



## Synthesis and Comparative Evaluation of Traditional Herbomineral Nanoceuticals for Their Haemopoietic Activity

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### ABSTRACT

The traditional System of medicine comprises a wide variety of formulations for various ailments. It is essential to Standardize Ayurvedic drugs so that drugs having good quality can be used for better effect. Standardization is not only limited to the final preparation, but it also includes the materials used during the development and SOP adopted and the equipment used. The present study is an attempt to prepare anti anaemic herbomineral nanoceutical by two different processes, and to standardize and evaluate the haemopoietic effect of the prepared formulations and compare with a marketed formulation. Preparation was done based on Ayurveda Pharmacopoeia. Formulations were characterized by conventional methods and by AAS, XRD, EDAX, SEM, TGA, and IR. The toxicity and anti-anaemic effectiveness are evaluated by using albino Wister rats and histopathology and cytotoxicity studies. Products have a crystalline nature (XRD). TGA shows the decomposition temp of ferric oxide. SEM EDAX revealed surface smoothness and nano to the micro size of particles. AAS shows that the elements are within the prescribed limits. The in-process Standardization shows the effectiveness of adjuvants. There is an increase of haemoglobin and body weight on day 75 for first preparation than the Second and were comparable to that of standard. No toxicity in all rats and indicated no renal and gastrointestinal toxicity. The size, smoother surface, and the therapeutic effectiveness of the herbominerals prepared by the two methods can be attributed to the calcination steps and adjuvants. Thus, maybe dose reduction and rapid effect can be achieved.



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### INTRODUCTION

Even though modern medicine has achieved heaps and developed newer technology, most of the rural

and remote areas around the world have got a keen interest in alternative Systems of medicines focusing on herbal medicine as the primary remedy for treating ailments. In India, the traditional medicinal systems comprising of Unani (The Arabian System), Ayurveda, and Siddha are famous for their effectiveness, and the present System of therapy has acknowledged the uniqueness of such a system of medicine. Many of these systems are practised from the Vedic period and are older than allopathic Systems of medicine (Kale and Rajurkar, 2019). There are several lacunae for mainstreaming of such medicine. The most important one is the lack of Standardization of the practising formulations, which is essential for quality control and the optimization of different parameters (Sen and

Chakraborty, 2017; Bhatambreyogesh and Pallavi, 2017).

The process of Standardization of any drug comprises of

1. Screening of Raw materials for ensuring their quality
2. Standardization during the processing of Formulation Here evaluation of the procedure and the intermediates formed during its preparation is done by doing specific tests (Drugs and Cosmetics, 2009).
3. Formulation Standardization Here, the final product or the prepared medicine is evaluated as per guidelines (Prashant, 2019; Singh *et al.*, 2016).

Ayurvedic formulations are prepared with many additives, which improve the therapeutic efficiency and are expected to reduce the toxicity. The steps involved in the processing also affect quality. Bhasma is one of the formulations used in Siddha and Ayurveda under Rasoushadhi. Bhasma is prepared by a unique process known as Bhamikaran involving Sodhana, Bhavana, and Marana. The particular process makes smooth nano-sized particles having a very potent therapeutic effect with a reduced dose. Bhasmas are unique in their properties, and these metal-containing nanomaterials are obtained by repeated heating and are administered together with plant juices/fruit juices. These types of ayurvedic formulations are prescribed for the treatment of a variety of diseases. Usually, the adjuvants like honey, butter, lime juice, milk ghee used may help the metal early assimilable and may reduce the harmful effect, and thereby biocompatibility is increased (Rasheed, 2016; Rasheed and Shivshankar, 2015). Kaseesa bhama is a famous herbs mineral formulation used in Ayurveda for its anaemic anti action. It contains iron metal in oxide form. Since the method of preparation affects the quality of the final product, the present study aims to standardize the process by selecting two different adjuvants for the Formulation of Kaseesa bhasma. It also includes the evaluation of the product by new analytical parameters and hemopoietic activity by using an animal model. The final products compared with the marketed formulation (Mukkavalli *et al.*, 2017; Rasheed, 2014).

