Original Article:
In vivo Toxicity Evaluation of Ethanolic Extract From Two Mangrove Plants

Afiya Aunjum[1], Rana Biswas[1], Md. Abdullah Al Munna[1], Md. Morsaline Billah[1], Md. Emdadul Islam[1], Kazi Mohammed Didarul Islam[1*]

1. Biotechnology and Genetic Engineering Discipline, Khulna University, Khulna, Bangladesh.

*** Corresponding Author:**
Kazi Mohammed Didarul Islam
Address: Biotechnology and Genetic Engineering Discipline, Khulna University, Khulna, Bangladesh.
Phone:+88 (172) 6852004
E-mail: didar950718@yahoo.com

Article info:
Received: 20 Dec 2020
Accepted: 01 May 2021

Keywords:
Swiss mice, Artemia, Toxicity, Lethal Dose (LD_{50}), Liver

ABSTRACT

Background: Mangrove plants, often possessing a unique collection of metabolites, have extensively been used for the primary healthcare of coastal residents.

Objectives: To determine the safety level and enrich the scientific data, the present study aimed to investigate the toxicity of *Avicennia officinalis* and *Excoecaria agallocha*.

Methods: Ethanolic leaf and stem extracts were evaluated for cytotoxicity by brine shrimp lethality assay. The obtained extracts were administered to Swiss albino in a single dose (200, 400, 800, 1600, & 3200 mg/kg body weight) by oral-gavage for acute toxicity assay. Furthermore, systematic observation was performed by close monitoring for any toxic manifestations and mortality after dosing for the first 4 h, at 24 h and twice daily for 6 days. Evaluating the adverse effects were estimated by comparing the test groups with the controls. After sacrificing all group animals, relative organ weight was measured and histopathological analysis was conducted.

Results: Having Lethal Concentration (LC_{50}) of 44.66 µg/mL, *E. agallocha* leaf was found with the highest toxicity against brine shrimp nauplii. The toxicological study data demonstrated no death and noticeable change in behavioral patterns in the test mice groups, compared with the control group. Moreover, no significant ($P>0.05$) differences were found in body weight and relative organ weights, compared to the controls. The histological structures of the liver in the treated mice displayed regular tissue configurations similar to the control group.

Conclusion: In this study, the mice model exhibited no harmful effects; thus, the reported results indicated that the ethanolic extracts of leaf and stem of these two mangrove plants are safe for therapeutic use. Further long-term toxicological impact of the extracts should be determined for well-founded confirmation.

Citation Aunjum A, Biswas R, Al Munna M A, Billah M M, Islam M E, Didarul Islam K M. *In vivo* Toxicity Evaluation of Ethanolic Extract From Two Mangrove Plants. Pharmaceutical and Biomedical Research. 2021; 7(3):209-216. http://dx.doi.org/10.18502/pbr.v7i3.7702

http://dx.doi.org/10.18502/pbr.v7i3.7702

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Pharmaceutical & Biomedical Research

Introduction

Medicinal plants have been a major recognized source of treatment for human maladies since time immemorial in different parts of the world. This is because of having several biological properties on microbial infections, cancer, oxidative stress-related diseases, and various other conditions [1, 2]. The salt-tolerant mangroves plants inhabit the intertidal zones between land and sea with specialized morphological and physiological features to survive in a hostile environment. Innumerable mangrove plants have traditionally and extensively been used for primary healthcare by coastal residents. Furthermore, enormous unique bioactive compounds from different parts of mangrove species are discovered and employed for therapeutic and nutraceutical purposes [3, 4].

The plant *Avicennia officinalis* (AO) is among the vital mangrove species having vast uses of its leaf, bark, roots, and fruits in traditional medicine by treating ulcers, skin problems (especially scabies), rheumatism, paralysis, asthma, snake-bites, and tumors. Moreover, in several studies, various parts of this plant suggested antimicrobial, antiulcer, anti-inflammatory, anticancer, and antioxidant activities [5].

Excoecaria agallocha (EO) is another notable mangrove plant. Besides, the toxic latex of this plant causes skin irritation and blistering and temporary blindness. Traditionally, the plant parts are used to treat rheumatism, ulcers, leprosy, and paralysis. Moreover, multiple studies reported considerable beneficial pharmacological properties of the species [6].

