Formulation, characterization and comparative evaluation of *Trivanga bhasma*: A herbo-mineral Indian traditional medicine

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Abstract: Bhasmas are unique Ayurvedic-metallic preparations with herbal juices/fruits, widely recommended to treat variety of chronic ailments. *Trivanga bhasma*, a calcinated preparation, is used to treat Diabetes mellitus and as Diuretic. In the present research an attempt has been made to carry out a comparative standardization of formulated *Trivanga bhasma* (TB1) prepared as per Ayurvedic formulary and marketed *Trivanga bhasma* (TB2) integrating conventional and modern analytical tools. The formulations were evaluated for physical properties, chemical characterization using FTIR, AAS, SEM, TGA, XRD and AFM along with anti-diabetic, diuretic and toxicology studies. The X-ray Diffraction analysis of both formulations exhibited crystalline nature and nano-sized particles by Scherrer's equation. In SEM studies, Lead, zinc and tin oxides show well-defined plate like structures while TB1 showed spongy, relatively compact microcrystalline aggregates with loss of grain boundaries. AFM analysis confirmed the spherical morphology of TB1 and TB2 with an average particle size of 500 nm. The present study is the first report of fingerprinting of *Trivanga bhasma* using sophisticated analytical techniques. *In vivo* pharmacological screening revealed that both TB1 and TBK2 possess anti-diabetic and diuretic activity and less toxicity, thereby facilitating standardization of *Trivanga bhasma*.

Keywords: *Trivanga bhasma*, Standardization, Anti-diuretic activity, SEM, AAS, AFM, herbal preparations.

INTRODUCTION

Ayurveda, the Indian traditional system of medicine means 'science of life and longevity'. It is a time tested medical system developed over a period of time since 500 BC in Indian sub-continent with continuous use by the national and international societies. The unbeaten heritage of this system is a treasure house of knowledge for both preventive and curative health care available to humankind. It has provided treatment to many diseases using herbs, metals and minerals, formulating them into potent dosage forms. out of all the ayurvedic formulations, Bhasmas are such kind of dosage forms which gained their reputation as effective formulations for any disease compromising the aspects of nanotechnology and overcoming the limitations of conventional dosage forms (Wadekar, 2005). They consider the therapeutic value of metals and modulate them into nontoxic formulations (Tripathi and Pandey, 2003).

Bhasmas are unique Ayurvedic metallic preparations with herbal juices/fruits, widely recommended for treatment of a variety of chronic ailments. Bhasmas are biologically produced metallic nanoparticles obtained by calcinations into ash and are taken along with milk, butter, honey, ghee, thus, making these elements easily assimilable, eliminating their harmful effects and enhancing their biocompatibility (Tripathi and Singh, 1996). The process of preparation of bhasma, bhasmikarana involves various steps termed as samskaras. Generally, shodhan, maran, chalan, dhavan, galan and puttan constitutes the samskaras.

Trivanga bhasma, calcinated metal and mineral based preparation is used to treat diabetes and as Diuretic (Arvind *et al*., 2010). The quality of bhasma depends on the raw materials used and formulation procedure adopted. Both these vary over space and finally reflect in the quality of bhasma. The wrong manufacturing and marketing practice pave way to the production of inferior quality products, which reduce efficacy and produce safety concerns. Thus, so as to minimize variability and to prevent adulteration, standardization of *bhasma* is must in all aspects. In this background, the present research aims at comparative standardization of formulated *Trivanga bhasma* (TB1) prepared as per Ayurvedic formulary and marketed *Trivanga bhasma* (TB2), integrating conventional and modern analytical tools. The standardization was attempted for physical, chemical and pharmacological properties.

MATERIALS AND METHODS

Plant Material

The plants used for the purification (sodhana) process were collected from hills of Tirumala (13.63 N latitude

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and 79.41 E longitudes), India and were duly authenticated by Dr. Jayaraman, Chairman, Plant Anatomy Research Center, Chennai, India. A specimen was kept in the Herbal Herbarium, Sree Vidyanikethan College of Pharmacy, A. Rangampet, Tirupati, India. Lead, Zinc and Tin are the ingredients used in the preparation of *Trivanga bhasma* and were procured from SD Fine Chem Ltd., Mumbai, India, marketed *Trivanga bhasma* from Mumbai, India and the other raw materials were obtained from the local ayurvedic market in Tirupati, India.

