Toxicological evaluation of methanol leaves extract of Vernonia bipontini Vatke in blood, liver and kidney tissues of mice

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Abstract

Background: Various medicinal plants have been studied using modern scientific approaches. These plants have a variety of properties and various biological components that can be used to treat various diseases. However, harmful effects of plants are common clinical occurrence.

Objective: This study was designed to investigate toxicological assessment of acute and chronic methanol leaf extract of Vernonia bipontini Vatke (V.bipontini V) on blood, liver and kidney tissues of mice.

Methods: Lethal dose (LD) at which 50% of experimental mice died and long term toxicity of methanolic leaf extract of V. bipontini V were determined. Some hematological and biochemical parameters were evaluated. Then, liver and kidney tissues of each animal were taken and processed for light microscopy.

Results: Almost all mice treated with 800mg/kg methanol leaf extract of V. bipontini V showed swellings on the left part of abdominal region related to location of spleen, mild diarrhea and enlargement of spleen. The LD50 of the methanol leaf extract of V. bipontini V was 2130.6±1.5mg/kg. Treatment with 800mg/kg body weight of methanol leaf extract significantly decreased body, liver and kidney weights, red blood cells (RBC), haemoglobin (Hgb), mean cell haemoglobin (Mch), Mchc, platelet and significantly increased serum aspartate transferance (AST), vatanine tranferance(ALT) and alkaline phosphate (ALP) levels while 400mg/kg dose had no effect on these parameters. The reduced organ weights did not correlate with loss of body weight at 800mg/kg of methanol leaf extract of the plant. Light microscope observations of liver tissue of mice treated with 800mg/kg of the methanol leaf extract revealed dilated sinusoids, nuclear enlargement, lots of bi-nucleation of hepatocytes, peripheral cramped chromatin, shrinkages (single cell death) of hepatocytes, fragmentation of hepatocytes while no histopathological changes were observed in liver and kidney of mice treated at 400mg/kg. Kidney tissue sections of mice did not show significant histopathological changes at 400mg/kg. However, at 800mg/kg kidney sections showed increased cellularity of glomerulus, urinary space obliteration and enlarged macula densa.

Conclusion: This study suggests that the methanol leaf extract may have been phytotoxic to liver that resulted in a rise in serum AST, ALT and ALP levels.

Key words: V. bipontini Vatke, Swiss Albino mice, liver, kidney, methanol, hematological and biochemical

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Introduction

Herbal medicine has become a topic of global importance and plays a central role in the health care system of large proportions of world's population[1]. The traditional use of plants in the treatment of different infections is widely practiced in developing countries. Ethiopian medicinal plants have also shown very effective medicinal value for treatment of some ailments of human and domestic animals.[2] Therefore, various medicinal plants have been studied using modern scientific approaches because many medicinal plants have a variety of properties and various biological components that can be used to treat various diseases[3] on the contrary, harmful effects of plants are common clinical occurrence[4].

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V. bipontini V is an herb claimed to be useful for the treatment of malaria and malaria related symptoms, and it was found to be effective at 400mg/kg/day of mice, and its inhibitions against Plasmodium berghei in both aqueous and methanol leaf extracts were 52.7% and 40%, respectively[5]. In Ethiopia, among medicinal plants, people use V. bipontini V against malaria[5]. Aqueous leaf extract of V. bipontini V may be safe even, when taken for 45 days at a dose of 800mg/kg[6]. V. bipontini V is a rarely erect and too straggly, woody herb, of 0.6-1.5m height. It is found in Shewa region above and to the west of 1000m up and down[7], Bale namely Delomenna and it is locally known as "Reji"[5]. Specimens of V. bipontini V, are found at Addis Ababa University Herbarium collected from different areas, are also indicated that it is widely distributed in the flora of Ethiopia and Eriterea such as Tigray, Asmara, Gondar, Wollo, Shewa, Ambo, Muger, Bale and Debre Birhan road.

V. bipontini V traditionally has the following indigenous use: significant anti- malaria, anti-spasmodic, anti-snake bite, anti-venereal diseases, purgative, and vermifuge[5]. People living in areas, where V. bipontini V grows use methods of water preparation of the plant leaves for treating malaria and malaria related symptoms and take in the form of drinking. From the species of Vernonia, V. amygdalina is one of the pharmacologically useful plants. Both aqueous and alcoholic extracts of the stem- bark, the roots, and the leaves of V. amygdalina are also reported to be extensively used as anti-malaria, purgative, and in the treatment of ecezema[8]. Microscopic observation of the tissue sections of liver and kidney has showed no morphological abnormalities as compared to the controls after 42 and 45 days of oral administration of aqueous leaf extract of V. amygdalina[9] and V. bipontini V[6], respectively. Histopathological studies of this plant didn`t reveal pathological lesions in the liver and kidneys even at 800mg/kg[6].

The phytochemical analysis indicated that the presence of antioxidant agents(sesquiterpene) such as saponins, flavornoids, oxalates, alkaloids and vernoniosides (glucosides) in the methanolic leaf extract[10-12]. However, in aqueous leaf extract vernodalin, vernolide, hydroxyvernolide, and glucosides (vernonioside) in related species (V. amygdalina)[13]. Nwanjo[12] in his study also showed the presence of tannins in addition to alkaloids, saponins, flavonoids, and glycosides chemical constituents in the fresh leaf of related plant (V. amygdalina). According to Diwan[14] the presence of saponin in the extract can cause from mild to severe diarrhea. The availability of active ingredient (Dichloromethane/DCM) in methanol leaf extract that can induce enlargement of spleen has also indicated in V. scorpioides (Lam.) Pers[15].

