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Full-Length Research Article

Luteolin Normalizes Blood Pressure Via Its Antioxidant Activity and Down-Regulation of Renal Angiotensin II Receptor and Mineralocorticoid Receptor Expressions in Rats Co-exposed to Diclofenac and Sodium Fluoride

Ajibade T.O.[a], *Akinrinde A.S.[a], Adetona M.O.[b], Adedapo A.D.A.[c], Oyagbemi A.A.[a] Larbie C.[d], Omobowale T.O.[e], Ola-Davies O.E.[a], Saba A.B.[f], Adedapo A.A.[f], Oguntibeju O.O.[g], Yakubu M.A.[h]

Departments of [a]Veterinary Physiology and Biochemistry, [e]Veterinary Medicine, [f]Veterinary Pharmacology and Toxicology, Faculty of Veterinary Medicine, University of Ibadan, Nigeria
Departments of [b]Anatomy, [c]Pharmacology and Therapeutics, University of Ibadan, Nigeria
[d]Department of Biochemistry and Biotechnology, College of Science, Kwame Nkrumah University of Science and Technology, Kumasi, Ghana.
[g]Phytomedicine and Phytochemistry Group, Oxidative Stress Research Centre, Department of Biomedical Sciences, Faculty of Health and Wellness Sciences, Cape Peninsula University of Technology, Bellville 7535, South Africa
[h]Department of Environmental & Interdisciplinary Sciences, College of Science, Engineering & Technology, Vascular Biology Unit, Center for Cardiovascular Diseases, Texas Southern University, Houston, TX, USA

Summary: This study was designed to investigate the modulatory role of Luteolin (Lut), a flavonoid phytochemical, on haemodynamic parameters and the potential mechanisms involving renal Angiotensin II (AT_2R) and Mineralocorticoid (MCR) receptors in renal toxicity induced by co-exposure to Diclofenac (Dcf) and sodium fluoride (NaF) in rats. Male Wistar rats were administered with either vehicle (control), Dcf only (9 mg/kg orally) or concurrently with NaF (300 ppm in drinking water). Other groups were treated with LutA (100 mg/kg) or LutB (200 mg/kg) along with Dcf and NaF exposures. All treatments lasted 8 days, following which blood pressure indices were measured using tail-cuff plethysmography. Renal expressions of AT_2R and MCR were studied with immunohistochemistry, while biomarkers of oxidative and antioxidant status were also measured in the kidneys. Systolic, diastolic and mean arterial pressures were significantly ($p<0.05$) reduced in Dcf-treated rats, compared to control values. However, co-treatment with NaF or Lut restored these parameters. While the expression of AT_2R and MCR was high in the Dcf and Dcf+NaF groups, treatment with Lut caused obvious reduction in the renal expression of these receptors. Increased lipid peroxidation (Malondialdehyde) and protein oxidation (protein carbonyls) with a lowering of reduced glutathione levels contributed to the renal toxicity of Dcf, and these were significantly ameliorated in Lut-treated rats. In conclusion, the preservation of haemodynamic indices by Luteolin in the experimental rats was probably mediated by mechanisms involving down-regulation of renal expressions of AT_2R and MCR, reduction of oxidative stress and an improvement of renal antioxidant status.

Keywords*: Renin-Angiotensin, hypotension, Diclofenac, fluoride, oxidative stress, polyphenol antioxidant.*

**Address for correspondence: E-mail: as.akinrinde@gmail.com; Tel: +2348051659095*

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INTRODUCTION

The toxicity of emerging anthropogenic pollutants such as residues of pharmaceuticals present in environmental compartments has become an important subject of interest in environmental toxicology not just for their individual toxicity, but also their presence in complex mixtures with other environmental stressors (Wieczerzak *et al.*, 2018). Diclofenac (Dcf) and many other Non-selective anti-inflammatory drugs (NSAIDs) are among the most widely prescribed drugs for analgesic and anti-inflammatory purposes. Residues of these drugs are increasingly detected in environmental matrices from their discharge as effluents from drug manufacturing companies or inappropriate disposal (Freitas and Radis-Baptista, 2021). The pharmacological use of non-selective NSAIDs, including Dcf is often hampered by life-threatening adverse effects including gastrointestinal toxicity, impairment of renal function, with consequent alterations of fluid/electrolyte balance and blood pressure changes (Gwanyanya *et al.*, 2011). While several studies have focused on the effects of exposure to single chemicals or drugs, it is now increasingly

recognized that environmental chemical agents usually affect body tissues in combination, rather than alone. Thus, a novel strategy in the study of the effects of environmental pollutants on critical physiological attributes is the evaluation of possible interactions between pollutants, leading to synergism or antagonism (Nica *et al*., 2017).

