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Safety and bioactivity studies of *Jasad Bhasma* and its in-process intermediate in Swiss mice

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ABSTRACT

Ethnopharmacological relevance: *Bhasma*, *Ayurvedic* medicinal preparations, are prepared using herbs and minerals on following long iterative procedures. However, industrially mercury and sulphur are more commonly used to prepare *bhasma* from its raw material. The end point of this iterative procedure is mainly judged by the traditional tests specifying physical appearance of the powders. They fail to give better idea about chemical nature of the material. Moreover, the differences in biological activity of final product verses intermediate are not addressed.

Aim of the study: To compare the physicochemical as well as biological properties of the *Jasad bhasma* and its in-process intermediate using modern science methods.

Materials and methods: The *Jasad bhasma* and its in-process intermediate are characterized for their physicochemical properties using electron microscopy, x-ray diffraction and CHNS(O) analysis. The biological effects of both the preparations are then studied. The bioaccumulation of zinc, effect on liver antioxidant status, liver and kidney function (by conventional tests as well as SPECT: Single Photon Emission Computed Tomography), effect on blood cells and effect on immune system are studied in mice model, *Swiss albino*. Since *bhasma* is given with an accompaniment (*anupan*), all the bioactivity studies were carried out by administering the preparation with and without *Amala* powder (*Phyllanthus emblica* L., fruit, dry powder) as *anupan*.

Results: The XRD results accompanied with Rietveld analysis indicate that the final *bhasma* is mainly oxide of zinc, whereas the intermediate is mainly sulphide of zinc. The animal studies show that the *bhasma* as well as its intermediate do not lead to any bioaccumulation of zinc in major organs, when administered with and without *anupan*. Both, *bhasma* and intermediate do not cause any deleterious effects on kidney and liver as indicated by blood biochemistry and SPECT studies. However, the intermediate perturbs antioxidant status more and affects the platelet turnover, in comparison with *bhasma*. On 28 day treatment, the *bhasma* treated animals show prominence of T_H1 mediated immune response whereas, intermediate treated animals show prominence of T_H2 mediated immune response.

Conclusion: A set of simple modern microscopy and diffraction techniques can affirmatively identify in-process intermediate from the final preparation. These can be used to decide the end point of long and iterative preparation methods in accordance with modern science practices. The differences in physicochemical properties of particles from the two preparations reflect in their different biological effects. Moreover, the *bhasma* affects several components of biological systems which again in-turn interact with each other, which emphasizes the need of multifaceted studies in this field.

1. Introduction

Jasad bhasma is an *Ayurvedic* herbo-mineral medicinal preparation. It is prepared using metallic zinc as the starting material. Such herbo-mineral preparation called *bhasma*, are prepared using

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several metals and minerals as starting material. The preparation method involves multiple cycles of heat treatment and trituration in presence of herbal juices, or other elements like mercury and sulphur. The final product obtained is fine powder of the oxide or sulphide of starting material. In case of *Jasad bhasma*, metallic zinc is surface cleaned (*Shodhana*) by several cycles of heating and quenching in Til Taila (sesame: *Sesamum indicum* L., seed oil), butter milk, cow urine (*Gomutra*), rice *kanji*, etc. The surface cleaned metal is then slowly converted to its compound during multiple cycles of heat treatment and triturations (*Marana*) in presence of herbal juice(s) like turmeric *Kwatha* (*Curcuma longa* L., root, dry powder, decoction) or mercury and sulphur (Sharma, 2000). The chemical conversion is brought about by multiple cycles of triturations and prolonged heating (*Gajaputa*). Trituration is essentially wet grinding in presence of herbal material like turmeric *Kwatha* or mercury and sulphur mixture for long time in mortar and pestle. The powder obtained from triturations is then subjected to prolonged heat treatment by packing it in clay saucers and placing them in earthen pit with. The cow dung is used as fuel for the heat treatment. The temperatures reached during these heat treatments can be as high 800 °C (Pandit et al., 1999; Wadekar et al., 2006). The final product, in the form of fine powder, is used to treat diabetes and diabetic complication such as diabetic polyuria, retinopathy, wound healing, and anaemia (Nanal, 1929a; b; Shastri, 1979). The *bhasma* is generally given with an accompaniment called *anupan* (Sudarshan, 2005), e.g. *Amala* powder (*Phyllanthus emblica* L., fruit, dry powder), *Amala* juice (*Phyllanthus emblica* L., fruit, juice), *Manjishtha* Powder (*Rubia cordifolia* L., root, dry powder) (Joshi, 2010b). However, before qualifying for the medicinal use, the *bhasma* powder traditionally is subjected to several tests to check the fineness of preparation and chemical conversion of starting material. The tests include 1. *Rekhapurita* – the powder should be very fine so as to fill in the finger crevices, 2. *Varitara* – powder should float on water, 3. *Nishchandika* – absence of metallic lustre etc. (Sheikh, 2004). These tests do not provide any clue about the chemical entity present and purity of the final preparation. The end point of the iterative process is decided by the ancient text referred or by the experience of the manufacturer. While manufacturing such products at large scale of industry it becomes vital to judge the end point more informatively, especially when heavy metals like mercury are used during the preparation. Moreover, the incompletely processed *bhasma* has been regarded as potentially harmful or without desired medicinal properties according to *Ayurveda*.

In recent times there have been several modern science studies taken up to understand these complex preparations with focus on physicochemical and medicinal properties. Previous physicochemical studies of *Jasad bhasma* reveal that the *bhasma* comprises of small fraction of nanoparticles and is incomplete zinc oxide with about 50% oxygen deficiency (Bhowmick et al., 2009b). Early studies to investigate its role in treatment of diabetes indicated the potentiation of Tolbutamide action when it was given together with *Jasad bhasma* to healthy rabbits (Kukarni and Gaitonde, 1962). When given to myopic individuals, the *Jasad bhasma* treatment led to improvement in visual activity of about 42.5% individuals in comparison with control group (Puri et al., 1983). However, these studies do not investigate the fundamental biochemical basis of the *bhasma* action. Studies with in vitro models such as yeast and mammalian cell line have shown that the *bhasma* protects the cellular macromolecules from oxidative damage resulting in prolonged stationary phase (Bhowmick et al., 2008; Pyrgiotakis et al., 2008). More importantly, the nanoparticle fraction from the preparation was found to be the most active one (Bhowmick et al., 2009a). Recently, 3 mg/kg dose of the *bhasma* has shown to lower the blood glucose in diabetic rats when compared with control. Also, the preliminary toxicity studies at 100X dose have shown no toxic effects of the preparation (Umrani and Paknikar, 2011). Few studies try to relate the effect of the *Jasad bhasma* with biochemical role of zinc ions. Prominent evidence was presented by treatment of diabetic Wistar rats by ZnO nanoparticles. The studies show increase in insulin secretion in type 2 diabetic rats treated with ZnO nanoparticles for 28 days resulting in lower fasting blood glucose levels (Umrani and Paknikar, 2013). Also, a systematic review by Jayawardena *et. al*. has shown that the zinc supplementation in type 2 diabetic patients help in reduction of fasting blood glucose, 2 hr post prandial blood glucose, glycated haemoglobin, improvement of lipid parameters and antioxidant status (Jayawardena et al., 2012).

Zinc is one of the most essential micronutrients known to play vital role in catalysis by associating with several enzymes. These proteins are involved in several important functions such as catalysis, gene expression, antioxidant defence, immune system, neuron function and storage and release of hormones (reviewed extensively in Salgueiro et al., 2000; Haase et al., 2008). Expression of several cytokines is linked to zinc status such as, IL-2, INFγ, IL-12, IL-10, TNFα affecting differentiation and functionality of cells from innate as well as adaptive immunity (Prasad, 2008). Zinc deficiency is known to suppress T_H1 response in healthy individuals and hence increases susceptibility to many infectious diseases. Therefore perhaps, zinc supplementation improves disease conditions such as diarrhoea, leprosy, leishmania, and common cold. However, excess zinc is also known to suppress the immunity by having adverse effect on interleukins leading to decrease in T_H1 cell response (Bao et al., 2006). Also, excess of zinc intake may result in copper deficiency by interfering with copper uptake through intestine (reviewed in detail in Maret and Sandstead, 2006). This shows that, though essential, there exists a narrow band where body zinc levels should lie: higher than this, results in deleterious effects likes copper mal-absorption; lower than this may be responsible for zinc deficient conditions like poor immune status. Therefore safety margins for zinc intake are narrow. Generally used zinc therapies use highly soluble salts of Zn such as sulphate, acetate, and gluconate (Haase et al., 2008). Considering the narrow therapeutic zone, the use of less soluble zinc therapies such as zinc oxide shall be more appropriate for long term treatments. Zinc oxide particles shall lead to slow release of soluble zinc in sera through cellular lysosomal stores once absorbed through intestine and blood stream (Umrani and Paknikar, 2013). Hence, study of absorption, accumulation, effect on vital organs and on immune system of these ZnO particles, especially through *Jasad bhasma*, is of immense importance to understand the therapeutic potential illustrated in *Ayurveda*.

The traditional use of *Jasad bhasma* is suggestive of antioxidant and immunomodulatory effects. Here in this article, we have studied its biodistribution in *Swiss albino* mice, effect on antioxidant status of liver, and its effects on liver, kidney and immune system. A comparison has been made between the effects when *bhasma* is given with and without its *anupan*. Considering the long, elaborate and iterative method of *bhasma* preparation we have also studied the effect of its in-process intermediate in similar manner. Owing to the traditional preparation procedure, the final product obtained in case of *bhasma*, varies with respect to composition/ purity and particle size distribution from the starting material as well as in-process intermediates. The use of mercury and sulphur during the preparation further increases the concerns about safety and efficacy of these preparations (Saper et al., 2008). Therefore, understanding the biological and physicochemical properties of in-process intermediates will help not only in order to define the end point of the process more scientifically but also to evaluate the ill-effects of an intermediate/ unfinished product, if any.

