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Physicochemical and Pharmacological Assessment of a Traditional Biomedicine: *Kukutandatwak Bhasma*

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ABSTRACT

Kukutandatwak bhasma (KTB) is a traditional Ayurvedic medicinal preparation. This biomedicine is synthesized through special calcination of eggshell as mentioned in classical Ayurvedic text. Physicochemical characterization of KTB was carried out using modern state-of-the-art techniques such as transmission electron microscopy, scanning electron microscopy, X-ray powder diffraction analysis, Fourier transform infra-red spectroscopy, inductively coupled plasma analysis, energy dispersive X-ray analysis and thermogravimetric analysis. The study showed that the raw material Kukutandatwak (egg shell) is a mineral-organic matrix containing calcium carbonate in calcite form. The calcite form of calcium carbonate remains stable during the process of bhasma formation and forms the main crystalline component of KTB. The heat treatment does result in partial conversion of calcite to calcium oxide, which appears as calcium hydroxide in the final product. The organic content of processed material degraded gradually. Physical evaluation revealed that KTB is a fine grayish white powder having poor flow property with narrow particle size distribution of 0.87 to 16.10 µm having a mean particle size of 5.38 µm. Trace element analysis of KTB revealed the presence of some other important metals like arsenic, lead, chromium, cadmium, mercury, and tin under regulatory acceptable limits at the prescribed dose of KTB. Energy dispersive X-ray analysis revealed calcium as the major element (44.07 wt %) in KTB. Microbial load for the formulation was found to be within limits. Animals were found to be safe up to a maximum dose of 2000 mg/kg body weight in acute toxicity studies.

Keywords: Ayurveda, calcium preparation, eggshell, mineral preparation
Abbreviations: KTB, *Kukutandatwak bhasma*

INTRODUCTION

Available literature from all the ancient civilizations indicates that man has used metals and minerals in disease treatment since time immemorial. As far as traditional Indian systems of medicine Ayurveda and Siddha are concerned, metals and minerals have been used mainly as *bhasma. Bhasma* literally means 'ash'. *Bhasmas* are inorganic preparations produced by an alchemic process which converts a metal or mineral into its compounds like carbonates, oxides, etc. *Bhasmas* of iron, calcium, copper, tin, silver, gold, lead and zinc are commonly used. The advantages of these preparations over plant preparation are their stability, lower dose and sustained availability (Anonymous 1978; Sharma *et al.* 1985; Mishra *et al.* 2004). Although *bhasmas* have been used as effective drugs for centuries without any noticeable side effects, certain factors related to their preparations have remained in disguise. The lack of understanding of traditional methods resulted in a difficulty to reproducibly produce authentic preparations.

Very few reports are available where attempts have been made to understand physico-chemical properties of *bhasma*. Dixit (1987) first gave the scientific basis for standardization of *bhasma*. Evaluation of chemical constituents and free-radical scavenging activity of *swarna bhasma* (gold ash) was reported by Mitra *et al*. (2002). Chemical and pharmacological evaluation of different ayurvedic preparations of *lauha bhasma* (iron ash) was studied by Pandit *et al.* (1999). Evaluation of *karpura shilajit bhasma* (asphalt ash) was carried out by Saleem *et al.* (2006). Traditional preparation and physicochemical evaluation of *godanti bhasma* (gypsum ash) was documented by Dubey *et al*. (2007). Physicochemical characterization of *jasada bhasma* (zinc oxide ash) was done by Bhowmik *et al*. (2009); they reported nanoparticles containing non-stoichiometric zinc oxide in *jasada bhasma*. The composition of copper ash was reported by Wadekar *et al*. (2005). Effect of calcination cycles on the preparation of *Vanga bhasma* (tin oxide ash) was reported by Wadekar *et al*. (2006). Gold nanoparticles were detected in *swarna bhasma* by Brown *et al.* (2007). Kumar *et al.* (2006) determined main constituent and trace element of twenty different *bhasma* of calcium, zinc, mercury, etc. by different analytical techniques. Saper *et al.* (2004) and Ernest *et al.* (2002) concluded that some Ayurvedic herbal medical products, including some *bhasma*, contain potentially harmful levels of lead, mercury, and arsenic. Further, they concluded that the users of Ayurvedic medicine may be at risk of heavy metal toxicity, and testing of Ayurvedic products for toxic heavy metals should be mandatory. The literature reveals the need of scientific methods for assessing and maintaining quality of Ayurvedic preparations (Mishra *et al.* 2004).