## MATERIALS AND METHODS

The critical ingredient of Kaseesa bhasma is ferrous sulphate powder, and it was procured from Nice Chemicals, Mumbai, India. Marketed Kaseesa bhasma and the other ingredients required were brought from the local market of Kannur district, Kerala, India. The purification (swedhana) pro-

cess was done by using plants obtained from the herbal Garden of Pariyaram Ayurveda Medical College, Kannur, India, and duly authenticated.

## Preparation

Kaseesa Bhasma prepared as per the Ayurvedic Pharmacopoeia. The steps involved are Shodhana/Purification, Bhavana/size reduction, and Puta/Calcination. Two different methods adopted for the Formulation of Bhasma by using different adjuvants (Elahi *et al.*, 2017; Ayurvedic Pharmacopoeia, 2008).

### Method 1 (Formulation 1:KaB1)

Shodhana was done by taking Ferrous sulphate 250gm and using Bringaraja Swarasa of pH 7.5 (2L) and then heating for 3hrs. The compounds were made into a paste by trituration using a vessel known as khalvam and a smooth pate of Ferrous sulphate. Further, the mixture was made into a soft powder by Marana process to make and Chakrikas of dimension: diameter: 1.5 – 2 cm. Thickness: 0.5 cm was prepared and transferred to a crucible named Sharava, and it is then covered and sealed by using sealing clay. The sharava is heated using Coconut husks for calcination of the contents. The calcination steps were repeated four times for 3hr, 4hr 2.15hrs, and for 2hrs respectively to get 20gm of product. The whole procedure repeated three times.

### Method 2 (Formulation 1:KaB2)

Ferrous sulphate 250gm then triturated and dried under sunlight for one day (Purification). Then using Nimbu Rasa 15ml, the compounds then made into a paste by keeping in khalvam. The obtained mixture powdered (Marana) Chakrikas of dimension: diameter: 1.5 – 2 cm. Thickness: 0.5 cm was prepared and transferred to a crucible named Sharava and then covered and sealed as method I. Finally, calcination has done by heating (Cow dung 75 Kg a fuel). The calcination steps were repeated for four times for 3hrs to get 20gm of product. The whole procedure repeated three times [Rasatharangini] (Sharma, 2014).

## Preparation Characterization

The formulations KaB1 and KaB2 was subjected to evaluation by the traditional method (Rasheed and Shivashankar, 2017; Wadekar *et al.*, 2006).

The prepared formulations were standardized initially by conventional means, which involve floating property determination, testing fineness, and metallic lustre for the preliminary analytical confirmation of Bhasma preparation. Physical evaluations like colour, odour, taste and pH and the determination of Ash values and LOD were also evaluated for the

**Table 1: Preliminary Standardization**

Preparations	°C	Colour after calcination			
		1st	2nd	3rd	Final
KaB1	428	Pale yellow	Brick red color-bottom Upper surface-dull white	Red	Dark red
KaB2	592	Light Pista green with a yellow tinge.	Dark red. With small amount of dull white color in between.	Red	Red

**Table 2: Preliminary Characterization of final Product**

Parameter	KaB1 (%)	KaB2 (%)	KaB3(%)
Colour,	Dark red	Red	Red
Rekhapoornatwa(Finenes)	100	100	98
Vareetaratwam(Floatness)	Floats on the surface	Floats on the surface	Floats on the surface
Taste	Metallic taste	Metallic taste	Metallic taste
Odor	Characteristic odor	Characteristic odor	Characteristic odor

**Table 3: Physical Standardization**

Parameter	KaB1 (%)	KaB2 (%)	KaB3(%)
pH	7.4	7.2	7.3
Total ash	100	100	98
Acid insoluble ash	68.4	50.35	66.2
Water-soluble ash	4.28	3.19	3.59
Loss on drying	1.0	2.0	1.5

