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Research Article

Acute and Subacute Oral Toxicity of Methanol Fruit Pulp Extract of *Azanza garckeana* (Tula Kola nut) in Adult Male Wistar Rats

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ABSTRACT

This study evaluated the safety profile of methanol fruit pulp extract of *Azanza garckeana* (MFEAG) in rats through single and 28-day oral administrations. Twenty-nine (29) adult male Wistar rats (weighing 130-240 g) were used for acute and subacute studies. In the acute study, MFEAG was administered sequentially at single doses of 300 and 2,000 mg/kg (48 hours apart) while its vehicle was administered to control at equal volumes (n = 3) and observed for 14 days for mortality or toxicity signs. For the subacute study, MFEAG was administered once daily for 28 days at 300, 600 and 1,200 mg/kg while the vehicle was administered to control (n = 5). The results revealed no death or treatment-related toxic signs and effects on body weight, Relative Organ Weights (ROW), biochemical parameters and tissue histology of brains, hearts, lungs, spleens, livers and kidneys of the rats after treatment with MFEAG compared to control, except for ROW of the lungs that was decreased (at 300 and 1,200 mg/kg), ROW of the hearts that was decreased (at 1,200 mg/kg) as well as the serum albumin and alanine aminotransferase levels that were respectively increased at 600 and 1,200 mg/kg doses of the extract after the subacute treatment. This study demonstrates the tolerability of MFEAG up to the dose of 2,000 mg/kg, except that it should be used with caution due to its possible systemic adverse effects on cardiopulmonary and hepatic systems when used at high doses and for long durations.

Keywords: *Azanza garckeana, fruit pulp, methanol, toxicity, rat*

INTRODUCTION

The use of herbs has been widely employed for centuries by local people, especially in Africa, for the treatment of diseases. It has been estimated that about 80% of people worldwide and up to about 95% of people in developing countries use traditional herbs as medicines. This is due to theirs ready availability and cheapness to these populations. Again, modern pharmacopoeias of scientific medicine contain at least 25% of drugs derived from plant parts (Kim, 2005; Woo *et al.*, 2012; Sajjad *et al.*, 2019). This compelled government to seek investments in complementary and alternative medicine, and the development of drugs from natural compounds isolated from medicinal plants is currently being encouraged (Mshelia *et al.*, 2016). However, it has been suggested that the use of conventional medicines for the treatment of diseases should be rationalized (Kim, 2005). Therefore, safety evaluation of foods, ingredients, chemical/pharmaceutical products, cosmetics, etc. has been stressed to be very crucial before their approval for human consumption and use (Oghenesuvwe *et al*., 2014).

Nigeria is blessed with many fruit-bearing plants such as cashew, pears, mangoes, and particularly Tula kola nut (i.e.

Goron Tula, in Hausa, scientifically called *Azanza garckeana*) (Ochokwu*et al.*, 2019). *Azanza garckeana* is a semi-deciduous tree/shrub native to North-Eastern parts of Nigeria, particularly in the Tula Village of Gombe State, where it is semi-domesticated and used locally as a source of nutrients and medication for various illnesses (Nkafamiya *et al.*, 2015; Maroyi, 2017). In the Tula Village, it is known for its use as an aphrodisiac and for increasing fertility. This is aside from its other uses which include its use as fodder, firewood and soil conservation. The fruits and leaves are said to be the most important parts of the plant. The leaves are cooked as a relish or as vegetables, also used as green manure to improve agricultural productivity. The fruits are eaten row while slightly green or ripe, soaked in a small quantity of water to make jelly, also boiled and used as a relish or made into porridge (Mojeremane & Tshwenyane, 2004; Ochokwu *et al.*, 2015). Various parts of the plant, especially the fruit pulp have been established as important sources of useful phytochemicals such as flavonoids, phenols, alkaloids, saponins, carotenoids, mansonone, terpenoids, phytosterols and sitosterols among others (Michael *at al.*, 2015a; Jacob *et al.*, 2017; Ochokwu *et al.*, 2019; Bioltif *et al.*, 2020). It is therefore pertinent to explore the potential benefits of *Azanza garckeana*.

Several studies have demonstrated the nutritional and therapeutic benefits of various parts of *Azanza garckeana* (Nkafamiya *et al.*, 2015; Maroyi, 2017; Ochokwu *et al.*, 2019; Yusuf *et al.*, 2020b). A study revealed a proximate composition of the seed extract and meal (fed to *Clariasgariepinus* juveniles at varying inclusion levels) having significant amounts of crude proteins, lipids, carbohydrates and minerals, with good cost-effectiveness compared to normal fish feed (Michael *et al.*, 2015b). Another study also revealed a significant nutritional content, at safe levels, of various parts of the plant. Large amounts of crude proteins were found in the stem bark (45.30%) and fruits (20.75%), with lower amounts of lipids in the leaves (2.56%) and roots (0.68%). Vitamins A, B_1, B_2, C and E were also found to be present respectively in the fruits (75.00±0.23, 1.28±0.97, 1.18±0.45, 319.09±0.45 and 3.08±0.55) and leaves (28.75±0.66, 1.00±0.67, 0.95±0.78, 98.02±0.65 and 2.09±0/77) – quantitatively more in the fruits (Nkafamiya *et al.*, 2015). In a similar development, the fruit was found to be rich in carbohydrates (49-56%), ascorbic acid (raging between 285.5-308.5mg/50g) and low-fat content (0.0541-0.0543%), metallic electrolytes Fe, Mg, Ca, and Mn (Jacob *et al.*, 2017) as well as pectin (Joel *et al.*, 2018).

