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ACUTE AND SUBCHRONIC TOXICITY STUDIES OF ETHANOL EXTRACT OF TERMINALIA MACROPTERA STEM BARK IN WISTAR ALBINO RATS

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ABSTRACT

This study evaluated the toxicity of ethanolic extract of Terminalia macroptera stem bark in female albino rats. Acute toxicity was determined by the Lorke Method while subchronic toxicity was determined by assessing some indicators of organ functions as well as lipid peroxidation levels after administering extract (50 - 1200 mg/kg) for 28 days. The extract exhibited LD_{50} > 5000 mg/kg. Subchronic investigation revealed slight cytolysis of hepatocytes but no significant effect on the hepatobiliary system. The synthetic role of the liver remained intact except in 400 - 1000 mg/kg dosed groups. Stable cardiological and nephrological states were indicated by heart and kidney function parameters respectively while malondialdehyde concentration was significantly elevated only in 200 mg/kg body weight dosed group when compared with control group. No distortions in liver tissue architecture were however observed. The study showed that extract from the stem bark of Terminalia macroptera is safe for consumption in rats but caution must be given to long-term repeated administration.

INTRODUCTION

Intensive study of plant extracts has come in the wake of the realisation that synthetic chemical treatment of ailments may lead to bioaccumulation of hazardous alien compounds. Plant phytochemicals are not only therapeutic, but being organic are readily absorbed in the body system. Traditional medicine practitioners who are engaged in the prescription of herbal preparations do so with no empirical evidence of the side effects associated with these medicinal plants or the safe dose just sufficient to eliminate the disease conditions. A common plant used in tropical Africa for various treatments is Terminalia macroptera (commonly called "Orin idi odan" among the Yorubas). It belongs to the Combretaceae plant family and it is a member of the guinea savanna biome[1]. Its leaves, flowers and stem have long been used in West Africa for the treatment of various diseases such as tuberculosis and hepatitis[2-7]. The extracts from the stem bark have been shown to depress food intake and decrease body weight[8]. Phytochemical evaluation of Terminalia macroptera stem bark extracts has revealed the presence of bioactive components[9-11]. However, detailed scientific studies of the plant's possible toxicity (within the available literature) have not been carried out. This among other reasons have made a number of pharmaceutical

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industries in Africa which should be using this herb to be doubtful about its potentials. There are arguments that raw parts of plants may be toxic due to the possible release of hydrocyanic acids or wild metabolites occasioned by special metabolic pathways in the body[12]. The aim of this work therefore, was to determine if the stem bark of Terminalia macroptera plant is safe for consumption when used for the various treatment of ailments.

MATERIALS AND METHODS

Collection of Plant Material

Matured stem barks of Terminalia macroptera were obtained from open forest in Ilorin and identified in the Department of Plant Biotechnology, University of Benin, Benin City (voucher number UBH_T 0232).

Preparation of Plant Extract

The bark was washed, cut into bits, dried under shade to constant weight and pulverized. Powdery sample obtained was then filtered with a 2 mm screen. The crude extract was prepared by adding 800 ml of absolute ethanol to 200 g of pulverized Terminalia macroptera stem bark and exhaustively extracted (at a temperature range of 8 - 15 ^{O}C in a refrigerator) for 72 hours by maceration and regular agitation of the sealed jar. The extract was evaporated to dryness in vacuum at 40 ^{o}C under a pressure of 175 mbar with a rotary evaporator (Model: SM-52 CS-1, Surgifield Medical, England) under reduced pressure.

Experimental Animals

Adult female Wistar rats of albino strain of approximate weight range (170 - 200 g) were obtained from a pure line breeder in Anatomy Department, School of Basic Medical Sciences, University of Benin, Benin City. The rats were maintained in well ventilated cages under standard laboratory conditions of temperature 23°C (± 3°C), photo sequence of 12 hours light and 12 hours dark, and relative humidity of 60 (± 5) % in the animal house of the Department of Biochemistry, University of Benin. They were allowed access to standard pelleted mash and water ad libitum. The rats were allowed to acclimatize for one week prior to commencement of experiment.

