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ORIGINAL ARTICLE

Mitigation of diazinon-induced cardiovascular and renal dysfunction by gallic acid

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ABSTRACT

Studies of the link between environmental pollutants and cardiovascular dysfunction, neglected for decades, have recently provided new insights into the pathology and consequences of these killers. In this study, rats were divided into four groups, each containing 10 rats. The rats in group one served as controls and were administered normal saline, whereas the rats in group two were orally gavaged with 3 mg/kg of diazinon (DZN) alone for twenty one consecutive days. The rats in groups 3 and 4 were administered respective 60 mg/kg and 120 mg/kg gallic acid (GA) in addition to DZN for twenty one consecutive days. Exposure of rats to diazinon significantly ($p<0.05$) reduced the activities of superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione-S-transferase (GST) and reduced glutathione (GSH) content. Malondialdehyde, hydrogen peroxide (H_2O_2) and nitric oxide (NO) contents were also significantly ($p<0.05$) elevated following DZN exposure. DZN further caused a significant ($p<0.05$) decrease of heart rate and QT interval prolongation. Hematologic analysis revealed significant reduction ($p<0.05$) in packed cell volume (PCV), hemoglobin concentration (Hb), red blood cell (RBC) count, and total white blood cell count of rats administered only DZN. Observations in this study suggest a modulatory role of gallic acid in diazinon-induced anemia and associated cardiovascular dysfunction in rats. Treatment with gallic acid reversed the oxidative stress markers studied, increased the antioxidant defence system and reduced deleterious effects on hematological parameters in rats. Pathologic findings of the heart and kidney were also found to be lessened.

KEY WORDS: diazinon; gallic acid; heart; kidney; oxidative stress; cardiovascular dysfunction

Introduction

The use of and exposure to pesticides in developing countries is becoming increasingly uncontrollable, making the environment more dangerous to the living system (Mostafalou & Abdollahi, 2013). The increasing incidence of hypertension, coronary heart disease and renal failure has become distressing over the last two decades (Sexton & Salinas, 2014). There is a probability of positive correlation between cardiorenal dysfunction and environmental pollutants such as organophosphate (Hutcheson & Rocic, 2012). Exposure to pesticides can be environmental, occupational, intentional or suicidal in nature (Paudyal, 2008). Since exposure of humans and animals to these agents is inevitable, it constitutes a major global health risk (Kumar *et al.,* 2008).

Diazinon (DZN) is an organophosphate insecticide used extensively in agriculture for control of pests (Jafari *et al.,* 2012). It is used also in veterinary medicine for prophylactic and therapeutic management of infestation with ticks, mites and fleas. Unfortunately, DZN is highly toxic to man and animals. The primary mechanism of toxicity for DZN to animals involves inhibition of acetylcholinesterase (Kousba *et al.,* 2007). Induction of free radical production and oxidative stress was reported in vital organs of rats, including the brain, heart and spleen (Jafari *et al.,* 2012). DZN causes deleterious effects on hematological and serum biochemical parameters in rodents (Kalender *et al.,* 2005). Decreased red blood cell count, hemoglobin concentration and packed cell volume were reported in DZN treated mice (El-Shenawy *et al.,* 2009).

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Acute organophosphate poisoning (OP) was reported to increase creatinine kinase and lactate dehydrogenase levels accompanied by myocardial necrosis (Repplinger *et al.,* 2014, Juliana *et al.,* 2009). Toxicities associated with OP may manifest as sinus tachycardia, sinus bradycardia, hypertension, hypotension, impaired heart rate and force of contraction (Juliana *et al.,* 2009). ECG changes associated with OP include prolonged QTc interval, ST segment elevation, low amplitude T waves, and prolonged PR interval (Yusuf *et al.,* 2009). Suicidal use of OP was reported by Hulse *et al.* (2014) and OP compounds have been linked with the highest incidence of suicidal poisoning (Tsai *et al.,* 2014). The typical toxidrome in OP poisoning comprises salivation, lacrimation, urination, defecation, gastric cramps, emesis, collectively known as ´SLUDGE´ symptoms (Peter *et al.,* 2014). Furthermore, high frequency of neurologic manifestations involving parasympathetic hyperactivity due to the accumulated acetylcholine (Ach) resulting from acetyl cholinesterase (AChE) inhibition has been documented (Rastogi *et al.,* 2010).

Different treatment regimens for OP have been adopted for decades to circumvent, delay or prevent toxicities associated with OP, with timely intervention yielding positive results (Moon *et al.,* 2015; Iyer *et al.,* 2015; Sun *et al.,* 2015; Kumar *et al.,* 2014; Buckley *et al.,* 2005; Sungur & Güven, 2001). Buckley *et al.* (2011) reported standard treatment to involve administration of intravenous atropine and oxime to reactivate inhibited acetylcholinesterase.

The use of medicinal plants to ameliorate various disease conditions has gained global acceptance in the last two decades. Humans and animals are constantly exposed to reactive oxygen species (ROS) and reactive nitrogen species (RNS) that are generated daily by endogenous and exogenous sources (Bak and Weerapana, 2015). These dangerous species are generated via different mechanisms/reactions, including mitochondrial electron transport chain, NADPH-oxidases, xanthine oxidase, metal dependent and independent pathways. Furthermore, ROS and RNS can damage the DNA, RNA and proteins and therefore serve as mediators of many disease conditions (Phaniendra *et al.,* 2015; Kielland *et al.,* 2009). In contrast, antioxidants of natural or synthetic origin can quench ROS, RNS and their products, thereby maintaining balanced redox signalling. The imbalance in the production of ROS and the antioxidant defence system is known as oxidative stress (Valko *et al.,* 2006).

Gallic acid (GA) is a natural phenolic agent endowed with antioxidant, anti-inflammatory, anticancer, and free radical scavenging activities (Subramanian *et al.,* 2014; Cedó *et al.,* 2014; Liang *et al.,* 2014; Nair & Nair, 2013; Bak *et al.,* 2013; Verma *et al.,* 2013). Furthermore, orally administered antioxidant agents are currently under intensive scientific investigation for their roles in the amelioration and/or prevention of wide varieties of cardiovascular diseases (Faure *et al.,* 2007). However, few reports exist on the amelioration of DZN-induced hemotoxicity and cardiovascular dysfunction with orally administered antioxidants. This study was designed to evaluate the presumed protective effect of GA on DZN-induced cardiorenal dysfunction in an experimental animal model and to assess the mechanism of its action.

Materials and methods

Chemicals

Hydrogen peroxide (H_2O_2), hydrochloric acid, sulphuric acid, xylenol orange, sodium hydroxide, potassium iodide, reduced glutathione (GSH), potassium dichromate, O-dianisidine, sodium potassium tartrate, copper sulphate, glacial acetic acid, ethanol, sodium azide, 2-dichloro-4-nitrobenzene (CDNB), thiobarbituric acid (TBA), trichloroacetic acid, Ellman's reagent (DTNB), ammonium ferrous sulphate, and sorbitol were purchased from Sigma (St Louis, MO, USA). All other chemicals used were of analytical grade and obtained from British Drug Houses (Poole, Dorset, UK).

