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Annona muricata mitigates glycerol-induced nephrotoxicities in male albino rats through signaling pathways of angiotensin conversion enzyme, kidney injury molecule-1, and antioxidant properties

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ABSTRACT

Objective: The ameliorative effect of *Annona muricata* in glycerol-induced acute kidney injury in laboratory rats was evaluated.
Methods: Thirty albino rats were used and were divided into five of six rats per group. The first (control) group was given distilled water (2 ml/kg) for seven days, while the second (toxicant or glycerol alone) group was given only glycerol (10 ml/kg) on day 8. The third, fourth, and fifth groups received methanol leaf extract of *Annona muricata* (100 mg/kg), 200 mg/kg dose of the extract, and enalapril (10 mg/kg) respectively for seven days, and on day eight all received glycerol (10 ml/kg). Serum chemistry, histopathology, immunohistochemistry, and changes in blood pressure were indices of toxicity.
Results: In the toxicant group, blood pressure increased significantly ($p<0.05$), but treatment with 200 mg/kg of the leaf extract reversed this. In biochemical analysis, the toxicant group had a significant increase in the levels of oxidative stress markers and decreased levels of some enzymatic and non-enzymatic antioxidants. Treatment with 200 mg/kg of the extract and enalapril reversed the trend. Histopathological changes were seen in the kidney of the toxicant group, but those treated with 200 mg/kg of the extract showed normal kidney histology with no observable lesion. The immunolocalization of angiotensin-converting enzyme and kidney injury molecule-1 in the kidney showed high expression of these proteins in the toxicant group. At the same time, enalapril and the extract caused significant downregulation of their expressions.

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Conclusion: Results showed that 200 mg/kg dose extract of *Annona muricata* could confer effective protection on the kidney due to its anti-inflammatory, anti-hypertensive and antioxidant properties.

Introduction

A sudden decrease in the functionality of the kidney with concomitant structural damage is known as acute kidney injury (AKI), with some of the potential causes having to do with a focal mismatch between oxygen and nutrient delivery to the nephrons. Increased energy demands on account of cellular stress is another cause [1]. AKI occurs when renal function is acutely decreased, so waste products accumulate within the body, making the body unable to maintain electrolyte, acid-base, and water balance [2]. Ischaemia, the most common cause of AKI, can occur for a number of reasons. Physiological adaptations as a compensatory response to blood flow reduction could occur to a certain degree. However, in the face of inadequate delivery of oxygen and metabolic substrates, the resulting cellular injury leads to organ dysfunction. Ischaemia-related injury has a profound negative effect on the kidney, especially in that ischaemia results in vasoconstriction, endothelial injury, and activation of inflammatory processes [3]. As a result of the decrease in the kidney's ability to carry out its perfusion activity, the epithelial cells lost the ability to maintain sufficient intracellular ATP for essential processes. This ATP depletion leads to cell injury. Depending on the severity of ATP depletion, cell death by necrosis or apoptosis could occur [4].

Rhabdomyolysis is characterized by the breakdown and necrosis of damaged skeletal muscle and subsequent myoglobin (sarcoplasmic proteins) release into extracellular fluid and circulation [5]. Filtration of these sarcoplasmic proteins through the glomeruli will lead to AKI through different processes, including intratubular obstruction as a result of protein precipitation. The resultant effect is renal vasoconstriction, which leads to inflammation and tubular damage associated with reactive oxygen species production. Glycerol-induced AKI is a commonly used model in laboratory animals, and this is considered an equivalent of human myoglobinuric AKI. The pathogenic mechanisms involve ischaemic injury, myoglobin-induced tubular nephrotoxicity, and the effects of cytokines released after rhabdomyolysis on renal actions [6].

Annona muricata Linn (Annonaceae), or soursop, found its origin in the hot tropical regions of the South and North Americas but now could be found in different parts of the globe [7, 8]. *Annona muricata* is an erect tree having large edible fruits [9]. Various phytoconstituents and compounds are found in different parts of this plant, such as alkaloids [10], megastigmanes [11], flavonol triglycosides [12], phenolics [13], cyclopeptides, and essential oils [14]. In addition, *A. muricata* is very rich in annonaceous acetogenin compounds [15] as well as other major minerals including K^{+}, Ca^{2+}, Na^{+}, Cu^{2+}, $Fe^{2+,}$ and Mg^{2+} indicating that the fruit of this plant, when consumed regularly, can assist in the supply of essential nutrients and elements to the human systems [16].

Since AKI is characterized by oxidative stress, the study aimed to evaluate the ameliorative effect of the methanol leaf extract of *Annona muricata* since it is rich in phenol and flavonol, which are sources of natural antioxidants.

Materials and methods

Collection of fresh leaves and preparation of methanol leaf extract

The fresh leaves of *Annona muricata* were collected in July 2019 from a backyard orchard in Ilorin, Nigeria. A taxonomist in the Department of Botany, University of Ibadan, Nigeria correctly identified the leaf sample of this plant, where a voucher specimen with ID number UIH 23095 was deposited in this department's herbarium. The leaves (82. 22 g) were dried, blended for processing, added to 1.5 L of pure methanol in a glass container, stirred intermittently, and allowed to stay for 72 hrs. This process was repeated twice, and the combined filtrate was further filtered using Whatman number 1 filter paper. The filtrate was then concentrated using a rotary evaporator set at 40 degrees Celsius. Finally, the amount of the crude methanol extract was taken, and its percentage yield was 22.12%.

Experimental animals

Thirty (30) male albino rats weighing 180 g on average and about eight weeks in age were obtained from and kept at the experimental animal house of the Department of Veterinary Pharmacology and Toxicology, University of Ibadan throughout this study. They were housed in good environmental conditions with access to good feed and clean water *ad libitum*. The Animal Care and Use Research Ethics Committee (ACUREC) of the University of Ibadan approved the study and the approval number is UI-ACUREC/17/0064.

