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Acute and sub-chronic oral toxicity studies on methanol leaf extract of *Gnetum africanum* Welw in wistar rats

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ABSTRACT

Aim: Acute and subchronic toxicity studies were performed on *Gnetum africanum* Welw leaves using well-established guidelines and methods. In spite of its use as a herbal remedy, there is a dearth of data on toxicity studies of the plant extract.

Method: *Gnetum africanum* leaves were identified by a plant taxonomist, air-dried, pulverized, and extracted with 80% methanol for 48 hours in July 2015. The extract was concentrated *in-vacuo* and stored as *Gnetum africanum* extract (GAE) at 4°C. Thirty-five male albino rats randomly divided into seven groups (n = 5) were used for acute toxicity test, which was done in August 2015. Rats in groups 1–6 were given GAE at 500, 1,000, 2,000, 3,000, 4,000 and 5,000 mg/kg, respectively, while group 7 rats received 10 ml/kg distilled water. Feed and water were provided and they were observed for signs of toxicity for 48 hours. A 90-day subchronic toxicity study was conducted using 56 rats of four groups (n = 14) in December 2015–March, 2016. Group 1 received feed without GAE, while groups 2–4 were given feed with GAE at 10, 20 and 40 mg/kg respectively. Four rats from each group were bled on days 30, 60, and 90 for clinical biochemistry and sacrificed for histopathological examination.

Result: The median lethal dose (LD_{50}) was greater than 5,000 mg/kg while no-observed-adverse-effect-level was 40 mg/kg. Serum marker enzymes and creatinine were not increased in rats given extract in feed, while there were no histopathological lesions in the organs studied.

Conclusions: The extract is safe at both the acute and subchronic levels.

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African jointfir; albino rats; serum biochemistry; histopathology; LD_{50}; NOAEL; safety

Introduction

Herbal remedies are therapeutic products and foods made from leaves, seeds, flowers, and roots of plants [1]. Herbs and other botanicals have been used by diverse people for medicinal and dietary purposes for centuries [2], and their use has increased globally in recent years [3]. Furthermore, it is estimated that 80% of the population of developing countries relies on herbal medicines for their primary healthcare because of the perceived low toxicity and availability of these remedies [4]. However, herbs elaborate a wide range of chemicals with beneficial properties when used in appropriate amounts, and this may become toxic when consumed in excess, either as tea or food additives, or manufactured into herbal products like herbal drugs and supplements [5]. Consequently, there is a growing concern about the safety of these botanicals as many studies have reported diverse levels of hepatotoxicity or herbs-induced liver injury [3,5,6]. This is because natural products are foreign to the body and need metabolic degradation to be

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eliminated. During this process, hepatotoxic metabolites may be generated causing liver injury in susceptible patients [7]. Oral toxicity studies including acute and subchronic tests using rodents have been recognized as a means of determining the safety or otherwise of herbal remedies before clinical studies [4]. Acute toxicity test, defined as the unwanted effects that occur either immediately or at a short time interval after a single or multiple administration of the test substance, is used to determine the median lethal dose (LD_{50}). This is the dose that kills 50% of the test population [8]. Subchronic test on the other hand, is a 90-day repeated daily treatment of rodents through feed, gavage, or drinking water from which the no-observed-adverse-effect-level (NOAEL) is often deduced [9].

