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Oral Safety Evaluation of the Aqueous Fruit Extract of Annona Muricata in Wistar Rats

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Abstract

Annona muricata fruit (i.e. Soursoup or custard apple a tropical plant species known for its edible fruit) has been attributed with numerous medicinal benefits (including antimicrobial, anti-inflammatory, anti-protozoan, antioxidant, anxiolytic, anti-stress, anti-ulcerogenic, wound healing, hepato-protective, anti-icteric, hepatoprotective, anticancer and antihyperglycemic activities) as well as some toxicological effects (neurotoxicity and neurodegeneration). Despite its ancestral use and wide applications in human health, scientific information on its oral safety remains scanty. In this study, the oral safety of 100 mg/kd/day, 200 mg/kg/day and 400 mg/kg/day of the aqueous fruit extract of Annona Muricata (AFAM) was evaluated in young adult, male and female white albino Wistar rats using standard acute and 42-days sub-chronic oral toxicity testing guidelines on anthropometric, biochemical, hematological and histo-pathological endpoints. In addition, preliminary qualitative and quantitative analyses of AFAM were undertaken using standard procedures. Results of the study showed that the estimated LD50 value for the acute oral toxicity study of AFAM calculated to be greater than 5 g/kg body weight/oral route although the testing was associated with transient but reversible behavioral toxicities. For its sub-chronic oral toxicity testing, AFAM treatment resulted in profound %weight gain, decreases in the serum triglycerides and very low density lipoprotein cholesterol and liver enzymes. Similarly, prolonged oral AFAM treatments caused significant decrease and increase in the differential neutrophils and platelet counts, respectively while the hist-opathological features of hepatic steatosis and renal tubule collapse in the AFAM-treated livers and kidneys, respectively suggested possible increased intrahepatic lipids biosynthesis and nephrotoxicity. The preliminary phytochemical analyses of AFAM showed the presence and relative amount of flavonoids, alkaloids, tannin, glycosides, saponin, and reducing sugars while the Gas Chromatography-Mass spectroscopy showed the relative abundance of thirteen compounds. Composite analyses conducted on the extract showed the presence of carbohydrate (64.65%), protein (2.14%), moisture content (8.07%), ash value (6.73%), lipid (14.22%) and fiber (4.19%). Overall, the study suggested that the prolonged AFAM oral treatments could predispose to the development of fatty liver disease from de novo intrahepatic biosynthesis of triglycerides, nephrotoxicity and hematotoxicity. In conclusion, the results of this study showed that prolonged consumption of AFAM should be with a great caution as it could pose serious health concerns.

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1. Introduction

Medicinal plants have long historical use in the management of both human and veterinary diseases. According to the World Health Organization Drugs Strategy (2002-2005), nearly 70%-80% of the world's population largely depend on one form of medicinal herbs or the other as either complementary or alternative medicine and this use has exponentially grown in both western and developing countries over the past 20 years [1-2]. Medicinal plants have been reported to serve the purpose of food (nutraceticals) [3], drug and drug discovery in ethnomedicine and ethnobotany [4] and in cosmetology (cosmeceuticals) [5], thus, forming an essential and integral component of primary healthcare globally. Although medicinal plants are generally considered to be safe for use in disease treatment but they are not entirely free of untoward effects or toxicity [6-8]. The toxicity profile of medicinal plants varies depending on a number of determinants such as their nature and amounts of their phytochemical constituents, dosage, route of administration, and the duration of exposure [9-11].

Annona muricata L. (of the family: Annonaceae) is an evergreen, terrestrial and erect fruit-bearing tree measuring up to 5-8 m high and ubiquitous to the tropical and subtropical regions including India, Malaysia and Nigeria [12,13] although was native to north and South America where it highly valued for its folkloric medicinal use [14,15]. Annona muricata tree is typically of an open, roundish canopy with large, glossy, and dark green leaves and of edible, green, and heart-shaped fruits, with a diameter of 15 and 20 cm [16]. Annona muricata, also known as Soursop, magic fruit, graviola, custard apple, Chop-chop, Sapi sapi [17-18], is locally known in Nigeria by various local names which include "Ebo or Apekan" (amongst the Yoruba tribe, Southwest Nigeria), "Sawansop" (amongst the Igbos, Eastern Nigeria), and "fasadarur" and "tuwon biri" (amongst the Hausas, Northern Nigeria) [19]. Its other local names include: Creole (saua sap, kowól); Dutch (soursap, sorsaka, zuurzak); Filipino (atti, llabanos, guayabano); French (corossol, corosselier, corossolier, corossel, corossol épineux, sappadillo, cachiman épineux, cachimantier); German (Sauersack, Stachelannone, stachliger); Indonesian (nangka seberng, sirsak); Javanese (nangka belanda, sirsak); Khmer (tiep banla, tiep barang); Lao (Sino-Tibetan) (khièp thét, khan thalot); Malay (durina makkah, durian benggala, durian belanda); Portuguese (coraçao-derainha, graviola); Spanish (coraçao de Rainha, araticu-ponhé, anona espinhosa, catuche, curassol, graviola, guanábana, jaca do pará, pinha azeda, zapote agrio, jaca de pobre); Swahili (mstafeli); Thai (riannam, thurian-khaek, thurian-thet); Tigrigna (anona); Vietnamese (mang câù xiêm) [20-21].

In beverage Industries, the fruits of Annona muricata are valuable raw materials for producing syrups, candies, beverages, ice creams and shakes [22]. Different ethnomedicinal activities have been attributed to different parts of Annona muricata, and these include antiparasitic, antispasmodic, astringent, anticancer, antihypertensive and hypoglycemic effect [23]. Similarly, different extracts prepared from Annona muricata parts have been documented to elicit diverse pharmacological activities which include antidiabetic [24-25] and hypolipidemic [26], hepatoprotective [27-28], antioxidant [29-34], anticancer [15, 34-36], hypotensive [37], wound healing [15, 38], antiplasmodial [39-41], anti-helminthic [42], anti-stress [43], anti-arthritis [44] and cytoprotective [15, 45] activities. The anticancer activity of Annona muricata has also being confirmed by two independent randomized controlled clinical trials evaluating the efficacy of Annona muricata of a daily oral intake of 5000 mg of powdered leaf and seed of Annona muricata together with other neutraceuticals and lifestyle modification in patients with colon carcinoma. The study showed substantial remission of the colon cancer cells in the treated patients [46]. In another study, 227 g of daily water infusion of Annona muricata leaves and capecitabina (2500 mg) orally administered on the basis of 2 weeks on/1 week off to metastatic breast cancer patients resulted not only in significant clinical improvement but attainment of so tumor markers and no major side-effects after therapy for 5 years [47].

Annona muricata L. is known to contain chemicals such as acetogenins (annomuricins and annonacin), alkaloids (coreximine and reticuline), flavonoids (quercetin), and vitamins, which are predicted to be responsible for the plant's biological activity [48]. However, despite the health benefits attributable to the consumption of Annona

muricata fruit, there are also concerns with the association of the consumption of A. muricata fruit and its homemade with the appearance of atypical Parkinsonism which have been attributed to the presence of the dopaminergic neurotoxins secondary metabolites, annonacin and reticuline, which are the two most abundant acetogenin and alkaloid in A. muricata, respectively [49-54]. Ensuring the safety, quality and effectiveness of medicinal plants and herbal drugs very recently became a topical issue and major concern in both industrialized and developing countries where use of herbal products has attained tremendous growth in recent times. Similarly, standardization as well as evaluation of active plant-derived/herbal drugs has been projected to usher in a new healthcare delivery era in the management of human and veterinary diseases in the nearest future [55].
Keeping in line with this, and fact that there have been cumulative evidence of the extensive consumption of the fresh, ripe fruits of Annona muricata as food, its extensive folkloric medicinal use and dearth of scientific studies on the safety profile of Annona muricata, a gap in the body of knowledge on the safety profile of Annona muricata was observed. This underscores the basis for the current study which seeks to evaluate effects of 42-days of oral administration of 100-400 mg/kg/day of the aqueous fruit extract of Annona muricata (AFAM) on biochemical, hematological and histopathological endpoints in treated Wistar rats.

2. Materials and Methods

2.1. Collection of Plant materials

Mature and ripe Annona muricata fruits were collected from the wild forest within Nsukka Local Government Area of Enugu, Nigeria, in October, 2018. The identification and authentication of Annona muricata was done by Dr. G.I. Nodza, a lecturer in the Department of Botany and Microbiology, University of Lagos, Akoka, Lagos Nigeria and the voucher number with the reference number 8545 (see Figure 1) was deposited in the Departmental Herbarium for referencing purposes.

Figure 1. Photograph of fresh mature fruit, florescence and aerial parts of Annona muricata processed for voucher specimen.

