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Reno- protective Effect of Graviola (Annona Muricata) Leaves Against Lead Acetate Toxicity on experimental Albino Rats

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Abbreviations

ABSTRACT

Background: Graviola, is commonly known as soursop. The leaves of this tropical fruit tree are traditionally used for its various medicinal benefits. The present study aims at estimating the anti-inflammatory and antioxidant activity of graviola leaf extract against nephrotoxicity caused by lead acetate in albino rats.

Methods: Forty rats were divided into four groups, (group I) control, (group II) graviola leaves extract 100 mg/kg BW, (group III) lead acetate 400 mg/kg BW and (group IV) lead acetate plus graviola leaves extract. After 8 weeks Blood and kidney tissue samples were collected for estimation biochemical, oxidative and inflammatory parameters in addition to histo-pathological examination.

Results: The lead acetate induced rats exhibited significant elevations in kidney function parameters (urea, creatinine, uric acid, cystatin A and LDH), pro-inflammatory markers (IL-6 and Pentraxin-3) and, markers of oxidative stress (MDA and 8-OHdG) and a significantly decrease in the activities of antioxidant enzymes (CAT, SOD, GR, GPx and GST) and reduced GSH. On the other hand, graviola leaves extract was significantly effective in lowering kidney dysfunction parameters, pro inflammatory markers and oxidative stress markers and significantly elevated the activity of the antioxidant parameters. Additionally, treatment with graviola leaves extract improved the histopathological abnormalities occurred in nephrotoxicity.

Conclusion: Our results provide an evidence of the benefit of using graviola leaves as a potent antioxidant and anti-inflammatory plant for treatment of nephrotoxicity induced by lead acetate.

INTRODUCTION:

Graviola is the fruit tree of Annona muricata L. (Annonaceae) commonly known as soursop is cultured in tropical countries and central to south America.(1) In the tropics all parts of this plant, including the bark, fruit-seeds, leaves and root are used in natural medicines. It is contributed to its various applications in folk medicine. (2) Traditionally, the leaves are used for cystitis, diabetes, headaches, hypertension, insomnia, liver problems and as an antidysenteric, and antispasmodic. In tropical Africa, leaves are usually used for skin diseases.(3)

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Environmental contamination is an incident in which a pollutant present in the environment such as air, water, soil, and hence in food which may lead to poisoning living things in the polluted areas. Lead is one of the heavy metals that is transmitted to man and animals through food chain. It is dangerous to most organs of human body if exposure exceed the safety levels. its toxicity depends on many parameters as age, sex, route of exposure, level of intake, solubility [4], Accumulation of lead produces damaging effects in the hematopoetical, hematic, renal nervous, cardiovascular gastrointestinal and immune systems. The inhalation of lead could permanently lower intelligence quotient (IQ), damage emotional stability and cause hyperactivity, poor school performance and hearing loss. [5]

With the important properties discovered from graviola, there is need to examine its reno-protective effects on the kidneys of adult albino rats. Therefore, the present study was designed to examine the efficacy of the leaves towards toxicity of lead acetate induced nephropathy on rats.

2. Materials and Methods

2.1. Animals

The study was carried out on forty healthy male Wistar albino rats with an average weight (150-200 gm). The rats were obtained from the Animal House of Pharos University in Alexandria. The animals were housed in plastic cages during the whole experimental period and maintained at uniform temperature of 23 ± 2°C, and 12 h hr in alternating light and dark cycles. The rats were given *ad libitum* feed and drink water during the experiment. All treatments and procedures were in accordance with the recommendations of the NIH guide for the Care and Use of Experimental Animals for the maintenance, treatment and killing of the animals.

2.2. Experimental design

The rats were randomly divided into four groups of ten rats in each group (Groups I, II, III, and IV) and were treated as follow:

Group I: control rats (10 rats) fed on normal diet and distilled water.

Group II: rats of this group (10 rats) orally received only graviola leaf extract 100 mg/kg rat body weight for 8 weeks.

Group III: rats of this group (10 rats) orally received lead acetate 400 mg/kg rat body weight once a day for 8 weeks.

Group IV: rats of this group (10 rats) orally received lead acetate 400 mg/kg rat body weight plus graviola leaf extract 100 mg/kg rat body for 8 weeks.

2.3. Chemicals

Lead acetate [Chemical formula: $Pb(C_2H_3O_2)_2$] was purchased from Sigma-Aldrich Co. (St. Louis, Mo, USA). Graviola leaves were purchased from Nallife company, USA.

