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Physicochemical characterization of *Suvarna Bhasma*, its toxicity profiling in rat and behavioural assessment in zebrafish model

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ABSTRACT

Ethnopharmacological relevance: Suvarna Bhasma is a gold-based Ayurved medicine that has a wide range of therapeutic indications like tuberculosis, diabetes mellitus, rheumatoid arthritis and nervous diseases. Suvarna Bhasma is also used in Suvarnaprashana, an Ayurved advocated therapy being practised to improve immunity in children.

Aim of the study: To augment traditional understanding, here we present an evidence-based study on Suvarna Bhasma regarding its physicochemical properties, toxicity and efficacy.

Materials and methods: Suvarna Bhasma was characterised by physicochemical characterization techniques such as scanning electron microscope (SEM), transmission electron microscopy (TEM), X-ray diffraction (XRD) and atomic emission spectroscopy (ICP-AES). Toxicity of Suvarna Bhasma was studied in Holtzman rats with daily oral dose from 3 mg/kg (therapeutic dose, TD) up to 30 mg/kg (10 TD) body weight for 90 days. Behavioural study, such as motor and geotactic behaviour were examined in zebrafish model to find out any sign of neurotoxicity or behavioural changes due to Suvarna Bhasma administration.

Results: Suvarna Bhasma has two types of gold particles, large ones (~60 μm) having irregular shapes, and nanosized spherical particles (starting from ~10 nm), the latter coated with Fe, Si, O, P and Na. XRD study revealed that all the peaks of Suvarna Bhasma match well with pure gold (face centred cube) with crystallites size 45 ± 2.8 nm. In rat studies, some change in biochemical parameters such as urea, creatinine and alanine aminotransferase (ALT) was observed mainly at the higher therapeutic dose; however, those parameters were within the normal range. There were no significant macroscopic as well as microscopic treatment-related alteration observed, in any of the organs and tissues evaluated. In zebrafish behavioural study, the motor parameters of Suvarna Bhasma treated fish showed normal behaviour analogous to the vehicle control group. Interestingly, the geotactic behaviour showed anxiolytic effects of Suvarna Bhasma as evidenced by the time spent in the upper zone, and average swimming height. The anxiolytic effects persisted for more than 30 days after withdrawing the Suvarna Bhasma treatment.

Conclusions: Suvarna Bhasma contained spherical gold nanoparticles. It was nontoxic in rat model at the does tested. Suvarna Bhasma has anxiolytic effects in zebrafish behavioural model.

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1. Introduction

Suvarna Bhasma (also spelt as Swarna Bhasma) is a gold-based Ayurved medicine. It is used in the treatment of diseases such as asthma, rheumatoid arthritis tuberculosis, diabetes mellitus, immune and nervous disorder (Singh, 2014; Yadav and Chaudhary, 2015; Patel and V Shah, 2013). In Suvarna Bhasma, gold in elemental form is the major element (> 98%) and it is the active ingredient. Suvarna Bhasma, along with honey, was also prescribed as a tonic for rejuvenation (Williamson, 2004). Suvarnaprashana is widely advocated by Ayurved practitioners and is gaining popularity as an Ayurved therapy to improve immunity of the child (Rao et al., 2012; Samant and Patil, 2014).

In this samskara (ritual), Suvarna Bhasma is administered and sometimes administered with pure honey and medicated ghee processed in Medhya (nootropic) and Rasayana (rejuvenating/anti-ageing) herbs as per the preference of the practitioners. Kashyap Samhita describes it to improve cognitive functions, digestive capacity, strength and longevity (Jyothy et al., 2014a). Likewise, in recent times, gold nanoparticles are attracting researchers, especially in the biomedical field, as these nanoparticles are biocompatible and having immense therapeutic and diagnostic applications. Suvarna Bhasma, comprising of nanogold particles, renewed the interest of the scientific community for finding its applicability in chronic diseases such as diabetes and cancer (Das et al., 2012; Kumar Pal, 2015).

Suvarna Bhasma is considered as one of the most prominent metal based medicine in Ayurved, which has both protective and curative properties on numerous health problems (Jyothy et al., 2014a). Gold based Ayurved medicines are traditionally used for centuries, however, a scientific evidence-based study is extremely required to find out the safety and efficacy of Suvarna Bhasma in the animal models. Therefore, this study was focused on a comprehensive assessment of Suvarna Bhasma by exploring its safety in rat model and efficacy in zebrafish behaviour model along with the physicochemical characterization of Suvarna Bhasma particles.

Interestingly, the physicochemical properties of Suvarna Bhasma may differ from manufacturer to manufacturer. This may happen because the traditional pharmacopoeia (texts) describe different preparation protocols for the same medicine. For example, approved Ayurved scripture, Rasatarangini has described five different methods for the preparation of Suvarna Bhasma. Also, the 5th volume of Bharat Bhaishajya Ratnakar (A compilation of Ayurved formulations from various Ayurved texts) has described 20 different methods for the preparation of Suvarna Bhasma. (Suvarna Bhasma used in these studies was manufactured using the method described in Bharat Bhaishajya Ratnakar 5/8357). Particles size, shape, and gold concentration vary largely for Suvarna Bhasma from a different origin. For example, Beaudet et al. (2017) showed that Au present in Suvarna Bhasma was approximately 57 wt%, Brown et al. Brown et al. (2007) reported 92 wt % Au in Suvarna Bhasma, whereas Thakur et al. (2017) found 98% gold in Suvarna Bhasma. Similarly, the Suvarna Bhasma crystallite size also varies largely from 23 nm (Brown et al., 2007) to 60 nm (Beaudet et al., 2017) according to the two different research articles. Therefore, before in-vivo studies, Suvarna Bhasma used in this work was physicochemically characterised. Here, the physicochemical study revealed some unique features about Suvarna Bhasma which were not reported in the literature. Additionally, it was found that for this particular Suvarna Bhasma, batch to batch variation was not significantly observed.

In the Suvarna Bhasma preparation process, metallic Hg is very frequently used, which is a common neurotoxin and Au is also not an essential element (Bhattacharya et al., 2016; Broussard et al., 2002), though a dose of 15–30 mg for an adult human is normal and the dose may be prescribed for a longer duration. Therefore, besides its traditional belief, a proper toxicological study was carried out in this work. In recent literature, some in-vitro and in-vivo toxicological studies were been reported, which concluded that Suvarna Bhasma was nontoxic (Beaudet et al., 2017; Paul and Sharma, 2011; Mitra et al., 2002; Khedekar and Priya, 2016; Jamadagni et al., 2015). The genotoxicity of Suvarna Bhasma was studied by Selkar et al. (2016) and they reported it is safe in terms of genotoxic and mutagenic activity. However, most of the in-vivo toxicity studies reported short term effects of Suvarna Bhasma in the rodent, for example, Khedekar et al. (Khedekar and Priya, 2016) reported the effect of Suvarna Bhasma in albino rats for 10 days treatments. On the other hand, Khan et al. (2018) administered Suvarna Bhasma for 20 days in albino rats. Jamadagni et al. (2015) did a 90 days toxicity study in Wistar rats with a maximum Suvarna Bhasma dose of 13.5 mg/kg. However, this group (Jamadagni et al., 2015) reported data only for the alteration of body/organ weight and histopathology of Suvarna Bhasma treated rats. In this present study, a comprehensive toxicity study was carried out with three different doses, a low dose (3 mg/kg), a medium dose (15 mg/kg) and a high

dose (30 mg/kg) of Suvarna Bhasma was administered orally for consecutive 90 days to male and female rat separately. Various haematological, biochemical and histopathological analysis was conducted after 45 and 90 days of Suvarna Bhasma treatment.

Additionally, Suvarna Bhasma was administered in a zebrafish model to observe behavioural changes. The behaviour of zebrafish is robust; abnormality in behaviour can be evoked by drug-induced toxicity (V Kalueff et al., 2013). With the aid of video tracking methodology, the behaviour of zebrafish can be analysed with high accuracy. In this study, zebrafish were efficiently tracked in the novel tank with the automated video tracking process. With the aid of indigenously build MatLab code, various motor and phenotype behaviour were accurately evaluated.

