

Sub-chronic Administration of Methanolic Whole Fruit Extract of *Lagenaria breviflora* (Benth.) Roberty Induces Mild Toxicity in Rats

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ABSTRACT

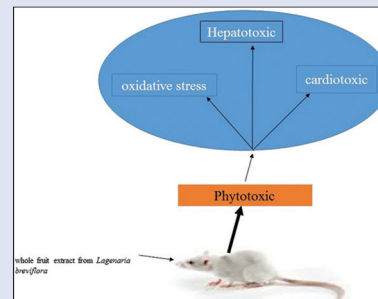
Background: The effect of the methanolic whole fruit extract from *Lagenaria breviflora* on vital organs and antioxidant enzymes was investigated in this study. **Materials and Methods:** *L. breviflora* (250, 500 and 1000 mg/kg/b.w.t./day/rat) was fed orally with the cannula to male albino rats for 28 days. At the end of the treatment, the rats were sacrificed and the effect of the extract on histology of the liver, heart, lipid peroxidation, tissue and serum antioxidant enzymes (superoxide dismutase, catalase, glutathione-s-transferase, glutathione peroxidase) activities, glutathione, myocardial marker enzymes (creatin kinase [CK], lactate dehydrogenase [LDH], alanine transaminase [ALT], and aspartate transaminase [AST]) in serum, and heart homogenate were assessed. **Results:** The extract demonstrated mild organ doses dependent (500 and 1000 mg/kg) pathological alterations in the architectural section of the liver and heart. At 250 mg/kg/b.w.t., the extract caused a significant ($P < 0.05$) increase in the level of thiobarbituric reacting acids substance and antioxidant enzyme activities, but causes ($P < 0.05$) decrease in serum and tissue antioxidant capacity at 500 and 1000 mg/kg/b.w.t., respectively. Also on these two doses, a significant ($P < 0.05$) increase in serum activity of CK, LDH, ALT, and AST and concomitantly decrease ($P < 0.05$) in heart homogenate were also observed. **Conclusion:** The results suggested that the Fruit of *L. breviflora* may contain phytotoxic Substances(s) which may be hepatotoxic, cardiotoxic or able to induce oxidative stress at high concentration. Hence, the consumption of the plant should be taken with caution.

Key words: Antioxidant enzymes, cardio-toxic, *Lagenaria breviflora*, lipid peroxidation, myocardial markers enzymes

SUMMARY

- Methanolic whole fruit extract from *Lagenaria breviflora* demonstrate dose

dependent mild toxicity on vital organs (Heart and liver) and anti-oxidant enzymes. The fruit of *Lagenaria breviflora* may contain Phyto-toxic substance (s) which may be hepatotoxic, Cardio-toxic or able to induce oxidative stress at high concentration. Hence, the consumption of the plant should be taken with caution.



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INTRODUCTION

Plants have great value to phytochemists because of their medicinal properties.^[1] They have been reported to play a significant role in maintaining human health, improving the quality of human life, and serve as valuable components of the medicine.^[2] Eighty percent of the world population still relies on medicinal plants because of their assumed minimal side effects and low-cost procurement.^[3] According to Adedapo *et al.*^[4] the use of herbal preparations is being gradually integrated into the primary and secondary health care systems in Nigeria. One of the numerous medicinal plants used in Nigeria is *Lagenaria breviflora* (Benth.) Roberty (family Cucurbitaceae). It is known as Wild colocynth in English, Gojinjima in Hausa, Anyūmmūō in Igbo and Tagiiri in Yoruba.^[5] *L. breviflora* is a perennial climber that occurs from Senegal to West Cameroon, and is generally widespread in Tropical Africa.^[6] The fruits are dark green with cream blotches, ovoid in shape, 9 cm long, and 15–25 cm in diameter.^[7] Traditionally, they are used for the prevention and treatment of Newcastle disease such as coccidiosis in poultry and measles in humans.^[8–10] Scientific reports showed that the plant demonstrated a considerable anti-bacterial,^[11] anti-implantation,^[12] miracidial and cercaricidal activities,^[13] hematinic and immunostimulatory activities,^[14,15] cytotoxic,^[16] anti-inflammatory,^[17] hepatoprotective, and cardiovascular effects.^[18] Phytochemical analysis of *L. breviflora* revealed that the fruit extract contains various types of bioactive compounds

