Original Research Article

Effect of ethanol Extract of *Xylopia aethiopica* Fruit on Oxidative Stress Indices of Wistar Rats

ABSTRACT

Background: The use of *Xylopia aethiopica* fruit in folklore medicine is on the increase without caution of its toxicity.

Aim: This present study tends to investigate its effect on the oxidative stress biomarkers of Wistar rats.

Methodology: The fruits of *Xylopia aethiopica* were air-dried and extracted using Soxhlet apparatus and ethanol as solvent. The median lethal dose (LD_{50}) of the extract was determined using standard method. Thirty Wistar rats were divided into five groups of six rats each. Animals in groups A, B, C, and D were administered 130, 259, 389 and 518 mg/kg body weight of *X. aethiopica* fruit extract respectively, while those in group E received normal feeds and water only. The administration was done once daily for 28 days via oral route. Oxidative stress indices were determined using standard methods.

Results: A significant reduction was observed in the activities of antioxidant enzymes (superoxide dismutase, catalase and glutathione peroxidase) in experimental animals compared with those in the control group (*P<0.05)*. Similarly, a significant reduction was observed in the concentration of reduced glutathione in experimental animals compared with those in the control group (*P<0.05)*. Lipid peroxidation was however observed to increase when experimental animals were compared with those in the control group. The elevation in lipid peroxidation was significantly different when animals treated with higher doses of 259, 389 and 518 mg/kg body weight of extract were compared with those in the control group (*P<0.05)*.

Conclusion: The adverse perturbation of antioxidant indices by *Xylopia aethiopica* fruit is suggestive that it could induce oxidative stress and thus unhinged the immune system. Oxidative stress has also been implicated in different diseases thus the consumption of *Xylopia aethiopica* fruit as well as its use in folklore medicine should be discouraged especially in high doses due to its toxic nature.

Keywords: Immune system; oxidative stress; toxic nature; *Xylopia aethiopica* fruit

1. INTRODUCTION

Antioxidants are substances that protect the body from damage caused by harmful molecules called free radicals [1]. Antioxidants help prevent oxidation, which can cause damage to cells and may contribute to aging. They may improve immune function and perhaps lower the risk for infection, cardiovascular disease, and cancer. Antioxidants exist as vitamins, minerals and other compounds in foods [2]. A diet containing plenty of fruits and vegetables, whole grains and nuts can supply all the antioxidants your body needs. Diets rich in antioxidants can be very beneficial. A few of the better known antioxidants include carotenoids (a form of

vitamin A) the substance that gives fruits and vegetables their deep rich colors [3]. Apricots, broccoli, pumpkin, cantaloupes, spinach and sweet potatoes are good choices. Foods containing vitamins C and E are also good sources of antioxidants, as well as selenium and zinc [4].

Oxidative stress induction generates free radicals including superoxides anions (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radical (OH^-), nitric oxide (NO), organic hydroperoxide (ROOH) through the production of reactive oxygen species (ROS) [1]. These ROS are very unstable atoms as they possess lone pair of electrons in their outermost shells. They have been implicated to play crucial roles in processes such as mutagenesis, carcinogenesis, aging, etc., which is mediated by their inherent capacity to cause cellular DNA damage [3]. Obviously, several activities of man generate free radicals endogenously and exogenously, which sadly result in debilitating pathological diseases. These pathological states are caused by the imbalance in the oxidative stress induction and the capacity of the biological system to neutralize the free radicals released following stress induction [5]. Of interest is the fact that the biological system has been packaged to nullify the destructive effect of these radicals generated through the inherent enzymatic antioxidants such as catalase, superoxide dismutase, and glutathione reductase [6]. However, there are also synthetic antioxidants that are taken into the body to enhance the activities of the enzymatic ones tert butyl hydroxyl toluene, butylated hydroxyl anisole, butylated hydroxyl toluene but regrettably, have been indicted to possess health-related risk [3]. This has led to the resurgence of the search for herbal antioxidants that will be compatible with the biological system as well as boost the capacity of enzymatic ones with the aim of averting the health-related problems accredited to synthetics [2].

Xylopia aethiopica also known as African Negro pepper enjoys a huge patronage both in nutrition and ethno medicine. Traditional medicine practitioners and traditional birth attendants (TBA) also uses decoction of the seed to induce placental discharge postpartum [7]. A decoction of the fruit or bark is useful in the treatment of bronchitis, asthma, stomach aches and dysenteric conditions [7]. An extract of the seed is also used as a vermifuge for roundworms [8]. A good number of postnatal women consume its aqueous decoction principally for its antiseptic properties. Some of them have occasionally presented to the clinic with features suggestive of organ complication [9]. Any herbal product with a therapeutic effect has the potential to be incorrectly prescribed or overdosed. The fact that *Xylopia aethiopica* is a natural product does not make it safe and may pose a risk to members of the public. Chemical components of *Xylopia aethiopica* have been helpful in the prevention and treatment of cancerous tumors [10]. *Xylopia aethiopica* fruits contain alkaloids, flavonoids, terpenoids, fixed oil and volatile aromatic oil. Key constituents are diterpenic and xylopic acids. *Xylopia aethiopica* oil contains carbohydrates and glycosides [11,12].

