Acute and Sub-chronic Toxicity Evaluations of Mallotus repandus Stem Methanol Extract in Female Sprague-Dawley Rats

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Abstract

Background

Mallotus repandus (*M. repandus*), a well-known medicinal plant, is traditionally used around the globe to treat or prevent numerous diseases and/or disorders such as inflammation, hepatic disorder, cirrhosis, rheumatic arthritis, tumor, and snake-bite. Some common names for it include Bon natai, Jhante, or Gunti. Some potential bioactive compounds from this plant have been identified, and hepatoprotective activities of the stem part have been also investigated by standard methods. However, there is no strong evidence about its toxicity profile that was performed in a systematic study using substantial animal models. We designed our current study with Swiss albino mice and Sprague-Dawley rat model using biochemical parameters, histological, and hematological evaluation to assess the toxic effects of *M. repandus* methanol stem extract (MMSE).

Methods

In response to determining the acute toxicity of *M. repandus* extract, we assigned Swiss albino mice to treat with *M. repandus* extract at the administration concentrations of 500, 1000, 2000, and 4000 mg/kg for 3 successive days. Within these 3 days, we checked out the behavioral changes, any responses to the acute toxicity, and the mortality of the selected mice at every moment. Conversely, to evaluate its sub-chronic toxicity of it, rats were allocated into four different groups to receive weight-based oral doses of MMSE (500, 1000, and 2000 mg/kg; once daily) for 28 days. Biochemical, hematological, and histological investigations were conducted after the treatment period.

Results

Findings disclosed that there were no substantial alterations in comparative organ weight, the water content percentage, and body weight. Moreover, results indicated that the MMSE provides no tenacious effects on hematological parameters, serum renal markers, and electrolytes in experimental rats. However, a reduction in triglyceride, low-density lipoprotein, atherogenic indices, and total cholesterol was observed. Similarly, a higher dose (2000 mg/kg) considerably decreased the hepatic marker enzymes such as alkaline phosphatase (ALP), lactic dehydrogenase (LDH), glycated hemoglobin, and alanine aminotransferase (ALT), and serum glucose level than the control group. Interestingly, in the histological investigation, necrotic and inflammatory features were also not found in normal cellular structures.

Conclusion

Taken all together, results suggest that MMSE is non-toxic, and bears cardio-protective, glucose-lowering capacity, and hepato-protective characteristics.

1. Introduction

Today, medicinal plants and products derived from them perform a significant role in health care services along with considerable quantities of medicinal compounds throughout the world in both developed and underdeveloped countries. It has been investigated that most of the people (about 80%) in developing and under-developing nations trust in the plant or animal-based traditional medications for primary health care necessities due to effectiveness, availability, fewer cost, and lesser side effects [1]. Medicinal plants were long been regarded as a huge repository of potential phytochemicals, featuring the primary emphasis on treatment discovery for serious ailments globally. These plants produce bioactive compounds that may be valuable to humans. Yet, some compounds of different medicinal plants are also well-known to be significantly toxic thanks to their carcinogenicity, toxicity, and teratogenicity [2]. With the frequent use of herbs and medicinal plants for health purposes, rigorous scientific investigations are needed to discover the efficacy and potential toxicity of these therapeutic plants. Generally, people perceive that natural compounds are safe and non-toxic with fewer side effects; however, many of these assumptions were proven to be false [3]. In this context, some earlier reports revealed that administration of extracts of medicinal plants deprived of their assessing potency, efficacy, efficiency, safety, and tolerability may lead to unanticipated incidents (i.e., toxic effects) that possibly affect the homeostasis of several organ systems [4, 5]. Therefore, appropriate scientific confirmation is essential to inaugurate the safety, potency, efficiency, and tolerability of plant extracts before using them for therapeutic purposes.

Mallotus repandus (Willd.), locally called ‘Bon natai’, ‘Jhante’, or ‘Gunti’, belongs to the family Euphorbiaceae and largely grows in different regions (Sundarbans, Savar, and Sylhet) of Bangladesh [6]. The genus Mallotus, containing approximately 150 species, is also dispersed in subtropical and tropical areas around the globe, especially in the Pacific-Ocean Archipelago, East, and North of Australia, and therefore in Asia. Most of the parts (roots, bark, stem bark, aerials, seeds, and leaves, as well as the whole plant of Mallotus species, were subjects of research. Based on the traditionally used treatment, this plant is functional to treat or prevent different diseases and/or disorders such as hepatic disorder, inflammation, liver cirrhosis, snake bite, tumor, and rheumatic arthritis [7]. Researchers have succeeded in the isolation of numerous effective bioactive chemicals from stem extract of *M. repandus* including amyrin, bergenin, furosin, mallotinic acid, lupeol, and ursolic acid [8, 9]. Rivière et al. (2010) validated that the existence of flavonoids and phenolic phytoconstituents in the ethyl-acetate extract of the stem of *M. repandus* displayed remarkable antioxidant activity [10]. Similarly, Lin and co-researchers reported the significant hepatoprotective effect of *M. repandus* stem (aqueous extract) against rats with tetrachloride (CCl_4)-treated hepatotoxicity [11]. Additionally, research findings by Mondal et al. (2020) revealed that the *M. repandus* stem (ethyl acetate extract) also showed hepatoprotective and antioxidant activity in contradiction to the rats with D-Galactosamine-administered hepatotoxicity [8]. However, there appears to be a lack of evidence related to the safety and the toxicological profile of the extract of *M. Repandus* stem.

By following the previous discussion, owing to the safety, bioactivity, and ethnopharmacological profile of *M. repandus*, and to avoid undesirable side effects, the goal of our current experiment was to examine the

conceivable toxicological effect of this plant. For this purpose, we allocated female Sprague-Dawley rats to appraise the acute and sub-chronic toxicity of MMSE expending with biochemical, histological, and hematological parameters.

2. Materials And Methods

2.1. Chemicals and reagents

Methanol for preparing plant extract was bought from a life science and biotechnology company namely Sigma Aldrich (St Louis, Missouri), and was considered as received. Ketamine was bought from a renowned conglomerate Advanced Chemical Industries (ACI) Pharmaceuticals Ltd. in Bangladesh. Contacting Human Laboratories, Germany, we have obtained kits for clinical investigations.

2.2. Plant material

In the course of the dry season (from November 2016 to December 2016), the fresh stem part of *M. repandus* was collected for extraction from a local market located at Savar, Dhaka, Bangladesh. From Bangladesh National Herbarium, the plant was authenticated and recognized, where an authenticated specimen (DACB accession no. 38733) of this plant was preserved.

2.3. Extract preparation

The stems that were previously purchased were taken to clean and wash properly with tap water. After that, fan aeration was applied to partially dry the cleaned stems, and then the complete drying was performed by using an oven at 40°C for 2 days. Subsequently, the powdered plant substantial (around 1500 g) was dissolved in 3000 mL ethyl acetate and then exposed to a laboratory apparatus Soxhlet extractor at 65°C for soxhlet extraction. It was considered to the completion of the extraction when applied solvent turned colorless in the Soxhlet apparatus. After filtering the solution with a fresh cotton bed, the solution was dried (40 ± 2°C) and concentrated in the gummy form with the aid of a rotary evaporator. Thus, obtained extracts were taken in the Petri dish at a suitable temperature (2 – 8°C) and then were used to screen for pharmacological activities.