**Table 4: Atomic Absorption spectroscopy**

Formulations	Iron Content (%)
KaB1 (%)	65.54 %
KaB2 (%)	68.92%
KaB3(%)	79.9%

**Table 5: Mean (%) of Haemoglobin level of rats in different treatment groups**

Day	Non Anemic	Anemic	KaB1	KaB2	KaB3
0	11.54±0.349	11.26±0.079	11.47±0.237	11.50±0.379	10.95±.067
4	11.69±0.099	11.03±-0163	11006±.399	10.34±-32	10.03±0.33
8	11.73±0.039	10.53±0.035	8.53±0.526	8.19±0.341	8.57±.255
10	12.57 ± 1.76*	9.67 ± 0.90	7.93 ± 0.21	7.90 ± 0.22*	7.89±.32
12	12.85 ± 1.63*	7.24 ± 2.01	6.50 ± 0.29	6.13 ± 1.00*	5.99±1.23
30	13.33 ± 1.17*	7.57 ± 1.30	8.82 ± 0.13*	8.43 ± 0.62*	8.00±1.21
45	13.77 ± 1.01**	7.73 ± 1.01	08.99 ± 0.11*	8.94 ± 0.71**	8.82±2.1
60	14.43 ± 0.65**	8.01 ± 0.02	12.42 ± 1.30*	11.24±1.90**	11.42±1.11
75	14.54 ± 0.75**	8.12 ± 0.13	13.91 ± 0.12*	13.60±1.17**	13.55±1.11

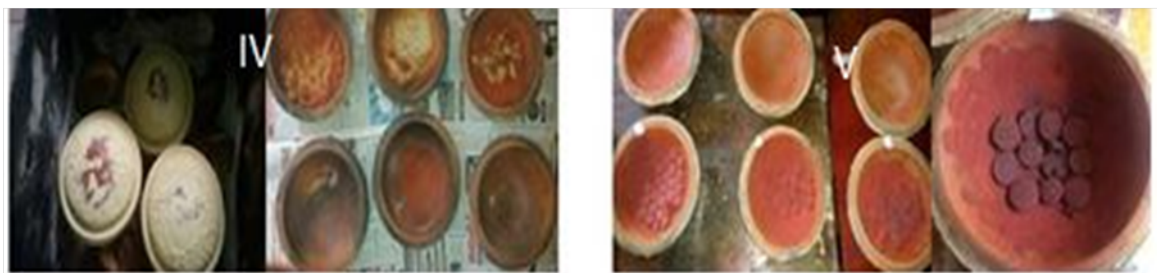
Each group contains six Animals. \*P<0.05. All values are expressed as mean ±S.D.



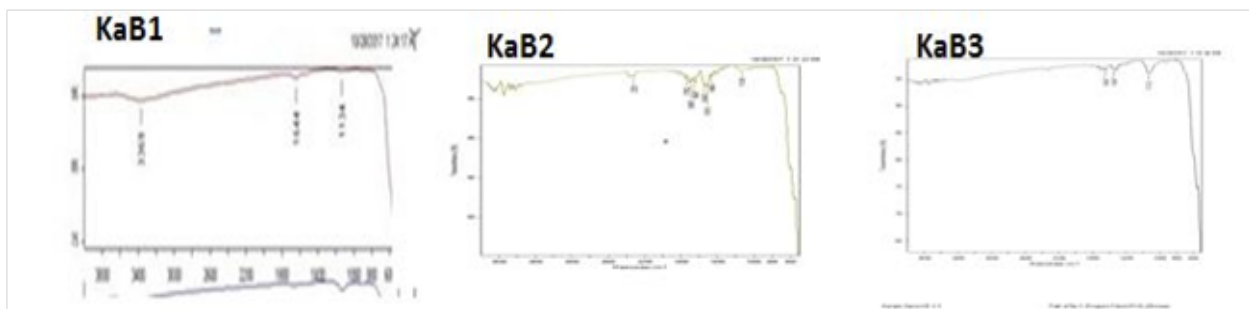
**Figure 1: Stages of Formulation: Kaseesa Bhasma: Method 1(KaB1) - I.RawFeO<sub>4</sub>. II.Purification.III.Purifiedwith bringaraja. IV.After Calcination**



