IJBPAS, December, 2024, 13(12): 6603-6615

ISSN: 2277–4998

International Journal of Biology, Pharmacy and Allied Sciences (IJBPAS)

'A Bridge Between Laboratory and Reader'

www.ijbpas.com

ACUTE ORAL TOXICITY EVALUATION OF *POTENTIATED YASHADA BHASMA* (ZINC OXIDE NANO-PARTICLES)

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Received 8th July 2024; Revised 5th Sept. 2024; Accepted 9th Oct. 2024; Available online 1st Dec. 2024

https://doi.org/10.31032/IJBPAS/2024/13.12.8557

ABSTRACT

Ayurvedic pharmaceutics deals with preparation and processing of metal and mineral based medicines. *Bhasmikarana* (incineration) is one such method to make the drug particles in nano scale aiming for better assimilation and bio-availability. Added on with such classical processing techniques, curating *bhasmikarana* along with mechanical size reduction techniques raises the question of greater bio-availability in lesser doses than recommended. Hence this paper attempted to evaluate the acute toxic effects of one such curated formula - Potentiated *Yashada Bhasma* (PYB) as a primary step in exploring the drug's safety and efficacy following OECD guidelines 420 with five female wistar rats. After confirmation of nil effects in sighting study (2000 mg PYB/kg) on one animal, rest four animals were also given the same dose and observed for adverse effects at different time points up to 14 days. On 15th day blood was collected for haematological and biochemical evaluation and later all the animals were euthanized. Major organs were dissected and sent for histopathological examination. The results revealed slight alterations in the total WBC count, neutrophils, SGOT and total bilirubin levels. Histopathological findings showed mild to moderate congestion in all major organs along with slight inflammatory changes especially in kidney, brain

and liver while symptomatically all the animals appeared completely normal at 2000mg/kg, which is almost ten times of therapeutic dose of *Yashada bhasma*. Thus, it can be concluded that PYB at 2000 mg/kg is found symptomatically safe in-spite of mild varied values with blood and histopathological investigations. However clinically this higher dose is very unlikely to occur, thus PYB can be considered safe.

Keywords: Zinc oxide nano particles, *yashada bhasma*, safety, adverse effects, metallic medicines

INTRODUCTION:

Rasashastra - the alchemy branch of Ayurveda, deals with metallic and mineral preparations to convert them into most potent, safe and assimilable forms for better therapeutic effects. The processes of drugs in *Rasashastra* are focused primarily to minimize the dose, to make the medicine completely imperceptible to taste and to produce instantaneous effects invariably **[1]**. The fact, that classically synthesized ayurvedic *bhasma* (nanoparticles) are capable enough of rendering large bio-availability in no time upon administration, is unquestionable. One of the key factors responsible for exhibition of such property is the size reduction during various processing techniques like *shodhana* (purification), *marana* (incineration), *jarana* (roasting) etc., that ultimately promotes the particles' fineness leading to increased absorption systemically **[2]**.

Yashada (zinc) in Ayurvedic system of medicine is a wonder drug in treating and managing respiratory illness, parkinsonism, diabetes etc., **[3]** Elmorsy EH *et al*. (2024) conducted an experimental study investigating the impact of high-fat diet (HFD) on spermatogenesis and testicular function in rats, with a focus on zinc's potential protective effects. The 12-week study revealed significant HFD-induced testicular dysfunction, oxidative stress, and inflammation, which were mitigated by zinc supplementation **[4]**. Seetha Chandran *et al*. (2019) assessed the anticancer efficacy of *Yashada Bhasma*, an Ayurvedic zinc-based preparation, against pancreatic cancer. Two formulations, *Parada Marita yashada Bhasma* (PMY) and *Vanaspati Jarita Marita Yashada Bhasma* (JMY), exhibited significant dose-dependent growth inhibition in human pancreatic cancer cells (MIA PaCa-2) in vitro. These findings suggest *Yashada Bhasma*'s potential as a bio-active nano-sized therapeutic agent for cancer therapy as well **[2]**.

With existing classical techniques for processing metals and minerals, additionally applying intermediate size reduction to raw mineral and metal drugs beyond already specified pharmaceutical techniques, presents an opportunity for enhanced efficacy. This

process potentially allows for lower doses, resulting in finer particles that promote swift absorption and optimal therapeutic outcomes. Alongside such creative horizons with Ayurvedic preparations, safety and adverse check is yet another important aspect to be analysed at every step made before introducing into clinical use.