Fig. 1: *Xylopia aethiopica* Fruit [13]

Xylopia aethiopica is known to have myriad chemical constituents with diverse therapeutic and pharmacological properties. These compounds, most of which have been isolated and characterized, include saponins, sterols, carbohydrates, glycosides, mucilage, acidic compounds, tannins, balsams, cardiac

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glycosides, volatile aromatic oils, phenols [9,14,15], alkaloids, rutin and fixed oils [16,17]. The plant also contains vitamins A, B, C, D, and E, and proteins together with high amounts of minerals like copper, manganese and zinc [15,17]. The effect of on body weight and glucose concentration of animals has been reported [18]. The fruit has also been reported to induce dyslipidemia [19], hepatotoxicity [20] as well as renal toxicity [21]. This present study tends to investigate its effect on the oxidative stress biomarkers of Wistar rats.

2. MATERIALS AND METHODS

2.1 Collection and Authentication of Plant Materials

The fruits of *Xylopia aethiopica* were obtained from new market in Aba, Abia State and were identified and authenticated by Prof. (Mrs) Margaret Bassey of the Department of Botany and Ecological Studies, University of Uyo with the voucher number UU/PH/4e. The plant was deposited in the Herbarium of the Department of Pharmacognosy and Natural Medicine, University of Uyo, Akwa-Ibom State, Nigeria.

2.2 Extraction of Plant Materials

The extraction was carried out in the Post-graduate Laboratory of Department of Pharmacognosy and Natural Medicine, Faculty of Pharmacy, University of Uyo, Nigeria. It was carried out according to the method described by Ogbuagu et al. [13]. The fruits were washed under running tap water to remove contaminants and air-dried. The plant material was pulverized using laboratory blender to provide a greater surface area. The pulverized plant material was macerated in 250 mL of 99.8% ethanol (Sigma Aldrich) contained in round bottom flask, which was then attached to a Soxhlet extractor coupled with condenser and heating mantle (Isomantle). It was then loaded into the thimble, which is placed inside the Soxhlet extractor. The side arm is lagged with glass wool. The mixture was heated using the heating mantle (Isomantle) at 60 °C and as the temperature increases it begins to evaporate, moving through the apparatus to the condenser. The condensate then drips into the reservoir containing the thimble. Once the level of solvent reaches the siphon it pours back into the flask and the cycle begins again. This continues until it is exhaustively extracted. The process runs for a total of 13 hours. Once it was set up, it was left to run without interruption as long as water and power supply were not interrupted. The equipment was turned on and off and overnight running was not permitted, and the time split over a number of days. The extract was poured into 1000 mL beaker and concentrated to dryness in water bath (A3672- Graffin Student Water Bath) at 35 °C. The total weight of the marc (residue) and the concentrated extract were recorded, these processes took several days. The dried extract was preserved in the refrigerator at 4°C for further analysis.

2.3 Determination of Median Lethal Dose (LD_{50})

The median lethal dose (LD_{50}) of the extract was estimated using albino mice according to the method described by Airaodion et al. [22]. This method involves two phases:

In Phase one, five groups containing five mice each weighing between 20 g and 27g were fasted for 18 hours. They were respectively administered 1000 mg/kg, 2000 mg/kg, 3000 mg/kg, 4000 mg/kg and 5000 mg/kg body weight intraperitoneally (i.p) and were observed for physical signs of toxicity and mortality for 24 hours. A dosage of 1000 mg/kg recorded 0% mortality while 2000 mg/kg, 3000 mg/kg 4000 mg/kg and 5000 mg/kg recorded 100% mortality within 24 hours. Based on the value of phase one, phase two was conducted.

In Phase two, twenty-five albino mice weighing between 20 - 27g were grouped into five of five mice per group and were fasted for 18 hours. Each group was administered 1200 mg/kg, 1400 mg/kg 1600 mg/kg, 1800 mg/kg and 2000 mg/kg body weight intraperitoneally (i.p) and was observed for physical signs of toxicity and mortality within 24 hours. 1200 mg/kg recorded 0% mortality while 1400 mg/kg, 1600 mg/kg,

1800 mg/kg and 2000 mg/kg recorded 100% mortality within 24 hours. The LD_{50} was calculated as geometrical means of the maximum dose producing 0% (a) and the minimum dose producing 100% mortality (b).

