Research Evidences of
R a s a s h a s t r a

PREFACE

Rasashastra is a branch of Ayurveda which explains the preparation and usage of minerals, metals, animal products and herbo– mineral products. Besides, the branch also deals with the quality standards of preparation.

Rasaushadhies being most efficacious in Ayurveda and known for its minimal dosage, palatability and quick action. However, the safety of Rasa preparations is being questioned, which hampering the practices of their useful preparations.

This EBook contains articles related to efficacy studies, characterization, standards of preparation and safety and toxicity of Rasa preparations. This will boost up the confidence and knowledge of practitioners for usage of Rasa preparations and also to educate the public regarding safety in usage of Rasa preparations.

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EFFICACY STUDIES

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Review Article

A critical appraisal on *Swarnaprashana* in children

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Abstract

Administration of processed gold in children is a unique practice mentioned in Ayurveda as "*Swarnaprashana*" by Acharya Kashyapa thousands of years back. He explained evidently the administration of *Swarna* (gold) in children for the benefits of improving intellect, digestion and metabolism, physical strength, immunity, complexion, fertility, and life span. There are various formulations of gold and even along with herbal drugs explained by different Acharya for prolonged usage in children. *Swarnaprashana* in children can be mainly implicated in two contexts of Ayurveda; *Lehana* (supplementary feeds) and *Jatakarma Samskara* (newborn care). This review is an effort to critically evaluate the available data, which may be helpful in clearing some of the existing fallacies on the topic. The age and method of administration, benefits and dosage as per various classical textbooks of Ayurveda are discussed here along with the reports of related scientific studies. This review proposes that the benefits of *Swarnaprashana* can be achieved at multiple levels like as a general health promoter and in specific to enhancement of intelligence, digestion, metabolism, immunity, physical strength, complexion, or fertility. Ayurveda recommends only purified and processed form of gold for internal administration. The age at which *Swarnaprashana* should be administered and its duration can be determined depending upon the desired effect in children as a positive health care program.

Key words: Ayurveda, children, gold preparation, immuno-modulator, *Swarnaprashana*

Introduction

The usage of metals and minerals was well-known to mankind since thousands of years. Gold has been given the status of the most precious metal, which was used to prepare sculptures of Gods, ornaments, coins, and so on all over the world. It is one of the most ancient metals even used for preventive and curative purposes. With regards to its early knowledge to Indians, an earliest reference is found during prehistoric period, mentioned as *Hiranya* (synonym with *Swarna*) in Vedic works.[1]

Manusmriti, the ancient Indian literature of rectitude, mentions that a *Dwija* (brahmin) has to undergo *Samskara* (purificatory rite) like *Jatakarma* (procedures at birth) and *Chudakarana* (ceremony of tonsure) to get rid of the impurities, which may be present in the *Bija* (sperm and ovum) and also due to the stay inside the womb as *Garbha* (fetus). Here, *Jatakarma Samskara* (newborn care) is told as a purification procedure, in which soon after cutting the umbilical cord the baby is made to lick the mixture of gold, honey, and ghee by chanting *Mantra* (spiritual hymns).[2]

Ayurveda, has imbibed itself many such references of gold pertaining to its medicinal properties and uses at different contexts. Gold is explained as one among the *Sapta Loha*[3] (seven metals). It is categorized under *Shuddha Loha*[4] (pure metal), which is said to be having both preventive and protective qualities. Gold is indicated for internal use even before conception due to its *Rasayana*[5] (rejuvenator) and *Vajikarana*[5] (aphrodisiac) properties so as to beget a healthy baby. After conception, it is used in *Pumsavana Karma*[6] (procedure done to get the desired sex of the baby and for the proper intra-uterine growth and development of the fetus). After birth in *Lehana*[7] (supplementary feeds) and *Jatakarma Samskara*[8-10] gold has been said to have a major role to play. As the child grows, gold is also being recommended to be given alone or along with various herbal drugs for procuring better *Agni* (digestive power and metabolism), *Bala* (physical strength and immunity), *Medha* (intellect), *Varna* (color and complexion), *Ayu* (lifespan),[7-9] etc., Even on the death bed, when *Arishta Lakshana* (fatal signs) are observed, gold is indicated for its protective action.[10] Gold is such a noble metal, which is having substantial outcomes in the human body starting before conception until demise.

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It is evidently mentioned in the texts of Ayurveda that any form of gold should be used internally only after proper purification failing to do, which may result in complications that may be even life-threatening.[3,4] The type of gold to be used, mode of administration of different forms, theirs benefits and therapeutic indications are also clearly explained.[3]

Methods

A methodical collection of data from classical texts of Ayurveda as well as related pharmaco-clinical research articles and dissertation works published in between May 1977 to November 2011 have been collected using PubMed and manual search of bibliographies as the sources.

Swarnaprashana

In *Lehana*

Lehana depicts administration various herbal drugs, ghee preparations, and gold alone as supplementary feeds wherein precise indications are stated. Acharya Kashyapa coined the term *Swarnaprashana* for administration of gold. This unique formula has been explained wherein gold should be triturated along with water, honey, and ghee on a clean stone facing eastern direction and made the *Shishu* (infant)[11] lick the same. The specific benefits ascribed to *Swarnaprashana* are as follows:[7]

- *Medha Agni Bala Vardhanam* (improvement of intellect, digestion, metabolism, immunity, and physical strength)
- *Ayushyam* (promoting lifespan)
- *Mangalam* (auspicious)
- *Punyam* (righteous)
- *Vrushyam* (aphrodisiac)
- *Varnyam* (enhancement of color and complexion)
- *Grahapaham* (protection from evil spirits and microorganisms).

The specific benefits of *Swarnaprashana* according to the duration of administration have been mentioned such as:

- If administered for 1 month, the baby will become *Parama Medhavi* (highly intelligent) and *Vyadhibhir Na Cha Drusyate* (will not be affected by any disease)
- If administered for 6 months, the baby will become *Srutadhara* (will be able to remember the things, which are just heard).[7]

All the above said benefits are indicating the enhancement of all favorable factors required for proper growth and development of a child, which is considered to be rapid during *Shaishavavastha* (infancy).

In *Jatakarma Samskara*

Acharya Sushruta cite administration of *Swarna* along with honey and ghee in one among the procedures of *Jatakarma Samskara* that is, as a single dose at birth in the procedure of new born care. He provided the rationale behind this practice that there will be no adequate secretion of breast milk for the first 4 days after delivery and so as to support the baby with respect to preventive and nutritive aspects such practices are indispensable.[8] Acharya Vagbhata advises to give a combination of herbal drugs in a specific shaped spoon as that of the leaf of holy banyan tree, made up of gold to the newborn for increasing *Medha* (intellect).[9] Administration of *Swarna* along with other herbs is also mentioned by Acharya Vagbhata in *Jatakarma Samskara*.[10]

Administration

In *Jatakarma Samskara*, it is told that *Swarna* should be administered soon after birth as one of the procedures of new born care. In *Lehana* it should be administered in *Shishu*. Acharya Sushruta suggests administration of *Swarna* along with other herbs like *Shankhapushpi* (*Convolvulus pluricaulis* Choisy), *Kushtha* (*Saussurea lapa* Clarke), *Vacha* (*Acorus calamus* L.), etc., with honey and ghee for a period of one year.[12] The term *Kumara* is also used in the same context that can be considered as a child of the age group in whom development of reproductive system is not yet complete.[13] In all above references, it is said that *Swarna* should be administered along with honey and ghee.

Acharya Vagbhata gives specific combinations of gold and herbal drugs as those who desire *Ayu*, *Medha*, *Lakshmi* (wealth), and *Kama* (sensual enjoyment) should consume it along with *Shankhapushpi* (*Convolvulus pluricaulis* Choisy), *Vacha* (*Acorus calamus* Linn.), *Padma Kinjalka* (stamen of *Nelumbo nucifera* Gaertn.), and *Vidari* (*Pueraria tuberose* DC.) respectively.[10] A glimpse of administration of *Swarna* in newborn is also found in the text Rasaratna Samuchaya,[4] which is very similar to above references. There is no reference of any specific day or time for *Swarnaprashana* in children.

Dosage

Acharya Kashyapa has not mentioned the dosage for *Swarnaprashana* in specific. However, he has given general dosage of children according to age in the same context from birth. The same can be followed to fix the dosage of *Swarnaprashana*. A few other available references regarding per day dose of *Swarna Bhasma* from various texts are as listed below:

- 1/4th–1/8th *Ratti* (15–30 mg) *Swarna Bhasma*[3]
- 2 *Gunja* (250 mg)[4]
- 1 *Gunja* (125 mg)/As per age[8]
- 1 *Harenu*[9]
- 1/32 *Ratti* (3.9 mg)[14]
- 15.5–62.5 mg of *Swarna Bhasma*.[15]

By taking these adult dosages as reference standards, dosage in children can be calculated.[16]

Forms of *Swarna*

Classics of Ayurveda mention specific forms of *Swarna* for internal administration such as *Patala/Mandala* (leaf/foil), *Churna* (powder), *Bhasma* (ash).[3]

Safety measures

Acharya were perceptive of all the serious adverse effects if gold is administered in impure form which is evident from the explanation of *Shodhana* (purification) of *Swarna* in specific. It is cited that if *Swarna* is administered without appropriate processing, it will destroy the happiness, potency, and strength of the individual and it will lead to manifestation of a group of diseases.[4] If *Swarna* is administered in impure form, it can also cause mental disturbances and even death.[3]

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Discussion

Acharya Kashyapa coined the term *Swarnaprashana*.[7] By considering various references it can be said that the term *Swarnaprashana* signifies the administration of gold alone or along with other herbs in a *Leha/Prasha* form. He describes *Swarnaprashana* in the context of *Lehana*. *Lehana* has been indicated for those children who are healthy, but have compromised breastfeeding and are having minor functional problems of metabolism. It is contraindicated in seriously ill children and also on daily basis.[7]

Swarnaprashana can be administered in all children as it acts at the level of nutrition, metabolism, growth and development, physical strength, and immunity. It is the only type of gold preparation described with its specific benefits according to the duration of administration. Even though *Lehana* is contraindicated on a daily basis, *Swarnaprashana* alone can be continued for a period of 1 or 6 months to get its specific benefits in children as stated by Acharya. Further research studies could reestablish the above said benefits of *Swarnaprashana* with respect to its duration.

In *Lehadhyaya* of Kashyapa Samhitha, the first mentioned formulation is *Swarnaprashana*. By considering the indication it can be said that *Shaishava Avastha* is the right period from which it can be commenced. The role of gold as a physiologically important element might have been understood by Acharya Kashyapa, thousands of years back, which made him to include gold as an important metal to be used in therapeutics. This precise mention may also be due to some specific action of gold in that age group of children, which is observed in the following studies. In a study, trace elements including gold were measured in human placenta and newborn liver at birth.[17] A trace elements like zinc, copper and gold were measured in the hair of newborn infants.[18] In a pharmco-clinical study on neonates *Madhu-Ghrita-Swarna-Vacha* combination showed a significant effect of humoral anti-body formation and it acted on immunological system, which was evident by triggering the response of immunological system by a rise in the total proteins and serum IgG levels.[19]

Basically, childhood is the period of growth spurts, which is considered to be until 16 years of age, as per Ayurvedic classics. Acharya Charaka mentions a period "*Vivardhamana Dhatu Avastha*" from 16 to 30 years of age in which growth and development is observed. According to him, this period comes under *Balyavastha* (childhood).[20] Administration of gold is suggested in new born care (*Jatakarma Samskara*), up to one year of age and in *Kumara*. This wide range of age is suggested for administration of gold always arise queries regarding the cited benefits of *Swarnaprashana* in children. It can be said that the benefits of *Swarnaprashana* can be attained from infancy to adulthood with a wide range of actions influencing the growth and development of a child. The age at which it can be administered should be dependent upon the expected effect in the body. Considering all the above cited references it can be said that the action of this noble metal might be at multiple levels like as a general health promoter or in specific to enhancement of intelligence, metabolism, immunity, physical strength, fertility, complexion, etc.

As a general tonic, it can be administered in any age group. For the benefit as an immuno-modulator, it can be administered in children in early ages as this period until one year is considered to be the most vulnerable time for infections due to immature immune system. It can be even administered in case of immunodeficiency states provided that there is no serious illness associated with. The action of gold in the immune system can be justified from the following study reports. Pharmacological studies showed specific and nonspecific immune responses, which were modified in a positive manner in *Swarna Bhasma*-treated mice. It also had a stimulatory effect on peritoneal macrophages, which may be helpful to fight against infections.[21,22]

In adolescence, there is the appearance of secondary sexual characters, which signify the development of the reproductive system. This could be the right period to get the benefit of *Swarnaprashana* as a fertility enhancer. In a study measuring trace elements in endometrium and decidua, looking for cyclic variations, including those during pregnancy, gold was found in human endometrium and decidua. The reported levels of gold were slightly lower around mid-cycle than at other stages of the cycle.[23] In male reproductive system, gold was measured in semen and reported that it is the richest source of gold reported in biological materials.[24] Another study suggests a significant stimulatory effect of gold chloride on female reproductive activity in immature rats.[25]

To get benefited as an enhancer of intelligence, *Swarnaprashana* can be given from birth and specifically for a period of 1 or 6 months continuously as mentioned in the classic. In a study colloidal gold was found to improve cognitive functioning, which was measured by IQ scores.[26]

As there are a number of forms of *Swarna* mentioned, picking the apt form is a major chore. Acharya Kashyapa has mentioned *Kanaka*[7] (a synonym of gold) triturated along with water, honey, and ghee. The idea behind this may be that as per the availability at that time in its pure form, gold could be administered directly and by rubbing on a stone micro particles of gold might be procured effortlessly. Later, other forms of *Swarna* were mentioned especially *Bhasma* which may be due to the advancement of pharmaceutical methods in Ayurveda like *Rasasastra* (metallurgy) as a separate branch of Ayurveda. Any form of gold, which is having better bioavailability should be the first choice for internal administration. The bioavailability of all the forms of gold is yet to be established. A few related study reports are cited below. *Swarna Bhasma* is said to have nanoparticles of gold which comprises gold-containing particles that are globular and have an average size of 56–57 nm.[27] In blood compatibility study, it was found that *Swarna Bhasma* contained gold nanoparticles with crystallite size 28–35 nm and was 90% pure gold.[28] Another study found that colloidal gold uptake in gastrointestinal tract is dependent on particle size that is, smaller particles cross the gastrointestinal tract more readily.[29]

It is very obvious in children that even slight negligence in the administered drug can cause serious adverse effects due to their highly sensitive body systems, which are still under rapid growth and development. Acharya had a very lucid idea regarding the significance of refinement of *Swarna* before internal administration. This is the reason that they have specifically pointed out the adverse effects of gold if administered without proper processing. Depending upon the form to be used, suitable processing should be adopted; that is, proper purification has to be performed if pure gold has to be administered and if *Bhasma*

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after proper purification and incineration. The particles of *Swarna Bhasma* were reported as non-cytotoxic.[28] In a study of chronic toxicity of *Swarnabindu prashana* no cytotoxicity was observed.[30]

There is no any specific day or time for administration of *Swarnaprashana*. *Acharya* Kashyapa mentions east direction to which one should face, while preparing *Swarnaprashana*. In the context of *Rasayana*[5] and *Pumsavana Karma*,[6] *Acharya* Susrutha and Charaka, respectively mention the usage of *Swarna* on *Pushya Nakshatra* (a star in Hindu calendar). The current popular practice of *Swarnaprashana* only on the day of *Pushya Nakshatra* may be due to the belief that it is an auspicious star to administer any medication due to its nourishing effect.[31] However, such administration once in a month in every age group of children without proper dosage fixation may not yield any benefit as mentioned in classics. Scientific studies in this direction maybe helpful to make this practice unambiguous.

Conclusion

Swarnaprashana does not signify administration of any specific form of gold on a specific day or time. Rather it is the administration of pure gold alone or different forms of gold along with various herbs where this specific word solely means the administration of gold. The selection of the form of gold should be based upon the factors such as better bioavailability, no adverse reactions, easier in preparation, and administration, which should be standardized by scientific studies. Imprecise usage in terms of dose, duration, and condition will definitely generate adverse effects in the body, especially in children. The benefits of *Swarnaprashana* in children as told by *Acharya* Kashyapa and others are to be studied with the help of various experimental models and also at clinical levels so as to re-establish the unaccepted facts due to lack of scientific support. The benefits of *Swarnaprashana* in children are multidimensional. In a nut shell, *Swarnaprashana* can be named as a positive health care program for children.

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AN EXPERIMENTAL STUDY ON MAKARADHWAJA

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ABSTRACT: *The authors report in this article an evaluation of the Rasayana effect of makradhwaja through biochemical parameters like – S.G.O.T., S.G.P.T and Blood urea along with E.C.G.*

INTRODUCTION

In the field of Ayurveda, there is a vast range of rasayana Preparations amongst them Makaradhwaja is a well known rasayana remedy which is a mercurial preparation and commonly used by physicians to maintain the health of a healthy person protecting him from various diseases and to cure various acute or chronic ailments, makaradhwaja is generally used with panaswasa (betal leaf juice), Ardakaswarasa (Zinger juice), madhu (Honey) or with other suitable anupanas (Adjuvents)[1] through oral route for therapeutic purposes.

The metals and minerals are in use since dates back to vedic period though their pharmaceutical and therapeutic development took place during medieval period. Makaradhwaja contains heavy metals like Mercury, Iron, Manganese, cobalt, Nickel and chromium where mercury and sulphur are its major constituents and remaining others are in traces analysed chemically as well as spectroscopically[2].

The review of Rasa shastra literature rerveals that impuremercury if used internally may likely produce many complications (unwanted effects) in the body viz. Aruchi (nausea), vidaha (Burning sensation), Kushtha (Skin diseases) and even death also[3]. So there are strict directions for samanya (general) and vishesa (specific) shodhana[4] (Purification) of parade (Mercury). Probably the ancient scholars were not quite satisfied with only shodhana process of parada regarding its therapeutic use so they adopted one more step forward in the form of Murchhana or Gandhaka Jarana[5]. Gandhaka Jarana is a process in which the Parada is triturated with gandhaka (Sulphur) and subjected to heat to allow burning of extra sulphur so that a suitable compound of the drug is obtained, which can be administered internally without any fear of side/toxic effects, hence murchhana is such an important process without which mercury may not become suitable for therapeutic use. Due to this reason there is no single ayurvedic physician who has any hesitation in prescribing mercurial drugs. After its use no toxic effect has been notices till date while modern scientists very much hestitate to recommend it for internal use considering it highly toxic agent.

Hence due to its high importance in therapeutics and to discard the objections of modern scientists regarding its internal use, an attempt has been made to establish the

effect of drug on experimental animals (Rabbits).

Material and Method

Experimental animals

Drug – Makaradhwaja

Suspension media- gum Acacia powder

Mortar and pestle – 200 ml capacity

Syringe of 10 ml

Rubber tube for feeding.

All the animals kept in laboratory for acclimatization for 7 days, these were weighed approx 1.5 kg to 2 kg each they were divided into two groups and kept in separate cages. The drug was administered in dose of 3 gm/100gm of body weight for a period of 6 weeks. Blood samples were collected after two weeks interval along with E.C.G report.

Preparation of Dosage form

The drug (Makaradhwaja) is not soluble in water therefore a suspension of gum acacia is made for oral administration.

The 10 gm. of gum acacia dissolved in 100 ml of distilled water by gradual trituration in a mortar. Then well prepared solution was taken according to the body weight of animal and added makaradhwaja at the dose of 3 mg/ml/100 gm. of body weight for the preparation of the suspension.

Procedure

After acclimatization period of 7 days the drug was administered by rubber tube according to the body weight for the period of two weeks. On 15^{th} day E.C.G recording was done and on the 16^{th} day the blood samples were collected from the marginal artery of ear by a small cut. These samples were analysed for estimation of S.G.O.T., S.G.P.T and blood urea for evaluation of any toxicity.

Table No.1: Effect of Makaradhwaja on circulating Blood Urea.

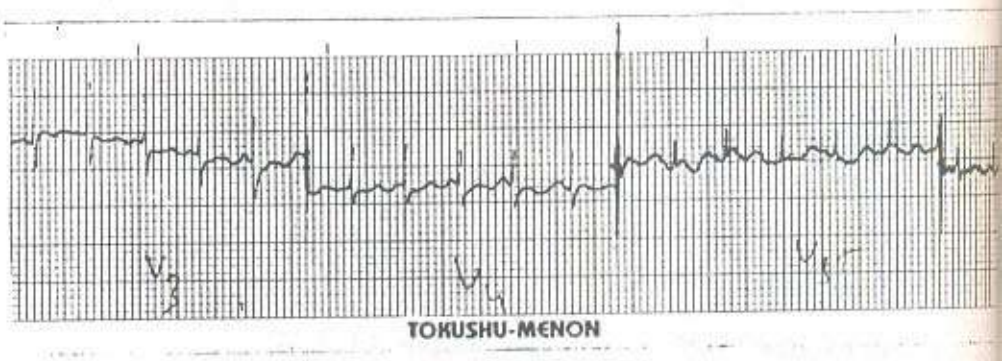
S. No	Initial (Normal)	After two weeks treatment	After four weeks treatment	After six weeks treatment
1.	75.0	77.5	60.0	45.0
2.	70.0	72.5	57.5	40.6
3.	62.0	71.0	70.0	55.0
4.	47.5	81.0	28.0	30.0
5.	50.0	70.0	30.5	-
6.	33.5	80.0	36.0	32.5
Mean	**56.33**	**75.33**	**47.08**	**40.62**
±S.E	**6.34**	**1.95**	**7.25**	**4.49**
No.	**(6)**	**(6)**	**(6)**	**(5)**

Table No.2: Effect of Makaradhwaja on circulating S.G.O.T.

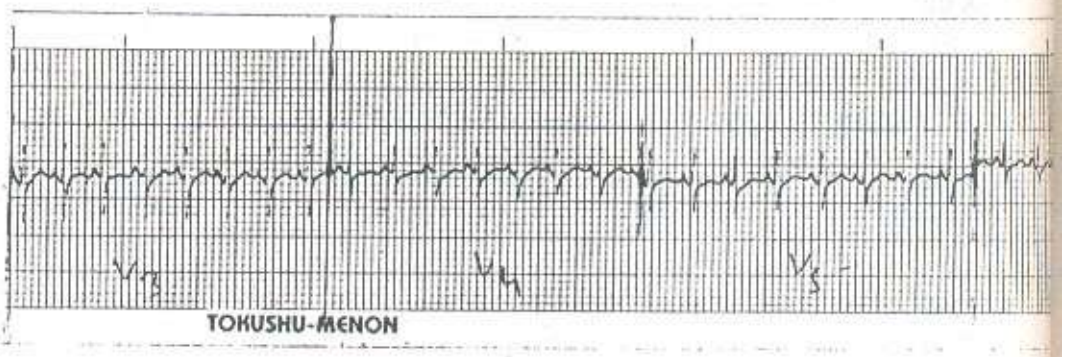
S. No	Initial (Normal)	After two weeks treatment	After four weeks treatment	After six weeks treatment
1.	24.0	40.0	62.0	72.0
2.	24.0	40.0	46.0	50.0
3.	76.0	28.0	41.0	72.0
4.	38.0	34.0	30.0	21.0
5.	30.0	34.0	30.0	-
6.	30.0	38.0	12.0	20.0
Mean	**37.00**	**35.67**	**36.83**	**49.17**
±S.E	**8.08**	**1.89**	**6.94**	**9.67**
No.	**(6)**	**(6)**	**(6)**	**(5)**

Table No.3: Effect of Makaradhwaja on circulating S.G.P.T.

S. No	Initial (Normal)	After two weeks treatment	After four weeks treatment	After six weeks treatment
1.	36.0	22.0	10.0	58.0
2.	32.0	28.0	24.0	47.0
3.	50.0	24.0	24.0	80.0
4.	28.0	37.0	35.0	24.7
5.	20.0	37.0	40.0	-
6.	28.0	35.0	32.0	25.3
Mean	**32.33**	**30.17**	**27.50**	**47.00**
±S.E	**4.14**	**2.79**	**4.34**	**10.43**
No.	**(6)**	**(6)**	**(6)**	**(5)**



Initial Electro Cardiogram before administration of Makaradhwaja



Electrocardiogram after six (6) weeks treatment of Makaradhwaja

Discussion

Observations of the experiment shows that after two weeks of treatment, there is initial increase in circulating blood Urea. It may possibly due to general vasodilatation action of the drug which reduces glomerular filteration by lowering blood pressure in glomeruli, It may not be taken as toxic effect of the drug as there is no significant increase is noticed in thelevel of S.G.O.T and S.G.P.T.

It is important to note that the readings of blood Urea levels after four weeks and after six weeks showed significant fall in blood urea levels which may be taken as the positive sign and may be explained as that after some time. There is a better clearance of drug due to compensatory mechanism adopted by the various living cells of kidney and against the unfavourable condition in initial stages. The gradual decrease in blood urea level after two weeks suggest the increase in renal blood flow along with increased catacholamine like substances which may help in maintaining the glomerular filteration rate. It also suggest the improved kidney function instead of probable damage, it was also deserved that there is simultaneous increase in S.G.P.T and S.G.O.T levels after four weeks and six weeks treatment but this increase is not beyond the normal levels and thus may not be taken as the toxic effects of the drug and there are suggestive of increase cellular activity with better clearance of waste product by the kidney is indicative of desired rasayana effect of makaradhwaja due to increase metabolic activity and suggesting improvement in the function of body organs and healthy state of life in the experimental animals.

The E.C.G taken after six weeks treatment shows a well defined Q.R.S complex specially in chest lead (V6) as compared to initial electro cardiogram as shown in figure. It is indicative to increase in force of contraction of ventricles and may to taken as positive for makaradhwaja effect on heart.

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Research Article

Anti-diabetic activity of traditional Indian gold containing preparation: *Shadguna Balijarita Makaradhwaja* on streptozotocin induced diabetic rats

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ABSTRACT

Background: *Makaradhwaja* a gold containing mercurial preparation used for diabetes mellitus in indigenous system of medicine. It is a popular aphrodisiac and rejuvenator traditional medicine. It is prepared by using processed gold, mercury and sulfur in different ratios by applying intermittent heating pattern in *Valuka Yantra*. **Objectives:** The aim of the study was to evaluate anti-diabetic effect of *Shadguna Balijarita Makaradhwaja* (SBM) on streptozotocin (STZ) induced diabetic rats. **Materials and Methods:** Diabetes was induced to normal rats by injecting STZ in dose 40 mg/kg. Powdered SBM and dried extract of *Tinospora cordifolia* were mixed with honey and administered orally for 20 days at dose 2.63 mg/kg and 42.34 mg/kg body weight, respectively. The effects of treatment on body weight changes and blood glucose levels were quantified on day 1, 5, 10, 15 and 21 of the experiments. On the 21st day, animals were sacrificed and gross histopathological changes in liver, kidney and pancreas were illustrated. Blood sugar level, glyacated hemoglobin, blood urea, serum cholesterol, serum creatinine, serum triglyceride and serum protein were estimated with standard methods. The study was conducted in the year 2011. **Results:** Test drug observed significant decrease ($P < 0.001$) in glyacated hemoglobin level compared to diabetic control rats. Blood sugar level of test drug group shown a significant decrease (279.11 ± 57.95) compared with diabetic rats. **Conclusion:** The present study demonstrates that SBM and dried extract of *T. cordifolia* with honey significantly reduces the blood glucose level and shows anti-diabetic effect.

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INTRODUCTION

Diabetes mellitus is threatening for the 21st century. It will be leading cause of morbidity and mortality in near future due to increasing incidence worldwide. Herbo-mineral-metallic drugs of Ayurveda having potential of decreasing blood sugar levels and found efficient on experimental animal models. *Makaradhwaja* [1] is such herbo-mineral-metallic composition.

Medieval classical texts of Ayurveda quoted that *Makaradhwaja* is one of the best rejuvenator and aphrodisiac [2] agent. It is prepared by using processed gold, mercury and sulfur in the ratio of 1:8:24 or 1:8:48 by sublimation in the traditional system of heating device *Valuka Yantra* [1,2]. Nowadays, it is prepared in modified vertical electrical muffle furnace (modified *Valuka Yantra*) [3]. It gives synergistic action with different herbs in the various disorders. It is therapeutically efficient in disorders such as *Madhumeha* (diabetes mellitus), *Jwara* (remittent fever), *Kushta* (skin disorders), and *Raktaja Vikara* (blood disorders) [2]. Previous studies claimed its safe use without any untoward effect and toxicity [4]. Specific action on cell-mediated immunity is proved by its immune-modulator

action [5]. In experimental and clinical studies, it is found effective in diabetes [6].

Guduchi (*Tinospora cordifolia* Linn.) is well-known *Madhumehahar* (anti - hyperglycemic) herb described throughout ayurvedic classics [7]. Most commonly, its stem is used for medicinal purposes. It is well-known anti-oxidant, anti-hyperglycemic, immune-modulatar, and rejuvenator [8]. Its different formulations are quoted in the ayurvedic classics like juice, decoction, powder and *Ghana* (dried extract) as per diseases.

Madhu (honey) is mentioned as *Madhumehahar* (~anti-diabetic) agent within classical texts of Ayurveda [9]. Its anti-diabetic activity is reported by Erejuwa *et al.* [10]. It is used as a vehicle drug (*Yogavahi*) in the numerous herbo mineral formulations. It is mentioned in the reference to *Makaradhwaja* as a vehicle drug [11]. In the present study, it was used as a vehicle drug as prescribed in texts of *Rasashastra*.

The present study was planned to evaluate anti-diabetic activity of *Shadguna Balijarita Makaradhwaja* (SBM) and *T. cordifolia* Linn with honey in streptozotocin (STZ) induced diabetic rats.

MATERIALS AND METHODS

Preparation of SBM and *Guduchi Ghana*

Test drug SBM was prepared as per the classical text reference in the Department of Rasashastra and Bhaishajya Kalpana, Institute of Post Graduate Teaching and Research in Ayurveda (IPGT and RA), Gujarat Ayurved University, Jamnagar in 2011 [1]. Raw Material *Swarna* (gold) was purchased from the local market, and *Hingula* (cinnabar) and *Gandhaka* (sulfur) were collected from Pharmacy of Gujarat Ayurved University, Jamnagar. Gold was subjected to *Shodhana* and after *Shodhana*, its foils were prepared. Cinnabar was processed to *Shodhana* (purification) and *Parada* (mercury) was procured from its sublimation by adopting *Nada Yantra* method [12]. *Gandhaka* (sulfur) was subjected to *Shodhana* by melting it and pouring in cow milk and continuously heated in same milk for 3 h [13]. Processed gold foils, mercury and sulfur were brought in ratio of 1:8:48 in weight. Amalgamation was done by adding gold foils to mercury. Fine lusterless powder *Kajjali* was prepared by triturating sulfur with above prepared amalgam. *Kajjali* was levigated with juice of *Kumari* (*Aloe barbadensis* Mill.) and *Japa* (*Hibiscus rosa-sinensis*) for 3 h consecutively. Levigated *Kajjali* was dried and powdered. The fine powder was filled in seven-layer mud smeared cotton cloth wrapped glass bottle (*Kacha Kupi*) and heated for 12 h in specially designed electrical muffle furnace. The heat was provided in controlled and gradually increasing temperature in modified electrical muffle furnace (modified *Valuka Yantra*). After the desired characteristic features of product preparation, the mouth of glass bottle was sealed; furnace was switched off and subjected for self-cooling. The highest recorded temperature during procedure was 600°C. Sublimed product was procured from neck of glass bottle, it was powdered and used for further analysis and study [14]. In analytical studies, it was observed that *Makaradhwaja* is consisted of red sulfide of mercury and having an empirical formula of HgS (mercury sulfide). Inductively coupled plasma optical emission spectrometry was observed that it contains 1.2% of gold with mercury and sulfur in finished product as a major element.

T. cordifolia's stems were collected from the herbal garden of Gujarat Ayurved University, Jamnagar. Stems of *T. cordifolia* were cut into small pieces and crushed. These crushed pieces were cooked with 4 times of potable water ant reduced at 1/4th of the same to prepare decoction. The decoction was sieved, cooked to semisolid consistency. The semisolid mass was dried in hot air oven at 45°C to prepare dry extract [15]. Average 5.21% yield was obtained from the stem extract. The *Guduchi Ghana* (dried extract) was powdered and stored [16].

Honey was collected from the local forest of Jamnagar.

Experimental Animals

Albino rats (160-220 g) of either sex were selected for this study. Animals were procured from the Animal house of Pharmacology laboratory of IPGT and RA, Gujarat Ayurved University, Jamnagar. Permission for the experiment was granted by Animal Ethical Committee of the Same Institute (IAEC-06/09-11/02). The animals were fed pellet diet and water. As per the guidelines of National Institute of Health's guide for the Care and Use of Laboratory Animals, the study was conducted [17].

Collection of Blood Samples

Tail veni-puncture method was applied for the collection of blood sample in the rats. For investigation Glucometer strip was used, and reading was noted down. By adopting this procedure blood glucose level of animals was estimated, prior injection of STZ, 5th, 10th, 15th, and 20th day during trials.

The sacrifice was carried out on the 21st day after completion of the study. Animals were anesthetized and stroked over tiles for sacrifice. The blood sample was collected by the dissection of jugular veins of animals. Biochemical parameters were investigated at pathology laboratory, IPGT, and RA, Jamnagar.

Experimental Induction of Diabetes

Diabetes was induced to rats by single intra peritoneal injection of STZ (40 mg/kg). STZ was weighed individually for each animal, according to its weight it was solubilized with 0.2 mL saline (154 mM NaCl) just prior to injection. After 72 h of STZ injection, rats with diabetic hyperglycemia (blood glucose more or equal to 250 mg/dL) were selected for experiment. Suspension of *Makaradhwaja* and *T. cordifolia* with honey was started fed to selected diabetic rats.

Anti-diabetic Activity

Anti-diabetic activity was evaluated by the effect of test drugs on the ponderal and biochemical parameters. Ponderal

parameters like gross body and different body organs weight were evaluated. Biochemical variables such as blood sugar, glycated hemoglobin (HbA1C), serum total cholesterol, serum high-density lipoprotein (HDL), serum triglyceride, serum creatinine, blood urea, serum glutamic-pyruvic transaminase (SGPT), serum glutamic oxaloacetic transaminase (SGOT), total protein, albumin, and globulin were estimated. Sacrificed animals gross and histological appearance of vital organs (liver, pancreas and kidney) were examined at the end of the study.

Experimental Design

Diabetic animals were divided into four groups. Food and water were provided to the animals.

- Group 1: Normal control (NC)
- Group 2: Diabetic control (DC)
- Group 3: DC + SBM and *Guduchi Ghana* with honey suspension (SBM)
- Group 4: DC + glibenclamide (reference standard [RS])

Dose Fixation

The dose of the drug was calculated by extrapolating the therapeutic dose to rat on the basis of body surface area ratio by referring to the table of Paget and Barnes (1964) [18].

Dose

Powdered SBM 2.63 mg/kg rat and dried extract of *T. cordifolia* 42.34 mg/kg rat were grinded with honey to prepare suspension. This suspension was administered orally to test drug group SBM diabetic animals. Glibenclamide, in the dose 0.45 mg/kg was administered to RS drug control group.

Statistical Analysis

All the values of were expressed as mean ± standard error mean. A statistical analysis was performed by using Student's *t*-test. It was calculated by using Microsoft excel programmer.

RESULTS

In NC rats, during the course of 21 days, 13.90% weight was increased. Insignificant decrease in body weight was observed in SBM and RS group in comparison to DC rats [Table 1]. In DC rats, weight of kidney and liver was increased up to significant extent. Treatment with test drug did not affect the weight of these organs to significant extent in comparison to the DC group [Table 2].

Initial blood sugar level was decreased by 44.27% and 44.04% in SBM and RS treated groups respectively [Table 3]. Raised levels of HbA1C were significantly attenuated by test drug SBM and RS [Table 4]. Increased blood sugar levels were insignificantly decreased by SBM and RS test drugs.

Non-significant rise in serum cholesterol, triglyceride and HDL were moderately decreased by administration of SBM. Blood urea levels decreased significantly in group SBM and moderately decreased in RS group. Elevated serum creatinine levels non-significantly decreased by administration of SBM which was non-significantly increased in RS group in comparison to DC group. Decreased SGOT parameter in DC group was significantly attenuated by SBM and RS drugs. Increased levels of SGPT were decreased up to significant extent by SBM and RS drugs [Table 5].

The accumulation of fat in liver was observed in histopathological study. SBM and RS treated animals restored the histological changes [Figure 1a-d]. Inflammation in blood vessels, increase in the thickness of bowman capsules, fat deposition, and change in size of the glomerulus were found in the kidney of

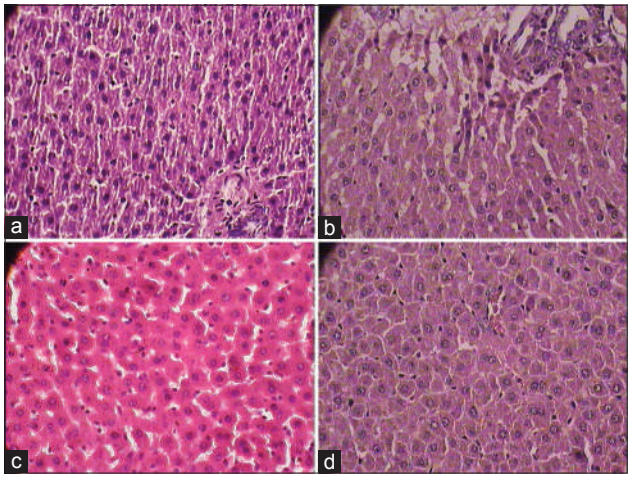

Figure 1: (a) Normal cytoarchitecture of liver in normal control group (1 × 400 magnification), (b) Photomicrographs of representative section of Liver. Macro and micro fatty changes, cell infiltration in almost all the sections streptozotocin control group (1 × 400 magnification) (c) *Shadguna Balijarita Makaradhwaja* treated rats shows almost normal cytoarchitecture of liver in comparison with streptozotocin control group diabetic rats (1 × 400 magnification), (d) Glibenclamide treated rat showed almost normal cytoarchitecture of liver sections in comparison with streptozotocin control group diabetic rats (1 × 400 magnification)

Table 1: Effect of test drug on body weight in STZ diabetic Wister strain albino rats

Group	0 day (g)	5th day (g)	10th day (g)	15th day (g)	21st day (g)	% change in comparison to 0 day
NC	187.00±10.95	192.00±11.27	196.33±11.03	205.67±10.28	213.00±10.57	13.90↑
DC (STZ)	170.67±11.73	165.33±12.49	165.67±14.00	167.00±13.61	166.67±15.85[α]	02.34↓
SBM	176.33±07.79	170.33±08.20	164.33±08.54	158.00±09.18	152.00±09.24	13.80↓
RS	172.67±04.15	162.00±09.62	158.33±14.04	161.17±14.38	161.33±17.15	06.57↓

Mean±SEM, NC: Normal control, DC: Diabetic control, SBM: *Shadguna Balijarita Makaradhwaja* control, RS: Reference standard control, STZ: Streptozotocin, ↑: Incease ↓: Decrease, SEM: Standard error of mean

diabetic rats. The treatment with SBM showed the normal histopathology of the kidney without any inflammatory vessels and fat deposition [Figure 2a-d]. Decreased Islets of Langerhans, smashed size of β cells and extensive necrosis, fibrosis and atrophy were observed in the pancreas of diabetic rats. STZ induced diabetic rat treated with SBM and glibenclamide restored the necrotic and fibrotic changes and up to moderate levels, raised the number of β cells. [Figure 3a-d].

DISCUSSION

Excessive breakdown of tissue proteins decreases body weight in diabetes. It was observed in DC rats in comparison to normal rats [19-21]. Treatment with SBM improved body weight to a certain extent, indicating that control over muscle wasting resulted from glycemic control.

Previous studies claimed that STZ destructs beta cells, which leads to cells less active that makes poor utilization by tissues [22,23]. This suggests that test drug may possess as insulin-like effect on peripheral tissues by inhibiting hepatic gluconeogenesis by promoting glucose uptake or metabolism [24]. Or it might be possible that test drug increases absorption of glucose into the muscles and adipose tissues [25]by stimulation of regeneration process and revitalization of remaining beta cells [26]. Hence, the hypoglycemic activity of SBM may be due to its protective action against damaged pancreatic beta cells and possibly because of increased insulin release or secretion or regeneration of damaged beta cell. *Makaradhwaja* is a well-known therapeutic medicine of rejuvenation in Indian system of medicine. Its immune-modulatory action was also established [5]. The observed effect may be attributed to the rejuvenation property of *Makaradhwaja*. The previous study supports its action too [27]. Moderate but insignificant decrease in blood sugar levels were also observed in test drug-treated animals.

Table 2: The effect of test drugs on weight of liver and kidney in streptozotocin induced diabetic Wistar strain albino rats

Organs	Kidney (g/100 g)	Liver (g/100 g)
NC	0.65±0.05	2.38±0.08
DC (STZ)	0.99±0.07$^{\alpha\alpha}$	3.49±0.19$^{\alpha\alpha\alpha}$
% change in comparison to NC	52.31↑	46.64↑
SBM	1.06±0.08	3.63±0.08
% change in comparison to DC	07.07↑	04.01↑
RS	0.92±0.08	3.36±0.17
% change in comparison to DC	07.07↓	03.73↓

Mean±SEM, $^{\alpha\alpha}P<0.01$, $^{\alpha\alpha\alpha}P<0.001$ (comparison to normal control group, unpaired '*t*' test) NC: Normal control, DC: Diabetic control, SBM: *Shadguna Balijarita Makaradhwaja* Control, RS: Reference standard control, SEM: Standard error mean, ↑: Incease ↓: Decrease

Higher levels of HbA1C were observed in the diabetic rats compared with those in normal rats, it might be due to poor glycemic control. SBM treated diabetic rats significantly decreased the level of HbA1C, may be glucose metabolism was improved. This action represents that SBM has an ability to prevent the development of diabetes associated complications.

Elevated levels of SGPT indicating impaired liver function due to hepato-cellular necrosis. Due to elevated transaminase activities leads to diabetic complications like increased ketogenesis and glucogenesis [28], test drug significantly restored this parameter toward normal levels.

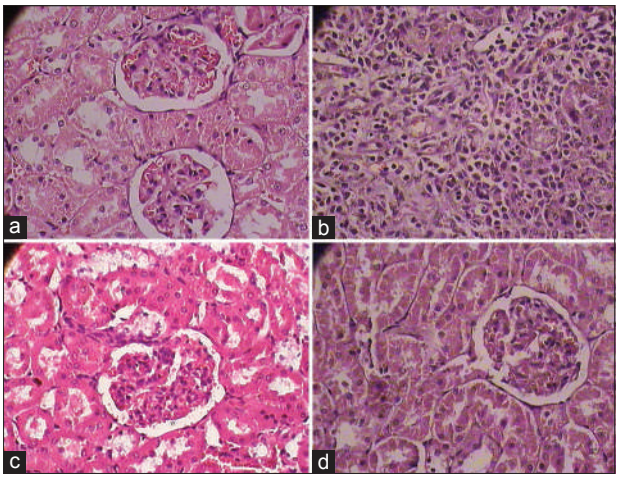

Figure 2: (a) Normal cytoarchitecture of sections of kidney in normal control group (b). (1 × 400 magnification), (b) Photomicrographs of representative section of kidney. Cell infiltration and micro-fatty changes in all the sections of streptozotocin control treated diabetic rats (1 × 400 magnification), (c) *Shadguna Balijarita Makaradhwaja* treated rat showed almost normal cytoarchitecture in comparison with streptozotocin control group diabetic rats (1 × 400 magnification), (d): Glibenclamide treated rat showed almost normal cytoarchitecture in comparison with streptozotocin control group diabetic rats (1 × 400 magnification)

Table 4: The effect of test drugs on HbA1c in STZ induced diabetic Wistar strain albino rats

Parameter	NC	DC (STZ)	SBM	RS
HbA1c	5.62±0.06	12.12±0.70$^{\alpha\alpha\alpha}$	6.04±0.71***	7.68±0.92**

Data: Mean±SEM, $^{\alpha\alpha\alpha}P<0.001$, (comparison to normal control group, unpaired *t*-test) $^{**}P<0.01$, $^{***}P<0.001$ (comparison to DC group, unpaired *t*-test) NC: Normal control, DC: Diabetic control, SBM: *Shadguna Balijarita Makaradhwaja* control, RS: Reference standard control, HbA1c: Glycated hemoglobin, SEM: Standard error of mean

Table 3: The effect of test drugs on blood sugar level in STZ induced diabetic rats at various intervals

Group	Blood sugar level (mg/dl)					
	0 day	5th day	10th day	15th day	21st day	% change in comparison to 0 day
DC (STZ)	383.67±51.44	380.33±37.26	355.33±28.09	372.83±34.49	328.17±30.67	14.67↓
SBM	500.83±42.61	503.67±31.81	455.67±35.60	486.67±51.93	279.11±57.95*	44.27↓
RS	512.83±21.43	374.33±22.16	334.83±32.03	314.83±29.68	287.00±33.78*	44.04↓

Mean±SEM, $^{*}P<0.05$, $^{***}P<0.001$ (comparison to diabetic control, unpaired *t* test). NC: Normal control, DC: Diabetic control, SBM: *Shadguna Balijarita Makaradhwaja* control, RS: Reference standard control, SEM: Standard error mean, STZ: Streptozotocin, ↑: Incease ↓: Decrease

Table 5: The effect of test drugs on various serum biochemical parameters

Parameters	NC	DC (STZ)	SBM	RS
Blood sugar (mg/dl)	117.50±2.50	321.00±27.80^{aaa}	261.22±60.01	286.33±29.52
Cholesterol (mg/dl)	57.17±4.72	62.00±4.03	45.67±4.46*	58.83±5.87
Triglyceride (mg/dl)	64.33±7.28	80.83±4.10	67.50±6.86	70.83±6.02
HDL (mg/dl)	25.00±3.22	34.17±2.60	29.50±3.77	28.33±3.26
Blood urea (mg/dl)	90.33±4.92	137.17±11.66^{aa}	57.00±5.49***	100.50±10.43*
Creatinine (mg/dl)	0.58±0.03	0.72±0.05^{a}	0.65±0.03	0.75±0.07
SGPT (IU/L)	75.50±8.60	323.00±21.29	88.83±10.37***	90.33±3.10***
SGOT (IU/L)	223.00±12.84	120.83±11.67^{aaa}	173.50±9.16**	304.67±20.66***
Total protein (g/dl)	7.35±0.11	6.70±0.23^{a}	6.70±0.28	6.08±0.31
Albumin (g/dl)	3.38±0.11	3.08±0.09	3.45±0.07**	3.00±0.18
Globumin (g/dl)	3.93±0.16	3.67±0.25	3.25±0.25	3.13±0.27

Data: Mean±SEM, $^{a}P<0.05$, $^{aa}P<0.01$, $^{aaa}P<0.001$, (comparison to normal control group, unpaired *t*-test) $*P<0.05$, $**P<0.01$, $***P<0.001$ (comparison to DC group, unpaired *t*-test), NC: Normal control, DC: Diabetic control, SBM: *Shadguna Balijarita Makaradhwaja* control, RS: Reference standard control, SEM: Standard error of mean, HDL: High-density lipoprotein

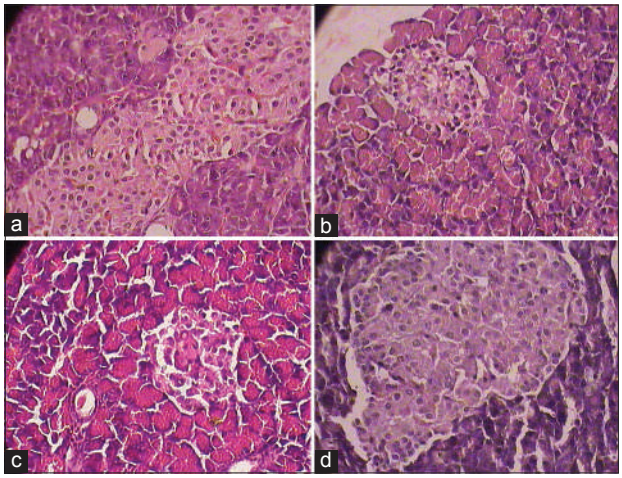

Figure 3: (a) Normal cytoarchitecture in normal control group, (b) Photomicrographs of representative section of Pancreas. Marked degeneration of Islets of Langerhans and degranulation of streptozotocin control treated diabetic rats (1 × 400 magnification), (c) SBM treated rats shows comparatively less degeneration of Islets of Langerhans with intact granules in comparison with streptozotocin control group diabetic rats (1 × 400 magnification), and (d) Glibenclamide treated rat showed normal cyto-architecture with intact granules in comparison with streptozotocin control group diabetic rats (1 × 400 magnification)

Elevation of urea and creatinine levels results due to renal dysfunction caused by free radical generation mediated stress in diabetes, persistent hyperglycemia, and hemo-dynamic changes within the kidney tissue [29-31]. Administration of SBM and glibenclamide to the diabetic rats significantly reduced the creatinine and urea levels, which represent the preventive action of SBM on kidney damages in diabetic condition perhaps due to the anti-oxidant properties. Blood urea level was significantly increase in diabetic rats compared to normal rats due to excessive breakdown of protein. This elevation was significantly decreased by test drug.

STZ induced diabetic rat's increases the level of lipid peroxidation, as an indirect evidence of production of free radical [32]. Hyperlipidemia commonly associated with diabetes [33]. Test drug significantly attenuated serum lipid profiles in diabetic rats. STZ induced diabetic groups treated with glibenclamide and SBM brought back the increased level of total cholesterol and triglyceride near to the normal levels.

Histopathological changes in liver, kidney and pancreas were well restored by the test drug in comparison with DC group. The observed results show anti-diabetic potential of SBM. Observed results may be due to synergistic action of *Makaradhwaja* with adjuvant *T. cordifolia* and honey.

CONCLUSION

The present study demonstrates that SBM and dried extract of *T. cordifolia* with honey significantly reduces the blood glucose level and shows anti-diabetic effect. Restoration histopathological changes in different organs support safe and effective anti-diabetic action of test drug. A significant decrease in glycated hemoglobin shows effect of *Makaradhwaja* on diabetes-related complications.

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Research Article

Analgesic activity and safety of ash of silver used in Indian system of medicine in mice: A reverse pharmacological study

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ABSTRACT

Objective: To study the analgesic activity of ash of silver used in Indian system of medicine and to explore its safety.

Materials and Methods: Albino mice of either sex (20-30 gm) were used to investigate the role of ash of silver against noxious stimuli: thermal (Eddy's hot plate and analgesiometer), mechanical (tail clip), and chemical (0.6% acetic acid induced writhing). An effort was made to find nature and site of action of ash of silver following naloxone pre-treatment. Maximum tolerated dose (MTD) and lethal dosage 50 (LD50) were also studied along with toxicological aspects of ash of silver.

Results: Test drug (ash of silver) at a dose of 50 mg/kg p.o exhibited analgesic activity against thermal, mechanical, and chemical stimuli. Analgesic effects were compared with the standard drug, morphine, in thermal and mechanical noxious stimuli and to aspirin in chemical stimulus. Analgesic activity of the test drug was reduced following naloxone pre-treatment. MTD was found out to be greater than 1.5 g/kg p.o. LD50 was 2 g/kg p.o. Fraction of mice showed symptoms of argyria as explained by autopsy reports.

Conclusion: Test drug exhibited moderate analgesic activity at 50 mg/kg p.o against all type of noxious stimuli, also suggesting a role of opioidergic system. The ash of silver was been found to be safe upto a dose of 1.5 g/kg p.o. in mice without any untoward toxicity. Further studies are required to explore the effect of ash of silver on pain mediators and excitatory neurotransmitters like glutamate, aspartate, or N-methyl-D-aspartic acid (NMDA).

KEY WORDS: Ash of silver, analgesic, argyria, nanosilver

Introduction

Ayurveda, an ancient system of medicine, advocates the use of herbal and elemental preparations. Many elements which are known to be toxic viz As, Cd, Hg, Fe, Ag, Au etc in modern medicine are also used in ayurvedic preparations.[1-3] One such metal is silver. It is considered to be non-essential accumulative trace element with wide distribution in body including the central nervous system, but with no known biological and physiological function. In ayurveda, ash of silver, also known as Raupya Bhasma, is used to treat many disease conditions like pain, neuralgias, inflammation, anxiety, convulsions, memory loss etc since years.[4,5] Since the safety of ash of silver is already established from traditional experience, the scope of reverse pharmacology is to understand the mechanism of action and to optimize the safety and acceptability of the lead compounds in natural products. In this approach, the candidate travels a reverse path from "clinics to laboratory" rather than classical "laboratory to clinic".[6]

Ash of silver is prepared by mixing pure silver leaves with equal quantity of sulphur by weight and one half quantity of arsenic trisulphide soaked in lemon juice and subjected to calcination process in sealed earthen containers at a temperature of 300°C.[1,7] The material is scrapped after cooling, triturated with lemon juice, pulverized, and calcined again. It undergoes the same process repeatedly (trituration with lemon juice, pulverization, and calcination) for 14 times to procure the ash of silver.[2-5,7] This process is chemical reduction of silver converting the silver molecule into uniform nano-particles, spherical in shape and with size of 16 nm without change in morphology of silver. Nanosize of silver particle is probably responsible for improving the penetration of silver in brain;

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hence, ash of silver has been used in past for the treatment of various pain and neurological conditions.[1,4,8,9]

To further confirm its central and peripheral analgesic property, reverse pharmacological studies were planned to find the effect of ash of silver and to explore whether it acts through opioidergic system or not. Safety of silver was also evaluated.

Material and Methods

Swiss strain of albino mice of either sex weighing between 20-30 gm were screened for study following the approval from Institutional Animal Ethics Committee. Mice were maintained under standard pellet diet and water *ad libitum*, housed in poly propylene cages under similar environmental conditions throughout the experiment. The test drug ash of silver was procured from M/s Baidyanath Ayurved Bhawan Ltd, Jhansi, India. Animals were divided into four groups consisting of six animals in each. Study protocol was as follows:

Group 1: Ash of silver (50 mg/kg) suspended in 1% solution of gum acacia, administered p.o. 20 min before exposure to noxious stimuli.

Group 2: Control, received gum acacia 1% solution p.o.

Group 3(a): Morphine hydrochloride (5 mg/kg) i.p. was administered 20 min before exposure to noxious stimuli.

Group 3(b): Aspirin (100 mg/kg) p.o was administered 20 min before exposure to noxious stimuli.

Once analgesic activity of ash of silver (50mg/kg) p.o was confirmed, further experiments were conducted to know its effect on opioidergic receptors as in group 4.

Group 4: Naloxone (1 mg/kg) i.p. was administered 30 min prior to ash of silver to study the mechanism and site of action of ash of silver (50 mg/kg) p.o. The responses of all drugs were assessed at 0, 30, 60, 90, and 120 min using analgesiometer in Tail flick test, Eddy's hot plate method, Haffner's tail clip method, and 0.6% acetic acid induced writhing methods, respectively.[10,11]

Systemic Toxicity Studies

Single dose toxicity studies were conducted in 18 mice of either sex in graded doses of drugs 25, 50, 100, 500, 1500, and 2000 mg/kg, respectively, within 24 hours. Animals were observed for 14 days for any signs of intoxication, any histopathological changes, gross behavioral changes, any weight loss and mortality.

Statistics

All data were expressed as mean ± SEM and analyzed using ANOVA followed by Dunnett's "*t*" test. *P*-value <0.05 was considered significant.

Results

The results have been shown in Tables 1-4. Ash of silver at a dose of 50 mg/kg p.o. produced significant analgesic response ($P<0.01$) compared to control (gum acacia 1% p.o.) but relatively less compared to standard drug (morphine 5 mg/kg i.p.) in the form of increased reaction time of mice to jump or lick paws in Eddy's hot plate method, increased tail flick latency in tail flick model, increased reaction time of mice to dislodge artery clip in tail clip model, all showing significant central analgesic activity. Ash of silver (50 mg/kg p.o.) suppressed

Table 1:

Assessment of central analgesic action of ash of silver by Eddy's Hot plate in albino mice

Treatment	Reaction time e (jumping/licking of paw) (Mean ± SEM) in seconds				
	0 min	30 min	60 min	90 min	120 min
1. Gum acacia(1% p.o.)	4.5±0.54	4.33±0.51	4.3±0.51	4.4±0.52	4.3±0.52
2. Morphine(5mg/kg i.p.)	4.5±0.54	4.8±0.75	8.3±0.81	7.1±0.98	6.0±0.63
3. Ash of silver (50mg/kg p.o.)	4.5±0.54	5.0±0.63	7.0±0.89	6.3±0.51	5.0±0.89
4. Naloxone(1mg/kg i.p.)+Ash of silver (50 mg/kg p.o.)	4.0±0.63	4.3±0.51	4.4±1.16	4.5±0.54	4.2±0.75
One way F	0.957	1.889	32.455	25.802	8.169
P-value	0.432 (>0.05)	0.164 (>0.05)	0.000 (<0.01)	0.000 (<0.01)	0.001 (<0.05)

n=6, ANOVA followed by Dunnett's Test, Data are (Mean ± SEM), $P<0.05$ (Statistically significant)

Table 2:

Assessment of central analgesic action of ash of silver by Tail flick method in albino mice

Treatment	Tail flick time (Mean ± SEM) in seconds				
	0 min	30 min	60 min	90 min	120 min
1. Gum acacia (1% p.o.)	2.6±0.51	2.8±0.75	2.5±0.54	2.8±0.75	2.6±0.81
2. Morphine (5 mg/kg i.p.)	3.0±0.54	6.3±0.51	9.0±0.89	8.6±0.51	6.3±0.51
3. Ash of silver (50 mg/kg p.o.)	3.2±0.75	3.5±0.54	7.2±0.54	6.8±0.75	4.0±0.63
4. Naloxone (1mg/kg i.p.)+ash of silver (50 mg/kg p.o.)	3.0±0.40	3.0±0.63	3.2±0.75	3.2±0.75	3.3±0.51
One way F	2.179	41.884	124.831	98.842	38.194
P-value	0.122 (>0.05)	0.002 (>0.05)	0.000 (<0.01)	0.000 (<0.01)	0.002 (<0.01)

n=6, ANOVA followed by Dunnett's Test, Data are (Mean ± SEM), $P<0.05$ (Statistically significant)

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Table 3:

Assessment of central analgesic action of ash of silver by nociception induced by Haffner's Tail Clip method

Treatment	Reaction time of mice (Mean ± SEM) in seconds at				
	0 min	30 min	60 min	90 min	120 min
1. Gum acacia (1% p.o)	21.5±0.81	21.8±0.75	22.0±0.89	22.1±1.16	21.8±0.98
2. Morphine (5mg/kg i.p)	21.3±0.54	23.8±0.75	28.2±2.04	30.5±1.04	26.3±1.03
3. Ash of silver (50mg/kg p.o)	21.8±0.98	21.5±1.04	26.3±0.51	25.6±0.81	21.0±0.75
4. Naloxone(1mg/kg i.p)+ash of silver (50 mg/kg p.o.)	22.0±1.26	22.8±1.60	21.8±0.75	22.1±1.16	22.5±0.83
One way F	0.314	5.556	41.571	83.00	38.973
P-value	0.815 (>0.05)	0.006 (>0.05)	0.001 (<0.01)	0.001 (<0.01)	0.002 (<0.01)

n=6, ANOVA followed by Dunnett's Test, Data are (Mean ± SEM), $P<0.05$ (Statistically significant)

Table 4:

Assessment of peripheral analgesic action of ash of silver by acetic acid (0.6%) induced writhing in albino mice

Treatment	Basal number of writhings in 1h after 0.6% acetic acid injection (mean±SEM)	Number of writhings in 1 hr (mean±SEM)	% inhibition
1. Gum acacia (1% p.o.)	7.66±0.51	7.16±0.75	6.25
2. Aspirin (100 mg/kg p.o.)	7.50±1.04	3.8±0.75	48.81
3. Ash of silver (50 mg/kg p.o.)	8.33±0.51	4.8±0.72	42.11
One way F	2.143±	30.980	
P-value	0.152 (>0.05)	0.001 (<0.01)	

n=6, ANOVA followed by Dunnett's Test, Data are (Mean ± SEM), $P<0.01$(Highly significant) when compared to control, $P<0.05$ (significant) when compared to standard.

acetic acid (0.6% i.p.) induced writhing episodes in mice ($P<0.05$) significantly showing peripheral analgesic activity compared to control, but standard drug aspirin (100 mg/kg p.o.) suppressed writhing episodes significantly more compared to ash of silver. Percentage inhibition of writhing episodes over 1 hour was 42.1% using ash of silver in contrast to standard drug aspirin (48.8%).

Safety Evaluation

Three out of eighteen mice died on day 11. No gross behavioral changes were observed in rest of the animals (up to a dose of 1500 mg/kg p.o.) except for some bluish black pigmentation of skin in four mice at the end of tenth day. Autopsy specimens from skin, spleen, liver, kidneys, and lymph nodes of dead mice showed deposits of silver granules in malpighian body of spleen, histiocytes of liver, renal glomerulus [Figures 1-3], and in the epidermal layer of skin without any changes in the morphology of skin. Maximum tolerated dose (MTD) was found to be >1.5g/kg. Lethal dosage 50 (LD50) was found to be 2 g/kg p.o as per the method described by Reed and Meuch.[10]

Discussion

In the present study, we have tried to explore the mechanism of action, therapeutic use, and safety of ash of silver (which is in use since traditional times as analgesic without any documented evidence) by reverse pharmacological study through screening in selected model to fast track drug discovery and development. Ayurvedic knowledge and experimental database can provide new functional leads to reduce time, money, and toxicity.[6]

Ash of silver (50 mg/kg p.o.) has shown to exhibit significant analgesic activity at 60 min ($P<0.01$) compared to control and significantly less than standard drug morphine (5 mg/kg i.p.) in Eddy's hot plate, Haffner tail clip, Tail flick methods in central pain producing models and comparable to aspirin (100 mg/kg p.o.) in peripheral pain producing method viz writhing episodes induced by using 0.6% acetic acid.[10,11] Ash of silver exhibited maximum analgesic effect at 60 min in contrast to morphine which exhibited maximum analgesic effect between 60 to 90 min [Tables 1-3] after which the effects started declining. Analgesic effect was observed in the form of increase in the reaction time of mice as paw licking or jump response, dislodging or biting of clip and tail flicking response in Eddy's hot plate, Haffner's tail clip and tail flick methods, respectively. Peripheral analgesic effect was tested by using 0.6% acetic acid [Table 4]. Number of writhing episodes before and after drugs administration was observed and percentage inhibition of writhing using ash of silver was compared with standard (aspirin 100mg/kg p.o.) and control (gum acacia) in the initial 1 hour. Percentage inhibition of writhing using ash of silver (42.1%) was comparable to that of the standard drug aspirin (48.8%), but not with control (gum acacia 6.25%) indicating that gum acacia does not have analgesic action of its own and does not potentiate the action of ash of silver in which it is suspended. The reduction in writhing episodes compared to basal number of writhings in first 60 min indicates an analgesic response.

To find the nature and site of action of analgesic action of ash of silver, we tried to block opioid receptors by pre treatment of mice with naloxone (1 mg/kg i.p.) in all central methods of nociception (Eddy's hot plate, Tail flick, and Tail clip methods) followed by the administration of ash of silver (50 mg/kg p.o.), morphine (5 mg/kg i.p.), and gum acacia (1% p.o.). There was significant reduction in analgesic responses elicited by drug ash of silver.

Ash of silver exhibited potent analgesic effect against thermal and mechanical noxious stimuli indicating that it as a predominant central analgesic, but also inhibited peripheral pain components as shown in writhing method. This is further

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Figure 1: Section of spleen of mouse showing silver deposits (A) In reticulo-endothelial meshwork. (Magnification 40 ×)

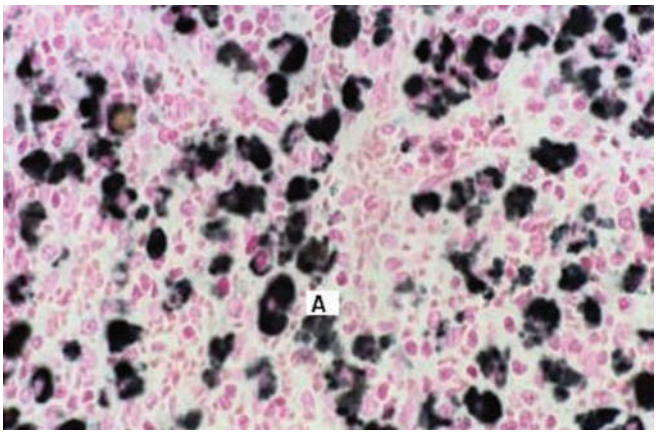


Figure 2: Section of mouse showing silver deposits (B) around whole thickness of portal vein (A) and hepatic artery (C) along with reticulum (D). (Magnification 40 ×)

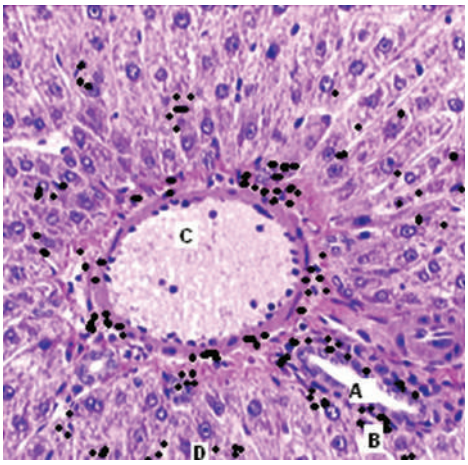
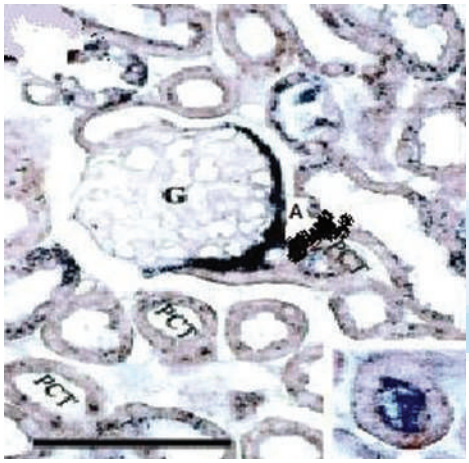


Figure 3: Section of kidney of mouse showing Argyrosis (A) in glomerular basement (G) and around proximal convulated tubules (PCT) (Magnification 100 ×)



supported by the fact that when we blocked opioidergic system using naloxone in central pain producing methods, the analgesic effect was reduced significantly. Any injury or tissue damage is associated with pain. Analgesics can act on peripheral or central nervous system to block pain perception. Peripherally acting analgesics act by inhibiting PGs and bradykinins at the site of pain, thereby suppressing the generation of impulses at chemoreceptor site of pain as shown in chemical method, while centrally acting analgesics not only raise the threshold of pain, but also alter the physiological response to pain by acting on CNS and suppress patient anxiety and apprehension.[12-14] Further, it can be hypothesized that ash of silver may be having an inhibitory action on excitatory neuro-transmitters glutamate, aspartate, or N-methyl-D-aspartic acid (NMDA) receptors which needs to be evaluated. As far as traditional system of medicine is concerned, ash of silver is being used for treating pain and inflammatory conditions since ancient times,[15] It might be having inhibitory action on certain pain and inflammatory mediators e.g. PGs, substance-p, bradykinin, LTs etc.

The previous studies by Khanna *et al.* suggest moderate to marked analgesic activity against chemical and electrical pain, but not against mechanical pain in contrast to our study which has shown moderate analgesic effect against mechanical pain which is also centrally mediated. The authors suggested a role of opioidergic system in pain suppression by silver. One of the studies has proposed that ashes of heavy metals used in traditional system of medicine act as catalyzer by their presence in intestine, plasma and blood, thereby acting as free radical scavengers.[16] Ash particles of heavy metals (gold, silver) in calcined form, being insoluble, exist as nanoparticles (16 nm) which are very tiny particles and found biocompatible, therefore, can cross blood brain barrier to produce various central actions viz analgesic, anti-inflammatory, anti-anxiety, cognitive, antidepressant, neuroleptic, and antiepileptic for which they are used in traditional Indian system of medicine.[1,3,4,17]

Use of metals in medicine is associated with toxicity.[18] The use of metal based medicines can be attributed to various causes including a need to revive a rich tradition, the dependency of 80% population of India on these drugs, their easy availability, comparatively low cost, and therefore, increasing world wide use. Calcined form of silver has been found to be non-toxic and exhibiting free radical scavenging activity by virtue of their antioxidant activity as shown in some studies. The quantitative analysis of ash of silver by spectroscopy has detected traces of heavy metals (in parts per million) like arsenic, lead, chromium, and iron. Ashes are associated with organic macromolecules show increased superoxide dismutase and catalase activity which reduce free radical concentration.[1-3] Nitrate based salt of silver has found to be toxic at 50 mg/kg p.o. in rats and mice leading to hepatic necrosis and deaths.[19-21] As far as safety of ash of silver is concerned, in our study, three mice died on day 11. Rest of mice did not show any weight loss or any morbidity. Ash of silver has been found to be safe up to 1.5 g/kg p.o. which is its maximum tolerated dose (MTD). Four among 18 mice showed characteristic bluish black pigmented patches over the skin. Autopsy reports of skin, spleen, liver, and lymph nodes from two dead mice have shown deposits of silver in these sites owing to their very small particle size. LD50 was found to be 2 g/kg p.o. The characteristic bluish black pigmentation is known as argyria is due to deposition of silver particles and

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melanocyte stimulating effect of silver.[22] Few studies have shown cyanocobalamin, vitamin E, and selenium antagonize the toxic effects of silver.[23,24] Such reports indicate the possibility that silver as such is not harmful in humans, but may be toxic in those deficient in vitamins and trace minerals.[25] A correlation between silver and vitamins like vitamin E and trace minerals need to be explored.

Further studies are required to establish the safety and efficacy of ash of silver. Metallic and herbal preparations offer advantages over plant drugs and allopathic preparations by virtue of their stability over a period, lower dose, easy stability, sustained availability, and fewer adverse effects.[4] The ashes of metals need to be thoroughly investigated with regard to their elemental content speciation and organic constituents so as to develop an understanding of their therapeutic effect.

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Ayurvedic Amalaki Rasayana and Rasa-Sindoor suppress neurodegeneration in fly models of Huntington's and Alzheimer's diseases

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We examined two Ayurvedic Rasayana formulations, claimed to facilitate 'healthy ageing', for their role in neuroprotection in fly models of polyQ (127Q and Huntington's) and Alzheimer's disorders. Our earlier findings showed that dietary supplement of Amalaki Rasayana, a preparation derived from Indian gooseberry fruits, and Rasa-Sindoor, an organo-metallic Bhasma prepared from mercury and sulphur, improves general well-being of fruit flies. Here we show that dietary supplement of either of these formulations during larval period substantially suppressed neurodegeneration in fly models of polyQ and Alzheimer's disorders without any side-effects. Dietary Amalaki Rasayana or Rasa-Sindoor prevented accumulation of inclusion bodies and heat shock proteins, suppressed apoptosis, elevated the levels of heterogeneous nuclear ribonucleoproteins and cAMP response element binding protein and at the same time improved the ubiquitin–proteasomal system for better protein clearance in affected cells. Our studies suggest, the potential of these Ayurvedic formulations in providing a holistic relief from the increasingly common neurodegenerative disorders.

Keywords: Ayurvedic formulations, dietary supplement, fruit fly, neurodegenerative disorders.

WITH improved healthcare and general hygiene and consequent longer life, the societal burden of the various late onset neurodegenerative diseases has substantially increased in recent times. Some of the inherited neurodegenerative diseases, known as codon reiteration disorders, are associated with a unique class of dynamic mutations which increase the number of trinucleotide repeats in certain genes beyond the gene-specific normal and stable threshold[1–3]. Several of these codon reiteration neurodegenerative disorders, which include Huntington's disease (HD) and several spinocerebellar ataxias (SCA), are grouped together as polyQ expansion disorders since they result from expansion of CAG repeats coding for polyglutamine (polyQ) tracts. Alzheimer's disease (AD), the other common form of senile dementia in humans, is associated with truncated Aβ peptides produced by aberrant proteolytic cleavage of the transmembrane receptor amyloid precursor protein (APP)[3–5]. A characteristic feature of these neurodegenerative diseases is the accumulation of protein aggregates, formed either by the repeat expanded or the truncated protein. AD patients[3–5] show presence of amyloid plaques formed by Aβ peptides and tau protein filament tangles in affected neuronal cells, while polyQ inclusion bodies (IB) are seen in polyQ disorders like HD and many of the SCAs[1–6]. The amyloid plaques or the polyQ IBs disrupt cellular homeostasis because a variety of critical cellular proteins like molecular chaperones, transcription factors, proteasome subunits and cytoskeletal components get sequestered with the IBs, which directly or indirectly cause cellular damage and consequent death of the target neuronal cells[1–6]. Therefore, these diseases are proteinopathies resulting primarily from a failure of the protein quality control mechanisms of the cell.

With a view to understand the molecular and cellular pathophysiology of neurodegeneration and to discover potential drug targets for therapeutic applications, several human neurodegenerative diseases like HD, different SCAs, AD, etc. have been modelled in yeast, *Caenorhabditis*, *Drosophila* and mouse model systems[1,2,5–7]. The *Drosophila* models offer many advantages because of the powerful genetic resources available in this organism[1]. Such studies have indeed helped in a better understanding of these neurodegenerative disorders and suggested several therapeutic approaches, although therapies that provide holistic relief with little side-effects continue to remain elusive.

Several traditional Ayurvedic formulations claim to facilitate 'healthy ageing'[8] and thus have the potential to mitigate the suffering from neurodegenerative diseases[9]. Although Ayurveda, the traditional medicine system of India, has been widely practised for several thousand years, very few systematic studies have been carried out to understand Ayurvedic formulations and practices in terms of contemporary science. With a view to fill this gap, we established *Drosophila* as a powerful model for

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studying the cellular and molecular biological bases of Ayurvedic formulations like Amalaki Rasayana (AR) and Rasa-Sindoor (RS)[10]. This study indeed showed that the biological effects of dietary supplement of AR or RS in flies were generally similar to those traditionally claimed for humans. The present study examines the effects of these two formulations on fly models of neurodegenerative disorders.

AR and RS are used as part of the rejuvenating Rasayana therapy, which is one of the eight major branches described in classical Ayurvedic texts like *Sushruta Samhita*[8,11]. Rasayana therapy is believed to promote long life with enhanced physical and mental strength so that the old age associated ailments are minimized. The Rasayana therapy involves, along with adoption of a certain lifestyle, oral administration of formulations based on plant and/or animal or mineral/metal sources. AR, a *kasthoushadhy*, is prepared from fruits of amla or Indian gooseberry (*Phyllanthus emblica*, synonym *Emblica officinalis*), while RS is a *Rasaoushadhi bhasma* in the form of mercuric sulphide with crystal size ranging from 25 to 50 nm, close to the nanocrystalline materials[12,13]. In our earlier study[10], we found that feeding of larvae and flies on food supplemented with 0.5% (weight/volume) of AR or RS significantly improved tolerance to thermal or starvation stresses and enhanced cellular levels of various heterogeneous RNA-binding proteins (hnRNPs), which have key roles in gene expression and RNA processing/transport[14,15]. Recently, we found that either of these formulations also enhances the levels of cAMP-response-element-binding protein (CBP/p300), a histone-acetyltransferase[16], in wild type *Drosophila* larval tissues (V. Dwivedi and S. C. Lakhotia, unpublished). Several earlier studies in different model systems[1,14,17–19] have shown that elevated levels of hnRNPs, CBP and better tolerance to thermal and/or oxidative stress suppress neurodegeneration. Therefore, we examined if dietary supplement of AR or RS, which enhances the levels of hnRNPs, CBP and stress tolerance, affects neurodegeneration in fly models of polyQ disorders or AD.

We used two well-established fly models for polyQ disorders, one expressing HA-tagged 127Q polypeptide (*UAS-127Q*)[20]; and the other expressing HA-tagged mutant human Huntingtin protein with a stretch of 93 glutamine residues (*UAS-httex1p Q93*)[21]. For AD, we used a fly stock carrying four copies of *GMR-Aβ42* transgene, which synthesizes the truncated Aβ polypeptide in developing eyes resulting in the formation of the amyloid plaques characteristic of AD[22]. Following the well-established practice in fly models of neurodegenerative disorders, the desired pathogenic polyQ transgene was expressed in developing eyes using the *GAL4-UAS* binary system of targeted gene expression[23] and the damage in the neuronal cells of eyes was assessed in the differentiating larval eye discs and/or adult eyes. We show that dietary supplement of 0.5% (weight/volume) of AR or RS results in significant suppression of neurodegeneration in parallel with greatly reduced accumulation of polyQ IBs in 127Q or the amyloid deposits in AD model. Levels of hnRNPs (like Hrp36 and Bancal, homologs of human hnRNP A1 and hnRNP K respectively), the CBP and the ubiquitin–proteasome (UPS) activity were significantly elevated in the 127Q transgene expressing eye discs in larvae reared on AR or RS supplemented food. Since these Ayurvedic formulations had no adverse side-effects in any of these fly models, further studies to examine the therapeutic potential of AR and RS in human neurodegenerative disorders like HD and AD would be rewarding.

Materials and methods

AR and RS, prepared by Arya Vaidya Sala (Kottakkal, Kerala, India), were separately mixed in fly-food (0.5% w/v) for rearing of experimental larvae and/or flies at 24° ± 1°C as described earlier[10], keeping controls on the standard agar–cornmeal–sugar–yeast food. Wild type (*Oregon R*$^+$), *w/w*; *UAS-127Q* (ref. 20), *w/w*; *UAS-httex1p Q93/CyO* (ref. 21), *w*; *GMR-GAL4* (ref. 24), w^{1118} *elav-GAL4* (ref. 25), *w; UAS-Ub*^{G76V}*-GFP* (ref. 26), w^{1118}*; GMR-Aβ42*^{K52}; *GMR-Aβ42*^{K53} (ref. 22) fly stocks were used. Appropriate crosses were carried out to obtain progenies of desired genotypes.

The surface organization of ommatidia in adult eyes was examined using nail-polish imprints[27], while the retinal rhabdomeres in adult eyes were visualized by pseudopupil technique[28] or phalloidin-TRITC staining[29]. Vision of 1, 5 or 10 days old wild type or *GMR-GAL4 > UAS-httex1p Q93* expressing flies, reared on different feeding regimes, was measured by the phototaxis assay as described earlier[29]. Apoptosis in eye discs was assayed by AO staining[30]. The total number of flies or larval eye discs of different genotypes and feeding regimes that were examined in each case is noted in the 'Results' section.

For assessing survival of individuals expressing *UAS-127Q* in the entire central nervous system under the *elav-GAL4* driver[29] on different feeding regimes, freshly hatched *elav-GAL4 > UAS-127Q* larvae were transferred to formulation-supplemented or standard food and the mean proportions (%) of larvae reaching pharate and adult stages were calculated from eight replicates of 25 larvae each.

Late third instar larval eye discs of desired genotypes were immunostained as described earlier[29,30] using P11 anti-Hrp36 (ref. 31), Q18 anti-Hrb57A or Bancal[31], 6E10 anti-amyloid plaque, mab22C10 anti-neuornal cells (DSHB, Iowa), SPA806 anti-Hsp60 (Stressgen), anti-CBP[32], SC-805 anti-haemagglutinin (Santa Cruz) for polyQ IBs, or 7Fb anti-Hsp70 (ref. 33) primary antibody. In many cases, the discs were also co-immunostained for HA-tagged polyQ IBs (SC-805 anti-haemagglutinin, Santa Cruz). Appropriate secondary antibodies conju-

gated with Cy3 (Sigma-Aldrich) or Alexa Fluor 488 (Molecular Probes) were used. Chromatin was counterstained with 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI). The immunostained discs were examined with Zeiss LSM 510 Meta confocal microscope.

For inhibiting the UPS activity, eye discs were dissected out from late third instar *GMR-GAL4 > UAS-127Q* expressing larvae and incubated in Poels' salt solution (PSS)[34] containing 1 μM proteasome inhibitor (clastolactacystin β-lactone, Sigma-Aldrich, India) for 2 h following which they were processed for immunostaining with the SC-805 anti-haemagglutinin Ab and confocal microscopy.

At least 20 eye discs were examined by confocal microscopy for each immunostaining. Four consecutive mid-level (along the *Z*-axis) optical sections, which distinctly showed the morphogenetic furrow, were used to generate the projection images of immunostained discs. These projection images were used to compare the levels of a given protein (expressed in arbitrary fluorescence units) in eye discs from differently fed larvae using the Histo option of Zeiss LSM 510 Meta software.

Western blots[10] of total proteins from eye discs (three replicates) were challenged with anti-Hrp36 or anti-β-tubulin and signals were detected using HRP conjugated anti-mouse or anti-rabbit IgG (Bangalore Genei, India) secondary antibodies respectively.

Levels of 127Q and G3PDH transcripts in *GMR-GAL4 > UAS-127Q* expressing eye discs from larvae reared on different feeding regimes were measured by semi-quantitative RT–PCR as described earlier[29].

Sigma Plot 11.0 software was used for statistical analyses. All percentage data were subjected to arcsine square-root transformation. One-way ANOVA was performed for comparison between the control and formulation-fed samples. Data are expressed as mean ± SE of mean of several replicates.

Results

Feeding on AR or RS supplemented (0.5%) food suppressed polyQ toxicity

The compound eye of the adult fly is a highly ordered array of nearly 800 ommatidial units, each having its own lens and 8 neuronal photoreceptor cells or rhabdomeres. The undifferentiated precursor cells of adult eyes are present in larvae as a pair of eye imaginal discs, which begin to differentiate and form the ommatidial units in an orderly manner during late larval and pupal stages.

It is known that *GMR-GAL4 > UAS-127Q* transgene expression in eye cells severely disrupts the regular arrays of ommatidia due to the polyQ toxicity-induced neurodegeneration[20,28]. We examined the organization of ommatidial units in eyes of one-day-old *GMR-GAL4 > UAS-127Q* flies, fed from the beginning of their larval life on normal or 0.5% AR or 0.5% RS supplemented food, through nail-polish imprints of eye surface or phalloidin staining of the retinal rhabdomeres. Compared to the regular arrays of ommatidia in eyes of wild-type flies reared on regular (Figure 1 *a*) or formulation supplemented food (Figure 1 *b*, *c*), nail-polish imprints of eyes of 75% flies ($N = 67$) expressing *GMR-GAL4 > UAS-127Q* and reared since larval stage on normal food showed near complete absence of the ommatidial arrays (Figure 1 *g*); eyes in the remaining 25% flies appeared slightly better, but the ommatidial pattern was still highly disorganized (not shown). Interestingly, a majority of *GMR-GAL4 > UAS-127Q* expressing flies reared during the larval and adult period on food supplemented with AR (58.34%, $N = 72$) or RS (71.6%, $N = 81$) showed some indication of arrays of ommatidia (Figure 1 *h*, *i*), more so in RS-fed flies. The ommatidial organization in the remaining formulation-fed flies was also better than in any of those reared on normal food.

Phalloidin staining of eyes of wild type flies reared on normal (Figure 1 *d*), or AR or RS supplemented food (Figure 1 *e*, *f*) showed the seven characteristically arranged rhabdomeres (photoreceptors) in each ommatidial unit. However, like the above noted complete disruption of ommatidial arrays, there was a near complete loss of photoreceptor neurons in each rhabdomeric unit in the eyes of all ($N = 17$) freshly emerged *GMR-GAL4 > UAS-127Q* expressing flies reared since the larval life on normal food, so that the phalloidin-positive F-actin formed irregular scattered aggregates (Figure 1 *j*). On the other hand, among those reared on formulation supplemented food since larval period, 43% ($N = 16$) of the AR and 67% ($N = 23$) of the RS-fed flies showed improvement in development of the photoreceptor neurons (Figure 1 *k*, *l*). The presence of at least some phalloidin-positive photoreceptor elements in ordered rows in AR or RS-fed 127Q expressing eyes (Figure 1 *k*, *l*) compared to the randomly distributed phalloidin-positive fragments in flies reared on normal food (Figure 1 *j*) and the presence of 2–3 more intense phalloidin-positive bodies in each cluster in their eyes clearly suggest an improvement in F-actin organization in rhabdomeres following the formulation feeding.

The overall structure of the eye surface and retina appeared significantly better in RS-fed flies (Figure 1 *i*, *l*).

As reported earlier[29], a pan-neuronal expression of the *127Q* transgene using the *elav-GAL4* driver resulted in substantial organismal lethality, mostly during pupal differentiation, so that on normal food only about 8% of eggs ($N = 200$ from 8 replicates of 25 larvae each) reached the pharate stage, none of which enclosed. Significantly, AR or RS supplemented food ($N = 200$ from 8 replicates of 25 larvae in each case) allowed 23.5% and 14% respectively, to reach pharate stage and 14.5% and 8% respectively, to actually eclose as flies with normal lifespan.

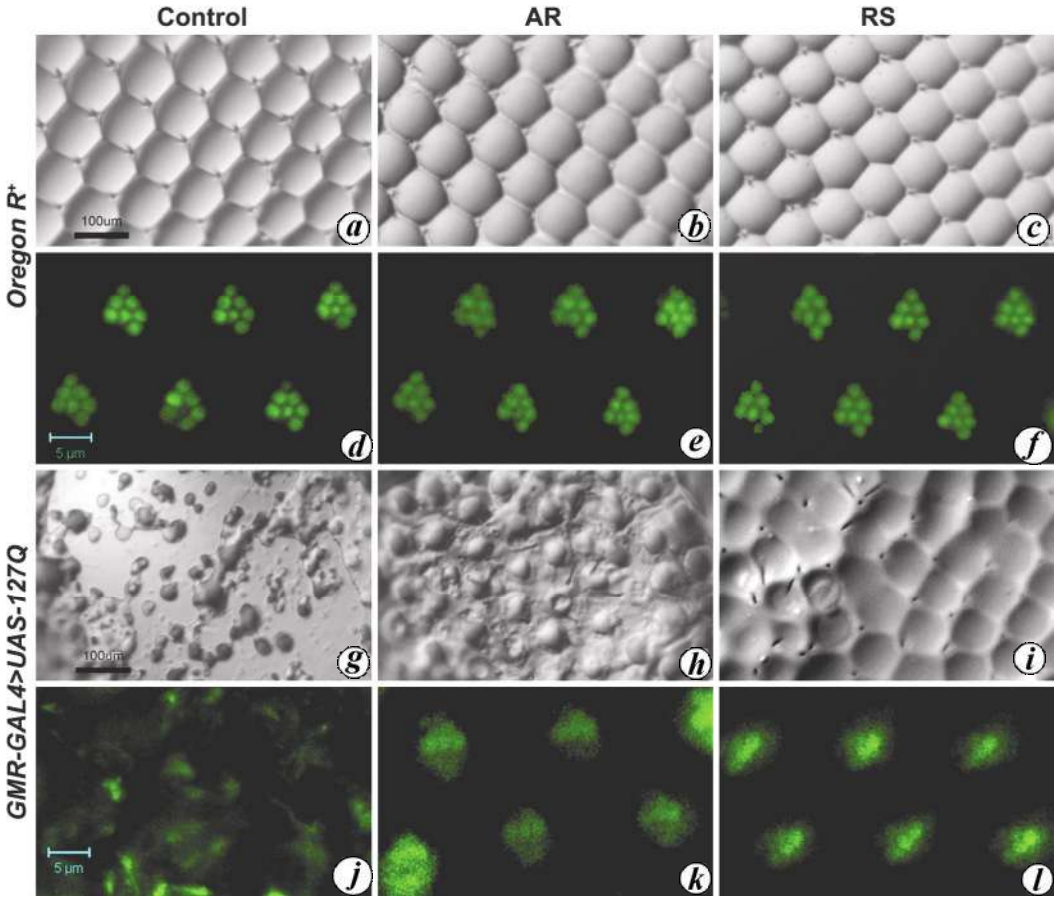

Figure 1. Dietary supplement of AR or RS substantially improved the ommatidial arrays damaged due to expression of *UAS-127Q*. Images of nail-polish imprints (***a***–***c*** and ***g***–***i***) and phalloidin-stained eyes (***d***–***f*** and ***j***–***l***) of one-day-old wild type *Oregon R*$^{+}$ (***a***–***f***) or *GMR-GAL4 > UAS127Q* adults (***g***–***l***) fed on normal (control, ***a***, ***d***, ***g***, ***j***) or AR (***b***, ***e***, ***h***, ***k***) or RS (***c***, ***f***, ***i***, ***l***) supplemented food since the beginning of larval life. Note the improved ommatidial arrays (***h*** and ***i***) and rhabdomeric units (***k*** and ***l***) in formulation-fed flies compared to those in (***g***) and (***j***) respectively. Scale bars in (***a***) and (***g***) correspond to 100 μm, while those in (***d***) and (***j***) correspond to 5 μm and each applies to all the images in that row.

To see if the above suppressive effects of AR or RS are applicable to other polyQ disorders, we used *GMR-GAL4* driver to express the *UAS-httex1p Q93* transgene which mimics the HD phenotype in flies[1,21,29,30]. It is known[1,29] that the *GMR-GAL4 > UAS-httex1p Q93* expressing freshly eclosed flies have near normal eyes and vision but show a progressive age-dependent degeneration, becoming almost completely blind by 10 days, even though the external eye surface of *GMR-GAL4 > UAS-httex1p Q93* expressing flies does not show any appreciable deterioration with age. Pseudopupil images of the rhabdomeres of 5 or 10-day-old *GMR-GAL4 > UAS-httex1p Q93* expressing flies revealed that while all the flies reared on normal food showed severely damaged retina with no detectable rhabdomere-like structures (Figure 2 *a*, *d*), those reared since larval stage on AR or RS supplemented food displayed at least some organized rhabdomere-like structures in 40–50% of 5-day-old flies (Figure 2 *b*, *c*) and 30–40% of 10-day-old flies (Figure 2 *e*, *f*). Interestingly, feeding on formulation-supplemented food during only the larval period also resulted in restoration of rhabdomere organization (Figure 2 *g*–*l*) comparable to that seen after larval as well as adult feeding. Significantly, however, when larvae were reared on normal food and AR or RS-supplemented food was provided after the flies emerged from pupal case, the retinal organization was as disrupted as in flies reared on normal food during larval as well as adult stages (Figure 2 *m*–*r*). This shows that these formulations can suppress neurodegeneration when it is taking place during development, but cannot restore the damage that has already occurred.

Assay for phototaxis, based on their preferential movement to the illuminated arm of a *Y*-maze, revealed, as reported earlier[1,29], that the *GMR-GAL4 > UAS-httex1p Q93* expressing 1-day-old flies reared on normal or formulation-supplemented food showed the expected near normal positive movement towards light (not shown here). However, with age, unlike the continuing positive phototactic behaviour of visually normal wild-type flies, which almost always moved to the lighted chamber, the *GMR-GAL4 > UAS-httex1p Q93* expressing flies reared on normal food became completely blind by day 10 so that

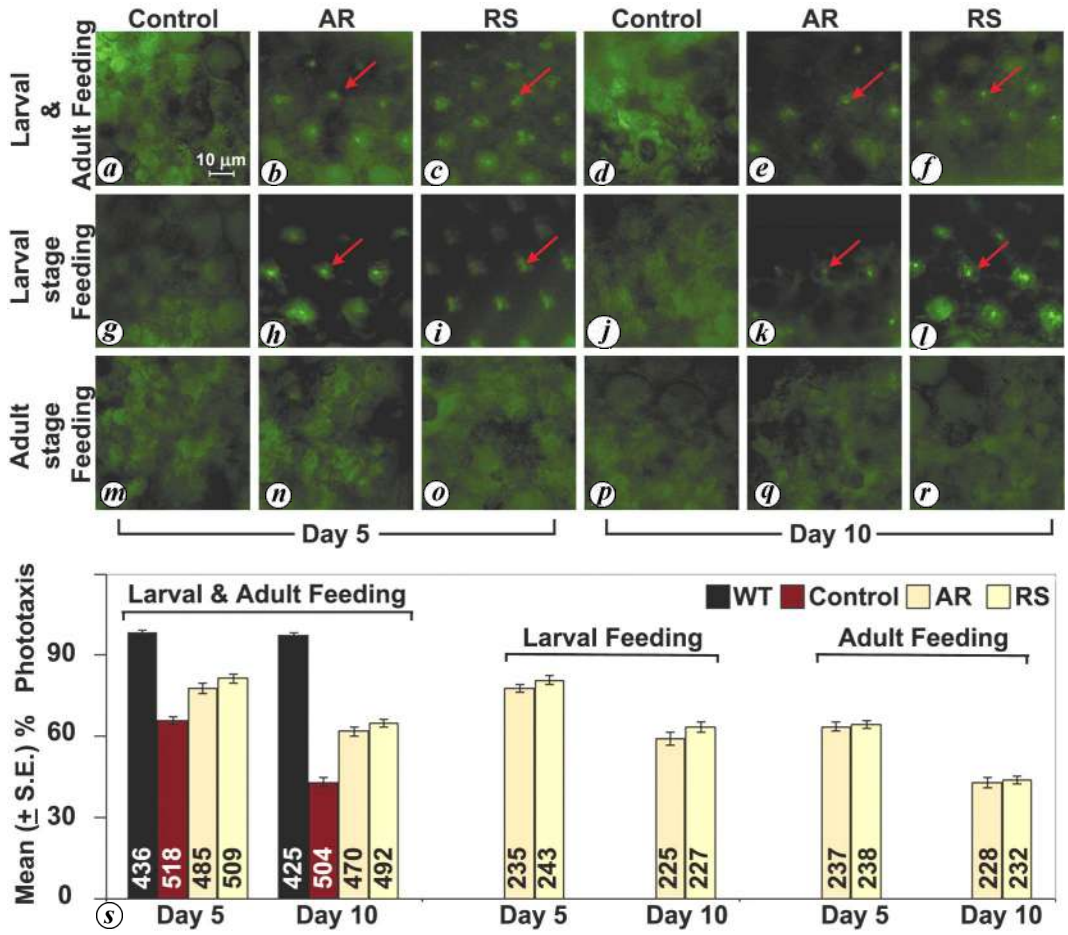

Figure 2. AR or RS feeding suppressed the progressive degeneration of eyes of adult flies expressing *GMR-GAL4 > UAS-httex1p Q93* as seen in the pseudopupil images of eyes (***a–f***, green pseudocolour) on day 5 (***a–c***) or day 10 (***d–f***) following rearing on normal food (control, ***a***, ***d***; *N* = 40 and 32 respectively) or AR (***b***, ***e***; *N* = 38 and 30 respectively) or RS (***c***, ***f***; *N* = 43 and 34, respectively) supplemented food since first instar larval stage; those reared on normal food showed severe disruption of rhabdomeres already on day 5, while those reared on AR or RS-supplemented food showed some rhabdomere-like structures (red arrows) even on day 10. A comparable improvement of ommatidial organization was seen in flies that received the AR (***h*** and ***k***) or RS (***i*** and ***l***) supplemented food only during larval period (*N* for control = 27, AR = 22 and RS = 29 for day 5 and control = 28, AR = 24 and RS = 21 for day-10 samples). Rearing of larvae on normal food and providing AR (***n*** and ***q***) or RS (***o*** and ***r***) supplemented food only from the day of fly emergence did not bring about any improvement in rhabdomere organization (*N* for control = 27, AR = 21 and RS = 25 on day 5 and control = 27, AR = 24 and RS = 28 on day 10). ***s***, Age-dependent loss of phototaxis in *GMR-GAL4 > UAS-httex1p Q93* flies is partially suppressed by larval feeding on formulation-supplemented food as seen in histograms of mean frequencies (± SE) of positively phototactic flies (*Y*-axis) fed on normal (control) or formulation supplemented food during larval and/or adult stages (feeding regime is shown above the histogram bars) on day 5 or day 10 (*X*-axis); frequencies of positively phototactic wild type (WT) flies reared on normal food are also shown for comparison. The number of flies examined for each data point is indicated within the frequency bars. The same set of flies was examined on day 5 and day 10 in each case, but since a few flies died in between, the numbers on day 10 were lesser than on day 5.

they moved randomly between the illuminated and dark chambers of the *Y*-maze (Figure 2 *s*). In contrast, more than 60% of the *GMR-GAL4 > UAS-httex1p Q93* expressing flies reared from first instar stage onwards on AR or RS supplemented food moved to the illuminated arm (Figure 2 *s*), indicating retention of some degree of functional rhabdomeres. The phototactic response of *GMR-GAL4 > UAS-httex1p Q93* flies reared on AR or RS-supplemented food only during the larval period was comparable to that of *GMR-GAL4 > UAS-httex1p Q93* flies that received the formulation-supplemented food during larval as well as adult stages. However, *GMR-GAL4 > UAS-httex1p Q93* flies that were fed on the supplemented food only after emergence from pupal case behaved like those that were reared all through on normal food (Figure 2 *s*).

Dietary AR or RS improved retinal organization, suppressed inclusion bodies and apoptosis

It is known[3,29,30] that *GMR-GAL4 > UAS-127Q* or the *GMR-GAL4 > UAS-httex1p Q93* transgene expression leads to accumulation of polyQ IBs posterior to the

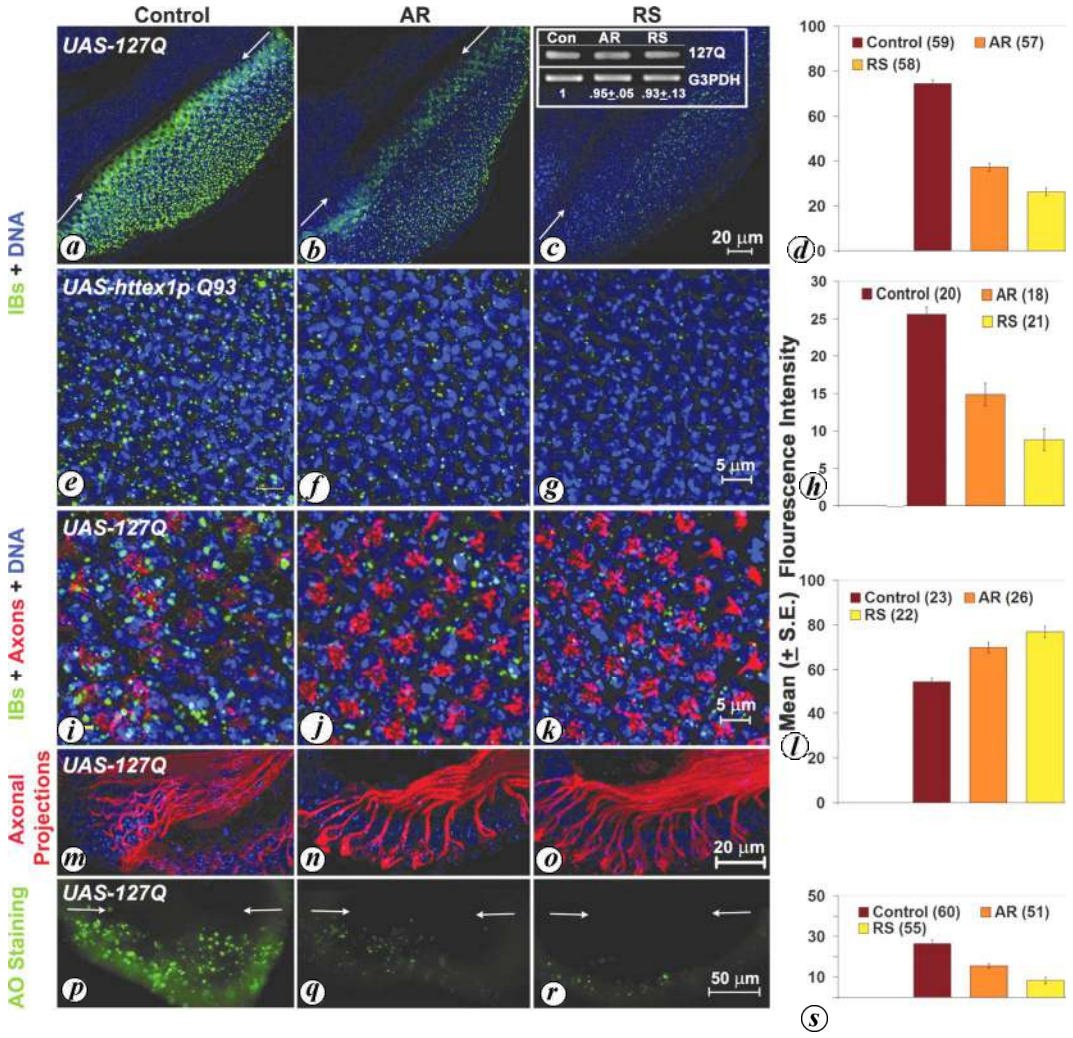

Figure 3. Formulation feeding reduced the accumulation of polyQ IBs (green, ***a***–***c***, ***e***–***g***), damage to rhabdomeric axons (red, ***i***–***o***) and cell death (***p***–***r***) in poly Q expressing eye discs. The specific polyQ transgene expressing in each case (*UAS-127Q* or *UAS-httex1p Q93*) is noted on the top left corner in column 1 of the row. Compared to normal food (***a***, ***e***), feeding on AR (***b***, ***f***) or RS (***c***, ***g***) substantially reduced the polyQ aggregates (***a***–***c***, ***e***–***g***) and improved the disarrayed axonal projections (immunostained with mab22C10, red) in the optic stalk (***i***–***o***). Images ***i***–***k*** show higher magnification confocal projections of four middle *z*-axis optical sections of eye discs immunostained for polyQ IBs (green) and axons (mab22C10 in red). Images ***m***–***o*** show axonal projection (mab22C10 staining, red) in optic nerve coming out of the *GMR-GAL4 > UAS-127Q* expressing eye discs from larvae reared on normal (***m***), AR (***n***) or RS (***o***) supplemented food. The nuclei were counterstained with DAPI (blue). Scale bars in ***c***, ***g***, ***k***, ***o*** and ***r*** apply to all images in the given row. White arrows in ***a***–***c*** and ***p***–***r*** indicate position of the morphogenetic furrow in eye discs. The inset in (***c***) shows the 127Q (upper row) and G3PDH (lower row) amplicons generated by semi-quantitative RT–PCR with total RNA from larval eye discs from larvae reared on Control, AR and RS-supplemented food (indicated on top of the columns). The values below each column indicate the mean (± SE, $N = 3$) levels of polyQ transcripts relative to that in control sample, which was taken as 1.0. ***p***–***r***, Compared to normal food (***p***), rearing on AR (***q***) or RS (***r***) significantly reduced apoptotic cell death as revealed by AO-stained live eye discs expressing *GMR-GAL4 > UAS-127Q*.. The scale bar (50 µm) in (***r***) applies to ***q***–***s***. Histograms in (***d***), (***h***) and (***s***) represent the mean (± SE) fluorescence intensities (measured in arbitrary fluorescence units) of polyQ IB (***d***), mab22C10 (***h***) and AO (***l***) staining in *GMR-GAL4 > UAS-127Q* expressing eye imaginal discs of late third instar larvae reared on different feeding regimes; numbers in parentheses after the bar legends indicate the figures of eye discs examined for each data point.

morphogenetic furrow in late third instar larval eye discs (Figure 3 *a*, *d*). The polyQ IBs were significantly reduced in eye discs from *GMR-GAL4 > UAS-127Q* (Figure 3 *b*, *c*) or *GMR-GAL4 > UAS-httex1p Q93* (Figure 3 *f*, *g*) expressing larvae that were reared on AR or RS-supplemented food, more so in RS-fed larvae (Figure 3 *c*, *g*). A quantitation of the polyQ immunofluorescence intensity in eye discs of each genotype confirmed that the accumulation of IBs in AR and RS-fed larvae was significantly reduced when compared to those reared on regular food (Figure 3 *d*, *h*).

In order to know if the greatly reduced polyQ IBs in formulation-fed samples were due to reduced transcription, levels of 127Q transcripts were measured through semi-quantitative RT–PCR using RNA isolated from *GMR-GAL4 > UAS-127Q* expressing larval eye discs of

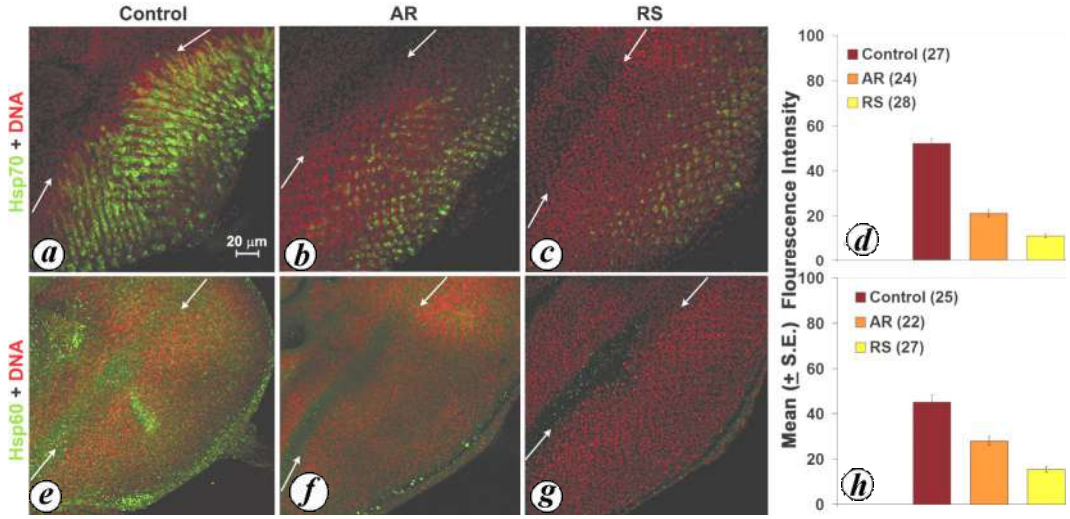

Figure 4. Compared to normal food (***a***, ***e***), feeding on AR (***b***, ***f***) or RS (***c***, ***g***) resulted in reduced levels of Hsp70 (***a–c***, green) and Hsp60 (***e–g***, green) in *GMR-GAL4 > 127Q* expressing larval eye discs. DAPI stained nuclei are shown in red (***a–g***). White arrows indicate position of the morphogenetic furrow in eye discs. The scale bar in (***a***) corresponds to 20 μm and applies to all the confocal projection image panels. Histograms in (***d***) and (***h***) show the mean (± SE) fluorescence intensities (in arbitrary fluorescence units) of Hsp70 and Hsp60 respectively, in *127Q* expressing larval eye imaginal discs under different feeding regimes; numbers in parentheses after the bar legends indicate the number of eye discs examined for each data point.

late third instar larvae reared on different feeding regimes. It was seen (inset, Figure 3 *c*) that the transcriptional activity of *GMR-GAL4 > UAS-127Q* transgene was not affected by feeding on any of the formulations since levels of polyQ transcripts in formulation-fed and normally fed samples remained similar (inset, Figure 3 *c*). This shows that the reduced load of polyQ IBs following AR or RS feeding is not due to reduced transcriptional activity of the *UAS-127Q* transgene.

In order to see if the reduced polyQ accumulation following the AR or RS feeding was also accompanied by restoration of ommatidial integrity and the axons projecting from rhabdomeres to the optic lobe in the brain, we co-immunostained *GMR-GAL4 > UAS-127Q* eye discs from larvae reared on normal or AR or RS-supplemented food with anti-HA (for polyQ IBs) and mab22C10 antibody, which specifically identifies the axons in fly retina (Figure 3 *i–k*). These clearly showed that along with the reduction in levels of polyQ IBs in eye discs from larvae reared on AR or RS-supplemented food, the differentiating arrays of ommatidial units were remarkably better organized, each with intact axons projecting out from the central region of each of the rhabdomeric complexes (Figure 3 *j*, *k*). Together with the disarrayed rhabdomeric complexes, the axons were also irregular or often missing in *GMR-GAL4 > UAS-127Q* eye discs from larvae reared on normal food (Figure 3 *i*). Quantitation of the mab22C10 fluorescence intensity in these discs also confirmed that there were more axons in the formulation-fed larval eye discs than in those from normally fed *GMR-GAL4 > UAS-127Q* larvae (Figure 3 *l*). The axonal projections from the rhabdomeres were also followed in the optic nerve from eye disc to optic lobe in the brain by immunostaining with mab22C10 antibody (Figure 3 *m–o*). In wild-type eyes, the axonal projections from different photoreceptor cells in each ommatidial unit follow a regular order with all the fibres running in a parallel pattern in the optic nerve (not shown, but see refs 27 and 28). It was clear that the axonal projections too were irregularly wavy and disarrayed in *GMR-GAL4 > UAS-127Q* larvae that were reared on normal food (Figure 3 *m*), while those reared on AR or RS-supplemented food showed an orderly arrangement of the axonal projections in the optic nerve (Figure 3 *n*, *o*) similar to that in the wild-type[27,28]. The polyQ IBs were not seen along the length of axons.

Since a high incidence of apoptosis is seen in eye disc cells expressing *GMR-GAL4 > UAS-127Q* transgene[30], we performed acridine orange (AO) staining of live 127Q expressing eye discs from larvae fed on normal or AR or RS-supplemented food to identify the apoptotic cells. This revealed a significant reduction in the incidence of apoptosis in differentiating eye discs from formulation-fed larvae (Figure 3 *q*, *r*) in comparison to control (Figure 3 *p*). Comparison of the fluorescence intensity of AO-stained eye discs confirmed that rearing on AR or RS formulation supplemented food (Figure 3 *s*) resulted in a significant reduction in cell death.

It is notable that RS feeding provided more pronounced suppressive effects in all these cases.

AR or RS feeding reduced the induction of Hsp70 and Hsp60 in 127Q expressing eye disc cells

As reported earlier[28,29], accumulation of IBs is accompanied by elevated levels of Hsp70 (Figure 4 *a*) and Hsp60 (Figure 4 *e*) in the polyQ-expressing eye discs. The

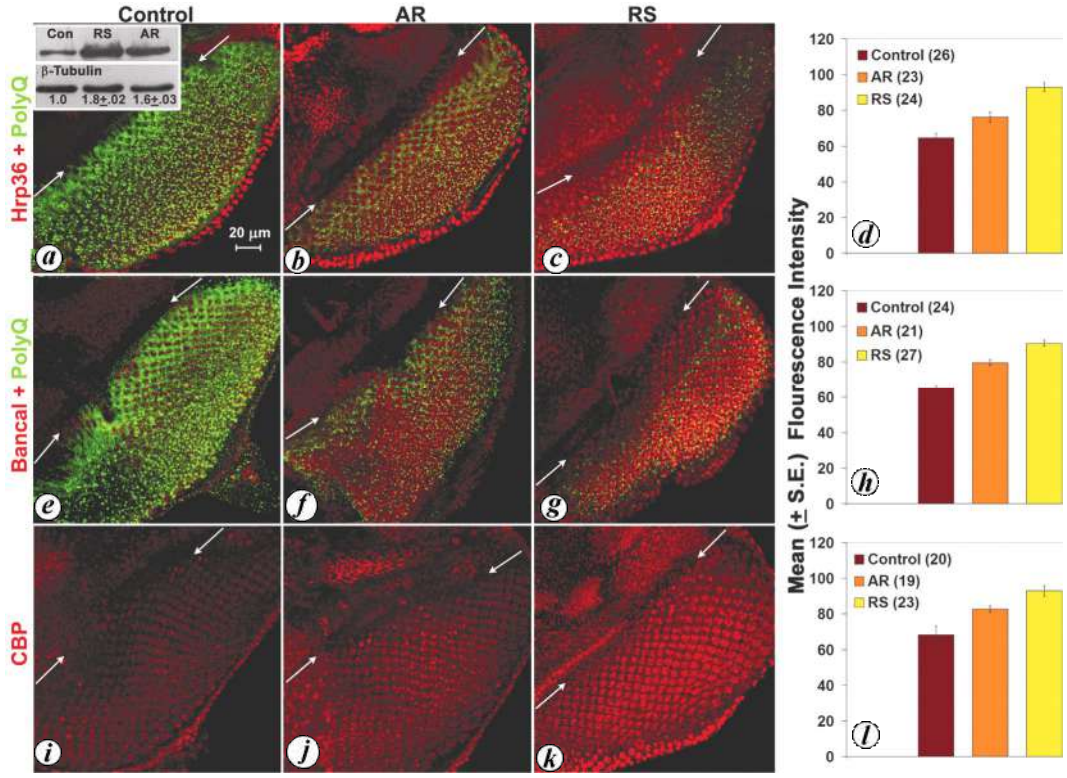

Figure 5. AR or RS-formulation feeding elevated cellular levels of Hrp36 (red, ***a–d***), Bancal (red, ***e–h***) and CBP (red, ***i–l***) along with the reduction in accumulation of polyQ IBs (green, ***a–g***) in *GMR-GAL4 > 127Q* expressing larval eye discs. The discs in (***i–k***) are immunostained only for CBP. The feeding regime, normal (control) or AR or RS-supplemented food, is indicated above each column of the confocal projections (***a–c***, ***e–g***, ***i–k***) of eye discs immunostained for the protein noted on the left of a row. White arrows indicate position of the morphogenetic furrow in eye discs. The scale bar in (***a***) corresponds to 20 μm and applies to all the confocal projection image panels. The inset in (***a***) is a Western blot of total protein from eye discs of 127Q expressing larvae reared on normal (control) or RS or AR-supplemented food to show the relative levels of Hrp36 (upper row in inset in ***a***); *β*-tubulin (lower row) was used as loading control; the values below each column indicate the mean (± SE, $N = 3$) levels of Hrp36 relative to those in control sample, which was taken as 1.0. Histograms in (***d***), (***h***) and (***l***) represent the mean (±SE) fluorescence intensities (in arbitrary fluorescence units) of Hrp36 (***d***), Bancal (***h***) and CBP (***l***) respectively, in *127Q* expressing larval eye imaginal discs following different feeding regimes; numbers in parentheses after the bar legend indicate the number of eye discs examined.

reduced levels of 127Q IBs in eye discs from AR or RS fed larvae (Figure 3 *b*, *c*) were paralleled by a substantial reduction in immunostaining for Hsp70 (Figure 4 *b*, *c*) as well as Hsp60 (Figure 4 *f*, *g*). Compared to AR (Figure 4 *b*, *f*), RS feeding (Figure 4 *c*, *g*) resulted in a greater reduction in immunostaining for Hsp70 as well as Hsp60. This is further confirmed by a quantitation of fluorescence intensities in eye discs immunostained for Hsp70 (Figure 4 *d*) or for Hsp60 (Figure 4 *h*).

AR or RS feeding enhanced levels of Hrp36, Bancal and CBP

Earlier studies[10] and other unpublished data have shown that dietary AR or RS significantly enhances the levels of various hnRNPs and CBP in different wild-type larval tissues and since levels of these proteins are known to modulate polyQ toxicity[1,30], we examined the cellular levels of two hnRNPs, viz. Hrp36 (hnRNP-A homolog) and Bancal (hnRNP K homolog) and CBP in *GMR-GAL4 > UAS-127Q* expressing eye discs in larvae reared on normal food and those reared on AR or RS-supplemented food. Immunostaining with appropriate antibody and fluorescence intensity values showed that compared to normally fed larvae (Figure 5 *a*, *e* and *i*), dietary supplement of either of the formulations resulted in significant increase in cellular levels of Hrp36 (Figure 5 *b–d*) and Bancal (Figure 5 *f–h*). The increase was more apparent in RS-fed larval eye discs (Figure 5 *c*, *d*, *g*, *h*). Co-immunostaining with antibody against polyQ also revealed that the increase in the cellular level of these hnRNPs following formulation feeding is associated with a reduction in the accumulation of IBs (Figure 5 *a–h*). Increase in levels of Hrp36 was further confirmed by Western blotting and in this case too, RS-fed larval samples showed a greater increase (inset, Figure 5 *a*).

Immunostaining for CBP/p300 in *GMR-GAL4 > UAS-127Q* expressing discs (Figure 5 *i–l*) showed that AR or RS feeding significantly enhanced the levels of CBP, more so in RS-fed samples (Figure 5 *k*, *l*).

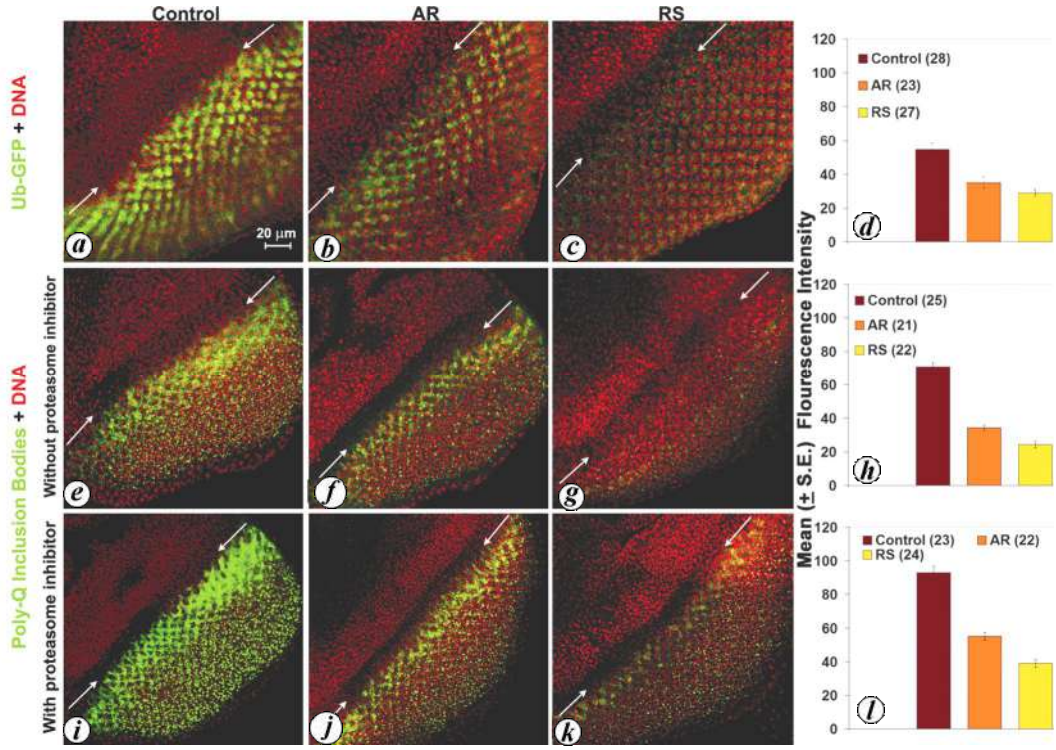

Figure 6. AR or RS feeding improved UPS activity in *–127Q* expressing eye discs. The feeding regime, normal (control) or AR or RS-supplemented food, is indicated above each column of the confocal projections of four mid *z*-axis optical sections of eye discs showing Ub-GFP (green, ***a***–***c***) or polyQ IBs (green, ***e***–***g*** and ***i***–***k***). ***e***–***g***, Confocal projection images of polyQ IBs (green) in *127Q* expressing larval eye discs without the 2 h *in vitro* exposure to proteasome inhibitors. ***i***–***k***, Confocal projection images of polyQ IBs in *127Q* expressing larval eye discs exposed to the proteasome inhibitors. DAPI-stained nuclei are shown in red (***a***–***k***). White arrows indicate position of the morphogenetic furrow in eye discs. The scale bar in (***a***) corresponds to 20 µm and applies to all the confocal projection image panels. Histograms in (***d***), (***h***) and (***l***) represent the mean (± SE) fluorescence intensities (in arbitrary fluorescence units) of Ub-GFP, polyQ IBs without and after treatment with proteasome inhibitor respectively, in *127Q* expressing larval eye imaginal discs following different feeding regimes; numbers in parentheses after the bar legends indicate the number of eye discs examined for each data point.

AR or RS feeding improved proteasome activity

The UPS activity, involved in degradation and clearance of unwanted proteins in cells[28], is compromised in the affected neuronal cells in polyQ/HD and AD, leading to enhanced accumulation of pathogenic protein[1,6]. Therefore, we examined the UPS activity in *GMR-GAL4 > UAS-127Q* expressing eye discs using the *UAS-Ub^{G76V}-GFP* transgenic line[26] in which the GFP is tagged with ubiquitin so that under conditions of compromised UPS activity, GFP fluorescence persists. As expected because of the compromised UPS activity, eye discs of normally fed *GMR-GAL4 > UAS-127Q* expressing larvae showed high levels of GFP fluorescence (Figure 6 *a*). However, in AR or RS-fed larval eye discs, the GFP fluorescence was significantly reduced, especially in RS-fed samples (Figure 6 *b–d*).

In order to further assess whether the improved UPS is indeed playing a role in reducing the accumulation of IBs and disappearance of *UAS-Ub^{G76V}-GFP* fluorescence, *GMR-GAL4 > UAS-127Q* expressing eye discs from differently fed late third instar larvae were incubated *in vitro* for 2 h in a medium containing proteasome inhibitor prior to immunostaining for polyQ IBs. As expected, the accumulation of IBs was much higher in discs from normally fed larval eye discs in which the proteasomal activity was inhibited for 2 h (Figure 6 *i*, compare it with Figure 6 *e*). Interestingly, however, the accumulation of IBs even in the presence of proteasome inhibitor was much less in discs from AR (Figure 6 *j*, *l*) or RS (Figure 6 *k*, *l*)-fed larvae, although they were slightly more abundant than in discs which were not exposed to the proteasomal inhibitor (Figure 6 *f*, *g*). Taken together, these results confirm that AR or RS feeding indeed improves the proteasomal activity.

AR or RS suppresses eye damage and accumulation of amyloid plaques in AD

In order to see if the protective effects of AR or RS feeding extend to AD associated with formation of amyloid plaques[5,6], we examined adult eye phenotypes (Figure 7 *a–c*) and accumulation of amyloid plaques in third

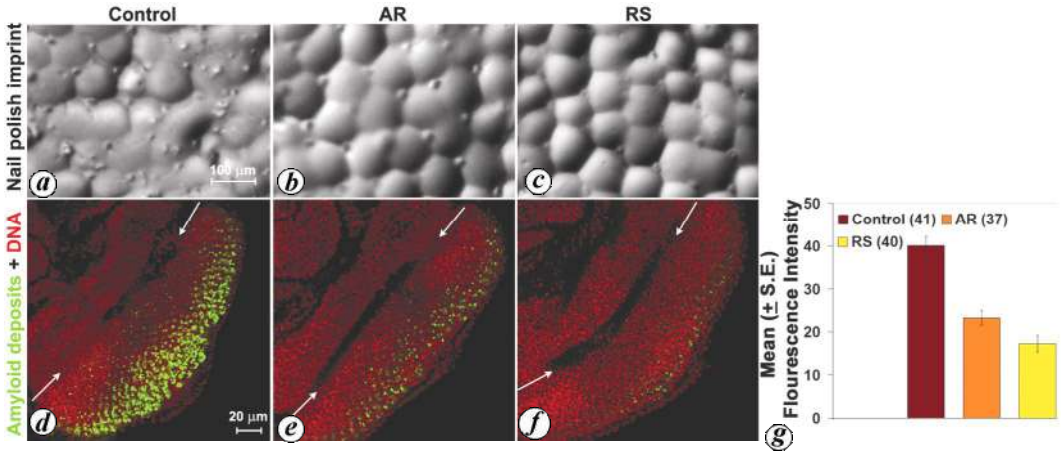

Figure 7. Dietary AR or RS reduced damage to eyes in *GMR-Aβ42* adult flies together with reduction in amyloid plaques. ***a–c***. Nail-polish imprints of eyes of 1-day-old *GMR-Aβ42* flies reared on normal (Control, ***a***) or AR (***b***) or RS (***c***) supplemented food. ***d–f***, Projections of four mid *z*-axis confocal optical sections showing amyloid deposits (green) in eye discs from larvae reared on normal (***d***) or AR (***e***) or RS (***f***) supplemented food. DAPI-stained nuclei are shown in red. White arrows indicate position of the morphogenetic furrow in eye discs. Scale bars in (***a***) (100 µm) and (***d***) (20 µm) apply to ***a–c*** and ***d–f*** respectively. Histogram in (***g***) shows the mean (±SE) fluorescence intensities (in arbitrary fluorescence units) of amyloid deposits in eye discs from differently fed larvae; numbers in parentheses after the legend bars indicate the number of eye discs examined in each case.

instar larval eye discs (Figure 7 *d–f*) of *GMR-Aβ42* (ref. 22) expressing flies/larvae reared on different feeding regimes. We found that damage to ommatidial arrays in flies expressing four copies of *GMR-Aβ42* transgene was substantially reduced when larvae were reared on food supplemented with AR or RS, so that a majority of *GMR-Aβ42* expressing flies reared on the formulation-supplemented food showed (67.22%, $N = 49$ in AR-fed and 71.23%, $N = 41$ in RS-fed) near normal or only mildly damaged eyes (Figure 7 *b* and *c*) when compared with those of control ($N = 40$). Immunostaining for the amyloid plaques in third instar eye imaginal discs and comparison of their respective fluorescence intensities revealed that unlike the very high levels of amyloid plaques in eye discs from *GMR-Aβ42* larvae reared on normal food (Figure 7 *d*), accumulation of plaques was greatly reduced in those from the formulation-fed larvae, especially those reared on RS-supplemented food (Figure 7 *e–g*). Thus, as in the case of the polyQ toxicity, dietary AR or RS suppresses AD pathology, with RS being more effective.

Discussion

A few earlier studies have examined efficacy of Ayurvedic and other herbal/traditional formulations in ameliorating neurodegenerative disease phenotypes[35–40]. However, most of these studies used individual constituents ('active principle') rather than the complete traditional formulation. As noted earlier[9,10,41], search for the so-called 'active principle' in an Ayurvedic formulation defies the holistic concept of Ayurveda which prescribes specific combinations of the herbal/organo-metallic components and *Anupana* for different ailments since different combinations are believed to have specifically varying effects. Therefore, we used the complete formulations as prepared for human consumption. As noted in our earlier studies with wild-type flies[10], the mercury-sulphide based RS had no toxic effects in the expanded polyQ or Aβ42 expressing genotypes as well. On the other hand, it has been seen (V. Dwivedi and S. C. Lakhotia, unpublished) that feeding on fly food supplemented (0.5% w/v) with dried *Kajjali*, an intermediary product which is subsequently sublimed at 600°C to produce the final RS[10], caused substantial developmental delay in wild type and more so in the polyQ expressing larvae. This confirms that the complex preparatory processes are necessary for activity of the Ayurvedic formulations and converting compounds like mercury sulphide into non-toxic but active formulation[42].

The substantial improvements in the different neurodegeneration phenotypes, viz. eye morphology, formation and organization of rhabdomeres, phototaxis, inclusion bodies/amyloid deposits, enhanced levels of Hsp70 and Hsp60, etc. following rearing on the AR or RS-supplemented diet clearly show that these two traditional Ayurvedic formulations effectively suppress neurodegeneration in fly models of polyQ toxicity and AD. The absence of a complete recovery in the eye phenotype of adult flies may be because while there was no intake of formulation during the 4–5 days of non-feeding pupal period between the larval and adult stages, the expanded polyQ or the amyloid protein continued to be synthesized in developing pupal eyes and cause neuronal damage. Our observation that feeding on formulation-supplemented diet during the adult stage only does not have any suppressive effect (Figure 2) and also indicates that these Rasayanas suppress neurodegeneration when it is being

inflicted, but do not restore the lost neurons in the fly model. This may partly be related to the fact that cell division in somatic cells is completely absent in adult flies, except in certain specific stem cells and, therefore, the neural cells that are lost during development because of the polyQ or mutant Aβ toxicity cannot be regenerated.

Abundance of the polyQ or amyloid aggregates is generally indicative of the degree of neurodegenerative manifestation. The significantly reduced accumulation of 127Q or the amyloid aggregates following the AR or RS feeding suggests that either these formulations inhibited the synthesis of the toxic proteins or facilitated clearance of the toxic proteins. Since we found the levels of polyQ transcripts in formulation-fed larvae to be similar to those in normally fed larvae, we believe that these formulations reduce the accumulation of IBs through clearance of toxic proteins rather than inhibiting transcription of the *polyQ* transgenes. The improved proteasomal activity in formulation-fed larval tissues is likely to be responsible for the significant reduction in polyQ IBS or the amyloid plaques through more efficient proteolysis.

Each of the two Ayurvedic formulations also enhanced cellular levels of hnRNPs and CBP, which are well known to suppress polyQ or amyloid plaque toxicity[1,14,17–20,30]. In another study (V. Dwivedi *et al.*, unpublished), it has been found that AR or RS supplement significantly suppressed induced apoptosis in eye discs caused by expression of the pro-apoptotic Reaper, Grim or Hid proteins. In the present study also we found a substantial suppression of apoptosis in *GMR-GAL4 > UAS-127Q* expressing discs from formulation-fed flies. Thus, besides the protection offered by elevated levels/activity of hnRNPs, CBP, 26S proteasomal components, inhibition of induced apoptosis by the dietary AR or RS also appears to contribute to suppression of neurodegeneration caused by polyQ or Aβ42 toxicity. It is significant that the generally greater suppressive effect of RS feeding in polyQ and AD models is paralleled by a greater enhancement in the levels of hnRNPs, CBP and UPS activity and a greater inhibition of apoptosis. An important role of hnRNPs in mediating the suppression of neurodegeneration by AR or RS feeding is supported by our other observations (V. Dwivedi and S. C. Lakhotia, unpublished) that if the Hrp36 is reduced or completely absent because of genetic mutation[43], neither of the Rasayanas brings about any suppression of the neurodegeneration caused by 127Q or Htt-ex1P Q93 toxic proteins. Further studies are needed to understand the pathways through which the dietary AR or RS elevate levels of hnRNPs, CBP and UPS activity. It also remains to be seen if any or both of these formulations affect the lysosomal activity as well, since autophagy too is involved in clearance of the toxic polyQ or amyloid aggregates[6,44–46].

Although we did not examine the levels of different proteins in the HD model, we believe that the AR or RS-induced changes seen in the 127Q model apply to this model as well since many previous studies have shown that the conditions that aggravate or ameliorate the polyQ toxicity have similar actions in the HD model[1,5].

The reactive oxygen species (ROS) are known to be significant causative factors in the neurodegenerative disorders[47,48]. Amalaki extracts are known to have very high antioxidant activity[49–52]. As expected, AR-fed larvae have also been found to display improved oxidative stress tolerance (V. Dwivedi and S. C. Lakhotia, unpublished). Thus boosting of oxidative stress tolerance may be an additional path through which dietary AR may ameliorate neurodegeneration. Although 'Makardhwaja', which like RS is a mercury-containing Ayurvedic preparation, is reported to significantly improve the oxidative stress scavenging system[53], we did not find any improvement in oxidative stress tolerance in RS-fed wild type larvae/flies (V. Dwivedi and S. C. Lakhotia, unpublished). This suggests that scavenging of ROS by RS may not be a contributing factor, but the greater enhancement in levels of hnRNPs, CBP and UPS activity following RS feeding may explain its greater suppressive effect on neurodegeneration in polyQ as well as AD than that of AR.

The traditional Ayurvedic literature does not appear to specifically indicate use of either of the two formulations for the polyQ or amyloid toxicity. It is quite likely that such ailments were not specifically identified in ancient times. However, these Rasayanas are indicated to generally improve health and brain functions, especially during ageing. Mercury-based *Bhasma* like the RS has been considered in traditional literature[54,55] as *Maharasa*, which promotes good physique, stable mind and good vision, improves memory and cures all diseases. RS has also been shown to significantly improve behaviour of geriatric dogs[53]. It has been reported[56] that WSHFD, a traditional Chinese drug containing 10% Cinnabar (HgS) and 10% Realgar (As_2S_4) together with certain plant products, exerts protection against LPS-induced neurotoxicity via the inhibition of microglial activation and the production of pro-inflammatory factors. This study[56] further showed that Cinnabar and Realgar in WSHFD are critical for the neuro-protection since removal or reduction of either of them rendered the treatment ineffective. Apparently, the mercury present in these traditional formulations is rendered non-toxic by the specific steps required for their preparation.

The multiple and varying phenotypes in patients of inherited neurodegenerative disorders due to mutation in a specific single gene, and the existence of a large number of genetic inter actors and possible therapeutic agents for such single gene defect disorders reflect the complexity of the underlying networks[1,5,31]. Unlike the target-specific chemicals/drugs which often have desirable therapeutic as well as undesirable side-effects, the Ayurvedic formulations containing a complex mix of mostly molecules of biological origin are likely to have more balanced effects on the systems biology of the body and thus help achieve

homeostasis[9,42]. Further, most of the oral Ayurvedic formulations are administered with one or more vehicle material/s or *Anupana*, which improve the main drug's acceptability and absorption of the main drug, besides acting as antidote. Our earlier studies[10] indeed showed that in agreement with the principles of Rasayana therapy[8,54], AR or RS supplements affect multiple pathways and thereby, each formulation improves homeostasis and general health. We believe that such multi-pronged actions of these Rasayanas provide a balanced defence to neuronal cells against the toxic protein aggregates.

The present study extends the beneficial effects of traditional Ayurvedic formulations in suppressing inherited neurodegenerative disorders. Since studies on fly models for diverse human neurodegenerative diseases have contributed significantly to our understanding of the genetic and cellular bases of these inherited disorders[1,2,5,57], it is expected that further studies on the Ayurvedic formulations in other model systems will be useful in developing them as convenient therapeutic formulations for combating the increasing burden of neurodegenerative disorders[9].

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Case Report

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AYURVEDIC MANAGEMENT OF RHEUMATOID ARTHRITIS: A CASE REPORT

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INTRODUCTION

The term '*Amavata*'is made up of two words *'Ama'*and '*Vata'*(Vitiated *vata dosha*) which are predominant pathological factors responsible for this disease Among *Ayurveda acharayas*, *Madhavkara* (700A.D) was the first one to describe the pathogenesis and features of *amavata* in Madhavnidana as amavata is caused by impairement of agni formation of ama and vitiation of vata and *ama* is being carried by vitiated *vata dosha* to *shleshmasthanas* (like joints etc.) producing features like *gatrastabdhata* (bodystiffness) *angamarda*(bodyache) *aruchi*(loss of appetite), *sandhiruk* (painful joints)and *sandhi shotha* (swollen joints) The treatment of *Amavata* was first explained by *Acharaya Chakradutta*.[2]

According to the clinical features described in *ayurveda*, *Amavata* very closely resembles with the Rheumatoid arthritis. Rheumatoid arthritis is a chronic, progressive autoimmune arthropathy and characterized by bilateral symmetrical involvement of joints with some systemic clinical features. Treatment in allopathic medicine involves NSAIDS and steroids, which carry their own collateral burden of Side effects in the long term whereas *ayurveda* treats root cause of *Amavata* (RA) as well as it has no side effects and patient get very good relief. The Ayurvedic treatment is safer, cost effective and traditionally proven. *Ayurveda* being India's traditional system of Healthcare has a wide pharmacopeia deriving from natural and organic sources including plants, minerals, mineral salts, marine salts. *Acharya Chakradatta* described the *Chikitsa Siddhant* for *Amavata*. It includes *Langhana*, *Swedana* and use of drugs having *Tikta*, *Katu Rasa* with *Deepana* property, *Virechana*, *Snehapana* and *kshara Vasti*. Here a case of *Amavata* was treated by using *virechan Chikitsa* given in this *Chikitsa Sutra*.

CASE REPORT

A 29 year old female patient visited *panchkarma* OPD of R.G.G.PG Ayurvedic hospital ,Paprola Distt.- Kangra (H.P) on 3/8/2019 with complaints of severe pain and swelling in wrist joint and interphalangeal joints of bilateral hands since 7 months associated with morning stiffness. Pain and stiffness was more in early morning hours. The patient was alright before 7 months and gradually developed the signs and symptoms .She also had associated complaints of loss of appetite, constipation, heaviness of body on and off since 2 months. For above same complaints she took allopathic treatment for about 5 months but did not get satisfactory results and for further management she came to R.G.G.PG Ayurvedic hospital. There was no history of Diabetes, Hypertension or any other major illness in the past.

Clinical Examination (at admission time)

Vitals:-	Personal history :-
B.P-130/80 mm of Hg	Appetite-Decreased
P.R- 78/min	Thirst- Normal
R.R-18/min	Urine- Normal
Temp.- 98.5 F	Stool- constipated

Sleep-Disturbed
Systemic examination
CVS-S1S2 heard
Chest –B/L equal air entry with no added sounds
CNS –patient conscious well oriented to time place person

Local examination revealed

- Swelling present in the interphalangeal joint of index finger ,ring finger and little finger of right hand and index middle and ring finger of left hand respectively. Swelling was of nonpitting type.
- Tenderness was present in all affected joints along with rise in temperature
- Range of movement-Restricted and painful movement of interphalangeal joint of B/L hands.

Grading of assessment of disease.

Pain 0 No pain 1 Pain but tolerable 2 Pain difficult to tolerate and take analgesic once a day 3 Intolerable pain and taking analgesics two times a day 4 Intolerable pain and taking analgesics more than two times a day
Swelling 0 No swelling 1 Feeling of swelling and heaviness 2 Apparent swelling 3 Huge (Synovial effusion)swelling
Tenderness 0 No Tenderness 1 Mild Tenderness 2 Moderate Tenderness 3 Severe Tenderness
Stiffness 0 No stiffness 1 20% limitation of normal range of mobility 2 50% limitation of mobility 3 75% or more reduction of normal range of mobility
Grip power and pressing power 0 = 200 mmHg 1= 198-120 mmHg 2= 118-70mmHg 3= <70 mmHg

Grading of subjective and objective parameters before treatment

Pain -2 Stiffness –1
Swelling-3 Grip power-2
Tenderness -2 Press power-2

Laboratory findings

Hb-9gm%, TLC-8,900/mm^3, FBS-90mg/dl, ESR -60mm at the end of first hour, Urea-15mg/dl, Creatinine-0.3mg/dl RA Factor-Positive, Uric acid-4.9mg/dl,

Treatment

Following Amavata chikitsa sutra [chakradutta 25/1]deepana pachana snehpana and virechan was done followed by shaman chikitsa.

PACHANA AND DEEPAN done by giving Chitrakadi Vati in dose of 2B.D for 2 days.

Snehapana With Panchtikta Ghrita

DAY	Dose
1st day	50ml
2nd day	100ml
3rd day	150 ml
4th day	200ml
5th day	250ml
6th day	300ml
7th day	350ml

After 7th day samyak snighda lakshana appeared and then for 3 consecutive days sarvanga abhyana with Saindhvadi taila followed by sarvanga swedana was done.

Virechana Karma

On 11th day, Virechan karma (Induced purgation) was done with triphala kwath (200ml) and Eranda taila (50ml) pana followed with luke warm water [dose was decided on the basis of rogi bala, roga bala and kostha (bowel habit)]

Some important instructions according to Ayurvedic view were given to the patient during the therapy, those were to avoid cold drinks, ice cream, curds, banana, coconut, black gram, cold water for bathing, sleep in day time. To use luke warm water for bathing.

After getting samyak lakshan of virechan,patient was told to follow samsarjan karma i.e specific diet for 5 days as the sudhi was madhayam.

In follow up following treatment schedule was selected for 15 days:-
Chitrakadi vati 2BD
Simhnaad guggulu 2BD (500mg)
Ashwagandha churna 3gm BD
1tsf Eranda taila with milk at night

RESULT

Patient started getting relief in pain during snehapana only. On first follow up after 15 days treatment there was reduction in stiffness and tenderness of joints and following changes in parameters as :
Pain- 0
Swelling – 0
Tenderness- 0
Stiffness- 0
Grip power – 2
Press power- 2

DISCUSSION

As amavata is caused by impairement of agni, formation of ama and vitiation of vata dosha, so these are the factors which are important to look upon while doing chikitsa of aamvata. First aim should be to bring out Niraamaavastha by giving tikta and katu dravya which act as pachana and deepan dravya.

Once niraamaavastha is attained patient should be subjected to snehana and virechan.

Snehana –Snehana act as Vata shamak, removes mala, and clear sanga caused by ama [CH.SI1/7] It also act as deepan [ch c.chi 15/201].

Virechan therapy helps to normalize the pratiloma gati of vata in aamavata.

Properties of shaman drugs
Simhanada guggulu - It bears the qualities like laghu ruksha ushna and teekshan. Owing to this property antagonism to kapha and ama it brings significant improvement in sign and symptom of disease.

Balya chikitsa –This disease is autoimmune disorder .So Rasyana chikitsa plays very important role to stop or reduce the frequency of occurrence of this disease.

CONCLUSION

From this case study it can be concluded that ayurvedic approach towards amavata shows satisfactory results not only in relieving sign and symptoms but also in frequency and time interval of reoccurrence of this disease.

ORIGINAL ARTICLE

Blood compatibility studies of *Swarna bhasma* (gold *bhasma*), an *Ayurvedic* drug

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ABSTRACT

Swarna bhasma (gold bhasma) preparations are widely utilized as therapeutic agents. However, in vitro biological evaluations of bhasma preparations are needed along with the physicochemical characterization for present day standardization of metallic bhasma preparations to meet the criteria that supports its use. Therefore, an attempt has been made to evaluate the protein adsorption, blood compatibility and complement activation potential of two batches of *Swarna bhasma* preparation, along with its physicochemical characterization. The particle size, morphology, elemental analysis, and in vitro cytotoxicity were evaluated initially. Red blood cell hemolysis, aggregation studies with blood cells, protein adsorption, complement C3 adsorption, platelet activation and tight junction permeability in Caco-2 cell line were investigated. The *Swarna bhasma* preparations with a crystallite size of 28–35 nm did not induce any blood cell aggregation or protein adsorption. Activation potential of these preparations towards complement system or platelets was negligible. These particles were also non-cytotoxic. *Swarna bhasma* particles opened the tight junctions in Caco-2 cell experiments. The results suggest the application of *Swarna bhasma* preparations as a therapeutic agent in clinical medicine from the biological safety point of view.

Key words: Blood compatibility, protein adsorption, *Swarna bhasma*

INTRODUCTION

From as early as 2500 BC, the therapeutic benefits of gold preparations have been reported in Indian, Arabic and Chinese literature.[1] *Swarna* (gold) *bhasma* has been utilized as a therapeutic agent in the traditional Indian *Ayurvedic* medicine for several clinical disorders including bronchial asthma, rheumatoid arthritis, diabetes mellitus, and nervous system diseases.[2-7] *Swarna bhasma* is usually given orally mixed with honey, ghee or milk.

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In recent years, there has been a renewed interest in drug discovery strategies where natural products and traditional medicines are re-emerging as attractive options[8] and hence renewed interests in agents like *Swarna bhasma.* Recent research has revealed that gold nanoparticles exhibit size-dependent absorption through rat skin and intestine, with smaller particles (~15 nm) absorbed more than larger particles (>100 nm).[9] Nanoparticles can also be absorbed through sublingual route directly into the blood stream.[10] Therefore, it can be presumed that some *Swarna bhasma* particles may get absorbed through the sublingual route directly into the blood stream. This has not been experimentally proved for *swarna bhasma*. However, the antioxidant/restorative effects of *Swarna bhasma* against global and focal animal models of ischemia have been reported.[6] Acute oral administration (continuous for 8 weeks on albino mice; 10 mg/20g b.w./day) of *Swarna bhasma* had not reported any toxic effects as assessed by liver function tests and histological investigations.[11]

In modern medicine, gold nanoparticles find significant applications in drug delivery as they are capable of encapsulating active drugs and targeting.[12] Colloidal gold nanoparticles represent a completely novel technology in the field of particle-based tumor-targeted drug delivery. The monolayer of polyethylene glycol (PEG) over gold nanoparticles has been found to improve the cellular internalization

properties.[13] Surface modification of gold nanoparticulate carriers with poly(ethylene glycol) has emerged as a strategy to enhance solubility of hydrophobic drugs, prolong circulation time, minimize non-specific uptake, and allow for specific tumor-targeting.[13] *Swarna bhasma* has been well-characterized physicochemically and since it contains more than 90% of gold particles[14] it may also be therapeutically applied in similar lines like gold nanoparticles. Cellular internalization of *Swarna bhasma* and/or its uptake *via* paracellular pathway have not been established yet.[15] Uptake of nanoparticles can occur not only *via* micro-fold (M)-cells, the highly specialized epithelial cells in the Peyer's patches and isolated follicles of the gut associated lymphoid tissue (GALT), but also across the apical membrane of enterocytes.[15] It has been demonstrated that uptake of gold nanoparticles occurred in the small intestine by absorption through single, degrading enterocytes in the process of being extruded from a villus and gold nanoparticles typically less than 58 nm in size ultimately reaches blood and various organs through blood.[16] Therefore, compatibility with blood is an extremely important factor when these particles are absorbed into the blood stream. Blood compatible materials can be defined as those materials which do not damage blood components when they come in contact with blood.[17] *In vitro* biological evaluations of *bhasma* preparations are also needed along with the physicochemical characterization and clinical evaluation for present day standardization of metallic *bhasma* preparations to meet the criteria that supports its use worldwide.

Therefore, an attempt has been made to study the physicochemical characterization and blood compatibility of two batches of *Swarna bhasma*.

Materials and Methods

Two bottles of *Swarna bhasma* were purchased from The Indian Medical Practitioners Co-Operative Pharmacy and Stores Limited, Chennai, India (*Swarna Bhasma* ED) and designated as SB1 and SB2. Complement protein C3 kit was from Orion Diagnostica, Finland. Platelet factor (PF4) kit, Asserachrom PF4, was from Diagnostica Stago, France. All other chemicals and other reagents used were of analytical reagent grade.

Particle size and zeta potential determination by dynamic light scattering

The particle sizes and the zeta potentials of *Swarna bhasma* samples were analyzed by photon correlation spectroscopy and laser Doppler anemometry, respectively, with a Zetasizer, Nano ZS (Malvern Instruments Limited, UK) at 25°C.[18]

X-Ray diffraction analysis

The X-Ray diffraction (XRD) powder diffraction pattern of *Swarna bhasma* was recorded on X-ray diffractometer (Siemens D5005 Diffractometer) using CuKα radiation, l = 1.5406 Å over the range 30.0–80.0°.

Scanning electron microscopy and energy dispersive spectroscopy

The morphology and elemental composition of the *bhasma* samples were determined by Environmental Scanning electron microscopy (SEM) (FEI Quanta) with energy dispersive spectroscopy (EDAX). A representative portion of each sample was sprinkled onto a double side carbon tape and mounted on aluminum stubs, in order to get a higher quality secondary electron image for SEM and EDAX examination.

In vitro cytotoxicity studies

The L929 fibroblast cells were seeded in 24 well plates at a density of 5×10^5 cells/well, cultured for 24 h in incubator at 37°C under 5% CO_2. The medium was replaced with SB1 and SB2 particle suspension in the medium at a concentration of 5 mg/ml/well and incubated for 20 h. Medium alone was used as control. The particles were removed and 3-(4,5-dimethythiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay was done.

Blood cell aggregation and hemolysis studies

RBCs were separated by centrifuging fresh blood at 700 rpm. This was washed with saline and diluted in saline in a ratio of 1:4. WBCs were isolated by centrifuging the fresh blood after layering with histopaque for 20 min at 700 rpm. Platelet-rich plasma (PRP) was collected by centrifuging the fresh blood at 1000 rpm for 20 min layered on histopaque solution. To 1 mg each of SB1 and SB2 particles, 100 µl of the diluted RBC, WBC suspension or PRP were added and incubated for 30 min at 37ºC. Polyethylene imine (PEI) and saline were taken as positive and negative controls, respectively, for all studies. Aggregations if any were observed through a phase contrast microscope (Leica DM IRB, Germany) at a magnification of 40×. Hemolysis assay was done on the particles as reported elsewhere.[19] Normal saline was used as negative control (0% lysis) and distilled water as positive control (100% lysis). The absorbance was measured at 541 nm by UV–Vis spectrophotometer (Varian).

Protein adsorption studies

The plasma was separated by centrifugation of fresh blood at 700 rpm. Ten milligram each of SB1 and SB2 particles were dispersed in 200 µl of saline. To this 200 µl of plasma was added and incubated for 1 h. After incubation the plasma was separated by centrifugation at 10,000 rpm and diluted with saline. The proteins in the plasma samples before and after incubation were separated by polyacrylamide gel electrophoresis (PAGE) using discontinuous native-PAGE method of Laemmli.[20] A resolving gel of 12% and a stacking gel of 4% were used. Electrophoresis was carried out at 100 V for 90 min using Mini-PROTEAN II electrophoresis cell (Bio-Rad, CA, USA). The gel was digitalized using an image analyzer (LAS 4000, Fuji) and the densitometry scans were

done with the software Multi-Gauge V3.

Complement activation

Complement activation by *Swarna bhasma* was determined by the turbidimetric method, assessing the depletion of complement protein C3 on incubation with the nanoparticles. The particle suspensions (100 µl) were incubated for 1 h at 37°C with 100 µl of citrated blood. The final concentration of the gold *bhasma* particles in the assay system was maintained at 10 µg/ml of blood. The assay was done as per the protocol provided by the kit manufacturer.

Platelet activation

Human blood (5 ml) was collected from consented voluntary donor in the morning hours after 25-min rest with slight or no stasis. It was immediately placed in the ice/water bath for 20 min. Platelet-rich plasma (PRP) was collected by centrifugation at 700 rpm for 20 min. Ten milligram each of *bhasma* samples were incubated with the fresh PRP for 15 min. This was centrifuged at 2500 g for 20 min. Plasma supernatant was collected by aspiration and PF4 was assayed by enzyme-linked immunosorbent assay (ELISA) kit (Diagnostica Stago, France) according to manufacturer's instructions. Samples were assayed in duplicates. PF4 levels were expressed in IU/ml. Precision of the assay was ±0.7 UI/ml in replicate determinations.

Visualization of tight junctions-actin and zona occludens 1(ZO-1)

Caco-2 cells were seeded (at 20,000 cells/well) onto four well cell culture plates (Nunc). The cells were maintained in incubator at 37°C under 5% CO_2 and used for transport experiments 6 days post-seeding.[21] Medium was replaced with Hank's buffered salt solution (HBSS) transport medium, and cells were equilibrated at least for 2 h before uptake experiments. Cells were treated with 500 µl SB particles at a concentration of 10 mg/ml for 1 h. The particles were removed by washing the cells three times with phosphate-buffered saline (PBS). The cells were fixed with 250 µl of 4% paraformaldehyde for 20 min at room temperature. Then the cells were permeabilized using 0.2% Triton X-100 in blocking solution, made of 1% (w/v) bovine serum albumin (BSA) in PBS, for 20 min, so as to make the cell wall permeable to the stain. The permeabilized cells were then washed twice with PBS and incubated with 250 µl of 1% BSA for 30 min.

For actin filament visualization, the blocking solution was removed and cells were incubated with 200 µl rhodamine phalloidin solution (0.2 µg/ml) for 20 min at room temperature. After removal of rhodamine phalloidin, the cells were treated with 1% BSA as before. The cells were washed with PBS, and dried overnight at 4°C. Images were obtained using Carl Zeiss LSM Meta 510 inverted confocal laser scanning microscope (Carl Zeiss, Germany), equipped with He/Ne laser 543. The visualization of rhodamine phalloidin was done using excitation and emission wavelengths of 543 and 605 nm, respectively. For ZO-1 staining the blocking solution was removed and cells were incubated with 200 µl of ZO-1 antibody (0.1 µg/ml) overnight at 4°C. After removal of ZO-1 antibody the cells were treated with 1% BSA as before. The blocking solution was removed and the cells were incubated with 250 µl FITC anti-rabbit IgG for one hour at room temperature. The cells were washed with PBS, and dried overnight at 4°C. Images were obtained using Carl Zeiss LSM Meta 510 inverted confocal laser scanning microscope (Carl Zeiss, Germany), equipped with Argon2 laser. The visualization of FITC was done using excitation and emission wavelengths of 488 and 505–530 nm, respectively.

Results

The particle size distributions of the two batches of *Swarna bhasma* particles evaluated by dynamic light scattering are

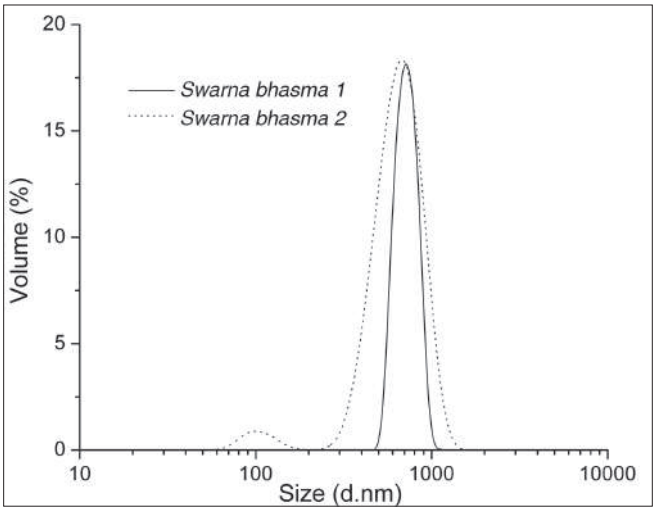

Figure 1: Particle size distributions of *Swarna bhasma* preparations SB1 and SB2

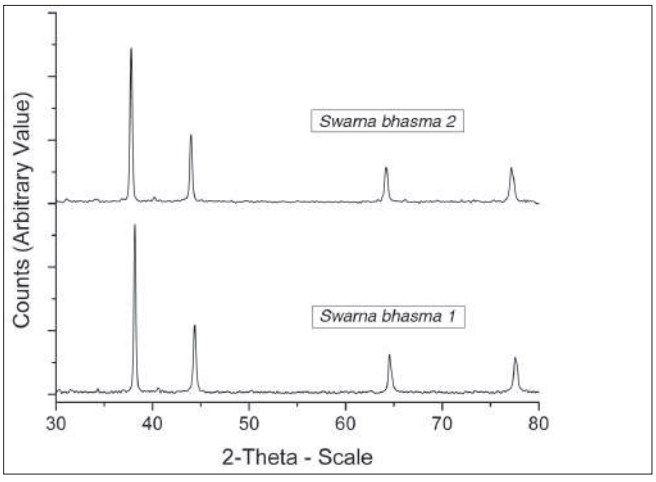

Figure 2: X-ray diffraction patterns of *Swarna bhasma* preparations SB1 and SB2

shown in Figure 1. SB1 had a mean particle diameter of 717 nm and SB2 had a mean particle diameter of 669 nm. The zeta potentials of nanoparticles at neutral pH (pH7.4) were found to be -17.4 ± 0.55 mV and -16.3 ± 0.37 mV, respectively, for SB1 and SB2 preparations.

The XRD patterns of *Swarna bhasma* are shown in Figure 2. The size of gold crystallites in *Swarna bhasma* was calculated from the XRD pattern using the Scherrer formula and determined to be the same (28 nm) for both SB1 and SB2. Morphologies of SB1 and SB2 by scanning electron microscopy are shown in Figures 3a and 3b. The elemental composition of the *Swarna bhasma* samples were analyzed by EDAX as shown in Table 1.

In vitro cytotoxicity of these particles has been done with L929 fibroblast cells as per ISO standard.[22] It has been confirmed by the *in vitro* cytotoxicity studies that the *bhasma* particles are non-toxic. As compared to control (medium) the particles showed 100% cell viability.

The aggregations of the blood cells on interaction with the nanoparticles are shown in Figures 4, 5 and 6, respectively, for RBC, WBC and platelets. It revealed no aggregation of blood cells on incubation of *Swarna bhasma* at a higher interaction ratio of 10 mg/ml. Polyethyeleneimine (PEI) which was used as positive control showed aggregation whereas saline used as negative control did not show any aggregation. The same was

Table 1: Elemental analysis of *swarna bhasma* by EDAX

Element	SB1 (%)	SB2 (%)
As	9.95	9.56
Nb	0.56	0.72
Au	88.10	91.2

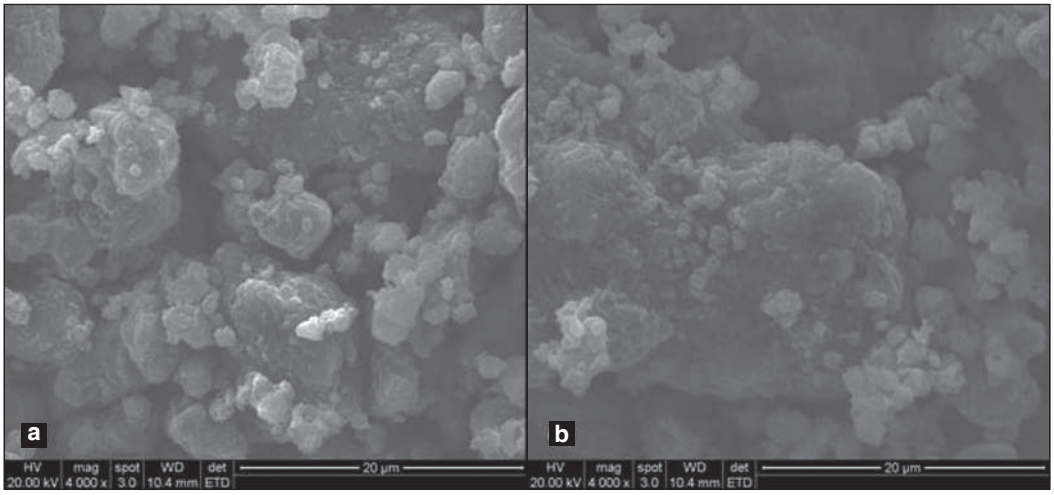

Figure 3: Morphology of *Swarna bhasma* preparations (a) SB1 and (b) SB2 by scanning electron microscopy

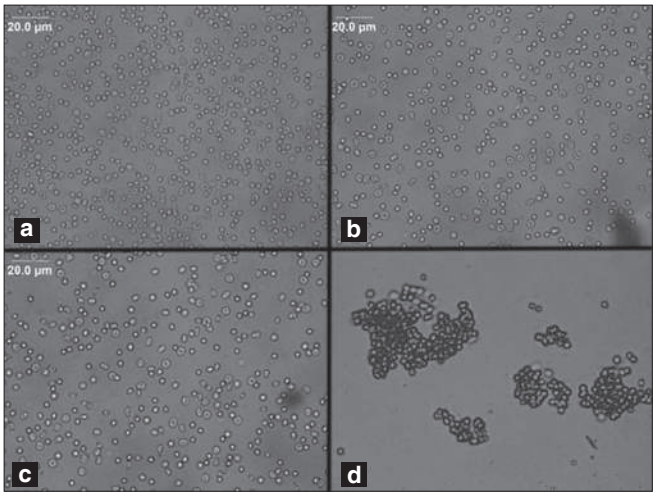

Figure 4: Aggregation of RBC by incubation of (a) SB1, (b) SB2, (c) normal saline (negative control) and (d) polyethylene imine (positive control)

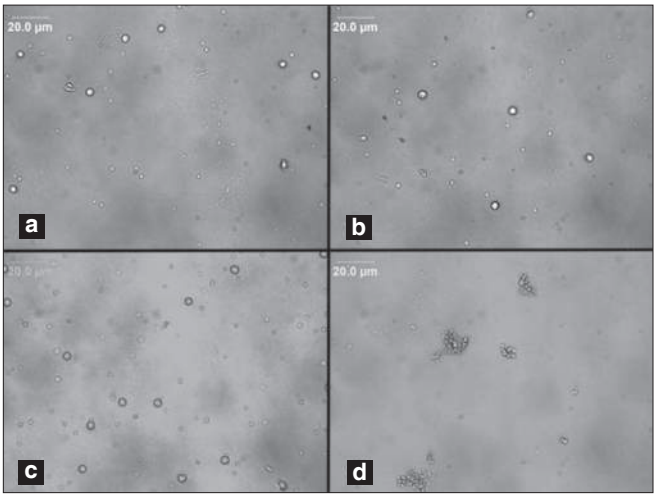

Figure 5: Aggregation of WBC by incubation of (a) SB1, (b) SB2, (c) normal saline (negative control) and (d) polyethylene imine (positive control)

visible with the hemolytic property of the nanoparticles. The hemolysis induced by SB1 was 0.05% and that for SB2 was 0.3% which was well within the acceptable limits of 1%.[23]

The protein adsorption studies evaluated using native-PAGE electrophoresis demonstrated no significant adsorption of proteins occurring onto SB1 or SB2 as shown in the densitometry scan of the treated plasma [Figure 7]. The figure shows the peaks of albumin, globulin region and fibrinogen. Compared to the densitometry scan of control plasma, the peak heights of albumin, fibrinogen or globulins of plasma treated with *bhasma* did not show any change indicating no significant adsorption of blood proteins.

Measuring C3a or C5a in blood or serum after contact with a material has been the most usual way of assessing complement activation. It has been claimed that a surface is biocompatible if these markers are not increased in the fluid phase.[24] Since C3 is cleaved to C3a and C3b by the contact of the surface with blood, irrespective of whether the activation occurs via classical or alternative pathways, and also C3a could be adsorbed on to the material surface just like any other proteins, C3 depletion in the medium can be taken as an indirect measure of complement activation. The amount of C3 in blood (pre-incubation) was 127 mg%. After incubation with SB1 and SB2, it was 126 mg% and 131mg%, respectively, indicating no significant changes in the complement protein level.

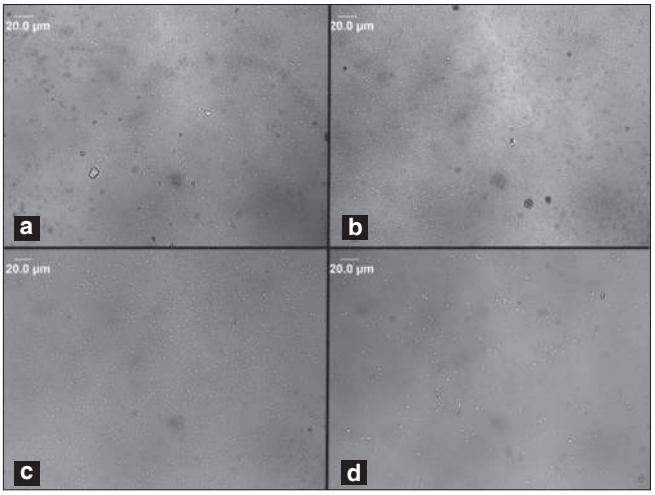

Figure 6: Aggregation of platelets by incubation of (a) SB1, (b) SB2, (c) normal saline (negative control) and (d) polyethylene imine (positive control)

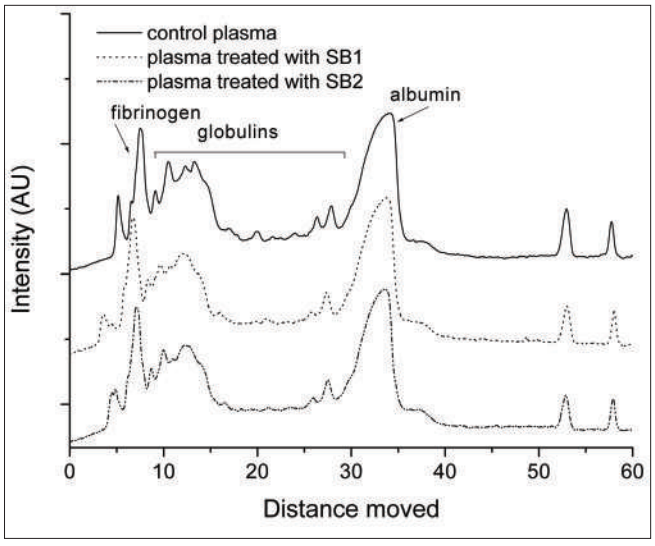

Figure 7: Densitometry scan of native-PAGE of plasma proteins before and after incubation with SB1 and SB2

Platelet factor 4 (PF4) is a platelet-specific protein secreted when a platelet is activated and belongs to the C-X-C chemokine family. Measurements of plasma levels of PF4 have been shown to be the marker of platelet degranulation, and increased level of PF4 is used to detect platelet activation of the circulating pool of platelets.[25] On incubation with *Swarna bhasma* the level of platelet factor 4 in plasma did not change appreciably compared to control plasma. The PF4 level in control plasma was 5.43 ± 0.10 IU/ml and after incubation with SB1 for 15 min it was 4.82 ± 0.4 IU/ml and for SB2 it was 5.07 + 0.3 IU/ml. The platelet adhered onto the bhasma particles were observed through scanning electron microscopy after incubating the SB particles with platelet-rich plasma. There were few cells observed adhering onto the particles. Only one cell was found adhered onto the SB2 sample with no activation or deformation of the platelets as shown in Figure 8.

The control cells stained with rhodamine phalloidin to visualize actin protein showed uniform staining pattern [Figure 9a]. Cells treated with SB1 and SB2 particles showed disrupted staining

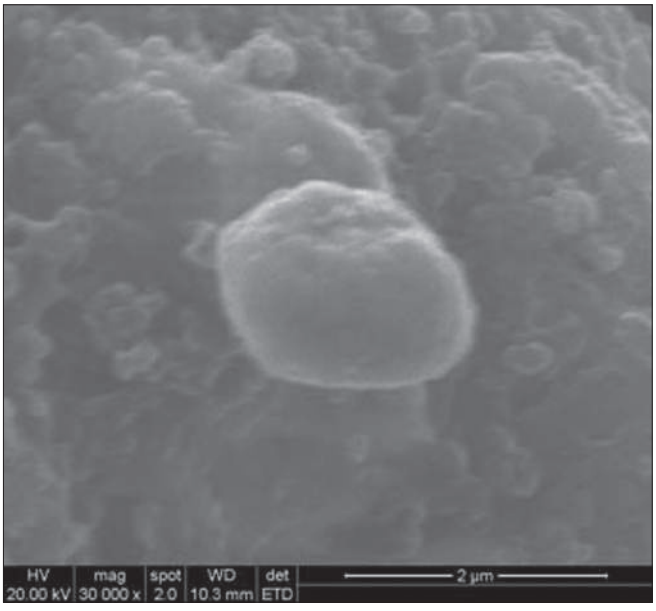

Figure 8: Morphology of platelet adhered onto *Swarna bhasma* preparation by scanning electron microscope

pattern, though the disruption was higher with SB1 as seen from Figures 9b and c. Actin filaments were observed to be discontinuous and disrupted as evidenced from the staining pattern and the clumping. To further investigate the effect on the tight junction proteins immunofluorescence studies using anti ZO-1 was done. ZO-1 is a tight junction associated protein, which plays an important role in tight junction functional regulation. Tight junctions are composed of transmembrane proteins occludin, claudins and junctional adhesion molecules which intercalate with corresponding proteins from adjacent cells to form the intercellular barrier. These proteins associate are with peripheral membrane proteins including the membrane proteins zonula occludens (ZO-1to3) which joins the transmembrane proteins to the actin cytoskeleton. ZO-1 and occluding phosphorylation are associated with stimulus-induced tight junction disassembly and paracellular permeability changes. The effect of SB1 and SB2 particles on ZO-1 tight junctional proteins was evaluated on Caco-2 cell monolayers as shown in Figure 10. In the untreated cells ZO-1 is observed as smooth lines at cell–cell junctions [Figure 10a]. The immunofluorescent staining intensity of *bhasma* particle treated cells were observed to be weaker compared to the control which indicated the loss of ZO-1 from sites of cell–cell contact [Figure 10a and b].

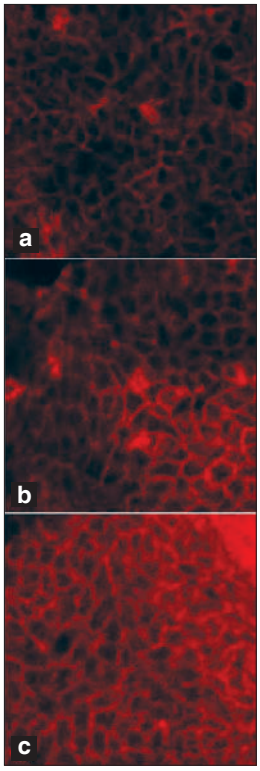

Figure 9: Confocal images (×20) of Caco-2 cell actin. (a) and (b) Caco cells exposed to 5 mg of SB1 and SB2, respectively, for 1 h; (c) Caco cells without any treatment (control)

Discussion

Different methods of preparing *Swarna bhasma* have been reported in various *Ayurvedic* texts.[2,3] This has been done by the incineration of gold with various compounds like mercury, mercury sulfide, sulfur, orpiment (As_2S_3), realgar (AsS), chalcopyrite, etc. out of which the procedure with mercury is considered to be therapeutically effective.[26] Various attempts have been done on the standardization of *Swarna bhasma* preparations for clinical applications.[27] However, the blood compatibility aspects have not been investigated till now.

Although the particle sizes of different batches showed similarity, it seems that these particles are aggregates of much smaller particles. When dispersed in an aqueous medium, gold colloids form a negatively charged hydrophobic particle suspension. This hydrophobicity gives these gold particles a tendency to aggregate together to form larger particles.[28] Both batches of *Swarna bhasma* exhibited larger sizes and agglomeration of the particles. However, the crystallite size calculated from XRD was much smaller. Therefore, the comparatively larger size may be due to the agglomeration of the particles by repeated cycles of calcinations involved in preparation as reported earlier.[29] Zeta potential has been suggested to play an important role in particle uptake because the surface of the intestinal mucosa is negatively charged owing to the presence of glycocalix. Particles with a high positive surface charge like chitosan are usually attracted by

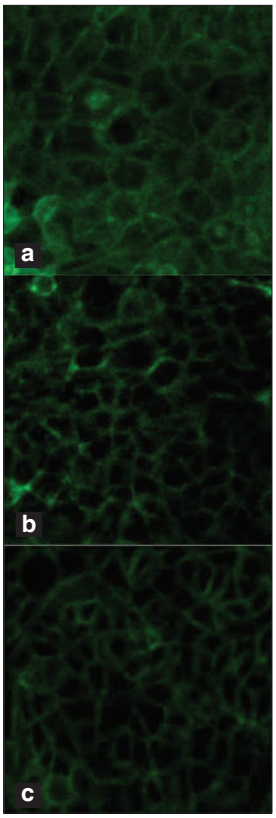

Figure 10: Confocal images (×20) of Caco-2 cell tight-junction protein ZO-1.(a) and (b) Caco cells exposed to 5 mg of SB1 and SB2, respectively, for 1 h; (c) Caco cells without any treatment (control)

the intestinal mucosa, which helps in increasing the intestinal absorption of the encapsulated drug. However, the strong electrostatic interaction between the positively charged particles and the negatively charged glycocalix may slow down the progression and penetration of these particles towards the epithelial cell surface reducing their uptake. Also it has been shown that non-ionized particles have a greater affinity for M cells than for ionized particles[30] and positively charged particles.[31] It has been suggested that gold *bhasma* particles with low negative zeta potential and nanosize may be uptaken by a similar manner.

The X-ray diffraction peaks at 2θ = 38.2°, 44.4°, 64.6° and 77.6° of *Swarna bhasma* were identical with those reported for the standard gold metal (Au°) (JCPDS File No. 04-0784). No other major diffraction peaks were observed confirming that the *Swarna bhasma* is composed of mainly gold nanoparticles. The high intensity of XRD lines in the XRD pattern suggests its crystalline nature. It has been reported that nanoparticles exhibited a size-dependent uptake from the intestine, and its passage via the mesentery lymph supply and lymph nodes to the liver,[32,33] with significant absorption for particles less than 100 nm. Therefore, uptake of *Swarna bhasma* particles of 28 nm through the intestine can be expected.

From the EDAX results it was confirmed that 90% of *Swarna bhasma* contains pure gold and is in correlation with XRD data. EDAX provide good estimate of the concentration of main elements in the sample in a significantly faster way and provides useful information on the distribution of the element forming the sample and their possible chemical form.[34]

The *Ayurvedic* multi-ingredient compounds are formulated in a way that the ingredients are capable of counter-balancing toxic effects, if any, present in the herbs or metals (*bhasma*).[35] These particles pass through extensive processing before they are declared fit for internal use. The processes consist of *Shodhan* and *Marana.* Culture of various cell types with colloidal gold showed no evidence of cytotoxicity.[36-39] No *in vivo* cytotoxicity has been reported with the use of colloidal gold administrated intravenously to ponies and pigs at doses of 400 mg of gold.[40] The initial event when a material comes in contact with blood is the adsorption of proteins. The nature of protein and amount of protein adsorbed will directly influence the compatibility of the particles with the blood. There was a correlation of the adsorption of the globular proteins with the blood cell aggregation showing no activation or aggregation of cells on incubation with *Swarna bhasma*. Activation of platelets initiates the deformation of the cells with pseudopod formation and ends with blood coagulation or thrombus formation.[41] In the present study platelets seem to be not activating and adhering onto the *bhasma* particles and even the very few platelets adhered are not activated as seen from their round shape. This is an indication of the very high platelet compatibility of *Swarna bhasma* preparations.

One of the negative effects of the clinical application of various blood-contacting materials is the activation of the complement system induced by the foreign surface. The response of blood in contact with the material depends on physicochemical features such as surface area, surface charge, hydrophobicity/hydrophilicity etc. The response depends directly on the surface area. Adsorption of C3 triggers complement activation.[41] It has been demonstrated in this study that the adsorptions of C3 on *Swarna bhasma* preparations are insignificant indicating that these preparations do not induce any complement activation when it reaches the systemic circulation.

Pharmacological effects exerted by the therapeutic agents depend upon its ability to cross the biological membranes into the systemic circulation and reach the site of action. This is usually occurred by one of the two pathways; paracellular or transcellular. Most drugs are transported transcellularly depending on their physicochemical properties; however, the paracellular route is usually the main route of absorption for nanoparticles. This is governed by the tight junctions (TJs). TJs are a multiple unit structure composed of multiprotein complex that affiliates with the underlying apical actomyosin ring. TJ proteins identified include transmembrane proteins; occludin and claudin, and cytoplasmic plaque proteins; ZO-1, ZO-2, ZO-3, cingulin, and 7H6. Although the adaptive mechanisms and specific regulation of these tight junctions are areas of active investigation and remain incompletely understood, it is known that some polymers can promote their widening, facilitating absorption of the particles into the systemic circulation. It has been established in this study by the tight junction visualization studies that the *Swarna bhasma* particles are capable of opening tight junctions, thus facilitating the *bhasma* particles to be absorbed into the systemic circulation and comes in direct contact with blood. Thus the *Swarma bhasma* particles should be highly compatible with blood.

Conclusion

Bhasmas are *Ayurvedic* metal-based preparations made by many systematic processes with herbs, converting raw metal into its therapeutic form. *Swarna bhasma*, a therapeutic form of gold metal of nano-sized particles found to be with a crystallite size of 28–35 nm and was 90% pure gold as visible from X-ray diffraction and elemental analysis. They had a low negative zeta potential in a physiological pH. The *Swarna bhasma* preparations did not induce any blood cell aggregation or any protein adsorption. Activation potential of these preparations towards complement system or platelets was negligible. These particles were also non-cytotoxic. Caco-2 cell experiments on tight junction integrity in the presence of *Swarna bhasma*

particles demonstrated their ability to open the tight junctions. It has been demonstrated that uptake of gold nanoparticles occurred in the small intestine by absorption through single, degrading enterocytes in the process of being extruded from a villus and gold nanoparticles typically less than 58 nm in size reaching various organs through blood,[16] which suggests the importance of the blood compatibility studies for the standardization of *bhasma* preparations. Since gold in the *Swarna bhasma* is approximately 28–35 nm in size, it can reach the affected site on oral administration via intestinal absorption and possibly can release Au(I) ions in a sustained manner required for therapeutic action.[42] These results reinforce the application of *Swarna bhasma* as a therapeutic agent in clinical medicine from the safety point of view. These testing protocols may be adopted as a screening test for all *bhasma* preparations to meet the criteria that supports its use worldwide.

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CASE REPORT Open Access

Case series and review of Ayurvedic medication induced liver injury

Christopher M. Karousatos[1], Justin K. Lee[2], David R. Braxton[2] and Tse-Ling Fong[2,3*]

Abstract

Background: Complementary and alternative medicine use among Americans is prevalent. Originating in India, Ayurvedic medicine use in the United States has grown 57% since 2002. CAM accounts for a significant proportion of drug induced liver injury in India and China, but there have been only three reports of drug induced liver injury from Ayurvedic medications in the U.S. We report three cases of suspected Ayurvedic medication associated liver injury seen at a Southern California community hospital and review literature of Ayurvedic medication induced liver injury.

Case presentations: Three patients presented with acute hepatocellular injury and jaundice after taking Ayurvedic supplements for 90–120 days. First patient took Giloy Kwath consisting solely of *Tinospora cordifolia*. Second patient took Manjishthadi Kwatham and Aragwadhi Kwatham, which contained 52 and 10 individual plant extracts, respectively. Third patient took Kanchnar Guggulu, containing 10 individual plant extracts. Aminotransferase activities decreased 50% in < 30 days and all 3 patients made a full recovery. Roussel Uclaf Causality Assessment Method (RUCAM) scores were 7–8, indicating probable causality. These products all contained ingredients in other Ayurvedic and traditional Chinese medicines with previously reported associations with drug induced liver injury.

Conclusions: These patients highlight the risk of drug induced liver injury from Ayurvedic medications and the complexity of determining causality. There is a need for a platform like LiverTox.gov to catalog Ayurvedic ingredients causing liver damage.

Keywords: Ayurveda, Ayurvedic medicine, Drug induced liver injury (DILI), Herb induced liver injury (HILI), Traditional Chinese medicine, Roussel Uclaf causality assessment method

Background

Originating in India thousands of years ago, Ayurveda is among the oldest healing systems in existence, with a focus on harmonious living and self-sustainability [1, 2]. Diseases are viewed in the context of their effects on an individual's dosha or mind-body type with respect to energies of the five elements: earth, water, fire, air, and ether [3]. Though illness prevention is promoted through lifestyle modifications, medicinal herbs are an integral part of Ayurveda [4]. Ayurveda is prominent in India, with the government recognizing and funding the practice, development, and research of Ayurvedic Medicine (AM) [5]. The Ayurvedic sector in India had an estimated market value of three billion USD in 2016 and its market value is expected to continue growing [6].

Though limited data exists quantifying the money spent on AM in the United States, recent trends suggest rising popularity [7]. Currently, the most popular Ayurvedic supplement in the United States is Ashwagandha (*Withania somnifera*), with sales in 2018 totaling over 7 million dollars, an increase of 165.9% from the year prior, according to a market report published by the

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American Botanical Council [8]. More broadly, the National Center for Health Statistics survey on Complementary and Alternative Medicine (CAM) in 2015 showed that 241,000 American adults used AM, a nearly 57% increase from 2002 [9]. A survey of South Asian Americans in Northern California estimated that about 59% had used or were currently using AM but only 18% mentioned this to their healthcare providers [10]. Given these indicators of rising popularity, awareness of AM and its potential dangers is increasingly important for physicians in the United States.

CAMs make up a larger percentage of drug-induced liver injury (DILI) in Asia where CAM is more prevalent [11, 12]. One study of cirrhotic patients in India reported that of 1666 patients with cirrhosis, 35.7% had acute-on-chronic liver failure secondary to CAM-related DILI on presentation [11]. Like AM, in China, traditional Chinese medicine (TCM) is officially state-supported and institutionalized [13]. A survey of cancer patients of a large urban hospital in China revealed that more than 80% of patients were using TCM in conjunction with Western medicine [14]. In a series of 1985 cases of DILI in China, TCM was implicated in 28% of cases and an additional 28% of cases where TCM was taken in conjunction with Western medication [15]. In contrast, herbal and dietary supplements accounted for only 15.5% of liver injury among cases recorded by the Drug Induced Liver Injury Network in the U.S. [16]. There have been only three cases where specific Ayurvedic medications have been linked to liver damage in the U. S [17, 18] and seven in Europe [18–22]. Other case reports outside of India have come from Canada [23], South America [24], and Israel [25].

Within a 3 month-span, three cases of suspected AM-induced liver injury were seen at a Southern California community hospital. Using the updated Roussel Uclaf Causality Assessment Method (RUCAM), we report these three patients and review the current literature of AM-associated DILI [26]. There are many plant species that are indigenous to India and China due to the proximities of these two countries. To highlight the common ingredients of AM and TCM, the herbal/plant ingredients of the AM supplements taken by these three patients were also cross-referenced with components of TCM that have been reported to be associated with liver injury. This report highlights the substantial challenges in assessing causality in cases of herbal product-induced liver injury.

Case presentation

Patient 1 is a 68-year-old South Asian female with a history of hypothyroidism, dyslipidemia and borderline diabetes mellitus who began taking an Ayurvedic supplement, Giloy Kwath, to improve her overall health. Her baseline blood work revealed mildly elevated alanine aminotransferase (ALT) 35 U/L and was otherwise normal. Four months later, routine follow-up blood work revealed acute hepatocellular injury: alkaline phosphatase (AP) 113 U/L, total protein (TP) 6.6 mg, albumin (alb) 4.1 g/dL, total bilirubin (t bili) 1.0 mg/dL, ALT 1016 U/L, aspartate aminotransferase (AST) 844 U/L, and international normalizing ratio (INR) 1.0. Viral hepatitis A, B, C and E serologies were negative. Antinuclear antibody (ANA) was weakly positive but anti-smooth muscle antibody (SMA) and anti-liver kidney microsomal antibody (LKM) were negative. Her physical exam was normal. She was asymptomatic until 1 week later when she became jaundiced. At this time, she immediately stopped the supplement. Within 1 month of stopping, her liver tests became normal and remain normal 1 year later (Fig. 1a).

Patient 2 is a 38-year-old South Asian female with a history of hypothyroidism who began taking Manjishthadi Kwatham and Aragwadhi Kwatham for a skin rash. For the preceding 4 years prior to taking AM, her liver tests were normal. Four months after starting AM, she was initially evaluated for jaundice, fatigue, anorexia, and right upper quadrant discomfort. Her physical exam was significant for marked scleral icterus. Abdominal exam was unremarkable, without hepatomegaly. Blood work on admission revealed AP 175 U/L, TP 7.5 mg, alb 3.7 g/dL, t bili 10.7 mg/dL, ALT 760 U/L, AST 1020 U/L, lactate dehydrogenase (LDH) 923 U/L, INR 1.3, and IgG immunoglobulin 1238 mg/dL. Abdominal ultrasound revealed normal liver echogenicity with a smooth contour. MRI/MRCP confirmed a normal biliary tree. Her gallbladder wall was thickened, measuring 13 mm. Viral hepatitis A, B, C and E serologies were negative. Epstein-Barr IgM antibody was negative. Autoimmune serologies including ANA, SMA, and LKM were negative. Liver biopsy showed severely active pan-lobular hepatitis with bridging necrosis and lymphoplasmacytic infiltrates. There was no definitive fibrosis (Fig. 2). She was treated with prednisone empirically for 6 weeks. Her liver tests normalized 6 weeks after presentation and have remained persistently normal 16 months after discontinuation of corticosteroids (Fig. 1b).

Patient 3 is a 46-year-old Hispanic female who is obese with menometrorrhagia who began taking Kanchnar Guggulu for her menses. Baseline liver tests taken 4 months prior to taking Kanchnar Guggulu were normal. Three months later, she became jaundiced with pruritus and a maculopapular rash. Her abdomen was nontender and without hepatosplenomegaly. The remainder of her physical exam was unremarkable. Her laboratory studies revealed AP 140 U/L, TP 6.4 mg, alb 4.1 g/dL, t bili 15.0 mg/dL, ALT 1382 U/L, AST 878 U/L, and INR 1.1. Viral hepatitis A, B, C and E serologies were negative. ANA was weakly positive but SMA and LKM were

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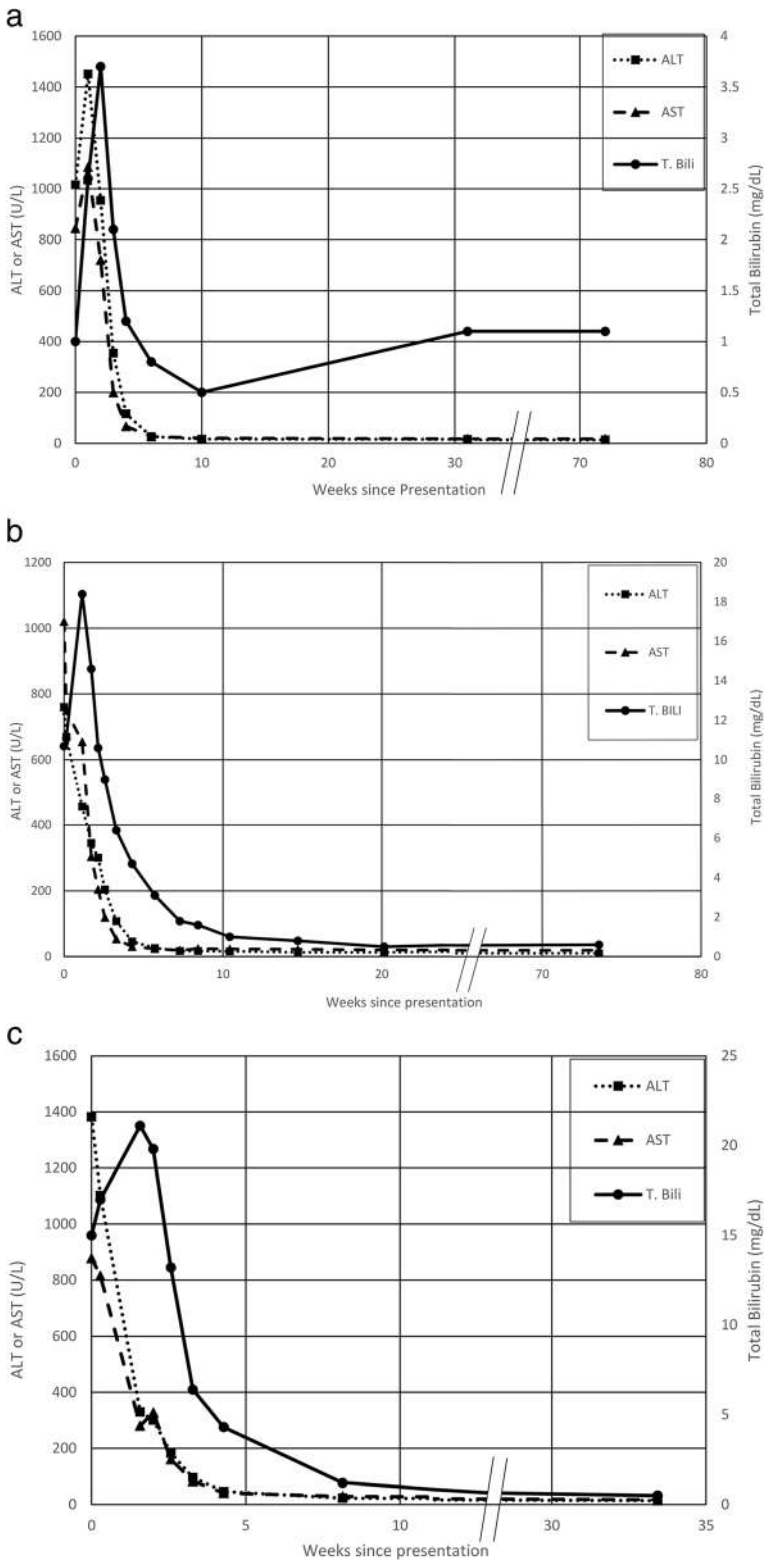

Fig. 1 (**a**, **b**, **c**) Alanine aminotransferase activity and bilirubin level of 3 patients

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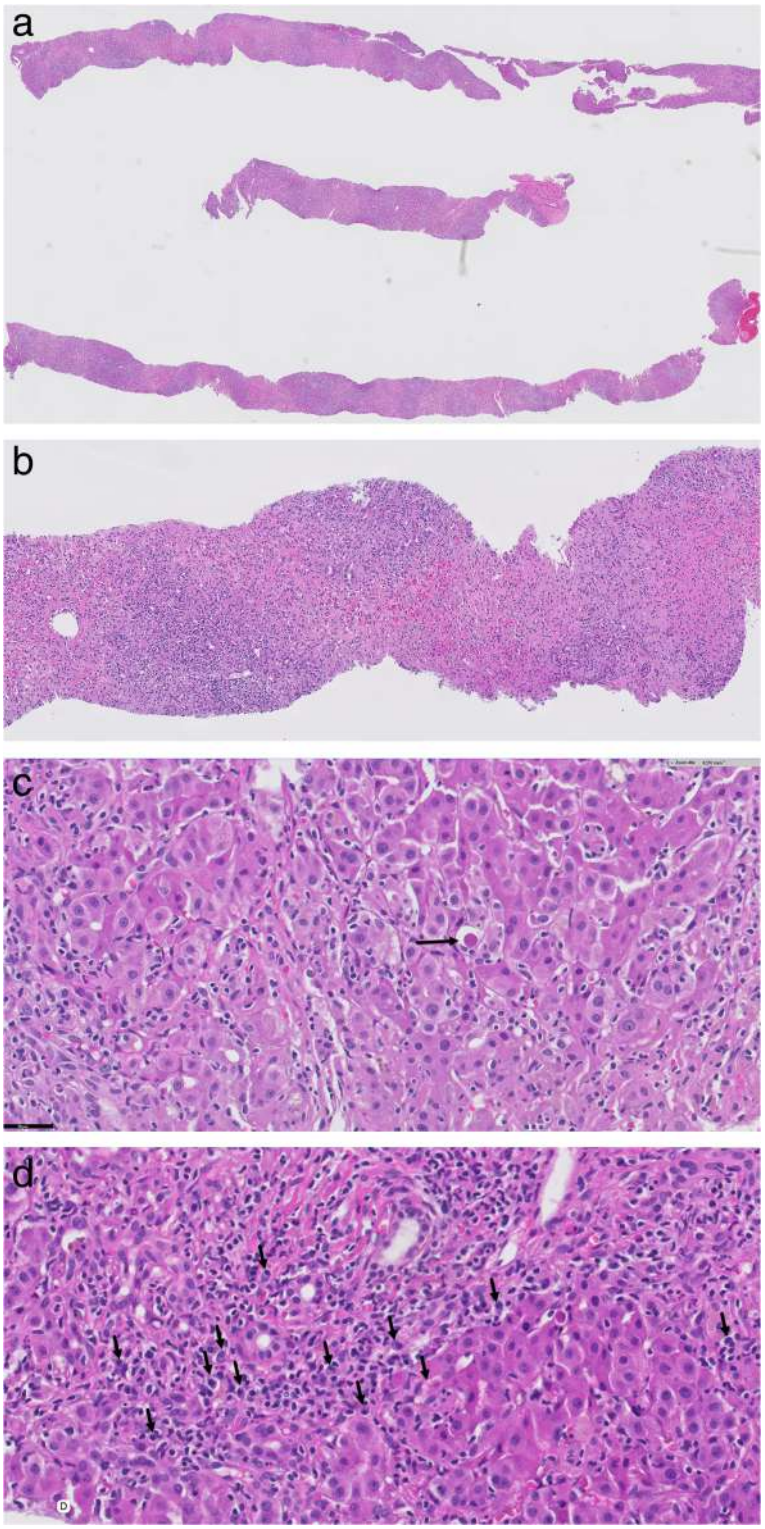

Fig. 2 Histopathology showing Severely active panlobular hepatitis with panacinar necrosis and lymphoplasmacytic infiltrates: **a**. Low power (2x Magnification) The portal tracts are expanded at low power with diffuse inflammatory infiltration. **b**. Collapse of hepatocyte lobules illustrate the presence of panacinar necrosis. Note the remnants of the portal tracts marked by the presence of biliary ductules. (Magnification 10x). **c**. Remnant hepatic lobule showing infiltration by predominantly lymphocytes. Apoptotic hepatocytes were conspicuous (arrow). (Magnification 40x). **d**. Numerous plasma cells (arrows) are present in portal tracts showing interface activity. (Magnification 40x)

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negative. Ultrasound showed a normal size liver with normal echogenicity. There were no gallstones or biliary dilatation. The supplement was discontinued and after 8 weeks her liver tests returned to normal. They remain normal 6 months later (Fig. 1c).

Summary of the clinical characteristics are shown in Table 1 and the compilation of RUCAM for the Ayurvedic supplements are shown in Table 2.

All three patients took Ayurvedic medications in quantities as recommended, which contained plant ingredients that have been previously associated with liver injury in a large cases series [27] and case reports of AM-associated liver injury [17–25, 28] (Table 3). Patient 1 took Giloy Kwath that contained a single ingredient, *Tinospora cordifolia*, that was a component in eight preparations of Ayurvedic medications associated with liver injury reported in a large single center case series [27]. Patient 2 took 2 ayurvedic preparations; Manjishthadi Kwatham and Aragwadhadi Kwatham that contained 52 and 10 individual plant extracts, respectively (Table 3). Twenty-three extracts found in Manjishthadi Kwatham were associated with liver injury in prior literature, including *Psoralea cordyfolia* which was reported in two cases where this herb was the lone ingredient in the supplements taken by the patients [20, 21]. In addition to *Tinospora cordifolia*, which was also present in Giloy Kwath taken by Patient 1 and Manjishthadi Kwatham also taken by Patient 2, there were 8 other plant extracts in Aragwadhadi Kwatham associated with liver injury reported in prior literature, detailed in Table 3. Patient 3 took Kanchnar Guggulu, one of the 3 supplements taken by a patient with DILI reported by Dalal et al. [29]. Nine ingredients were found in other preparations associated with DILI described in other case reports or reviews: *Cinnamomum tamala* [17, 27], *Cinnamomum zeylanicum* [27], *Elettaria cardamomum* [17, 27], *Emblica officinalis* [27], *Piper longum* [23, 27], *Piper nigrum* [27], *Terminalia belerica* [17, 23, 27], *Terminalia chebula* [22], *Zingiber officinale* [19, 27].

The individual ingredients of these AM supplements were cross-referenced with components of TCMs that have been reported to be associated with liver injury (Table 4). Although *Tinospora cordifolia* (contained in Gilroy Kvath taken by patient 1 and in Manjishthadi Kwatham taken by patient 2) was not reported among TCM, a closely related plant species, *Tinospora crispa*, is contained in Bo Ye Qing Niu Dan which has been associated with liver injury [31, 32]. *Azadirachta indica* contained in Manjishthadi Kwatham is found in Ku Lian Zi as well as *Cinnamomum tamala* (Indian bay leaf) that is contained in Sairei To, which has been reported to cause DILI in TCM supplements [31–33].

An acute hepatocellular injury pattern was seen in all three patients. Two patients were jaundiced but none developed clinical signs of liver failure. Complete recovery was seen in all three cases. The patients underwent an exhaustive evaluation including viral and autoimmune serologies and imaging to rule out biliary tract or infiltrative diseases that was negative. None of the patients were re-exposed to the supplements and RUCAM scores indicated probable causality for DILI in the three patients, as detailed in Table 2.

Discussion and conclusions

Use of ayurvedic medicine in the U.S. is more popular and extends beyond Indo-Americans as evidenced by one of the 3 patients in this cases series who is Hispanic [9]. Despite the widespread use of AM, there is a relative paucity of publications on AM-associated liver injury. With 94 patients, Philips et al. compiled the largest list

Table 1 Clinical characteristics

Patient Characteristics			
	Patient 1	**Patient 2**	**Patient 3**
Age (years)	68	38	46
Gender	F	F	F
Ethnicity	Asian	Asian	Hispanic
Herbal Product	Giloy Kwath	Manjishthadi Kwatham Aragwadhadi Kwatham	Kanchnar Guggulu
Time to onset (days)	~ 90	~ 120	~ 90
Peak bilirubin (mg/dL)	3.7	18.4	21.1
Peak alkaline phosphatase (U/L)	200	175	140
Peak ALT (U/L)	1451	760	1382
Peak AST (U/L)	1086	1020	876
R index	21.8	13	29.6
Peak INR	1.0	1.8	1.1
Presentation to peak ALT (days)	8	0	0

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Table 2 RUCAM for liver injury

	Possible Score	Patient 1	Patient 2	Patient 3
Time to onset	+ 2	+ 2	+ 1	+ 2
5–90 days				
< 5 or > 90 days	+ 1			
Course of ALT after cessation of herb		+ 2	+ 3	+ 2
Decrease > 50% within 8 days	+ 3			
Decrease > 50% within 30 days	+ 2			
Risk factors				
Alcohol +	−1			
Alcohol -	0	0	0	0
Age ≥ 55 years	+ 1	+ 1		
Age < 55 years	0		0	0
No concomitant drugs/herbs		0	0	0
All causes-group I and II-ruled out		+ 2	+ 2	+ 2
Previous hepatotoxicity		+ 1	+ 1	+ 1
Reaction labeled in the product characteristics	+ 2			
Reaction published but not labeled	+ 1			
Response to unintentional re-exposure		0	0	0
Total score for the case		+ 8	+ 7	+ 7

RUCAM score causality levels < 0 excluded causality; 1–2 unlikely; 3–5, possible; 6–8, probable; and > 9, highly probable (ref [26])

of Ayurveda and herbal medicine associated with severe liver injury from a single center from southern India. Five patients including one patient who underwent liver transplantation died [27]. Other clinical literature on the subject comprises case reports, case series, and broader reviews on herb-induced liver injury [17–25, 28, 31–34]. Initial patient complaints from herbal hepatotoxicity have ranged from asymptomatic liver function test abnormalities to acute liver failure requiring transplant and/or resulting in death [27, 34]. Our report of three patients with probable AM-induced liver injury highlights the pervasive challenges of evaluating causality in herbal-medication induced liver injury. One patient took the supplement Giloy Kwath (water extraction (decoction) of herb(s)), which contained only *Tinospora cordifolia*. This herb was also present in an AM, Manjishthadi Kwatham, taken by another patient in this report. *Tinospora cordifolia* was an ingredient found in eight different formulations of AMs associated with severe liver injury [27]. For a supplement with a single herb ingredient and a uniform pattern of liver injury across multiple occurrences, such as the recent case series involving *Withania somnifera* (also known as Ashwagandha) [18], assignment of causality can be determined with greater certainty. There are other case reports of AM-associated liver injury involving a single agent; Bakuchi Powder/Babchi Seeds/Bakuchiol which consist of *Psoralea corylifolia* [21], Bristly Luffa consisting of *Luffa echinate* [35], and *Cantella asiatica* [22, 24]. However, this kind of clear association is relatively uncommon.

As in two of the patients in this series, other case reports of AM-associated liver injury involve patients taking multiple preparations that may contain numerous ingredients [20, 23, 29]. For example, one of our patients took two preparations that contained 52 and 10 individual ingredients, respectively. The large number of components is not unusual for Ayurvedic preparations. On analysis of the compendium by Philips et al. [27], 8 of the listed 27 Ayurvedic medications reported to be associated with liver injury had detailed ingredient lists available online. The mean and median number of individual herbs in these medications was 19.8 and 9, respectively, ranging from 1 to 71 ingredients per supplement (see Supplement 1). Notably, this search for ingredients may be limited by conducting it retrospectively, as ingredient listings may have since changed since their usage in the review. There were 33 total components in the products taken by patient 2 that were listed as ingredients in the preparations associated with previous cases of AM-induced liver injury; 11 such ingredients were found in the AM products taken by patient 3. While this may indicate overlap in a component or components responsible for hepatic damage, it may also simply demonstrate the ubiquity of certain ingredients in Ayurvedic medications, which adds to the challenge of attributing causality.

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Table 3 Ingredients of Ayurvedic supplements

Patient 1	Patient 2		Patient 3
Giloy Kwath	**Manjishthadi Kwatham**	**Aragwadhadi Kwatham**	**Kanchnar Guggulu [29]**
Tinospora cordifolia[a] [17, 23, 27]	*Acacia catechu*[a] [17, 20]	*Alstonia scholaris*	*Bauhinia variegata*[a] [29]
	Acorus calamus[a] [27]	*Andrographis paniculate*[a] [27]	*Cinnamomum tamala*[a] [17, 27, 29]
	Azadirachta indica[a] [23, 27, 30]	*Azadirachta indica*[a] [23, 27, 30]	*Cinnamomum verum*[a] [17, 29]
	Cassia fistula	*Bridelia stipularis*	*Commiphora wightii*
	Cedrus deodara[a] [17, 29]	*Butea monosperma*	*Crataeva nurvala*[a] [29]
	Chonemorpha fragrans	*Calycopteris floribunda*	*Elettaria cardamomum*[a] [17, 27, 29]
	Citrullus colocynthis	*Cassia fistula*	*Embelica officinalis*[a] [23, 27, 29]
	Clerodendrum serratum[a] [17]	*Cyclea peltata*[a] [27]	*Piper longum*[a] [17, 23, 27, 29]
	Crataeva magna	*Holarrhena antidysenterica*[a] [23, 29]	*Piper nigrum*[a] [27, 29]
	Cullen corylifolium	*Marsdenia tenacissima*[a] [27]	*Terminalia belerica*[a] [29]
	Curcuma longa[a] [27, 29]	*Monringa oleifera*	*Terminalia chebula*[a] [23, 27, 29]
	Cyclea peltate[a] [27]	*Plumbago zeylanica*[a] [17, 27, 29]	*Zingiber officinale*[a] [19, 20, 27]
	Cyperus rotundus[a] [17, 29]	*Pongamia glabra*	
	Eclipta prostrata	*Stereospermuum suaveolens*[a] [27]	
	Embelia ribes[a] [23, 27, 29]	*Strobilanthes ciliatus*	
	Embelica officinalis[a] [23, 27, 29]	*Tephrosia purpurea*[a] [23]	
	Gentiana kurroo	*Tinospora cordifolia*[a] [17, 23, 27]	
	Hemidesmus indicus[a] [27]	*Trichosanthes cucumerina*	
	Holarrhena pubescens	*Trichosanthes tricuspidata*	
	Holoptelia integrifolia		
	Ichnocarpus frutescens		
	Justicia beddomei		
	Neopicrorhiza scrophulariiflora		
	Oldenlandia corymbosa		
	Operculina turpethum[a] [17, 27, 29]		
	Pavoni odorata		
	Psoralea corylifolia[a] [20, 21]		
	Phyllanthus emblica[a] [17, 27]		
	Piper longum[a] [17, 23, 27, 29]		
	Plectranthus vettiveroides		
	Pterocarpus marsupium[a] [17]		
	Rubia cordifolia[a] [17]		
	Santalum album[a] [17]		
	Streblus asper		
	Swertia chirayita		
	Tamarindus indica		
	Terminalia belerica[a] [27]		
	Terminalia chebula[a] [22, 27, 29]		
	Tinospora cordifolia[a] [17, 23, 27]		
	Tragia involucrata[a] [17, 27]		
	Trichosanthes cucumerina		
	Zingiber officinale[a] [19, 20, 27]		

[a] refers to herbs found to be a component of a compound previously associated with AM-induced liver injury

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Table 4 Ingredients common in AM and TCM associated liver injury

AM	Herb	TCM [31]
Bakuchi tablets [20]	*Psoralea corylifolia*	Bai Shi Wan
Babchi Seeds [21]		Shou Wu Pian
Avalguja[a]		Boh Gol Zhee
Liquorice [19]	*Glycyrrhiza glabra*	Bofu Tsu Sho San
		Bai Shi Wan
		Long Dan Xie Gan Tang
		Sairei To
		Da Chai Hu Tang
		Xiao Chai Hu Tang
		Gan Cao
Kaishore (Kishore) Guggal [27]	*Zingiber officinale*	Bofu Tsu Sho San
Pushpadhanva Ras [27]		Da Chai Hu Tang
Bhavana Dravya [27]		Kamishoyosan
Sunthi[a] [27]		Sairei To
Sonth [27]		Xiao Chai Hu Tang
Kaisoragulu vatika [27]		
Mahayograj Guggul [27]		
Shunthi[a]		
Kaishore (Kishore) Guggal [27]	*Piper nigrum*	Shen Min
Bhavana Dravya [27]		
Maricha[a]		
Pushpadhanva Ras [27]	*Cinnamomum verum*	Sairei To
Tej Patra[a]		
Tvak[a]		
Pushpadhanva Ras [27]	*Cinnamomum tamala*	Sairei To
Tej Patra[a]		
Tvak[a]		
Arishta[a]	Azadirachta indica	Ku Lian Zi
Bhringa[a]	*Eclipta prostrata*	Shou Wu Pian
Ghana[a]	*Cyperus rotundus*	Bai Fang
Trayanthi[a]	*Gentiana kurroo*	Long Dan Xie Gan Tang

[a] reported from the patients in this case series

Another inherent challenge in evaluating toxicity related to AM and other herbal supplements is the lack of accurate data regarding quantities and dosing. For most cases, the prevailing theory of the mechanism of herbal induced liver injury is idiosyncratic and therefore, dosing is not a parameter that considered in evaluating causation [26].

To tabulate a comprehensive list of Ayurvedic compounds associated with liver injury, we performed a literature search for all articles in English discussing Ayurveda and liver injury, damage, or hepatotoxicity in humans. Articles were excluded if they detailed hepatoprotective effects. The literature review for AM-associated herbal supplement induced liver injury in humans yielded 50 results with the following distribution according to PubMed archiving: 10 case reports/series, 9 reviews of AM, 2 clinical studies, and 29 journal articles. After eliminating off-topic matches, 12 were deemed to be directly relevant for the purpose of this summary. Articles excluded were among the following categories: discussions of uses of an herb or supplement unrelated to liver injury, pharmacologic properties of a substance unrelated to liver injury, heavy metal contamination in AM, cancer unrelated to AM hepatotoxicity, and liver physiology unrelated to hepatic injury. After analyzing the compounds listed and cross referencing their constituent ingredients from available sources online (shown in Supplement 1), a number of substances were found to

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be common ingredients in Ayurvedic medications causing liver injury: *Phyllanthus emblica* with 12 unique occurrences, *Withania somnifera* with 11, *Zingiber officinale* with 11, and numerous others (see Supplement 2). Importantly, these compounds were not all specifically implicated in their respective studies but rather recurrently present in substances taken by patients with DILI after examination of each's components through an online search.

With this information in mind, it is important to note the inherent limitations of this type of correlational summation. For instance, *Zingiber officinale*, though linked to 11 Ayurvedic medications [19, 27, 29] and TCM [31–33] associated with live injury, is ginger, a widely used household spice. While the ingredient could be hepatotoxic, its prevalence may also be explained by the fact that it is a common additive in Ayurvedic supplements. Similarly, *Piper nigrum* or black pepper, has 5 occurrences in medications causing liver injury, and several other compounds (see Supplement 2). This said, other commonly used products have been linked to acute liver injury, a notable example being green tea extract [36]. Until larger and more focused research is conducted, this list in Supplement 2 clearly limited in practicality by its correlational nature and should serve only as a starting ground for more focused future investigations and efforts to tabulate offending herbs or substances. Moreover, as previously mentioned, certain compounds' ingredients were retrospectively retrieved and may not accurately reflect the exact product taken at the time of each initial case.

Like Ayurveda, TCM, which includes the use of herbs, has evolved over thousands of years. Comparatively, more research exists on Chinese herbal medications and liver injury; however, Ayurvedic and Chinese herbal medications share many ingredients. In a compilation of 57 traditional Chinese medications associated with liver injury, 18 contained similar compounds in Ayurvedic medications associated with liver injury, shown in Table 4. Moreover, an additional 5 species of the same genus as those found in the Ayurvedic medications were identified. Teschke and others have created extensive compilations of TCM herbs associated with hepatoxicity [31–34]. Assigning causality to TCM is fraught with the same challenges as AM-associated liver injury. We discovered several compounds associated with liver injury in TCM contained in the AM products taken by our three patients: *Psoralea corylifolia*, *Zingiber officinale*, *Piper nigrum*, *Cinnamomum verum*, *Cinnamomum tamala*, *Azadirachta indica*, *Eclipta prostrata*, *Cyperus rotundus*, and *Gentiana kurroo* [31–33]. Again, as noted above, these simply represent shared associations with liver injury in AM and TCM, which may indicate hepatotoxicity, the popularity of certain herbs in traditional medicinal preparations, or both. A 2019 review by Byeon et al. of all documented herb-induced liver injury contained substances found *Psoralea corylifolia*, which was an ingredient of Manjishthadi Kwatham taken by patient 2 in this report, to be the second most common overall precipitant from the review, with 41 occurrences [37].

The spectrum of the histological findings on liver biopsy are just as diverse and varied as the ingredients of AM that are suspected to be causative for DILI. The most common finding present on liver biopsy has been a non-specific chronic hepatitis, present in 85% of cases reported by Philips et al. [27]. However, more severe findings have been reported and include sub-massive hepatic necrosis, pan-acinar necrosis, bridging necrosis, granulomatous hepatitis, steatohepatitis, and cholestatic hepatitis [17, 19, 21, 24, 27]. Features ranging from active cholangitis, biliary proliferation, and non-specific injury typical of DILI have also been reported [21, 25, 29]. Fibrosis was present in 77% of patients in one study [27], indicating a chronic injury in the vast majority of patients, which may be attributable to long term usage of AM. The variety of histological appearances of AM related DILI presents additional challenges in routine clinical diagnosis of this etiology and further complicates attribution of the injury to one specific ingredient in AM or TCM supplements.

As AM and TCM gain wider use in the West, there is a need to develop a central registry to document herbal supplements and their constituent ingredients that have been reported to cause liver injury similar to Livertox.nih.gov, a registry of medications associated with DILI. Complicating the matter is that supplements in the United States are unregulated. The chemical composition of herbal supplements is not verified and therefore analysis is additionally challenging.

In conclusion, our case series represents three of the few documented cases of AM-induced liver injury in the United States. As Ayurvedic supplement use becomes more popular in the U.S. and globally, recognition of AM induced liver injury will become crucial. However, the complexity of supplements taken by patients make causality difficult to discern. Investigating specific ingredients in Ayurvedic medications and other herbal supplements associated with liver injury would help evaluate current correlational relationships [11]. This effort would depend on a reliable catalog of constituent ingredients, in a platform similar to that of LiverTox.gov. This would facilitate quicker recognition of offending agents when diagnosing patients with herb-induced liver injury and safer consumption of Ayurvedic as well as other complementary and alternative medicine preparations by patients.

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Abbreviations
AM: Ayurvedic medication; USD: U.S. dollars; CAM: Complementary and alternative medicine; TCM: Traditional Chinese medicine; DILI: Drug-induced liver injury; RUCAM: Roussel Uclaf Causality Assessment Method; AP: Alkaline phosphatase; TP: Total protein; alb: Albumin; t bili: Total bilirubin; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase INR international normalising ratio; ANA: Anti-nuclear antibody; SMA: Anti-smooth muscle antibody; LKM: Anti-liver kidney microsomal antibody; LDH: Lactate dehydrogenase; MRI/MRCP: Magnetic resonance imaging/ magnetic resonance cholangio-pancreatography

Supplementary Information

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Additional file 1.

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Authors' contributions
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Availability of data and materials
All clinical data are available by contacting Dr. Tse-Ling Fong.

Declarations

Ethics approval and consent to participate
Ethics approval was waived by Institutional Review Board of Hoag Memorial Hospital Presbyterian.
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Competing interests
The authors declare that they have no conflicts of interest.

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Comparative anti-hyperlipidemic activity of ***Tamra Bhasma*** (incinerated copper) prepared from ***Shodhita*** (purified) and ***Ashodhita Tamra*** (raw copper)

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In Ayurveda, metals are converted into ***Bhasmas*** for internal consumption by processing them through various processes like ***Shodhana*** (purification and/or detoxification), ***Marana*** (incineration)***,*** etc. and then used in the treatment of various diseases. These procedures not only decrease the possible harmful effects of metals but also said to increase their bio-availability and thus efficacy. In ***Rasashastra*** classics, due emphasis has been given to the ***Shodhana*** procedure. One of the most popularly used metallic preparations is ***Tamra Bhasma*** (incinerated copper) and it is said to be very harmful if its ***Shodhana*** is not done or if it is improperly prepared. ***Tamra Bhasma*** has been advocated in the treatment of ***Medoroga*** (lipid disorders), ***Hridroga*** (cardiac disorders), etc. and role of copper in lipid disorders is well documented fact. In the present study, a comparative anti-hyperlipidemic activity of unpurified (***Ashodhita***) and purified (***Shodhita***) ***Tamra*** was carried out to know the effect of ***Shodhana*** on efficacy. The hyperlipidemia was induced by feeding high fat diet in Wistar strain albino rats. The parameters including body weight, weight of various organs, serum lipid profile and histopathology of liver, kidney, heart and aorta were studied. The results of this study suggests that ***Tamra Bhasma*** prepared from ***Shodhita Tamra*** is having significant anti-hyperlipidemic activity, while ***Ashodhita Tamra Bhasma*** is lack of such effects. Also ***Ashuddha*** sample proved to possess cardio-toxic effect. This shows that the ***Rasashastriya Shodhana*** procedure have definite role in not only increasing the efficacy of the drug but also in removing the toxicity.

Keywords: ***Bhasma***, Copper, Hyperlipidemia, ***Rasashastra***, ***Shodhana***, ***Tamra***.

IPC code; Int. cl. (2011.01)−C22B 15/14

Introduction

Hyperlipidemia is the presence of high levels of cholesterol in the blood. It is a metabolic derangement, not a disease. It can be secondary to many diseases and can contribute many forms of diseases, most notably cardiovascular diseases[1]. One percent drop in serum cholesterol reduces the risk for cardiac heart disease (CHD) by two percent. Low HDL cholesterol (<35 mg/dl); obesity (>30% overweight); HDL levels (>60 mg/dl) and high LDL (>160 mg/dl) are positive risk factors for CHD while negative risk factors include high HDL levels (>60 mg/dl)[2]. Large proportion of the deaths from cardiovascular (CV) diseases is attributed mainly to coronary heart disease and direct manifestation of atherosclerosis[3]. The treatment of hyperlipidemia depends on the patient's cholesterol profile. Statin, fibrates, niacin, bile acids, ezitimibe etc. are the antihyperlipidemic agents which reduce cholesterol level with different condition[1].

In Ayurveda, different formulations are in use for the treatment of ***Medoroga*** (lipid disorders) since centuries. Advent of ***Rasashastra*** (Ayurvedic pharmaceutics) from 7 A.D. onwards successfully made the usage of many metals, minerals, gems, poisonous substances, etc in the treatment of various diseases. To make them suitable for human use, these substances should undergo ***Shodhana*** (purification and/or detoxification) procedure as described in classics of ***Rasashastra***. These processes have dynamic effect on the pharmacological activities of the drug.

Tamra Bhasma (TB) (incinerated copper) is one of such drug which is widely used in treatment of ***Kushtha*** (Skin disorders), ***Kshaya*** (General debility), ***Pandu*** (Anaemia), ***Sthaulya*** (Obesity), ***Netrarogas*** (eye diseases), etc[4]. Various formulations having

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TB as an ingredient are indicated in the treatment of ***Hridrogas*** (cardiac disorders), viz. ***Hridayarnava Rasa***, ***Prabhakara Vati***, ***Kalyana Sundara Rasa***[5]. These formulations are frequently being practiced by the Ayurvedic physicians in cardiac disorders and the efficacy is anticipated because of the presence of TB. Lipids and cholesterol are directly related to obesity (***Sthaulya***)[6] ***Tamra*** (copper) has been described as ***Medopaha***[7] (destroyer of fat/lipids), ***Lekhana***[8] (scraps excessive fat), ***Sthaulyapaha***[9] (destroyer of obesity) in different texts of ***Rasashastra***. In modern researches also it is found that there is a special role of copper in lipid metabolism, its deficiency raises blood cholesterol and a diet high in copper has a beneficial effect on blood cholesterol[10]. These references directly indicate it as a lipid lowering agent.

Screening of literature revealed that, till date no pharmacological work has been reported on the role of ***Shodhana*** procedure of metals on their efficacy especially on TB. Hence to know the effect of ***Shodhana*** on efficacy, in the present study a comparative anti-hyperlipidemic activity of unpurified (***Ashodhita***) and purified (***Shodhita***) ***Tamra*** was carried out.

Materials and Methods

Test drugs

Copper wire of 99.89% purity was procured from local electrician. TB was prepared from this copper wire by subjecting it to ***Samanya*** (general), ***Vishesha*** (specific) ***Shodhana, Marana***[9] and ***Amritikarana***[11] procedures (coded as STB). Another sample of TB was prepared from the same copper wire by following only ***Marana*** and ***Amritikarana*** procedures without subjecting to the ***Shodhana*** procedure (coded as ATB).

Animals

Wistar strain albino rats of either sex, weighing 200 ± 20 g were obtained from the animal house attached to the pharmacology laboratory, I.P.G.T. & R.A, Jamnagar. They were exposed to natural day and night cycles, with ideal laboratory conditions in terms of ambient temperature (22 ± 02°C) and humidity (50-60%). Animals were fed ***ad libitum*** with Amrut brand rat pellet feed supplied by Pranav Agro Industries and tap water. The experiment was carried out after obtaining the permission from Institutional Animal Ethics Committee (Approval number: IAEC 07/2010/05/MD) and care of animals was taken as per the CPCSEA guidelines.

Dose fixation and schedule

The clinical dose of TB as mentioned in classics is 60 mg per day[11]. The suitable dose for rats was calculated by referring to table of Paget and Barnes (1964)[12] which becomes 5.5 mg/kg. The test drugs (ATB and STB) were made fine suspension in deionized water by adding few drops of gum acacia as suspending agent to suitable concentration depending up on body weight. The test drugs were administered orally with the help of gastric catheter sleeved to syringe.

Anti-hyperlipidemic activity evaluation[6]

The selected animals were divided into four groups of six animals each. First group was kept as normal control (NC) which received only deoinized water. To second group hyperlipidemic diet was administered and served as cholesterol control (CC) group. Third group received hyperlipidemic diet and ATB (5.5 mg/kg), while fourth group received hyperlipidemic diet and STB (5.5 mg/kg). Test drugs were administered at morning hour and hyperlipidemic diet was administered at evening hours for 20 consecutive days. The hyperlipidemic diet included hydrogenated vegetable oil (Vanaspati Ghee - 'Raag' brand, Batch No. BA 70, Adani Wilmar Ltd., Gujarat) and cholesterol extra pure powder (Batch No. 14022, Suvidhinath Laboratories, Baroda) made in to 20% suspension in coconut oil (Parachute coconut oil, Batch No. PSO73, Goa). The suspension was administered at the dose of 0.5 mL/100 g rat. On the 21st day, after overnight fasting, the animals were weighed and blood was collected from retro-orbital plexus under ether anesthesia. Serum was collected from blood for biochemical investigations like serum total cholesterol, serum triglyceride and serum HDL cholesterol by an auto analyzer (Fully automated Biochemical Random Access Analyzer, BS-200; Lilac Medicare Pvt. Ltd., Mumbai). References given in the kit literature mentioning the basis of the methods on which test procedures were as: serum total cholesterol[13], serum HDL cholesterol[14], serum triglyceride[15]. VLDL was calculated by using formula VLDL = TG/5, LDL by LDL = {TC-(HDL-VLDL}. Further, all the rats were sacrificed by overdose of ether anesthesia and from the sacrificed animals liver, kidney, heart and aorta were excised out. The liver, kidney and heart were weighed and fixed (including aorta) in 10% buffered neutral formalin solution. After fixation, tissues were embedded in paraffin and serial sections

were cut and each section was stained with hematoxylin and eosin[16]. The slides were viewed under trinocular research microscope (Germany) at various magnifications to note down the changes in the microscopic features of the tissues.

Statistical analysis

The data were expressed as mean ± standard error mean (SEM). The significance of differences among the groups was assessed using unpaired student's t as well as one-way ANOVA followed by Dunnett's test. ***P*** values less than 0.05 were considered as significant.

Results

In normal control group, progressive gain in body weight was observed in comparison to initial body weight (Table 1). In contrast significant increase in body weight was observed in cholesterol control rats in comparison to both initial values as well as water control group. Treatment with ATB failed to attenuate cholesterol rich diet induced weight gain, while treatment with STB apparently attenuated it. To know the effect of test drugs on various organs, the relative weight of liver, heart and kidney were measured and the outcome is provided in Table 2. Marked and statistically non-significant increase in relative weight of liver was observed in cholesterol control group in comparison to normal control group. Treatment with both ATB and STB failed to attenuate it to significant extent. Further, administration of hyperlipidemic diet did not affect the weight of heart and kidney to significant extent and treatment with test drugs also did not altered weight of these organs to significant extent.

Table 1—Effect on body weight

Groups	Initial body weight	Final body weight	Actual change in body weight (g)
NC	203.0 ± 6.4	210.00 ± 4.10	07.06 ± 2.26
CC	199.0 ± 6.9	213.30 ± 7.30	14.3 ± 2.40$^{\#}$
ATB	201.3 ± 8.5	229.30 ± 14.20	28.0 ± 7.60
STB	194.3 ± 5.5	203.30 ± 8.20	09.0 ± 7.40

Data: Mean ± SEM, $^{\#}$***P***<0.05 (Compared with normal control group)

Table 2—Effect on weight of liver, heart and kidney

Groups	Weight of liver (mg/100 g)	Weight of heart (mg/100 g)	Weight of kidney (mg/100 g)
NC	3061.8 ± 102.9	335.1 ± 6.3	755.0 ± 20.3
CC	3346.2 ± 209.8	300.19 ± 11.8	684.1 ± 18.6
ATB	3429.5 ± 103.5	370.5 ± 46.2	694.3 ± 23.6
STB	3550.2 ± 250.7	350.9 ± 28.4	756.2 ± 33.7

Data: Mean ±SEM

As an outcome, administration of exogenous cholesterol rich diet resulted in significant increase of various serum lipid profiles in cholesterol control group in comparison to control group (Table 3). Treatment with ATB did not attenuate any of parameters to significant extent, while treatment with STB significantly attenuated almost all serum lipid profiles in comparison to cholesterol control group. Histopathological section from control group shows normal cytoarchitecture of liver, kidney, heart and aorta (Plate 1a,e; Plate 2a,e). In contrast, hyperlipidemic diet produced macro and micro fatty changes in liver, cell infiltration and fatty changes in kidney and cell infiltration and fatty changes in majority of sections of heart (Plate 1b, f; Plate 2b). Some sections of aorta of cholesterol control group shows larger tunica adventitia (Plate 2f). ATB treated group does not show any significant attenuation of pathological changes in liver, heart and kidney caused due to hyperlipidemic diet (Plate 1c, g; Plate 2 c, g), whereas STB treated group showed almost normal cytoarchitecture of liver, kidney, heart and aorta (Plate 1d, h; Plate 2c, h).

Discussion

Elevated levels of different types of lipids have been implicated in the production of atherosclerosis. In this condition the blood vessel wall thickens due to accumulation of lipid in its wall ensuing inflammatory reaction. This leads to loss of elasticity of blood vessel wall and becomes the cause of many cardiovascular system (CVS) complications such as myocardial infarction, stroke, peripheral vascular disease which account for significant mortality in developed and developing countries. Through extensive studies it has been proved beyond doubt that lowering the elevated plasma lipid levels is highly effective in reducing coronary artery diseases (CAD) mortality and other CVS events mentioned

Table 3—Effect of test drugs on serum lipid profile

Groups	Cholesterol (mg/dL)	Triglyceride (mg/dL)	HDL (mg/dL)	LDL (mg/dL)	VLDL (mg/dL)
NC	66.3 ± 3.8	76.7 ± 6.9	40.3 ± 5.3	41.3 ± 10.9	15.3 ± 3.4
CC	86.2 ± 2.7$^{\#}$	176.2 ± 8.6$^{\#}$	44.0 ± 2.5	77.4 ± 10.0$^{\#}$	35.2 ± 4.2$^{\#}$
ATB	84.0 ± 2.7	139.8 ± 14.2	42.3 ± 1.6	69.6 ± 9.0	27.9 ± 6.9
STB	78.7 ± 2.8	139.2 ± 5.9**	43.7 ± 2.6	62.8 ± 6.6*	27.8 ± 2.9**

Data: Mean ± SEM, $^{\#}$***P***<0.05 One Way ANOVA with Dunnet's t test (Compared with normal control group);
*P<0.05, **P<0.01 Unpaired t test (Compared with cholesterol control group)

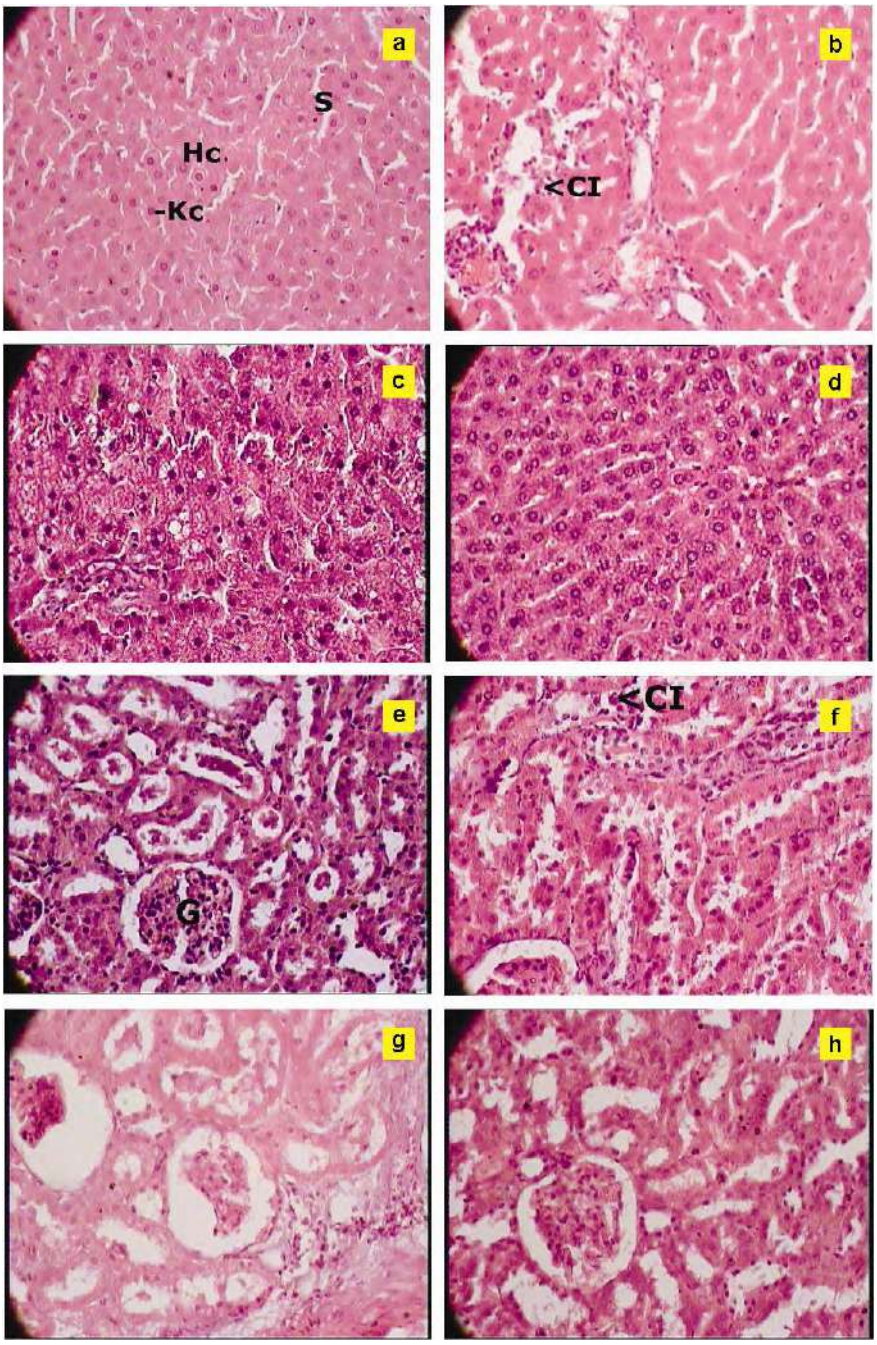

Plate 1–a. Photomicrographs of sections of liver from normal control group (1×400 magnification) (Hc-Hepatocytes; Kc-Kupffer cell; S-Sinusoid, Normal cyto architecture); b. Photomicrograph of sections of liver from cholesterol control group (1×400 magnification) (CI-Cell infiltration; Severe cell infiltration and micro fatty changes); c. Photomicrograph of sections of liver from ATB treated group (1×400 magnification, Comparatively less fatty changes); d. Photomicrograph of sections of liver from STB treated group (1×400 magnification, Normal cyto architecture); e. Photomicrographs of sections of kidney from normal control group (1×400 magnification) (G-Glomerulus; Ct-Convoluted tubule); f. Photomicrographs of sections of kidney from cholesterol control group (1×400 magnification) (Fc-Fatty changes; CI-Cell infiltration, Cell infiltration and micro-fatty changes); g. Photomicrograph of sections of kidney from ATB treated group (1×400 magnification); h. Photomicrograph of sections of kidney from STB treated group (1×400 magnification)

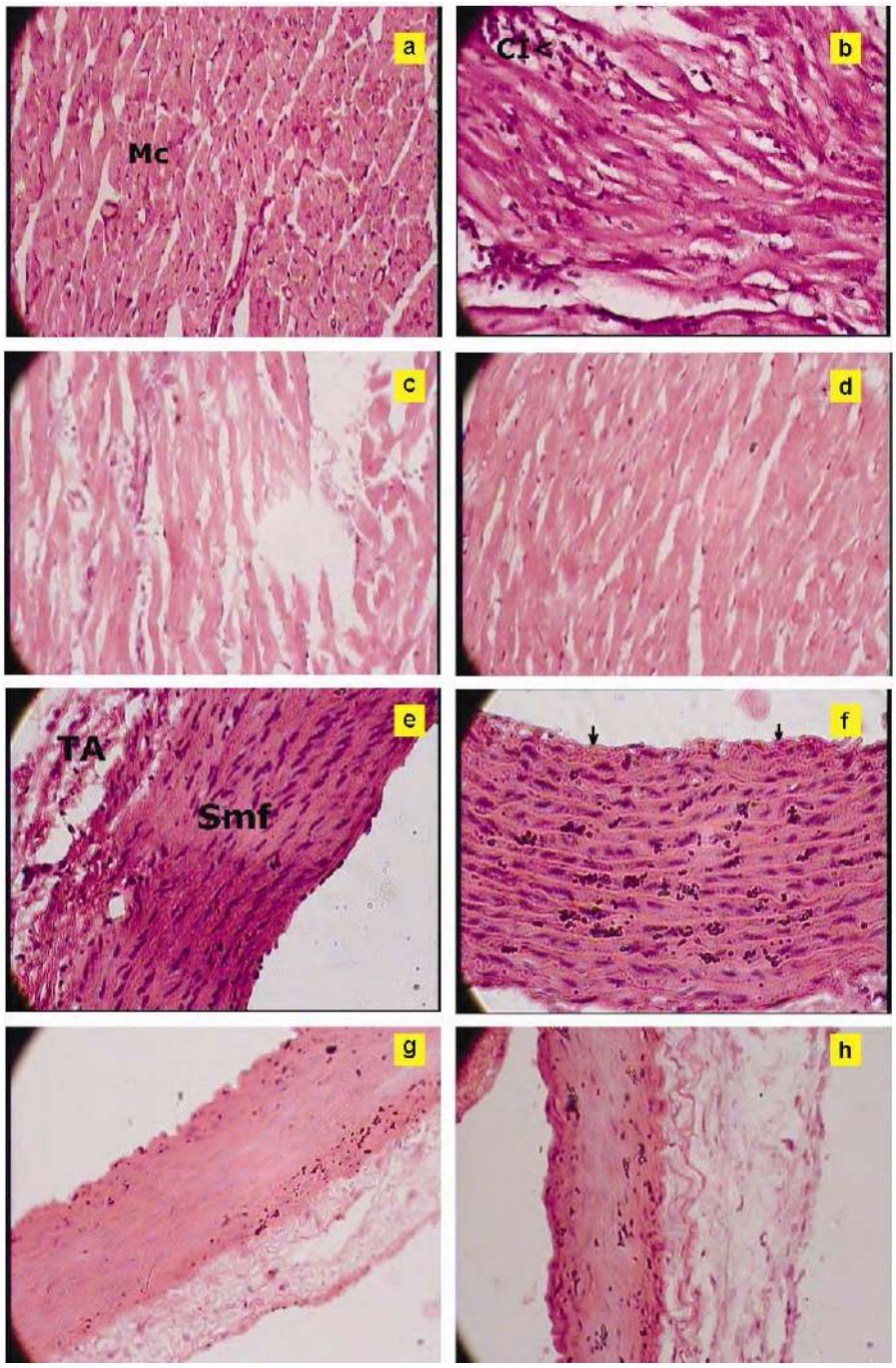

Plate 2–a. Photomicrographs of sections of heart from normal control group (1×400 magnification) (Mc-Myocardium; b. Photomicrograph of sections of heart from cholesterol control group (1×400 magnification) (CI-Cell infiltration); c. Photomicrograph of sections of heart from ATB treated group (1× 400 magnification); d. Photomicrograph of sections of heart from STB treated group (1×400 magnification); e. Photomicrographs of sections of aorta from normal control group (1×400 magnification); Smf- Smooth muscle fibre; TA- Tunica adventia; f. Photomicrographs of sections of aorta from cholesterol control group (1×400 magnification); g. Photomicrograph of sections of aorta from ATB treated group (1×400 magnification) ; h. Photomicrograph of sections of aorta from STB treated group (1× 400 magnification).

above[17]. Diet and when needed, drug therapy for hypercholesterolemia is clearly indicated for individuals with existing CAD, as well as for individuals with multiple risk factors for cardiovascular diseases. Thus there is scope for the introduction of effective hypolipidemic drugs in to existing therapeutic armamentarium.