2.2. Cold Aqueous Extraction

The fruit Annona muricata was first sorted and washed under running water to get rid of dirt. The fruit was then peeled and separated from the seeds and the pulp, after which it was cut diced and then seared in an aerated oven preset at 40 °C . After drying, the dried material was milled in a commercial blender to a deep brown fine powder and stored in an air-tight container for later use [56]. As previously described by Adeneye et al. [56], in preparing the cold aqueous fruit extract of Annona muricata, 400 g of the powdered fruit was dissolved in 1 L of distilled water in a 1 L Pyrex beaker and allowed to stand in a refrigerator at 4°C for 48 hours. After 48 hours, the mixture was shaken rigorously and intermittently for 3 hours and then rapidly filtered with a clean white cloth. The filtrate was then completely air-dried in an aerated oven preset at 40 °C to give a dark brown, thick, viscous, solid residue. The resultant residue was scrapped and stored in an air-tight container and kept in a refrigerator at 4 °C for use when required. This process was repeated for two more times. Fresh stock solution of 100 mg/ml of AFAM was prepared whenever needed. It was from this that the equivalent volume of the extract stock solution corresponding to the oral doses of 100 mg/kg, 200 mg/kg and 400 mg/kg of the extract was calculated as described by Adeneye et al. [56].

2.3. Care of Experimental Animals

Forty (40) young adult Wistar rats (aged 8-14 weeks) comprising of 10 females and 30 males were obtained from the Animal House, Lagos State University College of Medicine, Ikeja, Lagos, Nigeria after the required institutional ethical clearance was obtained. The rats were housed in metallic cages with bedding which were cleaned and replaced on daily basis. The rats were sham-handled and maintained at standard laboratory conditions (room temperature of 28 °C - 30 °C , relative humidity of 60-65%, 12 hour/12 hour of light/dark periodicity) [56].

2.4. Acute oral toxicity testing

In vivo acute oral toxicity test was done using the limit dose test of Up-and-Down Procedure according to OECD/OCDE Test guideline on acute oral toxicity, at a limit dose of 5 g/kg body weight/oral route and default of Sigma 0.5 [57]. In conducting the test, ten (10) nulliparous female Wistar rats aged 8-10 weeks were fasted overnight, from which a total of three (3) rats were selected by systematic randomization techniques. A rat was blindly hand-picked, weighed and orally gavaged with the equivalent volume of 5000 mg/kg of AFAM dissolved in distilled water at a time. The dosed rat was then closely observed for the first five minutes for possible signs of regurgitation and then kept under observation for close monitoring for lethality quarter hourly in the first 4 hours, then every half hourly in the successive 6 hours, then daily for the successive 48 hours for the short-term outcome and the remaining 12 days for the long-term outcome. In addition, the treated rat was closely monitored for behavioral manifestations of toxicity (including feeding and drinking pattern, restlessness, itching), neurological toxicity (including body posture, gait), fecal color and consistency). Each rat was monitored for a total of 14 days.

2.5. Sub-Chronic Oral Toxicity of AFAM

Twenty four (24) experimental male rats were randomly allotted into four groups of six rats in each group. Wistar rats in Groups II, III and IV were orally administered with 100 mg/kg/day, 200 mg/kg/day and 400 mg/kg/day of AFAM dissolved in distilled water, respectively, while Group I rats served as the untreated control and were only orally treated with 10 ml/kg/day of distilled water being a standard dose of water given to rodents and used in other studies [56]. The weight of the rats in each group was recorded pre-, mid- and post-experiment. The treatment lasted 42 days after which the rats were starved of food overnight.

2.6. Blood Samples Collection

On the 43th day, the rats were weighed and sacrificed after light inhalational anesthesia with diethyl ether. Blood samples were collected into clean sample bottles following cardiac puncture and assayed for hematological (full blood count) using heparinized sample bottles. Blood sample forbiochemical [(liver functions: liver enzymes (AST, ALT, ALP), total protein, albumin, total and conjugated bilirubin); and renal function: electrolytes (sodium, potassium, bicarbonate), urea and creatinine] were collected into the plain sample bottles and analyzed using procedures previously described by Adeneye et al. [56-57].

2.7. Hematological Assay

Hematological analyses were carried out using a three-part differential Hematological Autoanalyzer, SYSMEX KX-21N. 3-4mls of blood sample was collected into an EDTA-coated bottle and gently but thoroughly mixed so at to avoid coagulation. Approximately 20 µl of the mixed blood sample was aspirated by the auto-analyzer sampling probe and the result of analysis was displayed after about 30 s with a printout copy of the result released on the thermal printing paper. This procedure was repeated for other samples [57].

2.8. Bio-C hemical Analyses

The collected blood samples were collected into plain bottle and allowed to clot and then centrifuged at revolution of 5000 rpm at room temperature, to separate the clear sera from the blood sample [49-50]. Clear sera obtained were analyzed for lipids [high density lipoprotein-cholesterol (HDL-c), low density lipoprotein-cholesterol (LDL-c), very low density lipoprotein (VLDL-c), triglycerides (TRIG), total cholesterol (TC)]; liver enzymes [aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphate (ALP)], total bilirubin (TBil) and conjugated bilirubin (DBil), total protein (TP), albumin (ALB), electrolytes (Na+, K+, Cl-, HCO3-), urea and creatinine. These biochemical assays were conducted using the COBAS C111 Chemistry Auto-analyzer (manufactured by Roche Diagnostics, Rotkreuz, Switzerland). Calibration of the equipment for each analyte was done using

CFAS lipid for lipids and CFAS U for other parameters. Two level control runs were carried out using Percinorm (normal range) and Percipath (pathological range) (Boehringer Mannheim, Mannheim, Germany) as previously described [58]. Tests were carried out on samples by dispensing 200 µl of each sample into pre-labeled sample cup. Each sample cup was transferred into the corresponding slot in the rack accordingly. Clicking on 'ORDER', the sample identity was inputted and the test to be carried out was then selected. The results were then validated and the sample cup removed and replaced for the next run (COBAS C111 Equipment user manual).

2.9. Preliminary Phyto-Chemical Analyses

2.9.1. Preliminary Qualitative Analysis of AFAM

This test was done to determine either the presence or absence of secondary metabolites such as reducing sugar, triterpenoids, phenolics, flavonoids, tannins, alkaloids, and cardiac glycosides using methods earlier described by Adeneye et al. [57].

2.9.2. Preliminary Quantitative Analyses of AFAM

2.9.2.1. Estimation of alkaloids content

0.5 g of AFAM was dissolved in 10 ml of acetic acid solution in 100 ml of ethanol to form a ratio of 1:10 (10% acetic acid solution). The mixture was allowed to stand for 4 hours at 28 °C and later filtered using Whatman No. 42 filter paper. The filtrate was concentrated to one quarter of its original volume by evaporation and treated with drop-wise addition of concentrated aqueous NH4OH until the alkaloid content precipitated. The alkaloid precipitate was received on a weighed filter paper with 1% ammonia solution dried in the oven at 80 °C . Alkaloid content was calculated and expressed as a percentage of the weight of samples analyzed as previously described by Adeneye et al. [56].

2.9.2.2. Estimation of phenolic content in AFAM

AFAM's total phenolic content was determined using the Folin-Ciocalteu's reagent (FCR). Different concentrations of AFAM were mixed with 0.4ml FCR (diluted 1:10 v/v). After 5 minutes, 4 ml of concentrated sodium carbonate solution was added. The final volume of the tubes were made up to 10ml with distilled water and allowed to stand for 90 minutes at the room temperature of 23-25 °C. Absorbance of sample was measured against the blank at 750 nm using a spectrophotometer. A calibration curve was constructed using gallic acid solutions as standard and total phenolic content of the extract were expressed in terms of milligrams of gallic acid per gram of dry weight from the standard graph. The results were derived from the calibration curve equation (Y = 0.001X + 0.0321:R2= 0.9925) of gallic acid (µg/ml) and expressed in mg equivalent (GAE) per gram of dry AFAM.[56]

2.9.2.3. Estimation of total flavonoid content in AFAM

Aluminium chloride colorimetric method with some modifications was used to estimate flavonoid content in AFAM. An aliquant of AlCl3 solution (0.5 ml, 2%) was mixed with 1ml of AFAM test solution and subsequently with was thoroughly mixed with 0.5 ml of water, HCl, CH3COONa or CH3COONH4 (each at concentration of 1 M). The resulting mixture was incubated at room temperature for 15 minutes, after 100 µM of rutin (standard flavonoid) solution was prepared. This was then subjected to spectral analysis at the absorbance of 415 nm. Flavonoid content was expressed in terms of rutin equivalent (mg/g of extract). The results were derived from a calibration curve equation (y = 0.0009x + 0.0262; R2= 0.999) of rutin (µg/ml) and expressed in mg equivalents per gram of dry extract using the equation of y = mx + c, where y = Absorbance, m = slope, c = intersection, x = standard concentration [59].

