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Ayurvedic approach for Synthesis, Safety and Antacid Activity of Kapardika Bhasma: Marine Natural Calcium

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Abstract:

The widely used *rasaushadhi Kapardika bhasma*(calcined Cowrie) may have a potential of an active drug molecule to treat GIT diseases with proven safety and efficacy.

Objectives:

Present study was aimed at process standardization of 15 samples of Kapardika bhasma made by various permutations and combinations of *shodhan* ingredients and *maran* processes, physicochemical analysis, toxicity and in vitro antacid activity.

Materials and Methods:

*Cypraeamoneta***(**L), *Charachar* variety reported in *Rasashsatra* was processed using lemon juice, buttermilk, rice vinegar and *D. biflorus* decoction in Gajaputa and muffle furnace in our laboratory. The samples fulfilling standard bhasma testing criteria, physical tests and Ca percentage were studied for toxicity on Haffkin F-2 strain albino mice using OECD 420 fixed dose method for acute, and OECD 407 repeated dose 28 days method for sub chronic toxicity. The two safe samples E1 and E2 were tested in-vitro.

Results and conclusions:

Ten of the 15 samples complied with standard parameters, yet the samples E1 and E2, processed in lemon juice and triturated with Aloe vera juice with one more heating cycle matched best. Traditional *Gajaputa* method can be successfully mimicked by using electric muffle furnace to get standard product with reduced time and cost. No mortality or gross necropsy was observed in both acute and sub-chronic studies on 10 samples, yet some of the samples showed inflammatory lesions in the liver, intestine and kidneys on HPE except E1 and E2. E1 and E2 when studied in-

vitro may be labeled as antacid based on PAT though they did not comply with ANC, RRT and Reheis tests.

Key words:

Mollusk; Shell calcium; Ethno-medicine; Cowrie-bhasma; Bio inorganics; Toxicity

Introduction

Thousands of compounds are being isolated and developed by the chemists the world over, from which just about the few manage to hit the headlines yet experience a very short life span. It is an accepted fact that the few drugs that have survived for the past hundred years are of natural origins, such as aspirin, aminophylline, digitalis, atropine, local anesthetics derived from cocaine etc. Apart from herbals, natural products from marine source have also been studied for biological activities. About 1152 new compounds have been derived from various marine species like algae, seashells, mangroves, tunicates, intertidal plants and molluscs.[1]Often, we find that animal bodies are themselves used as the *living laboratories* which are capable of processing chemicals that sustain human well-being.[2] Medicinal cowries, specifically money cowries found in Indo-pacific region (*Cypraeamoneta,* Linn 1758*)* are one such example of the living laboratories that are seen to be a safe and effective source of calcium, a physiological mineral required by the human body almost every day.

Rapid industrialization and its concomitant pace of life has ushered numerous changes in the modern man's life style, mostly in the food habits. Hundreds of preprocessed fast food joints offering exotic food have been introduced. Consumption of such food results in various kinds of worm infestations, sluggish peristalsis, ulceration in the intestinal mucosa, inflammation superadded with bacterial infection of the intestines, acute and chronic colitis, distortion of the structure of the gastrointestinal tract, atonia, diverticulitis, reverse peristalsis etc. The most significant of these are the disturbances in the secretion pattern of the various digestive enzymes and Inflammatory Bowel Disease (IBD). The need, therefore, to introduce new drugs to rectify the odds of gastrointestinal tract is even more acute. Ayurvedic *Kapardika* or *Varatika* bhasma prescribed in all these conditions offers a promising solution to global population.

A careful scrutiny of the compendium *Bharat Bhaishajya Ratnakara* to search formulations containing Cowrie shell, revealed that is an essential ingredient of about 42 formulations that rectify the disorders of gastrointestinal tract (GIT),(Table-1 in supplementary data)[3].As viewed through the Ayurvedic paradigm, Ayurvedic physicians perceive a substantial difference between the calcium obtained from different sources, such as plants (Betel leaf), minerals (Calcium sulphate), and animal shells (Conch, Coral, Oyster etc.) due to very peculiar composition and *gunas* of each of these. Cowries are capable of supplying physiological calcium to human bodies without creating the detrimental after effects of depositions as are seen in the case of chemically pure calcium.[4]The natural Cowrie calcium associated with some other micronutrients like bio-

copper, zinc and magnesium may have a potential of an active drug molecule for correcting the gastrointestinal function.[5] Additionally, easy market availability and relatively less price may make the product Kapardika bhasma a value-added drug for GIT disorders.

Rasaratnasamucchaya and Rasatarangini disclose two shodhana (detoxification) methods, boiling and immersion using four processing agents, two fuel types cowdung and goat pellets (karisha), and two methods of heating, open burning and *Gajaputa* for bhasma making of cowries.[6,7] Some later references quote additional processing agents and also mixture of all products of*amlavarga*.[6]After the first *Gajaputa* many Ayurvedic pharmacies perform a step of trituration of the Kapardika bhasma using Aloe vera juice followed by additional *Gajaputa*.[8]Manual method of incineration is the source of batch to batch variation which can be minimized by using of electric muffle furnace. In order to test the Kapardika bhasma for any particular GI disorder, it is mandatory to ascertain its safety. It is also possible to test the bhasma for claimed activity using laboratory methods.

Therefore, in the present paper,possible variations of manufacturing Kapardika bhasma including use of electric muffle furnace have been discussed with a view to evolve a standardized manufacturing process. The results of acute and subacute toxicity of the standard Kapardika bhasma are presented. In vitro antacid activity of the same is also stated. (See Fig1 for study outline.)

MATERIALS AND METHODOLOGY

Procurement of Cowries and processing materials:

The cowries sourced from Arabian sea, sold in Mumbai Ayurvedic drug market were purchased. The oval shaped yellow cowries, slightly flattened dorso-ventrally; having pearly/ glossy luster; bearing nodules and bands on dorsum; heavily calloused sides; having length between 30 to 50 mm and weighing 3.5 to 4.5 gm. each, befitting the *Rasashara* selection criteria, were selected with a 15% rejection. The2.5 kg cowries were thoroughly washed with plenty of warm water and were scrubbed with plastic brush to remove the removable surface dirt and remnants of animal inside the shells. Use of detergents or soaps was avoided. Cleaned cowries were divided into 5 equal groups (viz. A to E), each group containing 500 gm. by weight. There were about 130 to 145 pieces in one lot. Each lot was processed in respective processing medium.

The processing drugs *Citrus limon* (L.,Osbeck) juice, sour buttermilk, sour *kanji* (edible vinegar of rice), decoction of seeds of horse-gram *Dolichosbiflorus*(L.) (syn. *Macrotylomauniflorum*), and *Aloe barbadensis* Miller (Aloe vera) juice were prepared in the laboratory by using standard methods(see notes for preparation methods). *Gajputa* pit, electric muffle furnace and other lab instruments, apparatus and standard chemicals were available in our laboratory.