Acute Toxicity Study

The rats were randomized into 3 groups comprising 3 animals each. Predetermined test doses (10, 100 and 1,000 mg/kg body weight) were administered orally in a single dose to the rats by means of a gavage according to the method of Lorke[13]. Thereafter, animals were observed for manifestation of toxicity and mortality for 24 hours. In the second phase, 3 rats were divided into 3 groups of 1 rat each and were also treated with the extract at doses of 1,600, 2,900 and 5,000 mg/kg body weight orally. The median lethal dose (LD_{50}) was then estimated by the geometric mean of the doses for which mortality ratios of 0/1 and 1/1 were obtained.

Subchronic Toxicity Study

The animals were randomized into 6 groups of 4 animals each representing the dose groups of 50 mg/kg, 200 mg/kg, 400 mg/kg, 1000 mg/kg, 1200 mg/kg and control. The crude extract was orally administered daily for 28 days, while control animals received distilled water in an equivalent volume of the maximum test dose. The animals were also observed for manifestation of toxicity and mortality as in acute test.

Blood and Organ Collection

Upon termination of the experiment on the 29th day, the animals were euthanized (by inhalation for 1-3 minutes) in a chamber containing cotton boll wetted with drops of $CHCl_3$. Blood was obtained by heart puncture by means of a 5 ml hypodermic syringe and needle and placed in 5 ml sterile sample container for biochemical assays. The blood was allowed to stand at room temperature for 30 - 45 minutes to clot and centrifugation performed at 1000 x g for 5 minutes to obtain serum with an electric centrifuge-12 buckets, model 80 - 2 (Finlab Nigeria Ltd). The liver tissues were extracted, rinsed in physiological saline and dried between layers of Whatman filter paper.

Biochemical Analyses

The separated serum was analyzed for various biochemical parameters: alanine aminotransferase and aspartate aminotransferase activities[14], total protein concentration[15], serum albumin concentration[16] while the amount of globulin was calculated as a difference between total serum protein and serum albumin. Other biochemical parameters include serum bilirubin concentration[17], y-glutamyl transferase activity[16], alkaline phosphatase activity[18], lactate dehydrogenase activity[19], creatine kinase activity[20], creatinine concentration[21], serum urea concentration[22], sodium[23-24], chloride[25], potassium[26] and evaluation of the liver homogenate for membrane lipid peroxidation[27].

Preparation of Tissue Homogenates

Tissues of liver were homogenized respectively in ice cold normal saline (1:10 w/v) and centrifuged at 10,000 rpm for 15 minutes. The supernatant was stored at 4 °C and analysized within 24 hours for malondialdehyde concentration.

Histopathological Study

The tissues (liver) were fixed with 10 % formal saline solution (3 - 5 days). They were later dehydrated by passing through varying (increasing) concentrations of alcohol, 70 % to 100 %, cleared in xylene and then embedded in molten paraffin. Five micron (5 μm) microtome sections were stained with hematoxylin and eosin dyes. The sections were examined under light microscope at high power magnifications and photomicrographs taken[28].

Statistical Analysis

Results were analyzed statistically by one-way ANOVA followed by Duncan's multiple range test by using SPSS version 19 (2010). All values were expressed as the mean ± SEM. $P < 0.05$ compared to control was considered to be statistically significant.

RESULTS

There was no record of mortality for the acute toxicity investigation. However, somnolence was observed in the groups administered 1000 - 5000 mg/kg body weight, which reverted to normal agility after 4 hours. Mean lethal dose of the extract was found to be above 5000 mg/kg body weight.

