Antihypertensive power of Naringenin is mediated via attenuation of mineralocorticoid receptor (MCR)/ angiotensin converting enzyme (ACE)/ kidney injury molecule (Kim-1) signaling pathway

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ABSTRACT

Hypertension is a condition with chronic elevation of blood pressure and a common preventable risk factor for cardiovascular disease with attendant global morbidity and mortality. The present study investigated the novel antihypertensive and neuroprotective effect of Naringenin on L-N^G-Nitro arginine methyl ester (L-NAME) induced hypertension together with possible molecular mechanism of action. Rats were divided into four groups. Rats in Group A were normotensive. The hypertensive group (Group B) received 40 mg/kg) of L-NAME alone while Groups C and D were concurrently administered Naringenin (50 mg/kg) or Lisinopril (10 mg/Kg) together with L-NAME orally for 3 weeks. Blood pressure parameters, markers of oxidative stress and renal damage were measured. The immunohistochemistry of kidney injury molecule 1, mineralocorticoid receptor and

angiotensin converting enzyme were also determined. Results indicated significant increases in malondialdehyde, advanced oxidation protein products, protein carbonyl contents and decrease in serum nitric oxide bioavailability in hypertensive rats. Furthermore, there were significant increases in serum myeloperoxidase, urinary creatinine, albumin and blood urea nitrogen in hypertensive rats in comparison to hypertensive rats treated with either Naringenin or Lisinopril. Immunohistochemistry reveal significant expressions of kidney injury molecule 1, mineralocorticoid receptor and angiotensin converting enzyme in hypertensive rats. However, co-treatment with either Naringenin or Lisinopril mitigated both renal and neuronal oxidative stress, normalized blood pressure and lowered the expressions of kidney injury molecule 1, mineralocorticoid receptor and angiotensin converting enzyme. Collectively, Naringenin offered a novel antihypertensive and neuroprotective effect through down regulation of kidney injury molecule 1, mineralocorticoid receptor and angiotensin converting enzyme.

Keywords: Hypertension; Oxidative stress; Naringenin; Novel-antihypertensive agent

1. INTRODUCTION

The global incidence of hypertension has risen significantly in the last few decades (Olsen et al., 2016). Hypertension is defined as a systolic blood pressure of at least 140 mmHg or a diastolic blood pressure of at least 90 mmHg, or both. However, it has been documented that millions of dollars are spent annually on the treatment of hypertension and its associated cardiovascular disease conditions (Condliffe et al., 2013; Heidenreich et al., 2011).

In United States alone, it has been reported that approximately one-fourth of the adult population is diagnosed with hypertension (Ahluwalia and Bangalore, 2017). Also, hypertension is associated with more cardiovascular disease deaths than any other modifiable disease conditions (Danaei et al., 2009). Hypertension is a major risk factor for cardiovascular morbidity and mortality including cardiovascular death, myocardial infarction, heart failure and stroke (Antza et al., 2016; Chaturvedi et al., 2014). Combination therapy such as the use of calcium channel blockers and inhibitors of the renin-angiotensin-aldosterone system (RAAS) have been adopted as means of maintaining blood pressure, thereby reducing cardiovascular morbidity and mortality (da Silva, 2010). Furthermore, genetic predisposition has been documented in some hypertensive patients (Sandra, 2018). Additionally, lifestyle factors including high sodium intake, obesity, excessive alcohol intake, and some drugs may induce or aggravate hypertension, hence, making it difficult to control (Lewington et al., 2002).

In recent times, the use of alternative medicine or phytochemicals has gained global acceptance for the treatment of hypertension (Ekor, 2014). Medicinal plants are naturally endowed with phytochemicals and phytonutrients with diverse biological and pharmacological activities (Lau et al., 2019; Nazifi et al., 2019; Yerlikaya et al., 2019; Menezes and Diederich, 2019; Shehadeh et al., 2019; Martínez-Rodríguez et al., 2019). Some of these identified phytochemicals in medicinal plants include saponins, tannins,

glycosides, flavonoids (Hafeez et al., 2017; Ghagane et al., 2017; Senguttuvan et al., 2014). Flavonoids are a class of plant secondary metabolites with a polyphenolic structure. They are also abundantly found in foods and beverages of plant origin, such as fruits, vegetables, tea, cocoa and wine (Kozłowska and Szostak-Wegierek, 2014). Flavonoids are known as potential drug candidates against chronic inflammatory and neurodegenerative disease conditions such as polycystic kidney disease, Alzheimer and Parkinson's disease (Panche et al., 2015; Jager and Saaby, 2011; Shoba et al., 2010). Again, the anti-hypertensive effect of flavonoids has been linked to significant improvement in endothelial function via vascular endothelial nitric oxide synthase and protein kinase B (Akt) activation (Brüll et al., 2015). The anti-Alzheimer disease and cholesterol reducing effect of flavonoid has been ascribed to its inhibitory effect on 3-hydroxy-3-methylglutaryl-Coa reductase, acetylcholinesterase and β active site cleavage enzyme-1 (BACE-1) activity, the rate-limiting enzyme responsible for the production of amyloid-β peptides (Paris et al., 2011).

Naringenin is one of the most important naturally-occurring flavonoids predominantly found in some edible fruits, like Citrus species and tomatoes (Zobeiri et al., 2018). Biological and pharmacological effects ascribed to naringenin including hepatoprotective, anti-atherogenic, anti-inflammatory, anti-mutagenic, anticancer, antimicrobial properties (Yin et al., 2018; Karim et al., 2018). However, medications available for the treatment of hypertension are with arrays of side effects. Furthermore, information is scarce on the comprehensive mechanism of action of antihypertensive effect of naringenin. Therefore, this explore novel antihypertensive effect of naringenin an its molecular mechanism of action.

2. MATERIALS AND METHODS

2.1. Experimental animals and design

Forty male Wistar rats were used for this study. Rats in Group A were normotensive rats. The hypertensive group (Group B) received 40 mg/kg of L-N^G-Nitro arginine methyl ester (L-NAME) alone while Groups C and D were concurrently administered Naringenin (50 mg/kg) or Lisinopril (10 mg/mg) together with L-NAME orally for 3 weeks. The dosage for L-NAME and Naringenin was chosen from previous study from our laboratory and other authors (Omóbòwálé et al., 2019; Ahmed et al., 2014). The animals were also fed with rat cubes *ad libitum*. The rats were kept in wire mesh cages under controlled light cycle (12 h light/12 h dark) and fed with commercial rat chow *ad libitum* and liberally supplied with water. The blood of the rats was taken on day 21 and rats were killed on the day 22. The Animal Care and Use Research Ethical Committee of the Faculty of Veterinary Medicine of Ibadan, approved the study with ethical approval number UI-ACUREC/18/0133.

2.2. Chemicals

Naringenin, Sulfosalicylic acid, acetylcholine iodide, guanidine hydrochloride, potassium chloride, ethanol, 2,4-dinitrophenylhydrazine, ethyl acetate, acetic acid, potassium hydroxide, reduced glutathione, sodium hydroxide, O-dianisidine, and hydrogen peroxide, xylenol orange (XO), 1,2-dichloro-4-nitrobenzene, oxidized glutathione, thiobarbituric acid, trichloroacetic acid, 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, Maryland, USA). The kidney injury molecule, mineralocorticoid receptor and angiotensin converting enzyme antibodies were purchased from (Bioss Inc. Woburn, Massachusetts, USA) while 3, 3'-Diaminobenzidine (DAB) tablets were purchased from (AMRESCO LLC. OHio, USA). All other chemicals were of analytical grade.

2.3. Blood pressure measurement

Twenty-four hours after the last administration, blood pressure parameters were taken using an automated blood pressure monitor (CODA S1, Kent Scientific Corporation, Connecticut, USA). The systolic blood pressure, diastolic blood pressure and mean arterial blood pressures were determined non-invasively in conscious animals by tail plethysmography.

2.4. Blood sample collection and serum preparation

An approximately 3 mL of blood were collected by retro–orbital venous puncture using heparinized capillary tubes into plain bottles and allowed to clot. The clotted blood was then centrifuged at 4, 000 ***g*** for 10 min. Clear serum was separated with Pasteur pipette into another plain tubes and then stored at 4 °C until needed.

2.5. Preparation of renal and neuronal post mitochondrial fractions

The organs (kidneys and whole brain) were excised, rinsed and homogenized using 50 mM Tris-HCl buffer (pH 7.4) containing 1.15% KCl. The homogenates were subjected to cold centrifugation at 4 °C using a speed of 10, 000 *g* for 15 min. The post mitochondrial fractions (PMFs) obtained from renal and neuronal homogenates were used for biochemical assays.

Biochemical assays.

2.6. Determination of renal and neuronal makers of oxidative stress

Hydrogen peroxide generation was determined according to the method of Wolff (1994). The reaction mixture was subsequently incubated at room temperature for 30 min. The mixtures were read at absorbance at 560 nm and H_2O_2 generated was extrapolated from H_2O_2 standard curve. The Malondialdehyde (MDA) contents as an index of lipid peroxidation were quantified in the PMFs of brain and renal tissue according to the method Varshney and Kale (1990). The absorbance was measured at 532 nm. Lipid peroxidation products were calculated with a molar extinction coefficient of 1.56×10^5/M/cm. Protein carbonyl (PCO) contents in the renal and brain tissues were measured using the method of Reznick and Packer (1994). The absorbance of the sample was measured at 370 nm. The carbonyl content was calculated based on the molar extinction coefficient of DNPH ($2.2\ 10^4\ cm^1\ M^1$). The vitamin C contents were measured as earlier described by Jacques-Silva et al. (2001). The advanced oxidation protein product (AOPP) contents as index of protein oxidation were determined as described by Kayali et al. (2006). Briefly, 0.4 ml of brain and renal PMFs were treated with 0.8 ml phosphate buffer (0.1 M; pH 7.4). The absorbance of the reaction mixture was immediately recorded at 340 nm wavelength. The content of AOPP for each sample was calculated using the extinction coefficient of 261 $cm^{-1}\ mM^{-1}$ and the results

were expressed as µmoles/mg protein. The activity of xanthine oxidase was determined according to method of Akaike et al. (1990). Serum myeloperoxidase (MPO) activity was determined according to the method described by Xia and Zweier (1997).

