Research Communication

Luteolin-mediated Kim-1/NF-kB/Nrf2 signaling pathways protects sodium fluoride-induced hypertension and cardiovascular complications

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Abstract

The use of sodium fluoride (NaF) as a major ingredient for tooth paste, mouth wash, and mouth rinse has become inevitable in our day-to-day life. However, flavonoids such as Luteolin might be of great value in the prevention of toxicity associated with accidental or inevitable ingestion of NaF. In the study, 40 male Wistar albino rats were randomly divided into four groups with 10 rats in a group. Group A was the control group and received normal saline, Group B was exposed to NaF at 300 ppm (300 mg/L) in drinking water daily for a week, Groups C and D were exposed to 300 ppm (300 mg/L) of NaF and coadministered with Luteolin orally daily at a dosage of 100 mg/kg and 200 mg/kg for the same time point. Our results indicated that NaF caused significant increases in systolic blood pressure, diastolic blood pressure, mean arterial pressure, malondialdehyde, protein carbonyl, myeloperoxidase, advanced oxidative protein products, together with significant reductions in glutathione peroxidase, superoxide dismutase, catalase, glutathione reductase, reduced glutathione, and nitric oxide (NO) bioavailability. The electrocardiogram results showed that NaF alone caused significant prolongation of QT and QTc intervals. Immunohistochemistry revealed that NaF caused increase expressions of Kidney injury marker 1 (Kim-1), nuclear factor

Abbreviations: ANOVA, One-way analysis of variance; AOPP, Advanced oxidation protein product; BUN, Blood urea nitrogen; CAT, Catalase; CDNB, 1, 2 Dichloro 4-nitrobenzene; CTnI, Cardiac troponin I; DAB, 3, 3′-Diaminobenzidine; DBP, Diastolic blood pressure; ECG, Electrocardiogram; GPx, Glutathione peroxidase; GRed, Glutathione reductase; GSH, Reduced glutathione; GST, Glutathione-S-transferase; H_2O_2, Hydrogen peroxide generation; HRP, Horse Radish Peroxidase; I/R, Ischemia/reperfusion; Kim-1, Kidney injury marker 1; MAP, Mean arterial pressure; MDA, Malondialdehyde; MPO, Myeloperoxidase; NaF, Sodium fluoride; NF-κB, Nuclear factor kappa bet; NO, Nitric oxide; Nrf2, Nuclear factor erythroid 2 related factors 2; ROS, Reactive oxygen species; SBP, Diastolic blood pressure; SOD, Superoxide dismutase; TBA, Thiobarbituric acid; TCA, Trichloro acetic acid; XO, Xanthine oxidase

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kappa bet (NF-κB), nuclear factor erythroid 2-related factors 2 (Nrf2), and cardiac troponin I (CTnI). Together, Luteolin coadministration with NaF improved NO bioavailability, reduced high blood pressure, markers of oxidative stress, reversed prolongation of QT and QTc intervals, and lowered the expressions of Kim-1, NF-κB, and CTnI. © 2018 BioFactors, 9999 (9999):1–14, 2018

Keywords: *sodium fluoride; Luteolin; hypertension; nutraceuticals*

1. Introduction

Sodium fluoride (NaF) is extensively used for the prevention of dental caries and tooth decay in the form of fluorinated drinking water, salts or milk, tooth pastes, mouth washes, and fluoride tablets [1]. Occupational exposure to organic fluoride has been reported to induce abnormal menstruation, increase the frequency of miscarriages and pregnancy complications among female workers in fluorine factories [2]. However, fluoride (F) anions are widely distributed in the environment and are naturally present in water sources and drinking water as they are released from the run-off fluoride containing rocks and soils, and then leach into groundwater [3]. Fluoride has been reported to cross the cell membranes and enter soft tissues [4]. Hence, fluorosis is a slow and progressive process of fluoride toxicosis causing metabolic, functional and structural damages affecting many tissues particularly musculoskeletal, dental systems [5,6], kidney [7], liver [8], and brain [9]. Previous studies reported that fluoride induced excessive production of free radicals with concomitant depletion in biological activities of some antioxidant enzymes [10]. Furthermore, Shivarajashankara *et al*. [11] reported that fluoride toxicity caused increased lipid peroxidation and disruption of antioxidant defense systems in brain, erythrocytes and liver in experimental animals.

Fluoride toxicity has been documented to increase the number of fetal resorptions and mortality due to oxidative damages [12,13]. Furthermore, it has been reported that high fluoride levels can cause accumulation of large amounts of free radicals and peroxides, thereby inhibiting superoxide dismutase (SOD) and glutathione peroxidase (GPx) activities with resultant cellular damage especially in areas endemic to fluorosis [14–16]. Another report documented that fluoride caused inhibition of SOD, GPx, and catalase (CAT) in the ovary coupled with enhancement of lipid peroxidation [17]. Also, various findings have suggested that fluoride can cross the placenta of rats [18], guinea pigs [19], rabbits [20], and Holstein cows [21]. Fluoride has also been reported to induce oxidative stress which plays an important role in progression and pathophysiology of a variety of cardiac disorders such as cardiac failure and ischemia [22].

Amini *et al*. [23] reported an ecological study on the relationship between fluoride concentrations in ground water and blood pressure in an Iranian population. Several human studies have also examined the relationship between excessive fluoride intake from drinking water and hypertension (HT). In another finding, Singh *et al*. [24] reported association between higher systolic blood pressure and increased left atrial diameter in patients with endemic skeletal fluorosis. Sun *et al*. [25] documented the effect on HT of high water fluoride and plasma endothelin 1 (ET-1) levels. Similarly, Susheela *et al*. [26] reported calcification and degeneration of smooth muscle fibers in the tunica media in the aorta of rabbits after being administered fluoride for 17–24 months; which in turn might cause an increase in blood pressure. Recently in our laboratory, we reported that NaF induced hypertension and cardiac complications through generation of reactive oxygen species and activation of nuclear factor kappa beta in a dose-dependent manner [27]. Flavonoids are a large and diverse group of phytochemicals with enormous protective roles [28]. Luteolin, 3′, 4′, 5, 7-tetrahydroxyflavone, belongs to a group of naturally occurring compounds called flavonoids that are widely found in the plant kingdom. It has been extensively documented that vegetables and fruits such as celery, parsley, broccoli, onion leaves, carrots, peppers, cabbages, apple skins, and chrysanthemum flowers are rich in luteolin [29–32]. Luteolin, is known to possess beneficial effects including anti-inflammatory [33], antidiabetic [34], cardio protective [35], and antiproliferative [36], and antioxidant properties [37]. Luteolin has been reported to ameliorate hypertensive complications such as vascular remodeling by inhibiting the proliferation and migration of vascular smooth muscle cells (VSMC) [38].

This study was designed to investigate the beneficial effects of Luteolin on NaF-induced hypertension and cardiac complications together with its molecular mechanism of action in experimental rat model.