2.9.2.4. Estimation of total tannin content in AFAM

The total tannin content in AFAM was determined using Folins-Ciocalteu spectrophotometric method. 0.1 ml of 100 mg/ml of AFAM was dispensed into 50 ml volumetric flask already containing 7.5 ml of distilled water and 0.5 ml of Folin-Ciocalteu phenol reagent, 1 ml of 35% sodium bicarbonate solution and diluted to 10 ml with distilled water. The mixture was rigorously shaken and incubated at the room temperature for 30 minutes with different concentrations (20 µg/ml, 40 µg/ml, 60 µg/ml, 80 µg/ml, and 100 µg/ml) of the standard solutions of tannic acid. The absorbance of the developed colour was measured at 760 nm wavelength with the reagent blank set at zero. The experiment was repeated two more times to get an average. Estimated tannin contents were derived from the calibration curve equation (y = 0.001x +

0.0321: R2=0.9925) of tannic acid (µg/ml) and expressed in mg/g of the dry extract [60].

2.9.2.5. Estimation of total anthraquinone glycosides content

The anthraquinone content was spectro-photometrically quantified in duplicates, after a suitable pre-treatment. A volume of 0.5 ml of ethyl acetate fraction was alkalinized with 50 mg of NaHCO3 and oxidized with an aqueous solution of 20 ml of 10.5% FeC13. The mixture was boiled under reflux for 5 minutes after which 1ml of concentrated HCI was added and the reaction medium was kept under the same condition for 20 more minutes, at room temperature. The mixture was partitioned with diethy1 ether three minutes. The ether phase was transferred into a 100 ml volumetric flask and the final volume was completed with this organic solvent to obtain the stock solution. Then, 10 ml of the stock solution was evaporated to dryness on a water bath (60 °C). The residual solid was dissolved in 10 ml of 0.5% magnesium acetate as methanol solution. The absorbance was analyzed at 515 nm with an Ultraviolet-visible (UV –Vis) spectrophotometer. 0.005 - 0.06 mg/ml of same analytical standard solutions of 1, 8-dihydroxyanthraquinone in ether was similarly evaporated and treated with 0.5% magnesium acetate in methanol to achieve the analytical curve. The methanol solution was used as a control. Results were expressed as milligrams of hydroxy1-anthracene derivatives per 100 g of the dry sample [61].

2.9.2.6. Estimation of total saponin content in AFAM

Estimated total saponin content was determined by vanillin reagent using diosgenin as the standard drug as described by Jain and Shrivastava [62]. 1 g of AFAM was dissolved in 10 ml of 80% methanol and to which 2 ml of vanillin in ethanol was subsequently added. This was thoroughly mixed following which 2 ml of 72% (v/v) tetraoxosulphate (VI) acid was added. The content was thoroughly mixed and heated up on a water bath preset at 60 °C for 10 min. Absorbance was measured at 544 nm against reagent blank. Diosgenin was used as a standard material and results were expressed as diosgenin equivalents. The results were derived from a calibration curve equation (y = 0.001x + 0.0321: R2=0.9925) of saponic acid (µg/ml) and expressed in mg equivalent of diosgenin.

2.9.2.7. Quantitative estimation of steroids in AFAM

1 ml of AFAM solution was pipetted into 10 ml volumetric flasks and 2 ml of 4N tetraoxosulphate (VI) acid and 2 ml of 0.5% w/v iron (III) chloride were added to it. 0.5 ml of 0.5% w/v potassium hexacyanoferrate (III) solution was subsequently added. The mixture was heated in a water-bath maintained at 70 ± 2 °C for 30 minutes with occasional shaking and diluted to the mark with distilled water. The absorbance was measured at 780 nm against the reagent blank. The results were derived from a calibration curve equation (y = 0.0009x + 0.0262; R2= 0.999) of cholesterol in (µg/ml) and expressed in cholesterol per gram of dry extract.

2.9.2.8. Determination of terpenoids content in AFAM

2 g of AFAM was weighed and dissolved in 50 ml of 95% (v/v) ethanol for 24 hours. The extract was filtered after which it was extracted with petroleum ether at 60-80 °C and later concentrated to dryness. The dried ether extract was treated as total terpenoids [63].

2.9.2.9. Estimation of cardiac glycosides content in AFAM

The cardiac glycosides content of AFAM was determined using the method described by Solich et al. [64] with some modifications. In doing this, a 10% solution of AFAM was mixed with 100 ml freshly prepared Baljet's reagent (95 ml of 1% picric acid + 5 ml of 10% v/v NaOH). After an hour, the mixture was diluted with 20 ml distilled water and the absorbance was measured at 495 nm by UV-Vis spectrophotometer model. For preparation of the standard curve, 10 ml of different concentrations (12.5-100 mg/l) of digoxin was used. Total glycosides from triple replicates were expressed as mg of digoxin per gram of dried extracts.

2.10. Proximate Analyses of AFAM

The proximate analyses were done using the standard method57 and all values were expressed in percentage.

2.10.1. Estimation of Moisture Content of AFAM

2 g of AFAM was taken in a pre-weight 50 ml size porcelain crucible and kept in a digital oven preset at 105 °C for 5 hours. The crucible was then removed for the oven and kept in a desiccator to cool at room temperature and weighed. The

procedure was repeated until a constant weight was attained. The percentage loss in weight of the AFAM was calculated.

2.10.2. Estimation of the Total Ash Content in AFAM

2 g of AFAM was taken into a previously ignited and tarred silica dish. The material was carefully spread evenly and ignited in a muffle furnace by gradually increasing the temperature to 500 °C until it glows white, indicating the absence of carbon. The dish was then cooled in desiccator and weighed and the percentage total ash of air dried material was then calculated.

2.10.3. Estimation of Crude Protein Content in AFAM

This was done using Kjeldahl method which involves a three-step approach of digestion, distillation and titration and described by Maehre et al. [65]. 2 g of AFAM was weighed and placed in digestion flask. 15 g Na2SO4, 1g CuSO4, one or two selenized boiling granules and 25 ml of concentrated H2SO4 was added sequentially to the flask and then digested until solution is almost colorless or light green (2 hours for inorganic material). It was later left to stand for at least a further 30 minutes. The mixture was allowed to cool without solidifying, following which 200 ml of distilled water was carefully added. 100 ml of 0.1 N HCl was slowly pipetted into a 500 ml Erlenmeyer flask. 1 ml Conway's indicator was then added and the flask was placed under the condenser and ensuring that the condenser tip was immersed in the acidic solution. The Kjeldahl flask containing the digested sample was tilted, and 100 ml of 50% NaOH solution slowly added, down the side of the Kjeldahl flask so that it forms a layer underneath the digestion mixture. The flask was immediately connected to the distilling bulb of the distillation apparatus and the flask was rotated to thoroughly mix contents. The content was then heated until all ammonia has passed over into the standard acid. The flask immediately removes to avoid bumping. The tip of the condenser was washed and the excess standard HCl in the distillate was titrated against NaOH standard solution. The percentage nitrogen was calculated as:

$$\%nitrogen\ (wet) = \left[\frac{(A-B)\times 1.4007}{Sample\ weight\ (g)}\right]\times 100 \quad \ldots(1)$$

where

$A = Volume\ (ml) standard\ HCl \times normality\ (HCl)$

and

$$B = Volume\ (ml) standard\ NaOH \times normality\ (NaOH)$$

The Nitrogen content on dry basis (when moisture content is known) was calculated as follows:

$$\%nitrogen\ (dry) = \left[\frac{\%\ nitrogen\ (wet)}{(100-\%moisture)}\right]\times 100 \quad \ldots(2)$$

Similarly, the percentage protein (wet or dry basis) was calculated as:

$$\%protien = \%nitrogen \times 6.25$$

where the 6.25 is the protein-nitrogen conversion factor for fish and fish by-products.

2.10.4. Estimation of Crude Lipids in AFAM

5 g of AFAM was dissolved and extracted with diethyl ether to dissolve fats, oils, pigments and other fat soluble substances contained in AFAM. The ether filtrate was then evaporated from the fat solution. The resulting residue was weighed and referred to as the crude fat. The percentage crude fat (ether extract) was calculated as:

$$\%crude\ fat\ (wet) = \frac{(W_{res.} - W_{ta.})}{sample\ weight\ (g)}\times 100 \quad \ldots(3)$$

where W_{ta} = tare weight of beaker (g) and $W_{res.}$ = weight of beaker and fat residue (g).

