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Full-length Research Article

Methanol Leaf Extract of *Azadirachta indica* Attenuates Lead Acetate-Induced Cardiorenal Toxicity in Rats

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Summary: The toxic effect of lead on heart and kidney tissues has been associated with its ability to cause oxidative stress. *Azadirachta indica* is a versatile medicinal plant with diverse pharmacological potentials. The ameliorative effect of the methanol extract of *Azadirachta indica* leaves (MEAI) was studied in male Wistar rats exposed to lead acetate (PbAc). Preliminary assays were carried out to assess the total phenolic, total flavonoid, and total flavonol contents of the extract using quantitative phytochemical assays. Cytotoxicity was tested on Vero cells using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide [MTT] assay. Wistar rats were grouped into control, toxicant, and treated groups and administered distilled water, 0.1/0.2% PbAc, and 0.1/0.2% PbAc + 100/200 mg/kg MEAI, respectively. Results showed that the total phenolic content of MEAI was negligible, while the flavonoid (300±0.12 mg Quercetin/g) and flavonol (80±0.06 mg Rutin/g) contents were significant. LC50 value of MEAI was 0.05 mg/mL. MEAI significantly ($p<0.05$) increased weight, reduced blood pressure, and increased blood flow/volume in the treated groups. There was significant ($p<0.05$) reduction in hydrogen peroxide (H_2O_2) and significant ($p<0.05$) increase in Nitric oxide (NO) in some of the treated groups. Histopathology revealed that MEAI reduced inflammatory cell infiltration in the heart and kidney tissues of the treated groups compared with the lead-exposed untreated groups. There was reduction in the expression of cytochrome C in the heart and kidney tissues of the lead-exposed untreated groups compared with the treated groups. This study demonstrates MEAI as a potential drug candidate for the amelioration of lead acetate-induced cardiorenal toxicity by inhibiting oxidative stress and inflammation and regulating apoptosis.

Keywords: *Lead acetate, Azadirachta indica , Cardiorenal toxicity, Oxidative stress, Inflammatory cells, Cytochrome C..*

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INTRODUCTION

Exposure to lead through drinking water, contaminated fish or meat, toys, paints, sand, and other sources predisposes humans and animals to various toxic effects (WHO, 2022). Contamination of the environment by toxins and metals is possibly related to various ailments. Lead has been linked to oxidative stress, resulting in tissue/organ damage, including in the heart and kidneys (Flora *et al*., 2012). The mechanism includes enhanced reactive oxygen species (ROS) generation and impaired antioxidant capacity, which further result in reduced nitric oxide availability and activation of Nuclear Factor-kB, a general transcription factor that promotes inflammation, fibrosis, and apoptosis (Vaziri et al., 2008). These result in renal tubulointerstitial inflammation, vasoconstriction, vascular remodelling, sodium retention, and platelet activation, all of which are implicated in the pathogenesis of cardiotoxicity and nephrotoxicity (Vaziri et al., 1999). A link has been established between lead exposure and the subsequent development of hypertension and cardiovascular disease in population studies (Almeida Lopes *et al.,* 2017).

Lead exposure causes oxidative stress in the kidneys and cardiovascular tissues of exposed animals and humans (Vaziri.,2012). Lead exposure has been shown to significantly increase malondialdehyde, a product of lipid peroxidation, and nitric oxide synthase (NOS), an enzyme that breaks down nitric oxide in tissues (Gonick *et al.,*1997). There has also been evidence of an increase in superoxide dismutase (SOD) without any significant increase in catalase and glutathione peroxidase in some tissues of lead-exposed animals (Farmand *et al*.,2005), thus resulting in a significant increase in both hydrogen peroxide (H_2O_2) and lipoperoxides, reactive oxygen species that are reduced by the antioxidant enzymes, catalase and glutathione peroxidase. Furthermore, the accumulation of H_2O_2 results

in inflammation and cardiovascular remodeling through the activation of NF-kB and stimulation of cellular growth (Vaziri *et al.,*2012). Infiltration of T-cells, macrophages, and apoptotic cells has been reported in increasing numbers in the histology of cells and tissues exposed to lead (Rodriquez-Iturbe *et al*., 2004).

Azadirachta indica , a versatile medicinal plant in the tropics is abundant with bioactive compounds, capable of chelating metals and ameliorating pathologies. *Azadirachta indica* has been shown to possess antioxidant properties (Ali *et al.,* 2022). Phenolic compounds and limonoids, amongst others, present in *Azadirachta indica* are attributed to high antioxidant and anti-radical scavenging activities (Ramamurthi *et al*., 2009; Nakamura *et al.,* 2022). GC-MS analysis of the methanol leaf extract of *Azadirachta indica* revealed the presence of 22 compounds; including 2-chlorobenzoic acid, Lauric acid, Myristic acid, 2,6-Di-O-pamitoyl-L-ascorbic acid, amongst others; which may be responsible for its vast pharmacological properties (Omobowale *et al.,* 2020). This study explores the ameliorative effect of methanol extract on lead-induced weight loss, cardiotoxicity, and nephrotoxicity via an antioxidant defensive mechanism, anti-inflammatory action, and modulation of apoptosis.

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MATERIALS AND METHODS

Plant material: Fresh *Azadirachta indica* leaves obtained from a Neem tree were identified and authenticated at the Department of Botany, University of Ibadan, air-dried in a cool room, and blended into a finer form. The leaves were defatted by soaking in n-hexane for 24 hours and then extracted in 100% methanol for 72 hours. The extract was concentrated in a rotary evaporator and dried.

Ethical approval: This was obtained from the University of Ibadan, Animal Care and Use Research Ethics Committee (UI-ACUREC) with Assigned number UI-ACUREC/05/003-0718/9.

Quantitative analyses of the phytochemical screening

Assessment of total phenolic content: The quantification of the phenolic content of the *A. indica* leaf *extract* was carried out using the procedure reported by Wolfe *et al*. (2005). An aliquot of extract (1 mL) was mixed with 5 mL Folin Ciocalteu reagent (previously diluted with water 1:10 v/v) and 4 mL (75 g/L) of sodium carbonate. The tubes were vortexed for 15s and allowed to stand for 30 min at 40°C for colour development. Absorbance was read at 765 nm using a spectrophotometer. Extract was evaluated at a final concentration of 1 mg/mL. Gallic acid was used as standard. Total phenolic content was expressed as mg/g gallic acid equivalent using the equation obtained from a calibration curve of gallic acid.

Determination of total flavonoids: The total flavonoid content of the extract was determined using the method of Chang *et al*. (2002). Briefly, 0.5 mL of the sample and standard (1 mg/mL), 2 mL distilled water, followed by 0.15 mL of 5% of $NaNO_2$ were mixed and allowed to stand at 25ºC for 5 - 6 min. 0.15 mL of 10% $AlCl_3$ was added and allowed to stand for another 6 minutes. After which 1 mL of 4% NaOH was added to the mixture and made up to 5 mL with distilled water, vortexed for 15 minutes, and colour change was observed. The absorbance was measured at 420 nm using a spectrophotometer. Total flavonoid content was calculated as quercetin (mg/g) equivalent using the equation obtained from a calibration curve of quercetin.

Determination of total flavonols: The total flavonol content was determined by the method described by Kumaran and Karunakaran (2007). Twenty grams of $AlCl_3$ and 50 g of sodium acetate anhydrous powder were separately dissolved in a small quantity of distilled water and made up to 1.0 L with distilled water respectively. The rutin calibration curve was prepared by mixing 2 mL of varying concentrations of rutin (0.2–0.1 mg/mL) with 2 mL (20 g/L) $AlCl_3$ and 6 mL (50 g/L) sodium acetate. The absorbance at 440 nm was read after 2.5 h at 20 °C. The same procedure was carried out with 2 mL of plant extract (0.2-1.0 mg/mL) instead of rutin solution. All determinations were done in triplicates. The flavonol content was obtained from the rutin calibration curve and expressed as rutin equivalents (mg/g).

Cytotoxicity assay: The cytotoxicity of the methanol extract, suspended in acetone, against Vero monkey kidney cells was determined by using the 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) reduction assay as previously described by Mosmann (1983) with slight modifications. Cells were seeded at a density of 1 x 105 cells/mL (100 µL) in 96-well microtitre plates and incubated at 37 °C and 5% CO_2 in a humidified environment. After overnight incubation, 100 µL each of varying concentrations of the extract was added to the wells containing cells. Doxorubicin was used as a positive reference. A suitable blank control with equivalent concentrations of acetone was also included and the plates were incubated for 48 h in a CO_2 incubator. Thereafter, the medium in each well was aspirated from the cells, cells were washed with PBS, and finally, 200 µL fresh media was added to each well. Thirty microlitre of MTT (5 mg/mL in PBS) was added to each well and the plates were incubated at 37 °C for 4 h. The medium was aspirated from the wells and DMSO was added to solubilize the formazan crystals. The absorbance was measured using a BioTek Synergy microplate reader at 570 nm. The percentage of cell growth inhibition was calculated based on a comparison with untreated cells.