Table 1 shows the effect of the extract on aspartate aminotransferase and alanine aminotransferase activities, total protein, albumin, globulin, direct and total bilirubin concentrations as well as y-glutamyltransferase and alkaline phosphatase activities of the rat liver enzymes present in serum after 28 days of administration. Extract doses of 200 mg/kg and 1000 mg/kg caused significant ($p < 0.05$) increase in AST activities when compared with control group. Extract concentrations of 200 mg/kg, 400 mg/kg,

Table 1: Liver Function Test of Rats after 28 Days of Administration

	Control	50 mg/kg	200 mg/kg	400 mg/kg	1000 mg/kg	1200 mg/kg
AST (U/L)	12.69±0.32 ^{ab}	13.19±1.12 ^{a}	19.37±1.26 ^{c}	12.92 ±0. 86 ^{a}	18.00 ±0.85^{c}	12.39±0.64 ^{ab}
ALT (U/L)	10.25±0.92^{a}	12.64±0.45 ^{a}	24.05±1.80 ^{bc}	19.72±1.80^{b}	23.25±1.24^{bc}	24.31±1. 87 ^{c}
T. P. (g/dl)	6.65± 0.06 ^{a}	6.13 ± 0.40 ^{a}	6.56 ± 0.26 ^{a}	3.38 ± 0.03 ^{b}	4.35 ± 0.19^{d}	7.56 ± 0.11 ^{c}
ALB. (g/dl)	3.85 ± 0.13 ^{ab}	3.73 ± 0.82 ^{abcd}	2. 83 ± 0.28 ^{cd}	2.27 ± 0.11 ^{d}	3.10 ± 0.06 ^{c}	3.72 ± 0.26 ^{ac}
GLOB. (g/dl)	2.80 ±0.08 ^{ab}	2.40 ± 0.44 ^{ad}	3.74 ± 0.33 ^{ce}	1.11 ± 0.09 ^{cf}	1.25 ± 0.25 ^{f}	4.01 ± 0.27 ^{e}
D.BIL. (mg/dl)	0.60±0.41 ^{abcd}	0.48±0.05 ^{bc}	0.41±0.01 ^{cd}	0.31±0.02 ^{e}	0.67±0.11 ^{b}	0.41±0.05 ^{cd}
T.BIL. (mg/dl)	0.74±0.08 ^{ab}	0.94±0.18 ^{abd}	0.79±0.06 ^{a}	0.94±0.10 ^{bc}	1.05±0.16 ^{c}	0.52±0.04 ^{d}
GGT (U/L)	3.53±0.37 ^{a}	2.47±0.47^{ac}	1.62±0.08^{c}	0.69±0.08^{b}	2.34±0.02^{a}	2.02±0.56 ^{ac}
ALP (U/L)	16.26±0.63 ^{a}	23.61±1.22 ^{b}	23.02±3.39 ^{ab}	10.71±1.86 ^{cd}	8.53 ± 0.78 ^{d}	15.19 ± 0.94 ^{ae}

Mean ± SE values on the same row with different superscripts differ significantly *(P<0.05)* for *n=4*. Where AST = Aspartate Aminotransferase, ALT = Alanine Aminotransferase, T. P. = Total Protein, ALB = Albumin, GLO = Globulin, D. BIL = Direct Bilirubin, T. BIL = Total Bilirubin, GGT = -Glutamyltransferase and ALP = Alkaline Phosphatase.

Table 2: Kidney Function Test of Rats after 28 Days of Administration

	Control	50 mg/kg	200 mg/kg	400 mg/kg	1000 mg/kg	1200 mg/kg
Creatinine (mg/dl)	1.73 ± 0.35[a]	4.20 ± 0.29[b]	2.79 ± 0.12[a]	2.08 ± 0.16[a]	2.47 ± 0.03[a]	2.72 ± 0.16[a]
Urea (mg/dl)	52.45 ± 3.71[a]	85.24 ± 4.46[b]	75.66 ± 2.64[b]	70.84 ± 3.07[b]	99.52 ± 6.81[b]	97.93 ± 5.49[b]
Sodium (mEq/L)	119.40 ± 4.38[a]	141.3 ± 3.34[b]	77.73 ± 5.60[b]	174.02± 11.20[b]	182.64 ± 0.64[b]	181.6 ± 10.70[b]
Chloride (mEq/L)	100.07 ± 0.19[a]	87.4 ± 5.00[a]	72.24 ± 3.76[b]	88.54 ± 2.68[b]	87.91 ± 2.85[b]	85.00 ±2.67[a]
Potassium (mEq/L)	5.73 ± 0.53[a]	3.10 ± 0.36[a]	1.14 ± 0.14[b]	1.50 ± 0.14[b]	3.87 ± 0.18[a]	6.94 ± 0.37[a]

Mean ± SE values on the same row with different superscripts differ significantly *(P<0.05)* for *n=4*.

