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Oral toxicity of elephant foot yam (*Amorphophallus paeoniifolius*) tuber in mice

[Evaluación de la toxicidad oral del tubérculo de la pata de elefante (*Amorphophallus paeoniifolius*) en ratones]

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Abstract

Context: *Amorphophallus paeoniifolius* tuber is an important constituent of Ayurvedic system of medicine. The tuber of this plant has high medicinal value and is consumed as a food. It is associated with acridity (itchy sensation in mouth and throat) upon oral consumption and presence of high oxalates raphides.

Aims: To evaluate the acute and subacute oral toxicity studies of methanolic (APME) and aqueous (APAE) extracts of *Amorphophallus paeoniifolius* tuber in Swiss albino mice according to OECD guidelines.

Methods: In acute oral toxicity study, the mice were orally administered a single dose of APME or APAE (2000 mg/kg) and clinical signs and mortality were observed for 14 days. In subacute (repeated dose) oral toxicity study, the mice were administered once daily, orally with APME or APAE (1000 mg/kg) up to 28 days. The parameters assessed were behavior, clinical signs, body weight, feed and water consumption, urinary, biochemical, hematological and major organ weights and histology.

Results: In acute toxicity study, there was no treatment related mortality and morbidity in any of the group. In subacute toxicity study, there were no significant changes in behavior, body weight, feed and water consumption, urinary, biochemical, hematological and organ weight and histological parameters compared to vehicle treated group. There was no treatment related mortality or morbidity.

Conclusions: Administration of methanolic and aqueous extracts of *Amorphophallus paeoniifolius* tuber, individually in acute and 28 days repeated dose in mice, did not exhibit any toxicity or adverse effect at the doses used.

Keywords: calcium oxalate raphides; oral toxicity; safety; suran.

Resumen

Contexto: El tubérculo de *Amorphophallus paeoniifolius* es un componente importante de la medicina ayurvédica. Este tiene un alto valor medicinal y se consume como alimento. Se asocia con acritud por el consumo oral (sensación de picor en boca y garganta) y la presencia de rafidios oxalatos.

Objetivos: Evaluar la toxicidad oral aguda y subaguda de los extractos metanólico (APME) y acuoso (APAE) del tubérculo de *Amorphophallus paeoniifolius* en ratones albinos suizos, según las directrices de la OCDE.

Métodos: En el estudio de toxicidad aguda por vía oral, a los ratones se les administró por vía oral una dosis única de APME o APAE (2000 mg/kg) y los signos clínicos y la mortalidad se observaron durante 14 días. En estudio de toxicidad oral subaguda (dosis repetidas), a los ratones se les administró una vez al día, con APAE o APME (1000 mg/kg), por vía oral, hasta 28 días. Los parámetros evaluados fueron la conducta, signos clínicos, el peso corporal, consumo de alimento y agua, urinarios, bioquímicos, hematológicos y el peso de los principales órganos y la histología.

Resultados: En el estudio de toxicidad aguda no hubo mortalidad o morbilidad relacionada con el tratamiento en ninguno de los grupos. En un estudio de toxicidad subaguda, no hubo cambios significativos en los parámetros de comportamiento, peso corporal, consumo de alimento y agua, urinarios, bioquímicos, hematológicos y peso de los órganos y los parámetros histológicos en comparación con el grupo tratado con el vehículo. No hubo mortalidad o morbilidad relacionada con el tratamiento.

Conclusiones: La administración de los extractos metanólico y acuoso de tubérculo de *Amorphophallus paeoniifolius*, de forma aguda y dosis repetidas durante 28 días en ratones, no presentó toxicidad o efectos adversos a las dosis utilizadas.

Palabras Clave: rafidios de oxalato de calcio; seguridad; suran; toxicidad oral.

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INTRODUCTION

Irrespective of the nature of medicaments, modern or herbal, patient safety is of paramount importance; hence the evaluation of drug safety has become matter of concern. The paucity of safety studies of the herbal drugs led to unwarranted use of herbal extracts without knowing their toxicity profile and adverse effects (National Pharmacovigilance Protocol, 2008). Charaka Samhita, Ayurvedic text describes lots of adverse reactions to medicines during their inappropriate uses (Thatte and Bhalerao, 2008). However, adverse drug reaction (ADR) reports from Ayurvedic drugs are rare; possibly due to unawareness of Ayurvedic practitioners to this information leading to poor documentation and reporting of ADR (Rastogi et al., 2007).

Amorphophallus paeoniifolius (Dennst.) Nicolson (Araceae) known as elephant foot yam is basically a crop of South East Asian origin. It is commonly known as 'suran' or 'jimmikand' in India. The tuber of this plant has high medicinal value and is consumed as a food. It is an important constituent of many Ayurvedic preparations. The tubers are used traditionally for the treatment of several ailments like elephantiasis, tumors, hemorrhages, cough, bronchitis, asthma, amenorrhea, dysmenorrhea, seminal weakness, fatigue and anemia. It has got remarkable effects on gastrointestinal system and known to correct various abnormalities viz. hemorrhoids, vomiting, anorexia, dyspepsia, flatulence, colic, constipation, carminative, digestive and hepatopathy (Nair, 1993; Dey et al., 2012). The tuber is used in ethnomedicinal practices as stomachic and for the treatment of piles (hemorrhoids), abdominal pain and constipation (Devi Prasad et al., 2013; Rahman et al., 2013). Pharmacologically, it has been demonstrated to exhibit gastrokinetic activity (Dey et al., 2016a), anti-hemorrhoidal activity (Dey et al., 2016b), anti colitic activity (Dey et al., 2016c), analgesic activity (Shilpi et al., 2005), CNS depressant activities (Das et al., 2009), anti-inflammatory activity (De et al., 2010), cytotoxic activity (Angayarkanni et al., 2007), antibacterial activity and antifungal activity (Khan et al., 2008) in experimental studies.

Chewing the raw tubers or their consumption cause itching, stinging and burning sensation in mouth and irritate the oral cavity (Quattrocchi, 2012; Kumar et al., 2013). This is referred as acridity, which is sometimes followed by pruritus, erythema and wheal on external skin and hands. This is due to presence of high amount of calcium oxalate raphides in tuber. It was also termed as "Nature's poisoned spear" for its acridity. As mammals are unable to degrade oxalate, their accumulation may have deleterious effect on kidney function.

