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SUB-ACUTE TOXICITY STUDIES OF ETHANOL AND N-HEXANE CRUDE SEED EXTRACTS OF *ANNONA MURICATA* IN ALBINO RATS

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ABSTRACT

The present study was aimed at evaluating the acute toxicity of ethanol and n-hexane seed extracts of *Annona muricata* in rats. The animals were treated with the extracts at the doses of 10mg/kg, 50mg/kg, 100mg/kg, 1000mg/kg, 1500mg/kg, 3000mg/kg and 5000mg/kg. The degree of toxicity was assessed using changes in biochemical parameters such as lipid profile, thiobarbituric acid reactive substances (TBARS), alanine transaminase (ALT), aspartate transaminase (AST), urea and creatinine. LD_{50} was determined for the extracts and there was no record of death in the treated animals. However, the treated rats had a significant decrease ($P<0.05$) in triglycerides and LDL-cholesterol when compared to the control group. There was also a decrease in malondialdehyde concentration in the treated animals compared to the control group. There were no significant ($P<0.05$) changes in alanine transaminase, aspartate transaminase, urea and creatinine concentrations in the treated animals compared to the control. A significant decrease ($P<0.05$) was observed in the final body weights of the treated animals. The results of the present study suggest that *Annona muricata* seed extracts (ethanol and n-hexane) could be considered safe within the doses of ≤ 5000mg/kg and exhibit hypolipidemic effect. This study also reveals that the extracts could be a potent agent for the management of coronary heart diseases and obesity.

Keywords: Acute toxicity, Hypolipidemic, *Annona muricata,* Low density lipoprotein.

INTRODUCTION

The use of complementary traditional medicines in the treatment of various diseases has expanded rapidly in both developed and developing countries, attributable to affordability, accessibility and efficacy. Medicinal plants have been used for centuries as remedies for human diseases because they contain components of therapeutic value (Arthur *et al*., 2011). Documented and undocumented adverse drug reactions associated with herbal medicines makes it pertinent that pre-clinical toxicological studies be carried out on these natural products.

Annona muricata (soursop) is a fruit native of southern part of Nigeria, North and South America, belonging to the genus *Annona* and of the family *Annonaceae*. A number of medicinal properties are attributed to the leaves and juice of the soursop plant. Toxic and insecticidal characteristics are reported for different parts of the tree and fruit (Rice *et al.*, 1991). The crushed seeds are used against internal and external parasites such as head lice, and worms (Data base for graviola).

Intensive chemical investigations of the leaves and seeds of this *A. muricata* have resulted in the isolation of a great number of acetogenins. The isolated compounds displayed some of the interesting biological and pharmacological activities, such as antitumoral, cytotoxicity, antiparasitic and pesticidal properties. Roots of

this species are used in traditional medicine due to their antiparasitic and pesticidal properties (Christophe *et al.,* 2011).

Phytochemical test revealed that *Annona muricata* seed extracts contains secondary metabolites such as saponins, alkaloids and triterpenoids. These compounds are natural defense chemical compounds of plants produced in the plant tissues. They are toxic and can also act as poison to animals' body system (Gajalakshmi *et al*., 2012). Therefore acute and Sub-chronic toxicity evaluation is required to establish the potential adverse effects of this valuable underutilized fruit plant as suggested by Arthur *et al*., 2011.

In the present study, the sub- acute toxicity of ethanol and n-hexane seed extracts of *Annona muricata* was investigated in albino rats.

MATERIALS AND METHODS

Preparation of Plant extract

The mature fruits of *Anonna muricata* were collected from Abocho in Dekina local government area and Lokoja vicinity of Kogi state, Nigeria. They were identified by Mr. Omolokun a botanist in the department of biosciences Salem University Lokoja. The seeds were removed from the fruits and air dried at room temperature. The hard outer covers of the seeds were then broken manually to expose the inner fleshy core of the seeds, which were also air dried. About 1.5kg of the dried seeds was first coarse blended with a sterilized iron blender and further blended with an electrical blender to obtain a more fine powder. Extraction was achieved usng absolute ethanol and n-hexane respectively in a soxhlet extractor. The extracts were concentrated using a rotary evaporator, followed by oven drying at 40^0c. The oven dried extracts were prepared into the different concentrations of 10mg/kg, 50mg/kg, 100mg/kg, 1000mg/kg, 1600mg/kg, 2900mg/kg and 5000mg/kg using olive oil as a vehicle.