Care of animals

All the experimental animals received humane care according to the criteria outlined in the Guide for the Care and the Use of Laboratory Animals prepared by the National Academy of Science and published by the National Institute of Health. The ethics regulations were followed in accordance with national and institutional guidelines for the protection of the animals' welfare during experiments according to Public Health Service (PHS, 1996).

Experimental design

In this study, forty male Wistar rats with the weight range of 130–180 g were used. The rats were divided into four groups each containing 10 rats. Rats in group one served as controls and were administered normal saline, whereas rats in group two were orally gavaged with 3 mg/kg of diazinon (DZN) alone for twenty one consecutive days. The rats in groups 3 and 4 were co-administered 60 mg/kg and 120 mg/kg gallic acid (GA) with DZN for twenty one consecutive days. Rats were sacrificed 24 hours after the last administration.

Blood collection, serum preparation and hematological analysis

From each rat, fresh whole blood (5 ml) was collected through the retro-orbital venous plexus into heparinized bottles and 2 ml were used for hematological analysis. Hematological analysis was done according to the instruction manual of an automated hematology analyzer (MINDRAY 3000) for the determination of packed cell volume (PCV), hemoglobin concentration (Hb), red blood cell count (RBC), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), and white blood cell counts. The remaining three milliliters (3 ml) of blood were also collected into sterile tubes and left for about 30 minutes to clot. The clotted blood was thereafter centrifuged at 4000 rpm for 10 minutes. Serum was harvested into sample bottles and stored at –20 °C till the time of analysis. The animals were not anesthetized.

Cardiac and renal tissue preparation

The rats were sacrificed 24 hours after the last administration. The hearts and the kidney (left and right) were removed, rinsed in 1.15% KCl and homogenized in potassium phosphate buffer (0.1 M, pH 7.4), centrifuged at 12 000 *g* for 15 minutes to obtain the post mitochondria fraction (PMF). The obtained PMF of the heart and kidneys was subsequently stored at –20 °C until the time of use.

Biochemical assays

Determination of cardiac and renal catalase (CAT) activity

Catalase (CAT) activity was determined according to the method of Sinha (1972). Briefly, 1ml portion from the reaction mixture – 2 ml of H_2O_2 solution, 2.5 ml of 0.01 M potassium phosphate buffer (pH 7.0) – and 1 ml of properly diluted PMF was blown into 1 ml dichromate/acetic acid solution by a gentle swirl at room temperature at 60-second intervals for 3 times. The mixture was incubated in the water bath at 100°C for 10 minutes. The absorbance was read at 570 nm using distilled water as blank. One unit of CAT activity represents the amount of enzyme required to decompose 1 µmol of H_2O_2/minute.

Determination of cardiac and renal superoxide dismutase (SOD) activity

Superoxide dismutase (SOD) was determined by the method of Misra and Fridovich (1972) with modification from our laboratory (Oyagbemi *et al.,* 2015; Omobowale *et al.,* 2014), giving credence to the validation of the method. Briefly, 100 mg of epinephrine was dissolved in 100 ml distilled water and acidified with 0.5 ml concentrated hydrochloric acid. This preparation prevents oxidation of epinephrine and is stable for 4 weeks. 0.01 ml of cardiac or renal PMF was added to 2.5 ml 0.05 M carbonate buffer (pH 10.2) followed by the addition of 0.3 ml of 0.3 mM adrenaline. The increase in absorbance at 480 nm was monitored every 30 seconds for 150 seconds. One unit of SOD activity was given as the amount of SOD necessary to cause 50% inhibition of the auto-oxidation of adrenaline to adrenochrome during 1 minute.

Determination of cardiac and renal reduced glutathione (GSH)

The cardiac and renal reduced glutathione (GSH) was estimated by the method of Jollow *et al.* (1974). Briefly, 0.5 ml of 4% sulfosalicylic acid (precipitating agent) was added to 0.5 ml of PMF and centrifuged at 4000 rpm for 5 minutes. To 0.5 ml of the resulting supernatant, 4.5 ml of Ellman's reagent (0.04 g of DTNB in 100 ml of 0.1 M phosphate buffer, pH 7.4) was added. The absorbance was read at 412 nm against distilled water as blank.

Determination of cardiac and renal glutathione peroxidase (GPX) activity

The cardiac and renal glutathione peroxidase (GPx) activity was also measured according to Buetler *et al.* (1963). The reaction mixtures contained 0.5 ml of potassium phosphate buffer (pH 7.4), 0.1 ml of sodium azide, 0.2 ml of GSH solution, 0.1 ml of H_2O_2, 0.5 ml of PMF and 0.6 ml of distilled water. The mixture was incubated in the water bath at 37 °C for 5 minutes and 0.5 ml of trichloroacetic acid (TCA) was added and centrifuged at 4000 rpm for 5 mins. One ml of the supernatant was taken and 2 ml of K_2PHO_4 and 1 ml of Ellman's reagent were added. The absorbance was read at 412 nm using distilled water as blank.

Determination of cardiac and renal thiobarbituric acid reactive substances (TBARS)

Thiobarbituric acid reactive substance (TBARS) was quantified as malondialdehyde (MDA) in the cardiac and renal PMF. The MDA was determined according to the method of Varshney and Kale (1990) To 1.6 ml of Tris-KCl, 0.5 ml of 30% TCA, 0.4 ml of samples and 0.5 ml of 0.75% thiobarbituric acid (TBA) prepared in 0.2 M HCl were added. The reaction mixture was incubated in the water bath at 80 °C for 45 minutes, cooled on ice and centrifuged at 4000 rpm for 15 minutes. The absorbance was measured against a blank of distilled water at 532 nm. Lipid peroxidation in units/mg protein was calculated with a molar extinction coefficient of 1.56×10^5 M/Cm.

Measurement of cardiac and renal nitric oxide (NO) contents

Cardiac and renal nitric oxide (NO) was measured as described by Olaleye *et al.* (2007) by indirectly measuring the nitrite concentration. After incubation at room temperature for 20 minutes, the absorbance at 540 nm was measured by spectrophotometer. The concentration of nitrite in the sample was determined from a sodium nitrite ($NaNO_2$) standard curve and was expressed as µmol nitrite/mg protein.

Measurement of cardiac and renal hydrogen peroxide (H_2O_2) generation

Hydrogen peroxide generation was determined according to Woff (1994). To 2.5 ml of 0.1M potassium phosphate buffer (pH 7.4), 0.250 ml of ammonium ferrous sulphate (AFS), 0.1 ml of sorbitol, 0.1 ml of xylenol orange (XO), 0.025 ml of H_2SO_4 and 0.050 ml of cardiac or renal PMF was added. The mixture was mixed thoroughly by vortexing till it foamed and a light pink color of the reaction mixture was observed. The reaction mixture was subsequently incubated at room temperature for 30 minutes. The absorbance was assessed at 560 nm, using distilled water as blank. The hydrogen peroxide (H_2O_2) generated was extrapolated from the hydrogen peroxide standard curve.

Determination of serum aminotransferases (ALT and AST) and alkaline phosphatase (ALP)

The activities of aspartate aminotransferase (AST), alanine aminotransferase (ALT) and alkaline phosphatase (ALP) were determined according to Reitma and Frankel (1957), using the Randox Kit (Randox Laboratories Limited, UK).

