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RESEARCH ARTICLE

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Crocin improves renal function by declining Nox-4, IL-18, and p53 expression levels in an experimental model of diabetic nephropathy

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Abstract

Oxidative damage, inflammation and apoptosis play significant roles in diabetic nephropathy. Previous studies demonstrated anti-inflammatory and anti-oxidative effects of crocin, but there is no evidence about its effects on IL-18, NOX-4, and p53 expression in diabetic kidneys. The aim of this study was to evaluate possible effects of crocin on improving main mechanisms underlying diabetic nephropathy. Male Wistar rats were randomly divided into four separate groups as normal (C), normal treated (CC), diabetic (D), and diabetic treated (DC) ($n = 6$). Diabetes was induced by a single dose of streptozotocin (40 mg/kg/intravenous). Treated groups received crocin (40 mg/kg, intraperitoneal) for 8 weeks. At the end of the 8th week of the study, all rats were sacrificed and urine, blood and tissue were collected. Levels of urea, uric acid, creatinine and glucose were determined collected sera, and proteinuria was measured in urine samples. Moreover, the contents of malondialdehyde (MDA), nitrate, and glutathione (GLT) as well as catalase (CAT) and superoxide dismutase (SOD) enzymes activities were measured. The expression of NOX-4, IL-18, and p53 at both mRNA and protein levels were also assessed. Hyperglycemia significantly increased proteinuria in diabetic rats (D). Also, depressed antioxidant defense system potency, but increased NOX-4 expression and free radicals production resulting in oxidative stress, were observed. Moreover, expressions of IL-18 (as a marker of inflammation) and p53 (as a marker of apoptosis) were increased. These outcomes were accompanied by enhanced histological damages and renal failure but, treatment with crocin improved these deteriorations, and ameliorated renal function. It potentiated renal cells antioxidant defense system and declined inflammation. Also, crocin lowered apoptosis and improved histological damages in renal cells. Oxidative stress, inflammation and apoptosis are considered three main mechanisms underlying diabetic nephropathy. Treatment with crocin prevented these deleterious effects and improved renal function under diabetic conditions.

KEYWORDS
antioxidant defense system, crocin, diabetic nephropathy, hyperglycemia, IL-18, NOX-4

1 | INTRODUCTION

Diabetes mellitus (DM) is known as a highly prevalent metabolic disorder worldwide.[1,2] It is estimated that in 2050, up to 21% of adult population will suffer from DM.[3] This chronic disorder can lead to many microvascular complications for example, retinopathy, neuropathy, and nephropathy.[4] Diabetic nephropathy (DN) is the most common cause of end-stage renal failure which might be experienced by up to the 40% of diabetic cases within 20-25 years after onset of diabetes.[5] DN makes many microstructural injuries in kidneys including basement membrane thickening, glomerulosclerosis, tubular atrophy, expansion of mesangial spaces, tubulointerstitial fibrosis, podocytes loss, increase of filtration barrier permeability, etc.[6–8] In fact, it has been declared that almost all types of kidney cells are affected by hyperglycemia resulting in various pathological consequences leading to proteinuria and eventually, renal failure.[9]

Remarkably diverse molecular pathways have been hold responsible for DN development including renin-angiotensin system, PKC[1] over-activity, TGF-β^2 over-production, TNF-α pathway, advanced glycation end products, oxidative stress, etc.[4,6,10,11] Also, more recent research have shown that inflammation has a pivotal role in this field.[7,12] Based on this hypothesis, over-production of inflammatory mediators during un-controlled hyperglycemia causes deleterious effects on kidney cells.[7] Among these mediators, IL-18 is a potent proinflammatory cytokine which have been shown to be more likely involved in onset of DN, compared to other cytokines.[7,10] IL-18, as a immunoregulatory agent has also direct destructive influences on renal glomerulus and can accelerate the DN progress.[10,13]