Animals and treatments

Fourty-four male albino rats (Wister strain) of about eight (8weeks) purchased from the biochemistry animal house of Salem University Lokoja, Nigeria were used. They were separated and acclimatized for one week prior to commencement of experiment. They were housed in plastic cages, and kept at room temperature of 25^0c. The room was maintained at 12 hours light and darkness condition and the animals were allowed access to food (growers' mash) and water ad libitum.

Experimental Design

The animals were divided into 11 groups of four animals each. After an overnight fast, the groups were treated with different doses both of the ethanol and n-hexane extracts, reconstituted with olive oil. They were then observed for acute toxicity. The different groups are as shown below: control (olive oil, 0.3ml/kg); Group1 (10mg/kg n-hexane extract);Group2: (50mg/kg n-hexane extract); Group 3 (100mg/kg n-hexane extract); Group 4 (1000mg/kg n-hexane extract); Group 5 (1500mg/kg n-hexane extract); Group 6 (3000mg/kg n-hexane extract); Group 7 (5000mg/kg n-hexane extract); Group 8 (1500mg/kg ethanol extract); Group 9 (3000mg/kg ethanol extract); Group 10 (5000mg/kg ethanol extract).

Administration of Extract

The extracts were orally administered using a canula (intubator). The animals were first treated with lower doses and observed for acute toxicity. Subsequently, they were treated with higher doses (1500mg/kg – 5000mg/kg). These groups were observed for two weeks. At the end of this period, the animals were fasted overnight prior to sacrifice. They were weighed and anesthetized using chloroform soaked in cotton wool and sacrificed by humane decapitation.

Preparation of Serum Sample

Blood were then collected into a non-heparinized tubes and centrifuged at 3000RPM (using a Microfield centrifuge, Model 90-2) for 10 minutes. The sera were then decanted into another sample tubes and stored in the freezer for subsequent analysis.

Preparation of Tissue Homogenates

The vital organs (liver, kidneys, heart, lungs and spleen) from each animal were rapidly excised during the sacrifice, washed with cold normal saline to remove excess blood, weighed and stored immediately at - 4^oc. subsequently, the liver, heart and kidneys were homogenized (using a Bocsh PSB 570-2 homogenizer) in ice-cold phosphate buffer (pH 7.4). The homogenates were centrifuged at 3000 RPM for 15 minutes and the supernatant decanted and kept in the fridge for subsequent analysis.

Acute Toxicity Study

The lethal dose (LD50) of the plant extract was determined by method of Lorke (1983). The animals were handled in accordance with international principles guiding the use and handling of experimental animals (United State National Institute for Health, 1985). In the first phase rats were treated with the extract at doses of 10, 50, 100 and 1000mg/kg body weight orally. They were observed for 24 hours for signs of toxicity. In the second phase, the animals were treated with the extracts at doses of 1500, 3000 and 5000mg/kg body weight orally. The median lethal dose (LD50) was calculated using the second phase.

Assessment of Lipid Peroxidation

Thiobarbituric acid reacting substances (TBARS) in tissues was estimated by the method of Torres *et al*., (2004).

Two milliliter of thiobarbituric acid (TBA) and trichloro acetate (TCA) was added to 50µl of the tissue homogenates respectively. The mixture was incubated for 30 minutes at 80^0c. The tubes were allowed to cool immediately under ice and centrifuged at 3000RPM for 15 minutes. The supernatant was measured using spectrophotometer at a wavelength of 535nm.

Determination of Liver Marker Enzymes in Serum

Serum was used for the determination of aspartate aminotransaminase (AST) (Reitman and Frankel, 1957), alanine aminotransaminase (ALT) (Reitman and Frankel, 1957), Agappe assay kit (U.S.A) according to the manufacturer's instructions.

Determination of Kidney Function

Serum was used for the determination of creatinine (Tanganelli *et al*., 1982) and urea using Agappe assay kit (U.S.A) according to the manufacturer's instructions.

Lipid Profile

Serum was used for the assessment of lipid profile (Allein *et al*., 1974) using Agappe assay kit (U.S.A) according to the manufacturer's instructions.

Statistical Analysis

The results are expressed as mean ± SD (n=4). The data was evaluated by one-way analysis of varince (ANOVA) using SPSS version 20. P values < 0.05 were considered statistically significant.

RESULTS

Acute Toxicity Studies

In the animals that were administered the extracts, no visible adverse reaction was observed up to 72 hour post-administration.

Body Weight, Organ Weights, and Relative Organ Weights

Tables 1 and 2 show the effects of *Annona muricata* n-hexane and ethanol seed extracts on body weight, organ weights and relative organ weights of the treated animals. There was a significant decrease in the final body weights of the animals treated with ethanol and n-hexane extracts.