***Kapardika Bhasma* making process**:

The first step includes either boiling or immersion for detoxification, followed by heating in kiln or furnace and trituration and repeat heating.

Detoxification process: The first lot 'A' of the cowries was boiled using traditional *dolayantra*in freshly squeezed lemon juice for 3 hrs and allowed to cool till room temperature(Fig.3a).[5] Similarly the lots B, C, and D were boiled in sour buttermilk, sour rice vinegar and decoction of horse gram respectively.[9]The lots E and F were immersed in fresh lemon juice for 12 hours. All the lots were washed thoroughly with normal water after the process and dried in shade (Fig. 2a).

Calcination/Incineration:The dried cowrie shells of the lots A, B, C, D and E were further divided into 3parts e. g. A1, A2, A3. Each sub-lot was filled in single layer in an earthen *sharavasamputa*, which was tightly closed using 7 layers of muslin band layered with fuller's earth.

The lots A1, B1, C1, D1 and E1were heated in the cubical pit of 30" using 100 cow-dung cakes each time, which is known as *Gajaputa*. The *sharavasamputas* were placed in the center of the pit on half of the cow-dung cakes and remaining half were placed above the pots. (Fig. 3c,d,e,f). The temperature was measured using nickel cadmium thermocouple device and was recorded at the interval of every 10 minutes during *Gajaputa* process. The pattern of *Gajputa* temperature graph was used to set the EMF (Fig.4).

The lots A2, B2, C2, D2 and E2 were similarly packed but processed in electric muffle furnace (EMF) at temperature maintained for the range 700 –950 -700^{0}c for 180 minutes and then turned off. The EMF heating chamber of dimension 4"x 4"x 12" was lined by fire bricks and insulated with glass wool. The temperature measurement accuracy was +0.1^{0}c.(Fig 3b)

The lots A3, B3, C3, D3, and E3 were also heated in *Gajaputa* kiln but using 36 kg. Goat pellets instead of cow-dung. Here the maximum temperature attained was 596^{0}C.

For all the 15 samples after natural annealing the *samput* was opened and observed. Cowrie powder was triturated using porcelain mortar pastel for 10 minutes to obtain uniform size and stored in airtight glass jars.

The lots E1 and E2 were further triturated with 250 ml of Aloe vera juice (for each lot) for 3 hours till thick paste was formed. Thin uniform flat tablets of 2-inch diameter were prepared and shade dried. Lot E1was heated in Gajaputa and E2 in muffle furnace for the second time. After annealing both bhasma samples were powdered and stored in airtight glass jars. The samples in polythene bags are shown in (Fig 2b).

Analysis of bhasma samples: All the samples were analyzed on Ayurvedic Bhasma testing criteria and the samples which did not comply with all the tests were rejected. The selected samples were analyzed for physical tests using standard methods and Ca percentage was tested by flame photometer.

Toxicity study:

The regular use of *Kapardika bhasma* by Ayurvedic physicians in India and neighboring countries since circa 1200 BC, obviates LD 50 study because the core concept of LD 50 or toxicity testing has been designed for chemicals which are primarily harmful.[10]The 10 bhasma samples that complied with the quality parameters were studied,after obtaining approval of the institute ethics committee, as follows:

Test substance: Kapardika bhasma samples A1, A2, B1, B2, C1, C2, D1, D2, and E1, E2.

Vehicle: Traditionally, Kapardika bhasma is administered with honey or sugar. Here 20% sugar solution was used to make suspension in order to feed the mice.

Test animals: Haffkine F-2 strain albino mice of age 8 weeks weighing 18-25 gm of both the sexes, were used. Total 72 animals were randomly allocated into 12 groups. 10 groups of study drug samples A to E, 11^{th} of sugar solution and 12^{th} of normal control were made. Six animals, 3 males and 3 females per group were housed in one stainless-steel cage having escape proof lid, feeding hopper and a water drip bottle. Each animal in a group was marked using picric acid on same body part e. g. front right limb, hind right limb etc. for group identification.The 12 cages were stacked in a steel shelf mounted over a trolley. The animals were maintained on animal food, procured from Amrut animal and bird feed, Market Yard Pune. They were provided with tap water ad libitum and were exposed to natural cycles of day and night. Ambient temperature and humidity were maintained throughout the experiment.

Test methodology:

Acute oral toxicity study OECD 420 fixed dose method: The mid-level dose as suggested in the guideline was selected for the test. The animals were overnight fasted prior to dosing but were provided with water ad libitum. Next day morning 300 mg. of Kapardika bhasma was mixed with sugar syrup and fed to each animal using 18 number oral gavage needle (rodent feeding). The animals were observed every 15 minutes for the first hour, every 30 minutes for next 3 hours and then at an interval of 3 hours for 24 hours. Further they were observed for 14 days and on day 15 representative animals from each group were sacrificed by cervical dislocation. The body was cut open to check gross necropsy and results were reported.[10]

Subacute oral toxicity study OECD 407 repeated dose 28 days method: The human dose of Kapardika bhasma is 250 mg for average body weight.[6] The dose was extrapolated using extrapolation factor for mice (0.0026) which was 0.65mg/20gm of mice.[11] Each animal was weighed every day morning prior to dosing which was done for 28 days. On day 29^{th} representative animals 1 male and 1 female from each group were randomly selected and sacrificed. Internal vital organs of the animal viz. liver, kidneys, stomach, piece of intestine (upper), piece of intestine (lower), lungs, brain, rectal canal, spleen, piece of skin, piece of bone (femur) hearth, testes or

ovaries, uterus, and eyeball were dissected out. These were preserved in 10 % Formal-saline and were sent for histopathological studies. Five mm sections of the tissue embedded in paraffin wax were taken out with the help of rotatory microtome, processed and stained with hematoxylin and eosin (Hand E) stain. The slides were prepared. Obtained reports were interpreted to see the effect of sample of Kapardika bhasma on the selected organs from each group and compared with those of the vehicle control and sham control (no treatment) groups.[12]

Evaluation of antacid activity:

In -vitro tests on two samples of Kapardika bhasma E1 and E2(bhasma processed using Aloe vera trituration), were performed using Preliminary Antacid Taste (PAT), Acid Neutralizing Capacity (ANC), Rosset-Rice Test (RRT) and Reheis test.[13,14]For the experiments, magnetic stirrer of Remi India Ltd. and pH meter manufactured by Toshniwal Instruments accuracy 0.01+1digit were used.