2. Materials and methods

2.1. Chemicals

The preparation of the Suvarna Bhasma (Batch no: P110600209) used in this study was carried out by Shree Dhootapapeshwar Limited, Mumbai, according to the classical Ayurved text (Bharat Bhaishajya Ratnakar 5/8357, Fig. 1). Suvarna Bhasma was prepared (Fig. 1) from gold bar (99.99% purity), following a rigorous Ayurved process consisting of Shodhana and Marana (special procedures meant for the conversion of metals into Bhasma). The gold bar was converted in the form of thin gold ribbons by passing through a machine by exerting pressure and these were further cut into small pieces. These gold pieces were then purified by quenching them in Taila (sesame oil), Takra (butter milk), Gomutra (Cow's urine), Kanji (fermented rice water), Kulattha Kwatha (decoction of Dolichos biflorus seeds) and Kanchanara Kwatha (decoction of Bauhinia variegata stem bark), for 7 times in each liquid. Then the purified gold pieces were amalgamated with Hg at 1:2 wt ratio (Au:Hg). The Au–Hg amalgamate was then covered with S powder in an earthen pot and heated slowly up

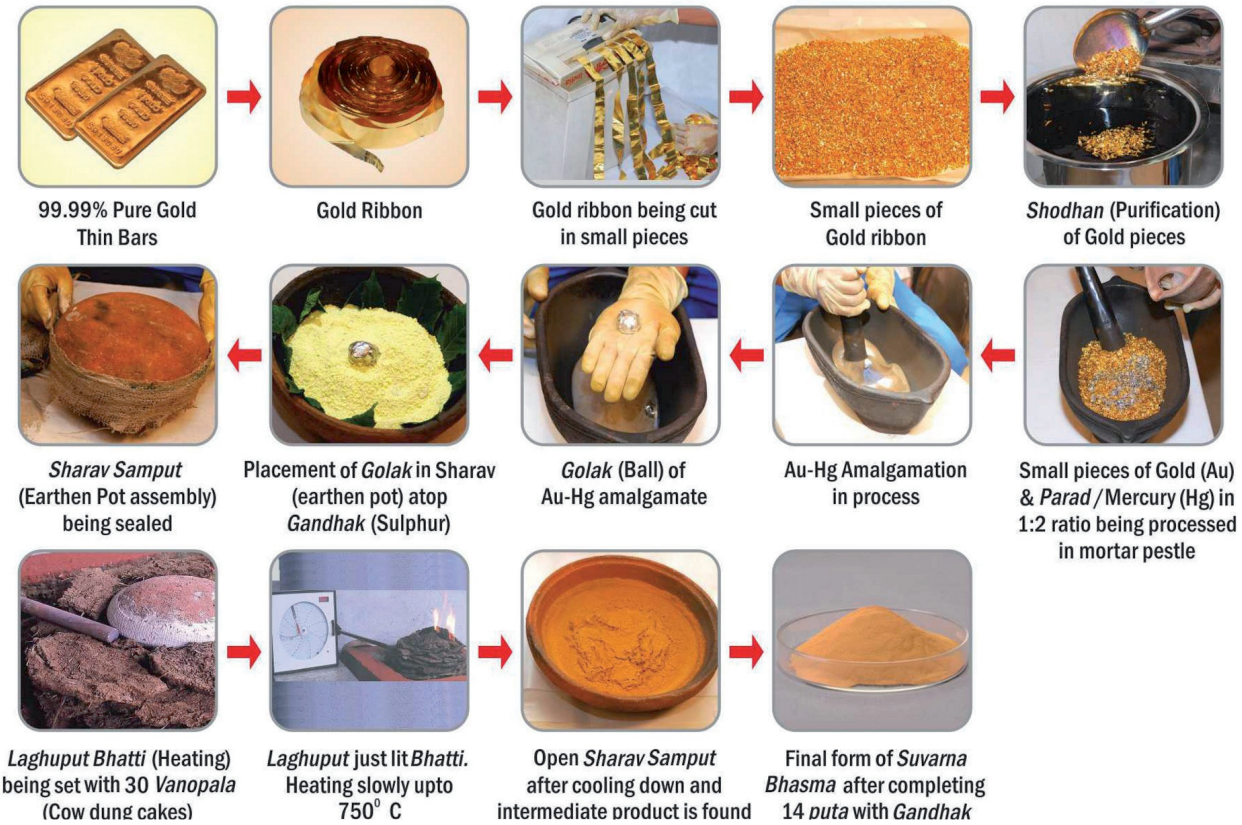

Fig. 1. Flow chart of Suvarna Bhasma preparation starting from gold bar. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

to 750 °C. After cooling down of the earthen pot, the intermediate product was found in a powder form. The heating process of the intermediate product with S powder was repeated for 13 times (total 14 times) which resulted in the final form of Suvarna Bhasma in the form of fine powder. The manufacturing process of Suvarna Bhasma took about 21 days. A quantitative detail for a typical Suvarna Bhasma batch (1 kg) has been provided in Supplementary File S2.

2.2. Physicochemical characterization of Suvarna Bhasma

Suvarna Bhasma was characterised to understand its physicochemical properties. The crystallographic details of Suvarna Bhasma were analysed by powder X-ray diffraction (XRD, Smart Lab, Rigaku, Japan) and peaks were compared with ICDD (International Centre for Diffraction Data) database. Transmission electron microscope (TEM, JEOL 2100, Japan) attached with energy dispersive spectroscopy (EDAX, OXFORD instrument, United Kingdom) was used to observe the morphology and elemental mapping of Suvarna Bhasma particles. TEM analysis was carried out after suspending Suvarna Bhasma particles in water; then the suspension was sonicated for 10 min and allowed to settle down for 5 min. After settling down, the lightweight suspended Suvarna Bhasma particles from the upper water level were pipetted out and fixed on the carbon coated copper grid for TEM analysis (Brown et al., 2007). The backscatter imaging of Suvarna Bhasma was grabbed by a scanning electron microscope (FEGSEM, JEOL, Japan). Elemental analysis of Suvarna Bhasma was carried out using inductively coupled plasma atomic emission spectroscopy (ICP-AES, ARCOS, SPECTRO Analytical Instrument, Germany) after digesting Suvarna Bhasma in aqua regia. The surface elemental analysis was conducted by high-resolution X-ray photoelectron spectroscopy (HRXPS, Kratos Analytical, Japan) equipped with a monochromatic X-ray source of 1486.6 eV. Thermogravimetric analysis was carried out with TGA analyser (PERKIN ELMER, USA). Details of physicochemical analysis carried out are enlisted in Table 1.

2.3. Animal ethics

Ethical clearance for the use of animals in the study was obtained from the Institutional Animal Ethics Committee of National Institute for Research in Reproductive Health (NIRRH), Parel (Study number 09/11) prior to the initiation of the study. The experiments were performed in accordance with the guidelines of the Committee for the Purpose of Control and Supervision of Experimental Animals (CPCSEA), India.

2.4. Toxicity assessment of Suvarna Bhasma in rat-model

Toxicological study of Suvarna Bhasma was conducted in the Holtzman rat model following OECD guidelines for sub-chronic toxicity. Total 90 rats (45 males and 45 females) of 9–10 weeks of age were randomly selected and assigned to the control and the treatment groups (Table 2) after the acclimatization of five days prior to the start of the study. The weight variation of animals used did not exceed±20% of the mean weight of each sex. The animals were kept in polypropylene cages and

Table 1
Details of physicochemical analysis carried out in this study.