2. Materials and methods

2.1. Chemicals

Luteolin, NaF, 1,2-dichloro-4-nitrobenzene, thiobarbituric acid (TBA), trichloro acetic acid (TCA), 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). Normal goat serum, Biotinylated antibody and Horse Radish Peroxidase (HRP) System was purchased from (KPL, Inc., Gaithersburg, MD). Kidney injury marker 1 (Kim-1), nuclear factor kappa bet (NF-κB), nuclear factor erythroid 2 related factors 2 (Nrf2), and cardiac troponin I (CTnI) antibodies were purchased from (Bioss Inc. Woburn, MA) while 3, 3′-Diaminobenzidine (DAB) tablets were purchased from (AMRESCO LLC., OH). All other chemicals were of analytical grade.

Experimental animals and design

In the study, 40 male Wistar albino rats were randomly divided into four groups with 10 rats in a group. Group A was the control group which was given normal saline, Group B was exposed to 300 ppm (300 mg/L) of NaF in drinking water daily for a week, Groups C and D were exposed to 300 ppm (300 mg/L) of NaF and concurrently administered Luteolin orally daily at a dosage of 100 and 200 mg/kg for the same time point. The dosage of Luteolin was chosen based on the previous studies but with slight modifications [39–41].

They 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 pressure and electrocardiogram (ECG) of the rats were taken on the 8th day and rats were sacrificed on the 9th day. All the animals received humane care according to the criteria outlined in the Public Health Service Policy on Humane Care and the Use of Laboratory Animals [42].

Blood pressure measurement

Twenty-four hours after the last administration of NaF, blood pressure parameters, including systolic (SBP), diastolic (DBP), and mean arterial (MAP) blood pressures were determined non-invasively in conscious animals by tail plethysmography using an automated blood pressure monitor (CODA S1, Kent Scientific Corporation, CT). The average of at least nine readings was recorded per animal in the quiescent state, following acclimatization period.

Electrocardiography (ECG)

Standard lead II ECG was recorded in conscious rats using a 7-lead ECG machine (EDAN VE-1010, Shanghai, China). The machine was calibrated at 20 mm / mV paper speed and 50 mm/s paper speed. From the ECG, parameters such as heart rate, P-wave duration, PR-interval, QRS duration, R-amplitude, QT segment, and Bazett's correction of the QT interval were determined. The rats were anesthetized with xylazine/ketamine (v/v) 0.1 ml/100 g of rats and administered intramuscularly.

Blood sample collection and serum preparation

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

Preparation of renal and cardiac homogenates

The organs (kidneys and hearts) excised were 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 cardiac and renal homogenates were used for biochemical assays.

2.2. Biochemical assays

Renal and cardiac makers of oxidative stress

Advanced oxidation protein product (AOPP) contents were determined as described by Kayali *et al*. [43]. 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. Hydrogen peroxide generation was determined according to the method of Wolff [44]. The malondialdehyde (MDA) content as an index of lipid peroxidation was quantified in the PMFs of cardiac and renal tissue according to the method Varshney and Kale [45]. Lipid peroxidation was calculated with a molar extinction coefficient of 1.56×10^5/M/cm. Protein carbonyl (PCO) contents in the renal and cardiac tissues were measured using the method of Reznick and Packer [46]. The PCO content was calculated based on the molar extinction coefficient of DNPH (2.2×10^4 cm^1 M^1) and expressed as nmoles/mg protein while vitamin C contents were measured as described by Jacques-Silva *et al*. [47].

Cardiac and renal antioxidant defense system

The SOD assay was carried out by the method of Misra and Fridovich [48], with slight modification [49]. The one unit of SOD activity was given as the amount of SOD necessary to cause 50% inhibition of the auto-oxidation of adrenaline to adrenochrome. Reduced glutathione (GSH) was estimated by the method of Jollow *et al*. [50]. Catalase (CAT) activity was determined according to the method of Shinha [51]. One unit of CAT activity represents the amount of enzyme required to decompose 1 μmol of H_2O_2/min. Glutathione peroxidase (GPx) activity was also measured according to Beutler *et al*. [52]. Glutathione-S-transferase (GST) activity was estimated by the method of Habig *et al*. [53] using 1-chloro-2, 4-dinitrobenzene as substrate. The activity of glutathione reductase (GRed) was determined according to the method of Racker [54]. The decrease in absorbance at 340 nm wavelength was measured and the enzyme activity was calculated with a molar extinction co-efficient of 6.1 $mmol/L^{-1}$ cm. The protein thiol (PSH) and non-protein thiol (NPSH) contents were determined as described by Ellman [55]. Protein concentration was determined by the Biuret method of Gornal *et al*. [56], using bovine serum albumin (BSA) as standard.

Determination of serum nitric oxide bioavailability, myeloperoxidase , xanthine oxidase, and blood urea nitrogen

The serum nitric oxide (NO) concentrations were measured spectrophotometrically at 548 nm according to the method of Olaleye *et al*. [57]. The serum myeloperoxidase (MPO) activity was determined according to the method of Xia and Zweier [58]. The activity of xanthine oxidase (XO) was determined according to method of Akaike *et al*. [59]. The blood urea nitrogen (BUN) was determined using Randox kits following manufacturer's instructions.

Histopathology

Small pieces of kidney and heart were fixed in 10% formalin, embedded in paraffin wax, and sections of 5–6 mm thickness

were made and thereafter stained with hematoxylin and eosin (H & E) for histopathological examination according to the methods described by Drury *et al*. [60]. Thereafter, the sections were examined with light microscopy.

Immunohistochemical staining for kidney injury molecule I, nuclear factor kappa beta, cardiac troponin I, and nuclear factor erythroid 2-related factor 2 expressions

The immunohistochemistry was described as reported by Oyagbemi *et al*. [27]. To determine the expression of kidney injury molecule I (Kim-1), nuclear factor kappa bet (NF-κB), cardiac troponin I (CTnI), and nuclear factor erythroid 2-related factor 2 (Nrf2) in the kidney and heart, fixed tissues were embedded in paraffin and sectioned at a thickness of 5 μm. The sections were subsequently deparaffinized in xylene and rehydrated with graded alcohol. Antigen retrieval was carried out by immersing the slides in 10 mM citrate buffer at 95–100 °C for 25 min with subsequent peroxidase quenching in 3% H_2O_2/methanol solution. The nonspecific binding was blocked in goat serum, then, followed by an overnight incubation at 4 °C with antirabbit Kim-1, NF-κB, Nrf2, and CTnI primary antibodies. Detection of bound antibody was carried out using biotinylated (goat anti-rabbit, 2.0 μg/mL) secondary antibody and subsequently, streptavidin peroxidase (Horse Radish Peroxidase–streptavidin) according to manufacturer's protocol (HistoMark®, KPL, Gaithersburg, MD). Reaction product was enhanced with diaminobenzidine (DAB, Amresco®) for 1–3 min and counterstained with high definition hematoxylin (Enzo®, NY). The sections were subsequently dehydrated in ethanol, cleared in xylene. The slides were covered with coverslips and sealed with resinous solution. The immunoreactive positive expressions of Kim-1, NF-κB, Nrf2, and CTnI antirabbit intensive regions were viewed starting from low magnification on each slide then with 400× magnifications using a photo microscope (Olympus) and a digital camera (Toupcam®, Touptek Photonics, Zhejiang, China). The immunoreactivity of antigens of interest was quantified with ImageJ software version 1.51p 22 (2018).

