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Evaluation of Median Lethal Dose and Subchronic Oral Toxicity Assessment of Ethanolic Leaf Extract of *Phyllanthus amarus*

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Authors' contributions

This work was carried out in collaboration among all authors. Author OEA wrote the first draft of the manuscript and performed the spectroscopy analysis. Author IO designed the study, wrote the protocol, managed the experimental process and vetted the draft manuscript. Author AOO managed literature searches and analysis of the study. Author JCM performed the statistical analysis and monitored plant authentication. All authors read and approved the final manuscript.

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ABSTRACT

Aims: To determine the median lethal dose (LD_{50}) of crude ethanolic leaf extract of *Phyllanthus amarus* and evaluate its sub-chronic oral toxicity in experimental mice ($BALB/_C$ strain).
Study Design: One-factor, one-control, one-test group experimental design.
Place and Duration of Study: Department of Medical Biochemistry, Delta State University, Abraka, Nigeria, between December, 2014 and November, 2015.
Methodology: Crude ethanolic leaf extract of *P. amarus* was prepared as previously described and twenty (20) Swiss albino mice ($BALB/_C$ strain) were randomly and equally divided into two (2) groups and administered 2000 mg/kg body weight (Group A) and 5000 mg/kg body weight (Group B) of the prepared extract as single oral dose in line with the limit dose method of determining LD_{50}. For the sub-chronic oral toxicity study, ten (10) mice were assigned into control (n=5) and experimental (n=5). The control animals were given placebo-normal saline, but the experimental mice were administered with nocebo – 300 mg/kg body weight of *P. amarus* crude ethanolic

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extract for twenty one (21) days. Thereafter, the animals in each group were sacrificed and then, serum and liver homogenate were obtained for the assay of total antioxidant capacity (TAC) and oxidative damage (Malondialdehyde-MDA) Using documented methods. Liver tissue was also processed for histopathological examination using H&E stain.

Results: Data showed LD_{50} of the extract to be greater than 5000 mg/kg. Assessment of the herb's sub-chronic oral toxicity indicates that the leaf extract significantly (*P*=.03) enhanced total antioxidant capacity (TAC) in both serum (Control: TAC = 0.10±0.03 mM, Experimental: TAC = 0.33±0.05 mM) and liver (Control: TAC = 0.12±0.09 mM, Experimental: TAC = 0.34±0.06 mM) but reduced (*P* = .01) the biomarker for liver tissue damage (Control: MDA = 41.89±3.36 µM, Experimental: MDA = 4.67±4.04 µM). In addition, hepatic cells were invigorated by *P. amarus* treatment as suggested by the histopathological features.

Conclusion: Collectively, *P. amarus* crude ethanolic leaf extract possesses high degree of tolerance and hepatic tonic potential with no identifiable toxic or side effects.

Keywords: Phyllanthus amarus; Median Lethal Dose (LD_{50}); sub-chronic toxicity; Total Antioxidant Capacity (TAC); Malondialdehyde (MDA).

1. INTRODUCTION

The use of plants, plant extracts or plant-derived chemicals to treat diseases is a therapeutic modality that has been explored for centuries. Over 40,000 species of tropical flowering plants are known to possess medicinal properties [1] and are currently in use for various medical conditions. Majority of Africans patronize herbal or traditional medicine for their health needs. It is estimated that 70-80% of patients in Africa are treated by traditional healers and herbal practitioners [2]. Modern medicine recognizes herbalism as a form of alternative medicine based on evidence derived from scientific methods [3]. Herbal medicine is, thus, gaining popularity and one of such herbs receiving wide patronage is *Phyllanthus amarus.*

Phyllanthus amarus is an herbal plant belonging to the Euphorbiaceae family. It has approximately 800 species which are found in tropical and subtropical countries of the world [4,5]. The plant has been found in Philippine, Cuba, Nigeria and India among others. Extract of the plant has been reported to possess pharmacological effects such as antibacterial [4,6], antiviral [7], anticancer [8], antiamnesic [9], antioxidative [10], antimicrobial [11], antileptospiral [12], anticonvulsant [13] and anti-inflammatory [14,15] activities. *Phyllanthus amarus* has been used as chemoprotective [16], antimutagenic [17], nephroprotective, cardioprotective [18], hepatoprotective [19] and hypoglycemic [20] agent. It is known to exhibit *in vivo* antiplasmodial property [21] in addition to its demonstrated ability to invigorate the pancreas [22] and restore renal function altered by *Plasmodium berghei* malarial parasite infection in experimental mice [21].