$LD_{50} = \sqrt{ab}$

2.4 Experimental Design

Thirty Wistar rats obtained from the University of Uyo, Nigeria were used for this study. They were acclimatized for seven days before the commencement of the experiment. They were weighed and divided into five groups of six rats each. Groups A, B, C, D served as the experimental groups, while group E served as the control. Animals in group A were administered 130 mg/kg body weight (10% of LD_{50}) of *X. aethiopica* fruit extract, those in group B were administered 259 mg/kg body weight (20% of LD_{50}) of *X. aethiopica* fruit extract, those in group C were administered 389 mg/kg body weight (30% of LD_{50}) of *X. aethiopica* fruit extract, those in group D were administered 518 mg/kg body weight (40% of LD_{50}) of *X. aethiopica* fruit extract, while those in group E (control) received normal feeds and water only. The administration was done once daily for 28 days via oral route. At the end of 28 days treatment, animals were sacrificed under ether anaesthesia in a desiccator after an overnight fast. Blood samples were collected via cardiac puncture.

2.5 Determination of Oxidative Stress Indices

2.5.1 Determination of Superoxide Dismutase (SOD) Activity

Principle: The method involves generation of superoxide radical of riboflavin and its detection by hydroxylamine hydrochloride. The nitrite reacts with sulphanilic acid to produce diazonium compound which subsequently reacts with naphthylamine to produce red azo compound whose absorbance is measured at 543 nm.

This assay was carried out according to the procedure of Mohammad *et al*. [23] in which 1.4 mL aliquot of the reaction mixture comprised 1.1 mL of 50 mM phosphate buffer (pH 7.4), 0.075 mL of 20 mML^{-1} methionine, 0.4 mL of 1% (v/v) Triton X-100, 0.075 mL of 10 mM hydroxylamine and 0.1 mL of 50 mM EDTA. The aliquot (1.75 mL) was added to 0.1 mL of tissue homogenate and incubated at 30^oC for 15 min. This was followed by addition of 80 µL of 50 µM riboflavin and then the tubes were exposed for 9 minutes to 200 watts Philip lamp. After exposure time, 1 mL of Greiss reagent was added and the absorbance of the colour formed was measured at 543 nm. One unit of enzyme activity was measured as the amount of SOD capable of inhibiting 50 % of nitrate formation under the assay condition.

2.5.2 Determination of Catalase (CAT) Activity

Principle: The method is based on the fact that dichromate in acetic acid is reduced to chromic acetate when heated in the presence of H_2O_2 with the formation of perchloric acid as unstable intermediate. The acetate produced is measured colorimetrically at 610 nm.

This was assayed by the method described by Sinha [24]. The reaction mixture (1.5 mL) contained 1.0 mL of 0.01 M phosphate buffer, 0.1 mL of tissue homogenate and 0.4 mL of 2 M H_2O_2. The reaction was stopped by the addition of 2.0 mL dichromate acetic acid reagent (5% potassium dichromate and glacial acetic acid mixed in 1:3 ratio). Then, the absorbance was measured at 610 nm. CAT activity was expressed as µmol of H_2O_2 consumed/min/mg protein.

2.5.3 Determination of Glutathione Peroxidase (GPx) Activity

Principle: The activity of GPx was determined by measuring the decrease in GSH concentration after incubating the sample in the presence of hydrogen peroxide and sodium azide.

$H_2O_2 + 2GSH \rightarrow 2H_2O + GSSG$

Glutathione peroxidase activity was assayed by the method described by Rotruck *et al.* [25]. The

reaction mixture contained 0.2 mL of 0.4 M Tris-buffer, pH 7.0, 0.2 mL of EDTA, and 0.1 mL of 10 mM sodium azide, 0.2 mL of 10 mM glutathione and 0.1 mL of 0.2 mM. H_2O_2. The content was incubated at 37 ^oC for 10 minutes. The reaction was terminated by the addition of 0.4 ml 10% (v/v) TCA and centrifuged at 5000 rpm for 5 minutes. The supernatant was assayed for glutathione by Ellman's method. Exactly 3.0 mL disodium hydrogen phosphate solution and 1.0 mL of DNTB reagent were added to 2.0 mL of the supernatant. The standard was taken and treated in similar manner. The absorbance was read at 412 nm and expressed in terms of glutathione consumed /min/mg protein.

2.5.4 Determination of Lipid Peroxidation

Principles: Lipid peroxidation in the supernatant fractions was determined spectrophotometrically by assessing the concentration of thiobarbituric acid reactive substances (TBARS) as described by Varshney and Kale [26]. The results were expressed in malondialdehyde (MDA) formed relative to an extinction coefficient of 1.56 x 10^6 mol/cm.

