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Sub-chronic toxicity of the leaf aqueous extract of *Bidenspilosa* Linn (Asteraceae) in male and female rats

Orélien Sylvain Mtopi Bopda[1]*, Frida Longo[2], Fidéliste Boade[2], Paul Desire Djomeni Dzeufiet[3] and Theophile Dimo[3]

[1]Department of Zoology and Animal Physiology, Faculty of Science, University of Buea, P.O. Box 63 Buea, Cameroon.
[2]Department of Biological Sciences, ENS, University of Yaounde I, P.O. Box 47, Yaounde, Cameroon.
[3]Department of Animal Biology and Physiology, Faculty of Science, University of Yaounde I, P.O. Box 712, Yaounde, Cameroon.

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Bidens pilosa **is used empirically for the treatment of various illnesses among which are: jaundice, conjunctivitis, cataract, malaria, ulcers and hypertension. This study is aimed at evaluating the sub-chronic toxicity of the leaf aqueous extracts of *B. pilosa*. The effects of the administration of the aqueous extracts of *B. pilosa* on physical (body weight, relative weight of organs), biochemical and histological parameters were studied in male and female albino Wistar rats. Daily doses of extract (100, 500 and 1000 mg/kg) and distilled water were administered orally for 4 weeks; during which signs of toxicity were checked. At the end of the experiments, the rats were sacrificed, their blood and organs collected for biochemical, histological and haematological analyses. No death was recorded. At the end of the treatment, there were no significant (P>0.05) variations of the body weight gain and the relative weights of detoxification organs (liver and kidney). The analysis of biochemical parameters showed a significant (p<0.001) variation of serum levels of ALT and creatinin. Histological analysis of liver and kidney, revealed no modification. The analysis of haematological parameters showed no significant difference between males and females, overall. However, when considering each sex in particular, it was observed that in males red blood cell distribution width-coefficient variation (RDWcv) significantly increased at the doses of 500 mg/kg (P=0.02) and 1000 mg/kg (P=0.04) (vs. control). Unlike in males, white blood cells (WBC) in females decreased significantly at 500 (P=0.01) and 1000 mg/kg (P=0.01) (vs. control). It can be concluded that *Bidenspilosa* has a relatively low toxic effects in both sexes, with some gender differences in the haematological results.**

Key words: *Bidenspilosa*, Asteraceae, sub-chronic toxicity, rat.

INTRODUCTION

Bidens pilosa is an Asteraceae, a family of plants with high importance contributing to medicinal species worldwide. The plant is indigenous to the Amazon rainforest and other tropical areas of South America,

*Corresponding author. E-mail: boresyl@yahoo.fr. Tel: (+237) 675072174.

Africa, the Caribbean and the Philippines (Ezeonwumelu et al., 2011). It is empirically used, as a decoction, for the treatment of various illnesses: in Cameroon for example, it is used for the management of hypertension (Dimo et al., 1999), in Taiwan for anti-oedemic and anti-inflammatory activity (Duke, 1997). Difficult access to conventional drugs in low/middle income countries, like Cameroon, favours the use of medicinal plants for the management of cardiovascular diseases and oxidative stress and *B. pilosa* has been extensively studied for those properties (Dimo et al., 1999, 2002; Chiang et al., 2004). In addition, the plant is used for curing many other diseases in and out of Cameroon (Dimo et al., 2002; Ezeonwumelu et al., 2011).

There are some variations in the level of activity of the different species of Bidens, although the general properties appear similar (Brandao et al., 1997; Andrade-Neto et al., 2004). These variations can be accounted for by the fact that, *B. pilosa* leaf aqueous extract contains different active principles responsible for it effects, as demonstrated by some researchers (Brandao et al., 2004; Chiang et al., 2004; Ezeonwumelu et al., 2011). However, it is very important to evaluate the safety margin of the plant, although it is popularly used in Africa in its aqueous form; which is also the most widely studied from the literature review. Despite the pertinent toxicological studies by Cárdenas et al. (2006) with a single dose of 1000 mg/kg, Ezeonwumelu et al. (2011) whose work only involved males and the preliminary study of its acute toxicity by Longo et al. (2008), there is insufficient information on the toxicity of this aqueous extract, especially when comparing both sexes. Therefore, this study aimed to evaluate the sub-chronic toxicity and important haematological, histological and biochemical effects of aqueous extract of *B. pilosa* in male and female rats.

MATERIALS AND METHODS

Preparation of plant extract

The *B. pilosa* plant was harvested in the Centre region of Cameroon in July 2010, early in the morning. It was identified by a botanist from Cameroon National Herbarium and a voucher specimen number 65112/HNC of the plant was deposited. The leaves were dried under shade and ground into fine powder. Decoction method of extraction was used: 670 g of the powder was weighed into an empty clean beaker and 4.5 L of distilled water was added and boiled for 5 min. It was cooled at room temperature and filtered using first a clean cotton cloth and then Whatmann No. 4 filter paper plugged in a funnel. The filtrate was poured into clean dry silver dishes, placed in oven and dehydrated to dryness at 60°C. The yield was 16.7%.

