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RESEARCH ARTICLE

SUBCHRONIC TOXICITY STUDIES OF BUTANOL FRACTION OF LEAVES OF *MORINGA STENOPETALA* IN EXPERIMENTAL RATS

Abdu Hassen Musa[1*], Prabhanjan Kumar Vata[2], Girmai Gebru[3], Yalemtsehay Mekonnen[4], Asfaw Debella[5] and Eyasu Makonnen[6]

[1,2]College of Health Sciences and Medicine, Dilla University, Dilla, Ethiopia
[3]Department of Human Anatomy, Medical Faculty, Addis Ababa University, Addis Ababa, Ethiopia
[4]Department of Biology, Faculty of Science, Addis Ababa University, Addis Ababa, Ethiopia
[5]Ethiopian Public Health Institute, Addis Ababa, Ethiopia
[6]Department of Pharmacology, School of Medicine Addis Ababa University, Addis Ababa

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ABSTRACT

Moringa stenopetala belongs to the family Moringaceae, and the family comprises a single genus Moringa. The genus Moringa comprises about 14 different species and is endemic to the Northeast tropical Africa. *Moringa stenopetala* is often referred to as the East African Moringa tree because it is native only to Southern Ethiopia and Northern Kenya, and is not as widely known as its close relative, Moringa oleifera of India. *M. stenopetala* is cultivated in terraced fields, gardens and small towns.

The current study was conducted as an laboratory based experiment, on experimental rats. The study has been carried out in School of Medicine, College of Health Sciences, Addis Ababa University (AAU), and Ethiopian Health and Nutrition Research Institute (EHNRI). The chronic toxicity study was conducted, based on the OECD 408 guideline (OECD, 1998), for nine weeks (63 days) to examine the toxicity of the fraction on some blood parameters and histopathology of the liver and kidneys. For this study healthy adult rats of both sexes were used. The present study was aimed to investigate the chronic toxic effect of the butanol fraction of leaves of *Moringa stenopetala* in experimental rats. The present study also helps us to know the histopathological changes due to butanol fraction of leaves of *Moringa stenopetala* in experimental rats.

The sub-chronic toxicity study was carried out by oral administration of the butanol fraction of the leaves at 500mg/kg and 1000mg/kg, for group II and III rats respectively, while the control group (I) received distilled water. Throughout the study period the general appearance and behavior of treated rats showed no significant differences as compared with the controls. Liver and kidneys of rats are used by many researchers to assess the safety or toxicity of drugs or plant materials (Graaf, 1995 and Satyapal *et al*., 2008.)

In the chronic toxicity study, results showed that the fraction did not produce adverse effects. These findings indicate that chronic exposure to the fraction does not lead to toxicity. The fraction did not significantly, induce severe toxic effects on the gross and histopathology of the liver and kidneys of treated rats, except infiltration of inflammatory cells around the portal area of the liver and Bowman's capsule of the kidney sections.

Further a brief bio chemical and hematological study has to be conducted to know in deep the effect of butanol fraction of leaves of *moringa stenopetala* in experimental rats.

INTRODUCTION

Medicinal plants have a long history and are still the mainstay of the world population for primary health care. Medicine from medicinal plants may come from any parts such as leaves, roots, bark, seeds, and flowers. The secondary metabolites include alkaloids, glycosides, polyphenols, tannins, phytosterols and with anoids.

Moringa stenopetala belongs to the family Moringaceae, and the family comprises a single genus Moringa. The genus Moringa comprises about 14 different species and is endemic to the Northeast tropical Africa. *Moringa stenopetala* is often referred to as the East African Moringa tree because it is native only to Southern Ethiopia and Northern Kenya, and is not as widely known as its close relative, Moringa oleifera of India. *M. stenopetala* is cultivated in terraced fields, gardens and small towns.