3. Statistical analysis

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

4. Results

4.1. Body weight and relative organ weight

Table 1 shows that NaF alone significantly increased the heart weight, kidney weight, and relative kidney weights, respectively. However, Luteolin at 100 and 200 mg/kg could not increase the kidney, heart, and relative organ weight to near normal values (Table 1).

4.2. Renal and cardiac markers of oxidative stress

The administration of NaF significantly increased H_2O_2 generation, MDA level, protein carbonyl (PCO) content, protein thiol (PSH), and nonprotein thiol (NPSH) contents, and significantly reduced vitamin C contents in both the cardiac and renal tissues (Tables 2 and 3). Also, reduced glutathione (GSH) contents were significantly increased in cardiac tissues but fell significantly in renal tissues as indicated in Tables 2 and 3. Further, NaF co-administration with Luteolin significantly lowered the cardiac and renal H_2O_2 generation, MDA level, PCO content, PSH, and NPSH and caused significant improvements in GSH and vitamin C contents when compared with the NaF-untreated group (Tables 2 and 3).

4.3. Renal and cardiac antioxidant enzymes

The results in Table 4 indicated that NaF alone caused significant reductions in the activities of both cardiac glutathione peroxidase (GPx), superoxide dismutase (SOD), catalase (CAT), but significantly reduced the activity of GRed in comparison to the control group. On the other hand, the activities of renal CAT and GRed were significantly higher than the control group following administration of NaF (Table 5). However, Luteolin coadministration with NaF at 100 and 200 mg/kg significantly increased the activities of cardiac GPx, SOD, CAT and GRed while 200 mg/kg of Luteolin-treated group showed significant improvement in the activities of renal CAT and GRed relative to the control and NaF only groups (Table 5).

4.4. Serum markers of inflammation, renal, and cardiac damage and hypertension

In another experiment, NaF alone significantly increased the serum MPO, advanced oxidative protein products (AOPPs), XO together with significant reduction in the serum nitric oxide (NO) bioavailability when compared with the control (Table 6). Interestingly, coadministration of experimental animals with Luteolin significantly reduced the serum MPO, AOPPs, XO in a dose-dependent manner while serum NO bioavailability was also improved significantly (Table 6).

4.5. The electrocardiographic (ECG) changes

The ECG results show that NaF alone caused a significant prolongation of QT and QTc intervals (Table 7). Similarly, P wave and QRS duration and R wave amplitude values increased though not significant in NaF alone group relative to the control (Table 7). The prolonged QRS duration, QT and QTc intervals were normalized by Luteolin coadministration at 100 and 200 mg/kg (Table 7).

4.6. Blood pressure parameters

From the present study, our results indicated that NaF alone caused significant increases in systolic blood pressure (SBP), diastolic blood pressure (DBP) and mean arterial pressure (MA) when compared to the control and other groups coadministered with Luteolin (Figs. 1–2 and 3). It is worth to note that Luteolin coadministration with NaF restored SBP, DBP and MAP to near control values (Figs. 1–3). Interestingly, in this study, NaF alone did not significantly affect the heart rate

TABLE 1 *The effect of sodium fluoride toxicity on organ weight and relative organ weight*

Experiments	*Group A (Control)*	*Group B (NaF)*	*Group C (NaF + luteolin 100 mg/kg)*	*Group D (NaF + luteolin 200 mg/kg)*
Heart weight (g)	0.5 ± 0.08	0.44 ± 0.05[a]	0.56 ± 0.05[a, b]	0.43 ± 0.15
Heart/body (g/g)	0.003 ± 0.0005	0.003 ± 0.0003	0.003 ± 0.0003	0.003 ± 0.001
Kidney weight (g)	0.88 ± 0.10	0.96 ± 0.11[a]	1.02 ± 0.04[a]	0.98 ± 0.13[a]
Kidney/body (g/g)	0.005 ± 0.0005	0.007 ± 0.0006[a]	0.006 ± 0.0004[a]	0.006 ± 0.0007[a]

Values are presented as mean ± standard deviation. Group A (Control), Group B (Sodium fluoride 300 ppm), Group C (Sodium fluoride 300 ppm + Luteolin 100 mg/kg), Group D (Sodium fluoride 300 ppm + Luteolin 200 mg/kg). Superscript ([a]) indicates significant difference at $P < 0.05$ *compared with control (Group A), while superscript ([b]) Indicates significant difference at* $P < 0.05$ *compared with Group B.*

relative to the control and groups co-administered with Luteolin (Fig. 4).

4.7. Histopathology and immunohistochemistry

The histopathology of the renal tissues following the administration of NaF shows disseminated glomerular messangialisation and a focal area of interstitial infiltration by inflammatory cells and necrosis while NaF administered with Luteolin revealed mild focal area of congestion and area of cellular inflammation indicating gradual restoration of ultrastructural anarchy (Fig. 5). On the one hand, the results from immunohistochemistry revealed that NaF alone caused increase in the expressions of Kidney injury marker 1 (Kim-1), nuclear factor kappa bet (NF-κB), nuclear factor erythroid 2-related factors 2 (Nrf2), and cardiac troponin I (CTnI) as indicated in Figs. 6–9. On the other hand, Luteolin administration reduced the expressions of Kim-1, NF-κB, and CTnI while the expressions of Nrf2 were reduced by Luteolin at higher dosage (200 mg/kg) compared to the NaF alone or the control.

5. Discussion

Oxidative stress has been linked to hypertension and other cardiovascular disorders [61]. From this study, NaF alone caused

TABLE 2 *Protective effect of Luteolin on cardiac markers of oxidative stress*

Experiments	*Group A (Control)*	*Group B (NaF)*	*Group C (NaF + luteolin 100 mg/kg)*	*Group D (NaF + luteolin 200 mg/kg)*
H_2O_2	27.56 ± 1.43	29.11 ± 1.00[a]	27.76 ± 2.52	25.59 ± 2.30[a]
MDA	2.09 ± 0.59	2.69 ± 0.42[a]	2.42 ± 0.41	2.15 ± 0.48[b]
Protein Carbonyl	15.97 ± 4.23	24.06 ± 4.37[a]	17.80 ± 3.09[b]	19.69 ± 4.26[ab]
Total thiol	267.41 ± 37.50	296.55 ± 72.14	341.18 ± 55.83[a]	283.42 ± 74.93
Non-Protein thiol	163.25 ± 35.23	193.52 ± 33.96[a]	181.95 ± 24.18	272.98 ± 31.00[ab]
GSH	83.96 ± 2.92	78.12 ± 1.50[a]	82.64 ± 2.53[b]	104.80 ± 3.40[ab]
Vitamin C	0.28 ± 0.04	0.24 ± 0.05[a]	0.28 ± 0.02[b]	0.26 ± 0.04

