Study of Subacute Toxicity in Wistar Rats Challenged with *Phyllanthusamarus*Schum and Thonn

Abstract

Phyllanthusamarus, a widely used plant in complementary and alternative medicine practice in Southern Nigeria, is used in treating and managing numerous metabolic disorders, and neurodegenerative diseases. This study is designedto assess the subacute toxicity of *P. amarus* in Wistar rats using body and relative organ weight, renal function, *in-vivo* antioxidant status and organ histology indices as a baseline. The twenty adult male rats weighing 120.00 ± 6.18 g wereapportionedinto four groups with five rats per group. Group A (Control)received1.0 mL of distilled water, group B received 1000 mg/kg group C received 1500 mg/kg and group D received 2000 mg/kg body weight of the crude ethanol extract. The extract from *P. amarus*was administered orally once daily at 8:30 am using an oral cannula attached to a 2 mL syringe. Subacute toxicity was evaluated after 14 days. The findings showed no visible and noticeable overt signs of toxicity throughout the experimental period, non-significant ($p > 0.05$)adverse change in body and relative organ weight, renal function and organ histopathology of the rats in the treated and control groups. However,*P. amarus* significantly ($p < 0.05$) improved*in-vivo* antioxidant status while significantly reducing ($p < 0.05$) the level of malondialdehyde, a biological indicator of oxidative stress in the living system.

Keywords:*Phyllanthusamarus*, antioxidants, oxidative stress,

1.0 Introduction

Medicines from plant sources commonly referred to as herbal medicine are an important alternative therapeutic aid for both curative and prophylactic purposes in developing and developed countries,primarily because of their wide range and diversity of phytochemicals inherent in them and their use dates back to antiquity. These phytochemicals include the different classes of flavonoids, tannins, saponins, and glycosides. Herbal medicine often involves using one or more plant component such as leaves, flowers, stem (bark) and roots, or a combination of two or more plants that work synergistically to heighten therapeutic potentials and other benefits. The active ingredients in these plant materials for medicinal purposes, if adhered to strictly in dose and regimen, are safe and reliable [1,2],cheap and nearly often available all year round [3]. Drugs from plants have been used with proven track records in the treatment and management of malaria, diabetes, hypertension, infertility, erectile

dysfunction and management of hypoactive sexual disorders in men and women, cardiovascular disease, neurological diseases etc. [4,5].

Phyllanthusamarus is one such plant frequently used in tropical and subtropical countries with ethnomedicinal applications as antimicrobial, anti-inflammatory, antidiabetic, anticancer, and antiplasmodial[6]. *P. amarus* also possesses potent antioxidant and diuretic properties [7].“*P. amarus*a member of the family *Euphorbiaceae* and the genus *Phyllanthus* and the species is *amarus*. it has nearly 800 species andiswidely distributed in tropical and subtropical countries” [8].“There is a general beliefby herbal practitioners and users of herbal medicine that because herbal products are sourced from nature they are therefore free of adverse or toxic effects unlike most synthetic drugs” [9,10]. Hence, the toxicity and adverse effects of most herbal products are often not evaluated and as such the users often look at the therapeutic advantage of the plant and disregard their toxic effects onthe body’s vital organs and tissues.

Arising from the widespread usage of *P. amarus* in folklore medicine, this study seeks to appraise the sub-acute toxicity of*P. amarus*for safety or possible toxic effects using alterations in body and organ weight, antioxidant status, renal function activities and liver histology as indices of toxicity in rats.

2.0 MATERIALS AND METHODS

2.1 Sample Collection and Identification

*P. amarus*washarvestedaround the Faculty Building (FB1) Laboratory of the Federal University Otuoke,Bayelsa State, Nigeriaon the 15th day of March 2024. The Plant Science section of the Biology Department, Federal University Otuoke, Bayelsa State identified and confirmed the plant sample.

2.2 Preparation of plant extract

The sampleswere painstakingly washed with distilled water to eliminate trash and contaminants, it was then air dried for 14 daysto give a persistent weight and then milled using an electric blender (Blender 462 Nakai Japan). 100 g of the powdered *P.amarus*was extracted in 300 mL of absolute ethanol for 24 hours at room temperature with continuous shaking using a flask shaker (Denly A 500). The extract was filtered with WhatmanNo.1 filter paper and the resultant filtrate evaporated to dryness using a rotary evaporator at 40 to give 4.33 g of the crude extract.