In vitro studies on various solvent extracts of the stem bark of *Azanza garckeana* revealed antioxidant potentials of the methanol and acetone extracts of the bark (Mshelia *et al.*, 2016). Aqueous leaf extract of the plant has been found to have antihyperglycemic activities in male guinea pigs, which confirmed its acclaimed diabetic use by local people (Amuri *et al*., 2017). The methanol and ethylacetate extracts of the fruit pulp have also been established to possess a myriad of therapeutic uses: antioxidant, antibiotic and anti-inflammatory/analgesic activities, with the methanol extract being more active (Yusuf *et al*., 2020a). Other reported uses of the fruits include antifungal, antimalarial and iron absorption effects (Maroyi, 2017). Chanda*et al*. (2020) also confirmed an acclaimed traditional use of the plant for improving labour, having been able to establish the uterotonic potentials of methanol and aqueous extracts of the plant's leaves.

A few researchers have attempted to evaluate the safety profile of Goron Tula (Mshelia *et al.*, 2016; Ochokwu *et al.*, 2019; Yusuf *et al.*, 2020b). Reports of a study revealed a significant increase in growth performance of *Clariasgariepinus* juveniles fed varying inclusion levels of *Azanza garckeana* seed (Michael, *et al.*, 2015b), although the study by Mshelia *et al*. (2016) revealed that the petroleum ether, ethyl acetate, acetone, methanol and water extracts of the plant's stem bark were toxic to brine shrimps, except for the petroleum ether extract, which was categorized as safe. A two weeks oral treatment of rats with methanol extracts of air- and sun-dried fruit pulp of the plant was also reported to cause some alterations in the functional integrity of the rats' kidneys, for which caution was recommended to be exercised when using the plant as an oral remedy (Yusuf *et al.*, 2020b). However, an in-depth systemic evaluation of the safety of *Azanza garckeana* to properly establish its safety is still required. Hence, there is a need for more in-depth toxicity studies of various parts of *Azanza garckeana* on various body systems, as also previously suggested by Maroyi (2017) and Bioltif *et al.* (2020).

Therefore, this study was carried out to evaluate the toxicity of *Azanza garckeana* on various body systems in laboratory animals. Acute and subacute oral toxicity studies of the plant's methanol fruit pulp extract were carried out in adult male Wistar rats.

MATERIALS AND METHODS

Drugs and reagents

The following drugs/reagents were used during the study: Tween 80 (CP: CAS No. 9005-65-6, manufactured by Guagdong Jinhuada Chemical Reagent Co. Ltd.), ketamine (Midazolam injection, USP) and diazepam (Centurion Healthcare Private Limited).

Experimental animals

Twenty-nine (29) healthy growing adult male albino Wistar rats (weighing 130-240 grams before commencement) were obtained from the animal house of the Department of Human Physiology, Ahmadu Bello University, Zaria-Nigeria where they were kept in standard small animal cages (5 per cage) and maintained on standard laboratory rodent feed and tap water *ad libitum*. All experimental protocols were in compliance with ARRIVE guidelines and in accordance with the U.K. Animals (Scientific Procedures) Act, 1986 and associated guidelines as approved by the Ahmadu Bello University Committee on Animal Use and Care (approval certificate number: ABUCAUC/2021/009).

Plant material

Azanza garckeana fruit was obtained in April, 2021 from Tula village in Kaltungo Local Government Area of Gombe State, Nigeria. The plant material was identified and authenticated by a taxonomist at the Department of Botany, Ahmadu Bello University, Zaria-Nigeria where a voucher specimen (with a number, ABU07276) was deposited.

Methanol fruit pulp extraction and preparation

According to the methods described by Gwarzo & Bako (2013), the fruit pulp was separated from the seed, shed-dried, shredded, a pulverized 1,000 g macerated with about 3,000 mL of methanol, allowed to stand for 48 hours, filtered and concentrated on a water bath at 50°C to get a jelly-like methanol fruit pulp extract of *Azanza garckeana* (MFEAG, 491 g, percentage yield = 49.2 %). Since the extract was jelly-like, it could not be dissolved in distilled water alone. Therefore, Tween 80, which is usually used to dissolve jelly-like or oily materials (Dholi *et al.*, 2011; Degu *et al.*, 2016; Dubo *et al.*, 2019; Malgave *et al.*, 2019), was used to dissolve the extract. The Tween 80 was first dissolved in distilled water in a ratio of 1:9, i.e. 10% to get the '10% v/v Tween 80' as a solvent for the extract.

Phytochemical screening

Phytochemical screening of the extract was carried out using previously described methods (Trease & Evans, 1989; Sofowora, 1993; Harborne, 2007; Boye *et al.*, 2021), the presence of free anthracenes, tannins, saponins, flavonoids, cardiac glycosides, terpenoids, steroids, alkaloids and carbohydrates using Bontrager's, bromine water, frothing, alkaline reagent, Keller-Kiliani, Liebermann's, Alkowski's, Wagner's and Molisch tests respectively were determined.

Acute oral toxicity study

An acute oral toxicity study was conducted using Up-and-Down-Procedure (UDP), as per the Organization for Economic Cooperation and Development (O.E.C.D.) 423 guideline (O.E.C.D., 2008), with 300 mg/kg as the lowest dose and 2,000 mg/kg body weight limit dose of the methanol fruit pulp extract of *Azanza garckeana*. Three (3) rats were used in each of the two treatment groups and a control group (administered the extract vehicle – 10% v/v Tween 80). The treatment was a one-time administration via oral gavage after overnight fasting. The volume was adjusted depending on the body weight of the rat using 10 ml/kg being the normal volume used for rats, as also used in other studies (Perret-gentil, 2005; Turner *et al.*, 2011). The treated rats were then observed closely for at least the first four hours after treatment for any signs of toxicity such as changes in skin and fur, eyes and mucous membranes, behaviour pattern, convulsions, tremors, lethargy, sleep, coma, salivation, diarrhoea and coma, then every day for 14 days for any delayed toxicity (O.E.C.D., 2008, 2009). Treatment of rats in the high dose (2,000 mg/kg) group was started 48 hours after the start of treatment of the low dose (300 mg/kg) group when there were no signs of toxicity or mortality observed among them. The body weights of the rats were recorded shortly before the administration of the extract and at the end of each week.