Procedure: Acetic acid 1.5 mL (20%; pH 3.5), 1.5 mL of 0.8% thiobarbituric acid and 0.2 mL of 8.1% sodium dodecylsulphate was added to 0.1 mL of supernatant and heated at 100 ^oC for 60 min. After centrifugation at 1200×*g* for 10 min, the organic layer was separated and absorbance measured at 532nm using a spectrophotometer. Malondialdehyde (MDA) is an end product of lipid peroxidation, which reacts with thiobarbituric acid to form pink chromogen–thiobarbituric acid reactive substance. It was calculated using a molar extinction coefficient of 1.56×10^6 mol/cm and expressed as nanomoles of MDA/tissue. The concentration of MDA (nmol/ml) was calculated by using the formula:

Concentration of the test = $\frac{\mathbf{Abs\ (test) - Abs\ (blank)}}{\mathbf{1.56\ x\ 10^6}}$

2.5.5 Determination of Glutathione Concentration

Principles: Glutathione (reduced) was measured according to the method of Jollow *et al*. [27]. Reduced glutathione (GSH) forms the bulk of non-protein sulfhydryl groups. This method is based on the formation of relatively stable yellow colour when Ellman's reagent is added to a sulfhydryl compound, 2-nitro-5-thiobenzoic acid, the chromophoric product resulting from the reaction of Ellman's reagent with reduced glutathione.

Procedure: Equal quantities of homogenate and 10% trichloroacetic acid were mixed and centrifuged at 4000 x g for 15 minutes to separate the proteins. To 0.5 mL of the supernatant, 4.5 mL of Ellman's reagent was added. The mixture was vortexed and the absorbance read at 412 nm within 15 min.

2.6. Statistical Analysis

Results are expressed as mean ± standard deviation. The levels of homogeneity among the groups were assessed using One-way Analysis of Variance (ANOVA) followed by Tukey's test. All analyses were done using Graph Pad Prism Software Version 5.00 and P values < 0.05 were considered statistically significant.

3. RESULT

3.1 Median Lethal Dose (LD_{50}) Result

The physical signs of toxicity observed in the animals included excitation, paw licking, increased respiratory rate, decreased motor activity, gasping and coma which was followed by death. In the first phase of the median lethal dose determination, no mortality was recorded in the group treated with 1000 mg/kg body weight of *X. aethiopica* fruit extract. However, 100 % mortality was recorded in the groups treated with 2000, 3000, 4000, and 5000 mg/kg body weight of *X. aethiopica* fruit extract respectively. Similarly, in the second phase of medial lethal dose determination, no mortality was recorded in the group treated with 1200 mg/kg body weight of *X. aethiopica* fruit extract while 100% mortality was recorded in the groups treated with 1400, 1600, and 1800 mg/kg body weight of *X.*

aethiopica fruit extract respectively as presented in table 1.

The median lethal dose (LD_{50}) was calculated as geometrical means of the maximum dose producing 0% (a) and the minimum dose producing 100% mortality (b).

$LD_{50} = \sqrt{ab}$

Where a = 1200 mg/kg

b = 1400 mg/kg

LD_{50}= 1296.15 mg/kg

3.2: Effect of ethanol extracts of *Xylopia aethiopica* Fruit on Oxidative Stress Indices of Animals after 28 days of Treatment

The effect of ethanol extract of *Xylopia aethiopica* fruit extract on oxidative stress parameters of animals after 28 days of treatment is presented in Figs. 2-6. A significant reduction was observed in the activities of antioxidant enzymes (superoxide dismutase, catalase and glutathione peroxidase) in experimental animals compared with those in the control group (*P<0.05)*. Similarly, a significant reduction was observed in the concentration of reduced glutathione in experimental animals compared with those in the control group (*P<0.05)*. Lipid peroxidation was however observed to increase when experimental animals were compared with those in the control group. The elevation in lipid peroxidation was significantly different when animals treated with higher doses of 259, 389 and 518 mg/kg body weight of extract were compared with those in the control group (*P<0.05)*.

Table 1: The Median Lethal Dose (LD_{50}) of *Xylopia aethiopica* Fruit Extract

Study Phase/ (Animal)	Dosage of Extract (mg/kg) b.w	No of Mice per Group	No. of Death Recorded	% Mortality
PHASE ONE				
I	1000	5	0	0
II	2000	5	5	100
III	3000	5	5	100
IV	4000	5	5	100
V	5000	5	5	100
PHASE TWO				
I	1200	5	0	0
II	1400	5	5	100
III	1600	5	5	100
IV	1800	5	5	100

