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Chromolaena odorata flavonoids attenuate experimental nephropathy: Involvement of pro-inflammatory genes downregulation

Olaposi Idowu Omotuyi [a,b,c,d,*], Oyekanmi Nash [d], Ojochenemi Aladi Enejoh [a,d], Eunice Iyanuoluwa Oribamise [a], Niyi Samuel Adelakun [a,c]

[a] Chemogenomics Research Unit, Department of Biochemistry, Adekunle Ajasin University, Akungba-Akoko, Ondo State, Nigeria
[b] Chemo-Genomics Research Institute, Afe Babalola University, Ado Ekiti, Ekiti State, Nigeria
[c] Phyto-Therapeutics Research Unit, Mols and Sims, Ado Ekiti, Ekiti State, Nigeria
[d] Phytomedicine Research Group, Center for Genomics Research and Innovation, National Biotechnology Development Agency, Abuja, Nigeria

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ABSTRACT

Nephropathy is a serious complication comorbid with a number of life-threatening diseases such as diabetes. Flavonoids are well known cytoprotective phytochemicals. Here, nephropathy associated with streptozotocin (STZ) treatment in experimental animals was challenged by flavonoids (CoF) isolated from *Chromolaena odorata.* Experimental animals were divided into control (n = 5), STZ (40 *mg/kg b.w. i.p.* n = 5) and STZ-CoF (CoF = 30 *mg/kg b.w. oral, 60 days,* n = 7) groups. Blood urea nitrogen (BUN) and serum creatinine (SC) levels were quantified using ELISA. Kidney function, inflammatory marker, and antioxidant gene expression levels were also evaluated using reverse-transcription and polymerase chain reaction protocols. Histological assessment was also performed using Haematoxylin and Eosin (H&E) staining protocols. CoF improved kidney function by restoring BUN/SC levels to pre-STZ treatment states. KIM-1, TNF-α, and MCP-1 but not TNF-R and IL-10 genes were significantly downregulated in STZ-CoF treated group in comparison with STZ-treated group ($p < 0.05$). Anti-oxidant genes (GPx-1, CAT) significantly ($p < 0.05$ vs. control) upregulated in STZ-treatment did not respond to CoF treatment. STZ treatment associated Bowman's space enlargement, thickened basement membrane, and glomerulosclerosis were completely reversed in STZ-CoF group. Finally, CoF has demonstrable anti-nephropathic via downregulation of proinflammatory genes and may represent new management option in clinical nephropathy.

1. Introduction

The kidney is anatomically structured to maintain body fluid levels, by eliminating excess water and other metabolic wastes via the urinary system [1]. In addition, its role is well defined in the maintenance of acid-base balance [2], production of hormones and as target organ for several hormones [3]. Kidney injury is therefore associated with several life threatening clinical conditions including anaemia, metabolic bone disease, and hypertension which result from defective renal erythropoietin, calcitriol, and renin-angiotensin-aldosterone system respectively [4]. Kidney injury/damage is an age-independent global health concern affecting between 1–66 % of world population, accounting for approximately 10 million deaths annually [5] and correlates negatively with quality of life (QoL) in patients [6]. Regardless of the initiator such as: chemical insult/toxin [7], genetic mutation (*e.g.* podocin) [8], or comorbidity with primary disease such as cardiovascular disease, diabetes, anaemia and hypertension [9], the connecting point is the reactive oxygen species build-up, loss of peritubular capillaries and hypoxia [10,11]. Since hypoxia damages tubular epithelial cells, promotes infiltration and activation of inflammatory cells [12], modulation of activation of inflammatory cells represent a key strategy for attenuating kidney injury [13,14]. Plants have proven to contain diverse secondary

Abbreviations: FLVs, Flavonoids; AKI, Acute kidney injury; STZ, Streptozotocin; CRD, Committee of Centre for Research and Development; Nrf2, Nuclear factor-erythroid 2-related factor 2; ROS, Reactive oxygen species; ARE, Antioxidant response element; GPx-1, Glutathioneperoxidase; OCC, Occludin; KIM-1, KidneyInjury Molecule-1; CAT, Catalase; MCP-1, Monocyte chemoattractant protein 1; TNF-α-R, Tumour necrosis alpha receptor; SOD, Superoxide dismutase; MKK-3, mitogen-activated protein kinase kinase 3; CoF, *Chromolaena odorata* is rich in flavonoids; QoL, Quality of life.