Lack of knowledge of the mechanisms and side effects of some herbal preparations as well as safety regulations for their usage may have serious consequences [23]. Many consumers believe that herbal medicines are "safe" because they are "natural", but, several adverse effects of herbs have been reported including allergic reactions, hepatotoxicity [24,25,26], nephrotoxicity [27,28,29], cardiac toxicity [30,31], neurotoxicity [32,33], and even death [34].

Since *Phyllanthus amarus* is currently gaining recognition in alternative medical practice, it has therefore become pivotal to evaluate the median lethal dose and subchronic toxicity of the ethanolic leaf extract of the plant cultivar wildly grown in the tropical rain forest zone of Abraka, Delta State, Nigeria. This freely growing variety of the plant is common and easily harvested in our environment for medicinal use.

2. MATERIALS AND METHODS

2.1 Harvesting and Preparation of Plant Extract

Fresh whole plants of *Phyllanthus amarus* wildly growing in uncultivated land space in Abraka, Ethiope East Local Government Area of Delta State, Nigeria were obtained in July, 2015 and authenticated (No: FHI: 109728) in the Herbarium Unit, Forestry Research Institute of Nigeria, Ibadan. Crude ethanolic leaf extract of the harvested fresh plant was prepared as earlier described [21]. The leaves were washed, air-

dried and pulverized using a sterile Electric blender (Kenwood Ltd, Hertfordshire, U.K) to produce a fine powder. The ethanolic extract of the plant sample was prepared by soaking 100 g of dry powdered sample in 200 ml of ethanol for 24 hours. The extract was filtered using whatman filter paper and the filtered extract were concentrated using the Soxhlet apparatus (Corning, U.S.A). The extract was evaporated to dryness using rotary evaporator (Buchi R-210 Hana, China) under reduced pressure and dissolved in distilled water which was then stored in a refrigerator until required for analysis.

2.2 Experimental Mice

Forty (40) Swiss albino BALB/$_c$ mice of mixed sexes weighing between 21.1 to 28.2 g were used for the entire study. They were maintained at the Laboratory Animal Centre, Faculty of Basic Medical Sciences, Delta State University, Abraka, Nigeria. The mice were fed on growers' mash (Top Feeds, Sapele, Delta State, Nigeria), and were given clean drinking water *ad libitum.* The animals were housed in plastic cages, under controlled condition of 12 hr light/12 hr dark cycle at a temperature of 29±2^{o}C. The animals were maintained in accordance with the guidelines provided by the Research and Bioethics Committee of the Faculty of Basic Medical Sciences, Delta State University, Abraka, Nigeria.

2.3 Evaluation of Lethal and Effective Doses (LD_{50} and ED_{50})

LD_{50} and ED_{50} were determined by the limit dose method [35]. A total of thirty (30) mice (20 for LD_{50} and 10 for ED_{50}) were used. In the phase of LD_{50} determination, the mice were divided into two groups of ten (10) mice each. They were treated with ethanolic leaf extract of *Phyllanthus amarus* at doses of 2000 and 5000 mg/kg body weight as oral single dose. The animals were observed for 24 hours first and then, for twenty one (21) days for any sign of toxicity and mortality.

2.4 Subchronic Study

For the subchronic study, the remaining ten (10) mice were divided into Control (n = 5) and Experimental (n = 5) Groups. The Experimental Group was administered 300 mg/kg/d *P. amarus* ethanolic leaf extract as single daily dose for 21 days. The dosing regimen was based on previous experience [22]. The animals were observed for any physical signs of toxicity, morbidity and mortality. Body weights were measured weekly throughout the 21-day study period.

2.5 Animal Sacrifice and Collection of Sample

On the 21st day of the experiment, the mice were fasted overnight and sacrificed the next day under chloroform anesthesia. The liver was excised and whole blood was collected by heart puncture and centrifuged (Cent 80D, Serico, China) to obtain serum which was used for the biochemical analyses of total antioxidant capacity (TAC) and malondialdehyde (MDA) levels. The excised liver was fixed in 10% formol saline for histological processing and examination. However, a portion (0.5 g) was homogenized and then, prepared for biochemical assay.