Animals: Male Wistar rats (n=70, weight 100-150g, eight weeks old) were purchased from the Animal House of the Faculty of Veterinary Medicine, University of Ibadan. They were allowed to acclimatize for 2 weeks by feeding them *ad-libitum* with pelletized rat feed and clean drinking water. All the rats were kept in the same environment and given the same feed throughout the 12 weeks of the experiment.

Experimental design: After the period of acclimatization, the rats were randomly divided into 7 groups (n=10). Group A (Control) received distilled water orally for 12 weeks. Group B, C and D received 0.1% PbAc in water for 6 weeks and were given distilled water, 100mg/kg MEAI and 200mg/kg MEAI, respectively for another 6 weeks. Group E, F and G received 0.2% PbAc in water for 6 weeks and

were given distilled water, 100mg/kg MEAI and 200mg/kg MEAI, respectively MEAI for another 6 weeks.

Lead acetate (PbAc) salt was weighed and dissolved in clean water at a concentration of 1 mg/mL and 2 mg/mL. It was administered to the rats *ad-lib*. The dried MEAI was weighed and dissolved in Corn oil at a stock concentration of 80 mg/mL. MEAI was administered to the rats according to their average weight. At the end of 12 weeks, blood pressure and electrocardiography readings were taken, blood was collected through the periorbital canthus, the rats were sacrificed by cervical dislocation and their organs harvested.

Weight gain: The rats were weighed at the onset of the experiment and every week till the 12th week.

Blood pressure and electrocardiography: Deep intramuscular administration of 0.1mL of a mixture of ketamine/xylazine (1:1) was given to each rat before non-evasive 5 ECG "leads" were attached to the limbs and chest for readings to be taken. An indirect method using an automated plethysmograph (Kent Scientific, USA) was used to take the readings of each rat. The systolic, diastolic, mean pressure, heart rate, blood volume as well as blood flow were recorded.

Biochemical analyses: Heart and kidney tissues were homogenized in phosphate buffer saline and centrifuged at 3000g for 20 min at 4°C. The supernatants obtained were used for biochemical assays. Reduced glutathione (GSH) was estimated using Ellman's reagent, and lipid peroxidation assessment was carried out according to the method of Varshney and Kale (1990) and Hydrogen Peroxide generation based on the Wolff method (1994). Other assays include Glutathione peroxidase assay, Glutathione-S-Transferase activity according to Habig *et al*. (1974), Superoxide Dismutase (SOD) activity based on the method of Misra and Fridovich (1972), and determination of Catalase activity was done according to the method of Sinha (1972).

Histology: The samples were kept in 4% phosphate-buffered formalin and refrigerated for 72 hours. They were then dehydrated with different concentrations of ethanol, absolute alcohol, and xylene. The clearing was done using xylene. They were infiltrated in paraffin wax in an oven at 60-65°C and then embedded in fresh molten wax in a mold containing the slides.

Immunohistochemistry: The tissue samples were mounted on charged slides, dewaxed twice in xylene for 5 minutes each and then rehydrated with decreasing concentrations of alcohol (80%, 90%, and 100%) for 5 minutes each. Slides were placed in a water tank containing distilled water for 5 mins and then incubated for 10 mins with endogenous peroxidase. The slides were afterwards rinsed with water and then placed for 5 mins, in wash buffer tank and rinsed with distilled water. The antigens were retrieved using the heat method with citrate buffer and then left to cool. They were rinsed and left in distilled water tank for 2 mins, cleaned with tissue paper, goat serum (KPL, Inc., Gaithersburg, Maryland, USA) added to prepare for antigen-antibody reaction, and then left to incubate in a humidifying chamber for 15 mins. Afterwards, excess goat serum was removed by shaking the slides. The slides were incubated with cytochrome C (Bioss Inc., Woburn, Massachusetts, USA) at 4°C overnight in a humidifying chamber. The next day, slides were rinsed with wash buffer, left in wash buffer for 5 mins, before the addition of Biotinylated antibody (KPL, Inc., Gaithersburg, Maryland, USA) and incubation in humidifying chamber for 30 minutes. The slides were furthermore rinsed and placed in wash buffer for 5 mins, incubated for 30 mins with Streptavidin HRP system (KPL, Inc., Gaithersburg, Maryland, USA), rinsed and then placed in phosphate buffer saline tank for 5 minutes.The reaction was stopped by rinsing with distilled water, then the sections were counter stained with HIGHDEF IHC hematoxylin (Enzo Life Sciences, NY, USA) for 3 seconds. Afterwards, the slides were placed 80%, 90%, and 100% ethanol for 3 minutes each and finally, twice in xylene (100%) tank for 5 minutes each.the slides were viewed with a digital microscope, under x400 magnification to assess the expression of cytochrome C. The degree of expression of Cytochrome C was evaluated using ImageJ (IJ 1.46r edition).

Data analysis

Data collected were analyzed using the student's T-test (Elston and Johnson, 2008) as well as the one-way Anova at the level of 5% significance and were presented as Mean ± standard deviation, using the PRISM software package (Version 5.0).

RESULTS

Phytochemical analysis and cytotoxicity profile of the Methanol leaf extract of *Azadirachta indica* : Table 1 shows the quantity of total phenols, total flavonoids, flavonols, and cytotoxicity profile (LD_{50}) of MEAI. MEAI has negligible total phenols and a significant amounts of flavonoids and flavonols. The LD_{50} value of MEAI was 0.05 mg/mL.

Table 1:
Phytochemical analysis and cytotoxicity profile of the Methanol leaf extract of *Azadirachta indica*

Phytochemical	Quantity
Total Phenols (mgGAE/g)	-21.76±0.07
Total Flavonoids (mg Quercetin/g)	300±0.12
Total Flavanols (mg Rutin/g)	80±0.06
Median Lethal Concentration [LC_{50}] (mg/mL)	0.05

mgGAE/g: milligram gallic acid equivalent per gram of extract, mgQuercetin/g: milligram quercetin equivalent per gram of extract, mgRutin/g: milligram rutin equivalent per gram of extract, mg/mL: milligram per mL

Effect of PbAc and MEAI on weight gain: Figure 1 shows the effect of lead and MEAI on weight gain across all the groups. PbAc caused a significant reduction in weight gain and MEAI caused increased weight gain in treated groups.

Effect of PbAc and MEAI markers on oxidative stress: H_20_2 was significantly increased at 0.1%PbAc in the cardiac tissues and at 0.2%PbAc in both the cardiac and renal tissues. It was however significantly reduced in some of the

treated groups. There was no marked alteration in the catalase and GPx levels of both the cardiac and renal tissues at both levels of lead exposure, however, in the 0.1%PbAc + 200mg/mL MEAI group, there was significant alteration in the levels of both enzymes in the cardiac and renal tissues . GST levels were significantly increased in all the toxicant and treated groups. There was a significant reduction in NO in the 0.1%PbAc group which increased significantly at 100 mg/mL and 200 mg/mL MEAI treatment (Tables 2 and 3).