Chemicals and reagents

Glycerol was provided from Sigma Biosciences, Egypt. On the other hand, Enalapril maleate was bought under the brand name Enalar 10mg from Aurobindo Pharma-Milpharm Ltd. Middlesex, HA4 6QD (England). Biuret's reagent and other reagents and chemicals used were of the analytical grade.

Experimental designs

Animals were randomly divided into five (5) groups of six rats:

Group A: Normal control group; Rats received no medication, but rats were given distilled water 2 ml/kg body weight orally for eight days once a day. So they served as a normal control group.

Group B: In this group, rats did not receive medication and were induced with 50% glycerol 10 ml/kg on day eight, and so they served as the toxicant group.

Group C: Treated group (plant extract): Rats were treated with plant extract in a dose of 100 mg/kg body weight/day orally for seven days and were given 50% glycerol (10 ml/kg) on the 8^{th} day.

Group D: Treated group (plant extract): Rats were treated with plant extract in a dose of 200 mg/kg body weight/day orally for seven days and were given 50% glycerol (10 ml/kg) on the 8^{th} day.

Group E: Treated group (standard drug): Rats were treated with standard drug (enalapril maleate) in a dosage of 10mg/kg per day orally for seven days and were given 50% glycerol 10ml/kg on the 8^{th} day.

Preparation of serum and tissues for biochemical assays

Blood samples collected from the retro-orbital venous plexus into dry clean plain tubes were allowed to clot. They were then centrifuged at 4,000 revolutions per minute (rpm) for 15 minutes, and the sera obtained were then stored until the time of analysis. After blood collection, the animals were sacrificed by cervical dislocation, and kidneys were thereafter harvested for histopathology and immunohistochemistry.

Homogenization

Kidney tissues were quickly excised and then chopped into bits and homogenized using the homogenizing buffer with the resultant homogenate was centrifuged to obtain post mitochondrial fraction (PMF). The supernatant obtained was thereafter used for all the biochemical analyses in this study.

Biochemical analyses

Biochemical analyses carried out in the study were: estimation of glutathione S-transferase (GST) activity [17]; determination of the activity of xanthine oxidase [18]; Renal nitric oxide (NO) measurement [19]; determination of hydrogen peroxide generation [20]; Quantification of thiobarbituric acid reactive substances (TBARS) as malondialdehyde (MDA) [21]; reduced glutathione concentration determination [22]. Other analyses carried out included the measurement of glutathione peroxidase (GPx) [23], determination of protein concentrations [24]; Superoxide dismutase (SOD) assay [23], and determination of serum myeloperoxidase (MPO) activity [25].

Histopathology

For proper fixation, renal tissues were collected into 10 % formal saline buffer and were thereafter processed for histology after being stained with H and E [26].

Immunohistochemistry

The immunolocalization of angiotensin-converting enzyme (ACE) and kidney injury molecule-1 (KIM-1) in the kidney was determined using the method of Oyagbemi et al. [27]. First, kidney tissues were fixed in 10% buffered formalin. These tissues were then embedded in paraffin and sectioned at a thickness of 5mm. Using a light microscope (Leica) equipped with a digital camera, the immunoreactive positive expressions of ACE and KIM-1 anti-rabbit intensive regions were observed and evaluated. The catalog numbers of anti-ACE antibody and anti-KIM-1 antibody are respectively ab108252 and ab228973.

Statistical analysis

The results were expressed as mean $\pm$ S.D., where the test of significance between two groups was estimated by Student's t test. Using GraphPad Prism version 4.00, one-way ANOVA with Tukey's post-test was also performed; $p < 0.05$ was considered the level of statistical significance.

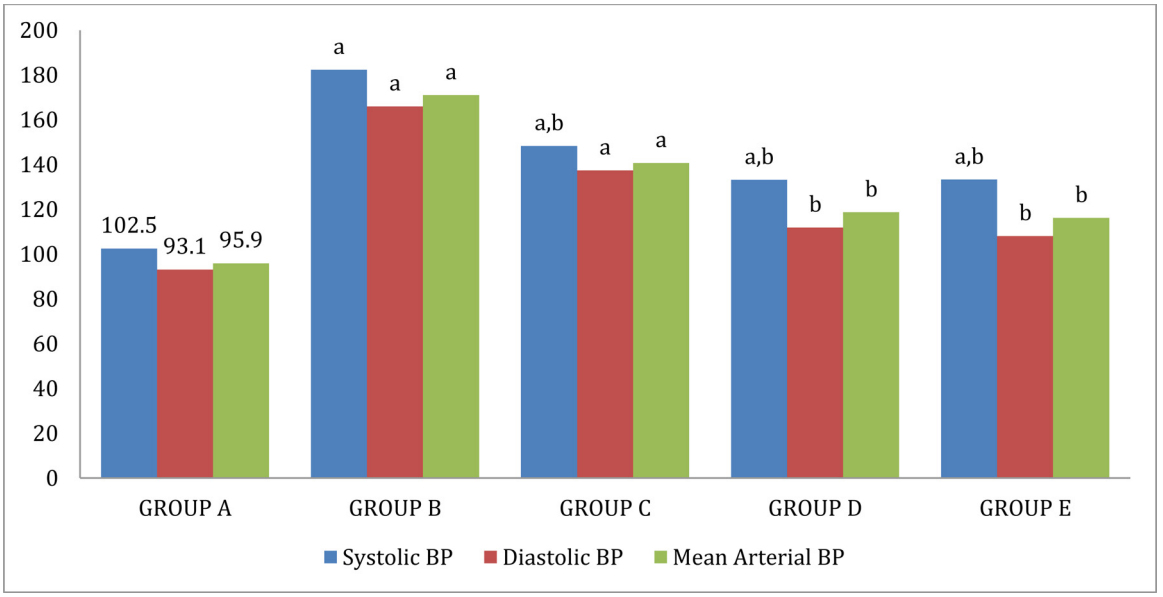

Figure 1. shows the effect of methanol leaf extract of *Annona muricata* and enalapril on systolic blood pressure (SDP), diastolic blood pressure (DBP) and mean arterial blood pressure (MAP). Superscript a indicates significant difference ($P<0.05$) when groups B, C, D and E were compared with A while superscript b indicates significant difference ($P<0.05$) when groups C, D and E were compared with B. A: control (distilled water 2 ml/kg) B: Glycerol alone (10 ml/kg), C: Glycerol + 100 mg/kg *Annona muricata*, D: Glycerol + 200 mg/kg *Annona muricata*, E: Glycerol + Enalapril (10 mg/kg).