* Corresponding author at: Chemogenomics Research Unit, Department of Biochemistry, Adekunle Ajasin University, Akungba-Akoko, Ondo State, Nigeria.
E-mail address: olaposi.omotuyi@aaua.edu.ng (O.I. Omotuyi).

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Table 1
List of Primers.

Primer Name	Forward Primer sequence (5′- 3′)	Reverse Primer sequence (5′- 3′)
TNF-α	CTCAAAACTCGAGTGACAAGC	CCGTGATGTCTAAGTACTTGG
IL-10	CAATAACTGCACCCACTTCC	ATTCTTCACCTGCTCCACTGC
KIM-1	AAGCCGAGCAAACATTAGTGC	TGAGCTAGAATTCAGCCACACA
MCP-1	TGCCAAGTAGCCACATCCAG	CACAGTGTGAGCAACTGGGA
GPX-1	CCGACCAGGGCATCAAAA	GAGGCCATAATCCGGATCTTC
OCC	CCCTTCTTTCCTTAGGCGACC	AGCAGACCTGCTGCGCC
CAT	AGTTCGGACATCAGGAGAATGGCA	TCACCATTCACCTCGCACTTCTCA
TNF-R	CTTTGCGACATGAACCTGCG	CCAAAGAGGAGGTTCACAGGG
Casp-3	TTGGAACGGTACGCGAAGAA	GCTTCCATGGATAGTCTTTGTTTCA
β-Actin	GTCGAGTCCGCGTCCAC	AAACATGATCTGGGTCATCTTTTCACG

metabolites with anti-inflammatory activities with demonstrable results in kidney injury [15,16], and cancer [17]. *Chromolaena odorata* is rich in flavonoids (CoF) [18]. Flavonoids are well reported to abrogate ROS-mediated tissue damage [19], inhibit inflammasome activation [20]. Flavonoids isolated from *C. odorata* has been tested in streptozotocin-induced acute kidney injury animal model in this study. The choice of this model was informed by its satisfaction of two key initiators of AKI; chemical insult and diabetes [21]. Having confirmed deranged kidney function by monitoring serum creatine level, a 2-month oral administration of CoF treatment was performed. Ultimately, kidney function, inflammatory mediators, anti-oxidant genes and histology were investigated in experimental and control groups. Biomarkers for monitoring kidney function or damage (e.g., kidney injury molecule-1 (KIM-1), neutrophil gelatinase-associated lipocalin (NGAL), interleukin-18, etc.) have been extensively reviewed [22,23]; making it possible to follow the reversal effect of CoF.

2. Materials and methods

2.1. Isolation of CoF

Flavonoid content of fresh *C. odorata* leaves was isolated using HCl (1 %, v/v) as previously documented [24] and purified using DOWEX-50 column resulting in CoF, the details of column purification and characterization of flavonoid composition has been previously reported by our group [18].

2.2. Induction and confirmation of kidney injury in rats

All protocols relating to animal studies were approved by the Animal Ethics Committee of Centre for Research and Development (CRD), Adekunle Ajasin University, Akungba- Akoko. Streptozotocin (STZ) used in the induction of kidney injury was prepared by dissolving in sodium citrate buffer (pH 4.5). Healthy male rats were divided into two groups. Group A (n = 4) served as the control, while groups B (n = 15) was administered freshly prepared STZ solution (40 mg/kg b.w., i.p.) once every other day for a total of three (3) times. 7 days after the termination of STZ treatment, animals blood samples were drawn from the tail vein as previously described[17] for blood glucose (BG) and serum creatine (SC) estimation using commercial assay kits following manufacturer's protocols. An animal should have the BG/SC values of ≥300 mg/dl / ≥ 80 μmol/L consistently for 14 days post STZ treatment to be used for the next experiment [25]. 12 animals which satisfied these preconditions were grouped into B (STZ-only, n = 5) and C (STZ + CoF, n = 7). CoF treatment (30 mg/kg b.w. oral) was performed once daily, for 60 days. All animals (treatment and control groups) were housed in a maximum of two (2) in polysulfonate cages, at 12:12-h reverse-lighting regimen, in a room maintained at 23 ± 2 °C and given water/chow ad libitum throughout the study. At the end of the study, animals were sacrificed under light anaesthesia and blood samples were drawn using venepuncture for SC as described earlier, and blood urea nitrogen estimation (commercial assay kit).

