The Effect of *Clerodendrum Myricoides* Aqueous Extract on Blood, Liver and Kidney Tissues of Mice

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ABSTRACT

Clerodendrum myricoides is a plant traditionally used for its medicinal value in many parts of the world including Ethiopia. Several human defects are treated in different regions of Ethiopia using the roots, leaves, twigs, fruits and root barks of *Clerodendrum myricoides* species. The objective of the present study is to investigate the effect of the aqueous extract of *Clerodendrum myricoides* root on different tissue of mice in chronic administration. The root of the plant were collected in Bale area, Ethiopia dried and crushed into powder and soaked in water to yield aqueous extract. The extract was then administered to mice at doses of 100 and 400mg/kg bw/day for six weeks and 1134mg/kg bw/day (LD_{50}) once. The 400mg/kg bw/day and LD_{50} treated mice showed hypoactivity (abnormally diminished activity), grooming, prostration (submissively prone position), piloroerection (elevation of hairs) and irritation, while 100mg/kg bw/day treated mice showed no behavioral changes. The dose of 100mg/kg bw/day produced significant weight gain, while 400mg/kg bw/day produced significant weight lost in chronic administration. The extract increased only urea at the dose of 100mg/kg bw/day, whereas it increases most hematological and biochemical parameters at 400mg/kg bw/day. The extract, however did not show significant change in platelet, HGB and MCHC value at both doses. Several histopathological changes in liver and kidney were also observed at both doses of the extract. There were inflammation and hydropic degeneration of hepatocytes at both doses. The LD_{50} of the extract produced hemorrhages and congestion in the glomeruli of the kidney.

Key words: *Clerodendrum myricoides*, Root, Aqueous extract, Toxicity, Mice.

1. INTRODUCTION

In the struggle for survival, man had to identify plants that are not only hazardous to health but also those that would serve for his well being. In Ethiopia, the long history of using medicinal plants for combating various ailments traditionally can be confirmed by referring to the recent collection of medico-religious manuscripts of the Axumite kingdom (Kibebew, 2001). Moreover,

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plant remedies are still the most important and sometimes the only source of therapeutics for nearly 80% of the population in Ethiopia (Abebe, 2001). One of the traditionally used medicinal plants in Ethiopia is *Clerodendrum myricoides*. *Clerodendrum myricoides* which is known as Marasissa, in Afaan Oromoo and Misirich, in Amharic (Desta, 1994) is a member of family Lamiaceae and erect or scandent shrub. In Ethiopia, *Clerodendrum myricoides* is widely distributed in the flora of Tigray, Gonder, Wollo, Shewa, Arsi, Welega, Illu Abba Boor, Kefa, Gamo Gofa, Sidamo, Harerge (Persson, 2006).

Ethnomedical value of *Clerodendrum myricoides* is enormous in Ethiopia as different parts of the plant are used as traditional medicine. The bark of the plant is used for abdominal pains, malaria and against snake bites (Persson, 2006). Seven human health defects were treated with the roots, leaves, twigs, fruits and root barks of *Clerodendrum myricoides* species (Getahun, 1976). Root decoction is also applied as antidotes in poisonings (Getahun, 1976). Bathing over the steam after boiling the leaves of *Clerodendrum myricoides* and *Clutia abyssinia* is used for the treatment of epilepsy. The root and the whole plant parts are used to treat leprosy and hemorrhoids, respectively (Tadesse, 1986). Roots and leaves of *Clerodendrum myricoides* are also used to treat gonorrhea, rabies, measles, glandular TB, colic, eye disease, malaria, swellings, in the body, wound dressings, hemorrhoids, asthma and as aphrodisiac (Abebe et al., 2003, Persson, 2006). This plant is also used for the treatment of pneumonia, dry cough, mental disorder, general malaise (mich), toothache, headache and diuretic (Dessissa, 2000). In spite of its wide traditional uses as medicinal plant, only few pharmacological and toxicological investigations have been carried out to date.