2.4. Animals for in vivo experiments

Through connecting with the Ayurvedic Medicine and Education (FRAME) Laboratory, Department of Pharmacy, Jahangirnagar University, Bangladesh, Swiss albino mice weighing 25 – 30 g (male) and Sprague – Dawley rats weighing 150 – 160 g (female) were collected and applied them for our proposed investigation. These animals were kept in a comfortable environment of 22 ± 2°C temperature and relative humidity (50 ± 5%), with a 12 h light/dark cycle respectively, and were served with water ad libitum and an experimental pellet diet. By following the approved guidelines provided by the Bangladesh Association for Laboratory Animal Science, all experiments were conducted. On the other hand, the Biosafety, Biosecurity, and Ethical Committee of the Faculty of Biological Sciences of Jahangirnagar University, Savar, Dhaka, Bangladesh also approved our experimental procedures [Reg. No. BBECJU/M2013(20)].

2.5. Acute toxicity study

Based on the guidelines approved by the Organization for Economic Cooperation and Development (OECD), the acute toxicity investigation of this extract was done. For this purpose, a total of 40 Swiss albino mice (female, BW: 25–30 g) were assigned to receive methanolic *M. repandus* stem extract at a dose concentration of 500, 1000, 2000, and 4000 mg/kg. In response to this study, these mice were divided into a total of four experimental groups containing 10 mice in each group and were treated with the selected extracts with the help of a stomach tube [12]. After that, we observed the animals for the first 30 minutes and the 1st hour, then 2nd, 3rd, and 4th hours of post-administration on the first day to determine any signs such as behavioral changes (agitation, ataxia, asphyxia, aggression, catatonia, convulsions, fasciculation, paralysis, prostration, somnolence and sedation, tremors, unusual vocalization, and unusual locomotion), signs of acute toxicity, and toxicity responses (changes in skin and fur texture, cardiovascular outcomes, and respiratory pattern), and finally mortality. After the first day, the observation was carried out for the next 3 days (once daily) following MMSE administration.

2.6. Experimental design for sub-chronic toxicity

Based on guideline 407 described by the OECD, the sub-chronic toxicity evaluation of MMSE was carried out by a slight alteration of this guideline [13, 14]. Animals (n = 24) were allocated into four groups containing six animals in each. Here, drinking water was used as a solvent for preparing desired dose concentrations of MMSE.

Group I (Normal Control): Animals orally received drinking water *ad libitum* and a normal diet.

Group II (MMSE 500): Animals orally received MMSE (500 mg/kg) for 28 days. (Drinking water *ad libitum* and standard laboratory diet were served to all animals).

Group III (MMSE 1000): Animals orally received MMSE (1000 mg/kg) for 28 days.

Group IV (MMSE 2000): Animals orally received MMSE (1000 mg/kg) for 28 days.

Here, drinking water *ad libitum* and a standard laboratory diet were served to all animals. Animals were observed daily for determining general morphological changes, drinking and feeding habits, and behavioral changes, and were also weighed weekly. After completing the experimental course, and after 18 hours of fasting, all animals were anesthetized with ketamine (500 mg/kg body weight, i.p.) [15]. Afterward, blood samples from the post vena cava of these anesthetized animals were taken for hematological analysis into Ethylene diamine tetra-acetic acid (EDTA) sample tubes. Conversely, remaining the blood samples were collected into plain sample tubes at environmental temperature for 30 min to clot the serum before centrifugation at 4000g for 10 min with the aid of a centrifuge (MSE Minor, England) for biochemical analysis. The supernatant was collected via a dry Pasteur pipette and was then frozen (− 20°C) for not more than 24 hours before analysis [1].

2.7. Measurements relative organ weight profile and percentage of water content

All the rats were sacrificed instantly. The following body organs: spleen, kidney, liver, fallopian tubes, heart, lung, and ovary were excised immediately from sacrificed rats and were then weighed these excised organs for calculating relative organ weight. The calculation of the relative organ weight was done according to the following mathematical equation [16]:

$$\text{Relative organ weight}(\%) = \frac{\text{wet organ weight}}{\text{body weight}} \times 1000$$

The water content percentage was calculated by subtracting each organs' dry weight from the respective each wet organs' weight [17].

2.8. Hematological analysis

Hematological parameters i.e., the hematocrit (HCT), lymphocytes, monocytes, mean corpuscular volume (MCV), hemoglobin (HGB), pro-calcitonin (PCT), platelets, mean platelet volume (MPV), platelet distribution width (PDW), neutrophil, pro-calcitonin (PCT), mean corpuscular hemoglobin (MCH), platelet larger cell ratio (P-LCR), red blood cells (RBC), platelet distribution width (PDW), and white blood cells (WBC) were measured employing an automated Sysmex KX-21 hematology analyzer (XS 1000i, Sysmex Corporation Ltd., Japan) according to the standard procedure reported by Wahed and coworkers [18].

2.9. Biochemical analysis

Biochemical parameters include alkaline phosphatase (ALP), aspartate transaminase (AST), alanine transaminase (ALT), albumin/globulin (A/G) ratio, albumin (ALB), creatinine (Crea), γ-glutamyl-transferase (GGT), high-density lipoprotein cholesterol (HDL-C), lactate dehydrogenase (LDH), triglyceride (TG), total cholesterol (TC), total protein (TP), total bilirubin (TB), calcium (Ca^{2+}), phosphate (PO_4^{3-}) ions, magnesium (Mg^{2+}), chloride (Cl^-), sodium (Na^+), potassium (K^+), blood glucose levels, total iron-binding capacity (TIBC), urea, iron (Fe), and uric acid (UA) were measured utilizing an automated chemistry analyzer (Human commercial kits and Humalyzer 3500) [1]. In addition, the glycated hemoglobin (HbA1c) test was accompanied by using an analyzer namely Bio-Rad D-10. Conversely, low-density lipoprotein (LDL) cholesterol and very-low-density lipoprotein (VLDL) cholesterol were measured based on the following formulas [19]:

VLDL cholesterol (mg/dL) = Triglyceride / 5

LDL cholesterol (mg/dL) = Total cholesterol − HDL cholesterol − (Triglyceride /5)

Additionally, atherogenic directories such as atherogenic index of plasma (AIP), atherogenic coefficient (AC), cardiac risk ratio (CRR), and Castelli's risk index-2 (CRI-2) were determined according to the following formulas [1]:

$$\text{AIP} = \log\left(\frac{\text{TG}}{\text{HDL-C}}\right)$$

$$\text{AC} = \frac{(\text{TC} - \text{HDL} - \text{C})}{\text{HDL} - \text{C}}$$

$$\text{CRR} = \frac{\text{TC}}{\text{HDL-C}}$$

$$\text{CRI-2} = \frac{\text{LDL-C}}{\text{HDL-C}}$$

2.10. Histological evaluation

After the investigational period rats were sacrificed, the following body organs: liver, kidney, spleen, fallopian tubes, heart, lung, thymuses, and ovary collected immediately, sliced into 5 μm thick slices, and washed in saline. Subsequently, the sliced sections were conserved in formalin (10%) for histopathological studies and were then embedded and processed in paraffin wax. The hematoxylin and eosin (H&E) dye were then applied in paraffinized sections for staining. Lastly, a fluorescent microscope (Olympus DP 72, 6100x) was used to image the stained slices [20].