Gnetum africanum Welw (African jointfir), family Gnetales, is an edible plant widely used as food. In Nigeria, it is called afang (Efik/ Ibibio) *ukazi*, *okazi* (Igbo) *yala* (Ogoja) *ajaabaje*; *ajakotale* (yoruba), in Cameroon it is known as *eru*, *okok*, *mfumbua* or *fumbua* and *koko* in Angola, Gabon, Central African Republic [10]. *G. africanum* has been used in Nigeria for the treatment of piles and high blood pressure [11]; enlarged spleen, sore throats, and as a cathartic [10]. In Ubangi (DR Congo), it is used for the treatment of nausea and is considered as an antidote to arrow poison made from *Periploca nigrescens* Afzel [10,12]. In Cameroon, the leaves are chewed to ameliorate the effects of drunkenness and are also taken as an enema against constipation and to ease childbirth. They are also used to treat diabetes, boils, and fungal infection in the fingers [12]. *Gnetum africanum* leaves have great culinary value in West Africa. They are eaten raw as local salad, and shredded and used in preparing soups and stews [10]. It is a good source of protein and has been noted as an anti-inflammatory, anti-carcinogenic, and antioxidant [13]. It is widely used by people of southern Nigeria as therapy and food for diabetics [12]. However, there is paucity of data on toxicity studies on this plant, especially the subchronic toxicity test. This study was therefore conducted to determine the safety or otherwise of *Gnetum africanum* using acute and subchronic oral toxicity tests.

Materials and Methods

Plant collection and extraction

Gnetum africanum Welw. leaves were collected between April and May 2015, from its natural habitat in Orba, Nsukka, Enugu State (6°5' 7°31'E). The plant sample was identified by a taxonomist, Mr Ozioko, of the Bioresources Development and Conservation Programmme, Nsukka, Enugu State. The plant materials were dried under mild sunlight. They were pulverized into coarse powder of about 1 mm in diameter. Plant material (2 kg) was extracted by cold maceration method in 80% methanol for 48 hours, with intermittent shaking at 2 hour intervals, after which they were filtered with Whatman® No. 1 filter paper. The filtrate was then concentrated *in vacuo* using rotary evaporator connected to a cold water circulator and a pressure pump at 40°C and 210 milibar. This extraction was carried out in July 2015. The extracts were stored in a refrigerator at 4°C as *Gnetum africanum* extract (GAE). The percentage yield was calculated as follows:

where a = weight of the original plant material used for extraction and b = weight of the recovered extract.

Experimental animals

Mature male albino rats bred in the Laboratory Animal Units of the Faculties of Veterinary Medicine and Pharmaceutical Sciences, University of Nigeria, Nsukka were used for the experiments. They were housed in an environment of normal ambient temperature and the lighting period was about 12 hours daily with a relative humidity of 40–60%. The weight of the rats varied between 97 and 150 g. The rats were kept in stainless steel cages, supplied with clean drinking water, and fed *ad libitum* with standard commercial pelleted feed (Vital® feed, Nigeria). Ethical conditions governing the conduct of experiments with life animals were strictly observed as stipulated by the National Institute of Health guide for the care and use of laboratory animals (NIH Publications No. 8023, revised 1978, updated 2011, eighth edition).

Acute toxicity test

The method of Karber [14] was adopted for this test, which was conducted in August 2015. Thirty-five mature albino rats of either sex were randomly grouped into seven groups (1–7) of five rats per group. Rats in groups 1–6 were given GAE (500, 1,000, 2,000, 3,000, 4,000 and 5,000 mg/kg), respectively, by oral gavage. Group 7 rats received distilled water (10 ml/kg) as the control. They were allowed free access to feed and drinking water and were observed for 48 hours for signs of toxicity and mortality. LD_{50} was calculated as follows:

$LD_{50} = LD_{100} - \sum axb/n$

where LD_{50} = median lethal dose

LD_{100} = least dose required to kill 100%

a = dose difference

b = mean mortality

n = group population.

Subchronic toxicity test

Preparation of experimental feed containing different concentrations of *G. africanum* extract.

G. africanum (10, 20 and 40 mg/kg) representing low, medium, and high doses was incorporated in feed (Grower mash, Vital® Feed, Jos Nigeria). To prepare the extract at each concentration, the amount (mg) was first dissolved in 20 ml of water and then uniformly made up to 2.5 l/3 kg feed. The feed and water containing extract were mixed thoroughly and the mixture was manually pelleted using a standard pelleting machine. This was done in the month of December 2015, being the harmattan period to enable efficient drying. The pelletized feed was dried for 5 days under mild sunlight. The feed was then stored in a dry environment with intermittent drying to prevent fungal growth.

