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Effect of intake of *Phyllanthus amarus* aqueous leaf extract on lipid peroxidation and some antioxidant factors in wistar rats

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Abstract
Different parts of *Phyllanthus amarus* is being used for therapeutic purposes in Nigeria by the rural dwellers in the treatment of various diseases. This study aimed at investigating effect of ingestion of *P. amarus* on lipid peroxidation and antioxidant parameters in Wistar rats. The acute oral toxicity and subchronic toxicity studies of the leaf extract was determined in Wistar rats according to the procedures of Organization for Economic Cooperation and Development (OECD 423 Limit test) and OECD 407 respectively. At the end of the procedures, the rats were sacrificed; blood samples were taken for determination of malondialdehyde (MDA), vitamins A, C and E; uric acid, albumin and total bilirubin using standard techniques. In the acute toxicity study, no death or sign of toxicities were observed in the rats after 24 hours and up to 14 days post-oral dosage, indicating that the LD_{50} is greater than 5000 mg/kg. Malondialdehyde (MDA) was significantly lower ($p<0.05$) while vitamins A and C were significantly higher ($p<0.05$) in study groups than the control group, no significant difference ($p>0.05$) was observed in other parameters. In sub-chronic toxicity study, there was no significant differences ($p>0.05$) in all the analyzed parameters between the control and study groups except malondialdehyde (MDA) which was significantly lower ($p<0.05$) in study groups than the control group. These findings indicate that *P. amarus* leaf extract did not provoke oxidative imbalance in Wistar rats, hence oxidative stress was not induced.

Keywords: *Phyllanthus amarus*, toxicity, lipid peroxidation, oxidative stress, therapeutic

Introduction
Medicinal plants have been used by all cultures throughout history as remedies for human diseases [1]. A large majority of rural and urban dwellers in Nigeria still rely on traditional medicines to meet their primary health care needs [1]. *Phyllanthus amarus* is a plant of the family Euphorbiaceae distributed in tropical and subtropical countries of the world [2]. It is widely used for its medicinal properties for a variety of ailments like jaundice, constipation, diabetes, kidney ailments, chronic dysentery, ringworm, ulcers, urogenital tract infections, hemorrhoids, gonorrhea, hepatic and urolithic diseases [3]. The plant extract was also found to possess anti-inflammatory, anti-carcinogenic, antioxidant and hypoglycaemic properties and was also active against hepatitis B virus (HBV) and HIV-1 [4].

Oxidative stress has been implicated in some pathological conditions like diabetes mellitus, cancer, cardiovascular and neurodegenerative disease, asthma, chronic obstructive pulmonary disease and aging [5]. Oxidative stress occurs when the balance between antioxidants and production of free radicals or reactive oxygen species (ROS) is disrupted either due to depletion of antioxidants, or accumulation of ROS [6]. *Phyllanthus amarus* have been widely used for the treatment of various human disorders [7]. Over the years, notable changes in herbal constituents were observed due to contamination with microorganisms, pesticides, heavy metal and fungal toxins such as aflatoxin [8]. Furthermore, presence of active ingredients that confer medicinal properties depends on a number of factors including the plant species, the time and season of harvest, the type of soil and the preparation method. Also, significant phytochemical diversity among this plant when collected from different geographical conditions has been reported [2, 9]. Despite widespread usage and extensive studies carried out on *Phyllanthus amarus*, limited information exist on the scientific evaluation of toxicity, oxidative and antioxidative potential of this species in the Sub-saharan region of Northern Nigeria. Therefore, this study was designed to investigate the effects of aqueous leaf extract of *Phyllanthus amarus* on the oxidative stress indices in Wistar rats exposed to the plant in acute and sub-chronic oral toxicity studies.

Materials and Methods

Plant collection and identification

Fresh leaves of *Phyllanthus amarus* were collected from Fadama, Dundaye area of Sokoto, Nigeria. The plant was identified and authenticated at the herbarium unit of the Department of Pharmacognosy and Ethnopharmacy, Faculty of Pharmaceutical Sciences, Usmanu Danfodiyo University, Sokoto. Voucher number was obtained to be PCG/UDUS/Phyl/001 and the specimen was deposited.

