Temidayo Olutayo Omóbòwálé, Ademola Adetokunbo Oyagbemi*, Bukola Ayokunmi Alaba, Olufunke Eunice Ola-Davies, Olumuyiwa Abiola Adejumobi, Ebunoluwa Racheal Asenuga, Temitayo Olabisi Ajibade, Adeolu Alex Adedapo and Momoh Audu Yakubu

Ameliorative effect of *Azadirachta indica* on sodium fluoride-induced hypertension through improvement of antioxidant defence system and upregulation of extracellular signal regulated kinase 1/2 signaling

https://doi.org/10.1515/jbcpp-2017-0029
Received March 8, 2017; accepted July 23, 2017

Abstract

Background: Toxicities due to fluoride exposure from natural and industrial sources occur commonly in man and animals with severe consequences ranging from mild cardiac derangements to sudden death. In this study, we investigated the protective effects of the methanol extract of *Azadirachta indica* (AI) against sodium fluoride (NaF)-induced hypertension and genotoxicity in rats.

Methods: Sixty rats were divided into six groups of ten rats each as follows: Group A, the control group received distilled water; Group B rats were administered NaF at 600 ppm in drinking water; Groups C and D rats were pre-treated with the methanol extract of AI and thereafter administered NaF at 600 ppm in drinking water for 7 consecutive days; Groups E and F rats were co-administered with AI and NaF.

Results: The administration of NaF caused significant ($p < 0.05$) increases in the blood pressure, markers of oxidative stress, serum myeloperoxidase, xanthine oxidase values in NaF-alone treated rats, compared with the control. Significant ($p < 0.05$) decreases were observed in cardiac and renal antioxidant defence system in rats administered NaF alone compared with the control group. NaF treatment also resulted in a reduction in the expressions of extracellular signal-regulated kinase (ERK) 1/2 in cardiac and renal tissues of NaF-treated rats. Moreover, NaF treatment elicited an increase in the frequency of micronucleated polychromatic erythrocytes when compared with the control group.

Conclusions: This study shows the protective effect of AI on NaF-induced hypertension and genotoxicity through antioxidant and ERK 1/2 signaling in rats.

Keywords: *Azadirachta indica* (AI); chemoprevention; genotoxicity; hypertension; oxidative stress.

***Corresponding author: Dr. Ademola Adetokunbo Oyagbemi,** DVM, PhD, FCVSN, Faculty of Veterinary Medicine, Department of Veterinary Physiology and Biochemistry, University of Ibadan, Ibadan, Nigeria, Phone: +234833639776, Fax: +28103043, E-mail: ademola.oyagbemi778@gmail.com; aa.oyagbemi@ui.edu.ng
Temidayo Olutayo Omóbòwálé, Bukola Ayokunmi Alaba and Olumuyiwa Abiola Adejumobi: Faculty of Veterinary Medicine, Department of Veterinary Medicine, University of Ibadan, Ibadan, Nigeria
Olufunke Eunice Ola-Davies and Temitayo Olabisi Ajibade: Faculty of Veterinary Medicine, Department of Veterinary Physiology and Biochemistry, University of Ibadan, Ibadan, Nigeria
Ebunoluwa Racheal Asenuga: Faculty of Veterinary Medicine, Department of Veterinary Physiology, Biochemistry and Pharmacology, University of Benin, Benin, Nigeria
Adeolu Alex Adedapo: Faculty of Veterinary Medicine, Department of Veterinary Pharmacology and Toxicology, University of Ibadan, Ibadan, Nigeria
Momoh Audu Yakubu: Department of Environmental and Interdisciplinary Sciences, College of Science and Technology, Vascular Biology Unit, Center for Cardiovascular Diseases, College of Pharmacy, Texas Southern University, Houston, TX, USA

Introduction

Fluoride in the environment originates from both natural and industrial sources [1]. When absorbed, fluoride is rapidly distributed via the systemic circulation into the intracellular and extracellular water of tissues [2]. The presence of fluoride in tissues is associated with both structural changes and disorders of function [3]. Occasionally, acute fluoride intoxication may result in sudden cardiac death and the mechanism is thought to result from profound hypocalcemia by the precipitation of calcium fluoride salt [4]. Chronic fluorosis which results from the excessive ingestion of fluoride over a long period of time is prevalent in many areas of the world and it endangers both human and animal health [5]. As exposure to fluoride has been shown to cause excessive production of reactive oxygen species (ROS) and a resultant disruption in the antioxidant defence system [6], the use of products with antioxidant properties may be beneficial in ameliorating fluoride-induced toxicity.

Azadirachta indica (AI) is a plant with a wide variety of clinical applications in traditional medicine [7]. Its extracts from young flowers and leaves also have strong antioxidant potential [8]. Administration of aqueous extract of neem along with deoxycorticosterone acetate salt prevented the development of hypertension in rats [9]. Also, the administration of the mature leaf extract of AI decreased serum cholesterol significantly without changing serum protein, protein urea, and uric acid level in rats [10]. Omobowale et al. recently demonstrated the antioxidant and cardioprotective effects of AI [11, 12]. In this study, we investigated the probable effects of AI on sodium fluoride (NaF)-induced cardiorenal dysfunction and genotoxicity in rats.

Materials and methods

Collection, identification, and extraction of plant material

Azadirachta indica leaves were botanically identified and authenticated at the Herbatium of the Department of Botany, University of Ibadan, Nigeria. The voucher number UIH-22527 was assigned. Fresh leaves of AI were cleaned, air-dried, and crushed into coarse powder using an electric blender. The powdered leaf was soaked in n-hexane for 24 h and agitated at intervals and then filtered. The ground leaves were afterwards soaked in methanol for 72 h and agitated at intervals. The mixture was filtered thereafter and the filtrate was concentrated at 37 °C using a rotary evaporator to give a residue methanol extract of AI.

Experimental animals

Some 60 male Wistar rats weighing 120–250 g were obtained from the animal house of the Faculty of Veterinary Medicine, University of Ibadan and housed in well-ventilated cages. The rats were fed with rat chow produced and water was provided ad libitum. The rats were subjected to a natural photoperiod of about 12 h light and 12 h darkness daily. The animals were acclimatized for 7 days prior to the commencement of the experiment. The protocols used were in conformity with the guidelines of the National Institute of Health for Laboratory Animal Care and Use [13].

Experimental protocol

The animals were randomly divided into six experimental groups each containing 10 rats, as follows: Group A (control) was administered distilled water for 2 weeks; Group B was administered NaF at 600 ppm (600 mg/L) in drinking water for 7 days; Group C was pre-treated with 100 mg/kg AI orally for 7 days and then given NaF at 600 ppm (600 mg/L) in drinking water for 7 days; Group D was pre-treated with 200 mg/kg AI orally for 7 days and then given NaF at 600 ppm (600 mg/L) in drinking water for 7 days; Group E was co-administered with both 100 mg/kg AI and NaF at 600 ppm (600 mg/L) in drinking water for 7 days; Group F was co-administered and treated with both 200 mg/kg AI and NaF at 600 ppm (600 mg/L) in drinking water for 7 days.

