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ANTIOXIDANT ACTIVITY AND TOXICOLOGICAL IMPLICATIONS OF THE AQUEOUS EXTRACT OF *Phyllanthus amarus* (Euphorbiaceae) LEAVES IN FEMALE WISTAR RATS

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ABSTRACT:
The aqueous extract of *Phyllanthus amarus* leaves (AEPAL) have been used in the treatment of female sexual dysfunction without recourse on its safety. Hence, the need to ascertain the safety of this plant by establishing the toxicological implication of consuming the plant and increasing their acceptability. Twenty-eight female Wistar rats (159.25 ± 3.32 g) were randomized into four groups (A - D) of seven animals each and orally administered 0.5 mL of distilled water, 20, 40 and 80 mg/kg body weight of *P. amarus* leaves extract respectively once daily for 21 days. The toxicological effects of the AEPAL were evaluated by assessing selected parameters in the liver, kidney and antioxidant system. All parameters measured were analyzed using standard procedures. The levels of liver total protein, calcium ion, catalase, superoxide dismutase, glutathione peroxidase and glutathione reductase increased significantly ($p < 0.05$), whereas the activities of glucose-6-phosphate dehydrogenase and concentration of reduced glutathione, urea, creatinine and sodium ion decreased significantly ($p < 0.05$). There were no significant changes in the activities of lactate dehydrogenase, liver and serum alkaline phosphatase as well as the levels of malondialdehyde and total antioxidant capacity. The results obtained in the present study indicate that the AEPAL is nephroprotective but may cause microvesicular steatosis in the liver at a dose of 80 mg/kg body weight.

Keywords: Antioxidant; Euphorbiaceae; hepatotoxicity; histoarchitecture; *Phyllantus amarus*; medicinal biochemistry

INTRODUCTION:

Medicinal plants, either as extracts, polyherbal mixtures, pure compounds or derivatives offer unlimited opportunities towards the discovery of new drugs [1]. The biological activities of various medicinal plants used in folklore medicine have been validated or refuted with scientific evidence. The general acceptability of herbal medicines has been limited by a lack of defined chemical characterization, dose regimen and adequate toxicity data to evaluate their safety [2]. Hence, there is a dearth of information on the toxicological implications of most of these plants majority of which are anecdotally perceived as safe. One of such plant is *Phyllantus amarus* Schum Thonn (Euphorbiaceae) commonly known as *eyin olobe* (Yoruba – Western Nigeria), *geeron tsutsaayee* (Hausa – Northern Nigeria) and ngwu ite kwowa nasu (Igbo – Eastern Nigeria) [3]. It is a small, erect, tropical, annual herb found in Philippines, Cuba, India, Malaysia and Nigeria. *P. amarus* can grow about 30 – 40 cm high and has slender leaves-bearing branchlets, anchored by distichous leaves which are subsessile, elliptically oblong, obtuse and rounded base. It is widely used in the treatment of various diseases such as diarrhoea, hepatitis, chronic dysentery, gonorrhea, diabetes, appendix inflammation, intermittent fever, scabies, gallstones, ulcers and prostate problems [4 – 6].

Nurudeen and Yakubu [7] reported the restoration of sexual competence in fluoxetine-induced female sexual dysfunction rats treated with the aqueous extract of *P. amarus* leaves at 80 mg/kg body weight. The acute and chronic toxicity study of *P. amarus* leaves revealed that there was no mortality at 5000 mg/kg body weight as well as the absence of functional and structural toxicity at 100, 400 and 800 mg/kg body weight respectively [8]. The extracts of *P. amarus* (aqueous, methanolic and hydromethanolic) and P. fraternus (hydroethanolic) had no toxic effect on WBC and RBC counts as well as the enzymes used for liver function tests (ALT, AST and ALP) [9]. The hepatotoxic assessment of the methanolic extract of P. amarus leaves at 1000, 2000 and 5000 mg/kg body weight revealed that the extract was relatively safe for consumption [6]. Despite the avalanche of studies on the toxicological evaluation of *P. amarus* leaves extract in open scientific literature, the efficacy and toxicity of plants vary by geographical location and soil composition. Hence, this study was designed to evaluate the toxicological implication of the aqueous extract of *P. amarus* leaves grown in Ilorin, Nigeria at 20, 40 and 80 mg/kg body weight on the functional capacity of the liver, kidneys and antioxidant system.

