IJPSR (2014), Vol. 5, Issue 7 (Research Article)

INTERNATIONAL JOURNAL OF PHARMACEUTICAL SCIENCES AND RESEARCH

Received on 26 January, 2014; received in revised form, 09 April, 2014; accepted, 27 May, 2014; published 01 July, 2014

TOXICITY STUDIES OF AQUEOUS EXTRACT OF *BRIDELIA MICRANTHA* STEM ADMINISTERED ON MICE AND RATS

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Keywords:

Intraperitoneal, Toxicity, Acute, Sub acute and Micrantha

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ABSTRACT: The World Health Organization (WHO) defines traditional medicine as "the health practices, approaches, knowledge and beliefs incorporating plants, animals and mineral-based medicines, spiritual therapies, manual techniques and exercises, applied singularly or in combination to treat, diagnose and prevent illness or maintain wellbeing. Various compounds have been isolated from *Bridelia micrantha* some of which are Friedelin, Taraxeronie, Epifiedelinol, Taraxerol, Garlic acid, Ellagic acid and sitosterol. Fresh stems of *B. micrantha* collected, dried, pulverized and mixed with sterile distilled water, extract left for 72hrs and stirred at 3hourly, filtered and concentrated in a water bath, stored in a refrigerator. Experiments were carried out using mice and wister rats of both sexes. The LD_{50} following the intraperitoneal route was estimated to be 20.3 mg/kg. The surviving animals following intraperitoneal route exhibited toxicological signs of writhing, decrease loco-motor activity, sedation and dose dependant mortality. Haematological parameters in the rat after 28days of daily oral administration show no significant difference between the treated groups and control. However, lymphocytes at the dose of 1000mg/kg indicate significant difference with control ($p<0.05$). The highest dose used in this study is 2g/kg which is quite high; the use of much higher doses in toxicological test gives an idea of the safety margin of the extract. The extract of the dose of 2g/kg caused significant increase in the level of GGT, ALP and significant decrease at the level of ALT and total protein at all doses. On the other hand the extract did not cause any significant change in the level of AST, Total bilirubin, direct bilirubin and albumin and blood glucose.

INTRODUCTION: Bridelia is named after Prof. S.E. Bridel (1761 - 1828), Micrantha means small flowered [1]. *Bridelia micrantha* is commonly known in English as "coastal golden leaf", Ogaofia" (the boss of the bush) in Igbo.

QUICK RESPONSE CODE

DOI: 10.13040/IJPSR.0975-8232.5(6).2856-65

Article can be accessed online on: www.ijpsr.com

DOI link: http://dx.doi.org/10.13040/IJPSR.0975-8232.5(6).2856-65

It is a semi – deciduous tree up to 20m tall with a dense rounded crown alternated leaves and spine stem indigenous to southern part of Nigeria. Its stem bark is grayish brown, flaky and slightly rough in order specimens, sometimes with small blunt spines at the bottom of trunk with its branches full of spines [2, 3].

It belongs to the Kingdom of plantain, order of Malpihiales, Family of Phyllanthaceale, genus of *Bridelia* and species of *B. Micrantha* [4].

The compounds isolated from *B. micrantha* include: Friedelin, Taraxeronie, Epifiedelinol, Taraxerol, Garlic acid, Ellagic acid and sitosterol [5]. Its stem bark which is yellow grey and smooth to slightly rough, has been of interest to researchers because of its use in the treatment of various disease condition in Nigerian traditional medicine. *B. micrantha* are used by the Uhavenda for the treatment of gynaecoloficalk complains [6]. Bark infusions of *B. micrantha* are taken by the zulu as emetics [7]. The East Africans use it to treat stomach ache, tapeworm infestations and diarrhea and as tonics for children [8]. In Zimbabwe, it is used to treat infant coughs [9]. Previous studies have demonstrated antibacterial activities [10,11], antimalarial activities [12], antidiarrhoeic activities [13], N – Hexane sub – fraction of *B. micrantha* back has antimycobacterium activity [14], ethylacetate extract of *B. micrantha* has hepatoprotective and anti – oxidants activities on istar rats [15]. Additionally, *B. micrantha* has been reported to inhibit lactamase activity, RNA dependent, DNA polymerase and ribonuclease activities of human immunodeficiency type 1 reverse transcriptase (HIV – 1 RT) [5, 16].

In this study, we evaluated the acute and subacute toxicological profile of *Bridelia micrantha*.

MATERIALS AND METHODS

Plants Material: Fresh stems of *B. micrantha* were collected from Afeye- Okpameri in Akoko-Edo Local Government Area of Edo State, Nigeria in November 2011. The plant was identified by Dr J.F. Bamidele in the department of Plant Biology and Biotechnology, Faculty of Life Sciences, University of Benin, Benin City. Immediately after collection, the stems were washed, chopped into bits and sundry for a week. The dried stems were pulverized (650g) was mixed with distilled water (5 litres) and left for 72 hours. The mixture was stirred at 3 hours interval using a sterile glass rod. At the end, the extract was passed through fitter paper. The filtrate was concentrated over a water bath and the concentrated extract was stored and refrigerated

Animals: All experiments were carried out using mice of both sex weighing 20-35g and adult Wistar rats weighing 150-250g. The animals were breed locally in the Department of Microbiology's animal house, University of Benin, Benin City, Nigeria. Animals were housed in a standard cages and allowed access to rat pellets and tap water. They were exposed to 12 hour light/dark cycle and were handled according to standard protocols for the use of laboratory animals (National Institute of Health USA; Public Health Service Policy on HUMANE care and use of Laboratory Animals 2002).