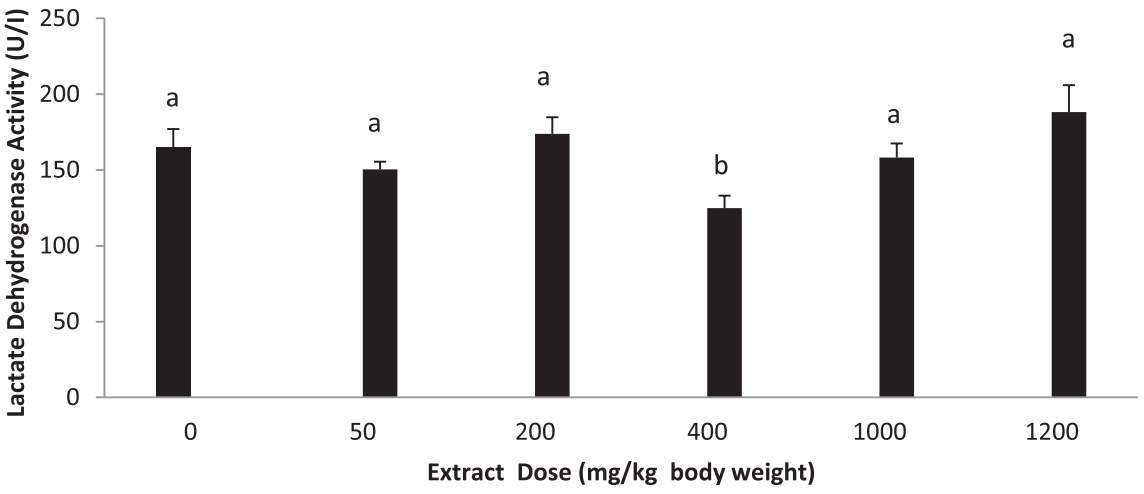

Fig. 1: Lactate dehydrogenase activity in rats administered ethanolic extract of *Terminalia macroptera* stem bark for 28 days. Height of each bar is mean value ± SE for *n=4* determinations. Statistical differences between control and tests were set at $P < 0.05$ and are indicated by different lower case alphabets.

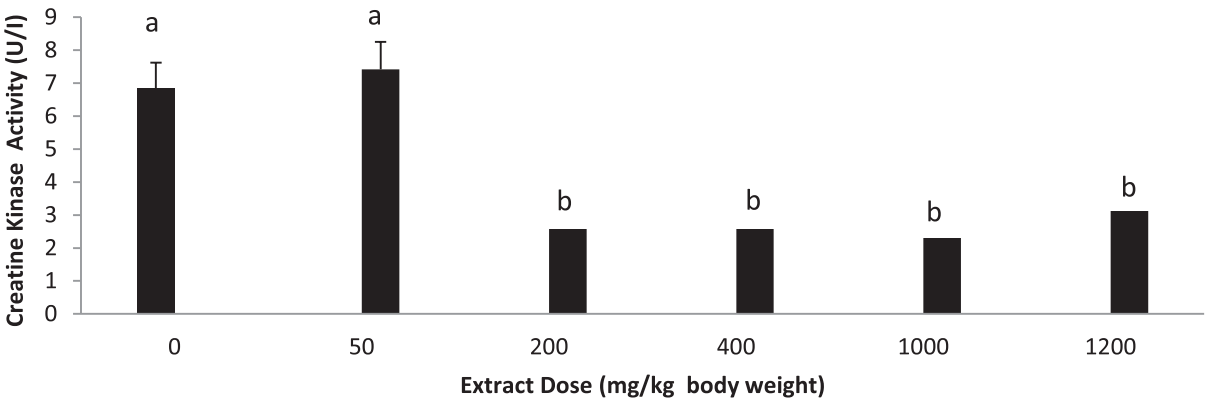

Fig. 2: Creatine kinase activities in rats administered ethanolic extract of *Terminalia macroptera* stem bark for 28 days. Height of each bar is mean value ± SE for *n=4* determinations. Statistical differences between control and tests at $P < 0.05$ are indicated by different lower case alphabets.