2.4. Preparation of graviola leaves aqueous extract

500 grams of graviola leaves were milled into fine powder in a blender. Then, the powdered leaves were soaked in 1000 ml boiling distilled water followed by filtration and extraction a second time with 1000 ml of boiling distilled water, with shaking at room temperature for 48 h. The aqueous extract was concentrated to dryness under reduced pressure at 60±1^{o}C in a rotary evaporator. The obtained aqueous extract was freeze-dried, finally yielding 15.1 g of a light green, powdery crude graviola aqueous leaf extract of. This obtained extract was refrigerated until use.

2.5. Blood collection

All rats were decapitated after 8 weeks from the previous treatments. Blood samples were taken by cardiac puncture into tubes and centrifuged at 3000 rpm for 15 minutes. Then, Sera were stored at −20°C until measurement of urea, creatinine, uric acd, cystatin A, LDH, IL-6 and pentraxin-3 levels.

2.6. Preparation of kidney tissue homogenate

Kidneys were homogenized in 50 mM sodium phosphate buffer (pH 7.4)

containing 0.1 mM ethylene diamine tetra acetic acid (EDTA) at 4°C. The supernatant was separated by centrifugation at 1000 rpm for 15 min at 4 °C. The supernatant was used for the analysis of MDA, 8-OHdG, SOD, CAT, GSH, GR, GPx and GST.

2.7. Biochemical analysis

Determination of serum kidney function parameters were performed using ready-made kits from Spectrum - Diagnostics, Egypt on semi-automated Photometer 5010 V5+ (RIELE GmbH & Co, Berlin, Germany).

2.8. Inflammatory markers assay

The serum IL-6 and Pentraxin-3 levels were analyzed using the enzyme-linked immunosorbent assay (ELISA) kits (Catalog no. ELR IL6, Raybiotech, and Catalog no. KT-32715, Kamiya)

2.9. Oxidative stress parameters assay

2.9.1. Determination of lipid peroxidation

Lipid peroxidation was measured as thiobarbituric acid reactive substance (TBARS). TBARS was estimated as malondialdehyde (MDA) and measured spectrophotometrically according to the method of Ohkawa et al. (1979).[6] 8-Hydroxy-2'-deoxyguanosine was assayed using the enzyme-linked immunosorbent assay (ELISA) kit (Catalog no. K4160-100, BioVision)

2.9.2. Assay of the antioxidant and nonantioxidant enzymes

The activities of different antioxidant enzymes, CAT, SOD, GR, GPx and GST were measured in kidney issue homogenates. Catalase activity was measured according to the method of Aebi (1974). [7] SOD activity was determined by the method of by pyrogallol method of Marklund S and Marklund G.[8] GR was assayed according to the method of Smith et al. [9] The total sGPx activity was determined by the method of Flohe and Gunzler.[10] The activity of GST was determined by the method modified by Carmagnol et al. [11] The non antioxidant enzyme GSH was determined spectrophotometrically by the method of Ellman.[12] Total protein was determined in the kidney homogenate by the method of Lowry et al. [13]

2.10. Histo-pathological study in the kidney

At the end of the experiment, from each sacrificed rat were dissected out. Then they were immediately fixed in formalin solution at 10%.the tissue samples were fixed for 48 h in 10% formalin solution, dehydrated in an ascending graded series of ethanol, cleared in toluene and embedded in paraffin. Sections of the tissue (5–6 mm thickness) were prepared by using a rotary microtome and stained with hematoxylin and eosin (H&E) (Lillie and Fullmer, 1976).[14] The stained sections were examined under a light microscope and images were captured by a digital camera.

2.11. Statistical analysis

All data were expressed as mean ± SEM of variables. Statistical analysis was carried out using available statistical soft ware (SPSS version 18). One-way (ANOVA) was used for multiple comparisons. In cases were ANOVA showed significant differences, Tukey test was applied. $P< 0.05$ was considered to be statistically significant.

3. Results

3.1. Effects of graviola on kidney dysfunction parameters

Serum kidney biomarkers including urea, creatinine, uric acid, cystatin C and LDH were mainly used in the evaluation of kidney damage. Lead acetate treated rats showed sever deviations in the renal parameters. As shown in Figure (1), administration of lead acetate (Group III) showed a significant elevation ($P<0.001$) in the serum levels of urea, creatinine, uric acid, cystatin C, and LDH when compared with normal control group (Group I). On treatment with graviola after oral administration of lead acetate (Group IV), the levels of these parameters were found to be significantly decreased ($P<0.001$). No significant change was recorded in

values of these parameters between animals given graviola alone and normal controls.