Experimental procedure

This was done between January and March 2016, according to the OECD guidelines for 90-day testing [9], with some modifications. Fifty six albino rats of both sexes were randomly grouped into four (1–4) groups (n = 14). Males and females were housed in different cages to avoid breeding. Group 1 rats received feed without extract (control), while groups 2, 3, and 4 were given GAE (10, 20 and 40 mg/kg in feed), respectively. The rats were fed in accordance with the normal feed consumption rates of rats at 10 g feed/100 g b. w/day [15], while clean drinking water was given *ad libitum*. On days 30, 60, and 90, four rats from each group was assayed for clinical biochemistry. Finally, the rats were humanely sacrificed using chloroform inhalation and internal organs (liver, kidney, heart, and pancreas) were collected in freshly prepared 10% formal saline for histopathology.

Liver function tests

The activities of serum aspartate aminotransferase (AST), alanine aminotransferase (ALT), and alkaline phosphatase (ALP) were analyzed using the methods described by Reitman and Frankel [16] and Kind and King [17], respectively, using Randox kits (Randox Laboratories, UK); serum albumin was estimated using the methods described by Northam and Widdowson [18]; total bilirubin was determined colorimetrically using the method described by Jendrassik and Grof [19], while the total protein was assayed by the direct Biuret method as described by Gornall, Bardawill and David [20].

Kidney function tests

Kidney function was evaluated using estimation of the serum urea and creatinine levels. Serum urea level was estimated using the method of Bauer, Brooks and Burch [21], while serum creatinine level was determined using the method described by Cockcrof and Gault [22] using Randox kits (Randox Laboratories, UK).

Histopathology

The tissues in 10% formal saline were processed using standard methods for histological examination [23]. Photomicrographs were taken using a moticam camera connected to a laptop.

Statistical analysis

Data generated were subjected to one-way analysis of variance and the variant means were separated using the least significant difference method. The differences in means were considered significant at $p < 0.05$.

Results

Extraction of Gnetum africanum

The methanol leaf extract of *Gnetum africanum* had a very dark green color and an oily consistency. The percent yield was 10.30% w/w.

Acute toxicity test

The rats were generally active and showed no visible signs of illness or toxicity. No mortalities were recorded throughout the period of the experiment. LD_{50} was greater than 5,000 mg/kg body weight of the rats.

Subchronic toxicity test: Clinical observations

The rats were generally active and showed no visible signs of illness, toxicity, nor morbidity. No mortalities were recorded during the experiment. The NOAEL of *Gnetum africanum* was 40 mg/kg in feed.

Liver function tests

On day 30, rats given GAE (40 mg/kg in feed) for 90 days had significantly ($p < 0.05$) lower AST than the control group and rats given GAE (10, 20 and 40mg/kg in feed) had significantly ($p < 0.05$) higher serum ALP activity. However, on day 60, rats in the control group had significantly ($p < 0.05$; $p < 0.01$) higher serum ALP activity compared to those that received GAE in feed at all the doses. There were no significant ($p > 0.05$) differences in the serum ALT activity in rats given GAE in feed and the control. Serum albumin was significantly ($p < 0.05$) higher in rats that received GAE (4 mg/kg in feed) on day 90, compared to the control. Also on day 90, rats in the control group had significantly ($p < 0.01$) higher levels of total serum bilirubin than those given GAE in feed (20 mg/kg) (Table 1). There were no significant differences between the serum protein of rats given GAE in feed and the control (Fig. 1).

Kidney function test

The results of the changes in serum urea and creatinine of rats given GAE in feed for 90 days is presented in Table 4. On day 30, rats that received GAE (10 mg/kg in feed) had significantly ($p < 0.05$) lower serum urea levels compared to the control. However, on day 90, rats given GAE in feed (10 and 40 mg/kg) had significantly ($p < 0.05$) higher levels of serum urea compared to the control. Serum creatinine levels were significantly reduced ($p < 0.05$; $p < 0.01$) in rats given GAE in feed than the control on days 30 and 60.