**Figure 2: Method2(KaB2): I.Raw FeO<sub>4</sub>. II.Purification.III.Purified with Nimbu rasa. IV.After Calcination.V.Final Product**



**Figure 2: Method2(KaB2): I.Raw FeO<sub>4</sub>. II.Purification.III.Purified with Nimbu rasa. IV.After Calcination.V.Final Product**



**Figure 3: FT IR Spectrum**

**Table 6: Mean body weight of rats in different groups of animal under study**

Treatment Group	Mean Body weight in gm±SD			
	0	15	45	75
Untreated	91±0.25	119.60±.12	176±8.66	204.83±11
2%gum Acacia	95±1.61	106±3.27	143±8.13	168±2.1
KaB1	97±7.083	106±5.83	186±7.36	213±4.85
KaB2	94±3.488	106±5.11	176±2.42	212±6.5
KaB3	92±3.32	104±3.18	176±3.18	212±4.4

Each group contains six Animals. P<0.001. All values are expressed as mean ±S.D.

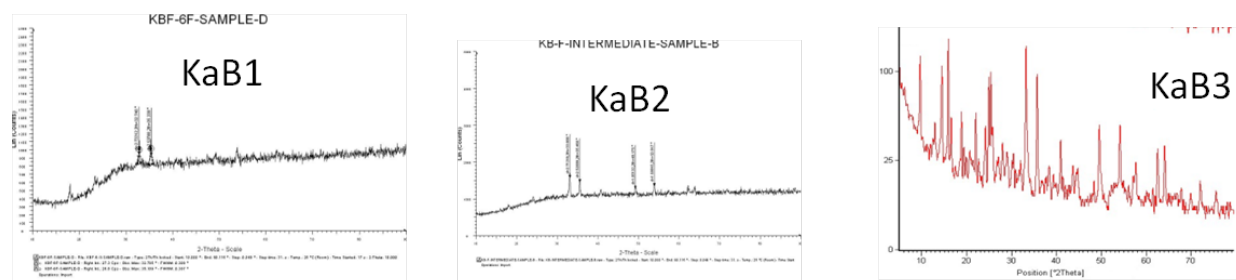
**Table 7: Mean serum ferritin, TIBC and serum Iron**

Treatment Gr	Mean Serum Ferritin(mg/l)		TIBC		Serum Iron	
	Day 15	Day75	Day 15	Day75	Day 15	Day75
Non Anaemic	17.15±.96	107.97±2	90.25±.56	99.62±1.6	18.50±1.33	18.07
Anaemic Group	72.35±1.8	57.63±1.7	101.95±1	110.41±2.6	9.56±0.09	8.78±0.73
KaB1	80.32±1.3	88.10±1.35	91.03±1.2	89.10±2.2	11.32±0.10	16.99±1.3
KaB2	79.5 ±3.21	84.9 ±2.70*	95.08 ±4.7	93.1 ± 3.0	10.79 ± 1.3	16.77±1
KaB3	84.3 ±3.90	83.6 ±2.32*	94.02 ± 3.5	92.7 ±6.03	11.6 ±1.72	16.82. ±03

Each group contains six Animals. P < 0.001. All values are expressed as mean ±S.D.