2. Materials and methods

2.1. Acquisition of Jasad bhasma and its intermediate

The *bhasma* samples for *Jasad bhasma* and its in-process intermediate (Batch No: P120200142), along with *Amala* powder (batch No.: 1PRMB00453, *Phyllanthus emblica* L., fruit, dry powder) as requested by authors, were received as kind gift from Shree Dhootpapeshwar Limited, Navi Mumbai, India.

Briefly, for *shodhana* row zinc is melt and quenched in *Til Taila* (sesame: *Sesamum indicum* L., seed oil), butter milk, cow urine, rice *kanji*, *Kulattha Kwatha* (*Dolichos biflorus* L., seed, decoction) and *Nirgundi* rasa (*Vitex negundo* L., leaves, juice). The *shodhit* zinc is then converted to the *bhasma* by *marana* cycles in presence of mercury and sulphur. The in-process intermediate for these studies is the product of half way through the *marana* cycles.

2.2. Physicochemical characterization of Jasad bhasma and its intermediate

The *Jasad bhasma* and its intermediate were studied for microstructure using Transmission Electron Microscopy, crystalline phase identification using X-ray Diffraction, and sulphur content using CHNS(O) analyser.

2.2.1. Particle characterization using Transmission Electron Microscopy (TEM)

Bright field images, dark field images and electron diffraction patterns for dry powders of *Jasad bhasma* and its intermediate were obtained using JEOL JEM-2100 operating at 200 kV. The dry powders were sprinkled on to carbon-formvar coated 200 mesh copper grids. The grids were then dehumidified under IR lamps for 5 min and observed in a standard room temperature sample holder.

2.2.2. Crystalline phase identification and quantification using X-ray Diffraction (XRD)

The powder XRD patterns of dry powders of *Jasad bhasma,* and its intermediate were recorded using X'pert Pro (PANalytical). The goniomter configuration used was $\theta/2\theta$, and the sample configuration was flat stage. The radiations used for obtaining diffraction pattern were Cu Kα (generator settings: 30 mA, 40 kV) within the θ range 10º to 90º (scan type: continuous, PSD mode: scanning). To analyse the obtained patterns, peaks were identified by specifying the peak search parameters (all in degree) as: minimum significance - 2.00 (2θ), minimum tip width – 0.01 (2θ), maximum tip width – 1.00 (2θ), peak base width 2.00 (2θ) using software X'Pert High Score Plus 2.1 (2.1.0). The prominent phases present in *bhasma* samples were recognized by matching the identified peaks with data base using the software. The preliminary quantitative analysis of different phases present is done using Rietveld method (software used: Maud 2.22, open source software by Luca Lutterotti from Department of Materials Engineering and Industrial Technologies, University of Trento, Italy).

2.2.3. CHNS(O) analysis

CHNS(O) analyser (FLASH EA 1112 series, ThermoFinnigan, Italy) was used for the analysis of C, H, N, S, and O content in *Jasad bhasma,* and its intermediate. For analysis of C, H, N, and S the samples were allowed to burn in oxygen supply at temperature 900 ºC in the instrument. The oxidised products of the reaction were then passed through the inbuilt GC system. The amounts of these elements were then estimated by analysing the respective areas under the curve. Oxygen was also analysed in similar manner, except the samples were subjected to pyrolysis in inert atmosphere at 900 ºC for about 10 min. The evolved oxygen from the samples was then passed through the inbuilt GC system.

2.3. Animal experiments

The design of experiment and all protocols for the animal studies were approved by Institutional Animal Ethics Committee (IAEC) at ACTREC (Animal Study Number. 23/2011).6–8 week old *Swiss albino* (CRL) male mice, weighing 25–30 g were acquired and maintained in the animal facility at ACTREC. The animals were grouped as 6 animals per group (total 7 groups) and housed in separate cages. The animals were maintained at 25 ± 2 °C with alternate light and dark cycles of 12 h each and were fed standard pelletized feed. Water was provided ad libitum. The *Jasad bhasma* (JB) and its intermediate (JBI) were administered to the animals orally by gavage. JB and JBI (with or without *anupan*) were given with 1% CMC in water as vehicle. Dry *Amala* powder (AP) was selected as *anupan* for these studies. The dose selected was 24.0 mg/kg body weight as human equivalent dose for mice (Center for Drug Evaluation and Research, 2005). In the groups, in which *anupan* was given along with JB (or JBI), a proportion of 1:2 of *Amala* powder:JB (or JBI) was maintained throughout the dosing regimen. The animals were dosed BID for 28 days. Two control groups were maintained in the study: An untreated control and a vehicle control treated with 1% CMC BID. The animals (three/group) were sacrificed on day 29 and organs namely duodenum, ileum, jejunum, liver, kidney, heart, lung, and bone were collected for monitoring the biodistribution of Zn and Hg. In addition, liver samples were collected for estimation of SOD, GSH and GSSH. These liver samples were stored at -80 °C till further analysis. Blood samples were also collected through retro-orbital puncture for complete blood count (CBC), Liver Function Test (LFT), and Renal Function Test (RFT). Whole blood from the remaining three animals in each group was collected and used for immunological studies. In addition to monitoring the LFT and RFT by biochemical tests, separate SPECT analyses for studying the liver and renal function were also performed on 6 animals (two groups of three animals each). The schematic representation of the experiment plan is given in Fig. 1.

The treatment groups were labelled as follows:

- *Jasad bhasma* (at 12X dose level): JB-12X
- *Jasad bhasma* (at 12X dose level) with *Amala* powder (at 6X dose level): JB-AP-12X
- *Jasad bhasma* intermediate (at 12X dose level): JBI-12X
- *Jasad bhasma* intermediate (at 12X dose level) with *Amala* powder (at 6X dose level): JBI-AP-12X
- *Amala* powder (at 6X dose level): AP-6X
- Control: Ctr.
- Vehicle control: V. Ctr.

2.3.1. Accumulation of Zn and Hg in tissue samples using Inductively Coupled Plasma – Atomic Emission Spectroscopy (ICP-AES)

All the tissues were weighed and wet weight of each tissue was recorded. The tissues were then digested in 1 ml of conc. HNO_3, at 90 °C for 4 h. Tissues were then treated with 30% H_2O_2 (1 ml) to digest the organic matter at 90 °C for 4 h. 1 ml of conc. HNO_3 was again added to the tissues and digestion was carried out at 120 °C. The heating continued for about 16 h (till about 0.5 ml of sample remained in the tubes). MilliQ water was added carefully to the pre-weighed sample tubes so as to make the final weight of the sample solution as 5 g. The solutions thus prepared were analysed on ARCOS from M/s. Spectro, Germany for Zn and Hg at wavelengths 213.856 nm and 184.950 nm respectively.

2.3.2. Analysis of liver SOD levels

The liver samples were thawed and lysed with the help of needled syringe in known volume of protein isolation buffer. The composition of buffer used was as follows: 20 mM of Tris-HCl

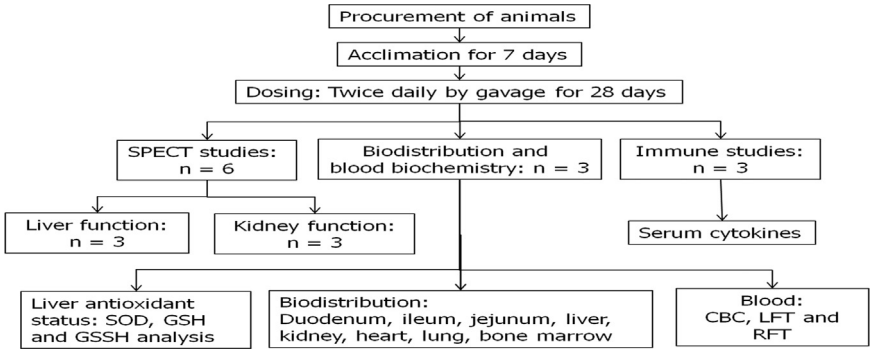

Fig. 1. Schematic representation of the experiment plan. Note: SPECT: Single-Photon Emission Computed Tomography, CBC: Complete Blood Count, LFT: Liver Function Test, RFT: Renal Function Test, SOD: Superoxide Dismutase, GSH: Reduced Glutathione, GSSG: Oxidised Glutathione.

buffer pH 8.0, 100 mMNaCl, 1 mM EDTA, 5 mM 2-Mercaptoethanol, 1 mM PMSF, 1 μg/ml Pepstatin, and 1 μg/ml Leupeptin. The lysed samples were then centrifuged at 12,000 rpm, 4 °C for 15 min. The supernatant was collected for protein estimation by Bradford assay (Bradford, 1976) and SOD assay (SOD Assay Kit by Fluca Analytical, Catalogue no. 19,160). The SOD assay was done as per the manufacturer's protocol. A multiplate reader, SpectraMax M2^e was used for measuring the absorbance at 595 nm in case of Bradford assay and 450 nm for SOD assay.

2.3.3. Analysis of liver Glutathione levels

The liver samples were thawed and lysed with the help of needled syringe in known volume of 5% metaphosphoric acid. The supernatants of the lysed samples were collected for assaying total glutathione on centrifugation at 5000 g for 10 min at 4 °C. The colorimetric assay kit, GT 30 from Oxford Biomedical Research was used for the assay. A multiplate reader, SpectraMax M2^e was used for measuring the absorbance at 412 nm.

2.3.4. Serum Liver and Renal Function Test (LFT-RFT)

The biochemical tests to assess the liver function and kidney function were carried out using an automated Beckman Coulter HMX-AZ model at composite laboratory ACTREC.

