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Evaluation of the acute and sub acute toxicity of *Annona senegalensis* root bark extracts

Theophine C Okoye[1*], Peter A Akah[1], Adaobi C Ezike[1], Maureen O Okoye[2], Collins A Onyeto[1], Frankline Ndukwu[1], Ejike Ohaegbulam[1], Lovelyn Ikele[1]

[1]*Department of Pharmacology and Toxicology, Faculty of Pharmaceutical Sciences, University of Nigeria, Nsukka 410001, Enugu State, Nigeria*
[2]*Department of Clinical Pharmacy and Pharmacy Management, Faculty of Pharmaceutical Sciences, University of Nigeria, Nsukka 410001, Enugu State, Nigeria*

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ABSTRACT

Objective: To investigate the safety profile of *Annona senegalensis* (*A. senegalensis*). **Methods:** Dried powdered root-bark of *A. senegalensis* was prepared by Sohxlet extraction using methanol-methylene chloride (1:1) solution and concentrated to obtain the methanol-methylene chloride extract (MME). MME was fractionated to obtain the *n*-hexane (HF), ethylacetate (EF) and methanol (MF) fractions. Acute toxicity (LD_{50}) test was performed with MME, HF, EF and MF in mice by oral route. The sub acute toxicity studies were performed in rats after 14 days of MME administration while haematological and biochemical parameters were monitored. **Results:** Medium lethal (LD_{50}) values of 1 296, 3 808, 1 265 and 2 154 mg/kg were obtained for the MME, MF, HF and EF, respectively. The sub-acute toxicity studies indicated a significant ($P<0.05$) increase in the body weight of both the treated rats and the control. The haematological tests indicated no change in the packed cell volume values but a significant ($P<0.05$) increase in the total WBC count at 100 and 400 mg/kg doses. The differential analysis showed a decrease in the nutrophils and a non-significant increase in the lymphocyte counts. The liver transaminase enzymes, alanin transaminase and aspartate transaminase showed no significant increase compared to the control. Histopathological examination of the liver sections also indicted no obvious signs of hepatotoxicity except with the 400 mg/kg dose that showed degeneration and necrosis of the hepatocytes. **Conclusions:** These results indicated that the root bark extracts of *A. Senegalensis* are safe at the lower doses tested, and calls for caution in use at higher doses in treatment.

1. Introduction

Annona senegalensis (*A. senegalensis*) Pers. (Annonaceae) is a shrub or small tree of about 2-6 m tall mostly found in the savanna and parts of tropical rain forest regions. It is found in Senegal, Nigeria, Cape Verde Island, Sudan and South Africa. *A. segalensis* is commonly known as African custard-apple, wild custard apple and wild sour sop[1,2]. It has aromatic flowers which are used to flavour food. The ripe fruit is yellow in colour and has a sweet edible jelly with pleasant odour. Common vernacular names are "Uburu ocha" (Igbo), "gwandar daajii" (Hausa), "arere" or "abo" (Yoruba), "Ukpokpo" (Igala), "ngonowu" (Kanuri), "uwu" (Idoma), "mkonokono" (Swahili)[3]. *A. senegalensis* is variously used in ethnomedicine for the treatment of different kinds of ailments and as such enjoys great reputation for its immense ethnomedicinal value. *A. senegalensis* has been reported to have antibacterial activity[4], antidiarrhoeal activity[5]. The anticonvulsant effects of the whole root extract has been reported[6]. The analgesic, anti-inflammatory activity[7,8], trypanocidal activity[9,10] and anti-snake venom activity[3], have been investigated. Also essential oils from the leaves of *A. senegalensis* exhibited antibacterial activity[11]. The plant decoction is used in the treatment of sleeping sickness in Northern Nigeria[12] as well as for the folkloric treatment

*Corresponding author: Theophine C Okoye, Department of Pharmacology and Toxicology, Faculty of Pharmaceutical Sciences, University of Nigeria, Nsukka 410001, Enugu State, Nigeria.
Tel: +234 803 668 4506
E-mail: theokuba@yahoo.com

of cancer[13,14] and malarial infection[15]. Recently, there are reports of the anticonvulsant, muscle relaxant and sedative effects of the root bark of *A. senegalensis*[16] and the neuro pharmacological activities of the leaf extracts[17]. In consideration of the diverse use of various parts of *A. senegalensis* for the management of many ailments in the Nigerian traditional medicine practice and the high edible application of the fruits, we set to evaluate the acute and sub-acute toxicity of its root bark extracts using experimental animals.