Blood pressure measurement

Indirect blood pressure was taken in conscious rats by tail plethysmography using an automated blood pressure monitor (CODA S1, Kent Scientific Corporation, CT, USA). The average of at least nine readings was taken in the quiescent state following acclimatization. The systolic blood pressure (SBP), diastolic blood pressure (DBP), and mean arterial pressure (MAP) values were recorded for each rat.

Electrocardiography

Standard lead II electrocardiogram was recorded in conscious rats using a seven-lead ECG machine (EDAN VE-1010, Shanghai, China). The machine was calibrated at 20 mm/mV amplitude and 50 mm/s paper speed. From the electrocardiogram, parameters such as heart rate, QRS duration, QT segment, and Bazett's correction of the QT interval were determined.

Preparation of serum and tissue samples for biochemical assays

The blood samples were then centrifuged at 400 rpm for 10 min. The serum was collected and stored in the refrigerator at 4 °C. The heart and kidney tissues were then minced with scissors in four volumes of ice-cold 0.1 M phosphate buffer, pH 7.4, and homogenized. The resultant homogenates were centrifuged at 10,000 *g* at 4 °C for 15 min. The cardiac and renal post mitochondrial fractions (PMFs) were collected and processed for biochemical assays.

Biochemical assays

The protein concentrations of the samples obtained from individual rats were determined by Gornal et al. [14]. The activity of superoxide dismutase (SOD) was determined by measuring the inhibition of auto-oxidation of epinephrine at pH 10.2 at 30 °C as described by Misra and Fridovich [15] with slight modification [16, 17]. Glutathione peroxidase activity was analyzed by the method of Beutler et al. [18]. Lipid peroxidation was determined by measuring the formation of thiobarbituric acid reactive substances according to the method of Varshney and Kale [19]. Levels of hydrogen peroxide in the samples were determined using the method described by Woff [20]. The activity of catalase was determined using the method of Sinha [21]. The production of NO was evaluated by measuring the level of nitrite, an indicator of NO, as described by Crespo et al. [22]. The sulfhydryl (total thiol) and non-protein thiol (NPT) content were determined as described by Ellman [23]. The activity of xanthine oxidase was determined according to the method of Akaike et al. [24] while serum

myeloperoxidase activity was determined according to the method of Xia and Zweier [25].

Genotoxicity

This was done using *in vivo* micronucleus assay technique as described [26]. The proximal ends of the femurs were carefully removed with a pair of scissors until a small opening to the marrow became visible. The femur was submerged in fetal calf serum and the marrow was flushed out gently by aspiration and flushing on glass slides. The marrow suspension was positioned on one end of a slide and spread by pulling the material behind polished cover glass held at an angle of 45°. Slides were fixed in methanol for 3–5 min; allowed to dry for 24 h and later stained with May-Gruenwald and later with 5% diluted Giemsa solution for at least 30 min. Slides were then rinsed in phosphate buffer for about 30 s and in distilled water. Slides were then air-dried. The dried stained slides were then mounted in DPX with coverslips. They were later viewed under the microscope at 100× magnification using oil immersion for the presence of micronucleated polychromatic erythrocytes (MNPCE). Scoring was done using a tally counter.

Immunohistochemistry of extracellular regulated kinase 1/2

Immunohistochemistry of paraffin-embedded tissue of the heart and kidneys was performed after the tissues were obtained from buffered formalin perfused rats as earlier described by Oyagbemi et al. [27].

Statistical analysis

All values were expressed as mean ± SD. The test of significance between two groups was estimated by Student's *t* test. One-way ANOVA with Dunnett's post-test was also performed using Graph Pad Prism version 4.00.

Chemicals and reagents

1,2 Dichloro 4 nitrobenzene, thiobarbituric acid (TBA), trichloroacetic acid (TCA), $CoCl_2$, sodium hydroxide, xylenol orange (XO), potassium hydroxide, reduced glutathione (GSH), O dianisidine, and hydrogen peroxide (H_2O_2) were purchased from Sigma (St. Louis, MO, USA). Normal goat serum, Biotinylated antibody and Horse Radish Peroxidase (HRP) System was purchased from KPL, Inc. (Gaithersburg, MD, USA). ERK1/2 antibodies were purchased from Bioss Inc. (Woburn, MA, USA) while 3,3′-diaminobenzidine (DAB) tablets were purchased from AMRESCO LLC. (OH, USA). All other chemicals used were of analytical grade and were obtained from British Drug Houses (Poole, Dorset, UK).

Results

In this study, NaF caused a significant ($p < 0.05$) increase in markers of oxidative stress (MPO – myeloperoxidase, XO, NO, malondialdehyde – MDA, and H_2O_2) in both cardiac and renal tissues. This was, however, reversed by both pre-treatment and co-treatment with AI (Tables 1–4). Similarly, a decline in the antioxidant defence system in cardiac and renal tissues of rats treated with NaF alone was observed when compared with the controls (Table 5). Significant ($p < 0.05$) decreases in the levels of nonenzymic antioxidants GSH, PT, and NPT (Tables 6–8) as well the activities of SOD, CAT, and GPx (Tables 9–11) were

Table 1: The effect of *Azadirachta indica* on serum myeloperoxidase (MPO) activity of NaF-treated rats.

Treatment group	MPO, µmol/mg protein
A (Control)	2.9 ± 0.1
B (NaF)	7.6 ± 0.9[a]
C (AI_1 + NaF)	3.2 ± 0.5[b]
D (AI_2 + NaF)	2.9 ± 0.6[b]
E (AI_3 + NaF)	4.9 ± 0.4[a,b]
F (AI_4 + NaF)	4.1 ± 0.9[a,b]

The results are shown as mean ± SD for each group of 10 rats per group. [a]$p < 0.05$ when compared with control group. [b]$p < 0.05$ when compared with NaF-treated group.

Table 2: The effect of *Azadirachta indica* on serum nitric oxide (NO) level of NaF-treated rats.

Treatment group	NO, µmol/mg protein
A (Control)	0.04 ± 0.02
B (NaF)	0.01 ± 0.00[a]
C (AI_1 + NaF)	0.04 ± 0.02[b]
D (AI_2 + NaF)	0.03 ± 0.01[b]
E (AI_3 + NaF)	0.04 ± 0.01[b]
F (AI_4 + NaF)	0.04 ± 0.02[b]

The results are shown as mean ± SD for each group of 10 rats per group. [a]$p < 0.05$ when compared with control group. [b]$p < 0.05$ when compared with NaF-treated group.