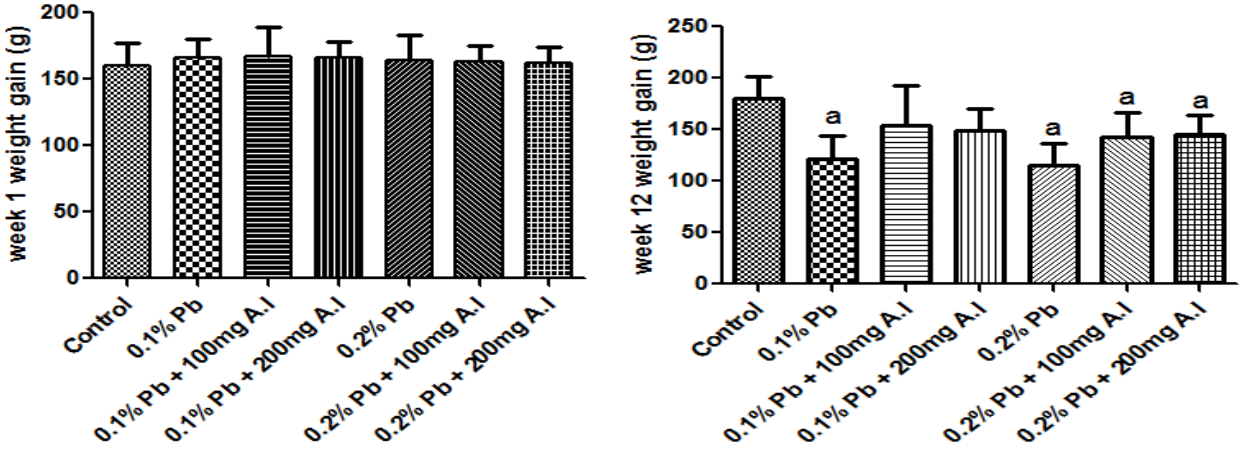

Figure 1:
Weight gain at week 1 and week 12. Group A: Control; Group B: 0.1% Pb; Group C: 0.1% Pb + 100 mg/kg MEAI; Group D: 0.1% Pb + 200 mg/kg MEAI; Group E: 0.2% Pb; Group F: 0.2% Pb + 100 mg/kg MEAI; Group G: 0.2% Pb + 200 mg/kg MEAI. Superscript a indicates group is significantly different at $p<0.05$ from control group (group A)

Table 2:
The effect of MEAI on markers of lead-induced oxidative stress in the cardiac tissues and serum NO levels

	Group A (Control)	Group B	Group C	Group D	Group E	Group F	Group G
Cardiac H_2O_2	11.40±0.50	10.50±0.10	11.50±1.50	11.00±0.50	12.50±0.90^{a,b}	11.05±0.60^{e}	11.15±0.50^{e}
Cardiac MDA	1.12±0.10	1.64±0.36^{a}	1.84±0.30^{a}	1.84±0.23^{a}	1.75±0.39^{a}	1.52±0.22	1.74±0.38^{a}
Cardiac CAT	1400±86.70	1333±126.70	1253±33.30^{a}	1466.70±186.70^{c}	1373.3±60.00	1373.0±120.00	1393.3±33.30
Cardiac GPx	424±20.00	416±12.00	390±6.00	442±44.00^{c}	416±2.00	420±22.00	404±20.00
Cardiac GST	0.029±0.02	0.074±0.02^{a}	0.09±0.02^{a}	0.11±0.01^{a,b}	0.098±0.06^{a,b}	0.11±0.02^{a}	0.10±0.01^{a}
Serum NO	2.64±0.56	1.64±0.33^{a}	3.62±0.82^{a,b}	3.88±0.46^{a,b}	3.31±0.38^{a}	3.24±0.38	3.46±0.38

Superscripts a,b, c and e indicate a significant difference ($p<0.05$) within rows from groups A, B, C and E. H_2O_2: Hydrogen peroxide generation (µL/mg protein); MDA: Malondialdehyde (µM/L); CAT: Catalase (µM/min/mg protein); GPx: Glutathione Peroxidase (µM/mg protein); GST: Glutathione-S-transferase (µM/min/mg protein); NO: Nitric Oxide (µM/L)

Table 3:
The effect of MEAI on markers of lead-induced oxidative stress in the renal tissues

	Group A (Control)	Group B	Group C	Group D	Group E	Group F	Group G
Renal H_2O_2	12.63±2.11	17.52±2.11^{a}	14.74±1.37^{a,b}	14.53±1.89^{b}	15.26±1.05^{a,b}	14.74±1.58^{a}	13.58±1.05
Renal MDA	2.09±0.23	1.76±0.90^{a}	1.98±0.65^{a,b}	1.43±0.46^{a,b}	1.52±0.23^{a,b}	1.94±0.42	1.82±0.55^{a}
Renal CAT	1166.67±71.43	1190.48±95.24	1238.1±100.00	1095.24±138.10^{c}	1142.86±104.76	1166.67±100.00	1219.05±109.52
Renal GPx	333.33±20.0	341±21.67	366.67±33.33	333.33±20.0	333.33±36.67	336.67±33.33	350±36.67
Renal GST	0.04±0.01	0.13±0.03^{a}	0.13±0.01^{a}	0.11±0.05^{a}	0.13±0.02^{a}	0.06±0.02^{e}	0.09±0.04^{a}

Superscripts a,b,c, and e indicate a significant difference ($p<0.05$) within rows from groups A, B, C, and E. H_2O_2: Hydrogen peroxide generation (µL/mg protein); MDA: Malondialdehyde (µM/L); CAT: Catalase (µM/min/mg protein); GPx: Glutathione Peroxidase (µM/mg protein); GST: Glutathione-S-transferase (µM/min/mg protein).

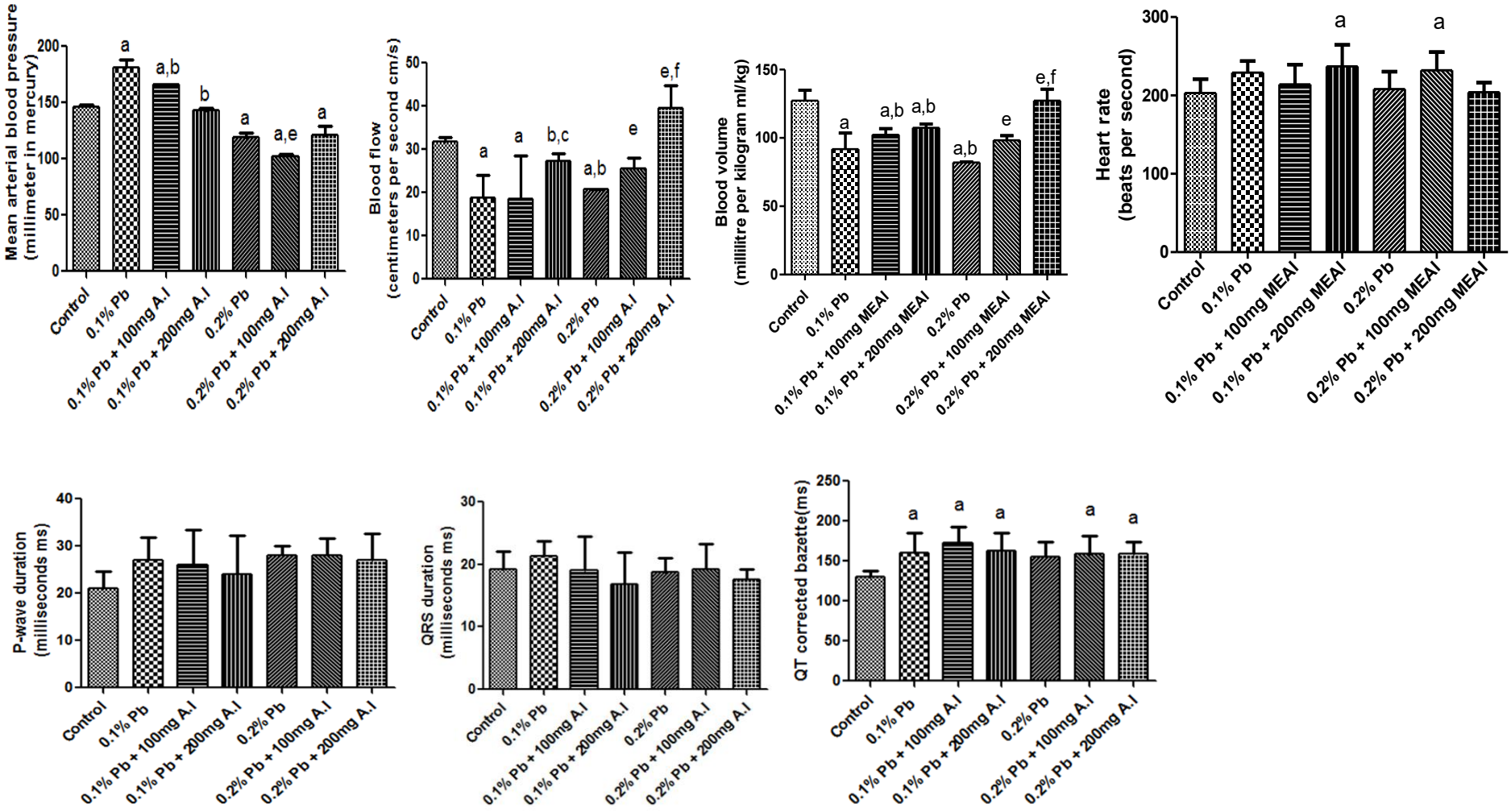

Figure 2:
Mean arterial Blood Pressure, Blood flow, Blood volume, Heart rate, P wave, QRS duration and QT corrected bazette of all groups at week 12. Group A: Control; Group B: 0.1% Pb; Group C: 0.1% Pb + 100 mg/kg MEAI; Group D: 0.1% Pb + 200 mg/kg MEAI; Group E: 0.2% Pb; Group F: 0.2% Pb + 100 mg/kg MEAI; Group G: 0.2% Pb + 200 mg/kg MEAI. Superscript a,b,c,e,f indicate group is significantly different $p<0.05$ from groups A, B, C, E and F

Effect of PbAc and MEAI on blood pressure, blow flow, blood volume, and ECG: There was a significant ($p<0.05$) increase in mean arterial blood pressure of the 0.1%PbAc group. Treatment with MEAI significantly reduced the blood pressure in both 0.1%PbAc + 100 mg/kg MEAI and 0.1%PbAc + 200 mg/kg MEAI groups. The opposite was observed with the 0.2% lead-exposed groups. The significant fall in blood pressure at 0.2% lead exposure compared with the control group was slightly increased with MEAI in the 0.2%PbAc + 200 mg/kg MEAI group. Lead exposure resulted in a significant decrease in blood flow. Treatment with 200 mg MEAI significantly increased the blood flow of both toxic groups. Blood volume was reduced at both levels of exposure and significantly increased with MEAI treatment while the heart rate was not significantly affected by lead exposure although significantly increased at 200mg MEAI treatment. Lead-induced increase in QT-corrected bazette was not reduced by MEAI in all the treated groups (Figure 2).