2.11. Data analysis

All data were statistically analyzed and displayed as mean ± standard error of the mean (SEM) and statistical analyzes were done with the help of SPSS (version 16, IBM software Inc, USA) and GraphPad Prism (version 6.02; GraphPad Software Inc., San Diego, CA, USA). Data were subjected to a one-way analysis of variance (ANOVA) for significance, followed by Dunnett's multiple comparisons to analyze data sets. The values were considered statistically significant if $*p < 0.05$ and $**p < 0.01$.

3. Results

3.1. Acute toxicity

Orally administered MMSE (4000 mg/kg) caused no toxic responses. No mortality occurred throughout the observational period. Interestingly, all the animals safely survived with no side effects such as hair loss, coma or convulsion, general irritation, respiratory suffering, and agitation during the acute toxicity study. Thus, the LD50 value of *M. repandus* can be considered to be above 4000 mg/kg. As a result, MMSE can be applied in experimental models up to the 4000 mg/kg dose without any doubt.

3.2. Effects of extract on rat organs body weight, relative organ weight, and percent water content

The findings of the effects of our selected extract MMSE on the organs of experimented rats are displayed in Tables 1 and 2, and Fig. 1. Here, we especially demonstrated the effects on relative organ

weight and the water content percentage of several important organs of the tested rats. Figure 1 showed that there were no momentous alterations occurred in the experimental rats compared with the control group, but there was an insignificant rise in body weights of the investigated rats. Additionally, Tables 1 and 2 represented that there were no considerable variances that happened between the observational models for the relative organ weight and the percentage of water content.

Table 1
Effects of *M. repandus* stem extract on the relative organ weight.

Organ	Control	MSM 500	MSM 1000	MSM 2000
Heart	0.312 ± 0.01	0.310 ± 0.03	0.273 ± 0.01	0.268 ± 0.01
Kidney	0.326 ± 0.02	0.321 ± 0.03	0.334 ± 0.04	0.311 ± 0.02
Lung	0.691 ± 0.01	0.617 ± 0.03	0.698 ± 0.01	0.615 ± 0.04
Liver	4.099 ± 0.10	3.945 ± 0.18	3.932 ± 0.09	3.896 ± 0.12
Spleen	0.411 ± 0.06	0.418 ± 0.01	0.425 ± 0.04	0.431 ± 0.05
Caecum	0.343 ± 0.07	0.359 ± 0.04	0.354 ± 0.05	0.361 ± 0.05
Pancreas	0.169 ± 0.05	0.176 ± 0.03	0.165 ± 0.05	0.170 ± 0.04
Brain	0.781 ± 0.04	0.788 ± 0.03	0.795 ± 0.04	0.787 ± 0.06
Thymus	0.235 ± 0.02	0.232 ± 0.03	0.242 ± 0.01	0.254 ± 0.01
Stomach	0.628 ± 0.05	0.642 ± 0.04	0.625 ± 0.08	0.622 ± 0.09
Ovary	0.033 ± 0.02	0.032 ± 0.02	0.037 ± 0.03	0.026 ± 0.04
Fallopian tube	0.256 ± 0.01	0.234 ± 0.07	0.268 ± 0.05	0.236 ± 0.08

Experimental results are expressed as mean ± SEM (each group containing six animals) and statistical data analyzes were done based on One-way ANOVA followed by Dunnett's multiple comparison test. Compared with the control group, values considered significant if $*p < 0.05$ and $** p < 0.01$.

Table 2
Effects of *M. repandus* stem extract on the percentage of the water content of different organs.

Organ	Control	MSM 500	MSM 1000	MSM 2000
Heart	75.52 ± 3.55	78.29 ± 2.63	77.56 ± 2.62	76.55 ± 3.37
Kidney	72.87 ± 2.85	69.16 ± 3.20	68.83 ± 9.57	74.37 ± 1.66
Lung	76.46 ± 4.62	81.65 ± 4.11	75.49 ± 4.97	78.78 ± 3.81
Liver	76.08 ± 3.93	77.74 ± 2.05	74.36 ± 5.23	73.73 ± 2.28
Spleen	76.32 ± 2.95	77.97 ± 4.43	79.49 ± 1.49	72.54 ± 2.55
Caecum	70.35 ± 3.46	69.44 ± 2.33	71.64 ± 2.39	70.65 ± 3.29
Pancreas	60.20 ± 3.56	62.36 ± 2.91	64.48 ± 4.96	63.51 ± 4.67
Brain	45.52 ± 3.05	45.44 ± 2.47	48.44 ± 3.14	49.66 ± 4.63
Thymus	77.75 ± 3.17	71.53 ± 2.19	77.75 ± 2.21	78.54 ± 2.33
Stomach	65.32 ± 2.96	67.45 ± 1.50	65.83 ± 2.15	68.13 ± 3.26
Ovary	53.96 ± 2.50	55.69 ± 4.64	51.60 ± 3.48	52.24 ± 4.02
Fallopian tube	71.74 ± 3.03	74.10 ± 1.98	75.71 ± 4.68	73.51 ± 3.51

Experimental results are expressed as mean ± SEM (each group containing six animals) and statistical data analyzes were done based on one-way ANOVA followed by Dunnett's multiple comparison test. Compared with the control group, values considered significant if $*p < 0.05$ and $** p < 0.01$.

3.3. Effects of extract on hematological parameters

Results demonstrated that no substantial effects of MMSE on hematological parameters were found among the observational groups, but MMSE (2000 mg/kg) only increased the neutrophil count (Table 3).

Table 3
Effects of *M. repandus* stem extract on the hematological parameters of rats.

Hematological parameters		Groups			
		Control	**MSM 500**	**MSM 1000**	**MSM 2000**
Erythrocyte count	RBC ($\times 10^{12}$/L)	6.12 ± 0.18	6.19 ± 0.15	6.25 ± 0.28	6.03 ± 0.23
	ESR(mm)	5.90 ± 0.73	6.17 ± 0.48	5.83 ± 0.40	6.05 ± 0.79
	HGB (g/dL)	13.85 ± 2.26	13.04 ± 2.17	12.65 ± 1.42	12.89 ± 2.14
	HCT (%)	40.05 ± 1.40	39.56 ± 1.30	39.19 ± 1.38	39.78 ± 1.01
	MCV (fL)	68.58 ± 1.74	69.62 ± 3.21	68.20 ± 2.29	64.62 ± 1.07
	MCH (pg)	19.08 ± 0.63	19.57 ± 0.91	19.48 ± 1.19	19.35 ± 0.80
	MCHC (g/dL)	27.86 ± 0.24	26.73 ± 1.43	28.16 ± 0.23	28.43 ± 0.28
	RDW-CV (%)	21.33 ± 2.75	21.65 ± 1.14	20.31 ± 1.42	20.55 ± 2.12
Leukocyte count	WBC(10^9/L)	5.63 ± 0.32	5.47 ± 0.67	5.77 ± 0.14	7.74 ± 0.15
	Lymphocyte (%)	21.50 ± 2.59	20.83 ± 0.75	22.83 ± 0.87	22.50 ± 0.92
	Monocyte (%)	2.67 ± 0.33	2.50 ± 0.22	2.33 ± 0.21	2.00 ± 0.26
	Neutrophil (%)	72.33 ± 1.98	75.67 ± 2.19	74.33 ± 3.05	72.17 ± 1.38
Platelet count	Platelet (10^3/µL)	403.50 ± 63.43	303.83 ± 34.99	255.83 ± 47.51	292.83 ± 50.19
	MPV (fL)	10.36 ± 0.38	10.17 ± 0.45	10.22 ± 0.16	10.24 ± 0.27
	PDW (fL)	12.57 ± 0.85	13.55 ± 1.12	12.40 ± 0.24	13.04 ± 0.65
	PCT (µg/L)	0.63 ± 0.05	0.67 ± 0.10	0.69 ± 0.06	0.62 ± 0.04
	P-LCR (%)	18.72 ± 2.38	17.72 ± 3.75	17.37 ± 2.46	18.57 ± 2.71