IL-18 as a known marker of endothelial cells injury, is produced by activated macrophages or vascular endothelial cells. This cytokine stimulates interferon-γ (INF-γ) production and INF-γ in turn, triggers a cascade of inflammatory responses by stimulating other inflammatory elements such as TNF-α, IL-1, and adhesion molecules, as well as inducing endothelial cells apoptosis, etc. which all exacerbate DN.[7,13–17] Thus, modification of inflammatory processes could be an interesting approach for prevention of DN.[7]

Oxidative stress is another mechanism that has been suggested to be involved in induction of DN during hyperglycemia.[4,11,55] During uncontrolled hyperglycemia, as a result of free radicals over-production, internal physiologic antioxidant defenses system are defeated leading to a condition known as oxidative stress.[19] Oxidative stress is strongly associated with different pathological processes underlying DN including inflammation, endothelial dysfunction, cell proliferation, vascular remodeling, extracellular matrix deposition, angiogenesis, fibrosis, etc.[20] In diabetic milieu, NADPH oxidase (NOX) enzyme is regarded as a main free-radicals generator.[18,21] Vicki et al[21] demonstrated that inhibition of NOX expression may delay the onset of DN. Also, Sedeek et al[22] showed that NOX-4 is the most abundantly expressed type of NADPH oxidases which is highly associated with oxidative stress in renal tissues. Various types of renal cells such as mesangial cells, endothelial cells, podocytes, fibroblasts, vascular smooth muscle cells, thick ascending limb, macula densa cells expressed different NOX-4 subunits including p47phox, p67phox, and p22phox.[23]

Free radicals can directly or indirectly harm different renal elements by oxidizing natural molecules such as DNA, lipids, proteins, and carbohydrates.[24] Oxidative stress has important roles both in micro and macrovascular complications of DN.[24] Glomerulus is one of the most susceptible tissues towards free radicals attacks.[24] So, pharmacological interventions which modify NOX-4 expression and improve redox state, may slow DN development.[11,24]

Strong evidence have demonstrated that apoptosis rate is increased during DN development.[25] Dinender et al[25] observed various features of apoptosis in different biopsies of diabetic subjects and suggested a potential role for apoptosis in DN progress. Susztak et al[26] stated that podocyte cells apoptosis increases by glucose-induced NADPH oxidase hyperactivation and oxidative stress during DN progress. Tikoo et al[27] noted that p53 protein is activated in DN and declared that this activation is strongly associated with renal cells injuries during hyperglycemia. p53 protein is a transcription factor which is activated by a wide range of agents and involved in apoptotic pathways in different tissues.[28]

Crocin, an active component of *Crocus sativus* L. (saffron) is known as an antioxidant[18,29,30] and anti-inflammatory[31,32] agent. There are numerous studies which have confirmed that crocin exerts beneficial effects against various conditions.[29–31] However, little is has been published[18] on the effects of crocin on renal function during uncontrolled hyperglycemia. Notably, Altinoz et al[33] revealed that crocin decelerates DN progression probably via its antioxidative effects. We found no further strong evidence about modulation of DN by crocin.

The aim of this study was to evaluate the role of oxidative stress, inflammation, and apoptosis processes in DN and to examine crocin effects on the above-mentioned features of DN.

2 | MATERIALS AND METHODS

2.1 | Animals

Male Wistar rats (200-220 g) were randomly divided into four groups as control, control treatment, diabetic, and diabetic treatment groups ($n = 6$). Animals were purchased from Pasteur institute (Tehran, Iran) and kept at 18-22°C with standard 12-12 hr dark—light cycles. All ethical rules related to animals experiments were considered and respected.

2.2 | Diabetes induction

Diabetes was induced by a single dose of streptozotocin (STZ; 45 mg/kg/iv). After 72 h, blood samples were collected from the tail vein and blood glucose was assayed using a glucometer (Bionime, Swiss) to verify diabetes induction. Rats with blood glucose >400 mg/dl were considered diabetic and randomly divided into four groups.

2.3 | Treatment

Crocin was purchased from Sigma-Aldrich (St. Louis, MO), diluted in distilled water and given daily to animals (40 mg/kg/ip) for 56 days.