Instruments

Instruments used for analysis include Perkin-Elmer FTIR spectrophotometer, U.K., NETZSCH Thermoanalyzer STA–409 model, U.K., Hitachi S-3000N Scanning Electron Microscope, Japan, Nanonics Multiview 1000 Atomic Force Microscope head with E scanner, Nanonics Imaging Ltd., Jerusalem, Israel, X'Pert Pro (Phillips) X-ray powder diffractometer, Netherlands and Atomic Absorption Spectrometer, Perkin Elmer, USA.

Animals

Adult male Wistar albino rats weighing 150-200 g, were kept under standard conditions such as temperature at 24 ± 10ºC, relative humidity at 45-55% and 12:12 h light:dark cycle. The animals were fed with standard rat pellet and water ad libitum. The animals were allowed to acclimatize to laboratory conditions 48 h before the start of the experiments. All the experiments on animals were performed with the approval from IAEC (Regd. No. IAEC/930/a/06/ CPCSEA).

Preparation of Trivanga bhasma

The preparation of *Trivanga bhasma* involves two steps, sodhana and marana processes. In sodhana, naga [lead], vang [tin] and yasad [zinc] (26 g each) was detoxified by adding madhuka thaila. The compounds were then triturated with small amounts of herbal powders such as ashawaganda, sathvari, yastimadhu, tintrivik using a khalvam till a homogenous paste was formed. The obtained mixture was powdered i.e., subjected to marana process, and transferred to an earthen crucible covered with a lid and sealed with sealing clay. Finally it was kept for calcination (40 cow dung cakes used to prepare 250g of formulation). The mixture is blended with kumari swars [Aloe vera juice] to form a cake. The cake on drying obtained yellow colour which is *Trivanga bhasma* (Pandit *et al*., 1999).

Characterization and Comparative Evaluation of Trivanga bhasma

Physical standardization

The preliminary analysis of the prepared bhasma involved determination of floating property, fineness and metallic lusture, which forms the basic analytical confirmation methods. The bhasma was evaluated for physical properties like colour, odour, taste and pH, and physical constants like total ash, acid insoluble ash, water soluble ash and loss on drying. The particle size determination was carried out by sieve analysis.

Chemical standardization

The chemical characterization of both formulated and marketed bhasma was carried out by FTIR (Fourier Transform Infrared Spectroscopy) using Perkin-Elmer FTIR spectrophotometer and Thermo Gravimetric Analysis (TGA) using NETZSCH Thermoanalyzer STA-409 model. Scanning Electron Microscopy (SEM) for surface morphology using Hitachi S-3000N SEM and Atomic Force Microscopy (AFM) for nanosized topography using Nanonics Multiview 1000 AFM head with E scanner (Nanonics Imaging Ltd., Jerusalem, Israel) were also carried out. Scanning was performed in tapping mode. Images were obtained with 20 nm radius AFM tips obtained from Nanonics Imaging. The cantilever is oscillated at its free resonance frequency (typically 80 kHz). The exact position of the tip onto the sample was controlled using an inverted microscope (Olympus, Japan) mounted above the AFM. All measurements were performed at ambient temperature (20°C) in air. AFM images were captured, processed and analyzed with QUARTZ software, version 1.00 (Cavendish Instruments Ltd., UK).

The chemical composition, crystallographic structure and crystalline phases of the prepared bhasma were evaluated using an X'Pert Pro (Phillips) X-ray powder diffractometer with sample placed in conventional cavity mounts. The crystallite size was calculated from XRD pattern following the Scherrer equation as given below

$t = \lambda \times 0.9 / (\beta \times \text{Cos}\ \theta)$

Where, t is the crystallite size for plane, λ is the wavelength, β is the full width at half maximum in radians and θ is the diffraction angle for plane.

An atomic absorption spectrometric (AAS) study was performed for quantitative estimation of iron in the prepared bhasma using Atomic Absorption Spectrometer (Perkin Elmer, USA). Bhasma (10mg) was digested in 2 mL of aqua regia and after complete digestion; the volume of the solution was made up to 25mL with distilled water. Appropriate dilutions were made and the concentration of iron was determined by flame AAS.

Anti diabetic efficacy

Adult male Wistar albino rats were divided into five groups as: (i) Normal Control (Normal Saline), (ii) Negative Control (Normal Saline), (iii) Standard (Glibenclamide [2.5mg/kg, p.o]) (iv) Test group (Dose 1) and (v) Test group (Dose 2). Six animals were taken in each group.

