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Emmanuel O Okwuofu
Department of Pharmacology & Toxicology, Faculty of Pharmacy, University of Benin, Benin City, Nigeria

Ray I Ozolua
Department of Pharmacology & Toxicology, Faculty of Pharmacy, University of Benin, Benin City, Nigeria

Oboma I Yibala
Department of Anatomical Pathology, Niger Delta University, Wilberforce Island Bayelsa State, Nigeria

Abigail M Akhigbemen
Department of Pharmacology & Toxicology, Faculty of Pharmacy, University of Benin, Benin City, Nigeria

Correspondence
Emmanuel O Okwuofu
Department of Pharmacology & Toxicology, Faculty of Pharmacy, University of Benin, Benin City, Nigeria

Evaluation of acute and sub-acute safety profile of *Syzygium guineense* (Wild) DC methanol stem bark extract in rodents

Emmanuel O Okwuofu, Ray I Ozolua, Oboma I Yibala and Abigail M Akhigbemen

Abstract
Herbal medicine has been widely accepted as an alternative or complement to orthodox medicines. The aim of this study is to evaluate the safety profile of methanol stem bark extract (ME) of *S. guineense.* Oral acute and 28-day toxicity tests were conducted in rats. The sub-acute evaluations included haematological, biochemical markers of toxicity and histology. The oral LD_{50} value was greater than 5000 mg/kg. The 28-day toxicity tests revealed significantly increased (P<0.01) liver/body weight ratio at 1600 mg/kg/day. Haemoglobin concentration and platelet count were significantly (P<0.05) increased in the group treated with 800 and 1600 mg/kg/day. Alkaline phosphatase (ALP), alanine aminotransferase (ALT), aspartate aminotransferase (AST), urea and creatinine were significantly ($P<0.05$) increased at the dose of 1600 mg/kg/day. There were no significant changes in the heart, lungs, brain and kidney/body weight ratios and other haematological parameters but there were distortions in hepatic and renal histology at doses of 1600 mg/kg. Acute doses up to 5000 mg/kg of ME appear safe but prolong use of high doses could lead to hepatotoxicity and nephrotoxicity.

Keywords: Organ/body weights ratios, haematological parameters, liver enzymes, serum electrolytes

1. Introduction
In The use of medicinal plants especially in developing countries has remained popular. The level of sensitization and mobilization by the World Health Organisation (WHO) has encouraged most African countries, including Nigeria to embrace and initiate developmental plans on Traditional African Medicine, the bulk of which is herbal [1]. An estimated three-quarter of the world's population uses herbs and other forms of traditional medicines to treat their illnesses [1-2]. Widespread use of herbal medicines has posed safety risks to consumers especially on prolonged usage. There exists among consumers a misleading impression that herbal medicines have fewer or no adverse effects [3]. This notion has been proven wrong by several researchers who have reported the toxicity level of many herbal preparations [4-5].
Syzygium guineense (Myrtaceae) (Wild) DC is a flowering plant found in the wild, native to the wooded savannah and tropical forest of Africa, with characteristics odour and edible fruits [6]. It is commonly called water berry, malmo in Hausa and aldere in Yoruba languages of West Africa. Various parts of this plant (leaves, roots, and stem bark) have been used in the management of different disease conditions. The analgesic and anti-inflammatory properties of the ethanol leaf extract has been reported in rats and mice [7]. Products from the shrub have been used by rural dwellers to make tooth brushes and in the treatment of amenorrhoea [8-9]. Omale *et al* [10] reported the anti-venom property of the methanol leaf extract. It is one of the useful antimalarial remedies in southern Uganda [11]. The role of plants in folkloric medicine is attributed to the presence of phytochemicals which are non-nutritive plant chemicals that have disease preventing or curative properties. A major disadvantage that has been linked with the use of herbal medicines is the absence of scientific evaluation of their safety profiles since many of them have turned out to be toxic [12]. In this study, we have evaluated the safety profile of methanol stem bark extract of the plant.