Laboratory animal acquisition and handling

Wistar rats were bred at the Animal House in the High Teacher Training School of the University of Yaounde I. Forty healthy young adult Wistar rats (2.5-3.5 months old), both sexes in equal number, weighing between 120 and 160 g were obtained and housed two per cage for 1 week to allow for acclimation. The animals' cages were lined with saw dust, at room temperature with adequate ventilation, under a naturally illuminated environment with 12 h of light and 12 h darkness. They were fed with standard chow. They had access to clean drinking water *ad libitum*. The animal experiments were conducted according to the USA National Institute of Health Guide for the care and use of laboratory animals (NIH, 1996) and ethical guidelines for investigation of experimental pain in animals (Zimmerman, 1993).

Sub-chronic toxicity test

Organization for Economic Cooperation and Development (OECD) Test Guidelines (TG) that describe short-term repeated-dose toxicity testing: Repeated Dose 28-day Oral Toxicity Study in Rodents (TG407) was used for the study (OECD, 2006). The forty acclimated healthy Wistar rats, the two sexes being in equal number, were weighed and grouped randomly into eight groups (n = 5) as follows: A low dose (100 mg/kg), a medium dose (500 mg/kg) and a high dose (1000 mg/kg, both sexes); the control group comprised of five rats of either sex, to which distilled water (10 mL/kg) was administered. They were given food thirty minutes after administration of the extract by gavage. Body weights of the animals were taken daily for 28 days before administration of the extracts. The end-of-week weights were used to plot the curve of the body weight variation. The rats were observed daily to detect differences in appearance, discoloured fur, diarrhoea, bloody stool and constipation, loss of appetite and thirst and lack of interest in the environment. After 28 days, all animals survived and were allowed to fast overnight before the sacrifice. To minimize pain and stress in animals, they were anaesthetized with pentobarbital (50 mg/kg, IP). About 3 mL of blood was first collected with the aid of a syringe for full blood count; this was followed by cervical dislocation allowing optimal collection of the rest of blood for biochemical parameters analysis. For the latter, blood samples were collected from the animal into non-heparinized vacutainers. Organs were removed, weighed and absolute and relative organ weights determined, and then organs were preserved separately for each animal in 10% neutral buffered formalin to prevent tissue autolysis and kept for histological analysis. Relative organ weight was determined using the following formula:

Relative organ weight = [Absolute organ weight (g) / Body weight of rat on sacrifice day (g)] x 100

Biochemical analysis

After the collection of about 3 mL of blood to be used for blood parameter analysis, the rest blood collected into non-heparinized tubes was allowed to clot for 30 min and then centrifuged at 3000 rpm for 10 min. The serum was separated and analysed, using the URIT- 810 Chemistry Analyzer for HDL-cholesterol, total cholesterol, triglycerides and proteins and some enzymes: alanine aminotransferase (ALT, MAK052 Sigma ALT Activity Assay, Germany), aspartate aminotransferase (AST, MAK055 Sigma AST Activity Assay Kit, Germany) and creatinine (MAK080 Sigma Creatinine Assay Kit, Germany) The readings were done at wavelength 450 nm and manufacturer's instructions were strictly followed.

Histological study

Preserved tissues were histologically analysed at the laboratory of Animal Physiology of the University of Yaounde I. Tissues (liver and

kidney) were processed with microtome (ErnstLeitzWetzlar GMBH 530497 No. 537, Germany) and automated tissue processor (USA), embedded, placed on a slide and stained with Haematoxylin and Eosin stain (HE) (Ganter and Jolles, 1970). The tissues were observed under light microscope for changes in structure and the pictures taken with digital camera (Kodak, USA) attached to the eyepiece of the light microscope.

Haematological analysis

With the aid of a syringe, blood (about 3 mL) was collected from both sexes of animal in a 5 mL heparinised and properly labelled tubes, then shaken gently to allow it homogenize. A blood count was ran using the Full Blood Count machine, URIT 3300 following the manufacturer's instruction, to automatically get the following indices: red blood cell distribution width-coefficient variation (RDWcv), haemoglobin (Hb), mean corpuscular haemoglobin (MCH), mean corpuscular volume (MCV) and mean corpuscular haemoglobin concentration (MCHC), white blood cells (WBC), haematocrit (HCT), red blood cell (RBC) and platelet (PLT).

Statistical analysis

Data are expressed as mean values±standard error of mean (SEM) and group data comparisons of test and control means were evaluated by ANOVA test while comparison between sexes was done by the paired T-test using SPSS version 20.0. Significance was set at $p<0.05$.

RESULTS

No death or considerable change of behaviour was noted in treated rats as compared to the control. Increases in bodyweights of rats were observed within each group of rats from days zero to 28. However, no significant variation of the mean % body weight gain was observed when comparing males and females, or comparatively with their controls (Figure 1).

Likewise, in either sex, no significant variation of the relative weight of liver and kidney was observed after 4 weeks of treatment. In males, the values for liver were 1.9, 1.9, 2.1 and 2.3% while in females, they were 2.4, 1.9, 2.0 and 1.6% in control, 100, 500 and 1000 mg/kg, respectively ($P>0.05$). With regards to the kidney, the relative weights were 2.1, 2.9, 2.1 and 2.8% in males while in females, they were 2.0, 2.5, 2.2 and 2.4% in the control, 100, 500 and 1000 mg/kg, respectively ($P>0.05$).