ranging from saponins, phenolics,^[19] and cucurbitacins.^[18,20] The dependence on natural products has its merits, but there are concerns by certain medical personnel that herbal medicines may be harmful to vital organs (liver, heart, and kidneys)^[21] and blood parameters.^[14] From the numerous publications on the pharmacology activities of *L. breviflora*, toxicological studies with laboratory animals, particularly in the cardiac and hepatocytes are scanty. Moreover, the effect of methanolic fruit extract from *L. breviflora*-induced redox changes in the liver, and heart has not yet been explored. Thus, the present study was designed to investigate the effect of the crude extract of *L. breviflora* on the histology of liver, heart, antioxidant enzymes, and myocardial marker enzymes in the Wistar albino rats.

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MATERIALS AND METHODS

The Fresh fruits of *L. breviliflora* were bought from a local market in Ogbomosho, Oyo State, Nigeria and were identified by Dr. Ogunkunle of the Department of Pure and Applied Biology, Ladoko Akintola University of Technology. A voucher (DSO 014) sample of the plant was also deposited at the university herbarium.

Extraction of the fruit of *Lagenaria breviliflora*

Fresh fruits of *Lagenaria breviliflora* were bought, washed and sliced. Two kilogram of the sliced fruits was blended with 4 L of methanol and soaked for 72 h at room temperature. The mixture was stirred, frequently and later filtered with a sack cloth and was stored at 40°C. The residue was re-soaked in 2 L of methanol for 24 h and then filtered. The filtrate obtained was concentrated in a rotary evaporator at 40°C. A greenish brown jell-like concentrate was obtained, weighing 69.6 g (3.48% of the fresh fruits). A stock solution was prepared by dissolving 100 mg of the extract in 100 mL of distilled water.

Experimental animals

Twenty-eight adult male rats (180–200 g) used for this study were housed at the Animal Housing Unit of the Department of Biochemistry, Ladoko Akintola University of Technology Ogbomosho, Oyo State, Nigeria, and were maintained under standard laboratory conditions at ambient temperature of $25 \pm 2^\circ\text{C}$ and $50 \pm 15\%$ relative humidity with a 12-h light/12-h dark cycle for 2 weeks before commencement of the experiment. The rats were fed with pelletized rat ration (Guinea Feed, Nigeria Ltd.) and water *ad libitum*. After acclimatization, the rats were divided into four groups (A, B, C and D) of seven animals each. Group A were administered with 0.9% physiological saline. While, the three treatment Groups B, C, and D were administered with methanolic extract of *L. breviliflora* at 250, 500, and 1000 mg/kg body weight of the rats, respectively. The rats were administered, orally with their various designated dosages once daily for 28 days using rat cannula.

Collection of tissue samples

After the experimental regimen, the animals were sacrificed under mild chloroform anesthesia. Blood was collected by cardiac puncture and serum was separated by centrifugation (for 20 min at 3500 rpm). Liver and heart were immediately dissected out, bloated, washed and 0.5 g of tissue was weighed and homogenized in 5 mL of 0.05 M of Tris-HCl buffer (pH 7.4) in 0.9% ice cold saline for various biochemical evaluations. The remaining portion of the tissues was put in 1% formalin for histopathological studies.

Estimation of biochemical parameters in serum and tissues

Serum marker enzymes such as alanine transaminase (ALT), aspartate transaminase (AST), lactate dehydrogenase (LDH), glutathione (GSH), and creatine kinase (CK) were assayed using diagnostic reagent kits manufactured by Merck India Ltd.

