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Hepatotoxic Assessment of *Phyllanthus amarus* leaf Extract in Wistar Rats

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Authors' contributions

This work was carried out in collaboration between all authors. Author TO design the study and reviewed the final draft of the manuscript. Author AAM managed the literature review. Author FA wrote carried out the laboratory analysis and wrote the initial draft of the manuscript. Authors MT and SOO managed the statistical analysis. All authors read and approved the final draft of the manuscript.

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ABSTRACT

Background and Aim: Different parts of *Phyllanthus amarus* are being used in the treatment of different diseases in several parts of Nigeria without considering its safety. This study was aimed at investigating the effect of ingestion of methanolic leaf extract of *Phyllanthus amarus* on the liver of Wistar rats.
Materials and Methods: The acute oral toxicity of the leaf extract (LD_{50}) was determined in 9 Wistar rats divided into 3 groups of 3 rats per group. Group 1 was the control and received distilled water. Different doses of 2000 mg/kg and 5000 mg/kg were administered orally once to the study groups 2 and 3 respectively. A sub-chronic toxicity study was carried out in 25 Wistar rats, divided into five groups of 5 rats per group. Group 1 served as control and received distilled water. The remaining 4 groups (2, 3, 4 and 5) served as the study groups and were administered different doses of 250

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mg/kg, 500 mg/kg, 750 mg/kg and 1000 mg/kg of methanolic leaf extract of *Phyllanthus amarus* respectively on a daily basis for 28 days. Total protein (TP), albumin (ALB), total and conjugated bilirubin (TB and CB), aspartate and alanine transaminase (AST and ALT), alkaline phosphatase (ALP) and gamma-glutamyl transferase (GGT) were assayed using standard techniques.
Results: In the acute oral toxicity study, no death or any sign of toxicities were recorded in the rats after 24 hours and up to 14 days post oral administration and there was no significant difference (P>0.05) in all the parameters analysed between the control and the study groups. In sub-chronic toxicity study, there was no significant difference (P>0.05) in all parameters analysed between the control and study groups. Histology of the liver of the rats in both the acute and sub-chronic study showed normocytic and normochromic cells.
Conclusion: Methanolic leaf extract of *Phyllanthus amarus* is relatively non-toxic and is not likely to induce liver damage.

Keywords: Phyllanthus amarus; hepatotoxicity; safety; wistar rats; toxicity study.

1. INTRODUCTION

Plant materials as sources of medicinal compounds continue to play a dominant role in the maintenance of human health since antiquity. At present, it is estimated that about 80% of the world population relies on herbal prescriptions and natural remedies for the treatment of various diseases, this practice being an alternative way to compensate for some perceived deficiencies in orthodox medicine like side-effects and cost [1]. The medicinal value of these plants lies in bioactive phytochemical constituents that produce definite physiological action on the human body [2].

Phyllanthus amarus, a widespread tropical plant belonging to the genus *Phyllanthus* of the family *Euphorbiaceae* is one of such herbal formulation used for various ethnomedicinal purposes [3]. It is known by the common names gale of the wind, stonebreaker, shatter stone and widely used in the treatment of liver diseases like hepatitis, diabetes, kidney and gallstones, hypertension, among others [4].

The liver plays a central role in the transformation and clearance of chemicals and it is susceptible to the toxicity from these agents [5]. Certain medicinal agents, when taken in overdose and sometimes even when introduced within therapeutic ranges, may injure the organ. Other chemical agents and herbal remedies can also induce hepatotoxicity. Chemicals that cause liver injury are called hepatotoxins and the damage done to the liver by chemicals or herbs is referred to as hepatotoxicity. The manifestation of hepatotoxicity ranges from an asymptomatic transient elevation of conjugated bilirubin, aspartate transaminase, alanine transaminase, gamma-glutamyl transferase and alkaline phosphatase with a decrease in the serum albumin and total protein to fulminant hepatic failure [6].

Most studies [7-9] focused on the hepatoprotective effects of *Phyllanthus amarus* after deliberate induction of hepatotoxicity in the experimental animals under study. Also few studies [10-11] have been conducted to assess the hepatotoxic effect of *Phyllanthus amarus*, results of which have shown the plant to be non-hepatotoxic. However, climatic, environmental and soil content variations from one region to the other have noticeable effects on the phytochemical distribution of the world's vegetation [12]. Hence, this study is designed to evaluate the hepatotoxic effect in Wistar rats of *Phyllanthus amarus* grown in Sokoto, North-Western, Nigeria.