Egg shell is a cheaply available rich source of calcium with obvious advantages especially for mothers of developing countries. *Kukutandatwak bhasma* (KTB) is a calcium-containing *bhasma*. This biomedicine is synthesized through special calcination of eggshell. KTB is used as antacid, anti-inflammatory and as a source of calcium. It is also used in tuberculosis, cough, asthma, dysmenorrhea, arthritis, rheumatism, conjunctivitis (Sharma *et al.* 1985). It is well known to increase the intestinal absorption of calcium which is beneficial for bone mineralization. It is also used in treatment of bone metabolic disorders associated with calcium deficiency (Sharma *et al.* 1985). Considering all these facts, it was found worthwhile to carry out a systematic and scientific study of KTB.

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Original Research Paper

MATERIALS AND METHODS

Raw materials

The chicken egg shells (*kukutandatwak*) obtained from Indian hen *Gallus gallus domesticus* were procured from a local market of Indore (Madhya Pradesh), India. *Nousadar* solution is freshly prepared aqueous potassium chloride solution (50% w/v). The *nousadar* solution was filtered using muslin cloth. *Aloe vera* gel is a colourless mucilaginous fillet of gel (parenchymatous cell mass) obtained by peeling off the outer layers of leaves including the pericycle cells of more than two-years-old *Aloe vera* (L.) Burm. f.

Chemicals

Potassium chloride was available as *nausader chikri* (S. K. Traders, Indore) in a local market. It is traditionally used for making *nousadar* solution i.e. potassium chloride solution (50% w/v). All chemicals used in various physico-chemical analyses were of analytical grade and procured from Merck (Mumbai, India).

Animals

Albino rats of Wistar strain of either sex weighing between 150-200 g were used. They were housed in standard cages at room temperature (26 ± 2°C) and 44-56% relative humidity, under a light/dark cycle of 10/12 h for 1 week before the experiments. Animals were provided standard rodent pellet diet (Amrut, India), and water *ad libitum*. The animals were deprived of food for 24 hrs before experimentation, but had free access to drinking water. All experiments were performed in the morning. The study was approved by the institutional ethical committee (465/01/96/ CPSCSEA), which follows the guidelines of CPSCEA (Committee for the Purpose of Control and Supervision of Experimentation on Animals), which complies with international norms of INSA.

Preparation of KTB

KTB was prepared under guidance of an authentic traditional practitioner, whose family has been synthesizing *bhasmas* for a few generations as per the method described in Ayurvedic texts (Anonymous 1978; Sharma *et al.* 1985). Good manufacturing practice (GMP) for manufacturing of ayurvedic medicine as per Schedule T to Indian Drug and Cosmetic Act & Rules was followed during entire preparation process. The process of synthesis of *bhasma* is divided broadly into three stages:

(i) Cleaning (*shodhana*): The egg shell fragments were gently crushed to smaller fractions of <10 mm using an agate mortar and pestle. Pieces of egg shell were first cleaned with hot water to remove dirty material. The eggshells were then immersed in aqueous solution of potassium chloride (50% w/v) and boiled for 90 min in specially prepared hanging sealed earthen pot (*dola yantra*). This process is known as boiling (*swedana*). The solution was filtered off to get the cleaned eggshells (*shodhit kukutandatwak*), which was subjected to first calcination. For calcination the cleaned eggshells were placed in sealed earthen pot (*sarava samputta*) and subjected to ignition in a traditional furnace (*gaja-puta*) as described in Ayurvedic literature to obtain an intermediate. The stable intermediate can be stored for indefinite time in sealed earthen pot till further use.

(ii) Trituration (*bhavana*): The intermediate obtained after first calcination was then treated with *Aloe vera* gel and triturated using an automated mortar and pestle at 1000 rpm (Pharmaceutical machinery Ltd., Mumbai). The total time of trituration was 8 hrs. The mixture was pressed in the form of cakes (*chakrikas*) and dried in shade for 48 hrs. These dried cakes were immediately subjected to further processing.

(iii) Calcination (*marana*): The cakes were calcinated as above to obtain the intermediate. The procedure was repeated two more times with *bhavana* till the sample showed positive to all the traditional tests for *bhasma* (**Table 1**), to obtain the final product KTB [QC 415K] (Anonymous 1978; Sharma *et al.* 1985).