Preparation of leaf extract

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 (1g) of the grinded plant material was soaked in 5mL of 80% methanol for 24 hours on a mixer to ensure maximum extraction by percolation using maceration technique under room temperature. This was followed by periodic stirring. Resulting crude extract was filtered using Whatman number 1 filter paper and the filtrate was concentrated in an oven at 40℃ to obtain 63.5g of green crude extract. The dried crude extract was stored in a refrigerator at 4℃ in a beaker until required for use. The crude extract was later dissolved in distilled water taking into consideration the average body weight of the Wistar rats.

Experimental animals

A total of thirty-four (34) Wister rats of both sexes, weighing 150g to 170g were purchased at the animal house of Faculty of Pharmaceutical Sciences, Usmanu Danfodiyo University, Sokoto. 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 daily and on a regular basis. They were housed in a clean metabolic cage-sand, placed in a well- ventilated conditioned room with a temperature of 26°C to 28°C, 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.

Experimental design

Acute oral 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 (OECD) Guidelines 423 [10]. Nine Wister rats of both sexes were used for this study. The rats were randomly divided into three groups of three rats per group with group 1 serving as control. The extract administered to the rats in groups 2 and 3 in single oral doses of 2000mg/kg and 5000mg/kg body weight respectively, was dissolved in 1mL of distilled water, by intra gastric gavage using oral cannula, one animal per day starting with group 2. The control group received an equal volume of distilled water as vehicle.

The animals were observed within the first 4 hours and subsequently 24 hours after administration of extract for toxic symptoms. Behavioural parameters and mortality were also closely monitored for 14 days.

Sub-chronic toxicity study

Sub-chronic toxicity study was carried out with OECD 407 guidelines [11]. Twenty-five (25) Wister rats of both sexes were divided into five groups of five 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 250mg/kg, 500mg/kg, 750mg/kg and 1000mg/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.

Blood sample collection and processing

At the end of the dosing period, the animals were fasted overnight and were anaesthetized in a glass jar containing wool soaked with chloroform. Ten milliliters of blood samples were collected from the animals using cardiac puncture into sterile lithium heparin containers. The blood samples collected were centrifuged at 4,000 rpm for 10 minutes. The plasma of each sample was separated and transferred into cryovial and stored frozen at -20°C until required for analysis.

Biochemical analysis

Malondialdehyde was estimated using the method described by Marbut *et al.* [12]. Vitamin A by the method of Rutkowski and Grzegorczyk [13] while vitamin C concentration was determined using the method of Singh and Singh [14] and Vitamin E concentration was estimated using the method described by Al-Kawaz and Al-Mashhady [15]. Uric acid concentration was estimated using the method described by Ochei and Kolhatkar [16], albumin using the method described by Cheesebrough [17] and total bilirubin using Malloy and Evelyn method [18].

Data analysis

The data obtained were analysed using Statistical Package for Social Sciences (SPSS) version 20. The results were expressed as mean ± standard deviation. Group comparisons were made using independent sample t-test, p-value less than or equal to 0.05 ($p \leq 0.05$) was considered as statistically significant.

Results

Acute toxicity study

Administration of aqueous leaf extract of *Phyllanthus amarus* (2000mg/kg and 500mg/kg) did not cause any adverse effect in treated rats. There was no change observed in physical parameters such as changes in fur, mucous membrane of the eyes, behavioral patterns, tremors, salivation and diarrhea in treated groups when compared to control. No mortality was observed in all treated groups throughout the dosing period.