Building on existing processing methods, this study investigates the acute toxicity of Potentiated *Yashada Bhasma* (PYB), a curated formulation prepared by incorporating additional size reduction techniques into the traditional manufacturing process of classical *Yashada Bhasma*. The study adheres to the guidelines outlined in OECD 420 (Organization for Economic Cooperation and Development), ensuring rigorous evaluation of PYB's safety profile.

MATERIALS AND METHODS:

PHARMACEUTICAL PART:

The test drug PYB was prepared in the laboratory of *Rasashastra* and *Bhaishajya kalpana* of KAHER's Shri BMK Ayurveda Mahavidylaya, Belagavi. For the same, raw sample of *Yashada* was procured from Shri Dhootpapeshwar Ltd (batch no. 1PRML00278/Jun/2021) with certificate of analysis having purity of 99.62% w/w. After authentication of *Yashada* (certificate no. CRF/Auth 42/2021) by the experts of the AYUSH Approved Drug Testing Laboratory at Shri BMK Ayurveda Mahavidyalaya, *shodhana* of *parada* (mercury)*, gandhaka* (sulphur) and *yashada* were carried out following the classical methods specified in the text *Rasatarangini* **[5]**. *Samanya shodhana* (general purification method) and *vishesha shodhana* (specific purification method) were done only to *yashada* followed by *Jarana* (roasting) **[1]**. As a first step of potentiation, the *jarita yashada* (*yashada* that has undergone *jarana* process), was subjected to additional size reduction with the aid of a ball mill for 24 hrs thus converting granular form of *jarita yashada* into a finer one. Following the reference of another classical text *Rasachandamshu* **[6]**, the above obtained ball-milled *jarita yashada* was further subjected to thorough grinding in wet grinder for palettization with the fresh juice of aloe vera and lemon juice later was processed in Electric Muffle Furnace (EMF) for *marana* (incineration) in *gajaputa* (950°C). The same way four such consecutive *marana* were carried out in EMF in-spite of attainment of all *bhasma siddhi lakshanas* (classical characteristic tests for nano sized particles) by the end of second *marana*. This was done to aid further size reduction after the second incineration. The next step followed is subjecting the obtained sample from the above

step for instrumental screening to confirm the desired particle size and compound formation and thus arrived at the end point of preparation of PYB.

EXPERIMENTAL PART:

Animal experimentation was carried out at CPCSEA approved Animal House Facility (License no. 1017/PO/Re/S/06/CPCSEA) of KAHER's Shri BMK Ayurveda Mahavidyalaya, Belagavi, Karnataka. Upon obtainment of ethical clearance (Approval no. BMK/IAEC/Res.No.-21/2021-11) from the Institutional Animal Ethical Committee, acute toxicity study of PYB was done following the guideline number 420 of OECD **[7]**. For the same, five female wistar rats (aged eight to 12 weeks) that weighed about 150 - 250 g were bought from licensed animal breeder at Pune. All the animals were acclimatized for five days before commencement of the experiment under specified ambient temperature (25 °C± 3°C) and humidity maintained at 40 - 60%. Rat food (Scientist's Choice rat pellets feed supplied by VRK Nutritional Solutions, Pune India) and tap water was provided for all the animals throughout the experiment.

Proper identification marks were made on each animal and one out the five animals was randomly chosen for sighting study to decide the dose for acute toxicity. Selected animal was kept under 12 hrs fasting (only water was fed) prior to the administration of sighting dose. 2000 mg/kg of PYB was administered orally (344 mg of PYB for 172 g weighed animal) along with 2 ml of 0.5% sodium CMC (carboxy-methyl cellulose) solution. Three hours of fasting was maintained post administration of sighting dose. Observations on signs of toxicity, changes in skin/fur, changes in eyes, mucus membrane, somatomotor activity, behavior pattern, tremor, convulsions, salivation, diarrhea, lethargy, sleep and coma were monitored after 2, 4, 6, 8, 12, 24 and 48 hours of sighting dose administration. After confirming the absence of any such sign in the animal, the same dose (2000 mg/kg) was decided as the dose for acute toxicity study.