Experimental results are expressed as mean ± SEM (each group containing six animals) and statistical data analyzes were done based on One-way ANOVA followed by Dunnett's multiple comparison test. Compared with the control group, values considered significant if *$p < 0.05$ and ** $p < 0.01$.

3.4. Effects of extract on biochemical parameters

Displayed in Fig. 2 are results related to the effects of oral administration of MMSE on serum hepatic marker enzymes. Investigational findings exposed no extensive changes in several biochemical parameters (such as LDH, ALT, GGT, ALP, and AST) of the investigational rats compared with the control group. Nevertheless, MMSE at a dose concentration of 2000 mg/kg significantly reduced the levels of

LDH, ALP, and ALT. Moreover, three different doses of MMSE did not cause any changes in the levels of globulin, albumin, total protein, total bilirubin, and therefore albumin/globulin (A/G) ratio that are important parameters in the evaluation of hepatic toxicity (Table 4). Furthermore, MMSE did not cause any substantial changes in the renal function biomarkers (urea, uric acid, creatinine, and blood urea nitrogen) or modify the electrolytes' levels (Ca^{2+}, $PO4_3^-$, Mg^{2+}, Cl^-, K^+, and Na^+) (Table 5). Conversely, MMSE potentially reduced the amounts of serum lipid profiles (TC, TG, and LDL-C) and atherogenic indices (CRI-2, ACC, CRR, and AIP) which are reliable indicators for the complications of cardiovascular (Fig. 3 and Table 6). In addition, the quantities of the pancreatic function markers (serum amylase and lipase) were not significantly altered by the MMSE, however, the quantities of HbA1c and blood glucose were substantially decreased by the MMSE (2000 mg/kg) (Table 7).

Table 4
Effects of *M. repandus* stem extract on the serum total globulin (GLB), albumin (ALB), total protein (TP), and bilirubin (TB) levels and the albumin/globulin (A/G) ratio.

Biochemical Parameters	Groups			
	Control	MSM 500	MSM 1000	MSM 2000
TB (mg/dL)	0.23 ± 0.016	0.26 ± 0.02	0.26 ± 0.02	0.26 ± 0.02
TP (g/L)	46.70 ± 1.24	44.50 ± 0.85	47.00 ± 1.34	48.33 ± 1.61
ALB (g/L)	28.00 ± 1.06	26.00 ± 0.97	28.33 ± 0.42	29.50 ± 0.62
GLB (g/dL)	18.00 ± 1.91	17.50 ± 0.85	19.67 ± 1.36	19.83 ± 1.94
A/G ratio	1.68 ± 0.24	1.46 ± 0.15	1.40 ± 0.07	1.49 ± 0.22

Experimental results are expressed as mean ± SEM (each group containing six animals) and statistical data analyzes were done based on one-way ANOVA followed by Dunnett's multiple comparison test. Compared with the control group, values were significant if *$p < 0.05$ and ** $p < 0.01$.

Table 5
Effects of *M. repandus* stem extract on the serum renal markers and electrolytes.

Serum Renal Markers And Electrolytes	Groups			
	Control	MSM 500	MSM 1000	MSM 2000
Urea(mmol/L)	29.05 ± 0.98	30.50 ± 2.60	29.60 ± 1.04	29.37 ± 1.45
Uric Acid (mmol/L)	1.95 ± 0.12	2.02 ± 0.06	1.92 ± 0.09	1.85 ± 0.07
Creatinine (mmol/L)	0.87 ± 0.03	0.80 ± 0.03	0.81 ± 0.003	0.92 ± 0.03
Blood Urea Nitrogen (mmol/L)	13.57 ± 0.46	13.83 ± 0.49	14.06 ± 1.22	14.71 ± 0.68
Na^+(mmol/L)	117.50 ± 4.67	115.36 ± 2.85	113.45 ± 1.44	114.00 ± 3.53
K^+(mmol/L)	6.19 ± 0.20	6.29 ± 0.34	6.28 ± 0.20	6.20 ± 0.10
Cl^-(mmol/L)	111.50 ± 3.11	112.64 ± 2.21	109.00 ± 3.42	113.38 ± 2.01
PO^{3+} (mmol/L)	4.35 ± 0.30	4.34 ± 0.26	4.27 ± 0.55	4.19 ± 0.23
Ca^{2+} (mmol/L)	2.89 ± 0.47	2.38 ± 0.20	2.51 ± 0.34	2.63 ± 0.27
Mg^{2+} (mmol/L)	0.90 ± 0.20	0.92 ± 0.10	0.95 ± 0.14	0.98 ± 0.15

Experimental results are expressed as mean ± SEM (each group containing six animals) and statistical data analyzes were done based on one-way ANOVA followed by Dunnett's multiple comparison test. Compared with the control group, values were significant if *$p < 0.05$ and ** $p < 0.01$.

Table 6
Effects of *M. repandus* stem extract on the atherogenic indices.

Atherogenic Indices	Groups			
	Control	MSM 500	MSM 1000	MSM 2000
CRR	2.02 ± 0.1	2.10 ± 0.01**	1.65 ± 0.04**	1.64 ± 0.01**
ACC	1.10 ± 0.01	1.09 ± 0.01	0.68 ± 0.02 **	0.64 ± 0.01**
AIP	0.484 ± 0.002	0.484 ± 0.001	0.486 ± 0.001	0.472 ± 0.001 *
CRI-2	0.432 ± 0.007	0.467 ± 0.010	0.065 ± 0.002**	0.053 ± 0.002**

Experimental results are expressed as mean ± SEM (each group containing six animals) and statistical data analyzes were done based on One-way ANOVA followed by Dunnett's multiple comparison test. Compared with the control group, values considered significant if *$p < 0.05$ and ** $p < 0.01$.

Table 7
Effects of *M. repandus* stem extract on the pancreatic function, blood glucose levels, and HbA1c.