2. Materials and Methods
2.1 Collection of Plant Materials
Fresh pieces of the stem bark of S. guineense were collected from a suburb of Abuja, Nigeria in August, 2015. Identification and authentication were done by Mr. Muazzam Ibrahim Wudil, an ethnobotanist at the National Institute of Pharmaceutical Research and Development

(NIPRD), Abuja where a voucher specimen with herbarium number NIPRD/H/6803 has been deposited. The pieces of stem bark were air-dried and powdered using an electric mill. The powder (500 g) was macerated in 2000 ml of methanol. The filtrate obtained was concentrated in a rotary evaporator and the resulting methanol extract (ME) was packed into an amber coloured bottle and stored in a refrigerator.

2.2 Animals

Adult males and females Wistar rats weighing 110 - 130g were obtained from the animal house of Ambrose Ali University, Nigeria and housed in the animal facility of the Department of Pharmacology and Toxicology, University of Benin. The animals had free access to water and standard pellets and were acclimatized for two weeks under natural lighting and room temperature conditions. All animal experiments were conducted in compliance with the guidelines of National Institute of Health for care and use of laboratory animals [13].

2.3 Phytochemical Studies

The methanol extract of S. guineense was screened qualitatively for phytochemical constituents using standard protocols [14].

2.4 Acute Oral Toxicity Studies

The oral LD50 was evaluated using method described by Locke [15]. Nine rats and nine mice were randomly allotted into three groups of three rats and three mice per group, and were orally administered with doses of 10, 100, and 1000 mg/kg of ME respectively. They were observed for 24 h for death and other gross toxicological symptoms. In the absence of death, 1600, 2900 and 5000 mg/kg doses of the extract were administered to another batch of the animals in the phase 2 consisting of one animal per dose per group and were observed for death and gross toxicological symptoms for 24 h and 14 days.

2.5 Sub-acute (28-day) Toxicity Tests

The animals were randomly divided into four groups of 10 animals each consisting of 5 females and 5 males kept in different cages. Group I received distilled water and served as control. Group II-IV: were administered ME at dose of 400, 800 and 1600 mg/kg p.o. respectively. On the 29th day after an overnight fast, blood samples were obtained from the abdominal aortae of the rats into plain bottles (for biochemical assays) and sodium EDTA bottles (for haematological assays). The brain, heart, lungs, liver and kidney were carefully excised kept in absorbent papers for 5 min, weighed and then preserved in 10% formosaline for histology.

2.6 Estimation of haematological parameters

Red blood cell (RBC), haematocrit (HT), haemoglobin (HB), mean corpuscular haemoglobin concentration (MCHC), platelet (PLT), plateletcrit (PCT), white blood cells (WBC) and the differentials [leucocytes (LT), monocytes (MO), granulocytes (GR)] were quantified using an automated haematology system (Sysmex Haematology-Coagulation Systems, Model KX21N, Sysmex Incorporated, Japan). The blood samples were first pipetted into a capillary tube, spun in a roller mixer for 2 - 3 min before values were read.

2.7 Determination of serum biochemical and Electrolyte parameters

The blood samples in the plain bottles was allowed to clot and then centrifuged at 5000 rpm to obtain sera. The sera were used for the assay of aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), urea, creatinine, total bilirubin and conjugated bilirubin. Serum AST, ALT and ALP were assayed using enzyme kinetic method [16-17]. Total bilirubin and conjugated bilirubin were determined by Jendrassik-Grof method [17]. Urea was assayed using modified diacetylmonoamine method [18], while creatinine was determined by the Jaffe's method [19-20]. Assay for bicarbonate was done using method described by Van Skye and Neil [21]. The assay method for chloride ion in the sample was determined using Schales and Schales method [22], while sodium and potassium was done using method described by Magoshes and Vallee [23].

2.8 Histology

Tissues were sectioned at 4 µm thickness on a rotary microtome (Leica RM 2125) and stained with Erhlich's haematoxylin and eosin for microscopy. The processing of the tissue was carried out in Niger Delta University Teaching Hospital, Okolobiri while photomicrographs were prepared in the Department of Anatomical Pathology, Niger Delta University, Wilberforce Island.