Materials and methods

Plant material collection and methanol extraction

The fresh leaves of V. bipontini V plant were collected based on Ethno-botanical description and with the help of local traditional healers around Shisha River in Harenna forest, Delomenna awraga, Bale region 524 kms southeast of Addis Ababa in December 2007. This plant grows with celtis, croton, and syzygium plants. The specimen of collected plant was identified and deposited at Herbarium of Department of Drug Research (DDR), Ethiopian Health and Nutrition Research Institute (EHNRI) and National Herbarium of the department of Biology, Addis Ababa University with a Voucher number of 05/MEB. Powdered V. bipontini V (1.8 kg) was macerated with 80% methanol for 48 hours with intermittent agitation by Orbital shaker DS-500. The supernatant part of agitated material filtered with 15 cm Whatman grade1 filter paper two times. The filtrate of V. bipontini V was then concentrated using Rotary evaporator (BÜCHI R-250) at 41oc to remove 80% methanol and further dried using Water Bath (BÜCHI B-490) to remove 20% of water. A yield of 15.5g of crude extract (0.86%) was obtained from 1.8kgs of dry powder and kept in refrigerator till use.

Experimental animal preparation

The animals used for this study were adult male and female Albino Swiss mice (25-35g). The mice were obtained from Department of Drug Research (DDR), Ethiopian Health and Nutrition Research Institution (EHNRI) and bred in the Research Animal Breeding Laboratory of DDR, EHNRI at Addis Ababa, Ethiopia. The mice were acclimatized to a laboratory condition for a week before the commencement of the experiment. Mice of the same sex were grouped into 8 experimental and 1 control groups for LD50 determination, and 2 experimental and 1 control groups for long term administration of methanol leaf extract. Finally, all mice were housed in common metallic cage under 23±20C. They had unrestricted access to a standard pellet diet and tap water. The animals were maintained under 12 hours light-dark cycle throughout the duration of the study.

Administration of the extract

Each group of mice was given different doses of methanol leaf extract. This extract was given once after they fasted for 18 hours for LD50 determination. However, in long- term toxicity study, mice were ad-

ministered with 400mg/kg and 800mg/kg doses of methanol leaf extract for 45 days[16] after 7 days acclimatization. The methanol leaf extract was dissolved in 4% tween. A volume of 2.5ml for 5 mice (0.5ml per mouse) of the extract was administered orally to experimental groups using a blunt intragastric catheter fitted to a 3ml syringe in 24 hours intervals for 45 days. Some volume of 4% tween was also given for control group in 24 hours intervals for 45 days. The blunt intragastric catheter was cleaned and placed in an oven after each administration to avoid any contamination.

LD50 determination

A lethal dose for fifty percent of the mice (LD50) for methanol leaf extract was determined using a total number of 90 Swiss Albino mice that were divided into 9 groups of 10 mice. Eight groups of mice were administered with methanol leaf extract of the plant at doses from 1250mg/kg to 2750mg/kg in 250mg/kg dose interval after fasting for 18 hours[17]. One control group was administered with 4% tween for methanol leaf extract.

The methanol leaf extract was dissolved in 4% tween for it was not fully miscible with distilled water. The number of death in each group within 24 hours was recorded. Besides, delayed mortality up to 3 days was considered as lethal dose. This was done by observing the mice for toxicity signs[18].

Long-term toxicity

The long-term toxicity study was carried out using 30 male and female Swiss Albino mice (25-35g). Animals were kept in a temperature-controlled environment 23±20C with 12 hours light-dark cycle. Food and water were freely available for a week before the beginning of administration of methanol leaf extracts of V. bipontini V. Out of the 30 mice, 20 were randomly assigned to 2 experimental groups of 10 mice each and the male and female mice were placed in separate cages. The remaining 10 mice were also randomly assigned to 1 control group. Then, the animals were randomly assigned into two (1st) control group and two (2nd, 3rd) experimental groups for methanol leaf extracts administration.

The 1st control group for methanol leaf extract received 0.5 ml of 4% tween. From the two experimental groups, two (2nd and 3rd) groups were administered with methanol leaf extract at doses of 400mg/kg and 800mg/kg, respectively. One male mouse died from the 3rd group one week before 45 days at which all mice sacrificed. Methanol leaf extract was administered in 24 hours intervals for 45 days[9,16.] Standard pellet (132g) was consumed within 24 hours intervals by a cage of 5 mice. All groups were closely observed for any physical, food intake, behavioral alterations and signs of abnormalities throughout the study. Blood samples were collected through heart puncture of each mouse into different sample bottles for blood parameter analysis. Finally, tissues were taken from all groups of mice for histopathological evaluation.

Body weight measurement

Body weight of all groups of mice was taken before the commencement of the first oral administration using SCIENTECH Mode No SL 3100D Rev-c accuracy class (II). These were considered to be the initial body weight. The body weights of all groups were also taken on the last day of oral administration and these were considered to be the final body weight.