Histopathology

There were neither gross nor histopathological lesions in the liver, kidney, heart, pancreas, and lungs of rats in all the days of the study. Photomicrographs of the liver, kidneys, heart, and pancreas of rats at day 90 are presented in Figures 2, 3, 4, and 5.

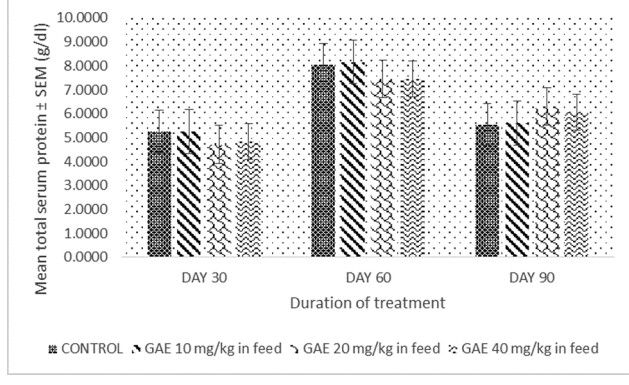

Figure 1. Total serum protein in rats given GAE in feed for 90 days.

Table 1. Liver function tests of rats given GAE in feed for 90 days.

Group	Treatment	Day 30	Day 60	Day 90
	AST ± SEM (U/L)			
1	Control (no GAE)	64.00 ± 3.09	24.32 ± 7.90	65.45 ± 5.58
2	GAE 10 mg/kg in feed	50.62 ± 6.00	40.52 ± 15.89	73.00 ± 1.91
3	GAE 20 mg/kg in feed	69.37 ± 1154	22.20 ± 4.87	73.25 ± 2.75
4	GAE 40 mg/kg in feed	40.70 ± 6.16*	34.45 ± 14.14	74.75 ± 2.86
	ALP ± SEM (U/L)			
1	Control (no GAE)	20.67 ± 4.44	180.78 ± 28.42	43.44 ± 7.85
2	GAE 10 mg/kg in feed	33.56 ± 4.61*	103.50 ± 20.70*	57.73 ± 5.2
3	GAE 20 mg/kg in feed	32.62 ± 1.82*	62.79 ± 3.45**	59.24 ± 4.74
4	GAE 40 mg/kg in feed	38.05 ± 1.75*	85.56 ± 21.10**	52.27 ± 7.62
	ALT ± SEM (U/L)			
1	Control (no GAE)	14.20 ± 3.22	20.12 ± 4.34	16.10 ± 1.52
2	GAE 10 mg/kg in feed	11.93 ± 4.24	22.28 ± 9.36	16.88 ± 2.92
3	GAE 20 mg/kg in feed	10.50 ± 3.36	14.92 ± 5.16	21.2 ± 3.84
4	GAE 40 mg/kg in feed	12.90 ± 2.91	16.71 ± 7.83	22.8 ± 2.49
	Albumin ± SEM (g/dl)			
1	Control (no GAE)	3.23 ± 0.09	3.60 ± 0.13	2.61 ± 0.28
2	GAE 10 mg/kg in feed	3.26 ± 0.11	3.78 ± 0.18	3.28 ± 0.28
3	GAE 20 mg/kg in feed	2.96 ± 0.19	3.46 ± 0.12	3.49 ± 0.20
4	GAE 40 mg/kg in feed	2.85 ± 0.17	3.29 ± 0.23	3.46 ± 0.11*
	Bilirubin ± (g/dl)			
1	Control (no GAE)	0.46 ± 0.02	0.32 ± 0.11	0.35 ± 0.07
2	GAE 10 mg/kg in feed	0.49 ± 0.07	0.11 ± 0.04	0.11 ± 0.03**
3	GAE 20 mg/kg in feed	0.43 ± 0.09	0.30 ± 0.05	0.30 ± 0.04
4	GAE 40 mg/kg in feed	0.46 ± 0.06	0.22 ± 0.02	0.22 ± 0.02

*$p < 0.05$, **$p < 0.01$ compared to the control; GAE = *Gnetum africanum* extract; SEM: standard error of mean.