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

Groups	No of death or Abnormal behavior (24hr)	No of death or Abnormal behavior (72hr)	No of death or Abnormal behavior (14 days)
Group 1 (Control) (Distilled water)			
Day 1:1st rat	None	None	None
Day 2:2nd rat	None	None	None

Day 3:3rd rat	None	None	None
Group 2 (2000 mg/kg)			
Day 1:1st rat	None	None	None
Day 2:2nd rat	None	None	None
Day 3:3rd rat	None	None	None
Group 3 (5000 mg/kg)			
Day 1:1st rat	None	None	None
Day 2:2nd rat	None	None	None
Day 3:3rd rat	None	None	None

Oxidative stress biomarkers in Wistar rats exposed to *P. amarus* aqueous leaf extract in acute oral toxicity study

Figure 1 shows the effect of administration of *Phyllanthus amarus* aqueous leaf extract on lipid peroxidation while figures 2-4 show its effect on antioxidant profile of Wister rats in acute oral toxicity. No significant difference ($p>0.05$) was observed in the analyzed parameters between the control and treated rats in group 2 except for malondialdehyde which was significantly lower ($p<0.05$) in treated rats than the control rats; whereas in group 3 (5000mg/kg), there was no significant difference ($p>0.05$) in the analyzed parameters between the control and treated rats except for vitamins A and C, which were significantly higher ($p<0.05$) in treated rats than the control rats respectively.

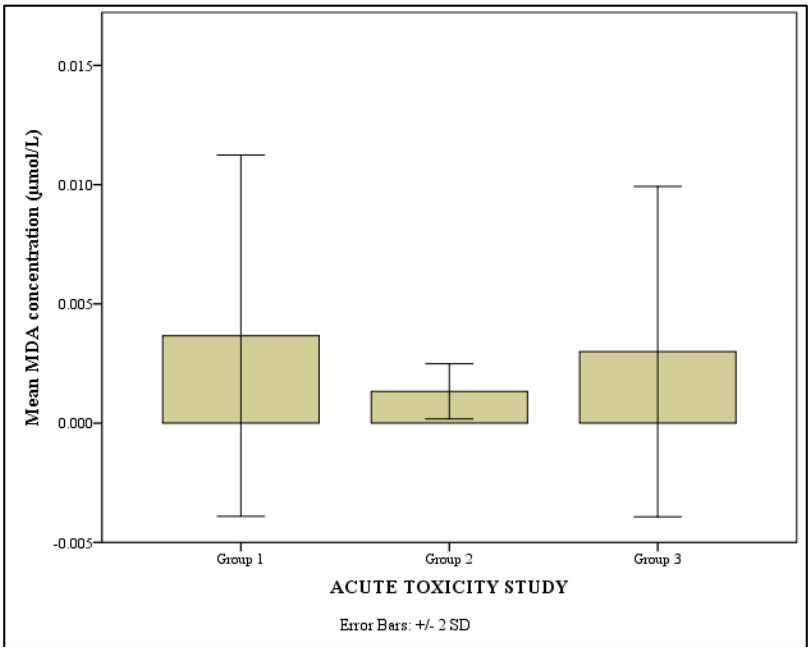

Fig 1: Effect of intake of *Phyllanthus amarus* aqueous leaf extract on lipid peroxidation of Wistar rats in acute oral toxicity study