2. MATERIALS AND METHODS

2.1 Plant Collection and Identification

Fresh leaves of *Phyllanthus amarus* were collected from Sokoto metropolis of Sokoto state, Nigeria. The plant was identified and authenticated at the Herbarium Unit of Department of Pharmacognosy and Ethno-pharmacy, Faculty of Pharmaceutical Sciences, Usmanu Danfodiyo University, Sokoto, by comparing with established herbarium specimen. Voucher number was obtained to be PCG/UDUS/Phyl/001 and the specimen was deposited.

2.2 Preparation and Extraction

Fresh leaves of *Phyllanthus amarus* were collected and air dried at room temperature over a period of 6 weeks. The dried leaves were

ground manually using mortar and pestle. One gram (1 g) of the grinded plant material was soaked in 5 mLs of 80% methanol for 24 hours on a mixer to ensure maximum extraction by maceration technique at room temperature. This is followed by periodic stirring, resulting crude extract was filtered using Whatman number 1 filter paper and the filtrate was concentrated in an oven at 40ºC to obtain 63.5 g of green crude extract [13].

2.3 Experimental Animals

A total of Thirty-four (34) adult Wistar rats of both sexes, weighing 150 g to 170 g purchased from the animal house of the Faculty of Pharmaceutical Sciences, Ahmadu Bello University, Zaria, Nigeria were used for the study. The rats were housed in well aerated cages under hygienic conditions in the animal house of Faculty of Pharmaceutical Sciences, Usmanu Danfodiyo University, Sokoto for the duration of the study. They were allowed to acclimatize for a period of 2 weeks before the commencement of the study. The animals were fed with pelletized growers feed (Vital®), obtained from Grand Cereal Soil Mills Limited, Jos, Nigeria. They were also allowed access to clean drinking water *ad libitum* throughout the experimental period. Cleaning of the animal cages was carried out on a regular basis. The animals were maintained as described by Aniagu et al. [13] in a clean metabolic cage-sand, placed in a well-ventilated room with a temperature of 26ºC to 28ºC, photoperiods of 12 hours light and 12 hours dark; humidity of 40% to 60%. All the experimental protocols were in compliance with our Institutional Animal Ethics Committee guidelines.

2.4 Experimental Design

2.4.1 Acute toxicity study

Acute oral toxicity study, Limit Test, was performed in accordance with the procedures outlined by the Organization for Economic Co-operation and Development Guidelines 425 [14]. Nine (9) Wistar rats of both sexes were used for this study. The rats were randomly divided into three (3) groups of three (3) rats per group with the first group serving as control. The extract was administered to the rats in groups 2 and 3 in single oral doses of 2000 mg/kg and 5000 mg/kg body weight respectively, by intragastric gavage using oral cannula (a feeding needle), one animal per day starting with group 2. The animals were observed within the first 4 hours and subsequently 24 hours for toxic symptoms. Also, behavioural parameters and mortality were closely monitored for 14 days. Lethal dose in 50% of total population (LD_{50}) was determined using OECD method as described by Aniagu et al. [13].

2.4.2 Sub-chronic toxicity study

Sub-chronic toxicity study was carried out following OECD 425 guidelines [14]. Twenty-five (25) Wistar rats of both sexes were divided into five (5) groups of five (5) rats per group. Group 1 served as the control and received distilled water as vehicle. Graded doses of crude extract were administered orally to the rats in groups 2, 3, 4 and 5. The doses given to the groups were 250 mg/Kg, 500 mg/Kg, 750 mg/Kg and 1000 mg/Kg body weight respectively daily for 28 days. All the rats had free access to feed and water throughout the period of the experiment and they were observed daily for general symptoms of toxicity and mortality.

2.5 Technique for Obtaining Blood and Serum Samples

Blood was collected by cardiac puncture from chloroform anaesthetized rats into heparinized bottles. The rats were later sacrificed using lumbar dislocation and their livers were removed. The blood samples collected were centrifuged at 4000 revolution per minute (4000 rpm) for 10 minutes. The plasma of each sample was separated and transferred into cryovial and stored (frozen) at -20ºC until required for analysis [13].

