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Research article

HPLC characterization, acute and sub-acute toxicity evaluation of bark extract of *Rhizophora mucronata* in Swiss Albino mice

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ABSTRACT

Aim: *Rhizophora mucronata*, commonly called as 'red mangrove' grows in the tropical and sub-tropical regions and on the sheltered shores. The bioactive compounds from the plant have been used in the treatment of wide range of diseases. Though the beneficial effects have been reported, the safety and toxicological studies are not carried out. Hence, major bioactives have been identified by HPLC and then acute and sub-acute toxicity studies of (BERM) in Swiss Albino mice have been carried out.

Main methods: HPLC fingerprinting was carried out of BERM for the characterization of bioactives. BERM as a single dose was given orally at 800, 1600 mg/kg and 3200 mg/kg by a stainless steel cannula to the mice. Then the mice were observed for 14 days for mortality and behavioural changes. Food, water intake and body weight changes were also observed throughout the study period. On the fifteenth day, the mice were anesthetized with isofluorane and blood was withdrawn for haematological and biochemical analysis. The animals were sacrificed by overdose of isofluorane and organs such as liver, kidney, lungs and spleen were dissected out for histopathological analysis. There was no mortality of the mice even in 3200 mg/kg dose, stating that the oral LD50 of BERM is more than 5000 mg/kg. In terms of Sub-acute toxicity, for a period of 28 days repeated dose of 400 mg/kg and 800 mg/kg as an optimum dose and a control group was kept with only distilled water at 5 ml/kg against the treated groups. On 29th day, the mice from all groups were sacrificed and blood was withdrawn and organs such as liver, kidney, lungs and spleen were dissected out for the assessment of internal tissues, wherein no abnormalities were observed in the treatment groups as compared to the control. The blood parameters, biochemical analysis of the treated groups were well within the range, histopathological confirmed the findings wherein the organs viz, liver, kidneys, lungs and spleen possessed normal architecture.

Key findings: Based on HPLC results, prominent 5 major compounds viz: Diadzein, Epicatechin, Hesperidin, Diosmin and Quercitrin respectively were identified. Isolated changes observed in the haematological, biochemical and histopathological studies were not dose related and showed the safety of the bark extract. Similarly, the sub-acute toxicity of BERM has been conducted for 28 days, wherein repeated dose of 400 mg/kg and 800 mg/kg and control group was given orally. There were no abnormalities found both in external and internal parameters.

Significance: Based on the study it is concluded that the bark extract of *Rhizophora mucronata* (BERM) is safe at 1000 mg/kg or less on repeated dosage can be considered as a safe dose for pharmacological efficacy studies.

1. Introduction

Rhizophora mucronata, commonly called as 'red mangrove', 'loop root mangrove' or 'Asiatic mangrove [1] belonging to the family *Rhizophoraceae* [2] is a true mangrove that grows in tropical and sub-tropical regions [3]. It is an evergreen, mangrove tree which grows about 25–30 m high, 70 cm in diameter with numerous arched branching stilt roots. The bark is either dark brown or blackish in colour, smooth with fissures horizontally. *Rhizophora* have been reportedly used both as medicine and food [4] in Indonesia and some other Asian countries. Various parts viz

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Figure 1. Bark of *Rhizophora mucronata*.

Table 1. HPLC chromatogram of BERM (Bark Extract of *Rhizophora mucronata*) at 250 nm@ 250 nm.

Peak	Retention Time Original	Ret Time of the Article	Wavelength	Expected Compound
1	24.28	24.9	248, 258sh, 299	Diadzein
2	33.54	————	————	unknown
3	34.96	————	————	unknown
4	45.28	————	————	unknown
5	46.31	————	————	unknown
6	51.16	————	————	unknown
7	86.24	————	————	unknown
8	104.29	————	————	unknown

(leaves, bark, stem, roots, fruits flowers) of *Rhizophora mucronata* finds its application in the treatment of various diseases in humans viz angina, haematuria, dysentery, etc [5] (Figure 1).

In folklore medicine, its bark and leaf extracts has been used as haemostatic, anti-septic and astringent with anti-ulcerogenic, anti inflammatory and anti-diabetic activities [6, 7]. In Burma, China and India, its bark and seeds are used to treat haemorrhage, angina, diabetes, haematuria and diarrhoea [8]. New triterpenoids, sesquiterpenoids [9] and diterpenoids [10] have been isolated from the fruits of this plant. Important studies such as anti-diabetic, anti-oxidant and anti-inflammatory activities associated with genus *Rhizophora* have been reported [11]. The many bioactive properties exhibited by *R. mucronata* could be accrued to the presence of different types of diterpenoids, steroids as well as triterpenoids in the species as reported in literature [12]. Triterpenoids from the stem bark of *Rhizophora mucronata* [13] was isolated. In spite of the fact that it is utilized as a researcher's medicines, no security or toxicological information is accessible.

The phytochemical investigation of the BERM was performed according to [14, 15] which clearly revealed the presence of eight major secondary metabolites viz, alkaloids, flavonoids, carboxylic acids, tannins, terpenoids, phyto-sterols, phenols and saponins respectively. Further, the results were supported by HPLC fingerprinting of BERM showed various peaks in the obtained chromatogram at different wavelengths viz, 250 nm, 280 nm and 320 nm, 5 prominent compounds have been identified on the basis of the retention time. The compounds obtained were, Diadzein, Epicatechin, Hesperidin, Diosmin and Quercitrin respectively. Out of 5 secondary metabolites, Quercitrin has been given more importance because of its hepatoprotective activity.

Moreover, mangroves viz *R. mucronata* bark in the present study, enjoying widespread use for the treatment of several ailments, but still little known about their toxicity and safety issue which are always a concern. Thus, the investigations leads to the evidence on the presence of substances that are offer potential human health benefits. However, it should be a vital requirement to determine the toxic effects of some of the substances contained in this plant and its parts [16].

Toxicity is considered as the expression of the poisonous status. It indicates the state of adverse effects of the interaction between the toxicant and the cells. This interaction may vary depending upon the cell membrane, chemical properties of toxicants which may affect the cells extracellular matrix, cell surface, beneath the cells and may affect the tissues. Therefore, the evaluation of the compound whether it is toxic or not is highly commended as it adversely affects the human health. In practise, evaluation may be carried out through various processes viz, acute, sub-acute, chronic, carcinogenic and reproductive effects [16].

Acute toxicity refers to the harmful/toxic effects of a substance that result either from a single or from multiple exposures (Oral/Dermal) in a short period of time (usually less than 24 h). In order to describe as acute toxicity the unfavourable effects should occur within 14 days of the administration of the substance [17]. It provides preliminary information on the toxic nature of the material for which no other toxicology information available. Such information can be used for:

- Poison control information: ingestion of large amount of material accidentally
- Determine the target organs to be examined or special tests to be conducted in the upcoming toxicity evaluation [17].

Table 3. HPLC chromatogram of BERM (Bark Extract of *Rhizophora mucronata*) at 320 nm@ 320 nm.

Peak	Retention Time Original	Ret Time of the Article	Wavelength	Expected Compound
1	17.58			unknown
2	18.23	————	————	unknown
3	34.96	————	————	unknown
4	36.96	————	————	unknown
5	45.08	————	————	unknown
6	62.54	61.4	251, 265, 344	Diosmin
7	104.08	————	————	unknown
8	104.42	————	————	unknown
9	104.85	————	————	unknown
10	105.49	————	————	unknown

Table 2. HPLC chromatogram of BERM (Bark Extract of *Rhizophora mucronata*) at 280 nm@ 280 nm.