Table 3: The effect of *Azadirachta indica* on serum xanthine oxidase activity.

Treatment group	XO, µmol/mg protein
A (Control)	0.10 ± 0.00
B (NaF)	0.26 ± 0.02[a]
C (AI_1 + NaF)	0.21 ± 0.01[a,b]
D (AI_2 + NaF)	0.21 ± 0.02[a,b]
E (AI_3 + NaF)	0.16 ± 0.00[a]
F (AI_4 + NaF)	0.17 ± 0.00[a,b]

The results are shown as mean ± SD for each group of 10 rats per group. [a]$p < 0.05$ when compared with control group. [b]$p < 0.05$ when compared with NaF-treated group.

Table 4: The effect of *Azadirachta indica* on malondialdehyde (MDA) levels in cardiac and renal tissues of NaF-treated rats.

Treatment group	MDA, µmol MDA formed/mg protein	
	Heart	Kidney
A (Control)	3.2 ± 0.7	1.1 ± 0.1
B (NaF)	8.7 ± 0.7[a]	3.5 ± 0.4[a]
C (AI_1 + NaF)	4.9 ± 0.07[a,b]	1.6 ± 0.2[a,b]
D (AI_2 + NaF)	4.6 ± 0.3[a,b]	2.1 ± 0.6[a,b]
E (AI_3 + NaF)	5.3 ± 0.7[a,b]	2.4 ± 0.3[a,b]
F (AI_4 + NaF)	5.4 ± 0.4[a,b]	1.9 ± 0.1[a,b]

The results are shown as mean ± SD for each group of 10 rats per group. [a]$p < 0.05$ when compared with control group. [b]$p < 0.05$ when compared with NaF-treated group.

Table 5: The effect of *Azadirachta indica* on hydrogen peroxide levels generated in the cardiac and renal tissues of the experimental rats.

Treatment group	H_2O_2, µmol/mg protein	
	Heart	Kidney
A (Control)	12.8 ± 0.1	19.4 ± 0.8
B (NaF)	14.9 ± 0.9[a]	22.4 ± 0.2[a]
C (AI_1 + NaF)	13.6 ± 0.1[a,b]	17.8 ± 0.9[a,b]
D (AI_2 + NaF)	13.2 ± 0.9[b]	19.1 ± 0.7[b]
E (AI_3 + NaF)	13.5 ± 0.5[a,b]	17.7 ± 1.0[a,b]
F (AI_4 + NaF)	13.5 ± 0.5[a,b]	18.0 ± 1.0[a,b]

The results are shown as mean ± SD for each group of 10 rats per group. [a]$p < 0.05$ when compared with control group. [b]$p < 0.05$ when compared with NaF-treated group.

Table 6: The effect of *Azadirachta indica* on protein thiol (PT) content in cardiac and renal tissues of NaF-treated rats.

Treatment group	PT, µmol/mg protein	
	Heart	Kidney
A (Control)	34.7 ± 7.9	66.9 ± 77
B (NaF)	23.2 ± 3.6[a]	56.1 ± 2.3[a]
C (AI_1 + NaF)	34.0 ± 4.6[b]	70.0 ± 13.1[b]
D (AI_2 + NaF)	38.3 ± 5.5[b]	76.3 ± 10.0[a,b]
E (AI_3 + NaF)	24.3 ± 1.9[a]	72.8 ± 11.6[b]
F (AI_4 + NaF)	32.4 ± 2.8[b]	77.4 ± 11.3[a,b]

The results are shown as mean ± SD for each group of 10 rats per group. [a]$p < 0.05$ when compared with control group. [b]$p < 0.05$ when compared with NaF-treated group.

Table 7: The effect of *Azadirachta indica* on cardiac and renal non-protein thiol (NPT) contents.

Treatment group	NPT, µmol/protein Heart	NPT, µmol/mg protein Kidney
A (Control)	100.2 ± 5.4	107.8 ± 9.2
B (NaF)	91.6 ± 0.8[a]	100.6 ± 6.2
C (AI_1 + NaF)	129.9 ± 9.3[a,b]	111.1 ± 2.0[b]
D (AI_2 + NaF)	114.3 ± 5.3[a,b]	98.7 ± 1.4[a]
E (AI_3 + NaF)	110.5 ± 5.1[a,b]	97.8 ± 4.8[a]
F (AI_4 + NaF)	121.3 ± 8.1[a,b]	96.0 ± 2.4[a]

The results are shown as mean ± SD for each group of 10 rats per group. [a]$p < 0.05$ when compared with control group. [b]$p < 0.05$ when compared with NaF-treated group.

Table 8: The effect of *Azadirachta indica* on reduced glutathione (GSH) levels in cardiac and renal tissues of NaF-treated rats.

Treatment group	GSH, µmol/mg protein	
	Heart	Kidney
A (Control)	14.6 ± 0.6	15.6 ± 0.5
B (NaF)	13.4 ± 0.7[a]	13.0 ± 0.7[a]
C (AI_1 + NaF)	17.2 ± 0.8[a,b]	15.0 ± 0.8[b]
D (AI_2 + NaF)	14.0 ± 0.6[b]	14.6 ± 0.2[a,b]
E (AI_3 + NaF)	14.8 ± 0.5[b]	16.0 ± 0.0[b]
F (AI_4 + NaF)	16.3 ± 1.3[a,b]	13.6 ± 1.0[a]

The results are shown as mean ± SD for each group of 10 rats per group. [a]$p < 0.05$ when compared with control group. [b]$p < 0.05$ when compared with NaF-treated group.

Table 9: The effect of *Azadirachta indica* on superoxide dismutase (SOD) activity in cardiac and renal tissues of NaF-treated rats.

Treatment group	SOD, units/mg protein	
	Heart	Kidney
A (Control)	1.04 ± 0.4	1.45 ± 0.1
B (NaF)	0.39 ± 0.2[a]	0.87 ± 0.3[a]
C (AI_1 + NaF)	1.32 ± 0.1[b]	1.45 ± 0.1[b]
D (AI_2 + NaF)	1.27 ± 0.1[b]	1.42 ± 0.1[b]
E (AI_3 + NaF)	1.53 ± 0.2[a,b]	1.29 ± 0[b]
F (AI_4 + NaF)	1.26 ± 0.4[b]	1.46 ± 0[b]

The results are shown as mean ± SD for each group of 10 rats per group. [a]$p < 0.05$ when compared with control group. [b]$p < 0.05$ when compared with NaF-treated group.

observed. Also, the decline in the activities of these antioxidant enzymes was reversed by both pre-treatment and co-treatment with AI.