Lipid Profile

Fig. 1 dipicts the effect of n-hexane seed extract on the lipid profile of the treated animals. There was a significant decrease in triglycerides concentration in rats treated with the n-hexane extract compared to the control group. There was also a significant decrease in LDL-Cholesterol concentration in rats treated with the extracts compared to the control group. No significant difference in HDL-Cholesterol concentration was observed in rats treated with the extract compared to the control group, except in the rats treated with 5000mg/kg of the extract.

Fig. 2 shows the effect of ethanol extract on the lipid profile of the treated rats.

A significant decrease in triglycerides concentration was observed in rats treated with the extract compared to the control group. There was also a significant decrease in LDL-cholesterol and cholesterol concentration in rats treated with the extracts compared to the control group. HDL was significantly high especially in rats treated with 5000mg/kg of the extract.

Lipid Peroxidation

Fig. 3 shows the effect of n-hexane extract on lipid peroxidation. There was a significant decrease in malondialdehyde concentration in the kidney and liver tissues of the treated rats compared with the control. No significant difference in malondialdehyde concentration was observed in the heart of rats treated with the extract compared to the control group.

Fig. 4 shows the effect of ethanol extract on lipid peroxidation.There was a significant

difference ($p < 0.05$) in malondialdehyde concentration observed in the liver, heart and kidneys of rats treated with 3000mg/kg and 1500mg/kg of the extract compared to the control group

Liver Function

Figs. 5 and 6 show the effect of n-hexane and ethanol extracts on liver function. There was no significant difference in alanine aminotransferase (ALT) and aspartate aminotransferase (AST) concentrations in rats treated with the extracts compared to the control group.

Renal Function

Figs. 7 and 8 show the effect of n-hexane and ethanol extracts on renal function. There was no significant difference in urea and creatinine concentrations in the treated rats compared to the control group; however, there was an observed increase in creatinine concentration in rats treated with 1500mg/kg and 3000mg/kg of the ethanol extract compared to the control group.

Table 1. Effect of *Annona muricata* n-hexane extract on body weights, organ weights and relative organ weight.

Dose	Initial weight (g)	Final weight (g)	Heart (g)	Liver (g)	Kidney (g)	Spleen (g)	Lungs (g)	Relative organ weight (as % body weight) Heart liver
Control	193.70±0.07	207.70±0.07	0.60±0.07	6.20±0.07	1.60±0.07	0.07±0.14	1.5±0.00	0.29±100.00 2.99±100.00
1500mg/kg	278.70±0.07	257.00±0.07	0.90±0.07	7.30±0.00	0.80±0.07	0.90±0.07	2.10±0.07	0.35±100.00 2.84±0.00
3000mg/kg	254.30±0.07	243.3±0.07	0.70±0.07	6.80±0.07	0.80±0.00	0.90±0.10	1.50±0.07	0.29±100.00 2.79±100.00
5000mg/kg	161.50±0.00	151.50±0.00	0.70±0.00	5.50±0.00	0.70±0.10	0.40±0.00	1.20±0.00	0.43±0.00 * 3.41±0.00*

*S.D at ($P<0.05$) from control.

Table 2. Effect of *Annona muricata* ethanol extract on body weights, organ weights and relative organ weight

Dose	Initial weight (g)	Final weight (g)	Heart (g)	Liver (g)	Kidney (g)	Spleen (g)	Lungs (g)	Relative organ weight (as % body weight) Heart liver
Control	193.70±0.07	207.70±0.07	0.60±0.07	6.20±0.07	1.60±0.07	0.07±0.14	1.5±0.00	0.29±100.00 2.99±100.00
1500mg/kg	253.70±0.00	221.00±0.07	0.70±0.00	6.20±0.00	0.70±0.07	0.80±0.07	1.30±0.07	0.32±0.00 2.81±0.00
3000mg/kg	258.30±0.07	188.3±0.07*	0.90±0.00	5.90±0.07	0.70±0.00	0.90±0.07	1.00±0.07	0.48±0.00 3.13±100.00
5000mg/kg	258.00±0.00	213.50±0.00*	0.70±0.00	6.10±0.00	0.70±0.07	0.90±0.00	1.50±0.30	0.33±100.00 2.86±0.00

*S.D at (P<0.05) from control.