Peak	Retention Time Original	Ret Time of the Article	Wavelength	Expected Compound
1	18.16	18.4	229sh, 277	Epicatechin
2	34.94	————	————	unknown
3	45.44	45.6	224sh, 281, 334sh	Hesperidin
4	51.15	————	————	unknown
5	56.98	56.9	253, 263sh, 344	Quercetin-3-O-rhamnoside (quercitrin)
6	73.41	————	————	unknown
7	86.26	————	————	unknown
8	104.29	————	————	unknown

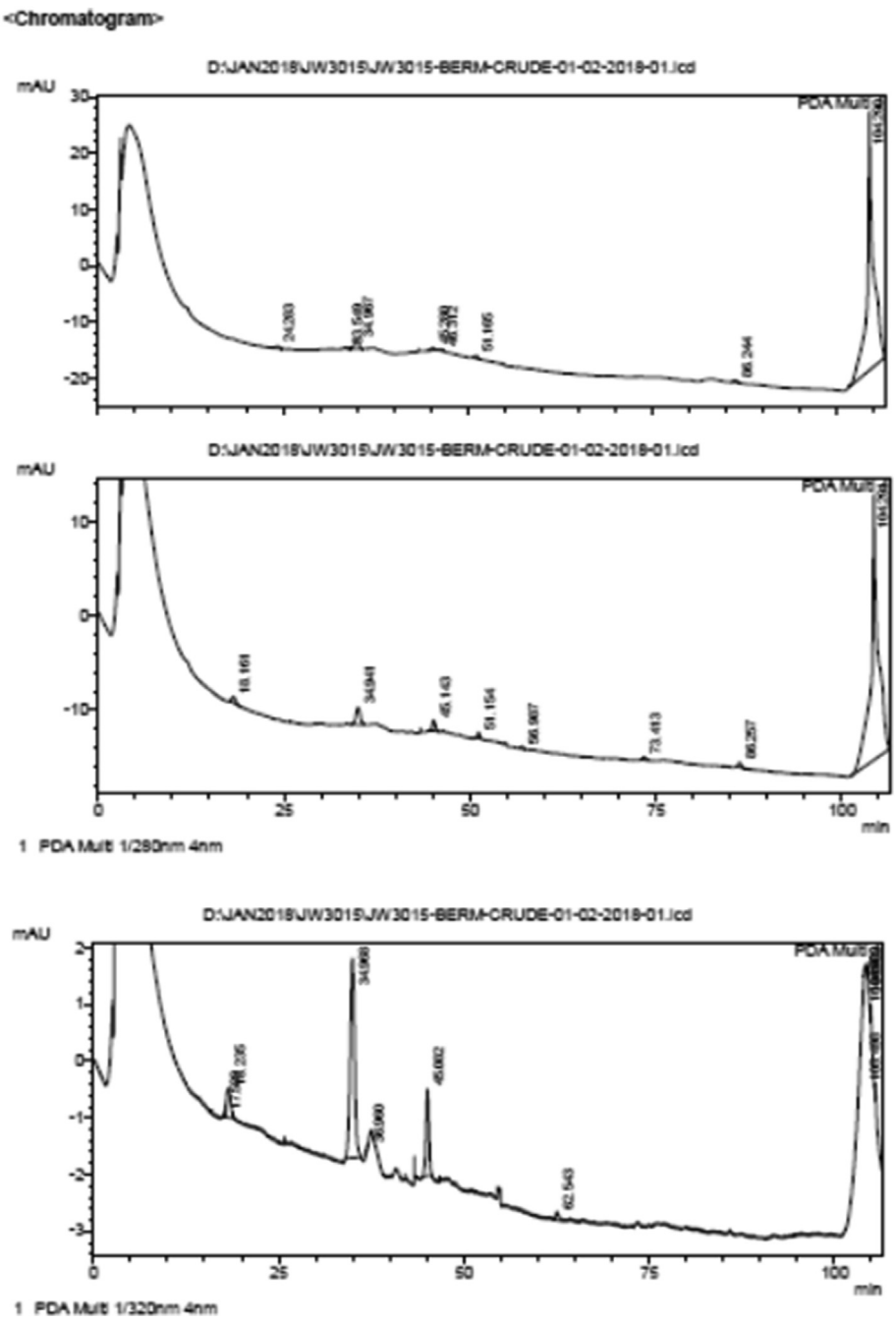

Figure 2. Illustrates the chromatograms obtained at different wavelengths viz, 250 nm, 280 nm and 320 nm respectively.

- Select doses for short term and sub-acute toxicity studies when there is no information available.

Sub-acute toxicity refers to the systemic effect of repeated doses of materials or their extracts occurring after multiple or continuous exposure between 24 h and 28 days. The study is performed in three different levels (low, mid and high dose), however studies have been restricted to 2 doses (mid and high) also. Sub-acute toxicity testing gives:

- valuable information on the cumulative toxicity of a substance
- Condition of the physiological organs
- metabolic of a compound at low dose prolonged exposure

Thus, the present study aims to determine secondary metabolites by HPLC fingerprinting [18]. As on date, there are no acute/Sub-acute toxicity studies reported using the bark extract of *Rhizophora mucronata* in mice/rats. This study is the first attempt in this regard. Thus, to check the toxicity of BERM using an acute oral toxicity test [19] and for sub-acute toxicity study, repeated dose 28 days oral toxicity study in rodents (TG-407) was performed in Swiss mice [20] with slight modifications.

2. Materials and methods

2.1. Collection of plant material

The bark of *Rhizophora mucronata* were collected during January 2018 from Pichavaram Mangrove forest (latitude: 11° 23′ to 11° 30′ N and longitude: 79° 45′ to 79° 50’ E) is located between Coleroon and Vellar estuary in the state of Tamil Nadu and identified in the herbarium

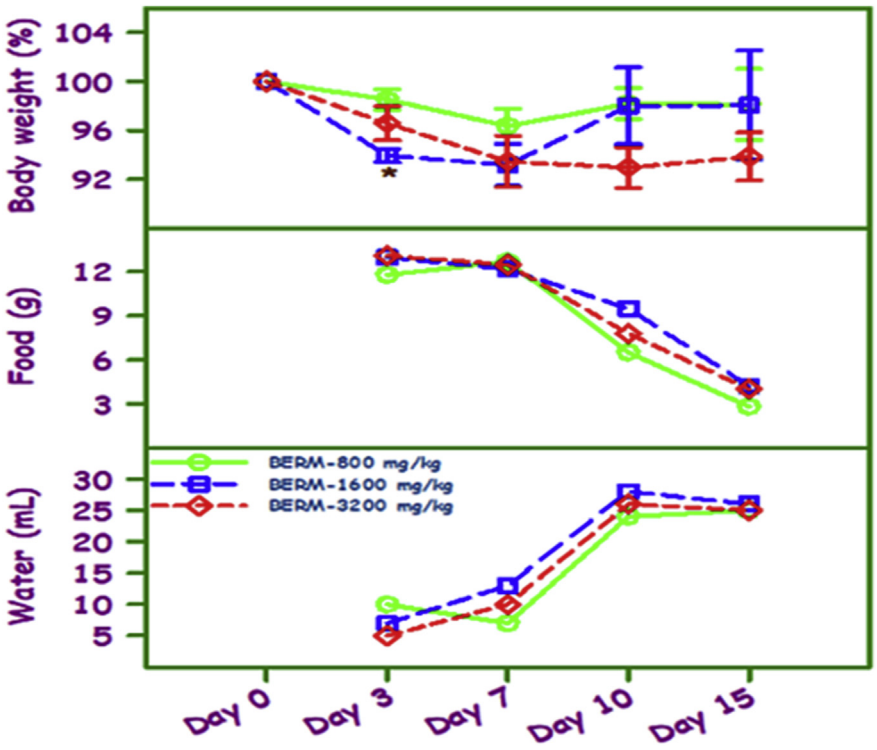

Figure 3. Change in the body weight, food and water intake of the animals of BERM 800 mg/kg b. w, BERM 1600 mg/kg b. w and BERM 3200 mg/kg b. w. respectively. P values for the body weight of the three groups on day 3–0.031, day 7–0.413, day 10–0.213 and day 15–0.593 respectively. Values were expressed as mean ± SEM, n = 4.

of Centre for Advanced Study (CAS) in Marine Biology, Annamalai University, and Parangipettai, India. The fresh barks were subjected to authentication by Prof. P. Jayaraman at Plant Anatomy Research Centre, West Tambaram, Chennai with the specimen no: PARC/2018/3854 for future reference. These barks were shade dried for 15 days. They were coarsely powdered and stored in air tight bottles for further work.

2.2. Cold extraction by maceration

Maceration is preferred over other extraction methods viz, Sohxlet's, microwave-assisted (MAE), ultrasound-assisted extraction (UAE) and supercritical fluid extraction (SFE), because of two reasons: mainly because of the cost effectiveness and requirement of sophisticated instrument is minimised. Therefore, in the present study maceration is employed for the extraction of the plant material. The powdered bark of *Rhizophora mucronata* weighing 500 gms was taken in a clean flat bottomed glass flask and soaked into ethanol: water (3:1) for 15 days with occasional shaking and stirring. On the 16th day the, the extract was filtered through Whatmann 1 filter paper. The filtrate was evaporated at room temperature to obtain the extract which was stored at 4 °C for further use.