Bhasma preparation and its physicochemical evaluation were performed in triplicate.

Table 1 Traditional Tests for formation of *bhasma*.

S. No.	Test
1	No metallic luster observed
2	Should fill the finger lines when taken between index finger and thumb
3	Sample floats on water
4	Should not regain luster on heating again at same temperature.
5	No gain in weight of Ag metal piece (sample +Ag metal piece, ignite)

Crystalline phase identification with X-ray diffraction (XRD)

All the samples were scanned on Phillips make X-pert powder diffractometer and 2θ scan was from 10 to 100° using Ni filter Cu K alpha radiation and NaI scintillator.

Fourier transform infra-red spectroscopic studies

The raw material and intermediates obtained after each calcination process and final product KTB were scanned using a Thermonicolet IR-200 spectrophotometer with a DTGS detector in the region of 400 to 4000 cm^{-1}. Each spectra was an average of 24 scans of 2 cm^{-1} resolution. Sampling was done using attenuated total reflectance (ATR) assembly with a sample holder of Zn-Se crystal.

Thermo gravimetric studies

The thermo gravimetric analysis was performed using a Perkin Elmer series TG analyzer. The thermogram was recorded from 40 to 1000° at the heating rate of 10°C min^{-1} under atmospheric air.

Elemental analysis with inductively coupled plasma (ICP)

A Perkin Elmer Optima 3300 RL ICP equipped with an As-91 auto sampler was used. The instrument was calibrated using reference standards of ICP-MS grade from SPEX CertiPrep, Metuchen, NJ. Approximately 0.1 g of KTB sample was accurately weighed into a metal-free container and dissolved in 1 ml of *aqua regia* and heated on a hot plate to extract the metal. The slurry was filtered using Whatman No. 1 filter paper (11 µm). The residue left after filtration was suspended in 5 ml of deionized water and refiltered using Whatman No. 1 filter paper. First and second filtrate were mixed and transferred to a volumetric flask and volume was made up to 10 ml. This freshly prepared solution was used for elemental analysis with ICP.

Elemental analysis with energy dispersive X-ray analysis (EDAX)

Quantitative determination of bulk elemental composition in the KTB sample was carried out by EDAX (EDAX Inc., Mahwah, NJ, USA). A sample of KTB was packed into a cavity of an aluminum stub (9 mm diameter, 9 mm depth). The operating parameters were: 30 kV, count-rate 1500 ± 500 counts/s, working distance 10 mm and accumulation time 50 s. The relative elemental compositions of the KTB particles were computed directly with EDAX software, using standard "ZAF" (atomic number, absorption, fluorescence) correction alogrithm. Analyses were performed on 13 different points for each KTB sample.

Particle size analysis using DLS technique

DLS was performed using a Brookhaven-Zeta plus (Holtsville, NY, USA) instrument to determine the mean particle size. The instrument was powered with an argon laser source, which produces 660 nm laser light for analysis. A suspension of 10 mg/mL concentration of KTB in MilliQ water was sonicated for 10 min to get the hydrodynamic diameter of the sample.

Scanning electron microscopy (SEM)

Surface analysis of particles was done using scanning electron microscope (JEOL-JSM 200). Carbon paste was applied on aluminum stubs and was allowed to dry overnight at room temperature. The powder KTB sample was sprinkled on the dried carbon paste. Aluminum stubs were placed in the vacuum chamber. The KTB samples were observed for morphological characterization using a gaseous secondary electron detector (working pressure: 2-5 mm Hg, acceleration voltage: 10.00 kV).

Evaluation of powder properties

Bulk density and tapped density were determined using standard methods. These values were used to indirectly calculate flow properties by deriving Carr's index. Static angle of repose was determined by the funnel method (Martin *et al.* 1991; Anonymous 1996).

The calcium carbonate and calcium hydroxide content in KTB was determined using tritrimetric procedure. The acid base titration was used to determine carbonate ion and complexometric titration was used to determine the calcium ion (Anonymous 1996).

Microbial evaluations

Microbial evaluation of KTB was carried out according to Indian pharmacopoeia (Anonymous 1996). The bhasma was tested for presence of contaminating fungus (yeast and moulds), *Staphylococcus aureus*, *Salmonella* sp., *Escherichia coli* and total aerobic microbial count.