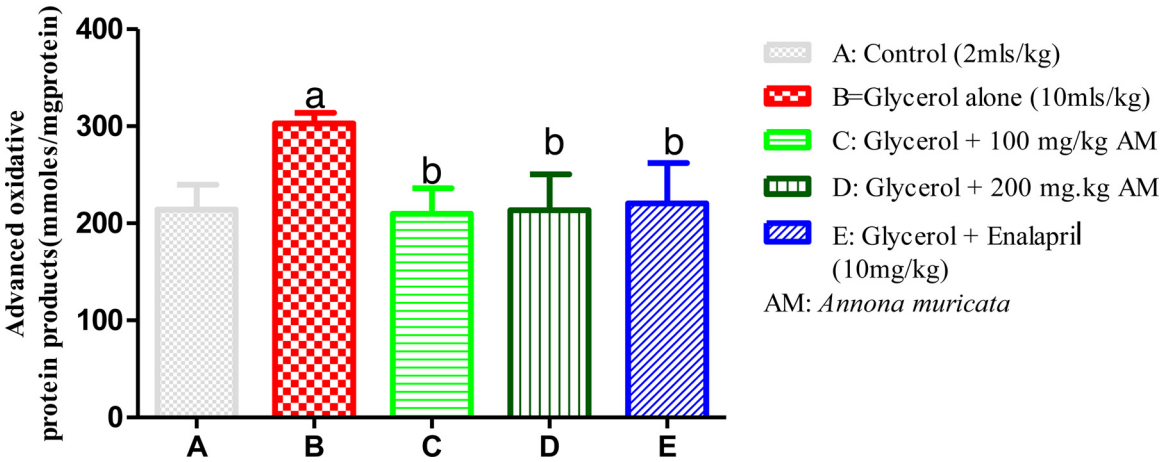

Figure 2. shows the effect of methanol leaf extract of *Annona muricata* and enalapril on advanced oxidative protein product (AOPP). Superscript a indicates significant difference ($P<0.05$) when groups B, C, D and E were compared with A while superscript b indicates significant difference ($P<0.05$) when groups C, D and E were compared with B. A: control (distilled water 2 ml/kg) B: Glycerol alone (10 ml/kg), C: Glycerol + 100 mg/kg *Annona muricata*, D: Glycerol + 200 mg/kg *Annona muricata*, E: Glycerol + Enalapril (10 mg/kg).

Results

Figure 1 shows the effect of methanol leaf extract of *Annona muricata* and enalapril on systolic blood pressure (SDP), diastolic blood pressure (DBP), and mean arterial blood pressure (MAP). The extract at the two doses and enalapril caused a significant decrease ($p<0.05$) in blood pressure indices compared to the toxicant group.

Figure 2 shows the effect of methanol leaf extract of *Annona muricata* and enalapril on advanced oxidative protein product (AOPP) where there was a significant decrease ($p<0.05$) in the level of this parameter when compared with the toxicant group.

Figure 3 shows the effect of methanol leaf extract of *Annona muricata* and enalapril on myeloperoxidase (MPO) with the extract at the two doses caused a significant decrease ($p<0.05$) in the level of MPO when compared with the toxicant group and enalapril group.

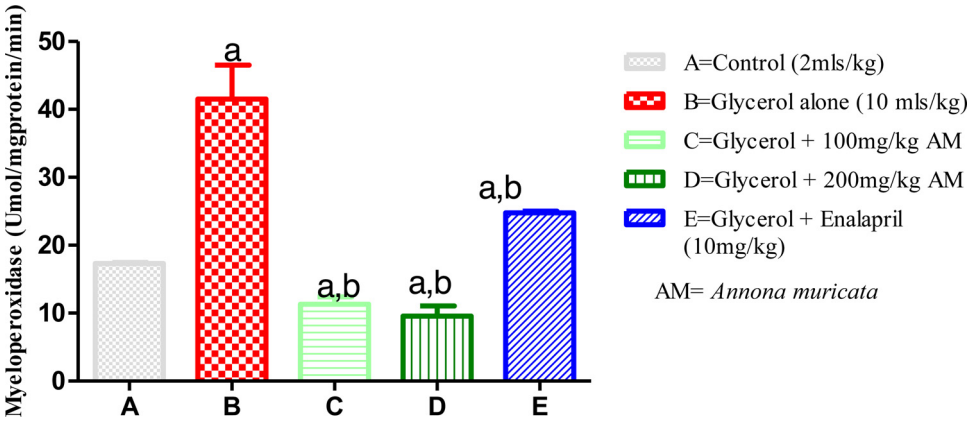

Figure 3. shows the effect of methanol leaf extract of *Annona muricata* and enalapril on myeloperoxidase (MPO). Superscript a indicates significant difference ($P<0.05$) when groups B, C, D and E were compared with A while superscript b indicates significant difference ($P<0.05$) when groups C, D and E were compared with B. A: control (distilled water 2 ml/kg) B: Glycerol alone (10mls/kg), C: Glycerol + 100 mg/kg *Annona muricata*, D: Glycerol + 200 mg/kg *Annona muricata*, E: Glycerol + Enalapril (10 mg/kg).