Despite the wide ethno-medicinal use of tuber of *Amorphophallus paeoniifolius* in several ailments and pharmacological findings, the studies delineating its oral toxicity are lacking. The present study demonstrated the acute and subacute oral toxicity study of extracts of *Amorphophallus paeoniifolius* in mice.

MATERIAL AND METHODS

Chemicals

Biochemical kits for estimation of aspartate transaminase (AST), alanine transaminase (ALT), alkaline phosphatase (ALP), albumin, total bilirubin, total protein, glucose, triglycerides, total cholesterol, urea, creatinine, calcium, phosphorus, chloride, sodium and potassium, and hematological reagents (Sysmex Corporation, Japan) like cell pack, stromatolyser, sulfolyser and cell clean were procured from Transasia Biomedicals Pvt. Ltd, Mumbai, India. All other common chemicals and reagents were procured from local scientific suppliers and of highest purity grade available.

Collection and authentication of the tuber

The tubers of *Amorphophallus paeoniifolius* were collected from the local market of Gwalior in December 2011. Tubers were identified by taxonomist of the institute and a voucher specimen No. 5-4/10-11/NRIASHRD/Tech/Survey/1611 was deposited in the herbarium of the institute.

Preparation of extracts

The tubers were separated for extraneous matter, chopped into thin pieces and subjected to shade drying, which were later subjected to grinding to obtain a coarse powder. The powdered tuber (100 g) in thimble was placed in Soxhlet extractor

(flask size 2000 mL) and extracted with methanol (1000 mL) for 48 h at the temperature of 40°C. The marc was finally macerated with distilled water to obtain aqueous extract. The filtered extracts were dried in a rotary evaporator. The extracts were stored in desiccators for further use. The methanolic extract (APME) of reddish brown semisolid consistency and aqueous extract (APAE) of brown solid consistency were obtained with percent yield of 9.48 and 6.16% w/w, respectively.

Preliminary phytochemical screening

Preliminary phytochemical screening was carried out to detect the presence and absence of various phytoconstituents like carbohydrates, proteins, steroids, flavonoids, tannins and other phenolic compounds, glycosides and alkaloids, amino acids, fats and oils in the extracts as per standard methods (Table 1) (Khandelwal, 2006). APME and APAE showed presence of carbohydrates, proteins, alkaloids, flavonoids, sterols, amino acids, phenolic compounds and tannins while glycosides, fats and oils were absent (Table 1).

Table 1. Phytochemical screening

Phytochemical	Phytochemical test	Observations	Inference	
			APME	APAE
Carbohydrates	Molish's test: To 2-3 mL extract solution, few drops of 5% α-naphthol solution was added and shaken. The conc. H_2SO_4 was added from side of test tube.	Appearance of violet ring at junction of two layers	+	+
Proteins	Biuret test: 1 mL of 4% NaOH and few drops of 1% $CuSO_4$ were added to 3 mL of extract solution.	Appearance of violet color	+	+
	Xanthoproteic test: 3 mL of extract solution was mixed with 1 mL conc. H_2SO_4.	Formation of white precipitate which turned to yellow when boiled.		
Amino acids	Ninhydrin test: To 3 mL of extract solution, 3 drops of 5% Ninhydrin solution were heated in boiling water bath for 10 min.	Appearance of purple color	+	+
Sterols	Salkowski reaction: To 2 mL of extract solution, 2 mL chloroform and 2 mL conc. H_2SO_4 were added and shaken well.	Appearance of red color in chloroform layer while greenish yellow color in acid layer	+	+
Fats and oils	Solubility test: The extract was dissolved in water, ether, benzene and chloroform to check the solubility.	Soluble in water and 90% ethanol	-	-
	Saponification test: To the extract, 25 mL of 10% NaOH was added and boiled in water bath for 30 min. Excess Na_2SO_4 solution (1 g in 10 mL distilled water) was added.	No appearance of soap		
Glycosides	Keller-Killani test: To 2 mL of extract, 2 mL glacial acetic acid, one drop of 5% $FeCl_3$ and conc. H_2SO_4 were added.	Appearance of reddish brown color at junction while bluish green color in the upper layer	-	-
Flavonoids	Shinoda test: To the extract 5 mL of 95% ethanol, few drops of conc. HCl and 0.5 g of magnesium turnings were added.	Formation of reddish to pink color	+	+
Alkaloids	To dry extract, dil. HCl was added, shaken well and filtered. Wagner's test: To 2-3 mL of filtrate obtained above, few drops of Wagner's reagent were added.	Formation of reddish brown precipitate	+	+
	Hager's Test: To 2-3 mL of filtrate obtained above, few drops of Hager's reagent (saturated solution of picric acid) were added.	Formation of yellow precipitate.		
Phenolic compounds and tannins	Lead acetate test: To 2-3 mL of extract solution, few drops of lead acetate solution (10%) were added.	Formation of white precipitate	+	+
	Ferric chloride Test: To 2-3 mL of extract solution, few drops of 5% ferric chloride solution were added.	Appearance of bluish black color		

+: Present, –: Absent

Animals

Healthy adult Swiss mice of both sexes and 25-30 g weight were used for the study. Animals were acclimatized for 7 days before initiation of the study. The health examination was performed during acclimatization period. The animals were housed at standard experimental conditions of temperature (25 ± 1°C) with relative humidity 50±5% under 12 h light: dark cycle. They were fed standard rodent chow (Ashirwad brand, Chandigarh, India) and water *ad libitum*. Experiments were performed in accordance with the guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) after the approval of the experimental protocol by the Institutional Animals Ethics Committee (IAEC) (IAEC Proposal No. NRIASHRD-GWL/IAEC/2013/01).

Acute oral toxicity study

Acute toxicity study was carried out by acute toxic class method of oral toxicity as per procedure described by Organization for Economic Cooperation and Development (OECD) 423 guideline (OECD, 2001). The mice were divided into three groups (n=3). Control group received 1% Tween 80 (prepared in double distilled water) as vehicle at a dose volume of 5 mL/kg body weight while the extract treated groups were orally administered APME or APAE in the limit test dose of 2000 mg/kg. The mice were observed continuously for behavioral, neurological and autonomic profiles for 2 h and after a period of 24, 72 h and thereafter up to 14 days for any lethality, moribund state or death. Cage side observations included change in fur, eyes and mucous membranes, and also respiratory, circulatory, autonomic and central nervous system, and somatomotor activity and behavior pattern. All the animals were observed once daily for morbidity and mortality. The limit test was repeated in another groups of mice (n=3) for confirmation and toxic class of LD_{50} determination. Animals were sacrificed in a CO_2 chamber on 15th day of the study and were subjected to detailed post-mortem examination.