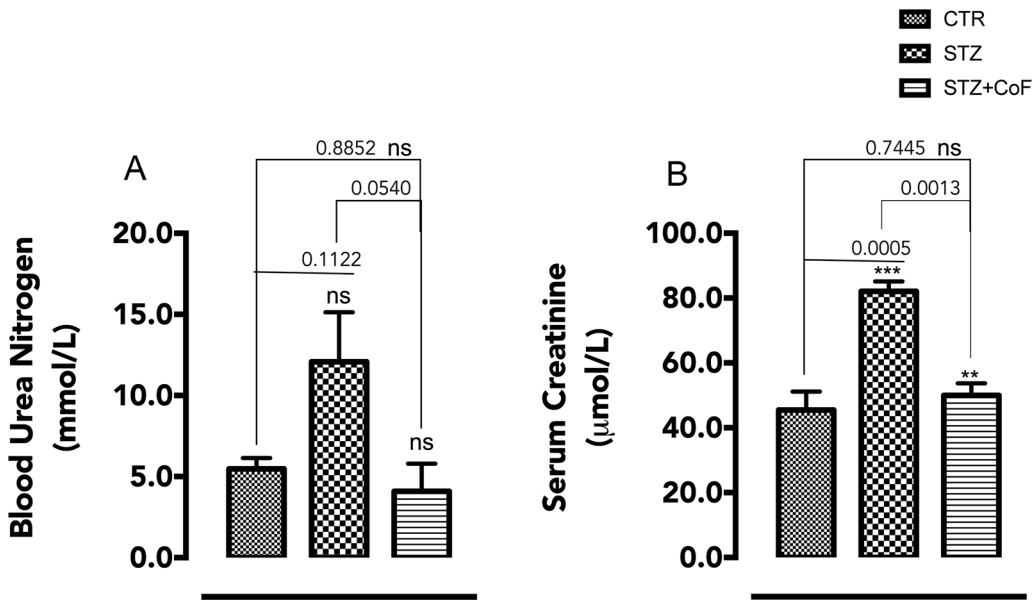

Fig. 1. Kidney function tests: Bar graph representing mean and SEM values the blood urea nitrogen (A) and serum creatinine in control (n = 5), STZ (n = 5) and STZ + CoF (n = 7) treatment groups. (Statistical comparison was done at $p < 0.05$), the calculated p values are displayed.