Protein determination

Protein concentrations were determined as described by Gornal *et al.* (1949). Briefly, 1 ml of diluted serum was added to 3 ml of the biuret reagent. The reaction mixture was incubated at room temperature for 30 minutes. The mixture was thereafter read with a spectrophotometer

at 540 nm using distilled water as blank. The final value for total protein was extrapolated from the total protein standard curve.

Electrocardiogram

Standard lead II electrocardiogram was recorded in conscious rats using a 7-lead ECG machine (EDAN VE-1010, Shanghai, China). From the electrocardiogram, parameters such as heart rate, P-wave duration, PR-interval, QRS duration, R-amplitude, QT segment and Bazett's correction of the QT interval were determined.

Histopathology

Small pieces of heart and kidney tissues were collected in 10% formal saline buffer for proper fixation. These tissues were processed and embedded in paraffin wax. Sections of 5–6 µm in thickness were made and stained with hematoxylin and eosin for histopathological examination (Drury *et al.,* 1976).

Statistical analysis

All values are expressed as mean±S.D. One-way ANOVA with Dunnett's post-test was also performed using GraphPad Prism version 4.00. The level of statistical significance was considered as $p<0.05$.

Results

In this study, administration of DZN in the dose of 3mg/kg led to a significant ($p<0.05$) increase in cardiac malondialdehyde (MDA) content, hydrogen peroxide (H_2O_2) generated and nitric oxide (NO) level, whereas co-administration of DZN and GA reversed the given markers of oxidative and nitrosative stress (Table 1). Similarly, rats exposed to a toxic dose of DZN showed a significant ($p<0.05$) increase in renal and cardiac MDA content and hydrogen peroxide (H_2O_2) generation compared to controls (Tables 1 and 2). Nitric oxide (NO) level, however, increased significantly in renal tissues but it significantly decreased in cardiac tissues (Tables 1 and 2). Furthermore, renal markers of oxidative stress were significantly decreased following GA (60 & 120 mg/kg) co-administered with DZN.

Our data showed that both cardiac and renal glutathione (GSH) reduced significantly following DZN treatment (Tables 3 and 4). The enzymatic antioxidants glutathione peroxidase (GPx) and glutathione-S-transferase (GST) decreased significantly in both cardiac and renal tissues in rats treated with DZN alone (Tables 3 and 4). The administration of GA dose-dependently improved the antioxidant defence system and attenuated the elevated markers of oxidative stress. The activities of cardiac superoxide dismutase (SOD) and catalase (CAT) activity decreased significantly following administration of diazinon compared to the control group (Figures 1 and 2). In contrast, rats co-administered GA (60 and 120 mg/kg) showed significantly improved activity of SOD as compared to DZN treatment only (Figures 1 and 2). Similar results were obtained in renal tissues with a significant reduction in both SOD and CAT activity (Figures 3 and 4). The cardiac SOD responded less than did the renal SOD following treatment with GA (Figures 1 and 3). DZN treatment also significantly reduced the antioxidant activity of catalase (CAT) compared to the control, whereas co-administration of DZN with GA (60 and 120 mg/kg) led to a significant increase in cardiac and renal CAT activity (Figures 2 and 4).

Table 1. Effect of Gallic acid on Diazinon-induced cardiac oxidative stress (Mean±S.D.).

Experimental Groups (n=10)	Control	Diazinon (3 mg/kg)	Diazinon + Gallic acid (60 mg/kg)	Diazinon + Gallic acid (120 mg/kg)
[a]LPO	9.65±0.32	16.73±0.38*	7.60±0.43*,#	7.28±0.37*,#
[b]H_2O_2 generated	15.33±0.26	16.00±0.35*	15.41±0.14#	15.38±0.18#
[c]Nitric oxide (NO)	0.25±0.03	0.19±0.04*	0.19±0.02*,#	0.17±0.03*,#

Superscripts (*, #) indicate significant difference at $p<0.05$. Superscript (*) indicates significant difference when Diazinon (3 mg/kg), Diazinon (3 mg/kg) + Gallic acid (60 mg/kg) and Diazinon (3 mg/kg) + Gallic acid (120 mg/kg) are compared to the control. Superscript (#) indicates significant difference when Diazinon (3 mg/kg) + Gallic acid (60 mg/kg) and Diazinon (3 mg/kg) + Gallic acid (120 mg/kg) are compared to the Diazinon (3 mg/kg).
[a]MDA/LPO (Malondialdehyde; µmole of MDA formed/mg protein), [b]H_2O_2 (Hydrogen peroxide generation; µmole/mg protein), [c]NO (Nitric oxide; µmole/L)

Table 2. Effect of Gallic acid on Diazinon-induced renal oxidative stress (Mean±S.D.).

Experimental Groups (n=10)	Control	Diazinon (3 mg/kg)	Diazinon + Gallic acid (60 mg/kg)	Diazinon + Gallic acid (120 mg/kg)
[a]LPO	6.42±0.65	9.47±0.51*	8.04±0.91*,#	8.82±0.23*
[b]H_2O_2 generated	15.44±0.13	17.13±0.18*	15.94±0.24*	15.42±0.14#
[c]Nitric oxide (NO)	0.40±0.03	1.31±0.25*	0.88±0.05*,#	0.71±0.03*,#

Superscripts (*, #) indicate significant difference at $p<0.05$. Superscript (*) indicates significant difference when Diazinon (3 mg/kg), Diazinon (3 mg/kg) + Gallic acid (60 mg/kg) and Diazinon (3 mg/kg) + Gallic acid (120 mg/kg) are compared to the control. Superscript (#) indicates significant difference when Diazinon (3 mg/kg) + Gallic acid (60 mg/kg) and Diazinon (3 mg/kg) + Gallic acid (120 mg/kg) are compared to the Diazinon (3 mg/kg).
[a]MDA/LPO (Malondialdehyde; µmole of MDA formed/mg protein), [b]H_2O_2 (Hydrogen peroxide generation; µmole/mg protein), [c]NO (Nitric oxide; µmole/mg protein)

Table 3. Effect of Gallic acid on Diazinon on cardiac non-enzymic and enzymic antioxidant defence system (Mean±S.D.).

Experimental Groups (n=10)	Control	Diazinon (3 mg/kg)	Diazinon + Gallic acid (60 mg/kg)	Diazinon + Gallic acid (120 mg/kg)
[a]GSH	60.67±0.14	60.00±0.25*	61.17±0.29*,#	61.19±0.13*,#
[b]GPx	43.75±0.18	37.77±2.63*	38.43±1.48*	39.37±1.79*
[c]GST	0.16±0.008	0.13±0.006*	0.17±0.002*,#	0.14±0.011*,#

Superscripts (*, #) indicate significant difference at $p<0.05$. Superscript (*) indicates significant difference when Diazinon (3 mg/kg), Diazinon (3 mg/kg) + Gallic acid (60 mg/kg) and Diazinon (3 mg/kg) + Gallic acid (120 mg/kg) are compared to the control. Superscript (#) indicates significant difference when Diazinon (3 mg/kg) + Gallic acid (60 mg/kg) and Diazinon (3 mg/kg) + Gallic acid (120 mg/kg) are compared to the Diazinon (3 mg/kg).
[a]GSH (Reduced glutathione; µmole/mg protein), [b]GPx (Glutathione peroxidase; (units/mg protein), [c]GST (Glutathione S-transferase; mmole1-chloro-2, 4-dinitrobenzene-GSH complex formed/min/mg protein).