Acute toxicological experiment: The intra-peritoneal and oral LD_{50} of the aqueous extract of B. *micrantha* were estimated by the Miller and Tainter method [17]. For the oral route two rodent species were used, mice and rats. Six groups of rats and mice comprising of 3 males and 3 females per group were used. Group 1 (control) was given the vehicle for extract reconstitution. Group 2-6 were administered 1, 2, 4, 6 and 8g/kg body weight of the extract respectively using Orogastric tube. Signs of acute toxicity and mortality were monitored for 72 hours and then 14 days. This protocol was repeated by administering extract intraperitoneally to another set of mice (6 per group) using a doses 10, 100, 200, 600, 800 and 1000mg/kg respectively.

Sub-acute toxicological experiment: Rats of both sexes were selected into four groups of control (n=6) and treated (n=6), such that the number of both sexes were the same in control and treated groups. The treated groups were given 0.5g/kg, 1g/kg and 2g/kg body weight (P.O) of extract daily for 28 days, the control group was given 10ml/kg of distilled water (P.O) daily for 28 days. The doses of the extract chosen were based on the accepted method [18]. On the 28th day, the animals were anaesthetized with chloroform in a chamber and blood collected from the abdominal aorta for biochemical and haematological assays. The heart, right kidney, liver and spleen were isolated, weighed and preserved for histological analysis.

Haematological assays: The haematological parameters were analyzed using auto haematology analyzer (Model BC-2800)

Biochemical assays: The blood samples were place in lithium heparin sample bottles and centrifuged at 3000 revolutions per minute (rpm) and plasma was separated using Pasteur pipettes into clear labeled bottles. The samples were stored

in deep freezer at -20°C until analyses were carry out. Total protein was assayed by Biuret method [19], albumin was assayed [20]. Alanine Aminotransferase (ALT) and Aspartate Aminotransferase (AST) were quantified [21]. Alkaline phosphate and GGT (Gamma Glutamyltransferase) were assayed using the standard method [22, 24], while Bilirubin was assayed by Jendrassik Grof method [23].

Histopathological Study: The isolated tissues of the liver, right kidney, heart and spleen were fixed in 10% formo saline for 18-24hour dehydration in an alcohol-xylene series and embedded in paraffin. Sections were stained with haematoxylin and eosin for histological examination.

Statistics: Results were expressed as Mean±SEM (Standard error of mean). Data were subjected to one way analysis of variance (ANOVA) and differences between samples were determined by newman-keuls multiple comparison test. All data were analyzed using Graph pad prism software (UK). P < 0.05 indicates statistically significant difference.

RESULTS: The oral LD_{50} of *B. micrantha* was indeterminable in rat and mice, as no deaths recorded in rats, one and two deaths recorded in mice at the dose of 6g/Kg and 8g/Kg respectively and the toxicology signs are dullness, writhing, decrease activity, sedation and fast breathing (**Table 1 and 2**). However, the LD_{50} following the intraperitoneal route was estimated to be 20.3 mg/kg (**Fig. 1**).The surviving animals following intraperitoneal route exhibited toxicological signs of writhing, decrease loco-motor activity, sedation and dose dependant mortality. Weight change and organ to body weight ratios were not significantly different between control and treated groups for 28 days oral daily administration (**Fig. 2 to Fig. 6**).

In **table 3,** the haematological parameter in the rat after 28days of daily oral administration show that white blood cell count (WBC) Monocyte, neutrophile, packed cell volume (PCV), haemoglobin (HB), red blood count (RBC) and platelet count (PLT) were all not significantly different between the treated groups and control. However, lymphocytes at the dose of 1000mg/kg indicates significant difference with control ($p<0.05$). Biochemical parameters in **table 4** indicate that total bilirubin, direct bilirubin, albumin, blood glucose, AST were all not significantly different between treated group and control ($p>0.05$). However, GGT, ALT, and ALP at the dose of 2000mg/kg shows significantly different with control ($p<0.05$). Total protein indicate significantly reduction with control at all doses ($p<0.05$).

TABLE 1: RESULT OF ACUTE TOXICOLOGICAL TEST OF AQUEOUS STEM EXTRACT OF *BRIDELIA MICRANTHA* IN RATS (OP)

Dose (g/kg)	Number of deaths	Mortality (%)	Symptoms
0	0/6	0	None
1	0/6	0	Writhing. Dullness after 1 hour of administration thereafter normalcy restored.
2	0/6	0	Writhing, dullness, sedation thereafter normalcy restored.
4	0/6	0	Writhing, dullness, sedation thereafter normalcy restored.
6	0/6	0	Writhing, dullness, decrease activity and sedation thereafter normalcy restored after 1 hr
8	0/6	0	Writhing, dullness, sedation, thereafter normalcy restored after 4.8 hours

Animals were first observed for 72hours and then for 14days after drug administration.

TABLE 2: RESULT OF ACUTE TOXICOLOGICAL TEST ON AQUEOUS STEM EXTRACT OF *BRIDELIA MICRANTHA* IN MICE (OP)

Dose (g/kg)	Number of deaths	Mortality (%)	Symptoms
0	0/6	0	None
1	0/6	0	Dullness, writhing after 30minutes follow by sedation
2	0/6	0	Dullness, writhing, sedation
4	0/6	0	Writhing, dullness, decrease activity and sedation.
6	1/6	16.57	Writhing, dullness, decrease activity sedation , fast breathing and death
8	2/6	33.33	Writhing, dullness, decrease activity, sedation, fast breathing and death.

Animals were first observed for 72 hours and then for 14 days after extract administration.