Blood collection for hematological and biochemical investigation

Blood collection was performed by placing each animal in airtight dissector jar with cotton soaked in diethyl ether. Blood was collected from each animal by cardiac puncture using sterile needle and 5ml syringe. The sample was put in an ethylene- diamine-tetra-acetic acid (EDTA) bottle to prevent adhesion proteins (coagulation factors) in cell-cell and cell-matrix interactions for hematological determinations[19] using automated hematological analyzer, SYSMEX KX- ZIN[20] at EHNRI, Addis Ababa, Ethiopia. Hematological parameters were measured[21].

Biochemical investigation was performed after blood sample was collected by using cardiac puncture and jugular veins with sterile needle and 5ml syringe. The sample was kept at 4 °C for 4 hours to let it clot. The clotted blood was centrifuged (using Humax 4k bench top Centrifuge with a capacity of 12x15ml; Germany, Max- Planck-ring 21D-65205 Wiesbaden) at 5000 RPM maximum speed for 10 minutes to obtain the serum. The serum samples were kept in -22oC refrigerator until used for biochemical analysis. Then, biochemical parameters were measured[21].

Animal dissection, organs weight measurement and tissue sampling

Animals of each group were sacrificed at the end of 45 treatment days after body weight of mice were taken one by one on a digital electronic balance while under diethyl ether anesthesia. Animals laid on a clean paper towel and had all four extremities pinned to thin corkboard. A vertical midline incision with scissors cut from the neck to pubis opened the peritoneum. Then, 3-4mm wide strips of tissue samples were randomly taken from right lobe of liver and coronal section of right kidneys were cut lengthwise with a scalpel through the renal pelvis after each of these organs was weighed with 0.001 precision automatic internal calibration CX series balance. These tissue samples were taken from each organ and transferred by a blunt forceps to a test tube containing 10% buffered formalin that completely immerses the tissues for the purpose of fixation.

Tissue processing and routine staining

Sample tissues were taken immediately after sacrifice from the right lobe of liver and coronal section of right kidney and immersed in 10% buffered neutral formalin over night at room temperature after blood collection performed. The formalin fixed tissues were washed in running tap water for 8 hours to allow paraffin wax to infiltrate into the tissue easily[22]. Following washing, tissues were dehydrated in a series of graded ethanol i.e. in 70%, 80%, 95%, 100% I and 100% II for 1 hour each[23].

In de-alcoholization step, two changes of xylene were used for one hour each to remove ethanol from the tissue and replace it with fluid that is miscible with paraffin[22].

Tissues were infiltrated by two changes of paraffin wax which had a melting point of 56^0C ($52\text{-}64^0$C) for 1½ hours in each change[23].

The tissues were embedded in paraffin wax with the help of Electro-thermal Wax Dispenser to form tissue blocks in squared metallic plate block moulds. The blocks were then labeled, sealed in plastic bags with examining surface downward prior to sectioning, and placed in refrigerator until sectioned[23]. This process enables the specimens too small and/or delicate to be surrounded with some suitable materials that impart firmness without producing any injuries on the tissue[22]. Rotary microtome was used for sectioning of tissue blocks manually at a thickness of 5 µm. The paraffin block having tissue was put in the rotary microtome. The ribbon of sections was carefully picked from the knife by a blunt forceps to float in a water-bath of 40^0C (slightly below the melting point of wax) to remove folds in the sections. Unfolded sections were picked by clean microscopic glass slides and were placed in an oven maintained at a temperature of 56^0C for 20-30 minutes for proper drying and better adhesion. At this stage, the sections are ready for staining[23].

Staining solutions were prepared using the formula given by Clopton[24]. The paraffin wax was removed from the tissue sections using xylene. The sections were then immersed in a series of descending alcohol concentration to remove xylene after which distilled water was used to hydrate the tissue. The hydrated sections were immersed in hematoxylin for 3-5 min then with acid alcohol to prevent over staining. Sections were immersed in a mixture of sodium bicarbonate, ethanol, and distilled water and tap water to give blue color to the nucleus. Finally, it was immersed in 95% alcohol and eosin to give pink color to the cytoplasm[24,22].

Finally, tissue sections were dehydrated in 95% alcohol, cleared in xylene, and mounted by adding a drop of DPX (Dibutyl phthalate in xylene) mounting medium on the section to cover the microscopic glass with cover glass and to increase the refractive index of the tissue under light microscope. This was done with care to prevent bubble formation between the tissue and the glass cover[22].

Statistical Method

Data were digitally analyzed using the statistical software package SPSS version 14. All values were expressed in mean ± SEM. Treatment effects over time were compared between control and treated groups by analysis of covariance. The results were analyzed statistically using probit analysis of regression to determine LD50 and analysis of variance one-way ANOVA to identify possible difference of body, liver, and kidney weights, and hematological and biochemical values. P values less than 0.05 were considered statistically significant.

Results

Physical signs of toxicity

Mice were observed for signs of abnormalities before and after sacrifice. Mice showed low locomotion, weakness, erection of hairs, and white color of the eyes in the course of acute study.

During long-term administration of the extracts both treated and untreated groups showed no physical changes in their appearances and signs of toxicity at 400mg/kg methanol leaf extract of the plant. However, almost all mice treated with 800mg/kg methanol leaf extract of V. bipontini V showed swellings on the left lateral part of abdominal region related to spleen,

weakness, frequent defecation, mild diarrhea, and enlargement of spleen as compared to the control group.