Values are presented as mean ± standard deviation. Group A (Control), Group B (Sodium fluoride 300 ppm), Group C (Sodium fluoride 300 ppm + luteolin 100 mg/kg), and Group D (Sodium fluoride 300 ppm + luteolin 200 mg/kg). Alphabets indicate significant difference across groups at $P < 0.05$. *H_2O_2 (hydrogen peroxide generation; μmol/mg protein), MDA (malondialdehyde; μmol of MDA formed/mg protein), GSH (Reduced Glutathione; μmol /mg protein), protein thiol (μmol /mg protein); nonprotein thiol (μmol/mg protein), protein carbonyl (μmol /mg protein), and vitamin C (μmol /mg protein).*
Superscript ([a]) indicates significant difference at $P < 0.05$ *compared with control (Group A), while superscript ([b]) Indicates significant difference at* $P < 0.05$ *compared with Group B.*

TABLE 3 ***Protective effect of Luteolin on renal markers of oxidative stress***

Experiments	*Group A (Control)*	*Group B (NaF)*	*Group C (NaF + luteolin 100 mg/kg)*	*Group D (NaF + luteolin 200 mg/kg)*
H_2O_2 generated	30.88 ± 2.26	33.44 ± 2.43 [a]	29.83 ± 2.86[b]	30.88 ± 2.67[b]
MDA	1.60 ± 0.17	1.89 ± 0.19[a]	1.78 ± 0.12[a]	1.78 ± 0.08[a]
Protein carbonyl	45.41 ± 3.03	63.40 ± 3.32[a]	24.95 ± 4.39[ab]	21.06 ± 4.43[ab]
Protein thiol	258.27 ± 26.23	277.51 ± 53.25	267.88 ± 59.72	263.24 ± 47.42
Nonprotein thiol	123.04 ± 14.80	147.70 ± 18.34[a]	132.54 ± 18.44[b]	125.25 ± 19.06[b]
GSH	116.74 ± 8.29	103.09 ± 2.54[a]	110.68 ± 3.47[ab]	108.73 ± 10.22[a]
Vitamin C	0.19 ± 0.01	0.17 ± 0.01[a]	0.20 ± 0.02[b]	0.19 ± 0.02[b]

Values are presented as mean ± standard deviation. Group A (Control), Group B (Sodium fluoride 300 ppm), Group C (Sodium fluoride 300 ppm + luteolin 100 mg/kg), and Group D (Sodium fluoride 300 ppm + luteolin 200 mg/kg). Alphabets indicate significant difference across groups at P *< 0.05. H_2O_2 (hydrogen peroxide generation; μmol/mg protein), MDA (malondialdehyde; μmol of MDA formed/mg protein), GSH (reduced glutathione; μmol /mg protein), protein thiol (μmol /mg protein); nonprotein thiol (μmol /mg protein), protein carbonyl (μmol/mg protein), and vitamin C (μmol /mg protein).*
Superscript ([a]) indicates significant difference at P *< 0.05 compared with control (Group A), while superscript ([b]) Indicates significant difference at* P *< 0.05 compared with Group B.*

increase in systolic blood pressure (SBP), DBP, and MAP as compared to rats coadministered with Luteolin and the control rats. Luteolin administration was potent enough as antihypertensive phytochemical to cause significant drop in the values of SBP, DBP, and MAP in hypertensive rats. Interestingly, Luteolin at the dose of 100 mg/kg reversed the high blood pressure than 200 mg/kg dosage. The observable increase in the values of the blood pressure parameters could also be supported with reduced serum NO bioavailability in rats that received only NaF. Since there is a causal link between hypertension and oxidative stress, the increase in malondialdehyde (MDA) content, hydrogen (H_2O_2) generation, advanced oxidative protein products (AOPPs), protein carbonyl (PCO), and reduction in the reduced glutathione (GSH) content signified the induction of oxidative stress by NaF. The MDA is the end-product of lipid peroxidation of polyunsaturated fatty acid with resultant damage

TABLE 4 ***Protective effect of Luteolin on cardiac antioxidant enzymes***

Experiments	*Group A (Control)*	*Group B (NaF)*	*Group C (NaF + luteolin 100 mg/kg)*	*Group D (NaF + luteolin 200 mg/kg)*
GPx	11.88 ± 1.57	8.23 ± 1.60[a]	10.34 ± 0.89[ab]	9.42 ± 1.10[ab]
SOD	43.12 ± 3.92	33.60 ± 3.43[a]	38.73 ± 3.55[ab]	38.23 ± 3.88[ab]
CAT	43.13 ± 0.36	31.12 ± 0.87[a]	35.52 ± 0.75[ab]	34.09 ± 0.78[ab]
G.Red	0.67 ± 0.10	1.52 ± 0.86[a]	3.06 ± 0.98[ab]	2.99 ± 0.25[ab]

Values are presented as mean ± standard deviation. Group A (Control), Group B (Sodium fluoride NaF at 300 ppm), Group C (NaF + luteolin 100 mg/kg), and Group D (NaF + luteolin 200 mg/kg). Alphabets indicates significant difference across groups at P *< 0.05. GST (Glutathione-S-transferase; mmole1- Chloro-2, 4-dinitrobenzene-GSH complex formed/min/mg protein), GPx (Glutathione Peroxidase; (units/mg protein); SOD (Superoxide Dismutase; units/mg protein); CAT (Catalase; mmole H_2O_2 consumed/min/mg protein).*
Superscript ([a]) indicates significant difference at P *< 0.05 compared with control (Group A), while superscript ([b]) Indicates significant difference at* P *< 0.05 compared with Group B.*

TABLE 5 ***Protective effect of Luteolin on renal antioxidant enzymes***

Experiments	*Group A (Control)*	*Group B (NaF)*	*Group C (NaF + luteolin 100 mg/kg)*	*Group D (NaF + luteolin 200 mg/kg)*
GPx	5.33 ± 0.60	6.50 ± 0.40[a]	6.76 ± 0.67[a]	6.41 ± 0.45[a]
SOD	19.57 ± 2.24	23.83 ± 1.18[a]	25.19 ± 2.06[ab]	24.00 ± 1.44[a]
CAT	22.44 ± 0.95	26.96 ± 0.96[a]	25.56 ± 1.71[a]	36.85 ± 2.72[ab]
G.Red	0.46 ± 0.02	1.29 ± 0.35[a]	1.33 ± 0.32[a]	1.71 ± 0.66[ab]