2. METHODOLOGY

2.1. Plant materials

Roots of *Clerodendrum myricoides* were collected from Bale-Delomena area about 550km away from Addis Ababa during September 2007. Specimens of the plant were identified by a taxonomist and a few samples were deposited at the National Herbarium in the Faculty of Science, Addis Ababa University (AAU) with a Voucher specimen number (002/TIG/PHARM).

2.2. Preparation of aqueous extract of *Clerodendrum myricoides*

The roots of the plant was rinsed, dried and crushed to powder at the Drug Research Department of the Ethiopian Health and Nutrition Research Institute (EHNRI), and 400g dry powder was obtained. The dried powder was macerated with 2 liters of water for 6 hours and placed in orbital shaker at room temperature. On the next day, the extract was filtered through guaze ($0.1mm^2$mesh) and Whatman filter paper (size 15cm). The extract was then placed in a petridish and deep freezed at-27^0C and lyophilized for one week (-52^0C, 133 x 10^{-3}mbar) to yield a solid residue. By such procedures, a total yield of 35g crude extract was obtained and was kept in a desecrator until used for the experiment.

2.3. Experimental Animals

The experimental animals used in this study were 40 Swiss albino mice of both sexes, each weighing 25-30g and aged 8-10 weeks. They were bred in the animal house of the Department of Drug Research, EHNRI. The mice were randomly distributed into four groups (group I, II, III & IV) each with 10 mice (five male and five female) per cage. All mice were maintained on a 12h light/dark cycle, at constant temperature (21^oc) and humidity with free access to water and food. They were all acclimatized prior to drug administration.

Group I and II were given 0.5ml/mouse of the aqueous extract at a dose of 100mg/kg body wt /day and 400mg/kg body wt /day, respectively for 44 consecutive days. The time table for the chronic treatment was according to WHO (2000). Group III was given 0.5ml/mouse of the aqueous root extract at the dose of 1134 mg/kg body wt /day which was its LD_{50} (Lethal dose that kills half of the animals tested) as determined by Belay (2008, unpubl. Data). Group IV were administered 0.5ml/mouse of distilled water (DW) and considered as control. In all cases, administrations of the extracts or distilled water were carried out using intragastric catheter. All the doses used for the experimental groups were those found to be effective against plasmodium berghie (Belay, 2008). Body weight of each animal was recorded just before the first day and after the last day of administration of the extract according to Tedong *et al*., (2007). Body weight taken on the first day of oral administration was considered initial weight and the weight taken on the last day of administration was considered final weight.

After 24 hours of the last day of extract administration the animals in Groups I, II and IV were sacrificed using diethyl ether inhalation in desiccators Jar. Blood for each animal was immediately withdrawn by cardiac puncture into test tubes with and without EDTA (Ethylenediaminetetraacetic acid). Blood samples from EDTA containing test tubes were immediately analysed for some hematological parameters. Blood samples from test tubes with no EDTA were allowed to clot and sera were obtained by centrifuging at 5000 revolution per minute for five minutes (Ogbu and Okechukwu 2001). The sera were used to analyse liver and kidney function tests. Following blood samples collections the right part of the liver and the right kidney were dissected out from mice in Groups I, II and IV. The selection to use the right part of the liver and the right kidney was random. As the mice in Group III died within 24 hours, only the right part of the liver and the right kidney were collected.

2.4. Tissue processing

Pieces of tissue samples from the right lobe of liver and the right kidney were immersed in 10% buffered neutral formalin (pH=7.0) overnight at room temperature. After overnight fixation, the tissue samples were washed for 6-8 hours in tap water then after, the tissue samples were dehydrated with graded series of alcohol: one hour each in 70% alcohol, 80% alcohol, 95% alcohol and absolute alcohol-I, and two hours in absolute alcohol-II. The tissues were cleared with two changes of xylene, one hour each. The tissues were then infiltrated with two changes of paraffin wax, for one and half hour each. Upon completion of infiltration, the tissues were embedded in the paraffin wax. All tissue blocks were labeled and placed in refrigerator until sectioned.