For the same, the remaining four animals were made to fast for 12 hours before commencement and later administered with 2000 mg/kg of PYB orally with 0.5% sodium CMC solution. Observations regarding signs of toxicity, changes in skin/fur, changes in eyes, mucus membrane, somatomotor activity, behavior pattern, tremor, convulsions, salivation, diarrhea, lethargy, sleep, coma were monitored at 2, 4, 6, 8, 12, 24 and 48 hours post administration of toxic dose. Observations regarding signs of toxicity, clinical signs and mortality were

monitored at least once daily for 14 days for all the animals including sighting study animal. Any signs of abnormal behavior or toxicity were scored and recorded on scale 0 to 5. Body weight was recorded on days 1, 7, 14 & 15 after dosing. On 15th day blood was withdrawn from retro-orbital vein of all the five animals and sent for assessing haematological and biochemical parameters after storing in EDTA non EDTA bulbs. Later all the animals were euthanized by inhalation of di-ethyl ether and all the major organs (ovaries, uterus, fallopian tubes, kidney, spleen, pancreas, heart, liver and brain) were dissected by taking mid-line incision in the abdomen, later weighed and then sent for examination of histopathological changes.

RESULTS:

Results of the sighting study indicated no evidence of adverse toxicity in the test animal. This finding was corroborated by the acute toxicity study, where no subjects exhibited adverse effects at the same PYB dose. Behavioral assessments revealed no alterations throughout the observation period. Moreover, all animals exhibited gradual weight gain by day 15 as summarized in **Table 1**. Additional results included onset of increased food consumption within 48 hrs of dosing and faecal colour change to light yellow with moderate softening that lasted up to one day.

Results of both biochemical as well as haematological parameters are shown in **Tables 2 and 3** respectively. It can be seen that none of the parameters are found to be alarmingly increased in all the test animals in-spite of gradual weight gain. Even when the values are to be compared between individual animals no any significant change seem to be present. There was not much of notable change in the weight of major organs as well **(Table 4)**.

The histopathological examination revealed a range of changes across major organs. Congestion was a common finding, affecting the ovaries, brain, heart, kidney and spleen **(Figure 01: (a) to (h))**. The brain also displayed mild oedema, inflammation, and mild neuronal eosinophilia. The heart showed cardiac muscle separation, while the kidneys suffered tubular desquamation. Liver damage included inflammation, ballooning degeneration, spotty necrosis, and cholestasis. Additionally, the spleen exhibited mild to moderate congestion and lymphoid hyperplasia.

Table 1: Weight of the animals during the study

Sl. No.	Animals	Weight of the animals in gram			
		Day 1	Day 7	Day 14	Day 15
1.	Tail - A	172	176	184	185
2.	Head - B	166	175	190	192
3.	Left arm- C	165	172	180	185
4.	Right arm- D	155	167	182	186
5.	Unmarked - E	153	156	169	173

Table 2: Effect of PYB on biochemical parameters of all animals

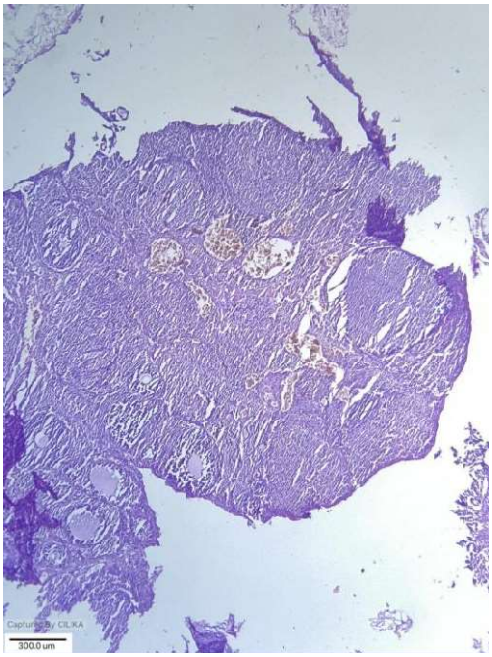
Parameter	A	B	C	D	E
Total bilirubin (mgs%)	0.4	0.4	0.5	0.4	0.5
Direct bilirubin (mgs%)	0.1	0.1	0.1	0.1	0.1
SGOT (U/L)	44	29	46	25	34
SGPT (U/L)	37	41	34	42	43
Alkaline phosphatase (U/L)	140	60	55	32	110
Total protein (gm%)	8.6	8.1	8.5	8	7.5
Albumin (gm%)	4.6	4	4.5	4.1	3.7
Urea (mgs%)	29	42	36	30	35
Creatinine (mgs%)	0.4	0.5	0.4	0.6	0.5