Values are presented as mean ± standard deviation. Group A (Control), Group B (Sodium fluoride NaF at 300 ppm), Group C (NaF + luteolin 100 mg/kg), Group D (NaF + luteolin 200 mg/kg). Alphabets indicates significant difference across groups at P *< 0.05. GST (Glutathione-S-transferase; mmole1- Chloro-2, 4-dinitrobenzene-GSH complex formed/min/mg protein), GPx (Glutathione Peroxidase; (units/mg protein); SOD (Superoxide Dismutase; units/mg protein); CAT (Catalase; mmole H_2O_2 consumed/min/mg protein).*
Superscript ([a]) indicates significant difference at P *< 0.05 compared with control (Group A), while superscript ([b]) Indicates significant difference at* P *< 0.05 compared with Group B.*

to the biological membranes [62,63]. The accumulation of MDA could also enhance generation of reactive oxygen species (ROS). Hence, the increase in the markers of oxidative stress might also contribute significantly to the development of hypertension, arterial stiffness, and endothelial dysfunction. Co-administration of NaF with Luteolin caused a significant reduction in the markers of oxidative stress and improvement in the renal and cardiac GSH content. Furthermore, increase in AOPPs contents have been reported to be associated with oxidative stress, inflammation, aging and acute renal damage [64,65]. The ability of NaF to increase the AOPPs content might be connected to the observed renal injury and histopathological lesions. We speculated that the renal injury induced by the administration of NaF might have precipitated hypertension. Furthermore, cardiac complication was also evident as indicated by the significant increase in serum MPO activity. The MPO is an enzyme that is located in the azurophilic granules of neutrophils [66,67]. Myeloperoxidase has been implicated in a wide range of pathologies and pathophysiology of disease conditions such as cancer, cardiovascular, neurodegenerative, renal and lung diseases, and other chronic inflammatory conditions [68]. In the presence of high levels of MPO, there is a concomitant increase in the conversion of low-density lipoprotein (LDL) to oxidized low-density lipoprotein (oxy-LDL), one of the major players in the pathogenesis of plaque formation and ultimately arteriosclerosis [69]. Increase in the levels of oxy-LDL might also enhance VSMC proliferation, migration, and adhesion [70]. The LDL cholesterol can also be

TABLE 6 ***Protective effect of Luteolin on serum markers of oxidative stress and inflammation***

Experiments	*Group A (Control)*	*Group B (NaF)*	*Group C (NaF + luteolin 100 mg/kg)*	*Group D (NaF + luteolin 200 mg/kg)*
MPO	35.81 ± 3.00	48.05 ± 2.64[a]	33.84 ± 3.64[b]	19.62 ± 2.00[ab]
AOPP	41.92 ± 2.61	91.87 ± 1.06[a]	77.62 ± 2.00[ab]	67.45 ± 2.77[ab]
Xanthine oxidase	1.95 ± 0.49	3.97 ± 0.82[a]	3.31 ± 0.45[ab]	2.07 ± 0.42[b]
Nitric oxide	2.49 ± 0.34	1.87 ± 0.48[a]	2.24 ± 0.20[ab]	2.32 ± 0.25[b]

Values are presented as mean ± standard deviation. Group A (Control), Group B (Sodium fluoride NaF at 300 ppm), Group C (NaF + luteolin 100 mg/kg), Group D (NaF + luteolin 200 mg/kg). Alphabets indicate significant difference across groups at P *< 0.05. MPO (Myeloperoxidase (μmol/min), AOPP (nmoles/mg protein), Xanthine Oxidase; (Units/min/mg protein), Nitric oxide (μmol/mg protein).*
Superscript ([a]) indicates significant difference at P *< 0.05 compared with control (Group A), while superscript ([b]) Indicates significant difference at* P *< 0.05 compared with Group B.*

TABLE 7 ***Protective effect of Luteolin on the electrocardiogram***

Experiments	*Group A (Control)*	*Group B (NaF)*	*Group C (NaF + luteolin 100 mg/kg)*	*Group D (NaF + luteolin 200 mg/kg)*
P wave duration	22.6 ± 3.85	26.00 ± 2.71	20.50 ± 1.00[b]	19.75 ± 1.71[b]
QRS duration	11.2 ± 3.19	13.00 ± 3.16	12.00 ± 1.83	13.00 ± 2.16
QT Interval	62.00 ± 7.07	68.50 ± 5.45 [a]	63.75 ± 7.97	57.75 ± 5.56[b]
QT corrected	135.2 ± 12.44	158.33 ± 10.02[a]	145.67 ± 15.50	126.33 ± 5.13[b]
R wave amplitude	0.39 ± 0.07	0.47 ± 0.10	0.40 ± 0.12	0.39 ± 0.14

Values are presented as mean ± standard deviation. Group A (Control), Group B (Sodium fluoride NaF at 300 ppm), Group C (NaF + luteolin 100 mg/kg), Group D (NaF + luteolin 200 mg/kg). Alphabets indicates significant difference across groups at P *< 0.05. P-wave (m/s), QRS duration (m/s), QT interval (m/s), QTC (m/s), R wave amplitude (m/s).*
Superscript ([a]) indicates significant difference at P *< 0.05 compared with control (Group A), while superscript ([b]) Indicates significant difference at* P *< 0.05 compared with Group B.*

modified by the activity of MPO thereby inhibiting the endothelia nitric oxide synthase (eNOS) that synthesizes NO. The inhibition of eNOS will ultimately lead to insufficient production of NO with ultimate reduction in NO bioavailability [71]. The reduction in NO bioavailability has also been identified as a prognostic marker of hypertension [72]. In this study, the level serum XO increased significantly in the rats administered only NaF. It can be extrapolated that the increase in serum level of XO was an indication of hyperuricemia which is major pointer to oxidative stress, inflammation, renal damage and hypertension, arteriosclerosis, and myocardial infarction [73–75]. The ability of Luteolin to inhibit the activities of MPO and XO confirms the antioxidant, anti-inflammatory, cardioprotective and reno-protective effect of Luteolin [76]. Cardiovascular complication was also evident from the ECG as indicated with increased P wave duration, prolonged QRS duration, QT and QTc intervals, R amplitude, and increase heart rate. Although, the increase heart rate was not significantly different from the control rats and rats coadministered with Luteolin. This implies NaF targets more of the renal system with little impact on the nervous conduction of the cardiovascular system.

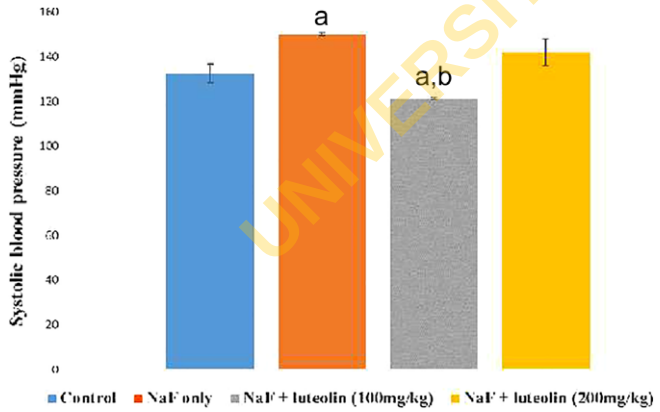

FIG 1 *The effect of sodium fluoride on systolic blood pressure. Values are presented as mean ± standard deviation. Group A (control), Group B (sodium fluoride 300 ppm), Group C (sodium fluoride 300 ppm + Luteolin 100 mg/kg), Group D (sodium fluoride 300 ppm + luteolin 200 mg/kg). Superscript ([a]) indicates significant difference at* P *< 0.05 compared with control (Group A), while superscript ([b]) Indicates significant difference at* P *< 0.05 compared with Group B.*