Tissue blocks were sectioned with a thickness of 5µm using Leica rotary microtome. After the sections were appropriately spread on a water bath, they were mounted on slides coated with egg albumin to maximize surface adhesion. The slides were arranged in slide racks and were placed in an oven with a temperature of 60^{o}c for 10-15 minutes. The tissue sections were then cooled dried and stained with routine Hematoxylin and Eosin staining method (H and E).

3. RESULTS

3.1. Effects of aqueous root extract of *Clerodendrum myricoides*

3.1.1. The behavioral changes of the experimental animals

Observation for any behavioral changes after treatment with the aqueous root extract revealed that there was no behavioral change at 100mg/kg body weight/day treatment as compared to the controls. However, the mice treated with 400mg/kg body weight/day of the aqueous extract showed some behavioral changes as compared to the controls. These changes were: hypoactivity, grooming and irritation during the administration of the drug after a month. Moreover, one mouse died on the 37th day of administration of the extract in the 400mg/kg body weight/day aqueous extract treatment study. Mice treated with a single dose administration of 1134mg/kg body weight/day aqueous extract showed behavioral changes which include: horripilation, difficult to breathe, grooming, and asthenia which were followed by the death of animals after some hours.

Table 1. Comparison of body weight gain among aqueous root extract of *Clerodendrum myricoides* treated groups, at doses of 100mg/kg body weight/day, 400mg/kg body weight/day, and control mice. Values are mean±SEM. P*<0.05, N_1= 10/group for 100mg/kg body weight/day and control group, but N_2=9/group for 400mg/kg body weight/day.

Group	***Initial weight (in g)***	***Final weight (in g)***	***Weight difference (final weight-initial weight) (in g)***	***% of body weight gain***
Control (DW)	26.43 ± 0.86	33.25 ± 3.26	6.82 ± 3.60	25.8
100mg/kg body weight/day (Aqueous)	28.40 ± 1.17	31.47 ± 2.38	3.07 ± 1.93*	10.8
400mg/kg body weight/day (Aqueous)	28.08 ± 1.65	24.9778 ± 1.71	-3.10 ± 3.15*	-11.0

3.1.2. The body weight of mice

The relative body weight gain in the mice treated with the aqueous extract was lower as compared with the controls. The body weight gain for mice treated with 100mg/kg body weight/day was 3.07gm which was significantly different from the controls which was 6.82 gm

(P<0.05). On the other hand, mice treated with 400mg/kg body weight/day aqueous extract has lost 3.10gm (Table 1). This was also statistically significant (P<0.05).

Table 2. Effects of the aqueous root extract of *Clerodendrum myricoides*, at doses of 100mg/kg body weight/day and 400mg/kg body weight/day on hematological parameters. Values are mean±SEM. P*<0.05, N_1= 10/group for 100mg/kg body weight/day and control group, but N_2=9/group for 400mg/kg body weight/day, DW= distilled water.

Haematological Parameters	***Control (DW)***	***100 mg/kg bwt/day (Aqueous)***	***% of mean diff.***	***400 mg/kg bwt/day (Aqueous)***	***% of mean diff.***
RBC (M/UL)	5.89 ± 0.49	6.51 ± 0.72*	10.53	5.53 ± 0.61	-6.11
WBC (K/UL)	6.92 ± 1.44	4.50 ± 2.02*	-34.97	3.51 ± 0.77*	-49.28
Platelets (K/UL)	343.30± 82.06	353.50 ±1 30.07	2.97	288.78 ±78.32	-15.88
HGB (g/dl)	11.00 ± 0.71	11.44 ± 1.91	4.00	9.84 ± 1.37	-10.55
HCT (%)	42.05 ± 5.40	38.29 ± 5.85	-8.94	32.20 ± 5.65*	-23.42
MCV (fl)	85.32 ± 3.00	83.78 ± 3.26	-1.80	54.91 ± 16.51*	-35.6
MCH (pg)	28.78 ± 1.25	28.83 ± 1.71	0.17	25.69 ± 3.89*	-10.75
MCHC (g/dL)	34.60 ± 2.12	35.41±2.36	2.34	32.43±3.45	-6.27
Lymphocyte (%)	47.24 ± 5.99	53.54 ± 23.47	13.33	65.31 ± 14.69*	38.25