Induction of Diabetes: Diabetes mellitus was induced in overnight fasted rats by a single intraperitoneal injection

of alloxan monohydrate dissolved in normal saline (0.9% w/v) at the dose of 120mg/kg. Hyperglycemia was confirmed by the elevated glucose levels determined at 72 h (Venkatesh *et al*., 2008). Animals were considered as diabetic. Rats found with permanent diabetes were used for the antidiabetic study. Tap water was allowed to the animals in this treatment group.

Treatment schedule

Diabetes induced animals of two test groups were given formulated *Trivanga bhasma*, TB1 (200 mg/kg) in honey, aqueous solution of the marketed formulation of *Trivanga bhasma*, TB2 (200 mg/kg). The other two groups were given with normal saline and Glibenclamide (2.5mg/kg, p.o). Normal control group was given normal saline. All the drugs were fed orally through feeding canula to each animal daily for fifteen days. Dosage of the different drugs for all the groups was calculated from prescribed human dosage using standard method (Freireich *et al*., 1966).

The effects of *Trivanga bhasma* in diabetic rats were observed by measuring fasting blood glucose levels and evaluating the serum lipid profile. Fasting blood glucose was estimated on days 0, 5, 10, 15 of Drug (*Trivanga bhasma*) administration. The biochemical parameters (TG, TC, LDL and VLDL) and histopathological studies of the pancreas were determined on day 15 after the animals were sacrificed by decapitation.

Diuretic efficacy

The method proposed by Lipschitz *et al*, was employed for the assessment of diuretic activity (Lipschitz *et al*,. 1943). Healthy albino Wistar rats of either sex were divided in to four groups of four animals each. *Trivanga bhasma* was evaluated for diuretic activity. Furosemide (20 mg/kg) was used as standard reference drug. Before the experiment, the rats were fasted for 18 hours with free access of water. On the day of experiment, the animals of group-1 were administered saline orally (2.5ml of 0.9% NaCl/100g body weight) and this group served as control. Group-2 rats were treated with standard drug Furosemide (20 mg/kg, i.p) in saline solution. Group-3 and Group-4 rats received *Trivanga bhasma* 200 mg/kg and *Trivanga bhasma* 400 mg/kg respectively. Immediately after the treatment, the animals were individually placed in metabolic cage (Kuppast & Nayak, 2005).

The urine was collected in measuring cylinder up to 5h for all control and treated groups. During this period no food and water was made available to the animals. The volume of urine, electrolyte (Na^+, K^+) content was estimated in the urine for assessment of diuretic activity. Na^+, K^+ estimation was carried out using flame photometry (ELICO CL361 flame photometer) (Jeffery *et al*,. 1989). The Diuretic index of tested drug was calculated by using the following formula.

$$\text{Diuretic Index} = \frac{\text{U.E. in test group}}{\text{U.E. in control group}}$$

Toxicology and histopathological studies

The toxicological studies were carried out following OECD 423 guidelines. The starting dose of *Trivanga bhasma* was 0.02 mg/kg body weight p.o. Dose volume was administered 0.02 mg per 100 g body weight to overnight fasted rats. Food was withheld for a further 3-4 h after administration of bhasma and observed for signs for toxicity. The body weight of the rats before and after administration was noted and any changes in skin and fur, eyes, mucous membranes, respiratory, circulatory, autonomic and central nervous system and motor activity and salivation, diarrhea, lethargy, sleep and coma were noted.

Histopathological studies were performed on the tissues of stomach, kidney and liver to observe the toxicity of the formulation. The rats were sacrificed and the organs were carefully isolated. The tissue of stomach, kidney and liver was fixed in 10% formalin embedded in paraffin wax. Histological sections were cut at 4-5 micron thickness and stained with routine hematoxylin and eosin. These were then examined by a consultant histopathologist. Photomicrographs of representative lesions at various magnifications were taken on a Zeiss optical microscope, Stemi 2000-C, with a resolution of 10x to 45x, with a trinocular camera attached.