Sub-acute oral toxicity study

Repeated dose (28 days) oral toxicity study was carried out as per procedure described in OECD 407 guideline (OECD, 2008). Total 30 mice (15 males and 15 females) were selected. The mice were divided into three groups (five males and five females per group) as follows:

Group I: Vehicle treated control, received vehicle (1% Tween 80);

Group II: Test (APME), received APME at a dose of 1000 mg/kg;

Group III: Test (APAE), received APAE at a dose of 1000 mg/kg.

The mice were orally administered vehicle, APME or APAE for 28 days in a dose volume of 5 mL/kg/day.

In-life phase observations

The following observations were made during the course of study up to 28 days.

Clinical signs and mortality

Mice were examined for clinical signs and mortality after dosing, at 5-10 min., 30-45 min, 1 (±10 min), 2 (±10 min), 4 (±10 min), 6 (±10 min) and 24 (±10 min) h post dosing followed by once daily throughout the 28 days. Cage side observations included change in fur, eyes and mucous membranes, and also respiratory, circulatory, autonomic and central nervous system, and somatomotor activity and behavior pattern. All the animals were observed once daily for morbidity and mortality.

Neurological examination

For neurological examination, the animals were taken in photo actometer (Model no. 600MT, Medicraft Electromedical Pvt. Ltd., Lucknow, India) for 10 min on 28th day of treatment. The locomotor count was recorded for individual mice.

Body weight

The body weight of each mouse was measured during the experimental period, every week during the treatment.

Feed and water consumption

The weekly feed consumption of mice was recorded by measuring the difference between feed offered and feed left over on subsequent weeks. The weekly water consumption of mice was recorded by

measuring the difference between water offered and water left over on subsequent weeks.

Hematological and biochemical analysis

Blood collection was carried on 29th day i.e. the end of the study to assess change in hematological and biochemical parameters of mice. The blood was collected from overnight fasted mice through retro-orbital plexus (Parasuraman et al., 2010) under light ether anesthesia. For hematology, blood was collected in K3 EDTA (Potassium Ethylene Di-amine Tetra Acetate) vacutainer whereas for biochemical analysis blood was collected in micro-centrifuge tubes containing heparin anti-coagulant. The following hematological parameters were evaluated using automated hematology analyzer (XT-2000iV, Sysmex Corporation, Japan) *viz.* white blood cells (WBC), red blood cells (RBC), hemoglobin (HBG), hematocrit (HCT), mean cell volume (MCV), mean cell hemoglobin (MCH), mean cell hemoglobin concentration (MCHC), platelets (PLT), red cell distribution width (RDW), platelet distribution width (PDW), mean platelet volume (MPV), platelet large cell ratio (P-LCR), plateletcrit (PCT), neutrophils, lymphocytes, monocytes, eosinophils and basophils. The plasma was separated by centrifugation of blood at 3000 rpm for 15 min and used for estimation of biochemical parameters like calcium, phosphorus, chloride, sodium, potassium, glucose, aspartate transaminase (AST), alanine transaminase (ALT), alkaline phosphatase (ALP), triglycerides, total cholesterol, urea, creatinine, albumin, total bilirubin and total protein using commercially available kits (ERBA Diagnostic, Mannheim, SPAN Diagnostic Ltd., Gujarat) on NANOLAB 240® clinical chemistry auto analyzer. The data of three replicates of readings were noted from individual animal.

Urine analysis

Individual animal of all groups was shifted to metabolic cages for urine collection for 16 h on 21st day of treatment. Feed was withheld during the phase of urine collection in metabolic cages and water was added *ad libitum*. The urine volume was measured using calibrated measuring cylinder manually. Color and visual appearance of the clarity was observed manually and noted. The other urine parameters viz. specific gravity, glucose, bilirubin, ketones, occult blood, pH, protein, urobilinogen, nitrite and leukocytes were estimated by Uridip 10C (Erba Mannheim, Germany). The visual observations of the strips were performed. The data of three replicates of readings were noted from individual animal.

Necropsy and histopathology

All surviving animals were sacrificed in a CO_2 chamber on 29th day of the study after blood collection and were subjected to detailed post-mortem examination. The organs such as heart, lungs, liver, kidneys, thymus, spleen, adrenals, brain, gastrointestinal tract and sex organs were removed. They were weighed immediately and stored in 10% neutral buffered formalin. Testes were collected in Davison's fluid and subsequently transferred to 10% neutral buffered formalin on next day. Organs were fixed in 10% neutral buffered formalin. Fixed tissues were processed i.e. dehydrated in graded alcohol, cleared in xylene and embedding bath. These tissue blocks were cut at 5-7 µm in thickness and stained with hematoxylin and eosin and finally mounted in DPX. These slides were then studied under binocular microscope (Model no. BM-400, Leedz Microimaging Ltd, London, England) with inbuilt CMOS camera (3 megapixel) and E Plan achromat objective lens. Test photomicrographs were taken and compared with control. The observations reported are representative findings from five males and five females of each group.

Statistical analysis

The software program GraphPad Prism 4.0 (GraphPad Software Inc., USA) was used. The data were expressed as the mean ± SEM. Comparisons between the different groups were performed by the one-way or two-way analysis of variance (ANOVA) wherever applicable. Differences were considered statistically significant at $p<0.05$.

RESULTS

Acute toxicity study

In the acute toxicity study, test doses of 2000 mg/kg of the APME and APAE did not cause death

of mice during 14 days observation period. The mice did not show any signs of toxicity or change in general behavior compared to vehicle control group. During necropsy, no gross morphological changes were observed in the internal organs from test groups compared to vehicle control mice.