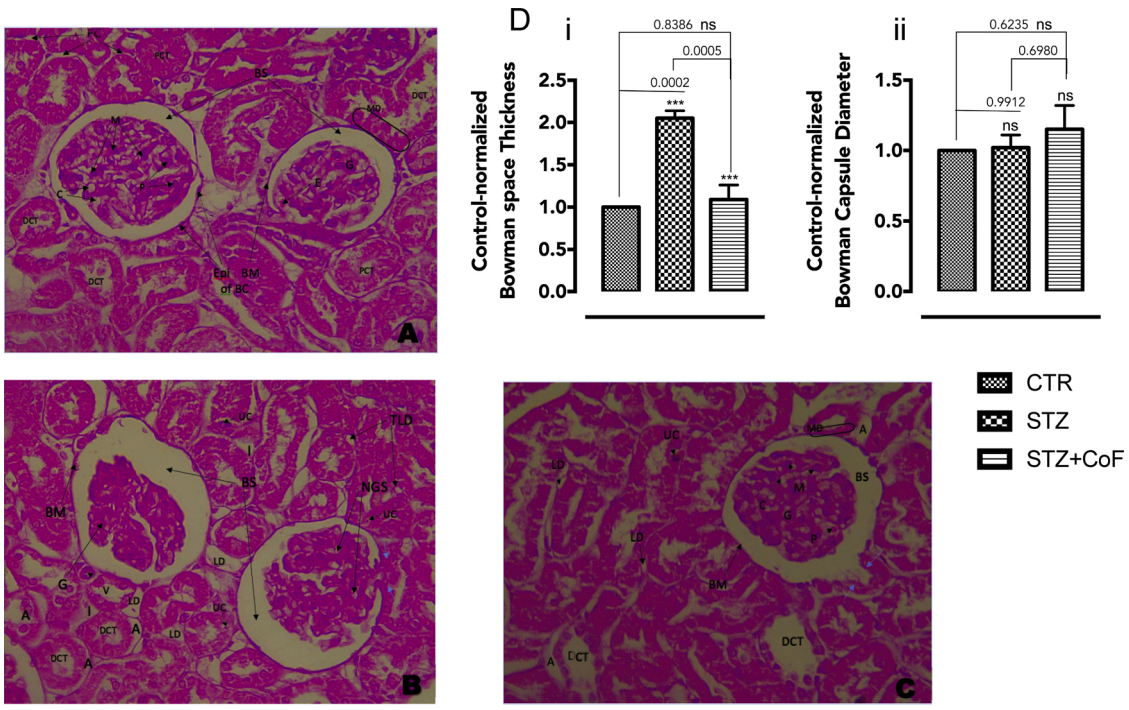

Fig. 2. Photomicrographs showing panoramic views of kidney general histomorphological presentations. Representative photomicrograph of control (A), STZ treated (B) and STZ + CoF co-administration group (C). Micro-morphometric values of Bowman's space thickness (D, *i*) and Bowman's capsule diameter (D, *ii*) are plotted as bar graph from mean and SEM values. (H&E Mag. = x400). (BC represents Bowman's capsule, BS represents Bowman's Space, PCT represents Proximal Convoluted Tubules, DCT represents Distal Convoluted Tubules, G represents Glomerulus, E represents Erythrocytes in glomerular capillaries, MD represents Macula densa, P represents Podocytes, M represents Mesengial cells, C represents Capillaries, PC represents Peritubular Capillaries, BM represents Basement Membrane, Interstitial space between tubules, Atrophy represents A, NGS - Nodules of Glomerular Scar, LD represents Lipid deposits, Blue arrows – FSGS tip variant, TLD represents Tubular Lipid Deposits, V – Vacoular, modifications, UC represents Unrecognized cells. (Statistical comparison was done at $p < 0.05$), the calculated p values are displayed on each bar.

2.3. Gene Expression Studies

For each group, the two kidneys were dissected into two halves longitudinally. One part was placed in TRIzol Reagent (ThermoFisher Scientific) for total RNA isolation following the manufacturer's protocol. DNA in the isolated RNA was removed following DNAse I treatment (ThermoFisher Scientific) as specified by the manufacturer. DNA-free RNA was converted to cDNA immediately using ProtoScript® First Strand cDNA Synthesis Kit (NEB). PCR amplification was performed using OneTaq® 2X Master Mix (NEB) using the following forward and reverse primer sets: TNF-a, IL-10, KIM-1, MCP-1, GPX-1, OCC, CAT, TNF-R, Casp-3, and β-Actin (Table 1). The bar graphs represent mean ± SEM (n = 5–7) values of gene/β-Actin ratio of the gel (1.5 % agarose in TAE buffer) image for each sample as estimated using (Image-J) while the images are representative snapshot of the pooled sample. Graph-pad Prism was used for statistical comparison ($p < 0.05$) using nonparametric (one-way ANOVA) test. Protein levels of catalase and GPX-1 were also determined spectrophotometrically as described by Gomathi et al. [26].

2.4. Histological examination

The other half of the kidney samples were immersed in freshly prepared 10 % neutral buffered formalin, and routinely processed and embedded in paraffin. 5 μm sections were cut and stained with hematoxylin and eosin (H&E) for routine histopathological examination and light microscopy (each plate represents x400). Bowman's space thickness and Bowman's capsule diameter were quantified using Image J [27], and the values were plotted as mean ± SEM (n = 5–7). GraphPad Prism ver 6.0e was used for statistical comparison ($p < 0.05$) using nonparametric (one-way ANOVA) test.