V	2000	5	5	100

LD_{50}= 1296.15 mg/kg

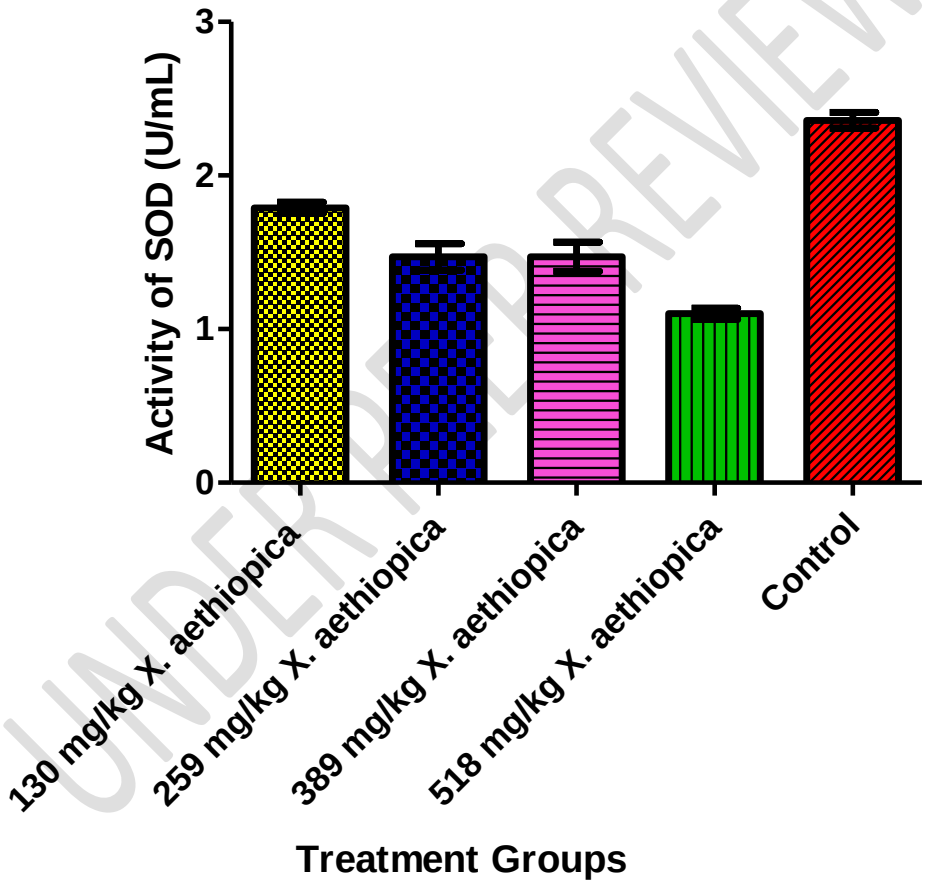

Fig. 2: Effect of *X. aethiopica* fruit extract on the Activity of Superoxide Dismutase (SOD) in Animals after 28 days of Treatment

Results are presented as mean ± SD with n = 6. Bars with different letters are significantly different at P<0.05

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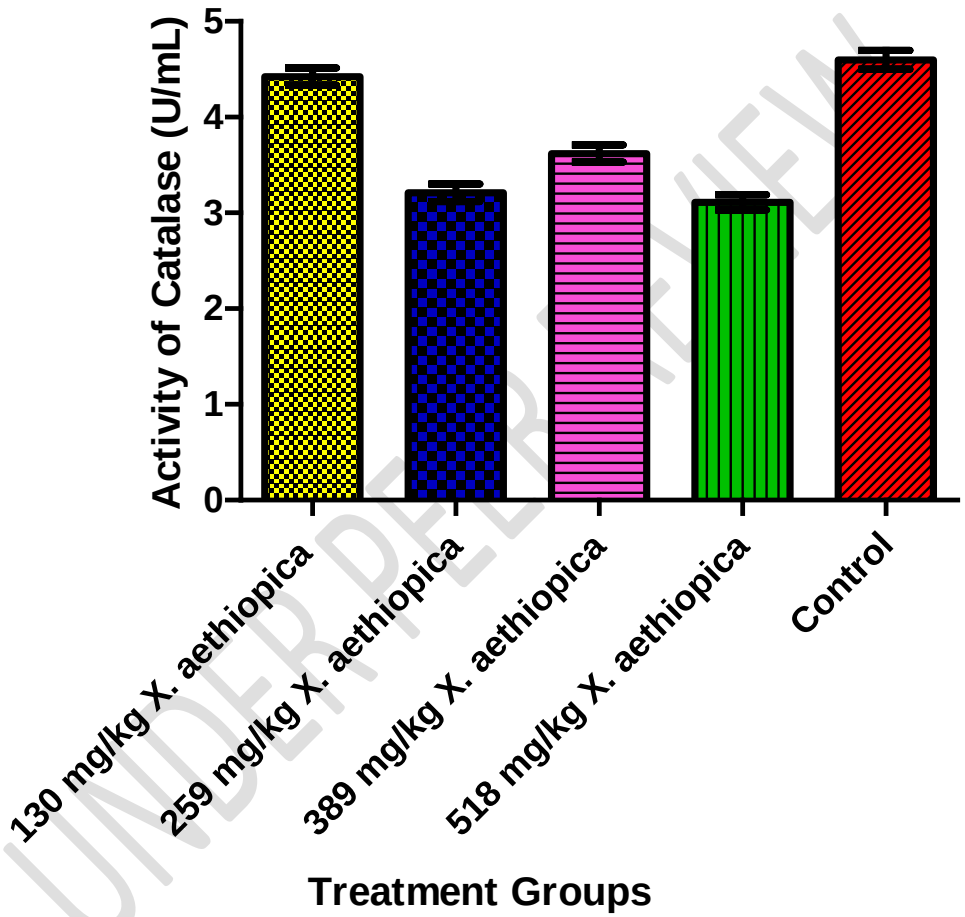