An increase of the level of HDL-cholesterol was noted in both sexes of rats, as compared to their respective controls. In males, the HDL- cholesterol levels were 32.04 ± 2.97, 21.16 ± 3.75, 22.62 ± 1.08 mg/dL, respectively. This corresponded to increases of 129.67, 51.68 and 62.15% for doses of 100, 500 and 1000 mg/kg, as compared to the control (14.01 ± 2.29 mg/dL). This increases were significant at the doses of 100 ($p<0.01$) and 1000 mg/kg ($p<0.001$). In females, changes observed in the levels of HDL-cholesterol were from 15.26 ± 1.64 to 23.53 ± 2.34, 20.26 ± 0.93 and 22.40 ± 2.39 mg/dL, corresponding to significant increases ($p<0.05$) of 54.19, 32.76 and 46.78% for the doses of 100, 500 and 1000 mg/kg respectively (Figure 2).

Counterwise, as shown in Figure 3, there was a decrease of the level of ALT in both sexes of rat, as compared to their respective controls. In males, the ALT levels were 3.23 ± 2.22, 3.88 ± 0.64 and 3.88 ± 0.64 IU/L, respectively. This corresponded to decreases of 40.51, 28.54 and 28.54% for doses 100, 500 and 1000 mg/kg, as compared to the control (5.43 ± 0.66 IU/L). The decrease was significant only at the dose of 100 mg/kg ($p<0.001$). In females, changes observed in the levels of ALT were from 4.65 ± 0.74 to 3.23 ± 2.22, 3.23 ± 2.22 and 3.88 ± 0.64 IU/L, corresponding to decreases of 30.53, 30.53 and 16.55% for the doses of 100, 500 and 1000 mg/kg, respectively. These decreases were significant at the doses of 100 ($p<0.05$) and 500 mg/kg ($p<0.001$).

Administration of *B. pilosa* extract for four weeks caused a decrease of the level of AST, at the lowest doses, in both sexes of rat, as compared to their respective controls. In males, AST levels were 2.52 ± 0.28, 4.65 ± 0.67, 4.71 ± 0.30 IU/L, respectively. This corresponded to a significant decrease of 34.88% ($p<0.001$), and non-significant increase of 20.15 and 21.70% for doses 100, 500 and 1000 mg/kg, as compared to the control (3.87 ± 0.46 IU/L). In females, non-significant decreases of 30.53, 30.53 and increase of 16.55% were found in the levels of AST after treatment with the doses of 100, 500 and 1000 mg/kg, respectively (Figure 4).

As concerns proteins, from a value of 4.75 ± 0.2 mg/mL, protein levels increased to 5.08 ± 0.15, 5.66 ± 0.15, 5.29 ± 0.48 mg/mL, in males. These corresponded to increases of 6.94, 19.15 and 11.36% for the doses 100, 500 and 1000 mg/kg of *B. pilosa* extract. The increase at the dose of 500 mg/kg was significant ($p<0.05$). In females in the contrary, no significant variation was observed. Increases were 4.20, 20.26 and 0.38% at the above doses, respectively (Table 1).

Creatinine levels decreased by 15.55 ($p<0.05$), 18.88 ($p<0.05$) and 3.61% ($p>0.05$) in males while in females significant ($p<0.01$) decreases of 20.32, 20.32 and 25.47% were observed at the respective doses of 100, 500 and 1000 mg/kg (Table 1).

After four weeks of treatment with the plant extract (100, 500 and 1000 mg/kg), no injury or inflammation of the liver or the kidney was observed. The figures 5 and 6 display the cross sectional areas of the kidney and liver, respectively.

As shown in Table 2, the paired sample T-test analysis of haematological parameters did not reveal any significant difference between males and females, overall. However, for RDWcv in males, there was a significant increase at the doses of 500 ($P=0.02$) and 1000 mg/kg ($P=0.04$) (vs. control). Unlike in males, WBC in females decreased significantly at 500 ($P=0.01$) and 1000 mg/kg ($P=0.01$) (vs. control).