Biochemical Parameters		Groups			
		Control	MSM 500	MSM 1000	MSM 2000
Pancreatic Function	Amylase (U/L)	700.45 ± 20.96	713.67 ± 19.57	704.55 ± 10.55	725.75 ± 14.45
	Lipase (U/L)	30.25 ± 2.40	28.50 ± 1.67	29.50 ± 1.73	29.70 ± 2.81
Blood Glucose		4.56 ± 0.05	4.52 ± 0.06	4.46 ± 0.03	3.81 ± 0.06 **
HbA1c (%)		2.70 ± 0.16	2.79 ± 0.20	2.62 ± 0.23	1.90 ± 0.12 **

Experimental results are expressed as mean ± SEM (each group containing six animals) and statistical data analyzes were done based on one-way ANOVA followed by Dunnett's multiple comparison test. Compared with the control group, values considered significant if *$p < 0.05$ and ** $p < 0.01$.

3.5. Effects of extract on histopathological evaluation

Figure 4 represents the findings of the histopathological investigation of selected body organs of the experimented rats after treatment with MMSE at several doses (500, 1000, and 2000 mg/kg; 28 days). There were not found any notable pathological and morphological variations between the treated and control groups.

4. Discussion

As a result of their natural origin, medicinal plants and their phytochemicals are often wrongly considered nontoxic for use. However, numerous reports recommend that plant-derived substances may modulate the complex mechanisms which initiate the pathological condition of chronic diseases including hepatic injuries, cardiovascular complications, cancer, and diabetes [21]. Thus, it is mandatory to evaluate the safety profiles of potential plants for pharmacological evaluation before being used by humans [13]. Like other medicinal plants, *M. repandus* is also traditionally used throughout the world to treat or prevent various diseases including inflammation, hepatic disorders, liver cirrhosis, snake bite, rheumatic arthritis, and tumor. The plant is a source of some bioactive chemicals that can act as anti-inflammatory, antimicrobial, antioxidant, and also protect against hepatic injuries [8]. Our current investigation deals with the appropriate knowledge about the toxicological effects of this plant for using it for therapeutic purposes.

Acute toxicity studies may provide information about the biological activity of chemicals, in addition to hazard identification and risk management associated with the use of these chemical compounds [22]. Our research findings presented that the MMSE (4000 mg/kg) provided no mortality rate or severe

adverse effects in rats. Thus, the LD_{50} value of *M. repandus* can be considered to be above 4000 mg/kg. These findings support the perception that MMSE is relatively safe and is suitable for oral administration in animal models for further investigation. In the sub-chronic toxicity (28 days) study, MMSE caused no deaths or physical anomalies along with the normal characteristics of rats, which highlights the safety profile of this plant.

Body weights are critical parameters for assessing organ damage in organ toxicity investigations [23]. Similarly, relative organ weight is a measure of the biological and pathological grade of animals which may be altered by toxic chemical compounds [24]. From this perspective, Demma and co-workers suggested that relative organ weight is a more crucial indicator of organ toxicity compared with absolute organ weight [25]. Reduction of organ weight is quite common in experimental studies dealing with chemically-induced toxicity [17]. Compared with the control group, our study presented that the MMSE did not demonstrate any substantial change in the relative organ weight and bodyweight of experimented rats. Thus, it has been suggested that the MMSE is safe to be used for therapeutic purposes along with maintaining relatively normal characteristics of organs. On the other hand, the percentage of water content is fundamental for controlling homeostasis which is another significant aspect in evaluating the noxious effects of compounds in animals [26]. Our current investigation signifies no changes in the water content percentage of principal organs, thus suggesting non-toxic properties of MMSE on the homeostasis system of rats.

Analysis of hematological parameters is beneficial to assess the extent of toxic effects of chemical compounds and herbal preparations on the blood compositions of the tested animal [1]. Various hematological tests in rodents were accomplished for the identification of several disease conditions which resemble unusual toxicity signs and symptoms in humans [27]. Erythrocyte counts and ESR are important parameters that can be used to ascertain the effect of biologically active compounds on the hematopoietic system [28]. Our research findings demonstrated that no conspicuous variations in erythrocyte parameters which eliminate the probability of incidence of the anemic condition, hemolysis, thalassemia, and polycythemia were observed [1]. Leukocytes, a biomarker for stress ailments and several inflammations, signify a substantial role in the body's immune system to fight against infections [29]. We found no serious deviations in the leukocyte counts which suggest the non-toxic effects of the extract against inflammatory conditions such as hemorrhage, bacterial infections, and leukemia. Similarly, Platelets are fundamental indicators to analyze atherosclerosis and thromboembolic diseases, which increase the duration of platelet activation [30]. Our prepared MMSE provided no substantial effects on the platelet counts in treated animals as compared with the controllable rats. Therefore, it is suggested that MMSE has no contribution to inducing thromboembolic disorders in the blood composition.

ALT, LDH, AST, GGT, and ALP enzymes are used in evaluating the degree of hepatic damage [20]. Higher quantities of these enzymes in the serum point out the hepatic dysfunctions [31]. Specifically, the liver allows higher amounts of ALT compared to other tissues, which catalyzes the transamination reaction and a rise in ALT serum levels indicating liver injury [32]. Instead, mitochondrial AST is mainly distributed

in the heart and liver, and elevated levels of this enzyme are observed in necrosis and chronic tissue degeneration [33]. Similarly, ALP largely presents in bone, epithelia of the liver, placenta, and kidney. Elevated serum ALP activity may be a marker for inflammation associated with liver dysfunctions including hepatitis and other pathologic conditions [34]. On the other hand, the microsomal enzyme GGT is found in the intestine, renal tubules, biliary epithelial cells, and hepatocytes. Higher GGT levels may cause severe liver diseases associated with fibrosis and bile duct damage [35], whereas lactate dehydrogenase, a cytosolic enzyme, is widely expressed in body tissues which indicate cell damage and necrosis [36]. Our findings from this study on hepatic marker enzymes showed that the extract, except for the 2000 mg/kg dose, caused irrelevant changes in plasma hepatocellular enzymes. However, MMSE (2000 mg/kg) caused a substantial decrease in the amounts of LDH, ALP, and ALT. These findings propose the protection potential of *M. repandus* to constrain the escape of hepatic enzymes from the cytosol. Our findings agree with an earlier study where *M. repandus* stem ethyl acetate extract displayed a defensive role against the D-Galactosamine-induced hepatic damage [8].

Serum albumin, total protein, globulin, and total bilirubin levels and the albumin/globulin (A/G) ratio are significant indicators of hepatocellular toxicity. Bilirubin is a heme metabolite that originates from RBC degradation and is transported to the liver by serum albumin, where it is glucuronide conjugated and discharged into the bile. Elevated levels of bilirubin are common consequences of jaundice and necrosis of hepatic cells where it is accumulated and excreted [37]. Assessment of total protein predicts the diagnostic status of liver and kidney functions [38]. In contrast, levels of serum albumin are tested for determining liver function because lower levels of albumin can indicate severe liver disease [32]. Similarly, Serum globulin (GLB) and albumin/globulin ratio (AGR) are diagnosed as prognostic markers for certain malignancies such as hepatocellular carcinoma [39]. Results obtained from our study did not show any significant alterations in these bio-markers levels among rats of different treatment groups. These outcomes indicate that MMSE contains safe components which did not cause any hepatic excretory functions.