2.3.5. Single Photon Emission Computed Tomography (SPECT) for liver and kidney function

The treated and control animals were anesthetized by continuous inhalation of 1–3% Isoflurane-air mixture during administration of radiopharmaceutical and scan. The radiopharmaceuticals $^{99\,m}$TC-Mebrofenin [(2,4,6-trimethyl-3-bromo)-iminodiacetic acid] and $^{99\,m}$TC-DTPA (diethylene-triaminepenta-acetic acid) were used for assessing the hepatobiliary function and renal function respectively. Out of 6 animals maintained per group, 3 animals each were scanned for liver and kidney. 100–350 μCi/75 μl of the corresponding radiopharmaceutical were injected to the mice by tail vein. The animals were scanned immediately after injection of radiopharmaceutical with liver and kidneys in the field of view for their respective functional scans. Dynamic images were obtained for 30 min during each scan. The hepatobiliary scans comprised of 360 frames of 1 frame/5 s. Whereas the renal scans comprised of three phases as following – Phase 1:60 frames of 1 frame/ second, Phase 2:30 frames of 1 frame/2 s, and Phase 3:27 frames of 1 frame/60 s. The HRES- Parallel hole collimator was used for the scans with a matrix size of 80×80 mm^2 and pixel size of 1.5×1.5 mm^2.

The counts for the liver recorded during hepatobiliary scans were normalized to 200 μCi dose (Eq. (1)). To normalize the counts for kidneys, the counts of both left and right kidneys were considered and then the background counts were deducted. The data was normalized to a dose of 200 μCi dose in similar manner (Eq. (2)).

$$\mathbf{NLC} = \frac{(\mathrm{LC} - \mathrm{BC})}{\mathrm{X}} \times 200 \ldots\ldots\ldots \quad (1)$$

$$\mathbf{NKC} = \frac{(\mathrm{LKC} + \mathrm{RKC} - 2\mathrm{BC})}{\mathrm{X}} \times 200 \ldots\ldots\ldots \quad (2)$$

Where, X is the actual dose in μCi of radioactivity injected to the animal during scan, LKC and RKC are the total counts of left kidney and right kidney respectively, BC are the background counts, LC are the liver counts, NKC and NLC are the normalized counts for kidney and liver respectively. The final normalized counts (NLC and NKC) of each animal were processed through a Pharmacokinetic modelling software WinNonlin 6.3 version to calculate organ clearance.

2.3.6. Complete Blood Count (CBC)

Several haematological parameters were determined using fully automated 3 part differential haematology analyser KX-21 model at composite laboratory ACTREC.

2.3.7. Analysis of cytokine levels in serum using Cytometric bead array (CBA)

2.3.7.1. Separation of serum:. Whole blood was collected from test and control animals through retro-orbital puncture. The blood was allowed to clot at room temperature. The serum was collected by centrifugation at 2000 rpm for 10 min at room temperature and the aliquots were stored at −80 °C till further use.

2.3.7.2. Cytokine estimation in sera:. The concentrations of IL-2, IL-4, IL-6, IL-10, IFN-γ, IL-17A, TNF in sera of test and control animals were measured using mouse $T_H1/T_H2/T_H17$ Cytometric Bead Array (BD Bioscience). The standard protocol as specified by the manufacturer was followed. Briefly, test samples, i.e. 50 μl serum, were incubated with the equal volume of suspension of antibody-coated cytokine capture beads for 2 h in dark at room temperature. The solution of phycoerythrin labelled secondary antibody was then added to the samples and were incubated for 30 min in dark at room temperature. All unbound antibodies were washed with Wash buffer. The beads were re-suspended in Wash buffer 300 μl before acquisition on BD FACS Aria Cytometer (BD Bioscience, San JoseCA, USA). Individual cytokine standard curve of the range 20 pg/ml to 5000 pg/ml prepared for each cytokine being assayed. The data generated was analysed using FCAP Array software version 1.0 (BD. Biosciences).

2.3.7.3. Statistical analysis:. The Cytometric Bead Array data, expressed as Mean ± SEM (Standard Error of the Mean), was

analysed using Graph Pad Prism software (version 5.0). The significance of the treatment was tested using unpaired *t*-test (two tailed) with $p \leq 0.05$ as the level of significance with respect to untreated control.

2.3.8. Statistical analysis

All the data were analysed for statistical significance using ANOVA considering inequality of variances (except analysis of cytokine levels). Statistical analytical software SPSS 16.0 was used for the same.

3. Results

We first present results of physicochemical characterization followed by biodistribution and bioactivity studies. The physicochemical characterization studies revealed the different nature of the particles from *bhasma* and its intermediate which reflects in the bioactivity studies.

3.1. Physicochemical characterization of Jasad bhasma and its intermediate

3.1.1. Particle characterization using Transmission Electron Microscopy (TEM)

Both *Jasad bhasma* and its intermediate have particles of irregular shapes and sizes. The particles size ranges from few microns to submicron scale. Small fraction of nanoparticles was also observed in bright field TEM images. The concentric ring pattern was recorded as electron diffraction pattern in both the cases, indicating that the particles are polycrystalline in nature. This was also evident from the dark field images (Fig. 2). However, no difference in particle sizes or shapes in case of final product and its intermediate was observed.

3.1.2. Crystalline phase identification and quantification using X-ray Diffraction (XRD)

X-ray diffraction patterns for *Jasad bhasma* and its intermediate showed peaks pertaining to Zincite (ZnO) (PDF: 00–036-1451), Sphalerite (ZnS) (PDF: 00–005-0566), Wurtzite (ZnS) (PDF: 00–036-1450), and mercury oxide (HgO) (PDF: 00-011-0584). To decipher the contribution of each of these phases in the diffraction patterns, Rietveld analysis was performed (Fig. 3(a) and (b)). In case of *Jasad bhasma*, Zincite (ZnO) was identified as major phase present in the preparation. Hence, it constitutes mainly of zinc oxide (~95.944%) with small amount of sulphide present (~3.742%) as Sphalerite. Though, few XRD peaks matched with Wurtzite, the Rietveld analysis shows that this phase does not contribute significantly to the XRD pattern of the preparation. ~0.313% mercury oxide is also present in the final preparation. Interestingly, Zincite is present in two forms having different unit cell parameters as compared with the reference phase. The details of phase quantification and corresponding unit cell parameters are listed in (Table 1(a)). In case of *Jasad bhasma* intermediate, zinc sulphides, present as Sphalerite and Wurtzite, were identified as major phases (~65.707%) contributing to the XRD pattern of the powder. The analysis indicates that the amount of Sphalerite in the powder is ~39.859% and that of the Wurtzite phase is ~25.848%. ~33.008% Zincite is also present in the intermediate. Moreover, the amount of mercury oxide is ~1.285% (Table 1(b)).

3.1.3. CHNS(O) analysis

The estimation of carbon, hydrogen, nitrogen, and sulphur is done in a CHNS(O) analyser by heating the sample in presence of oxygen at 900 °C. This leads to formation of oxides of these elements, which are further quantified using gas chromatography. Whereas for estimation of oxygen the samples are pyrolysed at 900 °C for 10 min followed by the gas chromatographic analysis of thus evolved oxygen. The CHNS(O) analysis of *Jasad bhasma* and its intermediate (Table 2) confirm the findings by XRD and Rietveld

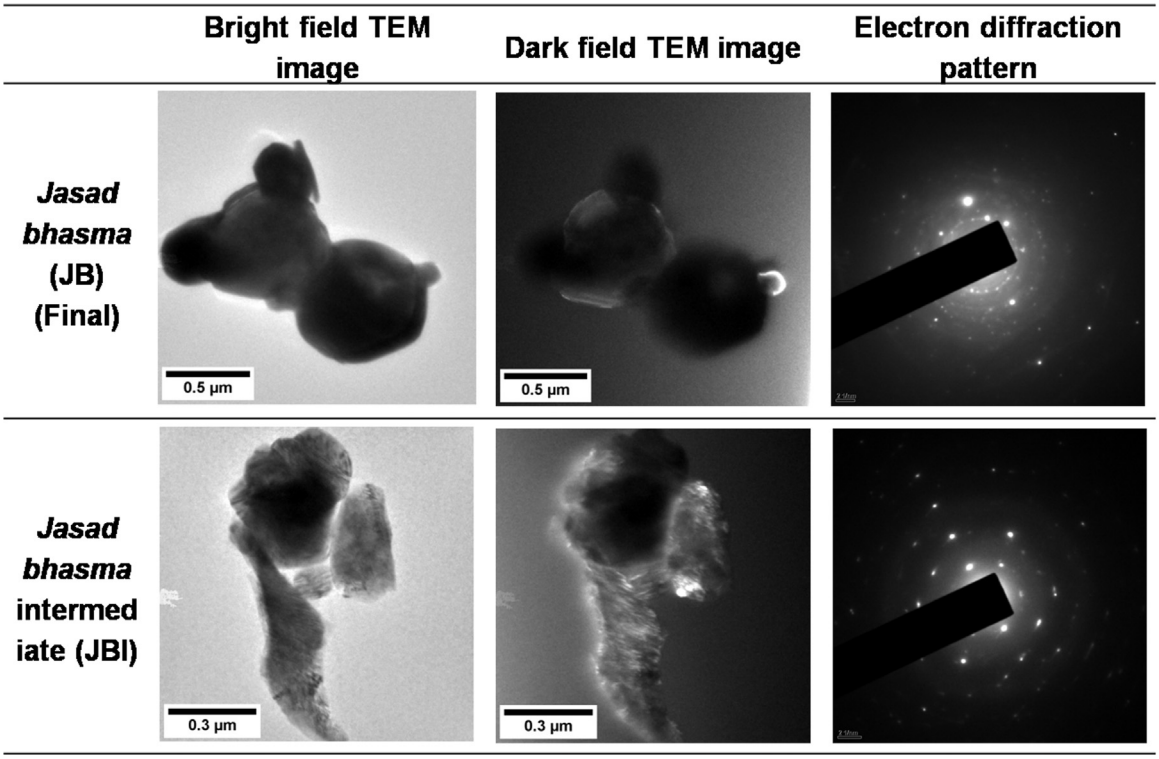

Fig. 2. Characterization of *Jasad bhasma* (final and intermediate) particles using TEM – The images show that the particles of final product as well as the intermediate are of submicron sizes, irregular shapes, and are polycrystalline in nature.

