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Single and repeated dose (28 days) intravenous toxicity assessment of bartogenic acid (an active pentacyclic triterpenoid) isolated from *Barringtonia racemosa* (L.) fruits in mice

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ABSTRACT

Bartogenic acid (BA), an active pentacyclic triterpenoid, has been reported for anti-diabetic, anti-inflammatory, anti-arthritic, anti-cancer, and anti-tumor activity. However, toxicity profiling of BA has not been reported till date. Hence, this study is designed to evaluate the single dose (12.5, 25, 50 and 100 mg/kg) and repeated dose (1.5, 6, and 24 mg/kg) intravenous toxicity of BA in BALB/c mice. Control group received vehicle. In single dose toxicity study, two mortalities were observed at 100 mg/kg of BA whereas lower doses were well tolerated. In repeated dose toxicity study, no mortality was observed. 1.5 mg/kg of BA was well tolerated in mice of both sexes. At 6 mg/kg of BA, female mice showed significant reduction in the body weight as compared to the control group however no significant change was observed in male mice. 24 mg/kg of BA showed significant reduction in the body weight in mice of both sexes. Further, these mice showed significant change in the relative organ weight. However, no toxicologically relevant changes were observed in hematology, biochemistry, and histopathology. Based on the findings, No-Observed-Adverse-Effect-Level (NOAEL) for BA were found to be < 24 mg/kg for male mice and < 6 mg/kg for female mice.

Introduction

Barringtonia racemosa (L.) is one of the plants, among accessible medicinal herbs, has been reported for high therapeutic value in traditional system of medicine in various countries. *Barringtonia racemosa* Linn. (Family Lecythidaceae) possess several bioactivities and is used in traditional medicine of India, Sri Lanka, Malaysia, Bangladesh, South Africa (Ong and Nordiana, 1999; Thomas et al., 2002; Deraniyagala et al., 2003; Bhat et al., 2012; Masoko, 2013; Osman et al., 2015; Kong et al., 2020). Although various species are recorded under the genus Barringtonia, only a few have been well-studied and documented. Approximately 15 species of Barringtonia are utilized as food (French, 2014), and only 3 species are extensively recorded to function as food and medicine, namely Barringtonia racemosa, Barringtonia asiatica, and Barringtonia acutangula. In Malaysia, apart from its medicinal value, the shoot, and young leaves of Barringtonia racemosa are usually consumed raw as a salad with various condiments (Ho et al., 2020).

Abbreviations: AAALAC, Association For Assessment And Accreditation Of Laboratory Animal Care; ALP, Alkaline Phosphatase; AST, Aspartate Aminotransferase; ALT, Alanine Aminotransferase; BA, Bartogenic Acid; BUN, Blood Urea Nitrogen; b.wt., Body Weight; FDA, Food And Drug Administration; GLP, Good Laboratory Practice; H&E, Hematoxylin–Eosin; HCT, Hematocrit; LC/MS, Liquid chromatography–mass spectrometry; MCV, Mean Corpuscular Volume; MCH, Mean Corpuscular Hemoglobin; MCHC, Mean Corpuscular Hemoglobin Concentration; NMR, Nuclear Magnetic Resonance; UHPLC, Ultra High Performance Liquid Chromatography; NOAEL, No Observed Adverse Effect Level; OA, Oleanolic Acid; OECD, Organization For Economic Co-Operation And Development; RBC, Red Blood Cells Count; RDW-CV, Red Cell Distribution Width - Coefficient Of Variation; SEM, Standard Error Of The Mean; TLC, Total Leukocyte Count; UA, Ursolic Acid; VLDL, Very Low Density Lipoprotein.

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Geographically, Barringtonia racemosa (L.) covers a broad range of area including Eastern Africa, Madagascar, India and other Indian Ocean islands, Asia and Southeast Asia, Northern Australia, Melanesia, Micronesia, and Polynesia (Kong et al., 2016).

Pharmacologically, extract of *Barringtonia racemosa* has been tested and reported for its efficacy in various ailments by the researchers such as anti-bacterial (Khan et al., 2001; Mmushi et al., 2009), anti-oxidant (Van et al., 2020), anti-cancer (Amran et al., 2016), anti-tumor (Thomas et al., 2002), anti-nociceptive / analgesic (Deraniyagala et al., 2003), anti-inflammatory (Patil and Patil, 2016), anti-diabetic (Gowri et al., 2007), and anti-proliferative effects (Ho et al., 2020). Further in toxicology aspects, the extract of *Barringtonia racemosa* has been reported for its toxicity in the rodents and found toxic above 24 mg/kg body weight (Thomas et al., 2002). Furthermore, alcohol and water extracts of the bark of this plant are toxic to aphis (Toxoptera aurantii Boy). The seeds and bark are used as fish poison. In Philippines the seeds are used to poison wild pig (Thomas et al., 2002).

A crude extract of aerial parts of *Barringtonia racemosa* plant extracted from solvents such as ethanol/methanol/ethyl acetate/chloroform/water comprised many active constituents and have been reported for numerous therapeutic activities. Patil and Patil, (2016) reported BREAF (fraction of ethyl acetate extract of fruits of *Barringtonia racemosa*) contained bartogenic acid (BA) as a major active constituent and belongs to the class of pentacyclic triterpenoids. Pentacyclic triterpenoids have been recognized for their therapeutic implications. BA has been reported for anti-diabetic (Gowri et al., 2007), anti-arthritic (Patil et al., 2011), anti-inflammatory (Patil and Patil, 2016), chemomodulatory (Ojha et al., 2016) and anti-tumor activities (Dubey et al., 2021). Apart from the reported therapeutic importance of BA in various diseases, there is a lack of toxicological profile for BA in the scientific literature, and it is not reported till date. Fundamentally, a toxicity assessment of BA in the rodents should be done.

Furthermore, many of the conventional drugs that are currently used are from herbs. Regardless of their consistent use, the stringent challenge faced by phytomedicines is the lack of scientific evidence for their mode of action and safety profile in vivo. This necessitates the conduct of meticulously planned toxicity studies based on stipulated guidelines to claim the safety of medicinal plants used in various traditional practices (Subramanian, 2018).

Bartogenic acid has close structural similarity with other pentacyclic triterpenoids such as oleanolic acid (OA) and ursolic acid (UA) (Fig. 1). OA and UA are currently being evaluated in clinical trial under cancer therapy through intravenous route (Shanmugam et al., 2014; Qian et al., 2015; Khwaza et al., 2020). BA has been reported for its chemomodulatory activity through oral route (Ojha et al., 2016) and anti-tumor activity through intravenous route (Dubey et al., 2021). Hence, in this study, intravenous route was selected to avoid limited systemic exposure due to the cellular barriers presented in the gastrointestinal tract. Further bioavailability of intravenous medication is for all time higher than that of the oral counterpart. Intravenous administration is desirable for first use in human studies since it eliminates variability related to the bioavailability. Furthermore, intravenous administration is an acceptable and recommended route of administration in humans. It is also the recommended route of administration according to the European Medicine Agency guideline for the evaluation of anticancer medicinal products for human use (EMA, 2017).

Therefore, in support to above statement, this study was conducted. The objective of this study was to determine the tolerability of BA in single dose toxicity test and No Observed Adverse Effect Level (NOAEL) for BA in 28-day repeated dose toxicity test. Under 28-day repeated dose toxicity assessment, emphasis was given to body weight change, food consumption, clinical signs, sensory reactivity, clinical biochemistry, hematology, urinalysis, relative organ weights, gross pathology, and histopathology followed 28 days daily intravenous administration of BA in BALB/c mice.

Methods and materials

Plant material

Fruits of *Barringtonia racemosa* were procured from the herbal drug market Delhi, India and authenticated from National Institute of Science Communication and Information Resources (NISCAIR), Council of Scientific and Industrial Research, (CSIR), Government of India, New Delhi, India with identification voucher number as NISCAIR/RHMD/Consult/2017/3122–71-1.

Extraction and isolation of BA

Fruits of *Barringtonia racemosa* were thoroughly washed and dried completely under the shade and powdered in a grinder. 1.5 kg of coarse powder of the fruits of *Barringtonia racemosa*was extracted through soxhlet extraction using 100% methanol for 7 days at 40 ± 2 °C. The extract was concentrated using rotary evaporator (BUCHI, Germany) under reduced pressure at 40 ± 2 °C yielded 24.5 g of dark brown colored thick paste (Gowri et al., 2009).

Characterization of BA by ^{1}H NMR and ^{13}C NMR

Characterization of BA was done by means of 1D ^{1}H NMR (400 MHz) and ^{13}C NMR (100 MHz). NMR spectra were recorded on a Bruker DRX-400 instrument (Rheinstetten, Germany), equipped with a dual 1H/13C probe, using standard Bruker pulse sequences. The chemical shifts were reported in parts per million (ppm), and J values were in Hz. Deuterated methanol was used as a solvent system.

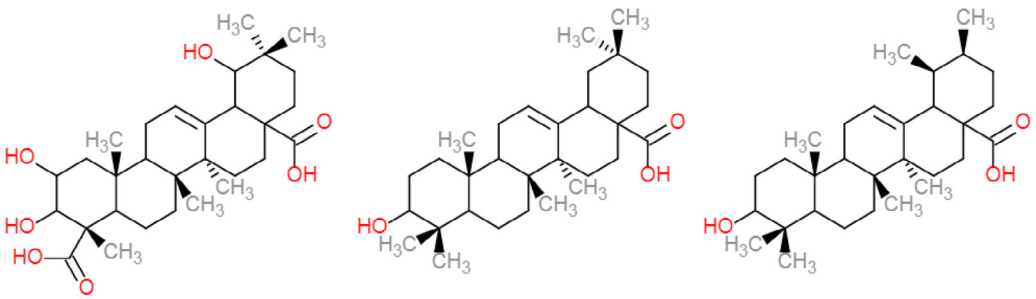


Fig. 1. Structure of Bartogenic acid (2α, 3β, 19β-trihydroxyolean-12-en-23, 28-dioic acid) and its structural resemblance with Oleanolic acid and Ursolic acid.

Characterization of BA by UHPLC

Test sample was dissolved in 100% methanol and analyzed by UHPLC (Thermo Scientific Ultimate 3000, USA). The system contained UV detector with 0.05 aufs (absorbance units full scale) sensitivity, LPG-3600 quat pump, TCC-3000 thermostatted column compartment, and WPS-3000 analytical thermostatted autosampler. Agilent Eclipse XDB C18 (250 × 4.6 mm, 5 μ) column equipped with automatic temperature controller module was used. A mobile phase of 0.1% formic acid: 100% methanol (30:70 v/v) with an elution volume of 0.5 mL/min was selected. The column temperature was maintained at 40 °C (±0.1 °C).

Characterization of BA by LC/MS

An LC/MS spectrum of BA was recorded on a triple quad 5500 + LC/MS system-QTRAP instrument (Sciex, USA). Acquisition information was as follows; polarity: negative, ion source: turbo spray, scan rate: 200 Da/s, injection volume: 10 μL, flow program: binary gradient program, Buffer A: 0.1% Formic acid, Buffer B: 100% Methanol, column oven temperature: 40 °C, Column: Agilent Eclipse XDB C18 (250 × 4.6 mm, 5 μ), flow rate: 0.7 mL/min. Mass spectrometry(m/z) were scanned in the negative ionization mode with the temperature source of 550 °C.