3.2. Effects of graviola on inflammatory markers

Regarding the inflammatory biomarkers, IL-6 and pentraxin-3 levels from rats exposed to lead acetate and/or graviola are presented in Figure (2). Oral administration of Lead acetate to rats(Group III) for weeks 8 weeks led to a significant increase(P<0.001) in serum IL-6 and pentraxin-3 levels as compared to normal control rats. However, Graviola treatment to rats following oral administration of lead acetate showed a significant decrease (P<0.001) in these inflammatory parameters.

3.3 Effects of graviola on oxidative stress markers in rat kidney

Figure (3) shows the effect of different treatments on renal oxidative damages. Statistical analysis of the obtained tissue values showed that MDA and 8-OHdG levels were similar in the control and graviola 100 mg/kg treated groups (p>0.05). Treating animals with lead acetate (Group III) induced a significant increase (P<0.001) in MDA and 8-OHdG levels as compared to control group. Graviola alleviated lipid peroxidation caused by lead acetate, it caused a significant decrease in the MDA and 8-OHdG levels (P<0.001) in comparison to lead acetate treated rat group.

3.4. Effects of graviola on enzymetic antioxidants in rat kidney

Figure (4) shows the effect of different treatments on renal enzymetic antioxidants. Lead acetate administration affected the antioxidant system. As shown in Figure (4), a significant decrease (P<0.001) in the activities of CAT and SOD was found in lead acetate treated rats when compared with normal control rats. While both SOD and CAT activities were significantly increased (P<0.001) in renal tissues treated with lead acetate plus graviola compared to lead acetate treated rats. The graviola alone (Group II) treated group didn't show significant changes in the SOD and CAT activities compared with control group.

3.5 Effects of graviola on non enzymetic antioxidants in rat kidney

As compared to the control group, exposure to lead acetate induced a significant reduction (p<0.001) in the GSH content. Administration of graviola to the lead acetate treated rats showed a significant increase (p<0.001) in the content of GSH (Figure (5)). Also, the results showed significantly lower (P<0.001) activities of glutathione metabolizing enzymes (GR, GPx and GST) in the lead acetate treated rats compared with control group (Figure (5)). However, there were significant elevations (P < 0.001) in the activities of these enzymes in rats co-administrated with graviola and lead acetate. Administration of graviola alone to rats showed no significant difference compared with control group.

3.6 Histopathological findings:

Histological studies showed normal structure of the renal cortex in the rat kidney of control group. Microscopic examination of normal kidney showed intact tubules and glomerulus. Each renal corpuscle was formed of a glomerular tuft of capillaries surrounded by Bowman's capsule. Proximal and distal convoluted tubules cells showed acidophilic cytoplasm and vesicular nuclei (Fig.6A). Administration of graviola (Group II) to normal rats did not produce any pathological changes in kidney when compared with normal control rats (Fig. 6B). Kidney sections in the lead acetate treated group (Group III) showed pathological changes in the renal glomeruli

and tubules when compared with the normal histological structure of control group. These changes were in the form of marked swelling and vacuolization of the endothelial cells lining the glomeruli, degeneration in renal tubules and inflammatory cells infiltration in between the glomeruli and tubules (Fig.6C). No sign of kidney damage was observed in the animals treated with lead acetate plus graviola (Group IV),the histological changes induced by lead acetate were markedly decreased (Fig.6D).

4. Discussion

Kidneys are the organ concerned with filtering waste products from the blood, regulating blood pressure, electrolyte balance and red blood cell production in the body.[15] Urea, creatinine and uric acid are indicators for renal function for routine analysis. In the present study, rats administrated lead acetate 400mg/kg body weight exhibited a significant increase ($p<0.001$) in the levels of urea, creatinine and uric acid compared to the control group (Fig.1) This elevation in uric acid level may be due to high catabolic rate of purines and pyrimidines, overproduction or decreased uric acid excretion. Creatinine is removed from the blood primarily by glomeluar filtration, in addition to proximal tubular secretion.[16] The significant increase in the serum urea and creatinine levels of rats may be due to the impairment of the glomrerular function and tubular damage of the kidneys. The increased levels of these end products in blood especially serum creatinine and serum urea indicate poor clearance of these substances by the kidneys. Lead toxicity was associated with alterations that cause nephrotoxicity.[17] LDH enzyme found in the proximal renal tubules, it is a sensitive indicator of tubular injury.[18] The activity of LDH was significantly ($p<0.001$) in the lead acetate treated rats compared to control group (Fig.1). The elevation in LDH level can be explained by the fact that administration of lead acetate induced an alteration in the redox status which proved by a decrease in the glutathione concentration and elevation in the lipid peroxidation end product 4-hydroxynonenal level that may be produced by damage in the red blood cell membrane.