2.3 Experimental Animals

Twenty healthy, male albino rats weighing 120.00 ± 6.18 g were purchased from the Animal House section, Department of Biochemistry, Federal University Otuoke, Bayelsa State, Nigeria. The animals were kept in separate investigational rooms, which were clean and well-ventilatedat a temperature between 28-30℃, under a natural dark/light cycle with free access to standard rat chow and water *ad-libitum* during the period of acclimatization which lasted for one week.

2.4 Animal Grouping

The twenty adult male rats were divided into four groups with five rats per group.Group A (Control),received 1.0 mL of distilled water,group B, received 1000mg/kg,group C, received 1500 mg/kg andgroup D, received 2000 mg/kg body weight of the extract.The extract from *P. amarus*was administered orally once daily at 8:30 am using an oral cannula attached to a 2 mL syringe. These doses were carefully chosen to avoid the LD_{50} but marginally above the effective dose of *P. amarus.* The rats were fed*ad libitum* with standard chow and tap water throughout the experimental protocol. The study was carried out for 14 days and on the 15th day, the animals were sacrificed.

2.5 Weekly Cage Side Surveillance for Physical Signs of Toxicity

This was done by physical examination of the rats from the cage sides for overt signs of toxicity such assalivation, lacrimation, eye dullness, eye opacity, diarrhoea, restlessness, red stained muzzle, lethargy, piloerection, skin appearance, subcutaneous swelling, loss of appetite, colour and consistency of faeces abdominal distension and mortalityarising from the administration of the different doses of *P. amarus.*

2.51 Changes in body weight

Rats in all groups were weighed on the first day and after the treatment protocols. Percentage change in body weight was evaluated by the expression below [11]

$$\%\ change\ in\ body\ weight\ = \frac{final\ body\ weight - initial\ body\ weight}{initial\ body\ weight} \times 100\%$$

2.52 Organ (kidney, liver and heart) as the ratio of body weight

Organs (kidney and liver) were removed and weighed immediately. The organ ratio was evaluated as a percentage with the expression below[11].

$$Organ\ ratio\ (\%)\ = \frac{weight\ of\ organ\ (g)}{body\ weight\ (g)} \times 100\%$$

2.6 Animal handling procedure and sample preparation technique

On day 15^{th} of the experimental protocol, the ratswere euthanized under anaesthesia using diethyl ether chamber,blood specimenwere obtained by cardiac puncture into plain sample bottles. The blood specimenwas allowed to stand for 20 minutes for coagulation to occur, afterwards, the specimenwas centrifuged at 2000 rpm for 10 minutes and the supernatant (serum) was collected and stored in the refrigerator before biochemical assay.The liver and kidney were dissevered out instantaneously for histological studies.

2.7 Biochemical Assay Kits

Assay kits for renal function indices and antioxidants are products of Randox Laboratories Ltd., United Kingdom. All other reagents/chemicals were obtained from standard suppliers and of analytical grade.

2.71 Biochemical Analysis of Antioxidant Enzymes

Catalase activity was estimated by the method of Cohen *et al*. [12]. "Superoxide dismutase (SOD) activity was by the methods of Misra and Fridovich"[13]. The assay method of Hunter *et al*. [14] as modified by Gutteridge and Wilkins [15]was adopted for the assay of Malondialdehyde (MDA) concentration.

2.72 Biochemical Analysis of Renal Function

Blood urea nitrogen (BUN)was evaluated by the Berthelot method as modified by Tobacco *et al.* [16]. Creatinine (CRT)was assayed by the colourimetric kinetic method of Bartels *et al.*[17]. Uric acid(UA)was assessed using the enzymatic colourimetric method of Duncan *et al*. [18].