Table 3: Effect of PYB on haematological parameters of all animals

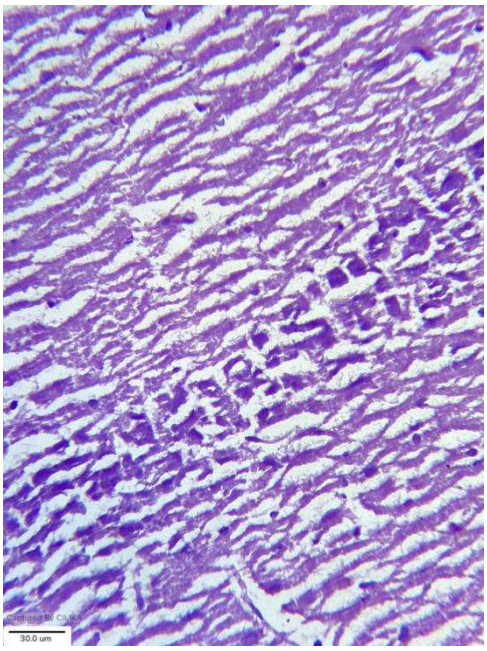
Parameter	A	B	C	D	E
Hemoglobin (gm%)	14.3	13.4	14.3	14.3	12.9
Total WBC (10^3 cells/cu.mm)	15.22	27.60	11.23	8.03	19.77
Neutrophils (%)	20	84	15	26	15
Lymphocytes (%)	71	40	76	68	74
Eosinophils (%)	01	02	00	01	01
Monocytes (%)	08	00	09	05	10
MCH (pg)	18	18.5	17.6	18	17.4
MCV (fl)	53.3	52.6	51.2	52.1	50.9
MCHC (gm/l)	33.8	35.1	34.3	34.5	34.1
RBC (million cells/cu.mm)	7.93	7.28	8.16	7.97	7.43
Platelets (10^5 cells/cu.mm)	5.24	6.01	4.84	5.08	4.39
PCV (%)	42.3	38.3	41.8	41.5	37.8
RDW (fl)	50.9	44.8	48.7	47.4	41.1

Table 4: Weight of major organs of all the animals

Organs	A	B	C	D	E
Ovaries, Uterus and Tubes	0.59 gm	1.16 gm	0.97 gm	0.75 gm	0.72 gm
Kidney	1.36 gm	1.18 gm	1.38 gm	1.24 gm	1.44 gm
Spleen	0.77 gm	0.59 gm	0.61 gm	0.59 gm	0.91 gm
Pancreas	0.69 gm	0.51 gm	0.48 gm	0.58 gm	0.46 gm
Heart	0.71 gm	1.00 gm	0.67 gm	0.89 gm	0.82 gm
Liver	7.19 gm	7.53 gm	8.18 gm	8.24 gm	7.64 gm
Brain	1.51 gm	1.47 gm	1.60 gm	1.52 gm	1.18 gm



(a)



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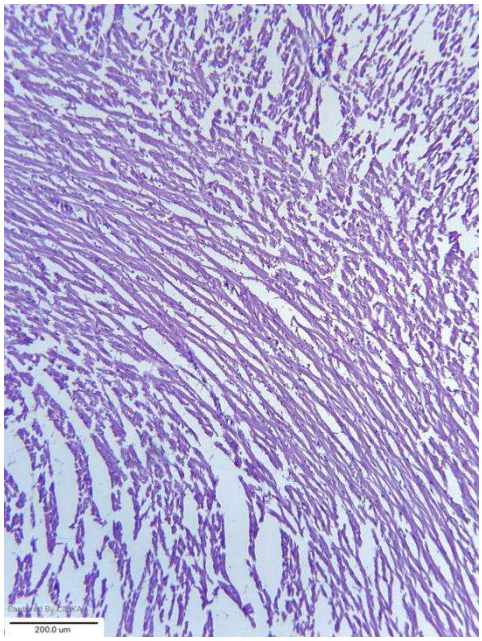