2.6 Biochemical Assay

Total antioxidant capacity, TAC in serum and liver homogenate as determined by the Trolox Equivalent Antioxidant Capacity (TEAC) method described by Miller *et al.*[36] and MDA levels were estimated by the Thio-Barbituric Acid Reacting Substances (TBARS) method earlier described by Ohkawa *et al.*[37]. TAC provides information on degree of antioxidant defense, and MDA indicates a measure of membrane lipid peroxidation, and hence, oxidative stress/damage.

2.7 Histological Studies

The portion of the liver tissue fixed in 10% formol saline was processed overnight using histokinette and embedded in paraffin wax. Three sections - four micron in thickness - were cut from each paraffin block.

2.8 Light Microscopic Examination

One section from each sample was stained with Heamatoxylin and Eosin (H&E) stain by the standard method for light microscopic (histological) examination.

2.9 Ethical Approval

The study was conducted in compliance to the guidelines provided by the Research and Bioethics Committee of the Faculty of Basic Medical Sciences, Delta State University,

Abraka, Nigeria – the body that approved the study.

2.10 Statistics

Data were presented as Mean ± S.D and analyzed by the Student's *t*-Test using SPSS software package version 20. Significant difference was set at *P*=.05.

3. RESULTS

Results obtained from evaluation of median lethal dose (LD_{50}) and subchronic oral toxicity study of the ethanolic leaf extract of *Phyllanthus amarus* grown freely in uncultivated land space in Abraka, Ethiope East Local Government Area of Delta State, Nigeria, are shown in Tables 1-2 and Figs. 1-2.

Table 1 shows the cage side physical observations of the control and experimental mice used in the determination of LD_{50}, while, Table 2 presents the biochemical data (TAC and MDA) obtained from both serum and liver tissues of the animals used to assess subchronic oral toxicity. Then, Figs. 1-2 are the histological features of the liver tissues excised from Control (Fig. 1) and *P. amarus* (300 mg/kg/d for 21 days) treated mice (Fig. 2).

4. DISCUSSION

This study attempted to evaluate the LD_{50} and subchronic oral toxicity of the crude ethanolic leaf extract of *Phyllanthus amarus*. Result of the limit dose test indicates that the LD_{50} of *P. amarus* crude ethanolic leaf extract is well above 5000 mg/kg with an ED_{50} of 200 mg/kg and hence, therapeutic index of 25. These observations show that the herb possesses very high phytotherapeutic efficacy with no demonstrated toxicity. These findings suggest that *Phyllanthus amarus* is safe and non-toxic with very high remedy potential in experimental mice. This agrees with previous documents [38].

Table 1. Cage side physical observations during the LD_{50} evaluation of *P. amarus* ethanolic leaf extract

Considerations		Cage side physical observations after 24 hours and 21 days					
		2000 mg/kg		5000 mg/kg		Control (0 mg/kg)	
		24 hours	21 days	24 hours	21 days	24 hours	21days
1	Condition of fur	Normal	Normal	Normal	Normal	Normal	Normal
2	Skin appearance	Normal	Normal	Normal	Normal	Normal	Normal
3	Subcutaneous swelling	Nil	Nil	Nil	Nil	Nil	Nil
4	Abdominal distension	Nil	Nil	Nil	Nil	Nil	Nil
5	Eye dullness	Nil	Nil	Nil	Nil	Nil	Nil
6	Eye opacity	Nil	Nil	Nil	Nil	Nil	Nil
7	Pupil diameter	Normal	Normal	Normal	Normal	Normal	Normal
8	Colour/consistency of faeces	Normal	Normal	Normal	Normal	Normal	Normal
9	Teeth condition	Normal	Normal	Normal	Normal	Normal	Normal
10	Gait	Normal	Normal	Normal	Normal	Normal	Normal
11	Weight gain (%)	0.3	5.0	0.5	7.0	0.1	3.0
12	Mortality	0	0	0	0	0	0

Evidence from observations (Table 1) indicates that the LD_{50} of P. amarus crude ethanolic leaf extract is greater than 5000 mg/kg. Trial doses cannot be increased beyond 5000 mg/kg because that is the limit dose. Effective dose (ED_{50}) = 200 mg/kg. Hence, therapeutic index, TI (LD_{50}/ ED_{50}) = 25.0