Effect of PbAc and MEAI on histology: The heart tissues of the control group showed no significant lesions while those of the 0.1%PbAc group showed myocardial necrosis and very mild pericardial inflammation. Heart tissue of the 0.1%PbAc + 100 mg/kg MEAI group showed mild inflammation in some of the tissues while others showed no visible lesions. 0.1%PbAc + 200 mg/kg MEAI group showed no visible lesions. There were signs of calcification, necrosis, and fibrosis of the aorta extending deep into the heart as well as focal areas of very mild necrosis of the myocardium of the 0.2%PbAc group. A focal area of infiltration of the myocardial interstitium by inflammatory cells was observed in the 0.2%PbAc + 100 mg/kg MEAI group while 0.2%PbAc + 200 mg/kg MEAI group showed extensive necrosis of the myocardium, narrowing of congested coronary artery with thickened wall and infiltration of the myocardium with inflammatory cells (Plate 1).

There were no significant lesions in the kidney tissues of the control group while those of the 0.1%PbAc group showed both mild and significant vessel congestions and mild peritubular infiltration by inflammatory cells. There was mild congestion of the vessels in the kidney tissues of some of the 0.1%PbAc + 100 mg/kg MEAI group, while some other tissues showed no significant lesions. However, no significant lesions were observed in all the kidney tissues of the 0.1%PbAc + 200 mg/kg MEAI group. No significant lesions were discovered in the kidney tissues of rats in 0.2%PbAc, however, the kidney tissues of the rats in the 0.2%PbAc + 100 mg/kg MEAI group showed mild and moderate peritubular infiltration by inflammatory cells and those of the 0.2%PbAc + 200 mg/kg MEAI group also showed some mild peritubular infiltration by inflammatory cells while others showed no significant lesions (Plate 2).

Effect of PbAc and MEAI on the expression of Cytochrome C: There were varied expressions of Cytochrome C in the cardiac and renal tissues. In the cardiac tissues, the expression of Cytochrome C reduced at both lead exposure and remained at lower levels compared to the control in the MEAI-treated groups. At 0.2% Pb exposure, MEAI significantly increased the expression of Cytochrome C. In the renal tissues, the expression of Cytochrome C was significantly reduced at 0.1% Pb exposure with a dose dependent increase in the MEAI treated groups. The reverse was observed at 0.2% Pb exposure, where the expression of Cytochrome C increased significantly with significant reduction in its expression in the MEAI-treated groups. (Plates 3 & 4)

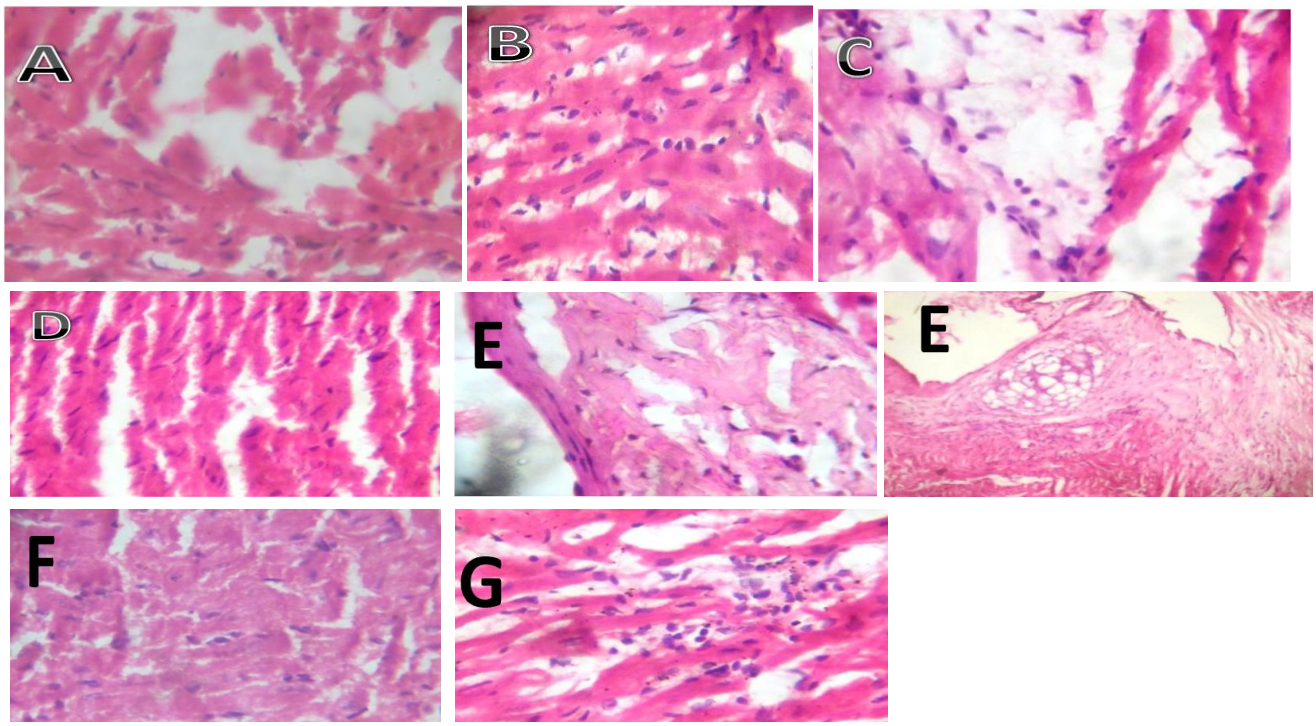

Plate 1:
Histology of the heart tissues with Haematoxylin and Eosin (H&E) staining (Magnification X400). Group A: Control; Group B: 0.1% Pb; Group C: 0.1% Pb + 100 mg/kg MEAI; Group D: 0.1% Pb + 200 mg/kg MEAI; Group E: 0.2% Pb; Group F: 0.2% Pb + 100 mg/kg MEAI; Group G: 0.2% Pb + 200 mg/kg MEAI.