Animals

The experiment was reviewed and approved by the Institutional Animal Ethics Committee (IAEC) of Dabur Research Foundation with reference number of IAEC/42/529. This study was performed at Dabur Research Foundation (Reg. No. 64/PO/RcBi/S/99/CPCSEA), accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) and certified with Organization for Economic Cooperation and Development (OECD) – Good Laboratory Practices (GLP). The experiment was performed in compliance with the appropriate care of all experimental animals in compliance with the National Research Council's Guide for the Care and Use of Laboratory Animals (NRC, 2011) and Reporting Animal Research: Explanation and Elaboration for the ARRIVE Guidelines 2.0 (Percie du Sert et al., 2020).

Healthy male and female BALB/c mice, procured from Vivo Biotech Limited, Hyderabad, India, were used in this study. BALB/c mice were maintained in polypropylene cages under standard conditions (temperature 22 ± 3 °C and relative humidity of 50 ± 20 °C) with 12 h of dark and light cycle with free access to food and water *ad libitum*.

Justification for selection of doses and dose preparation

Toxicity of BA has not been reported in the scientific literature till date hence, firstly, a single dose toxicity study was conducted in a stepwise manner, aiming to determine the tolerability of BA at the single intravenous dose of 12.5, 25, 50 and 100 mg/kg body weight. Mortality was considered as an endpoint. Two mortalities were observed at 100 mg/kg of BA treatment. Hence, doses lower than 100 mg/kg were selected in a 14-day repeated dose range finding study.

Prior to main study, a 14-day repeated dose range finding study was performed at the doses of 6, 12, 24 and 48 mg/kg body weight of BA. At 48 mg/kg of BA, post fourth dose of BA, severe toxic signs such as tremors and lethargy were observed. Later, all the animals died. Hence, main study was performed with the doses of 1.5, 6 and 24 mg/kg of BA. A wide range of doses were selected with the dose progression factor of 4.

BA was not soluble in 0.9% normal saline and water for injection hence, for intravenous administration, BA was formulated in US FDA approved vehicle. A mixture of Cremophor EL:ethanol (1:1, v/v) and sterile water for injection was used as vehicle. BA was formulated in Cremophor EL:ethanol (1:1, v/v) and sterile water for injection.

Study design

Single dose toxicity study

Single dose toxicity study was performed following the guidelines of Organization for Economic Co-operation and Development (OECD) for testing of chemicals, OECD 423 with some modifications (OECD, 2002) and Redbook 2000, IV.C.3.a. Short-term toxicity studies with rodents (US Food and Drug Administration, 2003). Twenty-five female mice (nulliparous and nonpregnant) were randomized and grouped into five groups (5 mice per group) viz., one control and four test groups. Control group received vehicle at a dose volume of 5 mL/kg b.wt. and the test groups received BA at single intravenous dose of 12.5, 25, 50 and 100 mg/kg b.wt. through tail vein in a stepwise manner. There was a 72 h dosing gap between the two test groups. Mortality was considered as an endpoint. All the mice were observed for mortality at 30 min, 1, 2 and 4 h post dosing and thereafter once daily for next 14 days. Body weights were recorded weekly once. On day 15, all the surviving mice were euthanized by CO_2 asphyxiation and subjected to gross pathological examination of the vital organs such as heart, lung, liver, kidney, and spleen. Mice died during the study were subjected to gross pathological examination.

Repeated dose toxicity study (Main study)

Repeated dose toxicity study was performed following the guidelines of the US FDA toxicological principles for the safety assessment of food ingredients (Redbook 2000) (US Food and Drug Administration, 2007) and Organization for Economic Co-operation and Development (OECD) for testing of chemicals, OECD guideline 407 with some minor modifications (OECD, 2008). First, animals were exposed to BA via intravenous administration to evaluate potential systemic toxicity. Second, additional satellite groups (control and high dose group) for observation of reversibility of toxic effects were not included in the study. Third, histopathology was performed for a limited number of organs i.e., mainly vital organs such as liver, heart, kidney, spleen, and lung however gross pathology was done for all the organs. Twenty male and twenty female mice (nulliparous and nonpregnant) were randomized and grouped into four groups. Each group comprised 5 male and 5 female mice. The study design is presented in Table 1. The test item (BA) and control item (vehicle) were administered to mice once-daily for 28 days through intravenous route (tail vein).

General clinical observations

Mice were observed once daily for clinical signs of toxicity and twice daily for mortality and morbidity throughout the study, special attention being paid during the first hour post dosing. The body weight was recorded daily. Food consumption was measured weekly once. Percent body weight change was calculated as final body weight (g) – initial body weight (g) x100/initial body weight (g).

Detailed clinical examination

Detailed clinical observation (outside the home cage in a standard arena) was performed weekly once for all the mice throughout the study. Clinical signs such as changes in skin color, fur, eyes, unusual secretions (lacrimation, piloerection, nasal discharge, diarrhea), unusual respiratory pattern, changes in gait, posture, response to handling, presence of clonic or tonic movements, and stereotypes (excessive grooming, repetitive circling, and bizarre behavior) were recorded.

Table 1
Experimental design of the study with the doses, dose volume, dose concentrations and group size.

Group	Treatment	Dose (mg/kg)	Dose Volume (mL/kg)	Dose Concentration(mg/mL)	Number of Animals/ Group	
					Male	Female
1	Vehicle	0	5	0	5	5
2	BA	1.5	5	0.3	5	5
3	BA	6	5	1.2	5	5
4	BA	24	5	4.8	5	5

Sensory reactivity examination

Sensory reactivity examination test was performed on day 28. Sensory reactivity to stimuli of different types (auditory, visual, and proprioceptive stimuli) was conducted for all the mice. Locomotor activity was assessed by using infra-red actophotometer. Individual animal was kept for free movement inside actophotometer for 5 min and the number of times it crosses the beam of light is displayed digitally and recorded.

Urinalysis

Urinalysis was performed post 28 days of dosing. On day 29, mice were placed in the metabolic cages for 6 h and urine of all the mice were collected for 6 h. Urine parameters like appearance, blood, bilirubin, ketone bodies, leukocytes, glucose, pH, specific gravity were analyzed.

Clinical biochemistry

At termination i.e., on day 29, before blood collection, local anesthesia (0.5% Paracaine) was given to avoid pain. Mice were anesthetized with isoflurane followed open drop method (AVMA, 2020) and blood was collected under isoflurane anesthesia by *retro*-orbital sinus puncture with the help of a fine capillary tube. Blood was collected in non-heparinized tubes, further, centrifuged at 2000 rpm/min for 10 min and serum was separated. The biochemical indicators such as alkaline phosphatase (ALP), aspartate aminotransferase (AST), alanine aminotransferase (ALT), albumin, total bilirubin, total protein, creatinine, urea, blood urea nitrogen (BUN), triglyceride, cholesterol, and very low-density lipoprotein (VLDL) were analyzed.

Hematology

For hematology, blood was collected into tubes containing EDTA. Standard operating procedures were used to determine the hematological parameters such as total leukocyte count (TLC), Lymphocyte, Neutrophils, Monocyte, Eosinophils, Basophils, red blood cells count (RBC), hemoglobin (HB), hematocrit (HCT), mean corpuscular volume (MCV), Mean corpuscular hemoglobin (MCH), Mean corpuscular hemoglobin concentration (MCHC), Red cell distribution width - coefficient of variation (RDW-CV) and platelet count (PLT).

Gross pathological examination

Post blood collection, mice were humanly sacrificed by overdose of thiopentone sodium through intravenous route (AVMA, 2020), organs such as liver, kidney, lungs, heart, spleen, brain, ovary, uterus, testes and epididymides were collected, and observed for external gross pathological changes.

Histopathological examination

The vital organs such as liver, kidney, lungs, heart, and spleen were fixed in 10% neutral buffered formalin (NBF), embeddedin paraffin,-sectioned at 5 μm thickness using a microtome and stained with haematoxylin and eosin (H&E). The sections were then evaluated for histological changes under light microscope (Nikon) and the images were taken using 100X, 200X and 400X magnifications. The stained sections were examined for any inflammatory changes like infiltration of cells, necrotic foci, damage to tissuestructure and scored on semi-quantitative scale of 0–5;scores were as follow: 0, no; 1, minimum (1–5%); 2, mild (6–20%); 3, moderate (21–35%); 4, marked (36–50%); 5, severe (51–100%) (Meyerholz et al. 2013).

Relative organ weight

The vital organs such as liver, kidney, lung, heart, and spleen were excised and weighed. Relative organ weight was calculated as absolute organ weight (g) x100/body weight on sacrifice day (g).

Statistical analysis

Statistical data analysis was performed using GraphPad Prism 8.4.1 Software (San Diego, CA, USA). Data expressed as mean ± SEM. The variance in the data of all parameters was checked for homogeneity by using Bartlett's procedure. When the data were homogeneous, ANOVA was used and for heterogeneous data, the Kruskal–Wallis test was used. ANOVA followed by Dunnett test or Bonferroni test were employed for comparisons between control and treated groups. Results were considered significant at $^{*}p < 0.05$.

Results

Characterization of BA

Analytical confirmation of BA was done by means of 1D ^{13}C NMR (100 MHz) and^{1}H NMR (400 MHz). ^{13}C NMR spectra of BA showed characteristics signals at δ 143.25, recognized as an olefinic carbon, signal at δ 123.31, revealed the presence of a double bond between C-12 and C-13 and signals at δ 179.20 and 180.90 were attributed to two acidic groups (–COOH). Further, ^{1}H NMR spectra of BA showed characteristics signals for the proton at defined region confirmed the presence of BA. ^{1}H NMR signals [δ 1.44 (3H, s), 1.03 (3H, s), 0.77 (3H, s), 1.32 (3H, s), 0.97 (3H, s), and 0.93 (3H, s), represented six methyl groups of BA (Fig. 2 a-b). This was in the agreement with the previously reported data of BA (Gowri et al., 2007a, 2009b). Purity of BA was established by using Ultra High Performance Liquid Chromatography (UHPLC) (Fig. 3 a-b) and LC/MS (Fig. 4). The LC/MS spectra showed molecular ion peak at *m/z* of 517.4 (at negative ionization mode) corresponding to molecular weight of BA ($C_{30}H_{46}O_7$) (Gowri et al., 2007). The purity of BA was found to be 98.45%. Structure of BA is presented in Fig. 1 (Patil and Patil, 2016).