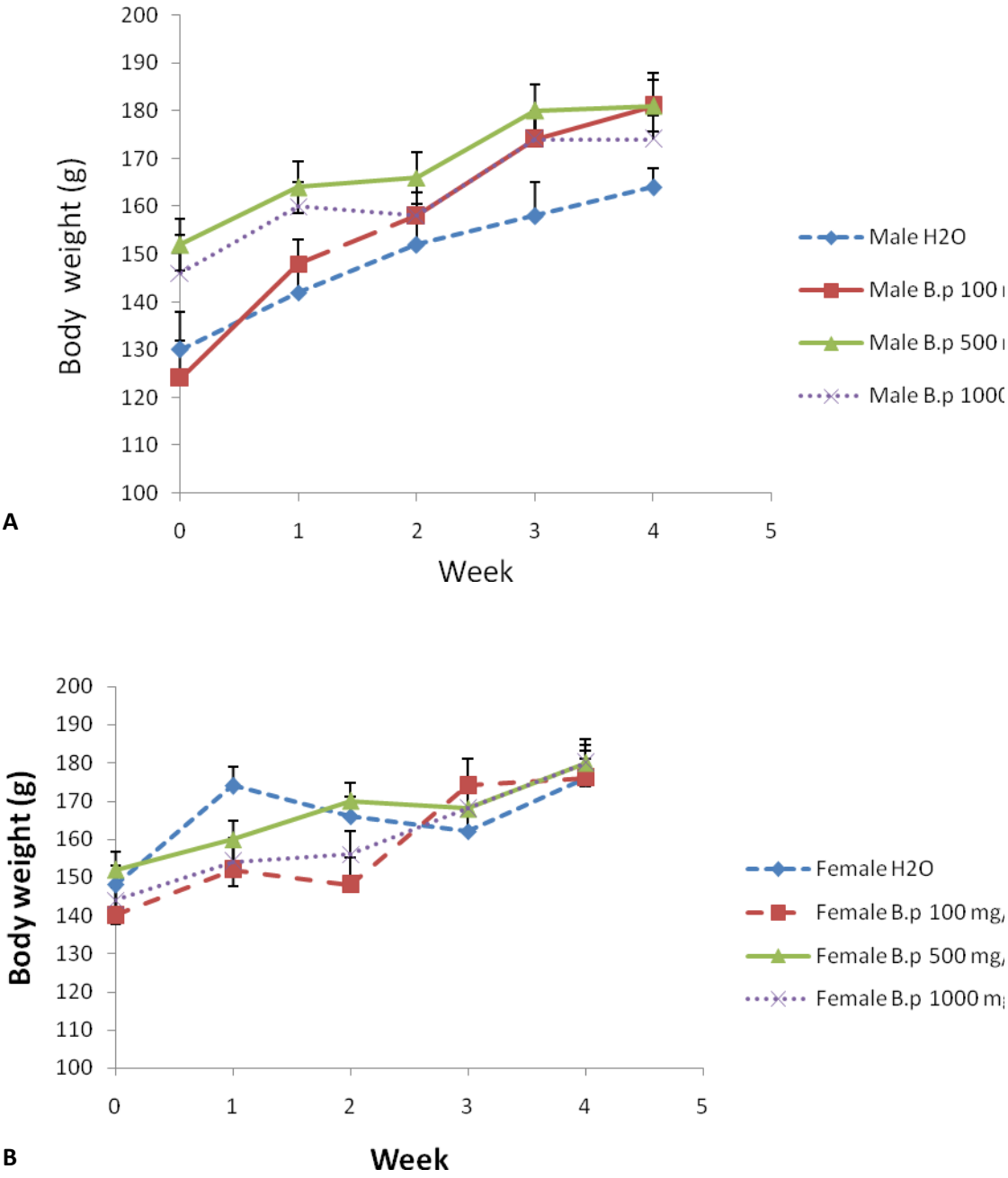

Figure 1. Variation of body weight during sub-chronic treatment with *B. pilosa.* A: males, B: females, each point represents the mean ± SEM, n = 5.

DISCUSSION

The toxicity study of *B. pilosa* did not elicit any mortality throughout the duration of the experiment, thus showing relatively high safety index in rats. Increases in body weights of rats were observed within each group of rats from days 0 to 28. The observed increases in body weight could be attributed to the nutritive components in their chow, as they were well fed and surely assimilated well their food. Since no significant difference was observed between controls (receiving H_2O) and test animals (receiving *B. pilosa*), the increase in body weight cannot be attributed to the components in the plant as revealed by Duke (1997). However, there was a general reduction in mean percentage body weight gain of rats administered with *B. pilosa* extract as compared to controls. Analyses of various species of *Bidens* have been conducted in several countries. Although, there is

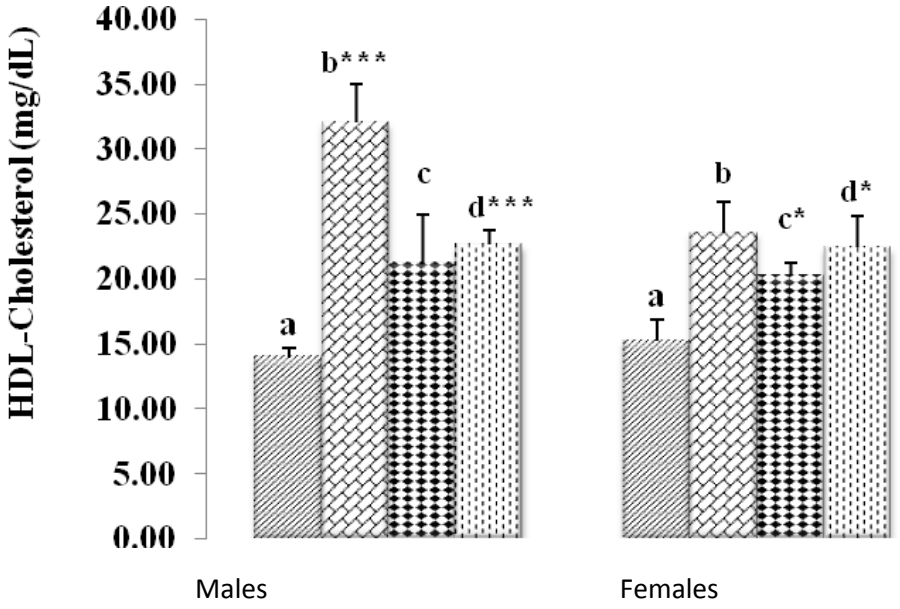

Figure 2. Effects of aqueous extract of *B. pilosa* leaf on the level of HDL-cholesterol after 4 weeks of treatment. Each bar represents the mean ± SEM, n = 5. *p<0.05, ***p<0.001, Significant difference with respect to control (distilled water). a, Distilled water; b, *B. pilosa* (100 mg/kg); c, *B. pilosa* (500 mg/kg); d, *B. pilosa* (1000 mg/kg)