3.1.3. Hematological parameters

Different values of hematological parameters were obtained between the controls and the aqueous extract treated groups. Such differences were, however, found to be not statistically significant for platelets, HGB (Hemoglobin) and MCHC (Mean corpuscular hemoglobin concentration) in mice treated at both doses of 100 and 400mg/kg body weight/day aqueous extracts. WBC (white blood cell) count was decreased (P<0.05) by 34.97% and 49.28% in mice treated with aqueous extract, respectively at 100 and 400mg/kg body weight/day doses as compared to the control mice. RBC (Red blood cell) count was significantly increased (P<0.05) by 10.53% in mice treated with 100mg/kg body weight/day of the extract, while no significant change was observed at 400mg/kg body weight/day aqueous extract treatment. In the contrary, HCT (Hematocrit), MCV (Mean corpuscular volume), MCH (Mean corpuscular hemoglobin) and lymphocytes values were not significantly different in mice treated with 100mg/kg body weight/day aqueous extract, while these were significantly different (P<0.05) at the dose of 400mg/kg body weight/day aqueous extract as compared to the controls. The values of HCT, MCV and MCH significantly decreased by 23.42%, 35.60% and 10.75%, respectively while that

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of lymphocyte was significantly increased by 38.25% as compared to the controls. The effect of the extract on the hematological parameters is shown in table 2.

3.1.4. Biochemical parameters

In general the biochemical parameters studied were found to increase in the aqueous root extract treated mice at both doses as compared to the control mice. Values of AST (Aspartate aminotransferase), ALT (Alanin aminotransferase) and ALP (Alkaline phosphatase) significantly increased ($P<0.05$) by 214.53, 537.13 and 141.95% respectively in the mice treated with doses of 400mg/kg body weight/day as compared to the controls. Such increases were insignificant with the 100mg/kg body weight/day aqueous extract treatment. In addition, there were significant increment ($P<0.05$) of urea at both 100 and 400mg/kg body weight/day as compared to the controls by 282.46 and 917.33% respectively (Table 3).

Table 3. The Effect of the aqueous root extract of *Clerodendrum myricoides*, at doses of 100mg/kg body weight/day and 400mg/kg body weight/day on biochemical parameters. Values are mean±SEM. P*<0.05, N_1= 10/group for 100mg/kg body weight/day and control group, but N_2=9/group for 400mg/kg/day. DW= distilled water.

Biochemical parameters	***Control (DW)***	***100mg/kg body weight/day (Aqueous)***	***% of mean difference***	***400mg/kg body weight/day (Aqueous)***	***% of mean difference***
AST (IU/L)	25.40 ± 5.21	44.00 ± 33.45	73.22	79.89 ± 69.83*	214.53
ALT (IU/L)	9.40 ± 3.60	8.90 ± 4.04	-5.32	59.89 ± 22.92*	537.13
ALP (IU/L)	107.00± 32.33	127.00 ± 76.46	18.69	258.89 ± 276.06*	141.95
Urea (mg/dL)	15.40 ± 4.81	58.90 ± 52.73*	282.46	156.67 ± 54.77*	917.33

3.1.5. Histopathology of the liver

Histopathological examinations of liver sections under light microscope revealed that there were several changes in the mice treated with all doses of the aqueous extract as compared with the control group. These changes include: inflammations around central vein in the 100mg/kg body weight/day treated mice (Fig 1A); cellular infiltrations and severe cytoplasm vacuolations (hydropic degenerations) of hepatocytes around central veins in which their nuclei were stained pale and pushed to the periphery in the 400mg/kg body weight/day aqueous extract treated mice (Fig 1B); and remarkable cytoplasmic vacuolations of hepatocytes around central veins in which their nuclei were stained pale and pushed away from the center were observed at 1134mg/kg

body weight/day treatment were observed (Fig 1C). In the liver sections of the mice from the control group no inflammations and cytoplasmic vacuolations was observed (Fig 1D).