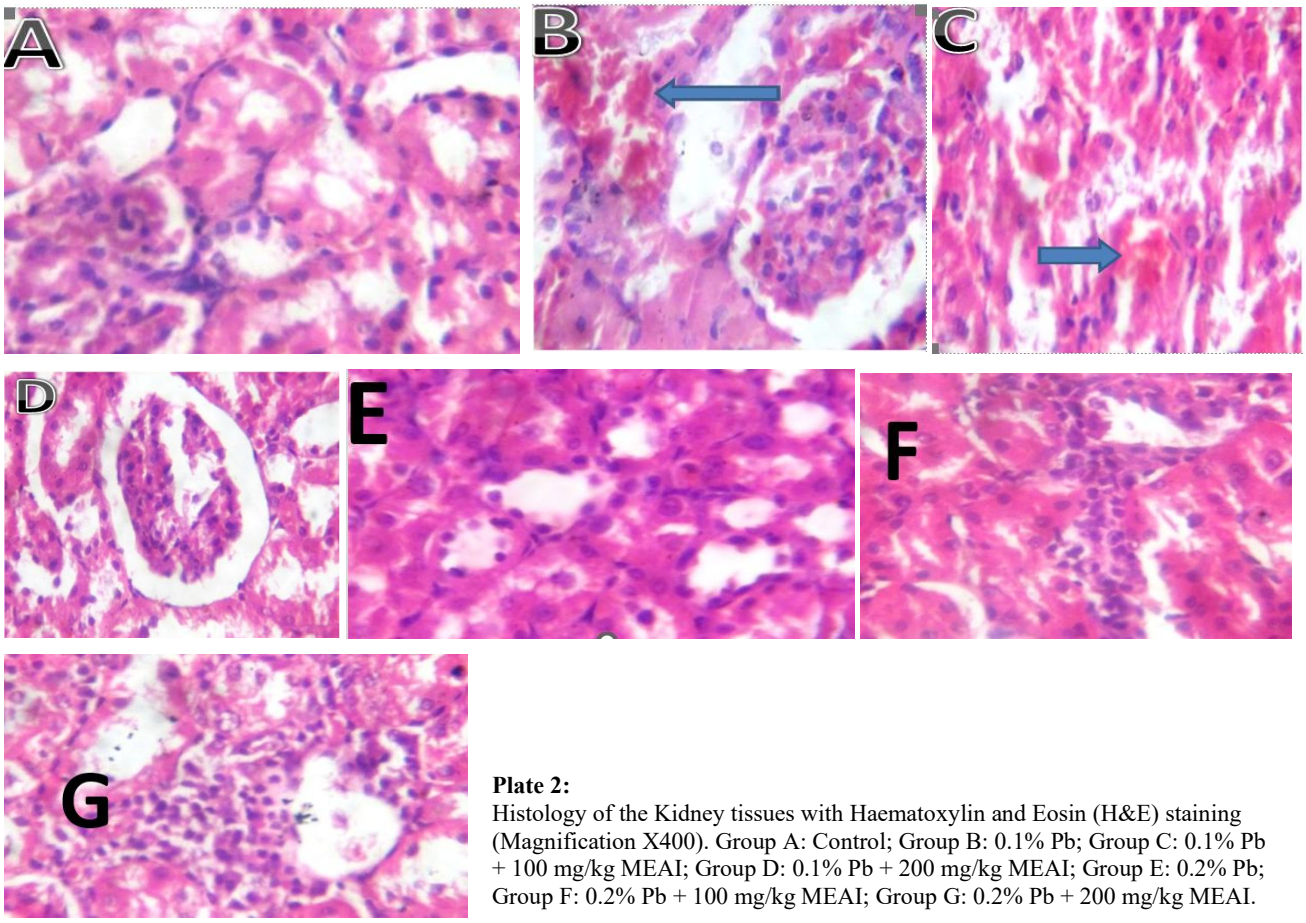

Plate 2:
Histology of the Kidney tissues with Haematoxylin and Eosin (H&E) staining (Magnification X400). Group A: Control; Group B: 0.1% Pb; Group C: 0.1% Pb + 100 mg/kg MEAI; Group D: 0.1% Pb + 200 mg/kg MEAI; Group E: 0.2% Pb; Group F: 0.2% Pb + 100 mg/kg MEAI; Group G: 0.2% Pb + 200 mg/kg MEAI.

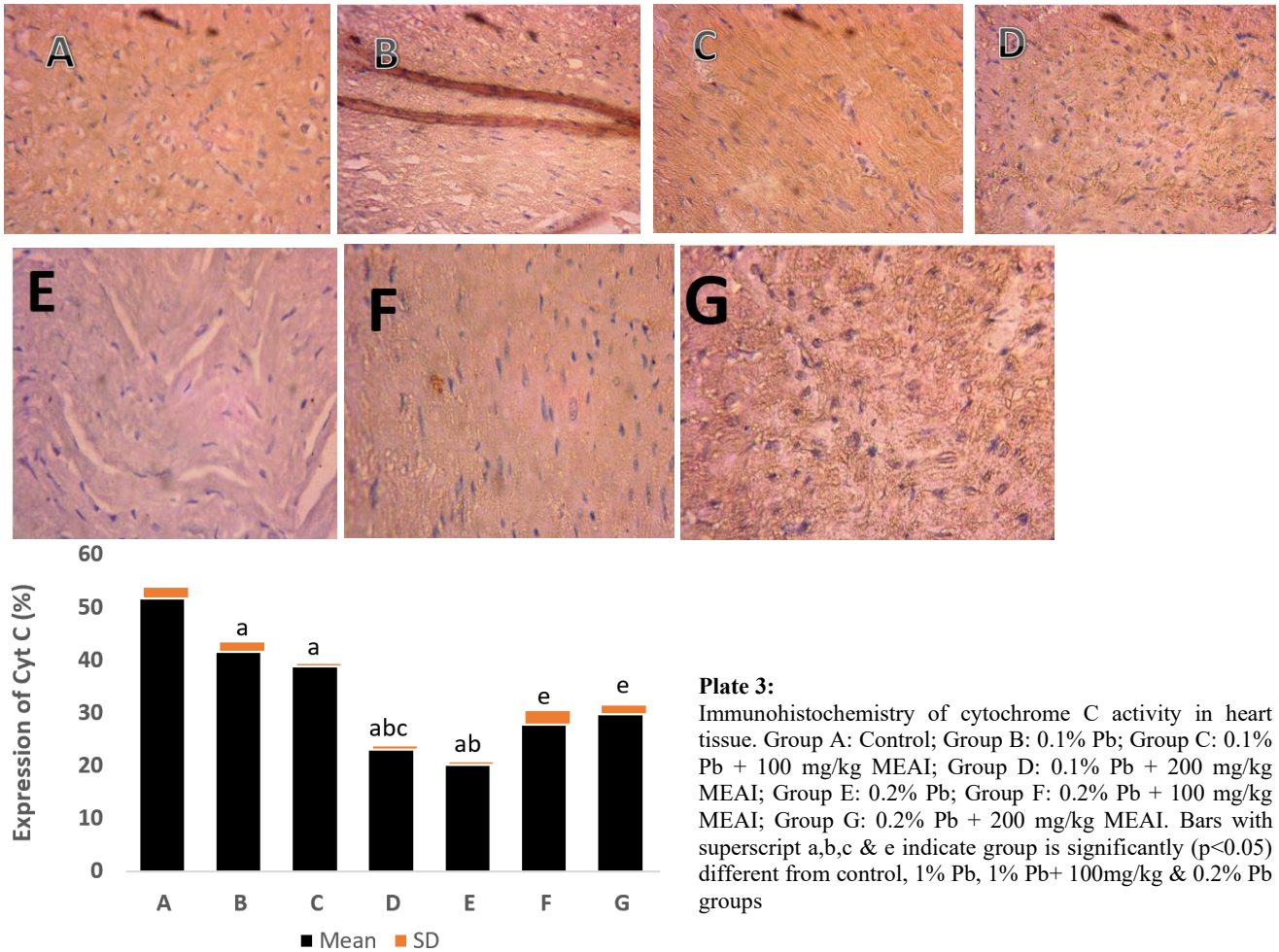

Plate 3:
Immunohistochemistry of cytochrome C activity in heart tissue. Group A: Control; Group B: 0.1% Pb; Group C: 0.1% Pb + 100 mg/kg MEAI; Group D: 0.1% Pb + 200 mg/kg MEAI; Group E: 0.2% Pb; Group F: 0.2% Pb + 100 mg/kg MEAI; Group G: 0.2% Pb + 200 mg/kg MEAI. Bars with superscript a,b,c & e indicate group is significantly ($p<0.05$) different from control, 1% Pb, 1% Pb+ 100mg/kg & 0.2% Pb groups