PAT test was performed as per the method described in USP 24. Kapardika bhasma250 mg was stirred with 40 ml water using magnetic stirrer for 1 minute at rpm 300± 1 to make a suspension. Then 10 ml of 0.5 N HCl was added to it, with continued stirring for 10 minutes further. The final pH of the solution was measured.

ANC test was conducted as per USP 23. Here 250 mg of Kapardika bhasma was mixed with 70 ml of water by continuous stirring for 1 minute at rpm 300 ± 1, using magnetic stirrer. To this mixture, 30 ml of 1 N HCL was added while stirring continued for 15 minutes. Within exactly 5 minutes the mixture was titrated. Excess of HCL was back-titrated using 0.5 N NaOH to attain a stable pH of 3.5. For the experiment solution temperature was maintained at 37^0C ± 1^0C. Acid neutralization capacity was calculated as 1 ml of 1N HCl is equal to 1 m.Eq. of consumed Acid.

RRT - 250 mg Kapardika bhasma mixed with 10 ml of water was taken in a beaker and 30 ml of 0.1 N HCl and 70 ml of distilled water was added with continuous stirring. When the temperature was maintained at 37^0 C, 0.1 N HCl was added to it at a rate of 4 ml/minute usinga burette with continuous monitoring of pH.

Reheis test: Measures the time taken by the antacid to partly neutralize the given amount of acid. 250 mg Kapardika bhasma was added to 100ml of 0.1N HCL at 37.5^0c and shaken. The time taken to reach 3.5 pH was recorded.

Results and Analysis

Manufacturing method:

Shodhana():After detoxification by boiling, the surface texture of cowries changed uniformly from smooth to rough in lemon juice, buttermilk and rice vinegar. In the remaining two groups it retained its smoothness. In the same three media the colour turned to opaque milky white while in horse gram decoction a faint reddish tinge was observed on the original yellow(Fig.2a). In immersion

process cowries showed slight roughness, like a thin powdered layer, much less as compared to the one boiled in lemon juice. Bulk weight of all five groups remain unchanged. Percent loss was recorded as nil.

Marana(Incineration process):The time taken to reach maximum temperature in *Gajaputa* (T*max*) was 30 minutes. Active heating phase of temperature range 700-950-700^{0}c was of 180 min followed by annealing phase of about 12 hours (Fig.4). At the time of lifting the *sharavsamputa* from the pit, temperature was 40^{0}c which was near to the atmospheric temperature. Total duration of puta process was of 15-16 hours.

Electric Furnace was preset to match the *Gajaputa* process hence similar observations were recorded, except the time span for annealing was reduced by about 4-5 hours.

In the third process using goat pellets the temperature was stable in the range of 500-596-500^{0}c for about 90 minutes and later on dropped gradually.At the time of taking out the *sharavsamputas*, the temperature was 40^{0}c. Total time was 15-16 hours.

Percent loss of raw material to finished product, including boiling/immersion, trituration, and puta process is calculated as 8 to 15.5 percent for GP and 4 to 5.25 percent for MF

AyurvedicBhasma Tests and physicochemical analysis:

All the 15 bhasma samples were tested on Ayurvedic parameters listed in table 2.Out of these, color and no burning sensation to buccal mucosa upon tasting are specific to cowrie and other marine shell bhasmas. The samples from Gajaputa process and Electric Furnace process fulfilled all the Ayurvedic parameters and did not show significant difference amongst and within the groups.

The bhasma samples A3, B3, C3, D3 and E3 processed using goat pellets turned out to be of grey color and half burned. Upon trituration also they failed to fulfill above mentioned criteria. Thus, they were discarded from the further study.

The 10 samples that complied with Ayurvedic parameters also gave similar values for physical tests (Table 3) which confirms the process standardization irrespective of processing materials (*shodhandravyas*).

Calcium percentage /amount:

Ca^{++} amount in the samples processed in muffle furnaceis higher as compared to those processed in Gajaputa. Similarly, trituration with Aloe vera juice does not affect the values in both the samples E1(GP) and E2 (MF).

3.3Toxicity studies:

3.3.1

Appearance and behavior: The animals of any of the test groups did not exhibit any significant change with peroral administration of the Bhasmas for 14 days and also for 28 days. There was no observable change in skin, fur, eyes, lacrimation, piloerection, pupil size, respiratory pattern, posture, gait or presence of clonic or tonic movements, excessive grooming, repetitive circling and self-mutilation and walking backwards. The appearance was similar to that of the control and sugar solution control group animals. No animal was dead till the end of the experiment.

There was no observable change in food consumption. There was steady increase in body weight.

The fecal matter i.e. pellets of animals from Kapardika bhasma treated groups were reduced in individual pellet size and also were very dry as compared to that of the control group. This observation was common amongst and within the groups under study.

On gross necropsy no congestion was seen in any of the organs.

3.3.2 Histopathology findings:

There was no congestion on gross necropsy in any of the animals after 28 days dosing. Histopathology examination showed following changes:

The liver showed 'cloudy changes' prominent in Kupffer cells in group A2while periportal infiltration of polymorphs was observed in groups A1 and C2 respectively. No significant change is observed in remaining 7 study groups as regards to liver. (Fig 5/1a,b, c)

The Kidney showed congestion in three groups viz. A2, C2, C1 while in other groups no significant change was observed. (Fig 5/2a,b)

The inflammatory cell infiltration in lamina propria of the intestine is observed in groups A1, D1, E2. Here also other groups reveal no change.(Fig5/3a, b)

In groups B1, B2, D2 and E1 none of the organs showed any morphological change. In remaining groups except the 3 organs none other showed any morphological change.

3.4 In vitro Antacid Activity

Results of the 4 tests are presented in table number 5.

Discussion:

The study was aimed at finding out astandard and replicable method of manufacture of Kapardika bhasma which will reduce batch to batch variation, be full proof by complying with Ayurvedic bhasma testing parameters coupled with physical tests as per Ayurvedic pharmacopoeia, Calcium content per 100 gm and economical. It was also the objective that the bhasma must be safe or

nontoxic to vital organsand effective in GI tract. Above mentioned experiments were conducted to fulfill each objective. Results of each are discussed below.

Validation of *Caracar* variety: The *Cypraeamoneta* L. found in Indo –pacific region shows two phenotypes bearing yellow and white colors with smooth or knobby dorsum sometimes having three or more bands.[15] The description of selection criteria of medicinal cowries, including weight range of each one, from *Rasashastra* text *Rasaratnasamucchaya* befits the variety *Cypraeamoneta*L. In Sanskrit it is labeled as *Charachar (Caracar)*.[16] It should be noted that many times on commercial scale *Cypraea annulus* L. is also used which is similar to the former except for the whitish colour and a thin dark yellow or golden ring on the dorsum. Apparently both the types share the same habitat, belong to the same genus and except for minor difference in external appearance by and large are similar. The paper by Devanathan et.al mentions the variety to be *C. moneta* in the text but picture resembles to *C. annulus*.[5]The starting material used in our study conforms to the ones used in other studies on Kapardika bhasma.[17,18,19] The length and weight of cowries indicate maturity of the animal and thereby ascertain the chemical composition of the procured shell which has implications towards efficacy. Full grown *C. moneta* has protein, carbs and elements Ca, Iron, Mg, Al, Mn, K, Na, Zn and Cu.[20,17] This is the first study to validate the selection criteria documented in Rasashstra.

