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ACUTE AND SUB-CHRONIC TOXICITY PROFILE OF *Annona muricata* (Sour sop*)* ON WISTER ALBINO RATS

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ABSTRACT
In drug development, determination of its toxic profile is paramount and usually the initial step in its evaluation. Phytochemical constituent's assessment can subsequently be elucidated. Annona muricata is an evergreen shrub that is endowed with ethno-medicinal values and also served as a source of food. The significant needs to evaluate the toxic profile of the plant lead to the design of this work. Therefore this work is designed to evaluate the phytochemical constituents, acute (LD_{50} as the index) and sub-chronic toxicity profile of the leaf extracts. The effect of the extracts on the serum levels of some biochemical parameters which include alkaline phosphatase (ALP), alanine aminotransferase (ALT) and aspartate aminotransferase (AST). Also include the effect on the kidney function parameters such as Urea, creatinine and electrolyte (K+, Na+, Cl- and HCO3-). The LD_{50} of the extract indicated that the plant extract is relatively safe with no mortality recorded at dose >5000mg/kg body weight of the drug. There was no significant (P > 0.05) significant difference in the serum level of ALP ALT and AST for the liver function test. In a related effect no significant variation recorded on the kidney function parameters. However, salient mild effect on the liver and kidney was observed from the histopathological examination, but can be effectively averted if extended use of higher concentration of the extract is avoided, as the effect is observed to be both concentration and time dependent. However the research findings observed and suggested, the possible hepato-protective and neuphro-protective potential of the plant methanol extract which should be further investigated
Keywords: Acute toxicity, Sub-acute toxicity, Annona muricata, Albino rats, Liver function and Kidney function.

INTRODUCTION

In recent times, there has been an increasing awareness and interest in medicinal plants and their preparations, commonly known as herbal medicines. The exclusive use of herbal drugs, prepared and dispensed by unscientifically trained herbalists, for treatment of diseases is still very common in rural communities in Nigerian and other African countries (Ogbonnia *et al*., 2008). These herbal drugs include herbs, herbal materials and preparations as well as finished herbal products that contain as active ingredients that are parts of plants, other plant materials or combinations (Larbie *et al*., 2011).

Medicinal plants constitute an important natural wealth of a country. They play a significant role in providing primary health care services to rural people. They serve as therapeutic agents as well as important raw materials for the manufacture of traditional and modern medicine. Substantial amount of foreign exchange can be earned by exporting medicinal plants to other countries (Adewole and Caxton-Martins, 2006).

The practice of traditional medicine is a worldwide custom that has preoccupied mankind in most civilized countries. In Nigeria, both herbal and orthodox medicines are practiced, though the conventionally trained medical doctors complement the former. Despite the fact that traditional approach to health care delivery system is popularly practiced and in spite of the availability of medicinal herbs in our local markets, there are very few if any in government health centers. Thus, problems evolved due to lack of adequate information on the subject, the crudity of the medicinal plants and the technique involved in its application traditionally (Tijani *et al*., 2013). Over 80% of the global population use plants as their primary source of medications (Yau *et al*., 1995), some of which have their constituents and therapeutic properties established scientifically while many others are yet to be subjected to such thorough investigations. In northern Nigeria variety of plant species are used in diseases management with little or no consideration to toxicological profile of the plant (Ogbonna *et al*., 2010). One of such plant is *Annona muricata* (Kim *et al*., 1998; Dada and Faleye, 2015).

Annona muricata being a member of family of *Annonaceae* commonly refers to as Custard apple trees are known mostly to produce edible fruits called Sour-sop due to it characteristic slight acidic taste when ripe. It is indigenous to warmest tropical areas in South and North America But grew natively in Caribbean and Central America (Ozolua *et al*., 2012), but are most recently cultivated widely in tropical climates globally. The fruit pulp is excellent for making drinks and sherbets and fruit juice. Tea made from it is taken for the management of fever, diarrhea, dysentery, worms and parasitic diseases such as lice, (Ozolua *et al*., 2012), to increase mother's milk after childbirth, and as an astringent (drying agent)(Owolabi *et al*., 2013). The barks, leaves, and roots are employed as antispasmodic, hypotensive, and sedative (Nwokocha *et al*., 2012; Dada and Faleye, 2015).