Single dose toxicity study

Single intravenous dose of BA at 12.5, 25, 50 and 100 mg/kg showed 0, 0, 0 and 2 mortalities, respectively. BA treated groups did not show significant change in the body weight as compared to vehicle treated

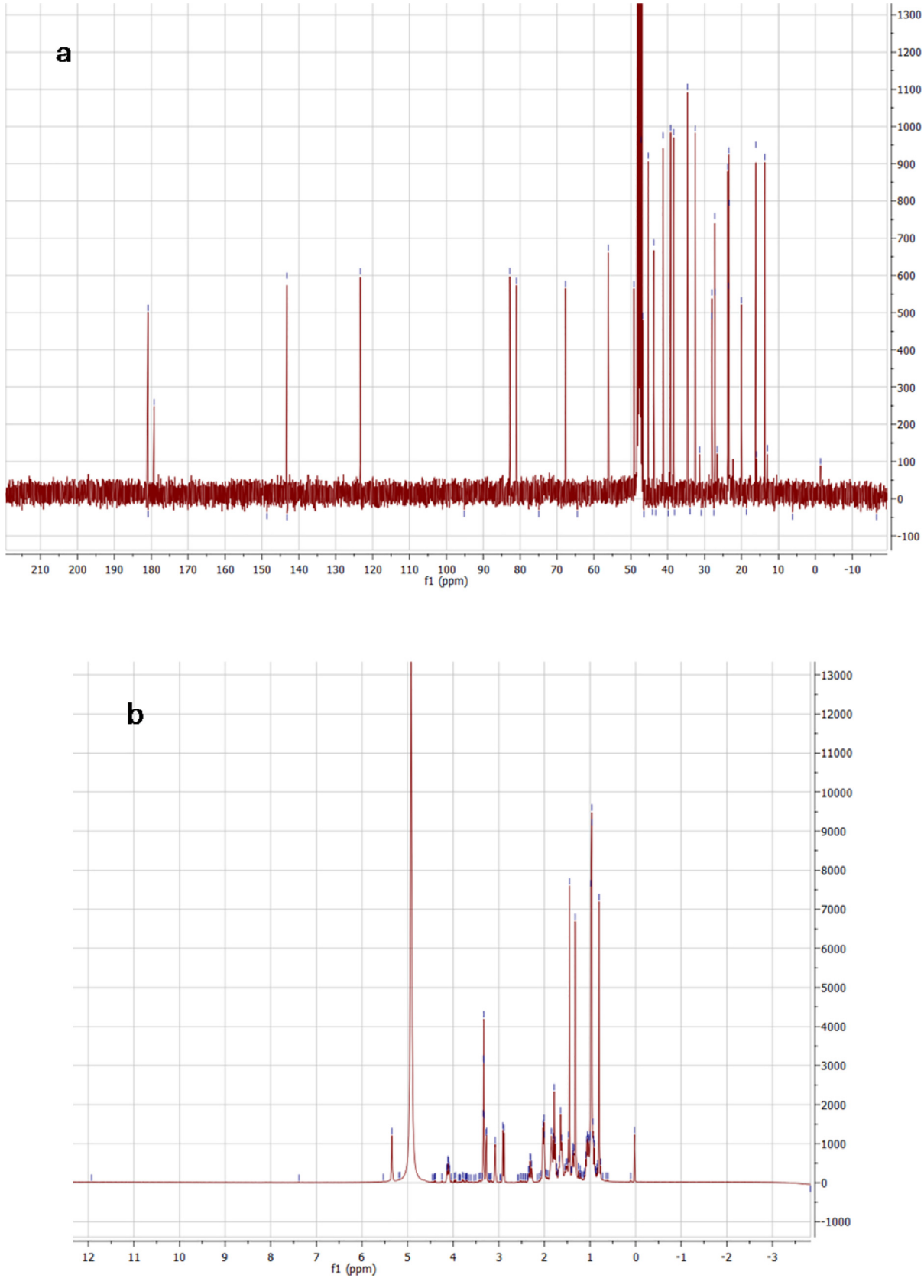
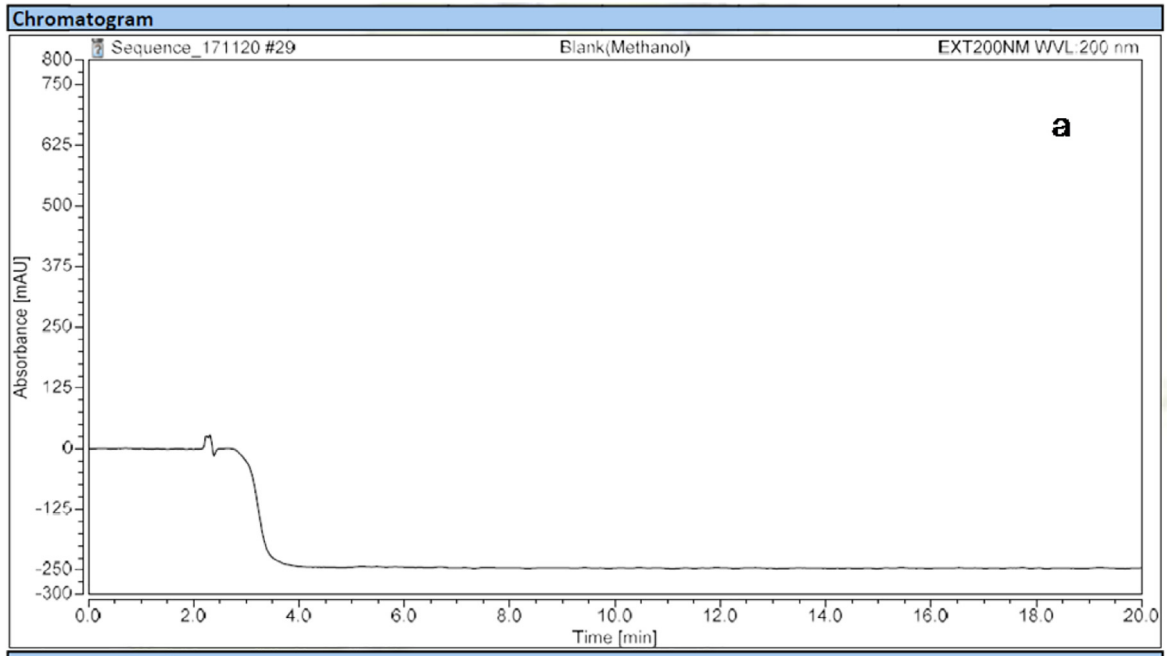
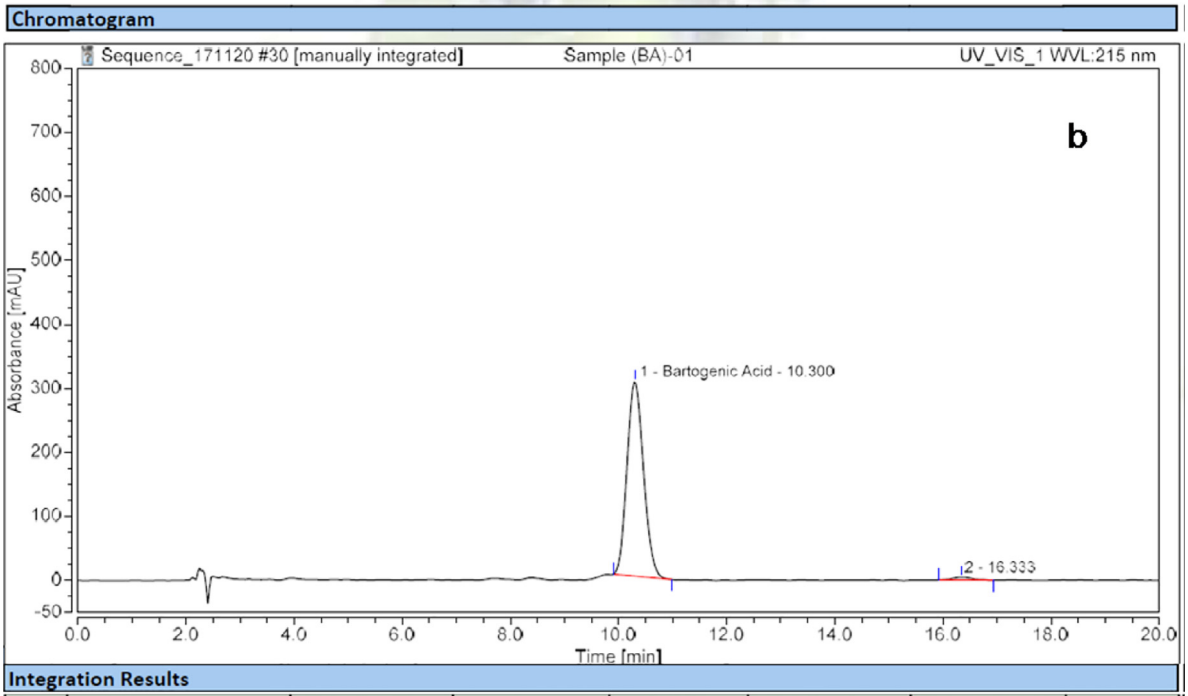


Fig. 2. NMR spectrum of BA. (a) ^{13}C NMR spectra of BA showed characteristics signals at δ 143.25, recognized as an olefinic carbon, signal at δ 123.31, revealed the presence of a double bond between C-12 and C-13 and signals at δ 179.20 and 180.90 were attributed to two acidic groups (–COOH). (b) ^{1}H NMR spectra of BA showed characteristics ^{1}H NMR signals for the proton at defined region confirmed the presence of BA. ^{1}H NMR signals [δ] 1.44 (3H, s), 1.03 (3H, s), 0.77 (3H, s), 1.32 (3H, s), 0.97 (3H, s), and 0.93 (3H, s), represented six methyl groups of BA.



Integration Results

No.	Peak Name	Retention Time min	Area mAU*min	Height mAU	Relative Area %	Relative Height %	Amount
n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
Total:			**0.000**	**0.000**	**0.00**	**0.00**	



Integration Results

No.	Peak Name	Retention Time min	Area mAU*min	Height mAU	Relative Area %	Relative Height %	Amount
1	Bartogenic Acid	10.300	107.756	304.330	98.45	98.68	n.a.
2		16.333	1.699	4.081	1.55	1.32	n.a.
Total:			**109.455**	**308.411**	**100.00**	**100.00**	

Fig. 3. (a) UHPLC chromatogram of Blank. (b) UHPLC chromatogram of BA Purity of BA was found to be 98.45%

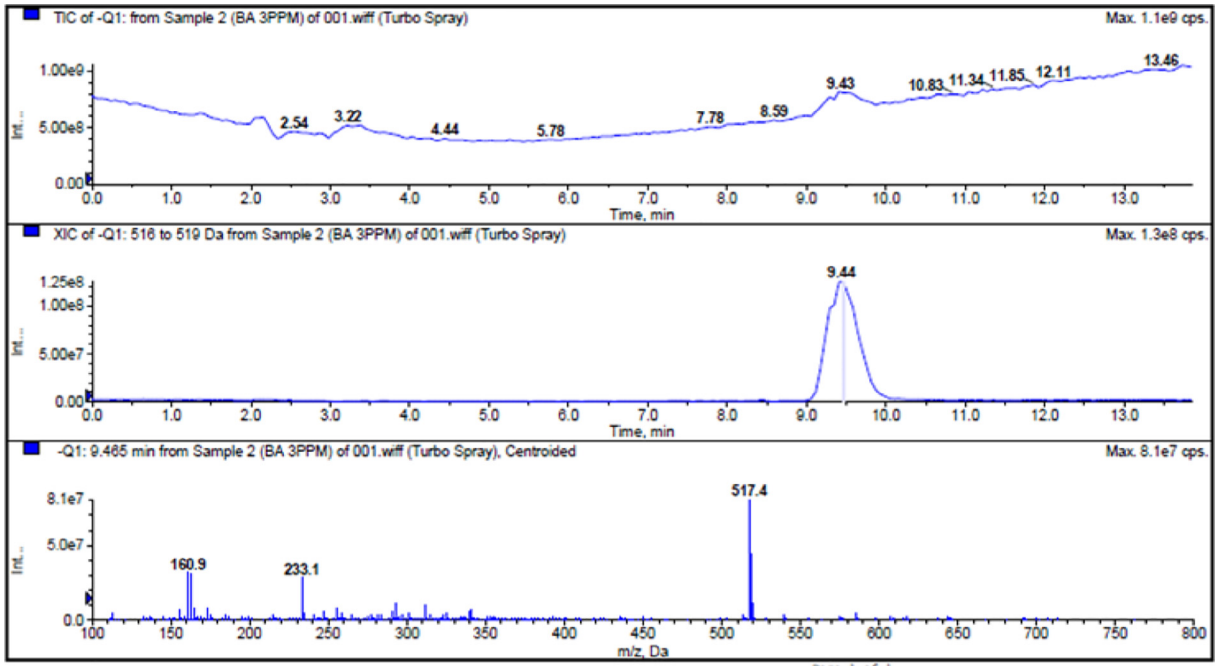


Fig. 4. LC/MS spectra of BA: Peak at 9.44 min indicated the *m/z* of 517.4 (At negative ion mode, *m/z* of 517.4 is equivalent to *m/z* of 518.7). Molecular weight of BA has been reported as 518.7.

control group (Data not shown). Gross pathological examination did not reveal any abnormality in all the surviving mice. Cause of the death of the mice died at 100 mg/kg of BA could not be determined.