Probable relevance of Process -

Detoxification / *shodhan*: By definition *shodhan* intends to convert the potentially harmfulsubstance nontoxic to the physiological system. Though calcium is physiologically essential element, safety of its compounds is very important owing to the Calcium-Alkali syndrome.[4]*Shodhan* implies to increase toxicity threshold of the compound by way of organic processing of the elements, which stands true for natural calcium carbonate in marine cowries.

The roughness of cowrie surfaces confirms the corrosive effect of *amla* rasa (acidic effect) of the media in A, B and C lots where external heatal so contributes. But the corrosion does not change bulk weight of the cowrie lot which may mean that external smooth layer of cowries reacts with various constituents from lemon juice, buttermilk and rice vinegar to form some organic complexes. Or some of them might get adsorbed on cowries as against dissolution of thin layer of cowrie calcium (carbonate) in the acidic media whereby the loss is compensated. But the fact that immersion in lemon juice for 12 hours does not corrode the surface while horse gram decoction imparts its color to the cowries, gives us every reason to propose that the rationale of *shodhan* process maybe beyond physical change. This particular process maybe the first step of successive biochemical change in the form of cowrie calcium.

It is common knowledge that the calcium carbonate disintegrates into calcium and $CO_2$when treated with citric acid (lemon), lactic acid (buttermilk) and acetic acid (rice vinegar).It is also possible that calcium citrate, calcium lactate and calcium acetate are formed as intermediates.

Besides the three ingredients are rich in mixture of four main organic acids citric, lactic, acetic and succinic in different proportions. Lemon predominantly has citric acid, vitamins A, C, B group and minerals Fe, Cu, Zn K and P with trace amounts of Mg and Mn.[21]Buttermilk is rich in lactic and acetic with small amount of pyruvic acids, lactobacilli and the yeast saccharomyces cerevisiae whereas rice vinegar dominates in acetic with small proportion of malic, oxalic, tartaric acids and phenolic group.[22,23]. In case of immersion of cowries in lemon juice the reaction product could be organic hydrate along with $CaCO_3$.[23]

Boiling with decoction of *Dolichosbiflorus* may yield alkaline reaction because it does not contain any of the above organic acids, and pH of the decoction is 7.4 unlike the above three. Further it contains phytochemicals like steroids, tannins, flavonoids, amino acids, saponins and some others which opens up the possibility of attachment of different functional groups to the cowrie calcium[24,25]. The study on Kapardika (Cowrie) bhasma intermediates by Bhagwat M. et al. has reported that different compounds are formed as intermediates having different thermal behavior and skipping the *shodhan* process yields calcium oxide.[18]These findings confirm that *shodhan* process leads development of specific molecule and cannot be omitted.

The Kapardika bhasma prepared by Pawale S.et al. have adopted boiling in lemon juice; Garde D.et al. used boiling in rice vinegar; Devanathan R.et al. have used decoction of *Dolichosbiflorus* for boiling.There is uniformity in all the studies regarding proportion of processing drugs to cowries and duration of boiling. Balmurugan S. etal. and Bhagwat M. etal have adopted the process of immersion in lemon juice where the duration differs considerably from 4 hours (Bhagwat M. etal.), to 24 hrs (Balamurugan S. etal.). We have maintained it at 12 hrs owing to local tradition. No one has used buttermilk for *shodhan* of cowries except us.

There is every possibility that the intermediate molecules are complexes having different functional groups which are further developed after trituration and heating to make bhasma which needs to be substantiated experimentally.

Calcination/ Bhasma making or *maran*: Maran process has two steps trituration (*bhavana*) and heating. The process is the source of variation in product standardization owing to no control over trituration intricacies, ambient temperature during process, uniformity of fuel, measurement of actual heating duration and human error.All the studies referred by us for discussion show uniformity in method such as first *Gajaputa* without trituration of *shuddha* cowries to obtain fine powder followed by 2 times subsequent trituration of Aloe vera juice and 2 *Gajaputa*. The proportion of juice to cowries and trituration duration is comparable in all studies including ours owing to the endpoint ‘thick paste convertible into pellet’. The maximum temperature recorded in our study is 950 whereas the other studies report it to be 860 and 1020 and time taken to attain it, is 30 min in all the studies. This similarity supported the temperature range we chose for MF. Rapid rise of temperature as against very gradual annealing is the salient feature of traditional *Gajaputa* process which was successfully mimicked for MF in our study.

We followed only one puta without trituration for samples A, B, C and D as mentioned in Rasashastra texts. For sample E we used one trituration followed by the second puta to compare theoretical text against practical experience for the purpose of validation. Predictable reaction of $CaCO_3$ in presence of oxygen, upon open burning of cowries using cow dung,prevented us from adding one more group to our study. Instead we could successfully use electric muffle furnace for minimizing all the processing errors and bring objectivity to monitor temperature range and duration.

Some studies have reported hydroxide impurities in bhasmas prepared in MF. The porosity of earthen *sharavsamputa* used in *Gajaputa* prevents formation of hydroxide by allowing the passage of CO_2 formed during decomposition of $CaCO_3$; which may not have happened in MF due to porcelain *sharavsamput*. However, this obstacle was overcome by replacing those by earthen *sharavas*. Traditional method can be suitably replaced by MF as it is easier to apply, standardized and quality of bhasma is similar to the one prepared using traditional method as tested using Ayurvedic parameters.

This study shows that percent loss in the yield of Kapardika bhasma obtained from Gajaputa method is slightly more than that of the MF.Aloe vera juice trituration compensates weight loss and also makes the bhasma assimilable. Specific test of non-burning buccal mucosa or tongue is indication of the process of not turning into calcium hydroxide. It could be the reason why the E1 and E2 did not show any undesired changes in histopathology.