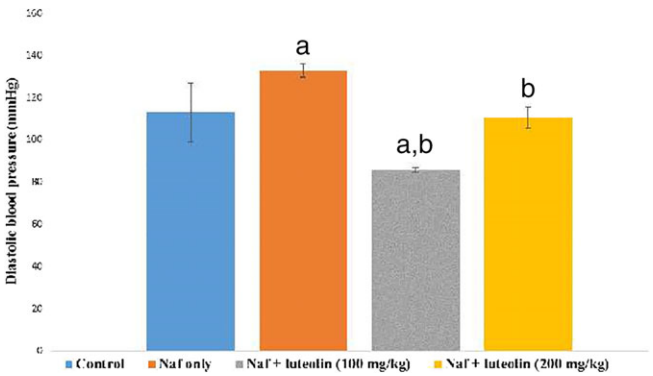

FIG 2 *The effect of Sodium fluoride on diastolic blood pressure. Values are presented as mean ± standard deviation. Group A (Control), Group B (Sodium fluoride 300 ppm). Group C (sodium fluoride 300 ppm + Luteolin 100 mg/kg), Group D (Sodium fluoride 300 ppm + Luteolin 200 mg/kg). Superscript ([a]) indicates significant difference at* P *< 0.05 compared with control (Group A), while superscript ([b]) Indicates significant difference at* P *< 0.05 compared with Group B.*

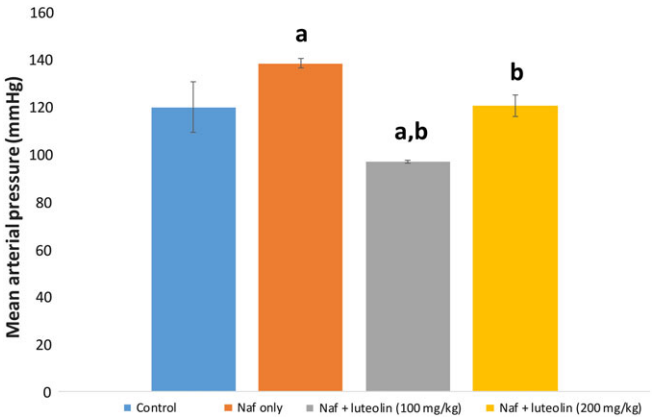

FIG 3

The effect of Sodium fluoride on Mean arterial pressure. Values are presented as mean + standard deviation. Group A (Control), Group B (Sodium fluoride 300 ppm). Group C (Sodium fluoride 300 ppm + Luteolin 100 mg/kg). Group D (Sodium fluoride 300 ppm + Luteolin 200 mg/kg). Superscript (^{a}) indicates significant difference at P < 0.05 compared with control (Group A), while superscript (^{b}) Indicates significant difference at P < 0.05 compared with Group B.

On the one hand, NaF reduced the contents of GSH, total thiol, non-protein thiol, and vitamin C. But, on the other hand, the activity of superoxide dismutase (SOD), glutathione peroxidase (GPx), catalase (CAT), and glutathione reductase (GRed) increased significantly in NaF alone treated rats when compared with the control. This is an adaptive mechanism to clear off the accumulation of lipid peroxidation products and other markers of oxidative stress as earlier indicated. However, our data showed that the Luteolin cotreatment increased the activity renal SOD, GPx, CAT, and GRed of experimental animals

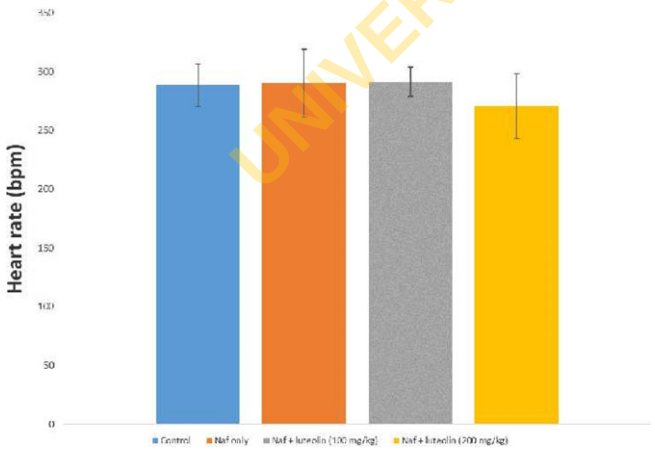

FIG 4

The effect of Sodium fluoride on Heart rate. Values are presented as mean + standard deviation. Group A (Control), Group B (Sodium fluoride 300 ppm), Group C (Sodium fluoride 300 ppm + Luteolin 100 mg/kg), Group D (Sodium fluoride 300 ppm + Luteolin 200 mg/kg).

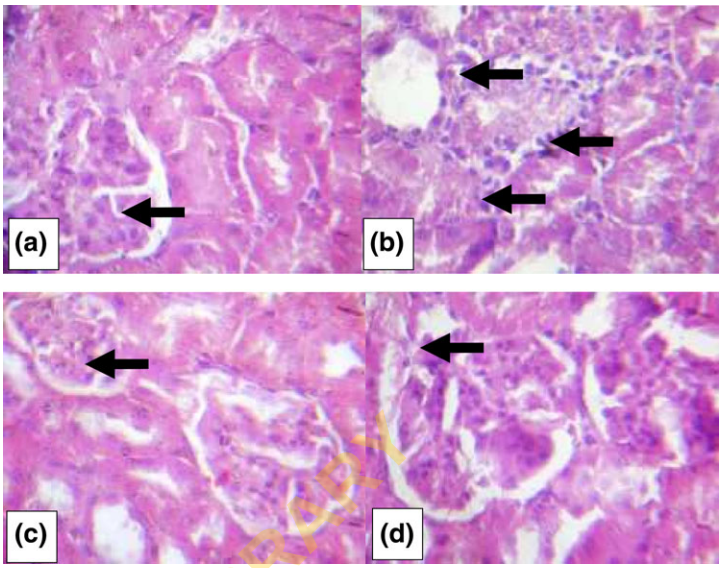

FIG 5

Photomicrograh showing kidney of rats (Group A)-Control: show focal area of congestion and mild rea of inflammation; (Group B) administered with (NaF 300 ppm alone) shows disseminated glomerular messangialisation and a focal area of very mild interstitial infiltration by inflammatory cells and necrosis (black arrow); (Group C: NaF 300 ppm + Luteolin 100 mg/kg) shows focal area of congestion and mild area of cellular inflammation, (Group D: NaF 300 ppm + Luteolin 200 mg/kg) focal area of congestion and mild area of cellular inflammation. Plates are stained with H and E stains and viewed with ×400 objectives.

coadministered with NaF. The beneficial effect of Luteolin as a potent antioxidant was observed in its ability to increase the levels of both enzymic and nonenzymic antioxidant defense system. The SOD is in first line of defense against free radical generation and oxidative stress. It dismutates superoxide anion radical (O_2-) to H_2O_2 while CAT and GPx participate in the final detoxification pathway of H_2O_2 to H_2O and O_2. In the GSH cycle, the GPx converts GSH to oxidized glutathione (GSSG) while GRed converts GSSG back to GSH with the help of reduced nicotinamide adenine dinucleotide phosphate (NADPH). The increase in the activity of GPx also correlates with the decrease in the GSH contents as increase in GPx consumes more of GSH with the increasing ratio of GSSG/GSH.