Diet induced hyperlipidemia is considered as better animal model for investigating antihyperlipidemic activity because the hyperlipidemia induced by diet is more similar to human situation. Serum cholesterol levels increase by accelerating the biosynthesis of saturated fats in the diet when taken in excess, whereas diet containing polyunsaturated fatty acids lowers the cholesterol level[18]. This may explain the significant elevation of serum cholesterol, serum triglycerides, serum LDL and serum VLDL levels in comparison to control rats on normal diet in the present study. Similarly, several other workers have also reported increased blood and tissue levels of cholesterol after feeding high fat diet for varying period[19-21].

Administration of hyperlipidemic diet led to significant increase in body weight of cholesterol control group albino rats when compared to normal control rats. Treatment with ATB failed to attenuate cholesterol rich diet induced weight gain, while treatment with STB apparently attenuated it. This indicates that the test drug STB has antagonizing effect against hyperlipidemic diet induced changes in the body weight. Administration of ATB failed to attenuate the increased serum lipid profile, while serum triglycerides, LDL and VLDL cholesterol levels were significantly attenuated by treatment with STB. The observed hypolipidemic activity of STB is further evidenced by histopathological examination of liver, heart, kidney and aorta. Organs from STB group showed significant attenuation effect on hyperlipidemic diet induced pathological changes whereas ATB not only failed to prevent hyperlipidemic diet induced pathological changes in these organs but it is also cardio toxic (evidenced by myocarditis) which is not present in cholesterol control group. The above profile indicates that STB can not only reverse hyperlipidemic diet induced changes in liver, heart and kidney, but also is devoid of cardio toxic effect.

Hypercholesterolemia, a high cholesterol diet and oxidative stress increase serum LDL levels resulting in increased risk for development of atherosclerosis[22]. The first line defensive enzymes against the free radical produced during the oxidative stress are the antioxidant enzymes, mainly superoxide dismutase (SOD) and catalase[23]. Like other metals copper is also considered as an essential element of body for normal physiological functions[24]. Deficiency of copper leads to anaemia, nervous weakness, weakness in connective tissue and the hypo activity of lysyl oxidase, cytochrome C oxidase, SOD, etc[25]. Researches have established the linkage between cholesterol metabolism and copper utilization[26,27]. Hypercholesterolemia from copper deficiency in several species has been found in at least 22 independent laboratories world wide[10]. TB is the rich source of copper. Previous studies have reported that, TB inhibits lipid peroxidation and induces the activity of SOD; thus proving it as a strong anti oxidant agent[28,29].

Observed anti-hyperlipidemic activity of STB may be attributed to involvement of one or more mechanisms, viz. by interfering with the absorption of the cholesterol from dietary sources, by interfering with the re-esterification or incorporation of fatty acids to form chylomicrons in the intestinal epithelial cells, by interfering with the formation of endogenous triglycerides in the tissues by inhibiting the enzyme diacylglycerol transferase, by interfering with the transport of triglycerides from endoplasmic reticulum to microsomal site which is by microsomal triglyceride transport protein, by inhibiting the activity of the lipoprotein lipase at different sites, by inhibiting the activity of the rate limiting enzyme in cholesterol bio-synthesis - HMG-CoA (3-hydroxy 3-methyl 3-methylglutaryl CoA).

Conclusion

Tamra Bhasma prepared from ***Shodhita Tamra*** possesses significant anti-hyperlipidemic activity, while ***Ashodhita Tamra Bhasma*** is lack of it besides it is having cardio toxic property. This shows that the ***Rasashastriya Shodhana*** procedure have definite role in not only increasing the efficacy of the drug, but also in removing the toxicity. Further comparative antihyperlipidemic activity with established antihyperlipidemic drug is required to provide some insight into the probable mechanism involved in activity profile.

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REVIEW ARTICLE

Critical review on the pharmaceutical vistas of *Lauha Kalpas* (Iron formulations)

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ABSTRACT

Iron is one among the major metals present in the earth's crust and is essential for sound sustenance of human body. Its deficiency leads to various health ailments. Contemporary medicine advises iron supplements in iron deficiency anemia. Ayurvedic classics also quote significant information about administration of iron. *Lauha Kalpas* are the unique compound herbo-mineral formulations where iron (*Lauha*) is used as a major ingredient. Relevant literature (Bhaishajya Ratnavali, Charaka Samhita, Rasendra Sara Samgraha etc.) reviewed to gather information about *Lauha Kalpas*. Critical analysis of these *Lauha Kalpas* reveals that ancient seers administered iron in a better acceptable form. Unlike popular understanding these are not only Khalviya preparations; but Churna (powders), Avaleha (confectionaries), Rasakriya (solidified decoctions), and Putapaka (incinerated) form of preparations are also found. Apart from solid dosage forms, semisolid dosage forms mentioned in classics are very much useful. Unfortunately most of the formulations are not found in the market. Hence Pharmaceutical firms may bring these unique dosage forms in to the market to supply the healthcare needs of the community. It is interesting that iron preparations are used in Ayurveda in different medical conditions apart from anemia (Pandu). This leaves a scope for further researches on different dosage forms of iron and their indications.

Key words: Ayurveda, Bhasma, Hematinics, Iron, Lauha Kalpa, pharmaceutics, Rasaushadhi

INTRODUCTION

Ayurveda is a well-documented Traditional system of Indian Medicine (TIM). *Rasa Shastra,* an offshoot of Ayurveda popular from medieval period, mostly deals with therapeutic utilization of metals and minerals.[1,2] Hundreds of formulations are explained in classical texts with the permutation combination of *Bhasmas* along with herbal ingredients. Careful review shows that there are around 30 types of *Bhasmas,* which are frequently used. A simple change in the method of preparation and ingredients can bring a change in the action, indication, and efficacy. Hence, it is the need of the hour to critically analyze the formulations and the rationality behind it. This may be a guideline for further research to unravel Ayurveda. Iron is a noncontroversial metal for therapeutic use since centuries in east as well as west. Iron containing drugs are widely used in modern medicine as hematinics. These drugs are known to induce some adverse drug reactions -- gastro intestinal symptoms (nausea, vomiting, epigastric pain, eructation, pyrosis, meteorism, borborygami, colic pain, flatulence, constipation, black feces, and diarrhea)[3] The hematinics market in India is currently worth around Rs. 900 crore and is growing at 15% per annum.[4] Hence it is the need of the hour to search some alternative from other systems of medicine like Ayurveda. *Lauha Kalpa*s can be a better alternative from Ayurveda. Till date, no scientific comprehensive review has been done on *Lauha Kalpa*s. Hence this study is undertaken.

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Lauha Kalpas (LK) are formulations which possess *Lauha Bhasma (calx of iron)* as the major ingredient along with the other herbal ingredients. These formulations have "*Lauha*" as suffix in their name. Some *Lauha Kalpas* possess other mineral ingredients including mercury along with *Lauha* as main ingredient.

Background

Charaka Samhita quotes the nonexistence of any entity

which is not a medicine (*Nanaushadhi*) in the universe. Acharya *Charaka* further elucidates that every object can be a medicine if used wisely with a logical thought (*Yukthi*).[5] Considering this fact, the three forms of natural materials -- herbal, mineral, and animal origins were subjected to trials and their therapeutic values were established. Due to the toxic nature of the minerals and metals, they were less used for the internal administration compared to herbs. Conversion of metals into suitable consumable medicinal form is found in the preparation of *Lohadi Rasayana*[6] and *Ayaskriti*.[7] In the *Samhita* period, iron (*Ayas-Lauha*) was used in the form of fine powder. Later *Rasa shastra* classical texts explained the *Shodhana* [i] and *Marana* [ii] methods.

Some researches have been carried out on individual *Lauha Kalpas* like *Navayasa Lauha*,[8] *Nayanamrita Lauha*,[9] *Sapthamrita Lauha*.[10]

According to Ayurvedic Formulary of India, *Lauha Kalpas* are the formulations of *Lauha Bhasma (LB)* as main ingredient added to other drugs. The drugs are reduced to fine powder and mixed with *Lauha Bhasma*. The *Bhavana* process is carried out with prescribed liquids if mentioned.[11] When well protected from moisture and heat, they preserve their potency for a period of about 10 years. Preparations containing mercury or its compounds preserve their potency indefinitely.[12]

MATERIALS AND METHODS

Important texts of *Ayurveda* commencing from *Charaka Samhita* (CS) to *Rasa Ratna Samucchaya* (RRS), *Rasendra Sara Samgraha* (RSS), and *Bhaishajya Ratnavali* (BR) have been the sources for *Lauha Kalpas*. BR being a comprehensive source for *LK*'s was the main source and the other important formulations are selected from other classics and enumerated according to the method of preparation.

Only the formulations having suffix as *Lauha* are considered for the study. The other *Lauha Bhasma* containing dosage forms such as *Asava* and *Arishta* (alcoholic formulations) are excluded from the study.

Quantity of *Lauha Bhasma* in few formulations is quoted as "*Sarva dravya samam Lauham*" which means 50% of the formulation will be *Lauha Bhasma*. In other formulations the quantity is calculated in accordance with the solid constituents, where in the ingredients like decoctions (*Kwatha*), fresh juices (*Swarasa*), ghee (*Ghrita* -clarified butter), and honey (*Madhu*) are not taken into consideration. Calculation is based on the raw ingredients taken prior to the pharmaceutical processes. Hence the percentage in the final product may change except in *Churnas*.

Method of calculation

Percentage of *LB* =100×weight of *LB*/weight of total solid ingredients (including *LB*). E.g., in *Mahamrityunjaya Lauha* (*BR* 41/136-45) total weight of all ingredients (mineral and herbal) is 17 *Tola*[iii], i.e., 17×12 = 204 g out of which 2 *Tola* (24 g) is *LB*. *Bhavana Dravya*, *Guduchi*, and *Ardraka Swarasa* are not considered for calculation.

Percentage of *LB* is 100×24 g/204 g = 11.76, i.e., approximately 12%

Churna Lauha Kalpa (Powder mixtures)

Churnas[13] (powder mixtures) are prepared by pounding the dry drugs and then sieving through a fine cloth or sieves. *Lauha Kalpa* in *Churna* form are prepared by adding the fine powder of the herbal drugs to the *Lauha Bhasma* and triturated (dry) until homogenous mixture is obtained [Table 1].

Khalviya Lauha Kalpa

Herbal juices advocated are added to mixture of the metals--minerals (*Bhasmas)* and herbs and then triturated till the liquid portion is totally dried. The liquid added should be optimum to form soft or soggy mass[15] [Table 2].

Some formulations do not contain herbal ingredients. The mineral ingredients are triturated with advocated herbal juices [Tables 3-6].

Lauha Rasakriya Kalpa

When primary liquid dosage forms are boiled until thicker consistency is attained, so that they can be rolled as pills, it is called *Rasakriya*. Here *Lauha Bhasma* is boiled along with different herbal juices and decoctions until it converts to solid dosage form[19] [Table 7].

Lauha Avaleha Kalpa

Literally, *Lehya* refers to that which is consumed by licking. It is a semisolid dosage form, prepared by solidifying the decoctions, etc. primary liquid dosage forms by boiling along with sugar-, jaggery-like sweetening agents and after it attains proper consistency fine powders of drugs (*Prakshepa Dravya*), ghee, and honey are added[21] [Table 8].

Putapaka Lauha Kalpa

The formulations which are prepared by the incineration method (*Puta Paka*) are included under this group. *Puta* indicates the specific quantum of heat required for conversion of a particular metal--mineral into an assimilable medicine using the suitable incineration method[22] [Table 9].

DISCUSSION

Ancient *Ayurveda* even after centuries is an inspiration

Table 1: *Churna Lauha Kalpas*

Compound name	Reference	Mineral ingredients
*[*Δ]Chandanadi Lauha*	*BR-(Jwara) 5/1145*	*LB (50%)*
Manasuranadya Lauha	*BR-(Arsha) 9/212*	*LB (50%)*
Vidangadi Lauha-1	*BR-(Pandu) 12/31*	*LB (50%)*
Darvyadi Lauha	*BR-Pandu 12/37*	*LB (50%)*
Dhatri Lauha	*BR-(Pandu) 12/30*	*LB (20%)*
*[*Δ]Navayasa (Lauha) Churna*	*CS Pandu Chikitsa 16/70-1*	*LB (50%)*
[*]Satamulyadi Lauha	*BR-(Rakta Pitta) 13/75-6*	*LB (50%)*
Sharkaradya Lauha	*BR- (Rakta Pitta) 13/77*	*LB (50%)*
[*]Raktapittantaka Lauha	*BR-(Rakta Pitta) 13/79-9*	*LB (50%)*
Yakshmanthaka Lauha	*BR-(Raja Yakshma) 14/84-5*	*LB (50%), Shilajit*
[*]Shilajitwadi Lauha	*BR-(Raja Yakshma) 14/86*	*LB (50%), SMB, Shilajit*
Rajatadi Lauha	*BR-(Raja Yakshma) 14/87-8*	*LB (50%), RB, AB*
Sama Sharkara Lauha (Kasa)	*BR-(Kasa) 15/116-20*	*LB (2%) , AB, YK*
[*]Guduchyadi Lauha	*BR- (Vatarakta) 27/59*	*LB (50%)*
Triphaladi Lauha	*BR-(Amavata) 29/99-101*	*LB (33%)*
Triphala Lauha	*BR-(Shula) 30/128*	*LB (50%)*
Vaishwanara Lauha	*BR-(Shula) 30/137-9*	*LB (50%) Shambhuka Bhasma, Saindava Lavana, Apamarga Kshara, Chincha Kshara*
Sharkara Lauha	*BR-(Shula) 30/129*	*LB (50%)*
Virdda darvyadi Lauha	*BR- Amavata (Parishishta) /110-1*	*LB (50%)*
Madhukadya Lauha	*BR-(Netra Roga) 64/231*	*LB (20%)*
Trikatwadi Lauha	*BR-(Shotha) 42/131-2*	*LB (67%)*
Varunadya Lauha	*BR- (Asmari) 36/44-47 (Parisishta) /119-22 Mutra kriccra*	*LB (2.75%), AB*
Tryushanadya Lauha	*BR-(Medoroga) 39/26-29*	*LB (50%), Chatur Lavana [x]*
[*]Pippalyadi Lauha-2	*BR-(Udara) 40/130*	*LB (50%), AB, Saindava Lavana*
*[*Δ]Rohitaka Lauha*	*BR- (Pliha Yakrit roga) 41/117*	*LB (50%),*
[Δ]Shothari Lauha	*BR-(Shotha) 42/133*	*LB (50%), YK*
Suvarchaladya Lauha	*BR-(Shotha) 42/134*	*LB (50%)*
[Δ]Amla Pittantaka Lauha	*BR-(Amlapitta) 56/33*	*LB (16.7), RS, TB,*
Pradarantak Lauha[14]	*RT-20/118-22*	*LB (11%), VB, Gairika,*
Tapyadi Lauha-2	*AH-{Pandu Ch)16/20-2*	*SMB, RB, LB (12%), MB, Shilajit*

Table 2: Khalviya formulations: Prepared by using water for *Bhavana*

Compound name	Reference	Mineral ingredients
*[*Δ]Sarvajwara hara Lauha*	*BR-(Jwara) 5/1170-4*	*LB (50%)*
Varunadya Lauha	*BR-(Medo roga) 39/22-5*	*LB (50%)*
[Δ]Chandramrita Lauha	*BR-(Kasa) 15/121-26*	*LB (50%) Saindava Lavana*
Vishamajwarantaka Lauha-1	*BR-(Jwara) 5/1146-50*	*LB (50%), AB, Rasaka Bhasma, Manashila*
*[*Δ]Yakridhari Lauha*	*BR- (Pliha Yakrit Roga) 41/129-31*	*LB (22%), AB, TB, Mriga Charma Bhasma*
*[*Δ]Pradarantaka Lauha*	*BR-(Udara) 66/79-83*	*LB (3.3), TB, VB, AB, Haratala, Kapardika Bhasma, Shanka Bhasma, Pancha Lavana*

Table 3: Khalviya formulations: Prepared by *Bhavana* with herbal juices having only mineral ingredients

Compound name	Reference	Mineral ingredients	Bhavana Dravyas
[*]Vishamajwarantaka Lauha -2	*BR-(Jwara) 5/1151-4*	*LB(50%), Kajjali, TB, SMB*	*Jayanti, Kokilaksha, Vasa, Ardhraka, Tamala Patra Swarasas*
[*]Sarvajwarahara Lauha (Brihat-2)	*BR- (Jwara) 5/1180-92*	*KLB (33%), Kajjali, SwB, RB, TB, SMB, AB, Haratala*	*Karavella, Dasamula, Parpata, Triphala,[vii] Guduchi, Nagavalli, Kakamachi, Nirgundi, Punarnava, Ardhraka Swarasas*
[*]Yakritplihodarari Lauha	*BR- (Pliha Yakrit roga) 41/162-66*	*LB (50%), SwB, RB, TB, VB, AB, SMB*	*Ardraka, Shephali (Parijata), Bilva Kirata Tikta, Tulasi Swarasas*
[Δ]Vadavagni Lauha	*BR-(Medoroga) 39/30-1*	*LB (25%), RS, Haratala, TB*	*Arka Swarasa*

Table 4: *Khalviya* formulations: Prepared with *Bhavana* of *Sneha dravya* (oil and ghee) and *Madhura dravya* (Sugars, Jaggery, and Honey) and *Drava Dravya* (Herbal juices and decoctions)

Compound name	Reference	Mineral ingredients	*Sneha dravya, Madhura dravya* and *Drava Dravya* for *Bhavana*
Ashtadashanga Lauha (Kiratadi Mandura)	*BR-Pandu 12/34-36*	*LB (50%)*	*Honey, Cow's Ghee*
Yograja	*C.S Chi. Pandu 16/78-86, BR-(Pandu) 12/109-15*	*LB (12.8), SMB, Rajata Makshika Bh, Shilajit*	*Sugar candy, Honey*
Maha Shwasari Lauha	*BR- (Hikka Shwasa) 16/39-41*	*LB (15.3%), AB, Vamsalochana*	*Sugar candy, Honey*
**ΔSaptamrita Lauha*	*BR-(Shula) 30/130 64/234-36*	*LB (50%)*	*Honey, Cow's Ghee*
Yakshmari Lauha	*BR-(Rajayakshma) 14/83*	*LB (50%), SMB, Shilajit*	*Cow's Ghee*
Shularaja Lauha	*BR-(Shula) 30/131-36*	*KLB (3.7%), AB*	*Sugar candy, Honey*
Trikatrayadi Lauha	*BR-(Pandu) 12/38-43*	*KLB (9%), MB*	*Honey, Cow's Ghee, five types of Sharkara (sugars)*
**Pippalyadi Lauha-1*	*BR-(Hikkashwasa) 16/42-3*	*LB (50%)*	*Honey, water*
Sarvatobhadra Lauha	*BR (Amlapitta) 56/42-53*	*LB (16%), TB, AB, Kajjali (Dviguna), SMB, Manahshila, Shilajit*	*Honey, Cow's Ghee*

Table 5: *Khalviya* formulations: prepared by *Bhavana* of both mineral and herbal ingredients (solid dosage form)

Compound Name	Reference	Mineral ingredients	Bhavana Dravya
Kamalantaka Lauha	*BR-(Pandu) 12/44-51*	*LB (23.5%), AB, MB, VB*	*Kesaraja, Bringaraja, Somaraji (Bakuchi), Manduka parni*
Kalamegha Navayasa Lauha[16]	*SYS-(Pandu)*	*LB (50%)*	*Kalamegha (Bhavana 7days)*
Langalyadya Lauha	*BR- (Vatarakta) 27/64-66*	*LB (50%)*	*Nimbu Swarasa, Triphala Kwatha*
**ΔDhatri Lauha(Shula)-1*	*BR-(Shula) 30/149-158*	*LB (28.5%)*	*Guduchi Kwatha*
Dhatri Lauha(Shula)-2	*BR-(Shula) 30/159-62*	*LB (4%), MB, AB*	*Yava, Satavari Swarasa, Amalaki Swarasa*
Shlipadari Lauha	*BR-(shlipada) 45/39-40*	*KLB (20%), Shilajit*	*Triphala Kwatha*
Karsyahara Lauha	*BR-(Rasayana) 73/55-6*	*LB (50%)*	*Bringaraja Swarasa*
Tandavari Lauha	*BR (Tandava Roga) 80/4-5*	*LB (75%), Shanka Pashana (Arsenic oxide), YB*	*Bhanga, Kupilu, Arjuna Kashaya*
Bala Yakruddhari Lauha[17]	*Bala roga Ayurved Vignan*	*LB (12.5%), 1000 Puti AB, RS*	*Guduchi Swarasa*
Nayanamrita Lauha[18]	*RSS 2-Netra roga/9-12*	*LB (5%), AB*	*Triphala Kwatha, Bringaraja Swarasa*

Table 6: *Khalviya* formulations: Prepared with mercurial ingredients (*Kajjali and Rasa Sindhura*) and *Bhavana* with herbal juices

Compound name	Reference	Mineral ingredients	Bhavana Dravya
Sarvajwarahara Lauha (Brihat-1)	*BR- (Jwara) 5/1175-9*	*LB (44.5%), Kajjali,*	*Ardraka Swarasa*
Jwarantaka Lauha (Brihat)	*BR-(Jwara) 5/1193-203*	*LB (1.7%), AB, RB, Kajjali, SwB, Shilajit, Saindhava, Vida Lavana*	*Ardraka Swarasa*
Pittantaka Lauha	*BR- (Vatarakta) 27/60-63*	*LB (50%)Kajjali, AB, TB*	*Guduchi Swarasa*
ΔYakritplihari Lauha	*BR- (Pliha Yakrit Roga) 41/123-28*	*LB (9%), Kajjali, AB, TB, Manahshila, Shilajit, Tankana*	*Danthimula, Trivrit, Chitraka, Sandhalu, Trikatu,*[viii] *Ardraka, Bringaraja swarasas*
Yakridari Lauha (Brihat)	*BR- (Pliha Yakrit roga) 41/132-5*	*LB (50%), Kajjali, AB,*	*Guduchi Swarasa*
Mahamrityunjaya Lauha	*BR- (Plihayakrit roga) 41/136-45*	*LB (12%), Kajjali, AB, TB, Sarja, YK, Saindhava, Vida Lavana, Varatika, Shankha Bhasma, Manashila, Haratala, Tuttha,*	*Ardraka Swarasa, Guduchi Swarasa*
Sarveswara Lauha	*BR- (Plihayakrit roga) 41/146-53*	*LB (7.4%), Kajjali, AB, TB, SMB*	*Ardraka Swarasa*

for the researchers to think beyond their imagination and knowledge. The same is true with pharmaceutical aspects. A better understanding of Ayurveda is adding up new things to the existent pharmaceutical excellence. *Rasaushadhis* are the formulations which are discussed in present era for right or wrong reasons. Even after this

Table 7: *Rasa Kriya Lauha Kalpas*: Formulations prepared by boiling decoctions until solidification

Compound name	Reference	Mineral ingredients	*Kwatha Dravya*
*[Δ]*Vidangadi Lauha-2*	*BR-(Pandu) 12/31*	*LB (50%)*	*Cow's urine*
[Δ]Tapyadi Lauha-1[20]	*AH-(Pandu Chi) 16/16-9*	*LB, Shilajit, SMB, RB, MB*	*Cow's urine*
Vidangadi Lauha (Amavata)	*BR-(Amavata) 29/102-10*	*Kajjali, Vajra, Pandyadi Loha (25%), AB,*	*Triphala Kwatha, Satavari, Cow's milk*
Panchanana Rasa Lauha	*BR-(Amavata) 29/111-19*	*Kajjali, LB (17%), AB*	*Satavari, Cow's milk*
Shothodarari Lauha	*BR-(Udara) 40/120-9*	*Kajjali, LB (50%), TB, Kankushta,*	*Punarnava, Guduchi, Chitraka, Indrayana, Mana Kanda, Shigru, Suryavarta, Arka Kwatha, Cow's Ghee, Arka, Snuhi Kshira, Guggulu,*
Chitrakadi Lauha	*BR- (Plihayakrit roga) 41/118-22*	*LB (50%), AB,TB, YK, Pancha Lavana [xi]*	*Cow's urine, honey*
Amla Pittantaka Lauha-2	*BR-(Amlapitta) 56/36-41*	*Kajjali, MB, KLB (20%), AB, MB, Pravala*	*Amalaki Swarasa*
Lakshmana Lauha	*BR-(Pradara) 66/84-6*	*LB (50%)*	*Lakshmana*
Pradarari Lauha	*BR-(Pradara) 66/74-8*	*LB (10%), AB*	*Kutaja Kwatha*

Table 8: *Avaleha Lauha Kalpas*: Prepared by boiling decoctions with sugar or jaggery

Compound name	Reference	Mineral ingredients	*Kwatha Dravyas*
Agnimukha Lauha	*BR-(Arsha) 9/213-21*	*LB (30% to Kwatha Dravya), Shilajit*	*Trivrit, Chitraka, Nirgundi, Snuhi, Mundi/Munditaki, Bhumyamlaki, Cow's Ghee, sugar*
Khandakadya Lauha (guda paka)	*BR- (Raktapitta) 13/80-90*	*LB (50% to Kwatha Dravya), Shilajit*	*Satavari, Vasa, Guduchi, Bala Mundi, Musali, Khadira, Triphala, Barangi, Pushkaramula, , Guda (Jaggery)*
Sama Sharkara Lauha	*BR (Raktapitta) 13/72-4*	*LB (80% to Prakshepa -Vidanga)*	*Cow's milk (4 times to LB), Cow's Ghee, (2 times to LB), Honey, Sugar candy*
Chatuhsama Lauha	*BR-(Shula) 30/140-148*	*LB (25% in mineral ingredients), Kajjali, AB, TB*	*Cow's Ghee, Cow's milk*
Rasayanamrita Lauha	*BR-(Gulma) 32/121-126*	*LB (44.5%), AB, Saindava Lavana*	*Sugar candy, Triphala Kwatha, Jambhiri Nimbhu, Cow's Ghee*
Yakritplihodarahara Lauha	*BR-(Pliha Yakrit roga) 41/154-61*	*LB (50% in mineral ingredients), AB, RS, Pancha Lavana, YK*	*Triphala Kwatha, Satavari Swarasa, Cow's Ghee*
Amritankura Lauha	*BR-(Kushtha) 54/186-93*	*LB (12%), RS, Gandhaka, TB, AB*	*Triphala Kwatha, Cow's Ghee*

Table 9: Formulations prepared with Puta Paka method

Name of the formulation	Reference	Mineral ingredients	Herbal drugs and method processing
[*]Puta Pakva Vishamajwarantaka Lauha [23]	*BR- (Jwara) 5/1162-9*	*LB (9.6%), Parpati, SB, TB, AB, VB, Gairika, Pravala, Mukta, Shankha, Shukti Bh*	*LAGHU PUTA*
Shankara Lauha (Durnamari Lauha) [24]	*BP Madyama Khanda-(Arsha) 5/96-125*	*LB, RB (prepared applying Manashila, Parada, SMB for Shodhana)*	*Triphala, Ardraka, Bhringaraja, Manakanda, Surana, Bhallataka, Chitraka, Cow's Ghee LAGHU PUTA*

*- Formulations mentioned in AFI part 1and2, Δ- Formulations available in the market (source -Ayurvedline) AH-Ashtanga Hridaya, YR-Yoga Ratnakara, SYS-Siddha Yoga Samgraha, RT- Rasa Tarangini, LB-Lauha (Iron or Steel) Bhasma, KLB-Kanta Lauha (Magnetite or Lodestone) Bhasma, MB-Mandura (Iron rust) Bhasma, RB- Rajata (Silver) Bhasma, VB-Vanga (Tin) Bhasma, SMB- Swarna Makshika (Copper pyrite) Bhasma, SwB- Swarna (Gold) Bhasma, YK - Yava Kshara

hue and cry it is impossible to discard them due to their effectiveness. *Rasaushadhis* (herbomineral formulations) are being prescribed by *Ayurvedic* physicians since long with a rare mention of toxicity. It is observed that herb--mineral complexes are more stable and more interactive compared to plain herbs as these result in faster therapeutic action and have a longer shelf life.[25] *Lauha Kalpas* are formulations which are safe, effective, and noteworthy compound formulations of iron. Prior to the period of *Rasa Shastra*, *Lauha* was used in the form of ultra fine powders (*Anjana sadrisha* -- collyrium like). A number of references for internal administration of *Lauha* and noble metals like gold and silver are found in our classics, as they are relatively less toxic than other metals in elemental form.[26,27] In the medieval period, the internal administration of all metals and minerals became possible because of the invention of pharmaceutical technology of converting metals and minerals into *Bhasmas*, i.e., detoxification, (*Shodhana*) converting into powders (*Jarana,* causing decay of metals) and incineration (*Marana,* killing metallic properties) methods.

After careful review of *Lauha Kalpas,* it is found that unlike popular understanding they all are not only *Khalviya* preparations but can be classified based on the methods of preparation into *Churna Lauha Kalpa*, *Khalviya Lauha Kalpa*, *Rasa Kriya Lauha Kalpa, Avaleha Lauha Kalpa*, and *Puta Paka Lauha Kalpa.* Other than these listed formulations, special

pharmaceutical preparations like *Parpati* (e.g., *Rasa Parpati*, *Panchamrita Parpati*), *Asava*, and *Arishta* are also described.

The percentage of LB varies from as less as 2–75% (*Jwarantaka Lauha* -- 1.7%, *Vidangadi Lauha* -- 2%, *Tandavari Lauha* -- 75%) in *Lauha Kalpa*s. Many formulations contain 50% of LB. Herbal ingredients found in maximum formulations are *Trika Traya*.[vi]

Khalviya Lauha Kalpas are prepared by wet trituration (*Bhavana,* impregnation) with liquid ingredients, i.e., decoctions and herbal juices. In few formulations, water is used as a media for *Bhavana*. Wet trituration (*Toya Sannikarsha*) facilitates particle size reduction and homogenization leading to modification of properties (*Gunantharadhana*) of the end product.[28] In the pharmaceutical preparation of *Chaushashta Prahara Pippali,* it was observed that the number of *Bhavanas* with *Pippali Kwatha,* was inversely proportional to the piperine content.[29] With this information inference can be drawn that reduction of piperine is anticipated in this particular formulation, justifying the significance of the *Bhavana* process. In another work, which was intended for the study of shelf life and interactions of *Vidanga* with iron-in-iron containing formulations like *Vidangadi Lauha*, *Chandanadi Lauha*, and *Navayasa Churna* showed the reduction of embelin content observed in 6 months shelf life studies. *Navayasa Lauha* decreases less compared with other two formulations. This study concludes that iron can interact and can react with embelin and form a complex.[30]

Triphala mainly consists of tannin, gallic acid, ascorbic acid (vitamin c), and phenolics. Ascorbic acid increases the bioavailability of iron by converting Fe^{3+} to Fe^{2+}, while phenolics can reduce the iron by binding to it. The presence of ascorbic acid or a lack of dietary tannins has both been suggested as contributing to clinical/pathological iron storage disease. Too much iron is toxic. It can damage the liver, heart, and pancreas and irritate the stomach and gut, causing constipation and diarrhea. In other words, this may also be taken as the various constituents of *Triphala* have antagonizing activity. Thus, too much iron absorption is prevented.[31] *Triphala* is a mild laxative and thereby counteracts the constipating property of iron and thus be beneficial due to which *Acharyas* might have mentioned *Triphala* in maximum *Lauha* formulations.

The findings from one study suggested that *Triphala* and its individual constituents have an inhibitory effect on metabolic enzymes when consumed along with therapeutic products. Further the inhibitory effects were relatively comparable to all the constituents tested, despite the variability of the content of biomarker. *Triphala* and its ingredients are likely to inhibit drug metabolizing enzymes, but less likely to produce significant drug interactions. Certain major factors of metabolism such as competition between coadministered drugs, unspecific interactions with proteins, and enzyme induction due to chronic intake are not addressed in that *in vitro* assay. However, one study clearly suggested that herbal products containing gallic acid may have the potential to inhibit the metabolism of certain coadministered drugs.[32] Hence a conclusion can be drawn that the presence of *Triphala* is a facilitator in the formulations.

Many formulations are said to be triturated with honey and sugar, due to which it becomes difficult to store them in tablet form, as honey and sugar (as liquids added while preparation) are hygroscopic and they become semisolid. Hence they have to be stored and consumed in semisolid form. Same is the problem with formulations containing Cow's *ghee*. *Sapthamrita Lauha* and *Madhukadi Lauha* both comprise *Triphala*, *Yashti* as ingredients. Honey and Cow's *ghee* are ingredients in *Sapthamrita Lauha* but they are *Anupana* (vehicle, adjuvant) in *Madhukadi Lauha*. Ideally *Sapthamrita Lauha* has to be prepared by adding honey and *ghee* and should be in semisolid dosage form and *Madhukadi Lauha* should be solid dosage form. But *Sapthamrita Lauha*, which is available in the market, is in solid dosage form[33] and devoid of honey and *ghee*; hence it will be rational to name it as *Madhukadi Lauha*.

It is understood that many proteins play significant role in absorption of iron from intestine (such as hespidin, DMT-1, ceruloplasmin) and are also required for efflux of iron from enterocytes. Iron supplements require bioavailability enhancers to minimize the side effects. Herbomineral formulations can be used to reduce various side effects as the processing of various herbal juices with already processed and micro-fined minerals lead to the formation of herbomineral complexes. These complexes upon interaction with digestive juices adopt a colloidal form, for faster absorption. Sometimes they play a catalytic role facilitating absorption of other nutrients and correcting a disease process.[34]

Few *Khalviya Lauha Kalpas* comprise *Parada* (Hg), *Gandhaka* (S) as ingredients. Initially *Kajjali* (HgS -- black) has to be prepared, later remaining powders are subjected for wet trituration and pills are to be made. These two types of formulations can be made into pills and stored; hence these are solid dosage forms. Few are prepared by adding mineral ingredients in decoctions, fresh juices and cow's urine, etc. and boiled, after it obtains proper consistency the remaining herbal ingredients (*Prakshepa dravya*) are to be added and pills are to be made. Cow's urine if taken has to be eight times to that of the quantity of *LB*.

Most of the LKs do not comprise mercury as an ingredient

(with few exceptions). *Dhanvanthari Bhaga* (share of the physician in the medicine prepared) has been told separately for *Loha Yogas* and *Rasa Yogas*.[35] Hence some may doubt the inclusion of *Lauha Kalpas* in *Rasaushadhis*. But while explaining the novel nature of *Rasaushadhis*, *Rasa Vagbhata* describes that *Rasaushadhis* are effective in low dose with better acceptability and potent to reinstate health in short duration in a diseased individual. The author defines *Rasaushadhis* as the formulations which possess *Rasa* (*Parada, Maharasa, Uparasa,* and *Sadharana Rasa*) or any metal and mineral or poisonous roots as an ingredient,[36] All *Lauha Kalpas* possess *LB,* an iron metal *Bhasma* as a major ingredient hence *LK* can be termed as *Rasaushadhis*.

Lauha by virtue of its potential can be administered in various dosage forms *(Bahu Kalpam)* and thus fulfilling the criteria of an ideal medicine.[37] It is understood that iron and its salts produce gastric intolerance. Hence *Acharyas* have used iron as a part of formulations. It is evident that other ingredients of LK, processes and dosage forms explained are in line to improve the acceptability by patients of different body types and ages in indicated disease conditions.

Lauha Kalpas are mentioned for different ailments like *Pandu* (anemia), *Arsha* (hemorroids), *Rakta Pitta* (bleeding disorders), *Shula* (pain of different origin), *Jwara* (fevers), *Yakrit Pliha Roga* (diseases of liver and spleen), *Udara* (dropsy), *Amavata* (rheumatic arthritis), *Vata Rakta* (gout), *Kushta* (skin disorders), *Medo Roga* (obesity), *Tandava Roga, Gulma, Kasa* (cough) and *Shwasa* (dyspnoea). The role of *Lauha* in a specific formulation has to be ascertained by an erudite researcher in a respective disease condition. There are many *LKs*, especially semisolid dosage forms which are mentioned in classical texts but these are not available in the market. It is need of the hour for the pharmaceutical houses to work in this aspect and make these formulations available to the needy population.

CONCLUSION

From pharmaceutical viewpoint all *Lauha Kalpas* are not only *Khalviya Aushadhis* (prepared by *Bhavana*, impregnation -- wet trituration with simple water to different herbal juices). They are *Churnas* (mixtures -- prepared by dry trituration), *Rasakriya* (solidified formulations -- prepared by boiling of decoctions, herbal juices and even cows urine), *Avaleha* (confectionery), and *Puta Paka* (calcination products) formulations. Based on the method of preparation, shelf life, pharmacological action, indication varies. There are number of formulations which are not available in the market and but suits the present day healthcare demand. Hence pharmaceutical houses can manufacture and make them available.

Herbs found repeatedly in the formulations irrespective of indication are *Trika Traya*. These might have some role (bio enhancer) in absorption, distribution, metabolism, and excretion of iron. This work may serve as preliminary data and can be a torch bearer for the further studies on *Lauha Kalpas*.

Notes:

i. *Shodhana*: It is a procedure, where the metal or mineral or other poisonous substances are subjected to specified pharmaceutical procedures like trituration etc. with required plant or animal products so as to remove unwanted properties, if any.
ii. *Marana*: Literal meaning of *Marana* is killing. In this process of *Marana*, minerals are incinerated to obtain *Bhasma*. The minerals which have undergone the process of *Shodhana* are ground with specified liquid to form a paste. This paste is flattened into small discs (*Chakrikas*) and dried. These discs are then kept in between two convex earthen plates (*Sharava*) and sealed with the help of mud soaked cloth strips. After complete drying this is kept in the pit of *Puta*, containing *Vanyopala* (cow dung cakes), and ignited. After self-cooling, it is taken out and the baked discs are removed and powdered. This whole process makes one Puta. It is repeated for several times to obtain the *Bhasma*.
iii. *Tola*: It is a measurement used in Ayurvedic classics (1 *Tola*=12 g).
iv. *Bhasma*: Bio-accessible forms of minerals and metals intended for internal administration obtained after *Shodhana* and *Marana*.
v. *Kshara*: Alkaline inorganic substances obtained from the ash of herbal drugs.
vi. *Trikatraya*: Formulation containing *Triphala, Trikatu, Trimada*.[ix]
vii. *Triphala:* Formulation containing *Amalaki (Embilica officinalis) Vibhitaki (Terminalia bellerica), Haritaki (Terminalia chebula)*.
viii. *Trikatu:* Formulation containing *Pippali (Piper longum), Shunthi (dry ginger- Zingiber officinale), Maricha (Piper nigrum)*.
ix. *Trimada:* Formulation containing *Vidanga (Embelia ribes), Musta (Cyperus rotundus), Chitraka (Plumbago zeylanica)*.
x. *Chatur Lavana* (four salts)*:* Formulation containing *Vida Lavana* (factious salt procured by boiling earth impregnated with saline particles), *Audbhida Lavana* (fossil salt), *Saindhava Lavana* (rock salt), *Sauvarchala Lavana* (sochal salt, prepared by boiling down soda with embelic myrobilon).
xi. *Pancha Lavana* (five salts): Formulation containing *Chatur Lavana* and *Samudra Lavana* (sea salt).

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Clinical Research

Effect of *Sameera Pannaga Rasa* (arsenomercurial formulation) in the management of *Tamaka Shwasa* (bronchial asthma) - Randomized double blind clinical study

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Abstract

Asthma represents a profound world-wide public health problem. The most effective anti-asthmatic drugs currently available include β2-agonists and glucocorticoids which can controls asthma in about 90-95% of patients. In Ayurveda, this miserable condition is comparable with *Tamaka Shwasa* type of *Shwasa Roga*. In the present study, 52 patients were treated with *Sameera Pannaga Rasa* at a dose of 30 mg twice a day for 4 weeks along with *Nagavallidala* (leaf of *Piper betel* Linn.) The results were assessed in terms of clinical recovery, symptomatic relief, pulmonary function improvement and on subjective and objective parameters. A significant improvement in subjective parameters, control on asthma, recurrence of asthma, increase in peak expiratory flow rate, considerable decrease in total and absolute, acute eosinophil count and erythrocyte sedimentation rate were observed. Overall marked improvement was found in 33.33%, moderate improvement in 44.44% and mild improvement in 20.00% was observed. The study reveals that *Sameera Pannaga Rasa* can be used as an effective drug in bronchial asthma.

Key words: Bronchial asthma, pulmonary function, *Sameera Pannaga Rasa*, *Shwasa*

Introduction

Asthma represents a profound world-wide public health problem. The past decade has witnessed phenomenal increases in the incidences of asthma, asthma-related deaths and Glucocorticoids are the drugs frequently used (about 95%) in the treatment of bronchial asthma.[1] Currently glucocorticoid dependent asthma presents a great clinical burden and reducing the side-effects of glucocorticoids using novel steroid-sparing agents is needed.[2] However, the future therapies will need to focus on the 5-10% patients who do not respond well to these treatments and who account for approximately 50% of the health-care costs of asthma.[3] The surveys in adults show high prevalence of asthma symptoms and reduced lung functions particularly in lower socio-economic groups of the sufferers.[4,5] Asthma causes recurring episodes of wheezing, breathlessness, chest tightness and coughing, particularly at night or in the early morning. Common risk factors for asthma include exposure to allergens (such as those for house dust mites, animal with fur, cockroaches, pollens and mold),[6] occupational irritants,[7] tobacco smoke,[8] respiratory (viral) infections,[9] chemical irritants,[7] food allergies such as milk, peanuts and eggs[10] and psychological stress.[11] When airways are exposed to any of these risk factors; broncho-constriction will get manifested leading to inflammation. The airflow becomes limited and the patient suffers with the symptoms of asthma. The disease is comparable with Tamaka Shwasa type of Shwasa Roga in Ayurveda.[12] Ayurveda prefers a number of formulations to treat *Tamaka Shwasa*, which include few metallic preparation. *Sameera Panaga Rasa* (SPR) is an among such preperation, which is indicated in *Tamaka Shwasa*.[13]

SPR, an arsenal mercurial formulation is mentioned in *Rasa Chandanshu* in which *Manahshila* is not a component and later on it has been added by Ayurveda Aaushadhi Guna Dharma Shashtra. This later version has been accepted by Ayurvedic Formulary of India but, justification regarding the addition of *Manahshila* has not been provided. In addition to this; there is controversy regarding the final product,

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i.e., whether to collect *Talastha* or *Ubhayastha (Galastha + Talastha)*. Considering this, it is planned to prepare SPR with and without *Manahashila* and collect *Talastha* and *Ubhayastha* one and compare their respective clinical efficacies in *Tamaka Shwasa*.

Materials and Methods

Selection of patients

For this study, 52 patients of bronchial asthma were registered from the out-patient department and inpatient department of Rasashastra and Bhaishajya Kalpana including Drug Research, IPGT and RA, Gujarat Ayurved University, Jamnagar. Of all, seven patients were dropped out and 45 completed the prescribed course of treatment. No direct or indirect drug related reason for discontinue of patient was noticed. All the patients registered in the study were informed about the nature of treatment. The study was started after obtaining approval from the Institutional Ethics Committee.

Criteria for inclusion

1. Age between 20 to 60 years
2. Difficulty in breathing
3. Paroxysmal attacks of dyspnea
4. Difficulty in expectoration
5. Wheezing sounds
6. Relief in upright position.

Criteria for exclusion

1. Age below 20 and above 60 years
2. Acute asthma requiring emergency medicines
3. History of Bronchiectasis, Tuberculosis, Pyothorax, Anemia, Malignancy, Diabetes Mellitus, Hypertention, Hepatic or Renal disease in the recent past
4. Dyspnea resulting from cardiac disease
5. *Maha Shwasa*, *Urdhva Shwasa* and *Chhinna Shwasa* (types of breathlessness explained in classics) which have been labeled as incurable in Ayurveda.

Posology

The trial drug (SPR) was prepared in the departmental laboratory by following standard manufacturing procedures (SMP). The formulation composition is shown in Table 1. SPR prepared without *Manahshila* was labeled as SPR and that prepared with *Manahshila* was labeled as SPRM. Groups for clinical trial were as follows:

- Group A: Treated with SPR prepared without *Manahshila* - *Talastha* (SPRT)
- Group B: Treated with SPR prepared without *Manahshila* - *Ubhayastha (Galastha + Talastha)* (SPRU)
- Group C: Treated with SPR prepared with *Manahshila* - *Talastha* (SPRMT)
- Group D: Treated with SPR prepared with *Manahshila* - *Ubhayastha (Galastha + Talastha)* (SPRMU).

A capsule of 250 mg (containing 30 mg SPR + 220 mg starch powder) was administered twice a day for 28 days along with juice of *Nagavallidala* (leaf of *Piper betel* Linn.) as *Sahapana* (adjuvant). Follow-up was done after 2 weeks. Patients were advised not to get exposed to the susceptible triggering or aggravating factors narrated in Ayurveda as well as in modern texts.

Table 1: Formulation composition of *Sameera Pannaga Rasa*

Ingredient	Chemical/ Botanical name	Proportion
Sameera Pannaga Rasa (*Rasa Chandanshu*)		
Parada	Mercury	1 part
Gandhaka	Sulfur	1 part
Somala	White Arsenic	1 part
Haratala	Orpiment	1 part
Tulasi Patra swarasa	*Ocimum sanctum* Linn.	Q.S
Sameera Pannaga Rasa (AFI-I 15:8)		
Parada	Mercury	1 part
Gandhaka	Sulfur	1 part
Somala	White Arsenic	1 part
Haratala	Orpiment	1 part
Manahshila	Realgar	1 part
Tulasipatra swarasa	*Ocimum sanctum* Linn.	Q.S

SPRM: *Sameera Pannaga Rasa* prepared with manahshila, SPR: *Sameera Pannaga Rasa*

Laboratory investigations

Routine hematological, biochemical investigations, and peak expiratory flow rate (PEFR) were done before and after the treatment. Sputum examination and chest X-ray were carried out to exclude pulmonary tuberculosis and other pulmonary diseases.

Assessment criteria

Registered patients were advised to visit the OPD at regular intervals of a week. Subjective and objective parameters were recorded in terms of improvement in pulmonary functions and other investigations. Overall assessment of the treatment was made on the basis of the results of the investigations as well as the symptomatic relief.

Results and interpretation

Overall effect of therapy on each scale was calculated with reference to percentage improvement in all symptoms, the relief was assessed on the below criteria:

1. <25% - Poor response/unchanged
2. 26-50% - Mild improvement
3. 51-75% - Moderate improvement
4. 76-99% - Marked improvement
5. 100% - Complete remission.

Statistical analysis

Wilcoxon signed rank test was applied to evaluate the overall effect of therapy. Paired *t*-test was applied to evaluate the effect on hematological, biochemical investigation and PEFR.

Observations and Results

Four patients (33.33%) of SPRT group, four patients (36.36%) of SPRU group, two patients (20.00%) of SPRMT group and five patients of (41.67%) group SPRMU showed marked improvement. Five patients (41.67%) of SPRT group, five patients (45.45%) of SPRU group, three patients (30.00%) of

SPRMT group and seven patients of (58.33%) group SPRMU showed moderate improvement and three patients (25.00%) of SPRT group, two patients (18.18%) of SPRU group and four patients (40.00%) of SPRMT group showed mild improvement. In SPRMT group, one patient (10.00%) did not respond to the treatment. Overall results have been tabulated in Table 2. All the groups have been found to be statistically highly significant providing in relief [Table 3].

The reduction in eosinophils count, erythrocyte sedimentation rate (ESR) and total leucocyte count are found to be insignificant [Tables 4-7]. It was found in the study that, the duration, paroxysm, wheezing, chest tightness, nocturnal symptoms and dosage of allopathic emergency medicines were drastically reduced.

Interestingly, most of the patients in their follow-up period did not require the need of any emergency medication, particularly in SPRMU group followed by SPRT and SPRU group [Table 8]. Level of asthma control was higher in SPRMT group (50.00%) and in SPRU group (45.55%) [Table 9].

As per ACT score, it was found that by SPRMU and SPRT groups provided statistically significant results in control of asthma [Table 10]. No recurrence of attacks was observed during follow-up period in SPRMU group [Table 11].

Table 2: Overall effect of the therapy (In percentage)

Groups	SPRT		SPRU		SPRMT		SPRMU		Total	
Relief	*N*	%	*N*	%	*N*	%	*N*	%	*N*	%
Unchanged	0	00.00	0	00.00	1	10.00	0	00.00	1	02.22
Mild improvement	3	25.00	2	18.18	4	40.00	0	00.00	9	20.00
Moderate improvement	5	41.67	5	45.45	3	30.00	7	58.33	20	44.44
Marked improvement	4	33.33	4	36.36	2	20.00	5	41.67	15	33.33
Complete remission	0	00.00	0	00.00	0	00.00	0	00.00	0	00.00

SPRT: *Sameera Pannaga Rasa* prepared without *Manahshila - Talastha*, SPRU: *Sameera Pannaga Rasa* prepared without *Manahshila - Ubhayastha (Galastha + Talastha)*, SPRMT: *Sameera Pannaga Rasa* prepared with *Manahshila-Talastha*, SPRMU: *Sameera Pannaga Rasa* prepared with *Manahshila - Ubhayastha (Galastha + Talastha)*

Discussion

Survey of available literature points out that, vitiation of *Vata*, *Kapha Dosha* along with *Prana*, *Udaka* and *Anna Vaha Srotas* and *Rasa Dhatu* are the responsible factors in the manifestation of *Tamaka Shwasa*. The disease *Shwasa* has its root in the *Pitta Sthana* endorsed by *Amashayodbhavaja Vikara*.[14]

Considering the aggravated *Vata and Kapha*, *Acharyas* have advised the use of *Vata kaphaghna*, *Ushna*, *Vatanulomaka* drugs as first line of treatment in *Shwasa*. However, adoption of certain specification is always required for the breakdown of the three pathways of *Samprapti*. Furthermore, drugs exhibiting quick control over vitiated *Vata* and *Kapha* are required during *Vegavastha*, while exerting action on *Agni* or *Pittasthana* along with *Vatakaphaghnata*. Hence, logically, the drug administered in the treatment of *Shwasa*, should be able to overcome *Vata* and *Kapha* for immediate and symptomatic relief but should also pacify the *Pitta* for relief. Vagbhata emphasizes that, a drug acts by its *Rasa*, *Vipaka*, *Virya*, *Guna* and *Prabhava*. Normally, the effect of *Rasa* is less than that of *Vipaka*. Effect of *Vipaka* is lesser than that of *Virya*, which further is lesser than *Prabhava*, provided all are present in equal proportions. The overall pharmacodynamics of SPR is *Katu Rasa*, *Ushna Guna*, *Ushna Virya*, *Katu Vipaka* and *Kapha*

Table 3: Effect of drugs on overall effect of therapy: (Applied Wilcoxon rank test)

Group (*n*=45)	*N*	Mean±SEM		Change		Actual rank (D)	'α'
		B.T	A.T	Mean±SEM	%		
SPRT	12	43.17±3.990	13.50±3.230	29.67±4.164	68.72↓	78***	<0.01
SPRU	11	41.36±3.270	13.82±3.46	27.55±3.361	66.59↓	66***	<0.01
SPRMT	10	48.90±5.271	23.70±5.676	25.20±2.719	51.53↓	55***	<0.01
SPRMU	12	48.42±4.222	15.17±2.528	33.25±3.740	68.67↓	78***	<0.01

Data: Mean±SEM, ↓: Decrease; *α<0.05, **α<0.02 ***α<0.01, SEM: Standard error of the mean, SPRT: *Sameera Pannaga Rasa* prepared without *Manahshila-Talastha*, SPRU: *Sameera Pannaga Rasa* prepared without *Manahshila-Ubhayastha (Galastha + Talastha)*, SPRMT: *Sameera Pannaga Rasa* prepared with *Manahshila-Talastha*, SPRMU: *Sameera Pannaga Rasa* prepared with *Manahshila-Ubhayastha (Galastha + Talastha)*

Table 4: Hematological results of SPRT (applied paired *t* test)

Parameter	*n*	Before treatment	After treatment	% Changes	*t* value	*P* value
Hb	12	12.36±0.47	12.93±0.93	04.51↑	2.33*	<0.05
TLC	12	7833.33±388.44	6733±217.539	08.80↓	1.46	>0.05
Eosinophils	12	3.75±0.28	5.00±0.73	33.33↑	0.22	>0.05
ESR	12	23.83±5.48	16.41±3.86	31.11↓	1.80	>0.05
AEC	12	279.17±25.72	354.17±55.89	26.87↑	1.28	>0.05
PEFR	12	178.33±32.497	255.00±31.515	42.99↓	3.56**	<0.01

Data: Mean±SEM, ↑: Increase, ↓: Decrease, *P < 0.05, **P < 0.01, ***P < 0.001, TLC: Total leucocyte count, ESR: Erythrocyte sedimentation rate, AEC: Acute eosinophil count, PEFR: Peak expiratory flow rate, SPRT: *Sameera Pannaga Rasa* prepared without *Manahshila - Talastha*, SEM: Standard error of the mean

Table 5: Hematological results of SPRU (applied paired *t* test)

Parameter	*n*	Before treatment	After treatment	% Changes	*t* value	*P* value
Hb	12	12.77±0.320	12.83±0.27	0.42↑	0.22	>0.05
TLC	12	8463.64±627.59	8081.82±630.41	4.51↓	0.59	>0.05
Eosinophils	12	5.00±1.140	5.19±0.84	3.64↑	0.88	>0.05
ESR	12	26.36±6.230	26.91±8.40	2.07↑	0.08	>0.05
AEC	12	450.00±122.47	454.55±104.33	1.01↑	0.03	>0.05
PEFR	12	185.45±35.275	209.09±31.979	12.74↓	1.12	>0.05

Data: Mean±SEM,↑: Increase, ↓: Decrease, *P < 0.05, **P < 0.01, ***P < 0.001, SPRU: *Sameera Pannaga Rasa* prepared without *Manahshila - Ubhayastha (Galastha + Talastha)*, TLC: Total leucocyte count, ESR: Erythrocyte sedimentation rate, AEC: Acute eosinophil count, PEFR: Peak expiratory flow rate, SEM: Standard error of the mean

Table 6: Hematological results of SPRMT (applied paired *t* test)

Parameter	*n*	Before treatment	After treatment	% Changes	*t* value	*P* value
Hb	12	12.01±0.67	11.94±0.50	0.58↓	0.26	>0.05
TLC	12	7300±614.46	7330±481.21	0.41↑	0.10	>0.05
Eosinophils	12	4.50±0.62	4.7±1.49	4.44↑	0.813	>0.05
ESR	12	17.20±3.79	27.1±10.04	57.56↑	1.150	>0.05
AEC	12	355.00±82.14	400.00±172.88	12.68↑	0.28	>0.05
PEFR	12	165.00±28.529	182.00±23.240	13.33↓	1.71	>0.05

Data: Mean±SEM,↑: Increase, ↓: Decrease, *P < 0.05, **P < 0.01,***P < 0.001, SPRMT: *Sameera Pannaga Rasa* prepared with *Manahshila - Talastha*, TLC: Total leucocyte count, ESR: Erythrocyte sedimentation rate, AEC: Acute eosinophil count, PEFR: Peak expiratory flow rate, SEM: Standard error of the mean

Table 7: Hematological results of SPRMU (applied paired *t* test)

Parameter	*N*	Before treatment	After treatment	% Changes	*t* value	*P* value
Hb	12	12.61±0.53	12.16±0.50	2.38↓	1.19	>0.05
TLC	12	74863.64±783.33	6681.82±529.87	11.83↓	1.83	>0.05
Eosinophils	12	4.55±0.64	4.00±0.83	5.5↓	0.264	>0.05
ESR	12	32.73±7.56	28.09±5.64	10.44↓	1.04	>0.05
AEC	12	381.20±88.55	259.09±51.80	32.14↓	1.17	>0.05
PEFR	12	166.67±24.005	197.50±23.327	18.50↓	2.02	>0.05

Data: Mean±SEM, ↑: Increase, ↓: Decrease, *P < 0.05, **P < 0.01,***P < 0.001, SPRMU: *Sameera Pannaga Rasa* prepared with *Manahshila - Ubhayastha (Galastha + Talastha)*, TLC: Total leucocyte count, ESR: Erythrocyte sedimentation rate, AEC: Acute eosinophil count, PEFR: Peak expiratory flow rate, SEM: Standard error of the mean

Table 8: Effect of drugs on withdrawal of emergency drugs (applied Wilcoxon rank test)

Group (*n*=45)	*N*	Mean±SEM		Change		Actual rank (D)	'α'
		B.T	A.T	Mean±SEM	%		
SPRT	06	4.83±0.401	1.00±0.632	3.83±0.946	79.31↓	21*	<0.05
SPRU	08	4.50±0.327	1.50±0.008	3.00±0.567	66.67↓	28**	<0.02
SPRMT	05	3.00±1.265	1.60±0.748	1.40±1.470	46.67↓	7	>0.05
SPRMU	08	4.50±0.412	1.25±0.696	3.33±0.527	74.07↓	36***	<0.01

Data: Mean±SEM, ↓: Decrease, *α<0.05, **α<0.02, ***α<0.01, SPRT: *Sameera Pannaga Rasa* prepared without *Manahshila - Talastha*, SPRU: *Sameera Pannaga Rasa* prepared without *Manahshila - Ubhayastha (Galastha + Talastha)*, SPRMT: *Sameera Pannaga Rasa* prepared with *Manahshila - Talastha,* SPRMU: *Sameera Pannaga Rasa* prepared with *Manahshila - Ubhayastha (Galastha + Talastha)*, SEM: Standard error of the mean, AT: After Treatment, BT: Before Treatment

Table 9: Level of asthma control before treatment and after treatment

Level of asthma control	SPRT				SPRU				SPRMT				SPRMU			
	BT	%	AT	%	BT	%	AT	%	BT	%	AT	%	BT	%	AT	%
Controlled	0	00	7	58	1	9	5	45.5	1	10	5	50	0	0	9	75
Partialy controlled	4	33	5	42	3	27	5	45.5	2	20	3	30	5	42	3	25
Un controlled	8	67	0	00	7	64	1	9	7	70	2	20	7	58	0	0

SPRT: *Sameera Pannaga Rasa* prepared without *Manahshila - Talastha*, SPRU: *Sameera Pannaga Rasa* prepared without *Manahshila - Ubhayastha (Galastha + Talastha)*, SPRMT: *Sameera Pannaga Rasa* prepared with *Manahshila - Talastha,* SPRMU: *Sameera Pannaga Rasa* prepared with *Manahshila - Ubhayastha (Galastha + Talastha)*, AT: After treatment, BT: Before treatment

Table 10: Effect of SPRT on asthma control† (applied 'χ^2)

Group	ACT score	<19	≥19	Row total	χ^2	*P*
SPRT	BT	9	3	12	4.17**	<0.02
	AT	3	9	12		
SPRU	BT	10	1	11	2.063	>0.05
	AT	6	5	11		
SPRMT	BT	9	1	10	3.516	>0.05
	AT	4	6	10		
SPRMU	BT	11	1	12	6.40***	<0.01
	AT	4	8	12		

χ^2 value for Df 1*P < 0.05, **P < 0.02, ***P < 0.01, ****P < 0.002, ******P < 0.001, SPRT: *Sameera Pannaga Rasa* prepared without *Manahshila - Talastha*, SPRU: *Sameera Pannaga Rasa* prepared without *Manahshila - Ubhayastha (Galastha + Talastha)*, SPRMT: *Sameera Pannaga Rasa* prepared with *Manahshila - Talastha*, SPRMU: *Sameera Pannaga Rasa* prepared with *Manahshila - Ubhayastha (Galastha + Talastha)*, AT: After treatment, BT: Before treatment

Table 11: Recurrence asthma in follow-up period (in percentage)

Follow-up (2 weeks)	SPRT		SPRU		SPRMT		SPRMU	
	n	%	*n*	%	*n*	%	*n*	%
Recurrence	1	8.33	1	9.09	3	30	0	00
No recurrence	11	91.67	10	90.91	7	70	12	100

SPRT: *Sameera Pannaga Rasa* prepared without *Manahshila - Talastha*, SPRU: *Sameera Pannaga Rasa* prepared without *Manahshila - Ubhayastha (Galastha + Talastha)*, SPRMT: *Sameera Pannaga Rasa* prepared with *Manahshila - Talastha*, SPRMU: *Sameera Pannaga Rasa* prepared with *Manahshila - Ubhayastha (Galastha + Talastha)*

Vataghna.[15] These dynamic actions are helpful in breaking the pathogenesis of *Tamaka Shwasa*. Asthma is now accepted as a T-helper type 2 lymphocyte-mediated chronic inflammatory disorder, characterized by airway eosinophilia and airway hyper responsiveness. Eosinophils appear to play a crucial role in the ongoing inflammation due to either an impaired clearance or a delayed apoptosis in the airways, where accumulation of a number of eosinophils cytotoxic proteins including major basic protein, cationic proteins and peroxidase could occur. As2o3 could alleviate the airway inflammation through promoting pulmonary eiosinophils (PE) apoptosis and lower PE infiltration. Low dose of As2o3 is proved to be effective with relative safety, it also has potential value in treating asthma.[16]

In modern point of view SPR is Mercury-Arsenical (*Ubhayastha*) or Arsenical preparation (*Talastha*).

In preparation of SPR, *Tulsipatra Swarasa* (leaf of *Oscimum sanctum* Linn.) as *Bhavana Dravya* is advocated. Studies have been proved that leaf extract of *Ocimum sanctum* is effective against arsenic induced toxicity. *Ocimum sanctum* has significant role in protecting animals from arsenic induced oxidative stress and in the depletion of arsenic concentration.[17] It has been proved that *Ocimum* extract can protect against mercury toxicity in mice. It significantly enhanced reduced glutathione, which is suggested that oral administration of *Ocimum* extract provides protection against mercury induced toxicity in Swiss albino mice.[18] Thus, Oscimum may help in nullifying possible ADRs of SPR.[19]

In the present study, *Nagavallidala* (*P. betel*) was taken as *Sahapana* for SPR. Studies have proved inhibitory effects of *P. betel* Linn. on production of allergic mediators by bone marrow derived mast cells and lung epithelial cells. PE significantly decreased histamine and granulocyte macrophage colony stimulating factors produced by an IgE mediated hypersensitive reaction and inhibited eotaxin and IL-8 secretion in a tumor necrosis factor-α and IL-4 induced allergic reaction.[20] Reduction of oxidative stress induced by free radicals by virtue of its anti-oxidant properties and chelation of heavy metals thereby minimizing its toxic potential and increasing safety margins were also found to be reported.[21] Considering all these, it is assumed that, *Nagavallidala* can reduce arsenic induced oxidative stress as well as the control of allergic diseases through inhibition of production of allergic mediators.

Conclusion

The results reveal that the SPR has a significant action in cases bronchial asthma and it could suppress total leukocyte count, eosinophil count, ESR and can improve PEFR along with providing symptomatic relief. Analysis of the data generated during the study shows that; all the groups of SPR have been highly significant in treating the condition. However, comparative evaluation shows that SPRMU group is better followed by SPRT and SPRU where SPRMT is less effective comparatively. None of the treated patients developed any adverse effects during the study period.

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Original Article

Effect of *Tamra Bhasma* (Calcined Copper) on Ponderal and Biochemical Parameters

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ABSTRACT

Introduction: *Tamra Bhasma* (TB) and its forms like *Somnathi Tamra Bhasma* (STB), etc., are in vogue since centuries in *Ayurveda*. The present study is carried out to evaluate the effect of TB and STB in different dose levels on ponderal and biochemical parameters in wistar strain albino rats to provide scientific basis for its safety profile. **Materials and Methods:** TB and STB were prepared as per the classical guidelines and administered to wistar strain albino rats for 45 consecutive days. Blood was collected and rats were sacrificed on the 46th day. Ponderal and biochemical parameters were studied. **Results:** Results showed significant decrease in serum cholesterol, High Density Lipoprotein (HDL) cholesterol, triglycerides, total protein, and serum alkaline phosphatase levels. Comparatively, all the differences in between the groups are insignificant and no pathological changes at ponderal and biochemical levels were observed. **Conclusion:** Based on these observations, it can be said that these formulations can be safely used in cases of hyperlipidemia.

Key words: *Bhasma*, biochemical parameters, copper, ponderal parameters, *Somnathi Tamra Bhasma*, *Tamra Bhasma*

INTRODUCTION

Metals (like mercury, iron, copper, lead, zinc, etc.) and minerals (like mica, arsenic, chalcopyrite, etc.) in the form of *Bhasmas* are an integral part of Ayurvedic therapeutics. As these *Bhasmas* are prepared by following the classical procedures of repeated calcinations, they are chemically mixed with oxides of one or more metals[1] and are associated with a number of trace elements. Therapeutic utility of properly processed *Bhasmas* and their hazardous effects under inappropriate use when used in impure form is well documented in Ayurveda.[2] Despite of this, concerns are being expressed frequently regarding the metal toxicity and safety of traditional preparations containing *Bhasmas*.[3-6] *Tamra Bhasma*, one of such metallic preparations of *Ayurveda* is useful in the treatment of *Udara* (ascitis), *Pandu* (anemia), *Svasa* (bronchial asthma), and *Amlapitta* (hyperacidity), etc.[7] It is an integral component in Ayurvedic formulations like *Kalyansundara Rasa*, *Hridayarnava Rasa,* etc., used for cardiac and lipid disorders.[8,9]

Tamra is attributed with *Ashtamahadoshas* (eight blemishes).[10] Hence, one has to be careful while handling this metal. Though, the role of incinerated copper in hepatoprotection and lipid peroxidation is reported, effect on biochemical parameters is not reported.[11] Considering this, the present study is aimed at screening the ponderal and biochemical changes in Swiss albino rats after administration of *Tamra Bhasma* (TB) and *Somnathi Tamra Bhasma* (STB) at different dose levels.

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MATERIALS AND METHODS

Test drugs

Both the trial drugs were prepared in the laboratory of *Rasashastra* and *Bhaishajyakalpana*, Institute for Post Graduate Teaching and Research in Ayurveda (I.P.G.T. and R.A), Gujarat Ayurved University, Jamnagar by following standard guidelines as prescribed in classical Ayurvedic literature.

Copper wire with 99.89% pure copper was procured from Amber Electricals, Jamnagar. It was processed through classical procedures of *Shodhana* (purification procedure), *Marana* (incineration process), and *Amritikarana* (necterization process) to prepare *Tamra Bhasma* and labeled as *Shodhita Tamra* (SHTB).[12-14] Another sample was processed for *Marana* avoiding the initial steps of *Shodhana* and labeled as *Ashuddha Tamra* (ATB). STB, another familiar copper formulation was prepared by *Kupipakva* method.[15]

Animals

Wistar strain albino rats of either sex weighing 200 ± 20 g were obtained from the animal house attached to the pharmacology laboratory, I.P.G.T. and R.A, Gujarat Ayurved University, Jamnagar and were exposed to natural day and night cycles with ideal laboratory conditions in terms of ambient temperature and humidity. Animals were fed *ad libitum* with Amrut brand rat pellet feed supplied by Pranav Agro Industries and tap water. The experiment was carried out after obtaining permission from Institutional Animal Ethics Committee (IAEC 07/2010/05/MD) and care of animals was taken as per the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) guidelines.

Dose fixation and schedule

The animal dose for rats was calculated by considering therapeutic doses of trial drugs (TB, STB) and referring to table of Paget and Barnes.[16] On this basis, dose of both the test drugs for rats was found to be 5.5 mg/kg and 13.5 mg/kg. The test drugs were administered in the form of suspension in distilled water orally with the help of rubber catheter attached to a disposable syringe. For the preparation of stock solution, both the test drug samples were taken in requisite quantity in small porcelain mortar and 0.5 ml of 5% gum acacia suspension was added, grounded for 5 minutes and the volume was made up with distilled water, so as to contain 5.5 mg/ml and 13.5 mg/ml test drugs.

Experimental design

Rats were randomly assigned into eight groups. Group I served as positive control (water control, WC) receiving tap water and normal food. Group II, III, IV received TB prepared from ATB in different doses and Group V, VI, VII received TB prepared from SHTB in different doses. Group VIII received STB at five Therapeutically Equivalent Dose (TED) levels [Table 1]. Body weight of all the animals was recorded initially and at the end of the study. General behavioral pattern was observed on every week by exposing each animal to an open arena. At the end of experimental period, all the animals were euthanized and gross pathological observations were performed.

Serum biochemical analysis

At the end of experimental period, animals were anesthetized with diethyl ether and blood was collected from supraorbital plexus in plain tube for serum biochemical investigations, including blood sugar, urea, creatinine, cholesterol, triglycerides, HDL, bilurubin, serum glutamic-pyruvic transaminase (SGPT), serum glutamic oxaloacetic transaminase (SGOT), S. alkaline phosphatase (SAP), total protein, and uric acid were analyzed by auto analyzer (Fully automated Biochemical Random Access Analyzer, BS-200; Lilac Medicare Pvt. Ltd., Mumbai)

Statistical analysis

The results were presented as Mean ± SEM in each group. Statistical comparisons were performed by both paired, unpaired Student's *t*-test, and one-way analysis of variance (ANOVA) with Dunnets' multiple *t*-test as post-hoc test by using Sigma stat software (version 3.1) for all the treated groups with the level of significance set at $P < 0.05$.

RESULTS AND OBSERVATIONS

Body weight

Insignificant weight gain was observed in control group while the weight was insignificantly reduced in other groups except Group II. Animals treated with ATB loose body weight significantly [Table 2].

Table 1: Test drug posology

Group	No of animals	Drug	Dose (mg/kg)	Duration
I	6	WC	-	45 days
II	6	ATB	5.5	
III	6		27.5	
IV	6		55	
V	6	SHTB	5.5	
VI	6		27.5	
VII	6		55	
VIII	6	STB	67.5	

Abbreviation: WC = Water control, ATB = *Ashuddha Tamra Bhasma*, SHTB = *Shodhita Tamra Bhasma*, STB = *Somnathi Tamra Bhasma*

Biochemical parameters

HDL was found to be reduced with all dose levels of ATB and with 10 TED of SHTB. Physiological levels of HDL were maintained with the treatment of other trial drugs. Insignificant changes were observed in blood glucose, serum cholesterol, and triglycerides in all groups. Animals treated with SHTB TED and SHTB 5 TED showed significant increase in serum creatinine and decrease in serum alkaline phosphatase (ALP). STB-treated group was also found be significant in decreasing ALP level. All other biochemical parameters were not affected to a significant extent in all the treated group in comparison to the control group [Table 3].

DISCUSSION

Metals may be toxic in their native or free form but not their *Bhasmas* because they have different compound forms. Thus, they are incorporated in herbomineral formulations for their specific therapeutic role and used successfully in the treatment of many diseases since a long period. Though metallic preparations are therapeutically used since long, there is a need to document their safety profiles.

TB is one among such herbometallic formulations used for treatment of anemia, cardiac, liver, and lipid-related disorders as an important ingredient in compound formulations or singly. As seers of Ayurveda claimed its therapeutic effectiveness in above pathological manifestations, the present study was designed to assess comparison of ponderal and biochemical parameters of TB and STB. Non-significant decrease in the body weight was observed in all samples. These results justify the role of TB in *Lekhana* (scraps excessive fat) property.

Significant decrease in serum HDL cholesterol level was found in ATB TED, ATB TED × 5 and SHTB TED × 10 groups. This showed that they may impair the transfer of cholesterol from both very-low-density lipoprotein (VLDL) and tissue to HDL fraction or it may be promoting the metabolism of this fraction by enhancing the activity of the key enzymes involved in HDL cholesterol metabolism. In contrast to this, SHTB TED and TED × 5 did not show any significant changes in HDL level [Figure 1].

Administration of TB in ATB TED, SHTB TED, and SHTB, STB-treated groups at TED × 5 dose levels showed significant decrease in serum ALP level but they did not affect this enzyme activity to significant extent at higher dose levels, hence, the extra hepatic cause for decreased activity of this enzyme may be involved. Changes in SGOT, SGPT, bilurubin level (total and direct) were found to be statistically insignificant [Figure 2]. Both ATB and SHTB group showed significant decrease in serum total protein level only at higher doses (TED × 10) but it is to be noted that they did not produce any significant changes at TED and even at 5 TED in all other test drugs, showing importance of dosage forms in drug toxicity [Figure 3].

Table 2: Effect of test drugs on the body weight of albino rats recorded during toxicological study

Group	Treatment	Body weight (g)		't' value	'P' value
		Initial	Final		
I	Water control	208.33±6.5	226.3±17.5	−1.046	0.344
II	TED ATB	215.0±14.3	190.7±16.8	5.255	0.003*@
III	TED×5 ATB	196.7±3.3	181.8±11.3	1.361	0.232
IV	TED×10 ATB	203.3±8.8	184.7±8.5	2.189	0.08
V	TED SHTB	215.0±5.0	212.4±9.1	0.581	0.593
VI	TED×5 SHTB	211.7±9.8	201.0±10.09	1.661	0.158
VII	TED×10 SHTB	208.3±10.1	209.3±7.5	−0.123	0.907
VIII	TED×5 STB	202.00±3.9	199.7±3.630	1.053	0.341

Data: Mean±SEM, *P<0.05 (Paired t-test), @P<0.05 (ANOVA test). ATB = *Ashuddha Tamra Bhasma*, SHTB = *Shodhita Tamra Bhasma*; STB = *Somnathi Tamra Bhasma*; TED = Therapeutically equivalent dose

Table 3: Effect of test drugs on biochemical parameters of albino rats recorded during toxicological study

Parameters	NC	TED ATB	TED×05 ATB	TED×10 ATB	TED SHTB	TED×05 SHTB	TED×10 SHTB	TED×5 STB
Blood glucose (mg/dL)	117.5±8.9	108.7±2.1	116.8±5.5	110.9±4.2	108.2±9.7	116.0±12.4	98.5±15.5	99.167±2.651
S. cholesterol (mg/dL)	77.5±9.9	63.3±6.02	51.5±4.4*	56.0±3.7	91.2±8.2	78.5±16.5	53.8±5.6	63.500±5.920
S. triglyceride (mg/dL)	97.5±11.9	61.7±6.9*	93.0±11.07	94.2±10.4	121.2±22.6	87.0±14.6	124.3±18.9	93.0±8.77
S. HDL (mg/dL)	39.2±5.4	26.3±2.2*	24.8±2.2*@	27.0±3.3	37.3±3.7	32.7±3.7	24.0±3.3*@	33.67±3.242
S. Urea (mg/dL)	100.3±11.4	112.0±10.5	114.8±11.8	115.8±12.6	96.0±5.7	97.3±6.9	83.0±3.4	83.33±7.149
S. creatnine (mg/dL)	0.6±0.2	0.6±0.07	0.6±0.03	0.6±0.06	0.7±0.03*	0.7±0.03*	0.6±0.03	0.67±0.03
S.G.P.T. (IU)	77.3±6.9	89.0±9.8	93.5±4.08	83.7±6.2	62.7±3.4	70.8±8.3	86.2±15.3	73.83±4.722
S.G.O.T. (IU)	332.0±42.2	279.2±9.9	321.7±25.6	335.3±54.9	246.5±9.9	302.0±19.9	309.0±36.6	283.33±27.85
Total protein (g/dL)	7.6±0.3	7.9±0.26	7.15±0.1	6.9±0.1*	7.7±0.2	7.5±0.1	6.7±0.2*@	7.45±0.118
S. Alkaline phosphatase (IU/L)	236.2±20.7	176.7±16.4*	300.3±60.3	250.0±29.8	146.7±11.4**@	146.8±30.3*@	170.3±34.72	146.5±5.277*@
S. bilirubin (T) (mg/dL)	0.7±0.2	0.9±0.2	0.6±0.1	0.9±1.1	0.5±0.04	0.5±0.04	0.5±0.04	0.467±0.0422
S. bilirubin (D) (mg/dL)	0.2±0.04	0.3±0.6	0.2±0.03	0.7±0.5	0.15±0.02	0.15±0.02	0.2±0.2	0.150±0.0224
S. Uric acid (mg/dL)	2.1±0.4	2.6±0.5	1.7±0.3	2.7±0.4	1.6±0.1	1.6±0.2	1.9±0.4	1.233±0.196

Data: Mean±SEM, *P<0.05 (unpaired t test), @P<0.05 (ANOVA test). ATB = *Ashuddha Tamra Bhasma*, SHTB = *Shodhita Tamra Bhasma*, STB = *Somnathi Tamra Bhasma*, TED = Therapeutically Equivalent Dose, SGPT = Serum glutamic pyruvic transaminases, SGOT = Serum glutamic oxaloacetic transaminase

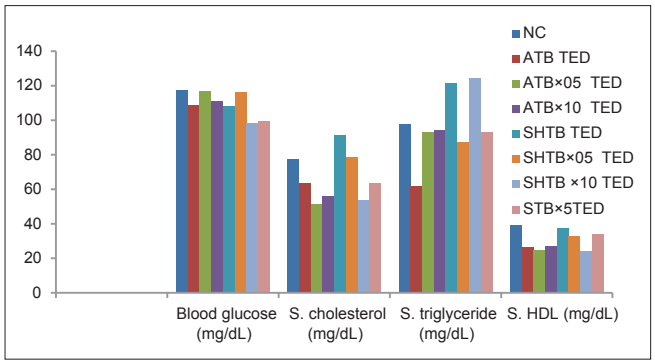

Figure 1: Effect of *Tamra Bhasma* on biochemical parameters related to lipid metabolism

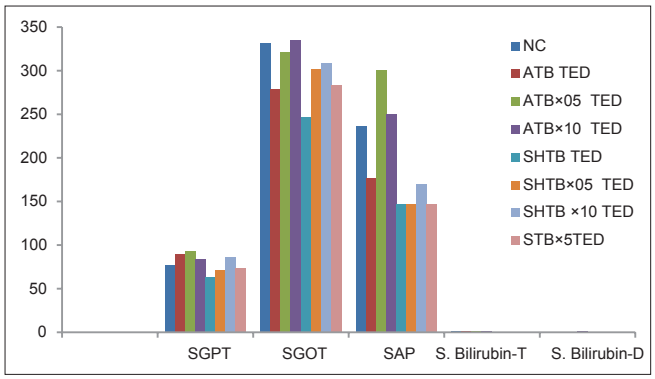

Figure 2: Effect of *Tamra Bhasma* on biochemical parameters related to liver functions

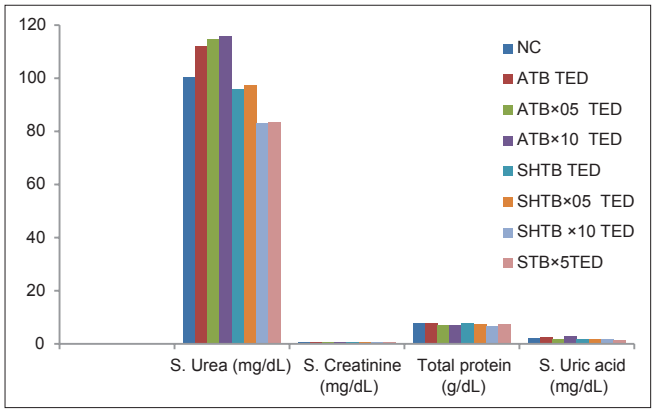

Figure 3: Effect of *Tamra Bhasma* on biochemical parameters related to renal functions

It indicates that the drug has no significant effect on parameters related to liver function when administered for 45 days. All these observations reveal safety of the formulations at therapeutic dose levels.

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Clinical Research

Effect of *Sameera Pannaga Rasa* (arsenomercurial formulation) in the management of *Tamaka Shwasa* (bronchial asthma) - Randomized double blind clinical study

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Abstract

Asthma represents a profound world-wide public health problem. The most effective anti-asthmatic drugs currently available include β2-agonists and glucocorticoids which can controls asthma in about 90-95% of patients. In Ayurveda, this miserable condition is comparable with *Tamaka Shwasa* type of *Shwasa Roga*. In the present study, 52 patients were treated with *Sameera Pannaga Rasa* at a dose of 30 mg twice a day for 4 weeks along with *Nagavallidala* (leaf of *Piper betel* Linn.) The results were assessed in terms of clinical recovery, symptomatic relief, pulmonary function improvement and on subjective and objective parameters. A significant improvement in subjective parameters, control on asthma, recurrence of asthma, increase in peak expiratory flow rate, considerable decrease in total and absolute, acute eosinophil count and erythrocyte sedimentation rate were observed. Overall marked improvement was found in 33.33%, moderate improvement in 44.44% and mild improvement in 20.00% was observed. The study reveals that *Sameera Pannaga Rasa* can be used as an effective drug in bronchial asthma.

Key words: Bronchial asthma, pulmonary function, *Sameera Pannaga Rasa*, *Shwasa*

Introduction

Asthma represents a profound world-wide public health problem. The past decade has witnessed phenomenal increases in the incidences of asthma, asthma-related deaths and Glucocorticoids are the drugs frequently used (about 95%) in the treatment of bronchial asthma.[1] Currently glucocorticoid dependent asthma presents a great clinical burden and reducing the side-effects of glucocorticoids using novel steroid-sparing agents is needed.[2] However, the future therapies will need to focus on the 5-10% patients who do not respond well to these treatments and who account for approximately 50% of the health-care costs of asthma.[3] The surveys in adults show high prevalence of asthma symptoms and reduced lung functions particularly in lower socio-economic groups of the sufferers.[4,5] Asthma causes recurring episodes of wheezing, breathlessness, chest tightness and coughing, particularly at night or in the early morning. Common risk factors for asthma include exposure to allergens (such as those for house dust mites, animal with fur, cockroaches, pollens and mold),[6] occupational irritants,[7] tobacco smoke,[8] respiratory (viral) infections,[9] chemical irritants,[7] food allergies such as milk, peanuts and eggs[10] and psychological stress.[11] When airways are exposed to any of these risk factors; broncho-constriction will get manifested leading to inflammation. The airflow becomes limited and the patient suffers with the symptoms of asthma. The disease is comparable with Tamaka Shwasa type of Shwasa Roga in Ayurveda.[12] Ayurveda prefers a number of formulations to treat *Tamaka Shwasa*, which include few metallic preparation. *Sameera Panaga Rasa* (SPR) is an among such preperation, which is indicated in *Tamaka Shwasa*.[13]

SPR, an arsenal mercurial formulation is mentioned in *Rasa Chandanshu* in which *Manahshila* is not a component and later on it has been added by Ayurveda Aaushadhi Guna Dharma Shashtra. This later version has been accepted by Ayurvedic Formulary of India but, justification regarding the addition of *Manahshila* has not been provided. In addition to this; there is controversy regarding the final product,

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i.e., whether to collect *Talastha* or *Ubhayastha (Galastha + Talastha)*. Considering this, it is planned to prepare SPR with and without *Manahashila* and collect *Talastha* and *Ubhayastha* one and compare their respective clinical efficacies in *Tamaka Shwasa*.

Materials and Methods

Selection of patients

For this study, 52 patients of bronchial asthma were registered from the out-patient department and inpatient department of Rasashastra and Bhaishajya Kalpana including Drug Research, IPGT and RA, Gujarat Ayurved University, Jamnagar. Of all, seven patients were dropped out and 45 completed the prescribed course of treatment. No direct or indirect drug related reason for discontinue of patient was noticed. All the patients registered in the study were informed about the nature of treatment. The study was started after obtaining approval from the Institutional Ethics Committee.

Criteria for inclusion

1. Age between 20 to 60 years
2. Difficulty in breathing
3. Paroxysmal attacks of dyspnea
4. Difficulty in expectoration
5. Wheezing sounds
6. Relief in upright position.

Criteria for exclusion

1. Age below 20 and above 60 years
2. Acute asthma requiring emergency medicines
3. History of Bronchiectasis, Tuberculosis, Pyothorax, Anemia, Malignancy, Diabetes Mellitus, Hypertention, Hepatic or Renal disease in the recent past
4. Dyspnea resulting from cardiac disease
5. *Maha Shwasa*, *Urdhva Shwasa* and *Chhinna Shwasa* (types of breathlessness explained in classics) which have been labeled as incurable in Ayurveda.

Posology

The trial drug (SPR) was prepared in the departmental laboratory by following standard manufacturing procedures (SMP). The formulation composition is shown in Table 1. SPR prepared without *Manahshila* was labeled as SPR and that prepared with *Manahshila* was labeled as SPRM. Groups for clinical trial were as follows:

- Group A: Treated with SPR prepared without *Manahshila* - *Talastha* (SPRT)
- Group B: Treated with SPR prepared without *Manahshila* - *Ubhayastha (Galastha + Talastha)* (SPRU)
- Group C: Treated with SPR prepared with *Manahshila* - *Talastha* (SPRMT)
- Group D: Treated with SPR prepared with *Manahshila* - *Ubhayastha (Galastha + Talastha)* (SPRMU).

A capsule of 250 mg (containing 30 mg SPR + 220 mg starch powder) was administered twice a day for 28 days along with juice of *Nagavallidala* (leaf of *Piper betel* Linn.) as *Sahapana* (adjuvant). Follow-up was done after 2 weeks. Patients were advised not to get exposed to the susceptible triggering or aggravating factors narrated in Ayurveda as well as in modern texts.

Table 1: Formulation composition of *Sameera Pannaga Rasa*

Ingredient	Chemical/ Botanical name	Proportion
Sameera Pannaga Rasa (*Rasa Chandanshu*)		
Parada	Mercury	1 part
Gandhaka	Sulfur	1 part
Somala	White Arsenic	1 part
Haratala	Orpiment	1 part
Tulasi Patra swarasa	*Ocimum sanctum* Linn.	Q.S
Sameera Pannaga Rasa (AFI-I 15:8)		
Parada	Mercury	1 part
Gandhaka	Sulfur	1 part
Somala	White Arsenic	1 part
Haratala	Orpiment	1 part
Manahshila	Realgar	1 part
Tulasipatra swarasa	*Ocimum sanctum* Linn.	Q.S

SPRM: *Sameera Pannaga Rasa* prepared with manahshila, SPR: *Sameera Pannaga Rasa*

Laboratory investigations

Routine hematological, biochemical investigations, and peak expiratory flow rate (PEFR) were done before and after the treatment. Sputum examination and chest X-ray were carried out to exclude pulmonary tuberculosis and other pulmonary diseases.

Assessment criteria

Registered patients were advised to visit the OPD at regular intervals of a week. Subjective and objective parameters were recorded in terms of improvement in pulmonary functions and other investigations. Overall assessment of the treatment was made on the basis of the results of the investigations as well as the symptomatic relief.

Results and interpretation

Overall effect of therapy on each scale was calculated with reference to percentage improvement in all symptoms, the relief was assessed on the below criteria:

1. <25% - Poor response/unchanged
2. 26-50% - Mild improvement
3. 51-75% - Moderate improvement
4. 76-99% - Marked improvement
5. 100% - Complete remission.

Statistical analysis

Wilcoxon signed rank test was applied to evaluate the overall effect of therapy. Paired *t*-test was applied to evaluate the effect on hematological, biochemical investigation and PEFR.

Observations and Results

Four patients (33.33%) of SPRT group, four patients (36.36%) of SPRU group, two patients (20.00%) of SPRMT group and five patients of (41.67%) group SPRMU showed marked improvement. Five patients (41.67%) of SPRT group, five patients (45.45%) of SPRU group, three patients (30.00%) of

SPRMT group and seven patients of (58.33%) group SPRMU showed moderate improvement and three patients (25.00%) of SPRT group, two patients (18.18%) of SPRU group and four patients (40.00%) of SPRMT group showed mild improvement. In SPRMT group, one patient (10.00%) did not respond to the treatment. Overall results have been tabulated in Table 2. All the groups have been found to be statistically highly significant providing in relief [Table 3].

The reduction in eosinophils count, erythrocyte sedimentation rate (ESR) and total leucocyte count are found to be insignificant [Tables 4-7]. It was found in the study that, the duration, paroxysm, wheezing, chest tightness, nocturnal symptoms and dosage of allopathic emergency medicines were drastically reduced.

Interestingly, most of the patients in their follow-up period did not require the need of any emergency medication, particularly in SPRMU group followed by SPRT and SPRU group [Table 8]. Level of asthma control was higher in SPRMT group (50.00%) and in SPRU group (45.55%) [Table 9].

As per ACT score, it was found that by SPRMU and SPRT groups provided statistically significant results in control of asthma [Table 10]. No recurrence of attacks was observed during follow-up period in SPRMU group [Table 11].

Table 2: Overall effect of the therapy (In percentage)

Groups	SPRT		SPRU		SPRMT		SPRMU		Total	
Relief	*N*	%	*N*	%	*N*	%	*N*	%	*N*	%
Unchanged	0	00.00	0	00.00	1	10.00	0	00.00	1	02.22
Mild improvement	3	25.00	2	18.18	4	40.00	0	00.00	9	20.00
Moderate improvement	5	41.67	5	45.45	3	30.00	7	58.33	20	44.44
Marked improvement	4	33.33	4	36.36	2	20.00	5	41.67	15	33.33
Complete remission	0	00.00	0	00.00	0	00.00	0	00.00	0	00.00

SPRT: *Sameera Pannaga Rasa* prepared without *Manahshila - Talastha*, SPRU: *Sameera Pannaga Rasa* prepared without *Manahshila - Ubhayastha (Galastha + Talastha)*, SPRMT: *Sameera Pannaga Rasa* prepared with *Manahshila-Talastha*, SPRMU: *Sameera Pannaga Rasa* prepared with *Manahshila - Ubhayastha (Galastha + Talastha)*

Discussion

Survey of available literature points out that, vitiation of *Vata*, *Kapha Dosha* along with *Prana*, *Udaka* and *Anna Vaha Srotas* and *Rasa Dhatu* are the responsible factors in the manifestation of *Tamaka Shwasa*. The disease *Shwasa* has its root in the *Pitta Sthana* endorsed by *Amashayodbhavaja Vikara*.[14]

Considering the aggravated *Vata and Kapha*, *Acharyas* have advised the use of *Vata kaphaghna*, *Ushna*, *Vatanulomaka* drugs as first line of treatment in *Shwasa*. However, adoption of certain specification is always required for the breakdown of the three pathways of *Samprapti*. Furthermore, drugs exhibiting quick control over vitiated *Vata* and *Kapha* are required during *Vegavastha*, while exerting action on *Agni* or *Pittasthana* along with *Vatakaphaghnata*. Hence, logically, the drug administered in the treatment of *Shwasa*, should be able to overcome *Vata* and *Kapha* for immediate and symptomatic relief but should also pacify the *Pitta* for relief. Vagbhata emphasizes that, a drug acts by its *Rasa*, *Vipaka*, *Virya*, *Guna* and *Prabhava*. Normally, the effect of *Rasa* is less than that of *Vipaka*. Effect of *Vipaka* is lesser than that of *Virya*, which further is lesser than *Prabhava*, provided all are present in equal proportions. The overall pharmacodynamics of SPR is *Katu Rasa*, *Ushna Guna*, *Ushna Virya*, *Katu Vipaka* and *Kapha*

Table 3: Effect of drugs on overall effect of therapy: (Applied Wilcoxon rank test)

Group (*n*=45)	*N*	Mean±SEM		Change		Actual rank (D)	'α'
		B.T	A.T	Mean±SEM	%		
SPRT	12	43.17±3.990	13.50±3.230	29.67±4.164	68.72↓	78***	<0.01
SPRU	11	41.36±3.270	13.82±3.46	27.55±3.361	66.59↓	66***	<0.01
SPRMT	10	48.90±5.271	23.70±5.676	25.20±2.719	51.53↓	55***	<0.01
SPRMU	12	48.42±4.222	15.17±2.528	33.25±3.740	68.67↓	78***	<0.01

Data: Mean±SEM, ↓: Decrease; *α<0.05, **α<0.02 ***α<0.01, SEM: Standard error of the mean, SPRT: *Sameera Pannaga Rasa* prepared without *Manahshila-Talastha*, SPRU: *Sameera Pannaga Rasa* prepared without *Manahshila-Ubhayastha (Galastha + Talastha)*, SPRMT: *Sameera Pannaga Rasa* prepared with *Manahshila-Talastha*, SPRMU: *Sameera Pannaga Rasa* prepared with *Manahshila-Ubhayastha (Galastha + Talastha)*

Table 4: Hematological results of SPRT (applied paired *t* test)

Parameter	*n*	Before treatment	After treatment	% Changes	*t* value	*P* value
Hb	12	12.36±0.47	12.93±0.93	04.51↑	2.33*	<0.05
TLC	12	7833.33±388.44	6733±217.539	08.80↓	1.46	>0.05
Eosinophils	12	3.75±0.28	5.00±0.73	33.33↑	0.22	>0.05
ESR	12	23.83±5.48	16.41±3.86	31.11↓	1.80	>0.05
AEC	12	279.17±25.72	354.17±55.89	26.87↑	1.28	>0.05
PEFR	12	178.33±32.497	255.00±31.515	42.99↓	3.56**	<0.01

Data: Mean±SEM, ↑: Increase, ↓: Decrease, *P < 0.05, **P < 0.01, ***P < 0.001, TLC: Total leucocyte count, ESR: Erythrocyte sedimentation rate, AEC: Acute eosinophil count, PEFR: Peak expiratory flow rate, SPRT: *Sameera Pannaga Rasa* prepared without *Manahshila - Talastha*, SEM: Standard error of the mean

Table 5: Hematological results of SPRU (applied paired *t* test)

Parameter	*n*	Before treatment	After treatment	% Changes	*t* value	*P* value
Hb	12	12.77±0.320	12.83±0.27	0.42↑	0.22	>0.05
TLC	12	8463.64±627.59	8081.82±630.41	4.51↓	0.59	>0.05
Eosinophils	12	5.00±1.140	5.19±0.84	3.64↑	0.88	>0.05
ESR	12	26.36±6.230	26.91±8.40	2.07↑	0.08	>0.05
AEC	12	450.00±122.47	454.55±104.33	1.01↑	0.03	>0.05
PEFR	12	185.45±35.275	209.09±31.979	12.74↓	1.12	>0.05

Data: Mean±SEM,↑: Increase, ↓: Decrease, *P < 0.05, **P < 0.01, ***P < 0.001, SPRU: *Sameera Pannaga Rasa* prepared without *Manahshila - Ubhayastha (Galastha + Talastha)*, TLC: Total leucocyte count, ESR: Erythrocyte sedimentation rate, AEC: Acute eosinophil count, PEFR: Peak expiratory flow rate, SEM: Standard error of the mean

Table 6: Hematological results of SPRMT (applied paired *t* test)

Parameter	*n*	Before treatment	After treatment	% Changes	*t* value	*P* value
Hb	12	12.01±0.67	11.94±0.50	0.58↓	0.26	>0.05
TLC	12	7300±614.46	7330±481.21	0.41↑	0.10	>0.05
Eosinophils	12	4.50±0.62	4.7±1.49	4.44↑	0.813	>0.05
ESR	12	17.20±3.79	27.1±10.04	57.56↑	1.150	>0.05
AEC	12	355.00±82.14	400.00±172.88	12.68↑	0.28	>0.05
PEFR	12	165.00±28.529	182.00±23.240	13.33↓	1.71	>0.05

Data: Mean±SEM,↑: Increase, ↓: Decrease, *P < 0.05, **P < 0.01,***P < 0.001, SPRMT: *Sameera Pannaga Rasa* prepared with *Manahshila - Talastha*, TLC: Total leucocyte count, ESR: Erythrocyte sedimentation rate, AEC: Acute eosinophil count, PEFR: Peak expiratory flow rate, SEM: Standard error of the mean

Table 7: Hematological results of SPRMU (applied paired *t* test)

Parameter	*N*	Before treatment	After treatment	% Changes	*t* value	*P* value
Hb	12	12.61±0.53	12.16±0.50	2.38↓	1.19	>0.05
TLC	12	74863.64±783.33	6681.82±529.87	11.83↓	1.83	>0.05
Eosinophils	12	4.55±0.64	4.00±0.83	5.5↓	0.264	>0.05
ESR	12	32.73±7.56	28.09±5.64	10.44↓	1.04	>0.05
AEC	12	381.20±88.55	259.09±51.80	32.14↓	1.17	>0.05
PEFR	12	166.67±24.005	197.50±23.327	18.50↓	2.02	>0.05

Data: Mean±SEM, ↑: Increase, ↓: Decrease, *P < 0.05, **P < 0.01,***P < 0.001, SPRMU: *Sameera Pannaga Rasa* prepared with *Manahshila - Ubhayastha (Galastha + Talastha)*, TLC: Total leucocyte count, ESR: Erythrocyte sedimentation rate, AEC: Acute eosinophil count, PEFR: Peak expiratory flow rate, SEM: Standard error of the mean

Table 8: Effect of drugs on withdrawal of emergency drugs (applied Wilcoxon rank test)

Group (*n*=45)	*N*	Mean±SEM		Change		Actual rank (D)	'α'
		B.T	A.T	Mean±SEM	%		
SPRT	06	4.83±0.401	1.00±0.632	3.83±0.946	79.31↓	21*	<0.05
SPRU	08	4.50±0.327	1.50±0.008	3.00±0.567	66.67↓	28**	<0.02
SPRMT	05	3.00±1.265	1.60±0.748	1.40±1.470	46.67↓	7	>0.05
SPRMU	08	4.50±0.412	1.25±0.696	3.33±0.527	74.07↓	36***	<0.01

Data: Mean±SEM, ↓: Decrease, *α<0.05, **α<0.02, ***α<0.01, SPRT: *Sameera Pannaga Rasa* prepared without *Manahshila - Talastha*, SPRU: *Sameera Pannaga Rasa* prepared without *Manahshila - Ubhayastha (Galastha + Talastha)*, SPRMT: *Sameera Pannaga Rasa* prepared with *Manahshila - Talastha,* SPRMU: *Sameera Pannaga Rasa* prepared with *Manahshila - Ubhayastha (Galastha + Talastha)*, SEM: Standard error of the mean, AT: After Treatment, BT: Before Treatment

Table 9: Level of asthma control before treatment and after treatment

Level of asthma control	SPRT				SPRU				SPRMT				SPRMU			
	BT	%	AT	%	BT	%	AT	%	BT	%	AT	%	BT	%	AT	%
Controlled	0	00	7	58	1	9	5	45.5	1	10	5	50	0	0	9	75
Partialy controlled	4	33	5	42	3	27	5	45.5	2	20	3	30	5	42	3	25
Un controlled	8	67	0	00	7	64	1	9	7	70	2	20	7	58	0	0

SPRT: *Sameera Pannaga Rasa* prepared without *Manahshila - Talastha*, SPRU: *Sameera Pannaga Rasa* prepared without *Manahshila - Ubhayastha (Galastha + Talastha)*, SPRMT: *Sameera Pannaga Rasa* prepared with *Manahshila - Talastha,* SPRMU: *Sameera Pannaga Rasa* prepared with *Manahshila - Ubhayastha (Galastha + Talastha)*, AT: After treatment, BT: Before treatment

Table 10: Effect of SPRT on asthma control† (applied 'χ^2)

Group	ACT score	<19	≥19	Row total	χ^2	*P*
SPRT	BT	9	3	12	4.17**	<0.02
	AT	3	9	12		
SPRU	BT	10	1	11	2.063	>0.05
	AT	6	5	11		
SPRMT	BT	9	1	10	3.516	>0.05
	AT	4	6	10		
SPRMU	BT	11	1	12	6.40***	<0.01
	AT	4	8	12		

χ^2 value for Df 1*P < 0.05, **P < 0.02, ***P < 0.01, ****P < 0.002, ******P < 0.001, SPRT: *Sameera Pannaga Rasa* prepared without *Manahshila - Talastha*, SPRU: *Sameera Pannaga Rasa* prepared without *Manahshila - Ubhayastha (Galastha + Talastha)*, SPRMT: *Sameera Pannaga Rasa* prepared with *Manahshila - Talastha*, SPRMU: *Sameera Pannaga Rasa* prepared with *Manahshila - Ubhayastha (Galastha + Talastha)*, AT: After treatment, BT: Before treatment

Table 11: Recurrence asthma in follow-up period (in percentage)

Follow-up (2 weeks)	SPRT		SPRU		SPRMT		SPRMU	
	n	%	*n*	%	*n*	%	*n*	%
Recurrence	1	8.33	1	9.09	3	30	0	00
No recurrence	11	91.67	10	90.91	7	70	12	100

SPRT: *Sameera Pannaga Rasa* prepared without *Manahshila - Talastha*, SPRU: *Sameera Pannaga Rasa* prepared without *Manahshila - Ubhayastha (Galastha + Talastha)*, SPRMT: *Sameera Pannaga Rasa* prepared with *Manahshila - Talastha*, SPRMU: *Sameera Pannaga Rasa* prepared with *Manahshila - Ubhayastha (Galastha + Talastha)*

Vataghna.[15] These dynamic actions are helpful in breaking the pathogenesis of *Tamaka Shwasa*. Asthma is now accepted as a T-helper type 2 lymphocyte-mediated chronic inflammatory disorder, characterized by airway eosinophilia and airway hyper responsiveness. Eosinophils appear to play a crucial role in the ongoing inflammation due to either an impaired clearance or a delayed apoptosis in the airways, where accumulation of a number of eosinophils cytotoxic proteins including major basic protein, cationic proteins and peroxidase could occur. As2o3 could alleviate the airway inflammation through promoting pulmonary eiosinophils (PE) apoptosis and lower PE infiltration. Low dose of As2o3 is proved to be effective with relative safety, it also has potential value in treating asthma.[16]

In modern point of view SPR is Mercury-Arsenical (*Ubhayastha*) or Arsenical preparation (*Talastha*).

In preparation of SPR, *Tulsipatra Swarasa* (leaf of *Oscimum sanctum* Linn.) as *Bhavana Dravya* is advocated. Studies have been proved that leaf extract of *Ocimum sanctum* is effective against arsenic induced toxicity. *Ocimum sanctum* has significant role in protecting animals from arsenic induced oxidative stress and in the depletion of arsenic concentration.[17] It has been proved that *Ocimum* extract can protect against mercury toxicity in mice. It significantly enhanced reduced glutathione, which is suggested that oral administration of *Ocimum* extract provides protection against mercury induced toxicity in Swiss albino mice.[18] Thus, Oscimum may help in nullifying possible ADRs of SPR.[19]

In the present study, *Nagavallidala* (*P. betel*) was taken as *Sahapana* for SPR. Studies have proved inhibitory effects of *P. betel* Linn. on production of allergic mediators by bone marrow derived mast cells and lung epithelial cells. PE significantly decreased histamine and granulocyte macrophage colony stimulating factors produced by an IgE mediated hypersensitive reaction and inhibited eotaxin and IL-8 secretion in a tumor necrosis factor-α and IL-4 induced allergic reaction.[20] Reduction of oxidative stress induced by free radicals by virtue of its anti-oxidant properties and chelation of heavy metals thereby minimizing its toxic potential and increasing safety margins were also found to be reported.[21] Considering all these, it is assumed that, *Nagavallidala* can reduce arsenic induced oxidative stress as well as the control of allergic diseases through inhibition of production of allergic mediators.

Conclusion

The results reveal that the SPR has a significant action in cases bronchial asthma and it could suppress total leukocyte count, eosinophil count, ESR and can improve PEFR along with providing symptomatic relief. Analysis of the data generated during the study shows that; all the groups of SPR have been highly significant in treating the condition. However, comparative evaluation shows that SPRMU group is better followed by SPRT and SPRU where SPRMT is less effective comparatively. None of the treated patients developed any adverse effects during the study period.

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"EFFECT OF SWARNA VANGA ON MADHUMEHA IN ALBINO RATS"

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ABSTRACT: *In this study on "Madhumeha" (Prameha) the authors discuss of minerlometallic preparations and its effect either as curative or as palliative measures in Prameha.*

INTRODUCTION

Besides herbal drugs, many a number of mineralo-metallic preparations are claimed to be effective either as curative or as palliative measures in Pramehas. In the present context we mean Madhumeha by the term prameha.

Swarna Vanga, a mosaic gold like Ayurveda preparation has been claimed to be effective in Pramehas since its introduction into the Ayurvedic Therapeutics i.e. from18th century A. D. onwards.

It is said to be bitter in taste and such bitter substances are generally claimed to be effective in pramehas by pacifying (vata and destroying kapha. Incidentally Madhumeha is one of the sub types of vatic pramehas; hence, in this, Vata is more predominating than kapha, though the pramehas are kapha dominated in general.

Keeping these points in view and also the claims of ancient Ayurvedic seers we have undertaken this study to assess the hypoglycaemic action of Swarna Vanga in four different doses on normogly-caemic and hyperglycaemic albino rats.

Efforts were also made to study its prophylactic effect against alloxan induced diabetes in experimental animals.

MATERIAL AND METHODS.

Swarna Vanga prepared with half mercury was selected for this study.

The chemicals required for blood sugar estimation and preparation of drug suspension are –

a) Sodium Tungastate – 10%
b) Sulphuric Acid – 0.67N
c) Alk. Copper Sulphate –
d) Phospho Molybdic Acid –
e) Alloxan
f) Insulin
g) Glucose
h) Gumacacia.

All the chemicals are of B. D. H. company A. R. group.

Borosil glasswares were used and readings were recorded in systronics photoelectric calorimeter.

Healthy adult albino rats of either sex weighing 100-150 gm. were procured from central animal house I. M. S., B. H. U.

The animals were allowed to acclamatise to he laboratory environment before starting the experiment. The animals were given normal laboratory diet and water ad-libitum.

In normoglycaemic study, diet was allowed till the starting of the experiment and in the diabetic experiments diet was withdrawn before 18 hrs. of induction of diabetes. During fast only water was allowed ad-libitum.

Preparation of Drug Suspension

Drug was made a suspension in 20 % Gum-acacia solution, given orally through '0' catheter and only gum-acacia solution was given to the controls.

Diabetes was induced by using alloxan following the usual method described by Leukens F. O.W. (1948).

Blood was collected in a wax block by puncturing the para orbital venous plexus with a thickish glass capillary. No anticoagulant was added.

Blood sugar was estimated by Folinswu method.

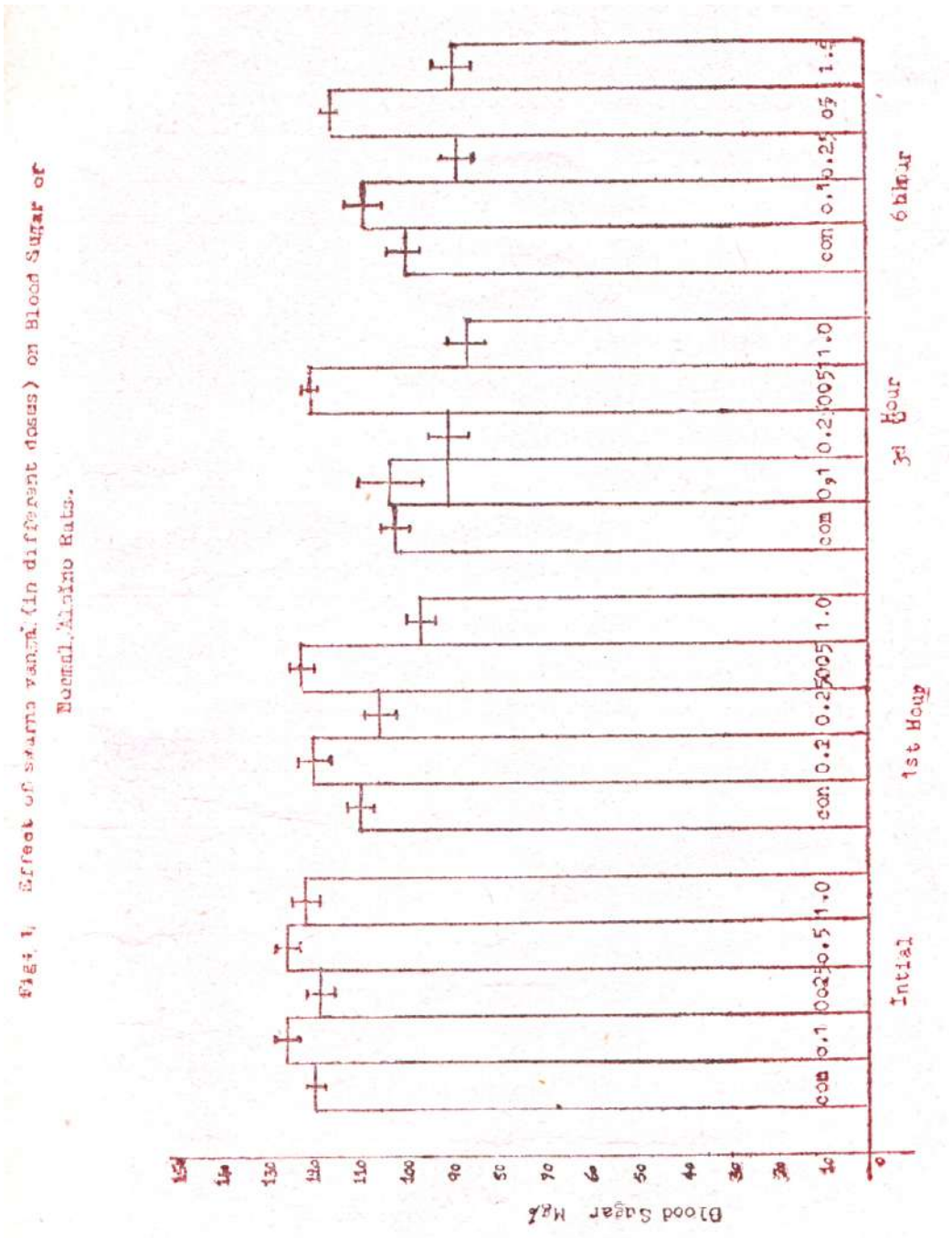
Normo Glyeaemic Study.

Forty albino rats were taken and grouped into four batches. Again each batch was subdivided into the two groups consisting 5 animals in each and housed in separate cages. One group was labeled as controls while other as treated. The treated group of each batch received 0.1; 0.25; 0.5 and 1gm/kg body weight doses, orally in suspension form. Control group was given only 20% gum-acacia solution.

The blood was collected at initial 'O' hours and after 1^{st}, 3^{rd} and 6^{th} hours of drug administration.

After suitable intervals (7days) the groups were reversed i.e. the previous control group was given the drug and treated group served as control. Thus the total numbers of animals assessed experimentally are 80.

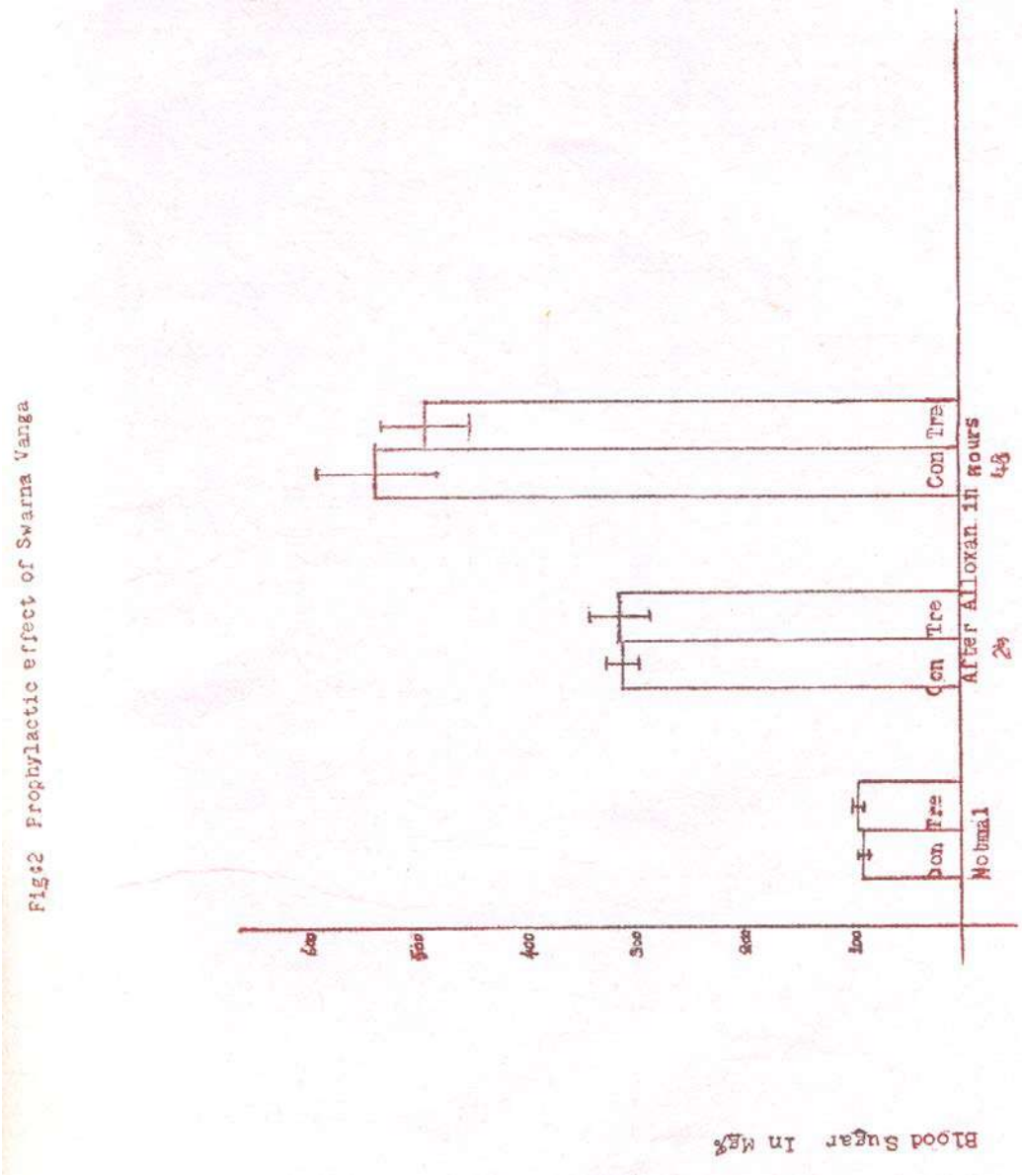
The results are shown in Table No. I. Fig. 1.



Prophylactic Study

20 albino rats were taken, grouped and kept in two (10+10) cages. The treated group was given the test drug in the dose of 0.5 gm/kg body weight (b. i. d.), for 3 days. The control group was given only gum-acacia solution. After 3 days diabetes was induced with alloxan after keeping the animals in 18 hrs. fast.

Blood sugar was estimated before and after 24 and 48 hours of alloxan administration. The results are shown in Table 2; fig. 2.



Hyperglycaemic [Anti-Diabetic – Experiment

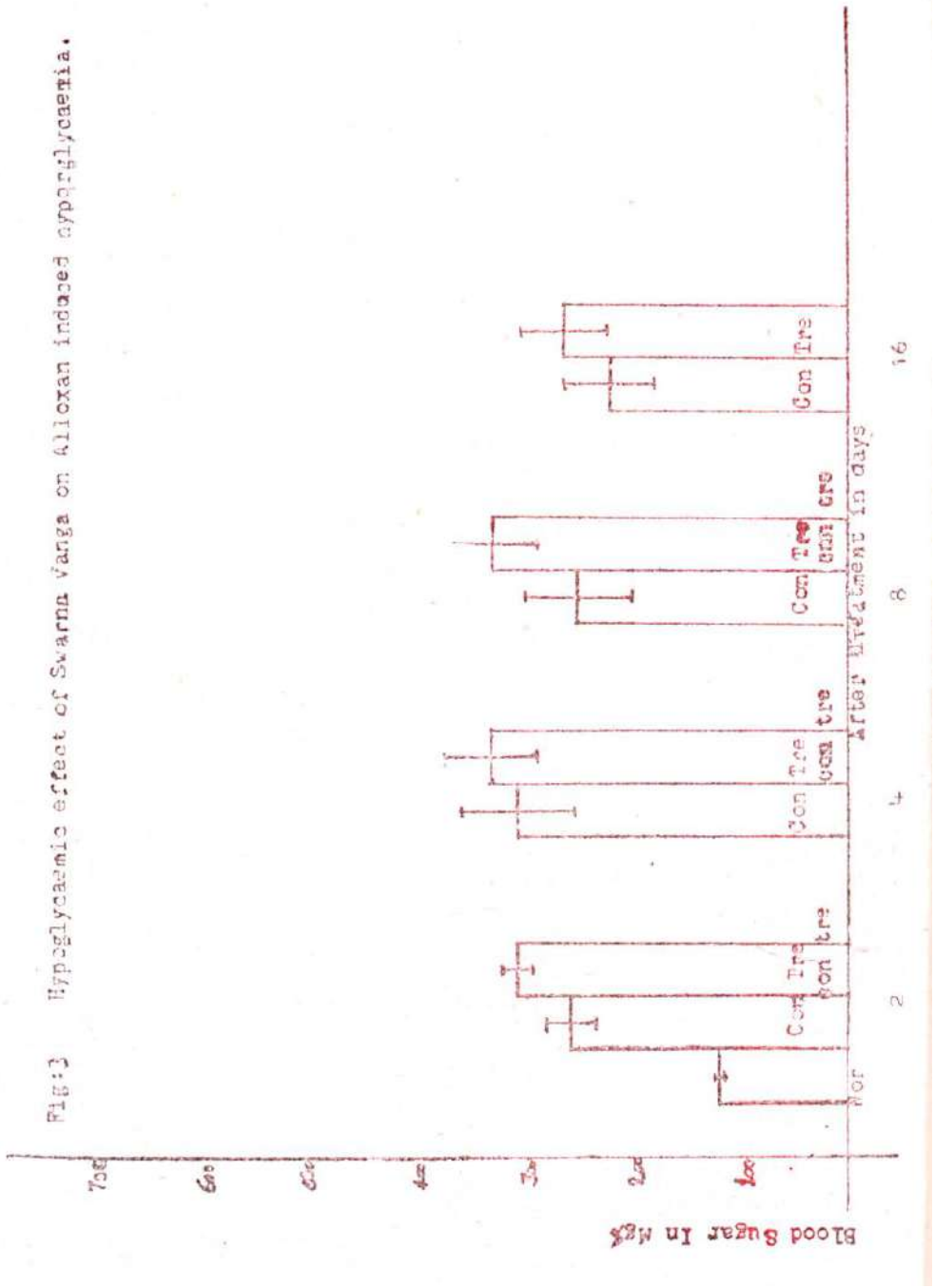
20 albino rats were kept under fast for 18 hours. The alloxan diabetes was induced in

usual manner. The blood sugar was estimated and the animals having blood sugar was estimated and the animals having blood sugar more than 200 mg% were taken for the study, and the rest were discarded. They were divided into control (7). and

treated groups (7). The treated group was given the test drug in 0.5 gm/kg body weight does b.i.d. for 15days and control group only gum-acasia solution.

The blood was collected before and after the 2^{nd}, 4^{th}, 8^{th} and 16^{th} day.

The results are shown in table 3; fig. 3.



Observation & Results

1) The rats which received the alloxan had passed pinkish coloured urine a few minutes after alloxan administration.

2) Later all the rats started passing large volumes.

3) Most of the animals have been emaciated.

4) In Antidiabetic experiment. Three rats did not become diabetic and 3 animals have died after developing convulsions.

5) The water consumption was also found increased by two times to the normal.

TABLE-I
NORMOGLYCAEMIC

The Effect of Swarna-Vanga on blood sugar levels of normal albino rats. Data represent mean ± S. E. blood sugar levels (Mg%) befoe) & after (1. 3, & 6) the drug and mean% fall (-) or rise (±) 1, 3 & 6) in blood sugar < levels in self conrol observations in cross over design P indicates Paired "t" value and "t" indicates studens 't' test.

Group	Dose in gm/kg.	No. of animals	Mean ± SE, Blood Sugar levels (Mg%)				Mean ± SE. fall (-) or rise (+) in Blood Sugar after (in hours)		
			O	1st	3rd	6th	1st	3rd	6th
Control	Gum-acacia suspension	39	119.03±1.59	109.39±2.61	100.32±2.85	99.97±3.24	3.78* 7.79 ±2.06	6.39** 15.48 ±2.42	6.15*** 15.87 ±2.58
Treated	0.1	10	125.01 ±2.72	118.2±3.81	102.6± 6.61	108.9±4.06	4.02±3.06 t.1.02N S	18.36±4.31 0.58N S	13.82±2.67 0.47N S
	0.25	10	118.00 ±3.13	105.7±3.45	90.9±3.59	88.3±3.65	4.16** 10.11±2.43 0.73NS	9.21*** 22.93±2.49 2.15<0.05	10.14*** 25.05±2.47 2.57<0.02
	0.5	10	125.5±2.73	121.5±2.09	119.3±1.44	115.6±2.39	1.69NS 2.97±1.75 t. 1.78NS	2.79NS 4.69±1.68 3.66<0.001	3.50* 7.67±2.19 2.42<0.02
	1.0	10	121.6 ±2.99	96.1 ±3.29	85.00 ±4.02	89.1 ±4.83	9.58*** 20.70 ±2.16 4.33<0.001	11.14*** 30.19 ±2.71 4.05<0.001	9.35*** 27.02 ±2.89 2.88<0.01

TABLE-II

Showing the Effect of Swarna-Vanga (0.5gm/kg body wt. oral) on blood sugar levels in albino rats when administered prior to and along with alloxan. Data represented mean blood sugar ± S.E. (Mg%). Number in parentheses indicate the number of observations.

Group	Normal	24 hours	48 hours	
Control	80.5±	309.2±	539.45±	
	2.25	15.51	56.49	
	(10)	(10)	(9)	
	A_1	A_2	A_3	
Treated	90.7±	312.9±	491.89±	
	4.14	25.89	41.47	
	(10)	(10)	(9)	
	B_1	B_2	B_3	
	A_1: A_2	14.59<0.001	B_1: B_2 8.48<0.001	A_1 : B1 2.17 < 0.05
	A_1: A_3	8.12<0.001	B_1: B_3 9.63<0.001	A_2 : B2 0.12 N.S.
				A_3 : B3 0.68 N.S.

TABLE-III

The effect of Swarna Vanga (0.5 gm/kg body wt. oral) on the blood sugar levels of alloxan induced hyperglycaemic albino rats. Data represent blood sugar mean ± S. E. (Mg%) Values. Number in parenthesis indicate the number of observations.

Group	Normal	After alloxan in days			
		2	4	8	16
Control	121.1±	259.14±	308.29±	252.00±	220.29±
	2.01	22.06	49.76	49.73	39.37
	(20)	(7)	(7)	(7)	(7)
	A_0	A_2	A_4	A_8	A_{16}
Treated	3	311.5±	333.67±	323.20±	266.40±
		12.85	44.35	43.08	41.21
		(7)	(7)	(6)	(6)
		B_2	B_4	B_8	B_{16}
	A_0:A_2 6.23 < 0.001		A_0:B_2 14.68 <0.001		A_2:B_2 2.05 N.S.
	A_2:A_4 0.90 N.S.		B_2:B_4 0.48 N.S.		A_4:B_4 0.38 N.S.
	A_2:A_8 0.13 N.S.		B_2:B_8 0.26 N.S.		A_8:B_8 1.09 N.S.
	A_2:A_{16} 1.16 N.S.		B_2: B_{16} 1.05 N.S.		A_{16}:B_{16} 0.81 N.S.

DISCUSSION

As per literature swarna vanga has been claimed to possess pramehaghna effect. Madhumeha is one of the subtypes of prameha, and so a systametic study has been carried out to verify this claim, on normoglycaemic , alloxan induced hyperglycaemic rats and also to assess its prophylactic effect.

The experiment on normoglycaemic rats showed that swarna vanga in 1 gm/kg dose produced a significant fall in blood sugar reading at 1st 3rd and 0.25 gm kg dose, the fall was obvious only in 3rd hour reading and in still lower doses the hypoglycaemia produced was not much significant statistically. This indicates that the drug is more effective in higher doses on normoglycaemic rats than lower doses.

1nprophylactic study though the drug has not prevented the hyperglycaemic condition completely, however, the results indicate that the increase in blood sugar level in treated group after 48 hour is less pronounced than controls, though statistically it is not found significant.

1n alloxan induced hyperglycaemia though the fall is statistically not significant but on comparison we can find a noticeable fall in treated group than the control.

By seeing the results of normoglycacaemic, prophylactic and antidiabetic experimens we can say that the drug has some role to play in lowering the blood sugar level. It may be of mild nature

CONCLUSION

It has shown significant hypoglycaemic effect in normoglycaemic rats, though in prophylactic and in alloxan induced diabetic rats the effect is not found statistically significant.

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Evaluation of the effect of conventionally prepared *swarna makshika bhasma* on different bio-chemical parameters in experimental animals

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ABSTRACT

Swarna makshika (chalcopyrite) *bhasma* (SMB) has been used for different therapeutic purposes since long in Ayurveda. The present study is conducted to evaluate the effect of conventionally prepared SMB on different bio-chemical parameters in experimental animals, for providing scientific data base for its logical use in clinical practice. The genuine SMB was prepared by following classical techniques of *shodhana* and *marana* most commonly used by different Ayurvedic drug manufacturers. *Shodhana* was done by roasting raw *swarna makshika* with lemon juice for three days and *marana* was performed by 11 *putas*. The experimental animals (rats) were divided into two groups. SMB mixed with diluted honey was administered orally in therapeutic dose to Group SMB and diluted honey only was administered to vehicle control Group, for 30 days. The blood samples were collected twice, after 15 days and after 30 days of drug administration and different biochemical investigations were done. Biochemical parameters were chosen based on references from Ayurvedic classics and contemporary medicine. It was observed that Hb% was found significantly increased and LDL and VLDL were found significantly decreased in Group SMB when compared with vehicle control group. This experimental data will help the clinician for the logical use of SMB in different disease conditions with findings like low Hb% and high LDL, VLDL levels.

Key words: Bio-chemical parameters, makshika bhasma, marana, puta, shodhana

INTRODUCTION

In Ayurveda, Swarna makshika (chalcopyrite) bhasma (SMB) is a very popular drug since hundreds of years.[1] It is used singly or as an important ingredient of many compound formulations for treating *pandu* (anemia), *hridaya roga* (cardiac diseases), *agnimandya* (impaired digestive capacity)[2] etc. The manufacturing of SMB involves two main processes i.e. *shodhana* and *marana*. *Shodhana includes* different techniques like roasting, heating and quenching. m*arana* is *puta* system of heating (please go through the glossary at the end). SMB can be manufactured using different *putas* like *varaha puta, kukkuta puta, gaja puta* etc. or *kupipakwa* method of heating can be used. Various processing materials of herbal origin like lemon juice and mineral origin like *gandhaka* (sulfur) are used during manufacturing process of SMB.

Biochemical parameters provide key points to diagnose and treat diseases for contemporary medicine. Though SMB is being used since long on the basis of Ayurvedic classical parameters, development of biochemical science can provide tools to strengthen its usage. The study was planned with an intention to enhance acceptability of SMB as a potent medicine by cross disciplinary scientists and practitioners. A database of biochemical values justifying its use in certain disease conditions was generated by this study on experimental animals.

MATERIALS AND METHODS

Genuine SMB was prepared in the laboratory of Dept. of Rasa Shastra, Faculty of Ayurveda, Banaras Hindu University by following classical references,[5] and earlier

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research work done in this line.[6] Standard operative procedures were strictly followed for manufacturing of SMB. *Shodhana* was done by roasting technique with frequent addition of lemon juice in it. *Marana* was done by *puta* system of heating using incineration of 04 kg cow dung cakes[1,6] each time. The incineration process was repeated 11 times (11 *putas*).

Steps in *Swarna Makshika Bhasama* manufacturing process

- Raw Swarna Makshika (Chalcopyrite)
- Powderization of Swarna Makshika using Mortar and Pestle
- Shodhana Process: Bharjana (Roasting with lemon juice at about 750°C for three days 24h)
- Swangashita (allowed to self cool)
- Shodita Swarna Makshika
- Bhawana (Wet trituration with lemon juice)
- Chakrika Nirman (Pelletization)
- Sharava Samputa (Sealing the dried pellets in earthen casseroles using cloth and fuller's earth)
- Putapaka (Firing the sharava samputa in puta system of heating using cow dung fuel and allowed to self cool)
- The processes of Bhawana, Putapaka and Swangashita were repeated 11 times
- Swarna Makshika Bhasma (Finished product)

Ayurvedic standardization of SMB

The finished product SMB was assessed on following quality control parameters advocated in Ayurvedic classical texts.[7,8]

Rekhapurnatvam

A pinch of SMB was rubbed between thumb and index finger. It was observed that the *bhasma* enters into the lines of the finger which was not easily cleansed out from the cleavage of finger lines.

Varitaratavam

Small amount of the SMB was taken and sprinkled over the silent water taken in a glass beaker. It was found that the *bhasma* particles float over the surface of the water.

Uttama

The SMB was sprinkled over water in a glass beaker and a rice grain was placed over it. Bhasma and the rice grain were found floating on water surface.

Nisvadutvam

The SMB was found tasteless when a small amount was kept over the tongue.

Amla Parikshya

A pinch of SMB was mixed with little amount *dadhi* (curd) taken in a clean and dry petri dish, kept for 24 h and then observed for any color change. No color change of *dadhi* (curd) was observed. The same procedure was followed with lemon juice taken in a test tube and same observation was found.

Dantagre na cha kacha kacha bhava iti (without gridding sensation on teeth)

When a small amount of the *SMB* was placed between the teeth, no sandy feeling was observed.

Avami

Intake of very small amount of the *SMB* did not produce any nausea / vomiting.

The SMB was also subjected to different modern quality control parameters like XRD (X-ray diffraction), TEM (transmission electron microscope) and EDAX (energy dispersive X-ray analysis), and reports were documented.

XRD of raw material was showing all the major peaks that of $CuFeS_2$ (chalcopyrite) and for finished product the peaks were indicating mixture of many compounds viz. Cu_2O, $FeSO_4$, Fe_2O_3, SiS_2, and Cu_2S.

TEM study showed that the particle size of the raw material was 5-10 μ and for the SMB it was 50-200 nm.

Analyzing both raw *Swarna Makshika* and SMB, using EDAX study revealed that, both contain, iron, copper and sulfur. In addition to it, finished product SMB contains potassium, magnesium, aluminum and silicon in small amount.

Dose determination

Dose of the trial drug was extrapolated (human dose to animal dose) using extrapolation factor and honey was used as vehicle for administration of the drug.[9]

Animal dose = total clinical dose (a) ×(extrapolation factor (b) 0.018 = (c) per 200 g of rat

Total clinical dose considered was 250 mg /60 kg of human per day[10]

250 mg /60 kg of human per day × 0.018 = 4.5 mg/200 g of rat/day

The actual dose administered to the animal was 4.5 mg/200 g of rat/day

Vehicle honey

Honey was used as the vehicle control as directed by the reference of the study. Other than this, honey is described as one of the best *yogavahi* substances, which facilitates assimilation of other substance in body, in Ayurvedic classics.

Also most of the time, Ayurvedic clinicians use SMB with honey. Hence it was used as the vehicle. Honey was diluted with de-ionized water for the convenience of administration.

Caring of animals

The study was conducted on Charles's foster strain, male albino rats weighing between 100 and 150 g. All the animals were kept in colony cages at an ambient temperature of 25± 2ºC, with relative humidity 45–55% and 10:14 h' light and dark conditions. The animals were kept on standard rodent feed and water was allowed *ad libitum*. Principles of laboratory animal care and use were followed throughout the study.[11] Animals were acclimatized for seven days before starting the experiment. The study was designed with due permission of Animal ethics committee.

Grouping of animals

The experimental animals were divided into two groups, with 6 animals in each group. SMB mixed with diluted honey was administered orally in therapeutic dose to Group SMB and diluted honey only was administered to vehicle control Group, for 30 days.

Group SMB

SMB was administered to the animals using rubber catheter in therapeutic dose, i.e. 4.5 mg/200 g of rat orally for 30 days with diluted honey (3 ml honey + 4.5 ml de-ionized water). The actual amount administered was adjusted between 0.5 ml and 1 ml containing calculated dose of the drug for individual animal.

Control group

Only diluted honey (3 ml honey + 4.5 ml de-ionized water) was administered to the animals in control group.

Evaluation of the effect of trial drug on biochemical parameters

As the trial drug is indicated for its use in *pandu* (anemia), *hridayaroga* (heart disease) and *agnimandya* (impaired digestive capacity) in classical texts,[3,4] biochemical parameters related to liver function, lipid metabolism and Hb% were chosen to evaluate in experimental animals.[12]

Biochemical parameters related to liver function

SGOT (serum glutamic oxaloacetic transaminase), SGPT (serum glutamic pyruvate transaminase), ALP (alkaline phosphatase), serum bilirubin (total), serum bilirubin (direct), serum protein, serum albumin.

Biochemical parameters related to lipid metabolism

Serum cholesterol, serum triglycerides, HDL (high density lipoproteins), LDL (low density lipoproteins), VLDL (very low density lipoprotein)

Hematological parameter:
Hemoglobin%

Collection of blood samples: Rationale and method

With an intention to study the effect of the drug intimately, the parameters related to liver function and Hb% were assessed twice i.e. after 15 days and after 30 days. However, the parameters related to lipid metabolism were studied only after 30 days because the parameters are not expected to change in a short duration of 15 days.

All the animals from both the groups were anesthetized using ketamine hydrochloride 24 mg/kg body wt.[11] and blood samples were collected from retro-orbital plexus. The samples were sent for biochemical analysis. The procedure was repeated both the times, after 15 days and after 30 days. After 30 days, the animals were sacrificed and disposed as per standard protocol for animal ethics.

The results of biochemical investigations were documented and analyzed statistically by using Student's paired 't' test.

OBSERVATIONS AND RESULTS

Comparison between biochemical values of both the groups SMB and control revealed following observation [Tables 1].

- Statistically significant increase in Hb% was observed in Group S in comparison with Group V. The observation was similar for both the phases of experiment.
- The changes in all the biochemical parameters related to liver function i.e. SGOT, SGPT, ALP, serum bilirubin (total), serum bilirubin (direct), albumin and protein were observed statistically insignificant. The observations were similar for both the phases of experiment.
- Serum cholesterol, triglyceride and VLDL were found significantly decreased after administration of trial drug for 30 days.
- After 30 days of administration, increased HDL level was observed in animals of treated group, but it was statistically insignificant.
- After 30 days of administration, the changes in LDL level were also found to be statistically insignificant.

DISCUSSION

Administration of SMB in therapeutic dose for 15 days significantly increased hemoglobin%. The observation advocates the logical use of the drug to achieve therapeutic effect even in short-term administration in disease conditions with low Hb%. This may be due to the presence of Fe (iron) in *makshika bhasma* as one of the major ingredients.

Table 1: Effect of SMB on various biochemical parameters after 15 and 30 days of administration and comparison between the groups (number of animals in each group: 6)

Biochemical parameters	After 15 days			After 30 days		
	Control group Mean ± SD	SMB group Mean ± SD	'Paired t' and '*P*'	Control group Mean ± SD	SMB group Mean ± SD	'Paired t' and '*P*'
Hb%	10.767 ± 0.5610	13.617 ± 1.1053	*t* = 5.632 *P*< 0.001	11.033 ± 0.7118	14.500 ± 0.7071	*t* = 8.463 *P*< 0.01
SGOT	25.40 ± 5.030	25.67 ± 4.412	*t* = 0.094 *P*>0.05	77.80 ± 11.819	66.80 ± 7.328	*t* = 1.769 *P*>0.05
SGPT	18.20 ± 2.588	19.33 ± 2.251	*t* = 0.778 *P*>0.05	41.40 ± 2.881	38.60 ± 2.191	*t* = 1.730 *P*>0.05
ALP	74.60 ± 5.413	78.17 ± 5.565	*t* = 1.071 *P*>0.05	85.60 ± 5.505	88.50 ± 2.380	*t* = 0.973 *P*>0.05
S. Bilirubin (T)	0.483 ± 0.0753	0.500 ± 0.0632	*t* = 4.15 *P*>0.05	0.483 ± 0.0408	0.450 ± 0.0837	*t* = 0.877 *P*>0.05
S. Bilirubin (D)	0.569 ± 0.0819	0.567 ± 0.0815	*t* = 000 *P*>0.05	0.450 ± 0.0837	0.400 ± 0.0894	*t* = 1.000 *P*>0.05
Protein (T)	9.25 ± 0.836	9.12 ± 0.760	*t* = 0.267 *P*>0.05	7.550 ± 0.9290	7.733 ± 0.6088	*t* = 0.404 *P*>0.05
Albumin	5.12 ± 0.366	5.07 ± 0.467	*t* = 0.204 *P*>0.05	4.383 ± 0.4262	4.300 ± 0.4472	*t* = 0.330 *P*>0.05
Total cholesterol	-	-	-	112.15 ± 18.965	90.84 ± 7.360	*t* = 2.352 *P*< 0.05
Triglycerides	-	-	-	114.16 ± 17.458	87.82 ± 14.353	*t* = 2.479 *P*< 0.05
LDL	-	-	-	10.58 ± 3.039	11.16 ± 3.303	*t* = 0.289 *P*>0.05
VLDL	-	-	-	22.88 ± 3.164	15.48 ± 4.016	*t* = 3.236 *P*< 0.05
HDL	-	-	-	68.48 ± 8.995	77.47 ± 15.602	*t* = 1.141 *P*>0.05

SGOT = Serum glutamic oxaloacetic transaminase, SGPT = Serum glutamic pyruvate transaminase , ALP = Alkaline phosphatase, LDL = Low density lipoproteins, VLDL = Very low density lipoprotein, HDL = High density lipoproteins

The changes in biochemical parameters like SGOT, SGPT, ALP, serum bilirubin (total), serum bilirubin (direct), serum protein, and serum albumin after 15 days were found to be statistically insignificant. It indicates that in short-term administration, the drug has no significant effect on parameters related to liver function.

Administration of SMB in therapeutic dose for 30 days showed significant increase in Hb% and significant decrease in serum cholesterol, triglycerides and VLDL level. It also showed increase in HDL levels, though it was not statistically significant.

Significant increase in serum cholesterol, triglycerides and VLDL levels elucidates that the SMB may be used very effectively in different health campaigning programs in the subject of high cholesterol, triglycerides and VLDL level leading to supportive treatment for obesity and cardiac problems.

Significant increase in Hb% in both the phases of study with liver function test values within the normal limits shows that the conventionally prepared SMB can be used very effectively in various disease conditions. The results advocate its use in conditions with hemoglobin deficiency e.g. anemia due to blood loss, malnutrition, pregnancy etc. which may be of great importance in public health perspectives.

CONCLUSIONS

The current research work justifies the therapeutic use of SMB in *pandu* (anemia), claimed in ancient texts, in experimental animals both for long-term and shot-term administration. Significant decrease in serum cholesterol, triglycerides and VLDL level in treated group rationalizes the thousands-years clinical use of SMB in *hridayaroga* in long-term administration.

This study gives explanation for the sayings of ancient *Acharyas* that the long-term use of the *bhasma* can provide strength and stout body[13] instead of causing untoward effect. It has no significant changes on parameters related to liver function, which indicates that the uses of traditional metal/mineral *bhasmas* are safe, even in long-term administration. Hence the conventionally prepared *bhasma* is a noble dosage form of Ayurvedic treatment and can be used very effectively if manufactured following the standard operative procedures and following the traditional quality control parameters strictly.

Glossary of important terms used in this article

Bhasma

Bhasma is a herbo-mineral manufactured from metal/mineral after typical Ayurvedic pharmaceutical processes like *shodhana* and *marana* documented in ancient texts. The finished product is expected to follow the standardization parameters quoted in texts and can be used as a safe drug if all the guidelines for its usage are followed.

Shodhana

Various pharmaceutical processes like heating and quenching in herbal juices, boiling in animal products like urine, roasting in pan are advocated with an intention to convert a metal-mineral into drug. The *shodhana* generates a product suitable for next pharmaceutical process or it may generate a finished

product. e.g. *shodhana* of *Abhraka* (mica) is done by heating till red hot and quenching it in *triphala* decoction.

Marana

The technique usually followed by *shodhana* is used to convert the metal/mineral into a potent therapeutic drug using ancient *Puta* system of heating

Puta

It is the measure of amount of heat required to convert or transform any metal or mineral into bhsama. This amount of heat is substance specific and measured in terms of fuel used (number of cow dungs or its weight).

Sharava

An earthen petri dish having specific measurements.

Bhavana

The pharmaceutical process of trituration of the drug with liquid medium e. g. *Hingula* with fresh *Zinzibar officinalis* juice.

Chakrika

These are disc-like pellets having approx. 1.5-2 cm in diameter and 0.5-8 cm in thickness, prepared during the *marana* process, after bhavana process.

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Pharmacological Study

Evaluation of subchronic genotoxic potential of *Swarna Makshika Bhasma*

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Abstract

Extremely diminutive published information is available on the mutagenic activity of Ayurvedic *Bhasmas*. Genotoxicity of few *Bhasmas* were reported on single maximum dose, but no reference is available on the sub-chronic level. Hence the present study was carried to generate and evaluate genotoxic potentials of *Swarna Makshika Bhasma* (mineral preparation) administered at therapeutic dose for 14 days. Chromosomal aberrations and abnormal sperm assay parameters were taken in this study. Cyclophosphamide (CP) was taken as positive group and results were compared. The results revealed a lack of generation of structural deformity in above parameters by tested drugs compared to CP treated group. Observed data indicate that the *Bhasmas* tested were non-genotoxic under the experimental conditions.

Key words: Abnormal sperm assay, chromosomal aberrations, cyclophosphamide, genotoxicity, *Swarna Makshika Bhasma*

Introduction

Ayurveda is known and carried forward as an ancient Indian heritage. It is a traditional medical system used by a majority of Indian population.[1] The drugs known as "*Bhasmas*" are well-known in the traditional Indian Ayurveda and these are chemically mixed oxides of one or more metals.[2] Their traditional preparation involves conversion of a pure metal into its oxide form following a typical procedure, available in the ancient literature of Ayurveda.[3] Recently, doubts have been raised about the safety of Ayurvedic preparations using *Bhasma* and concerns were expressed regarding the metal toxicity of traditional preparations containing *Bhasmas*. Ayurveda fraternity claims that these medicines, if properly prepared and administered are safe and therapeutic.[4] According to them toxicity can arise only from a metal in its free form, and that a *Bhasma* prepared according to the classical methods never contains a metal in free form. Despite these theories claiming *Bhasmas* are non-toxic, documented case reports of poisoning was noted.[5,6] Hence the current study of *Swarna Makshika Bhasma* (SMB) would serve as a database of baseline information for genotoxicity, since there is apparently no literature on this aspect of mineral preparations.

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Materials and Methods

Test drugs

Three different samples of *Swarna Makshika* (SM) were collected from different mines across India. *Bhasmas* were prepared according to classical reference.[7] Coded as mentioned below:

1. SMB prepared from samples collected from Khetri Mine, Rajasthan (SMBKR).
2. SMB prepared from samples collected from Hatti Gold Mine, Karnataka (SMBHK).
3. SMB prepared from Malharjkhand Mine, Madhya Pradesh (SMBMM).

Chemicals

Colchicine was obtained from Hi-media, Mumbai. Cyclophosphamide (CP) procured as Cyphos vial from Intas Pharmaceuticals, Mumbai. Potassium chloride, methanol, acetic acid, giemsa stains were obtained from Sissco Research Laboratory, Mumbai, India.

Study design

Animals

Adult Swiss albino mice (weighing 25 ± 5 g) procured from

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an institutional animal house attached to SSR College of Pharmacy, Silvasa, where the study was conducted. Animals were maintained with controlled temperature (23 ± 2°C), relative humidity of 50 ± 10% and 12 h light/dark photo-period. The animals were acclimatized for 7 days prior to the experiment and provided with standard mice feed and distilled water *ad libitum*. Study was carried out after obtaining approval from Institutional Animal Ethics Committee (IAEC/2011/01).

Animals were randomly divided into five groups (5 mice per group) after an acclimatization period. Group I served as positive control and challenged with CP single-dose of 25 mg/kg body weight intra-peritoneally 24 h prior to termination. Group II served as vehicle control. Vehicle was prepared in combination of honey and deionized water (with a ratio of 1:1.5) and administered in the dose of 0.5 ml/kg body weight as per CCRAS/NIN guidelines. Group III, IV, and V received SMBKR, SMBHK and SMBMM at therapeutic dose 4.5 mg/kg body weight respectively along with vehicle for 14 consecutive days and sacrificed on the 15th day. The doses of test drugs were calculated as per the reference of Paget and Barnes (1969).

Body weight

Animals were examined throughout the experimental period for signs of gross toxicity. Body weight was recorded initially and at the time of sacrificed on the 15th day.

Experimental procedure

Chromosomal aberration assay

Animals were injected colchicine intra-peritoneally at the dose of 4 mg/kg body weight, on the 15th day in order to arrest dividing cells in metaphase[8] and sacrificed by cervical dislocation, 90 min after the colchicine treatment. Bone marrow cells from both femurs were extracted, subjected to hypotonic shock treatment (KCl 0.075 M), for about 30 min, at room temperature and then centrifuged at 1000 rpm for 10 min. The cells were fixed 5 times using freshly prepared methanol-acetic acid (3:1). The cells were spread on clean glass slides that were dried on a hot plate at 40°C. One more drop of fixative was added on slides to see more reliable pictures of chromosomes and then the slides were air dried at room temperature and finally stained with a 5% dilution of Giemsa reagent in a phosphate buffer (pH 6.8) for 15 min. The chromosomes of 1000 cells in metaphase abnormalities were analysed with a ×100 oil immersion objective, using a Trinocular Research Carl Zeiss Microscope (Germany). Metaphases with chromosomes and chromatid breaks, gaps, rings, stickiness, centric fusion, and deletion were recorded.[9]

Sperm abnormality assay

The method of Wyrobek and Bruce[10] was used for investigating sperm morphology abnormality assay. The test preparations were administered for 14 days, to correlate the results with positive control group. On 15th day, the overnight fasted animals were sacrificed by cervical dislocation and dissected out. Both the cauda epididymus were removed and placed in a watch glass containing 1 ml phosphate buffered saline (pH 7.2). Then minced and teased carefully well with fine scissors and forceps to release the spermatozoa. After gentle pipetting, the suspension was separated from the tissue fragments and filtered through double layers of muslin cloth to remove the tissue debris. A drop of Eosin Y solution (10:1) was added to this suspension and kept for 30 min. Air dried smears were prepared on clean, grease-free glass slides and a uniform smear was made. About 1000 sperms per animal were examined at ×400 magnifications from each treatment and control groups for the presence of sperm morphological abnormalities.

Statistical analysis

Statistical methods were carried out only to assess change in body weight by applying paired *t*-test. Statistics were not applied in CA assay and sperm abnormality assay. Different kinds of morphological changes were observed in CAs and sperm abnormal aberrations.

Observations and Results

The effect of SMBs on body weight, chromosomal aberration and sperm abnormality assay are shown in Tables 1-3.

Discussion

In vivo CA assay is one of the most frequently used and sensitive tests for the detection of the genotoxic profiles of drugs. The test has been recommended for routine analysis and data obtained are considered highly relevant in human context.[11,12] In the present study a 14-day sub-chronic genotoxicity of SMBs prepared by different samples are evaluated by employing *in vivo* CA assay and abnormal sperm assay (ASA). Although the genotoxic profile of some of *Bhasmas* have been evaluated in various studies,[13] Till date no reports of sub-chronic genotoxicity studies on *Bhasmas* and SMB are available. With this view body weight of animals also recorded after 14 days of drug administration and compared with CP group [Table 1]. All treated groups exhibited significant gain in body weight in comparison to CP. Body weight loss is an indicator of marked tissue loss in the body protein degradation. Gain of body weight is indicating that test drugs are not bearing degenerative potentials.

Colchicine is effective in causing metaphase stasis in cell dividing matrix.[14] Thus used to arrest metaphase, when chromosome structure seen noticeably. It inhibits microtubule polymerization by binding to tubulin, one of the main constituents of microtubules. Availability of tubulin is essential to mitosis, and therefore, colchicine effectively functions as a "mitotic poison" or spindle poison. Hypotonic solution (KCl) causes the cells to swell and enhances eventual separation of the chromosomes to facilitate visual analysis.

Table 1: Effect of SMBs on body weight

Groups	Body weight		
	Before treatment	On 15th day	Actual % changes
CP	25.20±00.86	27.30±00.68↑	06.58±02.09
VC%	25.60±01.08	30.30±00.73↑	20.92±01.29***
SMBKR	26.00±00.89	30.50±00.67↑	20.13±01.65***
SMBHK	25.80±01.07	30.00±00.62↑	21.28±02.36**
SMBMM	26.60±00.81	29.60±00.78↑	20.16±01.66**

Data: Mean±SEM; ↓: Decrease; ↑: Increase; **P<0.01; ***P<0.001 (unpaired *t* test in comparison to CP group). CP: Cyclophosphamide; VC:Vehicle control; SMBKR: *Swarna Makshika Bhasma* prepared from Khetri mine, Rajasthan; SMBHK: *Swarna Makshika Bhasma* prepared from Hatti gold mine, Karnataka; SMBMM: *Swarna Makshika Bhasma* prepared from Malharjkhand mine, Madhya Pradesh; SEM: Standard error of mean

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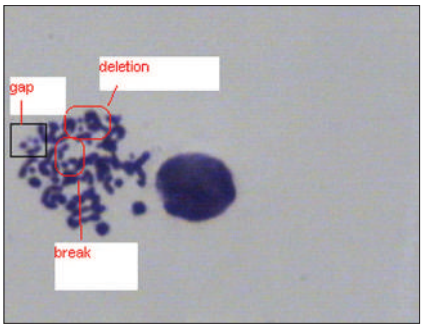

Figure 1a: CP-chromosomal and chromatid break and gap

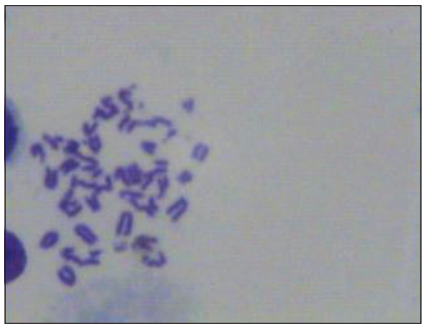

Figure 1b: CP-Deletion

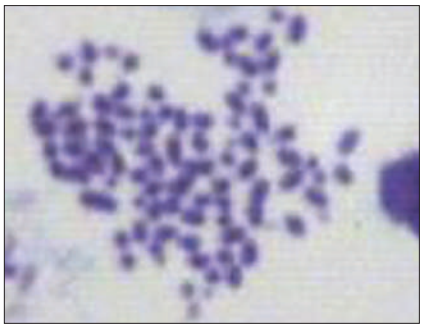

Figure 1c: CP- Pulverization

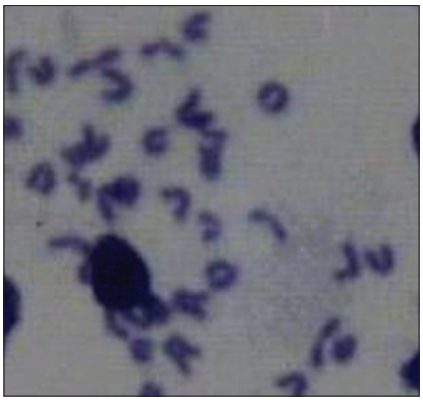

Figure 1d: CP-ring

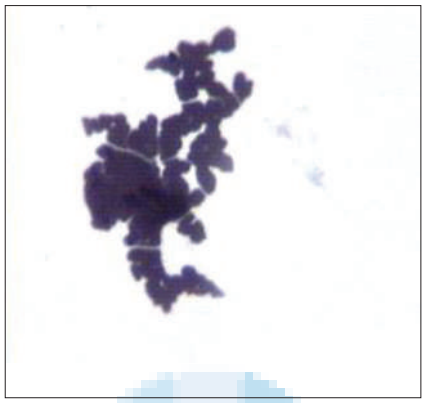

Figure 1e: CP-stickiness

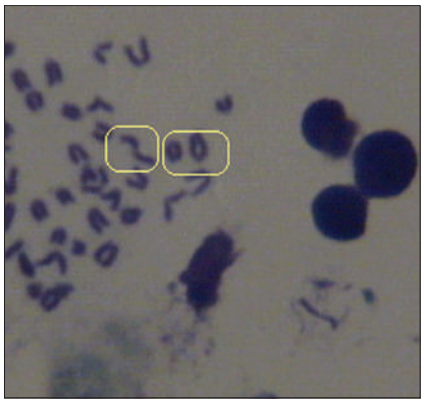

Figure 1f: CP-Translocation and ring

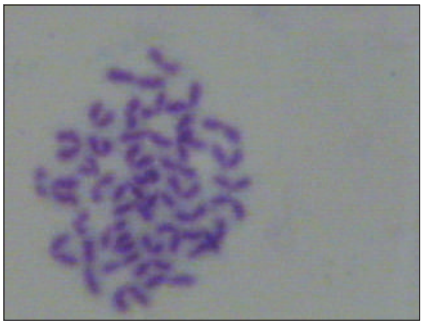

Figure 1g: Normal chromosomes

Figure 1h: Normal chromosomes – SMBKR

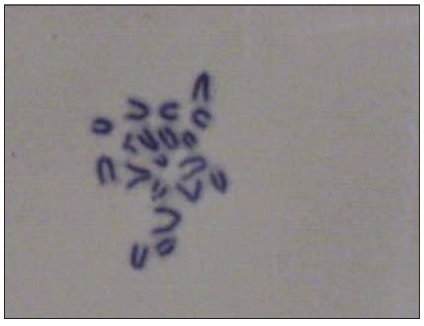

Figure 1i: Normal chromosomes – SMBHK

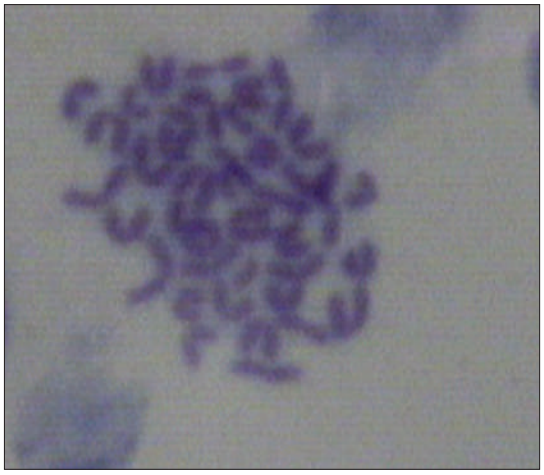

Figure 1j: Normal chromosomes – SMBMM

CP is an anticancer drug that is widely used in anti-neoplastic therapy as well as in the treatment of some non-malignant diseases like rheumatoid arthritis. It is also used as an immunosuppressive agent prior to organ transplantation.[15] In somatic cells, CP has been shown to produce gene mutations, chromosome aberrations, micronuclei and sister chromatid exchanges in a variety of cultured cells in the presence of metabolic activation as well as sister chromatid exchanges without metabolic activation. The compound also produced chromosome damage and micronuclei in rats, mice and Chinese hamster.[16] Its use as a positive control chemical in genotoxicity tests has been recommended.[17] It increased the number of chromosome aberrations in the given dose with relatively high frequencies of chromosome breaks, centric fusion compared with other types of chromosome abnormalities. Gap, ring formation as well as stickiness were also frequent in CP treated group [Figure1a-f]. This may be

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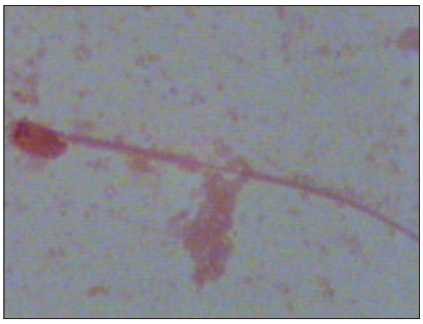

Figure 2a: CP – Hook less head

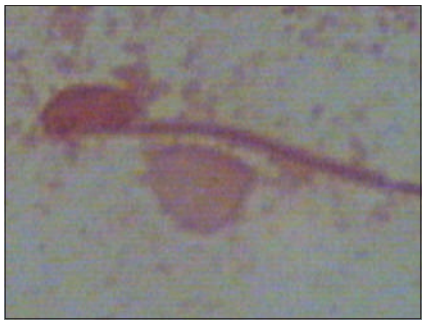

Figure 2b: CP – Deletion

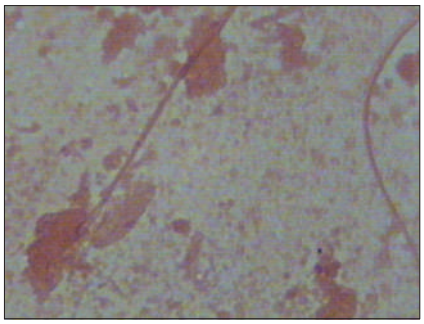

Figure 2c: CP – Banana shaped head

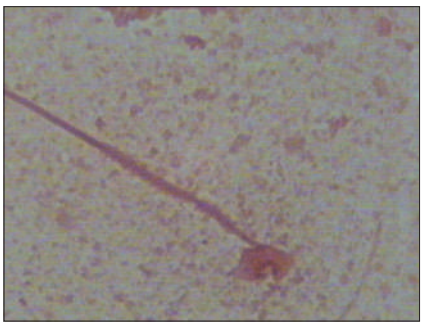

Figure 2d: CP – Amorphous shaped head

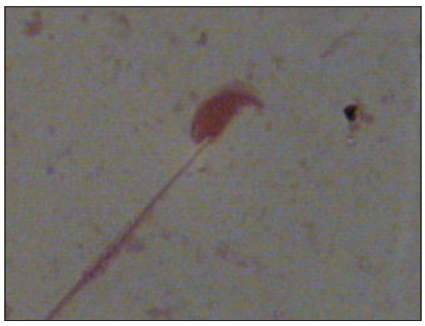

Figure 2e: Vehicle Control treated

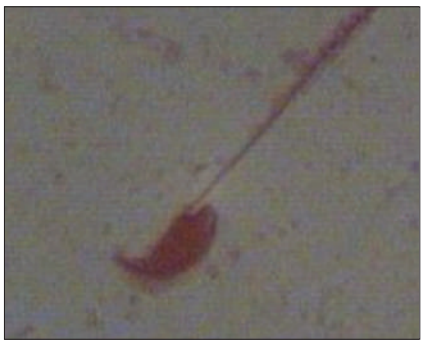

Figure 2f: SMB KR treated

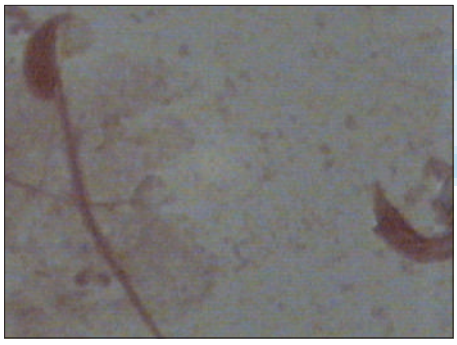

Figure 2g: SMB HK treated

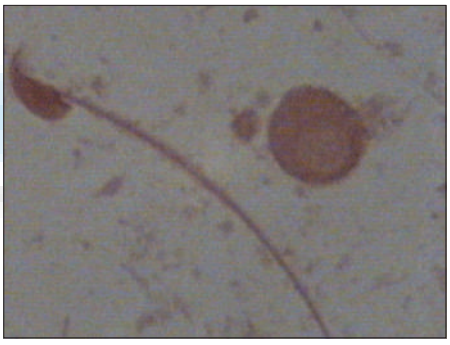

Figure 2h: SMB MM treated

Table 2: Effect of SMBs on chromosomal aberration

Groups	Chromosomal aberration									
	Cromatid		Chromosomal		De	Ex	Fg	PS	R	Dc
	Gap	Break	Gap	Break						
CP	+	+	+	+	+	+	+	+	+	+
VC	--	--	--	--	--	--	--	--	--	--
SMBKR	--	--	--	--	--	--	--	--	--	--
SMBHK	--	--	--	--	--	--	--	--	--	--
SMBMM	--	--	--	--	--	--	--	--	--	--

+: Presence; - Absence; De: Deletion; Ex: Exchange; Fg: Fragmentation; PS: Pulverization and stickiness; R: Ring; Dc: Dicentric; CP: Cyclophosphamide; VC: Vehicle control; SMBKR: *Bhasma* prepared from *Swarna Makshika* collected from Khetri mine, Rajasthan; SMBHK: *Bhasma* prepared from *Swarna Makshika* collected from Hatti gold mine, Karnataka; SMBMM: *Bhasma* prepared form *Swarna Makshika* collected from Malharjkhand mine, Madhya Pradesh; SEM: Standard error of mean

Table 3: Effect of SMBs on sperm abnormality assay

Groups	Sperm abnormality assay					
	Head abnormalities				Tail abnormalities	
	Amorphous shape	Hook less	Banana shaped	Folded	Double tailed	Coiled
CP	++	++	+	+	+	++
VC	--	--	--	--	--	--
SMBKR	--	--	--	--	--	--
SMBHK	--	--	--	--	--	--
SMBMM	--	--	--	--	--	--

--: Absence; +: Mild degree presence; ++: Moderate degree presence; CP: Cyclophosphamide; VC: Vehicle control; SMBKR: *Bhasma* prepared from *Swarna Makshika* collected from Khetri mine, Rajasthan; SMBHK: *Bhasma* prepared from *Swarna Makshika* collected from Hatti gold mine, Karnataka; SMBMM: *Bhasma* prepared form *Swarna Makshika* collected from Malharjkhand mine, Madhya Pradesh; SEM: Standard error of mean

because almost all mouse chromosomes are acrocentric. These types of chromosomes have the exceptional facility to merge with each other. Only structural aberrations were enumerated in all treated groups [Figure 1g-j] against CP treated group, with special emphasis on chromosome and chromatid gap, breaks and centric diffusions placed in Table 2.

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Morphological abnormalities of sperms are described as two types as head and tail abnormalities. The head abnormality included amorphous shape, without hook, banana shaped and folded head. CP treated group observed maximum number of abnormalities in both head and tail as shown in Table 3. Amorphous shaped head, hook less head and coil-tailed abnormalities were more frequent than other abnormalities of head and tail of CP treated group [Figures 2a-d]. The test preparations observed negative results in sperm abnormality showing non-toxic to sperms [Figures 2e-h]. Wyrobek[18] reported that large reductions in sperm number or mortality or large increases in sperm with abnormal shapes are associated with reduced fertility.

Conclusion

Present study revealed that SMB prepared from different samples were found to be safe after the administration for 14 days at the therapeutic doses. No abnormality was noticed in CA and sperm abnormal aberrations in all trial groups. Further, above findings provide new information that may be more imperative for the use of *Bhasmas*.

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Original Research Article (Experimental)

Explaining *Ayurvedic* preparation of *Rasasindura*, its toxicological effects on NIH3T3 cell line and zebrafish larvae

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ABSTRACT

Rasasindura is a mercury-based medicinal formulation that contains HgS (>99%). Although cinnabar ore was a well-known mineral in the past, the *Ayurvedic* practitioner adopted a critical and tedious procedure for the preparation of *Rasasindura*. Therefore, it is essential to understand the *Ayurvedic* process in the perspective of material science. Further, a toxicity study is also required as mercury is the main component in *Rasasindura*. Here, in the present study, we characterized *Rasasindura* and one of its intermediates (*Kajjali*) to understand the physicochemical changes that occur in the *Ayurvedic* process. Furthermore, we have assessed the toxicity of *Kajjali* and *Rasasindura* in NIH3T3 cell lines and zebrafish larvae. XRD analysis of *Rasasindura* confirms it as a highly pure α-HgS with size ranges from nano to micron sizes (starting from ~80 nm). Whereas, *Kajjali* is a β-HgS having lower size ranges (starting from ~30 nm). *Rasasindura* did not show significant cytotoxicity on NIH3T3 cell line up to 75 ppm, whereas for *Kajjali*, cytotoxicity was observed above 20 ppm. The higher toxicity of *Kajjali* is due to higher penetration of particles into the cells. However, in zebrafish larvae, even at too high concentrations (1000 ppm), both *Rasasindura* and *Kajjali* did not show any toxicity or morphological changes. This study concludes that *Rasasindura* is not toxic up to a reasonable concentration. Further, these two drugs did not contain toxic organic mercuric compound; otherwise, it could have been lethal to the zebrafish larvae.
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1. Introduction

Ayurveda is one of the oldest medicinal systems in human society which had originated more than 5000 years ago [1]. Interestingly, *Ayurveda* uses metal-based medicine in therapeutic applications. Numerous *Ayurvedic* medicines contain metals (or metal compounds) such as gold, silver, lead and arsenic, which are not essential elements for humans. The most astonishing fact is that mercury, which is a heavily toxic metal, is one of the most common ingredients in *Ayurvedic* medicines. Although WHO has advocated to restrict the use of mercury in medicinal applications, *Ayurveda* still uses these mercury-based medicines for multiple therapeutic purposes.

The importance of mercury in *Ayurveda* can be understood by the fact that a sub-branch of *Ayurveda*, *Rasa Shastra*, was named after mercury (in *Sanskrit*, *Rasa* means mercury) [2]. *Rasasindura* is a mercury-based medicine that is used to treat high fever, jaundice, sexual diseases, immune and nervous system related diseases [3]. *Kajjali* is another important medicine in *Ayurveda* which is an intermediate product in the *Ayurvedic* preparation process of *Rasasindura* and this is used as a rejuvenating agent [4]. Ayurvedic process of Rasasindura manufacturing is tedious and requires several days for preparing the final product as it involves several steps such as purification, mixing heat-treating steps. The raw materials required for the manufacturing process are liquid mercury and solid sulphur. Several physical and chemical transformations occur during the Rasasindura preparation. Therefore, the first objective of this study is to understand the *Ayurvedic* manufacturing process starting from raw mercury and sulphur. Moreover, various organic juices are used in the manufacturing process that could lead to the presence of organic mercury in *Kajjali* and *Rasasindura*. As organic mercury compounds such as methyl mercury are extremely toxic to the biological system, a small amount of them could lead to a severe adverse effects in patients. Furthermore, the presence of free mercury is also a concern.

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Therefore, to observe the toxicological effects, we have used NIH3T3 cell line and zebrafish larvae. We used very high concentrations of *Rasasindura* and *Kajjali* (up to 1000 ppm) and examined the alteration of various parameters such as viability, reactive oxidative species (ROS), particle uptake and morphology.

2. Materials and methods

2.1. Chemicals

Ayurvedic Kajjali and *Rasasindura* were gifted for research purpose by Shree Dhootapapeshwar Limited, Mumbai, India. The 2′,7′-dichlorodihydrofluorescein diacetate (DCFDA) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) were purchased from Sigma Aldrich. All chemicals for cell culture experiment was procured from Himedia, India.

2.2. Preparation of Rasasindura

The Ayurvedic preparation of Rasasindura has been described in our previous work [5]. A flow chart of the preparation steps has been included in the supplementary file (Fig. S1).

2.3. Physicochemical characterization

2.3.1. Crystallographic identification

The crystal phase identification of all samples was carried out using X-ray diffraction (XRD, SmartLab, Rigaku, Japan). XRD peaks were matched with the ICDD (International Centre for Diffraction Data) database. High-Temperature XRD (HTXRD) was carried out for Kajjali to understand the crystallographic changes occurring in the Rasasindura preparation procedure. The scanning was done at various temperatures between 25 °C and 325 °C in the air environment.

2.3.2. Particle size analysis

The particle size of *Kajjali* and *Rasasindura* was analyzed by dynamic light scattering (DLS) and transmission electron microscopy (TEM). For the DLS study, Malvern ZEN 1600 (Malvern Panalytical Ltd, United Kingdom) was used. For the DLS analysis, *Kajjali* and *Rasasindura* were suspended in isopropanol (1 mg/ml) and sonicated for 10 min prior to the analysis. TEM study was carried using a JEOL 2100 (JEOL, Japan) microscope operated at 200 kV. For the TEM sample preparation, suspension of particles was made similar to the DLS technique. The suspended particles were placed on a carbon-coated copper grid and dried before analysis.

2.3.3. Morphological and elemental analysis

The morphology of *Kajjali* and *Rasasindura* particles were analyzed using scanning electron microscopy (SEM, JEOL, Japan). For the elemental quantification, Energy-dispersive X-ray spectroscopy, (EDAX, Oxford instrument) was employed, which was attached with scanning electron microscopy (SEM).

2.3.4. Thermogravimetric analysis

Thermal analysis was carried out with thermogravimetric analysis (TGA) attached with differential thermal analysis (DTA). The DTA-TGA (Perkin Elmer, USA) experiment was conducted in an air atmosphere. The rate of temperature increase was 10 °C/min.

2.3.5. XPS

The surface elemental analysis was conducted by X-ray Photoelectron Spectroscopy (XPS, Kratos Analytical, Japan) equipped with a monochromatic X-ray source of 1486.6 eV. The peak position (binding energies) was calibrated with a standard gold peak (Au 4f7/2) at a position of 83.95 eV. The XPS peaks were analyzed and de-convoluted with the help of *ESCApe*^{TM} software, Kratos Analytical.

2.4. Exposure of Kajjali and Rasasindura to NIH3T3 cell line

The NIH3T3 (mouse fibroblast) cell line was obtained from National Centre for Cell Science, Pune, India. NIH3T3 cell line was cultured in Dulbecco's modified Eagle medium (DMEM) having 10% fetal bovine serum (FBS), 1% L-glutamine and 0.1% antibiotic. The cells were incubated in 5% CO_2 atmosphere at 37 °C. *Kajjali* and *Rasasindura* particles were suspended by sonicating it in the cell culture media for 10 min. The suspended *Kajjali* and *Rasasindura* particles were exposed to the adherent NIH3T3 cell line at various concentrations from 10 to 1000 ppm. For cell viability assessment, MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] assay was conducted in 96-well plate. Reactive oxygen species (ROS) was also studied in the black bottom 96-well plate by 2′,7′-dichlorodihydrofluorescein diacetate (DCFDA) method. Cell viability tests and ROS experiments were performed at various time points after drug exposure. For cell morphology study, the images were captured by confocal microscopy, followed by FITC and PI staining after 48 h from drug exposure. The fluorescence-activated cell sorting (FACS) flow cytometry study was conducted (48 h after treatment) after propidium iodide (PI) staining to confirm particles uptake by cells. SEM imaging of drug-treated cells was also conducted.

2.5. Exposure of Kajjali and Rasasindura to zebrafish larvae

The zebrafish experiment was done as per The Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), India. Adult zebrafish were maintained as per our previous studies [6,7]. Embryos were obtained after mating of adult male and female fishes. The embryos were maintained in E3 medium. Larvae were kept in reverse osmosis-filtered water (pH 6.5−7.5), whose salinity was reconstituted to ~500 μS. The zebrafish larvae (4 dayspost-fertilization, dpf) were exposed to various concentrations of *Kajjali* and *Rasasindura*. The *Kajjali -Rasasindura* water suspension was sonicated for 10 min and exposed to transparent zebrafish larvae to check the morphological and ROS changes. The images were captured using a stereomicroscope (SZX7, Olympus, Japan).

ROS of zebrafish larvae was carried out by the whole-mount method using DCFDA fluorescent probe [8]. For whole-mounted ROS detection, after 48 h of *Rasasindura* exposure, the larvae were anaesthetized using 0.05 mg/ml tricaine (MS-222) solution. After 10 min, the larvae were washed using PBS buffer (pH 7.4) twice and incubated with DCFDA for 30 min in 28 °C. The DCFDA labelled larvae were mounted on methylcellulose and photographed under a stereoscopic microscope.

2.6. Statistical analysis

Statistical analysis was carried out using one-way ANOVA technique in Origin-2018 (OriginLab) software. For statistical significance, Tukey's post hoc test was carried out with *$p < 0.05$, **$p < 0.001$ and ***$p < 0.0001$ v/s controls.

3. Results and discussion

3.1. Understanding the Rasasindura preparation process

In this section, the *Ayurvedic* process has been observed and documented in terms of changes in the physicochemical properties

of the materials involved during the *Rasasindura* preparation. The first step of *Rasasindura* preparation is mixing of purified solid sulphur (S) and purified liquid mercury (Hg) in a ball mill for 36 h, which yields *Kajjali* (black powder).

EDAX results of *Kajjali* at different regions showed that concentration of small size mercury particles was higher than that of large particles (Fig. 1c). Also, Hg to S ratio (Hg:S) for smaller particles was found to be 43.41:56.58, which is closer to the original stoichiometric composition of HgS (50:50). At the same time, large particles had a ratio of (Hg:S) was 18.09:81.90 (Fig. 1b). These observations can be explained as follows: in the mixing step, when liquid mercury was mechanically mixed with solid sulphur for 36 h, the HgS phase formed on the surface of sulphur particles (Fig. 1a) and the Hg converted to HgS completely having a wide range of particle sizes. The excess sulphur that remained combined with larger Hg particles.

In the next step, *Kajjali* was further heated in the glass vial with controlled temperature to obtain *Rasasindura*. The loss in mass of *Kajjali* with temperature were assessed with TGA-DTA. In the heating process, the excess sulphur (42% excess) was burned down at approximately 318 °C (Fig. 1d). After complete combustion of excess sulphur, the evaporation of *Kajjali* started (~350 °C) and it was completely decomposed at around 460 °C. But in the actual *Ayurvedic* process, after the completion of combustion of excess sulphur, the glass vial was sealed with a cap to restrict the evaporation of *Kajjali*. Without this step, the conversion of *Kajjali* (metacinnabar or β-HgS) to *Rasasindura* (α-HgS) could not be completed. This is evident in the HTXRD peaks of *Kajjali* (Fig. 1e). As the HTXRD of *Kajjali* was conducted on the open surface (on a glass plate in XRD instrument), it is inferred that *Kajjali* did not transform to *Rasasindura* up to 325 °C (Fig. 1e); and only above 350 °C, *Kajjali* started to evaporate. Since the glass vial was sealed in the *Ayurvedic* process, the evaporated *Kajjali* got sublimed at the neck of the glass bottle along with the phase transition from β-HgS to α-HgS. The sublimation is one of the reasons for the high purity of *Rasasindura*.

3.2. Physicochemical analysis of Kajjali and Rasasindura

SEM study (Fig. 2a) showed the particle size distribution of *Kajjali* powder. From the TEM (Fig. 2b) studies, it was confirmed that *Kajjali* had some nano-sized particles below 100 nm. The DLS study also found particles having nano sizes (supplementary file, Fig. S2). The high-resolution XPS (HRXPS) peaks (Fig. 2c) were found at 100 eV and 104.05 eV for Hg (Hg 4f peaks) having 4.05 eV $4f_{7/2}$ - $4f_{5/2}$ splitting. After deconvolution of the XPS $4f_{7/2}$ peaks, two peaks were obtained at 99.95 eV and at 100.65 eV, which are close to the peak position of β-cinnabar and α-cinnabar respectively [9–11] and no peaks of free Hg (Hg^0 < 99.8 eV) were obtained. The XRD (Fig. 2g) of *Kajjali* illustrated that major phases contained in *Kajjali* were the β-cinnabar (52.32 wt.%), orthorhombic sulphur (44.32 wt.%) and 3.32 wt. % α-cinnabar.

Rasasindura also contained nano-sized particles (<100 nm) which was confirmed by TEM (Fig. 2e) and DLS study (supplementary file, Fig. S3). From SEM images (Fig. 2d), it was observed that *Rasasindura* also contained large agglomerate particles (>1 μm). The agglomeration had been caused by the prolonged heat treatment of *Kajjali*. The XRD (Fig. 2g) profile of *Rasasindura* matched exactly with α-HgS. Therefore, it can be inferred that *Rasasindura* has a single crystalline α-HgS phase. The EDAX study at a different position (small and large particles) showed approximately similar Hg to S ratio (Hg:S = ~84:16 wt %), which was close to the stoichiometric concentration of HgS

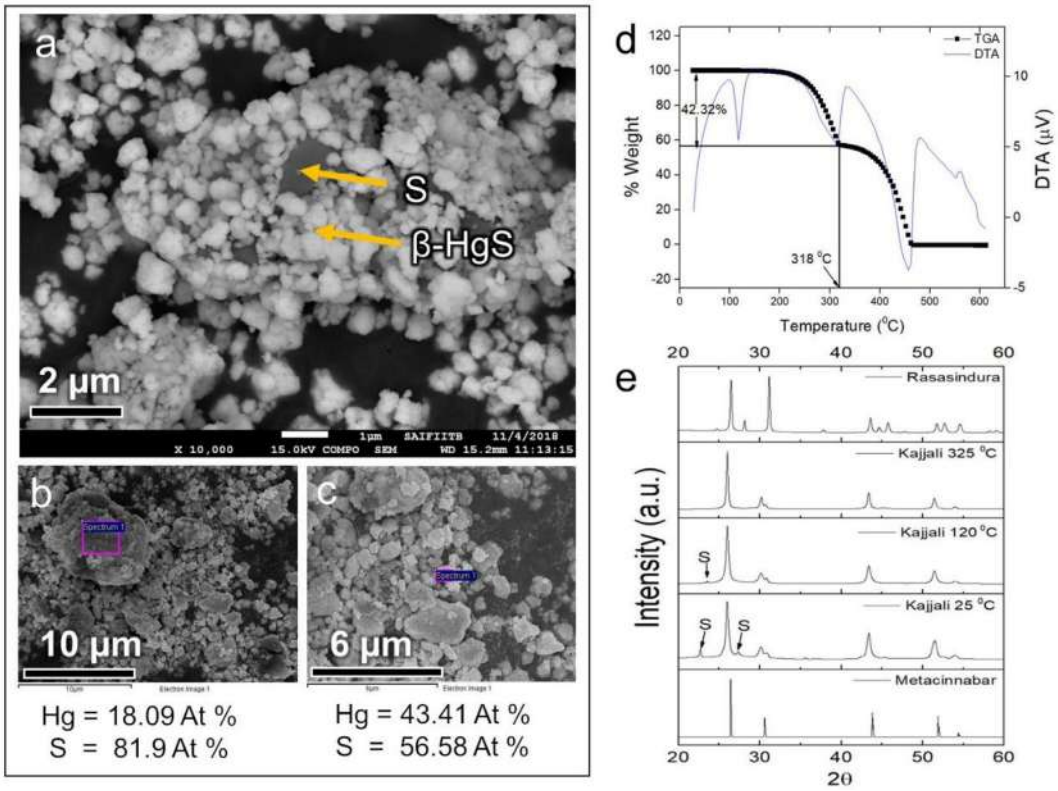
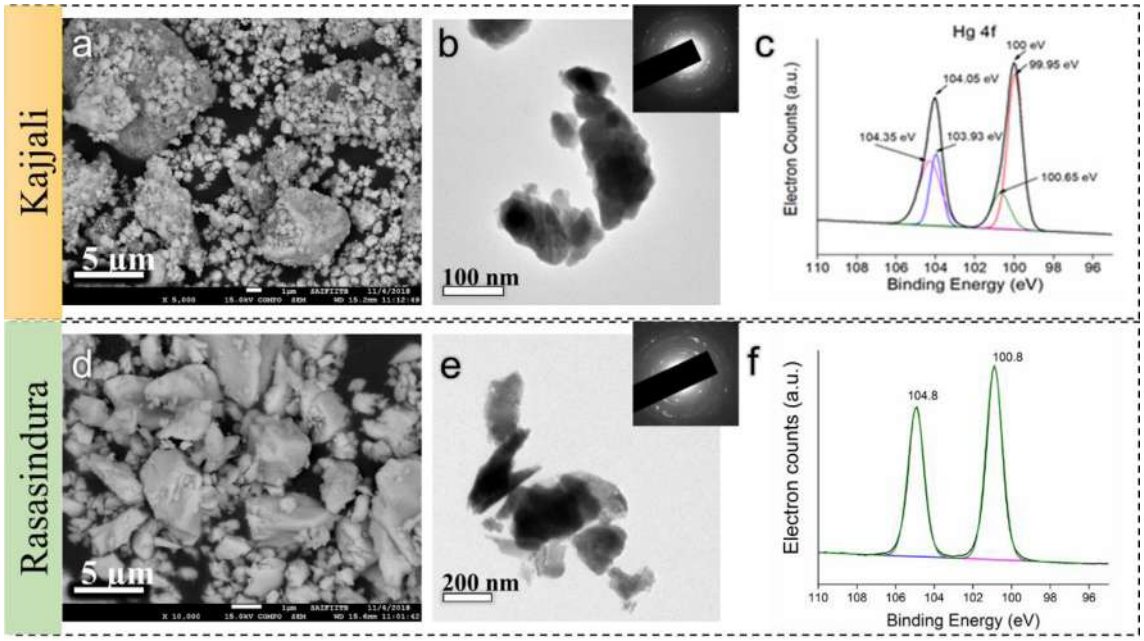


Fig. 1. Conversion of *Kajjali* to *Rasasindura*, a) SEM backscatter image of *Kajjali*, b) and c) selected area EDAX results on big and small particles in *Kajjali*, d) DTA-TGA of *Kajjali* e) High-temperature XRD of *Kajjali* (at 25 °C, 120 °C and 325 °C) with comparison to metacinnabar and *Rasasindura*.



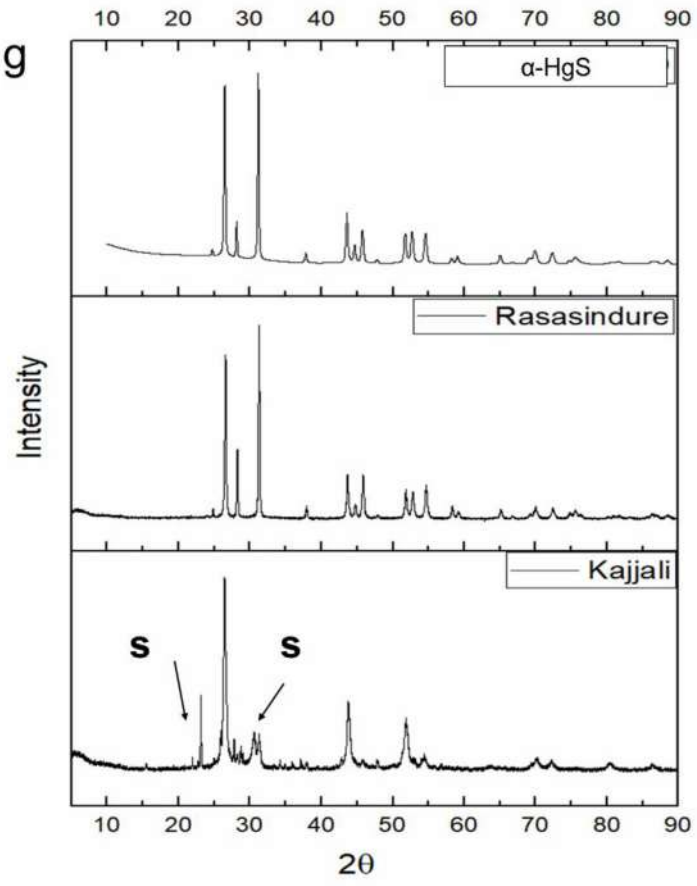


Fig. 2. Physicochemical characterization of *Kajjali* and *Rasasindura*. a) SEM of *Kajjali*, b) TEM of *Kajjali*, c) XPS of Hg 4f region of *Kajjali*, d) SEM of *Rasasindura*, e) TEM of *Rasasindura*, and f) XPS of Hg 4f region of *Rasasindura* and g) XRD of *Kajjali* and *Rasasindura*.

(Hg:S = 86.22:13.78 wt.%). HRXPS profile of *Rasasindura* (Fig. 2f) at Hg 4f region showed only one $4f_{7/2}$ peak (after de-convolution). The $4f_{7/2}$ XPS peak was obtained at 100.8 eV, which closely matched with α-HgS.

3.3. Cell viability and ROS study

To understand the biological effects of *Kajjali* and *Rasasindura*, cell culture study was carried out using NIH3T3 cell line. The cell

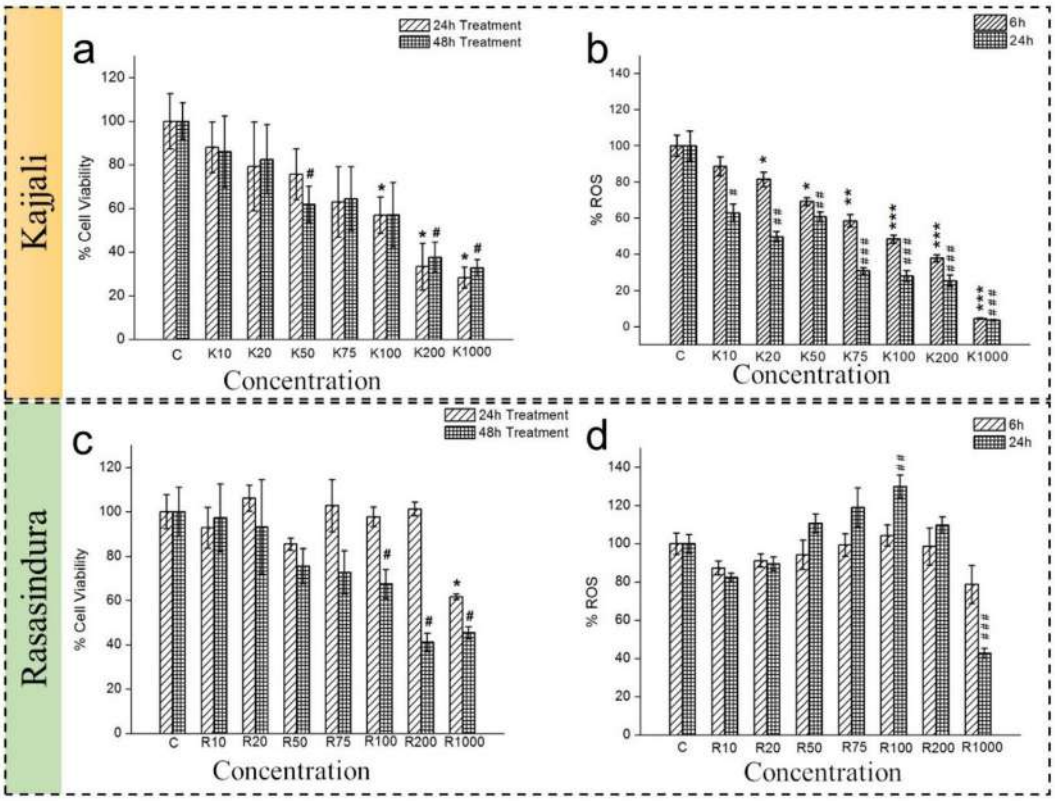


Fig. 3. MTT assay and ROS assay of NIH3T3 cell line after (a and c) *Kajjali* and (b and d) *Rasasindura* exposure. K10 indicates 10 ppm *Kajjali*, K20 indicates 20 ppm *Kajjali* and so on. Similarly, R10 equals to 10 ppm *Rasasindura* and so on. Values are expressed as mean ± SEM with *p < 0.05, **p < 0.001 and ***p < 0.0001 v/s control.

viability was examined using the MTT assay (Fig. 3a). The cells were treated by increasing the *Kajjali*/*Rasasindura* concentration from 10 to 1000 ppm. The cell viability was examined after 24 h and 48 h of drug exposure. At 50 ppm and concentrations above that, *Kajjali* showed significant cytotoxicity after 48 h (Fig. 3a). The ROS of *Kajjali* treated cells were found to be decreased with

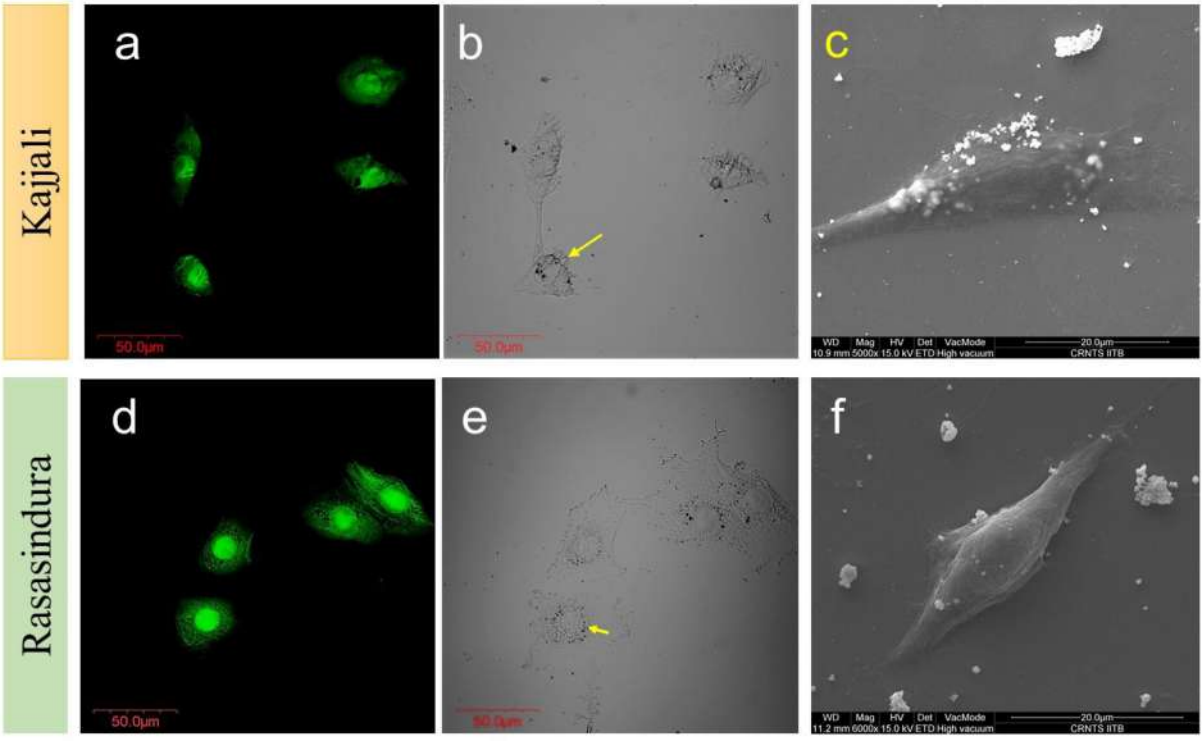


Fig. 4. High-resolution confocal image (a, b, d and e) and SEM (c and f) images of *Kajjali* and *Rasasindura* treated cells (50 ppm) after 48 h.

increasing concentration (Fig. 3b) due to cell death. The ROS results indicating ROS-independent cell death due to Kajjali exposure. Our ROS measurement time was 6 h and 24 h after Kajjali exposure. At the 6 h time point, we did not observe decreasing ROS with increasing concentration of Kajjali. It may happen due to cell death. The cell death could happen due to immediate ROS generation after Kajjali exposure (ROS may increase within the first few hours after exposure, hence it was not reflected in the 6 h ROS data). The confocal study further confirmed cell death due to *Kajjali* exposure (Supplementary file, Fig. S4).

On the other hand, it was observed that even at a very high concentration (up to 200 ppm), the *Rasasindura* was not cytotoxic after 24 h. However, at 48 h, 100 ppm and concentrations above that showed a significant reduction of cell viability for Rasasindura (Fig. 3c). The ROS study (Fig. 3d) at 6 h and 24 h showed that at 6 h, ROS change was insignificant up to 200 ppm concentration. However, after 24 h treatment, ROS increased as compared to the control for most of the concentrations (except 1000 ppm), but the significant variance was found at 100 ppm.

3.4. Cell morphology by confocal and SEM studies

The high-resolution confocal and SEM images revealed that after *Kajjali* treatment to the NIH3T3 cell line, the deformation was clearly seen in Fig. 4a–c. Deformation of the nucleus was also observed after Kajjali exposure (50 ppm).

On the other hand, *Rasasindura* particles were accumulated in the cells. The distribution of *Rasasindura* particles was not homogeneous among cells, as *Rasasindura* comprises a wide distribution of particle sizes. The confocal images demonstrated that particles were adsorbed on the cell membrane. The larger and agglomerated RS particles could not enter through the cell membrane; on the other hand, the smaller RS particles entered the intercellular cytosol and concentrated around the nucleus (Fig. 4e). However, the particles did not seem to enter the nucleus as no deformation was observed in the nucleus' shape (Fig. 4d–f). A considerable portion of *Rasasindura* particles accumulated in the endomembrane system surrounding the nucleus. The internalization of *Rasasindura* particles (smaller size) could occur via endocytosis [12].

3.5. Nanoparticle uptake measured by side scatter (SSC) vs forward scatter (FSC) signal

The cellular uptake of *Rasasindura* was further supported by the FACS study (Supplementary file, Fig. S5). It was observed from the SSC v/s FSC plot that the SSC signal increases as the concentration of *Kajjali/Rasasindura* increases [13].

3.6. Zebrafish larvae study

The effects of *Kajjali* and *Rasasindura* exposure on zebrafish larvae (4 dpf) at various concentrations were studied for

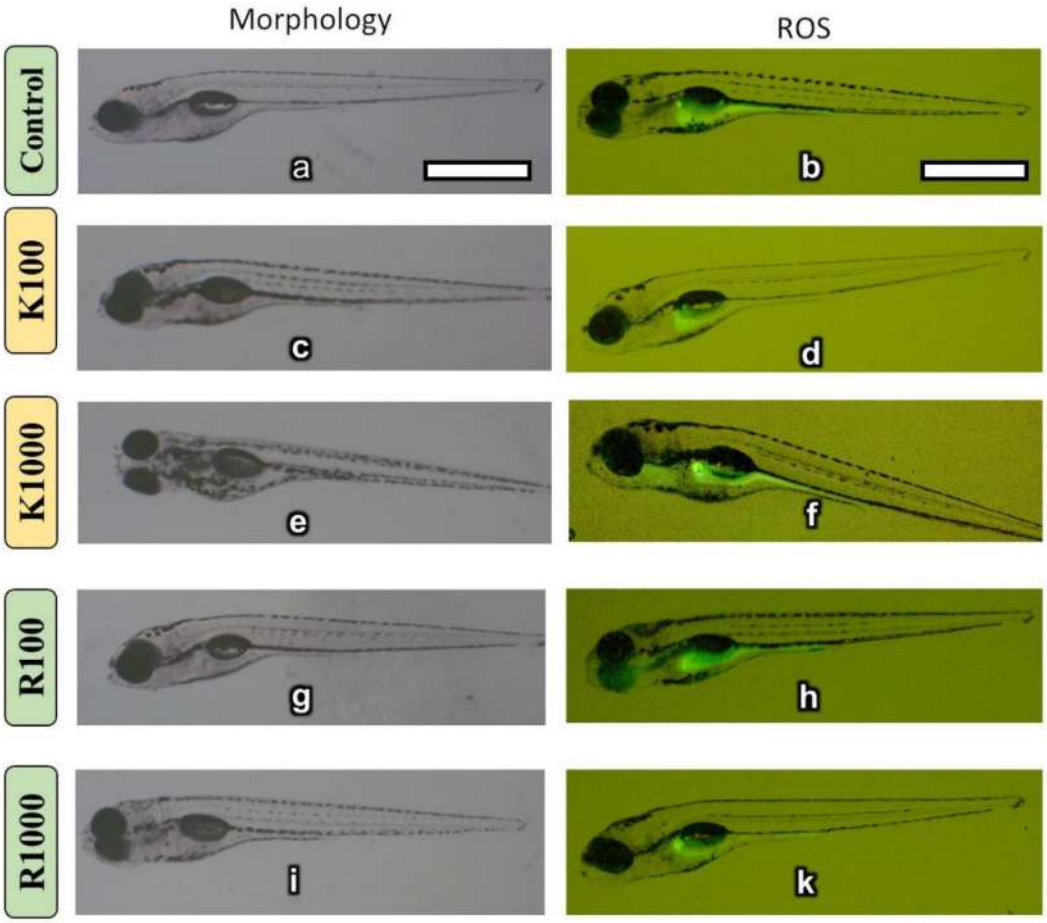


Fig. 5. Morphology (right-sided images) and ROS (left-sided images) of *Kajjali* and *Rasasindura* treated zebrafish larvae (K100 = 100 ppm and, K1000 = 1000 ppm *Kajjali*: R100 = 100 ppm, R1000 = 1000 ppm *Rasasindura*). Treatment was done at 4 dpf and images were taken after 48 h of treatment (at 6 dpf). Scale bar = 1 mm.

morphological changes and ROS generation (Fig. 5). The morphology of *Kajjali* and *Rasasindura* treated larvae were observed under a stereomicroscope and no significant difference was observed. Further, from the ROS study, it was observed that there were no significant changes in the reactive oxygen species between control and intervention groups. Therefore, it can be inferred that both *Kajjali* and *Rasasindura* were non-toxic to zebrafish larvae after 48 hours of treatment. This observation suggestes that there was no (or less minimally) soluble mercury (or organic mercury) released from *Kajjali* and *Rasasindura* to the larvae medium (water) that could induce toxicity to the larvae.

From the above results, it can be summarised that the interactions of *Rasasindura* with the biological system was not destructive in both cells (up to 75 ppm) and zebrafish larvae (up to 1000 ppm). Further, cell culture studies showed that *Kajjali* was not cytotoxic up to a reasonable concentration (20 ppm). In zebrafish larval study, both *Kajjali* and *Rasasindura* did not exert any toxicity up to 1000 ppm. In our previous study [6] on adult zebrafish, no toxic effect was observed up to 70 mg/kg dose for *Rasasindura* and *Kajjali*. Moreover, in some recent biological studies, *Kajjali* and *Rasasindura* showed some beneficial effects [14,15]. However, the concerns regarding the use of mercury cannot be ignored as it is a potential neurotoxin even at lower doses. However, some recent studies have showed that HgS (*Kajjali* and *Rasasindura* mainly contain HgS) is less toxic as compared to other Hg compounds such as methylmercury, $HgCl_2$ or diethyl mercury [16]. It is presumed that the low solubility of HgS could be the reason for its non-toxicity [5]. However, several mechanisms in the biological system can increase the solubility of HgS. The solubility may change due to the interaction with various enzymes which can lead to toxicity in organisms. Therefore, in the present study, the interaction of HgS with the biological system was shown that exhibits the non-toxic nature of *Rasasindura*. *Kajjali* shows higher toxicity in the cell line due to its smaller particle size as compared to *Rasasindura*. As *Kajjali* contains a higher portion of nano-sized particles, the penetration capacity of *Kajjali* particles in the cell and nucleus is far higher compared to *Rasasindura*, which makes it more toxic in the cell line study.

4. Conclusion

This study provides a detailed methodological insight into the *Rasasindura* preparation process. We have explained the transformation of *Rasasindura* from raw mercury and sulphur. Further, the physicochemical study of *Kajjali* and *Rasasindura* revealed their morphology, size distribution, crystallographic and elemental analysis. In summary, both *Kajjali* (up to 20 ppm after 48 h of exposure) and *Rasasindura* (up to 75 ppm after 48 h of exposure) were not found to be as toxic as compared to other Hg compounds (such as $HgCl_2$, methylmercury or Hg^0) reported in the literature. *Kajjali* and *Rasasindura* medicines, if prepared as per the *Ayurvedic* method, reduce the chances of toxicity as they do not possess any organic or soluble mercury counterparts.

Conflict of interest

None.

Author contributions

S.B. designed the study, collected experimental data, wrote the data, analyzed the data, and prepared the figures, and wrote the manuscript. J.B. designed the study, reviewed the manuscript, and supervision. All authors reviewed the manuscript.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jaim.2021.08.011.

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Original article

Exploratory studies on the therapeutic effects of *Kumarabharana Rasa* in the management of chronic tonsillitis among children at a tertiary care hospital of Karnataka

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ABSTRACT

The effect of an Ayurvedic poly-herbo-mineral formulation *Kumarabharana Rasa* (KR) in the management of chronic tonsillitis (*Tundikeri*) in children has been assessed in this study. This clinical study was a double-arm study with a pre- and post-test design at the outpatient level in a tertiary Ayurveda hospital attached to a teaching institute located in district headquarters in Southern India. Patients ($n = 40$) with chronic tonsillitis satisfying diagnostic criteria and aged between 5 and 10 years were selected from the outpatient Department of Kaumarbhritya, SDM College of Ayurveda and Hospital, Hassan. Among them, 20 patients were treated with *Kumarabharana rasa* (tablet form) at a dose of 500 mg once daily for 30 days (Group A). The other 20 patients were treated with *Godhuma Vati* (placebo) at a dose of 500 mg once daily for 30 days (Group B). In both groups, *Madhu* was the *Anupana* advised. After completion of 30 days of treatment, the patients were assessed on the following day and another investigation took place 15 days later. Statistically significant effects ($p < 0.05$) in the reduction of all signs and symptoms of chronic tonsillitis after KR treatment were observed. These results indicate that *Kumarabharana Rasa* has an ameliorative effect in reducing the signs and symptoms of chronic tonsillitis.
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1. Introduction

Chronic tonsillitis (CHT) is one of the most common otolaryngologic diseases.[1] CHT is rare in infants and older people. In young children, tonsillitis is one of the recurrent upper respiratory tract infections. CHT is a highly prevalent disease in the pediatric age group, and it peaks between 3 and 10 years of age and then declines.[2,3] In general practice children frequently visit with recurrent throat problems[4] and the incidence of this disease accounts for about 7% of all visits to the pediatrician.[3] Children with CHT experience discomfort, and the disease also impacts on social, emotional, and financial aspects for family members.[5] Tonsilitis is an infection of the tonsils.[6–8] Despite its high prevalence, the etiology of CHT has remained indistinct. The surface and deep bacterial flora of chronic inflamed tonsils consist of an abundance of probable pathogenic aerobic and anaerobic bacteria, primarily of streptococcal origin.[9–13] Tonsils are part of the immune system. Therefore, due to the decrease in immunity and the tonsils' incompetence in helping the immune system, they actually become a source of recurrent infections.[3] The current treatment option for CHT is tonsillectomy, but it is not the ultimate solution. The generally accepted criteria for tonsillectomy are at least three to seven episodes of tonsillitis per year in spite of medical therapy, but there is no international consensus.[14]

In Ayurvedic thought, tonsillitis can be correlated to *Tundikeri*, which is one of the *Urdhvajatrugata Roga* (diseases of the head and neck); it is mentioned in *Talugata Roga*[15] (diseases of the palate) as well as *Kanthagata Roga* (diseases of the throat).[16] Ayurveda explains that it is caused by the vitiation and imbalance of *Doshas*

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(bodily humors), i.e., *Vata, Pitta*, and *Kapha*. Mainly derangement of *Kapha* and *Rakta* (blood) is preceded by impaired digestive capacity (*Mandagni*/*Vishamagni*) and obstruction of channels (*Sroto Avarodha*) namely *Annavaha Srotas* (gastrointestinal tract) and *Pranavaha Srotas* (respiratory tract) which is manifested as difficulty in swallowing, mouth breathing, choking spells at night, etc.[15,16] The present study was conducted to explore the efficacy of *Kumarabharana Rasa* (KR) in the management of chronic tonsillitis in children. KR has a combined action over vitiated *Doshas* due to its anti-inflammatory, antimicrobial, immunomodulatory, and rejuvinative effects.

2. Materials and methods

2.1. Design

This study was an open-labeled double-arm setting with a pre- and post-test design.

2.2. Participants

Children presenting with any of the symptoms of chronic tonsillitis (*Tundikeri*), i.e., *Kathina Shotha* (enlargement of tonsils), *Ragatwa* (hyperemia), *Galoparodha* (dysphagia), *Mukha Daurgandhya* (halitosis), *Lasikagranthi Vriddhi* (enlargement of lymph nodes), and *Jwara* (fever) were selected and registered from *Kaumarabhritya* (Ayurvedic Pediatrics) outpatient department of SDM College of Ayurveda and Hospital, Hassan between June 2012 and December 2013. Before initiating the study ethical clearance was obtained from institutional ethics committee of SDM College of Ayurveda and Hospital, Hassan (IEC No. SDMAH/IEC/57/11-12 dated 01-04-2012). Written informed consent was taken from the parents of the study participants before any study-related procedures were performed. Inclusion criteria were: children of both sexes between 5 and 10 years of age and who had repeated attacks of tonsillitis (chronic infections) in the past year. Exclusion criteria were: patients with acute tonsillitis, peritonsillar abscess, tonsillar cyst, tonsillolith, or any other systemic disorders; patients who had taken systemic steroids and/or antibiotics in the past 4 weeks.

2.3. Study drugs

2.3.1. Kumarabharana Rasa

This is a compound drug comprising *Bhasmas* (purified calx) of Swarna (gold), Rajata (silver), Pravala (coral) and *Churna* (powder) of Yastimadhu (*Glycyrrhiza glabra* Linn.), Amlaki (*Emblica officinalis* Gaertn.), Ashwagandha (*Withania somnifera* Dunal.), Sunthi (*Zingiber officinale* Rosc.), Pippali (*Piper longum* Linn.), Haritaki (*Terminalia chebula* Retz.), Vacha (*Acorus calamus* Linn.). All these drugs were processed with *Swarasa* (extract juice) of Guduchi (*Tinospora cordifolia* Miers ex Hook. F. & Thoms), Brahmi (*Centella asiatica* Linn.), and Tulsi (*Ocimum tenuiflorum* Linn.) separately then prepared in tablet form.[17]

2.3.2. Godhuma Vati (placebo)

Wheat powder was processed and prepared in tablet form.

Raw drugs were obtained from SDM Pharmacy, Udupi and authenticated in the Department of Dravyaguna, SDM College of Ayurveda and Hospital, Hassan. The medicine was prepared in the Teaching Pharmacy, SDM College of Ayurveda and Hospital, Hassan. Tablets of 500 mg were prepared and preserved in air-tight, properly labeled plastic bottles containing 30 tablets in each.

2.4. Intervention

A total of 53 patients were screened for chronic tonsillitis. Among them 40 patients were enrolled into the study fulfilling the inclusion and exclusion criteria. A convenient sampling technique was adopted, with 20 patients each in study group (KR) and the control group (*Godhuma Vati*). The patients in the study group were treated with KR (tablet form) at a dose of 500 mg once daily for 30 days. The patients in the control group were treated with *Godhuma Vati* (tablet form) at a dose of 500 mg once daily for 30 days. Parents were advised to crush the tablet to a powder and to give it to the child using honey as *Anupana* (vehicle for drug administration) before food, in the morning, for both groups.

2.5. Observation-based assessment criteria

2.5.1. Subjective parameters

The assessment of the signs and symptoms were done on the Day 0 and Day 31. Severity was assessed by grading 1–5 (absence to severe) for each symptom. *Kathina Shotha* (enlargement of tonsils) – no enlargement, enlarged within anterior pillars, enlarged within posterior pillars, enlarged beyond pillars, kissing tonsils with sleep apnea. *Ragatwa* (hyperemia) – no hyperemia, hyperemia of tonsil surface, pinkish appearance of pillars, reddish appearance of surroundings, reddish appearance of surroundings and pharynx. *Galoparodha* (dysphagia) – no pain while swallowing, pain during swallowing solid food substances, pain during swallowing semi-solid food substances, pain during swallowing liquid food substances, continuous pain/unable to swallow. *Mukha Daurgandhya* (halitosis) – no halitosis, foul breath experienced by the patient only, foul breath experienced by the patient and friends/parents, foul breath is experienced by a group of surrounding people, foul breath is experienced as soon as the patient opens the mouth. *Lasikagranthi Vriddhi* (enlargement of lymph nodes) – no palpable lymph nodes, palpable lymph nodes unilateral/warm, palpable lymph nodes bilateral/soft/fluctuant, palpable lymph nodes bilateral which are hard, palpable lymph nodes bilateral with tenderness. *Jwara* (fever) was measured according in degrees Fahrenheit (normal was 98.6° Fahrenheit).

2.5.2. Objective parameters

Assessments were based on routine laboratory blood investigations – hemoglobin % (Hb%), total leukocyte count (TLC), neutrophils, lymphocytes, eosinophils, and erythrocyte sedimentation rate (ESR) were performed on Day 0 and Day 31.

2.6. Statistical analysis

For the statistical analysis, the Statistical Package for Social Sciences (SPSS) version 16 (SSPS Inc., Chicago. IL, USA) was used. The independent samples *t* test and Mann-Whitney *U* test (for between-subjects designs) and paired samples *t* test and the Wilcoxon test (for within-subjects designs) were done.

3. Results

In present study 40 patients were registered, of which only 37 participants completed the study. The sociodemographic profile of the participants shows that 57.5% (23) were male and the rest 42.5% (17) were females. The age groups of 5–6, 7–8, and 9–10 years consisted of 18 (45%), 27.5% (11), 27.5% (11) participants, respectively. The majority (90%, 36) of the participants belonged to the Hindu religion. Socioeconomic assessment revealed that 37.5% belonged to the lower-middle class followed by 30% in the upper-middle class strata. *Prakriti* (genetic phenotype) yielded the

majority 87.5% (35) were having *Pitta-Kapha Prakriti*. A mixed diet was consumed by 80% (32) of the participants. Sleep patterns showed the 60% (24 participants) slept for around 10 hours/day but 62.5% (25) had disturbed sleep, while 37.5% (15) had normal sleep. The family history of the participants highlighted that 25% (10) had a positive family history of chronic tonsillitis. Duration of tonsillitis revealed that 27.5% (11) participants had a duration of tonsillitis for a period of 3 years or more, 17.5% (7) had a duration of tonsillitis for a period of 2 years or more and 55% (22) had a duration of onset of tonsillitis for a period of 1 year or less. Poor oral hygiene was observed among 67.5% (27) participants and only 10% (4) had good oral hygiene.

Chief complaints reported were throat pain among 10% (4) patients, difficulty in deglutition in 17.5% (7) patients, halitosis in 12.5% (5) patients, fever in 10% (4) patients, breathing difficulty among 20% (8) patients (16.66%) with complaints of mouth breathing in 22.5% (9) patients. Only 5% (2) reported having Jugulodigastric lymphadenopathy and 2.5% (1) having enlarged tonsils. Clinical diagnosis revealed 62.5% (25) patients with chronic parenchymatous tonsillitis, 32.5% (13) patients with chronic follicular tonsillitis and only 5% (2) were diagnosed as chronic fibroid tonsillitis. Within the group (Wilcoxon signed ranks test) statistical significance ($p < 0.05$) on signs and symptoms of chronic tonsillitis was found for KR intervention, but nothing significant was observed with *Godhuma Vati* intervention (Table 1). However, between groups (Mann Whitney *U* test), the effects of KR on signs and symptoms of chronic tonsillitis was found to be significant ($p < 0.05$; Table 2). Routine laboratory blood investigations assessed within the KR (study group) showed statistical significance ($p < 0.05$) on Hb%, TLC, lymphocytes and ESR, whereas *Godhuma Vati* (placebo) were nonsignificant (Table 3). The comparisons on the effect of KR (study group) and *Godhuma Vati* (placebo group) on laboratory investigations and fever were nonsignificant ($p > 0.05$) as detailed in Table 4.

4. Discussion

CHT is an inflammation of the tonsils caused by a microbial infection. The symptoms are usually mild and often related to the common cold. In streptococcal infection the tonsils often swell and become coated and the throat is sore. The patient has a temperature, foul-smelling breath and may feel quite ill. Therefore drugs with anti-inflammatory and immunomodulatory effects may be useful for treatment of CHT. Ayurveda, as well as recent experimental results indicate the ingredients of a poly-herbo-mineral formulation KR as having anti-inflammatory, antimicrobial, and immunomodulatory effects. It is substantiated by the clinical observations among tonsillitis patients. The key ingredients being the metallic *Bhasmas* (purified calx), like *Swarna Bhasma*, is prepared by incinerating gold processed with herbal preparations.[18,19] *Swarna Bhasma* promotes immunity through phagocytosis.[20] Gold poly-herbal formulations are found to increases vitality and immunity.[21] The clinical applications of *Swarna Bhasma* and gold salts in Ayurveda are known for rejuvenation and immunomodulation for some chronic diseases.[22] Gold nanoparticles have significant applications in targeted drug delivery.[23,24] *Swarna Bhasma* contains gold particles in the size range of nanoparticles.[25] Recent studies shows that *Swarna Bhasma* contained not only gold, but also several microelements (Fe, Al, Cu, Zn, Co, Mg, Ca, As, Pb, etc.). Animal model studies show *Swarna Bhasma*-treated animals significantly increased superoxide dismutase and catalase activity, which are responsible for reducing free radical concentrations in the body.[19] The antioxidant effect of *Swarna Bhasma* is considered to be one of the mechanisms for the immunomodulatory mode of action of the formulation. *Rajata Bhasma* (silver calx) is a nanoparticulate complex of silver along with Sn, Cd, Mg, K, Na, S, Ca, P, Si, Al, Cl, Ar, In, Fe, Cu, Ba, Hg, or Cr.[26] Recent scientific trends indicate emergence of metallic silver in the form of silver nanoparticles as a potential antimicrobial agent, specially important, as several pathogenic bacteria have developed resistance against various antibiotics.[27]

Herbal components like Amalaki (*Phylanthus embellica*)[28,29] and Guduchi (*Tinospora cordifolia*)[30] are having nourishing and rejuvinative property. Experimental studies reveal their immunostimulant activity which combats sepsis in animals.[31–36] Vacha, Sunthi, Pippali, Ashwagandha, Yastimadhu, Amalaki, and Haritaki all have immunomodulatory properties.[37-40] Pippali is an important drug for the treatment of cough, common cold, and is useful as a digestive. The active principle piperine is known for its bioenhancing and anti-inflammatory effects.[41] Yasthimadhu is a prominent *Rasayana* known for its immunomodulatory, anti-inflammatory, anti-allergic and antiviral activities. It also enhances permeability in drug delivery systems.[42] Yastimadhu (*Glycerrhiza glabra*) also has cytoprotective and demulcent effects and is a popular home remedy for minor throat infections. Biologically active substances in liquorice root include glycyrrhizic acid (GL) and its aglycone (GA), phenolic compounds, oligosaccharides and polysaccharides, lipids, sterine, etc. Many researchers have suggested that the effects on the production of interferon (IFN) and Th2 cytokines might be one of the mechanisms involved in the anti-infective process. Recently, glycyrrhizin has been found to be active in inhibiting replication of the severe acute respiratory syndrome (SARS)-associated virus (FFM-1 and FFM-2) and also H5N1 influenza A virus-infected cells. GL is also reported to have modulatory effects on the complement system. Reports indicate that GL blocks C5 or a more distal stage of the complement cascade, suggesting that it might have a role in preventing tissue injury not only in chronic hepatitis, but also in autoimmune and inflammatory diseases. Chemical modification of GL and GA has been tried, and a significant improvement in anti-inflammatory, antiallergic, and antiulcer activities was observed. These observations indicate immune-modulating and biological response modifier activities associated with GL. *Tinospora cordifolia* (TC) is known for its immunomodulatory and cytoprotective activities Quaternary alkaloids and biotherapeutic diterpene glucosides of TC (syringin, cordiol, cordioside, and coriofolioside) showed an immunopotentiating activity. Research work has been conducted on berberine, jatrorrhizine, tinosporaside, and columbin, which shows a possible mechanism of immunomodulatory activity as an activation of macrophages, leading to increases in granulocyte–macrophage colony-stimulating factor (GM-CSF), leukocytosis, and improved neutrophil function. TC also inhibits C3 convertase of the classical complement pathway. Research on polysaccharide (α-D-glucan) derived from TC shows the activation of nuclear killer (NK) cells, complement system, and Th1 pathway cytokines, coupled with low nitric oxide synthesis.[43] Aswagandha has shown promising

Table 1
Effect of *Kumarabharana Rasa* and *Godhuma Vati* on signs and symptoms of chronic tonsillitis (BT & AT) – test statistics within the groups (Wilcoxon signed ranks test).

Sign and symptoms	*Kumarabharana Rasa*		*Godhuma Vati*	
	Z	*P* (2-tailed)	*Z*	*P* (2-tailed)
Kathina Shotha (enlargement of tonsils)	−3.100	0.002	−1.414	0.157
Ragatwa (hyperemia)	−3.256	0.001	−1.890	0.059
Galoparodha (dysphagia)	−3.580	0.000	−0.378	0.705
Mukha Daurgandhya (halitosis)	−3.341	0.001	0.000	1.000
Lasikagranthi Vriddhi (enlargement of lymph nodes)	−3.473	0.001	−1.633	0.102

Table 2
Comparison on the effect of *Kumarabharana Rasa* (study group) and *Godhuma Vati* (placebo group) on signs and symptoms – test statistics[b] between the groups (Mann Whitney *U* test).

BT & AT	*Kathina Shotha* (enlargement of tonsils)	*Ragatwa* (hyperemia)	*Galoparodha* (dysphagia)	*Mukha Daurgandhya* (halitosis)	*Lasikagranthi Vriddhi* (enlargement of lymph nodes)
Mann-Whitney *U*	60.000	101.500	50.000	54.000	65.500
Wilcoxon *W*	213.000	254.500	203.000	207.000	218.500
Z	−3.856	−2.335	−3.910	−3.932	−3.465
Asymp. Sig. (2-tailed)	0.000	0.020	0.000	0.000	0.001
Exact Sig. [2*(1-tailed Sig.)]	0.001[a]	0.036[a]	0.000[a]	0.000[a]	0.001[a]

[a] Not corrected for ties.
[b] Grouping variable: group.

Table 3
Effect of *Kumarabharana Rasa* and *Godhuma Vati* on laboratory investigations and fever in chronic tonsillitis (BT & AT) – paired samples test (within group).

Parameters	*Kumarabharana Rasa*				*Godhuma Vati*			
	Mean difference	Std. deviation	Std. error mean	*p*	Mean difference	Std. deviation	Std. error mean	*p*
Hb%	−0.34000	0.69008	0.15431	0.040	−0.16471	.86092	.20880	0.442
TLC	1.83700	3174.19878	709.77242	0.018	5.35294	2708.9991	657.02881	0.427
Neutrophils	4.00000	9.48683	2.12132	0.075	0.58824	7.53375	1.82720	0.752
Lymphocytes	4.30000	8.54770	1.91132	0.037	−3.35294	7.38191	1.79038	0.079
Eosinophils	0.40000	1.14248	0.25547	0.134	0.70588	1.53153	0.37145	0.076
ESR	1.08000	16.80413	3.75752	0.010	1.64706	10.32558	2.50432	0.520
Temperature	0.39500	0.87327	0.19527	0.057	−0.08235	2.47897	0.60124	0.893

Table 4
Comparison on the effect of *Kumarabharana Rasa* (study group) and *Godhuma Vati* (placebo group) on laboratory investigations and fever – independent samples test (between groups).

Differences BT & AT	*t*	Sig. 2-tailed (*p*)	Mean diff.	Std. error diff.
Hb%	−0.688	0.496	−0.17529	0.25496
TLC	1.328	0.193	1301.70588	979.95025
Neutrophils	1.560	0.128	4.31176	2.76446
Lymphocytes	−0.357	0.723	−0.94706	2.65088
Eosinophils	−0.695	0.492	−0.30588	0.44022
ESR	1.952	0.059	9.15294	4.68889
Temperature	0.778	0.442	0.46088	0.59237

immunomodulatory effect in inflammatory experimental models.[44] Sunthi (*Zingiber officinalis*) has anti-inflammatory property which inhibits LPS-induced NF-κB activation by preventing degradation of the IκB-α, as well as the phosphorylation of ERK1/2, SAPK/JNK, and p38 MAPKs which were associated with a decrease in the expression of inducible nitric oxide synthase (iNOS) and cyclooxyenase-2 (COX-2).[45] Vacha (*Acorus calamus*) is reported for its inhibitory effect on mast cell-dependent anaphylactic reactions in allergic reactions of the respiratory tract.[46] Amla (*Phyllanthus emblica*) has been reported to suppress the expression of LPS-induced pro-inflammatory genes (COX-2, iNOS, TNF-α, IL-16, and IL-6) in RAW 264.7 murine macrophage cells in a dose-dependent manner.[47] Honey is known to enhance the action of these medicines, and has a potential immunomodulatory effect.[48–51]

5. Conclusion

Thus the poly-herbo-mineral formulation KR has a combined mechanism of action on tonsillitis by reducing the number of attacks. From the Ayurvedic perspective it controls the imbalance of *Doshas* and thus effectively reduces the signs and symptoms of chronic tonsillitis. However, following the reverse pharmacological perspective we find scientific evidence of its components against inflammation and microbial invasion via multiple mechanisms mostly as immunomodulators, as discussed above. Future studies on KR formulation along the lines of reverse pharmacology may produce more conclusive information about its combined mechanism of action at the molecular level.

Conflicts of interest

All authors have no conflicts of interest to declare.

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Hypersensitivity with Ayurvedic Oils Under Inappropriate Use

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Dear Editor,

We are surprised after reading the case report entitled, "Allergic Contact Dermatitis (Type IV Hypersensitivity) and Type I Hypersensitivity Following Aromatherapy with Ayurvedic Oils (Dhanwantharam Thailam, Eladi Coconut Oil) Presenting as Generalized Erythema and Pruritis with Flexural Eczema" which was published in your esteemed journal.[1] Though the report explains the beliefs of Ayurveda medicines and tries to build newer thought of awareness about Ayurvedic treatments, but there are some points which need to be clarified.

First, a should validate that registered Panchakarma Physicians only can prescribe the oils, because it is the tendency of medical and non-medical community to take Ayurveda for granted. Today in the name of Herbal medicines, people are misguiding the global population. I would like to thank Author for her valuable work of testing hypersensitivity to Ayurvedic oil. This could convey the non-Ayurveda medical practitioner that Ayurveda drugs are not always natural or safe. Only Ayurveda physician can explain the efficacy, uses, adverse reaction and contraindications according to Prakriti (constitution), Rutu (season), Agni (appetite), Desha (location) and Vaya (age). These all the minute assumptions to be taken into account, it is having more significance in Panchakarma (Massage is the part of Panchakarma).

Ayurveda as Science has its eternal principles of healing. As per the basics of Abhyanga (Massage), there are specific conditions or oils which should be used.[2] It needs minute observation and keen thinking with deep knowledge. Not aroma therapist can prescribe Ayurveda oils for the so-called massage. It should be used with great caution. There are no explanations regarding patient's symptoms and prescription by physician. Anybody could get hypersensitivity with anything that we could not predict. It can be with ground nut or green gram also. So Dhanvanthara, Eladi oil can produce hypersensitivity of Type I-Type IV if they are not prescribed according to conditions of constitution, disease, season and Dosha.[3] That's why it is the need that those medicines should be prescribed by only authentic practitioners of Ayurveda.

We are using these both oils since years, why not a single patient developed hypersensitivity is due to proper drug selection. We want to convey that, these reactions happen due to wrong selection of drug in a wrong case. Going through the textual reference of Dhanvanthara oil[4] and Eladi oil,[5] is not advocated in the treatment of rheumatoid arthritis.

Many Ayurveda practitioners using Dhanvantharam and Eladi oil safely and successfully. These oils are indicated for Vata and Kapha vitiated conditions. According to Ayurveda Rakta (blood) is involved in pathology of rheumatoid arthritis where these oils are contraindicated.

Nowadays, most of non-Ayurvedic community are also prescribing massage. If any complication happens blame directly goes to Ayurveda. This is injustice with Science of Life-Ayurveda.

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Immuno-modulatory activity of *Triguna Makaradhwaja* — An Ayurvedic compound formulation

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The present study was carried out to evaluate immuno-modulatory activity of *Triguna Makaradhwaja* for humoral antibody formation and cell mediated immunity in established experimental models. Study was carried out in Wistar strain albino rats of either sex and test drug was administered orally at a dose of 3.15 mg/kg along with *Guduchi Ghana* and honey as adjuvant. Effect of test formulation on anti-body formation against sheep red blood cells was assessed for humoral antibody formation. The test drug and vehicles were administered for 10 consecutive days. On third day, 20% SRBC (Sheep Red Blood Cells) as sensitizing agent was injected subcutaneously to the rats of second, third and fourth groups in the dose of 0.5ml/100 g of body weight. Animals were sacrificed on 11th day. Parameters like haemagglutination titre; haematological, serum biochemical and histology of spleen, thymus and lymph nodes were studied to assess the effect on for humoral immunity. Immunological paw oedema was assessed for cell mediated immunity. Animals were sensitized with triple antigen subcutaneously on first day and the drug administration was continued for seven days. On 7th day animals were challenged with the same antigen by injecting to left hind paw and volume of immunological oedema thus produced was measured by volume displacement method after 24 and 48 hours. *Triguna Makaradhwaja* apparently enhanced antibody formation and cellularity of immunological organs, while it failed to show any significant impact on immunologically induced paw oedema. This study shows that *Triguna Makaradhwaja* is having marked immunostimulant effect and weak effect on cell mediated immunity (CMI). The adjuvant *Guduchi Ghana* and honey *per se* has immune-potentiating activity and it seems to be added to the therapeutic activity of the main drug.

Keywords: Cell mediated immunity, *Guduchi Ghana*, Haemagglutination, Honey, *Triguna Makaradhwaja*.

IPC code; Int. cl. (2011.01) — A61K 36/00, A61P 37/02

Introduction

Immunity is a biological term that describes a state of having sufficient biological defenses to avoid infection, disease, or other unwanted biological invasion. Immunity involves both specific and non-specific components. The non-specific components act either as barriers or as eliminators of wide range of pathogens irrespective of antigenic specificity. The main mechanism is to enhance different body defense mechanisms in a non-specific manner to endow it with better capacity to get adapted to different kinds of adverse conditions[1]. The term 'immunomodulation'[2] is used for describing, the effect of various chemical mediators, hormones and drugs on the immune system. Immunomodulation is a therapeutic approach in which intervene in auto regulating processes of the defense system is tried. Immunomodulation is very important in a homotoxicological treatment protocol, especially dealing with a chronic disease. To activate or regulate immune reactions, it not only put the defense system on the right purposeful level of action, but also keeps the clinical symptoms of inflammation within for the patient acceptable levels, or stimulates a non-reactive immune system[3].

Use of herbs or herbo-mineral formulations for improving the overall resistance of body against common infections and pathogens has been a guiding principle of Ayurveda[4] and for this there is a separate class of immunomodulatory drugs known as *Rasayana*. They are supposed to have the ability of

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protecting the body against external factors that induce disease. This implied resistance against disease may represent the modern concept of immunity[5]. *Makaradhwaja* is one such popular metalo-mineral formulation predominantly in manufacturing of which herb are also as inevitable and vital part, and is most popular and effective in *Kupipakwa* preparation (a specific type of pharmaceutical practice in which medicines are prepared by gradually increasing heating patterns using a vertical electric muffle furnace) of Ayurvedic medicine. The main components being *Swarna* (Gold), *Parada* (Mercury) *Gandhaka* (Sulfur) in the ratio of 1:8:16 generally[7]. As per classical literature, this drug is mainly used for *Rasayana* (Rejuvenator) purposes[8]. This drug is also well-known for its propensity to modulate immune system.

Triguna Makaradhwaja[9] (TM) is a herbo-minaral compound, levigated by two herbal drugs viz. *Gossypium arboreum* Linn. and *Aloe barbadensis* Mill. As the quantity of sulphur varies in the manufacturing of *Makaradhwaja* so the properties enhances, thus excess *Gandhaka* is assimilated/ digested in *Parada* (Mercury) to form a therapeutically extremely effective compound, a polysulfide form probably is termed as *Gandhaka Jarana*, which plays an important role in the detoxification of mercury. As the quantity of sulphur varies in the manufacturing of *Makaradhwaja* so the properties enhances and name of product also differs a bit. As in context to present research work *Gandhaka* was digested thrice the *Parada*. *Gandhaka Jarana* (The process of digestion of excess of sulphur in mercury) plays an important role in the detoxification of mercury. It has been claimed that *Parada* without the process of *Gandhaka Jarana*, cannot be able to cure the disease[10], so it must be treated with *Gandhaka* to make it highly effective i.e. to acquire many pharmacological and therapeutic properties. Here, *Makaradhwaja* was prepared using three times of *Gandhaka* than that of *Parada* i.e. *Triguna Makaradhwaja* and evaluated for immuno-modulatory activity.

Materials and Methods

All the raw drugs used in the formulation were procured and authenticated by pharmacy Gujarat Ayurved University, Jamnagar. *Triguna Makaradhwaja* was prepared using *Shuddha Swarna* (processed gold), *Parada* and *Gandhaka* in the proportion of 1:8:16 using levigating medias as *Rakta Karpasa Pushpa* (flowers of *Gossypium arboreum*) and *Kumari Swarasa* (expressed juice of *Aloe barbadensis* Mill.) by following the classical guidelines[11] in Electric muffle furnace with the help of gradual heating pattern in eighteen hours[11], in the department of Rasashastra and Bhaishajya Kalpana of IPGT & RA. *Guduchi Ghana*[12] (GG – water extract of *Tinospora cordifolia*), which is used as adjuvant as per classical guidelines was also prepared by crushing fresh stem pieces of *Guduchi* [*Tinospora cardifolia* (Willd.) Miers ex Hook.f.] and kept for overnight by soaking in water followed by preparation of *Kwatha* (decoction) using total eight times of water. The *Kwatha* is subjected to gradual heat to prepare *Guduchi Ghana*[12] as per classical procedure. Honey which was used as vehicle as advocated in classical texts was procured from local market.