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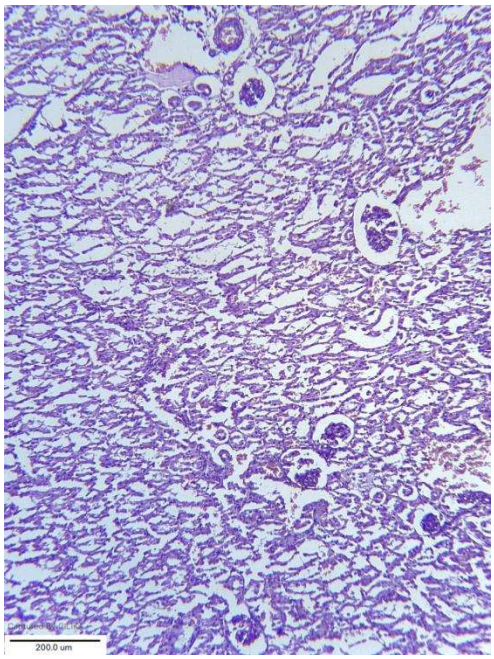
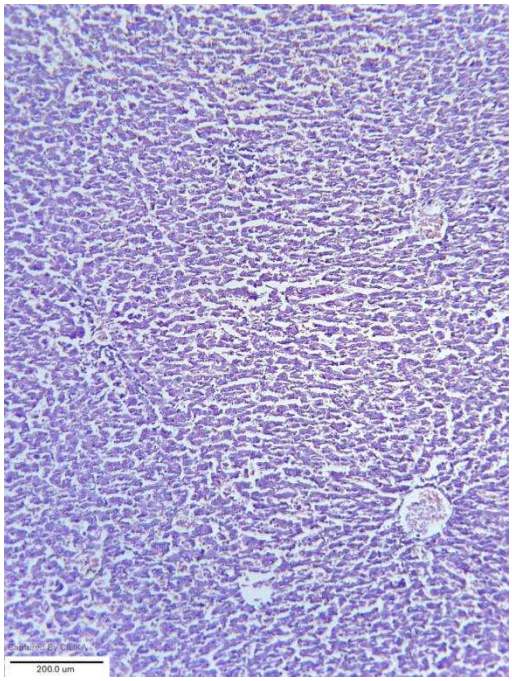
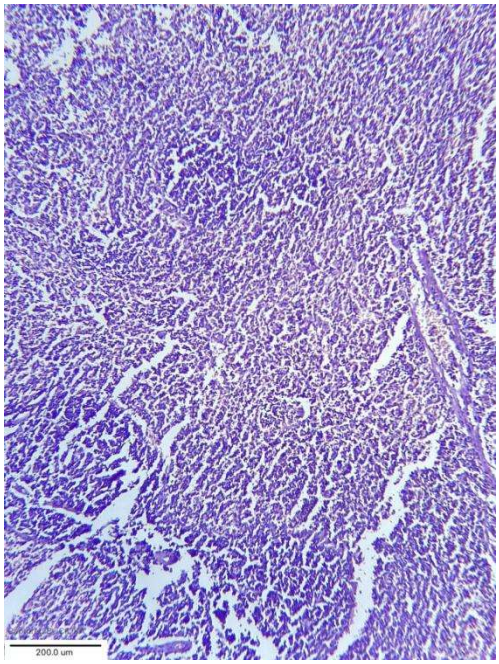


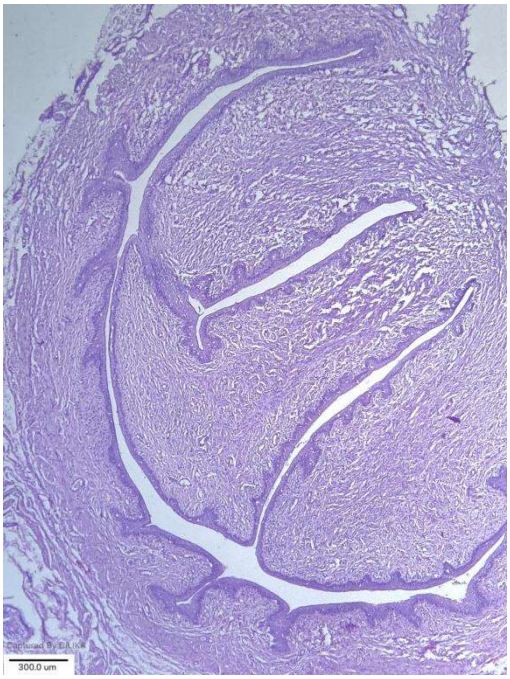
(d)