2.4 | Sampling

Blood sampling were performed from the tail vein for analysis of blood glucose and directly from the heart after sacrifice on the 56th day. Samples were centrifuged and sera were separated for blood tests. Urine samples were collected using metabolic cages during the last week of experiment. Tissue sampling were performed promptly after induction of deep anesthesia by ketamine and xylazine on the 56th day. Samples were kept at −70°C after freezing in liquid nitrogen.

2.5 | Tests

2.5.1 | Biochemical tests

Biochemical tests involved determination of glucose, creatinine, urea and uric acid concentrations in serum samples as well as measurement of creatinine and protein concentrations in urine samples using routine standard kits. After detecting creatinine in serum and urine samples and identifying 24-h urine volume, creatinine clearance was assessed using this formula:

$$\text{Creatinine clearance } (\text{mg/mL}/1.73\text{m}^2) = (\text{Serum cretinine} \times 1440)/(\text{urine creatinine}) \times (\text{daily urine volume})$$

2.5.2 | Oxidative stress tests

SOD activity assay

The activity of SOD enzyme was assessed according to Winterbourn method.[34] This method is based on SOD ability to inhibit the reduction of nitro-blue tetrazolium by superoxide.

In this assay, 0.067 mole of potassium phosphate buffer (pH 7.8) was added to 0.1 mole of EDTA[3] containing 0.3 mM sodium cyanide, 1.5 mM nitro-blue tetrazolium, and 0.1 mL of sample. Then, 0.12 mM riboflavin was added to each sample to activate the reaction and incubated for 10 min. The absorbance of each sample at 560 nm was recorded during 5 min using a spectrophotometer. The amount of enzyme required for 50% inhibition was considered 1 Unit (U) and results were expressed as U/mL.

CAT activity assess

The activity of CAT enzyme was measured by Aebi method.[35] Reaction mixture containing 0.85 mL of potassium phosphate buffer 50 mM (pH 7.0) and 0.1 mL of homogenate, was incubated at room temperature for 10 min. Reaction was triggered by addition of 0.05 mL H_2O_2 (30 mM prepared in potassium phosphate buffer 50 mM; pH 7.0) and absorbance at 240 nm was recorded during 3 min. Finally, enzyme activity was expressed as U/mL (1 μmole H_2O_2).

GLT content assessment

GLT content was assessed based on the method introduced by Tietz.[36] Cellular protein content was precipitated by addition of 5% sulfosalicylic acid, followed by centrifugation at 4000*g* for 15 min. GLT content in supernatant was assessed as follows: Hundred microliter of protein-free supernatant was added to 810 μL of 0.3 mM Na_2HPO_4 and 90 μL of DTNB[4] in 0.1% sodium citrate. The DTNB absorbance was recorded at 412 nm during 5 min. A standard curve for GLT was plotted and the sensitivity of measurement was determined to be between 1 and 100 μM. The level of GLT was expressed as nmol/mL.

MDA content assay

The amount of MDA as a marker of lipid peroxidation was assessed based on the method described by Satoh.[37] Here, 0.5 mL of tissue homogenate was mixed with 1.5 mL of 10% trichloroacetic acid and centrifuged at 4000*g* for 10 min. Next, 1.5 mL of supernatant and 2 mL of thiobarbituric acid (0.67%) were mixed and placed in a hot-water bath in sealed tubes for 30 min. Then, samples were allowed to chill at room temperature for 20 min. Afterwards, 2 mL N-butanol was added and centrifuged at 2000*g* for 5 min. The resulting supernatant was removed and its absorbance was measured at 532 nm using a spectrophotometer. MDA content was determined by using 1,1,3,3-tetraethoxypropane as a standard. Finally, MDA concentration was expressed as nmol/mL.