**Table 8: Toxicity studies**

Groups	Weight of Animals(g)		Toxicity	Duration Of Study(Days)
	Before Study	After Study		
Control	157	157	No Signs of toxicity	14
KaB1	150	150	No Signs of toxicity	14
KaB2	155	155	No Signs of toxicity	14
KaB3	150	150	No Signs of toxicity	14



**Figure 4: XRD Of Formulations**

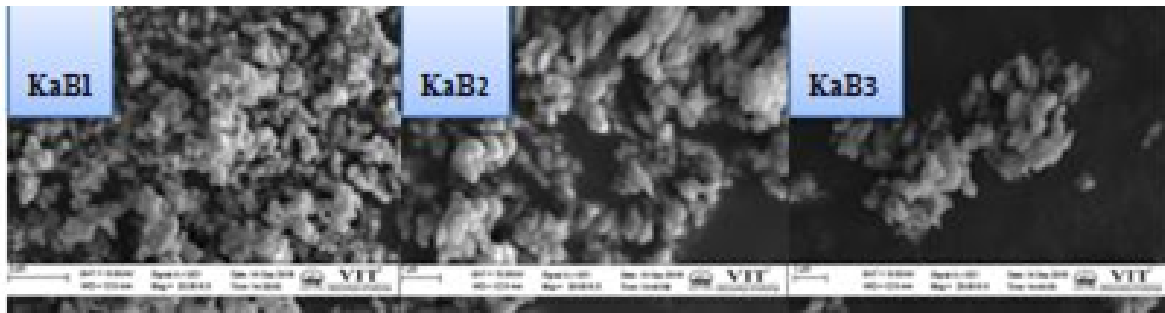


Figure 5: SEM Images for Formulations

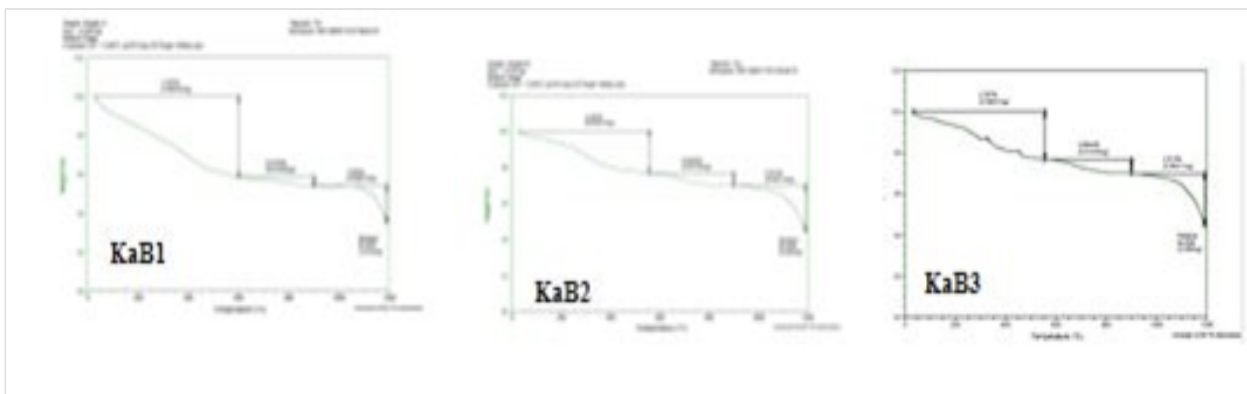
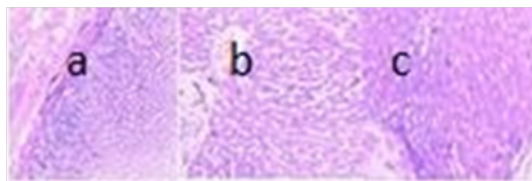
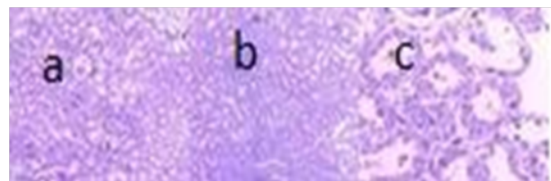


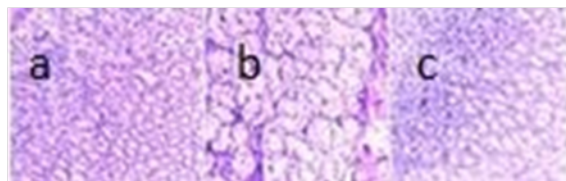
Figure 6: DTG Curve for the Formulations



Liver



Kidney



Stomach

Figure 7: Histopathology (a=Normal, b=KaB1, c=KaB2 )

formulations and compared with the standard marketed Formulation (Wadekar *et al.*, 2005; Sharma *et al.*, 2016).

