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Research Article

Evaluation of Toxic Effect of Hydro-Methanolic Root Extract of *Myrica salicifolia* A. Rich (Myricaceae) on Brain, Heart, Spleen and Blood Parameters in Swiss Albino Mice

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Abstract

Background: *Myrica salicifolia* A. Rich (Myricaceae) is a routinely used herbal medicinal plant for the treatment of various human diseases such as malaria, inflammation, infections, diabetes mellitus and gastrointestinal spasm. The aim of this study was to evaluate the hydro methanolic roots extract of the *M. salicifolia* toxic effect on brain, heart, spleen and some blood parameters in mice model.

Methods: Roots of *M. salicifolia* were collected from Gondar area, Northwest Ethiopia. The roots were dried and extracted with methanol. Swiss albino female mice, 25 - 40g weight and 8 - 12 weeks age, were randomly divided into four groups. The control group received 0.5 ml of distilled water orally and the treated group received the root extract of *M. salicifolia by* using intragastric tube at the doses of 100, 200 and 400 mg/kg body weight per day for 4 weeks. The hematological analysis was examined. The brain, heart and spleen were removed, stained and examined for histopathological effects. Hematological and histopathological features were compared with the control group.

Results: Most of the hematological parameters and all biochemical parameters of all the treated groups were statistically significant ($p < 0.05$) as compared to control group. While, the body weight and organ weight changes in tested doses didn't show any significant difference. Moreover, histopathology examination showed that the extract did not show any toxicity in brain, heart and spleen tissues. Overall, the result of the present study indicated that the plant extract is relatively safe to mice when given orally.

Keywords: *Myrica salicifolia; Sub-Acute Toxicity; Histopathology*

Introduction

Plants are known as a major source of modern medicines. From ancient times, human have utilized plants for treatment and prevention of diseases, leading to the dawn of traditional medicine. Traditionally, different parts of this plant are used by many communities to treat a number of ailments including infectious disease such as gonorrhoea, syphilis, trachoma, conjunctivitis, dysentery, malaria, inflammation, infections, diabetes mellitus and gastrointestinal spasm [1-3].

M. salicifolia (Myricaceae), known as *Shinet* in Amharic language, is one of the traditionally used medicinal plants in Ethiopia. It is widely distributed in the flora of Northern, Eastern and Southern part of the country. This plant fits in dry and moist agro climatic zones at an altitude ranges from 1,600 - 3,300 meter above sea level [4,5].

In the Ethno-medicine, different parts of *M. salicifolia* are used as a traditional remedy. The root and bark extracts are used with tea for aliment of different disease such as chest congestion, pneumonia, diarrhea, diabetes, hypertension, respiratory diseases and malaria

[6]. The barks are chewed for tooth ache problem. Whereas; the powdered young leaves are used to treat skin infections. Root and bark extracts of *M. salicifolia* are used for treatment of chest congestion, pneumonia, diarrhea, nervous disorder, hypertension, respiratory disease, headache and inflammation [7,8]. The leaf extract proved to have a central nervous system depressant effect [9,10]. In spite of its extensive traditional uses as medicinal plant, to the best of the literature, only few toxicological investigations have been carried out to date. Therefore, the aim of the present study was to evaluate the effects of the hydro-methanolic roots extract of the *M. salicifolia* on histopathology of brain, heart, spleen and some blood and biochemical parameters in mice.

Materials and Methods

Plant material collection and extraction

The roots of *M. salicifolia* were collected from Gondar area, Northwest Ethiopia, which is located about 740 km away from the capital city, Addis Ababa. The plant was then identified and authenticated by Mr. Abiyu Enyew, the Botanist, Department of Biology, College of Natural Sciences, and University of Gondar, where a voucher specimen (collection number BT001) was deposited for further reference.