Subacute oral toxicity study

In the subacute toxicity study, APME and APAE at the dose of 1000 mg/kg, orally for 28 days did not cause any mortality or moribund stage of the mice. No signs of toxicity were observed during the experimental period of 28 days as evident from unaltered ($p>0.05$) clinical signs and neurological examination (locomotor count) (Table 2) compared to vehicle control group. The extracts treatment did not cause any significant ($p>0.05$) change in weekly body weights of male and female animals in test groups as compared to vehicle control group (Table 2). There was no significant change in weekly feed consumption ($p>0.05$) and water consumption in male and female mice of test groups as compared to vehicle control group (Table 2). The hematological values of treated male and female mice were not significantly ($p>0.05$) different in test groups from those of the vehicle control group (Table 3). Blood biochemical values of male and female mice were not significantly ($p>0.05$) altered by extracts treatment as compared to vehicle control animals (Table 4). There was no noticeable change in any of the urinary parameters observed in test groups as compared to vehicle control group (Table 5).

During necropsy, no gross morphological changes were observed in the organs collected from test groups as compared to organs from vehicle control group. No treatment related effect on organ weights of males and females mice were observed in comparison to vehicle control group ($p>0.05$) due to 28 days repeated dose administration of APME and APAE (Table 6). The histological examinations of various organs from male and female mice did not reveal any treatment related changes (neither degenerative nor infiltrative) compared to vehicle control mice (Table 7; Figs. 1 and 2).

Table 2. Effects of 28 days repeated dose of APME and APAE on neurological behavior, body weight, feed intake and water intake.

Parameters	Week	Male			Female		
		Vehicle	APME1000	APAE1000	Vehicle	APME1000	APAE1000
Neurological behavior (Locomotor count)		414.2 ± 27.52	406.8 ± 36.01	414.8 ± 34.23	473.6 ± 62.38	430.0 ± 44.95	414.4 ± 42.00
Weekly Body weight (g)	1st	25.6 ± 0.24	25.8 ± 0.25	26.0 ± 0.32	25.8 ± 0.20	25.2 ± 0.20	25.6 ± 0.24
	2nd	27.0 ± 0.32	26.8 ± 0.37	27.2 ± 0.37	26.2 ± 0.37	26.4 ± 0.40	26.6 ± 0.40
	3rd	28.4 ± 0.25	28.4 ± 0.51	28.6 ± 0.4	27.2 ± 0.37	27.8 ± 0.37	27.4 ± 0.24
	4th	29.2 ± 0.37	29.0 ± 0.32	29.4 ± 0.24	28.2 ± 0.20	28.8 ± 0.20	28.0 ± 0.32
Weekly feed intake (g)	1st	69.2 ± 2.29	67.8 ± 1.86	68.2 ± 1.93	54.6 ± 1.69	54.4 ± 0.93	54.4 ± 1.33
	2nd	70.4 ± 1.43	68.4 ± 1.50	69.4 ± 2.04	55.8 ± 1.65	55.2 ± 0.86	54.2 ± 1.01
	3rd	70.8 ± 1.20	69.2 ± 2.28	69.8 ± 1.53	56.2 ± 1.16	56.4 ± 0.68	55.2 ± 1.59
	4th	71.4 ± 0.68	71.8 ± 1.24	72.6 ± 1.36	57.4 ± 1.08	57.0 ± 0.89	55.6 ± 1.63
Weekly water intake (mL)	1st	52.6 ± 1.96	53.2 ± 1.91	50.8 ± 1.91	47.2 ± 1.46	48.6 ± 2.06	49.7 ± 2.28
	2nd	53.2 ± 1.98	53.8 ± 1.66	51.2 ± 1.39	48.6 ± 1.29	49.4 ± 1.29	50.2 ± 1.59
	3rd	55.4 ± 1.86	54.8 ± 2.52	54.6 ± 2.23	49.2 ± 0.86	49.8 ± 1.85	51.0 ± 1.61
	4th	56.2 ± 2.03	56.4 ± 2.84	57.2 ± 1.66	50.8 ± 1.02	51.2 ± 1.43	51.6 ± 1.81

All values represent mean ± SEM, n = 5 per group. No significant differences were observed among groups, One-way or two-way ANOVA, wherever applicable, $p > 0.05$. APME: Methanolic extract of *Amorphophallus paeoniifolius* tuber; APAE: Aqueous extract of *Amorphophallus paeoniifolius* tuber. Vehicle or extracts were given for 28 days. Locomotor count was measured on 28th day while body weight, feed intake and water intake were assessed on weekly intervals up to 28 days.

Table 3. Effects of 28 days repeated dose of APME and APAE on hematological parameters.

Hematological parameters	Male			Female		
	Vehicle	APME	APAE	Vehicle	APME	APAE
WBC × 10^3/µL	7.61 ± 1.43	7.32 ± 0.81	7.47 ± 1.13	5.95 ± 1.28	6.18 ± 2.65	6.55 ± 2.28
RBC × 10^6/µL	10.74 ± 0.44	8.88 ± 2.07	10.25 ± 2.89	11.30 ± 0.48	10.99 ± 0.85	11.43 ± 0.36
HGB (g/dL)	14.54 ± 0.73	12.42 ± 2.77	13.38 ± 4.27	15.30 ± 0.40	15.00 ± 1.08	15.06 ± 0.19
HCT (%)	46.82 ± 1.76	41.42 ± 5.15	42.92 ± 11.21	47.34 ± 0.97	46.70 ± 2.33	46.28 ± 1.21
MCV (fL)	43.66 ± 1.76	47.82 ± 6.47	42.26 ± 1.94	41.98 ± 1.13	42.62 ± 2.18	40.52 ± 2.00
MCH (pg)	13.54 ± 0.43	14.04 ± 0.54	12.86 ± 0.99	13.54 ± 0.30	13.660 ± 0.38	13.20 ± 0.35
MCHC (g/dL)	31.06 ± 0.80	29.70 ± 3.28	30.54 ± 3.20	32.40 ± 0.58	32.12 ± 1.12	32.58 ± 0.89
PLT × 10^3/µL	2189.00 ± 185.17	1605 ± 806.00	1835 ± 650.16	1453.00 ± 307.70	1288.80 ± 251.90	1552.80 ± 374.00
RDW-SD (fL)	32.56 ± 1.14	37.36 ± 1.02	29.64 ± 2.57	29.46 ± 1.58	28.50 ± 1.31	28.16 ± 0.96
RDW-CV (%)	24.26 ± 0.51	24.28 ± 0.45	23.24 ± 1.15	23.64 ± 1.15	22.70 ± 1.96	23.82 ± 1.41
PDW (fL)	7.62 ± 0.45	8.20 ± 0.70	7.92 ± 0.52	7.56 ± 0.40	7.46 ± 0.45	7.68 ± 0.45
MPV (fL)	6.64 ± 0.15	7.20 ± 0.60	6.80 ± 0.20	6.40 ± 0.23	6.42 ± 0.27	6.46 ± 0.38
P-LCR (%)	6.10 ± 0.86	9.32 ± 3.16	6.96 ± 0.85	5.18 ± 0.94	5.00 ± 1.55	5.60 ± 1.47
PCT (%)	1.45 ± 0.15	1.16 ± 0.62	1.25 ± 0.47	0.92 ± 0.18	0.83 ± 0.15	1.00 ± 0.25
Neutrophil × 10^3/µL	0.81 ± 0.18	0.92 ± 0.38	0.84 ± 0.46	0.69 ± 0.11	0.50 ± 0.17	0.47 ± 0.05
Lymphocyte × 10^3/µL	6.35 ± 1.32	5.97 ± 0.92	6.25 ± 1.4	4.93 ± 1.26	5.45 ± 2.55	5.74 ± 2.24
Monocyte × 10^3/µL	0.24 ± 0.07	0.018 ± 0.09	0.19 ± 0.13	0.15 ± 0.07	0.10 ± 0.03	0.12 ± 0.05
Eosinophil × 10^3/µL	0.21 ± 0.12	0.24 ± 0.16	0.19 ± 0.19	0.17 ± 0.14	0.12 ± 0.01	0.21 ± 0.09
Basophil × 10^3/µL	0.006 ± 0.005	0.004 ± 0.005	0.004 ± 0.005	0.004 ± 0.005	0.004 ± 0.005	0.010 ± 0.007