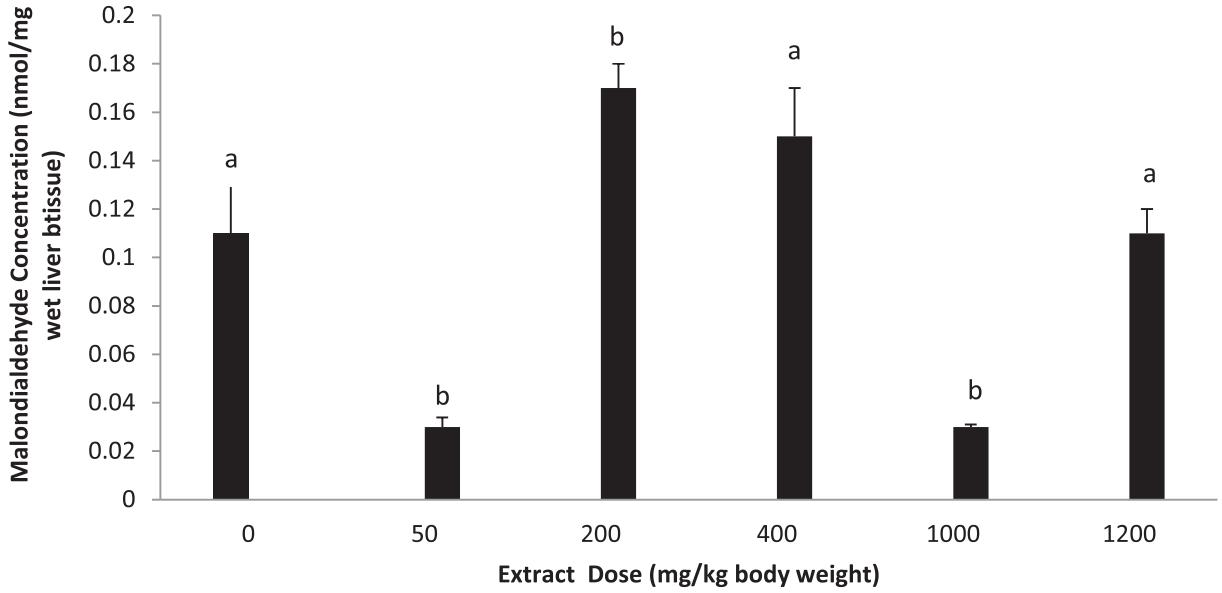

Fig. 3: Malondialdehyde concentration in rats administered ethanolic extracts. Height of each bar is mean value ± SE for *n =4* determinations. Statistical difference between control and extract tested groups at $P < 0.05$ are indicated by different lower case alphabets.