LD50 determination

The acute toxicity study in mice showed LD50 value of 2130.6±5.1 mg/kg body weight of mice for methanol leaf extract (Fig 1). The probit responses are indicated in vertical line and doses are indicated in horizontal line (Fig 1). The vertical arrow indicated the LD50 of the extract.

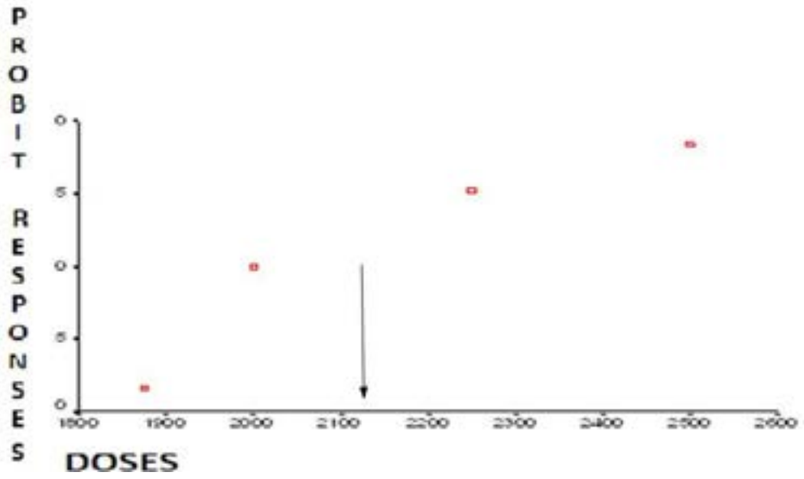


Figure 1: LD 50 curve for methanol leaf extract of *V. bipontini* V results which is marked by vertical arrow (2130.6±5.1 mg/kg)

Effects of methanol leaf extract of V. bipontini V on the body weight of mice

The changes in the mean values of the initial and final body weights of the mice treated with 400 and 800mg/kg of the methanol leaf extract of the plant is shown in Table 1. The result showed statistically boundary significant (P=0.06) in the body weights of mice treated at 400mg/kg bw (Table 1). Moreover, mice treated at a dose of 800mg/kg of the plant extract showed significant decrease (P=0.001) in the final body weight (Table 1).

Table 1: Effects of methanol leaf extract of *V. bipontini* V on the body weight of mice treated at doses of 400 and 800mg/kg

Group	Treatment (mg/kg)	Initial weight in g	Final weight in g	Weight change in g
Control	-	30.6±2.15	32.75±2.19	1.54±2.07
1	400	32.61±3.13	32.5±2.9	0.28±1.2* (0.06)
2	800	30.77±2.6	28.33±2.36*	-1.98±0.46* (0.001)

Values are mean ± SEM. *The mean difference is significant at the $P^* < 0.05$ level.

Effects of methanol leaf extract of V. bipontini V on hematological and biochemical parameters

The chronic effects of methanol leaf extract of V. bipontini Von hematological and biochemical parameters of blood are illustrated in Table 2. As can be seen from the Table, there was no significant difference in hematological and biochemical composition of blood between control and mice treated at a dose of 400 mg/kg of the extract. However, significant changes were observed in mice treated with 800mg/kg of the plant extract as compared to the control group. Red blood cell count (M/UL) significantly decreased (P=0.001) in mice treated with 800mg/kg of the extract. Platelet count (K/UL) also decreased considerably (P=0.001) from 1042.8±57.3 to 516.24±300 with 800mg/kg of the extract, while a dose of 400mg/kg showed no significant change in the platelet count as compared to the control group (Table 2). Moreover, at 800mg/kg of the extract considerably decreased (P=0.001) MCH and MCHC as compared to the control and at 400mg/kg MCHC is significantly decreased (P=0.027) Besides, 800mg/kg of the methanol leaf extract caused a significant increase in serum AST, ALT, and ALP levels, while 400mg/kg of the extract showed no change in AST, ALT and ALP levels as compared to the control group. Even though there was no statistically significant serum ALP, an increase in serum ALP level was observed at 400mg/kg methanol leaf extract of V. bipontini V. Moreover, blood urea concentration non-significantly increased in mice treated with 400 and 800mg/kg of methanol leaf extract of V. bipontini V. The results also revealed no change in the total WBC (K/UL) and lymphocyte percent at all doses (Table 2). Changes in Hgb, Hct and Mcv were not significant in mice treated with 400mg/kg of methanol leaf extract of V. bipontini V. However, these hematological parameters significantly decreased in mice treated with the extract at a dose of 800mg/kg as compared to the control (Table 2).

Table 2: Hematological and biochemical parameters between methanol leaf extract of *V. bipontini* V treated groups at doses of 400mg/kg, 800mg/kg, and control group