MATERIALS AND METHODS:

Collection and authentication of the plant material:

Fresh *Phyllanthus amarus* leaves were collected behind the Administrative building of Al-Hikmah University, Ilorin, Nigeria (Latitude 8° 29' 47.90" N; Longitude: 4° 32' 31.70" E) and authenticated at the Herbarium unit of the Department of Plant Biology, University of Ilorin, Ilorin, Nigeria. A voucher specimen was deposited under UIH 001/1109.

Experimental animals:

Twenty-eight healthy, female Wistar rats (*Rattus norvegicus*) weighing 159.25 ± 3.32 g were obtained from the Animal Holding Unit of the Department of Biochemistry, University of Ilorin, Ilorin, Nigeria. The animals were contained in their respective cages placed in a well-ventilated Animal House and maintained at a temperature of 25 ± 3oC, 12 hours light and dark cycle, relative humidity of 45-50%. The animals were maintained on rat pellets (Premier Feeds, Ibadan, Nigeria) and tap water ad libitum. All the animals were strictly handled in conformation to the Declarations of Helsinki in 1995 (as revised in Edinburgh 2000) and the University's guidelines on Care and Use of Laboratory Animals (approval number – UERC/ASN/2015/210).

Reagents and assay kits:

The assay kits used for the determinations of Albumin, Total and Conjugated Bilirubin, Alanine Aminotransferase (ALT), Aspartate Aminotransferase (AST), Alkaline Phosphatase (ALP), Lactate Dehydrogenase (LDH), Glucose-6-phosphate Dehydrogenase (G6PDH), Catalase (CAT), Glutathione Reductase (GRe), Glutathione Peroxidase (GPx), Reduced Glutathione (GSH), Urea, Creatinine, Electrolytes (Na, K, Ca, Cl and PO4), Superoxide Dismutase (SOD), Malondialdehyde (MDA) and Total Antioxidant capacity (TAC) were products of Randox Laboratory Ltd, Co-Atrim, UK. All other reagents used were of analytical grade and prepared using distilled water and stored in air-tight reagent bottles except otherwise stated.

Preparation of plant extracts:

Fresh leaves of *P. amarus* were removed from the stalks and rinsed in distilled water. Thereafter, the leaves were oven-dried at 40oC (Quincy Laboratory Oven, Model 30 GC, Chicago, USA) for 48 hours and pulverized in a blender (Master Chef Blender, Model MC-BL 1980, China). The powdered leaves (50 g) were extracted in 1000 ml of distilled water for 48 hours at room temperature with intermittent shaking. The resulting filtrate was concentrated in a rotary evaporator (Model RE 52A Zhengzhou, Henan, China) to give a yield of 6.97 g corresponding to a percentage yield of 13.94 %. This was reconstituted in distilled water to give the required doses of 20, 40 and 80 mg/kg body weight of the rats. The selected doses were

adopted from the previous study by Nurudeen and Yakubu [7] on the efficacy of AEPAL in the management and/or treatment of female sexual dysfunction.

Animal grouping and administration of plant extracts:

A total of 28 female rats were acclimatized for 2 weeks and randomly assigned into four groups (A-D) of seven animals each. The rats in group A were orally administered 0.5 ml of distilled water only (control group) whereas those of groups B, C and D (experimental groups) received equal volume of AEPAL corresponding to 20, 40 and 80 mg/kg body weight respectively for 21 days.