2.3. HPLC characterization

HPLC fingerprinting was carried out of the BERM according to the protocol by [18]. The hydroethanolic extract EtOH: Water (3:1), weighed about (50 mg) was mixed with 2 mL of 90% methanol containing 0.5% acetic acid, after adding 50 n mol of flavone in DMSO. Flavone was used mostly as an internal standard. The solution was allowed to stand in a sonicator for 1 min, and the supernatant was recovered by centrifugation at 3000 rpm for 10 min. After extraction three times, the extracts were dried with a centrifugal concentrator (VC–96N, Taitec Co., Saitama, Japan). The residues were dissolved in 0.5 mL of DMSO and filtered through a Millex-LG 0.2-μm membrane filter (Millipore Co., Bedford, MA) before the HPLC analysis. The treatment was repeated independently three times or more until the variation in the recoveries calculated with the internal standard was less than 5%.

The HPLC system employed was a Hitachi HPLC series D-7000 (Tokyo, Japan) equipped with auto sampler D-7200, column oven D-7300, and diode array detection system D-7450 to monitor at all wavelengths from 200 to 600 nm. For the column, Cap cell pak C18 UG120 (250 × 4.6 mm i. d., S-5, 5 μm, Shiseido Co., Ltd., Tokyo, Japan), joined with a guard column (10 × 4.0 mm i. d.), was used at 35 °C. Gradient elution was performed with solution A, composed of 50 mM sodium phosphate (pH 3.3) and 10% methanol, and solution B, comprising 70% methanol, delivered at a flow rate of 1.0 mL/min as follows: initially 100% of solution A; for the next 15 min, 70% A; for another 30 min, 65% A; for another 20 min, 60% A; for another 5 min, 50% A; and finally 0% A for 25 min. The injection volume for the extract was 10 μL.

2.4. Animals

Male Swiss albino mice for acute toxicity studies and female mice for Sub-acute toxicity studies, weighing 25–35 g (aged 8–10 weeks) obtained from Centre for Laboratory Animal Research (CLAR). The experiments were designed and conducted in accordance with the ethical norms approved by Committee for the Purpose of Control and Supervision on Experiments on Animals (CPCSEA, India) protocol number of approbation allocated for the Institutional Animal Ethical Committee (IAEC) of Saveetha Medical College, Thandalam, Chennai, Tamil Nadu, 602105 is (1183/PO/Re/S/08/CPCSEA). The approved protocol for the present study is (SU/CLAR/RD/002/2018, dated 26th June 2018).

The mice obtained were maintained on a natural light/dark cycle with a temperature of 22 °C (±3 °C). The relative humidity should be at least 30% and preferably not to exceed 70% other than during room cleaning, the aim should be 50–60%. Drinking water and food were provided ad libitum throughout the experiment, except for 2 h after oral

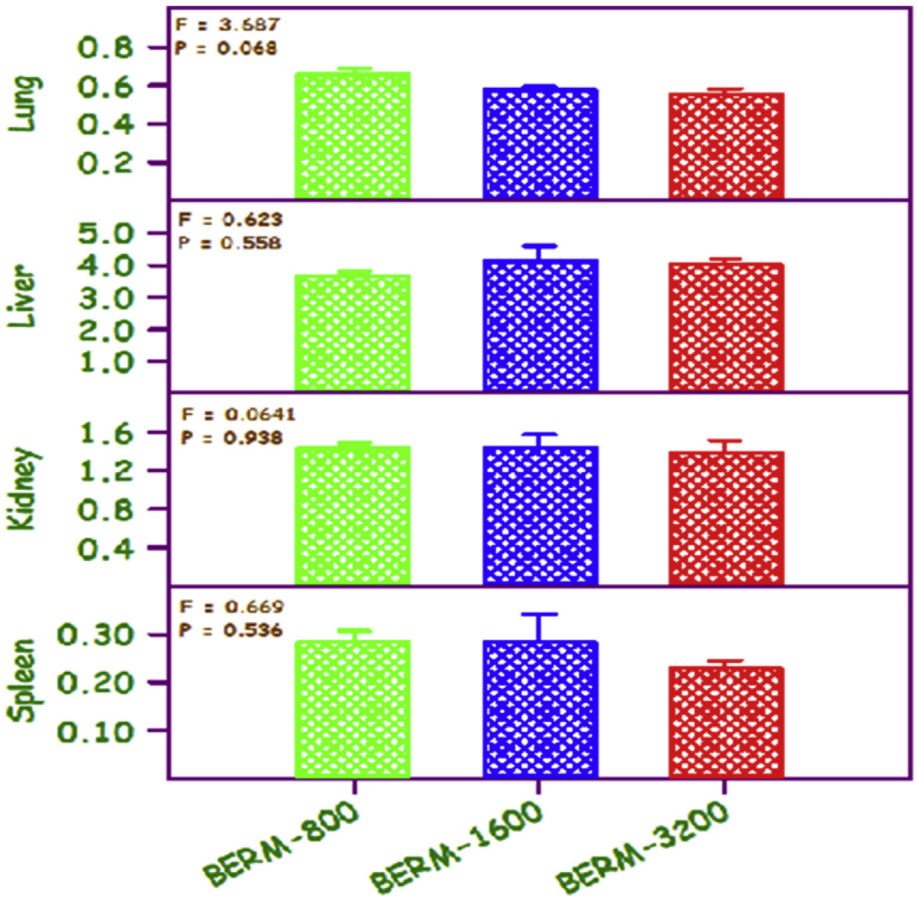

Figure 4. Organ: body weight ratio of the three groups of animals showed they are not statistically significant ($p < 0.05$). Values were expressed as mean ± SEM, n = 4.

feeding. They were marked in the tail for easy identification in the form of slashes.

2.5. Acute toxicity studies

The acute toxicity protocol was chosen based on [19] by moving average method. The 12 Swiss Albino mice (male) used for this experiment were divided into 3 groups of four animals each. They were marked in the tail for easy identification in the form of slashes. Each group 1, 2 and 3, received a single dose of 800 mg, 1600 mg and 3200 mg/kg b. w. respectively of the extract orally on day 1 only with the help of a sterilized stainless steel cannula. Since, only a single dose of extract was administered to the animals, 1600 mg/kg and 3200 mg/kg animals were compared with 800 mg/kg animals as internal control. The animals were then observed for 14 days. They were weighed daily, food and water consumption were monitored daily. They were also observed for any behavioural changes, mortality, if any. On 15th day, the animals were anesthetized with isofluorane and blood was collected in two tubes, with anti-coagulant and without anti-coagulants from orbital plexus. The animals were sacrificed by the overdose of isofluorane and vital organs viz, liver, lungs, kidneys and spleen were extracted.

2.6. Sub-acute toxicity studies

The assessment of sub-acute toxicity of BERM has been carried out as per [20] with slight modifications. The dosage was selected based upon the results obtained in the acute toxicity studies. The acute toxicity study was initially performed at the doses of 800 mg/kg, 1600 mg/kg and 3200 mg/kg b. w. respectively. The haematological, biochemical and histopathological results showed no toxicity at the levels of 800 mg/kg and 1600 mg/kg b. w. animals respectively, although mild inflammation was noticed in the kidneys of one mouse at 3200 mg/kg b. w. mice. The optimum dose selected was 800 mg/kg for the sub-acute study. Another dose lower than optimal dose was also chosen (400 mg/kg) for the sub-acute study for the statistical interpretation of data.

Healthy female Swiss Albino Mice (n = 15) were taken. They were divided into 3 groups viz: Group-1, Control (n = 5), Group-2 (n = 5) 400 mg/kg b. w and Group-3 (n = 5) 800 mg/kg b. w respectively. The Group-1 animal received only a single dose of distilled water at the concentration of 5 ml/kg b. w for 28 days orally. Group-2 and Group-3 animals received a single dose of 400 mg/kg b. w and 800 mg/kg b. w of BERM respectively for 28 days every day. The animals were monitored regularly for any adverse effects after dosing. On 29th day, the animals were anesthetized with isofluorane and above protocol for acute toxicity were followed to retrieve the blood samples and tissue samples from the animals.

2.7. Haematological studies

From the whole blood with anti-coagulants, haematological parameters such as haemoglobin, red blood cell count, white blood cell count, platelet count was determined by Complete Blood Cell Count (CBC) by Coulter Analyser.