TABLE 3: EFFECT OF 28 DAYS ADMINISTRATION OF AQUEOUS STEM EXTRACT OF *BRIDELIA MICRANTHA* ON SOME HAEMATOLOGICAL PARAMETERS ON RATS

Dose	PCV (%)	HC (gdL^{-1})	RBC ($\times10^{12}$/ul)	WBC(10^9/l	LYM (%)	NEU (%)	MONO (%)	PLT ($\times10UL^{-1}$)
Control	43.67±1.43	14.52±0.47	3.72±0.23	23.23±0.49	47.67±2.20	44.33±2.03	8.50±1.12	340.00±20.73
500mg/kg	42.17±0.65	14.07±0.22	3.34±0.12	21.33±0.01	51.83±0.60	38.83±1.42	8.00±0.26	359.30±16.45
1000mg/kg	42.33±0.95	14.10±0.32	3.46±0.14	23.47±0.93	42.00±1.29*	43.85±1.85	7.50±0.85	352.20±14.07
2000mg/kg	43.67±1.31	14.48±0.44	3.67±0.15	23.20±0.89	48.83±1.85	42.33±1.78	9.00±0.45	326.30±19.43

*p<0.05 compared to control value. PCV: packed cell volume: HB: Haemoglobin RBC: Red Blood Count; WBC: White Blood Count; LYM: Lymphocytes NEU: Neutrophils; PLT: Platelet Count, n=6 (central) or (treated).

TABLE 4: EFFECT OF 28DAYS DAILY ADMINISTRATION OF AQUEOUS STEM EXTRACT OF *BRIDELIA MICRANTHA* ON SOME BIOCHEMICAL PARAMETERS ON RATS

Dose	GGT (UL^{-1})	AST (UL^{-1})	ALT (UL^{-1})	Total Bilirubin (mg/dl)	Direct Bilirubin (mg/dl)	Albumin (g/dl)	Total Protein (g/dl)	ALP (UL^{-1})	Blood Glucose (mg/dl)
Control	13.70±5.39	18.17±4.41	57.33±5.49	6.52±1.76	4.22±1.20	3.71±0.72	10.20±1.28	31.90±0.34	133.80±18.73
500mg/kg	10.77±3.64	14.17±3.92	48.67±4.16	7.35±0.97	2.24±0.60	1.28±0.38	7.41±0.84**	49.78±11.96	123.80±9.12
1000mg/kg	11.93±1.01	15.00±1.81	45.17±6.17	4.93±1.12	4.82±0.51	4.09±0.13	4.77±0.41*	114.90±21.24	86.35±13.27
2000mg/kg	48.52±3.36**	20.00±3.36	34.67±4.52*	4.05±1.08	5.09±0.64	4.61±1.77	4.15±0.15*	287.20±123.5*	106.80±8.79

*p< 0.05, **p<0.01 Compared to control value in column GGT: Gamma Glutamyltransferase; AST: Aspartase Aminotransferase; ALT: Alamine Aminotransferase; ALP: Alkaline phosphatase; n=6 (control) or (treated).

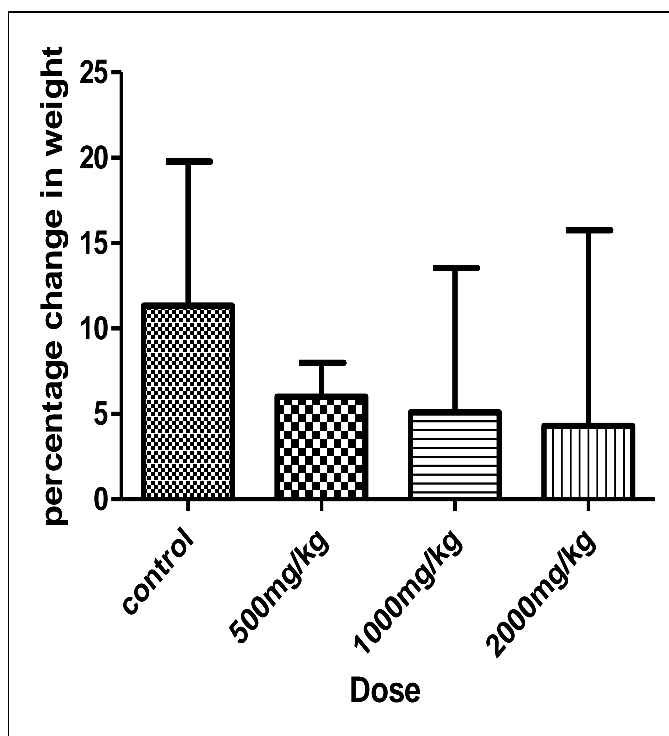

FIGURE 2: THE EFFECT OF AQUEOUS STEM EXTRACT OF *BRIDELIA MICRANTHA* ON WEIGHT CHANGE. Values are not significantly different (P>0.05) n = 6

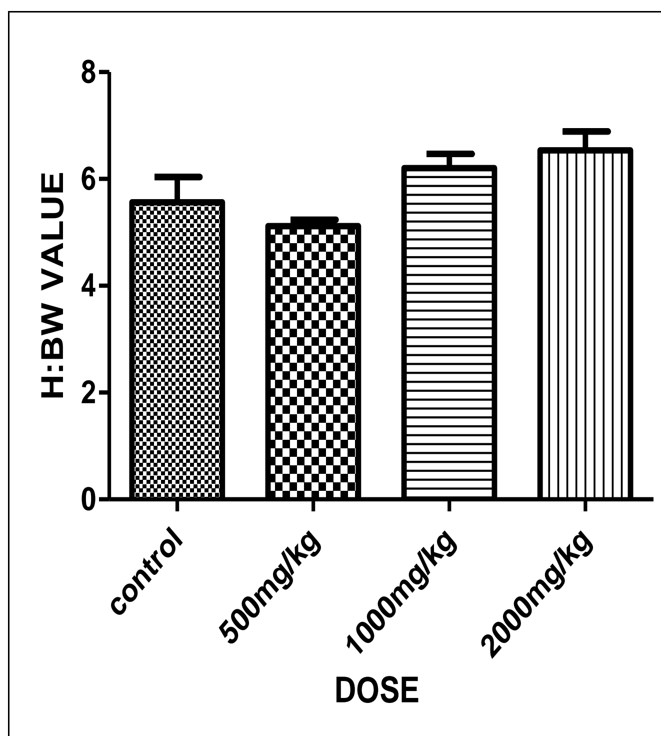

FIGURE 3: THE EFFECT OF AQUEOUS STEM EXTRACT OF *BRIDELIA MICRANTHA* ON HEART BODY WEIGHT RATIO. Values are not significantly different (P>0.05) n = 6