2.7. Determination of renal and neuronal antioxidants defence status

The Superoxide dismutase activity was carried out by the method of Misra and Fridovich (1972), with slight modification from our laboratory (Oyagbemi et al., 2015). The increase in absorbance at 480 nm was monitored every 30 s for 150 s. The one unit of superoxide dismutase activity was given as the amount of superoxide dismutase necessary to cause 50% inhibition of the autooxidation of adrenaline to adrenochrome. Reduced glutathione was estimated by the method of Jollow et al. (1974) while catalase activity was determined according to the method of Sinha (1972). One unit of catalase activity represents the amount of enzyme required to decompose 1 µmol of H_2O_2/min. Glutathione peroxidase activity was also measured according to Beutler et al. (1963). Glutathione S-transferase was estimated by the method of Habig et al. (1974) using 1-chloro-2, 4-dinitrobenzene as substrate. The activity of glutathione reductase (GRed) was according to the method of Racker (1954). The decrease in absorbance at 340 nm was measured and enzyme activity of calculated with a molar extinction co-efficient of 6.1 mmol/L^{-1} cm. The protein and non-protein thiol contents were determined as described by Ellman (1959). Protein concentration was determined by the Biuret method of Gornal et al. (1949), using bovine serum albumin (BSA) as standard.

2.8. Determination of serum nitric oxide concentration

The serum nitric oxide concentrations were measured spectrophotometrically at 548 nm according to the method of Olaleye et al. (2007).

2.9. Determination of urinary kidney function tests

Selected rats from each group were placed individually in metabolism cages for collection of urine before sacrifice. Urinary blood urea nitrogen (BUN), albumin, creatinine and total protein concentrations were determined using Randox kits following manufacturer's instructions.

2.10. Histopathology

Small pieces of kidney and brain were fixed in 10% formalin, embedded in paraffin wax, and sections of 5-6 mm in thickness and thereafter stained with Hematoxylin and Eosin (H&E) for histopathological examination as previously described (Drury and Wallington, 1976). Thereafter, the sections were examined with light microscopy.

2.11. Immunohistochemistry

The immunohistochemistry was determined as earlier described by Oyagbemi et al. (2019). We determined the immunolocalization of kidney injury molecule (Kim-1), mineralocorticoid receptor (MCR) and angiotensin converting enzyme (ACE) in the kidney, the fixed tissues were embedded in paraffin and sectioned at a thickness of 5 µm. The immunoreactive

positive expressions of kidney injury molecule 1, mineralocorticoid receptor and angiotensin converting enzyme were probed against anti-rabbit antibodies. The intensive immunoreactivity regions were viewed with light microscope (Leica LAS-EZ®, Germany) using Leica software application suite version 3.4 equipped with a digital camera. Immunoreactivity was quantified using Image J software.

2.12. Statistical analysis

Data obtained were analyzed with One-way ANOVA with Dunnett's post-test at a 95% confidence limit. All values were expressed as mean ±**S.D.** The test of significance between two groups was estimated by Student's *t*-test.

3. RESULTS

3.1. Body weight gain/loss and organ weight

The results from Table 1 show significant reduction in body weight with percentage weight loss of −11.71% of hypertensive rats relative to the control and hypertensive rats treated with Naringenin or Lisinopril. Also, there was significant increase in the kidney weight of the hypertensive rats relative to the control and hypertensive treated rats that were co-administered with either Naringenin or Lisinopril (Table 1).

Table 1. Body weight and relative organ weight.

Weight (g)	Ctrl	HTR	HTR + NAR	HTR + LISIP
Initial body weight (g)	77.5 ± 15.55	124 ± 6.52	113 ± 14.83	100 ± 8.16
Final body weight (g)	100 ± 11.40[a]	111 ± 14.14[a]	110 ± 14.14	107.5 ± 9.57
%body weight gain/loss (g)	22.5%	−11.71%	−2.72%	6.97%
Kidney weight (g)	0.71 ± 0.065	0.79 ± 0.044[a]	0.74 ± 0.077	0.69 ± 0.11
Relative kidney weight (g)	0.0072 ± 0.0014	0.0071 ± 0.00068	0.0081 ± 0.001	0.0081 ± 0.0041

3.2. Serum, renal and neuronal markers of oxidative stress and antioxidant status

In Table 2, the serum advanced oxidative protein products content and the myeloperoxidase activity increased significantly in hypertensive rats in comparison to the normotensive and hypertensive rats treated with either Naringenin or standard antihypertensive agent (Lisinopril) as indicated in Table 2. On the other hand, there was observable significant reduction in the serum nitric oxide bioavailability in hypertensive untreated rats (Table 2). In fact, hypertensive rats treated with either Naringenin or lisinopril had significant improvement in serum nitric oxide bioavailability which is necessary for the maintenance of blood pressure as recorded in Table 2.

Table 2. Serum markers of inflammation and hypertension.

Experiments	AOPP	NO	MPO
Ctrl	54.48 ± 3.17	0.49 ± 0.08	3.94 ± 0.44
HTR	66.6 ± 2.84[a]	0.29 ± 0.029[a]	4.25 ± 1.00[a]
HTR + NAR	57.33 ± 8.993[b]	0.58 ± 0.069[b]	3.62 ± 1.00[a, b]
HTR + LISIP	55.92 ± 3.67 [b]	0.55 ± 0.088[b]	2.90 ± 0.66[a, b]

Values are presented as mean ± **S.D.** (n = 10). Superscript([a]) indicate **statistically** significant **difference** when Groups B (**HTR**), C (**HTR + NAR)** and D (**HTR + LISIP)** compared with Group A at P < 0.05, Superscript ([b]) indicate **statistically** significant **difference** when Groups C and D compared with Group B. Group A (**Ctrl**; Control), Group B (**HTR;** L-NAME alone at 40 mg/kg), Group C (**HTR + NAR;** L-NAME (40 mg/kg) + Naringenin (50 mg/kg), Group D (**HTR + LISIP;** L-NAME (40 mg/kg) + Lisinopril (10 mg/kg).

AOPP (Advanced Oxidation Protein Products), NO (Nitric oxide; µmole/L), MPO indicating (Myeloperoxidase; µmole/mg protein), **HTR (Hypertensive rats), L-NAME (L-NG-Nitro arginine methyl ester (L-NAME), NAR (Naringenin), LISIP (Lisinopril).**

In the present study, the activity of renal catalase, superoxide dismutase, glutathione S-transferase, glutathione peroxidase and glutathione reductase reduced significantly in hypertensive rats (Table 3). The observed reduction the activity of these antioxidant enzymes was restored in hypertensive rats that received Naringenin or Lisinopril (Table 3). Similarly, the contents of non-enzymic antioxidants protein thiol, non-protein thiol and reduced glutathione reduced significantly in hypertensive rats relative to hypertensive treated rats (Table 4). The observed reduction in the contents of non-enzymic antioxidants received significant boost in hypertensive rats treated with Naringenin or Lisinopril (Table 4). This is indicative of the antioxidant power of Naringenin.

Table 3. Renal enzymic antioxidant defence system.

Experiments	CAT	SOD	GPx	GST	GR
Ctrl	694.81 ± 74.3	6.76 ± 0.49	190.64 ± 13.64	0.59 ± 0.15	0.33 ± 0.062
HTR	640.92 ± 27.70[a]	6.00 ± 0.27	167.08 ± 6.16[a]	0.41 ± 0.063[a]	0.18 ± 0.06 [a]
HTR + NAR	703.24 ± 2.93[a, b]	6.39 ± 0.36[b]	179.70 ± 9.34[a, b]	0.65 ± 0.18[b]	0.27 ± 0.036[b]
HTR + LISIP	840.81 ± 18.35[a, b]	7.71 ± 1.08[b]	220.28 ± 28.72[a, b]	0.74 ± 0.09[a, b]	0.20 ± 0.083[a, b]

Values are presented as mean ± **S.D.** (n = 10). Superscript([a]) indicate **statistically** significant **difference** when Groups B (**HTR**), C (**HTR + NAR)** and D (**HTR + LISIP)** compared with Group A at P < 0.05, Superscript ([b]) indicate **statistically** significant **difference** when Groups C and D compared with Group B. Group A (**Ctrl**; Control), Group B (**HTR;** L-NAME alone at 40 mg/kg), Group C (**HTR + NAR;** L-NAME (40 mg/kg) + Naringenin (50 mg/kg), Group D (**HTR + LISIP;** L-NAME (40 mg/kg) + Lisinopril (10 mg/kg).

CAT (Catalase activity; mmoleH_2O_2 consumed/min/mg protein), SOD (Superoxide dismutase; units'/mg protein), GPx (glutathione peroxidase; units'/mg protein), GST (Glutathione-S-transferase; mmole1-chloro-2,4-dinitrobenzene-GSH protein), GR (Glutathione reductase; units'/mg protein), **HTR (Hypertensive rats), L-NAME (L-NG-Nitro arginine methyl ester (L-NAME), NAR (Naringenin), LISIP (Lisinopril).**

Table 4. Renal non-enzymic antioxidant defence system.

Experiments	PSH	NPSH	GSH	Vitamin C
Ctrl	58.26 ± 8.48	50.44 ± 7.38	75.73 ± 3.24	0.087 ± 0.0077
HTR	41.85 ± 9.64[a]	39.82 ± 2.77[a]	79.13 ± 4.33[a]	0.083 ± 0.011
HTR + NAR	56.38 ± 7.72[b]	44.58 ± 3.32[b]	123.33 ± 5.85[a, b]	0.089 ± 0.015
HTR + LISIP	47.06 ± 6.99[b]	40.95 ± 3.69[a]	79.87 ± 0.57[a]	0.10 ± 0.024[a, b]

Values are presented as mean ± **S.D.** (n = 10). Superscript([a]) indicate **statistically** significant **difference** when Groups B (**HTR**), C (**HTR + NAR)** and D (**HTR + LISIP)** compared with Group A at P < 0.05, Superscript ([b]) indicate **statistically** significant **difference** when Groups C and D compared with Group B. Group A (**Ctrl**; Control), Group B (**HTR;** L-NAME alone at 40 mg/kg), Group C (**HTR + NAR;** L-NAME (40 mg/kg) + Naringenin (50 mg/kg), Group D (**HTR + LISIP;** L-NAME (40 mg/kg) + Lisinopril (10 mg/kg).