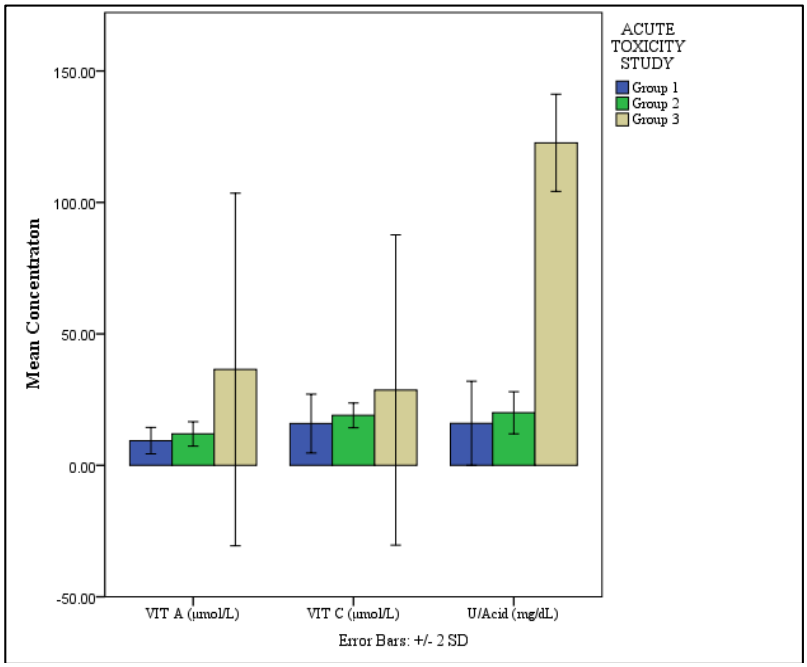

Fig 2: Effect of intake of *Phyllanthus amarus* aqueous leaf extract on antioxidant profile of Wistar rats in acute oral toxicity study

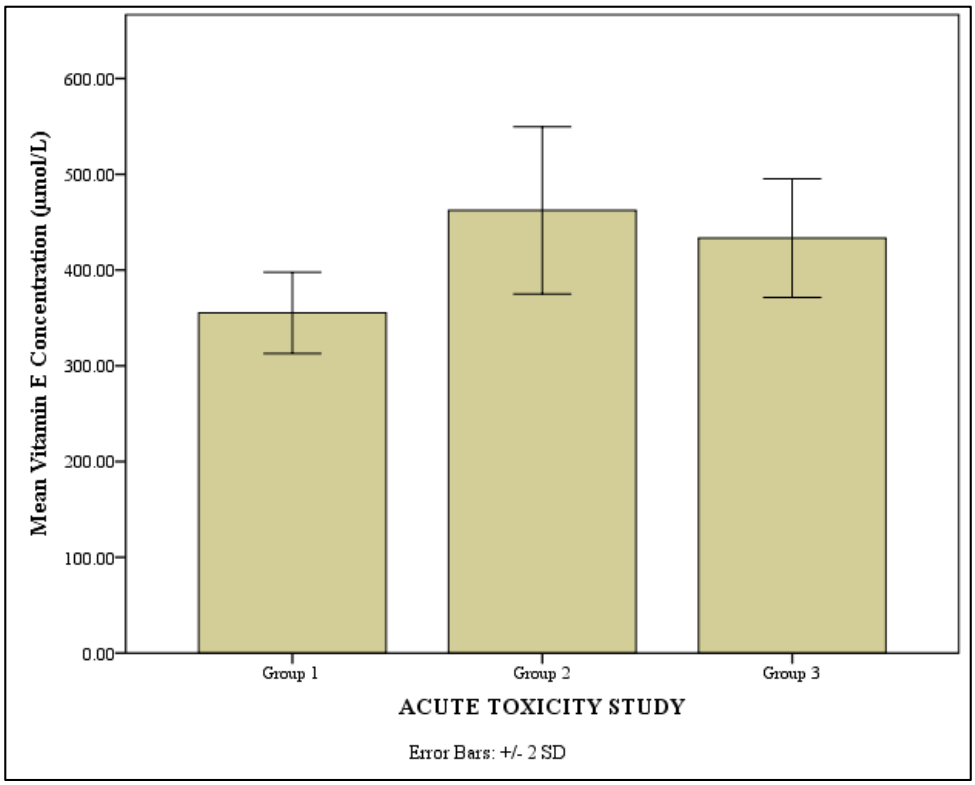

Fig 3: Effect of intake of *Phyllanthus amarus* aqueous leaf extract on antioxidant vitamin E level of Wistar rats in acute oral toxicity study