Animals

Wistar strain albino rats (*Rattus norvegicus*) of either sex were obtained from animal house attached to Pharmacology laboratory of our institute. Six animals were housed in each cage made up of poly-propylene with stainless steel top grill. The dry wheat (post hulled) waste was used as bedding material and was changed every morning. The animals were exposed to 12 h light and 12 h dark cycle with the relative humidity of 50 to 70% and the ambient temperature during the period of experimentation was 22 ± 03ºC. Animals were fed with Amrut brand rat pellet feed supplied by Pranav Agro Mills Pvt. Limited. For their drinking purpose tap water *ad libitum* was used. The experiments were carried out in conformity with the Insttitutional animals Ethics Committee (IAEC) after obtaining its permission (Approval number: IAEC/07/2010/01/MD).

Dose fixation

The general clinical dose of *Makaradhwaja* is 35mg[13] along with *Guduchi Ghana* (463 mg) as adjuvant and honey as *anupana* (Vehicle). The animal dose was calculated by extrapolating the human dose to animal dose based on the body surface area ratio by referring to the table of Paget and Barnes (1964)[14]. Thus the dose of *Triguna Makaradhwaja* was 3.15 mg/kg rat and the dose of *Guduchi Ghana* (GG) was 45 mg/kg. The test drug was weighed carefully in an electronic digital balance and stock solution was prepared in honey at suitable concentration to enable administration of 0.1 ml for 100 g rat and administered to animals orally based on the body weight. The amount of vehicle honey was decided on

the basis of observation of clinical study where the required quantity of it is to be added with the drug and given for licking. The previous research works have shown that honey as *Anupana* has considerable effect in reducing toxicity of mineral drugs[15].

Chemicals

All the chemicals and reagents used in the experimental study were procured from standard and reputed firms and are of analytical grade (EXLR), regularly used in the laboratory. Triple antigen was procured from Serum Institute of India, Pune.

Effect on humoral anti-body formation

The effect of test drugs on anti-body formation against sheep red blood cells (SRBC) was studied as described by Puri *et al*, (1994)[16]. Wister strain albino rats of either sex weighing between 200 ± 30 g were selected and divided into four groups. First group received tap water and served as the normal control to which SRBC was not injected. Second group received tap water and served as SRBC control to which SRBC was injected. Third group received GG with honey and served as vehicle control group. Calculated dose of TM was administered to fourth group. The test drug and vehicles were administered for 10 consecutive days. On third day, Sheep blood was collected from the city slaughter house in a sterilized bottle containing Alsever's solution (2% dextrose, 0.8% sodium citrate, 0.5% citric acid and 0.42% sodium chloride) aseptically so that agglutination of blood does not take place. The collected sheep blood was thoroughly washed with sterile normal saline through repeated centrifugation until the supernatant fluid became colorless and made to 20% SRBC solution. This sensitizing agent was injected subcutaneously to the rats of second, third and fourth groups in the dose of 0.5 ml/100 g of body weight.

On the 11th day blood was collected by puncturing supra-orbital plexus by capillary tubes under ether anaesthesia for estimation of haematological and biochemical parameters. Blood (0.08 ml) was mixed with 0.02 ml of EDTA (33.33 mg/ml) and fed to the auto analyzer (ERBA CHEM-5, Trans Asia) which was automatically drawn in to the instrument for estimating different haematological parameters. Then the animals were sacrificed by ether over dose and the blood was collected in sterile test tubes. Serum was separated from it and complement in it was inactivated by incubating it for 30 minutes at 56°C in a serological water bath. Serum total protein (Biurate method)[17] and serum albumin (BCG Dye method)[18] were also estimated. Serum globulin was calculated from serum protein and serum albumin values, A/G ratio was calculated from the above values. Serum IgG was estimated by using ELISA method.

Estimation of antibody titre

The micro-titer plate was filled with 0.1 ml sterile normal saline and serial two fold dilutions of 0.1 ml of the serum in sterile saline solution were made in the micro-titer plate up to 16 times. 0.1 ml of thrice saline washed 3% SRBC was added to each well of the tray. Blood from the same animal (Sheep) was used for both sensitization and to determine antibody titer. The trays were covered and placed in refrigerator overnight. Antibody titer (heamagglutination titer)[19] was noted on the next day. The titer was converted to $\log_2$ values for easy comparison.

Spleen, lymph node and thymus were dissected out from the sacrificed animals and transferred to 10% formaldehyde solution for fixation and later on processed for histological studies. The histopathological slides of different organs were prepared by referring standard procedure[20].

Effect on cell mediated immunity

Effect on cell mediated immunity was evaluated by following the procedure of Bhattacharya (1993)[21]. Wistar strain albino rats of body weight ranging from 180 ± 30 g were used as experimental animals. The selected animals were divided into three groups of six animals in each group. First group received tap water and served as the normal control. Second group received GG with honey and served as vehicle control group. Calculated dose of TM was administered to third group. All the animals were sensitized subcutaneously (0.5 ml/100g body weight) on first day of drug administration by following solution; Triple antigen (DPT)-1 ml, Normal saline (0.9%) -4 ml and potash alum (10%)-1 ml. The *p*H of this solution was maintained between 5.6 - 6.8 using 10% sodium carbonate. The drug administration was continued for seven consecutive days. On 7th day one hour after drug administration the initial paw volume of left hind paw was noted and 0.1 ml of above solution was injected in to plantar aponeurosis of same paw. Volume of immunological oedema thus produced was measured by volume displacement method (Bhatt *et al*, 1977)[22] after 24 and 48 h of injection using Plethysmometer (Electronic-IITC). Percentage increase in paw volume, which is the index of oedema formation over initial value, was calculated.

Statistical analysis

Results were presented as Mean ± SEM, difference between the groups was statistically determined by unpaired Student's 't' test[23]. $P<0.05$ was considered as statistically significant.

Results

Administration of GG and TM apparently increased the anti-body titre value in comparison to SRBC control rats, however only the observed increase of antibody titre of TM treated group is found to be statistically significant. Further when the value from TM treated group was compared with values of vehicle control (GG) group, test drug produced statistically non-significant increase in anti-body titre (Table 1).

SRBC sensitization significantly increased serum IgG, total protein and albumin levels. Treatment with test drugs did not affect the total protein level to significant extent in comparison to SRBC control group. TM treated group significantly decreased serum albumin level, while GG and SM did not affect it to significant extent. Treatment with GG and TM decreased serum IgG level, however the decrease observed in GG group is found to statistically significant (Table 1).

SRBC sensitization significantly decreased eosinophil and monocyte count to significant extent in comparison to normal control group. Administration of vehicle and test drug non-significantly attenuated eosinophil and monocyte counts. Other haematological factors are non-significantly affected by SRBC sensitization (Table 2).

Administration of GG and TM failed to suppress immunological paw oedema in 24 h, however, they suppressed paw oedema non-significantly at 48 h after triple antigen injection (Table 3).

Table 1—1: Effect on haemogglutination titre and serum biochemical parameters

Groups	Antibody titer (Log_2 values)	Total protein (g/dl)	Albumin (g/dl)	Globulin (g/dl)	Serum IgG
Control	---	6.35 ± 0.13	3.17 ± 0.062	3.18 ± 0.18	381.0 ± 5.26
SRBC	4.505 ± 0.52	7.08 ± 0.06$^{\#}$	3.67 ± 0.033$^{\#\#\#}$	3.41 ± 0.05	423.67 ± 10.55$^{\#}$
GG	6.007 ± 0.42$^{¥}$	6.85± 0.22	3.43± 0.17	3.41± 0.17	382.66± 12.36*
TM	6.354 ± 0.27$^{¥¥}$	6.80± 0.56	3.25± 0.17*	3.550± 0.41	381.50± 21.66

$^{\#}$ P<0.05, $^{\#\#}$P<0.001 (Compared with normal control), *P<0.05 (Unpaired t test Vs SRBC control)

$^{¥}$P<0.05, $^{¥¥}$P<0.01 (One Way ANOVA, P=5.37 Vs SRBC control)

Table—2: Effect on hematological parameters

Parameters	Control	SRBC	GG	TM
Hemoglobin (g/dl)	15.12 ± 0.22	15.28 ± 0.32	15.517±0.389	16.183 ± 0.37
WBC (10^3 L)	8250.0 ± 734.3	7766.6 ± 249.8	6016.67± 446.78*	7350.00± 684.96
Neutrophils (10^3 L)	22.17 ± 5.06	19.00 ± 1.82	22.33 ± 1.94	18.16 ± 1.86
Lymphocyte (10^3 L)	72.00 ± 5.03	78.00 ± 1.69	74.167± 2.22	79.33± 2.09
Eosinophil (10^3 L)	3.17 ± 0.17	1.83 ± 0.31$^{\#}$	2.000± 0.25	1.33± 0.21
Monocyte (10^3 L)	2.67 ± 0.21	1.17 ± 0.17$^{\#\#}$	1.50± 0.22	1.16± 0.16
RBC (10^6/ L)	8.45 ± 0.09	8.59 ± 0.31	8.82± 0.16	9.08± 0.17
MCV (C -M)	57.96 ± 0.38	58.05 ± 0.63	56.61± 0.66	57.55± 0.61
MCH (pg / l)	17.88 ± 0.19	17.85 ± 0.40	17.58± 0.16	17.81± 0.25
MCHC (g/dl)	30.85 ± 0.19	30.71± 0.37	31.06± 0.18	30.95± 0.23

$^{\#}$ P<0.05, $^{\#\#}$P<0.01 (comparison to normal control) *P<0.05 (comparison to SRBC control)

Examination of sections of thymus from normal control rats exhibited normal cytoarchitecture (Plate 1A). In sections from SRBC control group increase in cellularity was observed in comparison to normal control group sections (Plate 1B). In sections from GG and TM treated groups also mild increased cellularity was observed (Plate 1C & 1D).

Sections of spleen from normal control rats exhibited normal cytoarchitecture (Plate 2A). The cytoarchitecture of SRBC control group was found to be similar to normal control group sections (Plate 2B). In sections from GG and treated groups shows increase white pulp proportion in comparison to SRBC control group (Plate 2C & 2D).

Sections of lymph node from normal control rats exhibited normal cytoarchitecture (Plate 3A). The cytoarchitecture of SRBC control group was found to be similar to normal control group sections (Plate 3B). In sections from GG and TM treated groups moderate increased cellularity was observed (Plate 3C & 3D).

Discussion

Makaradhwaja is among the most popular and effective *Kupipakwa* preparation of Ayurvedic medicine. It is believed that after *Jarana* of three times of *Gandhaka* in it, effectiveness further potentiates and the formulation is termed as *Triguna Makaradhwaja*. As per classical literature, this drug is mainly used for *Rasayana* purposes. *Rasayana* drugs are well-known for their propensity to modulate immune system. The safety profile of this formulation was reported by Patgiri *et al*[24] on acute and chronic administration to the rats by adopting battery of parameters like ponderal changes, behavioural profile, hematological, biochemical and histopathological parameters and found to be relatively safe.

Table—3: Effect on immunological paw edema

Groups	24 h	% change	48 h	% change
Control	24.82 ± 4.74	--	15.99 ± 4.50	--
GG	33.95 ± 5.15	36.85↑	11.99 ± 1.46	25.04↓
TM	25.90 ± 4.30	04.40↑	14.28 ± 7.59	10.67↓

Data: Mean ± SEM ↑- Increase ↓- Decrease

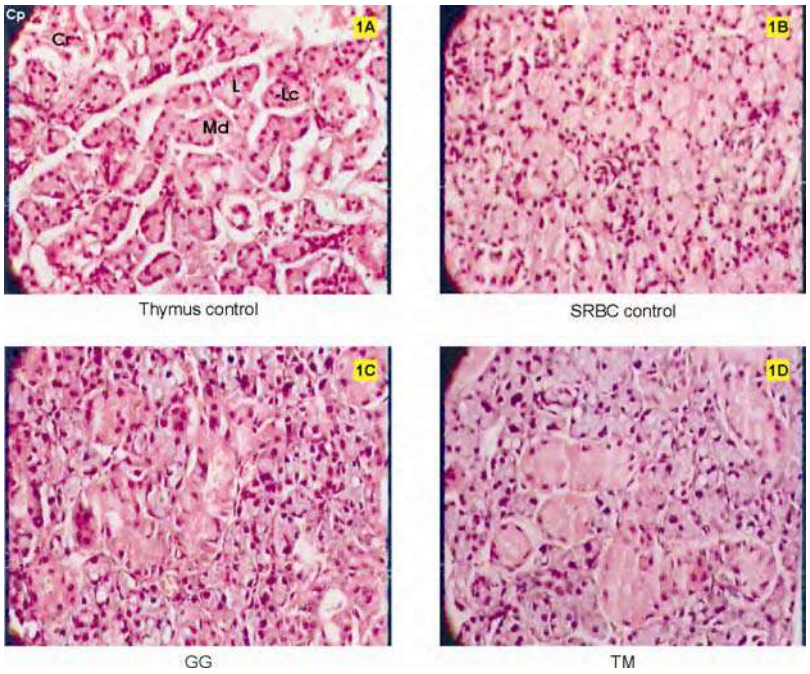

Plate 1 (A-D)— A. Photomicrographs of thymus from control group (1×400 magnification); **Cp**- Capsule. **Cr**- Cortex. **Md**-Medulla. **L**-Lobule. **Lc**-Lymphocytes; **Note:** Normal cytoarchitecture;
B. Photomicrographs of thymus from SRBC control group (1×400 magnification); **Note:** Slightly increased cellullarity;
C. Photomicrographs of thymus from GG treated group (1×400 magnification); **Note:** Increased cellullarity;
D. Photomicrographs of thymus from TM treated group (1×400 magnification); **Note:** Marked increase in cellullarity

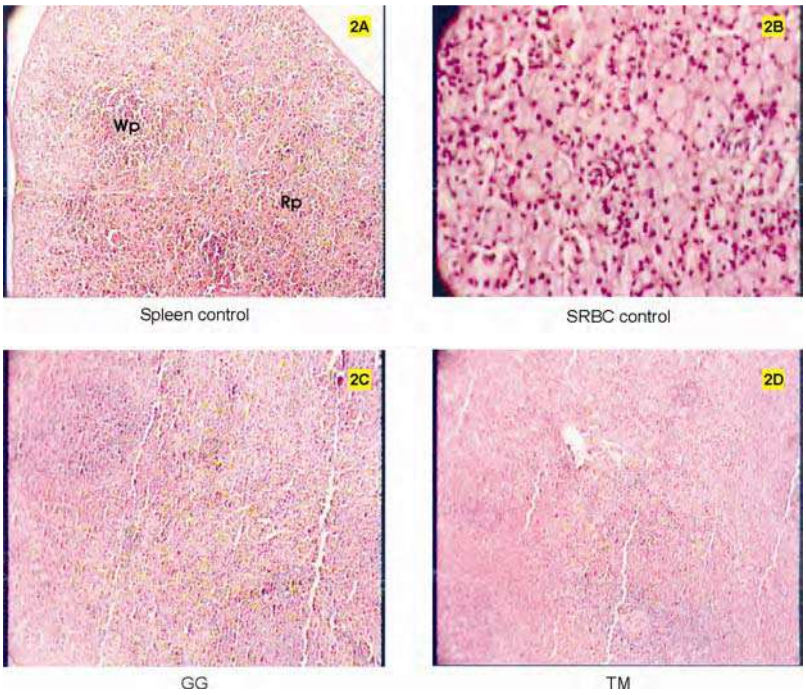

Plate 2 — A. Photomicrographs of spleen from control group (1×100 magnification); **Rp** -Red pulp, **Wp**-White pulp; **Note**: Normal cytoarchitecture;
B. Photomicrographs of spleen from SRBC control group (1×100 magnification); **Note:** Normal cytoarchitecture;
C. Photomicrographs of spleen from GG treated group (1×100 magnification); **Note:** Increased white pulp proportion;
D. Photomicrographs of spleen from TM treated group (1×100 magnification); **Note:** Increased white pulp proportion

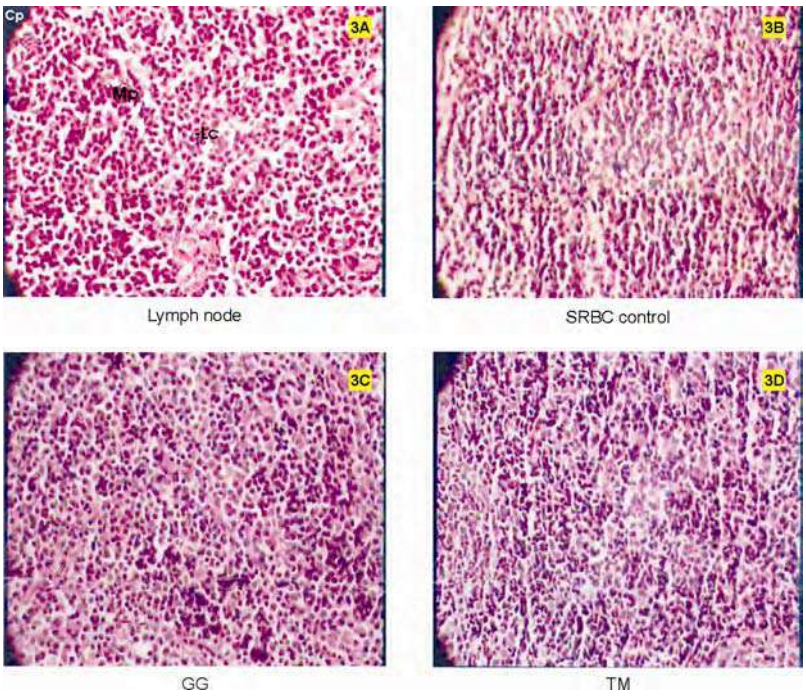

Plate 3 — **A.** Photomicrographs of lymph node from control group (1×400 magnification); **Cp**- Capsule, **Cr**- Cortex, **Md**-Medulla, **Lc**-Lymphocytes; **Note:** Normal cytoarchitecture;
B. Photomicrographs of lymph node from SRBC control group (1×400 magnification); **Note:** Slightly increased cellullarity;
C. Photomicrographs of lymph node from GG treated group (1×400 magnification); **Note:** Increased cellullarity;
D. Photomicrographs of lymph node from TM treated group (1×400 magnification); **Note:** Marked increase in cellullarity

Haemagglutination antibody titre is a primary parameter for studying the humoral response. Antibody molecules which are secreted by plasma cells mediate the humoral immune response. Treatment with TM significantly increased antibody titer, whereas in GG treated group only a non-significant increase was observed. It is to be noted that because of this non-significant increase the antibody elevation observed with test formulation was found to be statistically non-significant in comparison to GG group. This indicates that adjuvant does contribute, although moderately, to the antibody increasing effect. It may have contributed to the elevation observed in TM group.

Among the 10 hematological parameters studied, antigen injection leads to significant changes only in two parameters. They were significant decrease in eosinophil and monocyte counts in SRBC control group. Treatment with test drugs did not produce any significant impact on these two parameters.

Among the biochemical parameters, serum total protein and albumin levels are significantly increased in SRBC control group. Vehicle administration did not affect them to significant extent, while TM significantly reversed the antigen induced elevation in serum albumin level. Further serum IgG was found to be significantly elevated by sensitization of SRBC. However, this does not correlate well with anti-body titre changes since in GG group in which moderate elevation in antibody titre was observed a decrease in serum IgG level was observed. This may be reflective of effect on other types of antibodies also. As thymus and spleen were lymphoid organs and play a vital role in immune responses, administration of antigen may bring about some changes in their anatomical and physiological features. Administration of TM significantly enhanced cellularity of all the three organs studied as revealed by histological examination, this further indicates the immune stimulant property of the test drug.

Cell mediated immunity do not involve increased formation of antibodies. But through direct effect this reaction neutralizes intracellular pathogens (such as viruses), fungi, malignant cells and grafts of foreign tissue. The test drug did not affect immunological oedema which is used as representative test for cell mediated immunity. Normally those drugs which enhance anti-body formation depress cell mediates immunity, such an effect was not observed. This indicates complex nature of the immunomodulation observed with the test formulation.

The first step in the immune reaction is the recognition of the foreign antigen by macrophages and helper T cells, which become activated and are specific. These activated T cells, which are antigen specific, divide many times to form memory T cells and cytotoxic (killer) T cells (also called CD8 T cells). The memory T cells will remember the specific foreign antigen and become active if it enters the body again. Two pathways mediate immune response depending up on the type of T helper cells involvement. The two are Th1 helper and Th2 helper cell mediated pathways. Th1 pathway is involved in the elaboration of cell mediated immunity through secretion specific cytokines[25] where as Th2 pathway is involved in the enhancement of anti-body formation through plasma cell activation. In the present study no significant effect was observed on CMI while elevation in antibody titre and IgG secretion was observed. This indicates that the test formulation has got specific stimulatory effect on the Th2 pathway of immune reaction. Further studies would be required to elucidate the exact mechanism of this effect. However, it can be suggested that this stimulation may involve enhanced antigen processing by the antigen presenting cell and or through increased formation of cytokines like IL-$_4$ and tissue growth factor-b (TGB-b) both of which stimulate B-lymphocytes to proliferate[32]. Further, it is well established that *Guduchi*[26-28] and honey[29-31] are reputed for their immune-potentiating activity. Combining adjuvant with primary drug leads to further moderate increase in anti-body formation. The combination of these adjuvants with TM might have lead to further increase in observed immune stimulation activity. However, further studies in other models of immune reaction and evaluation in immune-suppressed state would throw more light on the probable mechanism involved. It would be useful to also study the effect on formation of different cytokines and modulation of factors involved in their expression.

Conclusion

Triguna Makaradhwaja was evaluated in two models of immune reactions. The data obtained indicate significant anti-body formation stimulation with the test formulation. This inference is supported by the elevation observed in serum IgG level. The immunological paw odema which was employed as representative of CMI was not affected to significant extent. Weak stimulation observed with the adjuvants indicates their role at least partially in the anti-body formation observed with the test formulation. It is

suggested that this increase in anti-body might be due to increased formation of cytokines mediating Th2 pathway of immune reaction.

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ORIGINAL RESEARCH ARTICLE

Immuno-Modulatory Effect Of Makaradhwaja

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ABSTRACT

The term immune-modulation is used for describing the effect of various chemical mediators, hormones and drugs on the immune system. The human body has the ability to resist almost all types of organism or toxins that tend to damage the tissue and organ. This capability is called immunity. Makaradhwaja is a metalo-mineral preparation, which is most popular and effective in Kupipakwa preparation of Ayurvedic medicine which is mainly used for Rasayana purposes. In this study total **18 Swiss albino mice** of either sex weighing between 20 - 36 g were divided into **three groups**, each having 3 males and 3 females. Sample of Makaradhwaja was taken and few drops of 5% gum acacia suspension were added, it was further grounded for 5 minutes and the volume was made up with distilled water, suspension form in 5% gum acacia orally with the help of plastic tube attached to a tuberculin syringe. Makaradhwaja comes to 16.25 mg kg^{-1} and was rounded of to 16 mg kg^{-1} dose, for 10 consecutive days. Study suggested **that the test drug has significant CMI (cell mediated immunity) enhancing effect** but has no significant effect on humoral anti-body formation.

Key words: Makaradhwaja, Immune-modulation, CMI, Humoral anti-body formation.

INTRODUCTION:

The term "immunomodulation"[i] is used for describing the effect of various chemical mediators, hormones and drugs on the immune system. The human body has the ability to resist almost all types of organism or toxins that tend to damage the tissue and organ. This capability is called immunity.

The immune mechanism: Basically there are two different types of lymphoid cells T and B cells which mediate "cellular" and "serologic" or humoral immunity, respectively. Both these types of cells are present in the circulating blood and in peripheral lymphoid tissues. The recognition of the antigen by the T cells leads to proliferation of these cells, infiltration of immune cells at the site of action and cellular immunity. These reactions may be manifested as delayed hypersensitivity reactions, tissue graft rejection or organ transplant rejection. The infiltrating T cells exert their cytotoxic action by the release of various lymphokines (transfer factor, TF; migration inhibitory factor, MIF; chemotactic factor, CF; lymphotoxin, LT; interleukin II (IL-2), interferon, IFN).The other limb of immune system involving B cells is responsible for the genesis of specific antibodies immunoglobulins (IgA, IgG, IgD, IgE, IgM).The recognition of antigen (Ag) by the B-cells lead to proliferation of these cells, conversion to plasma cells and generation of specific antibodies (Ab) (Igs). The specific antibody (Ab) binds with the specific antigen (Ag) leading to its inactivation or even phagocytosis. Besides the conventional T and B cells, the other cells of special significance are T_4 helper cells, T_8 suppressor cells, monocytes and macrophages. The existence of a heat labile serum component known as the complement system, which causes bacteriolysis and phagocytosis, is also well-known.

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Mechanisms of Immuno-modulation:

Drugs may modulate immune mechanism by either suppressing or by stimulating one or more of the following steps:

(1) Antigen recognition and phagocytosis.
(2) Lymphocyte proliferation
(3) Synthesis of antibodies
(4) Antigen - antibody interaction.
(5) Release of mediators due to immune response[ii]
(6)Modification of target tissue response.

Makaradhwaja is a metalo-mineral preparation, which is most popular and effective in Kupipakwa preparation of Ayurvedic medicine. As per classical literature, this drug is mainly used for Rasayana purposes. These drugs are also well-known for their propensity to modulate immune system hence the present study was planned to assess the effect of test drug on Immunomodulation activity.

MATERIALS AND METHODS:

Swiss albino mice of either sex weighing between **20 - 36 g** from the animal house of I.P.G.T. & R.A., G.A.U., Jamnagar were used in the studies. They were maintained on Nav Chankan Oil Mils "Amrut" brand animals feed and tap water given *ad-libitum.* The test drug (Makaradhwaja) was prepared in the department of Rasa Shastra and Bhaishajya Kalpana Including Drug Research, I.P.G.T. & R.A., G.A.U., sample of Makaradhwaja was taken in requisite amount in separate small porcelain mortars and few drops of 5% gum acacia suspension were added, it was further grounded for 5 minutes and the volume was made up with distilled water. The drug was administered in suspension form in 5% gum acacia orally with the help of plastic tube attached to a tuberculin syringe. Dose for this experimental study was calculated by extrapolating the therapeutic dose to mice dose on the basis of body surface area ratio (conversion factor 0.0026). Calculated this way the mice dose of Makaradhwaja comes to 16.25 mg kg^{-1} and was rounded of to 16 mg kg^{-1} dose. The experiment was carried out in accordance with the directions of the Institutional Animal Ethics Committee (IAEC) after obtaining its permission.

(A) **Effect on humoral antibody formation in mice[iii]:**

Total eighteen Swiss albino mice including nine male and nine females were divided into three groups each having 3 males and 3 females. Dose of the test formulations (Both A and B) was calculated by extrapolating the human dose to animals (1.8 g/kg) based on the body surface area ratio by referring to the standard table of Paget and Barnes (1969)[iv]- **(Table-1)**

Table 1: Grouping for effect on humoral antibody formation and CMI in mice:

S. No.	Name of the group	Dose
(a)	Group A (control)	Tap water (No drug given)
(b)	Group B (Therapeutic dose)	Makaradhwaja suspension 16 mg/kg body weight of mice.
(c)	Group C (Higher dose)	Makaradhwaja suspension 32 mg/kg body weight.

SRBC solution was prepared from the Sheep blood collected from the city slaughterhouse in a sterilized bottle containing Alsever's solution[v] (2% dextrose, 0.8% sodium citrate, 0.5% citric acid and 0.42% sodium chloride). SRBC was thoroughly washed with sterilized normal saline by centrifuging and stored in Alsever's solution in a refrigerator till experimentation. The drug was administered for 10 consecutive days, on 3rd day, 25% SRBC solution was **injected by i.p.route** in the dose of 0.1 ml/ 10 g of body weight. On the 10th day mice were sacrificed by **ether overdose** and blood was collected in separate test tubes. Blood from the same animals (Sheep) was used for both sensitization and determining antibody titre. From the collected blood, serum was separated and incubated in a serological water bath for 30 minutes at 55°C to inactivate the complement in it. Serial two fold dilutions of the serum in sterile saline solution were made in the volume of 0.1 ml in microtitre plate. 0.1 ml of thrice saline washed 2% SRBC was added to each well of the tray. The tray was covered and placed in refrigerator overnight. Antibody titre[vi] (haemaglutination titre) was noted next day. Titre was converted to $\log_2$ values for easy comparison. After scarifying, specimens of important organ related with Immuno-modulation activity like spleen, thymus and lymph nodes were collected from animals and their weight were recorded; tissues were transferred to 10% formaldehyde solution for fixation and histopathological study was carried out.

(B) **Effect of test drug on cell mediated immunity in mice[vii]:**

Total eighteen Swiss albino mice of either sex weighing between 20 - 36 g were selected and divided into three groups, each having 3 males and 3 females. Immunological inflammation was produced in mice by injection of Triple antigen with alum precipitate in the following proportion into sub planter tissue of mice hind paw. pH of the above solution was maintained between 5.6 - 6.8 using 10% sodium carbonate.

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Triple antigen : 1 ml

Normal saline (0.9% : 4 ml

Potash alum (10%) : 1 ml

Initially the mice were sensitized by injecting the triple antigen with alum precipitates **sub-cutaneously** in the nape of the neck in a dose of 0.1 ml / 10 g body weight. The test drug administration began on the day of sensitization and continued for the next five days. On 5th day, 1 hour after administration of the test drug, the mice were injected with 0.05 ml triple antigen with alum precipitates beneath plantar aponeurosis in the left hind paw.

The initial paw volume was measured in all the mice after marking in left leg and before sensitization of triple antigen in neck. Further paw volume was measured 24 and 48 hrs hour after injecting alum adjuvant. The paw volume was measured with the help of a Plethysmometer[viii].

Statistical analysis:

Student's t test for unpaired data has been used for analyzing the data generated during the study. A 'P' value less than 0.05 is considered as statistically significant and the value of P<0.01 or P<0.001 is considered statistically highly significant. Level of significance was noted and the results interpreted accordingly.

Observations and results:

The data pertaining to the effect of test drugs on spleen and thymus weight in SRBC sensitized mice are presented in **(Table-2).** The test drug did not affect spleen and thymus weight significantly. An apparent moderate 31.25% increase observed in thymus weight was found to be statistically non-significant.

(Table-3) depicts data related to the effect of test drug on antibody formation against SRBC. The apparent moderate increase observed at therapeutic dose level and slight decreases observed at higher dose level were found to be statistitically non-significant.

Table 2: Effect on humoral antibody formation- Effect of Makaradhwaja in different doses on spleen and thymus weight in SRBC sensitized mice.

Treatment	Dose mg/kg(po)	Spleen mg/10g body wt.mean ± SEM	% Change	Thymus mg/10g body wt .mean ±SEM	% Change
Control	-	22 ± 0.30	-	0.16 ± 0.02	-
Therapeutic dose	16	0.21 ±0. 01	4.54 ↓	0.21 ± 0.01	31.25 ↑
Higher dose	32	0.23 ± 0.05	4.54 ↑	0.18 ± 0.03	12.5 ↑

Table 3: Effect of Makaradhwaja on antibody formation against SRBC in mice

Treatment	Dose mg/kg	Haemaggulatination titre log_2 mean ± SEM	% Change
Control	-	4.04 ± 0.21	-
Therapeutic dose	16	4.96 ± 0.45	22.77 ↑
Higher dose	32	3.60 ± 0.33	10.86 ↓

Table 4: Effect of Makaradhwaja on alum adjuvant induced immunological paw oedema in pre sensitized mice:

Treatment	Dose mg/kg	% Increased in Paw volume after alum adjuvant injection			
		24 hours Mean ± SEM	% Change	48 hours Mean ± SEM	% Change
Control	-	19.06 ± 06.34	-	30.24 ± 8.93	-
Therapeutic dose	16	45.89 ± 12.63	140.76 ↑	71.22 ± 11.26*	135.51 ↑
Higher dose	32	39.15 ± 08.78	105.40 ↑	75.63 ±8.95 **	150.09 ↑

The following organs were subjected to histopathological study

(I) Spleen
(II) Thymus
(III) Lymphnodes

Fig- 1) Fig-15 to 15b Microscopic examination of Spleen: Sections obtained from different groups of rats subjected to immuno-modulation study did not show any significant difference in the cytoarchitecture. Show photomicrographs of representative sections from different groups.

Fig- 2) Fig-16 to 16b Microscopic examination of Thymus: Scanning of thymus sections obtained from different groups showed increased cellularity in-group A, while sections obtained from group B and C did not show any change in the cytoarchitecture. Show photomicrographs of representative sections from different groups.

Fig-3) Fig-17 to 17b Microscopic examination of Lymph node: Examination of sections of lymph node from different groups did not show any changes in the cytoarchitecture of the gland, respectively show photomicrographs of representative sections from different groups.

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Fig 1: 15 to 15b Microscopic examination of Spleen:

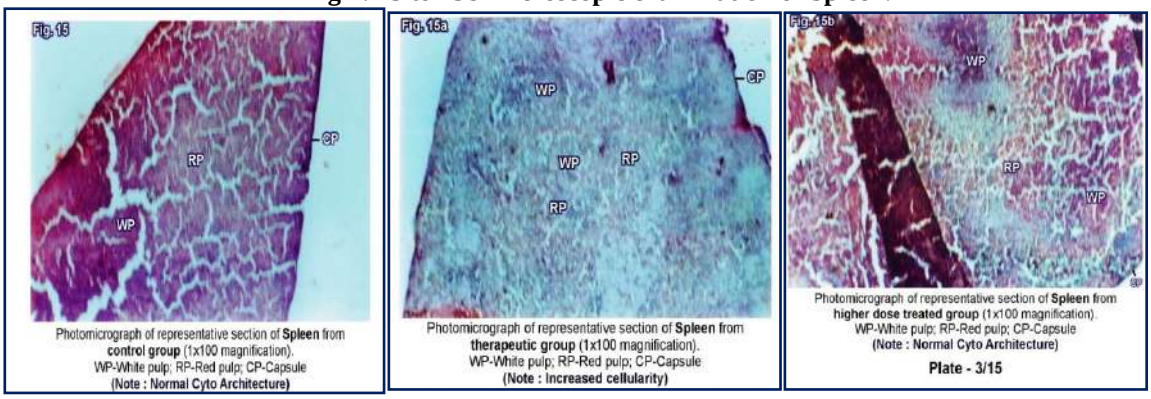


Fig 2: 16 to 16b Microscopic examination of Thymus:

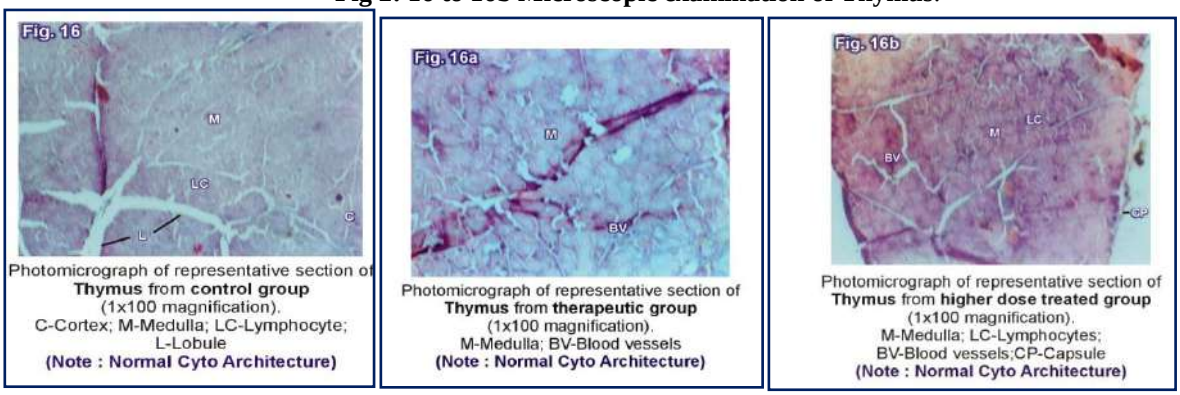
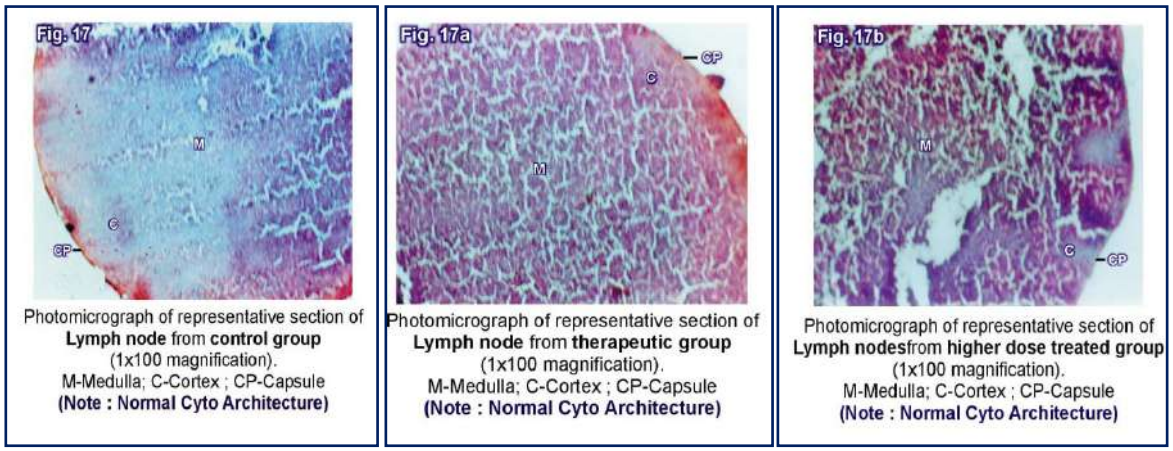


Fig 3: 17 to 17b Microscopic examination of Lymph node:



Effect on cell mediated immunity:

The data related to the effect of test drug on alum adjuvant induced immunological oedema studied at different dose level are presented in (Table-4). An apparent marked elevation in pedal oedema was observed in both the groups at both 24 and 48 hrs after the injection of the response eliciting dose of antigen. The increase with 16 mg/kg dose was 140.76% at 24h and 135.11% at 48 h. However due to variation in the data only the increase observed at 48 h was found to be statistically significant. At 32 mg/kg dose also similar type of results were observed. At 24 h 105.40% increase and at 48h 150.09% increase was observed with only the latter being statistically significant.

DISCUSSION:

From the above presented data it can be suggested that the test drug has no significant effect on humoral anti-body formation. But has significant CMI enhancing effect. CMI as mentioned earlier is mainly mediated through T-lymphocyte. The first step, which is common to both types of

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immune mechanism, is recognition of the antigen. The second step is the activation of cell involved in the CMI through elaboration of cytokines. The final mediator of this activity is the activated macrophage. The sequence of the events is that the allergen is taken up by antigen presenting cells, such as Langerhans cells in the skin, which then migrate to lymph nodes and present the allergen to T cells. This results in sensitization and proliferation of T-cells, some of which migrate via the lymph and blood to the site of allergen entry into the body. There they secrete cytokines, such as gamma interferon, which activates macrophages, and tumor necrosis factor (TNF), which stimulates an inflammatory response. Cell mediated immunity is amplified by γ-interferon by enhancing the process of antigen processing by macrophages. Macrophage migration inhibition factor inhibits movements of macrophages from the affected site. Interleukin-2 (IL2) acts on the activated T-lymphocyte and helps in their clonal expansion. It also activates cytotoxic lymphocytes and B-lymphocytes. T-lymphocytes modulate the adherence, locomotion and activation of eosinophils lading to accumulation at the site of immune reaction. Activated eosinophils further add to the tissue injury. In the light of the above it can be suggested that anyone or combination of the following mechanisms may be involved in the stimulation of CMI by the test drug:

(1) Facilitation of the presentation to antigen by macrophages.
(2) Stimulation of secretion of interleukin - 1 ($IL\text{-}_1$) from macrophages.
(3) Upgrading the $IL\text{-}_1$ receptor either through increase in their number or increasing the reactivity of the receptors
(4) Enhancement if release of $IL\text{-}_2$ from activated T cells
(5) Increase in the formation of alpha interferon or/ and facilitation its effect on T-lymphocytes
(6) Increasing the effect of $IL\text{-}_2$ on cytotoxic lymphocytes.

However to arrive at an unequivocal inference it is necessary to carry out further detailed studies employing appropriate experimental models.

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In Vivo Effects Of Traditional Ayurvedic Formulations in *Drosophila melanogaster* Model Relate with Therapeutic Applications

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Abstract

Background: Ayurveda represents the traditional medicine system of India. Since mechanistic details of therapy in terms of current biology are not available in Ayurvedic literature, modern scientific studies are necessary to understand its major concepts and procedures. It is necessary to examine effects of the whole Ayurvedic formulations rather than their ''active'' components as is done in most current studies.

Methods: We tested two different categories of formulations, a *Rasayana* (*Amalaki Rasayana* or AR, an herbal derivative) and a *Bhasma* (*Rasa-Sindoor* or RS, an organo-metallic derivative of mercury), for effects on longevity, development, fecundity, stress-tolerance, and heterogeneous nuclear ribonucleoprotein (hnRNP) levels of *Drosophila melanogaster* using at least 200 larvae or flies for each assay.

Results: A 0.5% (weight/volume) supplement of AR or RS affected life-history and other physiological traits in distinct ways. While the size of salivary glands, hnRNP levels in larval tissues, and thermotolerance of larvae/adult flies improved significantly following feeding either of the two formulations, the median life span and starvation resistance improved only with AR. Feeding on AR or RS supplemented food improved fecundity differently. Feeding of larvae and adults with AR increased the fecundity while the same with RS had opposite effect. On the contrary, feeding larvae on normal food and adults on AR supplement had no effect on fecundity but a comparable regime of feeding on RS-supplemented food improved fecundity. RS feeding did not cause heavy metal toxicity.

Conclusions: The present study with two Ayurvedic formulations reveals formulation-specific effects on several parameters of the fly's life, which seem to generally agree with their recommended human usages in Ayurvedic practices. Thus, *Drosophila*, with its very rich genetic tools and well-worked-out developmental pathways promises to be a very good model for examining the cellular and molecular bases of the effects of different Ayurvedic formulations.

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Introduction

Ayurveda represents the traditional medicine system of India which is widely practiced uninterruptedly at least from beginning of the Buddhist period in India. It continues to be a vibrant system of health care for millions, with over twenty thousand physicians being trained every year and which supports an industry producing drugs worth Rs. 6000 crores (US $1.3 billion) a year. By definition, Ayurveda signifies ''knowledge relating to life'', and regards diseases and medicine as no more than facets of the variegated theme of life. Ayurvedic texts like *Sushruta Samhita* [1] divide the discipline into eight branches, of which the rejuvenating *Rasayana* therapy aims at promotion of long life, enhancement of physical and mental strength, and strengthening of resistance against the infirmities and ailments of old age. *Rasayana* therapy calls for ethical living in conjunction with intramural or extramural protocols involving life style, diet, cleansing procedures and the intake of medicinal formulations. The intramural as well as extramural methods of *Rasayana* therapy require oral administration of drugs, which are mostly based on plant products but may also include drugs derived from animal and mineral/metal sources. Improvement in nutritional status and better qualities of body tissues (*dhatus*) are believed to lead to a series of secondary attributes like longevity, immunity against disease, improved mental and intellectual competence etc [2]. Etymologically, *Rasayana* implies supply of the nutrient sap (*Rasa*) resulting from the digestion of food to the target (*ayana*) body tissues. As described

in *Charak Samhita* [3], *Rasayanas* are believed to augment the transport and supply of "*Rasa*" to the tissues.

The two major groups of Ayurvedic drugs are *Kasthoushadhies* (herbal preparations) and *Rasaoushadhies* (Herbo-bio-mineral-metallic preparations). The *Bhasma*, belonging to the *Rasaoushadhies* group, has a metallic base but ordinarily does not contain active metal. The metal is converted into an ash or oxide and is usually in the form of an organo-metallic compound formed with a number of organic materials used for trituration as *Bhavana Dravya* [2]. Many a times, Ayurvedic drugs are administered orally with '*Anupana*', a vehicle material like honey, sugar, jaggery, Ghee, milk, warm water, juice of some medicinal herbs etc. *Anupana* as a medium of administration improves acceptability and palatability and helps in absorption of the main drug; additionally, it may also act as early antidote.

The ancient Ayurvedic literature available today does not elaborate the effect/s of any therapy in terms of our current understanding of biology/physiology. Given the antiquity, extensive use in health care, and the globally growing popularity of Ayurveda, it would be interesting and appropriate to initiate a new class of studies which apply the rigorous methods of modern science to understand its major concepts, procedures and mechanistic aspects of the products [4]. Although, there has been an increased interest in traditional and herbal medicine systems in recent times, most studies have used specific extracts or "active principles" derived from herbal or other traditional drugs/formulations. Since the Ayurvedic medicines/formulations are complex integrated derivatives involving several specific preparatory steps, studies using isolated active compounds may not really provide full insight into the efficacy or mode of action of the traditional formulations. In order to undertake scientific investigations on action/s of Ayurvedic drugs/formulations using experimental animals, there is an urgent need to develop good model systems which can permit in depth studies on the *in vivo* effects and mechanisms of actions of different Ayurvedic formulations.

With a view to get insight into the cell biological/biochemical/genetic bases of actions of the Ayurvedic formulations, we are using the fruit fly, *Drosophila melanogaster*, as a model. The advantages of the fly model as an experimental model, especially for examining the diverse factors that affect life-history traits and for understanding complex human disorders, are well known [5,6,7,8,9,10]. As a first approach in this direction, we used *Amalaki Rasayana*, a herbal derivative, and *Rasa-Sindoor*, an organo-metallic derivative of mercury, to examine if these formulations indeed affect some of the basic biological life parameters in the fly model. *Amalaki Rasayana* (AR) is a prominent drug in Ayurvedic classics like *Charak Samhita* [3] and *Ashtang Hridaya* [11] and continues to be widely used in view of the claim that it enhances life expectancy, body strength, intellect, fertility and gives freedom from illness. *Rasa-Sindoor* (RS), on the other hand is indicated, singly or in combination with other *Anupana*/formulation in a wide variety of disorders including chronic and recurrent infections (pneumonia/bronchitis), fistula-*in ano*, rheumatological diseases especially those of auto-immune origin, sexual and general debility and benign and malignant neoplasms [12,13].

AR is a *Rasayana* prepared from fruits of Amla or Indian gooseberry (*Phyllanthus emblica*, synonym *Emblica officinalis*). RS, on the other hand, is an organo-metallic *bhasma* in the form of mercuric sulphide with some other elements also present in micro/trace quantities [14]. RS has a crystalline nature with crystal size ranging from 25 to 50 nm, close to the nano-crystalline materials [14,15].

In the present report we have standardized the amounts of these two formulations for supplementation in the standard fly-food that have demonstrable effects on several general physiological parameters like life span, development time, fecundity, thermotolerance, starvation resistance etc. In our study, we did not attempt to characterize the "active principles" in the AR or RS since in Ayurveda, these formulations are believed to act as a whole, rather than through one or more of their compound/s in isolation. Our results show that feeding of the fly larvae and/or adults on food supplemented with non-toxic levels of the two very different classes of Ayurvedic formulations, viz., the herbal-derived AR and the organo-metallic RS, have distinct supplement-specific effects, which in general appear to be in agreement with those described in Ayurvedic literature. Thus, *Drosophila*, with its very rich genetic tools and well worked out developmental pathways promises to be a good model for examining the cellular basis of the effects of different Ayurvedic formulations.

Results

Standardization of dosages of AR and RS which affect life history parameters of *Drosophila*

In initial experiments, we fed wild type larvae on food supplemented with 1 or 2% of the AR (complete with honey and Ghee) to see effects on development and life span. While the development was not significantly delayed, the median life span of flies fed on 1 or 2% AR supplemented food since the 1st instar stage was reduced compared to those reared on regular food (Fig. 1A, Table 1). In view of the apparently toxic effects of higher concentrations, we used 0.5%, 0.25% or 0.125% AR supplemented food for the median life span assay. Flies fed, since 1st instar larval stage, on food supplemented with lower concentrations of AR showed a dose-dependent increase in the median life span, with 0.5% AR supplemented food resulting in maximal increase (40.4 days compared to 36 days for flies reared on regular food; Fig. 1B, Table 1). Supplementing fly food with only honey (0.286%) or only Ghee (0.072%) or with honey plus Ghee (0.36% in 1:0.25 ratio) at dosages equivalent to that in 0.5% AR supplemented food, did not significantly affect the median life span (data not presented). Therefore, in all subsequent experiments, we used 0.5% AR supplemented food.

In the case of RS, initially 1st instar larvae were fed on regular food supplemented with 2%, 1% or 0.5% RS to see effect on life span of adult flies. While 1% or 2% RS supplement reduced the median life span, 0.5% had no significant effect (Fig. 1C, Table 1). Lower concentrations of RS also did not affect the median life span (data not shown). Therefore, 0.5% RS was used in all subsequent experiments.

Rearing of larvae on 0.5% AR or RS supplemented food marginally hastens pupation and fly eclosion by a few hours

The 1st instar larvae that had hatched within one hour interval from the same batch of wild type eggs were distributed to plates with i) regular food (Control), ii) 0.5% AR supplemented food, iii) food supplemented with honey or Ghee or honey+Ghee or the triturated Amalaki powder only in the same proportion as in 0.5% AR supplemented food or iv) 0.5% RS supplemented food. The supplements in iii) served as additional controls for the AR-supplemented food (ii). The developmental assay revealed that larvae reared on 0.5% AR or 0.5% RS supplemented food developed a little faster since pupation in these dishes started a few hours earlier than in those having regular food or honey plus ghee supplemented food (Fig. 2A; Table 2). It is significant that the time

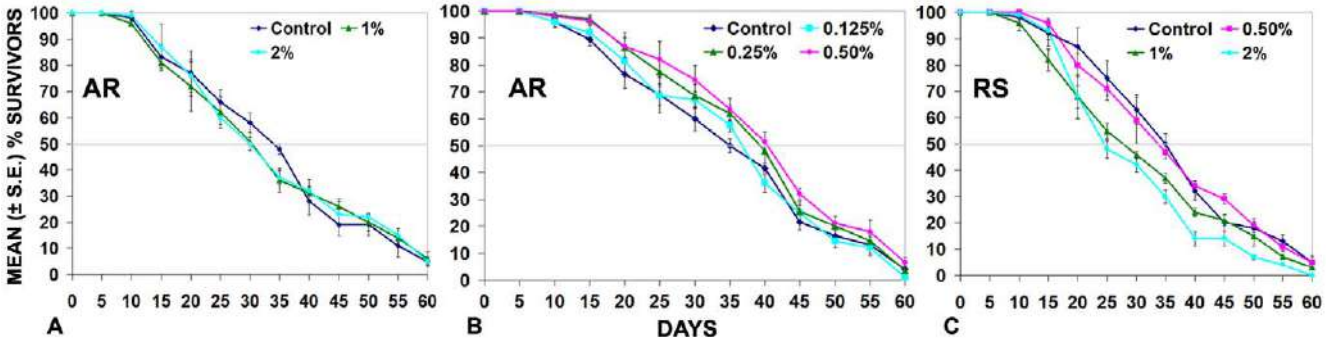

Figure 1. Viability assay of flies reared since embryo hatching on various concentrations of AR or RS supplemented or regular food. The survival curves of flies reared since the 1st instar stage on food supplemented with 1% (green) or 2% AR (light blue) are shown in **A**, for those grown on food supplemented with 0.125% (light blue) or 0.25% (green) or 0. 5% AR (red) are shown in **B; C** shows survival curves for those grown on food supplemented with 0.5% red) or 1% green) or 2% RS (light blue). Each of these also shows the survival curve for flies reared in parallel on regular food (Control, dark blue). Survival curves are based on observations on 200 (8 replicates of 25 flies each) adult flies in each set. Although surviving flies were counted on daily basis, the data are presented for 5 day intervals for the sake of convenience. The vertical bar at each data point indicates the ±S.E. of mean % survivors in the 8 replicates. Median life span is estimated as the day till which 50% (horizontal gray line at 50% on Y-axis) of original flies were still surviving.
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of earliest and last pupating larvae is consistently shifted a few hours earlier than in those growing in parallel on the regular food (see Table 2). Adult flies also emerged earlier in AR or RS fed samples. The faster development was more apparent in the AR fed larvae (Fig. 2B). Supplementing the food with honey and Ghee (H+G in Fig. 2, Table 2) or only Ghee or only honey (not shown) did not affect the normal rate of development. However, supplementing food with the triturated Amalaki powder only (0.143%, AP in Fig. 2) also hastened the development but it was less than that seen with the whole AR supplement (Table 2).

Dimensions of late 3rd instar larval salivary glands and their polytene nuclei are increased following feeding on formulations

In order to see if the AR or RS-supplement in food affected internal larval organs, we examined the different internal organs of late 3rd instar larvae. Various organs like the gut, imaginal discs, brain ganglia etc in larvae reared on the formulation supplemented food appeared generally comparable in size and disposition to those in larvae fed on regular food. Their SG, however, appeared somewhat larger in the formulation fed larvae. Therefore, we compared the dimensions (width and length) of SG of late third instar (spiracle eversion stage) larvae that were fed on regular (Fig. 3A) or 0.5% AR (Fig. 3B) or 0.5% RS (Fig. 3C) supplemented food since hatching. It is interesting that the length and width of the SG are significantly enhanced (Table 3 and Fig. 3A–C) in larvae grown on formulation supplemented food. The number of cells in each of the larval salivary glands is fixed during embryonic stage and the subsequent growth of this tissue occurs through endo-replication cycles that are highly regulated in relation to the anatomical location of each nucleus in the gland [16,17,18]. Therefore, we wanted to know if the increased dimensions of these glands correlated with change in nuclear size and increased DNA content. In order to obtain SG of same age, larvae that had just everted their anterior spiracles were selected and immediately dissected in Poels' salt solution (PSS) [19]. Measurement of diameters of nuclei in the posterior most 5–7 cells in these glands revealed that the nuclear size is significantly greater in the AR- (Fig. 3E) or RS-fed (Fig. 3F) larval SG compared to that in normally fed control larvae (Fig. 3D). Measurement of DNA-specific DAPI fluorescence of distal nuclei in salivary glands from larvae that had just begun the spiracle eversion (Fig. 3D–F, Table 3) revealed that increased nuclear size is paralleled by increase in the DNA content in these polytene nuclei. These effects are more pronounced in AR-fed larvae than in those receiving the RS supplement (Table 3 and Fig. 2G). These observations show that while the larvae reared on formulation supplemented food take a few hours less to pupate than those reared on regular food (see Fig. 2 above), the polytene nuclei in their SG undergo greater numbers of endoreplication cycles so that in parallel with increased DNA content per nucleus, the nuclear and SG dimensions also increase.

Table 1. Median Life Span of *Oregon R*$^+$ flies fed on regular or AR or RS supplemented food.

Food supplement	Median life span of flies under different feeding regimes (in days)					
	Control	0.125%	0.25%	0.5%	1%	2%
Amalaki Rasayana	36.0	37.4**	38.8*	40.4*	30.4*	30.0*
Rasa-Sindoor	35.1	35.2**	35.0**	35.3**	30.1*	25.4*

N = 200 for each feeding condition.
*P<0.001 when compared with the corresponding control (reared on regular food) set of flies;
**P>0.05 when compared with the corresponding control.
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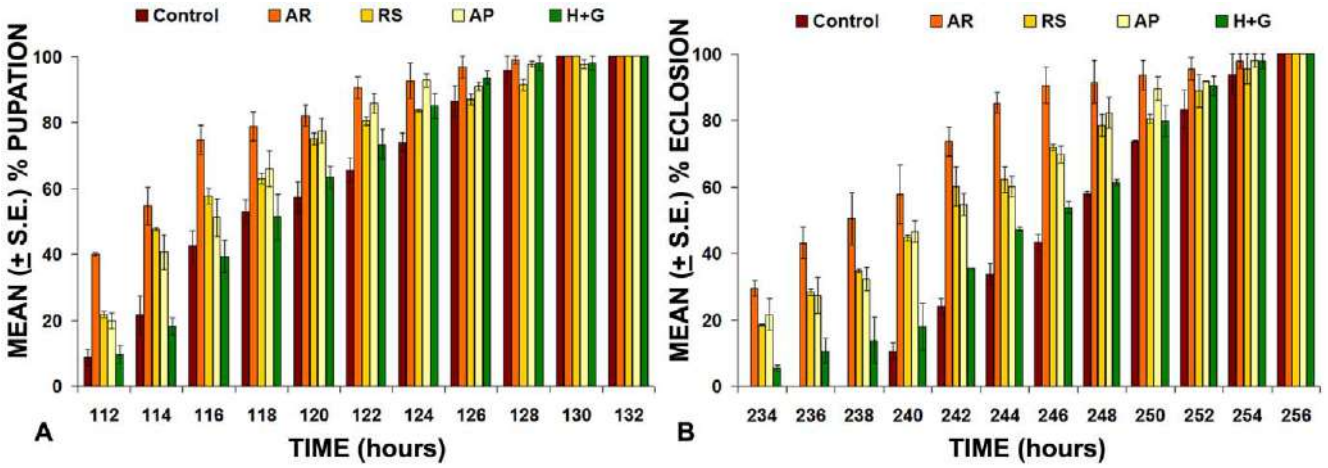

Figure 2. Feeding of *Oregon R*$^{+}$ first instar larvae on food supplemented with 0.5% of AR or RS hastens pupation (A) and fly eclosion (B). The bars represent mean (± S.E.) of 8 replicates (N = 25 in each replicate; total N for each feeding regimen = 200). Numbers of pupae (**A**) or flies (**B**) in a given sample were monitored every two hours between 112–132 and 234–256 hr (X-axis), respectively, and expressed as % of larvae that had pupated or flies that had eclosed by the given time period. Control – regular food, AR – 0.5% AR supplemented food, AP- 0.143% Amalaki Powder supplemented food. RS – 0.5% RS supplemented food and H+G – Honey plus Ghee (same proportion as in 0.5% AR) supplemented food.
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Differential effects of AR or RS feeding during larval or adult stage on fecundity

Synchronized 1st instar larvae were fed on regular (control) or 0.5% AR or RS supplemented food till pupation and the emerging flies were transferred, respectively, to regular or formulation supplemented food. Eggs laid by these females were monitored daily between day 5 and 25 after emergence (Fig. 4A, Table 4). In another set, larvae were reared on regular food and the freshly emerged flies were transferred to regular food (control) or 0.5% AR or RS supplemented food and eggs laid by these females were monitored as in the 1st set (Fig. 4B and Table 4). Flies and larvae reared on 0.5% AR supplemented food laid significantly increased number of total and hatched eggs per female fly but those fed on 0.5% RS produced reduced number of eggs (total as well as those that hatched, Fig. 4A). On the other hand, when only the flies were fed on the AR or RS supplemented food, those receiving RS, showed significantly enhanced fecundity since the total numbers of eggs per female as well as those hatched were significantly higher than in parallel controls (Fig. 4B). Interestingly, feeding on AR only during the adult stage did not result in any noticeable change in the numbers of total or hatchable eggs laid (Table 4, Fig. 4B).

Feeding on AR or RS supplemented food improves thermotolerance

With a view to see if feeding on AR or RS supplemented food affects thermo-tolerance, we exposed 100 hour old *Oregon* R^{+} larvae or 3-day old flies raised on regular or 0.5% AR or 0.5% RS supplemented food to different conditions of thermal stress and monitored their survival.

Late 3rd instar larvae (100 hr after hatching) reared on regular (control) or 0.5% AR or RS supplemented food were exposed to different regimens of heat shock (37°C for 60, 90 or 120 min, or at 38°C for 60 or 90 min or at 39°C for 30 min, see Table 5) following which they were restored to their respective food plates and allowed to develop at 24°C. Numbers of pupae surviving up to 72 hr after the thermal shock and those that later hatched as flies were counted. Wild type larvae reared on regular food are sensitive to a severe heat shock (e.g., exposure to 37°C for 60 min or longer or 30 min or longer at 38°C or at 39°C) and show a temperature and duration-dependent mortality (Table 5). In contrast, significantly larger numbers of those reared on either of the two formulation-supplemented food survived the milder (90 or 120 min at 37°C) as well as the more severe (38°C and 39°C) heat shock (Table 5). It is significant that while all of the larvae reared on regular food died soon after the 30 min exposure to 39°C, 6% and 17% of those reared on AR or RS supplemented food,

Table 2. Median pupation and adult eclosion times following rearing on regular or AR or RS supplemented food.

Food supplement	Median Pupation time (in hours) ±S.E.	Median Eclosion time (in hours) ±S.E.
Regular (Control)	116.1±2.2	246.1±1.6
Honey + Ghee	116.1±2.7**	245.6±1.5**
Amalaki Powder	114.3±1.9*	240.6±1.7*
Amalaki Rasayana	112.1±2.3*	236.7±3.3*
Rasa-Sindoor	114.1±2.1*	240.2±2.9*

N = 200 for each feeding condition (8 replicates of 25 each).
*P<0.001 when compared with the corresponding control (reared on regular food) set of flies;
**P>0.05 when compared with the corresponding control.
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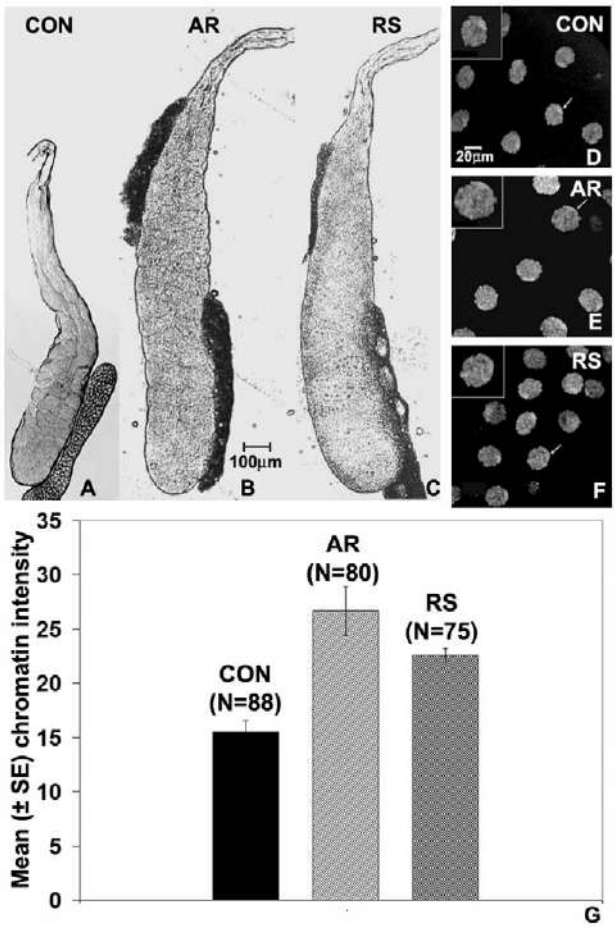

Figure 3. Larval salivary glands attain greater size following feeding on 0.5% AR (B) or RS (C) than in normally fed larvae (A) of same age (spiracle eversion stage). Individual polytene nuclei are also bigger in larvae reared on AR- (**E**) or RS-supplemented (**F**) food than in those fed on regular food (**D**); nuclei marked with arrows in **D**–**F** are shown at higher magnification in insets (also see Table 2). Histograms in **G** show the mean (±S.E.) DAPI fluorescence (in arbitrary fluorescence units on Y-axis) in distal polytene nuclei in salivary glands from larvae reared on regular (CON) or formulation (AR or RS) supplemented food (numbers of nuclei examined in each case are indicated in parentheses above the respective bars. Scale bar in **B** applies to **A**–**C** while that in **D**, applies to **D**–**F**.
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respectively, pupated and survived at least for 72 hr after the severe heat shock. However, none of them emerged as adult flies (Table 5). Interestingly, the thermotolerance of RS-fed larvae was better than that of the AR-fed larvae under all conditions of the thermal stress to larvae (Table 5).

Three day old adult flies, reared from 1st instar stage on normal (control) or AR or RS-supplemented food were exposed to 38°C or 39°C in vials which allowed monitoring of the flies during the stress. It is known that when exposed to these temperatures, the flies get knocked down or paralyzed to a motionless state from which they may or may not recover, depending upon their sensitivity and severity of the stress [20,21]. Therefore, flies in each vial were carefully watched and the numbers of flies that were paralyzed and, therefore, knocked down to bottom of the tube during exposure to the thermal stress were counted at 15 min intervals. As the data in Table 6 show, increasing duration and temperature resulted in a proportional increase in flies that got knocked down or paralysed. It is significant that flies reared, since 1st instar larval stage, on the AR or RS supplemented food showed significantly reduced incidence of paralysis after 45 or 60 min of exposure to 38°C. Further follow up of the recovered flies revealed that a significantly greater proportion of flies were alive in the AR- as well as RS-fed samples 24 hr after the 60 min exposure to 38°C (Table 6). Exposure to 39°C resulted in nearly all flies getting knocked down in all samples, especially after 30 min. It is, however, significant that in this case also, a greater proportion of flies that were reared on AR or RS supplemented food survived at least for 24 hr after the 30 min exposure at 39°C. These results clearly show that AR as well as RS feeding makes the larvae and flies more thermo-resistant.

0.5% AR- but not RS-feeding improves starvation tolerance

Flies reared on regular, AR or RS supplemented food since 1st instar stage were subjected to starvation stress and the LT_{50} was estimated in each case. As the data in Table 7 show, those reared on 0.5% AR supplemented food survived much longer than those fed on regular or 0.5% RS supplemented food (Table 7). Starvation tolerance of flies reared on 0.5% RS supplemented food since 1st instar stage remains comparable to those reared on regular food (Table 7).

Starvation tolerance of flies reared on food supplemented with honey and Ghee or only Ghee or only honey or only the triturated Amalaki powder was similar to that of normally fed larvae (data not presented).

Feeding on 0.5% AR or RS supplemented food during larval period enhances levels of heterogeneous nuclear ribonucleoproteins (hnRNPs)

The improved thermotolerance of formulation-fed larvae and flies may be a consequence of cellular stress caused by the dietary AR or RS since it is known that a milder stress significantly improves tolerance to a subsequent more severe stress [21,22]. One of the very sensitive indicators of cell stress in flies is the

Table 3. Feeding on 0.5% AR or RS supplemented food during larval period increases dimensions of salivary glands of late 3rd instar larvae.

Mean (±SE) dimensions (in μm)	Control	AR 0.5%	RS 0.5%
SG Length	1306.82±16.57 (N = 123)	1783.19±25.26* (N = 105)	1600.47±28.45* (N = 83)
SG Width	194.57±2.31 (N = 123)	268.98±4.16* (N = 105)	220.05±4.12* (N = 83)
Polytene nucleus diameter	46.41±0.48 (N = 100)	60.34±1.18* (N = 100)	53.79±2.68* (N = 100)

*P<0.001) when compared with corresponding control.
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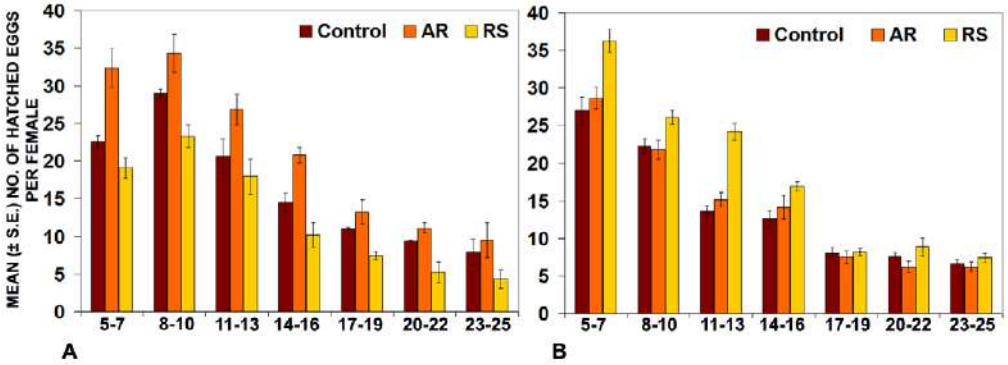

Figure 4. Fecundity of wild type flies (expressed as mean (±S.E.) numbers of hatched eggs on Y-axis) per female on given days (X-axis) reared on regular or 0.5% AR- or RS- supplemented food through larval and adult stages (A) or fed on 0.5% AR or RS only during the adult stage (B). For convenience of presentation, each bar represents the mean (± S.E.) number of eggs per fly that hatched during a window of three consecutive days.
doi:10.1371/journal.pone.0037113.g004

dramatic change in the nuclear distribution of the hnRNP family of proteins, which rapidly move away following cell stress from their normal locations on active chromatin and nucleoplasmic storage sites to the chromosomal site of the *93D* or *hsrω* gene [23,24,25]. Members of the conserved hnRNP family of proteins have important roles in gene expression, RNA processing and transport in eukaryotic cells and thus affect cell physiology in many different ways [26]. Therefore, we examined the sub-cellular distribution and levels of three different hnRNPs, viz., Hrp36 (also known as Hrb87F), Hrp38 (Hrb98DE) and Hrp40 (Squid) in SG of larvae that were fed on normal or AR- or RS-supplemented food.

In situ distribution of Hrp36 in intact SG cells in *Hrb87F-GFP* larvae reared under different feeding regimens was examined by confocal microscopy. The Hrp36 in SG was present in nucleoplasm and on specific chromatin regions in all the three cases (Fig. 5A–L). Interestingly, the SG nuclei from either of the formulation-fed larvae showed much higher nuclear GFP fluorescence, especially at certain chromatin regions than in corresponding tissues from larvae reared on regular food. Although the very intense signal from certain chromatin regions in nuclei from formulation-fed larvae appeared reminiscent of the strong presence of hnRNPs at the 93D site in heat shocked cells [27], a significant difference from the stressed cells is the continued presence of Hrp36 on other chromosome regions and in nucleoplasm (Fig. 5A–F). Parallel observations on polytene chromosome spreads revealed that the chromosome sites showing very intense Hrp36-GFP fluorescence in intact glands actually correspond to the early ecdysone puffs at 74DE and 75B on 3L, rather than the 93D puff (see below and Fig. 5L). Measurement of total nuclear GFP fluorescence in the distal salivary gland nuclei clearly showed that the levels of nuclear Hrp36 are significantly increased in AR- or RS- fed larvae (Fig. 5G). Comparison of the levels of Hrp36 in total proteins from *Oregon* R^+ larvae by western-blotting using the Hrp36-specific P11 antibody [28], confirmed that the relative levels of this proteins were significantly higher in the body of formulation-fed larvae (Fig. 5H).

In order to identify the specific chromosome regions which show very high presence of Hrp36 in SG nuclei from formulation fed larvae, we immunostained polytene chromosome spreads from 110 hr old larvae, that were reared on normal, AR- or RS-supplemented food, with the Hrp36-specific P11 antibody [28]. Our observations showed that unlike the very high level of the hnRNPs seen at the stress induced 93D puff in conventionally stressed cells with concomitant absence from almost all other chromosomal sites [27,28], the formulation feeding did not qualitatively alter the chromosomal locations of hnRNPs. However, the amount of these proteins on different puff and other sites

Table 4. Differential effects of AR or RS feeding during larval or adult stage on fecundity.

Feeding on formulation	Food	Mean (±S.E.) total no. of eggs per female in 25 days			N
		Hatched	Unhatched	Total	
A: Larval and adult stages	Control	115.1±0.9	40.8±0.7	155.9±1.0	2 replicates, each with 90 females and 60 males
	AR 0.5%	148.2±1.8*	39.2±0.8**	188.6±1.5*	
	RS 0.5%	87.5±1.4*	30.2±0.8*	117.7±1.1*	
B: Only adult stage	Control	97.7±0.9	42.2±0.7	139.8±1.3	2 replicates, each with 90 females and 60 males
	AR 0.5%	100.0±1.1**	43.7±1.1**	143.4±3.5**	
	RS 0.5%	127.9±0.9*	40.0±0.9**	167.4±1.1*	

*P<0,001 when compared with corresponding control.
**P>0.05 when compared with corresponding control.
doi:10.1371/journal.pone.0037113.t004

Table 5. AR or RS fed 100 hr old larvae show significantly improved survival following severe thermal stress.

Heat shock	Time (min)	% surviving 72 h after HS			% eclosion after heat shock			N
		CON	AR 0.5%	RS 0.5%	CON	AR 0.5%	RS 0.5%	
37°C	**60**	97.0±1.4	96.6±0.9**	97.5±1.1**	95.6±0.9	96.0±1.4**	96.5±0.8**	200
	90	67.2±0.9	84.7±1.5*	93.3±1.0*	25.1±1.8	47.2±1.7*	50.0±1.2*	200
	120	41.0±1.2	79.2*±2.1	87.8±3.1*	20. 6±2.0	43.7±3.6*	48.7±2.4*	200
38°C	**60**	7.5±1.3	30.5±2.3*	38.5*±1.1	4.34±0.3	6.7±0.5*	8.7±0.2*	200
	90	5.3±3.6	36.0±5.3*	61.3±4.8*	2.0±0.5	5.0±0.3*	6.0±0.5*	200
39°C	**30**	0	6.3±4.1*	17.0±5. 7*	No eclosion			200

*Values significantly different (P<0.001) from the corresponding control values.
**Values not significantly different from corresponding control values.
doi:10.1371/journal.pone.0037113.t005

was significantly higher in SG from larvae fed on 0.5% AR or RS-supplemented food than in those from larvae reared on regular food (Fig. 5I–K). A comparison of the site-wise distribution of Hrp36 on the left arm of chromosome 3 (3L) in polytene spreads from differently fed larvae (Fig. 5L) clearly revealed qualitative similarity in distribution of the Hrp36 on various developmental puffs. Significantly, however, the levels of Hrp36 present at each of these puff sites were much higher on chromosomes from formulation-fed larvae than in larvae reared in parallel on regular food (control). The stage of larvae used for these studies corresponds to puff stage 10 described by [29], which coincides with the activation of early ecdysone-responsive puffs like the 74EF and 75B in SG (Fig. 5H–J, L). Accordingly, and in agreement with an earlier report [30], a high level of Hrp36 was present at these puff sites. It is very interesting that there is a substantially greater presence of Hrp36 at the 74EF and 75B puffs in the formulation-fed larvae (Fig. 5I–K, L). Measurement of DAPI fluorescence (DNA content) and the P11 immuno-fluorescence (Hrp36) of the entire 3L showed (Fig. 5M), in agreement with the data on total nuclear DNA content presented in Table 2, that the amounts of DNA as well as Hrp36 were significantly greater in 3L from the formulation-fed larvae. Interestingly, a comparison of the amount of Hrp36 on per unit DNA on 3L (relative intensity shown in inset in Fig. 5M) revealed that compared to the increase in DNA content, the increase in Hrp36 on different chromosome regions is significantly greater, which shows that the increase in Hrp36 cannot be explained only by the increase in DNA content in formulation-fed larval polytene chromosomes. There is a net increase in the chromosome associated Hrp36 in formulation-fed larvae. Analysis of the distribution of Hrp36 on the right arm of chromosome 3 (3R) in these squash preparations showed results similar to those for the 3L (details not shown).

To determine the cellular levels of the Hrp40 (Squid) protein, we used the *Squid-GFP* protein-trap allele so that the sub-cellular distribution of the Squid protein can be monitored through the GFP fluorescence. Confocal microscopic examination of GFP fluorescence in SG from late 3rd instar *Squid-GFP* larvae reared since hatching on regular (Control) or 0.5% AR or RS supplemented food revealed significantly increased levels of Squid in the formulation-fed larvae, with several puff regions showing massive accumulation of Squid-GFP (Fig. 6A–F) in a manner comparable to that noted above for the Hrb87F protein (Fig. 5). Comparison of mean GFP-fluorescence intensity of distal polytene nuclei in SG from differently fed larvae (Fig. 6G) confirmed that the nuclear levels of Squid protein are indeed enhanced in larval tissues from the formulation fed larvae. Detection of Squid-GFP in total larval proteins by western blotting (Fig. 6H) also confirmed this protein's elevated levels in formulation-fed larvae.

The Hrb98DE (Hrp38) also shows a comparable increase in its levels in formulation-fed larval tissues (details not presented). Examination of Hrp36, Hrp38 and Hrp40 proteins in Malpighian tubules too showed a comparable increase in levels of these proteins in larvae that were fed on 0.5% AR or RS-supplemented food (not shown).

Table 6. *Oregon* R^+ flies reared on AR or RS supplemented food show significantly improved thermotolerance in the knockdown assay.

Heat Shock	Time (min)	% flies knocked down			% flies surviving after 24 hr			N
		CON	AR 0.5%	RS 0.5%	CON	AR 0.5%	RS 0.5%	
38°C	**15**	0	0	0	68.6±1.2	87.4±1.6*	88.4±2.6*	300
	30	0	0	0				
	45	56.7±1.92	27.9±0.8*	18.5±1.3*				
	60	76.0±2.7	56.0±0.6*	42.6±1.7*				
39°C	**15**	99.0±1.0	90.2±1. 6*	87.0±1.3*	27.0±0.9	43.0±1.7*	56.1±1.4*	200
	30	100	100**	100**				

*Values significantly different (P<0.001) from the corresponding control values.
**Values not significantly different from corresponding control values).
doi:10.1371/journal.pone.0037113.t006

Table 7. Flies reared on AR-supplemented food show greater tolerance to starvation.

LT_{50} (in hours)		
CON	**0.5% AR**	**0.5% RS**
56.8±2.2	70.5±2.0*	54.3±2.5**

*Values significantly different ($P<0.001$) from the corresponding control values.
**Values not significantly different ($P>0.05$) from the corresponding control values.
doi:10.1371/journal.pone.0037113.t007

A summary comparison of the different effects of AR or RS supplemented food on the parameters examined in this study is presented in Table 8.

Discussion

We tested two different categories of formulations, one being a *Rasayana* (*Amalaki Rasayana*) and the other a *Bhasma* (*Rasa-Sindoor*), for their effects on longevity, development, fecundity and stress-tolerance of *Drosophila melanogaster*. Both the formulations were prepared essentially as classically described with some modifications to ensure reproducibility and proper hygienic conditions. It may be noted that the available tools and preparatory methods have inevitably changed with the passage of centuries since the classical texts were written. This has also involved local variations in preparatory procedures and nomenclature of the formulations. The traditionally described elaborate practice of "*sandhanam*" which requires the AR mixture to be preserved in Ghee smeared vessel under ashes for one year and addition of other ingredients like sugar, *Pippali* etc were avoided in our method of preparation of AR to ensure reproducibility and hygienic conditions as required in current practices. Nevertheless, these two formulations were prepared essentially following the traditional practices. HPLC analysis (data not presented) confirmed that the different batches of the formulations were comparable in their constitution. It may also be noted that these batches of AR and RS were prepared solely for the coordinated research work directed to investigate and understand the scientific basis of Ayurveda [4]. The same AR has been used in the study of Swain et al [40] on effect of AR feeding on genomic integrity in rat brain.

A comparison of the observed effects (see Table 8) reveals that the two formulations affected life-history and other physiological traits in distinct ways. While the size of larval salivary glands, levels of hnRNPs in larval tissues and thermotolerance of larvae/adult flies improved following rearing on food supplemented with either of the two formulations, the median life span and starvation resistance improved only with AR-feeding. Interestingly, feeding on AR or RS improved fecundity, but the developmental stage at which the formulation was effective varied. Thus while larval and adult feeding with AR increased the fecundity, feeding larvae and adults with RS had the opposite effect. On the other hand, feeding only during the adult stage with AR supplement had no effect on fecundity but the RS-feeding during the same stage improved it. Such specific but different effects of the two Ayurvedic drugs tested in this study show that the observed effects are consequences of specific changes in the organism's metabolic activities following the dietary AR or RS supplement. Interestingly, the observed effects seem to generally agree with the reported usages of the two formulations in human [13].

Fruits of Amla or Indian gooseberry, the principal component of AR, are known to be very rich in anti-oxidants as revealed in several studies on different extracts of these fruits [31,32,33,34,35,36]. However, effects of complete AR on life history traits have rarely been examined. Two recent reports have claimed that dietary provision of a "modified" AR dramatically enhances longevity of flies [37,38]. Compared to the near doubling of life span claimed in these two publications, the increase in median life span observed in our study following 0.5% AR feeding is modest. It is, however, to be noted that these two reports [37,38] are based on rather small sizes of samples of control and formulation-fed flies; moreover, these studies do not provide the required details of the actual formulation used and the quantity provided to flies. Therefore, results claimed in these studies remain uncertain [39]. It is possible that some variations in the dietary delivery of AR may show more pronounced effects on longevity than observed in the present study. The significant point, however, is that feeding on 0.5% AR supplemented food enhanced the median life span of experimental flies. As noted earlier, one of the major usages of AR in human is to improve longevity and youthfulness. A recent study [40] has shown that the genomic integrity in ageing mice provided with AR is significantly better than control sibs.

It is interesting that higher concentrations of RS marginally reduced longevity of flies while 0.5% or lower concentrations of RS did not affect the median life span. In this context it is significant to note that although RS has varied applications in Ayurvedic treatment procedures [12], it is not claimed to improve longevity in human. RS is essentially mercuric sulphide with some organic components. Mercury salts are generally reported to be highly toxic and carcinogenic [13,41,42,43]. However, very few studies have examined the *in vivo* biological effects of *Rasa-Sindoor*. The mineral materia medica of Ayurveda claims mercury to have the power to assimilate (*rasanate*) all other metals [2]. A significant finding of our study is that feeding on RS-supplemented food did not elicit any evidence of heavy metal toxicity in larvae or flies since there was neither any evidence of lethality following RS-feeding nor of any developmental defects in the emerging flies. Supplementing food with 1% or higher concentration of RS resulted in a slightly reduced life span, but no developmental abnormalities or phenotypic consequences were noticed. Thus it appears that the processing of mercury in the specified Ayurvedic manner, which involves a systematic sublimation and grinding (see Materials and Methods), seems to convert the mercury sulphide to nano particles [14]. It may be noted that cinnabar, which too contains mercury sulphide, is widely used in traditional Chinese medicines [44]. Another recent study has also shown that the human gut flora does not convert mercury sulphide present in cinnabar into toxic derivates like methylmercury [45].

The observed differences in effects of the two formulations on fecundity are interesting. It is known that good larval nutrition/growth is associated with improved fecundity [46,47]. The anabolic effects of AR are reflected in faster larval development and increased size and polyteny levels in larval salivary glands. The overall elevated levels of hnRNPs, including those bound to active chromosome regions, also appear to reflect more robust gene expression patterns in the AR-fed larvae. All these may prepare the larval ovaries to produce more eggs during the adult stage. On the other hand, providing AR only during the adult stage fails to alter the state of gonads as established during the larval period. In this context, it is intriguing that RS had adverse effect on fecundity when provided during larval period but significantly improved fecundity when provided only during the adult stage. The physiological bases for such contrasting effects on fecundity need further cellular and molecular studies but it is interesting to note that in Ayurvedic practice, RS is not

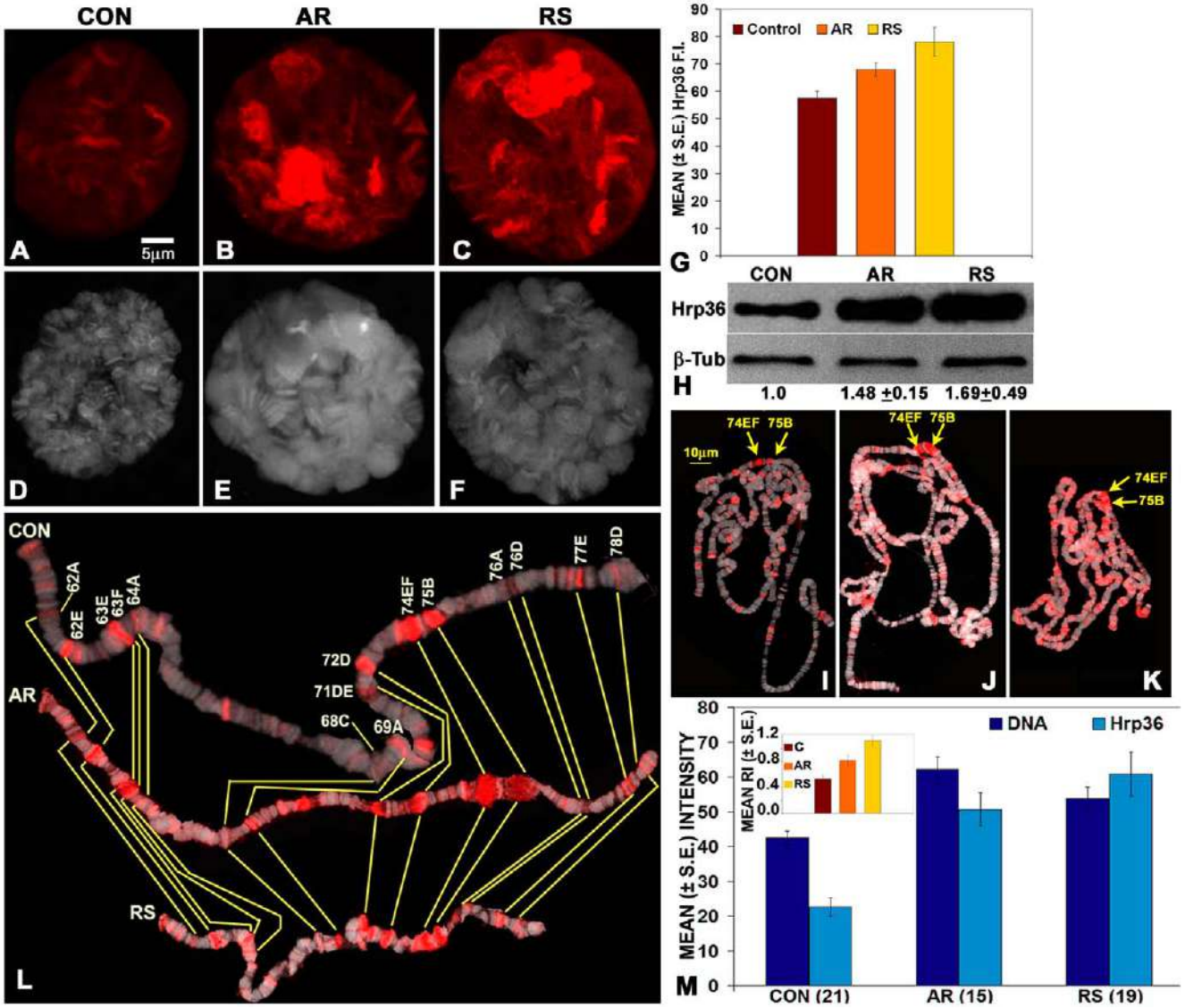

Figure 5. Feeding larvae on AR or RS supplemented food enhances the levels of Hrp36 in larval tissues. A–F Confocal projections show GFP (red, **A–C**) and DAPI (white, **D–F**) fluorescence in SG polytene nuclei from 110 hr old *HRB87F-GFP/Hrb87F-GFP* larvae. Histograms in **G** show that the mean levels of nuclear Hrp36 (GFP fluorescence) in polytene nuclei of SG (N = 100 for each sample) are significantly greater in AR or RS-fed larvae. Western blot in **H** shows the relative amounts of Hrp36 (detected by the P11 antibody) in total proteins from differently fed (control, AR and RS lanes) late 3rd instar larvae; the relative value of Hrp36 (ratio of the signal for Hrp36 and β-tubulin in a given blot) in normally fed (1st lane) larvae in each replicate was taken as 1. The normalized values of the mean (±S.E., N = 3) relative levels of Hrp36 in total larval proteins are noted below the AR and RS lanes. **I–K** are confocal projections of squashed SG polytene chromosomes from 110 hr old control (**I**) or AR- (**J**) or RS- (**K**) fed larvae immunostained with P11 antibody to localize Hrp36 (red fluorescence) and counterstained with DAPI (white fluorescence); the early ecdysone puffs at 75B and 74EF are marked in each case. **L** shows representative cut outs of the left arm of chromosome 3 (3L) from squash preparations as in **I–K** from salivary glands of 110 hr old larvae fed on regular (CON) or AR or RS supplemented food; some of the known puff sites [29] are marked on the top panel and the corresponding sites in the other two cut outs (middle and lower panels) are indicated by connecting yellow lines (the chromosome arm in these images was cut and re-positioned, as required, to provide a relatively straight orientation). Histograms in **M** show the mean fluorescence intensity for DNA and Hrp36 on the entire 3L in differently fed (noted on X-axis) larval salivary gland squash preparations as in **I–K**; inset shows the relative levels of Hrp36 in relation to the nuclear DNA content in the three sets of nuclei; the numbers of 3L examined for each sample is mentioned in parenthesis on the X-axis. The scale bar in **A** (5 μm) applies to **A–F**, while that in **I** (10 μm) applies to **I–K**.
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recommended for growing children but is indicated, among other things, for genital disorders and rejuvenation in adult subjects [13].

The significantly greater thermotolerance shown by larvae and adult flies that were reared on AR or RS-supplemented food is remarkable. It is known that a prior exposure to a mild stress makes the cells/organism better thermo-protected [21,22,48,49]. Therefore, we examined the possibility that these two formulations, especially the mercury based RS, may cause a mild/chronic stress which may result in improved tolerance to a more severe subsequent stress. Several studies in *Drosophila* have shown that heat shock brings about a rapid and dramatic change in the distribution of nucleoplasmic and chromosome associated hnRNPs so that soon after heat shock, almost all of them get aggregated at the 93D or *hsrω* locus [23,27,28,50]. Our present

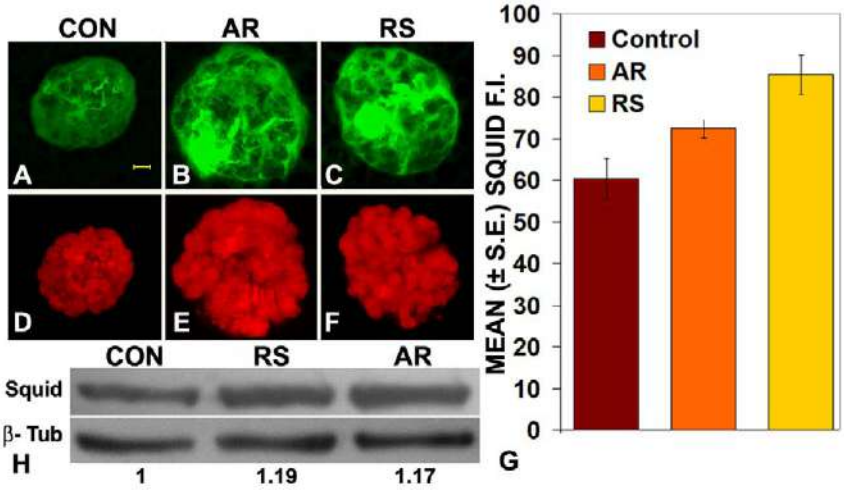

Figure 6. Levels of Hrp40 (Squid) are increased in formulation fed *Squid-GFP* larvae. Confocal projection images of GFP fluorescence (green, **A**–**C** and **G**–**I**) in polytene nuclei from late third instar larval salivary glands (**A**–**C**); DAPI-stained chromatin (**D**–**F**) is seen in red. Histograms in **G** show mean intensity of Squid-GFP fluorescence in SG polytene nuclei (N = 100 for each sample) from differently fed larvae. Western blot in **H** shows relative levels of Squid-GFP protein (Squid) and β-tubulin (β-tub) in whole proteins from differently fed (CON, RS or AR) larvae challenged with GFP antibody: the values below each lane indicate the relative levels (ratio of Squid:β-tubulin in the blot with the value for larvae reared on regular food (CON) taken as 1.0) of Squid-GFP protein. The scale bar in **A** represents 5 μm and applies to **A**–**F**.
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observation on the *in situ* distribution of three different hnRNPs, viz., Hrp36, Hrp38 and Hrp40, do not provide any evidence for the AR- or RS-fed larval cells being under stress since in all cases, the nuclear distribution of the three hnRNPs was qualitatively similar to that seen in normally fed larval cells. Heat shock does not increase the quantity of hnRNPs in cells [25] but the formulation-fed larval cells displayed significantly enhanced levels of these proteins, which as mentioned above, seems to reflect more robust developmental gene expression. Therefore, the improved thermotolerance of the formulation-fed larvae/flies is apparently not due to a mild stress inflicted by the formulations. The abundance of anti-oxidants in Indian gooseberry fruit [31,32,33,34,35,36] may partly explain the high thermotolerance of larvae/flies reared on AR-supplemented food but so far there is no evidence that RS also has similar properties. Studies are in progress to see if the induction of different heat shock proteins is modulated by the dietary AR or RS.

The improved starvation-tolerance of AR-fed larvae/flies agrees with earlier reported [46,47] correlation between better larval physiology and longevity of adults with starvation tolerance. In this context it is significant that while RS-feeding also improved the larval physiology, in terms of developmental time, salivary gland size and enhanced levels of hnRNPs, it had no effect on fly longevity and starvation tolerance.

The hnRNPs are a highly conserved group of primarily nuclear proteins that dynamically bind with the RNA-pol II transcribed nascent transcripts but do not stably associate with other RNA-protein complexes [51]. Mammalian cells have about 30 different hnRNPs, which are grouped into distinct sub-families; *Drosophila*, on the other hand, seems to have less diverse repertoire of these proteins [26]. The hnRNPs are involved, in conjunction with some other classes of RNA-binding proteins, in the synthesis and subsequent processing (splicing, capping and polyadenylation) of the pre-mRNAs, as well as in transport of the final mRNA to their destination [26]. Several of these functions are carried out by more than one hnRNP members in a combinatorial mode which results in a "RNA splicing code" [52]. In the present study we found that nuclear levels of the three examined members of hnRNP A/B family (Hrp36, Hrp38 and Hrp40) were significantly enhanced in AR or RS-fed larvae. It has been reported earlier that over- or

Table 8. Comparison of the observed effects of dietary supplement of AR or RS on different parameters in *Drosophila melanogaster*.

Parameter	Rearing on 0.5% AR supplemented food	Rearing on 0.5% RS supplemented food
Median life span	Increased	No effect
Developmental time	Shorter	Shorter
Larval salivary gland size and polytene level	Larger glands with additional cycle/s of endo-replication	Larger glands with additional cycle/s of endo-replication
Fecundity	Improved with larval feeding but no effect of adult feeding	Decreased with larval feeding but improved with adult feeding
Thermo-tolerance	Improved	Better than with AR-supplement
Starvation tolerance	Improved	No effect
Nuclear hnRNP levels in larvae	Increased, including on puffs	Greater increase than with AR-supplement

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under-expression of one hnRNP can adversely affect certain cell activities, although redundancy in functions of some of the hnRNPs acts as a compensating buffer system [26,52,53,54,55]. It is significant that either of the two Ayurvedic formulations enhanced the levels of all the three hnRNPs comparably so that activities that are dependent upon combinations of specific hnRNPs [9,52] are not adversely affected because of alterations in their relative abundance. We believe that elevation in levels of the hnRNPs in formulation-fed larvae provides for more robust developmental gene expression and RNA-processing etc. so that the overall physiology of the organism is better, as reflected in enhanced polyteny levels, slightly faster development and improved stress-tolerance. In this context, it is interesting to note that Hrp36-null mutants display similar larval or pupal development time irrespective of their rearing on normal or AR or RS-supplemented food (V. Dwivedi and S. C. Lakhotia, unpublished). This suggests that the enhanced levels of hnRNPs in the formulation-fed larvae are indeed involved in their faster growth.

Many earlier studies using *Drosophila melanogaster* have identified quantitative traits like longevity, development time, body/organ size, fecundity and starvation tolerance as indicators of individual and population fitness and these parameters have been utilized to study evolution in laboratory populations under selection for one or the other of these parameters to determine the tradeoffs between these measures of fitness [47,56]. Thermotolerance is also affected in a complex manner by quantitative trait loci (QTL)-linked genes [57]. It is obvious that the relationship between the different life history parameters is complex and therefore, specific experimental conditions may produce divergent results [34]. Although in our studies we did not examine any long-term population effects, the qualitative and quantitative differences in responses to the two Ayurvedic formulations within the same generation agree with the existence of complex regulatory networks that influence the diverse life history parameters. Notwithstanding the complexity of trade-offs between the different life history traits like longevity, fecundity and stress-tolerance, it is clear that these life history traits, which have bearing on individual's fitness, are affected in a manner largely comparable to that claimed in human.

A significant finding of the present study is that unlike the complete AR formulation, its individual ingredients, viz., Ghee or honey, when supplemented separately did not affect development time or starvation- or thermo-tolerance. Supplementing only the triturated Amalaki powder had only a marginal effect on development time but none on starvation- or thermo-tolerance. It is to be noted that the honey and Ghee are added to the triturated Amalaki powder as *Anupana*. Our results agree with the Ayurvedic principle that *Anupana* as a medium of administration improves acceptability and palatability and helps in action of the main drug.

In summary, our present results establish that, as for many other studies of relevance to human health and drug discovery [5,6,7,8,9,10], *Drosophila melanogaster* is a very good model for understanding the scientific bases of actions of different traditional formulations, like those of Ayurveda. Subsequent studies would be directed to understand the molecular and cellular bases of the observed effects on life-history and other traits. The very rich repertoire of mutants and other genetic analysis systems available in fly genetics would, in future studies, enable in-depth cellular and molecular analyses of the effect/s of the given Ayurvedic formulation. The presently available and emerging strategies in the fly model would enable specific studies on effect/s of the given formulation on general physiology, anti-oxidant and DNA repair status, stress-tolerance, protein-quality control, immune response, developmental plasticity etc. We expect that exploitation of the fly system with unbiased scientific rigour would help us understand the mechanistic details of actions of the different traditional medicinal systems and thereby improve their management and applications.

Materials and Methods

Preparation of formulations

The two formulations, viz., *Amalaki Rasayana* and *Rasa-Sindoor* were prepared at the Arya Vaidya Sala, Kottakkal, following the traditional methods as described below.

Amalaki Rasayana. Preparation of AR requires four steps. Dried gooseberry fruit was pulverised using Tyco Pulveriser (step 1) and parallely, gooseberry juice was prepared from fresh fruits with a juice extractor (step 2). In the 3rd step (trituration), products of the steps 1 and 2 were blended in 1:1 ratio and dried for about 24 hours at 55°C under low pressure (700 mm) in a Vacuum Tray Drier. The dry mass thus formed was pulverised and the steps 2 and 3 were repeated another 20 times (trituration for 21 times). Quantity of the additional gooseberry juice added at each trituration step remained the same as in the 1st trituration. These processing steps take about 2 to 3 months to complete. The AR was finally prepared at step 4 by blending the dry Amalaki powder (obtained after completion of 21 cycles of trituration) with commercially available honey (M/s. Hexa Apiarium Pvt. Ltd., Kannur, Kerala, or M/s. Galaxy Honey, Kottakkal, Kerala) and Ghee ("Milma" from Malabar Milk Marketing Federation, Govt. of Kerala, Kozhikode, Kerala or "Nandini" from Karnataka Milk Marketing Federation, Govt. of Karnataka, Mysore, Karnataka) in a 1:2:0.5 ratio resulting in a thick sticky paste.

Amla fruit and powder were authenticated by Centre for Medicinal Plants Research, Kottakkal and the Quality Assurance (QA) department of Arya Vaidya Sala (AVS), both of which are approved by Govt. of India for testing and issuing quality control certificates for Ayurvedic raw materials and finished products. Macroscopic/organoleptic (shape and taste of fruits) and microscopic parameters (nature of pericarp, mesocarp and vascular bundles of fruits) were compared with the standard values specified in Ayurvedic Pharmacopoeia of India (API). In addition, in-house developed HPTLC profiles of (a) raw Amalaki fruits, (b) Amalaki powder, (c) in-process samples at the conclusion of 1st, 10th, 15th and 21st steps of trituration and (d) the finished formulation were also compared with the standard HPTLC profiles of gallic acid and ellagic acid. Quality control assays on raw-materials and HPTLC profiles provided evidence for consistency of different batches used in this study. The final formulation was packed in sealed 45 g HDPE containers and stored under ambient conditions. HPTLC analysis indicated the shelf-life of finished product to be 12 months and, accordingly, each batch was used within this period.

Rasa-Sindoor. Preparation of RS also involves four steps. In step 1, sulphur was pre-processed in a three stage procedure in which sulphur and Ghee (1:1) were mixed and melted over fire. The molten mixture was mixed with milk (twice the quantity of sulphur). When cooled, the mix was drained and the residue repeatedly rinsed in water. The washed sulphur was subjected to the above three stages twice again. The pre-processed sulphur was dried by mild heating. Step 2 involved the mixing of pre-processed sulphur, mercury and *Aloe-vera* juice [12] in 1:1:0.7 ratio and grinding of the mixture in an electric wet grinder (85 RPM) for about 240 hours until the mixture became a thin paste, which is called *kajjali*. In step 3, the *kajjali* was spread on a wooden plank and kept for drying under shade. After about four days' time, the

dry mass was pulverised to a fine powder in domestic Mixer. In step 4, this powder was taken in a porcelain bowl whose mouth was covered with another inverted porcelain bowl of the same size. The junction of the two bowls was sealed with cloth smeared with wet clay. The twin bowls were thus made into a sealed sphere by complete coverage with clay smeared cloth. After drying of the clay, the sphere was charged into an open-hearth furnace and heated up to 620°C for 48 hours. Thereafter the bowl sphere was allowed to cool and the clay and clothing cover were removed and the two bowls were slowly separated. The *Rasa-Sindoor*, which is the sublimed product, sticks to the inner surface of the upper bowl. This sublimed deposit was carefully scraped out as flakes and further pulverised by grinding in a mortar and pestle. The final product is a fine dust of shining brick red colour. Appropriate chemical assays were carried out to check quality control of the RS preparations.

The quality control certificate for RS by the QA department of AVS was based on the following Pharmacopoeial parameters: (a) test for Hg and S, (b) assay of Hg and S, (c) acid insoluble ash, (d) water soluble ash and (e) loss on ignition at 500°C for 30 min. Additionally, the typical Ayurvedic parameters like *Nishchandrika* (lustreless), *Rekhapurnatwam* (fineness), *Varitaritwam* (water floating), etc were also applied. In conformity with the stoichiometry of HgS, mercury in the final preparation was 84.8% w/w while sulphur was 15.6% w/w. Ayurvedic Pharmacopoeia of India specifies the shelf life of RS as infinite since HgS (Cinnabar) is known to be highly stable. For the present set of studies, the final RS powder was packed and stored in 100 g sealed containers and used within a period of 12 months. All the test and assay reports for AR and RS are available with AVS, Kottakkal.

Fly rearing and food preparation

Oregon R^+ (wild type) and other fly stocks were maintained under uncrowded condition at 24°C±1°C. All studies on the effects of the dietary supplements on life-history traits were carried out with the *Oregon* R^+ flies while for some of the experiments to assess the status of heterogeneous RNA-binding proteins (hnRNP), the Hrb87F-GFP, Hrb98DE-GFP and Squid-GFP protein-trap lines [58,59] were used. In these protein-trap transgenic lines, the endogenous Hrb87F (Hrp36) or Hrb98DE (Hrp38) or the Squid (Hrp40) protein, respectively, gets tagged with GFP so that the sub-cellular distribution level of the given protein can be monitored through the GFP fluorescence. All the three GFP-tagged proteins function more or less like their original native proteins.

Flies were fed on standard fly food containing agar, maize powder, yeast and sugar. The AR or RS formulation was added at the desired concentration (weight/volume, see Results) to freshly prepared fly food while it was still in fluid state and thoroughly mixed with a glass rod. The mixed food was poured into containers (bottles, vials or Petri plates, as required) and allowed to cool and solidify before use. In some experiments, the fly food was supplemented with desired concentration of either a mixture of honey and Ghee (in 2:0.05 ratio) or honey or Ghee or the Amalaki powder (as obtained after 21 cycles of trituration) alone.

In all formulation feeding experiments, eggs were collected from fly stocks that had always been reared on regular food. For each experiment, the regular and the formulation supplemented foods were prepared from the same batch; likewise all larvae/adults for a given experiment were derived from a common pool of eggs of the desired genotype and reared in parallel on the regular or the supplemented food. The characteristic dark brownish red colour of RS was distinctly visible in the guts of larvae and adult flies. The AR-fed larvae, on the other hand, showed brownish-black colour in their gut. Thus the formulation mixed food was indeed taken up by the larvae/flies.

Viability assay

Viability assay was carried out with freshly eclosed wild type flies reared, since the 1st instar larval stage, on regular (control) or differently supplemented food at 23±1°C. Eight replicates were carried out with 25 flies per replicate. Flies were transferred, without anesthetization, to fresh vials after every 2–3 days and the number of surviving flies was recorded on daily basis until 60 days after eclosion. The median life span for each experimental condition was calculated as the day till which 50% of the original number of flies (N = 200 from 8 replicates for each feeding regime) survived in the vial.

Developmental assay

For developmental assay, eggs were collected at 1 hour interval and the freshly hatched larvae were gently transferred to food vials containing either 0.5% AR or 0.5% RS supplemented food or to vials with the regular food (control) and reared at 23±1°C. After 4 days of development, the time at which individual larvae pupated and subsequently emerged as flies was monitored at 2 hr intervals.

Sizes of salivary glands and polytene nuclei in formulation-fed late third instar larvae

In order to determine the sizes of larval organs or their cells in wild type larvae reared at 24±1°C on regular or AR or RS supplemented food, salivary glands (SG) were selected because these endoreplicated tissues have a defined morphology, fixed number of cells and follow a highly regulated pattern of endo-replication cycles [16,17,18]. In order to obtain SG of same age, larvae that had just everted their anterior pair of spiracles were selected and immediately dissected in Poels' salt solution (PSS) [60] fixed in 3.5% paraformaldehyde in phosphate buffered saline (PBS, 13 mM NaCl, 0.7 mM Na_2HPO_4, 0.3 mM NaH_2PO_4, pH 7.0), washed in PBS and stained with DAPI (4′,6-diamidino-2-phenylindole dihydrochloride, 0.5 µg/ml). The length and width of the each gland was measured using the Overlay and Histo options in the Zeiss LSM Meta 510 software. The diameter and DAPI fluorescence of 5–7 distal most polytene nuclei in each gland were also measured to estimate mean size and DNA content of polytene nuclei.

Fecundity

For examining the effect of formulation-feeding on fecundity of flies, two sets of feeding regimen were followed. In one set, first instar larvae were transferred to regular or formulation-supplemented food to develop at 23±1°C. Freshly eclosed males and virgin females from these cultures were separated and transferred to fresh regular or respective 0.5% formulation supplemented food for three days, followed by mixing on the fourth day; the mixed flies were transferred to population cages with regular or formulation supplemented food dishes, respectively, till the end of experiment. In the 2nd set, effect of feeding on formulation supplemented food only during the adult stage was examined. Freshly eclosed male and virgin female flies from larvae reared on regular food were separated and kept for three days in food vials containing the formulation supplemented food before mixing them for on the 4th day and transfer to population cages carrying the respective food plates. Food plates were replaced each day with fresh regular or formulation supplemented food plates, respectively. Total numbers of eggs in each of the plates and those that hatched or did not hatch were counted daily. Number of hatched

eggs per female fly was calculated by dividing the total number of hatched eggs with the total number of female flies alive during the given day.

Thermotolerance assay

Freshly hatched *Oregon* R^+ larvae were fed either with regular or 0.5% AR or 0.5% RS supplemented food and reared at 24±1°C. 100 hr old third instar larvae were kept in microfuge tubes lined with moist filter paper and subjected to heat shock (HS) at different temperatures (37°C, 38°C or 39°C) for 30, 60, 90 or 120 min. Following HS, the larvae were gently transferred to vials containing regular *Drosophila* food and allowed to develop further. The numbers of individuals dying at subsequent stages of development were counted till the emergence of flies. In another set, three day old *Oregon* R^+ flies fed through larval stages either on 0.5% formulation supplemented or regular food were lightly etherised and kept in plastic vials (25 flies per vial). The flies were allowed to recover from anaesthesia for at least 4 hr prior to the HS at 38°C for 60 min. or at 39°C for 30 min. The number of flies which fell down during HS was monitored every 15 min. Subsequently, the flies were transferred to food vials and the numbers of flies surviving after 24 hr were recorded. Eight replicates were carried out for each feeding condition.

Starvation resistance assay

Freshly hatched *Oregon* R^+ larvae were reared on 0.5% AR or 0.5% RS supplemented or regular (control) food at 24±1°C. Three day old flies (formulation fed and control) were starved in empty bottles containing filter paper strips soaked with water [61]. Mortality of starved flies was recorded every 12 hours. A total of 500 flies (5 replicates of 100 flies each) were tested for each group.

Distribution and quantification of different hnRNPs (Hrp36, Hrp38 and Hrp40) in larval tissues

Levels of the Hrp36, a HnRNP-A1 homolog in *Drosophila* [62] in intact SG or in squashed SG polytene chromosomes from 110 hr old larvae reared at 24±1°C on different feeding regimes were assessed either by measuring the GFP fluorescence in individual nuclei in the *HRB87F-GFP/HRB87F-GFP* [59] larval SG or by immunostaining of polytene chromosome squashes from wild type larvae with the Hrp36-specific P11 antibody 1:20 dilution, [28] as described earlier [25,27]. Western-blotting was carried out with total body proteins from differently fed larvae and separated by polyacrylamide gel electrophoresis to compare the levels of Hrp36 using the P11 antibody (1:200 dilution) as described earlier [27]. The levels of two other hnRNPs, viz., Hrp40 (hnRNP A1 or Squid) and Hrp38 (Hrb98DE) were examined using the respective protein-trap alleles (*Squid-GFP* or *Hrb98DE-GFP*) by measuring the GFP fluorescence in SG nuclei of differently fed late third instar larvae. Western-blotting was carried out with total proteins from differently fed *Squid-GFP/Squid-GFP* larvae using GFP antibody (1:500, Sigma-Aldrich, India). In each western blot, the levels of β-tubulin, detected with the E7 β-tubulin antibody (1:200, Developmental Studies Hybridoma Bank, Iowa), were used as the loading control. The secondary antibody for immunostaining was the Cy3 conjugated anti-mouse IgG, (1:200, Sigma-Aldrich, India) while for western blots, HRP conjugated anti-rabbit IgG (1:1500; Bangalore Genei, India) or HRP conjugated anti-mouse IgG (1:1500; Bangalore Genei, India) was used.

Microscopy and documentation

All GFP or immuno-fluorescence stained preparations were examined with a Zeiss LSM 510 Meta confocal microscope using appropriate lasers, dichroics and filters. The light/DIC microscopic examinations were carried out with a Nikon E800 microscope with appropriate filter combinations and the images were recorded with a Nikon DXM 1200 digital camera. The different objectives used for confocal or other microscopy were 10× (0.3NA, Plan Fluor), 20× (0.5NA, Plan, Fluor) or 60× oil (1.4NA, Plan Apo). All the images were assembled using Adobe Photoshop 7.0.

Statistical analysis

Sigma Plot 11.0 software was used for statistical analyses. All replicates for a given control or experimental sets were tested for homogeneity using the Holm-Sidak method. None of them showed any significant intra-group differences. For comparison between the control and formulation-fed samples, One-Way ANOVA was performed.

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Author Contributions

Conceived and designed the experiments: SCL MM MSV. Performed the experiments: VD. Analyzed the data: VD SCL MM. Contributed reagents/materials/analysis tools: EMA RSM TSM. Wrote the paper: SCL VD MM MSV.

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Review Article

Jasada bhasma, a Zinc-Based Ayurvedic Preparation: Contemporary Evidence of Antidiabetic Activity Inspires Development of a Nanomedicine

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The roles of metals in human physiology are well established. It is also known that many metals are required in trace amounts for normal metabolism and their deficiency leads to diseases. In Ayurveda, metal-based preparations, that is, *bhasmas*, are indicated for the treatment of several diseases. Standard textbooks of Ayurveda recommend *Jasada bhasma* (zinc based *bhasma*) as the treatment of choice for diabetes. Modern medicine also recognizes the important role of zinc in glucose homeostasis. Yet, studies that validate the use of *Jasada bhasma* are few and uncomprehensive. There is an imminent need for a systematic study on physicochemical characterization, pharmacological efficacy, and toxicity assessment of several *bhasma* preparations to generate scientific evidence of their utility and safety. Interestingly, recent studies suggest that *bhasmas* comprise submicronic particles or nanoparticles. Thus a *bhasma*-inspired new drug discovery approach could emerge in which several metal based nanomedicines could be developed. This would help in utilizing the age old, time-tested wisdom of Ayurveda in modern medicine. One such study on antidiabetic activity of *Jasada bhasma* and the corresponding new drug, namely, zinc oxide nanoparticles, is briefly discussed, as an example.

1. Diabetes

Diabetes mellitus is a metabolic disorder manifested by the presence of hyperglycemia, that is, fasting glucose levels >140 mg/dL and postprandial glucose levels >200 mg/dL. It is a heterogeneous group of disorders influenced by age, genetic composition, and environmental factors. The pancreatic β-cells and their secretory product, namely, insulin, are central in the pathophysiology of diabetes [1]. Based on the insulin levels and pancreatic function, two main types of diabetes are recognized. Insulin-dependent diabetes mellitus (IDDM) or type 1 diabetes is due to autoimmune destruction of the insulin-producing pancreatic β-cells resulting in an absolute deficiency of insulin. Therefore, type 1 diabetic patients need exogenous insulin for survival [2]. In noninsulin-dependent diabetes mellitus (NIDDM) or type 2 diabetes, muscle, liver, and fat cells become "resistant" to the actions of insulin. Also, the compensatory mechanisms that are activated in the β-cells to secrete more insulin are not sufficient to maintain blood glucose levels within a normal physiological range [1]. Besides type 1 and type 2 diabetes, several other forms of diabetes such as "maturity onset diabetes of the young" (MODY), "gestational diabetes," and "maternally inherited mitochondrial diabetes" (MIMD) are reported. However, the incidence of type 2 diabetes is the highest (~90%) followed by type 1 diabetes (~9%) among the diabetic population [2].

Currently, the only therapy for type 1 diabetes is administration of insulin and/or analogues (which differ from human insulin by one or two amino acids). Patient discomfort due to multiple injections a day and weight gain are major demerits. Considerable efforts have been made for the development of oral insulin for better patient compliance. However, such options are not yet available in the market and insulin remains the mainstay of treatment of type 1 diabetes.

Several oral antidiabetic agents are clinically used for the treatment of type 2 diabetes (Table 1). Life style and dietary changes are also recommended in early stage of the disease. Other than the listed agents, insulin and analogues are used

Table 1: Summary of the current therapeutic agents for type 2 diabetes and associated side effects.

Drug class/agent	Drug effect/action	Side effects/demerits
Metformin	Suppresses hepatic glucose output	Lactic acidosis and GI problems
Insulin secretagogues (sulphonylureas)	Increase insulin secretion	Hypoglycemia and weight gain
PPARγ agonists (thiazolidinediones)	Increase insulin sensitivity	Peripheral edema, weight gain, and anemia
Alpha-glucosidase inhibitors (acarbose)	Inhibit glucose absorption	Loose stools and flatulence
GLP-1 analogues (liraglutide)	Increase glucose stimulated insulin secretion	Patient compliance in case of peptide analogues (injection)
DPP-IV inhibitors (sitagliptin)	Enhance endogenous GLP-1 action	Specificity issues
SGLT2 inhibitors (canagliflozin)	Inhibition of glucose reabsorption in kidneys	Urinary tract infections

in the late stage of type 2 diabetes, especially in patients with poor glycemic control. However, these therapies are associated with several side effects based on their mechanism of action [3–5], summarized in Table 1.

2. Need for Newer Antidiabetic Drugs

Most of the antidiabetic agents cannot be used as a single therapy and are used in combination with each other or with insulin, increasing the treatment cost. Despite available polytherapy, current unmet needs are

(i) enhanced insulin secretion without the risk of hypoglycemia,

(ii) increased insulin sensitivity without body weight gain,

(iii) improvement in dyslipidemia that coexists with diabetes,

(iv) preservation of pancreatic beta cell action and delayed beta cell failure,

(v) delayed development of diabetes related complications, namely, retinopathy, nephropathy, neuropathy, and cardiomyopathy.

A single, cost-effective, oral, antidiabetic treatment with minimal side effects is the need of the day. As an important part of the continued research on developing newer antidiabetic agents, several metals are being investigated for beneficial effects in both type 1 and type 2 diabetes and associated complications. These include vanadium, chromium, magnesium, selenium, cobalt, zinc, tungsten, and molybdenum. Of these metals, zinc is of particular interest due to its pleiotropic role as discussed below.

3. Role of Zinc in Glucose Metabolism

Zinc is an essential micronutrient, found in all tissues of the body, 95% of it being intracellular [6]. Being a cofactor of more than 300 enzymes, zinc is involved in all cellular functions including signal transduction, transcription, and replication [7]. Zinc is also a cofactor in DNA, RNA, and protein synthesis and influences gene expression through transcription factors [8]. Zinc also plays a role in growth, development, apoptosis, immune function, reproduction, maintenance of vision, protein digestion, blood clotting, bone metabolism, and carbohydrate metabolism.

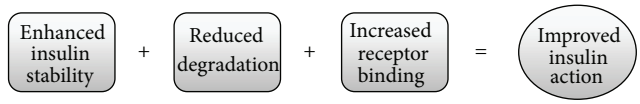


Figure 1: A schematic of the reported mechanisms by which zinc improves insulin action [9–13].

The pancreas is the site of insulin synthesis, storage, and secretion, and zinc is involved in each of these processes [9]. Even before any evidence of a relationship between zinc and insulin existed, it was known that addition of zinc extended insulin's duration of action [10]. As early as 1930's, zinc was known to be important for the integrity of the crystalline structure of insulin [11]. In the presence of zinc within the beta cell, insulin monomers assemble to a dimeric form for storage and secretion as the zinc crystal. Dimeric insulin assembles further into a hexamer that is relatively more stable form of insulin [12]. Zinc not only prevents the degradation of insulin hexamers but also improves the binding of insulin to its receptors and inhibits degradation by liver plasma membranes [13]. These reported mechanisms might be working together to improve insulin action (Figure 1).

Insulin mimetic actions of zinc are also known, reported as early as 1980, where zinc chloride stimulated lipogenesis in rat adipocytes [14]. It is now known that these insulin mimetic effects in adipocytes are through a complex interplay of improved insulin signaling, increased glucose transport, phosphodiesterase activation, and inhibition of free fatty acids release [15]. Haase and Maret [16] reported PTP1B inhibition by zinc, thus enhancing insulin signaling. Further, zinc enhances insulin signaling by increased insulin receptor tyrosine phosphorylation, enhanced PI3K activity, and inhibition of GSK3 [17].

Zinc also has beneficial effects on glucose metabolism. Inhibition of intestinal glucose absorption by zinc has been reported [18]. By inhibiting fructose 1,6-bisphosphatase [13], zinc favors glycolysis as opposed to gluconeogenesis in the cell. Zinc induces the translocation of glucose transporters (GLUT4) to plasma membrane in adipocytes [19, 20], thus increasing glucose uptake and reducing blood glucose levels.

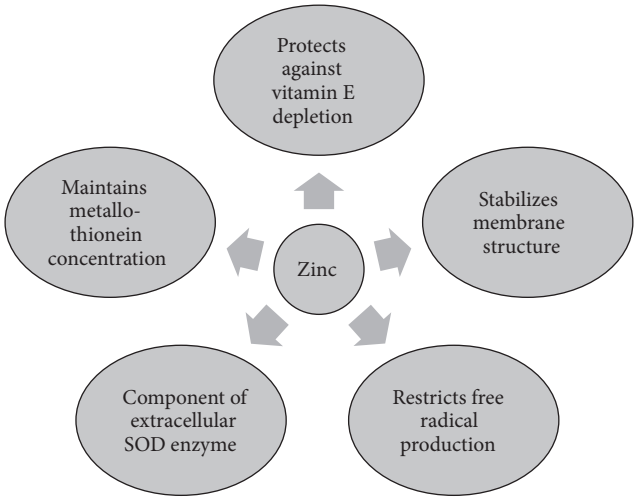


Figure 2: A schematic of the several antioxidant actions of zinc.

In the adipocytes, insulin regulates the activity of hormone sensitive lipase (HSL) and thereby inhibits lipolysis. This favors lipogenesis and storage of fat. It is known that, in diabetes, insulin's regulation over lipolysis is lost. This results in increased free fatty acids in blood. It is also known that these elevated FFAs impair beta cell function through ceramide production and induce apoptosis. The resulting pancreatic beta cell decompensation worsens the diabetic situation. Interestingly, zinc suppresses phosphorylation of hormone sensitive lipase (HSL), thereby inhibiting free fatty acids release [21]. Zinc deficiency may modify lipid metabolism and membrane integrity and these could impair glucose carrier function [13]. Inhibition of GSK3 by zinc [14] exerts beneficial effects on glycogen metabolism. Also, zinc is reported to inhibit glucagon secretion [22, 23], thus reducing gluconeogenesis and glycogenolysis.

Zinc ions also have pronounced effects on redox metabolism, although they are redox inert. Depending on how much zinc is readily available, zinc can either increase the cell's antioxidant capacity or elicit oxidative stress [24]. Zinc contributes to antioxidant defense as a component of Cu-Zn superoxide dismutase and metallothionein. Several mechanisms contribute to the antioxidant effects of zinc [25], summarized in Figure 2.

Zinc also protects beta cells from death, thereby ensuring higher plasma insulin levels. This is achieved by reduced production of interleukins and TNF-α, inflammatory mediators of cell death [17]. Ho et al. [26] reported that zinc protected beta cells from oxidative damage and death in streptozotocin and alloxan induced diabetic models.

Thus, zinc plays a pleiotropic role in the maintenance of glucose homeostasis. These metabolic actions of zinc and their mechanism are summarized in Table 2.

Interestingly, it is also known that zinc deficiency coexists with diabetes [12]. Patients with diabetes are more likely to have suboptimal zinc status and a negative correlation has been observed between zinc intake and prevalence of diabetes [27]. Whether zinc deficiency is cause or effect of hyperglycemia is still debatable. Diabetes itself affects zinc homeostasis in many ways resulting in decreases in total body zinc [12]. Zinc deficiency is associated with impairment in glucose tolerance and also an increased sensitivity to diabetogenic agents [26]. Zinc deficiency may also affect the progress of type 2 diabetes. Reduced zinc may also exacerbate the oxidative stress mediated complications of diabetes.

Several preclinical studies have demonstrated the antihyperglycemic effects of zinc supplementation in animal models of type 1 as well as type 2 diabetes [27–29]. Thus, it can be reasoned that a zinc based agent can be used for diabetes therapy. Orally active antidiabetic zinc complexes have also been developed [15, 18, 21, 30, 31]. Despite well proven results in animal models, these zinc complexes are not yet available as antidiabetic therapies in modern medicine.

4. Zinc Based Drug in Ayurveda: *Jasada bhasma*

Ayurveda is an ancient Indian system of medicine dating back to 5000 B.C. Ayurveda uses plant-, animal-, mineral-, and metal-based medicines for the treatment of diseases [32–34]. *"Rasashastra,"* an integral part of Ayurveda, deals with drugs of mineral origin and details their varieties, characteristics, processing techniques, properties, therapeutic uses, and management of adverse effects in a comprehensive way [35]. It was known in Ayurveda that metals as compared to animal and plant products were not compatible with human body constitution. They could not be consumed in their natural form, hence needed to be processed into fine and soft powder termed "*bhasma*" [36]. The preparation of *bhasmas* includes two main stages: *shodhan* (purification) and *maaran* (incineration). *Shodhan* process involves repeated trituration with herbal extracts, cow urine, milk, ghee, and so forth. *Maaran* process involves repeated cycles of incineration. Thus, *bhasmas* are metals that go through a purification and incineration process that turns them into ash [37, 38].

It was known that incomplete processing would result in metal ion impurities leading to adverse effects and toxicity. Therefore, tests were developed to evaluate the particle size, density, and physical and chemical stability of *bhasmas.* Compliance to these tests indicated complete conversion of metal to oxide form and desired size reduction, thereby implicating safety of the *bhasma* [39]. However, these tests are only qualitative and do not provide information about the chemical composition of *bhasmas* [40]. Pharmaceutical characterization is therefore necessary to identify all the active ingredients in *bhasmas* [41], since most of them are complex herbomineral preparations. Further, standardization of the raw materials, the synthesis procedure, and the finished product is also needed, which ultimately affects the purity, quality, and safety of the *bhasma* [42]. Several modern tools and techniques (namely, electron microscopy, X-ray diffraction, and atomic absorption spectrometry) can be employed to obtain detailed information on the size, structure, and elemental composition of *bhasmas*.

A condition similar to diabetes is recognized in Ayurveda, termed "*Madhumeha*" which means honey-like urine [43, 44]. Ayurvedic treatment of diabetes includes several herbal

Table 2: A summary of metabolic actions of zinc and their mechanism.

Metabolic action	Mechanism of action	References
Increases insulin action	Increases stability and receptor binding	[11, 12]
Improves insulin signaling	PTP1B inhibition	[16]
Enhances insulin signaling	Increases receptor phosphorylation and PI3K activity	[17]
Beneficial effect on glycogen metabolism	GSK3 inhibition	[14]
Increases glucose uptake	GLUT4 translocation in adipocytes	[19, 20]
Decreases lipolysis	Inhibition of HSL and FFA release	[21]
Inhibits glucagon secretion	Opening of K_{ATP} channels in pancreatic alpha cells	[22, 23]
Inhibits intestinal glucose absorption	Inhibits alpha-glucosidase enzyme	[18]
Reduces oxidative stress	Enhances SOD activity	[25]
Protects beta cells	Modulates NFκB activation	[26]

drugs and also a few mineral preparations including *bhasmas* [45]. In the texts of *Rasashastra* [46], *bhasmas* of *Mandura* (iron), *Vanga* (tin), *Naga* (lead), *Tamra* (copper), and *Jasada* (zinc) have been recommended for the treatment of diabetes. A few scientific reports on the clinical use of *bhasmas* in diabetic patients are available [47, 48].

Jasada bhasma (also known as *Yashada bhasma*) is also indicated in various disorders, namely, diabetes, anemia, cough, ulcers, depression, ophthalmic problems, and so forth [49–52]. Standard textbooks on *Rasashastra* recommend *Jasada bhasma* as the treatment of choice for diabetes. However, studies related to pharmacological/clinical investigation of *Jasada bhasma* as antidiabetic agent are few and not comprehensive [53–55]. Clearly, there is a need for undertaking a detailed and systematic study on the proclaimed antidiabetic efficacy of *Jasada bhasma*.

Recent renewed interest in Ayurveda has led to scientific investigations on therapeutic utility of several *bhasmas* [56–62]. Apart from pharmacological validation, pharmaceutical characterization of these *bhasmas* is also necessary. Interestingly, detailed investigation on the composition of *bhasmas* is being carried out by researchers, using modern analytical techniques. Recent reports have suggested the presence of submicronic particles or nanoparticles in *bhasma* preparations. Gold nanoparticles have been detected in *Swarna bhasma* and the formulation is effective antiarthritic agent in rats [63]. Bhowmick et al. [64] reported zinc oxide particles of size ~1 micron in traditionally prepared *Jasada bhasma*. Physicochemical characterization of *Naga* (lead) *bhasma* revealed micron sized particles of lead oxide [65]. Similarly, studies on *Swarna makshika bhasma* revealed that raw *Swarna makshika* is a complex compound that gets converted to simple oxides [66], with micron sized particles [67].

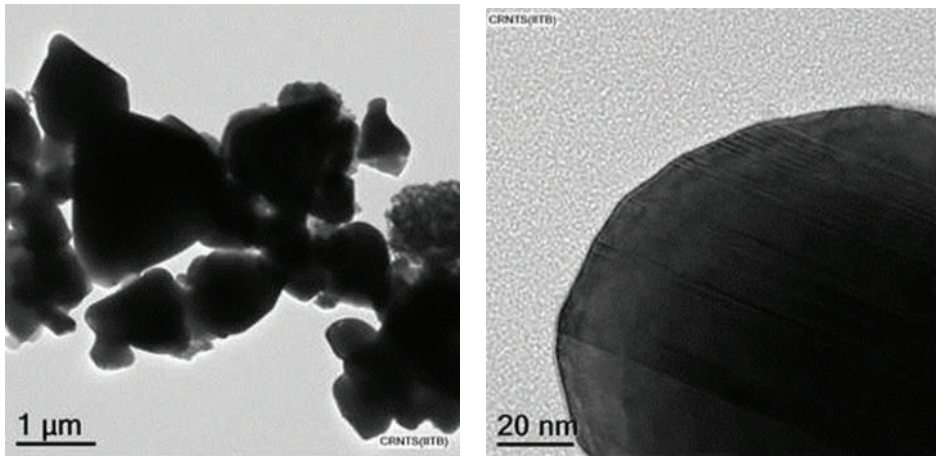
Reports are also available that provide scientific evidence of the incineration cycles during synthesis of *bhasmas*. For example, Wadekar et al. [38] evaluated tin based *bhasma* sample at various stages of calcination and found that the proportion of tin oxide increased with the number of calcination cycles while the particles size stabilized at ~1 micron after the final calcination cycle. Singh and Reddy [68] detected 300 to 500 nm sized particles in *Lauha bhasma*. Further, they observed successive decrease in particle size of *Lauha bhasma* with increase in the number of incineration cycles. In another study using *Lauha bhasma*, it was found that the incineration steps were critical for the formation of nanostructures [69]. Thus, it may be suggested that *bhasmas* contain submicronic particles or nanoparticles of the metal oxide if prepared extremely well.

It is well known that size reduction of particles increases solubility and hence bioavailability. Therefore *bhasmikaran* is expected to reduce the size of metal oxide particles enhancing their bioavailability and bioactivity. Interestingly, in modern science, several researchers have demonstrated enhanced bioavailability of nanoparticles as compared to their bulk form. For example, Ishihara et al. [70] reported higher bioavailability of micronized zinc oxide as compared to standard zinc oxide. In another report, poor water soluble iron compounds when formulated as nanoparticles displayed oral bioavailability similar to soluble salts [71]. Thus, it is safe to assume that *bhasmas* contain nanoparticles that lead to enhanced bioactivity.

5. Scientific Studies on *Jasada bhasma* and Its Use in Diabetes Treatment

Over this background of concordance of modern science and Ayurveda, as a case in point, we undertook a systematic study on the physicochemical characterization, antidiabetic efficacy, and safety assessment of *Jasada bhasma* (zinc *bhasma*). In an elaborate study conducted in our laboratory, *Jasada bhasma* was synthesized using traditional method and evaluated for its composition, size, shape, and morphology using several modern physicochemical techniques, namely, high resolution transmission electron microscopy (HRTEM) coupled with selective area electron diffraction (SAED), scanning electron microscopy (SEM) coupled with energy dispersive X-ray spectroscopy (EDS), X-ray diffraction (XRD), and atomic absorption spectrometry (AAS). Efficacy was evaluated using standard pharmacological methods in diabetic rats. Bioavailability and toxicity of *Jasada bhasma* were also assessed in rats.

SEM and HRTEM showed that the traditionally prepared *Jasada bhasma* consisted of 200–500 nm sized particles (Figure 3). EDS, SAED, AAS, and XRD analysis revealed that



(a) (b)

Figure 3: Presence of submicronic particles in *Jasada bhasma*. Images were taken by transmission electron microscopy at low (a) and high (b) magnification.

the preparation consisted predominantly of zinc oxide with hexagonal wurtzite crystal structure (details mentioned in [72]).

Dose range finding studies were carried out in normoglycemic Wistar rats. The dose used in Ayurveda (125 mg, twice a day) led us to simple calculation of 250 mg per day divided by average human body weight of 70 kg, for example, 3.5 mg/kg. To evaluate dose dependent effects, the doses of 3 mg/kg and above were selected. The effective dose range of *Jasada bhasma* in oral glucose tolerance tests was found to be 3–30 mg/kg. For efficacy evaluation, *Jasada bhasma* (1, 3, 10 mg/kg) was administered orally, once daily for 4 weeks, to streptozotocin (STZ) induced type 1 and type 2 diabetic rats. For induction of type 1 diabetes, adult rats were administered 45 mg/kg STZ intravenously whereas, for type 2 diabetes, 5-day-old pups were injected STZ intraperitoneally at the dose of 90 mg/kg.

In case of type 1 diabetic rats, *Jasada bhasma* treatment showed reduction of nonfasted (~20% at 10 mg/kg dose) as well as fasted blood glucose levels (~33% at 10 mg/kg dose). These results were comparable to glibenclamide, used as a positive control. In OGTT, *Jasada bhasma* showed a trend of suppressed glucose levels as well as reduced AUC values (~16%). Treatment also decreased the nonfasted serum insulin levels (~32% at 10 mg/kg dose), suggesting insulin sensitizing effects. In case of type 2 diabetic rats, treatment with *Jasada bhasma* resulted in improved glucose tolerance (~19%), lowered nonfasted (~20%) as well as fasted blood glucose levels (~27%), and reduced serum insulin levels (~27%). These effects were found to be comparable to pioglitazone, a drug widely used clinically.

Next, we evaluated the solubility of *Jasada bhasma* using a simple dialysis experiment. Several researchers have used this technique to estimate nanoparticle dissolution and predict bioavailability. It was found that the dialysability was around 30% in gastric pH, suggesting release of zinc ions. Systemic absorption was assessed by single dose pharmacokinetic study where serum zinc levels were found to be elevated (3.5 folds) after oral administration of *Jasada bhasma* [72].

Since there are lots of concerns related to the toxicity of metallic medicines, we also employed a comprehensive testing strategy for assessment of toxicity profile of *Jasada bhasma*. Cytotoxicity test revealed no loss of cell viability and no effects on cell morphology. Hemolysis was less than 5% (within acceptable limits) after oral administration of *Jasada bhasma* to rats. Acute and subacute toxicity tests demonstrated safety of *Jasada bhasma* up to 300 mg/kg dose in rats [73]. These findings provide concrete scientific evidence that justifies usage of *Jasada bhasma* in diabetes treatment.

6. Relevance of *Jasada bhasma* to Zinc Oxide Nanoparticles Based Drug

Encouraged by the results obtained in case of *Jasada bhasma*, we extended our work towards the development of a zinc based antidiabetic agent for modern medicine. Since our studies clearly demonstrated the presence of submicronic zinc oxide particles in *Jasada bhasma*, we hypothesized that nanoparticles of zinc oxide (≤10 nm size) should also be able to exert antidiabetic effects. An elaborate study was undertaken to investigate this possibility [74, 75].

In proof of concept studies, single administration of zinc oxide nanoparticles resulted in significant suppression of glucose levels in OGTT carried out in both type 1 and type 2 diabetic rats (~22% and ~30%, resp.). These effects appeared to be more prominent than those obtained with similar doses of *Jasada bhasma*. After 4 weeks of treatment (1, 3, and 10 mg/kg doses) to diabetic rats, significant reduction in glucose levels was seen in both nonfasted (~19% and ~29% in type 1 and type 2 diabetic rats, resp.) and fasted state (~26% and ~21% in type 1 and type 2 diabetic rats, resp.), suggesting multiple mechanisms. Reduction of nonfasted glucose levels can be attributed to insulin secretagogue effects. Reduction of fasted glucose levels may be due to glucagon inhibition, as is reported with zinc [22, 23]. Increased serum insulin levels (~35% and ~70% in type 1 and type 2 diabetic rats, resp.)

suggested insulin secretagogue effects. Reduction in serum TG (~48%) and FFA (~41%) levels was also observed after treatment indicating beneficial effects on lipid metabolism. Overall results suggested that zinc oxide nanoparticles were more potent and efficacious than *Jasada bhasma* [74].

Differences in efficacy of *Jasada bhasma* (200–500 nm particles) and zinc oxide nanoparticles (≤10 nm) suggested possible size dependent differences in bioavailability. Dialysability experiments revealed better dissolution and release of zinc ions from zinc oxide nanoparticles (~40%) as compared to *Jasada bhasma* (~30%). Further, it was found that >50% of the total zinc was left undialyzed. These results suggested that under *in vivo* conditions, *Jasada bhasma,* and zinc oxide nanoparticles are encountered as zinc ions as well as particulates, after oral administration [74].

The soluble fraction of *Jasada bhasma* and zinc oxide nanoparticles could get immediately absorbed from the intestine and result in initial spurt of zinc in blood. The particulates could be taken up by enterocytes through size limited endocytosis. It could be expected that the uptake of particulate fraction of zinc oxide nanoparticles from the intestine would be higher than that of *Jasada bhasma*. In circulation, very small particles (1–20 nm) can slowly extravasate into the interstitial spaces and then can be taken up by tissue cells [76]. Once in cells, particles encounter increasing acidic environment as they move from early to late endosomes and finally to lysosomes, resulting in dissolution of particulates [77]. Particulate component would thus result in slow and continuous release of zinc ions, acting as a depot.

As predicted, pharmacokinetic evaluation showed that serum and tissue zinc levels in zinc oxide nanoparticles treated rats were higher than *Jasada bhasma* treated rats. Further, serum zinc levels were maintained for 24 h in zinc oxide nanoparticles treated rats, whereas they declined within 4 h in *Jasada bhasma* treated rats. Long circulation of zinc oxide nanoparticles as compared to *Jasada bhasma* could possibly increase chances of their passage to tissues and hence higher cellular uptake, corresponding to the observed higher tissue zinc levels. These results correlated with *in vivo* efficacy studies where zinc oxide nanoparticles displayed a more potent and efficacious antidiabetic profile than *Jasada bhasma* [74].

Next, *Jasada bhasma* and zinc oxide nanoparticles were investigated in rat insulinoma (RIN5f) cell line to elucidate the possible mechanism of antidiabetic activity. *Jasada bhasma* did not enhance insulin secretion, whereas zinc oxide nanoparticles resulted in dose- and glucose- dependent insulin secretagogue effects. These results suggested that *Jasada bhasma* had poor cell permeability. Further, zinc oxide nanoparticles treatment *per se* enhanced SOD activity of RIN5f cells and also protected cells against H_2O_2 induced oxidative stress, suggesting antioxidant effects. Since uncontrolled hyperglycemia and oxidative stress contribute to development of diabetic complications, zinc oxide nanoparticles by virtue of antihyperglycemic and antioxidant effects may be expected to delay the progression of disease and development of associated complications [74].

Several toxicity tests were then performed to evaluate the safety of zinc oxide nanoparticles. Cytotoxicity was not seen up to 10 μg/mL concentrations of zinc oxide nanoparticles (concentrations resulting in insulin secretion) in RIN5f cells. Further to evaluate genotoxic effects of zinc oxide nanoparticles, *in vivo* micronucleus test was performed using Wistar rats. Micronuclei formation was not increased after zinc oxide nanoparticles treatment, indicating no risk of genotoxicity [75].

Since significant antihyperglycemic activity was seen at 3 mg/kg dose, toxicity studies were performed at 30 and 300 mg/kg, for example, 10 and 100 times the effective dose. In acute toxicity study, no behavioral abnormality or clinical signs or mortality was recorded after zinc oxide nanoparticles treatment at 300 mg/kg dose. Further, no effect was seen on body weight and major organ weights and tissue histology. In subacute toxicity test, 28 days of treatment with zinc oxide nanoparticles at two doses, 30 and 300 mg/kg, did not result in any significant effects on body weight gain or organ weight to body weight ratios. SGOT and SGPT activities and creatinine and urea levels were not altered in treatment groups as compared to control group, indicating no major organ damage. Histological examination of tissue sections did not reveal any necrotic damage. Taken together, toxicity studies revealed the safety of zinc oxide nanoparticles up to 100 times the effective dose [75].

Overall, it was clearly evident from our studies that zinc oxide nanoparticles can elicit potent antidiabetic activity in type 1 and type 2 diabetic rats [75]. Thus, taking inspiration from the usage of *Jasada bhasma* in diabetes, a new chemical entity (zinc oxide nanoparticles) is proposed in modern medicine warranting further investigation.

7. Conclusions and Future Prospects

The roles of metals in human physiology are well established. It is also known that many metals are required in trace amounts for normal metabolism and their deficiency leads to diseases. In Ayurveda, metal-based preparations, for example, *bhasmas*, are indicated for the treatment of several diseases. However, in present day Ayurvedic practice, the use of *bhasmas* is limited. This could be because the synthesis procedures of *bhasmas* are laborious, time consuming, and often difficult to interpret from ancient texts. Different protocols exist to get several types of *bhasma* of the same metal. Hence, selection of the synthesis protocol requires sound knowledge of the Ayurveda system. To address this issue, standardization of the synthesis procedure and its detailed documentation would be helpful.

Standards for manufacture and quality control are not yet properly defined and enforced. Development of detailed testing strategy using modern analytical tools would be useful for ensuring quality of *bhasmas*, especially absence of heavy metal impurities. FDA regulations for sale of *bhasmas* need to be clearly defined and executed to avoid the use of substandard drugs.

Parallely, scientific studies on all the *bhasmas* described in Ayurvedic texts are needed. Pharmacological validation studies can be undertaken to generate evidence of the efficacy of *bhasmas*. Further, detailed investigation of

the mechanism of action using modern research tools (namely, proteomics and genomics) will help solve the mystery of the observed effects of these Ayurvedic medicines. For example, a report on *Rasa-Sindoor* (mercury and sulfur) has detailed the various mechanisms by which it exerts a holistic effect in neurodegenerative disorders such as Huntington's and Alzheimer's disease [78]. Moreover, Ayurvedic practitioners should be encouraged to publish reports of patient outcomes. Such reports will add the much needed clinical evidence of the utility of *bhasmas*.

Most importantly, the knowledge gained out of Ayurvedic texts and evidence based studies should be extended to modern medicine. Although there are several drugs available for diabetes in the market, none of them is free from adverse effects. On the other hand, Ayurvedic medicines, namely, *bhasmas,* are known to be effective at very low doses and devoid of toxic effects. Once the active ingredients of *bhasmas* are identified, these metal oxides can be synthesized and evaluated as a new chemical entity in modern drug discovery. Taking inspiration from the fact that *bhasmas* contain submicronic or nanoparticles that enhance bioavailability, metal based nanomedicines can be developed for diabetes. This would help in utilizing the age old wisdom of Ayurveda for the development of newer drugs in modern medicine.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Management of functional gastrointestinal disorders

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ABSTRACT

Functional gastrointestinal (GI) disorders (eg irritable bowel syndrome and functional dyspepsia) are very common conditions which are associated with very poor quality of life and high healthcare utilisation. They are caused by disorders of GI functioning, namely altered gut sensitivity, motility, microbiota, immune functioning and central nervous system processing. They cause chronic symptoms throughout the gut (eg pain, dyspepsia and altered bowel habit), all of which are made worse by maladaptive patient behaviours, stress and psychological comorbidity. Management involves a biopsychosocial approach involving changes in lifestyle and diet, addressing coexisting psychological comorbidity and using medication to treat underlying pathophysiology. Pharmacological treatment with antispasmodics, neuromodulators, motility agents and antidepressants is effective. Psychotherapy in motivated individuals is equally effective. Success of treatment is increased by a good doctor–patient relationship and so this needs to be taken into account during the consultation.

KEYWORDS: gastrointestinal, irritable bowel syndrome, functional dyspepsia, functional disorders, pain

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Introduction

Functional gastrointestinal disorders (FGID) are a group of disorders characterised by chronic gastrointestinal (GI) symptoms (eg abdominal pain, dysphagia, dyspepsia, diarrhoea, constipation and bloating) in the absence of demonstrable pathology on conventional testing. Historically, they were defined as conditions which had no organic basis, but this definition has evolved with increasing understanding of these conditions and we now know that they arise due to alterations in brain–gut communication. The current classification system (ROME IV) divides them into 33 adult disorders and 20 paediatric disorders, the most common subtypes being irritable bowel syndrome (IBS) which causes abdominal discomfort, altered bowel habit and bloating; and functional dyspepsia (FD) which causes epigastric pain or discomfort, often related to eating which can be associated with fullness and satiety.[1]

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Epidemiology

FGID are very common with a worldwide prevalence of 40%, more common in women than men and this decreases with age.[2] They account for 12% of the workload in primary care and 30% of gastroenterology outpatient consultations.[3,4] More than two-thirds of patients with FGID will have seen a doctor in the last 12 months and 40% will use regular medication.[5] FGID pose a huge economic burden and treating them cost the NHS at least £72.3 million in the year 2014/2015, of which, two-thirds was on prescriptions, community care and hospital treatment.[6]

The presence of FGID is often associated with chronic pain (eg fibromyalgia) and other functional syndromes (eg chronic fatigue syndrome), and two-thirds will have psychopathology including anxiety and depression.[7] It is therefore not surprising that these patients have very poor quality of life, worse than other chronic medical conditions (eg grade III congestive cardiac failure and rheumatoid arthritis).[8]

Due to the very low quality of life (QOL) of patients with FGID and the fact that they incur a lot of healthcare costs, it is important that they are recognised and managed promptly.

Pathophysiology

It is now clear that there is abnormal physiological functioning in patients with FGID, thought to be due to underlying alterations in GI motility (either too fast or too slow), visceral hypersensitivity, altered microbiota, increased intestinal permeability, low grade immune infiltration and altered central nervous system processing of sensory input.[9] However, symptoms and healthcare seeking arise for complex reasons involving an interplay between early life events and coping styles, learned behaviour, alterations in GI physiology, and associated psychological morbidity as seen in the biopsychosocial model in Fig 1.[9] Management is therefore not simply directed at the abnormal physiology or symptoms but has to address behaviours, cognitions and beliefs.

Clinical approach

Assessment

The optimal approach involves a holistic assessment starting with a detailed history, taking care to exclude the presence of red

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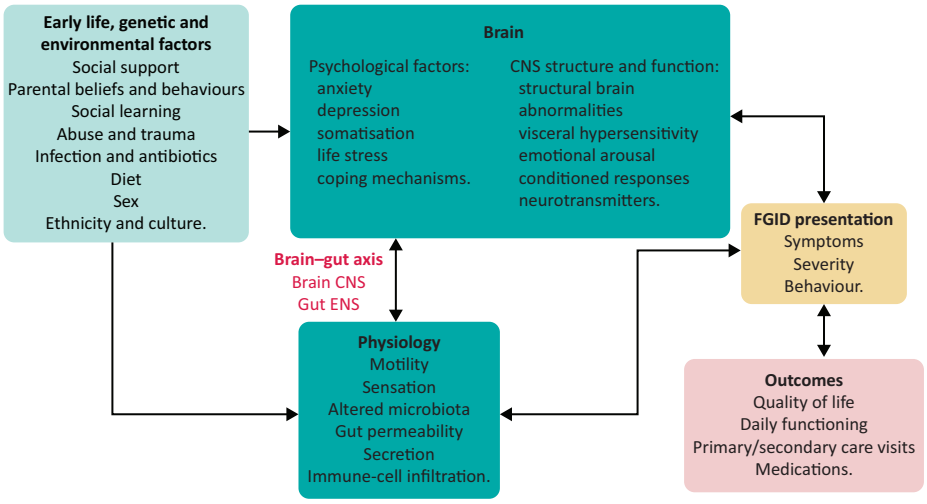

Fig 1. Biopsychosocial model of functional gastrointestinal disorders. CNS = central nervous system; ENS = enteric nervous system; FGID = functional gastrointestinal disorders.

flags (weight loss, family history of cancer, nocturnal symptoms, anaemia or GI bleeding) and organic differentials. It is important to ask about diet, lifestyle and psychological status, as this will enable you to target these as part of the management plan. Integral to all this is the doctor–patient relationship and the quality of the consultation (Box 1). This involves being empathic, avoiding jargon, being honest and admitting when you do not have the answers, which takes a lot longer than organising yet another futile test. In our opinion, 10 minutes is not sufficient for this kind of consultation, however, spending time addressing all these factors on the first visit and breaking the diagnosis of a functional disorder will save time (and money) on future visits.

Examination should include an assessment for abdominal masses, and quality of pain as well as a rectal examination. The latter is essential to rule out rectal masses and haemorrhoids, and to assess for anal tone and function. The latter can be assessed at baseline and by asking the patient to squeeze as if they are preventing themselves from emptying their bowels. Anal hypotonia is associated with faecal incontinence and hypertonia can be associated with dyssynergic defecation, itself a cause of constipation.

Box 1. Helpful questions during a consultation

Can you tell me your story starting at the very beginning? This helps work out the chronology of symptoms and can be therapeutic for the patient.

*What do **you** think is going on?* This helps identify abnormal health beliefs.

What concerns you? This allows you to address this and can relieve some patient stress.

What do you want help with? If we could change one thing what would it be? This helps you focus the management plan around it.

*What would you be doing in your life if these gastrointestinal symptoms were **reduced**?* – This helps to identify an incentive for getting better, which could be a goal that the patient works towards.

Are there other things in your life that are stopping you from this? This helps identify psychosocial issues which are contributing to the symptoms and which may need to be addressed by their general practitioner.

Investigations

With the current ROME classification, it is possible to make a positive diagnosis of FGID based on the pattern of symptoms, and so exclusion of all organic disease is not necessary. However, serious differentials that seem feasible after taking a good history (Box 2) should be ruled out.

All patients should get a basic blood and stool panel including:

- full blood count to look for anaemia
- urea and electrolytes to look for dehydration and evidence of electrolyte derangements with diarrhoea
- C-reactive protein or erythrocyte sedimentation rate to look for underlying inflammation, this should be normal in IBS
- coeliac serology
- thyroid function tests
- faecal calprotectin, if diarrhoea is present, to rule out inflammatory causes of this
- *Helicobacter pylori* testing (stool antigen test or urea breath test) for patients with dyspeptic symptoms.

Endoscopy: If a patient has typical IBS symptoms with a normal faecal calprotectin and there are no red flags to suggest a colorectal cancer (see earlier) then a lower GI endoscopy is not needed. There is little yield in performing a gastroscopy for *H pylori* negative dyspepsia in the absence of alarm symptoms (such

Box 2. Differentials for irritable bowel syndrome

Colorectal cancer

Ovarian cancer

Bile salt malabsorption

Microscopic colitis

Coeliac disease

Crohn's disease

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continuous pain, vomiting, anaemia and weight loss in patients under the age of 60), so this should not routinely be organised.[10]

Abdominal ultrasound: Abdominal ultrasound can be useful in IBS to screen for abdominal causes of pain and, in particular, for ovarian cancer which can cause pain, visible abdominal bloating and altered bowel habit. In dyspepsia, it can be useful to look for gallstones if the history is suggestive (ie colicky pain with fatty meals).

SeHCAT scan. If available, SeHCAT scans should be used to assess for bile salt malabsorption which is present in up to a third of patients with IBS-D.[11] Typical symptoms include watery diarrhoea, often yellow in colour, with or without nocturnal symptoms and faecal incontinence.

GI physiology. GI physiology is rarely indicated in IBS. One situation where it can be helpful is in patients who have severe constipation and are not responding to multiple laxatives. Lower GI physiology testing, particularly a colonic transit study and proctography can be useful at differentiating slow transit from a rectal evacuatory problem and can therefore help in fine tuning the management of constipation. In patients with functional dyspepsia, a gastric emptying study can be useful to look for severely delayed gastric emptying if there is persistent vomiting which is impacting on nutritional status, as this can help with decisions regarding feeding. For all physiological tests, it is important to be aware that medications, particularly opiates and anticholinergics, will alter GI motility and transit.

Management

General and initial approach

Once you have diagnosed a FGID, it is important to put a label on it, as patients often complain that they do not have a diagnosis or that 'nobody knows what is causing their symptoms', and then to explain in simple language what FGIDs are; information sheets or online resources can be very useful. In order to manage patient expectations, it is useful to reiterate the incurable nature of FGID and to explain that the aim of management is not to remove symptoms completely or return the patient back to 'normal', but to give them more control over their symptoms so that the GI symptoms do not dominate their life.

It is helpful when managing these patients to address the bio-psycho-social factors, in reverse:

- social/lifestyle factors: diet, exercise, sleep, and ingestion of caffeine, alcohol and other medication
- psychological factors: presence of stress, anxiety, low mood and history of eating disorders
- biological factors: physiological abnormalities and medication side effects which are contributing to symptoms.

The heterogeneity of FGID makes it difficult to design an algorithm to fit all patients, however, using a biopsychosocial approach and identifying factors which may have triggered symptoms and which ones are maintaining them enables the clinician to focus on modifying these factors as part of a personalised management plan.

Integrated multidisciplinary clinical care (eg gastroenterologist, nurse, dietitian and psychologist) appears to be superior to gastroenterologist-only care in terms of improving symptoms, psychological state, quality of life and cost of treating functional gastrointestinal disorders, so this should be offered where possible.[12]

Specific management

Lifestyle advice

Exercise

Exercise can improve bowel function, improve transit time (in females) and, therefore, help with constipation.[13] It can also reduce stress, improve mood and lead to better sleep, all of which impact on GI symptoms. In a randomised controlled trial (RCT), increased physical activity was associated with a greater reduction in IBS symptom severity scores.[14] Patients should be advised to do 20–30 minutes of exercise 3–5 times per week, even if this is just walking.

Sleep

Sleep disturbances are associated with both upper and lower GI symptoms and worse QOL.[15] Sleep disturbances can be initial (anxiety preventing onset of sleep), middle (typically broken sleep) and late (early morning awakening (can be a sign of depression)). Digital technology (eg 'Fitbits' and phone apps) can measure sleep patterns and quality, and then advise interventions to improve this (eg Sleepio app). Patients should be advised on sleep hygiene. If a drug is needed, benzodiazepines should be avoided but melatonin (3 mg nocte) can be used. This has been shown to reduce abdominal pain and rectal hypersensitivity but not bloating or overall IBS symptom scores.[16]

Phone apps

Apps can be useful for tracking lifestyle factors and monitoring changes to this. There are hundreds of healthcare apps available so it can be difficult to know what to recommend. Apps which are listed on the NHS Apps Library or ORCHA (www.orcha.co.uk) have been reviewed and can be more confidently recommended. We tend to recommend Sleepio for sleep and Headspace for mindfulness. Bowelle helps track symptoms in IBS. Zemedy is an IBS self-management app that uses a cognitive behavioural therapy (CBT) approach. The Monash University Low FODMAP Diet™ App helps users identify foods with high fermentable oligo-, di-, and monosaccharides and polyols (FODMAP) content and is useful for patients who are on the low FODMAP diet (further details follow later).

Stimulants and depressants

Caffeine increases diarrhoea, alcohol worsens reflux symptoms and heavy use is associated with increased risk of functional dyspepsia.[17] Both caffeine and alcohol can lead to disrupted sleep, so these should be minimised and not taken late at night in symptomatic individuals.

Cannabis misuse, with direct causative links to vomiting syndromes, has latterly been replaced by internet-bought cannabidiol and other plant derivatives. Even if the patient experiences none of the commonly associated GI side effects, such as vomiting and diarrhoea, there is an association with anxiety, fatigue, somnolence, risk of dependence and the potential for drug interactions, so this should not be recommended.[18,19]

Diet and nutrition

Eating behaviours

In FGID, symptoms are frequently associated with food intake, and so dietary assessment and optimisation should be part of the

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initial management strategy. Patients can be asked to keep a food diary to identify foods which trigger symptoms and to identify eating behaviours, however, the possibility of reporting bias should be noted.

IBS is associated with irregular dietary patterns and reduced diet quality and diversity (ie not having the recommended range and quantity of particular food groups), so education about *how* to eat as well as *what* to eat is important.[20,21] National Institute for Health and Care Excellence (NICE) has a useful information sheet for an 'IBS diet' which focuses on eating small regular meals, avoiding insoluble fibre, fatty foods, gas producing foods and caffeine.

Dietary restriction

There is evidence for symptom improvement following reduction in lactose and in high-starch and sugary foods and drinks, so these can be reduced if appropriate.[20]

The identification that consumption of foods high in FODMAP can exacerbate symptoms of IBS because of fermentation and osmotic effects (bloating/pain and diarrhoea, respectively) has led to the use of a low FODMAP diet as a dietary intervention for IBS, particularly in IBS-D.[22] However, this diet runs the risk of being overly restrictive and the long-term effects on nutrition and the colonic microbiome are unclear, so it is important that this is implemented by a trained dietitian.[23]

In two RCTs in Sweden and the USA, there was a similar reduction (40–50%) in IBS symptom scores in both a 'low FODMAP diet' and an IBS/NICE diet.[24,25] Thus, in the absence of dietetic support for the former, it is useful for a clinician to provide generic dietary advice in clinic based on NICE guidance.

The evidence for a gluten free diet is less clear cut. While minimising gluten intake is associated with a reduction in abdominal pain, it is unclear whether this is due to the fact that gluten is a high FODMAP food.[26] Patients can be asked to reduce their gluten intake if this is felt to be helpful but there is no clear evidence for restricting this completely.

Fibre

Soluble fibre – psyllium or isphagula husk (eg fybogel) – is cheap and useful for symptoms of IBS, particularly in IBS-C. This should be started at low dose and increased slowly. There is no evidence for insoluble fibre or for bran, both of which can exacerbate pain and bloating.[27]

Probiotics

Patients with FGID have altered microbiota, and this will be increased if patients have been on long-term or recurrent antibiotics. Microbial alteration with probiotics can be trialled and there is growing evidence for this. A meta-analysis demonstrated that probiotics improve global IBS symptom scores as well as individual symptoms of abdominal pain, bloating and flatulence.[28] Probiotics can improve stool consistency and frequency in both IBS-D and IBS-C but this is not as clear-cut as its effect on pain and bloating.[29] Combination probiotics are more likely to be beneficial but there is no consistent data to suggest what that combination should be, nor what the dose should be.[30] Although the long-term effect of probiotics on the microbiota is unknown, they are generally safe and so trialling them for 2–3 months can be considered early on in the treatment of IBS-D and possibly IBS-C.

There is no evidence to recommend faecal microbiota transfer, prebiotics (dietary supplements that result in specific changes in the composition and/or activity of the GI microbiota) or synbiotics (a mixture of probiotics and prebiotics that act synergistically to promote the growth and survival of beneficial organisms) at the current time.

Psychopathology: identification and psychological treatment

Although it may not be constructive to medicalise stress and distress by imposing diagnoses of anxiety and clinical depression, it is important to identify these as they are treatable and improving them will lead to an improvement in GI symptoms and QOL. Use of simple questionnaires (such as the hospital anxiety and depression scale) are a quick and easy way to identify this in an outpatient setting. Treatment is with pharmacological agents or with psychotherapy, both of which are effective treatments for FGID.[31]

Anxiety

Several symptoms of anxiety are similar to those in FGID (eg diarrhoea, vomiting, abdominal cramps and nausea) and patients with multiple functional syndromes often have an element of health anxiety. GI specific anxiety can be measured by the visceral sensitivity index (VSI) and this is the best predictor of IBS symptom severity.

Depression

It is important not to blame the GI disorder on low mood but explain how mood will contribute to GI symptoms and how it is treatable. Urgently refer patients with current suicidal ideation or plans to mental health professionals. For others, consider the role of psychotherapy versus pharmacotherapy for treating the low mood. As a general rule, unless there is suicidal ideation, we would consider psychotherapy (further details follow later) as a first line before medication.

Eating disorders and disordered eating

Although FGID are not typically associated with eating disorders, the presence of meal related symptoms can lead to disordered eating and, in severe cases, the development of food phobias. This is due to the conditioned pairing of an unpleasant GI symptom (eg abdominal pain) with specific foods which can then lead to avoidant restrictive food intake disorder (ARFID) which is an emerging category of eating disorders.[32]

Psychotherapy

A meta-analysis has confirmed that psychological therapies including CBT, gut-directed hypnotherapy, dynamic psychotherapy, and relaxation and mindfulness therapy are effective treatments for FGID (number needed to treat (NNT) 3–6).[30] Which of those is chosen will be determined by availability of local services. CBT is probably the easiest type of therapy to access (via Improving Access to Psychological Therapies (IAPT)) and most effective with an NNT of 3, meaning at least one in three referrals to CBT will experience significant reduction in symptoms.[31] Success

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of psychotherapy may be dependent on the expertise of the therapist; in some studies, psychotherapy delivered in specialised centres appears to have a better outcome.[33] However, CBT and mindfulness is effective even when delivered via the internet which will improve access to psychological therapies.[34,35] Psychotherapy can be time consuming, usually delivered over 12–14 weekly sessions. However, the effects appear to be long lasting and relatively free of side effects, so this form of therapy should be considered in willing patients.[36–39]

A meta-analysis has confirmed that antidepressants are also effective treatments for IBS, equally effective to psychotherapy, when patients are compliant with them.[31,40] The use of antidepressants for treating symptoms of FGID are discussed later.

Pharmacotherapy

Biological management of FGID involves either treating the underlying pathophysiology (ie neuromodulators to treat visceral hypersensitivity) or treating the symptoms (eg antiemetics to treat nausea or laxatives to treat constipation). Various algorithms exist to help guide this for functional dyspepsia and for IBS (Fig 2).[10,41] For the purposes of this review, a symptom-based approach is presented.

Pain

Opiates should be avoided as they are associated with dependence, tolerance and addiction, and can led to narcotic bowel syndrome which causes bloating, constipation, nausea and a paradoxical increase in pain with increasing doses of opiates.[42]

Instead, first-line management of pain involves antispasmodics for colicky pain. In more resistant cases, often characterised by chronic burning/neuropathic pain, neuromodulators are used as second-line treatment.

First line: antispasmodics

Antispasmodics are useful for colicky abdominal pain in IBS. There is good evidence for hyoscine (10–20 mg three times a day (tds); NNT 3) and dicycloverine (10–20 mg tds; NNT 4), though these can cause anticholinergic side effects of dry eyes and a dry mouth, and can worsen constipation. There is also evidence for peppermint oil (eg colpermin 2 capsules tds; NNT 4). It can cause heartburn, so may be best avoided for patients with coexisting GORD.[30] The evidence for mebeverine (135 mg tds) is not as good, however, it is well tolerated and can be very effective in a group of patients which do not respond to other antispasmodics.[30] Our practice is to trial a second antispasmodic if the first fails to control colicky abdominal pain.

Second line: neuromodulators

Antidepressants. The most commonly used antidepressants are tricyclic antidepressants (TCAs) and selective serotonin reuptake inhibitors (SSRIs). As a rule, antidepressants improve symptoms, particularly abdominal pain, as well as psychological distress in FGID, and the effect is increased in secondary compared with primary care, which probably reflects the greater psychological and pain comorbidity in the former.[31] For every four patients treated with an antidepressant, one will get better.

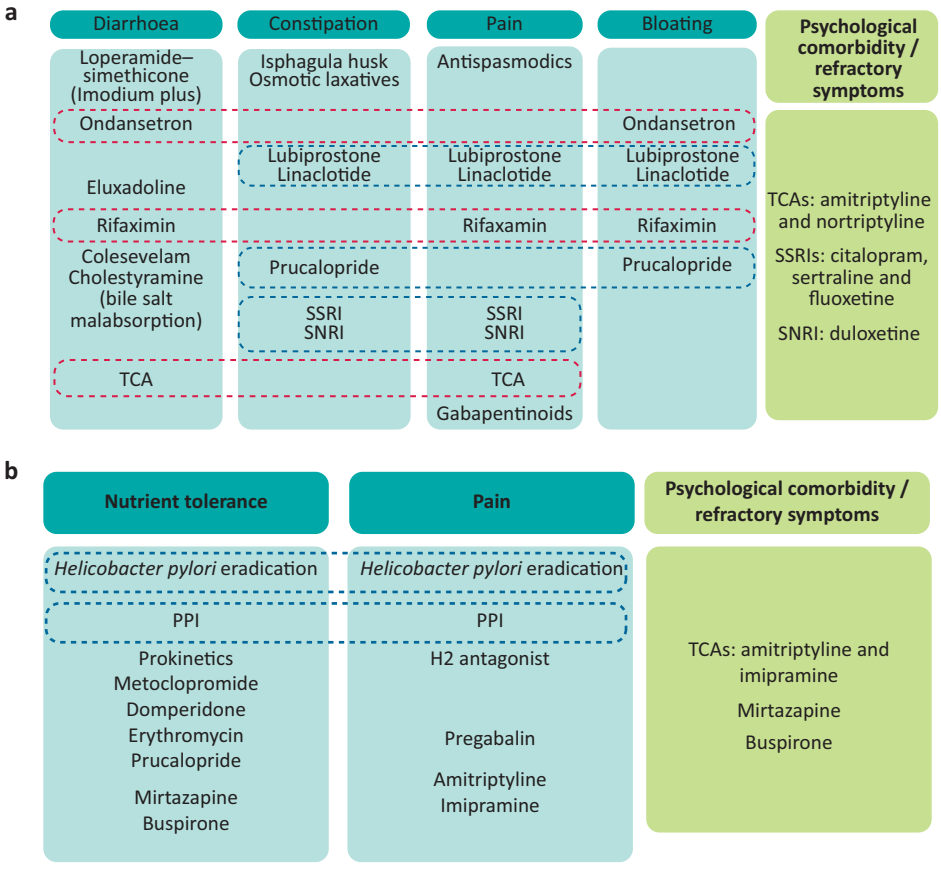

Fig 2. Algorithms for pharmacological management of irritable bowel syndrome and functional dyspepsia. a) Irritable bowel syndrome. Dotted red boxes identify medications that are useful for more than one symptom in IBS-D, dotted blue boxes identify medications that are useful for combination symptoms in IBS-C. **b) Functional dyspepsia.** Dotted blue boxes identify medicines that target for both nutrient tolerance and pain. PPI = proton pump inhibitor; SNRI = serotonin noradrenergic reuptake inhibitor; SSRI = selective serotonin reuptake inhibitor; TCA = tricyclic antidepressant.

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Most antidepressants will have GI side effects and can cause either constipation or diarrhoea (supplementary material S1), so choosing your antidepressant wisely can also help treat the altered bowel habit in IBS. TCAs (eg amitriptyline) are useful for patients with diarrhoea by slowing GI transit, whereas SSRIs (eg sertraline, citalopram, fluoxetine) are useful in those with constipation by accelerating transit. There are no RCTs of serotonin noradrenergic reuptake inhibitors (eg duloxetine) in FGID, however, open label studies for patients with IBS and comorbid anxiety and depression show that it is well tolerated at a total dose of 60 mg per day and improves IBS symptoms as well as depression and anxiety.[43,44]

Treatment with antidepressants is for an average of 18 months and stops once patients are symptom free for at least 6 months. In order to improve compliance, it is important to clarify to the patients that antidepressants are used primarily to target the IBS symptoms, notably pain, rather than the mood. It is important to pre-warn patients about the side effects and, if necessary, start at a very low (subtherapeutic) dose and work up slowly, in order to reduce adverse effects and therefore improve compliance. We would caution against using more than one antidepressant at a time unless there is expertise in doing this.

Gabapentinoids. Pregabalin and gabapentin are commonly used in chronic pain conditions and have a role in treating visceral hypersensitivity in FGID. Pregabalin is associated with an improvement in IBS symptoms (bloating, diarrhoea and abdominal pain) compared with placebo, so is a good choice for IBS-D.[45] This should be started at a low dose of 50 mg twice a day (bd) and increased gradually in line with symptomatic response, to a maximum of 300 mg bd, although it is not typically necessary to increase beyond 225 mg bd (3 × 75 mg tablets bd).[45] It causes weight gain, so may not be the best choice in obese individuals. Clinicians should be aware that it is addictive and is now considered a drug of abuse, so should be chosen with caution in certain patients. Gabapentin is an alternative but tends to have a worse side effect profile. Although there are no RCTs evaluating the efficacy of gabapentin on IBS symptoms nor the optimal dose for symptom improvement, one study demonstrated that, for patients using 300 mg gabapentin daily, there was a reduction in rectal sensitivity to distension and an increase in the thresholds for abdominal pain, bloating and discomfort.[46]

Diarrhoea

First line: loperamide

Loperamide (Imodium) reduces stool frequency and improves stool consistency in IBS-D, however, it is not effective at reducing abdominal pain or bloating, so it is poorly tolerated in IBS patients.[30] Loperamide–simethicone chewable product (Imodium plus) is much better tolerated and results in quicker relief from diarrhoea and greater relief from abdominal discomfort compared with loperamide alone or a placebo and, therefore, this should be preferentially recommended to patients who have IBS-D.[47] Patients can be asked to take two tablets to start with and then one tablet after each unformed stool until the diarrhoea stops (up to a maximum of eight tablets per day).

Second line: ondansetron

Ondansetron, a 5HT3 antagonist, improves stool consistency, frequency, urgency and bloating but not abdominal pain compared with placebo or to mebeverine for patients with IBS-D, so it is especially useful for patients who are more troubled with the altered bowel habit than the pain.[48] As it is an antiemetic, it can also improve nausea for patients who have overlapping dyspepsia. Alosetron was the precursor to ondansetron for IBS-D, however, it was associated with ischaemic colitis and therefore withdrawn. In a meta-analysis of patients with IBS-D and IBS-M, patients on 5HT3 antagonists did better than those on eluxadoline and rifaximin (see later), so it is worth considering the use of ondansetron early for patients with IBS-D.[49]

Third line: rifaximin and eluxadoline

Rifaximin is a non-absorbable antibiotic used in the treatment of GI disorders. A meta-analysis demonstrated that rifaximin is superior to placebo for reducing symptoms of diarrhoea and bloating in non-constipated patients with IBS, however, it had no effect on pain.[30,50] Recent unpublished evidence suggests that with 550 mg bd for 2 weeks, this effect may be sustained for at least 12 weeks after taking the antibiotic. However, rifaximin remains unlicensed for use in IBS in the UK and the long-term effects on the microbiota are unknown, so it is difficult to make evidence-based recommendations about this antibiotic at the current stage.

Eluxadoline is an opioid receptor antagonist which reduces stool frequency and improves global IBS symptoms, however, because of the association with pancreatitis, it is avoided for patients with a risk factor for acute pancreatitis (eg a prior history of pancreatitis, previous cholecystectomy, gallstones or alcohol).[30] The RELIEF RCT demonstrated that, in non-responders to Imodium who have an intact gallbladder, eluxadoline can be helpful in reducing stool frequency and improving pain over a 12-week period compared with placebo. A typical dose is 100 mg bd but this can be reduced to 75 mg bd in the case of side effects (eg nausea, abdominal pain, constipation and vomiting).[51]

Constipation

First line: osmotic laxatives

Polyethylene glycol (PEG)-based laxatives (such as Movicol and Laxido) as well as lactulose help to draw water into the bowel to soften the stool. Although they are associated with an increase in frequency of bowel movements, they do not alleviate pain in IBS. Lactulose can make bloating worse. In practice, it is useful to use osmotic laxatives to improve stool frequency for patients with constipation/IBS-C but this would need to be coupled with other agents to help with other symptoms such as pain.

Second line: prucalopride

Prucalopride is a highly selective 5HT4 agonist which acts as a prokinetic in the gut. An RCT demonstrated its efficacy in all patients with chronic constipation and in women with chronic constipation in whom laxatives have failed to provide adequate relief.[52] According to NICE, it is licensed for women in whom treatment with two laxatives have failed. A typical dose is 2 mg daily. A higher dose of 4 mg daily will result in improvement in straining than in stool frequency but will also be associated with greater side effects (such as headache, nausea and diarrhoea).[52,53] Patients who do not respond in the first 4 weeks are unlikely to do so with more treatment, so this can then be stopped. Prucalopride also acts as a gastric prokinetic, so it can be useful for patients with dyspepsia/gastroparesis type symptoms and would be a good choice for patients who have IBS-C and functional dyspepsia overlap.

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Third line: secretagogues

If the above measures fail, then the third-line option involves the use of secretagogues (linaclotide and lubiprostone) which improve bowel frequency and overall IBS symptoms. Some patients experience unwelcome diarrhoea with this so it may be worth reducing the dose in that case.

Linaclotide results in increased chloride and bicarbonate secretion into the gut lumen which leads to increased fluid secretion and intestinal transit. It improves bowel frequency and reduces bloating compared with placebo; this effect is similar even if the dose (290 µg) is reduced to 72 µg, which reduces diarrhoea (a common cause for discontinuation) and therefore may improve compliance.[54,55]

Lubiprostone at a dose of 8 µg bd improves abdominal pain, bloating and stool frequency in IBS-C more than placebo but can be associated with nausea.[56]

Functional dyspepsia

This is the second most common FGID and can be divided into epigastric pain syndrome (EPS), characterised by epigastric pain and burning unrelated to meals, and postprandial distress syndrome (PDS) which causes early satiety, postprandial fullness, nausea and epigastric bloating. A proportion of patients with FD will also have mild to moderate delays in their gastric emptying (Fig 2b).

First line: proton pump inhibitors and H pylori *eradication therapy*

A meta-analysis showed that *H pylori* eradication provides significant symptomatic benefits in the long term (>6 months) rather than short term (<6 months); but patients with functional dyspepsia are more likely to experience side effects including diarrhoea. It is therefore important to encourage them to remain compliant and complete the course and to ensure eradication following this if they have ongoing symptoms.

Patients who are proton pump inhibitor (PPI) responsive should be continued on the lowest dose needed to manage symptoms, and if this is not effective, it should be stopped.[10]

Second line treatment: H2 blockers and prokinetics

H2 blockers. In a meta-analysis of treatments for functional dyspepsia, H2 antagonists (such as ranitidine) are comparable to, if not more effective than, PPIs. This is not surprising in view of the increasingly important role played by mast cells and histamine in the development of functional dyspepsia.[10] Translating this to clinical practice, it is worth trialling H2 blockers for patients with FD even when PPIs have failed, although, in the current climate, the supply issues with these medications may make this difficult in practice.

Prokinetics. Prokinetics can be used in PDS, particularly if there is delayed gastric emptying. They improve symptoms but not quality of life.[57] Many prokinetics represented in RCTs are not available in the UK (eg itopride, acotiamide, cisapride and mosapride), however, these were no more effective than domperidone, which is available in the UK. Domperidone has been associated with increased QT intervals which has limited its use, however, for patients with a normal QT interval, domperidone can be safely trialled, although it is important to recheck electrocardiography once the patient is established on domperidone. There are no trials assessing other prokinetics (such as metoclopramide and erythromycin) in FD, however, theoretically these can be used in the short term.

Third line: neuromodulators

The evidence for antidepressants in functional dyspepsia is less clear but there seems to be a role for low dose TCAs such as amitriptyline (10–30 mg nocte) or imipramine (25 mg daily for 2 weeks then 50 mg daily) for patients with epigastric pain (ie epigastric pain syndrome).[58,59] Patients need to be warned about anticholinergic side effects, which can reduce compliance. Mirtazapine is increasingly gaining popularity, especially for patients with postprandial symptoms (ie discomfort, fullness and nausea post-meals). It improves symptoms and nutrient tolerance even in the absence of coexisting depression and anxiety and causes weight gain, so would be ideal for patients with symptoms of postprandial fullness who are underweight.[60] It also improves sleep and mood which will have a beneficial effect on global symptoms. Patients should start at 15 mg nocte and increase monthly to a maximum of 45 mg. A meta-analysis has demonstrated that there is no role for SSRIs (eg sertraline, fluoxetine or citalopram) in FD.[58] Buspirone is an anxiolytic which can improve gastric accommodation and can be useful for patients with postprandial fullness and early satiety but is associated with poorly tolerated side effects of dizziness and somnolence so not the first choice of neuromodulator.[10]

Conclusion

FGID are common but complex disorders which are associated with a lot of morbidity and associated psychopathology. As the aetiology of these disorders is still incompletely understood there remains no cure for them. Treatment involves a good therapeutic relationship and a holistic approach to treat the patient rather than disease, using a biopsychosocial model. ■

Supplementary material

Additional supplementary material may be found in the online version of this article at www.rcpjournals.org/clinmedicine:
S1 – Pharmacological agents that can be used for functional gastrointestinal disorders.

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ABSTRACT

Mercury is one of the elements which had attracted the attention of the chemists and physicians of ancient India and China. Among the various metal based drugs which utilize mercury, we became interested in the red sulfide of mercury which is known in ancient Indian literature as rasasindur (alias rasasindura, rasasindoor, rasasinduram, sindur, or sindoor) and is used extensively in various ailments and diseases. Following various physico-chemical characterizations it is concluded that rasasindur is chemically pure α-HgS with Hg:S ratio as 1:1. Analysis of rasasindur vide Transmission Electron Microscopy (TEM) showed that the particles are in nanoscale. Bio-chemical studies of rasasindur were also demonstrated. It interacts with Bovine Serum Albumin (BSA) with an association constant of $(9.76 \pm 0.56) \times 10^3\ M^{-1}$ and behaves as a protease inhibitor by inhibiting the proteolysis of BSA by trypsin. It also showed mild antioxidant properties.
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1. Introduction

Ayurveda, meaning the science of life, is one of the ancient medical systems of the Indian subcontinent. The principles and practices of the subject are documented in a large number of age-old texts; Charaka Samhita and Sushruta Samhita being two main Ayurvedic classics [1,2]. Ayurvedic material medica is dominated by substances of vegetable, animal and mineral origin. Metals and minerals used include mercury, gold, silver, copper, iron, tin, zinc etc. An extensive range of chemical and physical processing of these metals and their compounds has been elaborated in texts which are generally known as Rasashastra [3–7].

Mercury is one of the metals which attracted wide attention of ayurvedic chemists and physicians [8]. Indeed the documentation of chemical and physical processes involving mercury is truly enormous in ancient texts of which classics by Vagabhatta and Nagarjuna are noteworthy. Among the various procedures which utilize mercury, we became interested in the one that involves mercury and sulfur. The process is divided in three distinct steps, namely (i) pre-treatment of mercury and sulfur with herbal and milk products, (ii) mixing of mercury and sulfur along with other herbal ingredients resulting in the formation of black sulfide of mercury, (iii) thermal treatment of black sulfide of mercury at 600–650 °C [9]. The sublimed red sulfide of mercury is termed as rasasindur (alias rasasindura, rasasindoor, rasasinduram, sindur, or sindoor) in Rasashastra and is used extensively in various ailments and diseases [10].

The context of toxicity in metal based drugs in general, and of mercurial preparation in particular is an important issue [11]. It needs to be emphasized that the Rasashastra texts elaborately emphasize the concept of specific attributes in starting materials, intermediates, and products which lead to toxicity and adverse

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effects in a patient [12]. However, such narrations are at times difficult to interpret in equivalent modern scientific terms. The elaborate preparative protocol in the synthesis of rasasindur prompted us to study the physico-chemical properties of rasasindur. As presented below, we are delighted to find that the synthesis protocol described in the ancient text is indeed a case of bottom-up synthesis of red sulfide of mercury in nanoscale [13]. In light of the results of the present study and those by others [14,15] the context of nanotransformation (and its plausible implication on bio-efficacy/bio-availability/in-vivo toxicity) in metal-based ayurvedic drugs warrants a relook.

2. Experimental

2.1. Materials and methods

X-ray powder diffraction (XRD) patterns were obtained in a Bruker D8 Advance powder diffractometer using Cu Kα radiation (λ = 1.5406 A°). The optical absorption spectra of the sample were measured in the range of (500–800 nm) using PerkinElmer UV WinLab 5.2.0.0646/1.61.00 Lambda 900. Raman spectra were recorded with LabRAM HR800 micro-Raman spectrometer (Manufacturer HORIBA JobinYvon) using 632.8 nm laser excitation wave length. All measurements were made in a backscattering geometry, using a 50× microscope objective lens with a numerical aperture of 0.7. Typical laser power at the sample surface was 2.0 mW with spot size of 2 μm. The acquisition time for all the spectra was 2 s. Emission measurements were performed (excited at 270 nm) at room temperature with a Fluorolog-3 (HORIBA JOBINYVON) Spectrofluorimeter. Transmission electron microscopy (TEM) studies were carried out with a Jeol, Ultra high resolution Transmission Electron Microscope (PP resolution: 0.19 nm), at 200 KeV. Rasasindur powder was first dispersed in isopropanol to prepare a 0.1 mg/mL suspension, which was sonicated for 1 h. One drop of the suspension was taken on a copper coated grid, dried at room temperature and submitted for TEM. X-ray photoemission spectra (XPS) were recorded on a KRATOS AXIS 165 with a dual anode (Mg and Al) apparatus using the Mg Kα anode. Lens Mode: Electrostatic; Resolution: pass energy 80, Anode: A1 (150 W), step size is 100.0 meV. The pressure in the spectrometer was about 10^{-9} Torr. For energy calibration, we have used the carbon 1s photoelectron line. The carbon 1s binding energy was taken to be 285.0 eV. Spectra were deconvoluted using the Sun Solaris based Vision 2 curve resolver. The location and the full width at half-maximum (FWHM) for a species were first determined using the spectrum of a pure sample. The location and FWHM of the products, which were not obtained as pure species, were adjusted until the best fit was obtained. Symmetric Gaussian shapes were used in all cases. SEM analysis was carried out in a Zeiss Merlin Compact Oxford instrument. EDS analysis was performed at 20 kV. The working distance was set in the range 4–5 mm. The image was analyzed with secondary 1 detector. Energy of X-ray is characteristic of the difference in energy between the two shells or the atomic structure of the element.

2.2. Preparation of rasasindur

Rasasindur was prepared at Arya Vaidya Sala, Kottakkal, Kerala, India following validated standard operating procedure according to rasatarangini, which is one of the classics of Rasashastra [9]. The same procedure has been also cited in other reports [10,14]. Briefly, the preparation involves the following major steps each of which takes days to complete. Initially 350 g mercury and 350 g lime were ground on a mortar after which the mercury was filtered through a muslin cloth. The mercury thus obtained was again ground on a mortar with garlic and rock salt. Finally it was washed with water and kept ready for the next step. The processing of sulfur involved melting it, pouring the liquid sulfur in milk and in the juice of *Eclipta alba* in stages. After washing with water the sulfur was dried. 310 g of detoxified mercury and 310 g of detoxified sulfur, obtained as above, were ground together to a fine paste in the presence of the juice of *Ficus benghalensis*. The resulting black sulfide of mercury (called Kajjali in traditional texts) was then sun-dried. 250 g of Kajjali was taken in a porcelain reactor and the lid was closed. The reactor was smeared with five layers of clay and dried. Finally the reactor was heated in an open-hearth furnace at 650 °C for 33 h 15 min. The sublimed red crystals were milled on a mortar for such duration till the powder of Rasasindur passed through the standard quality control (QC) parameters as in Ayurvedic texts.

3. Results & discussion

3.1. Structure and morphological study

The crystalline behavior of rasasindur was established through Powder X-Ray Diffraction. The PXRD pattern was shown in Fig. 1. On comparison with standard JCPDS (Card No. 00-006-0256) data base values of all peaks, it is indexed to be a pure hexagonal lattice (cinnabar phase) and space group P3221. The lattice parameters are a = 0.415 ± 0.005 nm, c = 0.949 ± 0.005 nm. There are no impurities detected from the PXRD pattern, suggesting the high purity of rasasindur.

A complete analysis of elemental composition of rasasindur was possible by SEM-EDX (Fig. 2 (e)). EDX analysis concludes that the presence of only two elements e.g. Hg and S in the red HgS where Hg (80%) is present as a major element. Presence of other elements C and O are due to use of carbon tape for support and oxygen due to adsorption of atmospheric oxygen on the surface of the sample. Atom balance shows that both Hg and S are present ~50%. Thus from the EDX results we can interpret that the atomic ratio of Hg to S is 1:1.

HRTEM analysis indicated that the particles of rasasindur have spherical shape nanostructures. Particle size distribution shows that most of the particles are in the range of 8–16 nm range. From the high resolution HRTEM image (Fig. 2 (b)), the lattice spacing have been determined to be 0.337 ± 0.005 nm, 0.332 ± 0.005 nm which corresponds to (101) plane of HgS. The lattice parameters are a = 0.415 ± 0.005 nm and c = 0.949 ± 0.005 nm corresponding to Hexagonal crystal system, Space group-P3221 (PCPDF No. 060256).

From the diffraction pattern (Fig. 2 (c)), the interplanar distance has been calculated to be 0.281 nm, 0.231 nm and 0.171 nm which

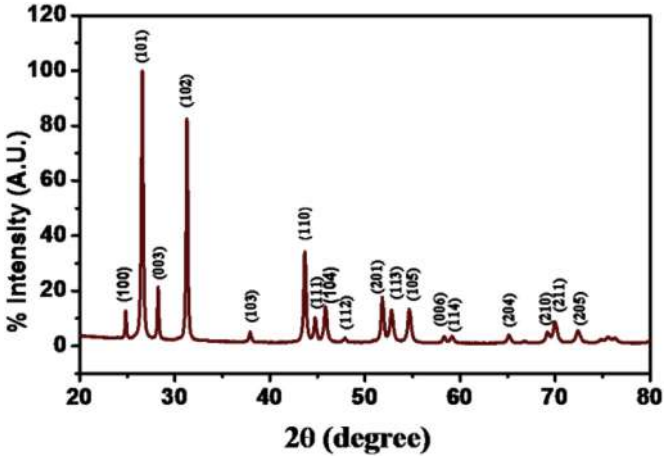

Fig. 1. Powder X-Ray Diffraction patterns of rasasindur.

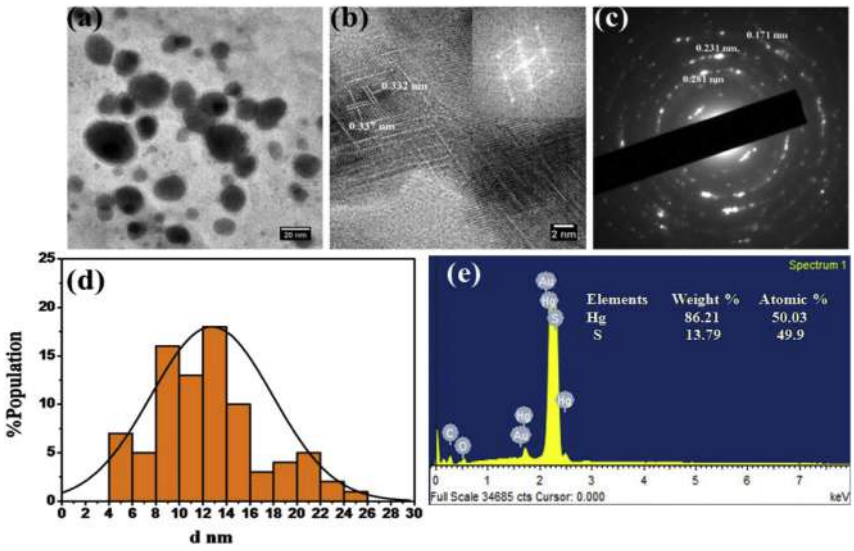

Fig. 2. (a) Low magnification image revealing the formation of nanoparticles; (b) HRTEM micrograph (inset shows the FFT of the HRTEM region); (c) Diffraction pattern taken on the nanoparticles; (d) Particle size distribution graph (e) EDX spectrum of rasasindur.

corresponds to (102), (103) and (113) planes of HgS (Crystal system-Hexagonal, Space group-P3221). The lattice parameters are a = 0.415 nm and c = 0.949 (error bars are similar to those described for Fig. 2(b)).

The binding energies obtained in the XPS analysis (Fig. 3) are corrected for specimen charging by referencing the C 1s to 285.0 eV. The two peaks obtained at 100.89 and 104.91 eV are for Hg (4f). Peaks at 161.89 and 163.29 eV are for S (2p). These values are well matched with the reported data of binding energies of HgS [16,17]. Hence from XPS it is attributed that it is a chemically pure material containing Hg and S.

Raman spectroscopy is an important technique to predict structural properties of nano materials. Raman spectrum of rasasindur (Fig. 4) gives the characteristic bands at 82 nm, 100 nm, 140 nm, 250 nm, 280 nm and 340 nm. Raman active modes are given as E mode at 82 nm, 100 nm, 280 nm, 340 nm; A1 mode at 250 nm and A2 mode at 141 nm which is well matched with the literature data [18].

3.2. Optical properties

In order to investigate the optical property of the rasasindur, the UV–vis adsorption spectrum of the product was measured. Fig. 5 (b) illustrates the DRS spectrum of rasasindur.

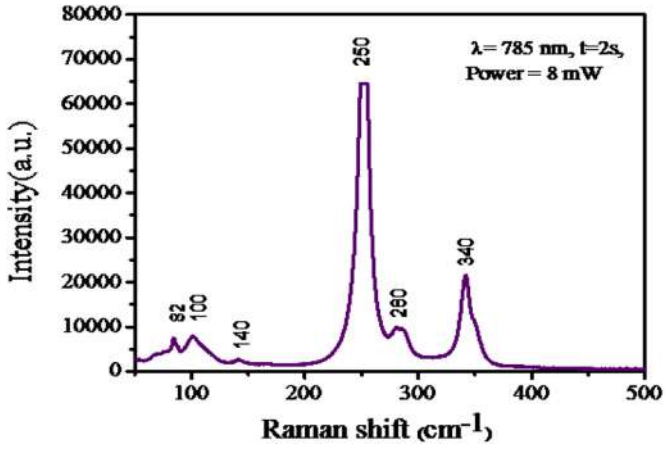

Fig. 4. Raman scattering spectrum.

Band Gap was calculated using the formula

$$\alpha h\nu = B(h\nu - Eg)^{1/2}$$

Where α is the absorption coefficient, $h\nu$ is the energy of the incident radiation Eg is the band gap, and B is a Constant [19]. Fig. 5

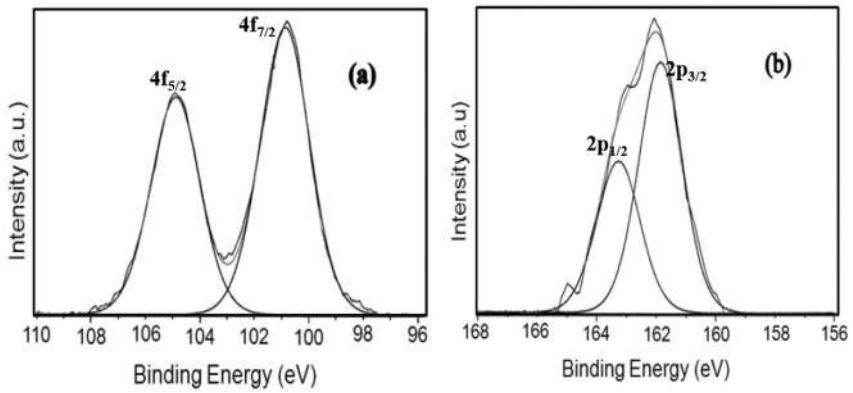

Fig. 3. X-Ray Photoelectronic Spectra (a) Hg 4f binding energy spectrum and (b) S 2p binding energy spectrum.

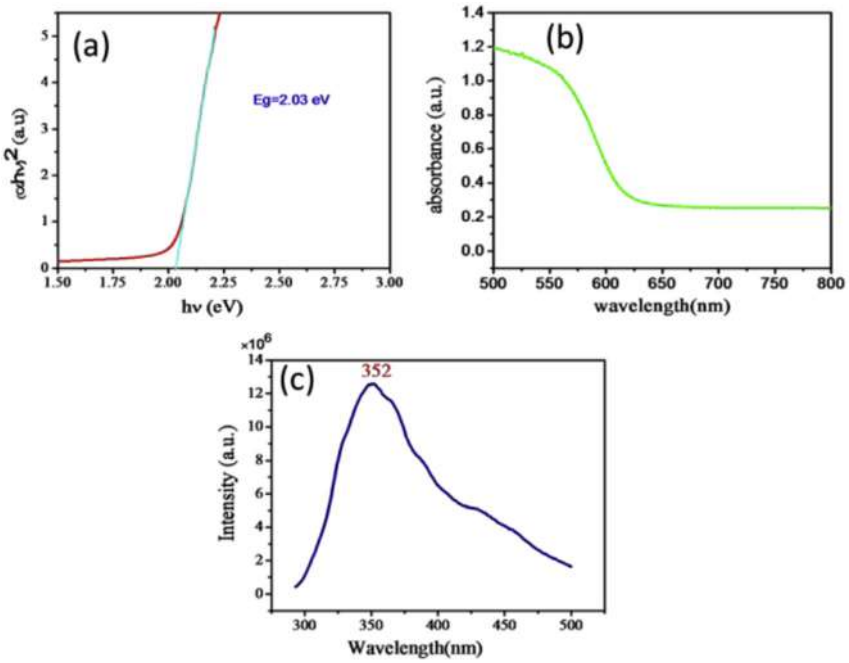

Fig. 5. (a) Band gap calculation graph, (b) UV–Vis DRS spectrum of rasasindur, (c) photoluminescence spectrum.

(a) shows the plot of $(\alpha h\nu)^2$ vs. hν which is linear over a wide range of photon energies indicating a direct type of transitions. The intercepts (extrapolations) of these plots (straight lines) on the energy axis reflect the energy band gaps. Calculated band gap is 2.03 eV. Photoluminescence spectrum is shown in Fig. 5 (c). It was carried out at excitation wavelength 270 nm and the emission maximum was found to be 352 nm.

3.3. Biochemical studies

DPPH is a stable free radical which when dissolved in ethanol exhibits violet coloration. In the presence of compounds which are able to donate hydrogen or electrons, the color of DPPH changes from violet to yellow. Such class of compounds which are able to quench the violet coloration of DPPH are known as antioxidants [20]. To evaluate whether rasasindur has potential antioxidant activity, we carried out DPPH scavenging activity with rasasindur in a range of concentration from 10 to 1000 μg/ml. It was observed that the free radical scavenging activity or percent (%) inhibition of the rasasindur increased in a concentration dependent manner which saturated at 800 μg/ml (Fig. 6). As depicted from Fig. 6, the highest antioxidant property obtained for rasasindur at 800 μg/ml was ~24%. Under similar condition, a standard sample of ascorbic acid showed ~82% free radical scavenging activity at a concentration of 200 μg/ml. Therefore as compared to ascorbic acid, rasasindur exhibited a lower antioxidant property.

Binding capacity of serum albumins has a great impact on the pharmaco kinetic properties of therapeutic drugs [21]. Since rasasindur is a traditional drug used in the Indian system of medicine [9] therefore we attempted to study its interaction with bovine serum albumin (BSA). BSA is highly homologous to human serum albumin (HSA) and is often chosen as a model protein to study small molecule albumin interactions [22,23]. Tryptophan fluorescence quenching experiment was carried out to study the interaction of the BSA with rasasindur. The intrinsic fluorescence of the protein is mainly due to the amino acids tryptophan, tyrosine and phenylalanine. BSA has two tryptophan residues and exhibits tryptophan fluorescence at an excitation of 295 nm with an emission maximum at 344 nm. The interaction of rasasindur with BSA was studied by monitoring the quenching of tryptophan fluorescence upon the addition of the rasasindur (Fig. 7 (a)). The intrinsic tryptophan fluorescence of BSA decreased gradually on increasing rasasindur concentration. The Stern–Volmer quenching constant (Ksv) as calculated from Eq. (1) was found to be $(3.21 \pm 0.19) \times 10^3\ M^{-1}$.

$$F_0/F = 1 + K_{SV}[Q] \quad (1)$$

where F_0 and F are the emission intensity of BSA in absence and in presence of rasasindur, [Q] is the concentration of rasasindur and K_{SV} is Stern–Volmer quenching constant.

The association of the rasasindur with BSA is directly correlated with the extent of fluorescence quenching [24]. Small molecules often bind to a set of sites in a protein molecule. The equilibrium

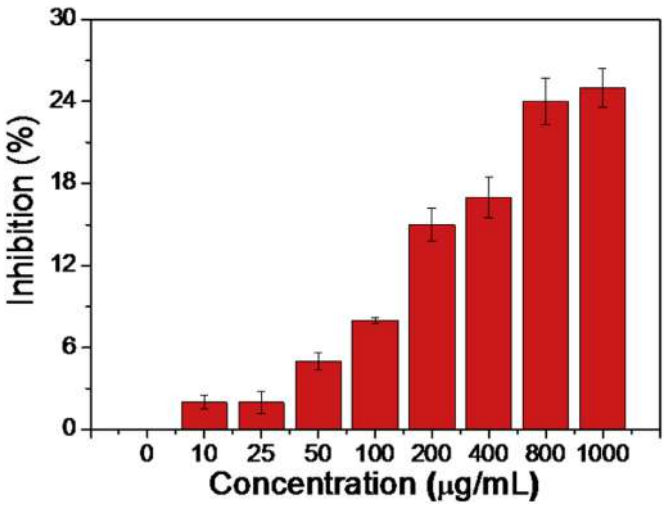

Fig. 6. Concentration dependent DPPH radical scavenging activity by rasasindur.

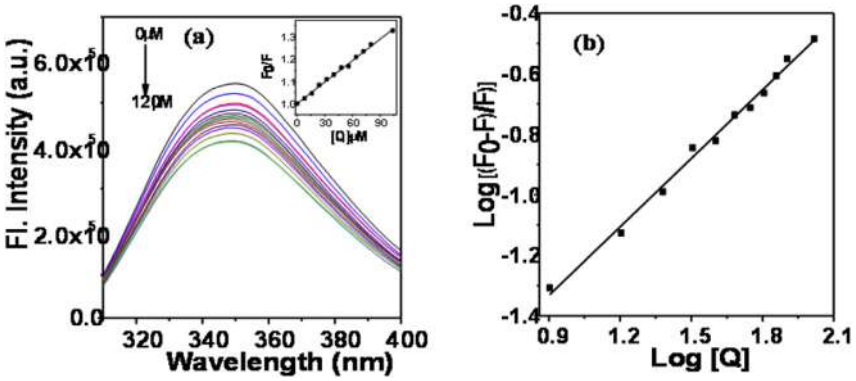

Fig. 7. Rasasindur interaction with bovine serum albumin (BSA). (a) Intrinsic tryptophan fluorescence spectra of BSA (2 μM) in the presence and absence of rasasindur [0–120 μM in 50 mM phosphate buffer (pH 7.5)]. The inset shows the linear fit of the F0/F vs. [complex] and Stern–Volmerquenching constant (KSV). (b) The plot represents the linear fit of log $[(F_0-F)/F]$ vs log [Q] for rasasindur and the association binding constant (KBSA).

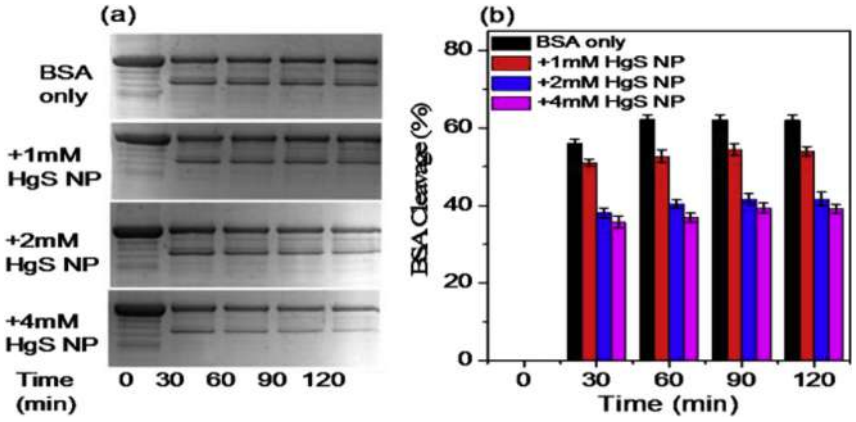

Fig. 8. Proteolysis of BSA in the presence and absence of rasasindur. (a) SDS-PAGE profile of the trypsin digest of BSA in presence and absence of rasasindur. The trypsin to BSA ratio was 1:1 (w/w). (b) Bar diagram representation of the net cleavage percent of BSA by trypsin at different time points in presence and absence of rasasindur.

between the free and the bound small molecule is given by the Scatchard analysis. The number of binding sites (n) and the association binding constant (KBSA) as calculated from Scatchard equation was found to be $(0.85 \pm 0.08) \times 10^3$ and $(9.76 \pm 0.56) \times 10^3\ M^{-1}$ respectively (Fig. 7 (b)).

Trypsin is a serine protease which is found in pancreas and helps in the proteolysis of the proteins. Inside the pancreas trypsin activity is inhibited by pancreatic secretory trypsin inhibitor (PSTI). But failure to inhibit trypsin activity can lead to damage of pancreatic cells. This can lead to pancreatitis, which may eventually develop into pancreatic cancer [25]. Therefore an imbalance between inhibition and activation of proteolytic enzymes may result in the damage of the extracellular matrix, which may lead to cancer, heart disease, neurodegenerative disease etc [26]. Thus, small molecules which can act as a potential protease inhibitor may prevent the unwanted cell damage and act as a drug against the disease caused by excessive proteolysis. Since rasasindur is used in traditional Indian medicinal system, therefore we attempted to explore its proteolytic inhibition activity by taking trypsin as the proteolytic enzyme and BSA as the substrate.

We carried out trypsin digestion of BSA in presence and absence of rasasindur at 37 °C at various concentration of the rasasindur i.e 1 mM, 2 mM and 4 mM. The SDS-PAGE profiles of the trypsin digested products of BSA in presence and absence of rasasindur is shown in Fig. 8 (a). In absence of rasasindur the proteolytic cleavage of BSA by trypsin after 2 h was ~61%. Interestingly, in presence of 1 mM rasasindur, the BSA cleavage decreased from ~61% to ~54%, which eventually saturated at ~39% when the rasasindur concentration was 4 mM (Fig. 8 (b)). Control experiments performed with BSA incubated with various concentrations of rasasindur at 37 °C for 2 h showed that rasasindur itself was cleavage inactive in absence of trypsin. These results clearly revealed that, rasasindur inhibited the proteolytic cleavage of BSA by trypsin.

4. Conclusion

Aided by a battery of experimental tools namely PXRD, HRTEM, SEM-EDS, XPS, Raman, UV–Vis-DRS, and PL-spectroscopy we conclude that:

(a) The Ayurvedic drug Rasasindur is nano-crystalline α-HgS with most of the particles in the range of 8–16 nm size.
(b) Rasasindur belongs to pure hexagonal lattice (cinnabar phase) and space group P3221.
(c) Rasasindur is a wide-band gap material having optical band-gap of 2.03 eV.
(d) Raman spectroscopy, SEM and XPS confirms the chemical purity of Rasasindur with Hg:S ratio as 1:1.

In addition to the above results, the following information from bio-chemical studies is noteworthy:

(a) Rasasindur interacts with Bovine Serum Albumin (BSA) with an association constant of $(9.76 \pm 0.56) \times 10^3\ M^{-1}$.

(b) Rasasindur behaves as a protease inhibitor by inhibiting the proteolysis of BSA by trypsin.
(c) This Ayurvedic drug also shows mild anti-oxidant property.

In light of the results of the present study and those by others, the context of nanotransformation and its plausible implication on bio-efficacy in metal-based ayurvedic drugs warrants a relook.

Conflict of interest

None declared.

Acknowledgement

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PERSPECTIVES OF KṢĀRA IN CARAKASAMHITA

Naveena Kodlady, Galib, Patgiri B. J. and Prajapati P.K.*

Abstract: Kṣāra, the alkaline group of medicinal substances, has emphasized throughout the classical literature for various therapeutic purposes. They are obtained from water soluble ash of the drugs of plant origin. These preparations possess corroding and dehydrating action and are mainly used for reduction of mass or lump, drying excess fluidity or vitiated kapha and in conditions such as arśa (haemorroid), gulma (abdominal lump), arbuda (tumour), grahaṇi (malabsorption syndrome) and śotha (oedema). A number of formulations and prescriptions containing kṣāra have referred to in Carakasamhita in different contexts. This review is expected to provide an insight about the introduction and evolution of kṣāra in āyurvedic therapeutics.

Introduction

Kṣāra (alkali) is a unique and potent dosage form described in the ayurvedic literature and is found advocated in various ailments as an independent medicine or contributory ingredient in several formulations. Kṣāra are the alkaline substances obtained from the water soluble ash of the drugs of plant origin.[1] However certain substances from mineral origin like taṅkaṇa (borax)[2] and animal origin like śaṅkha (conch shell)[3] are also used for the preparation of different kṣāra. The basic techniques involved in the preparation include burning of the raw drug into ash, dissolving the ash in specific quantity of water (6 parts of ash), discarding the water insoluble ash and concentrating the water soluble ash to obtain kṣāra.[1] As the name itself suggests, kṣāra is highly corrosive and is prescribed both internally as well as externally in right indications. The major indications of kṣāra include gulma, śotha, grahaṇi, arśa, arbuda and granthi.[4]

In spite a number of kṣāra preparations and their uses are described in āyurvedic texts, their use at present is limited to few areas like in kṣāra-sūtras (medicated threads coated with alkalis) and lepas (applications) indicated for anorectal disorders. Kṣāra being one of the aṇuśāstras (para-surgical instruments), the father of surgery, Suśruta has given special emphasis on this dosage form and has dedicated a separate chapter for kṣāra.[5] Descriptions about kṣāra found scattered across the classic, wherein its pharmaceutical procedure is not well explained. Caraka who known for the best medical treatment (carakastu cikitsite)[6] has advocated variety of kṣāra and their application in various disease conditions. An attempt is made in the current exercise to have an extensive compilation on ksara mentioned in Carakasamhita.

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Definition and grouping

The substance with corrosive (kṣāraṇa) properties is defined as kṣāra. It is produced by combination of many rasas, dominated by kaṭu and lavaṇa rasas and is prepared by some raw materials like yava, apamārga, etc. It is not a rasa (taste), instead a dravya (substance).[7] This clarification is given by Ātreya Punarvasu in reply to the argument that kṣāra is a kind of taste.[8] In another context while explaining the treatment of gulma, Caraka defines kṣāra as a substance that cuts and throws-out the morbid doṣas.[9] He has classified substances into three broad categories based on their origin viz. i) mineral (pārthiva), ii) animal (jaṅgama) and iii) vegetative (audbidha) and has placed kṣāra under audbidha category.[10] Based on taste, kṣāra is included under pungent group (kaṭuskandha).[11] Among the three broad categories of treatments viz. antarparimarjana (internal treatments), bahirparimarjana (external treatments) and śastrapranidhana (surgical treatments), kṣāra is considered as one of the śastrapranidhana treatment.[12]

Kṣāra in Carakasamhita

There are about 26 types of kṣāra mentioned in Carakasamhita. However, descriptions of individual kṣāra are not found except yavakṣāra. Yavakṣāara is useful in treatment of hṛdroga (cardiac diseases), pāṇdu (anaemia), grahaṇi, plīha (spleenomegaly), anāha (constipation), gaḷagraha (difficulty in swallowing), kāsa (cough), and kaphaja arsa (piles of kapha origin). Kṣāra are tīkṣṇa (sharp), uṣṇa (hot), laghu (light), rukṣa (dry), kḷedi (moistening), pakta (digestive), vidaraṇa (breaking), dahana (burning), chedana (cutting the morbid doṣas), dīpana (carminative) and are just like fire (agnisannibha).[13] Different types of kṣāra mentioned in Carakasamhita are shown in the Table 1.

Apart from these, Caraka has described certain substances that possessing kṣāra property viz. mahiṣamūtra (buffalo's urine),[14] pakva kūśmāṇḍa (ripened *Benincasa hispida*)[15] and audbidha

TABLE 1
Kṣāra referred to in Carakasamhita

Name of kṣāra/Botanical name	Ref. (Ci.)*
Yavakṣāra (*Hordeum vulgare*)	5/147
Sarjakṣāra (*Alhagi camelorum*)	2/23**
Tilakṣāra (*Sesamum orientale)*	3/14**
Jyotiṣmati kṣāra (*Celastrus paniculatus*)	1(3)/15-23
Iṅgudi kṣāra (*Balanites roxburghii*)	1(3)/15-23
Palāśakṣāra (*Butea monosperma*)	1(3)/32-35
Kamalakesarakṣāra (*Nelumbo nucifera*)	2/92-93
Kamalanāḷakṣāra (*Nelumbo nucifera*)	2/92-93
Priyaṅgukṣāra (*Callicarpa macrophylla*)	2/92-93
Madhūkakṣāra (*Madhuca longifolia* var. *latifolia*)	2/92-93
Asanakṣāra (*Pterocarpus marsupium*)	2/92-93
Nīlotpalakṣāra (*Monochoria vaginalis*)	5/177
Kadaḷīkṣāra (*Musa paradisiaca*)	7/88-89
Pāṭalakṣāra (*Stereospermum suaveolens*)	7/88-89
Nicūlakṣāra (*Barringtonia acutangula*)	7/88-89
Mālatīmukuḷa kṣāra (*Jasminum grandiflorum*)	7/168
Mūlakakṣāra (*Raphanus sativus*)	12/43-46
Ajapurīṣakṣāra (Goat's excreta)	13/162-165
Bilvakṣāra (*Aegle marmelos*)	13/169-170
Agnimantha kṣāra (*Premna integrifolia*)	13/170-171
Śyonākakṣāra (*Oroxylum indicum*)	13/170-171
Balakṣāra (*Sida corifolia*)	13/170-171
Apamārgakṣāra (*Achyranthus aspera*)	13/170-171
Aśvagandhakṣāra (*Withania somnifera*)	17/117
Eraṇḍapatrakṣāra (*Ricinus communis*)	18/171
Muṣkakakṣāra (*Scbrebera swietenioides*)	26/192-193

* Cikitsāsthānam; ** Sūtrasthānam

lavana.[16] Based on the similarity in the appearance, smell, taste and touch of urine, a subcategory of pittaja prameha has named as kṣārameha.[17]

Kṣāra formulations

A number of kṣāra formulations are described in the āyurvedic classics in different contexts (Tables 2-3).

Apart from above, Caraka describes different kind of compound kṣāra preparations [prepared by antardhūma (incineration in closed chambers) method] in the treatment of grahaṇi (disorders of small intestine). They include Pippalyamūlādi kṣāra,[18] Bhallātakadi kṣāra[19], Durālabhādi kṣāra,[20] Bhūnimbādi kṣāra,[21] Haridrādi kṣāra,[22] Kṣāra guṭika[23] and Pañcama kṣāra (Triphalādi kṣāra).[24] Caturthakṣāra (Vatsakādi kṣāra)[25] referred in this context is a preparation where palāśakṣārajala is used as a basic liquid media.

Kṣāra is suggested to be one of the pathyas in some of the ailments like sthaulya (obesity),[26] gulma,[27] grahaṇi[28] and śotha.[29] In the same line, several ill health conditions are treated by diet processed with kṣāra (Table 4).

Caution in administration

Kṣāra is one of the three drugs advocated not to be consumed in excess [other two are pippali (*Piper longum*) and lavaṇa (salt)]. On excess, it transforms properties like uṣṇa, tīkṣṇa and laghu into the body resulting in dryness, blindness, impotency, hair fall, yellowish discolouration of hair, cardiac diseases[30] and causes tridoṣa

TABLE 4

Kṣāra prescribed as independent medicine

Name of kṣāra	Indications	Vehicle	Reference**
1. All kṣāra	Sthaulya (obesity)	-	Su. 21/23
2. Kamalanāḷakṣāra	Kaphanubandha raktapitta	-	Ci.2/92-93
3. Kamalakesarakṣāra	-do-	-	Ci.2/92
4. Palāśakṣāra	-do-	-	-do-
5. Priyaṅgu kṣāra	-do-	-	-do-
6. Asanakṣāra	-do-	-	-do
7. Madhūkakṣāra	-do-	-	-do-
8. Palāśakṣāra	Raktagulma	Sesame oil, ghee	Ci.5/173
9. Nīlotpalakṣāra	Kaphaja-raktapitta	Honey, ghee	Ci.5/177
10. Palāśakṣāra	Śvitra	Phaṇita (sugar candy)	Ci.7/164
11. Yavakṣāra	Jalodara	Gomūtra (cow's urine)	Ci.13/93
12. Yavakṣāra	Arśa, vibandha (constipation)	Jaggery	Ci.14/98
13. Aśvagandhakṣāra	Hikka śvāsa (Hiccup and asthma)	Honey, ghee	Ci. 17/117
14. Eraṇḍapatrakṣāra	Kāsa	Trikaṭu*, sesamum oil, Jaggery/holy basil juice	Ci. 18/171
15. Yavakṣāra	Vātaja pīnasa (rhinitis of vāta-origin), kāsa and vaisvarya (loss of voice)	Ghṛta	Ci.26/134
16. Yavakṣāra	Ādhmāna (abdominal distention)	-	Si. 7/24

* Combination of black pepper, long pepper and ginger

**Carakasamhita: Si - Siddisthānam; Ci. Cikitsāsthānam

TABLE 2

Different types of kṣāra formulations described in Carakasamhita

Name/type of formulation	Name of ksara used	Indications/Use	Reference*
A. Prefix 'kṣāra' formulations:			
- Kṣāraguṭika	Yavakṣāra, sarjakṣāra and mūlakakṣāra	Śotha, udara (ascities), śvetakuṣṭha (leucoderma) and aśmari (calculi)	Ci.12/43-46
- Kṣāravaṭika	Ajapurīṣakṣāra	Udara and śotha	Ci.13/162-165
- Kṣāraghṛta	Yavakṣāra and sarjakṣāra	Grahaṇi	Ci. 15/171-182
- Kṣāragada guṭika	Palāśakṣāra	Viṣa (poisonous conditions)	Ci.23/101-104
- Kṣāraguṭika	Palāśakṣāra, muskakaksara and yavakṣāra	Kaṇṭharoga (diseases of throat)	Ci.26/192-193
- Kṣārataila	Mulakaksara, yavakṣāra and sarjakṣāra	Bādhirya (deafness), karṇanāda (tinnitus) and karṇaśūla (ear ache) for karṇapūraṇa (ear filling)	Ci.26/227-229
B. Topical applications:			
- Haridrādi lepa	Tilakṣāra	Kuṣṭha (skin diseases)	Su.3/14
- Udaraśūlaśamaka lepa	Yavakṣāra	Udaraśūla (abdominal pain)	Su.3/20
- Citrakādi lepa	Palāśakṣāra	Kuṣṭha	Ci.7/85-86
- Māmsyādi lepa	Palāśakṣāra	Kuṣṭha	Ci.7/87
- Prakṣāḷaṇa yoga	Kadaḷi, palāśa, pāṭala and nicūḷakṣāra	Kuṣṭha	Ci.7/88-89
- Lepa yoga	Kadaḷīkṣāra and mālatīmukuḷakṣāra	Śvitra	Ci.7/168
- Pratisāraṇa yoga	Yavakṣāra	Kaphaja viṣa	Ci.23/189
C. Cūrṇa formulations:			
- Śaṭhyādi cūrṇa	Yavakṣāra	Gulma and bastiśūla (pain in bladder)	Ci.5/86-90
- Nārāyaṇa cūrṇa	Yavakṣāra and sarjakṣāra	Udara, arśa, bhagandara (fistula-in-ano), śvāsa and kāsa	Ci.13/124-132
- Nīlinyādya cūrṇa	Yavakṣāra and sarjakṣāra	Udara and gulma	Ci.13/137-138
- Pippalyādi cūrṇa	Yavakṣāra	Grahaṇi and agnimāndya (low digestive power)	Ci. 15/106-107
- Maricādya cūrṇa	Yavakṣāra	Grahaṇi and aruci (loss of taste)	Ci. 15/108-110
- Pippalīmulādi cūrṇa	Yavakṣāra and sarjakṣāra	Kaphaja grahaṇi	Ci. 15/168-169
- Viḍaṅgādi cūrṇa	Yavakṣāra	Kapha vāta kāsa, hikka and agnimāndya	Ci. 18/47-48

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Table 2 continued

Name/type of formulation	Name of ksara used	Indications/Use	Reference*
- Vātaja kāsahara yoga	Yavakṣāra and sarjakṣāra	Vāta-kāsa	Ci. 18/48-49
- Sauvarcalādi cūrṇa	Yavakṣāra	Vāta-kapha kāsa	Ci. 18/122
- Cavyādi cūrṇa	Yavakṣāra	Kaphaja svarabheda	Ci.26/287
- Dhātakīpuṣpādi yoga	Yavakṣāra	Parikartika (fissure)	Si. 6/64-65
D. Vaṭi formulations			
- Hiṅgvādi guṭika	Yavakṣāra and sarjakṣāra	Vāta kaphaja gulma and mūtrakṛcchra (disurea)	Ci.5/79-84
- Citrakādya guṭika	Yavakṣāra and sarjakṣāra	Grahaṇi	Ci.15/96-97
E. Rasāyana/lehya formulations			
- Loharasāyana	Jyotiṣmatīkṣāra, iṅgudīkṣāra and palāsakṣāra	Rasāyana (rejuvenator), smṛtikara (memory enhancer), buddhi medha kara (increases intellect and intelligence)	Ci.1(3)/15-23
- Pippalīrasāyana	Palasakṣāra	Kāsa, kṣaya, śoṣa, svāsa, hikka, grahaṇi, pāṇḍu, pīnasa, śopha and gulma.	Ci.1(3)/32-35
- Indrokta rasāyana	Palasakṣāra	Vṛṣya (aphrodisiac), rasāyana	Ci.1(4)/13 -26
- Kamsaharītakīlehya	Yavakṣāra	Śvāsa, arocaka, jvara, prameha, gulma, udara, raktapitta, amlapitta, vivarṇata, mūtravikāra, vātavikāra and śukradoṣa	Ci.12/50-52
- Jīvantyādi lehya	Yavakṣāra	Sarva kāsa	Ci. 18/176-179
F. Sneha formulations			
- Sadyosnehana ghṛta	Yavakṣāra	Sadyo snehana (instant oleation)	Su. 13/94
- Hiṅgusauvarcalādya ghṛta	Yavakṣāra	Śūla, anāha and vāta-gulma	Ci.5/69-70
- Nīlinīghṛta	Yavakṣāra	Vāta-gulma	Ci. 5/105
- Tailapancakam	Yavakṣāra	Gulma and anāha	Ci.5/96
- Daśamūlī ghṛta	Yavakṣāra	Kaphagulma	Ci. 5/142
- Bhallātakādya ghṛta	Yavakṣāra	Kaphagulma, pāṇḍu and śvāsa	Ci. 5/143-146
- Kṣīraṣaṭpalaghṛta	Yavakṣāra	Kaphagulma, grahaṇi and pāṇḍu	Ci. 5/147-148
- Citrakādi ghṛta	Yavakṣāra	Śotha	Ci.12/57
- Pancakolaghṛta	Yavakṣāra	Udara, śotha, gulma and arśa	Ci.13/112-114
- Citrakaghṛta	Yavakṣāra	Udara	Ci.13/116-117
- Pippalyādi ghṛta	Yavakṣāra	Arśa	Ci.14/103-104
- Cavyādi ghṛta	Yavakṣāra	Arśa	Ci. 14/105

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Table 2 continued

Name/type of formulation	Name of kṣāra used	Indications/Use	Reference*
- Pippalīmūlādi ghṛta	Yavakṣāra	Arśa	Ci. 14/105
- Cavyādi ghṛta	Yavakṣāra	Arśa	Ci. 14/107-109
- Daśamūlādya ghṛta	Yavakṣāra and sarjakṣāra	Arśa	Ci. 15/82-85
- Pañcamūlādi ghṛta	Yavakṣāra and sarjakṣāra	Vāta-śleṣmāvṛta and sama arśa	Ci. 15/88-93
- Mṛdbhakṣyajanya pāṇḍuroga ghṛta yoga	Yavakṣāra	Mṛdbhakṣyajanya pāṇḍuroga	Ci. 16/121
- Daśamūlādi ghṛta	Yavakṣāra	Hikkaśvāsa	Ci. 17/140-141
- Pippalyādi ghṛta	Yavakṣāra	Kāsa, śvāsa, hṛtśūla, grahaṇi-doṣa and gulma	Ci. 18/36-38
- Kaṇṭakāri ghṛta	Yavakṣāra	Hikkaśvāsa, sarvakāsa and kaphavyādhi	Ci. 18/125-128
- Dvipañcamūladi ghṛta	Yavakṣāra and sarjakṣāra	Kṣaya and kāsa	Ci. 18/1580-160
- Ḍāḍimādi ghṛta	Yavakṣāra	Kṣayakāsa	Ci. 18/164-167
- Cāṅgeri ghṛta	Yavakṣāra	Atisāra and gudabhramśa ruja	Ci. 19/43
- Cavyādi ghṛta	Yavakṣāra	Gudabhramśa	Ci. 19/44
G. Kṣāra as anupāna (vehicle)			
- Eraṇḍa taila	Yavakṣāra	Vātaja-grahaṇi	Ci.15/79
- Tilvaka ghṛta	Yavakṣāra	Vātaja-grahaṇi	Ci.15/79
- Ativiṣādi kvātha	Yavakṣāra	Grahaṇi	Ci. 15/105
- Puṣkaramūlādi kalka	Yavakṣāra	Hṛdroga (cardiac diseases)	Ci.26/84,85
- Śilājatu	Yavakṣāra	Vātaja-gulma	Ci. 5/97
H. Therapeutic uses of kṣāra through pathya kalpana:			
- Takrasiddhakṛmighnayavāgu	Sarjakṣāra	Krimi	Su. 2/23
- Bhedini yavāgu	Yavakṣāra	Vibandha (constipation)	Su. 2/29
- Jīvantyādi yavāgu	Yavakṣāra	Arśa, atisāra, vātagulma, śopha and hṛdroga	Ci.12/60-61
- Takra	Yavakṣāra	Udara	Ci.13/101-102
- Mūlaka yūṣa	Yavakṣāra	Kaphaja grahaṇi	Ci. 15/144
- Kulattha yūṣa	Yavakṣāra	Kaphaja grahaṇi	Ci. 15/144
- Mātuluṅgādi yūṣa	Apamārgakṣāra	Hikka śvāsa	Ci. 17/97
- Śigrvādi yūṣa	Yavakṣāra	Hikka śvāsa	Ci. 17/98
- Mūlakādi yūṣa	Yavakṣāra	Granthī visarpa	Ci.21/128

*Carakasamhita - Ci. - Cikitsasthanam; Su. - Sutrasthanam; Si. - Sidhisthanam

TABLE 3

Kṣāra used in different formulations of pañcakarma and other procedures

Name of formulation	Procedure	Ingredient ksara	Indications	Reference
1. Palāśakṣārayukta-tilapiṣṭa	Yonikṣāḷana (vaginal douch)	Palāśakṣāra	Raktagulma	Ci.5/174
2. Uttarabasti yoga	Uttara basti (medicated enema through vagina)	Yavakṣāra	Raktagulma	Ci.5/178
3. Triphalādi taila	Nasya (nasal instillation)	Yavakṣāra	Udara	Ci.13/149
4. Dvidīya nirūha yoga	Basti (medicated enema through anus)	Yavakṣāra	Udara	Ci. 13/174-175
5. Citrakādi taila	Abhyaṅga (massage)	Yavakṣāra	Arśa	Ci.14/40
6. Pariṣekayoga	Pariṣeka (pouring)	Yavakṣāra	Granthi visarpa	Ci.21/122
7. Parama agada	Nasya, añjana lepa, pariṣeka	Yavakṣāra	Vṛścīkadamśa (scorpion bite)	Ci.23/212-214
8. Śatapadi viṣahara yoga	Nasya and añjana	Ajasakritkṣāra Sarjakṣāra	Śatapādi damśa (centipede bite)	Ci.23/215
9. Śigrvādi yoga	Nasya and kavaḷa	Yavakṣāra	Śiroroga (diseases of head)	Ci.26/186
10. Pippalyādi cūrṇa	Kavaḷa (gargling)	Yavakṣāra	Mukharoga (oral diseases)	Ci.26/188-189
11. Kṣāraguṭika	Gaṇḍūṣa (gargling)	Palāśa, muṣkaka and yava kṣāra	Kaṇṭha roga (diseases of throat)	Ci.26/192-193
12. Kāḷaka cūrṇa	Gaṇḍūṣa	Yavakṣāra	Danta, mukha and kaṇṭha rogas	Ci.26/194-195
13. Pītaka cūrṇa	Gaṇḍūṣa	Yavakṣāra	Dantaroga and mukharoga	Ci.26/196-197
14. Pariṣeka yoga	Kṣāra	Pariṣeka	Kaphaja vātarakta (gout-kapha origin)	Ci.29/147
15. Pippalyādi yoga	Virecana (purgation)	Yavakṣāra	kaphaja roga	K. 7/29
16. Triphalādi yoga	Virecana	Yavakṣāra	kaphaja roga	K. 7/37
17. Kośātakādi yoga	Basti	Yavakṣāra	Kaphaja roga	Si.3/57
18. Gomūtrādi yoga	Basti	Yavakṣāra	Vitiated kapha	Si. 7/63
19. Kaphanāśaka basti	Basti	Yavakṣāra	Vitiated kapha	Si. 10/24

*Carakasamhita - K - Kalpasthānam;, Si - Siddisthānam; Ci. Cikitsāsthānam

vitiation.[31] Caraka even warns that kṣāra is the top drug among those which causes infertility.[32] Apart from these, kṣāra is known to be a causative factor for many other diseases on excess consumption (Table 5).

Considering the contraindications in regular use, Caraka suggests that before prescribing kṣāra, certain factors like doṣa predominance, constitution of patient, season, strength of patient and diseases may be taken into account that and kṣāra should be administered with a gap of one day, two days or three days as suitable[33]. Kṣāra is contraindicated during śaratkāla (around August and September)[34] as it is the period of pittaprakopa (aggravation).

Discussion and conclusions

26 types of kṣāra and more than 100 kṣāra-contained formulations are mentioned in Carakasamhita. Though Caraka considers kṣāra under audbhidha category, Ajapurīṣakṣāra of animal origin is an exception. Among various kṣāra, yavakṣāra is found most frequently used, and often the word kṣāra is synonymously mentioned in place of yavakṣāra. Among kṣāra groups, kṣāradvaya (two kṣāra) - yavakṣāra and sarjakṣāra - has described in many places in the text, but other groups like kṣāratrya (three kṣāra) and kṣārapañcaka (five kṣāra) are not found though some of kṣāra under these groups like palāśakṣāra, muskakakṣāra, apamārgakṣāra, etc are described. Tańkaṇa, one among the kṣāra-traya (three kṣāra) is not found in Carakasamhita.

As kṣāra is rich with potassium and sodium contents, which are known for diuretic action, it is indicated in śotha. It is useful in treating conditions like gulma, grahaṇi, hikka and śvāsa due to its chedana (cutting) action on kapha and drying properties. However, kṣāra is mentioned as one of the aetiological factors for certain diseases like raktapitta, udara and arśa, it is indicated in such cases also; diseases of kapha origin are treated with specified kṣāra, but not pitta condition as kṣāra is tīkṣṇa and uṣṇa by nature.

Pharmaceutical aspect of kṣāra is not well explained in Carakasamhita; that is why Cakrapāṇidatta refers Suśrutasamhita in certain contexts. For e.g., in the context of treatment of granthi visarpa, he comments on the term 'pakya kṣāra' as that kṣāara prepared according to the procedure of kṣārapāka (kṣāra preparation) explained by Suśruta. While describing kṣāragada in viṣacikitsa, he comments palāśakṣāra to follow, the Suśruta's method of kṣāra preparation. However, Carakasamhita would help in understanding different types of kṣāra and their therapeutic applicability in different contexts.

TABLE 5
Kṣāra as actiological factor of diseases

	Disease	Reference*
1.	Pittaja śiroroga	Su.17/22
2.	Pittaja hṛdroga	Su.17/32
3.	Pittaja śotha	Su.18/2
4.	Jvara	Ni.1/22
5.	Raktapitta	Ni.2/4
6.	Pittaja prameha	Ni.5/24
7.	Udara	Ci.13/12
8.	Arśa	Ci.14/15
9.	Vātarakta	Ci.29/5
10.	Dvajabhaṅga (erectile disfunction)	Ci.30/163
11.	Pittaja pradara (menorrhagia of pitta origin)	Ci.30/214
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Review Article

RESEARCH WORKS DONE ON RASASINDURA (SUBLIMATED MERCURIAL PREPARATION) - A CRITICAL REVIEW

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Abstract

Rasasindura (RS) sublimated mercurial preparation often used in diseases like Madhumeha (Diabetus mellitus), Rajayaksma (Tuberculosis), Pandu (Anaemia), Sthoulya (Obesity), Mandagni (Impaired digestive fire) etc. Mercurial preparations are constantly targeted due to lack of documentation and published works. Till date several research works have been carried out at institutional level on RS. Aim of the present work is to compile such research works done on RS in Department of Rasashastra and Bhaishajya Kalpana, Institute for Post Graduate Teaching and Research in Ayurveda, Jamnagar. Various pharmaceutical experiments revealed yield of RS ranging from 11.70% to 92.52% and was prepared in 6 hrs to 168 hrs. RS is HgS Hexagonal cinnabar with percentage of Mercury ranging from 82-85%. Safety of RS was established through acute and sub-chronic toxicity studies. Efficacy of RS was studied in diseases like Shukra kshaya (Infertility), Shwasa (Bronchial Asthma), Madhumeha (Diabetes mellitus), Kshudra kushta (Skin diseases), shwitra (leucoderma), Hypertention etc. In all the studies encouraging results were reported with decrease in signs and symptoms. Augmenting effect of RS was established by clinical studies.

Key words: Ayurveda; Rasasindura; Sublimated mercurial preparation; I.P.G.T & R.A.

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INTRODUCTION

Ayurveda, a life science has contributed healthy lifestyle to humanity. Medicaments practiced in Ayurveda include herbal and herbo-mineral single and compound formulations.[1] Combination of minerals with herbs have not only enhanced the properties of these drugs but also played a vital role in reducing toxicity. Several claims in present day have alarmed one to prove the safety of these medicaments. Use of Mercury in Indian system of Medicine has been specially targeted as it is toxic when used in its native form.[2] Although these medicaments based on Traditional Knowledge are being used since ages, one cannot claim the safety of these medicaments without documented data. Lot of works have been carried out on these lines but only few of them are published and available for citation. For globalization and acceptance of these drugs, it is of great importance to make research works available to everyone so as to highlight the pros and cons of medicine. Reviewing previous work will definitely allow one to know regarding several factors associated with the drug discovery, like rectification of errors in earlier studies and developing better study design. Rasasindura (RS) a sublimated mercurial preparation is widely used by practitioners with different adjuvant and also in various compound formulations. RS is prepared by a unique process called as kupipakwa rasasyana. There have been more than 24 works carried out all over India on RS where as 13 works has been carried out in Institute for Post Graduate Teaching & Research in Ayurveda, Gujarat Ayurved University (IPGT&RA), Jamnagar alone.[3] Present study comprises summaries of the works on RS carried out in IPGT & RA.

MATERIALS & METHODS

Works carried-out in the Department of Rasashastra & Bhaishajya Kalpana including Drug Research, IPGT & RA, Jamnagar in MD and M.Pharma during 1964 - 2011 were compiled and screened to evaluate RS on Pharmaceutical, Analytical, Pharmacological and Clinical basis.