It is possible to map the transformation of calcium carbonate of the cowrie shells from aragonite form to calcite; however, we did not attempt to use sophisticated technique for the phase analysis of the Kapardika bhasma in this study. Bhagwat M. et al. have clearly shown using powder XRD and IR techniques that during the process of Kapardika bhasma making, decorbonation of calcium carbonate in aragonite form and its reformation in calcite form takes place.[26] They have used the method of immersion of cowries in dilute lemon juice, trituration with Aloe vera juice for 8 hours followed by lemon juice for 8 hours each time and 3 times calcination in *Gajaputa*; which is slightly different than the process of E1 sample in our study. We used lemon juice without dilution and attempted only one trituration with Aloe vera juice till the paste thickened as practiced by *vaidyas* in our area and did not add lemon juice trituration. Yet the bhasma complied with all test parameters. Our process of E1 significantly reduces processing time and costs.

Characterization:

In our study we adopted the basic and simple tests only for final products which are proved reliable and can be routinely used by individual manufacturers and ayurvedic pharmacies to ascertain completion of process.

However, it would be pertinent to mention that unprocessed C. moneta are reported to have Ca at highest followed by Fe and Al and Na with small amounts of Mn, Mg, K and Zn.[20]. Geochemical analysis of *shuddha* (processed in *D.biflorus*) cowries does not show any variation in main

chemical compounds and parameters like hardness, fracture etc.[5] ICP-OES analysis after immersion for 24 hours in lemon juice shows presence of Ca in highest amount (20 percent), followed by Na, K, P along with Fe (1850 ppm), Cu, Zn Mg and Mn.

Four studies have adopted analytical techniques SEM-EDS or EDAX, IR or FTIR, and powder XRD to study Kapardika bhasma samples self-prepared, procured from a known pharmacy or purchased from market. Particle size tested by simpler methods gives the size of 8.34μm and 9.69± 5.31μm. whereas using powder XRD and calculation by Scherrer equation, the bhasma particle size is calculated as 28-40 nm. SEM- EDS reveals presence of Calcium as main element, along with small amounts of Fe, Cu, Zn, Na, K, P, Mn, Mg. The samples of Kapardika bhasmas are heterogeneous agglomerations of either rod shaped or square particles. FTIR of two samples revealed presence of specific organic groups such as flavonoids, alkaloids, tannins, phenols consistent with *shodhan* and *maran*process; which substantiates our explanation and earlier studies regarding *shodhan a*nd *bhavana* processes. The reason for some differences in amounts of the elements should be attributed to the variation of processing ingredients and methods, which can be standardized for the particular path. Powder XRD has confirmed the nano-crystalline nature of Kapardika bhasma which is CaCO3 in calcite form.[22,27,28,29]

Vedhagiriet al. has reported the presence of Al, a toxic element found in raw cowrie, and also of barium and strontium, in the final bhasma sample unlike others. This clearly means that during process Al must get removed. Its presence co-relates with incomplete (*apakwa)* bhasma. Also retaining of aragonite form of $CACO_3$ as revealed by FTIR and XRDreported in the same study renders the process incomplete and calls for further *putas*. The manufacturing method of the sample is not revealed in this study.

In our study all the 10 samples complied with physicochemical parameters stated in Ayurveda and also standard tests. There is no difference in the values of loss on drying, acid insoluble ash etc. from the values reported in other studies. This again highlights process standardization. In our study pH of Kapardika bhasma samples A1, A2, B1, B2, C1, C2, D1 and D2 is in the range of 10.3 to 10.10 which is comparable to pH values of other studies, in spite of omission of the bhavana step using Aloe vera and two more Gajaputas. However, may be due to the use of undiluted lemon juice and Aloe vera with one more Gajaputa, concurrent to the study by Bhagwat M. et al. the pH of the two samples E1(GP) and E2 (MF) is 7.9 and 7.5 respectively. Further weagree with them that in case of Kapardika bhasma, monitoring of pH can be the simplest way to monitor quality. The similarity of method of preparation, also clarifies that standardization of Kapardika bhasma can be achieved by process specification and simple lab tests and also supports the anecdote ‘practice prevails theory’.

The range of Ca^{+2} part per million in the samples processed in GP is 104 – 109 whereas 150-180 in samples processed in MF. It means that amount of Calcium is better controlled in MF.

The findings of characterization should be viewed critically and comprehensively. On the basis of physical analysis and especially pH values anyone may say that any ingredient for *shodhan* and

bhavana will yield a similar kind of Kapardika bhasma molecule. But the FTIR results of above mentioned studies clearly show that even after trituration for hours together and burning up to 900 degree centigrade the samples contain functional groups corresponding to the ingredients. Also, SEM reveals the difference in the shape of nano particles which are dependent on the herbs used.[30] These findings support the Ayurvedic theory of drug development based on *gunas* of each herbal and animal product and their routing to target organs.

Toxicity

As expected, the bhasma samples A1, A2, B1, B2, C1, C2, D1, D2, E1 and E2 did not affect body weight or food and water consumption of the animals at the selected medium dose, in both the studies. No observable changes were seen on gross necropsy.

Sub-acute toxicity study was conducted in mice model, but we did not perform blood biochemistry to check hematological parameters, hepatic and renal parameters. The cloudy changes seen in Kuffer cells of liver, periportal infiltration by polymorphs, congestion in kidneys and inflammatory cell infiltration in intestinal tissue or any other degenerative changes were not seen in the two samples E1 and E2. These samples were treated with Aloe vera juice which highlight importance of *bhavana* of Aloe vera.[31]

The study by Kumar V. and Singh A. et.al reports no sub-chronic (sub-acute) toxicity of *Kapardika* (cowrie) bhasma at two dose levels, 15 mg and 45mg/per kg body weight in Charles Foster Albino rat model. In this methodically and statistically well-structured study no any toxicity was observed on gross necropsy as well as on hematology, hepatic and renal biochemical parameters and also histopathological findings. The authors have reported that the bhasma was prepared at 750^0c but no details of manufacture or temperature measurement are provided in this study.[32]Except this one, we could find no other toxicity study on Kapardika bhasma.

The methods developed for making Kapardika bhasma, in Ayurveda and Siddha systems are natural rather than chemical. The chemical process of extraction of fibrous cowrie protein from powder of raw shells using petroleum spirit and ether, phosphate buffer saline and citrate buffer reported to be immunogenic and toxic in mice models.[33,34]

Though we have followed the study protocol meticulously, in the absence of proper testing parameters; second rodent model (e.g. rats) and statistical methods our study can only serve as a preliminary study giving a basic impression of each sample. A robust study for each sample is necessary before drawing any negative or positive conclusions.

In vitro Antacid Activity

Since the Kapardika bhasma is administered in various diseases of GI tract including hyperacidity (~ *urdwagaamlapitta*) and ulcerative dyspepsia (~*Parinamshoola*); the very basic evaluation of its antacid activity was a relevant step. Samples E1 (GP) and E2 (MF)processed with lemon juice for *shodhan* and Aloe vera juice for bhavanawere selected based on safety profile.