2.6 Histopathology

The livers of the animals were fixed within 10% formalin solution in labelled bottles after which the tissues were processed routinely using automatic tissue processor. This was immediately followed by embedding in paraffin wax. Sections of 5-6 µm thick were cut, stained with haematoxylin and eosin and examined under the light microscope [15].

2.7 Determination of Biochemical Parameters

The biochemical analyses were performed at the Chemical Pathology Laboratory of Usmanu Danfodiyo University, Sokoto. The separated Plasmas were used for the assays of ALT and AST [16], GGT [17], ALP [18],

TB and CB [19], TP [20] and ALB [21], using Agape reagent kit (Agape Diagnostics, Switzerland GmbH)

2.8 Statistical Analyses

The results obtained from the study were expressed as a mean ± standard deviation. Statistical differences were compared with independent student t-test. P values < 0.05 were considered to be significant. All statistical tests were carried out using statistical package for social science (SPSS) for Windows, version 20.0 (SPSS Inc., Chicago, IL, USA).

3. RESULTS

3.1 Acute Toxicity

Table 1 shows the result of acute oral toxicity (LD_{50} determination) in Wistar rats. The result showed that no behavioural changes or death was recorded in both the control and the treated groups after 24 hours and up to 14 days. This indicates that the LD_{50} is greater than 5000 mg/kg.

Fig. 1 shows Liver Enzyme Profile in rats exposed to methanolic leaf extract of *Phyllanthus amarus* in acute toxicity study.

Fig. 2 shows Protein and Bilirubin Profile in rats exposed to methanolic leaf extract of *Phyllanthus amarus* in acute toxicity study.

3.2 Sub-acute Toxicity

Fig. 3 shows Liver Enzyme Profile in rats exposed to methanolic leaf extract of *Phyllanthus amarus* in sub-chronic toxicity study.

Fig. 4 shows Protein and Bilirubin Profile in rats exposed to methanolic leaf extract of *Phyllanthus amarus* in sub-chronic toxicity study.

3.3 Histological Results

Figs. 5, 6 and 7 represents the liver cells (H & E X100) of Groups 1, 2 and 3 in acute toxicity study.

Figs. 8, 9, 10, 11 and 12 represent the liver cells (H & E X100) of Groups 1, 2, 3, 4 and 5 in sub-chronic toxicity study.

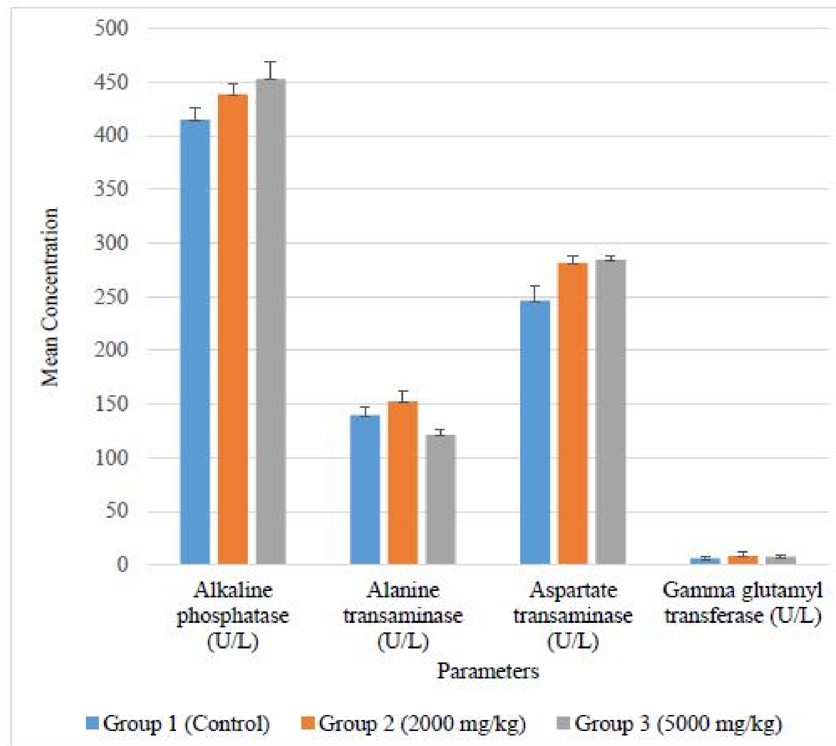


Fig. 1. Liver function profile in rats exposed to methanolic leaf extract of *Phyllanthus amarus* in acute toxicity study