Repeated dose toxicity study (Main study)

Body weight and % body weight change

Male mice treated with BA at the doses of 1.5 mg/kg and 6 mg/kg did not show significant change in the body weight as compared to the vehicle treated mice. 24 mg/kg of BA showed significant reduction in the body weight on day 11 (*P < 0.05), days 13–14 (*P < 0.05), days 15–18 (**P < 0.01) and days 19–28 (***P < 0.001). Male mice treated with 24 mg/kg of BA showed a maximum of 9.7% reduction in the body weight (Fig. 5 a-b).

Female mice treated with BA at the dose of 1.5 mg/kg did not show significant change in the body weight as compared to the vehicle treated mice. 6 mg/kg of BA showed significant reduction in the body weight on days 7–9 (*P < 0.05), day 10 (**P < 0.01), days 11–13 (*P < 0.05), day 14 (**P < 0.01), and days 15–28 (***P < 0.001). Apart from this, female mice treated with 24 mg/kg of BA showed significant reduction in the body weight on day 6 (**P < 0.01) and days 7–28 (***P < 0.001). Female mice treated with 24 mg/kg of BA showed a maximum of 12.9% reduction in the body weight (Fig. 5 c-d).

Food consumption

Mice of both sexes treated with BA at the dose of 1.5 mg/kg showed similar pattern of food intake throughout the study as compared to the control group. Male mice treated with BA at the doses of 6 mg/kg, and 24 mg/kg showed significant (*P < 0.05; **P < 0.01) reduction in the food intake on third and fourth week of the treatment period as compared to the control group. Female mice treated with BA at the doses of 6 mg/kg, and 24 mg/kg showed significant (*P < 0.05; **P < 0.01; ***P < 0.001) reduction in the food intake on first, second, third and fourth week of the treatment period as compared to the control group (Table 2).

General clinical signs and detailed clinical examination

None of the mice from the control group and BA treated groups showed any clinical signs of toxicity and mortality on cage side observation during experimental period. Vehicle and BA treated groups showed normal clinical signs throughout the study (Table 3).

Sensory reactivity and locomotor activity examination

Both male and female mice treated with BA at the doses of 1.5 mg/kg, 6 mg/kg, and 24 mg/kg showed normal sensory reactivity to various stimuli as compared to the control group (Table 4). Along with this, these mice did not show significant change in the motor activity as compared to the control group (Table 5).

Urinalysis

Both male and female mice treated with BA at the doses of 1.5 mg/kg, 6 mg/kg, and 24 mg/kg did not show significant change in the urine parameters as compared to the control group (Table 6).

Clinical biochemistry

Clinical biochemistry provides information on the metabolic/physiologic functioning of the organs which subsequently reflect health status of the organs. Due to the metabolic and excretory functions of the liver and kidney, these organs exposed directly. Therefore, under clinical biochemistry, renal function was assessed by estimating the urea and creatinine level in the blood (Gowda et al., 2010), and liver function was assessed by estimating the alkaline phosphatase (ALP), aspartate aminotransferase (AST), alanine aminotransferase (ALT), and total bilirubin level in the blood (Gowda et al., 2009).

Mice of both sexes treated with BA at the doses of 1.5 mg/kg, and 6 mg/kg did not reveal significant change in clinical biochemistry as compared to the control group (Table 6). At 24 mg/kg of BA, only female mice showed significant change in total bilirubin (*P < 0.05), AST (**P < 0.01), ALT (**P < 0.01), creatinine

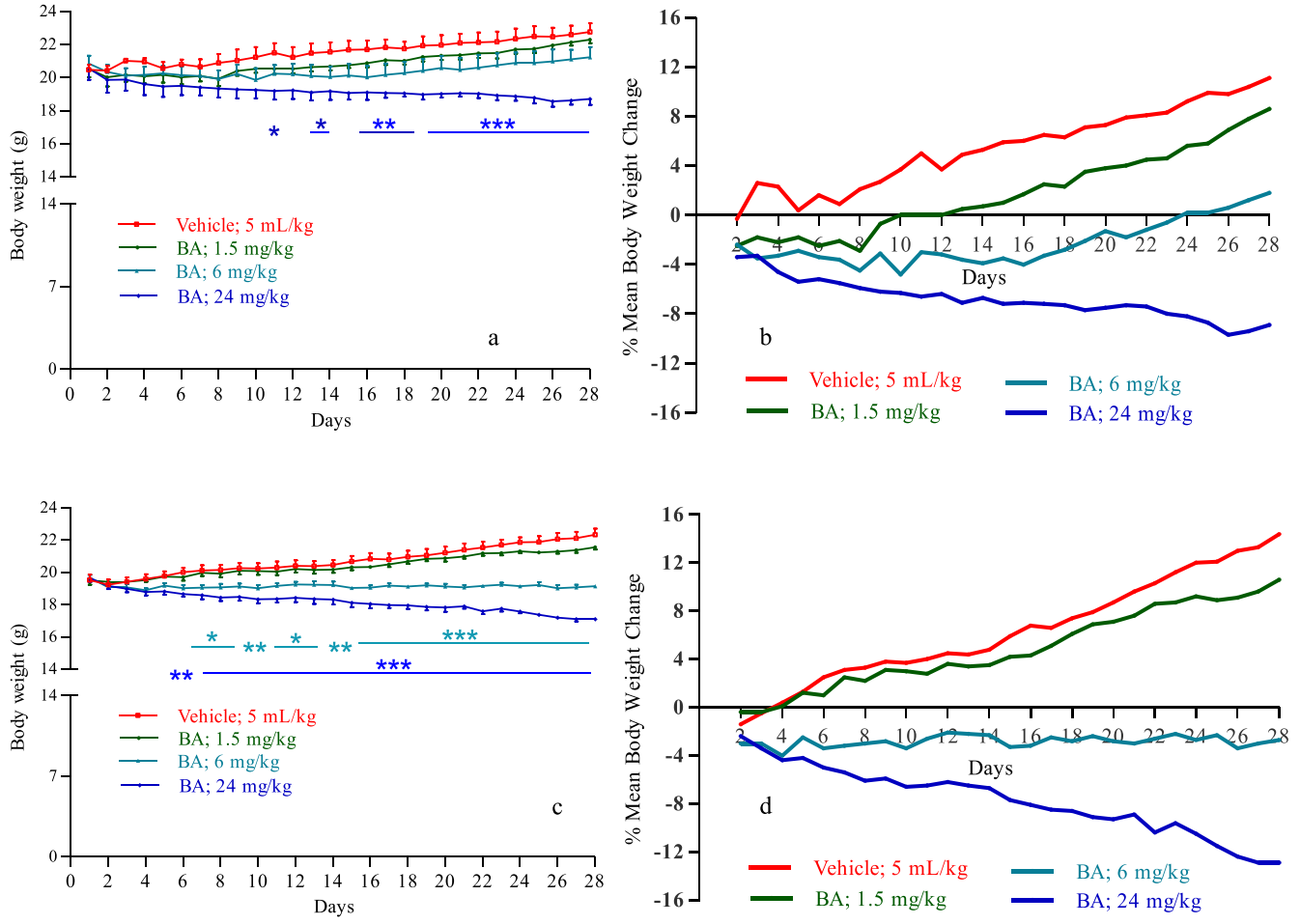


Fig. 5. Body weight data of the experimental animals. (a) Effect of BA treatment on body weight (Male). (b) Effect of BA treatment on % body weight change (Male). (c) Effect of BA treatment on body weight (Female). (d) Effect of BA treatment on % body weight change (Female). Values are presented in mean ± SEM, n = 5; statistical significance levels from control (vehicle) expressed as (*P < 0.05), (**P < 0.01), (***P < 0.001).

Table 2
Effect of BA treatment on weekly food intake (Male and Female)

Sex	Male				Female			
Treatment & Dose	Vehicle (5 mL/kg)	BA (1.5 mg/kg)	BA (6 mg/kg)	BA (24 mg/kg)	Vehicle (5 mL/kg)	BA (1.5 mg/kg)	BA (6 mg/kg)	BA (24 mg/kg)
First week	280.7 ± 6.1	278.9 ± 2.9	279.7 ± 2.1	272.3 ± 2.9	283.3 ± 4.5	272.5 ± 5.0	268.8* ± 3.5	266.7* ± 1.3
Second week	274.9 ± 7.1	280.0 ± 3.7	273.0 ± 1.7	268.7 ± 3.7	278.0 ± 4.2	269.3 ± 3.6	253.0*** ± 1.70	239.9*** ± 0.7
Third week	286.4 ± 2.4	271.5 ± 3.3	269.0* ± 4.7	262.4** ± 5.8	279.7 ± 0.3	279.9 ± 1.7	258.8*** ± 4.5	225.7*** ± 0.9
Fourth week	288.9 ± 6.2	286.7 ± 7.0	263.7** ± 2.0	263.3** ± 3.6	287.0 ± 2.0	280.3 ± 1.2	259.2*** ± 2.0	245.6*** ± 2.5

Values are presented in mean ± SEM, n = 5;*P < 0.05, **p < 0.01, ***P < 0.001 vs. Vehicle control group

(**P < 0.01), urea (*P < 0.05) and BUN (*P < 0.05) as compared to the control group (Table 6).

Hematology

Under hematology, erythrogram and leukogram were analyzed for the diagnosis of organ or tissue injuries and other pathologies (Kaid et al., 2019). Under erythrogram, hemoglobin, red blood cells count (RBC), hematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), and red cell distribution width - coefficient of variation (RDW-CV) were evaluated.

Under leukogram, total leukocyte count (TLC), differential leukocyte count (DLC) such as neutrophils, eosinophils, basophils, leucocyte, and monocyte were evaluated.

Mice of both sexes treated with BA at the dose of 1.5 mg/kg did not reveal significant change in the hematological parameters as compared to the control group. At 6 mg/kg of BA, only female mice showed significant (*P < 0.05) change in the neutrophils. Whereas at 24 mg/kg of BA, male mice showed significant change in MCHC (*P < 0.05) and female mice showed significant change in RBC (**P < 0.01), neutrophils (**P < 0.01) and hemoglobin (***P < 0.001) as compared to the control group (Table 7).

Relative organ weight

Both male and female mice treated with BA at the doses of 1.5 mg/kg and 6 mg/kg did not show significant change in the relative weights of the heart, liver, kidney, spleen, and lung as compared to the control group. However, at 24 mg/kg of BA, male mice showed significant

Table 3
Effect of BA treatment on detailed clinical examination at termination (Male and Female).