PSH (Protein thiol; nmole/mg protein), NPSH (Non-protein thiol; nmole/mg protein), GSH (reduced glutathione; micromole/g tissue), VITC (vitamin C; nmole/mg protein), **HTR (Hypertensive rats), L-NAME (L-NG-Nitro arginine methyl ester (L-NAME), NAR (Naringenin), LISIP (Lisinopril).**

The results obtained from the hypertensive rats indicated significant increase in renal markers of oxidative stress including protein carbonyl, malondialdehyde and hydrogen peroxide generated (Table 5). Also, co-administration of L-NAME with either Naringenin or Lisinopril caused significant attenuation of markers of oxidative stress to near normal values indicating the free radical scavenging action of Naringenin (Table 5). The cerebral GST and SOD activity and the content of GSH decreased significantly in hypertensive untreated rats in comparison to the normotensive rats, while the hypertensive rats treated with Naringenin or Lisinopril had improvement in cerebral antioxidant enzyme activity and GSH content better than the hypertensive rats (Table 6). Similarly, we recorded increase in the content of PSH and NPSH of hypertensive rats treated with Naringenin or Lisinopril (Table 6). Lastly, our results indicate statistically significant decrease in cerebellar GST and SOD activity and the content of GSH of hypertensive untreated rats similar to that of cerebrum when compared to the normotensive rats (Table 7). However, remarkable increase in the cerebellar enzymic (GST and SOD) and non-enzymic (GSH) content was obtained in hypertensive rats treated with Naringenin or Lisinopril (Table 7).

Table 5. Renal markers of oxidative stress.

Experiments	PCO	MDA	H_2O_2
Ctrl	8.07 ± 1.48	2.13 ± 0.34	26.79 ± 1.3
HTR	11.49 ± 1.32[a]	2.75 ± 0.45[a]	30.35 ± 1.24[a]
HTR + NAR	8.59 ± 0.54[b]	1.55 ± 0.14[a, b]	25.87 ± 2.22[b]
HTR + LISIP	7.61 ± 1.12[b]	1.40 ± 0.25[a, b]	29.10 ± 2.30[a]

Values are presented as mean ± **S.D.** (n = 10). Superscript([a]) indicate **statistically** significant **difference** when Groups B (**HTR**), C (**HTR + NAR)** and D (**HTR + LISIP)** compared with Group A at P < 0.05, Superscript ([b]) indicate **statistically** significant **difference** when Groups C and D compared with Group B. Group A (**Ctrl**; Control), Group B (**HTR;** L-NAME alone at 40 mg/kg), Group C (**HTR + NAR;** L-NAME (40 mg/kg) + Naringenin (50 mg/kg), Group D (**HTR + LISIP;** L-NAME (40 mg/kg) + Lisinopril (10 mg/kg).

PCO (Protein carbonyl; µmol/mg protein), MDA (Malondialdehyde; micromole MDA formed/mg protein), H_2O_2 (Hydrogen peroxide; µmol/mg protein), **HTR (Hypertensive rats), L-NAME (L-NG-Nitro arginine methyl ester (L-NAME), NAR (Naringenin), LISIP (Lisinopril).**

Table 6. Cerebral enzymic and non-enzymic antioxidant defence status.

Experiments	GST	SOD	GSH	PSH	NPSH
Ctrl	0.081 ± 0.008	29.26 ± 1.99	131.19 ± 6.43	2.30 ± 0.47	4.46 ± 0.10
HTR	0.029 ± 0.0O2[a]	25.44 ± 20.71[a]	109.43 ± 11.12[a]	2.97 ± 0.66	4.74 ± 0.73
HTR + NAR	0.040 ± 0.009[a, b]	28.30 ± 2.66	100.88 ± 2.97[a]	3.07 ± 0.58[a]	4.47 ± 0.67
HTR + LISIP	0.049 ± 0.009[a, b]	27.04 ± 3.90	100.33 ± 8.20[a]	2.64 ± 0.63	6.15 ± 0.63[a, b]

Values are presented as mean ± **S.D.** (n = 10). Superscript([a]) indicate **statistically** significant **difference** when Groups B (**HTR**), C (**HTR + NAR)** and D (**HTR + LISIP)** compared with Group A at P < 0.05, Superscript ([b]) indicate **statistically** significant **difference** when Groups C and D compared with Group B. Group A (**Ctrl**; Control), Group B (**HTR;** L-NAME alone at 40 mg/kg), Group C (**HTR + NAR;** L-NAME (40 mg/kg) + Naringenin (50 mg/kg), Group D (**HTR + LISIP;** L-NAME (40 mg/kg) + Lisinopril (10 mg/kg).GST (Glutathione S-transferase; mmole1-chloro-2,4-dinitrobenzene-GSH protein), SOD (Superoxide dismutase; units'/mg protein), GSH (reduced glutathione; micromole/g tissue), PSH (Protein thiol; nmole/mg protein), NPSH (Non-protein thiol; nmole/mg protein), **HTR (Hypertensive rats), L-NAME (L-NG-Nitro arginine methyl ester (L-NAME), NAR (Naringenin), LISIP (Lisinopril).**

Table 7. Cerebellar enzymic and non-enzymic antioxidant defence status.

Experiments	GST	SOD	GSH	PSH	NPSH
Ctrl	0.140 ± 0.020	11.63 ± 0.71	71.53 ± 2.01	3.06 ± 0.56	3.80 ± 0.57
HTR	0.118 ± 0.008 [a]	24.94 ± 4.94[a]	67.09 ± 2.41[a]	2.58 ± 0.58	2.97 ± 0.47
HTR + NAR	0.151 ± 0.031[b]	24.59 ± 1.33[a]	71.52 ± 2.53[b]	2.84 ± 0.56	3.34 ± 0.56
HTR + LISIP	0.147 ± 0.015[b]	25.61 ± 4.52[a]	70.48 ± 2.69[b]	2.74 ± 0.58	3.28 ± 0.43

Values are presented as mean ± **S.D.** (n = 10). Superscript([a]) indicate **statistically** significant **difference** when Groups B (**HTR**), C (**HTR + NAR)** and D (**HTR + LISIP)** compared with Group A at P < 0.05, Superscript ([b]) indicate **statistically** significant **difference** when Groups C and D compared with Group B. Group A (**Ctrl**; Control), Group B (**HTR;** L-NAME alone at 40 mg/kg), Group C (**HTR + NAR;** L-NAME (40 mg/kg) + Naringenin (50 mg/kg), Group D (**HTR + LISIP;** L-NAME (40 mg/kg) + Lisinopril (10 mg/kg).GST (Glutathione S-transferase; mmole1-chloro-2,4-dinitrobenzene-GSH/min/mg protein), SOD (Superoxide dismutase; units'/mg protein), GSH (reduced glutathione; micromole/g tissue), PSH (Protein thiol; nmole/mg protein), NPSH (Non-protein thiol; nmole/mg protein), **HTR (Hypertensive rats), L-NAME (L-NG-Nitro arginine methyl ester (L-NAME), NAR (Naringenin), LISIP (Lisinopril).**

3.3. Urinalysis and haemodynamic parameters

Urinalysis revealed significant increases in urinary creatinine, blood urea nitrogen, total protein with concomitant decrease in albumin (Fig. 1). The situation was reversed in hypertensive rats treated with either Naringenin or Lisinopril which is indicative of nephron-protective effect of Naringenin (Fig. 1). Similar to that was the results of haemodynamic parameters which indicated significant increase in the systolic blood pressure, diastolic blood pressure and mean arterial pressure of hypertensive untreated rats relative to the normotensive and the hypertensive treated rats (Fig. 2). However, the values of blood pressure parameters came to near normal in hypertensive rats treated with either Naringenin or Lisinopril as indicated in Fig. 2.

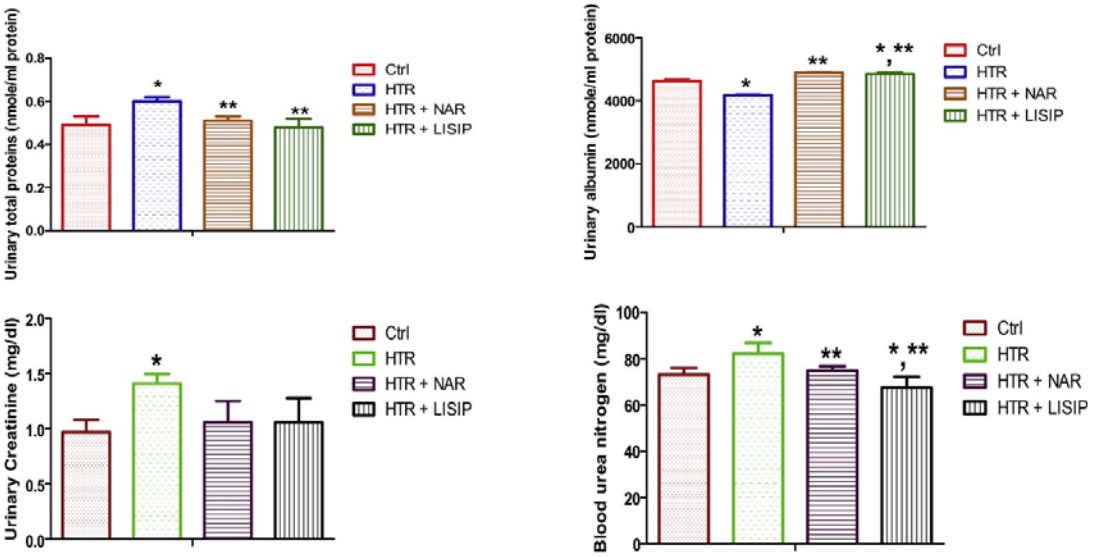

Fig. 1. Urinary markers of renal damage of Naringenin treated hypertensive rats. Values are presented as mean ± S.D. (n = 10). Asterisk (*) indicates statistically significant difference when Groups B (**HTR**), C (**HTR + NAR)** and D (**HTR + LISIP)** compared with Group A (Normotensive rats) at P < 0.05, while asterisks (**) indicate statistically significant difference when Groups C and D compared with Group B (hypertensive rats). Group A (**Ctrl**; Control), Group B (**HTR;** L-NAME alone at 40 mg/kg), Group C (**HTR + NAR;** L-NAME (40 mg/kg) + Naringenin (50 mg/kg), Group D (**HTR + LISIP;** L-NAME (40 mg/kg) + Lisinopril (10 mg/kg).