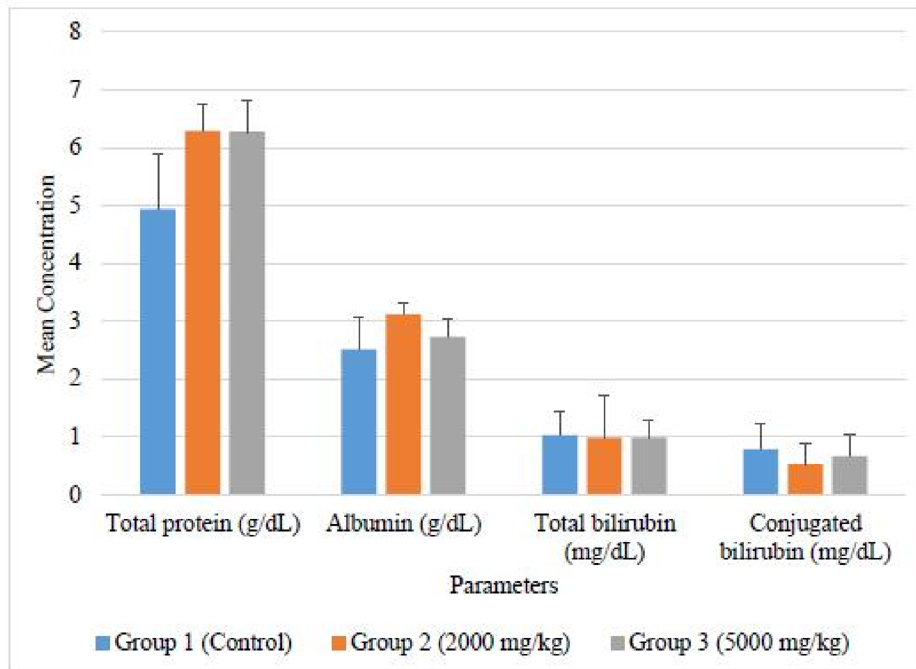


Fig. 2. Liver function profile in rats exposed to methanolic leaf extract of *Phyllanthus amarus* in acute toxicity study