2. Materials and methods

2.1. Animals

Adult albino rats (100-200 g) of either sex bred in the laboratory animal facility of the Department of Pharmacology and Toxicology, University of Nigeria, Nsukka, were used for the study. The animals were maintained at the normal room temperature (25±1)℃ with a 12 h light : 12 h dark cycle and allowed free access to standard pellets and water. All animal experiments were conducted in compliance with the National Institute of Health Guidelines for Care and Use of Laboratory Animals (Pub No. 85-23, revised 1985) and under approval of the University Ethical Committee on the use of laboratory animals.

2.2. Preparation of extracts

The fresh root barks of *A. senegalensis* were collected in June from Enugu Ezike, in Enugu State, Nigeria. The plant material was identified and authenticated at the International Centre for Ethnomedicine and Drug Development (InterCEDD), Nsukka, Enugu State, where a voucher specimen was deposited (specimen number: BDCP/INTERCEDD 64). The plant material was air dried under shade to a constant weight and later pulverized into coarse powder. About 2.95 kg of the pulverized plant material was extracted using soxhlet apparatus at reduced pressure with methanol-methylene chloride (1:1). The extract (MME) obtained was concentrated using a rotary evaporator to obtain a chocolate colored gummy residue 375 g (12.71% w/w). Subsequently 200 mg of MME was fractionated chromatographically with *n*-hexane, ethyl acetate and methanol to obtain hexane fraction (HF), Ethyl acetate fraction (EF) and methanol fraction (MF) respectively.

2.3. Phytochemical analysis

The extracts and fractions were subjected to phytochemical analysis using standard procedures[18,19].

2.4. Acute toxicity tests

The acute toxicity and lethality of the MME and fractions were determined using the method described by Lorke[20]. Briefly, 9 rats were randomly divided into three groups (n=3), and were orally administered with 10, 100, and 1 000 mg/kg of the MME, respectively. They were observed for 24 h for death. Since no death was recorded, 1 600 mg/kg, 2 900 mg/kg and 5 000 mg/kg of the extracts were administered to a fresh batch of animals at one animal per dose and the number of deaths in 24 h was recorded. This procedure was repeated for each of the various fractions HF, EF and ME.

2.5. Sub-acute toxicity tests

Rats were randomly placed in groups (n=5). While group 1 received daily oral administrations of the vehicle (1:1, 20% Tween 80 and propylene glycol, 10 mL/kg) groups 2-4 received 50, 100 or 400 mg/kg of the MME for 14 days, respectively. Body weights of the animals were recorded on day 0, 7, and 14. At the end of the experiment, blood was collected from the orbital sinus for biochemical and hematological analysis. Subsequently the animals were sacrificed and selected organs (liver, heart, spleen, kidney and lung) were removed for macroscopic analysis. The biochemical enzymes evaluated include alanine aminotransferase (ALT) and aspartate aminotransferase (AST) which were assessed using the commercial kits[21]. The hematological parameters determined included the packed cell volume (PCV), total white blood cell (WBC) count and differential WBC count. The liver tissue specimens were sectioned for histopathological studies[22].

2.5.1. Hematological analysis

The blood samples were placed in a well labeled sample bottles containing ethylene diamine tetraacetic acid (EDTA). The sample bottle was shaken gently to mix the blood with EDTA to prevent clotting.

The PCV was determined using microhematocrit centrifuge (Hawksley, England). A micro capillary tube sealed with plastacine at one end was nearly filled with blood sample and centrifuged in the equipment at 10 000 r/min for 5 minutes. After centrifugation, the PCV was read using a Hawksley microhematocrit reader.

The blood, 0.02 mL, was diluted in 0.38 mL of WBC diluting fluid (Turk´s solution) to make a 1:20 dilution of the blood. The diluted sample was loaded in a Neubauer Counting Chamber and all the cells on the 4 corner square (1 mm^2) areas were counted using a light microscope of ×10 objective. The number of cells counted for each blood sample was multiplied by 50 to obtain the total WBC count per micro liter of blood[22].