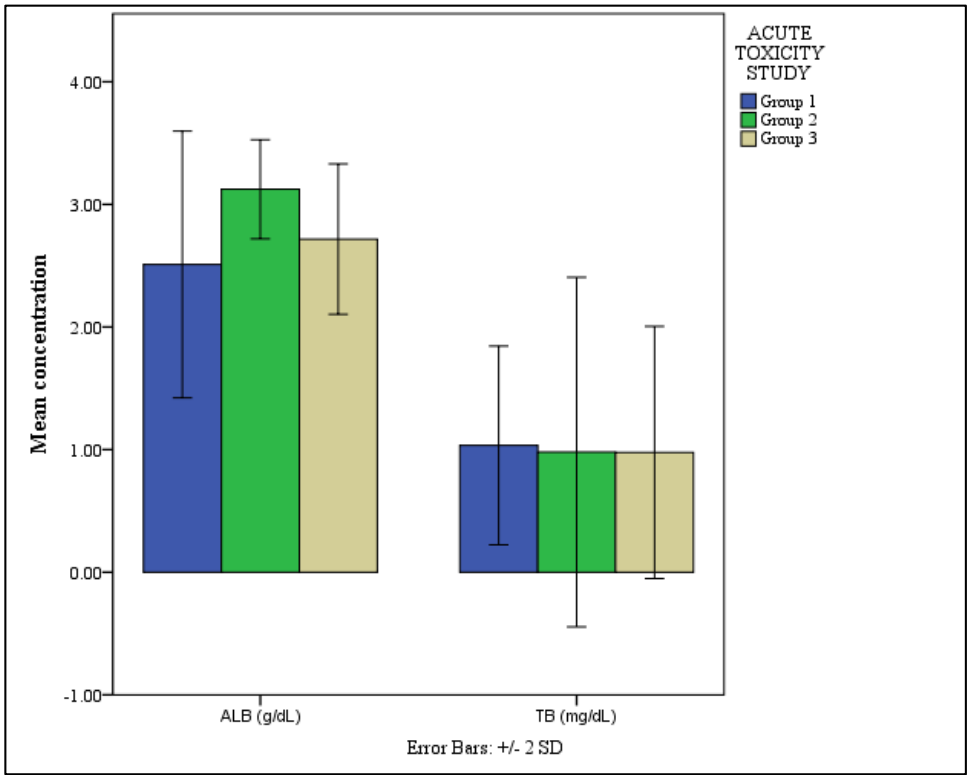

Fig 4: Effect of intake of *Phyllanthus amarus* aqueous leaf extract on antioxidant profile of Wistar rats in acute oral toxicity study

Oxidative stress biomarkers in Wister rats exposed to *P. amarus* aqueous leaf extract in sub-chronic toxicity study

Figure 5 shows the effect of administration of *Phyllanthus amarus* aqueous leaf extract on lipid peroxidation while figures 6-8 show its effect on antioxidant profile of Wister rats in sub-chronic toxicity study. Significantly reduced ($p<0.05$) values in malondialdehyde (MDA) were observed in all treated groups than control except for group 5 i.e. 1000mg/kg which was insignificant ($p>0.05$); whereas significantly higher ($p<0.05$) values in vitamin C were observed in treated rats of groups 2 and 3 (250mg/kg and 500mg/kg) respectively than control while others were insignificant.

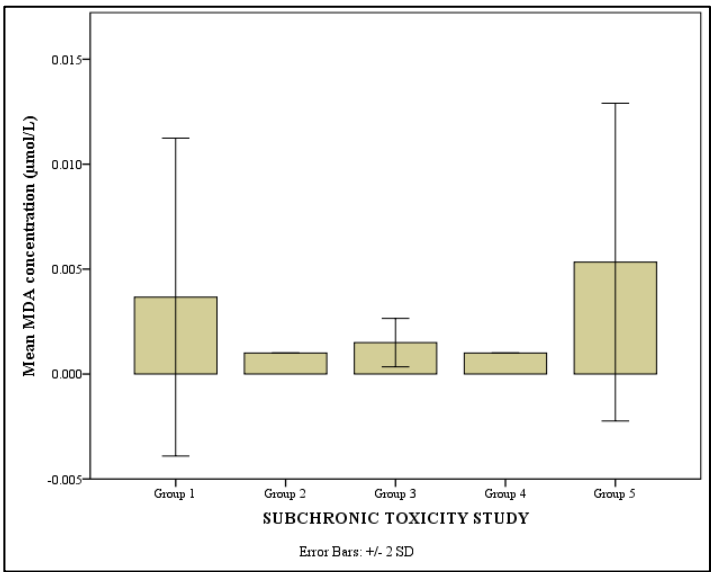

Fig 5: Effect of intake of *Phyllanthus amarus* aqueous leaf extract on lipid peroxidation of Wistar rats in sub-chronic toxicity study