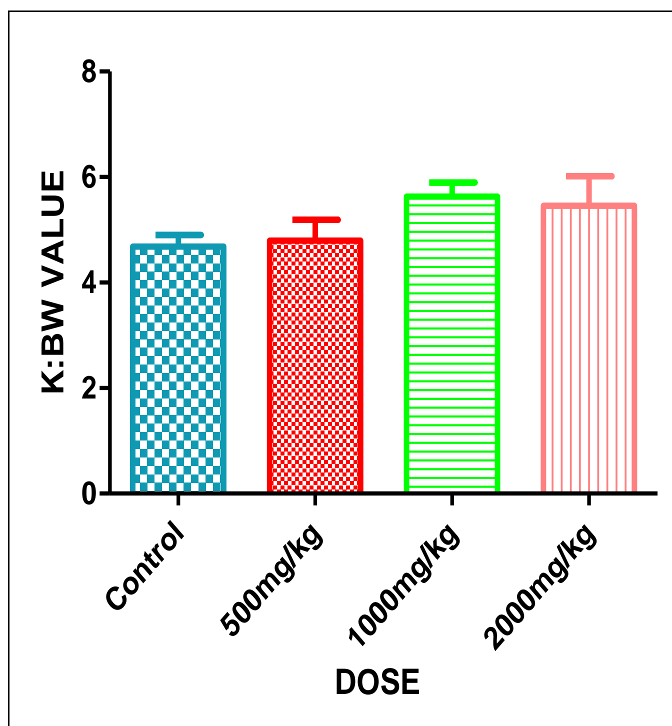

FIGURE 4: THE EFFECT OF AQUEOUS STEM EXTRACT OF *BRIDELIA MICRANTHA* ON KIDNEY BODY WEIGHT RATIO. Values are not significantly different (P>0.05) n = 6

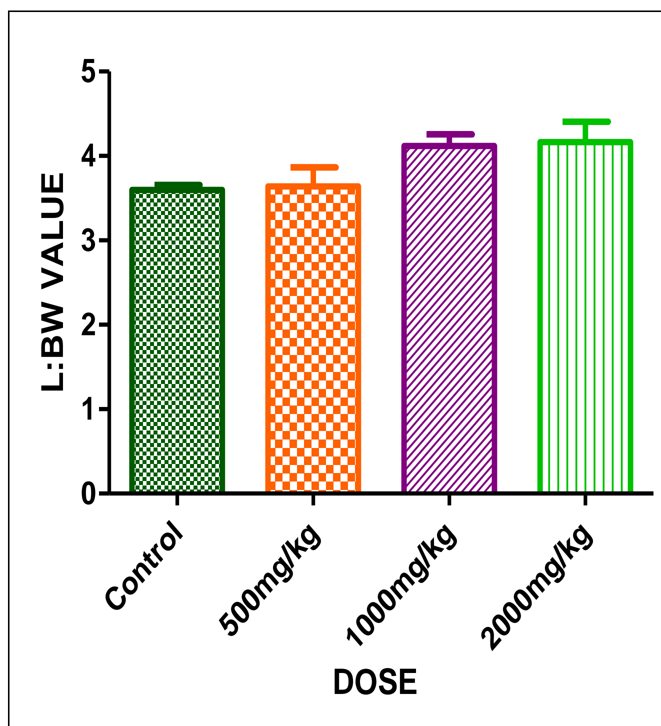

FIGURE 6: THE EFFECT OF AQUEOUS STEM EXTRACT OF *BRIDELIA MICRANTHA* ON LIVER BODY WEIGHT RATIO. Values are not significantly different (P>0.05) n = 6

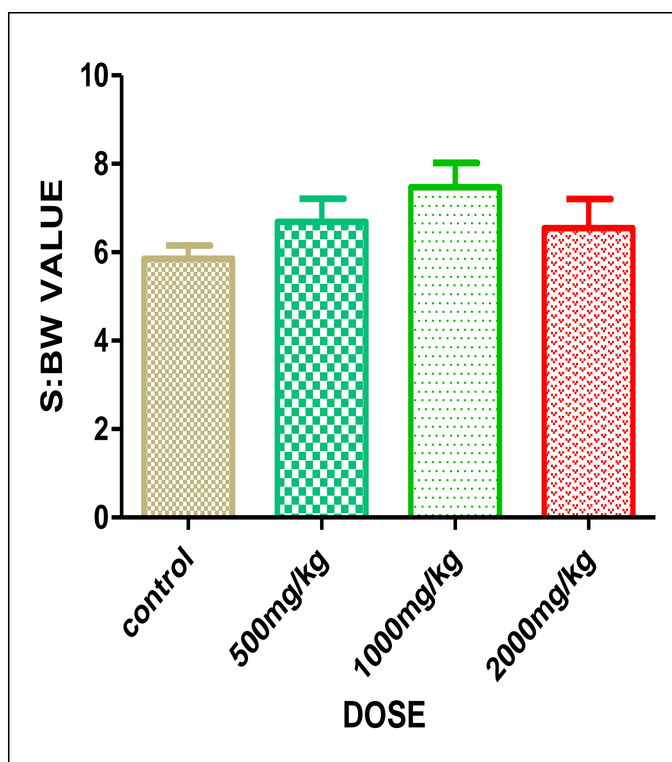

FIGURE 5: THE EFFECT OF AQUEOUS STEM EXTRACT OF *BRIDELIA MICRANTHA* ON SPLEEN BODY WEIGHT RATIO. Values are not significantly different (P>0.05) n = 6