3. Results

3.1. CoF restores STZ-induced loss of kidney function in experimental nephropathy

Regularly, the biomarkers that best predict kidney function are the levels of blood urea nitrogen (BUN) and serum creatinine (SC). Here, Fig. 1A shows that STZ treatment is associated with increased (not significant, $p < 0.05$) BUN, which is reduced to control levels with CoF intervention. Similarly, SC levels statistically ($p < 0.05$) increased following STZ treatment but not with CoF treatment (Fig. 1B) in comparison with the control group.

3.2. STZ treatment is associated with kidney histo-structural damages reversible by CoF treatment

Damages to the kidney are visually appreciated with the cytoarchitectural changes especially in comparison with undamaged controls. Fig. 2A shows normal cytoarchitecture of kidney; showing regular histological features (*Proximal Convoluted Tubules (PCT), Bowman's Space (BS), Glomerulus (G), Erythrocytes in glomerular capillaries (E), Macula densa (MD), Mesengial cells (M), Capillaries (C), Peritubular Capillaries (PC), Distal Convoluted Tubules (DCT), Podocytes (P), Bowman's capsule (BC), Basement Membrane (BM), Interstitial space between tubules (I)*). On the other hand, STZ-treatment group is associated with features such as *nodules of Glomerular Scar (NGS), enlarged BS*, *Tubular Lipid Deposits (TLD), shrunk glomeruli*, loss of *Macula densa (MD),* and *podocytes* (Fig. 2B). STZ-induced ultrastructural damages were completely reversed in CoF treated group (Fig. 2C) with features similar to those in the control group. This finding is best represented by the quantitative projection of the BS thickness (Fig. 2D, *i*) and BC diameter (Fig. 2D, *ii*) in