Preparation of serum and tissue supernatants:

The procedure described by Yakubu and Salimon [10] was adopted for the preparation of serum and tissue supernatants. Twenty-four hours after the last administration (Day 22), the rats were anesthetized in diethyl ether fumes to render them unconscious, and then weighed. The jugular veins were then cut for the collection of blood samples into clean, dry centrifuge tubes. The samples were allowed to clot for 15 minutes and then centrifuged (Biobase Laboratory Centrifuge, Model LC-4KA, Jinan, China) at 894 × g for 10 minutes. The sera were aspirated using Pasteur's pipette and kept frozen for 12 hours prior to biochemical analysis. The animals were then quickly dissected, and the liver and kidney were carefully removed, blotted and stored in ice-cold 0.25M sucrose solution. The organs were separately homogenized in ice-cold 0.25 M (1:5 w/v) sucrose solution and subsequently, the homogenates were centrifuged at 1789 x g for 10 minutes. The supernatants were frozen for 12 hours before being used for the determination of biochemical parameters.

Biochemical Analysis and Histological Examination:

The procedures described by Gornal *et al*. [11], Doumas *et al*. [12], Jandrassik and Grof [13], Tietz [14], Veniamin and Verkirtzi [15], Bartels and Bohmer [16] were adopted for the determination of total protein, albumin, bilirubin (total and conjugated), globulin, urea and creatinine respectively. The concentrations of electrolytes (Na^+, K^+, Ca^{2+}, Cl^- and PO_4^{2-}) were determined as described by Tietz [14] while the levels of GSH, MDA and TAC were determined following the procedures described by Ellman [17], Ohkawa *et al*. [18] and Miller *et al*. [19] respectively. The activities of ALT, AST, ALP, CAT, G6PDH, GRe, GPx, LDH and SOD were determined using standard procedures as described by Reitman and Frankel [20], Wright *et al*. [21], Aebi [22], Beutler [23], Goldberg and Spooner [24], Prabhu *et al*. [25], Wroblewski and La due [26] and Fridovich [27] respectively. Histological examination was carried out following procedures described by Krause [28].

Data Analysis:

Data were expressed as means ± standard error of mean (Mean ± SEM) of seven determinations. The Statistical Package for Social Sciences, Version 20.0 (SPSS Inc., Chicago, IL, USA) was used for data analysis and further subjected to Duncan Multiple Range Test. Differences were considered statistically significant at $p < 0.05$.

RESULTS:

There was dose dependent increase in the levels of liver total protein and calcium ions whereas the levels of serum total protein, albumin, globulin and conjugated bilirubin were not significantly ($p > 0.05$) altered by the aqueous extract of P. amarus at all doses investigated compared to the controls (Table 1).

Similarly, there was a significant ($p < 0.05$) increase in the activities of ALT and AST of both the liver and serum (Table 2). The activity of G6PDH was significantly ($p < 0.05$) increased in a dose-dependent manner when compared to the control group that received distilled water only, whereas the activities of LDH, liver-ALP and serum-ALP were not significantly ($p > 0.05$) altered (Table 2). There was a significant dose-dependent decrease in the concentrations of urea, creatinine and sodium ion (Table 3).

The levels of phosphate ion were significantly ($p < 0.05$) increased at 20 and 40 mg/kg body weight only, whereas there was no alteration in the levels of potassium and chloride ions at all doses investigated (Table 3). Furthermore, the activities of CAT, SOD, GPx and GRe were significantly ($p < 0.05$) increased in a dose-dependent manner (Table 4). There was a significant decrease in the levels of GSH at 40 and 80 mg/kg body weight of the extract while the levels of TAC and MDA were not significantly ($p > 0.05$) altered at all doses investigated (Table 4). The histo-architecture of the liver of rats treated with the aqueous extract of P. amarus at all doses investigated revealed a normal sinusoid with no hepatocellular infiltration (Plates 1 – 4). However, there was a mild, moderate and severe microvesicular steatosis in rats on 20, 40 and 80 mg/kg body weight of the extracts respectively (Plates 2, 3 and 4). Compared with the control that showed normal glomeruli, mesangial cells, tubules and interstitial space (Plate 5), there was no histoarchitectural changes in the kidneys of rats administered 20 and 80 mg/kg body weight of the extract (Plates 6 and 8). However, there was mild tubular necrosis in rats administered 40 mg/kg body weight of aqueous extract (Plate 7).