Estimation of malonyldialdehyde

The method described by Okhawa *et al.*^[22] was employed to determine the level of lipid peroxidation in the animal tissue. Briefly, the reaction mixture of 0.2 mL of 8.1% sodium dodecyl sulfate, 1.5 mL of 20% acetic acid solution adjusted to pH 3.5 with sodium hydroxide and 1.5 mL of 0.8% aqueous solution of thiobarbituric acid was added to 0.2 mL of 10% (w/v) of the homogenate. The mixture was brought up to 4.0 mL with distilled water and heated at 95°C for 60 min. cooling with tap water, 1.0 mL distilled water and 5.0 mL of the mixture of n-butanol, and pyridine (15:1 v/v) was

added and the mixture centrifuged at 2000 rpm for 10 min. The organic layer was removed, and its absorbance measured at 532 nm and compared with those obtained from malonyldialdehyde standards.

Determination of superoxide dismutase activity

The activity of superoxide dismutase (SOD) was determined as described by Misra and Fridovich^[23] The assay mixture contained 0.5 mL of hepatic PMS, 1 mL of 50 mM sodium carbonate, 0.4 mL of 25 μM nitroblue tetrazolium, and 0.2 mL of freshly prepared 0.1 mM hydroxylamine hydrochloride. The reaction mixture was mixed quickly by inversion followed by the addition of the clear supernatant of 0.1 mL of liver homogenate (10% w/v). The change in absorbance was recorded at 560 nm. The percentage of inhibition was calculated using this equation: Percentage superoxide dismutase inhibition = $([\text{normal activity} - \text{inhibited activity}]/[\text{normal activity}]) \times 100\%$.

Determination of catalase activity

Catalase (CAT) activity was measured, as described by Pari and Latha.^[24] Briefly, the tissue was homogenized in 0.01 M phosphate buffer (pH 7.0) and centrifuged at 5000 rpm. The reaction mixture consisted of 0.4 mL of hydrogen peroxide (H_2O_2) (0.2 M), 1 mL of 0.01 M phosphate buffer (pH 7.0), and 0.1 mL of liver homogenate (10% w/v). The reaction of the mixture was stopped by adding 2 mL of dichromate acetic acid reagent (5% $\text{K}_2\text{Cr}_2\text{O}_7$ prepared in glacial acetic acid). The changes in the absorbance were measured at 620 nm and recorded. The percentage of inhibition was calculated using the equation: Percentage catalase inhibition = $([\text{normal activity} - \text{inhibited activity}]/[\text{normal activity}]) \times 100\%$.

Determination of glutathione peroxidase

Glutathione peroxidase (GPx) was measured by the method described by Rotruck *et al.*^[25] Briefly, the reaction mixture contained 0.2 mL 0.4 M phosphate buffer (pH 7.0), 0.1 mL 10 mM sodium azide, and 0.2 mL tissue homogenized in 0.4M phosphate buffer pH 7.0. 0.2 mL tissue homogenized in 0.4 M, phosphate buffer, pH 7.0, 0.2 mL reduced GSH, 0.1 mL 0.2 mM H_2O_2 . The contents were incubated for 10 min at 37°C, 0.4 mL 10% TCA was added to stop the reaction and centrifuged at $3200 \times g$ for 20 min. The supernatant was assayed for GSH content using Ellman's reagent 19.8 mg 5,5'-dithiobisnitrobenzoic acid (DTNB) in 100 mL 0.1% sodium nitrate. The activities were expressed, as μg of GSH consumed/min/mg protein.^[25]

Estimation of reduced glutathione

The amount of reduced GSH in the samples was estimated by the method of Boyne and Ellman.^[26] 1 mL of the sample extracts were treated with 4.0 mL of metaphosphoric acid precipitating solution (1.67 g of glacial metaphosphoric acid, 0.2 g EDTA and 30 g NaCl dissolved in 100 mL water). After centrifugation, 2.0 mL of the protein-free supernatant was mixed with 0.2 mL of 0.4 M Na_2HPO_4 and 1.0 mL of DTNB reagent (40 mg DTNB in 100 mL of aqueous 1% trisodium citrate). Absorbance was read at 412 nm within 2 min. GSH concentration was expressed as nmol/mg protein.