Any antacid preparation has to comply with preliminary antacid test (PAT) and acid neutralization capacity test (ANC). During PAT the product should raise the pH above 3.5 within 10 minutes. E1 (GP) and E2 (MF)did achieve pH 7.42 and 7.70 respectively at the end of 10 minutes and hence these samples may be labeled as antacids. In the next step,ANCexpressed in m.Eq. of HCL consumed by E1 and E2 were found to be 10.623 m. Eq. and 15.3846 m.Eq. respectively. It means that the total m.Eq. of the acid neutralized by sample E2 in significantly greater than E1. Though both have low ANC, amongst the two E2 is better.

Rosset-Rice test is an in vitro method to evaluate the efficacy of the dose used. The normal acid secretion rate in the stomach is equal to about 4 ml of 0.1N HCL/min. which was followed in this study. During the test, sample E1 achieved pH 3.0 in6 minutes but failed to maintain that level up to one hour which is the Rosset-Rice time.E2 sample reached pH 3.0 within 8 minutes but it also failed to sustain it for one hour. For a good antacid, the level is expected to be maintained for more than an hour and a half. It means for both the samples rate of acid neutralization is significantly slow.

Reheis test is a reaction velocity test indicating time required to raise the pH to 3.0. It is the measurement of the speed of neutralization of acid by the drug. Weaker is the acid neutralizing capacity more is Reheis time. In this test both the samples E1 and E2 could not reachpH 3.0 during given time.

The known antacids aluminum hydroxide and magnesium trisilicate are known to pass all the above tests. Though both samples of Kapardika bhasma cleared PAT and ANC; the noncompliance to RRT and Reheis test show that Kapardika bhasmamay not work similar to the standard known antacids. In similar studies conducted on *Shankha Bhasma*; another marine shell product from Ayurvedic repertoire; for similar dose range results of RRT and Reheis test are negative.[13,35].It means that Ayurvedic products must have a different mode of action other than being antacids.

The pH of Kapardika bhasma prepared by us (10.3- 10.10) and also by others (10.0 -12) is alkaline. The samples E1 (7.9) and E2 (7.5) are also alkaline but near neutral. It clearly means that potential renal acid load (PRAL) of Kapardika bhasma will be negative. The standard samples of Kapardika bhasma should be experimented using other methods and in vivo models to find out mechanism of action in the diseases for which it is used clinically.

Proof of concept for Ayurveda:

Ayurveda has a different world view regarding actions of and interactions between biological systems. Ayurvedic theory of biological or bio-chemical properties is based on *rasa-veerya-vipaka-guna* of any substance. The normal (*prakrit*) and abnormal (*vikrit*) state of human physiology is analyzed on the basis of *gunas*. The common platform of *gunas* of natural substances and human body is the basis of drug development.

Action of a medicine depends on its *rasa veerya vipak* which are dependent on the *gunas*. In that sense, ingredient wise analysis of process of *Kapardika bhasma* can be broadly put into two categories:

a. Cowries of *katu-tikta rasa, ushna veerya and katuvipak* when treated with that of lemon juice or rice vinegar having *amla-ushna-madhura* and further triturated with Aloe vera of *tiktamadhur-sheeta-madhura* resultant bhasma has *tikta, katu, amlamadhurarasa* in descending order, *ushnaveerya andmadhurkatu vipak*. Here *kashay rasa* is absent. This can best work in the diseases *grahani*(~ IBD) and kshay(~ intestinal Koch's) by changing the environment in the target organ. The katu vipak of Kapardika bhasma prepared using this combination was seen on the mice pellets (turned dry and small) in our study.

b. Cowries when treated with buttermilk of *kashay-ushna-madhura* or *kulatthaquathkashay-usna-katu*and triturated with Aloe vera having *tikta,madhur-sheeta-madhura* combination the resultant bhasma has *kashaykatutikta rasas, ushna-sheeta veerya and katu vipak* . Here *amla* and *madhura rasa* are completely absent. Hence this might be more useful in inflammatory conditions like *parinamshul*(~ulcerative dyspepsia) and karnsrav(~otitis media) where specific pharmacological actions *shodhan, lekhan* and *ropan* are expected.

There is a lot of scope to study the Kapardika bhasma prepared using four different pathways of each ingredient lemon juice, sour buttermilk, rice vinegar and decoction of *Dolichosbiflorus* for *shodhan* and *Aloe vera* juice trituration in GP/MF and study its characteristics using sophisticated instruments to reveal complete morphology and composition. It would be interesting to map whether the *shodhan* process makes calcium citrate, calcium lactate, calcium acetate with lemon, buttermilk and rice vinegar and something else with D. *biflorus* and further during *maran* while reforming $CaCO_3$ in calcite form, the metals and minerals with organic functional groups from respective path get attached to it. Such standardized bhasmas should be tested clinically to check precise effects stated earlier.

Figures:

Figure 1:

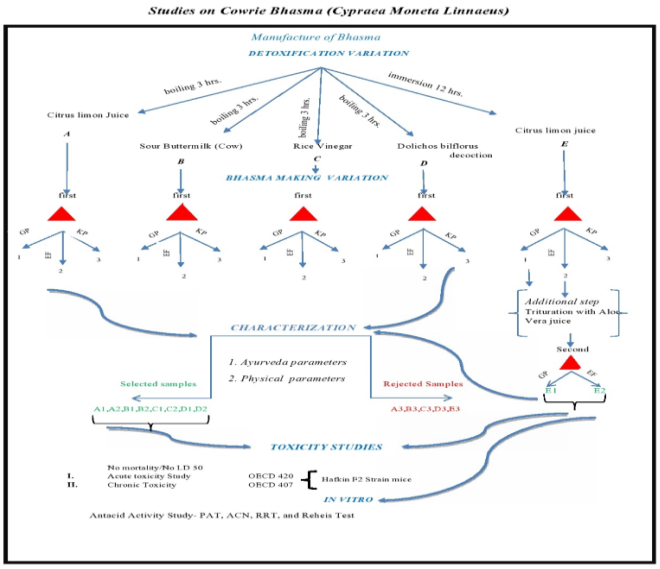


Figure 2:

Figure 2. **Raw and Suddha Cyprae Moneta Cowrie Bhasma Samples**

a. Raw and Suddha Cyprae Moneta; b. Cowrie Bhasma Samples;

Figure 3:

Figure 3: ***Cowrie Bhasma Making Process***

a. Dalayantra; b. Muffel Furnace; c. Sealing of Sharavasamput (Earthen Crucibles) containing Cowries; d. Placement of Earthen Crucibles on Cowdung; e. Gajaputa – Incineration using cowdung cakes f. Gajaputa pit filled with cowdung cakes

Figure 4:

Figure 4.