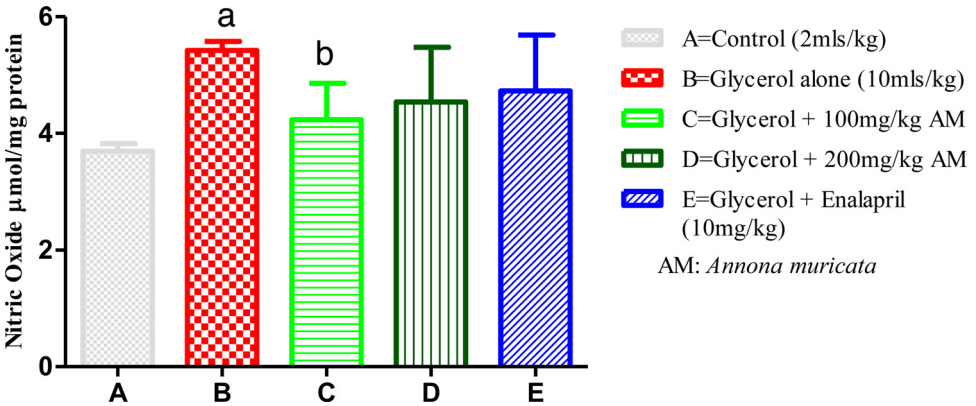

Figure 4. shows the effect of methanol leaf extract of *Annona muricata* and enalapril on nitric oxide (NO). Superscript a indicates significant difference ($P<0.05$) when groups B, C, D and E were compared with A while superscript b indicates significant difference ($P<0.05$) when groups C, D and E were compared with B. A: control (distilled water 2 ml/kg) B: Glycerol alone (10 ml/kg), C: Glycerol + 100 mg/kg *Annona muricata*, D: Glycerol + 200 mg/kg *Annona muricata*, E: Glycerol + Enalapril (10 mg/kg).

Figure 4 shows the effect of methanol leaf extract of *Annona muricata* and enalapril on nitric oxide (NO), where only the 100 mg/kg dose of the extract caused a significant decrease ($p<0.05$) in the level of NO compared to the toxicant group.

Figure 5 shows the effect of methanol leaf extract of *Annona muricata* and enalapril on hydrogen peroxide (H_2O_2), where the two doses of the extract and enalapril caused a significant decrease ($p<0.05$) in the level of hydrogen peroxide when compared to the toxicant group.

Figure 6 shows the effect of methanol leaf extract of *Annona muricata* and enalapril on malondialdehyde with the 200 mg/kg dose of the extract, causing the most significant decrease in the level of MDA relative to the toxicant group. The other treated doses all caused a significant decrease ($p<0.05$) compared to the toxicant group.

Figure 7 shows the effect of methanol leaf extract of *Annona muricata* and enalapril on protein carbonyl (PC), where enalapril caused the most significant decrease ($p<0.05$) in the level of PC, though the two doses of the extract also caused the same effect when compared to the toxicant group.

Table 1 shows the effects of methanol leaf extract of *Annona muricata* and enalapril on renal non-enzymatic antioxidant molecules (Reduced glutathione, Total thiol, and Non-protein thiol) where the 200 mg/kg dose of the extract caused a significant increase ($p<0.05$) in the level of total thiol and non-protein thiol when compared with the control and even the toxicant group.

Table 2 shows the effects of methanol leaf extract of *Annona muricata* and enalapril on renal enzymatic molecules (Glutathione-s-transferase, Glutathione peroxidase, and Superoxide dismutase) with the extract at the two doses and enalapril causing a significant increase in the levels of these parameters relative to the toxicant group. However, the control group showed a significant increase ($p<0.05$) when compared to the treated groups.

Figure 8 is the photomicrograph of the kidney where Group A shows normal kidney histology with no observable lesion; Group B shows tubular epithelial coagulation necrosis and luminal ectasia with the cellular cast (red arrows). Group C shows

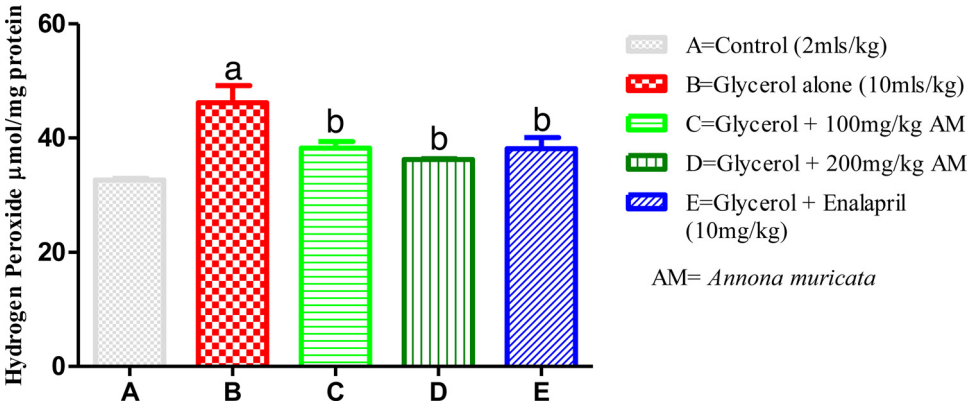

Figure 5. shows the effect of methanol leaf extract of *Annona muricata* and enalapril on hydrogen peroxide (H_2O_2). Superscript a indicates significant difference ($P<0.05$) when groups B, C, D and E were compared with A while superscript b indicates significant difference ($P<0.05$) when groups C, D and E were compared with B.
A: control (distilled water 2 ml/kg) B: Glycerol alone (10 ml/kg), C: Glycerol + 100 mg/kg *Annona muricata*, D: Glycerol + 200 mg/kg *Annona muricata*, E: Glycerol + Enalapril (10 mg/kg).