Table 4. Effect of Gallic acid on Diazinon on renal non-enzymic and enzymic antioxidant defence system (Mean±S.D.).

Experimental Groups (n=10)	Control	Diazinon (3 mg/kg)	Diazinon + Gallic acid (60 mg/kg)	Diazinon + Gallic acid (120 mg/kg)
[a]GSH	64.87±0.18	64.25±0.25*	64.33±0.29*	64.63±0.18#
[b]GPx	37.59±0.89	30.53±0.67*	33.18±1.01*,#	33.02±0.74*,#
[c]GST	0.209±0.031	0.131±0.012*	0.191±0.015#	0.194±0.008#

Superscripts (*, #) indicate significant difference at $p<0.05$. Superscript (*) indicates significant difference when Diazinon (3 mg/kg), Diazinon (3 mg/kg) + Gallic acid (60 mg/kg) and Diazinon (3 mg/kg) + Gallic acid (120 mg/kg) are compared to the control. Superscript (#) indicates significant difference when Diazinon (3 mg/kg) + Gallic acid (60 mg/kg) and Diazinon (3 mg/kg) + Gallic acid (120 mg/kg) are compared to the Diazinon (3 mg/kg).
[a]GSH (Reduced glutathione; µmole/mg protein), [b]GPx (Glutathione peroxidase; (units/mg protein), [c]GST (Glutathione S-transferase; mmole1-chloro-2, 4-dinitrobenzene-GSH complex formed/min/mg protein).

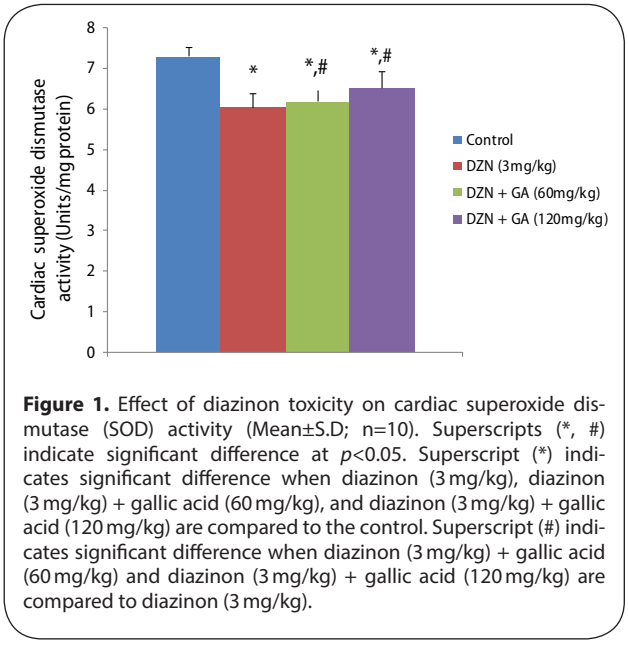

Figure 1. Effect of diazinon toxicity on cardiac superoxide dismutase (SOD) activity (Mean±S.D; n=10). Superscripts (*, #) indicate significant difference at $p<0.05$. Superscript (*) indicates significant difference when diazinon (3 mg/kg), diazinon (3 mg/kg) + gallic acid (60 mg/kg), and diazinon (3 mg/kg) + gallic acid (120 mg/kg) are compared to the control. Superscript (#) indicates significant difference when diazinon (3 mg/kg) + gallic acid (60 mg/kg) and diazinon (3 mg/kg) + gallic acid (120 mg/kg) are compared to diazinon (3 mg/kg).

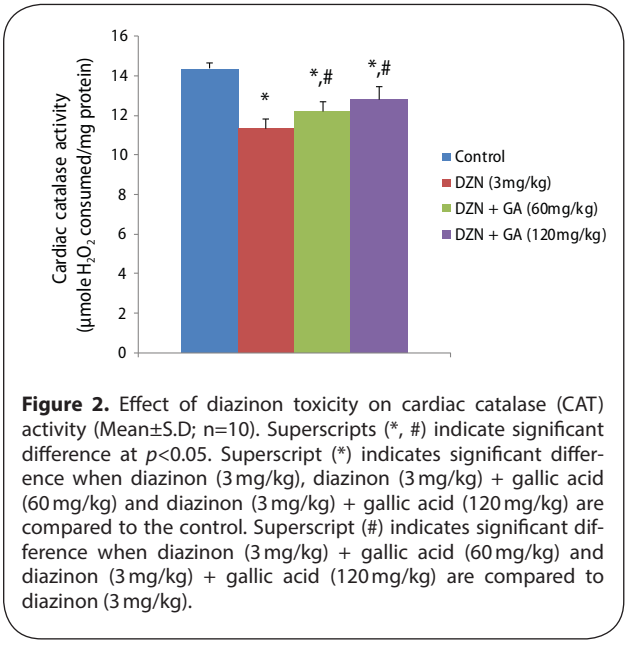

Figure 2. Effect of diazinon toxicity on cardiac catalase (CAT) activity (Mean±S.D; n=10). Superscripts (*, #) indicate significant difference at $p<0.05$. Superscript (*) indicates significant difference when diazinon (3 mg/kg), diazinon (3 mg/kg) + gallic acid (60 mg/kg) and diazinon (3 mg/kg) + gallic acid (120 mg/kg) are compared to the control. Superscript (#) indicates significant difference when diazinon (3 mg/kg) + gallic acid (60 mg/kg) and diazinon (3 mg/kg) + gallic acid (120 mg/kg) are compared to diazinon (3 mg/kg).

Aminotransferases and alkaline phosphatase

In addition, the administration of DZN led to a significant increase in serum alanine aminotransferase (ALT), aspartate aminotransferase (AST) and alkaline phosphatase (ALP), as shown in table 5. Treatment with GA (60 and 120 mg/kg) however significantly lowered these enzymes (ALT, AST and ALP) in rats compared to those on DZN alone and exhibited values close to normal compared to the control (Table 5). The toxicity of DZN was also demonstrated on cardiac and renal tissues. The toxic dose of DZN significantly increased the heart and kidney weight compared to the control and other treatment groups (Table 6).

