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Toxicological, Biochemical and Histopathological Evaluation of the Ethanolic extract of Seagrass-*Enhalus acoroides* in Albino wistar rats

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ABSTRACT

The purpose of this research was to examine the acute toxicity (14 days) and sub-acute toxicity (28 days) studies of ethanolic extract of *Enhalus acoroides* (EEEA) on male wistar albino rats. A single dose of 2000 mg/kg of the extract was administered to the male rats for acute study and for sub-acute study, dose levels of 100, 200 and 400 mg/kg/b.wt. of the extract was administered daily by oral gavage to the rats respectively. During the treatment periods, body weight, water and food consumption, clinical signs, mortality, biochemistry, hematology, organ weights, gross findings, and histopathology were examined. Imperative organs are subjected to histopathology. Upto 2 g/kg b.w. of EEEA did not show any mortality in the acute toxicity studies. Our results demonstrated that sub-acute treatment of the crude extract of *Enhalus acoroides* revealed there were no significant changes in the haematological and serum biochemical parameters seen in the treated groups compared to the control group. Histological studies revealed no abnormalities in liver, kidney and spleen tissue at the high dose level of EEEA. In conclusion, both the acute and sub-acute toxicity studies proved no clinical toxic symptoms and histopathological lesions, which show Ethanolic extract of *Enhalus acoroides* are safety at normal therapeutic doses.

1. Introduction

Medicinal plants are regularly used as therapeutic sources because of the simple and easy accessibility and also low in cost (Silva et al., 2014). As the natural herbal products are safe and free from side effects, the people consume it for medicinal purposes. Enormous different compounds are present in the medicinal plants, but some compounds with great complications are also seen (Nasri and Shirzad, 2013). Though herbs can be employed in treating most of the diseases, repeatedly they are consumed without knowing the scientific validation and substantiation of their pharmacological effects (Carneiro et al., 2014).

The current allopathic practices gradually turned to the site for good scientific and accomplished observational efforts of researchers in oxidative stress. During the last three decades, drugs based on antioxidants and finding the mechanism of action for the deterrence and management of complicated diseases like Alzheimer's disease and carcinoma have appeared (Aqil et al., 2006). However, the origin of its development remains embedded in traditional medications (Mcchesney et al., 2007).

Herbal products are devoid of toxic side effects which are derived from nature, are often coupled with allopathic drugs that are often consumed in established drugs (Pushpa Latha et al., 2010). However, the level of toxicity should be detected for accurate and acknowledged herbal drugs, like the established traditional drugs that are appropriately validated; the toxicity is not assessed often in the conventional herbal drugs (Smart et al., 2011). In general, people give importance to the remedial property of the medicinal drugs and abandon their toxic effects to different organs.

Due to effective pharmacological activities, less toxicity and cost effective and rich in antioxidant properties, this increases the recent scientific development of medicinal herbs, (Auddy et al., 2003). The research in herbal toxicity has increased anxiety among the globe to figure and implement the different guidelines to monitor and prevents toxicity due to herbal drugs. Currently, the research increases on searching novel compounds from seagrasses which are rich in antioxidants like polyphenols, terpenoids, flavonoid, tannins and saponins, since the mechanism of action begins at the target cellular level.

One such seagrass is *Enhalus acoroides* (Linn.f.) Royle, belongs to the family Hydrocharitaceae, has dark green long linear leaves with 1.0–1.5 cm width and 50–170 cm length. The calorific values of *E.acoroides* were comparable to sweet potato, peas, bengal gram, and potato. Literature also showed *E.acoroides* contains higher antioxidant activity, higher activity of scavenging free radicals and higher reducing

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power property and also effectively used for the treatment of various oxidative stress related problems. From the studies, *E.acoroides* present in the region of South China shows the properties like anti-feedant, antibacterial and anti-larval activities in the biochemical analysis and identified 11 pure compounds which include 5 sterols and 4 flavonoids (Ragupathi Raja Kannan et al., 2010). Recent studies statement reveals that antioxidant compounds are abundant in seagrass (Gillan et al., 1984).

In our past report we acknowledged the existence of phytochemicals from the EEEA which contains carbohydrates, terpenoids, steroids, alkaloids, flavonoid, and tannins (Amudha et al., 2017). Certain bio-active components were also isolated from *E.acoroides* including 1-nonadecene (17.15%), n-tetracosanol-1 (11.48%), 1-octadecene (10.06%), 2-pentadecanone (7.87%), behenyl alcohol (7.33%) (Amudha et al., 2018).

From the literature, it was proven the great potential and several applications of *E.acoroides* as a medicinal herb. However, data about toxicity of the crude extract from the whole seagrass of *E.acoroides* are still limited. The pharmacological and toxicological properties of the seagrass still need to be found better expose of the risks of their use, and ensuring the safety for the people. So for the safety evaluation of ethanolic extract of *E.acoroides*, acute and sub-acute toxicity studies were worked out to examine the toxicity after single dose of EEEA and 28-days repetitive oral dose of EEEA in wistar rats.

2. Materials and methods

2.1. Plant material

The whole seagrass of *Enhalus acoroides* was collected from Devipattinam, Ramanadhapuram District, Tamilnadu during the month of June 2016. The seagrass was authenticated in ICAR by Dr. N. Kaliaperumal M.Sc., Ph.D., Scientist-in-charge, CMFRI.

2.2. Obtention of extract

The collected *E.acoroides* leaves washed with distilled water carefully to get rid of the traces of impurities. The leaves were shade dried, mechanically grinded and powdered. The grinded powder was initially soaked into 1:2 ratio ethanol for three days with mild shaking. After 3 days, the macerate was filtered and concentrated in a rotary evaporator. Finally, lyophilize the extract and stored under refrigeration.

2.3. Experimental animals and diet

Wistar strains of albino male rats weighing between 180 and 200g were obtained from Sri Venkateswara Enterprises, Bangalore, India. The animals were kept for seven days in polypropylene cages and distributed randomly in various experimental groups with inhibited temperature (27 ± 2 °C) and regular cycle of 12 h light and dark. The animals were given with standard pellet diet (VRK Nutritional, Maharashtra, India) and water ad libitum. They were acclimatized to the environment for one week prior to experimental use. According to the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), New Delhi, India (IAEC No: XXI/VELS/PCOL/02/2000/CPCSEA/IAEC/01.12.2017) research was carried out.

2.4. Experimental design

According to the Guidance of OECD (Organization for Economic Co-operation and Development) numbers 423 (2001) and 407 (1995), acute and sub-acute toxicity experiments were performed respectively.