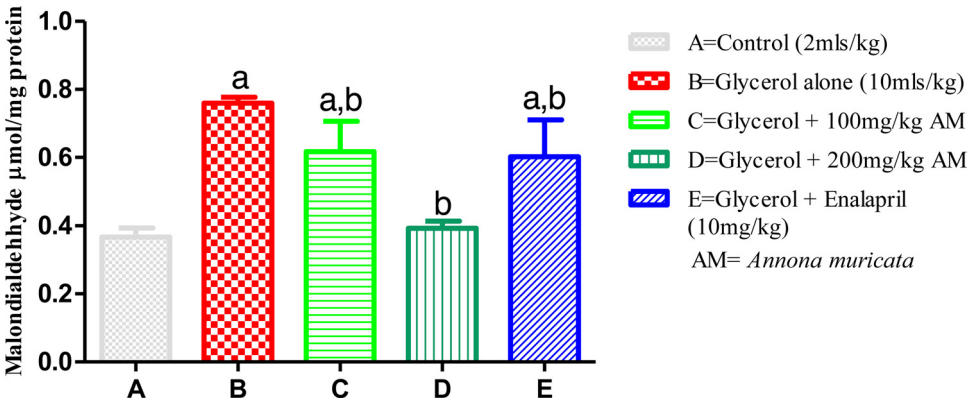

Figure 6. shows the effect of methanol leaf extract of *Annona muricata* and enalapril on malondialdehyde. Superscript a indicates significant difference ($P<0.05$) when groups B, C, D and E were compared with A while superscript b indicates significant difference ($P<0.05$) when groups C, D and E were compared with B. A: control (distilled water 2 ml/kg) B: Glycerol alone (10 ml/kg), C: Glycerol + 100 mg/kg *Annona muricata*, D: Glycerol + 200 mg/kg *Annona muricata*, E: Glycerol + Enalapril (10 mg/kg).

Table 1
Effects of methanol leaf extract of *Annona muricata* and enalapril on renal non-enzymatic antioxidants.

	A (Control)	B (Gly)	C (Gly + 100mg/kg AM)	D (Gly + 200mg/kg AM)	E (Enalapril)
Reduced Gluthatione	123.3±4.8	118±1.1	120±2.5	119.5±4.3	120.8±6.3
Total Thiol	92±19.7	50±2.0[a]	63.5±23.4[a]	136±8.7[a,b]	118±4.5[a,b]
Non protein thiol	13.6±1.7	10.2±0.3[a]	12±1.2	13.2±1.1[b]	12.6±2.5

Values presented as Mean ± STD. Superscript a indicates significant difference ($P<0.05$) when groups B, C, D and E were compared with A while superscript b indicates significant difference ($P<0.05$) when groups C, D and E were compared with B. Gly =Glycerol; AM= *Annona muricata*; A: control (distilled water 2 ml/kg) B: Glycerol alone (10 ml/kg), C: Glycerol + 100 mg/kg *Annona muricata*, D: Glycerol + 200 mg/kg *Annona muricata*, E: Glycerol + Enalapril (10 mg/kg).

tubular epithelial coagulation necrosis and a few cellular cast in the tubular lumen. Group D shows normal kidney histology with no observable lesion. Finally, group E shows tubular epithelial coagulation necrosis (yellow arrows) and proteinaceous cast in the tubular lumen.

Figure 9 is the immunohistochemistry of angiotensin-converting enzyme (ACE) expression in the kidney. While groups A, C, D, and E caused downregulation of this protein, group B caused its upregulation.

Figure 10 is the immunohistochemistry of kidney injury molecule-1 (KIM-1) expression in the kidney. While groups A, C, D, and E caused downregulation of this protein, group B caused its upregulation.

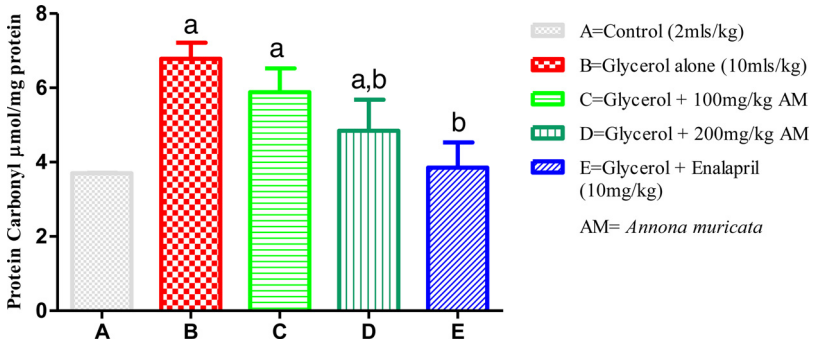

Figure 7. shows the effect of methanol leaf extract of *Annona muricata* and enalapril on protein carbonyl (PC). Superscript (a) indicates significant difference (P<0.05) when groups B, C, D and E were compared with A while superscript b indicates significant difference (P<0.05) when groups C, D and E were compared with B. A: control (distilled water 2 ml/kg) B: Glycerol alone (10 ml/kg), C: Glycerol + 100 mg/kg *Annona muricata*, D: Glycerol + 200 mg/kg *Annona muricata*, E: Glycerol + Enalapril (10 mg/kg).

Table 2
Effects of methanol leaf extract of *Annona muricata* and enalapril on renal enzymatic antioxidants.

	A (Control)	B (Gly)	C Gly + 100mg/kg AM)	D (Gly + 200mg/kg AM)	E (Enalapril)
Glutathione-s-transferase	7.8±0.9	2.4±0.6^{a}	5.4±0.8^{b}	4.8±0.2^{a}	6.8±3.4^{b}
Glutathione peroxidase	144.7±9.1	98.9±5.5^{a}	127.3±8.5^{a,b}	126.3±7.5^{a,b}	136.6±4.8^{b}
Superoxide dismutase	5.8±0.1	3.5±0.1^{a}	4.9±0.7^{a,b}	4.9±0.2^{a,b}	5.2±0.1^{a,b}

Values presented as Mean ± STD. Superscript a indicates significant difference (P<0.05) when groups B, C, D and E were compared with A while superscript b indicates significant difference (P<0.05) when groups C, D and E were compared with B. Gly =Glycerol; AM= *Annona muricata*; A: control (distilled water 2 ml/kg) B: Glycerol alone (10 ml/kg), C: Glycerol + 100 mg/kg *Annona muricata*, D: Glycerol + 200 mg/kg *Annona muricata*, E: Glycerol + Enalapril (10 mg/kg).