Fig. 3: Effect of *X. aethiopica* fruit extract on the Activity of Catalase in Animals after 28 days of Treatment

Results are presented as mean ± SD with n = 6. Bars with different letters are significantly different at P<0.05

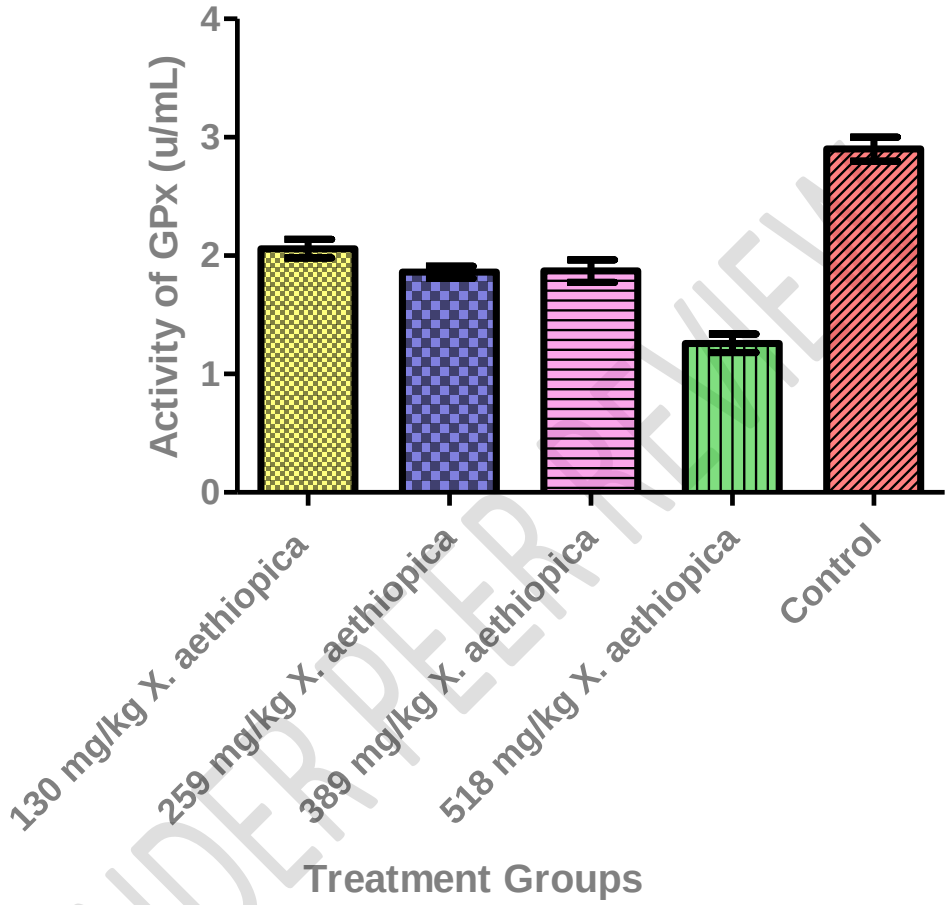

Fig. 4: Effect of *X. aethiopica* fruit extract on the Activity of Glutathione Peroxidase (GPx) in Animals after 28 days of Treatment

Results are presented as mean ± SD with n = 6. Bars with different letters are significantly different at P<0.05