RESULTS & OBSERVATION

RS in Tamaka Swasa (Bronchial Asthma)

In initial study carried out on RS in 1974, three batches of Samaguna RS were prepared with time duration of 32 hrs with an average yield of 55.6%. Samaguna RS was given bhavana with equal amount of Arkapatri Swarasa (leaf juice of *Calotropis procera* Linn.) and dried to form the compound. It was administered to 14 patients of Tamaka shwasa (Bronchial asthma) at a dose of 250-500 mg/day for 28 days. Encouraging results were obtained wherein 64.78% people got completely cured and 21.43% of subjects had symptomatic relief.[4]

RS in Swasa (Asthma)

A study conducted in 1977-78 was a continuation study where three treatment groups selected for treatment were Arkadala Choorna (leaf powder of *Calotropis procera* Linn.)-250 mg, Arkadala Choorna 87.5 mg with 12.5 mg Kajjali and Arkadala Choorna 87.5 mg with 12.5 mg RS for 7 days of duration in 20 subjects. Results showed that Arkadala Choorna alone caused some side effects like nausea, vomiting, giddiness, etc. but with Kajjali and RS it did not cause any untoward effect. Study concluded that Arkadala choorna along with RS showed better results compared to other groups.[5]

Pharmaceutical standardization of RS

Extensive study on the pharmaceutical aspect of RS with different proportion of Sulphur was carried out in 1985. All the samples were prepared in AIHORE yantra which is the easiest method of preparation of RS. Temperature was recorded by thermometer. Samaguna, Dwiguna, Triguna, Chaturguna,

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Panchaguna, Shadguna, Sardhasamaguna, Sapadasamaguna, Ardhaguna, Tritiyamsha, Chaturthamsha and Shadamsha RS with 57.17%, 27.57, 21.64%, 18.54%, 15.40%, 11.70%, 41.75%, 46.46%, 78.64, 81.15%, 86.28 and 92.52% yield respectively. Duration of flame increased according to increased quantity of Sulphur.[6]

RS in Male Infertility

Study conducted in 1986 includes four preparations of RS with 49.2% of product yield. Six Patients with male infertility were treated with 250mg of RS in first group, six patients were given 2 g of Atmagupta Beeja Choorna (Seed powder of *Mucuna pruriens* Linn.) in second and seven patients were subjected for combined treatment of both in the third group. Increase in motility and sperm count was observed in first and third group where as no difference was seen in second group. The difference found in first and third group were not statistically significant as the number of patients in each group was very small to conclude.[7]

RS in Skin manifestation

Comparative Study of RS with special reference to its preparation time and its clinical efficacy in Kshudra Kushthas was carried out in 1986. Samaguna RS of six hours duration and 168 hours duration were prepared. Four practical in each method was prepared to develop standard manufacturing procedure. 52.57% of yield was obtained in six hours and 53.33% in 168 hours preparation. Ointment was prepared with 500 ml of Sikta taila and 50 g of RS. In Clinical study, six patients of Group 1 received RS 250 mg of RS (6 hrs) internally with milk and Malahara (ointment) for external application for a duration of 30 days and six patients of Group 2 received RS 250 mg of RS (168 hrs) internally with milk and Malahara for external application for a duration of 30 days. Study concluded that both the groups showed significant results in Pama (Scabies), Dadru (Fungal infection) and Vicharchika (Eczema). RS prepared by 168 hrs showed better results in comparison to the other group, however the number of patients were very less to derive a definite conclusion.[8]

Pharmaceutical study of RS

An attempt was made to prepare Shadguna RS (RS prepared by 1:6 proportion of Mercury and Sulphur) by two different methods and evaluate its aphrodisiac effect in 1986. First method was to prepare RS in 7 days i.e. in 168hrs, ten such preparations were done with an average of 13.4% yield, in second method, Samaguna RS (RS prepared by 1:1 proportion of Mercury and Sulphur)was prepared and then mercury was separated by sublimation and once again equal Sulphur was added and Kajjali was prepared followed by preparing RS in 168hrs; likewise five more times the procedure was repeated to obtain Shadguna RS, following this method it yielded 6.4% final product. In Clinical study ten patients received 250mg capsule Of RS BD with milk for 50 days. Marked improvement was observed in signs and symptoms like insomnia, loss of appetite, weakness, night falls etc. Significant results were also seen in increased sperm motility; however increase in sperm count was not statistically significant.[9]

RS in Anti diabetic compound

Effect of Madhumeha hara yoga (Anti diabetic compound) with RS was studied in 1986. Samaguna RS was prepared by Ashtasamskarita Parada, adapting 6 hrs of time duration, average yield of preparations was 43.12%. Clinical study was done by prescribing tikta and ushna veerya dravyas alone and along with RS. Clinical study comprised of three groups of 6patients each, Group1included Herbal drugs where as Group 2 and 3 along with different proportion of RS. Herbal Ingredients used and proportion of RS used in second and third group was not

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mentioned clearly in the study. Study concluded that, in Group 1, 50% patients got relived of signs and symptoms where as in group 2 & 3, 75% and 80% patients got relived of signs and symptoms of Madhumeha.[10]

RS as Aphrodisiac

Augmenting effect of RS was screened in 1989. Samaguna RS was prepared in three batches with 12hrs of preparation with yield of 48.8%. Vrushya Yoga (Aphrodisiac compound) was prepared by 75 different ingredients of Herbal and Mineral origin in one group and along with RS in other group. Treatment duration was for 30days with milk as anupana (adjuvant). Group 2 showed significant results in libido, increased sperm count in patients of oligozospermia and increased motility of sperms. Study concluded that RS has augmenting effect to enhance property of Vrushya Yoga.[11]

RS as oral contraceptive

Experimental Study of oral contraceptive with special reference to augmenting property of RS was studied in 1990. Two batches of Samaguna RS were prepared by 11hrs with an yield of 17.5%. Compound A containing Vidanga (*Embelia ribes* Burm.f.), Pippali (*Piper longum* Linn.), Palasha beeja (seed of *Butea monosperma* Lam.), Tankana (Borax), Shuddha Haratala (Arsenic trisulphide) and Compound B which was made with addition of RS to the above drugs were screened for Anti-fertility activity,[12] vaginal cornification, vaginal opening, ovarian weight gain test on isolated rats, to rule out toxic effect. Results revealed that compound possess anti-implantation activity irrespective of dose, they also possess weak oestrogenic activity, but no anti ovulatory and oxytocic activity was evident. Both compounds did not produce any toxic effect on uterus and liver. On comparison of results, augmenting effect of RS was not significant.[13]

RS in Hypertension

Study of hypotensive formula alone and along with RS was studied in 1990. Three batches of samaguna RS was prepared with average yield of 44.23%, time taken for preparation was 7.18 hrs on an average. Hypotensive formula (A) was prepared with following ingredients i.e Tamra Bhasma (incinerated Copper), Abhraka Bhasma (incinerated Mica), Swarnamakshika Bhasma (incinerated copper pyrite), Shuddha Gandhaka (purified Sulphur), Shweta Chandana (*Santalum album* Linn.), Guduchi Satva (extract of *Tenospora cordifolia* Wild.), Sariva (*Hemidesmus indicus* Linn.), Mallika (*Jasminum officinale* Linn.), Parpataka (*Oldenlandia corymbosa* Linn.), Arjuna (*Terminalia arjuna* Roxb.), Ushira (*Vetiveria zizanoides* Linn.) and Brahmi (*Bacopa monnieri* Linn.) each one part and Sarpagandha Ghana (extract of *Rauvolfia serpentina* Linn.) four *parts.* Thus formed mixture was given bhavana (Levigation) with Musta (*Cyperus rotundus* Linn.), Shatavari (*Asparagus racemosus* Wild.), Murva (*Maerua oblongifolia* Forsk.), Parpataka (*Oldenlandia corymbosa* Linn.), Draksha (*Vitis vinifera* Linn.), Durva (*Cynodan dactylon* Linn.), Dadima (*Punica granatum* Linn.), Ketaki (*Pandanus tectorius* Parkinson.), Sahadevi (*Vernonia cinerea* Less.) and Kumari (*Aloe vera* L.) QS. Hypotensive formula (B) was prepared by adding 5% RS to the above mixture. Group A was given in 1.5 g dose twice a day and Group-B, 1 g twice a day with water for 6 weeks. Relief in Cardinal signs and symptoms of hypertention was 85.55% in Group B where as 67.64% in Group A. In both group A & B, systolic BP was lowered to highly significant level at weekly assessment as well as final results. Hence augmenting effect of RS is evident.[14]

RS in treatment of Leucoderma

Augmenting effect of RS with special reference to Svitraghna Yoga (SY) was

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studied in 1990. Samaguna RS was prepared in two batches with 51.5% yeild. Shvitraghna yoga 1 was prepared by combination of following drugs Aragwadha (*Cassia fistula* Linn.), Amalaki (*Emblica officinalis* Gaertn.), Bakuchi (*Psoralea corylifolia* L.), Bhringaraja (*Eclipta alba* L.), Bhallataka (*Semecarpus anacardium* L.), Chitraka (*Plumbago zeylanica* L.), Chakramarda (*Cassia tora* L.), Guggulu (*Commiphora wightii* Arn.), Haritaki (*Terminalia chebula* Retz.), Haridra (*Curcuma longa* L.), Kakodumbara (*Ficus hispida* L.), Khadira (*Acacia catechu* L.), Katuki (*Picrorhiza kurroa* Pk.), Mareecha (*Piper nigrum* L.), Nimba (*Azadirachta indica* A. Juss.), Pippali, Shunthi (*Zingiber officinale* Roscoe.), Vibhitaki (*Terminalia bellirica* Roxb.), Vidanga. Shvitraghna yoga 2 was prepared by combining RS to Shvitraghna yoga 1. Shvitraghna hara taila was prepared by using above herbal drugs and given for ext application. SY 2 showed statistically significant results compared to the 1st group. Thus it can be said that RS is having Augmenting effect.[15]

Toxicity and Bronchodilating study of RS

Toxicity and Bronchodilating effect of RS was studied in 2002. Samaguna and Shadguna Balijarita Rasasindura were prepared in 24 hours duration. The temperature was gradually increased after every 8 hour as follows; Mridu Agni (room temperature – 200°C), Madhyam Agni (200-450 °C) and Tikshan Agni (450-650 C). Yield of 50% observed in Samaguna RS where as 13.65% yield was obtained in Shadguna RS. Further Samaguna and Shadguna RS samples were subjected for Bhavana with Vasa Swarasa (Leaf juice of Adhatoda vasica L.). In Pharmacological study test drugs were subjected for the chronic toxicity study in 40 days duration, in Charles Foster albino rats, administering the dose of 22.5 mg/kg body weight. Also the 4 test drug samples Samaguna RS, Shadguna RS, Vasa Bhavita Samaguna RS and Vasa Bhavita Shadguna RS were assessed for their bronchodilating effect in the isolated guinea pig tracheal spirals. The study concludes that both Samaguna and Sadaguna Balijarita RS have little or no toxic effects. The tissue responses to drug and its modifying effect on the histamine induced contractile response in guinea pig were studied. Shadguna RS and Samaguna RS samples without Vasa Bhavana did not affect histamine induced contraction whereas Vasa Bhavita Samaguna RS and Vasa Bhavita Shadguna RS showed mild and moderate anti-spasmodic effect. Study concluded that, Shadaguna Balijarita RS difficult to prepare pharmaceutically and as its qualities are nearly similar to Samaguna Balijarita RS in terms of efficacy and safety.[16]

Analytical profile of Samaguna and Shadguna Balijarita RS

Pharmaceutico-Analytical profiles of Samaguna and Shadguna Balijarita RS was studied in 2004. Three batches of Samaguna RS of 6 hrs duration gave an average yield of 45.37%, whereas Shadguna RS 6hrs average yield was 12.36%. In Samaguna RS 24hrs, 48.14% yield was observed and Shadguna RS 24 hrs yielded 11.78%. Samaguna RS 7days gave 44.63% and Shadguna RS 12.41% yield. Analytical study revealed 82-85% of mercury detected in RS by volumetric method. X-ray diffraction studies showed the presence of Metacinnabar HgS in cubic form in Kajjali where as in Samaguna RS prepared in 6 & 24 hrs was found to be synthetic Cinnabar in Hexagonal form and Samaguna RS prepared in 7days and all the batches of Shadguna RS i.e 6 hrs, 24 hrs and 7 days was in Natural cinnabar form HgS with Hexagonal crystal structure.[17]

DISCUSSION

Indian system of medicine has abundant references of mercurial compounds and RS is one among them. It is of great importance that one should understand the pharmaceutical

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processing and judicial combination of different ingredients to make it safe and efficacious compound. In spotlight of current circumstances authors have tried to compile previous research works done on RS in IPGT to highlight pharmaceutical, Analytical, Pharmacological and Clinical findings.

Pharmaceutical

Initially AIHORE YANTRA was used to prepare RS where the bottle was wrapped with 7 layer of mud smeared cloth and kept directly over a coal chullika (burner) with specific diameter and length.[6] Bottle used to be in direct contact with burning coal due to which many a time there was failure due to breakage. Temperature pattern was measured by thermometer which had limitations as after boiling stage inner temperature could not be taken. Outer temperature also was limited to the maximum capacity of the thermometer i.e 400^0C hence the accuracy in ascertaining the changes at different temperature was not up to the mark. In later period RS was prepared in muffle furnace, where in controlled temperature pattern was adapted i.e Mridu Agni (room temperature – 200^oC), Madhyam Agni (200-450^oC) and Tikshan Agni (450-650^oC). Electric muffle furnace was definitely easy for process standardization, especially temperature control was possible as desired in various stages.[16][17]

Analytical

Detailed analytical study was carried out in all the research works however limitations of the time period in earlier works only physico-chemical tests were carried out with % of mercury in RS varying from 82-85%. In later period characterization of RS by XRD and revealed the presence of RS in HgS Hexagonal Natural cinnabar form in Shadguna RS and Synthetic Cinnabar form in Samaguna RS.[17]

Pharmacology

Toxicity study of Samaguna and Shadguna RS has proved least toxic effect. Histopathology studies have shown mild to moderate changes in the liver in both the groups. Though Shadguna RS has shown better tolerability compared to Samaguna RS but the same is not significant enough to claim. No Bronchodilating effect was obtained without Bhavita RS, whereas Vasa Bhavita Samaguna RS showed mild and Vasa Bhavita Shadguna RS moderate anti-spasmodic effect.[16]

Augmenting effect of RS had screened for Antifertility activity and toxic effect, the results revealed that compounds possess anti-implantation activity irrespective of dose, also possess weak oestrogenic activity, but no anti ovulatory and oxytocic activity was evident. Both compounds did not produce any toxic effect on uterus and liver. On comparison of results, augmenting effect of RS was not significant.[13]

Clinical

Three studies on Shukra kshaya related to male sexual problems, two studies on Shwasa, a study each on Kshudra kushta, Shwitra, Madhumeha and Hypertention have been carried out. In all the studies, RS was administered with suitable adjuvant. The therapeutic dose was ranging in between 12.5mg to 250mg. No adverse Effects were reported in any of the clinical studies. No statistical significance could be drawn as the studies contained less number of cases.

CONCLUSION

Rasasindura is a sublimed mercurial preparation prepared by different proportion of sulphur. Kramagni maintenance is very essential for the pharmaceutical process of Kupi paka. Yield of RS in various experiments ranges from 11.70% to 92.52% and can be prepared in 6hrs to 168hrs. Analytically RS is HgS Hexagonal cinnabar with percentage of Mercury ranging from 82-85%.

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Pharmacologically, safety of RS has been established through acute, sub-chronic toxicity studies. RS is proven to possess anti-implantation activity and anti-spasmodic properties in experimental rats. RS was administered along with various herbs and adjuvant, the dose ranging from 12.5-250 mg and the duration up to 30 days in diseases like Shukra kshaya, Shwasa, Madhumeha, Kshudra kushta, shwitra, Hypertention etc. In all the studies encouraging results were reported with decrease in signs and symptoms. Augmenting effect of RS was established by clinical studies. These results may be valuable for further studies in larger population.

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Review Article

Review of research works done on *Tamra Bhasma* [Incinerated Copper] at Institute for Post-Graduate Teaching and Research in Ayurveda, Jamnagar

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Abstract

The metal, *Tamra* though mentioned in Ayurveda with a wide range of therapeutic utilities; is attributed with *Ashta Maha Dosha.* Hence, one should be cautious while using *Tamra Bhasma.* Considering the significance of *Tamra* in therapeutics, many studies have been carried out at different centers of India. Aim of the present study was to compile such available research works done on *Tamra* in the Department of Rasa Shastra and Bhaishajya Kalpana (RS and BK), IPGT and RA, Jamnagar and provide brief information about pharmaceutical, analytical, and pharmacological studies. Total eleven studies on *Tamra Bhasma*, which revalidated the impact of classical guidelines, safety issues, and therapeutic utilities, were screened from PG Department of RS and BK, Institute for Post-Graduate Teaching and Research in Ayurveda, Gujarat Ayurved University, Jamnagar. All studies revealed that *Tamra Bhasma* is safe clinically, experimentally at Therapeutic Equivalent Dose (TED) levels as no toxic hazards were reported during the treatment period. In all aspects (pharmaceutical, pharmacological, and clinical) *Somnathi Tamra Bhasma* has proven to be better than *Tamra Bhasma.* The clinical efficacy of *Tamra Bhasma* has been evaluated in *Shvasa*, *Kasa*, *Yakrit Pliha Vriddhi*, *Grahani*, etc. conditions. Satisfactory responses with a decrease in the intensity of signs and symptoms were reported in all the studies. Though certain limitations were observed in these researches, the results can be considered as a lead for further well stratified studies covering larger population. No adverse effects were reported in any of these studies.

Key words: *Bhasma, Grahani, Rasa Shastra,* safety, *Somanathi Tamra, Tamra* toxicity

Introduction

Since the prehistoric period, man has been using different sources of drugs for protection of health and treatment of diseases. These drugs in Ayurveda are obtained from natural sources such as plants, animals, and minerals. Use of metals and minerals in therapeutics has been found since Vedic period, which became an important part of Ayurvedic therapeutics due to their additional advantages like smaller doses, quick action etc.[1] *Bhasmas* are microfine powders of incinerated metals and minerals that are therapeutically useful in different disease conditions since centuries without developing any noticeable side effects. Though these *Bhasmas* are being safely practiced in Indian scenario, concerns are being raised on safety issues in the recent past.[2] At the same time a number of studies have been conducted in different institutes of India, which provided safety aspects of *Bhasma.* Metals and minerals are the integral part of therapeutics in Ayurveda and *Tamra* (copper) is one of such metals, which, if properly processed and detoxified is useful in many diseases.[3] But if not processed properly, is dangerous to life.[4] It's toxic nature in improperly purified or incinerated state has been described as *Ashtamahadoshas* (eight blemishes) in Ayurvedic classics.[5] To make it fit for therapeutic use, *Tamra* has to pass through a set of classical pharmaceutical processes known as *Shodhana* (purification), *Marana* (incineration), and *Amritikarana*. *Tamra Bhasma* is useful in the treatment of *Udara* (ascitis), *Pandu* (anemia), *Shvasa* (bronchial asthma), and *Amlapitta* (hyperacidity), etc.[6]

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Considering the wide therapeutic utility of *Tamra Bhasma*, many research works have been carried out in the Department of Rasashastra and Bhaishajya Kalpana (RS and BK), Institute for Post-Graduate Teaching and Research in Ayurveda (IPGT and RA), Gujarat Ayurved University, Jamnagar with an intention to revalidate the classical principles of therapeutics.

The present study was to compile all available research works done on *Tamra* in the Department of RS and BK, and provide brief information about pharmaceutical, analytical, and pharmacological contributions.

Materials and Methods

Works carried-out in the Department of RS and BK, IPGT and RA, Gujarat Ayurved University, Jamnagar at PhD and PG levels during 1964-2011 were compiled and screened to revalidate the classical concepts of drug preparation.

Observations and Results

Initial studies carried out in 1964 evaluated clinical efficacy of *Tamra Bhasma*[7] in cases of *Shvasa* and *Amlapitta*.[8] The drug was administered at a dose of 65-125 mg. along with honey and other herbal powders such as *Guduchi* (*Tinosporia cordifolia* Willd.), and *Amalaki* (*Phyllanthus embilica* Linn.). Encouraging results were obtained in this preliminary study.

Another study in 1965 evaluated clinical efficacy of *Tamra Bhasma*[9] and *Somnathi Tamra Bhasma*[10] (STB) on *Shvasa* (bronchial asthma), *Kasa* (cough), *Yakrit-Pliha Vriddhi* (hepato-spleenomegaly).[11] *Tamra Bhasma* is reported to contain 37% Cu, while STB contains 24% Cu and traces of arsenic (80 ppm). *Tamra Bhasma* was administered in five clinically diagnosed cases of *Shvasa* for 30 consecutive days in a dose of 65 mg. along with *Talisadi churna* and *Madhu*. STB was administered for 30 consecutive days in six clinically diagnosed cases of *Shvasa* in a dose of 125 mg. along with *Pippali churna* and *Madhu*. Both the drugs were found to be significant in relieving the signs and symptoms of *Shvasa*. Both the drugs were also administered in cases of *Kaphaja Kasa* and *Yakrit Pliha Vriddhi* with promising results. On comparison of the clinical efficacy, STB was reported to have better results over *Tamra Bhasma*.

Pharmaceutical study of *Tamra Parpati*[12] and its clinical efficacy on *Yakrit Pliha Vridhhi* and *Grahani Roga* was evaluated in 1968.[13] *Tamra Parpati* was administered to the patients of *Yakrit Pliha Vridhhi* and *Grahani* in a dose of 65-250 mg twice a day with *Madhu* and other suitable adjuvants like *Takra* for 30 days. The size of the enlarged liver and spleen was found to be reduced up to 65% with the administration of *Tamra Parpati*. Relief was also reported by the patients of *Grahani*.

Role of *marana* media in the preparation of *Tamra Bhasma*[14] was studied during 1988.[15] *Bhasma* was prepared by using three different medias (*Parada*, *Gandhaka*, and *Kantakari*). *Gandhaka* (sulfur) was reported to be the simplest and precise media in securing safe media in preparing *Tamra Bhasma*. Difficulties were expressed by the researcher while preparing *Tamra Bhasma* in the presence of *Parada* media. It was also reported that, *Tamra Bhasma* prepared with *Parada* did not pass the curd test[16] and hence remaining both groups were used clinically in *Tamaka Shvasa* at the dose of 125 mg thrice a day along with honey for 21 days. *Tamra Bhasma* showed significant results in *Tamaka Shvasa*. Total symptomatic relief observed by *Tamra Bhasma* of *Gandhaka* and *Kantakari* (*Solanum xanthocarpum*) media are 96.3% and 90.8% respectively. X-ray reports of chest posterio anterior view have shown good responses including reduction in thickening of broncho-vascular marking in both groups.

Studies in 1991[17] attempted on revalidating anti-asthmatic activity of *Dhattura Mulatvak Swarasa Bhavita Kajjali*, *Gandhaka Marita Tamra Bhasma*,[14] and *Mallasindura*.[18] Comparative study of these three *Yogas* was done on 56 known patients of *Tamaka Shvasa* for 21 days. *Tamra Bhasma* was given in the dose of 125 mg thrice a day along with honey. In this group, 86.16% and 87.22% relief in main and associated symptoms were found. An increase in peak expiratory flow rate was reported in *Tamra Bhasma* group. Breath holding time was well responded to *Dhattura Mulatvak Swarasa Bhavita Kajjalli*. Maximum increase in chest expansion and hematopoietic action was found in *Mallasindura* group. From the overall effect of the three *Yogas*, it was concluded that *Tamra Bhasma* provided better results in pacifying the signs and symptoms of *Tamaka Shvasa*.

Experiments on *Tamra*, *Loha*, *Naga*, *Vanga*, and *Yashada Bhasmas* with respect to their media and to identify the composition and genuinity were carried out in 1991.[19] This study highlighted the importance of media used in the preparation of different *Bhasmas*. *Parada* media was used in preparation of *Tamra Bhasma*.[9] Classical parameters such as *Varna*, *Varitaratva*, *Rekhapurnatva*, and contemporary parameters such as chemical analysis, Namburi phased spot test, ICP-AES (Inductively Coupled Plasma Atomic Emission Spectroscopy) were used for evaluation of *Bhasmas*. On chemical analysis, acid insoluble ash 5.67% and 67.50% copper as Cu was found in *Tamra Bhasma*. Spectroscopy detected trace elements such as Ba, Cd, Hg, K, and Mg. Study revealed that *Bhasmas* prepared in different media having different compositions and thus show different chemical reactions.

Trial for pharmaceutical standardization of STB[10] was done in 2004.[20] Two samples of STB were prepared by *Kupipakva* method, one in *Valuka Yantra* and the other in electrical muffle furnace (EMF). EMF method was found more suitable than classical *Valuka Yantra* method considering the percentage yield of STB and other advantages of EMF. Manufacturing process of STB has been standardized in terms of time (total duration of 12 h) and temperature (*Mridu Agni* - 120-250°C, *Madhyama Agni* - 250-450°C, and *Tivra Agni* - 450-630°C). Analytically both samples of STB were found to contain Cu, As, S, and Fe. In addition, presence of Hg was reported (8.24%) in STB prepared in EMF while it was not found in the sample prepared in *Valuka Yantra*. This data reflects that, there is a difference in the chemical composition of both the samples prepared by different methods. Further, toxicity studies have been attempted in experimental animals. Chronic toxicity with five Therapeutic Equivalent Dose (TED) of both samples has shown mild degenerative changes in heart, kidney, liver, and spleen. Comparatively STB prepared in *Valuka Yantra* was reported to be safe in the study. Though the samples are

showing mild degenerative changes at five TED levels, they may be safe at TED levels.

Attempts to develop pharmaceutical profile and provide evidence about safety of *Tamra Bhasma*[21] and STB[10] on the basis of pharmacological grounds were done in 2005.[22] Pharmaceutically preparation of STB was found quite easier than *Tamra Bhasma*. Acute and chronic toxicities were studied in experimental animals. No mortality was reported in acute toxicity study even at 30 TED of *Tamra Bhasma* and STB. Chronic toxicity study with five TED has shown mild fatty degenerative changes in kidney, liver, and heart. However, these changes were observed only in kidney and heart with the treatment of STB. Both the drugs were used clinically in 12 patients of *Medoroga*. 30 mg of *Tamra Bhasma* twice a day with water for 21 days has shown a significant decrease in serum cholesterol and serum triglyceride levels. 75 mg of STB twice a day has found highly significant in decreasing serum triglyceride and high density lipoprotein level. This validated *Lekhana Karma* and lipid lowering capacity of STB. This activity was found significant in comparison to *Tamra Bhasma*.

Trial to develop standard manufacturing procedure of *Tamra Bhasma* by using *Rasa Bhasma* (*Kajjali*, *Rasa Sindura*, and *Rasa Parpati*) as media was done in 2006.[23] *Bhasma* was found to be cupric oxide in nature and associated with 30 other elements in different compound forms. In acute toxicity study, no exitus even at dose 16 TED was found, establishing the safety of the *Bhasma* at TED. Chronic toxicity studies (of 30 days duration) also established safety of *Bhasma* at five TED levels. Overall analysis of histopathological study indicates increased, though only modest, toxicity potential in *Tamra Bhasma* by *Rasa Sindura* media in comparison to other two groups. Clinically *Tamra Bhasma* prepared with *Kajjali* media was found better than *Tamra Bhasma* prepared with *Rasa Sindura* media in cases of *Sthaulya* (obesity).

Anti-hyperlipidemic and cardio-protective activity of *Tamra Bhasma*[21] and STB[10] in experimental models were evaluated in the studies of 2009.[24] *Tamra Bhasma* is identified as copper oxide (CuO); however, copper manganese oxide ($CuMn_2O_4$) was found in minor phase, whereas STB is reported to be a compound of copper, mercury, arsenic, and sulfur identified as aktashite ($Cu_6Hg_3As_4S_6$). Anti-hyperlipidemic activity and cardio-protective activities of STB were found significant in comparison to *Tamra Bhasma*. Significant decrease in serum triglyceride levels was reported with the treatment of *Tamra Bhasma*. The effect was statistically highly significant with STB in comparison to cholesterol control rats.

Trials on preparation of *Tamra Bhasma*[25] with *Kajjali* as media were done in 2011.[26] To evaluate the impact of *Shodhana* and establish safety, toxicity profile and anti-hyperlipidemic activity in experimental animals, *Tamra Bhasma* was prepared with *Shodhita* and *Ashodhita Tamra*. ICP-AES revealed that *Tamra Bhasma* prepared from *Shodhita Tamra* contained 56.42% copper and 23.06% sulfur while *Bhasma* prepared from *Ashodhita Tamra* (99.9% pure copper) had 44.91% copper and 45.35% sulfur. Traces of manganese, lead, arsenic, and zinc were found in both samples. Phase identification by X-ray diffraction revealed copper sulfate hydroxide [$Cu_4SO_4(OH)_6$] and copper sulfide (CuS) in both samples of *Bhasma*, respectively. It was opined that, presence of hydroxide in *Shodhita Tamra Bhasma* sample may be due to mishandling of the sample. *Tamra Bhasma*, which did not undergo *Shodhana* procedures, took more *Putas* for preparation of *Bhasma* indicating that *Shodhana* procedure helps in easy and early preparation of *Bhasma* by increasing brittleness of *Tamra* and reducing hardness and particle size. No mortality was observed in acute toxicity study with both the samples even at 180 TED levels. *Tamra Bhasma* prepared from *Shodhita Tamra* was reported to be safe in the sub-chronic study (of 45 days). In contrast, *Bhasma* prepared from *Ashodhita Tamra* was found to be toxic even at TED levels. *Bhasma* prepared from *Shodhita Tamra* has shown significant anti-hyperlipidemic activity which is almost absent in *Bhasma* prepared from *Ashodhita Tamra*. Exact reasons behind this profile need to be ascertained by adopting suitable analytical parmeters. This study focuses on the role of *Shodhana* procedure in preparation of good *Tamra Bhasma*.

Discussion

Metals are used in various disease conditions from Vedic period, but their use increased after development of *Rasashastra*, which is an integral part of *Ayurveda*. *Bhasma* of metals or minerals are one of the suitable pharmaceutical forms in *Rasashastra*. *Tamra Bhasma* is one among them. Though wide utility of *Tamra Bhasma* has been mentioned in *Rasa* classics, *Tamra* is said to be a poison or more than a poison if used in *Ashuddha* (impure) form. There is a need to revalidate these classical principles and develop safety profiles to generate evidences. In this course, a screening has been done through the works carried out in the Department of RS and BK, IPGT and RA, Gujarat Ayurved University, Jamnagar. Twelve studies have been carried out in the department. Initial study was done by Prof. HS Sharma under the guidance of Prof. Vasudeva Mulasankara Dwivedi in 1958. This work is familiarly known as "*Shulba Shastram*," a compilatory work including comprehensive information on *Tamra* from the classics of *Rasashastra*. Remaining 11 works were compiled in the current attempt.

Out of the twelve, five each studies are based on pharmacological and clinical trials and one is based on analytical study.

Pharmaceutical findings

Studies on evaluating the significance of *Tamra Shodhana* were reported that, more *Putas* are needed for the preparation of *Ashodhita Tamra Bhasma* in comparison to *Shodhita Tamra Bhasma*. Studies concluded that, *Shodhana* imparts increased brittleness, reduced hardness and particle size of *Tamra*.[25] Pharmaceutically, preparation of STB was found to be easier than preparation of *Tamra Bhasma*. Preparation of the product in EMF is reported to be convenient over *Valuka Yantra* posing to certain conveniences. *Kramagni Paka* for STB has been standardized in terms of time (of 12 h duration) and temperature (*Mridu Agni* – 120-250°C, *Madhyama Agni* – 250-450°C, and *Tivra Agni* – 450-630°C).[10]

Analytical findings

Initial studies reported the nature of *Tamra Bhasma* as copper sulfide,[8] while the *Bhasma* prepared in the presence of *Rasa Bhasma* was found to be cupric oxide in nature associated with 30 other associated elements.[23] Other studies identified *Tamra Bhasma* as copper oxide (CuO) along with copper

manganese oxide ($CuMn_2O_4$) in minor phases. STB was reported to contain Cu, As, S, and Hg and is identified as Aktashite ($Cu_6Hg_3As_4S_6$).[22] AES-ICP revealed 56.42% copper and 23.06% sulfur in *Tamra Bhasma* prepared from *Shodhita Tamra* while *Bhasma* prepared from *Ashodhita Tamra* (99.9% pure copper) found to contain 44.91% copper and 45.35% sulfur. Traces of manganese, lead, arsenic, and zinc were found in both samples. Phase identification of *Tamra Bhasma* by X-ray diffraction revealed copper sulfate hydroxide [$Cu_4SO_4(OH)_6$] and copper sulfide (CuS) in both samples of *Bhasma*, respectively.[26] All these studies, reveal that *Bhasmas* prepared in different media have different compositions and show different chemical reactions. This gives an idea that specific clinical efficacy of a particular *Bhasma* incinerated in specific media may be due to its specific chemical composition and trace elements present in it.

Pharmacological findings

No mortality with *Tamra Bhasma* was reported in acute toxicity at 30 TED[22] and 180 TED levels.[26] Sub-chronic toxicity studies evaluated the safety of *Tamra Bhasma* at five TED levels,[23] while chronic toxicity studies reported mild fatty degenerative changes in visceral organs with 5 TED. STB was reported to be safe, and no mortality was reported in acute toxicity at 30 TED.[22] Further studies attempted on evaluating the impact of *Shodhana* procedure. *Tamra Bhasma* prepared from *Shodhita Tamra* was reported to be safe in the sub-chronic study. In contrast, *Bhasma* prepared from *Ashodhita Tamra* was found to be toxic even at TED levels.[26] Significant anti-hyperlipidemic and cardio-protective activities of STB in comparison to *Tamra Bhasma* in experimental studies were reported.[22] Later studies also reported similar findings with *Tamra Bhasma* prepared from *Shodhita Tamra*, which was absent in *Bhasma* prepared with *Ashodhita Tamra*.[26]

Clinical findings

Therapeutic efficacy of *Tamra Bhasma* has been validated in cases such as *Shvasa*, *Amlapitta*, *Kasa*, *Yakrit-Pliha Vriddhi*, and *Grahani Roga*. In all the studies, the *Bhasmas* were administered by mixing with suitable herbal powders and adjuvants. The therapeutic dose was ranging in between 65 and 250 mg. On comparison, the effect of STB is reported to be more significant than the *Tamra Bhasma*. No adverse effects were reported in any of the clinical studies.

Conclusion

Metals and minerals are the integral parts of therapeutics in Ayurveda. *Tamra*, one of such metals been advocated in different forms in the management of various diseases. Classical texts emphasized on following classical guidelines (such as *Shodhana* and *Marana*) while preparing *Bhasmas* (incinerated powders) of the metals. This applies to *Tamra* too. Impact of *Shodhana* has been proven in studies which reported that *Ashodhita Tamra* poses inconvenience at pharmaceutical, pharmacological levels and proven to be toxic at TED level.

Analytically *Tamra Bhasma* is reported to be either sulfide or oxide of copper. The differences in chemical forms of incinerated copper reveal that *Bhasmas* prepared in different media have different compositions and show different chemical reactions. Pharmacologically, mild degenerative changes in visceral organs were observed in acute, sub-chronic, and chronic toxicity studies at higher dose levels. It implies that properly prepared *Tamra Bhasma* is safe to be used under the supervision. The samples were proven to be potent anti-hyperlipidemic and cardio-protective agent.

The clinical efficacy of *Tamra Bhasma* has been studied in *Shvasa*, *Kasa*, *Yakrit Pliha Vriddhi*, *Grahani*, etc., The dose of *Tamra Bhasma* administered was ranging from 65 to 250 mg, and the duration was up to 45 days. The *Bhasma* was administered along with various suitable herbal powders and adjuvants like honey. Positive results with a decrease in the intensity of signs and symptoms were reported in all the studies. These studies were limited to a few number of cases, hence statistical significance did not draw. Since no adverse effects were reported in any of these studies, and satisfactory responses were noted by the patients; the results can be considered as a lead for further well stratified studies covering larger population. In all aspects, (pharmaceutical, pharmacological, and clinical) STB is reported to be better than *Tamra Bhasma*. All these studies prove that, the classical principles have their own scientific rationale and need to be followed mandatorily while processing in order to avoid the possibilities of unwanted effects.

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Sedative-hypnotic Effect of *Ash of Silver* in Mice: A Reverse Pharmacological Study

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ABSTRACT

Ash of silver is used in traditional systems of medicine for various neurological conditions like insomnias, neuralgias, anxiety disorders, and convulsions. The present study was conducted to evaluate the sedative-hypnotic activity of *ash of silver* in comparison to pentobarbitone (standard drug) in albino mice. The mice were divided into four groups as follows: Group 1 (control): Gum acacia [GA; 1% per os (p.o.)], group 2 (standard): Pentobarbitone [50 mg/kg intraperitoneal (i.p.)], group 3 (test): *Ash of silver* (50 mg/kg p.o.), and group 4: *Ash of silver* (50 mg/kg p.o.) given 30 min prior to administration of pentobarbitone (50 mg/kg i.p.). Time of onset, recovery, and total duration of loss of righting reflex were studied. *Ash of silver* (test) produced significant sedation ($P < 0.01$) compared to control (GA 1%), but the effect was significantly less compared to that of standard pentobarbitone at the doses used. Also, significant potentiation ($P < 0.001$) of the sedative-hypnotic effect of pentobarbitone was observed with the test drug.

Key words: *Ash of silver*, Pentobarbitone, Sedation

INTRODUCTION

Traditional systems of medicines have been in use for promotive, preventive, and curative health services since centuries in many parts of the world. Being the oldest traditional system of medicine in India, Ayurveda caters to about 80% of the population in developing countries as per the estimate of World Health Organization (WHO). Despite their wide usage, research in this field is lagging behind with regard to their pharmacologic actions, safety, and efficacy. Ashes or *Bhasmas* used in traditional system of medicine contain heavy metal particles in varying proportions. It is not easy to write off the usage of these preparations just by presuming that heavy metals are toxic. Proper scientific documentation is required to validate the risks and benefits associated with use of such metallic Ayurvedic preparations. There are some specific methods for their detoxification and *Bhasma* preparation, making them suitable for clinical use in therapeutic doses, as claimed by *Rasa Shastra* experts. There is a need to ascertain whether the conventional *Shodhan* (purification) process of Ayurveda is being properly followed or not.[1-3]

Silver is one among the heavy metals which is considered to be a non-essential accumulative trace element with wide distribution in the body, including the central nervous system, but with no known biological and physiological function.[4-6] In Ayurveda, preparations like *Raupya Bhasma* and *Kusta Nukras* have been used to treat many clinical conditions such as pain, inflammation, insomnia, neuralgias, anxiety disorders, convulsions, memory loss, heat stroke, infections, pro-myelocytic leukemias, sexual disorders, etc., for many centuries. Apart from herbs/shrubs, ashes of silver are prescribed. This system also advocates the use of elemental or metallic preparations.[7-9] Metal *Bhasmas* of gold (e.g., gold disodium thiomalate and auranofin) and silver (*Raupya Bhasma,*

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Kusta Nukras, etc.) have been used for the treatment of rheumatoid arthritis, acute pro-myelo cytic leukemias, immunostimulation, and as analgesics in painful inflammatory conditions, and are prescribed with accompaniments such as ginger or cumin water, tulsi extract, lemon extract, etc., that have been shown to protect against unwanted toxicity due to various reasons which include high proportions of trace elements and have synergistic or protective effects due to buffering between various constituents or free radical scavenging property. Oxides of heavy metals are usually not toxic, as claimed by *Rasa Shastra*.[2,10,11]

Since the safety of *ash of silver* has already been established, reverse pharmacological studies are required to confirm the established facts regarding usage, safety, and efficacy in various clinical conditions mentioned above. Since raw silver ore is considered hazardous for health as mentioned in Ayurvedic literature, it needs to be converted into non-harmful form (*ash of silver*) by the process of trituration, pulverization, and repeated calcinations (at 300°C) for 14 times. Reduced form of silver thus obtained acquires spherical nanostructure with a size of 16 nm without any change in the morphology of silver, and is now called as *ash of silver*.[4,7,9] Nanosize of the silver particle is probably responsible for improving the penetration of silver in brain; hence, *ash of silver* had been used in the past for treatment of various neurological conditions, viz. insomnias, anxiety, and pain.[9,10] Being a heavy metal preparation, *ash of silver* bears cumulative potential after prolonged use and in overdoses, as seen in preliminary animal studies. After certain controversial reports of toxicity due to use of metallic/elemental drugs, it has now been made mandatory (WHO guidelines) that Ayurvedic drugs in any form should be tested for their heavy metal content prior to export, so that heavy metals remain within permissible limits.[11-13]

This study was conducted with an aim (a) to explore the sedative-hypnotic effect of *ash of silver*, if any, as claimed in Ayurvedic literature. Further, if *ash of silver* showed sedative-hypnotic effect, the study aimed (b) to observe whether the test drug *ash of silver* was potentiating the sedative-hypnotic effect of pentobarbitone at the doses used in mice when given 30 min prior to the standard drug.

MATERIALS AND METHODS

Swiss albino mice of either sex weighing between 20 and 30 g were screened for the study, after obtaining approval from the Institutional Animal Ethics Committee. Mice were fed on a standard pellet diet and water *ad libitum*, and were housed in polypropylene cages under similar environmental conditions in an animal room that was maintained at 24 ± 1°C and 55 ± 5% humidity with a 12 h light–dark cycle throughout the experiment. In case of oral administration, mice were fasted for 12 h before testing. Plexiglass chamber was used to observe the responses of mice.

Drugs and dosage forms

The test drug *ash of silver* was procured from M/s Baidyanath Ayurved Bhawan Ltd (Jhansi, India). *Ash of silver* [50 mg/kg per os (p.o.)] was suspended in 1% solution of gum acacia. Gum acacia (1% p.o.), procured from Arora Pharmacy (New Delhi, India), was labeled as cont rol and was administered in a volume of 1 ml/100 g. *Ash of silver* and gum acacia were administered orally using infant feeding pipe with a 1 ml syringe attached at the other end. Standard sedative-hypnotic pentobarbitone [50 mg/kg intraperitoneal (i.p.)] was procured from Nembutal Dainippon Pharmaceutical Co. (Osaka, Japan) and was administered as i.p. injection using 1 ml syringe.

Animals and their grouping

Animals (mice) were divided into four groups consisting of six animals in each. Study protocol was as follows:

Group 1: Received vehicle gum acacia (1% p.o.) as control, given in a volume of 1 ml/100 g p.o.

Group 2: Received pentobarbitone (50 mg/kg i.p.) as the standard drug

Group 3: Received the test drug *ash of silver* (50 mg/kg p.o.) suspended in 1% solution of gum acacia

Group 4: Received the test drug *ash of silver* (50 mg/kg p.o.) suspended in 1% solution of gum acacia, following which the standard drug pentobarbitone (50 mg/kg i.p.) was given after 30 min

The responses of all drugs [in terms of time of onset, time of recovery, and total duration of loss of righting reflex (LORR) in mice] were assessed by continuous observation of animals throughout the experiments from the time of administration of drug in the plexiglass chamber, using a stop watch.

Measurement of the duration of pentobarbital-induced LORR

The duration of LORR was measured according to the procedures described by Marley *et al*.[14] Mice were given an i.p. injection of pentobarbitone (50 mg/kg). When the mice became ataxic, they were placed on their backs on a pre-warmed (37°C) pad and the onset, recovery, and total duration of LORR [starting at the time of administration of the test drug (*ash of silver*), the standard drug pentobarbitone (50 mg/kg), and the test drug (*ash of silver*) followed 30 min later by the standard drug pentobarbitone (50 mg/kg)] were noted until they regained their righting reflexes. Mice were presumed to have regained the righting response when they could right themselves three times within 30 sec.

RESULTS

Findings of the present study are depicted in Table 1.

All values were expressed as Mean ± SEM and analyzed using analysis of variance (ANOVA) followed by Dunnett's "*t*" test. $P < 0.05$ was considered significant.

DISCUSSION

In the present study, we tried to explore the pharmacological effect of *ash of silver* as a sedative-hypnotic and its secondary effect to potentiate the sedative-hypnotic effect of pentobarbitone. Such studies help to fast track drug discovery and development when carried out in selected animal models through screening. Thus, Ayurvedic knowledge and experimental database are able to provide new functional leads, thereby reducing the toxicity of drugs and saving time and money.[13]

Table 1. Effects of various drug treatments on sleep in mice

Group no. (*n*=6 in each group)	Drug treatment with dose and route of administration	Righting reflex in mice (in min) (mean±SEM)		
		Time of onset of loss of righting reflex (O)	Time of recovery (R)	Total duration of loss of righting reflex (R-O)
1	Vehicle, gum acacia (1% p.o.)	No effect seen	No effect seen	No effect seen
2	Pentobarbitone sodium (50 mg/kg i.p.)	11.83±0.74**	33.13±0.43**	21.33±0.57**
3	Ash of silver (50 mg/kg p.o.)	29.33±0.99*	40.07±0.68*	10.74±0.52*
4	Ash of silver (50 mg/kg p.o.) + pentobarbitone sodium (50 mg/kg i.p.)	9.78±0.48**	37.10±0.57**	27.32±0.61**

***P*<0.001(highly significant), **P*<0.01(statistically significant)

In the present study, *ash of silver* was observed to possess sedative effect at a dose of 50 mg/kg (p.o.) in mice. The sedative effect was significant ($P < 0.01$) when compared with the vehicle gum acacia (1% p.o.) in mice. The sedative effect of the test drug *ash of silver* was significantly less ($P < 0.01$) compared to pentobarbitone (50 mg/kg i.p.), the standard drug ($P < 0.001$). Significant potentiation ($P < 0.001$) of the sedative-hypnotic effect of pentobarbitone (50 mg/kg i.p.) was observed with 30 min prior administration of the test drug *ash of silver* (50 mg/kg p.o.).

The above findings reveal that *ash of silver* had sedative effect at the doses used; therefore, it can be proposed that *ash of silver* might be acting as a sedative-hypnotic owing to its pharmacological effects probably mediated by inhibition of neuropeptide S (NPS) or *N*-methyl-d-aspartate (NMDA)/histamine/5-HT3/dopamine or potentiation of effects mediated through gamma-aminobutyric acid (GABA)/glycine or benzodiazepines (BZDs)/opioid receptors. NPS was recently identified as the endogenous ligand of an orphan receptor, now referred to as the NPS receptor. *In vivo*, NPS produces a unique behavioral profile by increasing wakefulness and exerting anxiolytic-like effects.[15-19]

To explore the mechanism of action of *ash of silver* as a sedative, antagonists/blockers need to be administered against the above-mentioned mediators. In our previous study, we tried to explore the analgesic activity of *ash of silver*, which is probably mediated through opioid receptors as it was observed after administering naloxone, the opioid antagonist, although the role of other mediators cannot be ruled out.[13] One of the studies has proposed that ashes of heavy metals used in traditional systems of medicine function as a catalyzer by their presence in intestine, plasma, and blood, thereby acting as free radical scavengers.[8,9,11] Ash particles of heavy metals (gold, silver) in calcined form, being insoluble, exist as nanoparticles (16 nm), which are very tiny particles and biocompatible, and therefore can cross the blood–brain barrier to exert various central actions as claimed in Ayurvedic literature, viz. analgesic, anti-inflammatory, sedative, anti-anxiety, cognitive, neuroleptic, and antiepileptic.[4-7,11,20] Lankveld *et al.* and Kim *et al*. have proved the distribution of nanosized silver particles in the central nervous system as well as in other tissues, e.g., liver, kidney and spleen, and intestine, when administered by the oral route.

As the findings of our study have shown significant potentiation of sedative-hypnotic effect of pentobarbitone with 30 min prior administration of *ash of silver* at the dose used, it can be hypothesized that there is a possibility of *ash of silver* acting as hepatic cytochrome P450 microsomal enzyme inhibitor. Further, it can be hypothesized that *ash of silver* increases the plasma levels of pentobarbitone by inhibiting its hepatic metabolism, so as to potentiate the sedative/anesthetic effect of pentobarbitone. Pentobarbitone is mainly metabolized in the liver. Further studies are required to confirm and establish this fact also by intracerebroventricular injection of *ash of silver* in the brain of mouse/rats.

Till date, hardly any studies have been conducted to explore the pharmacological effects of *ash of silver* on sedation/sleep in human body, in spite of its wide use in humans in Ayurvedic practice for many centuries. Studies conducted by Nadeem *et al*. on silver preparations showed their interesting anti-anxiety, anti-cataleptic, and anti-aggressive effects. No scientific reports are available to confirm these claims except for some preliminary experimental studies demonstrating slight diminution of discharge frequency in frog nerve-muscle preparation bathed in 3% suspension of *ash of silver*. The anti-anxiety and anti-aggressive effects observed by Nadeem *et al.* support the nerve-soothing properties (nervine tonic) of silver preparations.[11]

In one study, there is a mention of phytochelatins (PCs), produced from reduced glutathione present in green plants and legumes, which tend to chelate the heavy metals from soil. Therefore, it can be postulated that in the presence of vegetarian diet, a fraction of *ash of silver* is also liable to get chelated if taken with vegetarian food.[21-23] Also, the same theory can be applied to overcome the toxic effects produced by excess dose of *ash of silver*, as mentioned in the study of Inder *et al*. Pharmacokinetic and pharmacodynamic studies need to be carried out to find if there is any interaction of *ash of silver* if taken with vegetarian diet, so as to formulate and revise the dose for human use. The role of free radical scavengers needs to be established, which can help to reduce the adverse effects of *ash of silver*. One of the studies has shown the interaction of *ash of silver* with some biomolecules, proteins, vitamins, etc., thus affecting various physiological reactions.[24-28] From the observations and results of the study with the test drug *ash of silver*, it can be postulated that at the doses used in mice (50 mg/kg p.o.), it acts as a mild to moderate sedative and owing to this property, it might have potentiated the duration of LORR effect of pentobarbitone (50 mg/kg i.p.), i.e., synergistic effect. But as both these drugs are metabolized by the liver to a greater extent, inhibition of cytochrome P450 enzymes system in the liver by *ash of silver* cannot be ruled out, which is probably responsible for inhibiting the hepatic metabolism of pentobarbitone resulting in high plasma levels of pentobarbitone, which might have potentiated the sedative-hypnotic effect of pentobarbitone at the doses used in mice.

CONCLUSION

Ash of silver possesses significant sedative hypnotic potential at a dose used in present animal study. Therefore it can serve as a better alternative as sleep inducing drug with a better safety profile compared to conventional hypnotics. Further studies will provide adequate data to support this evidence.

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Steven–Johnson syndrome may NOT be due to ayurvedic drugs

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Quick Response Code:

Sir,

A recently published article in IJP[1] (Steven-Johnson syndrome due to Ayurvedic drugs) has a number of flaws. The medicine quoted by the authors fulfills the criteria for "misbranded" drug rather than anything else. Authors say that the pills resembled small balls and were white in color. Usually, Ayurvedic pills are herbal extracts and no dosage forms in

Ayurveda resemble this description. It is somewhat suggestive of Allopathic or Homeopathic pills (if they are sweet in taste). As per the case report, the patient was under medication for 12 years. Sudden appearance of SJS (within 3 days) suggests some other etiology. SJS is said to be frequently associated with recent drug ingestion or autoimmune disease. The authors have given a vague list of drugs under the heading "Ayurvedic drugs" (Ginko, Echinasia, St. John's wart, Ginsing, etc.). These are western herbal drugs and not Ayurvedic medicines. The safety of Ayurvedic drugs (including that of *Swarna Bhasma*[2]) has been documented in several useful research works. Regarding *Parad* (mercury) preparations, these are not used in elemental form and are usually prescribed as sulfides and safety of these is also established.[3]

It is requested that the information on adverse drug reaction to Ayurvedic medicines, if any, should be passed onto the regional or national pharmacovigilance centers of AYUSH for proper registration. Although a technical term equivalent to "pharmacovigilance" does not feature in Ayurvedic texts, the spirit of pharmacovigilance is vibrant throughout Ayurveda's classical literature. Pharmacovigilance aims to improve patient care and safety during treatment, and thus to promote rational use of medications. These are the recurrent themes of Ayurvedic pharmacology (*Dravyaguna*), pharmaceutics (*Rasa Shastra* and *Bhaishjya Kalpana*), and therapeutics (*Chikitsa*).

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Swarna Bindu Prashana—an Ancient Approach to Improve the Infant's Immunity

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Abstract

Swarna bindu prashana (SBP) is a metallic medicinal preparation widely used in *Ayurveda* pediatrics. The main ingredients of SBP are swarna prashan (gold nanoparticle), gou ghrita (cow ghee), madhu (honey), and other medhya dravyas (drugs which enhance intellectual, memory). According to the Indian classical text, SBP has been proposed as a potent medicine for immunotherapies and vaccine development due to its indefinite size, shapes, charges, and surface functionality. In this review, we describe the plausible mechanism of SBP in dendritic cells maturation and subsequent T cell activation. But being herbo-metallic preparation, its safety and efficacy are well supported by the classical publications of *Ayurveda*. To conclude, SBP is an immune booster for infants against any viral disease, and it is necessary to validate its safety and efficacy through systematic methodological research.

Keywords Swarna bindu prashan (SBP) · Pediatric · Immune booster · Herbo-metallic · Immune-modulatory · Vaccine

Introduction

In *Ayurveda*, administration of the gold particles in children is considered to be a unique practice termed as swarna prashana [1]. The term swarna refers to gold and prashana refers to consuming or ingesting. Therefore, swarna prashana refers to the act of consuming or ingesting gold in the prescribed dose and quantity [2]. Gold is one among seven metal categorized pure metals which is mainly used for preventive and curative purposes. The benefits of children, who consumed swarna prashana, improve their intellectual, digestion and metabolism, physical strength, immunity, fertility, and lifespan [3–5].

For the past several decades, experimental evidence proved that gold nanoparticles (AuNPs) have become better biocompatible metal nanoparticles in disease diagnosis and therapeutics [5]. These nanoparticles can be synthesized by using various methods which include chemical and electrochemical radiation, photochemical method, and biological techniques [1]. Nanoparticles synthesized for therapeutics using physical and chemical techniques have major limitation, namely, toxic and hazardous chemicals being produced during the synthesis process [6]. To overcome this limitation, synthesis of nanoparticles using biological conjugates such as proteins, peptides, oligonucleotide, polysaccharides, fatty acid, and amino acids might be done reducing the toxic nature of the prepared nanoparticles [1]. These gold nanoparticles also possess antibacterial, anti-cancer, and anti-inflammatory properties [7, 8]. Similarly, synthesis of swarna prashana was also done by using biological conjugates such as ghee, honey, and herbs. This will increase the immunity of the infant to acts on pathogens, cancer cell, and inflammatory agent [4, 9, 10].

In swarna prashana, gold particles are encapsulated by honey, ghee, and herbs, and it helps the gold particles to form into various size, shape, charges, and composition. This irregular form of the gold particles in swarna prashana may induce the non-specific immunity by activating both cellular and humoral immunity [4, 9, 11, 12]. In general, pathogens undergo several mutations naturally or induced by man. Therefore, human system which acquired non-specific immunity will be ready to defend against any pathogens and inflammatory substances that enter or develop in our system [13]. It is evident that gold nanoparticles are efficiently interacting with the target cells in terms of immunological responses and cytotoxicity [14].

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At present, the whole world is facing one of the greatest pandemics (COVID-19) in a century caused by a novel coronavirus. As a result, COVID-19 patient exhibits severe acute immune response causing cytokine release syndrome and acute respiratory distress. In the future, to avoid this kind of pandemic situation, ancient immunization technique, namely, SBP, could play a vital role in developing the resistance against any viral disease [15]. SBP not only has the capacity to attenuate the manifested disease but also produces resistance to the offspring of next generation [3]. The purpose of this mini-review is to highlight its role, preparations, salient features, mode of administration, and plausible mechanisms of SBP in manipulating both cellular and humoral immunity.

Swarna Bindu Prashan

When the swarna bhasma is administered in very low dose for a particular time, it is known to increase the memory power along with immunity [4, 16]. Swarna prashana is easily absorbable in oxide form. There are such confusions regarding the mixing and absorption of swarna bhasma although it is the simplest form. Then, how come the simple ashudha swarna in the crude form gets absorbed is a matter of discussion. So here, swarna may remain unabsorbed in the body and act as an incompatible substance or binding material by playing a significant role in the stimulation of the immune system [1, 5, 8]. Gold has already proved its immune-modulatory effects because of its antibacterial action against different organisms, but when it is mixed with kinds of honey and clarified butter, it widens its spectrum of action to stimulate body immune cells [4, 5, 16]. It is administered orally on an empty stomach, preferably in the early morning. It can be given from birth up to 16 years of age. It is given with clarified butter and honey in a dose of two drops up to 6 months and four drops after 6 months. It can be given daily for a minimum of 30 days and maximum of 180 days. Alternately, it can be given every pushya nakshatra (every 28 days) for a minimum of 30 doses [3, 4].

SBP can be administered to all children from day 1 up to 5 years. It will be more beneficial in children with low immunity, low intellect, low memory power, and dyslexia [2, 4]. It is contraindicated among children suffering from fever, dysentery, and indigestion, etc.

Properties and Benefits of SBP It enhances the immunity, improves memory intelligence and appetite, and tones up the skin [4]. As per Kashyapa Samhita, SBP will enhance medha, bala, agni, aayu, varnya, pavitra, and manglyakarak. Therapeutically, it is used as ghraha badha and vrushya [17].

Mode of Action SBP Swarna bhasma has moisturizing and unctuous effect on the body, as its madhura helps as rasayana, and through detoxification can treat vishamjwara, antra jwara (Enteric fever), weakness, etc. Scientifically, it has been proved that swarna bhasma also possesses antioxidant, antidepressant, anti-cancerous, antibacterial, and anti-rheumatoid property and acts as a nerve stimulant [7, 16, 18, 19].

Toxicity Clinical tests revealed that SBP is free from toxicity as the ingredients used for its preparation are only after their non-toxic certification [20].

Side Effects Sometimes due to smell of medicine and different taste, babies may vomit it. Except this, no other side effects have been reported [1, 4, 20, 21].

Till date, there are no published research data available regarding its safety and efficacy. But there are few studies presented in national and international seminars/conferences, which support its safety and efficacy. Besides, some animal studies support its safety and efficacy.

Plausible Mechanisms of Swarna Prashana

Swarna prashana is related to the smaller gold particles perhaps containing wide variation in shapes, sizes, charges, and bio-molecular compositions [4]. These particles retain high stability, low toxicity, and immunogenicity conjugation due to the molecular ingredients found in ghee and honey [21]. The molecular ingredients contain sugars, amino acids, proteins, lipids, vitamins, and other components [10]. Moreover, these components help in capping the gold particles in swarna prashan which exhibits multivalent interactions between the particle and membrane receptor of antigen-presenting cells (APCs) such as dendritic cells [1, 4, 8]. Targeting these dendritic cells is considered to be one of the efficient strategies in promoting immunotherapies and vaccine development [8]. Therefore, the plausible mechanism of swarna prashana in interacting with dendritic cells is as follows: dendritic cells opt several mechanisms in the internalization of swarna prashana particles including receptor-mediated endocytosis, pinocytosis, and phagocytosis [1, 4, 5, 20]. Immature dendritic cells will uptake and internalize the swarna prashana particles in the cytosol. As a result, immature dendritic cells differentiate into mature dendritic that causes expression of CD83 and CD86 and also morphological changes in the maturation of the dendritic cells [8] (Fig. 1). The internalized particles, namely, antigens, are processed in the cytoplasm and initiate T cell response based on antigen presented through MHC complex [4, 7, 19]. Interestingly, swarna prashana particles comprise of mosaic features in terms of size, shape, charge, and composition of the particles which results in intercellular trafficking in dendritic cells [1, 4]. Therefore, dendritic cells present multiple antigens effectively to the T cells [20]. It is believed that the activated dendritic

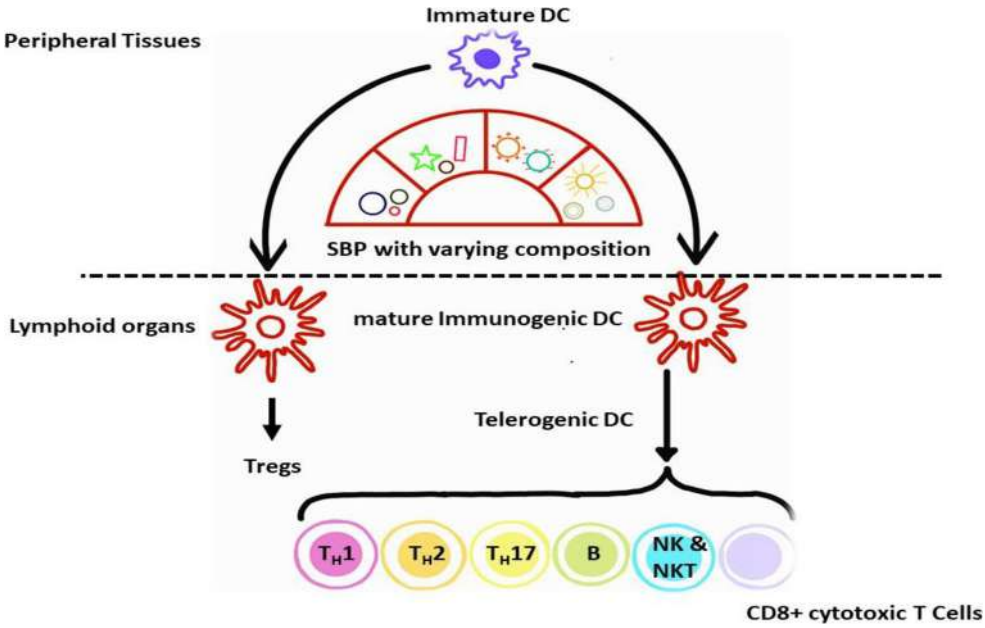

Fig. 1 Schematic representation of plausible mechanism of activation of immunogenic dendritic cells by SBP

cells and T cells require soluble cytokines including IL-7, IL-6, IL-10, IL-12, IL-23, TNF, and IFN to exhibit immunogenic response [4, 8]. The potential application of swarna prashana in immunomodulation is the development of both prophylactic and therapeutic vaccine [4, 8, 19]. Ancient scripts have suggested that the colloidal preparation of swarna (gold particles) with honey and ghee would significantly induce robust immunity like vaccines [4, 8, 10]. Gold particles are the most promising ones which do not affect living cells and do not produce adverse effects [20]. It is believed that gold is used in ayurvedic, herbal, and herbo-mineral preparations for the treatment of chronic and degenerative disease without any side effect [12, 13, 17]. The advantages of biodegradable gold particles are utilization in the vaccinated organism, high loading efficiency for the target substance, enhanced ability to cross various physiological barriers, and low systemic side effects. In all likelihood, the immune actions of biodegradable nanoparticles and gold nanoparticles as corpuscular carriers are similar. The recent data indicating low toxicity of gold nanoparticles makes it being used in the development of next-generation vaccines [1, 8, 16]. However, no extensive studies on animals or cell lines model are available, and further clinical trials are required on interaction of swarna prashana particles and human functions.

Conclusion

SBP is an immune booster used in pediatrics practice; its safety and efficacy are well supported by the classical texts of *Ayurveda*. In this review, we outlined the plausible mechanisms of swarna prashana in inducing immune system. It is also believed in older days that swarna prashana helps the child to grow up with a better immune system and intellectual performance. *Ayurveda* also explains about "vyadhikshamatwam" (immunomodulation), i.e., the individual's resistance to any infectious disease which includes both the capacity of attenuation against manifested disease and resistance to the offspring of the next generation. It is the ancient immunization technique with no adverse effect and provides a good life with physical, mental, and social health. So swarna prashana samskara should be accepted as immunization program. However, its safety and efficacy need to be validated through systematic methodological research.

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REVIEW ARTICLE

Therapeutic potentials of metals in ancient India: A review through *Charaka Samhita*

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ABSTRACT

The *Ayurvedic* system of medicine has stood the test of time for four millennia or more. The ancient seers found that drugs of different origin (herbal, metal or animal) in addition to codes of conduct and dietary regulations are suitable tools to maintain health in healthy and eradicating diseases in diseased. Use of metallic preparations in healthcare is a unique feature in this system. Processed metals including Mercury, Gold, Silver, Lead, Zinc, Copper etc. were used very frequently by seers of the Indian tradition in different disease conditions with great authority. It is generally claimed, that these metals are detoxified during the highly complex manufacturing processes described in *Ayurvedic*, especially *Rasashastra* texts. *Charaka Samhita*, one of the scheduled books of *Ayurveda* also holds ample of references regarding the use of metals for different purposes, which are summarized in the current paper.

Key words: *Ayurveda*, *Charaka Samhita*, lead, mercury, metals

INTRODUCTION

The *Ayurvedic* system of medicine has great antiquity, dating back to about 5000 years B.C. Its Materia Medica contain resources in the form of drugs derived from plant, animal, metal and mineral sources,[1] the use of which have been advocated in various different pathological manifestations. These drugs have also been converted in to poly-herbal, herbo-mineral and metallic compound formulations by the seers, who have documented their clinical experiences and passed on the knowledge to further generations.

During the medieval period, with the advent of *Rasashastra*, use of certain heavy metals and minerals in *Ayurvedic* therapeutics increased. *Rasashastra*, an integral part of *Ayurveda*, deals with the drugs of mineral origin, and details their varieties, characteristics, processing techniques, properties, therapeutic uses, possibilities of developing adverse effects and their management etc. in a comprehensive way. Although the roots of this science (*Rasa Shastra*) exist in the ancient texts of Indian civilization, its development as an independent system of therapy started around the 8th century A.D.. *Ayurvedic* classics written before that time, like *Charaka Samhita* and *Sushruta Samhita* etc. contain descriptions of metals and minerals, their processing techniques and their utilization in therapeutics etc.

In due course of time, herbo mineral and metallic preparations came to occupy a significant seat in *Ayurvedic* pharmacopoeia and have routinely been used in practice in different parts of India for many centuries. Such preparations are held to be safe, efficacious even in minute doses, and, when manufactured and used following specified classical guidelines, not to lead to any significant untoward effects.[2] The past decade, however, has witnessed concerns by the western scientific community, regarding the safety of *Ayurvedic* Herbal, Herbo-mineral and metallic preparations, which is a major concern for the age-old *Ayurvedic* heritage.[3]

This paper attempts to screen *Ayurvedic* classics for

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references emphasizing the utilization of metals for medicinal and other purposes. *Charaka Samhita* was scrutinized and found to contain referred frequent references to metals used for different purposes including medicinal and non medicinal ones. Seer *Charaka* advocated converting the metals in to fine powders before their utilization and observing great caution during the period of administration in different disease manifestations.

OBJECTIVES

The present paper is a compilation holding references pertaining to metals during the period of *Charaka Samhita* along with some information on the metals explained in textbooks of *Rasashastra* and modern science.

The references found in the classic were grouped into different categories with an intension to give an insight about the frequency of usage of the metals**.** The categories are as under:

- Utilization in therapeutics
  - Internal administration
  - External application
- Utilization in the preparation of equipment/instruments
- Other purposes etc.

Parada (Mercury)

Formulations containing mercury are only rarely mentioned in *Charaka Samhita*. The first reference pertaining to *Parada* and its utility in therapeutics mentioned in the classic is controversial,[4] only a few scholars interpret the term *Rasa* in the verse *chikitsasthana* 7/71 as *Parada*. The second reference is found in *Dwivraniya Chikitsa*, where the term *Rasa* is interpreted as *Parada* by the commentator *Chakrapani*.[5] It is interesting to note that, both these formulations are recommended for external application. [Table 1].

Though the term '*Rasa*' has been rendered as '*Parada*' by '*Chakrapani*', it is very difficult to claim that mercury was in practice during the period of *Charaka* for the following reasons.

1. The term '*Parada*' was not used by *Charaka* throughout the classic.
2. If the metal had been known to the physicians of those days, we might expect that it would have been discussed frequently, along with associated pharmaceutical procedures, and its combinations.
3. *Chakrapani* is silent and doesn't expressed an opinion on the term '*Rasa*' at *Chikitsa* 7/71, but interprets the same term in the earlier verse (*Chikitsa* 7/70) as 'expressed juice', while, at *Dwivraniya Chikitsa* (25/116), he interprets '*Rasa*' as *Parada*.
4. Later works like *Sushruta Samhita* (*Chikitsa* 25/39) and *Astanga Hridaya* (*Uttara* 13/36) preferred mercury as a component in topical applications. It was only later that the frequency of its internal utility increased. Probably, these developments might have been noticed by redactors and commentators like *Dridhabala* and *Chakrapani*, who expressed their opinions in their respective works.

Table 1: Depicting the references of Parada

Reference	Formulation	Uses
Utilization in therapeutics: External Application		
Chikitsa 7/71	*Lelitaka Prayoga*	*Kushtha* (Skin disorders)
Chikitsa 25/116	*Savarnikarana Lepa*	*Savarnikarana* (enhances complexion of the skin)

Swarna (Gold)

Swarna, the *Sara Lauha*[6] is an important, noble metal known to Indians since antiquity. References can be traced back to *Charaka* and *Sushruta Samhita* where the noble metal has been attributed with a wide range of applications. The '*Bhasma*' form of Gold is in metallic state. Quantitatively it is a combination of metallic Gold (96.76%), silica (1.14%), ferric oxide (0.14%), phosphates (0.78%), potash (0.16%), salt (0.078%), and traces of copper and magnesium.[7]

In its elemental form, Gold has been employed for centuries as an anti-pruritic agent to relieve itching palms. In 1980, Robert Koch observed that gold inhibits *Mycobacterium tuberculosis in vitro*. This led to trials on arthritis and lupus erythematosus. Also, previous studies carried out in 1973 on gold and its compounds observed beneficial activities at different levels. Gold compounds have the ability to decrease concentrations of rheumatic factors and influences the immunological responses.[53] It has also been established[54] that gold suppresses the anaphylactic release of histamine more effectively than gluco-corticoids. Sodium aurothio malate (water soluble preparation) was introduced around 25 years ago to treat arthritis, and is administered through IM injections. Its pharmacokinetics were not established but, its effects are probably due to its antimicrobial effects and stimulatIon of the Reticulo Endothelial system.[8]

Various formulations of '*Swarna*' are useful: *Vrishya*, *Balya*, *Rasayana*, *Medhya*, *Ayushya*, *Ojo Vardhaka*, *Vayah sthapaka* etc.[9] and disease alleviators particularly in chronic debilitating diseases like *Raja Yakshma*, *Swasa*, *Kasa*, *Pandu* etc.[10] Normal dose levels given for '*Swarna Bhasma*' is 15 mg. to 30 mg.[11] References pertaining to *Swarna* in *Charaka Samhita* have been depicted in Table 2.

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Table 2: Depicting the references of *Swarna* for different purposes

Reference	Formulation	Therapeutic uses
Utilization in therapeutics: Internal Administration		
*Chikitsa 1–1/58**	*Brahma Rasayana – II*	*Rasayana* (Rejuvenators)
Chikitsa 1–3/23	*Lohadi Rasayana*	
Chikitsa 1–3/25	*Indrokta Rasayana*	
Chikitsa 1–3/46	*Triphala Rasayana*	
Chikitsa 1–4/22	*Apara Indrokta Rasayana*	
Chikitsa 4/79	*Pana Yoga*	*Raktapitta hara* (Bleeding Disorders)
*Chikitsa 23/239***	*Curna Yoga*	*Visha hara* (Anti-poisonous)
Chikitsa 23/240	*Curna Yoga*	
Utilization in therapeutics: External Application		
Chikitsa 3/262	*Sheet of the metal*	*Pitta Jwara* (Fever of Pitta origin)
Chikitsa 21/131	*Curna Yoga*	*Granthi* (Abscess)
Preparation of Equipment / Instruments etc.		
Reference	**Description**	**Probable translation**
Sutra 5/74	*Jihva Nirlekhana Dravya*	Tongue Scrappers
Sharira 8/44	*Nabhi Kartana Dravya*	Scissors for cutting umbilical cord
Siddhi 3/7	*Vasti Netra Karnika Dravya*	Nozzle of enema pot
*Chikitsa 1-2/4****	*Suvarna Bhajana*	Vessels and Containers
Chikitsa 24/15 ****		
Chikitsa 24/154 ****		
Sharira 8/19	*Purusha Anupramanam under Pumsavana Karma*	A very minute sized idol of male gender
Sharira 8/34	*Teekshna Soochi Shastra*	Sharp instruments to be used in labor room
Other purposes		
Vimana 8/9	*Alankritam .....*	Wearing gold / gold ornaments
Vimana 8/11		
Sutra 5/18	--	General reference regarding Shodhana
Sutra 1/70	*Parthiva Dravya Ganana*	Sub-classification of the metal-based source

**Kanaka* one of the synonyms for gold referred even at *Brahma Rasayana* - I (*Chikitsa* 1–1/49) and at *Madhwasava* (*Chikitsa* 7/49), which has been clarified as *Nagakesara* by the commentator *Chakrapani*. **The dose of *Swarna Churna* (powder of gold) mentioned here is 1 *Shana* (3 g), ***Pharmaceutical procedure is to be carried out in Gold vessel. ****Water stored in gold vessel is to be consumed.

Rajata (Silver)

Rajata (Silver), another noble metal like gold, also attracted the attention of the ancient *Acharyas*. The use of silver in therapeutics dates back to the period of *Charaka* and his contempories. Though, its therapeutic applications are not as extensive as other metals like *Tamra* or *Loha*, the ancient classics reveal that silver also enjoyed an important place in Ayurveda therapeutics.

Classics of alchemy say s that, samples of *Rajata* which are clear, lustrous (*Swachha*), heavy (*Guru*), and with metallic sheen (*Snigdham*), and which also become bright white on heating or cutting (*Dahe Chede Samaprabham*), without any ridges or furrows (*Sphota rahitam*), is genuine, and can be considered acceptable for therapeutic purposes.[11] Quantitatively '*Rajata Bhasma*' is a combination of metallic silver (52 to 59%), free sulphur (0.675%), ferric oxide (14.33%), calcium (10.769%), silver chloride (0.479%), and traces of sodium, potassium and aluminium.[13] Various useful formulations of '*Rajata*' are: *Balya*, *Rasayana*, *Medhya*, *Ayushya*, *Ojo Vardhaka*, *Vayah sthapaka* etc. The normal dosage range given for '*Rajata Bhasma*' is 30 mg. to 120 mg.[14] The references of *Rajata* in *Charaka Samhita* as one of the components are listed in Table 3

Tamra (Copper)

Tamra (Copper) is another ancient metal known to human civilization. During pre-Vedic times, the metal was part of day-today livelihood functions. Further, it is the earlier known metal for the preparation of the stronger alloy metals brass and bronze of which it is a component. *Charaka* uses the term *Arka* in a few places which *Chakrapani* clarifies as synonymous with *Tamra*.[15] According to the descriptions of *Rasa* in *Vagbhata* Ref. there are two forms of *Tamra* viz. *Nepaliya* and *Mlechha*, only the former being acceptable. Samples with characteristic metallic sheen (*Snigdham*), soft (*Mridulam*), bright reddish in color (*Shonam*), having high tensile strength (*Ghanaghata Ksamam*), heavy (*Guru*), and devoid of impurities (*Nirvikaram*) are identified as best used for medicinal purposes.[16]

Formulations of '*Tamra*' are useful in a wide range of diseases like *Krimi*, *Sthaulya*, *Arsha*, *Ksaya*, *Pandu*, *Kusta*, *Swasa*, *Kasa*, *Amlapitta*, *Sotha*, *Sula*, *Yakrit Roga* and *Grahani dosha* etc.[17] In addition, *Charaka* advocates the use of *Tamra Patra* (copper

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Table 3: Depicting the references of Rajata for different purposes

Reference	Formulation	Therapeutic Uses
Utilization in therapeutics: Internal Administration		
Chikitsa 1–1/58	*Brahma Rasayana* - II	*Rasayana* (Rejuvenators)
Chikitsa 1–3/23	*Lohadi Rasayana*	
Chikitsa 1–4/22	*Apara Indrokta Rasayana*	
Chikitsa 16/78	*Tapyadi Loha*	*Pandu Roga* (Hematinics)
Chikitsa 16/82	*Yogaraja*	
Chikitsa 17/126	*Muktadi Curna*	*Hicca* (hiccup), *Swasa* (respiratory distress)
Preparation of Equipment / Instruments etc.		
Reference	**Description**	**Probable Translation**
Sutra 5/74	*Jihva Nirlekhana Dravya*	Tongue scrappers
Siddhi 3/7	*Vasti Netra Karnika Dravya*	Nozzle of enema pot
Siddhi 9/51	*Pushpa Netra Dravya*	
Sharira 8/9 **	*Rajata Patra*	Silver containers
Chikitsa 1-2/4 *		
Chikitsa 24/15 **	*Rajata Bhajana*	
Chikitsa 24/154 **	*Rajata Patra*	
Sharira 8/19	*Purusha ... anupramanam* under *Pumsavana Karma*	Idol of male gender
Sharira 8/34	*Teekshna Soochi Shastra*	Sharp instruments to be placed in labor room
Sharira 8/44	*Nabhi Kartana Dravya*	Scissors for cutting umbilical cord
Other purposes		
Vimana 8/9	*Alankritam .....*	Wearing gold / gold ornaments
Vimana 8/11		
Sutra 1/70	*Parthiva Dravya Ganana*	Classification of the metal based on the source

*Pharmaceutical procedure is to be carried out in Silver vessel, **Water stored in Silver vessel is to be consumed

vessels) in several pharmaceutical procedures.[18] Normal doses mentioned for '*Tamra Bhasma*' is 15 mg to 60 mg. [19] The references mentioned in *Charaka Samhita* that hold *Tamra* as one of the active components are listed in Table 4.

Aayasa or *Loha* (Iron)

Next to *Swarna*, *Rajata* and *Tamra*, *Loha* or *Ayasa* is another metal known to ancient civilizations. During the period of *Charaka*, it was used in different dosage forms named *Curna*, *Vati*, *Avaleha*, *Varti*, *Asavarishta* etc. either for external or internal administration in a number of pathological manifestations. Iron compounds were particularly employed in diseases suchas anaemia and other debilitating conditions, where functions of hemopoitic systems are disturbed and the blood has consequently become deficient in iron. *Rasa Shastra* classics explain that *Loha* is par excellence a rejuvenator as it stimulates functional activity of all the organs, promotes life, strength, destroys a number of diseases, and acts as a restorative.[20] The utility of this metal in therapeutics was only identified modern medicine in the first half of the 17th century, when its salts were recognized as the best haematenics. According to descriptions in *Rasa Vagbhata*, there are three varieties of *Loha* viz. *Munda*, *Teekshna* and *Kanta*, the latter being the best variety to use.[21]

As preparations of *Loha* are of foremost importance in Ayurveda therapeutics, proper care should be taken during procedures for its purification and incineration. *Chakrapani* stresses the need to take care when administering it.[22] *Charaka* emphasizes a special *Ayaskriti* procedure, which converts thin leaves of metal into a fine absorbable form.[23] In addition to these uses, iron vessels were specifically recommended to be used in certain pharmaceutical procedures (*Chikitsa* 1-3/43, 15/187, 16/83, 26/250, 26/274 etc.) Quantitatively, it is a combination of ferric oxide (96.5%), ferrous oxide (2.5%), magnesium oxide (0.8%), calcium oxide (0.3%), together with traces of phosphorus and potassium. Different formulations of '*Loha*' are useful in a wide range of diseases: *Sula*, *Arsha*, *Gulma*, *Pliha Roga*, *Yakrit Roga*, *Ksaya*, *Pandu*, *Kamala* etc.[24] Normal dose levels given for '*Loha Bhasma*' are 30 mg. to 240 mg.[25] List of the formulations mentioned in *Charaka Samhita* that hold *Loha* as one of the active components are depicted in the Table 5.

Mandura

Mandura, the second form of Iron, has been used for a wide range of therapeutic procedures in classical Ayurveda since antiquity. It is defined by *Madhava Upadhyaya* in the *Ayurveda Prakasha* as the debris collected after heating and beating processes of Iron around a blacksmith's anvil.[26] Generally, *mandura* is collected from sources like old anvils, and is considered to be very useful, if they are about 100 years old.

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Table 4: Depicting the references of Tamra for different purposes

Reference	Formulation	Therapeutic Uses
Utilization in therapeutics: Internal administration		
Chikitsa 1–1/58	*Brahma Rasayana* - II	*Rasayana* (Rejuvenators)
Chikitsa 1–4/22	*Apara Indrokta Rasayana*	
Chikitsa 17/126	*Muktadi Curna*	*Hicca* (hiccup), *Swasa* (respiratory distress)
Chikitsa 23/239	*Curna Yoga*	*Visha hara* (Anti-poisonous)
Utilization in therapeutics: External application		
Chikitsa 7/86	*Lepa Yoga*	*Kusta* (Skin disorders)
Chikitsa 21/131	*Curna Yoga*	*Granthi* (Abscess)
Chikitsa 26/246	*Sukhavati Varti*	Collyrium for *Akshi Roga* (Eye disorders)
Preparation of Equipment / Instruments etc.		
Reference	Description	Probable translation
Sutra 5/74	*Jihva Nirlekhana Dravya*	Tongue scrappers
Chikitsa 7/117*	*Tamra Bhajana*	Copper containers
Chikitsa 26/255 *		
Siddhi 3/7	*Vasti Netra Karnika Dravya*	Nozzle of enema pot
Other purposes		
Sutra 1/70	*Parthiva Dravya Ganana*	Classification of the metal based on the source
Sutra 1/131	*Visha Kwathita Tamra*	Simile for disrespect of a quack

*Pharmaceutical procedure is to be carried out in copper vessel.

Samples of ages 80 years and 60 years old are respectively considered moderately and least efficacious for therapeutic purposes.[27] According to the literature Mandura, which is smooth to touch (*Snigdha /Masruna*), heavy (*Guru*), strong (*Dridham*), without any fissures or furrows (*Kotaravarjitam*), and taken from age old constructions (*Jirna nasta purastham*) is genuine and can be used for therapeutic purposes.[28]

Purified *mandura*, when administered with proper justification is beneficial in inflammations, edematous conditions, jaundice etc. It is the drug of choice in cases of anaemia (*Pandu*), and *Charaka* refers to a number of its preparations. Chemically, *Mandura* is a combination of ferric oxide (59.14%), ferrous oxide (26.7%), chlorides (4.4%), magnesium (3.9%), sodium (1.7%) and a few other elements in trace quantities. Its unique constitution plays a pivotal role in therapeutics of anaemia and other associated disorders.[29] The normal dose given for '*Mandura Bhasma*' is 30 mg. to 240 mg. 30 Few of the formulations mentioned in *Charaka Samhita* that hold *Mandura* as one of the active components are depicted in Table 6.

Naga/Sisaka (Lead)

Naga is an important *Puti Loha* known since ancient times, also identified by other terms like *Sisaka* or *Sisa*. *Charak* emphasizes that medicinal uses of this metal should be external, partiicular in cases of *Mandala Kusta*. The *Brihad Rasa Raja Sundara* describes two varieties of *Naga* viz. *Kumara* and *Samala* the former being the acceptable variety for therapeutic applications. Samples which melt easily (*Drutadravam*), and are heavy (*Mahabharam*), externally black in color (*Bahihkrishnam*), and when incised shine with bright black luster (*Chede Krishna Samujwalam*) should be considered genuine and preferred for therapeutic purposes.[31] Quantitatively *Naga Bhasma* is a combination of lead oxide (75.6%), ferric oxide (7.5%), together with traces of calcium and magnesium chlorides and carbonates.

Different formulations of '*Naga*' are beneficial in diseases like *Prameha*, *Gulma*, *Arsha*, *Sweta Pradara*, *Grahani roga*, *Antra sotha* etc.[32] Therapeutic dosages given for '*Naga Bhasma*' range from 30 mg. to 120 mg. [33] References mentioned in *Charaka Samhita,* that hold *Naga* as one of the components are depicted in Table 7.

Vanga/Trapu (Tin)

Vanga, one of the *Puti Lohas* was known to ancient Indian physicians by the name of *Trapu*. In *Charaka Samhita*, the metal is categorized under *Parthiva Dravyas*. According to descriptions in *Rasa Vagbhata*, there are two varieties of *Vanga* viz. *Khuraka* and *Mishraka*, the former being acceptable for therapeutic applications. Samples with the characteristics, bright white in color (*Dhavalam*), soft (*Mridulam*), shiny, smooth (*Snigdham*), easily melts (*Drutadravam*), and heavy (*Guru*) are identified as *Khura Vanga* and should be preferred for therapeutic purposes.34 Quantitatively *Vanga Bhasma* is a combination of stannic oxide (i.e. of tin) (91.4%), ferric oxide (2.9%), potassium (2.9%), calcium oxide (2%), aluminium (2%) and magnesium (0.6%) oxides.

Formulations of '*Vanga*' are variously beneficial in diseases such as: *Prameha*, *Kasa*, *Shwasa*, *Krimi*, *Ksaya*, *Pandu*, *Pradara*, *Garbhashaya Chyuti* etc.[35] Singly or in combination with other *puti lohas*, it is beneficial in disorders of the Genito Urinary Tract. It has also been said that, Vanga Bhasma is

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Table 5: Depicting the references of *Loha* for different purposes

Reference	Formulation	Therapeutic uses
Utilization in therapeutics: Internal Administration		
Sutra 21/23	*Sthaulyahara Yoga*	*Sthaulya* (Obesity)
Chikitsa 1–1/58	*Brahma Rasayana* - II	*Rasayana* (Rejuvenators)
Chikitsa 1–3/16	*Lohadi Rasayana*	
Chikitsa 1–3/52	*Shilajatu Rasayana*	
Chikitsa 1–4/22	Apara Indrokta Rasayana	
Chikitsa 7/74	*Madhwasava*	*Kusta* (skin diseases), *Kilasa* (leukoderma)
Chikitsa 12/21	*Shophahara Yoga*	*Kaphaja Shopha* (inflammation of Kapha origin)
Chikitsa 12/39	*Triphaladyarishta*	*Arsha* (hemorrhoids), *Pandu* (anemia)
Chikitsa 12/42	*Shophahara Yoga*	*Chiraja Shopha* (chronic inflammation)
Chikitsa 12/43	*Ksara Gutika*	*Arsha* (hemorrhoids), *Pandu* (anemia) etc.
Chikitsa 13/73	*Shamana Yoga*	*Kaphaja Udara* (Ascitis of *Kapha* origin)
Chikitsa 15/188	*Panchama Ksara*	*Grahani* (malabsorption syndrome), *Pandu* (anemia)
Chikitsa 16/69	*Panduhara Yoga*	*Pandu* (anemia)
Chikitsa 16/70	*Navayasa Curna*	
Chikitsa 16/82	*Yogaraja*	
Chikitsa 16/97	*Kamalahara Yoga*	*Kamala* (jaundice)
Chikitsa 16/98		
Chikitsa 16/99		
Chikitsa 16/105	*Gaudarishta*	*Pandu* (anemia)
Chikitsa 16/119	*Panduhara Yoga*	*Mridbhakshana Pandu* (anemia of pica origin)
Chikitsa 17/126	*Muktadi Curna*	*Hicca* (hiccup), *Swasa* (respiratory distress)
Chikitsa 17/129	*Swasahara Yoga*	
Chikitsa 30/84	*Yonirogahara Yoga*	*Yoni roga* (disorders of female genital tract)
Utilization in therapeutics: External Application		
Sutra 14/26	*Swedana Dravya*	Material for sudation
Chikitsa 5/62	*Daha Karma*	*Gulma* (abdominal lump)
Chikitsa 7/88	*Kustahara Yoga*	*Kusta* (skin diseases)
Chikitsa 7/171	*Kilasahara Lepa*	*Kilasa* (leukoderma)
Chikitsa 9/80	*Sparsha Chikitsa*	*Unmada* (psychosis)
Chikitsa 21/131	*Curna Yoga*	*Granthi* (abscess)
Chikitsa 25/103	*Agni Karma*	*Kaphaja Granthi* (abscess of Kapha origin)
Chikitsa 25/115	*Savarnikarana Lepa*	*Savarnikarana* (provides complexion to the skin)
Chikitsa 26/246	*Sukhavati Varti*	Collyrium for *Akshi roga* (eye disorders)
Chikitsa 26/250	*Sukhavati Varti*	Collyrium for *Akshi roga* (eye disorders)
Chikitsa 26/254	*Dristiprada Varti*	Collyrium for *Akshi roga* (eye disorders)
Chikitsa 26/280	*Lepa Yoga*	*Khalitya* (alopecia)
Chikitsa 26/282		
Preparation of Equipment / Instruments etc.		
Reference	Description	Probable Translation
Sharira 8/19	*Purusha ... anupramanam* under *Pumsavana Karma*	Idol of male gender
Sharira 8/34	*Teekshna Soochi Shastra*	Sharp instruments to be placed in labor room
Sharira 8/44	*Nabhi Kartana Dravya*	Scissors for cutting umbilical cord
Chikitsa 25/82	*Shalaka Nirmana*	Metallic probes
Chikitsa 1–3/43 *	*Ayasa Patra* / *Ayasa Bhanda* / *Ayasa Bhajana* / *Loha Patra*	Iron Vessel / Container
Chikitsa 7/75 *		
Chikitsa 15/187 *		
Chikitsa 16/82 *		
Chikitsa 26/274 *		
Other purposes		
Sutra 1/70	*Parthiva Dravya Ganana*	Classification of the metal based on the source
Sutra 1/131	*Visha Kwathita Tamra*	Simile for disrespect of a quack
Chikitsa 12/7	*Swayathu Hetu*	Creator of inflammation

*Pharmaceutical procedure is to be carried out in Iron Vessel / Container

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Table 6: Depicting the references of Mandura

Reference	Formulation	Uses
Utilization in therapeutics: Internal Administration		
Chikitsa 16/74	*Mandura Vataka*	*Pandu* (anemia)
Chikitsa 16/78	*Tapyadi Yoga*	
Chikitsa 16/95	*Punarnava Mandura*	
Chikitsa 16/103	*Mandura Vataka*	

Table 7: Depicting the references of Naga for different purposes

Reference	Formulation	Uses
Utilization in therapeutics: Internal Administration		
Chikitsa 17/126	*Muktadi Curna*	*Hicca* (hiccup), *Swasa* (respiratory distress)
Utilization in therapeutics: External Application		
Chikitsa 7/88	*Lepa Yoga*	*Kusta* (skin diseases)
Other purposes		
Sutra 1/70	*Parthiva Dravya Ganana*	Classification of the metal based on the source

Table 8: Depicting the references of Vanga for different purposes

Reference	Formulation	Uses
Utilization in therapeutics: External Application		
Chikitsa 7/88	*Lepa Yoga*	*Kusta* (skin diseases)
Preparation of Equipment / Instruments etc.		
Reference	Description	Probable Translation
Sutra 5/74	*Jihva Nirlekhana Dravya*	Tongue scrappers
Siddhi 3/7	*Vasti Netra Karnika Dravya*	Nozzle of enema pot
Other purposes		
Sutra 1/70	*Parthiva Dravya Ganana*	Classification of the metal based on the source

the drug of choice in the case of *Prameha*.[36] Therapeutic doses given for '*Vanga Bhasma*' range from 120 mg. to 240 mg.[37] The references mentioned in *Charaka Samhita*, which hold *Vanga* as one of the components are depicted in Table 8

Pittala (Brass)

Pittala is an important *Misra Loha*, an alloy of *Copper* and *Zinc*, known since the period of *Samhita Kala*. *Charaka* used this metal to prepare *Vasti netra*. It is known as Brass. As per the descriptions available in *Rasa Ratna Samuchaya*, there are two varieties of *Pittala* viz. *Ritika* and *Kakatundi*.

Formulations of '*Pittala*' are beneficial in diseases like *Krimi*, *Kusta*, *Pandu* etc.[38] The therapeutic doses given for '*Pittala Bhasma*' range from 60 mg. to 120 mg.[39] Very few references mentioned in *Charaka Samhita* that hold *Pittala* as one of the components are depicted in Table 9.

Table 9: Depicting the references of Riti for different purposes

Reference	Description	Probable Translation
Sutra 5/74	*Jihva Nirlekhana Dravya*	Tongue scrappers
Siddhi 3/7	*Vasti Netra Karnika Dravya*	Nozzle of enema pot

Table 10: Depicting the references of Kamsya for different purposes

Reference	Description	Probable Translation
Sharira 8/9*	*Kamsya Patra*	Containers of Bronze
Siddhi 3/7	*Vasti Netra Karnika Dravya*	Nozzle of enema pot
Chikitsa 24/154*	*Kamsya Bhajana*	Vessels and containers

*Water placed in Kamsya vessel is to be consumed.

Kamsya (Bronze)

Kamsya is another important *Misra Loha*, an alloy of Copper and Tin known since the period of *Samhita Kala*. *Charaka* used this metal to prepare *Vasti netra*. It is known as Bell Metal or Bronze. According to the descriptions given in *Ayurveda Prakasha*, there are two varieties of *Kamsya* viz. *Pushpa* and *Tailika*, only the former being acceptable for therapeutic applications. Samples giving a sharp sound (*Teekshna Shabdam*), soft (*Mridu*), smooth to touch (Snigdha), slightly grayish (*Eshat Shyamalam*), clear from impurities (*Shubhram/Nirmalam*) and turning red on heating (*Dahe Raktam*) possess the characteristic features of the material preferred for therapeutic purposes.[40]

Formulations of '*Kamsya*' are beneficial in diseases like *Krimi*, *Kusta* etc.[41] Therapeutic doses iven for '*Kamsya Bhasma*' range from 60 mg. to 120 mg.[42] Very few references mentioned in *Charaka Samhita* that hold *Kamsya* as one of the active components are depicted in Table 10.

DISCUSSION

It becomes clear from screening the classic *Charaka Samhita* that metals like Gold, Silver, Iron, Copper, Lead, Tin etc. as well as some alloys were used to treat a wide range of diseases. Fine powders of these metals were prescribed for both internal and external applications. References also can be traced where the metallic powders were applied to the eyes.[43] Emphasis is given to purification of metals and their conversion to micro-fine powders by following specified guidelines, processes which were termed '*Ayaskriti*'. *Charaka* suggest using these metallic preparations with great authority, stating them to be safe and efficacious if used judiciously. He also emphasizes that great caution is needed when using such metallic powders in therapeutics.

In addition to the therapeutic utilization; different

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metals ranging from gold to iron were also used in preparing equipments like *Jihva Nirlekhana Yantra* (tongue scrappers),[44] *Nabhi Kartana Yantra* (sharp instruments to cut umbilical cord),[45] *Vasti Netra* (nozzle of enema pot),[46] different *Anjana Shalakas* (metallic applicator for application of medicaments into eyes)[47] etc. Besides this, Charak recommends preparing containers and vessels with different metals like gold[48], silver[49], copper[50], iron[51] and some alloys[52] etc. where instructions for pharmaceutical procedures like boiling etc. are given.

Close scrutiny makes it clear that such metallic preparations have held a significant place in *Ayurvedic* pharmacopoeia since antiquity. In the recent past, western scientists have begun to focus on the toxic nature of metals like mercury, lead etc. Reviewing the *Ayurvedic* literature reveals that ancient scholars had considered the possibility of toxicity of metallic preparations, and emphasized the necessity of taking great care over this point. They evolved specific methods using various pharmaceutical techniques like *shodhana*, *jarana*, *marana* etc. which have their own significance in detoxifying and increasing the therapeutic potential of metals.

CONCLUSIONS

Reviewing *Charaka Samhita* reveals thatAyurveda utilized metals for various therapeutic and non-therapeutic purposes. The text emphasizes the need to observe great caution while using metals, and directs that they should be reduced to micro-fine powders through the specially designed process '*Ayaskriti*'. The reduced metals may contain associated compounds together with major elements, which have their own significance in the process of disease pacification. In addition, a few of the metallic powders also may provide nourishment, as they are a combination of many trace elements and electrolytes. In the recent past, some researchers have suggested that these metallic/mineral preparations are anti-oxidants which fight free radicals, anddisease causing organisms and also help in developing immunity.[55] Studies carried-out in different parts of India have indicated that, when metals and minerals are converted into medicines strictly adhering to the classical guidelines specified in ancient texts, they are devoid of any toxicity even at the level of 100 TEDs. Histo-pathological studies of visceral organs in these studies revealed no apparent changes. For example, a study on *Rasa Karpura*, established safety of the compound even at 40 TED.[56]

Furthermore, the effectiveness of *Ayurvedic* medicines is not usually due to single active ingredients, but, usually to complex mixtures of compounds which target the pathological manifestation in several different ways. Therefore, *Ayurveda* differs from systems of medicine using single ingredients.

Keeping all these in mind, it can be said that the damning reports such as those of unacceptable levels of heavy metals etc. in *Ayurvedic* preparations should not be considered cause for alarm. The concepts, practices and products of the *Ayurvedic* system of medicine are unique, and its therapeutic values are needed to be explored by utilizing and adopting sophisticated technology, Only then can ailing humanity benefit from its age-old remedies.

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X-Ray Diffraction of different samples of *Swarna Makshika Bhasma*

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Abstract

Introduction: *Shodhana* and *Marana* are a series of complex procedures that identify the undesirable effects of heavy metals/minerals and convert them into absorbable and assimilable forms. Study on the analytical levels is essential to evaluate the structural and chemical changes that take place during and after following such procedures as described in major classical texts to understand the mystery behind these processes. X-Ray Diffraction (XRD) helps to identify and characterize minerals/metals and fix up the particular characteristics pattern of prepared *Bhasma*. **Aim:** To evaluate the chemical changes in *Swarna Makshika Bhasma* prepared by using different media and methods. **Materials and Methods:** In this study, raw *Swarna Makshika*, purified *Swarna Makshika* and four types of *Swarna Makshika Bhasma* prepared by using different media and methods were analyzed by XRD study. **Results:** XRD study of different samples revealed strongest peaks of iron oxide in *Bhasma*. Other phases of Cu_2O, FeS_2, Cu_2S, $FeSO_4$, etc., were also identified in many of the samples. **Conclusion:** XRD study revealed that *Swarna Makshika Bhasma* prepared by *Kupipakwa* method is better, convenient, and can save time.

Key words: *Bhasma*, *Makshika*, *Marana*, *Shodhana*, X-ray diffraction

Introduction

Ayurveda and other traditional medicines mainly depend on herbal, herbo-mineral formulations; have to change their track and method of approach to convince the scientific world. Some recent criticism from the West against the metallic preparations has created uproar from the Ayurvedic fraternity globally. An analytical study is one of the vital parts for drug standardization in traditional systems of medicine helps to interpret the pharmacokinetics and pharmacodynamics of Ayurvedic drugs.

Physico-chemical analysis provides objective parameters to fix up the standards for quality of raw drugs as well as finished products. Since *Rasa Shastra* has physics and chemistry as its close ally, there is scope to seek laws of chemistry and physics for providing a relationship for changes that take place in the pharmaceutical process.[1] In depth knowledge of imaging techniques and familiarity with the fundamental properties of matter are providing invaluable support for mapping the structure and function of drugs at all levels. Recent advances in data gathering techniques such as X-ray diffraction (XRD), field emission scanning electron microscopy, energy dispersive X-ray analysis provide an unprecedented view of the structure as well as cell function of the drug at the molecular and atomic level. These techniques are used in Ayurvedic pharmaceutical industries to characterize the raw material and final products and to establish this ancient science on modern scientific parameters. Hence, these tests can be put parallel to Ayurvedic *Bhasma Pariksha* (test) for ensuring genuine *Bhasma* production. Considering this, an effort has been made to analyze the raw *Swarna Makshika*, purified *Swarna Makshika* and four samples of *Swarna Makshika Bhasma* through XRD study. Prior to subjecting the material to XRD study, attempts were made to examine the *Bhasma* through classical parameters of analysis. In this study, emphasis has been given to find out the chemical changes takes place in *Swarna Makshika Bhasma* prepared by different methods by following X-Ray Diffraction.

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Materials and Methods

Materials and methods used in different samples of *Swarna Makshika Bhasma* preparations are based on availability, descriptions in *Rasa Shastra* classics, traditional values, and expert opinion. Raw *Makshika* is procured from the Ayurvedic Pharmacy of IMS, BHU. The raw drug is identified on the basis of its *Grahya Lakshanas* (acceptable characters) as mentioned in *Rasa* literatures,[2,3] and experts opinion.

Shodhana

Raw *Swarna Makshika* was taken in a clean and dry *Khalva Yantra* (mortar), pounded well to prepare fine powder, shifted to a clean and dry iron pan and subjected to intense heat at about a temperature of 750–900°C. The iron pan was then closed with an iron lid to avoid loss of material due to dusting. This process was continued for 3 days after complete cessation of sulfur fumes and until the mixture became red like fire.[4]

Marana

Four samples of *Swarna Makshika Bhasma* were prepared by following classical guidelines as described in Ayurveda classics.

Sample 1

Shodhita Swarna Makshika was triturated with lemon juice, and *Chakrika* (pellets) were made. Properly dried and weighed *Chakrikas* were arranged in a *Sharava*, closed by another *Sharava* and sealed by cloth smeared with clay. Properly sealed and dried *Sharava Samputa* was subjected to *Puta* system of heating. Twelve numbers of *Puta* were required to produce genuine *Bhasma*.[5]

Sample 2

Shodhita Swarna Makshika was mixed with equal amount of *Shodhita Gandhaka* and triturated with lemon juice; pellets were made and subjected to *Puta* system of heating. From second *Puta* onwards the amount of *Gandhaka* was taken half of the *Swarna Makshika*. Total 11 *Puta* were required to prepare *Swarna Makshika Bhasma*.[6]

Sample 3

Shodhita Swarna Makshika was mixed with 1/8th part of *Shodhita Hingula* and triturated with lemon juice; pellets were made and subjected to *Puta* system of heating. Total 09 *Puta* were required to prepare *Swarna Makshika Bhasma*.[7]

Sample 4

Shodhita Swarna Makshika was added with *Kajjali* and triturated with lemon juice till the material became homogenous and dried. The mixture was subjected for *Kupipaka* for 10 h. After breaking the *Kupi*, prepared *Swarna Makshika Bhasma* was collected from the bottom and *Rasa Sindura* was collected from the neck. Material collected from the bottom is further subjected to 6 *Putapaka* to prepare *Swarna Makshika Bhasma*.[8]

Analysis of samples

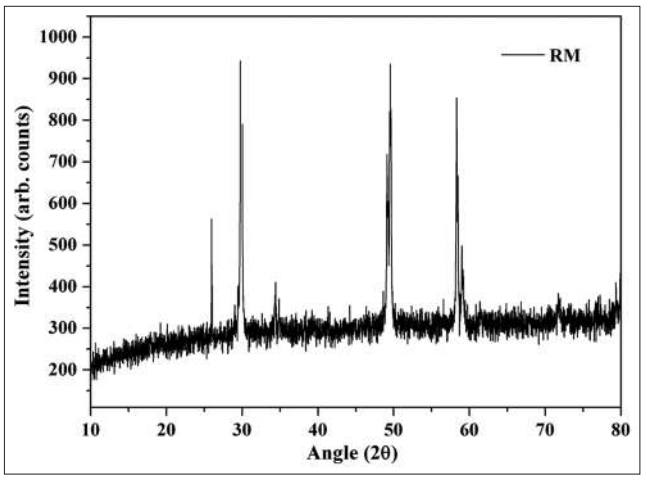
Samples of raw *Swarna Makshika*, *Shodhita Swarna Makshika*, and four samples of *Swarna Makshika Bhasma* were labeled and analyzed by XRD. The graph of each sample after comparing with Joint Committee on Power Diffraction Standards (JCPDS) data is illustrated in this study.

Observations and Results

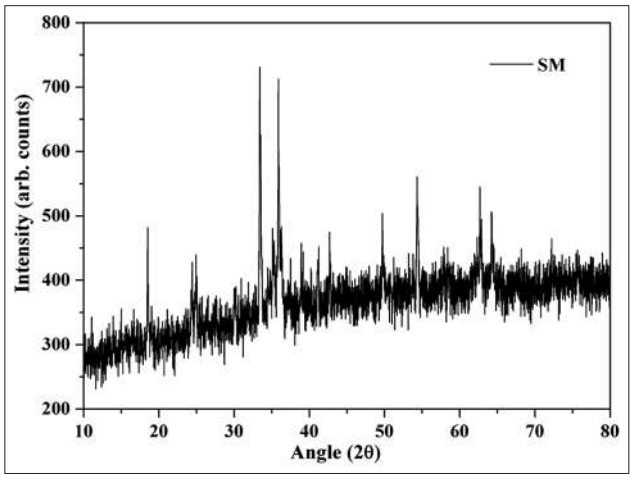
XRD study revealed that the strongest peaks identified in the raw material after comparing with JCPDS data was $CuFeS_2$ [Graph 1 and Table 1]. After *Shodhana*, the three highest peaks were identified as FeS_2, Fe_2O_3, and $FeSO_4$. Other peaks identified in *Shodhita Swarna Makshika* were Cu_2S and CuO [Graph 2 and Table 2]. Many complex compounds are also formed in *Shodhita Swarna Makshika*, but it is very difficult to detect them. Strongest peaks identified in sample 1 [Graph 3 and Table 3] sample 2 [Graph 4 and Table 4] sample 3 [Graph 5 and Table 5] of *Swarna Makshika Bhasma* is Fe_3O_4. In sample 4 of *Swarna Makshika Bhasma*, after *Kupipaka* [Graph 6 and Table 6] the peaks of $CuFeS_2$ again reappear and in the same sample, after *Putapaka* strong peaks of Fe_2O_3, Cu_2O, and $FeSO_4$ were identified [Graph 7 and Table 7].

Discussion

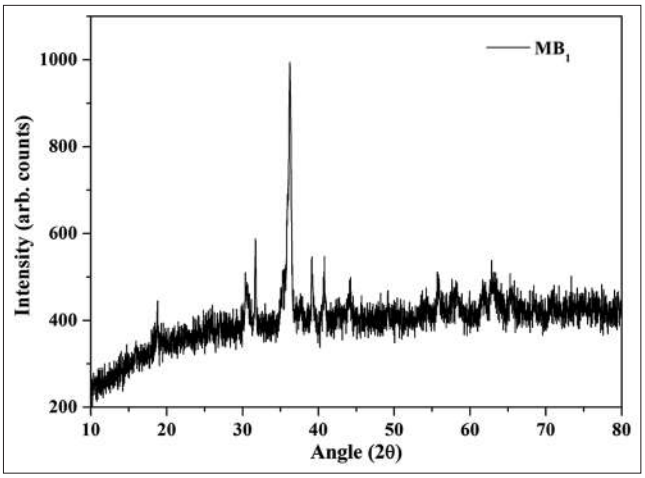
XRD of raw material reveals that the peaks obtained are corresponding to the peaks of $CuFeS_2$ in JCPDS file and hence the material is identified as copper pyrite. The strongest peak identified in *Shodhita* material (SM) was FeS_2. Other strong phases identified were Cu_2S, Fe_3O_4, $FeSO_4$, and SiO_2. The strongest peak identified in sample MB_1 was Fe_3O_4. In this sample, second and third strong peaks were identified as Fe_3O_4 and $FeSO_4$. The strongest peak identified in sample MB_2 was Fe_3O_4. Other strong peaks in MB_2 were identified as CuS and $FeSO_4$. Highest peak identified in MB_3 was Fe_3O_4. Other peaks identified in the sample were Cu_2O, $FeSO_4$, and Fe_2O_3. In partially prepared *Swarna Makshika Bhasma* (MBK_4) most of the highest peaks were identified as $CuFeS_2$. Regain of $CuFeS_2$ after *Kupipaka* was very surprising. As we know, SM contains mainly FeS_2, Cu_2S, Fe_3O_4, and $FeSO_4$. During *Kupipaka*, excess sulfur gets evaporated in the form of oxides of sulfur. Some of the sulfur reacted with mercury and converted into *Rasa Sindura* and some part of sulfur still remain un-reacted in the bottom of the bottle. On a specific temperature and conditions, this unreacted sulfur may react with copper and iron and get converted into copper pyrite. After further *Putapaka* of partially prepared *Swarna Makshika Bhasma*, highest



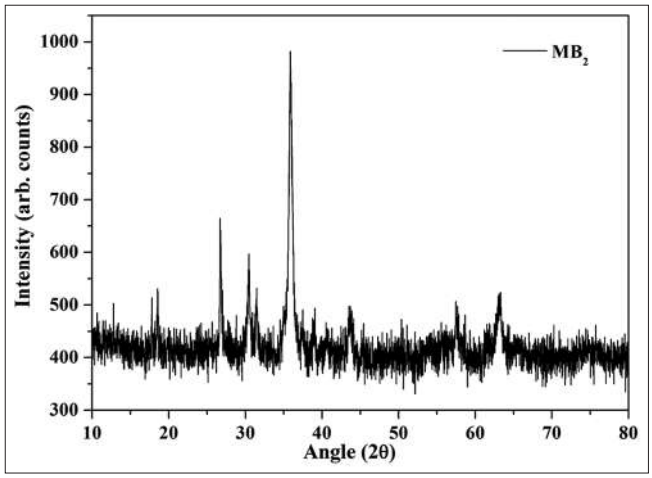
Graph 1: X-ray diffraction study of raw *Swarna Makshika*



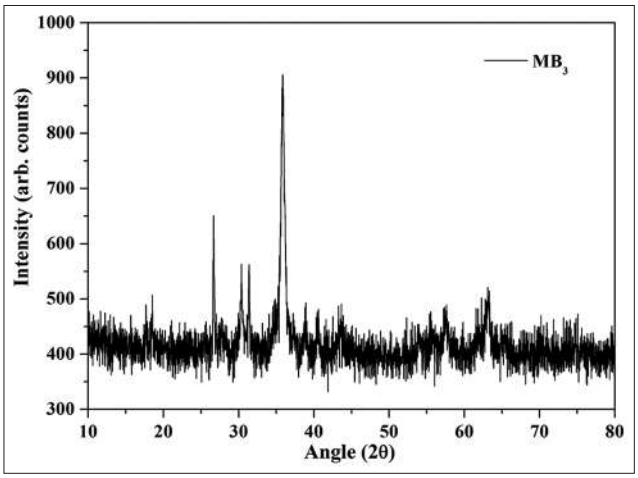
Graph 2: X-ray diffraction study of Shodhita *Swarna Makshika*



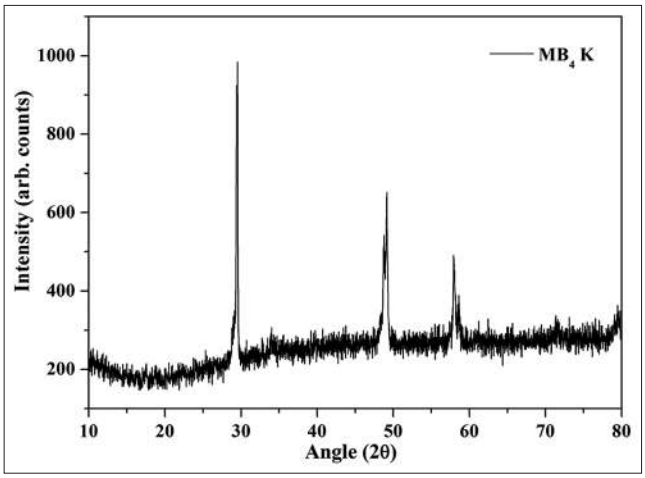
Graph 3: X-ray diffraction study of *Swarna Makshika Bhasma* (MB_1)



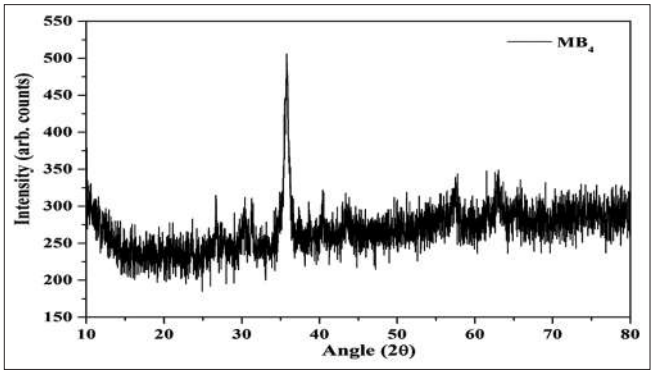
Graph 4: X-ray diffraction study of *Swarna Makshika Bhasma* (MB_2)



Graph 5: X-ray diffraction study of *Swarna Makshika Bhasma* (MB_3)



Graph 6: X-ray diffraction study of *Swarna Makshika Bhasma* (MB_4K)



Graph 7: X-ray diffraction study of *Swarna Makshika Bhasma* (MB_4)

peaks identified were Fe_2O_3, Cu_2O, and $FeSO_4$. Hence, many small peaks are seen in all samples, but these small peaks are very difficult to identify. According to the diffraction principle, small peaks are quantitatively very poor. On XRD report of the *Bhasma*, it can be assumed that the small peaks observed may be the trace elements or their compounds that may possibly incorporate into the prepared *Bhasma* due to repeated *Bhavana* (levigation) with herbal juices and firing in presence of oxygen and sulfur.

Table 1: The X-ray diffraction of raw *Swarna Makshika*

Compounds	Two theta	Intensity	Value of *d*
$CuFeS_2$	25.94	563	3.4311
$CuFeS_2$	29.75	943	3.0006
$CuFeS_2$	29.86	2960	2.9895
$CuFeS_2$	49.55	935	1.8379
$CuFeS_2$	58.31	854	1.5811

Table 2: The X-ray diffraction of *Shodhita Swarna Makshika*

Compounds	Two theta	Intensity	Value of *d*
Cu_2S	24.42	428	3.6418
	42.66	475	2.1174
	64.26	504	1.4483
Fe_3O_4	35.88	713	2.5002
	62.67	545	1.4810
FeS_2	33.42	731	2.6789
	40.18	419	2.2425
$FeSO_4$	18.49	482	4.7943
	54.31	561	1.6874
SiO_2	24.93	440	3.5687
	38.93	458	2.3111

Table 3: The X-ray diffraction of *Swarna Makshika Bhasma* (MB_1)

Compounds	Two theta	Intensity	Value of *d*
Fe_3O_4	31.48	588	2.8212
	36.24	1013	2.4764
	58.12	491	1.5857
CuS	30.37	510	2.9406
	62.84	538	1.4774
$FeSO_4$	18.97	445	4.7182
	43.6	498	2.0713
	55.69	512	1.690

Table 4: The X-ray diffraction of *Swarna Makshika Bhasma* (MB_2)

Compounds	Two theta	Intensity	Value of *d*
Fe_3O_4	31.48	532	2.8393
	35.86	982	2.5015
	57.46	507	1.6024
CuS	26.70	665	3.3360
	30.44	597	2.9335
	63.03	524	1.4697
$FeSO_4$	26.60	468	3.2635
	43.6	498	2.0713
	56.20	462	1.6031

Table 5: The X-ray diffraction of *Swarna Makshika Bhasma* (MB_3)

Compounds	Two theta	Intensity	Value of *d*
Fe_3O_4	35.85	906	2.5028
	57.63	489	1.5980
	63.11	489	1.5980
Cu_2O	31.38	562	2.8476
	40.55	476	2.2225
	53.98	447	1.7643
$FeSO_4$	18.52	507	4.7847
	26.66	651	3.3407
Fe_2O_3	38.93	493	2.3111

Table 6: The X-ray diffraction of *Kupipakwa Swarna Makshika Bhasma* (MB_4K)

Compounds	Two theta	Intensity	Value of *d*
$CuFeS_2$	29.46	1494	3.0287
	29.54	984	3.0212
	49.16	652	1.8518
	57.91	491	1.5909
Fe_3O_4	33.94	293	2.5018
	57.84	391	1.5912
	62.58	306	1.4071

Table 7: The X-ray diffraction of *Swarna Makshika Bhasma* (MB_4)

Compounds	Two theta	Intensity	Value of *d*
Fe_2O_3	35.78	506	2.5079
	57.75	344	1.5952
	62.56	346	1.4834
	65.84	336	1.4173
Cu_2O	31.27	311	2.8577
	35.45	424	2.7234
	40.40	322	2.2304
$FeSO_4$	26.66	315	3.3407
	44.64	400	1.9043
	56.35	319	1.7564
Cu_2S	30.409	312	2.9371
	43.36	318	2.0850
	48.18	288	1.9870

Conclusion

Particular benefit of diffraction analysis is that it discloses the presence of substances as that actually exists in the sample. This technique helps to identify and characterize the Ayurvedic raw material of mineral/metal origin and their processed form. XRD of different samples of *Swarna Makshika Bhasma* after comparing with JCPDS data revealed that raw *Swarna Makshika* contains $CuFeS_2$, which was converted into sulfides of copper and iron and oxide and sulfate of iron after *Shodhana*. Major compounds identified in *Bhasma* of different samples were Fe_3O_4, Fe_2O_3, FeS_2, $FeSO_4$, and Cu_2S. In *Bhasma* prepared by *Kupipaka* followed by *Putapaka*, Strongest peak of Fe_2O_3, Cu_2O and $FeSO_4$ were mainly identified.

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Conflicts of interest

There are no conflicts of interest.

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Application of Spectroscopic and Chromatographic Methods for Chemical Characterization of an Ayurvedic Herbo-Mineral Preparation: Maha Yograja Guggulu

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Abstract

Rasa Shastra is an exclusive branch of ayurveda that uses processed metals and minerals in various combinations. Though the formulations are time tested, safety and quality concerns are being raised since the past two decades. In view of this, it becomes mandatory to generate quality control profiles of such formulations by following available parameters. Considering this, we attempted to develop standard manufacturing procedures of *Maha Yogaraja Guggulu* and generate preliminary physicochemical profiles using inductively coupled plasma mass spectrometry, X-ray diffraction, and high-performance thin-layer chromatography. The results from high-performance thin-layer chromatography revealed presence of organic constituents from plant material. X-ray diffraction indicated that the prepared drug contained cinnabar (mercury sulfide; *Rasa sindhura*), cassiterite (tin oxide; *Vanga bhasma*), litharge (lead oxide; *Naga bhasma*), and iron dioxide and magnetite (di-iron oxide; *Loha and Mandura bhasma*). The observations of the present study are preliminary and first of its kind that may be considered as baseline data for future studies.

Keywords

bhasma, *loha*, *maha yogaraja guggulu*, *mandura*, *naga*, *rasa sindhura*, *vanga*

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Maha yogaraja guggulu (MYG) is a well-known ayurvedic herbo-mineral formulation known for its *Rasayana* (rejuvenative), *Shothahara* (anti-inflammatory), *Vedanahara* (analgesic) properties. It is in practice mainly for *Vatavyadhi* (specific disorders occurring due to *Vata dosha*), *Kustha* (skin diseases), *Arshas* (hemorrhoids), *Prameha* (diabetes), *Vatarakta* (gout), *Nabhi shula* (pain around umbilicus), *Bhagandara* (fistula-in-ano), *Gulma* (abdominal tumors), *Swasa* (dyspnea), *Kasa* (cough), *Aruchi* (anorexia), *Retasdosha* (seminal abnormalities), and *Rajodosha* (menstrual disorders).[1,2]

Considering its importance in clinical use, the detailed standard operative procedures for the preparation, standardization, and chemical characterization of this formulation should be well documented with substantial evidences for worldwide acceptance. Hence, this study was conducted with the objectives to evolve standard operative procedures for the preparation as per classical ayurvedic texts and physicochemical profile of *Maha Yogaraja Guggulu*.

Materials and Methods

Pharmaceutical Processing

Maha yogaraja guggulu was prepared as per standard methods mentioned in the Ayurvedic Formulary of India.[3] The details about the ingredients used are presented in Table 1. The whole process of preparation was divided into following steps.

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Table 1. Formulation Composition of *Maha Yogaraja Guggulu*[a].

Materials Used	Botanical/English Name	Part/Form Used	Proportion
Shunthi	*Zingiber officinale* Rose.	Dried rhizome	1 part
Pippali	*Piper longum* Linn.	Dried fruit	1 part
Chavya	*Piper chaba* Hunter.	Dried stem	1 part
Pippali mula	*Piper longum* Linn.	Dried root	1 part
Chitraka	*Plumbago zeylanica* Linn.	Dried root	1 part
Hingu	*Ferula foetida* Regel.	Exudates	1 part
Ajamoda	*Apium graveolens* Linn.	Dried fruit	1 part
Sarshapa	*Brasica compestris* Linn.	Dried seed	1 part
Shweta jiraka	*Cuminum cyminum* Linn.	Dried fruit	1 part
Krishna jiraka	*Carum carvi* Linn.	Dried fruit	1 part
Renuka	*Vitex agnus-castus* Linn.	Dried seed	1 part
Indrayava	*Holarrhena antidysentrica* Wall.	Dried seed	1 part
Patha	*Cissampelos pareira* Linn.	Dried root	1 part
Vidanga	*Embelia ribes* Burn. f.	Dried fruit	1 part
Gaja pippali	*Sciendapsus officinalis* Schott	Dried fruit	1 part
Katuki	*Picrorhiza kurroa* Royle ex Benth.	Dried root/rhizome	1 part
Ativisha	*Aconitum heterophyllum* Wall ex Royle.	Dried root	1 part
Bharangi	*Clerodendrum serratum* Moon.	Dried root	1 part
Vacha	*Acorus calamus* Linn.	Dried rhizome	1 part
Murva	*Marsdenia tenacissima* Wight and Arn.	Dried root	1 part
Triphala			40 parts
Amalaki	*Emblica officinalis* Gaertn.	Dried pericarp	
Haritaki	*Terminalia chebula* Retz.	Dried pericarp	
Bibhitaka	*Terminalia bellerica* Roxb.	Dried pericarp	
Guggulu	*Commiphora wightii* (Arn.) Bhandari	Oleo-gum resin	60 parts
Vanga	Tin	*Bhasma* (incinerated tin)	16 parts(320 g)
Rajata	Silver	*Bhasma* (incinerated silver)	16 parts
Naga	Lead	*Bhasma* (incinerated lead)	16 parts
Loha	Iron	*Bhasma* (incinerated iron)	16 parts
Abhraka	Biotite mica	*Bhasma* (incinerated mica)	16 parts
Mandura		*Bhasma* (incinerated sludge iron)	16 parts
Rasa sindoor	Sulfide of mercury (HgS)	A type of *kupipakwa rasayana* of mercury	16 parts

[a]Source: The Ayurvedic Formulary of India, Government of India, Part 1, 5:6.

Table 2. Details of Media Used, Nature and Number of *Puta*, and Temperature Used for the Preparation of Each *Bhasma*.

Name of *Bhasma*	Reference	Media Used	Nature of *Puta*	Number of *Putas*
Vanga	*Ayurved Sara Sangrah*	*Nimbu rasa*	*Ardha gaja puta*	10
Rajata	*Rasa Tarangini, Taranga*[16]	*Nimbuk swarsa*	*Urdhav patan yantra*	For 6 hours
Naga	*Sarangdhar Samhita*	*Kanjika*	*Ardha gaja puta* and *gaja puta*	60 (where last 10 *putas* are *gaja puta)*
Abhraka	*Rasa Tarangini, Taranga*[10]	*Arka ksheera*	*Gaja puta*	*Arka Ksheera*—7 *putas*
		Nyagrodha mula kwatha		*Nyagrodha mula kwatha*—3 *putas*
		Kadali rasa		*Kadali rasa*—7 *putas*
Mandura	*Rasa Tarangini, Taranga*[20]	*Triphala kashaya*	*Sadharana puta*	30
Lauha	*Rasa Tarangini, Taranga*[16]	*Triphala kashaya*	*Gaja puta*	60

Processing of Guggulu. One part (1700 g) of *Guggulu* was made into small pieces carefully by removing physical impurities like stone, glass, and so on, bundled into a *Pottali*, suspended in a vessel containing *Triphala kashaya* (3400 mL), and boiled on moderate flame maintaining the temperature at around 80°C. On complete dissolution of *Guggulu* in the liquid, the contents were filtered through mesh No. 60 (to remove possible insoluble impurities, if any) and the filtrate was again heated till the liquid converts into a semisolid mass. This semisolid mass was shifted into steel trays and dried in a tray drier at 50°C. The dried mass was taken out from the tray and used in the preparation of *maha yogaraja guggulu*.[4]

Preparation of Bhasmas (Vanga/Rajata/Naga/Lauha/Abhraka/Mandura/Rasa Sindhura)

Preparation of vanga bhasma. *Vanga* was melted and poured 3 times each in *Tila taila*, *takra*, *kanjika*, *gomutra*, and decoction of the seeds of *kulattha* (*Dolichus biflorus* L.).[5] It was further processed for *vishesha sodhana*, where molten *vanga* was poured in the leaf juice of *Nirgundi* (*Vitex negundo* Linn.) and powder of *haridra* (*Curcuma longa* Linn.) consecutively 3 times.[6]

Vanga collected at this stage was again molten in an iron pan, added with powder of *ashvattha* (bark of *Ficus religiosa* Linn.)

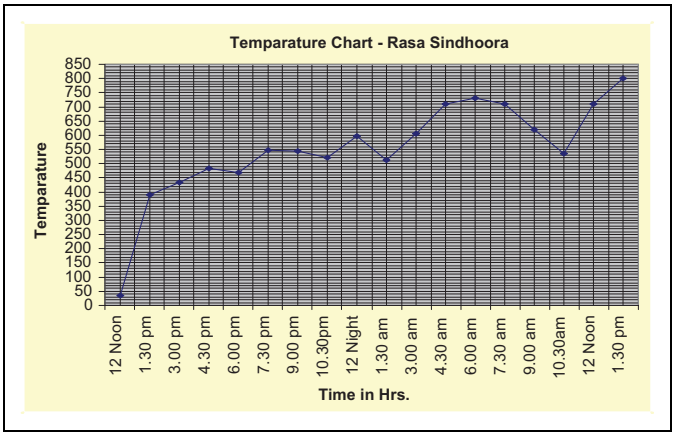

Figure 1. The temperature pattern for the preparation of *rasa sindhura.*

Table 3. Preliminary Physicochemical Profile of *Maha Yogaraja Guggulu*.

Parameter Tested	Observations
Organoleptic characters	
Color	Blackish
Taste	Acrid
Odor	Pleasant
Appearance	Tablet
Physicochemical	
Identification	Yields the reaction characteristics of silver, mercury, lead, tin, and iron
Loss on drying, % w/w	2.0-5.0
Total ash, % w/w	43.0-46.0
Acid-insoluble ash, % w/w	12.0-14.50
Water-soluble extractive, % w/w	11.0-14.0
Alcohol (90%) soluble extractive, % w/w	8.0-10.0
Resin content, % w/w	12.50-14.0
pH of aqueous extract	5.0-6.0
Organic and volatile matter, % w/w	55.50-57.50
Specific gravity	0.9980-0.9990
Particle size distribution	
10%	2.15-2.76 μm
50%	11.01-20.35 μm
90%	54.11-81.65 μm
Assay of elements	
Silver (% w/w)	1.5-2.0
Iron (% w/w)	4.0-5.0
Sulfur (% w/w)	2.0-2.5
Silica (% w/w)	0.5-2.0
Calcium (% w/w)	2.5-3.0
Copper(% w/w)	0.1-0.5
Boron (% w/w)	0.01-0.30
Manganese (% w/w)	0.05-0.07
Magnesium (% w/w)	0.50 -1.50
Chromium (% w/w)	0.03-0.05
Aluminum (% w/w)	0.50 -1.0
Mercury (% w/w)	1.0 -1.5
Lead (% w/w)	2.0-2.5
Arsenic (% w/w)	0.3-0.4
Cadmium (% w/w)	0.1-0.2
Tin (ppm)	350-750
Residual pesticide (μg/kg)	
Alpha and beta HCH	Not detected
Gamma HCH	Not detected
Delta HCH	Not detected
DDT and metabolites	Not detected
D.T.	18-20 minutes
Hardness	2.5 kg/cm^2
Friability	0.30-0.60%
Average weight	125-127 mg/tablet
Microbial contamination	
Total aerobic count	20 000-40 000
Coliform	Not detected
Escherichia coli	Not detected
Salmonella sp.	Not detected
Staphylococcus aureus	Not detected
Yeasts	Not detected
Molds	70-100
Pseudomonas aeruginosa	Absent
High-performance thin-layer chromatography	Incorporated

and *chincha tvak* (bark of *Tamarindus indica* Linn.) in small quantities, and stirred continuously with *loha darvi* (iron spatula). This process was continued till reduction of *vanga* to powder (*jarita vanga*).[7] Furthermore, equal quantity of orpiment powder was added to this *jarita vanga* and levigated with lemon juice; small *chakrikas* (flat cakes) were prepared, dried, placed in *sarava samputa*, and subjected to *gaja puta.* From the second *puta* onwards, one fourth part of the orpiment powder was added to *vanga*. The process of *puta* was repeated 10 times to obtain *vanga bhasma*.

Preparation of rajata bhasma. *Rajata patras* were heated to red hot and immersed consecutively in *tila taila*, *takra*, *kanjika*, *gomutra*, and decoction of the seeds of *kulattha.* The whole process was repeated 3 times. *Rajata patra* thus obtained was further processed to *vishesha sodhana* by processing in *agastya swarasa* (leaf juice of *Sesbania grandiflora*) 3 times.[8] In the next step, this was added with *suddha hingula* (processed cinnabar in lemon juice), ground well, and heated in *urdhva patana yantra* for 6 hours. On cooling, the apparatus was opened to collect *rajata bhasma* from the lower vessel.[9]

Preparation of naga bhasma. Molten *naga* was poured consecutively in *tila taila*, *takra*, *kanji*, *gomutra*, and decoction of the seeds of *kulattha* 3 times each in all the liquids. This was further collected in an iron pan and heated. On melting, powders of *chincha tvak* and *asvattha tvak* were sprinkled in small quantities and stirred with *loha darvi* (iron spatula). This process was continued till the molten *naga* is reduced to powder form (*jarita naga*). Furthermore, equal quantity of *manahsila* was added to *jarita naga* and levigated with *kanji*; small *chakrikas* were prepared, dried, and placed in *sarava samputa* and *ardha gaja puta* is given. This process was repeated 60 times to obtain *naga bhasma* of desired quality.[10]

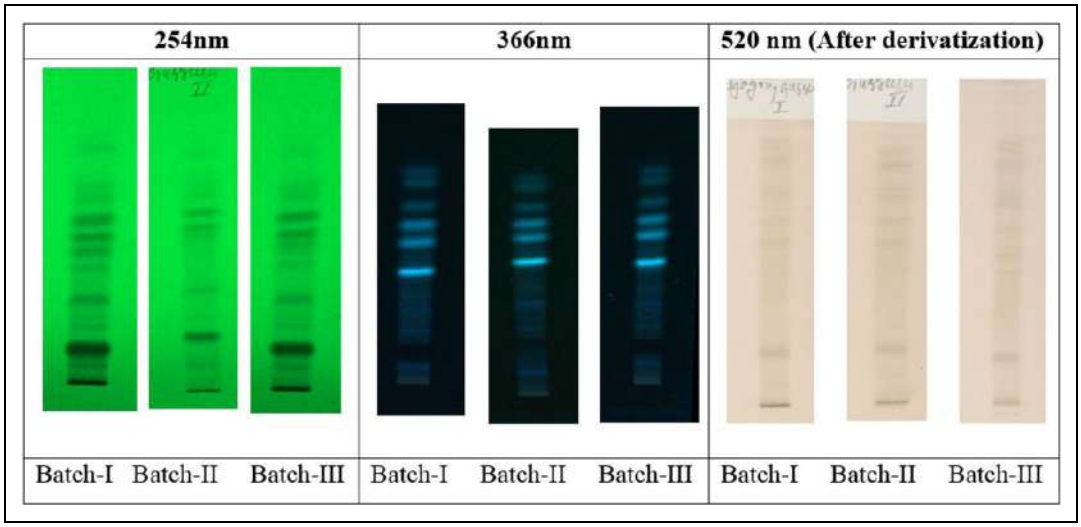

Figure 2. High-performance thin-layer chromatography profiles of *maha yogaraja guggulu*.

Preparation of abhraka bhasma. *Abhraka* was heated to red hot and immersed in decoction of *Triphala* (3 myrobalans) 7 times.[11] The *shodhita abhraka* was bundled in a jute bag (into *pottali* form) with one fourth quantity of paddy and immersed in *kanji*[12] for 3 days. Thereafter, the *pottali* was rubbed thoroughly and squeezed in the liquid itself so that only fine *abhraka* particles can escape through the holes of the bag. The bag was removed from the *kanji* and the contents were allowed to settle down. The supernatant liquid layers were separated carefully to collect fine particles of *abhraka* that had settled down in the container. These particles (*dhanyabhraka*) were dried and stored for further use.[13]

Dhanyabhraka was levigated with required quantity of *arka ksheera* (latex of *Calotropis procera* (Ait) R.Br.) for a day; *chakrikas* were prepared and dried in the sun. These *chakrikas* were placed in a *sharava samputa*, the junctions were sealed properly, and subjected to *gaja puta.*[14] The material thus obtained at the end of this *puta* was processed in similar way 6 more times. At the end of the seventh *puta*, the contents were levigated with *nyagrodha mula kwatha* (decoction of *Ficus bengalensis* roots), dried, and *gaja puta* was given. The process was repeated 3 times followed by levigation with *Rambha rasa* (juice of rhizome of *Musa paradisiaca*) and 7 *gaja putas* were given. Finally, this was grounded in *nyagrodha mula kwatha* and 3 *gaja putas* were given. After completion of these *putas*, the finished product *abhraka bhasma* was obtained.[15]

Preparation of mandura bhasma. *Shuddha mandura*[16] was levigated in *triphala kvatha* and *chakrikas* were prepared and dried that are placed in *sharava samputa* and subjected to heat in *sadharan puta*. The process was repeated 30 times to obtain red-colored *mandura bhasma*.[17]

Preparation of lauha bhasma. *Lauha* was heated to red hot and immersed 3 times each consecutively in *tila taila*, *takra*, *kanji*, *gomutra*, and decoction of the seeds of *kulattha*. This was further processed in equal quantities of *triphala kashaya* and *gomutra*.[18] *Lauha churna* thus obtained was further processed through *bhanupaka* (processed in sun rays), followed by *sthalipaka* (heated with decoction of *triphala* in stainless steel vessel).[19,20] This was further levigated with *triphala kwatha*, *Chakrikas* were prepared, dried, and placed in *sharava samputa*, and subjected to *gaja puta*. The same procedure was repeated 60 times to get *lauha bhasma* of desired quality.[21] The details of media used, nature, and number of *putas* and temperature used in the preparation of each *bhasma* are mentioned in Table 2.

Preparation of rasa sindhura. Preparation of *rasa sindhura* involves preparation of *kajjali*, *bhavana* (levigation) with *vatankura jala* (decoction of leaf buds of *Ficus bengalenesis* Linn.), and processing in *valuka yantra.*[22-24] *Kajjali* was prepared by triturating equal quantities of *hingulottha parada* (mercury obtained from cinnabar) and *shuddha gandhaka* (processed sulfur) in a *khalva yantra* (mortar pestle), till the formation of a black-colored, soft, lusterless fine powder like collyrium. This was further levigated with *vatankura jala* and then dried. This was filled in a strong amber-colored *kachakupi* (glass bottle) in *valuka yantra* (heating device) and subjected to heat by increasing the temperature gradually. Mild heat was applied for the first 6 hours, followed by moderate heat. The temperature pattern for the preparation of *rasa sindhura* is shown in Figure 1.

When the bottom of the bottle appears red, the mouth of the bottle was blocked with cork and sealed with mud mixed with lime and jaggery smeared cloth. This was followed by application of strong heat for the next 6 hours. Thereafter, the *valuka yantra* was allowed to cool down on its own (Figure 1). The bottle was then removed from the *valuka yantra* and the mud-smeared cloth was scrapped with a knife; the bottle was broken down carefully to collect crystallized *rasa sindhura* from the neck of the bottle.

Preparation of Finished Product. *Shuddha guggulu* (1.2 kg) was dissolved in 3 L of water to prepare a thick paste, to which fine powders of other components were added in end runner followed by levigation for 3 days. At the end of this process, the material was removed from the end runner, shifted to clean stainless steel trays, and dried at 50°C.

The dried material was converted into granules by passing through No. 40 sieve and shifted to the tablet section. Two percent of talcum powder was added as excipient to the granules and were compressed into tablets of 125 mg in a rotary tablet machine.

Physicochemical Analysis

Physicochemical analysis, that is, description, estimation of loss on drying, ash content, acid insoluble ash, water/alcohol soluble

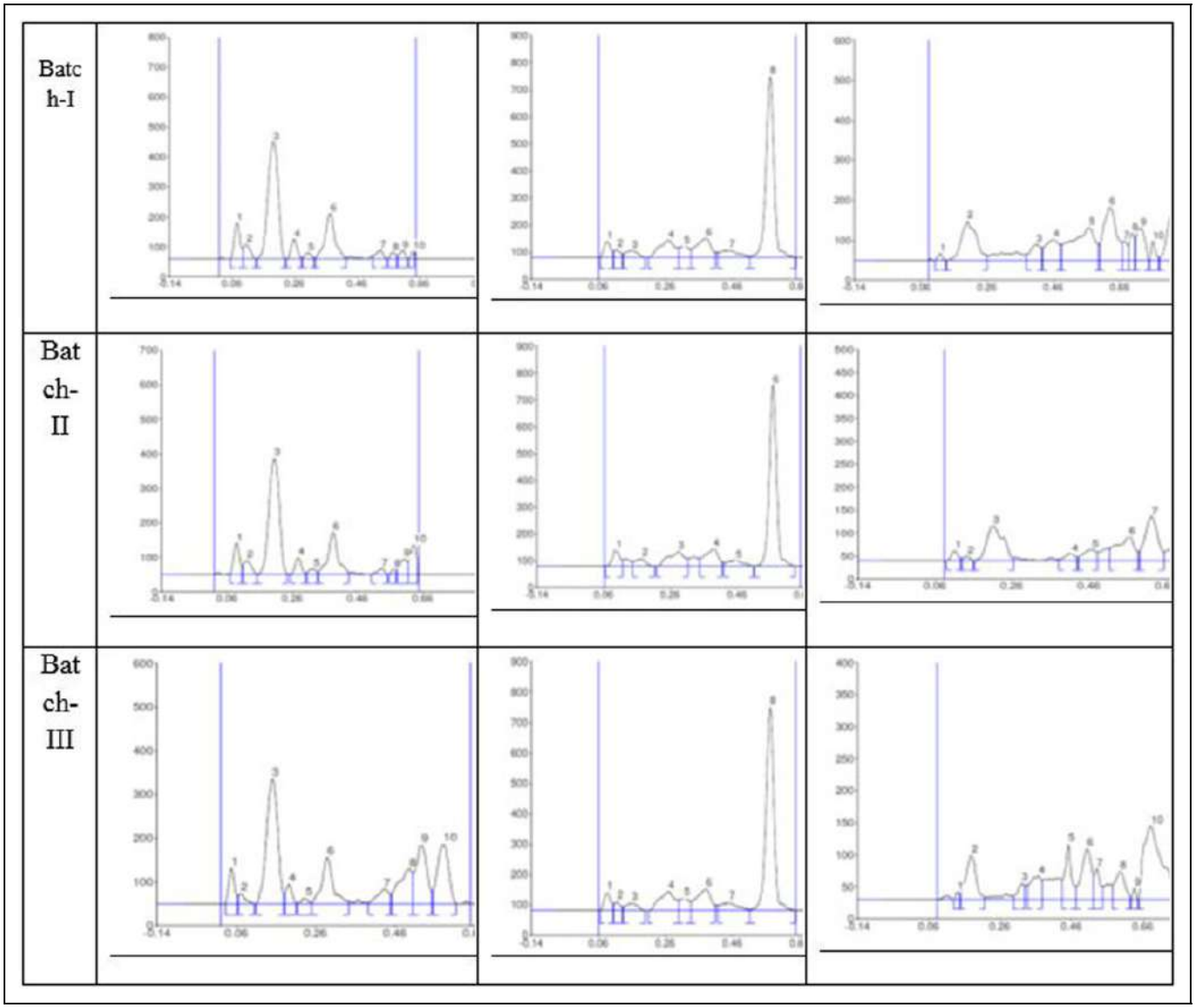

Figure 3. High-performance thin-layer chromatography fingerprints of *maha yogaraja guggulu.*

extractive, pH, and so on; qualitative/quantitative elemental testing; residual pesticide; microbiological examination; and tablet parameters, that is, hardness, friability, average weight, dissolution time, and so on, were carried out by following standard methods as per Ayurvedic Pharmacopoeia of India[25-27] guidelines. The quantitative estimation of heavy metals, that is, Pb, Cd, As, Hg, and Sn was carried out by atomic absorption spectrometer (Perkin Elmer Analyst 400), and the other elements that is, Ca, Mg, Cu, B, Mn, Al, Cr, Fe, and Ag were analyzed on ICP-AES (THERMO EECTRON Corporation's model IRIS INTREPRID II XDL). However, sulfur and silica were quantified by using conventional methods.[27]

High-Performance Thin-Layer Chromatography Method

Sample preparation. Two grams of powders each of the 3 batches of *maha yogaraja guggulu* were soaked overnight separately in 20 mL of methanol. The solutions were continuously stirred for 6 hours and kept for the next 18 hours and then the samples were filtered, dried, and made into 10% solution.

High-performance thin-layer chromatography was performed on thin-layer chromatography plates precoated with 0.25 µm thin layers of silica gel 60 F_{254} (E. Merck). Ten microliters of methanolic solution of formulation (3 batches) were applied on the plates as bands 8.0 mm wide by use of a Linomat-V applicator (CAMAG, Switzerland) fitted with a 100 µL syringe (Hamilton, Switzerland). The application positions X and Y were both 10 mm, to avoid edge effects. Linear ascending development to a distance of 80 mm with toluene–ethyl acetate–formic acid 10:3:1 (v/v) as mobile phase was performed in a twin-trough glass chamber previously saturated with vapors of the mobile phase for 20 minutes. The plates were dried in air and visualized under 254 nm and 366 nm for ultraviolet detection and taken the fingerprints as evident. The same thin-layer chromatography plate was also derivatized with anisaldehyde-sulfuric acid reagent and visualized in white light.

X-Ray Diffraction Study. Powder X-ray diffraction analysis was carried out using Rigaku Ultima-IV X-ray diffractometer with CuKα radiation

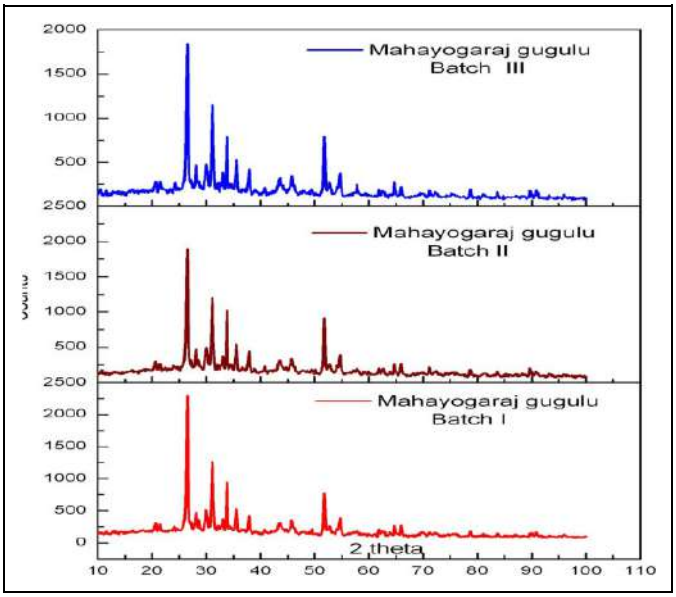

Figure 4. X-ray diffraction pattern of *maha yogaraja guggulu*.

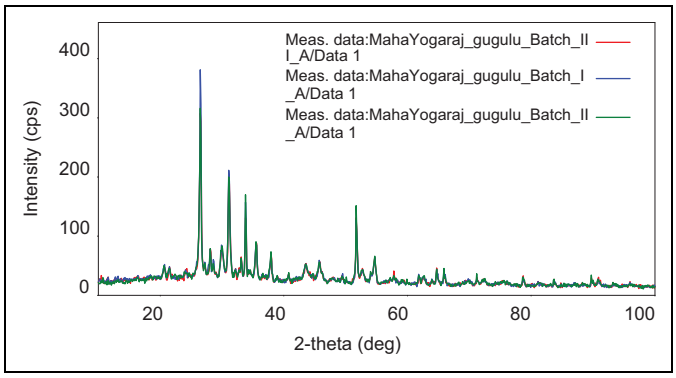

Figure 5. Overlay of X-ray diffraction pattern of *maha yogaraja guggulu*.

($\lambda = 1.54$ A) operating at 40 kV and 30 mA. The pattern was recorded for angle (2θ) ranging from 10° to 100° at a scanning rate of 1°/second and scan step of 0.1°. X-ray diffraction pattern of *maha yogaraja guggulu* (3 batches) is shown in the spectra. Sample identification was done by matching d-spacing with the standard database.

Results and Discussion

Organoleptic observations show that *maha yogaraja guggulu* is in the form of a brownish-colored tablet having characteristic pleasant odor and slightly acrid in taste. The qualitative analysis shows positivity for the presence of mercury, silver, sulfur, calcium, copper, iron, and lead. Chemical analysis revealed 2.39% of lead, 1.31% of mercury, 2.25% of sulfur, 4.36% of iron, 1.63% of silver, 2.73% of calcium, 1.08% of magnesium, and 1.18% of silica along with other trace elements like aluminum, manganese, arsenic, copper, tin, boron, chromium, and cadmium, which were found in <1.0% range. Moisture content 3.62% was found when determining loss on drying at 105°C.

Table 4. d-Spacing and 2-Theta (°) Values of X-Ray Diffraction Analysis.

2 theta	d (A)	Size (A)	Chemical Formula	Phase Data Name	cps
21.5	4.1	214.2	Sn	Tin (1,1,0)	12.7
26.5	3.4	381.0	HgS, SnO_2	Cinnabar (1,0,1), cassiterite, syn (1,1,0)	271.0
28.0	3.2	106.0	HgS	Cinnabar (0,0,3)	17.2
28.7	3.1	217.2	PbO	Litharge (1,0,1)	16.1
29.9	3.0	191.0	Fe_3O_4	Iron di-iron (III) oxide, magnetite HP, syn (2,2,0)	35.0
31.1	2.9	268.0	HgS	Cinnabar (1,0,2)	123.0
34.0	2.6	220.1	SnO_2	Cassiterite, syn (1,0,1)	122.3
35.4	2.5	336.0	Fe_3O_4, PbO	Iron di-iron (III) oxide, magnetite HP, syn (3,1,1), litharge (0,0,2)	47.0
37.9	2.4	297.0	HgS, SnO_2	Cinnabar (1,0,3), cassiterite, syn (2,0,0)	27.0
43.4	2.1	117.0	HgS, Fe_3O_4, Sn	Cinnabar (1,1,0), iron di-iron (III) oxide, magnetite HP, syn (4,0,0), tin (2,2,0)	17.8
45.7	2.0	228.0	HgS, PbO	Cinnabar (1,0,4), litharge (2,0,0)	21.5
49.5	1.8	231.7	Sn	Tin (3,1,0)	12.9
51.7	1.8	593.0	HgS, SnO_2	Cinnabar (2,0,1), cassiterite, syn (2,1,1)	95.0
52.8	1.7	235.0	HgS	Cinnabar (1,1,3)	17.4
54.7	1.7	224.0	HgS, SnO_2, PbO	Cinnabar (2,0,2), cassiterite, syn (2,2,0), litharge (2,1,1)	26.0
61.8	1.5	245.3	SnO_2	Cassiterite, syn (3,1,0)	14.3
62.5	1.5	246.2	Fe_3O_4, SnO_2, Sn	Iron di-iron (III) oxide, magnetite HP, syn (4,4,0), cassiterite, syn (2,2,1), tin (1,1,2)	6.8
64.8	1.4	249.2	HgS, SnO_2	Cinnabar (2,0,4), cassiterite, syn (1,1,2)	22.8
66.0	1.4	914.0	Fe_3O_4, SnO_2	Iron di-iron (III) oxide, magnetite HP, syn (5,3,1), cassiterite, syn (3,0,1)	32.0
69.7	1.3	29.0	HgS, SnO_2, Sn	Cinnabar (2,1,0), cassiterite, syn (3,1,1), tin (2,0,2)	3.0
71.2	1.3	258.9	Fe_3O_4, SnO_2	Iron di-iron (III) oxide, magnetite HP, syn (6,2,0), cassiterite, syn (2,0,2)	4.9
78.7	1.2	272.2	SnO_2	Cassiterite, syn (3,2,1)	12.7
83.7	1.2	282.6	SnO_2, PbO	Cassiterite, syn (2,2,2), litharge (1,1,4)	7.7
89.8	1.1	297.0	Fe_3O_4, SnO_2	Iron di-iron (III) oxide, magnetite HP, syn (7,3,1), cassiterite, syn (3,1,2)	5.9
90.9	1.1	212.0	HgS, SnO_2, PbO	Cinnabar (2,0,7), cassiterite, syn (4,1,1), litharge (3,2,1)	6.0

Total ash content (44.38%) is ash left after burning of organic and volatile matter (56.29%). As shown in the observations, water soluble (12.03%) and alcohol soluble (9.06%) matter were also present in this formulation. The drug also tested for residual pesticides and microbial contamination, which was found to be within permissible limits (Table 3). The results from high-performance thin-layer chromatography, as shown in Figures 2 and 3, revealed presences of organic constituents from plant material.

The X-ray diffraction patterns of the 3 batches, as shown in Figures 4 and 5 and Table 4, are nearly identical, showing clear crystalline phases of the inorganic constituents. The X-ray diffraction results have indicated that all the samples contained cinnabar (mercury sulfide added as *rasa sindhura*), cassiterite (tin oxide, *vanga bhasma*), litharge (lead oxide; *naga bhasma*), and iron dioxide and magnetite (di-iron oxide; *loha* and *mandura bhasma*). Some clear but weak signature of elemental tin is also seen. No signature of *rajat bhasma* could be identified in these spectra, indicating that the method used for its preparation did not yield any crystalline products or the signature is buried under the strong lines of other constituents. The X-ray diffraction lines at 21.5° and 49.5° could show the presence of elemental tin. The crystalline form of *abhrak bhasma* does not appear to be present. It can be seen that 3 X-ray diffraction patterns on the samples are qualitatively same in the relative intensities of the peaks, and the X-ray diffraction results indicate that the inorganic contents have remained intact over time.

Conclusion

This study reveals that *yogaraja guggulu* prepared following the classical guidelines seems to be very effective in converting the macro elements into therapeutically effective medicines in micro form. Well-prepared herbo-mineral drugs offer many advantages over plant medicines due to their longer shelf-life, lesser doses, easy storing facilities, better palatability, and so on. The inferences and the standards laid down in this study certainly can be utilized as baseline data of standardization and quality assurance of this herbo-mineral formulation. It will be helpful in laying down further pharmacopoeia standards of *maha yogaraja guggulu*.

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Author Contributions

Arjun Singh: Data generation, data interpretation, and drafted the chemical characterization part of the article.

Sarada Ota: Coordination of the project and drafted the ayurvedic part and standard operative procedures for the preparation of the formulation in the article.

Narayan Srikanth: Monitored the project work and revised the article critically.

Galib: Generated the data related to the standard operative procedures for the preparation of the formulation.

Sreedhar Bojja: Generated and analyzed the data related to chemical characterization.

Kartar Singh Dhiman: Supervision and final approval of the article for publication.

Declaration of Conflicting Interests

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Ethical Approval

Not applicable for this research study.

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Characterization and comparative physico-chemical studies of Manahshila (traditionally used arsenic mineral) and the corresponding polymorphs of realgar (As_4S_4)

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This communication presents characterization and comparison of the physico-chemical properties of different varieties of Manahshila with the corresponding polymorphs of realgar. Three varieties of Manahshila have been described in Ayurveda, viz. Shyamangi, Kanavirak and Khandakhya; the last two are acceptable therapeutically. Khandakhya contains high percentage of arsenic than Kanavirak. In this study, both samples of Manahshila have been collected. Their physical and chemical properties have been correlated with the polymorphs of realgar. XRD study classifies Kanavirak as alacranite and Khandakhya as realgar. EDXA study confirms 51.33% and 68.14% of arsenic in alacranite and realgar samples respectively. This work correlates the ancient description of Manahshila with contemporary mineralogical classification (polymorphs) of mineral realgar.

Keywords: Alacranite, Manahshila, physico-chemical studies, polymorphs of realgar, mineralogical classification.

REALGAR (red arsenic – an arsenic-containing mineral drug) has long been used in traditional Indian medicines for the treatment of diseases of respiratory and digestive systems, skin diseases, psychological disorders and certain eye disorders[1–3]. Recently, it has been demonstrated that it is clinically effective for the treatment of patients with refractory or relapsed acute promyelocytic leukaemia (APL) and other hematopoietic malignancies[4–6]; this has given rise to an upsurge of research on its oldest to newest forms. Generally, inorganic realgar is highly toxic and carcinogenic[7,8]; however, Ayurveda has emphasized that a strong poison may be converted into a safe and potent therapeutic drug by applying specific pharmaceutical processes as described in the Ayurvedic literature (e.g. shodhana, marana, etc.)[9]. The drug Manahshila, one of the arsenicals, has been identified as realgar due to its similar chemical and physical properties. Ayurveda has advocated proper method of shodhana (purification and detoxification from the unwanted elements by intervention

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Table 1. Description of mineral Manahshila from an Ayurvedic perspective

Category	Details	
Broadly classified group	Uparasa	
Acceptable characters	Devoid of stone, easily powdered, heavy in weight, red similar to the colour of lotus flower with shining luster.	
Shodhana	Process	Method
	Bhavana (levigation)	With Ardraka/Agasthya Patra Swarasa/Bringharaja/Matulunga Swarasa for seven times
	Swedana (boiling under liquid bath)	(a) In Dola Yantra with Churnodaka/goat urine for three days (b) With Bringharaja Swarasa for 12 h
Therapeutic uses	Agnimandya (digestive impairment), anaha (distension of abdomen due to obstruction to passage of urine and stools), kasa (cough), svasa (asthma), kshaya (phthisis), kandu (itching), bhuta badha (psychological disorders), bhuta upadrava (microbial infection) and jvara (fever).	
Dose	5.0–7.5 mg	
Adverse effects due to administration of unpurified Manahshila	Mandagni (digestive impairment), ashmari (renal calculi), malavishtambhakari (constipation), mutrakriccha (painful micturation), mutradaha (burning micturation), mutorodha (obstructed micturation).	
Antidote	Oral administration of Madhu (honey) and Godugdha (cow's milk) for three days	
Formulation	Internal use	Shwasakutthar rasa, trilokyachintamani rasa, kshayakesari rasa, manashiladhi ghrita, rasarajarasa mrutasanjeevani rasa, kalagnibhairava rasa and shilasinduram
	External use – used as anajan (collyrium) or lepa (external application)	For eye disorders – Manashil anjana/varti and candrodaye varti. For tropical application – paradadi lepa and tutthadi lepa for upadamsha roga (Syphilis/soft chancre/chancroid)

of some herbal juices or inorganic liquid media) before recommending it for therapeutic purposes[10]. However, the collection of genuine sample is prerequisite for desired therapeutic efficacy of any substance.

In ancient Indian alchemy, Manahshila has been described as an orange–red crystalline mineral categorized under the category of uparasa (a group of mineral drugs)[11]. More than 40 compound formulations for internal and external administration have been mentioned in the Ayurvedic formulary of India, in which Manahshila is used as a major ingredient[12,13] (Table 1). It is considered as one of the best rasayana drugs/rasayanaagrah[2] (best nutrient to body and mind with adaptogenic, neuroenhancing and immuno-endocrino modulator properties). Texts written after the 12th century AD provide a detailed description of Manahshila[14,15]. Most authors have identified three varieties of Manahshila, viz. shyamangi, kanavirak and khandakhya (Table 2); each has its own specific physical properties. The last two varieties display properties of shining red-coloured crystals, devoid of external impurity, heavy in weight and easily powdered[16]. Therapeutically, it is always recommended for its use only after purification process[17]. On the other hand, in contemporary science, three polymorphs (having similar chemical structure with different crystalline bonds of the atoms) of realgar (As_4S_4) have been identified[18] (Table 3), which match with the description of Manahshila described in the ancient rasa shastra (metal–mineral medicines) texts.

It is customary to use all the varieties of Manahshila by the name of realgar; however, this is not true in all cases. Thus, this study compares characteristics of different varieties of Manahshila with the corresponding polymorphs of realgar. Two samples of Manahshila collected in the present study (Figure 1), were characterized using X-ray diffraction (XRD), energy diffraction X-ray analysis (EDXA), and scanning electron microscopy (SEM) to fulfil the primary objective of the study. This work will also be helpful to recognize authentic samples of Manahshila using the current perspectives.

Two samples of Manahshila, identified according to characteristics as explained in Ayurveda were collected from the Department of Rasa Shastra, Faculty of Ayurveda, Institute of Medical Sciences, Banaras Hindu University (BHU), Varanasi, India, and coded as M1 and M2. Both samples have been selected for shodhana separately. For the shodhana process, raw sample was pulverized in a stony mortar and pestle. Then the powdered material was levigated (bhavana) with fresh extracted ginger juice in sufficient quantity until proper and complete drying of the material. The same procedure was repeated again by adding sufficient quantity of fresh juice of ginger. Thus, total seven bhavanas were performed to obtain detoxified (shuddha) Manahshila samples, which have been recoded as M3 (M2) and M4 (M1).

XRD study of all the four samples (M1, M2, M3 and M4) was carried out at the Centre of Advanced Study in Geology, Institute of Science, BHU. The diffraction pattern was obtained on a PANalytical X'Pert Pro diffractometer fitted with a copper tube (CuKα radiation) and xenon detector, scanned over a range 5°–70° 2θ using a 1/2° fixed divergence slit and 1/4° receiving slit with a step size of 0.0250, 1.20 sec/step, and total run time of 56 min 2 sec at 45 kV and 40 mA[19]. The standard Inorganic Crystal Structure Database (ICSD) was used for comparison of the measured data in a PANalytical X'Pert

Table 2. Classical description of physical properties of different types of Manahshila

Physical properties	Shyamangi	Kanavirak	Khandakhya (R.R.S.[14])/ Dwikhanda (A.P.[15])
Colour	Yellowish-red with blackish tint (shyama rakta sagaura; R.R.S.) Red like cinnabar (hingulavat rakta; A.P.)	Coppery red (tamraabha; R.R.S.) Red (A.P.)	Excessive red (atiraktangi; R.R.S.) Comparatively less red to pale (kinchitarakta ch rakta; A.P.)
Luster	Bright (atideeptika; A.P.)	Bright (tejaswani; R.R.S.)	–
Others	Heaviest in weight (bharadya; R.R.S.)	Brittle (churnarupa; A.P.) Heaviest in weight (atibharayukta; A.P.)	Heavy in weight (R.R.S. and A.P.) Brittle (churnibhuta; R.R.S.)
Acceptable variety	No	Yes (A.P.)[15]	Yes (R.R.S.)[14] due to comparatively large amount of satva

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Table 3. Description of mineral realgar As_4S_4 (arsenic sulphide) and its polymorphs

Parameters	Alacranite (M1)	Realgar (M2)	Pararealgar*
Chemical formula	As_4S_4	As_4S_4	As_4S_4
Chemical composition (using EDXA)	Arsenic 51.33%; sulphur 20.85%	Arsenic 68.14%; sulphur 31.86%	Not verified
Colour	Red, orange–yellow tint	Dark bright red	Yellow to orange
Streak	Yellow–orange	Orange to reddish-orange	Bright yellow
Cleavage	Indistinct	Good in one direction	None
Fracture	Conchoidal	Conchoidal	Uneven
Luster	Vitreous, resinous, greasy	Adamantine, resinous, sub-metallic	Vitreous to resinous
Tenacity	Very brittle	Slightly sectile	Brittle
Hardness	1.5	1.5–2	1–1.5
Specific gravity	3.4–3.46	3.5	3.5–3.6
Crystal system	Monoclinic	Monoclinic	Monoclinic

*Note: The mentioned physical properties of alacranite and realgar are verified in this study. However, data on pararealgar are taken from external sources[21].

Figure 1. Raw samples M1 and M2 corresponding to alacranite and realgar respectively.

High Score (Plus) v3.X database. The EDXA and SEM studies were carried out at Central Instruments Facility, Indian Institute of Technology (BHU), Varanasi. The samples were analysed on a Penta FET Precision OXFORD Instruments – X-act ZEISS model no. 51-1385-046 after gold grid coating by Coater – Sputter QUORAM Q-150RES. Particles with different patches (spots) were analysed by EDXA to ascertain the presence of elements. SEM study was done on very fine-grained powder of the sample (M3) to examine under electron microscope of 15000×. The surface photograph and thereof particle size estimation of a single particle as well as clusters of particle were analysed.

The colour, odour and texture of samples M1–M4 have been examined thoroughly (Table 4). M1 was red with orange–yellow streak, whereas M2 was dark red with orange streak. M1 and M2 were turned into smooth orange powder after shodhana. Figures 2 and 3 and Table 5 present XRD and EDXA data of the studied samples respectively. SEM study of M3 shows that the particles are homogenously mixed. Minimum size of half of the particles ranges from 400 to 800 nm (with maximum size 15.55 μm) (Figure 4). Various crystallites in different shapes like rod-shaped, cubical and square-shaped are embedded in lumps to form bigger particles.

The physico-chemical correlation of three varieties of Manahshila and three polymorphs of realgar (As_4S_4) has been discussed. Realgar melts at 320°C and burns with a bluish flame, releasing fumes of arsenic and sulphur. It is polymorphous with alacranite and pararealgar[20]. Pararealgar (β-polymorph of As_4S_4) gradually forms from realgar as a result of long exposure to light[21]. In the crystal structure of realgar, each arsenic atom is bonded to two sulphur atoms and another arsenic atom. As the As–As bonds are weaker than the As–S bonds, certain

Table 4. Organoleptic characteristics of alacranite (M1), realgar (M2), purified realgar (M3) and purified alacranite (M4) samples

Sample	Colour	Texture	Odour
M1	Red with orange–yellow precipitate	Smooth, fine	Metallic
M2	Dark red with orange precipitate	Smooth, fine	Metallic
M3	Orange	Smooth, fine, powder	Characteristic
M4	Orange	Smooth, fine, powder	Characteristic

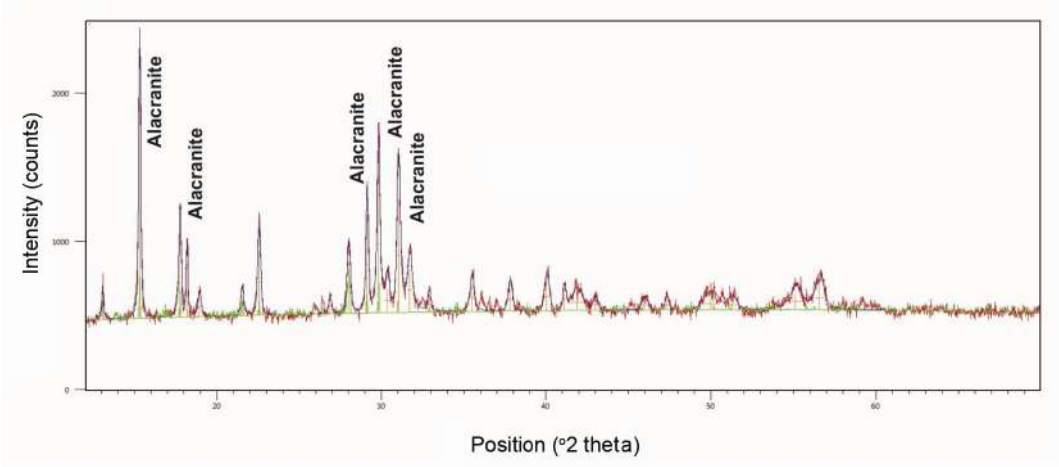

Figure 2. XRD scan of the sample M1, where five major peaks of alacranite are labelled. Other visible minor peaks also belong to alacranite.

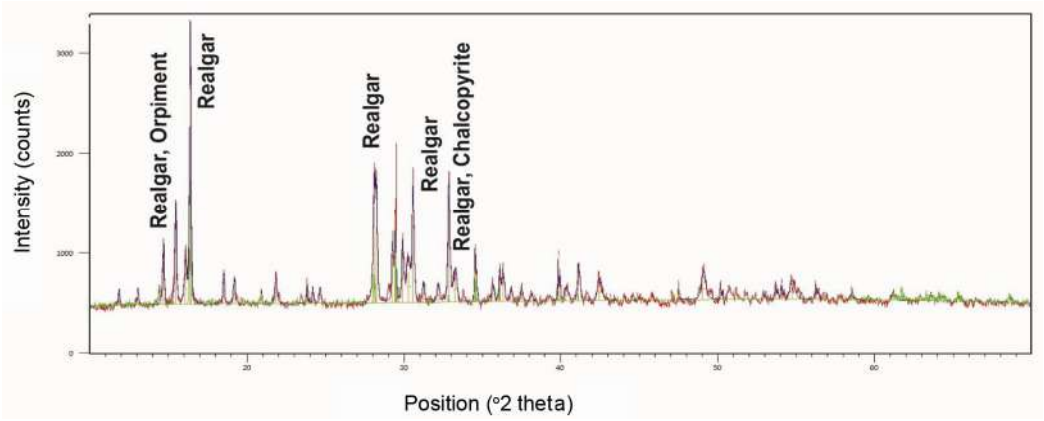

Figure 3. XRD scan of sample M2, where five major peaks of realgar, orpiment and chalcopyrite are labelled.

wavelengths of light interact with the crystal structure of realgar, breaking the weaker bonds between arsenic atoms and destabilizing the realgar structure, causing it to become powdery pararealgar without changing the overall chemical composition[22]. Alacranite (α-polymorph of As_4S_4) was first observed in association with barite–quartz calcite veins in the Alacran silver mine, Pampa Larga mining district, Chile in 1970 (ref. 23). In 1986, Popova *et al.*[24] have provided a detailed mineralogical description and a name for it. In alacranite, each arsenic atom is bonded to another arsenic atom and two sulphur atoms, while the sulphur atoms are bonded only to two arsenic atoms. The structural arrangement of molecules in alacranite is chemically similar to realgar bound together by van der Waals forces. The differences between alacranite and realgar have been demonstrated in the unit cell sizes and the packed structures of both minerals, which have been further confirmed through XRD study[25].

A wide range of internal and external applications of Manahshila is known since 200 BC. However, detailed description is available in the text of Indian alchemy only after the 12th century AD (Table 2). There are diverse opinions regarding the selection of genuine samples for therapeutic use. Some authors consider khandakhya variety to be the best because it contains high percentage of satva (i.e. somal)[14], whereas others recommend kanavirak

due to its physical properties[15]. By scrutinizing the properties of kanavirak and khandakhya varieties of Manahshila, it is observed that both varieties have similar characteristics. The established physical and crystallographic properties of polymorphs of As_4S_4 minerals have been correlated with the three varieties of Manahshila. Kanavirak has been considered as the best variety by Acharya Madhav, the author of *Ayurveda Prakash*, which has the similar properties to alacranite. On the other hand, Rasa Vagbhatta, the author of *Rasa Ratna Samucchaya* explained khandakhya variety as the best. In EDXA study, variation in arsenic has been observed from 51.33% to 68.14% in alacranite and realgar respectively. Thus, it is recommended that khandakhya may be considered as realgar.

All relative peaks are identified as alacranite with molecules of As_4S_4 in XRD analysis of M1 (Figure 2). The same sample was also studied after shodhana (M4), producing similar peaks as the raw sample (M1). In sample M2, most of the high-intensity peaks are identified as realgar (As_4S_4) with monoclinic structure. Some of the peaks (1 or 2) with low intensity are identified as orpiment (As_2S_3) and chalcopyrites ($CuFeS_2$), present in very low concentration (Figure 3). In nature, the sulphide minerals are generally associated with each other due to similar physico-chemical properties. The vast majority of sulphide minerals are part of hydrothermal sulphide ores[26]. After shodhana, XRD data of sample (M3) correspond to realgar, and no peak of associated minerals is identified. Moreover, some of the peaks also correspond to organometallic compounds, which may be added during shodhana through organic phyto-constituents and their reaction with metallic compound.

The objective of shodhana is detoxification of a mineral. This is achieved by a single or multiple processes like trituration followed by sublimation, roasting, etc. This concept modifies characteristics of a drug and enhances its therapeutic action for medicinal purposes[27]. For Manahshila, various pharmaceutical processes are described which can be performed by selecting the respective liquid medium mentioned under that pharmaceutical process. Fresh extracted juice of ginger (*Zingiber officinalis* Roscoe) is one of the commonly used media, in the process of Manahshila. In this study, an increase in the weight of Manahshila is observed after shodhana process, which is due to the addition of solid organic phyto-constituents from ginger juice. Similarly, levigation with ginger juice changes red colour of Manahshila powder into orange colour. EDXA study of shuddha Manahshila shows less percentage of arsenic. This may be due to addition of solid extract of ginger during the bhavana process. Thus 590 ml of ginger juice was absorbed in 300 g of Manahshila during the bhavana process seven times, resulting in an increase in weight by 36 g of Manahshila (final weight of shodhit Manahshila was 336 g). Shodhana of Manahshila by ginger juice involves several effective mechanisms. It may act as a phytochelation[28]. Phytochelatins are peptides which bind heavy metals by chelation, thus playing an important role in detoxification of heavy metals[29]. Ginger contains two significant sulphur-based amino acids (viz. cysteine and methionine), which can act as phytochelatins and render arsenic in the Manahshila nontoxic. Cysteine, a methyl donor peptide, helps in the process of methylation[30]. This is a process of detoxifying arsenic in the body through accelerated excretion. Ginger also preserves the level of glutathione, a natural antioxidant-recycling enzyme present in the blood. It acts as a detoxifying compound by combining with arsenic and excretes it via the bile. Arsenic poisoning reduces the level of glutathione and ingestion of ginger prevents the fall of glutathione in the blood. Hence, bhavana with ginger juice supports detoxification as well as combating its possible depletion due to arsenic. Nano-sized (10^{-9} m) range is known for its fast penetration and quicker action. In this study, half of the particles are in the nano range. It was found that nano-sized realgar particles could substantially enhance bioavailability[31].

The Ayurvedic Pharmacopoeia of India includes monograph of 21 metals and minerals; however, Manahshila is not included in this list. This detailed physico-chemical

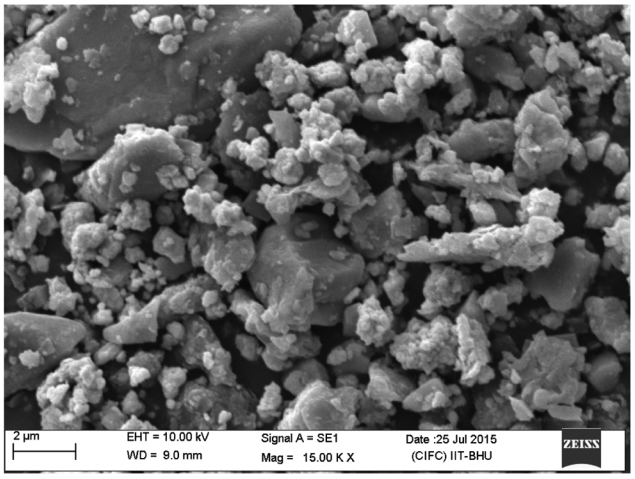

Figure 4. SEM image of sample M3 showing agglomerates of size range 400–800 nm.

Table 5. EDAX findings of alacranite (M1), realgar (M2) and purified realgar (M3) samples

	Weight %		
Element	M1	M2	M3
C K	27.81	–	43.97
O K	–	–	24.45
S K	20.85	31.86	9.31
As L	51.33	68.14	22.28
Total		100.00	

study of Manahshila may be helpful in the preparation of its monograph.

XRD and EDXA studies of two selected samples of Manahshila classified them as realgar (khandakhya) and alacranite (kanavirak) respectively, both accepted as the best varieties for therapeutic use. In the process of Manahshila, ginger juice has been used, which may subside the toxic effect of the drug by acting as a chelating agent. This has been verified through XRD study, as observed from the additional peaks of some organometallic compounds. Thus, this work provides a valid correlation between the traditional information on characterization and processing of Manahshila in toto and corresponding polymorphs of realgar, and confirms the most acceptable type, i.e. khandakhya to be realgar with formula As_4S_4.

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Original Article

Chemical Characterization of an *Ayurvedic* Herbo-Mineral Formulation - *Vasantakusumākara Rasa*: A Potential Tool for Quality Assurance

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Abstract

Background: Herbo-mineral formulations of Ayurveda contain specified metals or minerals as composition, which have their beneficial effects on biological systems. These metals or minerals are transformed into non-toxic forms through meticulous procedures explained in Ayurveda. Though literature is available on quality aspects of such herbo-mineral formulations; contemporary science is raising concerns at regular intervals on such formulations. Thus, it becomes mandate to develop quality profiles of all formulations that contain metals or minerals in their composition. Considering this, it is planned to evaluate analytical profile of *Vasantakusumākara Rasa*. **Objective:** To prepare *Vasantakusumākara Rasa* as per Standard operating Procedures (SoP) mentioned in classical text and to characterize it chemically using modern analytical techniques. **Materials and Methods:** The drug (*Vasantakusumākara Rasa*) in three batches was prepared in GMP certified pharmacy. Physico-chemical analysis, Assay of elements and HPTLC were carried out as per API. XRD was conducted using Rigaku Ultima-IV X-ray diffractometer. **Results:** The analysis shown the presence of Mercury, Tin, Gold, Silver, Iron, Zinc and Calcium etc., and HPTLC revealed presence of organic constituents from plant material. The XRD indicated the presence of cinnabar (mercury sulphide from *Rasa Sindhura*), cassiterite (tin oxide from *Vaṅga Bhasma*), massicot (lead oxide from *Nāga bhasma*) and Magnetite (di-iron oxide from *Loha bhasma*). **Conclusion:** The physico chemical analysis reveals that VKR prepared by following classical guidelines is very effective in converting the macro elements into therapeutically effective medicines in micro form. Well prepared herbo-mineral drugs offer many advantages over plant medicines due to their longer shelf life, lesser doses, easy storing facilities, better palatability etc. The inferences and the standards laid down in this study certainly can be utilized as baseline data of standardization and QC.

Keywords: *Lead, mercury, Rasa Sindhura, Vasantakusumākara Rasa, X-ray diffraction*

Introduction

Vasantakusumākara Rasa, a classical herbo-mineral formulation is in practice since long for various therapeutic purposes.[1,2] The drug said to bring new positive energy in body and life, glow to skin etc., and is known for its *Rasāyana* (rejuvenative), *vājīkaraṇa* (aphrodisiac), *Pramehanāśaka* (anti-diabetic) and *Jarā vyādhi nāśaka* (anti aging) properties. It is therapeutically known for *Prameha* (diabetes), *Vṛkkāmaya* (diseases of urinary tract), *Smṛti bhraṃśa* (memory loss), *Kārśya* (general debility), *Unmāda* (insanity), *Śvāsa* (bronchial asthma) etc.[3,4] Though it is an important formulation; standard manufacturing procedure (SMP) and preliminary analytical profiles are unavailable. Considering this, we planned to develop the physico-chemical profile along with possible characterization of *Vasantakusumākara Rasa*

Materials and Methods

Composition and preparation of *Vasantakusumākara Rasa*

Raw materials were procured from M/s. Maharishi Ayurveda Pharmacy, Noida and authenticated by Dravyaguna expert/ Botanist, while the metal/minerals were certified by Rasa Śāstra experts. The composition of the formulation is mentioned in Table 1.

Preparation of *Vasantakusumākara Rasa*

The drug was prepared in the following steps.

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Table 1: Formulation composition of *Vasantakusumakara Rasa*

Materials used	Botanical/English name	Part/form used	Proportion
Pravāla	Coral	*Bhasma* (incinerated coral)	4 parts (180 g)
Rasa Sindura	Mercury sulphide (HgS)	A type of *kupīpakva rasāyana* of mercury	4 parts (180 g)
Mauktika piṣṭi.	Processed pearl	Pisti (powder of pearl)	4 parts (180 g)
Abhraka	Biotite mica	*Bhasma* (calcined mica)	4 parts (180 g)
Rajata	Silver	*Bhasma* (calcined silver)	2 parts (90 g)
Svarṇa	Gold	*Bhasma* (calcined gold)	2 parts (90 g)
Loha	Iron	*Bhasma* (calcined iron)	3 parts (135 g)
Nāga	Lead	*Bhasma* (calcined lead)	3 parts (135 g)
Vaṅga	Tin	*Bhasma* (calcined tin)	3 parts (135 g)
Vasā	*Adhatoda vasica* Nees.	Leaf	QS
Haridrā	*Curcuma longa* Linn.	Stem	QS
Ikṣu	*Saccharum officinarum* Linn.	Stem	QS
Kamala (*Lotus*)	*Nelumbo nucifera* Gaertn.	Flower	QS
Mālatī	*Jasminum officinale* Linn.	Flower	QS
Kṣīra	Cow milk	Milk	QS
Kadalī	*Musa paradisiaca* Linn.	Stem	QS
Śveta candana	*Santalum album* Linn.	Heart wood	QS
Latā kastūrī	*Hibiscus abelmoschus* Linn	Seed	QS

QS: Quantum satis

- Preparation of *Rasa Sindhura*
- Preparation of *Pravāla, Abhraka, Rajata, Svarṇa, Lauha, Nāga* and *Vaṅga Bhasmas*
- Preparation of *Muktā piṣṭi*
- Levigation of the blend with specified liquids and converting into tablet.

Preparation of Rasa Sindhura

Preparation of *Rasa Sindhura* involves preparation *Kajjalī*, *Bhāvanā* (levigation) with *Vaṭāṅkura jala* (decoction of leaf buds of *Ficus benghalensis* Linn.) and processing in *vāluka yantra* Annexure 1.[5]

Kajjalī was prepared by triturating equal quantities i.e. 180 gm each of *Hiṅgulottha Pārada* Annexure 1 (Mercury obtained from cinnabar) and *Śuddha Gandhaka* (processed sulphur) in a *khalva yantra* (mortar pestle), till the formation of a black coloured, soft, lustreless fine collyrium like powder.[6-9] This was further levigated with *Vaṭāṅkura jala* and then dried. This was filled in a strong amber coloured *kāca kūpī* (glass bottle) in *Vāluka yantra* (heating device) and subjected to increasing heat gradually. Mild heat was applied for first 6 hours, followed by moderate heat. When the bottom of the bottle became red, the mouth of the bottle was blocked with cork and sealed with mud mixed with lime and jaggery smeared cloth. This was followed by application of strong heat for the next six hours. Thereafter, the *Vāluka yantra* was allowed to cool down on its own [Figure 1]. The bottle was then removed from the *Vāluka yantra* and the mud smeared cloth was scraped out using a knife. The bottle was broken down carefully to collect crystallized *Rasa Sindhura* of 180 gm from the neck of the bottle.

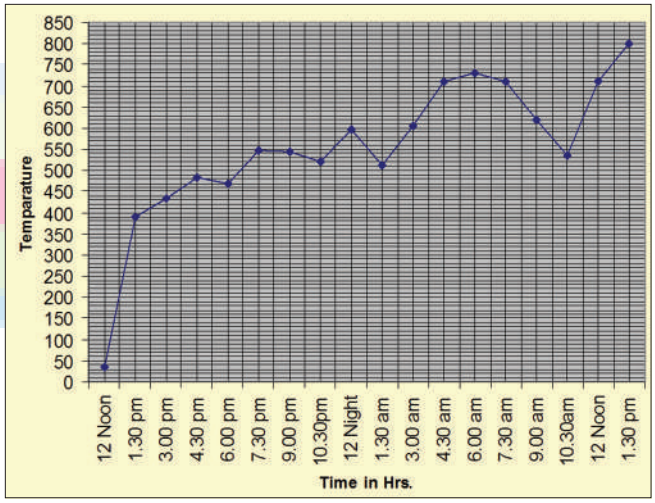

Figure 1: Showing the temperature pattern for the preparation of *Rasa Sindura*

Preparation of Bhasmas

Preparation of Pravāla Bhasma

260 gm of *śodhita Pravāla* (coral processed in *Sarjikṣāra* water for 3 hours*)* was levigated in the juice of *Aloe vera,* flat thin pellets were prepared, dried, placed in *śarāva sampuṭa* Annexure 1 and as heated in *Gajapuṭa* Annexure 1. At the end, 180 gm of *Pravāla bhasma* of desired quality was obtained.[10,11]

Preparation of Abhraka Bhasma

Vajrābhraka was processed in presence of *Triphalā* (three myrobalans) decoction by following classical guidelines. This was bundled in a jute bag with 1/4th quantity of paddy and immersed in *kāñji* Annexure 1 for 3 days, followed by

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thorough rubbing in *Kāñji,* so that fine *Abhraka* particles can enter into the liquid. The settled down contents known as *Dhānyābhraka* were collected carefully by decanting the liquid.

300 gm of *Dhānyābhraka* was levigated with *Arka kṣīra* (latex of *Calotropis procera*), flat thin pellets were prepared, dried, placed in *śarāva sampuṭa* and heated in *Gajapuṭa.* At the end of seventh *puṭa*, the contents were levigated with *Nyagrodha mūla kvātha* (decoction of *Ficus benghalensis* roots), dried and was subjected to *gaja puṭa*. The process was repeated three times followed by levigation with *Rambhā rasa* (juice of rhizome of *Musa paradisiaca*) and seven *gaja puṭas*. Finally, 180 gm of *Abhraka Bhasma* was obtained.[12-14]

Preparation of Rajata Bhasma

Rajata Patras were heated till red hot and immersed consecutively in *Tila taila, takra, kāñji, gomūtra* and decoction of the seeds of *kulattha.* The whole process was repeated three times. Śuddha Rajata patra (70 g) were further processed in *Agastya svarasa*, *Śuddha Hiṅgula* (processed cinnabar) was added to it and ground well to prepare a homogenous mass, placed in *Ūrdhva patana yantra* and heated for six hours. On cooling, the pot was opened to collect 90 g of *Rajata bhasma.*[15-17]

Preparation of Svarṇa Bhasma

Svarṇa patras were heated till red hot and immersed consecutively in *Tila taila, takra, kāñji, gomūtra* and decoction of the seeds of *kulattha*. This was amalgamated by triturating with *Śuddha pārada*. 95 g of śuddha svarṇa patra was placed in a *śarāva* along with half quantity of *Gandhaka* and covered by another *śarāva*. This *sampuṭa* was dried under sun and subjected to a *puṭa* with 30 cow dung cakes adding one part of *Gandhaka* each time. The process was repeated 14 times and 90 g. of *Svarṇa bhasma was obtained.*[18]

Preparation of Lauha Bhasma

Lauha was heated to red hot and immersed consecutively in *Tila taila, takra, kāñji, gomūtra* and decoction of the seeds of *kulattha*. This was further processed in equal quantities of *Triphalā kaṣāya* and *Gomūtra*. *Lauha cūrṇa* thus obtained was further processed through *Bhānupāka* (processed in sun rays), followed by *Sthālīpāka* (heated with decoction of *Triphalā* in stainless steel vessel). *Śuddha lauha patra* (125 g) was levigated with *Triphalā kvātha; Cakrikās* were prepared, dried and placed in *śarāva sampuṭa* subjected to *Gaja puṭa*. The same procedure was repeated for 60 times and 135 g of *Lauha bhasma* was obtained.[19-22]

Preparation of Nāga Bhasma

Molten *Nāga* was poured consecutively in *Tila taila, takra, kāñji, gomūtra* and decoction of the seeds of *Kulattha* for three times each in all the liquids. This was further collected in an iron pan and heated. On melting; powders of *Cincā tvak* (*Tamarindus indica* Linn.) and *Aśvattha tvak* (*Ficus religiosa* Linn.) were sprinkled in small quantities and stirred with *loha darvi* (Iron spatula). This process was continued till the molten *Nāga* is reduced to powder form.

165 g of *jarita nāga* was added to equal quantity of *Manaḥśilā* and levigated with *kāñji;* small *cakrikās* were prepared, dried and placed in *śarāva sampuṭa* and subjected to *ardha gaja puṭa* Annexure 1.[23] This process was repeated 60 times and 135 g. of *Nāga bhasma* was obtained.[24]

Preparation of Vaṅga Bhasma

Molten *Vaṅga* was poured consecutively three times each in *Tila taila, takra, kāñji, gomūtra* and decoction of the seeds of *kulattha*. In further processing, this was again melted and poured in *Sinduvāra svarasa* (juice of *Vitex negundo* Linn.) added with *haridrā cūrṇa* (powder of *Curcuma longa* Linn.) three times. The material obtained at the end of this process was converted into a powder (Jarita Vaṅga) by treating with powders of *Cincā* and *Aśvattha tvak.* 115 gm of jarita Vaṅga was added with equal quantity of orpiment powder and levigated with lime juice. Small *cakrikās* were prepared, dried, placed in *śarāva sampuṭa* and subjected to *ardha gaja puṭa*. This process was repeated 10 times and 13g of *Vaṅga bhasma* was obtained.[25,26]

Preparation of Muktā Piṣṭi

190 g of *Muktā* (pearl) was made into a bundle in a piece of cloth, suspended and heated in a vessel containing *Jayantī svarasa.* This was triturated with rose water for 21 days and 180 g. of *Muktā Piṣṭi* was obtained.[27,28]

Levigation of the blend with specified liquids and converting into tablet

All above ingredients were mixed together (1.305 kg) thoroughly to make a uniform blend and levigated with nine *Bhāvanā dravyas* in an end runner [Table 2]. At the end of this, 1.895 kg of contents were carefully collected, shifted to a tray drier and dried at 50°C. Dried material was shifted to tablet section and granules were prepared. 2% talcum powder was added to the granules and compressed

Table 2: Brief details of levigation process

Ingredient	Quantity of liquid for one *Bhāvanā* (ml)	Number of *Bhāvanās*	Time taken (h)
Vasā Svarasa	500	Seven	61
Haridrā Kvātha	150	Seven	56
Ikṣu Svarasa	245	Seven	49
Kamalapuṣpa Phāṇṭa	140	Seven	52
Mālatīpuṣpa Phāṇṭa	140	Seven	55
Kṣīra	150	Seven	59
Kadalīkāṇḍa Svarasa	140	Seven	52
Śvetacandana Phāṇṭa	180	Seven	54
Latākastūrī Hima	180	Seven	58

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into tablets of 125 mg size by passing through a Rotary Tablet Punching Machine.

Physico-Chemical Analysis

Physicochemical analyses, *viz.* estimation of loss on drying, ash content, acid insoluble ash, water/alcohol soluble extractive, *p*H, etc., qualitative/quantitative elemental testing, residual pesticide, microbiological examination and tablet parameters *viz.* hardness, friability, average weight, dissolution time etc**.,** were carried out by following standard methods as per Ayurvedic Pharmacopoeia of India (API) guidelines.[29-34] The quantitative estimation of heavy metals viz. Pb, Cd, As, Hg and Cr was carried out by Atomic Absorption Spectrometer (Perkin Elmer (USA) Analyst 400) and the other elements viz., Mg, Cu, B, Mn, Al, were analyzed on ICP-AES (Thermo Electron Corporation's model Iris Intreprid II XDL). However, Sulphur, Silica, Sn, Au, Ag, Fe, Zn, and Ca, were quantified by using conventional methods.[34]

High performance thin layer chromatography method

Sample preparation: 2 g powder each of three batches of VKR were soaked overnight separately in 20 ml of methanol. The solutions were continuously stirred for 6 hr and kept for next 18 hr and then the filtered samples were dried and made into 10% solution.

High Performance Thin Layer Chromatography was performed on TLC plates pre-coated with 0.25 µm thin layers of silica gel 60 F_{254} (E. Merck). 10 µL methanolic solution of formulation (three batches) were applied on the plates as bands 8.0 mm wide by use of a Linomat-V applicator (CAMAG, Switzerland) fitted with a 100 µL syringe (Hamilton, Switzerland). The application positions X and Y were both 10 mm, to avoid edge effects. Linear ascending development to a distance of 80 mm with mobile phase-Toluene: Ethyl acetate: formic acid 10:3:1 (v/v) was performed in a twin-trough glass chamber previously saturated with vapours of mobile phase for 20 min. The plates were dried in air and visualized under 254 nm and 366 nm for ultra violet detection and the fingerprints were taken. The same TLC plate was also derivatized with anisaldehyde-sulphuric acid reagent and visualized in white light.

X-ray diffraction study

Powder X-ray diffraction (XRD) analysis was carried out using Rigaku Ultima-IV X-ray diffractometer with CuK α radiation (λ=1.54 A°) operating at 40 kV and 30 mA. Pattern was recorded for angle (2θ) ranging from 10-100 degree at a scanning rate of 1 degree/second and scan step of 0.1 degree. XRD pattern of VKR (3 batches) is shown in spectra. Sample identification was done by matching d-spacing with the standard database.

Results and Discussion

The organo-leptic observation shows that the prepared VKR is a Brown colored tablet with aromatic odour and bitter, astringent taste. The qualitative analysis shows positive tests for the presence of Mercury, Tin, Gold, Silver, Iron, Zinc and Calcium. Chemical analysis revealed presence of 6.27% of Tin, 10.79% of Mercury, 2.89% of Sulphur, 5.66% of Iron, 3.67% of Gold, 4.47% of Silver, 9.5% of Calcium, 4.95% of Zinc, 1.196% of Magnesium, 1.05% of Lead and 2.08% of Silica with other trace elements such as Aluminum, Manganese, Copper, Boron, Chromium, Cadmium and Arsenic etc., Moisture content (4.18%), total ash (57.61%) is left after burning of organic/volatile matter (39.29%), water soluble (13.54%) and alcohol soluble (11.48%) extracts were evaluated (all these values are average analytical values of three batches). The drug was tested for residual pesticides and microbiological examination and they were found to be in permissible limits [Table 3]. HPTLC revealed presence of organic constituents from plant material [Figure 2 and Table 4].

Table 3: Analytical profile of *Vasantakusumākara Rasa*

Parameters	Observations
Organoleptic characters	
Colour	Brown
Taste	Bitter and astringent
Odour	Aromatic
Appearance	Tablet
Physico-chemical	
Identification	Yields the reaction characteristics of tin, gold, silver, iron, zinc and calcium
Loss on drying at 105°C	3.50-5.0
Total ash (%w/w)	56.50-58.50
Acid insoluble ash (%w/w)	16.0-19.50
Alcohol soluble extractive (%w/w)	10.50-12.50
Water soluble extractive (%w/w)	12.0-15.0
pH (1% of aqueous extract)	6.5-7.5
Organic and volatile matter	39.26 and 39.32
Specific gravity	0.9986-0.9990
Particle size distribution	
10%	0.188-0727
50%	1.332-3.855
90% (µm)	11.299-13.656
Assay of elements	
Tin (%)	6.0-7.0
Gold (%)	3.0-4.0
Silver (%)	4.0-5.0
Iron (%)	5.0-6.0
Zinc (%)	4.5-5.5
Calcium (%)	9.0-10.0
Mercury (%)	10.0-11.50
Sulphur (%)	2.5-3.5
Lead (%)	1.0-1.5
Silica (%)	2.0-4.0

Contd...

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Table 3: Contd...

Parameters	Observations
Magnesium (%)	0.50-2.0
Copper (%)	0.10-0.70
Boron (%)	0.05-0.25
Manganese (%)	0.01-0.06
Chromium (%)	0.01-0.04
Aluminium (%)	0.80-2.50
Arsenic (ppm)	35-40 ppm
Cadmium (ppm)	0.05-0.15 ppm
Residual pesticide (mcg/kg)	
Alpha and beta HCH	<200
Gamma HCH	<200
Delta HCH	<200
DDT and metabolites	<200
DT	15–20 min
Hardness	2 kg/cm^2
Friability	0.15%-0.25%
Average weight	125.05-125.25 mg/tablet
Microbiological examination	
Total aerobic count (CFU/g)	6000-9000
Coliform (*Enterobacteriaceae*) (CFU/g)	NAD
Escherichia coli (CFU/g)	NAD
Salmonella spp. (CFU/10g)	NAD
Staphylococcus aureus (CFU/g)	NAD
Yeasts (CFU/g)	NAD
Moulds (CFU/g)	NAD
Pseudomonas aeruginosa	Absent

NAD: No Abnormalities detected, DT: Dissolution Time

Table 4: Observations/visualization/detection (R_f values)

	VKR		
	Batch I	**Batch II**	**Batch III**
At 254 nm	0.11, 0.23, 0.71	0.11, 0.23, 0.71	0.11, 0.23, 0.71
At 366 nm	0.24, 0.72, 0.83	0.24, 0.72, 0.83	0.24, 0.72, 0.83
At 520 nm	0.55, 0.68, 0.83	0.55, 0.68, 0.83	0.55, 0.68, 0.83

VKR: *Vasantakusumākara Rasa*

The XRD patterns of the three batches of VKR as shown in spectra are nearly identical. Batch II showed slightly different phases. XRD results as shown in Table 5 indicate that all the samples contained cinnabar (mercury sulphide added as Rasa Sindhura); cassiterite (tin oxide, Vaṅga Bhasma); and, Massicot (lead oxide; Nāga bhasma) and Magnetite (di-iron oxide; Loha bhasma). No signature of *Raupya* or *Svarṇa Bhasma* could be identified in these spectra, indicating that the methods used for their preparation, did not yield any crystalline products or their signature is buried under the strong lines of other constituents. The XRD lines at 38.3, 44.4 and 64.7 degrees could show the presence of elemental gold. This has to be examined under light microcopy, for the presence of any shiny particles, which, if present, can indicate the reduction of *Svarṇa bhasma* added into metallic gold in the presence of organics. Elemental chemical analysis has

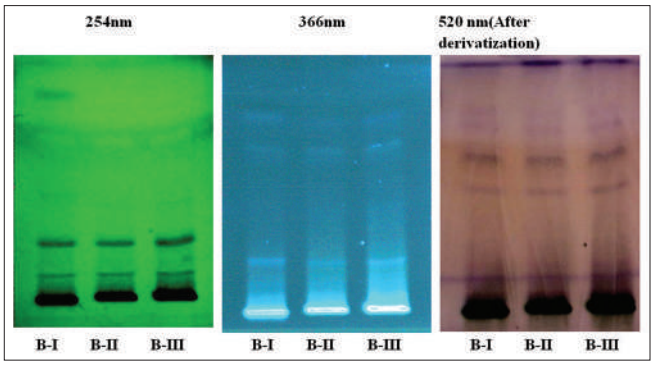

Figure 2: High performance thin layer chromatography Profiles of *Vasantakusumakara Rasa*

shown the presence of gold and silver. The crystalline form of *Abhraka bhasma* appears to be not present [Figures 3 and 4].

Despite the addition of *Pravāla bhasma* and *Mauktika piṣṭi* (essentially Calcium carbonate) has not shown any crystalline form such as aragonite or calcite in the XRD pattern despite high presence of calcium in the chemical analysis. The presence of crystalline magnesium oxide is not explained by the added inorganic chemicals in the preparation of VKR.

It can be seen that the three XRD patterns on the samples are qualitatively same in the relative intensities of the peaks. The assay of total ash content in these samples (app. 56% - 59% almost matching the expected fraction of inorganic constituents by weight) and the XRD results indicate that the inorganic contents have remained intact over time.

Conclusion

The physico chemical analysis reveal that VKR prepared by following classical guidelines is very effective in converting the macro elements into therapeutically effective medicines in micro form. Well prepared herbo-mineral drugs offer many advantages over plant medicines due to their longer shelf life, lesser doses, easy storing facilities, better palatability etc., The inferences and the standards laid down in this study certainly can be utilized as baseline data of standardization and quality assurance of this herbo-mineral formulation. It will be helpful laying down the further pharmacopoeial standards of *Vasantakusumākara Rasa*.

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Table 5: d-spacing and 2θ (°) values of X-ray diffraction analysis

2θ	d (A)	Size (A)	Chemical formula	Phase data name	cps
26.6	3.3	245.0	HgS, SnO_2	Cinnabar (1, 0, 1), cassiterite, syn (1, 1, 0)	145.0
28.1	3.2	87.0	HgS	Cinnabar (0, 0, 3)	15.0
29.5	3.0	211.9	PbO	Massicot, syn (1, 1, 1)	22.5
30.2	3.0	212.3	PbO, Fe_3O_4	Massicot, syn (0, 0, 2), iron diiron (III) oxide, magnetite HP, syn (2, 2, 0)	12.0
31.3	2.9	333.0	HgS, MgO	Cinnabar (1, 0, 2), magnesium oxide (2, 2, 0)	100.0
33.2	2.7	213.9	PbO	Massicot, syn (2, 0, 0)	11.9
33.9	2.6	214.3	SnO_2	Cassiterite, syn (1, 0, 1)	62.3
35.5	2.5	436.0	Fe_3O_4	Iron diiron (III) oxide, magnetite HP, syn (3, 1, 1)	25.0
38.2	2.4	180.0	HgS, PbO, SnO_2, Au, MgO	Cinnabar (1, 0, 3), massicot, syn (2, 1, 0), cassiterite, syn (2, 0, 0), gold, syn (1, 1, 1), magnesium oxide (2, 2, 2)	33.0
43.7	2.1	124.0	HgS, Fe_3O_4	Cinnabar (1, 1, 0), iron diiron (III) oxide, magnetite HP, syn (4, 0, 0)	14.0
44.4	2.0	176.0	HgS, Au, MgO	Cinnabar (1, 1, 1), gold, syn (2, 0, 0), magnesium oxide (4, 0, 0)	12.3
45.8	2.0	223.0	HgS, PbO	Cinnabar (1, 0, 4), massicot, syn (0, 0, 3)	24.0
51.8	1.8	227.9	HgS, PbO, SnO_2	Cinnabar (2, 0, 1), massicot, syn (2, 2, 0), cassiterite, syn (2, 1, 1)	71.8
52.8	1.7	228.8	HgS, PbO	Cinnabar (1, 1, 3), massicot, syn (1, 1, 3)	18.9
54.8	1.7	280.0	HgS, SnO_2	Cinnabar (2, 0, 2), cassiterite, syn (2, 2, 0)	21.2
61.9	1.5	239.0	SnO_2	Cassiterite, syn (3, 1, 0)	11.3
64.7	1.4	292.0	SnO_2, Au, MgO	Cassiterite, syn (1, 1, 2), gold, syn (2, 2, 0), magnesium oxide (4, 4, 0)	15.1
66.1	1.4	244.5	Fe_3O_4, SnO_2	Iron diiron (III) oxide, magnetite HP, syn (5, 3, 1), cassiterite, syn (3, 0, 1)	12.0

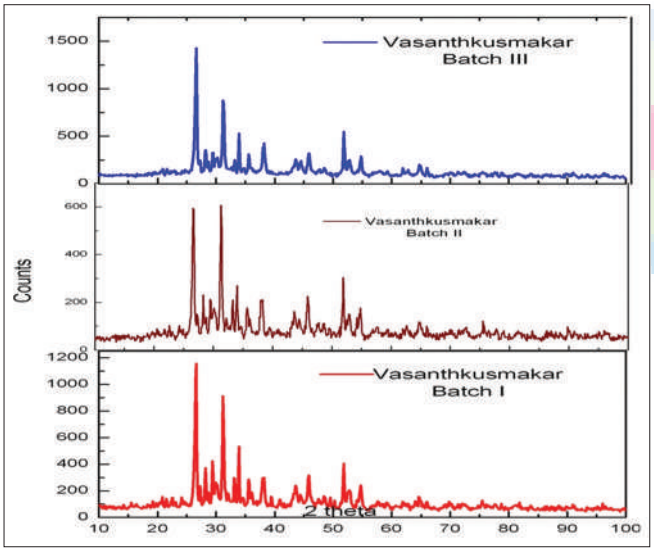

Figure 3: X-ray diffraction pattern of *Vasantakusumakara Rasa* (Batch –I, II, III)

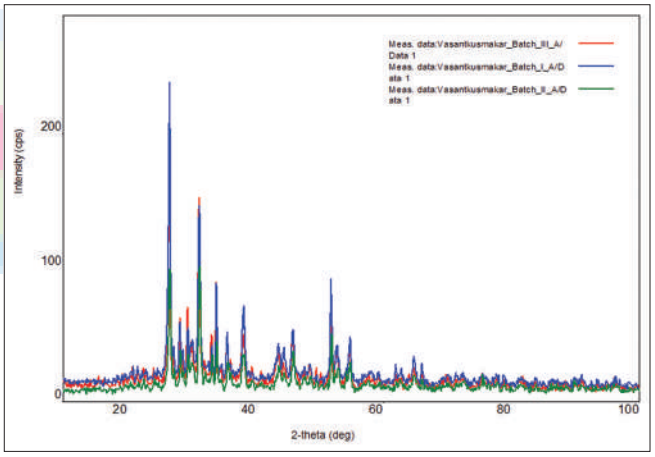

Figure 4: Overlay of X-ray diffraction pattern of *Vasantakusumakara Rasa* (Batch –I, II, III)

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Conflicts of interest

There are no conflicts of interest.

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Annexure-1

1. *Vāluka Yantra*
   Vāluka yantra is a sand bath heating vessel where in a wide mouth round pot is 1/4th filled with sand and then the glass bottle having the ingredients and covered with seven layered mud smeared cloth was lowered to the bottom of the pot. The empty space of pot was filled with sand upto the neck of the bottle; later heated for specified time.
2. *Hiṅgulottha pārada*
   Hiṅgula was levigated thoroughly in *khalva yantra* with lime juice. Small *cakrikās* (pellets) were prepared, dried and kept in the *tiryak patana yantra* and heat was applied. *Pārada* was collected then heating was stopped.
3. *Śarāva Sampuṭa*
   To reduce any metal or mineral into *bhasma* form, it is triturated with other prescribed drugs and made to a paste. Out of this paste, small round and flat cakes (*Cakrikās*) are prepared and dried under sun. Thereafter, these *cakrikās* are kept inside two earthen plates and the joint of these plates is sealed by wrapping seven layers of mud smeared cloth. This is dried under sun before being subjected to a *Puṭa*.
4. *Gajapuṭa*
   The *Gajapuṭa* is an arrangement of heating in a pit of 90 cm in length, breadth and depth. Half the pit is filled with cow-dung cakes. The *śarāva sampuṭa* is put upon it, the empty space above is filled again cow-dung cakes and ignited.
5. Kāñji
   01 kg powder of *Āśudhānya* such as *Kulmāṣa, Ṣaṣṭika* rice, etc., along with 250 gm of white radish (*Mūlaka*), cut into pieces, are placed in an earthen pot and 05 litres of water is added. The mouth of the pot is closed and kept for 10 days during which the fluid becomes sour. This sour fluid is called *Kāñjika, Dhānyāmla or Arṇala.*
6. *Ardha Gaja Puṭa*
   There is no such description of *Ardha gajapuṭa* mentioned in classical texts except mention of its name alone in in *Rasāmṛtam. Ardha gaja puṭa* can be considered as half of *gaja puṭa.* The *Ardha Gaja puṭa* is an arrangement of heating in a pit of 45 cms in length, breadth and depth. Half the pit is filled with cow-dung cakes. The *śarāva sampuṭa* is put upon it, the empty space above is filled again cow-dung cakes and ignited.

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Original Research Article (Experimental)

Chemical characterization of an *Ayurvedic* herbo-mineral preparation- *Mahalaxmivilas Rasa*

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ABSTRACT

Background: To protect the massive trust of patient in Ayurveda, a need aroused for the researches to ascertain the quality, safety & efficacy of herbo-mineral preparations on scientific lines. The rasa-aushadhis are having qualities such as instant effectiveness, requirement in very small dosage and ample therapeutic utility. *Mahalaxmi Vilas Rasa* [AFI, 20:27] has been used for treatment of a variety of ailments since time immemorial.

Objective: To prepare *Mahalaxmi Vilas Rasa* as per standard operating procedures (SoPs) mentioned in classical text and to characterize it chemically using modern analytical techniques.

Materials and Methods: The drug (*Mahalaxmi Vilas Rasa*) in three batches was prepared in GMP certified pharmacy. Physico-chemical analysis, HPTLC, Assay of elements by AAS & ICP-AES were carried out as per Ayurvedic Pharmacopoeia of India. Powder X-ray diffraction (XRD) was conducted using Rigaku Ultima-IV X-ray diffractometer.

Results: The elemental analysis shown the presence of Mercury, Sulphur, Calcium, Copper, Gold, Iron & Tin etc. and HPTLC revealed presence of organic constituents from plant material. The XRD had indicated that prepared drug contained free sulphur, cinnabar (mercury sulphide added as Kajjali), cassiterite (tin oxide, Vanga Bhasma), orpiment (Hartal, arsenic III sulphide) and mica (Leucite/ Zeolite, Abhrak Bhasma). The drug was also tested for residual pesticide and microbiological contamination which were found within permissible limits.

Conclusion: Classical pharmaceutical procedures of *Mahalaxmi Vilas Rasa* showed converting the macro elements into therapeutically effective medicines of micro form. Standards laid down in this study certainly utilized as an important tool for standardization and quality assurance of this herbo-mineral formulation.
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1. Introduction

A specialized branch in Ayurveda, which is “Rasa Shastra” having literal meaning as “Science of Mercury” deals with materials known as ‘Rasa dravyas’’. Rasa denotes mainly *Parada* (Mercury). Formulations made by mercury and incinerated metals and minerals are known as Rasa-aushadhis (Herbo-mineral-metallic preparations).

The rasa-aushadhis are having qualities such as instant effectiveness, requirement in very small dosage and ample therapeutic utility. There are four methods of preparation of these formulations i.e. *Khalviya Rasayana, Parpati Rasayana, Kupipakawa Rasyana, Pottali Rasayana. Mahalaxmivilas Rasa* is a herbo-mineral-metallic preparation comes under the *Khalviya Rasayana.*

It is well known for its use in *Urdhwa Jatrugata rogas* (Upper Respiratory Disorders) and *Amavata* (Rheumatoid arthritis). Important therapeutic uses are in *Kasa* (Cough), *Pinasa* (Chronic rhinitis/sinusitis), *Rajayakshma* (Tuberculosis), *Amavata* (Rheumatism), *Vajikarana* (Aphrodisiac), *Gala Roga* (Diseases of throat), lymphadenopathy, *Antra Vriddhi* (Hernia), *Kushtha* (Diseases of skin), non-healing wounds, *Atisara* (Diarrhoea), *Prameha* (Urinary disorders), *Shlipada* (Filariasis), *Vrana* (Ulcer), *Nadivrana* (Fistula), *Bhagandara* (Fistula-in-ano), *Arsha* (Hemorrhoids), *Udara* (Diseases of abdomen/enlargement of abdomen), *Raktavikara* (Disorders of blood), *Stri roga* (Gynaecological disorders), *Tvagroga* (Skin disease),

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Nasa Roga (Disease of nose), *Netraroga* (Eye disorder), *Mukha Roga* (Disease of mouth) and *Shula* (Colicky Pain) [1].

The adjuvant or *anupan* with which medicine should be taken depends on diseases to be treated (Table 1). It is used in Vati/tablet form. As it is an important and commonly used formulation, the documentation on chemical characterization and safety/toxicity studies is essential for its global acceptance. Hence, this study was conducted under Golden Triangle Partnership programme with the objectives to evolve physicochemical profile along with characterization. This paper is dealt only with preparation and chemical characterization.

2. Materials and methods

2.1. *Preparation of* Mahalaxmivilas Rasa

All the raw materials were procured by M/s. Maharishi Ayurveda Pharmacy, Noida and authenticated by Dravyaguna expert/Botanist, while the metal/minerals were certified by Rasa Shastra expert. The composition of *Mahalaxmi Vilas Rasa* is provided in the Table 2. The process involves following steps viz. Preparation of *Bhasmas* (*Abhraka/Swarna/Vanga/Tamra*), Preparation of *Kajjali,* Purification of *Haritala,* Purification of *Dhatura Bija,* Preparation of *Nagavalli Swarasa, Bhavana* with *Nagavalli Swarasa*.

2.1.1. Preparation of Bhasmas (Abhraka/Swarna/Vanga/Tamra)

2.1.1.1. Preparation of Abhraka bhasma [2]. The *Vajrabhraka* was heated to red hot in an iron pan and immersed in *Triphala kwatha.* This process was repeated for seven times. The processed *Abhraka* was bundled in a jute bag with 1/4th quantity of paddy and immersed in *kanjika* for 3 days. Thereafter, the bundle was rubbed thoroughly and squeezed in the liquid itself so that only fine *abhraka* particles can escape through the holes of the bag. Bag was removed from the *Kanji* and the contents were allowed to settle down. The supernatant liquid layers were separated carefully to collect fine particles of *Dhanyabhraka* [3] that were settled down in the container.

Dhanyabhraka (fine Biotite mica obtained through above process) was levigated with *Arka ksheera* (latex of *Calotropis procera* (Ait) R. Br.) for one day; *chakrikas* (thin, flat cakes) were made, and dried in sun rays. These *chakrikas* were placed in a *sharava samputa* (earthern plates) and the junctions were sealed properly. This sealed apparatus was subjected to *Gaja puta* (method of heating with specific temperature pattern). The material thus obtained at the end of the *puta*, was processed in the similar way for six more times. At the end of 7th *puta*; *abhraka* was levigated with *Nyagrodha-mula kwatha* (Decoction of root of *Ficus bengalensis* Linn.), dried and three *Gaja putas* were given. The *Abhraka* was next levigated with *Kadali rasa* (juice of rhizome of *Musa paradisiaca* Linn.) and 07 *Gaja putas* were given. After completion of *putas* it was grounded and preserved in air tight glass jar or porcelain containers [4].

2.1.1.2. Preparation of Tamra bhasma. Thin *patras* (plate) of *shodhita Tamra* [5,6], (processed copper) were boiled in *Nimbu swarasa* for 3 days. One fourth quantity of *shuddha parada* (processed mercury) was mixed with *Tamra patra* and grounded well for 3 h in presence of *Nimbu swarasa* (lemon juice). *Shuddha gandhaka* (processed sulphur) was added to this, levigated by adding *Nimbu swarasa* and made into bolus (*golaka*). This bolus was covered carefully with the paste of *Punarnava root* (*Boerrahvia diffusa* Linn.) and allowed to dry completely. This dried bolus was placed in *sharava* (earthen saucer), covered by another *sharava* and junction was sealed carefully with clay smeared cloth. This *sharava* was then placed in heating device and heated in *kramavriddhi agni (*gradually increased heat) for 12 h. When cooled, the *sharava* was removed from the pot. The product thus obtained was grounded with juice of *Surana kanda* (corm of *Amorphophallus campanulatus* Blume) for a day. A bolus of this was prepared by adding half part of *Gandhaka* and a little quantity of cow ghee. This was placed in *Sharava* and after sealing the joint of two *Saravas* heated in *Gajaputa*. This procedure was repeated for two times more [7].

2.1.1.3. Amrutikarana of Tamra bhasma [8]. The process was performed to remove the remnant impurities present in the *tamra bhasma* and to enhance the therapeutic efficacy by inducing nectar like properties [9]. For this, *Shuddha gandhaka* was added to *tamra bhasma* and levigated well with *Nimbu rasa* for 3 h. This was made into a bolus and placed into *S. kanda* scooped in the middle to accommodate the bolus. The upper portion was covered with *S. kanda* and wrapped with clay smeared cloth and dried. This was

Table 1
List of adjuvant or *anupan* to be taken for various diseases.

No	Diseases/Roga	*Anupan*
1	Heart palpitation, heart pain	Arjuna bark Kwath or Arjunarishta.
2	Sinusitis	Triphala decoction
3	Chronic Coryza	Mulethi powder and ghee, honey mixed unequal quantity
4	Tuberculosis/Kshaya roga	Chausath Prahri Pippal + Honey
5	Prameha, Impotency, Spermatorrhoea	Shilajita + Milk
6	Vitiation of Tridosha	Betel leaf juice
7	Rheumatism	Dashmoola Kwath
8	Diseases of abdomen	Punarnava Ras
9	Diarrhoea, Dysentery	Dry ginger powder + honey
10	Cold, coryza	Betel leaf juice
11	Fever due to cough	ginger juice + Honey.

Table 2
Composition and proportion of each ingredients of the formulation [1].

S. No.	Materials used	Botanical/scientific names	Part/form used	Proportion
1	*Abhraka*	Biotite mica	*Bhasma* (Incinerated powder)	16 parts
2	*Gandhaka*	Sulphur	Processed powder	8 parts
3	*Vanga*	Tin	*Bhasma* (Incinerated powder)	4 parts
4	*Vriddhdaru*	*Argyreia nervosa* (Burmf) Bojer.	Dried seeds	4 parts
5	*Dhatura*	*Datura metel* Linn.	Processed seeds	4 parts
6	*Parada*	Mercury	Processed Mercury	2 parts
7	*Haritala*	Orpiment	Processed powder	2 parts
8	*Karpura*	*Cinnamomum camphora* Linn.	Sublimated Extract	2 parts
9	Jatikosa	*Myristica fragnans* Houtt.	Dried Aril	2 parts
10	*Jatiphala*	*Myristica fragnans* Houtt.	Dried Seeds	2 parts
11	*Swarna*	Gold	*Bhasma* (Incinerated powder)	1 part
12	*Tamra*	Copper	*Bhasma* (Incinerated powder)	1 part
13	*Nagavalli*	*Piper betel* Linn.	Leaf juice	Q.S.(for bhavana)

subjected to *gaja puta*. On cooling, the ashes were removed and *tamra* was collected, powdered and preserved.

2.1.1.4. Preparation of Vanga Bhasma [10]. *Vanga* was taken in a metallic spoon and heated was given till it was melted. The melted *Vanga* was immersed in a pot containing *churnodaka*. After cooling, the *vanga* was collected from the pot and the above process was repeated again for 6 times to obtain processed *Vanga*. The Processed *Vanga* was kept in an iron pan and heated. While it was melting, powders of *cincha* and *Asvattha twak* were sprinkled in small quantities and stirred with *loha darvi* (iron spatula). This process was continued till the melted *vanga* was reduced to powder form called *Jarita Vanga* [11]. *Jarita vanga* was mixed with haritala churna and grounded well in nimbu swaras. Small thin *cakrikas* were prepared, dried and placed in *sarava samputa* and *ardha gaja puta* was given. This process was repeated for 10 times. One fourth of *haratala* is added to *vanga* from the 2nd *puta* onwards. On cooling, the ashes were removed and *vanga* was collected, powdered and preserved. Fig. 1

2.1.1.5. Preparation of Swarna Bhasma [12]. The *Svarna* patras were heated to red hot state and immersed thrice in each of the liquids viz. *Tila-taila* (til oil), *takra (butter milk), kanjika, gomutra* (cow urine) *and Kulatha kashaya* (decoction of horse gram) consecutively [13]. The Processed *patras* so obtained were used for further processing. In *suddha parada*, *sodhita svarna* was added and levigated to make a bolus. Half the quantity of *gandhaka* was kept in a *sarava* and *swarna golaka* was kept on it. The *golaka* was covered with another *sarava*. *Sandhi lepa* of *sarava samputa* was done and dried in sun rays. *Puta* with 30 *vanyopalas* was given adding *gandhaka* each time in 1 part and the process was repeated 14 times. On cooling, the ashes were removed and *svarna* was collected, powdered and preserved [14].

2.1.2. Preparation of Kajjali (black sulphide of mercury) [15]

Hingulotha Parada [16] (Mercury obtained from Cinnabar) and *shuddha Gandhaka* [17] (processed sulphur) were taken in equal quantities in a *khalva yantra* (mortar pestle). The mixture was triturated thoroughly till a black colour, soft, lustreless fine powder like collyrium (*kajjali*) is obtained.

2.1.3. Purification of Haritala [18]

Small pieces of *haritala* were bundled in a piece of cloth and subjected to svedana in *Dola yantra* containing juice of *Kusmanda (Benincasa hispida* Thunb.) for 3 h.

2.1.4. Purification of Dhatura Beeja [19]

Dhattura seeds were soaked in go-mutra (cow urine) for 12 h and then they were washed with water and dried in the sun and subjected to *svedana* in a *dola yantra* containing *go-dugdha* (cow milk) for 3 h.

Fig. 1. Images of finished drug of *Mahalaxmivilas rasa.*

2.1.5. Preparation of Nagavalli Swarasa

Nagavalli patra (leaves of *Nagavalli*) were collected from the local market, washed under tap water and made into small pieces. The pieces of leaves were grinded and a fine paste was prepared. The paste was strained in to a stainless steel vessel through a clean cloth. Total 1700 ml of juice was collected. This *swarasa* was utilized for *bhavana.*

2.1.6. Bhavana with Nagavalli Swarasa [preparation of finished product (Tablet)]

This liquid (*nagavalli swarasa*) was added to the ground material in the end runner and trituration process [*Bhavana*] was carried out. The trituration was continued till the material dries completely. At the end of all levigation process, the material is removed from the

Table 3
Physico chemical analysis (Observations of three batch analysis).

S. No.	Parameter tested	Observations of three batch analysis
1.	Organoleptic characters	
	Colour	Brownish Black
	Taste	Slightly bitter
	Odour	Aromatic
	Appearance	Tablet
2.	Physico-chemical parameters	
	Identification	Yields the reaction characteristics of Sulphur, Mercury, Gold, Copper, Tin, Arsenic & Iron
	Loss on drying	2.5–4.0
	Organic and volatile matter	40.0–41.0
	Total Ash %w/w	52.0–55.0
	Acid Insoluble Ash %w/w	22.0–27.0
	Water soluble extractive	3.0–6.0
	Alcohol (90%) soluble extractive	3.0–5.0
	pH of aqueous extract	6.0–6.5
	Specific gravity	0.9999 ± 0.0004
	Particle size distribution	
	10%,	3.46–15.29 μm
	50%,	25.35–64.27 μm
	90%	119.37–210.50 μm
3.	Assay of Elements	
	Mercury (%w/w)	4.0–5.0
	Gold (%w/w)	1.5–2.0
	Copper (%w/w)	1.5–2.0
	Tin (%w/w)	5.0–6.0
	Arsenic (%w/w)	1.0–2.0
	Iron (%w/w)	7.0–8.0
	Sulphur (%w/w)	18.0–20.0
	Calcium (%w/w)	2.0–4.0
	Silica (%w/w)	1.0–4.0
	Magnesium (%w/w)	1.0–1.50
	Lead (%w/w)	0.50–1.50
	Boron (%w/w)	0.10–0.20
	Manganese (%w/w)	0.05–0.10
	Aluminum (%w/w)	0.10–2.0
	Chromium (%w/w)	0.02–0.04
	Cadmium (ppm)	0.10–0.20
4.	Residual pesticide (mcg/kg)	
	Alpha and beta HCH	Not detected
	Gamma HCH	Not detected
	Delta HCH	Not detected
	DDT and metabolites	Not detected
5.	D.T.	16-17 min
	Hardness	1.5 kg/cm^2
	Friability	0.60–0.70%
	Average wt.	125.0–125.30 mg/tab
6.	Microbiological examination	
	Total aerobic count	8000–20 000
	Coliform	Not detected
	E.coli	Not detected
	Salmonella sp.	Not detected
	Staphylococcus aureus	Not detected
	Yeasts	Not detected
	Moulds	Not detected
	Pseudomonas aeruginosa	Absent

Table 4
Observations/visualization/detection (Rf values).

Mahalaxmi Vilas Rasa			
	Batch I	Batch II	Batch III
At 254 nm	0.37, 0.48, 0.75	0.37, 0.48, 0.75	0.37, 0.48, 0.75
At 366 nm	0.35, 0.45, 0.78	0.35, 0.45, 0.78	0.35, 0.45, 0.78
At 520 nm	0.74, 0.81	0.74, 0.81	0.74, 0.81

end runner, shifted in to the clean trays and dried completely in Tray Drier at 50 °C. Dried material was shifted to tablet section and granules were prepared. 2% talcum powder was added to the granules and was compressed in to desirable size of tablets [125 mg] by passing through Rotary Tablet Machine.

2.2. Experiments & results of chemical analysis

2.2.1. Physico-chemical analysis

Physicochemical analysis is the important characteristics to evaluate the quality, standardization and safety of Ayurvedic drugs and provide information about correct identification and authentication of the raw drugs & formulations and may help in preventing its adulteration. Ash values are important quantitative standards and criterion to analyse the identity and purity of crude drugs. Moreover the total ash of a crude drug also reflects the care taken in drug preservation, and the purity of crude and the prepared drug. Acid insoluble ash is a part of total ash and measures the amount of silica present, especially as sand and siliceous earth. Extractive values obtained using water and alcohol are useful for the evaluation of a crude drug as it gives an idea about the nature of chemical constituents present in it and is useful for estimation of chemical constituents, soluble in that particular solvent used for extraction. The pH conventionally represents the acidity and alkalinity.

Physicochemical parameters, *viz.* description, estimation of Loss on drying, Ash content, Acid insoluble ash, Water/Alcohol soluble extractive, *p*H, etc., qualitative/quantitative elemental testing, Residual pesticide, Microbiological examination and tablet parameters *viz.* Hardness, Friability, Average wt., Dissolution time etc. were carried out by following standard methods as per Ayurvedic Pharmacopoeia of India (API) [20–25] guidelines. The quantitative estimation of Heavy Metals viz. Hg, As, & Cd was carried out by Atomic Absorption Spectrometer (Perkin Elmer (USA) Analyst 400) and the other elements viz. Mg, Pb, Ca, B, Mn, Al, Cr were analysed on ICP-AES (THERMO EECTRON Corporation's model IRIS INTREPRID II XDL). However Sulphur, Silica, Sn, Au, Fe & Cu were quantified by using conventional methods [25]. The results of Physico-chemical analysis are mentioned in below Table 3.

2.2.2. HPTLC analysis

Sample preparation: 2 g powder each of three batches of MLV were soaked overnight separately in 20 ml of methanol. The solutions were continuously stirred for 6 h and kept for next 18 h and then filtered the samples, dried and made 10% solution.

High Performance Thin Layer Chromatography was performed on TLC plates pre-coated with 0.25 μm thin layers of silica gel 60 F_{254} (E. Merck). 10 μL methanolic solution of formulation (three batches) were applied on the plates as bands 8.0 mm wide by use of a Linomat-IV applicator (CAMAG, Switzerland) fitted with a 100 μL syringe (Hamilton, Switzerland). The application positions X and Y were both 10 mm, to avoid edge effects. Linear ascending development to a distance of 80 mm with Toluene: Ethyl acetate: formic acid 10: 3:1 (v/v) as mobile phase was performed in a twin-trough glass chamber previously saturated with vapors of mobile phase for 20 min. The plates were dried in air and visualized under 254 nm and 366 nm for ultra violet detection and taken the fingerprints as evident. The same TLC plate was also derivatized with anisaldehyde-sulphuric acid reagent and visualized in white light. The HPTLC profile alongwith the R_f values are shown in the mentioned Fig. 2 and Table 4

2.2.3. X-ray diffraction study

Powder X-ray diffraction (XRD) analysis of *Mahalaxmivilas Rasa* was carried out using Rigaku Ultima-IV X-ray diffractometer with CuKα radiation (λ = 1.54 A°) operating at 40 kV and 30 mA. Pattern was recorded for angle (2θ) ranging from 10 to 100° at a scanning rate of 1°/second and scan step of 0.1°. Sample identification was done by matching d-spacing with the standard database. The detailed XRD data and XRD pattern are given in below mentioned Table 4 and Figs.3 and 4

3. Discussion

The organo-leptic observation shows that the prepared *Mahalaxmivilas Rasa* is in the form of Brownish Black coloured tablet having aromatic odour and slightly bitter taste. The qualitative analysis shows the positive test for the presence of Mercury, Sulphur, Calcium, Copper, Gold, Iron and Tin etc. The Chemical analysis revealed that it contains 4.34% of Mercury, 19.12% of Sulphur, 7.29% of Iron, 5.33% of Tin, 1.85% of Gold, 1.74% of Copper, 1.43% of Arsenic, 2.75% of Calcium, 1.3% of Magnesium, 2.5% of silica together with

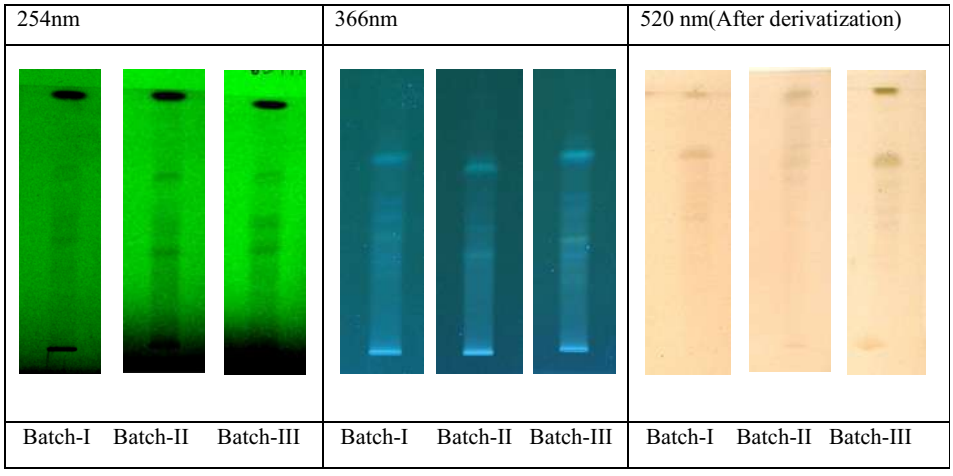

Fig. 2. -HPTLC profiles of *Mahalaxmivilas Rasa*.

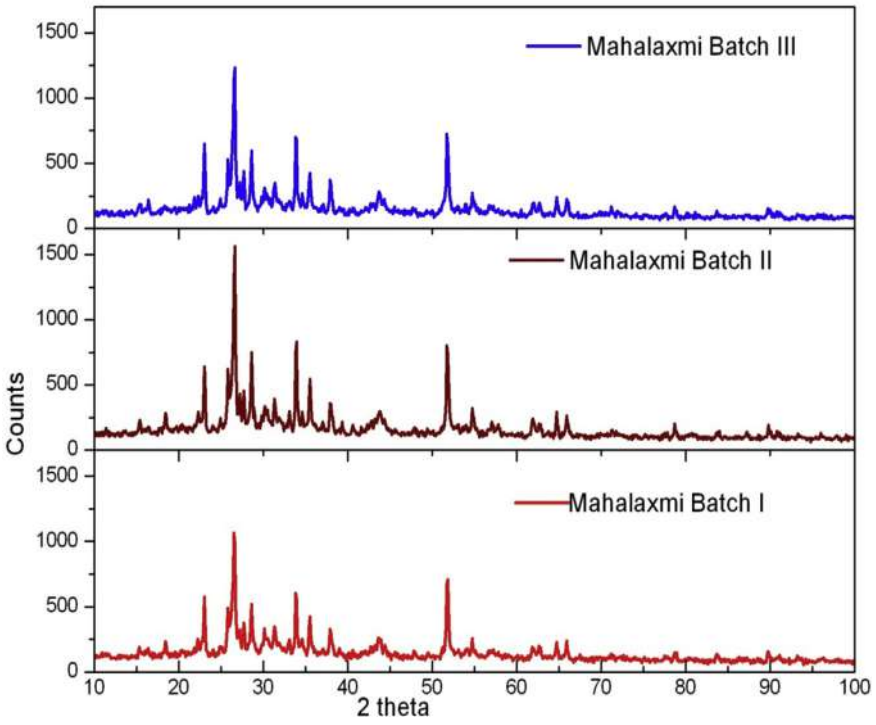

Fig. 3. XRD pattern of *Mahalaxmivilas Rasa* (batch –I, II, III).

trace elements *viz.*, Aluminium, Manganese, Lead, Boron, Chromium, Cadmium etc. which have been found in <1.0% range. Moisture content 3.70% was found when determined loss on drying at 105 °C. Total ash content (53.35%) representing the elemental composition is left after burning of organic/volatile matter (40.26%). The observations show that water soluble (4.67%) and alcohol soluble (4.12%) matter are also present in this formulation. The drug also tested as per API guideline for Residual pesticide and Microbiological examination which found in permissible limits.

The results from HPTLC profile as shown in Fig. 2 and Table 3 revealed the presence of few bands at different R_f representing organic constituents. Generally, the Herbo-Mineral preparations

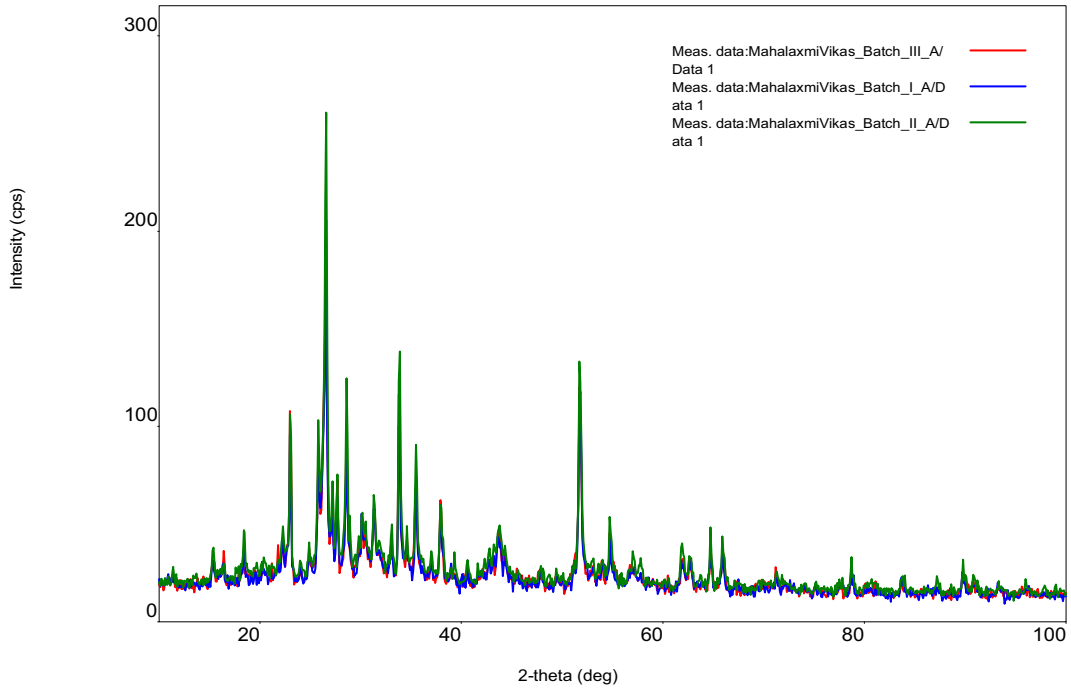

Fig. 4. Overlay of XRD pattern of *Mahalaxmivilas Rasa* (batch –I, II, III).

Table 5
d-spacing and 2-theta (deg) values of XRD analysis.