Genotoxicity shown by an increase in the frequency of MNPCE relative to the controls was observed in NaF-treated rats. This was, however, ameliorated by pre-treatment with AI at 100 and 200 mg/kg as well as co-treatment at 200 mg/kg as indicated in Table 12.

A significant ($p < 0.05$) increase in blood pressure parameters (SBP, DBP, and MAP) was observed in NaF-only treated rats relative to the controls. However, a significant ($p < 0.05$) decrease in SBP was observed with

Table 10: The effect of *Azadirachta indica* on catalase activity in cardiac and renal tissues of NaF-treated rats.

Treatment group	CAT, µmol of H_2O_2 consumed/min/mg protein	
	Heart	Kidney
A (Control)	1.34 ± 0.4	6.7 ± 1.0
B (NaF)	0.97 ± 0.6[a]	1.2 ± 0.4[a]
C (AI_1 + NaF)	3.2 ± 0.9[a,b]	2.7 ± 1.2[a,b]
D (AI_2 + NaF)	2.4 ± 0.5[a,b]	7.5 ± 0.6[b]
E (AI_3 + NaF)	3.5 ± 2.5[a,b]	3.8 ± 0.8[a,b]
F (AI_4 + NaF)	3.7 ± 2.2[b]	2.6 ± 0.9[a,b]

The results are shown as mean ± SD for each group of 10 rats per group. [a]$p < 0.05$ when compared with control group. [b]$p < 0.05$ when compared with NaF-treated group.

Table 11: The effect of *Azadirachta indica* on glutathione peroxidase (GPx) activity in cardiac and renal tissues of NaF-treated rats.

Treatment group	GPx, µmoles/mg protein	
	Heart	Kidney
A (Control)	220.7 ± 0.2	216.1 ± 1.1
B (NaF)	218.3 ± 0.25[a]	214.1 ± 0.4[a]
C (AI_1 + NaF)	220.0 ± 1.2[b]	218.3 ± 1.6[a,b]
D (AI_2 + NaF)	219.9 ± 0.6[a,b]	217.9 ± 1.9[a,b]
E (AI_3 + NaF)	219.7 ± 0.5[a,b]	217.4 ± 0.8[a,b]
F (AI_4 + NaF)	221.2 ± 0.8[b]	217.8 ± 1.0[a,b]

The results are shown as mean ± SD for each group of 10 rats per group. [a]$p < 0.05$ when compared with control group. [b]$p < 0.05$ when compared with NaF-treated group.

Table 12: The effects of *Azadirachta indica* on genotoxicity of NaF-treated Wistar rats.

Treatment group	Frequency of MNPCE
A (Control)	1.3 ± 0.6
B (NaF)	18.0 ± 1.8[a]
C (AI_1 + NaF)	15.8 ± 1.7[ab]
D (AI_2 + NaF)	8.5 ± 1.2[ab]
E (AI_3 + NaF)	16.5 ± 1.2[a]
F (AI_4 + NaF)	14.2 ± 0.8[ab]

The results are shown as mean ± SD for each group of 10 rats per group. MNPCE, micro nucleated polychromatic erythrocytes. [a]$p < 0.05$ when compared with control group. [b]$p < 0.05$ when compared with NaF-treated group.

both pre-treatment and co-treatment with AI. Again, only pre-treatment with AI at 200 mg/kg caused a significant ($p < 0.05$) reduction in DBP when compared with the NaF-treated group. Further, pre-treatment with AI at both 100 and 200 mg/kg caused significant ($p < 0.05$) reductions in MAP (Table 13). No significant effects were, however,

Table 13: The effect of *Azadirachta indica* on blood pressure of NaF-treated rats.

Treatment group	SBP, mmHg	DBP, mmHg	MAP, mmHg
A (Control)	127.6 ± 11.15	99.2 ± 12.83	108.2 ± 11.39
B (NaF)	166.0 ± 7.75[a]	128.2 ± 8.15[a]	140.4 ± 7.09[a]
C (AI_1 + NaF)	148.0 ± 16.97[a,b]	119.5 ± 21.92[a]	128.5 ± 9.19[a,b]
D (AI_2 + NaF)	127.0 ± 10.83[b]	100.6 ± 13.48[b]	109.2 ± 12.19[b]
E (AI_3 + NaF)	159.3 ± 1.15[a,b]	134.0 ± 3.61[a]	142.3 ± 2.52[a]
F (AI_4 + NaF)	149.5 ± 24.69[a,b]	118.8 ± 30.35[a]	128.8 ± 24.28[a]

The results are shown as mean ± SD for each group of 10 rats per group. SBP, systolic blood pressure; DBP, diastolic blood pressure; MAP, mean arterial pressure. [a]$p < 0.05$ when compared with control group. [b]$p < 0.05$ when compared with NaF-treated group.

Table 14: The effect of *Azadirachta indica* on heart rate, QT, and QTc of NaF-treated rats.

Treatment group	Heart rate, beats/min	QT segment, ms	QTc, ms
A (Control)	260.5 ± 13.4	65.0 ± 7.1	135.0 ± 11.3
B (NaF)	268.3 ± 31.5	61.0 ± 3.8	128.3 ± 10.6
C (AI_1 + NaF)	274.0 ± 15.4	68.8 ± 8.5	146.0 ± 15.0
D (AI_2 + NaF)	269.0 ± 8.9	62.3 ± 2.9	131.7 ± 8.4
E (AI_3 + NaF)	251.0 ± 17.3	72.7 ± 13.3	147.7 ± 26.4
F (AI_4 + NaF)	259.3 ± 14.4	69.8 ± 8.4	144.3 ± 15.7

The results are shown as mean ± SD for each group of 10 rats per group. [a]$p < 0.05$ when compared with control group. [b]$p < 0.05$ when compared with NaF treatment group.

observed with NaF treatment on heart rate, QT, and QTc (Bazett) values (Table 14). NaF treatment caused lower expressions of extracellular signal regulated kinase (ERK 1/2) in both cardiac and renal tissues of rats relative to the control. This was, however, ameliorated by treatment with AI (Figures 1 and 2).