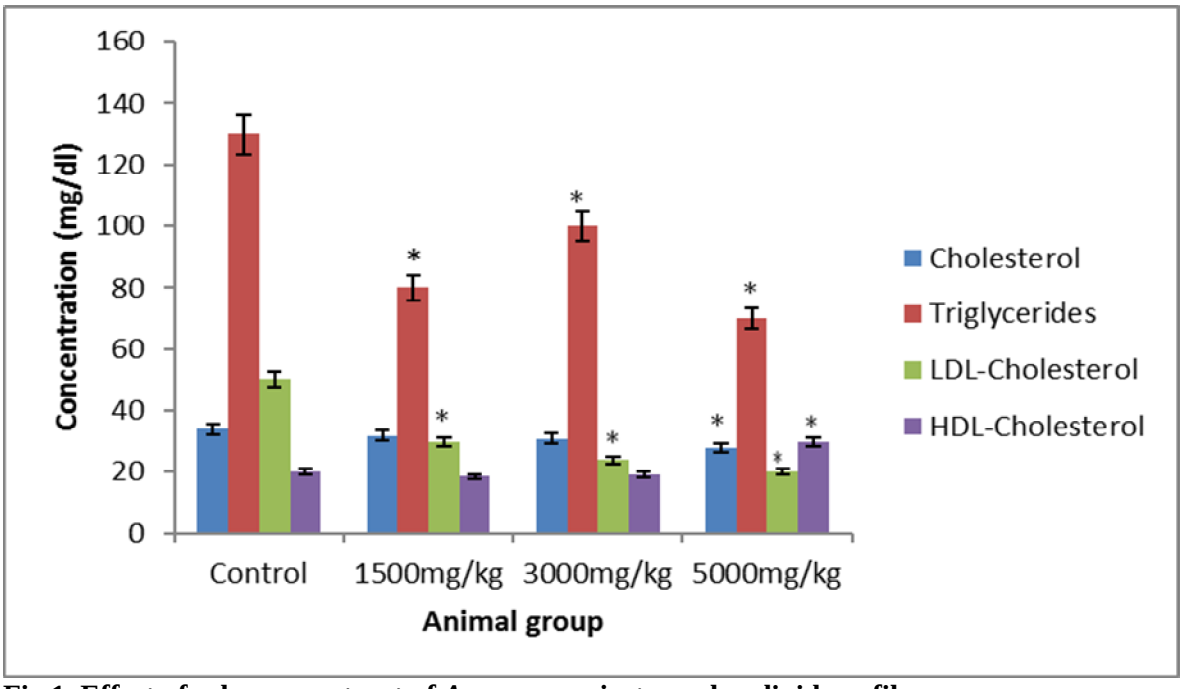

Fig.1. Effect of n-hexane extract of *Annona muricata* seed on lipid profile
*S.D at (P<0.05) from control.

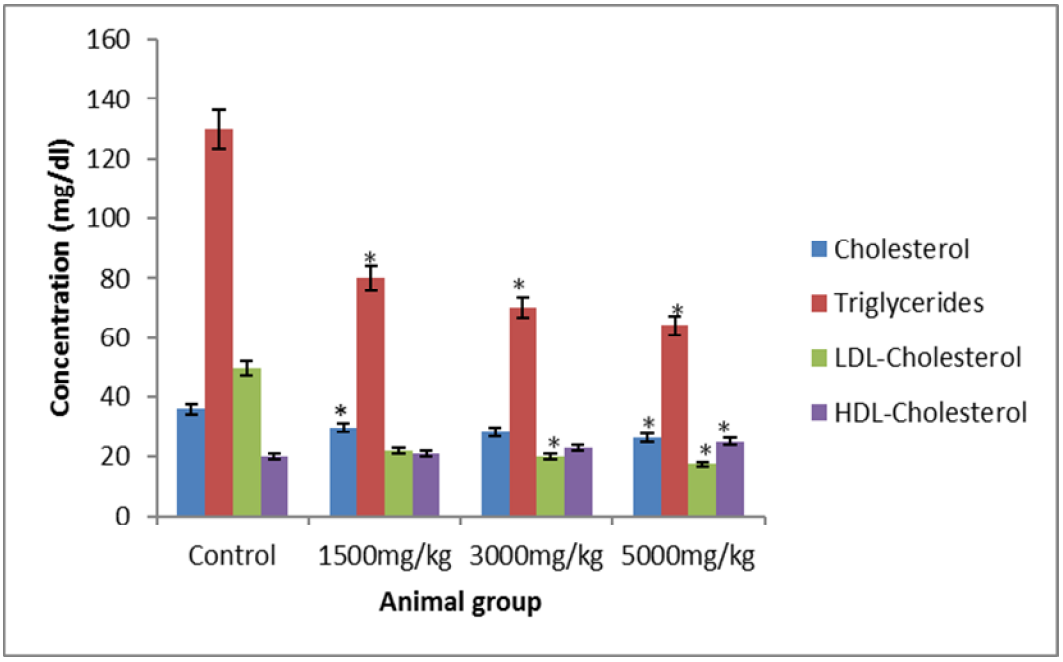

Fig 2. Effect of ethanol extract of *Annona muricata* seed on lipid profile.