Several prescription drugs are known to affect the blood pressure of patients or individuals being treated for hypertension. Although NSAIDs are among the most common classes of medication consumed by hypertensive patients, there exist wide variations regarding their effects on blood pressure and haemodynamics of exposed humans or experimental animals (Aljadhev *et al.*, 2012). Most studies associate an increased risk of hypertension with Dcf use, occurring via its inhibition of the cyclooxygenase pathway leading to reduction in production of natriuretic prostaglandins (e.g. PGE2), salt retention, as well as reduction in the vasodilatory effects of these prostaglandins (Harris, 2002). On the other hand, there are recent suggestions that upper or lower gastrointestinal bleeding resulting from acute exposure to NSAIDs may precipitate haematemesis, melena or haematochezia, with severe cases sometimes progressing into hypovolemia, hypotension and shock (Laine *et al*., 2021).

Exposure to fluoride salts is almost inevitable and can occur from different environmental sources such as drinking water, toothpastes and dental products (EFSA, 2013). A recent review has revealed that fluoride is often incorporated into pharmaceuticals in order to increase their biological half-lives, raising the likelihood of co-exposure with drugs, including Dcf (Yanac and Murdoch, 2019). Fluoride has been reported to cause elevation of systolic, diastolic and mean arterial blood pressures in rats (Oyagbemi *et al*., 2017), via mechanisms involving the induction of oxidative stress. However, the effect of co-exposure of fluoride and Dcf on blood pressure parameters is not known, although, both Dcf and NaF have been reported to increase the generation of reactive oxygen species (ROS) and oxidative stress in various tissues (Islas-Flores *et al*., 2013; Khan *et al*., 2013).

The kidney is a major organ involved in the control of salt and water homeostasis, and hence plays vital roles in the modulation of haemodynamic changes (Wadei and Tektor, 2012). Renal control of extracellular volume is closely linked to the regulation of urinary sodium excretion which is influenced by the activity of vasoactive systems, including the renin-angiotensin-aldosterone system (Granger and Schnackenberg, 2000). Renal angiotensin II increases blood pressure either directly by enhancing tubular transport of sodium, or indirectly through mineralocorticoid (aldosterone) stimulation. Therefore, AT_2R antagonists or inhibitors of the mineralocorticoid receptors (MCR) are expected to be effective in the treatment of hypertension by enhancement of natriuresis (Ivy and Bailey, 2014). The effects of co-exposures to Dcf and NaF on renal expression of AT_2R and MCR have not yet been studied. Additionally, the kidney is known to often accumulate fluoride at concentrations even higher than the concentration in the plasma (Guth*et al*., 2020).

Luteolin (Lut), chemically 3, 4, 5, 7-tetrahydroxyflavone, is a flavonoid component of many fruits and vegetables, and is known to exhibit antioxidant, anti-inflammatory and anticancer activities (Su *et al*., 2015). Available evidence suggests that luteolin causes reduction in blood pressure via stimulation of nitric oxide production and arterial relaxation (Si *et al*., 2014). Studies have shown that luteolin can influence blood pressure and cardiovascular protection by modulating proliferation of blood vessels and inhibiting hypertension-induced vascular remodelling (Qian*et al*., 2010). The effects of luteolin on renal regulation of vascular haemodynamics via the renin-angiotensin system are not yet fully known.

It is well known that NSAIDs, including Diclofenac (Dcf), produce nephrotoxicity with possible fluid retention and increase in blood pressure. Concurrent exposure to other agents that modulate blood pressure may either result in remission or aggravation of these undesirable effects. The present research was aimed to investigate how blood pressure indices and heart rates of Dcf-treated rats are affected during simultaneous exposure to NaF and Lut. The involvements of renal AT_2R and MCR were also studied by their expression levels by immuno-histochemical staining and the redox status of the renal tissues was assessed by measuring the levels of protein and lipid oxidation, glutathione concentration, as well as the activities of some antioxidant enzymes.

MATERIALS AND METHODS

Chemicals: Sodium fluoride, Luteolin, trichloroacetic acid (TCA), thiobarbituric acid (TBA), 5, 5'-dithio-bis-2-nitrobenzoic acid (DTNB), reduced glutathione (GSH), 1,2-dichloro-4-nitrobenzene (CDNB), adrenaline, sodium hydroxide, xylenol orange and other chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA). Diclofenac sodium, sold as Voltaren® was purchased from a reputable pharmacy in Ibadan, Nigeria. Angiotensin II receptor and Mineralocorticoid receptor antibodies were purchased from Bioss Inc. (Woburn, MA, USA). Normal goat serum, Biotinylated antibody and Horse Radish Peroxidase (HRP) System were purchased from KPL, Inc. (Gaithersburg, MD) and Diaminobenzidine (DAB) was purchased from AMRESCO LLC. (OH, USA). All other chemicals used were of analytical grade and obtained from British Drug Houses (Poole, Dorset, UK).