All values represent mean ± SEM, n = 5 per group. No significant differences were observed among groups, One-way or two-way ANOVA, wherever applicable, $p > 0.05$. APME: Methanolic extract of *Amorphophallus paeoniifolius* tuber, APAE: Aqueous extract of *Amorphophallus paeoniifolius* tuber; WBC: White blood cells; RBC: Red blood cells; HGB: Hemoglobin; HCT: Hematocrit; MCV: Mean cell volume; MCH: Mean cell hemoglobin; MCHC: Mean cell hemoglobin concentration; PLT: Platelets; RDW-SD: Red cell distribution width-standard deviation; RDW-CV: Red cell distribution width coefficient of variance; PDW: Platelet distribution width; MPV: Mean platelet volume; P-LCR: Platelet large cell ratio; PCT: Plateletcrit. Vehicle or extracts were given for 28 days. Hematological parameters were measured on 29th day.

Table 4. Effects of 28 days repeated dose of APME and APAE on biochemical parameters.

Biochemical parameters	Male			Female		
	Vehicle	APME1000	APAE1000	Vehicle	APME1000	APAE1000
AST (IU/L)	93.60 ± 17.34	80.60 ± 17.13	79.80 ± 13.59	85.00 ± 7.38	89.00 ± 5.92	96.60 ± 12.05
ALT(IU/L)	46.80 ± 14.01	34.60 ± 10.64	32.40 ± 8.99	36.24 ± 8.14	37.20 ± 7.95	37.20 ± 6.02
ALP (IU/L)	75.80 ± 15.50	69.54 ± 17.80	73.40 ± 5.22	70.60 ± 24.36	61.40 ± 7.27	71.00 ± 15.51
Total proteins (g/dL)	4.92 ± 0.60	4.72 ± 0.54	4.82 ± 0.57	5.20 ± 0.44	4.92 ± 0.37	4.58 ± 0.99
Albumin (g/dL)	3.32 ± 0.22	3.62 ± 0.19	3.32 ± 0.18	3.76 ± 0.09	3.86 ± 0.11	3.66 ± 0.11
Total bilirubin (mg/dL)	0.82 ± 0.12	0.58 ± 0.21	0.62 ± 0.19	0.91 ± 0.15	0.59 ± 0.35	0.75 ± 0.44
Total cholesterol (mg/dL)	65.22 ± 8.15	62.98 ± 4.78	63.36 ± 4.66	65.98 ± 7.37	62.40 ± 6.91	63.86 ± 6.11
Triglycerides (mg/dL)	88.75 ± 8.95	99.78 ± 8.61	92.00 ± 15.61	88.46 ± 6.78	87.58 ± 12.89	85.28 ± 13.67
Urea (mg/dL)	48.30 ± 5.43	48.29 ± 3.71	42.67 ± 1.92	50.04 ± 10.75	44.98 ± 2.61	42.13 ± 1.33
Creatinine (mg/dL)	0.80 ± 0.30	0.81 ± 0.28	0.59 ± 0.30	0.44 ± 0.41	0.47 ± 0.26	0.32 ± 0.05
Glucose (mg/dL)	101.96 ± 9.50	99.80 ± 5.52	104.34 ± 11.14	96.90 ± 10.17	96.58 ± 6.27	92.74 ± 5.26
Calcium (mg/dL)	9.60 ± 1.1	8.95 ± 1.82	8.32 ± 0.76	9.27 ± 0.58	10.26 ± 0.1.47	8.91 ± 0.35
Chloride (mEq/L)	115.94 ± 10.74	108.86 ± 1.38	106.12 ± 2.49	111.88 ± 8.40	108.44 ± 5.56	106.52 ± 1.86
Phosphorus (mg/dL)	5.64 ± 1.02	5.74 ± 1.99	5.95 ± 2.09	5.56 ± 0.67	5.47 ± 1.31	5.23 ± 0.22
Sodium (mEq/L)	167.49 ± 4.90	168.07 ± 5.01	157.13 ± 14.96	159.61 ± 21.41	166.01 ± 12.11	161.58 ± 15.83
Potassium (mEq/L)	7.48 ± 0.67	6.76 ± 0.74	7.70 ± 0.81	7.45 ± 1.31	8.61 ± 1.02	7.14 ± 1.18

All values represent mean ± SEM, n = 5 per group. No significant differences were observed among groups, One-way or two-way ANOVA, wherever applicable, $p > 0.05$. APME: Methanolic extract of *Amorphophallus paeoniifolius* tuber; APAE: Aqueous extract of *Amorphophallus paeoniifolius* tuber. AST: Aspartate transaminase; ALT: Alanine transaminase; ALP: Alkaline phosphatase. Vehicle or extracts were given for 28 days. Biochemical parameters were measured on 29th day.