2.8. Biochemical studies

From the tube without anti-coagulants, serum was separated and the serum biochemical parameters including total creatinine, urea, alanine amino transferase (ALT), aspartate amino transferase (AST), alkaline

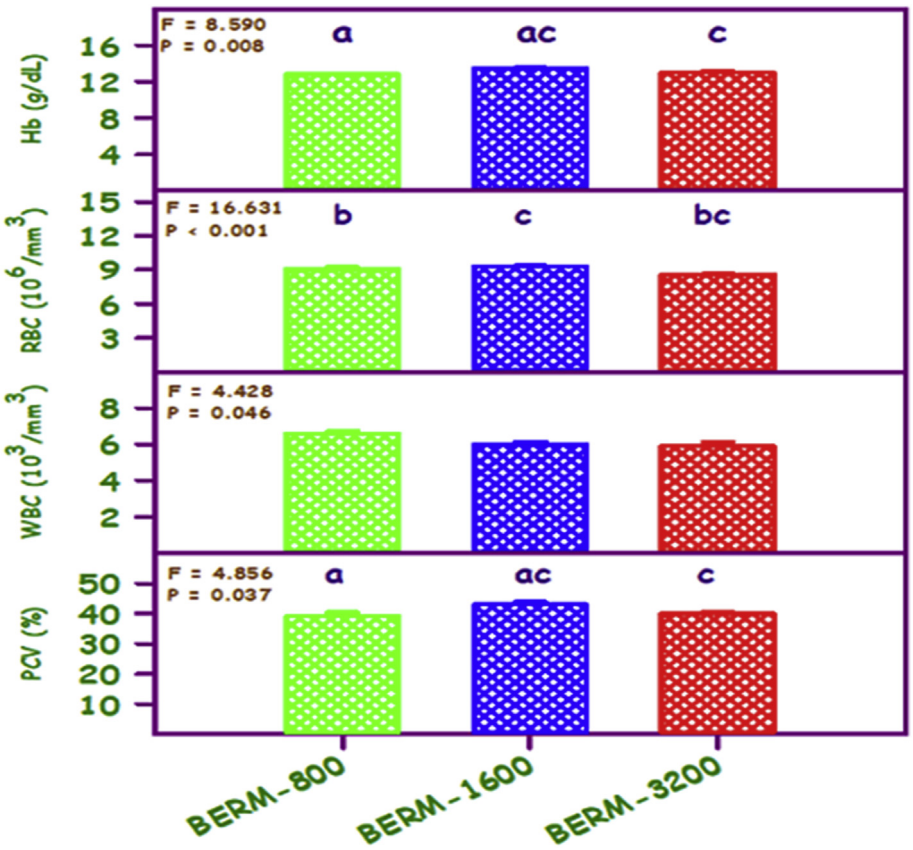

Figure 5. Shows the Haematological parameters of each group, BERM 800 mg/kg b. w, BERM 1600 mg/kg b. w and BERM 3200 mg/kg b. w. respectively. Values were expressed as mean per group ±SEM, n = 4.

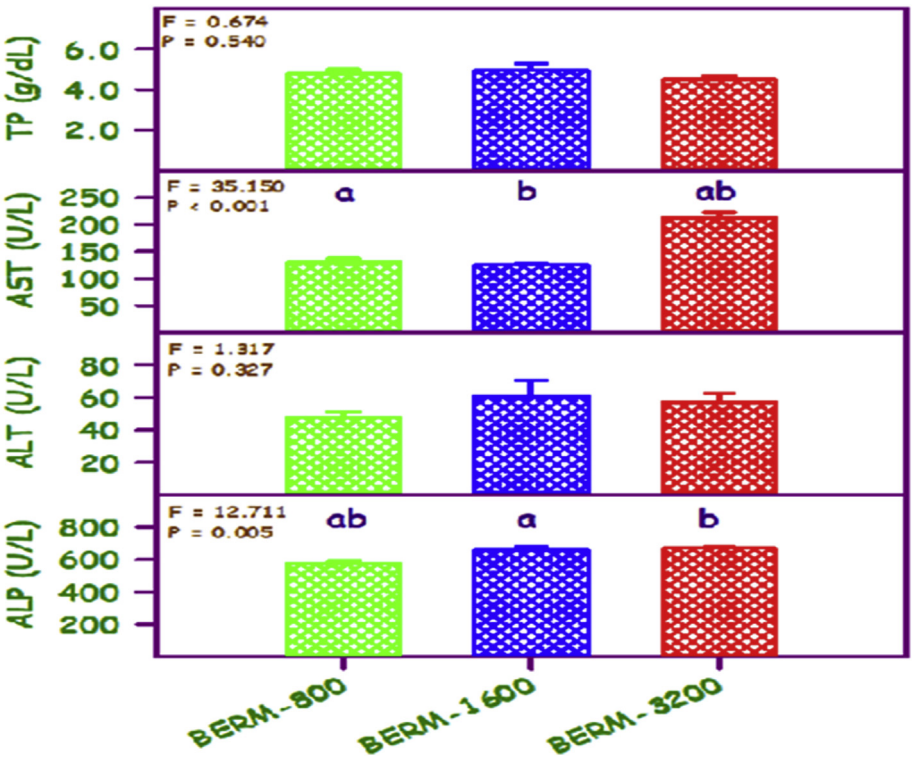

Figure 6. Shows the change in the liver enzymes AST, ALP, ALT and TP of the three groups of mice viz, BERM 800 mg/kg b. w, BERM 1600 mg/kg b. w and BERM 3200 mg/kg b. w. respectively. Alphabets a, b clearly indicates that they are statistically significant with ab ($p < 0.05$).

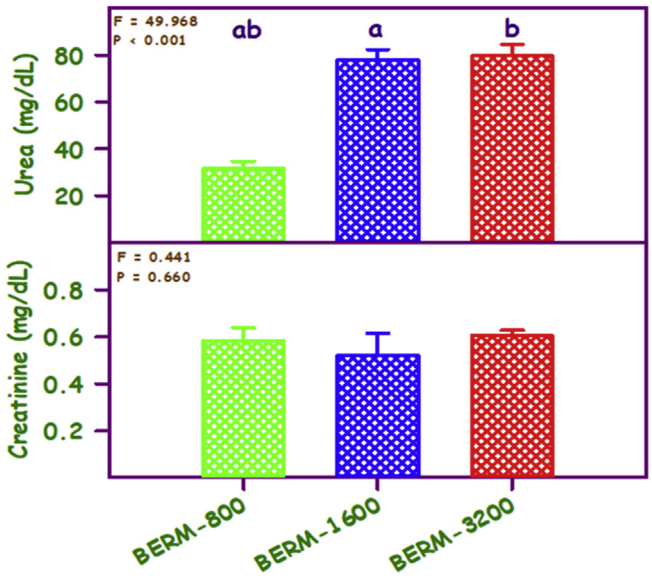

Figure 7. Shows the change in the urea and Creatinine levels the three groups of mice BERM 800 mg/kg b. w, BERM 1600 mg/kg b. w and BERM 3200 mg/kg b. w. respectively. Alphabets a, b clearly indicates that they are statistically significant with ab ($p < 0.05$).

phosphatase (ALP) and total protein were analysed by Accurex diagnostics kit.

2.9. Histopathological studies

The dissected organs viz, liver, lungs, kidneys and spleen were fixed in the 10% formalin, dehydrated in gradual ethanol (50–100%), cleared in xylene, and embedded in paraffin wax. The sections, which were 5–6 micron thickness, were then sectioned using rotary microtome and stained with haematoxylin and eosin dye for microscopic observation of histopathological changes in the liver.

2.10. Statistical analysis

The statistical analysis was carried out by using SigmaPlot-13 software (Systat, USA). The results are expressed as the mean ± SEM and evaluated by one-way ANOVA with Bunnett's comparison test from group-1 (800 mg) for acute toxicity studies and for sub-acute compared with the control group. Data were considered statistically significant if $p < 0.05$.

3. Results

3.1. HPLC results

The HPLC fingerprinting of the crude bark extract ethanol: water (3:1) showed various peaks in the obtained chromatogram at different wavelengths viz, 250 nm, 280 nm and 320 nm, 5 prominent compounds have been identified on the basis of the retention time with slight variation in the actual and theoretical value. But the compound named viz, Quercetin-3-O-rhamnoside (Quercitrin) fall under the same Rt value as mentioned in the related article [24] at 56.9 min. The Tables 1, 2 and 3 illustrates the Rt values obtained in actual and Rt value on theoretically, the wavelength and the expected compounds at 250 nm, 280 nm and 320 nm respectively. The different chromatograms obtained at 250 nm, 280 nm and 320 nm are shown in the Figure 2.