Subacute oral toxicity study (28-day repeated doses)

Here, as previously described (Al-Afifi*et al*., 2018), 20 rats were grouped into four (n = 5): Group 1 animals served as control (administered 10 ml/kg 10% v/v Tween 80), Groups 2-4 were administered the extract at doses of 300, 600 and 1,200 mg/kg (dissolved in 10% Tween 80) respectively. All administrations were done via oral gavage for 28 days to evaluate the multiple dosing effects of the extract. The rats were observed daily for any signs of toxicity or mortality, and body weights were recorded every week.

Sacrifice, collection of blood and organ samples and haematological/biochemical assays

At the end of the treatment periods of both the acute and subacute studies, the rats were fasted overnight before being anaesthetized with ketamine (75 mg/kg, i.p.) and diazepam (25 mg/kg, i.p.). From each rat, a blood sample was then collected via cardiac puncture and placed into EDTA and plain bottles for haematological and biochemical assays respectively. The vital organs (brain, heart, lung, liver, spleen and kidney) were removed through a midline incision in the rat's abdomen, cleaned of fat and blotted with clean tissue paper, while the brain was removed after decapitation and removal of the scalp and skull. The organs were then weighed to calculate their relative organ weights (using the formula below: Equation 1) and observed for any gross lesions.

$$\textit{Relative organ weight} = \frac{\textit{organ weigh}\ \text{(g)}}{\textit{body weigh}\ \text{ of the animal on sacrifice day (g)}} \times 100$$

The vital organs were then placed immediately in clean containers containing 10% buffered formalin for fixing. The blood samples in plain bottles were allowed to stand for 20-30 minutes for them to coagulate, centrifuged at 3,000 rpm for 10-15 minutes and then the serum was decanted into clean specimen tubes, which were taken for biochemical assays. Haematological parameters (RBC indices, differential WBC counts) were assayed from the blood samples in the EDTA bottles. Biochemical parameters from the serum (serum plasma proteins: total proteins, albumin and globulin; and liver enzymes: alanine transaminase, aspartate aminotransferase and alkaline phosphatase) were analyzed spectrophotometrically using Audicum auto-analyzer and Surechem spectrophotometer.

Histological examination

The isolated organs fixed in 10% buffered formalin were then taken for histological examination. The organs were routinely processed and embedded in paraffin wax, from which thin sections (5 μm) were made on glass slides and stained with haematoxylin and eosin. The slides were examined under a light microscope by an experienced pathologist who was not aware of the experimental groupings. These were carried out as done previously, with slight modifications (Al-Afifi *et al.*, 2018).

Data analysis

Data obtained were analysed using Statistical Package for Social Sciences (SPSS), version 23. Data were subjected to a one-way analysis of variance (ANOVA) followed by Tukey's *post hoc* test of multiple comparisons. Mean differences with $P < 0.05$ were considered statistically significant. Results are presented as means ± standard error of mean (SEM).

RESULTS AND DISCUSSION

Phytochemical constituents of MFEAG

In Table 1, the results of the phytochemical screening of MFEAG revealed the presence of carbohydrates, anthraquinones, steroids, cardiac glycosides, tannins, flavonoids, alkaloids, saponins and interpenes while phenols were found to be absent in the extract.

Table 1: Phytochemical Constituents of MFEAG

Phytochemical constituents	Tests	Result
Carbohydrate	Molish Test	+
Anthraquinones	Bontrager's Test	+
Steroids	Salkowski's Test	+
Cardiac Glycoside	Keller-killani Test	+
Tannins	Ferric chloride Test	+
Flavanoids	Alkaline reagent Test	+
Alkaloids	Mayer's Test	+
Saponin	Frothing Test	+
Interpenes	Lieberman's Test	+
Phenols	Lead acetate Test	–

+ Presence of phytochemicals
– Absence of phytochemicals

Toxicity signs and behavioural analysis

After both the acute and subacute studies MFEAG was found to have caused no treatment-related changes in the skin and fur, eyes and mucous membranes, behaviour pattern or caused any convulsions, tremors, lethargy, sleep, coma, salivation or diarrhoea in the treated rats. Also, no mortality was recorded during the study periods.

Effects of MFEAG on body and relative organ weights

Tables 2 and 3 show the results of the effect of MFEAG on the body weights of rats after acute and subacute treatments respectively. The results show no significant changes ($P \geq 0.05$) in the body weights of the rats when compared to control during both the acute and subacute treatment periods. Table 4 shows the results of the effect of MFEAG on relative organ weights of the rats after the acute and subacute studies. The result indicates that, after the acute study, there were no significant changes in the relative organ weights of all the isolated organs when compared to those of the control ($P \geq 0.05$). Also, after the subacute study, the relative organ weights of the organs were not significantly affected when compared to those of the control ($P \geq 0.05$) except for the hearts, which were significantly decreased by both 300 and 1,200 mg/kg and the lungs, which were also significantly decreased by 1,200 mg/kg of the extract when compared to those of the control rats ($P < 0.05$).