Discussion

In this study, the blood pressure parameters were significantly increased in the group treated with glycerol alone, while treatment with 200mg/kg of methanol leaf extract of *Annona muricata* and enalapril (10mg/kg) showed a significant decrease ($p<0.05$) in the DBP and MAP to a level comparable to the control group. Hollenberg and Raij [28] believed that the important pathophysiologic mechanisms associated with hypertension have to do with the mesangial and endothelial cell injury. While some suggest that glomerular hypertension is the primary pathogenetic mechanism, others were of the opinion that glomerular hypertrophy contributes to the injury. This result is similar to that carried out by Pablo *et al.* [29], where glycerol supplementation induced metabolic disorder leading to hypertension, but a contrasting result was seen when Drieman *et al.* [30] administered glycerol, and the SBP, MAP, and heart rate remained unchanged.

Increased levels of AOPP, MPO, NO were observed in the group that was given glycerol alone, while treatment with methanol leaf extract of *Annona muricata* at both doses (100 and 200 mg/kg) and enalapril (10mg/kg) significantly reduced the levels of this marker to levels comparable to that of the control. Studies have shown that AOPP is not only a marker of oxidative stress but is also known to be expressed as an inflammatory mediator; hence Assanga et al. [31] reported that the extract of *Bucida buceras* caused decreased levels in the expression of AOPP in carrageenin-induced oedema. Plasma proteins are the main targets of oxidants. As a result, AOPP is a risk factor in kidney disease and an important marker of oxidative stress in AKI, where AOPP acts as a mediator of oxidative stress. Witko-Sarsat *et al.* [32] suggested that AOPP could contribute to the monocyte-mediated inflammatory disorders associated with uremia.

MPO belongs to the subfamily of peroxidases and could be expressed in immune cells, such as lymphocytes, monocytes, and macrophages [33, 34], and produced in other body cells. MPO is stored in azurophilic granules [35], with oxidative stress playing a significant role in its release [36, 37]. Inflammation plays a role in its release into the extracellular medium [38] due to increased vascular permeability resulting in the accumulation of immunoglobulins and serum proteins at the inflammation site [39]. Migration of neutrophils to inflammation sites also leads to MPO release [40, 41]. The ability of methanol leaf extract of *Annona muricata* to significantly lower myeloperoxidase level could therefore indicate its anti-inflammatory effect with consequent nephroprotective effect.

NO is synthesized in the endothelium by the Ca^{2+}calmodulin-dependent nitric oxide synthase (eNOS) [42] and can regulate the vascular tone [43] as well as to attenuate smooth muscle cell proliferation [44]. For NO, a significant increase was seen in the group that was administered with glycerol alone, while treatment with methanol leaf extract of *Annona muricata* at both doses (100 and 200 mg/kg) and enalapril (10mg/kg) showed a significant decrease to a level comparable to the control group thus confirming its anti-antioxidant property. A significant increase in the glycerol-only group could bring about free radical generation because the bioavailability and actions of ˙NO are modulated by its fast reaction with superoxide radical (O•−2). This reaction is known to yield an unusual and reactive peroxide, i.e., peroxynitrite, which represents the

Effects of methanol leaf extract of *Annona muricata* and enalapril on renal histology.

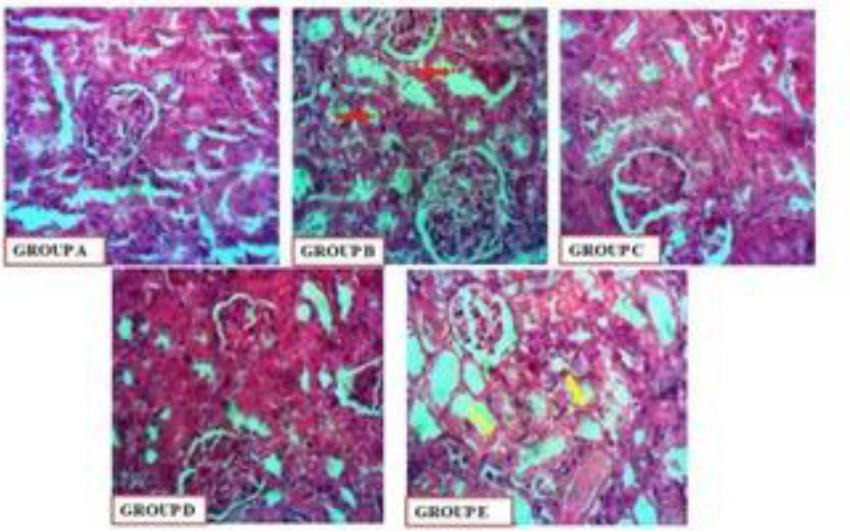

Figure 8. Photomicrograph of the kidneys. Magnification X 400, Hematoxylin and eosin staining Group A shows normal kidney histology with no observable lesion. Group B shows tubular epithelial coagulation necrosis, and luminal ectasia with cellular cast (red arrows). Group C shows tubular epithelial coagulation necrosis and a few cellular cast in tubular lumen, Group D shows normal kidney histology with no observable lesion and Group E shows tubular epithelial coagulation necrosis (yellow arrows) and proteinacious cast in tubular lumen.
A: control (distilled water 2 ml/kg) B: Glycerol alone (10 ml/kg), C: Glycerol + 100 mg/kg *Annona muricata*, D: Glycerol + 200 mg/kg *Annona muricata*, E: Glycerol + Enalapril (10 mg/kg).