2.4.1. Acute toxicity study

The experiment was based on the Guidelines OECD 423; a single dose of 2000 mg/kg of EEEA was dissolved in distilled water and administered through oral gavage, while the control group received only distilled water. The procedures were performed in duplicate, using 6 animals at each stage. After administration of the extract of EEEA, animals were continuously monitored for every 2 h for 24 h to detect the morphological and behavioral changes, diarrhea, lethargy, tremors, salivation, irritability, corneal refex, convulsion, and monitored also for any mortality during the experiment of the toxicity study. At the termination of the experiment, using chloroform vapour the rats were anesthetized prior to dissection and blood collected with and without anticoagulants (EDTA) by cardiac puncture for haematological and biochemical parameters, liver and kidney were removed for histopathological studies and examined macroscopically.

2.4.2. Sub-acute toxicity test

The experiment was based on the Guidelines OECD 407, wistar albino rats were divided into 4 groups (Group I-Group IV) of 6 animals each, three groups (Group II-IV) of them received various doses of EEEA (100 mg/kg b.w., 200 mg/kg b.w., and 400 mg/kg b.w.) and the control rats (Group I) was treated with 0.5 mL of normal saline once daily for 28 days. The drug was administered through oral gavage for 28 days. Throughout the experiment period, the rats were observed for physical and behavioral changes or any symptoms of abnormality during the period. At the termination of the experiment, the rats were fasted overnight, but with free access to water. Then they were anesthetized and the blood was collected by cardiac puncture for haematological and biochemical analysis, liver spleen, and kidney were removed for histopathological studies and examined macroscopically.

2.5. Hematology

The animals were fasted overnight prior to necropsy and blood collection. Blood samples were collected through Orbital Sinus Venipuncture technique from retro orbital sinus of rats by capillary tube. The blood samples were collected into tube containing EDTA-2K (Merk Pvt. Ltd., Mumbai, India) and analysis within 1 h. The haematological parameters including hemoglobin (HB) concentration using Beacon Diagnostic Kit (Dacie and Lewis, 1968), red blood cell (RBC) count, white blood cells (WBC) count, Packed Cell Volume (PCV) count (Ochei and Kolhatkar, 2000), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC) were examined for both acute and sub-acute toxicity studies.

2.6. Serum biochemistry

Blood for clinical chemistry was placed in tubes devoid of anticoagulant, allowed to clot at room temperature. Blood samples were centrifuged at 3000 rpm for 10 min within 1 h after collection, and then serum was separated. Serum biochemistry parameters including Alanine aminotransferase (ALT) and Aspartate aminotransferase (AST) by Reitman and Frankel (1957); Alkaline phosphatase (ALP) by Kind and King's (1954); total protein (TP) by Lowry et al. (1951); Urea (URE) by Natelson (1957); Creatine (CR) by Bonsnes and Taussky (1945); Sodium (Na), Potassium (K), Total bilirubin (T-BIL) by Malloy and Evenlyn (1937); Albumin (ALB) by Rodkey (1965); Albumin globulin ratio (A/G ratio), Triglycerides (TG) by Werner et al. (1981); Total cholesterol (TCH) by Allain et al. (1974); High density lipoprotein (HDL) by Allain et al. (1974); Low density lipoprotein (LDL) by Friedewald et al., (1972) were determined by Microlab-300 auto-analyzer (Merk Pvt. Ltd., Mumbai, India) were examined for both acute and sub-acute toxicity studies.

2.7. Serum antioxidants

Blood for clinical chemistry was placed in tubes devoid of

anticoagulant, allowed to clot at room temperature. Blood samples were centrifuged at 3000 rpm for 10 min within 1 h after collection, and then serum was separated. Serum antioxidant parameters including Reduced Glutathione (GSH), the reaction is described by Moron et al. (1979) in which the compound absorbs maximally at 412 nm. Catalase (CAT) antioxidant activity was determined according to the method described by Beers and Sizer (1952). This test is based on the conversion of H_2O_2 into H_2O and molecular oxygen by CAT, resulting in a loss of absorbance at 240 nm. Superoxide dismutase activity (SOD), the reaction is described by Kakkar et al. (1984) in which the compound absorbs maximally at 560 nm. The activity of Mitochondrial Glutathione Peroxidise (GPx) was assayed by the method of Rotruck et al. (1973). Vitamin C determined by the method described by Omaye et al. (1979) and Vitamin E by the method described by Baker et al. (1980) were measured for both acute and sub-acute toxicity studies.

2.8. Gross observation and organ weight

All the rats were sacrificed by cervical decapitation under anesthesia at the end of the experiment period and examine carefully for macroscopic abnormalities. The complete and relative (organ-to-body weight ratios) weights of major organs including liver, spleen, and kidney were measured for both acute and sub-acute toxicity studies.

2.9. Histopathology

After cervical decapitation under anesthesia, major organs include liver, spleen, and kidneys were preserved in neutral, phosphate-buffered 10% formalin. The organs were routinely processed, embedded in paraffin, and sectioned at 5–7 μm. The sections were stained with Hematoxylin–eosin stain for microscopic examination for both acute and sub-acute toxicity studies (Ochei and Kolhatkar, 2000).

2.10. Statistical analysis

Values were expressed as mean ± SD for six rats in the each group and statistical significant differences between mean values were determined by one way analysis of variance (ANOVA) followed by the individual comparison was obtained by Post-hoc Bonferroni test, a multiple comparison procedure by the SPSS software for Windows Version 20.0 (IBM Corp. Armonk, New York, NY, USA). A value of $p < 0.05$ was considered to indicate a significant difference between groups.

3. Results

The panel experts of OECD defines the acute toxicity study as “the adverse effect occurring within a short time of (oral) administration of a single dose of a substance or multiple doses given within 24 h”. And sub-acute toxicity as “the advance effects occurring as result of the repeated daily (oral) dosing of a chemical to experimental annual for part (not exceeding 10%) of the life span” (Lee and Dixon, 1978).

Acute toxicity tests provide preliminary information on the toxic nature of a material for which no other toxicological information is available. Sub-acute toxicity studies gives informative about the cumulative toxic effects of the drug, physiological organ and metabolites of a compound at minimum dose for prolonged exposure. The adverse effect of different substances can be analysed. From such studies can provide datas which will be helpful in fixing the level of doses.

3.1. Clinical sign and mortality

In the acute toxicity study, the body weight of animals, water and food consumption were found to be normal by the treatment of EEEA. The first sign of toxic nature of the drug is animal's general behavioral and mortality in the acute toxicity study (Carol, 1995). Even after the single highest dose level of 2000 mg/kg b.wt. of EEEA administered orally, there were no mortality or major changes in general behavior was observed (Table 1). That was the single maximum dose recommended by the OECD guidelines-423 (Echobicon, 1992). In order to evaluate the adverse effect of repeated daily exposure of drug, sub-acute toxicity study was carried out. Sub-acute toxicity studies are immensely valuable in measuring the safety profile of the drugs (Aniagu et al., 2005). To examine dose related toxic property, doses of 100 mg/kg b.wt, 200 mg/kg b.wt and 400 mg/kg b.wt, of EEEA extract were given orally for 28 days. EEEA at the individual doses did not produce any noticeable changes in the rats.