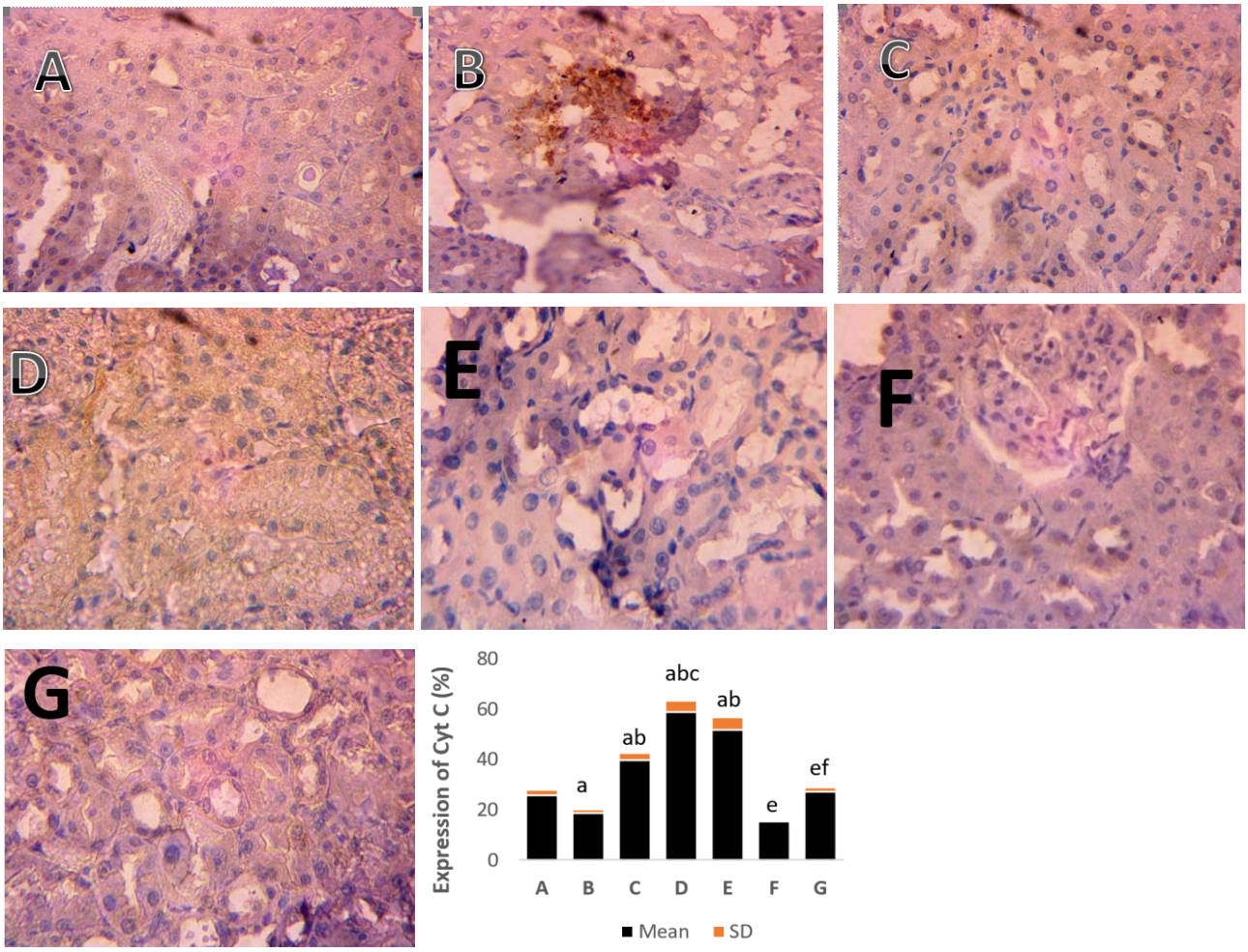

Plate 4
Immunohistochemistry of cytochrome C activity in Kidney tissue. Group A: Control; Group B: 0.1% Pb; Group C: 0.1% Pb + 100 mg/kg MEAI; Group D: 0.1% Pb + 200 mg/kg MEAI; Group E: 0.2% Pb; Group F: 0.2% Pb + 100 mg/kg MEAI; Group G: 0.2% Pb + 200 mg/kg MEAI. Bars with superscript a,b,c,e & f indicate group is significantly ($p<0.05$) different from control, 1% Pb, 1% Pb+ 100mg/kg 0.2% Pb & 0.2% Pb + 100 mg/kg MEAI groups

DISCUSSION

The presence of phytochemicals in plants is liable for their pharmacological properties. The solvent of extraction is an important determinant of the presence and quantity of phytochemicals present in an extract. Methanol, the solvent of extraction in this study, did not extract significant amount of phenols, but extracted flavonoids and flavonols in high quantity. According to Galanakis *et al.* (2010), the dissolution of phenols is determined by their stereochemistry; thus, phenols with simpler structures dissolve better in methanol than those with complex structures. This suggests the scarcity of simple phenols in the *Azadiracta indica* leaf. Numerous studies have demonstrated that methanol, due to its polarity, is an effective solvent for extracting flavonoids (Chaves *et al.,* 2020), thereby explaining the high flavonoid content in MEAI. Plant extracts used in therapy must be shown to be safe to target organs. The US National Cancer Institute (NCI) plant screening program states that after 48 hours of incubation in an *in vitro* experiment, crude extracts with LD_{50} values of 20 µg/mL or less are considered cytotoxic (Boik, 2001). MEAI, in this study, therefore, with an LD_{50} value of 0.05 mg/mL (50 µg/mL), may be considered safe for therapeutic use.

Weight loss caused by lead could result from several gastrointestinal symptoms, such as loss of appetite, nausea, diarrhea, constipation, and abdominal pain observed in lead poisoning (O'Connor *et al.,* 2020). The inclusion of *Azadirachta indica* leaf in lamb's diet enhanced energy use efficiency leading to improvements in food consumption and weight gain (Jack *et al.,* 2020; 2022). It has therefore been proposed to offer a substitute to enhance the efficiency of lamb fattening (Cordero-Mora *et al.,* 2024). In this study, lead-exposed rats treated with MEAI significantly gained weight compared to the untreated lead-exposed rats. The effect of the MEAI was more appreciable at the lower dose (0.1%) of lead exposure than at the higher dose (0.2%), pointing to the direct effect of MEAI on the reversal of lead-induced weight loss. Significantly higher percentages of proteins, crude fat, fiber, and carbohydrates have been demonstrated in feed supplemented with *A. indica* leaf extract (Abidin *et al.,* 2022), thus, supporting the potency of MEAI in enhancing weight gain.

Oxidative stress is a significant feature of lead toxicity (Lopez *et al.,* 2016). Oxidative stress causes damage to body cells by deluging and altering the functions of antioxidant

enzymes (Ray and Shah, 2005). In this study, this is indicated by increased H_2O_2, MDA and GST in the tissues of some of the 0.1%PbAc and 0.2%PbAc exposed rats. The normal response of the body to oxidative stress is an activation of antioxidant enzymes. In this study, there was the absence of significant difference in the levels of GPx and CAT in the 0.1%PbAc and 0.2%PbAc groups compared with the control group. This lack of upregulation of GPx and catalase; enzymes required for the breaking down of H_2O_2; suggests that the body might have been overwhelmed by the excessive production of ROS. This further resulted in the increase observed in H_2O_2 levels, which agrees with the report of Farmand *et al.* (2005). Increased MDA generated in the cardiac tissues of 0.1%PbAc and 0.2%PbAc groups agrees with the report of Gonick *et al.* (1997) and Upasani *et al.* (2001). Increased MDA is a reflection of reduced antioxidant status and cytotoxicity (Maurya *et al.,* 2021). Omóbòwálé *et al.* (2020) previously reported increased MDA levels in hypertensive rats compared with normotensive rats, thereby establishing a link between high blood pressure and oxidative stress. Reduced NO has been implicated in endothelial dysfunction that leads to hypertension (Armas-Padilla *et al.,* 2007). Gonick *et al.* (1997) reported an increase in NOS in the tissues of animals exposed to lead. The reduction in NO content observed in this study may therefore be attributed to increased NOS. Since reduced NO can lead to hypertension via vasoconstriction, its upregulation by MEAI suggests the antihypertensive potential of this extract.

Several studies have reported the anti-hypertensive effect of *Azadirachta indica* leaf extracts (Yarmohammadi *et al.,* 2021). Obiefuna and Young (2005) showed that DOCA-salt-induced hypertension and its accompanying ECG alterations were prevented by concurrent administration of 20mg/kg neem leaf extract with DOCA-salt for 5 weeks. Reports on the antihypertensive activity of neem leaf have been attributed to mechanisms that increase NO levels, reduce oxidative stress, and decrease inflammation (Omóbòwálé *et al.,* 2019). In this study, the antihypertensive potential of MEAI may also be linked with its antioxidative, nitric oxide increase, and anti-inflammatory activities. Furthermore, the composition of potassium (2%) and sodium (0.58%) in *Azadirachta indica* leaf (Ansari *et al*., 2012) is noteworthy. Ogbuewu *et al.* (2009) reported significant increase in potassium and a decrease in sodium sera levels, in rats treated with 5% neem leaf extract. An alteration in plasma and organ levels of sodium and potassium has also been reported in lead toxicity (Okediran *et al.,* 2019; Liu *et al.,* 2014), and a decrease in potassium: sodium ratio levels in diet have been attributed to the increased occurrence of hypertension in consumers of western diet (Houston *et al.,* 2008). It could be hypothesized that another mechanism by which MEAI reversed hypertension in this present study may be by restoring the altered plasma and organ levels of sodium and potassium in the treated rats. Increased P wave and QRS complex observed in the toxicant groups were not significant and were reduced insignificantly and in a few of the treated groups. A reduction in doxorubicin-induced increase in P wave and QRS complex by *Azadirachta indica* has previously been reported by Koul *et al.* (2014). The lead-induced increase in P wave and QRS complex, in addition to the lead-induced increased QT corrected bazette, were however not reversed by MEAI, suggesting the limitation of MEAI in reversing lead-induced cardiotoxicity. Lead-induced reduction in coronary blood flow rate has earlier been reported by Prentice and Kopp, (1985). The significant reduction in blood flow observed in the 0.1%PbAc and 0.2%PbAc groups might be linked to the reduction in the NO content observed, resulting in vasoconstriction. The blood vessels control the amount of blood that flows to a specific part of the body. The constriction of these vessels will cause resistance, hence, slowing down in the movement of blood through the vessels. It can also be associated with the reduction in blood volume observed in the same groups. This study reports a significant lead-induced reduction in blood volume. Although there is no previous report of lead toxicity causing hypovolaemia, this may be as a result of hemolysis which has been earlier reported by Corradi *et al.* (2011). MEAI increased both blood flow and blood volume in the 0.1%PbAc + 200 mg/kg MEAI, 0.2%PbAc + 100 mg/kg MEAI and 0.2%PbAc + 200 mg/kg MEAI groups, indicating its positive effect on hematology. This had earlier been insinuated by Ajayi *et al.* (2017), who reported an increase in the hematological parameters of broilers administered neem leaf in drinking water.