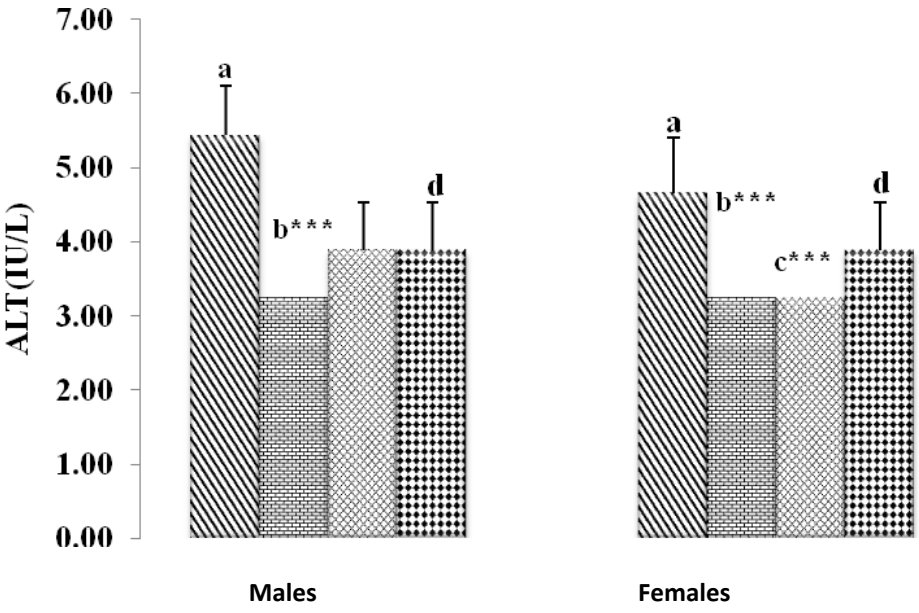

Figure 3. Effects of aqueous extract of *B. pilosa* leaf on the levels of ALT after 4 weeks of treatment. Each bar represents the mean ± SEM, n = 5. ***p<0.001, Significant difference with respect to control (distilled water). a, distilled water; b, *B. pilosa* (100 mg/kg); c, *B. pilosa* (500 mg/kg); d, *B. pilosa* (1000 mg/kg)

some variation in the level of activity of the different species of *Bidens* probably due to different levels of active constituents, the general property appears similar (Andrade-Neto et al., 2004). These phytochemical components have been found to be responsible for the various medicinal activities of *B. pilosa* including

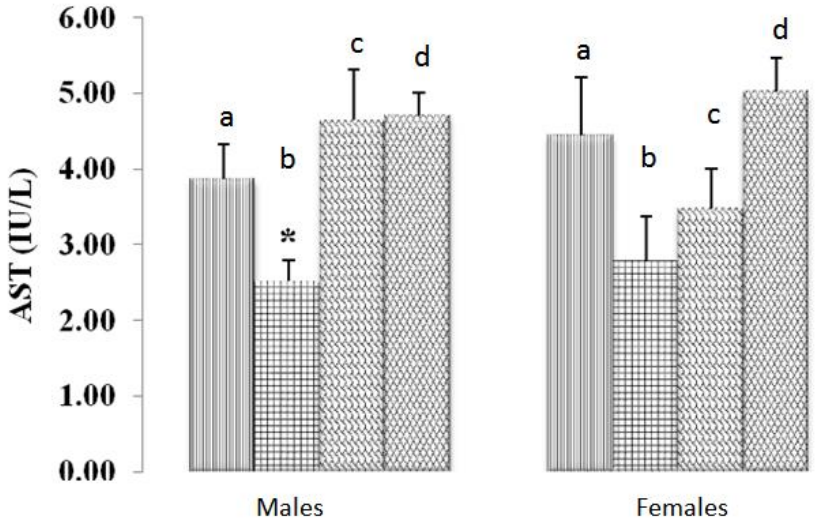

Figure 4. Effects of aqueous extract of *B. pilosa* leaf on the level of AST after 4 weeks of treatment. Each bar represents the mean ± SEM, n = 5. *p<0.05, Significant difference with respect to control (distilled water). a, distilled water; b, *B. pilosa* (100 mg/kg); c, *B. pilosa* (500 mg/kg); d, *B. pilosa* (1000 mg/kg).