Table 1
Acute toxicity study of EEEA in wellness parameters of rats.

Observations	Response	
	Group I (Control Rats)	Group II (EEEA treated rats)
Consciousness	+	+
Grooming	–	–
Touch response	+	+
Sleeping duration	+	+
Movement	+	+
Gripping strength	+	+
Righting re flex	+	+
Food intake	+	+
Water consumption	+	+
Tremors	–	–
Diarrhea	–	–
Hyper activity	–	–
Pinna reflex	+	+
Corneal reflex	+	+
Salivation	+	+
Skin color	+	+
Lethargy	–	–
Convulsion	–	–
Morbidity	–	–
Sound response	+	+

(+) indicate normal (−) indicate absent.

A morphological change gives a clear substantiation of serious changes of organ dysfunction due to the activity of the drug (Andrew and Krystal, 2003). Table 1 indicated the parameters after the administration of EEEA revealed no mortality as well as abnormality seen in the general behaviors of all the rats. The lethal dose (LD_{50}) of the extract is higher than 2000 mg/kg body weight and hence, in a single maximum dose administration, the EEEA had no adverse effects.

3.2. Body weight changes

The body weights of rats in all the groups were examined throughout the period of study and presented in Table 2 (Acute study) and Table 3 (Sub-acute toxicity study). Administration 2000 mg/kg/b.wt of EEEA was found to slight changes in the body weights of the rats but this was not statistically significant in the acute toxicity study. The observed weight gain in the EEEA treated rats' shows that the given

Table 2
Effect of EEEA on Body weight and Mortality rate in Acute toxicity study.

S.No	Animals	Group I (Control Rats)	Group II (EEEA treated Rats)
1	Initial (gm)	190 ± 6.32	195 ± 8.61^{NS}
	Final (gm)	200 ± 7.07	205 ± 9.17 ^{NS}
2	Animal live (Nos.)	6	6
3	Animal dead (Nos.)	Nil	Nil
4	% of Mortality	Nil	Nil

Values are expressed as Mean ± Standard Deviation for six rats in each group. Data were analysed by Independent samples *t*-test. Statistically significant analysis by two tailed variation, NS = Non-significant ($P < 0.05$).

Table 3
Effect of EEEA on Body weight in Sub-acute toxicity study.

S.No	Weight of the Animals	Group I (Control)	Group II (100 mg/kg/b.wt)	Group III (200 mg/kg/b.wt)	Group IV (400 mg/kg/b.wt)
1	Initial (gm)	183.33 ± 4.73	192.50 ± 6.35 ^{NS}	187.50 ± 5.65 ^{NS}	184 ± 6.22 ^{NS}
2	Final (gm)	212 ± 4.85	227 ± 5.89 *	224 ± 5.21 ^{NS}	218 ± 4.19 ^{NS}

Values are expressed as Mean ± Standard Deviation for six rats in each group. Data were analysed by one-way ANOVA followed by post-hoc Bonferroni test. Statistically significant variation was derived by comparing Group I versus Group II, Group III, and Group IV, $^{*}P = 0.013$ and NS = Non-significant ($P < 0.05$).

drug does not have any annoying action which affect the growth of the rats. To examine the detrimental effects of drugs and chemicals, change in the body weight is an uncomplicated and sensitive index. Changes in the body weight of animals indicates the toxic effects of the drug, not more than 10% of the initial body weight, the animals should survive (Teo et al., 2002). Control Group I rats and the EEEA treated Group II rats gained weight throughout the study period of 14 days and found there are no statistical ($P < 0.05$) significant changes in the weight of all the rats (Table 2). It goes without saying that a decrease in body weight may be an indicator of adverse effects (Tahraoui et al., 2010). Hence at a maximum dose of 2000 mg/kg body weight showed the non-toxic nature of *E.acoroides*. All over the period of 28 days, the mean weight of both EEEA treated rats (Group II- IV) and the control rats (Group I) increased. No significant ($P < 0.05$) increase in the animals weight which consumed EEEA extract when compared with the Control group I rats in the Sub-acute toxicity studies (Table 3).

3.3. Effect of EEEA on haematological parameters

3.3.1. Acute toxicity

In humans and animals, haematological system is one of the most responsive targets for toxic substances and a significant index for pathological and physiological status (Adeneye et al., 2006). The intake of toxic herbs will cause a modification in the haematological parameters (Sani et al., 2009). The toxic nature of drugs reported to produce haemolytic anaemia and dysfunction of organs which produces a significant change in the biomarkers of some haematological parameters (Echobicon, 1992). In the present investigation, Haematological parameters such as Hb, RBC and WBC were validated to found the any changes in the blood profile due to the toxicity of EEEA. Hb and RBC found to be normal in the EEEA treated Group II rats when compared with the Group I control rats and these results prove that EEEA was non-toxic to the circulating red cells and also not interfere with their production. PCV, MCV, and MCHC were significantly increased ($P < 0.05$) in the EEEA treated Group II rats depicted in Table 4. WBC level is slightly increased with the dose of 2000 mg/kg b.wt. when compared with Control Group I but this was not statistically significant.

Table 4
Effect of EEEA on Haematological profile in Acute toxicity study.

S.No	Parameters	Group I (Control Rats)	Group II (EEEA Treated Rats)
1	Hemoglobin (gm/dl)	13.59 ± 0.88	13.12 ± 0.87^{NS}
2	WBC(x10^3/mm^3)	7.11 ± 0.89	8.90 ± 0.95*
3	RBC(x10^6/mm^3)	4.71 ± 0.44	3.94 ± 0.21*
4	PCV (%)	23.78 ± 1.24	31.29 ± 2.40$^{\#}$
5	MCV(famato litre)	50.95 ± 7.18	79.97 ± 3.20$^{\#}$
6	MCH(pico gram)	30.67 ± 4.04	33.40 ± 3.40^{NS}
7	MCHC (%)	57.25 ± 4.72	42.14 ± 4.24$^{\#}$

Values are expressed as Mean ± Standard Deviation for six rats in each group. Data were analysed by Independent samples *t*-test. Statistically significant analysis by two tailed variation, $^{*}P = 0.001$, $\#P = 0.000$ and NS = Non-significant ($P < 0.05$).

3.3.2. Sub-acute toxicity

Haematological parameters are essential in finding the toxicity profile of drugs taken from herbs (Toyin et al., 2008). The reaction of the body to damage or stress is evaluated by the blood parameters and it is the most responsive index to determine the toxic nature of the drug in pre-clinical and clinical trials (Mukinda and Eagles, 2010). Oral administration of EEEA for 28 days at a dose of 100 mg/kg b.w, 200 mg/kg b.w. and 400 mg/kg b.w. did not cause any significant changes in haematological parameters when compared with Control Group I rats. Haematological Parameters include Hb, WBC, RBC, MCV, MCH, MCHC showed no significant ($P < 0.05$) differences observed between the EEEA treated rats (Group II– Group IV) when compared with the Control Group I rats shown in Table 5. Non-significant level of Hb, RBC, WBC, PCV, MCV, MCH and MCHC was observed, which may indicate the non-toxic beneficial effect of EEEA on the haematological parameters of sub-acute toxicity study. The slight increase in WBC level indices the reinforcement of the organism defence system (Chang-Gue et al., 2003). This elevation in WBC count suggests that EEEA possess immune potential.