Discussion

In this study, the administration of NaF induced both cardiac and renal toxicities as evidenced by a significant increase in MDA and H_2O_2 and decrease in the levels of PT, NPT, GSH, CAT, and GPx. Excessive formation of ROS, deoxyribonucleic acid (DNA)-damage, and decreased activity of antioxidant enzymes induced by sodium fluoride ingestion have been reported [28]. Also, the serum MPO increased significantly in this study. MPO is an enzyme stored in the azurophilic granules of polymorphonuclear neutrophils and macrophages [29]. At inflammatory sites, MPO is released into the cellular fluid,

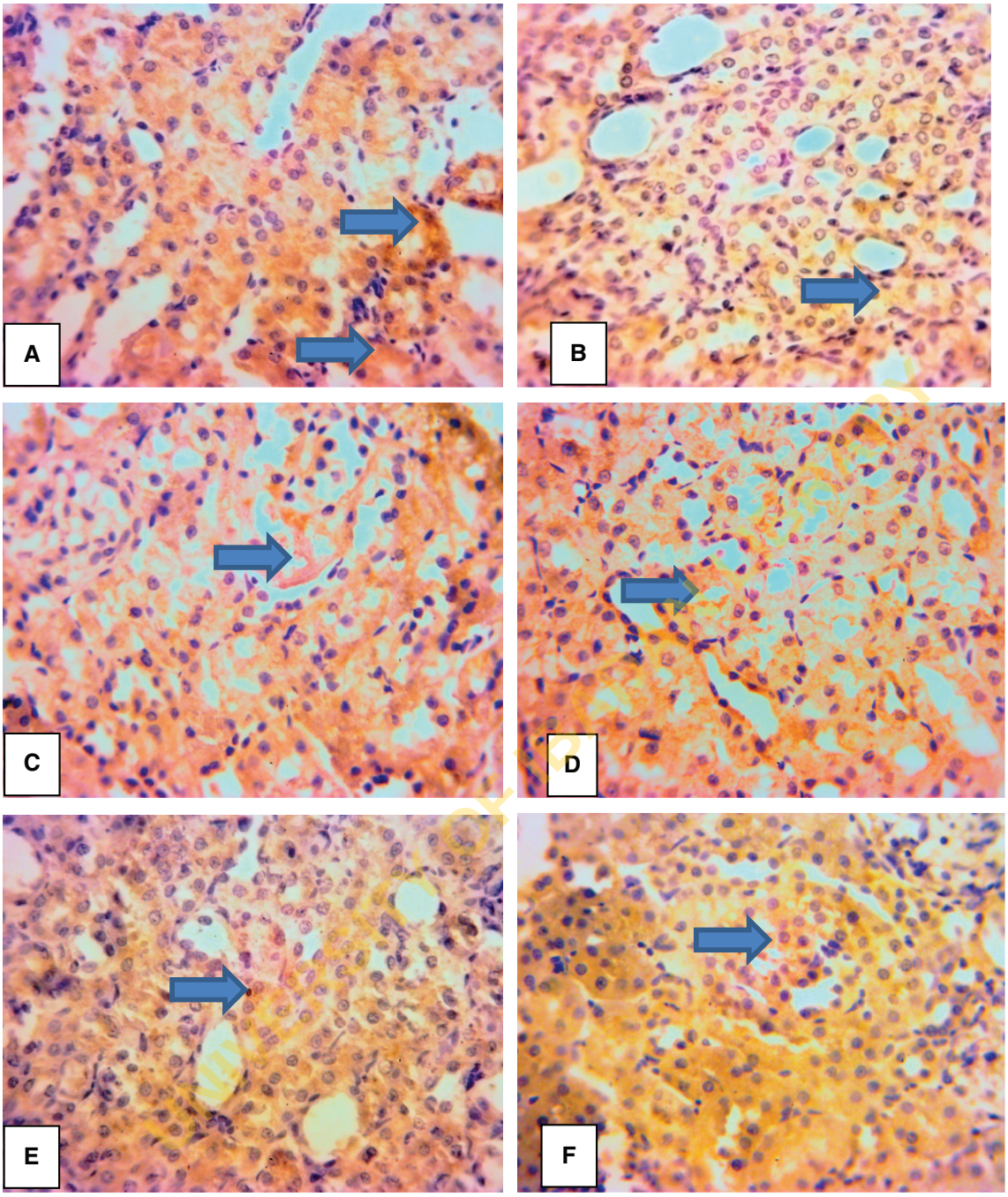

Figure 1: Lower expressions of ERK1/2 are observed in the renal and cardiac tissues of rats administered NaF alone relative to the control. Similarly, higher expressions of ERK1/2 are observed in rats pre-treated and co-treated with AI, respectively.
Group A (control) shows higher expressions of renal extracellular signal regulated kinase (ERK 1/2) expressions in the renal tissues. Group B (NaF only) shows lower expressions of ERK than the control. Group C [*Azadirachta indica*, AI + NaF (100 mg/kg pre-treatment)] shows higher expressions of ERK than Group B. Group D [AI + NaF (200 mg/kg pre-treatment)] shows higher expressions of ERK compared to Group B. Group E [AI + NaF (100 mg/kg co-treatment)] shows higher expressions of ERK similar to that of Group A. Group F [(AI + NaF (200 mg/kg co-treatment)] shows higher expressions of ERK similar to that of Group A. The slides were counterstained with high-definition hematoxylin and viewed 400× objectives (magnification: 100×).

consumes endothelial-derived nitric oxide (NO), an endogenous vasodilator, and thus reduces the bioavailability of NO with consequent impairment of the vasodilatory and anti-inflammatory activities of NO *in vivo*. MPO is also a marker of myocardial damage mediated by lipid peroxidation, endothelial dysfunction, modulation of proteases