3.2. Acute toxicity study

After the administration of a single dose of the bark extract of *Rhizophora mucronata* at different doses viz, 800, 1600 and 3200 mg/kg, there was no mortality seen in any of the groups. The general behavioural patterns and the appearance of mice were monitored regularly for the period of 14 days. In regards, to the body weight of the animals, a dose dependent decrease was observed 800, 1600 and 3200 mg/kg (Figure 3). The mean of body weight of 800, 1600 and 3200 mg/kg on 15th day was found to be 38.95 gms, 44.43 gms and 42.23 respectively. Statistically significant difference was observed on the on the third day at 1600 mg/kg. But in other days there were no significant difference.

The water and food consumption for 800, 1600 and 3200 mg/kg body weight are shown in Figure 3. The total water consumption for 800, 1600 and 3200 mg on 3rd day was 10 ml, 7 ml and 5 ml respectively, on 7th day, 7 ml, 10 ml and 13 ml, on 10th day was, 24,28 and 26 ml and on the 15 th day it was found to be 25, 26 and 25 ml respectively. Similarly, the total feed consumption for of 800, 1600 and 3200 mg/kg on 3rd day was 11.8 gm, 13.0 gm and 13.1 gm, on 7th day was 12.07, 12.2 and 12.5 gm, on 10th day was 6.5, 9.5, 7.8 gm and on the 15th day 2.8, 4.2 and 4.0 gms respectively. Thus, there is a dose dependent decrease was observed in terms of food consumptions with respect to the different groups.

The organ: body weight ratio of the three groups of 800, 1600 and 3200 mg/kg was given in Figure 4. The observed values for lungs for of 800, 1600 and 3200 mg/kg was 0.654 ± 0.06, 0.573 ± 0.03 and 0.552 ± 0.05 respectively. For liver, the observed values was found to be 3.66 ± 0.22, 4.11 ± 0.83 and 4.024 ± 0.28 respectively, for kidneys the observed values was 1.422 ± 0.1, 1.441 ± 0.22 and 1.386 ± 0.21 respectively and for spleen it was found to be 0.283 ± 0.04, 0.282 ± 0.103 and 0.228 ± 0.03 respectively for 800, 1600 and 3200 mg/kg body weight of the animals. The observed values of the relative organ: body weight ratio of the three groups 800, 1600 and 3200 mg were found to be non-significant ($p < 0.05$).

The haematological assay of all the three group mice was illustrated in Figure 5. The observed values for Haemoglobin (HB) for 800, 1600 and 3200 mg were 12.8 ± 0.25, 13.5 ± 0.18 and 13.02 ± 0.29 g/dL respectively. The observed values for RBC's was 9.1 ± 0.24, 9.3 ± 0.12 and 8.6 ± 0.13 (x10^6/mm^3) respectively. The observed values for WBC's for the groups were 6.6 ± 0.22, 6.0 ± 0.3 and 5.9 ± 0.5 (x10^3/mm^3) respectively. Similarly, for the value of Haematocrit (PCV) for the three groups were 39.1 ± 2.31, 43.0 ± 1.86 and 39.9 ± 1.3% for 800, 1600 and 3200 mg respectively. The values of basic haematological parameters viz, RBCs, WBCs, HB and PCV for all the three groups 800, 1600 and 3200 mg found to be similar in all the 3 different groups studied.

The biochemical investigations were performed to check the toxic effects of bark extract of *Rhizophora mucronata* (BERM) when a single dose was administered orally on liver, kidney, spleen and lungs. No significant change was observed in the serum enzyme ALT (SGPT), TP (total protein) and Creatinine levels. Instead, there is an increase in the level of AST (SGOT), ALP and Urea in the serum was observed. The statistical interpretation showed that as compared to the levels of ALP, 800 mg/kg BERM is statistically significant to 1600 mg/kg and 3200 mg/kg b. w, whereas the value of ALP between 1600 mg/kg and 3200 mg/kg was found to be statistically insignificant ($p < 0.05$). Similarly, the value of AST (SGOT) between 800 mg/kg BERM and 1600 mg/kg BERM was statistically insignificant. But the values compared to 800 mg/kg BERM and 3200 mg/kg BERM and the values of 1600 mg/kg BERM with 3200 mg/kg b. w was found to be statistically significant ($p < 0.05$). Figures 6 and 7 clearly depicts the statistical data of the different biochemical parameters analysed for the acute toxicity of bark extract of *Rhizophora mucronata* (BERM).

On the basis of histopathological investigation there is no significant change was observed in the organs viz, liver, lungs, spleen and kidneys of the treated groups. For liver, normal portal tract and normal architecture was observed in all the three groups of mice viz, BERM 800 mg/kg b. w, BERM 1600 mg/kg b. w and BERM 3200 mg/kg b. w. respectively. The

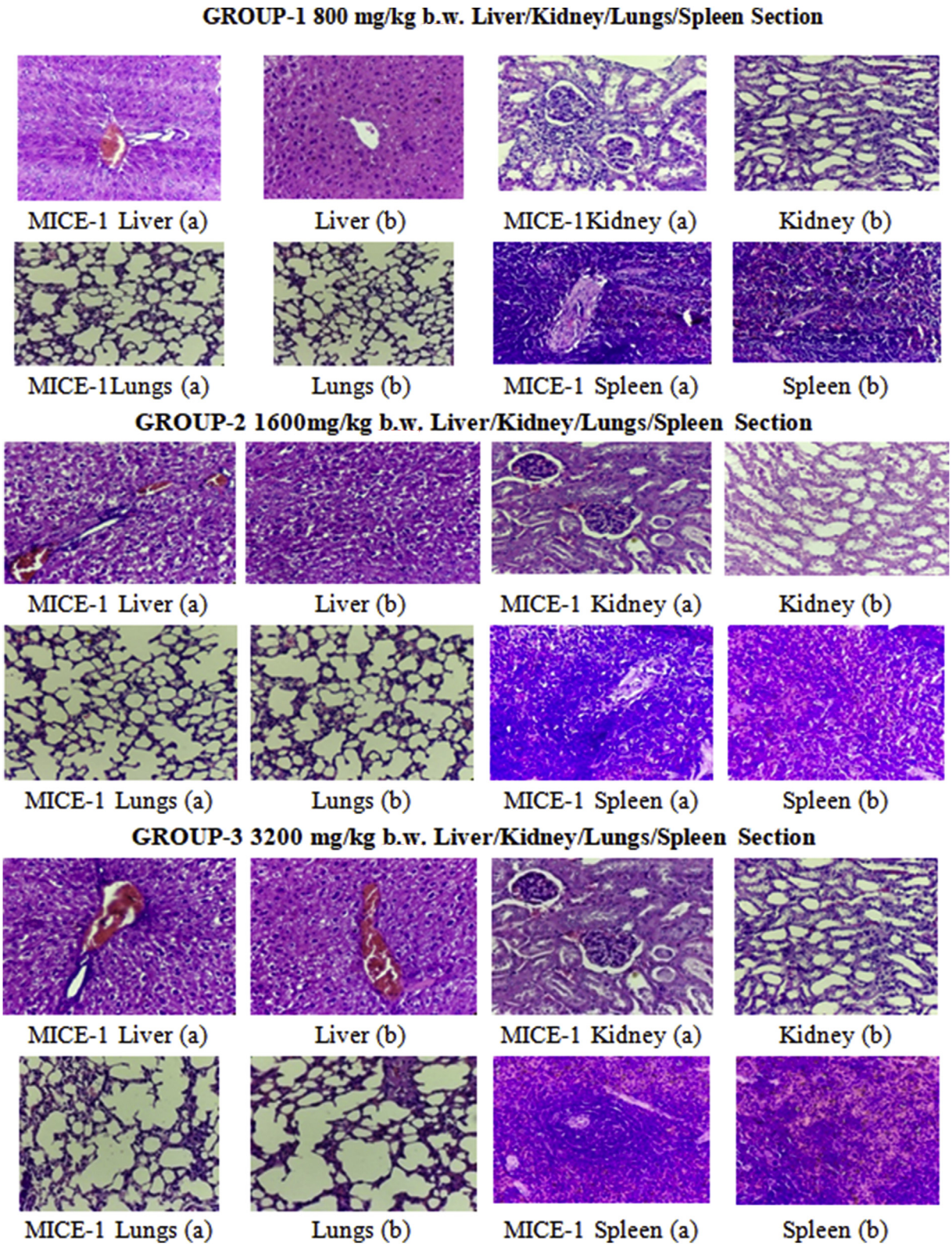

Figure 8. Histological sections of the organs viz, Liver, kidneys, lungs and spleen of the three groups of mice viz, BERM 800 mg/kg b. w, BERM 1600 mg/kg b. w and BERM 3200 mg/kg b. w respectively.