2.9 Statistical Analysis

The data are expressed as Mean ± Standard Error of Mean (S.E.M). They were compared using one way analysis of variance (ANOVA) with Dunnet's post hoc test. $P<0.05$ was considered significant.

3. Results

3.1 Phytochemical test

Phytochemical constituents found include flavonoids, tanins, saponins, sugars, proteins, terpinoids and glycosides.

3.2 Acute Oral Toxicity Test

The oral median lethal dose (LD50) of the extract was estimated to be greater than 5000 mg/kg in rats and mice. Gross toxicological symptoms observed include reduced alertness, slow response to touch and sedation. No death was recorded within 24 h and after 14 days of observation.

3.3 Sub-Acute Toxicity Tests

3.3.1 Effects of the Extract on Organ/body Weight Ratios

The organ/body weight ratios of the brain, heart, kidney and lungs were not significantly different across the three treated groups but there was a significant ($P<0.05$) increase in the liver/body weight ratio in the group treated with 1600 mg/kg/day (Table 1).

3.3.2 Effect of Extract on Electrolyte, Urea, Creatinine and Haematological Parameters

Table 4 shows that the levels of sodium, potassium, bicarbonate and chloride ions were not significantly altered across the groups. Urea and creatinine levels were significantly ($P<0.05$) increased in the group treated with 1600 mg/kg/day of the ME.

3.3.3 Effects of the Extract on Biochemical Parameters

The activities of ALP, AST and ALT were significantly (P<0.05) increased at the highest dose (1600 mg/kg/day) compared to control group. The level of total bilirubin and conjugated bilirubin were comparable across all the groups (Table 5).

3.3.4 Effects of the Extract on Tissue Histology

In all the groups, there were no obvious histological changes to the brain when compared to the control group (Figure 1). There were also no gross histological features observed in the heart (Figure 2) and lungs (Figure 3) of the animals. However, the kidneys of all ME-treated animals showed distorted glomeruli with varying level of cellularity (Figure 4) while the liver of animal in groups treated with 800 and 1600 mg/kg/day, had lipoid necrosis and infiltration of inflammatory cells around the central vein (Figure 5).

Table 1: The organ-body weight ratios of rats following 28-day daily treatment of rats with methanol stem bark extract of *S. guineense*

Treatment	Brain	Heart	Kidney	Liver	Lungs
Control	0.665±0.025	0.332±0.015	0.322±0.016	2.481±0.045	0.682±0.039
400 mg/kg	0.707±0.027	0.337±0.021	0.280±0.014	2.677±0.067	0.625±0.045
800 mg/kg	0.672±0.053	0.325±0.017	0.303±0.023	2.806±0.205	0.645±0.038
1600 mg/kg	0.606±0.021	0.385±0.068	0.279±0.014	3.083±0.160*	0.552±0.066

Values are not significantly different. n = 10 per group

Table 2: White blood cells and platelet counts following 28-day daily treatment of rats with methanol stem bark extract of *S. guineense.*

Treatment	TWBC ($x10^3/\mu L$)	LT ($x10^2/\mu L$)	MO ($x10^2/\mu L$)	GR ($x10^2/\mu L$)	PLT ($x10^3/\mu L$)
Control	12.3±0.5	63.1±0.3	10.0±0.1	39.1±1.4	669.4±36.6
400 mg/kg	14.7±2.1	77.8±0.8	14.3±0.3	36.1±1.8	669.5±30.4
800 mg/kg	13.3±1.2	69.3±0.9	16.9±0.3	43.1±1.3	851.7±6.9*
1600 mg/kg	14.5±0.3	82.6±0.3	14.3±0.1	44.5±0.2	762.8±17.3*

Values are not significantly different when compared with control group. Control = Distilled Water. TWBC: Total White Blood Cells, LT: Leucocytes, MO: Monocytes, GR: Granulocytes, PLT: Platelets, ME - methanol extract of *S. guineense.* n = 10 per group.