Hematological parameters

In this study, values observed for the packed cell volume (PCV), hemoglobin concentration (Hb), and red blood cell (RBC) count, were significantly ($p<0.05$) lowered in rats administered DZN alone and in those that received DZN and 60 mg/kg GA combination, compared to the control

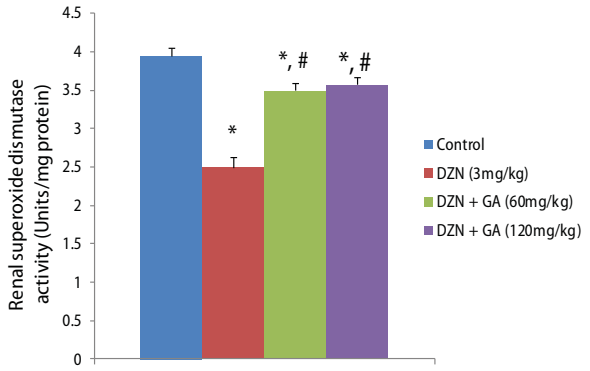

Figure 3. The effect of diazinon toxicity on renal superoxide dismutase (SOD) activity (Mean±S.D; n=10). Superscripts (*, #) indicate significant difference at $p<0.05$. Superscript (*) indicates significant difference when diazinon (3 mg/kg), diazinon (3 mg/kg) + gallic acid (60 mg/kg) and diazinon (3 mg/kg) + gallic acid (120 mg/kg) are compared to the control. Superscript (#) indicates significant difference when diazinon (3 mg/kg) + gallic acid (60 mg/kg) and diazinon (3 mg/kg) + gallic acid (120 mg/kg) are compared to diazinon (3 mg/kg).

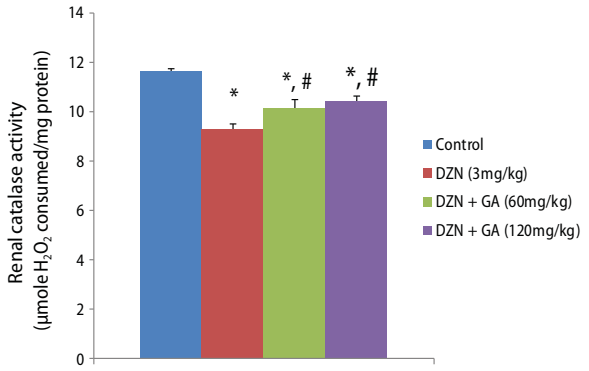

Figure 4. Effect of diazinon toxicity on renal catalase (CAT) activity (Mean±S.D; n=10). Superscripts (*, #) indicate significant difference at $p<0.05$. Superscript (*) indicates significant difference when diazinon (3 mg/kg), diazinon (3 mg/kg) + gallic acid (60 mg/kg) and diazinon (3 mg/kg) + gallic acid (120 mg/kg) are compared to the control. Superscript (#) indicates significant difference when diazinon (3 mg/kg) + gallic acid (60 mg/kg) and diazinon (3 mg/kg) + gallic acid (120 mg/kg) are compared to diazinon (3 mg/kg).

Table 5. Effect of Diazinon on serum aminotransferases and phosphatase (Mean±S.D.).

Experimental Groups (n=10)	Control	Diazinon (3 mg/kg)	Diazinon + Gallic acid (60 mg/kg)	Diazinon + Gallic acid (120 mg/kg)
ALT (U/L)	0.12±0.02	1.41±0.04*	0.58±0.07*, #	0.55±0.04*, #
AST (U/L)	161.77±1.99	175.28±2.60*	157.21±4.47#	121.42±7.31*, #
ALP (U/L)	289.80±19.52	358.80±39.03*	294.40±15.93#	266.80±31.87#

Superscripts (*, #) indicate significant difference at $p<0.05$. Superscript (*) indicates significant difference when Diazinon (3 mg/kg), Diazinon (3 mg/kg) + Gallic acid (60 mg/kg) and Diazinon (3 mg/kg) + Gallic acid (120 mg/kg) are compared to the control. Superscript (#) indicates significant difference when Diazinon (3 mg/kg) + Gallic acid (60 mg/kg) and Diazinon (3 mg/kg) + Gallic acid (120 mg/kg) are compared to the Diazinon (3 mg/kg).

Table 6. Effect of Diazinon on heart and kidney weight (Mean±S.D.).

Experimental Groups (n=10)	Control	Diazinon (3 mg/kg)	Diazinon + Gallic acid (60 mg/kg)	Diazinon + Gallic acid (120 mg/kg)
Heart weight (gram)	0.29±0.02	0.41±0.02*	0.35±0.03*, #	0.41±0.03#
Kidney weight (gram)	0.56±0.02	0.74±0.04*	0.77±0.03*	0.81±0.04#

Superscripts (*, #) indicate significant difference at $p<0.05$. Superscript (*) indicates significant difference when Diazinon (3 mg/kg), Diazinon (3 mg/kg) + Gallic acid (60 mg/kg) and Diazinon (3 mg/kg) + Gallic acid (120 mg/kg) are compared to the control. Superscript (#) indicates significant difference when Diazinon (3 mg/kg) + Gallic acid (60 mg/kg) and Diazinon (3 mg/kg) + Gallic acid (120 mg/kg) are compared to the Diazinon (3 mg/kg).

group. In contrast, reductions in the values of these hematological parameters were not significant in rats treated with 120 mg/kg GA and DZN. Furthermore, the PCV and hemoglobin concentration of these rats significantly increased relative to that of rats administered only DZN. Concerning hematometric indices, the following observations were also recorded: significant increase in the mean corpuscular volume (MCV) in rats administered only DZN and in rats with DZN and 60 mg/kg GA combination compared to the control group; significant decrease in MCV of rats administered DZN and 120 mg/kg, relative to that of rats administered DZN alone, and non-significant changes in the mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC), as shown in Table 7. The total white blood cell count (TWBC), lymphocyte and neutrophil counts of rats administered DZN alone was significantly ($p<0.05$) decreased relative to controls, but these changes were not significant in groups of rats administered 60 mg/kg and 120 mg/kg DZN. Further, lymphocyte and neutrophil count of these rats increased significantly compared to those of rats administered DZN alone.

ECG results

The ECG results showed that DZN treatment led to a significant reduction in heart rate (HR) compared to the

Table 7. Effect of Diazinon on haematological parameters of rats.

Parameter	Control	Diazinon	Diazinon+60 mg/kg GA	Diazinon+120 mg/kg
PCV %	38.42±5.39	31.59±1.22[a]	33.24±1.37[a]	34.07±2.22 [b*]
Hb g/dl	12.62±2.30	8.11±0.26[a]	8.11±1.00[a]	10.37±1.38 [b]
RBC × 10^{12}/l	7.90±1.01	5.37±1.44[a]	5.01±1.13[a]	6.84±1.00
MCV fl	48.63±3.12	60.40±2.03[a]	66.35±7.09[a]	49.81±1.08 [b]
MCH pg	15.97±1.02	15.51±0.95	16.19±1.44	15.16±0.27
MCHC g/dl	32.85±3.21	25.67±1.06	24.39±2.41	30.43±1.01
TWBC × 10^9/l	11.50±1.03	8.20±0.04[a]	10.19±2.34	10.53±1.62
Lymphocytes × 10^9/l	6.92±1.15	4.26±1.08[a]	6.03±0.21	6.11±0.23
Monocytes × 10^9/l	0.20±0.10	0.10±0.10	0.10±0.10	0.10±0.10
Neutrophils × 10^9/l	4.33±1.82	1.93±0.15[a]	4.22±0.13 [b]	3.92±1.08 [b]
Eosinophils × 10^9/l	0.10±0.01	0.10±0.01	0.10±0.01	0.10±0.01
Basophils × 10^9/l	0.01±0.00	0.01±0.00	0.01±0.00	0.01±0.00

[a]Significant as compared groups 2, 3 and 4 with group 1 (p<0.05)
[b]Significant as compared groups 3 and 4 with group 2 (p<0.05)

Table 8. Effect of Gallic acid on Diazinon-induced alteration of electrocardiograph (ECG) (Mean±S.D.).