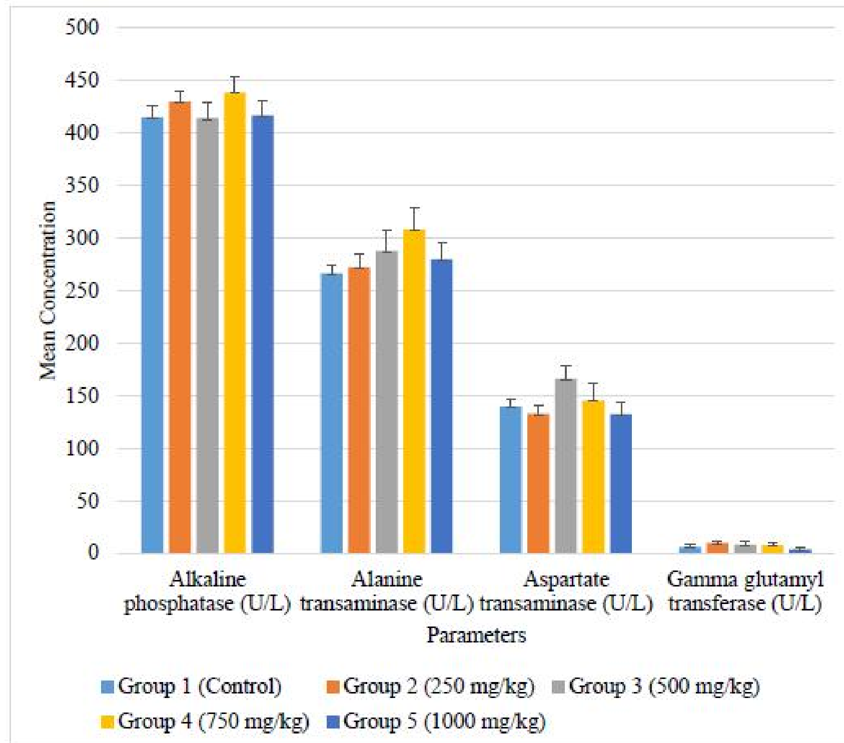
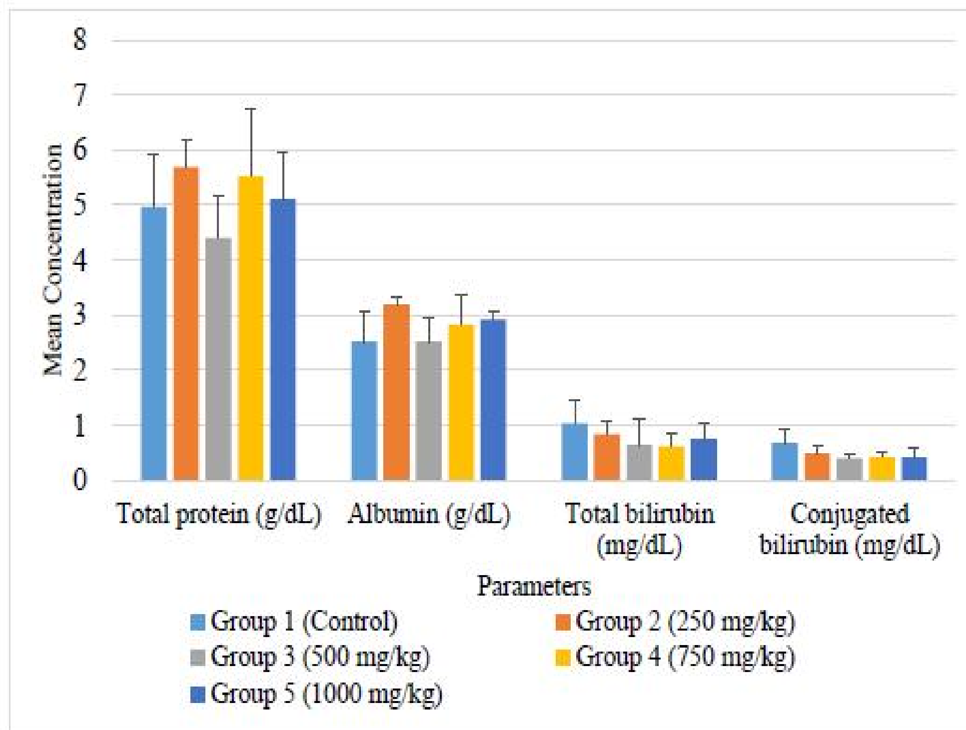


Fig. 3. Liver function profile in rats exposed to methanolic leaf extract of *Phyllanthus amarus* in sub-chronic toxicity study



Fi. 4. Liver function profile in rats exposed to methanolic leaf extract of *Phyllanthus amarus* in sub-chronic toxicity study

4. DISCUSSION

The present study evaluated the hepatotoxic effect of methanolic leaf extract of *Phyllanthus amarus.* No death or sign of toxicity was recorded in the experimental animals, Group 2 (2000 mg/kg) and Group 3 (5000 mg/kg) in acute oral toxicity after 24 hours and up to 14 days. This indicates that the LD_{50} of methanolic leaf extract of *Phyllanthus amarus* is higher than 5000 mg/kg, hence it is relatively safe. According to Globally Harmonised System of Classification and Labelling of Chemicals (GHS), a chemical is not classified as toxic, fatal or harmful if the LD_{50} is greater than 5000 mg/kg [22]. The result is in agreement with the work of Sirajudeen et al. [23], Lawson-Evi et al. [24] and Kushwaha et al. [11] who independently found the LD_{50} of *Phyllanthus*

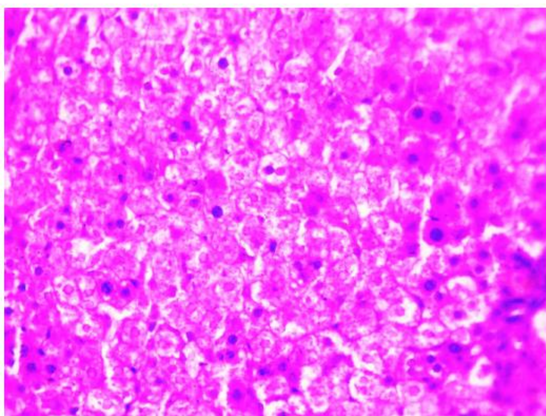


Fig. 5. Photomicrograph of liver cells (H & E X100) of group 1 (control) in acute oral toxicity study