The roots were cleaned from any irrelevant materials, dried at room temperature under shadow and crushed to coarse powder. The powdered plant material (672g) was macerated in 80% methanol for 72h with occasional stirring. The filtrate was separated from the mark by filtration (Whatman No.1, England), and the mark was re-macerated three times. The filtrates were combined and concentrated in a rotary evaporator (Buchi Rota- vapor type R.205, Switzerland). The concentrated extract was further kept in an oven at a temperature not exceeding 40°C, after removal of the solvent yielded brown powder of 145g (21.57%).

Experimental animals

Adult female mice weighing 25 - 40g and 8 - 12 weeks of age were obtained from the animal breeding house of Ethiopian Public Health Research Institution (EPHI), Addis Ababa, Ethiopia. All animals were housed in an air-conditioned room and were allowed to acclimatize for one week before commencement of the study. All the experiments were conducted in accordance with the internationally accepted laboratory animal use, care and guideline. Before and during the experiment, the mice were allowed free access to standard pellets and water *ad libitum* [11,12].

Acute toxicity test

Six nulliparous and non-pregnant female mice were equally divided into treatment and control group with three mice in each group as per OECD 425 guideline [12]. The extract was administered orally at a dose of 2000 mg/kg and the control group was given 0.5 ml distilled water. The mice were observed continuously for 1h after extract administration; intermittently for 4h, over a period of 24h and for 14 days. Gross behavioral changes such as loss of appetite, hair erection, laceration, salivation tremor, convulsion, diarrhea, mortality and other signs of toxicity manifestations were observed.

Sub-acute toxicity study

Forty mice were randomly assigned into four groups (Group I, Group II, Group III Group IV) with 10 mice each. Group I, II and III received 80% methanolic root extract of *M. salicifolia* at a dose of 100, 200and 400 mg/kg, respectively. While Group IV control group received 0.5 ml distilled water. The extract was administered orally using standard gavage per day for 28 days [13].

Body and organ weight measurement

Body weight was recorded at the beginning of treatment and once a week thereafter. The weight documented before administration of the test substance was considered as an initial and final body weight was recorded with an electronic balance with .001 precision on the last day just after 12 hours of the last dose [12,13]. Since neurons have high metabolic rate, which makes them extremely vulnerable to certain toxic substance insults that impairs the total intracellular energy for any metabolism [14]. Because of the heart tissues high-flying need of oxidative accomplishment, the tissue may be simply damaged by any compound that interrupts with its oxygen supply [15]. As spleen is highly vascular hematopoietic organ that prone to chemical toxic effect on its cellular morphology [16]. The weight of brain, heart and spleen might indicate sub-acute toxicity in the body.

Blood collection for hematological analysis

After 12 hours of receiving their last treatment doses. All of the mice were anesthetized by ketamine injection and the blood samples were collected by cardiac puncture, then to reduce suffering for euthanasia cervical dislocation was used. The test tubes containing anti-coagulant, ethylene diaminotetraacetic acid (EDTA), were used for the determination of the hematological parameters (WBC, RBC, HGB, HCT, MCH, MCHC and Platelets) by using an Auto mated Hematology Analyzer (Symex- RX, 21, Japan) [17].

Animal dissection, tissue collection and histological processing

The body cavities of the mice were opened by the vertical incision. The brain, heart and spleen were gently isolated; an extraneous tissue like fat was removed and immediately weighted by electronic balance and then a piece of tissue sections were randomly taken from each organ. The tissue samples were transferred by blunt forceps to labeled test tubes containing 10% neutral formalin buffer that completely immerse the tissue for 24 hours for fixation. After overnight fixation, the tissues were dehydrated with an increased concentration of ethanol alcohol accordingly with 70% for 15 minutes, 80% for 20 minutes, 90% for 20 minutes, absolute ethanol I and II for 20 minutes each and absolute ethanol III for 25 minutes. The tissues were cleared with a xylene: xylene I, II, and III for 30 minutes each. Finally, the tissues were impregnated in a paraffin wax: wax I, II and III for 40 minutes each. Then, tissue blocks were prepared by embedding with a paraffin wax in a square metal plate and all tissue blocks were labeled. Tissue blocks were sectioned in ribbons at a thickness of 5μm using a rotary microtome (LEE, GMBH, Germany) for proper tissue samples [18,19].