In the cardiac tissues, NaF significantly increased the MDA contents, H_2O_2 generation, PCO levels, total thiol and nonprotein thiol contents together with significant reductions in GSH and vitamin C content, SOD, and CAT activities, respectively, however, the activity of GPx and GRed increased significantly. The response of the cardiac tissue to NaF was completely different from what we obtained in the renal tissue at the level of antioxidant enzyme activity. The administration of Luteolin was not able to reverse the activity of SOD and CAT back to near normal values with the exception of significant improvement in the activity of GRed which a good indicator of antioxidant

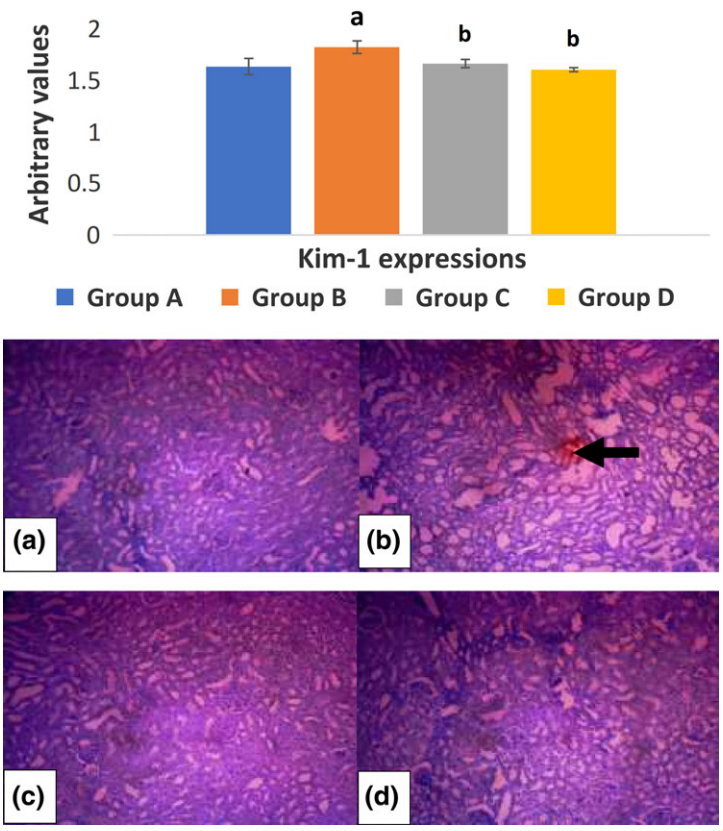

FIG 6

Immunohistochemistry of kidney injury molecule 1 (Kim-1) in rat kidney. A–Control: There is lower immune-positive expression of Kim-1. Group B (300 ppm NaF) shows higher immune-positive expression of Kim-1 when compared with the control. (Group C); NaF 300 ppm + Luteolin100 mg/kg) shows lower expressions of Kim-1 when compared with the NaF only treated group (black arrow) and (Group D) administered NaF 300 ppm + Luteolin 200 mg/kg; also shows lower expressions of Kim-1 when compared with the NaF only treated group (black arrow). The slides were counterstained with high definition hematoxylin and viewed ×100 objectives.

power of Luteolin. To our knowledge, this study is the first, till date to investigate the modulatory role of Luteolin in NaF-induced hypertension and cardiac complications.

The immunohistochemistry revealed higher expressions of kidney injury molecule 1 (Kim-1), nuclear factor-κB (NF-κB), nuclear factor erythroid 2-related factor 2 (Nrf2) and cardiac troponin I (CTnI) in NaF only treated rats. The NF-κB and Nrf2 are both transcription factors that regulate a number of genes responsible for oxidative stress, inflammation, and antioxidants [77,78]. From the results we obtained, NaF toxicity upregulated both NF-κB and Nrf2 indicating the capacity of NaF to modulate signaling associated with inflammation, oxidative stress and antioxidant response elements (ARE). Oxidative stress has been reported to be a major trigger of NF-κB signaling [79]. The observed upregulation of Nrf2 was also a confirmation of the significant increase in the activities of renal GRed, GPx, CAT, and SOD in NaF alone signifying adaptive response of these antioxidant enzymes through Nrf2/ARE pathway to NaF toxicity. Li *et al.* [80] reported the ameliorative effect of Luteolin in dextran sulfate sodium-induced colitis via activation of the Nrf2 signaling pathway. Similarly, Javkhedkar and Banday [81] documented that Resveratrol activated phase II antioxidant enzymes via Nrf2 signaling cascade, mitigated oxidative stress and normalized high blood pressure in spontaneously hypertensive rats (SHR). However, Luteolin co-treatment with NaF also led to a significant improvement in the activities of these enzyme even in the presence of NaF toxicity. Acute kidney injury (AKI) is a known to participate in the pathophysiology of many kidney disorders and dysfunction. Hence, the search for a more realistic and reliable diagnostic marker cannot be overemphasized as a therapeutic target for the treatment of hypertension and its

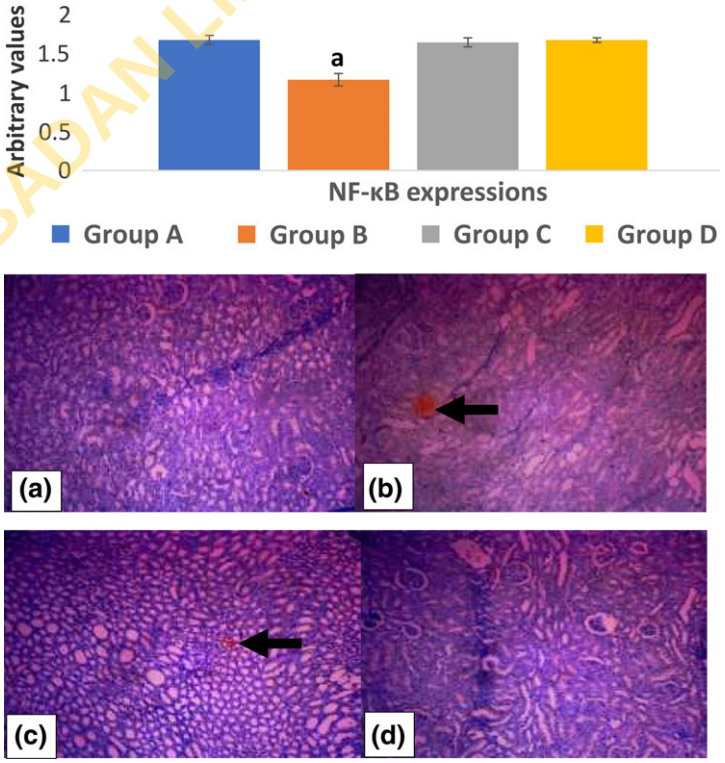

FIG 7

Immunohistochemistry of nuclear factor kappa beta (NF-κB) of Kidney of rats. A–Control: There is lower immune-positive expression of NF-κB. Group B (300 ppm NaF) shows higher immune-positive expression of NF-κB when compared to the control. (Group C); NaF 300 ppm + Luteolin 100 mg/kg) shows lower expressions of NF-κB when compared with the NaF only treated group (black arrow) and (Group D) administered NaF 300 ppm + Luteolin 200 mg/kg; also shows lower expressions of NF-κB when compared with the NaF only treated group (black arrow). The slides were counterstained with high definition hematoxylin and viewed ×100 objectives.