STATISTICAL ANALYSIS

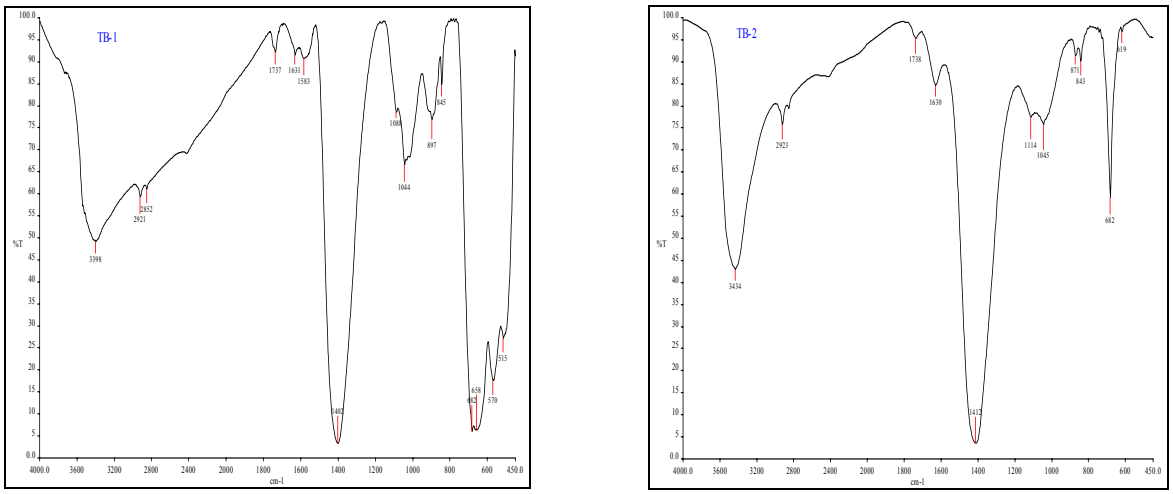
The significance of differences among the group was assessed using one way and multiple way analyses of variance (ANOVA). The test followed by Tukey-Kramer multiple comparison tests, the p values less than 0.05 were considered as significance.

RESULTS

Physical standardization

The various processes involved in the preparation of *Trivanga bhasma*, TB1 are shown in fig. 1. The physical examination using conventional methods showed TB1 as yellow colour, tasteless, having a characteristic odour and pH as 3.7. The formulation was found floating when sprinkled on the surface of water. Bhasma entered into the lines of the fingers when rubbed between the fingers so passing the fineness test. The loss of metallic lusture was confirmed when exposed to sunlight as there was no brilliance and shine of metal. The same characteristics were observed in marketed formulation TB2 as well. The other physical properties of bhasma TB1 and TB2 such as total ash, acid insoluble ash, water soluble ash and loss on drying were determined and given in table 1. The particle size determination was conducted by mesh test and the results are given in table 2. The results showed that the both bhasma came under very fine powder category with an amount of 46.55 % and 57.21 % respectively.

Fig. 1: Steps involved in the preparation of Trivanga Bhasma; (a) Putam used for formulation, (b) Shodana (c) Marana (d) Chalana (e) Dhavana (f) Galana (g) Puttan.



(i) (ii)

Fig. 2: FTIR spectra of (i) TB1 and (ii) TB2

Table 1: Physical parameters of TB1 and TB2

Parameter	TB1 (%)	TB2 (%)
Total ash	100	100
Acid insoluble ash	68.13	40.08
Water soluble ash	3.78	0.69
Loss on drying	1.0	2.0

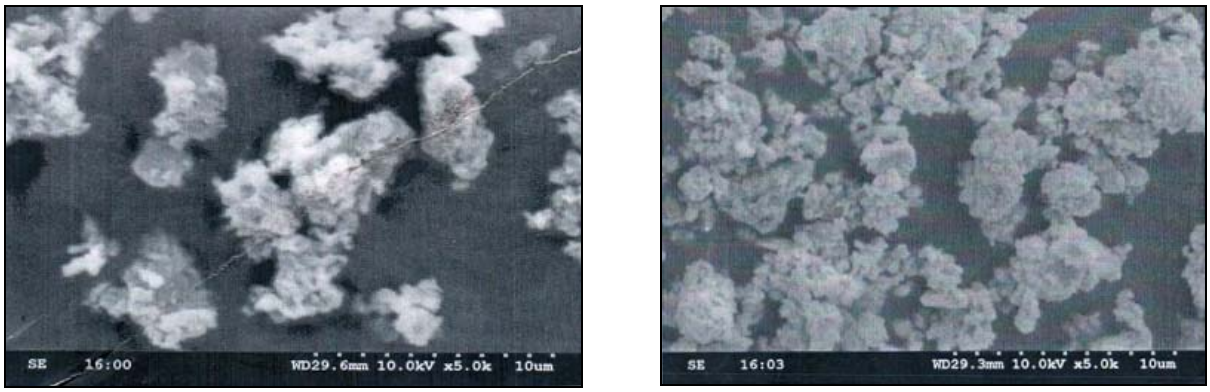
Table 2: Particle size variation between TB1 and TB2