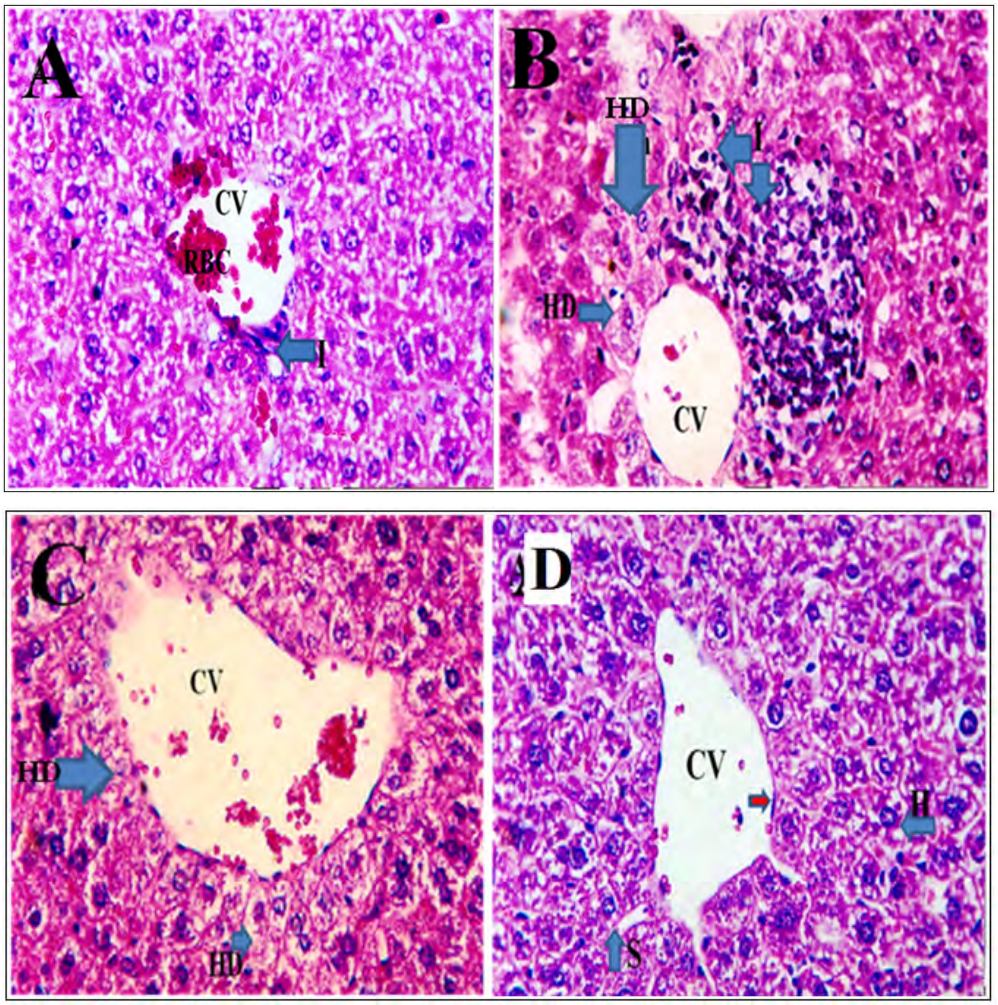

Figure 1. Photomicrographs of H and E stained liver sections from mice treated with aqueous root extracts of *Clerodendrum myricoides* at 100mg/kg body weight/day (A), 400mg/kg body weight/day (B), 1134mg/kg body weight/day (C) and control mice (D). Observe the following changes in the sections from the aqueous extract treated mice: inflammations around portal triads (I) in mice treated at 100mg/ kg body weight/day (A); inflammations around central vein (I), sever hydropic degenerations (HD), in mice treated at 400mg/kg body weight/day (B); sever hydropic degenerations (HD) in mice treated at 1134mg/kg body weight/day (C); and While there was no histopathological changes visible in the sections of the control mice (D). CV in A, B, C & D = Central vein, HD in B & C= Hydropic degenerations, I in A and B = Inflammations, RBC in A= Red blood cell. *Red arrow* in D indicates endothelial cells (Magnifications, all ×4200).