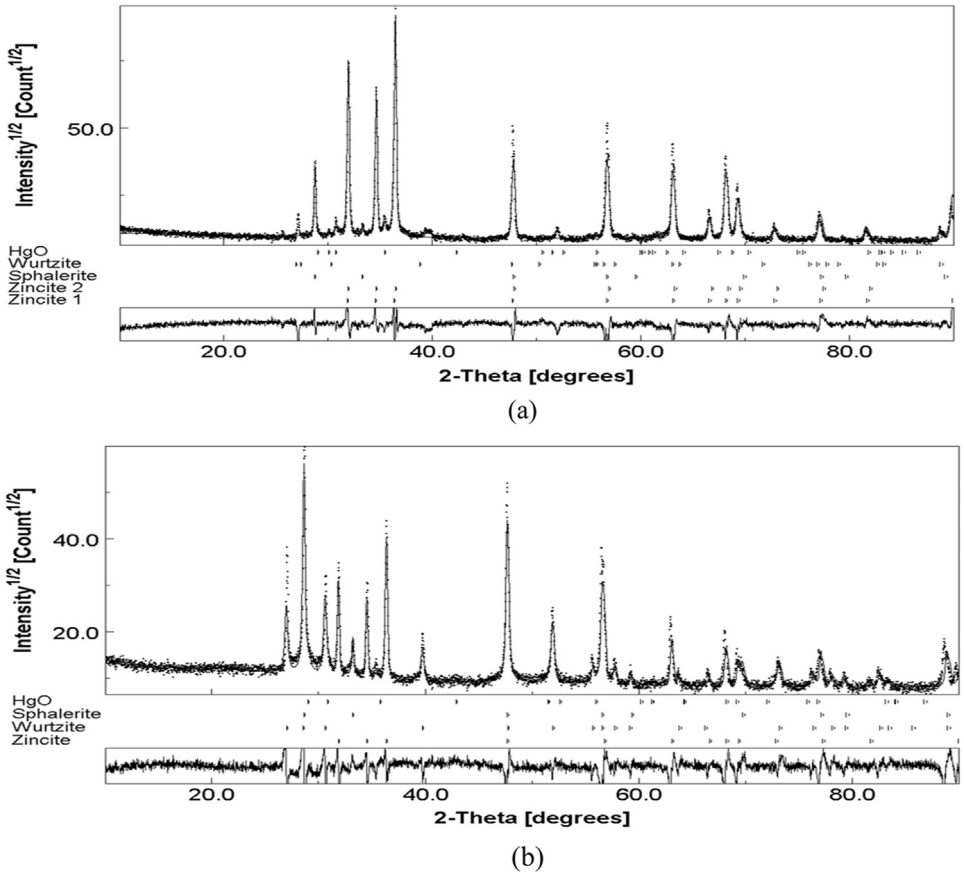

Fig. 3. Phase identification and quantification using XRD and Rietveld analysis. Powder XRD pattern and Rietveld analysis of *Jasad bhasma*(a) and its intermediate (b). The final preparation is ~95.944% ZnO with < 0.5% mercury oxide, whereas the intermediate is ~65.707% ZnS with ~1.285% mercury oxide.

Table 1
(a) Phase quantification and Unit Cell parameters for *Jasad bhasma.*

Sr. No.	Phase Name	Approx. Phase Quantity (wt%) Rietveld	Unit Cell parameters	
			Standard	***Jasad bhasma***
1	Zincite-1 (ZnO)	85.753 ± 4.071	a = b=3.24986 Å c =5.20662 Å	a = b=(3.2326 ± 0.0001) Å c =(5.178 ± 0.0002) Å
2	Zincite-2 (ZnO)	10.191 ± 0.323	a = b=3.24986 Å c =5.20662 Å	a = b=(3.2433 ± 0.0001) Å c =(5.1936 ± 0.0002) Å
3	Sphalerite (ZnS)	3.742 ± 0.160	a = b = c =5.409 Å	a = b = c =(5.3795 ± 0.0004) Å
4	Mercury Oxide (HgO)	0.313 ± 0.035	a = b=3.577 Å c =8.681 Å	a = b=(3.5488 ± 0.0019) Å c =(8.9158 ± 0.0091) Å

Table 1
(b) Phase quantification and Unit Cell parameters for *Jasad bhasma* intermediate.

Sr. No.	Phase Name	Approx. Phase Quantity (wt%) Rietveld	Unit Cell parameters	
			Standard	***Jasad bhasma* intermediate**
1	Zincite (ZnO)	33.008 ± 0.989	a = b=3.24986 Å c =5.20662 Å	a = b=(3.2399 ± 0.0002) Å c =(5.1902 ± 0.0005) Å
2	Sphalerite (ZnS)	39.859 ± 1.034	a = b = c =5.409 Å	a = b = c =(5.3899 ± 0.0002) Å
3	Wurtzite-2 *H*, syn (ZnS)	25.848 ± 0.899	a = b=3.8227 Å c =6.2607 Å	a = b=(3.8089 ± 0.0004) Å c =(6.2405 ± 0.001) Å
4	Mercury Oxide (HgO)	1.285 ± 0.22	a = b=3.577 Å c =8.681 Å	a = b=(3.5474 ± 0.0057) Å c =(8.6928 ± 0.0262) Å

analysis. The final *bhasma* preparation has 3.305% of sulphur whereas the intermediate contains 22.319% of sulphur. This sulphur content in the intermediate corresponds to presence of zinc oxide and zinc sulphide in the ratio 1:2. On the other hand the final preparation is > 90% pure zinc oxide. According to XRD and Rietveld analysis, the final preparation contains ~3.742% of zinc

Table 2
CHNS(O) analysis of *Jasad bhasma* and its intermediate.

	Carbon (wt%)	Hydrogen (wt%)	Nitrogen (wt%)	Sulphur (wt%)	Oxygen (wt%)
Jasad bhasma	0.061	0.000	0.018	3.305	5.589
Jasad bhasma intermediate	0.086	0.002	0.005	22.319	3.870

sulphide. However, CHNS(O) analysis estimates more sulphur content in the preparation than that estimated by XRD. This may correspond to presence of sulphide impurities other than zinc sulphide in minute amount in the preparation, which fail to contribute significantly to the XRD pattern. Moreover, in the specified conditions for estimation of oxygen, complete pyrolysis of zinc oxide to zinc does not happen (Karipidis et al., 2008). Hence, the method fails to estimate the total oxygen present in the final preparation as well as in intermediate.

The results of XRD and CHNS(O) analysis together confirm that during the elaborate method to prepare the *Jasad bhasma* from zinc metal, the metal is converted to its oxide via sulphide. And this conversion is mediated by mercury and sulphur added during the preparation.

3.2. Animal experiments

3.2.1. Accumulation of Zn and Hg in tissue samples using Inductively Coupled Plasma – Atomic Emission Spectroscopy (ICP-AES)

Tissue zinc levels in several tissues of animals treated with *Jasad bhasma* and its intermediate were measured to understand whether or not the zinc distribution within tissues is affected by the treatment. Zinc levels in duodenum, jejunum and ileum after treatment of *Amala* powder, *Jasad bhasma* and its intermediate do not change when compared with the zinc levels from corresponding control and vehicle control animals (Fig. 4). Though the level of zinc in ileum of animals treated with *Jasad bhasma* with *Amala* powder is significantly different than that in case of *Amala* powder and the intermediate treated animals, it is not significantly different than that in case of control and vehicle control animals. The tissue zinc levels in case of liver, kidney, heart and lung do not undergo any change after treatment when compared with the control and vehicle control animals. Moreover, the tissue zinc levels in all the tissues are within the previously reported range of zinc levels in corresponding tissues in mice (Tran et al., 1998; Verbanac et al., 1998). Also, the tissue zinc levels in case of bone did not show any significant change due to treatment. The bone zinc levels reported here are on the basis of wet weight and hence lower than those reported previously on the basic of dry weight (0.2 μg/mg of dry tissue) (Wastney and House, 2008). Importantly the mercury levels in tissues of animals treated with intermediate as well as final *bhasma* were below detection limit of the instrument; i.e. less than ~1 ng/gm of wet tissue. Thus, treatment of mice with the *bhasma* and its intermediate, even for 28 days and at human equivalent dose, does not lead to accumulation of zinc in the tissues.

3.2.2. Assessment of liver antioxidant status

Effect of *bhasma* and its intermediate on antioxidant status of liver has been studied by analysing liver SOD and reduced and oxidised glutathione levels. The SOD activity measured with respect to total protein decreases slightly when compared with control in case of the *bhasma* and its intermediate treated groups. At the same time, a slight increase is seen in treated animals in comparison with control animals when SOD activity is measured with respect to the weight of tissue. The amount of total protein per unit weight of tissue increases in treated groups with respect to control group, especially in case of animals treated with the intermediate alone. However, none of the changes are statistically significant (Fig. 5). This indicates marginal increase in synthesis of proteins in the treated groups; SOD activity per say does not increase. In case of reduced glutathione (GSH) levels, the levels increase in treated groups with respect to control and vehicle control groups. A statistically significant increase with respect to control is seen in case of *bhasma* with *Amala* powder group and also in case of intermediate treated group with respect to vehicle control group (Fig. 6). The oxidised glutathione (GSSG) does not show any such trend and statistically significant difference amongst the study groups.