Table 1: Effects of the aqueous extract of *Phyllantus amarus* leaves on the liver function indices of female Wistar rats

Parameters/Dose (mg/kg body weight)	Control	20 mg/kg body weight	40 mg/kg body weight	80 mg/kg body weight
Liver total protein (g/l)	8.92 ± 0.37^{a}	10.75 ± 0.45^{b}	10.25 ± 0.31^{b}	11.28 ± 0.23^{c}
Serum total protein (g/l)	12.86 ± 1.55^{a}	13.20 ± 1.20^{a}	13.99 ± 1.98^{a}	13.72 ± 1.45^{a}
Albumin (g/l)	1.19 ± 0.06^{a}	1.20 ± 0.03^{a}	1.20 ± 0.00^{a}	1.22 ± 0.02^{a}
Globulin (g/l)	11.67 ± 1.01^{a}	12.00 ± 0.15^{a}	12.79 ± 0.75^{a}	12.50 ± 1.33^{a}
Total bilirubin (mg/ml)	2.94 ± 0.10^{a}	2.93 ± 0.00^{a}	2.89 ± 0.01^{a}	2.75 ± 0.03^{c}
Direct bilirubin (mg/dl)	2.38 ± 0.05^{a}	2.35 ± 0.10^{a}	2.35 ± 0.02^{a}	2.29 ± 0.04^{a}
Calcium ion (mg/dl)	6.88 ± 0.03^{a}	7.68 ± 0.11^{b}	8.92 ± 0.04^{c}	9.32 ± 0.08^{d}

Values are mean of 7 determinations ± SEM, values with different superscripts (a-d) across the rows for each parameter are considered to be significantly different at $p < 0.05$.

Table 2: Effects of the aqueous extract of *Phyllantus amarus* leaves on some liver enzymes in female Wistar rats

Parameters/Dose (mg/kg body weight)	Control	20 mg/kg body weight	40 mg/kg body weight	80 mg/kg body weight
Liver alanine amino-transferase (U/l)	41.87 ± 0.27^{a}	42.75 ± 0.19^{b}	44.13 ± 0.25^{c}	44.53 ± 0.30^{c}
Serum alanine amino-transferase (U/l)	29.67 ± 0.99^{a}	37.17 ± 1.02^{b}	41.83 ± 0.67^{c}	43.17 ± 0.82^{c}
Liver aspartate amino-transferase (U/l)	65.50 ± 2.19^{a}	74.67 ± 0.89^{b}	75.83 ± 0.75^{b}	75.00 ± 1.03^{b}
Serum aspartate amino-transferase (U/l)	35.40 ± 0.11^{a}	38.40 ± 0.50^{b}	39.40 ± 1.00^{b}	37.73 ± 1.15^{b}
Liver alkaline phosphatase (U/l)	11.96 ± 0.25^{a}	10.12 ± 1.08^{a}	10.92 ± 0.73^{a}	10.46 ± 0.39^{a}
Serum alkaline phosphatase (U/l)	1.84 ± 0.02^{a}	1.86 ± 0.05^{a}	1.79 ± 0.03^{a}	1.92 ± 0.10^{a}
Lactate dehydrogenase (U/l)	201.57 ± 12.45^{a}	209.12 ± 13.33^{a}	193.57 ± 12.75^{a}	235.42 ± 10.33^{b}
Glucose-6-phosphate dehydrogenase (U/l)	11.22 ± 0.33^{a}	22.43 ± 1.35^{b}	23.10 ± 0.88^{b}	33.65 ± 3.02^{c}

Values are mean of 7 determinations ± SEM, values with different superscripts (a-d) across the rows for each parameter are considered to be significantly different at $p < 0.05$.