The traditional practice of using this plant in management of different ailment could be an alternative to compensate for some perceived deficiencies in orthodox pharmacotherapy (Sofowora, 1993). However, limited scientific evidence on the safety profile and it efficacy is required to back up the continued traditional therapeutic application of the plant extract. The aim of this research was therefore to screen for the phytochemicals, the acute toxicity based on Lorke's method, with LD_{50} as the index and the sub-chronic toxicity profile of methanol leaf extract of *Annona muricata* on albino rats.

MATERIALS AND METHODS

Sample and Sampling:

Annona muricata leaf samples were collected from Ungwan Mu'azu, Kaduna in April 2015 and was authenticated and identified by a botanist from Applied Science Department, Kaduna Polytechnic, and Kaduna State, Nigeria.

Sample Preparation

The leaf was shade dried at room temperature (25^{o}C) for two weeks and pulverized into a coarse powder using sterile mortar and pestle. The coarse powder was weighed, labeled and kept in an air tight container for the further analysis.

Extraction Procedure

The extraction of bioactive components of *Annona muricata* leaf was carried out by cold maceration method. About 500g of the powdered leaf sample was poured into a clean container after which 250ml of methanol was added. The mixture was labeled and kept for 48 hours with frequent intermittent agitation after which the mixture was filtered using filter paper (Whatman No. 1). The filtrate was then concentrated on steam bath to evaporate to obtain the crude extract (Iroha *et al*., 2010).

Phytochemical Screening

Phytochemical screening for the qualitative detection of alkaloids, tannins, saponin glycosides, flavonoids, unsaturated steroids and Triterpenes, carbohydrate, cardiac glycosides, and glycosides was carried out on the *Annona muricata* leaf extract using the conventional procedure as described by (Sofowora, 1993; Evans 2005).

Toxicological Studies

Experimental Animals

Animals: Adult rats of weight 100 to 256 were obtained from animal house in the Department of Pharmaceutical Science, Ahmadu Bello University, and Zaria. They were kept in a cage and fed with growers mash (Vital feed), and water ad-Libitum for two weeks to acclimatize before starting the experiment. They were also maintained under standard condition of humidity, temperature and 12 hours light/darkness cycle.

Animals Grouping

Group 1= Normal control (103g – 219g)

Group 2 = Treated with 1000mg/kg body weight per day (241g – 256g)

Group 3 = Treated with 100mg/kg body weight per day (157g – 185g)

Group 4 =Treated with 10mg/kg body weigh per day (155g – 179g)

Acute Toxicity Studies (Determination of LD50)

Acute Toxicity study was carried to determine the LD_{50}. The procedure involves two phases. The rats were weighted and grouped by randomizing method in to three groups of three rats each. The LD_{50} was carried out by adopting the method outlined by (Lorke, 1983) with a little adjustment. In the initial phase, nine (9) rats were grouped into three groups of three rats each and they were treated with extract at doses of 10, 100, and 1000mg/kg body weight orally. They were observed for 24 hours. In the second phase which is deduced from the first phase, four rats were grouped into four groups of one rat each and they were treated with doses of 1000, 1600, 2900 and 5000mg/kg body weight orally. They were also observed for 24 hours as in the first phase, and finally LD_{50} value was determined Lorke (1983).

Sub-chronic Toxicity Studies

Twenty rats were weighed, and randomly grouped into four groups of five rats each and treated with the extracts at the dose of 250, 500 and 1000mg/kg body weight for groups labeled B, C and D, while group A were treated with distilled water and served as the control group. The extract was administered for the period of 28 days during which food consumption and water intake of the groups were observed. Body weights were recorded on weekly basis. The animals were inspected for physical manifestation of toxicity and mortality. On the 29th day, the animals were starved overnight and weighed before sacrifice. The animals were anaesthetized with chloroform and sacrificed by decapitation. The blood samples were collected in sample bottles for biochemical assay. After the sacrifice, the kidney and liver were removed from the dissected animals and stored in 10% formalin for histopathology study.