complications. The kidney injury molecule-1 (Kim-1) has be found to be one of the recent markers of renal injury especially to the early tubular and glomerular dysfunction [82–85]. Administration of NaF upregulated the expressions of Kim-1 as indicative of acute renal damage while co-treatment of NaF and Luteolin lowered Kim-1 expressions asserting the nephroprotective effect of Luteolin as earlier described [86,87]. The inhibition of Kim-1 by Luteolin attested to the renoprotective effect of this potent dietary flavonoid as reported by various authors [86,88]. In this study, cardiac complication was also observed consequence of NaF administration as recorded by higher expressions of cardiac troponin I (CTnI). CTnI has been used clinically as diagnostic marker for various cardiovascular dysfunction [89–92]. It is worth to note that Luteolin co-administration lowered the expressions of CTnI in comparison to the control and NaF alone treated rats. More importantly, renoprotective, and cardioprotective effect of Luteolin was demonstrated by its antioxidant, anti-inflammatory, and free radical scavenging activities.

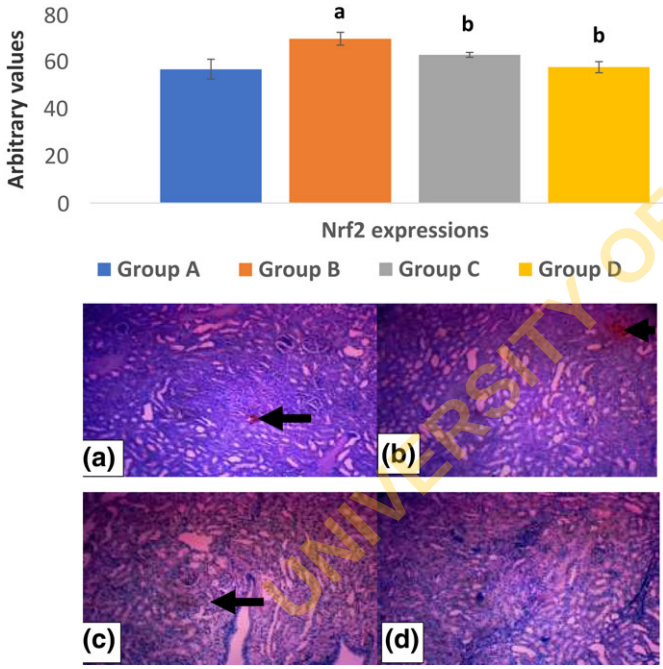

FIG 8

Immunohistochemistry of nuclear factor erythroid 2-related factor 2 (Nrf2) of Kidney of rats. A – Control: There is lower immune-positive expression of Nrf2. Group B (300 ppm NaF) shows higher immune-positive expression of Nrf2 when compared to the control. (Group C); NaF 300 ppm + Luteolin 100 mg/kg) shows higher expressions of Nrf2 when compared with the NaF only treated group (black arrow) and (Group D) administered NaF 300 ppm + Luteolin 200 mg/kg; also shows lower expressions of Nrf2 when compared with the NaF only treated group (black arrow). The slides were counterstained with high definition hematoxylin and viewed ×100 objectives.

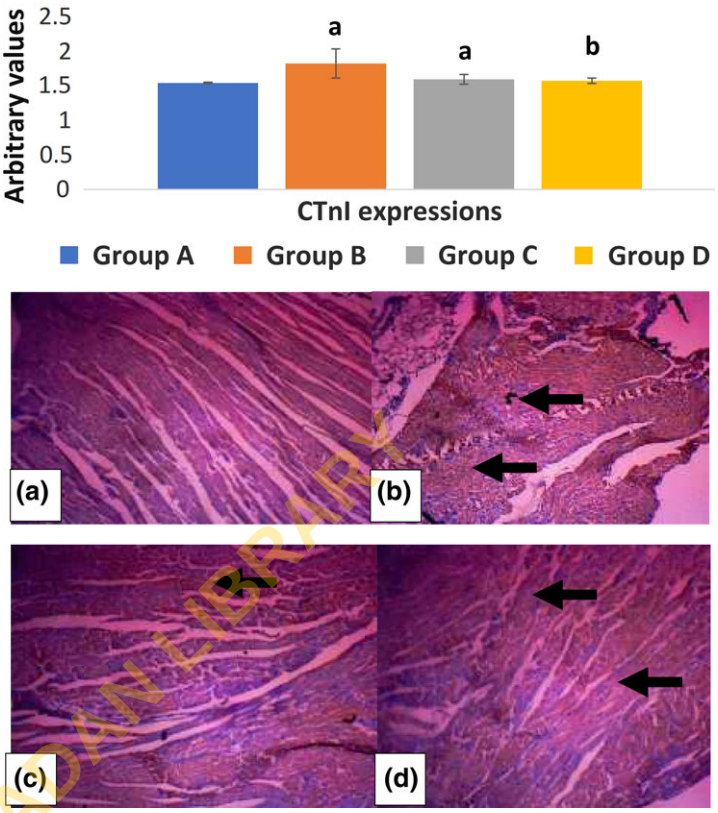

FIG 9

Immunohistochemistry of cardiac troponin I (CTnI) of rat's heart. A – Control: There is lower immune-positive expression of CTnI. Group B (300 ppm NaF) shows higher immune-positive expression of CTnI when compared to the control. (Group C); NaF 300 ppm + Luteolin 100 mg/kg) shows lower expressions of CTnI when compared with the NaF only treated group (black arrow) and (Group D) administered NaF 300 ppm + Luteolin 200 mg/kg; also shows lower expressions of CTnI when compared with the NaF only treated group (black arrow). The slides were counterstained with high definition hematoxylin and viewed ×100 objectives.

6. Conclusion

From this study, significant antihypertensive effect of Luteolin was demonstrated by reduction in high blood pressure, oxidative stress, improvement in antioxidant defense system, reduction of Kim-1, NF-κB, CTnI, and upregulation of Nrf2 expressions. The reduction in high blood pressure might be of paramount priority as hypertension is one of the silent killers globally. Furthermore, the use of Luteolin might also find relevance in clinical medicine against acute kidney injury and its associated cardiac complications. Hence, the use of dietary Luteolin in the prevention of acute kidney injury and cardiovascular disorders arising from daily exposure to NaF from mouth wash, mouth rinse, and tooth pastes might be of potential benefits.

Conflict of interest

We wish to confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

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