

Original Research Article**EVALUATION OF MEDIAN LETHAL DOSE AND
SUBCHRONIC ORAL TOXICITY ASSESSMENT OF
ETHANOLIC LEAF EXTRACT OF *PHYLLANTHUS
AMARUS*****ABSTRACT**

Phyllanthus amarus is a well-known tropical herb, recognized for its importance in the treatment of various ailments. This study was designed to evaluate the median lethal dose (LD₅₀) and subchronic toxicity of the crude ethanolic leaf extract of *Phyllanthus amarus*. Crude ethanolic fresh leaf extract of wild growing *Phyllanthus amarus* was prepared and used for study. The LD₅₀ and sub-chronic toxicity were evaluated using standard procedures and documented methods.

Aims: To determine the median lethal dose (LD₅₀) of crude ethanolic leaf extract of *Phyllanthus amarus* and evaluate its sub-chronic oral toxicity in experimental mice (BALB/c strain).

Study design: Randomized animal model laboratory experiment.

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 2000mg/kg (Group A) and 5000mg/kg (Group B) of the prepared extract as single oral dose in line with the limit dose method of determining LD₅₀. 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 - 300mg/kg of *P. amarus* of crude ethanolic 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 show LD₅₀ of the extract to be greater than 5000mg/kg. Assessment of the herb's sub-chronic oral toxicity indicates that the leaf extract significantly ($P=0.03$) enhanced total antioxidant capacity (TAC) in both serum (Control TAC=0.10±0.03, Experimental TAC=0.33±0.05) and liver (Control TAC=0.12±0.09, Experimental TAC=0.34±0.06) but reduced ($P=0.01$) the biomarker for liver tissue (Control MDA=41.89±3.36, Experimental =4.67±4.04). 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. Therefore, the structure and mechanism of the active chemicals need to be further elucidated.

Keywords: *Phyllanthus amarus*, Median Lethal Dose (LD₅₀), Sub-chronic Toxicity, Total Antioxidant Capacity (TAC), Malondialdehyde (MDA).

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*.

40 *Phyllanthus amarus* is an herbal plant belonging to the Euphorbiaceae family. It has
41 approximately 800 species which are found in tropical and subtropical countries of the world [4,5]. The
42 plant has been found in Philippine, Cuba, Nigeria and India among others. Extract of the plant has
43 been reported to possess pharmacological effects such as antibacterial [4,6], antiviral [7], anticancer
44 [8], antiemetic [9], antioxidative [10], antimicrobial [11], antileptospiral [12], anticonvulsant [13] and
45 anti-inflammatory [14,15] activities. *Phyllanthus amarus* has been used as chemoprotective [16],
46 antimutagenic [17], nephroprotective, cardioprotective [18], hepatoprotective [19] and hypoglycemic
47 [20] agent. It is known to exhibit *in vivo* antiplasmodial property [21] in addition to its demonstrated
48 ability to invigorate the pancreas [22] and restore renal function altered by *Plasmodium berghei*
49 malarial parasite infection in experimental mice [21].

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

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

60 MATERIALS AND METHODS

61 **Harvesting and preparation of plant extract:** Fresh whole plants of *Phyllanthus amarus* widely
62 growing in uncultivated land space in Abraka, Ethiope East Local Government Area of Delta State,
63 Nigeria were obtained in July, 2015 and authenticated (No: FHI: 109728) in the Herbarium Unit,
64 Forest Research Institute of Nigeria, Ibadan. Crude ethanolic leaf extract of the harvested fresh plant
65 was prepared as earlier described [21].

66 **Experimental mice:** Forty (40) Swiss albino BALB/c mice of mixed sexes weighing between 21.1 to
67 28.2g were used for the entire study. They were maintained at the Laboratory Animal Centre, Faculty
68 of Basic Medical Sciences, Delta State University, Abraka, Nigeria.

69 The mice were fed on growers' mash (Top Feeds, Sapele, Delta State, Nigeria), and were given clean
70 drinking water *ad libitum*. The animals were housed in plastic cages, under controlled condition of
71 12hr light/12hr dark cycle at a temperature of $29\pm 2^{\circ}\text{C}$. The animals were maintained in accordance
72 with the guidelines provided by the Research and Bioethics Committee of the Faculty of Basic Medical
73 Sciences, Delta State University, Abraka, Nigeria.