DISCUSSION: LD_{50} determination has remained a useful tool in safety assessment of substances, in spite of its several criticisms [17, 25]. The oral administration of the aqueous stem extract of *B. micrantha* in divided doses up to 8g/kg show no mortalities in rats and but showed mortalities at the dose of 6g/kg and 8g/kg in mice recording 16.6% and 33.33% mortality respectively indicating that the LD_{50} of the aqueous extract is greater than 5mg/kg. The LD_{50} greater than 5mg/kg put *B. micrantha* aqueous extract under the non-toxic category substance in using the Hodge and Sterner scale for toxicity classification of substance [26, 27]. In Fig. 1, following LD_{50} the intraperitoneal route is not safe. This result also indicates that the aqueous extract is better tolerated when administered orally than the intraperitoneal route. Thus, it is relatively safe through the oral route.

In the sub-acute toxicity study, all the haemotological parameters (except 1000mg/kg of lymphocyte) of all treated groups were not significantly different from the control group. The haematology result shows that the extract has no toxic effect on the haematology parameters. Some plants are known to cause destruction of red blood cells leading to anaemia; examples of such plants

include *Solanum tuberosum*, *S. lycopersicum*, *Mercuriatlis perennis, M. annua* [28, 29, 30]. The highest dose used in this study is 2g/kg which is quite high; the use of much higher doses in toxicological test gives an idea of the safety margin of the extract [31]. In fig. 2 to fig. 6 the body weight and the organs weight were not significantly altered, to show that the extract has no toxic effect on body and organ weight of the rats, it also indicate that the extract has no effect on the feed consumption.

The extract to the dose of 2g/kg caused significant increase in the level of GGT, ALP and significant decrease at the level of ALT and total protein at all doses. On the other hand the extract did not cause any significant change in the level of AST, Total bilirubin, direct bilirubin and albumin and blood glucose. GGT is a liver enzyme that is easily induced by drug [26, 32]. ALP, AST and ALT in tissue and blood are important marker enzymes which are used to access the integrity of cell membrane, cytosolic activity and cell death [33, 34].

Increase in ALP level suggests the possibility of the extract causing membrane damage in the level of high concentration. However, increased ALP activity is needed during stress to produce adequate amount of phosphate groups for oxidative phosphoryllation to generate ATP which, in turn, is required for the phosphoryllation of some biomolecules like ethanolamine and choline to form phosphaticyl ethanolamine and phosphatidyl choline which are vital phospholipids component of the plasma membrane, thereby trying to stabilize the integrity of the membrane [33].

Therefore higher concentration of the extract may enhance ALP activity during stress. The decreases in total protein shows that extract reduce the activity of total protein. When treated with 500 mg/kg, the heart showed mild vascular congestion, interstitial oedema and infiltrates of chronic inflammatory cells. These are signs of chronic inflammation (myocarditis) (**fig. 7 – 9**). Increasing the dosage to 1000 mg/kg and then to 2000 mg/kg produced little changes from the low dose apart from slight increase in the thickness of the wall of the blood vessels (**fig. 10**). When treated with 500 mg/kg and 1000 mg/kg *B. micrantha* (**fig. 11 – 13**), the liver showed mild signs of chronic inflammation around the portal region. However, with 2000 mg/kg, there was additional damage to the wall of the portal vein and the bile ducts (**fig. 14**). Treatment with (**fig. 15 – 17**) *B. micrantha*, the spleen showed activation of the lymphoid follicles, as well as the sinus histiocytes, which was most prominent with 1000 mg/kg dose and least evident with 2000 mg/kg dose (fig. 15 – 18). When treated with *B. micrantha*, there was mild chronic inflammation in the renal cortex. However, with increasing dosage, there appears to be progressive thickening in the wall of the cortical blood vessels (**fig. 19 – 22**).

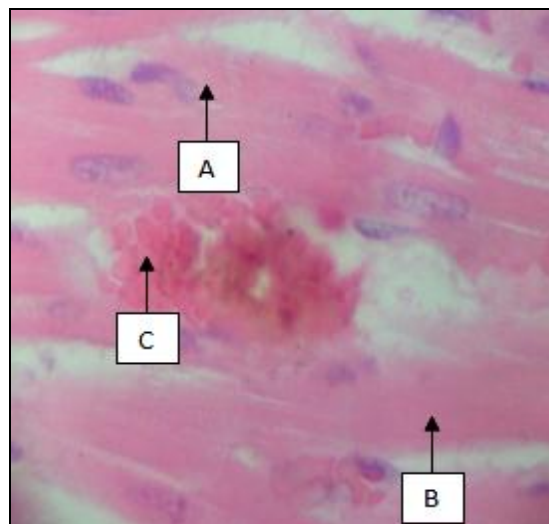

FIG. 7: RAT HEART (CONTROL) SHOWING MYOCYTES A, SEPARATED BY MILDLY *OEDEMATOUS INTERSTITIUM* B AND PIERCED BY CONGESTED CORONARY VESSEL C (H&E X 400)