3.4. Effect of EEEA on serum biochemical parameters

3.4.1. Acute toxicity

The essential function of normal liver cell is to synthesize protein, so the level of protein seems to be a trustworthy indicator of liver function while administering the drug (Hutchinson et al., 1980). In this study the level of protein shows no detrimental changes in EEEA treated Group II rats when compared to the Control Group I rats (Table 6). It indicates the non-toxic nature of EEEA on protein. Hepatic function has been determined by validating the marker enzymes like ALT, AST and ALP in serum. Transaminases and Alkaline Phosphatase is good indicator of liver and kidney damages, respectively (Martin et al., 1981). In the present study, the levels of ALT, AST and ALP present in the serum of Control Group I rats and EEEA treated Group II rats were examined. Any changes in the ALT, AST and ALP levels may reveal a change on the permeability of cells or damage in the cells (Kashaw et al., 2011). However, no such significant ($P < 0.05$) differences were found in the levels of serum marker enzymes of EEEA treated Group II rats when compared with the control Group I rats. Hence, the present data clearly showed that the treatment with EEEA did not produce any toxic biochemical effects in liver of rats.

In this present examination of renal function markers in serum, a non significant change was observed in Urea, Creatinine, Sodium, and Potassium levels of EEEA treated Group II rats when compared with Control Group I rats (Table 6). For continues and normal protein metabolism in the cell, Creatinine and Urea are the end products of protein metabolism which must be removed (Guyton and Hall, 1981). They derived from the proteins which are eliminated by the kidney. Renal dysfunction is connected directly with toxic substances of herbal remedies (Luyckx et al., 2002). Therefore it can be inferred that EEEA did not produce any toxicity on the kidneys. There was also no significant ($P < 0.05$) difference in serum total Cholesterol, Triglycerides, HDL, and LDL in EEEA treated Group II rats when compared with the control Group I rats (Table 6).

Table 5
Effect of EEEA on Haematological profile in Sub-acute toxicity study.

S.No	Parameters	Group I (Control)	Group II (100 mg/kg/b.wt)	Group III (200 mg/kg/b.wt)	Group IV (400 mg/kg/b.wt)
1	Hemoglobin (gm/dl)	14.37 ± 0.38	13.87 ± 0.41^{NS}	14.67 ± 0.74 ^{NS}	14.28 ± 0.52 ^{NS}
2	WBC(x10^3/mm^3)	7.10 ± 0.49	6.95 ± 0.66 ^{NS}	7.25 ± 0.54 ^{NS}	7.15 ± 0.44 ^{NS}
3	RBC (x10^6/mm^3)	4.54 ± 0.31	4.02 ± 0.34 ^{NS}	4.56 ± 0.34 ^{NS}	4.26 ± 0.28 ^{NS}
4	PCV (%)	24.89 ± 0.64	23.33 ± 1.07 ^{NS}	23.95 ± 1.02 ^{NS}	24.62 ± 1.10 ^{NS}
5	MCV(famato litre)	54.82 ± 6.87	58.03 ± 2.82 ^{NS}	52.52 ± 4.52 ^{NS}	57.79 ± 1.68 ^{NS}
6	MCH(pico gram)	31.65 ± 2.20	34.55 ± 1.94 ^{NS}	32.17 ± 2.06 ^{NS}	33.53 ± 2.49 ^{NS}
7	MCHC (%)	57.73 ± 2.88	59.45 ± 1.90 ^{NS}	61.25 ± 2.36 ^{NS}	58.06 ± 3.65 ^{NS}

Values are expressed as Mean ± Standard deviation for six rats in each group. Data were analysed by one-way ANOVA followed by post-hoc Bonferroni test. Statistically significant variation was derived by comparing Group I versus Group II, Group III, and Group IV, NS = Non-significant ($P < 0.05$).

Table 6
Effect of EEEA on Serum Biochemical Parameters in Acute toxicity study.

S.No	Assays	Group I (Control Rats)	Group II (EEEA Treated Rats)
1.	ALT (IU/L).	29.59 ± 2.27	30.18 ± 2.97 ^{NS}
2.	AST (IU/L).	52.47 ± 2.59	56.12 ± 3.52^{NS}
3.	ALP(IU/L)	52.84 ± 3.18	58.63 ± 4.43*
4.	Bilirubin (mg/dl)	0.72 ± 0.05	0.76 ± 0.05^{NS}
5.	Protein (gm/dl)	7.98 ± 0.71	6.67 ± 0.69$^{\#}$
6.	Albumin (gm/dl)	4.47 ± 0.60	3.56 ± 0.50$^{\$}$
7.	Globulin (gm/dl)	3.51 ± 0.35	3.11 ± 0.41^{NS}
8.	A/G ratio	1.27 ± 0.22	1.15 ± 0.21^{NS}
9.	Urea (mg/dl)	28.31 ± 2.16	30.47 ± 2.00^{NS}
10.	Creatinine (mg/dl)	0.61 ± 0.09	0.58 ± 0.10^{NS}
11.	Sodium (Meq/L)	152.97 ± 9.78	150.26 ± 10.05^{NS}
12.	Potassium (Meq/L)	4.16 ± 0.76	4.13 ± 0.86^{NS}
13.	Triglycerides (mg/dl)	113.94 ± 7.01	115.29 ± 8.43^{NS}
14.	Total cholesterol (mg/dl)	93.27 ± 6.96	96.61 ± 6.01^{NS}
15.	HDL (mg/dl)	33.12 ± 2.97	35.14 ± 2.89^{NS}
16.	LDL (mg/dl)	37.35 ± 6.66	38.01 ± 7.49^{NS}

Values are expressed as Mean ± Standard Deviation for six rats in each group. Data were analysed by Independent samples *t*-test. Statistically significant analysis by two tailed variation, $^{*}P = 0.027$, $\#P = 0.009$, $\$P = 0.018$ and NS = Non-significant ($P < 0.05$).

3.4.2. Sub-acute toxicity

Clinical biochemistry is mainly performed to evaluate the effect of EEEA on hepatic and renal functions as well as on Lipid profiles. Liver function tests are carried out to identify any inflammation or injury to the liver and also to check the working condition of the liver. The enzymes AST, ALT, and ALP play an essential role in the function of liver and used as biomarkers for predicting possible toxicity (Sangeetha et al., 2013). No significant ($P < 0.05$) increase in serum AST, ALT, ALP and total bilirubin and no significant ($P < 0.05$) decrease in total serum protein, albumin of EEEA treated Groups II - Group IV were observed when compared with the control Group I rats (Table 7). Hence ethanolic extract of *E.acoroides* is non-toxic to hepatocytes.