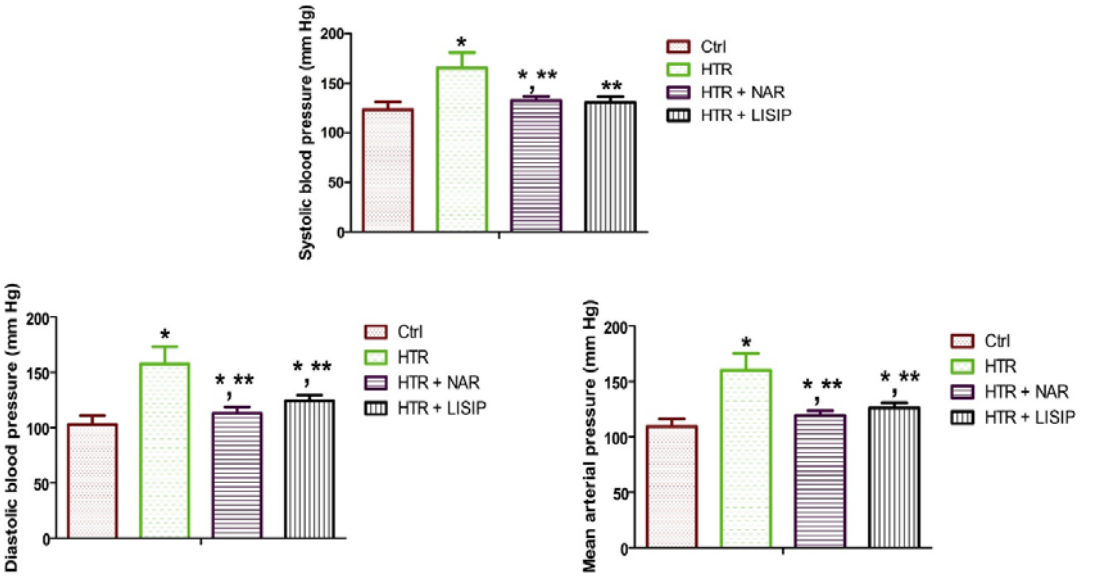

Fig. 2. Blood pressure parameters of Naringenin treated hypertensive rats. Values are presented as mean ± S.D. (n = 10). Asterisk (*) indicates statistically significant difference when Groups B (**HTR**), C (**HTR + NAR)** and D (**HTR + LISIP)** compared with Group A (Normotensive rats) at P < 0.05, while asterisks (**) indicate statistically significant difference when Groups C and D compared with Group B (hypertensive rats). Group A (**Ctrl**; Control), Group B (**HTR;** L-NAME alone at 40 mg/kg), Group C (**HTR + NAR;** L-NAME (40 mg/kg) + Naringenin (50 mg/kg), Group D (**HTR + LISIP;** L-NAME (40 mg/kg) + Lisinopril (10 mg/kg).

Our results from Fig. 3 show significant increase in cerebral malondialdehyde content, hydrogen peroxide generated together with insignificant reduction in the activity of acetylcholinesterase in hypertensive rats. The significant increase in the values of cerebral

markers of oxidative stress was significantly brought low in hypertensive rats treated with Naringenin or Lisinopril similar to that of normotensive rats (Fig. 3). Interestingly, there were significant increases in the cerebellar malondialdehyde contents and hydrogen peroxide generated with concomitant significant reduction in the activity of acetylcholinesterase as compared to the normotensive and hypertensive treated rats (Fig. 4).

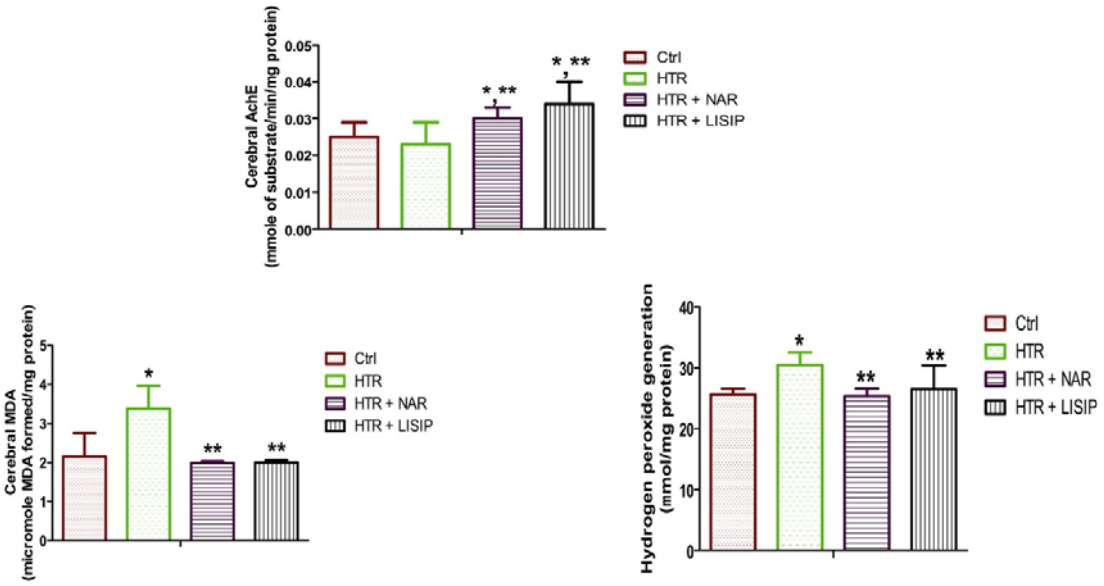

Fig. 3. Effects of Naringenin on cerebral markers of oxidative and acetylcholinesterase (AchE) activity in treated hypertensive rats. Values are presented as mean ± S.D. (n = 10). Asterisk (*) indicates statistically significant difference when Groups B (**HTR**), C (**HTR + NAR)** and D (**HTR + LISIP)** compared with Group A (Normotensive rats) at $P < 0.05$, while asterisks (**) indicate statistically significant difference when Groups C and D compared with Group B (hypertensive rats). Group A (**Ctrl**; Control), Group B (**HTR;** L-NAME alone at 40 mg/kg), Group C (**HTR + NAR;** L-NAME (40 mg/kg) + Naringenin (50 mg/kg), Group D (**HTR + LISIP;** L-NAME (40 mg/kg) + Lisinopril (10 mg/kg).

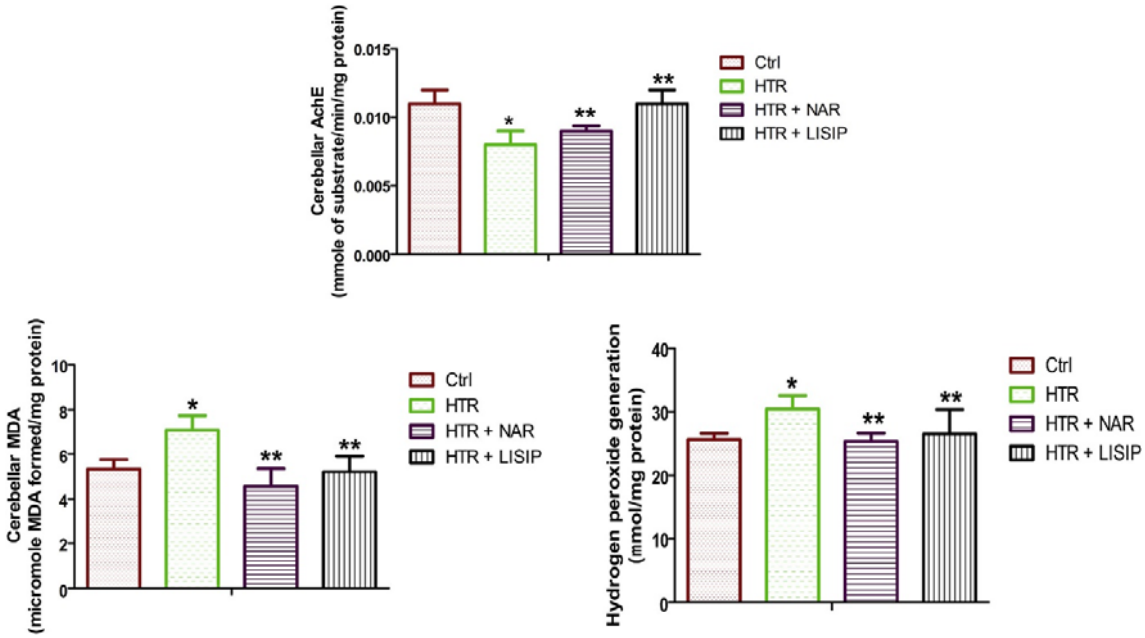

Fig. 4. Effects of Naringenin on cerebellar markers of oxidative and acetylcholinesterase (AchE) activity in treated hypertensive rats. Values are presented as mean ± S.D. (n = 10). Asterisk (*) indicates statistically significant difference when Groups B (**HTR**), C (**HTR + NAR)** and D (**HTR + LISIP)** compared with Group A (Normotensive rats) at $P < 0.05$, while asterisks (**) indicate statistically significant difference when Groups C and D compared with Group B (hypertensive rats). Group A (**Ctrl**; Control), Group B (**HTR;** L-NAME alone at 40 mg/kg), Group C (**HTR + NAR;** L-NAME (40 mg/kg) + Naringenin (50 mg/kg), Group D (**HTR + LISIP;** L-NAME (40 mg/kg) + Lisinopril (10 mg/kg).