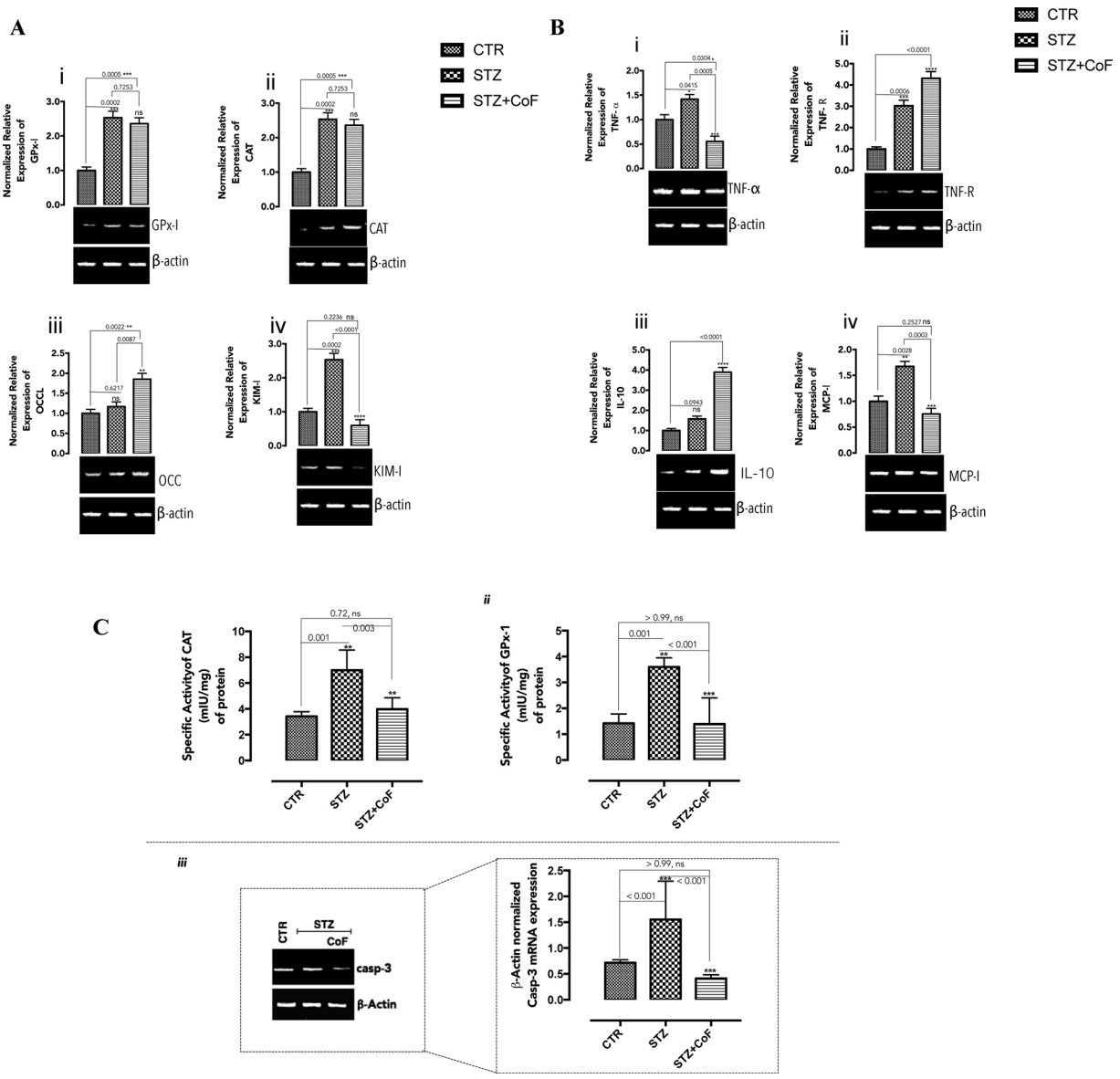

Fig. 3. Expression pattern of Antioxidant and Inflammatory genes in treatment and control groups.
(A, *i-iv*). Bar graph represents mean and SEM values of quantified band from each sample for specified antioxidant gene in control (n = 5), STZ (n = 5) and STZ + CoF (n = 7) treatment groups. The gel image is the representative snapshot of the pooled samples.
(B, *i-iv*). Bar graph represents mean and SEM values of quantified band from each sample for specified inflammatory gene in control (n = 5), STZ (n = 5) and STZ + CoF (n = 7) treatment groups. The gel image is the representative snapshot of the pooled samples. (Each bar represents control normalized relative expression (gene/β-actin). Statistical comparison was done at $p < 0.05$), the calculated p values are displayed. (C, *I & ii*). Bar graph represents the mean and SEM of specific activities of catalase and glutathione peroxidase enzymes from the tissue homogenate. (*iv*) Bar graph represents mean and SEM values of quantified band from each sample for caspase-3 gene in control (n = 5), STZ (n = 5) and STZ + CoF (n = 7) treatment groups. The gel image is the representative snapshot of the pooled sample.

the treatment and control groups. Indeed, significantly increased ($p < 0.05$ vs control) thickness of BS caused by STZ-treatment was reversed in CoF treated group.

3.3. STZ treatment is associated with upregulation of antioxidant and pro-inflammatory genes in the kidney: the ameliorative effect of CoF on pro-inflammatory genes

STZ treatment did cause negative ultrastructural changes in the kidney as shown in the photomicrographs, but the underlying mechanism is unknown. Here, we proposed underlying oxidative and pro-inflammatory mechanisms. Reactive oxygen species (ROS) build-up triggers nuclear factor-erythroid 2-related factor 2 (Nrf2) translocation and antioxidant response element (ARE) activation, which increases the expression of putative genes for antioxidant system. One of the genes upregulated by ARE is glutathione peroxidase (GPx-1) [28]. Catalase levels was also considered in this study as a result of its central role in detoxifying hydrogen peroxide generated during macrophage infiltration [29]. Under basal condition, the overall health of the kidney is also monitorable by the expression pattern of occludin (OCC) and Kidney Injury Molecule-1 (KIM-1). OCC is an important protein of the tight junction and renal tubule [30] while KIM-1, a known epithelial cell adhesion molecule Is up-regulated in renal cells injury episode [31]. Fig. 3A (*I, ii & iii*) show significantly upregulated GPx-1, CAT, and OCC-1 in STZ group in CoF irreversible manner. KIM-1 gene upregulation by STZ, on the other hand is completely reversed following CoF intervention TNF-R (Fig. 3A, *iv*). Finally, within the ROS-rich nephron, infiltration of macrophage and other inflammatory cells via monocyte