2.10.5. Estimation of Crude Fibers in AFAM

1 g of AFAM was weighed out and 150 ml of 1.25% tetraoxosulphate (VI) acid was added into a beaker and heated for 30 minutes. After preheating on a hot plate, 3-5 drops of n-octanol (antifoam agent) was added, boiled for 30 minutes and the tetraoxosulphate (VI) acid was drained using a vacuum system. This was later washed three times with 30 ml (crucible filled up to the top) of hot deionized water, connecting each time to compressed air for stirring the content in the crucible. After draining the last wash, 150 ml of preheated 1.25% potassium hydroxide (KOH) and 3-5 drops of antifoam was added. It was boiled for 30 minutes, filtered and washed with cold deionized water aimed to cool the crucibles and then washed three times (the crucible content with 25 ml of acetone, stirring each time by compressed air). The crucibles were removed and the dry

weight was determined after drying in an oven at 105 °C for an hour and then let cool in a desiccator. This weight (W2) represents the crude fiber plus ash content in comparison to initial weight (W1). Calculation of the percentage crude fiber (wet weight basis) as follows:

$$\%crude\ fiber\ (wet) = \left[\frac{(W_2 - W_1)}{W_1}\right] \times 100 \quad \ldots (4)$$

2.10.6. Estimation of Nitrogen-Free Extract (Digestible Carbohydrates)

This represents the soluble carbohydrate of the feed, such as starch and sugar while crude fiber represents insoluble carbohydrate.

% NFE = % DM - (% EE + % CP + % ash + % CF)

where NFE = nitrogen free extract, DM = dry matter, EE = ether extract or crude lipid, CP = crude protein, and CF = crude fiber

2.11. Gas Chromatography-Mass Spectro-Photometric Analyses of AFAM

This spectral study was performed using 7820A gas chromatograph coupled to 5975C inert mass spectrometer (with triple axis detector) and electron impact source (Agilent Technologies).

2.11.1. Column

The stationary phase of separation of the various compounds in AFAM was carried out on HP-5 capillary column coated with 5% v/v of phenylmethylsiloxane (30 m length × 0.32 mm diameter × 0.25 µm film thickness) (Agilent Technologies).

2.11.2. Carrier Gas

The carrier gas, helium, was used at a constant flow rate of 1.573 ml/min, an initial nominal pressure of 1.9514 psi and at an average velocity of 46 cm/s. 1 µl of AFAM solution was injected in split less mode at an injection temperature of 260 °C. Purge flow was 21.5 ml/min at 0.50 min with a total gas flow rate of 23.355 ml/min and was subsequently placed in the gas saver mode.

2.11.3. Oven

The oven was initially programmed at 60 °C for 1 minute, then ramped at 4 °C/min to 110 °C for 3 minutes, followed by temperature program rates of 8 °C/min to 260 °C for 5 minutes and 10 °C/min to 300 °C for 12 minutes with a run time of 56.25 min and 3 minutes solvent delay.

2.11.4. Mass spectrometer

This was operated in electron-impact ionization mode at 70 eV with ion source temperature of 230 °C, quadrupole temperature of 150 °C and transfer line temperature of 280 °C. Scanning for possible compounds in AFAM was from 30 to 550 amu at 2.62 s/scan, and identified by comparing measured mass spectral data with those in NIST 14 Mass Spectral Library.

2.12. Histopathological studies of vital organs

After sacrificing, vital body organs such as kidney, liver, stomach, heart, lungs, spleen and testis were identified, meticulously dissected out en bloc and preserved in a 10% formo-saline. The preserved organs were later dehydrated in absolute ethanol and the organs were embedded in paraffin blocks. From the paraffin blocks, 5µm thick sections of each tissue was prepared and stained with haematoxylin-eosin. The prepared slides were further examined under photomicroscope which was connected to host computer. The sections were illuminated with white light from 12V halogen lamp (100W) after filtering with a 520nm monochromatic filter. The slides were read and reported for associated histopathological lesions by an associate professor and consultant histopathologist, Dr. S.S. Soyemi in the Department of Pathology and Forensic Medicine, Lagos State University College of Medicine, Ikeja, Lagos State, Nigeria using a previously described procedure [66].

2.13. Statistical Analyses

Data were expressed as mean ± SEM of six (6) rats per group of four (4) groups in all. The statistical analyses was done using GraphPad Prism version 8.0.

3. Results and Discussion

3.1. Aqueous extraction of AFAM and calculation of %Yield

Complete extraction and in vacuo drying of AFAM produced a sweet smelling, sticky, deep brown solid residue with a percentage yield of 25.42 ±2.07 %. This is an indication of a good partitioning of the secondary metabolites of Annona muricata into water and ultimately yield of the extract from the original wet fruit material that was extracted through the water extraction process.

3.2. Gas Chromatography and Mass Spectrophotometry of AFAM

Presence and relative abundance of AFAM's secondary metabolites are indicated in Table 1 and Figure 2. AFAM contains secondary metabolites such as 5-hydroxymethyl furfural (29.1%), 4-hydroxyl pyran-4-one (9.3%), 2-furancarboxyl-aldehyde (4.9%), 1,3 dimethylimidazole-2(3H)-thione (4.1%), n-hexanedioic acid (4.0%), acetic acid (3.3%), 4-pyridinol (3.0%), 9-octadecanoic acid (2.3%), ethanol (1.5%), n-hexadecanoic acid (1.5%), furfural (1.3%), 2(3H)furanone (1.1%), dodecanoic acid (1.1%). Many of these identified secondary metabolites were at variance with those earlier identified from the ethanol leaf extract of Annona muricata which was reported to contain 7-tetradecenal, (Z), n-hexadecanoic acid, oleyl alcohol, phytol, cis-7,10,13-hexadecatrienal, 2-pentadecanol, 9,12-octadecadienoic acid, ethyl ester, 1,2-benzenedicarboxylic acid, butyloctyl ester, and 1,E11,Z-13-octadecatriene [67].

Table 1. Gas Chromatography and Mass Spectrophotometry study showing the secondary metabolites and their relative abundance in AFAM

S/N	Sample ID	Retention Time	% Total	Compound Name
1.	*Annona muricata* aqueous fruit extract (*AFAM*)	3.566	1.258	Furfural
		3.895	3.306	Acetic acid
		4.207	1.081	2(3H)furanone
		5.143	4.858	2-furancarboxaldehyde
		6.858	4.145	1,3-dimethylimidazole-2(3H)-thione
		7.851	7.8	4H-Pyran-4-one
		8.325	1.549	4H-Pyran-4-one
		9.22	29.114	5-hydroxymethylfurfural
		13.708	3.075	4-Pyridinol
		13.916	1.059	Dodecanoic Acid
		14.112	1.489	Ethanol
		15.677	1.489	n- Hexadecanoic Acid
		17.028	2.313	9-octadecanoic Acid
		18.83	4.023	Hexanedioic Acid

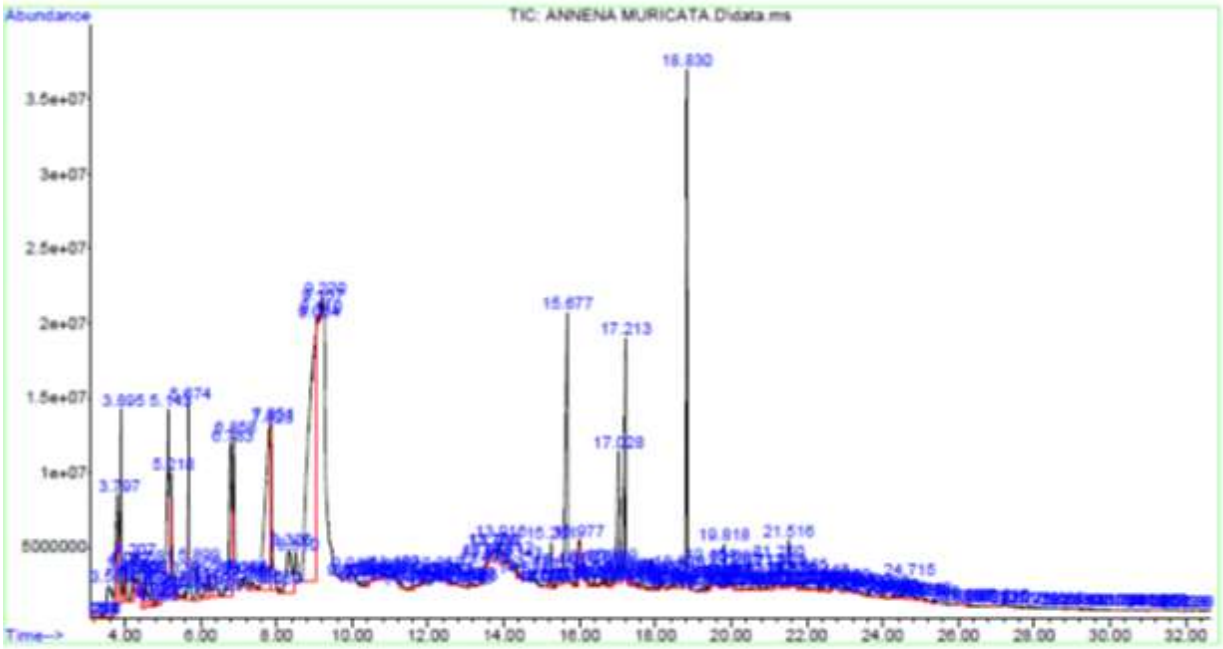

Figure 2. Gas Chromatography-Mass Spectrometry fingerprint of the secondary metabolites and their relative abundance in AFAM.