Hematological & Biochemical Parameters	Control with 4% tween in distilled water	Methanol leaf extract treated groups	
		400mg/kg	800mg/kg
RBC(M/UL)	7.85±0.73	6.44±1.16(0.07)	2.91±2.6* (0.001)
WBC(K/UL)	4.28±0.69	4.1±1.84(0.8)	3.36±1.82(0.21)
Platelet(K/UL)	1042.8±57.3	912.97±93.2(0.11)	516.24±300* (0.001)
Hgb (g/dl)	11.24±0.62	9.64±2.49(0.15)	7.58±3.4* (0.003)
Hct (%)	37.42±1.25	32.68±3.03(0.161)	24.56±12.78* (0.001)
Mcv(fl)	49.96±1.76	47.53±3.97(0.08)	46.8±2.83(0.031)
Mch (pg)	12.54±0.72	10.52±1.11* (0.001)	8.74±0.93* (0.001)
Mchc (g/dl)	23.78±1.16	22.22±1.72* (0.027)	19.2±1.53* (0.001)
L (%)	82.04±3.77	78.24±11.1(0.50)	75.5±18.9(0.27)
AST (IU/L)	115±23.68	88.5±15.99 (0.42)	211.5±128* (0.008)
ALT(IU/L)	40±4.71	45.5±8.95 (0.47)	69.4±28.33* (0.001)
ALP(IU/L)	65.5±28.4	108±159.56(0.35)	161±55.4* (0.04)
Urea(mg/dl)	53.5±6.25	58.5±8.51(0.14)	59.1±7.13(0.10)

Values are mean ± SEM. *The mean difference is significant at the $P^* < 0.05$ level.

Effects of methanol leaf extract of V. bipontini V on the weights of liver and kidney of mice

The mean values of the weights of the liver and kidney of both control and experimental groups treated with methanol leaf extract of the plant is indicated in Table 3. The result showed no significant change in the liver and kidney weights of those treated with 400mg/kg of the plant extract. However, mice treated at 800mg/kg dose showed significant decrease (P=0.001) in the liver and kidney weights as compared to the control group (Table 3).

Table 3: Effects of methanol leaf extract of *V. bipontini* V on the weights of liver and kidney of mice

Group	Treatment (mg/kg/bw)	Liver weight	Kidney weight
Control	-	1.71±0.06	0.24±0.01
1	400	1.67±0.07(0.21)	0.23±0.02(0.06)
2	800	1.33±0.1*(0.001)	0.13±0.03*(0.001)

Values are given as *mean ± SEM statistically significant at value P<0.05 level different from control by Post Hoc test. The mean difference is significant at the $P^* < 0.05$ level.

Correlation between body weight and organs weight of liver and kidney at 800mg/kg methanol leaf extract of V. bipontini V

Final body weight and organ weight of liver and kidney of mice treated with methanol leaf extract of V. bipontini V at a dose of 800mg/kg showed significant (P<0.05) decrease in mean values when compared with the control groups (Table 1 and 3). Even though the decrease is significant, the decrease in body weight did not correlate (P>0.05) with the decrease in liver and kidney weights at 800mg/kg body weight methanol leaf extract of the plant (Figure 2 & 3).

Figure 2: Pearson correlation between body weight and liver weight of mice treated with 800mg/kg of methanol leaf extract of V. bipontini V by Scatter plot

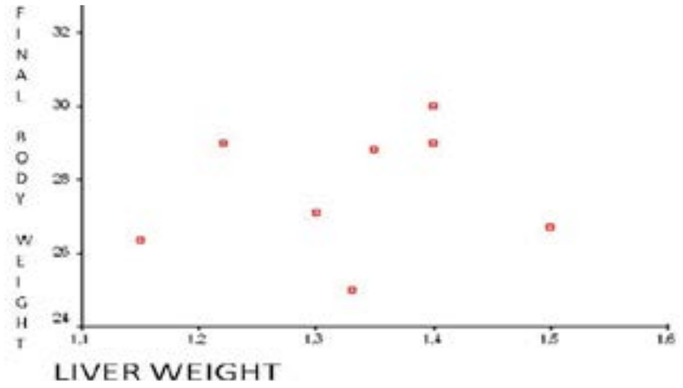
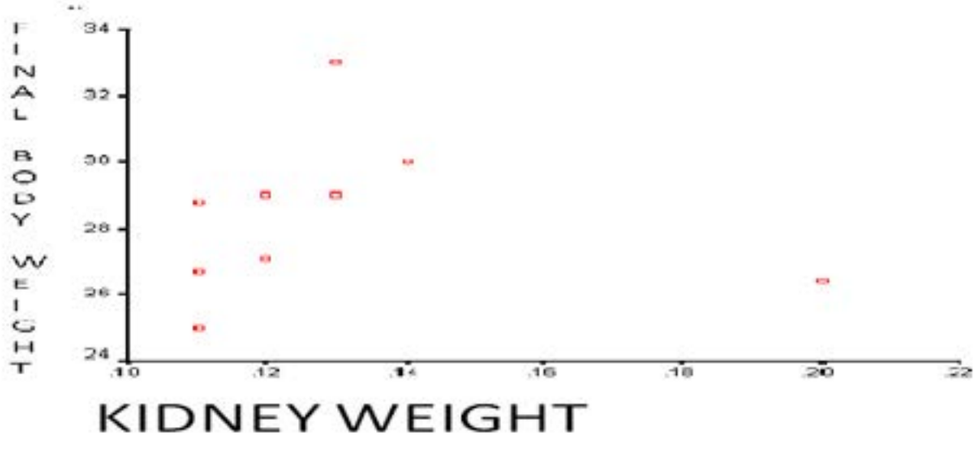


Figure 3: Pearson correlation between body weight and kidney weight of mice treated with 800mg/kg of methanol leaf extract of V. bipontini V by Scatter plot