Table 3: Effect of the aqueous extract of *Phyllantus amarus* leaves on the kidney function indices of female Wistar rats

Parameters/Dose (mg/kg body weight)	Control	20 mg/kg body weight	40 mg/kg body weight	80 mg/kg body weight
Urea (mm/l)	30.20 ± 0.81^{a}	18.49 ± 1.05^{b}	15.55 ± 0.15^{c}	11.09 ± 0.33^{d}
Creatinine (mg/ml)	2.96 ± 0.05^{a}	2.34 ± 0.09^{b}	1.79 ± 0.04^{c}	1.23 ± 0.12^{d}
Sodium ion (mEq/l)	129.55 ± 5.11^{a}	124.86 ± 3.08^{a}	118.04 ± 3.90^{b}	106.11 ± 10.88^{b}
Potassium ion (ppm)	6.10 ± 0.03^{a}	5.79 ± 0.25^{a}	5.55 ± 0.74^{a}	6.40 ± 0.32^{a}
Chloride ion (mg/dl)	111.25 ± 0.08^{a}	112.54 ± 0.19^{a}	112.11 ± 0.70^{a}	111.67 ± 0.82^{a}
Phosphate ion (mg/dl)	2.98 ± 0.02^{a}	3.53 ± 0.20^{b}	3.55 ± 0.05^{b}	3.07 ± 0.10^{a}

Values are mean of 7 determinations ± SEM, values with different superscripts (a-d) across the rows for each parameter are considered to be significantly different at $p < 0.05$.

Table 4: Effect of the aqueous extract of *Phyllantus amarus* leaves on enzymatic and non-enzymatic antioxidant parameters

Parameters/Dose (mg/kg body weight)	Control	20 mg/kg body weight	40 mg/kg body weight	80 mg/kg body weight
Catalase $\times 10^{2}$ (U/l)	20.00 ± 0.01^{a}	22.50 ± 0.05^{b}	22.55 ± 0.15^{b}	27.50 ± 0.50^{c}
Superoxide dismutase (U/l)	24.88 ± 0.58^{a}	49.75 ± 6.05^{b}	99.50 ± 7.55^{c}	149.75 ± 10.12^{d}
Glutathione peroxidase (U/l)	185.77 ± 0.39^{a}	132.63 ± 4.11^{b}	80.02 ± 3.89^{c}	35.54 ± 1.88^{d}
Reduced glutathione (U/mg)	8.75 ± 0.73^{a}	8.78 ± 1.05^{a}	8.05 ± 0.34^{ab}	7.05 ± 0.72^{b}
Glutathione reductase (U/mg)	569.71 ± 7.51^{a}	334.61 ± 8.23^{b}	105.68 ± 5.07^{c}	76.87 ± 2.29^{d}
Total antioxidant capacity (mg/dl)	82.80 ± 1.17^{a}	80.93 ± 0.91^{a}	84.85 ± 2.50^{b}	84.62 ± 1.85^{b}
Malondialdehyde x 10^{-5} (U/l)	2.30 ± 0.02^{a}	2.31 ± 0.02^{a}	2.33 ± 0.02^{a}	2.34 ± 0.01^{a}

Values are mean of 7 determinations ± SEM, values with different superscripts (a-d) across the rows for each parameter are considered to be significantly different at $p < 0.05$.

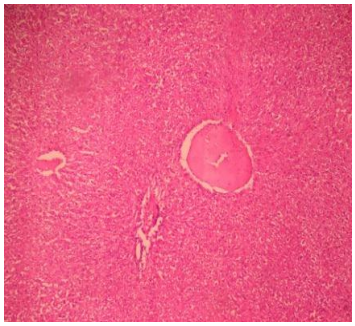

Plate 1: Cross section of the liver of control rat treated with distilled water (Mag. × 100; H & E)

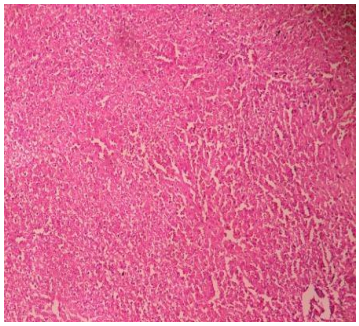

Plate 2: Cross section of the liver of rat treated with 20 mg/kg body weight of *P. amarus* leaves extract (Mag. × 100; H & E)