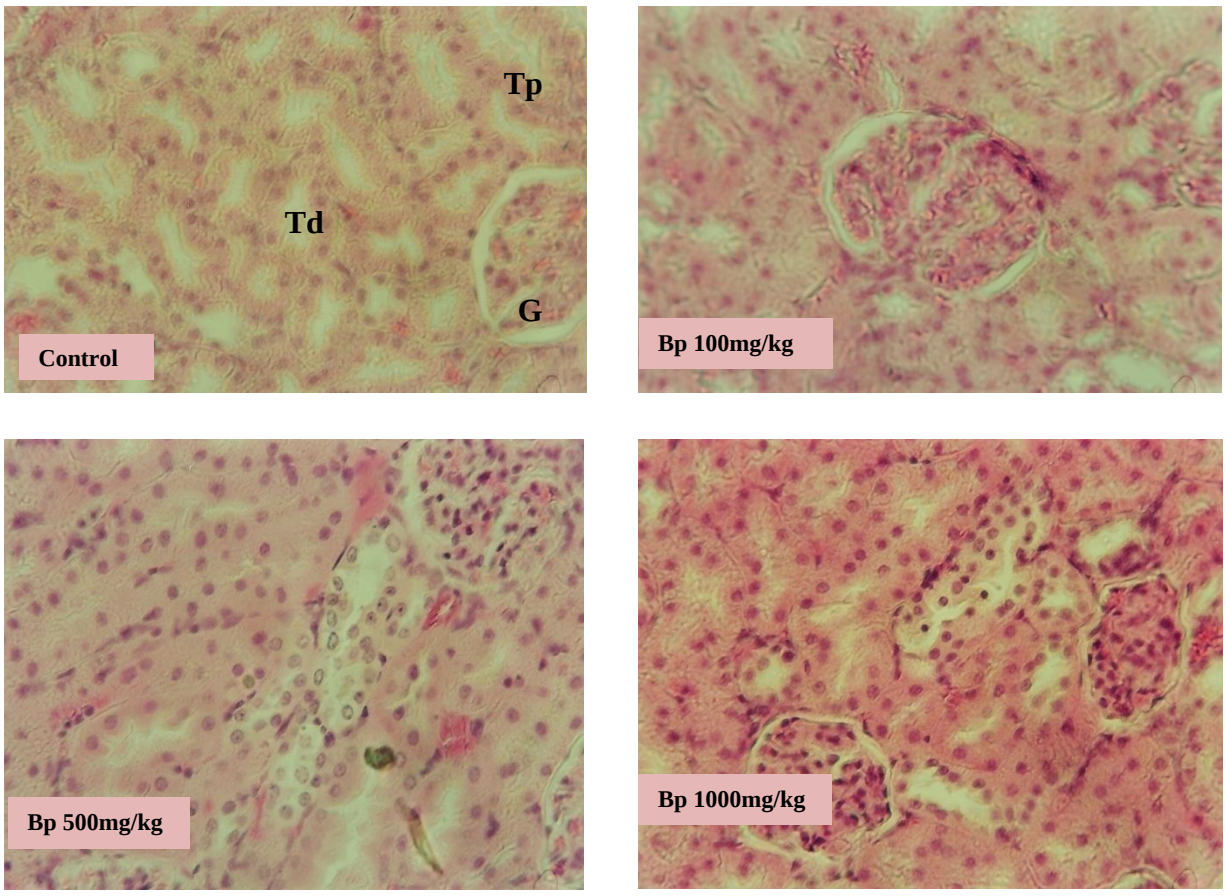

Figure 5. Light micrographs of kidney histological sections (HE x 400). G: glomerule; DCT: distal convoluted tube; PCT: proximal convoluted tube; Bp: *Bidenspilosa* extract.

Table 1. Effects of *B. pilosa* on serum proteins and creatinine in male and female rats.

Dose of *B. pilosa* extract	100 mg/kg	500 mg/kg	1000 mg/kg	100 mg/kg	500 mg/kg	1000 mg/kg
Effect	**Increase in serum proteins**			**Decrease in creatinine**		
Males	6.94% (p>0.05)	19.15% (p<0.05)	11.36% (p>0.05)	15.55% (p<0.05)	18.88% (p<0.05)	3.61% (p>0.05)
Females	4.20% (p>0.05)	20.26% (p>0.05)	0.38% (p>0.05)	20.32%(p<0.01)	20.32%(p<0.01)	25.47%(p<0.01)

P<0.05, P<0.01 significant difference between tests and control groups

Table 2. Haematological parameters.