3.3. Preliminary Qualitative and Quantitative Phytochemical Analysis of AFAM

Preliminary qualitative phytochemical analysis of AFAM done showed that the presence of alkaloids, saponin, reducing sugar, cardiac and steroidal

glycosides, tannin, flavonoids and terpenoids. This result is in tandem with those earlier reported by Agu and Okolie [68], and Hasmila et al. [69]. However, relative amount of the major secondary metabolites such as reducing sugars (307.30 ±0.05 mg/100 g), tannins (183.40 ± 1.06 mg/100 g), flavonoids (148.40 ± 1.00 mg/100 g), phenolic compounds (87.95 ± 0.54 mg/100 g), alkaloids (40.30 ± 0.03 mg/100 g), steroids (29.61 ± 0.83 mg/100 g), saponins (18.61 ± 1.52 mg/100 g), and cardiac glycosides (3.77 ± 0.02 mg/100 g) (Table 2). Again, this result is in complete agreement with that earlier reported by Onuah et al. [70].

Table 2. Preliminary Quantitative Analysis of AFAM

Secondary metabolites	Relative amount (expressed in mean ± S.E.M)
Alkaloids	40.30 ± 0.03 (mg/100 g of dry extract)
Saponins	18.61 ± 1.52 (mg/100 g of dry extract)
Reducing Sugar	307.30 ± 0.50 (mg/100 g of glucose)
Cardiac glycosides	3.77 ± 0.02 (mg/100 g of digoxin)
Steroids (mg/100 g of cholesterol)	29.61 ± 0.83 (mg/100 g of cholesterol)
Tannins (mg/100g of tannic acid)	183.40 ± 1.06 (mg/100 g of tannic acid)
Phenolic compound	87.95 ± 0.54 (mg gallic acid equivalent/100 g of dry extract)
Flavonoids	148.40 ± 1.00 (mg quercetin equivalent/100 g of dry extract)

3.4. Proximate Composition of AFAM

Proximate analyses of AFAM showed it contain 8.07% moisture, 2.14% crude protein, 6.73% ash, 4.19% crude fiber, 14.22% fat, and 64.65% carbohydrate (Table 3). This is in sharp contrast to the result of similar proximate analysis done in Anonna muricata seed that was reported to contain 8.5% moisture, 2.4% crude protein, 13.6% ash, 8.0% crude fiber, 20.5% fat, 47.0% carbohydrate, 0.2% water soluble ash, 0.79% titratable acidity and 17.0 mg calcium/100 g. Similarly, Anonna muricata pulp's content was found to be 81% moisture, 3.43% titratable acidity and 24.5% non-reducing sugar, results that similar to that of a previous study [64].

Table 3. Preliminary composite analysis of AFAM

S/N	Primary Metabolites	% Composition
1	Moisture content	8.07
2	Ash value	6.73
3	Protein	2.14
4	Lipid	14.22
5	Carbohydrate	64.65
6	Fiber	4.19

3.5. Acute oral toxicity testing in Wistar rats

Acute oral administration of 5000 mg/kg of AFAM to 3 sequentially treated female Wistar rats using 2001 OECD Guideline on Acute Oral Toxicity testing did not cause lethality in any of the treated rats although the extract administration caused initial restlessness which lasted for 3-4 hours but followed by calmness afterwards. Other observed toxicity signs include generalized body itching and piloerection. The sequence, short-term and long-term outcomes of the limit dose test of AFAM are as indicated in Table 4. According to Jothy et al. [71] substances with LD50 of 1 g/kg body weight/oral route are considered either safe or of low toxicity. In this present study, although the exact LD50 value was not determined but its computer generated estimated value was greater than 5 g/kg body weight/oral route indicating the low oral toxicity of AFAM.

Table 4. Sequence and result of limit dose test of Up-And-Down Procedure for AFAM in rats

Sequence	Animal Identification	Dose (mg/kg)	Short-term outcome (in the first 48 hours)	Long-term outcome (in the successive 12 days)
1.	01	5000	survived	Survived
2.	02	5000	survived	Survived
3.	03	5000	survived	Survived

3.6. Effect of prolonged oral treatment with 100-400 mg/kg/day of AFAM on the average body weight gain pattern and relative organ weights of treated rats

Repeated daily oral dosing with 100-400 mg/kg of AFAM was not associated with significant changes

in the average body weight gain pattern in the treatment groups (Groups II-IV) when compared with that of the control (Group I) on days 21 and 42 (Table 5). However, when compared to the initial average weights, there was profound weight gain on day 42 (Table 5). Conversely, repeated daily oral dosing with 100-400 mg/kg of AFAM was not associated with significant changes in the average relative organ weights in the vital organs of treated rats (Groups II-IV) when compared with that of the control (Group I) (Table 6). The % weight change increase could be due to high carbohydrate content of AFAM. An earlier study by Omotosho et al. [72] has equally reported high carbohydrate content (27.23%) through the composite analysis of the root extract of Annona muricata. Also, it is plausible that AFAM could also be appetite-stimulating in its activity, thus removing caloric restriction barrier, although the feeding pattern of AFAM-treated rats was not evaluated in this study.

Table 5. Effect of the administration of 100-400 mg/kg/day of AFAM on the average body weight gain pattern of treated rats

Groups	Average body weight (g) on:			%change in body weight on:	
	Day 1	Day 21	Day 42	Day 21	Day 42
I	143.90 ±04.70	179.60 ±08.70	211.90 ±14.91	24.0 ±05.43	46.66 ±09.59[a+]
II	156.80 ±24.85	196.20 ±15.47	225.30 ±13.79	26.48 ±15.47	51.18 ±20.79 [b+]
III	166.50 ±25.01	200.10 ±17.25	223.30 ±20.82	21.42 ±13.28	35.33 ±10.96 [a+]
IV	168.50 ±13.00	202.30 ±17.47	229.30 ±22.78	19.95 ±03.82	35.88 ±05.65 [a+]

a+ and b+ represent significant increases at $p<0.05$ and $p<0.001$ respectively when compared to respectively weight values on Days and 21; I = orally gavaged10 ml/kg/day of distilled water, II = orally gavaged 100 mg/kg/day of AFAM, III = orally gavaged 200 mg/kg/day of AFAM, IV = orally gavaged 400 mg/kg/day of AFAM

Table 6. Effect of the administration of 100-400 mg/kg/day of *AFAM* on the average relative organ weight of treated rats

	Treatment Groups			
	I	II	III	IV
Kidney	0.68 ± 0.07	0.69 ± 0.05	0.67 ± 0.11	0.69 ± 0.06
Liver	3.03 ± 0.25	3.26 ± 0.44	2.91 ± 0.30	3.08 ± 0.37
Lungs	0.70 ± 0.16	0.85 ± 0.17	0.83 ± 0.09	0.89 ± 0.14
Heart	0.33 ± 0.04	0.42 ± 0.18	0.28 ± 0.05	0.32 ± 0.05
Stomach	0.82 ± 0.10	0.72 ± 0.11	0.89 ± 0.11	0.82 ± 0.07
Spleen	0.37 ± 0.03	0.32 ± 0.05	0.32 ± 0.05	0.32 ± 0.06
Testis	2.34 ± 0.15	2.22 ± 0.23	2.03 ± 0.13	2.24 ± 0.29

I = orally gavaged 10 ml/kg/day of distilled water, II = orally gavaged 100 mg/kg/day of *AFAM* dissolved in distilled water, III = orally gavaged 200 mg/kg/day of *AFAM* dissolved in distilled water, IV = orally gavaged 400 mg/kg/day of AFAM dissolved in distilled water

3.7. Effect of prolonged oral treatment with 100-400 mg/kg/day of AFAM on serum lipids and liver function parameters

Repeated daily oral dosing with 100-400 mg/kg of AFAM caused significant ($p<0.05$) decreases in the serum triglyceride (TG) and VLDL-c levels while it caused no alterations in the serum TC, HDL-c and LDL-c levels when compared to the control (Group I) values (Table 7). Similarly, prolonged AFAM treatment caused non-significant ($p>0.05$) reductions in the serum levels of ALT and AST especially at the oral doses of 100 and 400 mg/kg/day while causing unappreciable alterations in the serum ALP (Table 8), total protein, albumin, total bilirubin and conjugated bilirubin levels (Table 9). The fact that AFAM treatment profoundly lowered the serum lipids especially triglyceride and VLDL-c hypolipidemia suggests that AFAM has hypolipidemic tendencies. The fact that there is an associated hepatosteatosis as indicated by the histological findings of AFAM-treated hepatic tissue at 400 mg/kg/day dose (Figure 3b) when compared normal hepatic histoarchitecture (Figure 3a) suggests that observed AFAM's hypolipidemic effect is probably mediated via enhanced mobilization of serum lipids and their deposition in the hepatic tissue or enhanced intrahepatic de novo triglyceride biosynthesis. However, the histological findings of hepatic steatosis appear to suggest possible mobilization of circulating lipids and their deposition in the hepatocytes or hepatic injury. The latter appear non-plausible as the key liver

enzymes (ALT and AST) were profoundly reduced by AFAM treatment (Table 8). Evaluation of the hepatic lipase activities in the liver could offer explanations to this observed 'phenomenon' in the nearest future. Hypertriglyceridemia is a well-known factor in the development of ischemic heart disease [73] and atherosclerosis [74-75]. The fact that AFAM lowered the circulating triglyceride level suggests that AFAM could possess cardioprotective potential. It is already documented that many toxic compounds accumulate in the liver where they are detoxified [71]. Plant parts such as fruit, leaves or bark can contain alkaloids, tannins or other compound that have toxic effect in various animal species [76]. The study of the liver function tests may prove useful in this study, especially the toxic effects of the extract on the liver.