Table 3: Red cell parameters of rats following 28-day daily treatment of rats with methanol stem bark extract of *S. guineense*

Treatment	HB (g/dl)	HCT (%)	MCHC (g/dl)	RBC ($x10^5/\mu L$)
Control	13.0±0.5	41.8±1.3	41.1±1.1	75.5±0.1
400 mg/kg	13.6±0.8	41.6±1.2	40.3±0.6	69.8±1.2
800 mg/kg	15.5±0.4*	42.5±1.3	41.0±1.2	71.2±0.3
1600 mg/kg	15.3±0.5*	41.5±1.7	41.0±0.9	74.6±0.2

*$P<0.05$ compared to control group.HB: Haemoglobin. HCT: Haematocrit MCHC: Mean Corpuscular Haemoglobin Concentration RBC: Red blood cells, ME - methanol extract of *S. guineense.* Control = Distilled Water. n = 10 per group.

Table 4: Serum electrolyte, urea and creatinineof rats following 28-day daily treatment with methanolstem bark extract of *S. guineense*

Treatment	Na^+ (mg/dl)	K^+ (mg/dl)	HCO_3 (mg/dl)	Cl^- (mg/dl)	Urea (mg/dl)	Creatinine (mmol/L)
Control	140.6±1.3	6.1±0.2	21.3±0.4	103.1±0.5	27.3±1.2	0.6±0.0
400 mg/kg	142.2±0.8	5.8±0.2	21.9±0.5	103.5±0.4	27.8±0.9	0.6±0.0
800 mg/kg	142.2±0.8	6.7±0.2	20.9±0.4	102.0±0.7	29.8±1.0	0.6±0.0
1600 mg/kg	144.8±0.6	6.7±0.3	21.9±0.6	102.1±0.5	31.1±0.9*	0.7±0.0*

ME, methanol extract, * $P<0.05$

Table 5: Effects of oral daily doses (x28) of the methanol stem bark extract of*S. guineense* on biochemical parameters of rats

Treatment	ALP (Units/L)	ALT (Units/L)	AST (Units/L)	TB $x10^{-2}$ (mg/dl)	CB $x10^{-2}$ (mg/dl)
Control	130.3±3.3	103.1±3.9	94.2±6.2	46±3.4	16±2.2
400 mg/kg	135.7±5.3	107.5±2.7	94.6±3.4	49±2.8	21±1.8
800 mg/kg	141.8±5.4	110.7±2.6	95.8±1.9	49±3.2	20±2.1
1600 mg/kg	153.6±1.9*	128.5±3.5*	129.4±3.8*	52±2.9	19±1.8

*$P<0.05$ compared to control group. ALP: Alkaline phosphatase, ALT: alanine aminotransferase, AST: aspartate aminotransferase, TB: total bilirubin, CB: conjugated bilirubin, ME - methanol extract of *S. guineense.* Control is distilled water treated group n = 10 per group.

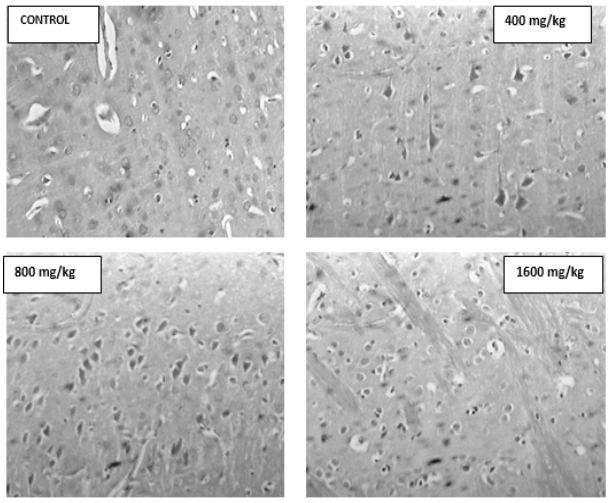

Fig 1: Representative photomicrographs of the brain of rats treated with the methanol extract of *S. guineense*. There are no obvious differences. H & E x 400