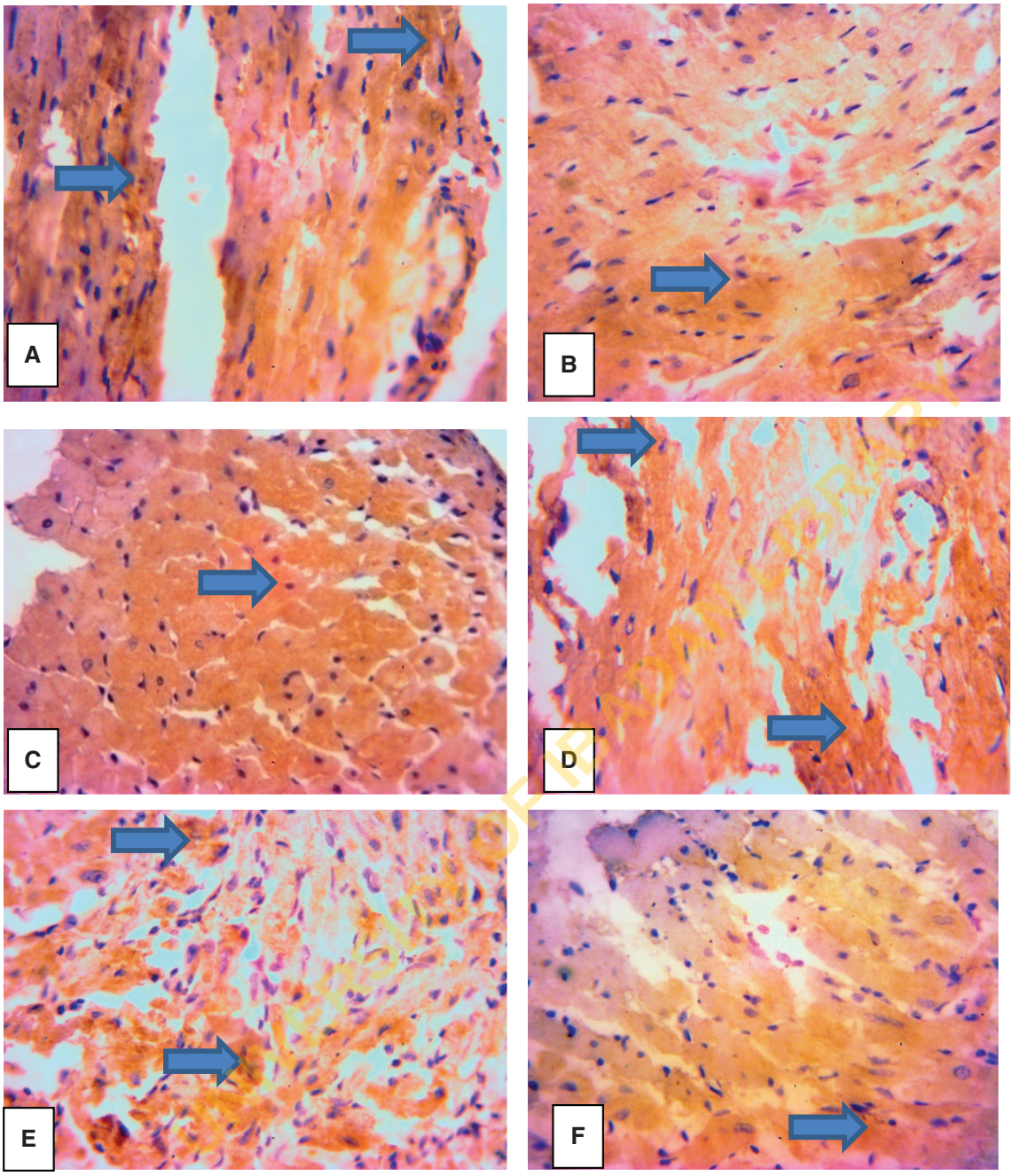

Figure 2: Group A (control) shows higher expressions of extracellular signal regulated kinase (ERK 1/2) expressions in the cardiac tissues. Group B (NaF only) shows lower expressions of ERK than the control. Group C [(*Azadirachta indica*, AI + NaF (100 mg/kg pre-treatment)] shows higher expressions of ERK than Group B. Group D [(AI + NaF (200 mg/kg pre-treatment)] shows higher expressions of ERK compared to Group B. Group E [(AI + NaF (100 mg/kg co-treatment)] shows higher expressions of ERK similar to that of Group A. Group F [(AI + NaF (200 mg/kg co-treatment)] shows higher expressions of ERK similar to that of Group A. The slides were counterstained with high-definition hematoxylin and viewed 400× objectives (magnification: 100×).

cascade, and increase in thrombogenicity [30]. The NaF-enhanced MPO activity, observed in this study, suggests an inflammatory process associated with the generation of free radicals and an induction of oxidative stress. Conversely, the decreased MPO activity associated with the pre-and co-treatment of AI at 100 and 200 mg/kg suggests an ameliorative action for AI in fluoride-induced oxidative stress.

Nitric oxide is actively involved in several protective functions of the intact endothelium and it maintains the blood vessel in a constant state of vasodilation [31]. In this study, significant decrease in the serum level of NO

was observed in rats exposed to NaF compared with the control. However, the pre-treatment of rats with AI and co-administration of NaF with AI caused a significant increase in the serum level of NO, compared with the untreated NaF exposed rats. This observation suggests a probable ameliorative role for AI on NaF-induced progressive loss of NO-associated endothelial protective functions.

Xanthine oxidase (XO) is an enzyme involved in the metabolism of purines; catalyzing the conversion of both hypoxanthine and xanthine to uric acid, a normal component of urine [32]. Unfortunately, oxidative stress-inducing free radicals are the by-products of XO activity [33]. Consequently, increased activity of xanthine oxidase is associated with significant oxidative stress-mediated endothelial dysfunction and tissue injury [34]. It has been demonstrated that mild uricemia has the ability to induce renal inflammation, activate the renin-angiotensin system, and downregulate nitric oxide metabolism, thereby leading to uric acid-mediated hypertension [35]. Hyperuricemia has also been linked to abnormalities such as afferent arteriopathy, glomerular hypertrophy, increased glomerular pressure, tubulointerstitial damage, and infiltration of the kidneys by macrophages [36]. In this study, NaF elicited a significant increase in the serum level of XO when compared with the control group, but pre-treatment with AI caused a significant ($p < 0.05$) decrease in the activity of serum XO, thus suggesting an effect of AI on free radical generation in both the pre-treatment and co-treatment groups.

A significant increase in MDA, which is a marker of oxidative stress [37], as observed in the untreated NaF-treated group of rats suggests an indication of oxidative stress. However, both the pre-treatment and co-treatment with AI caused a significant decrease in the cardiac and renal MDA levels in rats, thus suggesting potent antioxidant effect of AI and protection of the heart and kidney from lipid peroxidation. Liu et al. [38] reported increased production of lipid peroxides, reactive nitrogen and oxygen species, and concomitant cellular damage following fluoride exposure.

In this study, treatment with NaF elicited a significant increase in hydrogen peroxide (H_2O_2), one of the several ROS formed in the course of normal metabolism [39], whereas AI caused a significant decrease in the cardiac and renal H_2O_2 levels in both the pre-treatment and co-treatment groups, thus suggesting an amelioration of free radical generation. Moreover, thiols make up the major portion of the total body antioxidants and play a significant role in defense against ROS [40]. A decreased level of thiols has been noted in various disorders including chronic renal failure and cardiovascular disorders [41]. In this study, the levels of protein (PT) and NPT were significantly decreased following NaF exposure in the cardiac and renal tissues compared with the control group, probably due to an increased generation of free radicals induced by NaF. However, pre-treatment and co-treatment with AI caused a significant increase in PT and NPT in the cardiac and renal tissues of rats. This observation suggests prevention of the deleterious effect of NaF on the endogenous antioxidant of AI. Antioxidant enzymes, such as SOD, catalyze the conversion of superoxide radicals (O_2^-) into hydrogen peroxide (H_2O_2) and O_2, and further the detoxification of H_2O_2 into H_2O and O_2 [42]. Catalase (CAT) and glutathione peroxidase (GPx), also, are enzymes involved in the detoxification of H_2O_2. In this study, the activities of the antioxidant enzymes CAT, GPx, and SOD significantly decreased in cardiac and renal tissues of rats exposed to NaF when compared with the control. The decrease in the activities of these enzymes may be associated with their excessive utilization in the inactivation of the free radicals generated by NaF. The significantly increased activity of SOD, CAT, and GPx in the cardiac and renal tissues of rats pre-treated with and co-administered with the extract of AI suggest an inherent protective effect for AI on the observed NaF-induced depletion of the endogenous antioxidant enzymes. The observation in this study is similar to the report of Sinha et al. [43] who reported increased lipid peroxidation, but decreased antioxidant enzyme activities in NaF intoxication.