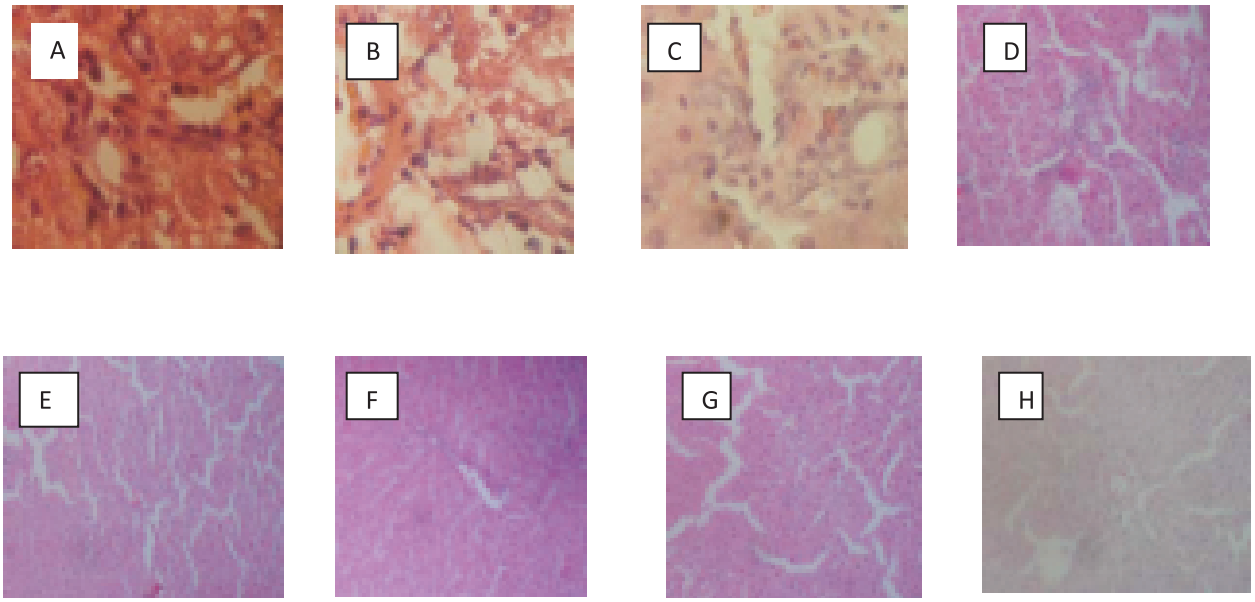

Fig. 4. A: Liver of control rat showing no visible histopathological lesion for 24 hours study period (H&E stain x 400). B: Liver of rat administered 5000 mg/kg ethanolic extract of *Terminalia macroptera* for 24 hours showing mild congestion and mild periportal lymphocytosis (H&E x 400) . C: Control rat liver showing portal triad, hepatocytes, sinusoids after 28 days study period (H&E x 400). D: Liver of rat administered 50 mg/kg ethanolic extracts of *Terminalia macroptera* for 28 days showing mild congestion and mild periportal lymphocytosis (H&E x 100). E: Liver of rat administered 200 mg/kg ethanolic extracts of *Terminalia macroptera* for 28 days showing mild congestion and mild periportal lymphocytosis (H&E x 100). F: Liver of rat administered 400 mg/kg ethanolic extracts of *Terminalia macroptera* for 28 days showing mild congestion and mild periportal lymphocytosis (H&E x 100). G: Liver of rat administered 1000 mg/kg ethanolic extracts of *Terminalia macroptera* for 28 days showing mild periportal lymphocytosis (H&E x 100). H: Liver of rat administered 1200 mg/kg ethanolic extracts of *Terminalia macroptera* for 28 days showing mild congestion (H&E x 100).

1000 mg/kg and 1200 mg/kg caused significant ($p < 0.05$) increases in ALT activities that were not dose dependent when compared with control group.

There was significant ($p < 0.05$) elevation in the concentration of total protein in the serum of rats dosed with 1200 mg/kg body weight extract of ethanol when compared with control. However, all other doses induced reduction in the concentration of serum total protein, where only those of 400 mg/kg and 1000 mg/kg were significantly reduced ($p < 0.05$), an observation which was similar for albumin and globulin. The administered dose of 200 mg/kg concentration of extract caused a significant ($p < 0.05$) depression in blood albumin with a contrasting significant ($p < 0.05$) elevation in

globulin content. This trend was also repeated in the group dosed with 1200 mg/kg body weight. No significant differences were observed in total bilirubin concentrations except in the group dosed with 1200 mg/kg which showed significant ($p < 0.05$) reduction with respect to control group.

The activities of y-glutamyl transferase were significantly decreased when compared to control group while those of alkaline phosphatase were significantly increased ($p < 0.05$) in groups administered lower doses. Their activities were not altogether altered in the highest dosed group.