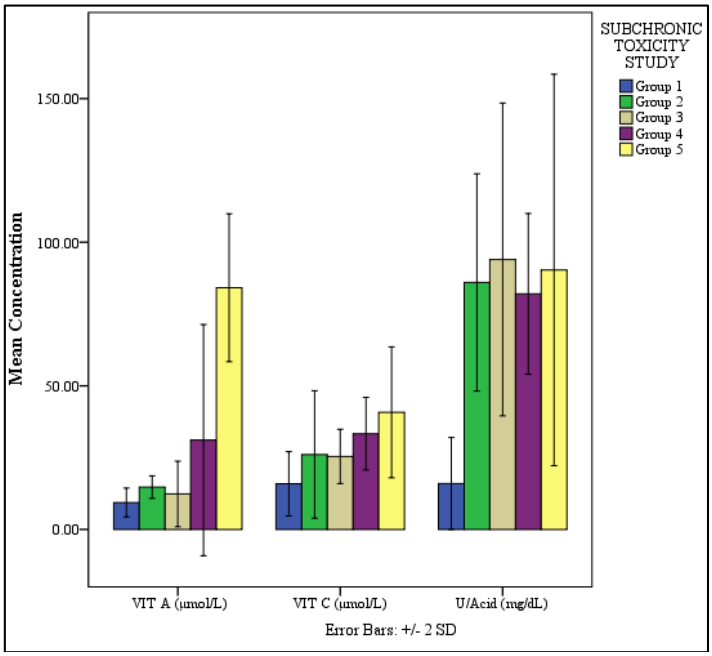

Fig 6: Effect of intake of *Phyllanthus amarus* aqueous leaf extract on antioxidant profile of Wistar rats in sub-chronic toxicity study

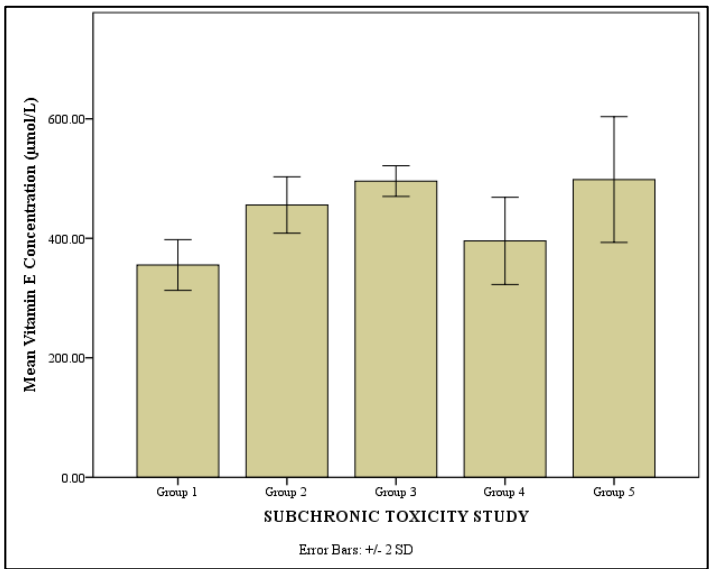

Fig 7: Effect of intake of *Phyllanthus amarus* aqueous leaf extract on antioxidant vitamin E level of Wistar rats in sub-chronic toxicity study