Clinical Examination	Treatment			
	Vehicle (5 mL/kg)	BA (1.5 mg/kg)	BA (6 mg/kg)	BA (24 mg/kg)
Skin Examination	Normal	Normal	Normal	Normal
Piloerection	Absent	Absent	Absent	Absent
Eye Examination	Normal	Normal	Normal	Normal
Lacrimation	None	None	None	None
Diarrhea	None	None	None	None
Nasal Discharge	None	None	None	None
Salivation	None	None	None	None
Arousal	Normal	Normal	Normal	Normal
Respiration	Normal	Normal	Normal	Normal
Clonic Movement	Absent	Absent	Absent	Absent
Gait	Normal	Normal	Normal	Normal
Mobility	Normal	Normal	Normal	Normal
Tonic Movements	Absent	Absent	Absent	Absent
Stereotype	Absent	Absent	Absent	Absent
Bizarre Behavior	Absent	Absent	Absent	Absent
Convulsion	Absent	Absent	Absent	Absent

n = 10 (5 male & 5 female).

Table 4
Effect of BA treatment on sensory reactivity at termination (Male and Female).

Sensory Reactivity	Treatment			
	Vehicle (5 mL/kg)	BA (1.5 mg/kg)	BA (6 mg/kg)	BA (24 mg/kg)
Approach Response	Moderate	Moderate	Moderate	Moderate
Touch response	Normal	Normal	Normal	Normal
Click response	Normal	Normal	Normal	Normal
Tail pinch response	Flinch	Flinch	Flinch	Flinch
Air righting reflex	Normal	Normal	Normal	Normal
Rears (Numbers)	93	93	88	89
Urination (Number ofurine pool in open Field)	10	10	8	12
Defecation (Number of boluses in open Field)	18	7	14	20

n = 10 (5 male & 5 female)

Table 5
Effect of BA treatment on locomotor activity at termination (Male and Female).

Sex	Male				Female			
Treatment & Dose	Vehicle (5 mL/kg)	BA (1.5 mg/kg)	BA (6 mg/kg)	BA (24 mg/kg)	Vehicle (5 mL/kg)	BA (1.5 mg/kg)	BA (6 mg/kg)	BA (24 mg/kg)
Locomotor activity	499.80 ± 84.04	509.00 ± 51.15	466.00 ± 72.41	454.00 ± 19.56	674.60 ± 58.09	642.80 ± 30.39	610.20 ± 63.59	611.80 ± 45.13

increase in the relative weights of the heart (**P < 0.01) and spleen (*P < 0.05) as compared to the control group whereas relative weights of liver, kidney, and lung were not statistically remarkable. Further female mice at 24 mg/kg of BA showed significant increase in relative weights of heart (***P < 0.001), liver (***P < 0.001), lungs (*P < 0.05), kidney (*P < 0.05) and spleen (*P < 0.05) (Table 8).

Gross pathological examination of the organs

No treatment-related gross pathological changes were observed in the liver, lungs, heart, kidneys, spleen, brain, ovary, uterus, testes and epididymides in mice of both sexes treated with 1.5 mg/kg, 6 mg/kg and 24 mg/kg.

Histopathological examination of the vital organs

Male and female mice treated with vehicle and BA at the doses of 1.5 mg/kg, 6 mg/kg and 24 mg/kg showed normal histological architecture of liver, kidney, lungs, heart, and spleen (Table 9) (Figs. 6-10).

Discussions

As not much information was available on the toxicity of BA, firstly a single dose tolerability study was performed. Based on the findings of the single dose toxicity study, further long-term repeated dose toxicity study was conducted to demonstrate the No-Observed-Adverse-Effect-Level (NOAEL) for BA. Single dose toxicity study revealed two mortalities at 100 mg/kg of BA whereas lower doses such as 12.5 mg/kg, 25 mg/kg, and 50 mg/kg of BA were found to be well tolerated. 28-day repeated dose toxicity study was performed at 1.5 mg/kg, 6 mg/kg, and 24 mg/kg. BA at the dose of 1.5 mg/kg was found to be well tolerated in mice of both sexes. Female mice treated with BA at 6 mg/kg showed significant reduction in the body weight as compared to the control group however male mice did not show significant change in the body weight. BA at the dose of 24 mg/kg showed significant reduction in the body weight in mice of both sexes as compared to the control group. Along with this, these mice showed significant reduction in the food intake as compared to the control group. It seems likely that this incident was related to the administration of BA and it could be directly correlated. BA at dose of 24 mg/kg showed a maximum of 12.9% reduction in the body weight during the study. Mice treated with BA at dose of 1.5 mg/kg showed progressive gain in the body weight and it was comparable to the control group.

Table 6
Effect of BA treatment on clinical biochemistry (male and female) compared with historical control data.

Parameters	Vehicle; 5 mL/kg	BA; 1.5 mg/kg	BA; 6 mg/kg	BA; 24 mg/kg	Historical control data (range)
Male					
Albumin (g/dL)	2.70 ± 0.34	2.31 ± 0.05	2.44 ± 0.05	2.41 ± 0.06	2.5–3.4
ALP (U/L)	300.02 ± 23.88	303.15 ± 15.26	317.40 ± 22.79	325.26 ± 27.25	251–353
Total Bilirubin (mg/dL)	0.07 ± 0.01	0.09 ± 0.01	0.08 ± 0.00	0.09 ± 0.00	0–0.1
Cholesterol (mg/dL)	53.71 ± 2.09	53.29 ± 5.61	62.52 ± 3.93	64.80 ± 1.01	44–72
Creatinine(mg/dL)	0.37 ± 0.03	0.39 ± 0.03	0.42 ± 0.01	0.43 ± 0.04	0.3–0.6
AST (U/L)	148.32 ± 5.78	147.18 ± 6.21	154.79 ± 10.37	163.38 ± 11.95	110–174
ALT(U/L)	45.36 ± 1.19	39.35 ± 9.99	46.38 ± 2.23	45.17 ± 6.79	36–68
Triglyceride(mg/dL)	56.89 ± 3.62	49.95 ± 8.16	49.28 ± 7.02	57.27 ± 5.93	42–88
Total Protein(g/dL)	3.88 ± 0.11	3.97 ± 0.13	3.99 ± 0.20	3.96 ± 0.17	3.2–5
Urea (mg/dL)	50.41 ± 2.22	50.16 ± 2.76	51.98 ± 3.46	53.51 ± 3.65	46–55
VLDL (mg/dL)	8.60 ± 1.29	8.60 ± 1.36	9.80 ± 1.53	11.60 ± 1.17	7–16
BUN(mg/dL)	19.62 ± 1.41	23.07 ± 2.50	24.29 ± 1.62	25.00 ± 1.71	14–28
Urine specific Gravity	1.071 ± 0.003	1.074 ± 0.003	1.072 ± 0.003	1.075 ± 0.003	1.065–1.085
Female					
Albumin (g/dL)	2.69 ± 0.23	2.59 ± 0.26	2.53 ± 0.17	2.64 ± 0.12	2.5–3.2
ALP (U/L)	397.36 ± 58.05	414.64 ± 53.45	441.24 ± 32.06	424.58 ± 34.26	350–464
Total Bilirubin (mg/dL)	0.10 ± 0.00	0.10 ± 0.00	0.11 ± 0.01	0.15 ± 0.02*	0.1–0.3
Cholesterol (mg/dL)	75.45 ± 7.33	77.17 ± 4.93	76.40 ± 3.76	79.17 ± 5.95	52–85
Creatinine(mg/dL)	0.35 ± 0.04	0.38 ± 0.01	0.41 ± 0.02	0.48 ± 0.02**	0.3–0.6
AST (U/L)	134.45 ± 10.05	147.25 ± 5.87	162.32 ± 16.58	191.78 ± 12.18**	99–210
ALT(U/L)	44.35 ± 3.34	49.78 ± 3.15	56.30 ± 2.22	62.39 ± 4.37**	40–74
Triglyceride(mg/dL)	63.44 ± 3.91	66.53 ± 7.11	59.66 ± 10.20	71.86 ± 8.90	48–97
Total Protein(g/dL)	5.05 ± 0.31	5.10 ± 0.31	5.23 ± 0.20	5.47 ± 0.17	3.7–5.8
Urea (mg/dL)	36.26 ± 1.04	37.13 ± 0.79	40.24 ± 2.26	44.03 ± 2.03*	34–46
VLDL (mg/dL)	12.80 ± 0.86	13.40 ± 1.47	12.00 ± 2.10	17.80 ± 1.46	9–20
BUN(mg/dL)	16.94 ± 0.49	17.35 ± 0.37	19.91 ± 1.11	20.24 ± 1.06*	13–24
Urine specific Gravity	1.074 ± 0.003	1.075 ± 0.003	1.076 ± 0.003	1.078 ± 0.002	1.065–1.085

Values are presented in mean ± SEM, n = 5; *p < 0.05, **P < 0.01 vs. vehicle control group

Table 7
Effect of BA treatment on hematological parameters (male and female) compared with historical control data.

Parameters	Vehicle; 5 mL/kg	BA; 1.5 mg/kg	BA; 6 mg/kg	BA; 24 mg/kg	Historical control data (range)
Male					
TLC ($x10^3/mm^3$)	4.76 ± 1.05	5.09 ± 0.89	4.27 ± 0.67	4.85 ± 0.69	4–7
Neutrophils (%)	22.50 ± 3.91	25.26 ± 3.76	20.24 ± 2.30	23.54 ± 0.90	16–31
Lymphocyte (%)	65.88 ± 4.68	60.30 ± 4.33	71.54 ± 3.76	67.32 ± 1.49	55–78
Monocyte (%)	6.66 ± 1.65	8.04 ± 2.90	4.86 ± 1.65	5.26 ± 0.95	0.1–8.5
Eosinophils (%)	4.36 ± 0.92	5.70 ± 1.13	2.42 ± 0.33	2.96 ± 0.35	0–6.1
Basophils (%)	0.60 ± 0.13	0.70 ± 0.11	0.94 ± 0.13	0.92 ± 0.12	0–0.9
RBC ($x10^6/mm^3$)	9.42 ± 0.34	8.63 ± 0.28	9.48 ± 0.30	9.54 ± 0.43	8.2–10
Hemoglobin (g/dL)	14.60 ± 0.32	14.02 ± 0.29	14.46 ± 0.43	15.00 ± 0.64	12.5–16.1
Hct (%)	43.76 ± 1.37	41.18 ± 1.30	43.04 ± 1.29	42.96 ± 1.78	38–46
MCV (fL)	46.56 ± 1.14	47.80 ± 1.42	45.42 ± 0.29	45.10 ± 0.23	43–51
MCH (pg)	15.54 ± 0.24	16.30 ± 0.45	15.28 ± 0.14	15.70 ± 0.07	15–17
MCHC (g/L)	33.38 ± 0.49	34.08 ± 0.42	33.64 ± 0.26	34.86 ± 0.19*	30–36
RDW-CV	14.32 ± 0.07	16.94 ± 2.32	13.80 ± 0.58	13.32 ± 0.11	12–18
Platelet count ($x10^3/mm^3$)	801.20 ± 81.55	917.60 ± 152.33	907.40 ± 265.87	893.20 ± 187.21	650–1150
Female					
TLC ($x10^3/mm^3$)	10.15 ± 0.51	10.82 ± 0.56	9.93 ± 0.34	8.29 ± 0.84	8–11
Neutrophils (%)	11.26 ± 2.66	13.74 ± 1.83	21.90 ± 1.18*	22.82 ± 2.85**	9–24
Lymphocyte (%)	74.52 ± 6.51	78.58 ± 3.15	68.50 ± 0.92	69.78 ± 2.06	62–83
Monocyte (%)	4.64 ± 1.70	5.14 ± 1.03	4.86 ± 1.10	4.08 ± 0.46	0.1–6.1
Eosinophils (%)	3.20 ± 0.37	2.16 ± 0.51	4.20 ± 1.66	2.58 ± 0.74	0–4.4
Basophils (%)	0.38 ± 0.16	0.38 ± 0.15	0.54 ± 0.22	0.74 ± 0.33	0–0.7
RBC ($x10^6/mm^3$)	9.91 ± 0.79	8.60 ± 0.19	8.73 ± 0.28	7.14 ± 0.57**	7.1–10.4
Hemoglobin (g/dL)	15.80 ± 0.68	14.16 ± 0.60	14.10 ± 0.34	12.24 ± 0.36***	11.5–16.5
Hct (%)	41.66 ± 3.70	40.12 ± 0.59	39.64 ± 2.58	33.92 ± 2.28	31–45
MCV (fL)	44.02 ± 0.63	46.76 ± 1.51	47.62 ± 0.94	47.88 ± 1.98	43–49
MCH (pg)	15.46 ± 0.29	16.00 ± 0.68	15.74 ± 0.48	17.46 ± 1.01	15–19
MCHC (g/L)	35.12 ± 0.17	34.34 ± 1.36	33.06 ± 0.63	36.50 ± 1.62	31–37
RDW-CV	18.04 ± 1.36	19.42 ± 1.57	18.66 ± 0.99	22.76 ± 2.99	14–20
Platelet count ($x10^3/mm^3$)	1030.20 ± 52.63	1052.40 ± 56.99	1030.20 ± 51.67	1099.80 ± 65.88	550–1250