Metabolic waste products are excreted by kidneys and regulate the concentration of the serum in different substances. The urea and creatinine are important biomarkers of renal toxicity (Raina et al., 2015). During kidney damage or inflammation, urea and creatinine become abnormal at certain stages and it is based on the degree of severity of the problem. Urea and Creatinine levels will increase, if the function of kidney is abnormal (Wang et al., 2006). Thus the present study reveals the normal levels of serum Urea, Creatinine, Sodium, and Potassium, indicate that EEEA did not interfere with renal function, and the renal integrity was preserved (Table 7). There was no significant difference in serum total Cholesterol, Triglycerides, HDL, and LDL in EEEA treated rats (Group II– Group IV) when compared with the control Group I rats (Table 7). Hence, the present data clearly showed that the treatment with EEEA did not produce any toxic biochemical effects in renal and lipid profile of rats.

3.5. Effect of EEEA on MDA and Antioxidant enzymes

3.5.1. Acute toxicity study

MDA levels in the biological system can be utilized as an imperative indicator of lipid peroxidation *in vitro* and *in vivo* analysis for several diseases (Ravindran et al., 2012). Test animals (Group II) administered 2000 mg/kg EEEA extract showed slight decrease in the concentration of MDA but not statistically significant ($P < 0.05$) when compared with the Control Group I rats (Table 8). The detrimental decrease of SOD may result in the damage to the structure of cell which causes oxidative stress (Qairunnisa et al., 2014). The role of SOD is to convert the superoxide radicals into hydrogen peroxide which is useful in the ROS scavenging reaction by the enzymes CAT and GPx. CAT and GPx combined to form the hydrogen peroxide. The decline in Catalase and GPx can be accredited to ineffective scavenging activity of H_2O_2 resulting in increasing levels of H_2O_2, which can react with oxygen to form the OH radicals which increases the LPO. NADPH is required for the inactive form of Catalase (Packer et al., 1997). There were no significant changes in the levels of antioxidant enzymes in the EEEA treated Group II rats when compared with the Control Group I rats depicted in Table 8. Ascorbic Acid is a water-soluble vitamin, which can decline the amount of free radicals through its antioxidant properties (Sutcu et al., 2006). Previous studies confirmed that Vitamin C reduced MDA levels and lipid peroxidation, and increased glutathione content in rat kidney (Abbasnejad et al., 2009). Also, simultaneous use of Vitamin E and C will significantly contribute to the improvement of lipid peroxidation and oxidative stress induced by diazinon in heart (Akturk et al., 2006), erythrocytes and brain tissues (Sutcu et al., 2007). Hence the non-enzymatic antioxidants did not show any detrimental changes in the EEEA treated Group II rats when compared with the Control Group I rats shown in Table 8. This proves that EEEA did not interfere with the activities of antioxidants and their mechanism of action and found to be safe.

3.5.2. Sub-acute toxicity

There is growing evidence to support a link between increased levels of ROS/RNS and deteriorated activities of enzymatic and non-enzymatic antioxidants in various diseases (Vaghasiya et al., 2011). Lipid peroxidation plays an important role in carcinogenesis (Banakar et al., 2004), is the most studied biologically relevant free radical chain reaction and measured as Malonaldehyde (MDA). SOD is the first line of defense in the antioxidant system against the oxidative damage mediated by superoxide radicals (Oberley and Oberley, 1986). Antioxidant defense molecules other than glutathione and glutathione related enzymes include SOD and CAT enzymes capable of removing or neutralizing free radicals. SOD is a metalloprotein, an enzyme of prime importance as it catalyses the dismutation of superoxide radicals into molecular oxygen (McCord and Fridovich, 1988). Further, CAT and GPx efficiently converted the H_2O_2 into FLO. SOD and CAT activities have played a very important role in the tumorigenesis in various experimental models (Jeon et al., 2007). Vitamin C is available in plenty of food products and can easily get the antitoxic property by daily

Table 7
Effect of EEEA on Serum Biochemical Parameters in Sub-acute toxicity study.

S.No	Assays	Group I (Control)	Group II (100 mg/kg/b.wt)	Group III (200 mg/kg/b.wt)	Group IV (400 mg/kg/b.wt)
1	ALT (IU/L).	28.51 ± 1.72	30.91 ± 1.49^{NS}	31.73 ± 2.26 ^{NS}	31.26 ± 2.96^{NS}
2	AST (IU/L).	58.69 ± 3.30	56.55 ± 3.27 ^{NS}	60.28 ± 3.32 ^{NS}	58.01 ± 3.59 ^{NS}
3	ALP(IU/L)	46.98 ± 2.62	45.90 ± 5.59 ^{NS}	51.53 ± 4.46 ^{NS}	49.38 ± 1.77 ^{NS}
4	Bilirubin (mg/dl)	0.82 ± 0.03	0.79 ± 0.07 ^{NS}	0.84 ± 0.08 ^{NS}	0.78 ± 0.04 ^{NS}
5	Protein (gm/dl)	8.03 ± 0.64	7.71 ± 0.47 ^{NS}	8.25 ± 0.47^{NS}	7.83 ± 0.43 ^{NS}
6	Albumin (gm/dl)	4.57 ± 0.72	4.02 ± 0.45 ^{NS}	4.92 ± 0.67 ^{NS}	4.44 ± 0.45 ^{NS}
7	Globulin (gm/dl)	3.46 ± 0.36	3.69 ± 0.13 ^{NS}	3.33 ± 0.52 ^{NS}	3.43 ± 0.33 ^{NS}
8	A/G ratio	1.32 ± 0.16	1.08 ± 0.08 ^{NS}	1.47 ± 0.28 ^{NS}	1.29 ± 0.18 ^{NS}
9	Urea (mg/dl)	26.90 ± 3.67	28.50 ± 2.93 ^{NS}	29.02 ± 2.42 ^{NS}	30.74 ± 1.60 ^{NS}
10	Creatinine (mg/dl)	0.69 ± 0.04	0.76 ± 0.14 ^{NS}	0.65 ± 0.02 ^{NS}	0.66 ± 0.09 ^{NS}
11	Sodium (Meq/L)	156.70 ± 12.29	156.47 ± 8.18 ^{NS}	157.30 ± 7.00 ^{NS}	162.90 ± 6.69 ^{NS}
12	Potassium (Meq/L)	4.74 ± 0.37	4.86 ± 0.42 ^{NS}	5.22 ± 0.82 ^{NS}	5.30 ± 0.42 ^{NS}
13	Triglycerides (mg/dl)	120.34 ± 9.77	120.80 ± 8.69 ^{NS}	117.90 ± 2.10 ^{NS}	121.30 ± 7.76 ^{NS}
14	Total cholesterol (mg/dl)	89.11 ± 4.84	91.02 ± 1.23 ^{NS}	89.50 ± 5.10 ^{NS}	94.16 ± 3.45 ^{NS}
15	HDL (mg/dl)	28.83 ± 4.06	30.46 ± 2.47 ^{NS}	32.29 ± 2.88 ^{NS}	33.46 ± 2.23 ^{NS}
16	LDL (mg/dl)	60.06 ± 1.68	60.24 ± 0.12 ^{NS}	60.69 ± 0.33 ^{NS}	60.60 ± 0.30 ^{NS}

Values are expressed as Mean ± Standard Deviation for six rats in each group. Data were analysed by one-way ANOVA followed by post-hoc Bonferroni test. Statistically significant variation was derived by comparing Group I versus Group II, Group III, and Group IV, NS = Non-significant ($P < 0.05$).