3.4. Histopathology

The histopathology of the kidney revealed focal area of tubular degeneration and necrosis, with severe interstitial cellular infiltrates and tubular degeneration in hypertensive rats while in Naringenin treated rats, there was a mild to moderate interstitial cellular infiltration, and oedema. Also, there was a moderate to severe periglomerular oedema, mild tubular degeneration with cellular infiltration in hypertensive rats administered Lisinopril (Fig. 5). There was severe capillary congestion in the cerebrum of hypertensive untreated rats. In the Naringenin treated rats, there was mild capillary congestion and very mild foci of haemorrhages while moderate congestion of the choroid plexus and a capillary was observed in Lisinopril treated rats (Fig. 6). In the cerebellum, few Purkinje cells are degenerated/rounded/distorted in the hypertensive untreated rats. Further, haemorrhagic foci within the brain parenchyma were observed in Naringenin treated rats while a moderate to mild congestion was observed in hypertensive rats treated with Lisinopril (Fig. 7).

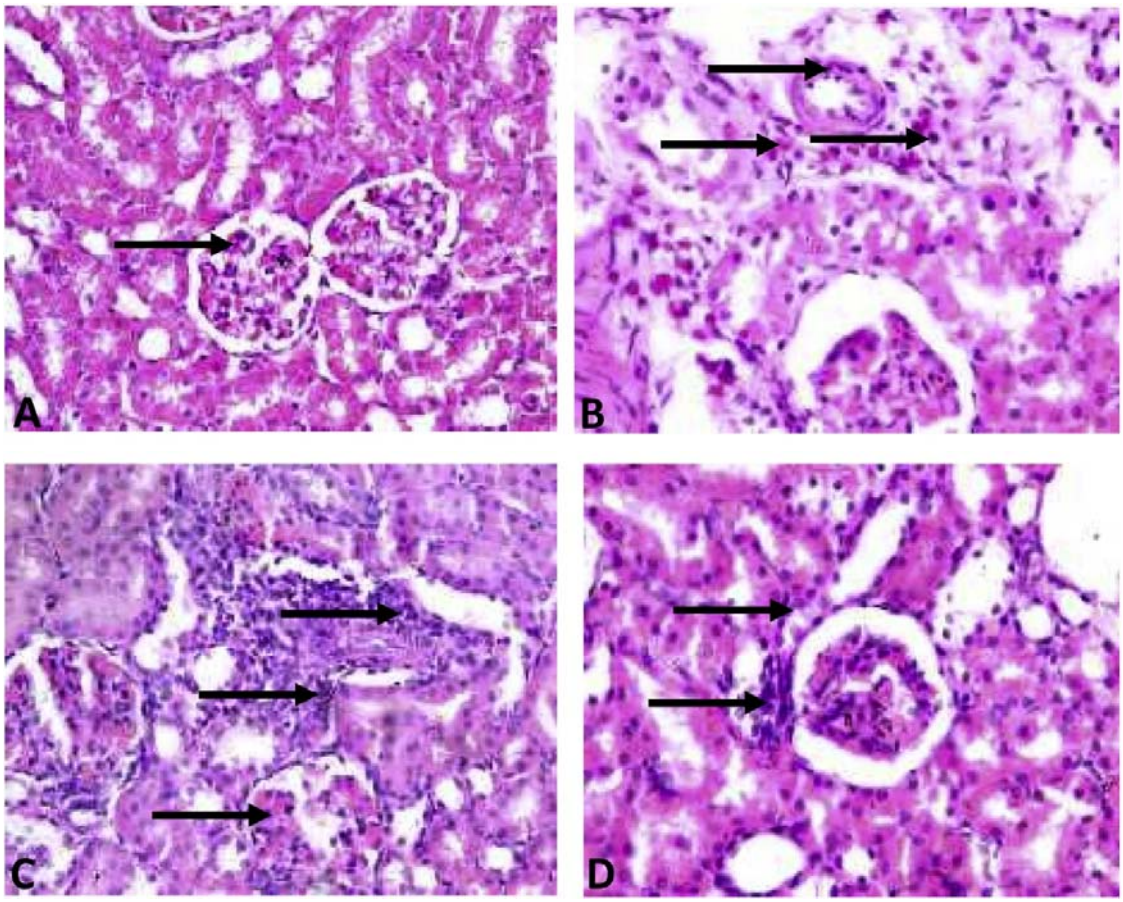

Fig. 5. Histology of the kidney: Group A (Control): There is a mild to moderate congestion at the renal cortex (arrows), Group B (L-NAME alone): There is a focal area of tubular degeneration and necrosis, with severe interstitial cellular infiltrates (arrows) Tubular degeneration; Group C (L-NAME + Naringenin): There is a mild to moderate interstitial cellular infiltration, and oedema. Group D (L-NAME + Lisinopril): There is a moderate to severe periglomerular oedema, mild tubular degeneration with cellular infiltration. Plates are stained with H and E stains and viewed with x 100 objectives.

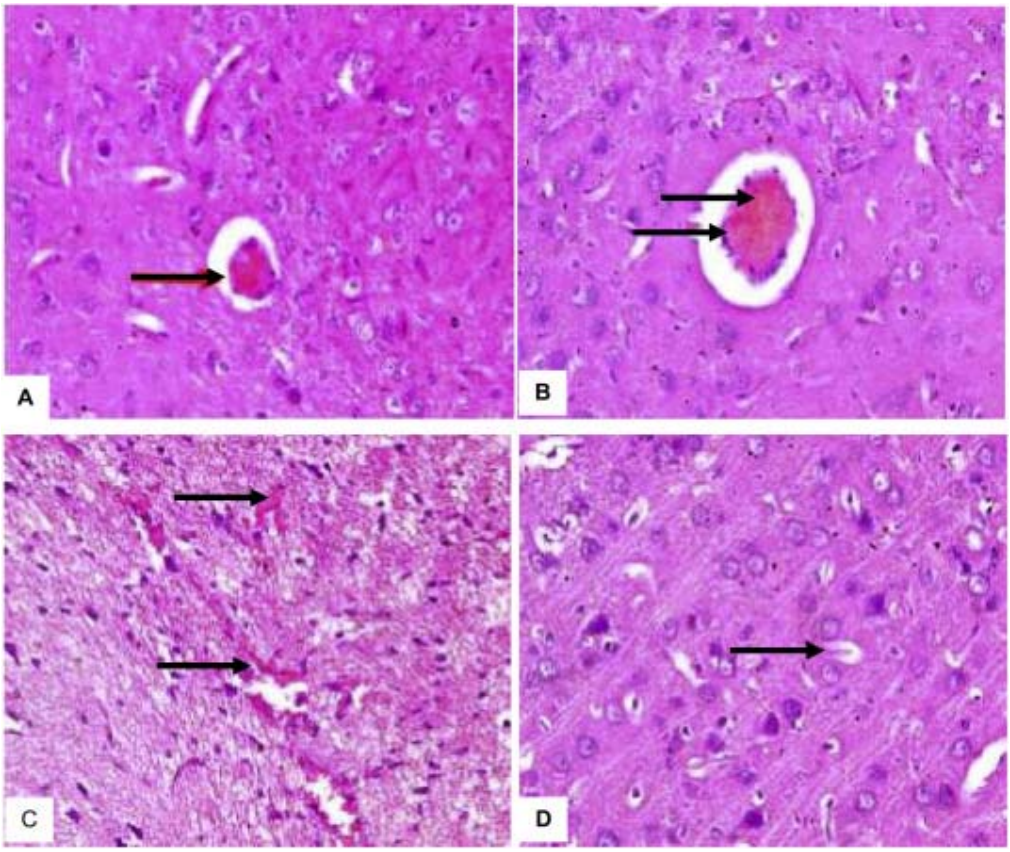

Fig. 6. Histology of the cerebrum: Group A (Control): Mild to moderate congestion of the capillaries. (cerebral cortex) (arrows), Group B (L-NAME alone): There is a severe capillary congestion (arrows); Group C (L-NAME + Naringenin): Mild capillary congestion (black arrow), very mild foci of haemo*rrhages* (red arrows). Group D (L-NAME + Lisinopril): Moderate congestion of the choroid plexus and a capillary. Plates are stained with H and E stains and viewed with x 100 objectives.