*S.D at (P<0.05) from control.

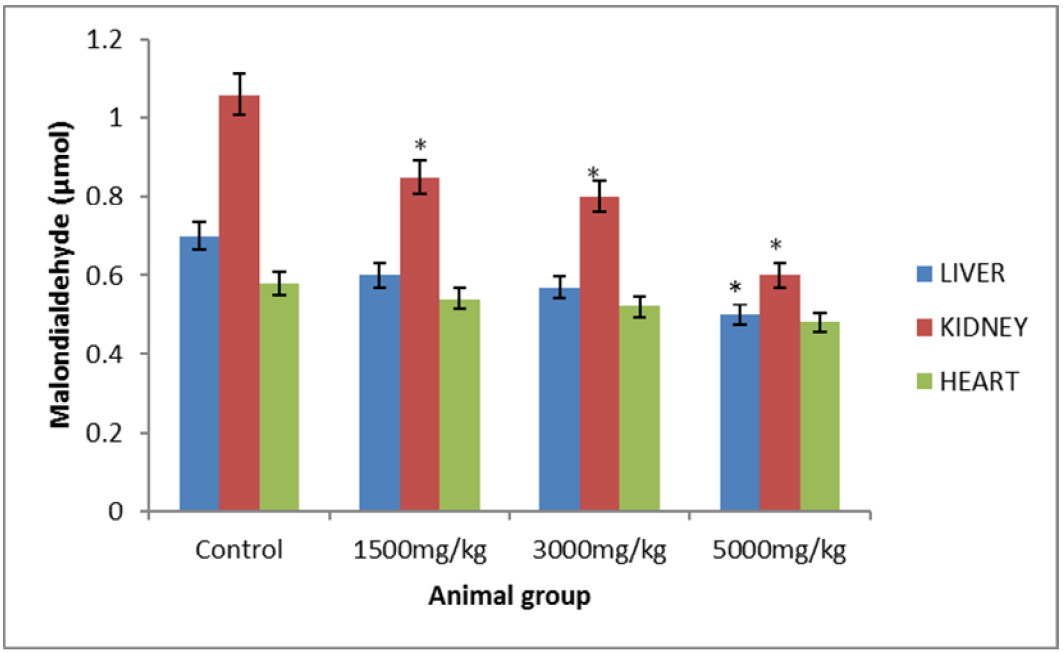

Fig.3. Effect of n-hexane extract of *Annona muricata* seed on lipid peroxidation.

*S.D at (P<0.05) from control.

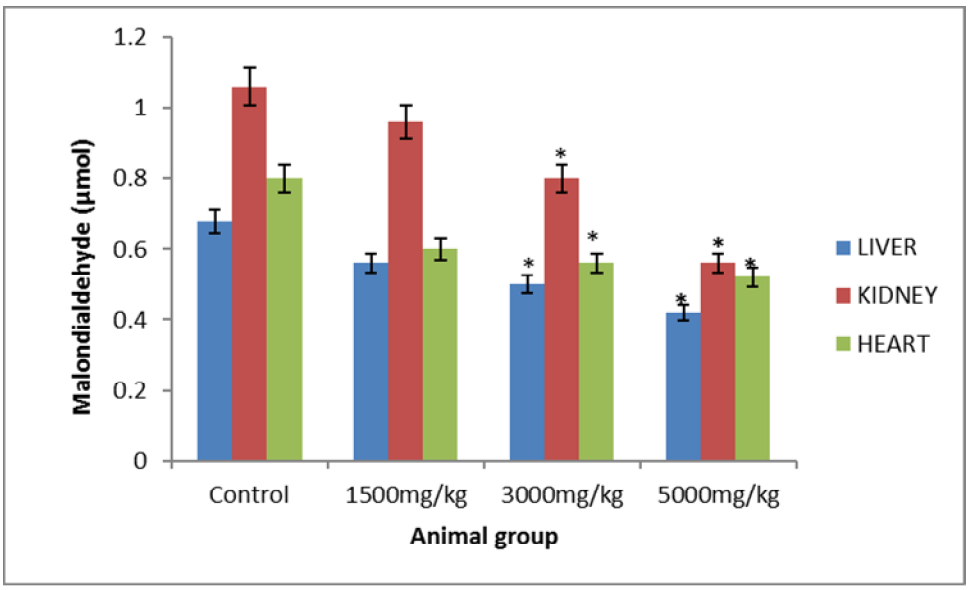

Fig.4. Effect of ethanol extract of *Annona muricata* seed on lipid peroxidation.