Analytical standardization involves IR, XRD analysis, after each stage of Formulation. The XRD pattern is used for determining the crystalline size using the Scherrer equation (Bhange, 2017; Madhavan *et al.*, 2016).

The final Formulation further subjected to instrumental analysis like AAS, TGA, SEM, TEM (Sharma *et al.*, 2009). Quantitative determination of iron present in the formulated bhasma was done by using Atomic Absorption Spectrometer (Perkin Elmer, USA) (Chavare *et al.*, 2017; Pal, 2015).

2.3 The therapeutic efficiency of formulations was checked for hematopoietic effect using albino Wistar Rats by inducing anaemia by phlebotomy

### Animals

Male Wistar albino rats weighing 90-100g, aged about  $45 \pm 2$  days, were kept separately in polypropylene cages at a temperature of  $25 \pm 10$  °C with average humidity ranging from 45-55 % and day and night cycle of 12:12 hrs. Standard rat pellets and water ad libitum fed to each rat of treatment groups. A period of 48 h acclimatization to the laboratory conditions made before the commencement experiments to experimental rats. Animal experiments were done with the approval and the guidelines as per IAECPC (Sumithra *et al.*, 2015; Luthy *et al.*, 2009).

### Antianemic efficacy

Male Wistar albino rats grouped as anemic and non-anemic were the treatment groups for the study. The anemic group of rats comprised of further four groups for the treatment schedule. Each group consists of six animals. Agar gel diet, prepared according to the standard method, were fed to the animals of the anemic group. The diet was changed every 7 days and restricted to once a day.

The diet was deprived of any trace of iron in it, and water was supplied ad libitum only after triple distillation to avoid any traces of iron contamination to the treated animal. Non-anemic group rats were given with a measured quantity of ferrous sulfate apart from an agar gel diet and sufficient water. Anemia was induced by phlebotomy (Sivakumar *et al.*, 1985).

The agar gel diet was given continually for 15 days, and then phlebotomy was performed on 1<sup>st</sup>, 4<sup>th</sup>, 8<sup>th</sup>, and 10<sup>th</sup> day. 0.6 ml of blood were bleed each day. The treatment schedule for the four anemic groups was as follows

### Group I

Formulated Kaseesa bhasma, KaB1 (0.02 mg/kg) using gum acacia 2% as medium

### Group II

Formulated Kaseesa bhasma, KaB2 (0.02 mg/kg) using gum acacia 2% as medium

### Group III

Marketed Formulation of Kaseesa bhasma, KaB3 (0.02 mg/kg) in an aqueous medium

### Group IV

Vehicle control used was a suspension of 2% gum acacia

All the treatments to each of the animals were orally given using feeding cannula and were given daily on the fifteenth day of starting the agar gel diet schedule and prolonged the treatment up to 30 days. Untreated control groups were the Non-anemic groups who had not fed with any drug. The standard method for human dosage used for calculating the dose of different treatments given to the anemic group. (Bennett *et al.*, 2012). On every 15<sup>th</sup> day, the body weight (in g) of animals in each group calculated.

### Estimation of Hemoglobin

The cyanomethemoglobin method used to determine hemoglobin level each animal from the blood collected on 0, 4, 8, 10, 12, 30, 45, 60, and up to the 75<sup>th</sup> day of the commencement treatment (Rasheed, 2014).

Determination of Serum ferritin: ELISA reader and conventional method were used for the estimation of serum ferritin level in every animal of each group by using the serum collected on 15<sup>th</sup> and 75<sup>th</sup> day

### Determination of Serum iron and total iron-binding capacity (TIBC)

On the 15<sup>th</sup> and 75<sup>th</sup> day, the blood serum collected from each animal of each group evaluated using spectrophotometry at 562 nm for estimating the concentration of Serum iron and TIBC (Kaur and Kawatra, 1994).