Acute toxicity studies

Rats were fasted for 24 hrs prior to drug administration. A total of five animals were used. KTB uniformly dispersed in 2% sodium carboxymethylcellulose suspension (500 mg/ml) was administered as a single oral dose equivalent to 2000 mg/kg body weight. Food was withheld for a further 4 hrs. Animals were observed individually at least once during first 30 min after dosing, periodically during the first 24 hrs (with special attention during first 4 hrs) and daily thereafter for a period of 14 days. Once daily cage side observation included changes in skin and fur, eyes and mucous membrane (nasal) and also respiratory tract, circulatory (heart and blood pressure), autonomic and cervical system changes. Mortality, if any, was determined over a period of 2 weeks (OECD, 2001). LD_{50} was calculated as per OECD guidelines.

RESULTS

Preparation of Kukutandatwak bhasma (KTB)

The KTB was strictly prepared as per method mentioned in ayurvedic text. The calcination was repeated till sample gave positive results to all tests for *bhasma* as mentioned in Ayurveda (**Table 1**).

Crystalline phase identification with X-ray diffraction (XRD)

The XRD pattern of KTB is shown in **Fig. 1A**. Sample identification was done by matching d- spacing with the standard JCPDS database. Diffraction pattern indicates calcite as the major crystalline phase present in KTB. Standard calcite was also analyzed with XRD and diffraction pattern is shown in **Fig. 1B**. Peaks at d = 3.04 A° (2θ =29.28), d = 2.28 A° (2θ = 39.32), d = 1.91 A° (2θ = 47.41), d = 1.87 A° (2θ = 48.42), and d = 1.42 A° (2θ = 65.44) confirm the presence of calcite as the major crystalline phase in the sample. Low intensity lines indicating the presence of calcium hydroxide d= 2.628 A° (2θ = 34.00), 4.9 A° (2θ = 17.94), 1.927 A° (2θ = 47.41) were also observed. This may be attributed to hydrolysis of calcium oxide formed due to partial decomposition of calcite during calcination cycles (Engin *et al*. 2006). Mean crystal size of the KTB particles was calculated from XRPD pattern (2θ for 100% intensity peaks) following the Scherer's Equation (Wadekar *et al.* 2006). The mean crystal size of KTB was found to be 39.08 nm whereas the mean crystal size of standard calcite was 39.80 nm (**Table 2**). KTB particles have smaller crystal size as that of standard calcite. The starting material in the preparation of KTB was powdered eggshell with the characteristic peak of calcite (**Fig. 1C**) appearing at d = 3.05 A° (2 θ = 29.23), d = 2.29 A° (2θ = 39.25), d = 1.91 A° (2θ = 47.36), d = 1.88 A° (2θ = 48.36), and d = 1.42 A° (2θ = 65.47). This result confirms the calcite in the final product of the KTB.

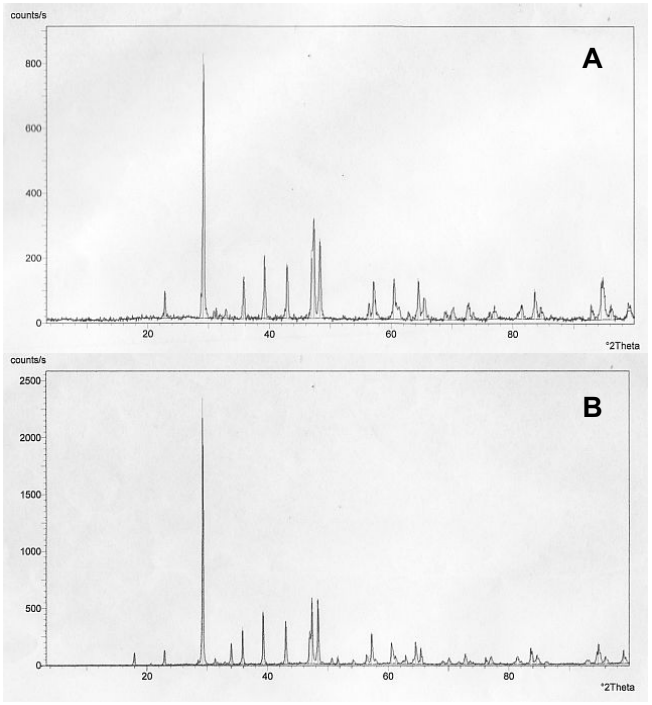

Fig. 1 (**A**) XRPD graph of egg shell. (**B**) XRPD graph of KTB.