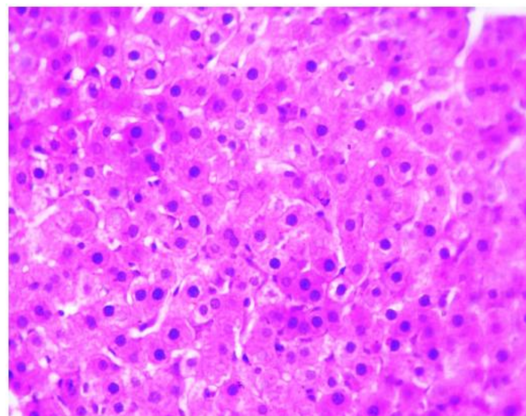


Fig. 6. Photomicrograph of liver cells (H & E X100) of group 2 (2000 mg/kg) in acute

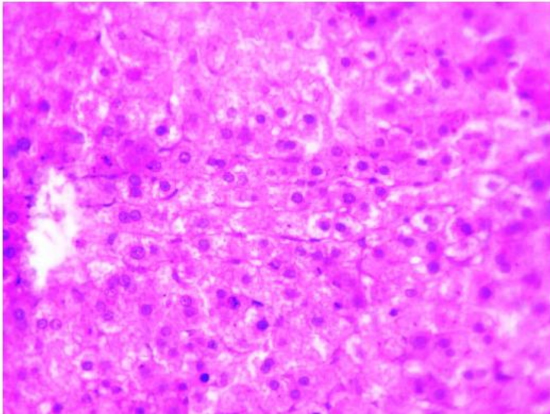


Fig. 7. Photomicrograph of liver cells (H&E X100) of group 3 (5000 mg/kg) in acute oral toxicity study

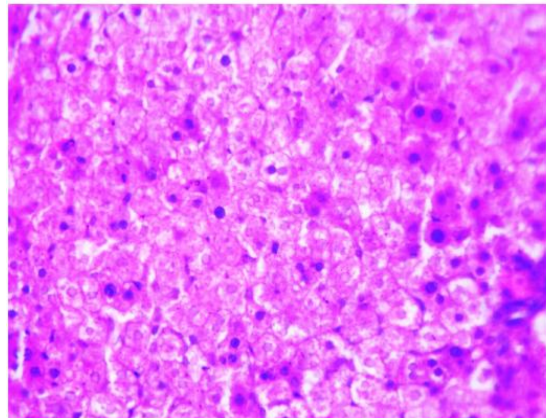


Fig. 8. Photomicrograph of liver cells (H & E X100) of group 1 (control) in sub-chronic

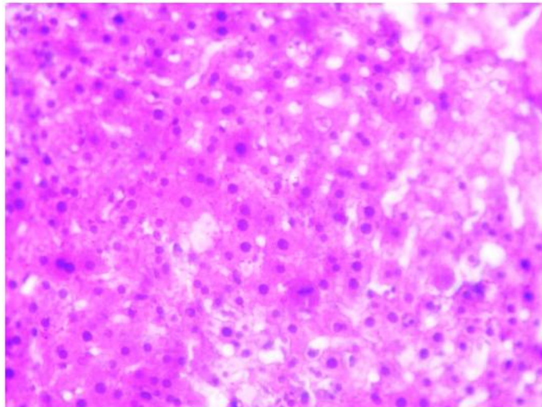


Fig. 9. Photomicrograph of liver cells (H & E X100) of group 2 (250 mg/kg) in sub chronic toxicity study

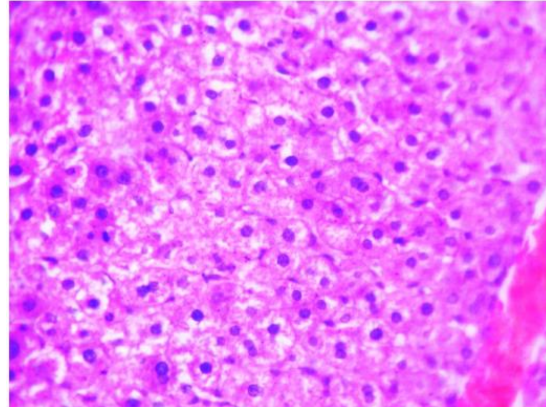


Fig. 10. Photomicrograph of liver cells (H & E X100) of group 3 (500 mg/kg) in sub