Table 8
Effect of EEEA on MDA and Antioxidant enzymes in Acute toxicity study.

S.No	Assays	Group I (Control Rats)	Group II (EEEA Treated Rats)
1	MDA (nmol of MDA formed/L)	7.49 ± 0.92	7.21 ± 1.01^{NS}
2	SOD (U/ml)	4.51 ± 0.52	4.33 ± 0.52^{NS}
3	Catalase (U/ml)	8.41 ± 0.71	7.78 ± 0.86^{NS}
3	GPx (U/ml)	8.27 ± 0.86	7.35 ± 0.79^{NS}
4	GSH (mg/dl)	6.11 ± 0.86	6.88 ± 0.82^{NS}
5	Vitamin C (μg/dl)	4.08 ± 0.65	4.41 ± 0.38^{NS}
6	Vitamin E (μg/dl)	2.12 ± 0.27	2.37 ± 0.18^{NS}

Values are expressed as Mean ± Standard Deviation for six rats in each group. Data were analysed by Independent samples *t*-test. Statistically significant analysis by two tailed variation, $^{*}P = 0.018$, $\#P = 0.032$ and NS = Non-significant ($P < 0.05$).

consumption. Vitamin E and Vitamin C should be a potent antioxidant which induces the LPO by inhibiting NO (Siu et al., 1999). As discussed above, in our present study, there were no significant ($P < 0.05$) changes in the serum antioxidants in the EEEA treated Groups II–Group IV rats when compared with the Control Group I rats (Table 9).

3.6. Gross observation and Organ weight

3.6.1. Acute toxicity

Liver of control rats had a normal morphological structure with a characteristic pattern of normal shape. When compared to the control Group I rats, the EEEA treated Group II rats shows no major morphological changes in the liver. Both the groups of liver show externally the normal brownish-red color. Kidneys of control Group I rats had a regular morphology structure with a typical pattern of bean shaped smooth, reddish-brown color and the EEEA treated Group II also shows the kidneys with normal morphological changes were found, when compared with the control group. Organ weight changes have long been accepted as a sensitive indicator of chemically induced changes to organs and in toxicological experiments, comparison of organ weights between control and treated groups have conventionally been used to predict toxic effect of a test material (Pfeiffer, 1968). Weight of the organ and the biochemical parameters are the important index for the toxicity of drug effects (Vaghasiya et al., 2011). As Pfeiffer (1968) stated, the EEEA treated Groups II rats showed no significant differences ($P < 0.05$) in the organ weight such as kidney, liver, and spleen compared with the control Group I rats depicted in Table 10. The present study reveals that EEEA does not induce any toxic effects in the organ weights of rats.

Table 10
Effect of EEEA on liver, kidney and spleen weight in Acute toxicity study.

S.No	Organ (s)	Group I (Control Rats)	Group II (EEEA Treated Rats)
1	Liver (g)	5.74 ± 0.55	6.94 ± 0.88 *
2	Kidney (g)	1.23 ± 0.05	1.57 ± 0.33 $^{\#}$
3	Spleen (g)	0.88 ± 0.07	0.92 ± 0.05 ^{NS}

Values are expressed as Mean ± Standard Deviation for six rats in each group. Data were analysed by Independent samples *t*-test. Statistically significant analysis by two tailed variation, $^{*}P = 0.018$, $\#P = 0.032$ and NS = Non-significant ($P < 0.05$).

3.6.2. Sub-acute toxicity

"Organ weight is an index of swelling, atrophy or hypertrophy" (Amresh et al., 2008). The adverse toxic effects are reflected in the

Table 9
Effect of EEEA on MDA and Antioxidant enzymes in Sub-acute toxicity study.

S.No	Assays	Group I Normal	Group II (100 mg/kg/b.wt)	Group III (200 mg/kg/b.wt)	Group IV (400 mg/kg/b.wt)
1	MDA (nmol of MDA formed/L)	8.01 ± 0.62	7.79 ± 0.43 ^{NS}	7.82 ± 0.18 ^{NS}	8.09 ± 0.31 ^{NS}
2	SOD (U/ml)	5.16 ± 0.47	4.69 ± 0.37 ^{NS}	5.42 ± 0.50 ^{NS}	4.85 ± 0.29 ^{NS}
3	Catalase (U/ml)	7.62 ± 7.79	7.79 ± 0.52 ^{NS}	8.40 ± 0.37 ^{NS}	8.16 ± 0.69 ^{NS}
3	GPx (U/ml)	9.26 ± 0.47	8.76 ± 0.40 ^{NS}	9.65 ± 0.62 ^{NS}	9.04 ± 0.46 ^{NS}
4	GSH (mg/dl)	5.81 ± 0.67	5.96 ± 0.29 ^{NS}	6.22 ± 0.52 ^{NS}	6.12 ± 0.35 ^{NS}
5	Vitamin C (μg/dl)	4.18 ± 0.21	3.93 ± 0.24 ^{NS}	4.61 ± 0.25 *	4.27 ± 0.22 ^{NS}
6	Vitamin E (μg/dl)	3.27 ± 0.12	2.73 ± 0.48 ^{NS}	3.47 ± 0.49 ^{NS}	3.18 ± 0.67 ^{NS}

Values are expressed as Mean ± Standard Deviation for six rats in each group. Data were analysed by one-way ANOVA followed by post-hoc Bonferroni test. Statistically significant variation was derived by comparing Group I versus Group II, Group III, and Group IV, $^{*}P = 0.031$ and NS = Non-significant ($P < 0.05$).

Table 11
Effect of EEEA on liver, kidney and spleen weight in Sub-acute toxicity study.