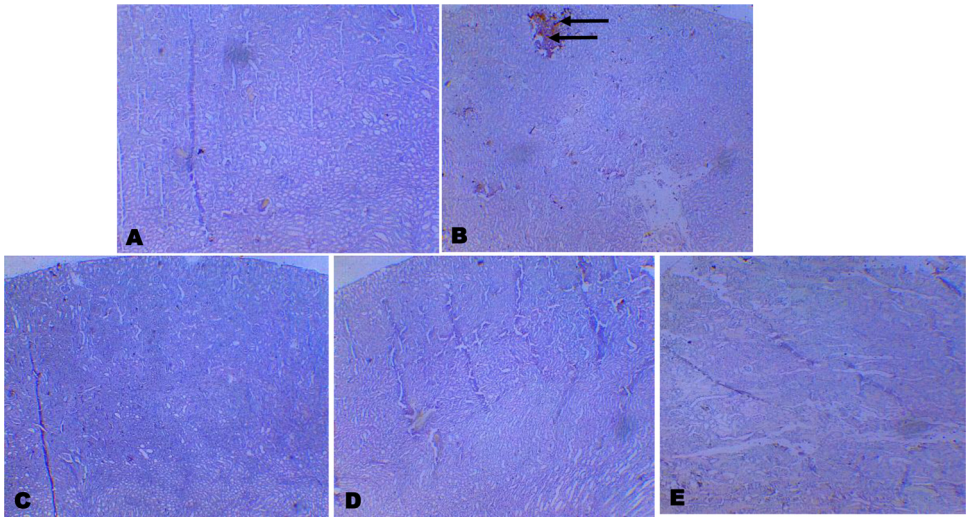

Figure 9. The immunohistochemistry of angiotensin converting enzyme (ACE). A (Control); B (10 ml/kg Glycerol); C 10 ml/kg Glycerol + *Annona muricata* 100 mg/kg); D (10 ml/kg Glycerol + Annona muricata 200 mg/kg); and E (10 ml/kg + Enalapril 10 mg/kg). Slides stained with high definition Haematoxylin. Magnification X 100

merging of the oxygen radicals and •NO pathways [45]. Thus significant reduction of NO by the extract and enalapril is very instructive.

In summary, it was observed that advanced oxidative protein product, myeloperoxidase, and nitric oxide were significantly increased in the toxicant group, while treatment with methanol leaf extract of *Annona muricata* at both doses (100 and 200 mg/kg) and enalapril (10 mg/kg) significantly reduced the effects of these biomarkers to levels comparable to that of the control. The results are in accord with the earlier study, which showed that pioglitazone (10 mg/kg) significantly blunted the effects of glycerol on the serum biomarkers in rats [46]. This nephroprotection has also been reported in other models using cyclosporine [47], nephritic syndromes [48], and cisplatin [49].

There was a significant increase in the levels of H_2O_2, MDA, and PC in the toxicant group, while treatment with methanol leaf extract of *Annona muricata* at both doses (100 and 200 mg/kg) and enalapril (10mg/kg) showed a significant decrease in

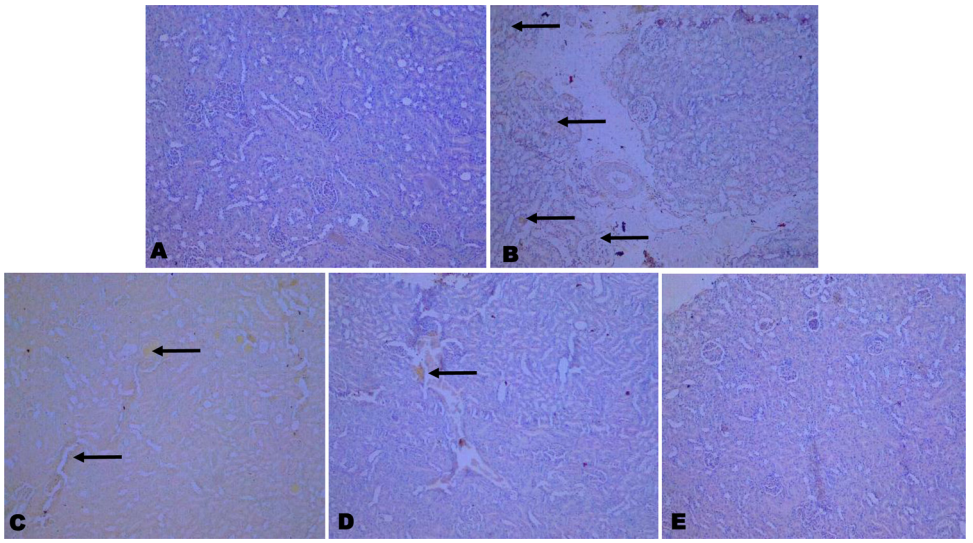

Figure 10. The immunohistochemistry of kidney injury molecule-1 (KIM-1). A (Control); B (10 ml/kg Glycerol); C 10 ml/kg Glycerol + *Annona muricata* 100 mg/kg); D (10 ml/kg Glycerol + *Annona muricata* 200 mg/kg); and E (10 ml/kg + Enalapril 10 mg/kg). Slides stained with high definition Haematoxylin. Magnification X 100

the levels of these markers relative to the toxicant group and some extent to the control group. H_2O_2 is not a free radical, but it is involved in the production of many reactive oxygen species. H_2O_2 is a by-product of oxygen metabolism and is neutralized by peroxidases. Lipid peroxidation affects cellular membranes in conditions with oxidative stress [50] because free radical extracts an electron from a lipid, bringing about the formation of a fatty acid radical [51]. Malondialdehyde is used as a biomarker for lipid peroxidation [52]. On the other hand, PC was suggested as a marker of free radical damage because protein oxidation results in protein carbonyls production [53]. Since all the treated groups experienced a significant decrease in the level of these markers, the plant extract might be said to have the ability to protect the kidney from oxidative stress.