Biochemical Assay

Sample bottles (plain bottles) were used to collect the blood for liver function tests, test for the liver enzymes which include AST, ALT, ALP; albumin and bilirubin. The Kidney function parameters, Urea cretinine and electrolytes tests were carried out using standard procedures adopted by (Sharif *et al*., 2015a).

Relative Organ Weight and Histopathology

The liver and kidney of each animal was excised, carefully examined for gross pathological changes and weighed. The Relative organ weight (ROW) was calculated by expressing absolute organ weight as a percentage of the total body weight. The liver and kidney were further taken for histopathology.

$$\text{Relative Organ Weight} = \frac{\text{Absolute organ weight (g)}}{\text{Body weight of rat on sacrifice day (g)}} \times 100$$

Histo-pathological Studies

Histo-pathological examination of the livers and kidneys was initiated by the fixation of which stabilizes the organs to prevent it decay. The organs were dehydrated, cleaned and impregnated with wax and finally embedded into paraffin wax which results into formation of block.

This block was trimmed and then mounted onto a rotary microtome for sectioning and fixed onto a glass side. De-waxing of sections was done by placing the shades on a water bath of temperature 40^{O}C. Staining with iron haemotoxylin and eosin was carried out for proper viewing of structures. The section were then mounted in Canada balsam and then covered with cover slip before viewing under X10, then X40 objectives of the microscope.

Statistical Analysis

The results were analyzed using SPSS 20 and Prism 5 software for the analysis of the in-vivo anti-plasmodia screening. The data there obtained are expressed as means and standard deviation of the mean (SD). Some of the data were analyzed as a completely randomized design using one-way analysis of variance (ANOVA) and Any significant differences between means are assessed by Duncan post hoc test at 95% level of significance ($P < 0.05$) would be used for the statistical analysis.

RESULTS

Physical Characteristics and Percentage Yield of *Annona Muricata* Leaf Extracts

The result of the physical characteristics and percentage yield of the leaf extract of *Annona muricata* as indicated in Table 1 below shows that the methanol extract was dark brown in colour and slightly gummy in texture. The percentage yield obtained was 7.26% after the extraction with methanol.

Phyto-chemical screening of *Annona muricata*

Phytochemical screenings for the bioactive metabolites present in the methanol extract of the leave of *Annona muricata* were determined as presented in Table 2. The result indicated the presence of Alkaloids, Cardiac, Glycosides, Carbohydrate, Glycosides, Flavonoids, Tannins, unsaturated steroids and Tri-terpenes in the leaf extracts while saponin and glycosides were absent.

Acute Toxicity profile of methanol leaf extracts of *Annona muricata*

The acute toxicity profile of the plants crude extract as measured using LD_{50} as the index indicates, the calculated LD_{50} of the methanol extracts of methanol leaf extracts of Annona muricata orally in rats is greater than 5000mg/kg body weight and cannot be calculated according to Lorkes (1983) (Table 3).

Effect of *Annona muricata* (L) Methanol extract on Some Serum Biochemical Parameters (Liver Function test)

The analysis of the results as represented in Table 4 indicated an elevation in mean values of alanine transaminase in all the groups including the control group as compared with the normal reference values. Other Parameters such as ALP, AST, T/B, C/B mean values do not differ significantly ($p>0.05$) with the values of the control and are all within normal reference values. Therefore the results are generally statistically insignificant ($P>0.05$). This signifies that the treatments over a long period time with the plant extract did not expressed any noticeable influence on the liver function parameters tested.