Determination of glutathione-s-transferase activity

The activity of glutathione-s-transferase (GST) was determined according to the method of Habig *et al.*^[27] Briefly, CDNB solution (0.1 mL) was pipetted into a conical flask before adding phosphate buffer (1 mL) and distilled water (1.7 mL). Next, the mixture was incubated at 37°C for 5 min. The serum sample (0.1 mL) and GSH solution (0.1 mL) were added (using an automatic micropipette), after the incubation. A blank devoid of the serum was prepared for background correction.

Absorbance readings at 340 nm were taken for 5 min at 60 s interval using a UV-VIS analyst spectrophotometer.

Histopathological analysis of the liver and heart

The liver and heart of the animals from each group were fixed in 10% formaldehyde, dehydrated, and paraffin blocks prepared for histopathological examination. The block was sectioned at 5–7 μm and stained with hemotoxylen.

Statistical analysis

Results are expressed as mean ± standard deviation of six individual experiments and the statistical significance was evaluated by one-way analysis of variance using SPSS software package, version (10.0) and the individual comparisons were obtained by the Duncan multiple range test. A value of $P < 0.05$ was considered to indicate a significant difference between groups.

RESULTS

Sub-chronic administration of methanolic fruit extract of *L. breviflora* to rats at 250 mg/kg/b.w.t., respectively cause a significant ($P < 0.05$)

dose-dependent increase in the serum and tissues (liver and heart homogenate) level of thiobarbituric reacting acids substance (TBARS), anti-oxidant enzymes (SOD, CAT, GPX and GST) and GSH, and concomitant reduction ($P < 0.05$) in antioxidant enzymes at 500 and 1000 mg/kg/b.w.t., respectively [Figures 1-6].

Table 1 shows the effect of graded doses (250, 500 and 1000 mg/kg/b.w.t.) of the extract on myocardial marker enzymes in serum and heart homogenate. The results revealed that the delirious effect of the extract on markers enzymes was more pronounced at 500 and 1000 mg/kg/b.w.t.

The histology results observed in the rats treated 500 and 1000 mg/kg/b.w.t. *L. breviflora* [Figure 7A and B] also agreed with the elevated serum and tissue antioxidant enzymes, lipid peroxidation, and makers of cardiotoxicity. At 250 mg/kg/body weight, the extract does not cause any alteration in the liver [Figure 7A, a and b] or heart [Figure 7B, a and b] architecture. However, the extract at 500 and 1000 mg/kg causes a dose-dependent mild inflammatory cells aggregate in the focal area, moderately dilated and very mild inflammatory cell infiltration of the sinusoids of the hepatocytes (1c-d). The heart section also revealed focal area of mild hemorrhage, perivascular infiltration and few adipose tissue at the pericardial layer and mild fibrosis in myocardial layers of the heart section at 500 and 1000 mg/kg/body weight [Figure 7B c and d].

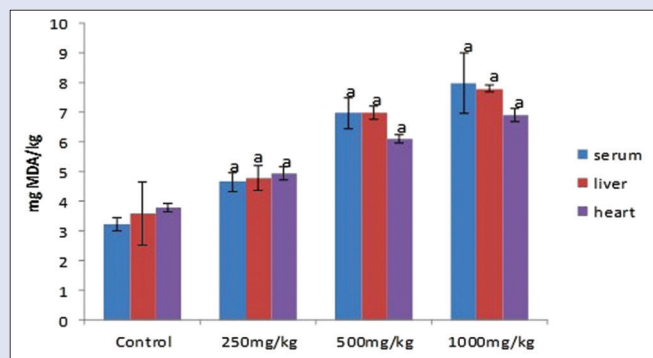


Figure 1: Lipid peroxidation (thiobarbituric reacting acids substance) in serum, liver and heart of control, and experimental groups. Values are expressed, as the mean ± standard deviation of six individual experiments. Values with superscript letter “a” differ significantly ($P < 0.05$) from the control