Many chemical compounds have been isolated from the leaves of *M. stenopetala*. The raw leaves contain proteins, fats, carbohydrates, crude fiber, vitamins such as vitamin C and β-carotene, minerals such as K, Fe, Ca, P, and Zn, in significant

concentrations (Abuye *et al*., 2003).

M. stenopetala is a widely used medicinal plant in the southern region of Ethiopia. There are claims that the leaves can cure malaria, hypertension, stomach pain, diabetes and other ailments (Mekonnen and Gessesse, 1998; Sileshi, 2010; and Toma *et al*, 2012). However, toxicity studies of this plant extract and fractions are limited.

This species (stenopetala) is known by different vernacular names such as Shiferaw (Amharic), Cabbage tree (English), Aleko, Aluko, Halako (Gamo Goffa/ Wollayta), Kallanki (Benna), Haleko, Shelchada (Konso), Telahu (Tsemay), and Haleko (Burgi, Derashe) (Mekonnen and Gessesse, 1998; Edwards *et al*., 2002; Shibru, 2002; Yisehak *et al*., 2011 and Seifu, 2012).

The present study was aimed to investigate the sub-chronic toxic effect of the butanol fraction of leaves of *Moringa stenopetala* in experimental rats. The present study also helps us to know the histopathological changes due to butanol fraction of leaves of *Moringa stenopetala* in experimental rats.

MATERIALS AND METHODS

The current study was conducted as a laboratory based experiment, on experimental rats. The study has been carried out in School of Medicine, College of Health Sciences, Addis Ababa University (AAU), and Ethiopian Health and Nutrition Research Institute (EHNRI).

The chronic toxicity study was conducted, based on the OECD 408 guideline (OECD, 1998), for nine weeks (63 days) to examine the toxicity of the fraction on gross histopathology of the liver and kidneys. For this study healthy adult rats of both sexes were used.

Eighteen rats were randomly distributed into three groups (I, II, &III) each consisting of six rats, three female and three males. The two sexes were kept in separate cages until the end of the study. Group-I served as control group and received distilled water or vehicle, whereas group II & III rats were treated with butanol fraction of the leaves at doses of 500 and 1000mg/kg body weight per day respectively. The doses were prepared based on their body weight and both the vehicle and test substance were administered via oral gavage.

On the 64th day the final weight of the rats were measured and then they were anesthetized under diethyl ether and sacrificed by cervical dislocationand blood samples were collected from each animal by cardiac puncture. The rats were sacrificed by cervical dislocation and parts of the liver and the kidney (they are a valuable indicators in evaluating the toxic effects of medicinal plants) were dissected out and gross pathological observations were performed to check for any gross lesions.

After gross observation, parts of the liver was taken and cut into smaller pieces and the right kidney was hemi-sectioned longitudinally and the left kidney was hemi-sectioned horizontally and placed into 10% neutral buffered formalin solution for 24 hours to preserve and prevent the tissue from degeneration. Then the tissues were washed in running tap water overnight to prevent over fixation. After washing, the tissues were dehydrated with increasing concentration of ethanol (50%, 70%, & 90%) for 2 hours each and followed with absolute alcohol I, II, & III for 1 and 1/2 hours each and in absolute alcohol IV overnight.

The tissues were then placed into two changes of xylene for clearing the tissues (in xylene-I for 2 and ½ hours and in xylene-II for 1 and 1/2 hours), and then the tissues were infiltrated with melted paraffin wax (wax-I for 2 and 1/2 hours and wax-II overnight). The infiltrated tissues were then placed in proper orientation into molds and the molds were then filled with melted paraffin wax and the wax was allowed to harden at room temperature. The hardened paraffin/ wax block, which contained the tissue conformed the shape of the mold, was then removed from the mold and sliced with a thickness of 4 - 5 µm (for hematoxylin and eosin staining) using a rotary microtome (LEICA RM-2125, Leica Microsystems Nussloch GmbH, Germany) (Bankroft *et al*.,1990).