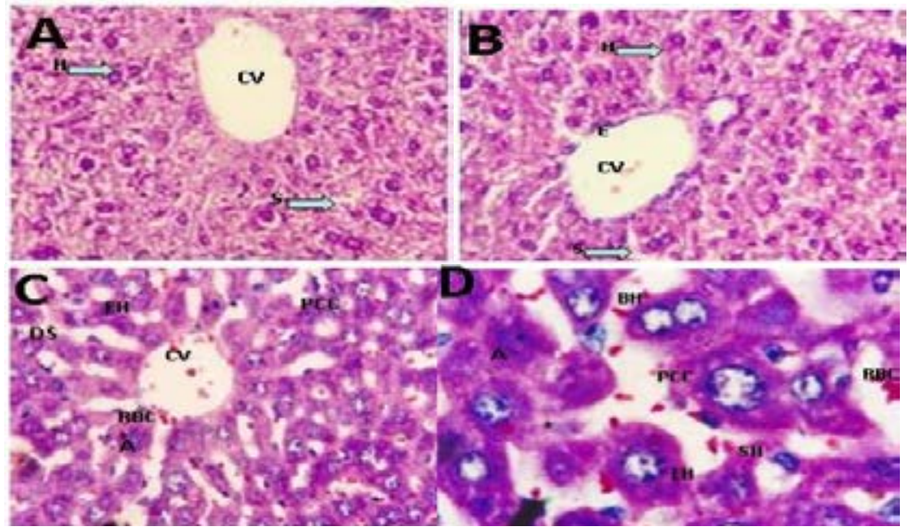


Microscopic observation

Histopathological evaluation of methanol leaf extract of V. bipontini V on liver in long-term toxicity.

The histopathological effect of methanol leaf extract of V. bipontini V on liver tissue (Fig. 4 C and D) of mice treated at 800mg/kg of the extract as compared to the control (Fig. 4 A) showed some histopathological abnormalities such as dilated sinusoid, nuclear enlargement, bi- nucleation of hepatocytes, peripheral cramped chromatin, shrinkage of hepatocytes (single cell death), fragmentation of hepatocytes (apoptosis) (Fig. 4. D, H&E, x10541). These findings are; however, not seen in the control (Fig. 4 A) and in mice treated with 400mg/kg of methanol leaf extract of the plant (Fig. 4. B, H&E, x4216).

Figure 4: Photomicrographs of liver sections A = control mice showing no histopathological change, (H & E, x4216).



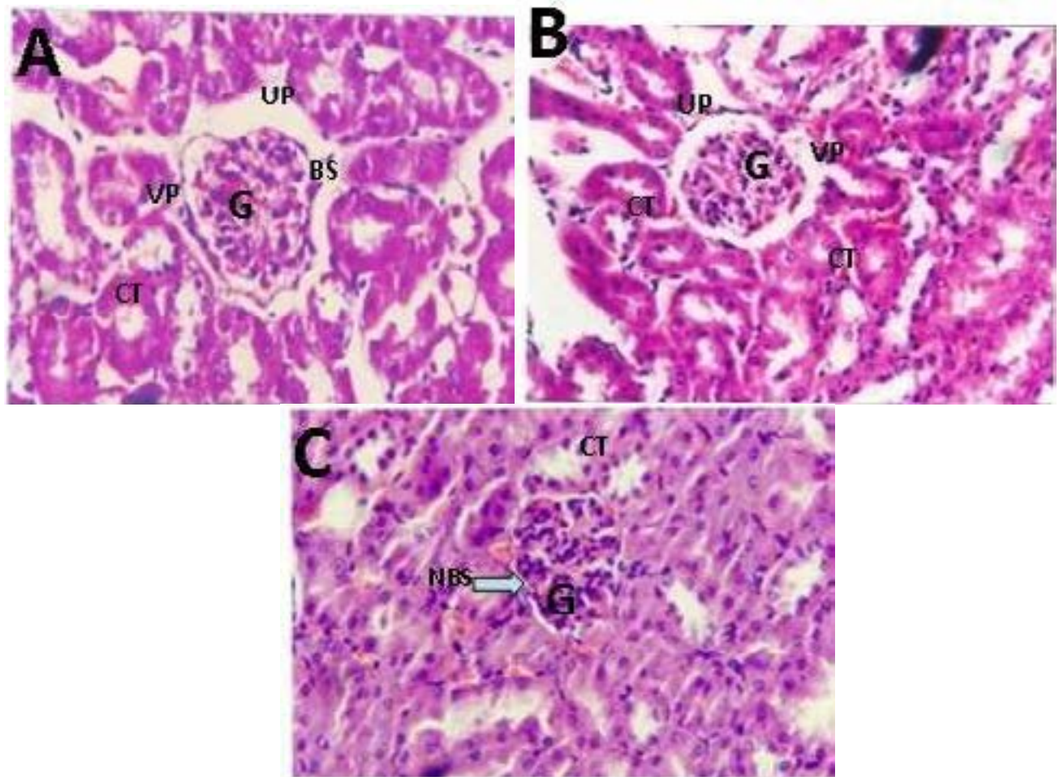
B = the mice treated with 400mg/kg methanol leaf extract of V. bipontini V, showing no histopathological changes. C and D = the mice treated with 800mg/kg of methanol leaf extract of the plant at (x4216, C) and (x10541, D) magnification showing dilated sinusoid, nuclear enlargement, bi-nucleation of hepatocytes, Peripheral cramped chromatin, shrinkage of hepatocytes (single cell death), and fragmentation of hepatocyte (apoptosis). H = Hepatocyte S = Sinusoid DS = Dilated sinusoid E= Endothelial cells SH = Shrinkage of Hepatocyte EH = Enlargement of hepatocyte CV = Central vein FH = Fragmented hepatocyte (A = Apoptosis) BH = Bi-nucleated hepatocyte PCC = Peripheral cramped chromatin RBC = Red blood cells

Histopathological evaluation of methanol leaf extract of V. bipontini V on kidney in long-term toxicity

Light microscopic observation showed that there was no significant difference between the kidney sections of the control (Fig. 5. A) and mice treated with methanol leaf extract of V. bipontini V at doses of 400mg/kg (Fig. 5 B).