On the other hand, elevated serum urea and uric acid concentrations are associated with kidney functions and renal dysfunctions [40]. Likewise, muscle creatine catabolism produces creatinine which acts as an important pointer to renal function. A blood urea nitrogen (BUN) test is usually used to evaluate the specific amounts of nitrogen in the blood samples that are generally obtained from the urea and indicates the activity level of the kidneys [41]. Our findings revealed that no substantial changes were obtained after MMSE administration for 28 days which corroborates the non-toxic effect of MMSE on normal kidney functions. For maintaining normal body functions such as muscle contraction, energy generation, pH, and fluid balance electrolytes are needed [42]. A rise or decline in the levels of serum electrolytes, possibly a result of hyper- or hypo-functioning of the disturbed tissue or organ, is used in evaluating the activity levels of the kidney [31]. In this current investigation, electrolytes such as Ca^{2+}, PO_4^{3-}, Mg^{2+}, K^+, Cl^-, and Na^+ levels were unchanged in all treated groups which could suggest that MMSE did not change the homeostasis of electrolytes and their associated activities.

The lipid profile is an imperative bio-marker of various ailments. Elevated plasma triglyceride level is the risk aspect for cardiovascular diseases, hypertension, and diabetes [1]. High total cholesterol levels can cause certain serious diseases including cardiovascular-related diseases and atherosclerosis [43]. Similarly, LDL and VLDL cholesterol lead to serious artery diseases and hypertension [44]. Results from this investigation showed a substantial decline in TG, TC, and LDL cholesterol levels which highlights the cardio-protective effect of MMSE. Cardiovascular conditions can also be explained by the important atherogenic indices which are used to quantify blood lipid levels. Elevated values of these indices may indicate high cardiovascular risk [45]. Our findings reveal that treatment doses support the protective effect of MMSE against cardiovascular-related complications.

According to the American College of Gastroenterology guidelines, serum lipase and amylase are two main markers of pancreatic assessment [31]. In acute pancreatitis, levels of these enzymes increase three to fivefold [46]. Results obtained from our investigation showed no potential changes in levels of these pancreatic enzymes in treated rats. Moreover, MMSE indicated a defensive role on the glycemic index because a high dose (2000 mg/kg BW) considerably decreased the levels of blood glucose that is widely used as a diagnostic tool for diabetes mellitus [47]. Likewise, HbA1c production is enriched in diabetes where extensive glucose levels in the blood samples react with serum hemoglobin and then glycated hemoglobin is produced [48]. An experimental dose concentration of 2000 mg/kg of MMSE, protected the tested animal from the substantial upsurge in HbA1c level in plasma.

Histological analysis signifies the basis of their safety assessment that evaluates pathological alterations in the organs of laboratory animals [49]. The histological inspection of the analyzed organs of this study revealed that organs from rats of different groups revealed no visible lesions including inflammation and necrosis in the cellular structure. So, histopathological findings advocate the results of the biochemical test of this pragmatic study.

5. Conclusions

In summary, our current experimental findings cleared that the oral administration of 4000 mg/kg BW of MMSE was safe in animals thanks to no responses of toxicity or mortality. Thus, the LD_{50} value of *M. repandus* can be considered to be above 4000 mg/kg. Results related to the sub-chronic toxicity (28 days) study revealed that MMSE repeated caused no momentous changes in body weight, the percent water content of organs, relative organ weight, and hematological parameter. In addition, different doses of MMSE have no significant deleterious outcome on biochemical parameters. Moreover, MMSE exhibited substantial protection against cardiac diseases along with significant defense against hepatic diseases. These findings are also supported by histological assessment. In conclusion, all experimental assessments indicate that *M. repandus* stem consumption is safe or non-toxic at relatively higher oral doses and advantageous for health care purposes. However, work that involves human subjects may be required before using this extract for therapeutic purposes.

Declarations

Authors' contributions

Conceptualization, Md. Rakib Hasan, Milon Mondal, Sherouk Hossein Sweilam **methodology**, Md. Habibur Rahman, Md. Rakib Hasan, Milon Mondal, Sherouk Hossein Sweilam; **validation**, Md. Habibur Rahman, Md. Rakib Hasan, Mohammad Mehedi Hasan, Chandan Sarkar; **formal analysis, investigation, data curation**, Md. Monir Hossain, Md. Abu Hanif, Sheik Md. Saiful Islam, Amit Kumar Roy, Kakoli Rani Mondal, Banani Mondal, Sarker Ramproshad; **writing**— Md. Rakib Hasan, Milon Mondal, Sherouk Hossein Sweilam, Md. Monir Hossain, Md. Abu Hanif; —**review and editing**, Mohammad Mehedi Hasan, Chandan Sarkar, Kakoli Rani Mondal, Banani Mondal, Sarker Ramproshad, Sandesh Panthi, Md. Habibur Rahman. All authors have read and agreed to the published version of the manuscript.

Data Availability

The data in this study that support the findings are available from the corresponding authors upon request.

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Conflicts of Interest

The authors declare no conflict of interest.