*S.D at (P<0.05) from control.

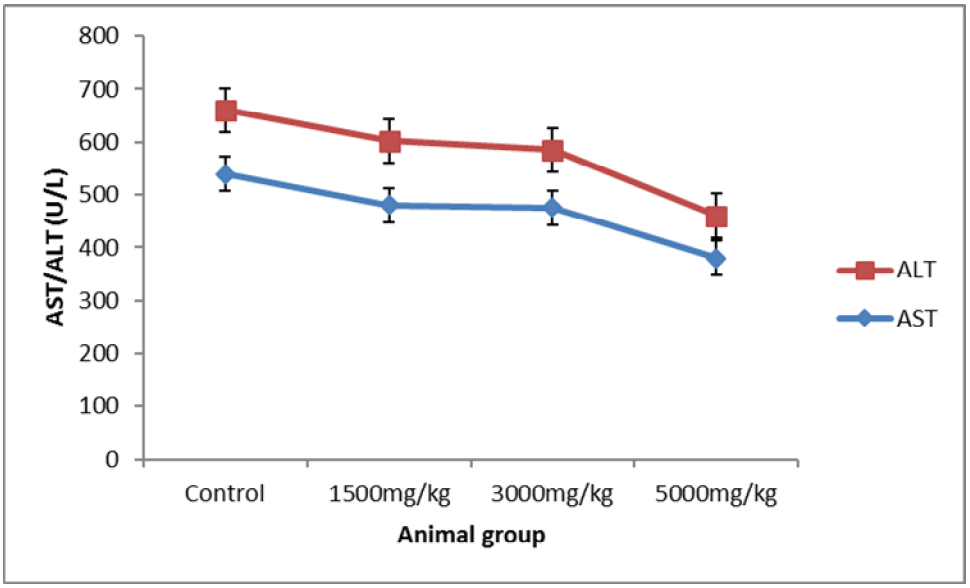

Fig.5. Effect of n-hexane extract of *Annona muricata* seed on liver function

*S.D at (P<0.05) from control.

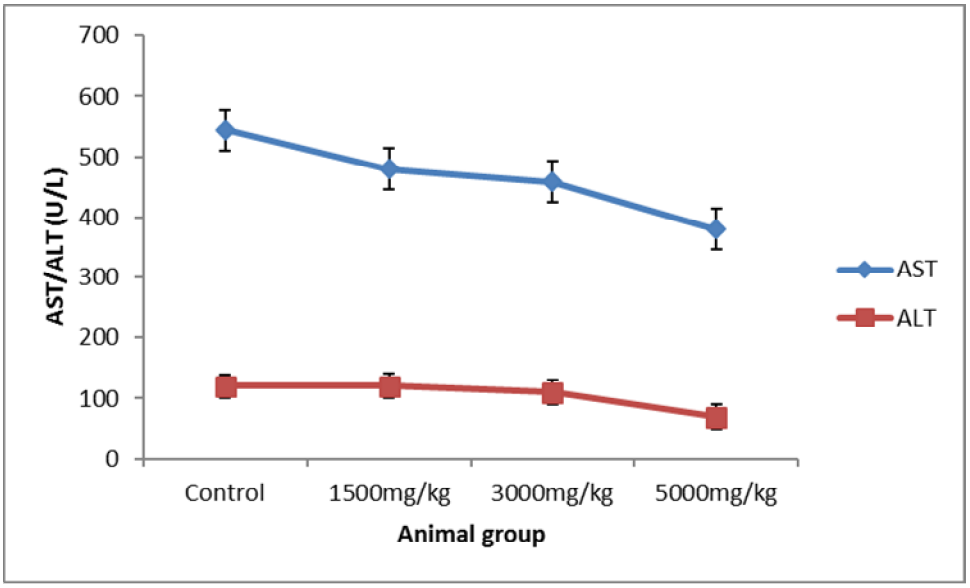

Fig.6. Effect of ethanol extract of *Annona muricata* seed on liver function.