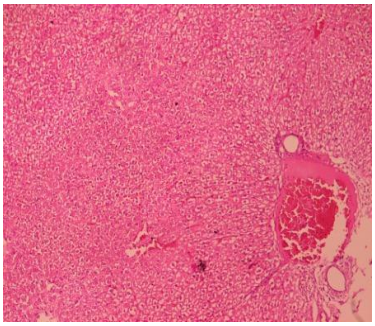

Plate 3: Cross section of the liver of rat treated with 40 mg/kg body weight of *P. amarus* leaves extract (Mag. × 100; H & E)

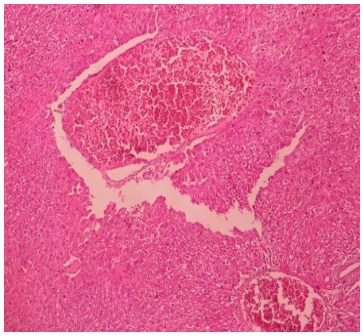

Plate 4: Cross section of the liver of rat treated with 80 mg/kg body weight of *P. amarus* leaves extract (Mag. × 100; H & E)

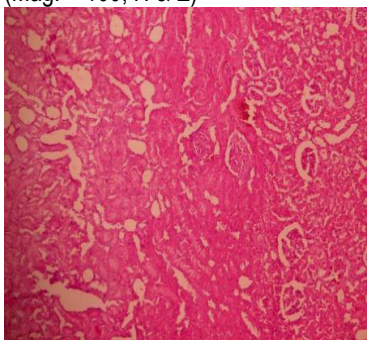

Plate 5: Cross section of the kidney of control rat treated with distilled water (Mag. × 100; H & E)

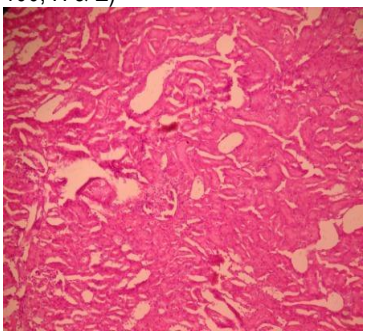

Plate 6: Cross section of the kidney of rat treated with 20 mg/kg body weight of *P. amarus* leaves extract (Mag. × 100; H & E)

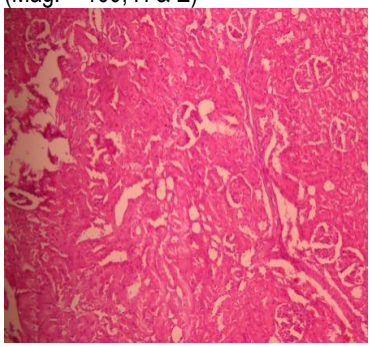

Plate 7: Cross section of the kidney of rat treated with 40 mg/kg body weight of *P. amarus* leaves extract (Mag. × 100; H & E)

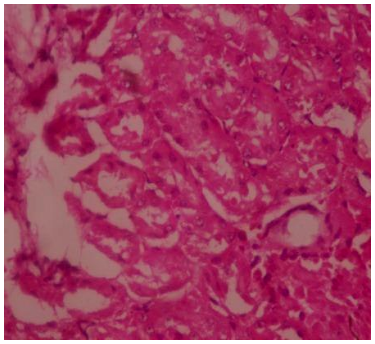

Plate 8: Cross section of the kidney of rat treated with 80 mg/kg body weight of *P. amarus* leaves extract (Mag. × 100; H & E)

DISCUSSION:

There is an ascendance in the rate of formulation and/or consumption of herbal products by the general populace due to the misconstrued belief that they are relatively safe unlike orthodox medicine with various known complications [29]. However, to declare a drug safe, it is imperative to determine the possible side effects, such as, the structural and functional integrity of the liver and kidney (the major organs involved in detoxification/biotransformation) following the use/consumption of the drug.