Values are presented in mean ± SEM, n = 5; *p < 0.05, **P < 0.01, ***P < 0.001 vs. vehicle control group

No mortality and no toxic clinical signs were observed during 28-day repeated dose toxicity study. Sensory reactivity examination test did not reveal any abnormality in all the mice treated with vehicle and BA at all the tested doses.

No toxicologically relevant changes were observed in the hematology in mice of both sexes at 1.5 mg/kg of BA as compared to the control group. However, at 6 mg/kg of BA, only female mice and at 24 mg/kg of BA, both male and female mice showed significant change

Table 8
Effect of BA treatment on the relative organ weight (g) (Male and Female).

Sex	Male				Female			
Treatment & Dose	Vehicle; 5 mL/kg	BA; 1.5 mg/kg	BA; 6 mg/kg	BA; 24 mg/kg	Vehicle; 5 mL/kg	BA; 1.5 mg/kg	BA; 6 mg/kg	BA; 24 mg/kg
Heart	0.696 ± 0.04	0.698 ± 0.03	0.783 ± 0.06	0.944** ± 0.06	0.737 ± 0.03	0.753 ± 0.04	0.909 ± 0.05	1.019*** ± 0.06
Kidney	1.96 ± 0.08	1.92 ± 0.03	2.05 ± 0.21	2.31 ± 0.17	1.99 ± 0.05	2.05 ± 0.08	2.24 ± 0.17	2.48* ± 0.16
Lungs	1.03 ± 0.10	1.01 ± 0.10	1.17 ± 0.14	1.31 ± 0.07	1.06 ± 0.08	1.04 ± 0.05	1.25 ± 0.12	1.44* ± 0.09
Liver	5.70 ± 0.38	5.59 ± 0.45	5.76 ± 0.29	6.88 ± 0.51	5.28 ± 0.26	5.34 ± 0.22	6.55 ± 0.46	7.08*** ± 0.34
Spleen	0.295 ± 0.02	0.353 ± 0.02	0.355 ± 0.02	0.394* ± 0.03	0.344 ± 0.03	0.388 ± 0.02	0.414 ± 0.02	0.444* ± 0.02

Values are presented in mean ± SEM, n = 5; *p < 0.05, **P < 0.01, ***P < 0.001 vs. control group

Table 9
Histopathological findings of the vital organs post BA treatment (Male and Female).

Organs	Histopathological Findings	Treatment			
		Vehicle; 5 mL/kg	BA; 1.5 mg/kg	BA; 6 mg/kg	BA; 24 mg/kg
Heart	Myocardial fiber degeneration	0/10	0/10	0/10	0/10
Liver	Inflammatory cells	0/10	0/10	0/10	0/10
	Steatosis	0/10	0/10	0/10	0/10
Lungs	Alveoli inflammation	0/10	0/10	0/10	0/10
Spleen	Diffuse white pulp	0/10	0/10	0/10	0/10
	Distorted lymphoid architecture	0/10	0/10	0/10	0/10
Kidney	Tubular degeneration	0/10	0/10	0/10	0/10
	Granular degeneration	0/10	0/10	0/10	0/10

n = 10 *(5 male and 5 female)*

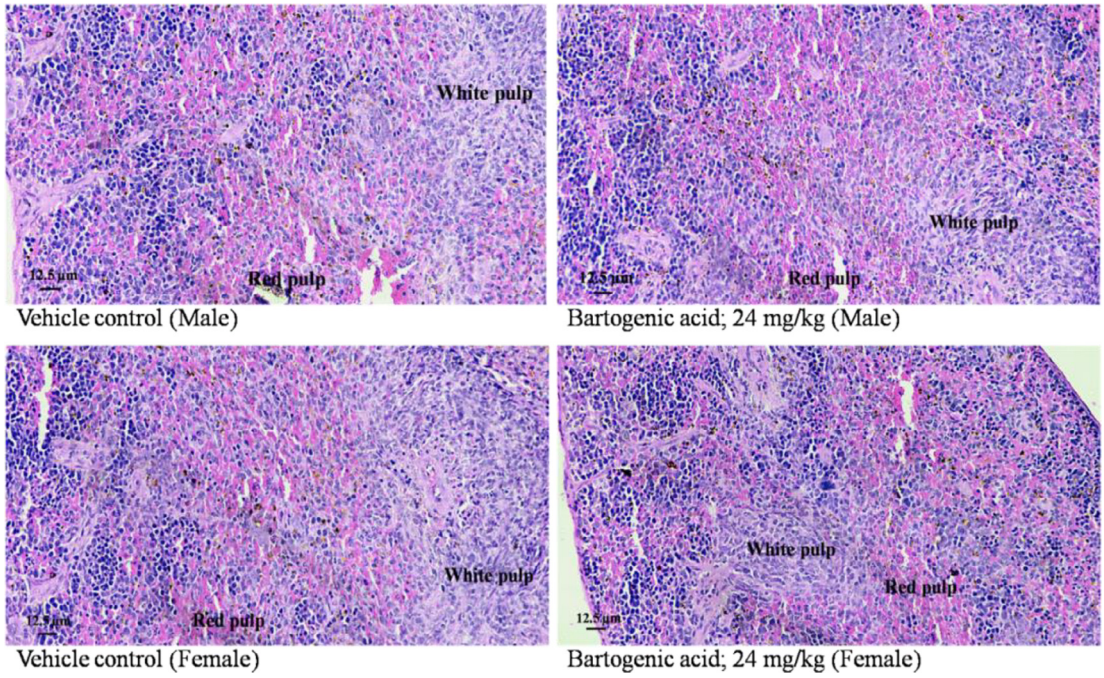


Fig. 6. Representative histopathological photomicrographs of spleen, sectioned transversely and stained with hematoxylin and eosin (Scale bar: 12.5 µm, Magnification; 200X). Both male and female mice treated with BA (24 mg/kg) and vehicle showed normal white and red pulp in the architecture of the spleen. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

in some hematological parameters in relation to the control group. These hematological changes were found within the range of the historical control values (Pessini et al., 2020, Kim et al., 2020). Hence, these changes were not considered as adverse effects.

At the systemic level, liver and kidneys are the most exposed organs to toxicants as their main function involves filtering toxins out of the blood into faeces and urine (Hamzah et al., 2020). No toxicologically relevant changes were observed in the clinical biochemistry in mice of both sexes treated with BA at the doses of 1.5 mg/kg, and 6 mg/kg as compared to the control group. However, at 24 mg/kg of BA, only female mice showed significant change in the liver and kidney function parameters as compared to the control group. These biochemical changes were found within the range of the historical control values (Kim et al., 2020, Silva-Santana et al., 2020) and in addition to this, no histopathological lesions were observed in the liver and kidney of the female mice at this dose. Hence, these changes were not considered as adverse effects.

Mice of both sexes treated with BA at the doses of 1.5 mg/kg, 6 mg/kg and 24 mg/kg did not reveal significant change in the absolute organ weight as compared to the vehicle treated control

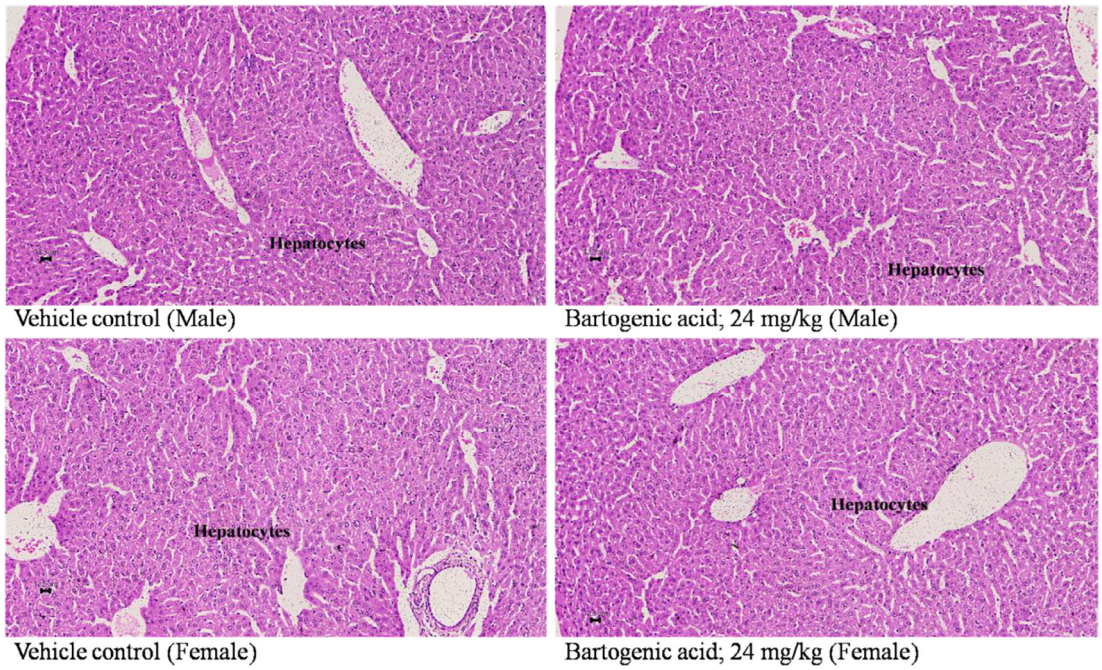


Fig. 7. Representative histopathological photomicrographs of liver, sectioned transversely and stained with hematoxylin and eosin (Scale bar: 12.5 μm, Magnification; 100X). Both male and female mice treated with BA (24 mg/kg) and vehicle showed normal hepatocytes and no inflammatory cells in the architecture of the liver.