Analysis	Instrument	Operation details	Sample Preparation
X-ray diffraction (XRD)	XRD, Smart Lab, Rigaku, Japan	Source: Cu K-α, wavelength = 1.5406 Å Scanning range 2theta = 10–90°	Powder sample was placed on a glass plate and pu scanning
Transmission electron microscope (TEM)	JEOL 2100, JEOL, Japan	Electron source 200 KV	Powder gold particles (Suvarna Bhasma) were susp water and sonicated for 10 min, after settling down particles from upper water layer were pipetted out the carbon coated grid
Scanning electron microscope (SEM)	JSM-7600F, JEOL, Japan	Field emission gun	Suvarna Bhasma powder was fixed on carbon tape, tape was placed on a sample holder.
Energy dispersive spectroscopy (EDS)	OXFORD instrument, UK, attached with TEM	Mapping in scanning transmission electron microscope (STEM) mode	Same as TEM sample preparation
Atomic emission spectroscopy (ICP-AES)	SPECTRO Analytical Instruments GmbH, Germany	Wavelength Range: 130 nm–770 nm. Detector: Charge Coupled Devices (CCD)	*Suvarna Bhasma* (~10 mg) was digested in *aqua reg* diluted with MiliQ water
Thermogravimetry (TGA)	PERKIN ELMER, USA	From room temperature to 1250 °C at 10 °C/min rate	Powder sample used directly
High-resolution X-ray photoelectron spectroscopy, (HRXPS)	Kratos Analytical, Japan	*X-ray source of 1486.6* eV	Suvarna Bhasma powder fixed on the carbon tape a on the sample holder

maintained under controlled temperature (23 ± 1 °C), humidity (55 ± 5%), and in a 14 h light/10 h dark cycle. Maximum three animals were housed in a single cage. All animals were acclimatized for five days prior to exposure of the test items.

Table 2
Grouping of rats and dose level of Suvarna Bhasma.

Group No	Groups	Group Name	Dose (mg/kg body weight)	No of Males	No of Females
I	Control	VC	Vehicle	10	10
II	Low	SB3	3 mg/kg (TD)	10	10
III	Mid	SB15	15 mg/kg (5 × TD)	10	10
IV	High	SB30	30 mg/kg (10 × TD)	10	10
V	[a]Recovery	SB30R	30 mg/kg (10 × TD)	5	5

[a] Recovery group was sacrificed 15 days after the completion of treatment. TD = therapeutic dose.

Suvarna Bhasma was administered orally to the animals with a syringe once daily for a period of 90 days. Animals from the control group were treated with vehicle alone (xanthine gum, 2% w/v). Dose volume was adjusted based on the weekly body weight of the individual animal. Except for the treatment with the test item, animals in the control group were handled in an identical manner to those in the test groups. The dose volume administered to each animal was calculated based on a constant factor of 2 ml/kg. Dose levels of 3, 15 and 30 mg/kg of Suvarna Bhasma were given for this study. Suvarna Bhasma was weighed and formulated with xanthine gum (2% w/v) freshly on each day of dosing. The concentration of Suvarna Bhasma was adjusted at 1.5, 7.5 and 15 mg/ml to administer the doses of 3, 15 and 30 mg/kg body weight. Body weight and feed intake of all the animals were monitored throughout study period.

Blood was collected from retro-orbital plexus under light anaesthesia (3–4% isoflurane) in two separate vials, one for haematology (EDTA used as anticoagulant) and other for serum biochemistry analysis. The haematological analysis was performed on freshly collected blood samples by using haematology analyser (Abacus, Diatron, Hungary) and, the serum samples separated after incubation at 37 °C of whole blood were stored at −20 °C for further analysis. The serum biochemistry analysis was performed by using fully automated serum biochemistry analyser (EM 200, ERBA).

At scheduled terminal necropsy, all surviving animals were humanely euthanized by CO2 asphyxiation and subjected to complete gross pathological examination. All collected organs were fixed in 10% neutral buffered formalin until processing. Organ weights of tissues were taken immediately after collection. These tissues were processed in an automatic tissue processor (ASP300, Leica, Germany) and embedded in the paraffin wax using a tissue embedding system (EG 1150H, Leica, Germany). The embedded tissues were further trimmed with the help of an automatic microtome (RM 2255, Leica, Germany) and sections were cut at 5 μm thickness and taken on a clean, greasefree slide for further staining with Haematoxylin and Eosin with automatic tissue stainer (Autostainer XL, Leica, Germany). Histopathological examination was performed on the specified list of tissues including all macroscopically abnormal tissues of all control and high dose group animals sacrificed at termination.

2.5. Behavioural experiments on zebrafish

Adult wild-type zebrafish (Danio rerio) of 5–6 months of ages were purchased from an authenticate zebrafish supplier (Vikrant Aquaculture, Mumbai, India). The zebrafish were kept in a lab-costumed housing system (Biswas et al., 2018), having biological, chemical and mechanical filtration facility to maintain the water quality preferred for zebrafish. The water temperature of zebrafish housing was kept consistent throughout the experimentation at 25–27 °C. The fish were maintained in 14 h light and 10 h dark cyclic period. The experiment was carried out after two months of acclimatization periods. Zebrafish were divided into four groups as Vehicle Control (VC), Suvarna Bhasma (SB), Comparative Control (CC, 1.5% alcohol) and Positive Control (PC, 50 mg/L caffeine) groups. For VC and SB groups, a total of 40 fish/group was allotted, whereas CC and PC group had 20 fish each. For the CC group, alcohol (1.5 vol %) and for PC group, caffeine (50 mg/L) was given for 30 min (n=20) to fish individually before behaviour tracking. In each experimental set, the male to female ratio was the same for VC and SB groups. Zebrafish from each group were kept as a cohort of 10 fish separately in a tank; therefore behaviour experiment was independently run in quadruplicate for VC and SB (Total n=40) groups, whereas behavioural experiment was done in duplicate for CC and PC groups with total n=20 fish/group.

All fish were given dry food twice a day (Tetra, Germany). TheSuvarna Bhasma was orally given mixed with dry granular food (Micro Wafers, Hikari Tropical, Japan) to the SB group daily for 15 consecutive days at the afternoon time. After the completion of Suvarna Bhasma treatment, on the 16th day at the afternoon time fish were placed individually in a novel tank (dimension:

L×H×W=200mm×170mm×130 mm) and the video was recorded from the top and side views with two webcams (Logitech B525). For CC and PC group, fish was treated with alcohol and caffeine respectively and behaviour was recorded in the same novel tank. Comparative control (CC) fish were individually exposed to 1.5 vol % alcohol in water for 30 min in a two-litter tank before novel tank tracking which has anxiolytic effects on zebrafish (Hamilton et al., 2017). Caffeine, an anxiogenic to zebrafish behaviour, was similarly treated for 30 min at a concentration of 50 mg/L water to the PC group (Egan et al., 2009; Collier, 2017).

Trajectories of all fish from individual videos were extracted with the help of open source MatLab software: idTracker (Pérez-Escudero et al., 2014). Each video was grabbed at a speed of 30 frames/sec. Videos of individual tracking were processed from frame number 1800 to 10800 (total 5 min/fish). From the trajectories, various motor parameters (speed, meander, and freeze point) and geotaxis phenotype behaviour (preference of being in the upper or lower zone) were calculated by indigenously developed MatLab code. To observe the after effect of Suvarna Bhasma, 30 fish from each SB and VC groups was kept in housing system and again tracked after 30 days (without any drug treatment) in the novel tank similarly as described above.

The oral drug dose for zebrafish was prepared by mixing Suvarna Bhasma with dry granular food and COD liver oil as sticking substance for 15 min so that the Suvarna Bhasma stuck to outside of the granular food. After ICP-AES quantification of the gold per gram of dry food + Suvarna Bhasma, the exact amount dose (dry food + Suvarna Bhasma) was given to the fish of SB group at a dose of 60 mg/kg fish weight. The calculated dose of the granules was added to the feed tank. Since Suvarna Bhasma is not soluble in water, granules retained their identity until consumed. The control groups were also provided dry food mixed with COD liver oil (without Suvarna Bhasma).