S.No	Organ (s)	Group I (Control)	Group II (100 mg/kg/b.wt)	Group III (200 mg/kg/b.wt)	Group IV (400 mg/kg/b.wt)
1	Liver (g)	4.35 ± 0.30	4.51 ± 0.48 ^{NS}	4.32 ± 0.29 ^{NS}	4.41 ± 0.21 ^{NS}
2	Kidney (g)	1.49 ± 0.23	1.57 ± 0.44 ^{NS}	1.56 ± 0.24 ^{NS}	1.58 ± 0.17 ^{NS}
3	Spleen (g)	0.89 ± 0.07	0.99 ± 0.10 ^{NS}	0.89 ± 0.07 ^{NS}	0.93 ± 0.05 ^{NS}

Values are expressed as Mean ± Standard Deviation for six rats in each group. Data were analysed by one-way ANOVA followed by post-hoc Bonferroni test. Statistically significant variation was derived by comparing Group I versus Group II, Group III, and Group IV, NS = Non-significant ($P < 0.05$).

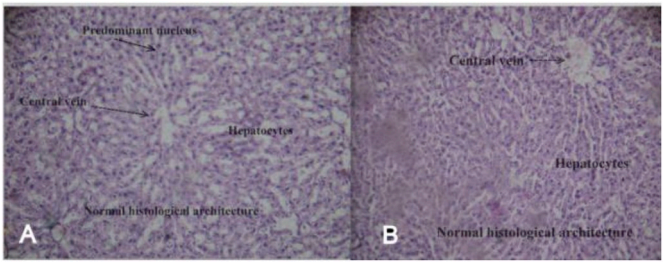

Fig. 1. Photomicrographs of liver sections stained with hematoxylin and eosin (40X) from Group I control rats (A) and Group II EEEA treated rats (B). The liver of control rats (A) rats showed normal histological architecture with predominant nucleus and central vein. Treated rats (B) shows the hepatocytes, central vein and visible nucleus similar to Control rats.

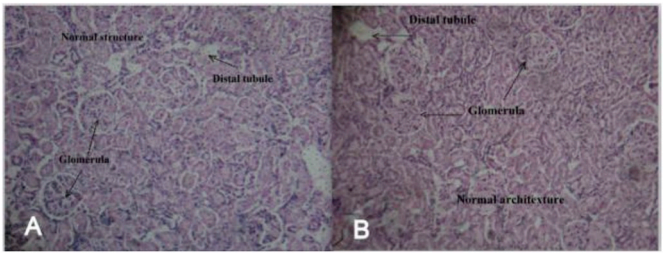

Fig. 2. Photomicrographs of kidney sections stained with Hematoxylin and eosin (40X) from Group I control rats (A) and Group II EEEA treated rats (B). The kidney of control rats (A) and treated rats (B) showed normal histological architecture. The renal corpuscle is a rounded or irregular structure which forms the glomerule that enveloped by Bowman's capsule.

metabolism of the structure and function of imperative organs like kidney, liver, and spleen (Dybing et al., 2002). There were no significant differences in relative organ weight between control rats and EEEA treated rats at a dose of 100 mg/kg b.w., 200 mg/kg b.w and 400 mg/kg b.w. The comparative organ weight of control rats (Group I) and EEEA treated rats (Group II-IV) were statistically insignificant. This result proves that the imperative organs such as kidney, liver, and spleen were not affected throughout the experiment (Table 11).

3.7. Effect of EEEA on histopathology

3.7.1. Acute toxicity

Histopathological examination was carried out to determine the biological response factors. The assessment of histopathology of liver and kidney in control group and EEEA treated animals showed normal architecture implied no damaging changes and morphological alterations (Fig. 1 and Fig. 2). The liver of control group and EEEA treated group showed normal histological architecture with predominant nucleus and central vein. EEEA treated rats shows the hepatocytes, central vein and visible nucleus similar to control group (Fig. 1). The kidney of control group and EEEA treated group showed normal histological architecture. A glomerule is formed by the rounded or irregular structure of renal and enveloped by the Bowman's capsule (Fig. 2). This indicates that the EEEA did not produce any toxicological effects on the rats.

3.7.2. Sub-acute toxicity

Histological examination was done to exemplify the toxic effects of the drugs in the organs. Histological analysis of kidney, liver, and spleen were done in male wistar rats subjected to sub-acute treatment with EEEA in different doses (100 mg/kg/b.w, 200 mg/kg/b.w and 400 mg/kg/b.w.) and in the control Group I rats. As represented in

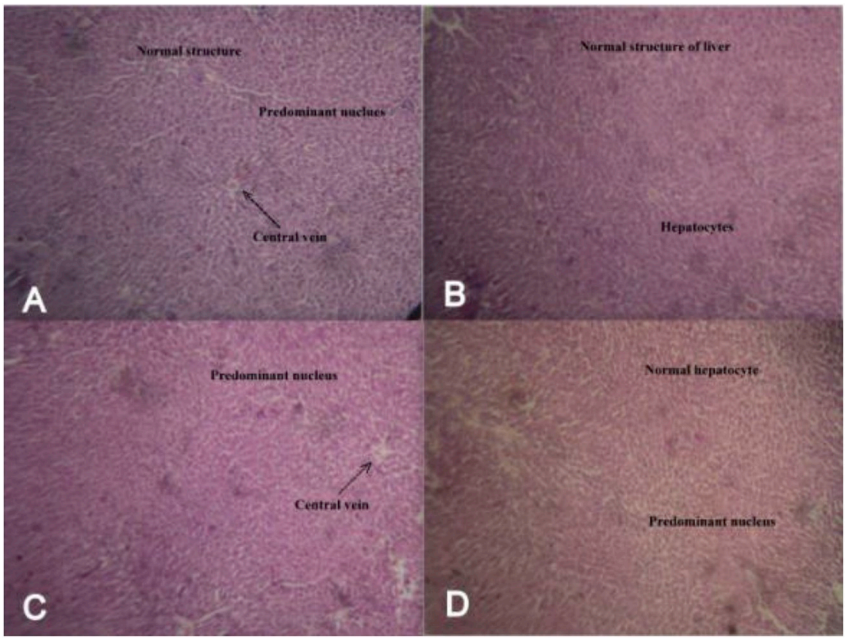

Fig. 3. Photomicrographs of liver sections stained with Hematoxylin and eosin (40X) of Group I control rats (A) and Group II treated with 100 mg/kg (B), Group III treated with 200 mg/kg (C) and Group IV treated with 400 mg/kg (D). Rats were observed that the liver cells are arranged into lobules in both control rats (A) and treated rats (B–D) slides. Liver cells hepatocytes are flat and arranged. A discontinuous layer of cells lines the sinusoids. Central vein is lined by epithelial cells predominant nucleus.