Table 2. Changes in total antioxidant capacity (TAC) and malondialdehyde levels (MDA) induced by subchronic oral toxicity study of *P. amarus* crude ethanolic leaf extract

Sample	Assay	Control	*P. amarus* (300 mg/kg/d)	P- value
Serum	TAC (mM)	0.10±0.03	0.32±0.05*	.03
	MDA (µM)	40.33±3.36	21.02±1.59*	.02
Liver	TAC (mM)	0.12±0.09	0.34±0.06*	.03
	MDA (µM)	41.89±2.27	4.67±4.04*	.01

Data are presented as Mean ±SD for n=5,

**Significantly different from comparable control values at P<0.05*

TAC-Total antioxidant capacity, MDA-Malondialdehyde, The subchronic oral toxicity of P. amarus crude ethanolic leaf extract was studied by administering 300 mg/kg/d of the plant extract to experimental BALB/$_c$ mice for 21 days

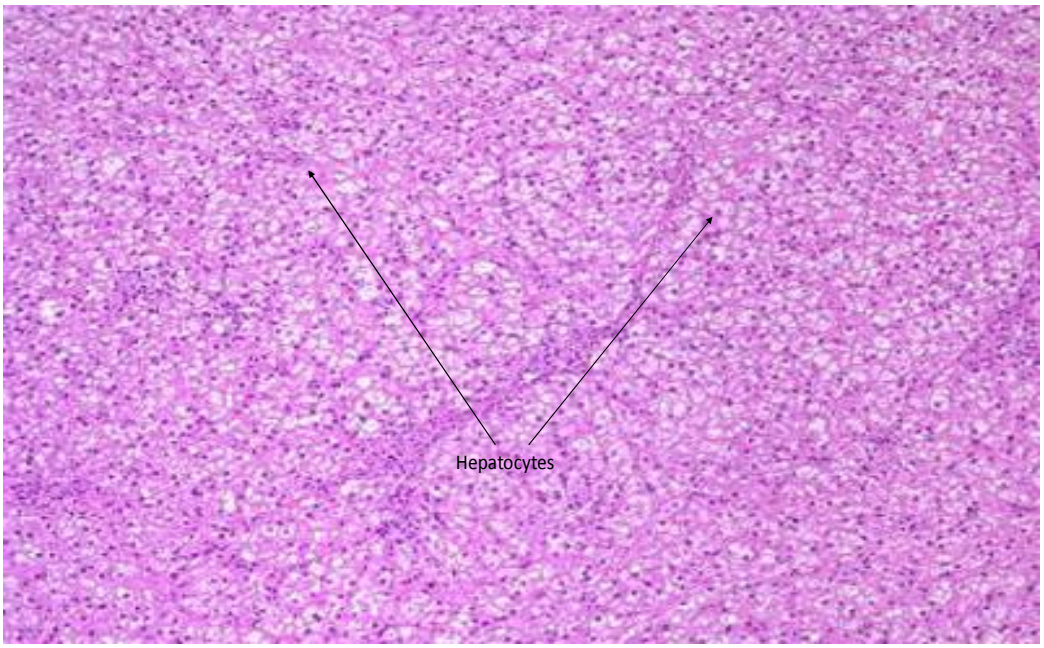

Fig. 1. Photomicrograph of liver tissue from control mouse showing normal hepatocytes. Magnification ×100 (H & E stain)