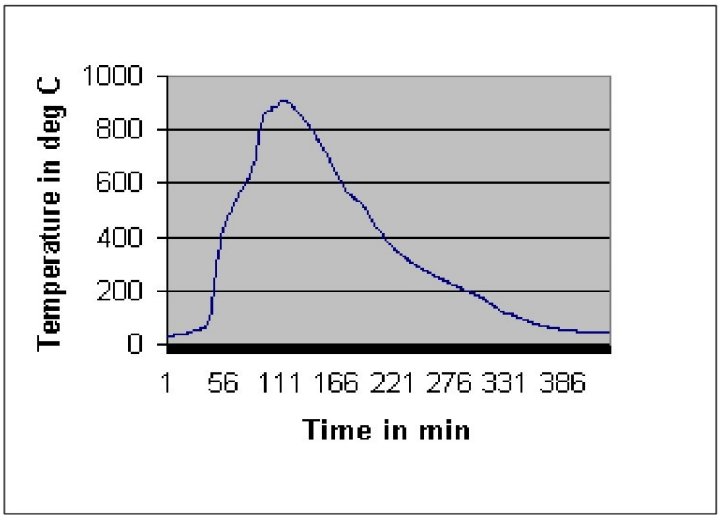
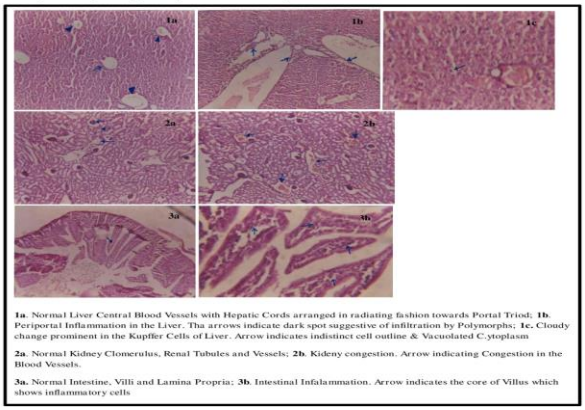


Figure 5:

Fig. 5: Histopathology findings after H&E staining of selected mice tissues



1a. Normal Liver Central Blood Vessels with Hepatic Cords arranged in radiating fashion towards Portal Triod; **1b**. Periportal Inflammation in the Liver. Tha arrows indicate dark spot suggestive of infiltration by Polymorphs; **1c.** Cloudy change prominent in the Kupffer Cells of Liver. Arrow indicates indistinct cell outline & Vacuolated C.ytoplasm

2a. Normal Kidney Clomerulus, Renal Tubules and Vessels; **2b**. Kideny congestion. Arrow indicating Congestion in the Blood Vessels.

3a. Normal Intestine, Villi and Lamina Propria; **3b**. Intestinal Infalammation. Arrow indicates the core of Villus which shows inflammatory cells

Tables:

Table 1: List of herbomineral formulations indicated for GIT containing cowrie bhasma (Supplementary data (not directly related to main objectives))

Sr.No.			
1.			
2.			
3.			

4.	AgnikumarRas (6)	1.B.N.R. 2.R.Rat 3.R.Chi 4.Y.R. 5. R.R.Sam 6.R.Cha 7. R.R.S. 8.Rasendra Chitamani	TridoshajShool - severe abdominal pain Vishuchiuka-cholera Samgrahanee-Inflammatory Bowel disease.
5.	KapardakRas	1.R.Rat.	Raktapitta-Purpura
6.	KamdudhaRas	1.Ras YOG Sagar	Amlapitta-hyperacidity
7.	Krimi KashthanalRas	1.R.S.S.	Krimirog-worm infestation
8.	GrahneeKapatRas (4)	1.R.Chi 2.Y.R 3.R.R.S 4.R.Cha 5.B.Y.T. 6.Ras Kamdhenu 7.B.R.	Grahanee Rog-Irritable Bowel Syndrome
9.	GrahneeKapatoRas	1.R.R.S. 2.B.N.R. 3.V.R.	Samgrahanee
10.	GrahanneKapardPottli	1.R.S.S.	Grahanee Rog
11.	GrahaneeKajKesari	RasRatnaSamuchhaya	Grahanee Rog
12.	PravalPanchmritaRas	1.B.N.R. 2.R.Cha 3.Y.R.	Gulma-abdominal mass
13.	PradarantakLoha	1.R.R.S. 2.R.S.S.	Pradar– vaginal discharge /vaginitis
14.	PranavallabhaRas	1.B.R. 2.R.Chi 3.R.Cha 4.R.R.S	Galganda-hyper thyroid Pleehavridhi-splenomegali Gulma

15.	Panchamrita Pottali Ras	R. Chi	Jwar Atisar Shool Agnimandya Ajeerna-indigestion Prameha - diabetes Vatvyadhi – Diseases included in vata domain
16.	Pottali Ras	RasRatnaSamuchhaya	TridoshajSangrahinee
17.	PleehashardoolRas	1.B.R. 2.R.Chi 3.R.S.S. 4.R.R.S	Pleeha Gulma Udar - ascitis Agnimandya
18.	BhaskarRas	1.R.S.S. 2.B.R.	Agnimandya Ajeerna
19.	YogeshwarRas	1.R.Chi 2.R.S.S 3.R.R.S	Pramena
20.	MrigankRas	1.R.Rat	Rajyakshma
21.	Raj MrigankRas	1.B.N.R. 2.R.Rat 3.R.Chi 4.Y.R. 5.R.R.Sam 6.R.Cha 7.R.R.S 8.R.S.S. 9.Sharangdhar 10.Rasendra Chitamani	Rajyakshma
22.	LavangadiVati	R.S.S.	Agnimandya
23.	Loknath Pottali	B.N.R.	Kasa-cough/ brhonchiatis

24.	Loknath Pottali Ras	RasKamdhenu	Prameha
25.	LoknathRas (8)	YogChitamani	Grahani
26.	LoknathRas	1.B.N.R. 2.R.Cha 3.Y.Chi 4.Sharangdhar Sanhita	Rajyakshma
27.	BrihadLoknathRas	1.B.N.R. 2.R.Cha 3.R.R.S. 4.R.S.S.	Pleehavridhhi
28.	LaghuLoknathRas	Sharangdhar Sanhita	Kshayavyadhi
29.	LoknathRas	1.B.R. 2.R.S.S. 3.R.Cha 4.R.R.S 5. DhanvantariNighantu	Udar
30.	LaghuLokeshwarRas	R.Rat 2.B.R. 3.R.S.S. 4.R.R.S 5.R.R.Sam	Atisar
31.	LoknathRas	R Chi	Sangrahani
32.	Lokeshwar Pottali Ras	1.R.R.S. 2.R.Cha 3.R.S.S 4.R.R.Sam	Kshaya
33.	LokeshwarRatnagarbha Pottali Ras	DhanvantariNighantu	Rajyakshma
34.	LokeshwarRas (2)	1.R.R.Sam 2.R.R.S	Atisar
		1.R.S.S. 2.R.R.S. 3.B.Y.T. 4. Y.R. 5.R.Chi 6.D.N.	Kshaya
35.	Loban Satva Yoga	1.Ras Cha. 2.R.R.S.	Shwas
36.	HootashanRas	1.R.R.S 2.B.Y.T. 3.Bhav Prakash R.Rat 3.R.Chi	Jwar, Gulma, Aruchee,Shool,Ajeerna
		1.R.Chi 2.Yog Ratnakar	
37.	Hiranyagarbha Pottali Ras	1.B.R. 2.R.R.S. 3.R.Cha. 4.R.S.S.	Grahani