Moreover, fluoride has been reported to inhibit various enzymes such as phosphofructokinase-1, thereby interfering with DNA repair mechanism and thus inducing mutagenesis [44]. An important biomarker of DNA damage is the detection of MNPCE in the bone marrow [45]. The observed increase in the frequency of MNPCE, observed in this study, following the administration of NaF suggests an alteration of the genomic integrity of rats, whereas the significant decrease in the frequency of MCPCE in NaF-exposed rats treated with 200 mg/kg of AI suggest a preservation of the genomic integrity and inhibition of DNA damage. The observation, in this study, of increased MNPCE in the untreated NaF-exposed rats agrees with an earlier report of Srilatha et al. [46] that oral administration of NaF induces DNA damage in rats.

Also in this study, the SBP which is a measure of blood pressure while the heart is relaxed between heart beats [47], increased significantly in the untreated NaF-exposed rats. The regulation of blood pressure and development of hypertension (increased blood pressure beyond the normal range for a given species) is a complicated multifactorial process involving the heart, autonomic nervous system, endothelial signaling intercellular mechanisms,

vasculature, and kidneys [48]. Fluoride has been shown to concentrate in the cardiovascular system and this can result in increased blood pressure [49]. In this study, the SPB, DBP, and MAP of the untreated NaF-exposed rats increased significantly when compared with the control group, but the pre-treatment and co-treatment with AI elicited a significant decrease in the blood pressure level, thereby suggesting that AI conferred some level of protection against the development of high blood pressure.

Extracellular signal regulated kinases are members of the mitogen-activated protein kinase superfamily involved in the ERK cascade, and they function to regulate cellular processes such as proliferation, differentiation, and cell cycle [50]. The activation of ERK 1/2 has been shown to inhibit apoptosis in response to a wide range of stimuli such as tumor necrosis factors, osmotic stress, nitric oxide, radiation, and hydrogen peroxide [51]. The result of this study showed that NaF administration elicited a lower expression of ERK 1/2 which caused a reduction in cell survival protein. However, pre-treatment and co-treatment with AI reversed the toxic effect of NaF as evidenced by a marked increase in ERK 1 and 2 expressions in cardiac and renal tissues. This suggests that AI has an antioxidant effect against the NaF-induced cell death. The pre-treatment and co-treatment with AI ameliorated the effect of fluoride-induced oxidative stress, DNA damage, hypertension, and reduction in cell survival protein in cardiac and renal tissues of rats. Therefore, AI holds great potential as a medicinal plant in the management of hypertension and its associated complications.

Author contributions: All the authors have accepted responsibility for the entire content of this submitted manuscript and approved submission.
Research funding: None declared.
Employment or leadership: None declared.
Honorarium: None declared.
Competing interests: The funding organization(s) played no role in the study design; in the collection, analysis, and interpretation of data; in the writing of the report; or in the decision to submit the report for publication.

References

1. Stevens DP, McLaughlin MJ, Alston AM. Phytotoxicity of aluminium-fluoride complexes and their uptake from solution culture by Avena sativa and Lycopersicon esculentum. Plant Soil 1997;192:81–93.
2. Inkielewicz I, Krechniak J. Fuoride content in soft tissues and urine of rats exposed to sodium fluoride in drinking water. Fluoride 2003;36:263–6.
3. Shashi A, Thapur SP. Histopathology of myocardial damage in experimental fluorosis in rabbits. Fluoride 2001;34:43–50.
4. McIvor ME, Cummings CC, Mower MM. Sudden cardiac death from acute fluoride intoxication: the role of potassium. Ann Emerg Med 1987;16:777–81.
5. Bouaziz H, Ketata S, Jammoussi K, Boudawara T, Ayedi F, Ellouze F, et al. Effects of sodium fluoride on hepatic toxicity in adult mice and their suckling pups. Pesticide Biochem Physio 2006;86:124–30.
6. Chlubek D. Fluoride and oxidative stress. Fluoride 2003;36:217–28.
7. Oyagbemi AA, Omobowale TO, Akinrinde AS, Saba AB, Ogunpolu BS, Daramola O. Lack of reversal of oxidative damage in renal tissues of lead acetate-treated rats. Environ Toxicol 2015;30:1235–43.
8. Sithisarn P, Supabphol R, Gritsanapan W. Antioxidant activity of Siamese neem tree (VP1209). J. Ethnopharmacol 2005;99: 109–12.
9. Obiefuna I, Young R. 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 2005;19:792–5.
10. Ogbuewu IP, Odoemenam VU, Obikaonu HO, Opara MN, Emenalom OO, Uchegbu MC, et al. The growing importance of neem (Azadirachta indica A. Juss) in agriculture, industry, medicine and environment: a review. Res J Med Plant 2011;5:230–45.
11. Omobowale TO, Oyagbemi AA, Oyewunmi OA, Adejumobi OA. Chemopreventive effect of methanolic extract of Azadirachta indica on experimental Trypanosoma brucei induced oxidative stress in dogs. Pharmacog Res 2015;7:249–58.
12. Omóbòwálé TO, Oyagbemi AA, Adejumobi OA, Orherhe VE, Amid AS, Adedapo AA, et al. Preconditioning with the methanol extract of Azadirachta Indica ameliorates intestinal ischaemia-reperfusion injury induced-cardiorenal dysfunction through the contribution of antioxidant and extracellular signal regulated protein kinase (ERK) signalling. J Ayurveda Integr Med 2016;7:209–17.
13. Garber JC. On the care and use of US lab animals. Nature 2011;476:152.
14. Gornal AG, Bardawill JC, David MM. Determination of serum proteins by means of Biuret reaction. J Biol Chem 1949;177: 751–66.
15. Misra HP, Fridovich I. The role of superoxide anion in the autoxidation of epinephrine and a simple assay for superoxide dismutase. J Biol Chem 1972;217:3170–5.
16. Omobowale TO, Oyagbemi AA, Akinrinde AS, Saba AB, Daramola OT, Ogunpolu BS, et al. Failure of recovery from lead induced hepatoxicity and disruption of erythrocyte antioxidant defence system in Wistar rats. Environ Toxicol Pharmacol 2014;37:1202–11.
17. Beutler E, Duron Oand Kelly BM. Improved method for the determination of blood glutathione. J Lab Clin Med 1963;61:882–8.
18. Varshney R, Kale RK. Effect of calmodulin antagonist on radiation-induced lipid peroxidation in microsomes. Int J Radiat Biol 1990;58:733–43.
19. Woff SP. Ferrous ion oxidation in presence of ferric ion indicator xylenol orange for measurement of hydroperoxides. Meth Enzymol 1994;233:182–9.
20. Sinha KA. Colorimetric assay of catalase. Anal Biochem 1971;47:389–94.