Due to over-dosage, improper use, and lack of adequate knowledge of every part and compound of the plants, detrimental impacts were observed through using local medicinal plants [7]. Thus, it is necessary to determine the toxicity of medicinal plants. The potentiality of mangrove plants, as a wellspring source of new bioactive principles, remains unexplored. Furthermore, there have been sporadic *in vivo* studies on the toxicological properties of mangrove medicinal plants from the Sundarbans Mangrove Forest, Bangladesh. Thus, as a part of the systemic approach in evaluating their efficacy and safety profile, the current study aimed at the potential toxicity assessment of the ethanolic leaf and stem extracts of AO and EA mangrove species.

Materials and Methods

Ethanol and Chloroform were obtained from Sigma Chemical Co. Ltd. (St. Louis, MO, USA). Moreover, Vincristine Sulfate (Criston 2) was purchased from Beacon Pharmaceuticals Ltd., Bangladesh. The rest of the chemicals were obtained from local commercial suppliers. All applied chemicals and solvents were of analytical grade.

The plant samples of AO and EA were collected from the Dhangmaree, Chadpai range, the east zone of the Sundarbans East Division, Khulna City, Bangladesh. The collected plant samples were deposited and authenticated at the herbarium of the Forestry and Wood Technology Discipline, Khulna University, Khulna City, Bangladesh (the given representative specimen numbers: AA-KU-2018012 & AA-KU-201801, respectively).

The studied plant parts were thoroughly cleaned with distilled water and sliced into small pieces. Those samples were then dried under sunshade and air till the completion of proper drying. Next, the plant materials were ground into powder. One hundred grams of powder from each of the 4 study samples were soaked in 250 mL of ethanol. They were then sealed and kept for 5 days in dark condition. The mixtures were coarsely filtered using double-layer Markin cloth, followed by Whatman filter paper 41. The filtrates were concentrated in a rotary evaporator for eliminating excess solvents. Next, they were kept at room temperature until entirely dried. The extracts yields were weighed, stored at 4°C and their yield percentage was calculated using the following formula: Extract yield (%)=R/S×100 (where R; the weight of the crude extract and S; the weight of the powdered plant material) [Reference No.: AA-KU-2018012 (*A. officinalis* L.) and AA-KU-201801 (*E. agallocha* L.)].

Brine shrimp lethality bioassay was performed for evaluating cytotoxicity where vincristine sulfate was taken as a standard drug [8]. We collected brine shrimp (*Artemia salina*) eggs and seawater from BRAC Prawn Hatchery, Bagerhat, Bangladesh. Different concentrations (5, 10, 20, 40, 80, 160, & 320 µg/mL) of extracts were prepared by dissolving with DMSO (10%). Each test tube was adjusted to the final volume of 10 mL using artificial seawater. Ten living nauplii were transferred into each tube followed by incubation for 24 hours and the number of survived nauplii was recorded. The 50% Lethal Concentration (LC_{50}) was estimated per plant sample.

Swiss albino mice (both genders) having a weight ranged 22-28 g were used in this study. The required mice were obtained from the Animal Resources Depart-

ment, International Centre for Diarrhoeal Disease Research, Bangladesh (icddr, b). The mice were acclimatized by keeping in the polypropylene cages with free access to standard rodent diet under controlled laboratory conditions (Mean±SD room temperature: 25±2°C, relative humidity 55±5%, & a 12:12 h light/dark cycle). Experiments mentioned in this study were conducted by following the guidelines of ethical ideologies and rules for the Scientific Experiments on Animals approved by the Swiss Academy of Sciences and the Swiss Academy of Medical Sciences (1995) and the animal ethics and regulation of Khulna University, Bangladesh (Research Ref. No. KUAEC-2017/08/15).

In total, 72 Swiss albino mice were weighed and randomly distributed into groups of 3 animals each. For each extract, 5 groups of mice were prepared for administration of 5 different concentrations of extract (200, 400, 800, 1600, & 3200 mg/kg body weight) with one control group. Thus, 4 extracts were tested. Each research group was supplied with a basal diet of mouse pellets (obtained from icddr,b) and water before commencing the study. During the experiment, normal saline was provided to the control group without extract.