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Figures

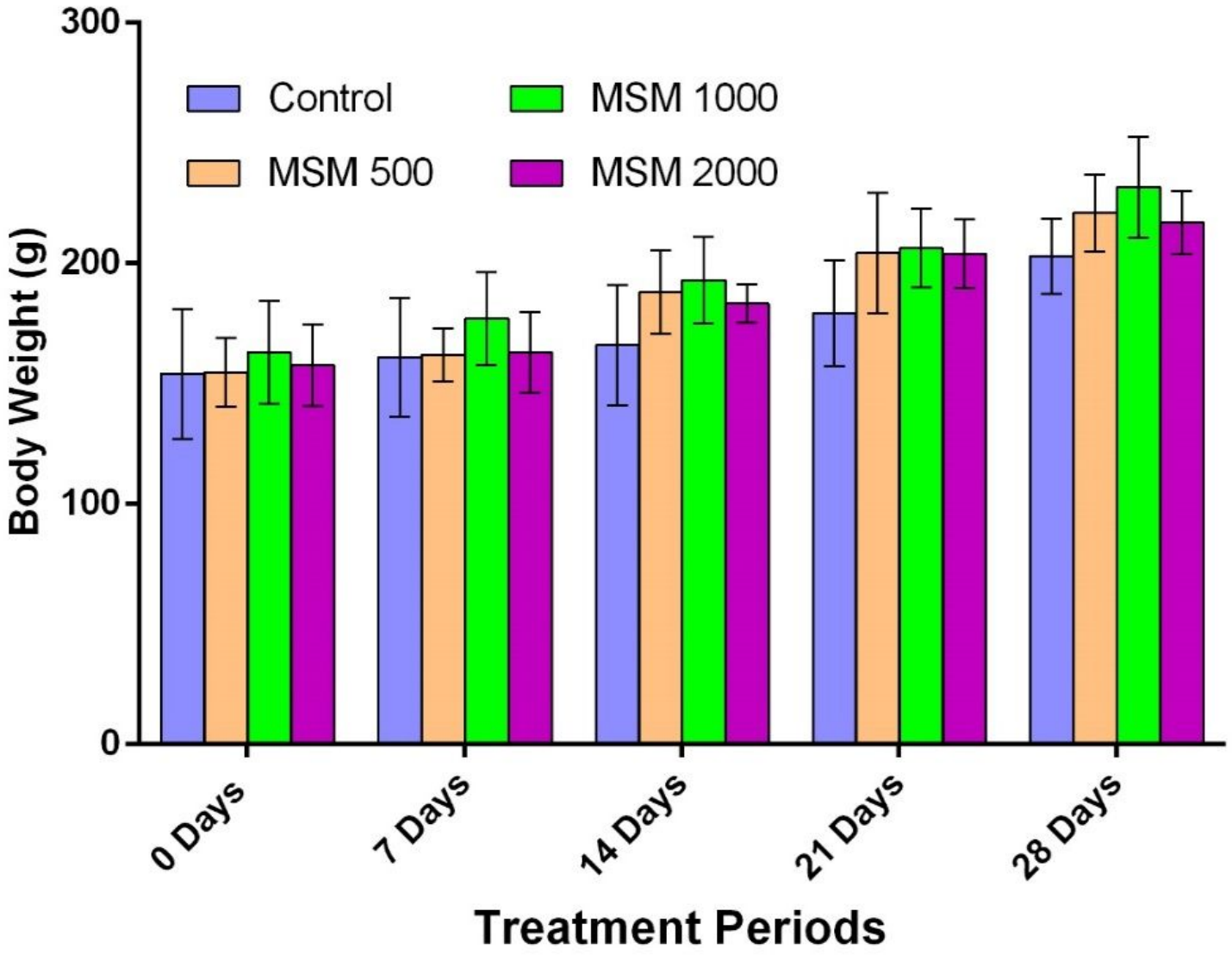

Figure 1

Effects of *M. repandus* stem extract on body weight (g) gain. Experimental results are expressed as mean ± SEM (each group containing six animals) and statistical data analyzes were done based on one-way ANOVA followed by Dunnett's multiple comparison test. Compared with the control group, values were significant if *p< 0.05 and ** p< 0.01.

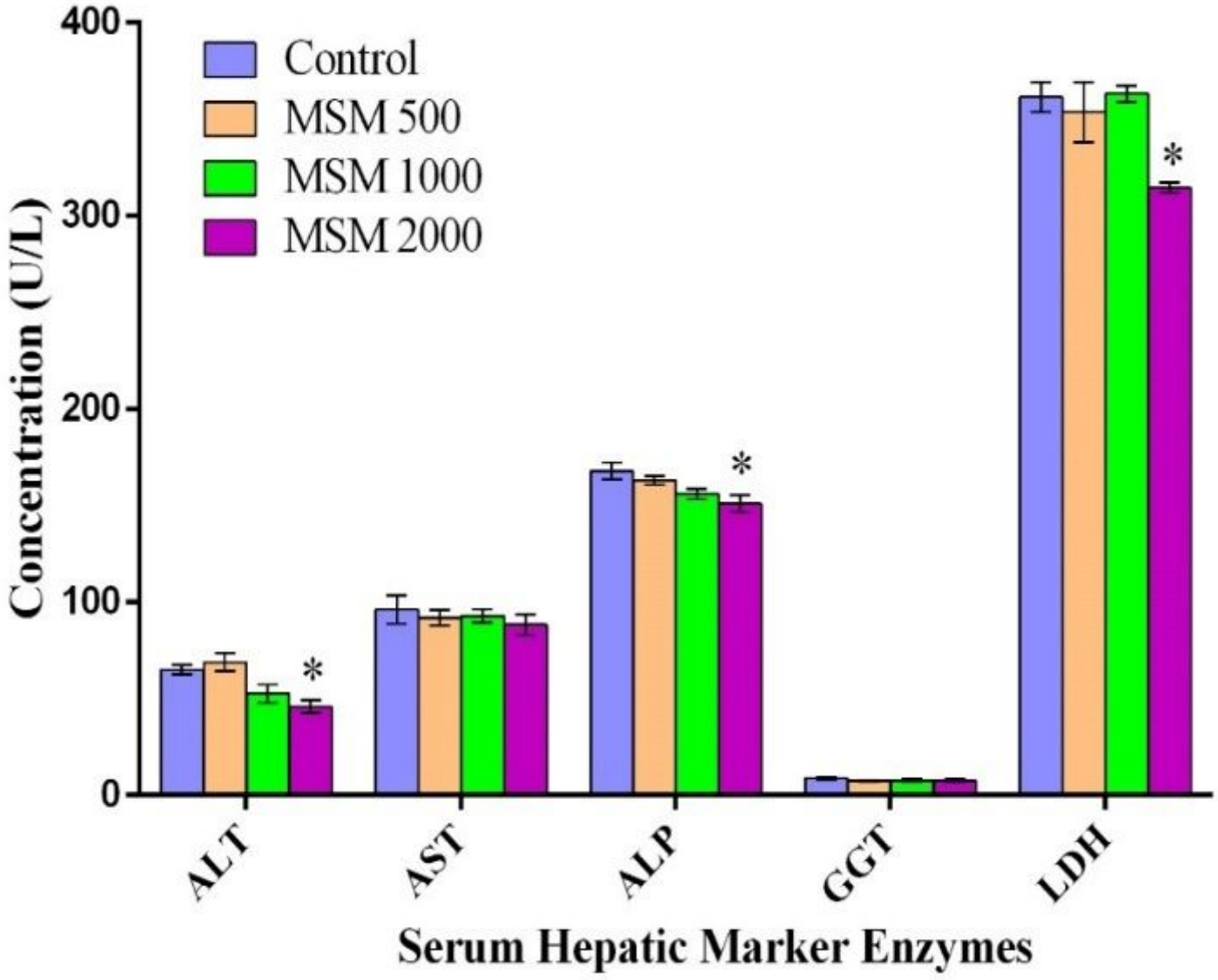

Figure 2

Effects of *M. repandus* stem extract on the serum hepatic marker enzymes. Experimental results are reported as mean ± SEM (each group containing six animals) and statistical data analyzes were done based on one-way ANOVA followed by Dunnett's multiple comparison test. Compared with the control group, values considered significant if *p< 0.05 and ** p< 0.01.