Animals and experimental design: A total of forty-five male Wistar rats weighing 120-150 g were obtained from the Experimental Animal Unit of the Faculty of Veterinary Medicine, University of Ibadan for this experiment. They were housed in plastic cages in a well-ventilated animal house facility and randomly grouped into five groups (A-E) consisting of nine animals each. The environmental conditions of the animal house included a 12 h light and12 h dark photoperiod and ambient temperature between 23-25ºC. The rats were maintained on a commercial rat feed and clean tap water ad libitum throughout the duration of the experiment, which included one week of acclimatization. All animals received humane treatment as outlined in the Guide for the Care and the Use of Laboratory Animals prepared by the National Academy of Science and published by the National Institute of Health (PHS, 1996). This study was also conducted according to local guidelines approved by the University of Ibadan's Animal Care and Use Research Ethics Committee (ACUREC) under the approval number: UIACUREC/ 19/124.

This study employed a previously applied model of Dcf-induced toxicity (Singh et al., 2017). The treatments administered to the rats were as follows: Group A (control) received normal tap water; Group B rats were orally exposed to Dcf (9 mg/kg) twice daily for three days; Group C was exposed to NaF (300 ppm) in drinking water for 8 days and concurrently with Dcf administration on the final three days; Group D was treated with Luteolin at 100 mg/kg (LutA) along with Dcf and NaF exposures, while Group D was treated with Luteolin at 200 mg/kg (LutB) concurrently with Dcf and NaF administration.

Measurement of Blood Pressure and Heart rate: Blood pressure parameters (systolic, diastolic and mean arterial pressures) and heart rates of rats were measured indirectly by tail plethysmography without anaesthesia using an electro-sphygmomanometer (CODA, Kent Scientific, USA). Following acclimatization of the rats to the sphygmomanometer conditions, rats were restrained carefully on lateral recumbency and placed on a well-padded platform with a tail cuff attached and an average of at least nine readings per animal were taken in the quiescent state.

Animal euthanasia and preparation of homogenates: All the rats were euthanized by cervical dislocation upon termination of the experiments, approximately twenty four hours after the last administration of the different chemicals. The kidneys were harvested immediately following euthanasia, rinsed and homogenized in phosphate buffer (0.1 M, pH 7.4). The homogenate was then centrifuged at 10,000 rpm for 10 min in a refrigerated centrifuge (4ºC). The supernatant was thereafter collected in separate bottles as the post-mitochondrial fraction and was used to assay for biochemical markers of oxidative stress.

Assessment of biochemical markers of oxidative stress and antioxidant status: The protein content of the kidney tissues was evaluated using the Biuret test as described by Gornal et al. (Gornal et al., 1949). The concentration of hydrogen peroxide in the kidney tissues was determined according to the Wolff (Wolff, 1994). The content of malondialdehyde (MDA) was used as an index of lipid peroxidation and was measured according to methods described by Varshney and Kale (Varshney and Kale, 1990), where a molar extinction coefficient of 1.56×10^5 $M^{-1}cm^{-1}$was used to compute the values. The renal activity of Glutathione peroxidase (GPx) was evaluated by the Rotruck et al. (Rotruck et al., 1973), while Glutathione S-transferase (GST) activity was measured as described by Habig et al. (Habig et al., 1974). The activity of Superoxide dismutase (SOD) was determined according to the methods described by Misra and Fridovich (Misra and Fridovich, 1972) by evaluating its inhibition of the autoxidation of adrenaline in an alkaline medium (pH 10.2), with slight modifications in our laboratory (Oyagbemi et al., 2015). The renal content of reduced glutathione was evaluated according to the method of Beutler et al. (Beutler et al., 1963), while the contents of total thiols and non-protein thiols were determined by the method of Ellman (Ellman, 1959).

Immunohistochemistry of Renal AT_2R and MCR: Kidney tissues were fixed immediately in 10% formalin after their harvest from the euthanized rats. The tissues were embedded and sectioned in paraffin and were processed for immunohistochemistry according to methods described by Todorich et al. (2011). Briefly, the paraffin sections were first melted at about 60ºC in an oven and dewaxing was done using xylene followed by passage of the tissues through ethanol solutions of decreasing concentrations (i.e. 100-80%). Thereafter, peroxidase quenching was carried out by applying 1% H_2O_2/methanol solution (v/v) and this was followed by antigen retrieval by microwave heating in citrate buffer (0.01 M; pH 6.0). The sections were blocked in normal goat serum (10%, HistoMark® Gaithersburg MD) and then probed overnight with Angiotensin II receptor antibody (Bioss, San Diego, CA, USA), while other sections were probed with Mineralocorticoid receptor antibody (Bioss, San Diego, CA, USA), all at room temperature. Bound antibody detection was carried out by using biotinylated (goat anti-rabbit, 2.0 mg/mL) secondary antibody and then Streptavidin peroxidase (HorseRadish Peroxidase-Streptavidin), according to the manufacturer's protocol (HistoMark® Gaithersburg MD). The product of the reaction was enhanced with diaminobenzidine (DAB, Amresco®, USA) for 2-3 min with counter-staining using high definition Haematoxylin (Enzo®, NY, USA), while the slides were subsequently dehydrated in ethanol, sealed with coverslips and resinous solution and the immunoreactive regions indicating positive expression of AT_2R and MCR were viewed with a light microscope (Olympus) and digital camera (Toupcam®, Touptek Photonics, Zhejiang, China).