The blood sample was shaken gently and a drop of blood

was placed on a clean grease-free slide. A thin smear of blood was made on the slide using a cover slip. The smear was allowed to air-dry and was stained using Leishman stain. The stained slides were later examined under oil immersion ×100 objective, using a light microscope. Result for each type of white blood cell was expressed as a percentage of the total count converted to the absolute value per micro liter of blood.

2.5.2. Liver function analysis

The effect of the extract on the ALT (SGPT) and AST (SGOT) was determined using Randox Assay kit. The GPT and GOT substrate solution (reagent A), 0.5 mL each, for ALT and AST respectively, was placed into the labeled test tubes and incubated for 5 min at 37 ℃. After the incubation, 0.1 mL of the serum sample was added in the test tubes and incubated for 30 min at 37 ℃. Then 0.5 mL of color developer (reagent B) was added and then allowed to stand for 20 min at room temperature (25-26 ℃). 5 mL of dilute NaOH solution (reagent C) was added and allowed to stand for 15 min at room temperature. The transmittance was read at 505 nm against water blank. The mixture was stable for up to 1 h. The ALT and AST activities (μ/L) were obtained by interpolating the transmittance into the calibration curve[21].

2.5.3. Histopathological examination of the liver

The rats were sacrificed, dissected and their livers carefully collected and fixed in 10% buffered formalin solution. Histologic sections were prepared from the livers and were fixed in bouin's solution (mixture of 75 mL of saturated picric acid, 25 mL of 40% formaldehyde and 5 mL of glacial acetic acid) for 12 h. This was embedded in paraffin using conventional method and cut into 5 μm thick sections and stained using hematoxylin-eosin dye and finally mounted in diphenyl xylene[23]. The sections were then observed under the microscope for histopathological changes in liver architecture and their photomicrographs were taken.

2.5.4. Organ toxicity

The gross examination (macroscopic analysis) of the target organs of the control and treated animals were done to check any significant change in weight, texture and shape. The major targeted organs include the liver, lung, heart, spleen and kidney.

2.6. Statistical analysis

The statistical analysis of data obtained were done by One Way ANOVA and subjected to Dunnett's *post hoc* Test, where the data were compared with the control. The mean difference is significant at $P<0.05$ level and values were stated as Mean±SEM.

3. Results

3.1. Phytochemical tests

Phytochemical tests of the extracts and fractions revealed the moderate presence of alkaloids, glycosides, carbohydrates and steroids, while flavonoids, terpenoids and resins were quite in abundance in the extracts and fractions (Table 1).

3.2. Acute toxicity tests

The acute lethality and toxicity (LD_{50}) tests of the extract and fractions showed an estimated LD_{50} values of 1 296, 3 808, 1 265 and 2 154 mg/kg for the MME, MF, HF and EF, respectively.

3.3. Effect of extract on body weight

The body weight of all the rats increased considerably with respect to their initial weight. At 400 mg/kg dose the extract exhibited significant ($P<0.05$) increase in body weight. The

Table 1
Phytochemical constituents of extract and fractions.

Constituent	MME	EF	HF	MF
Carbohydrate	++	−	−	++
Alkaloid	+++	++	++	++
Reducing sugar	++	−	−	++
Glycoside	+++	−	−	+++
Saponins	+	−	−	+
Tannins	−	−	−	−
Flavonoids	++++	++++	−	−
Resin	++++	++	++++	++
Fats and oils	+	+	+	−
Steroids	+++	−	++	+
Terpenoids	++++	++	+++	++
Acidic compounds	−	−	−	−

− = absent; + = present; ++ = moderately present; +++ = conspicuously present; ++++ = abundantly present.

weight gain in the extract treated groups was lower than in control group. The results show that the extract elicited a dose-dependent significant increase in body weight compared to the control (Table 2).

3.4. Effect of extract on organ weight

There was no significant (P>0.05) differences in the weight of heart, lung, spleen and kidney of treated and control rats, although, the liver weight of the extract treated rats was higher than the control (Table 3).