Effect of *Annona muricata* (L) Methanol extract on Some Serum Biochemical Parameters

The result as presented in table 5 showed that the mean value of urea for the groups treated with 250 and 1000mg/kg body weight of the extract are higher than the normal reference values, but the means values do not differ significantly ($p>0.05$) with the values of the control. The mean values of creatinine for the groups treated with higher doses (500 and 1000mg/kg) varies significantly with the control group ($p<0.05$) even though the values are far below the normal reference values including the value of the control group. The Sodium values of the treated groups differs significantly ($p<0.05$) from those of the control and chloride values did not, however both values are within the normal reference values. Potassium and bicarbonate both differ significantly ($p<0.05$), while former has it mean values higher than the reference values the later has its values lower Table 5.

Relative Organ Weight of the Rat Treated with *Annona muricata* (L) Methanol Extract

The result of the relative organ weight (ROW) indicated statistically that the mean values the liver varies significantly ($p<0.05$), while that of the kidney was insignificant ($p>0.05$) as compared with the control group (Table 6). In other words, various dose of the plant extract have influenced the relative organ weight of liver and not the kidney even though the ROW are within the normal reference value, hence the influence might not be of pathological significance.

Histopathology of Liver of rats Treated with *Annona muricata* (L) Methanol Extracts

The result of the liver histopathology showed that the control group of the extract showed normal histology of liver (Plate1A). Rat treated with 250, 500 and 1000mg/kg/day showed mild ballooning degenerations(Plate1B) Rats treated with 1000 mg/kg/day portal vascular congestion(Plate1IB).

Histopathology of Kidney of of rats Treated with *Annona muricata* (L) Methanol Extracts.

The result of the histopathology of kidney of control group and rats treated with doses 250mg/kg/day showed a similar pattern with majority of group members showing normal histology (Plate1IIA). Remarkably majority of the members of the groups treated with 500 and 1000mg/kg/day showed normal tubules with mild lymphocytic infiltrate (Plate1IIB).

Table 1: the % yield for the methanol extraction of *Annona muricata*

Plant	% Yield	Colour
Annona muricata	7.26%	dark brown

Table 2: The Phyto-chemical screening tests of *annona muricata*

Phytochemicals	*Annona muricata*
Alkaloids	+
Cardiac Glycosides	+
Carbohydrate	+
Glycosides	-
Flavonoids	+
Saponin	-
Tannins	+
Steroids	+
Triterpenes	+

Key: + = Presence of phytochemicals, = Absence of phytochemicals

Table 3: Acute toxicity tests of the methanol leaf extracts of ***Annona muricata***

Doses (mg/kg)	**Survival rate (phase 1)**
10	0/3
100	0/3
1000	0/3
Doses (mg/kg)	Survival rate (phase 11)
1000	0/1
1600	0/1
2900	0/1
5000	0/1
(LD_{50} for the extracts is higher than 5000mg/kgbw).	

Table 4 Effect of *Annona muricata* (L) Methanol extract on Some Serum Biochemical Parameters (Liver Function test) Assessment of Wistar Albino Rats

Parameter's	**Control**	**1000mg**	**500mg**	**250mg**
Aspatate transaminase(u/l) (Up to 218)	86.00 ± 6.93^{a}	116.67 ± 30.02^{a}	103.33 ± 27.23^{a}	95.00 ± 23.52^{a}
Alanine Transaminase(u/l) (up to 22UI)	26.67±14.05^{a}	32.00 ± 11.00^{a}	22.00 ± 8.89^{a}	24.33 ± 12.66^{a}
Alkaline Phosphotase(u/l) (60 - 170)	54.00 ± 7.00^{a}	45.00 ± 14.42^{a}	53.00 ± 7.21^{a}	48.33 ± 2.31^{a}
Conjugated Bilirubin(µmol /l) (1.7- 8.5)	2.66 ± 4.62^{a}	2.66 ± 4.62^{a}	2.66 ± 4.62^{a}	2.00 ± 2.00^{a}
Total Bilirubin(µmol/l) (1.7-17.1)	10.66 ± 9.33^{a}	10.66 ± 16.51^{a}	16.33 ± 16.51^{a}	2.00 ± 2.00^{a}

Values are expressed as mean ± SD for N = 5, Values in the same row carrying different super scripts (i.e. a, or b or ab) differ significantly from each other, therefore ($p<0.05$).