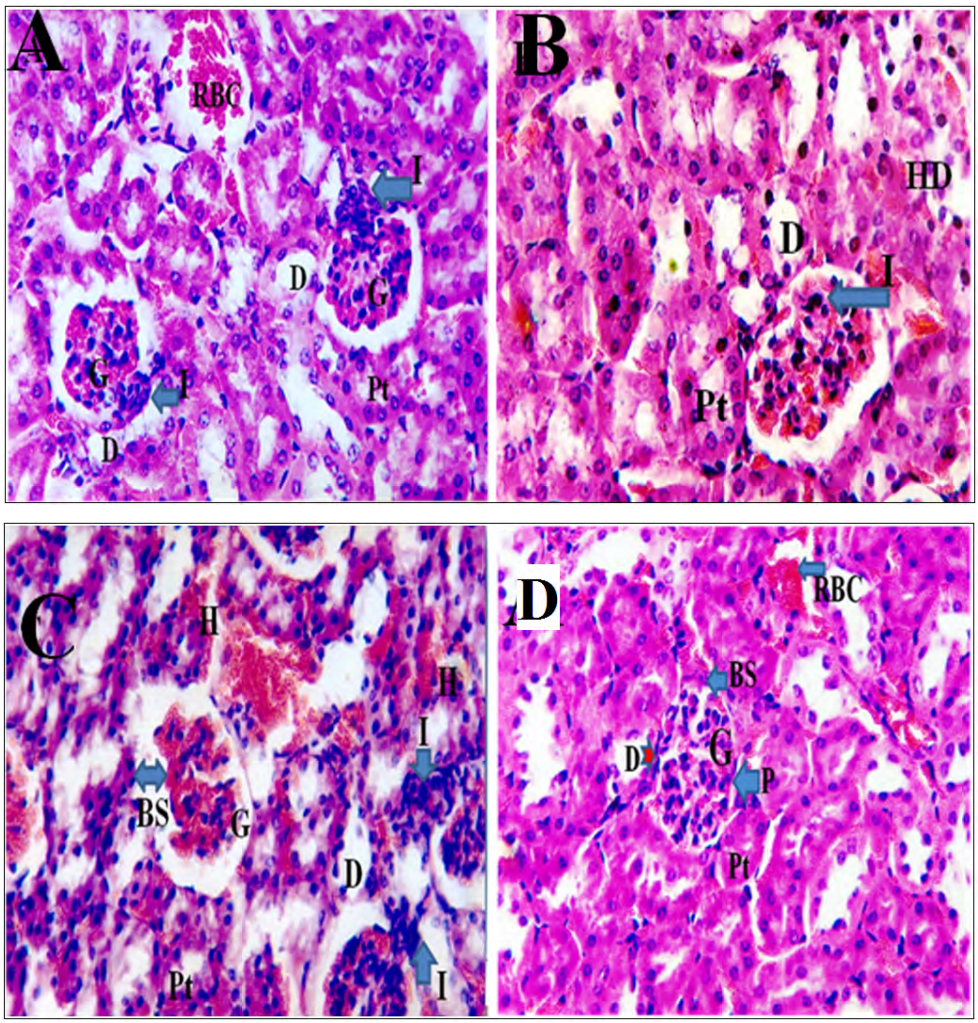

Figure 2. Photomicrographs of H and E stained kidney sections from the mice treated with aqueous root extracts of *Clerodendrum myricoides* at doses of 100mg/kg body weight/day (**A)**, 400mg/kg body weight/day (**B)**, 1134mg/kg body weight/day (**C),** and control mice (D). Observe the following changes in the sections from the extract treated mice: inflammations around the glomeruli and distal convoluted tubules (**I**) and signs in congestion of the glomeruli in mice treated at doses of 100mg/kg body weight/day (**A)** and 400mg/kg body weight/day (**B)**; haemorages (**H**), atrophied glomeruli and inflammations around distal convoluted tubules (**I**) in mice treated at 1134mg/kg body weight/day (**C)**. While there was no histopathological changes visible in the sections of the control mice (**D).** *Red arrow* in **D** indicates macula densa, BS in **C& D**= Bowman's space, D in all photomicrograph= Distal convoluted tubule, G in all photomicrograph= Glomeruli, H in **C** = Hemorrhage, HD in **B**= Hydropic degenerations, I in **A**, **B** & **C**= Inflammatory cells, Pt in all photomicrographs= Proximal convoluted tubule, RBC in **A**= Red blood cell, P in **D=** Podocyte (Magnifications: A – D = ×4200).

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3.1.6. Histopathology of the kidney

Several histopathological changes were observed in the kidneys of the mice treated with the aqueous extract as compared with those of the control mice. Such changes include: cellular infiltrations around the endothelial cells of glomeruli and the distal convoluted tubules, and signs of congestion in the glomeruli at 100mg/kg body weight/day treatment (Fig 2A); inflammations and signs of congestion in the glomeruli at 400mg/kg body weight/day treatment (Fig 2B); and atrophied, signs of congestion, hemorrhage and cellular infiltrations in the glomeruli and around distal convoluted tubules at 1134mg/kg body weight/day treatment (Fig 2C). None of the histopathological changes described above was observed in the kidney sections from the control mice (Fig 2D).

4. DISCUSSION

In this study it was found that mice treated with 100mg/kg body weight/day of the aqueous root extracts showed no distinct behavioral changes, while those treated with 400mg/kg body weight/day of the aqueous extract showed multiple behavioral changes including: hypoactive-asthenia, grooming and react vigorously during the oral route administration just after a month. Similarly, mice treated with a single dose administration of 1134mg/kg body weight/day (LD_{50}) aqueous root extracts showed similar multiple behavioral changes like reduction of motor activity, sedation, horripilation, asthenia, grooming, pileroerection and difficult to breathe after two hours of the drug administration. Behavioral changes such as hypoactive-asthenia and prostration were previously reported following acute & chronic treatment of *Dimorphandra mollis* (Feres et al., 2006).