2.8 Histopathological Scrutiny of the Organs

"The liverand kidney slices for histopathology were fixed in 10% formal saline and embedded in paraffin wax blocks, sections of 5μm thick were stained with hematoxylin and eosin (H&E) and then examined under a light microscope for determination of derangement and pathological changes" [19,20]

2.9 Statistical analysis

Experimental values were expressed as means ± SD. To determine differences between the groups studied, a one-way analysis of variance (ANOVA) with Duncan post hoc test was used to compare the group means, and $p < 0.05$ was considered statistically significant. SPSS for Windows version 23.0 (IBM Corp, USA) was used for the statistical study. The charts were plotted using GraphPad Prism 8.

3.0 RESULTS

3.1 The Effect of *P. amarus*extract onphysical signs of toxicity

Weekly observation for overt toxicity symptoms in the rats from the cage sides shows that the animals did not exhibit any sickly signs as presented in table 1

Table .1: Weekly Cage Side Scrutiny for Physical Signs of Toxicity of *P. amarus* Extract on Male Wistar Rats

S/N	PHYSICAL SIGNS OF TOXICITY	DAY 1				DAY 7				DAY 14			
		Control	1000 mg/Kg	1500 mg/Kg	2000 mg/Kg	Control	1000 mg/Kg	1500 mg/Kg	2000 mg/Kg	Control	1000 mg/Kg	1500 mg/Kg	2000 mg/Kg
1	Salivation	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil
2	Lacrimation	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil
3	Eye dullness	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil
4	Eye opacity	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil
5	Diarrhea	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil
6	Restlessness	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil
7	Red stained muzzle	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil
8	Lethargy	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil
9	Piloerection	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil
10	Skin appearance	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
11	Subcutaneous swelling	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil
12	Loss of appetite	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil
13	Colour and consistency of faeces	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
14	Abdominal distension	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil
15	Mortality	0	0	0	0	0	0	0	0	0	0	0	0

3.2 The Effect of *P. amarus*extracton Body Weight Indicators

The results of the*P. Amarus*extract on changes in body weight is depicted in Fig. 1.0.The oral administration of the extract caused increase in body weight but with no significant differences ($p > 0.05$) in the body with respect to the control and the respective working doses. Also non-significant changes ($p > 0.0$) was observed with respect to liver weight, relative liver weight, kidney weight, and relative kidney weight, heart weight and relative heart weight.

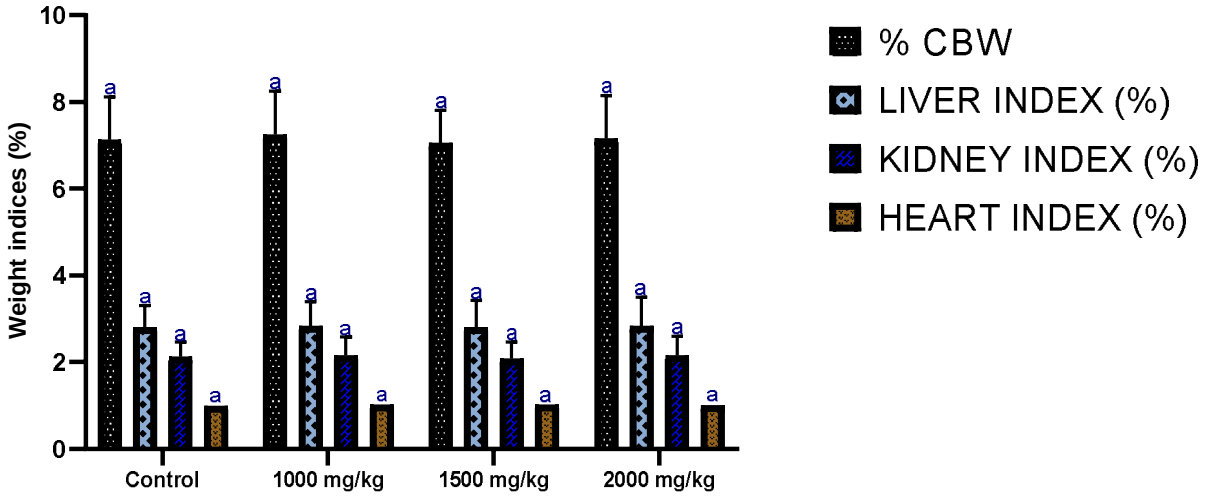


Fig 1.:Sub-Acute Effects of *P. amarus* Extract on Body Weight Indicators of Male Wistar Rats. Data are mean ± SD of triplicate determinations, values with identical bars with thesame superscript letter are not significantly different $p > 0.05$. One-way Analysis of Variance (ANOVA).