As drug dose was given in the water, there was always a chance of elution of Suvarna Bhasma. However, from ICP-AES analysis, it was confirmed that elution of Suvarna Bhasma particles was not more than 10% even after 5 min immersion in water. On the other hand, as

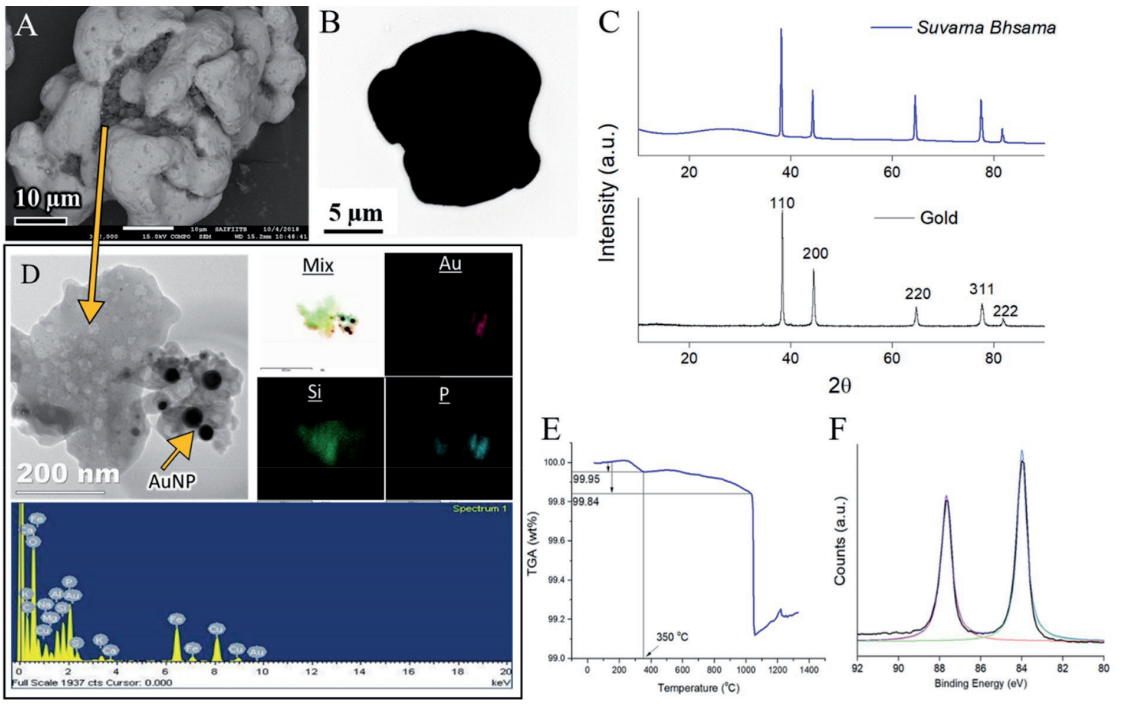

Fig. 2. Physicochemical characterization of Suvarna Bhasma. A) SEM backscatter image, B) TEM image of big Suvarna Bhasma particles, C) XRD profile of Suvarna Bhasma, D) TEM image and EDAX mapping of small Suvarna Bhasma particles, E) TGA profile of Suvarna Bhasma, and F) HRXPS (gold 4f region). XRD profile of Suvarna Bhasma perfectly matches with pure gold. Suvarna Bhasma contained encapsulate spherical nanoparticles starting from 10 nm size. EDAX mapping of small Suvarna Bhasma particles shows peaks of Au, Si, O, P, Fe, Ca etc. From TGA profile, it was observed that organic carbon may not present in Suvarna Bhasma. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

the oral dose was given to the fish cohort, so there is always a possibility of unequal distribution of drug dose. One could separately feed those fish, but as zebrafish is a social animal, separating them could lead to unnatural behaviour in them. Also, we observed that in isolation, attraction towards food in zebrafish decreases. The cohort was given dose which contains approximately 40–50 food granules (with Suvarna Bhasma), and within 2 min the whole bowl of the dose was consumed.

There was no avoidance towards Suvarna Bhasma dose observed in the fish cohort. Therefore, it was concluded that the amount of Suvarna Bhasma dose consumed by fish was proportional to fish's body weight.

2.6. Statistical analysis

Statistical analysis was performed using GraphPad Prism and Origin software. Data were analysed for dose wise comparison. One-way- analysis of variance (ANOVA) followed by Tukey's test was used to compare significance between various treatment groups.

3. Results

3.1. Physicochemical characterization of Suvarna Bhasma

Physicochemical study of Suvarna Bhasma revealed some new interesting features about it. Suvarna Bhasma contained very big particles as well as nanosize gold particles (Fig. 2A, B, and 2D). The nanosized gold particles were spherical, starting from ~10 nm in size (Fig. 2D). The spherical gold nanoparticles were encapsulated by an envelope of Si, O, P and Fe coating (Fig. 2D). These whole encapsulate gold particles were again embedded in the big gold particles (Fig. 2A). The large particles were agglomerated and irregular in shape having size up to 60 μm. The XRD peaks of Suvarna Bhasma matched with that of pure gold with no other extra peaks (Fig. 2C). The crystal size of Suvarna Bhasma, calculated using Sherrer's equation, was approximately 45 ± 2.8 nm. However, EDAX (Fig. 2D) and ICP-AES analysis show presence other elements as well, such as Fe, Ca, Si etc. Further analysis of Suvarna Bhasma using ICP-AES and ICP-AAS revealed that it contains approximately 98% Au (Table 3).

Up on thermal analysis (TGA), it was noticed that only 0.05% weight loss occur up to 350 °C and 0.16% weight loss up to ~1050 °C. HRXPS found only gold state (Au0), other gold peaks (such as gold salt) was not present in the Suvarna Bhasma.

Table 3
ICP-AES and ICP-AAS analysis of *Suvarna Bhasma* after digestion in *aqua regia* (ND = not detected).

Element	ICP-AES (Wt%)
Au	98.2 ± 1.82
Si	0.06
Fe	0.19
Ca	0.13
Cu	0.01
Zn	0.05
Pb	ND
As	ND
Hg	ND

3.2. Toxicological assessment in rat model

3.2.1. Haematology

The effects of Suvarna Bhasma on the haematological parameters are represented in Fig. 3 for male (Fig. 3A, C) and female (Fig. 3B, D) rats separately. There was no significant alteration observed in Suvarna Bhasma treated groups after 45 days (Fig. 3A and B) of treatment among any of the haematological parameters such as, haemoglobin level, counts of blood cells, mean corpuscular haemoglobin concentration (MCHC) etc. when compared with the vehicle control group (Details of other haematological parameters are enlisted in the Supplementary file S1-Table 3). Similarly, there were no significant alterations in any of the haematological parameters performed at the termination day (after 90 days) except percentage of red blood cells PCV % in SB3 group in female rats (Fig. 3A and B and Table 3d in Supplementary file S1).

3.2.2. Clinical chemistry

Serum biochemistry was similarly analysed at the two time points in rats (Fig. 4). After 45 days of treatment, significant alteration in some serum biochemical parameters was witnessed in the treated groups when compared with the control group. The significant variation in males included increased creatinine and triglyceride levels in the middose group (SB15) (Fig. 4A). The significant variations in females (Fig. 4B) included decreased creatinine and AST in the high dose group (SB30), increased glucose and DBIL (direct bilirubin) in the recovery group (SB30R), increased glucose level of the recovery group (SB30R), increased TBIL (total bilirubin) in the SB3 group, and increased phosphorus