*S.D at (P<0.05) from control.

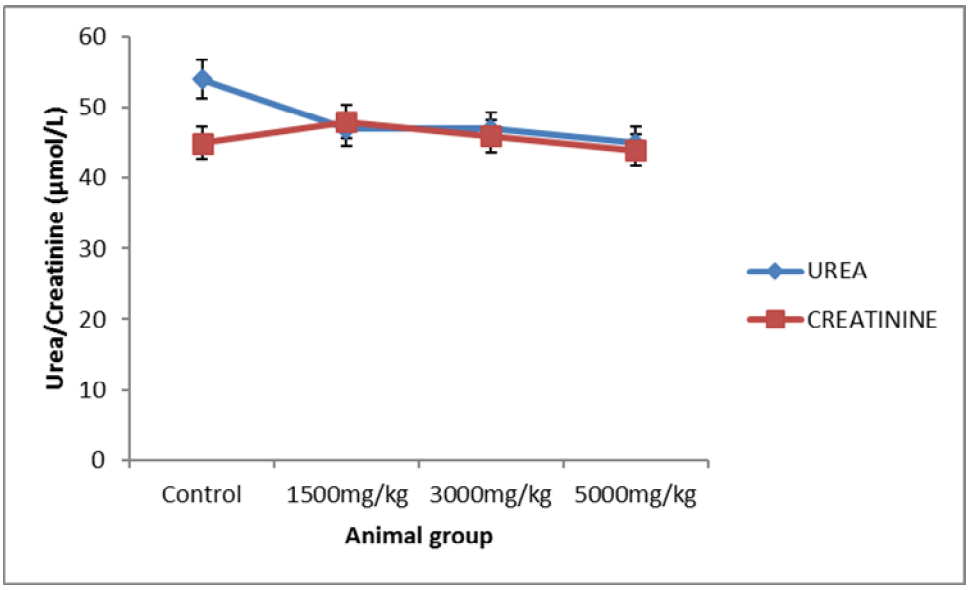

Fig.7 Effect of n-hexane extract of *Annona muricata* seed on renal function

*S.D at (P<0.05) from control.

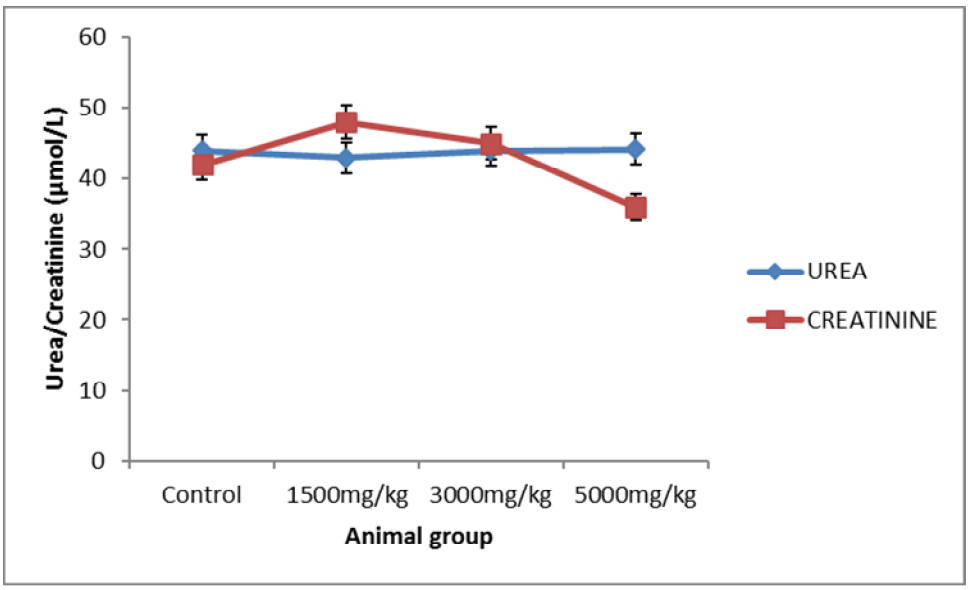

Fig.8. Effect of ethanol extract of *Annona muricata* seed on renal function

*S.D at (P<0.05) from control.

DISCUSSION

Medicinal plants such as Pacific yew (the original source of the cancer drug, taxol), opium poppy etc. have been used for centuries as remedies for human diseases because they contain components of therapeutic value (Musa *et al.*, 2011). However, plants are very complex in their composition and their therapeutic activity depends on their chemical constituents, these in turns according to age, geographical location and harvesting processes.