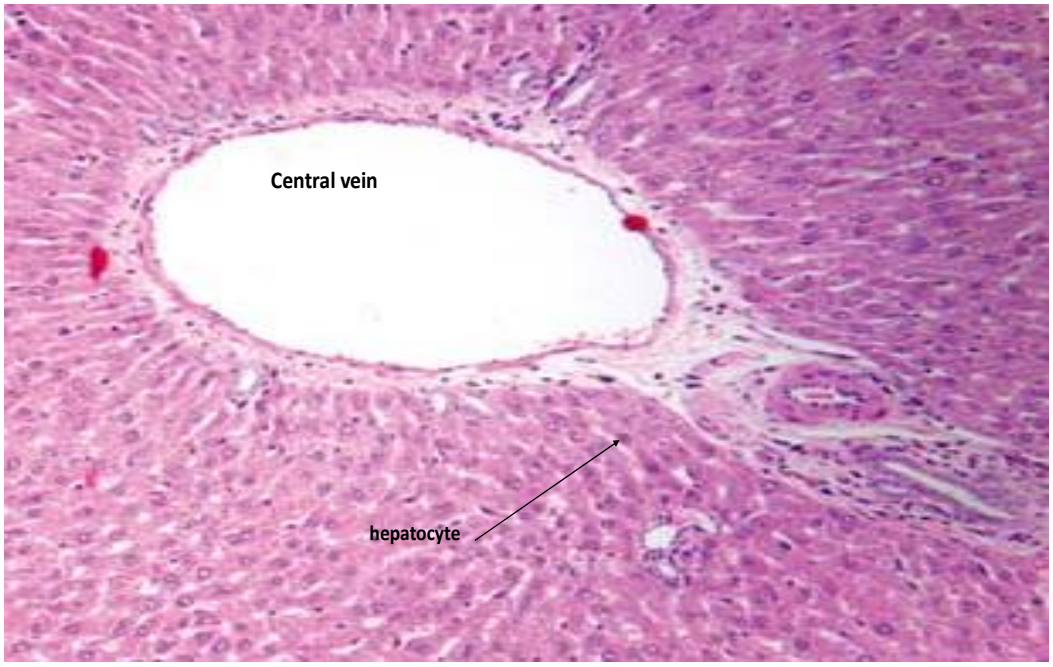

Fig. 2. Photomicrograph of liver tissue obtained from mouse administered 300 mg/kg body weight of crude ethanolic leaf extract of P. amarus for 21 days, indicating normal histological features of invigorated hepatocytes and central vein. Magnification × 100 (H & E stain)

Chronic toxicity study identifies and provides information on drugs that could possibly cause harm and pose health challenges [39]. The subchronic oral toxicity assessment of *P. amarus* crude ethanolic leaf extract during this study, reveals that the extract significantly (*P = .03*)

boosted antioxidant defense activity in both blood and liver tissue with associated reduction (*P = .01*) in overall membrane damage. The liver is the organ involved in several metabolic functions and is therefore prone to xenobiotic-induced injury because of its central role in xenobiotic metabolism [40]. Histopathological examination of the liver shows that *P. amarus* administered at 300 mg/kg/d body weight for 21 days invigorated liver cells. Hepatotoxic drugs could cause peroxidation of liver cell membrane lipids and increase the amount of end products such as MDA [39].

Data suggest that *Phyllanthus amarus* extract has a measure of health benefits as shown by the significant decrease in malondialdehyde (MDA) levels and associated increase in total antioxidant capacity, TAC (Table 2). The decrease in malondialdehyde level may be as a result of the increased antioxidant activities of *Phyllanthus amarus* [41]. Increased antioxidant activity in cells causes a decrease in free radicals thereby reducing lipid peroxidation and malondialdehyde production. The reduction in both serum and liver malondialdehyde levels suggests that the extract may contain mixture of biomolecules with hydroxyl groups that perhaps prevented the abstraction of hydrogen atom from the double bond of lipid bilayers thereby preventing lipid peroxidation. This suggestion corroborates previous report on the *in vitro* analysis of the plant extract [42].

Phytochemical studies of *Phyllanthus amarus* extract have shown that the plant contains chemicals such as flavonoids, tannins, saponins, alkaloids, terpenoids, glycosides and phenols [42,21]. Flavonoids present in the plant have been shown to possess several pharmacological properties such as antioxidant activities and anti-inflammatory activities [20,43]. Flavonoid as an antioxidant has a rejuvenating effect on cells and tissues [44], Tannin has demonstrated high activities against viral and bacterial infections as well as acting as strong antioxidant [45]. The antioxidant activity of this plant phytochemicals may have contributed to the decrease in MDA levels observed in this study. These findings are concurrent with previous studies conducted on the toxicological assessment of *Phyllanthus amarus* [46].

5. CONCLUSION

Findings indicate that *Phyllanthus amarus* plant materials have no significant toxic effect in Swiss albino mice.

6. RECOMMENDATION

Put together, the crude ethanolic leaf extract of *Phyllanthus amarus* is bestowed with very high phytotherapeutic efficacy and vitalizing property with no recognizable toxic effect. Therefore, the phytochemicals and nutrient quality of *P. amarus* need to be characterized for functional analysis.

CONSENT

It is not applicable.

ETHICAL APPROVAL

As per international standard or university standard, written approval of Ethics committee has been collected and preserved by the author(s).

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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