74 **Evaluation of lethal and effective doses (LD₅₀ and ED₅₀):** LD₅₀ and ED₅₀ were determined by
75 the limit dose method [35]. A total of thirty (30) mice (20 for LD₅₀ and 10 for ED₅₀) were used. In the
76 phase of LD₅₀ determination, the mice were divided into two groups of ten (10) mice each. They were
77 treated with ethanolic leaf extract of *Phyllanthus amarus* at doses of 2000 and 5000mg/kg body
78 weight as oral single dose. The animals were observed for 24 hours first and then, for twenty one (21)
79 days for any sign of toxicity and mortality.

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

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

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

97 **Histological Studies:** The portion of the liver tissue fixed in 10% formal saline was processed
 98 overnight using histokinette and embedded in paraffin wax. Three sections - four micron in thickness -
 99 were cut from each paraffin block.

100 **Light Microscopic Examination:** One section from each sample was stained with Heamatoxylin
 101 and Eosin (H&E) stain by the standard method for light microscopic (histological) examination.

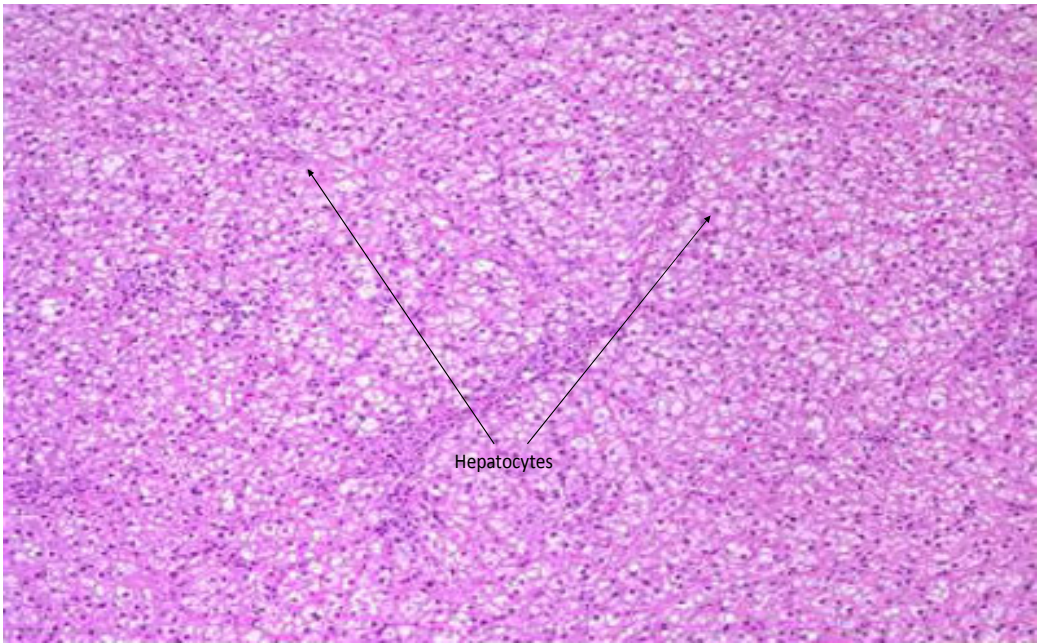
102 **Statistics:** Data were presented as Mean \pm S.D and analyzed by the Student's *t*-Test using SPSS
 103 software package version 20. Significant difference was set at $P=0.05$

104 **RESULTS**

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

109 Table 1 shows the cage side physical observations of the control and experimental mice used in the
 110 determination of LD_{50} , while, Table 2 presents the biochemical data (TAC and MDA) obtained from
 111 both serum and liver tissues of the animals used to assess subchronic oral toxicity. Then, Figures 1-2
 112 are the histological features of the liver tissues excised from Control (Fig. 1) and *P. amarus*
 113 (300mg/kg/d for 21 days) treated mice (Fig. 2).