The observed reduction in the activity and behavioral changes of the mice at higher doses of the extract may be due to the reduction of food intake by the mice which probably has affected the function of many organ-systems of the mice.

At the end of the experiment, both the 100mg/kg body weight/day aqueous extract treated and control mice gained body weight, while mice treated with the extract at 400mg/kg body weight/day lost body weight. The body weight gain by the 100mg/kg body weight/day aqueous extract treated mice was, however, much lower than that of the controls indicating the

suppression of the weight gain by the extract. Such changes in the body weight gain as compared to the controls is in line with the results of toxicity studies following treatments of aqueous extract of *Vernonia amygdalina* (Amole et al., 2006). The suppression of the weight gain in a dose dependent manner may be attributed to the presence of anorexigenic compounds in the extract.

Except platelets, HGB and MCHC, other hematological parameters significantly changed with the extract on the dose dependent manner. The increased number of lymphocytes in the extract treated mice may show the response of the different tissues to injuries or sign of underlying problems as the result of the extract treatment. Reduction in most of the different hematological parameters following extract administration observed in the present study are also found in other related toxicological studies by other workers, on *Bougainvillea spectabilis* leaves administration in rats (Adebayo et al., 2005), and treatment of rabbits with *Fagonia cretica* L. constituents (Saeed and Sabir, 2002). The reduction in WBC count with higher doses of the extract may be due to direct destructive effect of the extracts on these cells or their impaired production in the hematopoietic tissue (bone marrow). Moreover, the reduction of WBC following the extract treatment might be because of the mobilization of the leukocytes to the surrounding tissue outside the blood.

Like the changes in the body weight and hematological values, the increments of the biochemical parameters were on dose dependent manner. Similar trends of increments in these biochemical parameters were also observed by other researchers with aqueous extract of *Tithonia diversifolia* in rats (Oyewole et al., 2007), and *Dimorphandra mollis* in rats and mice (Feres et al., 2006).