The paraffin ribbons containing the tissue were allowed to float onto the surface of a warm water bath at 40°C to spread and remove folds in the sections. The slides were arranged in slide racks and were placed in an oven at temperature of 56°C for 1 hour for removal of paraffin. The tissue sections were allowed to cool, dry at room temperature and stained with a routine hematoxylin and eosin staining method. Hematoxylin and Eosin stained tissue slides of the brain, heart and spleen were examined for histopathologic effect of extract by using light microscope [20].

Data quality control

Each of the microscopic slides was coded and examined by authors.

Ethical clearance

This study was ethically reviewed by the Institutional Review Board of the University of Gondar. All protocols were performed in accordance with the international animal care and welfare guidelines.

Statistical analysis

Data were analyzed using the statistical software package IBM SPSS version 20. All the values in the test are presented as means and standard error of the means (mean ± SEM). The one-way analyses of variance (ANOVA) followed by Tukey's HSD post-hoc test, were used to compare results among and within groups and paired t-test for difference between initial and final body weight results. The results were considered significant when $P < 0.05$.

Results

Acute toxicity and LD_{50} determinations

In acute toxicity study, no sign of toxicity was shown in mice treated with the hydro alcoholic root extract of *M. salicifolia* at a dose of 2000 mg/kg. Furthermore, mortality was not observed at 2000 mg/kg during acute toxicity study, signifying that the oral LD_{50} was greater than 2000 mg/kg.

Effect of the plant extract on body weight

As shown in figure 1, during sub-acute administration, the weight of mice in lower two doses of extract treated groups were decreased insignificantly, particularly in the first two weeks. However, in the higher dose administration of extract was statistically significant decrement of weight of mice. As the paired t-test analysis showed that the mean of initial body weight and final body weight of control, 100 and

200 mg/kg group were not significantly different. However, at highest dose of 400 mg/kg there was a significant difference in initial and final weight (Table 1).

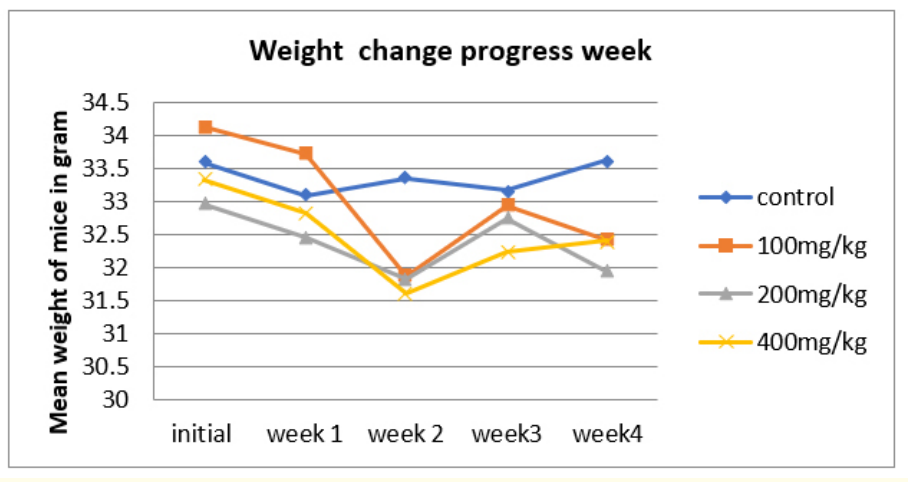

Figure 1: *Body weight of mice after sub-acute administration of the hydro-alcoholic root extract of M. salicifolia of administration period.*