Oral acute toxicity experiment of the extracts was performed following the Organization for Economic Co-operation and Development (OECD) guideline no. 423 with slight modification [9]. The explored mice were divided into groups for treatment and fasted prior to dosing with the extract; however, they were allowed free access to water. The study groups were each formed with 3 animals; each research group received a single oral dose of the extract by gavages using a feeding needle and a negative control group was maintained with normal saline. Five fixed level doses (200, 400, 800, 1600, & 3200 mg/kg body weight) per extract were administered for a particular group. The doses were taken according to OECD guideline no. 423. The obtained extracts were dissolved using normal saline. Before dose administration, the bodyweight of each tested animal was determined and the dose was calculated according to the body weight. All experimental animals were kept under close monitoring for any toxic manifestations and mortality after dosing for the first 4 h, at 24 h, and twice daily for 6 days. In this time, with a regular interval of 2 days, the bodyweight of each examined mouse was assessed. After overnight fasting with only access to water, on the seventh day, the explored mice were anesthetized with Chloroform and all the experimental animals plus the controls were euthanized by cervical dislocation. Major vital organs i.e., heart, liver, kidney, and spleen were collected, cleaned with saline, and weighed. The following formula [10] was used to determine relative organ weight:

Relative organ weight (%)=(weight of organ/bodyweight of the mouse on the day of sacrifice)×100

For additional histopathological inspection, the liver was stored in 10% formalin.

The processing of liver tissue samples for histological observation was accomplished with the established procedures [11]. Briefly, after dehydrating by a graded series of alcohols, the collected tissues were washed in xylene and embedded in paraffin; they were then cut by a microtome at 6 µm thickness. Hematoxylin and Eosin (H&E) staining method was followed by the cleaning in xylene twice (3 min each), hydrating in alcohol of 100%, 90%, and 70% for 3 min each, rinsing with water for 3 min; accordingly, this staining procedure was repeated. Furthermore, the stained slides were dehydrated using alcohol grades of ascending concentrations (70%, 90%, & 100%). Eventually, the sections were dried and mounted onto the slide to examine microscopically for the signs of toxicity.

Statistical analysis was performed by Mean±SD (n=3). GraphPad Prism 6.01 was used to assess the differences in the mean values by Analysis of Variance (ANOVA) followed by Dunnett's multiple comparisons test. $P<0.05$ was considered to be significant.

Results

For the toxicity analysis, the powdered leaf and stem of AO and EA were extracted by ethanol. The highest extract yield equaled 7.06%, i.e., obtained from EA stem. EA leaf, AO leaf, and stem extract yielded 4.67%, 3.56%, and 1.85%, respectively.

Regression analysis was conducted for analyzing the data obtained from brine shrimp lethality bioassay to observe the relationship between different samples and vincristine sulfate as standard. The brine shrimp lethality activity of the extracts is presented in Table 1. EA leaf extract possessed the lowest LC_{50} among all extracts tested, exhibiting an LC_{50} of 44.66 µg/mL, i.e., close to the standard value (LC_{50}=1.91 µg/mL). Furthermore, LC_{50} of 63.09 µg/mL was observed in EA stem and other extracts, suggesting LC_{50} of >100 µg/mL. Concentration-Dependent mortality details are presented in Figure 1.

There were no symptoms of toxicity or mortality in the acute toxicity study at any concentration of any extracts.