AST, ALT and ALP are the most widely used means of measuring hepatocellular injuries, although the precise levels of these enzymes do not correlate well with the extent of liver damage (Sforcin et al., 2002). In addition, increased AST, ALT and ALP are usually seen in conditions involving necrosis of hepatocytes, myocardial cells, erythrocytes, or skeletal muscle cells (Sforcin et al., 2002). Urea level in blood is also one of the determinant indicators for kidney damage (Feres et al., 2006). Usually urea is increased in acute and chronic intrinsic renal diseases, which is characterized by decrement in effective circulating blood volume within the kidney. The tremendous increment of these biochemical parameters observed in the aqueous root

extract treated mice in this study may, therefore, reflect liver and kidney damage caused by the extract.

The present study has also found the presence of histopathological changes in the liver and kidney tissue of the extract treated mice. There were cytoplasmic vacuolations (Hydropic degenerations) in the hepatocytes located towards the periphery of the hepatic lobules around the central veins in the 400mg/kg body weight/day of the aqueous extract and LD_{50} dose extract treated mice. Cytoplasmic vacuolations were also observed in the interstitial cells of the kidney of the mice that received 400mg/kg body weight/day and LD_{50} dose of the extracts. Such cytoplasmic vacuolations are said to occur when the cytoplasm becomes pale and swollen due to accumulation of fluid or lipids as the result of disturbance in lipid inclusions and fat metabolism (Ebaid et al., 2007). It could also occur when there is disturbance to the functions of ribosomes, uncoupling of lipid from protein metabolism or cytoplasmic alterations produced to collect the injured substances in the cell (Ebaid et al., 2007). In the kidney hydropic changes may appear whenever cells are incapable of maintaining ionic and fluid homeostasis as the result of loss of the function of plasma membrane energy-dependent ion pumps (Robbins and Cotran, 2004). It also occurs in hypoxic injury and various forms of toxic or metabolic injuries (Robbins and Cotran, 2004). It is, therefore, possible that the observed cytoplasmic vacuolations in the hepatocytes and interstitial cells of the kidney may have been caused by one or combinations of the above disturbance as the result of the extract treatments due to to the effect of the presence of the secondary metabolites in the extracts. These include: alkaloids, flavonoides and phenols (Belay, 2008) which contain free radical scavenging molecules that can cause substantial biological damage of the mice tissues (Rodriguez et al., 2008).

There was glomeruli atrophy in mice treated with higher dose than lower dose of aqueous root extracts. There were also signs of congestion and hemorrhage in the kidney of the LD_{50} of extract treated mice. The atrophy of the glomeruli may be due to sluggish circulation in the glomeruli or tissue hypoxia. Similarly, the signs of congestion and hemorrhage of the glomeruli may suggest, impaired out flow of venous blood from the tissue and sever vascular injury or depletion of coagulation factors respectively as the dose of the drug increases.

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The other histopathological changes observed were focal inflammations in the liver sections located around the portal triads in the 100mg/kg body weight/day, and around central veins in 400mg/kg body weight/day aqueous extract treated mice. In addition, such inflammations were observed near the nephrons of the kidney sections of the mice treated with both doses of the aqueous extracts. The increased inflammatory reactions observed in the present study may, therefore, be associated with the cellular and tissue damage caused by the extract in both liver and kidney, suggesting that these two organs are prone to be damaged by the extracts. Histopathological changes in these two organs were also reported by others following various herbal extract administrations in experimental animals (Sozmen et al., 2005; Effendy et al., 2006; Hassan et al., 2006; Dapar et al., 2007; Diwan et al., 2007).

In conclusion chronic treatments with *Clerodendrum myricoides* extracts in mice cause reduction in body weight gain, damage to liver & kidney and changes in some hematological & biochemical parameters in mice. Further studies are required to isolate and identify the active constituents of the roots of *Clerodendrum myricoides* for the observed changes as well as to elucidate the mechanism(s) of their toxic effect.

5. ACKNOWLEDGEMENTS

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