3.5. Effect of extract on hematological parameter

There was no significant (P>0.05) change in the neutrophil and lymphocyte counts, of the extracts treated rats when compared with the control. The extract exhibited a dose dependent increase in the PCV and WBC at same time showed a significant increase in WBC count at 100 mg/kg dose (Table 4).

3.6. Effect of extract on serum biochemical parameters

There was no significant (P>0.05) difference in the levels of ALT and AST between the treated and control groups (Table 5). This is an indication of no biochemical related changes in the hepatic cells.

3.7. Effect of extract on liver architecture

Histopathological examination of liver section of the control group and extract treated groups (50 mg/kg and 100 mg/kg dose levels), showed normal cellular architecture with distinct hepatic cells that are still intact, clear sinusoidal spaces and central vein (Figure 1 A-C). While the 400 mg/kg treated group showed a clear sinusoidal spaces and central vein, with mild sign of inflamed portal tracts between different lobules and showed degeneration and necrosis of the hepatocytes (Figure D). Gross anatomical observation of the

Table 2
Effect of extract on body weight (Mean±SEM).

Treatment	Dose (mg/kg)	Body weight (g)		
		Day 0	Day 7	Day 14
Control		102.00 ± 3.39	120.60 ± 3.22	131.00 ± 3.85
MME	50	100.00 ± 3.52	116.25 ± 3.15	126.00 ± 2.16
	100	109.00 ± 4.56*	120.80 ± 6.41	129.60 ± 7.91
	400	117.20 ± 2.33**	133.00 ± 3.86**	137.00 ± 4.12

Significance at *P<0.05; **P<0.001, *vs*. control group.

Table 3
Effect of extract on organ weight(Mean±SEM, n=5).

Treatment	Dose (mg/kg)	Weight (g)				
		Heart	Lung	Spleen	Liver	Kidney
Control		0.43±0.48	1.08±0.13	0.98±0.11	5.95±0.18	0.45±0.03
MME	50	0.40±0.00	0.93±0.08	1.08±0.13	6.75±0.10	0.38±0.03
	100	0.53±0.25	0.93±0.09	1.13±0.16	7.43±0.71	0.48±0.03
	400	0.48±0.03	0.98±0.08	0.80±0.11	6.33±0.43	0.43±0.03

Table 4
Effect of extract on haematological parameters(Mean±SEM, n=5).

Treatment	Dose (mg/kg)	PCV	WBC	Differential	
				Neutrophil	Lymphocyte
Control		37.50 ± 1.71	10.90 ± 1.58	28.50 ± 8.02	70.75 ± 8.01
MME	50	29.25 ± 4.15	9.08 ± 1.27	27.75 ± 6.36	72.00 ± 6.38
	100	37.25 ± 1.38	17.35 ± 2.08*	16.50 ± 1.94	83.25 ± 2.14
	400	38.00 ± 1.87	14.98 ± 0.98	17.25 ± 1.70	82.25 ± 2.17

*P<0.05; vs. control group.

Table 5
Effect of extract on liver enzymes(Mean±SEM, n=5) (IU/L).

Treatment	Dose (mg/kg)	ALT	AST
Control		33.00 ± 2.68	70.50 ± 6.45
MME	50	31.00 ± 7.80	56.75 ± 20.70
	100	33.25 ± 2.69	74.75 ± 5.20
	400	27.00 ± 2.74	72.75 ± 6.43

liver showed a distinct inflammed liver organ with 400 mg/kg treated group compared with the control group.

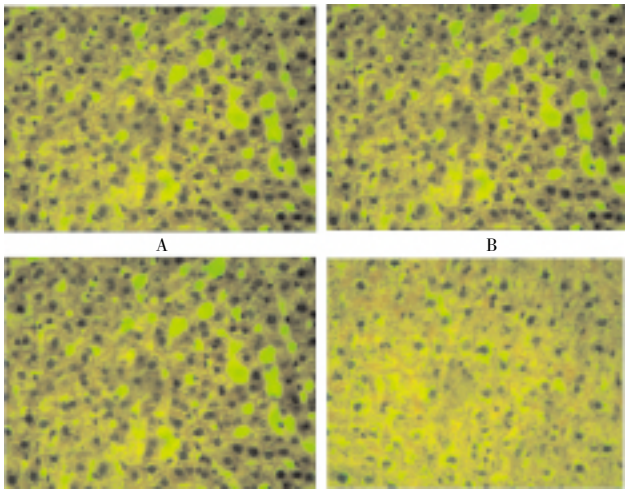

Figure 1. Photomicrographs of the Liver(×400, H & E).
A: Control; B: MME 50 mg/kg; C: MME 100 mg/kg; D: MME 400 mg/kg.