3.2.3. Serum Liver and Renal Function Test (LFT-RFT)

The treatment of *bhasma* and its intermediate leads to statistically significant effects as seen in case of serum total protein (TP). The *Amala* powder treatment shows significant effect from all other treatments. Though, treatment of intermediate results in

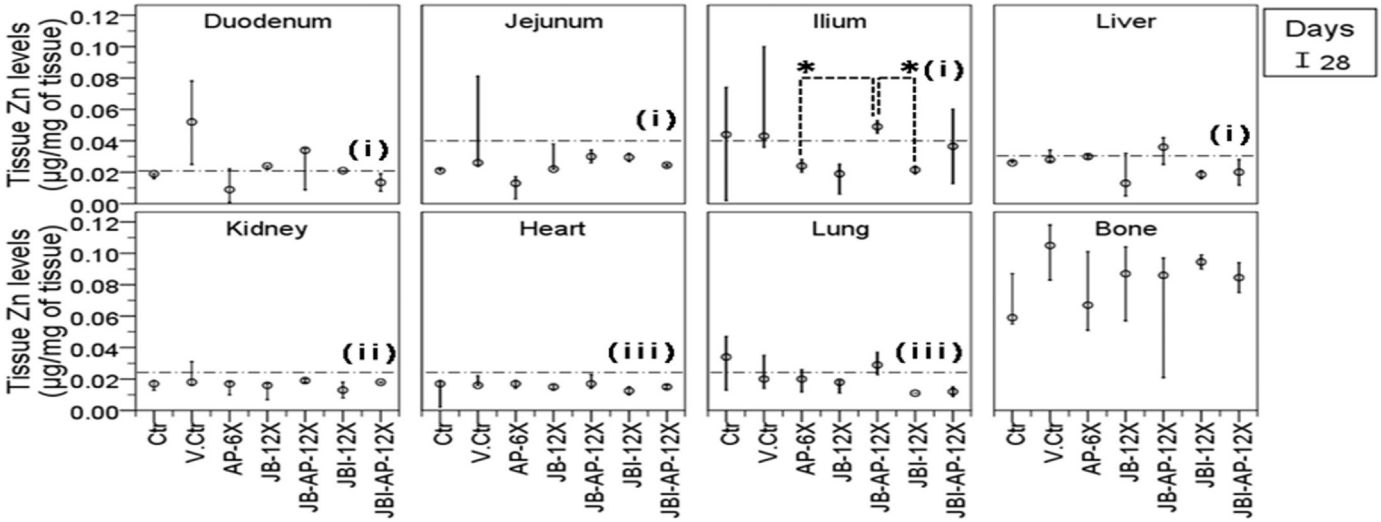

Fig. 4. Tissue Zn levels in organs from control animals and animals treated with *Jasad bhasma* and its intermediate with and without *Amala* powder. The data reported as median with maximum value and minimum value for each group. The data was analysed for statistical significance using ANOVA considering inequality of variances. The dotted line represents previously reported Zn levels in corresponding tissue. (i) Zn levels reported by Tran et al., 1998, (ii) Zn levels reported by Verbanac et al., 1998, (iii) Zn levels approximated to kidney Zn levels reported by Verbanac et al., 1998. In case of bone, no Zn levels on the basis of wet tissue have been reported.

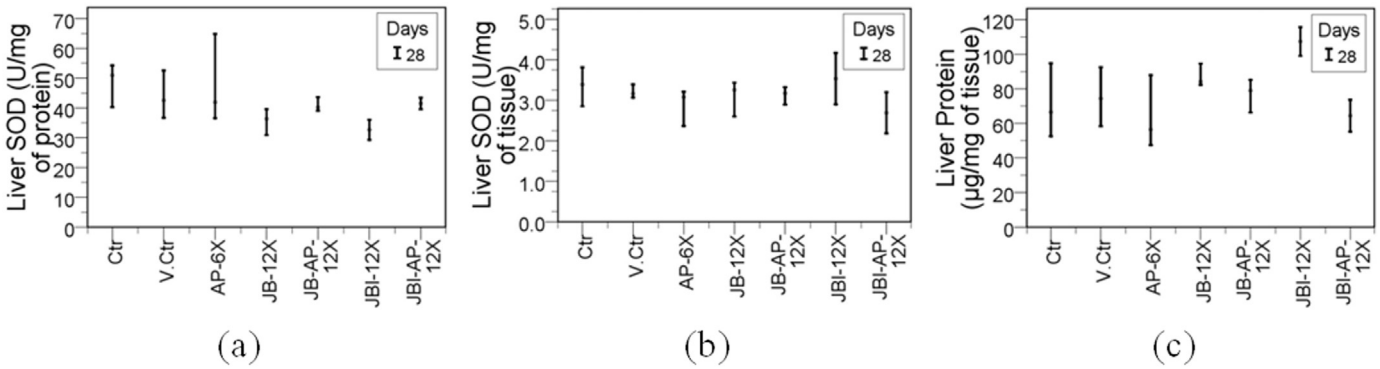

Fig. 5. Effect of Jasad bhasma and its intermediate with and without Amala powder on liver SOD levels – (a) Liver SOD levels per mg of total protein, (b) Liver SOD levels per mg of whole tissue, (c) Liver Protein levels per mg of whole tissue. Data plotted as median with maximum and minimum value for each group. The data was analysed for statistical significance using ANOVA considering inequality of variances.

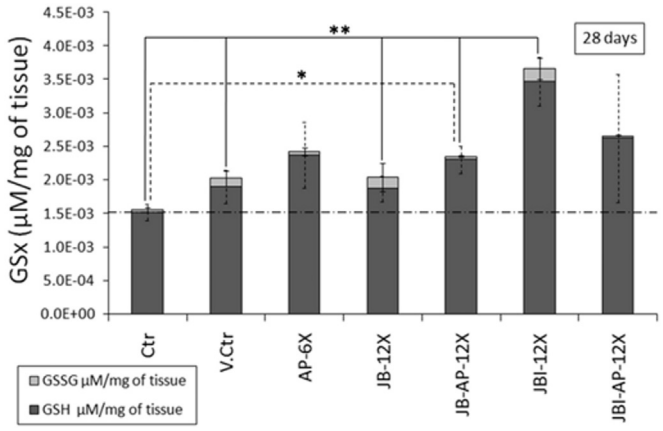

Fig. 6. Effect of *Jasad bhasma* and its intermediate with and without *Amala* powder on liver glutathione levels – Data plotted as mean and error bars represent the standard deviation for each group. The data was analysed for statistical significance using ANOVA after testing the homogeneity of the variance for the groups. The *bhasma* and its intermediate do not have any statistically significant effect on liver GSSG levels, where as the GSH levels are affected by treatments JBAP-12X and JBI-12X. *: p-value < 0.05,**: p-value < 0.001.

statistically significant change in TP with respect to vehicle control group, the change is within the normal range of the parameter (2003). The serum albumin (ALB) levels, in case of all the groups (including control) were found to be below the normal range. The levels of serum ALB in case of groups treated with *bhasma* and its intermediate were lower and statistically significantly different than that of control and vehicle control groups (Fig. 7(b)). However, total protein in liver tissue (Fig. 5(c)) shows no statistical difference. Hence, the reduced serum ALB due to treatment may not be specific to liver status (Thapa and Walia, 2007). The serum alanine transaminase (ALT) levels of groups treated with the *bhasma* with *Amala* powder show statistically significant elevation when compared with the vehicle control group. Similarly, the groups treated with the *bhasma* with *Amala* powder and intermediate alone show significant increase in ALT levels when compared with those treated with *Amala* powder alone. However, no difference is seen with respect to control group in any of the groups (Fig. 7(c)). No statistically significant difference in serum total bilirubin (TBI) levels was seen any of the treated groups when compared with control or vehicle control groups. The TBI levels in case of the intermediate treated groups show increase, though within the normal limits (Fig. 7(d)). This indicates no damage to the liver tissue due to the *bhasma* treatment. In case of the animals treated with the intermediate the GSH levels were found to be high, and hence damage due to oxidative stress was suspected. However, as per the findings of LFT, the treatment did not seem to affect the liver function.

To understand the effect of the treatments, *bhasma* and its intermediate with and without *Amala* powder on kidney function, the RFT parameters blood urea nitrogen (BUN), and serum creatinine (CREAT) were monitored. The treatment of *bhasma* and its intermediate, with and without *Amala* powder has led to increase in BUN levels than the normal range. The *Amala* powder treatment has resulted in significant reduction (within normal range) in BUN levels when compared with vehicle control, the *bhasma* and its intermediate with *Amala* powder. However, the BUN levels in case of control and vehicle control animals are above the normal range (Fig. 7(e)) (2003). Hence these changes may not be treatment specific. However, the key parameter to assess the kidney function, serum creatinine level (Vasudevan et al., 2011), is unaffected by any of the treatments mentioned here (Fig. 7(f)). This indicates no impairment of renal function due to the *bhasma* as well as its intermediate when given with and without *Amala* powder for 28 d to test animals.

3.2.4. SPECT for Liver and Kidney function

The hepatic and renal clearance were studied using SPECT imaging. The hepatic clearance measured in terms of half life of $^{99\,m}$TC-Mebrofenin shows a marked but statistically insignificant decrease with respect to control in case of animals treated with *Amala* powder alone and the *bhasma*, with and without *Amala* powder. The intermediate treated groups, with and without *Amala* powder does not result in any difference in hepatic clearance when compared with control group (Fig. 8(a)). In case of renal function, the half-lives of $^{99\,m}$TC-DTPA were comparable across the groups with no marked difference observed in any group compared to control (Fig. 8(b)).