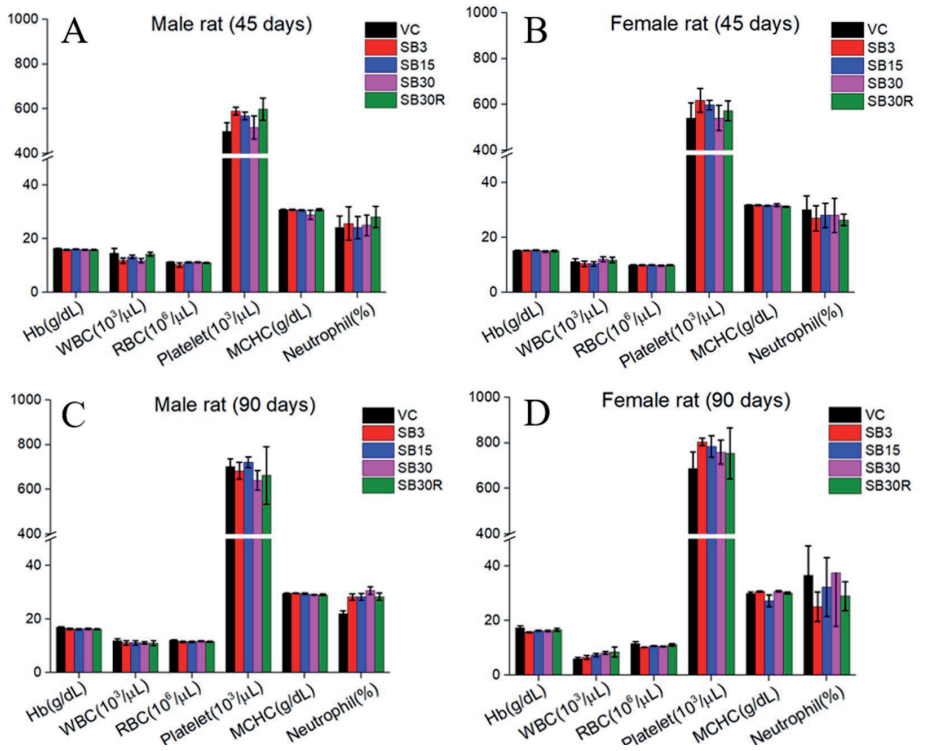

Fig. 3. Effects of Suvarna Bhasma on haematological parameters in rats. A) Male rats after 45 days of treatment, B) Female rats after 45 days of treatment, C) Male rats after 90 days of treatment, and D) Female rats after 90 days of treatment. Values are expressed as mean ± SEM (standard error of the mean) with *p < 0.05 vs vehicle control [n = 10 animal/ group, except Group V (n = 5)]. No significant alteration observed among any of the haematological parameters. Neutrophil counts for Suvarna Bhasma treated groups was increased for male rat after 90 days treatment without any statistical significance (p > 0.05).

levels in the groups SB30 and SB30R as compared to vehicle control females (Supplementary file S1-Table 4). All the above variations were well within the normal range, hence do not carry any toxicological significance.

Likewise, in the serum biochemistry performed at the termination (after 90 days), many biologically as well as toxicologically insignificant parameters either increasing or decreasing mere trends were observed (Fig. 4C and D). Those statistical significant variations in males included increased urea in mid and high dose (SB15, SB30 and SB30R), increased creatinine in high dose group (SB30), decreased TBIL at high dose (SB30), decreased creatinine and cholesterol in recovery dose group (SB30R) when compared to the vehicle control group (Fig. 4C and Supplementary file S1-Table 4c). Similar significant variations in females (Fig. 4D and Supplementary file S1-Table 4d) included decreasing trends AST and ALT in mid (SB15) and high (SB30) dose groups, increasing DBIL in SB15 and SB30, decreasing uric acid and increasing in glucose level in SB30 as compared to the female control group. However, all the above variations were well within the normal range, hence do not carry any toxicological significance.

3.2.3. Organ weight

No treatment-related adverse effects in absolute organ weight and relative organ weight were noticed during the study period Supplementary file S1-Table 5).

3.2.4. Gross pathology

There were no gross pathology observations recorded during the terminal necropsy examination of all animals including control and treatment groups. The only gross pathology observation recorded during the study was with the found dead animal (from SB30, Animal No. 459F, Datafile 1), it was suppuration in meninges and brain. This gross pathology observation was not related to treatment, but it was due to infection in meninges and brain.

3.2.5. Histopathology

Histopathological examination was performed on the specified list of tissues including macroscopically abnormal tissues of all control and high dose group animals sacrificed at termination. Tissues collected

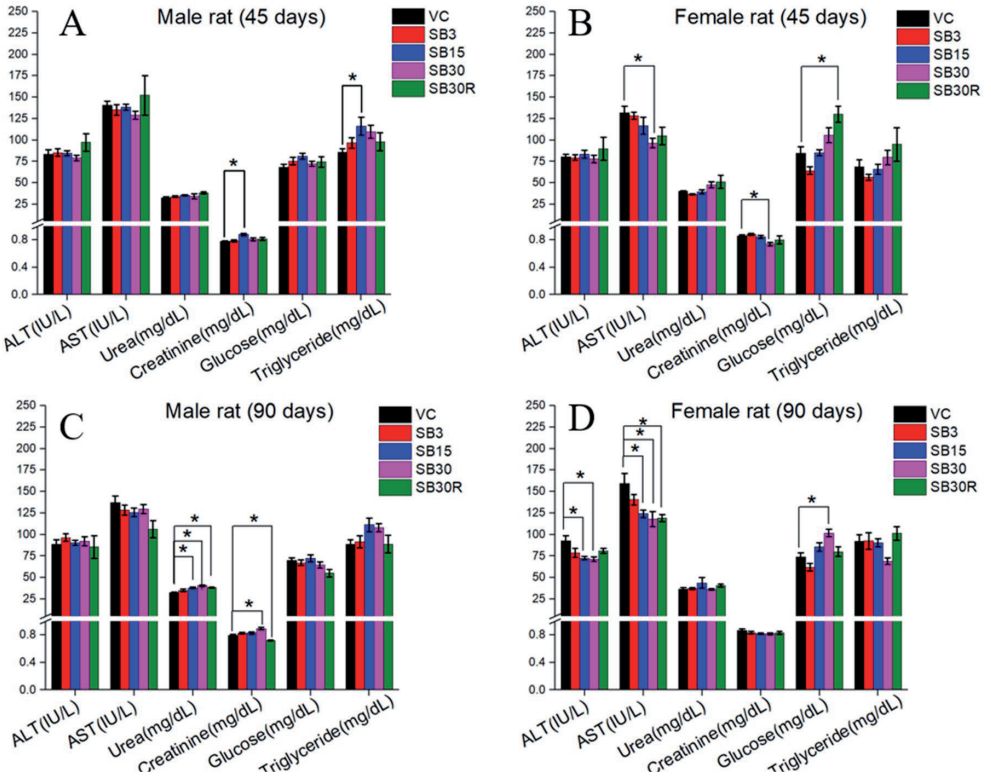

Fig. 4. Effects of Suvarna Bhasma on serum biochemistry in rats. A) Male rats after 45 days of treatment, B) Female rats after 45 days of treatment, C) Male rats after 90 days of treatment, and D) Female rats after 90 days of treatment. *p < 0.05 vs vehicle control [n = 10 animal/group, except Group V (n = 5)]. Alteration observed for few parameters such as urea, ALT, AST etc. However, the alterations were not consistent, for example, after 90 days treatment, urea and creatinine were significantly changed for male rats (SB15 and SB30) whereas for female rats the changes were not significant statistically for urea and creatinine parameters.

from a found dead animal (animal no. 459 F) were also processed for histopathology examination.

Four step grading system of minimal (+), mild (++), moderate (+++) and severe (++++) were used to rank microscopic findings for comparison among the groups. The histopathology lesions observed are summarized in the following table (Table 4) separately for males and females:

There were no significant macroscopic as well as microscopic treatment-related alteration observed, in any of the organs and tissues evaluated (Fig. 5). All above histopathological alterations that were observed were all considered as either spontaneous or known back-ground findings that are usually observed in laboratory rats of this strain and age under present experimental conditions.