Cystatin C is a 13 kD cysteine protease inhibitor synthesized in all nucleated cells, it involved in the measurement of renal function. [19] Cystatin C is filtered across the glomerular membrane and reabsorbed in the proximal tubules. In our study, Cystatin C level was significantly elevated ($p<0.001$) in the lead acetate treated rats compared to control group (Fig. 1). The increase in Cystatin C may be due to glomerular dysfunction and renal tubular damage induced by lead toxicity. Our results are in agreement with previous studies that showed that (CKD) in the general population.[20] In our study, graviola leaves aqueous extract group significantly decrease ($p<0.001$) urea, creatinine, uric acid, LDH and Cystatin C serum levels (Fig.1), which demonstrate its protective effect against lead induced renal toxicity.

In the current study, our data showed significantly elevations ($p<0.001$) in the serum levels of IL-6 and pentraxin-3 (Fig. 2). The pro-inflammatory effect of lead can be explained its ability to cause oxidative stress due to the increased generation or decreased utilization of ROS. Free radicals can lead to cell membrane damage, via lipid peroxidation, that in turn stimulates the signaling cascades of inflammatory process. Lead exposure increases the production of oxidative stress and inflammatory response, as seen by elevation of malondialdehyde (MDA) and pro inflammatory cytokines.[21] Additionally, ROS are responsible for activation of mitogen-activated protein kinases (MAPKs) and nuclear factor kappa B($NF_k\beta$) which are involved in the production of pro-inflammatory molecules.[22] Moreover, increased levels

of some pro-inflammatory cytokines were reported in occupationally lead-exposed humans. [23] Obviously, results of the current study confirm the ability of lead to elevate the levels of inflammatory markers, such as IL-6 and pentraxin-3. In addition in our work, we investigated the possible anti-inflammatory effect of aqueous extract from graviola leaves against lead induced nephrotoxicity. Results of the present study revealed a significant decrease ($p<0.001$) of both IL-6 and pentraxin-3 (Fig.2). our results are agreement with Roslida et al. 2010 [24] who demonstrated that, oral treatment in rats with grviola ethanolic leaf extracts significantly reduced carrageenan-induced edema in rat paws by 79% in a dose-dependent manner, exhibiting its anti-inflammatory activities.

A. muricata were previously found to possess chemical constituents of different alkaloids and essential oils.[25] Nonetheless, species of the Annonaceae family, including A. muricata are well known to have a variety of acetogenins compounds that act as major bioactive compounds in these species. [26] Antioxidant protection system contains mixture of enzymatic and non-enzymatic scavengers. The enzymatic antioxidants of cells, comprising SOD, CAT, GPx, glutathione reductase (GR),and glutathione-s-transferase (GST), SOD play an important role in the reduction of oxidative stress induced by free radicals [27] which is the first defensive mechanism against reactive oxygen species. It attenuates oxidative stress through dismutation of O^{-}_{2}. CAT enzyme has an essential role in converting H_2O_2 to water and oxygen. [28] As the accumulation of H_2O_2 in cells results in the production of highly reactive free hydroxyl radical (OH-) through Fenton reaction, which has a central role in oxidative damages.[29] Another essential antioxidant enzyme, GPx, degrades lipid peroxides to hydroxyl lipids and water through alteration of glutathione to glutathione disulfide The excessive production of reactive oxygen species and oxidative stress is known to cause lipid peroxidation in the respective tissue.[30] Lipid peroxidation of organelle and cellular membranes, being one of the destructive effects of oxygen radicals, is responsible for the defect in endothelial cells, fibroblast and collagen metabolism and keratinocyte capillary permeability. MDA and 8-OHdG are a critical biomarker for lipid peroxidation, and they have proposed to be an index of oxidative damage.[31] Our result showed a significant elevation in MDA and 8-OHdG levels (Fig. 3) and a significant reduction in all enzymatic and non enzymatic enzymes lead acetate groups while with graviola (Fig. 4) these result with in agreement with baskar et al. 2007 [32] who reported the antioxidant activity of A. muricata leaves in vivo study. Also Moghadamtous et al 2014 showed that showed that ethyl acetate extract of A. muricata leaves caused an increase in the activity of CAT, glutathione and SOD on gastric cells of ethanol-treated rats. [33]