The paraffin ribbon containing the tissue sections were collected with forceps and laid onto the surface of water bath heated at 40^{o}C. After the sections were appropriately spread on the water bath, they were mounted on the slides. In order to firmly attach the specimens onto the slides, the tissue slides were placed in an oven with a temperature of 60^{O}C for 10-15 minutes. After cooling the slides for 1 and ½ hours, the tissue sections were ready for staining. The staining process was carried out using Hematoxylin and Eosin staining method.

After tissue processing is done the slides was examined under a compound light microscope in laboratories of the Anatomy and Pathology Department, at AAU. Some of the slides were selected and their photographs were taken by microscope fitted with a digital camera (Nicon Coolpix-5000, Germany). Magnifications of 200X and 400X was used to examine the liver and kidney tissues for investigation of any histopathological changes.

The values after experiment , were expressed as Mean+SEM and statistical differences between the means of different groups were evaluated by a one- way analysis of variance (ANOVA) using the SPSS version 20 program followed by a Dunnet's t- test. P-values less than 0.05 were considered statistically significant.

RESULTS

The sub-chronic toxicity study was carried out by oral administration of the butanol fraction of the leaves at 500mg/kg and 1000mg/kg, for group II and III rats respectively, while the control group (I) received distilled water. Throughout the study period the general appearance and behavior of treated rats showed no significant differences as compared with the controls.

Table 1 Comparison of the effects of butanol fraction of the leaves of M. stenopetala on the absolute organ weights

Doses	Liver weight (g)	kidney weight (g)
Control (DW)	9.47 + 0.23	0.80 + 0.01
500mg/kg	9.68 + 0.29	0.82 + 0.02
1000mg/kg	11.06 + 1.05	0.86 + 0.04

Values are expressed as Mean ± SEM, n= 6/group

Throughout the study period no mortality occurred in the treated groups. Gross observation of the liver and kidneys of the treated rats showed no significant changes compared with the control group (figure-1); and no significant difference

(p>0.05) were observed in the absolute organs weight between the control and treated groups (Table 1). The mean absolute weights of the liver were 9.68 + 0.29 g (at 500mg/kg) and 11.06 + 1.05 g (at 1000mg/kg), as compared with the control (9.47 + 0.23 g). The mean absolute weights of the kidneys were 0.82 + 0.02 g (at 500mg/kg) and 0.86 + 0.04 g (at 1000mg/kg), as compared to the control (0.80 + 0.01 g). As summarized in table 2, there was a slight increment in the mean absolute weight of liver and kidneys of the treated rats, though, were not statistically (p>0.05) significant as compared with the control groups.

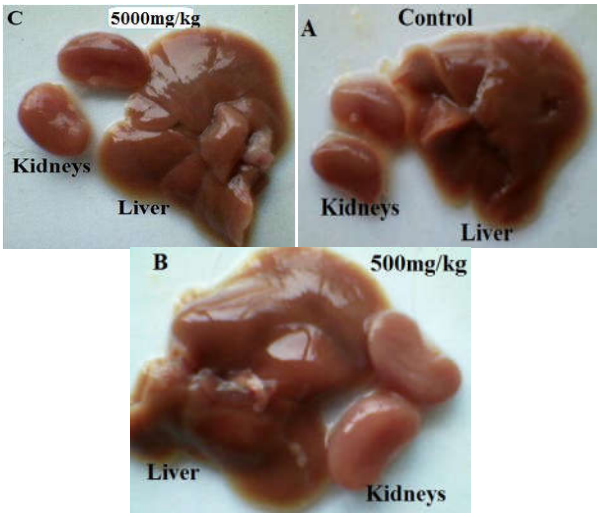

Figure 1 Photograph of liver and kidneys during sub-chronic toxicity study

During the experimental period all groups of rats showed gradual increase in their body weight (Table 2). The initial mean body weight of control group was 191.38 + 13.56 g, and final mean body weight was 251.27 + 11.55 g. The initial mean body weight of rats treated with the dose of 500mg/kg was 182.75 + 12.12 g, and final mean body weight was 245.04 + 8.21 g. The initial mean body weight of rats treated with the dose of 1000mg/kg was 172.39 + 6.39 g, and the final mean body weight was 241.04 + 6.74 g.