In this study, there was no significant change in the level of reduced glutathione in all groups. Glutathione exists as the thiol-reduced and disulfide oxidized forms [54], where the eukaryotic cells have three major reservoirs of GSH [55]. There was a significant decrease in the levels of total thiol and NPT in the toxicant group while treatment with methanol leaf extract of *Annona muricata* at both doses (100, 200 mg/kg) and enalapril caused a significant increase to a level higher when compared to that of the toxicant group and some extent to that of control. Thiols contain a sulfhydryl group, where some are found as low-molecular-mass disulphides [55]. Under the condition of moderate oxidative stress, oxidation of cystine residues can occur, leading to the reversible formation of mixed disulfides between protein thiol groups and low–molecular-mass thiols, particularly with GSH (S-glutathionylation) [56]. From this study, there was a significant decrease in the renal non-enzymatic antioxidant molecules in the toxicant group while treatment with methanol leaf extract of *Annona muricata* at both doses (100 and 200 mg/kg) and enalapril (10mg/kg) caused a significant increase in these markers. Oxidative stress has been implicated in renal failure, where studies have shown that, especially in chronic renal failure, thiols are consumed by the presence of ROS generated in these patients [56–59].

Antioxidant consumption is known to occur in the condition of oxidative stress; hence a decline in antioxidant levels is experienced [60]. It is to be noted that antioxidant enzymes like glutathione peroxidase (GPx) and superoxide dismutase (SOD) may be induced by oxidative stress to either increase or decrease their level of activity, especially in AKI [61]. However, some studies have shown that protective anti-oxidative effects through enzyme induction could be exerted by xenobiotics [62, 63]. However, Neelima et al. [64] obtained an increased level of these antioxidant enzymes in their study, albeit at $p<0.001$.

The results of this study showed that methanol leaf extract of *Annona muricata* markedly ameliorated renal injury occasioned by glycerol-induced AKI in rats. Moreover, all markers of oxidative stress used in this study were inhibited by this extract, implying that the methanol leaf extract of *Annona muricata* effect is most likely due to the antioxidant activity. It is to be noted that many bioactive compounds such as phenols, alkaloids have been reported to be found in the leaves and seeds of *A. muricata,* with the predominant one being acetogenins [65]. For instance, phenolic compounds such as quercetin [12] and gallic acid [7, 66, 67] were found in the leaves of *A. muricata*. The presence of flavonoids and lipophilic antioxidant compounds such as tocopherols and tocotrienols might thus be responsible for the pharmacological actions obtained in this study.

Regarding histopathology, the kidneys of rats that received glycerol alone showed marked histological changes, including tubular epithelial coagulation necrosis and luminal ectasia with the cellular cast. However, the kidney section of the group

that was treated with a 200 mg/kg dose of the extract showed no observable lesion, thus preserving its normal morphology. This result suggests that the 200 mg/kg dose of the extract showed the best result with respect to nephroprotection from glycerol-induced AKI in rats. The intramuscular administration of hypertonic glycerol induces myolysis and hemolysis with the integrated effects of three major pathophysiologic mechanisms, i.e., renal vasoconstriction, direct cytotoxicity, and cast formation [68], and these then results in acute renal failure [69]. This result is in agreement with an earlier report on similar work [64, 70]. It has also been reported that rats receiving glycerol alone showed extensive tubular damage, whereas, in N-acetylcysteine pretreated rats, the tubular injury was greatly reduced [71].

In immunohistochemistry, the two markers evaluated were angiotensin-converting enzyme (ACE) and kidney injury molecule-1 (KIM-1). ACE is known to function in the conversion of Ang I to Ang II (a vasoconstrictor). ACE is also known to break down bradykinin (BK), a vasodilator, thus bringing about vasoconstriction. The import of this is that both Ang II and bradykinin play a significant role in controlling blood pressure. It is for this reason ACE inhibitors are commonly recommended either as single or in combination with other agents in the treatment of high blood pressure because of their dual effects of lowering Ang II and slowing down BK degradation [72–75]. Ang II is a vasoconstrictor hence blood pressure. On the other hand, Bradykinin is a vasodilator, which is being degraded by ACE, thus contributing to high blood pressure. Enalapril is an ACE inhibitor implying that there is the blockade of conversion of Ang I to Ang II hence the availability of Ang I, which is a vasodilator. Since ACE is blocked, its ability to destroy bradykinin is also blocked, leading to further vasodilation. Since both enalapril and the plant extract caused significant inhibition of ACE expression, it showed that the anti-hypertensive property of the extract might be through a similar pathway.

Following renal injury, kidney injury molecule-1 (KIM-1) is highly upregulated in proximal tubular cells. Since the discovery of KIM-1 as a urinary biomarker of kidney injury in both rodent models and humans, its discovery has played a vital role in renal pathophysiology [76–80]. The downregulation of this protein in this study points to the ability of the methanol leaf extract of this plant to prevent kidney injury hence its nephroprotective effects.

Conclusions

Based on the results from this study, it could be concluded that the methanol leaf extract of *Annona muricata* has a nephroprotective effect, with the higher dose of 200 mg/kg being more effective. The abilities of this plant extract to also improve both enzyme and non-enzyme-based antioxidants, suppress inflammatory processes, and inhibit lipid peroxidation suggest its potent antioxidant and anti-inflammatory properties. Its downregulation of ACE may account for its anti-hypertensive property, while its similar downregulation of KIM-1 further justifies its nephroprotective action. These and others might have justified the use of this plant in many traditional settings for medicinal purposes. Finally, it needs to be stressed that glycerol from this study may precipitate cardiorenal syndrome, and its use may go a long way in unravelling many pathophysiologic processes involved in this syndrome.

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Authors contributions

AAA designed the experiments, and OAO, OOF, BSO, and IOO carried them out. AAO, TOO, OOO and MAY supervised the work, especially the immunohistochemistry and biochemical assay. AAA prepared the manuscript with contributions from all co-authors.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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