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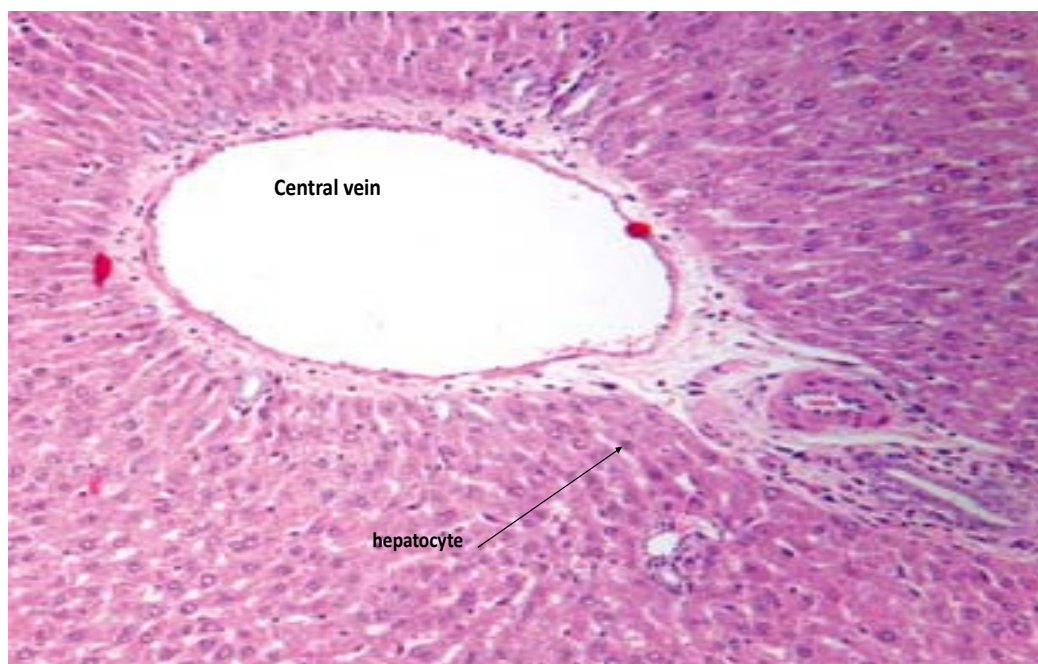
Fig. 1: Photomicrograph of liver tissue from control mouse showing normal hepatocytes. Magnification $\times 100$ (H & E stain).

135 **Table 2: Changes in total antioxidant capacity (TAC) and malondialdehyde levels (MDA)**
 136 **induced by subchronic oral toxicity study of *P. amarus* crude ethanolic leaf**
 137 **extract.**
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139	Sample	Test	Control	300mg	P-value
140	SERUM	TAC (mM)	0.10±0.03	0.32±0.05*	.03
141		MDA (µM)	40.33±3.36	21.02±1.59*	
142	.02				
143					
144	LIVER	TAC (mM)	0.12±0.09	0.34±0.06*	.03
145		MDA (µM)	41.89±2.27	4.67±4.04*	
146	.01				

147 *Data are presented as Mean ±SD for n=5*
 148 **Significantly different from comparable control values at P<0.05*
 149 *TAC-Total antioxidant capacity, MDA-Malondialdehyde.*
 150 *The subchronic oral toxicity of *P. amarus* crude ethanolic leaf extract was studied by administering*
 151 *300mg/kg/d of the plant extract to experimental BALB_c mice for 21 days.*

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

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DISCUSSION

161 Herbal medicine is the use and study of plants and their derived products for medicinal
 162 purposes. Plants have been the basis for medical treatments through much of human history and
 163 such traditional medicine is still being practiced today [38]. Herbal medicines are used in
 164 underdeveloped, developing and even in developed countries. Reports indicating that herbal drugs
 165 are safe and free from toxic side effects may not be absolutely true [39]. So, toxicological evaluations

166 of all medicinal plants are important in order to ascertain their safety. Therefore, clear understanding
167 of the adverse effect of herbs used by humans is necessary for the implementation of safety
168 measures. In this regard, this study attempted to evaluate the LD₅₀ and subchronic oral toxicity of the
169 crude ethanolic leaf extract of *Phyllanthus amarus*.