Blood parameter	*B. pilosa* (500 mg/kg)		*B. pilosa* (1000 mg/kg)		Control (distilled water)	
	f	m	f	m	f	m
	AV (SD)	AV (SD)	AV (SD)	AV (SD)	AV (SD)	AV (SD)
Platelet count(x10^9/L)	201.50 (79.90)	204.50 (40.31)	21.67(8.39)	304 (141.42)	80 (1.41)	177.50 (103.9)
Haemoglobin (g/dL)	14.45 (0.63)	15.70 (1.84)	15.37(1.35)	11.65(4.45)	14.10 (0.1)	11.95 (1.77)
Red Blood Cells (x10^{12}/L)	7.39 (0.03)	8.55 (.93)	7.87 (0.80)	5.89 (2.23)	7.27 (0.07)	5.81 (0.23)
White blood cells (x10^3/µL)	9.45 (1.63)*	8.80 (0.14)	9.90 (0.44)*	8.20 (3.68)	14.05 (0.07)	8.80 (0.04)
Haematocrit (%)	44.85(2.33)	48.90 (4.81)	46.67 (4.10)	36.20 (14.00)	47.7 (0.42)	37.50 (5.80)
Mean cell volume (fL)	60.70 (3.39)	59.85 (3.04)	63.23 (0.61)	61.45 (0.49)	65.85 (0.21)	62.25 (4.03)
Mean cell Hb concentration (g/dL)	32.20 (0.28)	32.6 (0.57)	30.90 (1.80)	32.20 (0.14)	29.15 (0.21)	31.50 (0.71)
Mean cell Haemoglobin (pg)	19.50 (0.99)	19.10 (0.28)	19.50 (1.23)	19.75 (0.07)	19.35 (0.21)	19.20 (0.28)
(RDWcv) (%)	15.25 (0.64)	15.65 (0.64)*	14.7 (1.56)	15.1 (0.14)*	15.35 (0.07)	13.56 (0.08)

*P<0.05 significant difference between tests and control groups, f = female, m=male, AV (SD) =average (standard deviation), red blood cell distribution width coefficient variation = RDWcv

antimalarial activity due to the presence of acetylene and flavonoids (Brandao et al., 2004), chemo protective activities of ethyl acetate and butanolic fractions (Chiang et al., 2004; Suzigan et al., 2009), anticancer properties by photoactivated polyacetylenes (Hou et al., 1989; Geissberger and Sequin, 1991; Sundararajan et al., 2006), anti-inflammatory properties (Pereira et al., 1999) among others.

The mean relative organ weights of rats did not vary significantly in either sex. Thus, the plant extract had no observable influence on those relative organ weights of the liver and kidney. These data demonstrate that, it might not be proper to conclude that *B. pilosa*, at the used doses, is capable within four weeks to affect either the detoxifying organs' weights or the body weight. The increase of body weight observed in all animal groups after four weeks can be explained by the normal growth process (Otimenyin et al., 2010), as the animals were still relatively young at the start of the experiment. These results are globally similar to those of Lakmichi et al. (2010) while studying the toxicological profile of hydro ethanolic extract of *Corrigiola telephiifolia* (Corryophyllaceae) in rat. Assuming that, a decrease of the relative weights of detoxifying organs is a marker of the harmful effect of a toxicant (Witthawaskul et al., 2003), this results demonstrate that, aqueous extract of the leaves of *B. pilosa* shall have very low toxic effect or none. This is in line with the findings from the study of acute toxicity of the plant by Longo et al. (2008).

Plasma protein level increased significantly only in males and at the dose of 500 mg/kg. Creatinine levels generally significantly decreased in both sexes. Creatinine is a biomarker of the kidney function (Mbaka et al., 2010). Injury of the kidney could have caused an increase of the level of creatinine. While the increase of protein level at the single dose of 500 mg/kg and decrease of creatinine levels at several doses (100-1000 mg/kg) are difficult to explain, one can at least say without risk of mistake that, the plant extract did not affect the renal function. This was confirmed by the histological architecture of kidney that did not change after treatment with *B. pilosa* extract.

Likewise, the aqueous extract of *B. pilosa* seems to have a protective effect on the liver. In effect, histological analysis of the liver also did not show any noticeable modification of the architecture of the liver of treated rats as compared to the controls. Treatment with aqueous extract of *B. pilosa* extract revealed significant decreases in the levels of ALT (at 100 mg/kg in males, 100 and 500 mg/kg in females), when compared with the control. The level of AST decreased significantly when animals (males) were treated with 100 mg/kg *of B. pilosa*; proving that only lowest doses of extract caused a significant effect on the liver. The two most important transaminases