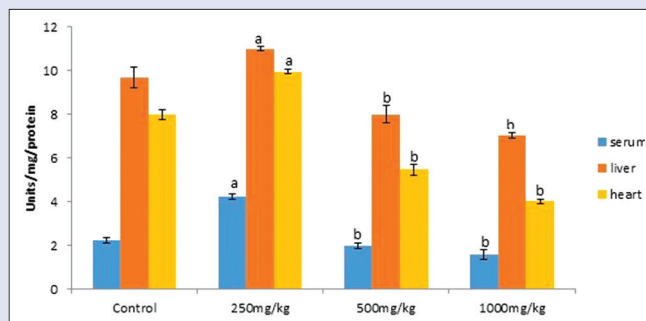


Figure 2: Activity of superoxide dismutase in serum, liver and heart homogenate of control and experimental rats. Values are expressed as the mean ± standard deviation of six individual experiments. Values with superscript letter “a” and “b” are significant ($P < 0.05$) different from the control

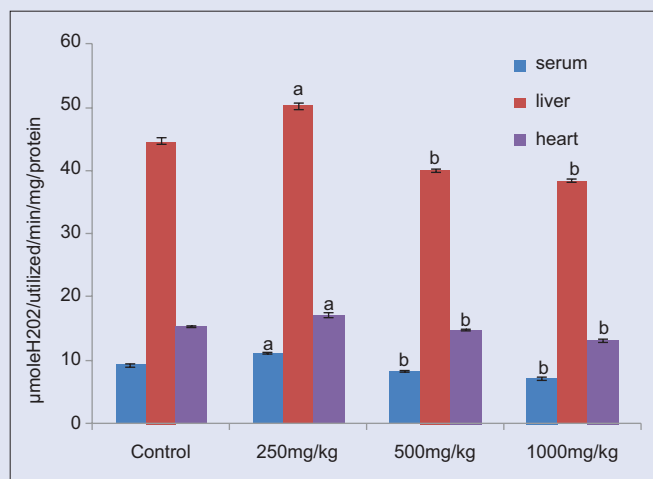


Figure 3: Activity of catalase in serum, liver and heart homogenate of control and experimental rats. Values are expressed as the mean ± standard deviation of six individual experiments. Values with superscript letter “a” and “b” are significantly ($P < 0.05$) different from the control

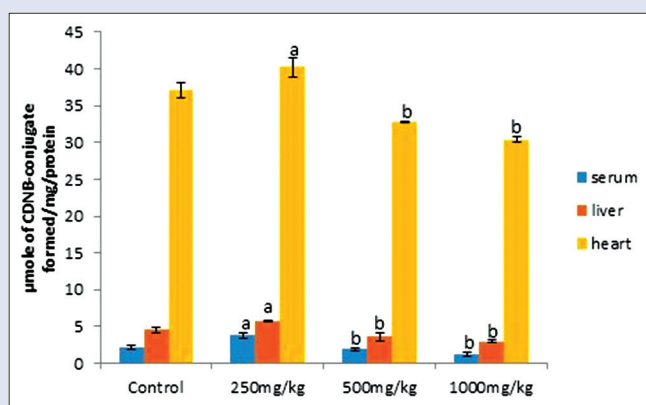


Figure 4: Activity of glutathione-s-transferase in serum, liver and heart homogenate of control and experimental rats. Values are expressed as the mean ± standard deviation of six individual experiments. Values with superscript letter “a” and “b” are significantly ($P < 0.05$) different from the control