3.3 The subacute effect of *P. amarus*extract on *in-vivo* antioxidant enzymes of male Wistar rats

The effect of*P. amarus*extract on *in-vivo* antioxidant enzymes of rats in unit/mg tissue is presented in Fig.2. Findings from the result indicateda significant differences ($p < 0.05$) between the control group and the respective working doses of 1500 and 2000 mg/kg body weight with regards to catalase, superoxide dismutase. Malondialdehyde was also significantly $p < 0.05$ reduced.

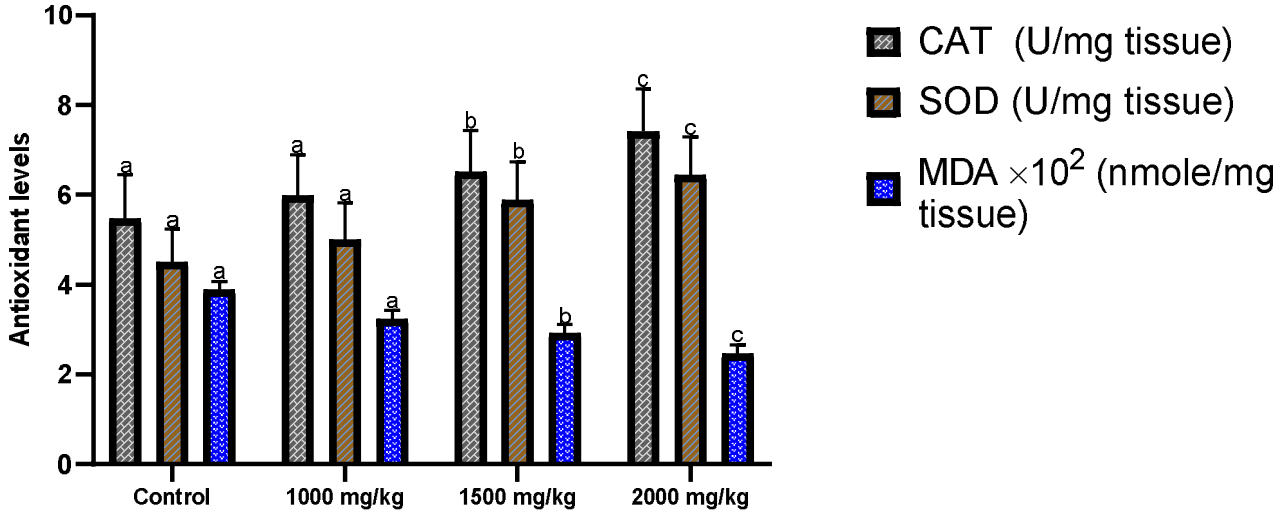


Fig 2.: Sub-Acute Effects of *P. amarus* Ethanol Extract on *In-vivo* Antioxidant Status of Male Wistar Rats. Data are mean ± SD of triplicate determinations values with identical bars but with different superscript letter are significantly different $p < 0.05$.

KEY: CAT-Catalase, SOD- Superoxide dismutase, MDA-Malondialdehyde

3.4 Sub-Acute Effects of *P. amarus* Extract on Renal Function Indices of Rats

The effect of*P. amarus*extract on the renal function of rats in mg/dL after 14 days' treatment regimen is presented in Fig. 3. The results obtained indicated a non-significant change ($p > 0.05$) on the concentrations of creatinine, blood urea nitrogen and uric acid.