Table 2: Effect of Methanol Fruit Pulp Extract of *Azanza garckeana* (MFEAG) on Body Weights of Wistar Rats during an Acute Oral Toxicity Study

Treatment	10% v/v Tween 80	300 mg/kg MFEAG	2,000 mg/kg MFEAG
Week			
Week 0 (g)	201.66±5.54	237.00±14.17	205.33±13.61
Week 1 (g)	227.66±8.41	263.00±17.67	241.00±13.11
Week 2 (g)	241.33±14.33	265.33±20.16	238.33±24.25

Table 3: Effect of Methanol Fruit Pulp Extract of *Azanza garckeana* (MFEAG) on Body Weights of Wistar Rats during a Subacute Oral Toxicity Study

Week Treatment	Week 0 (g)	Week 1 (g)	Week 2 (g)	Week 3 (g)	Week 4 (g)
10% v/v Tween 80	155.4±5.86	157.4±7.11	152.8±4.92	159.0±5.44	155.7±5.56
300 mg/kg MFEAG	163.6±9.13	146.8±9.27	147.6±9.92	148.2±8.66	152.0±11.51
600 mg/kg MFEAG	169.6±9.33	162.8±7.19	152.2±9.98	160.2±9.63	157.0±8.67
1,200 mg/kg MFEAG	151.0±5.00	139.8±5.53	151.6±6.34	154.8±7.73	154.2±7.18

Table 4: Effect of Methanol Fruit Pulp Extract of *Azanza garckeana* (MFEAG) on Relative Organ Weights of Wistar Rats after Acute and Subacute Oral Toxicity Studies

Treatment		Heart (%)	Liver (%)	Lung (%)	Spleen (%)	Right Kidney (%)	Brain (%)
Acute	10% v/v Tween 80	0.30±0.023	2.98±0.18	0.75±0.143	0.40±0.054	0.31±0.032	0.71±0.050
	300 mg/kg MFEAG	0.38±0.023	2.93±0.37	0.92±0.103	0.39±0.012	0.27±0.034	0.54±0.078
	2,000 mg/kg MFEAG	0.36±0.041	3.02±0.063	0.9±0.02	0.48±0.025	0.28±0.015	0.72±0.020
Subacute	10% v/v Tween 80	0.45±0.012	3.44±0.063	1.68±0.183	0.40±0.048	0.28±0.022	0.93±0.034
	300 mg/kg MFEAG	0.37±0.016*	2.89±0.140	1.20±0.198	0.39±0.013	0.29±0.026	1.03±0.089
	600 mg/kg MFEAG	0.4±0.013	3.44±0.250	1.23±0.178	0.39±0.074	0.32±0.013	1.00±0.095
	1,200 mg/kg MFEAG	0.36±0.013*	3.05±0.120	0.93±0.092*	0.43±0.016	0.30±0.015	0.98±0.034

Relative organ weight was calculated as (organ weight/body weight) ×100. * Significantly different from 10% Tween 80-treated group ($P < 0.05$).

Effects of MFEAG on serum plasma proteins and liver enzymes

Table 5 shows the serum biochemical parameters of control and MFEAG-treated rats after acute and subacute toxicity studies. No statistically significant differences were observed in all the parameters of the treatment groups compared to the control ($P \geq 0.05$) after both the acute and subacute studies, except for the albumin level of 600 mg/kg MFEAG-treated group and alanine aminotransferase (ALT) of 1,200 mg/kg MFEAG-treated group which were significantly increased when compared to those of the control ($P < 0.05$) after the subacute treatment period. However, the total protein levels of 600 and 1,200 mg/kg MFEAG-treated groups, the albumin level of 600 mg/kg MFEAG-treated group as well as the ALT and aspartate transaminase (AST) levels of 1,200 mg/kg MFEAG-treated group were significantly increased ($P < 0.05$) when compared to that of 300 mg/kg MFEAG-treated group after the subacute study. The ALT level of the 1,200 mg/kg MFEAG-treated group was also significantly increased ($P < 0.05$) when compared to that of the 600 mg/kg MFEAG-treated group after the subacute treatment.

Effects of MFEAG on haematological parameters

The results in Table 6 show no statistically significant differences in all the assayed haematological indices across the groups after both the acute and subacute studies.

Table 5: Effects of Methanol Fruit Pulp Extract of *Azanza garckeana* (MFEAG) on Serum Biochemical Parameters of Wistar Rats after Acute and Subacute Oral Toxicity Studies

Treatment		TP (g/dL)	Alb (g/dL)	Glb (g/dL)	ALT (IU/L)	AST (IU/L)	ALP (IU/L)
Acute study	10% v/v Tween 80	7.40 ± 0.32	3.03 ± 0.64	4.37 ± 0.32	30.67 ± 8.09	85.33 ± 18.62	14.90 ± 2.51
	300 mg/kg MFEAG	6.73 ± 0.65	3.47 ± 0.55	3.2 ± 71.19	28.33 ± 4.67	89.33 ± 25.98	25.30 ± 5.07
	2,000 mg/kg MFEAG	6.30 ± 0.40	2.00 ± 0.53	4.30 ± 0.25	39.00 ± 7.37	94.00 ± 18.52	13.30 ± 2.23

Subacute study	10% v/v Tween 80	3.30 ± 0.21	2.10 ± 0.16	1.20 ± 0.26	25.20 ± 2.39	10.80 ± 2.65	45.66 ± 8.56
	300 mg/kg MFEAG	2.84 ± 0.22	1.62 ± 0.18	1.22 ± 0.25	22.80 ± 3.10	6.00 ± 1.41	23.04 ± 3.72
	600 mg/kg MFEAG	4.52 ± 0.58[b]	3.22 ± 0.22[ab]	1.30 ± 0.49	24.00 ± 4.23	7.80 ± 1.77	27.42 ± 4.82
	1,200 mg/kg MFEAG	4.34 ± 0.29[b]	2.48 ± 0.37	1.86 ± 0.21	43.80 ± 7.02[abc]	32.2 ± 11.85[b]	47.80 ± 8.45

Superscripts 'a' and 'b' indicate significant differences from 10% Tween 80- and 300 mg/kg MFEAG-treated groups respectively ($P < 0.05$). TP: total protein; Alb: albumin; Glb: globulin; ALT: alanine transaminase; AST: aspartate transaminase; ALP: alkaline phosphatase.