3.3. Zebrafish behavioural study

3.3.1. Fish behaviour after 15-day Suvarna Bhasma treatment

Fig. 6 shows the behaviour alteration of various treatment. The zebrafish motor behaviours such as speed (Fig. 6A), meander (Fig. 6B) and freeze points (Fig. 6C) for Suvarna Bhasma treated (SB) group was similar to vehicle control group (VC) and no significant alteration ob-served between these two groups. Whereas, the alcohol-treated group (CC) showed a significant increase in speed and meander ($p < 0.05$). Caffeine treated (PC) group showed lower speed, meander and en-hanced number of freeze points ($p < 0.05$). In geotaxis behaviour, SB group preferred the upper zone as compared to the VC group, similar to the CC group (1.5% alcohol), although the motor behaviour of SB group was not similar to the alcohol-treated group. In contrary, caffeine-treated group (PC) preferred the upper zone least in the novel tank (see Fig. 7 for representative track plot). From the upper-lower zone tran-sitions, it was observed that for CC and PC group, the zone transition was lowest (Fig. 6E). The average height of swimming (Fig. 6F) for various groups showed that alcohol group swam at the maximum height throughout the tracking periods indicating anxiolytic effect, whereas, caffeine-treated anxious fish swam at the minimum height. Suvarna Bhasma treated group swam at more height than the control group, which indicates the anxiolytic behaviour.

Table 4
Grading of histopathological observation in the vehicle control group (VC) and 30 mg/kg *Suvarna Bhasma* treated group (SB30).

Tissue and Histopathological Observations	Male (numbers)		Female (numbers)	
	VC	SB30	VC	SB30
	(VC)	(30 mg/kg)	(VC)	(30 mg/kg)
Number of animals examined	10	10	10	10
Lungs				
Alveolar histiocytosis, focal (+)	1	0	1	0
Increased size of BALT (+) to (++)	1	1	1	1
Leukocytic infiltration in and around bronchiolar lumen (+++) to (++++)	2	0	0	1
Liver				
Hepatocellular hypertrophy, focal (+)	0	1	1	1
Spleen				
Increased EMH, focal (+)	1	1	1	1
Kidney				
Dilated medullary tubules, focal (+)	0	1	0	1
Vacuolar degeneration in cortical tubules, focal (+)	1	0	1	0
Mesenteric LN				
Increased histiocytes in medullary sinuses (+)	1	0		
Colon				
Enlarged GALT (+)	1	2	1	0
Testes				
Atrophy of seminiferous tubules near vasa recta (++)- unilateral	1	0		
Atrophy of seminiferous tubules, multifocal (++)- unilateral	0	1		
Cervical LN				
Increased histiocytes in medullary sinuses (+)			1	0

Key: (+) = Minimal, (++) = Mild, (+++) = Moderate and (++++) = Severe; MNC = Mononuclear Cells, LN = Lymph Node, GALT = Gut Associated Lymphoid Tissue, BALT = Bronchiole Associated Lymphoid Tissue, EMH = Extra Medullary Haematopoiesis.

3.3.2. Behaviour of zebrafish 30 days after the treatment completion

The treatment regime was daily dosing for 15 days. After behaviour tracking on Day 16, 30 fish from each VC and SB groups were further kept for 30 days in the housing tank without Suvarna Bhasma treatment. After 30 days without treatment, i.e. on day-46, the behaviour tracking was repeated to study recovery from treatment. The SB and VC groups showed analogous motor behaviour (speed, meander and freeze point, Fig. 8) to each other without any significant variation on day-46. However, here also the SB group preferred the upper zone significantly (spent 2.4 min) as compared to VC group (1.49 min, $p < 0.05$).

4. Discussions

Suvarna Bhasma is one of the most expensive medicine in Ayurved yet frequently prescribed by Ayurved practitioners for health improvement. According to the traditional Ayurved practitioners, to ensure safety and efficacy, textual manufacturing process needs to be followed stringently. However, due to the larger production urges and malpractices, many Ayurved drug manufacturers do not follow the traditional ways to manufacture Suvarna Bhasma, which could lead to toxic effects instead of its benefits. Variation of physicochemical properties of such metallic based medicines can lead to serious ill-effects in patients. Modern science has also established the vital role of physicochemical properties such as size, shape and chemical composition of the nanomedicines for its biological effects. (Liu et al., 2013). In view of this, this in-depth study was done.

The Suvarna Bhasma manufacturing process, which resembles the top-down method of modern nanoparticle preparation, the gold bar is reduced to gold particles, size varying from 10 nm to 60 μm. During rigorous annealing steps (14 heating and cooling cycles) the nanogold particles formed in Suvarna Bhasma were in spherical shape. EDAX mapping demonstrated that the nano-sized gold particles were surrounded by Si, O, P, Fe and Ca. The entrapment of nano-gold particles might happen in the heating steps, SiO2 mainly come from the earthen pot used during the preparation process. ICP-AES study revealed that Suvarna Bhasma used in this study contained approximately 98% Au along with Si, Fe, Na, and Ca. It is important to note that from a toxicological viewpoint that despite being used in the manufacturing process, Hg was not found in the finished product even after using three different elemental analytical methods, and this is in agreement with the toxicological data presented above. This can be explained as follows:

In the manufacturing process, the mercury in the Hg–Au amalgamate was extracted and eliminated with the help of S powder successively in each of the 14 cyclic heating and cooling steps. Due to the strong affinity between Hg and S and the repetitive heating and cooling steps, Hg was completely eliminated.

The toxicity assessment of Suvarna Bhasma in the rat model was conducted with the dose up to 30 mg/kg which was approximately 10 times higher as compared to the therapeutic dose in human. In haematological parameters, after 90 days, neutrophil counts in the male rats of all Suvarna Bhasma treated groups was increased compared to the control group. However, this alteration was not statistically significant. Moreover, neutrophil counts with similar dose did not causes similar changes in Suvarna Bhasma treated female rats. Therefore, haematology parameters in rats did not indicate any adverse effect of Suvarna Bhasma, even at a considerably high dose as compared to the therapeutic dose in human.

A few alterations in the serum biochemical parameters were observed in rats. ALT, AST parameters decreased significantly in the female rats at 15 mg/kg, and 30 mg/kg dose level after 90 days of treatment, however, such alteration was not observed in male rats. These liver enzymes are elevated only during cell damage. Urea and creatinine were significantly increased in the male rats after 90 days for higher dose Suvarna Bhasma treatment. In female rats such effects were not seen. At the low dose (3 mg/kg, therapeutic dose) treatment, both haematology and biochemical parameters did not change except bilirubin in the female rat after 45 days of treatment (Supplementary file S1-Table 4). Most importantly, the changes in the biochemical parameters were well within the normal range and acceptable limits. The histopathological alterations observed at a dose of 30 mg/kg are usually observed in the laboratory rats of this strain and age under present experimental conditions. The feed intake, body and organ weights were not changed due to the Suvarna Bhasma treatment. Therefore, in summary, from the rat study, it can be concluded that Suvarna Bhasma is safe up to a dose of 30 mg/kg (10 times the therapeutic dose).

Similar to our work, a previous study also reported no observed adverse effect level of Suvarna Bhasma up to 13.5 mg/kg dose in Wistar rats (Jamadagni et al., 2015). Whereas, several beneficial effects of Suvarna Bhasma are also reported in literature especially as an immunomodulator: In an experimental study in mice, Suvarna Bhasma significantly ($P < 0.001$) increased counts of peritoneal macrophages and stimulated phagocytic index of macrophages indicating its immunostimulant effect (Bajaj and Ahmad, 2001). Suvarnamalini vasanta a Suvarna Bhasma containing generic preparation exhibited immunomodulatory potential as evidenced by an increase in percent phagocytosis and protection against E. coli induced peritonitis in mice (Sangle et al., 2004). Madhu-Ghrita-Swarna-Vacha combination showed a significant effect on humoral antibody formation in neonates and it