### Evaluation of Toxicity

Toxicological studies were performed using 0.02 mg/kg as the starting dose Kaseesa bhasma. To overnight fasted rats, formulations were administered orally. After the administration also the food was withdrawn for 3-4 h and the animals were observed for any toxicity signs. It includes a reduction in body weight and any marked difference in eyes, mucous membranes, skin and fur, and in respiratory, ANS, and CNS and circulatory Systems. Phar-

macological parameters, like motor activity, salivation, sleep, and coma, were also observed. OECD 423 guideline has followed for the entire studies

Toxicity of formulations was further screened by Histopathological studies of tissues from the stomach, liver, and kidney. The study was done by isolating the organs immediately after scarification, and the stomach, kidney, and liver tissues were separated and fixed in 10 % formalin. Training done by using routine hematoxylin and eosin and sections were of 4-5 micron thickness. The histopathological sections examined with the cooperation of consultant histopathologist examination and images of sections were taken with optical microscope (Patanaik *et al.*, 2003; Pandit *et al.*, 1999).

### Statistical analysis

The significance of the study assessed by one way ANOVA analysis; the significance was evaluated for observations with p values less than 0.05.

### Observations And Reports

#### Physical characterization

##### Formulation 1(KaB1)

The adjuvant Bringarajawarasya has reduced its pH from 7.25 to 4.49 after swedana on heating for 3hrs to an average temperature of 454 °C. The temperature and appearance of Kaseesa after each calcination was different. [Table 1] The color of the product changed from pale green to red.

##### Formulation 1(KaB2)

The adjuvant Nimbu Rasa has reduced the color from light green to yellow [Table 1].

#### Preliminary Evaluation

The stepwise processes adopted for the Formulation of Kaseesa bhasma KaB1 and KaB2 were as per Figures 1 and 2. The traditional method of characterization for KaB1 and KaB2 showed them with brick red color, not having any taste, with a characteristic smell and having a pH of 7.4 [Table 2]. The Lightness formulations proved to sprinkle the bhasma over the water, and it has shown floating over the surface. Bhasma particle entry into the grooves of fingers when rubbed indicates the fineness. The bhasma showed no luster or brightness when exposed to sunlight, indicating that the metal has loosed its metallic shine. The marketed Formulation and KaB3 as well showed characteristics similarly.

The physio-chemical experiments for KaB1, KaB2, and KaB3 were different Ash values, and loss on drying performed. The results obtained were as per Table 3.

### Analytical standardization

Analytical Standardization involves FTIR, XRD analysis of Formulation of KaB1, KaB2, and KaB3 (Figures 3 and 4). The results show that the amorphous nature of particle changes to crystalline one after the preparation has completed. The FTIR spectroscopy plot does not show any peak for the presence of any organic molecule and that of any organic moiety bond.

Comparative analysis of XRD results demonstrates the characteristic reflection peaks of all the three samples, and all were at identical values. XRD pattern shows the sharp, high intensity of lines, which indicates that the chemical moiety is existing in crystalline form. The crystalline size calculated from each of the XRD patterns plotted and Scherrer formula used. The value was in the range of 53 - 57 nm. The peaks in the spectra correspond to that of Fe<sub>2</sub>O<sub>3</sub> form and in hydrated metal oxide (FeO or Fe<sub>2</sub>O<sub>3</sub>) of inorganic iron. From the AAS study was the concentration of elements seen in all three formulations was detected. From the results, it revealed that the iron was the element present in significant concentration with 65.54 %, 68.92%, and 79.91 % for KaB1 and KaB2 and KaB3, respectively, and the other entire element are within the specified limits (Table 4).