Inflammatory cells were reduced or absent in the heart and kidney tissues of the MEAI-treated groups as against the lead-exposed groups. The anti-inflammatory effect of neem leaf extract at 200mg/kg has been earlier reported by Chattopadhyay (1998) and Alzohairy (2016). Increasingly, research is pointing to oxidative stress and inflammation as major contributors to renal illnesses. Mitochondrial damage leading to the release of Cytochrome C and inflammation are also closely related (Myakala *et al.,* 2023). Cytochrome C has been shown to increase during inflammation and to reduce as healing progresses (Clayton *et al.,* 2021).

The stability of cells depends on apoptosis, or programmed cell death, which is linked to a range of illnesses, including several forms of cancer (Camp *et al.,* 2004) since a primary characteristic of cancer is its ability to avoid apoptosis. In mitochondria-mediated apoptosis, Cytochrome C is thought to be an important mediator and biomarker (Wen *et al.,* 2014). Cytochrome C expression is downregulated in the proliferation of neoplastic cells. Low expression levels of Cytochrome C have been linked to an advanced TNM stage and a bad prognosis, thereby showing Cytochrome C to be a tumor suppressor gene (Liu *et al.,* 2019). Extracts that induce the release of Cytochrome C, may, therefore, be adapted as cytotoxic drugs. This indicates that the better expression of Cytochrome C in the tissues of the MEAI-treated animals suggests that the extract used in this study could potentially act as a prophylactic or therapeutic agent in lead-induced neoplasms. On the other hand, over-expression of cytochrome C promotes apoptosis by the activation of caspase, thus suggestive of an ongoing cell death in the kidney tissues of the 0.2%Pb as well as the 0.1Pb + 100/200 mg/kg MEAI treated groups. This overexpression was not observed in the cardiac tissues. Increase in the expression of cytochrome C has also been linked to inflammation and reduction to the process of healing (Clayton *et al.,* 2021). The variation in the expression of cytochrome C in the Pb exposed and treated groups of both the cardiac and renal tissues conveys an alteration of inflammatory and healing processes on going in the exposed and treated tissues. The reduction in

inflammatory cells in the tissues of the MEAI-treated groups however, suggest that increased expression of cytochrome C in some of the treated tissues could be as a result of an initial on-going inflammatory process in these cells when they were exposed to lead acetate.

The antihypertensive, antioxidant, anti-inflammatory, and apoptotic actions of MEAI against lead-induced toxicity demonstrated in this study, point to its potential as an efficient antidote for lead-induced cardiorenal toxicity. Its use as a supplement or herbal preparation may therefore be employed in combating possible health challenges due to environmental toxicity.

REFERENCES

Abidin, Z. U., Hassan, H. U., Masood, Z., Rafique, N., Paray, B. A., Gabol, K., Shah, M. I. A., Gulnaz, A., Ullah, A., Zulfiqar, T. and Siddique, M. A. M. (2022). Effect of dietary supplementation of Neem, *Azadirachta indica* leaf extracts on enhancing the growth performance, chemical composition, and survival of rainbow trout, Oncorhynchus mykiss. Saudi J Biol Sci. 29(4): 3075-3081. 10.1016/j.sjbs.2022.01.046.

Ali, J., ur Rehman, I. and Bangash, J. A. (2022). Phytochemicals content and in-vitro antioxidant properties of *Azadirachta indica* seeds, leaves, and twigs prepared from different extraction techniques. IJEST 14(4): 12-20. Almeida Lopes, A. C., Silbergeld, E. K. Navas-Acien, A., Zamoiski, R., da Cunha Martins, A., Camargo, A. E. L., Urbano, M. R., Mesas, A. E. and Paoliello, M. M. B. (2017). Association between blood lead and blood pressure: a population-based study in Brazilian adults. Environ Health. 16(1): 27. 10.1186/s12940-017-0233-5.

Alonso, J. R., Cardellach, F., López, S., Casademont, J. and Miró, O. (2003). Carbon monoxide specifically inhibits cytochrome c oxidase of human mitochondrial respiratory chain. Pharmacol Toxicol. 93(3): 142–146. 10.1034/j.1600-0773.2003.930306.x.

Alzohairy, M. A. (2016). Therapeutic role of *Azadirachta indica* (neem) and their active constituents in diseases prevention and treatment. Evid Based Complement Alternat Med.: 7382506. 10.1155/2016/7382506

Ansari, J., Khan, S. H., ul Haq, A. and Yousaf, M. (2012). Effect of the levels of *Azadirachta indica* dried leaf meal as phytogenic feed additive on the growth performance and haemato-biochemical parameters in broiler chicks. J Appl Anim Res. 40: 336–345.

Armas-Padilla, M. C., Armas-Hernández, M. J., Sosa-Canache, B., Cammarata, R., Pacheco, B., Guerrero, J., Carvajal, A. R., Hernández-Hernández, R., Israili, Z. H. and Valasco, M. (2007). Nitric oxide and malondialdehyde in human hypertension. Am J Ther. 14(2): 172-176. 10.1097/01.pap.0000249914.75895.48.

Boik, J. (2021). Natural Compounds in Cancer Therapy. Princeton, Minn, USA: Oregon Medical Press. (2021).

Camp, R. L., Dolled-Filhart, M. and Rimm, D. L. (2004). X-tile: A new bio-informatics tool for biomarker assessment and outcome-based cut-point optimization. Clin Cancer Res. 10: 7252–7259. Chattopadhyay, R. R. (1998). Possible biochemical role mode of anti-inflammatory action of *Azadirachta indica* A. Juss. in rats. Indian J Exp Biol. 36(4): 418-420.

Chaves, J.O., de Souza, M.C., da Silva, L. C. Lacho-Perez, D., Torres-Mayanga, P. C., Machado, A. P., Forster-Carneiro, T. *et al*. (2020). Extraction of Flavonoids From Natural Sources Using Modern Techniques. Front Chem 8:2020. 10.3389/fchem.2020.507887.

Clayton, S.A., Daley, K. K., Macdonald, L., Fernandez-Vizarra, E. *et al*. (2021). Inflammation causes remodeling of mitochondrial cytochrome c oxidase mediated by the bifunctional gene C15orf48. Sci Adv 7(50): 1-19. Doi: 10.1126/sciadv.abl5182

Corradi, M., Goldoni, M., Sabbadini, F. and Mutti, A. (201). Intossicazione acuta da piombo: un caso singolare di anemia emolitica e colica saturnina [Acute lead poisoning: a singular case of hemolytic anemia and lead colic]. Med Lav. 102(3): 243-249. Cordero-

Mora, J. L., Martínez-Aispuro, J. A., Martínez García, J. A., Mendoza-Martínez, G. D., Sánchez-Torres, M. T. and Figueroa-Velasco, J. L. (2024). Neem (*Azadirachta indica* A. Juss) leaves as growth promoter in lambs' diets. Agro Productividad 17(2): 35-40. 10.32854/agrop.v17i2.2563

Elston, R. C. and Johnson, W. D. (2008). Basic biostatistics for geneticists and epidemiologists: A practical approach. John Wiley & Sons.

Farmand, F., Ehdaie, A., Roberts, C. K. and Sindhu, R. K. (2005). Lead-induced dysregulation of superoxide dismutases, catalase, glutathione peroxidase and guanylate cyclase. Env.res. 98(1): 33-39.