Table 5. Effects of repeated dose of APME and APAE on urine analysis (cumulative findings).

Urine Parameters	Control	Test	
	Vehicle	APME 1000	APAE 1000
Color	Pale to Dark yellow	No significant change	No significant change
Specific Gravity	1 to 1.005	No significant change	No significant change
Glucose	Negative	No significant change	No significant change
Bilirubin	Negative	No significant change	No significant change
Ketones	Negative	No significant change	No significant change
Occult Blood	Negative	No significant change	No significant change
pH	8 to 8.5	No significant change	No significant change
Protein	Negative	No significant change	No significant change
Urobilinogen (mg/dL)	0.1	No significant change	No significant change
Nitrite	Negative	No significant change	No significant change
Leukocytes	Negative	No significant change	No significant change

APME: Methanolic extract of *Amorphophallus paeoniifolius* tuber, APAE: Aqueous extract of *Amorphophallus paeoniifolius* tuber. Vehicle or extracts were given for 28 days. Urine parameters were measured on 21st day of study.

Table 6. Effects of 28 days repeated dose of APME and APAE on organ weights.

Organ weight (g)	Male			Female		
	Vehicle	APME 1000	APAE 1000	Vehicle	APME 1000	APAE1000
Heart	0.17 ± 0.02	0.17 ± 0.01	0.18 ± 0.03	0.13 ± 0.00	0.12 ± 0.02	0.13 ± 0.02
Liver	1.73 ± 0.18	1.60 ± 0.31	1.80 ± 0.11	1.4 ± 0.17	1.52 ± 0.21	1.39 ± 0.12
Kidneys	0.52 ± 0.05	0.53 ± 0.07	0.53 ± 0.07	0.31 ± 0.01	0.32 ± 0.01	0.32 ± 0.01
Spleen	0.15 ± 0.04	0.16 ± 0.09	0.16 ± 0.02	0.15 ± 0.02	0.15 ± 0.04	0.16 ± 0.04
Thymus	0.06 ± 0.01	0.05 ± 0.01	0.05 ± 0.003	0.06 ± 0.005	0.05 ± 0.01	0.06 ± 0.01
Brain	0.44 ± 0.01	0.43 ± 0.02	0.45 ± 0.03	0.44 ± 0.02	0.41 ± 0.02	0.43 ± 0.02
Epididymus	1.12 ± 0.22	1.22 ± 0.13	1.08 ± 0.22	-	-	-
Testis/Ovaries with uterus and oviduct	0.22 ± 0.01	0.21 ± 0.03	0.2 ± 0.05	1.79 ± 0.41	1.76 ± 0.57	1.53 ± 0.22

All values represent mean ± SEM, n = 5 per group. No significant differences were observed among groups, One-way or two-way ANOVA, wherever applicable, $p > 0.05$. APME: Methanolic extract of *Amorphophallus paeoniifolius* tuber, APAE: Aqueous extract of *Amorphophallus paeoniifolius* tuber. Vehicle or extracts were given for 28 days. Organs weights were measured on 29th day.

Table 7. Summary of histopathological findings (on the basis of severity of lesions) in mice treated with APME and APAE.

Organs/ lesions	Male			Female		
	Vehicle	APME1000	APAE1000	Vehicle	APME1000	APAE1000
Liver						
Vacuolation in hepatocytes	+	+	+	+	-	-
Kupffer cell proliferation/ hypertrophy	+	+	+	-	-	-
Necrosis/ apoptosis	+	+	+	+	+	+
Congestion	+	+	+	-	+	+
Kidney						
Degenerative changes	+	++	+	+	+	+
Tubular dilation	-	+	+	-	-	-
Heart /Lungs/Spleen	-	-	-	-	-	-
Brain						
Pyknosis in Purkinjee cell neurons	+	+	+	-	-	-
Congestion	+	+	+	-	-	-
Esophagus/ Stomach/ Deudonum/ Jejunum/ Caecum/ Colon/ Rectum	-	-	-	-	-	-
Adrenal	-	-	-	-	-	-
Testis/ Epididymus	-	-	-			
Ovaries				-	-	-

- Absent + Mild ++ Moderate

APME: Methanolic extract of *Amorphophallus paeoniifolius* tuber, APAE: Aqueous extract of *Amorphophallus paeoniifolius* tuber.