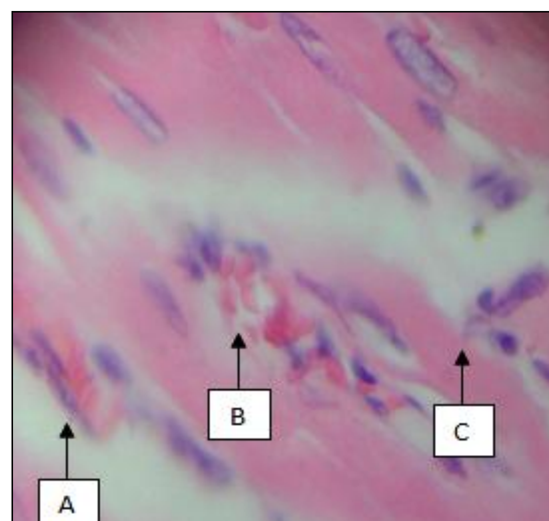

FIG. 8: RAT HEART TREATED WITH 500MG/KG *B.MICRANTHA* FOR 28 DAYS SHOWING MILD INTERSTITIAL OEDEMA A, MILD VASCULAR CONGESTION B AND INFILTRATES OF CHRONIC INFLAMMATORY CELLS C (H&E X 400)

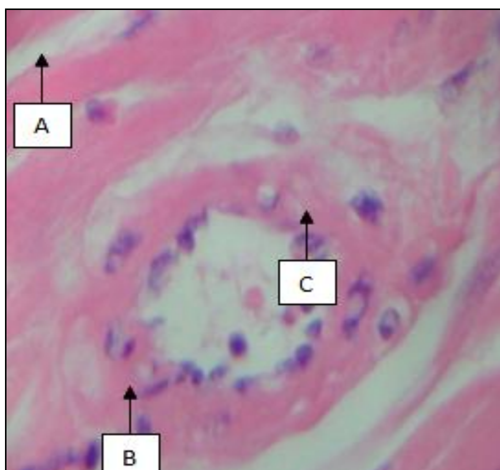

FIG. 9: RAT HEART TREATED WITH 1000MG/KG B.MICRANTHA FOR 28 DAYS SHOWING MILD INTERSTITIAL OEDEMA A, MILD VASCULAR HYPERTROPHY B AND INFILTRATES OF CHRONIC INFLAMMATORY CELLS C (H&E X 400)

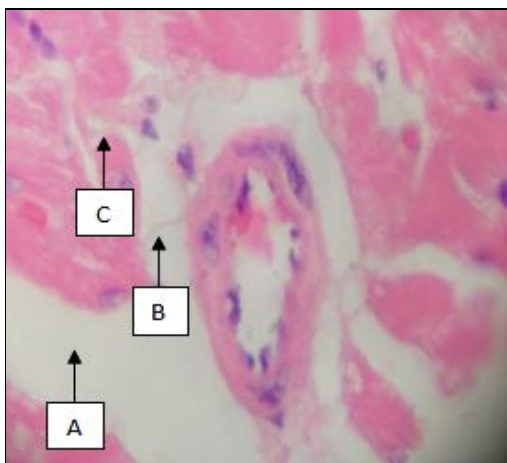

FIG. 10: RAT HEART TREATED WITH 2000MG/KG B.MICRANTHA FOR 28 DAYS SHOWING MILD INTERSTITIAL OEDEMA A, MILD VASCULAR HYPERTROPHY B AND INFILTRATES OF CHRONIC INFLAMMATORY CELLS C (H&E X 400)

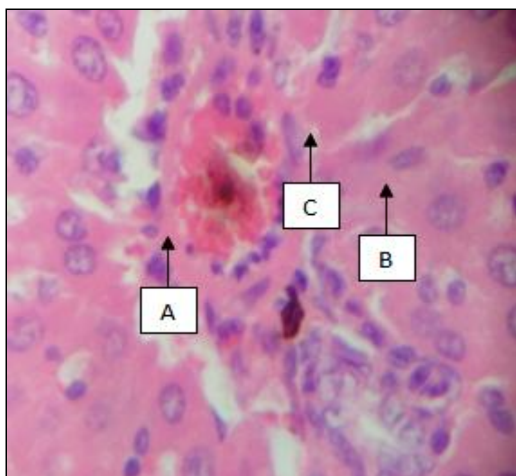

FIG. 11: RAT LIVER (CONTROL) SHOWING PORTAL TRIAD A, HEPATOCYTES B, SEPARATED BY SINUSOIDS C (H&E X 400)

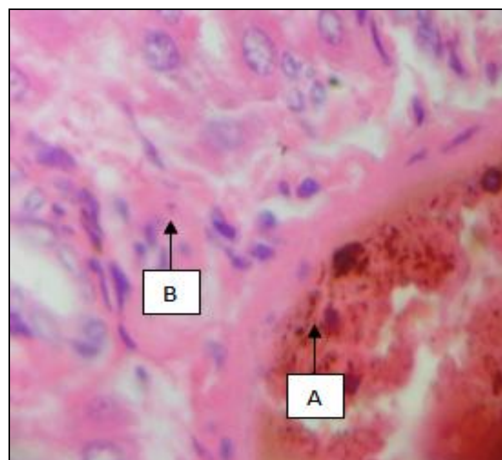

FIG. 12: RAT LIVER TREATED WITH 500MG/KG *B.MICRANTHA* FOR 28 DAYS SHOWING MODERATE PORTAL VASCULAR CONGESTION AND DILATATION A AND MILD PEREPORTAL INFILTRATES OF CHRONIC INFLAMMATORY CELLS B (H&E X 400)