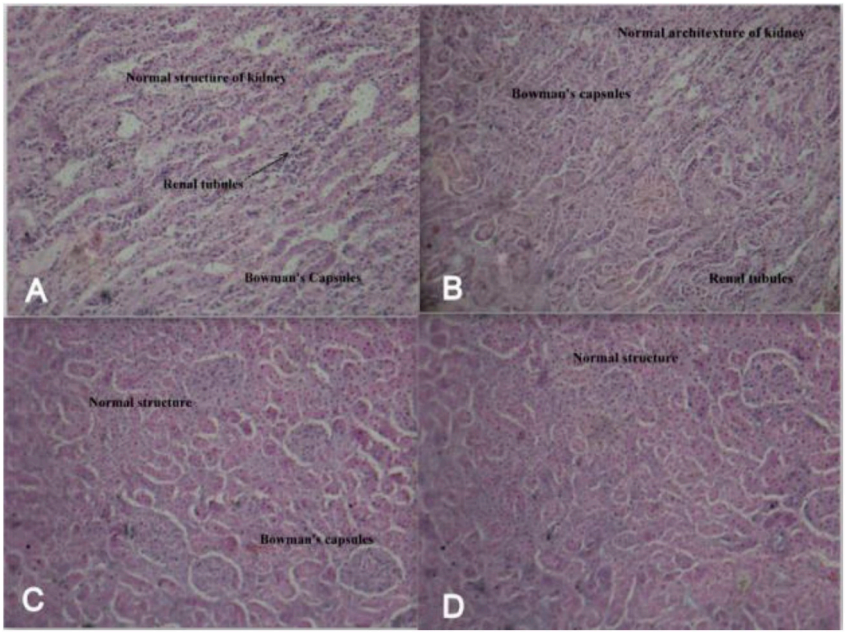

Fig. 4. Photomicrographs of kidney sections stained with Hematoxylin and eosin (40X) of Group I control (A) rats and Group II treated with 100 mg/kg (B), Group III treated with 200 mg/kg (C) and Group IV treated with 400 mg/kg (D).The Renal corpuscles in the center display a slight shrinkage artefact and thus clearly demonstrate Bowman's space. The renal corpuscles are surrounded by cross sections of proximal convulated tubules, distal convulated tubule and macula densa.

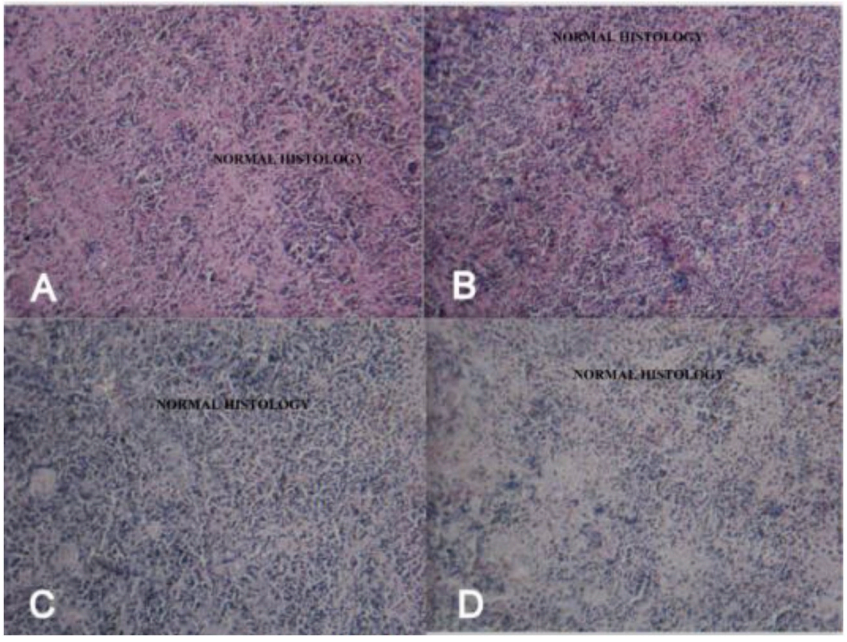

Fig. 5. Photomicrographs of spleen sections stained with Hematoxylin and eosin (40X) of Group I control (A) rats and Group II treated with 100 mg/kg (B), Group III treated with 200 mg/kg (C) and Group IV treated with 400 mg/kg (D). Rats were subdivided into red pulp and white pulp. White pulp is arranged as a cylindrical sheath of lymphocytes. It surrounds a blood vessel known as central artery as shown in both control and treated spleen. Red pulp consists of sinusoids. While treated spleen contains an area of germinal center.

Figs. 3–5 histological studies revealed no abnormalities in liver, kidney, and spleen tissue in EEEA treated rats. Liver of control Group I rats (Fig. 3A) and EEEA treated rats from Group II– Group IV (Fig. 3B, C and 3D) were observed that the liver cells are arranged into lobules. Liver cells hepatocytes are flat and arranged. A discontinuous layer of cells lines the sinusoids. Central vein is lined by epithelial cells predominant nucleus. Similar type of observation was also seen by Bello et al. (2016) in rat liver. In kidneys, the Renal corpuscle in the center displays a slight shrinkage artefact and thus evidently exhibits Bowman's space both in control Group I and EEEA treated rats. The renal corpuscles are bounded by cross sections of proximal convulated tubules, distal convulated tubule and macula densa shown in Fig. 4 which are comparable with the study made by Nabukenya et al. (2014). Spleen is sub-divided into red and white pulp (Control Group I rats and EEEA treated rats). White pulp is arranged as a cylindrical sheath of lymphocytes. It surrounds a blood vessel known as central artery as shown in both control Group I rats (Fig. 5A) and EEEA treated spleen of rats (Fig. 5B, C and 5D). Red pulp consists of sinusoids while treated spleen contains an area of germinal center. These observations agreed with that of Ping et al. (2013) in rat model that have been treated with *Euphorbia hirta*. Thus, histological examination reveals that the EEEA did not have any major effects in the tissue morphology and these examinations supported the biochemical parameter results mentioned.

4. Conclusion

The present investigation showed that the ethanolic extract of the whole parts of *Enhalus acoroides* did not present significant toxicity when administered in a single highest dose, being considered safe by the OECD. However, when various doses were given for 28 days, were found some changes in biochemical, haematological, and histological parameters, but not statistically significant. In conclusion, the current

investigation demonstrates that at doses consumed in the traditional medicine, the ethanolic extract of *Enhalus acoroides* considered as relatively protective, as it did not cause any mortality and not produced severe toxic effects on organs in the body, biochemical parameters and hematology of the animals during both the acute and sub-acute periods of study. Based on this scientific appraisal, it can be concluded that the extracts of the EEEA have a high margin of safety as it did not induce any toxicological effects and can be further used for pre-clinical trials. To our knowledge this is the first study of renal, hepatic, antioxidants, haematopoietic study and histopathologic biomarkers in the seagrass *Enhalus acoroides* using wistar albino rats in acute and sub-acute toxicity study.

Declaration of interest

The authors declare that there is no conflict of interest.

Submission declaration

The present work has not been published previously in any form and not under consideration for publication elsewhere.

Authors contributions

- The author P.Amudha executed the acute and sub-acute toxicity experiment, euthanasia of the animals and writing of the manuscript.
- The author Dr.V.Vanitha designed the protocol, assistance in animal euthanasia and supervised the research work.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bcab.2019.101082.

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