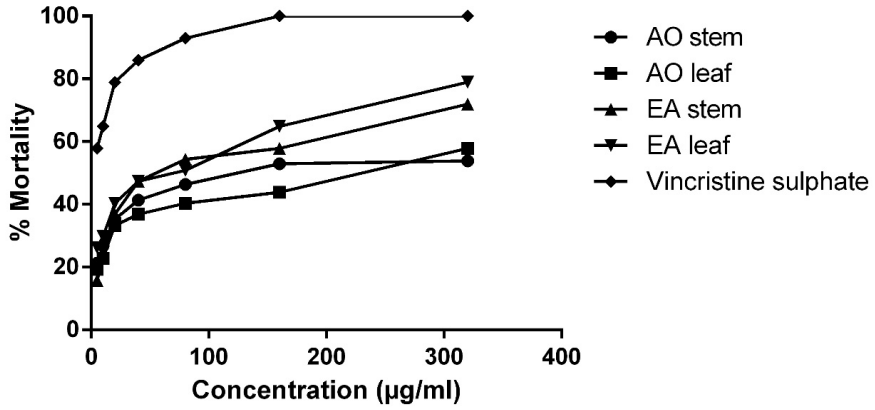

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Figure 1. Cytotoxicity of extracts and standard vincristine sulfate. AO: *Avicennia officinalis*; EA: *Excoecaria agallocha* Two-Way ANOVA data revealed significant differences between the test and control groups at P<0.05.

Normal health condition similar to the control group was noticed in all the test animals, as well as general behaviors. Moreover, natural movements were recorded and compared until the end of the experiment. All 4 extracts were safe for medicinal use without any adverse effects. Therefore, the 50% Lethal Dose (LD_{50}) of these plants was considered >3200 mg/kg body weight.

The body weights of mice in the treated group were not significantly (P>0.05) different from those of the control group (Figure 2). Normal weight gain was found in the extract-treated groups.

Tables 2 and 3 exhibit the relative organ's weight of the control and test animals administrated with AO and EA extract, respectively. The relevant results suggested that the research sample exerted no adverse impact on the vital organs (heart, liver, kidney, & spleen) during the experiment. The relative organ weight of the tested animals, compared to the control groups demonstrated statistically non-significant differences (P>0.05).

In the toxicity study, no differences were found in the histopathological examination of the liver treated with sample extracts, compared with the control group. The photomicrographs of the liver with the highest concentration of extract in all cases indicated normal architecture, the clear lumen of the central vein, and no evidence of lesion (Figure 3).

Discussion

More than 80% of the populations exploit indigenous natural products of their respective local area for a therapeutic purpose; however, safety/toxicity issues always get attention for validating traditional healthcare practices [12]. Moreover, cytotoxic compounds can be used as anticancer drugs, because of having the ability to prevent normal growth and the replication of a cell [13]. Thus, the present study evaluated the toxicity of two traditionally important mangrove plants, AO and EA.

As a preliminary test, a brine shrimp lethality assay was conducted to evaluate the cytotoxicity of the extracts. EA plant possessed LC_{50} <100 µg/mL (Table 1); thus, the extracts would contain potential compounds for antitumor or anticancer treatment.

Table 1. Brine-Shrimp bioassay of different extracts and standard

Extracts	LC_{50} (µg/mL)
AO stem	138.04
AO leaf	199.52
EA stem	63.09
EA leaf	44.66
Vincristine sulfate	1.91