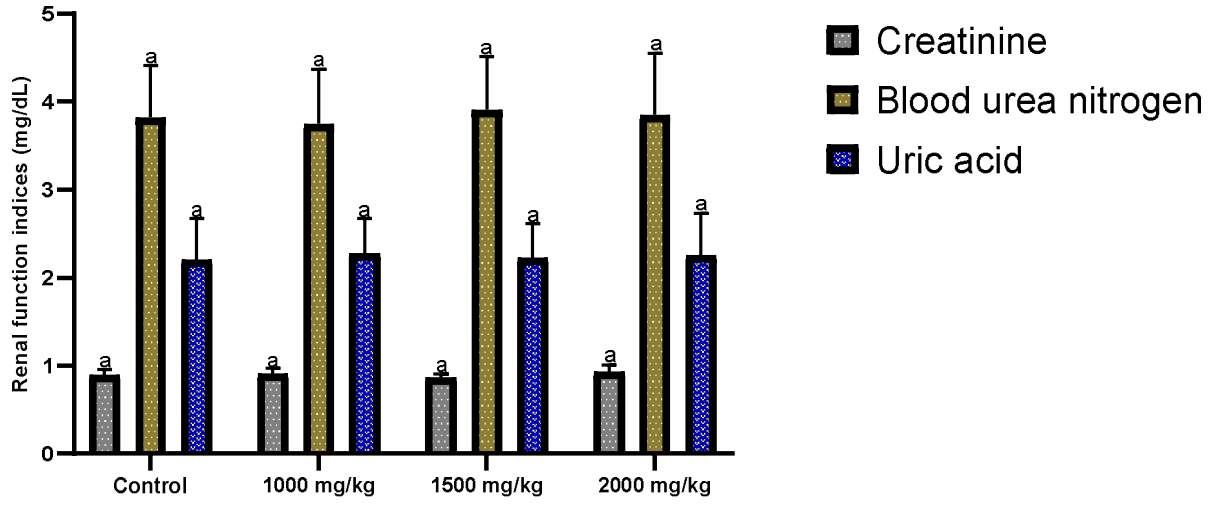
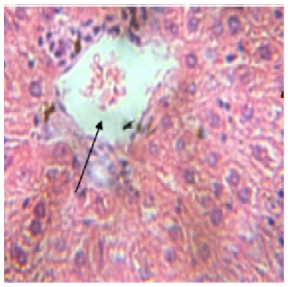
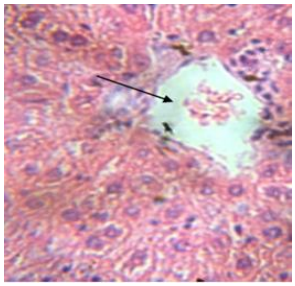
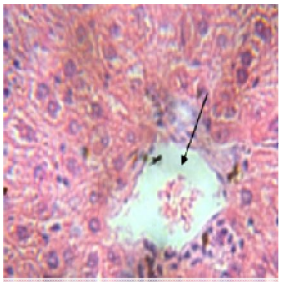
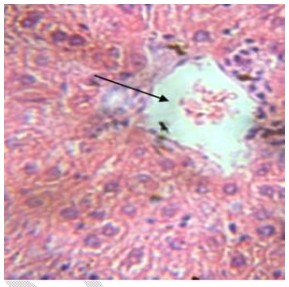
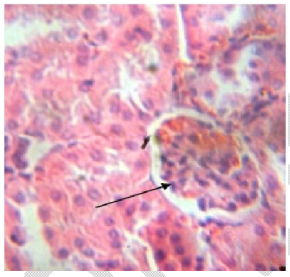
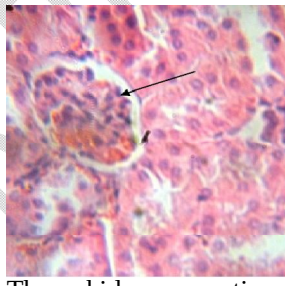
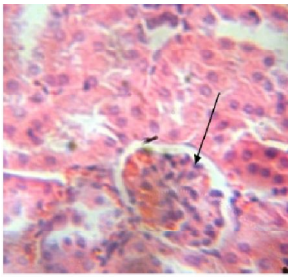


Fig 3: Subacute Effects of *P. amarus* Extract on Renal function indices of Rats. Data are mean ± SD of triplicate determinations values with identical bars with the same superscriptletter are not significantly different $p > 0.05$. One-way Analysis of Variance (ANOVA).