peri portal region composed of portal triad (portal vein, hepatic artery and bile duct, surrounded by cords of hepatocytes) and the central vein surrounded by cords of hepatocytes was normal in all the treated groups. In terms of kidneys, normal cortex, medulla and normal glomeluri was observed in animals belonging to the group BERM 800 mg/kg and BERM 1600 mg/kg respectively. The animals in the group of high concentration of BERM 3200 mg/kg b. w out of 4 animals' only one mouse found its kidney to be effected with interstitial inflammation predominantly in lymphocytes. Histological spleen section showed white pulp, central arteriole surrounded by sheets of Lymphocytes and red pulp, lymphocyte

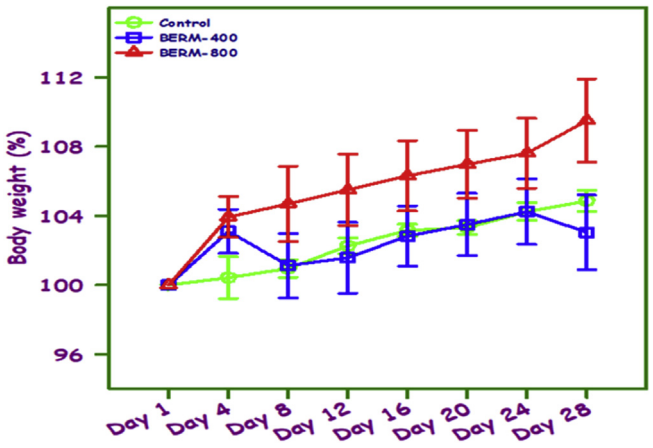

Figure 9. Show the change in % of body weight from day 1 till day 28th at Sub-acute toxicity study in Swiss Albino Mice. Values were expressed as mean (n = 5) for each group.

surrounded by RBCs, was normal and there is no variation observed in all the groups treated with different concentration of BERM. On observing the histopathological slides of the lungs, the section showed normal alveoli with normal bronchiole were observed in the treated groups of mice in 10 x and 40 x magnifications respectively. Figure 8 The representation of histopathological images of liver, spleen, lungs and kidneys of the treated groups 800, 1600 and 3200 mg body weight of the animals respectively.

3.3. Sub-acute toxicity

After administering the repeated dose of BERM for 28 days, 800 mg/kg animals showed there is an increase in the % of body weight of animals as compared with control. Wherein in case of 400 mg/kg animals there was a decrease in the % of body weight observed from day 4 to day 8, then there is a considerable increase was observed till 24th Day, later from 24th day till 28th day again there was a fall has been observed. Figure 9 clearly depict the results in terms of body weight of animals.

3.4. Biochemical parameters

Based on the biochemical parameters, the sub-acute toxicity assessment of BERM clearly indicated that there is no significant change was observed in both the groups as compared to control. Although they were statistically significant within themselves. Figure 10 indicated the values of liver enzymes viz AST, ALT and ALP respectively for three groups of animals. From, the graph it is clear that 800 mg/kg gives more protection than 400 mg/kg as the values of AST, ALT and ALP of 800 mg/kg is similar to that of control group.

3.5. Haematopoietic system

The haematopoietic system comprises RBCs, WBCs, Haemoglobin and PCV values, after repeated dose administration of 400 mg/kg and 800 mg/kg respectively, the values find to be non significant as compared to the control. However, they were mutually significant. Figure 11 showed the values of haematopoietic system.

3.6. Kidney parameters

The biochemical parameters pertaining to the kidney function refers Urea, Creatinine and Total protein levels, after the repeated dose for 28 days, clearly suggested that 800 mg/kg showed the similar values as compared to control. Although, both the groups 400 mg/kg and 800 mg/kg were non significant to control but they were mutually significant. Figure 12 refer the values of Urea, Creatinine and Total protein of all the three groups of animals.

3.7. Histopathological analysis

Based on the histopathological analysis, the liver, lungs, kidneys and Spleen found to be showed no abnormalities after repeated administration of BERM (400 mg/kg and 800 mg/kg) respectively for 28 days. Figure 13 depict the histopathological analysis of the three groups viz, control, 400 mg/kg and 800 mg/kg respectively.

4. Discussion

Various parts of *R. mucronata* plant like bark, collar, hypocotyl and stilt root were investigated for their anti-oxidant and hepatoprotective activity [21]. This plant has been extensively studied for its phytoconstituents with high therapeutic activity and the structure has been elucidated [22]. Benzophenone (16.09%) and 2-(2-ethoxyethoxy) ethanol (7.82%) were identified as predominant constituents of *R. mucronata* [23]. In another study, by [24] compounds viz, squalene (19.19%), n-Hexadecanoic acid (6.59%), phytol (4.74%), 2-cyclohexane-1-one, 4-hydroxy-3,5, (4.20%) and oleic acid (2.88%) was elucidated in this plant. But in the preliminary study for the characterization

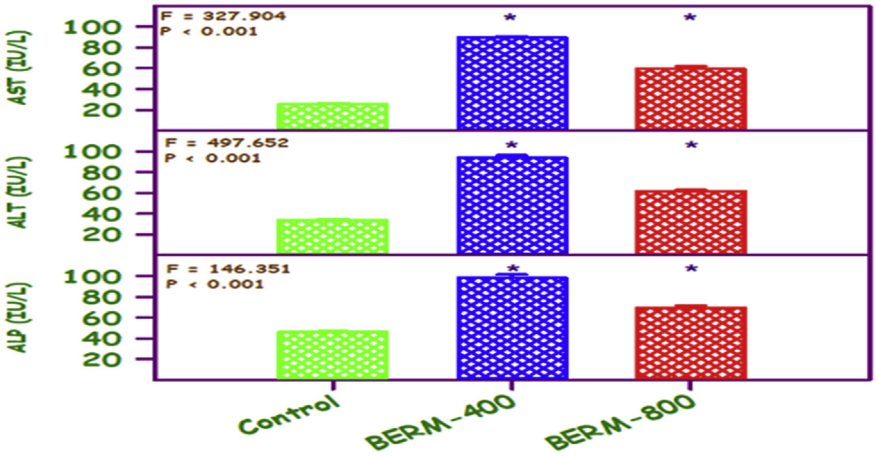

Figure: 10. Effect of hydroethanolic extract of bark of *Rhizophora mucronata* (BERM) on female mice (mean ± SE (n – Control = 5, 400 mg = 5; 800 mg = 5). AST = alanine aspartate transferase; ALT = alanineamino transferase; ALP = alkaline phosphatase. The 'F' and 'P' values are by one way ANOVA. * shows the groups are statistically significant.