2.6 | Nitrate assay

The nitrate content of the "cytosolic extract" as an indicator of free nitrate radicals was measured by the colorimetric reaction of the Griess method.[38] In this procedure, 0.4 mL of cytosolic extract was deproteinized by addition of 0.01 mL of zinc

TABLE 1 Forward and reverse primers used in RT-PCR protocol

Gene	Forward primer	Reverse primer
NOX-4	AGATGTTGGGCCTAGGATTGTG	AGCAGCAGCAGCATGTAGAAGA
IL-18	CGCAGTAATACGGAGCATAAATGAC	GTAGACATCCTTCCATCCTTCACAG
p53	TTCCGAGAGCTGAATGAGGCCTTG	ACTGACCGGATAGGATTTCGCTGG

sulfate followed by centrifugation at 4000*g* for 20 min at 4°C, to separate supernatant. Next, 0.3 mL of supernatant was added to 0.3 mL of chloride vanadium and incubated in the dark. Then, 0.15 mL of sulfanilamide (0.01%) and 0.15 mL of N-[1-naphthyl] ethylenediamine di-hydrochloride (NED, 0.01%) were added and incubated for 30 min at 37°C. Then, the absorbance of solution was determined at 540 nm. Nitrite concentration was estimated using a standard curve generated from the absorbance of different sodium nitrate solutions. Finally, the nitrate levels were expressed as nmol/mL.

2.6.1 | Histological assay

Histological assessment was performed by hematoxylin-eosin staining standard method. To fix the kidney tissue, dehydration (by 70, 80, 96, 100, and 100% ethanol) and clearing (by xylene) processes were performed and after paraffin embedding, coronal serial sections (5-μm thickness) were prepared for staining. The samples were placed in xylene solution for two times (each time for 15 min) for clearing. The histological changes were observed by an expert pathologist with a light microscope (Nikon, Japan) using an ×400 magnifier which was connected to a digital camera (CMEX, Netherland) for capturing the micro-graphs.

2.6.2 | mRNA expression assay

For assessment of various genes expression, we applied RT-PCR[5] technique using three sequential steps: (1) RNA extraction, (2) cDNA synthesis, and (3) amplification.