Figure 1 shows the lactate dehydrogenase activities in serum of control and tested rats. The activity of the enzyme showed no variance with that of the control, except for the enzyme activity in the group administerd 400 mg/kg which reflected significant ($p < 0.05$) reduction. Figure 2 shows creatine kinase (CK) activities in rats administered ethanolic extract of Terminalia macroptera stem bark for 28 days. Doses 200 mg/kg, 400 mg/kg, 1000 mg/kg and 1200 mg/kg ethanolic extract administered groups experienced significant reduction ($p < 0.05$) in CK activities when compared with control group respectively.

Table 2 shows the kidney function tests of rats after 28 days of extract administration. Creatinine concentration of the test groups administered ethanolic extract were not significantly altered ($p < 0.05$) when compared with control group except for 50 mg/kg dosed group that was significantly higher ($p < 0.05$) than control. However, significant elevations ($p < 0.05$) were reflected in the urea and sodium ion concentrations of all test groups administered the extract.

Administered doses of 400 mg/kg to 1,200 mg/kg showed contrasting changes in the alteration of concentrations between sodium and chloride ions. The extract doses (200 mg/kg and 400 mg/kg) caused significant reduction ($p < 0.05$) in the concentration of potassium ion, but no significant ($p < 0.05$) differences were noted in the groups administered 50, 1000 and 1200 mg/kg of the extract when compared with control group.

The concentrations of malondialdehyde in rats administered ethanolic extracts are shown in figure 3. Test animals administered 200 mg/kg extract showed significant ($p < 0.05$) elevated levels in concentration of MDA when compared to control group respectively.

Figure 4 shows photomicrographic plates A to H of liver sections of control and test rats administered extracts of Terminalia macroptera stem bark.

DISCUSSION

One of the toxicological indices used to assess the safety of plant extracts is Lethal Dose 50 % (LD_{50}), which is the amount of acute dose of the drug required to kill half of the test population. In this study, 5000 mg/kg body weight of the extract did not reflect any observable toxic symptoms under 24 hour monitoring. The result of this study clearly corroborated previous work that aqueous extract of Terminalia macroptera stem bark are relatively safe at 2000 mg/kg[10]. This present study showed no record of mortality with ethanolic extract, however, the group of rats dosed with 1000 mg/kg to 5000 mg/kg body weight crude extract showed somnolence (animals appeared drowsy, but on prodding were aroused, moved few

sluggish steps and resumed sleeping positions) for 1 to 4 hours after administration. This is in consonance with the findings of Adebiyi and Abatan[29], who reported that 1000 mg/kg - 3000 mg/kg ethanolic extracts of Enanthia chlorantha stem bark caused reduced mobility, dullness and general weakness in albino rats within 24 hours of administration.

Although alanine aminotransferase (ALT) activities were significantly elevated just as aspartate aminotransferase (AST) activities were staggeredly elevated in a few dosed groups (200, 1000 and 1200 mg/kg b. wt), ALT to AST ratios (not shown in table) remained within non-pathological range indicating that increased ALT levels may just be a slight insult on the integrity of the liver or a physiological cause at its best. This observation was further buttressed by the significant decrease in the concentrations of bilirubin and the non-alteration in the concentration of albumin, an indication of a stable synthetic function of the liver in the presence of the extract at its highest administered dose. In this present study, 1000 mg/kg body weight of extract had shown clear cut indication of hepatotoxicity by the significant increases in aspartate aminotransferase, alanine aminotransferase, total bilirubin and significant decreases in total protein, albumin and globulin, but definitely no hepatobiliary damage as reflected by y-glutamyltransferase (GGT) and alkaline phosphatase (ALP) activities. This observation seems to give support to our previous study where 1000 mg/kg body weight of extract caused the most of depression in body weight of rats[8].