Description	Mesh Size	Particle Retained (%)	
		TB1	TB2
Very Coarse Powder	2-10	5.34	0.00
Coarse Powder	20-40	9.27	0.84
Moderately Coarse Powder	40-80	26.72	26.85
Fine powder	80-120	12.08	15.06
Very fine powder	120-200	46.55	57.21

Chemical standardization

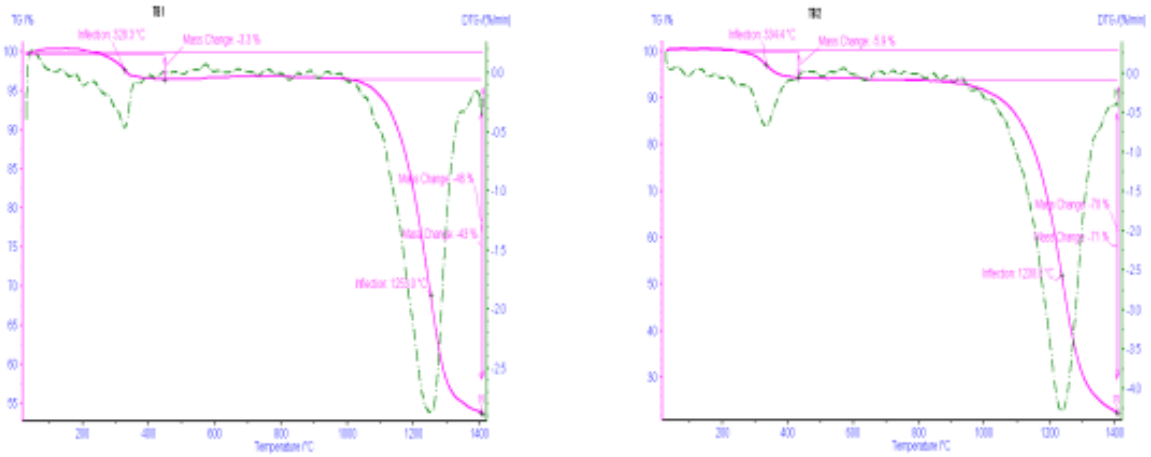
The chemical characterization of both formulations was carried out by sophisticated analytical techniques such as FTIR, AAS, SEM, TGA, XRD and AFM. The FTIR spectra of TB1 and TB2 are given in fig. 2. The observed absorptions correspond to inorganic metal, hydrated metal salt or oxide. The FTIR spectra showed no peak for any organic molecule or bond corresponding it, thereby confirming the absence of organic matter and external organic contamination.

The AAS study was conducted to determine the concentration of elements present in both formulations. The results showed that the elements Lead, Zinc and Tin were seen in major concentrations of 4.84%, 9.917% and 5.05% respectively for TB1 and 5.02%, 6.145% and 4.18% for TB2 respectively.



(1) (2)

Fig. 3: SEM of TB1 and TB2



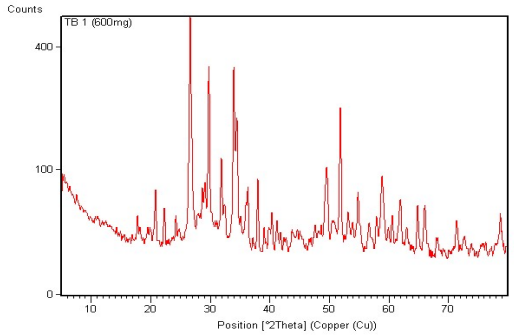
(1) (2)

Fig. 4: TGA curves of (i) TB1 and (ii) TB2

SEM images of TB1 and TB2 showed difference in size and agglomeration of the particles (fig. 3). The standard Lead, zinc and tin oxides (a) showed well-defined plate like structures while the formulated '*Trivanga bhasma*' (b) showed spongy, relatively compact microcrystalline aggregates with loss of grain boundaries. The average particle size of sample (a) was found to be about 1µ while that of sample (b) was in the range of 8-10µ obviously due to agglomeration of plate-like crystals which were also covered by the small dusty crystallites. Agglomeration of the particles is due to repeated cycles of calcinations involved in preparation. The influence of method of preparation on morphology, particularly the calcination temperature and duration, has also been reported in similar studies also (Wadekar *et al*., 2006).