3.5 Histopathology

	Control	1000 mg/kg	1000 mg/kg	1000 mg/kg
LIVER × 40 MAGNIFICATION	 Liver histology appears normal (arrow).Visible centrioles with properly fenestrated sinusoidal space. The hepatocytes appear distinct with well well-differentiated nucleus.	 Liver histology looksverynormal (arrow). Visible centrioles with properly fenestrated sinusoidal space. The hepatocytes appear distinct with well well-differentiated nucleus.	 Liver histology appears normal (arrow). Visible centrioles with well fenestrated sinusoidal space. The hepatocytes appear distinct with well well-differentiated nucleus.	 Liver histology appears normal (arrow). Visible centrioles with well fenestrated sinusoidal space. The hepatocytes appear distinct with well-differentiated nucleus
KIDNEY × 40 MAGNIFICATION	 The kidney section shows normal histological features. The section indicated a detailed cortical parenchyma and the renal corpuscles appeared as dense rounded structures (arrow)	 The kidney section shows normal histological features. The section indicated a detailed cortical parenchyma and the renal corpuscles appeared as dense rounded structures (arrow)	 The kidney section shows normal histological features. The section indicated a detailed cortical parenchyma and the renal corpuscles appeared as dense rounded structures (arrow)	 The kidney section shows normal histological features. The section indicated a detailed cortical parenchyma and the renal corpuscles appeared as dense rounded structures (arrow)

4.0 Discussion

Since primeval eras, nutraceutical products, like medicinal plants, have been the foundation for the treatment and management of diver's ailments. This study tries to reveal the effects of subacute doses of *P. amarus*, one of several natural products specifically distributed in the South geological zone of Nigeria [7]

"Alterations in body weight is a salient indicator of the overall health status and well-being of animals" [21]. The results obtained showed that all the animals in the respective experimental groups underwent an increase in body weight after the treatment regimen. This increase in body weight of the experimental groups administered with graded doses (1000, 1500 and 2000 mg/kg) of *P. amarus* was

not significantly different ($p > 0.05$) from the non-treated (control) group, an indication that *P. amarus* at the dose levels investigated did not alter normal metabolic activities of the experimental animals. Flavonoids and lignin in *P. amarus*have been associated with the improvement of glucose, and lipid metabolism, as well as insulin sensitivity [22,23]. The abundant presence of lignans (phyllanthin and hypophyllanthin), flavonoids (quercetin and rutin), tannin (ellagitannins and gallotannins), phenolic compounds (gallic acid and geraniin) and some alkaloids have been ascribed with potent anti-inflammatory and antioxidant properties [24]. The anti-inflammatory and antioxidant properties of these phytochemicals have been correlated with the maintenance of optimal body weight [25, 43]

"The kidney is actively involved in the routine metabolism of the cell utilizing its role in the excretion of waste products and toxins like urea, uric acid and creatinine, metabolic control of extracellular fluid volume, serum osmolality and electrolyte balance, combined with the production of hormones like erythropoietin and 1,25 dihydroxy vitamin D and renin"[26,27]. "Assessment of renal function markers is vital to the diagnosis, management and treatment of patients with kidney disorders. Pathological conditionsaffecting renal function may sometimes arise from the ingestion of certain synthetic drugs used in the management and treatment of other underlying health conditions" [19,28]. "Urea is the principal nitrogenous waste produced during protein metabolism and whose level in the blood is reliant upon the correlation between its production and excretion, increased levels beyond the reference range may suggest kidney disease, shock, dehydration, diabetes, acute myocardial infarction while a decreased value lower than normal may portend liver failure, impaired absorption and overhydrating"[29]. Creatinine is the breakdown product of creatine phosphate, primarily from muscle metabolic activities and then excreted by glomerular filtration during normal renal function. Higher values of creatinine above 1.5 mg/dL are pointers of impairment in liver function. Uric acid is a breakdown metabolite from purine metabolism, abnormally high levels of uric acid are associated with a condition called gout. The non-significant changes ($p > 0.05$) in values of urea, creatinine and uric acid by the various doses under investigation when compared to the control, is a positive clue that *P. amarus* ethanol extract had no adverse effect on the renal function indices of the rats. This positive stimulatory effect on the kidney is attributed to the high levels of antioxidant peptides in *P. amarus*[30,31] and other phytonutrients reported in literatures [32,33]