Table 7. Effects of the oral administration of 100-400 mg/kg/day of *AFAM* on serum lipids

Group	TG (mmol/l)	TC (mmol/l)	HDL-c (mmol/l)	LDL-c (mmol/l)	VLDL-c (mmol/l)
I	1.59±0.16	2.41 ±0.09	1.10 ±0.09	0.61 ±0.06	0.71 ±0.07
II	1.00±0.16*	2.10 ±0.23	0.93 ±0.13	0.73 ±0.12	0.46 ±0.07*
III	1.09±0.08*	2.35 ±0.14	1.00 ±0.09	0.69 ±0.04	0.50 ±0.03*
IV	1.06±0.04*	2.28 ±0.17	1.27 ±0.09	0.58 ±0.09	0.48 ±0.02*

*represents a significant decrease at $p<0.05$ when compared to the untreated control (Group I) values;
I = orally gavaged 10 ml/kg/day of distilled water,
II = orally gavaged 100 mg/kg/day of *AFAM*,
III = orally gavaged 200 mg/kg/day of *AFAM*,
IV = orally gavaged 400 mg/kg/day of *AFAM*

The inability of AFAM to show any of these effects on the liver enzymes indicates that the extract is non-toxic on the hepatocytes and as such could be considered relatively safe, though it must be taken with caution due to the observed hepatic steatosis. However, the observed toxicity could be attributed to the presence of active secondary metabolites in their respective proportions in AFAM.

Table 8. Effects of the administration of 100-400 mg/kg/day of *AFAM* on serum liver enzymes levels

Group	ATS (U/L)	ALT (U/L)	ALP (U/L)
I	451.7 ± 47.33	95.62 ± 11.20	277.20 ± 14.71
II	435.5 ± 31.66	77.52 ± 9.99*	280.00 ± 12.60
III	488.10 ± 50.70	88.50 ± 8.24	317.30 ± 22.18**
IV	419.70 ± 60.34*	84.90 ± 8.69	286.30 ± 28.20

*and ** represent significant decrease and increase at $p<0.05$, respectively, when compared to the untreated control (Group I) values;
I = orally gavaged 10 ml/kg/day of distilled water,
II = orally gavaged 100 mg/kg/day of *AFAM*,
III = orally gavaged 200 mg/kg/day of *AFAM*,
IV = orally gavaged 400 mg/kg/day of *AFAM*

Table 9. Effects of the administration of 100-400 mg/kg/day of *AFAM* on serum total bilirubin (TBIL), conjugated bilirubin (DBIL), total protein (TPROT) and albumin (ALB) in treated rats

Group	TBIL (µmol/l)	DBIL (µmol/l)	TPROT (g/l)	ALB (g/l)
I	21.23 ±1.72	12.91 ±1.54	61.02 ±2.19	34.27 ±0.74
II	20.27 ±1.64	12.33 ±0.67	61.30 ±2.25	32.13 ±1.88
III	23.46 ±2.14	15.59 ±1.20	60.42 ±1.35	34.32 ±0.53
IV	19.0 ±2.70	13.40 ±2.13	63.08 ±1.24	37.33 ±0.80

I = orally gavaged 10 ml/kg/day of distilled water,
II = orally gavaged 100 mg/kg/day *AFAM*,
III = orally gavaged 200 mg/kg/day of *AFAM*,
IV = orally gavaged 400 mg/kg/day *AFAM*
TBIL = total bilirubin; DBIL = direct bilirubin; TPROT = total protein; ALB = albumin

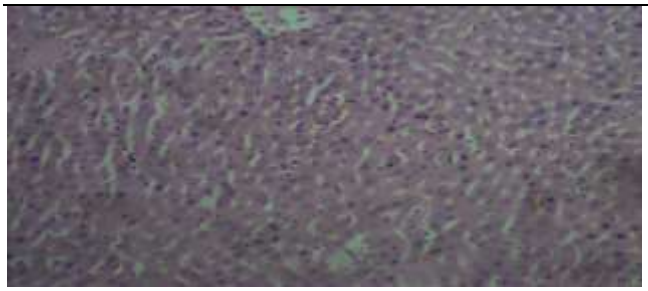

Figure 3a. Photomicrograph of the cross-section of normal rat liver showing normal cytoarchitecture (x100 magnification, Hematoxylin & Eosin stain)

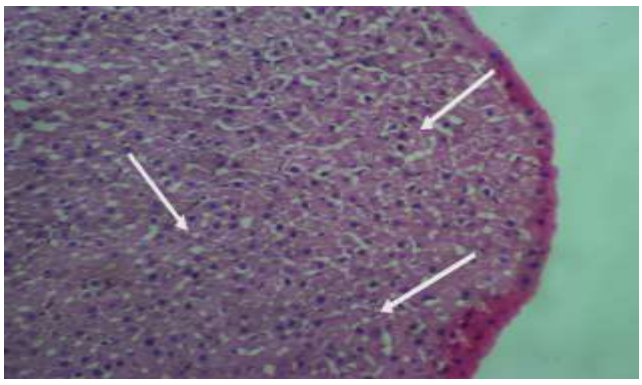

Figure 3b. Photomicrograph of the cross-section of 400 mg/kg/day of AFAM-treated rat liver showing mild fatty degeneration of peripherally located hepatocytes (hepatosteatosis) (x100 magnification, Hematoxylin & Eosin stain)

3.8. Effect of prolonged oral treatment with 100-400 mg/kg/day of AFAM on serum electrolytes (Na+, K+, Cl- and HCO3-), urea and creatinine levels in treated rats

Repeated daily oral treatments with 100-400 mg/kg/day of AFAM caused non-significant ($p>0.05$) increases in the serum K+ and Cl- levels while causing insignificant alterations in the serum Na+ and HCO3- when compared to the untreated control (Group I) values (Table 10). However, prolonged AFAM treatment caused a profound increase ($p<0.05$) in the serum urea levels at the oral dose of 400 mg/kg/day of the extract without any significant ($p>0.05$) alterations in the serum creatinine levels when compared to the untreated control (Group I) values (Table 11). The AFAM-induced alteration in the renal function parameters was associated with collapse of the collecting ducts and lumen of the treated rat kidneys histologically (Figure 4b) when compared to normal (Figure 4a). However, the histoarchitecture of the 100 and 200 mg/kg/day of AFAM-treated kidneys were essentially normal.

Table 10. Effects of the administration of 100-400 mg/kg/day of *AFAM* on serum electrolytes in treated rats

Group	Na^+ (mmol/L)	K^+ (mmol/L)	Cl^- (mmol/L)	HCO_3^- (mmol/L)
I	138.10 ±0.59	06.98 ±0.38	94.90 ±0.42	16.77 ±0.91
II	141.30 ±1.33	08.13 ±0.46	98.32 ±1.36	16.58 ±1.38
III	141.60 ±1.57	07.30 ±0.30	96.75 ±1.44	14.97 ±2.02
IV	137.10 ±0.80	06.29 ±0.24	95.43 ±0.66	17.77 ±1.39

I = orally gavaged 10 ml/kg/day of distilled water
II = orally gavaged 100 mg/kg/day of *AFAM*
III = orally gavaged 200 mg/kg/day of *AFAM*
IV = orally gavaged 400 mg/kg/day of *AFAM*
Na^+ = sodium ion; K^+ = potassium ion; Cl^- = chloride ion; HCO_3^- = bicarbonate ion

Table 11. Effects of the administration of 100-400 mg/kg/day of *AFAM* on serum urea and creatinine in treated rats

	Group	Treatment	Urea (mmol/L)	Creatinine (µmol/L)
I	orally gavaged	10 ml/kg/day distilled water	7.11 ±0.31	46.68 ±2.99
II	orally gavaged	100 mg/kg/day *AFAM*	7.54 ±0.31	55.90 ±6.24
III	orally gavaged	200 mg/kg/day *AFAM*	8.78 ±0.29	40.87 ±4.34
IV	orally gavaged	400 mg/kg/day *AFAM*	10.09 ±0.95**	40.80 ±1.30

*represents a significant increase at $p<0.05$ when compared to the untreated control (Group I) values

Chronic kidney disease is often indicated by decreased urinary excretion of creatinine and urea resulting in hypercreatininemia and

hyperuricemia, respectively which may both individually or jointly contribute to progression to chronic kidney disease [77-78]. Thus, it is plausible that chronic consumption of AFAM may result in hyperuricemia thereby resulting in chronic kidney disease over time. However, this hypothesis may be nullified by the non-alterations in the serum levels of other kidney function parameters especially serum electrolytes (sodium, potassium, bicarbonate) and serum creatinine. It is also possible for the observed hyperuricemia to be due to dehydration although the fluid intake/urinary output were not evaluated in this study. Dehydration is known to be associated with acute renal dysfunction and recently considered a major risk factor for chronic kidney disease [79]. Mechanisms of dehydration-induced chronic kidney disease include increased vasopressin release, cortical aldose reductase activation and stimulation of the central sympathetic nervous system [79-80]. Another possibility is increased catabolism of protein which was not evaluated in this study.