Statistical Analysis:
Data were expressed as mean ± standard deviation and analyzed using One-way Analysis of Variance (ANOVA), followed by the Tukey's post hoc test for multiple comparisons. Statistical analysis was performed using the GraphPad Prism software (Version 7.00). P-values<0.05 were considered statistically significant.

RESULTS

The effects of Dcf (Voltaren®), NaF and Lut exposures on blood pressure parameters and heart rates of the experimental rats are presented in Table 1. Rats exposed to Dcf alone at 9 mg/kg for three days had significant ($p<0.05$) reduction in systolic (SBP), diastolic (DBP) and mean arterial (MAP) pressures, but significant ($p<0.05$) increase in heart rates compared to the control rats. However, relative to exposure to Dcf alone, co-exposure to Dcf and NaF caused significant ($p<0.05$) increase in SBP, DBP and MAP, but reduced heart rates. Interestingly, rats treated with Lut along with Dcf and NaF showed significant ($p<0.05$) reduction in SBP, DBP and MAP compared to the group exposed simultaneously to the Dcf + NaF, with the values largely similar to those of control rats. It is important to note that SBP, DBP and MAP readings in the control and Lut-treated groups were similar to values recorded elsewhere in apparently healthy rats (Bunag and Butterfield, 1982), which reported 130 ± 5 mm Hg systolic, 100 ± 5 mm Hg mean, and 85 ± 5 mm Hg diastolic.

Table 1:
Blood pressure indices in rats exposed to Diclofenac, Sodium fluoride and Luteolin

Blood pressure parameters	Control	Dcf only	Dcf+NaF	Dcf+NaF +LutA	Dcf+NaF+LutB
SBP	127.91±5.82	103.13±0.84[a]	137.20±2.53[a,b]	122.40±4.16[c]	117.18±10.13[c]
DBP	99.27±5.29	66.38±2.67[a]	105.60±2.55[b]	106.00±3.32	99.00±8.78[c]
MAP	108.55±4.82	78.25±1.75[a]	115.90±2.38[b]	111.20±3.63	104.71±9.04[c]
Heart rate	350.09±42.91	388.13±8.37[a]	379.20±2.53[b]	336.00±28.39	355.53±30.79[c]

The presented values are the means±SD. Dcf, Diclofenac; NaF, Sodium fluoride; Lut, Luteolin. Superscript (a) indicates significant differences at p<0.05 when values in other groups are compared with group A; Superscript (b) indicates significant differences at p<0.05 when values in other groups are compared with group B; Superscript (c) indicates significant differences at p<0.05 when values in other groups are compared with group C.