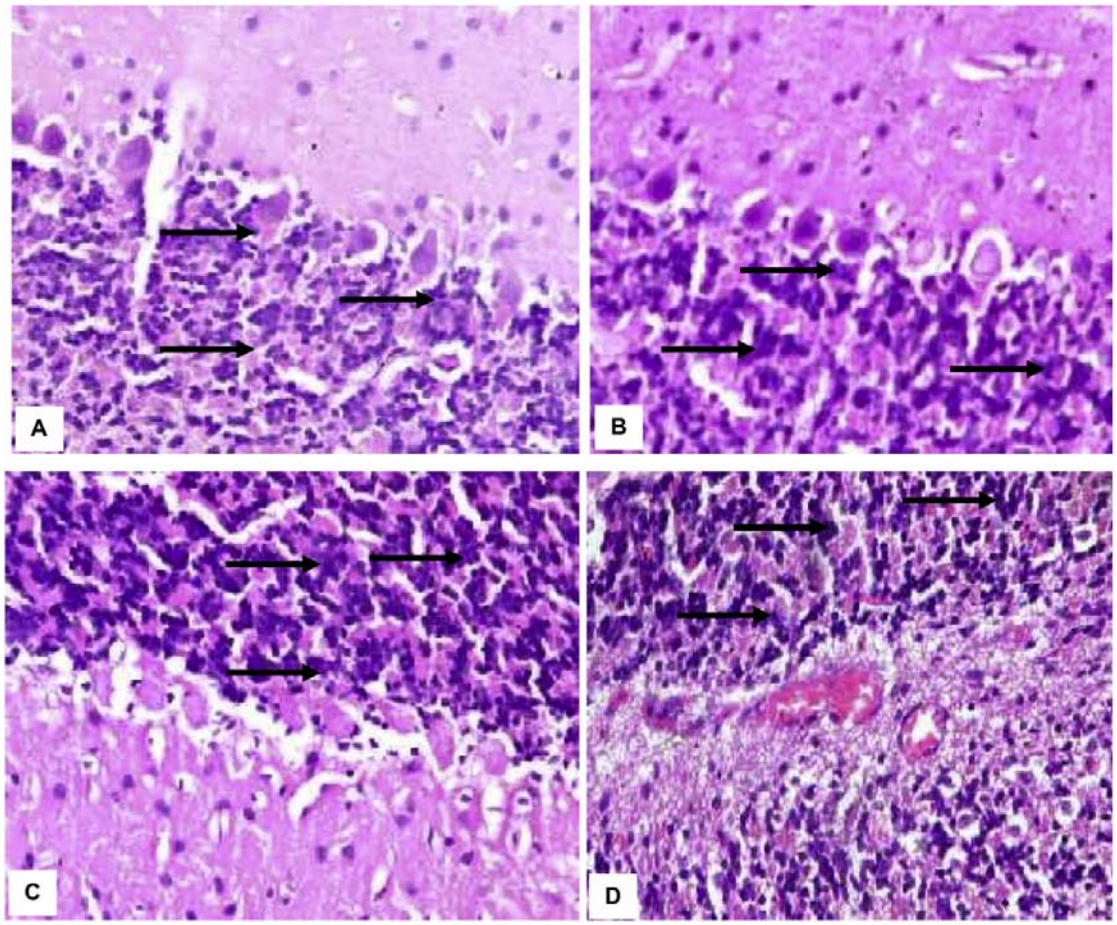

Fig. 7. **Histology of the cerebellum: Group A (Control):** There is No visible lesion (arrows), Group B (L-NAME alone): Few purkinje cells are degenerate/rounded/distorted (arrows); Group C (L-NAME + Naringenin): Haemorrhagic foci within the brain parenchyma. Group D (L-NAME + Lisinopril): There is a moderate to Mild congestion. Plates are stained with H and E stains and viewed with x 100 objectives.

3.5. Immunohistochemistry

There were significant expressions of the immunolocalization of Kidney injury molecule 1 in hypertensive rats. However, the expressions were significantly lowered in hypertensive rats treated with Naringenin or Lisinopril (Fig. 8). From this result, treatment of hypertensive rats with Lisinopril gave a better protection against renal damage associated with hypertension. We also observed significant increase in the expressions of angiotensin converting enzyme in hypertensive untreated rats (Fig. 9). The antihypertensive action of Naringenin was demonstrated with significant reduction in the expressions of renal angiotensin converting enzyme in hypertensive rats that received Naringenin with better reduction in the expressions of angiotensin converting enzyme with Lisinopril (Fig. 9). Also, immune-positive expressions of mineralocorticoid receptor increased significantly in hypertensive rats while the expressions of mineralocorticoid receptor were significantly down-regulated in hypertensive rats co-administered with either Naringenin or Lisinopril (Fig. 10).

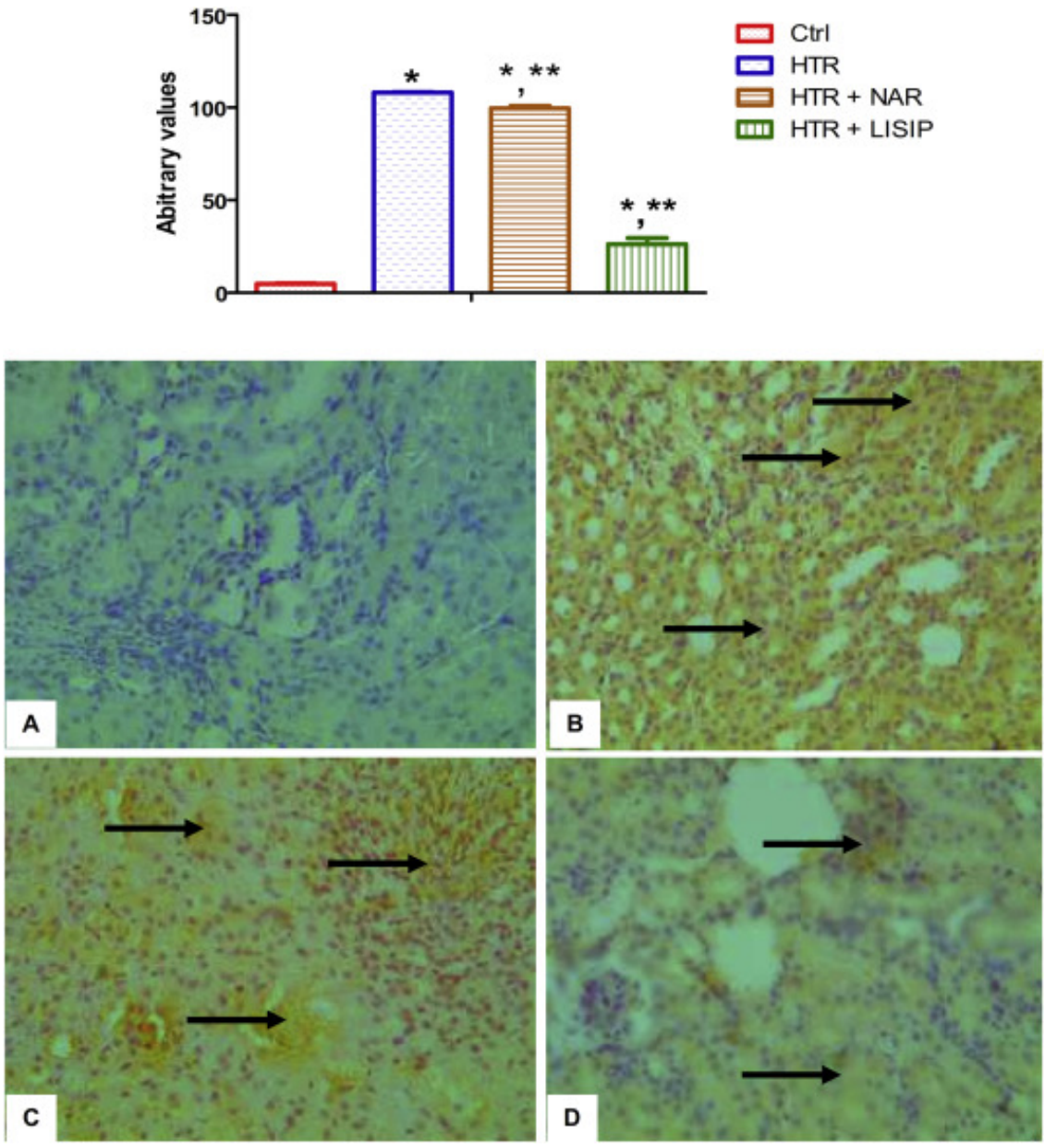

Fig. 8. The immunoreactivity of Kidney injury molecule 1 (Kim-1)**:** Group A (Control) shows lower expressions of ACE while Groups B (**HTR**) shows higher expressions of Kim-1 than the Control. However, Groups C (**HTR + NAR)** and D (**HTR + LISIP)** show lower expression of Kim-1 when compared Groups B (**HTR**). Asterisk (*) indicates statistically significant difference when Groups B (**HTR**), C (**HTR + NAR)** and D (**HTR + LISIP)** compared with Group A (Normotensive rats) at $P < 0.05$, while asterisks (**) indicate statistically significant difference when Groups C and D compared with Group B (hypertensive rats). Group A (**Ctrl**; Control), Group B (**HTR;** L-NAME alone at 40 mg/kg), Group C (**HTR + NAR;** L-NAME (40 mg/kg) + Naringenin (50 mg/kg), Group D (**HTR + LISIP;** L-NAME (40 mg/kg) + Lisinopril (10 mg/kg).