chemoattractant protein 1 (MCP-1) [32] is the next precondition for the generation of pro-inflammatory cytokines such as IL-10 and TNF-α and their cognate receptors. Fig. 3B (*i & iv*) shows significant upregulation of TNF-α and MCP-1 in STZ group, which is fully reversed with CoF treatment. TNF-R (Fig. 3A, *ii*) and IL-10 (Fig. 3B, *iii*) were both upregulated in STZ and STZ-CoF groups in comparison with basal control. To validate that the gene expression patterns are consistent with protein levels, Superoxide dismutase (SOD) and GPx-1 protein levels were assayed and shown to correlate (Fig. 3C, *i & ii*). Apoptotic marker, caspase-3 mRNA was significantly repressed only in STZ-induced animals only when CoF was administrated (Fig. 3C, *iii*).

4. Discussion

Acute kidney injury (AKI) often results from impaired renal perfusion, exposure to nephrotoxins, outflow obstruction, or genetic mutation, or as comorbid with other underlying diseases [5–7,12]. AKI currently affects reasonably large global population, and it is associated with fatal clinical outcomes especially in advanced stages; for survivors and their dependents, AKI is associated with brutal economic outlook and low quality of life (QoL) [33]. These factors, therefore, underscore an urgent need for immediate solution to AKI. In this study, nephropathy was induced using STZ, a well-known nephrotoxic agent with pro-diabetic potency [21,26,34] and the ability of *C. odorata* flavonoids (CoF) to reverse the biochemical, transcriptional and histological episodes associated with STZ treatment was evaluated.

Indeed, STZ treatment did result in elevated serum levels of creatine and blood urea nitrogen, clearly showing possible renal dysfunction, histopathological examination confirmed that renal tissue ultrastructure was damaged thus, establishing the basis for increased SC and BUN levels in the blood samples of the experimental animals.

In the STZ-treated group, the kidney ultrastructure depicts various abnormal ultrastructural features ranging from enlarged Bowman's space to thickened basement membrane to atrophied distal tubular epithelial cells and glomerulosclerosis. Enlarged Bowman's space observed here may have been caused by expansion of the Bowman's capsule and concurrent degeneration of the glomerulus [35–37]. Another abnormal feature observed in STZ-treated group is glomerulosclerosis characterised by the presence of nodules of Glomerular Scar (Kimmelstiel-Wilson nodules) in which the small capillaries that filter blood are distorted or compacted by nodular scarring. This abnormality is usually also manifested in cases of nephrotic syndrome and characterized by mesengial cell expansion. Mesangial expansion and basal membrane thickening are a consequence of extracellular matrix (ECM) accumulation [38].

Furthermore, glomerulosclerosis, characterized by segmental lesion between Bowman's capsule and the confluence of podocytes with parietal or tubular epithelial cells at the tubular lumen or neck is also observed following STZ treatment. These features are pronounced in clinical cases of renal failure [12].

Interestingly, just as CoF treatment reversed elevated levels of SC and BUN, all histological features characterizing renal failure in STZ group were markedly reduced in CoF treated groups. CoF treatment group appears essentially "repair-in-progress" state, showing a remarkable reduction in the Bowman's space, less thickened basement membrane and repair in the glomerulus of the renal corpuscle, with normal podocytes, mesangial cells, and glomerular capillaries. To better understand the underlying mechanism of action of CoF in the reversal of renal damage, two plausible mechanisms were suggested: anti-oxidant and anti-inflammatory mechanisms as previous studies strongly link flavonoids to reduced kidney histopathological damage [13,14,19–21] and perturbed expression of pro-inflammatory cytokines including TNF-α [39]. Taken together, flavonoids; exemplified by CoF may represent a blockage compound for inflammation-mediated renal damage with a note that inflammation is one of the key consequence of ROS accumulation [40,41].