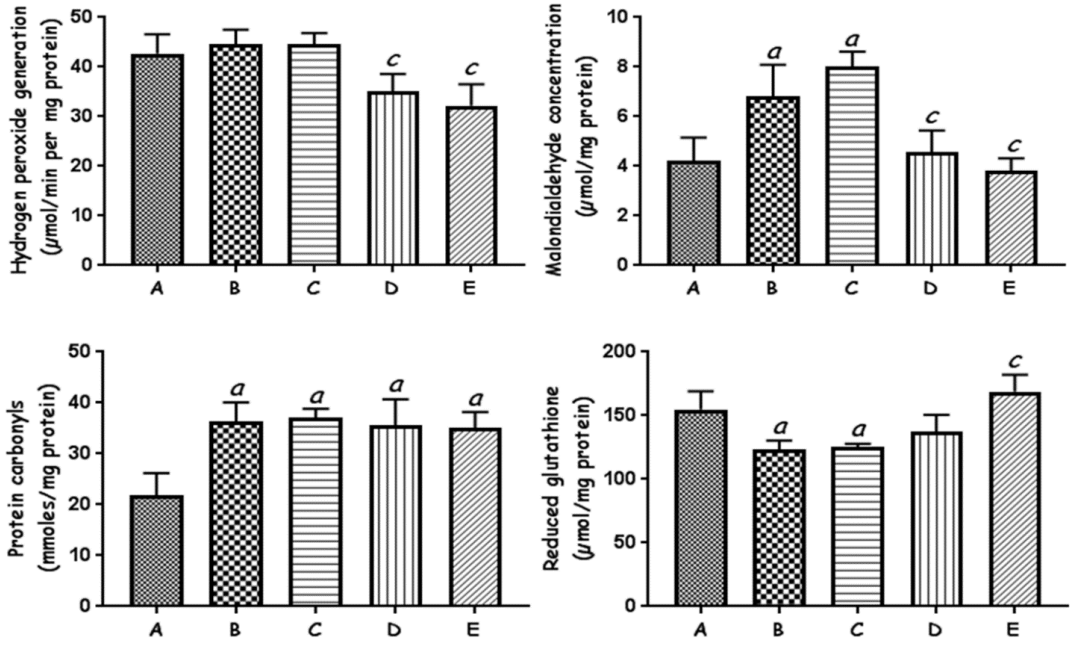

Figure 1:
Renal markers of oxidative stress following exposure to Diclofenac (Dcf), sodium fluoride (NaF) and Luteolin (Lut). Group A (control); Group B (9 mg/kg Dcf); Group C (9 mg/kg Dcf + 300 ppm NaF); Group D (9 mg/kg Dcf + 300 ppm NaF + 100 mg/kg Lut); Group E (9 mg/kg Dcf + 300 ppm NaF + 200 mg/kg Lut). Superscript (a) indicates significant differences at p<0.05 when values in other groups are compared with group A; Superscript (b) indicates significant differences at p<0.05 when values in other groups are compared with group B; Superscript (c) indicates significant differences at p<0.05 when values in other groups are compared with group C.

Table 2:
Levels of enzymic and non-enzymic antioxidants in rats exposed to Diclofenac, Sodium fluoride and Luteolin

Parameters	Control	Dcf only	Dcf+NaF	Dcf+NaF +LutA	Dcf+NaF+LutB
Protein thiols	118.77±7.43	118.35±5.66	125.49±10.98	122.40±6.68	125.38±12.54
Non-protein thiols	85.14±2.09	80.70±3.42[a]	80.62±2.54[a]	78.02±2.49[a]	79.39±4.40[a]
GST	0.26±0.05	0.38±0.05[a]	0.39±0.07[a]	0.40±0.12[a]	0.41±0.08[a]
SOD	18.26±2.65	17.58±2.31	15.79±2.31	17.59±3.71	17.11±5.19
Vit. C	1.51±0.12	1.55±0.06	1.57±0.09	1.68±0.25	1.72±0.12

The presented values are the means±SD. Dcf, Diclofenac; NaF, Sodium fluoride; Lut, Luteolin. Superscript (a) indicates significant differences at p<0.05 when values in other groups are compared with group A; Superscript (b) indicates significant differences at p<0.05 when values in other groups are compared with group B; Superscript (c) indicates significant differences at p<0.05 when values in other groups are compared with group C

The effects of Dcf, NaF and Lut exposure on renal markers of oxidative stress are depicted in Fig. 1. Although, the renal levels of hydrogen peroxide in the Dcf or Dcf + NaF groups remained unchanged, the same groups of rats showed significant (p<0.05) increase in the renal MDA content, when compared to the control group. The protein carbonyl levels in Dcf and Dcf + NaF groups were significantly (p<0.05) elevated, while the level of GSH in these groups was significantly (p<0.05) reduced compared to the control group. In the groups concurrently treated with LutA (100 mg/kg) or LutB (200 mg/kg), there was significant (p<0.05) lowering of hydrogen peroxide generation and MDA content relative to the Dcf + NaF group. Furthermore, treatment of rats with Lut resulted in significant (p<0.05) increase in GSH concentration compared to the Dcf + NaF combination, although, protein carbonyl level remained unaltered despite Lut treatment.

The results in Table 2 represent the activities and levels of enzymic and non-enzymic antioxidants, respectively. The concentrations of protein thiols and Vitamin C, as well as

the activity of SOD did not differ significantly among the various study groups. However, relative to the control group, all other groups had significantly ($p<0.05$) elevated levels of non-protein thiols and GST activity.

The immuno-histochemical staining of renal tissues revealed positive immuno-reactivity with higher expression of AT_2R in the groups exposed to Dcf alone and the group receiving a combination of Dcf and NaF, compared to the control group which showed lower expression of AT_2R (Plate 1). The rats in the control group had high expression of MCR which was also similar to the expression levels in the Dcf and Dcf+NaF groups (Plate 2). On the other hand, treatment of the rats with Lut resulted in down-regulation of AT_2R and MCR expression in the kidneys (Plates 2 and 3).