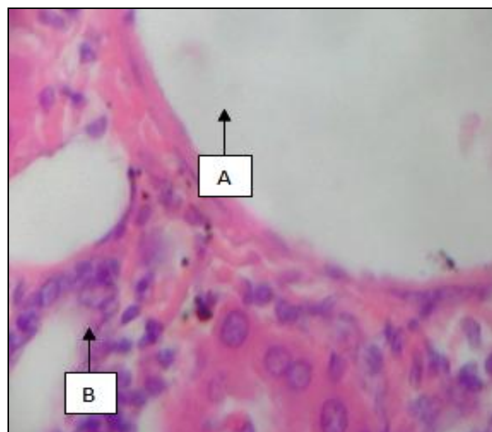

FIG. 13: RAT LIVER TREATED WITH 1000MG/KG *B.MICRANTHA* FOR 28 DAYS SHOWING MODERATE PORTAL DILATATION A AND MILD PEREPORTAL INFILTRATES OF CHRONIC INFLAMMATORY CELLS B (H&E X 400)

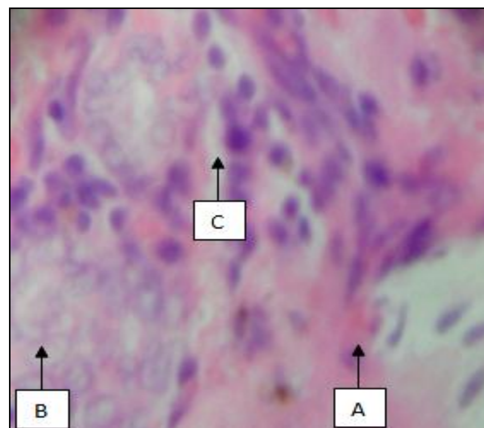

FIG 14: RAT LIVER TREATED WITH 2000MG/KG *B.MICRANTHA* FOR 28 DAYS SHOWING MILD PORTAL A AND BILE DUCT DEGENERATION B AND MILD PEREPORTAL INFILTRATES OF CHRONIC INFLAMMATORY CELLS C (H&E X 400)

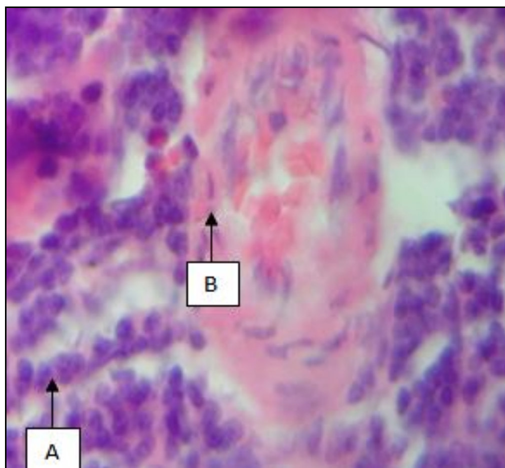

FIG. 15: RAT SPLEEN (CONTROL) SHOWING LYMPHOID FOLLICLES A AND SPLENIC ARTERIOLE B (H&E X 400)

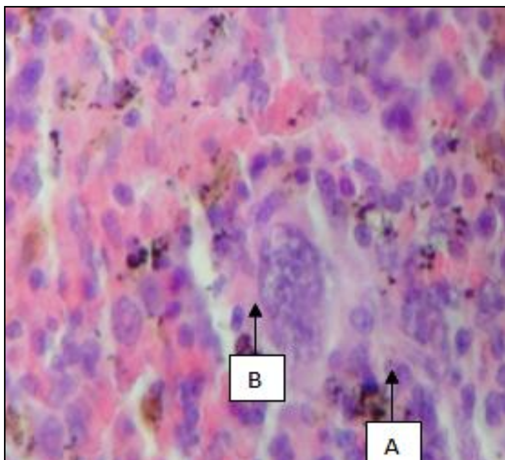

FIG. 16: RAT SPLEEN TREATED WITH 500MG/KG *B.MICRANTHA* FOR 28 DAYS SHOWING MILDLY ACTIVATED FOLLICLES A AND SINUS HISTIOCYTES B (H&E X 400)

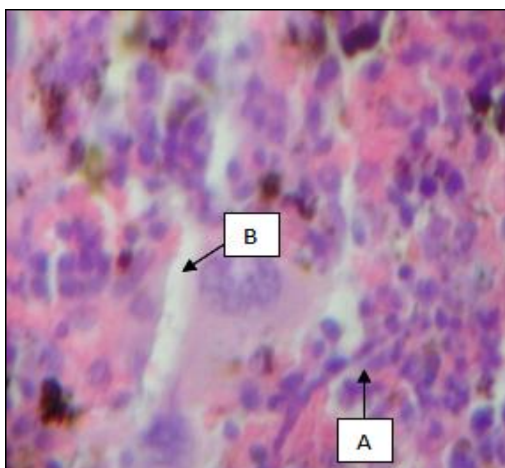

FIG. 17: RAT SPLEEN TREATED WITH 1000MG/KG *B.MICRANTHA* FOR 28 DAYS SHOWING MODERATELY ACTIVATED FOLLICLES A AND SINUS HISTIOCYTES B (H&E X 400)

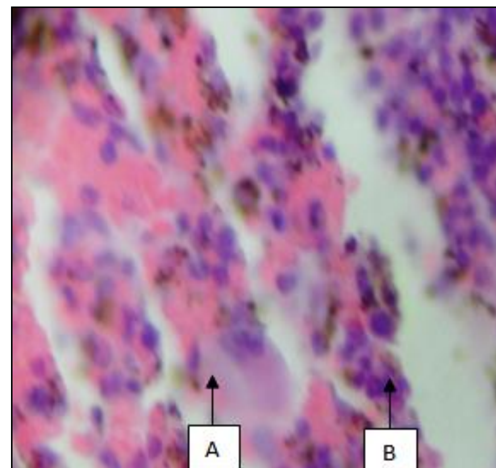

FIG. 18: RAT SPLEEN TREATED WITH 2000MG/KG *B.MICRANTHA* FOR 28 DAYS SHOWING MILDLY ACTIVATED SINUS HISTIOCYTES A AND MODERATE STROMAL OEDEMA B (H&E X 400)