2 theta	d (A)	size(A)	Chemical formula	Phase data name	cps
23.1	3.9	438.0	S	Sulfur (2,2,2)	62.0
25.8	3.5	437.0	S	Sulfur (0,2,6)	47.0
26.6	3.3	302.0	Hg S, SnO_2, $K_{86\cdot5}Al_{86\cdot5}Si_{105\cdot5}O_{384}$	Cinnabar (1,0,1), Cassiterite, syn (1,1,0), Zeolite X, (X) (6,4,2)	156.0
27.2	3.3	552.0	$K_{86\cdot5}Al_{86.5}$ $Si_{105\cdot5}O_{384}$	Zeolite X, (X) (7,3,1)	31.0
27.7	3.2	563.0	S, As_2 S_3	Sulfur (1,1,7), Orpiment (2,1,1)	36.0
28.6	3.1	492.0	Unknown	Unknown	74.0
30.1	3.0	175.0	K $Al(SiO_3)_2$, $K_{86\cdot5}Al_{86\cdot5}Si_{105\cdot5}O_{384}$	Leucite (4,0,2), Zeolite X, (X) (8,2,2)	16.7
31.3	2.9	524.0	Hg S, S, K $Al(SiO_3)_2$	Cinnabar (1,0,2), Sulfur (0,4,4), Leucite (3,2,3)	28.0
37.8	2.4	281.0	Hg S, SnO_2, S, $KAl(SiO_3)_2$	Cinnabar (1,0,3), Cassiterite, syn (2,0,0), Sulfur (4,2,2), Leucite (4,0,4)	25.0
43.8	2.1	61.0	Hg S, SnO_2, S, As_2 S_3, $KAl(SiO_3)_2$, $K_{86\cdot5}Al_{86\cdot5}$ $Si_{105\cdot5}O_{384}$	Cinnabar (1,1,0), Cassiterite, syn (2,1,0), Sulfur (3,1,9), Orpiment (0,4,1), Leucite (2,2,6), Zeolite X, (X) (10,6,2)	12.3
51.7	1.8	461.0	SnO_2, As_2S_3, $K_{86\cdot5}Al_{86\cdot5}Si_{105\cdot5}O_{384}$	Cassiterite, syn (2,1,1), Orpiment (1,3,-2), Zeolite X, (X) (14,2,0)	98.0
54.7	1.7	578.0	HgS, SnO_2	Cinnabar (2,0,2), Cassiterite, syn (2,2,0)	28.0
57.0	1.6	141.0	Sn O_2, S, As_2 S_3, $K_{86\cdot5}Al_{86\cdot5}Si_{105\cdot5}O_{384}$	Cassiterite, syn (0,0,2), Sulfur (2,2,14), Orpiment (4,5,0), Zeolite X, (X) (15,3,3)	7.9
61.8	1.5	206.0	SnO_2, K $Al(SiO_3)_2$	Cassiterite, syn (3,1,0), Leucite (7,5,2)	14.9
64.7	1.4	734.0	S	Sulfur (5,3,11)	32.0
65.9	1.4	518.0	SnO_2	Cassiterite, syn (3,0,1)	22.4

are insoluble in common organic solvents such as Chloroform, Acetone, Methanol and water. In this case, the alcohol soluble extractive is very less (<5.0%). This soluble portion did not show any defined behaviour on TLC may be due to its non-homogeneous nature.

XRD pattern of *Mahalaxmivilas Rasa* (3 batches) as shown in spectra Figs. 3 and 4 and results mentioned in Table 5, have indicated that all the samples contained free sulphur, cinnabar (mercury sulphide added as *Kajjali*); cassiterite (tin oxide, *Vanga Bhasma*); orpiment (*Hartal*, arsenic III sulphide); and mica (Leucite/Zeolite; *Abhraka Bhasma*). *Abhraka bhasma* is the major inorganic constituent added by weight followed by free sulphur and *Vanga bhasma*, in the *Mahalaxmivilas Rasa* samples. No signature of *tamra bhasma* or *Swarna Bhasma* could be identified in these spectra, indicating that the methods used for their preparation, did not yield any crystalline products or their signature is buried under the strong lines of other constituents. But their presence in the sample could be identified from the chemical analysis showing the presence of gold, boron, copper etc. The crystalline form of *abhraka bhasma* remains inconclusive. It can be seen that the three XRD patterns on the samples are qualitatively same in the relative intensities of the peaks. *Kajjali* is meta-cinnabar and in its preparation elemental mercury observed to be absent. The presence of free sulphur ensures that the *kajjali* does not decompose into mercury and sulphur and also some partial conversion into mercury oxide due to exposure to air; the presence of free sulphur is confirmed by XRD. The assay of total ash content in these samples (52%–55%) almost matching the expected fraction of inorganic constituents by weight and the XRD results indicate that the inorganic contents have remained intact over time.

4. Conclusion

The inferences and the standards laid down in this study certainly utilized as an important tool for standardization and quality assurance of this herbo-mineral formulation. It may be useful for Researchers/Scientists/Academicians and also may be considered for laying down the pharmacopoeial standards of *Mahalaxmivilas Rasa*.

This study reveals that *Mahalaxmivilas Rasa* prepared by ancient classical pharmaceutical processes is safe and very effective in converting the macro elements into therapeutically effective medicines of micro form. Further, the sub-chronic oral toxicity studies in male and female rats was studied and found non toxic. As this paper is dealt only with preparation and chemical characterization of *Mahalaxmivilas Rasa,* the detailed data on safety/toxicity studies will be published separately.

Conflicts of interest

There are no conflicts of interest.

Sources of funding

None declared.

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Comparative study on cellular entry of incinerated ancient gold particles (Swarna Bhasma) and chemically synthesized gold particles

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Gold nanoparticles (AuNPs) are used for a number of imaging and therapeutic applications in east and western part of the world. For thousands of years, the traditional Indian Ayurvedic approach to healing involves the use of incinerated gold ash, prepared with a variety of plant extracts and minerals depending on the region. Here, we describe the characterization of incinerated gold particles (IAuPs) in HeLa (human cells derived from cervical cancer) and HFF-1 (human foreskin fibroblast cells) in comparison to synthesized citrate-capped gold nanoparticles (AuNPs). We found that while individual IAuP crystallites are around 60 nm in size, they form large aggregates with a mean diameter of 4711.7 nm, some of which can enter cells. Fewer cells appeared to have IAuPs compared to AuNPs, although neither type of particle was toxic to cells. Imaging studies revealed that IAuPs were in vesicles, cytosol, or in the nucleus. We found that their nuclear accumulation likely occurred after nuclear envelope breakdown during cell division. We also found that larger IAuPs entered cells via macropinocytosis, while smaller particles entered via clathrin-dependent receptor-mediated endocytosis.

Gold nanoparticles (AuNPs) are used for a number of imaging and therapeutic applications including diagnosis and treatment of cancers[1,2]. Their physical and chemical properties are tunable, as they strongly depend on size, shape, aggregation state, and surface chemistry[3]. The use of AuNPs in modern medicine can be traced back to their use in the ancient traditional Indian Ayuverdic approach to healing[4–7] of many ailments. Ayuverdic (where Ayus means life principle, and veda refers to system of knowledge) is a philosophy, concerned with the protection of "ayus", by combining healthy living with therapeutic measures. Medicinal preparations typically consist of mixtures of plant- and animal -derived products, minerals and other metals[6,8,9]. Gold derived Ayuverdic medicine is called Swarna Bhasma (gold ash)[4,10–12], and testing their localization, entry and impact on human cells in comparison to chemically synthesized AuNPs will be the focus of this study.

Swarna Bhasma gold ash is prepared through a process called Putapaka, which involves heating and quenching gold with various plant extracts. Gold is hammered into a ribbon from a coarse powder, then ground with various herbal extracts and incinerated at high temperature ($\sim$1000 °C) in earthen crucibles. During the incineration phase, which is repeated several times, the size of gold particles is reduced more and more with each cycle, via mechanical comminution[10,13]. It is important to note that by this top down approach, the gradual reduction in size of the gold particles brings them increasingly closer to colloidal AuNPs. The gold ash, Swarna Bhasma, prepared according to Ayuverda texts will be called Incinerated Gold Particles (IAuPs) for this study.

Several comparisons have been made between IAuPs and colloidal AuNPs, which are chemically synthesized through the reduction of gold salts by various natural or chemical reducing agents[14]. Chemically synthesized AuNPs can be made with different surfactants and stabilizing groups, and their size can vary from 1 to 100 nm. Spectroscopic measurements of IAuPs revealed that they are comprised of individual particles of around 50–70 nm in size. Unlike chemically synthesized AuNPs, they are not well separated and form large aggregates

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over 2 μm in size[13,15,16]. The composition and size of IAuPs from different pharmaceutical companies is variable. They contain a range of compounds and elements, including heavy metals[17–19], derived from herbal extracts typically used for medicinal purposes. The presence of heavy metals has been associated with possible contamination of the soil, where the plants are grown, or from the crucibles used for the long calcination processes. Thus, the size, composition and morphology of AuNPs and IAuPs are different. However, given that both are used in medical applications, it is crucial to understand how they interact with, and impact, the core physiological functions of human cells[1,14,20].

Several studies have explored the entry mechanisms and cytotoxicity of colloidal AuNPs with different surface moieties, size and morphology *in vitro*, with variable outcomes. In general, small spherical particles (e.g. $<2\,nm$) were reported to cause cytotoxicity in different mammalian cell lines, when compared to rod shaped particles, although their toxicity varied depending on the surface coating (cationic vs anionic) and the cell line[14,21]. It is assumed that most AuNPs less than 50 nm in size enter cells via receptor-mediated endocytosis in a clathrin-dependent manner[21,22]. However, evidence suggests that they also enter cells via caveolin-mediated endocytosis or macropinocytosis[23–25] depending on their size, shape, surface coating, and if they form aggregates[1,26–29]. Also, they could enter via these different pathways depending on the cell type and/or receptors expressed at their surface[1,21,28,29]. It may also be desirable to target AuNPs to the cytosol or other subcellular locations inside the cell, such as the nucleus or mitochondria. However, to do this, they must escape from the endomembrane system, since particles are initially contained in vesicles, regardless of the mechanism of entry[30]. Surface functionalization could promote their escape from lysosomes into the cytosol, as in the case of encapsulation of AuNPs in a cationic core-shell polymer colloid that expands upon acidification in the lysosome causing rupture[31], while larger aggregates could mechanically disrupt vesicle membranes. There are reports of using protein tags with specific amino acid sequences known to mediate transport into the nucleus[32] or mitochondria[33,34]. Again, it is not clear how these tags have accessibility to the protein complexes that recognize them when the particles are retained in vesicles, particularly when they are in lysosomes or autophagosomes. However, once they are in the cytosol, the tags could become accessible to mediate transport. In addition, very small particles may not require tags to pass through the nuclear pores, or outer mitochondrial membrane.

This paper presents a comparative study of AuNPs and IAuPs in their (i) localization, (ii) physiological impact and (iii) entry in cancerous and non-cancerous human cells, which are crucial to understand for their design and therapeutic use. We found that IAuPs contain a large variety of elements, some of which are present at significant concentrations (e.g. Mg and Ca). While individual IAuP crystallites are 60 nm in size, they form large aggregates with a mean diameter of 4711.7 nm. IAuPs and AuNPs imparted no obvious toxicity to HeLa cells (human cervix adenocarcinoma) or HFF-1 cells (human foreskin fibroblasts, which are non-cancerous). Imaging revealed that while some IAuPs were in membrane-bound vesicles or vacuoles, others were in the cytosol and nuclei of cells, while AuNPs accumulated primarily in the endomembrane system. Mechanically disrupting IAuPs into smaller 100–200 nm particles increased their accumulation in cells where they localized to the endomembrane system similar to AuNPs. Interestingly, larger IAuPs accumulated in the nuclei of HeLa cells after nuclear envelope breakdown during cell division. Further studies revealed that IAuPs enter cells by more than one mechanism, as their entry was reduced after treatment with Cytochalasin D to block macropinocytosis, and Chlorpromazine to block clathrin-mediated receptor-mediated endocytosis. These studies show that IAuPs are large, inert aggregates, which could be explored for use as carriers.

Materials and Methods

Synthesis and Characterization of IAuPs and AuNPs. Citrate-capped spherical AuNPs were prepared by the reduction of chloroauric acid with sodium citrate using the Turkevich method[35,36]. Briefly 75 mL of chloroauric acid solution containing $45\,\mu g/ml$ gold was heated, and 5 mL of 1% sodium citrate was added to the boiling solution. After the solution turned purple, it was boiled for another 15 minutes, then left to cool to room temperature. The elemental composition of the synthesized AuNPs was measured by ICP-MS (Inductively-Coupled Plasma Mass Spectroscopy) on a 7700x Agilent and Energy Dispersive Spectroscopy (EDS)-SEM, and the shape and size of the AuNPs was determined by SEM using the Hitachi S 3400N.

IAuPs were obtained as a powder from Jaya Indian Medicine Pharmaceutical Pvt Ltd, Maduravoyal, Chennai, Tamilnadu, India. IAuPs were suspended in de-ionized water for use in all experiments. To determine particle size and shape, IAuPs were dried on pre-cleaned glass slides at room temperature and imaged by SEM using the Hitachi S 3400N. In addition, Dynamic Light Scattering (DLS) measurements were done using a Nicomb 380 instrument by Dynalene Lab Services. The elemental composition of IAuPs was measured using EDS-SEM and ICP-MS. For ICP-MS, the sample was oven dried and digested with aqua regia (3HCL: 1HNO3) at 110 °C for 3 hours. Then the sample was cooled and filtered through a $0.2\,\mu m$ PTFE filter. The sample was analyzed using "No gas" and "He" modes. To break the IAuPs into smaller particles, IAuPs in de-ionized water were broken mechanically (using Omni Mixer Homogenizer 20–25 minutes at a variable speed), and subsequently by ultrasound treatment (using Branson 200 Ultrasonic cleaner at 40 kHz, 8–10 times and 5 minutes each time) (Fig. 1).

Cell Culture. HeLa (human cervix adenocarcinoma) and HFF-1 (human foreskin fibrobast) cells were cultured in Dulbecco's modified Eagle's Medium (DMEM; Wisent) supplemented with 10 or 15% Fetal Bovine Serum (FBS; ThermoFisher Scientific), 100 U penicillin and 0.1 mg/mL streptomycin (Wisent), and 2 mM L-glutamine (Wisent). Cells were incubated at 37 °C in a humidified chamber with 5% CO_2, and passaged at 75–100% confluency or as needed for the analysis of IAuPs or AuNPs in cells.

Immunoflourescence and Microscopy. Cells were fixed for immunofluorescence using 10% trichloroacetic acid (TCA) as previously described[37]. Fixed cells were immunostained for microtubules using 1:250 mouse anti-tubulin antibodies (DM1A, Sigma-Aldrich) and anti-mouse Alexa 488 secondary antibodies were used at

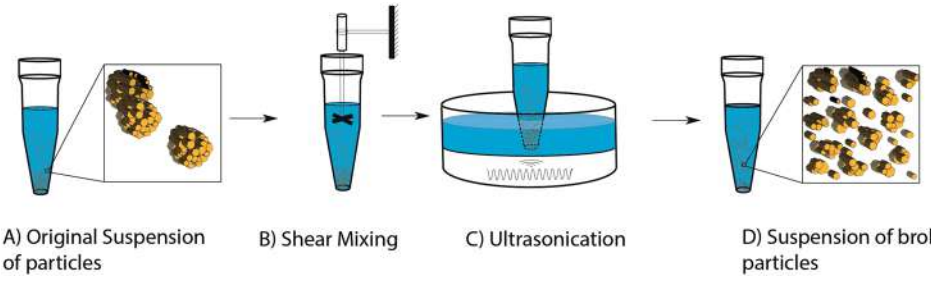

Figure 1. A Schematic diagram illustrating the process of mechanically disrupting IAuPs. IAuPs suspended in deionized water are broken with a homogenizer (Step 1) followed by ultrasound treatment (Step 2) to break apart larger particles into smaller particles.

a 1:400 dilution. DAPI (Sigma-Aldrich) was added at a 1:1000 dilution (1 mg/mL stock) for 5 minutes before mounting the coverslips onto slides. Fixed cells were imaged using a Leica DMI6000B epifluorescence microscope with the 63x/1.4 PL APO oil immersion objective (pixel size 0.102 μm), and z-stacks of 0.5 μm were acquired with a Hamamatsu OrcaR2 camera and Volocity software (PerkinElmer) using a piezo Z stage (MadCityLabs). Image files were exported as TIFFs, which were converted into maximum intensity z-stack projections in Image J (NIH). Hyperspectral microscopy was performed using the Cytoviva hyperspectral imaging system with enhanced dark-field optical illumination (Cytoviva, Inc) on AuNPs or IAuPs in HeLa cells. Using the 60x/1.4 or 100x/1.4 oil objectives, dark-field images were collected at oblique angles, and the reflective fluorescence was measured for selected pixels, using a spectrophotometer integrated CCD, with a spectral range of 400–1000 nm and spectral resolution of 2.8 nm.

Samples for SEM were fixed as above, but then were washed 3–4 times with TBST wash buffer (50 mM Tris pH 7, 150 mM NaCl, 0.5% Triton-X 100) and dehydrated, using a series of solutions ranging from 50–100% ethanol. Dehydrated samples were covered and placed in a fume hood overnight for complete drying. The cells were imaged using SEM (Hitachi S 3400 N) under a voltage of 15 kV and vacuum of 50 Pa, and images were collected after zooming in as indicated.

To perform live imaging, media was replaced with phenol red-free media. Cells were plated on 25 mm round coverslips (No. 1.5), placed in a 35 mm chamlide magnetic chamber (Quorum) and kept at 37 °C with 5% CO_2 using the INU-TiZ-F1 chamber (MadCityLabs). Cells were treated with either IAuPs or citrate-capped AuNPs for 24–48 hours prior to imaging. Cell membranes were stained using FM 4–64 lipophilic Styryl Dye (Invitrogen) 20–60 minutes prior to image acquisition. Live imaging was performed on an inverted Nikon TiE microscope using the 100x Plan APO WD oil immersion objective, and an Evolve (Photometrics) EMCCD camera using Elements 4.0 acquisition software (Nikon). Images were acquired with 30 ms exposures for brightfield, and 100 ms for fluorescence using the Heliophore LED with an excitation wavelength of 480 nm at 15% power (National Instruments). Z-stacks of 0.5 μm thickness were collected using a NI-DAQ piezo Z stage (National Instruments) every 3 seconds. Image files were exported as TIFFs, which were opened with Image J (NIH) and converted into maximum intensity z-stack projections.

Nuclear Entry Assay. To test for nuclear entry of the IAuPs, HeLa cells were blocked in S phase of the cell cycle using Thymidine as described previously[37]. A 'double' block was performed to ensure that the entire cell population was synchronized to be in S phase. To do this, HeLa cells were plated at 30–40% confluency on glass coverslips. They were treated with 2 mM Thymidine (Sigma) for 16 hours, then released for 8 hours after washing 3 X with PBS phosphate buffered saline (pH 7.4; 1.06 mM KH2PO4, 154 mM NaCl, 5.6 mM Na2HPO4), and adding and pre-warmed media. Cells were treated again with 2 mM Thymidine and after 1 hour, 200 μg of IAuPs was added to the cells. After 23 hours, the cells were fixed and immunostained as described above, and brightfield and fluorescence microscopy were used to assess localization of the IAuPs. Control cells did not receive the second Thymidine treatment to keep them cycling. This experiment was replicated for statistical analysis.

Cellular Entry Assay. To determine the mechanism by which IAuPs or AuNPs enter cells, HeLa cells were pre-treated with (i) 1% DMSO as control, (ii) 100 nM Cytochalasin D to block F-actin and disrupt macropinocytosis, (iii) 100 μm Genistein to block caveolin-mediated endocytosis, or (iv) 5 μm Chlorpromazine to block clathrin-dependent receptor-mediated endocytosis. HeLa cells were plated at 30–40% confluency on glass coverslips, and 100 μg IAuPs were added one hour after adding the drugs. After another 7 hours, cells were fixed and immunostained as described above for brightfield and fluorescence microscopy to assess localization of IAuPs. All treatments were replicated for statistical analysis.

Analysis. To determine the proportion of cells with IAuPs located in the nucleus, a minimum of 15 fields of view covering around 300–400 cells were imaged, and only cells with IAuPs were counted. Both the brighfield and DAPI images for multiple z-panes were used to access nuclear localization. Averages and standard deviations were calculated. To assess the entry mechanism of IAuPs, the total proportion of cells containing IAuPs was determined using a minimum of 10 fields of view covering 150–200 cells imaged per treatment. Averages and standard deviations were calculated and graphed. The student t-test was used to determine the statistical significance ($p < 0.05$).

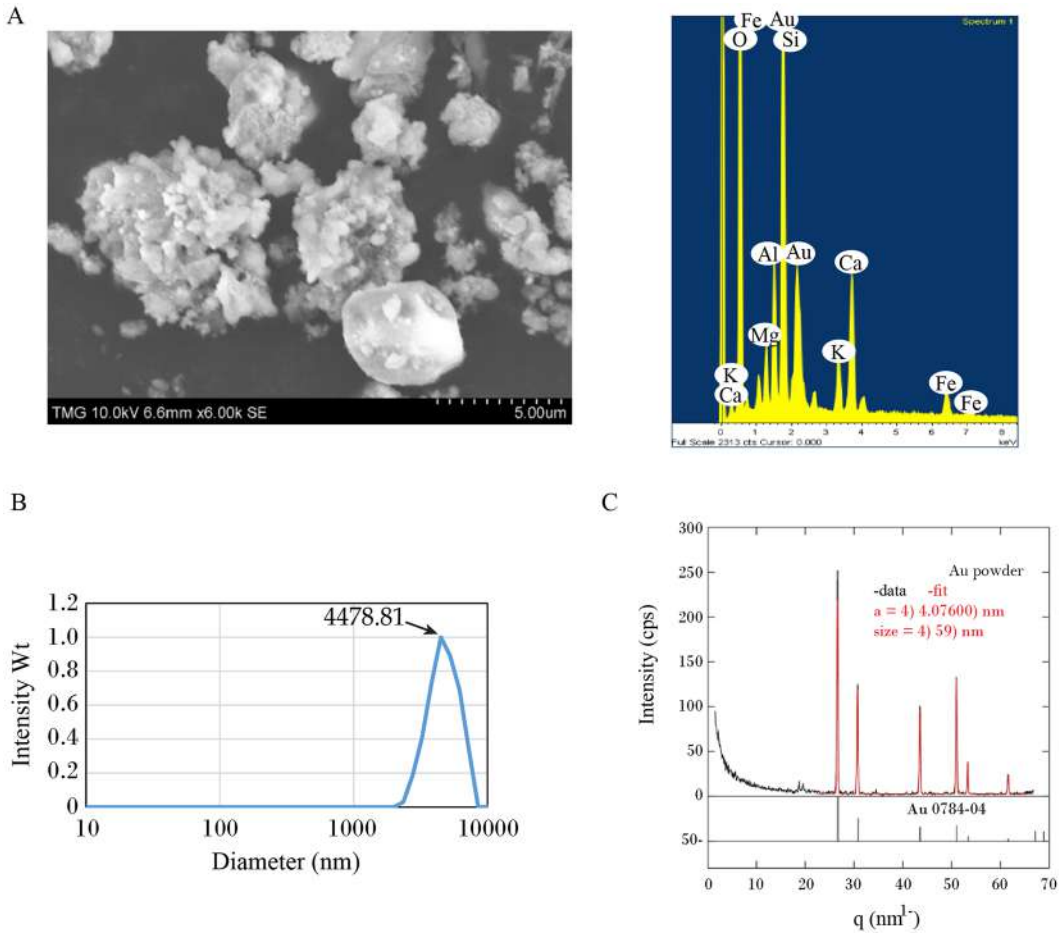

Figure 2. (**A**) IAuPs were imaged by SEM (left) and by EDS-SEM (right), which shows the shape and size of the particles, and the elemental composition, respectively. (**B**) A graph shows the mean size of the IAuPs by DLS. (**C**) A graph shows the XRD pattern of IAuPs in comparison to Au, and the size of individual particles is indicated.

Elements	*Concentration %*
Gold (Au)	56.88
Mg	1.8
Ca	1.4
Fe	0.29
Si	0.29
Trace Elements	
Mn	0.037
Ni	0.02
As	0.15

Table 1. The elemental composition of IAuPs determined by ICP-MS.

Results

Physicochemical characterization of Incinerated Gold Particles (IAuPs). Since the preparation and composition of Swarna Bhasma IAuPs can vary depending on the geographical region, the IAuPs used in this study were characterized *in vitro*[15–19]. DLS analysis revealed that IAuPs have a broad range in size with a mean diameter of 4711.7 nm (Fig. 2B). SEM imaging also showed the variation in size and irregular morphology of the particles (Fig. 2A). Further analysis of IAuPs using X-ray diffraction revealed that the size of crystallites within the IAuPs is approximately 60 nm (Fig. 2C). Therefore, IAuPs are likely large aggregates of smaller nanoparticles.

The composition of IAuPs in powder form was determined using EDS-SEM and ICP-MS. As shown in Table 1 and Fig. 2A, gold was found to be the most abundant element in IAuPs. Interestingly, this value was low in comparison to other samples that have been characterized[13,15–19]. ICP-MS revealed that the particles contained other elements, some of which were present at higher concentrations, such as Mg, Ca, Fe, and Si (0.29–1.9%), while

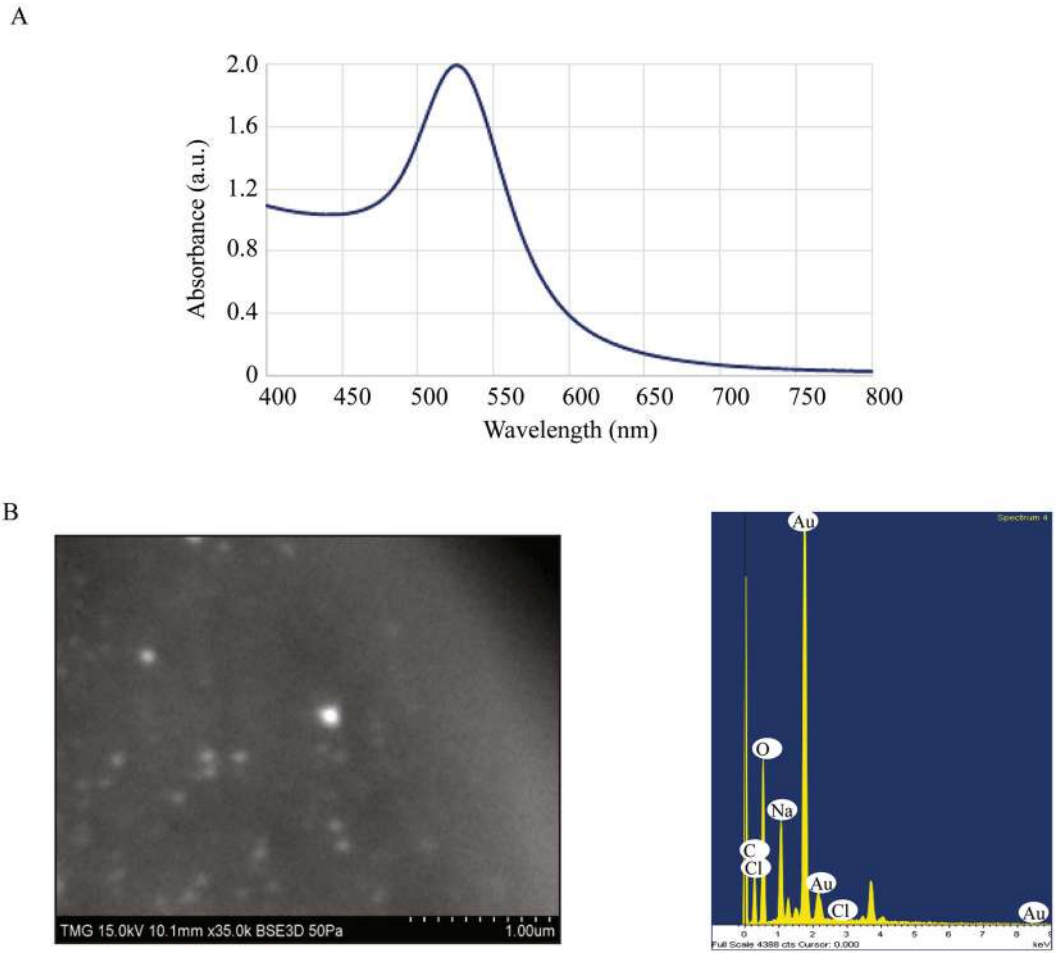

Figure 3. (**A**) The graph shows the LSPR band corresponding to AuNPs. (**B**) AuNPs were imaged by SEM (left) and by EDS-SEM (right), which shows the shape and size of the particles, and the elemental composition, respectively.

others (Mn, Ni, As) were found in trace amounts (Table 1). This was consistent with EDS-SEM results, which showed that the concentration of gold appeared to vary depending on the area used for analysis, with significant peaks corresponding to elements including Mg, Ca, Fe, and Si (Fig. 2A). Additional components in the IAuPs are likely oxygen and carbon caused by oxidation during the incineration process. The presence of oxygen in bhasmas was revealed by X-ray fluorescence spectroscopy[14].

The synthesized gold nanoparticles (AuNPs) generated for this study to compare cellular toxicity and localization with IAuPs was characterized *in vitro*. The size of the AuNPs was estimated from the UV-Visible spectrum of the colloidal solution (Fig. 3A). Their diameter was calculated using the following formula[38].

$$d = \frac{ln\left(\frac{\lambda_{SPR} - \lambda_0}{L_1}\right)}{L_2} \quad (1)$$

Where λ_{SPR} is the position of the band in the spectrum of the solution, $\lambda_0 = 512nm$ (Fig. 3A), $L_1 = 6.53$ and $L_2 = 0.0216$ are the fit parameters determined from the theoretical values. The average diameter of the colloidal AuNPs was calculated to be 32 nm. The elemental composition of colloidal AuNPs as determined by ICP-MS and EDS revealed that gold was the most abundant element as expected (Table 2; Fig. 3B). Imaging AuNP by SEM showed that they are spherical and may vary in size up to 100–200 nm (Fig. 3B).

Characterization of IAuPs in human cells. Since IAuPs have not been studied in human cells, their toxicity and subcellular location were characterized in comparison to AuNPs. Two well-characterized human cell lines were chosen for this study, namely HeLa cells, derived from human cervical adenocarcinoma, and HFF-1 (human foreskin fibroblasts) cells, which are non-cancerous. HeLa and HFF-1 cells were treated with citrate-capped AuNPs and IAuPs for 4 days. After, the cells were fixed and stained for tubulin, which is the core component of microtubules that controls cell architecture, and DAPI to visualize chromatin (Fig. 4). The bright-field and fluorescence microscopy images in Fig. 4A revealed that AuNPs accumulate in the endomembrane system surrounding the nucleus, which includes the golgi and endosomes. High levels of AuNPs accumulated

Elements	*Concentration (ppm)*
Gold (Au)	89.6
Mg	0.273
Ca	1.16
Na	20.9
Si	2.69

Table 2. The elemental composition of synthesized AuNPs determined by ICP-MS.

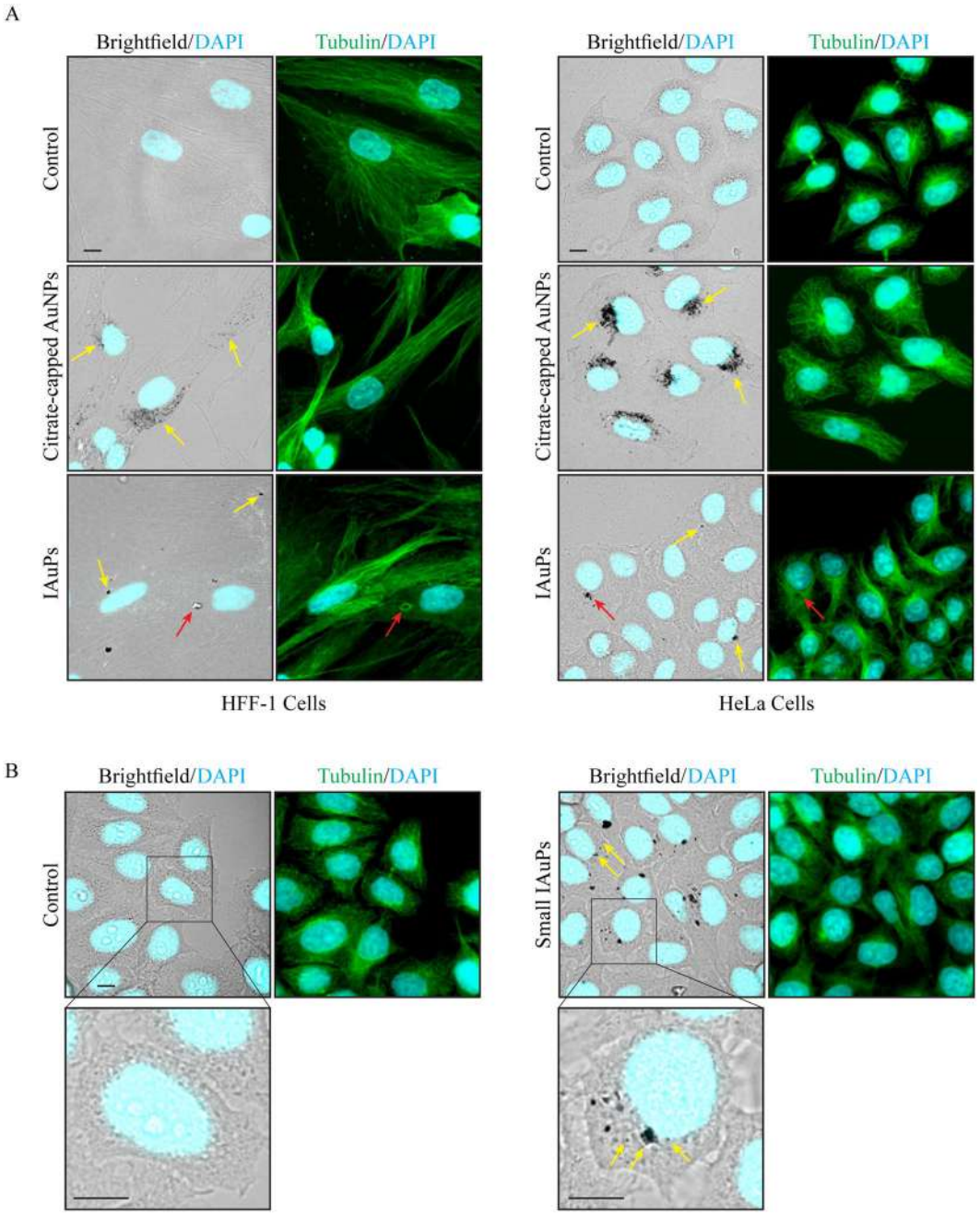

Figure 4. (**A**) Brightfield and fluorescence images of HFF-1 and HeLa cells co-stained for DAPI (to stain DNA; blue) and tubulin (to stain microtubules; green) show the location of AuNPs and IAuPs (yellow arrows). The red arrows point to microtubules that have been displaced around a particle. (**B**) Brightfield and fluorescence images of HeLa cells co-stained for DAPI (blue) and tubulin (green) show the location of mechanically disrupted IAuPs (small; yellow arrows). The scale bars are 10 μm.

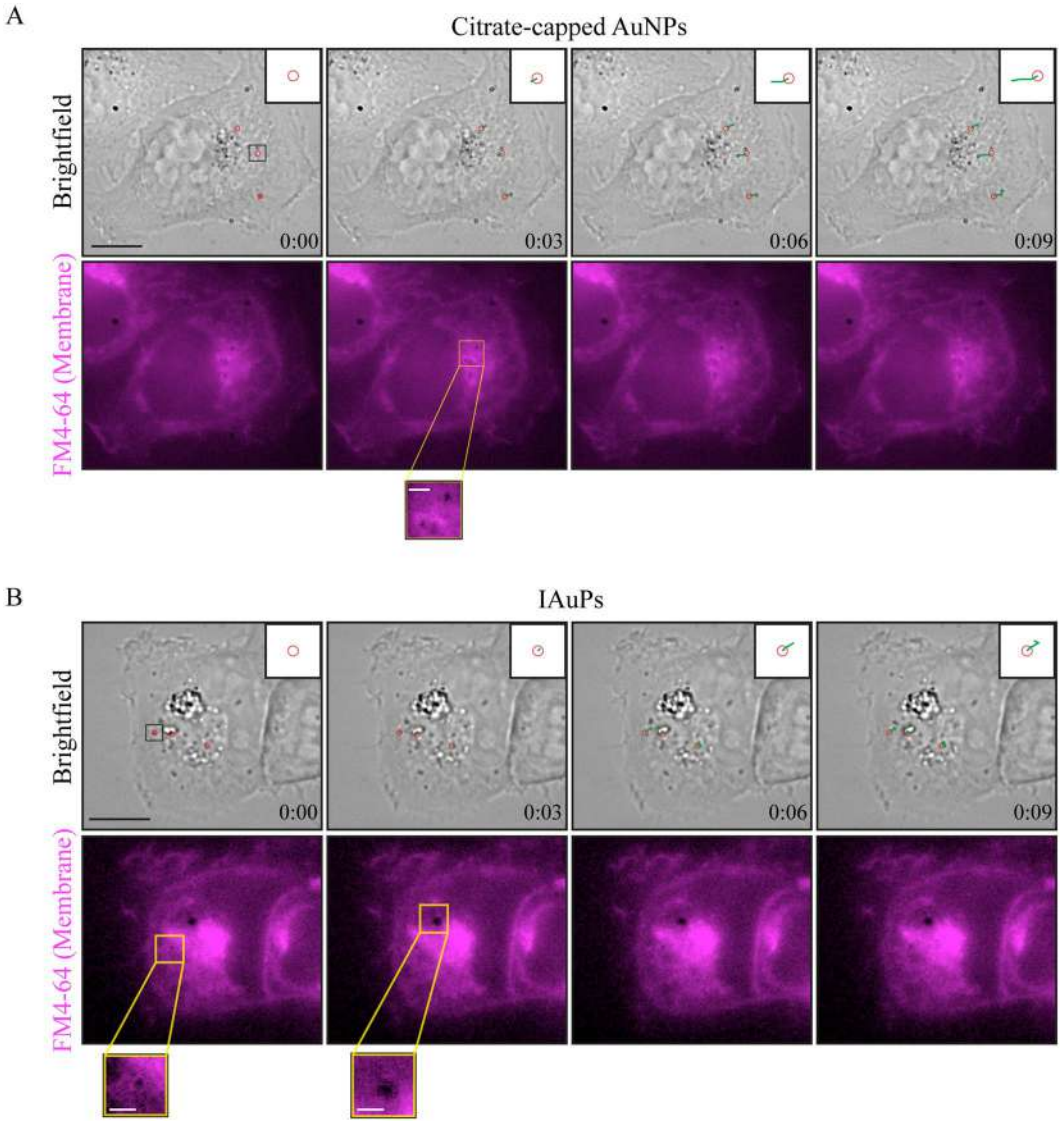

Figure 5. (**A**) Shown are time-lapse brightfield and fluorescence images of a HeLa cell treated with citrate-capped AuNPs and stained with FM 4-64 dye to show membranes. The inset in the top corner shows the start position of AuNPs (red circles) and the trajectory of their movement over time (green lines). The inset below shows a zoomed in image of particles surrounded by membrane. (**B**) Shown are time-lapse brightfield and fluorescence images of a HeLa cell treated with IAuPs and stained with FM 4-64 dye. The inset in the top corner shows the start position of IAuPs (red circles) and the trajectory of their movement over time (green lines). The lower insets show zoomed in images of a particle in a vesicle (left), and a particle that is not membrane-bound (right). The scale bars are 10 μm for the cells and 2 μm for the insets.

in cells and appeared to be non-toxic, as no cell death was observed up to a week after treatment. Fewer cells had IAuPs, and their distribution was more varied in comparison to the AuNPs. While some of the particles appeared to be in the endomembrane system surrounding the nucleus, others were in vacuoles, cytosol, or in the nucleus as seen in Fig. 4A. Interestingly, some of the larger aggregates disrupted the microtubule networks, which appeared to 'bend' around them (Fig. 4B). Similar to the AuNPs, the IAuPs appeared to be non-toxic, as no cell death was observed up to a week after treatment.

To better compare IAuPs with AuNPs, the IAuPs were mechanically disrupted to break them into smaller particles (see schematic in Fig. 1). HeLa cells were treated with the smaller IAuPs as above, and imaged by light microscopy. The cells appeared to have more particles in comparison to the larger IAuPs (Fig. 4A vs. 4B). In addition, the smaller IAuPs accumulated in the endomembrane system similar to AuNPs (Fig. 4B). Thus, the larger size of the IAuPs is likely responsible for their different subcellular locations.

In order to obtain more information about the subcellular localization of AuNPs and IAuPs in HeLa cells, live imaging was performed using a membrane-specific dye (FM-4-64), after treatment with AuNPs or IAuPs (Fig. 5). Similar to fixed cells, AuNPs appeared to be localized to membrane-bound vesicles surrounding the nucleus as shown in Fig. 5. Tracking vesicle movement revealed trajectories that are consistent with movement along

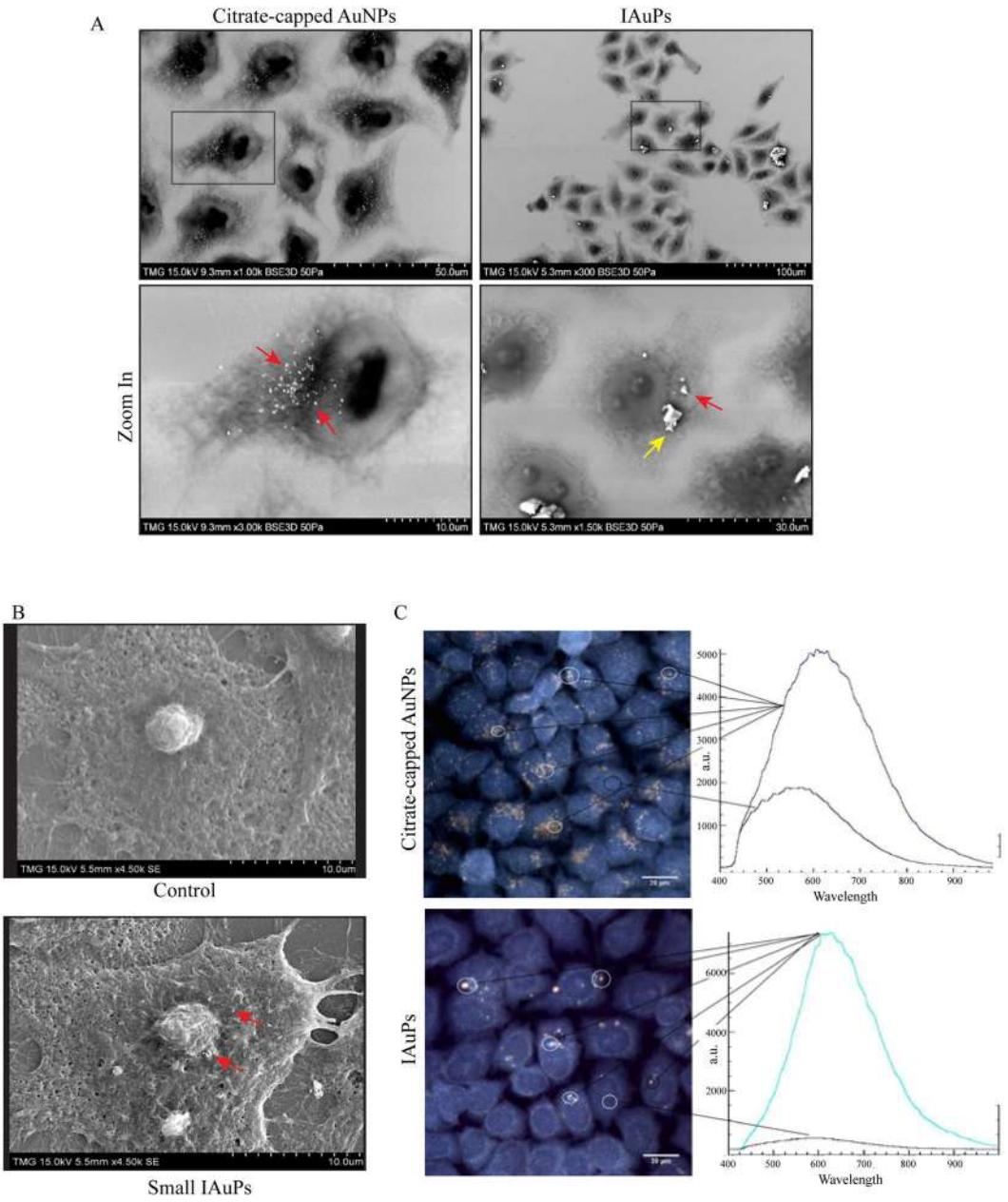

Figure 6. (**A**) The top panels show SEM images of IAuPs and citrate-capped AuNPs in HeLa cells. Single cells are shown at higher magnification, and the red arrow points to particles inside the cells, while the yellow arrow points to a particle outside of the cell. (**B**) SEM images show HeLa cells with either no-treatment (control) or after treatment with mechanically disrupted (small) IAuPs. The red arrows point to particles inside of the cell. (**C**) Cytoviva images of HeLa cells with IAuPs and citrate-capped AuNPs are shown. The corresponding spectral profiles for a selected particle vs. cytosol are shown for comparison. The scale is indicated for each image.

microtubule tracks, as expected for vesicles in the endomembrane system (green lines in Fig. 5). The localization of IAuPs was similar to what had been observed in fixed cells. Some particles appeared to be surrounded by membranes, while other particles were cytosolic, and their movement appeared to be random as shown in Fig. 5.

The physicochemical properties of AuNPs and IAuPs were characterized in cells. SEM of fixed HeLa cells after treatment with AuNPs revealed that, as expected, the particles were small and uniform in size, and had accumulated in the endomembrane system as indicated by the red arrows in Fig. 6A. However, IAuPs were larger and non-uniform in size as seen in Fig. 6A. Some of the larger particles had not entered the cells, as indicated by the shadow cast on cell marked by the yellow arrow, while others had entered the cells and were surrounded by membrane, or were in the cytosol (Fig. 6A). Imaging mechanically disrupted IAuPs in cells by SEM revealed that they were smaller and more uniform and size, and their location was similar to AuNPs, although there were fewer of them per cell (Fig. 6B).

Next, CytoViva imaging technology was used to compare the spectral profiles of AuNPs and IAuPs in HeLa cells using hyperspectral and dark-field illumination. As shown in Fig. 6C, the AuNPs had a broad peak between 600–700 nm, while IAuPs had a narrower peak between 600–625 nm. While the spectral shifts likely correspond to the difference in size between the two types of particles, differences in their spectral profiles also could be attributed to their interactions with different molecules inside the cells.

Nuclear localization of IAuPs. The nuclear localization of IAuPs was further investigated. Through functionalization, AuNPs can escape from lysosomes into the cytosol, where they could gain access to organelles such as mitochondria or the nucleus[30–34]. Although anti-cancer drugs like Doxorubicin can enter the nucleus without requiring a carrier, it may be desirable to use a carrier to have more control over drug targeting and/or release[39,40]. AuNPs can be further modified so that after their escape into the cytosol, they can be selectively targeted to the nucleus[32]. However, particles in the cytosol could also randomly incorporate into the nucleus during division. As shown by the schematic in Fig. 7A, as cells enter mitosis to divide, their nuclear envelope breaks down, and nuclear components mix with the cytosol until they are re-packaged during telophase. IAuPs were observed in the nucleus of a small proportion of HeLa cells (Fig. 7B). To determine if IAuPs can be sequestered in the nucleus by chance during cell division, DIC microscopy was used to image dividing HeLa cells containing citrate-capped AuNPs or IAuPs (Fig. 7C). During mitosis, AuNPs remained closer to the cell poles as indicated by the yellow arrows in Fig. 7C. This is consistent with their retention in vesicles of the endomembrane system, which remain near the centrosomes during mitosis. The distribution and movement of IAuPs was random, as some particles remained close to the condensed chromatin, while others moved to opposite sides of the cell as shown in Fig. 7C. To verify that IAuPs enter the nucleus randomly during mitosis, HeLa cells were arrested in S phase to prevent them from entering mitosis. To ensure that the majority of cells in the population were synchronized for S phase, they were treated twice with 2 mM Thymidine[37]. After the second treatment, IAuPs were added to the cells, then after 23 hours were fixed and stained with DAPI to visualize chromatin. Brightfield and epifluorescence microscopy revealed that there were no IAuPs in the nuclei of cells that were arrested in S phase (0%; n = 200 cells, standard deviation = 0), while IAuPs were observed in the nuclei of a small subset of cells that were permitted to enter mitosis (1.6%, n = 193 cells, standard deviation = 0.25). When cells were left to cycle randomly for several generations, 10% had nuclear IAuPs. Therefore, this data suggests that IAuPs are randomly sequestered in the nucleus during nuclear envelope reformation after cell division rather than being selectively targeted.

Entry mechanism of IAuPs. IAuPs differ from AuNPs in their physical properties and subcellular localization, and likely also vary in the mechanism by which they enter cells. Previous reports showed that AuNPs enter human cells via clathrin-dependent receptor-mediated endocytosis, where they remain in the endomembrane system[1,21,26,28,29]. While IAuP crystallites are 60 nm in size, they form large particles, and this study determined if IAuPs enter cells via macropinocytosis, clathrin-dependent receptor-mediated endocytosis, or clathrin-independent endocytosis[22–25]. As shown in Fig. 8A, HeLa cells treated with 100 nM Cytochalasin D to disrupt F-actin and block macropinocytosis had fewer IAuPs in comparison to control cells (4.7%, n = 727 cells vs. 9.2%, n = 628 cells for three replicates, respectively). Cells treated with 5 μm Chlorpromazine, which disrupts clathrin-dependent receptor-mediated endocytosis also had fewer IAuPs compared to control cells (4.9%, n = 647 cells vs. 11.1%, n = 630 cells for three replicates, respectively; Fig. 8A). Cells treated with both Cytochalasin D and Chlorpromazine similarly showed a strong reduction in the number of cells with particles compared to control (4%, n = 643 cells vs. 9.2%, n = 628 cells for three replicates, respectively; Fig. 8A). Cells treated with 100 μM Genistein to block caveolin-dependent endocytosis had no change in the number of particles compared to control cells (12.5%, n = 498 cells vs. 11.1%, n = 630 cells for three replicates, respectively; Fig. 8A). Thus, smaller IAuPs could enter cells via clathrin-dependent receptor-mediated endocytosis, while larger particles may rely more on macropinocytosis (Fig. 8B).

Conclusions

This study describes the toxicity, subcellular distribution and entry mechanism of Swarna Bhasma Incinerated Gold Particles, IAuPs, in human cells. This is the first study, to our knowledge, that has characterized IAuPs in human cells. IAuPs are large irregular-shaped particles formed from 60 nm crystallites that are not toxic to HeLa or HFF-1 cells. As shown in Fig. 8B, small IAuPs likely enter HeLa cells via receptor-mediated endocytosis, where they accumulate in vesicles, within the endomembrane system, similar to AuNPs. However, large IAuPs may enter cells by macropinocytosis and accumulate in vacuoles. Vesicles that are part of the endomembrane system, display restricted patterns of movement, which mirrors that of the microtubule tracks they traffic along. Since microtubules, emanate from the centrosomes, the vesicles tend to accumulate in the endomembrane system that hugs the nucleus, where the centrosomes are located. In support of this, vesicles containing AuNPs showed these types of movements. However, while some vesicles with IAuPs showed these types of movements, others were more random. In addition, IAuPs were observed in the cytosol and nucleus in some cells, suggesting that they had escaped from vesicles or vacuoles.

The localization of IAuPs in the nucleus occurs during cell division. As eukaryotic cells enter mitosis, their nuclear envelope breaks down exposing chromatin to the cytosol, and the nuclear envelope re-assembles as chromosomes finish segregating during telophase. Particles that are in the cytosol are randomly located, and could get sequestered in one of the daughter nuclei as they reform. We propose that this may be a mechanism by which cytosolic particles gain access to the nucleus, which is often overlooked in the literature. Another interesting question is how IAuPs gain access to the cytosol when they all presumably enter via membrane-bound vesicles. As outlined above, IAuPs enter HeLa cells by receptor-mediated endocytosis and macropinocytosis, where they accumulate in membrane-bound vesicles or vacuoles, respectively. These membranous networks protect cells from foreign material and molecules. Large particles could cause membranes to break via mechanical disruption,

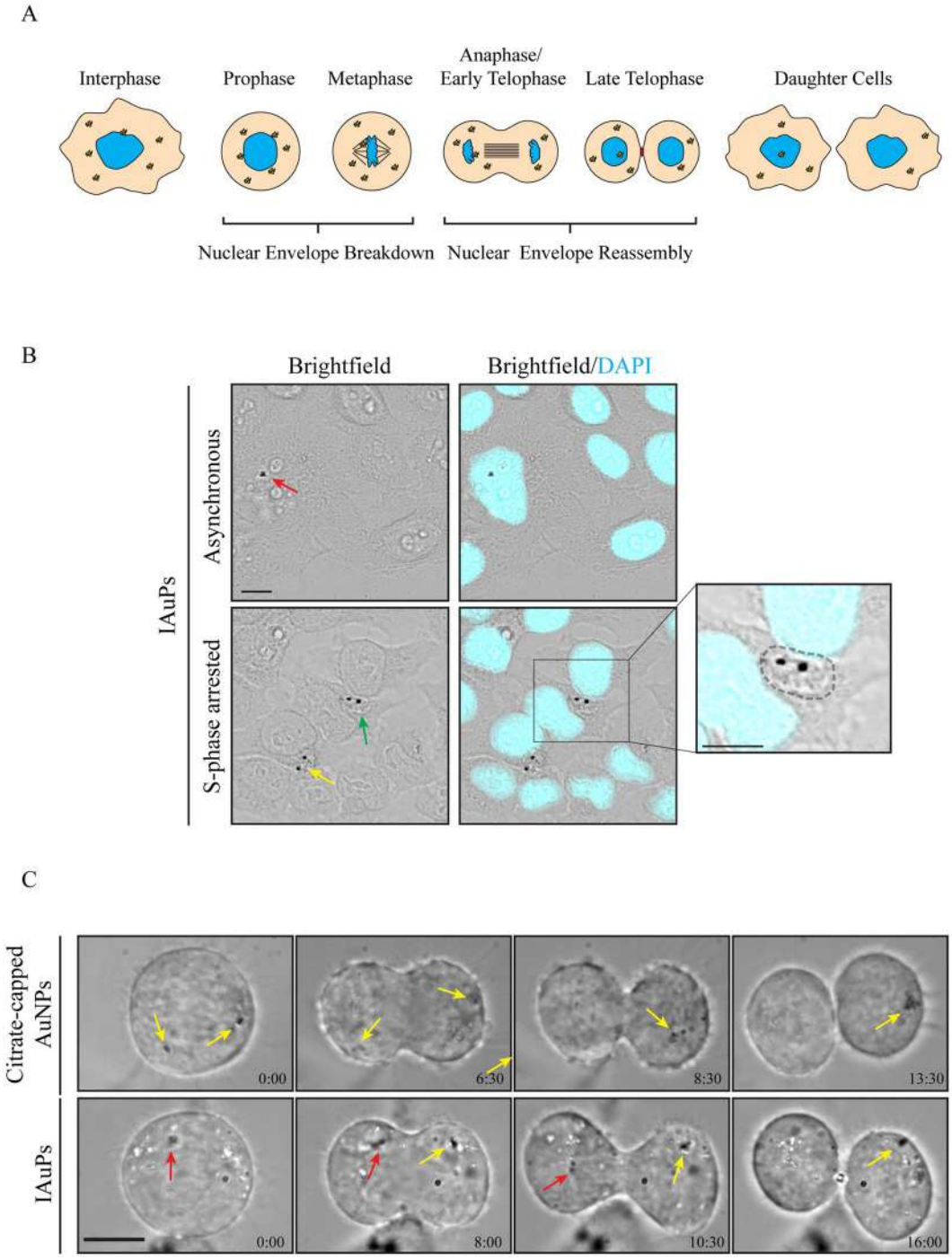

Figure 7. (**A**) A cartoon schematic shows the mechanism of nuclear entry for IAuPs during mitosis. As cells transition from prophase to metaphase, the nuclear envelope breaks down causing the nuclear and cytosolic contents to mix. The nuclear envelope reassembles in early telophase. (**B**) Images show asynchronous or S-phase arrested HeLa cells with IAuPs in the nucleus (red arrow), cytosol (yellow arrow) or in a vacuole (green arrow). The inset is a zoomed in region of a cell showing a vacuole, indicated by a dashed line, containing IAuPs. (**C**) Time-lapse images show dividing HeLa cells treated with citrate-capped AuNPs (top panel) or IAuPs (bottom panel). Yellow arrows indicate AuNPs or IAuPs that segregate to the poles of the cell, while red arrows point to IAuPs that stay near the chromatin and are likely incorporated into the nucleus. The scale bars are $10\,\mu m$.

or impede fission/fusion of the vesicles during remodeling of the membranous networks. Alternatively, IAuPs may contain elements or compounds that promote rupture of lysosomes or vacuoles. Once IAuPs gain access to the cytosol, they could be sequestered in the nucleus during division.

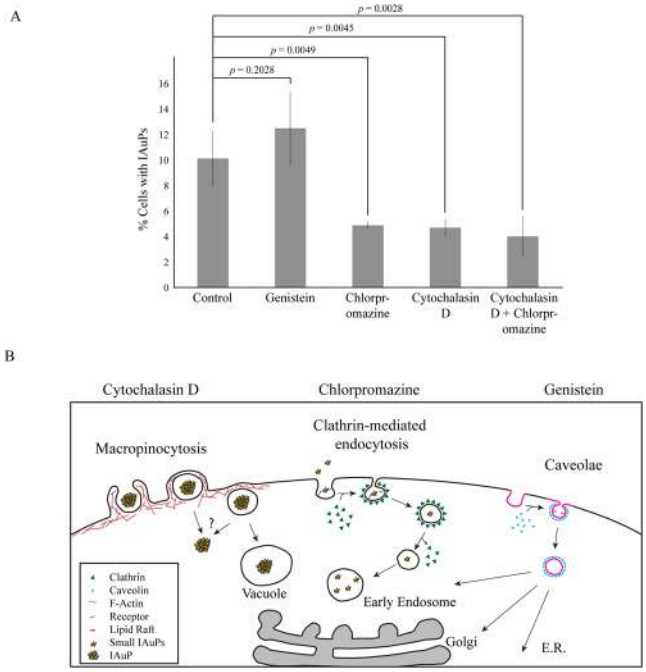

Figure 8. (**A**) The graph shows the proportion of cells with IAuPs after treatment with DMSO (control), 100 μ*M* Genistein, or 5 μ*M* Chlorpromazine or after treatment with 100 nM Cytochalasin D or both Cytochalasin D and Chlorpromorazine. Bars show standard deviation. (**B**) A cartoon schematic shows the different pathways by which the IAuPs enter HeLa cells, and those that are blocked by the drugs used in the assays in (**A**).

The composition of IAuPs varies between manufacturers even for those from a similar region. It is not clear how their composition impacts their entry, location or toxicity at the cellular level, or medicinal properties at the organismal level. It would be interesting to compare different IAuPs, as well as study their impact on different cell types than those studied here. For example, large particles may more successfully enter phagocytic cells (e.g. macrophages) causing an increase in the proportion of cells with IAuPs in comparison to HFF-1 or HeLa cells. IAuPs are typically administered by oral ingestion, and another question is how these particles are able to pass through the epithelial cells lining the digestive tract to enter the body, or if they enter the body at all and their medicinal properties are attributed to how they act as carriers for beneficial molecules from plant extracts. It would be interesting to explore the compounds and elements that are typically coupled with IAuPs to determine which ones confer medicinal properties. Some compounds could be small and amphipathic, permitting them to pass freely through the cell membranes. Given that IAuPs are inert, large particles, they could be further explored for use as carriers, imaging and/or temperature control for diagnostics or treatments.

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Author Contributions

S.B., M.P. and A.P. conceived the study and experiments. D.B., K.K., A.S.K., D.J., S.O. carried out experiments and contributed to the manuscript. All authors reviewed the manuscript.

Additional Information

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PHARMACEUTICAL STANDARDISATION OF SWARNA VANGA - A TRADITIONAL AYURVEDIC PREPARATION

Namrata Joshi[1], Khemchand Sharma[2], Manoj Kumar Dash[3] and Amit Verma[4]

Abstract

Swarna vanga is an important *Kupipakwa Rasayana*, prepared by using *Shodhita* (processed) *Vanga* (Tin), *Parada* (Mercury), *Gandhak* (Sulphur), *Navasadara* (Ammonium Chloride), *Kalmi Shora* (Potassium nitrate), in specified ratio. Number of methods are described in *Rasashastra* classics for preparation of *Swarna vanga*, but preparation of good quality *Swarna vanga* is a difficult task. Now a days due to the commercialization of Ayurvedic medicines and ignorance of classical methods, quality of drugs has deteriorated. Hence it is the need of hour to standardize Ayurvedic preparations especially *Kupipakwa Rasayana* for global acceptability, as by *Kupipakwa* method, different minerals and metals are transformed into potent medicines with quicker action on minimum dose in the present study, standardization of *Swarna vanga* was carried out by preparing three samples following same process, under similar environmental conditions. For preparation of *Kupipakwa Rasayana,* gradual rise in temperature is mandatory and specifically for *Swarna vanga*, maximum temperature required should not exceed 600 °C - 650 °C, as all required chemical reaction will be completed within this temperature range. Total time duration for the procedure is mentioned to be 12 hour as per different references. But in the present study, all the three samples took 18 hours for completion of the process. Heating pattern for *Swarna vanga* is standardized by preparing 3 samples with heating pattern as initial 2½ hour *Mruduagni* (upto 280 °C), next 7½ hour *Madhyamagni* (280 °C-580 °C) followed by 8 hour *Tivraagni* (580 °C-650 °C) in classical *Baluka yantra.*

Key words: *Swarna vanga, Kupipakwa Rasayana*, Standardization, *Shodhana*

Introduction

Kupipakwa Rasayana is an example of *Parada Murchhana* i.e. attainment of disease curing properties in *Parada*[1] that bears a unique importance in Rasa Shastra, due to its quicker action on minimum dose. It is a process in which *Parada* with or without *Gandhaka* is converted into suitable compound, which is classified in to *Sagandha Murchhana* (processed with *Gandhaka*) and *Nirgandha Murcchana* (processed without *Gandhaka*)[2]. By this method, different minerals and metals are transformed into effective medicines as association of Mercury with Sulphur, brings about the desired chemical changes in the final product with more potent efficacy[3]. *Swarna vanga* is one such example of *Sagandha* (with Sulphur), *Bahirdhuma* (Fumes generated during processing expelled outside), *Talastha* (Product sublimed at the bottom) *Murchhana* of *Parada* prepared by *Kupipakwa* method. *Bhaishajya Ratnawali* [4] is the first text to quote it by the name of *Swarna vanga,* although previously similar formula has been quoted by the name

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of *Vangeshwar Rasa* in different texts[5]. Basically, it is named so due to the appearance of golden colour, as similar to Gold. It is indicated in diseases like *Prameha* (Increased frequency and turbidity of urine), *Shukra roga* (Diseases of Semen), *Viryahani* (Azoospermmia)[6], *Shewta pradara* (Leucorrhoea)[7] etc. Primarily, its action is concentrated on different disorders of *Shukra* (Semen) like *Shukra taralya* (Increased liquidity of Semen), *Viryahani* (Azoospermmia), and *Daurbalya* (Weakness)[8]. That is why, *Swarna vanga* is generally considered as a drug of choice for seminal disorders. In preparation of *Kupipakwa Rasayana*, preparation of *Kajjali* and heating pattern plays most important role for proper sublimation of final product. Many researches have been carried out on different *Kupipakwa* preparations in this regard, but on *Swarna vanga* no such study has been validated so far. So, the present study was undertaken to develop the standard manufacturing process (SMP) for *Swarna vanga* prepared by traditional *Baluka yantra* focussing mainly on preparation of *Kajjali* and heating pattern.

Materials and Methods

(A) Pharmaceutical processing

Swarna vanga was prepared as per reference of Rasa Tarangini[9]. *Vanga* (Tin), *Parada* (Mercury), *Gandhak* (Sulphur), *Navasadara* (Ammonium Chloride) and *Kalmi Shora* (Potassium Nitrate) were collected from the Pharmacy, Rishikul Govt. Ayurveda College, Haridwar. *Gomutra* (Cow's urine) was procured from local *Goshala* while *Takra* (Buttermilk), *Tila taila* (*Sesamum* oil) were purchased from local market. *Kanji*[10] (Sour), *Kulattha kwath*[11] (decoction of *Dolichos biflorus*) and *Churnodaka*[12] (Lime water) were prepared as per standard methods. The whole process was divided into following steps

Processing (*Shodhana*) of Raw Material

Vanga Shodhana was done by following classical guidelines i.e *Samanya Shodhana*[13] and *Vishesha Shodhana*[14]. Similarly for *Parada Shodhana*[15], double distilled Mercury (500gm) from Merck company, was taken in a mortar. *Paan Svaras* (juice of *Piper betel* 100ml), *Aadrak Svaras* (juice of *Zingiber officinalis* 100ml) and *Kshartray* (combination of *Yavakshar, Sajjikshar* and *Tankan* 31.25gm each) were added and triturated. The process was continued for three days and *Parada* was then washed with potable water. For *Shodhana* of *Gandhak*, traditional method using cow's milk and clarified butter was employed. In this method, *Gandhak* was heated up to its melting temperature and was poured through a double layered cloth into a vessel containing boiled milk. *Gandhak* settled on the bottom and assiduously milk decanted off. It was collected in another stainless steel container. Process was repeated thrice. Finally, it was washed in water and dried to purified form[16]. *Navasadara Shodhana* and *Kalmi Shora Shodhana* was done in accordance to *Rasa Tarangini*. For *Navasadara Shodhana*, *Ashuddha Navasadara* was dissolved in desired quantity of water (3 times to *Ashuddha Navasadara*), filtered and evaporated on fire[17]. For *Shodhana* of *Kalmi Shora,* first of all the process of *Nirmalikarana* was done by dissolving it in boiled water and evaporating till it was converted into fine crystals followed by levigation (*Bhavana*) in Cold infusion (*Hima*) of *Ela* (*Elettaria cardamomum)*[18]. All purification procedures were carried out in the department of Rasashastra, Rishikul Govt. Ayurveda College, Haidwar.

Preparation of *Vanga pishti*

Shuddha Parada (50g) was taken in mortar and purified molten *Vanga* (100g) was added to it quickly. The mixture was triturated vigorously upto a homogenous form. *Saindhav lavana* (10g) was added in *Vanga pishti* and this mixture was triturated with *Nimbu Swarasa* (1 L). After 10-15 minutes of trituration, *Nimbu Swarasa* turned black; it was removed with the help of suction syringe and washed with hot water. Above procedure was repeated till black colour of *Nimbu Swarasa* was completely disappeared. The colour of mixture after 40 hours of trituration was light silver grey. Obtained material was again washed with hot water and kept for drying in sunlight for 2 days. Although *Saindhava lavana* is not an ingredient of *Swarna vanga* in accordance to the reference of *Rasa Tarangini* quoted here. However, it was added on the basis of another reference for the same formulation, for getting good quality of *Vanga Pishti* as per the advice of *Rasa Shastra* experts.

Preparation of *Kajjali* for *Swarna vanga*

Shuddha Gandhak (66.67g) was added to *Vanga pishti* and was subjected to the process of continuous trituration. During this process, the mixture changed from grey colour to dark grey colour. Trituration was done till this mixture became black, very fine and attains uniform consistency. *Shuddha Navasadara* (50g) was added in above mixture and again triturated properly. Lastly, *Shuddha Kalmi Shora* (4.1g) was added in above mixture. This mixture was triturated properly. Trituration was done till mixture became black, very fine, uniform and lustreless.

Preparation of *Swarna vanga*

Prepared *Kajjali* (254g) was slowly filled in mud smeared bottle (*Kanchkupi)* upto 1/3rd level of *Kanchkupi.* The inner surface of the bottle was cleaned thoroughly before filling the *Kajjali*. The *Kanchkupi* containing *Kajjali* was put in a special apparatus known as *Baluka Yantra* (sand bath), for *paka* (heating). The mouth of *Kanchkupi* was closed temporarily during filling of *Baluka,* in *Baluka Yantra* upto neck. A Pyrometer was inserted into the sand (*Baluka*) 3 inches above the base of *Baluka yantra*. The *Kupi* was fixed in *Baluka Yantra* (sand bath) in such a way that sand should surround it upto the neck and the heat would be applied through sand only. Heat treatment was given in gradual increasing way (*Kramagni*),[19] from mild temperature range to moderate and finally to excessive heat with the help of L.P.G gas which was used as a fuel. The whole procedure took 18 hrs. During the process, hot iron rod was regularly inserted to clean the bottle neck to avoid blockage by deposition of sublimed *Navasadara* and *Gandhak*. Different tests for the completion of *Paka* as copper-coin test, *Sheeta shalaka* tests were also done. After completion of *Paka,* corking of *Kanchkupi* was done cautiously and temperature was further maintained for 2 hrs. There after, the *Kupi* was left for self cooling. Next day after achievement of *Swangashita* (self cooled) state, *Kanchkupi* was taken out from the *Baluka Yantra* (sand bath) and *Kapadmitti* over the *Kupi* was removed carefully. The contents were visualized against light and the level of deposition of drug was marked that was about 2 cm from the base of the bottle. A jute string dipped in kerosene was wrapped around the bottle in the lower middle and set on fire. On complete burning ,the remnants of the string were immediately

removed and bottle was wrapped in a wet cloth. On gentle tapping at the level of string, the *Kanchkupi* broke into two parts. The material deposited at the base was collected by gentle tapping and with forceps. Absolute golden and light weighted material was collected. The compound obtained was powdered. The obtained *Swarna vanga* was washed with water to remove excess of *Kshara* added during preparation. Three batches of *Swarna vanga* were prepared in accordance to the method described above under similar environmental conditions.

(B) Physico-chemical analysis

pH value, loss on drying at 110ºC, were done as per guideline of Ayurvedic Pharmacopeia of India[20]. Estimation of total Sulphur and free Sulphur[21], XRD analysis[22] and assay for Tin[23] were also carried out using standard procedures.

Results and Discussions

Vanga Shodhana

For the preparation of *Kupipakwa rasayana, Dhatu pishti nirmana*, preparation of *Kajjali* and heating pattern are the most important factors to obtain best quality and maximum quantity of yield without any untoward effects.[24] The *Samanya* and *Vishesha Shodhana* of *Vanga* was done with the intention of eliminating *doshas* from raw drug *Vanga* and to make it suitable for further procedure. For *Samanya Shodhana* of *Vanga,* method of melting followed by pouring (*Dhalana)* sequentially in liquid media viz. *Tila taila, Takra, Gomutra, Aranala, Kulattha Kwatha* as depicted in *Rasa Ratna Sammucchya*. Similarly *Vishesha Shodhana* was done adopting the same procedure of melting followed by pouring. For this, *Churnodak* was taken as liquid media. It was found that after *Shodhana, Vanga* lose its lustre, became softer, fragile and converted into small pieces, even small quantity of *Vanga* turned to powder form (Figure-1). The rationality behind this can be, if we consider the *Rasa, Guna, panchabhoutikatva* constitution of the different medias those were used, they are dominated with *Agni* and *Vayu Mahabhuta* which may be helpful in breaking down the compact molecular structure of the metal resulting in decreasing the hardness. Alternate pouring in alkaline and acidic media during *Samanya Shodhana* also helps in reducing hardness of the metal. Also, the very fact that repeated and immediate alteration in the temperature plays an important role in annealing (i.e. breakdown on heat treatment of the metal, leading to fragility and pieces form)[25]. Loss in weight of *Vanga* was seen after *Shodhana* (Table No-1) may be due to removal of alloying elements or trace impurities.

Table No.1 Showing observations during *Samanya and Vishesha Shodhana* of *Vanga*

Initial wt of *Vanga* (in g)	800
Weight after *Shodhana* in *Taila*	795
Weight after *Shodhana* in *Takra*	775
Weight after *Shodhana* in *Gomutra*	755

Weight after *Shodhana* in *Kanji*	740
Weight after *Shodhana* in *Kulattha Kwatha*	700
Weight after *Shodhana* in *Churnodak*	690
Weight Loss	110
% loss	13.75

Figure No-1 Process of *Vanga Shodhana*

Fig. 1.1
Melting of *Vanga*

Fig. 1.2
Vanga Dhalan

Fig. 1.3
After *samanya shodhana*

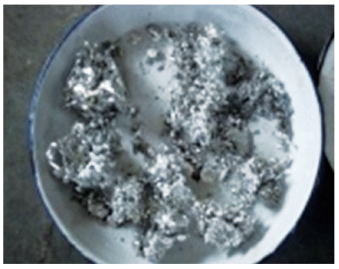

Fig. 1.4
After *vishesha shodhana*

***Parada Shodhana*:**

For *Shodhana* of *Parada*, triuration process (*Mardana)* was done in hot mortar[26] (*Tapta Khalwa)* which hasten the process and may help in removing the *Dosha* of *Parada* more frequently. (Figure-2) Loss of *Parada* during *Samanya Shodhana* might be due to removal of impurities and also might be due to various *Parada Gati* [27] (Table no-2).

Table No. 2 Showing observations during *Samanya Shodhana* of *Parada*

Total duration for *Shodhana* (in days)	**Total time taken for *Mardana***	**Weight of *Parada* before *Shodhana* (in g)**	**Weight of *Parada* after *Shodhana* (in g)**	**Weight loss (in g)**	**% loss**
3	24 hours	500	492	8	1.6

Figure No-2 Process of *Parada Shodhana*

Fig. 2.1
Mixing with *paan swaras*

Fig. 2.2
Mardan in *Tapt Khalwa*

Fig. 2.3
Separation of pure *Parada*

Fig. 2.4
Washing with hot *Adraka swarasa* and kshar traya water

***Gandhak Shodhana*:**

After dribbling down into *Godugdha, Gandhak* formed into granules due to the cold media of *Godugdha.* There will be a formation of new bond and change in crystal structure due to rearrangement of crystal

lattices[28]. There were physical impurities like mud etc on the cloth. Because these impurities remain unchanged at this temperature, this may be considered as purification at physical level. (Figure-3) Native Sulphur recovered from hot springs, volcanic vents tend to be rich in minerals, metals and trace ,the most notable of these being Arsenic, Antimony, Copper, Nickel, Tin, Lead etc.. These can be taken as toxic materials or *Vishas,* which are unwanted[29]. These chemical impurities may be removed by adsorbing over to colloidal fatty micro globules of *Godugdha*. *Godugdha* contain enzymes like *peroxidase, catalase, phosphatase, lipase*, gases like oxygen, nitrogen, protein, lactose, acid like citrate, formate, acetate, lactate, oxalate, minerals like Ca, P, citrate, Mg, K, Na, Zn, Cl, Fe, Cu, sulphate, bicarbonate and many other[30]. These constituents of *Godugdha* may have role in detoxifying the Sulphur[31]. The change in colour of *Godugdha* from white to yellowish cream and sulphur smell may indicate the dissolution of fat soluble sulphur content in the *Godugdha*. The process was repeated thrice to detoxicate it completely from any remnant *doshas*. *Shuddha Gandhaka* was thoroughly washed with hot water to remove remnant of *Godugdha*. Loss in weight might be due to removal of Impurities. (Table no-3)

Table No. 3 Showing observations during *Gandhak Shodhana*

Initial Weight of of crude *Gandhaka* (in g)	**Weight after 1st *Shodhana* (in g)**	**Weight after 2nd *Shodhana* (in g)**	**Weight after 3rd *Shodhana* (in g)**	**Weight loss (in g)**	**% loss**
1000	970	960	950	50	5

Figure No-3 : Process of *Gandhak Shodhana*

Fig.3.1
Melting in *Goghrita*

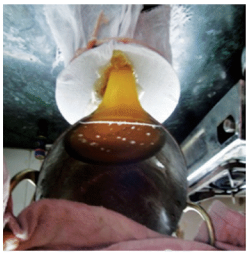

Fig.3.2
Pouring in *Gokshir*

Fig.3.3
Gandhak in *Gokshir*

Fig3.4
Washing in hot water

***Navasadara Shodhana*:**

In *Navasadara Shodhana,* dissolution of *Navasadara* into water helps in removal of foreign material (*Malas Doshas)* present in it (Table no-4).While filtering through cloth, these *Malas* or *Doshas* are removed (physical impurities). On heating (*Agni samsakara)*, there will be a transformation of certain qualities in *Navasadara*, i.e. after *Shodhana*, the *Tikshna, Sukshma, Laghu, Ushna guna* may increase in *Navasadara*. As per Rasatarangini, the text referred, for *Shodhana* of *Navsadar*, 1 part of *Navasadara* is to be added with 3 parts of water and then dissolved thoroughly. According to Chemistry, the solubility of Ammonium Chloride is 3. Loss in weight might be due to removal of impurities or due to sublimation of *Navasadara*. (Figure-4)

Table No. 4 : Showing observations during *Navasadara Shodhana*

Initial weight of *Navasadara* (in g)	Water added for dissolving (in ml)	Material obtained after complete drying (in g)	Loss in wt. (in g)	% loss
1000	3000	960	40	4

Figure No-4 : Process of *Navasadara* and *Kalmi Shora Shodhana*

Fig.4.1
Boiling of *Navasadara* in water

Fig.4.2
Suddha Navasadara

Kalmi Shora Shodhana

In case of *Kalmi Shora,* process of *Nirmalikaran* has been depicted prior to *Shodhana*, may be to remove external impurities like dust, sand etc. present in it. (Table no-5) It is alkaline in nature and thus named as *Surya Kshar*. According to *Rasarnava*, alkaline media (*Kshara)* possesses property of removing the *Malas* of *Dravyas* and this property may be helpful in *Swarna vanga* preparation. Loss in weight of *Kalmi Shora* may due to removal of impurities. (Table no-6)

Table No. 5 : Showing observations during *Kalmi Shora Nirmalikaran*

Initial Weight of of crude *Kalmi Shora* (in g)	Weight After *Nirmalikaran* (in g)	Appearance	Weight loss (in g)	% loss
100	90	White crystal like	10	10

Table No. 6 : Showing observations during *Kalmi Shora Shodhana*

Weight after *Nirmalikaran* (in g)	Weight after *Shodhana* (in g)	Appearance	Weight Loss (in g)	% loss
90	85	Greyish white	5	5.56

Figure No-5 : Process of *Kalmi Shora Shodhana*

Fig. 5.1
Kalmi Shora dissolved in hot water

Fig.5.2
Kalmi Shora after *Nirmalikaran*

Preparation of *Swarna vanga*

Molten *Shodhita vanga* was added to *Shodhita Parada* to make *Dhatu pishti*. It was a soft, smooth, grey colour paste. It was observed that after adding molten *Vanga* to *Parada,* it lost its consistency and mixed with *Vanga* completely within 15 minutes. The amalgamated mixture was triturated with *Nimbu Swarasa* and *Saindhava lavana* followed by washing with water till the black colour of the water disappeared. (Figure-6) By this unwanted impure materials may get loosen and removed, it may also induce *Deepana guna* in this mixture to get ready for next process. (Table no-7)

Table No. 7 Showing observations during *Dhatu pishti* Preparation

Weight of *Vanga* (in g)	Weight of *Parada* (in g)	Weight of *Dhatu pishti* (in g)	Weight of *Dhatu pishti* after trituration and washing (in g)	Weight Loss (in g)	% Loss
100	50	150	148	2	1.33

Figure No-6 : Process of *Dhatu Pisthi* Preparation

Fig.6.1 *Suddha Parada*

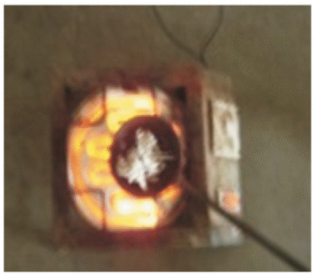

Fig.6.2 Melting of *Suddha Vanga*

Fig.6.3 Mardana of *Parada* and *Vanga*

Fig.6.4 *Mardana* with *Nimbu Svaras* and *Saindhava lavana*

Fig 6.5 Washing *Pisthi* with hot water

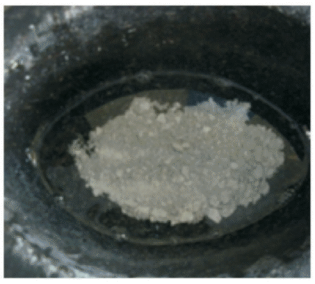

Fig.6.6 *Pisthi* after complete washing

Grey coloured above amalgamated mixture and 66.67 g *Shodhita Gandhak* was taken in *Khalvayantra* and triturated well. Within half an hour *Gandhak* completely mixed with above mixture and the colour of the mixture changes from dark grey to light grey colour that indicate the physical bonding of *Gandhak* and *Dhatupishti* in progress state. Trituration was done till the attainment of *Nishchandratwa* (lustreless). After 42 hours of continuous trituration, *Nishchandratwa* test became positive indicating absence of free mercury and along with the physical bonding, chemical bonding might have taken place in *Kajjali*. For better fineness and smoothness of *Kajjali*, *Mardana* was continued up to 62 hours. During the process,

spillage was more due to the increased fineness of *Kajjali*. (Table No-8) Then, 50gm Navasadara was added and triturated well. Here the Navasadara may help in potentiating the final product. (Figure-7) This mixture was triturated up to 3 hours, in which homogenous mixture take place. (Table No-9) 4.1 g *Kalmi Shora* was added to above mixture and triturated well. Although *Kalmi Shora* is not mentioned as a constituent drug in the preparation of *Swarna vanga* but it is added in tradition to impart bright yellow colour to the formula. Total loss in preparation of *Swarna vanga kajjali* was approximately 16.77 g. It might be due to spillage because of smooth and fine *Kajjali*. The green colour bottle of capacity 650 ml, devoid of air bubbles was selected as it is chemically inert and more pyro sensitive. *Kupi* was covered with 7 layer of *Multani Mitti* smeared cloth. Each layer was applied after complete drying of the previous one. Air bubbles in the bottle and air space between the layers may cause breaking of the *Kupi* during heating. Applications of *Multani Mitti* smeared cloth strengthen the bottle and help in regulation and maintenance of temperature inside the *Kupi* to facilitate the chemical reaction. Lower 1/3rd of the *Kupi* (after marking) was filled with *Kajjali*, as large quantity may cause over flow of boiling *Kajjali* from the *Kupi* mouth during *Madhyamagni* stage (280 °C-580 °C). Over the central hole of *Baluka yantra* sand was spread up to 2 angula thickness as direct heat to the *Kupi* may burst the bottle. *Kajjali* filled *Kupi* should be placed exactly at the centre of *Baluka yantra*. The remaining portion of the *Yantra* was filled with sand (*Baluka)*. The purpose of using the *Baluka* is to maintain the uniform and sustained heat to the *Kupi*. The system of applying *Kramagni* or ladder step heating procedure is recommended to give uniform, slow and steady rise in temperature[33]. By this, the ingredients are given enough time at each range of temperature allowing them for any kind of reaction to take place. After 2 hours, slight white colour fumes with *ugra, tikshna gandha* came. This may due to burning of *Navasadara*. After 3 hours, when red hot *shalaka* inserted semisolid state of *Kajjali* felt. This may be due to melting of *Kajjali*, which is usually between 420 °C - 430 °C. After 4 hours, dense yellow coloured fumes with smell of *tikshna Gandhak* came. This may be due to the temperature, which reaches the boiling point of *Gandhak* i.e. above 444 °C. At final stages, when temperature was increased upto 650 ºC, (Table No-10) (Figure-8) sand like feeling was observed when copper wire was inserted inside the *Kupi*. Corking was done and temperature was maintained for 2 hrs and then was kept for self cooling.

Table No. 8 Showing observations during addition of *Gandhak* to *Vanga Parada Pishti*

Weight of *Dhatu pishti* (in g)	Weight of *Gandhak* (in g)	Weight of *Kajjali* (in g)	Loss in Weight (in g)	% Loss
148	66.67	208	6.67	3.107

Table No. 9 Showing observations during addition of *Navasadara* to above *Parada* ,*Vanga*, *Gandhak* mixture

Weight of *Kajjali* (in g)	Weight of *Navasadara* (in g)	Weight of *Kajjali* after addition of *Navasadara* (in g)	Loss in Weight (in g)	% Loss
208	50	252	6	2.381

Figure No-7 : Preparation of *Swarna vanga Kajjali*

Fig.7.1 *Suddha Gandhak* to *Dhatupisthi*

Fig.7.2 After *Mardan* with *Gandhak*

Fig.7.3 Addition of *Suddha Navasadara*

Fig.7.4 After *Mardan* with *Navasadara*

Fig.7.5 Addition of *Suddha Kalmi Shora*

Fig 7.6 After *Mardan* with *Kalmi Shora*

Table No-10 Showing procedure and observations in *Swarna vanga* preparation

Stage of Process	Effect / Purpose	Duration	Time and Date	Temp.	Procedure and Observation
The stage of low temperature	For Liquefaction of material	00.00 hrs.	4.30 a.m/ 09.09.11	25 °C	*Kanchkupi* filled with *Kajjali* was kept in *Baluka Yantra* Heating process was started.
	For Liquefaction of material	1:00 hr	5.30 a.m	110 °C	Temperature was Maintained.
Stage of moderate temperature	Sublimation of *Navasadara*	2:00 hr.	6.30 a.m	280 °C	Appearance of white fumes that signifies beginning of burning of *Navasadara*
	Liquification of material	3.00 hr	7.30 a.m	420 °C	On insertion of red hot *shalaka,* semi solid state of *Kajjali* was felt. White fumes continue to appear.
	Start of *Gandhak jarana*	4.00 hr.	8.30 a.m	465 °C	White mixed yellow fumes appeared. Sulphur crystal started depositing around inner part of the neck, which was removed through red hot *shalaka* to avoid blockage of neck of the bottle.
	Gandhak jarana was in process	5.00 hr	9.30 a.m	465 °C	Yellow fumes appeared.

Stage of Process	Effect / Purpose	Duration	Time and Date	Temp.	Procedure and Observation
		6.00 hr	10.30 a.m	480 °C	When red hot *shalaka* was inserted, blue flames appeared.
		7.00 hr	11.30 a.m	532 °C	When red hot *shalaka* inserted, blue flames still persist.
		8.00 hr	12.30 a.m	544 °C	When red hot *shalaka* inserted, blue flames still persist.
	Gandhak jarana was in process	9.00 hr	1.30 p.m	556 °C	When copper coin was kept over the neck for 5 minutes and then removed, it turned black indicative of presence of sulphur and process of *Gandhak jarana*
		10.00 hr	2.30 p.m	580 °C	Again copper coin test was done. It turned black indicating *jarana* of *Gandhak* was still in process.
	Gandhak jarana was completed	11.00 hr	3.30 p.m	580 °C	On Copper coin test, it turned white, few white crystals were adhered to copper coin indicative of complete *Gandhak jarana* and evaporation of Hg.
		12.00 hr	4.30 p.m	580 °C	Temperature was maintained for 4 hrs
		16.00hr	8.30 p.m upto 10:30 p.m	650 °C	Sand like feeling when copper wire was inserted. Temperature was maintained for 2 hrs and then inserting *Kanchkupi* and corking was done.

Figure No-8 : Process of *Swarna vanga* Preparation

Fig.8.1 Filling of *Kajjali*

Fig.8.2 Kupi in *Valuka Yantra*

Fig.8.3 Appearance of white fumes

Fig.8.4 Appearance of white mixed yellow fumes

Fig.8.5 Wrapping & Burning of jute string with kerosene

Fig.8.6 *Swarna vanga*

Fig.8.7 *Swarna vanga* after washing

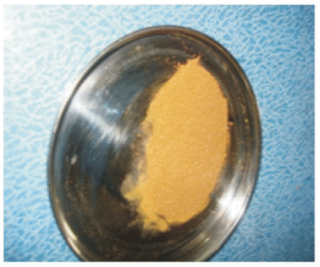

Fig.8.8 After complete dryingll

In the present research work for the purpose of standardization, three samples of *Swarna vanga* were prepared under similar conditions and were analyzed on various parameters. Final yield in all three samples of SV were found to be same. (Table No-11) All three samples complied with traditional methods for Bhasma Pariksha and were found to be same organoleptically. (Table No-12) When analyzed physicochemically, identical values were found. (Table No-13). On AAS analysis mercury was found to be absent whereas assay for tin showed nearly 66% (mean 65.53±0.2905) of tin in the formulation as shown in Table No-14 and Table No-15 respectively. On XRD analysis, final product was found to be Tin Sulphide in major phase and rest are found in minor phase. (Table No-16) Thus, the present study reveals that the three samples prepared by following the SOP have showed identical results which means the SOP if followed give uniform results.

Table No. 11 Showing final yield for three samples of *Swarna vanga*

Sample No.	Weight of *Kajjali*	Weight of *Swarna vanga* before washing	Weight of *Swarna vanga* after washing	Loss in wt.	% loss
1.	254g	115g	100g	154g	60.63%
2.	254g	120g	105g	149g	58.63%
3.	254g	118g	103g	151g	59.44%

Note: 4.1 g of *Kalmishora* was added to the *Kajjali* to impart bright colour.

Table No. 12 Showing classical parameters for *Swarna vanga* (SV)

S.No.	Parameter	SV1	SV2	SV3
1.	Colour (*Varna*)	Golden *(Swarnabha)*	Golden *(Swarnabha)*	Golden *(Swarnabha)*
2.	Touch(*Sparsh)*	Fine powder	Fine powder	Fine powder
3.	Odour (*Gandha*)	Odourless	Odourless	Odourless
4.	Floating test (*Varitarata*)	Positive	Positive	Positive
5.	Fineness test (*Rekha purnatva*)	Positive	Positive	Positive
6.	Grain floating test (*Unnam)*	Positive	Positive	Positive
7.	Tastelessness (*Gatarasatva*)	Positive	Positive	Positive

Table No. 13 Showing Physico-chemical analysis of *Swarna vanga* (SV)

Name of test	SV1	SV2	SV3	Mean (±SE)
pH value	7.56	7.57	7.62	7.5833 ± 0.0185
Loss on drying at 110°C %w/w	4.48	4.51	4.21	4.4 ± 0.0953
Estimation of Total Sulphur %w/w	18.80	17.95	17.70	18.15 ± 0.3329
Estimation of free Sulphur %w/w	1.2628	1.2328	1.2624	1.2526 ± 0.0099

Table No. 14 Showing percentage of Mercury in different samples of *Swarna vanga*

S.No.	Drug Sample	Mercury (µg/ml)
1	SV1	ND
2	SV2	ND
3	SV3	ND

Table No. 15 Showing percentage of Tin in different samples of *Swarna vanga*

S.No.	Drug Sample	Tin (%)	Mean of Tin (±SE)
1	SV1	65.0	65.53±0.2905
2	SV2	66.0	
3	SV3	65.6	

Table No.16 Showing results of XRD Study of 3 samples of *Swarna vanga*

Sample	d-Standard	d-identified	Name and composition
SV1	5.878	5.84108	Tin Sulphide SnS_2
	2.944	2.92977	
	1.964	1.95704	

Sample	d-Standard	d-identified	Name and composition
SV2	5.878	5.94038	Tin Sulphide SnS_2
	2.944	2.91935	
	1.964	1.95335	

Sample	d-Standard	d-identified	Name and composition
SV3	5.878	5.85438	Tin Sulphide SnS_2
	2.944	2.93819	
	1.964	1.95988	

Conclusion

Kupipakwa Rasayanas are product of complex chemical processes in which preparation of *Kajjali* and heating pattern plays most important role. Heating pattern for *Swarna vanga* is standardized by preparing 3 sample with heating pattern as initial 2½ hour *Mruduagni* (upto 280 °C), next 7½ hour *Madhyamagni* (280 °C- 580 °C) followed by 8 hour *Tivraagni* (580 °C - 650 °C) in classical *Baluka yantra.* For preparation of *Kupipakwa Rasayana,* gradual rise in temperature is mandatory and specifically for *Swarna vanga*, maximum temperature required should not exceed 600 °C- 650 °C, as all required chemical reaction will be completed within this temperature range.

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Original Article

PHARMACEUTICAL STANDARDIZATION OF *RAJATA BHASMA* (INCINERATED SILVER) BY TWO DIFFERENT METHODS

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Abstract

Rajata bhasma (incinerated silver) potentially utilized in various formulations such *as Jayamangala Rasa, Mahamraganka Rasa, Lakshmivilasa Rasa, Vatagajankusha Rasa, Vijaya Parpati* etc. Silver being a noble metal is said to be superior among metals after Gold. Works emphasizing on pharmaceutical standardization of *Rajata Bhasma* (RB) are very few. Though, standards of *Rajata Bhasma* prepared by *Kupipakva* method followed by *puta* (RB2) are available, comparison with direct *puta* (RB1) has not been found. Present study attempts to provide a comparative pharmaceutical and physico-chemical profile of RB1 & RB2.

Authentic raw materials were collected and classical guidelines were followed in preparing both the samples of *bhasma*. pH, Ash value, Acid insoluble ash, Water soluble ash, Loss on drying, percentage of Silver and Sulphur were evaluated in both the samples. RB1fulfilled all the classical parameters of *Bhasma* after 17th *puta*, whereas RB2 took 11 *putas* for the same. The physico-chemical profiles of both samples were found to be identical. Considering pharmaceutical observations, RB2 is found to be better in comparison to RB1. As, there is no physico-chemical profile available for RB, the current data may be considered as standard in future studies.

Key words: Standardization, *Rajata Bhasma*, *Puta*, *Kupipaka*

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Introduction:

Rasashastra a branch of *Ayurveda,* mainly developed for converting metals and minerals in to therapeutically potent from, the formulations which can deliver quick desired effect in minimal dosage.[1] Among various metals described in *Rasashastra*, Silver is regarded as noble metal with vast therapeutic utilities.[2] Therapeutic repertory of *Rajata Bhasma* ranging from *Madhumeha* (diabetes mellitus), *Jwara* (fever), *Pandu* (anaemia), *Yakrit vikara* (liver disorders) and *Manasa rogas* (Psychological disorders) etc.[3] *Bhasma* (incinerated metals and minerals) is prepared by combination of different herbo-mineral drugs as media followed by incineration. Process involved in *Rajata Bhasmeekarana* (process of incineration) differs in different classics. Around 115 different methods are available for preparation of *Rajata Bhasma* (RB).[4] Common procedure of *Bhasmeekarana* can be described in following steps i.e; purification of raw materials, levigation with prescribed herbal juices, pellet formation and incineration either in classical *puta* (repeated incineration cycle) with cow dung or in muffle furnace. However, certain other procedures can also be followed like *Kupipaka* followed by *puta* etc. Standard manufacturing procedure of RB prepared by *Kupipaka* followed by *Putapaka* has been attempted earlier, but no physico-chemical profile has been established.[5] In present study, two samples of RB were prepared by following two different methods to evaluate the significance of pharmaceutical procedures. Physico-chemical analyses of the samples were also attempted, which can be accounted as standards for RB.

Materials and Methods:

Hingula (Cinnabar), *Gandhaka* (Sulphur) and 99.98% pure *Rajata* (Silver) were procured from authentic sources and confirmed for their authenticity based on classical parameters. *Kajjali* was prepared in the Department of *Rasashastra,* TGAMC, Bellary. *Kumari* (*Aloe vera*

Linn.) was collected form botanical garden of the Institute and *Nimbu* (*Citrus limon* Linn.) was collected from local market. Equipments like S. Steel vessel, *Khalwa yantra* (mortar and pestle), *Sharava* (earthen saucer), *Upala* (cow dung cakes) were used from department. Specifications of the instruments are given in Table No.1.

Preparation of RB involves the following steps:

1. Purification of raw materials i.e. *Rajata, Gandhaka*
2. Extraction of *Parada*
3. Preparation of *Rajata Bhasma* by *puta* method (RB1)
4. Preparation of *Rajata Bhasma* by *kupipaka* followed by *puta* method (RB2)

Rajata Shodhana: Purification of Silver was done by *Nirvapa* (quenching) method.[6] Thin silver foils were heated to red hot and dipped in *Tila Taila* (Sesame oil), *Takra*[7] (Butter milk), *Gomutra* (Cow's urine), *Aranala*[8] (Sour gruel prepared out of rice), *Kulattha kwatha* (decoction of *Dolichos biflorus* Linn.) consecutively for *Samanya Shodhana*. Similarly quenching in *nimbu swarasa* (juice of *Citrus limon* Linn.) is followed for *Vishesha Shodhana.*[9] (Table 2)

Gandhaka Shodhana: Purification of *Gandhaka* was carried out by *dhalana* method.[10] Raw *Gandhaka* was taken in a stainless steel container containing 1/4th *Ghee* and melted over mild flame. A muslin cloth of four layers was tied to the mouth of the container containing milk. As soon as *Gandhaka* is melted, the mixture was poured into the container through the cotton cloth. The above process was repeated for two more times. Thus obtained *Gandhaka* was washed with hot water and dried. (Table 3).

Extraction of *Parada:* *Parada* was extracted from *Hingula* by *Nadayantra* method.[11] *Hingula* was levigated with *Nimbu swarasa* and allowed to dry, made in to fine powder. Uniform layers of the powder was made on a cotton cloth (equal to the weight of *Hingula*), tied with thread and ignited. While igniting, an earthen pot was placed inverted to facilitate the collection of *Parada*. Thus obtained *Parada* was collected carefully and filtered through four folded cotton cloth (Table 4).

Preparation of RB1: The method adopted is from *Rasa Tarangini.*[12] *Rajata* foils were cut into small pieces and amalgam was formed with *Parada* in a mortar. Purified *Gandhaka* was added to the amalgam and triturated till formation proper *Kajjali* (lustreless fine black powder). [Figure 1] This was followed by impregnation with *kumari swarasa*[Figure 2] to preparation of *chakrikas* (pellets). [Figure 3] Dried *chakrikas* were placed in *sharava* and *laghu puta* was given. [Figure 4&5][Graph 1] After first *puta, Rajata* was in completely powder form. In subsequent two *putas,* half the amount of *Kajjali* was added, triturated with *kumari swarasa* and *puta* was given. From forth to ninth *puta,* half part of *Gandhaka* was added in place of *Kajjali.* The remaining *putas* were followed without addition of *Kajjali* or *Gandhaka*. Thus 17 *putas* were given to obtain RB1 that is passing all classical parameters.[Figure 6] Cow dung cakes required for each *Laghu puta* were 22-26 weighing 2.5Kg.

Preparation of RB2: The same reference of RB1 is followed for RB2 with slight modification i.e. *Kupipakwa* process was done which was followed by the *Puta* process. *Kajjali* was filled in *kacha kupi,* placed in *Valuka yantra,* subjected to *Kramagni paka* (gradual rise of temperature).[Graph 2] [Figure 7 & 8] Corking was done after the appearance of chief desired characteristics (CDC) i.e. complete cessation of Sulphur fumes, *Suryodaya Lakshana* (appearance of red bottom) and positive copper coin test etc. *Kupi* was removed from *Valuka yantra* after self cooling, removed the external layers carefully, and broken. Partly formed *Rajata Bhasma* [Figure 9] procured from the bottom of *kupi* collected and triturated with *Gandhaka,* levigated with *kumari swarasa,* and pellets were formed. [Figure 10 & 11] *Sharava Samputa* was done and *Laghu puta* was given. In subsequent five *putas*, addition of half part of *Gandhaka* and levigation with *kumari swarasa* was followed. Thus 11 *putas* were given to obtain RB2.[Figure 12]

Physical and Chemical Tests:

Rajata Bhasma samples were analysed for classical parameters like *Rekhapoorna*[13] (*Bhasma* entering creases of finger), *Varitara*[14](floats on water surface), *Nischaandratwa*[15] (devoid of shine), *Niswadu*[16] (no metallic taste), *Apunarbhava*[17] (not able to reproduce in metallic form), *Niruttha*[18] (does not mix with liquefied

silver) etc. and physicochemical tests like pH[19] Value, Ash Value,[20] Acid insoluble ash,[21] Water soluble ash[22], Loss on drying,[23] tests for presence of Total Silver,[24] Total Sulphur.[25]

Observations & Results:

Time taken for heating *Rajata* foils to red hot was 12-18 seconds. Slight hissing sound was heard on quenching. After completion of *Shodhana, Rajata* became soft and malleable. Metallic lustre was slightly diminished. RB1 was found *Rekhapoorna* after fifth *puta* where as *Varitara* was positive from 15th *puta*. *Niswadu* was evident from tenth *puta* where as *Nichandratwa* was observed after 15th *puta*. *Apunabhava* and *Niruttha* tests were negative at 15th & 16th *puta* but found positive after 17th *puta*. (Table 5). In RB2 *Rekhapoorna* and *Varitara* were positive after tenth *puta* whereas *Apunabhava* and *Niruttha* tests were positive after 11th *puta* (Table 6).

The percentage yield of RB1 and RB2 are placed at Table 7 & 8. RB subjected to various physico-chemical parameters (Table 9).

Discussion:

During *Rajata shodhana,* colour changes were observed from white metallic shine to dull blackish due to acidic (*Takra, Kanji* & *Kulattha kwatha*) and alkaline nature (*Gomutra*) of media used during the procedure. Silver foils were tarnished with serrated margins indicating oxidation and corrosion.

Role of media: Media also helps to convert metal in to specific chemical form. If sulphur is used as a media then the *Bhasma* gets converted in to sulphide form.[26] RB1 and RB2 were found to be sulphides of silver.

Phase of pellet formation: In this phase levigated doughy mass is converted in to small pellets. The shape of the pellets are said to resemble *nuxvomica* seeds in context of *Abhraka Bhasma*.[27] Homogeneous heat pattern can be achieved with preparation of pellets of uniform size and shape.

Laghu Puta: There is no specific *puta* mentioned for preparation of *Rajata Bhasma.* Considering the hardness of *Rajata, laghu puta* has been considered as source of heat. Accordingly a square pit of 30 cm in height, width and depth was prepared for *laghu puta*. 22-26 cow dung cakes of 2.5 Kg. were found to be needed for each *puta*. Constant peak temperature (596^{o}C to 625^{o}C) was achieved approximately after 80min.

The addition of medias like *Kajjali*, *Gandhaka* in different *putas* and *Bhavana* with *Kumari swarasa* is followed as per the classical reference.[28] A previous research work also followed the similar pattern.[29] RB2 was prepared in 11putas. Partially formed material at the bottom of the *kupi* was triturated with half part of *Gandhaka* and levigated with *Kumari swarasa, subjected to puta.* The same method was followed for the next five subsequent *putas. Gandhaka* was not added from 6th *puta* onwards. *Kumari swarasa bhavana* was done till 11th *puta*. *Rekhapoorna* and *Varitara* were positive after ten *putas* where as in 11th *puta* other CDC like *Apunarbhava* and *Niruttha* tests were observed. Both RB1 and RB2 were fine powder, black in colour, which fulfilled all the classical parameters set for *Bhasma*. Colour of the *Rajata Bhasma* is because of formation of black sulphide.[30] *Bhasma* fulfilled *Varitara* & *Rekhapoorna* suggesting lightness and particle size reduction, *Apunarbhava* and *Niruttha* tests were positive indicating complete formation of *Bhasma*. Partial conversion of product was evident after each *puta*, whereas at the end of 17th *puta* in RB1 and 11th *puta* in RB2, all the criteria's were fulfilled indicating complete formation of *bhasma*. Here *Rekhapoorna*, *Varitara*, *Nischandrata*, *Niswadu, Apunarbhava* and *Niruttha* tests are indicating physical and chemical stability of final product. In RB1, 22% of weight gain was observed after *puta*, whereas 20.6% gain in RB2. RB2 is pharmaceutically better as it is subjected to less number of *putas* consuming less time. In addition, this procedure can yield *bhasma* along with *Rajata Sindura*, which is an added benefit.

Physico-chemical values of RB1& RB2 were as follows; pH 7.34 & 7.36, suggestive of mild basic nature, ash value was found to be 98.32 & 97.85 major portion of *Bhasma* is inorganic compound. Percentages of Silver in RB1 & RB2 were 81.77 & 80.82 and percentage Sulphur 13.15 & 14.32 respectively, shows the major portion of *Bhasma* may be in sulphide form.

Conclusion:

RB1 was prepared in 17 *putas* whereas for RB2, 11 *puta* were required. Both RB1 and RB2 yielded black coloured *bhasma* containing, Silver in 81.77% & 80.82% and Sulphur 13.15% & 14.32% respectively. RB2 is an easier method and can be prepared in less number of *putas* with similar physico-chemical profiles. As no standard physic-chemical profiles on these *Bhasmas* are reported till date, the current data may be considered in future studies.

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Soure of Support : Nil
Conflict of Intrest : None Declared

Table 1 Equipment specifications

S.No.	Name of the Equipment	Nature	Dimension (cm.)	Capacity
1.	Khalwa	Black Granite	40(l) 24(b) 13(h)	5lit
2.	Pestle	Black Granite	20(l)	-
3.	Pot for melting	Stainless Steel	18(d), 50(c), 16(h)	2lit
4.	Pot for collecting	Stainless Steel	20(d), 54(c), 20(h)	5lit
5.	Saucer	Earthen		-
6.	Laghu puta	Square pit	30(l) 30(b) 30(h)	-

Table 2 Observations made during *Samanya & Vishesha Shodhana* of *Rajata*:

S. No	Name of liquid	Quantity in ml	Initial wt in gm	Final wt in gm	Loss in gm
Samanaya Shadhana					
1.	*Tila taila*	650	650	650	0
2.	*Takra*	650	650	650	0
3.	*Gomutra*	650	650	649.75	0.25
4.	*Kanji*	650	649.75	649.75	0
5.	*Kulattha Kwatha*	650	649.75	649.75	0
Vishesha Shodhana					
6	*Nimbu Swarasa*	650	649.75	649.6	0.15

Table 3 Observation during Gandhaka Shodhana

Initial weight of Sulphur(g)	Yield (g)	Wt. loss (g)	% of wt loss
1000	962	38	3.8%
1000	960	40	4.0%
1000	964	36	3.6%
Average		38	3.8%

Table 4 Extraction of Mercury from Cinnabar

Initial weight of Cinnabar	Duration	Mercury extracted	Yield in %
500 g	6hrs 30min	362 g	72.4%
500 g	6hrs 22min	356 g	71.2%
500 g	6hrs 40min	350 g	70%
500 g	6hrs 46min	342 g	68.4%
500 g	6hrs 32min	370 g	74%
Avg	**6hrs 34min**	**356g**	**71.2%**

Table 5 Results of Chief desired characteristics of RB1(1st to 17th *puta*)

Tests	Before *marana*	No. of *Puta*					
		01	05	10	15	16	17
Colour	Dull Silver	Black Shiny	Black Shiny	Black Shiny	Black	Black	Black
Taste	Metallic	Metallic	Metallic	Tasteless	Tasteless	Tasteless	Tasteless
Appearance	Powder	Powder	Powder	Fine powder	Fine powder	Fine powder	Fine powder
Odour	Sulfur	Sulfur	Faint	Faint	Odourless	Odourless	Odourless
Rekhapo-ornatwa	-ve	-ve	+ve	+ve	+ve	+ve	+ve
Varitaratwa	-ve	-ve	-ve	-ve	+ve	+ve	+ve
Apunar-bhava	-	-	-	-	-ve	-ve	+ve
Nirutha	-	-	-	-	-ve	-ve	+ve

Table 6 Results of Chief desired characteristics of RB2 (1st to 11th *puta*)

Tests	**Before *marana***	**Observations during *Puta***			
		01	**05**	**10**	**11**
Colour	Silvery black	Black	Black	Black	Black
Taste	Metallic	Metallic	Metallic	Tasteless	Tasteless
Appearance	Coarse powder	Powder	Fine Powder	Very Fine Powder	VeryFine Powder
Odour	Faint	Faint	Faint	Faint	Faint
Rekhapoor-natwa	-ve	-ve	-ve	+ve	+ve
Varitaratwa	-ve	-ve	-ve	+ve	+ve
Apunar-bhava	-	-	-	-	+ve
Nirutha	-	-	-	-	+ve

Table 7 Weight changes in RB1

Batch	**Initial weight of Silver**	**Weight after 17 *putas***	**% Wt gain**
Batch 1	100g	125g	25%
Batch 2	100g	122g	22%
Batch 3	100g	119g	19%
Avg		122g	22%

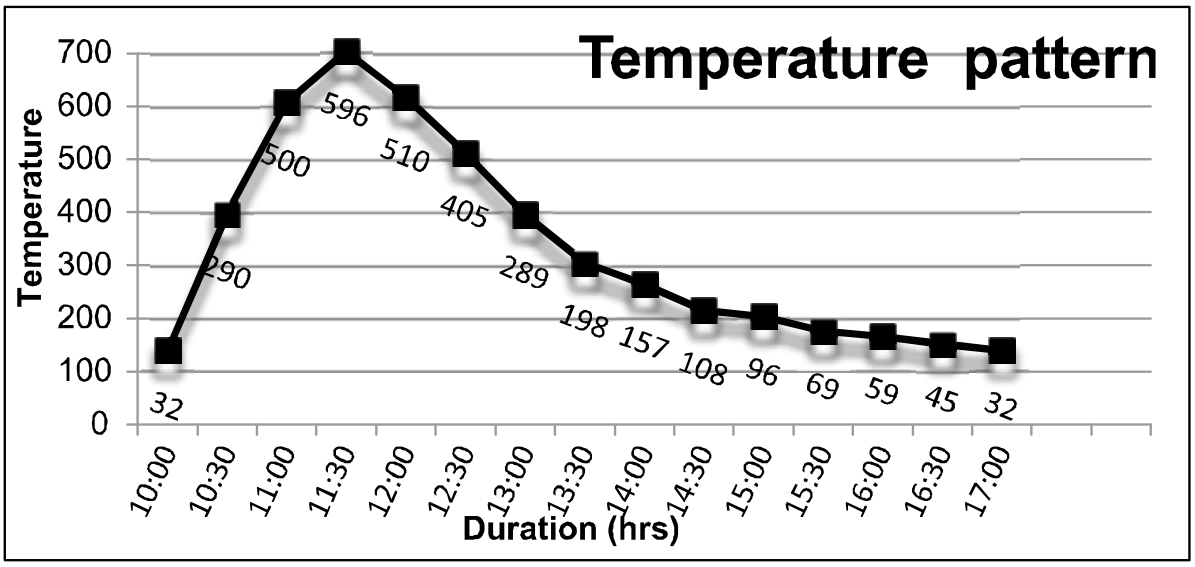
Table 8 Weight changes in RB2

Batch	**Initial weight of Silver**	**Weight after 11 *putas***	**% Wt gain**
Batch 1	100gm	120gm	20%
Batch 2	100gm	12gm	24%
Batch 3	100gm	118gm	19%
Avg		120.6gm	20.6%

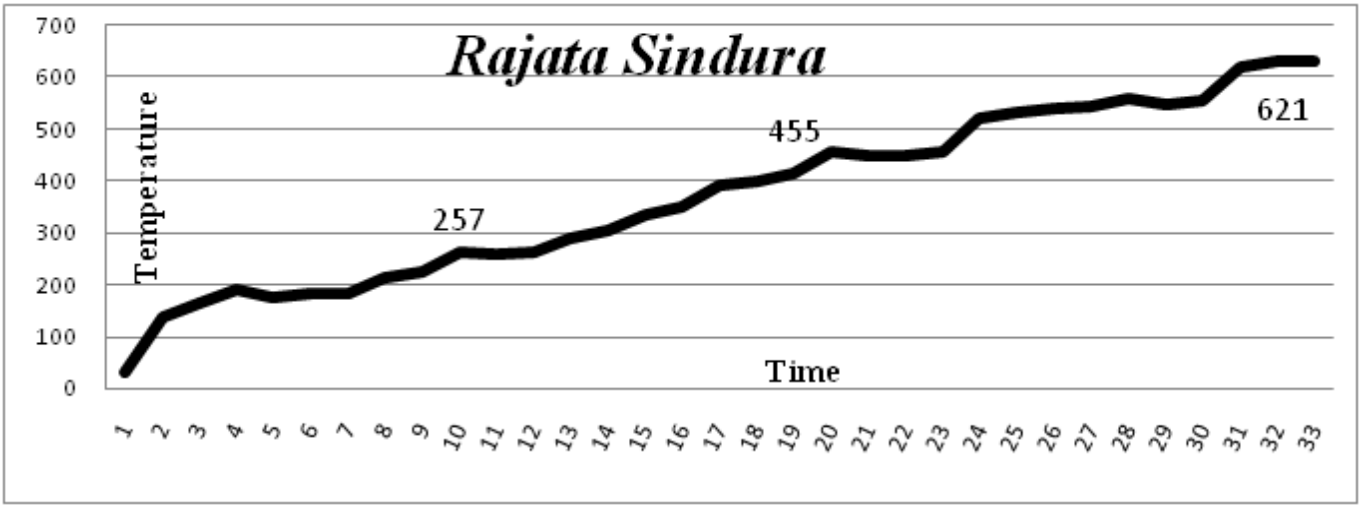
Table 9 Physico-chemical characteristics of RB1 & RB2

Contents	P^{H} Value	Ash Value	Acid insoluble ash	Water soluble ash	Loss on drying at 110^{0}C	%Ag	%S
R B1	7.34	98.32	9.32	0.10	0.54	81.77	13.15
RB2	7.36	97.85	10.30	0.12	0.62	80.82	14.32

Graph 1 Temperature of *Laghu puta* for RB



Graph 2 Temperature graph of RS preparation



Rajata Bhasma by Puta Method

Fig.1: Mixing Parada, Rajata and Gandhaka

Fig.2: Bhavana

Fig.3: Chakrika

Fig.4: Sharava in Laguputa

Fig.5: Igniting puta with cow dung

Fig.6: Rajata Bhasma

Rajata Bhasma Prepared by Kupi followed by puta

Fig.7: Bhatti

Fig.8: Kupi in Valuka Yantra

Fig.9:Rajata after Kupipaka

Fig.10: Bhavana

Fig.11: Chakrika

Fig.12: Rajata Bhasma

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Pharmaceutical standardization of *Samaguna Bali Jarita Rasasindura* prepared by conventional and modified method

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ABSTRACT:

Introduction: *Rasasindura* (RS) is one of the unique mercurial preparations commonly used in *Ayurveda* therapeutics. The traditional procedure followed for preparing RS in the classical furnace has several drawbacks, such as difficulty in controlling temperature, consumption of large quantity of fuel, and excess human effort.

Aims & Objective: The present study was conducted to evaluate an alternate method to prepare RS by using an electrical muffle furnace (EMF) and to standardize this method.

Materials and Methods: RS was prepared by adapting similar temperature pattern in both the traditional and EMF methods, and observations were recorded. Obtained product was subjected to orgennoleptic and physico-chemical analysis.

Result: No remarkable differences were seen in observations of both preparations, except the melting of *Kajjali*, appearance of flame, and appearance of desired characteristics of product formation, which were observed at 190, 496, and 567°C in the traditional method and at 180, 473, and 560°C in EMF, respectively. Yield of the traditional method was 53.5% whereas in EMF, it was 50.83%. Organoleptic and physicochemical analysis revealed no significant deviation in values.

Conclusion: The present study reveals that the traditional method of preparing RS can be effectively replaced by the EMF with some added advantages.

Key words: Electrical muffle furnace, *Rasasindura*, traditional furnace

INTRODUCTION

Rasashastra, the pharmaceutical science, deals mainly with the processing and therapeutic utilization of mercury, metals, and minerals. It aims at designing novel drugs with better curative attributes at minimum doses.[1] *Kupipakwa rasayana*[2] is a unique pharmaceutical preparation wherein the drug is prepared in a glass bottle called *kupi* and the processing is done in a traditional furnace with a gradual rise in temperature. *Rasasindura* (RS), most commonly used in *Kupipakwa Rasayana* is a sublime product of a mixture of mercury and sulfur, and due to its wide therapeutic utility, it is used in several formulations. The therapeutic repertory ranges from treating diseases like diabetes mellitus, indigestion, fever, urinary tract infection, and respiratory infections to alleviating aging as an aphrodisiac and rejuvenator.[3] The traditional furnace used to manufacture RS is known as *bhatti* and fuel used is either hard or soft coal. Difficulty in controlling the temperature, large amount of fuel consumption, exposure of the personnel to heat, and air pollution are the drawbacks in the traditional method. With advancement in pharmaceutics, replacement of the fuel and instrument by more efficient instruments such as the electric muffle furnace (EMF) may be considered, which has benefits like easy handling, temperature control, control of air pollution, and reduction in human effort. The above points can prove the edge of EMF over the traditional method, but the real facts can only be revealed by a detailed comparative study. Previously, a study had been carried out on the standard manufacturing process of RS on a large scale in the traditional method.[4] However, they did not replicate the same on EMF. Therefore, a comparative study was conducted in both the traditional furnace with coal as fuel and vertical EMF to evaluate the gradation of temperature and total duration of *paka*, and variation in the processes and yield; a comparative pharmaceutical standardization of the same was carried out.

MATERIALS AND METHODS

Collection of raw materials

Ashuddha Hingula (raw cinnabar), *Ashuddha Gandhaka* (raw

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sulfur), and cow's ghee were procured from the Pharmacy, Gujarat Ayurved University, Jamnagar. *Nimbu* (*Citrus medica* Linn.) was purchased from the local market in Jamnagar. *Vatajata (Ficus benghalensis)* was collected from the botanical garden of the Gujarat Ayurved University, Jamnagar.

Materials

Parada (mercury) extracted from cinnabar,[5] *Shodhana* of *Gandhaka*,[6] and preparation of *Kajjali*[7] was carried out as per classical references [Figures 1a-c]. *Kupi* (glass bottle coated with 7 layers of mud-smeared cloth, capacity: 750 mL), *Bhatti* [Figure 2a] (diameter: 5 cm, height: 85 cm, upper hole: 18×18 cm^2 for filling coal, lower hole: 20×20 cm^2 for collection of ash and connecting blower), *Valuka yantra* (iron vessel filled with sand for placing the *kupi*) [Figure 2b] (diameter inner: 20 cm, brim: 30 cm, height: 30 cm, depth: 27 cm, thickness: 03 cm), *Valuka* (sand with mesh, size: 10), charcoal, coal, and EMF- [Figures 3a and 3b] inner hearth (length: 15 cm, breadth: 15 cm, depth: 30 cm, max. temp. capacity: 1000°C) were collected as per the classical references and requirement.

Method

The preparation of RS is divided into three stages, namely, preoperative (*Purvakarma*), operative (*Prdhanakarma*), and postoperative (*Paschatkarma*) stages. RS was subjected to various organoleptic and physicochemical analyses like texture, color, taste, odor, pH, ash value, acid-insoluble ash, water-soluble ash, loss on drying, and percentages of mercury, free mercury, sulfur, and free sulfur.

Figure 1: *Rasasindura* pictures: Preparation of *Kajjali*. (a) Shuddha *Parada*, (b) *Shuddha Gandhaka*, (c) *Kajjali*

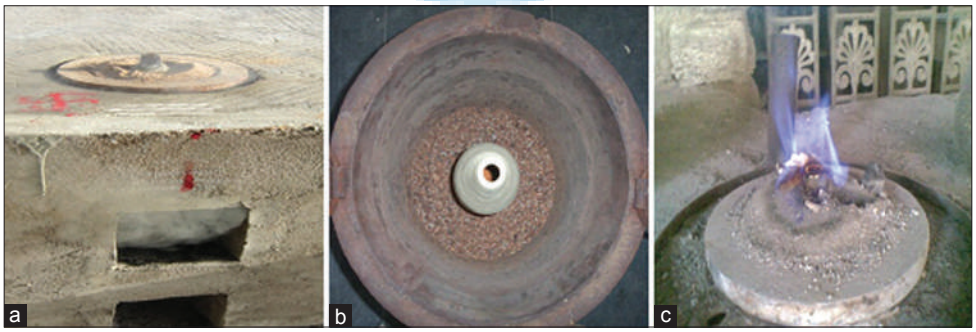

Figure 2: *Rasasindura* pictures: Preparation of RS by traditional furnace. (a) *Bhatti*, (b) Placing *Kupi*, (c) *Flame*

Figure 3: *Rasasindura* pictures: Preparation of RS in EMF. (a) EMF, (b) Placing Kupi, (c) Flame

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Purvakarma

Extracted mercury and purified sulfur were taken in an appropriate ratio [Tables 1 and 2] and triturated in an iron mortar till the whole mixture was converted into a fine black, lustreless powder (*Kajjali*). Then this mixture was levigated three times with *Vatajata swarasa (*juice of *F. benghalensis)*. Four hundred grams of *Kajjali* was filled which was upto one-third of the *Kupi* and was placed on a traditional furnace in such a way that the neck of the *kupi* remained just outside the sand, and in EMF, the *Kupi* was placed in the center in such a way that the *kupi* could receive equal distribution of heat.

Pradhankarma

Preparation of RS by the traditional furnace[8] was begun by igniting charcoal in a controlled intermittent manner and gradually increasing the temperature. Initially, 4 kg of coal and 2 kg of charcoal were used and at definite intervals, a fixed amount of fuel was added for maintenance and increment of temperature. A pyrometer was inserted into the sand of the *Valuka Yantra* employed to record the temperature of the furnace at regular intervals. A blower was also utilized to facilitate the increase in temperature; initially, three hours of mild (120–250°C) and moderate temperature (250–450°C), and lastly, an intense fire at a temperature of 450–600°C were used [Table 3]. A red-hot iron rod was repeatedly inserted in the neck of the bottle so as to burn any accumulated sulfur there. After observation of the confirmative test, the mouth of the *Kupi* was corked and the temperature was increased to around 50°C to facilitate sublimation of the final product inside the neck of the *Kupi*. This temperature was maintained for a minimum of two hours. After adequate cooling, the bottle was removed and thus RS was prepared in three batches of 400 g of *Kajjali* in each.

In the preparation of RS by EMF,[8] the temperature pattern standardized by the traditional method was followed and the pattern of gradual rise in temperature was also used. Observations were recorded, and in the same way, two more batches were prepared to ensure a standard manufacturing process.

Paschatkarma

On the next day, after self-cooling, the bottle was carefully scraped, broken, [Figure 4a] and the product deposited at the neck [Figure 4b] was collected and weighed.

OBSERVATION AND RESULTS

Different phases of the desired characteristics during the process were observed, namely, sulfur fuming [Figure 5a], melting and boiling of *Kajjali*, flame, and confirmative test (flame disappearance, *Shita shalaka* test, red-hot appearance of the bottom) [Figure 5b], and copper coin test [Figure 5c] were observed and recorded in both the traditional and EMF method [Tables 4 and 5] [Graph 1].

RS, collected from the neck of the *Kupi* from all the batches by both the methods were weighed and calculated for the percentage of absolute and relative yield and tabulated [Table 6] and also subjected to various organoleptic [Table 7] and physicochemical parameters [Table 8].

Table 1: Ingredients and observations during preparation of *Kajjali*

Ingredient	Batch 1	Batch 2	Batch 3
Purified mercury	200 g	200 g	200 g
Purified sulfur	200 g	200 g	200 g
Wt. of *Kajjali*	395 g	389 g	393 g
Vatajata Swarasa (juice of *Ficus benghalensis*)	70 mL	75 mL	70 mL
Wt. after *Bhavana*	402 g	406 g	403 g

Wt: Weight

Table 2: Ingredients and observations during preparation of *Kajjali*

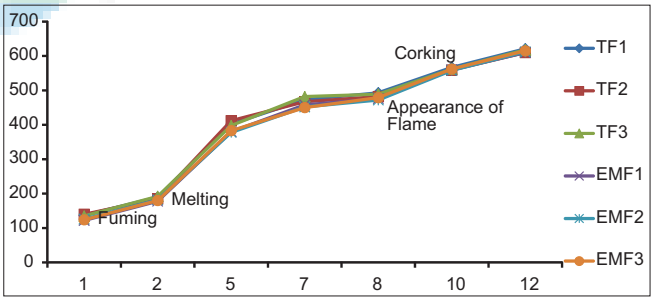
Ingredient	Batch 4	Batch 5	Batch 6
Purified mercury	200 g	200 g	200 g
Purified sulfur	200 g	200 g	200 g
Wt of *Kajjali*	391 g	390 g	394 g
Vatajata Swarasa (juice of *Ficus benghalensis*)	70 mL	75 mL	70 mL
Wt. after *Bhavana*	405 g	403 g	401 g

Wt: Weight

Table 3: Temperature gradation and time duration

Grade	Traditional and EMF (hr)
Mild (120–250°C)	3
Moderate (250–450°C)	3
Intense (450–600°C)	6
Total	12

EMF: Electrical muffle furnace; hr: Hour



Graph 1: Temperature pattern of traditional furnace and electrical muffle furnace (EMF)

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DISCUSSION

In the traditional method, after first three hours of mild temperature, light white fumes were observed initially that gradually thickened. On probing with an iron spoke, it was observed that the *Kajjali* melted at around 190°C, whereas the same stage was observed at 180°C in the EMF method. During moderate temperature, dense yellow fumes were observed and the bottom of the *kupi* was not seen. In later stages, the bottom appeared maroon in color as seen using a torch. On gradually increasing the temperature, the *Kajjali* started to boil and frequent deposition of sulfur was observed at the neck of the *Kupi*. Dense fumes were

Table 4: Observations at specific time and temperature in traditional furnace

Time (hr)	Observations	Temp (°C) in CF1	Temp (°C) in CF2	Temp (°C) in CF3
1	White fumes observed	137°C	140°C	135°C
2	*Kajjali* melted	190°C	185°C	192°C
5	A thick yellow fume; bottom of the *Kupi* not seen	402°C	412°C	398°C
6	Maroon red color liquid at the bottom of the *Kupi* visible. *Kajjali* started boiling	452°C	447°C	457°C
7	Dense fumes followed by flame at the neck of the *Kupi*.	473°C	467°C	482°C
8	Hot *Shalaka* inserted, flame risen about 6–8 inches.	493°C	482°C	489°C
10	Flame at the neck of the bottle stopped. Copper coin test was +ve. Bottom of *kupi* appeared red; corking was done	567°C	559°C	562°C
12	Heating was stopped	621°C	610°C	618°C

hr: Hour

Table 5: Observations at specific time and temperature in three batches of EMF

Time (hr)	Observations	Temp (°C) in EMF1	Temp (°C) in EMF2	Temp (°C) in EMF3
1	White fumes observed	122°C	126°C	124°C
2	*Kajjali* melted	178°C	182°C	180°C
5	A thick yellow fume; bottom of the *Kupi* not seen	380°C	378°C	383°C
6	Maroon red color liquid at the bottom of the *Kupi* visible. *Kajjali* started boiling	440°C	442°C	438°C
7	Dense fumes followed by flame at the neck of the *Kupi*	458°C	452°C	450°C
8	Hot *Shalaka* inserted, flame risen about 4–6 inches	476°C	472°C	479°C
10	Flame at the neck of the bottle stopped. Copper coin test was +ve. Bottom of *kupi* seen red; corking was done	560°C	558°C	562°C
12	Heating was stopped	610°C	612°C	615°C

EMF: Electrical muffle furnace; hr: Hour

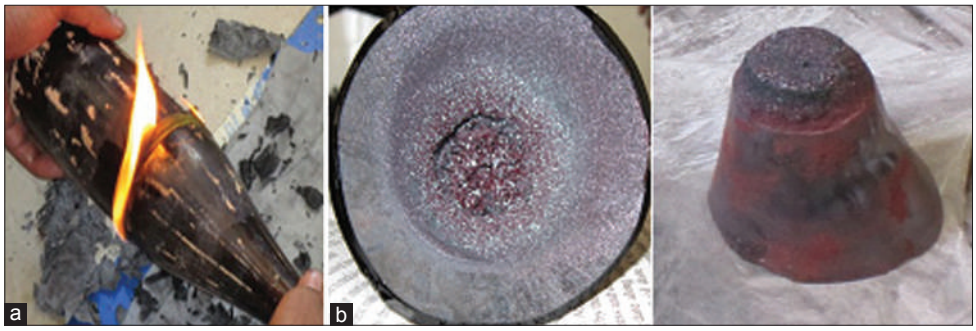

Figure 4: *Rasasindura* pictures-2: Breaking of Kupi and product. (a) Breaking of Kupi, (b) Product.

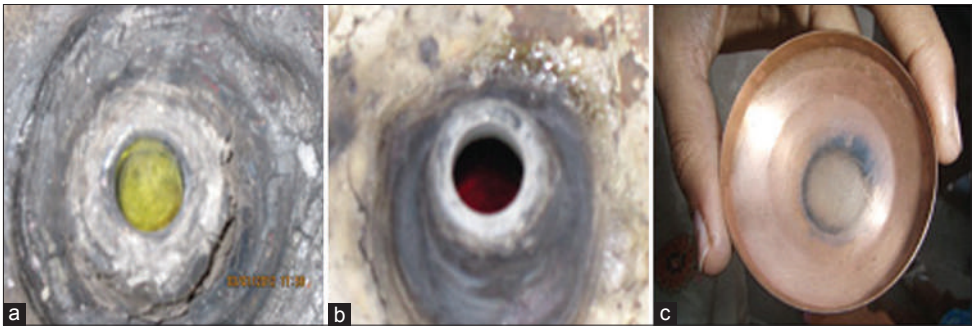

Figure 5: *Rasasindura* pictures-2: Different stages of product formation. (a) Sulfur fumes, (b) Red hot appearance of bottom (c) copper coin test.

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Table 6: Weight and yield (%) of traditional and EMF methods

Wt of Kajjali	Wt of product	Yield %	Yield % (as per law of definite proportion)
Traditional method			
400	212	53	90.98
400	210	52.5	90.12
400	220	55	94.42
Avg	214	53.5	91.84
Electrical muffle furnace			
400	197	49.25	84.54
400	203	50.75	87.12
400	210	52.5	90.12
Avg	203.33	50.83	87.26

Wt: Weight; avg: Average

Table 7: Results of organoleptic tests

Parameters	RS prepared in traditional furnace	RS prepared in EMF
Texture	Compact	Compact
Color (after trituration)	Vermillion	Vermillion
Taste	Tasteless	Tasteless
Smell	Indistinct	Indistinct

RS: Rasasindura; EMF: Electrical muffle furnace

Table 8: Results of physicochemical tests

Tests	RS (*bhatti*)	RS (EMF)
pH value	6.20	6.39
Ash value	0.01%	0.11%
Acid-insoluble ash	Nil	0.09%
Water-soluble ash	Nil	0.01%
Loss on drying	0.05%	0.39%
% mercury	82.48%	83.25%
% free mercury	Nil	Nil
% sulfur	13.12%	13.52%
% free sulfur	Nil	Nil

observed in the traditional and EMF methods at a range of 467–482°C and 450–458°C, respectively followed by flame. With the increase in temperature, the height of the flame increased by 6–8 inches [Figure 2c] and 4–6 inches [Figure 3c] in the traditional and EMF methods, respectively. This variation of height of the flame in the traditional method may be due to environmental factors like open air circulation. Duration of the flame was for two hours 10 minutes and two hours 24 minutes on an average in the traditional and EMF methods, respectively. Confirmatory tests like disappearance of flame, *Sheeta Shalaka* test (dry appearance of iron spoke inserted into the *kupi* and taken out), copper coin test (completion of product formation confirmed by placing a copper coin which is determined by color changes on it), appearance of red-hot bottom and movement of the product in the *kupi* were observed at around 559–567°C and 558–562°C, respectively, in the traditional and EMF methods. Disappearance of flame and *Shita Shalaka* test (+ve) during the process indicated no free sulfur available inside the *Kupi*. Reddish color at the bottom of the *Kupi* indicated that material was converted into compound form. The copper coin test (+ve) is also one parameter of free sulfur test.

Here, the not able point is that the observations in EMF were seen slightly earlier with respect to temperature than the traditional method, which may be due to installation, designing, and operational differences in the instruments. The heating pattern of the both methods differ, as in the traditional method, indirect heating process is adopted whereas in the mechanized method, a direct heating process is used. In the traditional method, the heat reaching the bottle is through *Valuka* (sand) which maintains a steady temperature throughout the procedure. Here in the traditional furnace, the difference in temperature gradient from the bottom (relatively more hot) to the neck (relatively cool) of the bottle was observed, whereas in EMF, the electric coils heat the bottle directly and distribute the temperature equally throughout the bottle.

The time required to complete the procedure was 12 hours in both the traditional and EMF methods. Although *Kramagni*[9] is mentioned in the classics which is interpreted as intermittent gradual rise of temperature and equal amount of heat at all three stages of heating, mild and moderate stages are for three hours each and increased heat for six hours to facilitate enough time for digestion of sulfur and to avoid delaying of the procedure. Practically, moderate and intense stages are more essential in the heating process, as in this stage, maximum chemical reaction and compound formation take place.

Average yield in the traditional method was 53.5%, whereas it was 50.83% in EMF. Removal of sand around the neck of the *kupi* in the traditional furnace facilitates deposition of the sublimated product by providing a cooling surface, whereas in the case of EMF, there is equal distribution of temperature from bottom to neck, which may result in dissociation of some product at the neck at high temperatures (beyond sublimation), if not controlled appropriately. This may be one of the reasons for relatively less yield by the EMF method.

In the traditional furnace where the temperature is maintained manually and is dependent on the amount of coal used, it is very difficult to maintain the desired temperature, whereas in EMF, the system supports maintaining the temperature through automated control; the desired temperature once set will be maintained till

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further operation. This precision in temperature ensures the proper formation of the product.

The amount of coal required is 5 kg of charcoal and 15 kg of hard coal (15 Rs/kg); so, the cost is about Rs 300/procedure as per existing rates. Moreover, in the traditional furnace, the energy required is more, as loss of heat due to radiation walls, sand, and iron vessel will add to greater consumption of fuel. The usage of electricity is 1.8 units/hour × 12 hours at 8 Rs/unit; hence, total cost is around Rs 172.8/procedure. So, EMF, along with other advantages over the traditional furnace, is also cost effective.

Heat radiating through the traditional furnace during moderate and intense stage is very high, which will harm the personnel if exposed for a long duration of time.[10] This problem in EMF is solved through an insulating layer, which prevents escape of heat. Working with such a modified method not only serves for better fuel efficiency and a pollution-free process, but also reduces human labor and exposure to heat.

In the traditional method, all the observations and stages are clearly distinguished, whereas it is not so in EMF. For yield and observation, the traditional method has an edge over EMF, but in terms of fuel, human effort, and pollution, EMF is a better option.

However, the few drawbacks in EMF like even distribution of temperature and not sufficient cooling surface over the neck of the bottle for collection of the product can be solved by modification of the technical design.

Results of the organoleptic studies of RS were similar [Table 7], whereas physicochemical studies [Table 8] showed a slight deviation in the values which is not significant enough to comment. However, advanced analytical studies maybe carried out to detect minute differences. Clinical study of RS prepared by both the methods can be studied comparatively to ascertain efficacy.

CONCLUSION

RS can be prepared in 12 hours by adopting a pattern of three hours of mild and moderate and six hours of intense temperature in both traditional and EMF methods. The traditional method is better in terms of yield, but EMF has advantages such as better maintenance of temperature, saving of energy, cost effectiveness, less human effort, and it is environment friendly. By comparing yield, and organoleptic and physicochemical studies, it is clear that the traditional method can be effectively replaced by EMF.

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ORIGINAL ARTICLE

Physicochemical characterization of Ayurvedic *bhasma* (*Swarna makshika bhasma*): An approach to standardization

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ABSTRACT

Swarna makshika [SM], a mineral having various therapeutic uses, has been used since long in Ayurveda . The present study was conducted to generate a fingerprint for raw and processed SM using techniques which can be used by pharmacies. Powdered SM was heated in an iron pan by adding lemon juice for 3 days, till liberation of sulfur fumes stopped. *Bhasma* of this *shuddha* SM was obtained by triturating it with *shuddha gandhaka* and lemon juice. It was then subjected to heat in 09* *putas,* and for firing in each *puta,* 4 kg cow dung cakes were used. To assure the quality of *bhasma, rasa shastra* quality control tests like *nischandratva*, *varitara, amla pariksha,* etc., were used. After the *bhasma* complied with these tests, the *bhasma* was analyzed using X-ray Diffraction (XRD) analysis of raw SM and SM *bhasma* revealed that raw SM contains $CuFeS_2$, and SM *bhasma* contains Fe_2O_3, FeS_2, CuS and SiO_2. Scanning Electron Microscope (SEM) studies showed that the grains in SM *bhasma* were uniformly arranged in agglomerates of size 1-2 microns as compared to the raw SM which showed a scattered arrangement of grains of size 6-8 microns. It may be concluded that raw SM is a complex compound which gets converted into a mixture of simple compounds having very small particle size after the particular process of marana. This is the first report of fingerprinting of SM bhasma prepared using this particular method.

Key words: Scanning electron microscope, *Swarna makshika bhasma*, X-ray diffraction

INTRODUCTION

Swarna makshika [SM] *bhasma* has been used for *pandu* (anemia), *anidra* (insomnia), *apasmara* (convulsions), *mandagni (poor digestion), kustha* (skin diseases),[1] etc., as well as a potent *rasayana*[2] drug. Generally, SM *bhasma* is prepared in two steps: *shodhana,* by different techniques like fomentation, heating and quenching and roasting, etc.; and *marana,* by *puta* system of heating in different types of *putas,*[3] like *varahaputa, kukkutaputa, gajaputa,* etc.; and *kupipakwa* procedures,[4] etc. During *marana, bhavana* with lemon juice, *kulottha* decoction, *eranda taila, snuhi ksheera,*[5] etc., are given with addition of *shuddha gandhaka* and *shuddha hingula,*[6] etc., as associated materials.

SM *bhasma* is used as a single constituent formulation or in multi-ingredient formulation. However, there is variation in collection of raw materials and the pharmaceutical procedure followed, which generates the same *bhasma* with different characters. As a result, reproducibility is often not achieved. In many cases, wrong manufacturing and marketing practice leads to the production of inferior quality products, which reduces efficacy or produces safety concerns. In order to minimize variability and to check adulteration, standardization of a *bhasma* is a must.

Ayurvedic texts have described methods for quality control of finished products through different parameters like *nischasndratva, varitara, nirutha*, *apunarbhava,* etc., to achieve a specific acceptable standard *bhasma*. This study was performed to characterize the *bhasma* using sensitive tools and techniques . These fingerprints generated for the raw material and *bhasma* could be used as standards to for ensuring quality and reproducibility of standards of the medicines.

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MATERIALS AND METHODS

Swarna makshika processing

This was done using standard procedures and included two steps, namely *Shodhana* and *marana* of *swarna makshika*.[7]

The *Swarna makshika* was procured from the pharmacy (I.M.S., BHU) and lemon juice (sufficient quantity) was obtained from the market

Iron mortar and pestle, charcoal furnace, iron pan, iron ladle, pyrometer, etc.

At first the *swarna makshika* was powdered in an iron mortar with an iron pestle. A clean and dry iron pan was then heated on a charcoal furnace onto which was poured the powdered *swarna makshika* and subjected to intense heat with frequent addition of lemon juice till the liberation of sulfur fume stopped and it turned red. The process was completed in 3 days and the final product called *Shodhita swarna makshika* obtained.

For the *Marana* of *swarna makshika*[7] *Shuddha gandhaka* and lemon juice (q.s.) were procured. Equal amounts of *shuddha swarna makshika* and *shuddha gandhaka* were triturated with lemon juice till a homogenous paste was formed. After triturating, small pellets of uniform size and thickness were prepared and dried in sunlight. Pellets were kept inside a *sarava* (shallow earthen disc) and another *sarava* was inverted over it. The joint between the two discs was sealed with a rag and mud*/kapad mitti:* a ribbon of fine cloth uniformly smeared with fuller's earth seven times and dried in sunlight.

The properly sealed and dried *samputa* was subjected to *puta* system of heating with 4 kg cow dung cake. The process was repeated using *shuddha gandhaka* in equal proportion to *swarna makshika* for the first cycle and then in half the proportion for subsequent 8 cycles. *Bhasma* of the desired quality was obtained in 9 *putas.* The *bhasma* obtained from the above process was taken for analysis.

Analysis using parameters described in Ayurveda texts

The final *bhasma* was analyzed for quality control as described in Ayurvedic texts as follows and found suitable:

1. *Nischandratva:* The *bhasma* was taken in a Petri dish and observed for any luster in daylight through magnifying glass. No luster was observed in the *bhasma.*
2. *Rekhapurnatvam:* A pinch of *bhasma* was taken in between the thumb and index finger and rubbed. It was observed that the *bhasma* entered into the lines of the finger, and was not easily washed out from the cleavage of the lines.
3. *Varitaratavam:* A small amount of the prepared *bhasma* was sprinkled over the still water in a beaker. It was found that the *bhasma* particles floated over the surface of the water.
4. *Nisvadutvam:* The prepared *bhasma* was found to be tasteless when a small amount was kept on the tongue.
5. *Amla pariksha:* A pinch of prepared *bhasma* was mixed with a little amount of *dadhi* (curds) in a clean and dry Petri dish and observed for any color change. No color change of *dadhi* was observed. The same procedure was followed with lemon juice taken in a test tube, and the same result was observed.
6. *Avami:* Ingestion of 5-10 mg of the *bhasma* did not produce any nausea/ vomiting.

Analysis using modern parameters

The *bhasma* as well as the starting material (raw *swarna makshik*) was also analysed using the following techniques:

1. X-ray diffraction (qualitative)
2. Scanning electron microscopy (qualitative)

X-ray diffraction study[8]

X-ray diffraction studies were performed in the Regional Research Laboratory (CSIR), Bhubaneswar.

The powdered sample was spread onto a double-side tape with a spatula, which was then placed on an aluminum sample holder. All the peaks were recorded on the chart, and the corresponding 2 theta values were calculated. Results are summarized in Figures 1 and 2 as well asTables 1 and 2.

The strongest peak identified in the raw material was $CuFeS_2$ while that in the final product was Ferrous oxide of Iron (Fe_2O_3). In the raw material, only one phase of Copper iron Sulphide ($CuFeS_2$) was identified while in the final product, different phases were identified including Fe_2O_3, FeS_2, Copper sulphide (CuS) and SiO_2.

Study using scanning electron microscope[9]

Scanning Electron Microscopy[9] (SEM) was performed in the Dept. of Physics, BHU.

Table 1: X-ray diffraction of the raw *Swarna makshika*

Compounds	d' value	R. Intensity
$CuFeS_2$	3.03	100
	2.64	4
	1.85	21.3
	1.59	12
	1.57	6

Table 2: X-ray diffraction of the *bhasma*

Compounds	d' value	R. intensity
Fe_2O_3	2.51	100
	2.95	32.4
FeS_2	2.69	82. 2
	1.69	24.3
CuS	3.03	50.6
	3.16	26.6
	1.45	12.0
SiO_2	3.67	31.9
	1.60	8.9

N.B.: 'd' value-'d' space; R. Intensity- Relative intensity

The mounted sample was placed inside the microscope's vacuum column through an airtight door, and then the air was pumped out. After the air was pumped out of the column, a beam of electrons was emitted by an electron gun from the top. This beam travels downward through a series of magnetic lenses designed to focus the electrons to a very fine spot. Near the bottom, a set of scanning coils made the focused beam to move back and forth across the mounted sample, row by row.

As the electron beam hits each spot on the sample, secondary electrons are backscattered from its surface. A detector counts these electrons and sends the signals to an amplifier. The final image was built up from the number of electrons emitted from each spot on the sample [Figures 3 and 4].

The particles in the raw material and in the sample of intermediary process were not uniformly arranged while in the final product the particles uniformly arranged.In the final

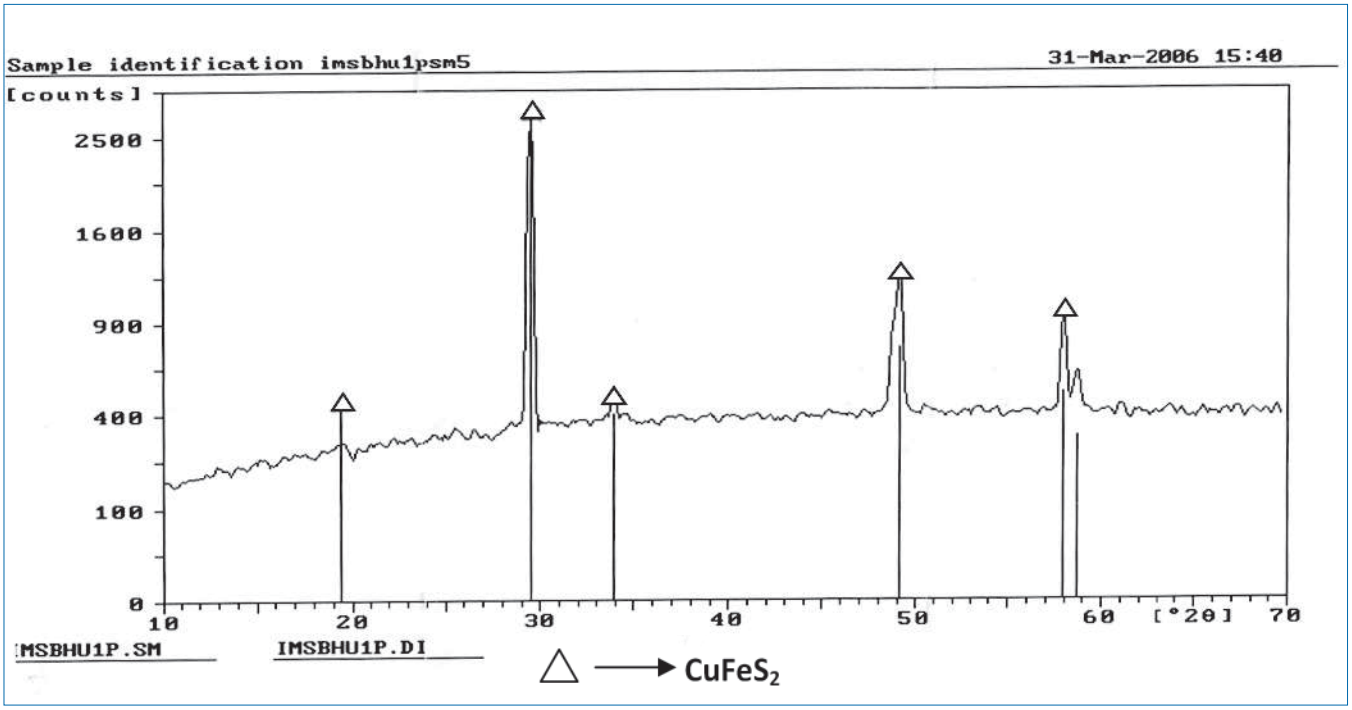

Figure 1: X-ray diffraction of the raw *Swarna makshika*

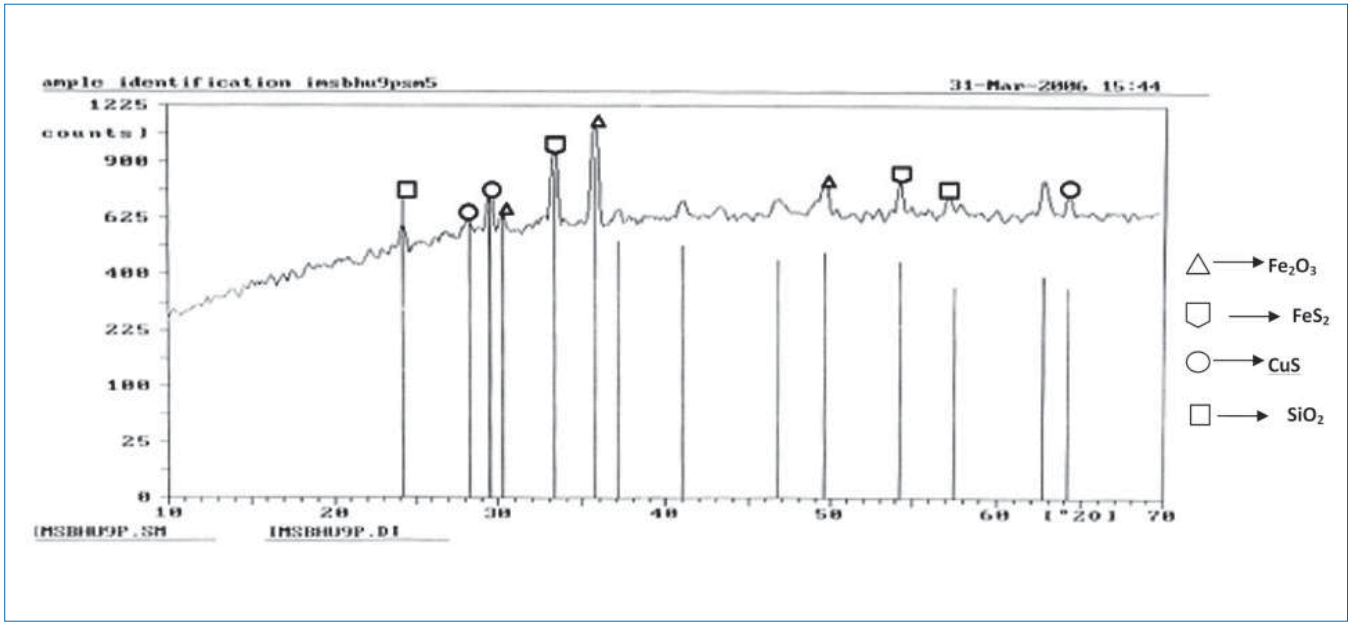

Figure 2: X-ray diffraction of the *Swarna makshika bhasma*

product, more agglomerates of grain were observed, whereas in the raw material the grains were found scattered. The size of the particles was less in the final product.

The particle size of the raw material was between 6 and 8 μ while that for the *bhasma* particles was 1-2 μ.

Discussion

It is noteworthy that there are very specific pharmaceutical procedures and techniques described in the *rasa shastra* literature which convert the toxic metals/ minerals into a suitable dosage form. The *bhasmas* prepared are well tolerated both for short-term and long-term use; moreover, it is claimed that their prolonged administration is required to achieve the rejuvenation effect.[10] According to the need of time, characterization of *bhasma* using scientific techniques is necessary to determine the effect of the process and to judge its safety and efficacy. SM *bhasma* was prepared and studied with this objective. X-ray diffraction study of the raw material showed a sharp peak, indicating its crystalline nature; whereas the final product did not give sharp peaks, indicating the loss of crystalline nature. This is suggested by the test described in Ayurveda namely, as loss of luster *(nischandrika)* in the final product. Thus the sharp crystalline structure of the raw material reflects light rays whereas loss of crystalline nature in the final product prevents it from doing so.Hence the "loss of luster" *(nischandrika)*described in Ayurveda as a quality to be looked for in the final product.

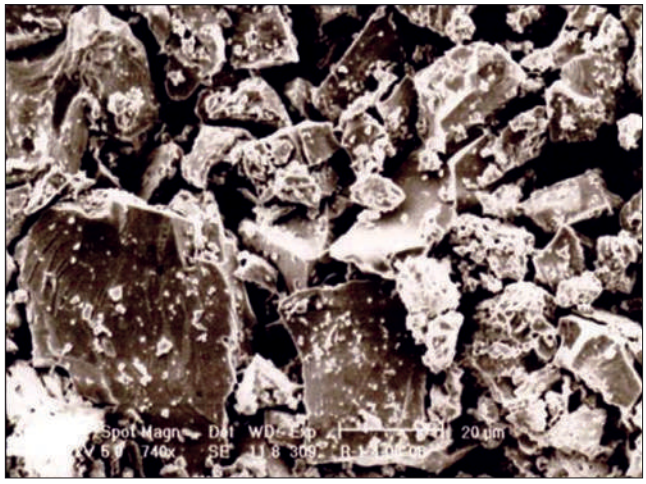

Figure 3: Scanning electron microscopy feature of raw *Swarna makshika*

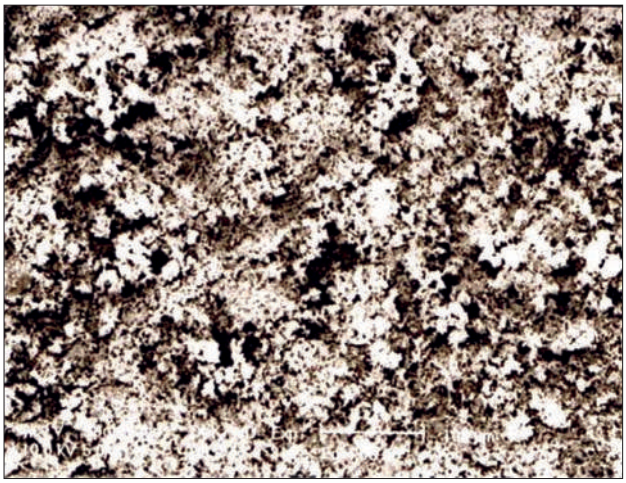

Figure 4: Scanning electron microscopy feature of *Swarna makshika bhasma*

The study also revealed peaks of $CuFeS_2$ in the raw material and Fe_2O_3, Iron sulphide (FeS_2), CuS and silicon oxide (SiO_2) in the final product. The formation of some different compounds in the final product may be due to oxidation and reduction reaction of Cu, Fe with sulfur in the presence of oxygen. It is likely that the lack of change of colour in an acidic medium (*amla pariksha*) in the final product is due to the absence of free metallic groups as free copper reacts with lemon juice to give a blue color. Sulfides and oxides of iron and copper present in *makshika bhasma* do not show any unwanted effect in experimental study,[11] and the *bhasma* has been used over a long period of time in clinical practice and no toxic effect has been recorded so far. Presence of SiO_2 may be due to the use of earthen casseroles, which may have a reaction with oxygen. Scanning electron microscopy study showed that the particle size reduced from 6-8 μ to 1-2 μ after *bhasma* process. It is this significant reduction of size and that allows the phenomenon of *rekhapurna* and *varitara* to develop. Reduction in particle size facilitates absorption and assimilation of the *bhasma* in the system. Again the clusters of particles are regular and uniform in the final *bhasma* in comparison to the raw material. The particle size recorded can be characterized as the desired specification of the final *bhasma.*

Conclusion

Thus, *Swarna makshika* which contains iron (Fe), Copper (Cu) and sulfur The manufacturing process plays a specific role to convert the $CuFeS_2$ in the raw material mixture of Fe_2O_3, FeS_2, CuS and SiO_2, in the final product. These could be important chemical markers for SM *bhasma* prepared using this particular method. As a result of different stages of processing techniques like *shodhana* (which involves roasting, with addition of herbal juices and continuous stirring) and *marana* [which involves *bhavana* (wet trituration) and *puta* system of heating], the particle size reduces significantly, which may facilitate absorption and assimilation of the drug into the body system. The particle size in the final *bhasma* was 1-2 μ, which could be specified as the criterion for the final product conforming to all the traditional parameters under *bhasma pariksha* (examination of properly prepared *bhasma*).

This can be one of the important factors for standardization of *bhasmas*.Thus,modern techniques can assist in proper characterization of *Ayurvedic* dosage forms and standardization of *Ayurvedic* medicines.

Glossary of Important Terms used in this Article

Tests of *bhasma*

Varitara- The *bhasma* that floats on water is termed as *varitara.*
Apunarbhava- Bhasma when mixed with *mitrapanchaka* and heated at high temperature should not undergo any change in its physical properties. The *bhasma* should not regain its original state.
Niruttha- Bhasma is heated at high temperature in a *koshthi* along with measured quantity of silver. At the end of the process, the quantity of silver should not increase.
Niswadu- Bhasma should be tasteless. If *bhasma* has any taste, it is considered as semi-finished and should be subjected to *puta* again.
Nischandra- The sparkling particles *(chandrika)* in a *bhasma* indicate a semi-finished product.
Avami- The *bhasma* should not produce nausea on administration.
Puta- In continuation with the etymological meaning, *puta* is the measure of the amount of heat required to convert or transform any metal or mineral. This amount is substance specific and measured in terms of number or weight of fuel.
Sharava- Earthen Petri dish having specific measurements.
Bhavana- Trituration of the drug with liquid medium, e.g., *hingula* with juice of fresh zinzibar officinalis.

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Original Research Article (Experimental)

Preparation and physicochemical characterization of ingredients of Indian traditional medicine, *Mahamrutyunjaya Rasa*

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ABSTRACT

Background: *Mahamrutyunjaya rasa* is an ayurvedic formulation used in the treatment of cardiac disorders. It contains the purified roots of *Visa* (*Aconitum ferox*), *Brihati* (*Solanum indicum*), fruits of *Pippali Kana* (*Piper longum*), *Marica* (*Piper nigrum*), *Gandhaka* (Sulfur), *Hingula* (Cinnabar) and *Tankana* (Sodium metaborate) as per *Bhaishajya Ratnavali*. The purification (*shodhana*) process changes the physiochemical properties of the raw materials which need to be studied and understood.
Objective: The present work aims to perform a comprehensive physicochemical characterization of raw materials, intermediates and the final product obtained during purification, using modern analytical techniques.
Materials and methods: The standard methods as per traditional text were followed and the physicochemical changes were also investigated by collecting samples at different steps of purification. The samples were analysed using various techniques, viz. Fourier transform infra-red spectroscopic (FTIR), X-ray diffraction (XRD), Differential Scanning Calorimeter (DSC) and High Performance thin Layer chromatography (HPTLC).
Results: The FTIR and HPTLC analysis of the alkaloidal extracts of *Visa* showed loss of an ester group with shift in the peaks from 1720 cm^{-1} (C=O stretching of esters) to 1676 cm^{-1} (C=O stretching of Ketone) which signifies the conversion of alkaloid Aconitine (LD_{50} – 0.08 mg/kg) to Benzoylaconine (LD_{50} – 24 mg/kg) improving its safety. The analysis of *gandhaka* by XRD and DSC showed that purification brought about transformation of orthorhombic sulphur into monoclinic sulphur and it reverted back to original form with higher purity. The treatments given to *gandhaka* and *hingula* with organic compounds made them homologous to the body tissues. Analysis of purified *tankana* showed that the processing led to loss of water and slight change in the crystal structure with the shift in the endothermic peak from 110.6 °C to 104.2 °C.
Conclusion: Thus, the present study provides a scientific backing to the methodologies used by Ayurvedic practitioners. The study also provides physicochemical fingerprints for the standardization as well as characterization of raw materials and forms a technical platform for manufacturers to develop quality control standards.
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1. Introduction

Ayurvedic medicine originated in India more than 2000 years ago and it makes use of herbs, metals and minerals for curative effects. Therapeutic effectiveness of the Ayurvedic drugs has been established and well documented in the form of classics attributed to them. However, lots of changes have occurred since the time these classics were written and the impact of these changes on the therapeutic efficacy of the preparations formulated has not been ascertained [2]. Further the art of preparing the formulations requires certain amount of expertise and no information is documented about the likely impact of changes in the manufacture techniques or improper preparation on the expression of biological activity including possibility of production of undesirable effects [15].

Ayurveda does not use heavy metals or minerals without extensive processes, like *shodhana* or purification to render them fit

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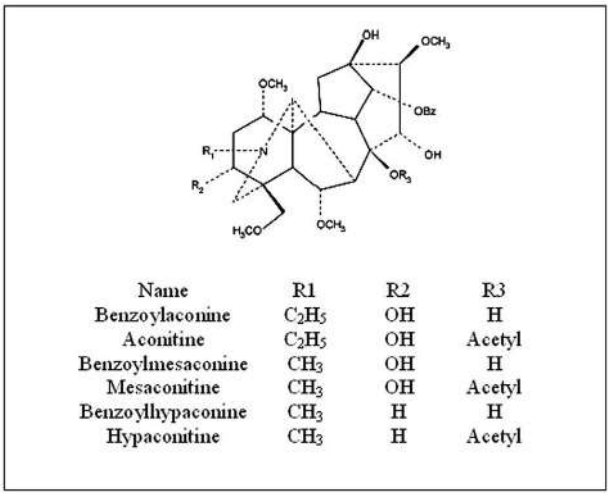
for human consumption. In this regard, it uses drugs medicinally but in a careful, complex and safe manner [13]. In this way, Ayurveda can employ the great healing power of minerals while avoiding their side effects [1]. It is believed that the *shodhana* process converts the metal into its specially desired chemical compound which eliminates the toxicity of the metal and has the necessary medicinal benefits [14].

Mahamrutyunjaya Rasa (MHR) is a compound herbal-mineral formulation often used to treat cardiac disorders. *Bhaishajya ratnavali* records the formula of MHR tablet as 1 part each of processed *Visa* (*Aconitum ferox*), *Brihati* (*Solanum indicum*), *Pippali Kana* (*Piper longum*), *Marica* (*Piper nigrum*), powdered and sieved through 100 mesh sieve. It is then mixed with 1 part purified *Gandhaka* (Sulfur), 1 part purified *Tankana* (Sodium metaborate) and 2 parts of purified *Hingula* (Cinnabar) [4].

The ingredients like *Visa*, *Gandhaka*, *Hingula* and *Tankana* have to be processed before internal administration as per the ayurvedic literature [17]. The principal active ingredients in *Visa* are C19-diterpenoid alkaloids, including aconitine, mesaconitine and hypaconitine (Fig. 1). However, these alkaloids are toxic with a very narrow safety range, because they easily induce ventricular tachycardia and fibrillation even at therapeutic dose levels. Down the ages, various processing methods were developed to reduce their toxicity during which the drug still retains pharmacological properties while their toxicity is reduced about a hundred-fold [8]. Hingula (HgS) is another ingredient in MHR. Hingula is insoluble, has very low bioavailability and its long-term use is major cause of mercury intoxication, but at the therapeutic doses, the adverse effects hingula-containing traditional medicines seem to be tolerable and reversible [6]. Gandhaka is also one of the ingredients. Gandhaka is mild laxative, detoxifying. Improperly purified gandhaka medicine if consumed over a long period causes toxic effects like dyspepsia, flatulence. Tankana is also added in MHR. In ayurveda, it is given internally in acidity of the stomach, amenorrhoea and to promote uterine pains during labour. Tankana is not acutely toxic with the LD_{50} 2.66 g/kg in rats. Consumption over a long duration of time may cause gastrointestinal distress including nausea and diarrhoea [23].

Extensive literature is available regarding the pharmacological actions of all these components. The purification procedures are also well documented but none of the methods have been studied in detail to determine the structural and chemical changes taking place in the ingredients, which is essential requirement to discuss the non-toxicity and therapeutic value of such formulations. Since these preparations are sustaining themselves since centuries in clinical use, therefore one cannot exclude its use [11]. In this report, an attempt was made to derive certain standard data which may form the basis of quality control of the raw materials present in the formulation. The standard methods as per traditional text were followed and the physicochemical changes were also investigated by collecting samples at different steps of purification. The samples were analysed using various techniques, viz. Fourier transform infra-red spectroscopy (FTIR), X-ray diffraction (XRD), Differential Scanning Calorimeter (DSC) and High Performance Thin Layer Chromatography (HPTLC).



Name	R1	R2	R3
Benzoylaconine	C_2H_5	OH	H
Aconitine	C_2H_5	OH	Acetyl
Benzoylmesaconine	CH_3	OH	H
Mesaconitine	CH_3	OH	Acetyl
Benzoylhypaconine	CH_3	H	H
Hypaconitine	CH_3	H	Acetyl

Fig. 1. Chemical structures of benzoylaconine, aconitine, benzoylmesaconine, mesaconitine, benzoylhypaconine and hypaconitine.

2. Experimental

2.1. Materials

Dried roots of *Visa*, were purchased from the local store and identified by NISCAIR, Delhi. Hingula was purchased from a local ayurvedic store. Gandhaka, Tankana, Chloroform, Toluene, and all other solvents of analytical grade were purchased from Qualigens (Mumbai).

2.2. Method of preparation

The following methods of purification were used as per the reported methods [14] and samples were withdrawn at intermediate and final steps for analysis.

2.3. Purification of aconite alkaloids

The aconite roots were washed with water and soaked in cow urine for 48 h. It was then washed with water and boiled in milk. The drug was again washed with water and dried. The samples of crude drugs were collected and the alkaloid fraction was subjected to HPTLC and IR studies. The samples were collected at following steps (i) Pure Drug (A-1), (ii) Washed with water (A-2), (iii) Soaked in cow urine for 24 h (A-3), (iv) Soaked in cow urine for 48 h (A-4), (v) Washed with water and boiled with milk (A-5), (vi)Washed with water and dried (A-6).

2.4. Purification of Gandhaka

Gandhaka mixed with ghee (a cow milk preparation) in an iron vessel was heated up to its melting temperature and the resulting liquid was poured through a filter into a vessel containing boiled milk. The final product was taken out, washed with hot water and dried. The samples at following steps were collected and subjected to XRD and DSC studies. (i) Crude Gandhaka (S-1), (ii) Gandhaka treated with cow ghee (S-2). (iii) Melted mixture in iron vessel (S-3), (iv) Mixture washed with cow milk (S-4). (v) The mixture washed with water and dried (S-5).

2.5. Purification of Hingula

Crude hingula was soaked in lemon juice until the colour of the powder became dark red. It was then washed with water and the same process was repeated seven times. After final treatment hingula was dried. The samples were collected at following steps and subjected to XRD. (i) Crude hingula (H-1), (ii) Hingula treated

with lemon juice-three times (H-2), (iii) Hingula treated with lemon juice-seven times (H-3).

2.6. Purification of Tankana

The purification of *Tankana* was done by heating till constant weight was obtained. The moisture free *Tankana* was stored in air tight container. The samples were collected at the following steps and analysed using XRD and DSC. (i) Crude *tankana* (B-1), (ii) *Tankana* heated for 1 h at 80 °C (B-2), (iii) *Tankana* heated for 2 h at 80 °C (B-3) (iv) Sample weight was constant in three consecutive weighing (B-4).

2.7. Characterization

For powder X-ray diffraction (XRD) a Philips 1710 X-ray diffractometer with CuKα radiation (λ = 1.5418 **Å**) operating at 30 KV and 20 mA was used. Pattern was recorded for the angle (2θ) ranging from 5 to 80° at a scanning rate of 3°/second. The results were compared with the values of standard substances [7].

IR spectra in the region (4000–450 cm^{-1}) were recorded on Perkin Elmer FTIR spectrophotometer (Perkin Elmer – 377) in KBr pellets.

Thermograms were obtained using DSC. Mettler Toledo DSC 821° module was used. Heating range: 30 °C–550 °C; Rate of heating: 10 °C/min; Rate of nitrogen flow: 100 ml/min. The DSC of the selected samples was done by using Aluminium crucibles. The system was purged with nitrogen gas to maintain inert atmosphere.

For the HPTLC studies, the alkaloid fraction of each sample was used. It was prepared by treating 1 gm sample with ammonia and extracting with ethyl acetate. The extract was concentrated and evaporated under vacuum. A 10 mg/ml solution of alkaloid fraction was prepared in chloroform. A Camag microlitre sample (Hamilton, Bonaduz, Switzerland) syringe was used for sample application on pre-coated silica gel aluminium plate 60F-254, 20 cm × 10 cm with 0.2 mm thickness, (E. Merck, Darmstadt, Germany) using a Camag Linomat-V (Switzerland). The linear ascending development was carried out in Toluene: Ethyl Acetate: Diethyl amine (7:2:1 v/v). Densitometric scanning was performed on Camag TLC scanner III in the reflectance–absorbance mode for all measurements and operated by CATS software (V1.4.3 Camag). The plate was scanned at 235 nm. The plate was sprayed with Dragendorffs reagent immediately scanned at 500 nm and data of peak area and height of each band were recorded.

3. Results and discussion

3.1. HPTLC studies of Visa alkaloids

The contents of *Visa* alkaloids in different extracts of processed aconite roots and unprocessed aconite roots were compared using HPTLC. The representative HPTLC chromatograms are shown in Fig. 2(a and b). The tracks from 1 to 6 show that there was a gradual chemical degradation with decrease in the concentration of two alkaloids, and increase in concentration of other alkaloids (Table 1).

3.2. IR studies of V alkaloids

The IR spectra of the six samples have been shown in Fig. 3. IR (KBr): 3435 (O–H stretch), 2929 and 2819 (Alkyl C–H stretch), 1720 (C=O stretching of esters), 1676 (C=O stretching of Ketone), 1601 (C=C stretching of ring), 1514 (N–H bending vibrations), 1462 (C–H Symmetrical bending vibrations of cycloalkanes), 1362 (C–H Asymmetrical bending vibrations of cycloalkanes), 1294 (C–O or O–H stretch), 1271 (Asymmetrical C–O–C stretch), 1224 (C–O stretch, 1177 (C–C(=O)–O saturated esters)), 1096 (Symmetrical C–O–C stretch), 1024 (Aromatic ethers ($O–CH_2$)), 985 and 765 (Out of plane –C–H bend). The peak at 1720 cm^{-1} disappears gradually till A-6 and is replaced with a peak at 1676 cm^{-1} depicting the conversion ester to keto-group. The peak at 1294 cm^{-1} is also lost which depicts the C–O or O–H stretch. Thus, there is an alteration in the ester group of the alkaloids.

3.3. XRD studies of gandhaka

Fig. 4 shows the XRD patterns of gandhaka samples. The d-spacing values of gandhaka samples were compared with coinciding values of the reference standards of various allotropes of gandhaka. The pattern of S-1 shows that the raw material *gandhaka* (sulphur) has a number of peaks coinciding with the reference orthorhombic sulphur in the Fddd space group (Table 2). The diffraction pattern of S-2 shows that the number of peaks coinciding with orthorhombic sulphur increase, depicting the increase in that form of gandhaka. The pattern of S-4 shows the presence of only two intense peaks which are found to be present in monoclinic type of gandhaka crystal. Again in S-5, the peaks coincide with the orthorhombic sulphur. However, the peaks are even sharper which reflect higher purity of the final product.

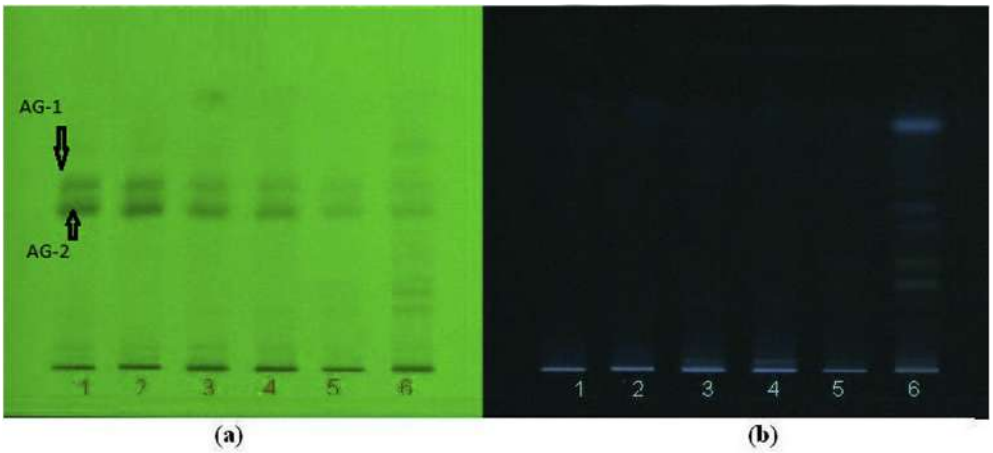


Fig. 2. HPTLC chromatograms of *Visa* alkaloids (a) Image at 254 nm and (b) Image at 366 nm.

Table 1
Results of the finger printing analysis of Aconitum alkaloids.

Sample	Concentration µg per spot	Peak	Rf value	Height	Area	% Height	% Area
A-1	100	AG-1	0.42	210.3	6295.1	44.77	41.28
	100	AG-2	0.48	149.7	5618.0	31.86	38.58
A-2	100	AG-1	0.42	160.6	6092.4	41.12	40.21
	100	AG-2	0.48	144.3	5294.0	29.26	32.79
A-3	100	AG-1	0.42	148.9	5748.1	30.00	35.40
	100	AG-2	0.48	135.3	5070.5	26.25	33.28
A-4	100	AG-1	0.42	129.9	5309.5	24.88	28.74
	100	AG-2	0.48	126.8	5142.2	24.01	32.98
A-5	100	AG-1	0.42	120.2	5018.5	20.30	25.22
	100	AG-2	0.48	116.5	5093.7	21.04	29.14
A-6	100	AG-1	0.42	114.6	4974.6	16.39	22.06
	100	AG-2	0.48	100.3	5325.6	14.89	24.72

3.4. Thermal studies of Gandhaka

All the thermograms (Fig. 5, Table 3) show two sharp peaks up to the temperature of 122 °C. The peaks displayed for S-1, S-2 and S-5 are similar with changes in the number and sharpness of the peaks. A small endothermic peak is observed at 115.61 °C in the S-5 sample which may be due to a different type of allotrope of gandhaka formed during the heating procedure.

3.5. XRD studies of hingula

The pattern of H-1(Fig. 6) when compared with the reference XRD pattern shows that hingula is present along with other components. In the intermediate sample (H-2), the peaks of other components are reduced. Further in the final XRD spectra of H-3, a number of peaks are absent and the intensity of remaining peaks is increased. The d-spacing values of H-3 matched with the reference data showing high purity of hingula in the trigonal trapezohedral crystalline form. (Table 4)

3.6. XRD studies of Tankana

The XRD spectra of tankana (Fig. 7) depicts that there was a gradual loss in the sharpness and number of peaks from B-1 to B-4.

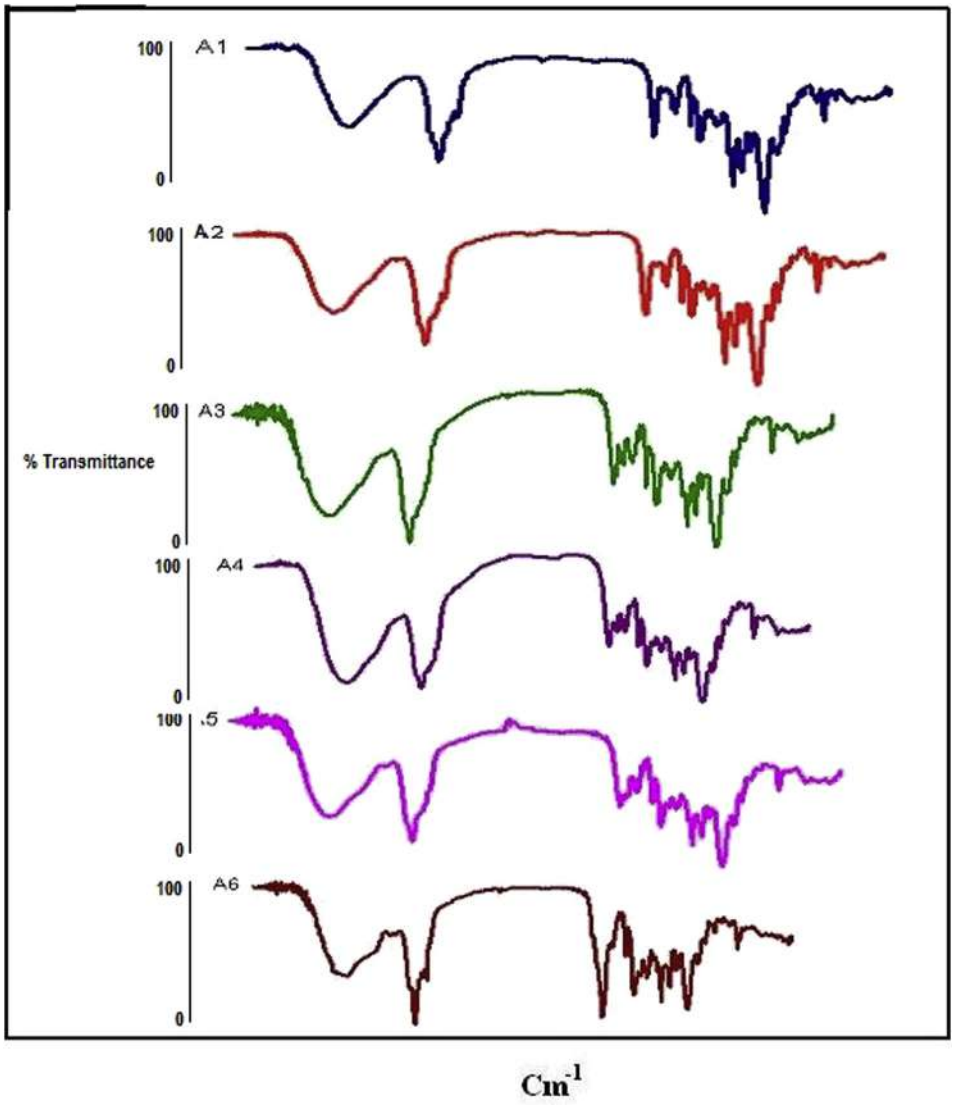


Fig. 3. IR spectra of samples of *Visa* extracts.

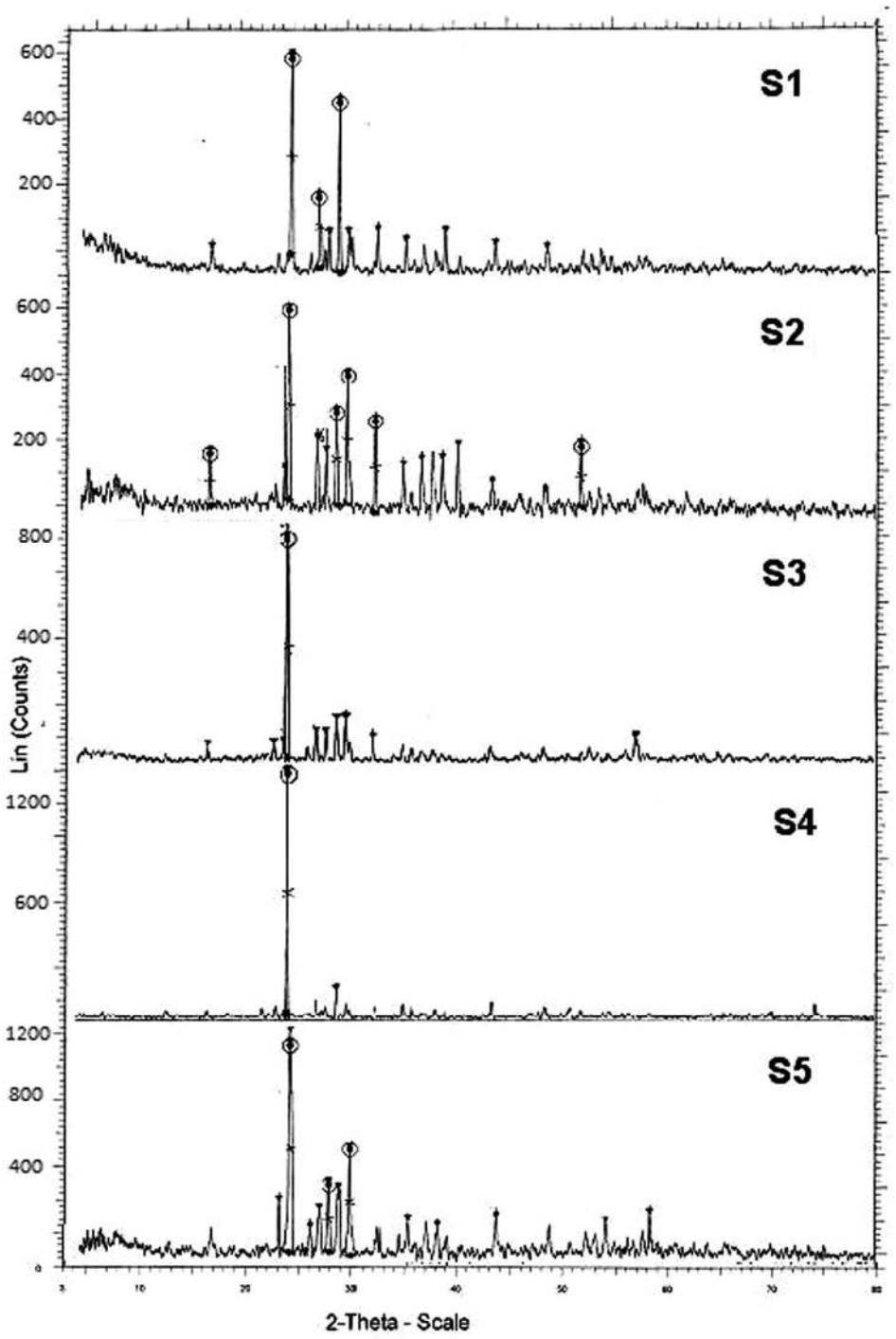


Fig. 4. X-ray diffraction spectra of Gandhaka samples.

Table 2
d-Spacing values of samples and standard sulphur.

S-1 d(Å)	S-2 d(Å)	S-3 d(Å)	S-4 d(Å)	S-5 d(Å)	Orthorhombic sulphur d(Å)	Monoclinic sulphur d(Å)
3.825 (a = 23.235)	3.844 (a = 23.114)	3.834 (a = 23.178)	3.781 (a = 23.505)	3.853 (a = 23.174)	3.85 (a = 23.083)	3.803 (a = 23.372)
3.198 (a = 27.871)	3.102 (a = 28.757)	3.099 (a = 28.77)	3.166 (a = 28.156)	3.084 (a = 28.927)	3.113 (a = 28.653)	3.168 (a = 28.145)
	3.215 (a = 27.721)	3.321 (a = 26.823)		3.312 (a = 26.891)	3.219 (a = 27.690)	
	3.331 (a = 26.743)			3.205 (a = 27.81)	3.336 (a = 26.315)	
	2.838 (a = 31.499)				2.848 (a = 31.385)	

a = 2 Theta (deg).

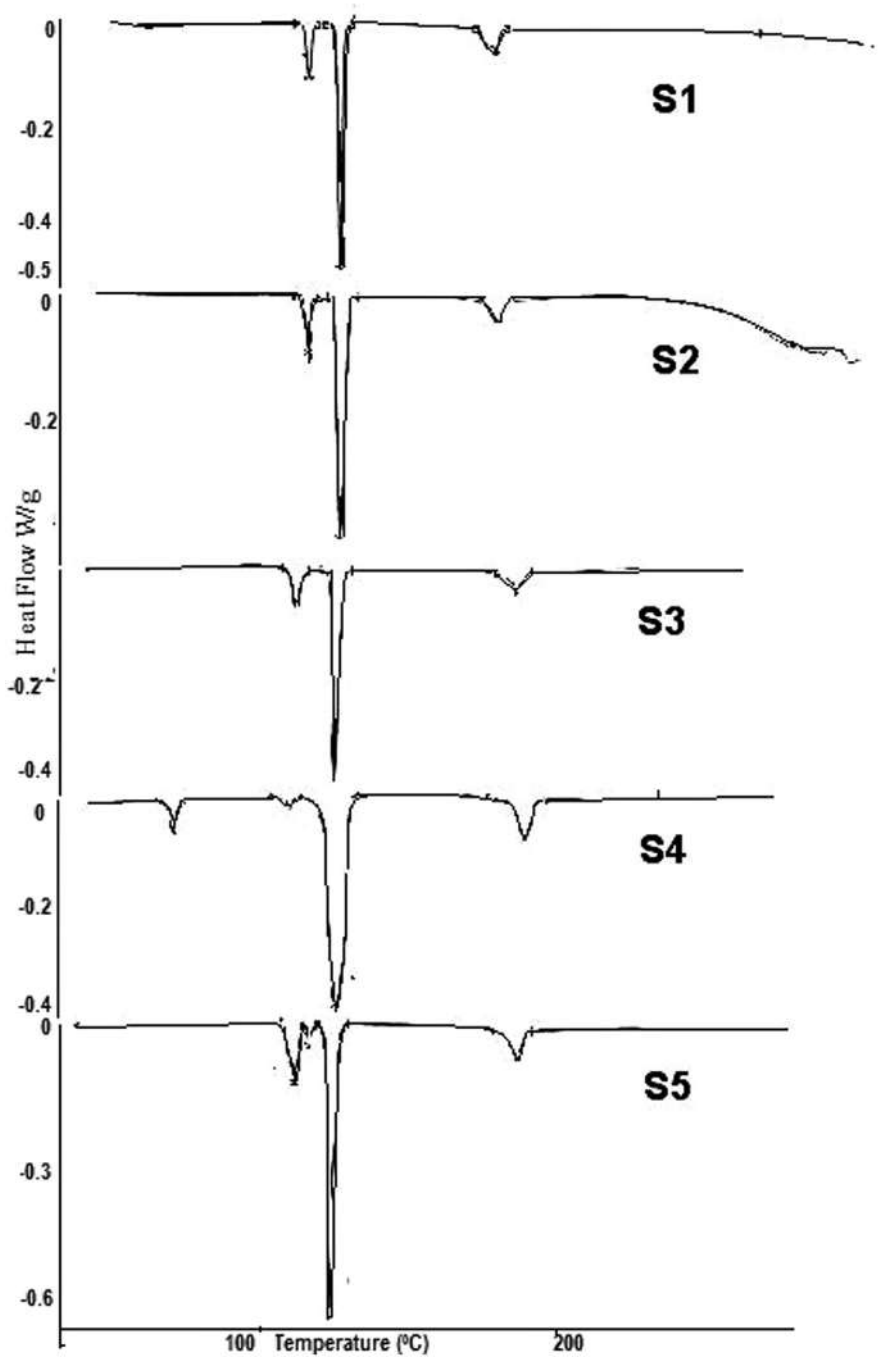


Fig. 5. Differential thermograms of Gandhaka samples.

Table 3
Values of endothermic peaks of sulphur in DSC.

Sr. no.	S-1	S-2	S-3	S-4	S-5
1	–	–	–	75.97 °C	–
2	108.60 °C	110.93 °C	110.04 °C	108.49 °C	111.55 °C
3	–	–	–	–	115.61 °C
4	119.97 °C	121.10 °C	121.29 °C	121.01 °C	121.31 °C
5	186.83 °C	174.17 °C	174.73 °C	173.96 °C	174.74 °C
6	410.87 °C	–	–	–	–

3.7. Thermal studies of Tankana

The thermograms (Fig. 8) of B-1 show an extra peak at 74.71 °C which may be due to the presence of solvates of water, while the peak is missing in the differential thermogram of B-2. Further, the range of peak onset and endset is narrow in the B-3 thermogram as compared to B-1 and B-2. A gradual increase in the sharpness of an endothermic peak at 137 °C is observed. The peak at 110.62 °C of B-2 is slightly shifted to 104.22 °C in B-3, which may be due to some change in the crystal structure of the final product (Table 5).

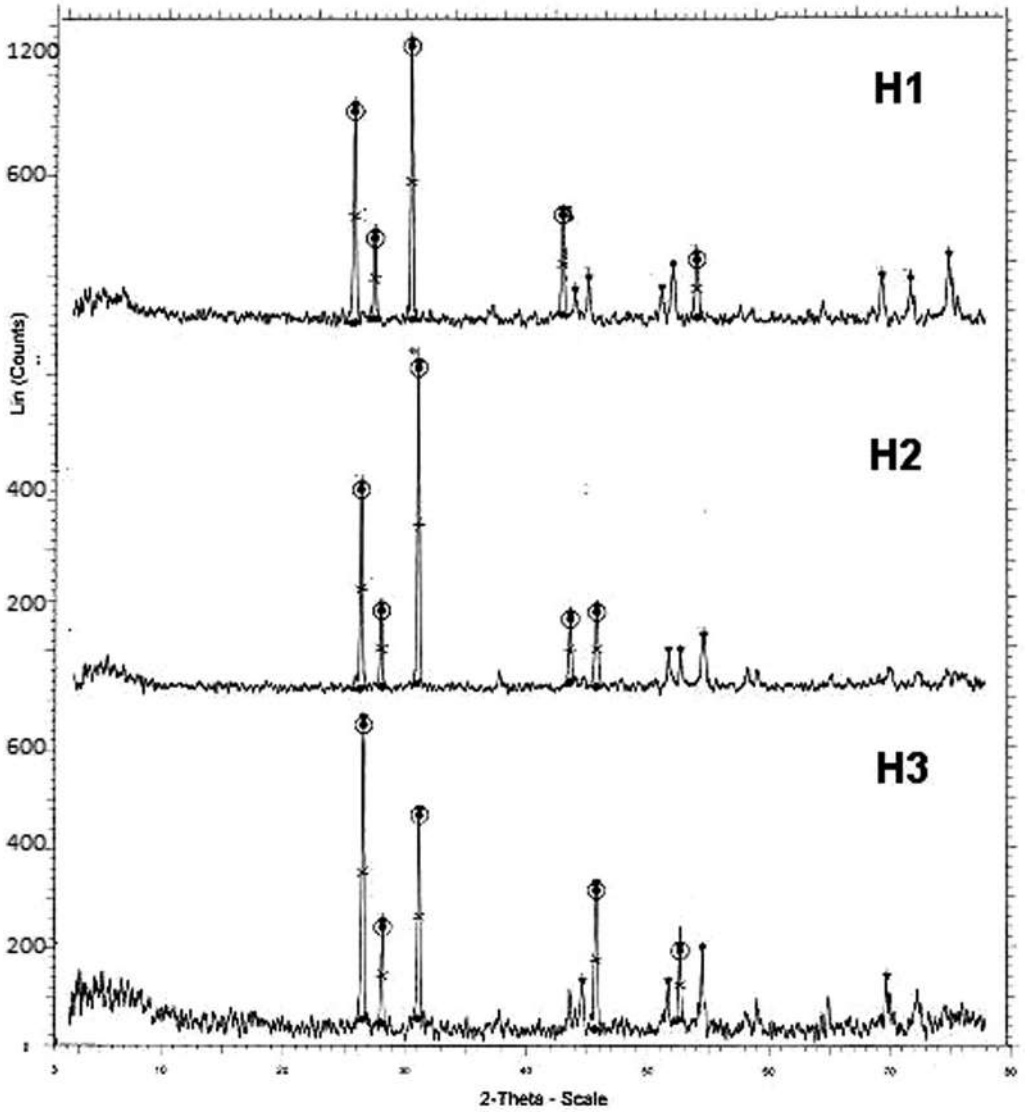


Fig. 6. X-ray diffraction spectra of Hingula samples.

Table 4
d-Spacing values of samples and standard Cinnabar.

d-Spacing values	
H-1 d(Å)	**HgS d(Å)**
3.337 (a = 26.363)	3.36 (a = 26.507)
3.181 (a = 28.024)	3.181 (a = 28.218)
2.877 (a = 31.060)	2.85 (a = 31.362)
2.078 (a = 43.50)	2.06 (a = 43.917)
1.682 (a = 54.508)	1.67 (a = 54.937)
H-2 d(Å)	**HgS d(Å)**
3.370 (a = 26.422)	3.36 (a = 26.507)
3.171 (a = 28.109)	3.181 (a = 28.218)
2.874 (a = 31.089)	2.85 (a = 31.362)
2.030 (a = 44.593)	2.06 (a = 43.917)
1.682 (a = 54.490)	1.67 (a = 54.937)
H-3 d(Å)	**HgS d(Å)**
3.358 (a = 26.517)	3.36 (a = 26.507)
3.162 (a = 28.199)	3.181 (a = 28.218)
2.862 (a = 31.224)	2.85 (a = 31.362)
2.071 (a = 43.665)	2.06 (a = 43.917)
1.677 (a = 54.677)	1.67 (a = 54.937)

a = 2 Theta (deg).

3.8. Discussion

The above study was performed in order to understand the physicochemical changes taking place in the raw materials due to the purification methods reported. The HPTLC and IR studies for *Visa* alkaloids show that the alkaloids undergo degradation on being processed for purification. The results show that there were significant differences in alkaloid contents between the processed and unprocessed aconite roots. It is observed from the IR spectra that during the purification there was loss of an ester group (peak at 1720 cm^{-1}), which may have been replaced with a Keto-group (1676 cm^{-1}). However, due to the presence of a number of alkaloids in the alkaloid fraction, the exact chemical changes cannot be predicted. These findings are in accordance with the earlier reports [5,10,19,21] that the di-ester alkaloids of *Visa* are prone to hydrolysis. The purification procedure may thus be responsible for the chemical degradation of the diester alkaloids. It has also been reported that the di-ester alkaloids (aconitine, hypaconitine and mesaconitine) are toxic as compared to the monoester alkaloids (benzoylaconine, benzoylhypaconine and benzoylmesaconine)

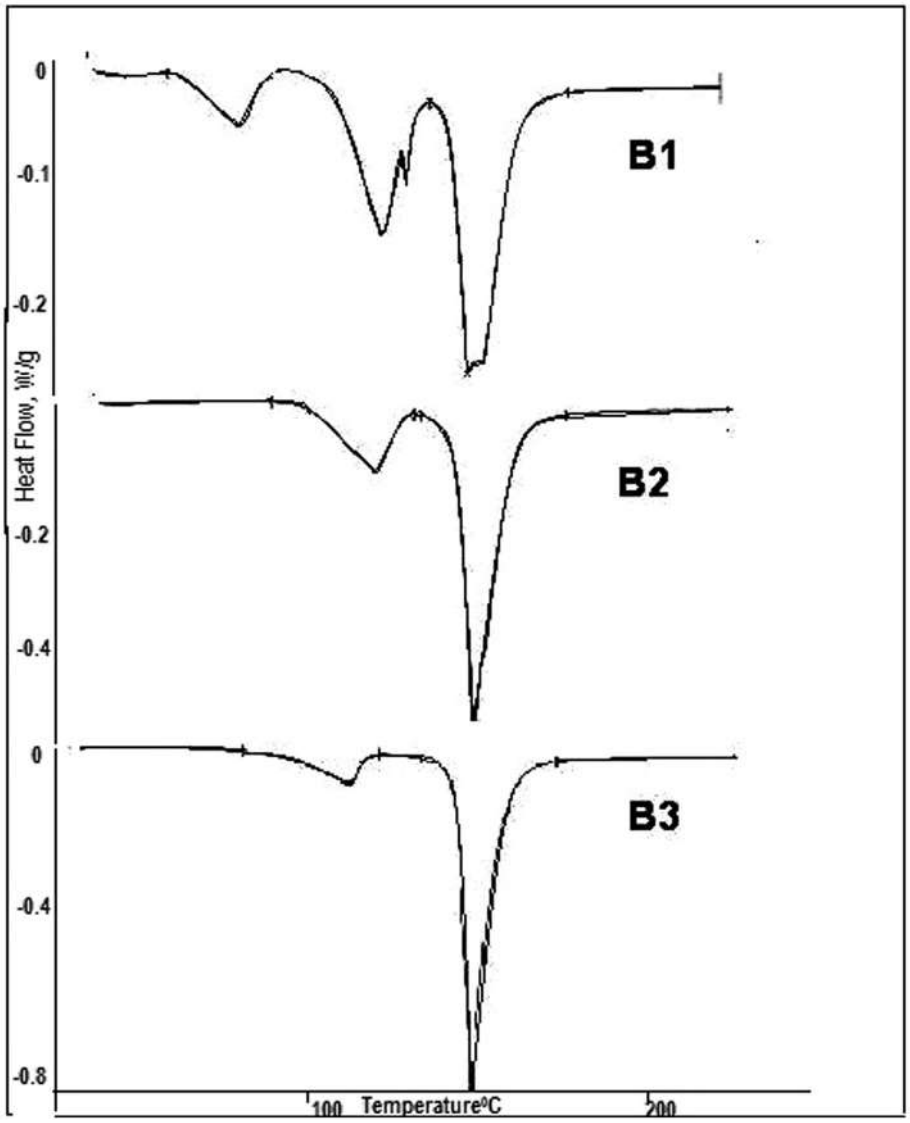


Fig. 7. X-ray diffraction spectra of Tankana samples.

[12]. The monoester alkaloids are significantly effective in inflammation and pain [8]. Toxicological studies have demonstrated that the toxicity of diester alkaloids is almost the same with LD_{50} values of about 0.08 mg/kg body weight, while the hydrolysed monoester alkaloids show much lower toxicity (LD_{50} – 24 mg/kg) in rats [22].

Studies were also performed on gandhaka using XRD and DSC. In nature, Gandhaka atoms aggregate, with each other forming long chains. In any sample of gandhaka, thermal agitation is constantly breaking and reassembling these chains. The S_8 molecule is stable and their two crystalline forms compete, orthorhombic α-sulphur and monoclinic β-sulphur. Below 96 °C, the orthorhombic form is more stable and the conversions between the two forms are slow [18]. If the liquid is cooled slowly, needle-like monoclinic crystals form. When the temperature falls below 96 °C, these crystals slowly change to orthorhombic microcrystal [3].

The gandhaka used as the crude raw material in our study as is evident from XRD, is largely of orthorhombic crystalline nature and is probably the mixture of α-Sulphur and small amounts of β-sulphur, displayed by two sharp endothermic peaks at about 109 °C and 122 °C in the differential thermogram. There is no significant difference between the DSC as well as XRD of S-1 and S-2, suggesting no interaction between gandhaka and ghee. This also suggests amorphous nature of cow ghee, with absence of any additional peak in XRD pattern of sample S-2. XRD and DSC patterns of sample S-3 and S-4 is more or less same as that of S-2. Significant change is observed in DSC of S-4 wherein additional endothermic peak is observed at 76 °C which may be due to the removal of the hydrates. The hydrates may have been formed during the washing of the gandhaka with hot water. All endotherms of S-4 are with lesser energy changes. As also seen in XRD patterns, the sample tends to display the presence of monoclinic sulphur. The XRD and DSC patterns of S-5, display that the structure of S-4 reverts back to the S_8 orthorhombic sulphur. Further, the unwanted components are also reduced which may be observed by the sharpness of the peaks in the X-ray diffraction pattern of S-5. Thus, from the above study it can be concluded that the processing of gandhaka brings about purification, reducing the toxic nature of gandhaka. By impregnating with organic material, like ghee, gandhaka is made homologous to the tissue cells and their toxicity is reduced and acceptability to the cell is increased.

One of the toxic components in MHR is hingula containing mercury. Mercury's toxicity and bioavailability are reduced in the

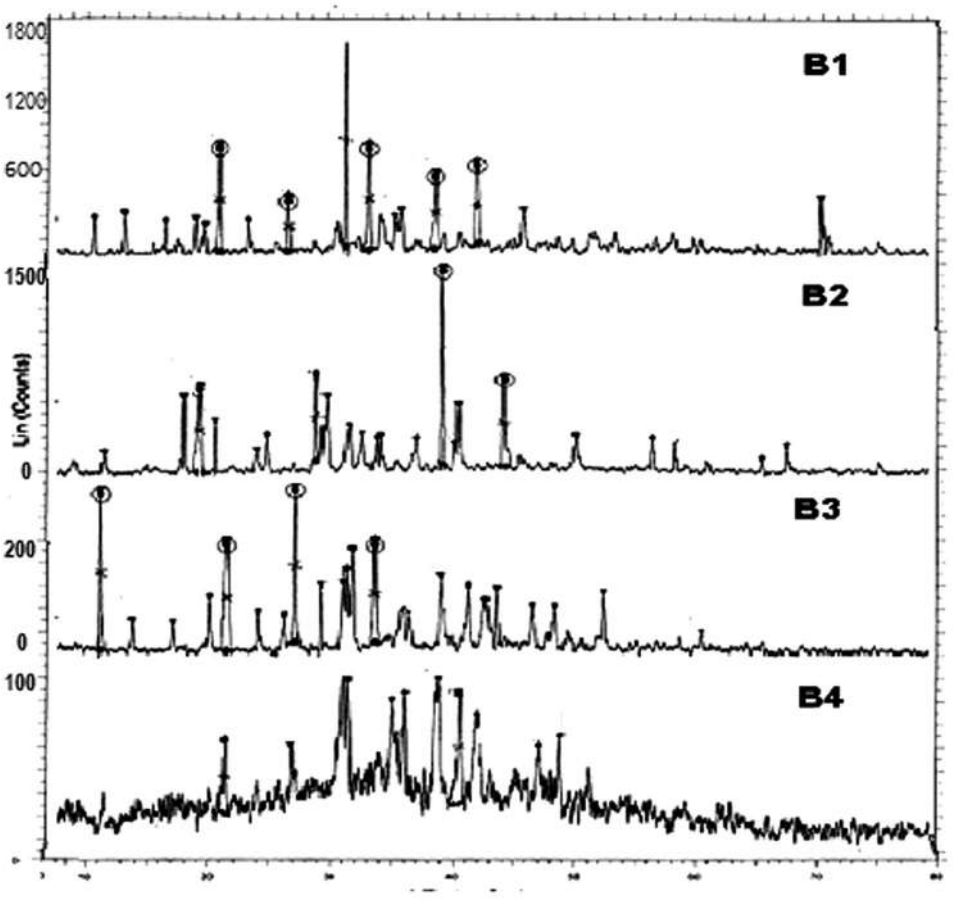


Fig. 8. Differential thermograms of Tankana samples.

form of hingula because it is relatively insoluble. The dissolution of hingula, however, is significantly enhanced in the presence of dissolved organic matter. In nature hingula is seldom available in its physically and chemically pure form. The contamination takes place naturally in the mine during the process of extraction with extraneous material which should be removed to avoid toxicity [16]. Metals, even in their physically and chemically pure form might produce adverse effects because they are inorganic in nature and they are heterogeneous to the body tissue [20]. By impregnating and triturating with organic material, like juices, decoctions of herbs etc., they are made homologous to the tissue cells and their toxicity is reduced and acceptability to the cell is increased. During this process certain organic materials are added to mercury, which help to increase its medicinal efficacy and safety. From the XRD pattern of hingula it is evident that there is no chemical change taking place due to the processing done. However, when compared with reference values of trigonal trapezohedral crystalline form of hingula, it is clear that the final product is of very pure nature. Further, on treatment with lemon juice the organic material make hingula more homologous to the body for its assimilation and the therapeutic effect.

Table 5
Values of endothermic peaks of sodium metaborate in DSC.

Sr. no.	B-1	B-2	B-2
1	74.71 °C	–	–
2	114.65 °C	110.62 °C	104.22 °C
3	138.67 °C	138.39 °C	137.54 °C

In case of Tankana study, marked changes are observed in the XRD and DSC patterns. The purity of the raw material can be ascertained from the DSC study as the sharpness of the endothermic peak at 137 °C increased markedly even when low concentration of sample was analysed. However, the slight shift in the endothermic peak at 104.22 °C in final product shows certain changes taking place in the crystal structure which further needs to be studied.

The data obtained certainly proves that all the procedures had marked effect on the nature of the raw materials. It indicates that the traditional methods of purification are responsible for making the formulation therapeutically useful [8,9] with less toxicity and thus should be followed very carefully. Even after all, the actual biological role of the metal present in such drugs is not very clear. In order to accept such kind of herbo-metallic drugs especially containing heavy metals, an extensive research is needed for the complete pharmacokinetic study on the animal system regarding its safety and efficacy.

4. Conclusion

The characterization techniques like FTIR, XRPD, DSC, HPTLC which have been used in the present studies can be used as a physico-chemical fingerprint for characterization of the raw materials in industry not only to check uniformity but also to ensure that each step is been followed as per the standard text. A routine use of such scientific techniques will lead to standardization of the product to a certain extent and would definitely help in building confidence in use of such products for medication.

Conflict of interest

None declared.

Acknowledgement

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Preparation and characterization of a mercury based Indian traditional drug—*Ras-Sindoor*

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The mercury based Indian traditional drug Ras-Sindoor is administered for the various ailments such as syphilis, genital disorders, and for rejuvenation. Pharmaceutical processing of *Ras-Sindoor* involves treating metallic mercury with sulfur and the juice of the aerial root of Banyan tree *(Ficus benghalensis* Linn.*)* and then controlled intermittent heating so that the metallic state is transformed into the corresponding sulfide form. In the study, synthesis and systematic characterization of this drug using various techniques, viz. X-ray diffraction (XRD), transmission electron microscopy (TEM), X-ray photoelectron spectroscopy (XPS), far infrared spectroscopy (FIR), Fourier transform infrared spectroscopy (FTIR), differential thermal analysis (DTA), thermogravimetry analysis (TGA), energy dispersive X-ray analysis (EDAX) and atomic absorption spectroscopy (AAS) have been reported. Drug contains mercury in the mercury sulfide form (Hg^{2+}) being nanocrystalline (20-50 nm) in nature and associated with the organic contents of the aerial root of the *Ficus benghalensis* Linn. Some specific findings were also made which could be of help for the interpretation of therapeutic value, none-toxicity of *Ras-Sindoor* and for the standardization of such kind of traditional herbo-metallic drugs.

Keywords: *Ayurvedic* drug, *Bhasma*, Traditional medicine, *Ras-Sindoor*
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Ayurveda make use of herbal preparations for their curative effects. Use of metallic herbal preparations (*bhasma*), in which a process termed *bhasmikarana* used to prepare the drug, is unique to the *Ayurveda*. It is believed that the *bhasmikarana* process converts the metal into its specially desired chemical compound, which eliminates the toxicity of the metal and has the necessary medicinal benefits[1-2]. *Ayurvedic* texts provide a list of tests for the efficacy of the *bhasmikarana* process. The tests which are essentially qualitative ensure that the resulting drug is very fine (small grains), has no metallic shine and does not alloy with silver even at higher temperature to which it was subjected[3-6]. However, these qualitative tests do not provide any quantitative information about the composition and the structure of the final drug. For any drug containing heavy metals (for example mercury), such structural information is an absolute necessity[7]. *Ras-Sindoor* is a well known mercury based *bhasma* prescribed for certain diseases, viz. syphilis, genital disorders and also for rejuvenation purposes[4]. The combination of sulfur is believed to have neutralized the toxicity of mercury[8]**.** Some researchers utilized Hg^{203} as tracer to study the pharmaco-kinetics and bio-distribution of *Kajjali* (a sulfur and mercury preparation)[9]. Several others have also worked on the efficacy and safety aspects of mercurial preparations in such traditional drugs[10,11]. None of the work presents the elemental and structural characterization of the drug, which is essential requirement to discuss the non-toxicity and therapeutic value of the mercurial preparations. In the report, the composition and the structure of *Ras-Sindoor* using various techniques, viz. X-ray diffraction (XRD), transmission electron microscopy (TEM), X-ray photoelectron spectroscopy (XPS), far infrared spectroscopy (FIR), Fourier transform infrared spectroscopy (FTIR), differential thermal analysis (DTA), thermogravimetry analysis (TGA), energy dispersive X-ray analysis (EDAX) and atomic absorption spectroscopy (AAS) have been characterized.

Methodology

Preparation of *Ras-Sindoor*

Ras-Sindoor sample was prepared from raw materials obtained from the pharmacy of the Institute

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of Medical Sciences, Banaras Hindu University, Varanasi. Mercury so obtained was purified through sublimation. For purification of the sulfur, the traditional method using cow's milk and ghee (a milk preparation) was employed. In this method, sulfur mixed with *ghee* was heated up to its melting temperature and the resulting liquid is poured through a filter into a vessel containing boiled milk. Sulfur was on the bottom of this vessel. This process was repeated seven times and the final deposited product was taken out, washed with hot water and dried. Mercury and sulfur thus purified in the ratio (1:6) were mixed with the juice of the aerial root of Banyan tree (*Ficus benghalensis* Linn.). This mixture was placed in an iron mortar and crushed till the whole mixture was converted into a fine black, lusterless powder (*Kajjali*). This fine powder *Kajjali* was filled in a glass bottle (*Kach Kupi*) and heated in a controlled intermittent manner with gradually increasing temperature till the blue flame emerging from the pot disappear and the bottom of the bottle becomes red hot. A red hot iron rod was repeatedly inserted in the neck of the bottle so as to burn any accumulated sulfur at the neck of the bottle. After adequate cooling, the sublimate deposited at the neck of the bottle was collected. The whole heating process required is 7-8 hrs and the highest needed temperature was 650^{o}C.

Structural and chemical characterization of *Ras-Sindoor*

For powder X-ray diffraction (XRD) a Philips 1710 X-ray diffractometer with CuKα radiation (λ=1.5418 A°) operating at 30 KV and 20 mA was used. Pattern was recorded for the angle (2θ) ranging from 5-80 degree at a scanning rate of 3 degree/second. For the characterization of nanostructure if any and the defined phases in the sample, a transmission electron microscope (TEM) was used. X-ray photoelectron spectra (XPS) measurement was performed on ESCLAB MKII instrument, using none monochromatized MgKα X-ray as the excitation source.

The Infrared (IR) spectrum in the low frequency region (50-400 cm^{-1}) was recorded on a Bruker IFS 66 V/S vacuum Fourier transform interferometer, where as the spectra from 400-4,000 cm^{-1} region were recorded using FTIR spectrometer. Thermograms DTA and TGA were recorded in a Nitrogen atmosphere on a Pyris Diamond thermal analyzer EXSTAR 6000, Perkin Elmer. The sample was placed in an alumina crucible and the temperature was varied from 40-400^{0}C. EDAX attached to TEM (CEM, CM-12) was used for the detection of various elements in the sample. For quantitative detection of trace metals in parts per million (ppm) an atomic absorption spectrophotometer was utilized. Sample preparation for AAS was done as per the laid procedure[12].

Results

XRD, TEM and XPS analysis

XRD pattern of *Kajjali* shows peaks due to free sulfur, mercury oxide and mercury sulfide (JCPDS File number-20-1227, 01-0896, 02-461, respectively) while the XRD pattern of *Ras-Sindoor* shows peaks only due to mercury sulfide ((JCPDS File number-02-461) (Fig.1). No extra diffraction peaks were observed in the case of *Ras-Sindoor* confirming that while in the initial stages of the processing of the medicine (before the heat treatment) mercury oxide and free sulfur are present in significant amount while after heat treatment only mercury sulfide remains in the product. The diffraction peaks in the XRD pattern of *Ras-Sindoor* corresponding to mercury sulfide become sharper and intense compared to *Kajjali* sample as well as some new peaks appears due to mercury sulfide, which were not present in the *Kajjali* sample. This observation confirms that the heat treatment of *Kajjali* helps in the formation of mercury sulfide and increases the crystallinity in the sample. The crystallite size was calculated from XRD pattern following the Scherrer equation $t = \lambda \times 0.9/ (\beta \times \text{Cos}\theta)$. Here, t is the crystallite size for (h k l) plane, λ is the wavelength of the incident X-radiation [CuK_{α} (0.1542 nm)], β is the full width at half maximum (FWHM) in radians and θ is the diffraction angle for (h k l) plane. The above equation yields t = 25-50 nm. It is notable here that the FWHM in case of *Kajjali* is high in comparison to the finally prepared *Ras-Sindoor* samples that confirms that the size of the crystallite increases. It is obviously due to heat treatment of the *Kajjali* sample.

TEM image of the drug sample shows spongy like structure with the particle size lying in the micro range (Fig.2). From the image it is clear that several crystallites are agglomerated in a signal particle giving rise to microcrystalline structure with loss of grain boundaries. XPS analysis provides valuable information for the surface state of the drug sample. A typical survey spectrum of the drug *Ras-Sindoor* confirming the presence of mercury and sulfur was

observed (Fig.3). In addition, it also shows the presence of C peak as well as O peak. Although the presence of the Mg, Ca, and Fe was shown in EDAX analysis, these ions were not observed in XPS analysis, indicating their absence on the surface. Presence of C and O, which are the building blocks or the organic molecules, on the surface of the drug by XPS supports the idea of the coating of organic molecules on the surface of the metallic compounds. High resolution spectra (Fig. 4) at Hg core level showed the presence of the peaks at 100.28 eV and 104.32 eV corresponding to Hg ($^4f_{5/2}$) and Hg ($^4f_{7/2}$) while S core level showed at 161.8 eV corresponding to S ($^2P_{3/2}$), respectively for HgS phase[13]. Thus the XPS analysis also confirms the presence of Hgs phase in the sample.

IR spectra analysis

FIR spectrum of *Ras-Sindoor* in the region (50-400 cm^{-1}) was studied (Fig.5). Crystalline mercury sulfide (HgS) is known to have absorption at 83, 92 and 100 cm^{-1} and their presence in the present FIR spectra indicate that *Ras-Sindoor* is essentially mercury sulfide[14]. This observation supports the XRD analysis. FTIR spectrum of *Ras-Sindoor* in the region from 400-4,000 cm^{-1} is shown (Fig. 5). There are fairly sharp peaks at 768, 1285, 1364, 1422, 2724 and 2870 cm^{-1} which indicate the presence of the organic compounds in the drug. These arise probably from the aerial root of the *Ficus benghalensis* Linn.[15-17]. FTIR spectrum of the powder of the aerial root also seems to confirm the conjection. The presence of appreciable concentrations of C, N, and O (Table 1) also suggests the presence of organic molecules in the drug. It would not be unexpected if the organic molecules also play an important role in the medicinal properties of these drugs. Therefore, a systematic study for their pharmacological activity would be desirable.

DTA and TGA analysis

The thermal analysis (DTA) plot for *Ras-Sindoor* shows three endothermic peaks in the range of 100-170 °C which could be indicative for the decomposition of water molecules (Fig. 6). On further heating another sharp peak is seen at 354°C which also corresponds to a weight loss of 9.54% (TGA). This may correspond to melting of mercury sulfide (melting temperature 344°C) present in the sample. Weight loss could also be due to the burning away of some attached organic molecules. Thus, thermal analysis supports the presence of mercury sulfide observed by XRD and FIR analysis and organic matter observed by FTIR analysis.

EDAX and AAS analysis

In addition to the metal Hg used in the drug, other metals are also expected in the drug which enters in it during its pharmaceutical processing. EDAX has been used to detect the elements present a considerable amount where as the AAS method is used to detect any elements in trace amount. Chemical compositions of *Kajjali* and *Ras-Sindoor* using EDAX (Table 1) and trace metal composition of Ras-Sindoor and for the aerial root of *Ficus benghalensis* Linn detected by AAS have been listed (Table 2). Abundance of C, N, and O in the drug was observed which is obviously from the plant extract used in the preparation of the drug. Sulfur is also abundant in *Kajjali* but proportion is greatly reduced in *Ras-Sindoor*. This is obvious as heat treatment removes a large amount of free sulfur and only the sulfur bound in mercury sulfide remains in the product. XRD analysis of *Kajjali* and

Table 1—Macroelement composition of *Ras-Sindoor* by Energy dispersive X-ray analysis

Element	*Kajjali*		*Ras-Sindoor*	
	Weight %[a]	Atomic %[a]	Weight %[a]	Atomic %[a]
C	35.69	44.66	25.74	30.36
N	27.44	29.45	41.37	41.84
O	23.56	22.14	30.14	26.68
Mg	0.56	0.34	1.12	0.65
S	6.13	2.88	0.88	0.39
Ca	0.07	0.03	0.04	0.04
Fe	0.04	0.01	0.08	0.02
Hg	6.51	0.49	0.63	0.04
Total	100.00	100.00	100.00	100.00

[a]Based on ZAF quantification (standardless)

Table 2— Trace element composition of *Ras-Sindoor* and Banyan aerial root

Element (in ppm)	*Ras-Sindoor*	Aerial root of Banyan tree
Na	27.5±2.062	71.50±2.22
K	19.8±1.039	59.66±1.74
Cu	0.64±0.039	5.66±0.311
Zn	04.3±0.352	33.06±0.353
Ni	0.07±0.005	00
Mn	0.21±0.015	3.28±0.82
Cd	00	00
Cr	0.09±0.018	0.60±0.0
Pb	01.7±0.135	3.66±0.84

Values are arithmetic mean ± standard deviation of three determinations in each caseppm- parts per million

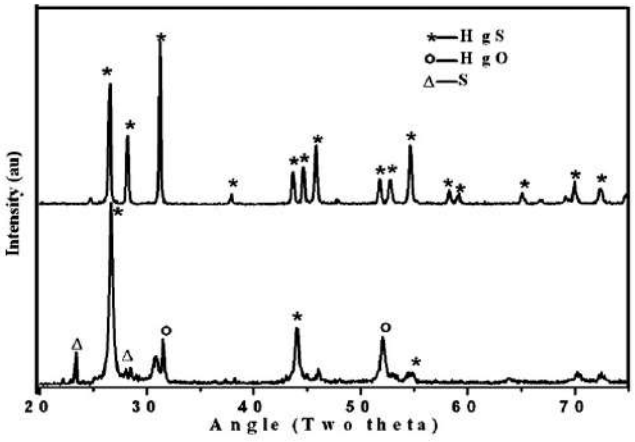

Fig.1XRD pattern of *Kajjali* & *Ras-Sindoor*

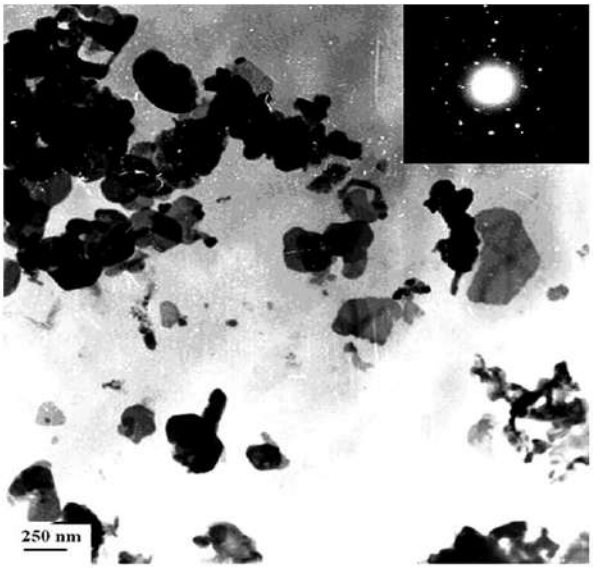

Fig.2 TEM image of *Ras-Sindoor*

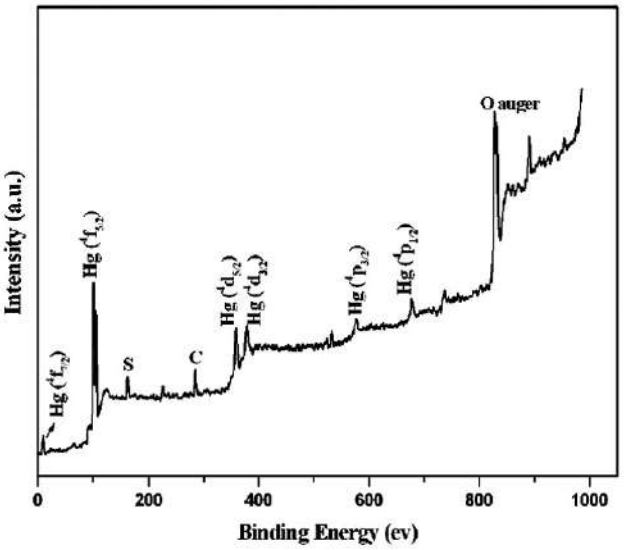

Fig.3 XPS spectrum of *Ras-Sindoor*

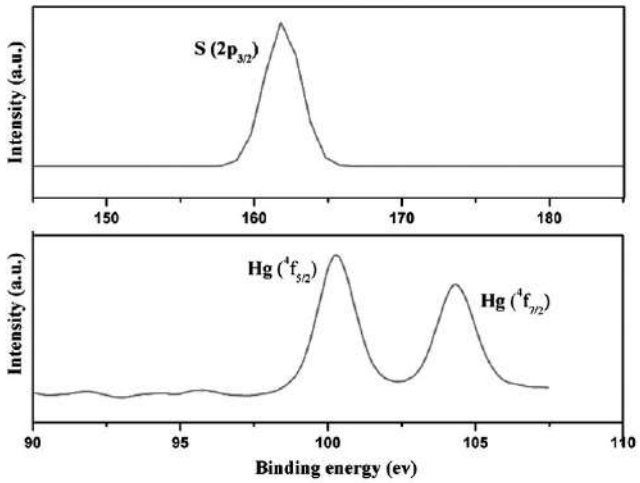

Fig.4 XPS spectrum of drug samples

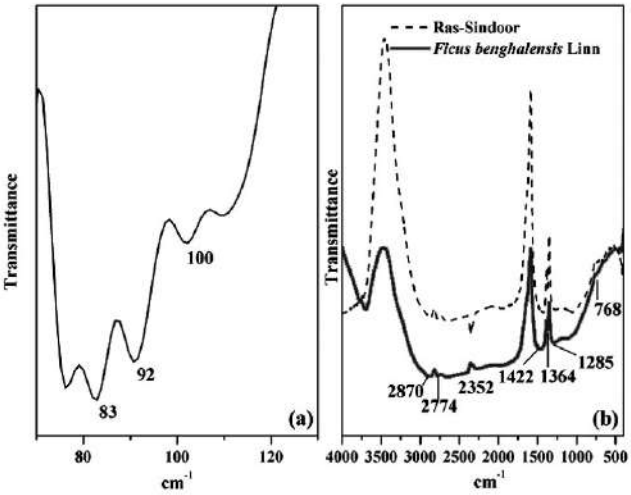

Fig.5 FIR and FTIR spectra

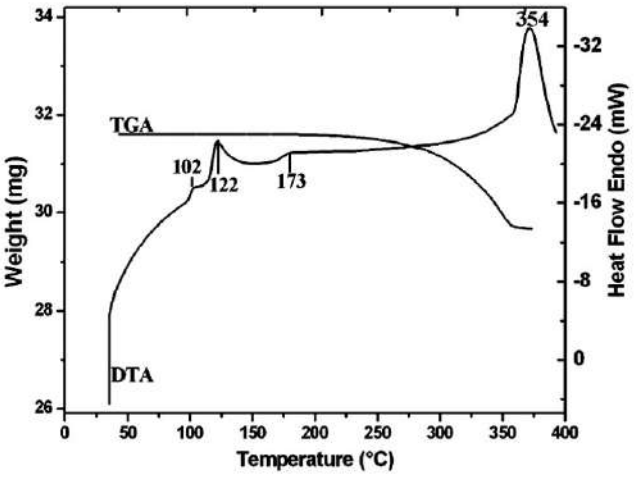

Fig.6 DTA & TGA plot of *Ras-Sindoor*

Ras-Sindoor also confirms this. Mg, Ca and Fe, conducive to healthy metabolism and preventives for stomach lesions, were also found to be present in the final *Ras-Sindoor* product in significant concentrations[18]. Na and K, needed for maintaining normal fluid balance are also present in the final product as is Zn useful for proper growth and immunity (Table 2). These elements (Mg, Ca, Fe, Na, K and Zn) act as additional supplement improving the curative properties of the drug. Several other heavy metals, e.g. Pb, Cd, Cr, Cu and Ni were also tested for their presence but their concentration (Table 2) was found to be well within the safe limits recommended by WHO[19]. The above beneficial elements are also present in significant concentrations in the aerial root of the *Ficus benghalensis* Linn. indicating that these elements enter in the *bhasma* along with other organic matter from the aerial root of *Ficus benghalensis* Linn. used in the preparation of the drug (Table 2). Thus, the additional element present in the drug is clearly due to plant part used and so may be called as bioavailable. It is notable that the proportion of mercury in *Kajjali* and in *Ras-Sindoor* does not seem to follow a consistent trend, though some of it is certainly lost during the preparation through direct spillage, vaporization or in the ignition process. This variability in the amount of mercury raises the safety concerns regarding the use of the drug and requires further work on the processing technique.

Discussion

Fundamental reaction for the generation of Mercury sulfide in a mixture of mercury and sulfur is $S + Hg \leftrightarrow HgS$ with $\Delta G° = -46$ KJ/ mol. This negative free energy change $\Delta G°$ shows the theoretical feasibility of making mercury sulfide by mixing elemental mercury and sulfur. Thus, even before the heat treatment some mercury sulfide is present in *kajjali* sample. To increase its proportion heat treatment seems essential. The pharmaceutical processing up to 650°C seems reasonable since sulfur boils at 392°C and mercury at 630°C. Juice of aerial root of *Ficus benghalensis* Linn serve as acidic medium and this acidic medium helps in formation of mercury sulfide[20]. Macro particle size of the preparation may be attributed to the grinding of raw materials for a long duration as well as the heat treatment which causes the change in the chemical nature of the raw materials. It is in general expectation that organic molecules will burn out at the processing temperature of the *bhasma* (above 400°C in most of these kinds of preparations). Here, the IR and thermal analysis shows the possibility of organic matter in the sample. This could be due to the formation of organo-metallic complexes in the drug sample that can sustain even at the high processing temperature of herbo-metallic drugs.

The studies discussed here are quite promising. Several significant possibilities and future prospects of the drug could be debated with these results. The macro particle size of the drug matches well with the colloidal size and this suggest the possibility that these colloidal particles are get attached to the human intestine and provide a large surface area thereby increasing the absorption of other nutrients and drugs, which are added to it during the process of preparation or prescribed to the patient along with them. Thus, these drugs act as the absorbent. Further, the presence of the organic matter on the surface of the drug suggests that these organic matter acts as the coating material on the surface of the metallic compound present in the drug and metal compound acts as the carrier of the organic mater (just like the concept of novel drug delivery in the modern medicine) derived from the herbs/plant used during the pharmaceutical processing. In short, *Ras-Sindoor* acts as a carrier for the organic contents from the aerial root of the *Ficus benghalensis* Linn. which is styptic and immunomodulator and is known to be useful in treatment of syphilis, dysentery, inflammation of lever, etc[21]. It could be concluded that mercury sulfide (HgS) in nano crystalline (20-50 nm) form associated with organic molecules probably plays an important role in making it biocompatible and non-toxic at low doses (dose of *Ras-Sindoor* is<125 mg/day). Other essential elements present in *Ras-Sindoor* act as additional supplement and help in increasing the efficacy of the drug. Even after all, the actual biological role of the metal present in such drugs is not very clear. In order to accept such kind of herbo-metallic drugs especially containing heavy metals, an extensive research is needed for the complete pharmacokinetic study on the animal system.

Conclusion

Ras-Sindoor is shown to contain mercury sulfide (crystalline in nature with crystallite size ranging from 25 to 50 nm) associated with several organic macromolecules derived from the plant extract used during the processing of the drug. Several macro/trace elements are also found to be present in different

amounts, which were bio-available and responsible for adding to the medicinal value of *Ras-Sindoor*.

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ORIGINAL RESEARCH ARTICLE EXPERIMENTAL

Preparation and characterization of mercury-based traditional herbomineral formulation: *Shwas kuthar rasa*

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ABSTRACT

Background: *Shwas kuthar rasa* is a prestigious and potential herbomineral formulation of Ayurveda tested on 100 years of time scale for the treatment of asthma, allergy, and other respiratory problems. However, there is a lack of scientific work on *Shwas kuthar rasa.* **Objective:** To prepare and physicochemically evaluate mercury-based *Shwas kuthar rasa* herbomineral formulation of Ayurveda for asthma and allergy. **Materials and Methods:** *Shwas kuthar rasa* was prepared as per Ayurvedic text and characterized by various modern analytical techniques, viz., transmission electron microscopy (TEM), X-ray diffraction (XRD), far infrared (IR) spectroscopy, fourier transform IR spectroscopy, energy dispersive X-ray analysis, and inductively coupled plasma-mass spectroscopy. **Results:** Study clearly revealed that prepared *Shwas kuthar rasa* formulation shows several crystallites agglomerate into a single particle. It yields submicron size particle structure (1.22 μ) with TEM analysis. The usage of mercury in the formulation found in the form of mercuric sulfide (HgS) and reaching to nanocrystalline (31–56 nm) size by XRD analysis. **Conclusion:** The present study indicates *Shwas kuthar rasa* is nanocrystallite with submicron size particle. Trituration of *Kajjali* helps in the formation of HgS and increases the crystallinity in the formulation.

Key words: Formulation, herbomineral, mercury, *Shwas kuthar rasa*

INTRODUCTION

Rasa Shastra means the science of mercury but also refers to the preparation of minerals/metals suitable for the body so that they can be used as medicines.[1,2] Minerals such as mercury and arsenic are considered toxic[3] but with proper *shodhana* (detoxification) process, they can be turned into therapeutic medicines. The formulation was prepared and processed properly, the mercury balances all three *Doshas*, has a soothing effect and protects the body from diseases and aging process. It nourishes all vital body parts and increases the strength of the eyes.[4,5] It is a *vrishya* (aphrodisiac), *balya* (tonic), *rasayana* (rejuvenating), *vrana shodhana, ropana* (wound cleaner and wound healer), and *krimighna* (anthelmintic and antimicrobial).[6] Mercury compounded with herb, the mercury heightens the medicinal potential of the particular herb. Mercury is said to give a firm physique, a stable mind, and considered to be the destroyer of diseases.[7] Herbs were used in the combination with *rasa*, the efficacy spectrum of herbs increases to a

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great extent enabling them to treat complicated diseases. *Rasa Shastra* has been placed with great importance in Ayurveda.

Shwas kuthar rasa a reputed preparation of Ayurveda valued for the treatment of asthma and allergy is a herbomineral formulation contains herbs, purified *Aconitum ferox* (*Aconite*), *Piper longum* (long pepper), *Piper nigrum* (black pepper), and *Zingiber officinale* (ginger), and minerals that is, *parada* (mercury), *gandhaka* (sulfur), *tankana* (borax), and *manahsila* (arsenic disulfide) in purified form as per Ayurvedic text.[8] *A. ferox* inhibited the biosynthesis of leukotriene B4 in bovine polymorphonuclear leukocytes.[9] *P. longum* have a traditional claims of Ayurveda for antiallergic and antiasthmatic activity[10] *P. nigrum* suppressed and reduced the infiltration of eosinophils, hyper responsiveness, and inflammation in mice.[11] *Z. officinale* are capable of inhibiting allergic reactions and is useful for the treatment and prevention of allergic diseases[12] Review of literature revealed that *Shwas kuthar rasa,* apart from treating asthma and allergy, is used for the cure of cough, laryngitis, tuberculosis, unconsciousness, mental disorders, comma, chest burn, and heart diseases.[8] None of the work presents the elemental and structural characterization of herbomineral formulation which is an essential requirement to discuss the therapeutic value of mercurial preparations. The present study aims to study the composition and the structure of *Shwas kuthar rasa* a herbomineral formulation using various techniques, viz., transmission electron microscopy (TEM), X-ray diffraction (XRD), far infrared spectroscopy (FIR), fourier transform infrared spectroscopy (FTIR), energy dispersive X-ray analysis (EDAX), and inductively coupled plasma-mass spectroscopy (ICP-MS).

MATERIALS AND METHODS

Plant materials

The dried fruit of *P. longum and P. nigrum*, root of *A. ferox,* and rhizome of *Ginger officinale* were obtained from Amruth Kesari Herbs, Bangalore in February. The drugs were identified by Prof. K. Prabhu, Botanist, S. C. S. College of Pharmacy, Harapanahalli (Karnataka), India. The herbs voucher specimens (No: SCSCOP/Ph.cog/Herb-652, 717, 691, and 572, respectively) were deposited in the botany herbarium of the institute.

Preparation of *Shwas kuthar rasa*

Shwas kuthar rasa was prepared using herbal and mineral ingredients as prescribed in Ayurvedic text,[13] initially detoxifying the *parada* (mercury), *manahsila* (arsenic disulfide), *gandhaka* (sulfur), *tankana* (borax), and *A. ferox* (vastanabha) as per given Ayurvedic text. Equal quantities of *shodhit* (detoxified and pure) *parada* and *gandhaka* were taken (1:1) in a stone mortar in reference amount, triturated for 40 h or until it attained the required *Kajjalabha* (blackish appearance) and *Nishchandra* (lusterless) state, that is, shining of *parada* is lost. This state of formulation is called *kajjali*.[14] *Kajjali* was then triturated, with the reference amount of powdered *manahsila*, *vastanabha, tankana,* and *trikatu* (equal part of black pepper, long pepper, and ginger), for 72 h to obtain fine powdered herbomineral formulation *Shwas kuthar rasa* and it was allowed for drying and stored in glass jar.