Table 6: Effect of Oral Administration of Methanol Fruit Pulp Extract of *Azanza garckeana* (MFEAG) on Haematological Parameters of Adult Male Wistar Rats after Acute and Subacute Toxicity Studies

Treatment		WBC (10^3/µL)	PCV (%)	RBC (10^6/µL)	Hb (g/dL)	MCV (fL)	MCH (pg)	MCHC (g/dL)
Acute study	10% v/v Tween 80	3.80±0.49	38.33±2.40	5.63±0.27	12.53±0.74	85.00±3.27	32.97±2.82	33.20±0.30
	300 mg/kg MFEAG	4.57±0.35	34.67±0.67	5.50±0.29	11.33±0.49	88.43±0.98	29.73±0.62	34.97±1.42
	2,000 mg/kg MFEAG	4.87±0.47	38.33±1.76	5.93±0.07	13.43±0.48	91.60±2.83	31.63±2.11	31.83±0.91
Subacute study	10% v/v Tween 80	5.02±0.34	37.40±1.87	5.70±0.20	12.52±0.89	86.74±1.89	30.58±0.83	34.72±0.84
	300 mg/kg MFEAG	4.52±0.21	40.00±1.41	5.76±0.17	13.30±0.52	91.66±2.37	32.76±1.89	32.30±0.59
	600 mg/kg MFEAG	4.64±0.42	40.60±1.47	6.08±0.07	13.34±0.37	83.22±3.61	35.68±3.87	33.60±0.25
	1,200 mg/kg MFEAG	5.06±0.33	42.40±1.89	6.34±0.19	13.86±0.23	88.48±0.52	33.08±1.S97	33.40±1.56

WBC: White Blood Cells; PCV: Packed Cell Volume; RBC: Red Blood Cells; Hb: Haemoglobin; MCV: Mean Corpuscular Volume; MCH: Mean Corpuscular Haemoglobin; MCHC: Mean Corpuscular Haemoglobin Concentration

Histological observations

Histological studies were carried out to confirm biochemical findings and to identify any structural changes in various vital organs. Light microscopic examinations of the vital organs including brain, lung, spleen, liver and kidney of the rats in all the MFEAG-treated and control groups for acute oral toxicity (Figure 1) and subacute oral toxicity (Figure 2) studies did not reveal any gross pathological lesions that could be related to the treatments.

Photomicrographs of brain tissues of both the control and MFEAG-treated rats show relatively normal morphological architecture, with, however, moderate vacuolation (V) and slight neuronal necrosis (NN) observed in the brain of the control rat after the acute toxicity study. Normal cardiac muscle fibres were observed in the heart of both control and MFEAG-treated rats after both the acute and subacute studies, but myocardial adhesion (MA) and slight myocardium necrosis (MN) were noted after the subacute treatment with 300 mg/kg MFEAG. Slight to moderate alveolar congestion (AC) was noted in the lungs of both the control and MFEAG-treated rats, with alveolar necrosis (AN) also seen in that of the 300 mg/kg MFEAG-treated rat after the acute study. Normal alveolar features were observed in the lungs of all the rats after the subacute study. The spleens of control and MFEAG-treated rats show slight to moderate hyperplasia of inflammatory cells (LH) after both the acute and subacute studies, except that of the 2,000 mg/kg MFEAG-treated rat, which shows intense LH after the acute study. Slight LH in the liver of 300 mg/kg MFEAG-treated rat was observed, with normal features in the others after the acute study. No gross signs of injury, necrosis, congestion, fatty acid accumulation, or haemorrhage around the central vein or sinusoids were noticed. However, there were slight indications of LH, red pulp necrosis (RPN) and haemorrhage (H) in the liver of the control rat, vacuolation in that of the 300 mg/kg MFEAG-treated rat and slight LH in that of the 1,200 mg/kg MFEAG-treated rat, while those of the other groups shows normal features. The hepatocytes are arranged in cords and visible, with no lyses in the blood cells. For the kidneys, a tubular distortion (TD) and slight LH were noted in the kidney tissue of the control rat after the acute study.

Hyperplasia of inflammatory cells was noted in the kidneys of the control rat after the acute study, as well as in the 300 and 1,200 mg/kg MFEAG-treated rats after the subacute study. Slight tubular necrosis (TN) in the kidney of 300 mg/kg MFEAG-treated rat was noted after the acute study, while moderate TN was seen in those of the control and 300 mg/kg MFEAG-treated rats. Glomerular necrosis (GN) and tubular adhesion (TA) were manifest in the kidney of the 1,200 mg/kg MFEAG-treated rat.