In the control and mice treated with 400mg/kg urinary pole, vascular pole, glomerulus, convoluted tubules were normal and clearly visible (Fig. 5. A and B, H&E, x4216). The common toxicity structural changes such as tubular necrosis, inflammation, fatty changes, and WBC infiltration were not observed. Tissue section of mice treated with the methanol leaf extract of V. bipontini V at a dose of 800mg/kg/bw showed narrowing of Bowman's space, increase in cellularity of glomerulus and enlarged macula densa (Fig. 5. C). However, convoluted tubules and Bowman's space were normal (Fig. 5 C) as compared to the control (Fig. 5 A) and to mice treated with 400mg/kg methanol leaf extract of the plant (Fig. 5. B, H&E, x4216).

Figure 5: Photomicrographs of kidney of control mouse (Fig. 5. A) and of mice treated with 400mg/kg of methanol leaf extract of V. bipontini V showing no histopathological change (B) (H&E, x4216). Mice treated with 800mg/kg showed some morphological changes, cellularity of glomerulus and narrowing of Bowman's space (Fig. 5. C, H&E, x4216). G = Glomerulus UP = Urinary pole VP = Vascular pole NBS = Narrow Bowman's space BS = Bowman's space CT = Convoluted tubule



Discussion

Medicinal plants are precursors for the synthesis of useful drugs[25]. Over 5000 plants are known to be used for medicinal purposes in Africa, but only a few have been studied[26]. Thus, knowledge of uses and side effects of medicinal plants provide a vital contribution to human health care. Vernonia species are the sources of many local medicines[9]. People living in areas, where V. bipontini V grow use the plant for treating malaria and malaria related symptoms. According to Ashenafi[5] leaf extract of V. bipontini V in vivo anti-malarial activity, in 4-day, suppressive assays against Plasmodium berghei in mice reduced parasitemia by more than 50% when tested at an oral dose of 400mg/kg/day indicating that the median effective dose (ED50) is 400mg/kg/day. The inhibition of this plant in methanol leaf extract was 40%[5] . LD50 of aqueous leaf extract of the plant was found to be 2500.62±5.24 mg/kg6 which was higher than 2130.6±1.5mg/kg methanol leaf extract.

This study is primarily designed to determine LD50, and long-term effects of methanol leaf extracts of V. bipontini V at doses of 400mg/kg and 800mg/kg that might probably have effects on hematological and biochemical parameters and on liver and kidney tissues.

Mice treated with 400mg/kg of the plant methanol leaf extract didn`t show: any physical signs of toxicity, hematological and biochemical parameters, and histopathological abnormalities of the liver and kidney. These results go in line with the study of aqueous leaf extract of Vernonia bipontini V even at higher dose (800mg/kg)[6]. These findings are supported by previous published articles described the absence of any significant effect on the hematological and biochemical parameters[6,9,10,8] after long term administration of the same species and related species (Vernonia amygdalina).

Physical signs of toxicity showed that frequent defecation, mild diarrhea, weakness, and enlargement of spleen were observed at a dose of 800mg/kg methanol leaf extract of the plant during long-term experiment. The mild diarrhea might be due to the presence of bioactive chemical (saponins) in methanol leaf extract[14]. The presence of saponins may create some health hazard[27]. The observed splenomegaly may also be induced by the active ingredients (Dichloromethane/DCM) found in the plant extract[34].

The acute toxicity study in LD50 determination showed that methanol leaf extract of V. bipontini V is more toxic than the aqueous leaf extract of the plant. This might be due to active ingredients responsible for toxic effects, which were more abundant in methanol extract of the plant leaves than in aqueous leaf extract. The methanol leaf extract of V. bipontini V decreased body weight in mice treated at 800mg/kg. The decrease in body weight of the treated animals with methanol leaf extract of V. bipontini V at 800mg/kg might be due to the fact that V. bipontini V leaf extract contains some anti-nutritional factors, which reduce body weight. This is in agreement with the previous studies done on related species (V. amygdalina) by Igile[27] and Eleyinmi[10] who reported the presence of anti-nutritional factors especially, phytic acid, tannin, and oxalate, which inhibit the activities of digestive enzymes. Phytic acid, tannin, and oxalate which form complexes with metals (Ca++, Zn, Mg and Fe) and proteins, reduce mineral and protein bioavailability[10,11]. In turn, this may lead to low growth, notably reduced mean body weight. Kumar[28] also reported that weight of mice treated with methanol leaf extract of Caesalpinia bonducella and Bauhinia racemosa decreased significantly as a reflection of low growth.