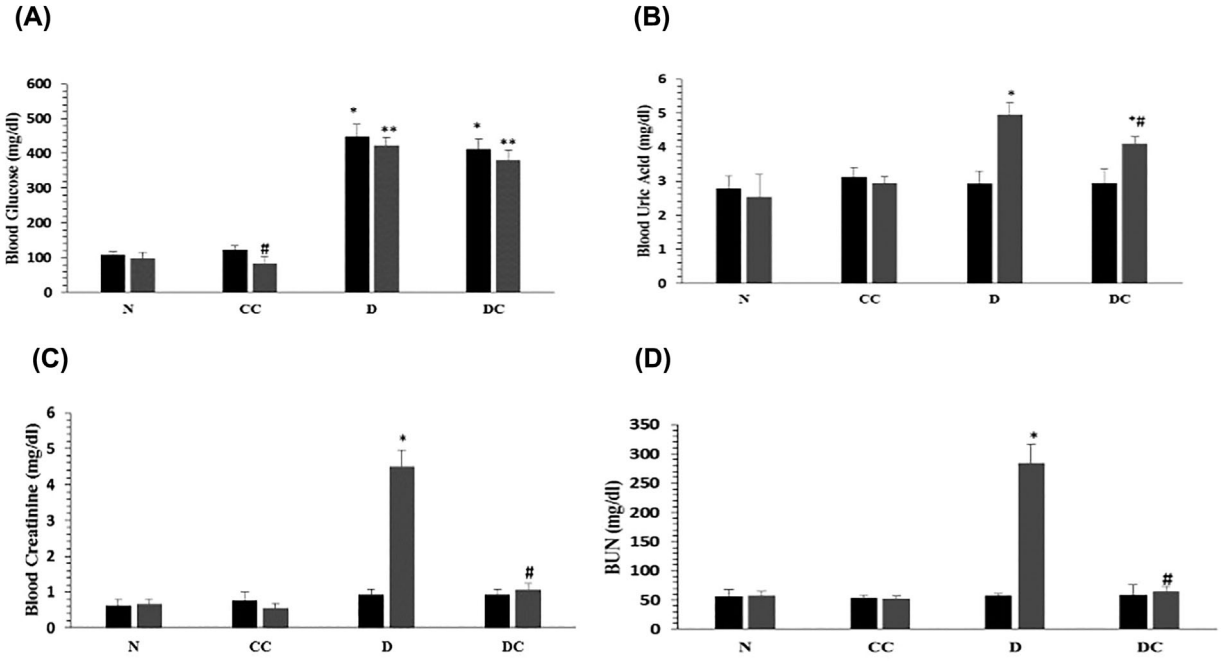

FIGURE 1 Changes in Blood levels of glucose (mg/dl), uric acid (mg/dl), creatinine (mg/dl), and BUN (mg/dl) in normal (N), normal + crocin (C), diabetic (D), and diabetic + crocin (DC) groups. All values are presented as mean ± SD. Black and gray bars indicate the values at the beginning (day 1) and end (day 56) of the study. In section (A): #($P = 0.05$) and *($P = 0.001$) show significant differences compared to control group (N) on the 1st day. **($P = 0.001$) shows significant differences compared to control group (N) on the 56th day. In section (B): *($P = 0.00$) shows significant differences compared to control group (N) on the 56th day. #($P = 0.03$) shows significant differences compared to diabetic group (D) on the 56th day In section (C): *($P = 0.00$) shows significant differences compared to control group (N) on the 56th day. #($P = 0.00$) shows significant differences compared to diabetic group (D) on the 56th day In section (D): * ($P = 0.00$) shows significant differences compared to control group (N) on the 56th day. #($P = 0.00$) shows significant differences compared to diabetic group (D) on the 56th day

In this process, 100 mL of tissue was mixed with 1 mL of Topazol solution and after 15 min, 200 μL chloroform was added and incubated at room temperature for about 10 min followed by centrifugation at 120 00*g* for 15 min at 4°C) and the supernatant was removed. Afterwards, 500 μL isopropanol was added and the mixture was centrifuged at 120 00*g* for 15 min at 4°C). Then, liquid was separated and 1 mL ethanol was added and centrifuged at 7500*g* for 8 min at 4°C. By adding 70 μL of DEBS solution into micro tube and incubating at 55°C for 5 min, total RNA was extracted.

cDNA synthesis was performed as follows: 3 μL of RNA and 17 μL of distilled water were added to cDNA synthesis commercial kit. Then, cDNA was synthesized during 12 rounds of 3 steps by thermo-cycler (step 1: 20°C for 30 s; step2: 45°C for 4 min; and step 3; 55°C for 30 s) plus one round of heat activation step (55°C for 5 min).

For cDNA amplification, 3μL of cDNA, 2 μL of primers (forward and reverse; Table 1) and 17 μL of distilled water were added to commercial PCR kit and then inserted in thermo cycler for 6 heating steps of which, steps 2-5 were repeated for 35 cycles (step 1: 95°C for 2 min; step 2: 95°C for 30 s; step 3: 53°C for 30 s; step 4: 72°C for 1 min; step 5: 72°C for 10 min and step 6: 30°C for 30 s). For running amplified genes, agarose gel was used and a house keepinggene (*beta-actin*). After running, gels were kept in ethidium bromide for 20 min and finally, photos were obtained in Gel-Doc.