"Oxidative stress arises when the equilibrium between reactive oxygen species (ROS) formation and detoxification promotes an increase in ROS levels leading to agitated cellular function. ROS causes injury to cellular components leading to lipid peroxidation, nucleic acid, and protein modifications. The formation of lipid peroxidation and the subsequent alteration of nucleic acid and protein are primary etiological factors in the initiation and progression of various metabolic and

neurodegenerative diseases"[21,34]. "Oxidative stress is correlated with disturbed redox control mechanisms and cellular signallingpathways, leading to the formation of various forms of cancer and oncogenic initiation and propagation. Antioxidants in cells include catalases, superoxide dismutases (SOD), and glutathione peroxidases (GPX), their induction is usually in response to specific toxicants and pollutants that can induce oxidative stress.Superoxide dismutase (SOD) is essential in protecting cells from oxidative damage by catalysing the dismutation of superoxide radicals into oxygen and hydrogen peroxide, which other antioxidant enzymes like catalase and glutathione peroxidase can further detoxify"[35]. This act is key in preserving redox homeostasis and alleviating oxidative stress, which is implicated in various diseases and ageing progressions. Catalase is an important enzyme that plays a crucial function in defending cells from oxidative damage by disintegrating hydrogen peroxide, a toxic derivative of numerous metabolic activities, into water and oxygen. This reaction is vital in inhibiting oxidative damage to cellular components such as DNA, proteins, and lipids [36]. The significant increase $(p < 0.05)$ in the activity of the SOD and catalase by *P. amarus* in this study is key to its optimization of cardiovascular health [37], reproductive health [38], neuroprotection [39], anti-ageing and skin health [40] reported in literature.

Malondialdehyde is a reactive organic compound and a byproduct of lipid peroxidation, it occurs when reactive oxygen species (ROS) attack polyunsaturated fatty acids in cell membranes. It is extensively employed as a biomarker to evaluate the level of oxidative stress in biological systems. Elevated levels of MDA imply increased lipid peroxidation and oxidative damage, which are correlated to numerous disease conditions and ageing[41]. The significantly decreased $(p < 0.05)$ MDA of the rats treated with 1500 and 2000 mg/kg of *P. amarus* is a positive indication that the extract has a special ability to mitigate lipid peroxidation.

Histological investigation of the overall architecture of the liver of all groups studied shows that the integrity of the hepatic lobules was well maintained, with clear demarcation between lobules, an indication of normal liver function and the absence of pathological changes such as steatosis (fatty liver), inflammation, fibrosis, or cirrhosis. The overall architecture of the kidneys of the rats in the respective groups also revealed a very distinct cortex and medulla well-delineated with an appropriate cortical-medullary ratio with the kidney capsule being thin and intact without any signs of thickening or inflammation. Kidney histology reflects the absence of pathological changes such as glomerulosclerosis, tubular atrophy, interstitial fibrosis, and vascular changes, which are indications of several kidney diseases. *P. amarus* demonstrates significant histopathological advantages, chiefly through its antioxidant, anti-inflammatory, hepatoprotective, and nephroprotective activities. These properties make it a valuable therapeutic agent in managing various pathological conditions [42].

5.0 Conclusion

This study investigated the toxicological profile of the crude ethanol extract of *P. amarus*. The findings submit that the daily single administration at doses of 1000, 1500 and 2000 mg/kg body weight for two weeks is nontoxic to the albino rats, as depicted by no visible and noticeable overt signs of toxicity, non-significant $(p > 0.05)$adverse change in body and relative organ weight, renal function and organ histopathology of the rats. *P. amarus* also significantly boasts*in-vivo* antioxidant status while significantly reducing the level of malondialdehyde

Ethical Approval

Etiquettes for the use of these animals were endorsed by the Directorate of Research and Quality Assurance, Federal University Otuoke, Bayelsa State vied an approval DRQA/FUO/0100/13/03/24 and the its guidelines were meticulously adhered to.

Disclaimer (Artificial intelligence)

Author(s) now declare that NO generative AI technologies such as Large Language Models (ChatGPT, COPILOT, etc) and text-to-image generators have been used during the writing or editing of this

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