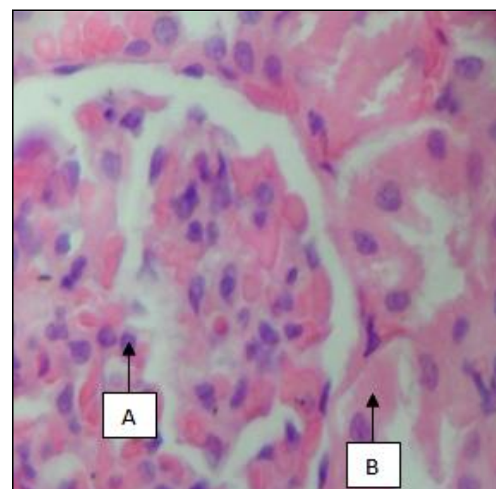

FIG. 19: RAT KIDNEY (CONTROL) SHOWING CORTICAL GLOMERULUS A, AND TUBULES B

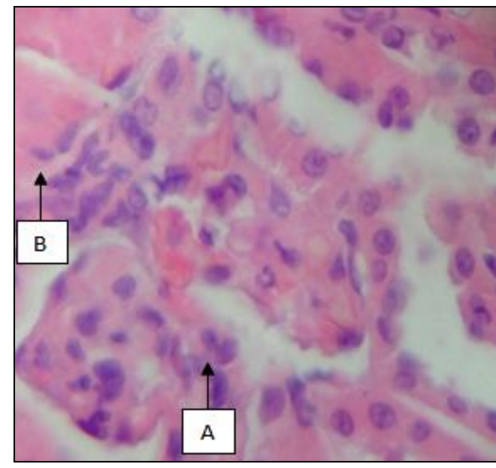

FIG. 20: RAT KIDNEY TREATED WITH 500MG/KG *B.MICRANTHA* FOR 28 DAYS SHOWING MILD INTERSTITIAL OEDEMA A, MILD INTERSTITIAL INFILTRATES OF CHRONIC INFLAMMATORY CELLS B (H&E X 400)

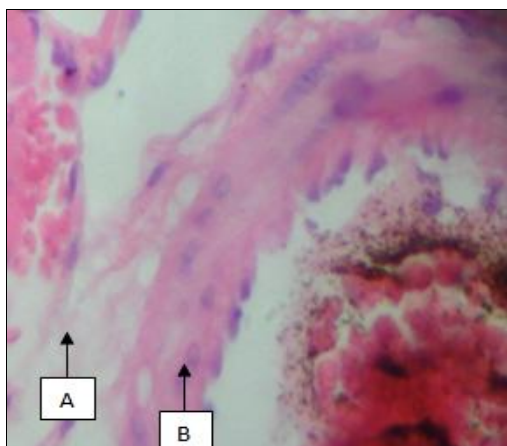

FIG. 21: RAT KIDNEY TREATED WITH 1000MG/KG *B.MICRANTHA* FOR 28 DAYS SHOWING MODERATE INTERSTITIAL OEDEMA A, MODERATE INTERSTITIAL VASCULAR HYPERTROPHY, DILATATION AND CONGESTION B (H&E X 400)

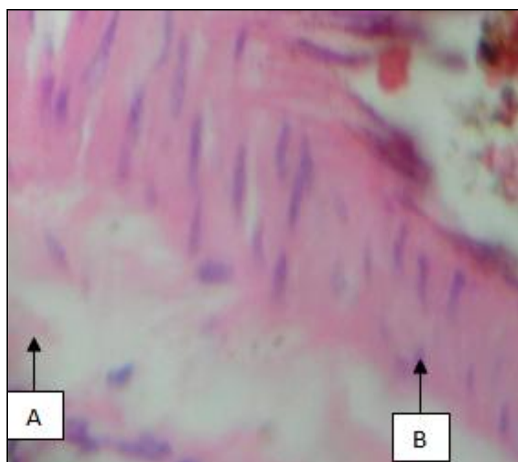

FIG. 22: RAT KIDNEY TREATED WITH 2000MG/KG *B.MICRANTHA* FOR 28 DAYS SHOWING MODERATE INTERSTITIAL OEDEMA A, MODERATE INTERSTITIAL VASCULAR HYPERTROPHY, DILATATION AND CONGESTION B (H&E X 400)

CONCLUSION: The LD_{50} of *B. micrantha* was indeterminable in rat and mice, as no deaths recorded in rats, one and two deaths recorded in mice at the dose of 6g/Kg and 8g/Kg, LD_{50} following the intraperitoneal route was estimated to be 20.3 mg/kg (Fig. 1).. The sub-acute toxicity studies carried out could be considered with a wide margin of safety for oral use at doses below 2000mg/kg as there were no significant alterations in the haematological and biochemical parameters.

The extract administered caused no damage to the tissue analyzed, however it produced mild inflammation in all the tissues as well as activating the local immune system, with changes been most apparent at a very high dose (2000mg/kg).

Since toxicity in humans cannot always be entirely extrapolated from animal studies, clinical evaluation should be performed to precisely define the safe dosage to administer in humans.

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How to cite this article:
Oshomoh EO, Idu M and Uwaya D: Toxicity studies of aqueous extract of *Bridelia micrantha* stem administered on mice and rats. Int J Pharm Sci Res 2014; 5(7): 2856-65.doi: 10.13040/IJPSR.0975-8232.5(7).2856-65

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