38.	Hemagarbha Pottali Ras (4)	1.R.Cha 2. R.S.S. 3.D. N. 4.R.R.S. 5.R.R.Sam. 1.B.N.R. 1.Rasasar 1.Sharangdhar Samhita 2.Rasprakash Sudhakar 3.Raskamdhenu	Rajyakshma Kshaya Kshaya Sammgrahani, JwarKshaya
39.	HemaMrigankRas	Bharat Bhaishajya Ratnakar	Shoth, Udhar, Arsh, Grahanee, Jwara, Gulma
40.	Chandra SooryatmakaRas	1.B.R. 2. R.S.S. 3.D.N. 4.R.R.S.	Pandoo-Anaemia
41.	GrahaneeKapatPanchanan	1.Raskamdhenu	Grahanee

Table 2: Ayurvedic parameters for testing of bhasma. (* indicates specifically important tests for Samudrapanchaka or Sudhavarga drugs.)

Sr. no.	Ayurvedic parameters	Expected characteristic of Bhasma
1.	Grittiness (shabda)*	Absent
2.	Texture (sparsha)	Fine and smooth
3.	Color (roop)*	White
4.	Taste (rasa)*	No specific taste or burning sensation in mouth.
5.	Odor (gandha)	Odorless
6.	Flame test	Burns with no Smoke
7.	*Rekhapurnatva*	Lodges in finger lines

8.	*Varitaratva*	Floats on water
9.	*Unam/Uttam*	Floats on water with a rice grain

Table 3:Physical parameters of the samples

Sr. no	Sample code	Solubility in dilHCl	Total Ash (%)	Water insoluble Ash (%)	Acid insoluble Ash (%)	Loss on Drying (%)	pH
1.	A1	+	2.06	3.6	122	0.6	10.7
2.	A2	+	2.05	3.6	1.2	0.6	10.8
3.	B1	+	2.06	3.8	1.25	0.58	10.5
4.	B2	+	2.06	3.9	1.26	0.58	10.5
5.	C1	+	2.05	3.9	1.2	0.48	10.8
6.	C2	+	2.05	3.9	1.2	0.46	10.10
7.	D1	+	2.06	3.8	1.27	0.65	10.3
8.	D2	+	2.06	3.8	1.27	0.65	10. 4
9.	E1	+	2.06	3.7	1.2	0.6	7.9
10.	E2	+	2.06	3.8	1.27	0.6	7.5

Table 4: **Calcium from the Cowrie bhasma in PPM measured by flame photometry.**

Sample Code	A1	A2	B1	B2	C1	C2	D1	D2	E1	E2
Ca^{++} ppm	108	163	108	173	109	170	109	150	104	180

Table 5: Results of in-vitro antacid activity tests

Sr. no	Product Dose 250 mg	PAT Average pH	ANC Total mEq.	RRT Time required to reach pH 3	Reheis Test
10.	Cowrie bhasma E1	7.42	10.623 ± 0.2733	6 min	pH < 3
11.	Cowrie bhasma E2	7.70	15.3846 ± 1.1421	8 min	pH < 3

Conclusion:

From this study following conclusions are dawn:

5.1 Synthesis: Kapardika bhasma prepared using *Charachar* (C′arac′ar) i. e. *Cypraeamoneta* L. variety of cowries mentioned in *Rasashastra*,; by meticulously following manufacture process yields a standard drug. *Shodhan* is a very important step which cannot be skipped. Traditional, yet non textual method of trituration with Aloe vera juice adds to the quality of the final product which is proven by near neutral pH of the two samples and no adverse effect on tissues.Traditional *Gajaputa* method can be successfully mimicked by using electric muffle furnace with earthen *shravsamputa* to control batch to batch variation of the products.

5.2 Characterization: All the samples complied with standard ayurvedic and physical parameters, yet the samples E1 and E2, processed in lemon juice and triturated with Aloe vera juice with one more heating cycle matched best.

5.3 Toxicity: Some of the samples showed inflammatory lesions in the animal tissues except the E1 and E2.

5.4 Antacid activity: The two samples E1 and E2 maybe labeled as antacid but did not comply with the standard antacid tests viz. PAT, ANC, RRT and Reheis test.

Notes:

Following standard procedures are practiced in Ayurvedic pharmacies to make liquid media**.**

Lemon juice: Fresh yellow lemons were squeezed using mechanical squeezer. The expressed juice was used for boiling. In this study amount of lemon juice was four ltrs.

Buttermilk: Three liters of boiled and fat separated cow milk was inoculated with 60 gm culture of cow curd and allowed to settle for 6 hours. It was continued to ferment further. After 30 more

hours, it turned sour with specific smell when it was thoroughly churned with addition of one liter of potable water and was allowed to mature for another 12 hours till it became very sour. It was homogenized and used for boiling. The total time was 48 hours.(Bhavprakash, purvakhanda, chapter 6 and pharmacy practice)

Rice Vinegar: The Vinegar (Kanjika) was prepared from 500 gm. of rice flour. The flour was thoroughly mixed with 5 liters of potable water. To the mixture 500 gm. rock salt, 500 gm. powder of mustard (*Brassica juncea*, L.) and 1 ltr horse gram decoction was added. The mixture was fermented in cool dry place in a porcelain jar (mouth covered with cotton cloth) for three days (72hrs). At the end of fermentation supernatant clear liquid was siphoned. Four liters were used for boiling the cowries. (Rasayansar, page 59 and Sharangdhar Samhita, chapter 10, verse 12)

Horse gram decoction: One kg of *Kulattha*seeds (*Dolichosbiflorus*) was coarsely pulverized. This powder was soaked in 16 liters of potable water for an hour. The mixture was boiled on medium flame until 4 liters remained in the pot. It was filtered through muslin cloth and used in *dolayantra* for boiling. (Sharangdar Samhita)

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