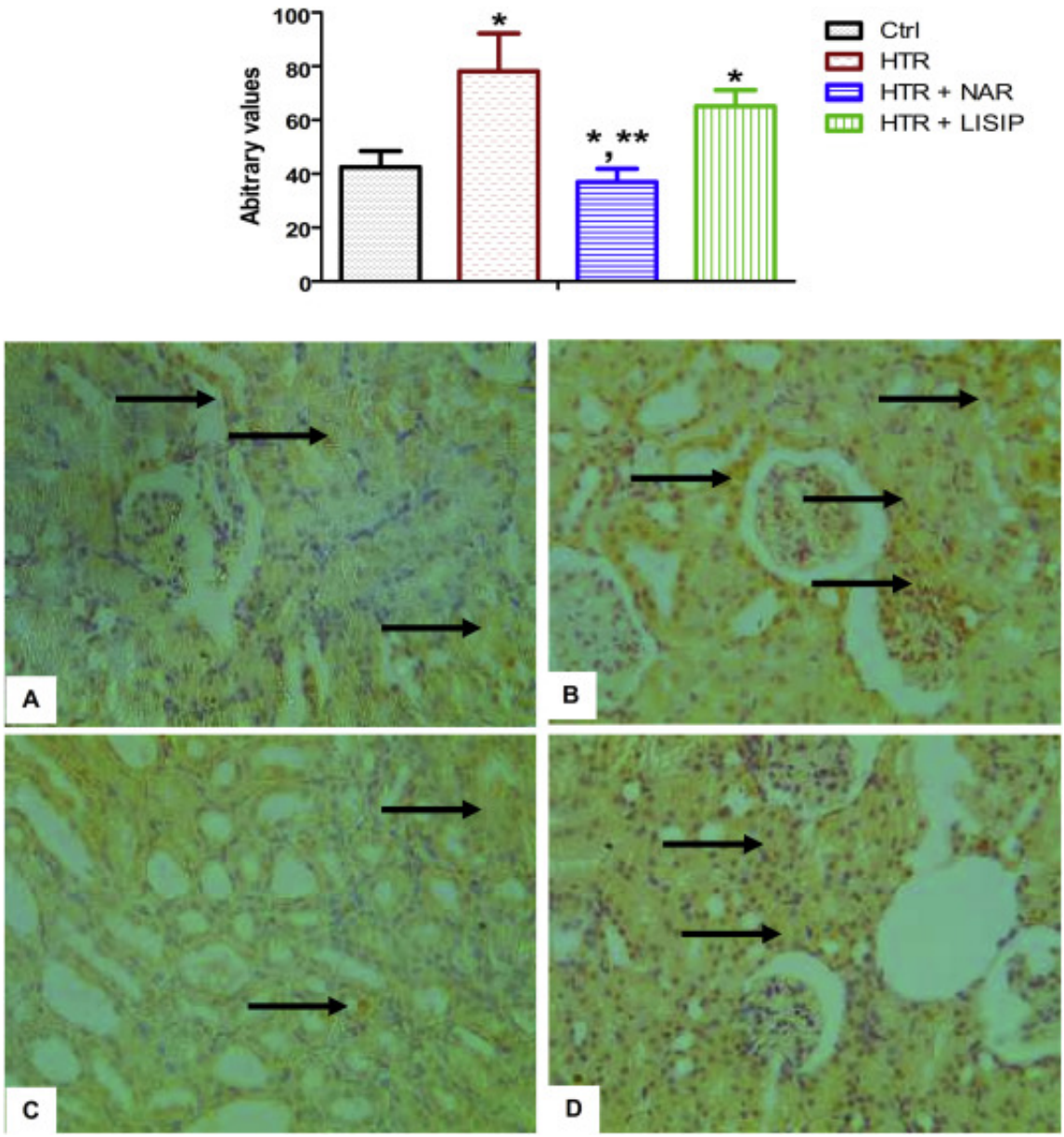

Fig. 9. **The immunoreactivity of Angiotensin Converting Enzyme (ACE):** Group A (Control) shows lower expressions of ACE while Groups B (**HTR**) shows higher expressions of ACE than the Control. However, Groups C (**HTR + NAR)** and D (**HTR + LISIP)** show lower expression of ACE when compared Groups B (**HTR**). Asterisk (*) indicates statistically significant difference when Groups B (**HTR**), C (**HTR + NAR)** and D (**HTR + LISIP)** compared with Group A (Normotensive rats) at $P < 0.05$, while asterisks (**) indicate statistically significant difference when Groups C and D compared with Group B (hypertensive rats). Group A (**Ctrl**; Control), Group B (**HTR;** L-NAME alone at 40 mg/kg), Group C (**HTR + NAR;** L-NAME (40 mg/kg) + Naringenin (50 mg/kg), Group D (**HTR + LISIP;** L-NAME (40 mg/kg) + Lisinopril (10 mg/kg).

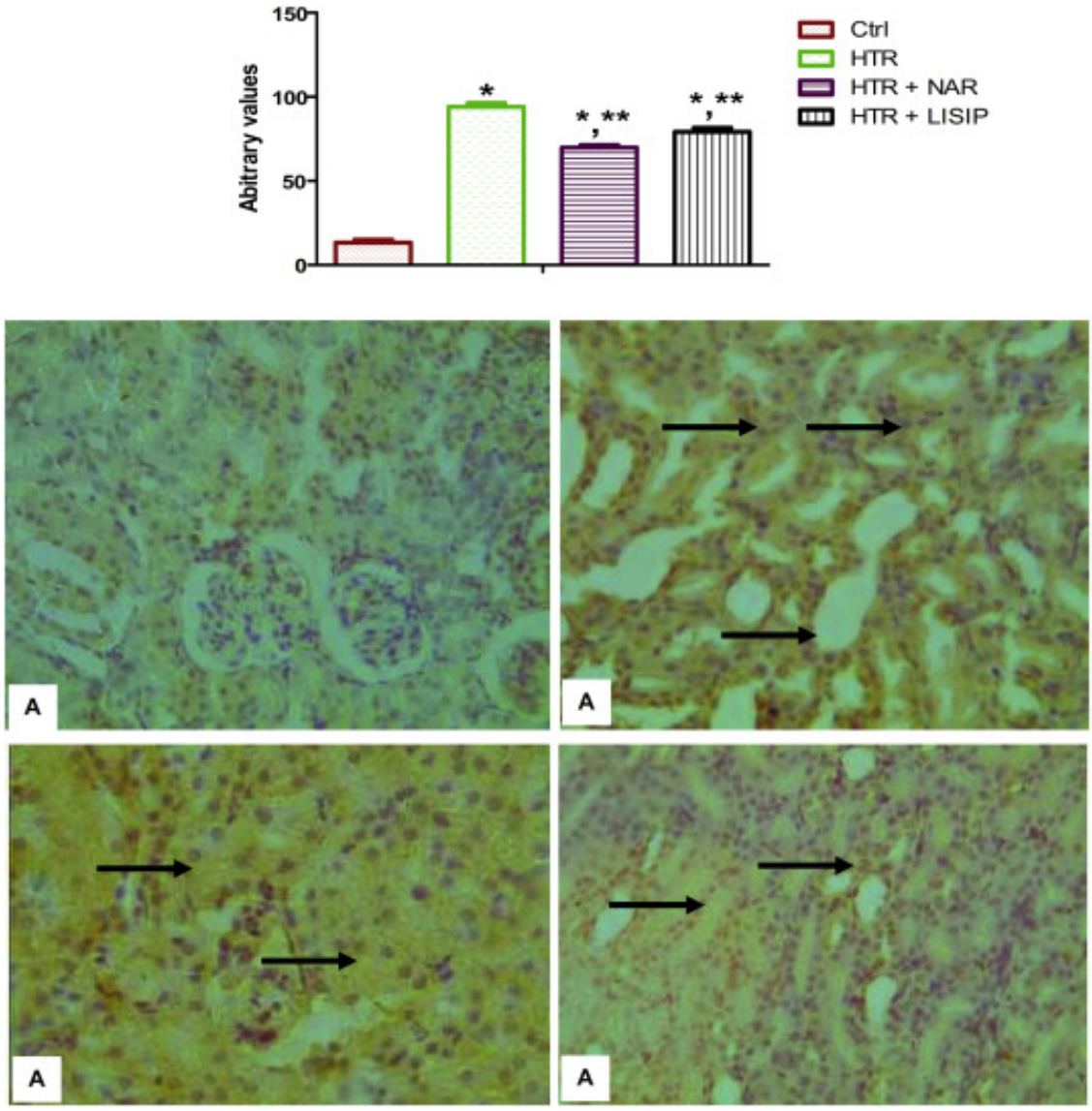

Fig. 10. The immunoreactivity of Mineralocorticoid receptor: Group A (Control) shows lower expressions of MCR while Groups B (**HTR**) shows higher expressions of MCR than the Control. However, Groups C (**HTR + NAR)** and D (**HTR + LISIP)** show lower expression of MCR when compared Groups B (**HTR**). Asterisk (*) indicates statistically significant difference when Groups B (**HTR**), C (**HTR + NAR)** and D (**HTR + LISIP)** compared with Group A (Normotensive rats) at $P < 0.05$, while asterisks (**) indicate statistically significant difference when Groups C and D compared with Group B (hypertensive rats). Group A (**Ctrl**; Control), Group B (**HTR;** L-NAME alone at 40 mg/kg), Group C (**HTR + NAR;** L-NAME (40 mg/kg) + Naringenin (50 mg/kg), Group D (**HTR + LISIP;** L-NAME (40 mg/kg) + Lisinopril (10 mg/kg).

4. DISCUSSION

Oxidative stress is known to play an important role in the development of hypertension (Loperena and Harrison, 2017). Increased blood pressure has been shown to promotes vascular smooth muscle cell proliferation, hypertrophy and collagen deposition, thickening of the vascular media and narrowing of the vascular lumen (Liu et al., 2019; Zhang et al., 2018). It has also been observed that increased oxidative stress may damage the endothelium and impair endothelium-dependent vascular relaxation with resultant increase

in vascular contractile activity (Ali et al., 2019; Žėkas et al., 2019). Elsewhere, it was reported that reactive oxygen species (ROS) production particularly in the kidney and the centers within the brain participate in blood pressure regulation (Harrison et al., 2007).

From the present study, ultrastructural anarchy such as focal area of tubular degeneration, necrosis and severe interstitial cellular infiltrations accompanied by severe weight loss and significant increase in kidney weight were observed in hypertensive rats. Severe capillary congestion and Purkinje cell degeneration was observed in the cerebrum and cerebellum, respectively, in hypertensive untreated rats. All these were ameliorated in hypertensive rats treated with Naringenin. We therefore speculate that enhanced production of ROS and depletion of antioxidant defence system might have contributed significantly to the observed renal and neuronal pathology in hypertensive rats. The involvement of oxidative stress in hypertension as demonstrated in this study was accompanied with significant depletion in renal and neuronal antioxidant defence system coupled with exaggerated levels of markers of oxidative stress (malondialdehyde, protein carbonyl and hydrogen peroxide) generation in hypertensive untreated rats. Furthermore, numerous studies have linked excessive amounts of ROS in essential hypertensive patients and various animal models of hypertension (Saheera et al., 2019). Similarly, activation of NADPH oxidase and xanthine oxidase, the uncoupling eNOS and mitochondrial dysfunction have been directedly corelated with chronic hypertension and production of superoxide anion radicals (Brito et al., 2015). However, previous evidence reported hypertensive stimuli such that high-salt diets and angiotensin II, promoted the production of ROS in the brain, the kidney, and the vasculature (Harrison and Gongora, 2009). Interestingly, inactivation of signaling molecules such as nitric oxide by superoxide anion radicals and impairment endothelium-dependent vasodilation has been previously reported in hypertension (Hasdan et al., 2002).