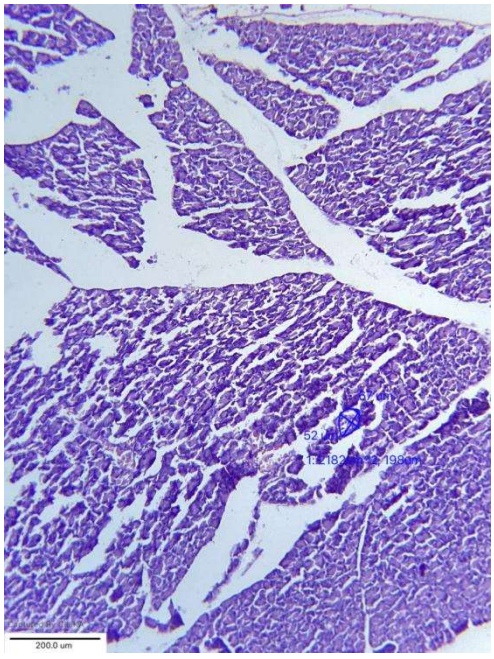
(e)



(f)



(g)



(h)

Figure 1: Images of histopathological examination of major organs. (a) Ovary (b) Brain (c) Heart (d) Kidney (e) Liver (f) Spleen (g) Uterus (h)

DISCUSSION:

There has always been a concern of heavy metal toxicity for metallic and mineral based preparations. To overcome such fear and also to gain clarity with respect to careful dose fixation in clinical practice, assessing the toxic potential of any formulation is invariably essential. In the present study, toxicity of PYB was assessed as there was additional size reduction hours compared classical *bhasma* preparation. The OECD Guidelines 420 is a globally recognized standard protocol for acute oral toxicity testing, ensuring scientific validity, animal welfare, and regulatory compliance. It provides a standardized, step-by-step approach to evaluate chemical toxicity, minimizing animal numbers and distress while promoting reliable and reproducible results. In rodent toxicity testing, rats are the species of choice, and females are commonly used. Research has shown that while sex-related differences in sensitivity are generally minimal, females are slightly more sensitive than males in instances where differences are observed **[7]**. Due to this reason the present study specifically employed female species to test acute toxicity. During the ethical permission approval total of 18 animals were sanctioned for any mortality during the sighting study for step down procedure as per OECD 420 guidelines. As no mortality was observed, so the study was completed with initial five animals only.

Ashish Arora *et al*., (2023) in their study explored the toxic effects of *yashada bhasma* and *yashada pushpa bhasma* in swiss albino mices at doses each 15.6 mg/kg and 78 mg/kg in comparison with control group. It was reported that *yashada bhasma* at both the doses did not show any signs of toxicity justified by insignificant variation in body weight, hematological and biochemical parameters **[8]**. The same was even found supporting with the findings of Rinku D Umrani *et al*., (2013), where traditionally prepared *yashada bhasma* was found to be non-toxic at the dose of 300 mg/kg **[9]**. Considering the data from above works, the dose for sighting study in the present attempt was arrived at 2000 mg/kg which is almost ten times the therapeutic dose (125 - 250 mg) **[10]**. Body weight fluctuations serve as a sensitive indicator of animal health. Specifically, a weight loss exceeding 10% is a reliable marker of toxicity, even in the absence of other observable adverse effects **[11]**. Present study did not show derangement in the body weight, instead has shown a steady increase. The probable reason for this weight gain can be attributed towards *yashada bhasma* effect in improvement of appetite and

use of sodium CMC for dissolving the test trug PYB in dosing. Findings made by Alper Baran *et al*., (2020) says that Sodium carboxymethyl cellulose (CMC) was found to be non-toxic in zebrafish embryos, but its increased lipid accumulation in a dose-dependent manner and altered gene expression related to obesity-linked lipid metabolism **[12]**. The second reason due to CMC can be denied to normal weights and HPC reports of liver after the dissection of animals.