Thirty-seven phenolic compounds have been reported to be present in A. muricata. The important phenolic compounds found in A. muricata leaves include quercetin and gallic acid. [34] Phenolic compounds are considered as the major phytochemicals responsible for the antioxidant activity. In addition, Correa-Gordillo et al. (2012) [35] compiled studies on the antioxidant activity of A. muricata considering different assays, The antioxidant activity has been evaluated in fresh and frozen pulp, juice, and fresh or dried leaves. Also, A positive correlation between antioxidant activity and the total polyphenol content was reported (George et al., 2012).[36] Our findings in the present study strongly suggested the protective effect of graviola against toxicity of lead acetate in rats obviously increased the antioxidant markers and reduction of inflammatory and oxidant parameters

Histopathologically, administration of lead acetate showed signs of

inflammation as vacuolization of the endothelial cells lining the glomeruli, degeneration in renal tubules and infiltration of inflammatory cells in between the glomeruli and tubules (Fig 6C). Treatment with graviola leaves extract revealed all these changes (Fig. 6D) . This anti-inflammatory action of graviola leaves may be due to the inhibition of one or more signaling intracellular pathways involved with these mediators effects. Moreover, the ability of graviola leaves to neutralize free radicals. due to the presence of flavonoids and other polyphenols.

Our data are further supported by De Sousa et al (2010) [37] who reported the anti-inflammatory effects of graviola leaves.

Conclusion

In conclusion, our findings confirm that lead acetate induced nero-toxicity which can be improved by the significant potential protective effect of graviola leaves evidenced by the improvement in the kidney function, reduction of lipid peroxidation, inflammation markers and elevation of all antioxidant enzymes in addition to the restoration of the kidney architecture so the results can provide the possible use of graviola leaves against oxidative stress induced nephrotoxicity.

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Biochemistry letters, 13(1) 2018, Pages: 1-13