4. Discussions

The use of medicinal plants to manage ailments is very common especially in rural areas of Nigeria, where about 70% of the populace reside. Preparations of *A. senegalensis* are used in the management of a wide range of ailments ranging from gastrointestinal disturbances, central nervous system disorders to cancer. In addition the fruit offers an edible source to the people. The importance of the plant in folk medicine, its popularity and high demand, as well as its promising pharmacological potentials make this toxicological studies on it imperative. The acute lethality, LD_{50}, of the extracts and fractions indicated a relatively high safety profile[20]. The increase in body weight after drug administration usually indicate absence of toxicity as decrease in body weight is an index of toxic effect of a compound[24-29]. In this study, the weight of the vital organs (the heart, lung, spleen and kidney) after the treatment period indicated no significant obvious change. However, the liver showed slight non dose dependent increase in weight. The macroscopic examination of these target organs showed no significant changes in colour, shape and texture when compared with the control. This probably may have ruled out any organ toxicity at the doses tested. The extract showed no obvious alterations in the haematological parameters at the doses tested. The results indicated no significant ($P>0.05$) increase in the PCV values but exhibited dose dependent increase in the total WBC count which was significant ($P<0.05$) at 100 mg/kg dose. This increase might probably be as a result of increase in the lymphocyte level which was evident in the differential WBC count. The degree of immunological stimulation is one of the important factors responsible for the increased circulating lymphocytes in the body[30] and this might suggests possible immune modulatory effects of the extract, which is a point for further research. Moreover, the differential test showed no increase in the number of circulating neutrophils. On the other hand the serum biochemical parameters did not show any significant increase. The activities of the liver marker enzymes ALT and AST remain insignificant variations compared with the control animals. Although there was insignificant increase in AST values as observed in 100 and 400 mg/kg treated groups, this could not amount to any noticeable alterations in the activity and morphology of the liver cells in the treated animals. High values of serum ALT and AST have been implicated in liver necrosis, hepatitis, toxic liver diseases and acute myocardial infarction and ALT is one of the plasma cardiac markers that increase sequentially after acute myocardial infarction[31,32]. A rise in the activities of ALT and AST is a sensitive indicator of damage to cytoplasmic and mitochondrial membranes and their relative plasma activities may help to indicate the type of hepatic damage whenever it manifested[33]. The histopathological studies showed no prominent hepatic damages/injuries in the control and treated animal groups (50 mg/kg and 100 mg/kg) while disclosing some degree of degeneration and necrosis of the hepatocytes. Therefore, the histopathological findings of the liver in this study, calls for caution when using the extract at higher doses as this may lead to damage of the liver cells as found in the animals that received 400 mg/kg dose. Meanwhile the phytochemical tests revealed the abundant presence of flavonoids, terpenoids, resins, alkaloids and moderate presence of glycosides, steroids in the extracts and fractions. The presence of these phyto-constituents is correlated with those present in the methanol root bark extract of *A. senegalensis*. The observed toxicity with the 400 mg/kg dose could not be ascribed to a particular constituent(s) at this stage of the work. The variations in the degree of presence of these phytochemicals in the fractions may have been responsible for the different lethality values exhibited by the fractions, though they all manifested high level of safety.

In conclusion, the root bark extract of *A. senegalensis* possesses no obvious significant toxic effects and the doses treated excerpt at 400 mg/kg dose exhibit no significant acute lethality on the treated animals. This overall findings buttress the fact that local dwellers in different parts of the savanna and rainforest zones have been using parts of *A. senegalensis* for medicinal purposes and the juicy fruits as edible for many years without obvious complaints or toxicity.

Conflict of interest statement

We declare that we have no conflict of interest.

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