Dosage Group	Mean weight mice (gram)as mean ± SEM)				Mean weight organs (gram) as mean ± SEM		
	Initial weight	Final weight	difference	t- test	Brain	Heart	Spleen
Control	33.6 ± .54	33.6 ± .41	0	.958	.43 ± .01	.16 ± .01	.124 ± .03
100 mg/kg	33.34 ± 1.17	32.45 ± 1.16	+0.89	.244	.43 ± .01^{NS}	.16 ± .01^{NS}	.129 ± .05^{NS}
200 mg/kg	32.45 ± 1.17	31.94 ± 1.28	+0.51	.140	.43 ± .01^{NS}	.16 ± .01^{NS}	.126 ± .04^{NS}
400 mg/kg	34.14 ± .64	32.42 ± .81	+1.72	.036*	.43 ± .01^{NS}	.16 ± .01^{NS}	.127 ± .05^{NS}

Table 1*: The mean initial and final weight of mice in all groups and mean of brain, heart and spleen weight changes after sub-acute administration of the hydro-alcoholic root extract of Myrica salicifolia.*

*Value mean of brain, heart and spleen weight changes es expressed as mean ± SEM, N = 10/group, *paired t- test (p. values) in each group, NS= non-significant (P > 0.05) as compared with control group.*

Effect of the plant extract on organ weight (brain, heart and spleen)

The weight of organs such as brain, heart and spleen were analyzed at the end of the experiment. As revealed in table 1, statistical insignificant weight change in organ was observed when compared to that of the control group.

Effect of the plant extract on hematological parameters

As shown in table 2, the effects of sub-acute administration of 80% methanolic root extract of *M. salicifolia* affected the hematological parameters of treated mice. The WBC counts of mice treated with 400 mg/kg of *M. salicifolia* extract significantly decreased whereas the RBC counts of extract treated mice significantly decreased at the dose of 200, and 400 mg/kg. Moreover, MCHC level was declined significantly at all tested doses when compared to the control. Meanwhile, towards the highest dose, insignificant decrement in MCH and a significant decrement in PLT at 400 mg/kg were observed.

Parameters	Control	100 mg/kg	200 mg/kg	400 mg/kg
WBC (K/µl)	4.42 ± 0.8	4.41 ± 0.13	4.32 ± 0.6	2.18 ± 0.18*
RBC (M/ µl)	7.45 ± 0.23	6.97 ± 0.26	6.05 ± 0.22*	5.76 ± .14*
HGB (g/dl)	10.05 ± 0.23	10.65 ± 0.55	9.66 ± 0.52	9.13 ± .69
MCV (fl)	47.21 ± 0.53	49.22 ± 0.62	46.7 ± 0.61	45.22 ± .1.12
MCH (pg)	17.66 ± 0.31	16.56 ± 0.33	16.87 ± 0.43	15.86 ± 0.51
MCHC (g/dl)	37.3 ± 0.38	32.96 ± 0.6*	33.2 ± 0.5*	31.86 ± 0.51*
PLT (K/µl)	732.96 ± 0.64	737.30 ± 0.38	731.86 ± 0.51	133.5 ± 0. 46*

Table 2*: Hematological parameter change of mice after sub-acute administration of the hydro alcoholic root extract of Myrica salicifolia.*

The mean value is significant (P < 0.005) when compared with control; data are expressed as means ± SEM for ten mice per a group. WBC: White Blood Cell; RBC: Red Blood Cell; HGB: Hemoglobin; MCV: Mean Cell Volume; MCH: Mean Corpuscular Hemoglobin; MCHC: Mean Corpuscular Hemoglobin Concentration; PLT: Platelets;* ***µl:* *Microlitre; K: 10^3; M: 10^6; fl: Femtolitre; pg: Pictogram.*

Effect of the extract on the histology of the brain

In addition to the body and organ weight changes and hematological effects, the histopathological effects of the plant extract on the brain tissue were also investigated. Light microscopic examinations of the sections of brain tissue of the control mice and treated groups showed that a normal architecture of cerebellum (Figure 2A, 2B, 2C and 2D) and cerebrum (Figure 2E, 2F, 2G and 2H). The mice treated with 80% methanolic root extract of *M. salicifolia* at all tested doses of the extract showed no histopathological changes as compared to the control group.