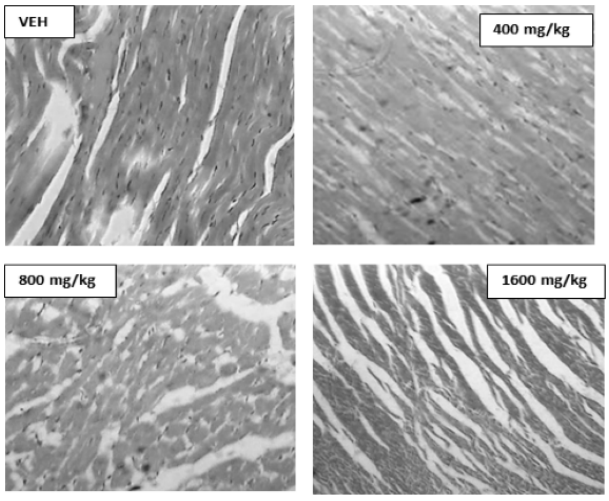

Fig 2: Representative photomicrographs of the heart of rats treated with the methanol extract of *S. guineense*. There are no obvious differences. H & E x 400

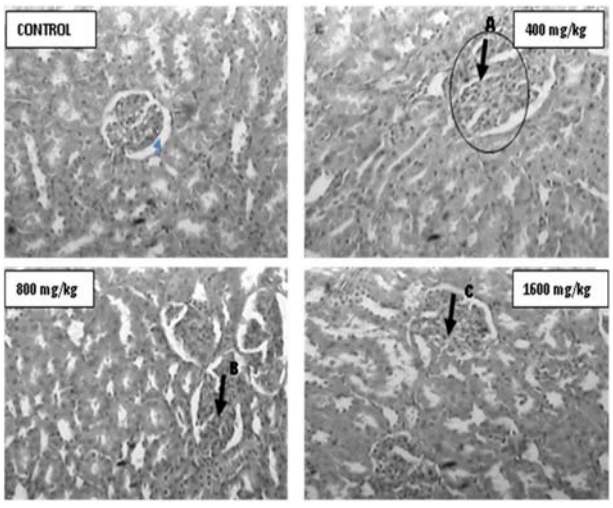

Fig 3: Representative photomicrographs of the kidney of rats treated with the methanol extract of *S. guineense*. Obvious distortion in the glomerula cells in all the treated groups (H & E x 400).

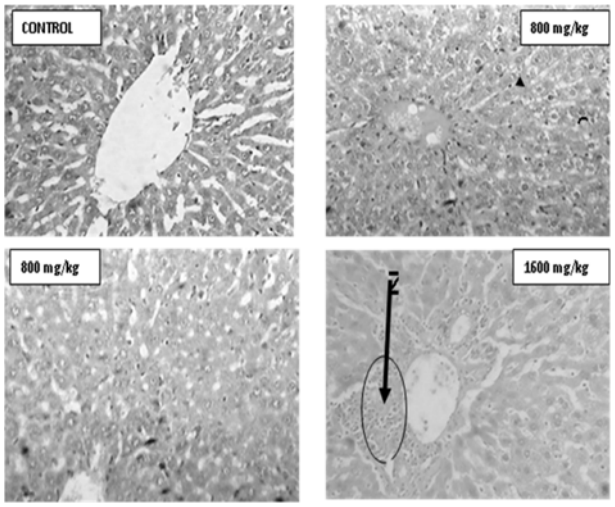

Fig 4: Representative photomicrographs of the liver of rats treated with the methanol. Z, showing lipoid necrosis and infiltration of inflammatory cells around the central vein in the group treated with 1600 mg/kg extract of *S. guineense* (H & E x 400).