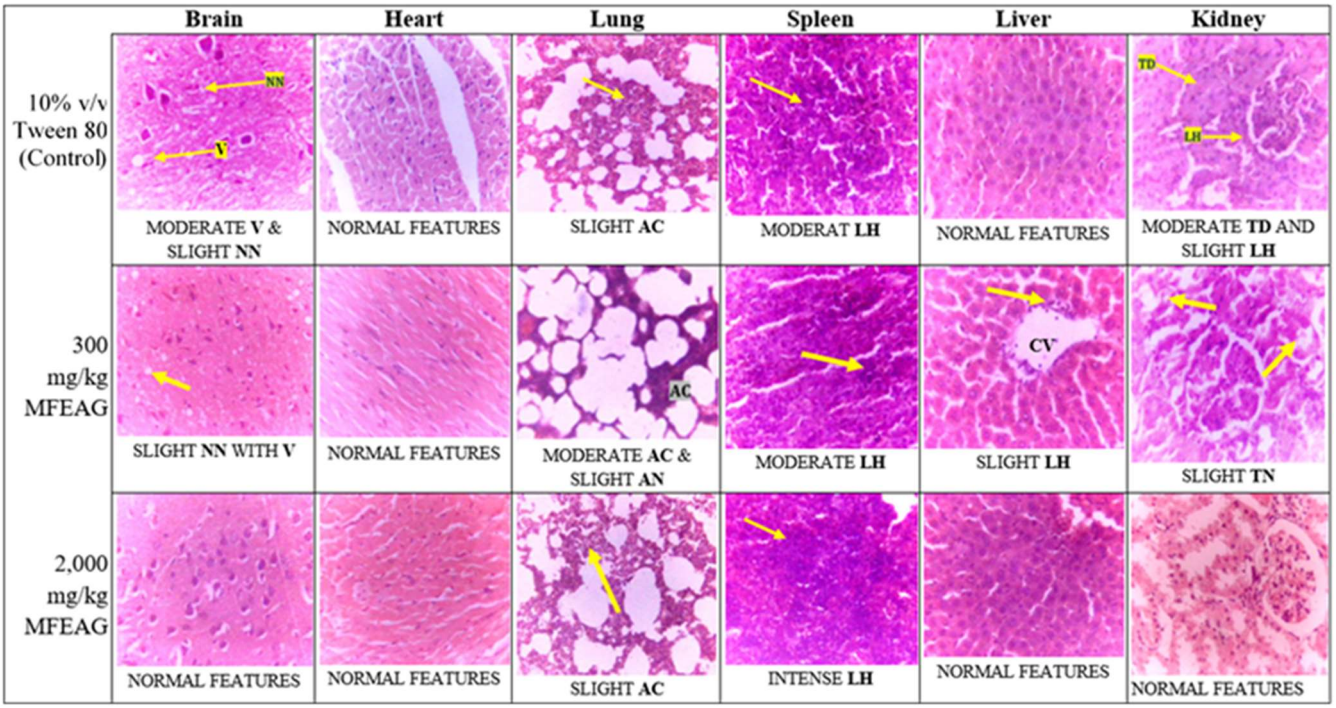

Figure 1: Histological Sections of the brains, Lungs, Spleens and Livers of Control (10% Tween 80-treated) and Methanol Fruit Pulp Extract of *Azanza garckeana* (MFEAG)-treated Rats after an Acute Toxicity Study.

Arrows indicate mentioned changes. V: Vacuolation; NN: neuronal necrosis; AC: alveolar congestion; AN: alveolar necrosis; LH: hyperplasia of inflammatory cells; CV: central vein; TN: tubular necrosis; TD: tubular distortion. Hematoxylin and eosin stains; 250× magnification

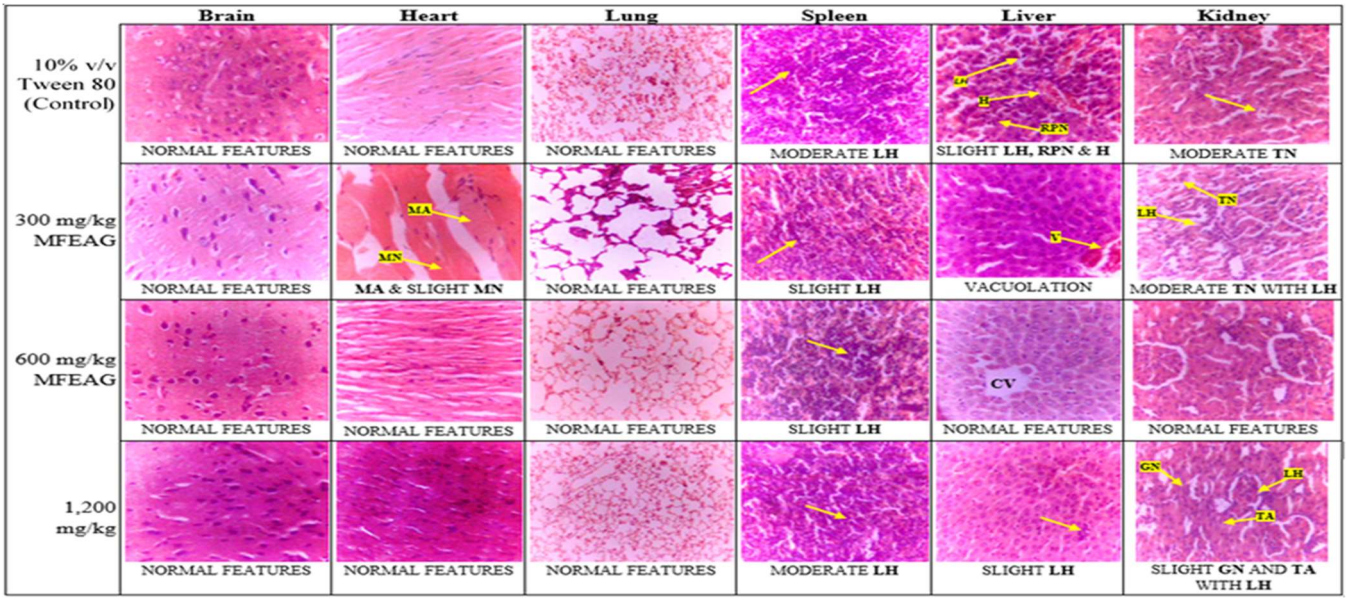

Figure 2: Histological Sections of the Brains, Lungs, Spleens and Livers of Control (10% Tween 80-treated) and Methanol Fruit Pulp Extract of *Azanza garckeana* (MFEAG)-Treated Rats after a Subacute Toxicity Study.