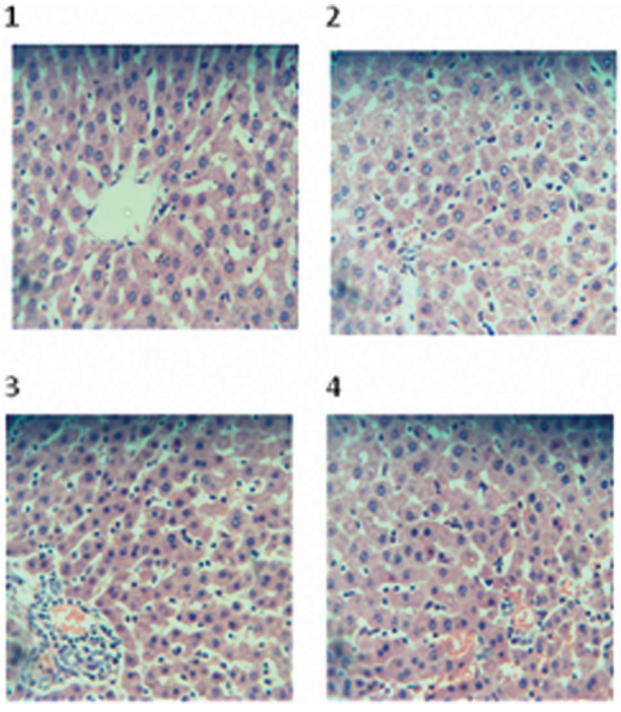

Figure 2. Liver photomicrograph of rats given GAE in feed at day 90, showing normal hepatocyte tissue (1—control; 2—GAE 10mg/kg in feed; 3—GAE 20 mg/kg in feed; 4—GAE 40 mg/kg in feed H and E (x400).

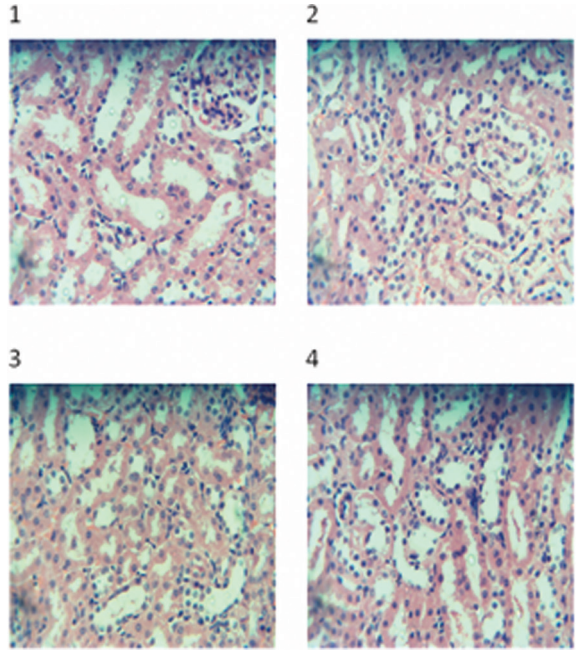

Figure 3. Kidney photomicrograph of rats given GAE in feed for 90 days showing normal renal tissues (1—control; 2—GAE 10 mg/kg in feed; 3—GAE 20 mg/kg in feed; 4—GAE 40 mg/kg in feed. H and E. (X400).