Structural and physicochemical characterization

Shwas kuthar rasa was subjected to evaluation for physicochemical characters such as loss on drying, ash value, and acid insoluble ash[15] followed by TEM (Philips, CEM, CM-12) was used to study the particle size of *Shwas kuthar rasa* whereby a beam of electrons transmits through an ultrathin specimen, interacting with the formulation. As the beam passes through, an image was formed from the interaction of the electrons transmitted through the specimen, the image was magnified and focused on fluorescent screen, on a layer of photographic film (SAIF, Chandigarh, Punjab).

The powder XRD patterns of the *Shwas kuthar rasa* were recorded on X'pert pro panalytical X-ray diffractometer with CuKα radiation (λ =1.5406 A°) operating at 45 KV and 40 mA for the angle (2θ) ranging from 5° to 50° at a scanning rate of 3°/s. A representative portion of *Shwas kuthar rasa* was placed in an alumina crucible, and the temperature was varied from 40°C to 400°C. EDAX (EDAX Inc., Mahwah, NJ, USA) attached to TEM (CEM, CM-12) was used for the detection of various elements in *Shwas kuthar rasa* (SAIF, Chandigarh, Punjab).

For the quantitative determination of heavy metals in *Shwas kuthar rasa* in parts per million (ppm), an ICP-MS, PerkinElmer ELAN-6000 was used. The infrared (IR) spectrum in the low- frequency region (50–400/cm) was recorded on a Bruker IFS 66 V/S vacuum Fourier transform interferometer; whereas the spectra from 400 to 4000/cm region were recorded using FTIR spectrophotometer (Spectrum RXI, PerkinElmer). For IR spectra, powdered samples were mixed in KBr to make translucent pellet and spectrum was recorded (SAIF, Chandigarh, Punjab).

RESULTS

Evaluation of *Shwas kuthar rasa* for organoleptic characters revealed that preparation possess no metallic sound, it is black colored, tasteless, and odorless with no coarse particle. The data of loss on drying, ash value, and acid insoluble ash values were recorded [Table 1].

Modern analytical techniques were used to observe the effect of the procedure employed in processing of *Shwas kuthar rasa*. Particle shape and size from the TEM photograph of *Shwas kuthar rasa* shows spongy structure with the irregular particle size lying in the submicron range [Figure 1]. From the image, it is clear that nanosize crystallites are agglomerated giving rise to micro sized particles with the loss of grain boundaries. These studies confirm that *Shwas kuthar rasa* is nanocrystallite with submicron size particle (1.22 μ).

XRD pattern of *kajjali* [Figure 2a] shows the peaks due to the presence of free sulfur, mercuric oxide, and mercuric sulfide (HgS) (Joint Committee on Powder Diffraction Standards [JCPDS] file number-20-1227, 01-0896, and 02-461, respectively) while the XRD pattern of *Shwas kuthar rasa* [Figure 2b] shows the peaks due to major presence of HgS (JCPDS File number-02-461), mercuric oxide (JCPDS File number-01-0896), and very low intensity of sulfur (JCPDS file number-20-1227). No extra diffraction peaks were observed in case of final *Shwas kuthar rasa* formulation confirming that while in the initial stages of the processing of the formulation free sulfur is present in significant amount; however, after trituration process, the major amount of HgS and mercuric oxide remains in the product. The diffraction peaks in the XRD pattern of *Shwas kuthar rasa* corresponding to HgS become sharper and intense compared to *Kajjali* sample as well as some new peaks appeared due to HgS, which were not present in the *Kajjali* sample. This observation confirms that the trituration of *Kajjali* helps in the formation of HgS and increases the crystallinity in the sample. The crystallite size was calculated from XRD pattern following the Scherrer equation $t = \lambda \times 0.9/(\beta \times \text{Cos}\theta)$. Here "t" is the crystallite size for (h k l) plane, λ is the wavelength of the incident X-radiation (CuKα [λ =1.5406 A°]), β is the full width at half maximum (FWHM) in radians, and θ is the diffraction angle for (h k l) plane.[16] It is notable, here, the FWHM in case of *Kajjali* is high in comparison to the finally prepared *Shwas kuthar rasa* confirms that the size of the crystallite increases. It is obviously due to the trituration process of the *Kajjali* sample. Thus, the XRD study concludes the presence of nanocrystalline structure of the drug.[17]

In addition, the metal Hg and As used as ingredients, other metals like calcium is also expected in the drug that enters in it during its trituration process while carryout the detoxification of individual herbs and metals. EDAX has been used to detect the presence of elements in considerable amount,[17] whereas ICP-MS was used to detect elements Hg and As in trace amount. Chemical compositions of *Shwas kuthar rasa* using EDAX and trace metal composition of *Shwas kuthar rasa* using ICP-MS have been listed [Table 1]. Abundance of C (31.24%), N (12.40%), and O (42.63%) in the drug was observed which is obviously from the herbs used in the preparation of the formulation. Ca (1.62%) conducive to healthy metabolism and preventive for stomach lesions was also found to be present in the final *Shwas kuthar rasa* product.

Table 1: Physicochemical characters of *Shwas kuthar rasa*

Parameters	*Shwas kuthar rasa*
Loss on drying (%)	0.73
Ash value (%)	98.29
Acid insoluble ash (%)	44.58
Particle size (μm)	1.22
Phase identification	HgS and HgO
Element contents (weight %)	C - 31.24, S - 0.89, N - 12.40, O - 42.63, Na - 2.37, Ca - 1.62, Cl - 3.46, H - 4.65.
Heavy metals (ppm)	Hg - 0.94, As - 8.78
Organic macromolecules	9 sharp peaks

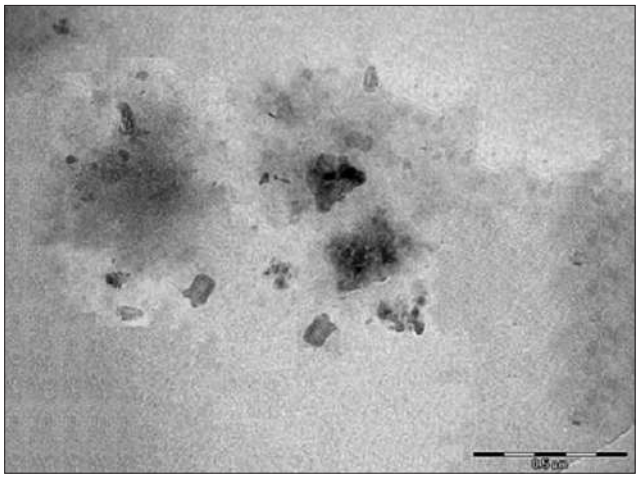

Figure 1: Transmission electron microscopy image of *Shwas kuthar rasa*

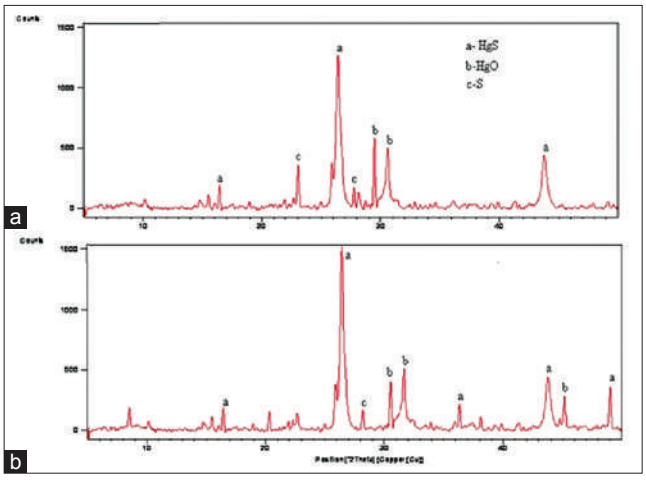

Figure 2: X-ray diffraction pattern of (a) *Kajjali* and (b) *Shwas kuthar rasa*

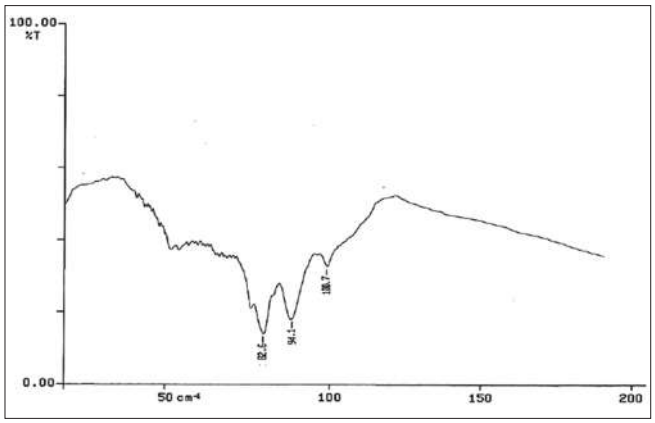

Figure 3: Far infrared spectroscopy spectrum of *Shwas kuthar rasa*

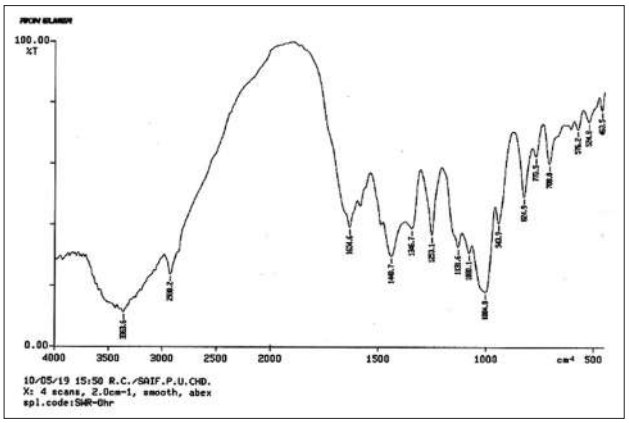

Figure 4: Fourier transform-infrared spectrum of *Shwas kuthar rasa*

Na (2.37%) needed for maintaining normal fluid balance is also present in the final product [Table 1]. These elements (Ca, Na, and S) act as additional supplement improving the curative properties of the formulation.[18] Other elements such as H (4.65%) and Cl (3.46%) were also found in the formulation. Concentration of heavy metals was found 0.94 ppm for Hg and 8.78 ppm for As, which were well within the safe limits recommended by WHO.[19,20] Thus, the additional element present in the drug is clearly due to the botanical origin. It is notable that the proportion/concentration of mercury in *Shwas kuthar rasa* does not seem to follow a consistent trend, and some of mercury is certainly lost during the preparation through direct trituration process. This raises the safety concerns pertaining to the use of mercury and may require additional work on the processing technique employed in the preparation of *Rasas*.

FIR spectrum of *Shwas kuthar rasa* in the region from 50 to 400/cm was studied [Figure 3]. Crystalline HgS is known to have absorption at 83, 92, and 100/cm and their presence in the present FIR spectra indicate that *Shwas kuthar rasa* is essentially HgS. This observation supports the XRD analysis. FTIR spectrum of *Shwas kuthar rasa* in the region from 400 to 4000/cm is shown [Figure 4]. There are fairly sharp peaks at 708, 1080, 1131, 1253, 1346, 1440, 1634, 2930, and 3363/cm which indicate the presence of the organic compounds in the formulation. These arise probably from the usage of the source of herbs. The presence of appreciable concentrations of C, H, O, and N [Table 1] also suggests the presence of organic molecules in the drug. It would not be unexpected if the organic molecules also play an important role in the medicinal properties of these drugs.

DISCUSSION

Macro particle size of the preparation may be attributed to the trituration of detoxified metals, nonmetals, and herbs for a long duration which causes the change in the chemical nature of materials. FT-IR analysis shows the possibility of organic matter in the formulation. This could be due to the formation of organometallic complexes in the drug sample that can sustain even at the high processing temperature of herbomineral drugs. Several significant possibilities and future prospects of the drug could be debated with these results. The macro particle size of the drug matches well with the colloidal size and this suggest the possibility that these colloidal particles are get attached to the human intestine and provide a large surface area thereby increasing the absorption of other nutrients and drugs, which are added to it during the process of preparation or prescribed to the patient along with them.[18] Further, metal ingredients act as the carrier of the herb derived organic matter used during the pharmaceutical processing. In short, metals as a carrier for the organic contents from *A. ferox, P. nigrum, P. longum,* and *Z. officinale* are known to be useful in the treatment of asthma, allergy, cough, inflammation, etc.[21-24] From XRD studies, *Shwas kuthar rasa* concluded that HgS in nanocrystalline range (31–56 nm), in association with organic molecules probably plays an important role in making it biocompatible and nontoxic at therapeutic doses. Other elements present in *Shwas kuthar rasa* act as additional supplement and possibly help in increasing the efficacy of the formulation.

CONCLUSION

Shwas kuthar rasa herbomineral formulation revealed the presence of crystalline HgS associated with several organic macromolecules derived from the herbs as basic ingredients of formulation. In addition, several elements were also found in varying concentrations, which bioavailability enhanced and thus responsible for therapeutic value addition of *Shwas kuthar rasa.*

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Conflicts of interest

There are no conflicts of interest.

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Process standardization of *Swarna Makshika Shodhana* (purification) in *Triphala Kwatha* (decoction)

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Abstract

Background: *Swarna Makshika* (SM) is a brassy golden yellowish mineral with chemical composition of $CuFeS_2$ that is widely used in therapeutics to treat various disease conditions such as *Prameha* (diabetes), *Paṇḍu* (anemia), *Kushtha* (skin diseases) and *Jwara* (fever). This mineral needs to be processed by the following specified Ayurveda guidelines in order to make it therapeutically safe and more potent. These processes include *Shodhana* (preliminary process of eliminating unwanted substances), *Marana* (incineration) and *Amritikarana* (nectorization) that are mandatory and play a crucial role in therapeutics. However, till date, no published reports are available on standard manufacturing procedure of SM *Shodhana*. **Objective:** The objective of this study is to develop the standard manufacturing procedure of SM *Shodhana*. **Materials and Methods:** Methods described in Rasaratna Samuchhaya were followed to perform *Shodhana* process. *Shodhana* of SM was carried out in three batches (600 g in each batch) by seven quenching in *Triphala Kwatha* (TK, decoction of *Terminalia chebula* Retz., *Terminalia bellirica* Roxb. and *Phyllanthus emblica* Linn.) maintaining batch manufacturing records. Organoleptic and physicochemical analysis of media, i.e., TK and SM was carried out. **Results:** After *Shodhana*, golden yellowish luster of SM was completely lost and it turned into dark black coarse powder. The hardness went on decreasing and brittleness went on increasing. Average 532 g of *Shodhita* SM (88.67%) from 600 g of SM was obtained. Average time required for achieving red hot stage was 24.81 min. Analysis of the media revealed an increase in pH, specific gravity, and total solid contents. **Conclusion:** The adopted method for *Shodhana* of 600 g of SM can be considered as easy, convenient and standard.

Keywords: Chalcopyrite, Rasa Shastra, *Shodhana*, standardization, *Swarna Makshika*

Introduction

Ayurvedic system of medicine has its own methodology of drug manufacturing with the highest care toward safeguarding of products. The quality of prepared *Bhasma* (incinerated mineral) depends on the quality of raw material and standard manufacturing practices followed during its preparation. Standardization techniques help in ensuring safety and efficacy of the product. Different pharmaceutical processing techniques such as *Shodhana* (preliminary process of eliminating unwanted substances), *Marana* (incineration), *Jarana* (roasting) and *Amritikarana* (nectorization) are mandatory for manufacturing of Ayurveda medicines, especially when a formulation contains metals or minerals or some poisonous substances in its composition. *Shodhana* process is said to remove soluble impurities from raw material, adds some organic materials, and reduces toxicity to the great extent.[1]

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Swarna Makshika (SM) is an important mineral used in Ayurveda medicines and *Swarna Makshika Bhasma* (SMB) possesses *Vrishya* (aphrodisiac), *Rasayana* (immunomodulation), *Yogavahi* (targeted drug delivery), *Swarya* (good for voice) and *Chakshushya* (helpful in eye diseases) properties. It alleviates disorders caused by *Tridosha* (*Vata*, *Pitta,* and *Kapha*) and is widely used in the treatment of various diseases such as *Kshaya* (emaciation), *Prameha* (diabetes), *Basti Shoola* (bladder pain), *Pandu* (anemia), *Shotha* (edema) and *Kushtha* (skin disorders).[2] This mineral needs to be processed (*Shodhana*) meticulously before its conversion

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into *Bhasma*, and if used in impure form, it manifests a few untoward effects such as *Agnimandya* (dyspepsia), *Vanti* (vomiting), *Vibandha* (constipation), *Krimi* (worm infestation), *Vrana* (ulcer), *Kushtha* (skin disorders), *Netraroga* (eye diseases), *Daurbalya* (generalized weakness), *Kshaya* (emaciation), *Balanasha* (loss of strength) and even *Marana* (death).[3,4] Hence, its processing in terms of *Shodhana* becomes must to make it more potent therapeutically. Earlier scholars have documented on the pharmaceutical methods, but their observations were not comprehensive. Hence, this study is designed to put forward and ensure that those shortcomings are addressed. Earlier scholars have worked on pharmaceutical preparation of SM *Bhasma* with *Nimbu Swarasa* (fresh lemon juice) and *Saindhava Lavana* (rock salt) as *Shodhana* media.[5,6] Around 26 different methods of SM *Shodhana* are found in Ayurveda texts that can be categorized into five basic methods, namely *Swedana* (boiling), *Bharjana* (frying or roasting), *Nirvapa* (heating and quenching), *Mardana* (trituration) and *Putapaka* (incineration). These procedures use more than 35 liquid media.[7] Among these liquids, *Triphala Kwatha* (TK) is more frequently used, easily available, and affordable too. *Triphala* is also emphasized as an *Anupana* (vehicle) for SMB.[8] Hence, using TK as media in the processing may also enhance therapeutic qualities of SMB. Considering this, an attempt has been made to develop standard manufacturing procedures of SM *Shodhana* by *Nirvapa* in TK by following the classical methods.

Materials and Methods

Procurement of raw drugs

Raw samples of SM were collected from central hang wall of Malanjkhand copper project, Balaghat district, Madhya Pradesh, India,[9] and identified to have all acceptable characters such as *Swarna Sannibham* (having golden color and luster), *Guru* (heaviness), *Snigdha* (smooth), *Nishkona* (devoid of angle), and *Kalimam Vikirettattu Kare Ghrushtam* (leaves a black impression when rubbed on palm or white paper) [Figure 1].[10,11] Ground level of the sample collection area was 580 mean reduced level (mRL) and sample was collected from 380 mRL. Collected samples were quartz reef mineralization of chalcopyrite and pyrites. Dried *Triphala* (pericarp of *Terminalia chebula* Retz., *Terminalia bellirica* Roxb., and *Phyllanthus emblica* Linn.) was procured from Pharmacy, Gujarat Ayurved University, Jamnagar.

Method of preparation

The procedure of *Shodhana* was divided into two stages.

Preparation of Triphala Kwatha (decoction)

Individually coarse powder (#10) of *Triphala* was prepared and collected into a stainless steel vessel. The contents (4.2 kg) were added with eight parts (33.6 l) of potable water and left undisturbed overnight. On next morning, the contents were boiled over mild flame maintaining temperature in between 85°C and 95°C and reduced to one-fourth (8.4 l) of its initial volume with constant stirring. The contents were filtered through clean muslin cloth to obtain decoction.[12]

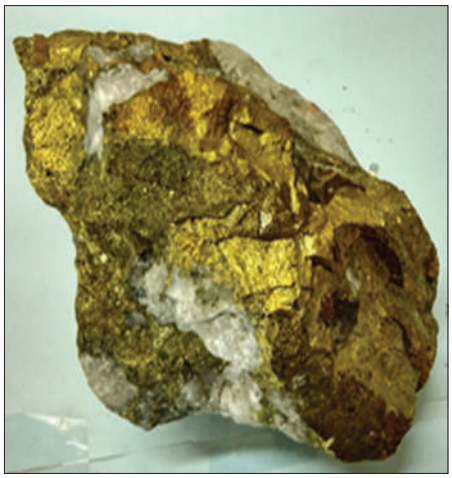

Figure 1: Unit Operative procedure of *Swarna Makshika Shodhana*. Raw *Swarna Makshika* as per the classical characteristics

Shodhana of Swarna Makshika by Nirvapa (quenching)

Raw SM was taken in a clean and dry *Khalva Yantra* (mortar and pestle), pounded well to prepare ½–1-cm small crystals [Figure 2], shifted to a clean and dry iron pan, and subjected to intense heat in charcoal furnace at about a temperature of 800°C–850°C [Figures 3 and 4]. Subsequently after achieving red hot stage [Figure 5], it was quenched in another vessel containing TK [Figure 6]. After self-cooling, SM was collected from the *Kwatha* and dried and the process of quenching was repeated for six more times. At the end of the seventh procedure, the contents were collected carefully, shade dried, powdered, labeled, and stored in airtight containers for further use [Figures 7-9].[13] The brief video of Unit Operative procedure of *Swarna Makshika Shodhana* was also created for clear perception.[14] The whole process was repeated in two more batches. Each time fresh and same amount of TK was taken. Temperature and volume of media before and after quenching were noted. Temperature of coal and SM was monitored by electric digital pyrometer. Weight of SM before and after quenching was noted.

Analysis

Organoleptic parameters

Organoleptic characters, i.e., color, odor, taste and feel of drug upon touch by sensory observations of SM as well as TK before and after *Shodhana* were noted.[15]

Physicochemical parameters

Physicochemical parameters of TK such as pH,[16] specific gravity (Sp. Gr.)[17] and total solid content[18] were determined before and after each *Nirvapa*. pH (5% aqueous suspension), loss on drying[19] and ash value[20] of raw and *Shodhita* SM were carried out according to standard methods as mentioned in the Ayurvedic Pharmacopoeia of India.

Results

During SM *Shodhana*, specific crackling sound was observed when SM was heated during the first quenching process, and few small particles of SM were escaping

Figure 2: *Swarna Makshika* converted to ½–1-cm particles

Figure 3: *Swarna Makshika* subjected to heat

Figure 4: Roasting of *Swarna Makshika* in iron pan

Figure 5: *Swarna Makshika* achieving the red hot stage

Figure 6: Quenching of *Swarna Makshika* in *Triphala Kwatha*

Figure 7: Decantation of *Swarna Makshika*

from the iron pan. Characteristic smell and fumes of sulfur (sulfur dioxide) were observed during the process. Sulfur fumes were found increased at the time of red hot stage of SM and complete cessation of sulfur fumes was observed during the seventh *Nirvapa*. As sulfur fumes produce irritation to the nose and throat, precautions should be taken during the *Shodhana* process. Brassy golden yellowish SM changed to black after the first and second *Nirvapa* and turned to blackish red by the fourth *Nirvapa*. By the end of the fifth *Nirvapa*, it turned

to dark black coarse powder. Brown-colored TK was turned to dark black and its consistency became thicker. Characteristic odor was observed during quenching.

Some particles were stuck to the vessel after quenching which needed to collect carefully. Details along with results obtained during *Nirvapa* process, changes observed in the media, and organoleptic characters of SM and TK are depicted at Tables 1-3, while the changes in physicochemical profiles of media and SM has been presented in Tables 4 and 5 and the equipment specifications in Table 6.

Discussion

Shodhana is a procedure of elimination of *Dosha* (impurity/toxicity/flaw) from the drug.[21] The term *Dosha* indicates not only impurities but also all that which makes the drug unsuitable for further process or therapeutic use.

Figure 8: *Swarna Makshika* after the third *Nirvapa*

Figure 9: *Swarna Makshika* after the seventh *Nirvapa*

Table 1: Observations during *Swarna Makshika Shodhana*

Batch	Number of *Nirvapa*	Temperature of coal (°C)	Temperature of SM in red hot stage (°C)	Total time taken to reach red hot stage (min)	Weight of SM (g)		
					Before quenching (g)	After quenching	Weight loss
I	1	845	436	35	600	595	5
	2	832	440	28	595	586	9
	3	847	410	20	586	578	8
	4	825	445	18	578	564	14
	5	816	423	16	564	558	6
	6	850	451	15	558	549	9
	7	812	431	15	549	542	7
	Average	832.43	433.71	21.00		-	58
II	1	811	412	40	600	593	7
	2	837	450	34	593	581	12
	3	822	426	29	581	570	11
	4	832	435	21	570	559	11
	5	819	444	20	559	543	16
	6	849	395	18	543	534	09
	7	820	418	19	534	521	13
	Average	827.14	425.71	25.86			79
III	1	848	428	42	600	592	8
	2	808	408	36	592	583	9
	3	825	430	31	583	577	6
	4	838	385	26	577	564	13
	5	815	433	21	564	553	11
	6	850	390	19	553	546	7
	7	828	441	18	546	533	13
	Average	830.28	416.43	27.57			67
Average of three batches (%)		829.95	425.28	24.81	600	532 (88.67)	68 (11.33)

Table 2: Weight loss and temperature of *Triphala Kwatha* after each *Nirvapa*

Batch	Number of *Nirvapa*	Temperature of *Kwatha* before *Nirvapa* (°C)	Temperature of *Kwatha* after *Nirvapa* (°C)	Loss of media after *Nirvapa* (ml)			
				Before quenching	After quenching	Weight loss	Percentage loss
I	1	33	82	1200	1150	50	4.16
	2	32	79	1200	1140	60	5.00
	3	30	84	1200	1160	40	3.33
	4	31	86	1200	1155	45	3.75
	5	33	78	1200	1150	50	4.16
	6	34	88	1200	1130	70	5.83
	7	32	90	1200	1145	55	4.58
	Average	32.14	83.85	1200	1147.14	52.86	4.40
II	1	36	80	1200	1152	48	4.00
	2	37	83	1200	1126	74	6.17
	3	34	75	1200	1143	57	4.75
	4	35	90	1200	1120	80	6.66
	5	36	85	1200	1132	68	5.67
	6	33	82	1200	1165	35	2.92
	7	35	76	1200	1140	60	5.00
	Average	35.14	81.57	1200	1139.71	60.28	5.02
III	1	38	84	1200	1134	66	5.50
	2	39	89	1200	1121	79	6.58
	3	37	79	1200	1144	56	4.67
	4	35	90	1200	1136	64	5.33
	5	36	85	1200	1150	50	4.16
	6	34	80	1200	1130	70	5.83
	7	36	78	1200	1115	85	7.08
	Average	36.43	83.57	1200	1132.85	67.14	5.59
Average of three batches		34.57	82.99	1200	1139.90	60.09	5.00

Table 3: Organoleptic characters of *Swarna Makshika* and *Triphala Kwatha*

Parameters	*Swarna Makshika*		*Triphala Kwatha*	
	Before *Shodhana*	After *Shodhana*	Before *Shodhana*	After *Shodhana*
Color	Brassy golden yellow	Blackish	Dark brown	Dark black
Odor	Odorless	Odorless	Characteristic	Sulfurous
Texture	Hard crystalline, Shiny	Brittle, course powder	Liquid	Viscous liquid
Taste	Metallic	Metallic	Astringent	Astringent

In case of metals and minerals, it is a physicochemical and therapeutic transformation of a substance making it feasible for the next process (*Marana*) or directly for therapeutic use. It is a mandatory process of metals and minerals that help to expose maximum surface area of drug for chemical reactions and also in impregnation of organic materials and their properties into the drug. This makes the mineral brittle and helps in particle size reduction. There are different types of *Shodhana* methods mentioned in Ayurveda, and *Nirvapa* is one among them.

During *Shodhana,* gravimetrically double amount of TK was taken for quenching. This requirement may change depending on the vessel specifications. Ideally, cylindrical vessels with suitable wide mouth will be justifiable. Ideally, quantity of liquid should be sufficient enough to completely immerse the material being quenched. The process of *Nirvapa* was carried out first by making pieces of SM crystals approximately in ½–1-cm dimensions. Big pieces of SM could not get red hot because of their size during *Nirvapa* process. Considering this, a powder (60# mesh) form of SM was attempted that was adhering to the iron pan and leading to difficulty in quenching and significant loss. Hence, in subsequent practicals, crystals of approximately ½–1 cm were prepared, and the same process was repeated.[22] It was observed that particles of SM became red hot uniformly and do not adhere to the iron vessel and suitable for *Nirvapa.* While performing *Nirvapa*, strong sulfur smell was observed from the first to the fourth cycle of quenching that was due to sulfur dioxide (SO_2) vaporization and oxidation of SM.[23] From the fifth cycle onward, the intensity of odor was reduced, and by the seventh cycle, no odor was observed.

Table 4: Changes in the physicochemical constants of *Triphala Kwatha* before and after *Nirvapa*

Batch	Before/after *Nirvapa*		pH	Specific gravity	Total solid contents (%)
I	Before *Nirvapa*		3.25	1.057	15.5
	After *Nirvapa*	1	3.34	1.058	17.0
		2	3.37	1.063	16.0
		3	3.40	1.067	18.0
		4	3.46	1.074	19.0
		5	3.53	1.079	23.0
		6	3.67	1.080	20.0
		7	3.88	1.086	25.0
		Average	3.52	1.072	19.71
II	Before *Nirvapa*		3.24	1.050	15.1
	After *Nirvapa*	1	3.36	1.062	15.1
		2	3.38	1.060	16.0
		3	3.47	1.072	18.0
		4	3.54	1.076	18.0
		5	3.59	1.079	19.0
		6	3.63	1.081	20.0
		7	3.74	1.084	23.0
		Average	3.53	1.074	21.86
III	Before *Nirvapa*		3.25	1.057	15.3
	After *Nirvapa*	1	3.30	1.059	16.0
		2	3.37	1.060	17.0
		3	3.47	1.075	19.0
		4	3.51	1.070	20.0
		5	3.64	1.080	22.0
		6	3.69	1.080	23.0
		7	3.84	1.090	24.0
		Average	3.55	1.077	20.14
Average of three batches			3.53	1.074	20.57

Table 5: Physicochemical parameters of *Swarna Makshika* before and after *Shodhana*

Parameter	*Swarna Makshika*	
	Before *Shodhana*	After *Shodhana*
pH value (5% aqueous solution)	4.24	4.52
Loss on drying at 110°C (%w/w)	0.67	0.54
Ash value (%w/w)	96.66	91.48

$$2CuFeS_2 + 7.5O_2 \rightarrow 2CuSO_4 + Fe_2O_3 + 2SO_2$$

$$2CuFeS_2 \rightarrow CuS + 2FeS + S$$[24]

At early stages of *Shodhana,* cracks were seen on the surfaces of SM flakes, and finally, some coarse powder was observed. Repeated heating and cooling of SM flakes causes disruption in compression-tension equilibrium and leads to cracks on the flake surface (stress corrosion theory).[25] During red hot state, different compounds such as pyrrhotite ($Fe_{1-x}S$), pyrite (FeS_2), bornite (Cu_5FeS_4) and chalcocite (Cu_2S) will be formed on the surface of SM flakes.[26] Theory of thermal expansion states that expansibility differs from metal and mineral to compound on heating. In general, expansibility of compound is less than metal.[27] Hence, repeated heating leads to breaking of SM flakes into coarse to fine powder. Copper and iron are converted to oxide form at red hot state by reacting with atmospheric oxygen.[28] Studies reported oxidized iron and copper compound as Fe_2O_3 (ferric oxide) and CuO (cupric oxide), and rest sulfides are FeS_2 (iron disulfide), $FeSO_4$ (ferrous sulfate), Cu_2S (cuprous sulfide) and CuS (copper sulfide).[29] Raw SM may contain physical impurities such as unwanted rock or gangue minerals, typically silicate minerals, or oxide minerals for which there is often no value.[30] These visible impurities were removed by hand picking method before *Nirvapa*. SM is often found with a variety of other trace elements such as cobalt (Co), nickel (Ni), manganese (Mn), zinc (Zn) and tin (Sn) substituting for copper and iron (Fe), selenium (Se) and arsenic (As) substitute for sulfur and trace amount of silver (Ag), gold (Au), platinum (Pt), lead (Pb), vanadium (V), chromium (Cr), indium (In), aluminum (Al) and antimony (Sb).[31,32] It is most likely that many of these elements are present in finely intergrown mineral within the SM (chalcopyrite). During the process of red hot in iron pan on high temperature, chemical impurities such as extra sulfur in the form of sulfur dioxide (SO_2) and arsenic may be get vaporized[13] while other get oxidized and after heating, it was instantly quenched in the TK. It facilitates the media to enter inside the drug easily by which the remaining blemishes will get separated or dissolved in the liquid media as well as the therapeutic property of TK introduced into SM. Instant quenching is important because repeated immediate cooling after heating leads to breaking of the material. Yellowish and golden shining of SM was lost completely after *Shodhana* process and turned into dark black coarse powder.

An average temperature of SM in red hot stage was noted as 425.28°C. Average time required to get red hot was noted around 24.81 min. There was a continuous decrease in the period of reaching to the red hot stage of SM during first to seventh process of *Nirvapa*, which may be due to the conversion of oxides and sulfides of SM. At a later stage of *Shodhana*, surface area of SM was increased due to repeated heating and quenching. Hence, SM took less time to become completely red hot [Table 1]. An average loss of SM was noted as 68 g (11.33%) after *Nirvapa*. After seventh *Nirvapa*, fine particles of SM were found settled to the bottom of vessel. Loss was observed during *Nirvapa* as some fine particles were lost through filtering and a fraction might have entered into TK. Documentation of weight changes is an essential part for the drug standardization. Assessment of final weight with relation to initial weight will give an idea to choose the weight of the initial material to procure the desired quantity of finished product. Large-scale production will substantially decrease the expenditure in relation to the quantity as the expenditure on labor is not going to change by increasing quantity up to some level.

The formation of oxides scales is also responsible for increase in pH after every purification steps. Oxides (Fe and Cu oxides) are mainly basic in nature, so it can raise the pH after every

Table 6: Equipment and their specifications in the process of SM *Shodhana*

Equipment and their specifications	Value
Iron pan/Kadhai (for *Swarna Makshika*)	
Depth	25 cm
Diameter	100 cm
Circumference	250 cm
Weight	4.5 kg
Stainless steel vessel (for *Triphala Kwatha* during quenching)	
Depth	21 cm
Diameter	17.5 cm
Circumference	55 cm
Capacity	5 l
Stainless steel vessel (for *Triphala Kwatha* preparation)	
Depth	45.72 cm
Diameter	58.42 cm
Circumference	194.31 cm
Capacity	50 l
Thermocouple	
Ceramic made with 1200^{o} with digital display	
Iron ladle	
Length	106.68 cm
Stainless steel ladle	
Length	30 cm
Cotton cloth	
Length	100 × 100 cm
Weighing balance	
Maximum capacity	25 kg
Minimum capacity	10 g
Measuring jar	
Maximum capacity	2 l
Stainless steel tray	
Length	20 cm
Breadth	20 cm

quenching. Specific gravity of TK before *Nirvapa* was 1.057 and after *Nirvapa* was 1.074. Specific gravity is defined as the weight of a given volume of the liquid at the stated temperature as compared with the weight of an equal volume of water at the same temperature.[17] Increase of specific gravity in TK after *Nirvapa* is due to the presence of SM particles in TK. Total solid content of the TK before *Nirvapa* was 15.5 and after *Nirvapa* was 20.57. The total solids are the measure of the combined content of all inorganic and organic substances contained in a liquid. The soluble content determines the amount of constituents in a given sample of drug.[18] Total solid content of the media after *Shodhana* procedure were increased slightly just because of the fine particles of SM which came into it during *Shodhana* procedure. Analysis of the media revealed that increase in pH, specific gravity, and total solid contents [Table 4] justifies this. An average 60.09 ml (5%) loss was observed in TK, the loss which might be due to the intense heat of the material at the level of which some fraction of moisture gets evaporates. As fuel, an average 2-kg soft coal and 1-kg hard coal for each process of *Nirvapa* is needed. Average temperature of coal during red hot stage was noted as 829.95°C.

Role of *Triphala*

Triphala mainly consists of tannins, gallic acid, chebulinic acid, ascorbic acid (Vitamin C) and phenolics.[33] Ascorbate (one of a number of mineral salts of ascorbic acid [Vitamin C]) has been known to antagonize the intestinal absorption of copper. More recent studies have characterized a post absorption role for ascorbate in the transfer of copper ions into cells. The vitamin reacts directly or indirectly with ceruloplasmin, a serum copper protein, specifically stabilizing the bound copper atoms and facilitating their cross-membrane transport. Ascorbate at physiological levels and above impedes the intracellular binding of copper to Cu, Zn and superoxide dismutase. The mechanism is unclear but nonetheless suggests both positive and negative regulatory functions for ascorbate in copper metabolism.[34,35] Ascorbic acid increases the bioavailability of Fe by converting Fe^{+3} to Fe^{+2}, while phenolics can reduce the bioavailability of Fe by binding, for example, tannins. Excess of ascorbic acid and a lack of dietary tannins have both been suggested as contributing to clinical/pathological Fe storage disease.[36] *Triphala* is a mild laxative and thereby counteracts the constipating property of iron and copper. Thus, it is mentioned in maximum *Shodhana* procedures of various metals and minerals.

Precautions

1. Procurement of raw SM should be done as per the classical characteristics of acceptable variety
2. Preparing crystals of around ½–1 cm will facilitate *Nirvapa* procedure
3. Protective measures should be taken to avoid contact of red hot particles of SM coming out of the iron pan
4. Mouth and nostrils are to be covered with a mask to avoid sulfur fumes. Eyes are also to be covered appropriately (protective glasses)
5. On observing red hot stage, contents are to be quenched immediately without delay
6. While quenching, maximum care should be taken to avoid loss and accidents
7. Level of liquid should be sufficiently maintained to immerse the mineral completely
8. Collection of SM should be done carefully after quenching. Fresh liquid should be used in each experiment
9. All observations should be recorded carefully during each experiment.

Conclusion

Process standardization and quality control is a mandatory task and an essential requirement for good manufacturing practices to assure the quality and quantity of final product. Repeated quenching in the decoction of *Triphala* imparts some microelements that contribute to nullify possible toxic nature of raw material and help in increasing therapeutic attributes of SM. Average 88.67% yield was obtained after

Shodhana. Results of the present study ensure the uniformity of the operative procedures, and those can be followed in SM *Shodhana* successfully.

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Conflicts of interest

There are no conflicts of interest.

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Scientific Insights in the Preparation and Characterisation of a Lead-based *Naga Bhasma*

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Nagarajan, *et al.*: Science of Preparation of Naga Bhasma

***Naga bhasma* is one of the herbo-metallic preparations used in *Ayurveda*, a traditional Indian System of Medicine. The preparation of *Naga bhasma* involves thermal treatment of *'Naga'* (metallic lead) in a series of quenching liquids, followed by reaction with realgar and herbal constituents, before calcination to prepare a fine product. We have analysed the intermediates obtained during different stages of preparation to understand the relevance and importance of different steps involved in the preparation. Our results show that *'Sodhana'* (purification process) removes heavy metals other than lead, apart from making it soft and amenable for trituration. The use of powders of tamarind bark and peepal bark maintains the oxidation state of lead in *Jarita Naga* (lead oxide) as Pb^{2+}. The repeated calcination steps result in the formation of nano-crystalline lead sulphide, the main chemical species present in *Naga bhasma*.**

Key words: *Sodhana* (purification), *naga bhasma*, lead, lead oxide, lead sulphide, calcination

Ayurveda, an ancient system of medicine, has been practiced in India since time immemorial. Plants, minerals, molecules from animal sources are used for the preparation of *Ayurvedic* drugs. *Bhasmas* are one of the main components in *Ayurvedic* system of medicine and are used to treat various chronic ailments and maintain good health of an individual. *Bhasmas* are herbo-metallic ashes in which the metal is calcined along with various herbal ingredients to form complexes[1,2]. These complexes should neither contain free metal nor contain free organic constituents, whose presence in *bhasma* indicates improper calcination[3]. Lead is one of the seven metals used for preparation of *bhasma*. Several studies have reported the presence of heavy metals like lead, mercury, arsenic and others in high amounts in *bhasma*[4-6]. Owing to its insoluble nature, lead sulphide (PbS) is the least toxic form of lead[7].

Naga bhasma, an *Ayurvedic* lead-based herbo-metallic medicine, has its history of medicinal applications dating several centuries back. *Naga bhasma*, with its predominant chemical species being PbS, administered at 6 mg/kg body weight was found to be nontoxic in animal model[7]. *Naga bhasma* has specific regenerative potential on germinal epithelium of testes in $CdCl_2$ administered albino rats[8]. In addition to treating diabetes mellitus, *Naga bhasma* has been prescribed for certain disorders related to liver, spleen and skin. Few clinical trials have also shown that *Naga bhasma* considerably reduces blood glucose level in diabetic patients[9]. Singh *et al*. reported the presence of hydrogenated amorphous carbon in *Naga bhasma*, an indication for possible presence of organic moieties[10]. Preparation protocol plays a major role in deciding the therapeutic efficacy as well as the toxic effects of *bhasma*s. According to *Ayurvedic* experts, nonconformity to the preparation protocol causes toxicity, probably due to incomplete transformation of free metal[11]. *Naga bhasma* procured from different manufacturers revealed differences in their composition, which could be attributed to different procedures adopted for preparation[3,12]. Hence, a standard operating procedure (SOP) for the preparation of *Naga bhasma* is essential to standardise the preparation[13]. Understanding the physico-chemical changes that occur during various stages of preparation, through characterisation of intermediates, will promote conformity to preparation

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protocol. This paper focuses on such a study for preparation of *Naga bhasma*.

MATERIALS AND METHODS

Preparation of *Naga bhasma*:

Naga bhasma was prepared using the procedure described in *Ayurvedic* Formulary of India[14]. The process flow diagram for the preparation of *Naga bhasma* is shown in fig. 1. The first step in the preparation of *Naga bhasma* is *sodhana* (a purification step), which involves sequential quenching in *tila thaila* (gingelly oil), *takra* (butter milk), *kanjika* (rice gruel), *gomuthra* (cow's urine) and *kulatha kasaya* (horse gram decoction). About 2 kg of metallic lead was melted and immersed in 2 l of treating liquid. The quenched material was filtered out and this was repeated thrice with each treating liquid listed above. The *Naga* obtained at this stage is called *Suddha Naga*.

About 500 g of *Aśvatthacūrṇa* (peepal bark powder) and 500 g of *cincātvakcūrṇa* (tamarind bark powder) were mixed with 2 kg of *Suddha Naga* and heated until fine powders were formed. This is referred to as *Jarita Naga*. About 1.8 kg each of *Jarita Naga* and *manashila* (realgar; arsenic sulphide) were mixed with 1.5 l of *kanjika* and triturated well. This mixture was made into *cakrikas*, (thin flat disks), sun dried and subjected to *puta*. In traditional literature, *puta* refers to a process of controlled heating and cooling (calcination) of herbo-mineral mixture to achieve *bhasmikaran* or ashing. The material to be subjected to *puta* was taken in an earthen vessel and closed with another inverted earthen vessel. The interface between the two vessels was sealed with a clay-smeared cloth. This arrangement is known as *Saravasamputa*. *Puta* process for preparation of *Naga bhasma* involves 50 cycles of *arddha gajaputa* and 10 cycles of *gajaputa*, performed in a brick-lined calcination chamber measuring 90×90×90 cm. In a typical *Arddha gajaputa* step, *saravasamputa* was placed inside a heap of 125-150 cow dung cakes such that, equal number of cow dung cakes were above and below the *Saravasamputa*. The cow dung cakes were ignited to supply the thermal energy required for calcination. The *gajaputa* step is similar to *arddha gajaputa*, which utilises double the number of cow dung cakes (250-300) for calcination. The intermediate obtained after each *arddha gajaputa* and *gajaputa* step was triturated with a mixture of purified *manashila* (realgar; arsenic sulphide) and sufficient quantity (1.5 l) of *kanjika*. This mixture was sun dried to prepare *cakrikas* (thin flat disks) for subsequent *arddha gajaputa* and *gajaputa* steps.

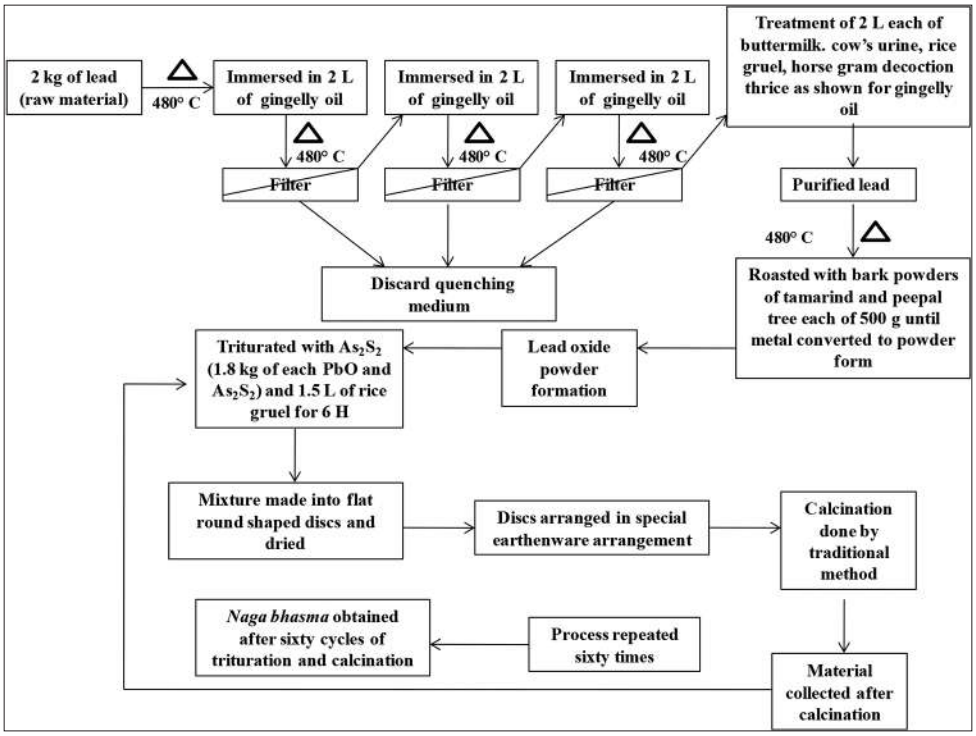

Fig. 1: Flow sheet for the preparation of Naga bhasma.

Elemental analysis:
An X-ray fluorescence spectrometer (S8 Tiger, Bruker AXS, Germany) equipped with 4 kW, Rh anode X-ray tube was used to determine the elemental composition. The aluminium cups (sample holder) were filled with 2 g of boric acid, on top of which 1 g of the sample was added. These were pelletised using a 25-tonne hydraulic press to obtain 34 mm diameter pellets of moderate thickness.

Powder X-ray diffraction analysis:
A powder X-ray diffractometer (D8 Focus, Bruker AXS, Germany) equipped with Cu anode (40 kV, 30 mA) and photo scintillation detector was used to record the diffraction patterns of the samples. Scan was performed over an angular range (2θ) of 10-60° at the rate of 0.01°/sec.

Morphological analysis:
The surface morphology of samples was observed using a cold Field Emission Scanning Electron Microscope (JSM 6701F, Jeol, Japan) at an acceleration voltage of 3 kV. A small quantity of the sample was sprinkled on a carbon tape mounted on a brass stub and sputter coated with gold, before imaging.

Spectroscopic analysis:
Fourier Transform Infrared (FTIR) spectra of the samples were recorded between 4000 and 400/cm in FTIR spectrometer (Spectrum 100, Perkin Elmer, USA). The samples were prepared by mixing with KBr and pelletising them for analysis using diffuse reflectance accessory.

Electrospray ionisation mass spectrometry:
For identification of key compounds present in the bark of *Tamarindus indica* and *Ficus religiosa*, their aqueous extracts were analysed by liquid chromatography/electrospray ionisation mass spectrometry (LC/ESI-MS) using Bruker UHPLC 3000 chromatography coupled to quadrupole ToF mass selective detector (micrOTOF-QII). The experimental conditions used for LC-MS analysis of *Tamarindus indica* and *Ficus religiosa* were followed as per procedure described by Krishnamachary *et al.*[15].

RESULTS AND DISCUSSION

Table 1 show the elemental composition of various intermediates obtained during *samanya sodhana*, from which it is was observed that the relative mass percentage of lead in the intermediates increased during the *samanya sodhana* treatment. The raw material (lead) contained other metals like iron, ruthenium, silicon, molybdenum as impurities. The treating liquids used (gingelly oil, butter milk, cow's urine, rice gruel and horse gram decoction) have been shown to form soluble-chelates with metals, leading to their removal. This resulted in the increase of relative mass percentage of lead from 97.13% in the raw material to 98.98% in the intermediate obtained after treatment with the horse gram decoction. Elemental composition of *Jarita Naga*, given in Table 2, shows that more than 90% of lead exists in oxide form.

Tables 3 show the elemental composition of *cakrikas* and the intermediate obtained after the first cycle of *Arddha gajaputa*. It is interesting to note that the arsenic content present in the *cakrikas* due to addition of realgar is reduced to below detectable limit after the first cycle of *Arddha gajaputa*.

Fig. 2 shows the scanning electron micrographs

TABLE 1: ELEMENTAL COMPOSITION OF RAW MATERIAL AND INTERMEDIATES OBTAINED DURING *SODHANA*

Element	Elemental composition in mass percentage					
	Raw material	*Taila*-treated sample	*Takra*-treated sample	*Kanjika*-treated sample	*Gomuthra*-treated sample	*Kulatha kasaya*-treated sample
Pb	97.13	98.97	98.27	99.33	98.49	98.98
Fe	0.45	ND	0.16	ND	0.18	ND
Ru	0.27	ND	ND	ND	ND	ND
Ca	0.27	ND	ND	ND	ND	ND
Si	0.26	0.06	0.26	ND	0.25	0.09
Na	0.25	ND	ND	ND	ND	ND
K	0.20	ND	0.04	ND	ND	ND
Mo	0.18	ND	ND	ND	ND	ND
As	ND*	ND	ND	ND	ND	ND

*ND=Not detected by X-ray fluorescence spectroscopy

TABLE 2: ELEMENTAL COMPOSITION OF *JARITA NAGA*

Sample	Elemental composition in mass percentage						
	PbO	CaO	K_2O	MgO	P_2O_5	Fe_2O_3	SiO_2
Jarita Naga	94.21	2.79	1.08	0.43	0.25	0.26	0.15

TABLE 3: ELEMENTAL COMPOSITION OF CAKRIKAS FOR THE FIRST *ARDDHA GAJAPUTA* CYCLE AND THE INTERMEDIATE AFTER FIRST *ARDDHA GAJAPUTA* CYCLE

Sample	Composition in mass percentage							
	Pb	O	Ca	K	Mg	S	Si	As
Cakrikas	13.04	35.08	0.30	0.45	3.56	12.55	4.78	19.99
Intermediate after 1st cycle of Arddha gaja puta	30.92	25.32	0.51	0.73	0.31	5.27	10.54	ND*

*ND=Not detected by X-ray fluorescence spectroscopy

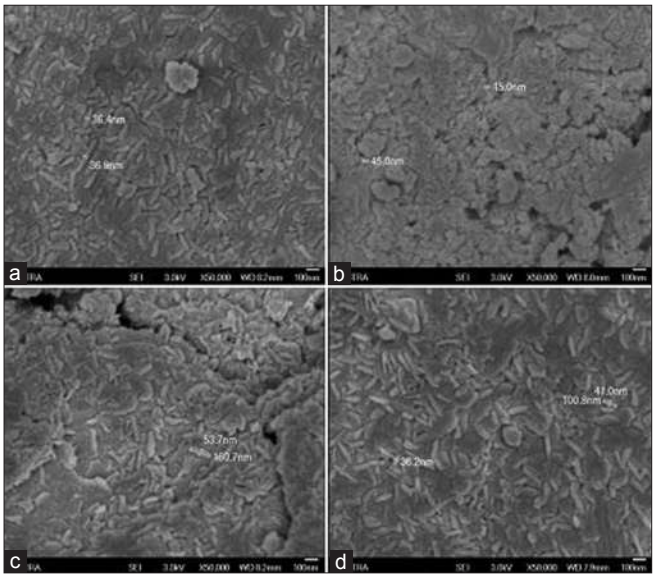

Fig. 2: Surface morphology of the intermediates obtained during various stages of sodhana.
(a) Taila-treated intermediate; (b) gomutra-treated intermediate; (c) kanjika-treated intermediate; (d) kulatha kasaya-treated intermediate.

of the intermediates obtained after each stage of *sodhana* during the preparation of *Naga bhasma*. These micrographs help to understand the changes in gross morphology during various stages of *sodhana*. It may be observed that there are substantial changes in the morphology of intermediates during *sodhana* (fig. 2). A common feature in the micrographs of intermediates is the appearance of rod-shaped, nanoscale structures on the surface. The width of the nanostructures lies between 30 and 50 nm, while the length ranges between 100 and 160 nm. High thermal stresses induced in the material during quenching are expected to form microcracks leading to increased surface area[16,17]. *Sodhana* has also led to substantial softening of the material transforming ductile lead to a brittle form, enabling ease of subsequent processing.

The characterisation of bark of *Tamarindus indica* and *Ficus religiosa* is essential to identify the key organic components present in them and their possible role in the preparation of *Naga bhasma*. The LC-MS/MS spectra of aqueous extract of bark of *Tamarindus indica* are shown in fig. 3. It was observed that the aqueous extract contained characteristic markers (proanthocyanidin B1 Dimer, proanthocyanidin C1, catechin and bergenin) reported for *Tamarindus indica* (fig. 3a-d). Bergenin has been reported to possess immumomodulatory effects. Also the polyphenols from *Tamarindus indica* possess excellent chelating ability that can serve to form complexes with metal ions.

Fig. 4 shows the LC-MS/MS spectra of aqueous extract of bark of *Ficus religiosa*. It was observed that the extract contained proanthocyanidin B1, proanthocyanidin C1, chlorogenic acid, kaempferol-3-galactoside-6''-rhamnoside-3'', caffeic acid and epicatechin, which are characteristic markers for *Ficus religiosa* (fig. 4a-f). The proanthocyanidins, chlorogenic and polyphenols from *Ficus religiosa* can form metal ion chelates. The flavonoids epicatechin and kaempferol present in *Ficus religiosa* are well-known antioxidants and antiinflammatory agents.

Fig. 5a shows the X-ray diffraction pattern of *Jarita Naga*, which is observed to be a crystalline material with diffraction peaks at 2θ of 26.5°, 30.5° and 54.0° characteristic of lead oxide (PbO). The analysis of various oxides present in *Jarita Naga* revealed the presence of PbO to the extent of 94.21%, with other oxides being CaO, K_2O and MgO (Table 2). These minerals may be incorporated from the bark of *Tamarindus indica* and *Ficus religiosa* during treatment of purified lead with the plant ingredients. The FTIR spectra of *Jarita Naga* reveal the presence of absorption band around 640/cm, which may be attributed to Pb–O bond (fig. 5b). This correlates very well with the X-ray diffraction patterns and elemental analysis.

From the spectroscopic and diffraction analyses of *Jarita Naga*, it is observed that the treatment of intermediate after *sodhana* with the powders of peepal bark and tamarind bark results in oxidation of metallic lead to lead oxide (*Jarita Naga*). The modification of

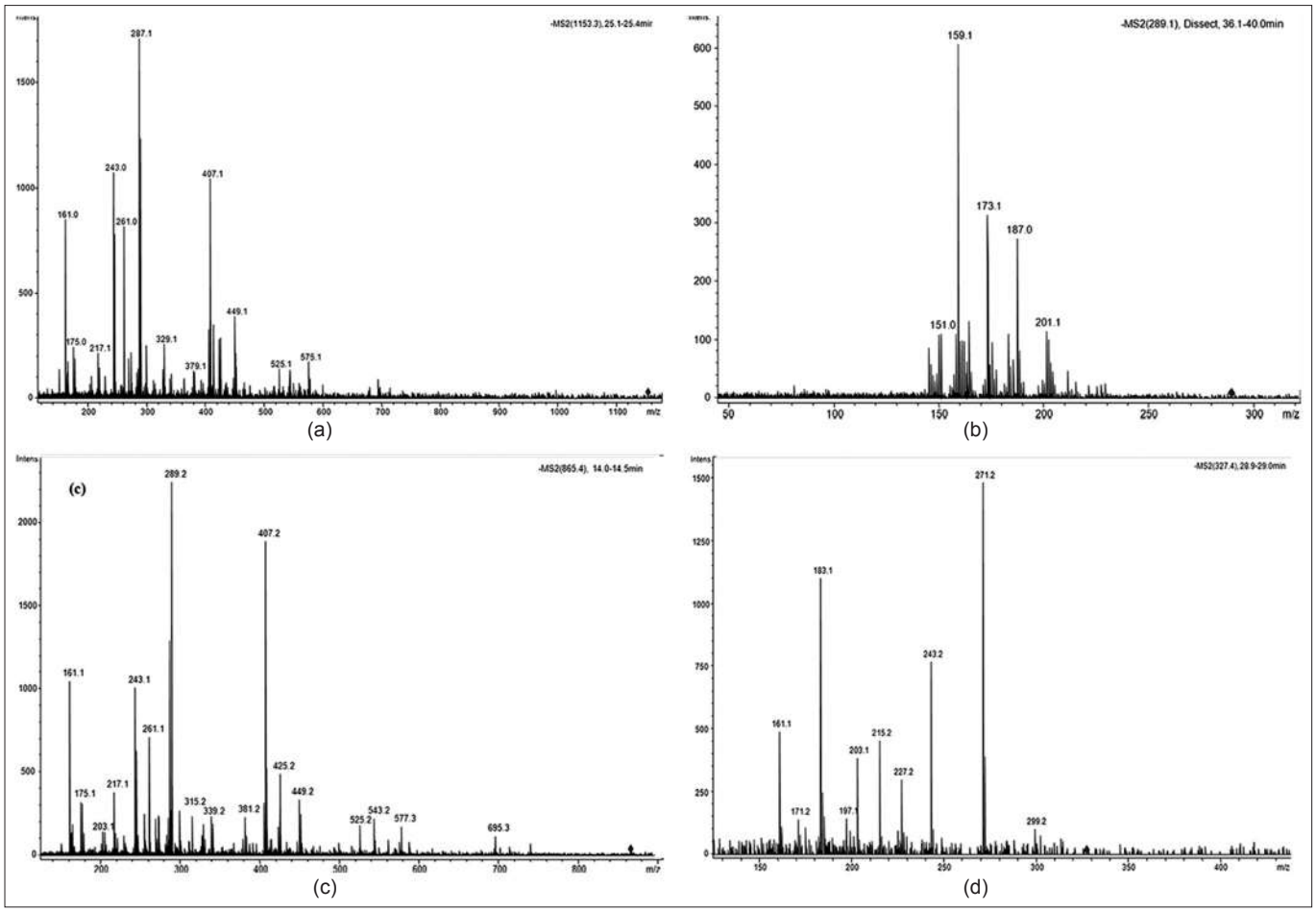

Fig. 3: LC-MS/MS pattern of aqueous extract of bark of *Tamarindus indica*.
(a) Proanthocyanidin B1 Dimer; (b) proanthocyanidin C1; (c) catechin and (d) bergenin.

morphology of metallic lead as a result of *sodhana* facilitated the oxidation reaction induced by treatment with these plant ingredients. When molten lead is exposed to air for longer duration of time without these plant ingredients, the same gets converted to its oxides, PbO and PbO_2. Of these two forms of lead oxide, PbO is useful for further transformation to *Naga bhasma*. The use of peepal bark and tamarind bark in the preparation of *Jarita naga* might have been probably aimed at reducing PbO_2 to PbO.

As evident from the XRF, XRD and FTIR results, major constituent of *Jarita Naga* is PbO. The *cakrikas* for the first *arddha gajaputa* cycle were made by grinding *Jarita Naga* (PbO) with realgar (As_2S_2) and *kanjika* (rice gruel). During this step, PbO is converted to PbS and As_2O_3 is formed through a slow reaction. The use of *kanjika* (rice gruel) facilitates wet grinding, leading to increased contact between the solid reagents (*jarita naga* and realgar) through decrease of particle size and increase of surface area[17-20]. The elemental compositions of *cakrikas* for first cycle of *arddha gajaputa* (made by grinding *jarita naga* with realgar and *kanjika*) and the intermediate after the first *arddha gaja*puta cycle are compared in Table 3.

A comparison of elemental composition of *cakrikas* for the first *arddha gajaputa* cycle and the intermediate from the first *arddha gajaputa* cycle (Table 3) indicates that though arsenic was present in the *cakrikas* that were subjected to *arddha gajaputa*, the intermediate obtained after first *arddha gajaputa* cycle did not contain arsenic. This may be understood from the study of temporal variation of temperature during a typical *arddha gajaputa* cycle shown in fig. 6. High temperature prevails during calcination facilitating conversion of arsenic sulphide (As_2S_2) to arsenic oxide (As_2O_3) and the conversion of PbO to PbS[21]. The boiling point of arsenic oxide (As_2O_3) is about 465° and hence would have vaporised during the *Arddha gajaputa* cycle where

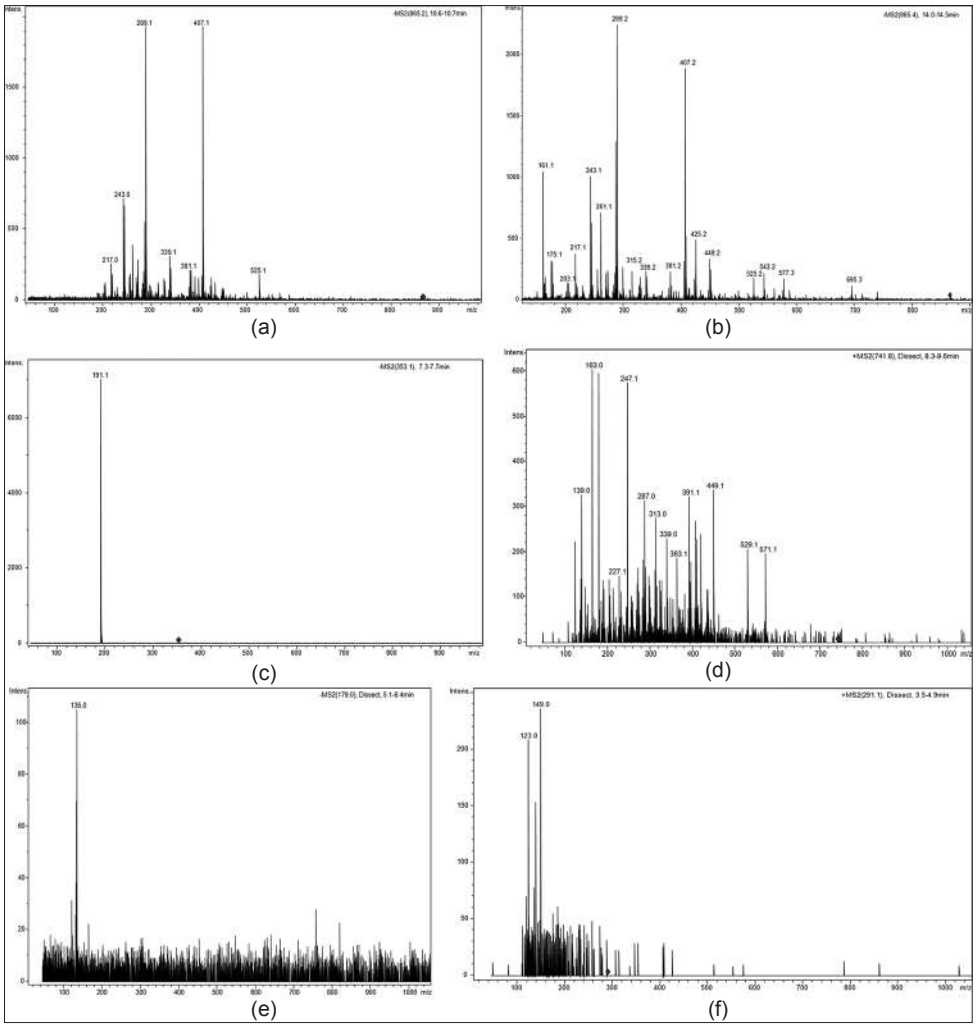

Fig. 4: LC-MS/MS pattern of aqueous extract of bark of *Ficus religiosa*.
(a) Proanthocyanidin B1; (b) proanthocyanidin C1; (c) chlorogenic acid; (d) kaempferol-3-galactoside-6′′-rhamnoside-3′′; (e) caffeic acid and (f) epicatechin.

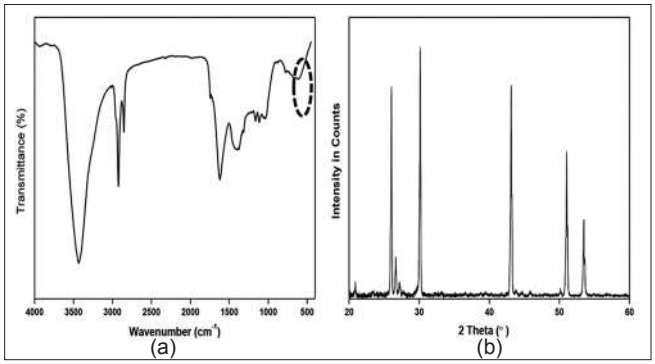

Fig. 5: Characterisation of jarita Naga.
(a) X-ray Diffraction pattern and (b) FTIR spectrum of jarita Naga.

temperature of above 800° was reached (fig. 6). This is confirmed from the X-ray diffraction patterns of the intermediate after the first *Arddha gajaputa* cycle, which shows diffraction peaks characteristic of PbS only (fig.7).

PbS is known to react with PbO when heated, leading to formation of metallic lead and sulphur dioxide[22].

$$PbS+2PbO \rightarrow 3Pb+SO_2$$

The addition of realgar during each *gaja puta* cycle may probably be aimed at suppressing the above reaction. The addition of realgar in excess quantity reacts with any unreacted PbO transforming the same to PbS. This ensures that the intermediates do not contain elemental lead.

Fig. 8 shows the powder X-ray diffractogram obtained after different *Arddha gajaputa* cycles. It was observed that the intermediates were crystalline PbS and with increase in *arddha gajaputa* cycle, there was increase in degree of crystallinity. Fig. 9 shows the morphology of the intermediates obtained after different *arddha gajaputa* cycles exhibiting

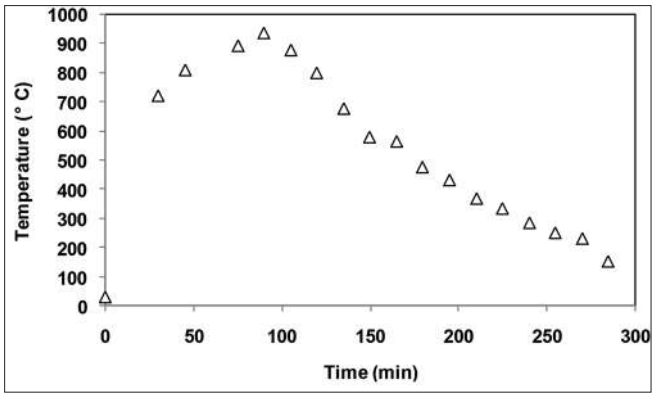

Fig. 6: Temporal variation of temperature during Arddha gajaputa. The maximum temperature of 937° was observed at about 90 min.

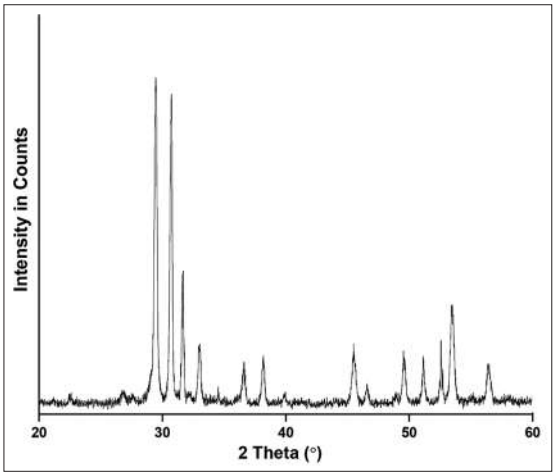

Fig. 7: X-ray Diffraction pattern of intermediate obtained after first *arddha gajaputa* cycle showing the presence of lead sulphide.

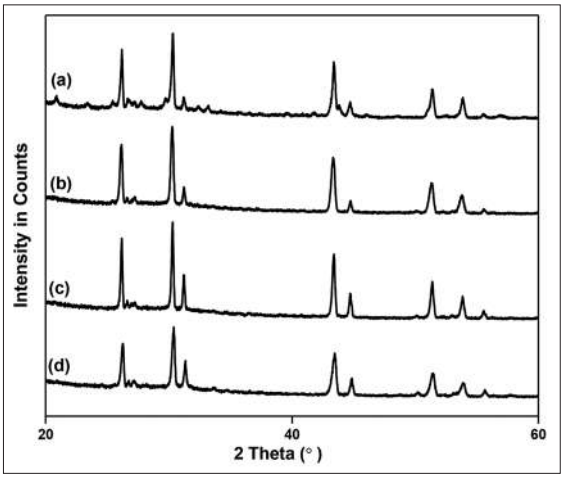

Fig. 8: X-ray diffraction patterns of intermediate obtained after different *arddha gajaputa* cycles.
(a) 20th *puta* cycle, (b) 25th *puta* cycle, (c) 30th *puta* cycle and (d) 35th *puta* cycle.

nanoscale features. The average size of the nanoparticles was observed to decrease, accompanied by an increase in monodispersity during subsequent *arddha gajaputa* cycles. Some of our results are in qualitative agreement with those reported in literature for preparation of *naga bhasma*[7,21]. Comparison of characteristics of intermediates could not be carried out due to lack of such information in earlier works. The existing *bhasma* preparation techniques follow different procedures for *Naga bhasma* and the protocol may differ in terms of raw materials utilised, variety of plants used and number of calcination cycles performed[7,13,21]. The present study has followed the standard preparation protocol mentioned in *Ayurvedic* Formulary of India and scientifically validated the steps involved in the preparation[14].

In conclusion, it is evident from the present study that the treatment of raw material (crude metallic lead) with various treating liquids removes heavy metals such as lead, iron, molybdenum, copper and aluminium through chelation, apart from causing substantial changes in morphology. In addition, *sodhana* (purification) steps improve the processability of lead facilitating further treatment. Initial preparation of *jarita naga* (PbO) from *naga* (Pb) enables the conversion of lead to PbS through the formation of intermediate (PbO). The use of realgar during *arddha gajaputa* cycles enables the conversion of PbO to PbS along with elimination of arsenic as arsenic oxide vapours. Calcination steps play a predominant role in attaining monodispersity of the *bhasma* particles. Each steps of the preparation should be done with utmost care to get the good quality of *bhasma*. It is also important to understand each and every step to set up the gold standards for *bhasma* preparation. This work has resulted in better understanding of the preparation protocol through chemical, crystallographic and morphological characterisation of intermediates. The role of herbal ingredients (*Tamarindus indica* and *Ficus religiosa*) in ensuring the transformation of Pb to PbO (lower oxidation state of Pb) has also been elucidated.

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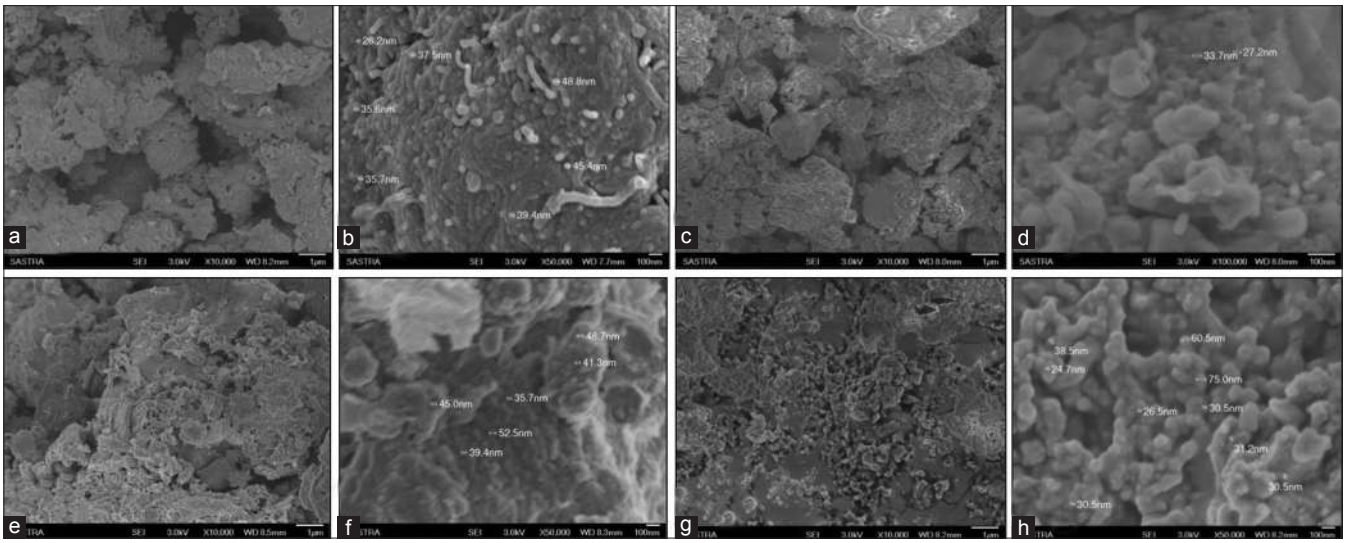

Fig. 9: Scanning electron micrographs of intermediates obtained after different arddha gajaputa cycles.
Monodispersity of the nanoparticles are observed with increase in puta cycle. (a) 1st *puta* cycle (lower magnification), (b) 1st *puta* cycle (higher magnification), (c) 5th *puta* cycle (lower magnification), (d) 5th *puta* cycle (higher magnification) and (e) 15th *puta* cycle (lower magnification), (f) 15th *puta* cycle (higher magnification) (g) 50th *puta* cycle (lower magnification), (h) 50th *puta* cycle (higher magnification).

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Pharmaceutical Standardization

Standard manufacturing procedure for *Laghu Malini Vasanta Rasa* in context of *Bhavana* (levigation)

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Abstract

Introduction: *Laghu Malini Vasanta* (LMV) *Rasa* is a well-known *Vasanta Kalpa* (formulation). As per reference of *Yoga Ratnakara*, *Rasaka* and *Maricha* are chief ingredients in 2:1 proportion. *Bhavana* (levigation) is said to be given first with *Navaneeta* (freshly prepared cow's butter) and then with *Nimbu Swarasa* (lemon juice) until *Ghrita Vimukta* stage is reached. Quantity of *Bhavana Dravya* (levigating media) and duration of levigation are not mentioned. **Aims:** To develop standard manufacturing procedure of LMV *Rasa*. **Materials and Methods:** The study was carried out in two stages - preparation of *Yashada Bhasma* and preparation of LMV *Rasa* and its tablet. A pilot study was carried out to fix quantity of cow's butter as levigation media. Based on results of the pilot study, LMV *Rasa* was prepared in two groups, that is, LMV 50 (LMV *Rasa* - prepared with weight of freshly prepared butter in 50% quantity of total ingredients) and LMV 75 (LMV *Rasa* prepared with weight of freshly prepared butter in 75% quantity of total ingredients). Complete drying of levigated mass and minimal spreading of fatty portion on filter paper was considered as the end point of levigation. Tablets of both samples were prepared by adding *Pippali* and honey in it and analyzed for their quality control parameters. **Results:** Twenty-eight hours duration of repeated levigation was required in LMV 50 which was prolonged up to 48 h in batch carried out in rainy season. In LMV 75, comparatively maximum duration of 54 h was required for levigation which was prolonged in the rainy season to 88 h. In both groups, lemon juice required for repeated levigation was 10 times of quantity of butter added initially. **Conclusion:** From pharmaceutical point of view, preparation of LMV *Rasa* tablets with quantity of butter in 50% of total ingredients is more convenient.

Key words: *Bhavana, Laghu Malini Vasanta Rasa, Rasaka*

Introduction

Laghu Malini Vasanta (LMV) *Rasa*, a renowned *Vasanta Kalpa*, comes under *Kharaliya Rasayana* that is drugs prepared by trituration in mortar and pestle. Use of processed metal or mercurial compound has been advised in least doses that too with specific herbal drugs as *Anupana* (adjuvant) for a specific period. Herbal drugs and media used in *Kharaliya Rasayana* induce the organic quality in the final product for its better therapeutic efficacy and least toxic effect. It also helps in target specific action of drug. In Ayurvedic Formulary of India (AFI), LMV *Rasa* has been cited under *Rasa Yoga* section. As per the reference in *Yoga Ratnakara*, *Rasaka* and *Maricha* are the basic ingredients in 2:1 proportion. *Navaneeta* (freshly prepared butter) and *Nimbu Swarasa* (lemon juice) are the levigating media. The first levigation is to be carried out with *Navaneeta* followed by lemon juice until levigated mass become *Ghrita Vimukta* (devoid of greasiness).[1]

Rasaka has been mentioned as the chief ingredient in the formulation. AFI in the context of preparation of this formulation suggests to use *Yashada Bhasma* in case of non-availability of genuine *Rasaka*;[2] hence, here an attempt has been made to standardize the formulation with *Yashada*

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Bhasma as chief ingredient, also to determine the proportion of freshly prepared cow's butter and lemon juice and period of repeated levigations with lemon juice.

Materials and Methods

The whole pharmaceutical study was arranged in two unit processes - preparation of *Yashada Bhasma* and preparation of LMV *Rasa* and its tablet.

Collection and authentication of raw materials

Raw *Yashada* of Binani mark in metal sheet form, *Tila Taila* (sesame oil), *Maricha* (*Piper nigrum* Linn. fruits), and *Pippali* (*Piper longum* Linn. fruits) were procured from Pharmacy, Gujarat Ayurved University, Jamnagar. *Kulattha* (*Dolichos biflorus* Linn.) seeds, *Nimbu*, and Amul gold brand milk and honey were purchased from local market of Jamnagar. After collection, *Yashada* was authenticated as per classical *Grahya Lakshana*[3] (acceptable qualities).

Takra (freshly prepared buttermilk),[4] *Kanji* (sour gruel),[5] *Kulattha Kwatha* (decoction of *D. biflorus* seeds),[6] preparation of *Yashada Bhasma*,[7] and extraction of freshly prepared cow's butter were carried out as per classical reference. Equipment specification needed to develop SMP of LMV *Rasa* is given in Table 1.

Preparation of *Yashada Bhasma*

For preparation of *Yashada Bhasma*, first *Samanya Shodhana* of *Yashada* was carried out by quenching it in five media successively namely *Tila Taila*, *Takra*, *Kanji*, *Gomutra* (cow's urine), and *Kulattha Kwatha*; with three consecutive quenching in each media. *Shodhita Yashada* was then subjected to *Jarana* for which raw *Apamarga* (*Achyranthes aspera* Linn.) *Panchanga* (entire plant) was used as *Avapa* media. *Kumari* (*Aloe barbadensis* Mill.) *Swarasa* (leaf juice) was used as *Bhavana Dravya* (levigation media) to prepare *Chakrikas* (pellets). Successive *Putas* were given to *Jarita Yashada* in electric muffle furnace (EMF) with 650°C as peak temperature until the attainment of chief desired characteristics (CDC) of *Yashada Bhasma* – white in color with a yellowish tint.

Preparation of *Laghu Malini Vasanta Rasa*

For the preparation of LMV *Rasa*, a pilot study was carried as per reference of AFI[2] to find out actual proportion of freshly prepared butter, lemon juice required to neutralize the fat content and also to determine the duration of levigation. Details of ingredients used for the pilot study are listed in Table 2. First *Yashada Bhasma* was taken in porcelain mortar and pestle (*Khalva Yantra*). Fine powder of *Maricha* (85#) was added to it, and levigation process was done till mixture became homogenous. Freshly prepared butter was added little by little till whole mixture became moist. Levigation up to 6 h was carried out until mixture turned into doughy consistency. Lemon juice was added in repetitive manner to the mass, and levigation was carried out simultaneously in mechanical wet grinder until greasiness of mixture disappeared.

On the basis of inferences from the pilot study, LMV *Rasa* was prepared in two samples [Figure 1]. These samples were identified as LMV 50 (LMV *Rasa* prepared with weight of freshly prepared butter in 50% quantity of total ingredients) and LMV 75 (LMV *Rasa* prepared with weight of freshly prepared butter in 75% quantity of total ingredients).

To assess *Ghrita Vimukta* (devoid of unctuousness) stage, two criteria were fixed. Complete drying and minimal or no spreading of oily layer were assessed by spreading test. In this test, 20 g of sample was separated from the mixture at regular interval of 6 h of levigation, respectively. Ten grams of this sample was kept for drying, and remaining 10 g was made into pellet form kept over Whatman filter paper no. 40 to assess fat content of mass by virtue of spreading. Each sample was observed for 48 h. Levigation was continued until complete drying of the sample and minimal or no spreading of oily portion on filter paper which was assessed by spreading test [Figure 2]. The whole mixture was then kept in an oven at 50°C until it got completely dried.

Preparation of *Laghu Malini Vasanta Rasa* tablets

LMV *Rasa* and *Pippali Churna* in equal proportion were taken and mixed homogenously. This mixture was transferred to

Table 1: Equipment specification for the preparation of *Yashada Bhasma* and LMV *Rasa*

Equipment	Specification
For *Shodhana* of *Yashada*	
Iron ladle	Length: 89.5 cm, diameter of body: 15 cm, depth: 5 cm, capacity: 800 ml
Pithara Yantra	Body: Height: 30 cm, diameter: 17 cm Lid: Shape: Conical, diameter of the hole: 2 cm, capacity: 5800 ml
For *Jarana*	
Iron ladle	Length: 89.5 cm, diameter of body: 15 cm, depth: 5 cm; capacity: 800 ml
Lauha Kadhai	Diameter: 57.5 cm, depth: 23.5 cm, circumference: 183.5 cm
For preparation of *Yashada Bhasma* and LMV *Rasa*	
Porcelain *Khalva*	Mortar: Inner diameter: 20.5 cm Outer diameter: 24.5 cm Depth: 12.5 cm Inner circumference: 62 cm Outer circumference: 75 cm
Butterfly matchless table top wet grinder with three conical roller stone	Depth: 16 cm, diameter: 26 cm, roller stone: Outer diameter of middle roller stone: 9 cm, height of middle roller stone: 8 cm, outer diameter of lateral roller stone: 15 cm, height of lateral roller stone: 9 cm, capacity: 2 kg

LMV: *Laghu Malini Vasanta*

stainless steel (SS) vessel. In LMV 50%, 30% honey was added as binding agent and converted into granules with the help of a 20# sieve. Prepared granules taken into a SS tray and kept in the oven at 50°C until complete drying. Granules were passed through a tablet punching machine to prepare tablets of 325 mg [Figure 3]. A 16 station rotary tablet machine was used for tableting. Details of equipment and their respective specifications used for preparation of tablet are listed in Table 3. The tablets were collected, weighed, and stored in air tight sterile glass containers along with small pieces of cotton in them. In LMV 75%, the same procedure was followed with 20% honey as a binding agent.

Table 2: Details of ingredients of LMV *Rasa* for pilot study

Drug	Botanical name	Part used	Proportion
Yashada	Calcinated zinc	*Bhasma*	1 part
Maricha	*Piper nigrum* Linn.	Fruit	2 parts
Navaneeta	Freshly prepared butter		Q.s.
Nimbu	*Citrus acida* Linn.	Fruit	Q.s.

LMV: *Laghu Malini Vasanta*

Observations and Results

Quantitative analysis of raw *Yashada* revealed the presence of 99.99% pure zinc in the sample. Total five *Putas* were required to achieve CDC of *Yashada Bhasma* such as *Rekhapurnatva* (fineness), *Gatarasatva* (tasteless), *Varitaratva* (lightness), and *Bhasma* white in color with yellowish tint. The details of the preparation of *Yashada Bhasma* are listed in Table 4.

The pilot study inferred that minimum 50% of weight of butter was required of total ingredients for levigation, whereas with 75% quantity of butter, levigation can be carried out more conveniently. Observations and results of the LMV *Rasa* preparation pilot study are given in Table 5. On the basis of inferences of pilot study, LMV *Rasa* was prepared in two samples LMV 50 and LMV 75; observation profile of media used in the preparation of LMV is presented in Table 6.

In preparation of LMV 50, 180 g of butter was used while in LMV 75, 270 g of butter was used for levigation. On addition of butter, the mixture turned greasy making levigation laborious. On addition of lemon juice, greasiness decreased to some extent

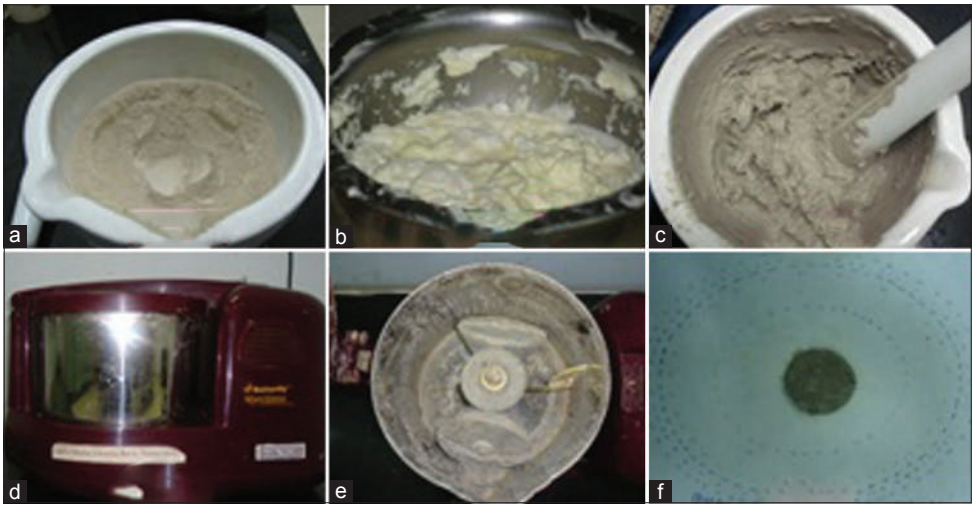

Figure 1: (a) *Maricha* added to *Yashada Bhasma*. (b) Freshly prepared butter. (c) Trituration after addition of *Navaneeta* for 6 h. (d and e) Butterfly matchless table top wet grinder with three conical roller stone. (f) Ten grams pellet kept over Whatman filter paper (no. 40) to assess fat content of mass by virtue of spreading

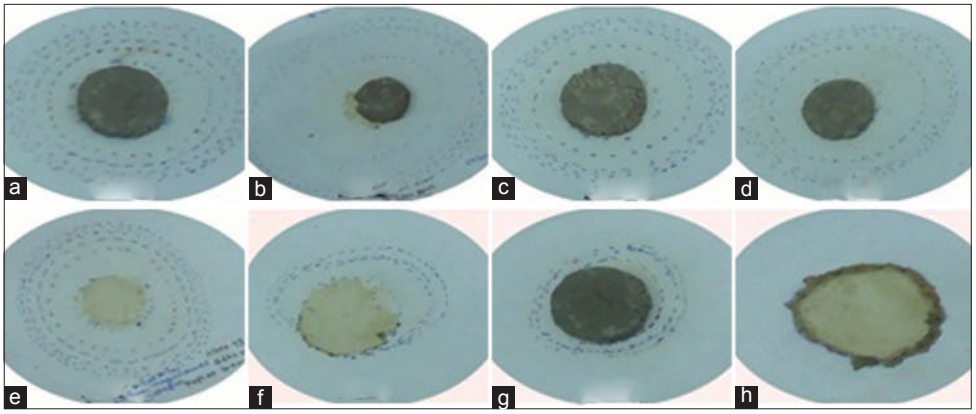

Figure 2: (a) Spreading on Whatman filter paper at end of 6 h. (b) Spreading on Whatman filter paper at end of 12 h. (c) Spreading on Whatman filter paper at end of 18 h. (d) Spreading on Whatman filter paper at end of 24 h. (e) Spreading on Whatman filter paper at end of 30 h. (f) Spreading on Whatman filter paper at end of 36 h. (g) Spreading on Whatman filter paper at end of 42 h. (h) Spreading on Whatman filter paper at end of 48 h

Figure 3: (a) *Laghu Malini Vasanta Rasa* tablets. (b) Packing of *Laghu Malini Vasanta* tablets. (c) Label of *Laghu Malini Vasanta* tablets

Table 3: Equipment specification for the preparation of LMV *Rasa* tablet

Name of equipment	Specification
Stainless steel tray	Length - 8 inch, breadth - 8 inch
Stainless steel sieve: Number 20	Diameter 5.5 inch
16 station rotary tableting machine	12 mm diameter, maximum capacity up to 15 mm Speed variable
Hot air oven	Inner chamber stainless steel, outer body mild steel Size of chamber 12.0×12.0×12.0 inches Temperature range up to 300°C, accuracy ±10°C Temperature control-thermostatic
Weighing balance (for tablet)	Analytical balance model - 200d, range - 200 g

LMV: *Laghu Malini Vasanta*

making levigation convenient. To remove greasiness of mixture, the quantity of lemon juice utilized in repeated levigation was observed to be 10 times of butter used in both samples. As levigation advanced fatty portion within mass was decreased gradually when assessed by spreading test [Figure 2]. In LMV 50, 73.92% average weight gain was observed after completion of levigation [Table 7]. Twenty-eight hours of levigation with lemon juice was required to attain end point of levigation which was prolonged up to 48 h in the rainy season.

In LMV 75, 54 h of duration was required for levigation with lemon juice and 88 h in rainy season which were comparatively higher when compared to LMV 50. 84.99% average gain was observed in final product comparatively higher than LMV 50 (73.92%) [Table 7]. In both groups, the final product was grayish in color, pungent in taste with characteristic butter smell and was completely dry. The smell was observed more in LMV 75 sample.

In preparation of tablet, 30% quantity of honey was required for LMV 50 as the binding agent and 20% in LMV 75. About 14.24% and 22.91% of loss were observed in the preparation of tablets of LMV 50 and LMV 75, respectively [Table 8].

Organoleptic characters of tablets showed that the tablets of both LMV 50 and LMV 75 were greenish in color, pungent in taste, and circular with flat facets. The average weight of tablet was 312 and 335 mg in LMV 50 and LMV 75 with disintegration time of 45 min and 41 min, respectively [Table 9]. Physicochemical analysis of LMV *Rasa* and its tablet was carried out details of which are presented in Table 10.

Discussion

LMV *Rasa* has been described in 30 texts with the earliest citation in *Rasa Paddhati*.[8,9] As per reference of *Rasa Paddhati*, levigation with butter is said to be done for 3 days, but quantity has not been specified. Levigation with lemon juice is said to be done until levigated mass becomes dry. Whereas *Yoga Ratnakara* has not mentioned duration of levigation with butter, and levigation with lemon juice is said to be done until *Ghrita Vimukta* stage. Composition of formulation mentioned as per reference of *Yoga Ratnakara* has been quoted by Maximum texts including AFI, hence followed in the current study.

In the pilot study, addition of freshly prepared butter was started in 1/8th proportion of total ingredients and was gradually increased in same manner until the mixture became thoroughly wet. The pilot study inferred that minimum 50% of total ingredient weight of freshly prepared butter was required for levigation. Whereas with 75% quantity of freshly prepared butter levigation can be carried out more conveniently. Based on these inferences, two final samples of LMV *Rasa* were prepared, that is, LMV 50 and LMV 75. As period of levigation with freshly prepared butter was not mentioned in *Yogaratnakara*, it was done manually for 6 h in porcelain *Khalvayantra* due to inconvenience of levigation in butterfly grinder machine because of more greasy nature of compound. Whereas the repeated levigation with lemon juice was carried in the wet grinder machine. Interpretation of *Ghrita Vimukta* stage can be assumed as removal of exceeding amount of butter from mass by lemon juice. In the context of preparation, it has been mentioned that *"Shushka Churnam Yadavadhi"*[9] (until mass gets completely dried) as the end point of levigation with lemon juice. Spreading test developed in the department was utilized to assess unctuousness more precisely. Negligible or no spreading of the fatty portion on filter paper along with complete drying was considered as the end point of levigation with lemon juice. In the later stage of repeated levigation with lemon juice, spreading of fatty portion on filter paper got decreased. It signifies that repeated levigation with lemon juice decreases greasiness of compound. Generally, blotting paper is used to absorb greasiness of material. However, it is found difficult to standardize the method due to weight variation of blotting paper. It was observed in the pilot study that the spread of oleaginous contents was not uniform on blotting paper, but there was no such difficulty with Whatman filter paper. Therefore, to standardize method, Whatman filter paper (no. 40) has been used to assess extent of greasiness of compound.

The extent of levigation was observed to be higher in rainy season in both samples. Increased humidity within atmosphere

Table 4: Details of preparation of *Yashada Bhasma*

Details of *Shodhana*					
Media	**Processing stage**	**Weight of *Yashada* (g)**			**Average loss/gain of *Yashada* (%)**
		B1	**B2**	**B3**	
Tila Taila	Before	700	700	600	0.22↓
	After	712	695	590	
	Percentage of ↑/↓	1.71↑	0.71↓	1.66↓	
Takra	Before	712	695	590	2.91↓
	After	695.4	698	584	
	Percentage of ↑/↓	2.33↓	0.43↑	1.01↓	
Kanji	Before	695.4	698	584	1.36↓
	After	685	687	578	
	Percentage of ↑/↓	1.49↓	1.57↓	1.03↓	
Gomutra	Before	685	687	578	2.50↓
	After	659	672	569	
	Percentage of ↑/↓	3.79↓	2.18↓	1.55↓	
Kulattha Kwatha	Before	659	672	569	0.49↓
	After	665.8	664.8	560.8	
	Percentage of ↑/↓	1.03↑	1.07↓	1.44↓	
Percentage of gain/loss in total					5.47↓

Details of *Jarana*												
Weight of *Shodhita Yashada* (g)			**Weight of *Apamarga Panchanga* (g)**			**Weight of *Jarita Yashada* (g)**			**Weight of *Jarita Yashada* (after *Prakshalana*) (g)**			**Average weight increase (%)**
B1	**B2**	**B3**	**B1**	**B2**	**B3**	**B1**	**B2**	**B3**	**B1**	**B2**	**B3**	
615	615	615	148	154	140	650.92	645	655	635	628	630	2.59↑

Details of *Marana*										
Number of *Puta*	**Weight of *Jarita/Marita Yashada* (g)**			***Kumari Swarasa* (ml)**			**Weight of *Yashada* after *Puta* (g)**			**Percentage of loss or yield**
	B1	**B2**	**B3**	**B1**	**B2**	**B3**	**B1**	**B2**	**B3**	
1	625	625	625	250	255	250	544	588	550	22.53↓
2	540	554	546	230	230	240	522	535	520	
3	515	530	515	220	220	220	503.5	518	510	
4	500	515	505	190	210	210	491	508	495	
5	485	500	490	190	195	195	478	491.5	483	

Table 5: Observations and results of pilot studies of LMV *Rasa*

Parameters	LMV 50	LMV 75
Weight of *Yashada Bhasma* (g)	50	50
Weight of *Maricha Churna* (g)	25	25
Weight of freshly prepared cow's butter for levigation (g)	37.6	56
Total quantity of lemon juice required (ml)	335	540
Total duration of levigation (h.min)	15.45	25.30
Weight of final product after complete drying (g)	90.6	120.5
Color of final product	Grayish white	Grayish white

LMV: *Laghu Malini Vasanta*

might be the reason for prolonged duration of trituration. Average yield (84.99%) was maximum in LMV 75 in comparison to LMV 50 (73.92%) which signifies that increase in proportion of freshly prepared butter increases extent of levigation with lemon juice, and as period of levigation increases more solid contents of levigation media gets impregnated within compound, increasing percentage of yield. Freshly prepared butter was weak base and lemon juice was weak acid in nature. There might be possibility of neutralization[10] taking place when these two media inter-react during levigation. For the neutralization as proportion of base and acid remains definite, this could be the reason for fixed ratio (1:10) of butter and lemon juice that was observed during levigation in both samples. By virtue of degreasing property of citric acid,[11] lemon juice cleanses greasiness of compound which gets separated in oily tinge over surface. Freshly prepared butter is water in oil emulsion, the continuous phase being the oil and the dispersed phase being the water. Agitation and elevated temperature are one of the several methods that break water in oil emulsion into oil in water emulsion with water as continuous phase. During agitation, mixing increases the collision number among particles and their coalescence decreases stability of emulsion. An elevated temperature accelerates the water separation by increasing the probability of the water

Table 6: Mean observation profile of media used in the preparation of LMV *Rasa*

Media	Quantity obtained (g)	Color	Odor	Taste	pH
Cow butter	139.66 (per liter milk)	White	Milky	Slight sour	4.57
Lemon juice	23.20 (per lemon)	Yellowish	Sour	Lemon specific	3

LMV: *Laghu Malini Vasanta*

droplets to collide and decreasing the viscosity of the continuous phase.[12] During levigation, both agitation and slight elevation in temperature of compound takes place. These sequences act as demulsifying agent causing breaking of water in oil emulsion of freshly prepared butter which increases its viscosity and separation of water portion from butter. This breaking of emulsion further allows its interaction with lemon juice and compound.

For preparation of tablets, *Pippali Churna* was added in equal proportion to LMV *Rasa* as per reference of *Sharangdhara*

Table 7: Details of observation obtained during levigation of LMV *Rasa*

Ingredients	Batch code					
	LMV 50			LMV 75		
	Batch I	Batch II	Batch III	Batch I	Batch II	Batch III
Weight of *Yashada Bhasma* (g)	240	240	240	240	240	240
Weight of *Maricha Churna* (g)	120	120	120	120	120	120
Quality of *freshly prepared butter* utilized in 1st *Bhavana* (g)	180	180	180	270	270	270
Lemon juice utilized in 2nd *Bhavana* (ml)	1785	1820	1750	2680	2720	2685
Duration of levigation in 2nd *Bhavana* (h)	28	28	54	54	54	82
Final weight of product after complete drying (g)	598.56	603	676.8	645	648	725.76
Percentage weight gain	66.26	67.5	88	79.16	80	101.6
Average weight gain (%)	73.92			84.99		
Ratio of freshly prepared butter: Lemon juice	1:9.9	1:10.1	1:9.72	1:9.9	1:10.07	1:9.9
Average	1:9.9			1:9.9		
Color of final product	Grayish	Grayish	Grayish	Grayish	Grayish	Grayish
Taste of final product	Pungent	Pungent	Pungent	Pungent	Pungent	Pungent
Greasiness of final product	Absent	Absent	Absent	Absent	Absent	Absent

Note: Batch III of both samples was prepared in rainy season. LMV: *Laghu Malini Vasanta*

Table 8: Details of observation obtained during preparation of LMV *Rasa* tablet

Group	Weight of LMV *Rasa* (g)	Weight of *Pippali Churna* (g)	Honey (g)	Weight of tablets (g)	Weight of residue (g)	Total loss (%)
LMV 50	1000	1000	600	2229.8	273	14.24
LMV 75	1000	1000	400	1850	142	22.91

LMV: *Laghu Malini Vasanta*

Table 9: Details of physical analysis of LMV *Rasa* tablet

Group	Shape	Size (mm)		Hardness (kg/cm²)	Average weight of tablet (mg)	Disintegration time (min)
		Diameter	Width			
LMV 50	Circular with flat facets	9	3	Na	312	45
LMV 75	Circular with flat facets	9	3	Na	335	41

LMV: *Laghu Malini Vasanta*

Table 10: Details of physiochemical analysis of LMV Rasa and tablet

Group	pH	Loss on drying (%)	Ash value (%)	Water soluble extractive (% w/w)	Methanol soluble extractive (% w/w)
Laghu Malini Vasanta Rasa					
LMV 50	6.5	4.13	33.14	14.56	14.07
LMV 75	6.5	3.6	34.83	15.10	15.68
Laghu Malini Vasanta Rasa tablet					
LMV 50	6.5	8.62	17.44	34.67	25.15
LMV 75	6.5	10.14	14.15	36.54	49.80

LMV: *Laghu Malini Vasanta*

Samhita.[13] In authentic textual reference of formulation as mentioned in *Yogaratnakara Jwaradhikara*, both *Pippali* powder and honey have been indicated as adjuvant for therapeutic action of the drug. Though there is no any published data on interactions of *Pippali Churna* and honey with LMV at pharmacokinetic–dynamic levels; to avoid the inconvenience to take *Pippali* and honey each time at time of administration, the tablet has been formulated by combining these two adjuvants with the base drug. Quantity of honey was fixed (30% in LMV 50 and 20% in LMV 75) on the basis of appropriate formation of granules. Granules prepared with same proportion as that of LMV 50 in LMV 75 did not dry completely. This signifies that either moisture content or fat content in LMV 75 is comparatively higher, and hence proportion of honey has to be decreased until formation of appropriate granules.

Conclusion

From pharmaceutical point of view, preparation of LMV *Rasa* tablets with quantity of butter in 50% of total ingredients was more convenient. To assess *Ghrita Vimukta* stage of LMV, spreading test can be utilized. Complete drying of levigated mass and minimal or no spreading of fatty portion on filter paper can be considered as end points of levigation. In both groups, approximately 10 times of lemon juice of the quantity of butter used is required to neutralize excess fat within compound. The extent of repeated levigation with lemon juice was increased with percentage of butter used for levigation and the moisture content of the atmosphere.

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Conflicts of interest

There are no conflicts of interest.

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Original Research

STANDARD MANUFACTURING PROCEDURE OF LAUHA BHASMA USING *TRIPHALA* MEDIA AND BY EMPLOYING ELECTRIC MUFFLE FURNACE HEATING

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Abstract
Lauha (Iron) Bhasma is one of the important metallic preparations used in Āyurveda for therapeutic purposes. A popular method for the preparation of lauha bhasma is trividha pāka method and this method was employed in this study. Āyurvedic purification (śodhanā) of iron was done by heating iron up to red hot and quenching in various prescribed liquid media. Then it was subjected for sun drying with triphala decoction (Bhānupāka). Then it was taken in iron vessel with triphala decoction and exposed to intense heat until total decoction was evaporated (sthālipāka). Then incineration cycles were given (Putapāka). It took 20 incineration cycles for the procurement of desired Bhasma which fulfils the set standards of the classical literature like color (pakva zambhū phala, purple), floating on water (vāritara), fineness (rekhāpūrna) etc. It was found that there was substantial weight increase in the final product. It took about 100 working days for the preparation.
Key Words: Lauha Bhasma, Muffle furnace,
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Introduction

Āyurveda, Traditional system of Indian Medicine (TIM) is popular in Indian sub continent since time immemorial. rasa śāstra, a new offshoot of Āyurveda is popular since medieval period deals with mostly therapeutic utilization of metals and minerals.[1][2] Iron is a common metal which has been used since time immemorial for therapeutic purpose in both western and Indian diaspora. Iron predominant formulation, Āyaskruti is used in Brihatrayi. Emergence of rasa śāstra revolutionized the Āyurvedic pharmaceutics. Pharmaceutical Methods like Śodhanā, Mārana, amritīkarana etc. converted hard metals, minerals and poisonous substances into safe and effective bio accessible medicaments. Earlier physicians used to prepare Bhasmas for their personal use in the clinical practice. Nowadays their dependence on the large scale manufacturers lead to several quality related problems. Revalidation of pharmaceutical procedures of all rasa dravyas with contemporary parameters became essential for the drug standardization. In tune to it, the advanced heating devices like Electric Muffle Furnaces replaced conventional puta system for heating. For scientific understanding, utility and rationality of them has to be documented. Earlier scholars have documented on the pharmaceutical methods but their observations were not comprehensive. Hence this study is designed to put forward and ensure that those shortcomings are addressed. Earlier scholars have worked on pharmaceutical preparation of Lauha bhasma with Triphala as media. Study reported followed the EMF method, where in, it was documented that it requires 22 incineration cycles (Puta) with 600^0C peak temperature for one hour for the procurement of lauha bhasma. [3] Chemical form of lauha bhasma is found as Fe_2O_3. [4] One another study identified that iron was available in ferrous as well as ferric form of oxides in the lauha bhasma. Then stochiometrically approximate weight gain in bhasma in between 28 to 42% is anticipated with initial iron weight. But going through the earlier studies this weight gain was not properly documented. Hence considering all these points this study is planned.

Material and methods

Materials: Present pharmaceutical study is planned to validate the ancient methods of Lauha bhasma preparation by employing electric muffle furnace for heating instead of classical Puta system of heating. For that tīkshna lauha i.e. raw iron scrap, Triphala, Sesame oil etc. were procured from Āyurvedic Pharmacy, Gujarat Āyurveda University. Cow urine, procured from Goshāla, Jamnagar. Sour gruel (Aranāla) was prepared according to the method described in AFI, buttermilk (takra), Horse gram for decoction (kuluttha kvatha) procured from the market.

Methods

Process of Śodhanā: Lauha was subjected to both sāmānya and viśesha śodhanā.

Sāmānya śodhanā: Materials required - mild steel turnings (1750 gm), Sesame oil (1750 ml), Butter milk (1750 ml), cow urine (1750 ml), Sour gruel (1750 ml), horse gram decoction (1750 ml)

Procedure: Lauha procured after sthālīpāka was heated to red hot (at 800-850°c) and immediately quenched into sesame oil. The process was repeated for seven times, taking fresh oil every time. The process of heating and quenching was continued in Takra (buttermilk), gomūtra (cow urine), kānjī (sour gruel) and decoction of horse gram (Kuluttha, Dolichus biflorus) seven times in each liquid

Viśesha Śodhanā (specific purification process) is performed in triphala kvātha (8/4 reduced decoction) for seven times.

Procedure: Iron turnings are heated up to red hot (at 800-850 ° C) and quenched in Triphala decoction, this procedure was repeated for seven times. Fresh decoction was used for quenching every time.

Precautions: While doing quenching one should take care and keep face away from the liquid, as the hot vapors and fine particles coming from the liquid may cause injury. Oil catches flame immediately after quenching.

Liquids should be taken in equal quantity for immersion of the material completely.

Observations

Table 1 Observation of Sāmānya and Viśesha Śodhanā of Lauha.

Liquid (pH)	Changes in iron	Changes in liquid
Sesame oil (6.5)	Color turned more Blackish, brittleness not altered	Oil takes flame just after quenching Color became blackish
Butter milk (4.3)	Iron turning started breaking in pieces	Consistency disturbed. Color slight blackish
Cows urine (7.8)	Size of iron scrap reduced much more	Ammoniac smell was spread in the surrounding area
Sour gruel (3.1)	Rate of breaking of iron scrap was increased	It produces its typical smell
Horse gram decoction (6.1)	Rate of breaking of iron turning was still more increased more	Brownish red decoction turned into slight blackish
Triphala decoction (2.3)	Color of iron turning changed to blackish. It became more brittle and particle size decreased.	Brownish red decoction turned into slight blackish

Lauha Mārana After Sāmānya and Viśesha Śodhanā, Lauha is subjected for three phases of Mārana. A specific method for Lauha Mārana is mentioned in 'Rasendra Sara Sangraha' by Krishna Gopala Bhat which is popular and in vogue. This consists of three main steps

1. Bhānupāka 2.Sthālīpāka and 3.Putapāka

Bhanupaka

In Bhānupāka, the Śodhita Lauha is kept in 2/4th reduced Triphala decoction (two times water is added to the drug [v/wt] and boiled until one fourth of the water

remains i.e. in the end only half to the quantity of Lauha remains) in a vessel in the sunlight.[5]

Procedure: in this process, 250 g. iron scrap was mixed with 500 ml of Triphala decoction and kept under the sun till complete evaporation of liquid. For getting more brittle and fine particles, on complete drying of the mixture again 250 ml. Decoction of Triphala was added and dried under the sun. This was repeated for seven times.

Observation: Iron turnings became more brittle and can be coarse powdered easily by pounding.

Sthalipaka

In second step Sthālīpāka, the Lauha is procured after washing from first phase is taken in a vessel immersed in 16/8th reduced Triphala decoction (prepared by 3 times quantity to Lauha added 16 times water and reduced until one eighth portion remains) and intense heat is given until the total decoction evaporates and the Triphala remnants are also burnt out. [6]

Procedure: Iron scrap procured after Bhānupāka was taken in iron pan, mixed with 1 liter decoction of Triphala, and subjected to mild heat till complete evaporation of liquid. Finally strong heat was given then residue of Triphala decoction was burnt to some extent.

Observation: During boiling Triphala decoction takes dark black color. Iron turnings became black. Again weight of iron turnings increased because of Triphala decoction.

Putapāka

Lauha procured after Sthālīpāka is subjected for incineration (Gajaputa) of 20 cycles. Confirmation of completion of incineration is by conventional test and modern analytical tests.

Procedure: Lauha procured after Sthālīpāka triturated with decoction of Triphala. The process of trituration was conducted in a mortar pestle for six hours. Then the pellets (Chakrika) were prepared little bigger than the size (0.5 cm x 2 cm) and shape of poison nut (Kupeelu, strychnos nuxvomica L.) and) dried. These are kept in between earthen casseroles and sealed with clay cloth; this is incinerated by keeping it in the EMF. Then pellets of were made, dried in sun/oven and finally Puta (heat treatment) was given. Heat treatment was given in electric muffle furnace. Maximum temperature and duration of heat treatment was decided on the basis of temperature required for Mārana of the material taken for comparison with results of previous research work done in department of Rasa śāstra. So in this method the maximum temperature given was 650^{o}c which was maintained for one hour. This procedure was repeated for 20 times. Bhasma was tested after each Puta.

Observation: Weight loss observation during initial 2 Puta, may be because of burnt Triphala remnants. Changes in color and hardness of the pellets were observed and are mentioned in the table given below.

Table : 2 observations and results of Lauha Mārana process:

Number of Puta	Color of Pellets	Other observation
1st	Black	Varitaratva - not present
5th	Purplish Black color	Rekhāpūrnatva test fulfilled Vāritaratva - not present
10th	Dark Purple Color	Varitaratva – present
15th	Dark Purple Color	Varitaratva – present
20th	Purple Color (pakwa zambū phala varna	Varitaratva – present

Color of Bhasma was black up to in first three Putas. Color turned towards purple black after fourth Puta.

Bhasma became rekhāpūrna on wet impregnation after third puta. Maximum Varitaratva was present after ten puta.

Weight changes: initial weight took for the 250gm iron scrap is taken for the study. The final weight depicted in the table is the initial weight of the next step of the pharmaceutical study as no sample is taken out in middle of the study.

Table : 3 Weight changes during the pharmaceutical steps

	Pharmaceutical Phase	Final Weight (g)/(-) Loss / (+) gain (g)		
		Batch 1	Batch 2	Batch 3
1	Asuddha Lauha	250	250	250
2	After Sāmānya Śodhanā	230/(-20)	225/(-25)	230/(-20)
3	After Viśesha Śodhanā	235/(+5)	230/(+5)	235/(+5)
4	After Bhānupāka	310/(+40)	300/(+40)	325/(+100)
5	After Sthālipāka	255/(-60)	250/(-50)	255/(-70)
6	After Putapāka	275/(+20)	270/(+20)	275/(+20)
	End **Product (Lauha bhasma)**	**275/(+25)**	**270/(+25)**	**280/(+25)**

Figure : 1 Graphical representation of weight changes in different steps pharmaceutical procedures

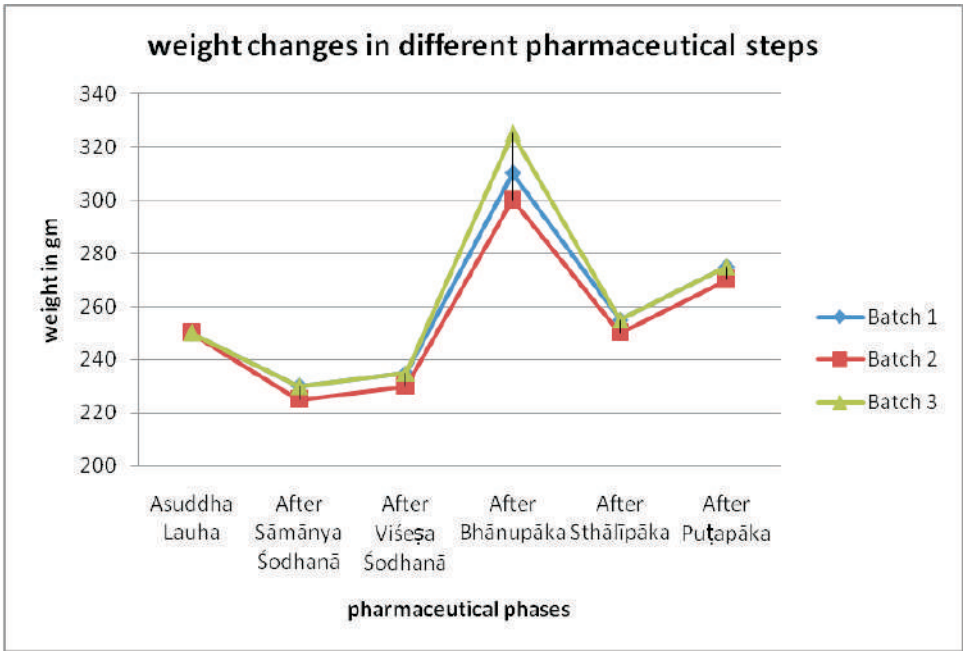

Table 4 : Weight changes during the incineration cycles

Pharmaceutical Phase	Final Weight (g)		
	Batch 1	Batch 2	Batch 3
After Sthālipāka	255	250	255
After 1st Puta	235	230	235
After 5th Puta	245	235	245
After 10th Puta	255	245	260
After 15th Puta	265	265	270
After 20th Puta	275	265	280
End Product (Lauha bhasma)	**275**	**270**	**280**

Figure 2 :

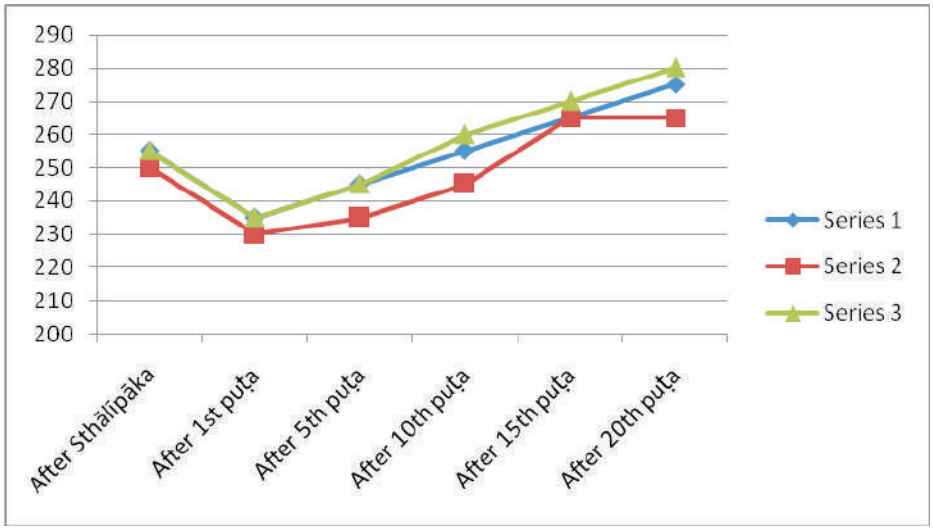

Figure 3 : lauha bhasma cakrika

Figure 4 : Electric Muffle Furnace employed for incineration

Table 5 :

Pharmaceutical Step	Duration in days
Śodhanā	3
Viśesha Śodhanā	1
Bhānupāka	3 x 7 = 21
Sthālipāka	3
Putapāka	4 x 20 = 80
Total duration	108

Discussion: Iron and its compounds were in use in different medical conditions including anemia (Pāndu) in Āyurveda since the period of Brihatrayi. But the modern medical science was totally ignorant about its utility in anemia until eighteenth century. The rational use of salts of iron in the treatment of anemia was not developed until the nineteenth century.[7] In recent years many compounds of iron have been used in modern medicine to develop body strength but in Āyurveda; Lauha bhasma is still in use due to its wide range of utilities. Lauha bhasma is a main ingredient in many formulations termed as Lauha Kalpas.

Selection of raw material: In ancient times either naturally occurring iron oxide or Bhasma prepared from wrought iron (iron produced by satvapātana), was used for medicinal purposes. Abandoned or pieces of swords, instruments were used for the preparation of Lauha bhasma. Hence it will be rational to consider mild steel as Tīkshna Lauha. Considering this earlier scholar conducted a study on the preparation of Lauha bhasma with mild steel scrap. [8] The flow sheet of quenching the raw material in six different liquids from a temperature of 750^{0}C and repeated in the iron pieces and it could be easily converted into fine powder by intermittent trituration after each quenching. During repeated heating, the iron surface got oxidized and the layer of this chemical compound was removed during trituration, thus exposing fresh surface for the chemical reaction.

Selection of quantity: rasa ratna samucchaya mentioned the quantity has to be taken for Śodhanā as minimum of 5 pala to maximum of 13 pala i.e. the quantity used for heating and quenching. [9] Quantity of five pala of Lauha is mentioned for viśeʔa śodhanā as per the reference of rasendra sara sangraha. Hence 250 gm i.e. 5 pala (one pala equal to 48gm, rounded to 50gm) is taken for the study. Regarding Triphala kvātha in different steps the ratio and reduction factor differs. The quantity of liquid media is not mentioned for Sāmānya Śodhanā in the classics; hence equal quantity of liquid media is taken for the quenching following the common rule of unexplained (Anukta Mana). But in Viśesha Śodhanā specific quantity of the drug and reduction factor while preparing decoction is mentioned, hence that is followed. [10]

Sodhana

In the quenching process of an iron–nickel alloy, the structure of simple phase, the austenite, is changed ideally into martensite. In the case of carbon-steel, the austenite is ideally changed into perlite, bainite and martensite, depending on the time of transformation, the temperature and the cooling rate. [11] Mild steel scrap consists of pearlite and ferrite crystals, due sudden heating and quenching this crystals are converted into hard and brittle martensite crystals[12] due to sudden cooling, by which it facilitate further processing such as improvement in grindability. Initial quenching in olium sesame causes catch of fire due to oil. Hence there sudden cooling did not occur. But in remaining liquids after cooling, temperature reaches to below 100^{0}C. Hence possibility of conversion into different types of crystals of iron is possible.

Thermodynamics of śodhanā: Any metal including iron if heated, at certain temperature they start to emit radiation, this is known as thermal radiation and this phenomena means conversion of thermal energy into light is called incandescence. At that time color of the metal becomes red. Iron shows thermal radiation means become red hot at above 750 ^{0}C. Hence iron turnings were heated up to 750^{0}C while doing cycles of heating and quenching. Sudden temperature decrease happen during quenching. Quenching media possess alternate increase and decrease in the pH. This may also contribute for the processing.

Mārana

Role of Bhāvanā dravya: bhāvanā dravya forms an herbal coat on the surface of particles and forms a surfactant and thus facilitates the further processing. This is similar to the surfactant mediated production of nano particles. [13]

Why muffle furnace instead of classical puta? : Standardization mainly aims at reproducibility. The temperature variations are observed in the routine puta system of heating taccording to the seasons due to variation in humidity, temperature as puta system is an open method where there is every possibility of loss of heat by which it require more energy to meet the loss. For standardization it is necessary to document and reproduce the temperature patterns. With muffle furnace heating, the temperature pattern can be maintained according to the needs with precision and with minimal loss of energy as it is a closed system. Hence this study is planned to establish the utility and viability of muffle furnace heating in Bhasma preparation and document the weight changes in pharmaceutical study of Lauha bhasma. Chances of contamination are also less with EMF method.

Role of triphala: *Triphala* mainly consists of tannin,

gallic acid, ascorbic acid (vitamin c), and phenolics. Ascorbic acid increases the bioavailability of iron by converting Fe^{3+} to Fe^{2+}, while phenolics can reduce the iron by binding to it. The presence of ascorbic acid or a lack of dietary tannins has both been suggested as contributing to clinical/pathological iron storage disease. Too much iron is toxic. It can damage the liver, heart, and pancreas and irritate the stomach and gut, causing constipation and diarrhea. In other words, this may also be taken as the various constituents of *Triphala* have antagonizing activity. Thus, too much iron absorption is prevented. *Triphala* is a mild laxative and thereby counteracts the constipating property of iron and thus be beneficial due to which *ācārya* might have mentioned *Triphala* in maximum *Lauha* formulations. [14]

Changes in weight: The Documentation of weight changes are essential part for the drug standardization. Assessment of final weight with relation to initial weight will give pharmacist an idea to choose the weight of the initial material to procure the desired quantity of finished product. Inference can be drawn basing on the weight changes due to loss after heating and quenching and loss of iron particles in the liquid media. Loss is observed due to inability to procure the fine particles dispersed in the quenched media The weight gain has occurred in sun drying (Bhānupāka) due to addition of Triphala remnants. Again weight loss in vessel heating is due to combustion of Triphala. In first incineration cycle, it is observed that substantial weight loss occurred, but later gradually weight gain observed. The initial weight loss can be substantiated due to complete burning of Triphala. But later weight gain is due to compound formation (Fe_2O_3, Fe_3O_4) with oxygen.

Incineration cycles: Puṭapāka is done in two steps one is Bhāvanā, in this step the purified Lauha is taken with herbal juices i.e. Triphala kvātha and triturated until drying. Due to attrition the particle size of the Lauha decrease during Bhāvanā and herbal coat will form on the superficial surface of the particle. The flat disc shape of the pellets facilitates maximum exposure of heat during the incineration cycles. When it is subjected for incineration due to the presence of herbal material the superficial surface of the iron particles react and oxides may get formed. These oxides will again get reduced to iron and get separated with the core particle. When again we subject it for Bhāvanā then again due to attrition separated iron particles may be separated from the core particle. Repeated incineration cycles can cause repeated oxidation and reduction of superficial iron atoms. They gradually get separated from the core particles to ultimately produce the nano particles. It is found that Bhasma possess significant percentage of nano particles along with micro particles. Nano particles due to the separation of iron atoms from the core iron particles due to oxidation, reduction and separation iron atoms occur during the incineration cycles. The micro particles may be the core iron particles.

Fe (Core) + O_2 æ∆ Fe (core) + FeO, Fe_2O_3, Fe_3O_4 (superficial coat formed on the core particle)

FeO, Fe_2O_3, Fe_3O_4 +Herbal coat (carbon) æ∆ Fe (reduced which will be separated from core particle)+CO_2

This is evident that Bhasma are having more density than water they have to dip in the water, but bhasma float on the water. Bhasma float how boat or ship sails in water. This is due to increased surface area and surface tension. Boat floats on the water basing on the displacement theory. This too applied for the Bhasma flotation.

Costing: Large scale production will substantially decrease the expenditure in relation to the quantity as the expenditure on labor is not going to change by increasing quantity up to some level.

Conclusions:

Development of standard manufacturing methods in drug making is essential for following Good Manufacturing Practice. For the preparation of metallic Bhasma the processing technology is complex. The metal pieces have to go through a number of steps and processes by cyclic heating and quenching in different liquids to obtain purified and nontoxic metal powder. Preparation of Lauha bhasma is a laborious and time consuming process. Trividha Lauha Pāka is a unique

method explained in classics to convert the hard iron metal into bio accessible Lauha bhasma. Triphala is found very useful in the lauha bhasma pharmaceutical study in viśesha śodhanā and as well as Bhāvanā dravya. Total duration required for the preparation is about 100 days. Total weight gain observed is 10 percent. This can be attributed due to loss during śodhanā process. Restricting weight loss in that stage can produce up to 40 percent of weight gain.

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Pharmaceutical Standardisation

Standard manufacturing procedure of *Tamra Bhasma*

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Abstract

Tamra Bhasma (incinerated copper) is one of the main weapons in the archery of Ayurvedic practitioners. Though several methods of preparation of *Tamra Bhasma* (TB) are found in *Rasashastra* classics, several difficulties occur during the preparation of a good-quality *Bhasma*. In this study, TB was prepared and analyzed to develop the standard manufacturing procedure. Each unit operative procedure was considered as an independent processing and an attempt was made to validate each procedure. Wire used for the purpose of electrical earthing was taken for the preparation of *Bhasma*. Procedures of *Shodhana*, *Marana*, and *Amritikarana* were followed as per the classical references. Specific temperature pattern was adopted for *Puta* in the electrical muffle furnace. From 500 g of *Tamra*, 483.4 g of black colored TB was obtained after subjecting to three *Putas*. Final product was detected to be cupric sulfide in X-ray diffraction. In particle size distribution analysis 10% of the material was below the size of 2 μm, while in inductive coupled plasma - atomic absorption spectrometry 58.56 wt% copper and 22.48 wt% of sulfur were found present in the final product along with the elements such as arsenic, lead, zinc, mercury, and manganese in traces.

Key words: *Bhasma*, *Puta*, standard manufacturing procedure, *Tamra*, X-ray diffraction

Introduction

Present day demand of Ayurvedic formulations has been raised globally due to increased response toward Ayurvedic system of medicine. Hence, commercialization of Ayurvedic drug manufacturing has taken place. Therefore, certain things like standardization, quality control, and safety became essential requirements for Ayurvedic formulations. It is the need of the time to present, understand, and implement these things into Ayurvedic formulations more accurately for globalization of Ayurveda. Thus, the production of standard, effective, genuine, safe drugs of utmost quality should be given prime importance by the processing units of Ayurvedic drugs.[1] Unfortunately, Ayurvedic formulations prepared in above-said terms are fewer. This may be because of the scarcity of standard parameters to compare a prepared drug.

Metals and minerals, as such in an elemental form, cannot be used for the therapeutic purpose, since many of them are toxic to the human body.[2] But the *Rasashastriya* pharmaceutical processes like *Shodhana* (purification/detoxification), *Marana* (incineration/calcination), etc., make them into such form (compound) that they are highly effective without any untoward effects in the therapeutic dose.[3] *Tamra Bhasma* (incinerated copper) is important amongst the metallic *Bhasmas* used for the treatment of many diseases. Though numbers of methods of *Tamra Bhasma* (TB) preparation are described in *Rasa* literature; its preparation has always been a practical problem. Moreover, improperly prepared (*Apakwa*) TB has been quoted as poison or more than a poison, because of its hazardous effects on the body.[4] To indicate its toxic potential, *Ayurveda Prakasha* have quoted *Ashtamahadoshas* (eight major ill effects).[5] Therefore, it is extremely important to prepare TB of good quality by following the classical procedures, which include *Shodhana*, *Marana*, and *Amritikarana*. This particular work is the modest attempt in the direction of development and establishment of Standard Manufacturing Procedure (SMP) for TB. For validation, each unit operative procedure was considered as an independent processing. Final product (TB) was analyzed by classical tests, physicochemical parameters, and by applying some advanced analytical techniques.

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Materials and Methods

Tamra wire of 0.5 mm diameter was collected from an electrician. There was no coating over the wire. This wire was converted to thin sheet of 36 gauge thickness by passing it through a roller press. The sheet was then converted to pieces

of 1 × 1 cm. A brief description of the materials used during the preparation of TB is given in Table 1.

Tila Taila (sesame oil), *Parada* (mercury), and *Gandhaka* (sulfur) were collected from Pharmacy, Gujarat Ayurved University, Jamnagar. *Kulattha* (*Dolichos biflorus* Linn.) seeds, *Surana Kanda* (corm of *Amorphophyllus campanulatus* Linn.) and *Nimbu* (*Citrus medica* Watt.) were procured from the local market of Jamnagar. All the herbal materials were identified and authenticated in the Pharmacognosy Laboratory of Institute for Post Graduate Teaching and Research in Ayurveda, Gujarat Ayurved University, Jamnagar. *Gomutra* (cow urine) was collected from local cow shed.

Process validation of *Samanya Shodhana* (general purification) of *Tamra*

Samanya Shodhana of *Tamra* was carried out by *Nirvapa* (heating and quenching).[6] *Kanji* (sour gruel), *Kulattha Kwatha* (decoction of horse gram), and *Takra* (buttermilk) were prepared as per the references of *Parada Samhita*,[7] *Sharangadhara Samhita*,[8] and *Sushruta Samhita*[9] respectively. The 500 g of *Tamra* (pieces of 1 × 1 cm and 36 gauge thickness) was heated in an iron ladle to red-hot stage and quenched in each liquid media for 7 times. Temperature at the time of red-hot stage was taken by a thermocouple. Each time, liquid media was taken fresh and gravimetrically equal to the *Tamra* (500 ml × 1 × 7 = 3500 ml).

Process validation of *Vishesha Shodhana* (specific purification) of *Tamra*

Vishesha Shodhana of *Tamra* was carried out as per the reference of *Rasaratnasamucchaya*.[10]) *Samanya Shodhita Tamra* (3 batches) was boiled in freshly collected *Gomutra* (3 L × 3 batches = 9 L) for 3 h in *Dola Yantra*. It was then washed with warm water and dried.

Process validation of *Parada Shodhana*

Parada Shodhana was done as per the classical reference of *Rasatarangini*.[11] *Ashuddha Parada* (impure mercury): 500 g × 3 batches = 1500 g, *Sudha Raja* (lime powder): 500 g × 3 batches = 1500 g, *Nistusha Lashuna* (garlic): 500 g × 3 batches = 1500 g, and *Saindhava Lavana* (rock salt): 250 g × 3 batches = 750 g were taken as ingredients. *Parada* was triturated with *Sudha Raja* for 3 days. On the 4th day, it was filtered through a cotton cloth and procured *Parada* was triturated with *Nistusha Lashuna* and *Saindhava Lavana* until the *Kalka* (paste) became black. Then it was washed with hot water and filtered through a cloth, weighed and stored in a glass bottle.

Process validation of *Gandhaka Shodhana*

The classical reference of *Rasatarangini* was followed throughout the procedure.[12] *Ashuddha Gandhaka*: 1 kg × 6 batches = 6 kg, *Godugdha* (cow milk): 2 L × 3 times × 6 batches = 36 L (gravimetrically double to the *Gandhaka* each time), *Goghrita* (cow ghee): 250 g × 3 times × 6 batches = 4500 g (gravimetrically 1/4th to *Gandhaka* each time) were taken for the practical work. In the reference, volume of *Godugdha* is not mentioned. Here, the volume was considered, so as to dip the melted *Gandhaka* completely. Furthermore, the amount of *Goghrita* was not taken as per the reference, because it was observed in pilot study that only 1/4th of *Goghrita* is sufficient to melt the *Gandhaka*. Powdered *Gandhaka* was heated with *Goghrita* over *Mandagni* (mild heating). After melting, it was poured into hot *Godugdha* through *Goghrita* smeared cotton cloth. A solid mass with some granular *Gandhaka* was taken out and washed with hot water. The procedure was repeated twice. After drying, it was powdered, weighed and kept in a glass bottle.

Process validation of preparation of *Kajjali*

Shuddha Parada: 500 g × 3 batches = 1500 g, *Shuddha Gandhaka*: 500 g × 3 batches = 1500 g were taken for the preparation of *Samaguna Kajjali*. In a *Khalvayantra*, *Shodhita Parada*, and *Shuddha Gandhaka* were taken in equal quantity and triturated. It was continued until the powder became black, smooth, and lusterless.

Process validation of *Tamra Bhasma*

TB was prepared as per the reference of *Rasaratnasamucchaya* by the principle of *Puta* (incineration) in an Electrical Muffle Furnace (EMF).[13] *Samaguna Kajjali* equal to the amount of *Shuddha Tamra* was taken in mortar and *Nimbu Swarasa Bhavana* (wet trituration with lemon juice) was given. When paste like consistency appeared, the mixture of *Shuddha Tamra* was added in it and triturated. After drying in shade, it was kept in *Sharava* (earthen saucer) and covered by another *Sharava* and junction was sealed by double fold of *Multany Mitty* smeared clothes. It was subjected for *Puta* in EMF. On the next day, after *Swangasheetikarana* (self-cooling) *Sharava Samputa* was removed, material was collected and triturated. In subsequent *Puta* equal amount, to that of one *Puti Bhasma*, of *Samaguna Kajjali* was added and triturated well by giving *Bhavana* of

Table 1: Brief description of drugs used in preparation of *Tamra Bhasma*

Name of drug	Latin name/English name	Purpose
Tila Taila	Oil of *Sesamum indicum* Linn.	*Samanya Shodhana*
Takra	Buttermilk	
Gomutra	Cow urine	
Kanji	Sour gruel	
Kulattha Kwatha	Decoction of seeds of *Dolichos biflorus* Linn.	
Gomutra	Cow urine	*Vishesha Shodhana*
Shuddha Parada	Mercury (Hg) – purified	*Marana*
Shuddha Gandhaka	Sulfur (S) – purified	
Nimbu Swarasa	Fruit juice of *Citrus limon* (Linn.) Burm. f.	*Bhavana*
Surana Kanda	Corm of *Amorphophyllus campanulatus* Linn.	*Amrutikarana*

sufficient quantity of *Nimbu Swarasa*. After drying, this powder was subjected to *Puta*. Total three *Puta* were given to complete the preparation of *Bhasma*. Following temperature pattern was followed: 1st *Puta* – 700°C for 20 min, 2nd *Puta* – 600°C for 25 min, 3rd *Puta* – 500°C for 30 min.

Process validation of *Amrutikarana* of *Tamra Bhasma*

Amrutikarana of TB was done as per the reference of *Rasa Tarangini*.[14,15] *Surana Kanda*, *Shuddha Gandhaka*, and *Nimbu Swarasa* were used in this procedure. TB was triturated with ½ part of *Shuddha Gandhaka* in mortar and *Bhavana* of *Nimbu Swarasa* given. After proper trituration, a round bolus was prepared and dried in sunlight. *Surana* weighing 2.5 kg was cut into two halves horizontally. A round pit was made in the middle of both the halves. Dried bolus was kept in it and the two halves were joined together. A thick layer of *Kapadmitty* (mud smeared cloth) was done over it. It was then dried in sunlight. This was kept in EMF at the temperature of 500°C for 15 min. After *Swangasheetikarana* it was removed, triturated and stored in an airtight glass bottle.

Analysis of final product

To lay down the standards, raw *Tamra*, in process material and final product were analyzed physico-chemically. Final product (TB) was analyzed by using different organoleptic parameters like *Varna* (color), *Rasa* (taste), etc.; classical tests like *Varitaratwa*, *Rekhapurnatwa*, *Apunarbhavatwa*, *Uttama/Unama*, *Niruttha*, etc.,[16] and *Dadhi Pariksha* (curd test)[17] were performed. Other parameters like *Avami*, *Niswaduta*, etc., were also applied.[18] Modern physico-chemical parameters like loss on drying, ash value, acid insoluble ash, and water soluble extractive, etc., were performed on three samples of TB.[19] Sophisticated instrumental analytical techniques like scanning electron microscopy (SEM), particle size distribution (PSD), and inductive coupled plasma - atomic absorption spectrometry (ICP-AES) of one sample of TB was carried out. Instrument used for the particle PSD was Sympatec HELOS (H1004) SUCELL. Instrument used to perform quantitative elemental evaluation (ICP-AES) of the sample was - Inductive Coupled Plasma Spectrometer, Model: ARCOS from M/s. Spectro, Germany, R.F. Generator: Maximum of 1.6 KW, 27.12 MHz.

Observations and Results

During the *Samanya Shodhana* of *Tamra*, all *Tamra* flakes became red-hot at 460°C. As soon *Tamra Patra* were quenched in media typical "hissing" sound was produced with boiling of that media. After every quenching some residue in the form of powder was observed. After complete *Shodhana*, *Tamra* turned into a mixture of black coarse powder and pieces of *Tamra*. The average time taken by *Tamra* to became red and change in weight during the procedure is depicted in Table 2.

During the *Vishesha Shodhana* of *Tamra*, froth was observed on *Gomutra* after 10 min of boiling. Temperature of *Gomutra* was recorded between 96°C and 102°C throughout the procedure. After *Swedana*, *Tamra* became greenish in color. Color of *Gomutra* turned to blackish green with a strong irritating smell. The weight loss of *Tamra* after *Vishesha Shodhana* is depicted in Table 3.

In *Parada Shodhana* procedure, after trituration with *Sudha*, almost all *Parada* mixed with it after 4 h of trituration and color of the mixture turned gray. White *Lashuna Kalka* changed to black within 2 h of trituration. After this procedure, 451.5 g of *Shuddha Parada* was procured and 48.5 g (9.7%) of weight loss was observed in *Parada*.

During the *Gandhaka Shodhana* procedure, *Gandhaka* melted in 14 min (average) and the temperature recorded at its time of melting was 116°C. Crystalline dark yellow *Gandhaka* turned granular and dull yellow after *Shodhana*. Average 16.40 g (1.64%) decrease in weight was observed.

During the *Kajjali* preparation, color of the mixture started to become black after 12 h of trituration which turned dark black after 16 h trituration. *Varitaratva* (floating on stagnant water), *Rekhapurnatva* (filling the furrows when rubbed between two fingers), and *Nischandratva* (lustreless) were found after average 26.33 h of *Mardana* (trituration). On an average, 960 g of *Kajjali* was obtained and 40 g (4%) loss in weight was observed.

During the *Bhasma* preparation, cracks were observed on *Sharava* after taking out from EMF. Whitish layer of mercury was observed near the door of EMF. After first *Puta*, *Tamra Patra* became so brittle that it was getting powdered on rubbing between two fingers. Black, smooth, and very fine *Bhasma* was obtained after 3 *Puta*. Particulars of *Marana* procedure, duration of temperature given to *Puta*, specific observations and other observations regarding change in weight, etc., are depicted in Tables 4-7.

In the process of *Amritikarana*, three hours of trituration was required for the consistency of bolus formation. After *Putapaka*, *Surana* was found totally burnt. Bolus inside was black and breakable by some pressure. After trituration it converted to black, smooth, and fine powder [Table 8]. When different samples of *Tamra* analyzed quantitatively, 99.89%, 90.84% of copper and 0.10%, 5.53% of iron was detected in raw and

Table 2: Average time to become red hot and change in weight after *Samanya Shodhana*

Media	Avg. time taken to become red hot (h:min)	Wt. of residue after 7 *nirvapas* (g)	Avg. wt. (g)	Avg. wt. ↑/↓ (g)	Avg. wt. ↑/↓ (%)
Tila Taila	7:46	4	514.18	14.18 ↑	1.42 ↑
Takra	8:53	NM*	489.47	11.53 ↓	2.30 ↓
Gomutra	8:28	53.8	478.38	22.62 ↓	4.52 ↓
Kanji	8:54	112.9	465.10	34.90 ↓	6.98 ↓
Kulattha Kwatha	8:59	90.4	460.20	39.80 ↓	7.38 ↓

*Not measurable, ↑: Increase, ↓: Decrease, Wt.: Weight, Avg. wt.: Average weight

Shuddha Tamra respectively. TB complied with all the classical parameters [Table 9]. Physicochemical and advances analytical characters of TB are depicted in Table 10.

Discussion

Aim of the present study was to standardize the preparation method of TB. The *Bhasmas* prepared with *Parada* or *Parada Bhasma* as media are considered superior to others.[20] In this study, *Kajjali* (black sulfide of mercury - HgS)[21] was used as media for TB preparation.

99.89% of copper was found in raw *Tamra* which indicates the high purity of raw material. Thin sheets of copper were satisfying almost all the classical parameters for *Nepalaka Tamra*.[22] Copper wire taken for the study is used for electrical earthing and that is why it was in a very pure form. Slight impurity in it decreases its electric conductivity.[23] Hence these types of wires should be used as raw material for the preparation of TB.

For *Samanya Shodhana* of *Tamra*, it was heated and after the red-hot it was quenched for 7 times in *Tila Taila*, *Takra*, *Gomutra*, *Kanji*, and *Kulattha Kwatha* in order. These are acidic, acidic, basic, acidic and basic media in order. This specific order disrupts the internal structure of *Tamra* during the process. During *Shodhana*, color of *Tamra* became black. This is because during red-hot state, *Tamra* reacts with atmospheric oxygen and steam to form cupric oxide (CuO) which is black in color,[24] and reaction of *Tamra* occurs mainly on surface. It was observed that as *Shodhana* procedure advances *Tamra* takes comparatively more time to get complete red hot [Table 4]. Conductivity of heat is more in metallic form than compound state. So at later state of *Shodhana*, *Tamra* and its compound (CuO which is formed on the surface of *Tamra* flakes) took more time to become complete red hot.

At early stage of *Shodhana*, cracks were seen at the surface of *Tamra* flakes and finally, some coarse powder was observed. Repeated heating and cooling of *Tamra* flakes causes disruption in compression-tension equilibrium leads to cracks on the flake surface (stress corrosion theory).[25] During red hot state, compounds are formed on the surface of *Tamra* flakes. Expansibility differs from metal to compound on heating [generally expansibility of compound is less than metal (theory of thermal expansion).[26] So on repeated heating cracks are seen on the surface leading to breaking of *Tamra* flakes into coarse and some fine powder. After every seven *Nirvapas* this powder was found as sediment in media. Maximum amount of sediment (average 112.9 g) was found in *Kanji* medium [Table 3]. *Kanji* falls under the strong acidic category and also it has *Tikshna* property.[27] In *Takra* medium sediment was not observed but black particles were seen suspended in it, which was difficult to collect. In *Tila Taila* media, 1.42% weight gain was observed in spite of weight loss after *Nirvapa* process. This may be because of the adhered *Tila*

Table 3: Average weight loss after *Vishesha Shodhana* of *Tamra*

Avg. wt. of *Samanya Shodhita Tamra* (g)	Avg. amount of *Gomutra* required (ml)	Avg. wt. loss (g)	Avg. wt. loss (%)
450	3000	2.3	1.96

Avg. wt.: Average weight

Table 4: Particulars of *Tamra Marana*

Batch no.	Materials	Quantity		
		1st *Puta*	2nd *Puta*	3rd *Puta*
1	*Shuddha Tamra*	450.0 g	1st *Puta Marita Tamra*	2nd *Puta Marita Tamra*
	Samaguna		465 g	472.5 g
	Kajjali	450.0 g	465 g	472.5 g
	Nimbu Swarasa	200 ml	330 ml	335 ml
2	*Shuddha Tamra*	440.0 g	1st *Puta Marita Tamra*	2nd *Puta Marita Tamra*
	Samaguna		458 g	467.8 g
	Kajjali	440.0 g	458 g	467.8 g
	Nimbu Swarasa	200 ml	320 ml	330 ml
3	*Shuddha Tamra*	455.0 g	1st *Puta Marita Tamra*	2nd *Puta Marita Tamra*
	Samaguna		466 g	480.3 g
	Kajjali	455.0 g	455.0 g	480.3 g
	Nimbu Swarasa	200 ml	320 ml	340 ml

Table 5: Duration and temperature given for *Putas*

Puta no.	Maximum temp. given in EMF (°C)	Time taken to reach that temp. (h:min)	Duration of maximum. temp. (min)	Total duration of giving temp. (h:min)	Time taken for *Swangasheetikarana* (h:min)
1	700	2:5	20	3:0	20:0
2	600	2:1	25	2:25	18:0
3	500	2:0	30	2:20	15:0

EMF: Electric muffle furnace, temp.: Temperature

Taila; which could not be removed even after thorough wash with hot water.

Out of six liquid media used for *Shodhana* procedure, three were acidic (pH ranging from 3 to 3.5) and others were weak bases (pH ranging from 7.3 to 8.9). The alternate heating and quenching in these media lead to corrosive changes in the metal and may also cause removal of acid and alkali soluble impurities from the metal. It should also be noted that these media were among the naturally and easily prepared source of acid and base at the ancient times.

Vishesha Shodhana of *Tamra* is a simple process of *Swedana* in *Dola Yantra*, specific to *Tamra*. Possible hypothesis for conduction of this process may be further micropurification and impregnation of qualities for *Bhasma* preparation. In this process, components of *Gomutra* may pierce through the micropores and cracks created during *Samanya Shodhana* and may produce the required change specific to *Tamra* for further process. After *Vishesha Shodhana*, observed weight loss was may be due to the reaction between ammonia from *Gomutra* and copper to form the cuprammonium ion $[Cu(NH_3)_4]$, a chemical complex which is water-soluble.[28] This complex gets washed away during washing with hot water.

Ingredients used in the *Parada Shodhana* contained *Kshariya* (alkaline) materials like *Sudha* (lime) and *Saindhava* (rock salt). They may be removing the alkaline soluble impurities along with trace elements present in *Parada*. It was observed that when *Parada* was triturated with *Sudha*, it was converted to powder form, which may be referred as Grey powder (calomel). It is difficult to procure the whole amount of *Parada* by *Vastra Galana* (straining through cloth) process as mentioned in classics. So it was washed with hot water. During this procedure loss of *Parada* with water should be checked.

For the process of *Gandhaka Shodhana*, powdered sulfur was taken for the sake of easy melting. *Mandagni* was given to avoid burning of sulfur. Cloth was smeared with *Ghee* to avoid sticking of *Gandhaka* to the cloth. Hot *Godugdha* was taken for pouring of melted *Gandhaka* since it facilitates the granule formation while cold milk forms hard mass of *Gandhaka*. After each *Dhalana* (melting and pouring), *Gandhaka* was thoroughly washed with hot water to remove fat contents of milk and *Ghee*.

Loss in weight of *Kajjali* was observed because of dusting of mixture during trituration and some of it remained adhered to *Khalva* (mortar) which was difficult to collect.

Due to unavailability of *Jambira* (*Citrus limon* Linn. Burm.f.) in Jamnagar region, *Nimbu* (*Citrus medica* Watt.) was used. EMF was used for *Puta* to avoid variations such as weight and quality of cow dung cakes, seasonal variations that occur in classical *Puta*. EMF is a modified instrument by which a desired highest temperature and duration of heat can be adjusted; chance of heat loss is less and a homogeneous temperature pattern can be provided. Nowadays, many of the Ayurvedic pharmacies are using this instrument for preparing *Bhasmas*. Therefore it was preferred for *Putapaka* to provide a standard heating pattern.

Chakrikas were not prepared. In pilot study, it was observed that when *Chakrikas* were prepared, they became hard after every *Puta* and took nine *Putas* to form *Bhasma*. However, in a batch where *Chakrikas* were not prepared and only mixture was layered; it took only four *Putas* to form *Bhasma*. Thickness of layered mixture was around 1 cm. Although, thickness of the layer (1 cm) was more than that of traditional *Chakrika* (0.5 cm), which may cause less heat flow through the layered mixture according to Fourier's law.[29] However, in layered method also temperature was given for more time to complete the chemical reaction and to form a desired compound.

Table 6: Specific observations and results of *Tamra Bhasma* preparation after each *Puta*

Batch no.	*Puta* no.	Wt. of *Bhasma* (g)	Color of *Bhasma*	Taste	Curd test
1	1	465.0	Blackish brown	Strongly metallic	–ve
	2	472.5	Blackish	Slightly nauseating	–ve
	3	476.3	Black	Tasteless	+ve
2	1	458.0	Blackish	Strongly metallic	–ve
	2	467.8	Blackish	Slightly astringent	–ve
	3	470.7	Black	Taste less	+ve
3	1	455.0	Blackish brown	Strongly metallic	–ve
	2	480.3	Black	Tasteless	–ve
	3	482.6	Black	Tasteless	+ve

Wt.: Weight

Table 7: Increase in weight of *Tamra Bhasma*

Batch no.	Weight (g)		Weight increased (g)	Weight increased (%)
	Initial	Final		
1	450	476.3	26.3	5.8
2	440	470.7	30.7	6.9
3	455	482.6	27.6	6.06
Average	448.33	476.53	28.2	6.25

Table 8: Observations during *Amrutikarana* of *Tamra Bhasma*

Batch no.	TB taken for *Amrutikarana* (g)	*Surana kanda* (g)	*Shuddha gandhaka* (g)	*Nimbu swarasa* (ml)	TB after *amrutikarana* (g)	Wt. gain (g)	Wt. gain (%)
1	470	2550	235	230	483.6	13.6	2.9
2	465	2730	232.5	210	480.8	15.8	3.4
3	475	2390	237.5	210	485.7	10.7	2.3
Avg.	470	2556.7	235	216.7	483.4	13.4	2.9

Wt.: Weight; Avg.: Average; TB: *Tamra Bhasma*

For the first *Puta*, *Nimbu Swarasa* was required gravimetrically 1/4th of the total amount of *Shuddha Tamra* and *Samaguna Kajjali*. This amount of *Nimbu Swarasa* increased from second *Puta* onwards. This is because of reduced particle size and increased surface area of the material.

In *Rasamrutam*, Yadavaji has told that higher temperature in initial *Putas* and lower temperature in later should be given during preparation of TB.[30] Hence, EMF was selected for the *Putas* to regulate the temperature owing to its convenience. First, *Puta* was given 700°C and maximum temperature of *Putas* was reduced to 600°C and 500°C in later *Putas* [Table 5]. This particular temperature pattern was followed as a result of a pilot study for preparation of TB, which was carried out before going through this study.

The color of TB is black (*Krishna*).[31] In the process of TB preparation, copper is converted to its sulfide form in major since the sulfur (dissociation product of HgS) is an accompaniment to the metal in the processing. During the heat treatment for multiple times (*Puta*), some sulfides may get converted to oxide.

Table 9: Organoleptic and other classical characters of *Tamra Bhasma*

Parameters	*Tamra Bhasma*
Shabda (sound)	No sound when rubbed between fingers
Sparsha (touch)	*Mridu* (smooth)
Rupa (color/appearance)	*Krishna* (black), powder
Rasa (taste)	*Niswadu* (tasteless), *Awami* (non-nauseating/vomiting)
Gandha (odor)	Not specific
Varitaratva (floating on water)	+ve
Rekhapurnatva (filling the furrows when rubbed between two fingers)	+ve
Nishchandratva (lusterless)	+ve
Dantagre Kachkachaabhava (no grating sensation when chewed between teeth)	+ve
Dadhi Pariksha (curd test)	Passed

+ve: Compiled

Table 10: Physicochemical and advanced analytical characters of *Tamra Bhasma*

Parameters	*Tamra Bhasma*
Loss on drying (%w/w)	0.13
Ash value (%w/w)	98.10
Acid insoluble ash (%w/w)	2.32
Water soluble extract (%w/w)	0.78
CS_2 soluble extract (%w/w)	1.33
Phase identification (XRD)	CuS
Particle size distribution (VMD)	28.70 μm
Element content (ICP-AES) wt%	Cu: 58.56 S: 22.48, Fe: 0.31 As, Pb, Hg, Mn, Zn: Traces Cd, Se: Not detected

XRD: X-ray diffraction;,Wt.: Weight;,VMD: Volumetric mean diameter, ICP-AES: Inductive coupled plasma-atomic emission spectrometry, CuS: Cupric sulfide

Because metallic sulfides when heated in air get converted to oxide of the metal and sulfur dioxide.[32] Therefore, TB may be considered as a mixture of copper oxides (Cu_2O, CuO), copper sulfides (Cu_2S, CuS) and other trace elements.CuO is brown in color;[33] hence after first two *Putas* color of *Bhasma* was brownish black. It can be said that it was a combination of all these compounds. When *Bhasma* was properly formed, it was black in color. CuO and cupric sulfide (CuS) are black in color.[31] Therefore, it can be inferred that TB is either CuO or cuprous sulfide or combination of both. Average 476.53 g of TB was obtained from 448.33 g of *Shuddha Tamra*. 6.25% increase in weight after *Marana* may be attributed to formation of the oxides and sulfides of copper as mentioned earlier. Inorganic contents of *Nimbu Swarasa* (9.45% w/w) also lead to increase in weight of TB.

For the *Amrutikarana* 470 g of TB *Surana Kanda* of 2.5 kg was required. Same temperature was applied at which the *Bhasma* was formed. 2.9% increase in weight after *Amrutikarana* may be attributed to inorganic contents (mainly calcium oxalate crystals) of *Surana Kanda*. Organic contents of *Surana* act as a source of carbon. An unstable metallic compound (especially oxides) can be reduced to metallic state during this procedure by the carbon reduction process.[34]

$$CuO + C \xrightarrow{\Delta} Cu + CO$$

This metallic copper can be further reduced to sulfide in presence of sulfur. However, as metallic compound should not be changed on the particular temperature in which it is formed. Hence it can be inferred that the process of *Amrutikarana* removes any unstable compound (CuO in this case) and makes the product more stable (CuS).

The color of TB obtained was black [Figure 1]. Color indicates the formation of specific compound. Here black color indicates the formation of either CuO or CuS of copper or both. Tests like *Rekhapurnatva* and *Varitaratva* indicate the lightness and fineness of the *Bhasma*. *Awami* nature of TB indicates that there is no free copper or any unwanted compound (like copper sulfate) in it. Further no discoloration in curd test even after 48 h proves that there is no free copper or copper sulfate in final product. *Awami* and no discoloration in curd test should be considered as main tests to assess the properly prepared TB.

Figure 1: Appearance of final product (*Tamra Bhasma*)

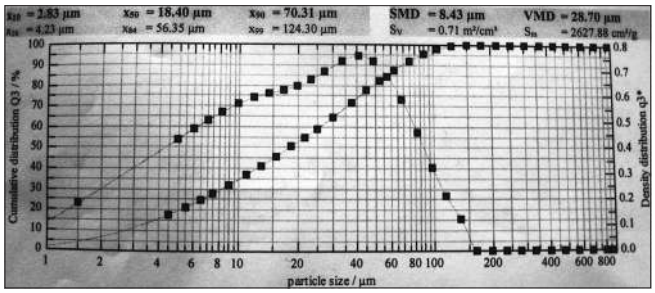

Figure 2: Particle size distribution of *Tamra Bhasma*

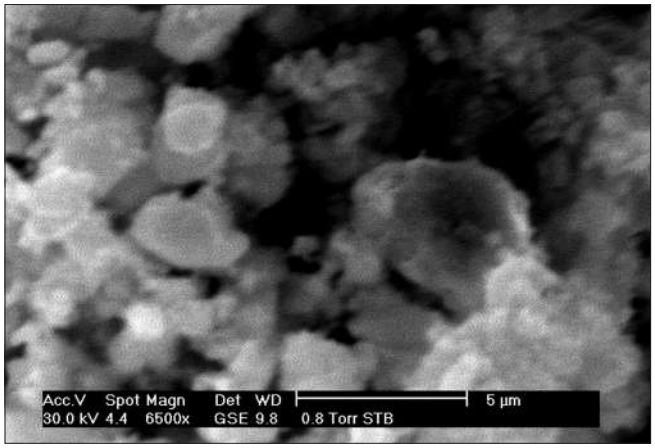

Figure 4: SEM images of Tamra Bhasma (at resolution 5 µm and magnification 6500×)

In PSD analysis 10% of the material was found below 2.83 μm where as maximum material was below 124.30 μm [Figure 2]. Small particle size enhances the absorption,[35] hence the bioavailability and thus potency of the drug increases resulting in decrease in its dose. This eventually leads to decrease in dose-related side effects of the drug.

Bhasma analyzed under scanning electron microscope showed particle size less than 2μm [Figures 3 and 4]. In X ray diffraction [Figure 5] prominent peaks of CuS were seen, which confirms that final product is sulfide form of copper. Presence of oxides cannot be denied because other peaks were also present. Furthermore, in ICP-AES 58.56 wt% of copper against 22.48 wt% of sulfur was present. As the procedure of *Tamra Shodhana* was carried out in an iron pan 0.31 wt% of iron was traced in *Bhasma*. Heavy metals like cadmium, selenium were not detected while others like arsenic, lead and mercury were present in traces. Hence TB can be considered as blend of micronutrients.

Conclusion

The adopted method for preparation of TB can be considered as easy, convenient and SMP. The temperature pattern adopted in EMF to prepare TB in this study can be considered as a standard heating pattern. TB should be considered as combination of copper and sulfur in CuS form though presence of oxides cannot be denied. Curd test is the simple household test to finalize the preparation of TB. After three *Puta* and *Amritikarana*, particle size of TB reaches up to micron level. In TB, along with copper and sulfur as main elements other elements like manganese, zinc, lead, and arsenic are also present in traces.

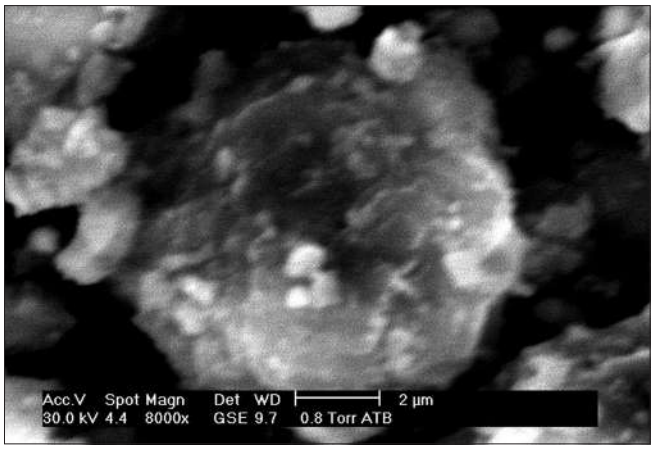

Figure 3: SEM image of *Tamra Bhasma* (at resolution 2 µm and magnification 8000×)

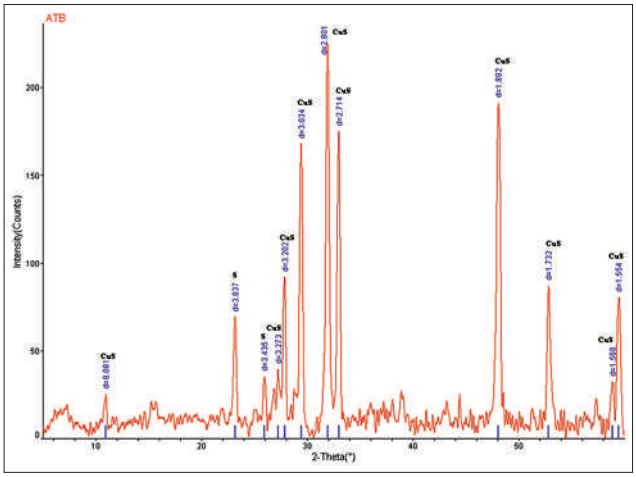

Figure 5: XRD analysis of *Tamra Bhasma*

Acknowledgment

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Original research article (Experimental)

Standard manufacturing procedure of *Teekshna lauha bhasma*

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ABSTRACT

Background: *Lauha bhasma* is one of the herbo-metallic preparations used in *Ayurveda*, a traditional Indian system of medicine for treating various ailments such as anemia, diarrhea, hyperlipidemia and diabetes.
Objective: To establish standard manufacturing procedure of *Teekshna lauha bhasma* and analyze its physico-chemical properties.
Materials and methods: The preparation of *T. lauha bhasma* (calx of iron [Fe] turning) involves *samanya shodhana*, *vishesha shodhana* followed by *bhanupaka*, *sthalipaka* and *putapaka* with *Triphala kwatha* as a medium under temperature of 650 °C in electric muffle furnace (EMF) and maintained for 1 h. *T. lauha bhasma* were subjected to different physico-chemical characterization using X-ray fluorescence spectrophotometer and scanning electron microscopy.
Results and discussion: The results suggest that these steps are necessary to obtain a good quality of *bhasma* and also make it acceptable for trituration during *Bhasmikarana* process. It is found that *T. lauha bhasma* was prepared properly in 20 *puta* at a temperature of 650 °C. The particle size of 20 *puta T. lauha bhasma* is 100–500 nm in range.
Conclusion: Pharmaceutical procedures given in *Ayurvedic* texts are necessary to prepare *pakwa jambu phala varna T. lauha bhasma* that complies with all the classical *bhasma pariksha* and modern analytical parameters in 20 *puta* at a temperature of 650 °C maintained for 1 h in EMF.
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Introduction

Ayurveda, the science of life, is a comprehensive medical system that has been the traditional system of healthcare in India for more than 5000 years. The basic aim of this science is to maintain healthcare by balancing the physical, mental, and spiritual functions of the human body [1,2]. *Rasashastra*, an integral part of *Ayurveda*, deals with the drugs of mineral origin, and details their varieties, characteristics, processing techniques, properties, therapeutic uses, possibilities of developing adverse effects and their management, etc. in a comprehensive way [3]. *Ayurvedic* experts have estimated that 35–40% of the approximately 600 medicines mentioned in the *Ayurvedic* formulary may contain at least one metal [4]. *Ayurvedic* medicines are mostly *Rasoushadhies* (herbo-mineral) and they play an important role in *Ayurvedic* therapeutics because of their qualities such as *Alpamatropayogitvat* (low dose), *Arucher-aprasangata* (good palatability) and *Kshipramarogayadayitvat* (fast acting) [5]. *Rasayana* (immunomodulation and anti-aging quality) and *Yogavahi* (ability to target drugs to the site) are characteristics of a properly made herbo-mineral preparation, which is also nontoxic, readily absorbable, adaptable, and assimilable in the body [6]. *Bhasmas* are herbo-metallic ashes in which the metal is calcined along with various herbal ingredients to form organometallic complexes [7]. These complexes should neither contain free metal nor contain free organic constituents, whose presence in *bhasma* indicates improper calcination [8].

Iron (Fe) is an essential element for almost all living organisms as it participates in a wide variety of metabolic processes, including oxygen transport, deoxyribonucleic acid synthesis, and electron transport [9]. The incinerated Fe preparations of *Ayurveda* are known as *lauha bhasma* (Fe calx) [10]. It is a herbo-metallic calx that has several therapeutic applications. Pandit et al. reported that, *lauha bhasma* for hematinic activity and hemoglobin regeneration efficacy on agar gel diet and phlebotomy induced Fe deficiency anemia in rats and reported significant hematinic and hemoglobin

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regeneration efficiency in comparison to control and standard ferrous sulfate containing drug [11]. Antibacterial activity of *lauha bhasma* was reported by Tambekar and Dahikar [12], In the *Samhita* period Fe (*Ayas-Lauha*) was used in the form of fine powder. Later, *Rasashastra* classical texts explained the *shodhana* (purification) and *marana* (incineration) methods [13]. According to *Rasa Ratna Samuchchhaya*, *kanta lauha* (magnetite Fe ore) is considered as best raw material variety of Fe-for *lauha bhasma* [14]. However in the absence of *kanta lauha*, *Teekshna lauha* (Fe turning) is used for the preparation of *lauha bhasma*. Now-a-days, in many *Ayurvedic* pharmacies and industries *lauha bhasma* is prepared from *T. lauha* and the preparation protocol for *bhasma* varies from manufacturer to manufacturer; there are many *Ayurvedic* texts describing different methods of preparation of *lauha bhasma* [15,16] and it plays a major role in deciding the therapeutic efficacy, as well as the toxic effects of *bhasmas*. The conventional *puta* (using electric muffle furnace [EMF]) method of heating is very easy and convenient to regulate temperature in closed atmosphere as comparative to traditional *puta* (using cow dung). Hence, in this study standard manufacturing procedure of *T. lauha bhasma* (calx of Fe turning) was established by following the guidelines of *Ayurvedic* formulary of India by adopting various procedures such as *samanya shodhana* (normal purification), *vishesha shodhana* (special purification), *trividh lauhapaka,* that is, *bhanupaka* (exposure to sunlight), *sthalipaka* (roasting in an Fe pan), and *putapaka* (calcination) using EMF. This study also attempts to characterize physico-chemical properties of *T. lauha bhasma* through conventional studies for studying the quality of *bhasma Nischandratvam* (lusterless), *Apunarbhava* (metal irreversibility test), *Varitaratvam* (floating test), and detailed information on elemental composition and particle size of *T. lauha bhasma* has been evaluated by Bhargava et al. using modern analytical instruments like scanning electron microscope (SEM) and X-ray fluorescence (XRF) spectrophotometer [17,18].

Materials and methods

Procurement of raw material

The authenticated raw materials; *T. lauha* (Fe turnings) were collected from the Department of Metallurgy, IIT (BHU); *Tila taila* and *Triphala* were collected from the *Ayurvedic* pharmacy, BHU; *Kulattha* collected from local market, Varanasi and *Gomutra* (cow's urine) were collected from Dairy farm, Institute of Agricultural Sciences, BHU, Varanasi.

Materials

EMF – inner hearth (length: 15 cm, breadth: 19 cm, depth: 30 cm, and maximum temperature capacity: 1000 °C), *khalva yantra* (mortar – length: 36 cm, breadth: 21 cm, thickness: 3 cm, depth: 11 cm; pestle – length: 13 cm, and diameters: 7 cm).

Methods

The preparation of *T. lauha bhasma* (calx of Fe turning) was carried out in Laboratory of Department of Rasa Shastra, Institute of Medical Sciences, Banaras Hindu University, Varanasi, India by following the procedure described in the *Ayurvedic* formulary of India [19]. It involves the following major steps; *samanya shodhana* (normal purification), *vishesha shodhana* (special purification), *trividh lauhapaka*, that is, *bhanupaka* (exposure to sunlight), *sthalipaka* (roasting in an Fe pan), and *putapaka* (calcination) (Fig. 1).

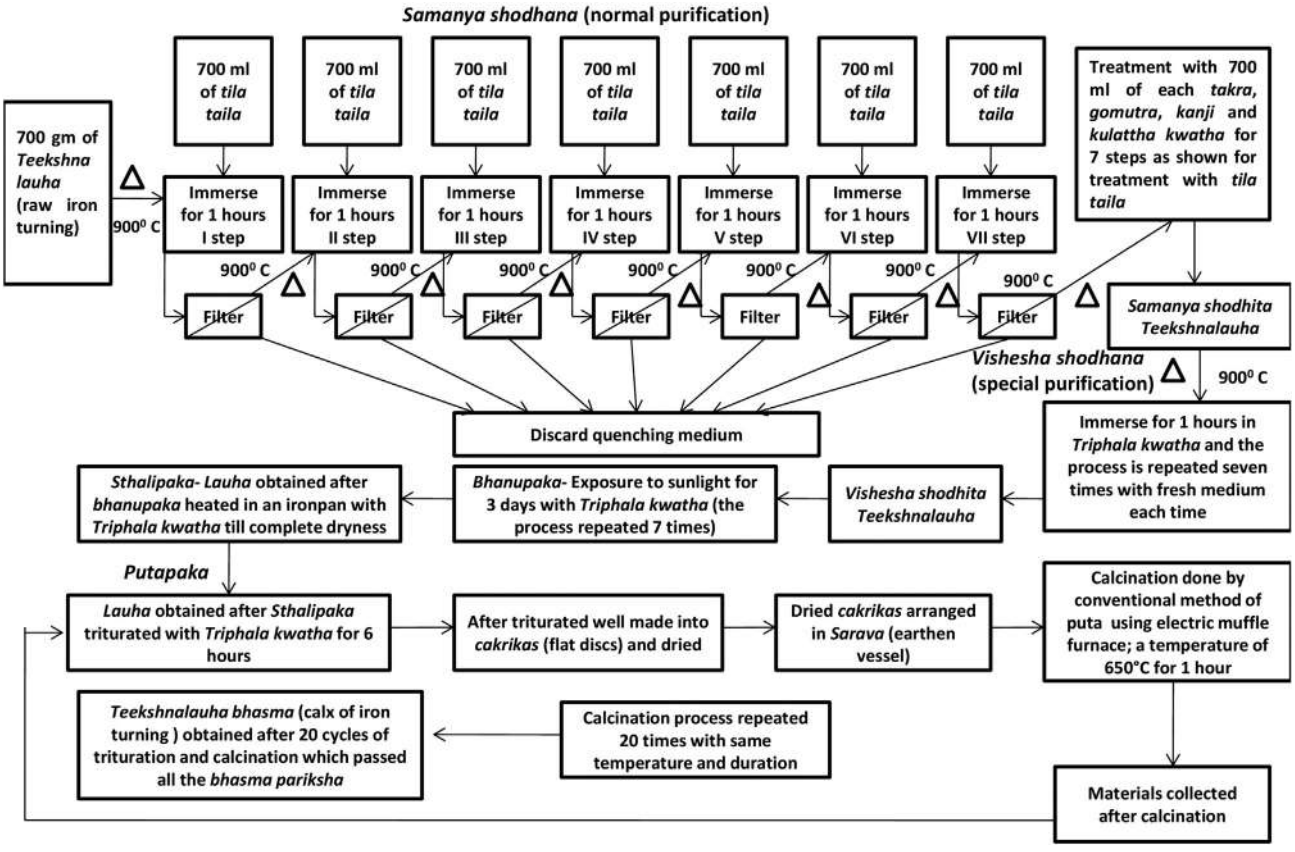

Fig. 1. Flow diagram for the preparation of *Teekshna lauha bhasma*.

T. lauha bhasma was subjected to various organoleptic and physico-chemical analysis such as color, taste, texture, loss on drying [20], ash value [21], acid insoluble ash [21], and water soluble ash [21]. Modern analytical instruments such as XRF and SEM were employed to determine the elemental composition and particle size respectively.

Samanya shodhana

In *samanya shodhana* process, 700 g of raw material (Fe turning) was heated in EMF till red hot condition (~875–900 °C) and immersed in 700 ml of each medium viz. *tila taila* (sesame oil), *takra* [22] (buttermilk), *Gomutra* (cow's urine), *kanji* [23] (rice gruel), and *kulattha kwatha* [24] (decoction of horse gram) and kept for self-cooling (approximately 1 h) at room temperature (Fig. 2). This quenching process was repeated for seven times consecutively in *tila taila* followed by seven times consecutively in *takra*, *gomutra*, *kanji,* and *kulattha kwatha* by using fresh media every time. After completion of the process, material was filtered by Fe mesh and dried under sunlight. The material obtained at this stage is called *samanya shodhita lauha*.

Vishesha shodhana

In this purification step, quenching was done in *Triphala kwatha*. It was prepared by taking coarse powders of three myrobalans, taken without seed: *Haritaki* (*Terminalia chebula* Retz.), *Bibhitaki* (*Terminalia bellirica* [Gaertn.] Roxb.), and *Amalaki* (*Phyllanthus emblica* L.) in equal quantity (each 1 kg) and boiled in 24 L of water till reduction to 1/4th of the original volume of water to obtain *Triphala kwatha*. Using this, repeated quenching process of *samanya shodhita lauha* was done. This purification step was repeated seven times using freshly prepared *Triphala kwatha*. The *lauha churna* (coarse powder of Fe turning) obtained at this stage is called *vishesha shodhita lauha*.

Bhanupaka

Triphala kwatha was prepared by heating equal quantity of *Triphala* to vishesha *shodhita lauha* churna with two parts of water and reduced to 1/4th of original volume. This *Triphala kwatha* was added to *lauha* obtained after *vishesha shodhana* and allowed to dry under sunlight. It took a maximum of 3 days for complete drying of *Triphala kwatha*. This process was repeated seven times to yield intermediate after *bhanupaka* (Fig. 3a).

Sthalipaka

In this step, *Triphala kwatha* was prepared by taking *Triphala* 3 times of *lauha* obtained after *bhanupaka* and 16 times of water was added to it. The whole material was boiled in a stainless steel container to reduce the volume to 1/8th of the original volume of water. *Lauha* obtained after *bhanupaka* was washed with hot water and placed in a sthali (Fe pan), to which above freshly prepared *Triphala kwatha* was added and intense heating was given for complete evaporation of water contents of *Triphala kwatha*. On complete drying of the material, again *Triphala kwatha* was added and subjected to heat. This process required 4 h 30 min for complete drying of *Triphala kwatha*. The whole process was repeated seven times to yield intermediate after *Sthalipaka* (Fig. 3b and c).

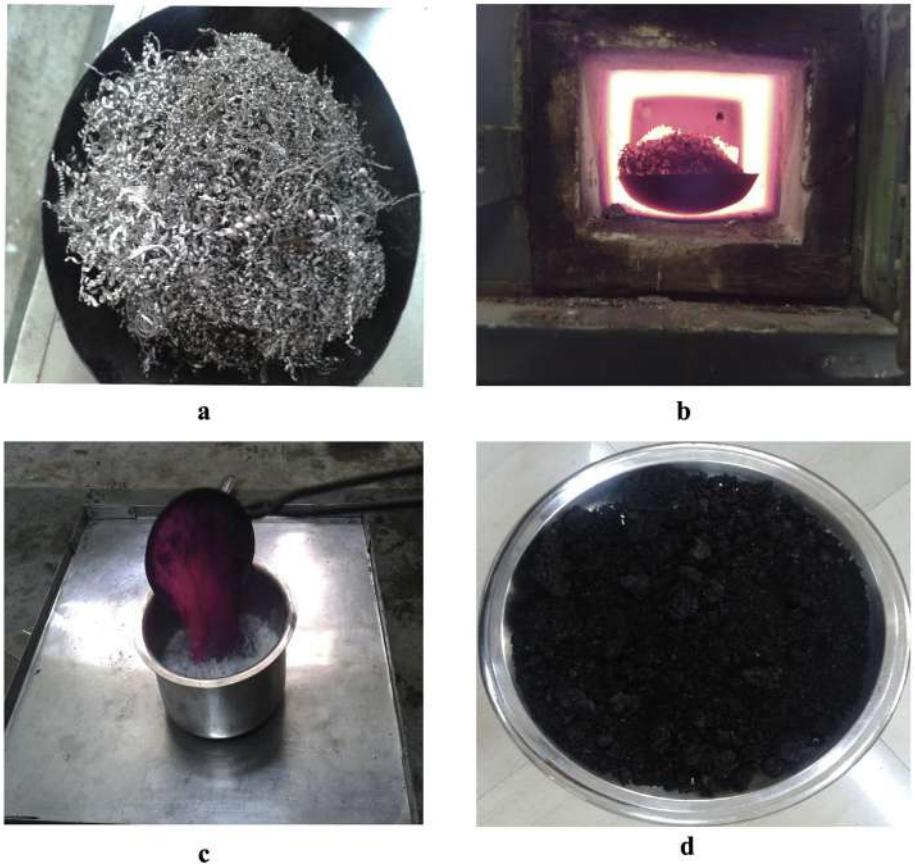

Fig. 2. *Shodhana* process of *Teekshna lauha* (iron turning). (a) Raw *Teekshna lauha* (iron turning), (b) red hot iron turning, (c) quenching process, (d) *Teekshna lauha* after *shodhana*.

Fig. 3. *Trividh lauhapaka*. (a) *Bhanupaka*, (b) *Sthalipaka*, (c) after *Sthalipaka*, (d) after 1 *puta*, (e) after 5 *puta*, (f) after 10 *puta*, (g) after 15 *puta*, (h) after 20 *puta*.

Putapaka

In traditional literature, the process of *puta* (calcination) refers to controlled heating of herbo-mineral preparations and allowing the preparation to cool to room temperature. [25] According to *Rasa tarangini, lauha bhasma* should be prepared by triturating with specific media according to disease and subjecting to *puta*, but in case of unavailability of the specific media the *bhasma* should be prepared by *Triphala kwatha*. [24,26] In this process, freshly prepared *Triphala kwatha* was mixed with *lauha* obtained after *sthalipaka* in mechanized *khalva yantra* and trituration was done with a frequency of 60 times/min. The paste formed during this trituration

Fig. 4. Scanning electron micrographs of 20 *puta Teekshna lauha bhasma.*

was made into *cakrikas* (pellets) and dried under sunlight. After complete drying of *cakrikas*, it was taken in an earthen vessel (*sarava*) and covered with another inverted earthen vessel. The space between the two earthen vessels was covered with clay smeared cloth; this specific process is known as *Sarava samputikarana* (sealed earthen pot). After this, it was subjected to *puta* in horizontal EMF and temperature was allowed to gradually rise to 650 °C in 2 h and maintained for 1 h [27]. The furnace was then switched off and allowed for self-cooling. The next day, pellets were collected from *sarava* and again triturated with *Triphala kwatha*. Same process of *puta* was repeated for 20 times to obtain *T. lauha bhasma* of desired quality.

Observations and results

Samanya shodhana of *T. lauha* (Fe turning) was carried out as per *Ayurvedic* formulary of India where the sequential quenching process was done in different media by following conventional order. Metallic luster was lost after quenching in *tila taila* and prominent cracks and flakes were seen after quenching in *takra* and *gomutra*. The maximum part was in powder form after quenching in *kulattha kwatha*. At the end of *samanya shodhana,* it was observed that, the hard shining Fe metal was converted into black colored lusterless powder of brittle material. After the process of *vishesha shodhana,* brittleness and weight of *T. lauha* increases and the dark black color powder was formed. It was observed that pH of media increases after every quenching of *shodhana* process (Tables 1 and 2).

During the process of *bhanupaka* while adding *Triphala kwatha* in *shodhita lauha* churna, color changes from greenish brown to black (Fig. 3a). After complete sun drying, *lauha churna* gets converted into a big cluster. The product obtained after *bhanupaka* was more brittle in nature and big particles of *lauha churna* were converted into fine particles. In *sthalipaka*, during heating *lauha churna* was adhering to the surface of container and fumes increased with the duration of heating (Fig. 3b). At the end of *sthalipaka* the color changes from dark black to brick brown in color and *lauha churna* was converted into powder form completely (Fig. 3c). Various observations during *bhanupaka and sthalipaka* are tabulated in Table 3.

During *Putapaka* (calcination), *lauha churna* obtained after *sthalipaka* was triturated with *triphala kwatha*. In first *puta,* it was taking 8 h to get converted into paste like structure and also difficult to make pellets. After *putapaka* the color of pellets changed from brick brown to dark brown (Fig. 3d–h). After every *puta,* it was easy to make pellets due to reduction in particles size and also color changes. Physical characteristics of the material recorded down after every *puta* which was shown in Tables 4 and 5. *T. lauha bhasma* was passed by various classical *bhasma pariksha* (Table 6). The results of various physiochemical parameters color, taste, texture, loss on drying, ash value, acid insoluble ash, water soluble ash, particle size (Fig. 4), and elemental composition are tabulated in Tables 7 and 8.

Table 2
Changes in pH of media before and after quenching during *samanya* and *vishesha shodhana* of *Teekshna lauha* (iron turning).

Media	Before	After
Takra	3.5	4
Gomutra	7.5	8.3
Kanji	3	4
Kulattha kwatha	6.4	7.4
Triphala kwatha	2.8	3.3

Discussion

Bhasma preparations involve the conversion of the metal into its mixed oxides, during which, the zero valent metal state is converted to a higher oxidation state. The significance of this "*Bhasmikarana*" is that the toxic nature of the resulting metal oxide is completely destroyed while introducing the medicinal properties into it [28]. A *bhasma* means a fine ash obtained though incineration [29]. Selection of raw material is the most important step in the *bhasma* preparation. Authentic raw material with high quality assures producing safe and efficacious finished product. In traditional literature, *kanta lauha* (magnetite Fe ore) and *T. lauha* (Fe turnings) is considered as best raw material for *lauha bhasma* preparation [30]. The availability of *kanta lauha* (magnetite Fe ore) is rare, that is why, in many *Ayurvedic* pharmacies *and* industries, *lauha bhasma* is prepared from *T. lauha*. Hence in this study, a step is made to find out the best quality of *T. lauha bhasma* using EMF. In this regard, raw material *T. lauha* was tested by classical method, as well as by modern analytical techniques [18,31]. In classical method, *Kalka* of *Kasisa* (ferrous sulphate, $FeSO_4.7H_2O$) and *Amalaki* (*P. emblica* L.) fruit pulp was applied over the surface

Table 1
Observations made during *samanya* and *vishesha shodhana* of *Teekshna lauha* (iron turning).

Media	Quantity (ml)	Initial weight (g)	Final weight (g)	Gain/loss (g)	Actual percentage change
Tila taila	700	700	740	40 ↑	5.7 ↑
Takra	700	740	780	40 ↑	5.7 ↑
Gomutra	700	780	746	34 ↓	4.4 ↓
Kanji	700	746	740	6 ↓	0.9 ↓
Kulattha kwatha	700	740	740	No change	No change
Triphala kwatha	700	740	850	110 ↑	15% ↑

Table 3
Observations made during *bhanupaka* and *sthalipaka* of *vishesha shodhita Teekshna lauha*.

Pharmaceutical procedure	Media (*Triphala kwatha* in ml)	Initial weight (g)	Final weight (g)	Gain/loss (g)	Actual percentage change
Bhanupaka	425	850	1250	400↑	47↑
Sthalipaka	7500	1250	885	365↓	29↓

Table 4
Changes in weight of *Teekshna lauha bhasma* before and after *puta* during *putapaka.*

Puta	Media (*Triphala kwatha* in ml)	Initial weight (g)	Final weight (g)	Loss (g)	Actual percentage change
1st	325	885	839	46 ↓	5 ↓
2nd	325	839	836	3 ↓	0.35 ↓
3rd	325	836	832	4 ↓	0.47 ↓
4th	325	832	830	2 ↓	0.25 ↓
5th	325	830	828	2 ↓	0.25 ↓
6th	350	828	815	13 ↓	1.5 ↓
7th	350	815	813	2 ↓	0.25 ↓
8th	350	813	813	No change	No change
9th	350	813	810	3 ↓	0.35 ↓
10th	400	810	810	No change	No change
11th	400	810	790	20 ↓	2.4 ↓
12th	400	790	786	4 ↓	0.47 ↓
13th	400	786	783	3 ↓	0.35 ↓
14th	400	783	783	No change	No change
15th	425	783	780	3 ↓	0.35 ↓
16th	425	780	770	10 ↓	1.2 ↓
17th	425	770	769	1 ↓	0.1 ↓
18th	450	769	766	3 ↓	0.35 ↓
19th	450	766	765	1 ↓	0.1 ↓
20th	450	765	765	No change	No change

of *T. lauha* and after some time conical protrusion (*girisringa*) are seen over the surface of *lauha*, it shows the sample of *T. lauha* is genuine. The elemental analysis through XRF study also revealed that raw Fe turning contains 98.10% of Fe (Table 8) [18]. All the other necessary media for pharmaceutical procedure such as *kanji, takra, Triphala kwatha,* etc., that were used at various stages were prepared under observation.

Shodhana (purification/detoxification) is the foremost step in the preparation of *lauha bhasma* because impure *lauha bhasma* on administration causes *Hritpida* (chest-pain), *Agnimandya*

Table 5
Observations recorded before and after *puta* of *Teekshna lauha bhasma* during *putapaka.*

Puta cycle	Before *puta*	After *puta*
1st	Brick brown color, on trituration color changes to black	Dark brown color
2nd	Pellets were made easily and rough in consistency	Pellets were easily breakable by hand and color was bluish black but surface of pellets was brown
3rd	After trituration color turns to grayish black	Pellets become soft and color changes to more bluish black
4th	Particle size increases and soft in consistency	Pellets were fragile and color was same as previous *puta*
5th	Duration of trituration decreases and pellets were made easily	Color of pellets were nearer to blackish red, that is, *pakwa jambu phala varna* and soft in consistency
6th	*Lauha bhasma* was very soft and quantity of media increases for trituration	Color was blackish red and pellets were easily breakable
7th	Again on trituration, color was black and very soft in consistency	Color was very much similar as previous *puta* and 15% *rekhapurnatva* test positive
8th	Color was bluish black before trituration	Pellets were easily broken by mild pressure of finger and color was blackish red. 20% *rekhapurnatva* passed
9th	*Lauha bhasma* was very soft in consistency	25% *rekhapurnatva* and other findings are same as previous *puta*
10th	More quantity of liquid media required for trituration and it was easily to make pellets	Hardness of pellets was increased. Color of pellets was turned to blackish red and black spots were found over few pellets. 35% *rekhapurnatva* passed
11th	*Bhasma* was brownish red color and became more *rekhapurnatva*	Pellets were still very hard and color was blackish red and 45% *rekhapurnatva* test positive
12th	Color was same and after trituration material becomes stickier	Pellets were hard and *bhasma* achieved 50% *rekhapurnatva* and 30% *varitar*
13th	*Bhasma* was blackish red colored and metallic taste present	Color maintained and pellets were mild hard
14th	Same as previous	Color was blackish red and *bhasma* achieved 60% *rekhapurnatva* and 40% *varitar*
15th	Quantity of liquid media increases for trituration	Pellets were little hard and blackish spots were found on the surface. Color of *bhasma* was blackish red and 50% *varitara* test positive
16th	After trituration material were stickier in nature and difficult to make pellets	Color of *bhasma* was completely blackish red, that is, *pakvajambuphalavarna.* 65% *rekhapurnata and* 60% *varitara* test positive
17th	On trituration gritty appearance of *bhasma* was observed. Color maintained	Color of *bhasma* maintained and metallic taste present. 70% *rekhapurnata.* Pellets were now soft
18th	Color of *bhasma* maintained and soft in consistency. On trituration quantity of media increases	75% *rekhapurnata and* 70% *varitara* test positive. Metallic taste present
19th	Color and softness of pellets maintained	90% *rekhapurnata and* 75% *varitara* test positive. Metallic taste absent
20th	Same as previous	*Bhasma* passed all *bhasma pariksha* with 95% *rekhapurnata* and 80% *varitara* test positive

Table 6
Analysis of *Teekshna lauha bhasma* by ancient methods.

Test	*Teekshna lauha bhasma*
Rekhapurnatvam	The *Lauha bhasma* was rubbed in between index finger and thumb. It enters into the ridges of the finger – positive
Varitaratvam	A small amount of *Lauha bhasma* was carefully sprinkled on the water. It was found that 80% *bhasma* was floating on the water surface – positive
Nirdhumatvam	The *Lauha bhasma* was sprinkled on red hot coal. It did not emit smoke – positive
Nischandratvam	It was not having any lustre found positive
Apunarbhava	The *Lauha bhasma* was triturated with *Gunja* (*Abrus precatorius* L.), *Goghrita* (cow ghee), *Madhu* (honey), *Tankana* (Borax), *Guggulu* (*Commiphora wightii* [Arn.] Bhandari), and made pellets. Then it was get subjected to *puta* on 650 °C in EMF maintained for 1 h. Next day after self-cooling pellets were triturated and not any agglomeration was found

EMF = Electric muffle furnace.

Table 7
Physico-chemical analysis of *Teekshna lauha bhasma.*

Test	Result
Color	*Pakva jambu phala varna*
Taste	Tasteless
Texture	Amorphous
Loss on drying (%)	0.31
Ash value (%)	98.15
Acid insoluble ash (%)	27.50
Water soluble ash (%)	30.26
Particle size	100–500 nm

(indigestion), *Apasmar* (epilepsy), *Shotha* (oedema), *Napunsakata* (impotency), *Prameha* (diabetes), *Ashmari* (renal calculus), and even death also [32,33]. For the process of *samanya* and *vishesha shodhana*, the process of *nirvapa* (quenching) was adapted. It is one of the foremost step in the purification process of *T. lauha* in which the metal is heated up to red hot condition and immediately immersed in various plants and animal media (sesame oil, butter milk, cow urine, cow milk, etc.) and kept for self-cooling [34]. It removes the inorganic impurities and incorporates beneficial organic moieties into the metal which render them suitable for further process of preparation of *bhasma* (grinding with plant drug and repeated calcination) [35]. In this study, *T. lauha* (Fe turning) was heated till they were red hot and poured in different media, that is, *tila taila, takra, gomutra, kanji, kulattha kwatha,* and *Triphala kwatha* (7 times each). At each time, sufficient quantity of media was taken which was approximately 700 ml. The average temperature of the heating device and surface of container was 1000 °C and 950 °C, respectively. The average temperature of red hot Fe turning recorded in EMF was 900 °C. The use of a particular media and particular sequence is notable. The probable concept behind using such variation may be removal of impurities from the drug in a particular acidic or alkaline media and also reduction in particle size of drug [36]. After heating, immediate cooling in liquid media leads to decrease in tension and increase in compression force. Repetition in heating and cooling causes disruption in compression tension equilibrium and leads to increased brittleness, reduction in hardness, and finally reduction in the particle size [37]. After removing the hydrophobic impurities using sesame oil (*tila taila*) treatment, oxide scales are formed due to atmospheric oxidation of the raw material, are removed by treatment with aqueous media viz., butter milk (*takra*), cow urine, rice gruel (kanji), and horse gram decoction, which are traditionally known for this property [25]. The formation of oxides scales are also responsible for increase in pH after every purification steps. Oxides (Fe oxides) are mainly basic in nature, so it can raise the pH after every quenching. In each of the steps in *samanya shodhana* (normal purification), progressive increase in surface area and reduction in particle size, probably due to micro cracks formed during heat treatment is observed. At the end of *shodhana* process weight gain was observed, it may be due to addition of contents of quenching media (Tables 1 and 2).

During the process of *bhanupaka, Triphala kwatha* was added to *vishesha shodhita lauha* and allowed to dry under sunlight. The role of sunlight during *bhanupaka* has very specific reason. It has been widely established that the metallic Fe is toxic [38]. Hence, Fe supplements should contain Fe in the form of complex. The ultraviolet radiation present in the sunlight reduces the oxidation state of Fe in the presence of Vitamin C present in the *Triphala* decoction thereby improving the bioavailability [39]. In this process, more time is available for the reaction between *Triphala* and *lauha churna*, it causes an increase in the weight of *lauha churna*, which may be due to the addition of solid contents of *Triphala kwatha*. In *sthalipaka*, *lauha* obtained after *bhanupaka* was mixed with *Triphala kwatha* in an Fe pan and intense heating was given up to complete dryness. After *sthalipaka* weight of *lauha churna* was decreased due to burning of solid contents of *Triphala kwatha* (Table 3).

Triphala mainly consists of tannins and ascorbic acid. The absorption of food Fe can be greatly influenced by other constituents in the diet, such as ascorbic acid (Vitamin C) and phenolics. Ascorbic acid increases the bioavailability of Fe by converting Fe^{3+} to Fe^{2+}, while phenolics can reduce the bioavailability of Fe by binding to its phenolics (e.g., tannins). Excess of ascorbic acid and/or a lack of dietary tannins have both been suggested as contributing to clinical/pathological Fe storage disease [36]. Five hundred milligram of Fe causes severe toxicity, leads to shock, metabolic acidosis, and liver damage [38]. In other words, this may also be taken as the various constituents of *Triphala* have antagonizing activity. Thus, too much Fe absorption is prevented. *Triphala* is a mild laxative and thereby counteracts the constipating property of Fe and thus be

Table 8
Results of XRF analysis showing elemental composition.

Element	Raw iron turning (%)	After *sthalipaka* (%)	After 10 *puta* (%)	After 20 *puta* (%)
Fe	98.10	58.18	80.92	70.26
Si	0.40	3.74	1.44	0.96
Al	0.13	1.26	0.49	0.31
Ca	0.074	0.30	1.32	1.50
Mn	0.75	1.36	1.77	1.63
Others elements[a]	0.54	35.16	14.06	74.66

[a] Other elements are P, Cl, Ni, Ar, S, K, Tb, Sm, W, Dy, Cu, Zn, Gd, Co, Rb, Sr, Ti, Er, Ga, Y, and Na. XRF = X-ray fluorescence.

beneficial due to which ancient scholars of *Ayurveda* might have mentioned *Triphala* in maximum *lauha bhasma* preparations [13]. For the *putapaka* process instead of traditional method, to provide controlled and regulated heat and with a view of standardizing a modified EMF was used. *Lauha bhasma* were prepared on same temperature, that is, 650 °C and the duration of peak temperature was 1 h for all *puta*. The amount of liquid media (*Triphala kwatha*) for trituration increases after every successive *puta*, this may be because of decrease in particle size causes increases in surface area which are responsible for more absorption of *Triphala kwatha* during trituration (Table 4). [40] During first few *puta,* metallic luster was observed on the surface of pellets, later on it disappeared. Luster is the physical character of metal, when the metal transforms to compound form then its luster is lost. Appearance of luster after first few *puta* indicates *lauha* was still persistent in metallic form, later on it completely transformed to lusterless compounds. After trituration *cakrikas* (pellets) were formed and *sarava samputikarana* was done by earthen vessels that are responsible for homogenous distribution of heating and also does not react with the material because of inert property of earthen vessel. After every *puta*, weight of *lauha bhasma* decreases due to burning of solid contents of *Triphala kwatha* (Table 4). After 5th *puta*, color was nearer to blackish red and pellets were fragile and soft. In the 7th *puta* 1st time *rekhapurnatva* (the *bhasma* enter the ridges of finger) test presence which shows the reduction in particle size of *bhasma*. In 10th *puta,* pellets were hard and after 11th *puta* color was almost blackish red, that is, *pakwa jambu phala varna* which suggests the formation of an entirely new compound. On 15th *puta,* it was observed that hardness of pellets decreases and *bhasma* passed 50% *varitara* (the *bhasma* floats on the still water surface) test. After 20th *puta bhasma* passed all *bhasma pariksha* with 95% *Rekhapurnata and* 80% *varitara* test and color of *bhasma* was blackish red, that is, *pakwa jambu phala varna* (Tables 5 and 6). The color of *lauha bhasma* is purple (*Pakwa jambu phala varna*), it may be considered as a mixture of ferrous oxide, ferrous sulphide, ferric oxide and other trace elements**.** Ferrous oxide and ferrous sulphide are black in color, and ferric oxide is red in color. Combination of all these compounds makes the *lauha bhasma* purple in color. Several physical and chemical parameters have been described for ascertaining the purity of *lauha bhasma* [19]. Physical parameters include luster, color, fineness, floatability, etc., while chemical parameters include test of *lauha bhasma* for irreversibility to metallic state, floatability taste, etc. [19] In this study, after quality check done by classical parameters (Table 6), *T. lauha bhasma* were subjected to different physico-chemical characterization studies using modern analytical tools. Our results showed negligible moisture content (0.31% loss on drying), total ash value (98.15%), water soluble ash (30.26%), and lower solubility in acid (27.50%) (Table 7). The results are comparable to the reported values [41]. The total ash value is useful in determining the purity of *bhasma* and indicates the absence of free organic moieties. During the preparation of *lauha bhasma*, large quantity of *Triphala* were added resulting in the formation of complexes between the constituents of *Triphala* and the metal. The formation of coordination compounds will be precluded however, if the *bhasmas* are not prepared properly, resulting in lower total ash content [41]. Lower acid-insoluble ash indicates higher bioavailability of the drug [42] and lower value of loss on drying indicates the absence of moisture in the drug. The results of XRF study revealed that, Fe present in the elemental form along with its impurities in the raw Fe turnings is converted into different forms of Fe oxide in final *bhasma* were estimated by X-ray diffraction [18,43]. Other impurities such as Si, Al, etc., are also get oxidized after repeated trituration and calcination. *Triphala* which is used extensively in preparation of the *bhasma* for *puta* and also for trituration between *puta* consist of a number of minor elements such as Na, K, Mg, Ca, Cl, and P; and 23 trace AI, Ba, Br, Cd, Co, Cr, Cs, Cu, Fe, Eu, Hf, Hg, La, Mn, Ni, P, Pb, Rb, Sb, Se, Th, V, and Zn elements [18]. These get oxidized during *puta* and remain as integral part of the final *bhasma*. The SEM of 20 puta *T. lauha bhasma* showed irregular aggregates of various sizes and shapes with nano structure on the surfaces (100–500 nm) (Fig. 4) [17]. The role of nano structured materials as therapeutic agents has been reasonably established [44,45] and we also presumed that the efficacy of *lauha bhamsa* may be attributed to the presence of nanostructures.

Limitations of the study: Particle size after each *puta* was not measured. It can be analyzed at regular intervals to provide leads for further studies.

Conclusion

Lauha bhasma preparation includes major steps such as *samanya shodhana* (normal purification), *vishesha shodhana* (special purification), and *trividh lauhapaka,* that is, *bhanupaka* (exposure to sunlight), *sthalipaka* (frying in an Fe pan), and *putapaka* (calcination). It is essential to follow all these procedures as per *Ayurvedic* guidelines to get good quality of *bhasma*. It is also very important to understand each and every step to set up the standards for *bhasma* preparation. This work has resulted in establishing standard manufacturing procedure of *Teekshna lauha bhasma* (calx of Fe turning) by adapting conventional method of *puta* using EMF. This study revealed that a temperature of 650 °C with peak duration of temperature 1 h in 20 *puta* is sufficient to obtain purple colored, that is, *pakwa jambu phala varna Teekshna lauha bhasma* which passed the *bhasma parikshas*. Our results, also evaluated the physico-chemical properties of *Teekshna lauha bhasma*, based on AYUSH guidelines, as well as modern analytical tools.

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None.

Conflicts of interest

None declared.

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Pharmaceutical Standardization

Standard manufacturing process of *Makaradhwaja* prepared by *Swarna Patra – Varkha* and *Bhasma*

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Abstract

Makaradhwaja is an important *Kupipakwa Rasayana*. It is prepared by using *Swarna* (gold), *Parada* (mercury) and *Gandhaka* (sulfur) in different ratios, i.e. 1:8:16, 1:8:24 and 1:2:4, respectively. The amount of *Gandhaka* in the *Jarana* process is directly proportional to the increase in therapeutic efficacy and reduces the toxicity of the product. Specific temperature pattern for the preparation of *Makaradhwaja* has been followed. In the present study *Swarna*, *Parada* and *Gandhaka* were taken in the ratio 1:8:24, respectively, and 12 h of heating for a specified amount of *Kajjali* (i.e., 400 g) in a *Kacha Kupi* 1/3rd of its capacity. There are some controversies regarding the form of *Swarna* (i.e., *Swarna Patra Swarna Varkha* or *Swarna Bhasma*) used in the preparation of *Makaradhwaja*. Therefore, in the present study, the samples of *Makaradhwaja* were prepared by *Swarna Patra*, *Varkha* and *Bhasma* in different batches. It was found that the use of *Varkha* produced a good-quality product along with the maximum amount of gold, i.e. 268 ppm, in comparison with *Patra*, i.e. 131 ppm, and *Bhasma*, i.e. 19 ppm, respectively.

Key words: *Bhavana*, *Hingulottha Parada*, *Kupipaka*, Standard manufacturing process, *Shodhana*

Introduction

Makaradhwaja is a popular *Kupipakwa Rasayana*, prepared with the *Swarna* (gold), *Parada* (mercury) and *Gandhaka* (sulfur) in a specified ratio. It was first described by Rasendra Chintamani[1] by the name of *Chandrodaya Rasa*, although the word *Makaradhwaja* was first coined by Rasaratnakara. The term *Makaradhwaja* is composed of two words, i.e. *Makara* and *Dhwaja*. The term *Makaradhwaja* is also a synonymous of *Kamadeva*,[2] the God of beauty. When it is used for therapeutic purposes, it produces *Rasayana* and *Vrishya* (in literary meaning, *Makara* indicates the aphrodisiac because *Makara Retasa* has been described as best *Shukrala*[3] while *Dhwaja* [straight rod or flagpole] indicates its sexual potency. It is directly correlates the *Dhwajabhanga*, i.e. non-erection of penis.) property. A total of 30 formulations[4] are found described by the name of *Makaradhwaja* and *Chandraodaya*, and two types of instruments are used for its preparation, i.e. *Valuka Yantra* and *Khalwa Yantra*. Nowadays, *Makaradhwaja* is generally prepared as per the reference of Rasendra Chintamani due to easy and convenient preparation method; here, *Swarna*, *Parada* and *Gandhaka* are used in 1:8:16 ratio, but in different *Rasa* classics it is found described that the amount of *Gandhaka* is directly proportional to the therapeutic activity.[5] Therefore, in this study, the ratio was followed as per the *Bhaishajya Ratnawali*, i.e. 1:8:24.

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Aims and objective

The aim of the study was to develop the Standard Manufacturing Process (SMP) for *Makaradhwaja* prepared by *Swarna-Patra-Varkha* and *Bhasma* by Electrical Muffle Furnace (EMF).

Materials and Methods

Swarna was collected from a local authentic hallmark-certified Jeweler from Jamnagar. *Hingula*, *Tila Taila*, *Gandhaka* and *Kulattha* were collected from the Pharmacy, Gujarat Ayurved University, Jamnagar. *Takra*, *Kanji*, *Kulattha Kwatha*, *Japakusuma Swarasa*, *Kumari Swaras*, *Nimbu Swarasa* etc. were taken as per the classical reference and processed through prescribed methods. The whole process has been divided into the following:

Shodhana of raw materials

Kantakavedhi Swarna Patra was prepared from 24 carat gold biscuits on the machine that had 2.5 cm breadth, 150 cm length and 0.5 mm diameter. For *Samanya shodhana*, these *Kantakavedhi Swarna Patra* were heated up to red hot through a Gas blower and dipped into prescribed liquid media three times.[6] *Gandhaka*[7] was melted along with *Goghruta* and poured into the *Godugdha*. Then, *Swedana* (heating under liquid bath) was done for 3 hrs. After that, it was washed with hot water, dried and powdered [Table 1].

Hingulottha Parada Nirmana

Shodhana of *Hingula* was done by giving *Bhavana* of lemon juice three times.[8] After that, it was washed, dried and stored. The required amount of *Parada* was extracted from *Hingula* by *Nada Yantra* method. Fine powder of *Shuddha Hingula* was wrapped in cotton cloth (equal weight of *Hingula*) and burnt under the pot. Thus, due to heat, the sulfur part of *Hingula* burns and leaves the *Parada*, which gets evaporated as vapor and is collected on the inner side of the pot. *Parada* was collected by rubbing with cloth and then washing with hot water and filtering through four folders of cloth. This *Parada* was used to prepare *Makaradhwaja* [Table 2].

Preparation of *Swarna Varkha*

Shodhita Swarna Patra was cut into small pieces (2 cm × 4 cm length). Then, these *Patra* were continuously hammered with the help of an iron hammer in the leather bags up to the *Varkha* formation. This procedure was performed in Ahmedabad under supervision. The thickness of the *Varkha* was not measured because it was too thin. This *Varkha* was then used in the preparation of *Swarna Pishti*.

Preparation of *Swarna Bhasma*

Shuddha Swarna Patra was cut into fine pieces and triturated with the *Hingulottha Parada* up to *Pishti* (amalgam) formation and then this amalgam was kept between *Shuddha Gandhaka* in *Sharava*, i.e. *Swarna Pishti* was covered completely by *Shuddha Gandhaka*. *Sharava Samputa* was prepared and after complete drying, it was subjected to heat. After each *Puta*, *Parada* was reduced by 1/16th from the quantity of the initial *Parada* and the *Gandhaka* was in equal quantity of *Swarna* up to the last *Puta*. But, here, a total of 30 *Puta* were required for the preparation of *Swarna Bhasma* up to *Kumkum* colored without shining of particles.[9]

Preparation of *Swarna Pishti*

Shudha Swarna patra were cut into small pieces. *Hingulottha Parada* was taken in *Simaka Khalvayantra* and, after that, *Shuddha Swarna Patra* pieces were added one by one to it with proper trituration. The mixture was triturated up to a homogeneous form. The *Nimbu Swarasa* was added for proper preparation of amalgamation. Thus, semisolid, soft *Swarna Pishti* (amalgam of gold) was formed.[10]

The second batch of *Swarna Pishti* was prepared from *Swarna Varkha* and *Hingulottha Parada*; *Nimbu Swarasa* was not needed because it was prepared within few minutes by simple trituration.

The third batch of *Swarna Pishti* was prepared from *Swarna Bhasma* and *Hingulottha Parada*, with the addition of *Nimbu Swarasa*. But, for this, a large quantity of *Nimbu Swarasa* and a longer period of trituration was required even it was not properly prepared so that an equal amount of *Saindhava Lavana*[11] was added to it and again triturated till the formation of *Pishti* [Table 3].

Preparation of *Kajjali* of *Makaradhwaja*

The *Swarna Pishti* (amalgam of gold) was taken in a *Simaka Khalvayantra* and *Shuddha Gandhaka* was added to it in the prescribed quantity. *Mardana* was done for 24 hrs till fine, soft, *Nishchandra Kajjali* was formed [Table 4]. This was subjected to *Bhavana Dravya* of *Japakusuma Swarasa*, which was added to it in an adequate amount to wet the *Kajjali* (*Rasapankavat*) semisolid and *Mardana* was carried out for 3 hrs, till the homogeneous, soft mass was formed and then it was dried in sunlight[8] [Table 5]. The same procedure was repeated by adding the *Bhavana Dravya*, i.e. *Kumari Patra Swarasa*, and it was taken 1/4th of the total amount of *Kajjali* as it was found to be sufficient to wet the total material.[10] The observations of *Kupibharana* of *Makaradhwaja* are shown in Table 6.

Preparation of *Makaradhwaja* Apparatus

Mortar and pestles, *Kanchakupi*, *Multani mitti*, cloth, *Loha shalakas*-2, kerosene oil, matchbox, thread, enamel tray, glass container, cork, copper coin, torch, knife; Electric muffle furnace: Outer length: 40 cm, breadth: 40 cm, height: 50 cm, inner hearth length: 15 cm, breadth: 15 cm, depth: 30 cm, *Kanchakupi*: Amber-colored beer bottle, capacity: 625 ml, total height: 28 cm, cylindrical part: Height: 14 cm; circumference: 24.5 cm; diameter of the bottom: 6.5 cm, conical part: Height: 14 cm; diameter of the mouth: 2cm, weight: Before *Kapadamitti*: 450 g; after *Kapadamitti*: 590 g.

Table 1: Observation of *Shodhana* of the raw materials

Drug	Media	Method	Initial weight (ml)	Final weight (ml)	Loss/gain (ml)	Duration (days)
Swarna Samanya Shodhana	*Tila taila*	*Nirvapa* 3 times in each	155	155	00↓	1
	Takra, *gomutra*, *Kanji*, *Kulattha Kwatha*					
Gandhaka	*Godugdha*	*Dhalana*	5000	4810	190↓	1

Table 2: Observation of extraction of *Parada* from *Hingula*

Quantity of *Hingula* (g)	Quantity of cotton cloth (g)	Method	Time taken (hrs)	Procured amt. of *Parada* (g)	Procured amount of *Parada* (%)
3000	3000	*Urdhvapatan* by *Nada yantra*	8	2196	73.20

Table 3: Observations of *Swarna Pishti* from *Swarna Patra*, *Varkha* and *Bhasma*

Batch	Weight of *Swarna Bhasma* (g)	Weight of *H.Parada* (g)	*Nimbu Swaras* (ml)	Weight of *Saindhava Lavana* (g)	Duration (h)
MP	125	1000	90	-	12
MV	30	240	-	-	1
MB	40	320	150	40	32

MP- *Makaradhwaja* prepared by *Swarna Patra*, MV- *Makaradhwaja* prepared by *Swarna Varkha*, MB- *Makaradhwaja* prepared by *Swarna Bhasma*

Table 4: Observations during preparation of *Kajjali* of *Makaradhwaja*

Batch	Weight of *Swarna Pishti* (g)	Weight of *S.Gandhaka* (g)	Total	*Nischandratva*, *Rekhapurnatva* of *Kajjali* (h)	Total *Mardana* period (h)	Weight of *Kajjali* after *Mardana* (g)	Weight loss during *Mardana* (g)
MP	1098	3000	3098	16	24	3028	70
MV	270	720	990	12	24	973	17
MB	360	960	1320	16	24	1292	28

Table 5: Observations during *Bhavana* processing of *Kajjali* of *Makaradhwaja*

Batch	Weight of *Kajjali* after *Mardana* (g)	*Japakusuma Swaras* (ml)	Total *Mardana* period (hrs)	*Kumari Swaras* (ml)	Total *Mardana* period (hrs)	Weight of *Kajjali* after *Bhavna* (g)	Weight increased due to *Bhavna*	
							Weight (g)	%
MP	3028	750	3	750	3	3183	155	4.86
MV	973	250	3	250	3	993	20	2.10
MB	1292	325	3	325	3	1341	49	3.79

Table 6: *Kupikabharana* of *Makaradhwaja*

Batch	No. of *Kupi*	Sub batch	Weight of *Kajjali* (g)	Duration of heat (hrs)
MP	9	MP1	424	12
		MP2	424	12
		MP3	424	12
		MP4	424	12
		MP5	424	12
		MP6	424	12
		MP7	424	12
		MP8	424	12
		MP9	424	12
MV	3	MV1	330	12
		MV2	330	12
		MV3	330	12
MB	4	MB1	330	12
		MB2	330	12
		MB3	330	12
		MB4	330	12

Procedure

The *Bhavita Kajjali* of *Makaradhwaja* was taken, triturated well in *Khalvayantra* and filled in *Kanchakupi* [Table 6]. The *Kupi* was placed exactly at the center of the electric muffle furnace and fixed in proper position with the help of firebrick blocks. The heating process was carried out in a *Kramagni* pattern, i.e. increasing order but intermediate heating. Heat was gradually increased over a period as per the schedule, i.e. 3 hrs *Mandagni* (120-250°C), 6 hrs *Madhyamagni* (250-450°C), 3 hrs *Tivragni* (450-600°C). The temperature of the furnace was recorded after intervals of 30 min. During the course of heating, the hot *Shalaka* was repeatedly inserted into the mouth of the *Kupi* to burn the accumulated sulfur at the neck of the bottle to prevent blocking. After achieving the confirmative test, the mouth of the *Kupi* was corked and the temperature was increased up to 600°C and it was maintained for the next 2 hrs. [Table 7]. Then, the Muffle furnace was switched off and left for self-cooling. After *Swangsheeta*, *Kanchakupi* was taken out from the muffle furnace and the outer covering was removed; a thread (which was soaked in kerosene) was tied below 1 inch from the final product and ignited. Then, a few drops of water were sprinkled, which leads to break the *Kupi*, and finally, *Makaradhwaja* was collected from the neck of the *Kupi*. *Swarna* powder was collected from the bottom of the *Kupi*. The *Makaradhwaja* was triturated well in *Khalva yantra* up to a fine red color powder [Table 8]. *Makaradhwaja* samples were analyzed by employing various possible organoleptic, physical and chemical parameters [Tables 9 and 10]. The same procedures were followed for all three samples of the *Makaradhwaja*, i.e. for *Swarna Patra*, *Swarna Varkha* and *Swarna Bhasma*. A total of 17 batches were prepared to determine the SMP [Tables 8 and 11].[10]

Discussion

For the preparation of *Kupipakwa rasayan*, preparation of *Kajjali* and heating pattern are the most important factors to obtain maximum quantity of yield and to increase efficacy of the product without any untoward effect. As per classical texts, the *Kramagi*[12] heating pattern should be provided during processing of any *Kupipakwa rasayana*. It means temperature pattern should

Table 7: Observations during *Kupipaka* of *Makaradhwaja* (MP, MV and MB avg)

Time (hrs)	Temp. setting (°C)	Temp. recorded (°C)	Observations
00:00	100	37	Switch on the furnace
00:30	150	105	Slight sulfur aroma was smelt at the *Kupi* mouth White fumes started
01:00	200	156	Fumes turn slightly yellowish
01:30	250	204	Fumes turn slightly yellowish
02:00	250	251	Melting of *Kajjali* started and yellowish fumes continued
02:30	300	254	Melting of *Kajjali* with yellowish fumes continued
03:00	300	305	Yellowish color deposition at the neck
03:30	350	302	*Kajjali*-semi liquid form, yellowish fumes increased
04:00	350	347	*Kajjali*-molten and yellowish fumes increased
04:30	400	352	Complete molten *Kajjali* and yellowish fumes increased
05:00	400	399	Persisting yellow fumes
05:30	400	398	Persisting yellow fumes
06:00	450	403	Yellowish fumes increased, stickiness was found inside the *Kupi*
06:30	450	452	Profuse dark yellowish fumes started
07:00	475	454	Profuse yellowish fumes
07:30	475	478	Fumes disappeared and reddish blue-colored flame started
08:00	500	476	Flame increases to about 4-5 inches height
08:30	500	501	Flame gradually decreased and slight sulfur deposit found at *Kupikantha* (neck of the *Kupi*). Red tinge at *Kupitala* (bottom of the *Kupi*) was observed
09:00	550	499	Slight bluish flames persisting at the neck of the *Kupi* and red tinge at the bottom gradually increased
09:30	550	554	Flame disappeared, bottom of the *Kupi* was found bright red, *Sheeta Shalaka* test and copper coin test were found to be positive. Corking was done immediately at 9.25 hrs
10:00	600	553	Temperature maintained till the completion of heating
10:30	600	599	Temperature maintained till the completion of heating
11:00	600	598	Temperature maintained till the completion of heating
11:30	600	604	Temperature maintained till the completion of heating
12:00	stop	599	Furnace was switched off and left for self-cooling

MP- *Makaradhwaja* prepared by *Swarna patra*, MV- *Makaradhwaja* prepared by *Swarna Varkha*, MB-*Makaradhwaja* prepared by *Swarna Bhasma*

Table 8: Results of preparation of *Makaradhwaja* of the different batches

Batch	No. of *Kupi*	Sub batch	Weight of *Kajjali* (g)	*Makaradhwaja* (g)	% of *Makara* obtained	Residue (g)	% res
MP	9	MP1	424	92	86.85	20	4.7
		MP2	424	89	83.96	20	4.7
		MP3	424	96	90.56	15	3.5
		MP4	424	101	95.28	15	3.5
		MP5	424	102	96.22	19	3.5
		MP6	424	95	89.62	21	4.4
		MP7	424	109	102.83	21	4.9
		MP8	424	101	95.28	30	7.0
		MP9	424	114	107.54	18	4.2
Avg.				99.88	94.22	19.88	4.6
MV	3	MV1	330	55.6	59.78	31	9.3
		MV2	330	77	82.79	12	3.6
		MV3	330	81	87.09	12	3.6
Avg.				71.2	76.2	18.3	6.1
MB	4	MB1	330	79	84.94	11	3.6
		MB2	330	82	87.86	12	3.9
		MB3	330	86	92.14	12	3.9
		MB4	330	81	86.78	13	4.2
Avg.				82	87.8	12	3.9

be an increasing order but intermediate heating process. In can be divided into three stages, i.e. *Mridu*, *Madhya* and *Tivra agni*. Here, *Mriduagni* indicates the melting stage of *Kajjali*, *Madhyam agni* indicates the boiling stage of *Kajjali* and *Tivra agni* means immense heating, which takes place a confirmative test of the final product. One such effort had been done by Prajapati *et al*.,[13-15] who has given the temperature range for the particulars of *Agni*, such as-*Mridu Agni* 120-250°C (6 hrs), *Madhyama Agni* 250-450°C (6 hrs), *Tivra Agni* 450-630°C (6 hrs). This standardization was done in an electric muffle furnace for the preparation of *Makaradhwaja* in the ratio of 1:8:16, and the same was also followed by Chinta Durga *et al*.[16] and Patgiri *et al*.[17,18]

But, for the present study, to prepare the *Triguna Balijarita Makaradhwaja* in minimum heat duration, the heating pattern was changed. As the proportion of *Gandhaka* increases with *Parada*, i.e. *balijarana*, it was observed that there is an increase in the heating duration for the *Jarana*. Here, an attempt was made to prepare the same in minimum time with least consumption of energy while maintaining its therapeutic efficacy. Therefore, the duration of *Mriduagni* and *tivraagni* period was decreased without disturbing the *madhyamagni* duration (i.e., *Mriduagni* for 3 hrs, *Madhyamagni* for 6 hrs and *Tivragni* for 3 hrs). Because of a higher amount of *Kajjali* in *Makaradhwaja* prepared by the *Swarna Patra* sample, each batch was prepared with 424 g of *Kajjali* whereas in the *Varkha* and *Bhasma* samples, because of the lesser amount, it was prepared with 330 g of *Kajjali* in the same heat duration for the standardization purpose.

The *Hingulottha Parada* was used because as per classical text, its properties are equal to those of *Astasamskarita Parada*.[19] For the preparation of *Swarna Pishti* of *Varkha*, only 1 hr was required as compared with 12 hrs required for *Swarna Patra*, which may be because the particle size of *Swarna Varkha* is too fine as against that of *Swarna Patra*. In the preparation of *Pishti* of *Swarna Bhasma* 32 hrs were required, which was too much in comparison with that required for *Swarna Varkha* and *Swarna Patra*; the reason behind this may be that the *Swarna Bhasma* is not in elemental form, which leads to the extra time taken for the amalgamation with *Parada* [Table 3]. The *Nischandratva* and *Rekhapurnatva* tests of *Kajjali* were passed in 16 hrs, but 24 hrs trituration was done for the fineness. The weight of *Kajjali* was found to be increased (3.5% on an average) after *Bhavana*, which may be due to the added solid contents of *Japakusuma Swarasa* and *Kumari Swarasa* [Table 5].

It was observed during a trial and error study that the amount of *Gandhaka* is directly proportional to the burning period. Therefore, as per the classical notes, a specific temperature pattern was mentioned for the *Samguna Kajjali Sindura*, i.e. *Mridu Agni*, *Madhyama Agni* and *Tivraagni* in an equal ratio. In this, *Gandhaka* was just in the melting stage in the *Mriduagni* while in the *Madhyama Agni* stage, *Gandhaka* boils and burns and in the *Tivra Agni* period, *Parada* with *Gandhaka* sublimes. Thus, the temperature required for the melting of *Gandhaka* and sublimation of the product is not dependent on the amount of *Gandhaka*. Taking note of this, the period of *Mridu* and *Tivra Agni* was not changed but the period of *Madhyama Agni* was increased twice of the normal ratio due to which an excess amount of *Gandhaka* gets more period for the burning, which is the main aim. Also, in the previous study, it was found that *Samaguna* and *Dwiguna Sindura* were prepared in 16-20 hrs. Here, an attempt was made to prepare the *Triguna*

Table 9: Classical analytical tests of the *Makaradhwaja* samples

Pariksha	MP	MV	MB
Varna	Red	Red	Red
Sparsh	Slakshana	Slakshana	Slakshana
Gandha	Not specific	Not specific	Not specific
Rasa	Tasteless	Tasteless	Tasteless
Shabda	+ve	+ve	+ve
Rekhapurnata	+ve	+ve	+ve

+ve - Compiles as per classics, MP- *Makaradhwaja* prepared by *Swarna patra*, MV- *Makaradhwaja* prepared by *Swarna Varkha*, MB-*Makaradhwaja* prepared by *Swarna Bhasma*

Table 10: ICP analysis of the *Makaradhwaja* sample

Element	Sample results of MP mg/kg (ppm)	Sample results of MV mg/kg (ppm)	Sample results of MB mg/kg (ppm)
Au mg/kg (ppm)	131	268	19
Hg (%)	82.9	80.1	81.6

MP- *Makaradhwaja* prepared by *Swarna patra*, MV- *Makaradhwaja* prepared by *Swarna Varkha*, MB-*Makaradhwaja* prepared by *Swarna Bhasma*

Table 11: Standard manufacturing procedure for the preparation of *Makaradhwaja*

No. of process		Quantity of ingredient	Method	Yantra	Temp.	Duration (hrs)
Swarna Pishti	*Swarna Patra*	*Shuddha Swarna Patra + Hingulottha Parada*	*Mardan*	*Khalva yantra*	-	12:00
	Swarna Varkha	*Shuddha Swarna Varkha + Hingulottha Parada*	*Mardan*	*Khalva yantra*	-	01:00
	Swarna Bhasma	*Swarna Bhasma + Hingulottha Parada*	*Mardan*	*Khalva yantra*	-	32:00
Kajjali		*Swarnapishti* + 24-times *Shuddha Gandhaka*	*Mardan*	*Khalva yantra*	-	24:00
Bhavana		1. *Japakusuma Swarasa* 2. *Kumari Swarasa*	*Mardana*	*Khalva yantra*	-	03:00 hrs each
Kupipaka		*Makaradhwaja kajjali*	*Kupipaka*	EMF	*Kramagni* up to 600°C	12:00

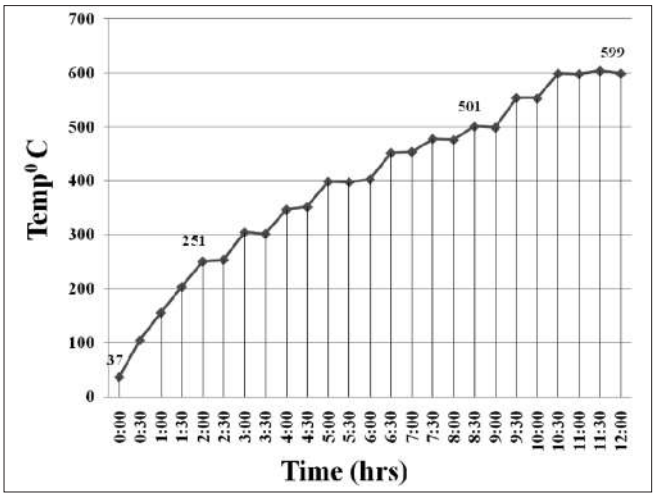

Figure 1: The average temperature pattern for preparation of *Makaradhwaja* through the electrical muffle furnace

Balijarita Makaradhwaja in a minimum heating period, which was 12 hrs [Tables 6 and 7]. For this, many trial and error studies were carried out to finalize the temperature pattern. During the *Kupipaka*, flame of sulfur occurred at the neck of the *Kupi* after 7.30 hrs on an average, which was continued for 1.45 hrs [Table 7].

An average of 94.22% of the *Makaradhwaja* was prepared by the *Swarna Patra* with residue (gold powder) of 4.6%, also 76.2% of *Makaradhwaja* and 6.1% of gold powder was prepared by *Swarna Varkha* with 87.8% of *Makaradhwaja* and 3.9% gold powder was prepared by *Swarna Bhasma* [Table 8].

The first sample of *Makaradhwaja* prepared by *Swarna Varkha* was not properly sublimed due to irregular electrical power so that the average percentage of the final product of *Makaradhwaja* was decreased and residue (gold powder) was increased compared with the other two samples.

Analytically, there were no changes found in loss on drying, ash value, acid-insoluble ash and carbon disulfide extract.

But, in the ICP analysis for gold content, it was found to be 268 ppm in *Makaradhwaja* prepared by *Swarna Varkha* whereas in a previous study (Patgiri *et al.*),[20] *Makaradhwaja* prepared by *Swarna Patra* found only 7.5 ppm and also by author prepared *Makaradhwaja* prepared by *Swarna Patra* and *Bhasma* was found to be 131 ppm and 19 ppm. This variation found in the gold content may be due to the particle size of the raw material (gold), because the particle size of *Swarna Varkha* is least compared with the *Swarna Patra* and, in the *Bhasma* form, gold is not as elemental as the form of gold. Thus, here, it is observed that least particle size of elemental gold increases the gold content in the sublimed *Makaradhwaja*. This increased concentration of gold content in sublimed *Makaradhwaja* enhances the therapeutic efficacy of *Makaradhwaja*, which has been also observed in comparative clinical trials of *Makaradhwaja* prepared by *Swarna Patra-Varkha* and *Bhasma* on *Madhumeha* (diabetes mellitus),[21] and pharmacological studies also supports this.

Conclusion

Makaradhwaja has been standardized in terms of time and temperature for 330-424 g. *Kajjali* (average), i.e. *Mridu Agni*: 100-250°C (2.5 hrs), *Madhyamagni*: 250-450°C (5 hrs), *Tivragni*: 450-600°C (4.5 hrs). *Makaradhwaja* prepared by least particle size of elemental gold increases the concentration of gold content in the sublimed *Makaradhwaja* [Figure 1].

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Original Research Article (Experimental)

Swarna Makshika Bhasma preparation using an alternative heating method to traditional *Varaha Puta*

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ABSTRACT

Background: Metals, minerals and gemstones are used to prepare drugs in combination with various herbal materials in ayurvedic treatments. During the process of preparation, metals, minerals or gemstones are converted into special form known as *bhasma* by series of pharmaceutical processes; *shodhana*, *bhavana* and *marana*. *Puta* is the amount of heat required to produce specific *bhasma* in a cycle of treatment in the process of *marana*. Traditionally, heat is produced by burning cow dung cakes and the amount of heat to be provided is described in terms of cow dung cakes (fuel) burnt.
Objective: The present study was aimed to obtain the temperature profile of the traditional *Varaha Puta* and to establish a complementary temperature profile in a muffle furnace.
Materials and methods: The temperature profile of *Varaha puta* was determined using dried cow dung cakes (which were prepared using cow dung and paddy husk) with an average calorific value of 15.44 MJ/kg as the fuel. Then temperature profile of traditional *Varaha Puta* was mapped with an electric muffle furnace and *Swarna Makshika* (Chalcopyrite) *bhasma* was prepared using both traditional method and in electric muffle furnace.
Results: Bhasma prepared using both *Varaha Puta* and muffle furnace have shown similar properties according to classical tests of Ayurveda and laboratory techniques.
Conclusion: The results show the possibility of using a muffle furnace to prepare *Swarna Makshika bhasma* instead of using traditional *Varaha Puta*.
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1. Introduction

Bhasmas are very fine ayurvedic medicinal powders prepared from minerals, metals, poisonous materials and gems combining with various herbs in treatments. The conversion of metals and minerals into acceptable form for oral administration is done by a series of pharmaceutical processes; *shodhana*, *bhavana* and *marana* and preparation procedures of these medicines are time-consuming and complicated [1,2].

Shodhana is the process in which the external and internal impurities of metals and minerals are removed. It involves elimination of harmful matter from the drug, modification of undesirable physical properties of the drug, conversion of some of the characteristics of the drug to different stages and enhancement of the therapeutic action [3,4]. The second process, *bhavana* is the wet grinding process in which materials are ground with specific liquid media for a particular period and this process leads to unique and suitable physico-chemical changes and potentiate the efficacy of material. In the final process, *marana,* purified metals or minerals are made in to pellet form with required other ingredients and herbal extracts as prescribed in classical texts and then subjected to fire treatment in closed earthen crucibles in a pit by burning dried cow dung cakes to obtain *bhasmas* [5]. This process is repeated many times (number of heating cycles) as prescribed in classical texts for each preparation. The amount of heat required in a cycle of treatment in the preparation of specific *bhasma* is known as *Puta*. Amount of heat required for one *Puta* is described in terms of cow dung cakes. However, the temperature profiles or the amount of heat to be supplied are not defined for traditional *Puta*. According to the type of metal or mineral to be incinerated in *bhasma* preparation, dimensions of the pit, the number of cow dung cakes to be

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burnt and number of heating cycles to be employed are described in Ayurvedic texts.

This work was carried out with the objective of studying the temperature profile of the traditional *Varaha Puta* and to establish a complementary temperature profile in a muffle furnace. The established temperature and heating profile of the muffle furnace was compared with the properties of *Swarna Makshika Bhasma* produced using both *Varaha Puta* and the muffle furnace.

2. Materials & methods

2.1. Materials

Imported *Swarna Makshika* mineral, *Swarna Makshika Bhasma* and Copper Sulphide were purchased from a local supplier. Cow dung, paddy husk and saw dust were purchased from the local market.

2.2. Analysis of Swarna Makshika

Imported *Swarna Makshika* mineral samples purchased from three different suppliers and imported *Swarna Makshika Bhasma* available in the market were analyzed for its composition of Cu, Fe and Si using Atomic absorption Spectrophotometer (GBC933AA).

2.3. Cow dung cake preparation

Cow dung cakes were prepared using different compositions of cow dung, paddy husk and saw dust to identify suitable composition. The sample no. 1, 2 & 3 were prepared using cow dung and paddy husk and the compositions were 60:40, 82:18 and 76:24 respectively. The sample no. 4 was prepared using cow dung, paddy husk and saw dust and the composition was 63:20:17. Diameter and height of the mould used to prepare cow dung cakes were 6″ and 1” [6] respectively. Calorific value of each sample was measured using a Bomb calorimeter (Parr 1341EE).

2.4. Establishment of temperature profile of Varaha Puta

In order to measure *Puta*, the pit for *Varaha Puta* was built using bricks and clay. According to Vagbhatacharya [13], *Varaha Puta* is defined as 1 *Aratne Pramana* and it is equal to 22 *Angulas* [7] (1 *Angula* = 0.75 inches and then 1 *Aratne* is approximately equal to 16.5 inches or 42 cm in SI units). Therefore, length, width and the depth of the pit are 16.5 inches each. The total number of cow dung cakes burnt to establish the temperature profile was 120 and the weight of the cow dung cakes was 12.7 kg.

Initially 80 cow dung cakes were filled to the *Varaha Puta* pit, sealed earthen crucible containing *Swarna Makshika* pellets was placed in it and then covered with the remaining 40 cow dung cakes. Thermocouple wire was inserted to the crucible containing *Swarna Makshika* pellets in order to record the temperature inside the crucibles using the Temperature Data Logger (Lutron TM-947SD) before placing the crucible in the pit. Partly filled *Varaha Puta* with cow dung cakes is shown in Fig. 1.

Then cow dung cakes were ignited from all four sides and in the middle of the pit. When burning was over, the contents in the crucible was allowed to self-cool completely.

2.5. Verification of temperature profile of Varaha Puta from muffle furnace

The temperature of the traditional *Varaha Puta* was mapped in the muffle furnace by following the temperature profile obtained for the *Varaha Puta*. Temperatures were set manually and the set temperatures with time are shown in Table 1.

Fig. 1. Partly filled Varaha Puta with cow dung cakes

2.6. Swarna Makshika Bhasma by traditional method and muffle furnace

Swarna Makshika Bhasma was prepared following the procedures given in the traditional method and using the muffle furnace for the verification of the temperature profile obtained from the traditional *Varaha Puta*.

Among the various methods described in different Ayurvedic classical texts, method described by Vagbhatacharya [13] was selected for the preparation of *Swarna Makshika Bhasma.*

i. Analysis of composition of initial mineral and imported *bhasma* samples

Three *Swarna Makshika* mineral samples those were purchased from different suppliers in local market and two imported *bhasma* samples were analyzed using Atomic Absorption Spectrophotometer (GBC933AA) to identify the composition of minerals and *bhasmas*. Since the samples did not contain the required Cu content of *Swarna Makshika* mineral, Cu was externally added in the form of Copper Sulphide. Therefore, 90 g of powdered *Swarna Makshika* mineral was mixed with 95 g of Copper Sulphide to adjust the Copper content and the sample was analyzed again to verify the composition.

ii. *Swarna Makshika Shodhana* process

Prepared *Swarna Makshika* mixture (180 g) was roasted in an open pan with 200 mL of lime juice for 48 min as proposed by Vagbhatacharya [13].

Table 1
Temperatures with time duration for muffle furnace to prepare *Swarna Makshika bhasma.*

Time (min)	Temperature (°C)
0	25
20	60
30	120
40	275
50	500
60	650
70	800
80	850
95	off

Table 2
Materials used for *marana* of *Swarna Makshika* pellets in *Varaha Puta* and in muffle furnace.