Table 5 Effect of *Annona Muricata* (L) Methanol Extract On Some Serum Biochemical Parameters for Kidney Function Assessment of Rats After Treatment

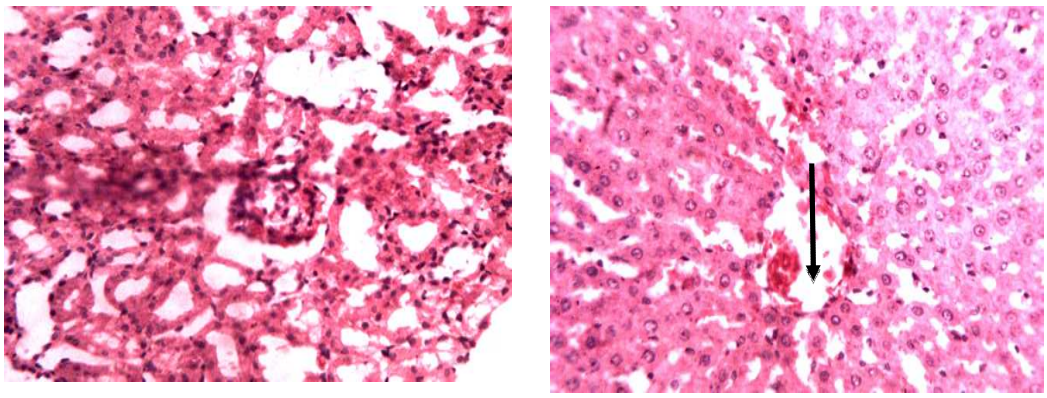
Parameters	**Control**	**1000mg/kg**	**500mg/kg**	**250mg/kg**
Urea (mmol/L) (2.5-6.5)	6.1 ± 0.95^{a}	7.600 ± 2.9^{a}	5.93 ± 0.81^{a}	8.33 ± 1.61^{a}
Creatinine (mmol/L) (90.0-126)	36.90 ± 1.90^{a}	24.00 ± 1.00^{b}	38.00 ± 2.00^{c}	28.66 ± 8.1^{a}
Sodium (Na) (mmol/L) 135-150	137.66 ± 0.577^{ab}	136.00 ± 1.00^{a}	138.66 ± 1.53^{bc}	140.33 ± 0.577^{c}
Potassium (K^{+}) (3.4-5.3mmol/L)	6.233 ± 0.51^{a}	8.366 ± 0.057^{b}	7.066 ± 1.52^{ab}	5.966 ± 1.17^{a}
Chloride (Cl^{-}) (mmol/L) 95-110	108.33 ± 1.55^{a}	107.00 ± 1.732^{a}	106.33 ± 1.53^{a}	108.33 ± 2.52^{a}
Bicarbonate(HCO_3^{-}) (24-32mmol/L)	22.00 ± 0.00^{ab}	21.33 ± 0.577^{a}	22.66 ± 1.2^{ab}	23.00 ± 1.00^{b}

Values are expressed as mean ± SD for N = 5, Values in the same row carrying different super scripts (i.e. a, or b or ab) differ significantly from each other, therefore ($p<0.05$

Table 6: Effects of the *A. muricata*, methanol extract on the relative organ weight of liver and Kidney of rat after treatment