For TGA, formulated and marketed bhasma were heated in presence of air and TGA curves were obtained which are shown in fig. 4. The analysis showed complete decomposition of TB1 and TB2 at 1253ºC and 1238ºC respectively. Lead, Zinc and Tin, when heated in presence of air were converted to Lead oxide, Zinc oxide and Tin oxide, after then these are converted as lead sulphide, zinc sulphide and tin sulphide. Since TGA curves showed the complete decomposition at 1253ºC and 1238ºC, it confirms the presence of sulphide forms of lead, zinc and tin.

The XRD pattern of both TB1 and TB2 are shown in fig. 5. The diffraction peaks of TB1 and TB2 are at 2θ=34.8º, 36.4º, 49.7º, 55.3º, 63.9º, 65.1º and 11.2º, 15.6º, 16.1º, 34.5º, 36.9º, 42.1º, 50.9º, 56.4º, 63.1º, 65.6º respectively. Comparative analysis of XRD results between TB1 and TB2 shows the major reflection peaks of both samples are at identical positions. The high intensity of lines in the XRD pattern suggests that the drug is present in crystalline form. The size of crystallites in *Trivanga bhasma* was calculated from the XRD pattern using the Scherrer formula and found to be in the range 52.7 nm. The Scherrer equation is not always a reliable measure of particle size; in fact sharp peaks indicate slightly higher sizes. Hence, other means of characterization were investigated.



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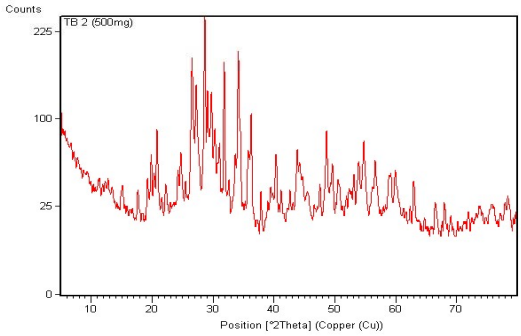
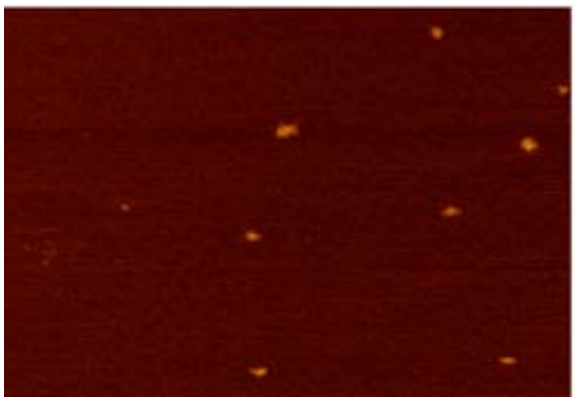


(2)

Fig. 5: XRD pattern of (i) TB1 and (ii) TB2



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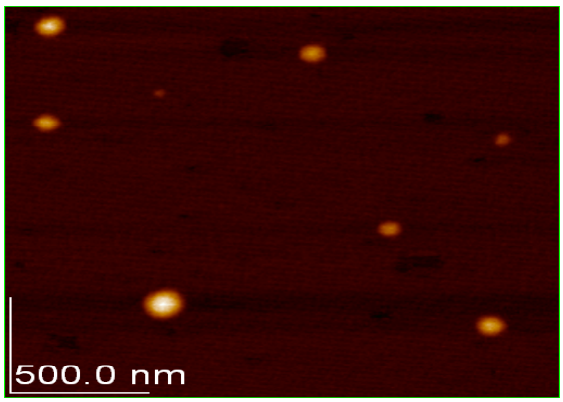

(2)

Fig. 6: AFM photograph of TB1 and TB2

AFM analysis confirmed that both formulations have spherical morphology with an average particle size of 60 nm for both TB1 and TB2. The spherical morphology was due to the aggregation of the nanocrystals of the metallic oxides. The image of AFM of both formulations is shown in fig. 6.

Pharmacological standardization

Anti-diabetic Activity

Trivanga bhasma at the dose of 200 and 400 mg/kg, produced a dose-dependent fall in fasting blood glucose level (FBG). After 15 days of treatment, the maximum reduction in FBG levels was observed in the treated group rats (Group-IV and V) as compared with untreated group (Group-II). On the progression of treatment with TB1, FBG levels reduced from 5th day. At the end of experiment (15th day) FBG levels was found to be 127.16 and 120.5 mg/dl in the rats treated with TB1 at the dose of 200 and 400mg/kg respectively. Comparison of FBG levels of various groups was also represented in the fig. 7.