Table 2 Comparison of the effect of n-butanol fraction of the leaves of M. stenopetala on body weight of treated and control rats during sub-chronic toxicity study

Doses	Initial body weight (g)	Final body weight (g)	Mean weight increment (g)	% of body weight gain
Control (DW)	191.38 + 13.56	251.27 + 11.55	60	31.29
500mg/kg	182.75 + 12.12	245.50 + 8.21	62.5	34.34
1000mg/kg	172.39 + 6.39	241.04 + 6.74	72.65	39.81

Values are expressed as Mean ± SEM, n= 6/group.

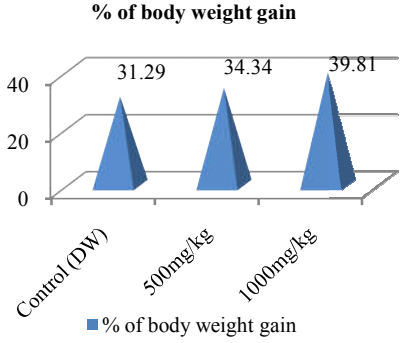

Figure 2 Comparison of percent of body weight gain between treated and control group

As summarized in table 5, the mean body weight gain for rats treated with doses of 500mg/kg and 1000mg/kg were 62.75 g (34.34%) and 72.65 g (39.81%) respectively, as compared with the controls 60 g (31.29%). Both groups of treated rats showed increment in body weight than the control group (Figure 2). However, there was no statistically significant (p>0.05) weight gain differences between the treated and control groups.

Effects of the fraction on histology of the liver

Histopathological studies of the liver sections in the control group (Figure 3 and 4 - A) showed normal appearance of central vein (CV) and hepatic sinusoids (S) lined by endothelial cells (EC) with normal radiating hepatocytes.

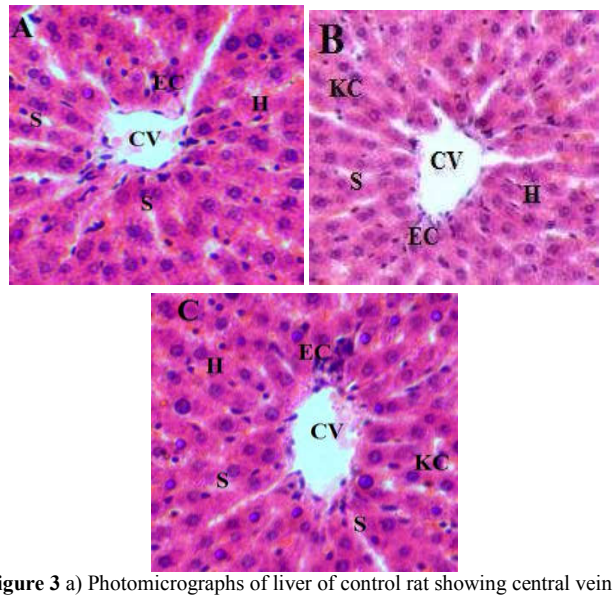

Figure 3 a) Photomicrographs of liver of control rat showing central vein (H and E, X200). b) Rats treated with 500 mg/kg body weight per day of butanol fraction of the leaves of M. stenopetala (H and E, 400x). c) Rats treated with 1000 mg/kg body weight per day of butanol fraction of the leaves of M. stenopetala (H and E, X400).

CV= Central vein, EC=Endothelial cells, H= Hepatocytes, KC=Kupffer cells, S= Sinusoids.