Arrows indicate mentioned changes. MN: myocardium necrosis; MA: myocardium adhesion; LH: hyperplasia of inflammatory cells; RPN: red pulp necrosis; H: haemorrhage; V: vacuolation; CV: central vein; GN: glomerular necrosis; TA: tubular adhesion. Hematoxylin and eosin; 250× magnification

DISCUSSION

The use of herbal products for the treatment of diseases has over time gained a greater deal of acceptance than conventional therapy. However, screening of plant products has been considered an initial step to evaluate their toxicities before wider usage (Olson *et al.*, 2000; Parasuraman, 2011; Ifeoma & Oluwakanyinsola, 2013). *Azanza garckeana* is an indigenous fruit tree with antibacterial, antifungal, antioxidant, cytotoxicity and aphrodisiac activities (Williams *et al.*, 2020). This study evaluated the acute and subacute oral toxicities of methanol fruit pulp extract of *Azanza garckeana* (MFEAG) in adult male Wistar rats.

The presence of many active phytochemicals in the extract confirms the importance of *A. garckeana* in the treatment of several diseases, as practised by local people in whose habitats the plant is abundant. This also confirms the findings of several studies that reported the presence of these chemicals in various parts of the plant (Nkafamiya *et al.*, 2015; Michael *et al.*, 2015a; Jacob *et al.*, 2017; Ochokwu *et al.*, 2019; Bioltif *et al.*, 2020), most of which were widely reported to be medicinally active (Adamu *et al.*, 2013; Nkafamiya *et al.*, 2015; Koche *et al.*, 2016). Even though these compounds are medicinally useful, they limit the wide use of many tropical plants due to their ubiquitous occurrence as natural compounds capable of eliciting deleterious effects (Osagie and Eka, 1998; Nkafamiya *et al.*, 2015), hence the need to evaluate the effects of these phytochemicals on various body systems. However, the absence of phenols in MFEAG seen in this study contradicts the findings of some of the previous studies in which the phytochemical was found to be present in the seeds (Michael *et al*., 2015a) and fruit pulp meal inclusion (Ochokwu *et al.*, 2014) of *Azanza garckeana*. It has been reported that ethanol extracts more polyphenolic compounds, especially higher molecular weight polyphenols, than methanol (Diem *et al.*, 2013). The use of ethanol, as against methanol, in further studies may be employed in order to elucidate if phenolic compounds can be extracted from the fruit pulp using ethanol.

An initial assessment of toxic manifestations in laboratory animals is one of the screening experiments performed during the evaluation of the toxic characteristics of medicinal plants (Ukwuani *et al.*, 2012). Hence, these assessments were carried out in this study. Again, establishing a safety margin between different dose levels and the doses having therapeutic effects and or adverse effects are essential and usually only possible using experimental animal models (Prajapati *et al.*, 2006). Therefore, the use of the rat models was employed in this study. All administrations of the extract were done orally, as it is the most useful and normally used route for toxicity studies – the absorption is usually slow, but it is less expensive and less invasive to the animals. The rats were fasted before administration to avoid any reaction of the extract with food substances, as food and other chemicals within the digestive system have been known to be able to react with drugs administered through the oral route (Kumar & Lalitha, 2013). Also, the test dose of 300 mg/kg is used when an investigator does not have any information indicating that the test material is likely to be toxic (O.E.C.D., 2001), hence the choice of this as the lowest dose in this study. The observation of toxic signs (which are considered as clinical symptoms) is among the most important aspects of evaluating the toxicities of substances. Therefore, these toxic signs were cardinal in the evaluations used in this study. The demonstration of no treatment-related deaths or toxic signs after the acute study reveals that the extract could be well tolerated up to the dose of 2,000 mg/kg body weight (Jothy *et al.*, 2011). According to the Globally Harmonized Classification System, the O.E.C.D. test guideline 423 for oral toxicity tests, MFEAG can be classified as Category 5, which is the lowest toxicity class of drugs/chemicals that are found to be safe at doses ranging between 2,000-5,000 mg/kg. This means that MFEAG can be used at doses of up to 2,000 mg/kg on rats when scientific investigations having direct relevance to protecting animal and human health or the environment using the extract are desired (O.E.C.D., 2021). Acute toxicity testing has limited clinical applications because cumulative toxic effects can occur even at a very low dose of a chemical (Chitra *et al.*, 2015). Hence, the subacute test with multiple administrations was also carried out to get information on possible health threats and cumulative effects on various body systems that could arise on exposure over a long period. There were no deaths or any signs of toxicity recorded throughout the subacute study, further indicating the tolerability of MFEAG.

After both the acute and subacute toxicity studies, no significant body weight changes were observed in the rats treated with all doses of the extract compared to the control. Significant body weight changes are indicative of adverse side effects, and more than 20% of the animal loss of body weight is regarded as critical and has been defined as one of the humane endpoints in several international guidelines (O.E.C.D., 2001). This implies that MFEAG may not have any potential to cause changes in body weight or cause obesity and therefore safe in that regard. Tannins (commonly referred to as tannic acids) are water-soluble polyphenols that are present in many plant foods and reported to cause a decrease in net metabolizable energy, and protein digestibility in experimental animals. In large

amounts, tannic acid can cause side effects such as stomach irritation, nausea, vomiting, and liver damage (Chung *et al.*, 1998). It may be expected that the tannins present in MFEAG exhibit a similar effect in this study; but to the contrary. It is possible that the level of tannins in MFEAG is not high enough to elicit that effect. Hence, even with the presence if tannins in MFEAG, its intake may neither cause malnourishment nor lead to obesity, as evidenced from the finding of this study.