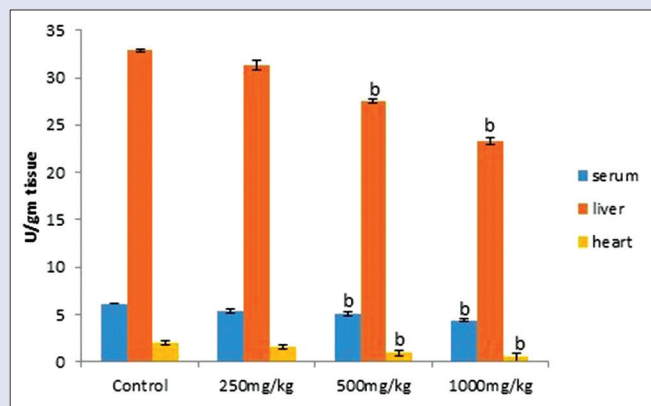


Figure 5: Activity of glutathione in serum, liver and heart homogenate of control and experimental rats. Values are expressed as the mean \pm standard deviation of six individual experiments. Values with superscript letter "b" are significantly ($P < 0.05$) different from the control

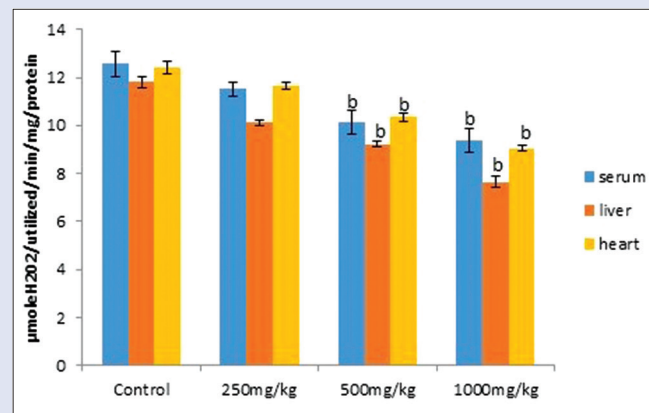


Figure 6: Activity of glutathione peroxidase in serum, liver and heart homogenate of control and experimental rats. Values are expressed as the mean \pm standard deviation of six individual experiments. Values with superscript letter "b" are significantly ($P < 0.05$) different from control

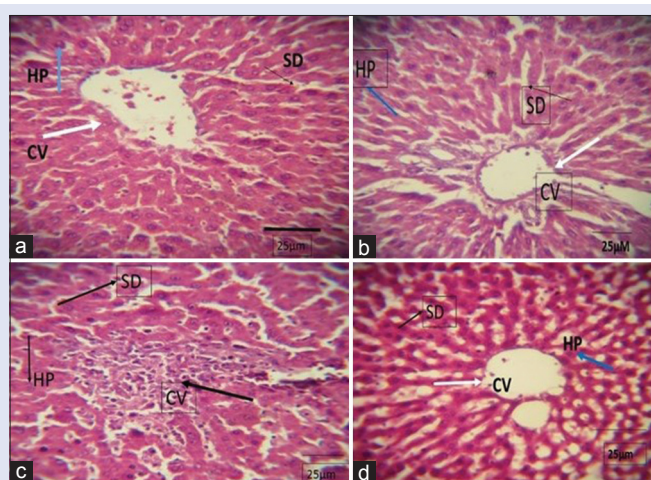


Figure 7A: Photomicrograph of rat liver. (a) The liver of control rat; (b) liver of rat fed with 250 mg/kg/b.w.t. of methanolic extract of *Lagenaria breviflora*; (c) liver of rat fed with 500 mg/kg/b.w.t., of methanolic extract of *L. breviflora*; (d) liver of rat fed with 1000 mg/kg/b.w.t. of methanolic extract of *L. breviflora* showing focal area of mild inflammatory cells aggregate (black arrow), the sinusoids (standard deviation) (slender arrow) show very mild infiltration, the central vessels (CV) (white arrow) are not congested. The hepatocytes (HP) (blue arrow) appear normal with normal nuclei and cytoplasmic morphology. Scale bar: 20 μ m, H and E, $\times 400$)

The results of the histology investigation also showed that the extract demonstrate mild toxicity at various concentration considered, especially at 500 and 1000 mg/kg [Figures 7A and B].