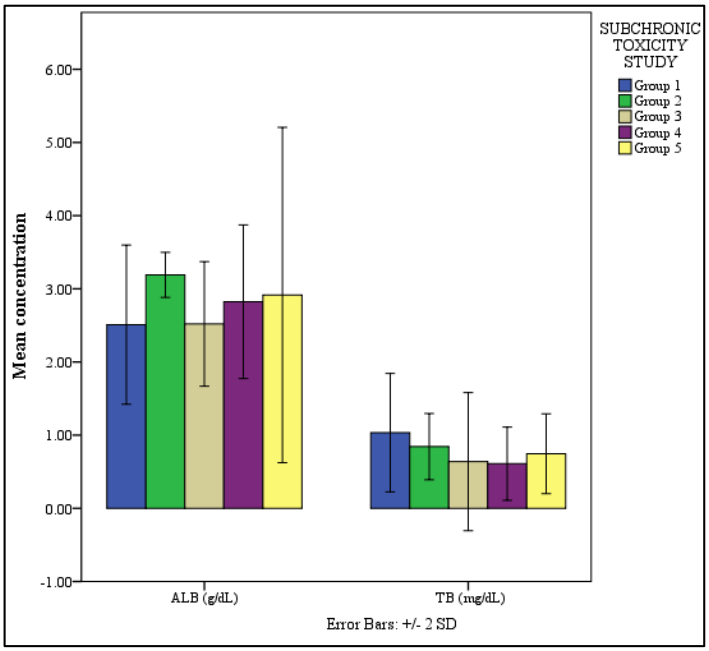

Fig 8: Effect of intake of *Phyllanthus amarus* aqueous leaf extract on antioxidant profile of Wistar rats in sub-chronic toxicity study

Discussion

In this study, oral administration of single dose of *Phyllanthus amarus* to Wistar rats at both 2000mg/kg and 5000mg/kg body weight had no effect on mortality and toxicity signs such as changes in fur, mucous membrane of the eyes, respiratory rate, effects on autonomic (salivation, perspiration, piloerection, urinary incontinence and defecation) and central nervous system (ptosis, drowsiness, gait, tremors and convulsion). No toxic effect was observed in rats treated with *Phyllanthus amarus* and therefore could be classified as non-toxic because the median lethal dose (LD_{50}) was higher than 5000mg/kg body weight. According to the globally harmonized system of classification (GHS) and Organization for Economic Co-operation and Development (OECD) method of classification and labeling of chemicals, any substance with LD_{50} higher than 5000mg/kg body weight could be generally regarded as safe [10, 19]. This finding is consistent with the reports of Lawson-Evi *et al.* [4] Pingale *et al.* [7] and Kushwaha *et al.* [20] in which the LD_{50} is greater than 5000mg/kg body weight.

Ingestion of *Phyllanthus amarus* leaf extract did not induce lipid peroxidation, as plasma malondialdehyde (MDA) concentration in both acute and sub-chronic toxicity studies did not show significant increase; rather significantly low MDA values were obtained in experimental rats as compared to control except 1000mg/kg and 5000mg/kg groups which were insignificant. This finding is in agreement with the study of Karuna *et al.* [21] in which reduced MDA values was observed post-oral treatment with aquoeus leaf extract at a dose of 200mg/kg body weight. This shows the ability of the plant to reduce lipid peroxidation and as such protects against oxidative stress induced damage. The mechanism by which *P. amarus* lowers plasma lipid peroxidation could be due to presence of the antioxidant phytochemical like phyllanthin that was shown to exhibit free radical scavenging activity and thus prevents the auto-oxidation of lipids [2, 3].

In both acute oral and sub-chronic toxicity studies, concentration of antioxidant parameters (vitamin E, albumin and total bilirubin) in the experimental rats were not significantly different ($p>0.05$) compared to control groups. Concentration of vitamins A and C of group 3 (5000mg/kg); vitamin C and uric acid values of groups 2 and 3 (250mg/kg and 500mg/kg) respectively were significantly higher ($p<0.05$) in the experimental rats than the control. This finding was similar to the study of Karuna *et al.* [21] in which higher values for antioxidant vitamins were observed post-oral treatment with aquoeus leaf extract at a dose of 200mg/kg body weight. Increase in concentration of antioxidant vitamins in treated rats is an indication that *Phyllanthus amarus* possess antioxidant activity. This might be responsible for the observed decrease in plasma lipid peroxidation.

In summary, MDA which is an indicator of lipid peroxidation was reduced in the animals treated with *P. amarus* and the antioxidant vitamins (vitamins A, C, E) were increased in the treated animals. These findings revealed that the leaf extract of *Phyllanthus amarus* did not induce oxidative stress in the rats. It also showed that the leaf extract can be useful in the management of antioxidant imbalance in humans.

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Competing interests

Authors have declared that no competing interests exist.

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