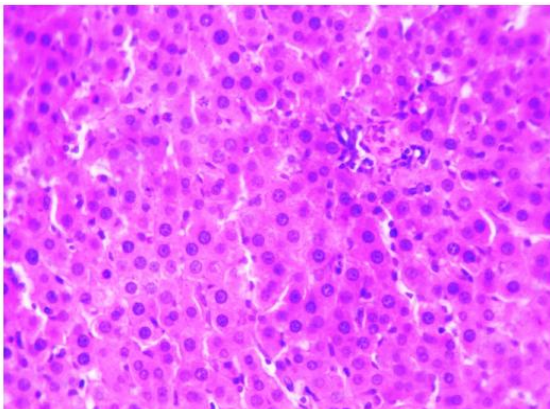


Fig. 11. Photomicrograph of liver cells (H & E X100) of group 4 (750 mg/kg) in sub chronic toxicity study

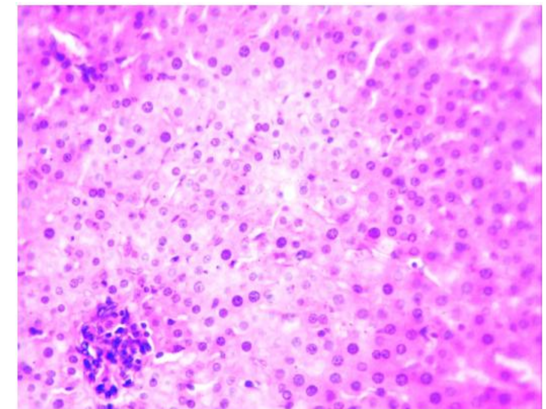


Fig. 12. Photomicrograph of liver cells (H&E X100) of GROUP 5 (1000 mg/kg) in sub chronic toxicity study

Table 1. Acute oral toxicity (LD_{50}) study of methanolic leaf extract of *Phyllanthus amarus* in wistar rats

Groups	Dosage/kg body weight	Observation period	Behavioural changes	Mortality
Group 1 (n=3)	Distilled water	After 24 hours and up to 14 days	None	0
Group 2 (n=3)				
Day 1: 1st rat	2000 mg/kg	After 24 hours and up to 14 days	None	0
Day 2: 2nd rat	2000 mg/kg	After 24 hours and up to 14 days	None	0
Day 3: 3rd rat	2000 mg/kg	After 24 hours and up to 14 days	None	0
Group 3 (n=3)				
Day 1: 1st rat	5000 mg/kg	After 24 hours and up to 14 days	None	0
Day 2: 2nd rat	5000 mg/kg	After 24 hours and up to 14 days	None	0
Day 3: 3rd rat	5000 mg/kg	After 24 hours and up to 14 days	None	0

Keys: n= number f rats per group, Group 1 = control

amarus to be above 5000 mg/kg. However, Zbinden and Roversi [25] suggested that variables such as animal species, strain, age, gender, diet, bedding, ambient temperature, caging conditions and time of the day can all affect the LD_{50} values obtained and as such are considerable uncertainties in extrapolating the LD_{50} obtained for species to other species.

Liver integrity is often determined by measuring the blood levels of ALT, AST, ALP and GGT.