	Puta 1/Cycle 1	*Puta 2/Cycle 2*	*Puta 3/Cycle 3*	*Puta 4/Cycle 4*	*Puta 5/Cycle 5*
Wt of cow dung cakes used for (kg) *Varaha Puta*	12.5	12.9	12.4	12.6	12.4
No. of pellets used for *marana* in *Varaha Puta*	38	15	10	8	7
No. of pellets used for *marana* in muffle furnace	37	15	11	8	6
Wt of pellets before *marana* (g) in *Varaha Puta*	149.642	55.403	35.980	29.245	24.234
Wt of pellets before *marana* (g) in muffl furnace	148.214	54.45	35.065	28.825	23.578
wt loss (%) in traditional *Varaha Puta*	59.38	25.02	13.74	9.84	8.72
wt loss (%) in muffle furnace	60.44	26.20	13.20	9.52	8.34

iii. Sulphur *Shodhana* process

Approximately 2 L volume clay pot was filled with 1 L of cow's milk and the mouth of the clay pot was tied with a piece of cloth. Cow's ghee was applied on the cloth and then 245 g of powdered Sulphur was placed on the cloth. Clay pot was covered with another similar size clay pot and sealed using clay-smeared cloth. Then the pot containing milk was buried and the upper clay pot was heated using 2.5 kg of cow dung cakes (approximately 20 cow dung cakes). Finally, Sulphur that was melted and dropped in to the milk was washed with warm water and the process was repeated three times using the resultant Sulphur.

iv. Preparation of pellets

Equal amounts of and *shoditha* Sulphur (200 g of each) were put into the mortar and lime juice was added until all the solids were covered. Then the mixture was ground until all the lime juice was evaporated, pellets were made (approximately 2 cm diameter and 3 mm thick) and sun dried.

v. Marana of *Swarna Makshika* pellets in Varaha Puta

38 numbers of sun-dried pellets (149.642 g) were placed in the earthen crucible and was sealed with another crucible using clay smeared cloth. Then it was incinerated in *Varaha Puta (Puta 1)* using 120 cow dung cakes and allowed to self-cool. The resultant *bhasma* from *Puta* 1 was ground again with lime juice, pellets were prepared and incinerated in *Varaha Puta*. This process was repeated 5 times up to *Puta* 5 and materials (no. of pellets & their weight) and weight of cow dung cakes used in each *Puta* is shown in Table 2.

vi. *Marana* of *Swarna Makshika* pellets in muffle furnace

37 number of sun dried pellets (148.214 g) were placed in the earthen crucible and sealed with another crucible using clay smeared cloth. Then it was incinerated in a muffle furnace in which the temperature was adjusted manually as described in 2.5.

The resultant *bhasma* from Cycle 1 was ground with lime juice; pellets were prepared and incinerated in the muffle furnace. This process was repeated 5 times and the number and the weight of *Swarna Makshika* pellets used in each cycle is shown in Table 2.

vii. Analysis of final bhasma

Final *bhasmas* prepared in both traditional *Varaha Puta* and in the muffle furnace were analyzed using traditional tests mentioned in ayurvedic texts such as colour (*varna*), floatability (*varitara*), fineness (*Rekhapurnata*), *nirutta.* Further, loss on drying, total ash, acid insoluble ash, chemical composition and particle size distribution were done.

a) Floatability (*Varitara*)

Small amount of *bhasma* was taken and sprinkled slowly on to a stagnant water surface from a short distance [8].

b) Fineness (*Rekhapurnata*)

Small amount of *bhasma* was rubbed in between index finger and thumb to observe whether particle can fill furrows of finger tips [8].

c) *Nirutta*

In this test, *bhasma* was mixed with a fixed weight of silver leaf, kept in earthen pot and similar grade of heat was applied and after self-cooling, weight of the remaining silver leaf was measured [8,9].

d) Loss on drying

One gram of *bhasma* was taken in a crucible and dried in an oven at 105 °C for about 5 h. The sample was allowed to cool and the dry mass was determined [10].

$$\text{Loss on drying} = \left(\frac{\text{Initial weight} - \text{Final weight}}{\text{Initial weight}}\right) \times 100$$

e) Total ash

Two grams of each ash samples were weighed accurately in silica crucibles. The samples were spread uniformly on the bottoms of the crucibles, incinerated, cooled and weighed. Difference between the weight of the crucible with incinerated *bhasma* and the empty crucible gives the total ash value [10].

f) Acid insoluble ash

The residues from total ash estimations were boiled with hydrochloric acid. The insoluble matter was washed with hot water, transferred to a crucible, dried and weighed. The

Table 3
Characterization of *Swarna Makshika* samples.

Constituent	Mineral samples			*Bhasma* samples	
	Sample 1	Sample 2	Sample 3	Sample 1	Sample 2
Fe (wt %)	37.3	44.10	40.11	60.44	69.83
Cu (wt %)	<0.01	<0.01	0.02	<0.01	<0.01
Si (wt %)	3.95	2.28	2.8	–	–

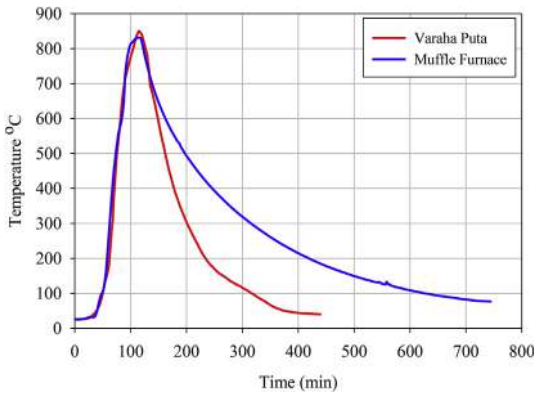

Fig. 2. Temperature profile of traditional *Varaha Puta* and Muffle Furnace.

weight difference between the crucible with incinerated *bhasma* and the empty crucible gives the acid insoluble ash value [10].

g) Chemical composition

The chemical composition of *bhasmas* were analysed using Atomic Absorption Spectrophotometer (GBC933AA).

h) Particle size distribution

Particle size distributions of *bhasmas* were analyzed using Laser Particle size analyzer (Chengdu Jingxin, JL-1177).

3. Results & discussion

3.1. Analysis of composition of Swarna Makshika

Cu is one of the major elements in *Swarna Makshika* ($CuFeS_2$) and it contains 34.5% Copper, 30.5% Iron and 35% Sulphur by weight [11]. *Swarna Makshika* is not available in Sri Lanka, and generally, it is imported from India for preparation of medicines. However, three *Swarna Makshika* mineral samples purchased from three different suppliers contained <0.02% Cu by weight. Further, two of the imported *Swarna Makshika bhasma* samples purchased from the local market also showed a considerable low amount of Cu. Fe, Cu and Si contents of initial mineral samples and imported *bhasma* samples are shown in Table 3. Since it was difficult to find good quality *Swarna Makshika* in Sri Lankan market, it was decided to use artificially prepared *Swarna Makshika* for *bhasma* preparation. Therefore, CuS was added externally to adjust the required Cu percentage in the initial mineral.

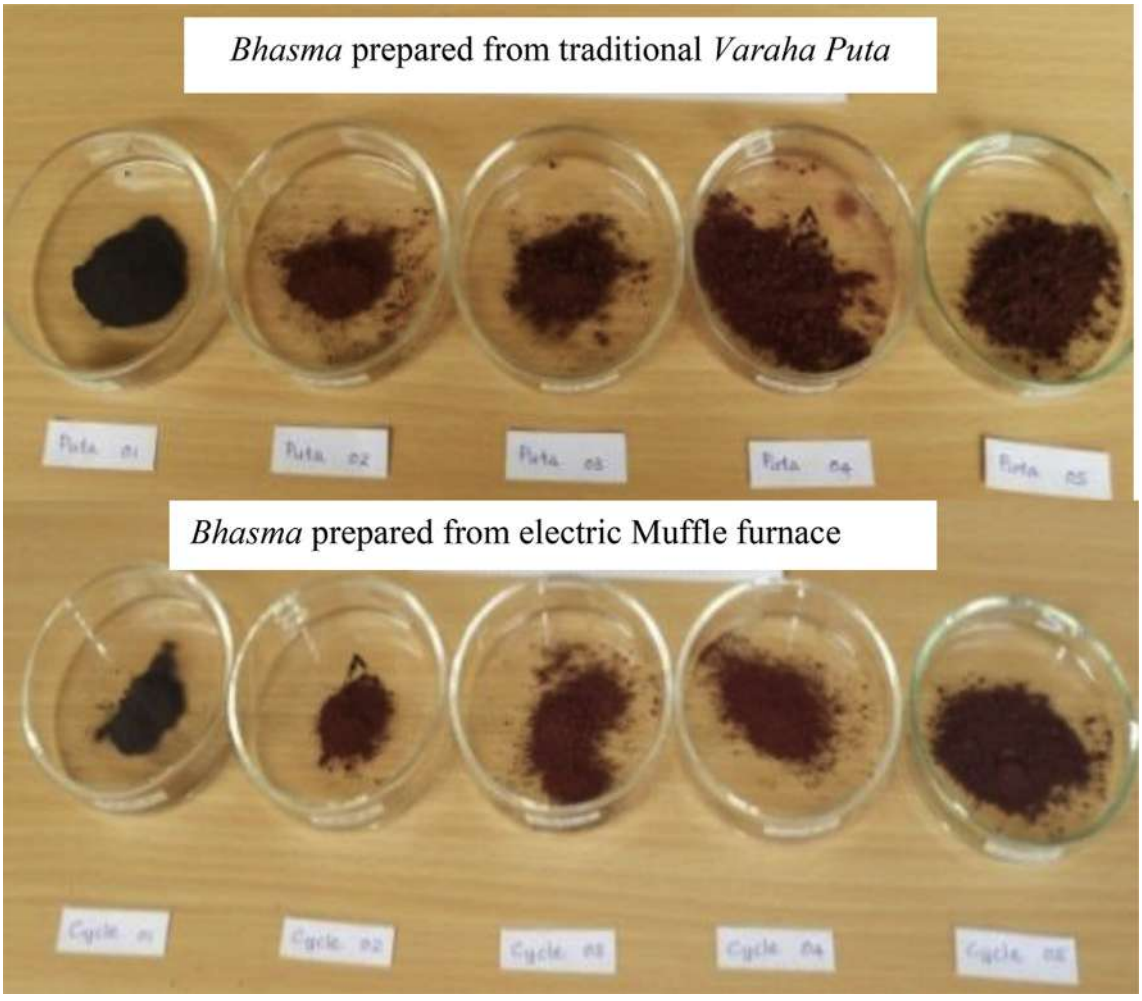

Fig. 3. Colour of resultant *Swarna Makshika bhasmas* after each puta and after each heating cycle in the muffle furnace.

Table 4
Results for traditional tests and physic-chemical tests of final *bhasmas*.

Property	Traditional *Varaha Puta*	Muffle Furnace	Recommended values
Colour	Reddish Brown	Reddish Brown	Reddish Brown [12]
Rekapurnata	Fills the space between finger lines	Fills the space between finger lines	Fills the space between finger lines [12]
Varitara	Floats on surface of water	Floats on surface of water	Floats on surface of water [12]
Nirutta	Silver wt remain unchanged	Silver wt remain unchanged	Silver wt remain unchanged [12]
Loss on drying	0.16% (w/w)	0.25% (w/w)	Not more than 0.5% (w/w) [12]
Total ash	99.84% (w/w)	97.29% (w/w)	
Acid insoluble ash	19.39% (w/w)	16.95% (w/w)	Not more than 21% (w/w) [12]

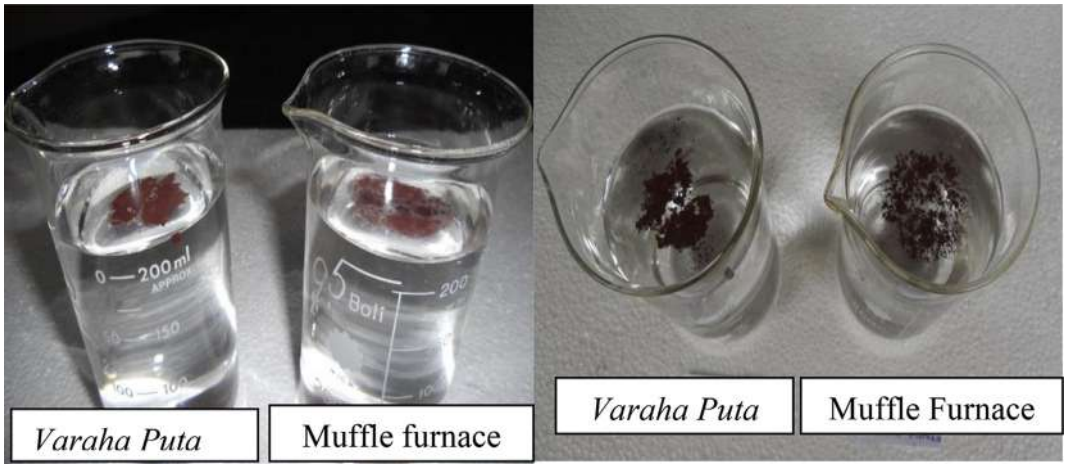

Fig. 4. Floatability test for *Swarna Makshika bhasma* prepared from *Varaha Puta* and Muffle Furnace. Side view (left) and top view (right).

3.2. Analysis of cow dung cakes

According to the Nishteswar, K., & Vidyanath, R. (2010), bits and fragments of cow dung collected from a cow-pen is defined as *Gobara* and cow dung cakes are different from *Gobara.* Therefore, paddy husk and saw dust were mixed with cow dung to prepare cow dung cakes. The average calorific values of 4 cow dung cake samples prepared in this work were 15.18, 15.30, 15.44 and 14.65 MJ/kg respectively and the moisture contents (% dry basis) were 15.84, 14.97, 15.56 and 14.28 respectively.

Maximum Calorific Value, 15.44 MJ/kg was obtained from cow dung cakes prepared using 76% cow dung and 24% paddy husk mixture (dry basis) and it was the most stable mixture among the prepared samples. Therefore, all the cow dung cakes used as the fuel for traditional *puta* furnace in this work were prepared by mixing 24% paddy husk and 76% cow dung.

3.3. Establishment of temperature profile of Varaha Puta

Temperature profiles of the traditional *Varaha Puta* and the muffle furnace are shown in Fig. 2.

As can be seen from Fig. 2, temperature of the *Varaha Puta* furnace started to increase 35 min after ignition and gradually reached the peak temperature 850 °C, 120 min after ignition. Temperatures above 750 °C and above 600 °C were maintained for 35 min and 70 ± 5 min respectively. It takes about 5 h to self-cool the *Varaha Puta* to 40 °C.

3.4. Verification of temperature profile of Varaha Puta from muffle furnace

As shown in Table 1, temperature of the muffle furnace was adjusted manually at specified times in order to establish a similar temperature profile as in the *Vahara Puta* and the temperature profile of the muffle furnace is also shown in Fig. 2. It clearly shows that the heating of the *puta* and the muffle furnace follows the same profile however, muffle furnace takes a longer time to cool compared to *Varaha puta.*

3.5. Swarna Makshika Bhasma by traditional method and muffle furnace

Moisture, volatile matter and Sulphur in the form of SO_2 can be escaped during heating in the *puta* as well as in the muffle furnace resulting in the reduction in the weight of pellets. Weight loss after incineration of pellets in the traditional *Varaha Puta* and in the muffle furnace is shown in Table 2.

The weight loss during each cycle in *Varaha Puta* and muffle furnace prepared *bhasmas* are approximate to each other. Therefore, this is an indication of the similarity of heat supplied in each cycle in both traditional *puta* method and electric muffle furnace. Further, it can be seen that the amount of weight losses have been reduced with the increase of incineration stage. This is due to the amount of Sulphur remaining in the pellets has reduced with the *marana* process and therefore amount of SO_2 liberated during the *marana* is reduced with the *marana* stage.

Table 5
Chemical Composition of raw mineral and resultant *bhasmas.*

Chemical constituent	Wt (%)			
	Raw mineral	Pellets	Final *bhasma*	
			Puta	Muffle
Fe_2O_3	15	7.5	47	38
Cu	38	19.0	44	35
SiO_2	1.8	0.9	2	traces

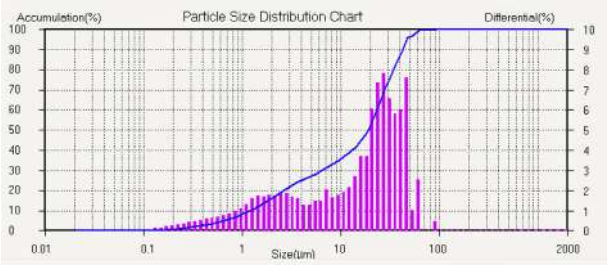

Fig. 5. Particle size distribution of *Swarna Makshika bhasmas* prepared in *Varaha Puta*.

There was a colour change in the ashes after each stage of heat treatment and the resulted ashes is shown in Fig. 3. It was observed that in both methods, colour has gradually changed from dark brown to reddish brown during 5 incineration stages. Therefore, colour of the final ashes (*bhasma)* obtained from both methods were reddish brown and it is similar to the colour of Chalcopyrite ash specified in Ayurvedic text [12]. This test further indicates the similarity of heat supplied and heat treatment.

Results obtained for traditional tests and physic-chemical tests are summarized in Table 4.

Both *bhasmas* filled the space between finger lines and floated on the surface of water (Fig. 4) confirming that they have achieved the required fineness required in the final *bhasma*.

In *nirutta* test, weight of silver leaf has not increased in both *bhasmas* and it indicates that there was no free metallic portion in the *bhasma* samples. If free metals were present in the *bhasma*, it formulates different compounds with silver and hence weight of silver in the *bhasmas* should be increased. Loss on drying of final *bhasmas* should not more than 0.5% w/w [12] and *bhasmas* prepared from both traditional *Puta* and from electric muffle furnace showed 0.16% and 0.25% of loss on drying respectively. Therefore, results obtained were within the required level of loss on drying and these values of loss on drying are an indication of the absence of moisture in the final product. The total ash contents of *Varaha Puta* and muffle furnace prepared *bhasmas* were 99.84% and 97.29% respectively and it indicates the presence of very high inorganic content in both *bhasmas*. Acid insoluble ash indicates the presence of silica and oxalates in drugs and values obtained for *Varaha Puta* and muffle furnace prepared *bhasmas* were 19.39% (w/w) and 16.95% (w/w) respectively. Recommended acid insoluble ash content in *Swarna Makshika bhasma* should not be more than 21% (w/w) [12] and the results obtained from this study are within the recommended range.

Chemical composition of raw mineral and resultant *bhasmas* is shown in Table 5.

Initial compositions of formulated *Swarna Makshika* by externally adding CuS were 15% Fe_2O_3, 38% Cu and 1.8% SiO_2. In this preparation, more attention was paid to maintain correct Cu % in the initial sample as Cu is the main element in *Swarna Makshika*. The compositions of Fe, Cu, and SiO_2 in pellets become approximately half of the initial raw mineral composition since *Swarna Makshika* was mixed with equal amount of Sulphur in the preparation of pellets. After marana, Fe, Cu & SiO_2 contents in *bhasmas* prepared in *Varaha Puta* were 47%, 44% & 2% respectively while they were 38%, 35% & traces respectively in the *bhasma* prepared in the muffle furnace. The required copper content in final *bhasma* should be in the range of 15–18% [12]. Deviation in the results obtained could be due to formulation of *Swarna Makshika* mineral and therefore the chemical structure/bonding of artificially prepared mineral can be different from the original mineral.

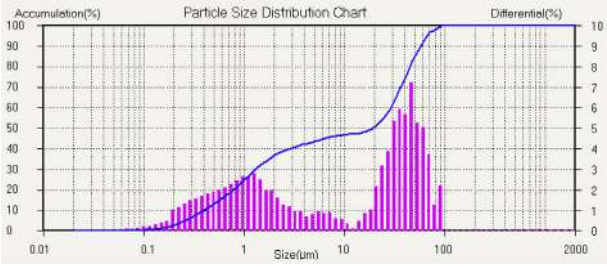

Fig. 6. Particle size distribution of *Swarna Makshika bhasmas* prepared in Muffle furnace.

It is well known that reduction of particle size increases the solubility and hence bioavailability of these *bhasmas* when used in therapeutics. The particle size distributions of *bhasmas* prepared in *Varaha Puta* & muffle furnace are shown in Figs. 5 and 6 respectively.

Swarna Makshika bhasmas prepared from both methods have particles in the range of 0.05–92.57 μm in diameter. 35.71% of total *bhasma* prepared using traditional *Varaha Puta* is below 10 μm in diameter while 47.03% of particles are below 10 μm in diameter in *bhasma* prepared in muffle furnace. Further it showed that 50% of particles of both *bhasmas* were below 20 μm. Previous study has shown that particle size of the Swarna M*akshika bhasma* is in the range of 3–100 μm in diameter. Therefore, particle size distribution of both *bhasmas* obtained in this study is comparable with previous studies.

4. Conclusion

Cow dung cakes prepared using 76% cow dung and 24% paddy husk mixture (dry basis) resulted the most stable mixture for cow dung cakes and gave a maximum calorific value of 15.44 MJ/kg.

Temperature profile of *Varaha Puta* was determined using dried cow dung cakes as the fuel and the same temperature profile can be established using a muffle furnace. *Swarna Makshika. bhasma* prepared using both traditional method and the electric muffle furnace, showed comparable properties according to classical tests of Ayurveda and laboratory techniques, demonstrating heating employed in both methods are complementary. Therefore, muffle furnace can be used to prepare Swarna Makshika *bhasma* instead of using traditional *Varaha Puta* and hence muffle furnace can be used to prepare *bhasmas* that are generally produced in *Varaha Puta.*

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Conflicts of interest

None.

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Variations in Physicochemical Properties of a Traditional Mercury-Based Nanopowder Formulation: Need for Standard Manufacturing Practices

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Kamath, *et al.*: Physicochemical Properties of a Mercury-based Nanopowder Formulation

***Rasasindura* is a mercury-based nanopowder synthesized using natural products through mechanothermal processing. It has been used in the *Ayurvedic* system of medicine since time immemorial for various therapeutic purposes such as rejuvenation, treatment of syphilis and in genital disorders. *Rasasindura* is said to be composed of mercury, sulphur and organic moieties derived from the decoction of plant extracts used during its synthesis. There is little scientific understanding of the preparation process so far. Though metallic mercury is incorporated deliberately for therapeutic purposes, it certainly raises toxicity concerns. The lack of gold standards in manufacturing of such drugs leads to a variation in the chemical composition of the final product. The objective of the present study was to assess the physicochemical properties of *Rasasindura* samples of different batches purchased from different manufacturers and assess the extent of deviation and gauge its impact on human health. Modern characterization techniques were employed to analyze particle size and morphology, surface area, zeta potential, elemental composition, crystallinity, thermal stability and degradation. Average particle size of the samples observed through scanning electron microscope ranged from 5-100 nm. Mercury content was found to be between 84 and 89% from elemental analysis. Despite batch-to-batch and manufacturer-to-manufacturer variations in the physicochemical properties, all the samples contained mercury in the form of HgS. These differences in the physicochemical properties may ultimately impact its biological outcome.**

Key words: Mercury, sulphur, chemical composition, elemental, field emission, heavy metals

The synthesis of *Rasasindura* (RS) involves meticulous process engineering. It involves several unit operations and processes on a wide variety of materials, ranging from metals to plant ingredients, to transform into a product with therapeutic values. These processes are expected to exert subtle control over the morphology, fineness, surface area, chemical reactivity, solubility as well as biological interactions that decide the therapeutic efficacy and safety of this nanopowder formulation[1]. The synthesis of RS involves the following steps (i) Treatment of mercury; (ii) treatment of sulphur; (iii) mechanochemical processing of purified mercury and sulphur to obtain a black lusterless powder; (iv) addition of herbal extracts and (v) thermal processing to obtain the final product. Various treatment processes prescribed in classical texts are hypothesized to detoxify the metal and convert it into a biocompatible form. Though metallic mercury is incorporated deliberately for therapeutic purposes, it certainly raises toxicity concerns[2]. Several groups have attempted to characterize such nanopowders in order to ascertain their safety. The elemental composition of *Rasa parpati*, another traditional mercury-based powder, was evaluated using inductively coupled plasma-optical emission spectrometry (ICP-OES) and the results indicated a mercury content greater than 10 g/kg in the samples[3]. Similarly, yet another metallic preparation, *Siddha Makardhwaja* was analysed using instrumental neutron activation analysis (INAA) and was reported to contain mercury (85.3%) and sulphur (14.1%)[4]. Saper *et al.* had reported mercury concentration to be 20.23 mg/g in the herbal medicine products manufactured in South Asia and sold in stores in Boston[5]. Similarly, 1/5th of 193 *Ayurvedic* medicines purchased via the internet were found to

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contain mercury, lead or arsenic[6]. These findings have been countered by the traditional medicine practitioners. It is believed that these metallic ingredients are not contaminants and are deliberately added key constituents in these preparations. In spite of the metallic content, these powders have been employed as therapeutic formulations, as it is believed that these metals do not exist in their elemental form in the preparations, but rather in a complex biocompatible form. However, no concrete scientific evidence has been presented to justify these claims. Although the debate on the origin and the role of metallic constituents in the traditional medicine formulations rages on, another crucial aspect with respect to traditional herbo-metallic preparations has come to the fore in the recent years. Wide variations in the mercury content were observed in the commercial samples, which could be attributed to the differences in preparation procedures adopted by the manufacturers. The lack of gold standard for quality control and multiple procedures available for preparation of these formulations further contribute to the variations in composition and possibly both therapeutic and toxic effects of these preparations. The purpose of this study was to carry out a comprehensive physicochemical characterization of RS preparations available in the Indian market, using modern characterization techniques.

MATERIALS AND METHODS

Samples of different batches of RS were procured randomly from three different manufacturers. To maintain discretion, the manufacturers were designated as X, Y and Z. The container labels of all three manufacturers mentioned the presence of mercury, but not its concentration. The drug name and dosage details are listed in Table 1.

TABLE 1: NAME OF THE DRUGS AND DOSAGE INDICATIONS

Name of the drug	Sample ID	Dosage (mg)
Ekaguna Sindura	X-B1	100-200
Rasa Chenduram	X-B2	50-100
Rasa Chendooram	Y-B1	100-200
Rasa Chendooram	Y-B2	100-200
Rasa Chendooram	Y-B3	100-200
Rasa Sindur	Z-B1	Not mentioned
Rasa Sindur	Z-B2	Not mentioned

Details of commercially available samples of *Rasasindura* where X-B1 and X-B2 are samples obtained from X-manufacturer with Batch 1 and Batch 2 preparation. Y-B1, Y-B2 and Y-B3 obtained from Y-manufacturer with Batch 1, Batch 2 and Batch 3 preparation. Z-B1 and Z-B2 obtained from Z-manufacturer with Batch 1 and Batch 2 preparation

Particle size, zeta potential and surface area:
The average particle size and zeta potential of the aqueous dispersion of the samples were determined using a Zetasizer (Zetasizernano, Malvern Instruments Limited, USA). The particle size distribution was carried out using laser diffraction technique (Bluewave Microtrac, Nikkiso, Japan). Surface area was determined based on the adsorption of nitrogen on particle surfaces using surface area analyzer (ASAP 2020, Micromeritics, USA). The powder samples were also passed through different sieves ranging from ASTM 20 – ASTM 325 and the samples retained on each sieve were weighed and the percentage retained was calculated.

Electron microscopy:
The surface morphology of the commercial samples of RS was qualitatively assessed using a cold field emission scanning electron microscope (JSM 6701F, Jeol, Japan). The ultrafine structure of RS was analysed using a field emission transmission electron microscope (JEM 2100F, Jeol, Japan). The selected area electron diffraction (SAED) patterns were recorded by dispersing the sample over a carbon coated copper grid.

Elemental analysis:
The commercial samples of RS were mixed with boric acid and pelletized using a 25-tonne hydraulic press to obtain thin discs of 34 mm diameter. The elemental composition of the samples was determined using an X-ray fluorescence spectrometer (S8 Tiger, Bruker AXS, Germany) using a 4 kW Rhodium anode X-ray tube. Energy dispersive X-Ray spectroscopy (EDAX, Oxford, UK) was also used to identify the elements.

Crystallinity:
The crystal phases of all the samples were analysed using an X-ray diffractometer (D8 Focus, Bruker AXS, Germany). The XRD patterns were recorded at '2θ' ranging between 10° and 60° at a scan rate of 0.01°/s.

Thermal analysis:
The melting point, thermal stability and degradation temperature of the samples were analyzed using a Thermogravimetry-Differential Thermal Analyzer (SDT Q600, TA Instruments, USA).

RESULTS

All samples from the manufacturers 'X' and 'Y' exhibited orange red hue characteristic of the vermillion or red cinnabar colour. However both samples from manufacturer Z showed a dark pink colour. The difference in colour between the samples may be attributed to the different particle sizes of the samples[7]. The samples from manufacturer X and Y were very fine powders whereas those from manufacturer Z were coarse. It was observed that there was a huge variation in the size distribution between the samples (fig. 1). A considerable percentage of the population in all the samples existed in the micron range. The sieve analysis of the samples shown in the Table 2 also suggested the wide size ranges of the particles. Interestingly, the samples from manufacturers Z showed particle sizes between 2 to 900 µ, which were much higher than those observed with the samples from manufacturers X and Y. This agrees with the difference in colour observed for the samples from manufacturers X, Y and Z. The agglomeration of the particles could be one of the factors contributing to the wide variations in the particle size distributions. Agglomeration is the result of attractive forces between the particles. The shape and the size of the particles as well as their surface charge play key roles in determining their agglomeration. Generally, irregular shaped particles tend to agglomerate to a larger extent as

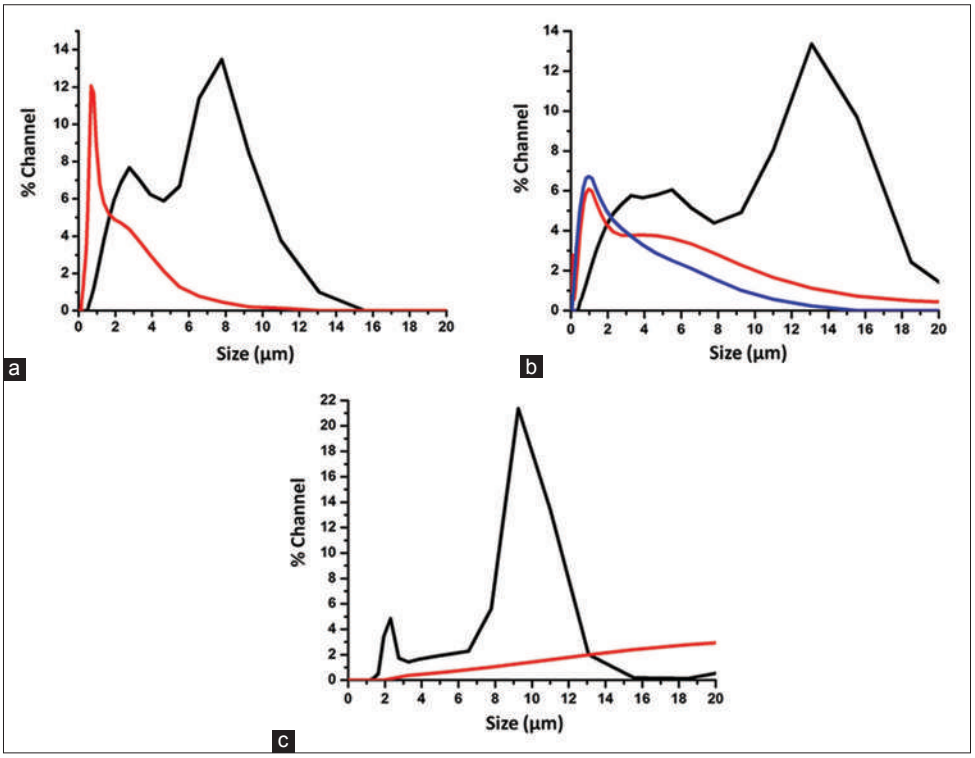

Fig. 1: Particle size distribution of each sample.
Particle size distribution of samples obtained from (a) X-manufacturer where black line denotes X-B1 and red line denotes X-B2, (b) Y-manufacturer where black line denotes Y-B1, red line denotes Y-B2 and blue line denotes Y-B3, (c) Z-manufacturer where black line denotes Z-B1 and red line denotes Z-B2.

TABLE 2: SIEVE ANALYSIS OF RS SAMPLES

Sample ID	Retained on ASTM 20 (%)	Retained on ASTM 45 (%)	Retained on ASTM 60 (%)	Retained on ASTM 80 (%)	Retained on ASTM 120 (%)	Retained on ASTM 325 (%)	Less than 0.045 mm (%)
X-B1	0.00	0.00	1.50	5.13	7.40	22.60	63.37
X-B2	0.00	0.00	3.30	4.40	8.00	25.30	59.00
Y-B1	0.00	0.00	1.13	7.10	11.03	21.60	59.14
Y-B2	0.00	0.00	1.80	10.80	13.70	23.00	50.70
Y-B3	0.00	0.00	1.53	11.40	15.90	24.70	46.47
Z-B1	4.00	13.80	17.10	18.80	21.60	23.40	1.30
Z-B2	1.10	11.23	16.80	19.40	25.00	25.40	1.07

ASTM: American society for testing and materials

reported for alumina powders[8]. Since calcination and milling processes generally lead to formation of irregular shapes and sizes, it is probable that the solid-state processes leading to the synthesis of RS might have resulted in irregular particle shapes and sizes.

All samples were insoluble in water. The variation in colour and particle size indicates difference in the preparation procedures among the *Ayurvedic* manufacturers of RS. Increase in particulate size will ultimately affect pharmacokinetics of the drug. Smaller particle size will enable drugs to circumvent barriers that are impenetrable to larger particles[9]. Reduction in size will increase the surface to volume ratio, which in turn can enhance the interactions with biological components[10]. Another factor that

TABLE 3: AVERAGE PARTICLE SIZE AND ZETA POTENTIAL OF RS SAMPLES

Sample ID	Average particle size (nm)	Zeta potential (mV)
X-B1	514.2	−23.5±5.43
X-B2	434	−26.2±7.49
Y-B1	532.6	−34.4±6.38
Y-B2	402.6	−36.6±7.02
Y-B3	435.6	−26.9±5.92
Z-B1	519	−25.6±7.90
Z-B2	446.1	−25.9±8.00

Values of zeta potential are expressed as mean±standard deviation

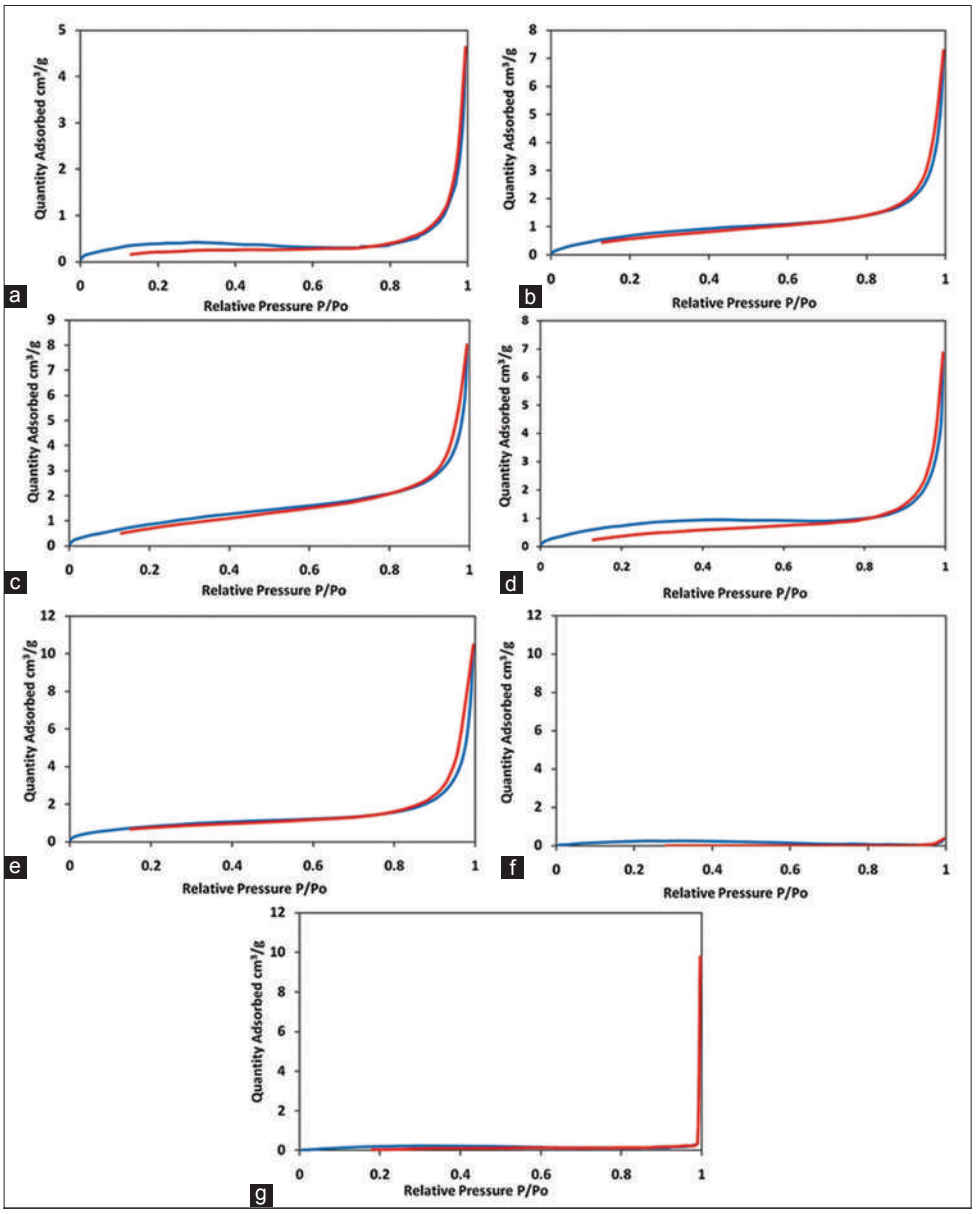

Fig. 2: Adsorption–desorption isotherms of RS samples.
(a) X-B1 (b) X-B2 (c) Y-B1 (d) Y-B2 (e) Y-B3 (f) Z-B1 and (g) Z-B2 where blue line denotes adsorption and red line denotes desorption.

can influence particle stability in solvent is its zeta potential. Table 3 shows the average particle size and zeta potential of the RS samples. Average particle size of the samples ranged between 400 and 535 nm. The zeta potential ranged between -23 and -37 mV and there was no significant batch-to-batch and manufacturer-to-manufacturer variation in zeta potential. The zeta potential values indicate an incipient to moderate colloidal stability for these particles according to the DLVO theory and therefore formation of agglomerates is possible. The mechanism of cellular uptake of RS has not yet been established. Zeta potential plays an important role in particle uptake by cells, especially in intestinal mucosa whose extracellular surface is negatively charged due to presence of glycocalyx, which is a polymeric material excreted by epithelial cells[11]. The negative zeta potential indicates that the cell uptake of RS might be less. The surface charge also determines the electrostatic interactions, which in turn determines the mechanism and magnitude of protein adsorption[12]. Intestinal uptake of RS may be low due to the presence of anionic groups on its surface, which affects protein adsorption in negatively charged extracellular surface. Fig. 2 shows the nitrogen adsorption-desorption patterns for the different samples of RS. The adsorption profiles show large deviation from the Langmuir adsorption isotherms indicating occurrence of a multilayer adsorption in the samples. The adsorption pattern also exhibits characteristics of weak adsorbate-adsorbent interactions. The absence of a knee in the absorption profile indicates absence of micropores in the sample. The absence of significant hysteresis also suggests absence of pores in the sample. It is observed that all samples showed an inflection at P/P_o>0.9. This is a trend associated with macroporous samples with large voids. The incomplete adsorption-desorption profiles obtained for Z-B1 and Z-B2 suggests the coarse nature of this sample, thus implying the inapplicability of BET analysis for these samples.

Fig. 3 shows the scanning electron micrographs of the different samples of RS. All the samples exhibited inhomogeneous surface morphology as evident from the scanning electron micrographs. X-B1 (fig. 3a) revealed more spherical particles on strip-like surface, whereas X-B2 (fig. 3b) contained aligned fine grains on lamellar surface. The particle size ranged from 5-100 nm in X-B1 and X-B2 samples. The samples from manufacturer Y were irregular in shape and size with agglomeration (fig. 3c and d) (Y-B3 not shown). Z-B1 and Z-B2 samples showed particles with more-defined morphology (fig. 3e and f). Such wide variations clearly demonstrate difference in synthesis of these drugs by various manufacturers especially during the mechano-chemical processing, which plays an important role in modifying the physicochemical characteristics.

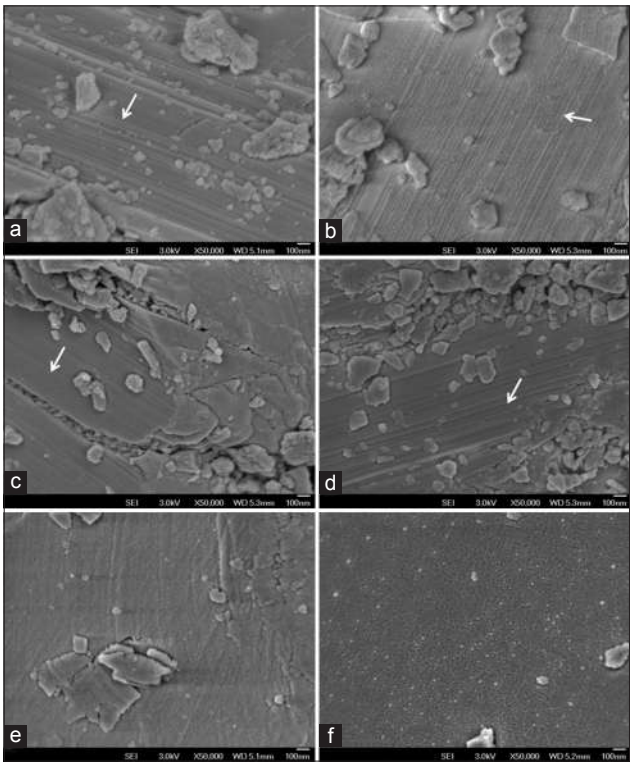

Fig. 3: Morphology of RS samples by scanning electron microscopy. (a) X-B1 (b) X-B2 (c) Y-B1 (d) Y-B2 (e) Z-B1 (f) Z-B2. The arrow marks indicate fine grains.

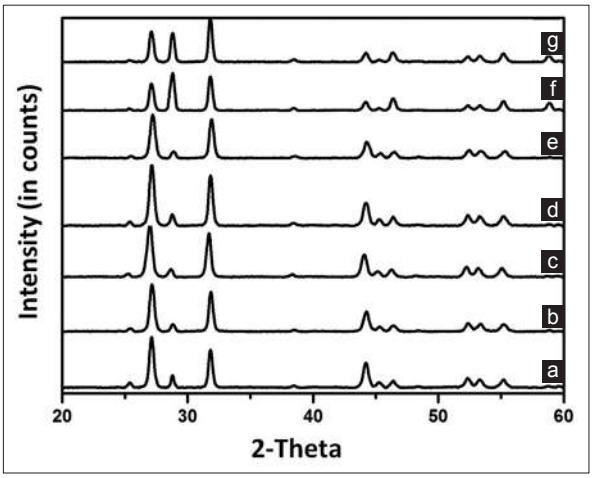

Fig. 4: X-ray diffraction pattern of RS samples.
XRD pattern of RS samples, where (a) X-B1 (b) X-B2 (c) Y-B1 (d) Y-B2 (e) Y-B3 (f) Z-B1 (g) Z-B2.

The X-ray diffractograms of the samples are shown in fig. 4, in which peaks corresponding to mercuric sulphide [01-080-2192, ICDD], with a hexagonal system and primitive lattice structure are observed. The minimum crystallite size, calculated from Scherrer formula, ranged between 16.7 and 22.7 nm with minimum variation between batches (Table 4). The mass percentage of Hg in pure mercuric sulphide (HgS) can be determined from stoichiometry as 86.2%. Interestingly, this is in accordance with the mass percentage of mercury (84-89%) determined from X-ray fluorescence spectroscopy, which indicates that the mercury in RS is in the form of HgS. Fig. 5 shows the selected area electron diffraction (SAED) pattern of RS samples. The SAED pattern indicates the hexagonal lattice characteristic of mercuric sulphide and the d values ranged from 0.108-1.226 nm. This correlates with the x-ray diffraction studies, which also indicated a primitive hexagonal lattice arrangement in the samples.

TABLE 4: GRAIN SIZE OF EACH SAMPLE OF RS

Name	FWHM	2θ	Minimum crystallite size (nm)
X-B1	0.397	27.11	20.35
X-B2	0.439	27.15	18.40
Y-B1	0.482	26.98	16.74
Y-B2	0.430	27.13	18.80
Y-B3	0.465	27.20	17.37
Z-B1	0.426	28.79	19.03
Z-B2	0.360	31.80	22.70

FWHM: Full width at half maximum; 2θ values are expressed in degrees

Table 5 shows the elemental composition of the RS samples. Elements such as Hg, S, Mn and Fe were found in all the samples. Mercury and sulphur were found to be the major constituents of all the samples, with concentration ranging from 84 to 89% and 9 to 11% respectively, which is further confirmed from the EDAX spectra (Table 6). Mercury level was very high with reference to WHO standards. However, mercury may not be present in free form but in its sulphide form along with other macro- and micronutrients[13]. It is yet to be demonstrated experimentally whether mercury dissociates from the sulphide after it is ingested in the body.

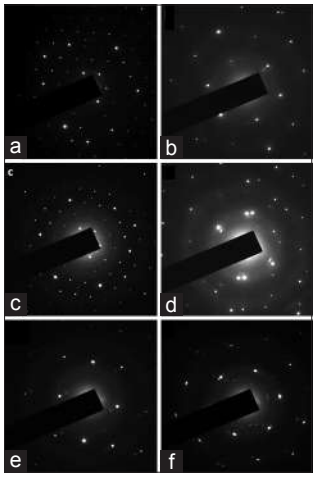

Fig. 5: SAED pattern of RS samples.
Selected area electron diffraction pattern of RS samples, where (a) X-B1 (b) X-B2 (c) Y-B1 (d) Y-B2 (e) Z-B1 (f) Z-B2.

TABLE 5: ELEMENTAL COMPOSITION OF RS SAMPLES DETERMINED USING X-RAY FLUORESCENCE SPECTROSCOPY

Element	Mass (%)						
	X-B1	X-B2	Y-B1	Y-B2	Y-B3	Z-B1	Z-B2
Mercury	88.5±1.42	84.7±2.24	84.8±1.36	85.4±1.13	85.5±0.90	89.2±0.71	88.7±0.70
Sulphur	10.05±1.52	10.69±2.24	11.20±1.57	10.6±1.14	11.05±0.94	9.61±0.75	10.01±0.75
Manganese	0.42±0.08	0.41±0.04	0.39±0.04	0.38±0.02	0.38±0.02	0.37±0.01	0.37±0.02
Calcium	0.01±0.01	0.05±0.01	0.04±0.005	-	0.01±0.01	-	0.01±0.01
Iron	0.01±0.01	0.06±0.01	0.02±0.00	0.02±0.02	0.01±0.005	0.02±0.01	0.01±0.01
Silica	0.02±0.02	0.04±0.04	0.01±0.00	0.006±0.01	0.02±0.02	0.01±0.01	0.02±0.02
Potassium	0.006±0.01	-	-	0.05±0.05	0.08±0.07	-	-

Values are expressed as mean±standard deviation

TABLE 6: ELEMENTAL ANALYSIS OF RS SAMPLES BY ENERGY DISPERSIVE X-RAY ANALYSIS

Element	Mass (%)						
	X-B1	X-B2	Y-B1	Y-B2	Y-B3	Z-B1	Z-B2
Mercury	65.04±6.37	55.57±2.07	67.29±1.31	56.28±0.59	57.83±5.64	72.86±0.15	73.92±0.48
Sulphur	14.70±0.23	10.76±0.93	15.63±0.62	10.85±0.54	12.66±1.18	15.38±0.49	14.66±0.33
Oxygen	20.25±6.14	33.67±1.14	13.63±0.50	32.86±0.04	29.50±6.82	11.76±0.33	11.42±0.82
Arsenic	a	a	3.45±0.18	a	a	a	a

Values are expressed as mean±standard deviation; a – denotes element not present in the sample

Humans require macronutrients (calcium, magnesium,) and micronutrients (iron and manganese) to carry out a range of physiological functions[14]. Calcium is needed for blood cell function, bone, muscle, heart and digestive system functions, while magnesium is required for processing of ATP[15]. Iron is utilized by haemoglobin[16]. Hence the presence of these elements even in small quantities might serve to impart therapeutic value to the preparation. These elements are most likely introduced in RS during the elaborate treatment processes designed to purify mercury and sulphur. Interestingly, there was no considerable variation in the elemental composition between the various commercial samples investigated implying that the composition chiefly is dependent on the ingredients introduced during the purification stages and not on the mechano-chemical grinding processes during the preparation. The thermal analyses of all the samples are shown in fig. 6. The TG-DSC results indicate degradation point of 460° for X-B1 and 500° for X-B2. The degradation point of Y-batch samples ranged from

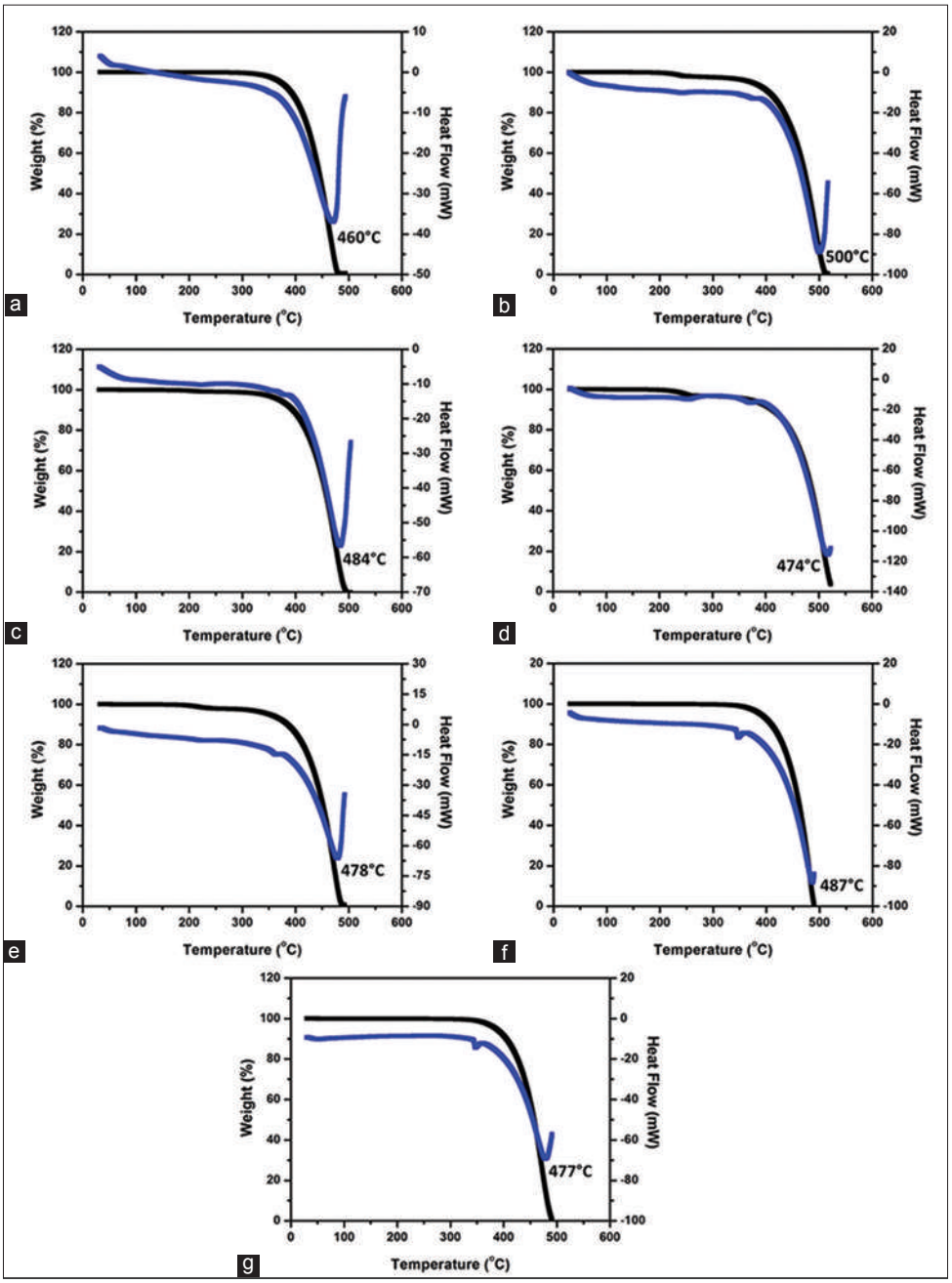

Fig. 6: TG-DSC of commercial samples of RS.
Thermogravimetry-differential scanning calorimetry patterns of RS samples where (a) X-B1 (b) X-B2 (c) Y-B1 (d) Y-B2 (e) Y-B3 (f) Z-B1 (g) Z-B2. Black curve denotes TGA and blue curve denotes DSC.

474-484°. The degradation point of Z-batch samples ranged from 477-487°.

DISCUSSION

The need for characterization of *Rasasindura* samples arises due to recent reports on toxicity of herbo-metallic preparations containing more than the permissible limit of heavy metals. Although use of metals in *Ayurveda* has been in practice since ages, the lack of pharmacovigilance raises questions on the scientific use of heavy metals as a form of medication. The physicochemical characterization of RS samples of different batches from different manufacturers has been investigated. Noticeable differences in surface morphology, surface area and particle size indicated probable variations in manufacturing procedure. These results indicate necessity for standardization of preparation procedures. Elemental analyses revealed high mercury content (84-89%) present as HgS, confirmed later from the X-ray diffractogram. Although elemental concentration is high and more than the permissible limits, it is also evident that mercury is not in the free form, which may be attributed to the stringent purification steps followed to detoxify metals and convert them into biocompatible form. The drug may not get absorbed through the intestinal tract due to its negative zeta potential and also due to its insolubility. This may be the reason the drug is said to be non-toxic. Since absorption through gastrointestinal tract may be minimal or nil, questions arise over its therapeutic potential. On the contrary, these drugs are administered with honey as a vehicle, which may aid in absorption of the drug through the intestinal tract after digestion. Honey has been reported to provide stability and act as capping agent for delivery of platinum nanoparticles[17]. The human dose of RS is 125 mg for a 60 kg adult, which can be considered to be minimal. All these factors necessitate the need for its preclinical toxicity as well as therapeutic efficacy studies in laboratory animals. Characterization of commercial samples has only lead to a better understanding of the physicochemical properties of RS. But a vast lacunae still exists on the therapeutic implications these properties may impart as well as their toxicological manifestations which can only be answered by *in vitro* and *in vivo* studies to confirm the safety of this traditional form of medication. Even though such drugs have been in clinical practice in the Indian subcontinent for thousands of years, the lack of pharmacovigilance has dent a severe blow on the assessment of patient safety.

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SAFETY & TOXICITY

Research Paper

Acute and Chronic Toxicity of *Rasamanikya*, an Ayurvedic Arsenical Formulation in Rats

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***Rasamanikya* (an arsenical formulation of Ayurveda) contains *Haratala* (arsenic trisulphide) as an integral component. Concerns are being raised on such Ayurveda formulations with heavy metals in their composition for safety aspects. Though, these are being used safely in Ayurvedic clinical practice since ages without any noticeable untoward effects; there is a need to generate scientific evidence that these are safe and non-toxic. In the present study, safety profile of *Rasamanikya* prepared from *Kushmanda Swarasa Shodhita Haratala* (arsenic trisulphide processed in juice of *Benincasa hispida*) was evaluated through acute and chronic toxicity studies. In acute toxicity, *Rasamanikya* was administered at a maximal dose of 2000 mg/kg to overnight fasted rats and observed closely for behavioral changes, signs of toxicity and mortality if any, continuously for the first six hours and thereafter periodically up to 14 days. In the chronic toxicity evaluation, the drug was administered daily at the doses of 22.5, 112.5 and 225 mg/kg along with honey and ghee as an adjuvant to rats for 90 days followed by a 30-day recovery period. Animals were sacrificed on the 91st day and hematological, serum biochemical parameters and histopathology of organs were studied. In acute toxicity, *Rasamanikya* at the dose of 2000 mg/kg did not produced any observable toxic effects or mortality. Safety of *Rasamanikya* at therapeutic and five-fold therapeutic dose level has been revealed in the chronic toxicity study. Mild to moderate pathological changes on different haematological, serum biochemical and cytoarchitecture of different organs were observed at ten-fold therapeutic dose level. Based on these observations, it can be concluded that *Rasamanikya* is safe at therapeutic dose levels when used judiciously along with specified adjuvants.**

Key words: Arsenic, *Haratala*, *Rasamanikya*, safety, *Shodhana*, toxicity

Ayurveda utilizes natural resources of plant, animal, metal and mineral origin in therapeutics of different pathologies. These resources are converted into formulations based upon the need by following specified classical guidelines. Herbo-mineral and metallic formulations are an important part of Ayurveda that are attributed to be safe and efficacious when manufactured and used judiciously. *Rasamanikya*, one such metallic formulations, attracted controversies in scientific community due to the presence of arsenic as an integral component. It is being used by Ayurveda physicians to treat fever (*Jwara*), cough (*Kasa*), asthma (*Shwasa*), fistulous tracts (*Nadi Vrana*) and skin diseases (*Kushtha*)[1]. In general, arsenicals are toxic and produce untoward effects on administration[2]. Anticipating such untoward effects; seers of Ayurveda have explained meticulous handling procedures and administration modalities for all such metals and minerals including arsenicals[3]. Importance of following traditional pharmaceutical procedures in preparation of Ayurvedic formulations have been well-established[4,5]. Ayurveda emphasizes on administration of metallic formulations orally in specified quantities with great caution along with requisite *anupana* (vehicle) that is anticipated to play a key role in safety aspects of *Rasaushadhies. Anupana* facilitates drug administration, improves palatability and also reduces toxic nature of the drug[6,7]. Though, there are many

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evidences that indicate the safe nature of metallic formulations; there is a need to generate scientific data-based evidences for all such formulations that contain heavy metals. As, *Rasamanikya* contains *Haratala* (arsenic trisulphide) as a component; it became necessary to evaluate its safety profile. Earlier studies established safety of *Rasamanikya* prepared by *Haratala* processed in *Churnodaka* (lime water)[8]. But, whether the safety profiles will be the same, if processing media is changed is not known. Considering this, safety profiles of *Rasamanikya* prepared with *Kushmanda Swarasa Shodhita Haratala* (arsenic trisulphide processed in fruit juice of *Benincasa hispida*) was evaluated in the current study.

MATERIALS AND METHODS

Haratala Shodhana (processing of arsenic trisulphide):

Raw *Haratala* was procured from the Pharmacy, Gujarat Ayurved University, Jamnagar, Gujarat; made into small pieces (40#), bundled in a cotton cloth (*pottali*), suspended in a stainless steel vessel taking care not to touch the bottom and walls of the vessel assuring free movement. Quantity sufficient amount of *Kushmanda Swarasa* (fruit juice of *Benincasa hispida*) to completely immerse the *Pottali* was added into the vessel and subjected to heat at 100° for three hours. Care was taken to immerse *Pottali* completely in *Kushmanda swarasa* throughout the boiling process. At the end of three hours boiling, *Haratala* was removed carefully from the *Pottali*, washed with hot water and dried to obtain *Shuddha* (processed) *Haratala*[9] (fig. 1a-e).

Preparation of *Rasamanikya*:

Thin layers of completely dried and powdered *Shuddha haratala* were placed in between two mica sheets and the boundaries were locked with 'U' pins. This was held with the help of tongs and heated over a gas stove until *Haratala* melted completely. Heating was stopped, the 'U' clips were carefully removed to open the mica layers to expose the ruby-colored product, *Rasamanikya* was collected carefully by avoiding the mica particles. This was coded as RM (fig. 1f-h)[10].

Experimental animals:

Charles-Foster rats of either sex weighing 200±20 g were obtained from the animal house attached to the pharmacology laboratory, Institute for Postgraduate Teaching and Research in Ayurveda, Gujarat Ayurved University, Jamnagar. The animals were exposed to natural day and night cycles under ideal laboratory conditions in terms of ambient temperature (23±2°) and humidity (50-60 %). Animals were fed *ad libitum* with Amrut brand rat pellet feed supplied by Pranav Agro Industries and tap water. The experiment was carried out after obtaining permission from Institutional Animal Ethics Committee (IAEC/15/2013/39) and care of animals was taken as per the Committee for the Purpose of Control and Supervision of Experiments on Animals guidelines.

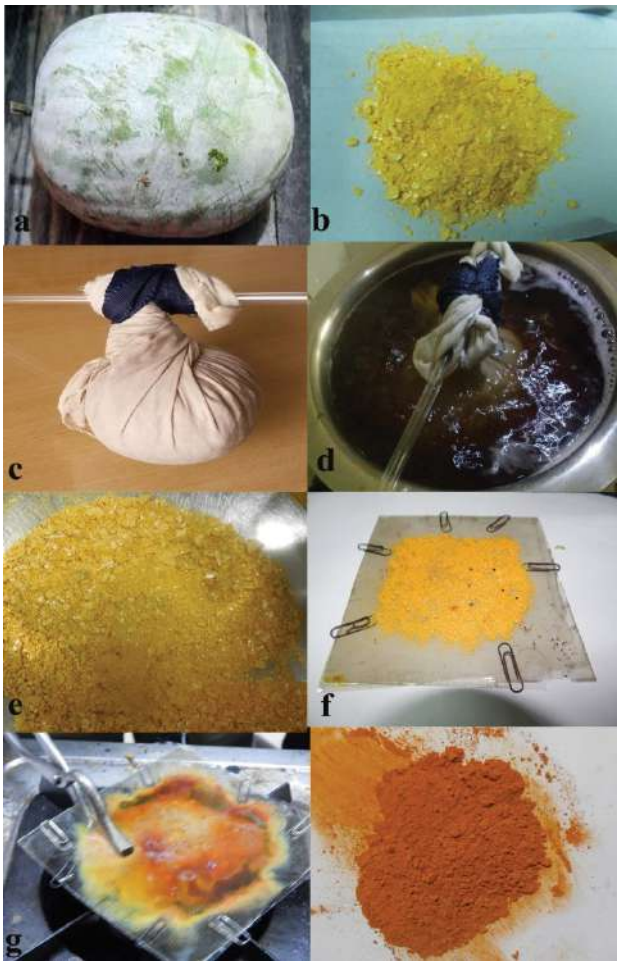

Fig. 1: Photomicrographs of preparation steps of *Rasamanikya* (a) Raw *Kushmanda* (*Benincasa hispida*), (b) raw *Haratala,* (c) *Pottali* of raw *Haratala*, (d) boiling of raw *Haratala*, (e) *Shodhita Haratala*, (f) clipped *Haratala* powder within mica sheets, (g) heating on LPG gas stove, (h) prepared *Rasamanikya* powder

Dose fixation and schedule:

The therapeutic dose (TED) of RM is 250 mg[1]. Rat dose was calculated by referring to table of Paget and Barnes and was found to be 22.5 mg/kg of rat[11]. As classics advocate using RM along with honey and ghee as adjuvant[12]; RM was administered orally along with honey and ghee with the help of oral cannula.

Acute toxicity study:

Young, healthy, nulliparous, and non-pregnant Charles-Foster female rats were selected and acclimatized for seven days before the experiment. RM along with adjuvant were orally administered at the limit dose of 2000 mg/kg to overnight fasted rats by following Organization for Economic Cooperation and Development (OECD) 425 guidelines[13]. The rats were observed closely for behavioural changes, signs of toxicity, and mortality, if any continuously for the first six hours and thereafter periodically up to 14 d.

Chronic toxicity study:

The study was carried out by following OECD 408 guidelines[14]. Charles-Foster rats were selected and randomly grouped into six, each consisting of six rats comprising three males and three females. Animals of group-I received tap water and normal food and served as normal control (NC), while animals in group-II received vehicle (1 ml/kg of honey and ghee orally) and served as vehicle control (VC). Group-III to V received RM along with adjuvant at TED (22.5 mg/kg orally), TED×5 (112.5 mg/kg orally) and TED×10 (225 mg/kg orally) dose levels, respectively. Animals of group-VI also received test drug at the level of TED×10 (225 mg/kg orally) along with adjuvant and is served as recovery group (Table 1).

Initial body weight of all animals was recorded. General behavioural pattern was observed once a week by exposing each animal to open arena. On 90th d, animals of group I-V were weighed again and anaesthetized with diethyl ether. Supraorbital plexus was punctured and blood was collected using capillaries in two different tubes, one containing anticoagulant fluid for haematological parameters and another plain tube for serum biochemical investigations. Then the rats were sacrificed with overdose of diethyl ether and the abdomen was opened through midline incision to observe the autopsy changes followed by dissecting out the important organs.

TABLE 1: TEST DRUG POSOLOGY FOR CHRONIC TOXICITY

Group		No of animals	Drug	Dose (mg/kg)
I	Normal control	6	NC	--
II	Vehicle control	6	VC	1
III	Therapeutic dose	6	RM	22.5
IV	TEDx5	6	RM	112.5
V	TEDx10	6	RM	225
VI	Recovery group	6	RM	225

NC: normal control, VC: vehicle control, RM: *Rasamanikya*, TED: therapeutically equivalent dose

Haematological analysis was performed by using an automatic haematological analyser (Swelab, Sweden). Total red blood cell (RBC), hemoglobin, hematocrit, mean corpuscular volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, white blood cell, neutrophils, percent lymphocytes, eosinophil's and monocytes, packed cell volume (PCV) and platelet count were measured from the blood samples.

Serum biochemical parameters were carried out by using fully automated biochemical random access analyser (BS-200, Lilac Medicare Pvt. Ltd., Mumbai). The studied parameters were blood glucose, total cholesterol, triglyceride, high density lipoproteins (HDL) cholesterol, very low density lipoprotein cholesterol, serum glutamic oxaloacetic transaminase, serum glutamic pyruvic transaminase (SGPT), alkaline phosphatase, total bilirubin, direct bilirubin, blood urea, creatinine, total protein, albumin, globulin, uric acid and serum calcium[15-29].

Liver, kidney, heart, lungs, trachea, intestine, spleen, thymus, lymph node, ileum, testis, seminal vesicle, prostate, uterus and ovary were dissected carefully. After noting signs of gross lesions and ponderal changes of these organs; all were transferred to 10 % phosphate buffered formalin solution for fixation and later on subjected to dehydrating, wax embedding, sectioning and staining with haematoxylin and eosin for histological evaluation. The slides were viewed under trinocular research Carl Zeiss microscope at various magnifications to note down the changes in the microscopic features of the tissues.

Statistical analysis:

The data is expressed as mean±standard error (SE) of each experimental group. Statistical comparisons were carried out by both unpaired Student's *t* test and one-way analysis of variance to compare the mean values of quantitative variables among the groups followed by Dunnett multiple *t* - test for unpaired data by using Sigma stat software (version 3.5, Systat Software Inc.) to determine significant difference between groups at $p<0.05$.

RESULTS AND DISCUSSION

The results of acute toxicity showed no changes in gross behaviour in any of the animals. RM along with

adjuvant did not showed any signs of toxicity and mortality up to 14 d when given at a dose of 2000 mg/kg. Behavioural changes were not observed in any of the treated groups during the course of chronic toxicity in comparison to NC group. No mortality was observed in any of the experimental groups. Weight gain was observed in all treated groups, but percent change in body weight pattern in treated groups did not differ significantly from the changes observed in control group (Table 2). Administration of RM at different dose levels resulted in insignificant changes in relative weight of nine organs except significant increase in weight of kidney at TED, TED×10 and thymus at TED×5 dose levels. Significant increase in liver, kidney, thymus and uterus was observed in recovery group (Table 3).

Significant increase in neutrophils and decrease in lymphocytes was observed in group-II and group-III, but they showed insignificant changes at higher dose levels and in recovery group. Significant increase was observed in RBC, PCV and hemoglobin at lower dose level and VC group in comparison to NC group. However, there was insignificant change at higher dose levels in comparison to NC and VC (Table 4). The results of test drugs on serum biochemical parameters showed significant decrease in blood urea at all dose levels including in recovery group. Significant increase in serum creatinine and albumin was observed at lower

TABLE 2: EFFECT OF *RASAMANIKYA* ON BODY WEIGHT

Groups	0 day	4th week	8th week	12th week
NS	180.00±8.76	192.50±7.04	197.50±8.3	205.83±11.50
VC	196.67±3.33	208.33±4.77	223.33±9.54*	238.33±20.07
RM-TED	195.00±8.47	203.33±9.54	230.00±11.83*	244.00±13.64*
RM-TED×5	190.00±7.75	198.33±10.77	221.67±12.22	226.67±18.74
RM-TED×10	208.33±9.46	205.00±7.19	235.00±6.71	228.33±16.00
Recovery	198.33±4.014	201.67±5.43	193.33±3.33	228.33±7.49

Data presented as mean±SEM, *p<0.05

TABLE 3: EFFECT OF *RASAMANIKYA* ON RELATIVE ORGAN WEIGHT

Relative weight	NC	VC	*Rasamanikya*			
			TED	TED×5	TED×10	Recovery
Heart (mg/100 g)	0.616±0.02	0.581±0.04	0.597±0.02	0.681±0.03	0.675±0.02	0.656±0.03
Liver (g/100 g)	6.025±0.19	6.162±0.64	5.694±0.27	7.376±0.67	7.277±0.53	7.527±0.61
Spleen (mg/100 g)	0.394±0.01	0.337±0.03	0.339±0.01	0.447±0.03	0.441±0.03	0.459±0.04
Kidney (mg/100 g)	1.334±0.04	1.495±0.10	1.504±0.06	1.547±0.10	1.604±0.06*	1.645±0.06*
Thymus (mg/100 g)	0.305±0.02	0.340±0.02	0.303±0.00	0.370±0.01	0.375±0.02	0.385±0.02
Testis (mg/100 g)	2.385±0.08	2.410±0.04	2.104±0.04	2.104±0.14	2.258±0.17	2.374±0.09
Seminal vesical (mg/100 g)	0.798±0.15	0.741±0.11	0.650±0.06	0.966±0.13	1.083±0.09	1.108±0.00
Prostate (mg/100 g)	0.257±0.02	0.237±0.04	0.218±0.01	0.324±0.02	0.321±0.02	0.387±0.08
Uterus (mg/100 g)	0.168±0.00	0.247±0.05	0.269±0.05	0.208±0.04	0.271±0.03	0.327±0.03*

*P<0.05 when compared with control group (ANOVA followed by Dunnett's multiple 't' test), NC: normal control, VC: vehicle control

TABLE 4: EFFECT OF *RASAMANIKYA* ON HEMATOLOGICAL PARAMETERS

Parameters	NC	VC	*Rasamanikya*			
			TED	TED×5	TED×10	Recovery
TWBC ($10^3/\mu l$)	7700.00±699.05	6766.67±725.10	6980.00±563.38	8750.00±834.96	7366.67±1006.53	8600.00±610.46
Neutrophil (%)	14.17±2.59	29.17±4.44*	35.20±2.94	20.17±4.08	22.67±2.79*	13.67±3.76
Lymphocyte (%)	81.83±2.53	67.00±4.70*	63.00±1.64	76.50±4.37	73.17±2.63	82.50±4.07
Eosinophil (%)	2.33±0.21	2.17±0.31	2.40±0.24	1.83±0.17	2.33±0.33	2.17±0.31
Monocyte (%)	1.68±0.21	1.67±0.21	1.40±0.24	1.50±0.22	1.83±0.17	1.68±0.21
Hb (g/dl)	14.40±0.36	17.67±0.56	17.20±0.62	14.70±0.18	14.73±0.22	15.33±0.25
PCV (%)	43.97±0.62	52.75±1.91	53.26±2.50	45.58±0.81	46.12±0.85	46.90±0.96
TRBC ($10^6/\mu l$)	7.69±0.14	9.26±0.34	9.53±0.47	8.12±0.22	8.33±0.19	8.36±0.18
MCV (fl)	57.22±0.63	56.97±0.30	55.84±0.47	56.23±0.78	55.38±0.51	56.10±0.58
MCH (pg/red cell)	18.72±0.30	18.67±0.17	18.12±0.31	18.13±0.32	17.77±0.28	18.35±0.29
MCHC (g/dl)	32.73±0.44	32.75±0.21	32.46±0.39	32.25±0.21	32.05±0.23	32.70±0.68
Platelets ($10^3/\mu l$)	1201.83±56.13	1284.17±68.67	1254.60±74.64	1217.50±137.16	1291.33±79.78	1218.50±37.49

Data: Mean±SEM, *p<0.05, when compared with control group; NC: normal control, VC: vehicle control

dose level but was within normal range. However, similar changes were not observed at higher dose levels and in recovery group in comparison to control and VC groups. Significant decrease was observed in serum globulin at TED×5 and in recovery group. Total protein level was not affected by the drug at all doses, while albumin level was increased and globulin level was decreased by test drugs, but, were within normal range, hence are not of serious in nature. The test drug at all dose levels decreased total cholesterol, increased triglycerides and HDL-cholesterol in comparison to control group, but no changes were observed when compared to VC group (Table 5).

Histopathological studies revealed pathological changes in stomach, heart, liver, kidney and ileum on administration of RM at TED×10 dose levels. Mild to moderate epithelial erosions were observed in stomach (fig. 2), while liver showed micro fatty changes (fig. 3). Mild to moderate inflammation was observed in kidney (fig. 4). Villous shortening at ileum (fig. 5) and fatty changes in heart was observed (fig. 6). These changes were recovered and no pathological changes were observed in any of the organs in the animals of group-VI.

OECD 425 guidelines for oral acute toxicity study was employed to record immediate adverse signs and symptoms after administration of single dose of drug at 2000 mg/kg dose level that is several folds higher than the actual therapeutic equivalent dose. Female rats were used for acute toxicity study to reduce variability. This is because there is little difference in sensitivity in LD_{50} between the sexes; however, in those cases where differences were observed; females were generally slightly more sensitive[30]. Oral administration of RM at the dose level of 2000 mg/kg did not produced any observable toxic effects and all female rats were survived for 14 d of observation suggesting that LD_{50} value of *Rasamanikya* may be higher than 2000 mg/kg.

Significant increase was observed in RBC, PCV and haemoglobin in group-II and group-III in comparison to NC group. The observed changes cannot be considered as toxicity of test drugs as these changes were not observed at higher dose levels. Arsenic likely causes both direct cytotoxic effect on blood cells and suppression of erythropoiesis through bone marrow toxicity and may inhibit haem synthesis[31]. Test drug did not showed any such toxic effects on cellular constituents in present study. Test drug did not hamper the erythropoiesis rather enhanced the formation of RBC's suggesting possible therapeutic role of *Rasamanikya* along with honey and ghee in conditions like anaemia. Role of arsenical compound in blood disorders was also reported earlier[32].

Result of test drugs on serum biochemical parameters showed significant decrease in blood urea at all dose levels including recovery group of animals. Increase in urea has significant role in toxicity, while low level represents low turnover of protein and nitrogenous

TABLE 5: EFFECT OF *RASAMANIKYA* ON BIOCHEMICAL PARAMETERS

Parameters	NC	VC	*Rasamanikya*			
			TED	TED×5	TED×10	Recovery
FBS (mg/dl)	62.20±3.72	51.00±6.21	59.20±4.19	66.50±4.31	70.00±3.53	71.33±1.02
Serum cholesterol (mg/dl)	46.83±2.97	41.50±1.43	37.00±3.18	41.00±3.03	43.00±0.73	39.17±3.31
Triglycerides (mg/dl)	67.50±4.31	78.50±13.55	69.60±20.05	85.00±11.48	90.33±13.57	87.50±9.94
HDL (mg/dl)	31.00±1.53	33.17±1.83	29.60±2.84	34.17±2.07	36.50±1.15	29.67±1.91
VLDL (mg/dl)	13.50±0.86	15.70±2.71	13.92±4.01	17.00±2.29	18.07±2.71	17.50±1.99
SGPT (IU/L)	43.50±3.57	49.83±3.46	46.20±2.75	54.33±4.88	43.33±2.85	43.00±3.71
SGOT (IU/l)	135.50±9.63	157.67±12.95	134.40±8.15	157.17±7.97	128.00±6.72	117.83±9.25
ALP (IU/l)	166.00±24.59	134.50±23.11	106.40±19.00	163.33±24.21	187.50±23.32	134.83±13.81
Total-value bilirubin (mg/dl)	0.60±0.73	0.40±0.04	0.46±0.07	0.57±0.08	0.48±0.05	0.47±0.05
Direct bilirubin (mg/dl)	0.22±0.02	0.13±0.02	0.16±0.02	0.20±0.02	0.17±0.02	0.13±0.02
Blood urea (mg/dl)	56.33±2.08	37.33±2.73*	35.80±1.11*	30.33±1.43*	30.00±1.39*	28.00±1.21*
Creatinin (mg/dl)	0.63±0.02	0.83±0.02*	0.80±0.00*	0.58±0.05	0.63±0.04	0.65±0.03
Protein (g/dl)	6.88±0.21	7.43±0.16	7.14±0.16	6.72±0.20	6.98±0.22	6.62±0.11
Albumin (g/dl)	3.40±0.086	4.00±0.15	4.02±0.15	3.87±0.09	3.90±0.12*	3.88±0.17*
Globulin (g/dl)	3.48±0.15	3.43±0.12	3.12±0.04	2.85±0.16*@	3.15±0.24	2.73±0.11@
Uric acid (mg/dl)	1.10±0.15	1.50±0.19	1.66±0.20	1.00±0.17	0.87±0.11	0.82±0.08
Serum calcium (mg/dl)	10.05±0.60	9.83±0.37	9.92±0.33	10.12±0.16	10.45±0.19	10.57±0.31

Data: mean±SEM, *p<0.05, when compared with control group, @p<0.05 compared to control group (ANOVA followed by Dunnett's multiple 't' test), NC: normal control, VC: vehicle control

material in rats or may be due serious liver toxicity. In present study, liver function tests didn't elevated to significant extent but mild to moderate changes were observed in histopathological studies suggesting decrease in urea level may be due to low turnover of

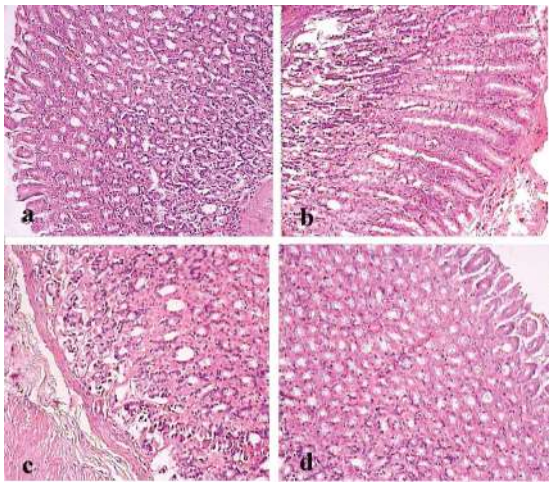

Fig. 2: Photomicrographs of gastric mucosal sections at x200 magnification

(a) Normal cyto-architecture in control group, (b) epithelial erosion and inflammation in RM-TED×10, (c) and (d) normal cyto-architecture in RM recovery TED×10 group

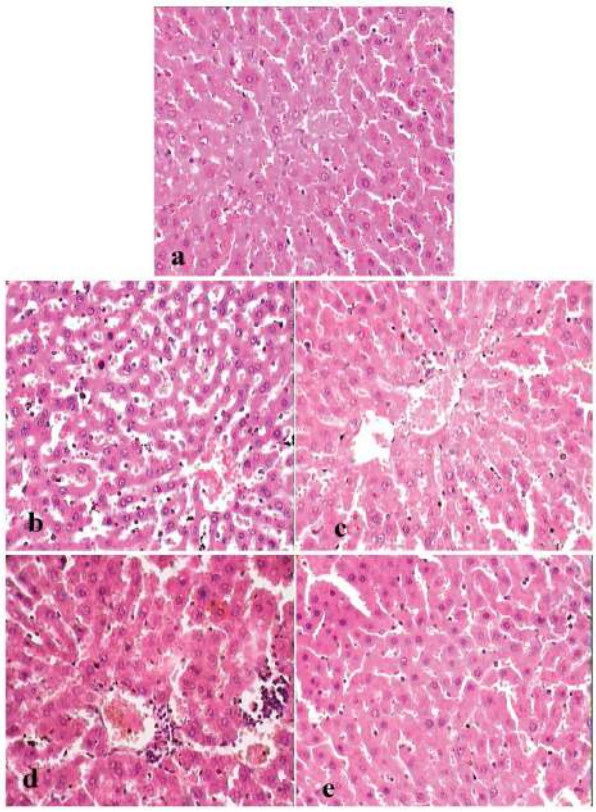

Fig. 3: Photomicrographs of sections of liver taken at x400 magnification

(a) Normal cyto-architecture in control group, (b), (c) and (d) fatty degenerative changes, cell infiltration, sinusoidal inflammation in RM-TED×10, (e) almost normal cyto-architecture in RM recovery TED×10 group

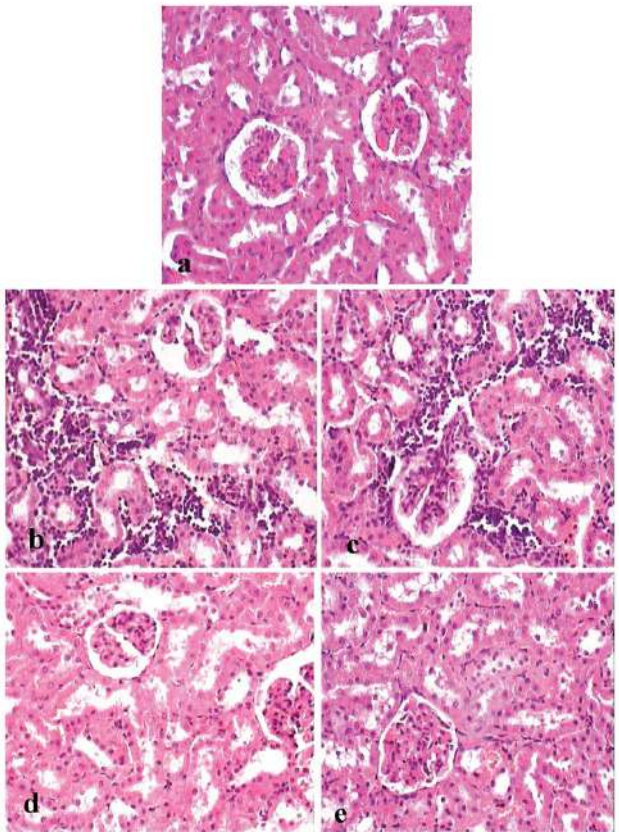

Fig. 4: Photomicrographs of sections of kidney taken at x400 magnification

(a) Normal cyto-architecture in control group, (b) and (c) fatty degenerative changes, cell infiltration and oedema in RM-TED×10, (d) and (e) almost normal cyto-architecture in RM recovery TED×10 group

protein level and nitrogenous material in rats or low magnitude of liver toxicity. Significant increase in serum creatinine was observed at TED level in group-III, but was in the physiological range. This increase in serum creatinine was not observed at higher dose levels and in animals under recovery study in comparison to control or VC groups. Significant decrease was observed in serum globulin at TED×5 and in recovery group. Total protein level was unaffected at all doses. Albumin level was increased while globulin level was decreased by test drugs. All these values are within normal range.

The test drug at all dose levels decreased total cholesterol, while triglycerides and HDL cholesterol were increased in comparison to control group, but no changes were observed when compared to VC group. This indicates possible interference with the lipid turnover in the experimental animals. HDL plays very important role in preventing the atherogenesis by taking away the cholesterol from the arterial wall and by inhibiting the oxidation of atherogenic

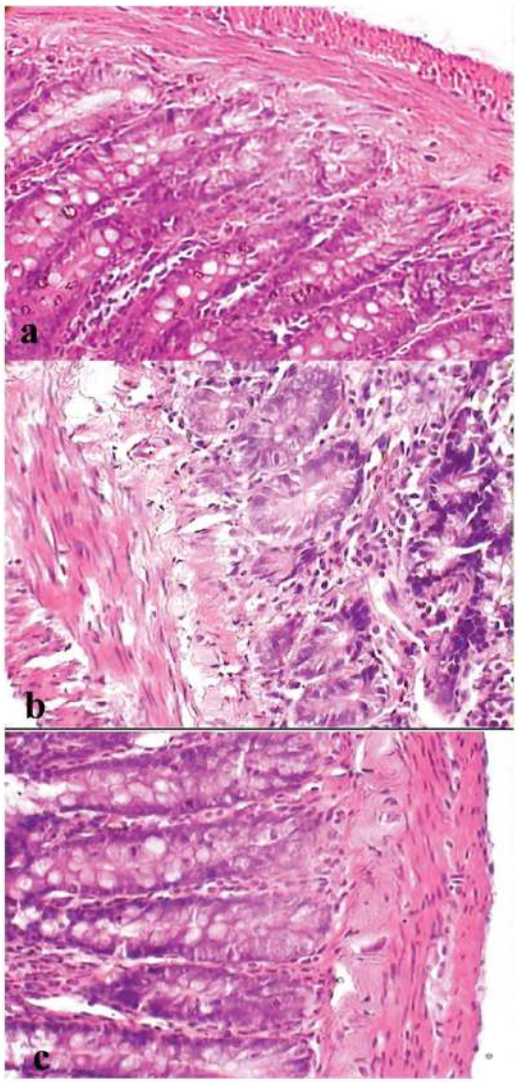

Fig. 5: Photomicrographs of sections of ileum at x400 magnification
(a) Normal cyto-architecture in control group, (b) decrease in villous height and cell infiltration in RM-TED×10, (c) normal cyto-architecture in RM recovery TED×10 group

lipoproteins[33]. Decrease in total cholesterol level with concomitant increase in HDL cholesterol may suggest therapeutic utility of *Rasamanikya* in the hyperlipidemic conditions.

Whenever there is injury in organs like liver; SGPT level gets elevated. Serum aminotransferases are elevated in most liver disorders and these are one of the most reliable markers of hepatocellular injury or necrosis[34]. In present study, serum transaminases insignificantly increased at lower dose level including VC, however values are within normal range. All these observed changes are reverted in recovery group and not observed at higher dose level in comparison to control and VC group. The decrease in bilirubin is still within normal range. The observed serum biochemical changes are mild and within normal ranges, hence it is inferred that drug is devoid of any serious toxic effects on serum biochemical parameters in rats.

Mild to moderate pathological changes in heart, liver, kidney and GI tract at higher dose levels were observed in histopathological study. The observed changes were not seen in group-I, where RM was administered at therapeutic dose, suggesting safety nature of the drug. The effects of biochemical parameters also match and support the histopathological findings of liver, kidney, stomach and ileum at higher dose level of the test drug validating safety nature of Ayurvedic arsenical formulations.

As few pathological changes appeared at TED×10 dose levels; physicians should be cautious while using this drug in patients with hepatic, cardiac and renal

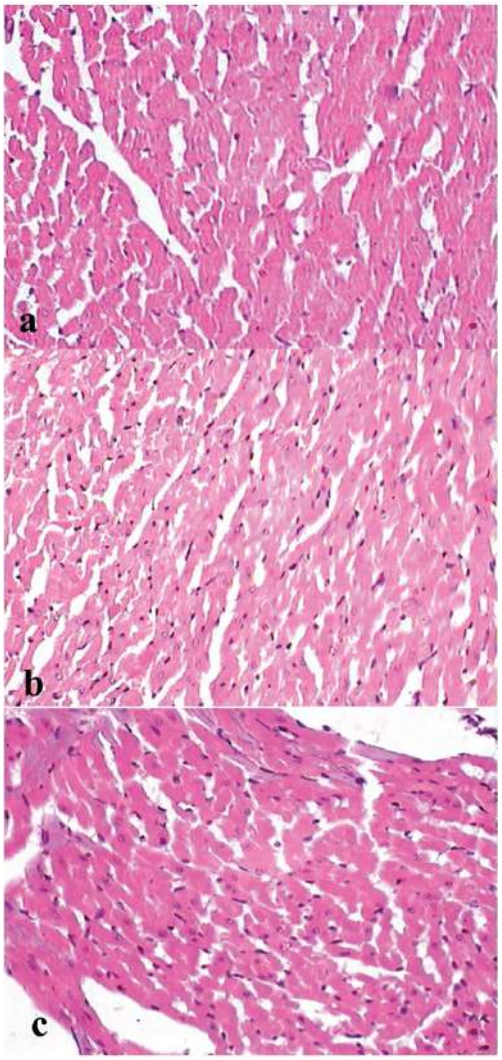

Fig. 6: Photomicrographs of sections of heart at x400 magnification
(a) Normal cyto-architecture in control group, (b) micro fatty changes in RM-TED×10, (c) normal cyto-architecture in RM recovery TED×10 group

impairments. Interestingly, these pathological changes were reversed in the recovery group indicating reversal of drug-induced adverse changes. Main treatment of heavy metal toxicity is to prevent or terminate the exposure[35]. Hence, after termination of RM, normal histopathology was appeared in the organs of animals in the recovery group.

The current study demonstrated safety of *Rasamanikya* processed in fruit juice of *Benincasa hispida* when administered at therapeutic and at TED×5 dose levels along with honey and ghee as adjuvants. RM may produce mild to moderate pathological changes when administered for longer duration at higher (TED×10) doses. Though the observed pathological changes were reverted after withdrawal of the drug; it is advisable to administer RM in specified TED along with a suitable vehicle (honey and ghee) for shorter periods. Care need to be observed while administering the drug in patients with hepatic, cardiac and renal impairments. It is also inferred that such formulations are not to be administered continuously for a longer period and observing a gap of few days is advisable[36].

Conflicts of interest:

There are no conflicts of interest.

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Research Paper

Acute and Subchronic Toxicity Study of *Tamra Bhasma* (Incinerated Copper) prepared from *Ashodhita* (Unpurified) and *Shodhita* (Purified) *Tamra* in Rats

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The use of metals in traditional medicines is very often seen as matter of concern these days, especially the *Bhasma* preparations which are always under stringent observations for containing highly reactive inorganic elements such as lead, mercury, arsenic and others. One of the *Bhasma* extensively used in routine Ayurvedic practice is *Tamra* (copper) *bhasma*. If it is not prepared properly or *Shodhana* procedure is not done properly, it acts as a poison. To indicate its toxic potential, *Ashtamahadoshas* (eight major ill effects) have been quoted in classics and due emphasis have been given to its *Shodhana* procedure. In the present study, *Tamra bhasma* prepared from *Shodhita* and *Ashodhita Tamra* was subjected to oral toxicity study to ascertain the role of *Shodhana* process on safety profile of *Tamra bhasma* on subchronic administration to albino rats. Both the samples were administered to rats for 45 consecutive days at the doses of 5.5, 27.5, and 55 mg/kg. Animals were sacrificed on 46th day and parameters like hematological, serum biochemical, and histopathology of various organs were studied. Results showed that *Tamra bhasma* prepared from *Ashodhita Tamra* has pathological implications on different hematological, serum biochemical and cytoarchitecture of different organs even at therapeutic dose level (5.5 mg/kg). Whereas, *Tamra bhasma* prepared from *Shodhita Tamra* is safe even at five-fold to therapeutic equivalent doses (27.5 mg/kg). These observations emphasize the role of *Shodhana* and importance of dose in expression of toxicity of the medicinal preparations.

Key words: *Amrutikarana*, copper, herbomineral formulation, LD_{50}, *Marana, Shodhana*

In traditional medicines, use of metals is very often seen as matter of concern these days. Especially the *Bhasma* preparations; which are always under stringent observations for containing highly toxic inorganic elements such as lead, mercury, arsenic and others[1,2]. In this present era of scientific validation and good manufacturing practices, the metals and minerals that are transformed into drugs must have the excellent quality, safety and therapeutic efficacy. These metallic preparations have unique procedures of their preparation, involving *Shodhana* (purification and/or detoxification), *Marana* (incineration and/or calcination), and are sustaining themselves since centuries in clinical practices. These methods were developed by practitioners of this science to detoxify the raw material by chemical transformations and modify the properties of therapeutic materials to enhance their potential[3,4]. Hence their use cannot be denied just for their heavy metal content. But unfortunately some metals and minerals have the potential to produce adverse effects[5]. Therefore, during their transmutation to drugs, it is essential to evaluate the margin of safety between the dose level that produces the therapeutic effects and that produces the adverse effects. Animal experimentation is the only way through which this evaluation can be made.

Tamra (copper) *bhasma* is used in various Ayurvedic preparations. If it is not prepared properly or *Shodhana* procedure is not done; it acts as a poison[6]. To indicate its toxic potential, *Ashtamahadoshas* (eight major ill effects) have been quoted in classics and due emphasis has been given to its *Shodhana* procedure[6]. Previously Nayak *et al.* found that *Tamra Bhasma* has the tendency of producing toxicity[7] on oral administration. Pattanaik *et al*. observed that *Tamra bhasma* given for longer period (90 days), in 5 mg/kg induced lipid peroxidation without any effect on the survival[8]. Vahalia *et al*. suggested that the safety of *Tamra bhasma* may be attributed to *Bhasmikarana* (Incineration) procedure which

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converts the metal into its specially designed chemical compound which eliminates the toxicity of metal and has the necessary medicinal benefits[9]. Although several such studies were reported, till date no research work on role of *Shodhana* (Purification) on *Tamra bhasma* has been reported to provide scientific basis to this. Hence in the present study, *Tamra bhasma* prepared from *Shodhita* and *Ashodhita Tamra* was subjected to oral toxicity studies to ascertain the role of *Shodhana* on safety profile of *Tamra bhasma* on subchronic administration to albino rats.

MATERIALS AND METHODS

Copper wire containing 99.89% copper, which is used for the purpose of electrical earthing, was procured from local electrician. In one sample raw copper wire was directly subjected to *Marana* and *Amritikarana* procedures and coded as ATB (*Tamra bhasma* prepared from *Ashodhita Tamra*). In another sample raw *Tamra* was subjected to *Samanya* (general) *Shodhana*, *Vishesha* (special) *Shodhana*, *Marana*[10] and *Amritikarana*[11] procedures as per the classical references and coded as STB (*Tamra Bhasma* prepared from *Shodhita Tamra*). These two coded samples were subjected to acute and sub-chronic toxicity studies.

Wistar strain albino rats of either sex, weighing 200±20 g were used as per the guidelines of the Institutional Animal Ethics Committee (IAEC). The animals were obtained from the animal house attached to the Pharmacology laboratory, Institute for Post Graduate Teaching and Research in Ayurveda, Gujarat Ayurved University, Jamnagar, and were exposed to natural day and night cycles, with ideal laboratory conditions in terms of ambient temperature and humidity. Temperature during the time of carrying out the experiment was between 24±2º and humidity around 50-60%. Animals were fed *ad libitum* with Amrut brand rat pellet feed supplied by Pranav Agro Industries and tap water. The experiment was carried out after obtaining the permission from institutional animal ethics committee (Approval number: IAEC 07/2010/05/MD) and care of animals was taken as per the CPCSEA guidelines.

Dose fixation and schedule:
Clinical dose of *Tamra bhasma* is 30 mg twice a day (60 mg per day)[12]. The suitable dose for rats was calculated by referring to table of Paget and Barnes[13] and was found to be 5.5 mg/kg (considered as therapeutic equivalent dose, TED). The test drugs were administered in the form of suspension in distilled water orally with the help of rubber catheter attached to a disposable syringe. For the preparation of stock solution, both the test drugs samples were taken in requisite quantity in small porcelain mortar and 0.5 ml of 5% gum acacia suspension was added, the formed mixture was further grounded for 5 min and the volume was made up with distilled water, to obtain a concentration of 5.5 mg/ml test drug.

Acute toxicity study:
Young, healthy, nulliparous, and nor pregnant Wistar albino female rats were selected and acclimatized for seven days before the experiment. The test drug was administered to overnight fasted animals at graded doses by following 'Up and Down method' with 2000 mg/kg (OECD TG 425). The animals were observed continuously for 6 h and then intermittently up to 48 h after the dosing to record changes in behavior pattern and mortality if any.

Study protocol for subchronic toxicity:
Animals were divided in seven groups, each comprising three male and three females. First group was kept as control where as the second to fourth groups were administered with test drug ATB at the dose of 5.5 mg/kg (TED), 27.5 mg/kg (TED×5), and 55 mg/kg (TED×10). Fifth to seventh groups were administered with test drug STB at the dose of 5.5 mg/kg (TED), 27.5 mg/kg (TED×5) and 55 mg/kg (TED×10). Initial body weight of all the groups was recorded and test drugs were administered for 45 consecutive days to overnight fasted rats. The general behavioral pattern was observed once every week by exposing each animal to open arena. On 46th day, animals were weighed again and anaesthetized with diethyl ether. Blood was drawn from supraorbital plexus by puncturing and collected in two different types of tubes, one containing anticoagulant fluid for hematological parameters and another plain tube for serum biochemical investigations. Then the rats were sacrificed by overdose of diethyl ether anesthesia and important organs were dissected out and transferred immediately to a glass bottle containing 10% formalin for histopathological studies.

Hematological analysis was performed using an automatic hematological analyzer (Swelab AC 970^{EO+}, AC 920^{EO+}, AC 910^{EO+} , Boule Medical AB, Stockholm, Sweden). Hemoglobin (Hb),

hematocrit, total red blood cell (RBC), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), white blood cell (WBC), neutrophils, lymphocyte percentage, eosinophils percentage, monocyte percentage, packed cell volume (PCV), and platelet count were measured from the blood samples.

For estimation of biochemical parameters serum was separated from collected blood. Requisite quantity of serum was fed to the auto analyzer (Fully automated Biochemical Random Access Analyzer, BS-200; Lilac Medicare Pvt. Ltd., Mumbai) which was automatically drawn in to the instrument for estimating different parameters. The biochemical parameters were recored a per the commercial kit instructions for blood glucose[14], blood urea[15], serum creatinine[16], serum total cholesterol[17], serum high density lipoproteins (HDL) cholesterol[18], serum triglyceride[19], serum total protein[20], serum alkaline phosphatase[21] activity, serum glutamate oxaloacetate transaminase (SGOT)[22], serum glutamate pyruvate transaminase activity (SGPT)[23], uric acid[24], direct bilirubin[25], and total bilirubin[26].

The histopathological slides of different organs like liver, kidney, heart, lung, trachea, jejunum, spleen, thymus, lymph node, testis, seminal vesicle, prostate, uterus, ovary, pituitary, brain, and adrenal gland were prepared by referring to standard procedure of Raghuramulu *et al*.[27]. The slides were viewed under trinocular research Carl-Zeiss's microscope at various magnifications to note down the changes in the microscopic features of the tissues studied.

Statistical analysis:

The results were presented as Mean±SEM for six rats in each group. Statistical comparisons were performed by both unpaired Student's *t* test and one way ANOVA with Dunnets' multiple *t* test as post-hoc test by using Sigmastat software (version 3.1) for all the treated groups with the level of significance set at $P<0.05$.

RESULTS

No change in gross behavior was observed in any group during acute toxicity study. Both ATB and STB did not show any mortality up to 14 days when given in dose up to 2000 mg/kg.

Subchronic administration of ATB at TED dose level leads to significant increase in total RBC count, neutrophil percentage and PCV, while significantly decrease in lymphocyte percentage. However, it did not produce any significant impact at TED×5 and TED×10 dose levels. In contrast, administration of STB did not affect the majority of hematological parameters except for significant increase in platelet count at TED×5 and significant decrease in total RBC count at TED×10 dose level (Table 1).

Among the 13 serum biochemical parameters, ATB at all the three levels significantly decreased HDL cholesterol. Other changes observed were

TABLE 1: EFFECT ON HEMATOLOGICAL PARAMETERS

Parameters	NC	ATB TED (5.5 mg/kg)	STB TED (5.5 mg/kg)	ATB TED×5 (27.5 mg/kg)	STB TED×5 (27.5 mg/kg)	ATB TED×10 (55 mg/kg)	STB TED×10 (55 mg/kg)
Hb (g %)	14.8±0.5	16.9±0.8	15.5±0.3	15.6±0.5	14.8±0.6	16.04±0.6	14.2±0.3
Hematocrit (%)	48.7±1.2	54.7±2.9	47.8±2.0	50.3±2.1	45.9±2.5	50.8±2.5	43.9±2.2
RBC ($\times10^6$ cells/mm^3)	8.6±0.1	9.7±0.5a	8.5±0.3	8.9±0.4	8.1±0.4	8.8±0.4	7.8*±0.2
MCH (pg)	17.3±0.6	17.3±0.2	18.2±0.5	17.5±0.4	18.4±0.2	18.2±0.2	18.2±0.4
MCHC (g/dl)	30.5±0.9	30.9±0.3	32.4±0.8	31.2±0.3	32.4±0.8	31.6±0.4	32.2±0.5
MCV (fl)	56.9±1.1	56.1±0.6	55.4±0.7	56.2±0.7	56.8±0.4	57.4±0.3	56.2±0.6
WBC ($\times10^3$/mm^3)	7966.0±656.59	8390.3±847.00	8933.33±868.58	8696.00±996.2	8166.66±841.29	7040.0±518.3	8240.00±700.40
Neutrophils (%)	27.12±0.44	36.66±3.09[a]	25.40±2.87	26.00±2.62	27.66±2.81	29.40±6.76	26.16±2.08
Lymphocytes (%)	68.83±0.4	58.0±3.3[a]	68.40±3.76	69.8±2.7	67.8±2.9	66.4±7.6	69.2±2.4
Eosinophils (%)	2.25±0.16	3.0±0.0	2.20±0.2	2.20±0.2	2.50±0.50	2.40±0.51	2.50±0.22
Monocytes (%)	2.00±0.18	2.33±0.21	2.0±0.31	2.0±0.0	2.0±0.0	1.8±0.37	2.16±0.16
PCV	47.38±1.23	54.73±2.89[a]	47.83±2.03	50.28±2.10	45.93±2.53	50.82±2.47	43.95±1.37
Platelet count (10^3/µl)	1047.7±49.3	1202.5±72.9	1215.8±70.7	949.2±125.3	1317.6±58.4[ab]	1208.0±140.2	1005.7±47.9

Data presented as=Mean±SEM, SEM is standard error of means, [a]$P<0.05$ (Unpaired *t* test), [b]$P<0.05$ (Dunnett's multiple *t* test), PCV=Packed cell volume, WBC=White blood cell, MCV=Mean corpuscular volume, MCHC=Mean corpuscular hemoglobin concentration, MCH=Mean corpuscular hemoglobin, RBC=Red blood cell, TED=therapeutic equivalent dose.

decrease in serum triglyceride level and serum alkaline phosphatase activity in TED dose levels of ATB, decrease in serum alkaline phosphatase activity in TED×5 dose level of ATB, decrease in total protein in TED×10 dose level of ATB in comparison to control group. Changes that occurred in STB-administered groups were; significant decrease in serum alkaline phosphatase activity in TED and TED×5 doses, significant decrease in total protein and HDL cholesterol at TED×10 dose (Table 2).

Microscopic examination of all the organs obtained from control group exhibited normal cytoarchitecture (figs 1a, 2a, 3a, 4a and 5a). All the doses of ATB and STB did not affect the cytoarchitecture of brain, pituitary, spleen, lung, stomach, lymph node, ovary, thymus, and uterus. Administration of ATB at all the three doses lead to myocarditis and fatty changes in heart (figs 1b, 1d, and 1f), cell infiltration and microfatty changes in liver (figs 2b, 2d, and 2f), cell infiltration and degenerative changes in kidney (figs 3b, 3d, and 3f), and decrease in spermatogenesis (figs 5b, 5d, and 5f). It also caused cell depletion and erosion of gastric mucosa at TED×10 dose (fig. 4b). In contrast, STB at TED and TED×5 dose showed normal cytoarchitecture in all of these organs; however, at TED×10 dose it caused pathological changes in myocardium (figs 1c, 1e and 1g), liver (figs 2c, 2e, and 2g), kidney (figs 3c, 3e, and 3g) stomach (fig. 4c) and testicular tissue (figs 5c and 5e) similar to that of ATB.

DISCUSSION

The right dose distinguishes the drug from a poison. So the safety and toxicity profile of the drug is important which is achieved through animal experimentation. Acute toxicity study was done

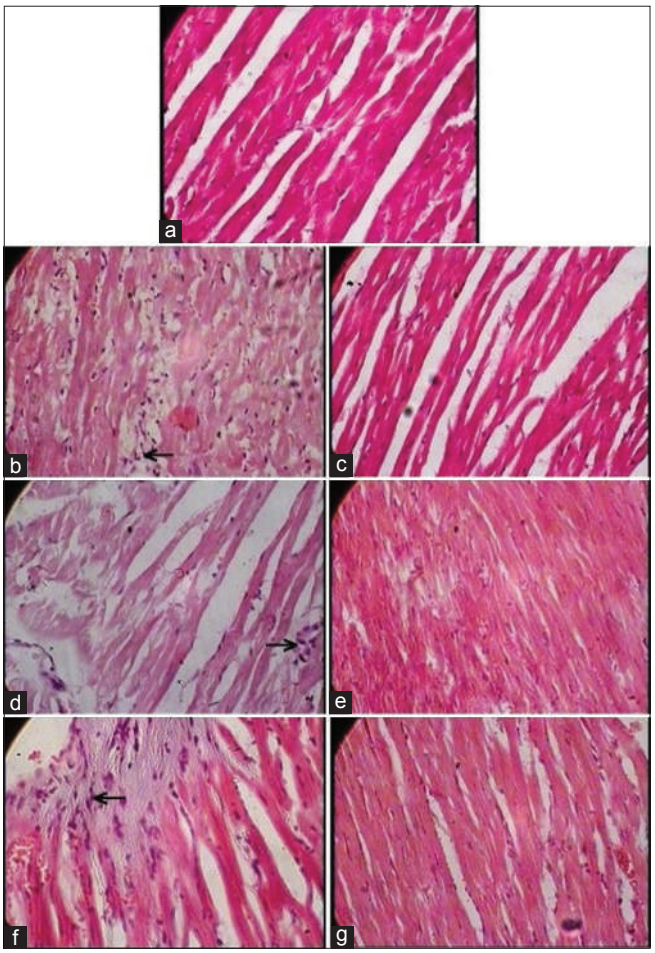

Fig. 1: Photomicrographs of sections of myocardium.
Photomicrographs of heart tissues taken at ×400 magnification. (a) Normal cytoarchitecture (Control group), (b) myocarditis and fatty changes (ATB TED), (c) Normal cytoarchitecture (STB TED), (d)myocarditis and fatty changes (ATB TED×5), (e) Normal cytoarchitecture (STB TED×5), (f) myocarditis and fatty changes (ATB TED×10), (g) Normal cytoarchitecture (STB TED×10).

TABLE 2: EFFECT ON SERUM BIOCHEMICAL PARAMETERS

Parameters	NC	ATB TED (5.5 mg/kg)	STB TED (5.5 mg/kg)	ATB TED×5 (27.5 mg/kg)	STB TED×5 (27.5 mg/kg)	ATB TED×10 (55 mg/kg)	STB TED×10 (55 mg/kg)
Blood glucose (mg/dl)	117.5±8.9	108.7±2.1	108.2±9.7	116.8±5.5	116.0±12.4	110.9±4.2	98.5±15.5
Total cholesterol (mg/dl)	77.5±9.9	63.3±6.02	91.2±8.2	51.5±4.4[a]	78.5±16.5	56.0±3.7	53.8±5.6
Triglyceride (mg/dl)	97.5±11.9	61.7±6.9[a]	121.2±22.6	93.0±11.07	87.0±14.6	94.2±10.4	124.3±18.9
HDL cholesterol (mg/dl)	39.2±5.4	26.3±2.2[ab]	37.3±3.7	24.8±2.2[ab]	32.7±3.7	25.0±1.2[a]	24.0±3.3[a]
Blood urea (mg/dl)	100.3±11.4	112.0±10.5	96.0±5.7	114.8±11.8	97.3±6.9	115.8±12.6	83.0±3.4
Serum creatinine (mg/dl)	0.6±0.2	0.6±0.07	0.6±0.05	0.6±0.03	0.6±0.02	0.6±0.06	0.6±0.03
S.GPT (IU)	77.3±6.9	89.0±9.8	62.7±3.4	93.5±4.08	70.8±8.3	83.7±6.2	86.2±15.3
S.GOT (IU)	332.0±42.2	279.2±9.9	246.5±9.9	321.7±25.6	302.0±19.9	335.3±54.9	309.0±36.6
Total protein (g/dl)	7.6±0.3	7.9±0.26	7.7±0.2	7.15±0.1	7.5±0.1	6.9±0.1[a]	6.7±0.2[ab]
Alkaline phosphatase (IU/l)	236.2±20.7	176.7±16.4[a]	146.7±11.4[a]	300.3±60.3	146.8±30.3[a]	250.0±29.8	170.3±34.72
Total bilirubin (mg/dl)	0.7±0.2	0.9±0.2	0.5±0.04	0.6±0.1	0.5±0.04	0.9±1.1	0.5±0.04
Direct bilirubin (mg/dl)	0.2±0.04	0.3±0.6	0.15±0.02	0.2±0.03	0.15±0.02	0.7±0.5	0.2±0.2
Uric acid (mg/dl)	2.08±0.4	2.6±0.5	1.6±0.1	1.7±0.3	1.6±0.2	2.7±0.4	1.9±0.4

Data presented as=Mean±SEM, SEM is standard error of means, [a]P<0.05 (Unpaired t test), [b]P<0.05 (Dunnett's multiple t test), TED=therapeutic equivalent dose, ATB=*Ashodhita Tamra bhasma*, STB=*Shodhita Tamra bhasma*, GPT=Glutamate pyruvate transaminase, GOT=Glutamate oxaloacetate transaminase

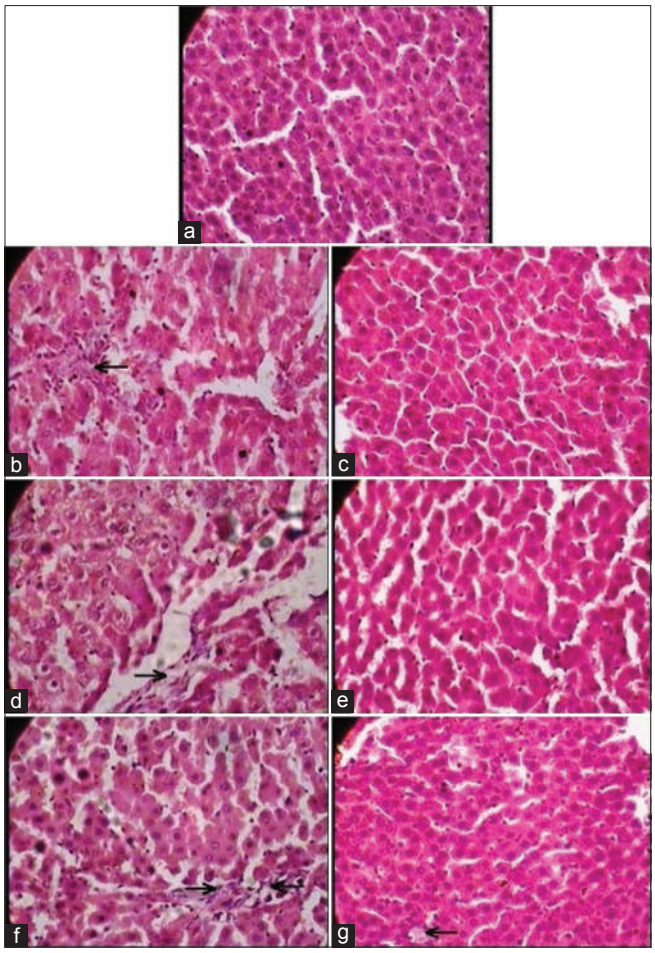

Fig. 2: Photomicrographs of sections of liver.
Photomicrographs of liver tissues taken at ×400 magnification. (a) Normal cytoarchitecture (Control group), (b) cell infiltration and microfatty changes (ATB TED), (c) Normal cytoarchitecture (STB TED), (d) cell infiltration and microfatty changes (ATB TED×5), (e) Normal cytoarchitecture (STB TED×5), (f) cell infiltration and microfatty changes (ATB TED×10), (g) cell infiltration and microfatty changes (STB TED×10).

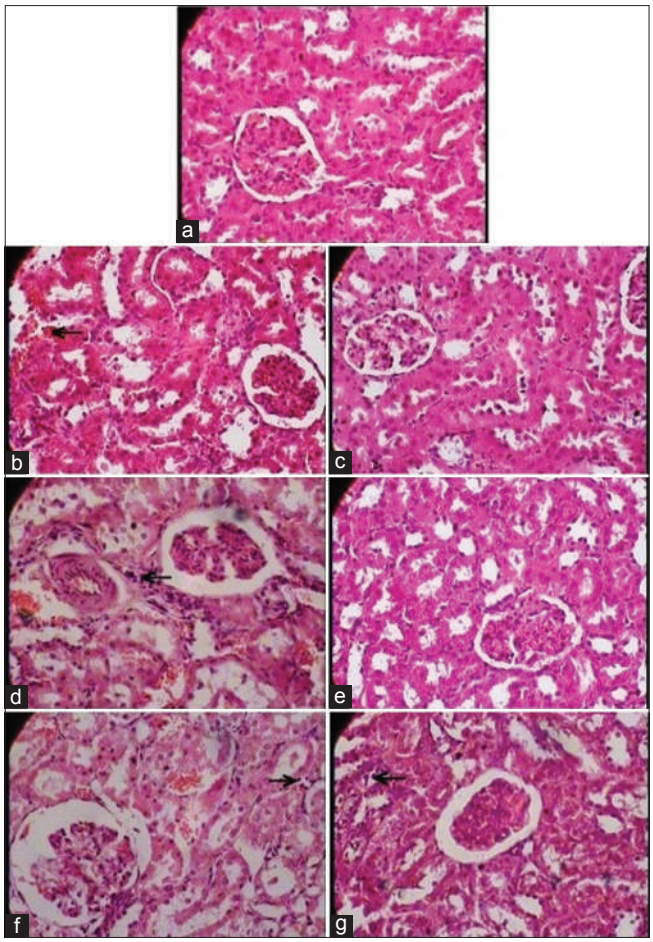

Fig. 3: Photomicrographs of sections of kidney.
Photomicrographs of kidney tissues taken at ×400 magnification. (a) Normal cytoarchitecture (Control group), (b) cell infiltration and degenerative changes (ATB TED), (c) Normal cytoarchitecture (STB TED), (d) cell infiltration and degenerative changes (ATB TED×5), (e) Normal cytoarchitecture (STB TED×5), (f) cell infiltration and degenerative changes (ATB TED×10), (g) cell infiltration and degenerative changes (STB TED×10).

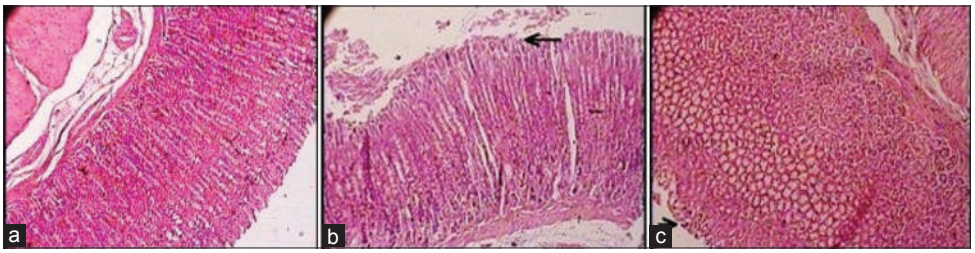

Fig. 4: Photomicrographs of gastric mucosal sections.
Photomicrographs of stomach tissues taken at ×100 magnification. (a) Normal cytoarchitecture (Control group), (b) cell depletion and erosion of gastric mucosa (ATB TED×10), (c) cell depletion and erosion of gastric mucosa (STB TED×10).

to record immediate adverse signs and symptoms after the administration of single dose of drug at dose levels that are several folds higher than the therapeutic equivalent dose and 2000 mg/kg was set as limit test. In this test, mortality was not observed in any of the groups which suggest that the LD_{50} is much higher than this dose for both ATB and STB.

Globally Harmonized System (GHS) is an internationally agreed system of classification and labeling of chemicals, which was developed under the auspices of the United Nations (UN)[28]. The GHS document, which is known as the "purple book", describes the harmonized classification criteria and the hazard communication elements

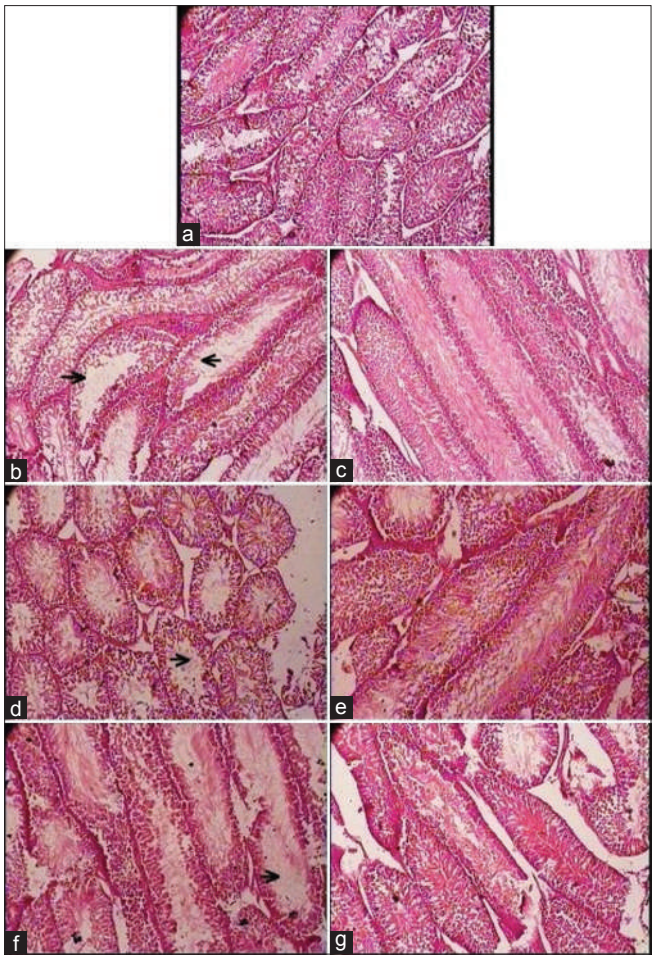

Fig. 5: Photomicrographs of testicular tissue.
Photomicrographs of testicular tissues taken at ×100 magnification. (a) Normal cytoarchitecture (Control group), (b) decrease in spermatogenesis (ATB TED), (c) Normal cytoarchitecture (STB TED), (d) decrease in spermatogenesis (ATB TED×5), (e) Normal cytoarchitecture (STB TED×5), (f) decrease in spermatogenesis (ATB TED×10), (g) decrease in spermatogenesis (STB TED×10)

by the type of hazard. It provides decision logics for each hazard, examples of classification of chemicals and mixtures and illustrates how to apply the criteria. The GHS includes harmonized criteria for the classification of physical hazards (e.g., flammable liquids); health hazards (e.g., carcinogens); and environmental hazards (e.g., aquatic toxicity). Since the test formulation is used for health care it was analyzed for probable health hazards. According to this classification if the LD_{50} in acute oral toxicity test is more than 2000 mg/kg it can be considered as hazardous and put in category 4. Material containing heavy metals are categorized as hazardous[29]. As per UN Classification any substance which has oral LD_{50} of more than 200 mg/kg is considered as low hazard potential and categorized as UN 6.1 PG III[30]. Thus as per the above criterion both ATB and STB can be categorized as substances with low health hazard potential (Class 4 of GHS and UN 6.1PGIII).

On subchronic administration ATB nonsignificantly increased WBC count in all the three doses. Increased WBC count is indicative of inflammatory conditions of certain organs especially of liver[31]. Histopathological studies showed severe fatty changes and degenerative changes not only in liver but in other organs like kidney and heart also. This may be the reason for the elevation of WBC count. Although significant changes occurred in other hematological parameters like total RBC count, neutrophil percentage, and PCV, they cannot be taken as pathological as they were not occurred in dose-dependant manner.

ATB administration at all the three levels significantly decreased HDL cholesterol. Smoking, anabolic steroids, beta-blockers, malnutrition and obesity lead to low HDL[32]. However these factors in the experimental conditions studied are unlikely to be the cause of the observed decrease in serum HDL-cholesterol level in treated groups. Two other possibilities that can be proposed to explain the decrease are the test drugs may impair the transfer of cholesterol from both very low density lipoproteins and tissue to HDL fraction or they may be promoting the metabolism of this fraction by enhancing the activity of the key enzymes involved in HDL cholesterol metabolism. In contrast to this observation, administration of STB at TED and TED×5 did not alter the HDL cholesterol to significant extent, however only at TED×10 dose levels it decreased the HDL cholesterol to significant extent. Significant decrease in serum total protein only at higher dose level (TED×10) of both ATB and STB indicates possibility of hypoproteinemia which is most commonly associated with the decreased production of albumin in liver due to significant destruction of hepatocytes[33]. It can also be seen in nephritic syndrome due to excessive loss in urine[34]. Observed decrease in serum protein level may be attributed to impairment of liver function because histopathological study of liver sections from this dose level shows cell infiltration and microfatty changes. It is interesting to note that at TED and even TED×5 dose levels both the test samples did not produce any significant changes in total protein level. This clearly indicates importance of drug dosage in production of toxicity.

Tamra bhasma prepared from *Ashodhita Tamra* is toxic even in the dose of therapeutic equivalent dose and highly toxic at TED×5 and TED×10 doses on subchronic administration for 45 days as revealed by hematological, biochemical, and histopathological parameters. *Tamra bhasma* prepared from *Shodhita Tamra* is safe even five-fold to therapeutically equivalent doses. However at ten fold dose it is not only prone to cause haepatotoxicity and nephrotoxicity, but also cause gastric mucosal damage. These observations reveal the role of *Shodhana* and importance of dose in expression of toxicity of the medicinal preparations.

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Efficacy & safety evaluation of Ayurvedic treatment (*Ashwagandha* powder & *Sidh Makardhwaj*) in rheumatoid arthritis patients: a pilot prospective study.

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***Background & objectives*: In the traditional system of medicine in India *Ashwagandha* powder and *Sidh Makardhwaj* have been used for the treatment of rheumatoid arthritis. However, safety and efficacy of this treatment have not been evaluated. Therefore, the present study was carried out to evaluate the efficacy and safety of Ayurvedic treatment *(Ashwagandha* powder and *Sidh Makardhwaj)* in patients with rheumatoid arthritis.**

***Methods*: One hundred and twenty five patients with joint pain were screened at an Ayurvedic hospital in New Delhi, India. Eighty six patients satisfied inclusion criteria and were included in the study. Detailed medical history and physical examination were recorded. Patients took 5g of *Ashwagandha* powder twice a day for three weeks with lukewarm water or milk. *Sidh Makardhwaj* (100 mg) with honey was administered daily for the next four weeks. The follow up of patients was carried out every two weeks. The primary efficacy end point was based on American College of Rheumatology (ACR) 20 response. Secondary end points were ACR50, ACR70 responses, change from baseline in disease activity score (DAS) 28 score and ACR parameters. Safety assessments were hepatic function [alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), bilirubin and β2 microglobulin], renal function (urea and creatinine and NGAL) tests and urine mercury level.**

***Results*: The study was completed by 90.7 per cent (78/86) patients. Patients with moderate and high disease activity were 57.7 per cent (45/78) and 42.3 per cent (33/78), respectively. All patients were tested positive for rheumatoid factor and increased ESR level. *Ashwagandha* and *Sidh Makardhwaj* treatment decreased RA factor. A significant change in post-treatment scores of tender joint counts, swollen joint counts, physician global assessment score, patient global assessment score, pain assessment score, patient self assessed disability index score and ESR level were observed as compared to baseline scores. ACR20 response was observed in 56.4 per cent (44/78) patients (American College of Rheumatology criteria) and moderate response in 39.74 per cent (31/78) patients [European League Against Rheumatism (EULAR) criteria]. Ayurvedic treatment for seven weeks in rheumatoid arthritis patients showed normal kidney and liver function tests. However, increased urinary mercury levels were was observed after treatment.**

***Interpretation & conclusions*: The findings of the present study suggest that this Ayurvedic treatment *(Ashwagandha* powder and *Sidh Makardhwaj*) has a potential to be used for the treatment of rheumatoid arthritis. However, due to small sample size, short duration, non randomization and lack of a control group as study limitations, further studies need to be done to confirm these findings.**

Key words *Ashwagandha* powder (*Withania somnifera*) - clinical trial - efficacy - rheumatoid arthritis - safety - *Sidh Makardhwaj*

Rheumatoid arthritis (RA) is a chronic, systemic, inflammatory, progressive disease. The prevalence of rheumatoid arthritis has been reported to be 0.75 per cent in Indian population[1]. The disease is more common in women than in men and occurs between the ages of 40 and 60 yr[2]. It leads to irreversible joint damage and systemic complications, and is associated with substantial morbidity and increased mortality[3,4]. Patients with active RA suffer from significant decline in functional capacity and 40 per cent become work disabled within five years from onset of symptoms[5]. Direct and indirect costs are also enormous[6].

The goals of RA management are to control pain and swelling, delay disease progression, minimize disability and improve quality of life. Non steroidal anti-inflammatory drugs (NSAIDs) have both analgesic and anti-inflammatory properties but do not change disease outcomes[7] and side effects are gastrointestinal ulcers (15-20% of patients), ulcer with bleeding and perforations (2-4% of patients over 70 yr of age)[8]. Glucocorticoids have greater action on joint pain than NSAIDs but have numerous side effects including adrenal suppression, ulcers and osteoporosis[9]. Disease modifying antirheumatic drugs (DMARDs) reduce the progression of joint erosion but they have slow onsets and no analgesic activity. Methotrexate has been shown to cause pulmonary complications[10].

Ayurveda is a widely practiced system of traditional medicine in India. It has been shown that 60-90 per cent of persons with arthritis use complementary and alternative medicine (CAM)[11]. Stress is widely recognized as an important risk factor in the aetiology of inflammatory rheumatic diseases[12,13]. *Withania somnifera* (*Ashwagandha*) exhibits anti-inflammatory, anti-tumour, anti-stress, antioxidant, immunomodulatory, haematopoietic and rejuvenating properties[14,15]. Thamaraiselvi *et al*[16] have shown *Ashwagandha* powder to be effective in patients with RA. *Sidh Makardhwaj* is a formulation mentioned in Ayurvedic Formulary of India[17]. It is a sublimed product made from pure mercury, sulphur and gold. It is used in rheumatoid arthritis, and neurological disorders, as *rasayana* for vigour and longevity of life[17]. Though *Ashwagandha* and *Sidh Makardhwaj* have been used in the treatment of patients with RA for many decades, their safety and efficacy have not been evaluated. The present study was, therefore, undertaken to evaluate the efficacy and safety of *Ayurvedic* treatment *i.e. Ashwagandha* and *Sidh Makardhwaj* in patients with RA.

Material & Methods

The present study was a prospective, open-label, non-randomized, outpatient-based, single centered drug trial conducted in the department of Pharmacology, All India Institute of Medical Sciences (AIIMS), New Delhi. It was conducted during October 2009 to December 2010 after obtaining approval from the Institute Ethics Committee. The study was registered with Clinical Trial Registry of India, (CTRI- 2009 000699). Single batches of *Sidh Makardhwaj* (Maharshi Ayurveda Pharmaceutical Pvt. Ltd., India), *Ashwagandha* and honey (Dabur, India) were procured for the entire study. Patients of either sex between the age group of 18 to 60 yr, who were diagnosed with rheumatoid arthritis by the American College of Rheumatology (ACR) 1987 criteria[18], able to provide written informed consent were included in the study. Exclusion criteria were medical history of unstable angina, myocardial infarction, heart failure or stroke within three months of the study, uncontrolled hypertension (diastolic blood pressure >100 mm Hg), uncontrolled diabetes mellitus, alanine and aspartate aminotransferases (ALT and AST) > 2 x upper limit of normal, impaired renal function (creatinine ≥2.0 mg/dl), pregnancy/lactation, or patients on any other Ayurvedic drugs during the last 15 days.

The first 125 patients with joint pain were screened in the OPD at CGHS Ayurvedic Hospital, Lodhi Road, New Delhi during the study period. Eighty six patients satisfied the inclusion criteria and were willing to participate in the study, signed the informed consent. Detailed medical history, general physical examination and rheumatologic evaluation were recorded by the designated Ayurvedic physician. Laboratory tests were carried out as per protocol. Subsequently, all patients were examined by the physician at every visit during the trial.

Medications: The patients took 5g of *Ashwagandha* powder twice a day for three weeks with lukewarm water or milk. *Sidh Makardhwaj* (100 mg) with honey was administered daily for the next four weeks. Records of dispensed drugs were maintained in drug inventory form. Concurrent analgesics/ NSAIDs in any form, oral, injectable or topical were not permitted. However, there was facility of rescue treatment with NSAIDs. Patients who received rescue medications were excluded from the study. Patients were allowed to continue with their ongoing lifestyle and diet.

Clinical assessment: The primary efficacy end point was the proportion of patients with a 20 per cent improvement as per ACR criteria (ACR20)[19] response at the end of treatment. ACR 20 is defined as 20 per cent improvement in tender joint counts, swollen joint counts and 20 per cent improvement in 3 of the 5 areas, *i.e.* physician global assessment score, patient global assessment score, pain assessment score, patient self assessed disability index score, ESR[18]. Secondary end points included ACR50 and ACR70 responses, change from baseline in the Disease Activity Score in 28 joints (DAS28), categorical analyses of DAS28/European League Against Rheumatism (EULAR) response[20], change from baseline in each of the ACR core set of parameters. Safety assessments included hepatic function [alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP) and bilirubin] and renal function (urea and creatinine) tests were analyzed separately using individual kit by semi auto analyzer (Mini techno, USA). Early sensitive marker of liver (β2 microglobulin) and kidney (NGAL: neutrophil gelatinase associated lipocalin) were reported as the instructions of the manufacturer of ELISA assay kits (Logitech India Pvt, Delhi, India) to evaluate the effect of the *Ashwagandha* and *Sidh Makardhwaj* treatment. Urinary mercury level was estimated by ICP-AES (Jobin Horiba, JY 2000-2, France)[21].

Sample size determination: The sample size was determined by the following specifications: *(i)* there would be an 80 per cent power for detecting a change from baseline in the DAS in 28 joints; *(ii)* 20 per cent improvement as per ACR criteria (ACR 20) response at fourth week; *(iii)* the test of the null hypothesis was conducted at a 2-sided 5 per cent significance level; *(iv)* acceptable margin of errow will be 5 per cent; and *(v)* population size of 200 and expected response of 10 per cent. Under these assumptions, the required sample size was 75 subjects. However, considering high dropout rate (20%) and lack of sufficient data from published rheumatoid arthritis drug trials using Ayurvedic medicines, it was then decided to enrol 90 patients.

Statistical analysis: Chi-square test was applied for patients showing improvement/cure after therapy. Laboratory measurements were compared with baseline using an analysis of variance.

Results

Efficacy evaluation of Ashwagandha and Sidh Makardhwaj: A total of 90.7 per cent (78/86) patients adhered to study protocol and completed seven weeks of treatment (three weeks of *Ashwagandha* powder followed by four weeks of *Sidh Makardhwaj)*. Eight patients prematurely discontinued due to lack of efficacy and refusal of continued treatment (2 = due to unknown reason, 2 = shifted to allopathic medication, 4 = concomitant use of other medication). There were 57.7 per cent (45/78) female and 42.3 per cent (33/78) male subjects and mean ages were 45.7 ± 8.6 (range 19-59) and 49.8 ± 7.9 (range, 25-59) yr, respectively. At baseline, majority of patients tested positive for rheumatoid factor (RF), its values in male and female patients were 31.2 ± 3.1 and 50.9 ± 16.1 IU/ml, respectively and post-treatment levels were 22.1 ± 1.4 and 41.7 ± 14.4 IU/ml. The levels of ESR (erythrocyte sedimentation rate) at baseline in male and female were 28.8 ± 3.3 and 43.8 ± 16.16.3 mm/h and post-treatment levels were 21.6 ± 1.9 and 35.4 ± 14.3 mm/h, respectively. The results showed significant decrease in post-treatment levels of ESR and RA factor as compared to baseline levels in male and female (Table I).

Seven weeks treatment effectiveness was assessed by *(i)* ACR 20 response; *(ii)* change in DAS28 (baseline DAS28 to 4 wk DAS28); *(iii)* non response, moderate and good response according to the EULAR response criteria; and *(iv)* the proportion of patients achieving disease remission (DAS28 < 2.6), according to the EULAR criteria.

There were significant changes in post-treatment scores of tender joint counts, swollen joint counts, physician global assessment score, patient global assessment score, pain assessment score, patient self assessed disability index score and ESR level as compared to baseline scores in male and female patients (Table I). ACR 20 response was observed in 48.5 per cent (16/33) male and 62.2 per cent (28/45) female patients.

In our study, DAS28 score in male and female patients at baseline were 5.01±0.36 and 5.12 ± 0.33, respectively and at post-treatment were 4.29 ± 0.21 and 4.28 ± 0.19 showing significant decrease in DAS28 score. DAS28 score of higher than 5.1 is indicative of high disease activity, whereas a DAS28 below 3.2 indicates low disease activity[18]. A total of 45 (57.7%) patients were in moderate disease activity with DAS28 score between 3.2 and 5.1. High disease activity was observed in 42.3 per cent (33/78) patients with DAS28 score >5.1.

In moderate disease activity, DAS28 improvement over the time in the range of 0.6-1.2 was observed in 55.6 per cent (25/45) patients and <0.6 improvement over the time in DAS28 was observed in 44.4 per

Table I. Clinical features in rheumatoid arthritis patients (male=33, female=45) on *Ashwagandha* and *Sidh Makardhwaj* treatment

Disease parameters	Sex	Baseline	Post-treatment
Tender joint counts	M	6.55 ± 1.27	4.82 ± 0.81***
	F	6.6 ± 1.21	4.75 ± 0.55***
Swollen joint counts	M	3.36 ± 1.73	2.45 ± 1.03***
	F	3.87 ± 1.80	2.73 ± 1.01***
Physician global assessment score	M	46.36 ± 7.83	33.64 ± 6.99***
	F	47.56 ± 8.02	6.0 ± 6.54***
Patient global assessment score	M	52.12 ± 11.11	35.15 ± 7.95***
	F	53.56 ± 11.52	34.44 ± 7.85***
Patient self assessed disability index score	M	3.3 ± 1.09	2.47 ± 0.91***
	F	3.32 ± 1.25	2.57 ± 0.91***
Pain assessment score	M	6.21 ± 0.66	4.41 ± 0.42***
	F	6.17 ± 0.72	4.44 ± 0.48***
Visual analogue scale (VAS) score	M	58.79 ± 8.20	41.21 ± 7.39***
	F	58.89 ± 8.85	40.44 ± 7.37***
ESR (mm/h)	M	28.8 ± 3.3	21.6 ± 1.9***
	F	31.2 ± 3.1	22.1 ± 1.4***
RA factor (Unit/ml)	M	43.8 ± 16.3	35.4 ± 14.3***
	F	50.9 ± 16.1	41.7 ± 14.4***
DAS activity	M	5.01 ± 0.36	4.29 ± 0.21***
	F	5.12 ± 0.33	4.28 ± 0.19***

Data are expressed as mean ± SD, *** $P<0.01$ as compared to baseline values.
ESR, erythrocyte sedimentation rate; DAS, disease activity score

cent (20/45) patients. In high disease activity, >1.2 improvement over the time in DAS28 was observed in 18.2 per cent (6/33) patients and DAS28 improvement over the time in the range of 0.6-1.2 was observed in 81.8 per cent (27/33) patients. In summary, *Sidh Makardhwaj* treatment for four weeks in rheumatoid arthritis patients showed moderated response in 39.74 per cent (31/78) patients.

Safety evaluation of Ashwagandha and Sidh Makardhwaj: There was no significant change in serum levels of ALT, AST, ALP, bilirubin, urea, creatinine, β2MG and NGAL at post-treatment as compared to baseline (Table II). Urinary mercury level increased from 6.9 ± 1.3 to 32.5 ± 2.4 and 8.2 ± 1.6 to 41.7 ± 3.1 µg/l in male and female patients, respectively. Mercury levels increased significantly after Ayurvedic treatment as compared to baseline level.

In our study, significant improvements were observed in patients' tender joint counts, swollen joint counts, ESRs, physician's rating of disease activity, physical function and pain. For the ACR20 response, there was a moderate improvement while ACR50 and ACR70 responses were not observed. In agreement with earlier reports[15], *Ashwagandha* and *Sidh Makardhwaj* were found to be effective and safe treatment for patients with RA. Seven weeks after starting Ayurvedic treatment, mean DAS28 scores significantly decreased in male and female patients. Only 39.74 per cent of the patients were EULAR responders (moderate). The results of the present study are in concordance with several studies on Ayurvedic treatment[22-28].

Sidh Makardhwaj has been used in the Indian System of Medicine with claimed efficacy and safety[29]. Ayurvedic physicians often avoid prescribing it as a medicine particularly for longer periods. But there has

Table II. Safety evaluation of liver and kidney of rheumatoid arthritis patients (male=33, female=45) on treatment with *Ashwagandha* and *Sidh Makardhwaj*

Biochemical parameters	Sex	Visit 1 (Day 0)	Visit 2 (Day 21)	Visit 3 (Day 35)	Visit 4 (Day 50)
ALT (IU/l)	M	28.07 ± 4.15	24.74 ± 3.07	26.47 ± 3.47	19.18 ± 3.98
	F	26.80 ± 4.81	24.39 ± 3.94	26.13 ± 2.72	28.76 ± 3.11
AST (IU/l)	M	28.28 ± 4.91	26.97 ± 4.22	28.49 ± 4.28	29.65 ± 4.33
	F	27.54 ± 4.29	26.07 ± 4.25	24.98 ± 4.42	29.09 ± 4.26
ALP (IU/l)	M	137.80 ± 18.26	135.85 ± 18.19	137.99 ± 17.83	139.64 ± 17.72
	F	143.62 ± 17.14	142.27 ± 17.32	144.71 ± 16.83	146.18 ± 16.36
Bilirubin (mg/dl)	M	0.78 ± 0.17	0.80 ± 0.15	0.83 ± 0.15	0.85 ± 0.15
	F	0.73 ± 0.16	0.75 ± 0.16	0.77 ± 0.16	0.80 ± 0.16
Urea (mg/dl)	M	27.46 ± 5.04	28.23 ± 4.92	28.91 ± 4.74	30.01 ± 5.21
	F	27.58 ± 5.77	28.37 ± 5.48	28.92 ± 5.41	29.69 ± 5.18
Creatinine (mg/dl)	M	0.79 ± 0.10	0.82 ± 0.10	0.84 ± 0.11	0.88 ± 0.10
	F	0.87 ± 0.09	0.83 ± 0.09	0.85 ± 0.03	0.88 ± 0.10
β2 Micro-globulin (µg/ml)	M	1.07 ± 0.41			1.12 ± 0.38
	F	1.05 ± 0.41			1.27 ± 0.38
NGAL (ng/ml)	M	35.16 ± 8.5			35.79 ± 8.40
	F	35.52 ± 9.53			36.09 ± 8.90

Data are expressed as mean ± SD.
NGAL, neutrophil gelatinase-associated lipocalin; ALT, alanine aminotransferase; AST, aspartate aminotransferase; ALP, alkaline phosphatase

always been ambiguity in use of this mercury sulphide containing Ayurvedic medicine and often associated with the question of toxicity. The US Environmental Protection Agency (EPA) has adopted a reference dose (RfD) for methyl mercury of 0.1 µg/kg body weight/day[30]. The total mercury content of *Sidh Makardhwaj* formulation used in the present study was 35454.2 µg/g. The calculated total ingested mercury per day was 3545.4 µg *(Sidh Makardhwaj* dose = 100 mg per day). Thus, in its therapeutic dose, the per day ingested mercury was many fold higher than the reference dose. It was observed that with this high concentration of mercury in *Sidh Makardhwaj* given for 28 days did not cause significant change in liver and kidney functions of the patients. Urinary mercury levels were significantly increased after Ayurvedic treatment. Hence, mercury present in *Sidh Makardhwaj* was slowly eliminated from the body.

Lande in 1927 used aurothioglucose for the first time in the treatment of RA[31]. In another study, Forestier in 1935[32] used gold thiopropanol sodium sulphanate in 550 cases of RA with beneficial results. Gold containing drugs comprise a class of distinctive anti-arthritic agents (DMARDs) used when NSAIDs are insufficient to treat severe cases of rheumatoid or psoriatic arthritis. The reported remissions of rheumatoid arthritis was of the order of 30 per cent with gold therapy[33]. Gold content in *Sidh Makardhwaj* formulation used in the present study was 29.1 mg/g. The calculated total ingested gold per day was 2.91 mg *(Sidh Makardhwaj* dose = 100 mg per day). Thus, the per day ingested gold in *Sidh Makardhwaj* was in therapeutic dose.

There were a few limitations of our study. This was a seven week study with a sample size of 78 patients carried out in a single Ayurvedic hospital at New Delhi. Randomization was not done. Also, there was a lack of standard therapy group as a control group due to ethical issues.

In conclusion, our findings demonstrated the potential efficacy and safety of *Ashwagandha* (3 wk) and *Sidh Makardhwaj* (4 wk) in the treatment of RA. *Ashwagandha* and *Sidh Makardhwaj* should be further tested in long term randomized placebo controlled trial to establish its clinical use.

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Pharmacological Study

Evidence for safety of Ayurvedic herbal, herbo-metallic and *Bhasma* preparations on neurobehavioral activity and oxidative stress in rats

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Abstract

Heavy metals in Ayurvedic formulations have been used for centuries with claimed efficacy and safety. However, concerns are often raised about the toxicity due to heavy metals used in Ayurvedic formulations. The aim of present study is to explore the effect of Calcury tablet, Energic-31 capsule and *Basanta Kusumakara Rasa* (BKR) on neurobehavioral activity and oxidative stress in rats. Male wistar rats weighing 200-250 g were used and divided into normal control, positive control (mercury chloride, lead acetate, cadmium chloride, sodium arsenite, each 10 mg/kg, p.o for 28 days) and treated group (Calcury tablets at doses of 130, 650, 1300 mg/kg, Energic-31 capsule at doses of 150, 750, 1500 mg/kg and BKR at doses of 26, 130, 260 mg/kg, p.o. for 28 days). After performing the behavioural parameters on the 29th day, homogenate of rat's brain was used to determine malondialdehyde (MDA) and glutathione (GSH) levels and heavy metal level in brain. Results showed that there were no significant change in cognitive function, motor coordination, MDA and GSH levels as compared to normal control group at all doses of Calcury tablet, Energic-31 capsule and *Basant Kusumkar Rasa*. However, heavy metals level in rat's brain was higher as compared to normal control group at all doses of Calcury tablet, Energic-31 capsule and BKR. In conclusion, Calcury tablet, Energic-31 capsule and BKR in doses equivalent to the human dose does not have appreciable adverse effects on brain which demonstrates the non-toxic nature of metal based Ayurvedic formulations.

Key words: Ayurvedic formulations, neurobehavioral activity, oxidative stress, heavy metals

Introduction

In India, it has been estimated that about 14% sick persons utilizes Indian system of medicine. On the basis of preference, 18.7% population uses Ayurveda for normal ailments, 7.1% in case of sickness and 5% in case of serious ailments.[1] A report by the World Health Organization (WHO) indicates that many people in developing countries still rely on herbal medicine.[2] Majority of people believe that herbal medicines are safe and nontoxic, unlike modern chemotherapeutic agents. Individuals generally use herbal medicine for prolonged periods to achieve a desirable effect. On contrarily, it has been reported that herbal drugs used in the Indian subcontinent and China contain higher concentration of heavy metals than in other areas.[3-5] Another study showed that one of five Ayurvedic herbal medical products, produced in South Asia contains high levels of lead, mercury, and arsenic.[6,7] However, heavy metals are integral to some formulations and are been used for centuries.[8] Ayurvedic formulations are produced after different processes like detoxification, trituration and heating etc., of raw material. Therefore, elements present in finished products do not produce toxicity. Ayurvedic textbooks takes note of the toxicity of heavy metals and recommend special pharmacological process to detoxify them. Those metals which are obtained from ores may contain several impurities. These impurities are removed by *Shodhana* process. The *Shodhana* process removes unwanted part from the raw material and separate out impurities. In context of *Bhasma*, *Shodhana* means purifying and making the product suitable for the next step i.e. *Marana*. Ayurveda classifies *Shodhana* into (a) general process and (b) specific process. In general process for *Shodhana*, the sheets of metals are heated till red hot and are successively dipped into liquids like oil, buttermilk, cow's urine etc., and the procedure is repeated 7 times. In specific process

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for *Shodhana*, for some metals a specific process is described for *Shodhana* e.g. for purification of *Jasada*, the molten mass is poured in cow's milk for 21 times.[9] Ayurvedic text books emphasize the role of heavy metals in the proper function of the human body. In *Rasa Shastra*, the metals and the minerals are also termed as "*Dhatus*" and "*Updhatus*" because of their specific role in biological systems i.e. they can sustain body tissues by supplementing some of the essential elements to the tissues, whose deficiency causes many undesired problems or disease in the body. The available Ayurvedic literature emphasis the need of metals in maintaining the metabolic equilibrium of the human body. These metals are mercury, gold, silver, copper, iron, tin, lead, zinc etc., Any deficiency, excess or imbalance in the composition of these metals leads to certain metabolic and anabolic disorders. Equilibrium state of metals in the human body provides the basis for strong immunity.[10] Therefore, any imbalance in the composition of these metals could cause diseases and equilibrium of these metals is seen as a preconditioning for a normal immune defense and general health. Therefore, heavy metals from outside are deliberately added and processed with herbal plants to form herbo-metallic drugs.[11] Each time before burning, the metallic powders are processed with fresh herb juices to neutralize their toxicity. Some of the metals are burnt up to 100 times to make sure the heaviness or toxic effect of the metal is nullified. Once the "*Bhasma*" is ready it is tested for toxicity. One of the numerous tests the *Bhasma* has to pass through is called "*Varitar*" which means the *Bhasma*, once ready for internal use, floats on water indicating non-existence of heavy metal in it. The "*Bhasma*" are then transformed to compound formulas by mixing herbal powders. Special herbal juices are used for processing the compound formula for no more toxic metals but for non-toxic herbo-metallic compounds. Therefore, it is claimed that heavy metals are detoxified with herbal extract and excreted out from the body without any harm to body.[12] *Bhasmas* are metal preparations which are subjected to physico-chemical processing called *Samskaras* to purify, detoxify and retain the therapeutic properties. Ayurvedic experts have estimated that 35-40% of the approximately 600 medicines in the Ayurvedic formulary, intentionally contain at least one metal.[13] On the other hand, there are certain plant species, which has high affinity to absorb certain traces of metals from the soil. There are more than 60 plant species which has a natural tendency to absorb traces of metals from the soil which could be used as a natural ingredient and may be important for therapeutic efficacy. Here, trace metal might be working as an active ingredient in the plant material[14] and the possibility of presence of heavy metals in herbal, herbo-metallic and *Bhasma* are unavoidable. Hence, the aim of present study was to evaluate the effect of chronic administration of Calcury tablet (herbal), Energic-31 capsule (herbo-metallic) and *Basanta Kusumakara Rasa* (BKR) on neurobehavioral activity and oxidative stress in rat.

Materials and Methods

Experimental animals

Male wistar rats weighing 200-250 g were used in the present study. Rats were randomly divided into 14 groups with 6 rats in each group. The rats were obtained from the Central Animal House Facility of All India Institute of Medical Sciences, New Delhi and housed in the departmental animal house. The rats were group housed in polyacrylic cages (38 × 23 × 10 cm) with not more than 4 animals per cage and maintained under standard laboratory conditions with natural dark and light cycle. They were allowed free access to standard dry rat diet (Ashirwad, Punjab, India) and tap water *ad-libitum*. However, 12 h before the behavioral test, the rats were deprived of food as this is known to enhance their motivation to perform the test.

Permission of institutional animal ethics committee

The protocol of the work mentioning details of the experimental technique, justification of the use of animals, number of animals to be used, type of anesthesia, surgical procedure to be used were reviewed and approved by the Institutional Animal Ethics Committee, All India Institute of Medical Sciences, New Delhi India (497/IAEC/09).

Drugs preparation, dose and duration of treatment

Calcury tablet (herbal), Energic-31 capsule (herbo-metallic) and BKR were purchased from market (New Delhi, India) and suspended in normal saline. Mercury chloride, lead acetate, cadmium chloride, sodium arsenite were purchased from Merck (USA) and dissolved in normal saline. These three Ayurvedic formulations have been selected on the basis of preliminary study in which seventy eight drugs were analyzed for heavy metals content. These Ayurvedic formulations contained heavy metals above permissible limit. Hence, these formulations were considered for toxicological evaluation in rats.

Each tablet of Calcury contains *Saxifraga ligulata* (150 mg), *Saccharum officinarium* (75 mg), *Boerhaavia diffusa* (75 mg), *Hazarat Yahud Pishti* (37.5 mg), *Yava Kshara* (15 mg). Extracts derived from *Parmelia perlata* (150 mg), *Crataeva nurvala* (150 mg), *Tribulus terrestris* (75 mg), *Picrorrhiza kurroa* (75 mg), *Tinospora cordifolia* (75 mg) and preservatives used were sodium Methlparaben and Sodium Propylparaben. The drug manufacturer was Charak Pharmaceutical Pvt.(I) Limited (Batch no. CA177H). Weight of each tablet was 625 mg. Human dose indicated on package insert was one tablet twice a day.

Energic-31 capsule contains *Shudha Shilajita* (450 mg), *Shankha Bhasma* (10 mg), *Tribang Bhasma* (30 mg), *Shudha Kupeelu* (50 mg), *Kukkutandtwak Bhasma* (20 mg), *Muktashukti Bhasma* (10 mg), *Swarnamakshik Bhasma* (10 mg), *Shatavari* (20 mg), *Konch ke beez* (10 mg), *Asgandh* (20 mg), *Dalcheeni* (10 mg), *Nagkesar* (10 mg), *Gokhru* (10 mg), *Sonth* (10 mg), *Loh Bhasma* (10 mg), *Lodh Pathani* (10 mg), *Chhoti Ilaichi* (10 mg), *Jabitri* (10 mg), *Suranjan Meetha* (10 mg), *Bidhara* (10 mg), *Jaiphal* (10 mg), *Moosli Safed* (10 mg), *Samundra Sosh* (10 mg), *Long* (10 mg), *Babool ka Gond* (10 g), *Talamkhana* (10 mg), *Chhoti Papal* (10 mg), *Kali Mirch* (10 mg), *Safed Chandan* (10 mg), *Akarkara* (10 mg) and *Konkol Mirch* (10 mg). Weight of each capsule content was 725 mg. The drug manufacturer was Ayurved Vikas Sansthan (Batch no. 997). Human dose indicated on package was one capsule twice a day.

Basanta Kusumakara Rasa (BKR) consists of *Prawal Bhasma*, *Chandrodaya* or *Ras Sindoor*, *Moti Pishti*, *Abhrak Bhasma*, *Raupya Bhasma*, *Suvarna Bhasma*, *Shatavari*, *Adulasa Swarasa*, *Ganna*, *Kamal Ke Phool*, *Mahuti Ke Phool*, *Kadali-Kanda*, *Malati Phool*,

Chandan, and *Kasturi*. The source of composition is Siddhayoga Sangraha. The drug manufacturer was Baidyanath (Batch no. 10). And the human dose is 125 mg/day.[15]

Animal dose were calculated from human dose per day according to the method followed by Center for Drug Evaluation and Research, Food and Drug Administration (USA), 2005.[16] Three dose of each drug (Calcury tablet, Energic-31 capsule, BKR) were selected for toxicological study according to Schedule Y of Drugs and Cosmetics Acts, 2005.[17] Three doses were human equivalent Therapeutic Dose (TD), 5 times of human equivalent Therapeutic Dose (5TD) and 10 times of human equivalent Therapeutic Dose (10TD).

Animals dose for Calcury tablets were 130, 650, 1300 mg/kg, for Energic-31 capsule were 150, 750, 1500 mg/kg and for BKR were 26, 130, 260 mg/kg. All the solutions were prepared in such a way that each animal was administered solution less than 1 ml. Solutions for Calcury tablet were 50, 200, 300 mg/ml, for Energic-31 capsule were 50, 200, 400 mg/ml and for BKR were 10, 50, 100 mg/ml. All the drug solutions were administered orally to rats for 28 days. The doses and concentration for mercury chloride, lead acetate, cadmium chloride, sodium arsenite was 10 mg/kg/day and 5 mg/ml were administered orally to rat for 28 days.[18]

Experimental design

On day 1st (Baseline, pre-treatment) and on 29th day (post-treatment) neurobehavioral activity was assessed by elevated plus maze, foot fault apparatus, photoactometer, rota rod and passive avoidance apparatus. Animals were decapitated under anaesthesia after neurobehavioral activity test. Brain was removed and washed with ice-cold normal saline and stored at −70°C. Brain tissue was thawed and homogenized with 10 times (w/v) ice cold 0.1M phosphate buffer (pH-7.4). Aliquots of homogenate from rat's brain were used to determine glutathione (GSH), MDA level and heavy metal concentration.

Behavioral tests

Cognitive impairment was evaluated by using passive avoidance and elevated plus maze.[19] The motor incoordination was tested by using rota rod and photoactometer.[20] Only one animal was tested at a time.

Estimation of biochemical markers of oxidative stress

The oxidative stress markers, malondialdehyde (MDA) and reduced GSH levels were estimated in whole brain tissue of rats. The rats were anaesthetized under chloroform anesthesia to decapitate and the brains were quickly removed, cleaned by rinsing with chilled saline and stored at −70°C. The biochemical analysis was performed within 48 h.

Measurement of lipid peroxidation

Malondialdehyde (indicator of lipid peroxidation) was estimated by - Brain tissues were homogenized with 10 times (w/v) 0.1M sodium phosphate buffer (pH 7.4). The reagents acetic acid 1.5 ml (20%, v/v) pH 3.5, 1.5 ml thiobarbituric acid (0.8%, w/v) and 0.2 ml sodium dodecyl sulfate (8.1%, w/v) were added to 0.1 ml of processed tissue sample. The mixture was then kept in boiling water for 60 min. The mixture was then cooled with tap water and 5 ml of n-butanol: pyridine (15:1, v/v) and 1 ml of distilled water were added to it. Then the mixture was vortexed and centrifuged at 4000 rpm for 10 min. The organic layer was withdrawn and absorbance was measured at 532 nm using a spectrophotometer (specord 2000, Analytik Jena, Germany). The concentration of MDA was determined by the linear standard curve.[21]

Estimation of glutathione

Reduced GSH was measured by - Equal quantity of homogenate was mixed with 10% trichlororacetic acid and centrifuged to separate the proteins. To 0.1 ml of this supernatant, 2 ml of 0.3 M phosphate buffer (pH 8.4), 0.5 ml of 5'5-dithiobis (2-nitrobenzoic acid) and 0.4 ml of double distilled water were added. The mixture was vortexed and the absorbance was read at 412 nm within 15 min.[22]

Estimation of heavy metals

Lead, cadmium, mercury and arsenic levels were estimated in brain tissue by inductively coupled plasma –atomic emission spectrophotometer (ICP-AES, JY 2000-2, France). Brain tissues were digested by cold vapors digestion procedure. Metal levels were expressed in $\mu g/g$-wet tissue.[23,24]

Statistical analysis

All datas are expressed as mean ± SEM. Drugs treated groups were compared to normal control and positive control group using one way ANOVA with posthoc Tukey test. Difference with a $P < 0.05$ was accepted as statistically significant. All the statistical analyses were performed using software (SPSS, version 15).

Observations and Results

Effect of chronic administration of Ayurvedic formulations on learning and memory in rats

One trial passive avoidance

There was significant decrease in mean retention latencies of mercury, lead, cadmium and arsenic treated group as compared to normal control group ($P < 0.001$). However, there was no significant change in mean retention latencies of Calcury (TD, 5TD, 10TD), Energic-31 (TD, 5TD, 10TD) and BKR (TD, 5TD, 10TD) treated group as compared to normal control group ($P > 0.05$) [Table 1].

Elevated plus maze

There was significant increase in mean retention transfer latencies of mercury, lead, cadmium and arsenic treated group as compared to normal control group ($P < 0.001$). On contrary, there was no significant change in mean transfer retention latencies of Calcury (TD, 5TD, 10TD), Energic-31 (TD, 5TD, 10TD) and BKR (TD, 5TD, 10TD) treated group as compared to normal control group ($P > 0.05$) [Table 1].

Effect of chronic administration of Ayurvedic formulations on locomotor activity in rats

Photoactometer (spontaneous locomotor activity)

There was significant decrease in spontaneous locomotors activity of mercury, lead, cadmium and arsenic treated group as compared to normal control group ($P < 0.001$). However, there was no significant change in spontaneous locomotors activity of

Calcury (TD, 5TD, 10TD), Energic-31 (TD, 5TD, 10TD) and BKR (TD, 5TD, 10TD) treated group as compared to normal control group ($P > 0.05$) [Table 1].

Rota rod

There was significant decrease of retention time on rod of mercury, lead, cadmium and arsenic treated group as compared to normal control group ($P < 0.001$). However, there was no significant change in retention time on rod of Calcury (TD, 5TD, 10TD), Energic-31 (TD, 5TD, 10TD) and BKR (TD, 5TD, 10TD) treated group as compared to normal control group ($P > 0.05$) [Table 1].

Effect of chronic administration of Ayurvedic formulations on oxidative stress in rats

Glutathione estimation

There was significant decrease of GSH level in brain of mercury, lead, cadmium and arsenic treated group as compared to normal control group ($P < 0.001$). However, there was no significant change in GSH level in brain of Calcury (TD, 5TD, 10TD), Energic-31 (TD, 5TD, 10TD) and BKR (TD, 5TD, 10TD) treated group as compared to normal control group ($P > 0.05$) [Figure 1].

Malondialdehyde estimation

There was significant increase of MDA level in brain of mercury, lead, cadmium and arsenic treated group as compared to normal control group ($P < 0.001$). However, there was no significant change in MDA level in brain of Calcury (TD, 5TD, 10TD), Energic-31 (TD, 5TD, 10TD) and BKR (TD, 5TD, 10TD) treated group as compared to normal control group ($P > 0.05$) [Figure 1].

Effect of chronic administration of Ayurvedic formulations on rat's brain heavy metals

Mercury estimation

There was significant increase of mercury concentration in brain

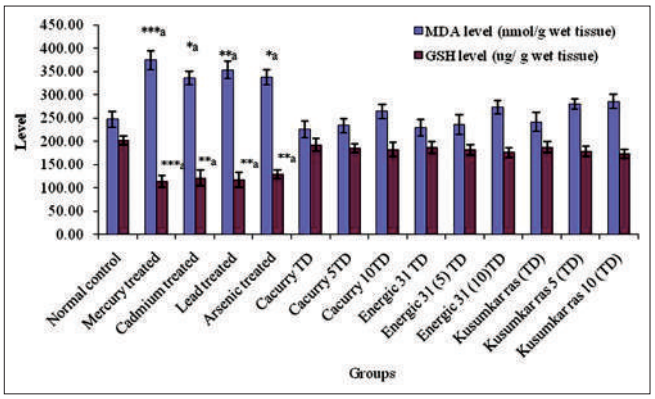

Figure 1: Effect of Ayurvedic formulations on brain malondialdehyde and glutathione levels in rats *$P<0.05$, **$P<0.01$, ***$P<0.001$, [a]As compared to normal control

Table 1: Effect of Ayurvedic formulations on learning and memory and locomotor activity

Experimental groups	Passive avoidance		Elevated plus maze		Rota rod		Photoactometer (counts/10 min)	
	ITL (s)	RTL (s)	IL (s)	RL (s)	IL (s)	RL (s)	Counts pre-treatment	Counts post-treatment
Normal control	44.52±7.57	203.33±37.22	36.3±11.12	10.5±2.3	144±19.48	156.5±10	206.75±21.06	204.25±25.6
Mercury treated	40.8±5.42	68.48±4.23***[a]	25.5±5.62	52.5±4.6***[a]	127±15.44	94±26.8*[a]	218.5±50	119.3±39.89*
Cadmium treated	36.4±7.39	119.53±6.08***[a]	26.8±7.74	41.2±6.4**[a]	144.7±19.11	109.5±22.3*[a]	238.5±34.5	123±28.79*
Lead treated	38.9±6.2	93.6±2.23**[a]	33.2±4.2	44.5±4.9**[a]	129.7±7.28	101.8±9.8*[a]	234.7±32.61	117±14*
Arsenic treated	27.4±2.57	105.02±7.49**[a]	28.2±6.38	46.0±3.2**[a]	137±16.96	107±6.4*[a]	205±18.45	132.2±5.34*
Calcury (TD)	45.95±10.51	198.62±13.22	29.33±4.9	14.33±2.74	181±21.27	145.33±15.02	216±11.37	248.17±9
Calcury (5TD)	43.25±7.34	180.1±19.98	32.33±5.4	17.33±5.45	196.17±25.81	142.5±19.71	227±14.16	228.67±13.45
Calcury (10TD)	34.12±11.65	169.68±21.1	33.0±6.4	14.67±4.13	128±31.19	146.83±24.51	246±10.76	256.33±7.7
Energic-31 (TD)	27.98±3.67	185.12±17.57	31.8±4.37	14.33±8.03	110.83±27.74	157.17±21.46	208±9.5	226.33±15.77
Energic-31 (5TD)	38.33±10.61	186.5±20.39	32.7±2.14	16.33±8.64	122.83±9.36	133.83±14.05	204.8±16.18	223.83±15.72
Energic-31 (10TD)	27.6±7.47	177.53±14.5	34.6±6.34	18.67±5.48	125.5±15.33	140±15.82	238.83±7.48	218.5±16.04
BKR (TD)	35.42±7.4	195.83±23.74	37.83±7.32	15.33±3.15	118.67±18.49	132.67±12.23	223.83±10.46	195.33±23.35
BKR (5TD)	42.17±9.32	166.57±31.7	32.83±8.37	15.17±6.24	165±9.28	168.33±12.45	257.5±9.59	192.17±17.47
BKR (10TD)	31.53±3.64	185.8±24.9	32.17±5.1	18.33±4.54	140.83±13.13	118.17±15.21	248±11.21	194.5±5.37

IL: Initial latency, RL: Retention latency, *$P<0.05$; **$P<0.01$; ***$P<0.001$; [a]As compared to normal control, ITL: Initial transfer latency, RTL: Retention transfer latency, TD: Therapeutic dose, BKR: *Basanta Kusumakara Rasa*

of mercury treated groups (203.70 ± 5.15 μg/g) as compared to normal control group (2.01 ± 0.18 μg/g) ($P < 0.001$). However, there was also significant increase of mean mercury concentration level in brain with increasing doses of Calcury (TD, 5TD, 10TD), Energic-31 (TD, 5TD, 10TD) and BKR (TD, 5TD, 10TD) treated group as compared to normal control group ($P < 0.001$) but on contrary, the mercury in brain of Calcury (TD, 5TD, 10TD), Energic-31 (TD, 5TD, 10TD) and BKR (TD, 5TD, 10TD) treated group was significantly less as compared to mercury treated group ($P < 0.001$) [Table 2].

Lead estimation

There was significant increase of lead concentration in brain of lead treated groups (421.90 ± 6.5 μg/g) as compared to normal control group (1.6 ± 1.5 μg/g) ($P < 0.001$). However, there was also significant increase of mean lead concentration in brain with increasing doses of Calcury (TD, 5TD, 10TD), Energic-31 (TD, 5TD, 10TD) and BKR (TD, 5TD, 10TD) treated group as compared to normal control group ($P < 0.001$) but on contrary, the lead in brain of Calcury (TD, 5TD, 10TD), Energic-31 (TD, 5TD, 10TD) and BKR (TD, 5TD, 10TD) treated group was significantly less as compared to lead treated group [Table 2].

Cadmium estimation

There was significant increase of cadmium concentration in brain of cadmium treated groups (289.19 ± 5.35 μg/g) as compared to normal control group (0.44 ± 0.08 μg/g) ($P < 0.001$). However, there was also significant increase of mean cadmium concentration in brain with increasing doses of Calcury (TD, 5TD, 10TD), Energic-31 (TD, 5TD, 10TD) and BKR (TD, 5TD, 10TD) treated group as compared to normal control group ($P < 0.001$) but on contrary, the cadmium in brain of Calcury (TD, 5TD, 10TD), Energic-31 (TD, 5TD, 10TD) and BKR (TD, 5TD, 10TD) treated group was significantly less as compared to cadmium treated group [Table 2].

Arsenic estimation

There was significant increase of arsenic concentration in brain of arsenic treated groups (88.4 ± 4.9 μg/g) as compared to normal control group (0.25 ± 0.05 μg/g) ($P < 0.001$). However, there was also significant increase of mean arsenic concentration in brain with increasing doses of Calcury (TD, 5TD, 10TD), Energic-31 (TD, 5TD, 10TD) and Kusumkar ras (TD, 5TD, 10TD) treated group as compared to normal control group ($P < 0.001$) but on contrary, the arsenic in brain of Calcury (TD, 5TD, 10TD), Energic-31 (TD, 5TD, 10TD) and BKR (TD, 5TD, 10TD) treated group was significantly less as compared to arsenic treated group [Table 2].

Discussion

Ayurveda is a widely practiced system in India and the used formulations are herbal, herbo-metallic and *Bhasma*. Ayurvedic text books emphasize the role of metals in proper functioning of the human body. Therefore, metals are inevitable part deliberately added and processed with herbal plants to form herbo-metallic drugs.[11,25] Ayurvedic experts have estimated that 35-40% of the approximately 600 medicines in the Ayurvedic formulary intentionally contain at least one metal.[13] *Bhasma* contains only metals which are detoxified during processing (*Shodhana*). So possibility of presence of heavy metals in herbo-metallic and *Bhasma* are unavoidable. Therefore, objective of our study was the determination of effect of chronic administration of Ayurvedic formulations on neurobehavioral activity and oxidative stress in rats.

The case reports suggests that lead content is a bigger problem with Indian HMPs and poisoning due to heavy metals have been regularly reported in the last three decades.[26-28] The mercury poisoning occurs by deposition in human cortical neuron and in a scattered group of neurons in the brain stem and cerebellum by generation of free radicals, release of intracellular calcium, lysozomal enzyme or cytoskeleton disorganization.[29] Neurological deficits due to organic mercury exposure includes encephalopathy with persistent neurological disabilities while inorganic mercury exposure produce polyneuropathy and tremor and further results decrease in visual acquity, ataxic gait and involuntary jerk movements. Most of the cognitive and emotional problems have been found in patients exposed to inorganic or organic mercury.[30]

Table 2: Effect of Ayurvedic formulations on brain heavy metal levels

Experimental group	Mercury (μg/g)	Lead (μg/g)	Cadmium (μg/g)	Arsenic (μg/g)
Normal control	2.01±0.18	1.6±1.5	0.44±0.08	0.25±0.05
Mercury treated	203.70±5.15***[a]	4.02±0.6	0.59±0.05	0.33±0.02
Cadmium treated	3.59±0.25	3.87±0.4	289.19±5.35***[a]	0.19±0.03
Lead treated	3.04±0.38	421.9±6.5***[a]	0.52±0.18	0.32±0.08
Arsenic treated	3.61±0.1	3.7±3.7	0.61±0.9	88.4±4.9***[a]
Calcury (TD)	6.08±1.31	4.9±1.4	0.48±0.06	0.38±0.16
Calcury (5TD)	6.80±1.1	5.1±1.7	0.58±0.11	0.42±0.12
Calcury (10TD)	7.61±1.14	5.9±0.3	0.61±0.14	0.46±0.04
Energic-31 (TD)	12.14±1.26	17.6±3.5	0.81±0.16	0.34±0.11
Energic-31 (5TD)	12.31±1.35	23.9±2.5	0.84±0.15	0.37±0.05
Energic-31 (10TD)	14.38±2.08	28.8±3.1	0.88±0.28	0.38±0.08
BKR (TD)	24.79±1.80	8.7±3.4	0.69±0.10	0.29±0.11
BKR (5TD)	27.04±1.30	9.7±3.6	0.74±0.22	0.34±0.07
BKR (10TD)	31.29±1.09	9.8±2.5	0.89±0.27	0.36±0.05

***$P<0.001$; [a]As compared to normal control, BKR: *Basanta Kusumakara Rasa*, TD: Therapeutic dose

On contrary, our study shows that the animals treated with Calcury (TD, 5TD, 10TD), Energic-31 (TD, 5TD, 10TD) and BKR (TD, 5TD, 10TD) containing heavy metals does not cause cognition impairment and motor incoordination while positive control group in which mercury, lead, cadmium and arsenic salts were given orally to rats for 28 days caused cognition impairment and motor incoordination. The oxidative stress markers like MDA and GSH level has not been altered as compared to normal control group while there was significant decrease in GSH level and increase in MDA level in brain of positive control in which mercury, lead, cadmium and arsenic salts were given orally to rats for 28 days. We observed the higher level of heavy metal concentration in rat's brain of positive control, Calcury (TD, 5TD, 10TD), Energic-31 (TD, 5TD, 10TD) and BKR (TD, 5TD, 10TD) treated group as compared to normal control group. However, mercury, lead, cadmium and arsenic level in brain of Calcury (TD, 5TD, 10TD), Energic-31 (TD, 5TD, 10TD) and BKR (TD, 5TD, 10TD) treated group were significantly very less as compared to positive control in which mercury, lead, cadmium and arsenic salts treated group. However, no sign and symptoms, toxic manifestations were observed in these groups. Possibility of raised heavy metals level in brain could be[1] low heavy metals level exposure to rats[2] heavy metal levels were estimated immediately after 28 days administration of drugs[3] heavy metals has longer half life and[4] elimination from the body is slow. Hence, rats treated with Ayurvedic formulations showed raised heavy metal levels in brain.

The results of present study showed that herbo-metallic formulation and *Bhasma* are non-toxic even though these drugs contain metals. The reason for nontoxic nature of herbo-metallic formulation and *Bhasma* in animal could be,[1] metals in Ayurvedic formulation are not present in elemental form.[2] Physico-chemical state of the heavy metals in the form of Ayurvedic medicine is totally different from the known Physico-chemical forms of that metal.[12,13,28,31]

Heavy metal preparations (*Bhasma*) have been used in Indian System of Medicine for centuries with claimed efficacy and safety. Processed mercury shows excellent therapeutic activities in low doses without producing toxic effect in the human subjects. The toxic effects are due to impure mercury or improper use of processed mercury. The complication and toxic effects of metals has already been mentioned in Ayurveda.[8,31] However, Ayurvedic literature also mentions that metals are subjected to *Samskaras* which attributes to purification, detoxification and restoration of its therapeutic properties.[32]

Conclusion

The results of present study are coherent with the Ayurvedic literature. There were no significant changes in cognitive and motor functions and biochemical parameters of Calcury, Energic-31 and *Basanta Kusumkara Rasa* treated rats, demonstrates the safety of Ayurvedic formulations. These drugs are clinically used by a large number of populations without showing heavy metals toxicity. Hence, Calcury, Energic-31 and *Basanta Kusumkara Rasa* can be used at recommended dose and duration.

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Is mercury really toxic? The way forward for its judicious medicinal applications based on the therapeutic doctrines of Ayurveda

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Mercury-based formulations have been extensively used in Indian and Chinese systems of medicine with high therapeutic index. However, at present mercury is considered as a global pollutant having potent neurotoxic effects. Also, it has been proposed to ban the export, import and manufacture of a range of products containing mercury from 2021. This situation compels one to ponder how mercury-based formulations were safe during ancient times, but are now considered to be toxic. Naturally mercury occurs in three forms, viz. elemental (Hg), inorganic (HgS, $HgCl_2$) and organic (MeHg). Organic mercury is completely absorbed by gastrointestinal tract, and elemental mercury is 75–80% absorbed by the lungs only in vapour state, while 10% of inorganic mercury is absorbed by the gastrointestinal tract. Additionally, organic mercury crosses the blood brain barrier and placenta, producing neurotoxic symptoms and foetal toxicity. In Ayurvedic science, mercury is converted only into inorganic form with special stabilization technology and this stabilized mercury is converted to mercuric polysulphides under gastrointestinal conditions, whereas elemental mercury is converted to organic form by specific bacteria. Thus mercury available in the atmosphere is converted into organic form that is highly toxic, while using Ayurvedic methods it is converted to inorganic form having potent therapeutic activity. Judicious and injudicious application of mercury has advantageous and disadvantageous effect respectively.

Keywords: Ayurveda, mercury, neurotoxic effects, pharmacokinetic profile, stabilization.

MERCURY is a hazardous metal causing serious health problems[1], especially in children and during embryonic development[2]. A study showed that more than 2265 individuals had died and infants were born with severe developmental disabilities due consumption of fish contaminated by mercury[3]. The United States Environmental Protection Agency reported that mercury is a potent neurotoxic that negatively impacts human health and environment around the world[4]. The agency has also started mercury policy project (MPP) to eliminate its use, reduce its export and trafficking, and significantly reduce mercury exposure at the local, national and international levels[5]. The European Commission estimates current mercury demand at 260–400 tonnes/year and projects that it will be reduced up to 40–220 tonnes/year in 2025–2030. It is proposed to ban the use of new products containing mercury in manufacturing processes from 2018, restricted use in certain manufacturing processes in dental amalgam from 2019, and ban the export, import and manufacturing of a range of products containing mercury from 2021 (ref. 6).

Ancient Ayurvedic scholars were attracted to mercury for its positive characters; they were also aware of its negative impacts (toxicological profile)[7]. In *Samhita* period (800 BC–200 AD) mercury (or its compounds) was rarely used for the benefit of mankind, in spite of its therapeutic value. However, with the development of *Rasa Shastra* procedures like *Shodhan*, *Maran*, etc. it was frequently used for therapeutics. In the medieval period, mercury (or its compounds) was used for the treatment of common disorders such as *jwara* (fever), *kasa* (cough), etc. to critical situation. It is also used in the Chinese system of medicine for therapeutic purposes[8].

The herbomineral compound containing silver, mercury, sulphur and arsenic trisulphide has been used for the treatment of leukaemia, without any side effects[9–11]. After critical analysis of ancient and contemporary document on mercury, there is a state of dilemma that mercury is blessing for mankind or not, so a hypothesis is assumed that mercury is boon for human.

Materials and methods

To find out if mercury is a boon or bane, we searched various domains of knowledge such as Ayurvedic science, contemporary medical science, environmental science, etc. along with World Health Organization (WHO) documents, reputed national/international publications and other available resources. After obtaining data we critically analysed mercury and its compounds and their potential role, both positive and negative.

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Table 1. Pharmacokinetic profile of mercury

Mercury	Elemental (metallic)	Inorganic (mercuric chloride)	Organic (methyl, ethyl)
Sources of exposure	Environmental: volcanic explosions, weathering of rocks, degassing; anthropogenic inadvertent combustion: fossil fuels (coal), waste incineration; industrial gold/silver mining, batteries, switches, fluorescent lights, thermometers, sphygmomanometers; anthropogenic intentional; dental amalgams; ritual and folk medicine.	Environmental none; industrial products disinfectants, antimicrobials alternative; medicines, cosmetics vapour lamps, embalming photography latex paint (pre-1990s). Example: mercuric chloride.	Environmental conversion fish and shellfish (e.g. methyl mercury); Industrial production fungicides, bactericides (e.g. phenyl mercury); vaccine preservatives (e.g. thiomersal).
Routes of exposure	Inhalation (volatile at room temp): 75–85% absorption; ingestion and skin: almost no absorption.	Ingestion: 10% absorbed; skin: can be high and fatal.	Gastrointestinal: rapid and complete absorption; parenteral: 100% absorbed; transplacental: concentrated in cord blood.
Elimination	Urine and faeces	Renal	Faeces: $T_{1/2}$ 45 to 70 hours
Toxicity	Lungs, eyes, gingival, skin, central nervous system, kidneys, immune system.	Primary: kidneys, gastrointestinal tract; secondary: central nervous system.	Primary: central nervous system; secondary: cardiovascular system.

Source: World Health Organization. Mercury: children's health and the environment. 2008; available from: http://www.who.int/ceh/capacity/Mercury.pdf.

Pharmacokinetic profile of mercury

Among the three forms of mercury, viz. elemental, inorganic and organic, 75–85% of elemental mercury is absorbed by the lungs in the form of mercury vapour, organic mercury is completely absorbed by the gastrointestinal tract, while only 10% of inorganic mercury is absorbed by gastrointestinal tract (Table 1)[12].

Observations

From Tables 2 to 4, it can be seen that inorganic mercury (sulphide form) is extensively used in Ayurvedic science; some references for the use of elemental mercury are available, but none for organic mercury[13–47].

Discussion

In Africa, artisanal small-scale gold mining (ASGM) is a common practice; it is used for the extraction of gold from its ore through 'amalgamation'. This process is responsible for 37% global mercury emissions[48]; ASGM workers and their families are directly exposed to mercury vapours. In addition, dental amalgam is the main source of mercury toxicity because individuals with amalgam have 2–12 times more mercury in their body tissues compared to those without amalgam. Mercury, particularly mercury vapour, is known to be the most toxic even in very low doses and is non-radioactive[49]. It is reported that approximately 80% of inhaled mercury vapour is absorbed via the lungs[50]; so individuals living in or near an ASGM community and those with dental amalgam suffer from neurologic disorders, kidney and toxic to immune system and autoimmune dysfunction. It might be possible that these abnormalities occur due to inhalation of elemental mercury vapour. In Ayurveda, only few references are available where mercury (elemental) is used externally (in the form of *lepa*) for therapeutics[51]. From Table 1, it is clear that absorption of mercury (elemental) through skin is almost negligible[12]. Thus, almost negligible amount of mercury may be entering the body system when it is used externally in the form of *lepa*; this is also supported by contemporary science[52].

In Ayurveda, mercury is subjected to specific procedures like *Shodhan*, *Samskar*, etc.[53], where it is either subjected to triturating by specified liquid medium with specified plant material, or fomentation process. It has been reported that boiling and frying reduced Hg bioaccessibility by 40% and 60% respectively, rendering Hg–protein complexes less available for solubilization during digestion, due to strong affinity for proteins[54]. It might be possible that during the processing of mercury using decoction of plants, it forms Hg–protein complex which is poorly absorbed by gastrointestinal tract[55] before use of mercury as medicine it is converted into suitable dosages with herbs, metals and minerals and animal product.

Furthermore, before therapeutic use, mercury is converted into sulphide form by mixing solid sulphur with liquid mercury to form *Kajjali* (black powder of mercury sulphide)[56], or by dissolving mercury in liquid sulphur by applying specific process to form *Parpati*[57], or in a gas-phase reaction between gaseous mercury and gaseous sulphur with unique equipment (*Kupi pakwa*)[58]. Mercury is converted into a black-coloured mercurial compound (non-stoichiometry mixture of elemental mercury and sulphur permits the formation of meta-cinnabar) in the presence of sulphur through mechanical pressure[59] and

Table 2. Properties of mercury and mercury-based formulations

Formulation	Form of mercury	Properties
Lelitak prayog	Elemental	*Kushtha* (skin disorders)[13].
Savarnikaran lepa	Elemental	*Savarnikaran* (enhancement of skin completion)[14].
Lepa	Elemental	*Nilika*, *sphota* on *mukha roga*[15].
Poorna chandrodayam	Inorganic	Particle size in the range 60–70 nm; irregular size[16].
Mercury preparations	Inorganic	Vital in *Siddha* medicine for debilitating disease[17].
Ayurvedic and Chinese traditional medicines	Inorganic	Mercurial medicine may not pose a problem, unless due to misuse, abuse, over dosage and improper storage[18].
Cinnabar	Inorganic	Used as a sedative in traditional Chinese medicine[19].
Anxioloytic	Inorganic	High-dose HgS (1.0 g/kg/day) intoxication reduced the activity of Na(+)/K(+)-ATPase in the brainstem neurotoxicity, but toxicity by HgS completely disappears after cessation of its administration[20].
Garbha chintamani rasa	Inorganic	Improved liver synthetic activity, reduced lipids level and increased kidney function parameters used to improve the complications in diabetic condition[21].
Sidh Makardhwaj	Inorganic	Neurotoxic potency of mercuric sulphide (HgS) is about 1000 times less than soluble methyl mercury (MeHg)[22].
Hridayarnava Rasa	Inorganic	Safe from genotoxic hazards[23].
Swasa kuthara rasa and *smriti sagara rasa*	Inorganic	Free from genotoxicity abnormality[24].
Kajjali	Inorganic	Does not have genotoxic potential[25].
Makaradhwaja	Inorganic	Attributed to anti-stress activity. Improves the quality of life[26].
Mahamrutyunjaya rasa	Inorganic	Cardiotonic property[27].
Rasa sindur	Inorganic	Human gut flora does not convert mercury sulphide into toxic derivates like methyl mercury[28].
Mercury in Indian and Chinese systems of medicine	Inorganic	Chronic ailments like syphilis, high fever, pneumonia, insomnia, nervous disorders, deafness and paralysis of the tongue[29].
Arogyavardhini vati	Inorganic	Does not produce toxicological effects on brain, liver and kidney up to 10 times higher dose[30].
Rasa sindhura	Inorganic	Increases lifespan and fecundity[31].
Arogyavardhini vati	Herbomineral	Reduces oxidative stress (decreasing MDA and increasing GSH), increases serum HDL level, efficacious in dyslipidemia[32]. Reduces the risk factors of CVD[30].
Sidh makardhwaj	Inorganic	Does not show toxic effect on brain (cerebrum), liver and kidney[33], effective in the treatment of rheumatoid arthritis[34].
Mahamrutyunjaya rasa	Inorganic	Cardiotonic property[27].

indicated as single therapeutic agent as well as used in various herbomineral formulations for the treatment of different disorders. Pharmaceutical process of *Kupi pakwa* involves four steps (*Shodhan*, *Kajjali* preparation, *Bhavana* and *Kupi pakwa*). Before the preparation of *Kajjali*, *Shodhan* (purification and potentiation) of mercury, sulphur, etc. is done as mentioned in the Ayurvedic literature. The prepared *Kajjali* is levigated by specified liquid media for a certain period and then filled in glass bottles (*Kachkupi*) followed by indirect and homogenous heating for a certain period[60]. Mercury is then converted into non-toxic, stabilized form and used in therapeutics[61]. In Ayurvedic science, compounds of mercury are used along with specified organic liquid media in the form of *Anupana*. It has been reported that foodstuff like green tea, black tea and coffee simultaneously ingested with fish meal (containing mercury) decreased fish mercury bioaccessibility. Thus one can ensure that before the administration of mercury as medicine, it is converted into a form which is least toxic and absorbed to some extent by gastrointestinal tract, so that only small amounts enter the biological fluid that may not show toxic effect.

Three inorganic mercurial compounds are used for therapeutic purposes in Ayurveda, namely chloride (*Rasa Pushap*, *Rasa Karpura*), sulphide (*Parpati*, *Rasa Sindur*, etc.) and oxide form (*Mridarsing*)[62]. Among them, sulphide form is frequently used. The chloride form is not used because of accumulation and also since it acts as poison when used in excess dose[58]. It is supported by contemporary science that cinnabar (mercuric sulphide) is less nephrotoxic and hepatotoxic than $HgCl_2$ (*Rasa Pushap*, *Rasa Karpur*)[63]; this may be due to strong affinity of mercury for the thiol (SH) groups (such as GSH, cysteine), which provide the major intracellular defence mechanism against mercury-induced toxicity. Mercury is useful in many cosmetic preparations in its inorganic form for skin lightening by suppression of melanin production by the skin[64]. Furthermore, inorganic mercury does not pass the blood brain barrier and placenta[65]. Inorganic mercury used in Ayurveda does not enter the brain and does not goes into foetal circulation, hence it does not produce any adverse effects on the brain and foetus. It is reported that only 10% of inorganic mercurial compounds are absorbed through the gastrointestinal tract[66] and therapeutic dose mentioned for sulphide of mercury (inorganic form) is less[58]. So small amount of mercury is available for therapeutic action and it may not show any negative effects.

Table 3. Hazardous effects of mercury

Use of mercury	Form	Hazardous effects
Mercury	Inorganic and methyl mercury	Disrupts the endocrine system[35]
Mercury	Elemental	Inhibits the production of neurotransmitters[36]
Mercury toxicity	Elemental	Causes damage to the immune system[37]
Mercury	Elemental	Damage to kidney[38]
Mercury (Hg)	Inorganic mercury	Causes adverse effects on nervous system[39]
Mercury	Elemental	Cognitive deficits, liability, fatigue, decreased stress tolerance[40]
Mercury	Organic	Mental retardation, cerebellar ataxia deformed limbs, hyper-salivation growth disorders[41]
Methyl mercury	Organic	Increase blood pressure[42]
Methyl mercury	Organic	Neurotoxic[43]
Methyl mercury	Organic	Induces oxidative stress and mitochondrial dysfunction[44], inhibits cardio-protective activity of paraoxonase[45], kidney injuries[46], immune, neurological and behavioural dysfunctions[47]

Table 4. Indications of mercury based formulations

Formulation	Dosages form	Indications
Rasa sindur	Powder	Bronchial asthma, pleurisy with effusion.
Rasraj rasa	Liquid	Stroke, hypertension, diabetes, erectile dysfunction, oligospermia, kidney disorders, vata disorders.
Rasraj raas	Tablet	Paralysis, hemiplegia, lockjaw.
Destone	Capsule	Urinary tract infections, kidney stones, prostate gland inflammation and leucorrhoea.
Ekangvir ras	Capsule	Paralysis, Bell's palsy, hemiplegia, brachial palsy and sciatica.
Carwin	Capsule	Deep-seated wounds; tumours; loss of appetite, diminished growth, strength and vital elements along with haematopoiesis; physical and general ill-health due to radiation and chemotherapy.
Addyzoa	Capsule	Increases sperm count and motility; improves sperm morphology (prevents DNA damage to sperms); increases sexual desire.
Rasa sindur	Powder	HIV–AIDS.
Brento	Tablet	Impaired cognitive function, improves overall mental, performance, memory, concentration and learning abilities.
Mahayograj guggul	Tablet	Used in muscular–skeletal disorders; maha yogaraj *guggul* is not only anti-inflammatory and safe in the long run, but it medicinal herbs strengthen the system and extend remission.
Rhumayog	Tablet	Coronary insufficiency and ischaemic heart disease; the oleoresin of guggul has a cholesterol-lowering effect. It also has hypolipidaemic and anti-inflammatory effects.
Vrihat Vatchintamani rasa	Tablet	Improves sensory and motor performance in chronic neurological conditions such as hysteria, insomnia and paralysis.
Purnachandra rasa	Tablet	Rejuvenator; improves strength, stamina and energy.
Vasant Kusumakar rasa	Tablet	Diabetes, diabetic carbuncle, diabetic neuropathy, diabetic retinopathy.
Trailokya chintamani rasa	Tablet	Chronic and recurrent respiratory tract infections such as influenza, pneumonia, cachexia, emaceration associated with fever.

Source: ref. 76.

Ingested mercury on the effect of gastrointestinal tract

It has been reported that MeHg is not formed in human intestinal flora under anaerobic conditions because of the low redox potential from cinnabar, HgS or $HgCl_2$ (ref. 67). The intestinal bacteria may produce soluble mercuric polysulphides from HgS instead of methylmercury[68]. It was found that cinnabar is most likely converted into mercuric polysulphides under simulating gastrointestinal tract condition and that the latter exhibited relatively high membrane permeability as well as high affinity to plasma protein, but low cytotoxicity in HK-2 cells[28].

The Minimata disaster in Japan was due to the release of industrial wastewater containing mercury into the Minimata Bay, where it was converted to methyl mercury (MeHg) by bottom sediment microorganisms in the aquatic environment and entered the ecosystem through the food chain[43]. The methyl mercury can be biomagnified rapidly, leading to high concentration in top predators in the aquatic ecosystems[69]; i.e. higher concentration of MeHg is present in humans because, they are usually the top predatory in many food webs[70]. When people of particular region consumed contaminated fishes, they exhibited signs of neurological damage; their offspring were also severely affected by such disorders, because this form of mercury is completely absorbed by gastrointestinal tract and higher mercury levels are available in the biological fluid that may be responsible for such adverse effects. MeHg causes imbalance in calcium homeostasis due to dysregulation of intracellular calcium stores and/or increased permeability of the biomembranes to this ion[71]. It preferentially accumulates in astrocytes and inhibits glutamate uptake causing neuronal dysfunction[72]. MeHg and glutamate concentrations lead to the typical appearance of neuronal lesions associated with toxic stimulation[73]. MeHg causes neurotoxicity induced by the formation of reactive radicals. It also causes oxidative stress, resulting in

CNS damage by several interacting mechanisms, including mitochondrial damage with increase in intracellular free calcium ion, activation and inhibition of enzymes, release of excitatory amino acids, metallothioneins expression, and microtubule disassembly[74]. It inhibits mitochondrial enzyme and subsequently depolarizes mitochondrial membrane, thus reducing ATP production[75].

Conclusion

Thus, the rational use of mercury in its inorganic form (HgS, $HgCl_2$) will be beneficial for humans and wildlife, whereas irrational use in the organic form (MeHg) will produce hazardous effects.

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Mercury in traditional medicines: Is cinnabar toxicologically similar to common mercurials?

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Abstract

Mercury is a major toxic metal ranking top in the Toxic Substances List. Cinnabar (contains mercury sulfide) has been used in traditional medicines for thousands years as an ingredient in various remedies, and 40 cinnabar-containing traditional medicines are still used today. Little is known about toxicology profiles or toxicokinetics of cinnabar and cinnabar-containing traditional medicines, and the high mercury content in these Chinese medicines raises justifiably escalations of public concern. This minireview searched the available database of cinnabar, compared cinnabar with common mercurials, such as mercury vapor, inorganic mercury, and organic mercury, and discusses differences in their bioavailability, disposition, and toxicity. The analysis showed that cinnabar is insoluble and poorly absorbed from the gastrointestinal tract. Absorbed mercury from cinnabar is mainly accumulated in kidney, resembling the disposition pattern of inorganic mercury. Heating cinnabar results in release of mercury vapor, which in turn can produce toxicity similar to inhalation of these vapors. The doses of cinnabar required to produce neurotoxicity are thousands 1000 times higher than methyl mercury. Following long-term use of cinnabar, renal dysfunction may occur. Dimercaprol and succimer are effective chelation therapies for general mercury intoxication including cinnabar. Pharmacology studies of cinnabar suggest sedative and hypnotic effects, but the therapeutic basis of cinnabar is still not clear. In summary, cinnabar is chemically inert with a relatively low toxic potential when taken orally. In risk assessment, cinnabar is less toxic than many other forms of mercury, but the rationale for its inclusion in traditional Chinese medicines remains to be fully justified.

Keywords

Cinnabar; Traditional medicines; Elementary mercury; Mercuric chloride; Methylmercury; Bioavailability; Disposition; Toxicology

Introduction

Cinnabar (contains mercury sulfide) has been used for 2000 years in traditional Chinese medicines and in Indian Ayurvedic medicines (1–3). Mercury is a well-known toxic heavy metal, ranking high on the CDC Toxic Substances List (http://www.atsdr.cdc.gov). Mercury content found in traditional medicines justifiably alarms the public (4–7), and many mercury-

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containing traditional medicines have been banned. However, some are still in use today (1–2).

Mercury in traditional Chinese medicines mainly comes from cinnabar, deliberately included for therapeutic purposes based on Pharmacopeia of China (1). Chinese Ministry of Health has paid close attention to the mercury contents in traditional Chinese remedies, and the allowable amounts of cinnabar in these preparations have been dramatically decreased by as much as 65%, from a daily allowable dose of 0.3 – 1.5 g in the 1977 Pharmacopeia to 0.1 – 0.5 g in the 2005 Pharmacopeia of China (1,8), but the mercury in these traditional medicines can still be thousands folds higher than what is considered safe in Western countries including the USA. The question becomes, is cinnabar toxicologically similar to common mercurials?

Mercurials are commonly grouped as elementary, inorganic, and organic mercurials (Figure 1). Mercury disposition and toxicity are highly dependent on the chemical forms and physical status, and the three major mercurial forms must be distinguished when discussing their toxicity (9,10). For examples, mercury vapor (Hg^0) is much more hazardous than the liquid form of elementary mercury (Hg, also called *Shui Yin* and *Quicksilver*). Mercury ores are often found as cinnabar which contains mercuric sulfide, HgS) (11). Mercury binds to other elements, such as chlorine, sulfur, or oxygen, to form inorganic mercurous (Hg^{1+}) or mercuric (Hg^{2+}) salts, such as mercury sulfide (HgS, purified from cinnabar), mercurous chloride (Hg_2Cl_2, also called *calomel*) and mercuric chloride ($HgCl_2$). Mercury can form a number of stable organic metallic compounds by attaching to one or two carbon atoms. Methyl mercury (CH_3Hg^+) is the toxicologically most important organic form (11), but dimethyl mercury [$(CH_3)_2Hg$] is the most toxic mercurial (12). Ethyl mercury ($C_2H_5Hg^+$) is the major component of *thimerosal*, used as preservative in many vaccine preparations routinely administered to infants (13). However, little is known about the disposition and toxicity of cinnabar used in traditional medicines. Based on available data on cinnabar from the literature, this minireview compared the human exposure, disposition, and toxicology of cinnabar with mercury vapor, mercuric chloride, and methyl mercury. All mercurial compounds have characteristic toxicokinetics and quite different health effects depending on oxidation state, physical status, and associated organic species (9–13). Thus, total mercury content alone seems to be insufficient for safety evaluation of cinnabar and cinnabar-containing traditional medicines, and their chemical characteristics should be taken into consideration.

Human exposure of mercurial compounds

Elementary mercury is the pure form of mercury, also called metallic mercury. Metallic mercury is a shinny, silver-white metal that is in liquid state at room temperature, so called *Quicksilver* or *Shui Yin*. The first emperor of China, *Qin-Shi-Huang*, was driven insane and killed by mercury pills intended to give him eternal life and buried in a tomb fill with mercury (http://wikipedia.org). Mercury amalgam has been used for centuries in tooth filling and it is now gradually being replaced by other materials. In the processing of gold, especially in developing countries, large quantities of metallic mercury are used to form a gold amalgam. The amalgam is then heated to drive off the mercury, resulting in a substantial atmospheric mercury release (11). Metallic mercury is also used in some religious practices, and is sold under the name “azogue” in botanicas stores. Botanicas are common in Hispanic and Haitian communities where azogue may be used as an herbal remedy or for spiritual practices (11). However, metallic mercury is not used in traditional Chinese medicines.

Mercury vapor is colorless and odorless. The higher the temperature the more vapor will be released from liquid metallic mercury. Inhalation of large amount of mercury vapor can be fatal (14). Elemental mercury spills can occur in many ways, such as from broken elemental mercury containers, medicinal devices, barometers, and from melting tooth amalgam fillings

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to recover silver (14), or from smelting gold-mercury amalgam (11). When cinnabar is inappropriately overheated, mercury vapor can also be released, and in Chinese Pharmacopeia, heating cinnabar is never a part of preparation technique (1,8)

Cinnabar

Cinnabar is the naturally occurring mineral with mercury in combination with sulfur, and is red in color so called red mercury sulfide, *Zhu Sha* or *China Red*. Cinnabar ores are the major source for metallic mercury production. Cinnabar is insoluble and stable, and cinnabar powder has been used as an important ingredient in traditional Chinese medicines (1) and in Indian Ayurvedic medicines (3,15). Cinnabar-gold was used as an alchemical drug of longevity, called *Makaradhwaja* in India (16). Cinnabar is used to color paints and as one of red coloring agents used in tattoo dyes. Approximately 40 traditional Chinese medicines contain some cinnabar according to Pharmacopeia of China (1), and it is the major source of mercury found in traditional medicines.

Mercurous mercury also called *calomel*, was used as diuretics, antiseptics, skin ointments, vitiligo, and laxatives for centuries. Calomel was also used in traditional medicines, but now theses uses have largely been replaced by safer therapies. Other preparations containing mercury are still used as antibacterials (11). Very few traditional remedies contain calomel, and no calomel-containing oral Chinese remedy is listed in Pharmacopeia of China (1).

Mercuric mercury was once used as a disinfectant and antiseptic agent (9,11). Occupational exposure may occur from chloralkali industry where mercury is used as a cathode in the electrolysis of brine or from manufacturing scientific instruments and electrical control devices. Some degree of exposure of mercuric mercury may also occur from diet, such as from consumption of mercury-contaminated fish (11). Mercuric chloride is not used in traditional medicines.

Methyl mercury is produced primarily by microorganisms (bacteria and fungi) in the environment, rather by human activity. Fish consumption is the major route of exposure to methyl mercury. Until the 1970s, methyl mercury and ethyl mercury compounds were used to protect grain seeds from fungal infection. This use was banned after consumption of organomercury-treated grains in Iraq and China in 1970s leading to mass poisonings with hundreds of deaths (12,13). No methyl mercury is used in any of traditional medicines.

Dimethyl mercury is the most toxic form among mercurial compounds. Contact with even a small amount of dimethyl mercury can penetrate laboratory gloves and resulted in rapid transdermal absorption, causing delayed cerebella damage and death (13). The use of dimethyl mercury as a laboratory standard is now strictly regulated.

Ethyl mercury was used as preservatives. Thimerosal contains the ethyl mercury radical attached to the sulfur group of thiosalicylate (49.6% mercury by weight as ethyl mercury), and has been used as a preservative in many children vaccines since 1930s. Thimerosal has been removed from US vaccines by switching to single-dose vials that do not require any preservatives, but it is approved by WHO to use as preservatives in multidose vials in developing countries (17). No ethyl mercury is used in any of traditional medicines.

Thus, cinnabar is the only form of mercury used in traditional Chinese medicines today. How cinnabar differs from other forms of mercury will be discussed below.

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Disposition of cinnabar as compared to mercury vapor, mercuric chloride and methyl mercury

The solubility and bioavailability of cinnabar are quite low. The water solubility of mercuric chloride is 30–70 g/L, but cinnabar is less than 0.001 g/L at 20°C (11,18). In the stomach, the lower pH the more cinnabar dissolution as Hg_2SOH^+ formation occurs. In the intestine, sulfur (Na_2S and S^0) increase cinnabar dissolution as mercury-sulfur complexes such as $Hg(SH)^+$, $HgS(OH)^-$, $HgS_2(OH)^-$, and $HgS_3(OH)^-$ are formed (19). Sonochemical dissolution of cinnabar was investigated by measuring sulfur oxidation products and dissolved Hg^{2+} released into the aqueous solution (20). Dissolved S^{2-} was not detected and SO_4^{2-} was the main S species, but Hg^{2+} release was much lower than S species. Ultrasound can reduce cinnabar particle size and increase cinnabar surface area and isoelectric point. Humic acid acts synergistically to enhance cinnabar dissolution (20). The insoluble property of cinnabar or mercury sulfide made its accumulation in the body quite different from common mercurials (Table 2).

Absorption

Absorption of cinnabar (0.2%) from the gastrointestinal tract is much less than mercuric chloride (7–15%), and methyl mercury (>95%). Oral administration of powered cinnabar or mercury sulfide to mice resulted in 10- to 100-fold less tissue mercury accumulation as compared to the similar dose given as mercuric chloride either acutely (21), or chronically (18). When powdered cinnabar or mercury sulfide-containing diet was given to mice for 5 days, less than 0.02% of the dose was found in the kidney and liver (22). In general, bioavailability of cinnabar is 30- to 60-fold less than mercuric chloride (23). In comparison to methyl mercury, oral cinnabar or mercury sulfide administration results in at least 1000-fold less tissue mercury accumulation in mice (24–27), and in rats (28). Synthetic mercury sulfide was reported to have better bioavailability than cinnabar in mice (18,21), but in other studies, synthetic mercury sulfide was reported to have less oral bioavailability than cinnabar in mice (27) and in guinea pigs (29). This discrepancy could be due to differences in cinnabar processing methods, as well as to animal species or strain variation. Nonetheless, both crude cinnabar and synthetic mercury sulfide have very low oral bioavailability and are poorly absorbed from the gastrointestinal tract as compared to mercuric chloride and methyl mercury, but are better than liquid elementary mercury (less than 0.01%). Mercury vapor is readily absorbed (80%) through diffusion in the lungs. When cinnabar is heated, mercury vapor is released, and is easily absorbed to produce local and systemic toxicity. This is why in Pharmacopeia of China (1), heating cinnabar is restricted. Cinnabar is not used in injectable preparations. Little is known about cinnabar absorption via the skin, or from parenteral administration.

Distribution and biotransformation

The distribution of mercury from absorbed cinnabar basically follows the distribution pattern for inorganic mercurials. The highest concentration of mercury is found in kidney, a major target of inorganic mercury exposure (18,21–27). Renal uptake of mercury salts is through two routes: from luminal membranes in renal proximal tubule in the form of the cysteine S-conjugates (Cys-S-Hg-S-Cys) or from the basolateral membrane through organic anion transporters (30). Inorganic mercury salts do not readily pass blood-brain barrier or placenta. However, a small portion of absorbed inorganic mercury can be reduced in tissues and exhaled as mercury vapor. A significant portion of mercury vapor crosses the blood-brain barrier and placenta before it is re-oxidized to divalent inorganic mercury by tissue and erythrocyte catalase (9–12,17). Oral cinnabar or synthetic mercury sulfide administration results in brain distribution (about 10% of renal accumulation), mainly to the cerebral cortex and cerebellum

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(26–29). Accumulation of mercury from cinnabar in liver ranged from 5% to 50% of that in kidneys depending on experimental conditions (18,21;26–29). In comparison, methyl mercury is more uniformly distributed to various tissues upon absorption (9,13). Methyl mercury is bound to thiol-containing molecules such as cysteine (CH_3Hg-S-Cys), which mimic methionine to cross the blood-brain barrier and placenta through the neutral amino acid carrier (30). Methyl mercury is slowly metabolized to inorganic mercury by microflora in intestine (about 1% of the body burden per day), resulting in increased kidney accumulation (13).

In the Chinese literature, it is assumed that cinnabar could be converted to methyl mercury in the intestine under anaerobic conditions at pH 7 (8). However, no evidence is available to support this assumption. Unlike arsenic methylation reactions, mercury methylation reaction does not occur in humans, and little is known about biotransformation of inorganic mercurial salts to methyl mercury in the body; instead, intestinal bacteria can convert methyl mercury to inorganic mercury (9–13). From over 1000- to 5000-fold differences in bioavailability between cinnabar and methyl mercury (28), such assumed reaction, if it exits, is very minor accounting for less than 0.02% of dosed cinnabar.

Inorganic mercury salts non-uniformly distributed to kidney and are excreted in urine and feces, with a half-life of about 2 months. Methyl mercury undergoes extensive enterohepatic recycling which can be interrupted to enhance fecal excretion. 90% of the methyl mercury is eliminated from the body in the feces, and less than 10% is in the urine, with a half life of 45–70 days (9–13).

It is quite clear that solubility and bioavailability of cinnabar is quite different from mercury vapor, mercuric chloride, and methyl mercury. The bioavailability is a critical determinant of toxicity of mercurial compounds. Thus, it is not surprising that cinnabar has quite different toxicology potentials from common mercurials. To better understand toxicokinetics of cinnabar is very important for appropriate safety assessment of mineral cinnabar used in traditional medicines.

Toxicological profiles of cinnabar, mercury vapor, mercuric chloride and methylmercury

The toxicity potentials for mercurial compounds, including cinnabar and cinnabar-containing Chinese medicines, vary greatly dependent on the chemical forms of these mercurials (9–13).

Cinnabar-containing traditional medicines are generally relatively non-toxic at therapeutic doses. The correct preparation methods, appropriate doses, disease status, age and drug combinations are important factors impacting cinnabar toxicity (1,8,31). In general, the adverse effects at therapeutic doses of cinnabar-containing traditional medicines are rare and are largely tolerable and reversible. The cinnabar poisoning cases are associated with overdose, long-term uses, and improper processing such as heating, decocting, fumigating, or in combination with other drugs (31). For example, heating cinnabar resulted in mercury vapor release, and acute inhalation of mercury fumes can be fatal (32). Grinding cinnabar using aluminum utensils or in combination of iodide- and bromide-containing drugs could increase mercury toxicity (31), but the mechanisms of such interactions are not completely known. The long-term use of cinnabar-containing traditional medicines could result in renal dysfunction due to accumulation of mercury in the kidney. Blurred vision due to accumulation of mercury in brain is possible, gastrointestinal symptoms also often occur following long-term administration (9–11,31). Skin allergic reaction may occur when cinnabar is used in tattoo dyes (33).

Oral administration of cinnabar at a high dose (1.0 g/kg/d for 7d) produced reversible hearing dysfunction, learning memory deficit, and other behavioral abnormalities in mice (24), rats

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(25,28), and guinea pigs (29). In comparison, the ototoxicity produced by methyl mercury was so dramatic and irreversible, even at doses 1/1000 to 1/5000 of cinnabar (24–29). It should also be pointed out that the dose of cinnabar or mercury sulfide (1.0 g/kg) used in these studies is at least 100–500 times higher than human daily dose (i.e., 50 g/50 kg person, while allowable daily human oral dose is 0.1 – 0.5 g) (1). At lower cinnabar doses (10 mg/kg/d) for a longer time (up to 11 weeks), cinnabar did not produce neurotoxic effects in mice until 7 weeks of continuous administration (27). The cerebellum appeared to be the most vulnerable brain region (27). Long-term (4 weeks) oral administration of mercury sulfide in mice increased renal mercury burden, and decreased circulating thyroxin (T_4) levels (34). However, no data on nephrotoxicity was reported from this study.

Inhalation of mercury vapor produces acute corrosive bronchitis and interstitial pneumonitis and, if not fatal, may be associated with central nervous system effects such as tremor or increased excitability (9–11). With chronic exposure to mercury vapor, the major effects are on the central nervous system. The triad of tremors, gingivitis and erethism (memory loss, increased excitability, insomnia, depression and shyness) has been recognized historically as the major manifestation of mercury poisoning from inhalation of mercury vapor. Sporadic instances of proteinuria and even nephrotic syndrome may occur in persons with exposure to mercury vapor, particularly with chronic occupational exposure (9–11). A case report of chronic mercury poisoning from burning a traditional medicine mixture composed of cinnabar and calomel in the treatment of vitiligo, blood mercury levels were elevated to 1100 μg/L (normal < 20 μg/L), and central nervous system toxicity and renal toxicity typical to chronic mercury poisoning occurred. After chelation treatment with dimercaprol for 4 weeks, her blood mercury levels decreased with improved mercury intoxication symptoms (32).

Kidney is the major target organ for inorganic mercury in humans and in experimental animals (9–11). Although a high dose of mercuric chloride is directly toxic to renal tubular cells, chronic low-dose exposure to mercuric salts may induce an immunologic glomerular disease (36). Exposed persons may develop proteinuria that is reversible after workers are removed from exposure. Experimental studies have shown that the pathogenesis has two phases: an early phase characterized by an anti–basement membrane glomerulonephritis, followed by a superimposed immune-complex glomerulonephritis with transiently raised concentrations of circulating immune complexes (37). The pathogenesis of the nephropathy in humans appears similar, although antigens have not been characterized. In humans, the early glomerular nephritis may progress to interstitial immune-complex nephritis (36). Acrodynia has occurred in children chronically exposed to inorganic mercury compounds in teething powder and diaper disinfectants, as well as to organomercurials. Acordynia is characterized by pink hands and feet (also called Pink Disease). These subjects are photophobic and suffer from joint pains (11–13). The long-term use of cinnabar-containing traditional medicines could result in accumulation of mercury in the kidney and renal dysfunction similar to mercuric mercury exposure may occur (8,31,35).

The major human health effect from exposure to methyl mercury is neurotoxicity. Clinical manifestations of neurotoxicity include paresthesia (a numbness and tingling sensation around the mouth, lips) and ataxia, manifested as a clumsy, stumbling gait, difficulty in swallowing and articulating words. Other signs include neurasthenia (a generalized sensation of weakness), vision and hearing loss, and spasticity and tremor. There may finally progress to coma and death (9–13). Neuropathological observations have shown that the cerebral cortex and cerebellum are selectively involved with focal necrosis of neurons, lysis and phagocytosis, and replacement by supporting glial cells. These changes are most prominent in the deeper fissures (sulci), as in the visual cortex and insula. The overall acute effect is cerebral edema, but with prolonged destruction of gray matter and subsequent gliosis, cerebral atrophy results (9–13, 17). There is as yet no available report on cinnabar-induced neurotoxicity in humans.

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Children are sensitive to mercury toxicity

Early life stages are particularly vulnerable to mercury intoxication (38). In Minamata, Japan, pregnant women who consumed fish contaminated with methyl mercury manifested mild or minimal symptoms, but gave birth to infants with severe developmental disabilities, raising initial concerns for mercury as a developmental toxicant. Methyl mercury crosses the placenta and reaches the fetus, and is concentrated in fetal brain at least 5 to 7 times that of maternal blood (13). Prenatal methyl mercury exposure at high levels can induce widespread damage to the fetal brain. However, the effects from low-level exposures are inconsistent (38,39). In the Seychelles Children Development Study, a group with significant methyl mercury exposure from a diet predominantly of fish was studied for developmental adverse effects. These children were examined 6 times over 11 years using extensive batteries of age-appropriate developmental endpoints, but no convincing associations were found except for delayed walking (38). The National Research Council reviewed the epidemiologic studies relating *in utero* methyl mercury exposure and fetal neurological development. It concluded that the current EPA reference dose for methyl mercury of 0.1 μg/kg per day or 5.8 μg/L cord blood is scientifically justifiable for protection of human health (40). The RfD is equivalent to 12 ppm methyl mercury in maternal hair (10,40).

More than 12 cinnabar-containing Chinese medicines are used in pediatrics, mainly for their sedative and hypnotic effects. Toxicity has been reported from inappropriate use of cinnabar and cinnabar-containing medicines in infants and preschool children (7,31). Thus, caution should be taken when cinnabar-containing Chinese medicines are used for children, as children are susceptible to mercury toxicity.

Treatment

Therapy for mercury poisoning should be directed toward lowering the concentration of mercury at the critical organ or site of injury. For the most severe cases, particularly with acute renal failure, hemodialysis may be the first measure, along with administration of chelating agents for mercury, such as dimercaprol (BAL), 2, 3-dimercaptosuccinic acid (DMSA, succimer), EDTA (calcium disodium, edentate calcium disodium), or D-penicillamine (NAP). Chelation therapy is not very helpful for methyl mercury exposure (9,13,17). Biliary excretion and reabsorption by the intestine can be interrupted by oral administration of a nonabsorbable thiol resin, which can bind mercury and enhance fecal excretion (17). Succimer (DMSA) is a FDA-approved pediatric use in the treating mercury poisoning (41).

Pharmacology studies of cinnabar

The effects of cinnabar on anxiety-like behaviors in mice were studied using the elevated plus maze test. Cinnabar at the oral dose of 50 and 100 mg/kg/d for 10 days significantly improved the performance in the elevated maze test, but at the 1000 mg/kg, a dose 100-fold higher than the human daily dose, it was ineffective (42). This pharmacological effect is associated with the decreased in serotonin levels in mouse brain, but the dose-dependent relationship is not clear (42). In mice given low dose of cinnabar (10 mg/kg/d) for 11 weeks of continuous administration, the locomotor activity was reduced and pentobarbital sleeping time was increased, suggesting sedative or hypnotic effects (27). Induction of renal metallothionein in rats by cinnabar is dose- and time-dependent, but induction of hepatic metallothionein was lower and independent of dose and time (43). This fortifies the notion that cinnabar is poorly absorbed and the kidney is the major organ of mercury accumulation, despite the fact that the doses used in this study were 1000 times higher than human daily dose (2.5– 5.0 g/kg, po, for 2–4 weeks). In general, little is known about the therapeutic effects of cinnabar, and available pharmacology literature is limited.

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Cinnabar is not used alone in traditional medicines, and it is usually used as an ingredient in traditional Chinese medicine recipes (1). Some pharmacological studies on cinnabar-containing traditional medicines are available in the Chinese literature, but not in PubMed. The available studies on the pharmacological effects of cinnabar-containing traditional medicines are inconsistent. For example, the inclusion of cinnabar in An-Gong-Niu-Huang Wan, a famous cinnabar-containing Chinese medicine, has been reported to be essential (44), to have some beneficial effects (45), or not to be important at all (46). Considering the very poor bioavailability of mercury in cinnabar, whether the mercury in these preparations is of any therapeutic value is highly questionable (47). To extensively comment on these studies is beyond the scope of this minireview, and much more studies are needed to fully justify the therapeutic basis for inclusion mercury in any form of traditional medicines.

Summary

This minireview comments on natural mineral cinnabar used in traditional medicines. Cinnabar is insoluble, has very low bioavailability and thus is poorly absorbed from the gastrointestinal tract. Once absorbed into the blood, the mercury disposition from cinnabar follows the pattern for inorganic mercury salts and preferentially distributed to kidney, with a small portion to the brain. The heating, overdose and the long-term use of cinnabar are major causes of mercury intoxication, but at the therapeutic doses, the adverse effects cinnabar-containing traditional medicines seem to be tolerable and reversible. In safety evaluation of cinnabar-containing traditional medicines, total mercury content alone is insufficient, and chemical forms of mercurial compounds should be taken into consideration. Toxicologically, cinnabar or synthetic mercury sulfide should be distinguished from mercury vapor, mercuric chloride, and methyl mercury.

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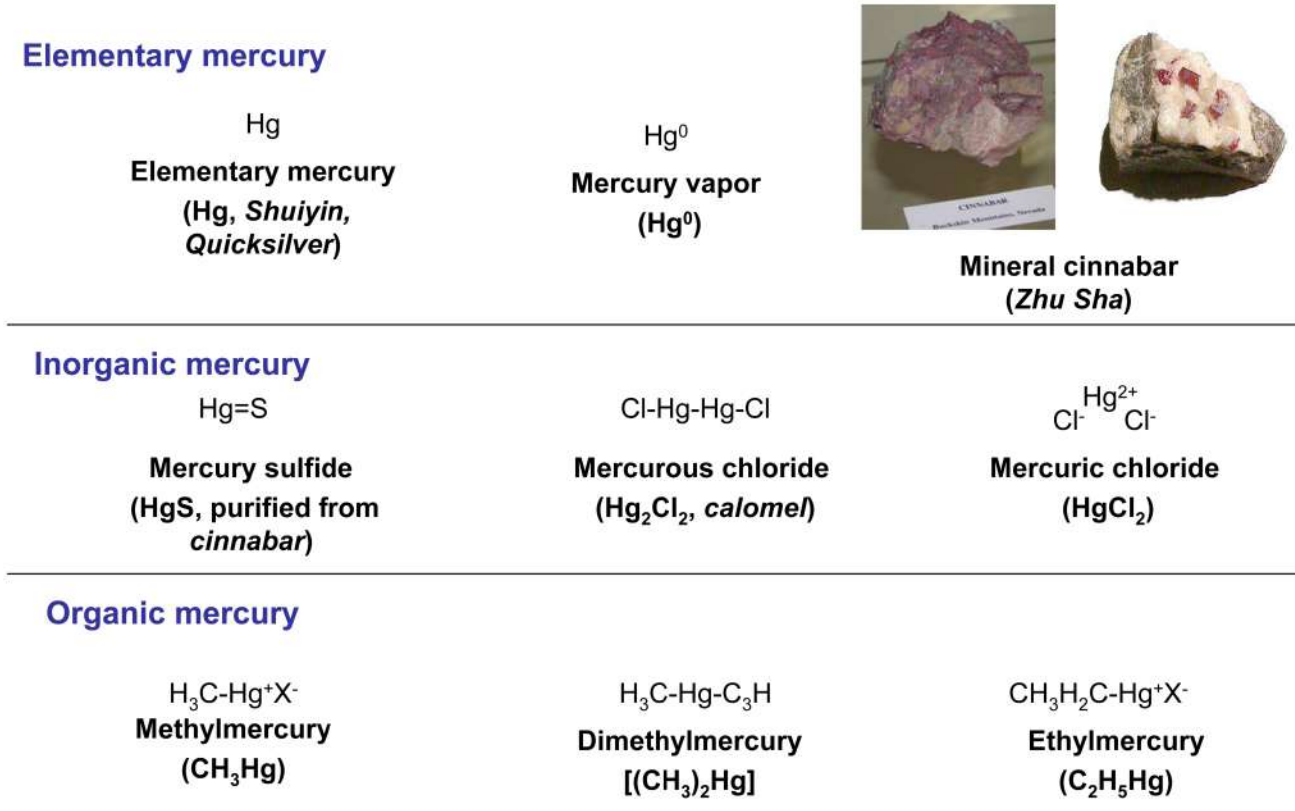

Fig. 1.
Chemical structure of common mercurials

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Table 1

Human Exposure of Mercurial Compounds

Name	Symbol, popular names	Major source of exposure	Traditional Uses	References
Elementary mercury	Hg, *Quicksilver*, *Shui Yin*	Barometer, Dental amalgams	Religious	11
Mercury vapor	Hg^0	Occupational, accidental		14
Cinnabar	*Shu Sha*, *China red* Contains mercury sulfide, HgS	Traditional medicines	Sedative, disinfection	1–3,15–16
Mercurous chloride	Hg_2Cl_2, *Calomel*	Medicinal uses External use	Antiseptics, diuretics Anti-parasites, detoxication	9,11
Mercuric chloride	$HgCl_2$	Chloralkali industry	Industry	9–11
Methyl mercury	CH_3Hg	Fish consumption	Preservative in vaccine	9–13,17
Ethyl mercury	C_2H_5Hg	Preservatives,	Vaccines, agriculture	9–13

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Table 2

Disposition of cinnabar, mercury vapor, mercuric chloride, and methyl mercury

Name	Absorption	Distribution	Biotransformation	Excretion	References
Cinnabar	GI, <0.2%	Kidney, spleen, liver	HgS to Hg^{2+}	Feces and urine	21–29
Mercury vapor	Lung 80%, GI<0.01%	Lung, brain, kidney	Hg^0 to Hg^{2+}	Urine and feces	9–10,14
Mercuric chloride	GI, 7–15%	Kidney, liver, spleen	Hg2+ to Hg^0	Urine and feces	9–11
Methyl mercury	GI, > 95%	Brain, kidney, liver	CH_3Hg to Hg^{2+}	Feces and urine	9–13,17

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Table 3

Toxicological profiles of cinnabar, mercury vapor, mercuric chloride, and methyl mercury

Name	Acute toxicity	Chronic toxicity	Treatment	References
Cinnabar	Heating cinnabar, death	Neurotoxicity, Renal and GI symptoms	BAL	8,31–33
Mercury vapor	Death, lung and brain	Pneumonitis, bronchitis Neurotoxicity, nephrotoxicity	DMSA, BAL	9–11,14,41
Mercuric chloride	Renal failure	Kidney injury and immunopathy Skin irritation, Acordynia	BAL, EDTA	9–11
Methyl mercury	Death, brain	Neuropathy, developmental toxicity	No Chelators	9–13,17,41

BAL= dimercaprol; DMSA= 2,3-dimercaptosuccinic acid, succimer; EDTA = Ethylenediaminetetraacetic acid, its sodium salt edentate disodium, and closely related edentate calcium disodium.

Ninety days repeated dose oral toxicity study of *Makaradhwaja* in Wistar rats

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Abstract

Context: *Makaradhwaja* is a *Kupipakwa Rasayana*. Since it contains two heavy metals, namely mercury and gold, it is essential to evaluate its safety. Hence, the present study was undertaken with an objective to evaluate toxicity and target organ of toxicity of *Makaradhwaja* if so. **Aims:** The objective was to evaluate toxicological profile, the target organ of toxicity and to find no observed effect level (NOEL) or no observed adverse effect level (NOAEL) in rats after oral administration for ninety consecutive days. **Materials and Methods:** *Makaradhawaja* preparation was administered to male and female Wistar rats for ninety consecutive days at 2.7, 13.5, and 27 mg/kg body weight. All relevant biochemical and hematological changes were observed. At termination, all the rats were sacrificed and necropsy was performed. Histopathological evaluation was also performed. **Statistical Analysis Used:** Dunnett's test followed by analysis of variance. **Results:** There was a significant increase in high-dose group kidney weight of both sexes which could not be correlated with histopathology findings and serum biochemistry. Therefore, the change was not considered as an adverse effect. **Conclusions:** The dose level 27 mg/kg of *Makaradhwaja* was found as NOAEL and dose level 13.5 mg/kg of *Makaradhwaja* was found as NOEL.

Keywords: Gold, *Makardhwaja*, mercury, rats, toxicity

Introduction

Makaradhwaja is a classical Ayurvedic preparation categorized as *Kupipakwa Rasayana* (rejuvenating mercurial formulation which is prepared in glass bottle by sublimation through sand bath with specific temperature pattern). The oldest reference of its use is found in classical text of *Rasendra Chintamani* by *Acharya Dhundhuknath* in the 16th century.[1] According to Chopra,[2] its first reference can be attributed to *Vaidya Bhva Mishra* of 16th century. *Kupipakwa Rasayana* is basically powdered minerals and metals heated gradually in glass flask. They have often characteristic red (or yellow color) and their potency persists indefinitely if stored in well-stoppered bottles.[3] Gold and mercury are the heavy metal contents of the *Makaradhwaja* along with sulfur.[4] *Makaradhwaja* is aphrodisiac and nutrient to body and mind with adapto-immuno-neuro-endocrino-modulator properties. It is indicated for dyspepsia, weakness of heart, senility and fever.[4]

Users of Ayurvedic medicine may be at risk for heavy metal toxicity.[5] Repeated exposure to heavy metal containing products may lead to cumulative toxicity. Regulatory guidelines also require the product to be tested for its toxicity in rodents carrying single and repeated dose studies so as to establish the no observed effect level (NOEL) and no observed adverse effect level (NOAEL).[6] The present study was a part of multicentric toxicity studies of the Central Council for Research in Ayurvedic Sciences, hence drug was provided in a coded manner.

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Materials and Methods

Test drug preparation and analysis

Makaradhwaja was provided by the Central Council for Research in Ayurvedic Sciences, New Delhi, in a coded manner that was decoded along with chemical analysis report after completion of the study. The drug was prepared as per the

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method described in *Rasa Tarangini*[7] a classical Ayurvedic text.

Details of the ingredients are given in Table 1.

All the ingredients were collected and the final drug was as given below.

Purified gold was taken into mortar and purified mercury was added to it and triturated. Trituration was continued till amalgam of *Swarna* (gold) and *Parada* (mercury) was formed. The amalgam of *Swarna* and *Parada* and powder of *Shodhit Gandhaka* (purified sulphur) in specified quantity was taken and grinded to obtain homogenous, soft, fine (non lustrous) powder. The fresh juice of flower of *Rakta Karpasa* (Gossypium arboreum Linn) was prepared and added to the *Swarna Kajjali* in adequate quantity and levigated. After completing the process, whole mixture was dried. The fresh juice of root bark of *Ankol* (*Alangium salvifolium* Linn.) was prepared and added to the *Swarna Kajjali* in adequate quantity and levigated. After completing the process, the whole mixture was dried. Pulp collected from fresh leaves of *Kumari* (*Aloe vera* Linn) was processed to obtain fresh juice. The fresh juice was added to the *Swarna Kajjali* in adequate quantity and levigated. After completing the process, whole mixture was dried. The dried *Swarna Kajjali* was taken in a glass bottle and placed in *Valuka Yantra* (sand bath). Then, the *Valuka Yantra* was subjected to heat adopting *Kupipakva* method. The temperature was increased gradually in a phased manner. At the end, *Valuka Yantra* was allowed to self cool and bottles were taken out. The bottles were observed for deposition of *Makaradhwaja* at the neck (i.e., *Kanthastha Makaradhwaja*). A thread soaked in kerosene was tied around the bottle just below the level of deposited *Makaradhwaja* and burnt to break the bottle. After this, *Kanthastha Makaradhwaja* was collected in mortar and was grinded to convert it in to fine powder, which was reddish (*Sindoor Varneeya*) in color. The residual of gold obtained from the bottom of the bottles was collected separately.

Physicochemical analysis, namely morphological description, estimation of moisture content, qualitative elemental testing, and Ayurvedic parameters, were carried out by following standard methods as per the Ayurvedic Pharmacopoeia of India Guidelines.

The quantitative elemental composition was performed on JY 2000 sequential inductively coupled plasma-atomic emission spectrometry (ICP-AES) spectrometer (Horiba Jobin Yvon, France). The operating parameters of ICP-AES were – RF power: 1000 w at 40.68 MHz, plasma gas flow rate: 12 L/min, nebulizer gas flow rate: 0.1 L/min, Sample uptake rate: 1.2 L/min, slit with: 20 micron/20 micron (entrance/exit), and monochromator: 0.64 m focal length, 2400 groves/mm, Czerny turner mounting.

Table 1: Ingredients used for preparation of *Makaradhwaja*

Ingredients	Latin name	Part used	Quantity
Shodhit Swarna	Gold	-	1 part
Shodhit Parada	Mercury	-	8 part
Shodhit Gandhaka	Sulphur	-	16 part
Juice of *Raktakarpas* flower	*Gossypium arboreum* Linn	Flower	QS
Juice of *Ankol* root bark	*Alangium salvifolium* Linn	Root bark	QS
Juice of *Kumari*	*Aloe vera* Linn	Leaf	QS

QS: Quantum sates

Study protocol

The study protocol was provided by the sponsor Central Council for Research in Ayurvedic Sciences, New Delhi, which was designed by broadly following principles of Schedule Y of Drugs and Cosmetic Act, 1940.

Housing and environment

A total of 80 Wistar rats (40 male and 40 female) with body weight ranging from 150 g to 200 g were obtained from animal house. Ethical clearance was taken from the Institutional Animal Ethical Committee (IAEC) with letter number 6-17/2003-CRI/Tech/777 dated 02.07.2009. All the rats were maintained as per the guidelines of the committee for purpose of control and supervision of experiments on animals for laboratory animal facility. Rats were acclimatized for 7 days. Temperature and relative humidity were maintained at 25 ± 1°C and 40%–70% respectively and illumination was controlled to give approximately a sequence of 12 h light and 12 h dark. Rats were individually housed in polycarbonate cages (43 cm × 28 cm × 21 cm) with lids and rice husk bedding. Pelleted rodent diet obtained from National Institute of Nutrition, Hyderabad, was provided along with deionized water using plastic bottles with stainless steel nozzle *ad libitum*. Females selected were nulliparous and nonpregnant.

Experimental study design

The animals were divided into four groups of 10/sex/group. Ninety days repeated dose oral toxicity study of *Makaradhwaja* was conducted by daily single administration of the drug at 27 mg/kg (high dose [HD]), 13.5 mg/kg (mid-dose) and 2.7 (low dose) mg/kg body weight along with vehicle control. The test drug was administered as suspension in vehicle, that is, honey mixed with water by gavage and control group received the vehicle. To make the drug and vehicle suspension easy to gavage, it was diluted with water in 2:3 ratio. The suspension was administered @10 ml/kg body weight. The dose calculation was as follows:

Therapeutic dose for human (70 kg body weight approximately): 30 mg/day. Dose conversion factor from human to rats as per the Paget and Barnes[8] is 0.018.

Hence, the dose for rats (200 g body weight approximately) will be 30 × 0.018 = 0.54 mg. Hence, therapeutic equivalent dose for rats per kg/body weight will be 2.7 mg which was considered as low dose. The dose that was 5 times more than therapeutic, that is 2.7 × 5 = 13.5 was considered as mid-dose, and 10 times therapeutic dose, that is, 2.7 × 10 = 27 was considered as highest dose for the study.

All animals were observed for morbidity and mortality twice daily. General clinical observations were made twice a day at

the same time throughout the study. The animals were observed for changes in skin, fur, eyes, mucous membrane, occurrence of secretions and excretions. For neurological examination, the animals were taken outside the cage in a standard arena and their behavior was recorded. Body weights and feed consumption of each animal were recorded at the start of the study and thereafter at weekly intervals. At the termination of the study, that is, on 90th day, serum glucose, total protein, SGOT, SGPT and creatinine along with hematological parameters which were white blood cell and red blood cell count, hemoglobin, hematocrit (%), platelet count, mean cell volume (MCV), mean corpuscular hemoglobin (MCH) and MCH concentration were measured for all animals. Animals were sacrificed on the 91st day using CO_2 euthanasia and were subjected to a detailed postmortem examination and histopathological analysis. The adrenals, heart, kidney, spleen, testis, epididymis, ovaries, uterus, liver, brain and thymus were weighed and collected along with jejunum, duodenum, colon, cecum, ileum, stomach, lungs, pancreas, esophagus and trachea in 10% neutral-buffered formalin. All collected organs from control group and HD group were processed as per the Registry of Industrial Toxicology Animal data guidelines[9] and subjected to histopathological evaluation.

Test drug physicochemical analysis

Makaradhwaja prepared was in the form of reddish powder and had no characteristic odor. Assay of element showed 82% of mercury and 13% of sulphur. Moisture content was 0.08% which was found when loss on drying was determined at 105°C.

Results

No abnormality in clinical signs was detected across all the groups throughout the study. No mortality was found in any of the study groups. No abnormality was detected across the groups during neurological examination. No treatment-related as well as dose-dependent effect on body weights, feed consumption, water consumption, fecal consistency and biochemical parameters was observed as compared to the control group. MCV of HD group male was significantly increased but was within biological limits. However, significant increase in the weights of kidneys from the male and female animals of HD group was recorded. There was no toxicity induced or any other lesion observed during the histopathology evaluation which could be correlated with the increase in weight of the kidneys. Further, there was no significant change in serum biochemistry parameters of HD group females which could be attributed to increased weight of kidneys. The results are described in Tables 2-9. No treatment-related and dose-dependent adverse changes were observed on detailed histopathological evaluation [Figures 1-6]. Photomicrographs of the selected organs/tissues from vehicle control group and high-dose group are provided in Figures 1-6.