The physiological range of hematological parameters - hemoglobin (mgs%), total WBC count (10^3 cells cu.mm), neutrophils (%), lymphocytes (%), eosinophils (%), monocytes (%), MCH (pg), MCV (fl), MCHC (gm/l) and RBC (million cells/cu.mm), in female wistar rats aged about eight to 12 weeks is found between the ranges 13±1 to 15±1, 7±2 to 10±3, 14±65 to 45±22, 81±6 to 43±23, 1±1 to 1±2, 2±1 to 1±1, 20±1 to 18±1, 57±4 to 60±2, 20±1 to 18±1 and 6±1 to 8±1 respectively **[13]**. Based upon the results fetched from present study we can say that almost all of the animals had normal ranges in their haematological parameters. Only the second animal's total WBC count and neutrophil is observed to be higher than the normal values. On the other hand, the normal limits of total bilirubin of female wistar rats is 0.08 - 0.24 mg/dl as reported by Suresh Patel *et al*., (2024). Normal range of total bilirubin, SGOT (U/L), SGPT (U/L), alkaline phosphatase (U/L), total protein (gm%), albumin (gm%), urea (mgs%) and creatinine (mgs%) are reported as 0.1 - 0.2, 72.94 - 204.13, 16.53 - 37.95, 36.47 - 108.52, 5.2 - 8.2, 3.7 - 5.6, 27.35 - 60.74 and 0.35 - 0.87 respectively **[14]**. Although some biochemical parameters - total bilirubin, exhibited slightly abnormal values in the present study, these variations did not translate to clinically significant adverse events or symptomatic manifestations. Prolonged stool discoloration beyond 24 hours may indicate a risk of jaundice made evident by increased total bilirubin levels. Conversely, increased food and water consumption suggests the absence of clinically manifested pathology, despite altered haematological and biochemical parameters.

With the changes found in histopathological examination of the tissues from major organs, it can be witnessed that the overall toxic effect of PYB at 2000 mg/kg is found at the range of mild to moderate severity. The increased leukocyte and neutrophils count from the hematological parameter can be substantiated with the inflammatory and congestive changes in the

brain tissues **[15]**. The neurotoxic effects of zinc at higher concentrations might have contributed to such changes histopathologically **[16]**. Toxicity effects at a low grade witnessed in the heart tissues is substantiated with previous findings of zinc causing untoward effects in the high-density lipoprotein levels and also causing elevated risks of suffering cardio-vascular diseases **[17]**. There are various researches that state the beneficial effects of zinc in managing chronic kidney disease. But owing to the caustic nature, certain zinc compounds are found suggestive of causing injuries to the renal structures in the form of interstitial nephritis and tubular necrosis **[18]**. Structural changes in the liver tissue is directly attributed towards the exposure of increased levels of zinc leading to jaundice, cholestatis and hepatic failure **[19]**. This again corroborates the elevated total bilirubin levels in all the animals. The spleen's lymphoid hyperplasia suggested immune system activation justifiable with leukocytosis observed in the second animal. The euthanizing factor also has to be considered with respect to the changes found at the tissue levels of all the major organs. When euthanasia is done with di-ethyl ether, the chemical inhalation is prone to cause tissue necrosis at brain and damages liver and kidney as well **[20]**. Hence in the present work, the range of damage seen in histopathology might have contributed by euthanasia also.

The current study focused on the acute toxicity potential of PYB. Testing of blood parameters for all the animals at baseline could have fetched comparable data in the present work. Future research directions include conducting chronic toxicity studies on PYB or other potentiated metallic and mineral drugs to generate clinically relevant data, enhancing the safe therapeutic application of such metals.

CONCLUSION:

The present work has successfully established acute toxicity profile of PYB that was curated based on the ideology of size reduction to potentially increase bio-availability. From the obtained results it can be concluded that at the dose of 2000 mg/kg, PYB is found to be completely safe symptomatically in-spite of certain alterations in hematological, biochemical and histopathological observations. It is also to be reminded that the dose handled in present study is ten times the therapeutic dose of *yashada bhasma* and hence the scope of PYB to produce adverse toxic effects very much unlikely. Findings of the present study can be interpreted at clinical level to comply the

safety of such metal-based formulations of Ayurveda.

CONFLICTS OF INTEREST: Nil

SOURCES OF FUNDING: The work did not receive any sort of financial assistance from any body or organization.

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