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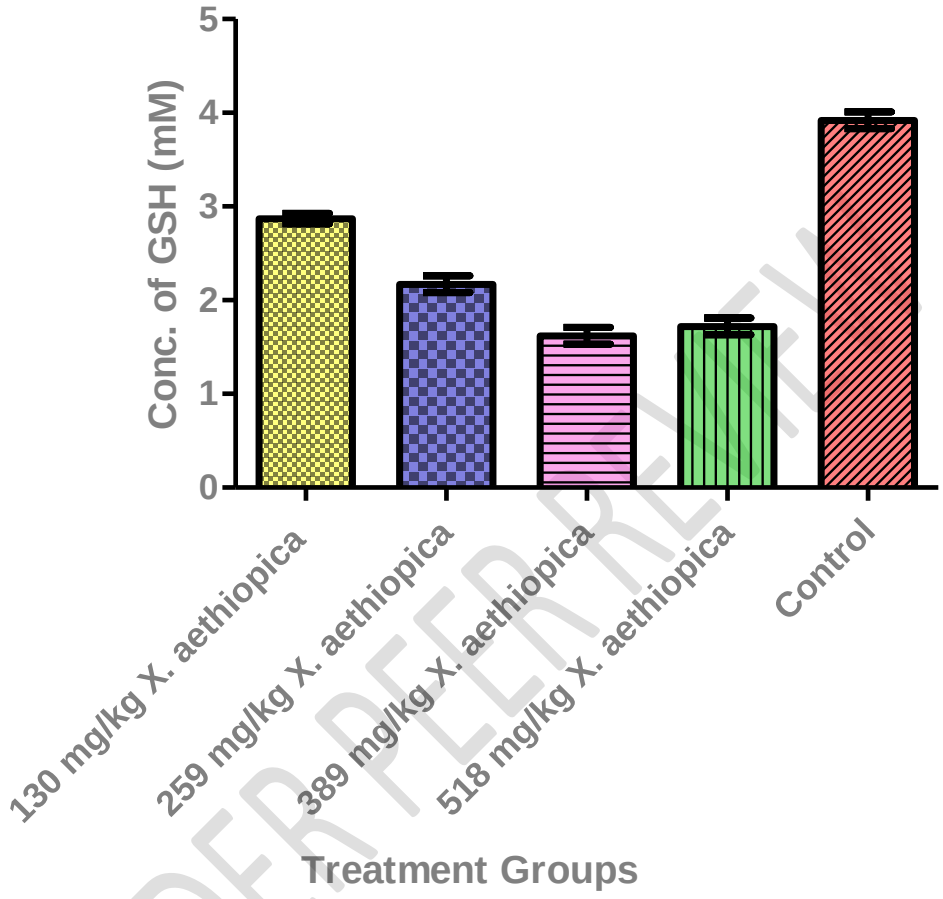

Fig. 5: Effect of *X. aethiopica* fruit extract on the Concentration of Reduced Glutathione (GSH) in Animals after 28 days of Treatment

Results are presented as mean ± SD with n = 6. Bars with different letters are significantly different at P<0.05

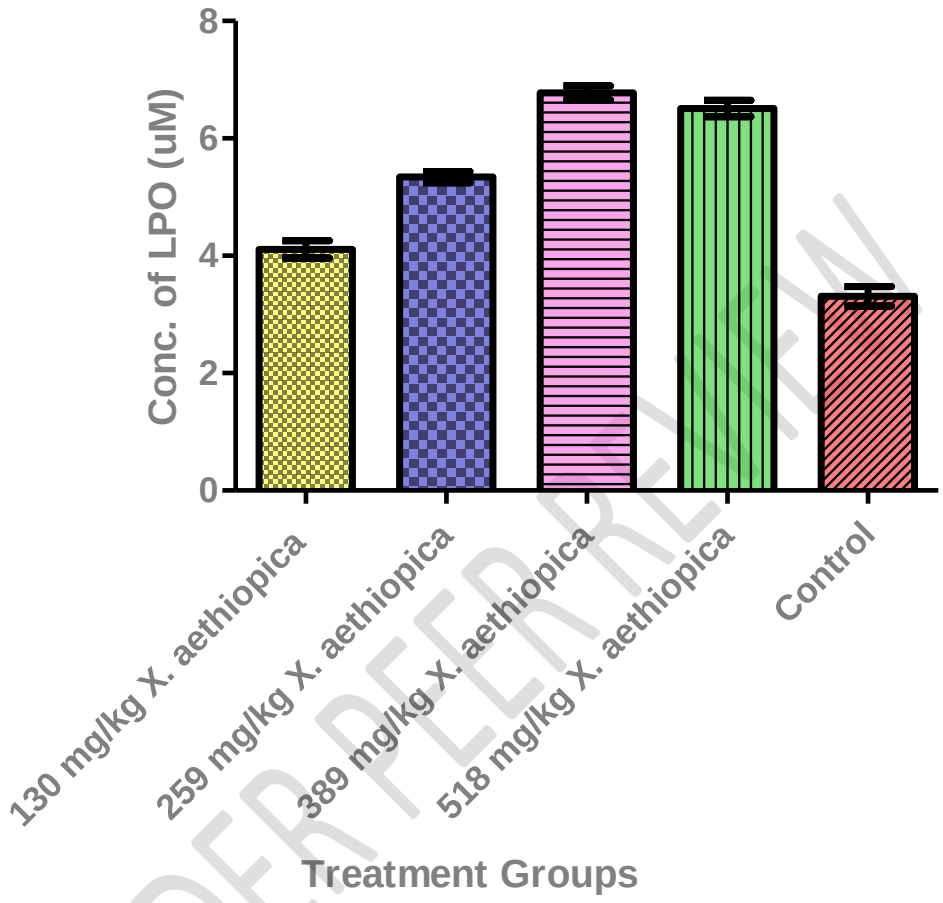

Fig. 6: Effect of *X. aethiopica* fruit extract on Lipid Peroxidation of Animals after 28 days of Treatment