Discussion

Acute and chronic toxicity studies of the methanol leaf extract of *Gnetum africanum* were conducted using rat models. The absence of mortalities and signs of toxicity in rats treated with a single dose of *Gnetum africanum* up to 5,000 mg/kg and observed for 48 hours suggest that the plant was well tolerated and safe. However, *Gnetum africanum* is used for the treatment of diabetes, which is a chronic condition. Therefore, oral subchronic toxicity tests using this plant were conducted on albino rats to determine the long-term effects of its oral consumption. There were no signs of drug toxicity in rats over the period of the test; there were also no mortalities recorded. NOAEL (40 mg/kg) is the highest experimental point that is without adverse effect [24]. Although *G. africanum* has been reported to contain anti-nutritional and toxic components, they occur at levels below their toxicity thresholds. Ali, Assanta and Robert [13] reported that whereas the toxicity level for hydrocyanic acid is 35 mg/10 g dry matter, the level of this acid in the leaves of this plant is only 0–5.4 mg/100g. Also for oxalate to be toxic, it must be present in concentrations of the order of 2–5 g/100 g dry matter, whereas in *G. africanum* concentrations of 16–68 mg/100 g DM were detected [13]. However, many toxicity studies on *Gnetum africanum* in the literature were done using 3–30-day toxicity tests [25–27]. Data on subchronic tests are scarce and as such are provided by this work.

The liver is the organ involved in metabolic degradation of both natural and synthetic chemicals, during which process hepatotoxic metabolites may be generated, leading to liver injury [7]. Liver function tests performed including assay of activities of marker enzymes AST, ALP, ALT as well as determination of the levels of total bilirubin, albumin, and protein enable more incisive information to be obtained in a live animal on the state of the liver. Assay of activities of body fluid enzymes is a major aspect of toxicological investigation and an indicator of probable tissue damage [28]. Harmful substances put stress on the liver and this can be detected as increases in liver enzyme activities. AST and ALT are cytosolic enzymes involved in transamination reactions during amino acid metabolism [29]. The absence of high levels of these enzymes in the treated rats suggests that the extract did not induce inflammatory injury on the hepatocyte membrane and, thus, there were no leakages of this cytosolic enzyme. ALP, on the other hand, is useful for diagnosis of obstructive liver diseases [30]. The

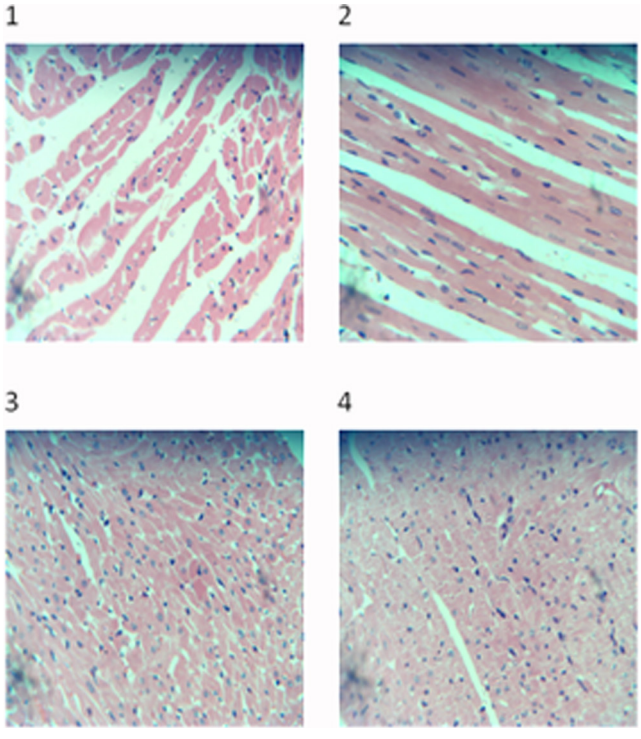

Figure 4. Photomicrograph of the heart showing normal cardiac tissue (1—control; 2—GAE 10 mg/kg in feed; 3—GAE 20 mg/kg in feed; 4—GAE 40 mg/kg in feed. H and E . (X400).

initial increase in ALP of treated rats suggests an obstruction, which was later counteracted as the rats adapted to the extract. On day 90, rats given GAE in feed had significantly ($p < 0.05$) higher serum albumin than the control (Table 3). This suggests that *G. africanum* does not have adverse effects on the ability of the liver to produce albumin needed for the transport of substances in the blood [30]. Bilirubin levels were significantly ($p < 0.05$) higher in the control rats than in the treated rats (Table 3). Bilirubin is the final product of heme degradation and at physiologic pH, it is insoluble in plasma and requires protein binding with albumin.