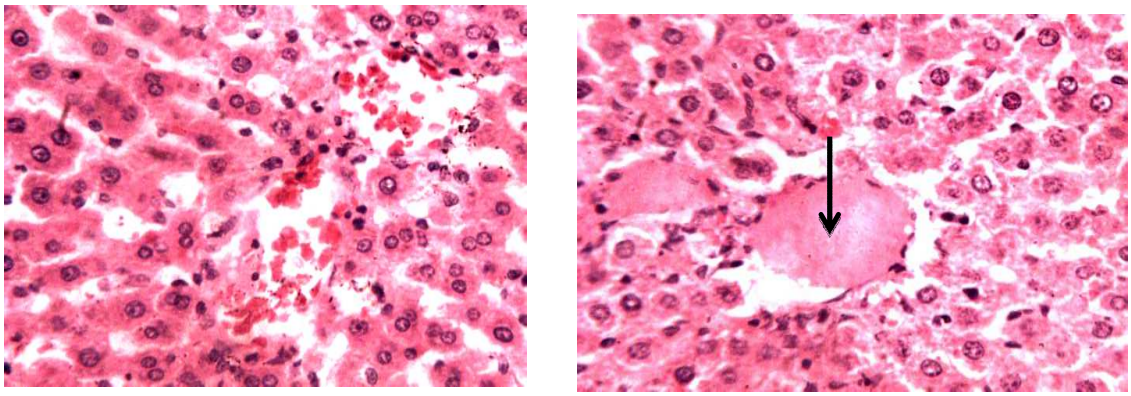
Parameters	**Concentration (mg/kg body weight)**			
	control	250	500	1000
Liver	0.9567±1.36^{a}	3.1100±0.64^{ab}	3.2233±0.37^{b}	1.8433±1.57^{ab}
Kidney	0.5833±0.059^{a}	0.5400±0.085^{a}	0.5800±0.061^{a}	0.5700±0.026^{a}

Values are expressed as mean ±SD. Data in the same row carrying different super scripts (i.e. a, or b or ab) differ significantly from each other therefore ($P<0.05$)



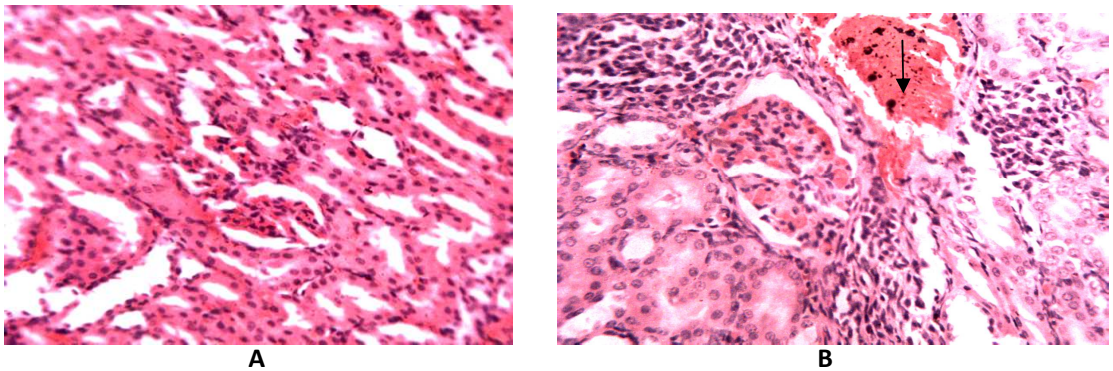
A B

Plate IA: Micrograph of the liver section from the control rat showing normal portal triads and centrilobular B: Micrograph of the liver section from rats treated with 250, 500 and 1000mg/kg arrow showing mild ballooning degeneration (Mag x40).



A B

Plate IIA: Micrograph of the liver section from the control rat showing normal portal triads and centrilobular **B:** Micrograph of the liver section from rats treated with 1000mg/kg arrow showing Portal vein Congestion (PVC) (Mag x40).



A B

Plate IIIA: Micrograph of the kidneysection from the control rat showing normal tubules and glomerulus **B:**Micrograph of kidney section from rats treated with 500 and 1000mg/kg showing mild lymphotic infiltrate

DISCUSSION

Medicinal plant are considered as natural heal and are extensively employed in the treatment of wide ranges of disease on the pretext that they are readily available, more effective, less toxic and much affordable in terms of synthetic orthodox drugs . The versatility of plants like *Annona muricata* coupled with the fact that documented and undocumented adverse effects associated to the used of the plants are grossly inadequate (Arthur, Woode, Terlabi, & Larbie, 2011), it become pertinent that the toxicological effect of such plant be ruled out. The 7.26% percentage yield obtained by the use of methanol in this present research suggested the solvent even though is organic with high polarity index *was* not better solvent of extraction compared to the aqueous, as thus obtained by Arthur *et al*., (2011) which was 14.96% w/w twice the value from methanol extract. This could further justify the traditional use of aqueous as solvent of extraction.