Diuretic activity

Trivanga bhasma (200mg/kg and 400 mg/kg body weight) caused dose-dependent increase in urine volume.

Electrolyte excretion

Table 3 shows the urinary electrolyte content following the administration of the extracts. 400 mg/kg dose of TB1 produced a significant increase in Na^+ excretion when compared with the control group ($P<0.01$). Only Furosemide and the 400 mg/kg dose of TB1 produced significant increases in potassium excretion.

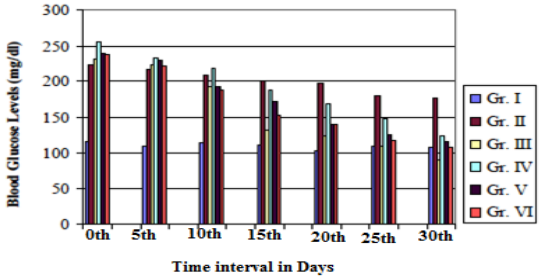

Fig. 7: Comparison of FBG levels of various groups

Toxicological studies

TB1 and TB2 showed no toxicity in all rats and their body weights remained constant before and after administration of formulations indicating no renal and gastro intestinal toxicity. The body weight variations are tabulated in table

Table 3: Effect of oral administration of TB and furosimide on urine volume, diuretic index, conductivity and pH.

Treatment	Dose (mg/kg p.o.)	Volume of urine (ml)	Sodium (mmol/L)	Potassium (mmol/L)	Na^+/K^+ ratio	Diuretic action
Control	---	2.1±0.08	138.9±0.50	54.73±0.60	54.73±0.60	1.00
Standard	20	21.65±0.25**	150.1±0.55*	71.06±0.75*	71.06±0.75	10.21
TB-1	200	9.6±0.43*	148.3±1.46	72.01±0.34	72.01±0.34	4.53
TB-1	400	12.2±0.26**	150.16±1.47*	72.21±0.37*	72.21±0.37	5.75

Values are expressed as mean ± SEM (n = 6); *p < 0.05 and **p < 0.01 compared with control (One way ANOVA followed by Tukey-Kramer's multiple comparison test).

Table 4: Toxicity studies

Groups	Weight of animals (g)		Toxicity	Onset of Toxicity	Duration of Study (days)
	Before Test	After Test			
TB1	150	150	No signs of Toxicity	Nil	14
TB2	150	150	No signs of Toxicity	Nil	14
Control	155	155	No signs of Toxicity	Nil	14