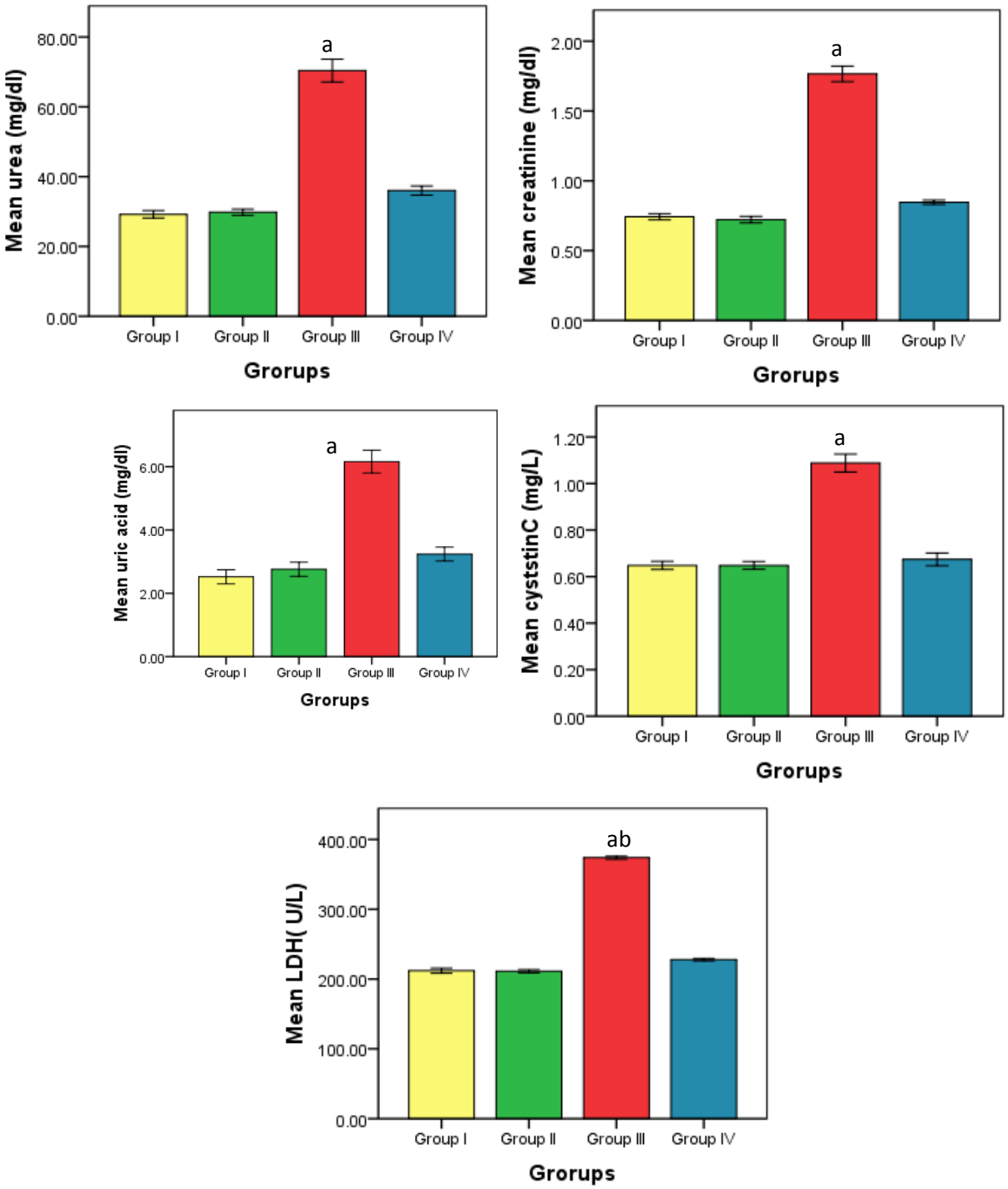

Figure 1: Mean values of kidney markers in serum for the experimental rat groups.
a: indicates the significant difference ($^{a}p< 0.001$)between the normal control and lead acetate treated group. b: indicates the significant difference ($^{b}p< 0.001$) between the lead acetate treated group and the group treated with graviola after lead acetate exposure.Each column represents mean ± SEM, n = 10

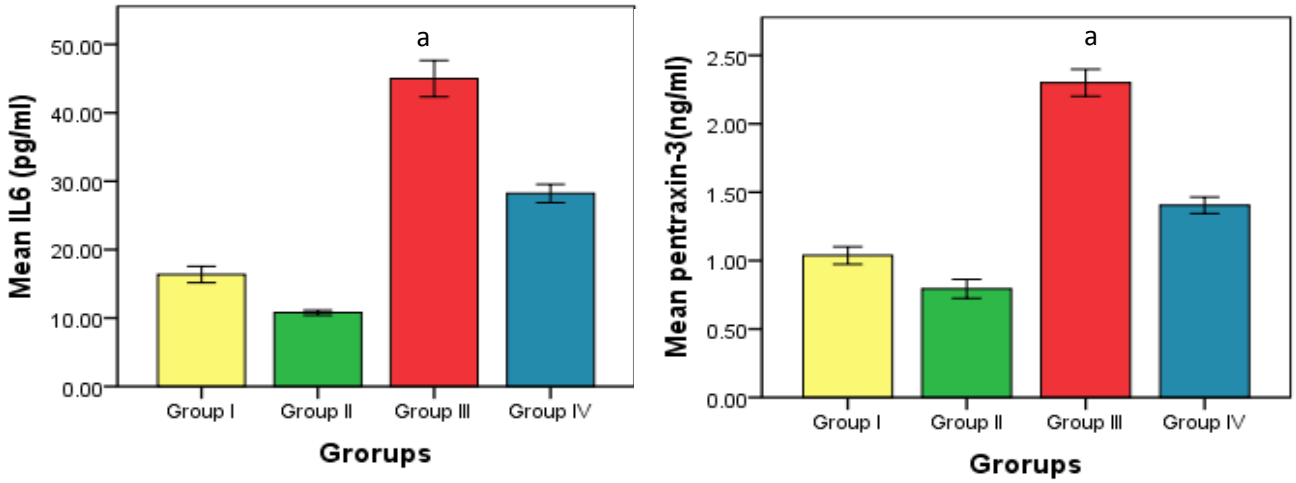

Figure 2: Mean values of serum inflammatory markers for the experimental rat groups. a: indicates the significant difference ($^{a}p< 0.001$)between the normal control and lead acetate treated group. b: indicates the significant difference ($^{b}p< 0.001$) between the lead acetate treated group and the group treated with graviola after lead acetate exposure. Each column represents mean ± SEM, n = 10