Figure 4a. Photomicrograph of the cross-section of normal rat kidney showing normal cytoarchitecture (x40 magnification, Hematoxylin & Eosin stain)

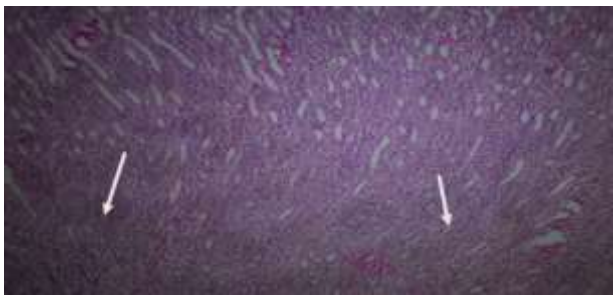

Figure 4b. Photomicrograph of the cross-section of 400 mg/kg/day of AFAM-treated rat kidney showing collapsed collecting ducts and lumen (indicated in white solid arrow) (x40 magnification, Hematoxylin & Eosin stain)

3.9. Effect of prolonged oral treatment with 100-400 mg/kg/day of AFAM on complete blood count of treated rats

Repeated daily oral treatments with 100-200 mg/kg/day of AFAM caused non-significant ($p>0.05$) increases in the platelet (PLT) count, % lymphocyte (LYM) and % mixed cell count (MXD) and non-significant ($p>0.05$) decrease in %neutrophils (NEUT). However, at 400 mg/kg/dose of the extract, there was a significant ($p<0.05$) increase and decrease in the platelet count and differential neutrophil count, respectively, when compared to the untreated control (Group I) values (Tables 12a & 12b).

Table 12a. Effect of prolonged oral treatment with 100-400 mg/kg/day of *AFAM* on White blood cell (WBC), Red blood cell (RBC), Hemoglobin (Hb), Packed cell volume (PCV), Mixed corpuscular volume (MCV) and Mean corpuscular haemoglobin (MCH) in treated rats

Group	WBC (µ/L)	RBC (µ/L)	Hb (g/L)	PCV (%)	MCV (fl)	MCH (pg)
I	12.25 ±1.88	8.68 ±0.16	14.33 ±0.22	51.18 ±1.28	58.98 ±0.73	16.55 ±0.09
II	11.48 ±0.79	8.70 ±0.10	14.40 ±0.18	51.10 ±0.93	58.82 ±0.81	16.60 ±0.25
III	11.85 ±0.80	8.81 ±1.20	14.13 ±0.24	49.72 ±1.05	56.48 ±0.63	16.07 ±0.20
IV	12.18 ±0.67	8.87 ±0.87	14.56 ±0.14	50.58 ±0.38	57.10 ±0.96	16.57 ±0.17

I = orally gavaged 10 ml/kg/day of distilled water
II = orally gavaged 100 mg/kg/day of *AFAM*
III = orally gavaged 200 mg/kg/day of *AFAM*
IV = orally gavaged 400 mg/kg/day of *AFAM*
WBC = total white blood cell count; RBC = total red blood cell count; Hb = hemoglobic concentration; PCV = packed cell volume; MCV = mean corpuscular volume; MCH = mean corpuscular hemoglobin concentration

Table 12b. Effect of prolonged oral treatment with 100-400 mg/kg/day of *AFAM* on the mean corpuscular hemoglobin concentration (MCHC), Platelet (PLT) and differential blood counts %lymphocytes (LYM), %mixed cell count (MXD) and %neutrophils (NEUT) in treated rats

Group	MCHC (g/dL)	PLT (µL)	LYM (%)	MXD (%)	NEUT (%)
I	29.72± 1.52	882.2 ±94.2	77.13 ±2.44	9.63 ±1.93	13.23 ±1.31
II	28.22 ±0.50	990.5 ±55.57	78.95 ±4.89	10.30 ±3.29	9.57 ±1.94
III	28.43 ±0.27	841.8 ±49.71	80.47 ±3.59	10.60 ±2.16	8.93 ±2.19
IV	29.05	931.8±	80.77	12.27	6.97

	±0.33	66.88**	±3.74	±2.81	±1.09*

* and ** represent significant decrease and increase, respectively, at $p<0.05$ when compared to the untreated control (Group I) values,
I = orally gavaged 10 ml/kg/day of distilled water, II = Administered 100 mg/kg/day of *AFAM*, III = orally gavaged 200 mg/kg/day of *AFAM*, IV = orally gavaged 400 mg/kg/day of *AFAM*

Blood is generally considered an important indicator of physiological and pathological status in man and animals and its parameters include hematocrits, red blood counts, white blood cell, platelet count and their differentials [57]. In this study, oral treatment at 100 mg/kg/day and 200 mg/kg/day for 42 days was generally safe on the hematological parameters measured except for the highest dose (400 mg/kg/day) of AFAM that was associated with thrombocytosis and neutropenia highlighting the propensity of AFAM to predispose its users to hypercoagulabity state and infections, respectively. The observed hematotoxicity could be due to presence in high amount of anti-nutritive and pro-oxidant secondary metabolites such as flavonoids, tannins and saponin in AFAM that have been documented to be hematotoxic [81-83]. Saponins have been reported to induce hematotoxicity and behavioral toxicity in rats administered extracts containing high concentration of saponin [84]. It is, therefore, possible that the high saponin content of AFAM is responsible for the observed behavioral toxicities of AFAM. However, this assertion remains to be validated. It is worthy to note that AFAM-induced thrombocytosis reported in this study is at complete variance with that previously reported by Awodele et al. [85] where oral dosing with 2000 mg/kg/day of lyophilized fruit extract of Annona muricata for 60 days resulted in profound but reversible thrombocytopenia. The reason for this variance could be adduced to differences in dose (400 mg/kg/day versus 2000 mg/kg/day) and treatment duration (42 days versus 60 days).

3.10. Histopathological effects of prolonged AFAM treatment on the lungs, stomach, testes, spleen and heart

Prolonged daily oral administration of 400 mg/kg AFAM induced marked bronchial lymphoid aggregates, mild vascular congestion and pulmonary edema in the AFAM-treated rat lungs (Figure 5b) when compared to untreated normal rat lung (Figure 5a) while 100 and 200 mg/kg/day AFAM –treated lungs showed mild alveoli interstitial lymphocytosis. This strongly suggests that prolonged AFAM use could predispose to the development of lung infections. Neutropenia has been reported to be associated with bacterial and fungal lung infections [86-88]. Thus, the hematological report of neutropenia which was associated with prolonged AFAM treatment is a strong pointer to the tendencies of AFAM causing pulmonary infection which was corroborated by the histological features of interstitial alveolitis. On the gastric tissue, prolonged 200-400 mg/kg AFAM treatment induced moderate gastric submucosa infiltrations by neutrophils and lymphocytes although the gastric mucosa showed no remarkable histological lesions in the AFAM-treated rat stomach (Figure 6b) when compared to normal gastric histoarchitecture (Figure 6a). Literature has shown gastric mucosal injury to be associated with neutrophilic and lymphocytic infiltration of the gastric mucosa and submucosa [89-92]. Thus, the result of our study is in complete agreement with histological findings of earlier studies, suggesting AFAM's tendency to either induce gastritis or gastric dysplasia. In the same vein, prolonged oral 400 mg/kg AFAM dosing caused a more diffused, large and better-formed follicles in the AFAM-treated rat spleen (Figure 7b) when compared to that of untreated normal spleen which showed normal histoarchitecture (Figure 7a). Conversely, prolonged 100-400 mg/kg/day of AFAM showed no appreciable changes in the AFAM-treated rat testes and heart (Figures 8b and 9b, respectively) when compared to that untreated normal testes and heart histoarchitecture (Figures 8a and 9a, respectively). The different histological features seen in some the of the other vital body organs such as the spleen, lungs, stomach, testes and heart after histopathological examination indicates the relatively low or no toxicity of the fruit extract, which could be attributable to "organ toxicity-sparing phenomenon", as AFAM relatively spared organs that are vital for the animal survival. Although organ toxicity-sparing phenomenon are commonly seen in iron-deficiency anemia [93-94] and prolonged starvation as seen in kwashiorkor [95-96] and marasmus [97], the animal in this case were neither iron deficient

based on the unaltered hematological parameters nor malnourished based on the satisfactory serum total protein and albumin levels of the AFAM-treated rats. In this instance, the organ-sparing effects may be attributed to the presence of secondary metabolites having high affinity for those organs and thus, protecting them.