3.2.5. Complete Blood Count (CBC)

The number of WBC (Fig. 9(a)) and RBC (Fig. 9(c)) was unaffected by the treatments; *Amala* powder, the *bhasma* and its intermediate, given with and without *Amala* powder. The values fall within the normal range (2003). Though, the treatment with *Amala* powder and vehicle has resulted in low WBC count than normal range, the change is not statistically significant with respect to control (Fig. 9(a)). Similarly, lymphocyte percentage of all the treatment groups lies within the normal range, except that in case of the animals treated with the intermediate with *Amala* powder. In this case, the increase in lymphocyte percentage is significant with respect to the lymphocyte percentage in case of animals treated with *Amala* powder alone as well as those treated with the *bhasma* with the *Amala* powder (Fig. 9(b)).

The haemoglobin levels in case of animals treated with the

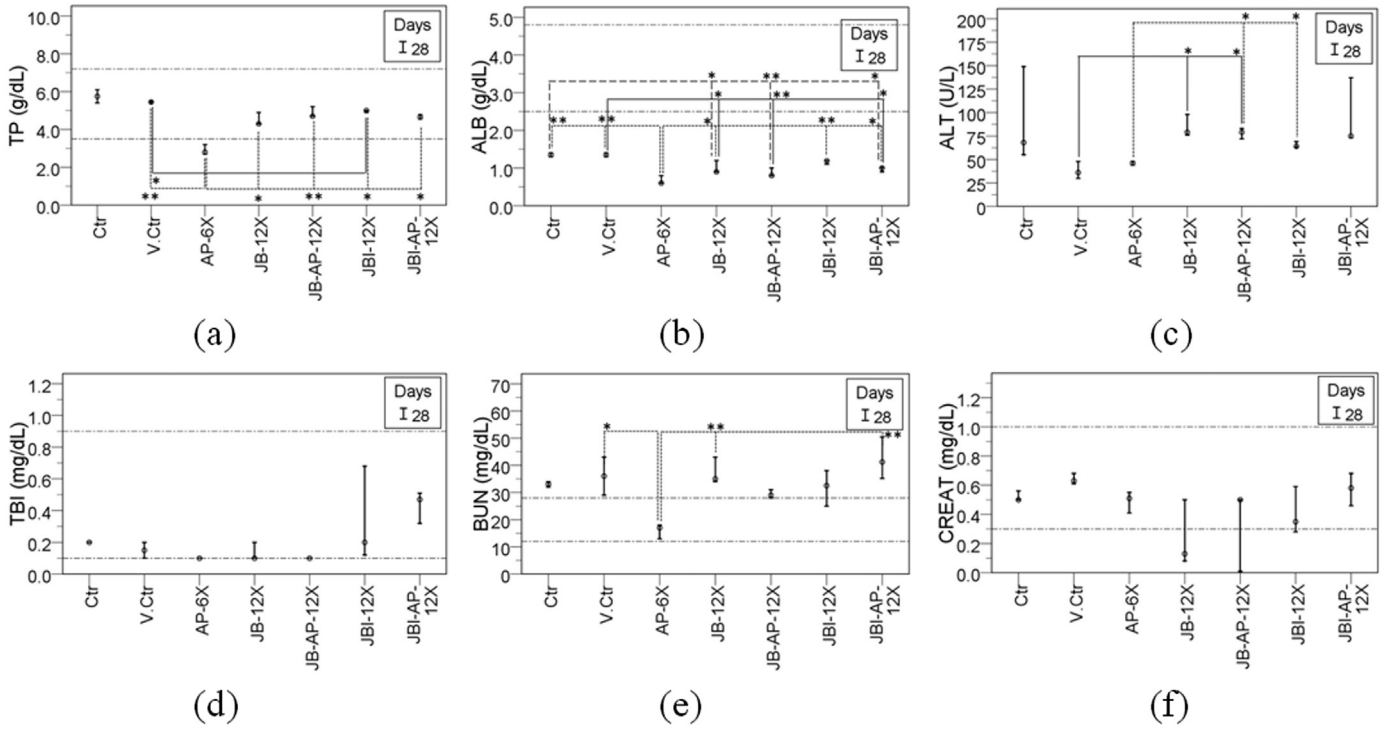

Fig. 7. Effect of *Jasad bhasma* and its intermediate with and without *Amala* powder on Liver Function and Renal Function parameters– Data plotted as median with maximum and minimum value for each group. The data was analysed for statistical significance using ANOVA considering inequality of variances. *: p-value < 0.05,**: p-value < 0.01.

bhasma, and with the intermediate and *Amala* powder show significant reduction in comparison with that in case of control group. However, the levels are within the normal range (Fig. 9(d)). The hematocrit levels in case of *Amala* powder treated animals do not show any change when compared with control animals. The hematocrit levels in case of *bhasma* treated animals, with and without *Amala* powder, show reduction. But this change is not statistically significant when compared with control animals. On the other hand, the animals treated with the intermediate, with and without *Amala* powder, show significant reduction in the hematocrit levels when compared with those in case of control animals (Fig. 9(e)). The increase in mean corpuscular haemoglobin concentration (MCHC) in case of animals treated with the *bhasma*, with and without *Amala* powder is not statistically insignificant. The levels are unaffected in animals treated with *Amala* powder when compared with control. Similarly, the treatment of intermediate alone does not affect the MCHC in significant manner. On the other hand, the treatment of the intermediate with *Amala* powder does lead to significant increase in MCHC when compared with control animals [Fig. 9(f)]. The mean corpuscular volume (MCV) shows significant decrease in case of animals treated with the *bhasma*, with and without *Amala* powder when compared with control and *Amala* powder alone. The treatment of intermediate with and without *Amala* powder, however, does not cause any statistically significant change in comparison with that in case of control animals (Fig. 9(g)).

The platelet count remains unchanged in treated animals (Fig. 9 (h)). However, there is significant reduction in mean platelet

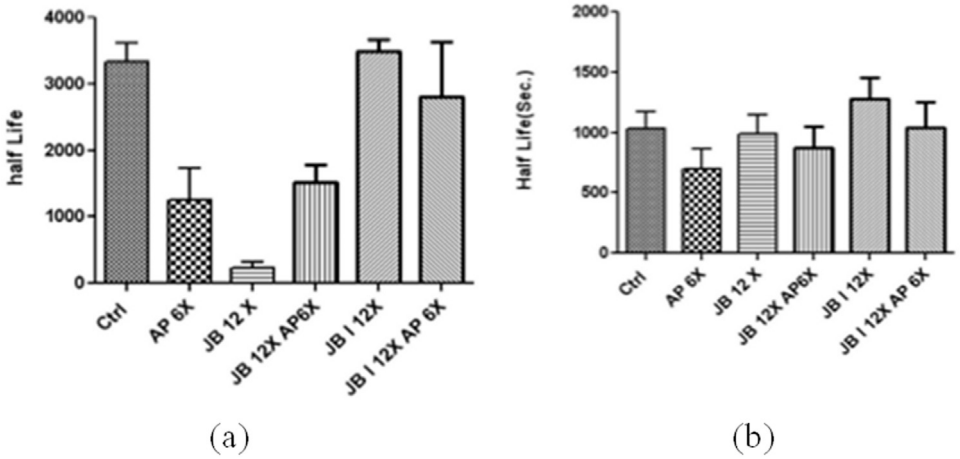

Fig. 8. (a) Hepatic clearance of $^{99\,m}$TC-Mebrofenin and (b) Renal clearance of $^{99\,m}$TC-DTPA by SPECT in animals treated with *Jasad bhasma* and its intermediate with and without *Amala* powder. The data was expressed as Mean ± Standard Error of the Mean. The significance of the treatment was tested using unpaired *t*-test (two tailed) with $p \leq 0.05$ as the level of significance with respect to untreated control.