Assessment of organ weights, absolute or relative, are sensitive indicators in determination of early toxicity of chemicals. Changes in organ weight may reveal target organ toxicity. It may not be evident after acute toxicity test, but usually seen after a subchronic or chronic tests (King, 2002). Even though there is a decrease in ROW in hearts and lungs of rats treated with 1,200 mg/kg MFEAG after the subacute study, the inability of MFEAG to affect the ROW of the other organs (brain, spleen, liver and kidney) indicates that the extract may not be entirely toxic. However, caution must be taken during its use, especially at high doses and for a long period due to its possible adverse effects on the cardiopulmonary system as evident from the toxicity-related effects it exhibited on the hearts and lungs of rats by decreasing their ROWs after subacute daily administrations in this study. This is because a decrease in ROW of hearts and lungs is usually seen in cardiopulmonary diseases such as chronic bronchitis, congestive heart failure, emphysema, cardiac hypotrophy, chronic obstructive pulmonary disease (Garcia *et al.*, 2011), as well as conditions that interfere with heart and lung tissue growth such as pulmonary hypoplasia, pulmonary fibrosis, cardiac cachexia, heart failure, cardiac myopathy, cancer and other lung/heart diseases pointed out in the literature (Matsuura & Fett-Neto, 2015).

Haematological values could serve as the baseline for information on understanding the physiological and pathological state of the body. Evaluation of haematological and biochemical parameters plays a major role in determining the toxicities of drugs (Petterino & Argentino-Storino, 2006). More so, the analysis of haematological parameters is important in risk evaluation as the haematological system being the main medium of transport of drugs and xenobiotics has a high prognostic value for toxicity of drugs (Olson *et al.*, 2000). Hence, any damages to haematological values are considered as detrimental changes to the body. Oyawoye and Ogunkunle (2004) reported that haematological constituents which consist of red blood cells, white blood cells, mean corpuscular volume, mean corpuscular hemoglobin, and mean corpuscular haemoglobin concentration are valuable in monitoring feed toxicity, especially with feed constituents that affect the blood and overall health status of farm animals. In this study, the observation of no significant changes in all the haematological parameters by the extract after both acute and subacute periods indicates the safety of the fruit pulp of *Azanza garckeana* on the haematological system.

The liver is a target organ and primary site of detoxification and is generally the major site of intense metabolism and therefore prone to various disorders as a consequence of exposure to toxins of extrinsic and intrinsic origins (Hodgson, 2004). Plasma proteins are produced in the liver. In case of liver damage, production of these proteins is reduced or completely ceased (Chukwudoruo *et al.*, 2020). Plasma proteins interact with virtually all body tissues or cells and are ultimately related to protein metabolism in the liver. Albumin constitutes approximately 60% of normal plasma proteins (Alshafi, 2002). From the results of this study, MFEAG did not affect the total protein levels. However, it caused an increase in the albumin level at the dose of 600 mg/kg after the subacute study. Albumins can bind with tannic acid-related compounds, which have been reported to markedly increase their normal levels. Hence, the increase in the normal level of albumin after the subacute study could be due to the presence of tannins in the extract. Although the liver histology indicates no gross treatment-related toxicity signs, the increase in serum albumin level after subacute treatment by MFEAG indicates that the extract may have some level of toxicity to the liver. This is in concordance with the adverse-related effects of the extract seen on the hearts and lungs of the treated rats in this study. Alanine transaminase (ALT) is an enzyme that helps metabolize proteins. When the liver is damaged, the production of ALT is increased in the liver and released into the bloodstream. Both AST and ALT are increased in liver damage. However, increase in ALT is more specific to the liver damage, because AST can also be released due to damage of other organs such as the heart and kidneys, and in the liver only 20% of AST is cytosolic (the remaining 80% is mitochondrial) while ALT is solely in the liver cytosol. Hence, a rise in ALT level is more specific to hepatocellular damage (Giannini *et al.*, 2005; W. R. Kim *et al.*, 2008; Lala *et al.*, 2022). From the results, AST was not affected by MFEAG, but at the high dose of 1,200 mg/kg ALT was increased after the subacute treatment. This further indicates that MFEAG may be toxic to the liver at a high dose, especially after treatment for a long period. Exposure to unusual amounts of anthraquinone and its toxic metabolites have been implicated in liver toxicity (Vanderperren *et al.*, 2005; Chen *et al.*, 2015; Wang, 2015; Liu *et al.*, 2020; Kang *et al.*, 2022). The increase in albumin and ALT caused by the high doses of MFEAG after a subacute treatment seen in

this study that suggests liver damage could be a result of the effects of anthraquinones found present in the extract.

Assessments of histological alterations of vital organs are considered essential in evaluating the toxicity of chemicals (Greaves, 2012). Hence, the histological examinations of the brains, lungs, spleens, livers and kidneys of the rats were also carried out. The observation of slight neuronal necrosis and moderate vacuolations in the brains of both the control rats and those treated with the low dose of MFEAG after the acute study shows that the changes are not treatment-related, as supported by the normal features seen in the organ tissues of all treated rats compared to that of the control after the subacute study. Similar observations were also made on the heart, lung, spleen, liver and kidney tissues of the rats, as all the changes seen were only slight to moderate in nature and mostly seen in both the control and MFEAG-treated rats. Therefore, the observation of no treatment-related gross abnormalities in the tissue histology of these vital organs suggests the safety of MFEAG in that regard.

Nevertheless, it is pertinent to say that sub-chronic toxicity and genotoxicity studies on MFEAG are required based on the oral doses used in the acute and subacute studies to ascertain the safety of the extract.

CONCLUSION

From the findings of this study, it can be concluded that the methanol fruit pulp extract of *Azanza garckeana* is safe for oral administration and can be classified as Category 5 according to Globally Harmonised Classification System as it was well tolerated up to the dose of 2,000 mg/kg body weight after an acute toxicity study in adult male Wistar rats. The extract can also be considered safe on various body systems, as evidenced by its ability to cause no mortality or changes in body weight, relative organ weights, biochemical parameters and vital organs histology of these animals after both acute and subacute oral toxicity studies, except that the extract may cause some systemic adverse effects on the cardiopulmonary and hepatic systems when administered at high doses and for a long period, as seen in its ability to decrease relative organ weights of the hearts and lungs and to increase serum albumin and ALT levels in the rats after the subacute treatment.

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CONFLICT OF INTEREST

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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