AO: *Avicennia officinalis*; EA: *Excoecaria agallocha*

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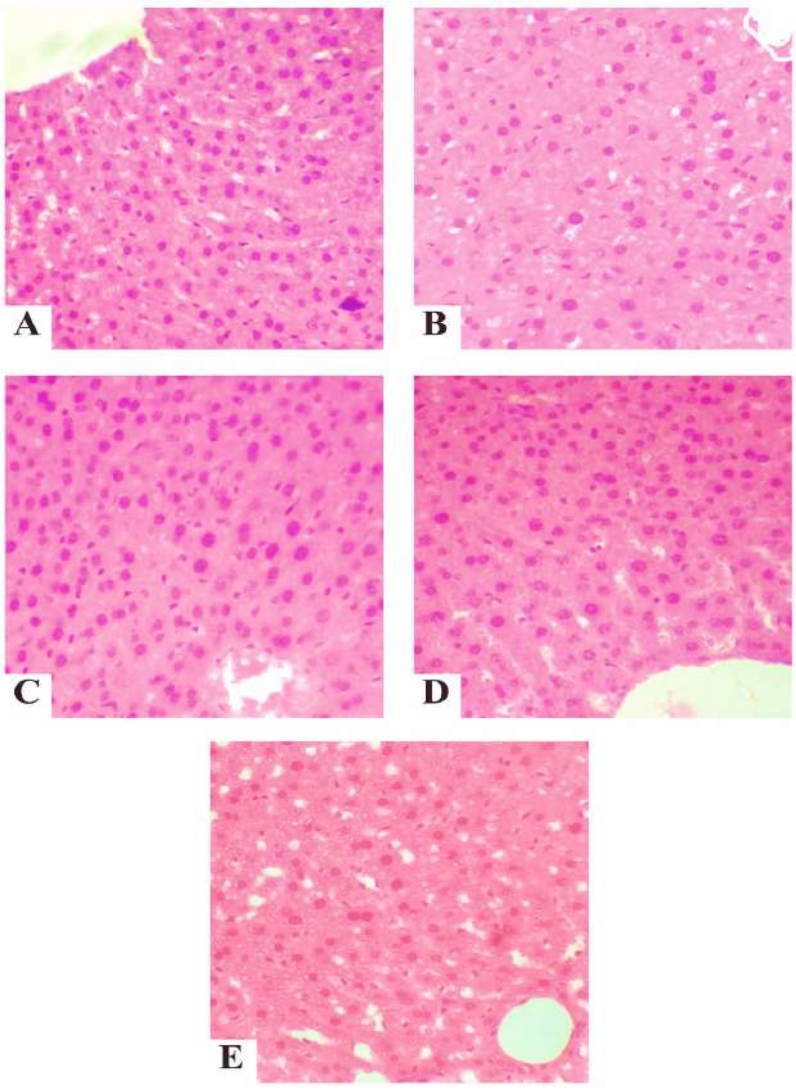

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Figure 3. Photomicrographs of liver sections of (A) AO leaf, (B) AO stem, (C) EA leaf, (D) EA stem treated, and (E) control mice stained with hematoxylin-eosin under light microscopy

AO: *Avicennia officinalis*; EA: *Excoecaria agallocha*. All liver sections presented healthy histology architecture along with clear lumen. No lesion was present (at magnification 40X).

Table 2. Relative organ weight (%) of AO stem and leaf extract-treated mice

Organs	Control	AO Stem Extract Conc. (mg/kg b.w)					AO Leaf Extract Conc. (mg/kg b.w)				
		200	400	800	1600	3200	200	400	800	1600	3200
Liver	5.96±0.41	5.77±0.26	6.02±0.13	5.10±0.48	5.73±0.37	5.37±0.82	5.53±0.68	6.11±0.21	6.77±0.27	6.43±0.12	5.71±0.61
Heart	0.65±0.08	0.52±0.02	0.62±0.14	0.49±0.01	0.55±0.02	0.51±0.06	0.53±0.08	0.49±0.07	0.45±0.05	0.51±0.01	0.53±0.04
Kidney	1.35±0.25	1.37±0.35	1.40±0.38	1.29±0.17	1.62±0.02	1.33±0.21	1.50±0.32	1.22±0.28	1.54±0.08	1.36±0.09	1.28±0.21
Spleen	0.39±0.07	0.45±0.02	0.37±0.05	0.40±0.06	0.55±0.02	0.43±0.01	0.62±0.19	0.42±0.04	0.47±0.08	0.43±0.04	0.51±0.12

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Data indicate Mean±SD, n=3. There are no significant (P>0.05) differences between the control and treated mice in their relative organ weight; AO: *Avicennia officinalis*; EA: *Excoecaria agallocha*; conc.: concentration; b.w: body weight.