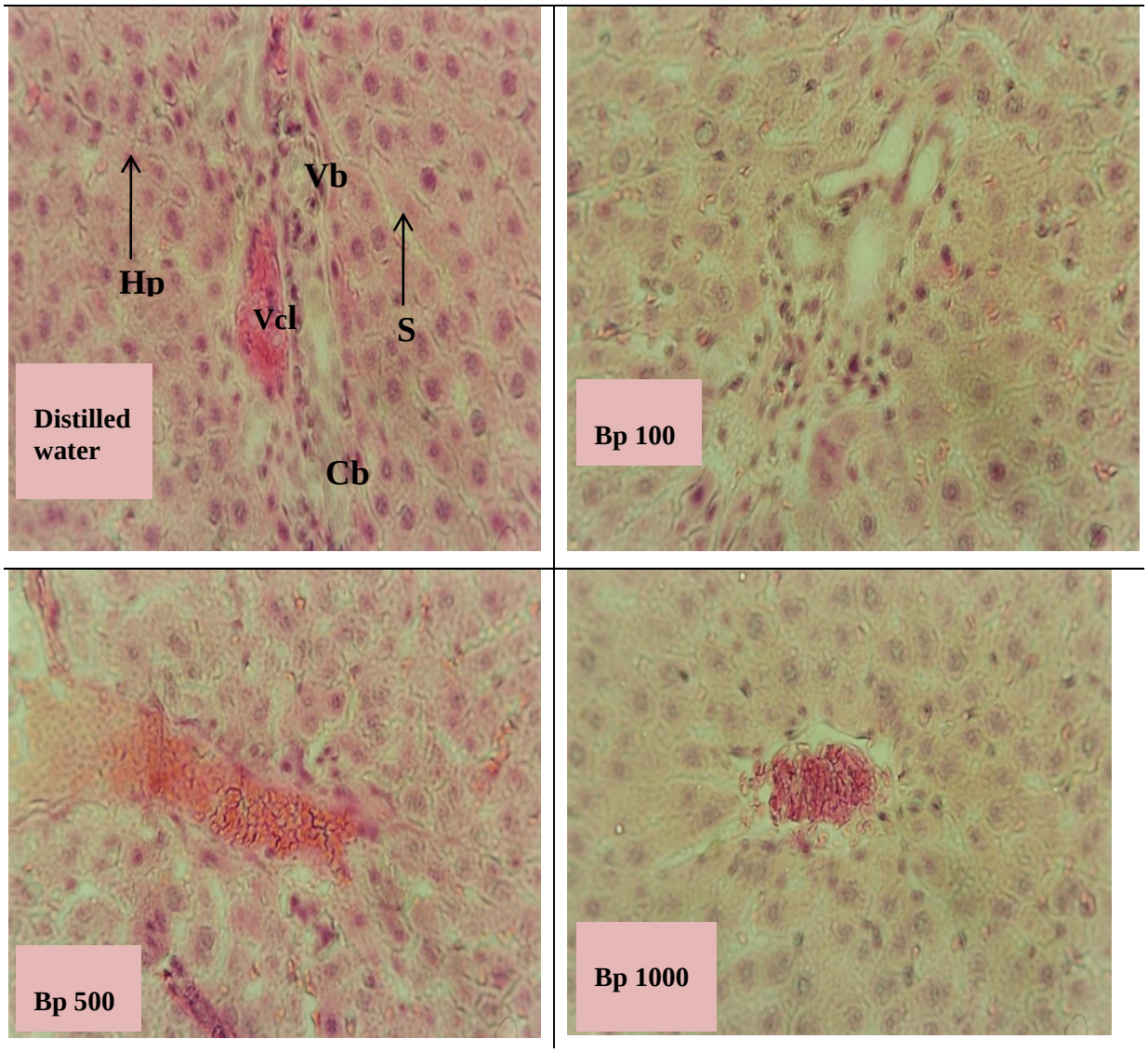

Figure 6. Micro section of Liver from control and treated rats (HEX40). Cb: Bile duct ; Vb: bile vessel ; Vcl: centro-lobularvein; Hp: hepatocyte, S: sinusoid. No difference was observed between the control and treated rats

are aspartate amino transferase (AST) and alanine amino transferase (ALT). They are present in high concentrations in liver and muscles. An increase of ALT level is a strong indicator of liver disease (Hartwell and Schwartz, 2009). These results reinforce the idea that, *B. pilosa* might have hepatoprotective activity. Such activity of the plant was actually demonstrated by other researchers (Suzigan et al., 2009; Li-Ping et al., 2008; Chin et al., 1996), meanwhile, they opposed to the findings of Ezeonwumelu et al. (2011). But according to the latter, there is a likelihood that, reversible damages might be caused by short term administration of *B. nitens* aqueous extract.

Lipid profiles were also evaluated during this study. Prevention of coronary diseases requires low triglycerides, LDL- cholesterol, associated with high HDL-cholesterol (Cerisier et al., 2004). As compared to the controls, it was found that, 28-day treatment of rats with the extract induced significant increase of HDL-cholesterol. At the dose of 100 and 1000 (in males) and 500 and 1000 mg/kg (in females), significant increases were recorded. These results are similar to those of Adewole and Ojewole (2008) who reported on the protective effect of *Annona muricata* on the lipid profile and the oxidative stress of hepatocytes in streptozotocine diabetic rats.

Males displayed significant increases of their RDW_cv in test groups as compared to control, but their mean corpuscular volume remained similar with control. Processes which are known to cause elevation of the RDW in the face of normocytic or microcytic indices

include iron deficiency, red blood cell fragmentation, certain hemoglobinopathies, myelofibrosis and sidero-blasticanemia; of which the most frequent is iron deficiency (Morgan and Peck, 1988). Elevated RDW aids differentiating between uncomplicated iron deficiency anemia (elevated RDW, normal to low MCV). Elevated RDW and normal MCV can be associated with any of the following conditions: 1)-Early iron, vitamin B12, or folate deficiency; 2)-Dimorphic anemia (for example, iron and folate deficiency); 3)-Sickle cell disease; 4)-Chronic liver disease or 5)-Myelodysplastic syndrome (Mates, 2004).

A significant decrease of white blood cells (WBC) was observed exclusively in females. WBC is naturally involved in the defence mechanisms of the body. Therefore, females could be considered more vulnerable to the plant extract, with regards to their immune capacity.

Conclusion

It can be concluded from this study that, the aqueous extract of the leaves of *B. pilosa* has a relatively good margin of safety in both sexes, but haematological indices like RDW or WBC were differently affected in males and females. It will be worth carrying out, an important study on this plant to determine its long term effect in animals.

Conflict of interests

The authors have not declared any conflict of interests.

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