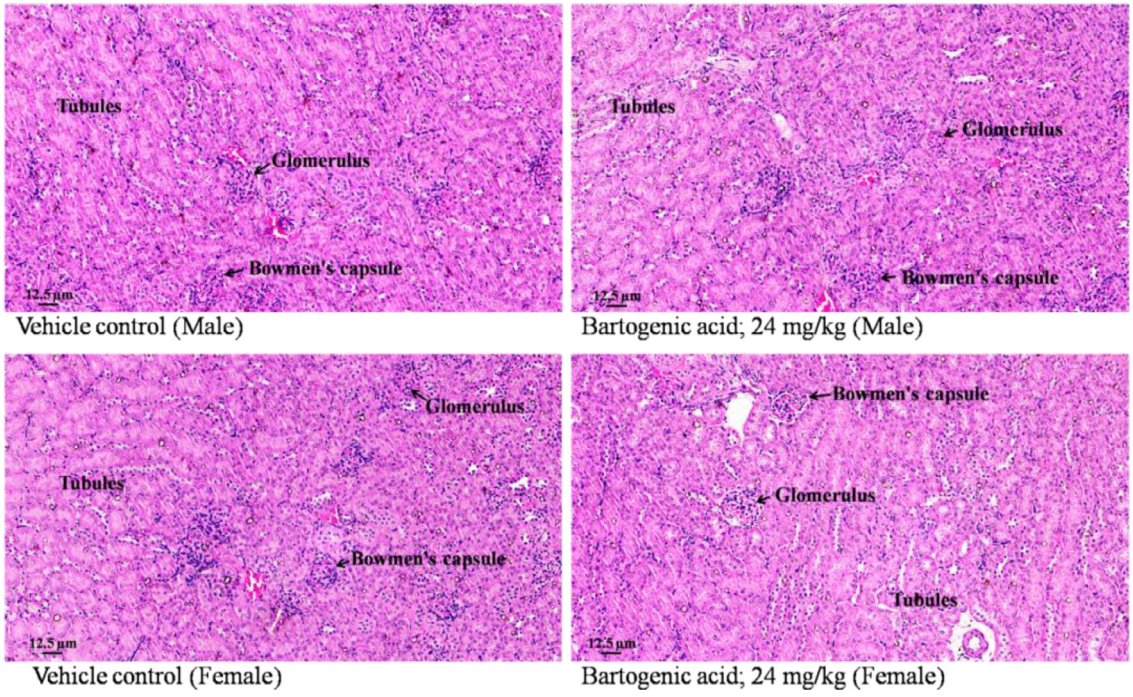


Fig. 8. Representative histopathological photomicrographs of kidney, sectioned transversely and stained with hematoxylin and eosin (Scale bar: 12.5 μm, Magnification; 100X). Both male and female mice treated with BA (24 mg/kg) and vehicle showed normal glomerulus, bowmen's capsule and tubules in the architecture of the kidney.

mice. Further in context to the relative organ weight, BA at 1.5 mg/kg, and 6 mg/kg did not reveal significant change in the relative organ weight in mice of both sexes as compared to the control group. However, at 24 mg/kg of BA, female mice showed significant change in the relative weights of the heart, liver, spleen, kidney, lungs and male mice showed significant change in the relative weights of the heart and spleen. The possible reason for this could be the noteworthy change in the body weight in both male and female mice treated with BA at the dose of 24 mg/kg. Gross pathologic examination did not reveal any abnormality in all the mice. On histopathological investigation, no microscopic and macroscopic lesions were observed in the liver, kidney, lungs, heart, and spleen in all the mice treated with vehicle and BA at the tested doses.

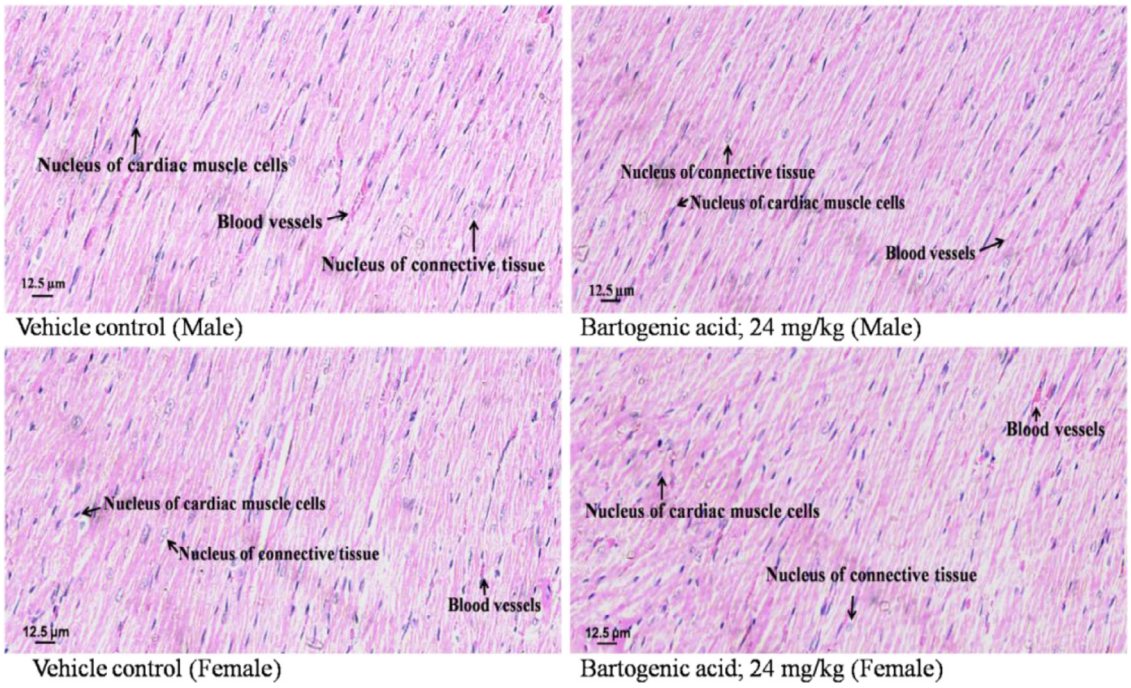


Fig. 9. Representative histopathological photomicrographs of heart, sectioned transversely and stained with hematoxylin and eosin (Scale bar: 12.5 µm, Magnification; 100X). Both male and female mice treated with BA (24 mg/kg) and vehicle showed normal cardiac muscle cells and connective tissues in the architecture of the heart.

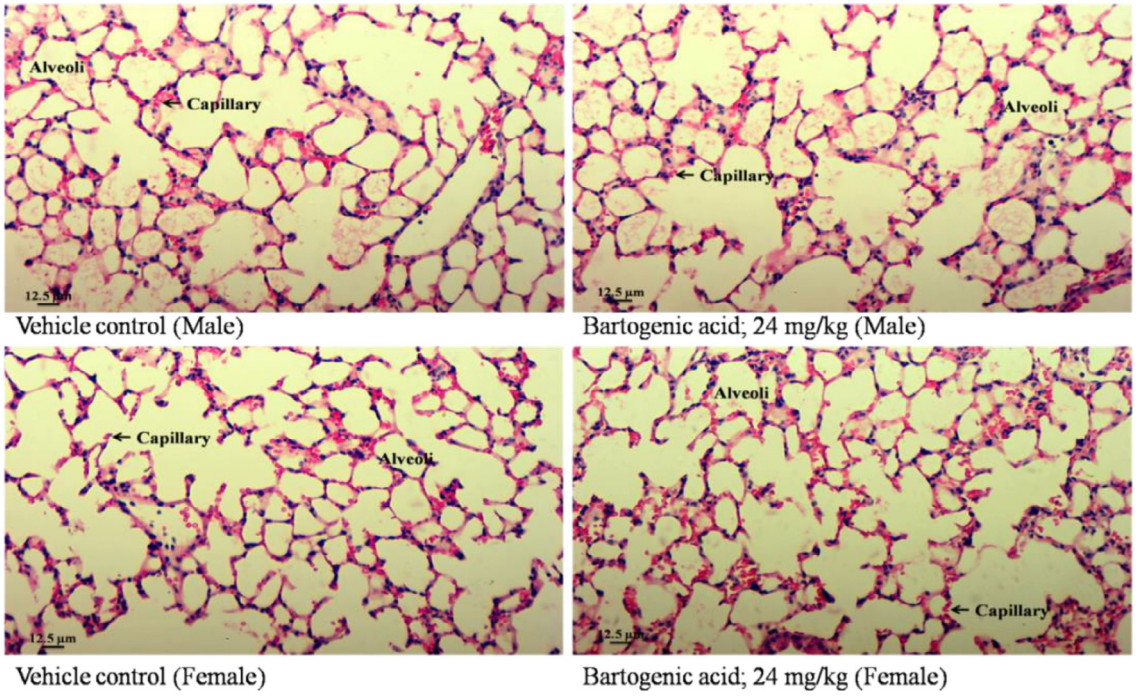


Fig. 10. Representative histopathological photomicrographs of lungs, sectioned transversely and stained with hematoxylin and eosin (Scale bar: 12.5 µm, Magnification; 400X). Both male and female mice treated with BA (24 mg/kg) and vehicle showed normal alveoli and capillaries in the architecture of the lungs.

Conclusions

In conclusion, based on the findings, single dose intravenous toxicity test revealed toxicity at the dose of 100 mg/kg of BA. The No-Observed-Adverse-Effect-Level (NOAEL) was determined in the 28-day repeated dose intravenous toxicity study in BALB/c mice. Based on the findings, NOAEL for BA were found to be <24 mg/kg for male mice and <6 mg/kg for female mice. The data generated in this study will surely be productive in designing further long-term repeated dose studies.

Author contributions

The study was designed by Vishal Kumar Dubey, Swati Madan, Satyendra K Rajput. Vishal Kumar Dubey and Swati Madan and

Satyendra K Rajput performed the activity and analyzed the data. Vishal Kumar Dubey drafted the manuscript. Swati Madan, Satyendra K Rajput, Anu T Singh, and Manu Jaggi provided comments on the content and interpretation of the manuscript. All authors have read and approved the manuscript.

Credit Author Statement

Vishal Kumar Dubey: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing – original draft. **Swati Madan:** Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Supervision, Writing – review & editing. **Satyendra K Rajput:** Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Supervision, Writing – review & editing. **Anu T Singh:** Conceptualization, Project administration, Supervision, Writing – review & editing. **Manu Jaggi:** Conceptualization, Project administration, Supervision, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.crtox.2021.10.004.

References

American Veterinary Medical Association (AVMA), 2020. Guidelines for the Euthanasia of Animals. edn. pp. 1-121.

Amran, N., Rani, A.N., Mahmud, R., Yin, K.B., 2016. Antioxidant and Cytotoxic Effect of Barringtonia racemosa and Hibiscus sabdariffa Fruit Extracts in MCF-7 Human Breast Cancer Cell Line. Pharmacognosy Res. 8 (1), 66–70. https://doi.org/10.4103/0974-8490.171104.

Bhat, P., Hegde, G., Hegde, G.R., 2012. Ethnomedicinal practices in different communities of Uttara Kannada district of Karnataka for treatment of wounds. J. Ethnopharmacol. 143 (2), 501–514. https://doi.org/10.1016/j.jep.2012.07.003.