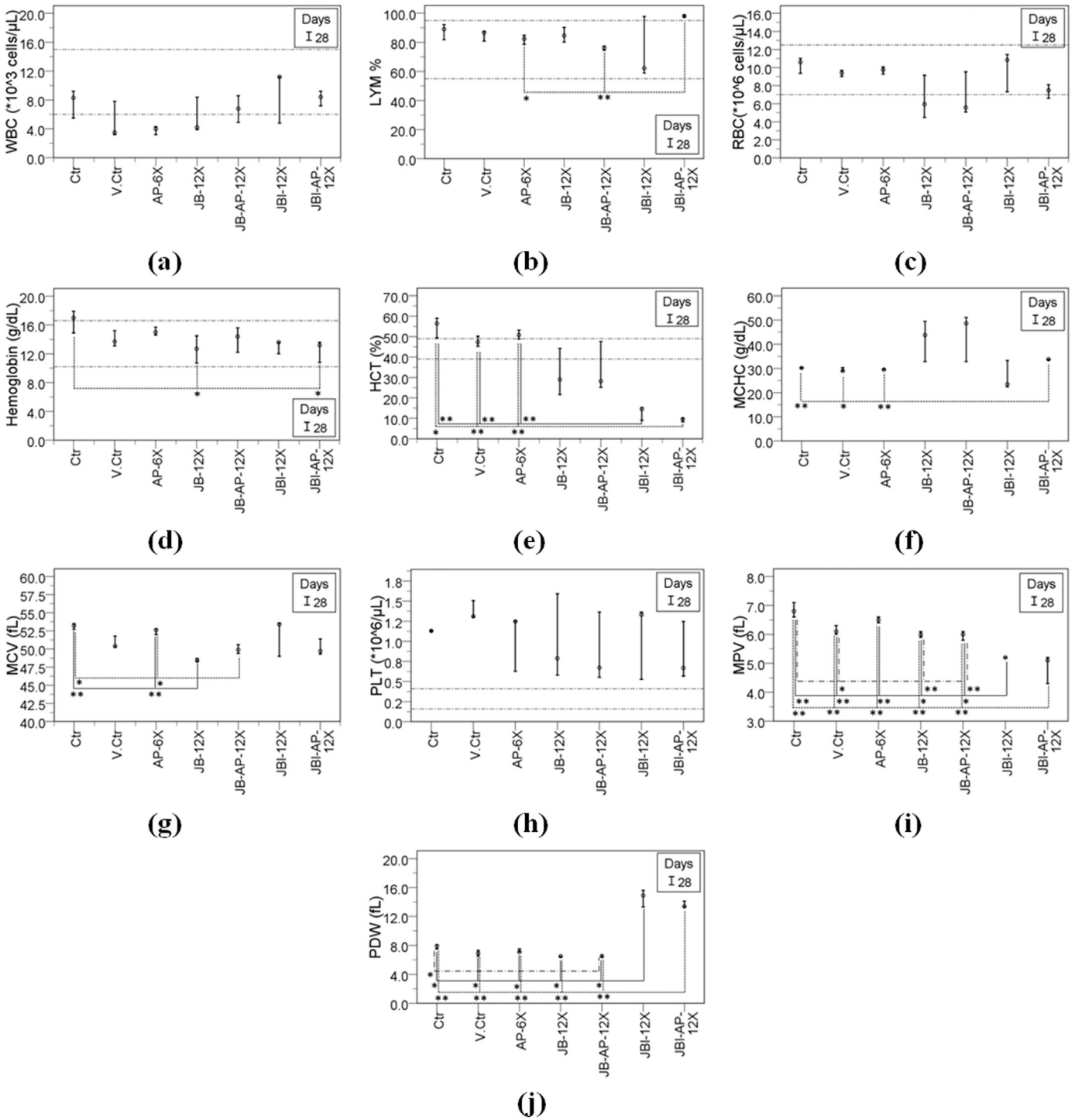

Fig. 9. Complete Blood Count of animals treated with *Jasad bhasma* and its intermediate with and without *Amala* powder– Data plotted as median with maximum and minimum value for each group. The data was analysed for statistical significance using ANOVA considering inequality of variances. *: p-value < 0.05,**: p-value < 0.01.

volume (MPV) in case of animals treated with *bhasma*, with and without *Amala* powder in comparison with control animals. The MPV shows further significant reduction in case of animals treated with the intermediate (with and without *Amala* powder) in comparison with control animals. However, *Amala* powder treated animals shows no significant change in MPV when compared with control animals (Fig. 9(i)). The platelet distribution width (PDW) is unaffected due to the treatment of *Amala* powder and the *bhasma* (with and without *Amala* powder) when compared with control groups. The intermediate treated groups (with and without *Amala* powder), however, show significant increase in the parameter when compared with control group (Fig. 9(j)).

3.2.6. *Analysis of cytokine levels in serum using Cytometric bead array (CBA)*

Blood was collected from control and test mice by retro-orbital puncture at various time points as indicated and sera were separated. The cytokines representing T_H1(IFN-γ, IL-2, TNF-α), T_H2(IL-4, IL-10) and T_H17 (IL-17A) in the sera of these mice were analysed using Cytokine Bead Array on flow Cytometer.

Fig. 10 shows the changes in serum cytokine levels in case of treatment as well as control groups. Also, the fold increase in each

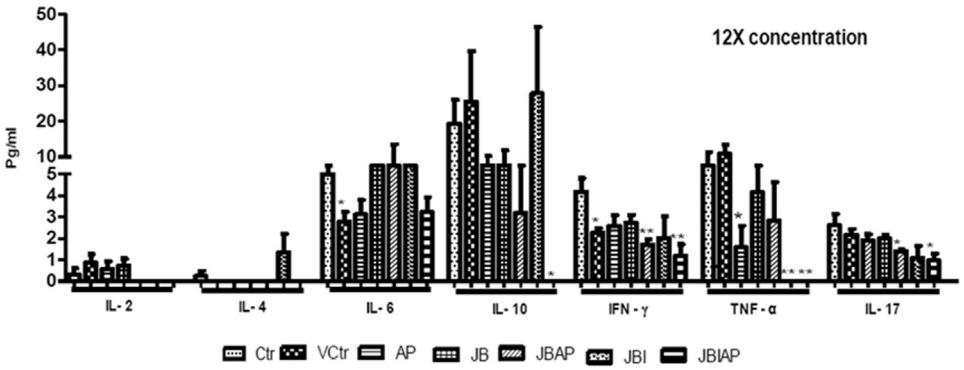

Fig. 10. Effect of *bhasma* and its intermediate, when administered with and without *Amala* powder on serum cytokine levels – data plotted as mean and error bars represent standard error over mean for each group, for respected cytokine. The data was analysed using unpaired *t*-test (two tailed) with $p \leq 0.05$ as the level of significance. *: p-value < 0.05,**: p-value < 0.005 and ***: p-value < 0.0005.

Table 3
Fold increase in case of animals treated with *bhasma* and its intermediate, with and without *anupan* in serum cytokine levels over untreated mice.

Representing cell/ function	Cytokine	AP-6X	JB-12X	JB-AP-12X	JBI-12X	JBI-AP-12X
T_H1	**IL-2**	1.11	2.45	0	0	0
	IFNγ	0.62	0.65	0.41	0.48	0.28
	TNFα	0.18	0.47	0.32	0	0
T_H2	**IL-4**	0	0	0	5.53	0
	IL-10	0.32	0.38	0.17	1.44	0
T_H17	**IL-17A**	0.74	0.76	0.53	0.42	0.37
Inflammatory	**IL-6**	0.25	0.15	0.48	0.3	0.16

cytokine production observed in sera of mice treated with *Amala* powder, *Jasad bhasma* with and without *Amala* powder, the intermediate with and without *Amala* powder over untreated (control) mice shown in Table 3.

The IL-2 levels do not show any statistically significant changes in case of animals treated with the *bhasma* and its intermediate, with and without *Amala* powder (Fig. 10). However, when fold change is considered, the *bhasma* treatment causes about 2-fold increase in the IL-2 levels. The treatment of *Amala* powder does not cause any change IL-2 levels. On the other hand, in case of all other treatment groups, there is marked decrease in the IL-2 levels when compared with control. The other two cytokines representing T_H1 cells, IFNγ and TNFα show marked decrease in the levels when compared with control (Table 3). The decrease in IFNγ levels is statistically significant in case of animals treated with *bhasma* with *Amala* powder and intermediate with *Amala* powder in comparison with control animals. The decrease in TNFα levels is statistically significant n case of animals treated with *Amala* powder, intermediate and intermediate with *anupan* in comparison with control animals. Even if no statistically significant change is seen in case of IL-4 and IL-10 in case of all treatment groups, the fold change in case of IL-4 and IL-10 levels in intermediate treated animals show marked increase. The levels of IL-17A decrease in statistically significant manner on treatment with the *bhasma* with *Amala* powder and intermediate with *Amala* powder on comparison with control. The inflammatory cytokine, IL-6 levels decrease in case of all treatment groups in comparison with control. Though, the decrease is not statistically significant, it is considerable in terms of fold change.

4. Discussion

4.1. Difference in particle attributes in case of Jasad bhasma and its intermediate

From XRD it is clear that, the final *bhasma* preparation is mainly of zinc oxide (~95.944%). On the other hand, in case of intermediate zinc sulphide constitutes the major portion (~65.707%). The CHNS(O) studies, though support the XRD findings, estimates more sulphur than that indicated by XRD studies. This may correspond to presence of elemental sulphur along with sulphide impurities other than zinc sulphide in minute amount in the preparation, which fail to contribute significantly to the XRD pattern. Moreover, in the preparation method mercury and sulphur are used in order to convert metallic zinc to its oxide. During this process HgS may react with Zn. Also, Hg may further be removed by volatilization. However, sulphur may remain in the preparation as elemental sulphur. In further cycle(s) of heating, this sulphur too may leave the preparation in form of oxide. It may be understood that during the elaborate method to prepare the *Jasad bhasma* from zinc metal, the metal is converted to its oxide via sulphide. And this conversion is mediated by mercury and sulphur added during the preparation. Hence, though the particles of *Jasad bhasma* do not differ significantly from particles of its intermediate with respect to size and shape, the chemical composition of the two is distinct.

4.2. Effect of anupan (Amala powder) alone on mice

Amala powder (*Phyllanthus emblica* L., fruit, dry powder) was chosen as an *anupan* (accompaniment) for *Jasad bhasma* in this study since it is one of the commonly prescribed accompaniments for the *bhasma* (Joshi, 2010a). However, *Amala* by itself is known to have antioxidant (Hari Kumar et al., 2004) and immunomodulatory (Suja et al., 2009) properties. Hence to understand the effect of *Amala* powder on healthy mice, in isolation from *Jasad bhasma* on study parameters separate group of animals was maintained. The results indicate that the powder when given at dose 12 mg/kg body weight, daily twice, for 28 d to mice does not have any effect on bioaccumulation of zinc (Fig. 4). Also the powder does not lead to any change in SOD and GSH levels in liver tissue (Figs. 5 and 6); hence it does not cause any significant effect on antioxidant status of the tissue. The LFT and RFT results indicate that there is no adverse effect of the treatment on liver and kidney function (Fig. 7). However, according to SPECT studies; *Amala* decreases hepatic half life of the radiotracer which is suggestive of improved hepatic function (Fig. 8). In this study, the treatment of

Amala powder did not caused any change in any of the CBC parameters (Fig. 9), indicating that the powder does not have considerable effect on formation, circulation and destruction of any of the blood cells. However specific analysis of serum cytokine levels show that, the cytokines specific for T_H2 and inflammatory cytokine (IL-6) show marked decrease, and hence suppression of T_H2 and inflammatory immune responses. The levels of IL-2 and IL-17A are comparable with those in case of control animals; hence the alteration in T_H1 and T_H17 responses is minimal (Table 3).