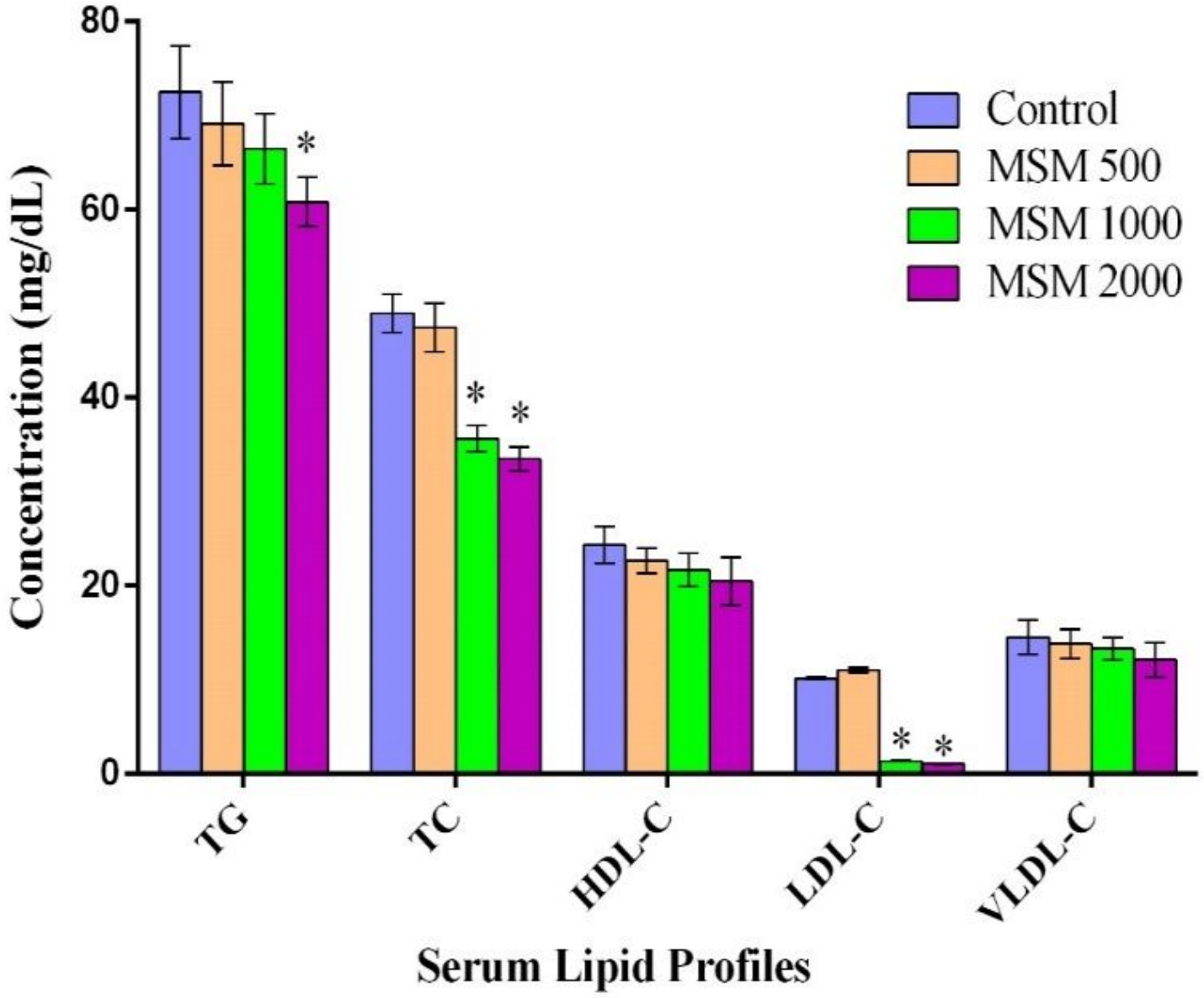

Figure 3

Effects of *M. repandus* stem extract on the serum lipid profiles. Experimental results are expressed as mean ± SEM (each group containing six animals) and statistical data analyzes were done based on one-way ANOVA followed by Dunnett's multiple comparison test. Compared with the control group, values considered significant if *p< 0.05 and ** p< 0.01.

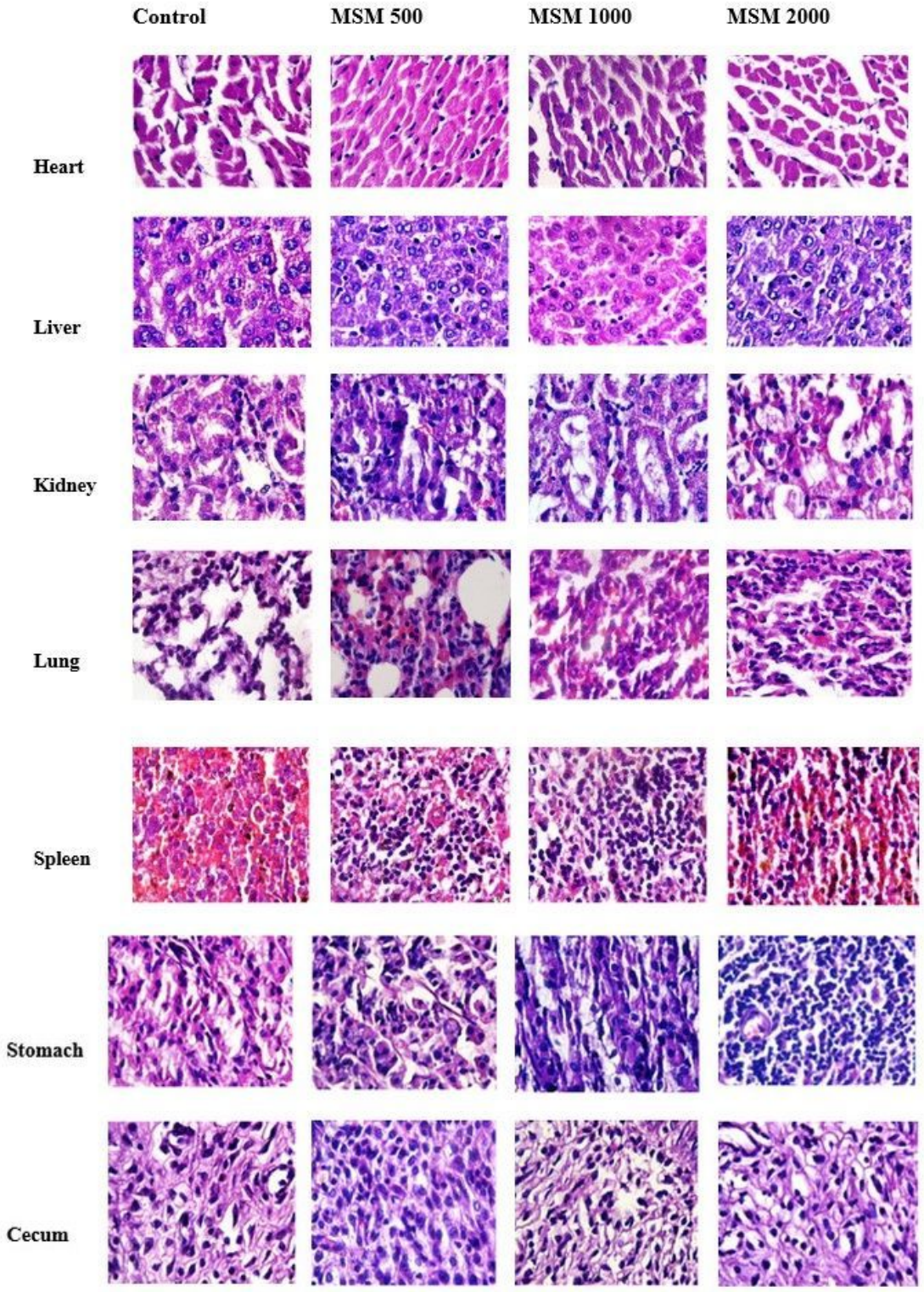

Figure 4

Histological photomicrographs of control and three different doses (500, 1000, 2000 mg/kg) of *M. repandus* (×100 magnification, Scale bar: 20 μm). Compared with the control group, no histological variations were found.