Statistical analysis

Study observations such as body weight gain, feed consumption, blood biochemistry and hematology parameters and organ weights were recorded and analyzed statistically. One way analysis of variance and Dunnett's test were applied to compare the dose groups over the control arm.[10]

Discussion

The test drug *Makaradhvaja* was prepared in compliance with Ayurvedic literature and contained heavy metal mercury (82%) and sulphur (13%). However, no treatment related adverse effect was observed up to 10 times therapeutic dose levels, that is, 27 mg/kg body weight in animal experiment. G. Kumar *et al*.[11] studied 28 days repeated dose toxicity of mercury in Ayurvedic formulation of *Aarogyavardhini Vati* in rats at dose of 50, 250, and 500 mg/kg of body weight with mercury content dose equivalent to 1 mg/kg to $HgCl_2$ in HD group and reported that there was no adverse effect on cerebellum,

Table 2: Body weight (g) of male rats

Group	Mean±SD			
	Control	Hd (27.0 Mg/kg)	5Td (13.5 Mg/kg)	Td (2.7 Mg/kg)
Initial body weight	127.24±32.00 (10)	127.20±28.60 (10)	127.10±32.39 (10)	127.46±39.09 (10)
Week 1	153.92±37.23 (10)	155.40±26.23 (10)	165.46±37.46 (10)	160.94±44.41 (10)
Week 2	186.28±29.92 (10)	179.46±26.26 (10)	191.52±36.00 (10)	179.04±48.44 (10)
Week 3	210.18±26.11 (10)	198.80±26.85 (10)	215.14±37.89 (10)	199.76±48.85 (10)
Week 4	231.60±23.84 (10)	220.96±27.40 (10)	233.92±34.35 (10)	217.06±50.20 (10)
Week 5	244.82±24.55 (10)	228.95±30.43 (10)	253.61±36.30 (10)	235.70±56.97 (10)
Week 6	257.42±26.58 (10)	242.68±30.67 (10)	267.92±34.72 (10)	246.62±57.65 (10)
Week 7	269.78±24.70 (10)	256.44±31.59 (10)	279.46±32.85 (10)	258.58±50.84 (10)
Week 8	280.50±27.23 (10)	266.86±33.95 (10)	291.30±32.56 (10)	266.51±51.59 (10)
Week 9	289.20±28.72 (10)	272.88±34.60 (10)	301.10±31.93 (10)	274.72±52.63 (10)
Week 10	296.72±29.55 (10)	274.04±36.91 (10)	308.68±34.20 (10)	276.60±55.87 (10)
Week 11	297.36±28.88 (10)	276.88±40.50 (10)	314.04±33.40 (10)	282.58±51.23 (10)
Week 12	307.62±26.32 (10)	284.20±40.25 (10)	320.94±33.31 (10)	282.12±51.46 (10)
Week 13	311.99±29.12 (10)	289.86±38.26 (10)	325.06±33.27 (10)	279.60±50.10 (10)

Figures in parenthesis indicate number of animal. SD: Standard deviation

Table 3: Body weight (g) of female rats

Group	Mean±SD			
	Control	HD (27.0 mg/kg)	5TD (13.5 mg/kg)	TD (2.7 mg/kg)
Initial body weight	103.34±14.39 (10)	103.36±27.85 (10)	103.08±8.57 (10)	103.34±16.07 (10)
Week 1	111.66±22.36 (10)	118.48±25.61 (10)	113.28±21.03 (10)	129.68±12.20 (10)
Week 2	118.92±24.74 (10)	129.50±21.58 (10)	124.64±17.95 (10)	137.70±11.57 (10)
Week 3	130.28±19.19 (10)	137.42±19.73 (10)	133.26±16.27 (10)	145.88±12.66 (10)
Week 4	134.26±17.74 (10)	144.32±18.23 (10)	140.00±16.85 (10)	151.36±12.34 (10)
Week 5	136.48±19.12 (10)	143.50±19.33 (10)	145.18±15.79 (10)	154.74±11.74 (10)
Week 6	143.30±19.23 (10)	142.42±22.43 (10)	147.68±16.80 (10)	158.30±11.13 (10)
Week 7	149.91±16.66 (10)	144.46±22.33 (10)	153.10±16.90 (10)	162.86±10.84 (10)
Week 8	151.62±16.02 (10)	148.78±26.78 (10)	156.24±18.07 (10)	165.86±10.53 (10)
Week 9	154.24±14.81 (9)	148.16±27.76 (10)	158.46±17.43 (9)	169.08±10.27 (10)
Week 10	154.98±14.92 (9)	150.56±29.50 (10)	160.98±18.62 (9)	167.96±11.26 (10)
Week 11	159.44±14.21 (9)	159.36±20.26 (9)	161.62±17.77 (9)	169.60±11.49 (10)
Week 12	161.93±12.91 (9)	161.44±18.11 (9)	161.76±19.20 (9)	171.02±12.14 (10)
Week 13	162.82±14.24 (9)	162.69±18.55 (9)	163.81±19.59 (9)	170.40±10.73 (10)

Figures in parenthesis indicate number of animals. SD: Standard deviation, HD: High dose, TD: Therapeutic dose

Table 4: Relative organ weight of male rats

	Adrenals	Liver	Brain	Thymus	Heart	Kidneys	Testes	Spleen	Epididymis
Control									
n	10	10	10	10	10	10	10	10	10
Mean±SD	0.03±0.01	2.98±0.31	0.58±0.04	0.07±0.02	0.37±0.03	0.78±0.05	1.01±0.10	0.37±0.05	0.41±0.03
HD (27.0 mg/kg)									
n	10	10	10	10	10	10	10	10	10
Mean±SD	0.04±0.02	3.09±0.30	0.65±0.09	0.09±0.03	0.37±0.03	0.91*±0.06	1.02±0.15	0.34±0.05	0.42±0.05
5TD (13.5 mg/kg)									
n	10	10	10	10	10	10	10	10	10
Mean±SD	0.03±0.01	3.13±0.17	0.60±0.06	0.07±0.01	0.36±0.04	0.87±0.06	0.99±0.15	0.33±0.04	0.42±0.08
TD (2.7 mg/kg)									
n	9	9	9	9	9	9	9	9	9
Mean±SD	0.03±0.02	3.07±0.26	0.71±0.13	0.09±0.03	0.38±0.05	0.87±0.08	1.11±0.12	0.36±0.02	0.41±0.04

*Significant at 5% level. Figures in parenthesis indicate number of animals. SD: Standard deviation, SD: Standard deviation, HD: High dose, TD: Therapeutic dose

Table 5: Relative organ weight of female rats

Group	Adrenals	Liver	Brain	Thymus	Heart	Kidneys	Ovaries	Spleen	Uterus (with cervix)
Control									
n	9	9	9	9	9	9	9	9	9
Mean±SD	0.06±0.04	3.05±0.33	1.05±0.09	0.12±0.06	0.43±0.09	0.84±0.12	0.13±0.15	0.36±0.08	0.31±0.08
HD (27.0 mg/kg)									
n	9	9	9	9	9	9	9	9	9
Mean±SD	0.05±0.02	3.15±0.22	1.04±0.10	0.12±0.04	0.44±0.05	0.96±0.07*	0.09±0.03	0.45±0.06	0.33±0.09
5TD (13.5 mg/kg)									
n	9	9	9	9	9	9	9	9	9
Mean±SD	0.09±0.12	3.16±0.20	1.07±0.11	0.15±0.05	0.41±0.07	0.93±0.09	0.19±0.26	0.43±0.05	0.34±0.08
TD (2.7 mg/kg)									
n	10	10	10	10	10	10	10	10	10
Mean±SD	0.05±0.03	3.19±0.42	1.01±0.06	0.12±0.04	0.41±0.04	0.89±0.07	0.08±0.03	0.44±0.09	0.29±0.06

Figures in parenthesis indicate number of animals. *Significant at 5% level when compared with control group. SD: Standard deviation, SD: Standard deviation, HD: High dose, TD: Therapeutic dose

liver and kidneys and the drug was safe up to 500 mg/kg body weight. Whereas $HgCl_2$ at 1 mg/kg body weight alone showed pyknosis in the brain neurons and congestion of blood vessels was reported in the kidney. G. Kumar *et al*.[11] also attributed the

Table 6: Hematological analysis of male rats at 90th day

Group	WBC (10^3/mm^3)	RBC (10^6/mm^3)	Hb (g/dl)	HCT (%)	PLT (10^3/mm^3)	MCV (μm^3)	MCH (pg)	MCHC (g/dl)
Control								
n	10	10	10	10	10	10	10	10
Mean±SD	5.34±1.83	9.96±2.09	15.78±3.27	49.9±11.41	597.4±136.36	49.7±1.34	15.9±0.91	31.9±1.94
HD (27.0 mg/kg)								
n	10	10	10	10	10	10	10	10
Mean±SD	5.64±2.47	9.49±3.42	16.28±4.24	50.95±15.22	572.7±180.10	51.60±1.17*	16.72±1.08	32.38±2.03
5TD (13.5 mg/kg)								
n	10	10	10	10	10	10	10	10
Mean±SD	5.47±3.25	9.75±3.50	16.31±3.91	51.43±14.80	653.6±156.39	50±1.16	16.09±0.83	32.1±1.67
TD (2.7 mg/kg)								
n	10	10	10	10	10	10	10	10
Mean±SD	5.78±5.32	10.75±1.95	17.24±3.09	54.4±10.55	696.4±190.11	50.7±1.49	16.07±0.84	31.83±1.64

Table 7: Hematological analysis of female rats at 90th day

Group	WBC (10^3/mm^3)	RBC (10^6/mm^3)	Hb (g/dl)	HCT (%)	PLT (10^3/mm^3)	MCV (μm^3)	MCH (pg)	MCHC (g/dl)
Control								
n	9	9	9	9	9	9	9	9
Mean±SD	3.76±2.14	9.82±1.34	16.85±2.39	52.36±7.66	511.13±115.32	53.38±1.92	17.18±0.92	33.08±1.65
HD (27.0 mg/kg)								
n	9	9	9	9	9	9	9	9
Mean±SD	3.5±1.81	8.71±1.56	15.32±2.52	46.3±8.84	509.56±101.78	53.22±1.39	17.68±0.67	33.29±1.24
5TD (13.5 mg/kg)								
n	9	9	9	9	9	9	9	9
Mean±SD	2.43±1.26	8.73±1.58	14.94±2.55	46.22±9.00	522.33±193.94	52.89±1.54	17.12±0.48	32.46±1.25
TD (2.7 mg/kg)								
n	10	10	10	10	10	10	10	10
Mean±SD	3.55±1.79	8.642±1.77	14.9±2.87	45.33±9.84	561.1±195.94	52.3±1.16	17.29±.51	33.04±1.15

Figures in parenthesis indicate number of animals. SD: Standard deviation, WBC: White blood cells, RBC: Red blood cells, Hb: Hemoglobin, HCT: Hematocrit, MCV: Mean corpuscular volume, MCH: Mean corpuscular hemoglobin, MCHC: Mean corpuscular hemoglobin concentration, HD: High dose, PLT: Platelet, TD: Therapeutic dose

Table 8: Serum biochemical analysis of male rats at 90th day

Group	Glucose (mg/dl)	Total protein (g/dl)	SGOT (U/L)	SGPT (U/L)	Serum creatinine (mg/dl)
Control					
n	10	10	10	10	10
Mean±SD	70.1±21.59	7.18±1.28	218±89.91	62.3±8.18	0.44±0.14
HD (27.0 mg/kg)					
n	10	10	10	10	10
Mean±SD	77±16.23	6.09±0.78	167.3±52.99	64.3±14.07	0.39±0.15
5TD (13.5 mg/kg)					
n	10	10	10	10	10
Mean±SD	86.1±35.48	5.99±0.90*	150±58.57	51.3±8.81	0.35±0.15
TD (2.7 mg/kg)					
n	10	10	10	10	10
Mean±SD	65.2±19.83	5.81±1.25*	158±40.88	61.7±27.19	0.42±0.17

Figures in parenthesis indicate number of animals. *Significant at 5% level. SD: Standard deviation, SGOT: Serum glutamic oxaloacetic transaminase, SGPT: Serum glutamic pyruvic transaminase, HD: High dose, PLT: Platelet, TD: Therapeutic dose

nontoxic character of mercury in *Arogyavardhini Vati* to process of *Shodhana* due to which, mercury may not retain its original physiochemical form and hence toxic character. According to an experimental toxicity study,[12] *Makaradhwaja* when given orally did not produced mortality up to the dose of 480 mg/kg in mice. A 28 days repeated dose oral toxicity study of *Siddha*

Table 9: Serum biochemical analysis of female rats at 90th day

Group	Glucose (mg/dl)	Total protein (g/dl)	SGOT (U/L)	SGPT (U/L)	Serum creatinine (mg/dl)
Control					
n	9	9	9	9	9
Mean±SD	73.78±13.99	6.29±0.47	143.44±38.18	47.89±7.01	0.48±0.21
HD (27.0 mg/kg)					
n	9	9	9	9	9
Mean±SD	80.78±27.19	6.38±0.56	155.89±64.58	45.22±11.17	0.38±0.21
5TD (13.5 mg/kg)					
n	9	9	9	9	9
Mean±SD	87.78±21.21	6.54±0.99	142.56±51.92	43.78±9.88	0.44±0.25
TD (2.7 mg/kg)					
n	10	10	10	10	10
Mean±SD	75.4±24.24	6.07±1.57	149±48.13	42.1±13.61	0.42±0.19

Figures in parenthesis indicate number of animals. SD: Standard deviation, SGOT: Serum glutamic oxaloacetic transaminase, SGPT: Serum glutamic pyruvic transaminase, HD: High dose, PLT: Platelet, TD: Therapeutic dose

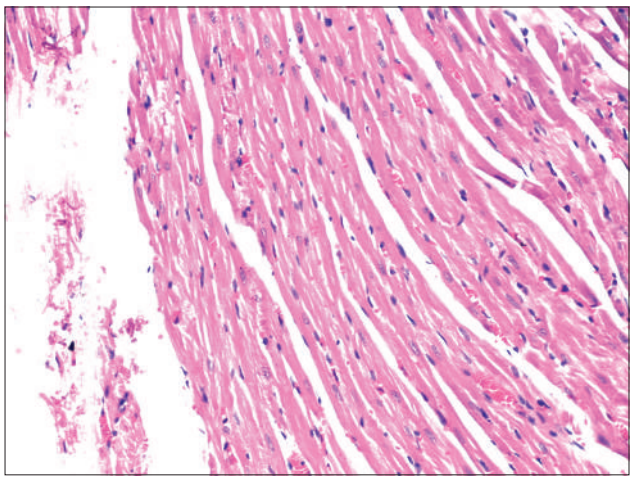

Figure 1: Heart control group: No abnormality detected (10 × 10)

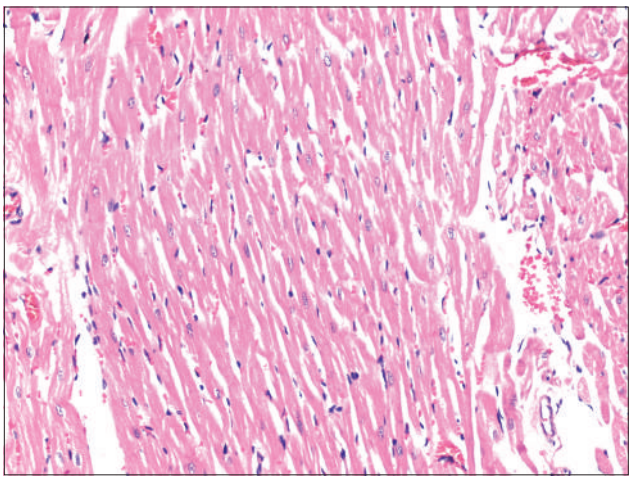

Figure 2: Heart high-dose group: No abnormality detected (10 × 10)

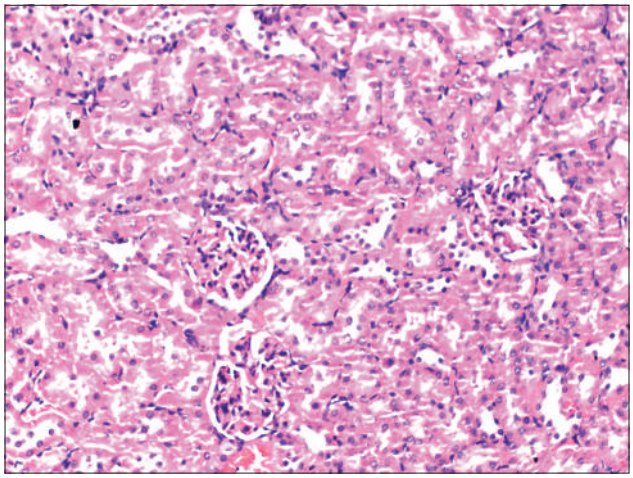

Figure 3: Kidney control group: No abnormality detected (10 × 10)

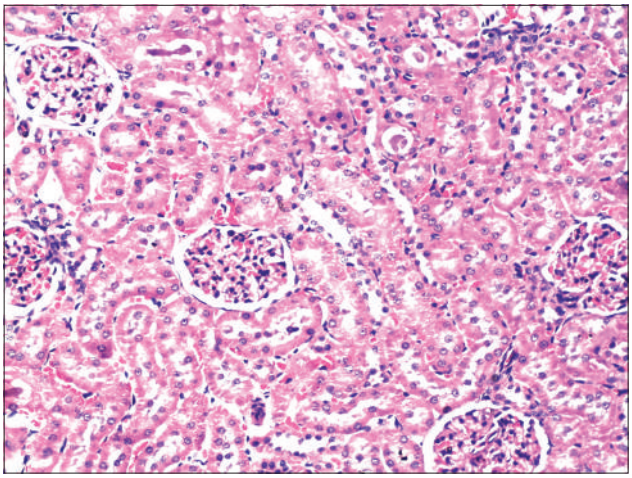

Figure 4: Kidney High Dose Group: No abnormality detected (10× 10×)

Makaradhwaja in rats showed neurodegenerative changes in brain at the dose of 100 mg/kg, but no histopathological changes were seen at dose level of 50 mg/kg in the kidney, liver and brain. Further no changes were seen in serum alanine aminotransferase, aspartate aminotransferase, alanine phosphatase, bilirubin, urea and creatinine implying the safety of *Siddha Makaradhwaja* on hepatorenal system of the rats.[13] Both *Triguna* and *Shadaguna Makaradhwaja*

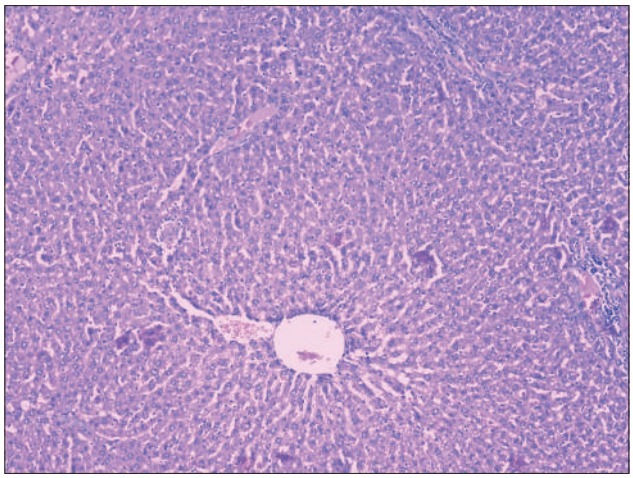

Figure 5: Liver control group: No abnormality detected (10 × 10)

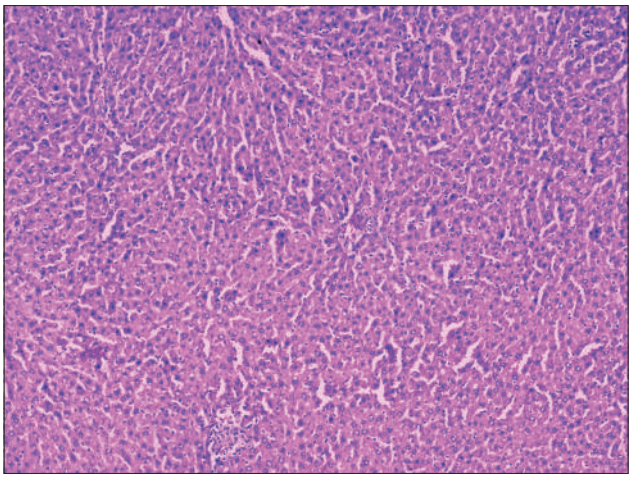

Figure 6: Liver high-dose group: No abnormality detected (10 × 10)

offered *Madhumehahara* property (antidiabetic) in clinical trials, but *Shadguna Balijarita* possessed more effect than *Triguna Balijarita Makaradhwaja*. It has experimentally shown antihyperglycemic, antidiabetic, renoprotective, cardiac stimulant activity and is a known immunomodulating agent in experimental studies with its use being reached in veterinary practices too.[14] A pilot clinical trial of *Makaradhwaja* has shown promising results for treating rheumatoid arthritis, but the study also showed mercury excretion in urine.[15] In the present study, the results are in line with earlier studies and histopathological evaluation also indicated no evidence of treatment-related or dose dependent lesions in any organ. It is apparent that *Makaradhwaja* is safe at dose rate of 27 mg/kg/day for ninety consecutive days. However, there was significant increase in kidney weights of females from HD group, which could not be corroborated with serum creatinine or other biochemical data and histopathological evaluation findings. Therefore, HD level, that is, 27 mg/kg body weight was decided as NOAEL and mid-dose level, that is, 13.5 mg/kg body weight was decided as NOEL was. Thus, the output of the study is in accordance with the results and conclusion by Kumar *et al*.[11] which underlined that the mere presence of very high proportion of mercury in *Makaradhawaja* does not possess any risk or toxic potential to animals or humans. The purification process adds several organic chemicals to the crude form of mercury which may lead to altered physicochemical properties of mercury and hence alters its toxic potential.[11,16-18]

Conclusion

When *Makaradhwaja* was administered daily for ninety consecutive days at dose levels 27, 13.5 and 2.7 mg/kg/day by oral route to Wistar rats, it was found that there was no effect on body weights, feed consumption and biochemical parameters. There was significant increase in kidney weight of male and females treated with HD which could not be correlated with histopathological and serum biochemical changes. There was no treatment-related changes observed in the histopathological evaluation at dose level 27 mg/kg. There was significant increase in MCV value of males treated with HD but was within biological limits. The dose level 27 mg/kg of *Makaradhwaja* was found as NOAEL and dose level 13.5 mg/kg of *Makaradhwaja* was found as NOEL. Ayurvedic preparation method of *Makaradhwaja* could be altering physicochemical properties of raw mercury rendering it to become significantly less harmful.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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Physicochemical characterization of *Suvarna Bhasma*, its toxicity profiling in rat and behavioural assessment in zebrafish model

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ABSTRACT

Ethnopharmacological relevance: Suvarna Bhasma is a gold-based *Ayurved* medicine that has a wide range of therapeutic indications like tuberculosis, diabetes mellitus, rheumatoid arthritis and nervous diseases. *Suvarna Bhasma* is also used in *Suvarnaprashana*, an *Ayurved* advocated therapy being practised to improve immunity in children.

Aim of the study: To augment traditional understanding, here we present an evidence-based study on *Suvarna Bhasma* regarding its physicochemical properties, toxicity and efficacy.

Materials and methods: Suvarna Bhasma was characterised by physicochemical characterization techniques such as scanning electron microscope (SEM), transmission electron microscopy (TEM), X-ray diffraction (XRD) and atomic emission spectroscopy (ICP-AES). Toxicity of *Suvarna Bhasma* was studied in Holtzman rats with daily oral dose from 3 mg/kg (therapeutic dose, TD) up to 30 mg/kg (10 TD) body weight for 90 days. Behavioural study, such as motor and geotactic behaviour were examined in zebrafish model to find out any sign of neurotoxicity or behavioural changes due to *Suvarna Bhasma* administration.

Results: Suvarna Bhasma has two types of gold particles, large ones (~60 μm) having irregular shapes, and nano-sized spherical particles (starting from ~10 nm), the latter coated with Fe, Si, O, P and Na. XRD study revealed that all the peaks of *Suvarna Bhasma* match well with pure gold (face centred cube) with crystallites size 45 ± 2.8 nm. In rat studies, some change in biochemical parameters such as urea, creatinine and alanine aminotransferase (ALT) was observed mainly at the higher therapeutic dose; however, those parameters were within the normal range. There were no significant macroscopic as well as microscopic treatment-related alteration observed, in any of the organs and tissues evaluated. In zebrafish behavioural study, the motor parameters of *Suvarna Bhasma* treated fish showed normal behaviour analogous to the vehicle control group. Interestingly, the geotactic behaviour showed anxiolytic effects of *Suvarna Bhasma* as evidenced by the time spent in the upper zone, and average swimming height. The anxiolytic effects persisted for more than 30 days after withdrawing the *Suvarna Bhasma* treatment.

Conclusions: Suvarna Bhasma contained spherical gold nanoparticles. It was nontoxic in rat model at the does tested. *Suvarna Bhasma* has anxiolytic effects in zebrafish behavioural model.

1. Introduction

Suvarna Bhasma (also spelt as Swarna Bhasma) is a gold-based Ayurved medicine. It is used in the treatment of diseases such as asthma, rheumatoid arthritis tuberculosis, diabetes mellitus, immune and nervous disorder (Singh, 2014; Yadav and Chaudhary, 2015; Patel and V Shah, 2013). In Suvarna Bhasma, gold in elemental form is the major element (> 98%) and it is the active ingredient. *Suvarna Bhasma,* along with honey, was also prescribed as a tonic for rejuvenation (Williamson, 2004). *Suvarnaprashana* is widely advocated by *Ayurved* practitioners and is gaining popularity as an *Ayurved* therapy to improve immunity of the child (Rao et al., 2012; Samant and Patil, 2014).

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In this *samskara* (ritual), *Suvarna Bhasma* is administered and sometimes administered with pure honey and medicated ghee processed in *Medhya* (nootropic) and *Rasayana* (rejuvenating/anti-ageing) herbs as per the preference of the practitioners. *Kashyap Samhita* describes it to improve cognitive functions, digestive capacity, strength and longevity (Jyothy et al., 2014a). Likewise, in recent times, gold nanoparticles are attracting researchers, especially in the biomedical field, as these nanoparticles are biocompatible and having immense therapeutic and diagnostic applications. *Suvarna Bhasma*, comprising of nanogold particles, renewed the interest of the scientific community for finding its applicability in chronic diseases such as diabetes and cancer (Das et al., 2012; Kumar Pal, 2015).

Suvarna Bhasma is considered as one of the most prominent metal based medicine in *Ayurved,* which has both protective and curative properties on numerous health problems (Jyothy et al., 2014a). Gold based *Ayurved* medicines are traditionally used for centuries, however, a scientific evidence-based study is extremely required to find out the safety and efficacy of *Suvarna Bhasma* in the animal models. Therefore, this study was focused on a comprehensive assessment of *Suvarna Bhasma* by exploring its safety in rat model and efficacy in zebrafish behaviour model along with the physicochemical characterization of *Suvarna Bhasma* particles.

Interestingly, the physicochemical properties of *Suvarna Bhasma* may differ from manufacturer to manufacturer. This may happen because the traditional pharmacopoeia (texts) describe different preparation protocols for the same medicine. For example, approved Ayurved scripture, *Rasatarangini* has described five different methods for the preparation of *Suvarna Bhasma*. Also, in the 5th volume of *Bharat Bhaishajya Ratnakar* (A compilation of Ayurved formulations from various Ayurved texts) has described 20 different methods for the preparation of *Suvarna Bhasma.* (Suvarna Bhasma used in these studies was manufactured using the method described in Bharat Bhaishajya Ratnakar 5/8357). Particles size, shape, and gold concentration vary largely for *Suvarna Bhasma* from a different origin. For example, Beaudet et al. (2017) showed that Au present in *Suvarna Bhasma* was approximately 57 wt%, Brown et al. Brown et al. (2007) reported 92 wt % Au in *Suvarna Bhasma,* whereas Thakur et al. (2017) found 98% gold in *Suvarna Bhasma.* Similarly, the *Suvarna Bhasma* crystallite size also varies largely from 23 nm (Brown et al., 2007) to 60 nm (Beaudet et al., 2017) according to the two different research articles. Therefore, before *in-vivo* studies, *Suvarna Bhasma* used in this work was physicochemically characterised. Here, the physicochemical study revealed some unique features about *Suvarna Bhasma* which were not reported in the literature. Additionally, it was found that for this particular *Suvarna Bhasma*, batch to batch variation was not significantly observed.

In the *Suvarna Bhasma* preparation process, metallic Hg is very frequently used, which is a common neurotoxin and Au is also not an essential element (Bhattacharya et al., 2016; Broussard et al., 2002), though a dose of 15–30 mg for an adult human is normal and the dose may be prescribed for a longer duration. Therefore, besides its traditional belief, a proper toxicological study was carried out in this work. In recent literature, some *in-vitro* and *in-vivo* toxicological studies were been reported, which concluded that *Suvarna Bhasma* was nontoxic (Beaudet et al., 2017; Paul and Sharma, 2011; Mitra et al., 2002; Khedekar and Priya, 2016; Jamadagni et al., 2015). The genotoxicity of *Suvarna Bhasma* was studied by Selkar et al. (2016) and they reported it is safe in terms of genotoxic and mutagenic activity. However, most of the *in-vivo* toxicity studies reported short term effects of *Suvarna Bhasma* in the rodent, for example, Khedekar et al. (Khedekar and Priya, 2016) reported the effect of *Suvarna Bhasma* in albino rats for 10 days treatments. On the other hand, Khan et al. (2018) administered *Suvarna Bhasma* for 20 days in albino rats. Jamadagni et al. (2015) did a 90 days toxicity study in Wistar rats with a maximum *Suvarna Bhasma* dose of 13.5 mg/kg. However, this group (Jamadagni et al., 2015) reported data only for the alteration of body/organ weight and histopathology of *Suvarna Bhasma* treated rats. In this present study, a comprehensive toxicity study was carried out with three different doses, a low dose (3 mg/kg), a medium dose (15 mg/kg) and a high dose (30 mg/kg) of *Suvarna Bhasma* was administered orally for consecutive 90 days to male and female rat separately. Various haematological, biochemical and histopathological analysis was conducted after 45 and 90 days of *Suvarna Bhasma* treatment.

Additionally, *Suvarna Bhasma* was administered in a zebrafish model to observe behavioural changes. The behaviour of zebrafish is robust; abnormality in behaviour can be evoked by drug-induced toxicity (V Kalueff et al., 2013). With the aid of video tracking methodology, the behaviour of zebrafish can be analysed with high accuracy. In this study, zebrafish were efficiently tracked in the novel tank with the automated video tracking process. With the aid of indigenously build MatLab code, various motor and phenotype behaviour were accurately evaluated.

2. Materials and methods

2.1. Chemicals

The preparation of the *Suvarna Bhasma* (Batch no: P110600209) used in this study was carried out by Shree Dhootapapeshwar Limited, Mumbai, according to the classical *Ayurved* text (*Bharat Bhaishajya Ratnakar* 5/8357, Fig. 1). *Suvarna Bhasma* was prepared (Fig. 1) from gold bar (99.99% purity), following a rigorous *Ayurved* process consisting of *Shodhana* and *Marana* (special procedures meant for the conversion of metals into *Bhasma*). The gold bar was converted in the form of thin gold ribbons by passing through a machine by exerting pressure and these were further cut into small pieces. These gold pieces were then purified by quenching them in *Taila* (sesame oil), *Takra* (butter milk), *Gomutra* (Cow's urine), *Kanji* (fermented rice water), *Kulattha kwatha* (decoction of *Dolichos biflorus* seeds) and *Kanchanara kwatha* (decoction of *Bauhinia variegata* stem bark), for 7 times in each liquid. Then the purified gold pieces were amalgamated with Hg at 1:2 wt ratio (Au:Hg). The Au–Hg amalgamate was then covered with S powder in an earthen pot and heated slowly up to 750 °C. After cooling down of the earthen pot, the intermediate product was found in a powder form. The heating process of the intermediate product with S powder was repeated for 13 times (total 14 times) which resulted in the final form of *Suvarna Bhasma* in the form of fine powder. The manufacturing process of *Suvarna Bhasma* took about 21 days. A quantitative detail for a typical *Suvarna Bhasma* batch (1 kg) has been provided in Supplementary File S2.

2.2. Physicochemical characterization of Suvarna Bhasma

Suvarna Bhasma was characterised to understand its physicochemical properties. The crystallographic details of *Suvarna Bhasma* were analysed by powder X-ray diffraction (XRD, Smart Lab, Rigaku, Japan) and peaks were compared with ICDD (International Centre for Diffraction Data) database. Transmission electron microscope (TEM, JEOL 2100, Japan) attached with energy dispersive spectroscopy (EDAX, OXFORD instrument, United Kingdom) was used to observe the morphology and elemental mapping of *Suvarna Bhasma* particles. TEM analysis was carried out after suspending *Suvarna Bhasma* particles in water; then the suspension was sonicated for 10 min and allowed to settle down for 5 min. After settling down, the lightweight suspended *Suvarna Bhasma* particles from the upper water level were pipetted out and fixed on the carbon coated copper grid for TEM analysis (Brown et al., 2007). The backscatter imaging of *Suvarna Bhasma* was grabbed by a scanning electron microscope (FEGSEM, JEOL, Japan). Elemental analysis of *Suvarna Bhasma* was carried out using inductively coupled plasma atomic emission spectroscopy (ICP-AES, ARCOS, SPECTRO Analytical Instrument, Germany) after digesting *Suvarna Bhasma* in *aqua regia.* The surface elemental analysis was conducted by high-resolution X-ray photoelectron spectroscopy (HRXPS, Kratos Analytical,

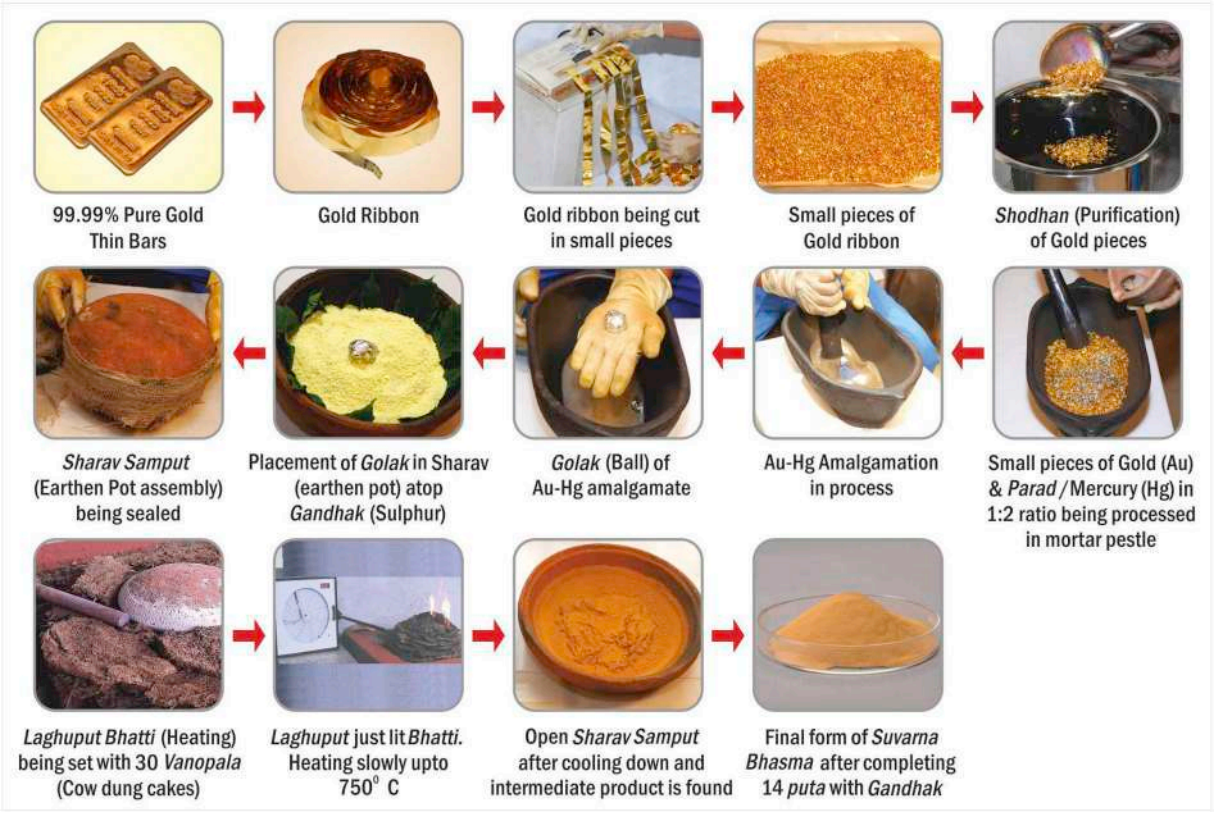

Fig. 1. Flow chart of *Suvarna Bhasma* preparation starting from gold bar. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Japan) equipped with a monochromatic X-ray source of 1486.6 eV. Thermogravimetric analysis was carried out with TGA analyser (PERKIN ELMER, USA). Details of physicochemical analysis carried out are enlisted in Table 1.

2.3. Animal ethics

Ethical clearance for the use of animals in the study was obtained from the Institutional Animal Ethics Committee of National Institute for Research in Reproductive Health (NIRRH), Parel (Study number 09/11) prior to the initiation of the study. The experiments were performed in accordance with the guidelines of the Committee for the Purpose of Control and Supervision of Experimental Animals (CPCSEA), India.

2.4. Toxicity assessment of Suvarna Bhasma in rat-model

Toxicological study of *Suvarna Bhasma* was conducted in the Holtzman rat model following OECD guidelines for sub-chronic toxicity. Total 90 rats (45 males and 45 females) of 9–10 weeks of age were randomly selected and assigned to the control and the treatment groups (Table 2) after the acclimatization of five days prior to the start of the study. The weight variation of animals used did not exceed ± 20% of the mean weight of each sex. The animals were kept in polypropylene cages and maintained under controlled temperature (23 ± 1 °C), humidity (55 ± 5%), and in a 14 h light/10 h dark cycle. Maximum three animals were housed in a single cage. All animals were acclimatized for five days prior to exposure of the test items.

Table 1
Details of physicochemical analysis carried out in this study.

Analysis	Instrument	Operation details	Sample Preparation
X-ray diffraction (XRD)	XRD, Smart Lab, Rigaku, Japan	Source: Cu K-α, wavelength = 1.5406 Å Scanning range 2theta = 10–90°	Powder sample was placed on a glass plate and put for XRD scanning
Transmission electron microscope (TEM)	JEOL 2100, JEOL, Japan	Electron source 200 KV	Powder gold particles (Suvarna Bhasma) were suspended in water and sonicated for 10 min, after settling down, the particles from upper water layer were pipetted out and put on the carbon coated grid
Scanning electron microscope (SEM)	JSM-7600F, JEOL, Japan	Field emission gun	Suvarna Bhasma powder was fixed on carbon tape, then the tape was placed on a sample holder.
Energy dispersive spectroscopy (EDS)	OXFORD instrument, UK, attached with TEM	Mapping in scanning transmission electron microscope (STEM) mode	Same as TEM sample preparation
Atomic emission spectroscopy (ICP-AES)	SPECTRO Analytical Instruments GmbH, Germany	Wavelength Range: 130 nm–770 nm. Detector: Charge Coupled Devices (CCD)	*Suvarna Bhasma* (~10 mg) was digested in *aqua regia* and then diluted with MiliQ water
Thermogravimetry (TGA)	PERKIN ELMER, USA	From room temperature to 1250 °C at 10 °C/min rate	Powder sample used directly
High-resolution X-ray photoelectron spectroscopy, (HRXPS)	Kratos Analytical, Japan	*X-ray source of 1486.6* eV	Suvarna Bhasma powder fixed on the carbon tape and placed on the sample holder

Table 2
Grouping of rats and dose level of Suvarna Bhasma.

Group No	Groups	Group Name	Dose (mg/kg body weight)	No of Males	No of Females
I	Control	VC	Vehicle	10	10
II	Low	SB3	3 mg/kg (TD)	10	10
III	Mid	SB15	15 mg/kg (5 × TD)	10	10
IV	High	SB30	30 mg/kg (10 × TD)	10	10
V	[a]Recovery	SB30R	30 mg/kg (10 × TD)	5	5

[a] Recovery group was sacrificed 15 days after the completion of treatment. TD = therapeutic dose.

Suvarna Bhasma was administered orally to the animals with a syringe once daily for a period of 90 days. Animals from the control group were treated with vehicle alone (xanthine gum, 2% w/v). Dose-volume was adjusted based on the weekly body weight of the individual animal. Except for the treatment with the test item, animals in the control group were handled in an identical manner to those in the test groups. The dose volume administered to each animal was calculated based on a constant factor of 2 ml/kg. Dose levels of 3, 15 and 30 mg/kg of *Suvarna Bhasma* were given for this study. *Suvarna Bhasma* was weighed and formulated with xanthine gum (2% w/v) freshly on each day of dosing. The concentration of *Suvarna Bhasma* was adjusted at 1.5, 7.5 and 15 mg/ml to administer the doses of 3, 15 and 30 mg/kg body weight. Body weight and feed intake of all the animals were monitored throughout study period.

Blood was collected from retro-orbital plexus under light anaesthesia (3–4% isoflurane) in two separate vials, one for haematology (EDTA used as anticoagulant) and other for serum biochemistry analysis. The haematological analysis was performed on freshly collected blood samples by using haematology analyser (Abacus, Diatron, Hungary) and, the serum samples separated after incubation at 37 °C of whole blood were stored at −20 °C for further analysis. The serum biochemistry analysis was performed by using fully automated serum biochemistry analyser (EM 200, ERBA).

At scheduled terminal necropsy, all surviving animals were humanely euthanized by CO_2 asphyxiation and subjected to complete gross pathological examination. All collected organs were fixed in 10% neutral buffered formalin until processing. Organ weights of tissues were taken immediately after collection. These tissues were processed in an automatic tissue processor (ASP300, Leica, Germany) and embedded in the paraffin wax using a tissue embedding system (EG 1150H, Leica, Germany). The embedded tissues were further trimmed with the help of an automatic microtome (RM 2255, Leica, Germany) and sections were cut at 5 µm thickness and taken on a clean, grease-free slide for further staining with Haematoxylin and Eosin with automatic tissue stainer (Autostainer XL, Leica, Germany). Histopathological examination was performed on the specified list of tissues including all macroscopically abnormal tissues of all control and high dose group animals sacrificed at termination.

2.5. Behavioural experiments on zebrafish

Adult wild-type zebrafish (*Danio rerio*) of 5–6 months of ages were purchased from an authenticate zebrafish supplier (Vikrant Aquaculture, Mumbai, India). The zebrafish were kept in a lab-costumed housing system (Biswas et al., 2018), having biological, chemical and mechanical filtration facility to maintain the water quality preferred for zebrafish. The water temperature of zebrafish housing was kept consistent throughout the experimentation at 25–27 °C. The fish were maintained in 14 h light and 10 h dark cyclic period. The experiment was carried out after two months of acclimatization periods.

Zebrafish were divided into four groups as Vehicle Control (VC), *Suvarna Bhasma* (SB), Comparative Control (CC, 1.5% alcohol) and Positive Control (PC, 50 mg/L caffeine) groups. For VC and SB groups, a total of 40 fish/group was allotted, whereas CC and PC group had 20 fish each. For the CC group, alcohol (1.5 vol %) and for PC group, caffeine (50 mg/L) was given for 30 min (n = 20) to fish individually before behaviour tracking. In each experimental set, the male to female ratio was the same for VC and SB groups. Zebrafish from each group were kept as a cohort of 10 fish separately in a tank; therefore behaviour experiment was independently run in quadruplicate for VC and SB (Total n = 40) groups, whereas behavioural experiment was done in duplicate for CC and PC groups with total n = 20 fish/group.

All fish were given dry food twice a day (Tetra, Germany). The *Suvarna Bhasma* was orally given mixed with dry granular food (Micro Wafers, Hikari Tropical, Japan) to the SB group daily for 15 consecutive days at the afternoon time. After the completion of *Suvarna Bhasma* treatment, on the 16th day at the afternoon time fish were placed individually in a novel tank (dimension: L × H × W = 200 mm × 170 mm × 130 mm) and the video was recorded from the top and side views with two webcams (Logitech B525). For CC and PC group, fish was treated with alcohol and caffeine respectively and behaviour was recorded in the same novel tank. Comparative control (CC) fish were individually exposed to 1.5 vol % alcohol in water for 30 min in a two-litter tank before novel tank tracking which has anxiolytic effects on zebrafish (Hamilton et al., 2017). Caffeine, an anxiogenic to zebrafish behaviour, was similarly treated for 30 min at a concentration of 50 mg/L water to the PC group (Egan et al., 2009; Collier, 2017).

Trajectories of all fish from individual videos were extracted with the help of open source MatLab software: idTracker (Pérez-Escudero et al., 2014). Each video was grabbed at a speed of 30 frames/sec. Videos of individual tracking were processed from frame number 1800 to 10800 (total 5 min/fish). From the trajectories, various motor parameters (speed, meander, and freeze point) and geotaxis phenotype behaviour (preference of being in the upper or lower zone) were calculated by indigenously developed MatLab code. To observe the after effect of *Suvarna Bhasma*, 30 fish from each SB and VC groups was kept in housing system and again tracked after 30 days (without any drug treatment) in the novel tank similarly as described above.

The oral drug dose for zebrafish was prepared by mixing *Suvarna Bhasma* with dry granular food and COD liver oil as sticking substance for 15 min so that the *Suvarna Bhasma* stuck to outside of the granular food. After ICP-AES quantification of the gold per gram of dry food + *Suvarna Bhasma*, the exact amount dose (dry food + *Suvarna Bhasma*) was given to the fish of SB group at a dose of 60 mg/kg fish weight. The calculated dose of the granules was added to the feed tank. Since *Suvarna Bhasma* is not soluble in water, granules retained their identity until consumed. The control groups were also provided dry food mixed with COD liver oil (without *Suvarna Bhasma*).

As drug dose was given in the water, there was always a chance of elution of *Suvarna Bhasma*. However, from ICP-AES analysis, it was confirmed that elution of *Suvarna Bhasma* particles was not more than 10% even after 5 min immersion in water. On the other hand, as the oral dose was given to the fish cohort, so there is always a possibility of unequal distribution of drug dose. One could separately feed those fish, but as zebrafish is a social animal, separating them could lead to unnatural behaviour in them. Also, we observed that in isolation, attraction towards food in zebrafish decreases. The cohort was given dose which contains approximately 40–50 food granules (with *Suvarna Bhasma*), and within 2 min the whole bowl of the dose was consumed. There was no avoidance towards *Suvarna Bhasma* dose observed in the fish cohort. Therefore, it was concluded that the amount of *Suvarna Bhasma* dose consumed by fish was proportional to fish's body weight.

2.6. Statistical analysis

Statistical analysis was performed using GraphPad Prism and Origin software. Data were analysed for dose wise comparison. One-way-

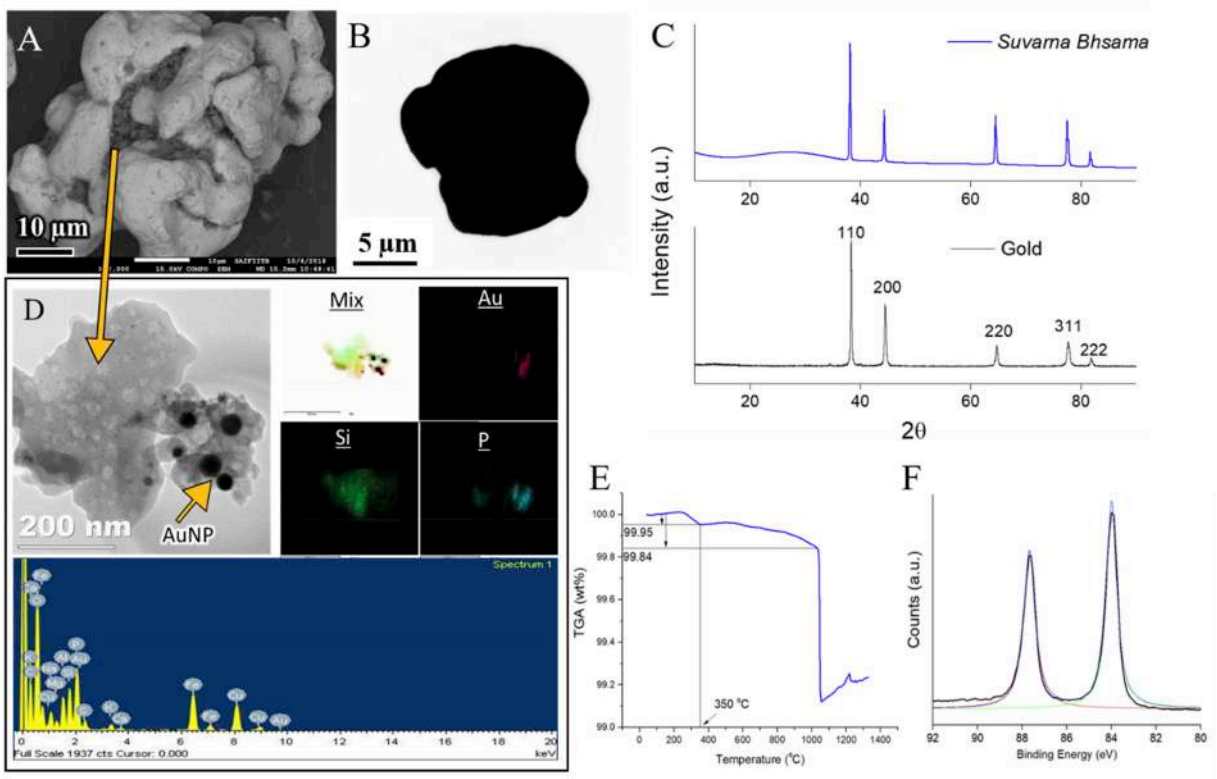

Fig. 2. Physicochemical characterization of *Suvarna Bhasma*. A) SEM backscatter image, B) TEM image of big *Suvarna Bhasma* particles, C) XRD profile of *Suvarna Bhasma*, D) TEM image and EDAX mapping of small *Suvarna Bhasma* particles, E) TGA profile of *Suvarna Bhasma*, and F) HRXPS (gold 4f region). XRD profile of *Suvarna Bhasma* perfectly matches with pure gold. *Suvarna Bhasma* contained encapsulate spherical nanoparticles starting from 10 nm size. EDAX mapping of small *Suvarna Bhasma* particles shows peaks of Au, Si, O, P, Fe, Ca etc. From TGA profile, it was observed that organic carbon may not present in *Suvarna Bhasma*. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

analysis of variance (ANOVA) followed by Tukey's test was used to compare significance between various treatment groups.

3. Results

3.1. Physicochemical characterization of Suvarna Bhasma

Physicochemical study of *Suvarna Bhasma* revealed some new interesting features about it. *Suvarna Bhasma* contained very big particles as well as nanosize gold particles (Fig. 2A, B, and 2D). The nanosized gold particles were spherical, starting from ~10 nm in size (Fig. 2D). The spherical gold nanoparticles were encapsulated by an envelope of Si, O, P and Fe coating (Fig. 2D). These whole encapsulate gold particles were again embedded in the big gold particles (Fig. 2A). The large particles were agglomerated and irregular in shape having size up to 60 µm. The XRD peaks of *Suvarna Bhasma* matched with that of pure gold with no other extra peaks (Fig. 2C). The crystal size of *Suvarna Bhasma*, calculated using Sherrer's equation, was approximately 45 ± 2.8 nm. However, EDAX (Fig. 2D) and ICP-AES analysis show presence other elements as well, such as Fe, Ca, Si etc. Further analysis of *Suvarna Bhasma* using ICP-AES and ICP-AAS revealed that it contains approximately 98% Au (Table 3).

Up on thermal analysis (TGA), it was noticed that only 0.05% weight loss occur up to 350 °C and 0.16% weight loss up to ~1050 °C. HRXPS found only gold state (Au^0), other gold peaks (such as gold salt) was not present in the *Suvarna Bhasma*.

3.2. Toxicological assessment in rat model

3.2.1. Haematology

The effects of *Suvarna Bhasma* on the haematological parameters are represented in Fig. 3 for male (Fig. 3A, C) and female (Fig. 3B, D) rats separately. There was no significant alteration observed in *Suvarna Bhasma* treated groups after 45 days (Fig. 3A and B) of treatment among any of the haematological parameters such as, haemoglobin level, counts of blood cells, mean corpuscular haemoglobin concentration (MCHC) etc. when compared with the vehicle control group (Details of other haematological parameters are enlisted in the Supplementary file S1-Table 3). Similarly, there were no significant alterations in any of the haematological parameters performed at the termination day (after 90 days) except percentage of red blood cells PCV % in SB3 group in female rats (Fig. 3A and B and Table 3d in Supplementary file S1).

Table 3
ICP-AES and ICP-AAS analysis of *Suvarna Bhasma* after digestion in *aqua regia* (ND = not detected).

Element	ICP-AES (Wt%)
Au	98.2 ± 1.82
Si	0.06
Fe	0.19
Ca	0.13
Cu	0.01
Zn	0.05
Pb	ND
As	ND
Hg	ND

3.2.2. Clinical chemistry

Serum biochemistry was similarly analysed at the two time points in rats (Fig. 4). After 45 days of treatment, significant alteration in some serum biochemical parameters was witnessed in the treated groups when compared with the control group. The significant variation in

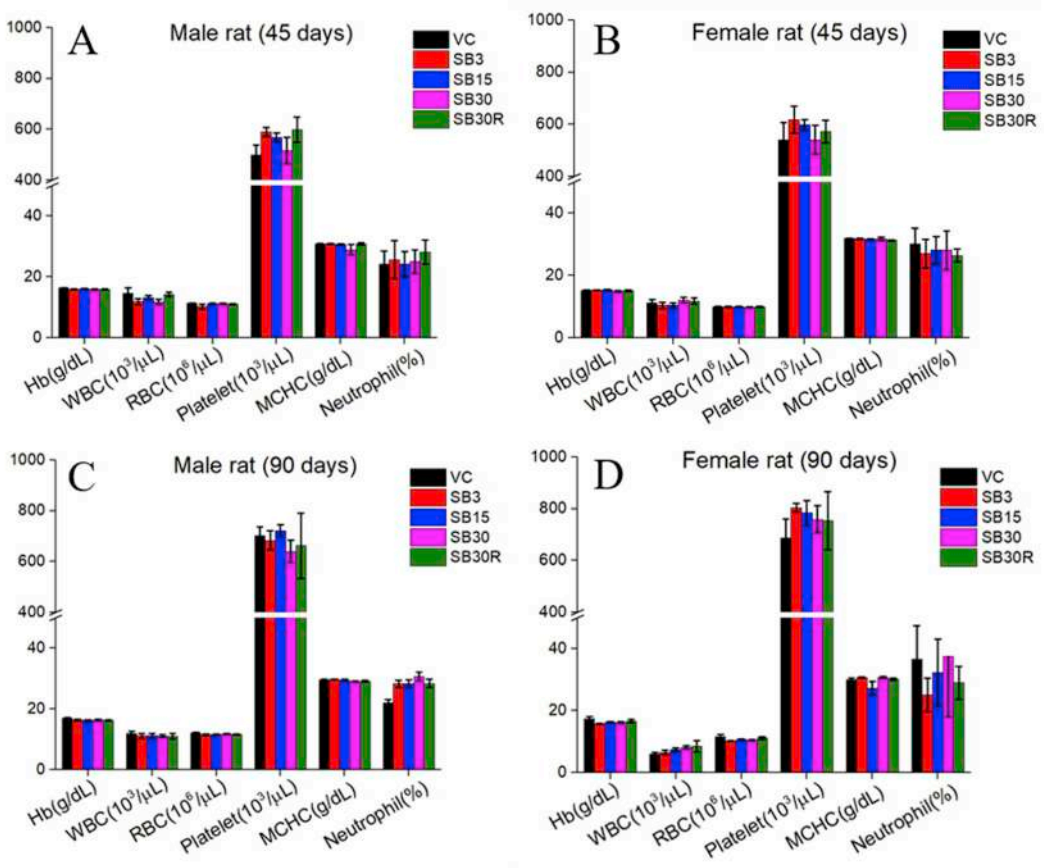

Fig. 3. Effects of *Suvarna Bhasma* on haematological parameters in rats. A) Male rats after 45 days of treatment, B) Female rats after 45 days of treatment, C) Male rats after 90 days of treatment, and D) Female rats after 90 days of treatment. Values are expressed as mean ± SEM (standard error of the mean) with *p < 0.05 vs vehicle control [n = 10 animal/group, except Group V (n = 5)]. No significant alteration observed among any of the haematological parameters. Neutrophil counts for *Suvarna Bhasma* treated groups was increased for male rat after 90 days treatment without any statistical significance (p > 0.05).

males included increased creatinine and triglyceride levels in the mid-dose group (SB15) (Fig. 4A). The significant variations in females (Fig. 4B) included decreased creatinine and AST in the high dose group (SB30), increased glucose and DBIL (direct bilirubin) in the recovery group (SB30R), increased glucose level of the recovery group (SB30R), increased TBIL (total bilirubin) in the SB3 group, and increased phosphorus levels in the groups SB30 and SB30R as compared to vehicle control females (Supplementary file S1-Table 4). All the above variations were well within the normal range, hence do not carry any toxicological significance.

Likewise, in the serum biochemistry performed at the termination (after 90 days), many biologically as well as toxicologically insignificant parameters either increasing or decreasing mere trends were observed (Fig. 4C and D). Those statistical significant variations in males included increased urea in mid and high dose (SB15, SB30 and SB30R), increased creatinine in high dose group (SB30), decreased TBIL at high dose (SB30), decreased creatinine and cholesterol in recovery dose group (SB30R) when compared to the vehicle control group (Fig. 4C and Supplementary file S1-Table 4c). Similar significant variations in females (Fig. 4D and Supplementary file S1-Table 4d) included decreasing trends AST and ALT in mid (SB15) and high (SB30) dose groups, increasing DBIL in SB15 and SB30, decreasing uric acid and increasing in glucose level in SB30 as compared to the female control group. However, all the above variations were well within the normal range, hence do not carry any toxicological significance.

3.2.3. Organ weight

No treatment-related adverse effects in absolute organ weight and relative organ weight were noticed during the study period (Supplementary file S1-Table 5).

3.2.4. Gross pathology

There were no gross pathology observations recorded during the terminal necropsy examination of all animals including control and treatment groups. The only gross pathology observation recorded during the study was with the found dead animal (from SB30, Animal No. 459F, Datafile 1), it was suppuration in meninges and brain. This gross pathology observation was not related to treatment, but it was due to infection in meninges and brain.

3.2.5. Histopathology

Histopathological examination was performed on the specified list of tissues including macroscopically abnormal tissues of all control and high dose group animals sacrificed at termination. Tissues collected from a found dead animal (animal no. 459 F) were also processed for histopathology examination.

Four step grading system of minimal (+), mild (++), moderate (+++) and severe (++++) were used to rank microscopic findings for comparison among the groups. The histopathology lesions observed are summarized in the following table (Table 4) separately for males and females:

There were no significant macroscopic as well as microscopic treatment-related alteration observed, in any of the organs and tissues evaluated (Fig. 5). All above histopathological alterations that were observed were all considered as either spontaneous or known background findings that are usually observed in laboratory rats of this strain and age under present experimental conditions.

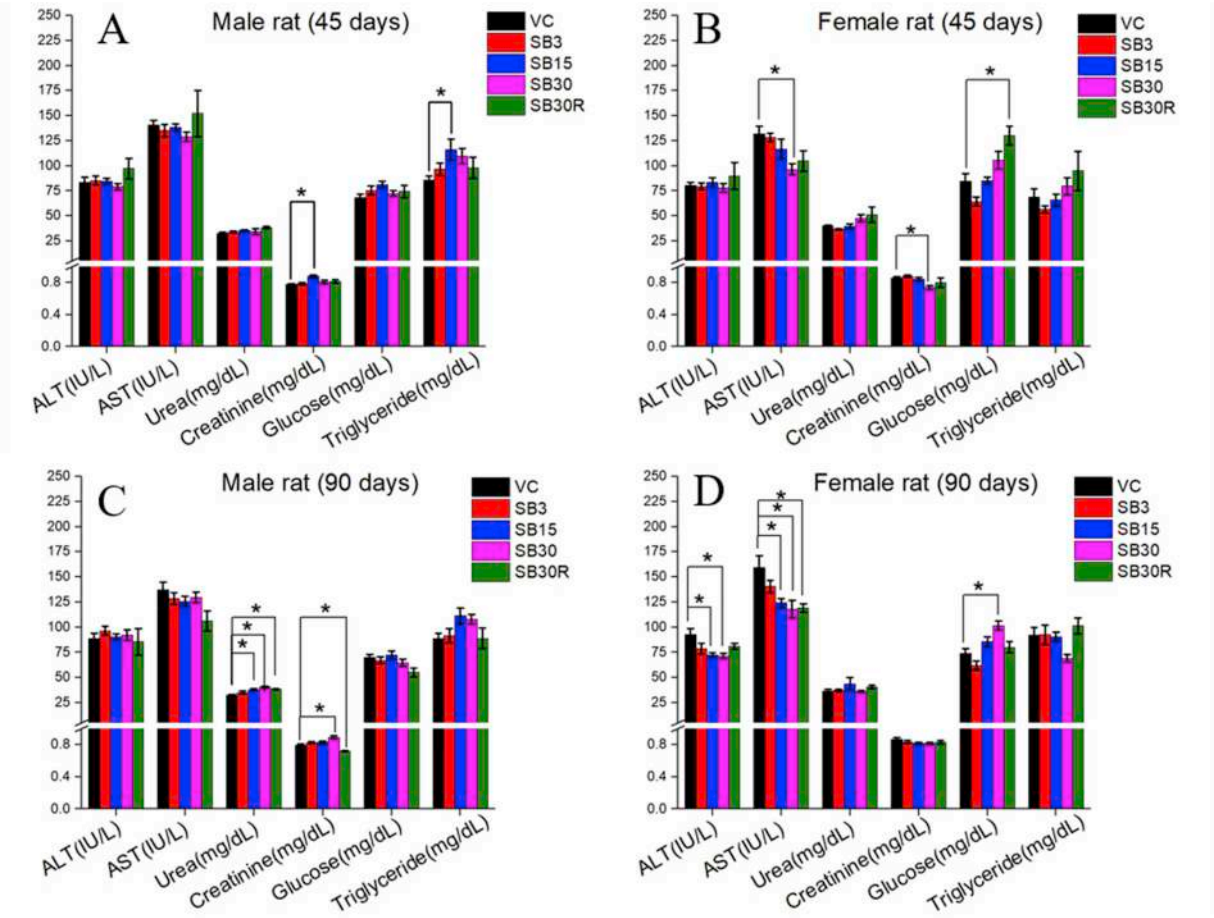

Fig. 4. Effects of *Suvarna Bhasma* on serum biochemistry in rats. A) Male rats after 45 days of treatment, B) Female rats after 45 days of treatment, C) Male rats after 90 days of treatment, and D) Female rats after 90 days of treatment. *$p < 0.05$ vs vehicle control [n = 10 animal/group, except Group V (n = 5)]. Alteration observed for few parameters such as urea, ALT, AST etc. However, the alterations were not consistent, for example, after 90 days treatment, urea and creatinine were significantly changed for male rats (SB15 and SB30) whereas for female rats the changes were not significant statistically for urea and creatinine parameters.

3.3. Zebrafish behavioural study

3.3.1. Fish behaviour after 15-day Suvarna Bhasma treatment

Fig. 6 shows the behaviour alteration of various treatment. The zebrafish motor behaviours such as speed (Fig. 6A), meander (Fig. 6B) and freeze points (Fig. 6C) for *Suvarna Bhasma* treated (SB) group was similar to vehicle control group (VC) and no significant alteration observed between these two groups. Whereas, the alcohol-treated group (CC) showed a significant increase in speed and meander ($p < 0.05$).

Caffeine treated (PC) group showed lower speed, meander and enhanced number of freeze points ($p < 0.05$). In geotaxis behaviour, SB group preferred the upper zone as compared to the VC group, similar to the CC group (1.5% alcohol), although the motor behaviour of SB group was not similar to the alcohol-treated group. In contrary, caffeine-treated group (PC) preferred the upper zone least in the novel tank (see Fig. 7 for representative track plot). From the upper-lower zone transitions, it was observed that for CC and PC group, the zone transition was lowest (Fig. 6E). The average height of swimming (Fig. 6F) for various groups showed that alcohol group swam at the maximum height throughout the tracking periods indicating anxiolytic effect, whereas, caffeine-treated anxious fish swam at the minimum height. *Suvarna Bhasma* treated group swam at more height than the control group, which indicates the anxiolytic behaviour.

3.3.2. Behaviour of zebrafish 30 days after the treatment completion

The treatment regime was daily dosing for 15 days. After behaviour tracking on Day 16, 30 fish from each VC and SB groups were further kept for 30 days in the housing tank without *Suvarna Bhasma* treatment. After 30 days without treatment, i.e. on day-46, the behaviour tracking was repeated to study recovery from treatment. The SB and VC groups showed analogous motor behaviour (speed, meander and freeze point, Fig. 8) to each other without any significant variation on day-46. However, here also the SB group preferred the upper zone significantly (spent 2.4 min) as compared to VC group (1.49 min, $p < 0.05$).

4. Discussions

Suvarna Bhasma is one of the most expensive medicine in *Ayurved* yet frequently prescribed by *Ayurved* practitioners for health improvement. According to the traditional *Ayurved* practitioners, to ensure safety and efficacy, textual manufacturing process needs to be followed stringently. However, due to the larger production urges and malpractices, many *Ayurved* drug manufacturers do not follow the traditional ways to manufacture *Suvarna Bhasma*, which could lead to toxic effects instead of its benefits. Variation of physicochemical properties of such metallic based medicines can lead to serious ill-effects in patients. Modern science has also established the vital role of physicochemical properties such as size, shape and chemical composition of the nanomedicines for its biological effects. (Liu et al., 2013). In view of this, this in-depth study was done.

The *Suvarna Bhasma* manufacturing process, which resembles the top-down method of modern nanoparticle preparation, the gold bar is reduced to gold particles, size varying from 10 nm to 60 μm. During rigorous annealing steps (14 heating and cooling cycles) the nanogold

Table 4
Grading of histopathological observation in the vehicle control group (VC) and 30 mg/kg *Suvarna Bhasma* treated group (SB30).

Tissue and Histopathological Observations	Male (numbers)		Female (numbers)	
	VC	SB30	VC	SB30
	(VC)	(30 mg/kg)	(VC)	(30 mg/kg)
Number of animals examined	10	10	10	10
Lungs				
Alveolar histiocytosis, focal (+)	1	0	1	0
Increased size of BALT (+) to (++)	1	1	1	1
Leukocytic infiltration in and around bronchiolar lumen (+++) to (++++)	2	0	0	1
Liver				
Hepatocellular hypertrophy, focal (+)	0	1	1	1
Spleen				
Increased EMH, focal (+)	1	1	1	1
Kidney				
Dilated medullary tubules, focal (+)	0	1	0	1
Vacuolar degeneration in cortical tubules, focal (+)	1	0	1	0
Mesenteric LN				
Increased histiocytes in medullary sinuses (+)	1	0		
Colon				
Enlarged GALT (+)	1	2	1	0
Testes				
Atrophy of seminiferous tubules near vasa recta (++)- unilateral	1	0		
Atrophy of seminiferous tubules, multifocal (++)- unilateral	0	1		
Cervical LN				
Increased histiocytes in medullary sinuses (+)			1	0

Key: (+) = Minimal, (++) = Mild, (+++) = Moderate and (++++) = Severe; MNC = Mononuclear Cells, LN = Lymph Node, GALT = Gut Associated Lymphoid Tissue, BALT = Bronchiole Associated Lymphoid Tissue, EMH = Extra Medullary Haematopoiesis.

particles formed in *Suvarna Bhasma* were in spherical shape. EDAX mapping demonstrated that the nano-sized gold particles were surrounded by Si, O, P, Fe and Ca. The entrapment of nano-gold particles might happen in the heating steps, SiO_2 mainly come from the earthen pot used during the preparation process. ICP-AES study revealed that *Suvarna Bhasma* used in this study contained approximately 98% Au along with Si, Fe, Na, and Ca. It is important to note that from a toxicological viewpoint that despite being used in the manufacturing process, Hg was not found in the finished product even after using three different elemental analytical methods, and this is in agreement with the toxicological data presented above. This can be explained as follows: In the manufacturing process, the mercury in the Hg–Au amalgamate was extracted and eliminated with the help of S powder successively in each of the 14 cyclic heating and cooling steps. Due to the strong affinity between Hg and S and the repetitive heating and cooling steps, Hg was completely eliminated.

The toxicity assessment of *Suvarna Bhasma* in the rat model was conducted with the dose up to 30 mg/kg which was approximately 10 times higher as compared to the therapeutic dose in human. In haematological parameters, after 90 days, neutrophil counts in the male rats of all *Suvarna Bhasma* treated groups was increased compared to the control group. However, this alteration was not statistically significant. Moreover, neutrophil counts with similar dose did not causes similar changes in *Suvarna Bhasma* treated female rats. Therefore, haematology parameters in rats did not indicate any adverse effect of *Suvarna Bhasma,* even at a considerably high dose as compared to the therapeutic dose in human.

A few alterations in the serum biochemical parameters were observed in rats. ALT, AST parameters decreased significantly in the female rats at 15 mg/kg, and 30 mg/kg dose level after 90 days of treatment, however, such alteration was not observed in male rats. These liver enzymes are elevated only during cell damage. Urea and creatinine were significantly increased in the male rats after 90 days for higher dose *Suvarna Bhasma* treatment. In female rats such effects were not seen. At the low dose (3 mg/kg, therapeutic dose) treatment, both haematology and biochemical parameters did not change except bilirubin in the female rat after 45 days of treatment (Supplementary file S1-Table 4). Most importantly, the changes in the biochemical parameters were well within the normal range and acceptable limits. The histopathological alterations observed at a dose of 30 mg/kg are usually observed in the laboratory rats of this strain and age under present experimental conditions. The feed intake, body and organ weights were not changed due to the *Suvarna Bhasma* treatment. Therefore, in summary, from the rat study, it can be concluded that *Suvarna Bhasma* is safe up to a dose of 30 mg/kg (10 times the therapeutic dose).

Similar to our work, a previous study also reported no observed adverse effect level of *Suvarna Bhasma* up to 13.5 mg/kg dose in Wistar rats (Jamadagni et al., 2015). Whereas, several beneficial effects of *Suvarna Bhasma* are also reported in literature especially as an immunomodulator: In an experimental study in mice, *Suvarna Bhasma* significantly ($P < 0.001$) increased counts of peritoneal macrophages and stimulated phagocytic index of macrophages indicating its immunostimulant effect (Bajaj and Ahmad, 2001). *Suvarnamalini vasanta* a *Suvarna Bhasma* containing generic preparation exhibited immunomodulatory potential as evidenced by an increase in percent phagocytosis and protection against *E. coli* induced peritonitis in mice (Sangle et al., 2004). *Madhu-Ghrita-Swarna-Vacha* combination showed a significant effect on humoral antibody formation in neonates and it acted on the immunological system, which was evident by triggering the response of immunological system by a rise in the total proteins and serum IgG levels (Jyothy et al., 2014b). *Suvarna bhasma* was evaluated in a global and focal model of ischaemia in albino rats (Shah and Vohora, 2002). Enzymatic parameters (lipid peroxidase, reduced glutathione, catalase, glutathione reductase, glutathione-S-transferase, glutathione peroxidase, superoxide dismutase, and glucose-6-phosphate dehydrogenase) were used to assess the ischaemic brain damage and its modulation by using *Suvarna Bhasma. Suvarna Bhasma* (25 mg/kg, orally for 10 days) significantly restored the altered values to near normal levels suggesting its potential in cerebrovascular diseases.

On the other hand, zebrafish behavioural assessment is able to screen various toxic or neurotoxic drugs. Drug-induced anxiogenic or anxiolytic behaviours are well defined in the literature for zebrafish (V Kalueff et al., 2013). Motor behaviour, scototaxis, geotaxis and cohort behaviour of zebrafish are studied extensively in recent literature (Maximino et al., 2010). In this study, we find that the motor behaviour of zebrafish did not alter significantly due to the action of *Suvarna Bhasma* treatment. Average speed, meander, freezing points etc. for *Suvarna Bhasma* treated and control fish were similar. However, geotaxis behaviour such as swimming height and preference of height zone was significantly changed ($p < 0.05$) in *Suvarna Bhasma* treated fish compared to the vehicle control group. The SB and CC groups spent more time in the upper zone (> 2 min) as opposed to the VC and PC groups (less than 2 min). Although, the motor behaviour of CC group differ from SB group. Usually, zebrafish in the novel tank initially prefer the bottom of the tank due to protective intuition. Preference to upper zone in the novel tank for *Suvarna Bhasma* treated groups indicated anxiolytic-like behaviour similar to alcohol-treated groups (CC) which is anxiolytic to zebrafish (Tran et al., 2016). Anxiogenic caffeine-treated fish, in contrast, preferred lower zone.

There is always a chance that toxicity of any drug may appear long after completion of the drug treatment. To address this issue, in this study, zebrafish were tracked 30 days after completion of the drug treatment, which resulted in no unnatural motor behaviour in the fish. However, similar to the previous novel tank experiment, *Suvarna Bhasma* treated fish preferred upper zone compared to the VC group.

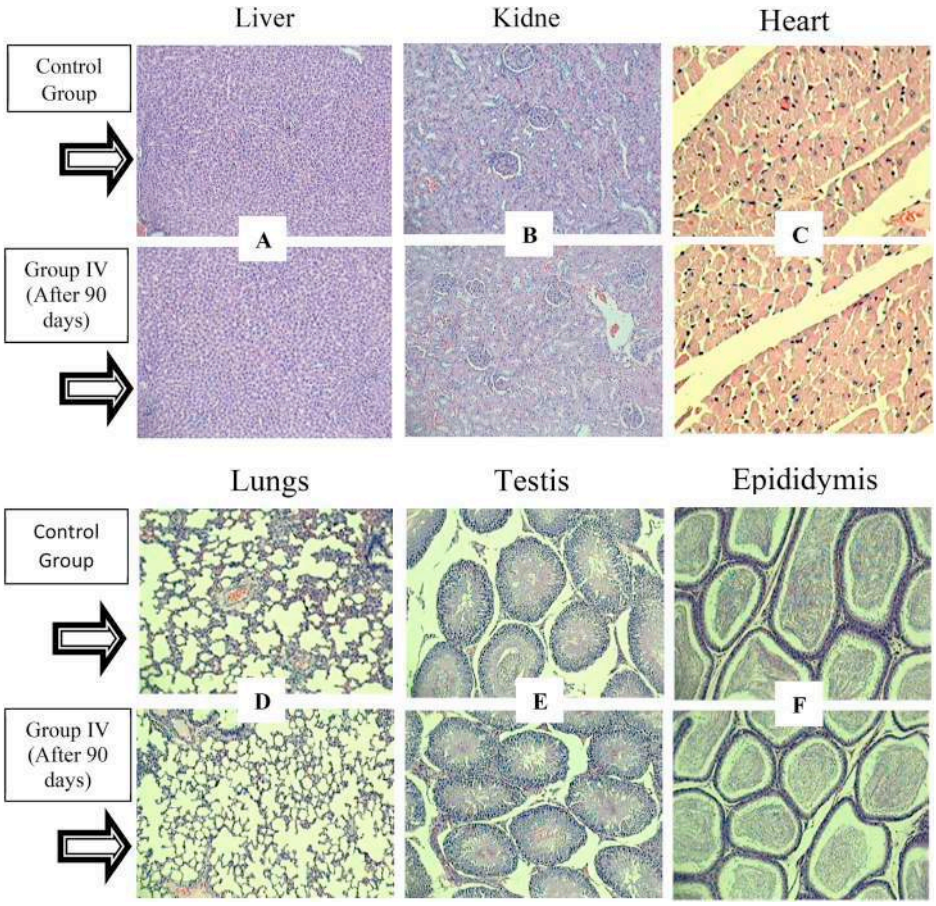

Fig. 5. Histopathology of vehicle control (VC) and 30 mg/kg SB treated rat (SB30). Various organs were imaged, such as A) Liver B) Kidney C) Heart, D) Lungs, E) Testis, and F) Epididymis. No treatment related alterations in tissue morphology were observed.

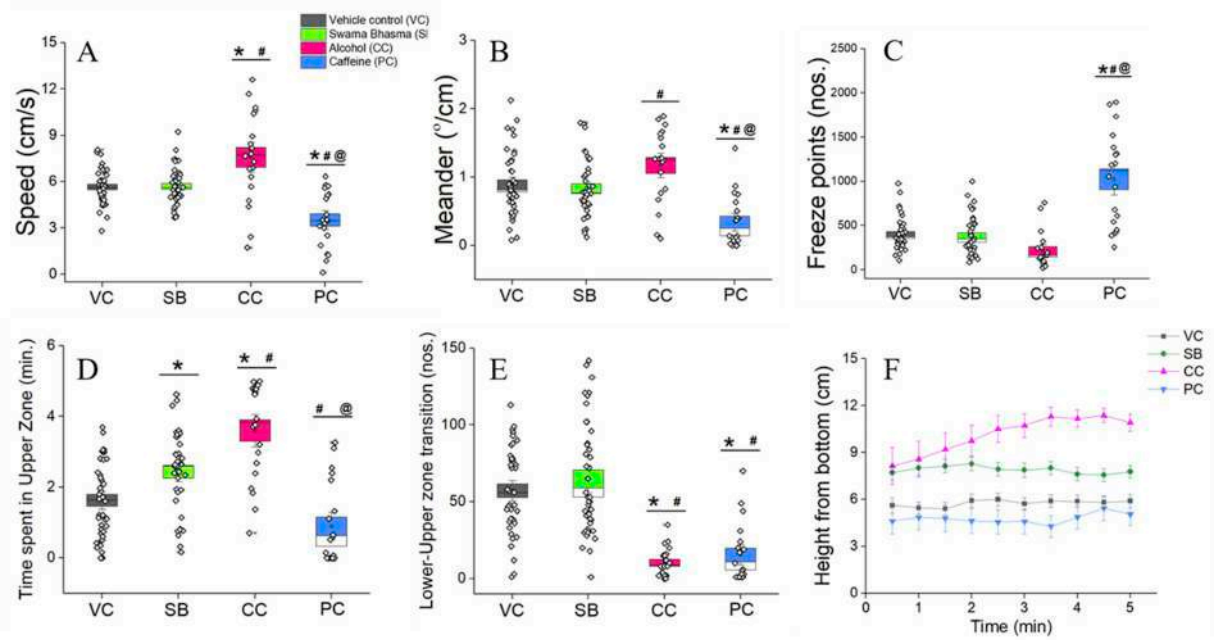

Fig. 6. Behavioural study of *Suvarna Bhasma* treated zebrafish. The fish were tracked a day after 15 days of *Suvarna Bhasma* treatment. A) Speed, B) Meander, C) Freeze points, D) Time spent in the upper zone, E) Number of transition between upper zone and lower zone, and F) Average swimming height from the bottom of the novel tank. VC = vehicle control group, SB = *Suvarna Bhasma* treated group, CC = comparative control, and PC = positive control group. Values are expressed as mean ± SEM with $^{*}p < 0.05$ vs VC group, $^{\#}p < 0.05$ vs SB group and, $^{@}p < 0.05$ vs CC group. No statistical variation was observed between VC and SB groups in motor parameters. However, geotactic behaviour such as the preference of swimming zone significantly varied between SB and VC groups.

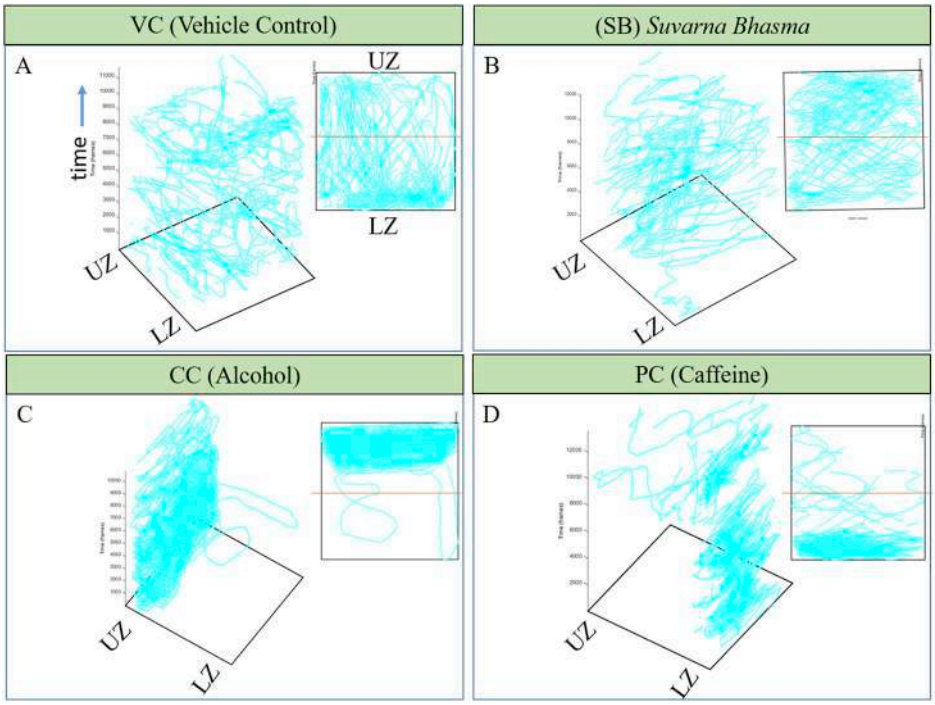

Fig. 7. Representative 3D and 2D track plots of zebrafish behaviour (taken from front view videos) showing the swimming traces and time spent in upper and lower zone. 3D track plot is showing fish position in x-y coordinate with time (frame number). A) VC = vehicle control, B) SB = *Suvarna Bhasma* treated, C) CC = comparative control, and D) PC = positive control group.

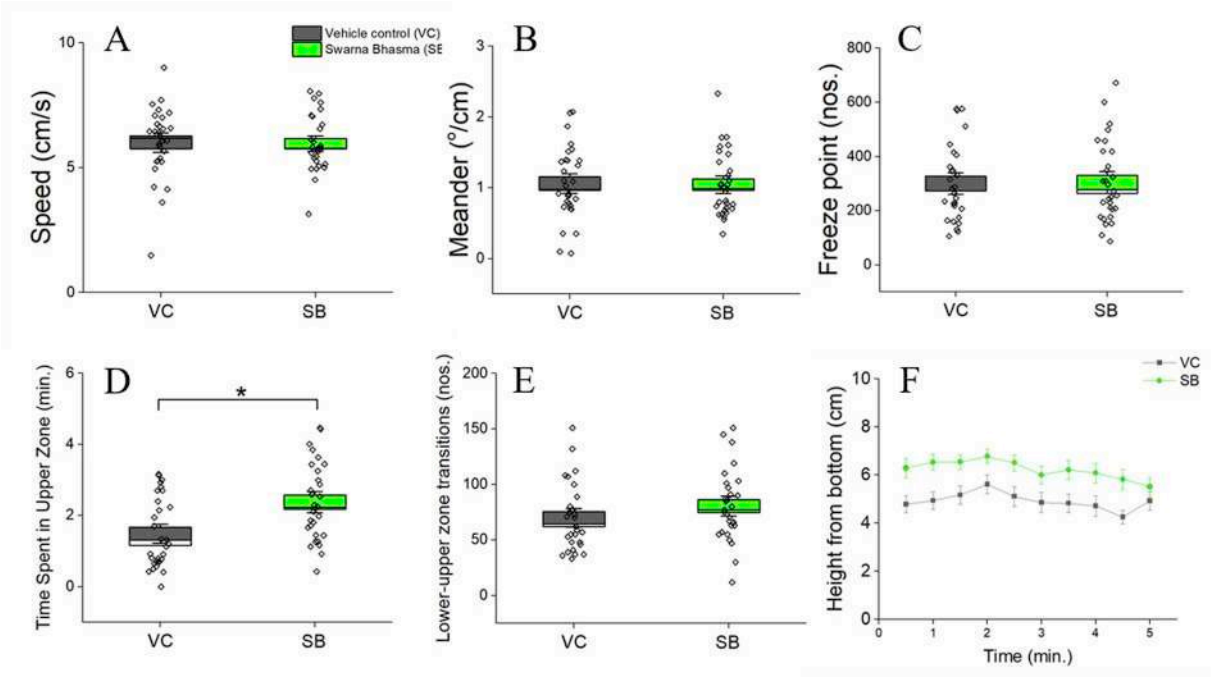

Fig. 8. Zebrafish behaviour of *Suvarna Bhasma* treated groups 30 days after completion of the drug treatment. A) Average speed, B) Meander, C) Freeze points, D) Time spent in the upper zone, E) Number of transition between upper zone and lower zone, and F) Average swimming height from the bottom of the novel tank. VC = vehicle control group, and SB = *Suvarna Bhasma* treated group. Values are expressed as mean ± SEM (n = 30 animal/group) with *$p < 0.05$. All motor parameters of *Suvarna Bhasma* treated fish were normal and analogues with the vehicle control group. However, here also SB group preferred upper zone compared to the VC group.

For zebrafish, preference towards the bottom of the tank is in response to the novel environment. However, for SB treated fish, it swims at a higher level in compared to VC group. Therefore, it can be inferred that *Suvarna Bhasma* has an anxiolytic effect on zebrafish behaviour model and this anxiolytic effect presumed long after completion of the drug treatment.

Gold, in modern medicine, is used mainly in the therapy of rheumatoid arthritis. Additionally, bio-liberated gold ions from gold implants mediate antiapoptotic, anti-inflammatory and neuroprotective effects (Østergaard et al., 2010). Ionic gold inhibits of proinflammatory mediators such as tumor necrosis factor alpha (TNFα), interleukin-1 and interleukin-6, leukotrienes, prostaglandins, nitrogen oxide and lysosomal proteases (Østergaard et al., 2010). Gold ion also heals injured neurons by increasing neurotrophin (NT-4), transforming growth factor-beta 3 (TGF-β3), leukemia inhibitory factor (LIF), and metallothionein (MT-I + II) which may be the reason for the

neuroprotective effect of gold. Interestingly, in Ayurveda *Suvarna Bhasma* is also used as a nerve tonic and to treat various neuronal diseases. In recent studies, several neurons related beneficial effect has been reported in the literature, such as neuroprotective against cognitive impairment or as anxiolytic and antidepressant agent (Bajaj and Vohora, 2000). Although *Suvarna Bhasma* contains gold particles (Au^0), it can release ionic gold in biological medium (e.g. gastric fluid). This bio-released gold ion may contribute its neuroprotection.

Anxiety, depression and various neurodegenerative diseases are widely prevalent in the modern stressful lifestyle. Anxiety is a part of the normal behavioural repertoire of defence mechanism to deal with novel environment. GABAergic neurotransmission in the amygdala altered anxiety-driven response (Nuss, 2015). Many research found that fear and anxiety in several animals decreases due to the infusion of GABA into the amygdala. Other neurotransmitters also recognised as anxiety modulators, such as opioid peptides, serotonin oxytocin and endocannabinoids. *Suvarna Bhasma* affects opioidergic mechanism (Bajaj and Vohora, 2000). In a previous behaviour study in rat, *Suvarna Bhasma* was found as anxiolytic, antidepressant and anti-cataleptic (Bajaj and Vohora, 2000). In our study, we find anxiolytic behaviour in zebrafish repeatedly in every set of experiment for *Suvarna Bhasma* treated fish. However, the exact mechanism for the anxiolytic effect was unknown, and further investigation is required to establish the pathways which *Suvarna Bhasma* acts in anxiolysis.

5. Conclusions

This study finds that gold in *Suvarna Bhasma* is present in two forms, one consisting of larger gold particles with crevices and second form consisting of gold nanoparticles, some of which are present in the crevices along with small amounts of Si, O, Fe etc. The larger gold particles consist of polycrystalline agglomerated gold of up to 60 µm size having crystallites of size 45 ± 2.8 nm. In the *Suvarna Bhasma* manufacturing process, Hg is used, however the finished product did not show the presence of Hg by any of the elemental detection methods (EDAX, and ICP-AES). The *in-vivo* studies of *Suvarna Bhasma* suggest no major adverse effects in rat model. In the zebrafish behavioural model, *Suvarna Bhasma* exhibited anxiolytic effects. Therefore, it can be inferred that the *Suvarna Bhasma* used in this study is safe and has an anxiolytic effect.

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Author contributions

Conceptualization: Snehasis Biswas (SB^1), GV, JB.
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Project administration: MC, KT, RG, GV, JB.
Supervision: GV, JB.
Writing: original draft: SB^1.
Writing, review and editing: SB^1, MC, GV, JB.

Declaration of competing interest

Three co-authors are from Shree Dhootapapeshwar Limited, which has also funded the study. The other authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jep.2019.112388.

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Safety evaluation of mercury based Ayurvedic formulation (*Sidh Makardhwaj*) on brain cerebrum, liver & kidney in rats

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***Background & objectives*: *Sidh Makardhwaj* (SM) is a mercury based Ayurvedic formulation used in rheumatoid arthritis and neurological disorders. However, toxicity concerns due to mercury content are often raised. Therefore, the present study was carried out to evaluate the effect of SM on brain cerebrum, liver and kidney in rats.**

***Methods*: Graded doses of SM (10, 50, 100 mg/kg), mercuric chloride (1 mg/kg) and normal saline were administered orally to male Wistar rats for 28 days. Behavioural parameters were assessed on days 1, 7, 14 and 28 using Morris water maze, passive avoidance, elevated plus maze and rota rod. Liver and kidney function tests were done on day 28. Animals were sacrificed and brain cerebrum acetylcholinesterase activity, levels of malondialdehyde (MDA), reduced glutathione (GSH) in brain cerebrum, liver, kidney were estimated. The levels of mercury in brain cerebrum, liver and kidney were estimated and histopathology of these tissues was also performed.**

***Results*: SM in the doses used did not cause significant change in neurobehavioural parameters, brain cerebrum AChE activity, liver (ALT, AST, ALP bilirubin) and kidney (serum urea and creatinine) function tests as compared to control. The levels of mercury in brain cerebrum, liver, and kidney were found to be raised in dose dependent manner. However, the levels of MDA and GSH in these tissues did not show significant changes at doses of 10 and 50 mg/kg. Also, there was no histopathological change in cytoarchitecture of brain cerebrum, liver, and kidney tissues at doses of 10 and 50 mg/kg.**

***Interpretation & conclusions*: The findings of the present study suggest that Sidh Makardhwaj upto five times the equivalent human dose administered for 28 days did not show any toxicological effects on rat brain cerebrum, liver and kidney.**

Key words Brain cerebrum - kidney - liver - mercury - oxidative stress - *Sidh Makardhwaj*

Sidh Makardhwaj is a popular *Kupipakwa rasayan*, prepared with *swarna* (gold), *parada* (mercury), *gandhaka* (sulphur) in a specific ratio (1:8:24) mentioned in Ayurvedic Formulary of India[1]. It has been used in the Indian Systems of Medicine for centuries with claimed efficacy and safety for the treatment of rheumatoid arthritis, neurological disorders, as a *rasayana* for vigour and longevity of

life[2]. It is prepared by a specific process of constant heating for more than 24 h converting it in a stable compound (mercury sulphide)[1].

Recent reports on the presence of heavy metals in Ayurvedic or herbal preparations have raised concern and controversy. Saper *et al*[3] have reported that one out of five Ayurvedic herbal medicine products (HMPs), produced in South Asia contains potentially harmful levels of lead, mercury, and arsenic. However, mercury along with sulphur (mercury sulphide) is one of the ingredients used in many traditional Ayurvedic medicines. Ayurvedic experts have estimated that approximately 20 per cent of the Ayurvedic formulations contain mercury sulphide as an ingredient[1,4]. Therefore, heavy metals content in *bhasma* can be thousand folds higher[2]. As per the classic Ayurvedic text, the processed mercury along with sulphur is converted to mercury sulphide and in low dose shows good therapeutic activities without producing toxic effects in the human subjects[5]. However, safety issues have been raised about mercury content present in herbo-mineral and *bhasma* preparations[6]. We, therefore, undertook this experimental study to evaluate safety profile of *Sidh Makardhwaj* mercury based Ayurvedic formulation.

Material & Methods

This study was conducted in the department of Phamacology, All India Institute of Medical Sciences (AIIMS), New Delhi, India. Male Wistar adult rats (150-200 g) were obtained from the Central Animal Facility of AIIMS, and stock bred in the departmental animal house. The rats were group housed in polyacrylic cages (38x23x10 cm) with not more than four animals per cage and maintained under standard laboratory conditions with natural dark and light cycle. They were allowed free access to standard dry rat diet (Ashirwad, Punjab, India) and tap water *ad libitum*. However, 12 h before the behavioural testing, the rats were deprived of food. The study protocol was approved by the Institutional Animal Ethics Committee, All India Institute of Medical Sciences, New Delhi, India.

Drugs preparation and duration of treatment: *Sidh Makardhwaj* (Batch number, MDR 022; date of manufacturing, September 2009; Maharshi Ayurveda Pharmaceutical Limited, New Delhi, India) was suspended in honey (Dabur Pharmaceuticals Pvt. Ltd, Gaziabad, India) and mercuric chloride (Sigma, USA) solution made in distilled water. Rats were randomly divided into five groups consisting of six rats each *i.e.* normal control, mercuric chloride (1 mg/kg/day) and *Sidh Makardhwaj* (10, 50 and 100 mg/kg) treated. The doses of *Sidh Makardhwaj* (10, 50 and 100 mg/kg/day) for rat were calculated by extrapolating the equivalent human dose (1, 5 and 10 times)[7] and were administered orally between 10.00 and 11.00 h every day for 28 days, in a volume not exceeding 1 ml/100 g rat weight. On 28th day, the rats were subjected to the behavioural tests and then sacrificed for biochemical and histopathological studies.

Neurobehavioural activity (cognition and motor coordination)

One trial passive avoidance task: Passive avoidance (Ugo Basile, USA) was used to evaluate the memory retention deficit and was evaluated according to the method described by Nakahara *et al*[8]. In acquisition trial, the rat was placed in a lighted chamber and guillotine door separating the light and dark chambers was opened. Initial latency (IL) to enter the dark chamber was recorded. Immediately after the rat enters the dark chamber, the guillotine door was closed and an electric foot shock (75 V, 0.2mA, 50 Hz) was delivered to the floor grids for 3 sec. The rat was removed from the dark chamber 5 sec later and returned to its home cage. After 24 h, retention latency (RL) time was noted in the same way as in the acquisition trial.

Morris water maze: Morris water Maze (Ugo Basile, USA) was use to evaluate the learning and memory. The Morris water maze consisted of a large circular pool filled with water (1.8 m in diameter, 0.6 m in height) and a platform (10 cm in diameter) submerged 1 cm below the water's surface. An automated tracking system (Video tracking system, Stoelting, USA) analyzed the total path lengths. Rats were given four acquisition sessions with an inter-trial interval of 10 min. Once a rat located the platform, it was allowed to remain there for 10 sec before being removed from the tank. If a rat failed to locate the platform within 120 sec, it was manually guided to it[9].

Elevated plus maze: Elevated plus maze was used to evaluate the memory retention deficit[10]. Rats were placed at one end of an open arm, facing away from the central square. Time taken by the rat to move from open arm to closed arms was recorded and marked as "initial transfer latency" (ITL). Animal was allowed to explore the maze for 30 sec after recording initial transfer latency. Retention transfer latency (RTL) was recorded by placing the rats similarly on the open arm at specified intervals.

Rota rod: Rota rod was use to evaluate the muscle coordination of rats. Rats were conditioned to the accelerating rod (Ugo Basile). Each animal received a training session on the rota rod at constant speed of 8 rpm and was tested until learned to remain on the rotating spindle for 60 sec. Each rat received single base line trial on the accelerating rota rod in which the spindle speed increased from 4 to 40 rpm over a period of 5 min. After administration of selected drugs for 28 days, each rat received a test trial[11].

Biochemical estimation and histopathology: At the end of behavioural experiments, blood was withdrawn by puncturing retro-orbital sinus for biochemical estimations. Serum was separated to measure liver and kidney functions test parameters. Animals were then sacrificed under ether anaesthesia and the brain cerebrum, liver, kidney were quickly removed. Tissues (n=50%) were cleaned with ice cold saline and stored at -80° C to determine the level of melondialdehyde (MDA), glutathione (GSH), acetyl cholinesterase (AChE) activity, mercury level and the remaining tissues were kept in 10 per cent formalin for histopathological study.

AChE activity was measured in the brain according to the method of Ellman *et al*[12], and was expressed as change in optical density/min/mg protein. Protein estimation was carried out by the method of Lowry *et al*[13]. MDH was determined by the method of Ohkawa *et al*[14], and its concentration was expressed in nmol/g wet tissue. Glutathione was measured according to the method of Ellman[15] and concentration was expressed as mg/g wet tissue.

Mercury level was estimated in tissue by inductively coupled plasma – atomic emission spectrophotometer (ICP-AES, JY 2000-2, France). Brain cerebrum, liver and kidney tissues were digested by cold vapors digestion procedure according to the method of Jacob *et al*[16]. Mercury levels were expressed in µg/g wet tissue.

Serum aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), bilirubin, urea and craetinine levels were estimated separately using individual kit by semi auto analyzer (Mini techno, USA). The enzyme activities were reported as the instructions of the manufacturer of assay kits (Logitech India Pvt. Ltd, Delhi, India).

Histopathological study: Tissue specimens from brain cerebrum, liver, and kidney fixed in 10 per cent formalin were processed by conventional method, embedded in paraffin, sectioned at 4-5 µm and stained by haematoxylin and eosin[17]. Tissues were examined under a light microscope (Nikon, Japan). Histopathological study was carried out in the department of Pathology, AIIMS, blinded to the groups.

Statistical analysis: Data were expressed as mean ± SEM. A one-way analysis of variance (ANOVA), followed by Post-hoc multiple comparisons of Tukey test was used for statistical analysis. SPSS (version 16) statistical software USA was used for the analysis of data and $P<0.05$ was taken as the level of significance.

Results

Effect of Sidh Makardhwaj on behavioural parameters

Passive avoidance task - There was no significant change in the initial latencies and retention latencies of *Sidh Makardhwaj* (10, 50 and 100 mg/kg) treated groups as compared to normal control. However, there was significant decrease in mean retention latencies of mercuric chloride treated group as compared to normal control group ($P<0.001$) (Table I).

Table I. Effect of of *Sidh Makardhwaj* (SM) on passive avoidance and elevated plus maze test

Treatment groups	Passive avoidance test		Elevated plus maze test	
	Initial latency	Transfer latency	Initial latency	Transfer latency
Normal control	13.92 ± 3.21	236.98 ± 28.1	41.12 ± 4.35	6.37 ± 1.08
Mercuric chloride (1 mg/kg)	12.10 ± 2.11	3.11 ± 0.81***	35.61 ± 4.51	64.91 ± 5.92*
SM (10 mg/kg)	8.55 ± 2.42	248.53 ± 29.43†	38.22 ± 4.51	6.85 ± 0.68†
SM (50 mg/kg)	10.95 ± 2.25	250.31 ± 16.48†	37.03 ± 5.11	5.03 ± 0.43†
SM (100 mg/kg)	8.46 ± 1.72	238.67 ± 21.74†	38.68 ± 4.47	4.47 ± 0.41†

Values are ± SEM (n=6)
*$P < 0.001$ compared to normal control; †$P<0.001$ compared to mercury chloride treated group

Table II. Effect of of *Sidh Makardhwaj* (SM) on moris water maze and rota rod test

Treatment groups	Moris water maze test		Rota rod test	
	Acquisition trial	Probe trial	Pre-drug treatment	Post-drug treatement
Normal control	12.22 ± 2.12	3.84 ± 0.94	171.2 ± 7.83	185.44 ± 8.23
Mercuric chloride (1 mg/kg)	11.24 ± 1.83	17.08 ± 2.32*	173.59 ± 1.28	60.88 ± 3.32*
SM (10 mg/kg)	10.85 ± 1.92	1.93 ± 0.84*#	170.42 ± 3.45	205.15 ± 6.84*#
SM (50 mg/kg)	10.92 ± 1.78	1.79 ± 0.66*#	171.24 ± 5.36	240.18 ± 9.64*#
SM (100 mg/kg)	11.26 ± 1.94	1.85 ± 0.56*#	169.67 ± 6.35	234.72 ± 8.54*#

Values are mean ± SEM (n=6)
*P<0.001 as compared to normal control; #P<0.001 as compared to mercury chloride treated group

Morris water maze test - *Sidh Makardhwaj* (10, 50 and 100 mg/kg) treated groups did not show significant change in total distance travelled during the acquisition trials and probe trial to reach the platform as compared to normal control group. However, there was increase in distance travelled during probe trial to reach the platform of mercuric chloride treated group as compared to normal control (P<0.001) (Table II).

Elevated plus maze test - There was no significant change in initial transfer latencies and retention transfer latencies of *Sidh Makardhwaj* (10, 50 and 100 mg/kg) treated groups as compared to normal control group. However, there was significant increase in mean retention transfer latencies as compared to normal control group (P<0.001) (Table I).

Rota rod test - There was no significant change in the time spent on the spindle of the rota rod before drug treatment and post drug treatment as compared to normal control group. However, there was significant decrease in time spent on spindle of mercuric chloride treated group as compared to control group (P<0.001) (Table II).

Effect of Sidh Makardhwaj on biochemical parameters

AChE activity of frontal cortex and hippocampus - There were no significant difference in AChE activity of *Sidh Makardhwaj* (10, 50 and 100 mg/kg) groups as compared to normal control group in frontal cortex as well as in hippocampus. However, there was significant (P<0.001) decrease in AChE activity in frontal cortex as well as in hippocampus of mercuric chloride group as compared to normal control group (Table III).

MDA and GSH levels in brain cerebrum, liver and kidney - There were no significant difference in brain cerebrum's MDA and GSH levels at studied doses of *Sidh Makardhwaj* while increased levels were observed in mercuric chloride group as compared to normal control group (P<0.001). Significantly increased MDA and decreased GSH levels in liver and kidney of mercuric chloride treated group (P<0.001) and *Sidh Makardhwaj* (100 mg/kg) treated group (P<0.05) were observed while no significant change was observed at lower doses of *Sidh Makardhwaj* (10 and 50 mg/kg) as compared to the control group (Table IV).

Mercury level in brain cerebrum, liver and kidney - There was significant increase in rat's brain, liver and kidney mercury levels of *Sidh Makardhwaj* (10, 50 and 100 mg/kg) groups (P<0.05, P<0.001) as well as in mercuric chloride treated group (P<0.001) as compared to normal group (Table V).

Liver and kidney function test parameters - There was no significant change in the serum ALT, AST,

Table III. Effect of of *Sidh Makardhwaj* (SM) on rat frontal cortex and hippocampus acetylcholinesterase activity

Treatment groups	AChE activity (μM/g protein/min)	
	Frontal cortex	Hippocampus
Normal control	24.94 ± 0.74	29.36 ± 0.69
Mercuric chloride (1 mg/kg)	14.12 ± 1.03*	12.32 ± 0.92*
SM (10 mg/kg)	23.83 ± 0.53†	28.88 ± 0.81†
SM (50 mg/kg)	23.70 ± 0.46†	27.94 ± 0.88†
SM (100 mg/kg)	23.21 ± 0.45†	27.81 ± 1.08†

Values are mean mean ± SEM. (n=6)
*P<0.001, compared to normal control; †P<0.001 compared to mercury chloride treated group

Table IV. Effect of *Sidh Makardhwaj* (SM) on MDA and GSH of rat brain cerebrum, liver and kidney tissue

Treatment group	MDA (nmol/g wet tissue)			GSH (mg/g wet-tissue)		
	Brain cerebrum	Liver	Kidney	Brain cerebrum	Liver	Kidney
Normal control	84.6 ± 4.3	64.66 ± 1.48	180.29 ± 3.48	2.23 ± 0.06	3.31 ± 0.21	4.02 ± 0.09
Mercuric chloride (1 mg/kg)	122.4 ± 16.99**	176.15 ± 6.79**	242.49 ± 1.64**	1.07 ± 0.15**	1.71 ± 0.18**	1.64 ± 0.21**
SM (10 mg/kg)	82.1 ± 3.2	65.83 ± 2.98	181.72 ± 1.64	2.24 ± 0.03	3.28 ± 0.13	3.96 ± 0.08
SM (50 mg/kg)	83.9 ± 5.2	66.72 ± 2.63	188.89 ± 4.42	2.25 ± 0.02	3.15 ± 0.07	3.66 ± 0.15
SM (100 mg/kg)	84.2 ± 3.7	92.70 ± 6.03*	216.76 ± 20.54*	2.27 ± 0.02	2.28 ± 0.20*	2.57 ± 0.23*

Values are mean ± SEM (n=6)
P<0.05, **<0.001 compared to normal control

Table V. Effect of of *Sidh Makardhwaj* (SM) on mercury level of rat brain cerebrum, kidney and liver

Treatment groups	Mercury level (µg/g wet tissue)		
	Brain cerebrum	Liver	Kidney
Normal control	1.84±0.22	46.49±5.64	115.94±4.95
Mercuric chloride (1 mg/kg)	151.03±14.13**	775.02±34.07**	948.78 ±19.70**
SM (10 mg/kg)	33.15±3.61*	143.88±10.23*	181.91±4.39*
SM (50 mg/kg)	40.59±5.34**	198.88±8.61**	377.04±13.03**
SM (100 mg/kg)	49.49±3.65**	302.73±8.56**	500.01±32.94**

Values are mean ± SEM (n=6)
*P<0.05, **P<0.001 compared to normal control

ALP, bilirubin, urea and creatinine levels of *Sidh Makardhwaj* treated groups at doses of 10, 50 and 100 mg/kg as compared the normal control group, while significant change was observed in mercuric chloride treated group (Table VI).

Effect of Sidh Makardhwaj on brain cerebrum, liver and kidney histology: The brain cerebrum, liver and kidney of normal control and those treated with lower doses of *Sidh Makardhwaj* (10, 50 mg/kg) showed no abnormal histopathological changes but mild histopathological change was observed with higher dose of *Sidh Makardhwaj* (100 mg/kg). Microscopically, necrosis of neurons in cerebrum (Fig. 1), inflammed periportal zone in liver (Fig. 2) and disruption of epithelium in proximal convoluted tubules in kidney (Fig. 3) were observed at higher dose (100 mg/kg). However, mercuric chloride treated group showed expected toxicities *i.e.* necrosis of neurons in cerebrum, inflammed periportal zone in liver and disruption of epithelium in proximal convoluted tubules in kidney.

Discussion

Neurotoxicity, hepatotoxicity and nephrotoxicity due to mercury exposure are well known[18-19]. However, mercury based Ayurvedic formulations (*Sidh Makardhwaj*) have been widely used in India for centuries. The US Environmental Protection Agency (EPA) has adopted a reference dose (RfD) for methyl mercury of 0.1 µg/kg body weight/day[20]. However, the total mercury content of *Sidh Makardhwaj* formulation used in the present study was 35454.2 µg/g. The calculated total ingested mercury at doses of 10, 50 and 100 mg/kg were 354.5, 1772.5 and 3545.4 µg/kg. Thus, in therapeutic dose of *Sidh Makardhwaj* (10 mg/kg), the per day ingested mercury was many fold higher than the reference dose. It was interesting to note that even this high concentration of mercury in *Sidh Makardhwaj* for 28 days did not cause significant toxicity in liver, kidney and brain cerebrum. The absence of toxicity could be due to the fact that Ayurvedic detoxification process (*Sodhana*) might have contributed in some modification of metal property resulting in abolition

Table VI. Effect of of *Sidh Makardhwaj* (SM) on liver and kidney function test parameters of rat serum

Treatment groups	Liver functions tests				Kidney function tests	
	ALT (IU/l)	AST (IU/l)	ALP (IU/l)	Bilirubin (mg/dl)	Urea (mg/dl)	Creatinine (mg/dl)
Normal control	129.48 ± 1.39	106.50 ± 1.74	41.92 ± 0.53	0.13 ± 0.11	14.15 ± 1.91	0.39 ± 0.07
Mercuric chloride (1 mg/kg)	223.78 ± 12.01*	191.64 ± 7.38*	104.20 ± 6.46*	1.09 ± 0.09*	42.98 ± 3.07*	1.19 ± 0.09*
SM (10 mg/kg)	127.75 ± 1.82	109.12 ± 1.64	44.10 ± 1.56	0.16 ± 0.02	15.63 ± 4.78	0.40 ± 0.07
SM (50 mg/kg)	128.83 ± 2.85	110.38 ± 2.42	45.37 ± 1.66	0.18 ± 0.01	17.93 ± 3.93	0.47 ± 0.04
SM (100 mg/kg)	130.10 ± 1.56	109.42 ± 1.95	44.08 ±1.45	0.17 ± 0.01	19.20 ± 3.96	0.54 ± 0.17

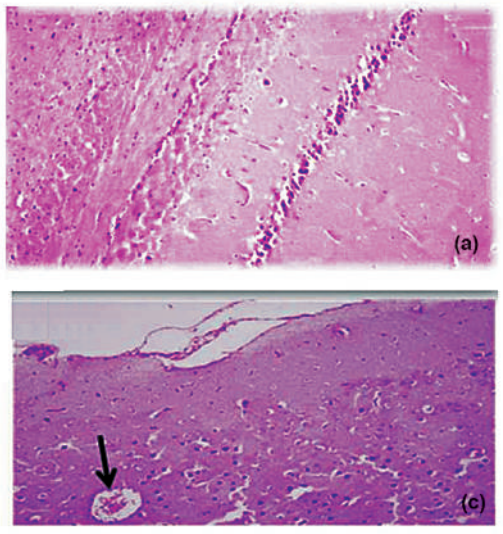
Values are mean ± SEM (n=6)
*P<0.001 as compared to normal control group
ALT, alanine aminotransferase; AST, serum asparate aminotransferase; ALP, alkaline phosphatase

of toxicity, yet retaining its pharmacological property. Another reason could be due to mercuric sulphide content in *Sidh Makardhwaj* because Son *et al*[21] have reported that mercuric sulphide at the dose of 2 g/kg did not cause hepatotoxicity in the biochemical and histological examination in mice.

Previous studies have shown behavioural and spatial learning deficits in animals due to mercury exposure[22]. Baraldi *et al*[23] showed cognitive impairment in chronically mercury exposed rats and there was decreased ability to learning in water maze model. Mercuric chloride (1 mg/kg/day, p.o) caused impairment of memory and motor activity in our study and also reported in literature[24]. However, *Sidh Makardhwaj* (10, 50 and 100 mg/kg), admininistered orally for 28 days did not affect cognitive and motor function in rats.

Acetylcholine, acetyl cholinesterase (AChE) and choline acetyltransferase (ChAT) are involved in cognition function and motor activity. Several studies have shown decreased ChAT and AChE activity after mercury exposure[24-26] which could be the reason for impairment in cognition function and motor control. In our study, decreased acetylcholinesterase activity was found in hippocampus and frontal cortex in mercuric chloride treated group while *Sidh Makardhwaj* treatment for 28 days did not cause significant change in rat frontal cortex and hippocampus AChE activity as compared normal control group.

Mercuric chloride administration reduces renal and hepatic GSH content and increases lipid peroxide formation[27,28]. The results of the present study were in conformity with the earlier studies which showed that mercuric chloride caused oxidative stress in



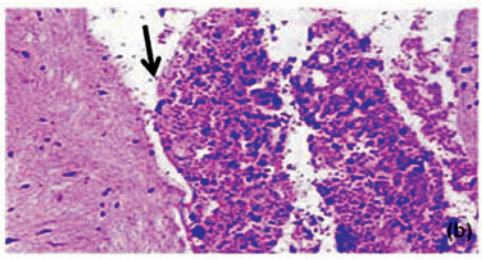
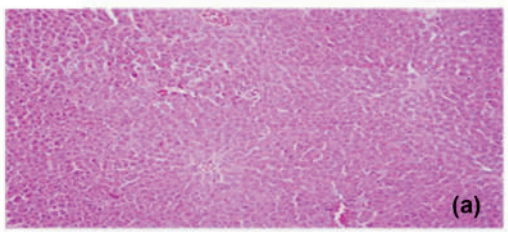
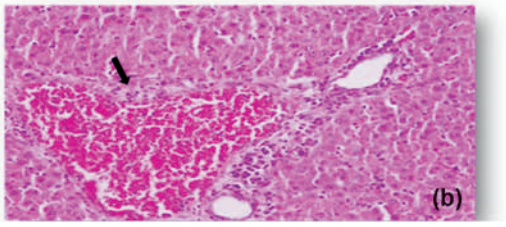


Fig. 1. Effect of *Sidh Makardhwaj* on cerebrum histology. Lingh micrograph of **(a)** control brain cerebrum showing normal architecture **(b)** 1 mg/kg/day, $HgCl_2$ treated rat showing pyknosis, congestion of blood vessels and necrosis of Purkinje cells (arrow) **(c)** 100 mg/kg/day, *Sidh Makardhwaj* treated showing pyknosis of neurons and congestion of blood vessels (arrow). Original magnification 20X.





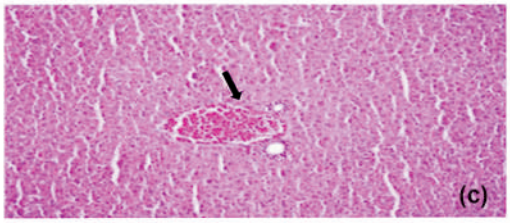
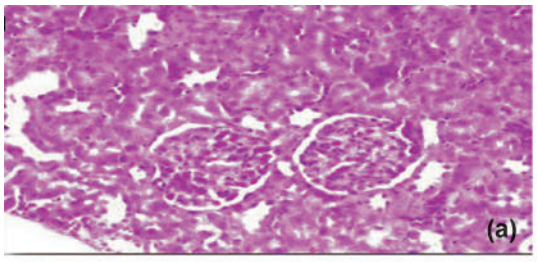
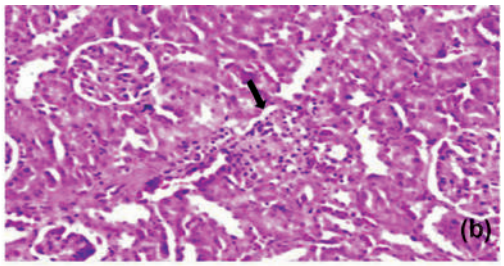


Fig. 2. Effect of *Sidh Makardhwaj* on rat's liver histology. Light micrograph of **(a)** control liver showing normal architecture **(b)** 1 mg/kg/day, $HgCl_2$ treated showing pyknosis, vascular, degenerative and necrotic changes in the liver (arrow) **(c)** 100 mg/kg/day, *Sidh Makardhwaj* treated showing pyknosis and congestion of blood vessels (arrow). Original magnification 20X.





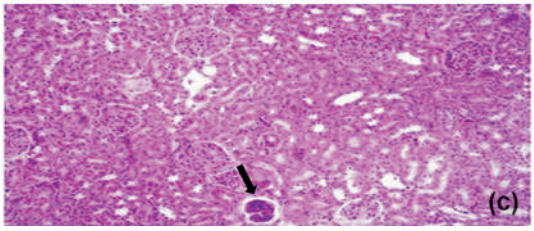


Fig. 3. Effect of *Sidh Makardhwaj* on rat's kidney histology. Light micrograph of (a) control liver showing normal architecture (b) 1 mg/kg/day, $HgCl_2$ treated showing pyknosis, vascular, degenerative and necrotic changes in the liver (arrow) (c) 100 mg/kg/day, *Sidh Makardhwaj* treated showing pyknosis and congestion of blood vessels (arrow). Original magnification 20X.

brain cerebrum, liver and kidney. However, *Sidh Makardhwaj* (10, 50 and 100 mg/kg) did not affect brain cerebrum's MDA and GSH levels indicating that *Sidh Makardhwaj* in therapeutic doses does not cause oxidative stress.

There is evidence that chronic exposure to low concentration of mercury causes tissue or organ damage[29]. In the present study, there was significant increase in brain cerebrum, liver and kidney mercury levels at all doses of *Sidh Makardhwaj* (10, 50 and 100 mg/kg) as compared to normal control group. However, levels of mercury in brain cerebrum, liver and kidney were significantly lower as compared to mercuric chloride treated group.

Several studies have shown significant elevations in serum ALT, AST, ALP, bilirubin due to mercury exposure[19,28-30]. In the present study also rats exposed to mercuric chloride showed elevated levels. However, no significant change in serum ALT, AST, ALP, and bilirubin was observed in *Sidh Makardhwaj* treated groups.

Kidney damage is indicated by elevated serum urea and creatinine levels. Mercuric chloride treatment has been shown to cause a significant increase in serum creatinine and serum urea nitrogen indicating an impaired renal function[31]. In our study, *Sidh Makardhwaj* administered orally for 28 days did not cause nephrotoxicity in rats.

Inorganic mercury (mercuric chloride) has been shown to accumulate in the renal cortex and affect the morphology and function of the proximal tubules[32]. Jadhav *et al*[33] have observed dose-dependent vascular, degenerative and necrotic changes in the brain cerebrum and liver of male rats exposed to mercury via drinking water. In the present study, congestion of blood vessels, neuronal degeneration, necrosis of hepatic cells, cellular necrosis involving primarily the pars recta of proximal tubules was observed at higher dose of *Sidh Makardhwaj* (100 mg/kg) and mercuric chloride treated group. Normal architecture of brain cerebrum, liver and kidney was seen at lower dose of *Sidh Makardhwaj* (10 and 50 mg/kg).

Mercury sulphide is one of the ingredients of many traditional Ayurvedic medicines. Liu *et al*[34] have reported that cinnabar is chemically inert with a relatively low toxic potential when taken orally. In risk assessment, cinnabar is less toxic than many other forms of mercury. There are many studies showing the safety of cinnabar and mercury sulphide which could be the reason for non toxic nature of *Sidh Makardhwaj*[21,34,35].

In conclusion, the findings of the present study suggest that *Sidh Makardhwaj* in the doses equivalent to human dose given for 28 days does not have any adverse effects on brain cerebrum, liver and kidney. Importantly, there were no changes in biochemical parameters at therapeutic dose. The histopathological examination also showed normal cytoarchitecture of brain cerebrum, liver and kidney ruling out its toxic potential at the therapeutic dose levels. However, mild histopathological changes were observed at higher dose (10 times of therapeutic dose) of *Sidh Makardhwaj*.

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***Conflict of interest*:** The authors declare no conflicts of interest.

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Analytical methodology

The chemical speciation, spatial distribution and toxicity of mercury from Tibetan medicine *Zuotai* , β-HgS and $HgCl_2$ in mouse kidney

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ABSTRACT

Zuotai, a famous Tibetan medicinal mixture containing β-HgS, has been used to combine with herbal remedies for treating diseases for more than 1 300 years. The target organ for inorganic mercury toxicity is generally considered to be the kidney. Therefore, it is crucial to reveal the chemical speciation, spatial distribution and potential nephrotoxicity of mercury from *Zuotai* in kidney. To date, this remains poorly understood. We used X-ray absorption spectroscopy (XAS) and micro X-ray fluorescence (μ-XRF) imaging based on synchrotron radiation to study mercury chemical forms and mercury special distribution in kidney after mice were treated orally with *Zuotai*, β-HgS or $HgCl_2$. Meanwhile, the histopathology of kidney was observed. Mice exposed with *Zuotai* showed kidney with significant proportion of mercury ions bound to sulfydryl biomolecules (e.g. Cys-S-Hg-S-Cys) plus some of unknown species, but without methylmercury cysteine, which is the same as β-HgS and $HgCl_2$. The mercury is mainly deposited in renal cortex in mouse treated with *Zuotai*, β-HgS or $HgCl_2$, but with a low level of mercury in medulla. The total mercury in kidney of mice treated with $HgCl_2$ was much higher than that of β-HgS, and the later was higher than that of *Zuotai*. And, $HgCl_2$ cause severe impairments in mouse kidney, but that was not observed in the *Zuotai* and β-HgS groups. Meanwhile, the bio-metals (Ca, Zn, Fe and Cu) micro-distributions in kidney were also revealed. These findings elucidated the chemical nature, spatial distribution and toxicity difference of mercury from *Zuotai*, β-HgS and $HgCl_2$ in mouse kidney, and provide new insights into the appropriate methods for biological monitoring.

1. Introduction

Mercury is one of the ubiquitous environmental pollutants with seriously hazardous effects on living organisms [1,2]. People are exposed to mercury through the food we eat, the water we drink, the polluted air we breathe, and the medicine we take. In traditional medicines, metallic mercury often undergo specific processing procedures (e.g.: washing, grinding, heating) [3–5]. The addition of the processed mercury to herbal mixture is thought to assist the efficacy or reduce the toxicity in a given remedy [3,4,6]. *Zuotai*, a famous Tibetan medicinal mixture composed of 54.5% cubic crystal mercuric sulfide (β-HgS) [7,8], has long been used for more than 1300 years and still widely used in Tibetan Plateau today [9,10]. According to traditional empirical knowledge, *Zuotai* is used for treating stroke, leprosy, glomus tumor, anthrax, joint disease, gout, yellow inflammations and poisoning disease, hypertension, heart disease and others [3,6,9,11]. Recently, it has been found that *Zuotai* not only can allay excitement, promote sleep, and produce an antipyretic effect [12,13], but also can have anti-inflammatory effects, extending the life of fruit flies, inhibiting the expression of caspase-3, and regulating the biological clock [13–16]. Overall, these findings show a great contrast to the common conception that any substance containing mercury is toxic.

The toxicities of different chemical forms of mercury vary greatly. Organic mercury (e.g. methylmercury and ethylmercury) and vapor mercury (Hg^0) possess strong toxicity on the central nervous system [2,17,18]. Soluble inorganic mercury [e.g., mercuric chloride ($HgCl_2$) and mercurous chloride (Hg_2Cl_2)] are characterized by severely renal toxicity and gastrointestinal toxicity [18,19]. The ingestion of liquid metallic mercury or “quicksilver” does not appear to be toxic in itself, and the health hazards from quicksilver are due to its potential to release mercury vapor [17,18]. Mercuric sulfide (HgS) is the reputed insoluble mercuric compound, and there is no definite evidence to

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prove that it is toxic [20–25].

Cubic crystal mercuric sulfide (β-HgS) is insoluble with a solubility product constant (K_{sp}) of 1.6×10^{-52} [26] and usually disregarded to be significantly absorbed from the gastrointestinal tract following oral administration, but it must be aware of its potential toxic effects under long-term exposure. The estimated human therapeutic priming dose of *Zuotai* in traditional medicine used is approximately 0.66 mg/kg/day [11]. Our previous study found that mercury from *Zuotai* was absorbed and transported into mice tissues, particularly in kidney, after the oral administration under the equivalent dose (6.67 mg/kg/day) for 4.5 months [27]. The mRNA expression of kidney injury molecule-1 (Kim-1) and metallothionein-1 (MT-1) in rat kidney were increased obviously after 180 days oral administration with *Zuotai*, and these indicators also can be recovered to normal level after 30 days discontinuance [28]. This indicated that *Zuotai* may have some potential adverse effects on kidney under long-term exposure. The chemical forms of mercury in kidney and its spatial distribution are of significant interest for understanding its toxicological behavior and the potential ramifications. However, this remains poorly known.

The X-ray Absorption Spectroscopy (XAS) and micro X-ray fluorescence (μ-XRF) imaging based on synchrotron radiation provide the methods for detecting the chemical species and 2D spatial distribution *in situ* of an element in complex biological sample with nondestructive, respectively [29–32]. Here, we used XAS and μ-XRF to investigate the chemical nature and micro-distribution of mercury in kidney tissues of mice treated with *Zuotai*, β-HgS and $HgCl_2$ for 14 weeks. And, the nephrotoxicity was observed through pathological histology. Besides, the spatial micro distributions of bio-metals (Ca, Zn, Fe and Cu) in kidney also were investigated.

2. Materials and methods

2.1. Reagents

Zuotai was prepared by the Company of Tibetan Medicine in Tibetan Autonomous Region according to a China Patent (88107006.8) [3], and was labeled ZT20110705 and deposited at the Qinghai Key Laboratory of Tibetan Medicine Pharmacology and Safety Evaluation. The storage solution (1.0 mg/mL Hg) was purchased from National Standard Research Center (Beijing, China). β-HgS (98%) and α-HgS (98%) was purchased from Alfa Aesar Chemical Co. Ltd, UK; $HgCl_2$ was purchased from Guizhou Tongren Chemical Reagent Factory, China. Hg_2Cl_2 was purchased from Sinopharm Chemical Reagent Co., Ltd., China. HgO (red) and HgO (yellow) were purchased from Guizhou Province Tongren Yinhu Chemical Co., China. $HgSO_4$, $HgAc_2$ and CH_3HgCl (MeHgCl) were purchased from Aladdin reagent (Shanghai) Co., Ltd., China. L- Cysteine (L-Cys) was purchased from Sigma-Aldrich Corp., China. Ultra-pure water (ρ = 18.2 MΩ*cm) was obtained from a UP Water Purification System (Milli-Q Reference pure, Millipore Corporation, US). $HgCys_2$ (Cys-S-Hg-S-Cys) and MeHgCys (CH_3-Hg-S-Cys) were synthetized according to the following methods.

2.1.1. Preparation of $HgCys_2$

The mole ratio of L-Cys to $HgCl_2$ was 2.2: 1 to make sure L-Cys was excessive to the free mercuric ions (Hg^{2+}). The 0.5 g (1.8 mmol) $HgCl_2$ and 0.4909 g (3.96 mmol) L-Cys were dissolved in pure water, respectively. Then, the both solutions were mixed together. All the procedures were under the protection of pure argon gas (Ar) to prevent the polymerization of L-Cys molecules. Finally, the sold powder of $HgCys_2$ was prepared through vacuum freeze drying.

2.1.2. Preparation of MeHgCys

0.3983 mmol (0.1 g) MeHgCl was firstly dissolved in 3 mL ethanol, then added 7 mL pure water to dilute. Additionally, 0.4782 mmol (0.05793 g) L-Cys was dissolved in pure water. Then, the above both solutions were mixed. All the procedures were under the protection of Ar gas. Finally, the sold powder of MeHgCys was prepared through vacuum freeze drying. The mole ratio of L-Cys to MeHgCl was 1.2: 1. L-Cys is excessive to ensure that MeHgCl in solution were reacted completely with no residue.

2.2. Apparatuses

The XAFS experiment endstation 1W1B, Beijing Synchrotron Radiation Facility (Beijing, China); the micro X-ray fluorescence beamline BL15U at Shanghai Synchrotron Radiation Facility (Shanghai, China); fully automatic direct mercury analyzer (DMA-80, Milestone Co., Ltd, Italy); electronic balance (ME204, Mettler Toledo Co., Ltd., Switzerland); infrared tablet presser (HY-12, Tianjin skylight Optical Instrument Co., Ltd.); vacuum freeze dryer (FD-1D-50, Beijing Boyikang Laboratory Instrument Co., Ltd.); frozen microtome (CM 1950, Leica Co., Ltd., Germany); positive optical microscope imaging system (E200, Sony Co., Ltd., Japan); UP water purification system (Milli-Q Reference pure, Millipore Corporation, US).

2.3. Animal subjects

SPF female KM mice (age: 12 weeks) were purchased from Gansu University Traditional Chinese Medicine, SCXK (Gan) 2011-0001, housed in the laboratory animal facility of Northwest Institute of Plateau Biology, Chinese Academy of Sciences, under specific pathogen-free conditions, and maintained with 12 h light/dark cycle (lights on at 8:00 a.m.) at 22 °C–25 °C. Mice growth maintenance feed and sawdust pads were purchased from Beijing Ke'ao Xieli Feed Co., Ltd. (Beijing, China). The animals had free access to standard diet and water.

2.4. Pharmacological treatments

Zuotai, β-HgS and $HgCl_2$ were suspended or dissolved in a 2.5% starch solution. Female mice were allowed to acclimate to their surroundings for 7 days prior to initiation of the experiment. 32 mice were randomly allocated to one of the following six groups (n = 8 for each group): Control (2% starch solution), *Zuotai* (66.7 $mg{\cdot}kg^{-1}$), β-HgS (36.35 $mg{\cdot}kg^{-1}$) and $HgCl_2$ (1 $mg{\cdot}kg^{-1}$) groups. *Zuotai* group and β-HgS group have same mercury dose, 31.36 $mg{\cdot}kg^{-1}$ Hg. Mercury dose of $HgCl_2$ group was 0.74 $mg{\cdot}kg^{-1}$ Hg, which is about 1/42 the dose of *Zuotai* group or β-HgS group. All groups were orally administrated for 14 consecutive weeks to ensure the mercury level in mouse kidney to reach the threshold of XAS analysis. The weights of the mice in each group were measured every week, and the volume of the drugs was adjusted according to their weight. At the end of animals experiment, the mice were sacrificed using a euthanasia method, the dislocation of cervical vertebra. And, the right and left kidneys of each mice were collected. The Animal Experimentation Committee of Northwest Institute of Plateau Biology, Chinese Academy of Sciences approved the protocol for all animal experiments performed in this study. The procedures involving mice and their care were conducted in conformity with the international guidelines, the European Community guidelines (EEC Directive of 1986; 86/609/EEC), and the US guidelines (NIH Publication #85-23, revised in 1985).

2.5. X-ray absorption spectroscopy

2.5.1. Sample preparation and reference materials

The left kidneys (n = 4) of each group mice were freeze-dried by vacuum, then were made into little round tablet with 1 cm diameter. Samples were coded and examined by X-ray absorption spectroscopy. The reference materials, β-HgS, α-HgS, red HgO, yellow HgO, $HgCl_2$, Hg_2Cl_2, $HgSO_4$, HgAc, $HgCys_2$ and MeHgCys, were used.

2.5.2. Work conditions of facility

X-ray absorption spectroscopic (XAS) measurements were

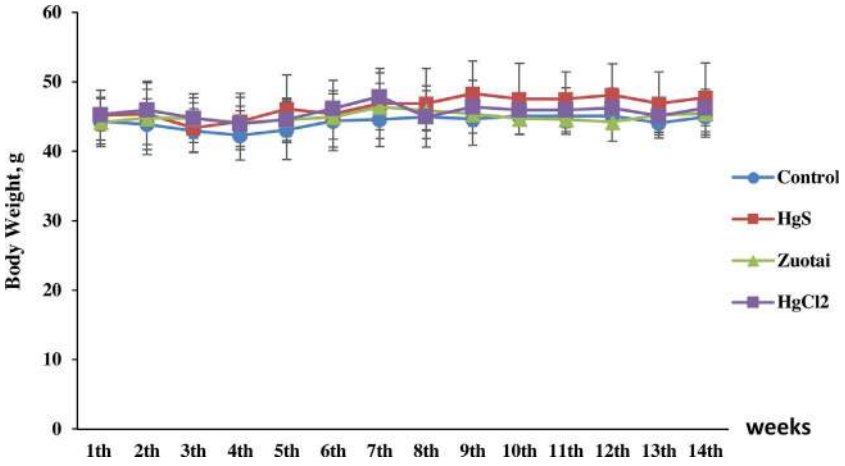

Fig. 1. Body weight change of each group mice of 14 weeks treatment. Mice in control group were treated orally with 2.5% starch solution; Mice in *Zuotai* group were orally treated under the dose of 66.7 mg·kg^{-1} *Zuotai*; Mice in β-HgS group were orally treated under 36.35 mg·kg^{-1} β-HgS; Mice in $HgCl_2$ group were orally treated under the dose of 1 mg·kg^{-1} $HgCl_2$. All data were expressed as the mean ± SD of each group (n = 8). The body weights of each group were compared using One-Way ANOVA Post Hoc Multiple Comparisons with Duncan method. At each time point, each group was compared with control group, *P < 0.05.

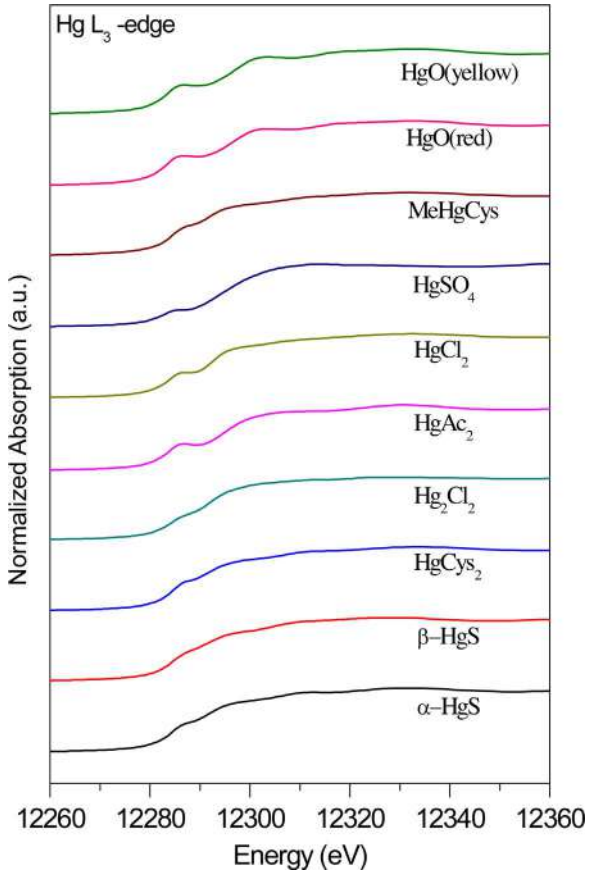

Fig. 2. Comparison for Hg L_3 near-adge spectra of standard species. β-HgS, α-HgS, red HgO, yellow HgO, $HgCl_2$, Hg_2Cl_2, $HgSO_4$, HgAc, $HgCys_2$ and MeHgCys were run as solids by monitoring transmittance.

conducted at Beijing Synchrotron Radiation Facility (BSRF, Beijing, China). Mercury L_3-edge data was collected on the XAFS experiment endstation 1W1 B under room temperature. Transmission mode was used for reference compounds and fluorescence mode was used for samples with Lytle detectors or 19-element Ge solid state detector employing a Si (110) double-crystal monochromator. Harmonic rejection was accomplished by setting the cutoff angle of the mirrors to reject energies above 15 keV. Photon flux was about 4 ∗ 1011 photon/s. Facula area was 0.9(H) × 0.5(V) mm. The storage ring ran at the energy of 2.5 Ge V with current intensity of 250 mA.

2.5.3. Data analysis

The raw XAS data of kidney samples and the references were preprocessed through conventional procedures by normalizing to the unit edge jump after removing the atomic background as implemented in Demeter package (developed by Dr. Bruce Ravel) [33]. The Fourier Transforms of k1-weighted EXAFS were conducted in over the k range [3–12 Å^{-1}] for all samples. According to reference materials, the speciation of the samples was analyzed in the XANES range through mathematical methods called principal component analysis (PCA) and linear combination fit (LCF), which were implemented in the Demeter − Athena package [33].

2.6. Micro X-ray fluorescence imaging

2.6.1. Sample preparation

The right kidneys of four mice in each group were made into frozen sections with 50 μm thickness, and were pasted on the XRF tape (TF-500) for μ-XRF imaging.

2.6.2. Facility work conditions

The distribution and relative contents of Hg, Ca, Zn, Fe and Cu in mouse kidney sections were analyzed with μ-XRF at the beamline BL15U at Shanghai Synchrotron Radiation Facility (SSRF, Shanghai, China). The continuous synchrotron X-rays were monochromatized by a Si (111) double crystal. A monochromatic X-ray beam with photon energy of 14 keV was used to excite the samples. The cross-section of the beam irradiating on the samples was adjusted to about 200 * 200 mm^2 with an about 10^{11} phs/s photons flux. The sample was placed at a 45° angle to the incident X-ray beam, and X-ray fluorescence was detected with a 50 mm^2 silicon drift detector (Vortex, USA) oriented at a 90° angle to the incident beam. A light microscope was coupled to a computer for sample viewing. The sample platform was moved by a motorized x-y mapping stage. The Hg, Ca, Zn, Fe and Cu distributions in the sections of stomach and duodenum were continuously scanned at a step of 200 μm for both x and y direction. Each spot was irradiated for 5 s.

2.6.3. Data analysis

The X-ray spectra were analyzed by the AXIL program (Canberra Benelux, Belgium) and all the element fluorescence intensities and the Compton scattering intensity were normalized to the collecting time and the changes in I_0, which was measured by an upstream ion chamber. The relative quantitative images of metals were obtained using software Igor Pro 6 (Wave Metrics Inc., USA).

2.7. Validation of total mercury analysis in kidney

The total mercury in right kidney of each group mice (n = 4) was

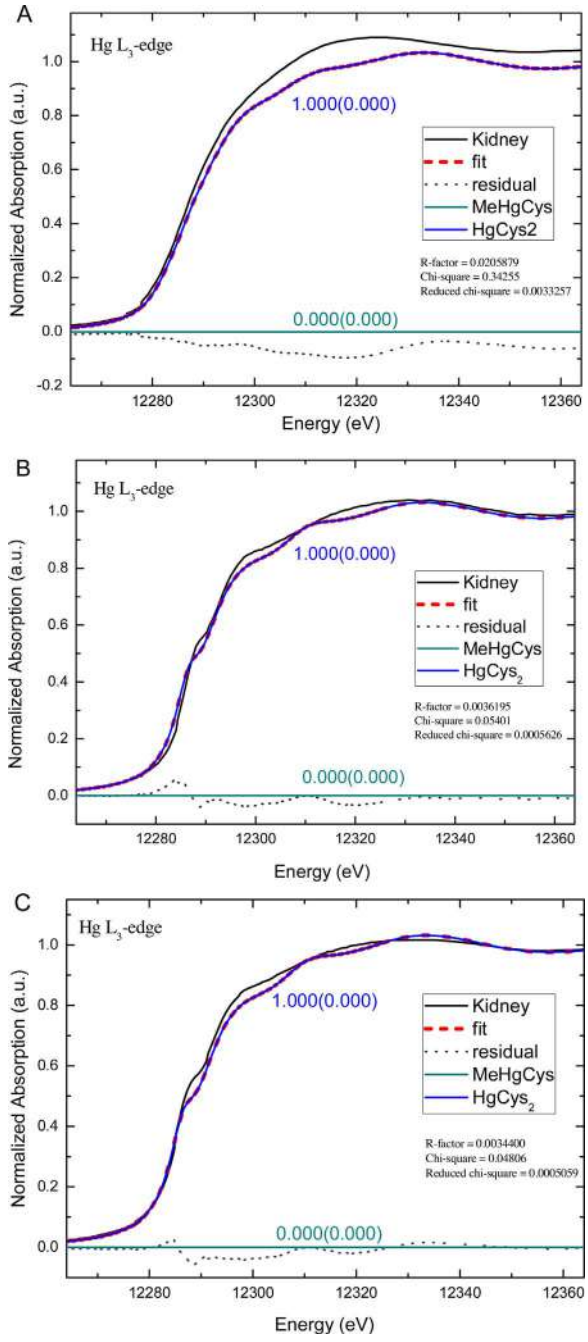

Fig. 3. The least-squares fitting of Hg L_3 X-ray absorption near edge spectra obtained from kidney samples. (A) The fitting of Hg L_3 X-ray absorption near edge spectra in kidney of *Zuotai* group mice; (B) The fitting of Hg L_3 X-ray absorption near edge spectra in kidney of β-Hg group mice; (C) the fitting of Hg L3 X-ray absorption near edge spectra in kidney of $HgCl_2$ group mice.

validated by automatic direct mercury analyzer (DMA-80, Milestone Co., Ltd, Italy), which can directly determine total Hg amount on solid, liquid and air samples without pre-treatment step. Standard curve functions were established as following: $A = 9.91148\,e^{-4}$ Hg ($R = 0.9999$) in a high mercury amount range of 20 − 1000 ng; $A = 0.05835222$ Hg − 0.00094396 Hg^2 ($R = 0.9999$) in a low mercury amount range of 0 − 20 ng. A represents the absorbance of mercury, Hg represents the mercury amount (ng), and R represents regression coefficient. Work condition of DMA-80: decomposition temperature is 650 °C, decomposition time is 120 s, the drying temperature is 200 °C, the drying time is 60 s, gold amalgam heating time is 15 s, the recording time is 45 s, the waiting time is 60 s, and the oxygen flow is 0.4 MPa, wavelength of atomic absorption measurements at 253.7 nm. In details, about the range of 0.005 g − 0.1 g kidney tissues were weighted and detected, according to the sample concentrations of different groups. And, each sample was tested for three repeats.

2.8. Kidney pathological observation

The left kidney of three mice in each group were fixed in 4% neutral formalin solution and embedded with paraffin, then cut into 2 μm thick section and stained with hematoxylin-eosin (HE). The microscope data of kidney histological structure were observed under 200 times amplification using optical microscope.

2.9. Statistical analysis

All data were expressed in the form of Mean ± SD for each group. The body weights of each group were compared using One-Way ANOVA Post Hoc Multiple Comparisons with Duncan method. The Independent-Samples *t*-Test was used to compare the mercury amounts in kidney of each group. Statistical analysis and graphs were performed using SPSS20.0 statistic software (IBM Corp., USA). A P value of < 0.05 was considered statically significant.

3. Results

3.1. Body weight change

Fig. 1 shows the body weight changes of each group mice ($n = 8$) during 14 weeks administration. At each week point, there was no significant changes between the body weights of control group and that of *Zuotai* group ($P > 0.05$), β-HgS group ($P > 0.05$) and $HgCl_2$ group ($P > 0.05$). The mental statuses of the mice in control, *Zuotai* and β-HgS groups were well, and their fur or hair were lustrous. However, the mental states and hair of mice in $HgCl_2$ group were abnormal at the end of administration period, comparing with control group.

3.2. X-ray absorption spectroscopy

X-ray absorption spectra arise from excitation of a core electron (e.g., a $2p_{3/2}$ electron for an L_3 − edge or a 1 s electron for a K-edge). They can be arbitrarily divided into two overlapping regions: the near-edge spectrum, which is the structured region within about 50 eV of the absorption edge, and the extended X-ray absorption fine structure (EXAFS), which is an oscillatory modulation of the absorption on the high-energy side of the absorption edge and can be interpreted in terms of a local radial structure. Near-edge spectra are comprised of transitions from the core level to unoccupied molecular orbitals of the system. Intense transitions are dipole allowed, $\Delta l = \pm 1$, and thus for K and L_3 edges are to levels with a lot of p and d orbital character, respectively. Near-edge spectra are therefore sensitive to electronic structure and give a fingerprint of the type of chemical species of the metal or metalloid concerned.

The Hg L_3-edge XANES spectra of the reference samples, β-HgS, α-HgS, red HgO, yellow HgO, $HgCl_2$, Hg_2Cl_2, $HgSO_4$, HgAc, $HgCys_2$ and MeHgCys are shown in Fig. 2. The speciation analysis was conducted on the XANES region, the 20 eV below and 80 eV above the absorption edge, for all kidney samples using the standard compounds. Firstly, we screened the useful candidate standards by comparing the spectral fingerprint of the sample and that of the standards in χ (k) function space using a method named PCA in Demeter-Athena software package. We finally narrowed to two compounds, i.e. $HgCys_2$ and MeHgCys, each of which showed distinctive fingerprints in the XANES region. Then, we analyzed the speciation of the samples through a mathematical method called linear combination fit (LCF), which is implemented in the Demeter-Athena package. By carefully calibrating the energy and allowing a small shift of energy as a fitting parameter, the proportion of mercury

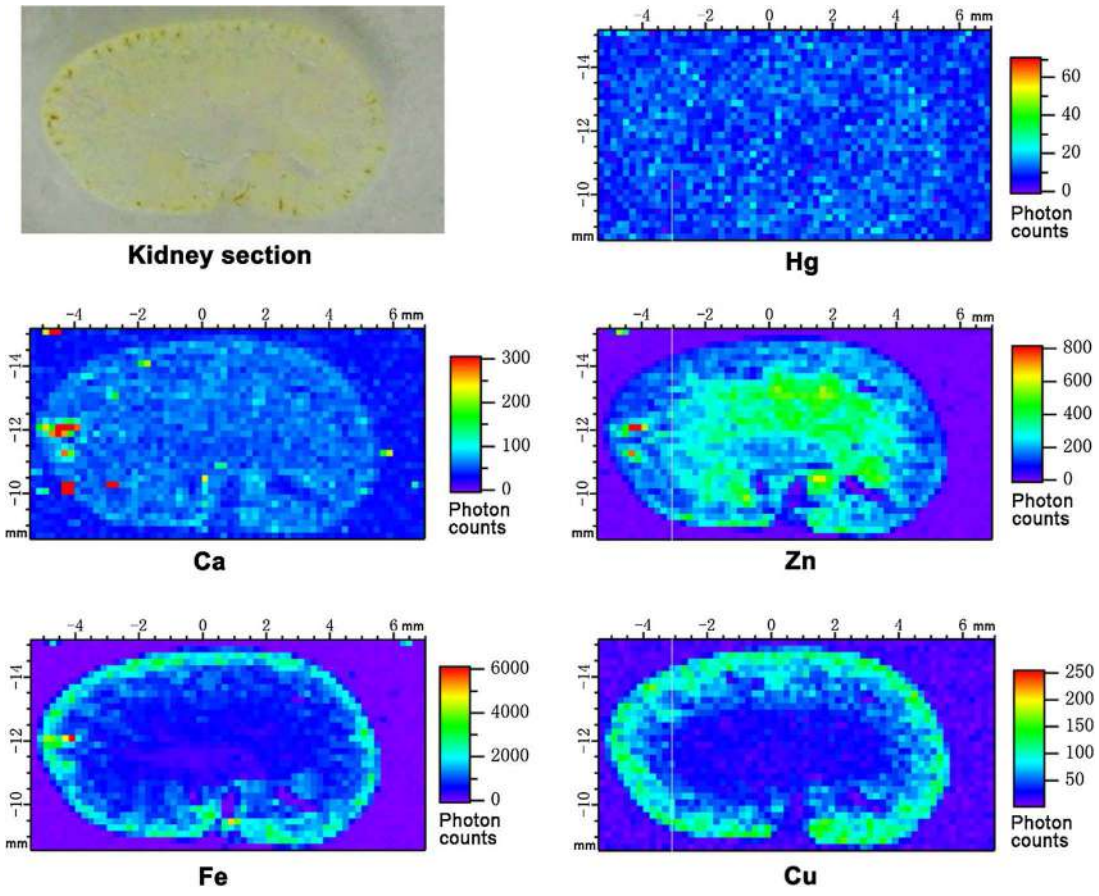

Fig. 4. The μ-XRF images of Hg, Ca, Zn, Fe and Cu in the kidney section of control group mouse after 14 weeks treatment. The images were normalized by the beam current intensity.

species can be obtained by linear combinations of the selected standards. The acceptable criteria for LCF is to obtain fitting with minimum residual and full physical meaning.

Fig. 3 shows the Hg L_3 near-edge XAS of kidney tissue samples. The kidneys of *Zuotai*, β-HgS and $HgCl_2$ groups mice showed that the chemical speciation of mercury mainly was in the form of a mercuric ion (Hg^{2+}) bonded to sulfydryl groups of two Cys molecules (Cys-S-Hg-S-Cys), plus some of unknown species, but with no MeHgCys. XAS determines the presence not of specific molecules but rather of classes of chemical species with similar local environment around the central absorbing atom. Therefore, the speciation of mercury in kidney may be not limited to Cys-S-Hg-S-Cys, and may be of the same category of molecules, R-S-Hg-S-R [$Hg(SR)_2$]. However, some potentially unknown reference standards may not be included in our standard library here, because we found that there has some level of residual after the spectra were fitted by the above candidate forms of mercury. Fortunately, the major constituents were included in our analysis. We underline that the spectral features cannot be fully reproduced, considering difference between the natural/complex samples and the synthesized/simpler standards. Unfortunately, we cannot analyze the EXAFS data for this series due to that most of the data have no good S/N ratio.

3.3. Micro X-ray fluorescence imaging of Hg and bio-metals

The kidney sections of each group mice were imaged using synchrotron radiation micro X-ray fluorescence (μ-XRF). The relative level of mercury (Hg) and biological metal elements (Ca, Zn, Fe and Cu) were represented by the intensity or counts of fluorescence photon. The Figs. 4–7 show the Hg micro-disribution in kidney of each group. In control group, mercury was very tiny and relative even in both cortex and medulla. But, in the *Zuotai*, β-HgS and $HgCl_2$ groups, the mercury was deposited in a large amount in renal cortex, and the renal medulla was distributed just only with a much low level of mercury. And, according to the μ-XRF relative quantification, the mercury level in kidney of $HgCl_2$ group was significantly higher than that of β-HgS group, the latter was higher than that of *Zuotai* group, which was also higher than that of control group.

Besides, bio-metals (Ca, Zn, Fe and Cu) spatial distributions are investigated and also shown as in Figs. 4–7. Ca was with a relatively even distribution in mouse kidney. Zn was mainly distributed in the inner stripe of the renal cortex and the outer stripe of renal medulla, and the outer layer of cortex and the inner layer of medulla in kidney were much low. The levels of Fe and Cu were the highest in renal cortex, which was followed by the outer stripe of renal medulla, and the lowest was in the inner stripe of renal medulla.

3.4. Validation of total mercury in kidney

The results of total mercury in kidney of each group mice are shown as in Fig. 8. Comparing with control group, the total mercury in kidneys of *Zuotai* group, β-HgS group and $HgCl_2$ group were extremely increased ($P < 0.01$ for *Zuotai*; $P < 0.001$ for β-HgS and $HgCl_2$) after 14 weeks consecutive administration in oral. Meanwhile, we found that the total mercury in kidney of the β-HgS and $HgCl_2$ groups mice were also remarkably higher than that of *Zuotai* group mice ($P < 0.05$ for β-HgS; $P < 0.001$ for $HgCl_2$). Therefore, it needs to note that *Zuotai* can result in a significantly low level of mercury deposition in mouse kidney under the high dose and long-term administration.

3.5. Pathological observation

Fig. 9 shows the histological images (×200) of kidney sections

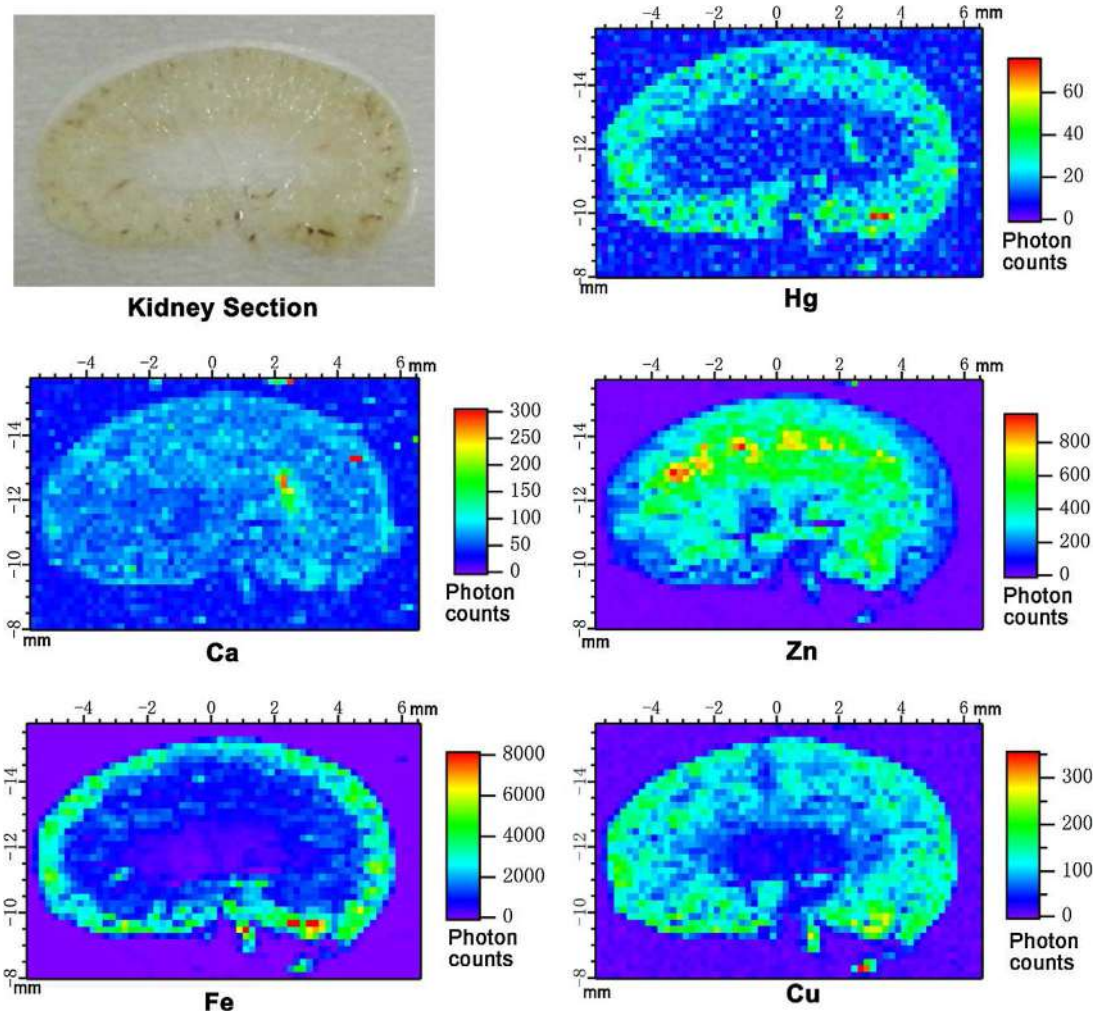

Fig. 5. The μ-XRF images of Hg, Ca, Zn, Fe and Cu in the kidney section of *Zuotai* group mouse after 14 weeks treatment. The images were normalized by the beam current intensity.

stained with HE dye. The kidneys of the *Zuotai* and β-HgS groups mice appear clear boundaries and structural integrity for renal glomerulus, glomerular cysts wall cell, proximal tubule and distal tubule, just with a little of infiltration inflammatory cell in renal interstitium, that was the same as control group. However, in $HgCl_2$ group, proximal tubule epithelial cells necrosis and falling in large amount of cells were observed; necrosis matters and tube-shape protein were filled in the proximal tubule of necrosis; glomerular atrophy or necrosis, cystic dilatation, glomerular wall cell necrosis and abscission with a large number of inflammatory cell infiltration in renal interstitium also were found. This findings suggest that renal toxicity of *Zuotai* (dose of 66.7 $mg{\cdot}kg^{-1}$) and β-HgS (dose of 36.35 $mg{\cdot}kg^{-1}$) is far lower than that of $HgCl_2$ (dose of 1 $mg{\cdot}kg^{-1}$).

4. Discussion

The present study has investigated the chemical speciation, spatial distribution, and toxicity of mercury from *Zuotai*, β-HgS and $HgCl_2$ in mouse kidney. The XAS data presented herein provide clear evidence concerning the chemical speciation of mercury in kidney. The speciation of mercury in kidneys of *Zuotai*, β-HgS and $HgCl_2$ groups was mainly the conjugates of mercuric ion (Hg^{2+}) bound to sulfydryl biomolecules (e.g. L-Cys) plus some unknown species, but with no MeHgCys. The mercury from *Zuotai*, β-HgS and $HgCl_2$ was mainly deposited in the renal cortex, and with a low level in renal medulla. The total mercury in kidney of $HgCl_2$ group mice was far higher than that of HgS group, the later was significantly much than that of *Zuotai* group, which was also higher than that of control group. There were no difference among the body weights of control, *Zuotai*, β-HgS and $HgCl_2$ groups at each week point during 14 weeks treatment. Besides, the current research also has revealed the spatial distributions of bio-metals (e,g., Ca, Zn, Fe and Cu) in mouse kidney. In details, Zn was in highest level of distribution in the inner stripe of cortex and the outer stripe of medulla; Fe and Cu were primarily distributed in the cortex; the level of Ca was relative uneven in both cortex and medulla.

As a classic traditional Tibetan medicinal mixture containing mercury, *Zuotai* is prepared from quicksilver, sulfur, *Nengchi* Eight metals (gold, silver, bronze, copper, brass, iron, lead, and tin), *Nengchi Eight Minerals* (gold ore, silver ore, magnet ore, tufa, pyritum, orpiment, realgar, and red mica), and other natural medicinal materials through complex processing procedures [3]. Physicochemical analysis had found that mercury in *Zuotai* was present in divalence (Hg^{+2}), and existed in the form of cubic crystal (β-HgS) without metallic mercury [8].

β-HgS is a typical insoluble substance with solubility product constant (K_{sp}) of 1.6×10^{-52} [26], but it still can dissociate a trace of free mercuric ions (Hg^{2+}), theoretically. Hg^{2+} ions can be bound to sulfydryl biomolecules (e.g. L-Cys) or small organic anions which are a large amount of in food digested in gastrointestinal tract, and generate mercuric conjugates which may be transported into body through mimicking endogenous biomolecules in intestine [34]. Our previous research had suggested that there had significant mercury deposition in mouse kidney after the long-term oral administration with *Zuotai and* β-HgS. The present study found that the chemical form of mercury from

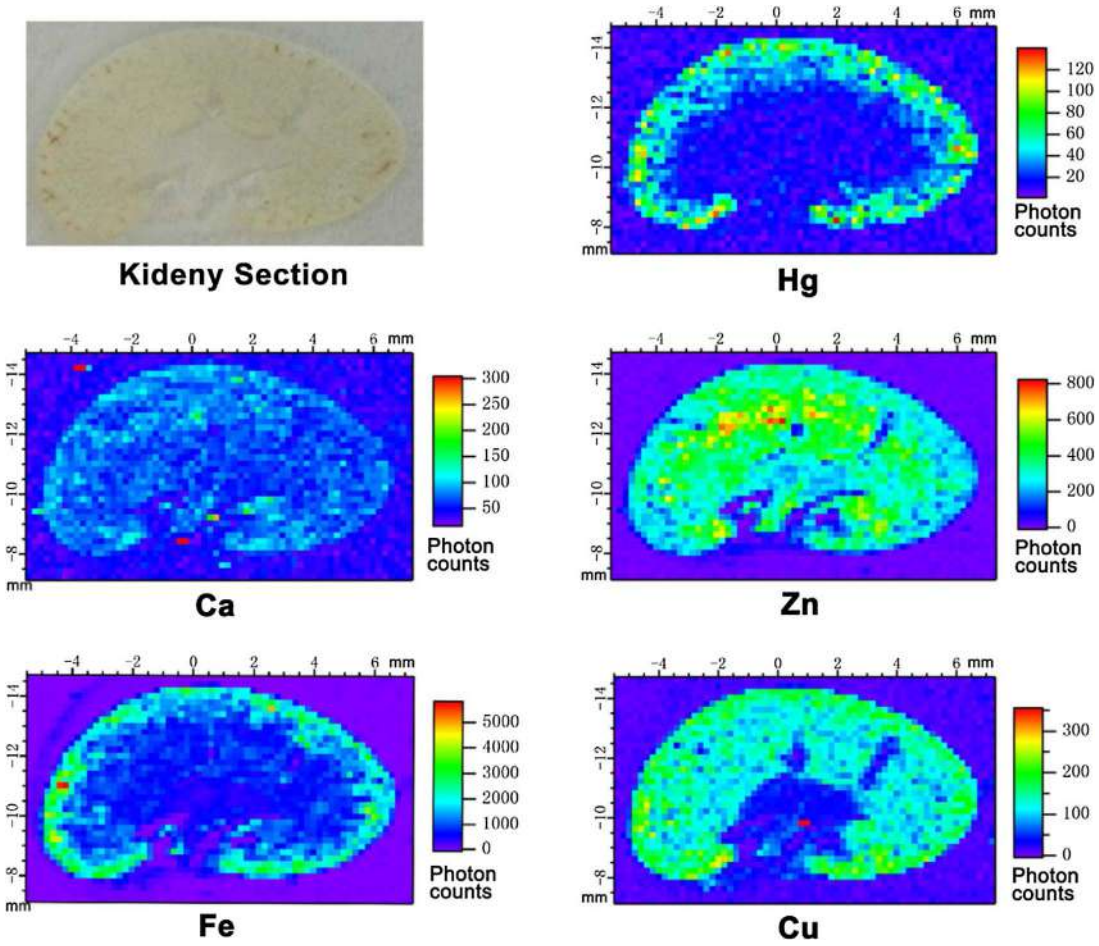

Fig. 6. The μ-XRF images of Hg, Ca, Zn, Fe and Cu in the kidney section of β-HgS group mouse after 14 weeks treatment. The images were normalized by the beam current intensity.

Zuotai, β-HgS and $HgCl_2$ in kidney was mainly the complex of and mercuric ion (Hg^{2+}) bound to L-Cys, Cys-S-Hg-S-Hg. *In vivo*, Hg^{2+} ion nephrotoxicity is hypothesized to involve the interaction of Hg^{2+} ion with sulfhydryl groups located on critical proteins and enzymes [35,36]. It has been reported that the complex of a mercuric ion bonded to two Cys molecules was one of important chemical forms in mammalian tissues. Human individuals poisoned with high levels of methylmercury species showed elevated cerebral cortical selenium with significant proportions of nanoparticulate mercuric selenide plus some inorganic mercury and methylmercury bound to organic sulfur [32]. Huang et. al reported that after rats were treated with $HgCl_2$ and β-HgS, these mercury binding forms in liver and kidney could be protein/polypeptide-bound mercury compounds, and about 71.8% of Hg was bound to one particular type of protein, possibly [37]. In organisms, almost the L-Cys molecules primarily exist in the form of residues in proteins or polypeptides [34]. Hg^{2+} ion has a very high affinity for sulfhydryl groups, with the formation constants of Hg^{2+} and the anionic form of a sulfhydryl group, R-S–, being $\geq \times 10^3$ higher than Hg^{2+} affinity constants for carboxyl or amino groups [38,39]. Thus, it is not surprising that mercury compounds identified in biological sample have been complexed with the molecules containing at least one sulfhydryl group. Therefore, the mercury in mouse kidney in the current study may be most of in the complexes of mercuric ions bound to the proteins or polypeptides containing L-Cys residues.

In the present study, there was no MeHgCys found in mouse kidney in the *Zuotai*, β-HgS and $HgCl_2$ groups, that was consistent with previous studies [40–42]. For example, Zhou et al. had reported that cinnabar, HgS and $HgCl_2$ cannot be transformed into highly toxic methylmercury under gut flora conditions[40]. Organisms that convert inorganic mercury salts (e.g. HgS, HgO, $HgCl_2$) into methylmercury are mainly low organisms, such as bacteria, algae and fish [43–47]. Not all bacteria can make mercury methylated, and the bacteria that are capable of mercury methylation are mainly sulfate reducing bacteria (SRB), iron reducing bacteria(IRB) and methanogens [45–47]. Although the mercury chemical species in mouse kidney in $HgCl_2$ group was similar as that of *Zuotai* and β-HgS, the kidney mercury level in mouse treated with $HgCl_2$ was far higher than that of *Zuotai* and β-HgS. So, a large amount of Hg^{2+} ions from $HgCl_2$ would combine to function proteins or enzymes containing sulfydryl (-SH) in kidney, and generate massive superoxide free radical to induce severely renal injure, which has been observed in current research.

Besides, we found that the L_3 near X-ray absorption spectra fitted of mercury in mouse kidney of *Zuotai*, β-HgS and $HgCl_2$ groups have some level of residue, which indicates that there may have some other unknown mercury species in kidney except for $HgCys_2$. It had been found that mercury and selenium exist in the form of HgSe in the liver of northern fur seal [48]. In black-footed albatross, XAFS analysis disclosed an existence of chalcogenide containing both Hg-Se and the Hg-S bonds, suggesting the existence of a solid solution Hg (Se, S) as granules in tissues [48]. In the striped dolphin, the SR-micro XRF spectra and the SR-micro XRD pattern of the hot spots confirmed the presence of tiemannite, HgSe, and XAFS analysis confirmed the presence of HgSe in liver, kidney, lung, spleen, pancreas, muscle and brain [49].

The target organ for inorganic Hg(II) ions toxicity is generally considered to be the kidney, which is the dominant site of Hg(II) deposition [36,50]. And, the renal injury might be caused following acute and chronic exposure to various form of mercury [51]. However, different forms of mercury have different accumulation and toxicity on

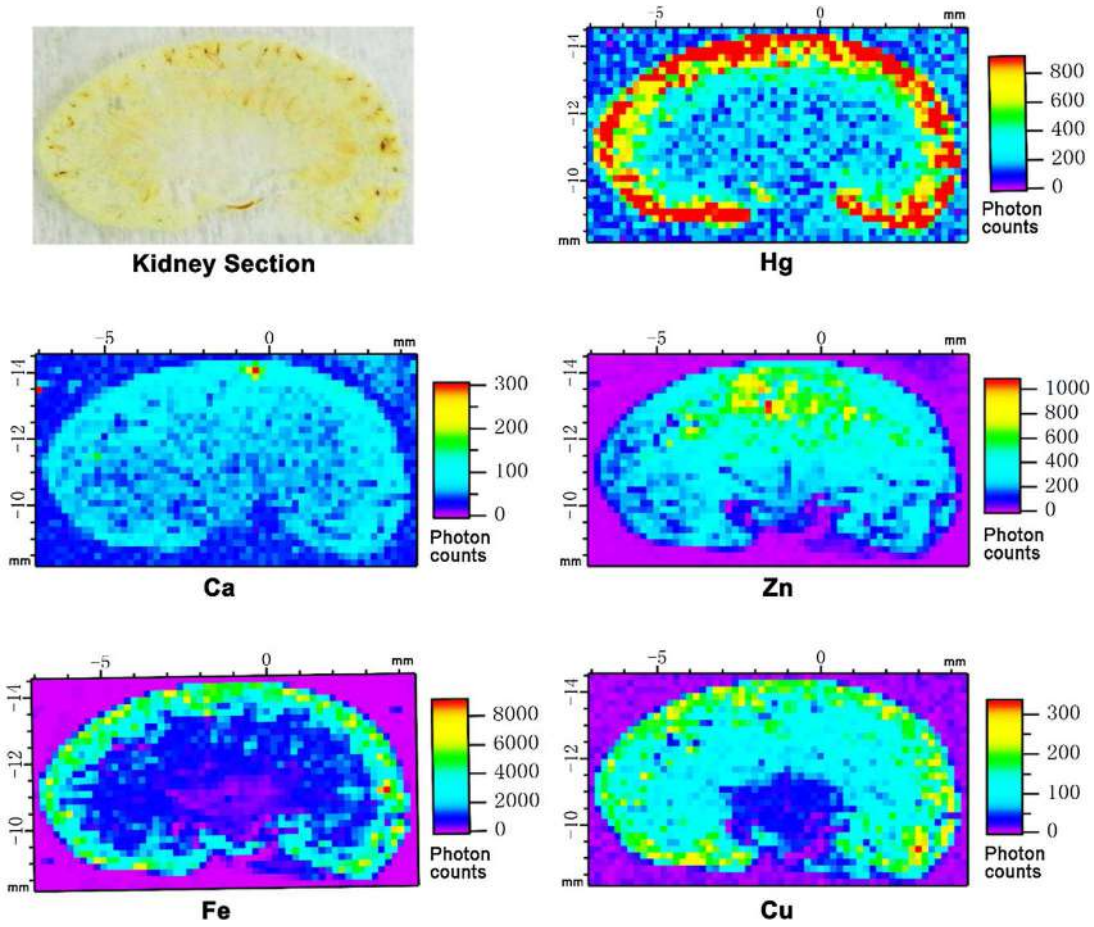

Fig. 7. The μ-XRF images of Hg, Ca, Zn, Fe and Cu in the kidney section of $HgCl_2$ group mouse after 14 weeks treatment. The images were normalized by the beam current intensity.

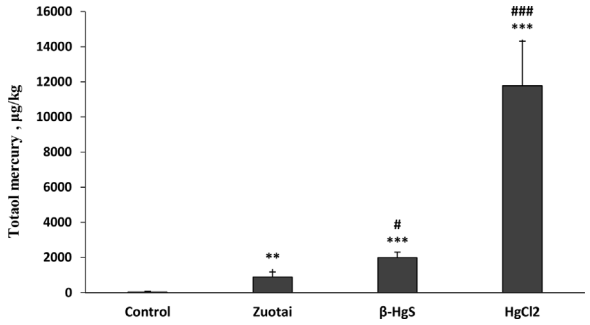

Fig. 8. Total mercury (μg/kg) in kidney of each group. Mice in control group were treated orally with 2% starch solution; Mice in *Zuotai* group were orally treated under the dose of 66.7 $mg{\cdot}kg^{-1}$ *Zuotai*; Mice in β-HgS group were orally treated under 36.35 $mg{\cdot}kg^{-1}$ β-HgS; Mice in $HgCl_2$ group were orally treated under the dose of 1 $mg{\cdot}kg^{-1}$ $HgCl_2$. All data were expressed as the mean ± SD of each group ($n = 4$). The Independent-Samples *t*-Test was used to compare the mercury amounts in kidney of each group. Compared with control group, *P < 0.05 , **P < 0.01 , ***P < 0.001; Zuotai group was compared with β-HgS group and $HgCl_2$ group, #P < 0.05 , ##P < 0.01 , ###P < 0.001.

kidney, of them free divalence mercuric ions (Hg^{2+}) possess the most toxicity to kidney [19,52]. The present study suggested that the $HgCl_2$ resulted in a very high level of deposition and serious nephrotoxicity (e.g. proximal tubule epithelial cells necrosis and falling, the proximal tubule filled with tube-shape protein, glomerular necrosis, cystic dilatation, glomerular wall cell necrosis, and a large number of inflammatory cell infiltration renal interstitium) in mouse kidney, but that was not found in *Zuotai* and β-HgS groups. *In vitro* studies indicate that the Hg^{2+} ions initially affects the pars recta of the proximal tubule, suggesting that this section of the nephron is the most sensitive to the toxic effects of mercury [53–55]. Due to renal tubules and glomeruli are mainly located in the cortex, therefore the layer is the mostly sensitive to free mercuric ions (Hg^{2+}). The micro X-ray florescence analysis in this paper found that highest mercury level in kidney was mainly located in cortex, the lowest is in medulla. This finding provides a direct evidence for that the cortex is the most important target site of mercury toxicity in kidney from a new perspective.

In summary, the present study has elucidated that the chemical speciation of mercury from *Zuotai*, β-HgS and $HgCl_2$ in mouse kidney mainly was in the form of mercuric ions (Hg^{2+}) bound to sulfydryl group biomolecules, but no methylmercury cysteine, with a high level of mercury distribution in renal cortex. The deposition and toxicity of mercury from *Zuotai* and β-HgS in kidney were far less than that of $HgCl_2$. However, we should be aware of that *Zuotai* can induce a low level of mercury accumulation, which may have some potential adverse effects on kidney, after long-term and high dose exposure. Besides, some level of residue in the fitting spectra of Hg L_3 near-edge X-ray absorption of kidney indicates that there may have some unknown mercury species in kidney. That is very worthy to further investigate in the future. Taken together, these findings are importantly scientific significance for the recognition of the safety of Tibetan medicine and its compound preparation.

Conflict of interests

The authors declare that there are no conflicts of interest.

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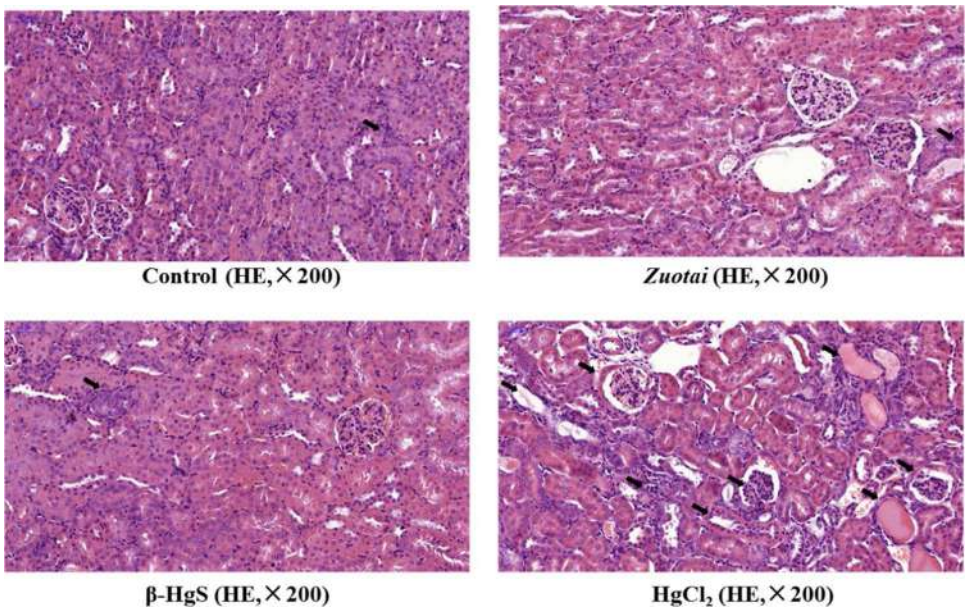

Fig. 9. The histological images of kidney sections of each group mice. These kidney sections were stained with HE dye, and observed under 200 times of magnification.

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Toxicity study of *Lauha Bhasma* (calcined iron) in albino rats

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ABSTRACT

Background: *Lauha Bhasma* (LB) is a complex herbomineral preparation widely used as an Ayurvedic hematinic agent. It is an effective remedy for chronic fever (*jīrṇa jvara*), phthisis (*kṣaya*), Breathlessness (*śvāsa*) etc., and possesses vitality enhancing (*vājīkara*), strength promoting and anti aging (*rasāyana*) properties.

Objectives: The present work was conducted to establish the safety aspects of the use of *Lauha bhasma*.

Setting and Design: LB was prepared by Ayurvedic procedures of purification (*śodhana*), sun drying (*bhānupāka*), *sthālīpāka*, followed by repeated calcination (*māraṇa*) and "nectarization" (*amṛtīkaraṇa*). The resultant product was subjected to acute and sub acute toxicity studies.

Materials and Methods: Acute and subacute toxicity study of LB was conducted in albino rats. Criteria for assessment included ponderal changes, change in biochemical parameters viz., LFT and KFT and hematological parameters. Histopathological studies of different organs including liver, kidney, spleen, testis etc., were also conducted to observe pathological changes if any.

Results: In the acute toxicity study, the animal group did not manifest any signs of toxicity and no mortality was observed up to 100 times the therapeutic dose (TD). Significant increase in blood urea (27.83%, $P < 0.01$), serum creatinine (30.92%, $P < 0.05$), Aspartate aminotransferase (15.09%, $P < 0.05$), and serum alkaline phosphatase (27.5%, $P < 0.01$) was evident in group IV (10 TD). A significant increase in serum total protein (6.04%, $P < 0.05$) level was observed in group III (5 TD). Histopathological examination of livers in group IV (10 TD) showed mild inflammation in terms of bile stasis, peri-portal hepatic inflammation and sinusoidal congestion; lymphocyte infiltration in kidney and intracellular deposits in the splenic tissue.

Conclusion: *Lauha Bhasma* was found to be safe at the therapeutic dose and also at five times the therapeutic dose levels. However, alteration in some of the biochemical and haematological parameters along with histopathological findings were evident at the highest dose level.

KEYWORDS: Biochemical, histopathology, *Lauha bhasma*, liver, spleen *Triphala*, toxicity

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INTRODUCTION

Traditional medicines are used in the treatment of various chronic disorders and for the improvement of well-being of individuals.[1] In Ayurveda, metals such as Iron, Copper, Zinc, and Lead, etc., are used in many preparations, after transforming the metals into nonmetallic forms.[2] The medicines so prepared are classified under a group called *Rasauṣadhis*. *Rasauṣadhis* have been prescribed by Ayurvedic physicians since long with rare mentions of toxicity. It is observed that herbomineral complexes are more stable and more interactive compared to plain herbs as these result in faster therapeutic action and have a longer shelf life.[3] *Bhasmas* are unique preparations involving metallic/mineral preparations calcined using heat to transform metals into non-toxic organometallic forms.[4] In preparations of these *Bhasmas*, the preliminary

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process which causes detoxification without harming its medicinal properties (*guṇas*) is called as '*Śodhana*'.[5,6] The process of *śodhana* was well accepted by the pioneers of *Rasaśāstra* for the purification of herbomineral drugs. These methods were developed to detoxify the raw material by chemical transformations and enhance the properties and therapeutic potential.[7,8] There have been concerns raised regarding the use of metals and minerals in therapeutics.[9] Raising safety concerns about traditional preparations, the WHO has issued guidelines regarding toxicity studies of herbal drugs.[10] The present study was designed to establish the toxicity profile of *Lauha Bhasma* (LB) in experimental animals.

MATERIALS AND METHODS

Raw materials

Iron flakes were procured from a local market of Jaipur. Raw *Triphalā* used in the pharmaceutical processing was procured from pharmacy of NIA Jaipur and was made into a decoction. *Sesamum* oil (*tila tailam*), buttermilk (*takra*), seeds of *Dolichos biflorus* (*kullatha*) used for *śodhana* were purchased from local market and authenticated at Pharmacognosy Laboratory, National Institute of Ayurveda, Jaipur. Cow urine (*gomūtra*) was procured from a local *gośālā*.

Preparation of *Lauha bhasma*

Iron flakes were subjected to the Ayurvedic process of purification which included general purification (*sāmānya śodhana*),[11] specific purification (*viśeṣa śodhana*),[12] followed by sun drying (*bhānupāka*),[13] *sthālīpāka*,[14] levigation and calcination (*puṭapāka*)[15] and "nectarization" (*amṛtīkaraṇa*)[16] as per the classical references mentioned in the Ayurvedic Formulary of India (AFI). The final product was named as LB and was subjected to acute and sub-chronic toxicity studies.[17]

Experimental animals

Adult albino rats of Wistar strain of both sex weighing between 150 – 200 g were used in this study. They were procured from the animal house attached to the Department of Zoology, Rajasthan University, Jaipur. The animals were kept in standard conditions of 22°C ± 2°C and relative humidity 55% ±15%. The rats had free access to food (Pranav agro mills "Amrut" brand rat pellets) and water *ad libitium* with 12 hours light and dark cycle. All animals were acclimatized for at least 5 days before the start of the study. All the experimental protocols were approved by Institutional Animal Ethics Committee (IAEC) and performed according to the CPCSEA guidelines for the care and use of animals. Dose of *Lauha bhasma*[18] was escalated as per conversion of human dose to experimental animal dose.[19]

Acute toxicity study of *Lauha bhasma*

Totally, 15 Wistar albino rats of either sex, weighing 150–250 g were divided randomly into five groups, containing three animals each. All animals in group I were treated with *Lauha bhasma* orally at the dose of 10 times the therapeutic dose. Animals of Group II were given 20 times the therapeutic dose while, group III were given 40 times, Group IV were given 80 times, and Group V were given 100 times the therapeutic dose of *Lauha Bhasma* respectively. Single doses of drugs was administered orally according to the stated dosage schedule. Gross behaviour and exitus (death) were recorded for 14 consecutive days.

Sub-acute toxicity study of *Lauha bhasma*

Twenty four Wistar rats (weighing 150–250 g) were randomly assigned into four groups of six each as shown in Table 1. First group was treated as control whereas other three groups were administered therapeutic dose (TD) i.e., 4.16 mg/kg, five times of TD (20.80 mg/kg) and ten times of TD (41.60 mg/kg) along with 0.5 ml of honey as vehicle respectively. Initial body weights of all the animals was recorded and blood was drawn from supraorbital plexus[20] for laboratory testing. The same procedure was repeated at the end of study. At the end of study the rats were sacrificed by stunning and severing the neck vessels.

Histopathological study

All the vital organs were carefully dissected, cleaned, weighed and transferred to Bouin's solution for preservation and sent to a commercial laboratory for preparation of histopathological slides. The slides were scanned in trinocular Carl Zeiss's microscope (Germany) under different magnifications. Changes if any, in cytoarchitecture were noted down.

Table 1: Dose selection for subacute toxicity of *Lauha Bhasma* (L.B.)

Group	No. of animals	Dose (mg)/kg	Anupana (honey) ml
Control	6	-	0.5
Therapeutic dose (T.D.)	6	4.16	0.5
Intermediate dose (5 T.D.)	6	20.80	0.5
Higher dose (10 T.D.)	6	41.60	0.5

T.D: Therapeutic Dose

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Laboratory investigation

All the hematological, histopathological as well as biochemical studies were conducted in a private well equipped laboratory in Jaipur.

Hematological and biochemical analysis

Hematological analysis was performed using an automatic hematological analyser. Samples of blood were drawn at the beginning and termination of the experiment. Parameters studied included red blood cell (RBC) count which was done by Haymes method, white blood cell (WBC) count by Turkish method, haemoglobin (Hb) by Sahil's method. Packed cell volume (PCV), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC) and platelets count were investigated by complete blood count (CBC). For biochemical analysis, blood sugar, total cholesterol, triglyceride, high-density lipoproteins (HDL), urea, creatinine, total protein, bilirubin, aspartate aminotransferase (AST), alanine aminotransferase (ALT) and alkaline phosphatase (ALP) were analysed by rapid photoelectric method.

Statistical analysis

Values reported are mean ± standard error. The data were analysed by unpaired Student's *t*-test and ANOVA. A level of 0.001 was considered to be highly significant whereas $P < 0.05$, 0.01 were considered to be significant. Level of significance was noted and interpreted accordingly. All the statistical analyses were performed using SPSS, version 15 (SPSS Inc., Chicago).

RESULTS AND DISCUSSION

Iron based drugs are widely used in modern medicine as haematinics. These drugs are known to induce adverse drug reactions including gastro intestinal symptoms such as nausea, vomiting, epigastric pain, eructation, pyrosis, meteorism, borborygami, colic pain, flatulence, constipation, black faeces, and diarrhoea.[21] The haematinics market in India is currently worth around Rs. 900 crore and is growing at 15% per annum.[22] *Lauha Bhasma* can be an effective hematinic provided its safety can be established. In the acute toxicity study, none of the animal groups manifested any signs of toxicity and no death was observed even at a dose which was 100 times the therapeutic dose. There was only mild behavioural hyperactivity noticed. From this it can be concluded that the approximate LD_{50} value is more than 416 mg/100 g of the weight of the experimental animal.

In sub-acute toxicity study, in all groups at varying test doses, a considerable non-significant increase in body weight was evident. Body weight is indicative of increased appetite and food intake and adverse effects of a drug may become expressed as decrease in body weight.[23] As there was no decrease in body weight observed, it may be presumed that LB at even its highest dose level did not have any deleterious effect. A non-significant increase or decrease in organ weight was evident in all groups as shown in Table 2. Eleven biochemical parameters [as shown in Tables 3 and 4] were measured in all three test groups, out of which significant increase in blood urea (27.83%, $P < 0.01$), serum creatinine (30.92%, $P < 0.05$), Aspartate

Table 2: Change of weight in different organs

Group	Weight of liver (g)	Weight of spleen (g)	Weight of heart (g)	Weight of kidney (g)	Weight of brain (g)	Weight of lungs (g)	Weight of testes (g)	Weight of uterus ovaries (g)	Weight of testes (g)
Control	5.41±0.15	0.44±0.03	0.58±0.02	1.11±0.03	1.34±0.03	1.25±0.01	1.70±0.06	0.67±0.02	1.70±0.06
Therapeutic dose (T.D.)	5.44±0.17*	0.39±0.02*	0.55±0.02*	1.08±0.04*	1.39±0.01*	1.19±0.01*	1.63±0.07*	0.68±0.03*	1.63±0.07*
Intermediate dose (5 T.D.)	6.08±0.16*	0.47±0.02*	0.53±0.02*	1.21±0.03*	1.40±0.01*	1.21±0.05*	1.77±0.05*	0.74±0.02*	1.77±0.05*
Higher dose (10 T.D.)	5.27±0.26*	0.37±0.02*	0.53±0.03*	1.14±0.05*	1.38±0.01*	1.08±0.03*	1.59±0.07*	0.69±0.04*	1.59±0.07*

*P<0.05, **P<0.01, ***P<0.001. Values are mean±SEM from 6 animals in each group. Statistical significant test for comparison was done by ANOVA, followed by *t*-test. Comparison between: a-Group I versus Group II, b-Group I versus Group III, c-Group I versus Group IV, *: Nonsignificant, SEM: Standard error of mean, T.D.:Therapeutic dose

Table 3: Change in biochemical parameters in different groups

Group	Blood sugar (mg/dl)	Serum cholesterol (mg/dl)	Serum triglyceride (mg/dl)	H.D.L. cholesterol (mg/dl)	Blood urea (mg/dl)
Group I (control group)	84.19±1.17	53.83±1.17	184.17±1.25	49.50±0.85*	36.83±0.60
Group II (T.D.)	84.83±1.19*	54.50±1.76*	160.50±11.28*	50.17±2.67*	34.83±2.03*
Group III (5 T.D.)	85.42±0.99*	54.00±1.28*	179.83±3.42*	54.50±2.32*	39.67±1.12*
Group IV (10 T.D.)	87.58±1.06*	49.50±2.23*	178.50±4.17*	56.33±3.99*	50.50±3.23**

*P<0.05, **P<0.01, Values are mean±SEM from 6 animals in each group. Statistical significant test for comparison was done by ANOVA, followed by *t*-test. Comparison between: a-Group I versus Group II, b-Group I versus Group III, c-Group I versus Group IV, *: Nonsignificant, **: Significant, SEM: Standard error of mean, T.D.:Therapeutic dose

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Table 4: Change in biochemical parameters in different groups

Group	Serum creatinine (mg/dl)	Serum total protein (g/dl)	Serum bilirubin (ml/dl)	S.G.O.T. (IU/L)	S.G.P.T. (IU/L)	Serum A.L.P. (IU/L)
Group I (control group)	0.94±0.06	6.90±0.14	0.75±0.02	383.17±5.99	115.50±7.33	275.00±4.08
Group II (T.D.)	0.85±0.01*	7.17±0.10*	0.73±0.01*	375.83±10.3 *	116.17±8.62*	285.00±24.05*
Group III (5 T.D.)	1.04±0.06*	7.28±0.05**	0.76±0.01*	417.83±27.76*	109.50±6.86*	289.17±13.48*
Group IV (10 T.D.)	1.24±0.11**	7.18±0.07*	0.79±0.02*	441.00±24.03**	99.67±3.28 *	346.83±32.14**

*P<0.05, **P<0.01, Values are mean±SEM from 6 animals in each group. Statistical significant test for comparison was done by ANOVA, followed by *t*-test. Comparison between: a-Group I versus Group II, b-Group I versus Group III, c-Group I versus Group IV, *: Nonsignificant, **: Significant, SEM: Standard error of mean, T.D.:Therapeutic dose

aminotransferases (15.09%, $P < 0.05$), and serum alkaline phosphatase (27.5%, $P < 0.01$) was evident in group IV (highest dose level). A significant increase in serum total protein (6.04%, $P < 0.05$) level was observed in group III. Urea contributes most of the body's non-protein nitrogen. It is the major end product of protein catabolism synthesized in liver, released in blood and excreted by the kidneys. It is a chief indicator of renal and hepatic integrity.[24] Elevated serum urea level may be due to pre renal, renal or post renal etiology. Similarly, serum creatinine is a product of creatine and phosphocreatine, which are important components of muscle. Creatinine is freely filtered and therefore the serum creatinine level depends on the glomerular filtration rate.[25] Renal dysfunction diminishes the ability to filter creatinine and raises its serum level. The only condition that causes a significant increase in serum creatinine level is damage to a large number of nephrons. The increase in serum urea and creatinine in animals which received higher dose of *Lauha bhasma* with evident inflammatory histopathological changes indicate that as the dose is increased from intermediate dose level (i.e., 5 times to T.D.) there is a likelihood of kidney damage even when absolute values were still within normal range. However, utmost precautions are needed in prescribing higher than equivalent doses in human beings. This feature is totally dose dependent and result of excess of free iron, which acts as a free radical and is tissue toxic.[26] Aspartate aminotransferases (APT) is a liver enzyme that aids in producing proteins. Besides liver, it is also found in other organs such as heart, muscle, brain and kidney. Injury to any of these tissues can cause an elevated blood level.[27] It also helps in detecting hepatocellular necrosis but is considered a less specific biomarker enzyme for hepatocellular injury[28] as it can also signify abnormalities in heart, muscle, brain or kidney.[29] On the other hand serum alkaline phosphatase (ALP) is an important parameter in distinguishing hepatobiliary disease.[30] It is particularly present in the cells which line the biliary ducts of the liver.[31] Cholestatic injury is characterized by predominantly initial alkaline phosphatase level elevations.[32] Significant but not marked increase in APT at the highest dose (H.D.) which was 10 times the Therapeutic Dose (T.D.) level with simultaneous increase in ALP, as well as histopathological evidence of bile stasis reflects a stage of hepatobiliary damage, although again it is a dose dependent finding as increase in alkaline phosphatase and/or bilirubin with little or no increase in ALT is primarily a biomarker of hepatobiliary effects and cholestasis.[32,33] The estimation of total proteins in the body is helpful in differentiating between normal and damaged liver functions. This is because the majority of plasma proteins such as albumins and globulins are produced in the liver.[34] Low total protein level is suggestive of kidney disorder or disorder where protein is not absorbed or digested properly.[26] Thus, increase in total body protein without any untoward histopathological findings is suggestive of anabolic effect of test drugs. Analysis of blood parameters is relevant to risk evaluation of alterations of the haematological system in humans.[35] A highly significant increase in haemoglobin percentage at T.D (5.25%, $P < 0.001$) and significant increase in other two tested groups shows haematinic potential. Just as haemoglobin concentration, the total R.B.C. count is an indicator of ready bioavailability of iron. At therapeutic dose level, an increase in total R.B.C. count was observed thus suggesting better absorbability and ready availability of iron for R.B.C. formation. Other red cell indices as shown in Table 5 were not much affected except for a change in haematocrit which showed a significant increase (8.68%) at therapeutic dose level. None of the groups showed any effect on W.B.C. and platelet count except for their raised levels at 10 T.D. Increased WBC count is indicative of inflammatory conditions of certain organs, especially of liver.[36] Histopathological findings showing normal tissue structures after administration of *Lauha Bhasma* is shown in Figure 1, however histopathological examination showed some notable changes in liver, kidney and spleen at highest dose treated L.B. group as shown in Table 6. Liver findings of the 10 T.D. group were suggestive of inflammatory infiltrate [Figure 2c], mild inflammation in terms of bile stasis [Figure 2d], and sinusoidal congestion. [Figure 2f], mild inflammation with peri-portal ballooning [Figure 2e] was also seen in 5 T.D. group. Similarly, 10 T.D. group

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Table 5: Change in Hematological parametes in different groups

Group	Hb% (g/dl)	T.R.B.C. (10^6/µl)	P.C.V. (%)	M.C.V. (fl)	M.C.H (pg)	MCHC	Total W.B.C. count (10^3µl)	Platelet count (10^3µl)
Group I (control group)	14.97±0.13	8.48±0.09	44.50±0.91	52.18±1.35	17.66±0.28	33.68±0.46	6.22±0.03	951.75±3.77
Group II (T.D.)	15.88±0.13***	8.60±0.07*	49.00±1.73**	57.13±2.05*	18.49±0.16**	32.43±1.30*	7.26±0.80*	972.07±6.00*
Group III (5 T.D.)	14.97±0.50**	8.25±0.12*	45.83±1.35*	55.98±1.91*	18.42±0.59*	32.53±1.32*	7.26±0.34**	900.02±32.83*
Group IV (10 T.D.)	13.60±0.50**	8.15±0.11**	44.80±1.13*	54.67±1.61*	16.07±2.25*	28.47±1.02***	7.17±0.17***	931.57±30.58*

*P<0.05, **P<0.01, ***P<0.001. Values are mean±SEM from 6 animals in each group. Statistical significant test for comparison was done by ANOVA, followed by *t*-test. Comparison between: a-Group I versus Group II, b-Group I versus Group III, c-Group I versus Group IV, *: Nonsignificant, **: Significant, ***: Highly Significant, SEM: Standard error of mean, T.D.:Therapeutic dose

Table 6: Histopathological finding of different organs in albino rats

Organ	*Lauha bhasma* (L.B.)
Liver	Bile stasis 10 T.D. (Fig-2d) Periportal ballooning 5 T.D. (Fig-2e) Sinosoidal congestion (Fig-2 f)
Spleen	Intra cytoplasmic pigments 10 T.D. (Fig-2g)
Heart	NE
Kidney	Granular deposit in the lumen 10 T.D. Lymphocytic infiltration 10 T.D. (Figure 2a)
Brain	NAE
Lung	NAE
Testes	NAE
Uterus + Ovary	NAE

NAE: No adverse effect, T.D.: Therapeutic dose

showed lymphocyte infiltration [Figure 2a] in kidney which in all of the other dose was found to be of normal cytostructure. The same group showed intracellular deposits in the splenic tissue that might be due to deposition of excess of iron that failed to get metabolized [Figure 2g]. Increase in some of the indicators for liver and kidney functions i.e. urea, creatinine, ALT etc., along with histopathological findings may contribute to elevation of WBC count and was only seen in group treated with highest dose. None of the findings could be attributed to toxic effect of the drug as most of the findings observed are mild in nature and thus are self-limiting. Critical analysis of the observations mentioned above reveals that *Lauha bhasma* did not impart any untoward effect when administered at therapeutic dose level but on increasing the dose level further, features suggestive of altered physiology (not toxicity) were evident. So, it may be concluded that the untoward effect of *Lauha* is totally a dose dependant phenomenon. The famous phrase of Paracelsus "all things are poisonous and there is nothing that is harmless, the dose alone determines that something is no poison" is true for Ayurvedic metallic preparations. Use of *Lauha Bhasma* alone at high doses is not recommended in Ayurvedic texts. Wherever *Lauha bhasma* is administered in high doses, it is administered in one of the following ways: (i) *Kalpa Krama*[37] i.e., administration in gradual increasing order

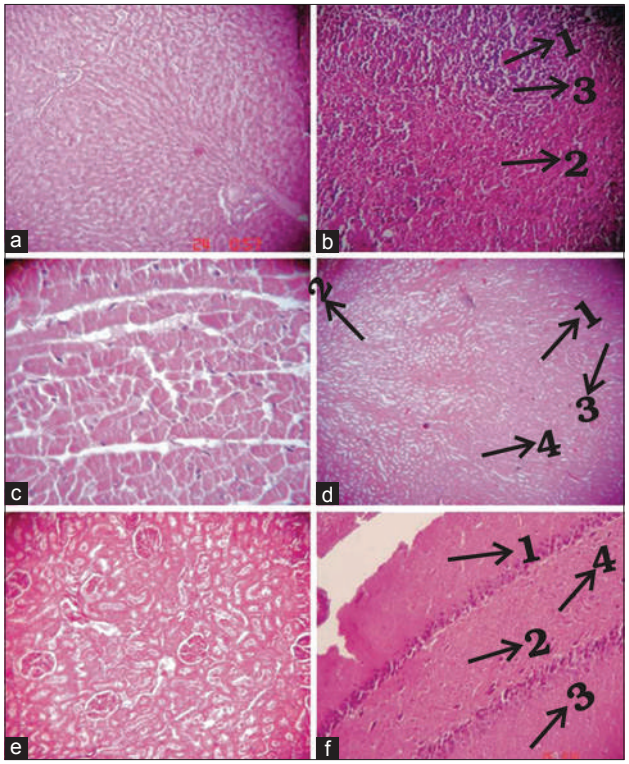

Figure **1:** Normal cytostructure of histopathological findings of toxicological study of *Lauha Bhasma*. (a) Photograph of liver showing normal cytostructure of portal tract control group (H and E, ×10) (portal vein hepatic artery, bile duct hepatocyte). (b) Photograph of spleen showing normal follicular cytostructure control group (H and E, ×10). (1) Central sinusoid (2) red pulp space (3) white pulp space. (c). Photograph of heart showing normal cytostructure control group (H and E, ×40). (d) Photograph of kidney showing cortical and medullary areas control group (H and E, ×10). (1) Cortex (2) medulla (3) glomerulus (4) renal tubule. (e) Photograph of kidney showing normal cytostructure of glomerulus control group (H and E, ×40). (f) Photograph of brain showing normal cytostructure control group (H and E, ×40). (1) Grey matter (2) white matter (3) astrocyte (4) oligodendrocyte

so as to make the body adapt to the large dose or (ii) as a *kalpa*[38] i.e., with sufficient amount of adjuvant drugs that help in counteracting the ill effect of metal. Further, these manoeuvers are chiefly employed for *rasāyana karma* of *kṣetrīkaraṇa* (making body suitable for excess amounts of

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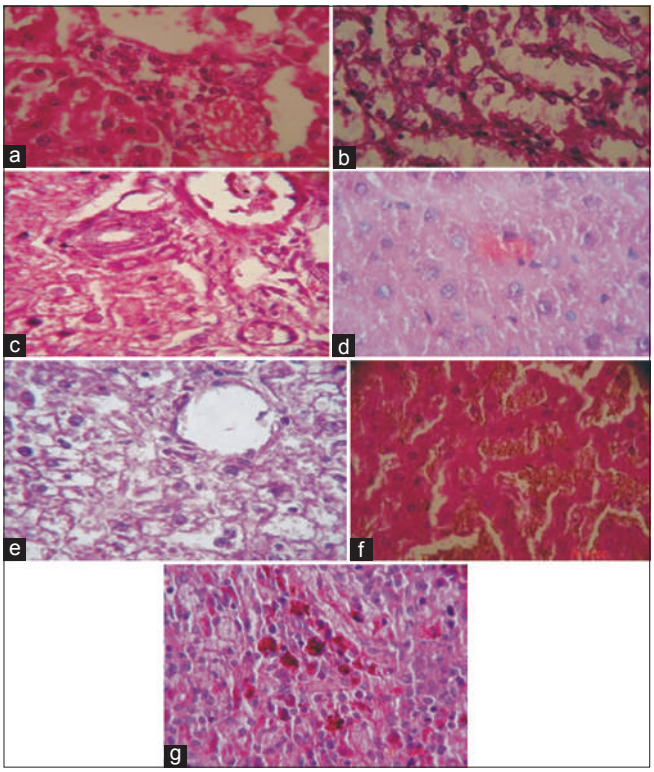

Figure **2:** Histopathological findings in toxicoogical study of *Lauha Bhasma*. (a) Photograph of kidney showing periportal lymphocytic infiltrations L.B. 10 T.D. (H and E, ×40). (b) Photograph of renal tubule showing normal cytostructure L.B. 5 T.D. (H and E, ×40). (c) Photograph of liver showing inflammatory infiltrate L.B. 10 T.D. (H and E, ×40). (d) Photograph of liver showing bile stasis L.B. 10 T.D. (H and E, ×10). (e) Photograph of liver showing mild inflammation with periportal ballooning L.B. 5 T.D. (H and E, ×10). (f) Photograph of liver showing sinusoidal congestion L.B. 10 T.D. (H and E, ×40). (g) Photograph of spleen showing intra cytoplasmic pigments (normal cytostructure) L.B. 10 T.D. (H and E, ×40)

metal) as necessary. But for routine therapeutic application, such a large amount of metal is not usually prescribed. Moreover, *Lauha kalpas* are administered along with suitable *kramaṇa dravyas* (vehicle or *anupāna*) rather than the *Lauha bhasma* being administered alone. These *kramaṇa ~ Anupāna* drugs act as on adjuvants to *Lauha* and exert synergistic effects to potentiate the therapeutic action and also eliminate the innate toxic effects of the metal. A great emphasis is laid on dosage and *anupāna* (vehicle) with which a *bhasma* should be administered. In *anupāna* may possibly lie the key to the safety of the *bhasma*. In absence of such caution, adverse reaction is likely.[39] *Madhu* (honey) as *anupāna* (vehicle) brings about quick action due to its *Yogavāhi* (super-advenient) property.[40]

Metals, while being made into *bhasma,* get chelated with organic molecules (ligands) present in the herbs which leads to better assimilation. Chelation therapies have been used to bring down the levels of toxic metals in patients. During pharmaceutical processing of *Lauha bhasma,* right from *śodhana* till *amṛtīkaraṇa, Triphalā* is used as an organic media to convert metal *Iron* (*Lauha*) into a herbomineral complex. *Triphalā* mainly consists of tannins, gallic acid, ascorbic acid (Vitamin C), and phenolics. Ascorbic acid increases the bioavailability of iron by converting Fe^{3+} to Fe^{2+}, while phenolics can reduce the iron by binding to it. The presence of ascorbic acid or a lack of dietary tannins has both been suggested as contributing to clinical/pathological iron storage disease. Excess Iron causes Iron overloading in the body[41] and can damage the liver, heart, and pancreas and irritate the stomach and gut, causing constipation and diarrhoea. In other words, the various constituents of *Triphalā* have antagonizing activity and thereby too much iron absorption is prevented.[42] In Ayurvedic texts, terms like *Viṣaghna*[43] (antitoxic), *Giridoṣa nāśaka* (remover of metallic properties) are abundantly used for *Triphalā,* and this is an indication of the above phenomenon. *Triphalā* is a mild laxative and thereby counteracts the constipating property of iron and thus is beneficial. This may be the reason due to which *ācāryas* might have recommended *Triphalā* in a maximum number of *Lauha* formulations.[44] The findings from one study suggested that *Triphalā* and its individual constituents have an inhibitory effect on metabolic enzymes when consumed along with therapeutic products. Further the inhibitory effects were relatively comparable to all the constituents tested, despite the variability of the content of biomarker. *Triphalā* and its ingredients are likely to inhibit drug metabolizing enzymes, but less likely to produce significant drug interactions. Certain major factors of metabolism such as competition between coadministered drugs, unspecific interactions with proteins, and enzyme induction due to chronic intake are not addressed in that *in vitro* assay. However, one study clearly suggested that herbal products containing gallic acid may have the potential to inhibit the metabolism of certain coadministered drugs.[45] The present study clearly shows that use of *Lauha Bhasma* does not cause any adverse reaction, provided the drug is properly processed in suitable herbal media (as in the present case, *Triphalā*), taken in suitable dose, for a limited period of time and that too with proper *anupāna* (vehicle).

CONCLUSION

Observations of the present study did not show any untoward effect of *Lauha bhasma* at therapeutic dose and at a dose five times the therapeutic dose, thus proving its safety. However, alteration in some of the

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biochemical and haematological parameters along with histopathological findings at highest dose level were evident. As these changes were dose dependent, and none of the Ayurvedic text suggests taking great amounts of *Lauha bhasma*, precautions are needed in prescribing higher than equivalent doses in human beings. Moreover, more detailed studies are still required to come to a final conclusion about the safety of *Lauha bhasma*.

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Conflicts of interest

There are no conflicts of interest.

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Research Paper

Toxicological Studies of *Rasasindura*, an Ayurvedic Formulation

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***Rasasindura* is a unique, Ayurvedic mercurial preparation widely used by practitioners. This investigation is an attempt to perform acute and chronic oral toxicity evaluation of *Rasasindura* along with an adjuvant *Guduchi Ghana* (solidified aqueous extract of *Tinospora cordifolia* Will.) in rats. Oral acute toxicity study of test drug was carried at the limit dose of 2000 mg/kg orally in rats. For chronic toxicity, *Rasasindura* with adjuvant was administered at therapeutic equivalent dose (45 mg/kg, orally), therapeutic equivalent dose×5 (225 mg/kg, orally), therapeutic equivalent dose×10 (450 mg/kg, orally) for 90 days and an additional recovery group of therapeutic equivalent dose×10 for 30-day observation after the treatment period. Acute toxicity result showed that drug did not produce any signs and symptoms of toxicity or mortality up to an oral dose of 2000 mg/kg in rats. Chronic toxicity results showed that *Rasasindura*, even at a level as high as therapeutic equivalent dose×10 level, had no significant effect whatsoever on the ponderal and hematological parameters. Although the drug produced mild to moderate adverse changes (in kidney, liver, intestine, and stomach) at therapeutic equivalent dose×10 dose level, equivalent of which are unlikely to be ever employed in a clinical trial. The observed changes were not seen at the lower dose levels as well as in the recovery study. Hence, it is suggested that the *Rasasindura*, along with the adjuvant prepared as per the customary method, is safe for consumption at the therapeutic dose level.**

Key words: *Rasasindura, Guduchi ghana, Tinospora cordifolia, bhasma,* toxicity

Ayurveda, the traditional system of Indian medicine, has enriched the historical background and is one of the great living traditions. Use of processed metals, minerals, and mercurial-processed herbs as a medicament has been an integral part of Ayurvedic practice. These metallic preparations have unique process of preparation, involving *Shodhana* (purification and/or detoxification) and *Marana* (incineration and/or calcination). Practitioners developed these methods to detoxify the raw material by chemical transformations and thus modify the properties of materials to enhance therapeutic potential[1,2]. Their extensive use of these medicaments since more than a millennium without any reports of any untoward events can be considered as a testimony to their safety; but no objective-verifiable data exists to support such claims. Pre-clinical studies of Ayurvedic drugs provide scientific basis for their traditional use and to prove that they are safe and efficacious[3].

Rasasindura is one among such mercurial preparations widely used by Ayurveda practitioners. Pharmaceutical processing of *Rasasindura* involves treating purified mercury with purified sulphur and juices of *Aloe vera* (Linn.) Burm. f. to form black sulphide of mercury, which is further treated with gradual, intermittent heat to transform into more stable form, i.e. cinnabar by *Kupipakwa* method[4]. *Kupipakwa* is a specialized heating system, i.e. gradual, intermittent heat by vertical electrical-muffle furnace. *Rasasindura* is said to be mercury sulphide, associated with several organic macro-molecules derived from processing with plant extracts[5]. Toxic effects of purified mercury were said to

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be neutralized in the presence of purified sulphur[6]. As per the classics, *Rasasindura* is administered with the adjuvant such as *Guduchi Ghana* (solidified aqueous extract of *Tinospora cordifolia* Will.)[7]. *Rasasindura* has unique properties to pacify diseases such as diabetes, fistula, fever, lack of appetite, anaemia, oedema etc.; and it is considered equivalent to the elixir, which is known to overcome death[4,7]. Since reports of toxicity evaluation of this classical preparation (along with the adjuvant) was not available during extensive literature review, it was thought worthwhile to undertake the detailed toxicity assessment in albino rats.

MATERIALS AND METHODS

Aloe vera and *T. cordifolia* were collected from the botanical garden of Gujarat Ayurved University, Jamnagar. The plant materials were authenticated and voucher specimens of each submitted to Pharmacognosy laboratory of Institute. *Rasasindura* and *Guduchi Ghana* (solidified aqueous extract of *T. cordifolia*) were prepared in Department of Rasashastra and Bhaishajya Kalpana, Gujarat Ayurved University, Jamnagar; and SOPs were prepared and documented[4,8]. All chemicals used in the study were of analytical grade.

Experimental animals:

Wistar albino rats of either sex weighing 200±20 g body weight were used for the study. The animals were maintained under ideal husbandry conditions in terms of standard conditions of temperature (23±2°), relative humidity (50 to 60%), and exposed to 12 h light-and-dark cycles. All animals were exposed to the same environmental conditions and were maintained on standard diet and drinking water *ad libitum.* The experimental protocol was approved by the Institutional Animal Ethical Committee (IAEC/10/2012/08, Ph.D.) as per the guideline of Committee for the Purpose of Control and Supervision on Experiments on Animals, India.

Dose selection:

As per the classical guideline, the therapeutic dose of *Rasasindura* is 125 mg/d[9] and should be administered with the adjuvant as solidified aqueous extract of *T. cordifolia* (375 mg/d). Hence, total dose of drug with adjuvant is 500 mg/d. The suitable dose for rats was calculated by referring to table of Paget and Barnes[10] and was found to be 45 mg/kg rat (considered as therapeutic equivalent dose, TED). The test drug along with adjuvant was administered orally with the help of cannula, in the form of suspension in honey and distilled water solution.

Acute toxicity study:

Young, healthy, nulliparous, and non-pregnant Wistar-strain albino, female rats were selected and acclimatized for seven days before the experiment. The *Rasasindura* along with adjuvant was orally administered at limit dose of 2000 mg/kg to overnight fasted female rats in sequential manner as per the OECD 425 guideline[11]. The rats were observed closely for behavioural changes, signs and symptoms of toxicity, and mortality continuously for the first six hours; and thereafter, periodically up to 14 d. The body weight of each rat was noted on the last day and the rats were sacrificed. The abdomen was opened through mid-line incision to record the autopsy changes, followed by dissecting the important organs for histopathological changes.

Chronic toxicity study:

The chronic toxicity study was carried out followed by standard guideline with modification as per experimental need[12,13]. Rats were randomized into six groups of six rats in each with three males and three females. Group (I) was kept as control group, received vehicle as honey solution in distilled water (5 ml/kg, orally). Group (II) to (IV) were administered with test drug *Rasasindura* along with adjuvant at TED (45 mg/kg, orally), TED×5 (225 mg/kg, orally), and TED×10 (450 mg/kg, orally), respectively. The suspensions of test drugs were administered orally once-a-day for 90 consecutive days in main study. Additional six animals were kept in satellite control group (V) and in the recovery TED×10 treated group (VI) for observation after the treatment period, for reversibility or persistence of any toxic effects. The duration of post-treatment period was fixed as 30 d (total of 120 d, including 90 d treatment period and 30 d recovery period). All the animals were dosed with constant dose volume of 5 ml/kg, orally.

The rats were observed daily, carefully for any overt and apparent signs and symptoms of toxicity. The body-weight change of an individual rat was noted initially and thereafter weekly during the study period. At the end of experimental periods, blood was withdrawn by the retro-orbital puncture under light-ether anaesthesia using capillary tube for estimation of serum biochemical and haematological parameters. The body weight of each rat was noted on last day and rats were sacrificed. The abdomen was opened through mid-line incision to

record the autopsy changes followed by dissecting out the important organs.

Haematological analysis was performed using an automatic haematological analyser (Swelab, Sweden). The parameters were total red blood cell (RBC), haemoglobin (Hb), packed cell volume (PCV), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC), white blood cell (WBC), neutrophils percentage (%N), lymphocyte percentage (%L), eosinophil's percentage, monocyte percentage, and platelet count (PC).

Serum bio-chemical parameters were carried out using fully automated biochemical random access analyser (BS-200, Lilac Medicare Pvt. Ltd., Mumbai). The parameters were blood glucose[14], urea[15], creatinine[16], total cholesterol[17], HDL-cholesterol[18], triglyceride[19], VLDL-cholesterol, LDL-cholesterol, total protein[20], albumin, globulin[21], alkaline phosphatase[22], SGOT[23], SGPT[24], uric acid[25], direct bilirubin[24], total bilirubin[26], and serum calcium[27].

Bone-marrow smear from the femur bone was prepared using standard procedure. All the important internal organs were carefully dissected namely brain, pituitary, liver, heart, thymus, spleen, kidney, lung, stomach, intestine, testis, prostate, seminal vesicle, uterus, ovary, adrenal gland, trachea, aorta, lymph node, and skin. After noting for any signs of gross lesion and ponderal changes of major organs, all were transferred to 10% phosphate buffered formalin solution for fixation and later on subjected to dehydrating, wax embedding, sectioning, and staining with haematoxylin and eosin (H and E) for histological evaluation by light microscopy. The slides were viewed under trinocular research Carl-Zeiss's microscope at various magnifications to note down the changes in the microscopic features of the tissues.

Statistical analysis:

The data is expressed as mean±standard error of mean for six rats per experimental group. One-way analysis of variance (ANOVA) was used to compare the mean values of quantitative variables among the groups, followed by Dunnet's multiple *t*-test for unpaired data to determine significant difference between groups at $P<0.05$.

RESULTS AND DISCUSSION

Acute toxicity study of test drug was carried out to record immediate adverse signs and symptoms of drug in female rats at dose levels that are several folds higher than the therapeutic equivalent dose. Administration of *Rasasindura* along with adjuvant did not affect any behavioural changes and other parameters observed during the acute toxicity test in female rats. No signs and symptoms of toxicity and mortality were observed up to oral dose of 2000 mg/kg of test drug in rats. Further, drug did not affect the cytoarchitecture of major organs like heart, kidney, liver, uterus, and ovary which suggest that LD_{50} value may be higher than 2000 mg/kg by oral route. As per UN classification, any substance, which has oral LD_{50} of more than 2000 mg/kg is considered as low hazard potential and categorized as UN 6.1 PG III[28]. Thus as per the above criterion *Rasasindura* along with adjuvant can be categorized as substances with low health hazard potential (Class 4 of GHS and UN 6.1 PG III).

There were no behavioural changes observed in *Rasasindura* treated groups during the course of chronic toxicity study. No symptoms of toxicity and mortality was observed in treated groups at TED×10, TED×5, and TED dose levels in the main study and TED×10 in the recovery study. Normal body weight gain was observed in control rats during main study (90 d) as well as recovery study (120 d). An increase in body weight was found in *Rasasindura* treated groups at all dose levels. Changes in body weight are an important factor to monitor the health of an animal. Loss of body weight is usually the first sign indicating the onset of an adverse effect. The dose, at which body weight loss is by 10% or more, is considered to be a toxic dose, irrespective of whether or not it is accompanied by any other changes[29]. The percentage change in body weight pattern in test drug treated groups did not differ significantly from the changes observed in the control groups, which suggest the absence of serious toxic effect of *Rasasindura* during chronic administration in rats.

Out of the nine organs for which relative weights were recorded (Table 1), *Rasasindura* treated group showed significant decrease in relative weight of liver in TED treated group in comparison to the normal control, whereas in other dose levels the changes were non-significant. A significant increase was observed in relative weight of testis at TED, uterus weight at TED×5, and kidney and testis at TED×10 dose levels; however, the changes were almost reversed in the drug treated recovery group at TED×10 dose level. Normally, decrease in the weight of an organ is indicative of loss of

tissue mass in that organ, exception being the secretory organs in which the decrease in weight sometimes is seen along with the increased activity. In this case, the increase in the weight of reproductive organs may be indicative of stimulation of hormone secretion. In the present study, there were no remarkable changes observed in the relative weight of the organs at higher doses of test drugs. Hence, it may be, understood that the drugs do not tend to produce any serious toxic effect on the relative weight of the important internal organs in chronic toxicity studies.

Analysis of the effects of *Rasasindura* on haematological parameters (Table 2) revealed Non-significant increase in WBC count at each of the dose level studied in the main study; however, contrary effect was observed in the recovery phase in the drug treated group in comparison to the control group. However all the values were within the normal range[30]. The test drug at all dose level did not affect the RBC related parameters. This clearly indicates that the test drug did not affect the cellular and non-cellular elements of the blood to significant extent. Further, with the discontinuation of the drug, most of the values were similar to that observed in the recovery control group, which suggests that the drug is devoid of any serious haematological toxicity, even at higher dose on repeated administration.

The effects of *Rasasindura* on serum bio-chemical parameters are presented in Table 3. Out of eighteen parameters studied, significant decrease was observed in blood glucose level at TED and TED×5 treated groups, while significant increase in creatinine level at TED dose level and triglyceride level at TED×5 dose level in comparison to the control group in main study. However, similar significant changes were not observed in higher dose of *Rasasindura*. After discontinuation of test drug in the recovery group, the observed changes in glucose level, creatinine, and

TABLE 1: EFFECT OF TEST DRUGS ON RELATIVE WEIGHT OF ORGANS OF RATS RECORDED DURING CHRONIC TOXICITY STUDY

Relative weight	Control group	Drug treated groups			R Control group	Recovery TED x 10
		TED	TED×5	TED×10		
Liver (g/100 g)	3.57±0.17	3.12±0.10*	3.27±0.06	3.26±0.09	2.72±0.07	2.99±0.11
Heart (mg/100 g)	280.41±8.88	266.04±5.36	289.12±9.69	281.65±6.78	240.44±8.42	269.80±12.81
Kidney (mg/100 g)	720.10±26.28	675.17±19.26	766.08±30.88	804.74±25.63*	691.42±18.42	714.44±10.76
Spleen (mg/100 g)	194.50±9.32	173.56±8.14	206.28±18.26	189.69±10.14	158.85±7.44	179.45±8.87
Thymus (mg/100 g)	172.54±6.73	165.65±5.21	167.91±7.71	158.91±7.60	145.56±10.91	146.15±12.89
Testis (g/100 g)	648.26±28.37	818.50±41.74*	684.25±123.10	868.99±45.83	792.01±61.68	661.95±39.86
Prostate (mg/100 g)	146.97±21.19	128.77±11.70	129.53±9.04	180.36±7.43	145.07±24.68	167.09±16.38
S. vesicle (mg/100 g)	504.08±95.14	504.55±55.60	481.70±32.76	527.32±47.38	421.09±62.60	477.01±58.91
Uterus (mg/100 g)	230.03±17.72	215.88±28.98	323.70±26.45*	222.81±21.52	197.89±8.69	178.48±9.04

The results are expressed as mean±SEM, where n=6. SEM: Standard error of mean. *P<0.05, compared with control group

TABLE 2: EFFECT OF TEST DRUGS ON HEMATOLOGICAL PARAMETERS IN RATS RECORDED DURING CHRONIC TOXICITY STUDY

Hematological Parameters	Control group	Drug treated groups			R control group	Recovery TED×10
		TED	TED×5	TED×10		
RBC (10^6/ml)	7.55±0.08	7.69±0.17	7.41±0.10	7.86±0.22	8.67±0.26	8.74±0.26
Hemoglobin (g/dl)	13.85±0.34	13.95±0.21	13.60±0.16	14.00±0.21	15.38±0.24	15.38±0.33
PCV%	42.18±0.58	42.48±0.95	41.40±0.43	44.41±1.01	48.76±1.22	49.60±1.30
MCV (fl)	55.86±0.41	55.25±0.47	55.84±0.48	56.54±0.75	56.32±1.18	56.75±0.80
MCH (pg/red cell)	18.35±0.32	18.15±0.24	18.35±0.27	17.84±0.37	17.38±0.53	17.63±0.38
MCHC (g/dl)	32.81±0.49	32.87±0.25	32.84±0.24	31.58±0.33	30.84±0.42	31.03±0.34
WBC (10^3/ml)	6816.66±630.03	8114.28±1217.61	8114.28±1068.91	7585.71±570.47	7020.00±575.67	6233.33±276.48
Neutrophil%	24.50±4.32	28.42±5.47	19.57±2.32	17.14±1.79	23.00±4.93	23.66±2.10
Lymphocyte %	71.66±4.27	66.57±5.81	76.85±2.13	79.42±2.04	75.80±5.06	72.00±2.51
Eosinophil%	2.33±0.21	2.71±0.28	2.00±0.21	2.00±0.21	2.60±0.24	2.33±0.21
Monocyte%	1.50±0.22	2.28±0.28	1.57±0.20	1.42±0.20	2.60±0.24	2.00±0.36
PLT (10^3/ml)	1152.83±54.10	1267.57±47.57	1154.00±62.82	1267.85±51.82	1115.80±45.11	1057.50±83.48

The results are expressed as mean±SEM, where n=6. SEM: Standard error of mean

triglyceride were almost same as seen in the recovery control group. Cholesterol level and HDL-cholesterol level in rats were unaffected by the test drug at all dose level in comparison to the control group; hence, it can be inferred that the observed changes do not cause any serious toxic effect (Table 4).

Significant decrease in blood urea was observed in recovery TED×10 treated rats in comparison to the recovery control group however same was not observed in main study. Test drugs did not influence the level of serum transaminases, urea, and creatinine to a significant extent in main study; which suggests that the test drug may not affected the liver and kidney function in the treated rats. The observed values of above bio-chemical parameters are within the normal range[30].

The histopathological studies of twenty organs showed that *Rasasindura* along with adjuvant at highest dose level exhibited mild to moderate changes in kidney, liver, intestine, stomach, and lung in comparison to the control group. *Rasasindura* produced mild pigment deposition, fatty changes in epithelium, and oedematous changes in kidney tubule, whereas recovery group showed normal cytoarchitecture (fig. 1). *Rasasindura* TED×10 and TED×5 treated groups exhibited pigment deposition, mild necrosis, and fatty changes in the liver in comparison the control group, while the recovery group showed almost normal cytoarchitecture (fig. 2). Test drug at highest-dose level exhibited mild to moderate loss of villi and cell infiltration in intestine (fig. 3) and displayed mild to moderate sub-mucosal inflammation in the stomach (fig. 4). Mild effusion was seen in cytoarchitecture of lung of one of the rat at higher dose of TED×10 dose level in comparison to the control group; whereas, in other dose levels the changes were almost normal (fig. 5). Bone marrow smear and the other organs exhibited normal cytoarchitecture in the *Rasasindura*-preparation treated groups, both in the main study as well as in the recovery study, in comparison to the control groups.

The observed histopathological changes were not seen at therapeutic dose level and not in the recovery study. The result of bio-chemical parameters reveal that the drugs do not seem to produce any drastic changes in

TABLE 3: EFFECT OF TEST DRUGS ON BIOCHEMICAL PARAMETERS IN RATS RECORDED DURING CHRONIC TOXICITY STUDY

Biochemical Parameters	Control group	Drug treated groups			R control group	Recovery TED×10
		TED	TED×5	TED×10		
Glucose (mg/dl)	92.83±8.63	68.57±2.61*	70.85±4.11*	107.28±3.63	117.50±4.86	111.66±3.34
Urea (mg/dl)	69.83±4.88	81.00±2.43	64.00±4.35	69.14±8.74	88.60±5.00	65.16±3.15
Creatinine (mg/dl)	0.58±0.03	0.70±0.03*	0.62±0.03	0.55±0.02	0.56±0.02	0.60±0.02
Total protein (g/dl)	7.08±0.17	6.94±0.19	6.95±0.24	7.12±0.10	6.90±0.24	7.05±0.10
Albumin (g/dl)	3.58±0.16	3.61±0.15	3.67±0.23	3.72±0.08	3.36±0.14	3.35±0.08
Globulin (g/dl)	3.50±0.17	3.12±0.10	3.28±0.17	3.40±0.09	3.33±0.15	3.70±0.17
ALP (IU/L)	233.25±45.02	149.00±11.82	143.33±23.78	263.42±37.67	261.40±37.64	281.33±51.96
SGOT (IU/L)	138.16±7.85	153.28±9.11	142.85±8.42	148.85±9.83	124.50±8.65	143.83±5.17
SGPT (IU/L)	67.00±7.82	53.28±1.37	58.28±5.16	62.00±6.92	57.00±2.70	57.00±4.20
Uric acid (mg/dl)	0.70±0.13	0.81±0.09	0.84±0.09	0.72±0.06	0.95±0.16	1.05±0.09
D. Bilirubin (mg/dl)	0.13±0.03	0.11±0.01	0.10±0.00	0.12±0.02	0.13±0.03	0.13±0.02
T. Bilirubin (mg/dl)	0.45±0.05	0.48±0.07	0.41±0.04	0.48±0.06	0.45±0.07	0.48±0.08
Calcium (mg/dl)	9.58±0.30	8.98±0.17	9.81±0.25	9.22±0.22	9.35±0.22	9.48±0.09

The results are expressed as mean±SEM, where n=6. SEM: Standard error of mean. *P<0.05 compared with control group; @P<0.05 compared with recovery control group

TABLE 4: EFFECT OF TEST DRUGS ON SERUM LIPID PROFILE IN RATS RECORDED DURING CHRONIC TOXICITY STUDY

Serum Lipid profile	Control group	Drug treated groups			R control group	Recovery TED x 10
		TED	TED x 5	TED x 10		
Total cholesterol (mg/dl)	34.33±3.45	38.42±1.81	37.28±3.92	30.71±2.01	37.50±3.59	28.16±1.86@
HDL-cholesterol (mg/dl)	33.16±3.15	37.14±4.50	37.85±5.70	27.85±2.08	31.66±3.41	25.16±1.60
Triglyceride (mg/dl)	61.33±3.67	76.14±6.55	78.42±6.07*	70.28±6.80	70.16±5.29	73.83±8.56
VLDL-cholesterol (mg/dl)	12.26±0.73	15.22±1.31	15.68±1.21	14.05±1.36	14.03±1.05	14.76±1.71
LDL-cholesterol (mg/dl)	11.10±1.68	13.32±3.86	14.40±3.30	11.20±0.80	7.43±1.90	11.76±2.26

The results are expressed as mean±SEM, where n=6. SEM: Standard error of mean. *P<0.05 compared with control group; @P<0.05 compared with recovery control group

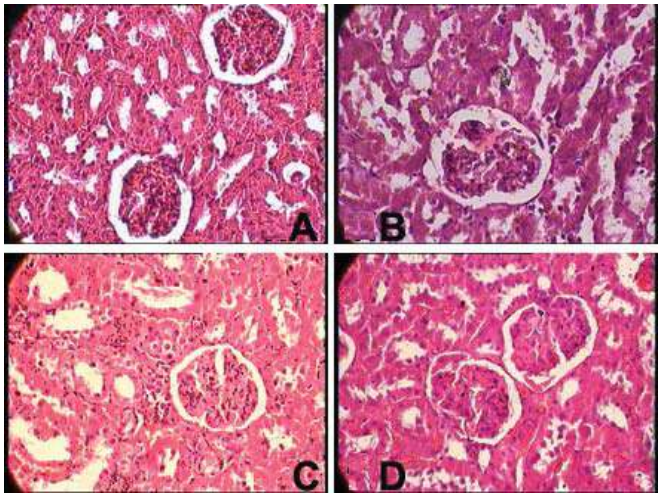

Fig. 1: Histopathology of kidney tissues (x400 magnification) (A) Normal cytoarchitecture (control group), (B) edema and fatty changes (TED×5), (C) oedema, pigment deposition and fatty changes (TED×10), (D) almost normal cytoarchitecture (recovery TED×10)

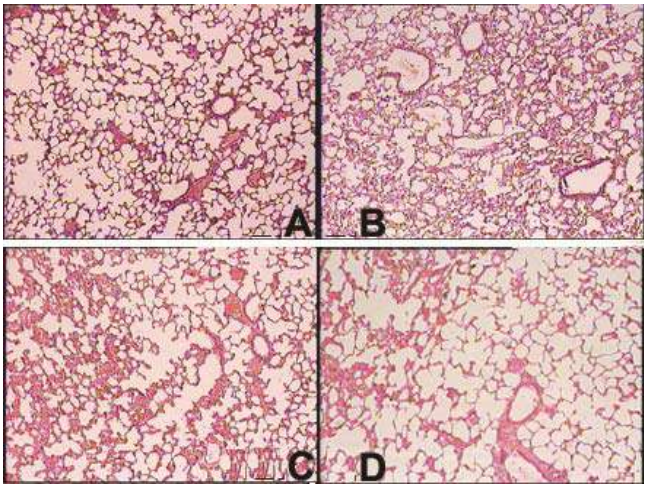

Fig. 2: Histopathology of liver tissues (x400 magnification) (A) Normal cytoarchitecture (control group), (B) pigment deposition, mild necrosis and fatty changes (TED×5), (C) pigment deposition, mild necrosis and fatty changes (TED×10), (D) fatty changes (recovery TED×10)

the liver and kidney function parameters in rats; which suggests that the organ damage as mentioned above in chronic toxicity is of mild intensity at higher dose level, however drug is relatively safe at therapeutic dose level. In previous 28-d toxicity study also demonstrated that *Rasasindura* (50-100 mg/kg) in Wistar-albino rats did not have any adverse effect on kidney[31]. Further, feeding on *Rasasindura* supplemented food did not elicit any evidence of heavy metal toxicity in larvae or flies, since there was neither any evidence of lethality, nor of any developmental defects in the emerging flies[32].

The results reiterates the fact that *Bhasmas*, despite their trace heavy metal content, are safe when appropriately manufactured and consumed as per directed instructions[33]. Toxic effects of mercury were said to be neutralized in the presence of sulphur[6]. In Ayurvedic system of medicine, *Anupana* (called vehicle, as a medium of administration) improves acceptability and palatability and helps in absorption of the main drug; additionally, it may also act as early antidote[32]. *Guduchi* used as adjuvant in current study is having antioxidant property acts as hepatoprotective drug and has potential against aflatoxins and heavy metal toxicity[34,35].

From the present study, it is concluded that *Rasasindura* along with adjuvant is not toxic on acute administration at a maximum oral dose level of 2000 mg/kg in female

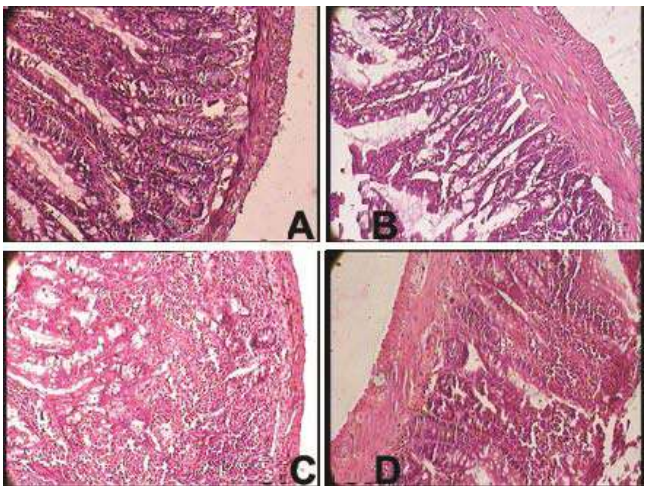

Fig. 3: Histopathology of intestine tissues (x200 magnification) (A) Normal cytoarchitecture (control group), (B) almost normal cytoarchitecture (TED×5), (C) mild to moderate loss of villi and cell infiltration (TED×10), (D) almost normal cytoarchitecture (recovery TED×10)

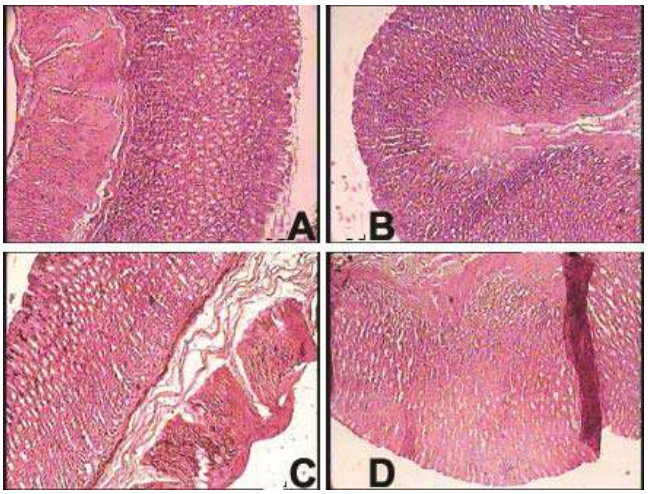

Fig. 4: Histopathology of stomach tissues (x100 magnification) (A) Normal cytoarchitecture (control group), (B) normal cytoarchitecture (TED×5), (C) mild to moderate sub-mucosal inflammation (TED×10), (D) almost normal cytoarchitecture (recovery TED×10)

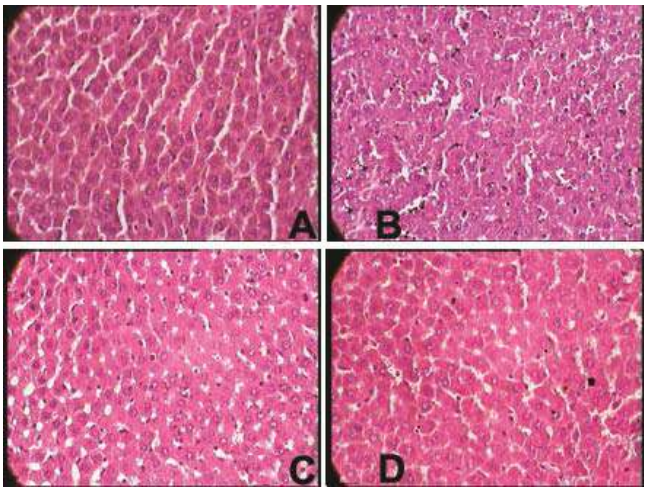

Fig. 5: Histopathology of lung tissues (x100 magnification) (A) Normal cytoarchitecture (control group), (B) normal cytoarchitecture (TED×5), (C) mild effusion (TED×10), (D) almost normal cytoarchitecture (recovery TED×10)

rats. However, on chronic administration of test drug for 90 d produced mild to moderate adverse changes in the kidney, liver, intestine, and stomach of rats at TED×10 dose level, equivalent of which are not likely to be ever employed in clinical conditions, conversely *Rasasindura* at TED dose has no toxic potential. *Rasasindura* prepared as per customary method and administered with appropriate adjuvant is safe to consume at therapeutic dose level.

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Conflict of interests:

None declared.

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