4.3. Effect of bhasma and its intermediate (process effect) on mice

Jasad bhasma being micro and nano-particulate zinc compound, in long term treatment it was suspected that, there will be increase in elemental zinc in several organs. Zinc from the *bhasma* can be absorbed in soluble as well as particulate form. However, the body pool of soluble zinc is subjected to homeostasis by regulating its absorption and excretion (Lowe et al., 2009; Whittaker, 1998). Hence, no bioaccumulation of zinc is seen, in this study, in any of the major organs studied on treatment of *bhasma* and its intermediate (Fig. 4). Though the mercury was present in the form of mercury oxide as impurity in *bhasma* as well as intermediate above the allowable daily intake levels (20 μg/day for human), as provided by the American National Standards Institute/National Sanitation Foundation International Dietary Supplement Standard 173 (ANSI 173) (2006), no accumulation of mercury was observed in any of the organ samples studied here.

Even though zinc is known to affect the liver SOD levels, in our studies, the *bhasma* and its intermediate does not cause significant alterations in the liver SOD levels. Previous studies have shown that the *bhasma* improves cells capacity to combat oxidative stress by boosting its antioxidant system (especially SOD and peroxidase) in yeast (Bhowmick et al., 2009a). However, the SOD gene expression is controlled by several factors such as zinc levels (Prasad, 2008), oxidative stress, cytokines and growth factors in mammalian cells (Rojo et al., 2004). Though statistically insignificant, the treatment of intermediate shows increase in tissue total protein levels (Fig. 5). Hence, the results of this study too indicate the possibility of multifaceted regulation of the gene involving oxidative trigger by the particles, and transient changes in zinc pool, and zinc induced immunomodulation. The treatment of intermediate causes significant increase in liver GSH levels. This indicates better ability of the treated tissue to fight with the oxidative stress. However, there exists a threshold of oxidative stress for cells and tissues to handle (Shull et al., 1991). Very high levels of GSH may relate with presence of excessive oxidative stress beyond the cells capacity to neutralise (Fig. 6). This may result in tissue damage. However, no such damage resulting in loss of function is indicated by the liver and renal function tests (Fig. 7). Also, the treatments of *bhasma* as well as intermediate do not lead to any significant change in case of hepatic clearance in comparison with control. This indicates that the liver function is unaffected by the treatments (Fig. 8(a)). It has been reported that the SPECT imaging using $^{99\,m}$Tc-Mebrofenin and $^{99\,m}$Tc-DTPA is more sensitive method of assessing the liver function (Erdogan et al., 2004) and renal function (Waller et al., 1991) than the serum biochemistry. Thus the study provides conclusive evidence for the safety of *Jasad bhasma* and its intermediate on chronic administration in mice.

The treatment of *bhasma* and its intermediate does not have any effect on WBC count, lymphocyte percentage, RBC count and haemoglobin levels. However, the reduction in HCT is significant in case of intermediate treatment and reduction in MCV is significant in case of *bhasma* treatment, which may indicate possibility of anaemia due to these treatments. In case of anaemia, the drop in MCV and HCT levels marks the early stages. The haemoglobin and RBC levels drop subsequently when iron stores are further depleted (Lokwani, 2013). To understand further development of anaemia due to the treatment, even longer duration studies are needed. Though the platelet count is unaffected by the treatments, the MPV shows significant decrease in case of *bhasma* and intermediate treated groups. The PDW shows marked increase in case of intermediate treated animals (Fig. 9). This may result due to rapid turnover of platelets (Erhabor and Adias, 2013).

The animals treated with the *bhasma* show two fold increase in serum IL-2, a cytokine produced by T_H1 cells. All other cytokines studied show decrease in serum levels on comparison with control animals, indicating prominence of T_H1 mediated (cell mediated) immune response and suppression of T_H2 mediated (humoral) immune response (Kindt et al., 2007). The IL-17A levels and hence contribution of T_H17 regulated responses are unaffected by the *bhasma* treatment. T_H17 is a type of T-helper cells which have major role in organ specific autoimmunity and chronic inflammatory conditions (Mills, 2008). Moreover, the *bhasma* treatment has reduced expressions of IL-6, an inflammatory cytokine. Interestingly, the intermediate treatment, on the other hand, leads to suppression of cytokines representing T_H1, T_H17 and inflammation. The T_H2 mediated (humoral) immunity is induced by the intermediate treatment (Table 3). The hyper-activation of humoral response is known to lead to the allergic reactions (Kindt et al., 2007). It has been reported that Zn deficiency causes suppression of T_H1 mediated immune response, a condition which is reversed by the Zn supplementation (Prasad, 2008; Prasad et al., 2002). Our results indicate that the *bhasma* might be working as Zn source and hence modulate the immune response towards cell mediated immunity. Since, the intermediate is mainly sulphide of Zn, may be less soluble at gastric as well as physiological pH (Kapoor, 2010). It may not be as bioavailable as ZnO or *Jasad bhasma*. And therefore, it fails to maintain the cell mediated immune response. However, the more studies are needed to understand the reason for activation of humoral response in case of intermediate treatment.

The method to prepare *bhasma* is an iterative one. According to *Ayurveda*, *bhasma* are supposed to gain the medicinal properties during these iterations. It is believed that the *bhasma* preparation is inadequate if the iterations are not carried out in sufficient number. Moreover, in most of the cases, the starting materials as well as incompletely processed *bhasma* are considered to cause ill effects (Sheikh, 2004). In our study, the intermediate treatment does not show extreme ill effects, especially on liver and kidney. But differences in immune activation due to the *bhasma* and intermediate treatments are prominent. Also, some effect on the platelet homeostasis is seen in this study, specific to the treatment with intermediate. However, further studies should be carried out to understand the exact effect of the preparation on activation of immune response and platelet turnover.

4.4. Effect of bhasma and its intermediate when given with anupan

Anupan when given with the *bhasma* is expected to facilitate its absorption and hence the treatment should affect animals differently than that when given alone. Since zinc homeostasis is maintained by controlling absorption and excretion rigorously, the treatment of *bhasma* and intermediate, when given with *Amala* powder too does not lead to bioaccumulation of zinc. As mentioned in the previous section, the treatment of *bhasma* and its intermediate cause perturbance in antioxidant status. The increase in GSH levels is significant when treated with the *bhasma*, with and without *Amala* powder in comparison with control group. Moreover, the intermediate alone causes very prominent increase in GSH levels, but when given with *Amala* powder, the GSH levels are lowered to the values comparable with animals treated with *Amala* powder alone (Fig. 6). Both, *Jasad bhasma* and *Amala*

powder have shown antioxidant activities in previous studies. A substance can have antioxidant activity in two ways; by enhancing the synthesis of cellular antioxidants such as SOD, peroxidase and by scavenging the reactive oxygen species. *Jasad bhasma* is known to induce the antioxidant effect by enhancing the synthesis of SOD and peroxidase (Bhowmick et al., 2009a), whereas the *Amala* powder exerts the antioxidant effect by scavenging the ROS (Sharma et al., 2009). The GSH levels can increase as a response to increased oxidative stress due to the intermediate treatment (Shull et al., 1991). Our results suggest that, this increased oxidative stress is reduced by scavengers from *Amala* powder, when given with the intermediate. And probably, no such oxidative stress is imparted by the final *bhasma*. However, further detailed studies are needed to conclude.

The other parameters affected by the treatment of *bhasma* and its intermediate such as, MCV, MPV, and PDW are affected in similar manner by the treatments; *bhasma* with *Amala* powder and intermediate with *Amala* powder. Also, the SPECT studies show no change in liver and renal function in case of animals treated with the *bhasma* or its intermediate with *Amala* powder in comparison with control animals (Fig. 8). On the other hand, the *bhasma* and its intermediate when given with *Amala* powder show marked suppression of all the immune responses (Table 3). This indicates that the *Amala* powder affects the immune response dominantly than the *bhasma* and the intermediate.

5. Conclusions

The *Jasad bhasma* is zinc oxide with nano to micron sized particles. The particles are polycrystalline. Its in-process intermediate on the other hand is mixture of zinc sulphide and oxide, with predominance of sulphide phase. Intermediate too has nano to micron sized particles, similar to the final product. This study demonstrates use of diffraction and microscopy techniques for wholesome understanding of the process endpoint. The intermediate contains about 1.28% of mercury oxide. However, no bioaccumulation of mercury was seen. Also, the treatment of *bhasma* and its intermediate, with and without *Amala* powder does not lead to bioaccumulation of zinc. None of the treatments studied show drastic toxic effect on liver and kidney tissue. However, the intermediate affects platelet turnover and antioxidant status. *Anupan* studied here is *Amala* powder. It does not affect liver and kidney function. However, it affects antioxidant status of the tissue. In our study, it is evident that the antioxidant enzyme (SOD) and glutathione levels are regulated in complex multifaceted manner. The regulation is brought about by several factors which are induced by the treatment and they interact amongst themselves too. Hence to understand the interactions of *bhasma* particles with several components and components themselves under the *bhasma* treatment, multifaceted studies as reported here are necessary. This is essential so as to develop better understanding of the preparations as therapeutic entities, which will be acceptable to modern medicinal sciences and practices.

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Conflict of Interest

No actual or potential conflict of interests exists.

Author Contribution

The study has been designed, executed and inter-institutionally coordinated by Aparna Chavare. The author has also drafted this manuscript. Praneeth Chowdari has maintained and dosed the animals; and has coordinated this inter-departmental study at ACTREC, Navi Mumbai. Sandipto Ghosh has provided the technical expertise in generating the SPECT scans. Priyanka Pawar, Meena Patkar, and Suresh Dakawe from Department of Immunology, ACTREC have analysed the serum cytokine levels in treated animals. Dr. Vikram Gota, Dr. Pradeep Chaudhari, and Dr. S. V. Chiplunkar from ACTREC, Navi Mumbai have provided the guidance in planning, execution and reporting the biological component of this study. Dr. Shantaram Kane and Prof. A. K. Suresh have guided the physicochemical characterization studies reported here. Prof. Jayesh Bellare (corresponding author) has provided the guidance in designing and execution of the study. Apart from reviewing the manuscript critically, the inter-institutional study has been harmonized by him.

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