Experimental Groups	Heart rate (rates/min)	P-Duration (ms)	PR (ms)	QRS (ms)	QT (ms)	QTc (ms)	R-Amplitude (mv)
Control	286±28.66	26.83±5.78	45.17±6.49	20.00±2.68	77.33±16.60	146.00±12.88	0.20±0.08
Diazinon (3 mg/kg)	231±18.88*	29.33±4.27	49.00±9.36	26.40±4.51*	83.33±11.81	173.25±15.97*	0.33±0.04*
Diazinon + Gallic acid (60 mg/kg)	245.75±5.12*, #	26.25±5.44	47.75±3.59	18.00±3.81#	79.00±9.76	150.33±9.61#	0.22±0.02#
Diazinon + Gallic acid (120 mg/kg)	241.25±7.59*	23.00±2.65	40.25±4.88#	19.75±1.71#	57.25±6.608, #	120.67±11.02*, #	0.24±.08#

Superscripts (*, #) indicate significant difference at p<0.05. Superscript (*) indicates significant difference when Diazinon (3 mg/kg), Diazinon (3 mg/kg) + Gallic acid (60 mg/kg) and Diazinon (3 mg/kg) + Gallic acid (120 mg/kg) are compared to the control. Superscript (#) indicates significant difference when Diazinon (3 mg/kg) + Gallic acid (60 mg/kg) and Diazinon (3 mg/kg) + Gallic acid (120 mg/kg) are compared to the Diazinon (3 mg/kg).

values obtained from control animals and rats administered GA (60 and 120 mg/kg) body weight (Table 8). Similarly, other ECG changes observed included prolongation of QRS, QT and QTc intervals as indicated by significant (p<0.05) increase in the values of the these parameters compared to GA treated rats and controls (Table 8).

Histopathology

The histology of the cardiac tissue showed mild infiltration of inflammatory cells in cardiomyocytes of the DZN treated group (Figure 5). However, administration of GA (60 and 120 mg/kg) reduced the level of cellular infiltration accompanied with no visible lesion on cardiomyocytes (Figure 5). This cellular infiltration might also indicate inflammation. A similar result was obtained from the histology of the kidney of rats exposed to DZN with renal tissue showing infiltration of the perivascular and interstitial spaces with infiltration of inflammatory cells (Figure 6). However, the renal tissues could not recover from the toxicity of DZN, as evidenced by the presence of infiltration of inflammatory cells with tubular degenerative changes (Figure 6). The glomeruli exhibited a normal appearance, with normal urinary space in rats that obtained DZN co-administered with GA (Figure 6).

Discussion

The increase in the incidence of organophosphate (OP) poisoning in developing countries can be attributed to lack or inadequate control, to ignorance, poverty and illiteracy. However, abuse and misuse of these killer agents cannot be underestimated. This work sought to understand the relationship between cardiovascular dysfunction and OP and the use of phytonutrients present in medicinal plants. Induction of oxidative stress has been implicated in the pathogenesis of OP (El-Demerdash *et al.,* 2014, Oksay *et al.,* 2013, Messarah *et al.,* 2013). Accumulating evidence is emerging on the benefits of antioxidants that reside in medicinal plants to quench free radicals and oxidative stress associated with OP poisoning (Kristofco *et al.,* 2015, Oğuzhanoğlu *et al.,* 2014, Razavi *et al.,* 2013, Hariri *et al.,* 2010).

NO has been extensively documented as a signalling molecule. It has been implicated both in physiology and

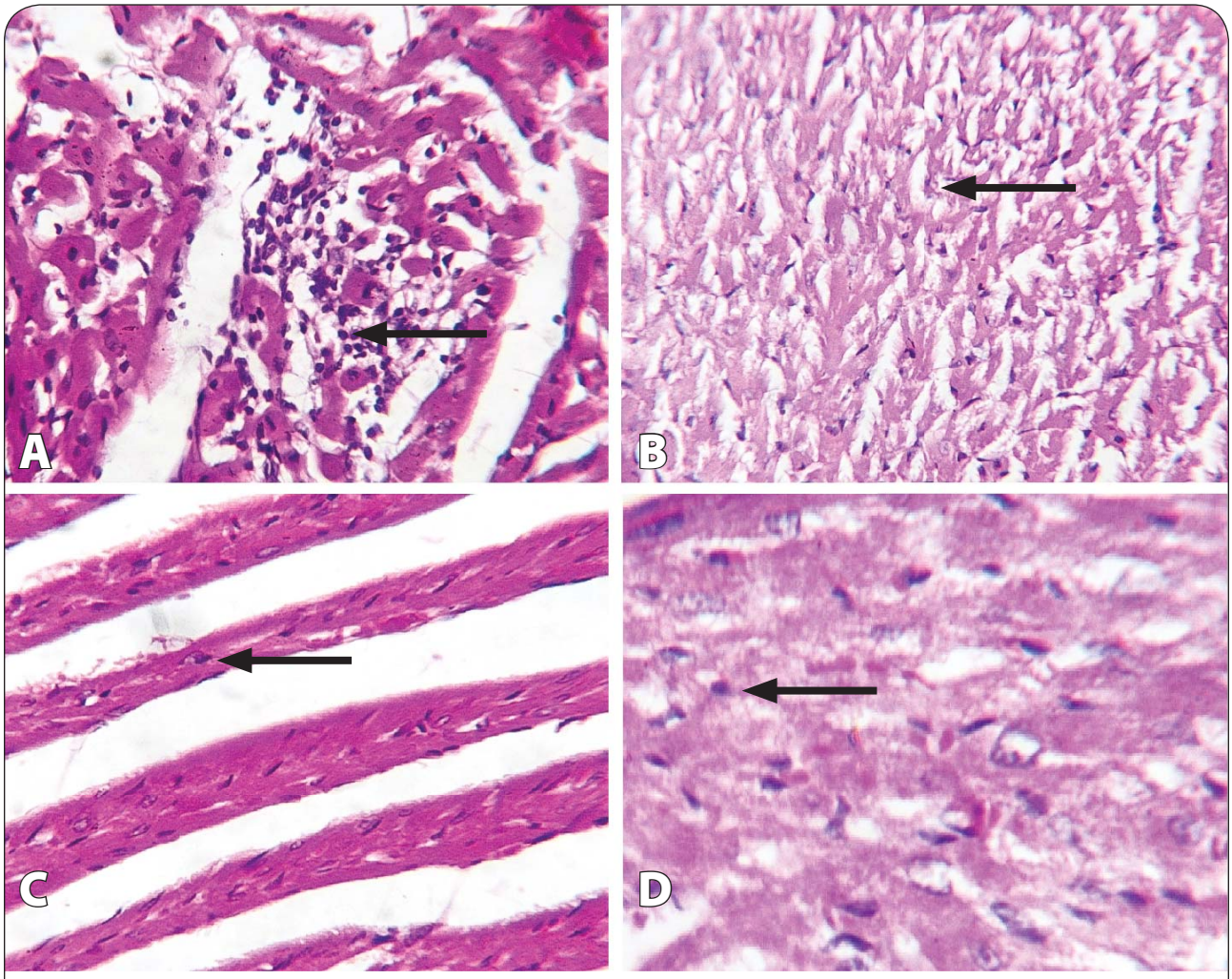

Figure 5. Group A (control) cardiac tissue shows mild infiltration of inflammatory cell (black arrow) into the myocardium. Group B (diazinon 3 mg/kg) indicates cardiac cell swelling (black arrow) of the cardiac tissue. Group C (diazinon 3 mg/kg + 60 mg/kg gallic acid) shows photomicrographs of cardiac tissue with no significant lesion. Similarly, rats in Group D (diazinon 3 mg/kg + 120 mg/kg gallic acid), show cardiac tissue with no significant lesion. Histologic slides were stained with hematoxylin & eosin (magnification X400).