The outcome of the phytochemical screening of this current research revealed the presence of Alkaloids, Cardiac

Glycosides, Carbohydrate, Glycosides, Flavonoids, Tannins, unsaturated steroids and Tri-terpenes, which was in variation with that obtained by Arthur *et al*., (2011) as Alkaloids and sterols present in this research outcome were conspicuously absent, While saponin and glycoside absent in the work are present in reasonable amount. This could imply that methanol can better be used to extract alkaloids and steroids while phytochemicals such as saponin, glycoside and tannins could best extracted using aqueous.

The acute toxicity (LD_{50}) test the extract in this work indicated that the extract was relatively safe upon administration within a short period of time with no mortality or any possible signs of the toxicity recorded at dose of 5000mg/kg body weight of the experimental model. This is in conformity with the finding of Arthur *et al.*, (2011), which indicated the plant extract to be practically nontoxic even though the solvent of extraction was aqueous. (Theophine *et al*., (2012) also observed that the root bark extract and fractions of *Annona muricata* does not show acute toxicity. It can be inferred that within the standard range of 500 – 5000mg/kg body weight the methanol extract of the plant can be described as nontoxic on the scale proposed by (Lorke, 1983).

In the sub-chronic toxicity study the current work reccoded an elevation in mean values of alanine transaminase(ALAT) (Cheesbrough, 2006) in all the groups including the control group as compared with the normal reference values (Table 4), however, the results were generally statistically insignificant (P>0.05) which signifies absence of noticeable influence on the liver function parameters tested by the extract even over a long period of treatments with the plant extract. This was corroborated with the result of liver ROW which was found to be within the normal reference value, even though statistically, the mean values varies significantly ($p<0.05$), various dose of the plant extract have insignificance pathological influenced on the relative organ weight of liver. On the same direction the results of the liver histopathology showed that the rat treated with higher doses over a long period especially with 1000 mg/kg/day of the extract showed mild ballooning degenerations(Plate1B) and portal vascular congestion(Plate1IB) which is quite amazing as that could possibly translate to deleterious effect on the liver (Sharif *et al*., 2015b). However, such danger can be averted if the extract is used over a short period of time, as the effect is observed to be time and concentration dependent. The research find is in line with the finding of (Arthur *et al*., 2011; Holanda *et al*., 2014) in which both expresses insignificant effect on the liver function parameters and the later even reported histo-protective potential of the plant extract. However the variations noted with the current work are, both authors worked on aqueous extract. It is therefore recommended that further work be designed to look into the hepato-protective potential of the methanol extract of *A. moricata.*

The results of kidney function parameters indicated that urea, creatinine and the electrolytes do not express any significant difference translatable to deleterious effect on the kidney, although the mean values of creatinine for the groups treated with higher doses varies significantly with the control group ($p<0.05$) the values are far below the normal reference values suggesting probable neuphroprotective potential which is worth investigable. Furthemore ROW and Histopathological finding do not significantly corroborate deleterous effect on kidney, although mild lymphocytic infiltrate is noticed (Plate1IIB) an indication of mild effect, which can be effectively averted by avoiding extended use of higher concentration of the extract as the effect is observed to be both concentration and time dependent.

Conclusion

The LD_{50} obtained was a clear indication of the safety profile of *A. muricata.* This validated the traditional claim of it safe use within short period of time for both internal and external use. However the study showed that *A. muricata at* higher doses over a longer period suggested a speculative danger to the liver; however the research findings suggested the probable neuphro-protective potential of the methanol extract of the plant and should further be investigated.

Contribution of Authors:

- Hauwa B. Sharif – Research initiator, bench work and write up supervisor.
- Gabi Baba – Bench work supervisor and results interpretation.
- Sadiya M. Abdullahi – Bench work and the write up.

Conflict of Interest

- There is no conflict of interest in this research work.

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