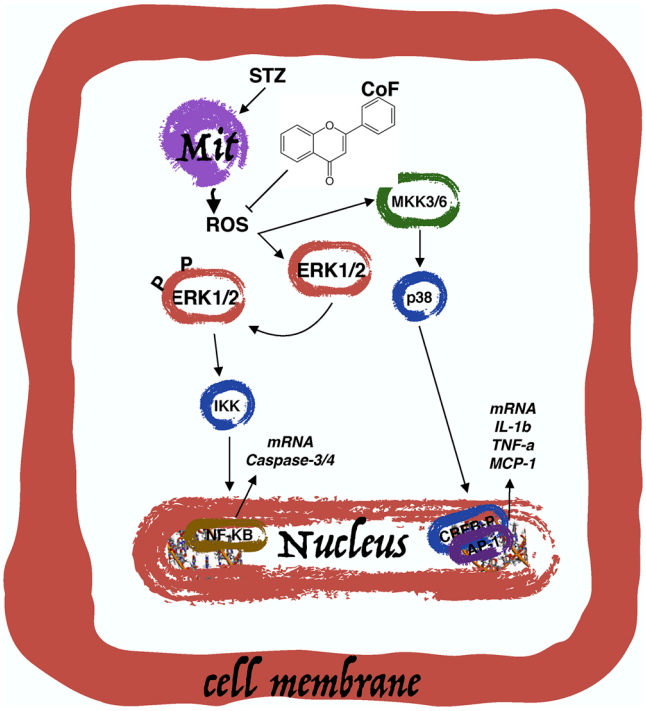

Fig. 4. XXX.

STZ-treated group exhibited significantly high expressions of TNF-α and monocyte chemoattractant protein (MCP-1), which account for damaged ultrastructure observed. CoF significantly downregulated TNF-α and MCP-1 and upregulated TNF-R and IL-10 as previously reported. Our findings here agree with previous report that flavonoid-rich preparations could inhibit production of renal TNF-α and MCP-1 following LPS treatment [42] and in cadmium toxicity [45].

Catalase (CAT) and glutathione peroxidase 1 (GPx-1) are well documented part of the battery of antioxidant system utilized by the body in combating ROS generation via NRSF/ARE mechanisms [25–28]. Since expression of GPX-1 and CAT is insensitive to CoF treatment, it may seem that once STZ initiates ROS build-up, the cellular mechanism for detoxification is initiated regardless of the presence of CoF. This finding is consistent with previous findings that diabetic hepatopathy caused by STZ treatment is associated with increased expression of antioxidant enzyme genes, activation of caspase 9/3 and apoptosis via AIF-caspase independent/mitochondrial apoptotic pathway

CoF is however beneficial as the tight junction molecules such as occludin is being re-assembled as shown by the increased expression levels, and more importantly, the injury is being resolved as kidney injury molecule-1 (KIM-1) [32,43] and pro-apoptotic marker caspase-3 expression levels [44] were declining following intervention of CoF; these are key indicators of renal epithelial cell regeneration. Our findings suggest improved kidney architecture and function following CoF administration.

5. Conclusion

Nephropathy is a severe complication comorbid with a number of life-threatening diseases such as diabetes. Flavonoids represent a class of phytochemicals capable of reversing nephropathic phenotypes in experimental models. Here, nephropathy associated with streptozotocin (STZ) treatment in experimental animals was challenged by previously characterized flavonoids (CoF) isolated from *Chromolaena odorata*. From our results, the likely model of CoF induced nephroprotection in STZ-treated rats is the blockage of ROS generation from the mitochondria, which inhibits downstream ERK-1/2-NF-kappa-B-mRNA activation

resulting in repressed caspase-3 mRNA expression; MKK-3/6-p38-CREB-P/AP-1 activation following STZ-induced ROS is also blocked which results in repression of IL-1β, TNF-α, MCP-1 mRNA (Fig. 4). We have demonstrated that nephropathy is reversible by CoF administration in experimental animals via countering of inflammatory processes. CoF may represent new management option in clinical nephropathy.

Author statement

Olaposi Idowu Omotuyi and Oyekanmi Nash (Conceptualization and Design of experiments), Ojochenemi Aladi Enejoh (Performed molecular biology Methods and gene expression), Eunice Iyanuoluwa Oribamise (Performed H&E experiments) Niyi Samuel Adelakun (Enzyme assay). All authors wrote and approved the manuscript. OIO and ON have equal contribution to the experiments.

Declaration of Competing Interest

The authors declare no conflict of interest.

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