Deraniyagala, S.A., Ratnasooriya, W.D., Goonasekara, C.L., 2003. Antinociceptive effect and toxicological study of the aqueous bark extract of Barringtonia racemosa on rats. J. Ethnopharmacol. 86 (1), 21–26. https://doi.org/10.1016/s0378-8741(03)00015-1.

Dubey, V.K., Budhauliya, A., Jaggi, M., Singh, A.T., Rajput, S.K., 2021. Tumor-suppressing effect of bartogenic acid in ovarian (SKOV-3) xenograft mouse model. NaunynSchmiedebergs Arch Pharmacol. 394 (8), 1815–1826. https://doi.org/10.1007/s00210-021-02088-0.

European Medicines Agency. Committee for Medicinal Products for Human Use (CHMP), 2017. Guideline on the evaluation of anticancer medicinal products in man. Available from: http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2017/11/ WC500238764.pdf.

French, B., 2014. Edible Barringtonia nuts and leaves. Food Plants Int. 15.

Gowri, P.M., Tiwari, A.K., Ali, A.Z., Rao, J.M., 2007. Inhibition of alpha-glucosidase and amylase by bartogenic acid isolated from Barringtonia racemosaRoxb. seeds. Phytother. Res. 21 (8), 796–799. https://doi.org/10.1002/ptr.2176.

Gowri, P.M., Radhakrishnan, S.V., Basha, S.J., Sarma, A.V., Rao, J.M., 2009. Oleanane-type isomeric triterpenoids from Barringtonia racemosa. J. Nat. Prod. 72 (4), 791–795. https://doi.org/10.1021/np8007396.

Gowda, S., Desai, P.B., Kulkarni, S.S., Hull, V.V., Math, A.A., Vernekar, S.N., 2010. Markers of renal function tests. N. Am. J. Med. Sci. 2 (4), 170–173.

Gowda, S., Desai, P.B., Hull, V.V., Math, A.A., Vernekar, S.N., Kulkarni, S.S., 2009. A review on laboratory liver function tests. Pan. Afr. Med. J. 3 (17).

Hamzah, H., Reduan, F.H., Shaari, R.M., Sayuti, N.S.A., Mustapha, N.M., Abu-Bakar, M. Z., Sithambaram, S., 2020. Acute and subacute dermal toxicity of ethanolic extract of Melastomamalabathricum leaves in Sprague-Dawley rats. Toxicol. Res. 36 (3), 203–210. https://doi.org/10.1007/s43188-019-00013-5.

Ho, I.Y.M., Abdul-Aziz, A., Mat Junit, S., 2020. Evaluation of Anti-proliferative Effects of Barringtonia racemosa and Gallic Acid on Caco-2 Cells. Sci. Rep. 10 (1), 9987. https://doi.org/10.1038/s41598-020-66913-x.

Kong, K.W., Mat Junit, S., Aminudin, N., Abdul Aziz, A., 2020. Phytochemicals in Barringtonia species: Linking their traditional uses as food and medicine with current research. J. Herbal Med. 19, 100299. https://doi.org/10.1016/j.hermed.2019.100299.

Khwaza, V., Oyedeji, O.O., Aderibigbe, B.A., 2020. Ursolic Acid-Based Derivatives as Potential Anti-Cancer Agents: An Update. Int. J. Mol. Sci. 21 (16), 5920. Doi: 10.3390/ijms21165920..

Kim, H.-Y., Zuo, G., Lee, S.K., Lim, S.S., 2020. Acute and subchronic toxicity study of nonpolar extract of licorice roots in mice. Food Sci. Nutr. 8 (5), 2242–2250. doi: 10.1002/fsn3.v8.510.1002/fsn3.14658.

Kong, K.W., Abdul-Aziz, A., Razali, N., 2016. Aminuddin N, Mat Junit S. Antioxidant-rich leaf extract of Barringtonia racemosa significantly alters the in vitro expression of genes encoding enzymes that are involved in methylglyoxal degradation III. PeerJ. Doi: 10.7717/peerj.2379..

Khan, S., Jabbar, A., Hasan, C.M., Rashid, M.A., 2001. Antibacterial activity of Barringtonia racemosa. Fitoterapia 72 (2), 162–164. https://doi.org/10.1016/s0367-326x(00)00264-1.

Kaid, F., Alabsi, A.M., Alafifi, N., Ali-Saeed, R., Ameen-Al-Koshab, M., Ramanathan, A., Ali, A.M., 2019. Histological, Biochemical, and Hematological Effects of Goniothalamin on Selective Internal Organs of Male Sprague-Dawley Rats. J. Toxicol.. https://doi.org/10.1155/2019/6493286.

Masoko, P., 2013. Ethnobotanical study of some selected medicinal plants used by traditional healers in Limpopo Province (South Africa). Am. J. Res. Commun. 1, 8–23.

Meyerholz, D.K., Gibson-Corley, K.N., Olivier, A.K., 2013. Principles for valid histopathologic scoring in research. Vet. Pathol. 50 (6), 1007–1015. https://doi.org/10.1177/0300985813485099.

Mmushi, T., Masoko, P., Mdee, L., Mokgotho, M., Mampuru, L.J., Howard, R., 2009. Antimycobacterial evaluation of fifteen medicinal plants in South Africa. Afr. J. Tradit. Complement. Altern. Med. 7 (1), 34–39. https://doi.org/10.4314/ajtcam.v7i1.57230.

NRC, 2011. National Research Council (US) Committee for the Update of the Guide for the Care and Use of Laboratory Animals. Guide for the Care and Use of Laboratory Animals,. National Academies Press Washington, D.C..

Osman, N.I., Sidik, N.J., Awal, A., 2015. Pharmacological Activities of Barringtonia Racemosa L. (Putat), A Tropical Medicinal Plant Species. J. Pharm. Sci. & Res. 7 (4), 185–188.

Ong, H.C., Nordiana, M., 1999. Malay ethno-medico botany in Machang, Kelantan, Malaysia. Fitoterapia 70 (5), 502–513.

Organization for Economic Cooperation and Development (OECD), 2002. Guidelines for the Testing of Chemicals/Section 4: Health Effects Test No. 423: Acute Oral toxicity - Acute Toxic Class Method. Paris, France..

Organization for Economic Cooperation and Development (OECD), 2008. Guidelines for the Testing of Chemicals and Food Ingredients, Section 4 (Part 407): Repeated Dose 28-day Oral Toxicity Study in Rodents. Organization for Economic Cooperation and Development, Paris, France.

Patil, K.R., Patil, C.R., 2016. Anti-inflammatory activity of bartogenic acid containing fraction of fruits of Barringtonia racemosa Roxb. in acute and chronic animal models of inflammation. J. Tradit. Complement Med. 7 (1), 86–93. https://doi.org/10.1016/j.jtcme.2016.02.001.

Patil, K.R., Patil, C.R., Jadhav, R.B., Mahajan, V.K., Patil, P.R., Gaikwad, P.S., 2011. Anti-Arthritic Activity of Bartogenic Acid Isolated from Fruits of Barringtonia racemosaRoxb. (Lecythidaceae). Evid. Based Complement. Alternat. Med. 2011, 1–7. https://doi.org/10.1093/ecam/nep148.

Patil, C.R., Sonara, B.M., Mahajan, U.B., Patil, K.R., Patil, D.D., Jadhav, R.B., Goyal, S.N., Ojha, S., 2016. Chemomodulatory Potential of Bartogenic Acid Against DMBA/Croton Oil Induced Two-Step Skin Carcinogenesis in Mice. J. Cancer 7 (14), 2139–2147. https://doi.org/10.7150/jca.16446.

Pessini, P.G.D.S., Knox de Souza, P.R., Chagas, C.D.S., Sampaio, E.G., Neves, D.S., Petri, G., Fonseca, F.L.A., da Silva, E.B., 2020. Hematological reference values and animal welfare parameters of BALB/C-FMABC (Mus musculus) inoculated with Ehrlich tumor kept in the vivarium at ABC Medical School. Animal Model. Exp. Med. 3 (1), 32–39. https://doi.org/10.1002/ame2.12099.

Percie du Sert, N., Ahluwalia, A., Alam, S., Avey, M.T., Baker, M., Browne, W.J., Clark, A., Cuthill, I.C., Dirnagl, U., Emerson, M., Garner, P., Holgate, S.T., Howells, D.W., Hurst, V., Karp, N.A., Lazic, S.E., Lidster, K., MacCallum, C.J., Macleod, M., Pearl, E. J., Petersen, O.H., Rawle, F., Reynolds, P., Rooney, K., Sena, E.S., Silberberg, S.D., Steckler, T., Würbel, H., Boutron, I., 2020. Reporting animal research: Explanation and elaboration for the ARRIVE guidelines 2.0. PLoS Biol. 18 (7), e3000411.

Qian, Z., Wang, X., Song, Z., Zhang, H., Zhou, S., Zhao, J., Wang, H., 2015. A phase I trial to evaluate the multiple-dose safety and antitumor activity of ursolic acid liposomes in subjects with advanced solid tumors. Biomed Res Int. https://doi.org/10.1155/2015/809714..

Silva-Santana, G., Bax, J.C., Fernandes, D.C.S., Bacellar, D.T.L., Hooper, C., Dias, A.A.S. O., Silva, C.B., de Souza, A.M., Ramos, S., Santos, R.A., Pinto, T.R., Ramão, M.A., Mattos-Guaraldi, A.L., 2020. Clinical hematological and biochemical parameters in Swiss, BALB/c, C57BL/6 and B6D2F1 Mus musculus. Animal Model Exp Med. 3 (4), 304-315. Doi: 10.1002/ame2.12139..

Subramanian, K., 2018. Natural Products and Drug Discovery 491–505..

Shanmugam, M.K., Dai, X., Kumar, A.P., Tan, B.K., Sethi, G., Bishayee, A., 2014. Oleanolic acid and its synthetic derivatives for the prevention and therapy of cancer:

preclinical and clinical evidence. Cancer Lett. 346 (2), 206-216. https://doi.org/10.1016/j.canlet.2014.01.016..

Thomas, T.J., Panikkar, B., Subramoniam, A., Nair, M.K., Panikkar, K.R., 2002. Antitumour property and toxicity of Barringtonia racemosaRoxb seed extract in mice. J. Ethnopharmacol. 82 (2–3), 223–227. https://doi.org/10.1016/s0378-8741(02)00074-0.

US Food and Drug Administration, 2003. IV.C.4.a Short-Term Toxicity Studies with Rodents. Redbook 2000: Toxicological Principles for the Safety Assessment of Food Ingredients. US Food and Drug Administration (FDA), Washington, DC..

US Food and Drug Administration, 2007. IV.C.4.a Subchronic Toxicity Studies with Rodents. Redbook 2000: Toxicological Principles for the Safety Assessment of Food Ingredients. US Food and Drug Administration (FDA), Washington, DC..

Van, Q.T.T., Vien, L.T., Hanh, T.T.H., Huong, P.T.T., Cuong, N.T., Thao, N.P., Thuan, N.H., Dang, N.H., Thanh, N.V., Cuong, N.X., Nam, N.H., Kiem, P.V., Minh, C.V., 2020. Acylated flavonoid glycosides from Barringtonia racemosa. Nat. Prod. Res. 34 (9), 1276–1281. https://doi.org/10.1080/14786419.2018.1560290.