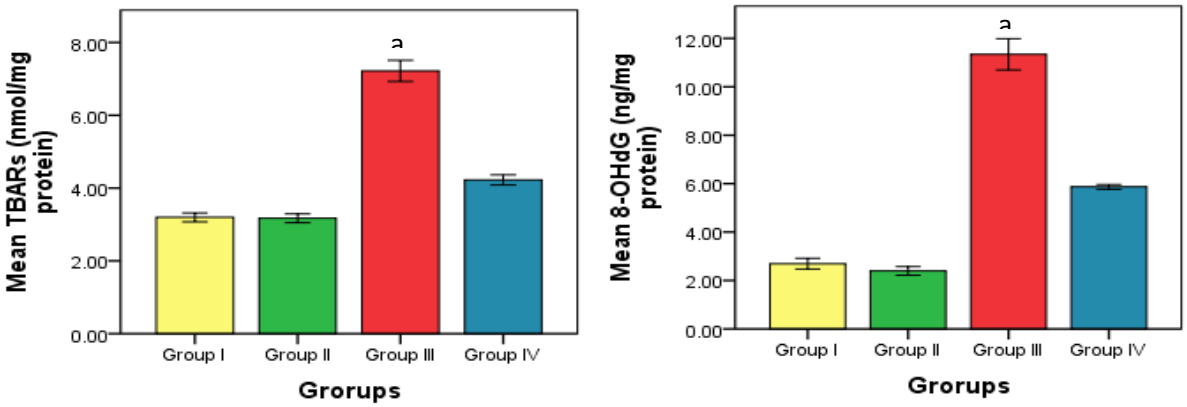

Figure 3: Mean values of oxidative stress markers in kidney homogenate of experimental rat groups. a: indicates the significant difference ($^{a}p< 0.001$)between the normal control and lead acetate treated group. b: indicates the significant difference ($^{b}p< 0.001$) between the lead acetate treated group and the group treated with graviola after lead acetate exposure.

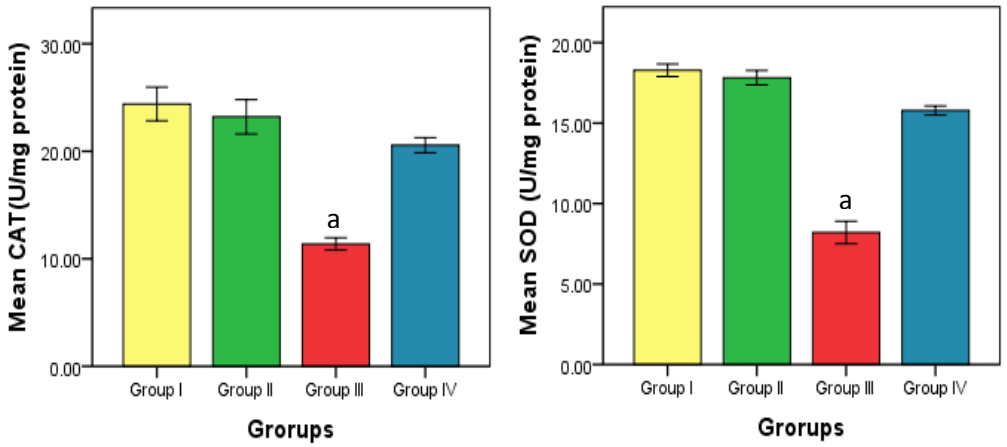

Figure 4: Mean values of antioxidant enzymes in kidney homogenate of experimental rat groups. a: indicates the significant difference ($^{a}p< 0.001$)between the normal control and lead acetate treated group. b: indicates the significant difference ($^{b}p< 0.001$) between the lead acetate treated group and the group treated with graviola after lead acetate exposure. Each column represents mean ± SEM, n = 10