pathology. Its low level was reported to elicit hypertension, whereas excessive production could also precipitate hypotension. Administration of DZN might cause overproduction of NO in renal tissues and this may be attributed to cardiovascular dysfunction. Surprisingly, administration of GA reversed the excessive production of NO and reduced the tissue level to near normal values.

In parallel, our findings showed that administration of DZN significantly reduced the activity of SOD, CAT, GPx, GST activities and GSH content. SOD is the first line of defence against free radical generation. SOD dismutates superoxide anion radical ($O_2^{\bullet -}$) to hydrogen peroxide (H_2O_2). The reduction in the activity of SOD by DZN will allow $O_2^{\bullet -}$ to accumulate and then to react with NO to generate peroxynitrite ($ONOO^-$), which is more reactive and damaging. $ONOO^-$ can damage DNA, RNA, lipids and proteins.

From this study, administration of DZN to rats led to significant elevation of MDA and H_2O_2 in both cardiac and renal tissues and of NO only in renal tissues. These are markers of oxidative and nitrosative stress. On balance, this is an indication of peroxidation of membrane phospholipid via oxidation. The increase in these markers in renal tissues has been associated with oxidative and nitrosative stress, which is an imbalance between generation of reactive oxygen or nitrogen species and the antioxidant defence system. This imbalance favors the production of ROS and RNS rather than the antioxidant defence system. Furthermore, we speculate that DZN enhanced the production of ROS and RNS, reducing thereby the *in vivo* antioxidant capacity in the heart and kidney, vital organs of the body. In parallel, the ROS and RNS generated by DZN could reduce the ability of the kidney to excrete sodium, thus facilitating sodium retention accompanied by hypertension. The functional capacity of the nephron could also be disrupted via reduction in the glomerular filtration rate (GFR) and enhancement of sodium retention. However, co-administration of GA was able to significantly reduce these markers of ROS and RNS. The ability of GA to quench these radicals is

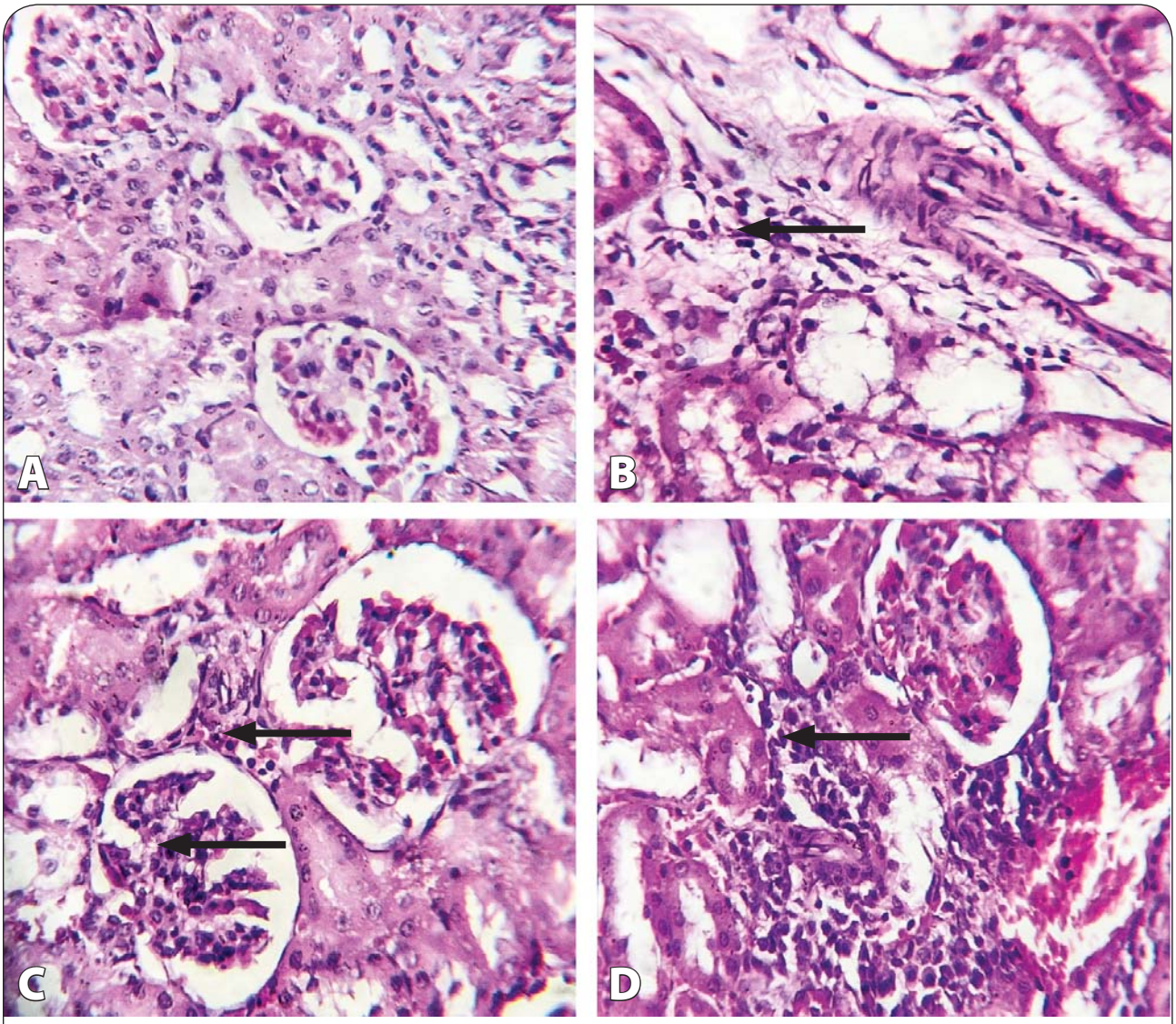

Figure 6. Group A (control) shows no visible lesion. Group B (diazinon 3 mg/kg) indicates renal tissue infiltration of the perivascular and interstitial spaces with inflammatory cells (black arrow). Group C (diazinon 3 mg/kg + 60 mg/kg gallic acid) shows the architecture of renal tissue with slight periglomerular inflammation and desquamation of the tubules, whereas in rats in Group D (diazinon 3 mg/kg + 120 mg/kg gallic acid), the renal tissue shows severe infiltration of inflammatory cells (black arrow) with tubular degenerative changes (black arrow). However, the glomeruli look normal with normal urinary space. Histologic slides were stained with hematoxylin & eosin (magnification X400).

an indication of its cardioprotective and renoprotective property. DZN also reduced myocardial contractility and precipitated left ventricular hypertrophy with resultant heart failure. These results thus corroborate the report of Cakici and Akat (2013), Boroushaki *et al.* (2013), Shah and Iqbal (2010), Gokel (2010), Wu *et al.* (2008), Stonys *et al.* (2007), and Agostini and Bianchin (2003) who reported renal failure and hepatic damage following DZN toxicity.