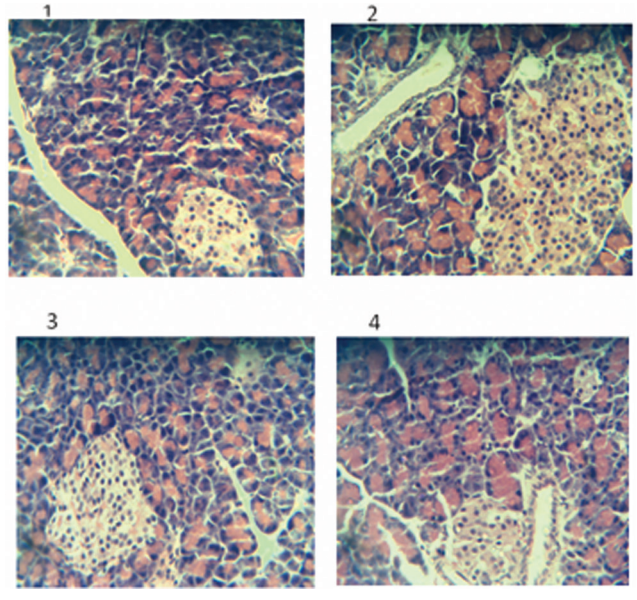

Figure 5. Photomicrograph of the pancreas showing normal pancreatic tissues (1—control; 2—GAE 10 mg/kg in feed; 3—GAE 20 mg/kg in feed; 4—GAE 40 mg/kg in feed. H and E (x400).

After conjugation in the liver, it is excreted in the bile. Toxic hyperbilirubinemia is a type of unconjugated hyperbilirubinemia that occurs following toxin-induced liver dysfunction which impairs conjugation [31]. The lower bilirubin levels recorded in treated rats as opposed to the control is also is an indication of the nontoxic nature of GAE after a 90-day subchronic exposure. The total protein levels in control and treated rats were not significantly ($p > 0.05$) different (Fig. 1). This equally suggests that GAE has no deleterious effect on the liver as abnormal protein levels could be indicative of liver injury. Urea and creatinine are indices of renal function and kidney damage and can be used to diagnose impaired renal function. Although serum creatinine levels were not adversely affected by the extract, care should be taken in kidney dis-

Table 2. Kidney function tests for rats given GAE in feed.

Group	Treatment	Day 30	Day 60	Day 90
	Urea ± SEM (mg/dl)			
1	Control (no GAE)	24.75 ± 0.97	29.92 ± 2.58	19.47 ± 1.60
2	GAE 10 mg/kg in feed	18.08 ± 0.64*	32.98 ± 1.22	29.21 ± 1.17*
3	GAE 20 mg/kg in feed	24.16 ± 3.55	24.10 ± 3.76	22.63 ± 2.61
4	GAE 40 mg/kg in feed	24.91 ± 0.98	29.57 ± 1.38	27.73 ± 2.62
	Bilirubin ± (g/dl)			
1	Control (no GAE)	0.54 ± 0.05	1.12 ± 0.09	1.26 ± 0.08
2	GAE 10 mg/kg in feed	0.38 ± 0.03*	1.06 ± 0.15*	1.06 ± 0.05
3	GAE 20 mg/kg in feed	0.34 ± 0.01**	0.90 ± 0.60	1.10 ± 0.05
4	GAE 40 mg/kg in feed	0.39 ± 0.05*	1.03 ± 0.07*	1.09 ± 0.6

*$p < 0.05$, **$p < 0.01$ when compared with the control; GAE= *Gnetum africanum* extract; SEM: standard error of mean.

ease patients due to increases in the serum urea observed on day 90.

Conclusion

The findings from this study suggest that *G. africanum* is safe both at the acute and subchronic levels. The extract did not elicit any herb-induced hepatic injury. However, patients with impaired renal function should take it with caution, bearing in mind the increase in urea levels which was observed. However, histological examinations showed that the kidneys were not affected by the plant. Further study is needed to identify and characterize the beneficial bioactive principles in this plant which can be said to be nontoxic.

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