21. Crespo E, Macías M, Pozo D, Escames G, Martín M, Vives F, et al. Melatonin inhibits expression of the inducible NO synthase II in liver and lung and prevents endotoxemia in lipopolysaccharide-induced multiple organ dysfunction syndrome in rats. FASEB J 1999;13:1537–46.
22. Ellman GL. Tissue sulfhydryl groups. Arch Biochem Biophy 1959;82:70–7.
23. Akaike T, Ando M, Oda T, Doi T, Ijiri S, Araki S, et al. Dependence on O2-generation by xanthine oxidase of pathogenesis of influenza virus infection in mice. J Clin Invest 1990;85:739.
24. Xia Y, Zweier JL. Measurement of myeloperoxidase in leukocyte-containing tissues. Anal Biochem 1997;245:93–6.
25. Oyagbemi AA, Omobowale TO, Asenuga ER, Adejumobi AO, Ajibade TO, Ige TM, et al. Sodium fluoride induces hypertension and cardiac complications through generation of reactive oxygen species and activation of nuclear factor kappa beta. Environ Toxicol 2017;32:1089–101.
26. Bonassi S, El-Zein R, Bolognesi C, Fenech M. Micronuclei frequency in peripheral blood lymphocytes and cancer risk: evidence from human studies. Mutagenesis 2011;26:93–100.
27. Nabavi SM, Nabavi SF, Eslami S, Moghaddam AH. In vivo protective effects of quercetin against sodium fluoride-induced oxidative stress in the hepatic tissue. Food Chem 2012;132: 931–5.
28. Loria V, Dato I, Graziani F, Biasucci LM. Myeloperoxidase: a new biomarker of inflammation in ischemic heart disease and acute coronary syndromes. Mediators Inflamm 2008;2008:135625.
29. Roman RM, Wendland AE, Polanczyk CA. Myeloperoxidase and coronary arterial disease: from research to clinical practice. Arqui Bras Cardio 2008;91:12–9.
30. Teixeira BC, Lopes AL, Macedo RC, Correa CS, Ramis TR, Ribeiro JL, et al. Inflammatory markers, endothelial function and cardiovascular risk. J Vasc Bras 2014;13:108–15.
31. Pacher P, Nivorozhkin A, Szabo C. Therapeutic effects of xanthine oxidase inhibitors: renaissance half a century after the discovery of allopurinol. Pharmacol Rev 2006;58:87–114.
32. Rashmi R, Aiki K, Pushpa B, Aparna M, Misra MK. Xanthine oxidase as a marker of myocardial infarction. Ind J Clin Biochem 2007;22:90–2.
33. Feig DI, Kang DH, Nakagawa T, Mazzali M, Johnson RJ. Uric acid and hypertension. Curr Hypertens Rep 2006;8:111–5.
34. Sanchez-Lozada LG, Tapia E, Santamaria J, Avila-Casado C, Soto V, Nepomuceno T, et al. Mild hyperuricemia induces vasoconstriction and maintains glomerular hypertension in normal and remnant kidney rats. Kidney Int 2005; 67:237–47.
35. Lima MC, Guido M, Iandara SS, Baldomero AK, Lourdes ZZ, Gabriel B. Evaluation of oxidative stress in mice subjected to aerobic exercise. Acta Cir Bras 2012;27:544–51.
36. Yamada S, Kumazawa S, Ishii T. Immunochemical detection of a lipofuscin-like fluorophore derived from malondialdehyde and lysine. J Lipid Res 2001;42:1187–96.
37. Liu G, Chai C, Cui L. Fluoride causing abnormally elevated serum nitric oxide levels in chicks. Environ Toxicol Pharmacol 2003;13:199–204.
38. Shacter E. Quantification and significance of protein oxidation in biological samples. Drug Metab Rev 2000;32:307–26.
39. Mungli P, Jeevan K, Shetty, Sambit D, Bijay KB, Abhirup S, et al. Urinary protein thiols in different grades of proteinuria. Indian J Clin Biochem 2008;23:404–6.
40. Lu Z, Xu S. ERK1/2 MAP Kinases in cell survival and apoptosis. IUBMB Life 2006;58:621–31.
41. El-Shafey AF, Armstrong AE, Terrill JR, Grounds MD, Arthur PG. Screening for increased protein thiol oxidation in oxidatively stressed muscle tissue. Free Radic Res 2011;45:991–9.
42. Oyedemi OS, Bradley G, Afolayan AJ. In-vitro and in-vivo antioxidant activities of aqueous extract of Strychnos henningsii Gilg. Afr J Pharm Pharmacol 2010;4:70–8.
43. Sinha M, Manna P, Sil PC. Terminalia arjuna protects mouse hearts against sodium fluoride-induced oxidative stress. J Med Food 2008;11:733–40.
44. Wang AG, Xia T, Chu QL, Zhang M, Liu F, Chen XM, et al. Effects of fluoride on lipid peroxidation, DNA damige and apoptosis in human embryo hepatocytes. Biomed Environ Sci 2004;17:217–22.
45. Luzhna L, Palak K, Olga K. Micronuclei in genotoxicity assessment: from genetics to epigenetics and beyond. Front Genet 2013;4:131.
46. Srilatha K, Banji D, Banji OJ, Vinod KR, Saidulu A. Investigation on the anti-genotoxic effect of ocimum sanctumin fluoride induced genotoxicity. Int Res J Pharma 2013;4:160–4.
47. Zheng L, Sun Z, Li J. Pulse pressure and mean arterial pressure in relation to ischemic stroke among patients with uncontrolled hypertension in rural areas of China. Stroke 2008;39:1932–7.
48. Cain AE, Khalil RA. Pathophysiology of essential hypertension: Role of the pump, the vessel and the kidney. Semin Nephrol 2002;22:3–16.
49. Varol E, Varol S. Water-borne fluoride and primary hypertension. Fluoride 2013;46:3–6.
50. Yoon S, Seger R. The extracellular signal-regulated kinase: multiple substrates regulate diverse cellular functions. Growth Factors 2006;24:21–44.
51. Kitagawa D, Tanemura S, Ohata S, Shimizu N, Seo J, Nishitai G, et al. Activation of extracellular signal-regulated kinase by ultraviolet is mediated through Src dependent epidermal growth factor receptor phosphorylation. Its implication in an anti-apoptotic function. J Biol Chem 2002;277:366–71.