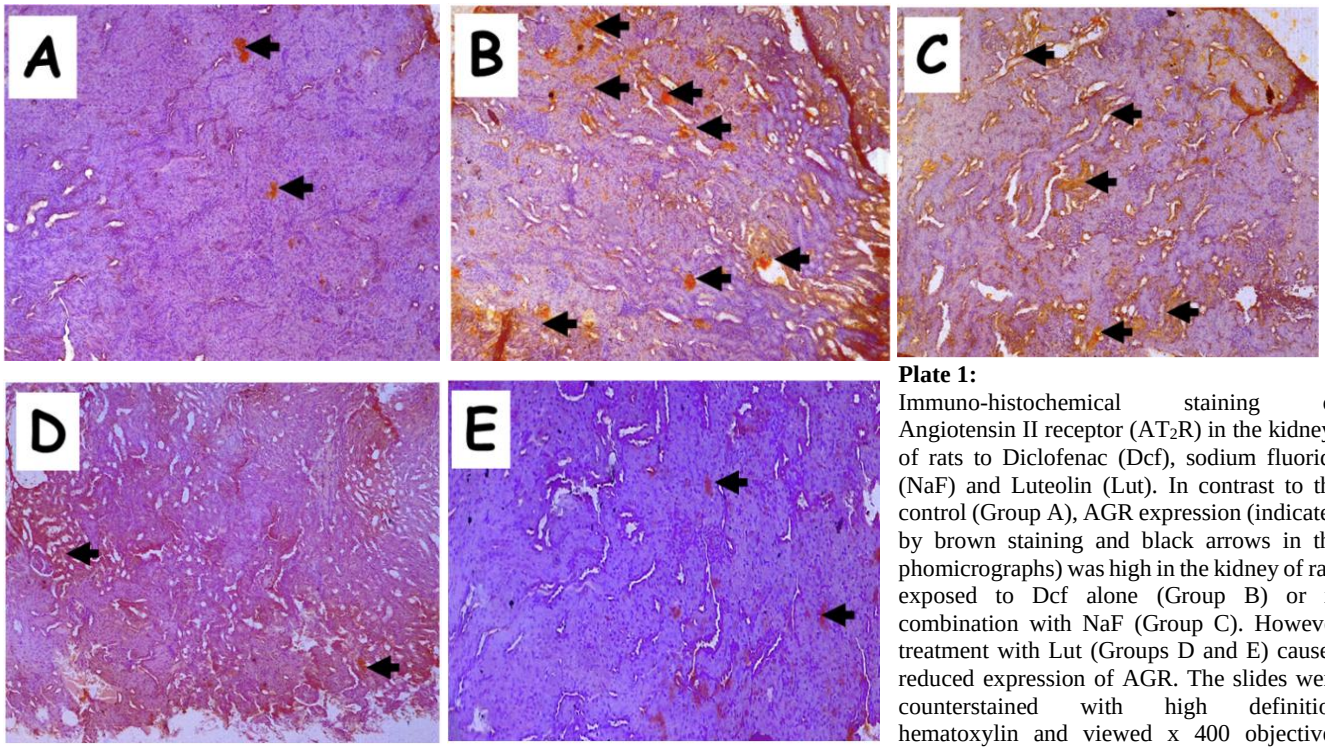

Plate 1:
Immuno-histochemical staining of Angiotensin II receptor (AT_2R) in the kidneys of rats to Diclofenac (Dcf), sodium fluoride (NaF) and Luteolin (Lut). In contrast to the control (Group A), AGR expression (indicated by brown staining and black arrows in the phomicrographs) was high in the kidney of rats exposed to Dcf alone (Group B) or in combination with NaF (Group C). However treatment with Lut (Groups D and E) caused reduced expression of AGR. The slides were counterstained with high definition hematoxylin and viewed x 400 objectives (magnification X100).