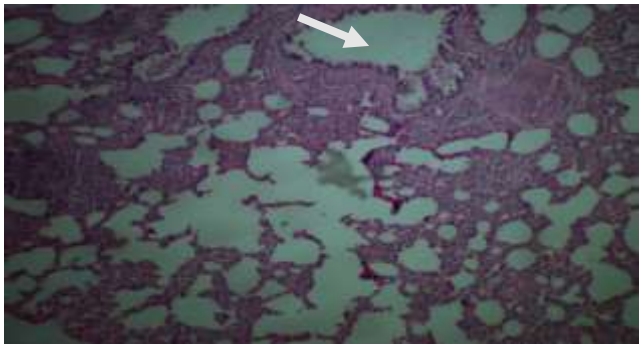

Figure 5a. Photomicrograph of the cross-section of normal rat lungs showing normal cytoarchitecture (x100 magnification, Hematoxylin & Eosin stain).

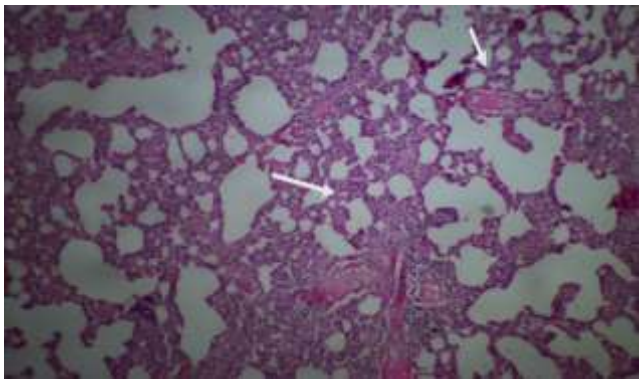

Figure 5b. Photomicrograph of the cross-section of 400 mg/kg/day of *AFAM* on treated rat lung showing bronchial aggregate, mild vascular congestion and pulmonary edema (indicated in the white solid arrow) (x100 magnification, Hematoxylin & Eosin stain)

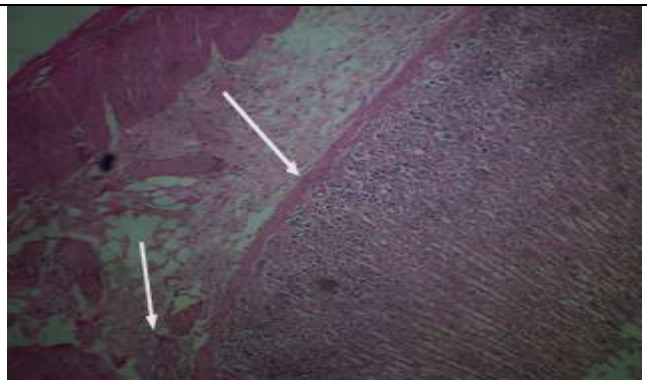

Figure 6b. Photomicrograph of the cross-section of 400 mg/kg of *AFAM*-treated rat stomach showing moderate infiltration of the submucosa by neutrophils and lymphocytes and an unremarkable gastric mucosa distortion (x100 magnification, Hematoxylin & Eosin stain)

Figure 7a. Photomicrograph of the cross-section of normal rat spleen showing normal white follicle and red pulp (x100 magnification, Hematoxylin & Eosin stain)

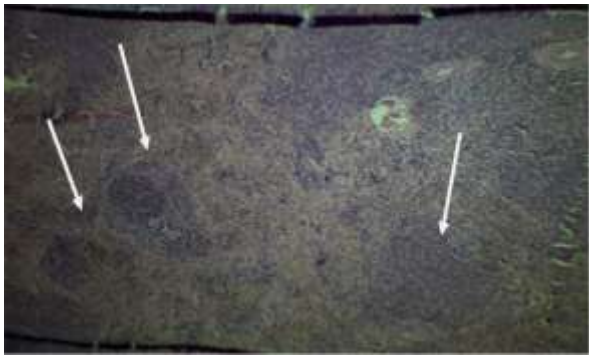

Figure 7b. Photomicrograph of the cross-section of 400 mg/kg extract-treated rat spleen showing a more diffuse, large and better formed white cell follicle and red pulp (x100 magnification, H & E stain)

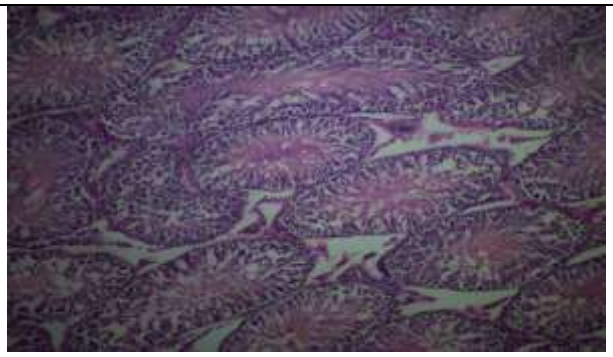

Figure 8a. Photomicrograph of the cross-section of normal rat testis showing normal testicular histoarchitecture (x400 magnification, Hematoxylin & Eosin stain)

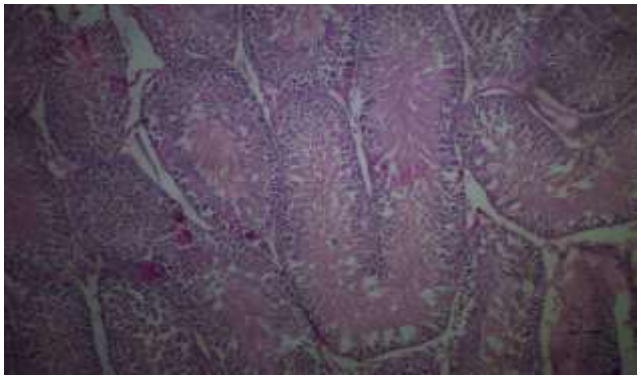

Figure 8b. Photomicrograph of the cross-section of 400 mg/kg extract-treated rat testis showing normal testicular cytoarchitecture (x400 magnification, Hematoxylin & Eosin stain)

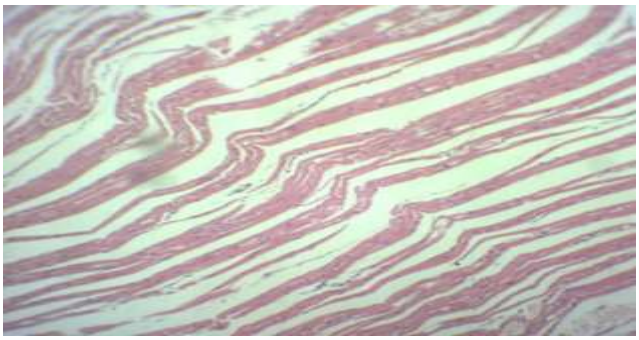

Figure 9a. A photomicrograph of the cross-section of normal rat heart showing a normal cytoarchitecture (x100 magnification, H & E stain)

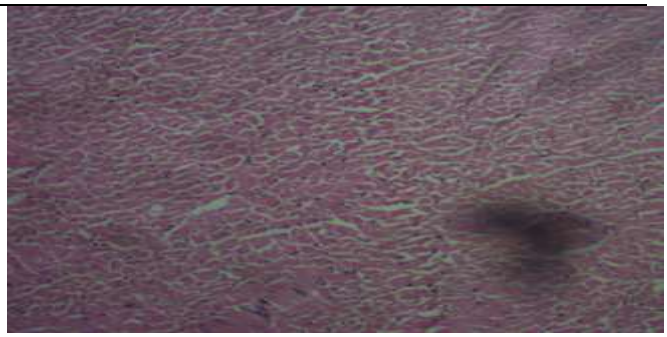

Figure 9b. Photomicrograph of the cross-section of the 400 mg/kg/day of AFAM-treated rat heart showing a normal histoarchitecture (x100 magnification, Hematoxylin & Eosin stain)

4. Conclusions

Overall, results of this study showed that although AFAM could be considered relatively safe for consumption but this should be done with a great caution as its prolonged use and consumption at high dose could be deleterious to human health.

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