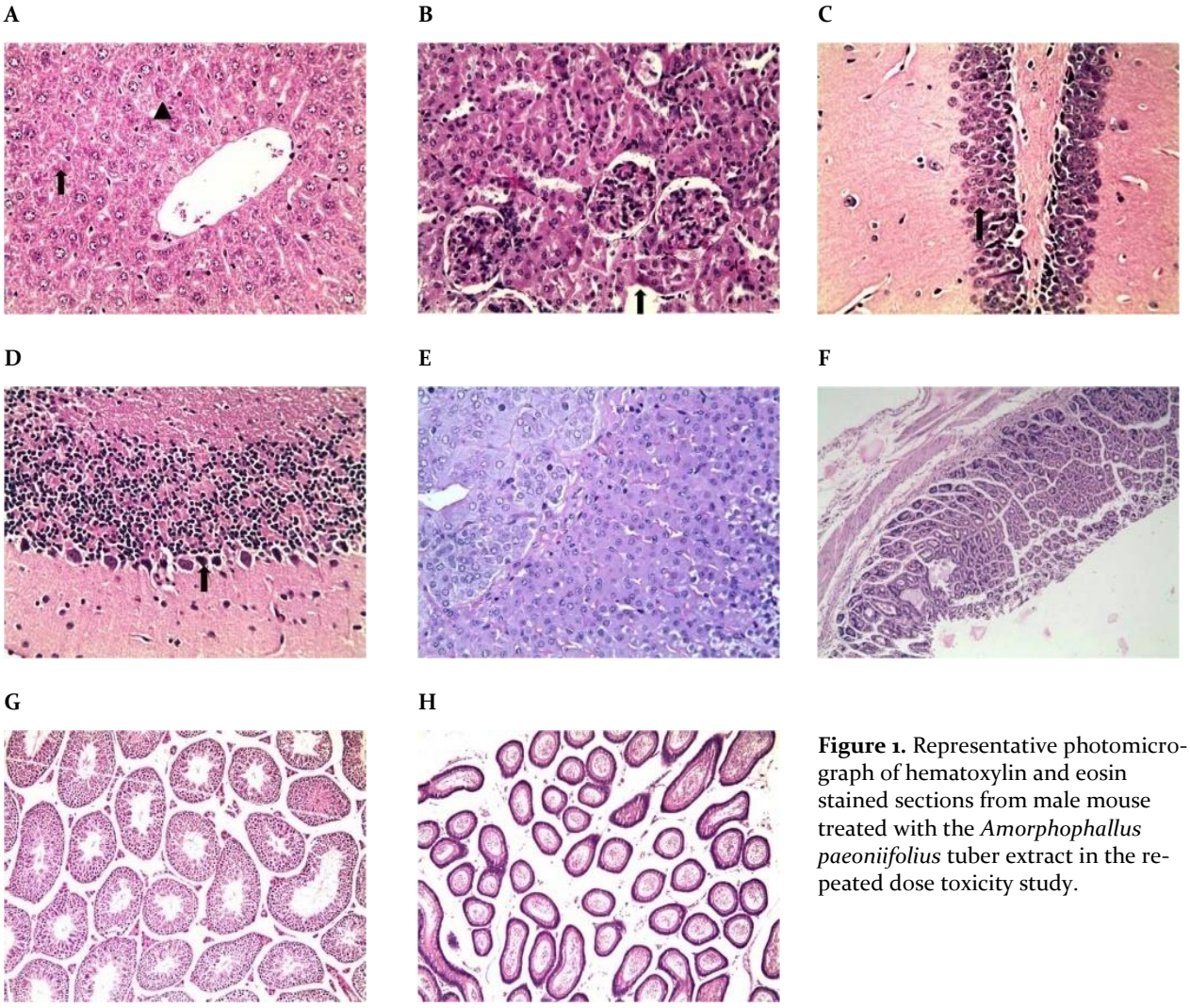

Figure 1. Representative photomicrograph of hematoxylin and eosin stained sections from male mouse treated with the *Amorphophallus paeoniifolius* tuber extract in the repeated dose toxicity study.

(A). Liver section from control mouse showing hepatocyte vacuolization (arrowhead) and necrosis (arrow). **(B).** Kidney section from control male mouse, showing degenerative changes in the renal tubules (arrow) **(C).** Hippocampus of control mouse brain showing pyknosis (nuclear condensation) in the pyramidal neurons (arrows). **(D).** Cerebellum of control mouse brain showing pyknosis in purkinjee neurons (arrow). **(E).** Section of male mouse (control) adrenal showing normal anatomy with capsule, zona glomerulosa, zona fasiculata and zona reticularis clearly distinguishable from medulla. **(F).** Section of male mouse jejunum showing normal histology in methanolic extract treated group. **(G and H)**. Section of mouse (treated with methanolic extract) testis and epididymus respectively depicting normal histology.

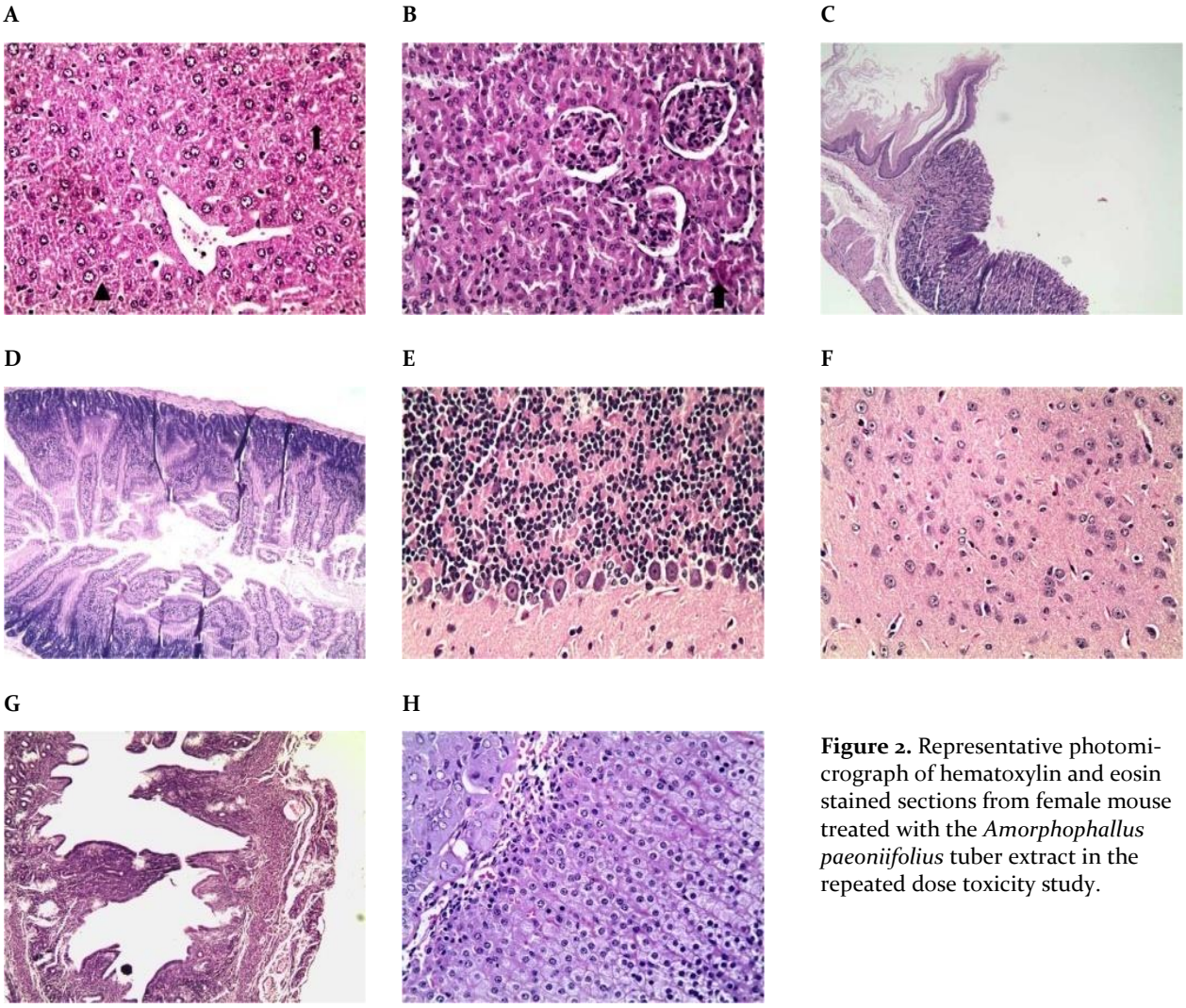

Figure 2. Representative photomicrograph of hematoxylin and eosin stained sections from female mouse treated with the *Amorphophallus paeoniifolius* tuber extract in the repeated dose toxicity study.