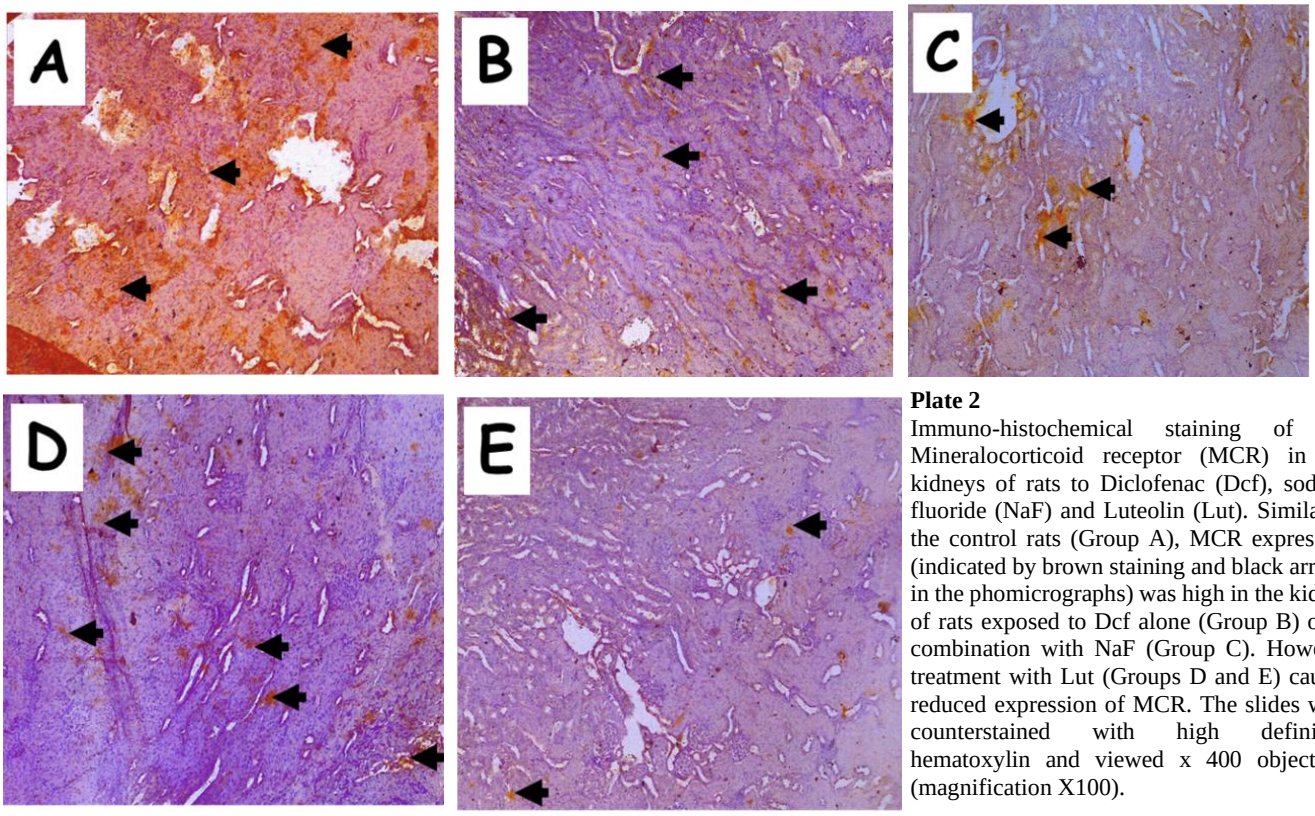

Plate 2
Immuno-histochemical staining of the Mineralocorticoid receptor (MCR) in the kidneys of rats to Diclofenac (Dcf), sodium fluoride (NaF) and Luteolin (Lut). Similar to the control rats (Group A), MCR expression (indicated by brown staining and black arrows in the phomicrographs) was high in the kidney of rats exposed to Dcf alone (Group B) or in combination with NaF (Group C). However treatment with Lut (Groups D and E) caused reduced expression of MCR. The slides were counterstained with high definition hematoxylin and viewed x 400 objectives (magnification X100).

DISCUSSION

NSAIDs, such as Dcf and Ibuprofen, have been associated with an increased risk of hypertension due to their inhibition of Cyclooxygenase (COX)-derived vasodilatory prostaglandins (Harris, 2002; Izhar *et al*., 2004), although, other contrary reports have also indicated that certain NSAIDs such as Dcf do not necessarily increase the risk of hypertension (Sherve *et al*., 2014). The present study hypothesized that combined exposure to Dcf and sodium fluoride, a compound known to modulate blood pressure (Oyagbemi *et al*., 2017), could lead to interactions that affect blood pressure dynamics. The study, therefore, sought to investigate the effect of treatment with Luteolin, a food-derived anti-oxidative and anti-inflammatory flavonoid (Nakayama *et al*., 2015) on relevant biomarkers involved in blood pressure regulation in Dcf- and NaF co-exposed rats. Our findings indicate a reduction in the systolic, diastolic and mean arterial pressures in rats that were exposed orally to Dcf twice daily for 3 days. In addition, there was an increase in the heart rates of this group of rats. However, combined exposure of rats to Dcf and NaF, resulted in an increase in the blood pressure parameters, representing a reversal of the effects obtained with administration of Dcf alone.

Despite the documented anti-natriuretic and vasoconstrictor effects that results from non-selective COX inhibition by NSAIDs, it appears that the findings of Dcf-induced reduction in blood pressure in the present study may be linked to separate mechanisms such as a likely reduction in extracellular fluid volume and/or hypovolaemia caused by severe haemorrhagic diarrhea observed in the rats treated with Dcf as reported in our earlier study (Akinrinde *et al*., 2020). Differences in pharmacokinetics and dosage of the drug used in different studies have been suggested to be responsible for the conflicting reports on how NSAIDs affects blood pressure parameters (Stempak *et al.,* 2002). Furthermore, the observed drop in blood pressure in Dcf-treated rats was corroborated by a corresponding increase in the heart rate of these animals. Increased heart rate and vasoconstriction are normal compensatory responses directed at reversing hypotension, and this occurs via an increase in sympathetic stimulation of the heart, causing increase in cardiac output (DiBiona, 2004).

It was obvious from the current results that co-exposure of rats to Dcf and NaF resulted in a reversal of the above effects, probably due to reported blood pressure-enhancing effects of NaF (Yousefi *et al.,* 2018), suggesting an antagonistic interaction between the two compounds on blood pressure in the present study. Increased blood pressure following NaF administration in rats has been associated with increased vascular generation of reactive oxygen/nitrogen species (ROS/RNS) and oxidative stress (Oyagbemi *et al*., 2018). Reactive oxygen species may increase blood pressure in the short-term by stimulating heart rate and inducing vasoconstriction, while in the long term, ROS contributes to hypertension by promoting inflammation, myocardial hypertrophy, vascular remodeling and endothelial dysfunction (Touyz and Brimes, 2011, Rodrigo *et al*., 2011). In the present study, we examined the effect of Dcf or its co-administration with NaF on renal oxidative stress and antioxidant markers and found obvious indications of oxidative stress such as increased levels of malondialdehyde and protein carbonyls, as well as reduction in GSH levels in the kidneys of rats treated with Dcf and NaF.