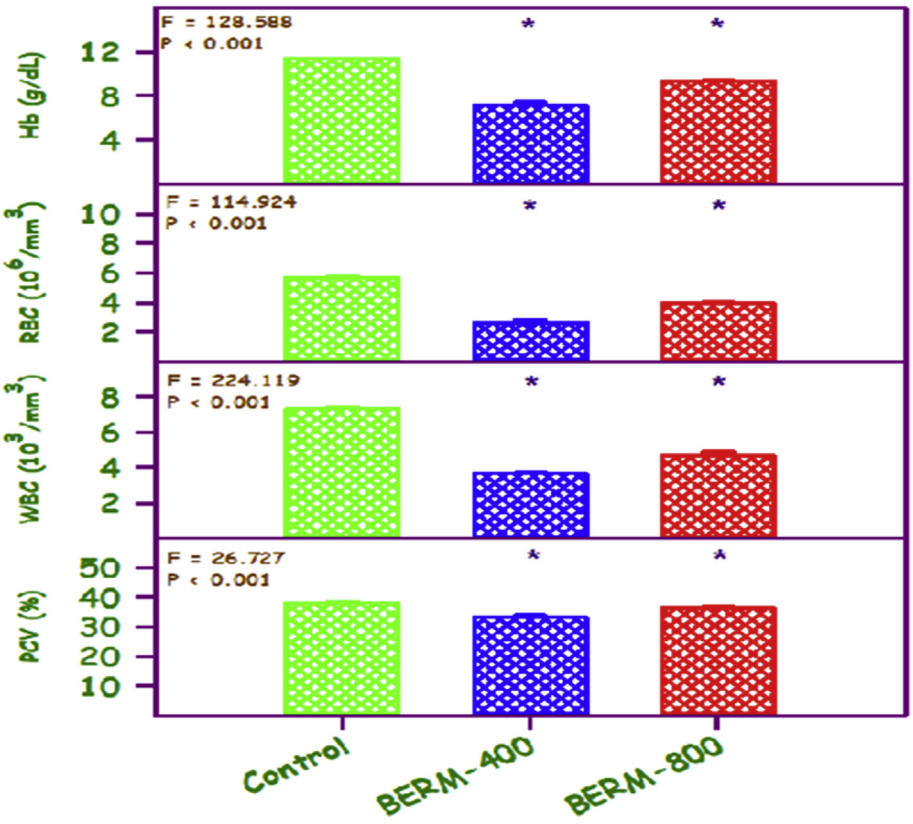

Figure 11. Effect of hydroethanolic extract of bark of *Rhizophora mucronata* (BERM) on female mice (mean ± SE (n – Control = 5, 400 mg = 5; 800 mg = 5). RBCs, WBCs, Hb: Haemoglobin and PCV: Packed Cell Volume/Haematocrit. The 'F' and 'P' values are by one way ANOVA. * shows the groups are statistically significant.

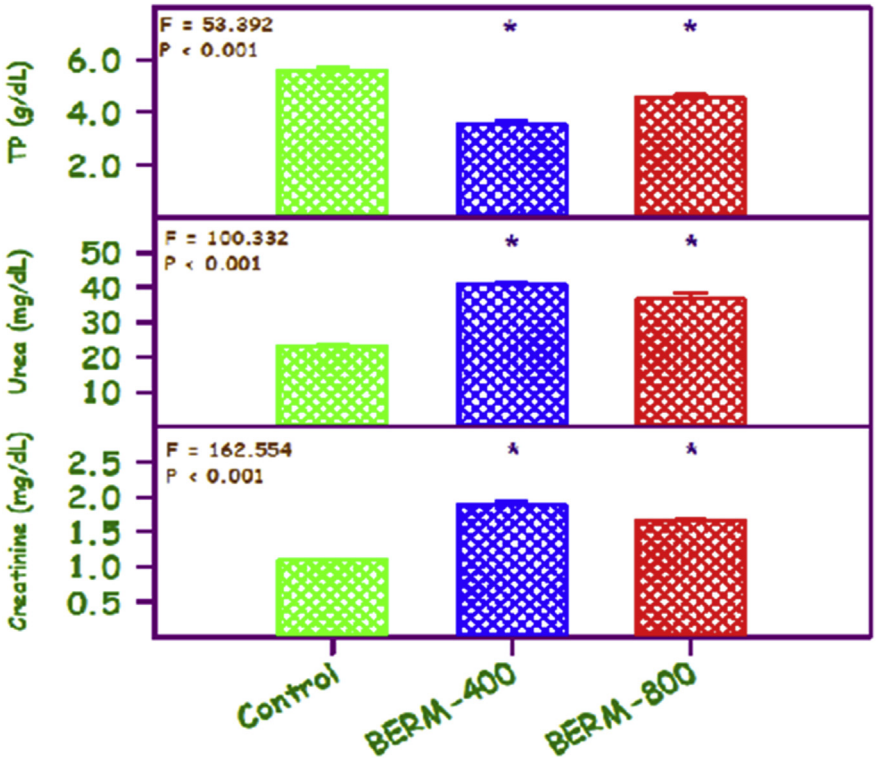

Figure 12. Effect of hydroethanolic extract of bark of *Rhizophora mucronata* (BERM) on female mice (mean ± SE (n – Control = 5, 400 mg = 5; 800 mg = 5). Urea, Creatinine and TP: Total protein. The 'F' and 'P' values are by one way ANOVA. * shows the groups are statistically significant.

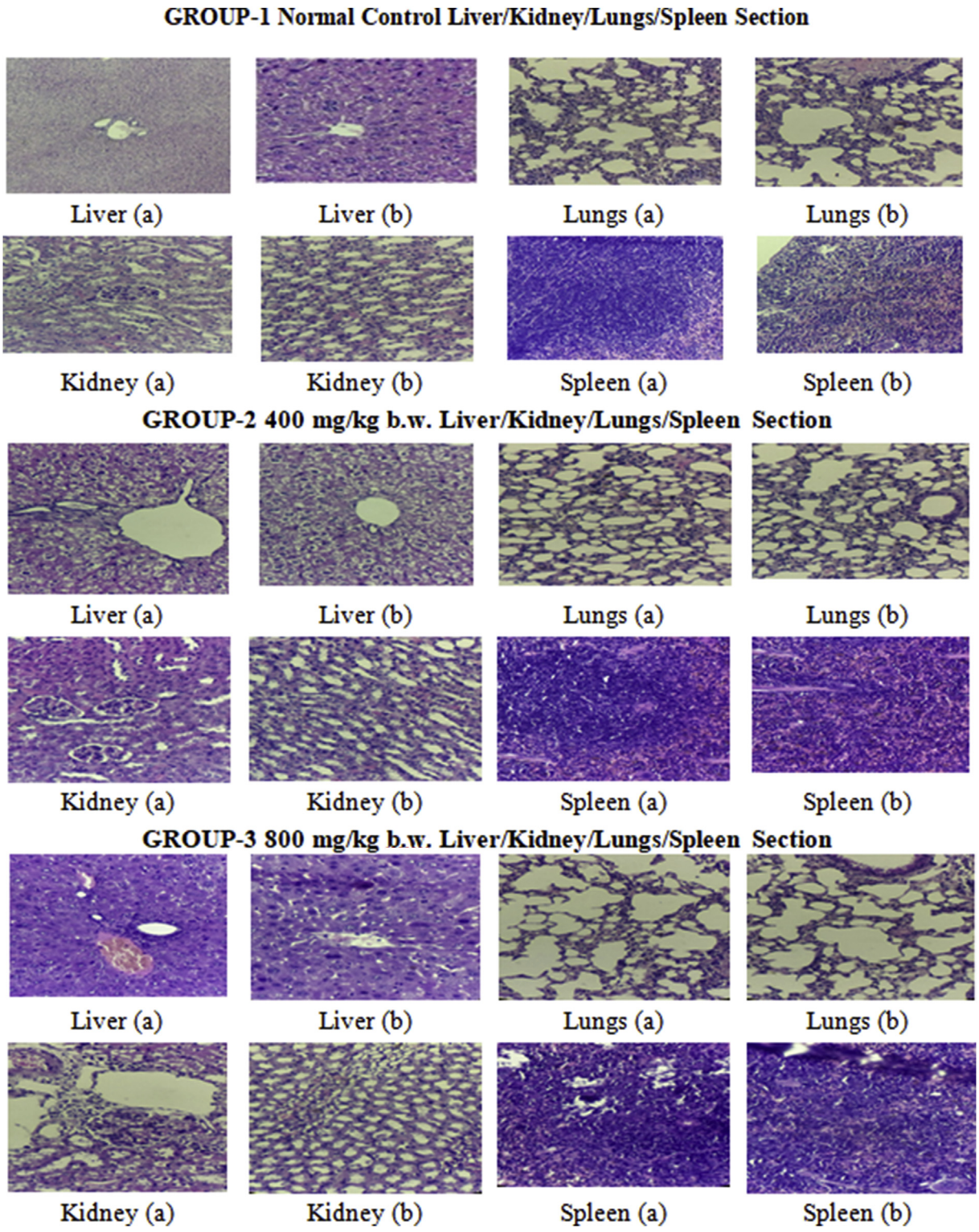

Figure 13. Shows the histological section of the organs viz, Liver, kidneys, lungs and spleen of the three groups of mice viz, Control, BERM 400 mg/kg b. w and BERM 800 mg/kg b. w respectively. All the mice pertaining to the groups showed normal histology (n = 5), after repeated administration for 28 days. Where Liver (a), Kidney (a), Lungs (a) and Spleen (a) refers to 10X magnification and other refers to 40X magnification.

of secondary metabolites, BERM found to contain 5 compounds, viz: Diadzein, Epicatechin, Hesperidin, Diosmin and Quercitrin respectively. These compounds are mainly fall in the category of flavonoids, flavones, flavonones and flavonoid + Deoxy ribose sugar.