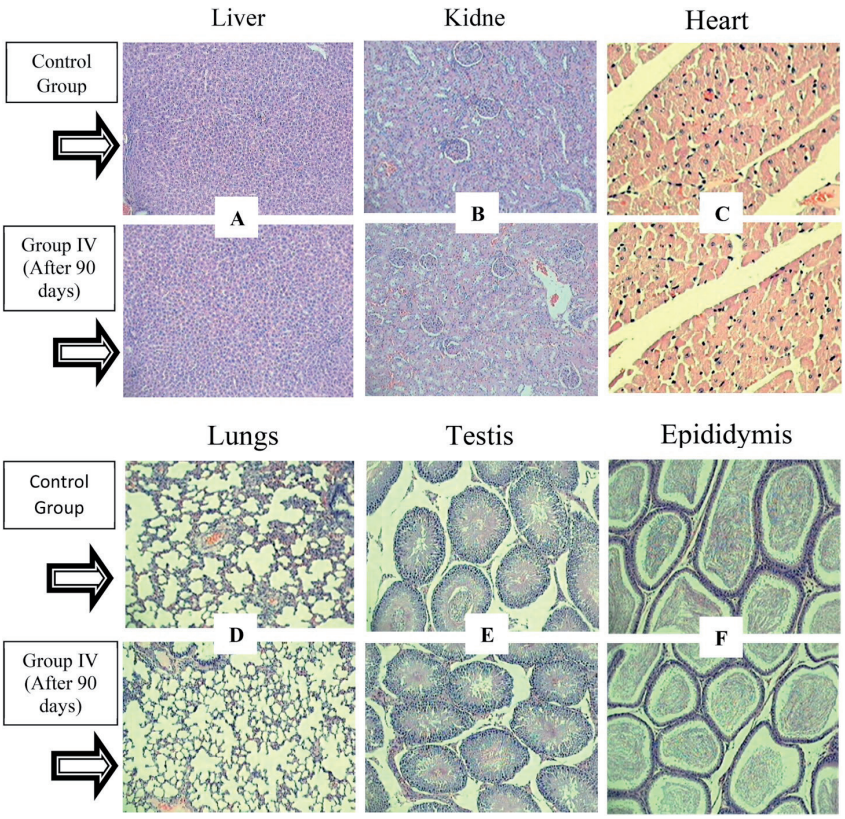

Fig. 5. Histopathology of vehicle control (VC) and 30 mg/kg SB treated rat (SB30). Various organs were imaged, such as A) Liver B) Kidney C) Heart, D) Lungs, E) Testis, and F) Epididymis. No treatment related alterations in tissue morphology were observed.

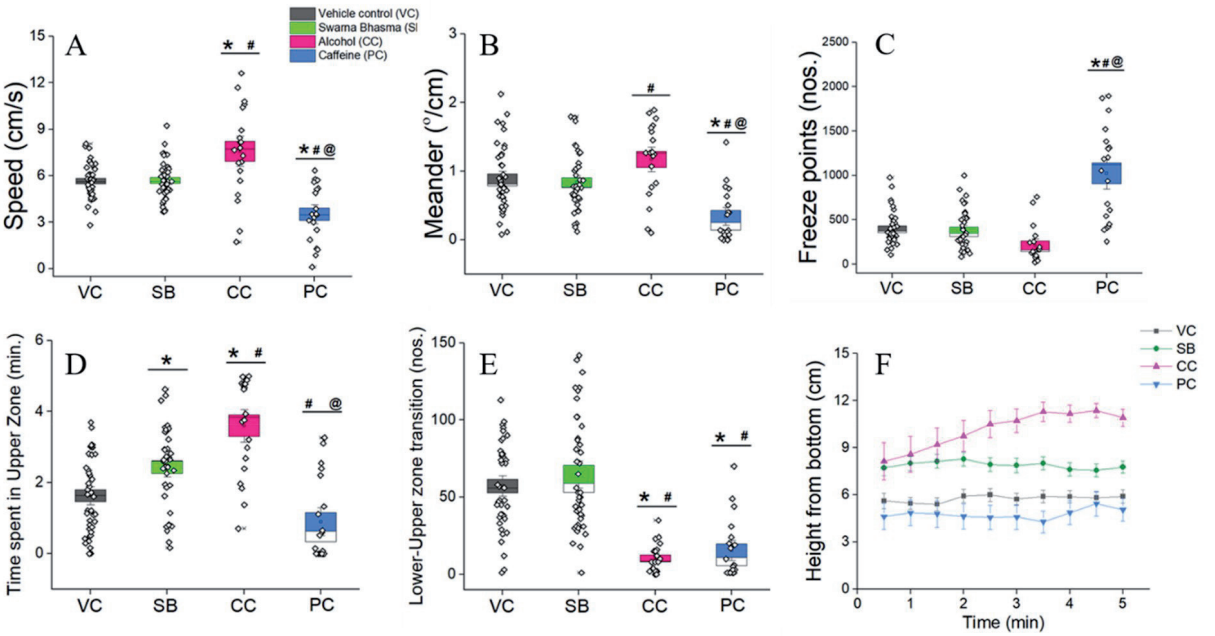

Fig. 6. Behavioural study of Suvarna Bhasma treated zebrafish. The fish were tracked a day after 15 days of Suvarna Bhasma treatment. A) Speed, B) Meander, C) Freeze points, D) Time spent in the upper zone, E) Number of transition between upper zone and lower zone, and F) Average swimming height from the bottom of the novel tank. VC=vehicle control group, SB = Suvarna Bhasma treated group, CC = comparative control, and PC = positive control group. Values are expressed as mean ± SEM with *p < 0.05 vs VC group, #p < 0.05 vs SB group and, @p < 0.05 vs CC group. No statistical variation was observed between VC and SB groups in motor parameters. However, geotactic behaviour such as the preference of swimming zone significantly varied between SB and VC groups.

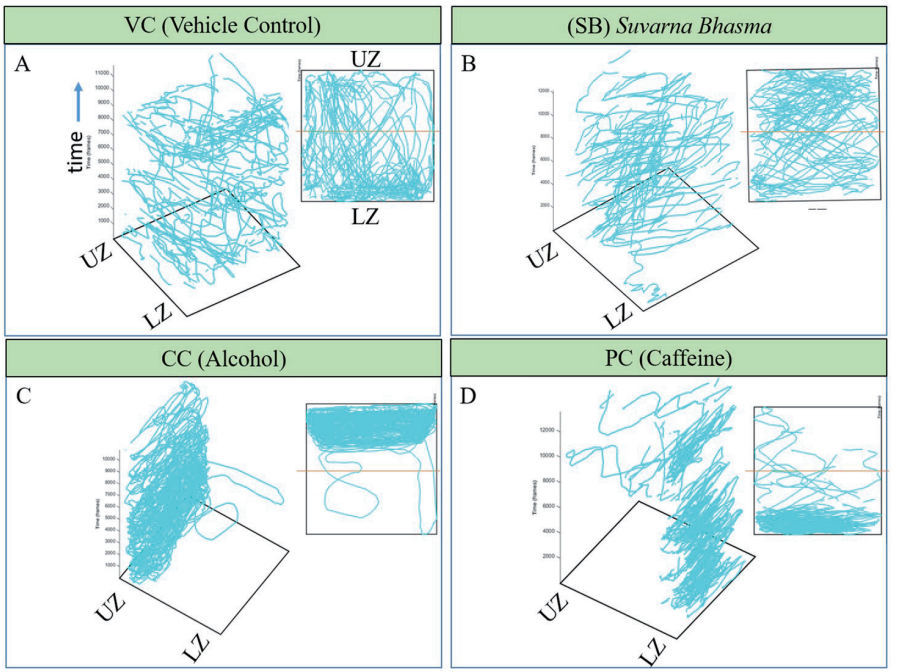

Fig. 7. Representative 3D and 2D track plots of zebrafish behaviour (taken from front view videos) showing the swimming traces and time spent in upper and lower zone. 3D track plot is
showing fish position in x-y coordinate with time (frame number). A) VC=vehicle control, B) SB= Suvarna Bhasma treated, C) CC=comparative control, and D) PC=positive control group.