GGT is of clinical value in identifying the source of obscure ALP elevations whose activity is usually high in hepatobiliary disorder and bone diseases[30]. Consequently, y-glutamyltransferase determination is considered more sensitive when compared to alkaline phosphatase since it is primarily from the hepatobiliary system[31]. The results showed reduced activities observed in the y - glutamyltransferase (GGT) enzyme assay which did not complement the increase in alkaline phosphatase (ALP) activities seen only in rats administered 50 mg/kg (significant) and 200 mg/kg extracts (insignificant). The lack of consistency in the enzyme activities across the doses may be due to intrinsic factors in vivo which clearly did not point to hepatobiliary damage, but rather a probable bone effect, an argument that can only be substantiated by bone histomicrography (not considered in this study). The liver parameters showed much isolated increase and decrease in dosed animals that reflected a case of nonmonotonic dose response[32-33], however, the group administered the maximum dose showed no evidence of hepatotoxicity from the assay results.
The activities of lactate dehydrogenase and creatine kinase were completely stable in the presence of ethanol extract of Terminalia macroptera, an indication of no toxic effect on the heart muscle.

The insignificant increase ($p < 0.05$) observed in the creatinine concentration did not confirm the suspicion that the ethanolic extract is toxic to the ultrafiltration apparatus of the kidney as posed by the significantly elevated serum urea concentration of the test groups. However, the creatinine result may foretell of possible deleterious effect in the kidney upon prolonged administration. The observation with urea concentration may be due to decreased blood flow to the kidneys arising from stress in handling the animals. This appeared to be supported by the hypernatraemia observed in the extract dosed groups.

However, an onset of renal dysfunction resulting from the inability of the kidney to regulate electrolyte balance may not be totally denied since low chloride and potassium concentrations were recorded for the test groups, except for the non-significant changes reflected by the highest dose group which summarily portrayed a current stable kidney function.

Physiologically, the circulatory system of test animals provides a unique transport route for the plant phytochemicals making them come in contact with the organs that are most susceptible to their chemical influence. On arrival at a choice organ, a dorminant component or group of components present in the administered bolus, may have enjoyed an advantage in terms of large quantity or an effective bioactive structure when compared to other phytocomponents and consequently exhibited a progressive or regressive trend in observed effect under the dose response experiment. Sudden deviation in the trends observed upon the administration of the graded doses could be explained by the presence of an active component (initially minor and inactive at a lower dose), that just attained its effective bioactive level (EBL) at a higher dose in vivo irrespective of its ratio to the domineering component at the former dose(s)[34]. At this point, 'the nouveau active' either strengthened the observed dose response trend initiated at the lower doses or altered it. The effective bioactive component at the new dose became the dorminant component or at its best competed with the initial dose frontliner and where possible shared their dorminance at the receptors in the manipulation of the target organ. This most likely explains the multimodal effect of plant extracts[35] and may be responsible for the generality of inconsistency observed in the nonmonotonic dose response of the investigated extract[32].

Malondialdehyde (MDA) is a secondary product of lipid peroxidation, that causes cross-linkage of membrane components containing amino groups which makes the membrane fragile and easily perforated[36]. The monitoring of malondialdehyde levels in biological system can be used as an important indicator of lipid peroxidation in vitro and in vivo for various diseases. The mild elevation in concentration of liver MDA observed in a few dosed groups of rats may be due to peroxidation activity arising from injury to hepatic cells occasioned by repeated daily dosing. Consequently supporting the aforementioned, liver biomarkers (alanine aminotransferase) might probably have leaked through membranes of hepatocytes.

However, changes were not observed in the architectural layout of the liver tissue probably as a result of the minuteness of the membrane puncture. The presence of mild inflammatory cells detected in the liver slides auguments this argument. Equally, the congestion and lymphocytosis so observed in the photomicrographs indicated a diffused activation of the local immune system of the liver, which is a desirable effect of the extract, though. These observations however, send precautionary signals on the repeated administration of ethanolic extract of Terminalia macroptera stem bark.

CONCLUSION

The study has shown that the LD_{50} of ethanolic extract of Terminalia macroptera stem bark is greater than 5000 mg/kg body weight of Wistar albino rat

and therefore not acutely toxic. Moreso, the administration of this extract did not produce overt subchronic toxicity, however consumption for longer periods of time and at higher doses would require corresponding liver and kidney assessment especially when it is used for the treatment of hepatitis.

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