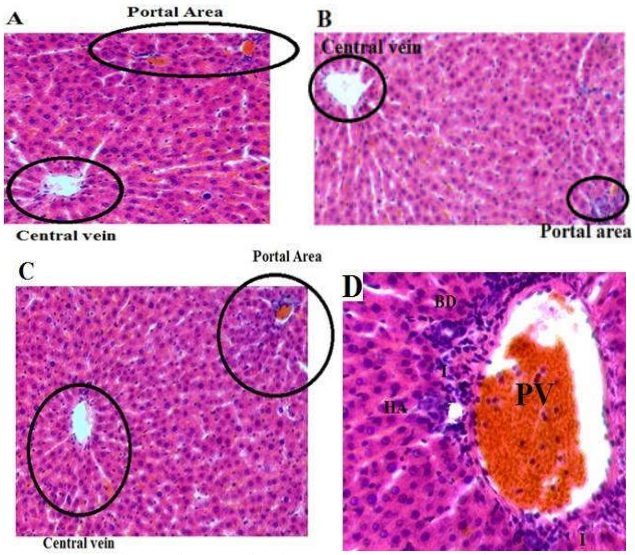

Figure 4 a) Photomicrograph of control rat liver around portal tract and central vein, showing no histopathological changes (H and E, X200). b) Section of rat treated with 500mg/kg of the fraction showing central vein (H and E, X200). C) Section of rat treated with 1000mg/kg of the fraction, showing central vein and mild inflammatory cell infiltration around the portal triad (H and E, X200). d) Portal area of rat treated with 1000mg/kg showing mild infiltration of inflammatory cells (H and E, X400).

BD= Bile duct, HA=Hepatic artery, I=Inflammatory cell infiltration, PV=Portal triad.

There was also normal appearance of the portal triad including hepatic portal vein, interlobular bile duct, and branches of hepatic artery. Rats treated with butanol fraction of the leaves of *M. stenopetala* at both doses of 500mg/kg (Figure 3 and 4 - B) and 1000mg/kg (Figure 3 and 4 - C) showed normal appearance of the central veins (CV) and hepatic sinusoids (S) lined with endothelial cells (E) with normal radiating hepatocytes. However, rats treated with dose of 1000mg/kg (Figure 4 - C and D) showed mild inflammatory cell infiltration around the portal area. However, in both treated groups no significant histopathological changes/abnormalities were noted in the hepatocytes, hepatic sinusoids, central vein, Kupffer and endothelial cells as compared with the controls.

Effects of the fraction on histology of the kidney

In the present sub-chronic toxicity study, histopathological examination of kidney sections of rats treated with doses of 500mg/kg (B) and 1000mg/kg (Figure 5 - C) showed no significant microscopic changes as compared with the controls (Figure 5 - A). As shown in the figure 5-B and C, longitudinal sections of the cortex, revealed normal glomerulus, Bowman's capsule lined with outer parietal layer/squamous cells (SC) and inner visceral layer/podocyte (P), urinary space (US), proximal convoluted tubules (PCTs) lined by simple cuboidal epithelium with brush border, distal convoluted tubules (DCTs) lined by simple cuboidal epithelium with more nuclei per cross-section, macula densa (MD) with taller cells around the vascular pole. However, there was mild infiltration of inflammatory cells around the vascular pole of the Bowman's capsule of both treated groups (500mg/kg and 1000mg/kg).