Treatment of Dcf- and NaF-exposed rats with Luteolin effectively reduced the renal contents of Hydrogen peroxide, MDA protein carbonyls, along with increase in the level of GSH, indicating the antioxidant effects of Lut in the renal tissues. This radical scavenging and antioxidant role of Lut might be responsible for maintaining the blood pressure of the affected rats. In this study, blood pressure values in the Lut-treated rats were not significantly different from those of the control rats. Indeed, Oyagbemi *et al*. (2018) reported similar values of systolic (124.3±12.64 mmHg), diastolic (92.41±16.05 mmHg) and Mean arterial (102.66±14.76 mmHg) pressures as those recorded in the control rats used in the present study. In effect, the blood pressure of rats treated with Lut exhibited a tendency to be maintained at values close to that of normal or control rats.

The renin-angiotensin-aldosterone system (RAAS) is important in the control of blood pressure and fluid/electrolyte balance. Increased activation of the RAAS is a major contributor to the development of hypertension via stimulation of Na^+ reabsorption and K^+ excretion (Yatabe *et al*., 2011); therefore, blockade of components of this system has been a useful strategy for the therapeutic control of blood pressure (Parichatikanond *et al.,* 2012). However, the usefulness of this strategy during concurrent exposure to chemicals and drugs that are capable of modulating blood pressure is not fully known. In this study we examined the effects of Dcf administration on major receptors of the RAAS i.e. AT_2R and MCR. Early reports have shown that certain NSAIDs actually possess intrinsic mineralocorticoid receptor agonist activity (Feldman and Couropmitree, 1976), while other reports also indicated that Dcf, like some other commonly prescribed NSAIDs may inhibit the glucuronidation of aldosterone in human liver and kidneys (Winner *et al*., 2005). However, to the best of our knowledge, the effect of Dcf on the expression of the mineralocorticoid receptor has not been reported.

In this study, an obvious increase in the expression of AT_2R was observed in rats treated with Dcf alone, although this was inconsistent with the reduced blood pressure in the same group of rats. It appears, however, from our results that the effects of Dcf on these receptors of the RAAS may be masked, at least in the early stages of Dcf usage, by other haemodynamic factors including the drug's ability to induce severe gastrointestinal bleeding and possible hypovolaemia as previously reported (Akinrinde *et al.,* 2020). Hypovolemia resulting from acute loss of circulating blood volume after hemorrhage may result in low cardiac output and hypotension (Noori *et al.,* 2017). A potential limitation to our study was the inability to assess the independent effects of NaF on AT_2R and MCR expression, due to the study design employed. Nevertheless, the reversal of blood pressure in the rats given a combination of Naf and Dcf with corresponding increase in the expression of AT_2R appears to indicate that the enhancement of the RAAS pathway by NaF may have taken a more dominant role over the effects observed in rats given Dcf alone.

More significantly, in this study, we demonstrated a profound down-regulation of both AT_2R and MCR in Lut-treated rats, a finding that was also consistent with its ability to reduce the blood pressure of these rats, compared to those

co-exposed to Dcf and NaF. Dietary components and natural bioactive products from plants, including flavonoids such as quercetin or anthocyanins e.g. delphinidin, have been shown to reduce blood pressure (Parichatikanond *et al*., 2012), although the molecular mechanisms of many of these compounds are not yet fully understood. Previous evidence supporting the blood pressure lowering effects of Lut have suggested the involvement of Lut-mediated regulation of hypertensive vascular remodeling via its inhibition of proliferation and migration of angiotensin II-induced vascular smooth muscle cells (Su *et al*., 2015). In the present study, we provide new insights into the molecular mechanisms by which Lut can exert anti-hypertensive effects via inhibition of receptors involved in RAAS signaling. This is in addition to its ability to regulate the production of ROS and inhibition of lipid and protein oxidation.

Although the present study reveals for the first time the protective roles of Lut against nephrotoxicity and haemodynamic alterations in rats induced by co-exposure to Dcf and NaF, there may be a limitation. The experimental design does not include separate groups of rats treated with NaF or Luteolin alone, results from which further interpretations could otherwise be made. However, the ethical use of animals as approved by our local ethics committee considerably limits the use of larger numbers of animals than that used in the present study.

In conclusion, our data supports an antihypertensive effect of Luteolin mediated by inhibition or down-regulation of receptors of the RAAS pathway. The model of Dcf and NaF co-exposure showed potential for antagonistic effects between the two compounds, probably obtainable with short-term exposure. Although not usually associated with NSAIDs, Dcf-induced hypotension, in this study, may probably be a result of severe gastrointestinal bleeding and resultant hypovolemia. The nature of interactions during prolonged co-exposure to the two compounds may form the focus of future studies.

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