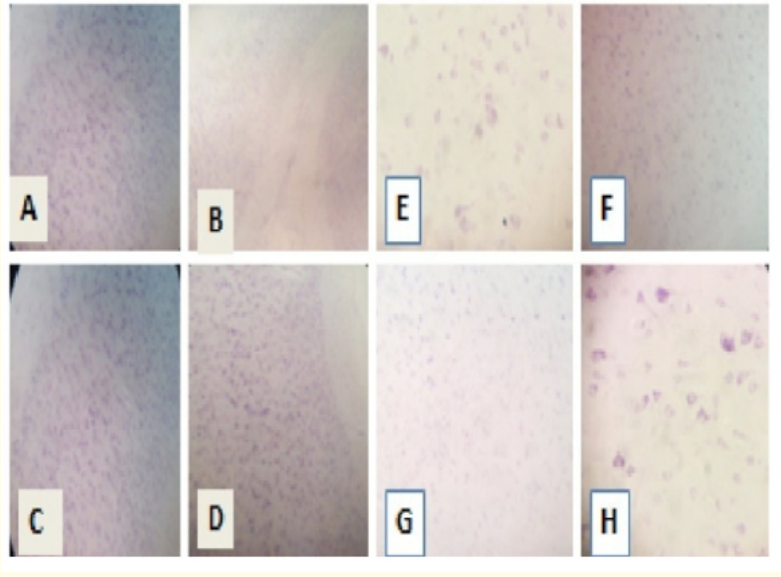

Figure 2: *Photomicrographs of the cerebellum and cerebrum of control mouse (A, E) (H and E, x40). (B, F) Mice treated with 100 mg/kg of M. salicifolia root extract. (H and E, x40). (C, G) Mice treated with 200 mg/kg of M. salicifolia root extract. (H and E, x40). (D, H) Mice treated with 400 mg/kg of M. salicifolia root extract (H and E, x10) indicated respectively.*

Effect of the plant extract on the histology of the heart

The histopathological effect of hydro alcoholic root extract of *M. salicifolia* was conducted on the histological sections of the heart that was stained with H&E staining technique. The light microscopic examinations of the sections of heart for all control mice and all treated groups showed that a tissue with normal anatomical features of myocardium (Figure 3A, 3B, 3C and 3D).

Effect of the plant extract on the histology of the spleen

The splenic examination for histopathological effect of hydro alcoholic root extract of *M. salicifolia* was conducted on the histological sections of the spleen that was stained with H&E staining technique. The light microscopic examinations of the spleen from the control mice showed a tissue with characteristic normal structural features white pulp consists of lymphocytes with periarterial lymphatic sheath surrounded by rep pulp. All hydro alcoholic root extract of *M. salicifolia* treated mice showed similar structural features as compare with control group (Figure 3E, 3F, 3G and 3H).