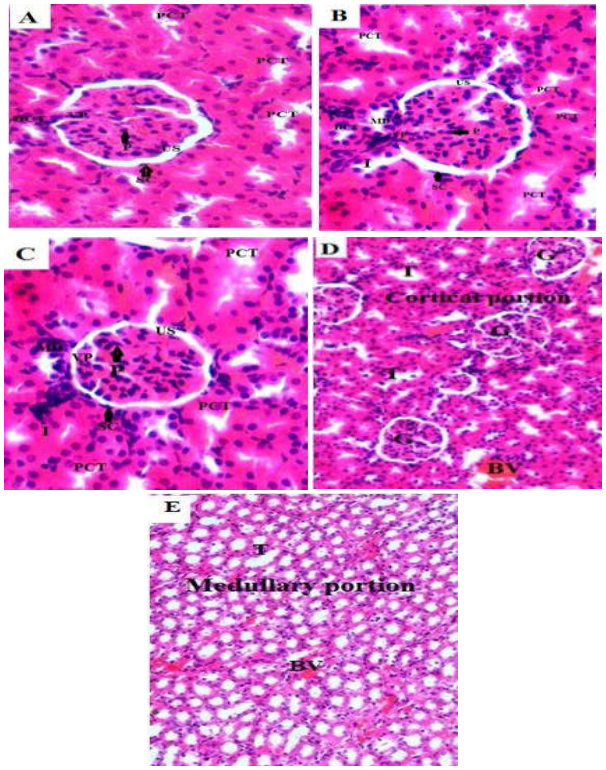

Figure 5 Photomicrographs of the kidney sections of control rats (A), rats treated with 500mg/kg (B), and rats treated with 1000mg/kg body weight per day of butanol fraction of the leaves of M. stenopetala (C) (H&E, 400X). Cortical portion (D) and medullary portion (E) of control group (H&E, X200). PCT=proximal convoluted tubule DCT=distal convoluted tubule MD=macula densa, G=glomerulus, I = Infiltration, US =urinary space, SC=Squamous cell, VP=Vascular pole, P=Podocyte.

Kidney section of the control group (Figure 5 - A, D, and E) showed normal glomerulus, Bowman's capsule, blood vessels and normal tubules.

DISCUSSION

M. stenopetala is a multipurpose tree which grows in the southern part of Ethiopia, and almost, all of its parts are used traditionally for the treatment of various ailments. Its leaves are traditionally used for treatment of diabetes mellitus, malaria, hypertension, asthma, stomach pain, and sometimes to expel retained placenta (Mekonnen and Gessesse, 1998). Fresh leaves are also eaten as vegetables in the southern part of Ethiopia (Mekonnen, 2005). Because of the widespread use of *M. stenopetala* as a food and medicinal plant in the southern Ethiopia, it was felt necessary to investigate the potential toxicity of the extracts and fractions of the leaves on experimental animals. The present study was, therefore, aimed at evaluating the effects of the butanol fraction of the leaves of *M. stenopetala* on histopathology of liver and kidney in laboratory-bred rats.

Liver and kidneys of rats are used by many researchers to assess the safety or toxicity of drugs or plant materials (Graaf, 1995 and Satyapal *et al*., 2008). The rat liver is a large multilobulated gland that represents approximately 5% of the total body weight. In rats weighing between 200 – 300 g, the liver mean weight measures about 6–15 g (Martins and Neuhaus, 2007). The rat kidney is bean- shaped organ that lie in a retroperitoneal position. In rats weighing between 140 – 300 g, the kidney mean weight measures about 0.7 – 1.9 g (Webster *et al*., 1947; Dirikolu *et al*., 2011). Though not significant, the absolute liver and kidney weight of treated rats appeared to increase as compared with the control group (Table 2) perhaps due to presence of active compounds (such as glucosinolates) in the butanol fraction of the leaves of *M. stenopetala.*

During the 63 days of sub-chronic toxicity study the rats that were treated with butanol fraction of the leaves at the doses of 500mg/kg and 1000mg/kg showed no signs of morbidity and mortality. Both treated rats (500mg/kg and 1000mg/kg) were healthy with normal appearance, behavior, and motor activities. During the experimental period no death or apparent behavioral changes were observed as compared with the control group. This might suggest the non-toxic nature of the fraction. The current result was in agreement with the findings of Sileshi, (2010), Ghebreselassie *et al*., (2011), Nardos *et al*., (2011) and Toma *et al*., (2012).