DISCUSSION

A large population in Africa still relies on the use of medicinal plants for the treatment of various diseases without any consideration for their side effects. It is generally believed that they are safe. However, recent scientific reports have shown that most of these plants are injurious to animal cells. For example, the aqueous leaf extract of *Eucalyptus globulus* was reported to demonstrate the deleterious effects on the liver membrane structure and functional integrity.^[28]

TBARS or malondialdehyde is a major indicator usually employed in the assessment of the degree of lipid peroxidation. The dose-dependent

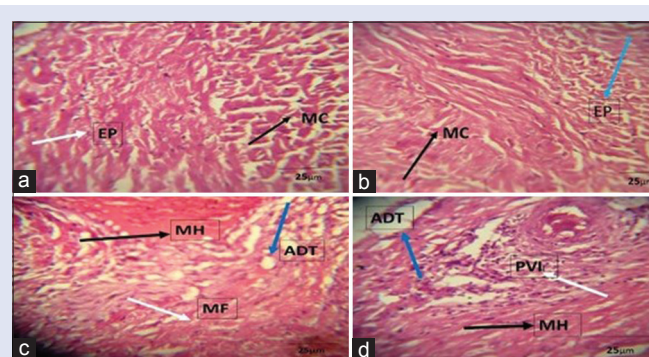


Figure 7B: Photomicrograph of rat heart. (a) The heart of control rat; (b) heart of rat fed with 250 mg/kg/b.w.t. of methanolic extract of *Lagenaria breviflora*; (c) heart of rat fed with 500 mg/kg/b.w.t. of methanolic extract of *L. breviflora* showing focal area of mild hemorrhage (MH) (black arrow) and moderate adipose tissue (blue arrow) at the pericardial layer (PV) and mild myocardial fibrosis (white arrow) (scale bar: 25 μ m, H and E, $\times 400$)

increase ($P < 0.05$) observed in the serum and tissues level of TBARS of rats treated with the extract [Figures 1-6] suggested membrane lipid peroxidation. According to Pandey and Rizvi,^[29] TBARS react with the free amino group of proteins, membrane phospholipids, and nucleic acids leading to structural modification and oxidative stress.^[28] The significant increase in serum and tissue antioxidant enzymes activities, especially SOD and CAT [Figures 2 and 3] at low concentration (250 mg/kg/b.w.t.) may be due to early response to lipid peroxidation induced oxidative stress caused by the extract of *L. breviflora*. At this concentration, the endogenous detoxification system (GST and GSH) is comparable with the control and is still sufficient to cope with the level of the toxicant from the extract [Figures 4 and 5]. However, at high concentration of 500 and 1000 mg/kg/b.w.t., decrease ($P < 0.05$) serum and tissue antioxidant enzymes activities observed suggest depletion of antioxidant enzymes occasioned by elevated TBARS [Figure 1], decreased endogenous detoxification system [Figures 4 and 5], and increased level of oxidant influx caused by the extract. SOD, CAT, GPx are enzymes that destroy the peroxides. SOD act to dismutate superoxide radicals to H_2O_2 which is then converted to water and oxygen by CAT.^[30,31] Furthermore, the observed decrease in the

Table 1: Effect of extracts *Lagenaria breviflora* on myocardial marker enzymes in serum and heart homogenate

Parameters	NC	Extract treated groups (mg/kg/b.w.t.)		
		250	500	1000
Serum (µmol/L)				
AST	98.2±0.35	100.8±0.44	132.3±0.31 ^a	155.7±0.11 ^a
ALT	79.03±0.41	90.43±0.27	115.2±0.22 ^a	130.4±0.26 ^a
LDH	102.4±0.71	123.6±0.51	140.1±0.12 ^a	165.9±0.16 ^a
CK	95.46±0.52	102.3±0.13	132.6±0.42 ^a	145.0±0.14 ^a
Heart homogenate (µmol/L)				
AST	49.37±0.21	31.21±0.32	28.57±0.44 ^a	19.03±0.18 ^a
ALT	89.94±0.33	70.53±0.41	61.45±0.23 ^a	49.97±0.24 ^a
LDH	72.11±0.42	60.01±0.22	51.02±0.41 ^a	39.07±0.19 ^a
CK	34.32±0.14	27.44±0.51	20.39±0.35 ^a	17.99±0.11 ^a