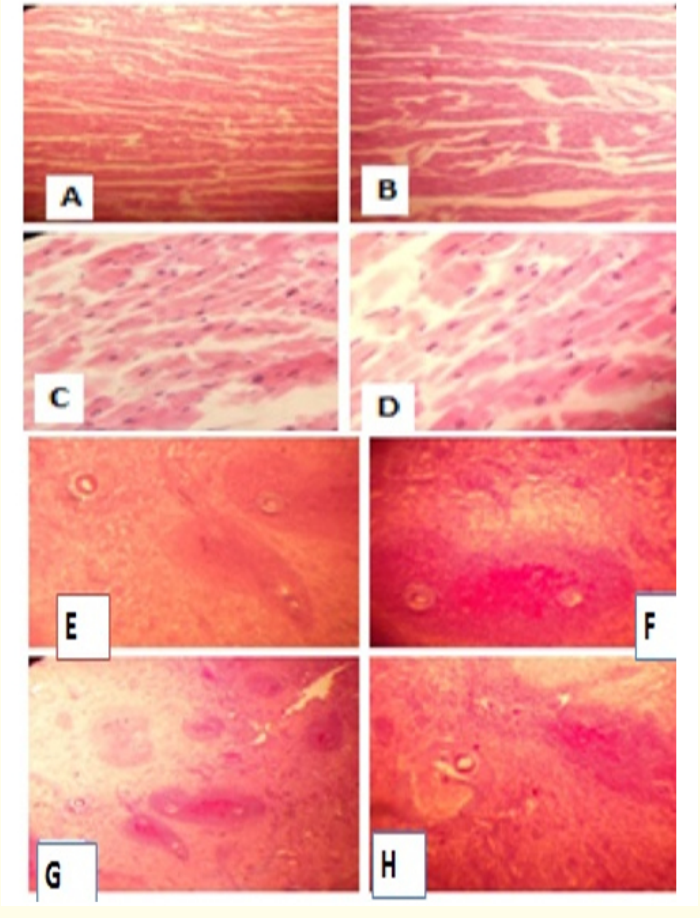

Figure 3: *Photomicrographs of heart specimen section of control mouse (A) (H and E, x10). (B) Mice treated with 100 g/kg of M. salicifolia root (H and E, x40). (C) Mice treated with 200 mg/kg of M. salicifolia root extract (H and E, x40). (D) Mice treated with 400 mg/kg of M. salicifolia root tract (H and E, x40), Photomicrographs of spleen sample section of control mouse (E) (H and E, x10). (F) Mice treated with 100 g/kg of M. salicifolia root (H and E, x10). (G) Mice treated with 200 mg/kg of M. salicifolia root extract (H and E, x10). (H) Mice treated with 400 mg/kg of M. salicifolia root tract (H and E, x10).*

Discussion

The circumstance not damaging the tissues by any substance used considered as safety, whereas efficacy is the competence to provoke a clinical advantage. The indispensable attributes of safety and efficacy for any administered medication is very significant, particularly safety has taken superiority than efficacy [21]. Estimation of histopathological changes induced by plant extract indicates their safety assessment. Herbal preparations have addressed greater values to any medical therapy. These need of plants for herbal remedies give better attention now a day. Despite of the fact that the use of herbal plants as a medicinal importance for many years, only recently has toxicity studies intention for researchers. The present study groundwork evaluation showed chief hand-outs to the advance of toxicology of plant extract.

Globally, many plants including *M. salicifolia* are commonly engaged by local traditional healers, as a single or combinations of medicinal plants to have synergic end product for a number of alignments. Toxicity studies are undertaken to differentiate any toxicity of a substance, thereby to ensure their safeties. In all conditions, the toxic effects are usually manifested either in an acute, sub-acute or a chronic manner that occurs mostly as a result of time exposure to a toxic compound by oral ingestion, inhalation or absorption following skin contact [8,22].

In the present study, a single dose administration of crude extract of 80% methanolic root extract of *M. salicifolia* at 2000 mg/kg to female mice did not show any behavioral changes such as salivation, diarrhea, piloerection and depression in all 14 days of follow up period. LD_{50} of the crude extract to be greater than 2000 mg/kg, which could be possibly accepted as safe [12].

During sub-acute administration for four weeks, the general weight change, organ weight change, biochemical and blood parameters as well as histopathology of brain, heart and spleen were analyzed. Significant decrement in final weight of mice at a highest dose as compare with initial body weight and insignificant decrement in lower doses of *M. salicifolia* treated mice was observed. However, there was no statistically significant difference among groups as compare with control group. In the first two weeks, the body weight of extract treated groups highly declined then after slight increments were observed. The body weight increment of mice are allied with luggage compartment of fat more willingly than the toxicological effect of plant extract [23]. On the contrary decrement in the body weight may be correlated with normal well-designed response of animals to extract that results in appetite lost and lower the nutrient intake by experimental animals [24]. Similarly, no significant weight change in brain, heart and spleen of treated mice was observed as compared to control mice. This finding is in line with the study done for evaluation of the acute and long term safety of hydroalcoholic extract of *sapthaparna* (*Alstonia scholaris*) in mice and rats [25]. This shows that the plant extract might have caused insignificant change in their food intake and utilization of food. Thus, the absences of significant difference in the weight of organs provide support for the safety of the plant extract under investigation.