Fourier transform infra-red spectroscopic studies

The characteristic bands of carbonate ion in standard calcite are evident in **Fig. 2A**. Similar peaks were reported by Engin *et al*. (2006) in the IR spectrum of eggshell in which they obtained the carbonate ions of the mineral with the internal vibration modes of CO_3^{2-} ν_3 mode (asymmetric stretch) at 1415 cm^{-1}, ν_2 mode (out of plane bend) at 879 cm^{-1} and ν_4 mode (in plane bend) at 700 cm^{-1}, respectively. A broad absorption around 3400 cm^{-1} indicates the stretching vibration of a structural water molecule. Absorption peaks for organic matter were observed between 3000 and 2500 cm^{-1}.

The IR spectrum of powdered eggshell (**Fig. 2B**) obtained during the study was in confirmation with reported data (Engin *et al*. 2006). It showed characteristic bands for

Table 2 Comparison of crystal size of standard calcite and KTB using XRPD.

Sample name	2θ values at I/I0 = 100 (degree)	FWHM (radian)	d-Value (Å)	Crystallite size[(a)] (nm)
KTB	29.28	0.0036	3.04282	39.08
Standard calcium carbonate (calcite)	29.405	0.0036	3.03288	39.80

[(a)] Scherrer's equation, d = 0.9λ/βcos θ, where d is diameter of crystal, λ is X-ray wavelength of analysis, β is FWHM in radian, θ is theta in Braggs equation for X-ray diffraction.

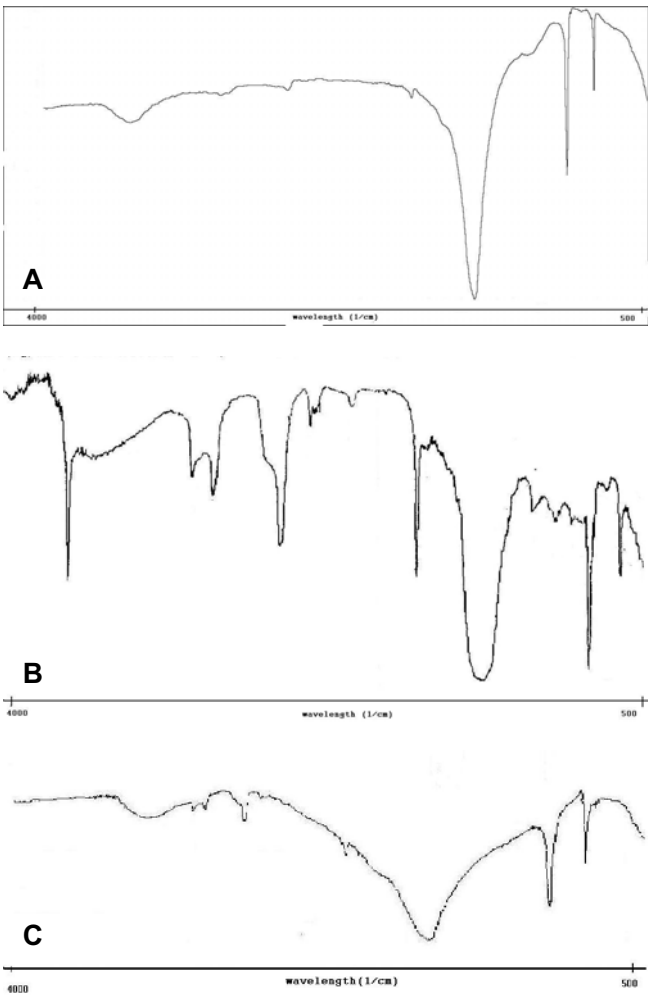

Fig. 2 (**A**) FTIR spectrum of calcite. (**B**) FTIR spectrum of KTB. (**C**) FTIR spectrum of egg shell.

natural calcite in the region of 4000 to 400 cm^{-1}. The carbonate ions of the mineral were shown by the internal vibration modes of CO_3^{2-} ν_3 mode (asymmetric stretch) at 1417 cm^{-1}, ν_2 mode (out of plane bend) at 878 cm^{-1} and ν_4 mode (in plane bend) at 709 cm^{-1}, respectively. The stretching modes of water group [ν_3 and ν_1] were found at 3405 and 3555 cm^{-1}. Significant peaks in the 2500-3000 cm^{-1} region correspond to C-O and C-N of the organic matrix. The studies confirmed that powered eggshell is a calcite crystal of calcium carbonate embedded in an organic matrix.