SEM images of KaB1, KaB2, and KaB3 are given in Figure 5 indicates size variations and particle agglomeration within the formulations. Repeated calcination cycles involved in the preparation is responsible for the agglomeration. Depending upon the temperature achieved during heating and the adjuvants used, the particle size and agglomeration may occur. In KaB2 with lemon juice adjuvant, ferric oxide shows well-defined structures while the KaB1 showed spongy, microcrystalline aggregates without marked boundaries. The large particle size of KaB2 and KaB3 than KaB1 can be attributed to the preparation methodology that includes several calcination steps. The method adopted for preparation, calcination temperature and duration of preparation may influence the morphology and were reported in previous studies.

TGA curves were obtained for all the preparations of Kaseesa Bhasma by heating in air and were obtained as given in Figure 6. The complete decomposition of KaB1 and KaB2 and KaB3 formulations were occurred at 1210 °C 1232 °C and 1279 °C respectively. Heating of ferrous sulphate in air converted the compound to ferric oxide. As the TGA curves showed the complete decomposition at 1210 °C, 1232 °C, 1279 °C respectively therefore confirms the presence of ferric oxide.



### Pharmacological standardization

In this study, Kaseesa Bhasma KaB1 showed an appreciable increase of hemoglobin (13.01 %) on the 75th day, and KaB2 showed an increase close to that of marketed Formulation treated group (13.70 %) indicating prominent Anti-anemic activity (Table 5). Rats of Bhasma treated groups showed a significant rise in average bodyweight that was comparable to the standard non-anemic group (Table 6). As loss of body weight is the significant clinical indications of iron deficiency anemia, it may conclude that treating with Kaseesa bhasma may help in overcoming the weight loss.

The serum ferritin level is determined to assess the total available iron, which is essential to determine the anemic state. As the stored iron is high in the formulated and standard bhasmas, the increase in serum ferritin level indicates that Kaseesa bhasma can be used effectively in iron deficiency anemia as well as for other iron-deficient diseases. The serum ferritin level is comparatively more in KaB1 compared to the other two Bhasmas. The clinical study on iron deficiency anemia showed that the binding of serum proteins with the free iron and the TIBC (Total iron binding Capacity) increases as the disease progress. This work shows that the treatment with Kaseesa Bhasmas understudy has produced a significant decrease of TIBC level, which indicates the role of bhasma in the dissociation of bound iron from serum protein and thereby producing an increase in iron level in serum (Table 7). When compared to the result, the KaB1 slightly better than KaB2 and marketed Bhasma. Another significant result found was the inflation level of serum iron in KaB1 KaB2 and Marketed Bhasma, and that attributed to their effectiveness in iron deficiency anemia.

### Toxicological studies

KaB1 KaB2 and Marketed Bhasma had not showed any toxicity symptoms in any of the treated rats and constant body weights was noted before and after the administration of formulations and that indicated the absence of renal and gastro intestinal toxicity for the Kaseesa Bhasma (Table 8). The histopathological sections shown in Figure 7 does not revealed any signs of toxicity in liver and kidney cells.

### CONCLUSIONS

The main component of Kaseesa bhasma found as iron. The various stages of formulation techniques involved, like *shodhana* (purification) *Marana* [consisting of *Bhavana* (wet trituration) and the cal-

ination process (*puta*), reduces the particle size remarkably, and thereby improved absorption, metabolism, and distribution of the drug into the various systems of the body. By the analytical techniques studies, the size of the formulations revealed as micro to the nano range. From the findings of the present study, we can conclude that Kaseesa Bhasma effectively used for the treatment of iron deficiency anemia, and the method of preparation has a significant role in the absorbability of the Formulation. Out of the three formulations under the studies, KaB1 has shown comparatively more haemopoietic activity. The reason may be attributed to the adjuvant used (*Bringaraja swarasa*) and the process of preparation. This work is an attempt to carry out in-process Standardization for Herbometallic nanoceuticals.

### Ethical Issues

The study was approved by the CPSEA.

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### Conflict of Interest

The authors declare that they have no conflict of interest for this study.

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