Table 3. Relative organ weight (%) of EA stem and leaf treated mice

Organ	Control	EA Stem Extract Conc. (mg/kg b.w)					EA Leaf Extract Conc. (mg/kg b.w)				
		200	400	800	1600	3200	200	400	800	1600	3200
Liver	5.96±0.41	5.27±0.16	5.90±0.59	5.54±0.11	5.34±0.21	5.92±0.22	5.36±0.34	5.26±0.20	5.00±0.87	5.96±0.37	5.67±0.25
Heart	0.65±0.08	0.51±0.06	0.52±0.04	0.42±0.00	0.46±0.06	0.73±0.20	0.50±0.01	0.47±0.02	0.52±0.01	0.51±0.02	0.55±0.01
Kidney	1.35±0.25	1.17±0.23	1.11±0.13	1.34±0.00	1.32±0.27	1.50±0.23	1.25±0.07	1.28±0.00	1.41±0.17	1.34±0.46	1.56±0.51
Spleen	0.39±0.07	0.35±0.07	0.38±0.09	0.37±0.07	0.37±0.06	0.42±0.00	0.44±0.01	0.50±0.10	0.44±0.05	0.41±0.10	0.53±0.13

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Data indicate Mean±SD, n=3. There are no significant (P>0.05) differences between the control and treated mice in their relative organ weight; AO: *Avicennia officinalis*; EA: *Excoecaria agallocha*; conc.: concentration; b.w: body weight

For establishing the notable importance of medicinal plant extract, *in vivo* model, especially mouse is considered standard for preclinical toxicological evaluation [13]. Neither signs of toxicity nor mortality were presented at any concentration of both extracts, including the highest dose of 3200 mg/kg b.w during the 7 days of experiment in the acute toxicity study. During the experiment, all the tested mice exhibited well physical conditions with normal general appearance (skin, fur, eyes, & mucous membranes), respiratory, circulatory, and nervous system as well as behavioral patterns. None of the tested groups exhibited any toxicity signs or death even at the maximum concentration; thus, LD_{50} would be greater than 3200 mg/kg b.w; accordingly, these data supported estimating a therapeutic dosage range of the plant extracts. Moreover, our results were in line with those of Sura et al.; they reported no toxicity in Wistar

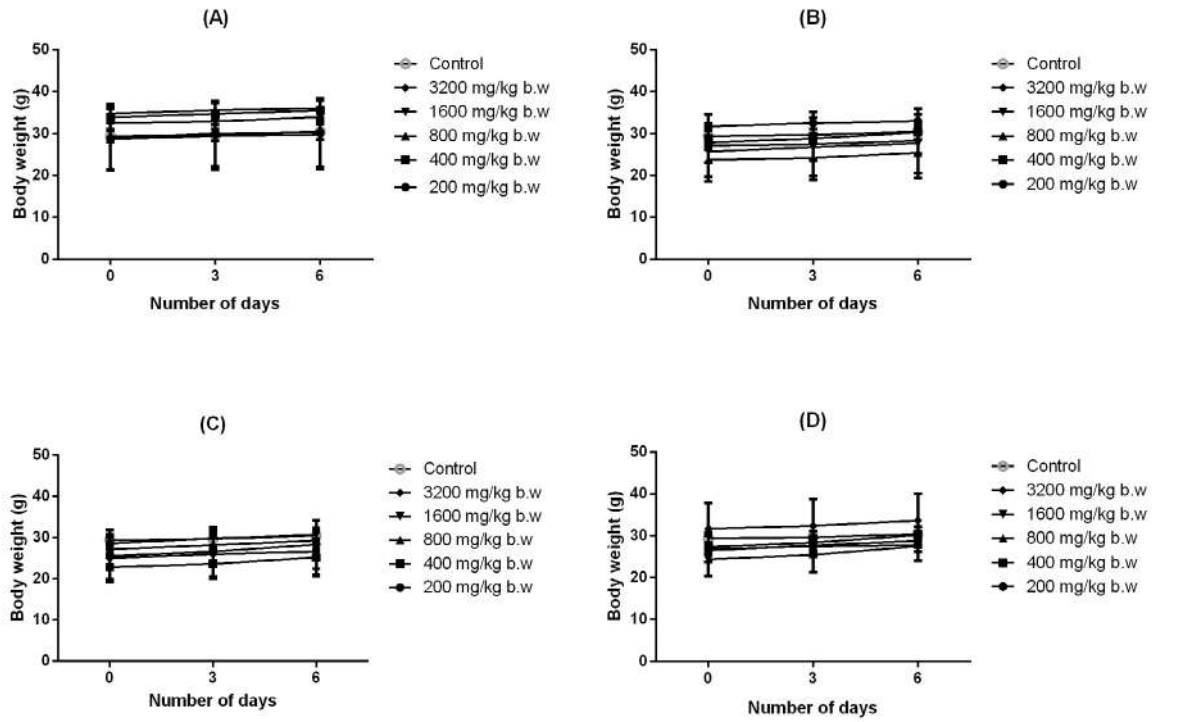

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Figure 2. Bodyweight of (A) AO leaf, (B) AO stem, (C) EA leaf and (D) EA stem treated group mice with control in the acute toxicity