In this study, hematological parameters such as RBC, WBC, MCH, MCHC and platelets were investigated. Blood parameters as RBC and MCHC were significantly different from the control group suggesting that the long-term use of the crude methanolic root extract of *M. salicifolia* has an effect on the rate of production of RBC and imply that there might be a change in the oxygen carrying capacity of the blood and the amount of oxygen delivered to the tissue following the administration [26]. The total count of WBC has decreased significantly at a higher dose of 400 mg/kg of treated group. The current study was in supportive comparable trend with a previously recorded study on the effect hydromethanolic leaf extract of *Grewia crenata* [27]. This reductions of WBC count may be due to decline in immune system of animals. In this study, the total count of the platelets was significantly decreased at a higher dose (400 mg/kg) as compared with the vehicle administered group. This finding was similar with the research done on the sub-chronic evaluation of hydro-methanolic seed extract of *Coriandrum sativum* in mice that showed decrement in the platelet count of the treated groups [28]. This decrement in platelet count may be due to the ability of the plant extract interruptive effect in thrombopoietin activity of platelet cells production.

Histopathological studies of selected organs

As response of toxicological, numerous changes occur in neurons and their cellular processes. These include contraction of the soma (cell body), nuclear pyknosis, vanishing of the nucleolus, and loss of chromatophilic substance with intense basophilic of the cytoplasm.

Injured axons undergo swelling and show disruption of axonal transport. The swellings can be recognized on H&E stains. Axonal injury also leads to cell body enlargement and rounding, peripheral displacement of the nucleus [35]. On the histopathological examination of current study on the brain tissue of mice treated with the 80% methanolic root extract of *M. salicifolia* did not show any morphological changes of the tissues architecture of treated mice as compared with their controls. this study was supported by similar study that evaluates the acute and sub-acute toxic effect of ethanolic aerial parts extract of *Leucas aspera* (*Lamiaceae)* at similar a doses of the present study [36]. Another similar study conducted to evaluate the toxic efect of *Jatropha curcas* phorbol esters in mice also support the present study [37].

The myocardium could be scratched by a large quantity of toxic agents with minimal ability of response. Cardiac muscle fibers toxic injury signs include a cytoplasmic variation such as necrosis that occur along with hypertrophy of neighboring cardiac myocytes, the total number of cardiac myocytes is reduced. The cytoplasmic necrosis is accompanied by a variable degree of inflammation that depends to some extent on the injurious agent [38]. In this study, the histological examination of the heart sections of the treated mice at all tested doses revealed no histological changes as compared to the control group. The current study was in line with the previous similar study conducted to assess the acute oral toxicity of methanolic seed extract of *Cassia fistula* L. (*Caesalpinioideae*) in mice [39].

Toxicological damage of splenic tissue is characterized by expansion of red pulp with small basophilic erythroid cell, enlargement venous sinusoid, hypocellular white pulp and vacuolation of cytoplasm [40]. However, histological examination of the spleen sections of the treated mice at all tested doses of present study revealed no histological changes as compared with the control group. This study was in notion with a similar study conducted to evaluate the acute and sub-acute toxic effect of ethanolic aerial parts extract of *Leucas aspera* (Lamiaceae) in experimental rats [36]. The current study also in line with the previous similar study conducted to assess the acute oral toxicity of methanolic seed extract of *Cassia fistula* L. (*Caesalpinioideae*) in mice [39]. Overall, the result of the present study indicated that the plant extract is relatively safe to mice when given orally.

Conclusion

In this study, the acute and sub-acute toxicity of hydro-alcoholic root extract of *M. salicifolia* were carried out. No sign of toxicity was observed during acute toxicity study follow-up. Moreover, during sub-acute oral administration, no significant organ weight change as well as histological change was observed at all tested doses. However, a significant change was observed in certain hematological parameters. Overall, a further toxicological study is warranted to conclude about safety nature of this plant extract.

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