In the present study, total protein, albumin, globulin and bilirubin (total and direct) were monitored to assess the secretory and excretory functions of the liver [10]. The absence of significant changes in the levels of albumin, globulin and bilirubin implies that the functional capacity of the liver was not affected by any of the components in the extract. The dose-dependent increase in the levels of protein and calcium ions can be attributed to the abundance of essential amino acids and minerals in P. amarus leaves [30], which could result in improved bone density, muscle development, organ and tissue repair. Our findings were consistent with those of Oduola *et al*. [6] and Sirajudeen et al. [9] where the aqueous extract of P. amarus leaves was found to be relatively safe for consumption.

Tissue and serum enzyme activities were evaluated to monitor toxicity at cellular level [31]. The enzymes ALT and AST are important markers for hepatocellular impairment or necrosis [10]. The elevation in the levels of ALT and AST in both liver and serum might imply enhanced de novo synthesis [32]. This was further corroborated by the absence of changes in the activity of liver and serum ALP, a plasma membrane enzyme that characterizes the leakage of cytosolic content (ALT and AST) in the biliary tract to the external milieu [31].

The activity of LDH often increases during tissue degeneration, a catabolic process occurring due to increased anaerobic respiration [33]. The absence of alterations in the activity of LDH at 20 and 40 mg/kg body weight suggests that the extract is safe at these doses whereas the significant increase in activity at 80 mg/kg body weight might be an indication that the extract have caused tissue breakdown or increased hypoxic events in the experimental animals. G6PDH is a cytoplasmic enzyme in the Pentose phosphate pathway; it is involved in the biosynthesis of Ribose-5-phosphate (a precursor for the biosynthesis of Purines and Pyrimidine) and the production of Reduced Nicotinamide Adenine Dinucleotide Phosphate (NADPH) which is required for the maintenance of GSH [34]. Various intracellular processes as well as

antioxidant system requiring reduction depends on the adequate supply of NADPH. The upregulation in the expression of G6PDH at all doses of the extract can be attributed to the protective role of some components in the plant against oxidative stress. The dose-dependent decrease in the concentrations of urea and creatinine in plasma is an indication of the nephroprotective effect of the plant as it stimulates the excretion of nitrogenous waste products. This was in corroboration with the absence of alterations in the concentrations of potassium, calcium and chloride ions. This study contrasts previous findings by Yakubu and Salimon [10] where the aqueous extract of Mangifera indica adversely affected the glomerular and tubular function in female rats. The reduction in the activities of GRe and GPx could be related to the superfluous mobilization of antioxidant enzymes towards the mop up of oxidative stress [31]. The decrease in the level of GSH might be consequence of the depletion of GRe and GPx [35]. Elevation of TAC at 40 and 80 mg/kg body weight as well as the lack of alteration in MDA levels at all doses investigated confirms the aqueous extract of P. amarus leaves as an antioxidant modulator as the extract was able to mitigate oxidative damage and protect membrane lipids [31].

The absence of gross distortion in the histo-architecture of the kidney implies that there was no treatment related structural toxicity. However, the presence of microvesicular steatosis at 80 mg/kg body weight might have resulted from the accumulation of lipids which can be attributed to impaired synthesis and/or elimination of triglycerides in the hepatocytes. Chemical profiling of AEPAL revealed the presence of anti-nutrients such as cyanide, oxalate, phytate and polyphenols in trace amounts [30]. Hence, the structural toxicity reported in this study might be attributed to the presence of these constituents due to accumulation over the duration of the study. This result was in tandem with the findings of Adebayo et al. [36] where the administration of Hepacare at 750 and 2500 mg/kg body weight inflicted mild – severe microvesicular steatosis in rat liver.

CONCLUSION:

The present study has demonstrated that the aqueous extract of P. amarus leaves may not induce significant toxic effects when administered at 20, 40 and 80 mg/kg body weight and thus may be safe for use at the recommended therapeutic dose. However, intake of higher doses should be avoided by consumers, especially patients with protracted liver diseases to avoid additional complications.

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