(A). Liver section from control mouse showing hepatocyte vacuolation (arrowhead) and necrosis (arrow). **(B).** Kidney section from control female mouse, showing tubular degeneration (arrow). **(C)**. Normal histology of gastric mucosa of female mouse treated with methanolic extract of *Amorphophallus paeoniifolius* tuber. **(D).** Section of female mouse jejunum showing normal histology in methanolic extract treated group. **(E).** cerebellum of control mouse brain showing normal histology of neurons and basket cells (arrow). **(F).** Cerebellar cortex of control mouse brain showing normal histology of neurons and glial cells arranged in various layers. **(G).** Section of fallopian tube of female control mouse depicting normal histological features. **(H).** Adrenal of methanolic extract group exposed mouse showing normal anatomy with capsule, zona glomerulosa, zona fasiculata and zona reticularis clearly distinguishable from medulla.

DISCUSSION

In the present study, the acute and 28 day repeated dose subacute toxicity studies of the methanolic and aqueous extracts of *Amorphophallus paeoniifolius* tuber were evaluated in mice as per OECD guidelines. The extracts were standardized by HPLC as per our previous report (Dey et al., 2016a). Acute oral toxicity study of the APME and APAE revealed that there was no toxicity of any nature, mortality or moribund stage during the observation period. The clinical signs were normal and no gross morphological changes in the organs of test group mice as compared to vehicle control mice during necropsy. This indicates that no observed adverse effect level (NOAEL) of APME and APAE is more than 2000 mg/kg and the approximate LD50 of the extracts was more than 2500 mg/kg.

Subacute toxicity was conducted because tubers are used prolong durations for treatment of chronic disease conditions like piles and constipation. According to OECD guideline, if a limit test at the dose of 1000 mg/kg produce no observable toxic effects then a full toxicity study using three doses is not necessary (OECD, 2001). Previous studies also evaluated the oral subacute toxicity studies of plant extracts using the limit dose of 1000 mg/kg/day (Witthawaskul et al., 2003; Ferrero et al., 2007). The present study indicates that repeated oral dose treatment of APME and APAE for 28 days did not cause any mortality or moribund stage during the observation period. The clinical signs were normal and there were no significant effects of the extracts on neurological behavior. Das et al. (2009) and Dey et al. (2011) reported the CNS depressant activity of petroleum ether extract of the tuber in mice with the calculated lethal dose of 2500 mg/kg body weight. However, in the present study there was no significant effects were observed in neurological behavior and brain histology by APME and APAE. It suggests that methanolic and aqueous extracts do not cause any toxicity in the nervous system. The body weight gained by the treatment animals along with their feed and water intake were not significantly affected compared vehicle treated mice. It indicates that the extracts did not show any alteration in cellular biosynthesis and metabolism which was also evident from the insignificant changes in glucose, cholesterol and triglycerides levels. Further, no hepatotoxicity was seen due to APME or APAE treatment at the selected dose evident from the non-significant changes on plasma AST, ALT, ALP and total protein levels along with liver weight and histological observations compared to vehicle control mice.

In the present study, repeated dose of APME and APAE did not show any significant effect on hematopoietic system as indicated by the unaffected hematological parameters when compared to vehicle treated mice. It was supported by the unchanged histological observation of the spleen in the test animals, which are known to be the source of PLT.

The tuber contains calcium oxalate, which may cause urinary oxalate excretion (Kumar et al., 2013). However, in the current study, repeated doses of APME and APAE did not cause any type of nephrotoxicity in mice as indicated by the normal plasma urea and creatinine levels in test group as compared to control, which is also evident from the unaffected urine volume, pH, kidney weights and histology. Adrenal gland is a very important endocrine gland which release aldosterone, epinephrine and norepinephrine, which are essential for normal physiological functions. The organ weights and histology of heart, adrenal and thymus did not show significant changes in test mice as compared to control mice. These findings suggest that APME and APAE did not produce any adverse effect on cardiovascular and urinary systems.

In case of gastrointestinal system, APME and APAE did not show any toxicity as seen by the normal clinical signs. There were no alterations in the histological structures of stomach and intestine in both sexes of mice. However, it was found that the fecal content and moisture in feces in test mice were more than that of control. It may be due to the laxative property of the extracts (Dey et al., 2016a) which may be due to the presence of glucomannan (Nguyen et al., 2009), a laxative phytoconstituent, in the tuber extracts (Loening-Baucke et al., 2004; Widjanarko et al., 2013).

In the present study, repeated dose of APME and APAE did not show any significant difference in the organ weight, necropsy and histology of testes and epididymus of male mice as well as ovary in female mice. It suggests that the extracts did not show any toxicity in reproductive system of animals. Tradi-

tionally, the tuber is used to cure asthma (Nair, 1993) so it is very important to reveal the effect of the extracts on histology of lungs in the test mice. Here in our study, APME and APAE treatments did not cause any histological changes in lungs which indicate that the extracts can be evaluated for chronic respiratory diseases.

CONCLUSIONS

Administration of single and repeated dose treatment of methanolic and aqueous extracts of tuber of *Amorphophallus paeoniifolius* in mice did not exhibit any toxicity or adverse effects, which could be compromise the medicinal use of the tuber in ethno medicinal practices. This suggests that no observed adverse effect level (NOAEL) of APME and APAE is more than 2000 mg/kg in acute dose and more than 1000 mg/kg in 28 days repeated dose study.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Contribution	Dey YN	Wanjari MM	Kumar D	Lomash V	Gaidhani SN	Jadhav AD
Concepts or Ideas	X	X	X		X	X
Design	X	X			X	
Definition of intellectual content		X				
Literature search	X	X	X			X
Experimental studies	X	X		X		
Data acquisition	X			X		
Data analysis	X			X	X	
Statistical analysis	X	X				
Manuscript preparation	X	X				
Manuscript editing	X	X				
Manuscript review	X	X	X			X

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