Characteristic peaks of the calcite form of calcium carbonate with few modifications were evident in the IR spectra of KTB (**Fig. 2C**). Absorption bands of CO_3^{2-} molecules shifted to higher energies (ν_3 mode to 1470 cm^{-1}, ν_2 mode to 1040 cm^{-1}, ν_4 mode to 820 cm^{-1}) in the final product KTB as compared to powdered eggshell, probably due to the decrease in reduced mass of the functional groups associated with the CO_3^{2-} ions. Furthermore, the vibrations of the CO_3^{2-} ions in a crystalline structure may have been considerably affected by its environment. The absorption band of water molecules decreases with increasing temperature. The absorption bands, attributed to organic matter, were found to decrease slowly with increasing calcination temperature and number of calcination cycles. A sharp OH^- stretching band at 3643 cm^{-1} appeared in the final product KTB indicating formation of calcium hydroxide (Engin *et al*. 2006). The results of FTIR studies indicating presence of calcium hydroxide were in agreement with the findings of XRD studies as mentioned above.

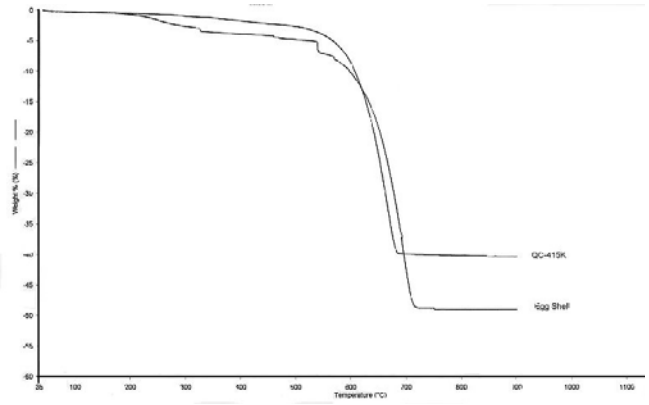

Fig. 3 TGA curves of egg shell and KTB.

Thermo-gravimetric studies

A thermograph (**Fig. 3**) of KTB showed a small weight loss up to 600°C, which may be attributed to the loss of moisture content of the crystal. A gradual weight loss up to 43% w/w was also observed between 800 and 900°C due to gradual conversion of calcium carbonate to calcium oxide. The thermal behaviour of KTB was comparable to that of standard calcium carbonate. The raw material (powdered eggshell) showed a weight loss of slight higher percentage up to 600°C and above, when compared to KTB; which may be attributed to the loss of organic material.

Chemical composition analysis by ICP and EDAX

An average elemental content of triplicate batches of KTB using EDAX and ICP analysis was shown in **Table 3A** and **3B**, respectively. EDAX analysis revealed calcium as the major element (44.07 wt %) in KTB. The element analysis revealed the presence of heavy metals like arsenic, lead and tin in KTB. Other heavy metals like chromium, cadmium and mercury were below the detection limit of ICP analysis. The detection limit in ICP analysis is much lower than the acceptable concentration of heavy metals as per standard regulation given in **Table 3B** (Anonymous 2006). Thus, it can be concluded that KTB complies with regulatory permissible limits for heavy metals in natural drug product (Anonymous 2006).

Table 3A Comparison of elemental composition of eggshell and KTB detected by EDAX analysis.

Particulars	Element	EDAX (wt%) (Mean± S.D.)
Kukutandatwak (egg shell)	Ca	23.66 ± 0.97
	C	45.45 ± 0.64
	O	24.96 ± 2.74
Kukutandatwak bhasma	Ca	44.07 ± 0.05
	C	12.92 ± 0.04
	O	43.01 ± 1.08

Table 3B Comparison of elemental composition of eggshell and KTB detected by ICP analysis.