Ferreira O. and Pinho, S. P. (2012). Solubility of flavonoids in pure solvents. Ind Eng Chem Res. 51(18): 6586–6590. Doi: 10.1021/ie300211e.

Flora, G., Gupta, D. and Tiwari, A. (2012). Toxicity of lead: A review with recent updates. Interdiscip Toxicol. 5(2): 47-58. doi:10.2478/v10102-012-0009-2.

Galanakis, M., Goulas, V., Tsakona, S., Manganaris, G. A. and Gekas, V. (2013). A Knowledge Base for The Recovery of Natural Phenols with Different Solvents. Int J Food Prop. 16(2): 382-396. Doi: 10.1080/10942912.2010.522750

Gonick, H. C., Dinq, Y., Bondy, S. C., Ni, Z. and Vaziri, N. D. (1997). Lead-induced hypertension: interplay of nitric oxide and reactive oxygen species. Hypertension. 30(6): 1487-1492.

Habig, W. H., Pabst, M. J. and Jakoby, W. B. (1974). Glutathione S-transferases the first enzymatic step in mercapturic acid formation. Journal of Biological Chemistry. 249(22): 7130-7139.

Jack, A. A., Oghenesuvwe, O., Adewumi, M. K., Omojola, A. B., Adegbeye, M. J., Faniyi, T. O., Salem, A. Z. M., Elghandour, M. M. Y., Cuevas-Barragán, C. E., Barbosa-Pliego, A. and Ekanem, D. E. (2022). Conversion of Neem fruit biomass for rumen manipulation, meat fatty acid profile improvement of rams. Biomass Convers. Biorefin.14(10161): 1-10. 10.1007/s13399-022-02629-4.

Jack, A. A., Adewumi, M. K., Adegbeye, M. J., Ekanem, D. E., Salem, A. Z. M. and Faniyi, T.O. (2020). Growth promoting effect of water-washed neem (*Azadirachta indica* A. Juss) fruit inclusion in West African dwarf rams. Trop. Anim. Health Prod. 52(6): 3467-3474. 10.1007/s11250-020-02380-w.

Koul, A., Goyal, R. and Bharati, S. (2014). Protective effect of *Azadirachta indica* A. Juss against doxorubicin-induced cardiac toxicity in tumour bearing mice. Ind. J. Exp. Biol. 52(4): 323-331.

Liu, D. J., Wu, J., Liu, Y. Q., Ouyang, L. and Wang, Y. J. (2014). Effects of lead exposure on 18 elements in blood and excretions in rats. Beijing Da Xue Xue Bao Yi Xue Ban. 46(2): 232-236. Chinese. PMID: 24743812.

Liu, Z., Zhao, X., Zhang, L. and Pei, B. (2019). Cytochrome C inhibits tumor growth and predicts favorable prognosis in clear cell renal cell carcinoma. Oncol Lett. 18: 6026-6032. https://doi.org/10.3892/ol.2019.10989.

Lopez, A., Peixe, T. S., Mesas, A. E. and Paoliello, M. M. (2016). Lead Exposure and Oxidative Stress: A Systematic Review. Rev Environ Contam Toxicol. 236: 193-238. 10.1007/978-3-319-20013-2_3.

Maurya, R. P., Prajapat, M. K., Singh, V. P., Roy, M., Todi, R., Bosak, S., Singh, S. K., Chaudhary, S., Kumar, A. and Morekar, S. R. (2021). Serum Malondialdehyde as a Biomarker of Oxidative Stress in Patients with Primary Ocular Carcinoma: Impact on Response to Chemotherapy. Clin Ophthalmol. 15: 871-879. 10.2147/OPTH.S287747.

Misra, H. P. and Fridovich, I. (1972). The role of superoxide anion in the autoxidation of epinephrine and a simple assay for superoxide dismutase. J Biol Chem. 247(10): 3170-3175.

Myakala, K., Wang, X. X., Shults, N. V., Krawczyk, E., Jones, B. A., Yang, X. and Rosenberg, A. Z. (2023). NAD metabolism modulates inflammation and mitochondria function in diabetic kidney disease. J Biol Chem. 299(8): 104975.

Nakamura, K., Katafuchi, A., Shimamoto, S., Ogawa, G., Khandelwal, N., Tatsugawa, K., Fujita, Y., Ohtsuka, A. and D. Ijiri. (2022). Effects of a dried neem leaf extract on the growth performance, meat yield and meat quality in skeletal muscle of broiler chickens under high temperature conditions. Front. Anim. Sci. 3: 914772.

Obiefuna, I. and Young, R. (2005). Concurrent administration of aqueous *Azadirachta indica* (neem) leaf extract with DOCA-salt prevents the development of hypertension and accompanying electrocardiogram changes in the rat. Phytother Res. 19(9): 792-795.

O'Connor, D., Hou, D., Ok, Y. S. and Lanphear, B. P. (2020). The effects of iniquitous lead exposure on health. Nat Sustain. 3: 77-79.

Okediran, B. S., Sanusi, F. and Suleiman, K. Y. (2019). Electrolytes alteration in some organs due to lead exposure. Advances in clinical toxicology. Adv Clin Toxicol. 4(1): 000150.

Omóbòwálé, T. O., Oyagbemi, A. A., Ogunpolu, B. S., Ola-Davies, O. E., Olukunle, J. O., Asenuga, E. R. *et al*. (2019). Antihypertensive effect of polyphenol-rich fraction of *Azadirachta indica* on Nω-Nitro-L-arginine methyl ester-induced hypertension and cardiorenal dysfunction. Drug Res. 69: 12–22.

Omobowale, T. O., Oyagbemi, A. A., Adejumobi, O. A., Ugbor, F., Asenuga, E. R. and Ajibade, T. O. (2020). Antihypertensive effect Of methanol leaf extract Of *Azadirachta indica* is mediated through suppression of renal Caspase 3 expressions on Nω nitro l arginine methyl ester induced hypertension. Pharmacog Res. 12(4): 460-465.

Oyagbemi, A. A., Omobowale, T. O., Asenuga, E. R., Adejumobi, A. O., Ajibade, T. O., Ige, T. M. *et al*. (2017). Sodium fluoride induces hypertension and cardiac complications through generation of reactive oxygen species and activation of nuclear factor kappa beta. Environ Toxicol. 32 (2017), pp. 1089 1101.

Prentice, R. C. and Kopp, S. J. (1985). Cardiotoxicity of lead at various perfusate calcium concentrations: Functional and metabolic responses of the perfused rat heart. Toxicol Appl Pharmacol. 81(3)(1): 491-501. Doi: 10.1016/0041-008X(85)90420-X.

Ray, R. and Shah, A. M. (2005). NADPH oxidase and endothelial cell function. Clin Sci.(Lond) 109(3): 217-226. Rodriquez-Iturbe, N.D. and Vaziri, J. (2004). Herrera-Acosta, R.J. Johnson. Oxidative stress, renal infiltration of immune cells, and salt-sensitive hypertension: all for one and one for all. Am J Physiol Renal Physiol 286: 606–616.

Sinha, A. K. (1972). Colorimetric assay of Catalase. Anal. Biochem. 47: 389-394

Upasani, C. D., Khera, A. and Balaraman, R. (2001). Effect of lead with Vitamins E, C, or Spirulina on malondialdehyde: conjugated dienes and hydroperoxides in rats. Ind. J. Exp. Biol. 39: 70–74.

Varshney, R. and Kale, R. K. (1990). Effects of Calmodulin Antagonists on Radiation-induced Lipid Peroxidation in Microsomes. Int. J. Radiat. Biol. 58(5): 733-743.

Vaziri, N. D. (2012). CKD impairs barrier function and alters microbial flora of the intestine: a major link to inflammation and uremic toxicity. Curr Opin Nephrol Hypertens 21(6): 587-592. Vaziri, N. D.,

Ding, Y. and Ni, Z. (1999). Nitric oxide synthase expression in the course of lead-induced HTN. Am. Heart J. 34: 558–562. Vaziri, N. D. (2008). Mechanisms of lead-induced hypertension and cardiovascular disease. Am. J. Physiol. Heart Circ. Physiol. 295(2): 454-465.

Wen, Q., Zhang, X., Cai, J. and Yang, P. H. (2014). A novel strategy for real-time and in situ detection of cytochrome c and caspase-9 in Hela cells during apoptosis. Analyst. 139: 2499–2506.

Yarmohammadi, F., Mehri, S., Najafi, N., Salar, A. S. and Hosseinzadeh, H. (2021). The protective effect of *Azadirachta indica* (neem) against metabolic syndrome: A review. Iran J Basic Med Sci. 24(3): 280-292. Doi: 10.22038/ijbms.2021.48965.11218.