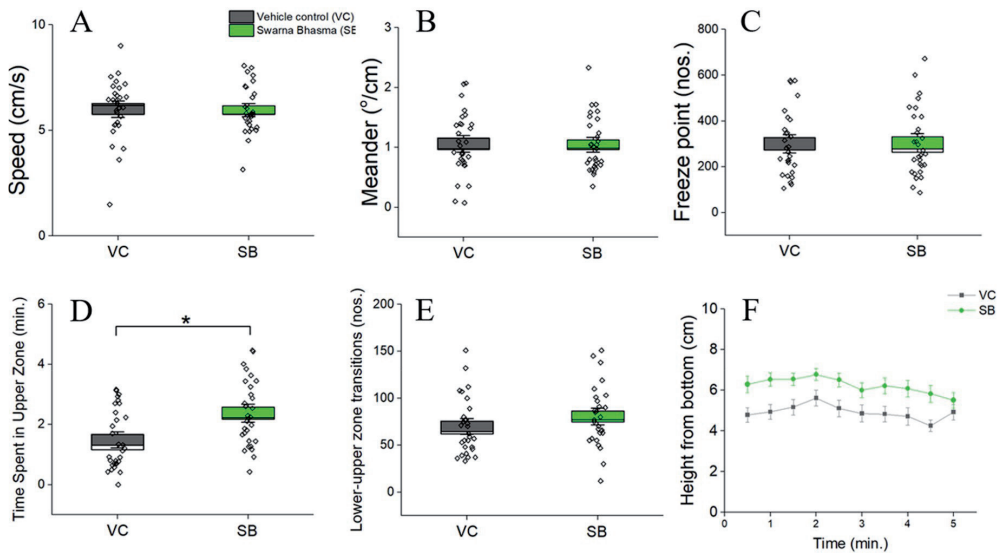

Fig. 8. Zebrafish behaviour of Suvarna Bhasma treated groups 30 days after completion of the drug treatment. A) Average speed, B) Meander, C) Freeze points, D) Time spent in the upper zone, E) Number of transition between upper zone and lower zone, and F) Average swimming height from the bottom of the novel tank. VC=vehicle control group, and SB = Suvarna Bhasma treated group. Values are expressed as mean ± SEM (n = 30 animal/group) with *p < 0.05. All motor parameters of Suvarna Bhasma treated fish were normal and analogues with the vehicle control group. However, here also SB group preferred upper zone compared to the VC group.

acted on the immunological system, which was evident by triggering the response of immunological system by a rise in the total proteins and serum IgG levels (Jyothy et al., 2014b). Suvarna bhasma was evaluated in a global and focal model of ischaemia in albino rats (Shah and Vohora, 2002). Enzymatic parameters (lipid peroxidase, reduced glutathione, catalase, glutathione reductase, glutathione-S-transferase, glutathione peroxidase, superoxide dismutase, and glucose-6-phosphate dehydrogenase) were used to assess the ischaemic brain damage and its modulation by using Suvarna Bhasma. Suvarna Bhasma (25 mg/kg, orally for 10 days) significantly restored the altered values to near normal levels suggesting its potential in cerebrovascular diseases.

On the other hand, zebrafish behavioural assessment is able to screen various toxic or neurotoxic drugs. Drug-induced anxiogenic or anxiolytic behaviours are well defined in the literature for zebrafish (V Kalueff et al., 2013). Motor behaviour, scototaxis, geotaxis and cohort behaviour of zebrafish are studied extensively in recent literature (Maximino et al., 2010). In this study, we find that the motor behaviour of zebrafish did not alter significantly due to the action of Suvarna Bhasma treatment. Average speed, meander, freezing points etc. for Suvarna Bhasma treated and control fish were similar. However, geotaxis behaviour such as swimming height and preference of height zone was significantly changed ($p < 0.05$) in Suvarna Bhasma treated fish compared to the vehicle control group. The SB and CC groups spent more time in the upper zone (> 2 min) as opposed to the VC and PC groups (less than 2 min). Although, the motor behaviour of CC group differ from SB group. Usually, zebrafish in the novel tank initially prefer the bottom of the tank due to protective intuition. Preference to upper zone in the novel tank for Suvarna Bhasma treated groups indicated anxiolytic-like behaviour similar to alcohol-treated groups (CC) which is anxiolytic to zebrafish (Tran et al., 2016). Anxiogenic caffeine-treated fish, in contrast, preferred lower zone.

There is always a chance that toxicity of any drug may appear long after completion of the drug treatment. To address this issue, in this study, zebrafish were tracked 30 days after completion of the drug treatment, which resulted in no unnatural motor behaviour in the fish. However, similar to the previous novel tank experiment, Suvarna Bhasma treated fish preferred upper zone compared to the VC group. For zebrafish, preference towards the bottom of the tank is in response to the novel environment. However, for SB treated fish, it swims at a higher level in compared to VC group. Therefore, it can be inferred that Suvarna Bhasma has an anxiolytic effect on zebrafish behaviour model and this anxiolytic effect presumed long after completion of the drug treatment.

Gold, in modern medicine, is used mainly in the therapy of rheumatoid arthritis. Additionally, bio-liberated gold ions from gold implants mediate antiapoptotic, anti-inflammatory and neuroprotective effects (Østergaard et al., 2010). Ionic gold inhibits of proinflammatory mediators such as tumor necrosis

factor alpha (TNFα), interleukin-1 and interleukin-6, leukotrienes, prostaglandins, nitrogen oxide and lysosomal proteases (Østergaard et al., 2010). Gold ion also heals injured neurons by increasing neurotrophin (NT-4), transforming growth factor-beta 3 (TGF-β3), leukemia inhibitory factor (LIF), and metallothionein (MT-I + II) which may be the reason for the neuroprotective effect of gold. Interestingly, in Ayurveda Suvarna Bhasma is also used as a nerve tonic and to treat various neuronal diseases. In recent studies, several neurons related beneficial effect has been reported in the literature, such as neuroprotective against cognitive impairment or as anxiolytic and antidepressant agent (Bajaj and Vohora, 2000). Although Suvarna Bhasma contains gold particles (Au^0), it can release ionic gold in biological medium (e.g. gastric fluid). This bio-released gold ion may contribute its neuroprotection.

Anxiety, depression and various neurodegenerative diseases are widely prevalent in the modern stressful lifestyle. Anxiety is a part of the normal behavioural repertoire of defence mechanism to deal with novel environment. GABAergic neurotransmission in the amygdala altered anxiety-driven response (Nuss, 2015). Many research found that fear and anxiety in several animals decreases due to the infusion of GABA into the amygdala. Other neurotransmitters also recognised as anxiety modulators, such as opioid peptides, serotonin oxytocin and endocannabinoids. Suvarna Bhasma affects opioidergic mechanism (Bajaj and Vohora, 2000). In a previous behaviour study in rat, Suvarna Bhasma was found as anxiolytic, antidepressant and anti-cataleptic (Bajaj and Vohora, 2000). In our study, we find anxiolytic behaviour in zebrafish repeatedly in every set of experiment for Suvarna Bhasma treated fish. However, the exact mechanism for the anxiolytic effect was unknown, and further investigation is required to establish the pathways which Suvarna Bhasma acts in anxiolysis.

5. Conclusions

This study finds that gold in Suvarna Bhasma is present in two forms, one consisting of larger gold particles with crevices and second form consisting of gold nanoparticles, some of which are present in the crevices along with small amounts of Si, O, Fe etc. The larger gold particles consist of polycrystalline agglomerated gold of up to 60 μm size having crystallites of size 45 ± 2.8 nm. In the Suvarna Bhasma manufacturing process, Hg is used, however the finished product did not show the presence of Hg by any of the elemental detection methods (EDAX, and ICP-AES). The in-vivo studies of Suvarna Bhasma suggest no major adverse effects in rat model. In the zebrafish behavioural model, Suvarna Bhasma exhibited anxiolytic effects. Therefore, it can be inferred that the Suvarna Bhasma used in this study is safe and has an anxiolytic effect.

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Declaration of competing interest

Three co-authors are from Shree Dhootapapeshwar Limited, which has also funded the study. The other authors declare no conflict of interest.

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Appendix A. Supplementary data

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