Results are presented as mean ± SD with n = 6. Bars with different letters are significantly different at P<0.05

4. DISCUSSION

The acute toxicity study of the plant extracts recorded 100% mortality at a dose of 1400 mg/kg bodyweight and above. This shows that the fruit of *Xylopia aethiopica* might be highly toxic. The physical signs of toxicity observed in the animals included excitation, paw licking, increased respiratory rate, decreased motor activity, gasping and coma which was followed by death.

In this study, a significant decrease was observed in the activity of superoxide dismutase (SOD) in animals treated with *Xylopia aethiopica* fruit extract at all doses when compared with control animals at *P<0.05* (Fig. 2). This suggested that *Xylopia aethiopica* fruit extract might be toxic and possibly involved in the generation of free radicals. This agreed with the findings of Nnodim *et al*. [28] who reported that *Xylopia aethiopica* fruit induced oxidative stress.

In this study, administration of ethanol extract of *Xylopia aethiopica* fruit was observed to significantly decrease the activity of Catalase in animals treated with 259, 389 and 518 mg/kg of *Xylopia aethiopica* fruit extract when compared with control animals at *P<0.05* (Fig. 3). This suggested that *Xylopia aethiopica* fruit extract at these doses might be toxic and possibly lead to induction of oxidative stress. This agreed with the findings of Nnodim *et al*. [28], who reported that *Xylopia aethiopica* fruit generated free radicals in treated animals.

Administration of *Xylopia aethiopica* fruit extract significantly down-regulated the activity of glutathione peroxidase (GPx) at all doses (Fig 4). The biochemical function of glutathione peroxidase is to reduce lipid hydroperoxides to their corresponding alcohols and free hydrogen peroxide to water [29,30].This is in consonance with the findings of Nnodim *et al*. [28], who reported that *Xylopia aethiopica* fruit significantly reduced the activity of glutathione peroxidase of treated animals. Glutathione peroxidase is an enzymatic antioxidant that acts as a defense mechanism against oxidative stress [31]. The reduction in the activity of glutathione peroxidase observed in this study might be an indication that *Xylopia aethiopica* fruit has the propensity to induce oxidative stress.

In this study, administration of ethanol extract of *xylopia aethiopica* fruit caused a reduction in the concentration of reduced glutathione at all doses when compared with control group (Fig 5). The significant decrease observed in the reduced glutathione level in this study might be an indication that *Xylopia aethiopica* fruit enhances the conjugation of GSH with acetaldehyde [32]. This might be due to the direct reactive oxygen species (ROS) producing effect of *Xylopia aethiopica* fruit or a decrease in GSH synthesis.

In this study, administration of *Xylopia aethiopica* fruit extract significantly elevated the malondialdehyde (MDA) levels indicating enhanced peroxidation and breakdown of the antioxidant defense mechanisms. Malondialdehyde is a product of lipid peroxidation of polyunsaturated fatty acids [33,34]. Furthermore, extensive damage to tissues in a free radical mediated lipid peroxidation results in membrane damage and subsequently decreases the membrane fluid content. This is consistent with the study of Nnodim *et al*. [28], who reported that *Xylopia aethiopica* fruit significantly elevated lipid peroxidation (Fig. 6).

The depletion in the activities of antioxidant enzymes observed in this study is in line with the work of Somnez *et al*. [35]. The biochemical mechanism by which *Xylopia aethiopica* caused reduction in enzymatic antioxidants is currently unclear. It might be postulated that the consumption of this plant extract could cause oxidative stress and hence generate free radicals. These free radicals could then cause membrane damage through lipid peroxidation and protein oxidation [36-38]. Therefore, the decrease in the activities of superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx), enhanced lipid peroxidation. This is evidenced by the increased level of MDA observed in this study.

5. CONCLUSION

The adverse perturbation of antioxidant indices by *Xylopia aethiopica* fruit is suggestive that it could induce oxidative stress and thus unhinged the immune system. Oxidative stress has also been implicated in different diseases thus the consumption of *Xylopia aethiopica* fruit as well as its use in folklore medicine should be discouraged especially in high doses due to its toxic nature.

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