Aminotransferases are markers of hepatocellular damage and injury to the liver, whether acute or chronic, eventually results in an increase in their serum concentrations. AST and ALT are enzymes that catalyse the transfer of α-amino groups from aspartate and alanine to the -keto group of ketoglutaric acid to generate oxaloacetic and pyruvic acids respectively, which are important contributors to the citric acid cycle [26]. Both aminotransferases are highly concentrated in the liver. AST is also diffusely represented in the heart, skeletal muscle, kidneys, brain and red blood cells, and ALT has low concentrations in skeletal muscle and kidney, an increase in ALT serum levels is, therefore, more specific for liver damage [27]. Both AST and ALT are released into the blood in greater amounts when hepatocytes are damaged [28]. In this study, there was no statistically significant difference (P>0.05) in the AST and ALT levels of the treated rats in acute and sub-chronic toxicity study when compared to their respective control, this observation may infer that the plant extract did not induce hepatocellular damage in Wistar rats. On the other hand, ALP and GGT are markers of cholestasis. ALP is an enzyme that transports metabolites across cell membranes. Liver and bone diseases are the most common causes of pathological elevation of ALP levels, although ALP may originate from other tissue, such as the placenta, kidneys or intestines, or from leukocytes. The third trimester of pregnancy (placenta origin) and adolescence (bone origin) are associated with an isolated increase in serum ALP levels. Hepatic ALP is present on the surface of bile duct epithelia. Cholestasis and accumulating bile salts enhance the synthesis and release of ALP from the cell surface subsequent increase in its serum concentrations [29]. When the bone disease is excluded, an elevation suggests biliary obstruction, injury to the bile duct epithelium, or cholestasis [28]. Ingestion of the extract did not affect alkaline phosphatase levels because there was no statistically significant difference (P>0.05) between the values of control and treated rats in the acute and sub-chronic study. GGT is an enzyme that occurs mainly in the liver, kidney and pancreas but occurs in particularly high concentrations in the epithelial cells lining the biliary ductules. The enzyme is also found in the hepatic parenchyma, in the smooth endoplasmic reticulum. Serum or plasma elevation of the enzyme is however from the liver. This increase occurs in cholestatic liver disease and found in parallel with the elevated serum or plasma level of ALP. GGT increase can also be induced by several drugs, such as anticonvulsants and oral contraceptives [29]. There was no statistically significant difference (P>0.05) between the values of control and experimental animals in the acute and sub-chronic toxicity study.

Albumin and proteins involved in secondary haemostasis and fibrinolysis, including vitamin K–dependent coagulation proteins are exclusively synthesized by the liver, and their plasma concentrations are, therefore, used as indirect indicators of liver synthetic function. In liver disease, there is a decrease in the synthesis of albumin and coagulation factors [29]. There was no statistically significant difference (P>0.05) in plasma total protein and albumin levels of experimental rats and the control rats in the acute and sub-chronic toxicity study. The ingestion of the extract did not impair the hepatic synthetic function in the animal.

Bilirubin is the product of haemoglobin catabolism within the reticuloendothelial system. Haem breakdown determines the formation of unconjugated bilirubin, which is then transported to the liver [30]. Plasma bilirubin concentration provides indirect information on the uptake, conjugation, and excretory function of the liver. Elevated plasma concentrations of bilirubin are specific markers for serious liver injury and, therefore, loss of liver function (27). After formation of unconjugated bilirubin, it is bound to albumin for transport to the liver. Bilirubin is taken up by hepatocytes, where it is bound by a group of cytosolic proteins, mainly glutathione S-transferases, to prevent efflux from the cell [31]. Bilirubin is conjugated to glucuronic acid under the catalytic activity of UDP glucuronosyl-transferase 1-1, which converts conjugated bilirubin from a highly hydrophobic molecule to a relatively hydrophilic molecule [31]. Plasma bilirubin may increase in severe hemolysis, hepatocellular damage and biliary obstruction [27]. In the present study, total bilirubin values did not show any significant difference (P>0.05) between the control and experimental rats, hence the excretory function of the liver is not impaired.

Histological examination of liver tissues of control and experimental rats in both acute and sub-chronic toxicity study reveal no malignancy. The liver cells were essentially the normal, an indication that methanolic leaf extract of *Phyllanthus amarus* did not induce any lesion in the liver cells of the rats.

The findings of the biochemical indices and histological examination of the liver in both acute and sub-chronic toxicity studies in the present study is in agreement with the work of Sirajudeen et al. [23], Lawson-Evi et al. [24], Chopade and Sayed [32] and Kushwaha et al. [11], who separately reported no statistical significant difference in the biochemicals and histology of the liver of the treated animals when compared with the control. However, the result is at variance with the findings of Adedapo et al. [33] who reported statistical significant increase in the levels of aspartate aminotransferase (AST), total and conjugated bilirubin, total protein and albumin of the treated groups when compared with control and a histological report of foci of lymphocytic infiltration at the portal areas of the liver.

5. CONCLUSION

In conclusion, the present findings have shown methanolic leaf extract of *Phyllanthus amarus* to be relatively safe for consumption and non-hepatotoxic and is not likely to induce liver damage in humans.

CONSENT

It is not applicable.

ETHICAL APPROVAL

As per international standard or university standard ethical approval has been collected and preserved by the authors.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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