Results from hypertensive rats showed significant accumulation of toxic lipid by-product (malondialdehyde), protein oxidation product (protein carbonyl) and hydrogen peroxide levels in both renal and brain tissues signifying the impact of hypertension on the central nervous system. In fact, both cerebral and cerebellar markers of oxidative stress increased astronomically in hypertensive rats. However, inhibition of superoxide dismutase, catalase and glutathione peroxidase activity ultimately resulted in accumulation of superoxide anion radicals (O^{-}_{2}) and hydrogen peroxide which might have contributed significantly to the development of oxidative stress as observed in this study. The enhanced production of superoxide anion radicals has the potential to combine with available nitric oxide thereby depleting serum nitric oxide bioavailability with the formation of highly toxic, reactive and cytotoxic peroxynitrite ($ONOO^{-}$) as also reported by other authors (Sárközy et al., 2018). More so, enhanced production of $ONOO^{-}$ has been implicated to mediate vascular remodeling, arterial stiffness, endothelial dysfunction, right-ventricular dysfunction and hypertension (Jankov et al., 2010). Also, significant increases in the markers of oxidative stress were accompanied by significant depletion of reduced glutathione, non-protein thiol (NPSH), protein thiol (PSH) and vitamin C contents, respectively. The reduced glutathione is a tripeptide that consists of glycine, cysteine and glutamate. The free sulfhydryl (SH) group of the cysteine molecule within reduced glutathione accounts for its capacity to neutralize ROS and increase antioxidant capacity (May et al., 2003 & 2001). Interestingly, treatment of hypertensive rats with Naringenin caused a significant improvement in the non-enzymic antioxidant status both in the renal and neuronal tissues, respectively. Worthy of note, in

this study, there were significant decreases in the makers of oxidative stress and improvement in the antioxidant in hypertensive rats that received Naringenin. Our data bring to light the importance of antioxidant therapy in the treatment of hypertension. This study therefore confirms the earlier reports on the antioxidant power of Naringenin (Rashmi et al., 2018; Butkhup et al., 2017; Martinez et al., 2015).

In the present study, hypertension precipitated significant elevation of serum advanced oxidative protein product levels and myeloperoxidase activity. This observation supports previous finding that accumulation of advanced oxidative protein products and myeloperoxidase may enhance oxidative stress and facilitate inflammatory response (Meng et al., 2019; Skoie et al., 2019). The advanced oxidation protein products as end-products of protein oxidation are known to participate in the pathophysiology and pathogenesis of hypertension (Xu et al., 2015; Meloche et al., 2013; Zhou et al., 2012). Hence, possible involvement of critical proteins following enhanced lipid peroxidation by hypertension seemed to have contributed to the observed high contents of protein carbonyl and advanced oxidative protein product in the brain and renal tissues. The increased activity of myeloperoxidase has been documented to enhance production and accumulation of advanced oxidative protein product that can ultimately trigger inflammation and immune dysregulation (Cristani et al., 2016). The levels of these two biomarkers may have negative impact on the CNS including chronic fatigue and ageing as reported by Lee et al. (2018) and Cristani et al. (2016). The anti-inflammatory property of Naringenin was demonstrated as it significantly inhibited myeloperoxidase activity. The involvement of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) signaling and vascular cell adhesion molecule 1 (VCAM-1) in an experimental model of metabolic syndrome with fructose-rich diet-induced hypertensive rats has been documented (Viedt et al., 2002). Of particular interest is the strong association and positive correlation between blood levels of advanced oxidative protein products and myeloperoxidase with uremia, arteriosclerosis and oxidation of low-density lipoprotein (ox-LDL) as previously reported (Guo et al., 2009; Liu et al., 2006). We therefore propose that the exaggerated high levels of advanced oxidative protein products and myeloperoxidase inhibited endothelial nitric oxide synthase (eNOS) activity, thereby accelerating the development of hypertension and renal damage.

For the past few decades, hypertension has been reported as a major risk factor for cardiovascular diseases, chronic kidney disease and death (Kario et al., 2016; Abdi et al., 2018). From the present study, it was observed that in the hypertensive rats, the urinary creatinine, blood urea nitrogen and total protein increased significantly while the albumin decreased significantly. Previous documentation has reported hypertension to precipitate renal damage and chronic kidney disease (van Duijl et al., 2019). However, novel biomarkers of renal damage with high specificity have been extensively documented (Waring and Moonie, 2011). The abnormal elevation of renal function tests has shown positive correlation with reduced glomerular filtration rate and deterioration in renal function (Kobayashi et al., 2019; Onohara et al., 2018; Wang et al., 2019). The observed exaggerated increase in urinary creatinine, blood urea nitrogen and total protein might be an indication of renal damage together with a sustained elevation of blood pressure in hypertensive untreated rats. However, the urinary biomarkers of renal damage were lowered significantly in hypertensive rats that received Naringenin similar to those that were administered Lisinopril. Again, the elevated levels of systolic, diastolic and mean arterial pressures

observed in hypertensive rats were normalized to near normal values of normotensive rats in hypertensive rats administered Naringenin. The recorded elevated blood pressure could be supported by significant decrease in nitric oxide bioavailability in hypertensive rats. The reduced serum nitric oxide bioavailability has been positively correlated with hypertension (Gheibi et al., 2018; Mels et al., 2016; Li et al., 2015). Our data indicated that co-treated with Naringenin significantly improved serum nitric oxide bioavailability, thereby mitigating the inhibitory effect of hypertension on (eNOS) activity. This study therefore confirmed the antihypertensive effect of Naringenin in monocrotaline-induced pulmonary hypertension as previously reported by Ahmed et al. (2014). It has been reported that plant-based dietary phytonutrients such flavonoids, anthocyanines, and phenolic are known to have potential health benefits for the treatment of hypertension, metabolic syndrome, cardiovascular diseases, and obesity (Martin and Appel, 2010). In this study, we observed that Naringenin significantly reduced the expressions of Kidney injury molecule 1 in hypertensive treated rats. The kidney injury molecule 1 is highly expressed in the proximal tubule in acute renal injury and benign renal tumour (Kushlinskii et al., 2019; Waring and Moonie, 2011). The kidney injury molecule 1 is a type 1 transmembrane protein, with an immunoglobulin and mucin domain, and its expressions have been shown to be markedly up-regulated in the proximal tubule in the post-ischemic rat kidney (Han et al., 2002; Khan et al., 2019). The observed significant reduction in the expressions of kidney injury molecule 1 in hypertensive treated rats is an indication of nephroprotective effect of Naringenin. Hence, kidney injury molecule 1 could serve as a molecular therapeutic target of nephroprotection for drug candidates against renal damage associated with hypertension. Similarly, the observed proteinuria might have also contributed significantly to renal damage, enhanced expressions of kidney injury molecule 1 and angiotensin converting enzyme and overstimulation of mineralocorticoid receptor. Previously, Holtkamp et al. (2011) documented that intervention in the renin-angiotensin-aldosterone-system (RAAS) pathway was associated with slowing the progressive loss of renal function. Therefore, there is a cross-talk between mineralocorticoid receptor and angiotensin converting enzyme signaling pathway as a novel molecular therapeutic target for maintenance of blood pressure in hypertensive individuals. We speculated that blockage of both mineralocorticoid receptor and angiotensin converting enzyme as a double-edged sword is an added advantage over inhibition of angiotensin converting enzyme alone for the treatment of hypertension and associated complication such as renal damage. Naringenin as a novel antihypertensive agent significantly reduced the expressions of both mineralocorticoid receptor and angiotensin converting enzyme which is indicative of the molecular mechanism of antihypertensive effect of Naringenin. From our findings, the antihypertensive effect of Naringenin is superior over Lisinopril which is a classic angiotensin converting enzyme inhibitor. The renin-angiotensin-aldosterone system (RAAS) has been reported to play critical roles in the pathogenesis of arterial hypertension (Horký, 2007). Also, the pathogenesis of mineralocorticoid receptor - associated hypertension with normal plasma aldosterone levels has been reported to be mediated via mineralocorticoid receptor overstimulation as observed in the present study (Shibata and Itoh, 2012). From this study, we recorded proteinuria, this might be linked to overstimulation of mineralocorticoid receptor in the kidney, which was in line with the report of Nagase and Fujita (2009) in which overstimulation of mineralocorticoid receptor resulted in proteinuria and glomerulosclerosis. The renin-angiotensin system (RAS) is a major regulator of blood pressure and vascular response to injury, and that inhibition of RAS might provide a protection against an end-stage organ damage (Weir, 2007). The inhibition

of the RAS has been shown to have antihypertensive and renoprotective (Nishiyama et al., 2010). It is therefore not surprising that Naringenin normalized blood pressure and offered protection against renal damage as observed in the present study.

Taken together, findings from this study demonstrated the beneficial effects of fruits and vegetables that are naturally laden with Naringenin as novel sources of antihypertensive agents. Naringenin, from the present study proved that it could be recommended as drug candidate for the management and treatment of hypertension due to its antioxidant, anti-inflammatory and nephroprotective property. Similarly, the use of Naringenin as antihypertensive agent could open a novel therapeutic window for the use of plant-derived phytochemicals against hypertension. Collectively, we report for the first time novel antihypertensive and neuroprotective of Naringenin through down regulation of kidney injury molecule, mineralocorticoid receptor and angiotensin converting enzyme signaling pathways.

AUTHOR CONTRIBUTION LIST

AAO conceived and designed the study. FOH, OEO, TOO, OSA, AOA, ABS, AAA, MOO, SMN, LJM, KOS, IOO, and BOS analyzed the data, and AAO and FOH drafted the manuscript. All authors read and approved the final version of the manuscript.

Declaration of competing interest

The authors declare no conflicts of interest.

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RESEARCH DATA FOR THIS ARTICLE

Data not available / The authors do not have permission to share data

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