In terms of their biological activity, Hesperidin possess hypoglycaemic and hypolipidemic effect, anti-inflammatory, cholesterol lowering and anti hypertension activity [25]. Diadzein found to contain anti-oxidant and phytoestrogenic properties [26]. Diosmin is effective against chronic venous insufficiency (CVI) including spider and varicose veins, leg swelling (oedema), stasis dermatitis and venous ulcers. Epicatechin has Gastro-protective, anti-oxidant, antinociceptive effect [27]. Similarly, Quercitrin, of potential interest found to possess anti-oxidant

activity, anti venom, anticancer, and antitumor activity, hepatoprotective activity, anti-inflammatory activity, anti-diabetes activity, antiviral activity [28]. The diverse biological activities manifested by BERM are due to the presence of various types of flavonoids, tannins, flavones, etc.

The non-toxic nature of the bark extract of *Rhizophora mucronata* (BERM) is evident by the absence of mortality amongst the animals at the highest oral dose of 3200 mg/kg body weight in this study; the LD_{50} is estimated to be more than 5 gm/kg. Similar study conducted by [29] showed that the methanolic extract of water hyacinth at dose of 500 mg/kg body weight is non-toxic. In another study, by [30] in the acute toxicity study, a single administration of the extract at doses of 2000 and 5000 mg/kg, respectively, was given to the mice which did not cause any mortality or significant behavioural changes. Thus, LD50 value of the extract was found to be greater than 5000 mg/kg which co relates with our present study. In another study [31], a single dose of methanolic leaf extract of *Rhizophora mucronata* at the dose of 2,000 mg/kg bw for acute toxicity in Wistar rats caused neither treatment related signs of toxicity nor mortality during 14 days of the study which co-related the present study. Thus, LD50 is more than 2000 mg/kg whereas in the current study LD50 was found to be greater than 5 gm/kg which clearly indicated the bark extract of *Rhizophora mucronata* (BERM) is non-toxic and safe to use.

Haematopoietic system is considered to be the important index of pathological and physiological status and is the most sensitive target for the toxicants. That's why the haematological parameters were analysed in this study. No abnormalities were observed in the concentration of RBC's, WBC's, HB and PCV and were all within the range when treated with the BERM extract, thus indicating the extract to be non-toxic and safe. Similar results were obtained by [30, 31] wherein the haematological parameters showed no statistical difference which co relates with our present study.

Biochemical parameters viz, TP, ALP, AST, ALT and kidney parameters viz, urea and creatinine were analyzed quantitatively to check the internal damage of the specific organs. There is a dose dependent increase in the level of AST has been observed which co-related the study by [31] and the level of AST for 3200 mg/kg body weight was found to be significant to 800 mg/kg and 1600 mg/kg b. w ($p < 0.05$). The increase in the levels of AST may be attributed to the inflammation of the liver cells. In the similar way, for the kidney parameters, creatinine levels are well within the limits and were normal in range. But there is a dose dependent increase in the concentration of Urea levels was observed. This can be attributed to dehydration in animals.

The body weight changes and organ weight plays a vital role in checking the toxicity of the compound. The body weight changes are markers of adverse effects of drugs and chemicals and if the body weight loss observed is more than 10 % of initial body weight, it will be considered as statistically significant [32]. In this study, there was a dose dependent decrease in the animal's body weight. This may be due to the fact that the extract BERM, may contains some nutritive substances which led the animals to be active. On the contrary, the results obtained by [33] clearly suggested that there is an increase in the body weight of the animals was observed. In terms of organ weight ratio, the results found to be normal and all within the range. Food consumption was found to be decreased with the increasing concentrations; however the water consumption showed the dose dependent increase throughout the period of study. Since, there is a less intake of food in the animals were observed, which showed a dose dependent decrease in the weight of the corresponding animals. However, this can be taken as a positive response because despite of less feed intake, animals were active and normal and no mortality was observed throughout the 14 days.

Historically, histopathology has been the most consistent method of establishing the no-observed-adverse-effect level (NOAEL). Histopathological analysis of acute toxicity clearly indicated that the internal organs viz, liver, spleen and lungs showed no abnormalities and tissues showed normal morphology. The kidney tissues (one animal in 3200 mg/kg b. w) showed interstitial inflammation, predominantly in the lymphocytes. This can also be the reason for the higher amount of urea in these animals. Alveoli sacs and bronchioles showed normal histology and do not had any abnormal state. No necrosis, cirrhosis or any abnormalities were observed in the liver tissue, which is the clear indication that the liver was healthy in the treated cells. In terms of kidneys, the 11 animals showed normal morphology, medulla and cortex regions, Glomeruli were found to be normal, indicating the BERM extract to be non-toxic and safe to use. The spleen, the white pulp; central arteriole surrounded by sheets of Lymphocytes and the Red pulp; Lymphocytes with RBC's showed no abnormalities indicating the BERM extract for its non-toxic and safe to use. The results obtained by [34] wherein the acute and sub acute toxic study of aqueous leaf extract of *Combretum molle* showed some infiltration of inflammatory cells at the level of the portal vein in the liver. The inflammation caused may be due to passive immune response to the extract, which contradicted with our present study. As per the current study all the liver sections treated with a single dose of BERM 800 mg/kg b. w, BERM 1600 mg/kg b. w and BERM 3200 mg/kg b. w showed no significant changes in hepatic cells. All the liver sections showed normal histology upto the highest dose of 3200 mg/kg dosage.

The sub-acute toxicity guideline [20] with slight modifications, two doses viz 400 mg/kg and 800 mg/kg b. w. has been studied, wherein both does not show toxicity effects. No significant change was observed in both the doses w.r.t. the haematological and biochemical parameters when compared with control which co-related the study [31].

Histopathological analysis of sub-acute toxicity assessment revealed that the repeated dose of BERM for 28 days did not produce any adverse effects. The liver, lungs, kidney and spleen showed normal histology of cells even at 800 mg/kg. There were no signs of necrosis, damaged cells, inflammation in the cells were observed. Since the oral dose of 400 mg/kg and 800 mg/kg bw/day of BERM administered for 28 consecutive days did not induce any biochemical, haematological, and histopathological signs of toxicity, it can be defined as the NOAEL for Swiss Albino mice under the experimental conditions used. Thus, can be concluded that BERM is safe, non-toxic and can be used as alternative for liver protection.

5. Conclusion

The HPLC fingerprinting clearly indicated the presence of polyphenolic compounds, viz: flavonone glycoside, flavonoids, flavones, isoflavones and flavonoid plus Deoxy ribose sugar respectively. All pertains to high anti-oxidant value and as hepatoprotectant. The acute toxicity study of the bark extract of *Rhizophora mucronata* (BERM) clearly indicated that the extract at the maximum concentration 3200 mg/kg b. w was found to be non-toxic and safe to be used as a drug. Since, there was no mortality observed in the 14 days study, it can be stated that the extract has some medicinal and nutritive value. Although the biochemical parameters viz AST and Urea were found in higher concentrations but it did not cause any harm to the animals. They can be neutralized by animals own immune system. The haematopoietic and histopathology investigation clearly stated that the animal is normal and no abnormalities were seen in serum and tissues. The liver, lungs and spleen were showed normal histology. A small or minute inflammation was observed in one out of 12 in terms of kidney histology. Similarly, in terms of Subacute toxicity, on giving a fixed but repeated dose of 400 mg/kg and 800 mg/kg BERM clearly indicated that at higher dose, the protection found to be maximum. According to the findings of the current study, the bark extract of *Rhizophora mucronata* (BERM) at 800 mg/kg was safe and non-toxic when given orally repeatedly for 28 days. The biochemical, haematopoietic and histopathological investigations pertaining to sub-acute toxicity supported our study and confirmed our findings. This study is a first attempt in terms of this plant part (BERM) made to study the toxic nature of the plant for 28 days. Thus, from our study we can conclude that the bark extract of *Rhizophora mucronata* which provides assurance on the safety of the extract can be widely used as one of the ingredient in the pharmaceutical formulation of drugs.

Declarations

Author contribution statement

Chitra Jairaman: Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Syed Ali Mohamed Yacoob: Conceived and designed the experiments.

Senthil Kumar Sivanesan, Vijayaraghavan Rajagopalan: Contributed reagents, materials, analysis tools or data.

Anuradha Venkataraman: Performed the experiments.

Yogananath Nagarajan: Analyzed and interpreted the data.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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