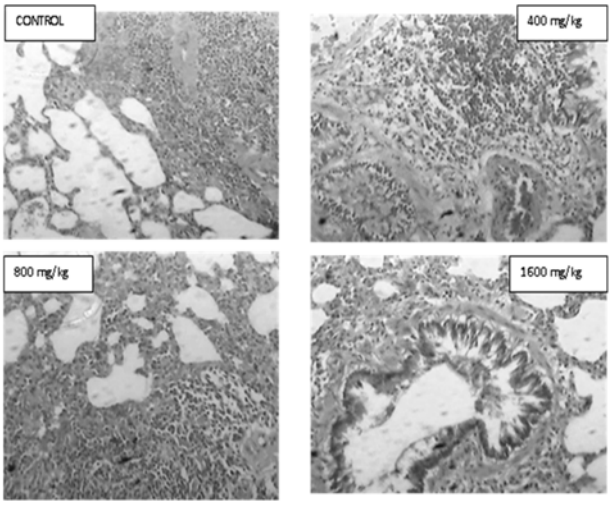

Fig 5: Representative photomicrographs of the lungs of rats treated with the methanol extract of *S. guineense*. H & E x 400.

4. Discussion

The methanol extract contained saponins, tannins, flavonoids, sugars, cardiac glycosides and proteins and agrees with the report by Nigatu [24]. These phytoconstituents are believed to be responsible for the wide array of biological actions of the plant. The median lethal dose (LD_{50}) determination has become a valuable index in evaluating the safety of substances [25]. The absence of death at the acute dose of 5000 mg/kg indicates that the extract may be very safe and unlikely to cause serious toxicity in humans [26]. The use of doses higher than are required for pharmacological actions in toxicological evaluation enables an appreciation of the safety margin of the substance [27]. Although there were no deaths at the dose of 5000 mg/kg, behavioural symptoms such as reduced alertness, decrease locomotion and sedation were observed.

An increase in organ/body weight ratio is an indication of hyperplasia/hypertrophy and inflammation, while a reduction can be attributed to atrophy [28]. In this study, liver/body weight ratio increased significantly. Liver function tests provide vital information about the state of the liver,

describing its functionality and cellular integrity [29-31]. ALT and AST are transaminase enzymes produced within the cells of the liver, especially in condition where liver cells have been inflamed or are dead [29-30]. Damages to liver parenchyma cells result in the leakage of ALT, AST and ALP into the circulation leading to an increase in serum concentrations [32-33]. However of these hepatic enzymes, ALT is the most sensitive and reliable biomarker of hepatocellular injury useful in predicting possible toxicity [34]. AST is less specific and is present in abundance in the cardiac muscles, skeletal muscles, kidneys and testes, while ALP is also found in growing bone [35]. In any case, any disease state affecting any of these extrahepatic tissues significantly elevates the serum levels of these enzymes [35]. The increase in the levels of ALT, AST and ALP which occurred with 1600 mg/kg/day of the extract indicates damage to the liver and possibly other internal organs. This result corroborates lesions seen in the photomicrographs of the liver of rats given 1600 mg/kg/day.
Serum electrolytes, urea and creatinine are reliable indicators of renal function [31]. The levels of urea and creatinine were increased significantly at the dose of 1600 mg/kg/day thereby suggesting possible nephrotoxicity. These renal effects are further supported by the histology which showed distortion in the Bowman's capsule and its surrounding organelles at 1600 mg/kg/day. Aside from indicating deleterious effects, plant extracts can also indicate positive haematological changes [36]. Increased hemoglobin concentration seen at doses of 800 and 1600 mg/kg/day suggests stimulation of haemoglobin synthesis by unknown mechanisms [37-38]. The elevated platelet count could result in increased thromboembolic disorders [33]. The extract seems safe at an acute dose up to 5000 mg/kg in rats and mice and sub-acute doses of 400 and 800 mg/kg in rats. Chronic administration of high doses may cause renal and hepatic toxicities.

5. Acknowledgement

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6. Conflict of Interest

There is no conflict of interest associated with this work.

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