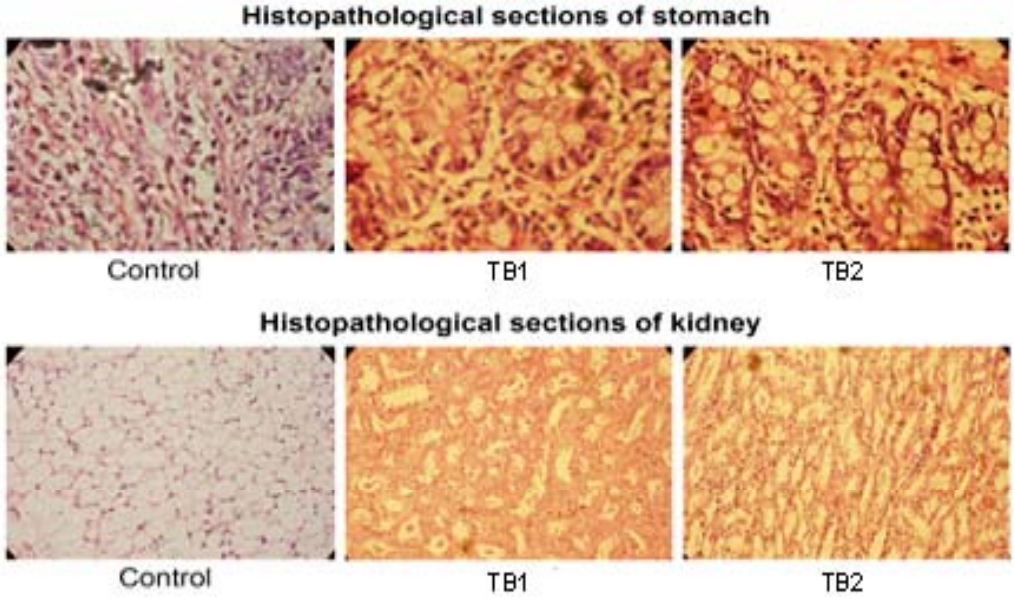

Fig. 8: Histopathology of stomach and kidney

4. The histopathological results in fig. 8 revealed the normal structure of cells of stomach and kidney before and after administration. A detailed histopathological examination was carried out for liver. The liver sections of normal group showed normal cellular architecture with distinct hepatic cells, sinusoidal spaces and central vein. Disarrangement of normal hepatic cells with centrinobular necrosis, vacuolization of cytoplasm and fatty degeneration were observed in CCl_4 intoxicated rats. The liver sections of the group III and IV rats treated with TB1 and TB2 at the dose of 200 mg/kg p.o. showed a sign of protection as it was evident by the moderate accumulation of fatty lobules, absence of necrosis and vacuoles in a dose dependent manner which is shown in fig. 9. Almost similar sign of protection was shown in the liver sections of silymarin at a dose of 100 mg/kg p.o treated rats.

DISCUSSION

Trivanga bhasma contain Lead, Zinc and Tin as the active ingredients with additional herbal constituents, *Withania somnifera, Asparagus racemosus, Glycerizza glabra* and *Tamarindus indicus*. The various stages of formulation techniques like *shodhana* (which involves roasting, with addition of herbal juices and continuous stirring) and *marana* [which involves bhavana (wet trituration) and *puta* system of heating], the particle size reduces significantly, which may facilitate absorption and assimilation of the drug into the body system. The micro to nano size of the formulations was confirmed by the analytical techniques, which could be specified as the criterion for the final product conforming to all the traditional parameters under *bhasma pariksha*

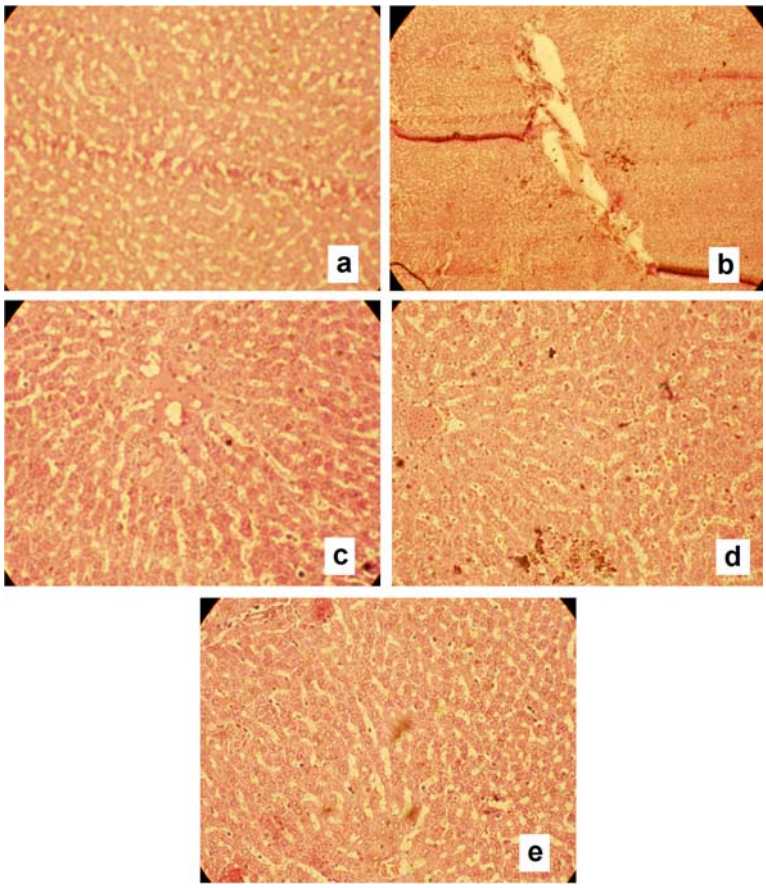

Fig. 9: Histopathology of liver treated with (a) control (b) CCl_4 (c) TB1, (d) TB2 and (e) Silymarin

(examination of properly prepared *bhasma*). The results of the present study revealed that *Trivanga bhasma* is an efficient herbo-metallic preparation for the management of Diabetes mellitus and also due to its diuretic effect; it can be used in the management of certain cardiovascular diseases. So far very few attempts have been made to evaluate metallic ayurvedic formulations using sophisticated analytical tools and the present work is a step towards the development of quality control methods for metallic ayurvedic preparations.

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