The $ONOO^-$ that is formed via reaction between $O_2^{\bullet -}$ and NO will reduce the bioavailability of NO and ultimately precipitate hypertension. We have also suggested that DZN could stimulate NADPH oxidase-mediated $O_2^{\bullet -}$ production. This pathway was found to be upregulated in hypertension, atherosclerosis and diabetes (Griendling *et al.,* 2000). Although, blood pressure was not recorded in this study, we hypothesized that reduction in the bioavailability of NO induced hypertension. NO is known to mediate vascular tone, prevent platelet activation, limit leukocyte adhesion to the endothelium, and regulate myocardial contractility. Furthermore, it plays a critical role in the pathogenesis of common cardiovascular disorders, including hypotension, hypertension, and atherosclerosis. Yet co-administration of GA with DZN improved the antioxidant capacity of cardiorenal SOD. Similarly, the activities of CAT and GPx were significantly inhibited following DZN treatment. CAT and GPx are enzymes involved in the second line of defence against ROS and RNS. CAT and GPx catalyze the conversion of H_2O_2 to H_2O and O_2. However, reduction in the activity of these enzymes will cause accumulation of H_2O_2. The accumulated H_2O_2 will disrupt the functional capacity of the heart and kidney with resultant vascular remodeling

and endothelial dysfunction. Thus our study exhibits important mechanisms that underlie the exposure to environmental pollutants such as DZN with a negative impact on the cardiovascular system. Cardiotoxicity associated with DZN toxicity was reported (Pizzurro *et al.,* 2014, Razavi *et al.,* 2014, Watson *et al.,* 2014, Razavi *et al.,* 2013). We further proposed a positive correlation between exposure to OP such as DZN and the incidence of hypertension/hypotension, heart failure, arteriosclerosis and renal failure. Karasu-Minareci *et al.* (2012) reported that OP poisoning could result in myocardial infarction. Interestingly, our data showed that GA administration reversed and increased the activity of CAT and GPx that appeared so far decline by the toxicity of DZN. GST is a phase 2 detoxification and antioxidant enzyme. The activity of GST and its co-factor GSH was reduced by DZN. In contrast, GA by its strong antioxidant capacity increased the activity of GST and GSH content of the heart and the kidney. GSH belongs to the intracellular antioxidant defence system. It is important to note that the ability of GA to increase GSH content might occur through increase in the novel synthesis of GSH. Other observable cardiotoxic effects of DZN in this study included reduction in heart rate accompanied with both QT QTc interval prolongations. Accumulating evidence has shown that QTc interval prolongation is a predictive indicator of the severity of poisoning and is also associated with cardiovascular collapse (Lin *et al.,* 2014; Liu *et al.,* 2012; Anand *et al.,* 2009; Yurumez *et al.,* 2009). However, administration of GA corrected the QT interval prolongation by reducing the QTc interval caused by DZN administration.

We also found that DZN caused increase in the heart and kidney weight. Co-administration of GA was able to reduce the size of the heart yet not of the kidney, as compared to the control, suggesting that the anti-inflammatory property of GA exerts a more profound effect on the heart than on the kidney. We may also speculate that the toxicity of DZN affects more the kidney and that the heart has the capacity to withstand the toxicity of DZN better than the kidney. On balance, DZN toxicity could also reduce cardiac output, thereby affecting normal cardiovascular function and renal perfusion.

An increase in alkaline phosphatase and in the aminotransferases ALT and particularly AST has been associated with cardiotoxicity. The elevation of this enzyme in blood or tissues is known to occur due to membrane breakdown and subsequent leakage into the systemic circulation. An elevated AST level in particular is a pointer of cardiac damage, suggesting that DZN disrupted the membranes covering the heart (pericardium) allowing AST to accumulate in blood. The above results suggest that GA provided a cardioprotective effect through improvement of the cardiorenal antioxidant defence system and its capacity to quench and scavenge ROS and RNS.

Baydin *et al.* (2007) reported a negative correlation between blood cholinesterase and QTc interval in patients with acute organophosphate poisoning.

In this study, the establishment of anemia in the group of rats administered DZN alone can be inferred from the significant reduction observed in the values of PCV, Hb, and RBC counts of this group compared to the control group. The significant increase in MCV and normal values of MCH and MCHC suggest macrocytic normochromic anemia. This anemia was effectively reversed in rats following co-administration of GA, especially at the 120 mg/kg dose. Observations in this study are similar to the reports of El-Shenawy *et al.* (2009) and Kalender *et al.* (2006) who reported amelioration of DZN-induced hemotoxicity following administration of vitamin E, an orally active antioxidant, in mice and rats. The ameliorative activity of GA on DZN-induced hemotoxicity observed in this study might be due to a direct inhibition of oxidative stress and free radical production in the erythocyte membrane. GA has been reported to suppress lipid peroxidation in erythrocytes (Nabavi *et al.,* 2013) and to increase the resistance of the erythrocyte membrane to hydrogen peroxide-induced peroxidation (Ramkumar *et al.,* 2014). Inhibition of lipid peroxidation may expectedly lead to amelioration of anemia since lipid peroxidation is an important step before cell membrane blebbing, hemolysis, and consequent shortening of the lifespan of erythrocytes (Betrosian *et al.,* 1995; Bowman *et al.,* 2005).

Another important component of the ameliorative potential of GA on DZN-induced anemia observed in this study was the restoration of reduced hemoglobin concentration to near normal values in rats in which DZN was co-administered to rats with 120 mg/kg body weight GA. Concomitant reduction in hemoglobin and red blood cell counts is sometimes attributable to the depressant effects of toxicants on hemopoietic organs, especially bone marrow, and interference with specific steps in the heme synthetic pathway (Ray, 1992). Thus, results from this study suggest the ability of GA to counteract or ameliorate the toxic effects of DZN on hemopoietic organs and processes. Moreover, the observed decrease in total white blood cell count, lymphopenia and neutropenia in rats administered DZN alone relative to that of the control group, suggests a depression of bone marrow. A potential anti-immunosuppressive effect of GA is reflected in this study by the significantly increased lymphocyte and neutrophil count of rats in which GA was co-administered with DZN, in contrast to those that received DZN only.

The study concludes that GA holds great promise in the amelioration of DZN-induced hemotoxicity and associated cardiovascular dysfunction. On balance then, inclusion of GA-rich feed supplements in diets of humans and animals potentially at risk of DZN exposure is advocated.

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Conflict of interest. *There is no conflict of interest.*

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