Values are mean±SD of the mean of six Wistar rats. ^a $P < 0.05$ different from the control. ALT: Alanine transaminase; AST: Aspartate transaminase; LDH: Lactate dehydrogenase; CK: Creatine kinase; SD: Standard deviation; NC: Normal control

activities of GPx, SOD, CAT, GST and GSH in serum, liver, and heart homogenate in the extract (500 and 1000 mg/kg/b.w.t.) treated rats compared to the control suggested that the extract may contain toxic agent (s) which increased lipid peroxidation and tissue damage leading to excessive demand and consumption of antioxidant machinery.^[32] According to Banjo *et al.*^[33] the fruit extract of *L. breviflora* is rich in phytochemicals such as tannin, flavonoids, alkaloids, terpenoids, anthraquinone, and reducing sugar. Hence, the observed toxic effect of the plant extract may be due to the presence of alkaloids or other constituents,^[33] as flavonoid has been reported not to be toxic to antioxidant enzymes.^[34,35]

The etiopathogenesis of cardiovascular diseases is multifactorial, however, oxidative stress resulting from increase free radicals generation, and decrease antioxidants status has been implicated.^[36,37] The results of oral administration of a methanolic extract of *L. breviflora* at 500 and 1000 mg/kg/b.w.t. caused a significant ($P < 0.05$) dose-dependent increase in serum level of myocardial marker enzymes CK, AST, ALT, and LDH and a concomitant decrease in heart homogenate compared with the control [Table 1]. However, the extract did not present any significant change when administered at 250 mg/kg/b.w.t. while the effect of the extract was more pronounced at 1000 mg/kg/b.w.t. The observed increase in the activities of myocardial marker enzymes in serum and concomitant reduction in the cardiac homogenate may be due to enzymes leakage occasioned by lipid peroxidation induced oxidative stress or hyperlipidemia and hyperglycemia effect of the plant extracts.^[38] The results suggested that the extract of *L. breviflora* might contain phytotoxic agent (s) which mediated peroxidative myocardial damage resulting in increased release of these diagnostic marker enzymes into the serum.^[39] Our results supported Saba *et al.*^[40] who advice that higher doses of the extract should not be taken for long period because of its hyperglycemic and dyslipidemic effects and tendency to easily predispose to diabetes mellitus, atherosclerosis and cardiopathy among other sequel. Although, pretreatment of rats with ethanolic fruit extract of *L. breviflora* at 100, 250 and 500 mg/kg/b.w.t. demonstrate hepatoprotective and enhance *in vivo* antioxidant activities in CCL₄ induced hepatic damage and antioxidant depletion, the antioxidant potential of the extract may only be effective at lower doses. The histopathological evidence suggested mild pathological alterations in the architectural section of the liver [Figure 7A, c and d] and the

heart [Figure 7B, c and d], therefore, fruit extract of *L. breviflora* may be hepatotoxic and cardiotoxic. Although, the mechanism by which the plant produces these toxicity are not clearly known, it is thought to result from the presence of phytotoxic agent (s) which induces lipid peroxidation in biomembrane with a resultants depletion in antioxidant capacity.

CONCLUSION

Administration of *L. breviflora* for a longer period at higher doses (500 and 1000 mg/kg) may cause oxidative stress by depleting antioxidative mechanisms or by enhancing prooxidant components of tissues, leading to cardiac, and hepatic injuries.

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Conflicts of interest

There are no conflicts of interest.

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