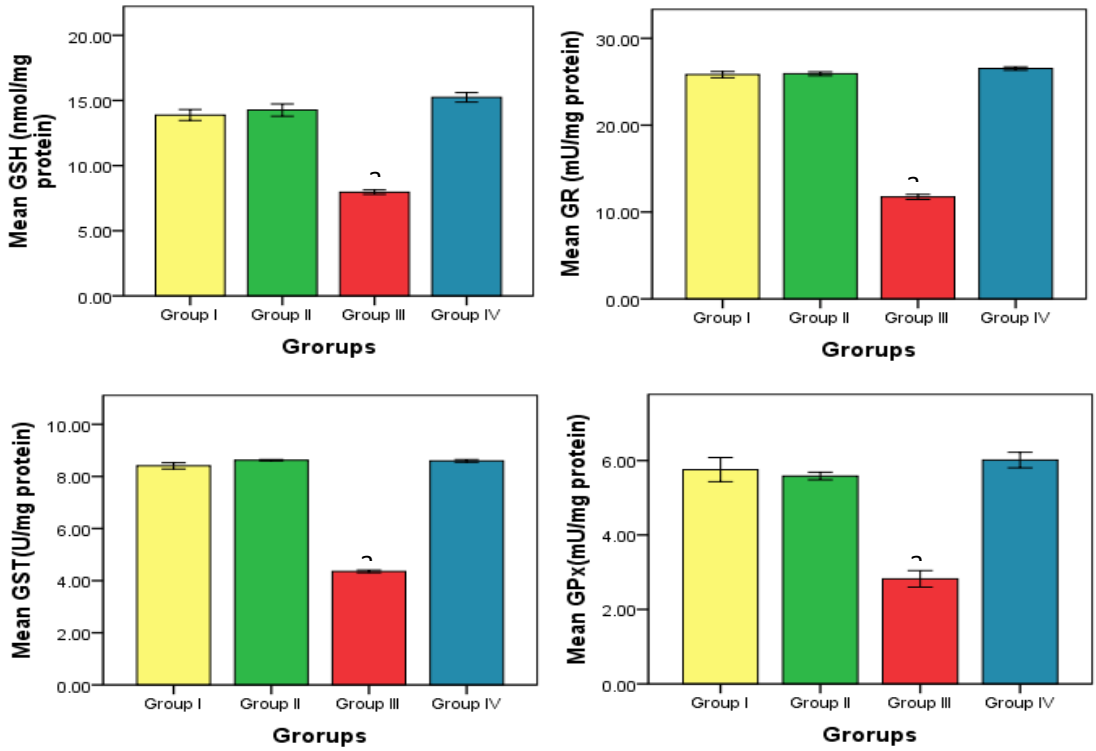

Figure 5: Mean values of glutathione and glutathione metabolizing enzymes in kidney homogenate of experimental rat groups. a: indicates the significant difference ($^{a}p< 0.001$)between the normal control and lead acetate treated group. b: indicates the significant difference ($^{b}p< 0.001$) between the lead acetate treated group and the group treated with graviola after lead acetate exposure. Each column represents mean ± SEM, n = 10.

Biochemistry letters, 13(1) 2018, Pages: 1-13

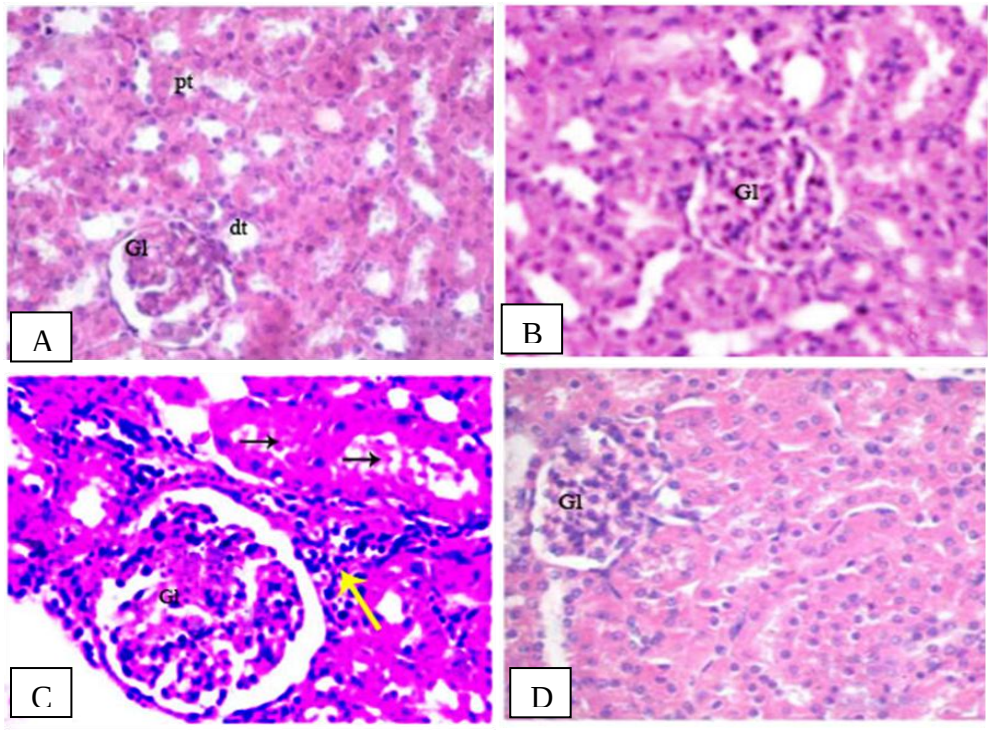

Figure 6: Photomicrographs of kidney sections of rats in the different groups stained with Haematoxylin & Eosin. (A): rat kidney section in control group (Group I) showing the intact histological structure of the glomeruli (Gl), proximial (pt) and distal convoluted tubules (dt) in renal cortex. (C): Rat kidney section in lead acetate treated group (Group III) showing glomerular damage with marked swelling and vacuolization of the endothelial cells lining the glomeruli, degeneration in renal tubules (Black arrows) and inflammatory cells infiltration in between the glomeruli and tubules (Yellow arrow). (B)& (D): rat kidney sections in graviola treated group (Group II) and lead acetate plus graviola co administrated group (Group IV) respectively showing approximately normal renal histological structure.