In the gross pathological examination of the liver and kidneys, the treated rats that used to receive the butanol fraction of the leaves, showed no change in color, shape, size, and texture, as compared to the control group (Figure 7). The slight increments, though not significant ($p>0.05$), observed in the absolute weight of liver and kidneys of treated rats (Table 4) might be due to the presence of active compounds (such as glucosinolates) in the fraction which may result in an increase in the weight of these organs. This was in agreement with the findings of Mekonnen and Dräger, (2003) and Ghebreselassie *et al*., (2011), who reported that treatment of rodents with the extract containing glucosinolates did not adversely affect the vital organs. In the sub-chronic toxicity study treated rats that

received the fraction of the leaves of *M. stenopetala* showed gradual increase in their body weight (Table 5 and Table 8).

In this study, administration of butanol fraction of the leaves of *M. stenopetala* showed no harmful effect on body weight of the treated rats. The result was in agreement with the previous studies (Mekonnen, 2005; Jiru, *et al*, 2006; Sileshi, 2010; Ghebreselassie *et al*., 2011 and Toma *et al*., 2012), who reported the non-toxic effects of the leaves on the body weights in rodents.

In the current histopathological examination of the liver, rats treated with doses of 500mg/kg and 1000mg/kg of the butanol fraction of the leaves of *M. stenopetala* showed no significant changes in the microscopic structures of the liver (Figure 3 and 4 - B and C). The general architecture of the liver, appearance of the hepatocytes, the hepatic sinusoids, and the central veins are normal as compared with the controls (Figure 3 and 4 - A). However, rats treated with dose of 1000mg/kg (Figure 4 - C and D) showed mild inflammatory cell infiltration around the portal area. These changes might have resulted from active components of the fraction which might have induced the liver cells to perform various metabolic processes. However, these changes did not alter the general structure of the liver. This finding is in agreement with the findings of Hassen *et al*., (2006), Ghebreselassie *et al*., (2011), and Getachew (2012), who reported that after treatment of mice with fenugreek oil, aqueous extract of *M. stenopetala* and fractionated aqueous extract of G. stenophylla showed mild inflammatory cell infiltrations around the portal triad, and a slight activation of Kupffer cells within the sinusoids.

Thus, it may be concluded that the butanol fraction of the leaves of *M. stenopetala* may have minimal effect on the general architecture of the liver, the hepatocytes, the hepatic sinusoids, and appearance of the central vein. Drugs and drug-products, including plant products, may also cause damage to the kidney components such as the glomeruli, tubules, interstitium and blood vessels. Therefore, it is essential to assess the effect of plant extract and fraction in the kidney components and some metabolic waste products excreted by the kidneys, which will provide useful information about the health status of the kidneys (Robbins and Cotran, 2005).

In the current histopathological study of the kidney, rats treated at both doses (500mg/kg and 1000mg/kg) of the fraction showed no significant changes (Figure 5 – B and C) as compared with the control group (Figure 5 – A, D, and E). The present finding of sections of the kidneys of treated rats showed normal general structure of the kidney, the normal appearance of glomeruli and tubules. The proximal convoluted tubules, distal convoluted tubules and macula densa are intact. However, mild inflammatory cell infiltrations were observed around the glomeruli. This may indicate that some active compounds in the fraction may pose their effect on the kidney that may result in mild inflammation or allergic reaction. However, the fraction did not affect normal histology of the kidney. Therefore, it may be concluded that the butanol fraction of the leaves of *M. stenopetala* did not alter the general architecture of the kidneys.

CONCLUSION

In the Sub-chronic toxicity study, results showed that the fraction did not produce adverse effects. These findings indicate that Sub-chronic exposure to the fraction does not lead to toxicity. The fraction did not significantly, induce severe toxic effects on the gross and histopathology of the liver and kidneys of treated rats, except infiltration of inflammatory cells around the portal area of the liver and Bowman's capsule of the kidney sections. Further a brief bio chemical and hematological study has to be conducted to know in deep the effect of butanol fraction of leaves of *moringa stenopetala* in experimental rats.

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