Intensive chemical investigations of the leaves and seeds of *Annona muricata* have resulted in the isolation of a great number of acetogenins. The isolated compounds displayed some of the interesting biological and pharmacological activities, such as antitumoral, antiparasitic and pesticidal properties (Christophe *et al.*, 2011).

In the present study, a decrease in final body weight was observed in rats treated with both n-hexane and ethanol extracts compared to the control group. The observed weight loss in the treated animals could be as a result of increased activity of lipoprotein lipase or due to increased metabolic reaction as a result of the administered extracts. This is an indication that these extracts could be useful in the management and treatment of obesity.

High serum levels of total cholesterol, LDL-cholesterol and triglycerides leads to cardiovascular diseases and atherosclerosis (Adeyemi *et al.*, 2009). Decrease in LDL-cholesterol, cholesterol and triglycerides were observed in rats treated with n-hexane and ethanol extracts. There is a substantial evidence that lowering the total cholesterol, particularly LDL-C level will lead to a reduction in the incidence of coronary heart disease (Adeyemi *et al.*, 2009). The probable mechanism of cholesterol reduction could be by the inhibition of the 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase which is the rate-limiting enzyme of cholesterol biosynthesis or reduction in the absorption of cholesterol from the intestines. The ability of these extracts to selectively reduce total cholesterol through the reduction of LDL-C components could be beneficial in preventing atherosclerotic conditions and thereby reducing the possibilities of coronary heart diseases in general.

In the human body, high levels of triglycerides in the bloodstream have been linked to atherosclerosis and, by extension, the risk of heart disease and stroke (Lampe *et al.*, 1983). The reduction in the triglycerides levels as observed in the present study could be as a result of the increased activities of both lipoprotein

lipase and the hormone sensitive lipase. This result is similar to that of Raman *et al*. (2014) on methanolic extract of edible mushroom *pleurotus djarmor var. roseus*. This is also a pointer that *Annona muricata* seed extracts could be a potent chemotherapeutic agent in the treatment of heart diseases as well as stroke.

There was also a significant increase ($p< 0.05$) in the HDL-cholesterol levels, especially at the dose of 5000mg/kg of the treated rats. However there was no significant increase in HDL-C of other treated groups compared to the control group. This suggests that the anti atherogenic effects of these extracts may not necessarily be by changes in the proportions of lipoprotein cholesterol fractions. This result conforms to the reports of (Aouadi *et al*., 2000) on fresh garlic.

Lipid peroxidation (LPO) was investigated in this study by assessing the levels of TBARS (malondialdehyde) in the liver, heart and kidneys of the experimental animals. Increased lipid peroxidative status in membranes indicates membrane and tissue damage as a result of the generation of free radicals. In addition, end-products of lipid peroxidation may be mutagenic and carcinogenic (Marnett, 1999). In the present study, there was a significant decrease ($p< 0.05$) in malondialdehyde levels in the heart, liver and kidneys of rats treated with both ethanol and n-hexane extracts. The observed trend in LPO of the treated rats indicates a decrease in oxidative stress and membrane damage of the treated rats.

Figs. 5 and 6 represent the changes in the activities of aspartate and alanine transaminases (AST and ALT). Elevated levels of these serum enzymes are indicative of cellular leakage and loss of functional integrity of the liver cell membrane (Watkins and Seef, 2006). In the present study, there was no significant difference in the activities of AST and ALT between the control, the n-hexane and ethanol extract treated groups. This suggests that both extracts have no damaging effect on the liver.

A simultaneous increase in serum creatinine and urea concentrations could be a sign of impaired renal function. But as shown in figs. 7 and 8, there was no significant difference in urea and creatinine concentration of animals treated with 3000 and 5000mg/kg of n-hexane extract compared to the control group. However there was an observed increase in creatinine levels of the 1500 and 3000mg/kg of the ethanol extract treated groups. This observation does not pose a physiologic problem, because urea level was not simultaneously increased. This suggests that the extracts have no deleterious effect on renal function.

The present study revealed that ethanol and n-hexane seed extracts of *Annona muricata*, influenced lipid metabolism in the experimental rats. The extract significantly reduced LDL-cholesterol and triglycerides in the treated rats. There was also a significant decrease in the final body weights of the treated animals. Therefore *Annona muricata* seed extracts could be a potent therapeutic agent for the management of free radical mediated chronic diseases such as coronary heart diseases and obesity.

However, further work is required to unravel the mechanism of hypolipidemic effect of these extracts.

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