There are no significant (P>0.05) differences between the control and extracts treated mice in their body weight.

AO: *Avicennia officinalis*; EA: *Excoecaria agallocha*; b.w: body weight

albino mice at 250 and 500 mg/kg b.w dosages of ethanolic leaf extract of AO [14].

Introduction to potentially poisonous substances retards the body weight gain of animals [15]. In our study, all mice continued to gain weight at each dosage group during the experiment (Figure 2). No significant differences were noted between the control and treated groups. This recommends that the exposure to extracts did not hamper the growth of the examined mice.

To determine whether the size of the organ has changed related to the weight of the whole animal, relative organ weight is usually observed. If the extract did not cause any form of swelling, atrophy, and hypertrophy on the organs, no effect would be noticed on the estimated organs and body weight ratios [16]. According to Demma et al., this ratio is more supportive regarding comparative analysis [17]. Therefore, we also recorded the relative organ weight of all the tested groups (Tables 2 and 3). No significantly toxicological effect was found between the control and treated groups in this case.

The liver is a very sensitive organ to toxic substances as well as drug or bioactive compounds, as it metabolizes these substances to other compounds [18]. The liver can assist as a key index of the physio-pathological state of the animals and humans; thus, we decided to focus on the liver histopathology test. The section of liver tissue from all the tested mice manifested normal histology with typical hepatocytes having intact cell margins and normal nucleus, compared with the controls (Figure 3). Apart from exhibiting no inflammation, there was a regular appearance of central vein and sinusoids (no congestion).

No lesions in histology or attributable changes in any other traits of mice in the experimental groups indicated that the samples up to a maximum concentration of 3200 mg/kg b.w are safe to be used without any adverse effects.

Conclusion

This study provided crucial data on the toxicity outline of the ethanolic extract of *Avicennia officinalis* and *Excoecaria agallocha*. To use these two plant extracts as nutraceuticals or pharmaceutical products, including antineoplastic drugs, the function of a particular isolated compound should be evaluated. Besides, the absence of any significant toxic impacts on the mice model not only provides support for the safety of these extracts but also explains the extensive use of the plants as traditional medicine. For performing any future *in vivo* and clinical trials of this plant medicine, the present study results can be very helpful. Moreover, further long-term toxicity studies should be established for ensuring a complete safety profile.

Ethical Considerations

Compliance with ethical guidelines

Ethical ideologies and rules for Scientific Experiments on Animals originated by the Swiss Academy of Sciences and the Helsinki Declaration in 2000 and the animal ethics and regulation of Khulna University, Bangladesh (Research Ref. No.: KUAEC-2017/08/15) were strictly followed.

Funding

The research was funded by grants for Advanced Research in Science, Ministry of Education, Government of the People's Republic of Bangladesh, and Khulna University, Bangladesh (Grant No.: 2016-17/64).

Authors' contributions

Resources, Methodology, Software, Formal analysis, Writing – Original Draft: Afiya Aunjum, Rana Biswas and Md. Abdullah Al Munna; Conceptualization, Validation, Investigation, Writing – Review, and Editing, Supervision, Project administration, Funding acquisition, Md. Morsaline Billah, Md. Emdadul Islam and Kazi Mohammed Didarul Islam.

Conflict of interest

The authors declared no conflict of interest.

Acknowledgments

Experimental facilities were provided by Biochemistry and Molecular Biology Laboratory, Khulna University, Bangladesh. We acknowledge the Sundarbans east division, Ministry of Environment, Forest and Climate change, Government of Bangladesh for necessary administrative and logistic support in collecting plant sample material.

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