Element	Acceptance limit of elemental concentration of product (µg/mg)	Concentration of elements of egg shell (µg/mg ± SD)	Concentration of elements in KTB. (µg/mg ± SD)
As	0.0196	BDL(x)	0.0052295 ± 0.0010
Cr	1	BDL(x)	BDL(x)
Cd	0.0126	BDL(x)	BDL(x)
Pb	0.0406	BDL(x)	0.0018991 ± 0.0014
Hg	0.0406	BDL(x)	BDL(x)
Sn	40	BDL(x)	0.1882 ± 0.0094

(x) Below detection level of the instrument

Particle size analysis with Dynamic light scattering (DLS)

The particle size of KTB ranges between 0.87 and 16.10 µm with a mean particle size of 5.38 µm. 6% of the particles were also found to have a particle size less than 50 nm.

Scanning electron microscopy

SEM images of standard calcite and KTB samples after different cycles of calcinations are shown in **Fig. 4**. It was found that: (i) samples at different calcination cycles showed a higher degree of agglomeration than that shown by standard calcite sample. (ii) Morphology of KTB samples was remarkably different from that of standard calcite. (iii) Spongy and relatively compact microcrystalline aggregates of calcite were observed after the first calcination cycle, which were covered by small dusty crystallites. (iv) Second calcination cycles resulted into a spongy nature of the crystallites with increased agglomeration as indicated by the increased particle size. (v) A distinct change in the morphology was also observed with last calcination cycles as several well-defined rod-shaped particles were seen in the SEM of KTB. Higher numbers of smaller agglomerates were also observed in finished product indicating that the last calcination had disintegrated bigger agglomerates to a stable particle size. This simply means that repeated calcination cycles are necessary to stabilize the particles to a minimum particle size. This finding supports the requirement of multiple calcination cycles recommended in classical Ayurvedic texts.

Evaluation of powder properties

KTB is non-lustrous white powder having a bulk density of 0.893 ± 0.038, tapped density 1.031± 0.035 and an angle of repose of 34.94 ± 1.127°. The value of Carr's index was found to be 45.86 ± 0.18. These data clearly indicated that KTB is a fine powder with poor flowability that needs to be granulated for improving the flow characteristics. The calcium carbonate and calcium hydroxide content of KTB determined by tritrimetic methods was found to be 97.4 ± 1.79 and 2.3 ± 0.61% (w/w), respectively. The difference in assay value of calcium carbonate obtained by acid-base titration and complexometric titration gives the amount of calcium hydroxide.

Microbial evaluations

Microbial load of the KTB was found negative for the presence of *Escherichia coli*, *Salmonella* spp. and *Staphylococcus aureus.* Total aerobic count was under the acceptance limit.

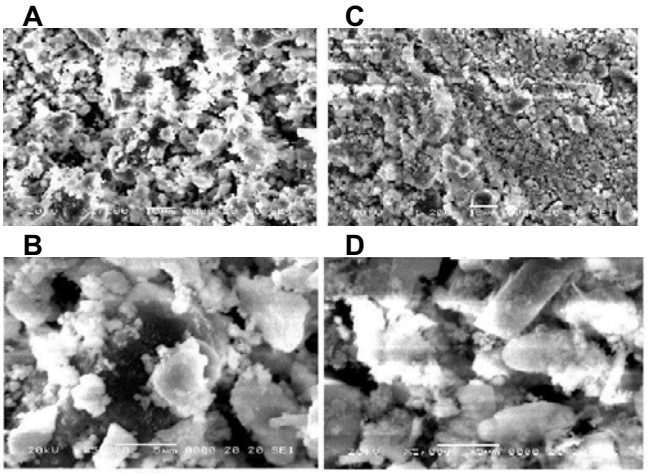

Fig. 4 (**A**) SEM of standard calcite. (**B**) SEM of KTB after 1st calcination cycle. (**C**) SEM of KTB after 2nd calcination cycle. (**D**) SEM of KTB after 3rd calcination cycle.

Acute toxicity studies and dose determination

The LD_{50} of KTB as per OECD guideline falls under class four with no signs of acute toxicity up to a maximum dose of 2000 mg/kg. Any changes in normal behavioral pattern or signs and symptoms of toxicity and mortality were not observed up to this dose level.