170 Result of the limit dose test indicates that the LD₅₀ of *P. amarus* crude ethanolic leaf extract is
171 well above 5000mg/kg with an ED₅₀ of 2000mg/kg and hence, therapeutic index of 25. These
172 observations show that the herb possesses very high phytotherapeutic efficacy with no demonstrated
173 toxicity. These findings suggest that *Phyllanthus amarus* is safe and non-toxic with very high remedy
174 potential in experimental mice. This agrees with previous documents [40].

175 Chronic toxicity study identifies and provides information on drugs that could possibly cause
176 harm and pose health challenges [41]. The subchronic oral toxicity assessment of *P. amarus* crude
177 ethanolic leaf extract during this study, reveals that the extract significantly ($P=.03$) boosted
178 antioxidant defense activity in both blood and liver tissue with associated reduction ($P=.01$) in overall
179 membrane damage. The liver is the organ involved in several metabolic functions and is therefore
180 prone to xenobiotic-induced injury because of its central role in xenobiotic metabolism [42].
181 Histopathological examination of the liver shows that *P. amarus* administered at 300mg/kg/d for 21
182 days invigorated liver cells. Hepatotoxic drugs could cause peroxidation of liver cell membrane lipids
183 and increase the amount of end products such as MDA [41].

184 Data suggest that *Phyllanthus amarus* extract has a measure of health benefits as shown by
185 the significant decrease in malondialdehyde (MDA) levels and associated increase in total antioxidant
186 capacity, TAC (Table 2). The decrease in malondialdehyde level may be as a result of the increased
187 antioxidant activities of *Phyllanthus amarus* [43]. Increased antioxidant activities in cells causes a
188 decrease in free radicals thereby reducing lipid peroxidation and malondialdehyde production. The
189 reduction in both blood and liver malondialdehyde levels suggests that the extract may contain
190 mixture of biomolecules with hydroxyl groups that perhaps prevented the abstraction of hydrogen
191 atom from the double bond of lipid bilayers thereby preventing lipid peroxidation. This suggestion
192 corroborates previous report on the *in vitro* analysis of the plant extract [44].

193 Phytochemical studies of *Phyllanthus amarus* extract have shown that the plant contains
194 chemicals such as flavonoids, tannins, saponins, alkaloids, terpenoids, glycosides and phenols
195 [44,21]. Flavonoids present in the plant have been shown to possess several pharmacological
196 properties such as antioxidant activities and anti-inflammatory activities [20,45]. Flavonoid as an
197 antioxidant has a rejuvenating effect on cells and tissues [46], Tannin has demonstrated high
198 activities against viral and bacterial infections as well as acting as strong antioxidant [47]. The
199 antioxidant activity of this plant phytochemicals may have contributed to the decrease in MDA levels
200 observed in this study. These findings are concurrent with previous studies conducted on the
201 toxicological assessment of *Phyllanthus amarus* [48].

202 CONCLUSION

203 From the results of this study, oral administration of *Phyllanthus amarus* extract is considered
204 non-toxic to mice at all doses (2000mg/Kg body weight to 5000mg/Kg body weight). Toxicity studies
205 of *Phyllanthus amarus* extract administration showed the absence of cumulative toxicity as reflected in
206 the absence of mortality recorded even at the highest dose level (5000mg/Kg body weight) of the
207 plant extract as well as results from the histological studies. In the light of these findings, we can
208 conclude that *Phyllanthus amarus* plant materials have no significant toxic effect in Swiss albino mice
209 for all the doses studied herein.

210 RECOMMENDATION

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

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Considerations	Cage side physical observations after 24 hours and 21 days					
	2000mg/kg		5000mg/kg		Control (0mg/kg)	
	24hours	21 days	24hours	21days	24hours	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 distention	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

UNDER PEER REVIEW

323 **Table 1: Cage side physical observations during the LD₅₀ evaluation of *P. amarus* ethanolic leaf extract**

324 Evidence from observations (Table 1) indicates that the LD₅₀ of *P. amarus* crude ethanolic leaf extract is greater than 5000mg/kg. Trial doses cannot be
 325 increased beyond 5000mg/kg because that is the limit dose. Effective dose (ED₅₀) =200mg/kg. Hence, therapeutic index, TI (LD₅₀/ ED₅₀)=25.0