In the present study, mice treated with methanol leaf extract of V. bipontini V at a dose of 400mg/kg showed no changes in their hematological and biochemical parameters. However, significant changes in the blood parameters were observed in mice treated at a higher dose of the methanol leaf extract of the plant. Higher dose (800mg/kg) of the extract decreased RBC, Hgb, platelet count, Mch and Mchc and increased serum AST, ALT and ALP levels in treated animals. Other investigators following chronic treatment with different agents in plant extracts also noted reductions in hematological profiles of blood. Thus, the findings of the present study is consistent with the previous reports[29,30] which suggested that methanol leaf extract of related species of V. bipontini V (V. amygdalina) possesses the potential of adversely affecting hematological indices. According to Choudhari and Deshmukh[29] the decreased number of RBC count and Hgb content may be due to defective haematopoiesis, inhibited erythropoiesis or an increase in destruction of red blood cells[29,21]. Methanol leaf extract of V. bipontini V may induce inhibition of RBC formation, which reduced hemoglobin content. The fall in hemoglobin content and RBC count can be correlated with induction of anemia in mice treated with methanol leaf extract of the plant at 800mg/kg[29]. The reduced number of platelets (thrombocytopenia) and RBC in the circulating blood might be due to the observed enlarged spleen which could probably trap and store them excessively[31,32] and the platelets deficiency might also induce hemorrhage[31].

Low hematological values obtained in mice treated with 800mg/kg of methanol leaf extract of V. bipontini V might also be due to the characteristics of bioactive components present in V. bipontini V as if sesquiterpene lactones and vernonisides may have been responsible10. Moreover, the results of the current study showed chronic treatment of methanol leaf extract of V. bipontini V induced increase in biochemical parameters (AST, ALT and ALP) at a dose of 800mg/kg. These results are in agreement with the previous report by James[33]. Saponin, flavonoids and tannin might elicit adverse biochemical actions when ingested by animals[34]. According to James[33], increase in chemistry of serum ALP, a membrane- bound enzyme, is due to release of the enzyme following a pathological phenomenon. Similarly, ALT, a cytoplasmic enzyme, found in hepatocytes normally at very low concentration, is also released into the plasma following hepatotoxic damage[35,36] and apoptosis[37].

There was no change in the actual organ weights of liver and kidney seen in all animals treated with methanol leaf extract of V. bipontini V at a dose of 400mg/kg. However, the organ weights of the animals treated with the plant extract at a dose of 800mg/kg significantly decreased. The decrease in the weight of these organs might be due to the anti-nutritional bioactive components (phytic acid and tannin) probably present in the plant extract. The reduced organ weights of liver and kidney did not correlate with loss of body weight at 800mg/kg of methanol leaf extract of the plant. The

decrease in body weight might be due to the reduced weight of other organs.

Histological examination of the liver and kidney of mice treated with methanol leaf extract at a higher dose revealed some histopathological changes. The changes in the liver were characterized by dilated sinusoids, nuclear enlargement, bi- nucleation of hepatocytes, and peripheral cramped chromatin. The methanol leaf extract also induced apoptosis in hepatocytes as demonstrated by fragmentation of hepatocytes, cell shrinkage and destruction of the cytoskeleton.

Other investigators following treatment with different agents[38-41,37,42] noted similar histopathological changes in liver. The histopathological abnormalities of liver and kidney tissues might be due to the presence of bioactive compounds (alkaloids, tannins, saponins, flavonoids, oxalate, and glycosides)[10,43,12] which are not dissolved in methanol during extraction of the plant because such histopathological abnormalities of the tissues didn`t find in aqueous leaf extract of the same species[6]. The presence of oxalate in the food is also associated with acidity and toxicity[10]. Retention of water inside hepatocyte resulting in cell enlargement (swelling) may be due to reduction of energy necessary for ion regulation in the cells[38,44]. The observed apoptosis may be an important pathophysiologic mechanism for the maintenance of liver tissue, allowing hepatocytes to die without provoking a potential harmful inflammatory response through a tightly controlled and regulated process[45,39]. Previous studies have also reported that phenolic compounds (e.g. flavonoids), nitrogen compounds (e.g. alkaloids), saponins, and tannin present in the plant extracts have antiradical activities46. Free radicals setup a chain reaction that can cause biological damage by stimulating glycation of protein, inactivation of enzymes and alteration in the structure and function of collagen basement and other membranes and alcoholic leaf extracts were more effective stable free radical scavengers than aqueous leaf extracts[46.] This may lead to the observed apoptotic hepatocytes.

The histopathological changes observed in the kidney sections showed increase in cellularity of glomerulus, urinary space obliteration and enlarged macula densa. This finding may agree with the reports of Ebaid[38] that showed similar alterations in the structure of glomerulus as a result of the treatment different toxic substances (piroxicam). However, necrosis, tubular degradation, fatty changes as well as inflammatory cellular infiltration, which are signs of renal toxicity, were not observed. This shows the plant extract has no marked effect on kidney of mice at higher dose.

The above investigation showed that the methanol leaf extract of the plant at higher dose (800mg/kg) might induce anemia and some histopathological alterations in liver and kidney.

Conclusion

From this investigation, it can be concluded that the methanol leaf extract of the plant at 800mg/kg might induce anemia and some histopathological alterations in liver and kidney. For these reasons, it is better to recommend further investigation on the histopathology of other organs especially on spleen because enlargement of spleen was seen during physical observation of dissected mice treated with methanol leaf extract of V. bipontini V at 800mg/kg and studies on metabolism of action of the extract for the toxic effects and to find out the active ingredients found in this study and examine studies in prenatal mice.

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