DISCUSSION

Kukutandatwak [egg shell] represents around 11% of the total weight of eggs and is constituted of calcium carbonate (94%), calcium phosphate (1%), organic matter (4%) and magnesium carbonate (1%). Cleaning of eggshell by potassium chloride solution for 90 min results in a porous surface with the complete loss of luster. Cleaning thus helps to remove dirt and any other organic layers at the surface. The time of treatment with potassium chloride solution should not be more than 90 min, as longer treatment will result in excessive loss of calcium in the eggshell as calcium chloride.

Marana, the process of calcination of an intermediate sealed in earthen pots was carried out in a traditional furnace (*gaja-puta*). The temperature inside the sealed earthen pots placed in the gajaputa varies between 600 and 900°C; these observations are in agreement with the studies of Ketkar *et al.* (2003). Such a high temperature supports the conversion of calcium carbonate into calcium oxide, which should result into a high concentration of calcium oxide in the final product (Engin *et al*. 2006); however, KTB contains mainly calcium carbonate in calcite form along with less intense peaks for calcium hydroxide. This phenomenon may be attributed to the bhavana with *Aloe vera* gel. Incineration of *A. vera* gel might have helped in maintaining carbon and oxygen atmosphere inside sealed earthen pots which prevents decarbonation of calcium carbonate to calcium oxide (Ketkar *et al.* 2003). The bands for organic matrix were modified at different stages of the process. The partial destruction of the organic matrix was indicated by weight loss during synthesis with decreased band intensities. Thus, KTB is chemically a mixture of calcium carbonate in calcite form with not more than 4% w/w of calcium hydroxide.

It is evident from XRD studies (**Table 2**) that repeated trituration and calcination decreased the crystal size of the KTB to a value less than that of standard calcite. The scanning electron micrograph (**Fig. 4**) revealed size stabilization of particles on repeated calcination. From all the above observations, it can be concluded that repeated trituration and calcination cycles definitely impart specific physico-chemical characters to KTB which might be responsible for the potent therapeutic activity of this unique class of medicine.

The element analysis (**Table 3B**) revealed the presence of heavy metals like arsenic, lead and tin in KTB. But these elements were present in very minor amount as compared to percentage of calcium present in KTB hence considered trace elements. It was also observed that all the elements were quite below regulatory permissible limits for trace elements in a natural drug product (Anonymous 2006). The microbial load of the preparation was under permissible limits for drug products as per Indian pharmacopeia (Anonymous 1996). The LD_{50} of KTB as per OECD guideline falls under class 4 with no signs of acute toxicity up to a maximum dose of 2000 mg/kg.

CONCLUSION

Although *bhasmas* are complex materials, physicochemical analysis (**Table 4**) using modern techniques will assist to unravel their mode of action. Altogether, these characterization techniques will be most attractive techniques for the standardization of herbomineral medicines. This would definitely help in building confidence in use of such products for medication by ensuring safety, efficacy and batch to batch uniformity.

Table 4 Quality assessment of KTB.

Test parameter	Test	Inference	
Identity	Macroscopic	Non lustrous, grayish white, fine powder.	
	Physical properties (mean ± SD)	Bulk density	0.893 ± 0.038
		Tapped density	1.031 ± 0.035
		Particle size range	0.87-16.10 μm
		Angle of repose	34.94 ±1.127°
		Carr's index	45.86 ± 0.18
		Loss on drying at 110°C	< 0.5 % w/w
		loss on ignition	< 0.05% w/w
	XRPD	Characteristic d-spacing value at 3.04 Å, 2.28 Å, 1.91 Å, 1.87 Å, 1.42 Å. (calcite form of calcium carbonate)	
	TGA	40.17 % weight loss at 800°C	
	IR spectroscopy	882 cm^{-1}, 714 cm^{-1} (calcite form of calcium carbonate)	
Purity	Contaminating fungus (Yeast and mould)	<1 X 10^4 CFU/g	
	Total Aerobic Count	<1 X 10^5 CFU/g	
	Escherichia coli	Absent	
	Salmonella spp.	Absent	
	Staphylococcus aureus	<1 X 10^2 CFU/g	
	Arsenic	<0.14 μg/Kg body weight/day	
	Cadmium	<0.09 μg/Kg body weight/day	
	Lead	<0.29 μg/Kg body weight/day	
	Total mercury	<0.29 μg/Kg body weight/day	
Quantity	Quantitative test	Calcium content: 44.07 ± 0.05%	
		Calcium carbonate: 97.4 ± 1.79 % w/w	

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