2.6.3 | Protein expression assays

For detection of the protein expression, we utilized IHC[6] method. In this method, tissues were washed four times with PBS[7], with 5 min intervals. Paraformaldehyde 4% was added and incubated for 20 min and hydrochloric acid (2 normal) was added and incubated for 30 min. Then, borate buffered saline was added for neutralization. Tissues were washed again. Trition 3% was added for cells disruption and samples were washed again with PBS. Goat serum 10% was added and incubated for 30 min. Afterwards, diluted primary antibody (1/100 in PBS) was added and incubated at 4°C for 12 h. Next, tissues were washed again four times with PBS. Then, diluted secondary antibody (1/200) was added and incubated at 37°C

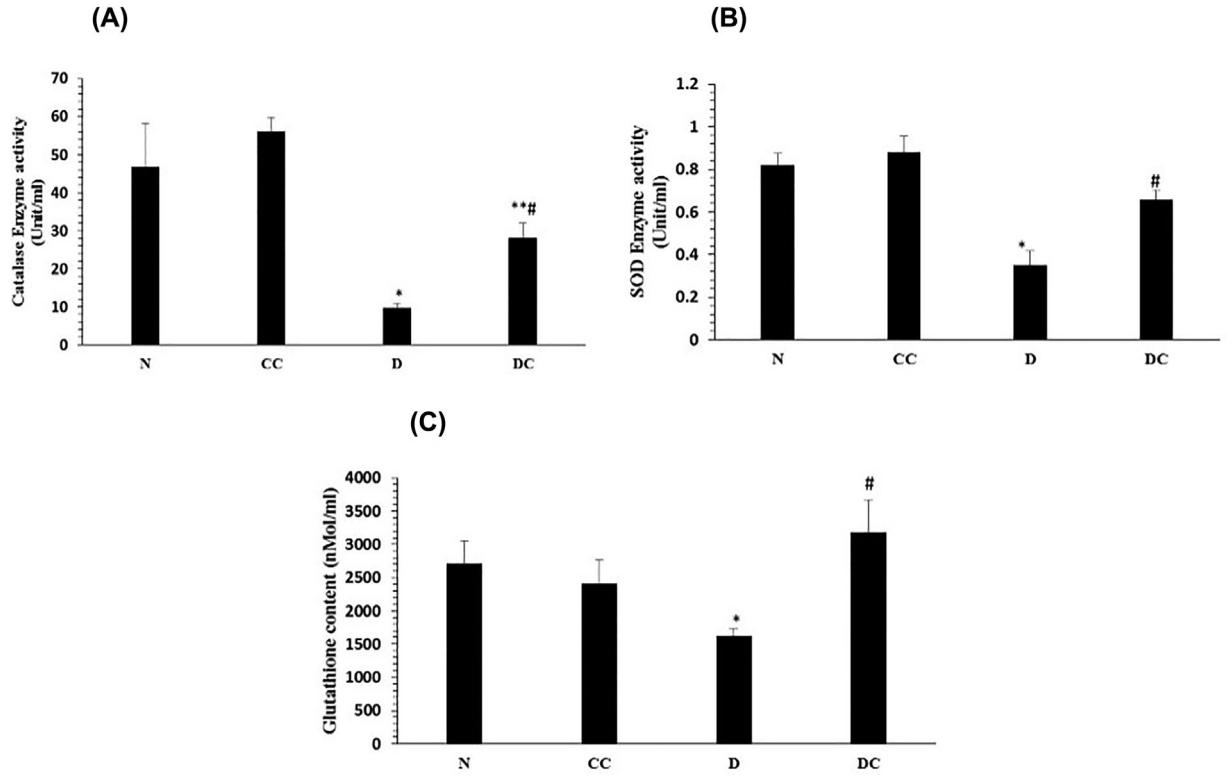

FIGURE 2 Catalase enzyme activity (Unit/mL), SOD Enzyme activity (Unit/mL), and glutathione content (nmol/mL) in normal (N), normal + crocin (C), diabetic (D), and diabetic + crocin (DC) groups at the end of study. All values are presented as mean ± SD. In section (A): * ($P = 0.00$) and **(P = 0.05) show significant differences compared to control group (N). #($P = 0.01$) shows significant differences compared to diabetic group (D). In section (B): *($P = 0.00$) shows significant differences compared to control group (N). #($P = 0.01$) shows significant differences compared to diabetic group (D). In section (C): *($P = 0.00$) shows significant differences compared to control group (N). #($P = 0.00$) shows significant differences compared to diabetic group (D)

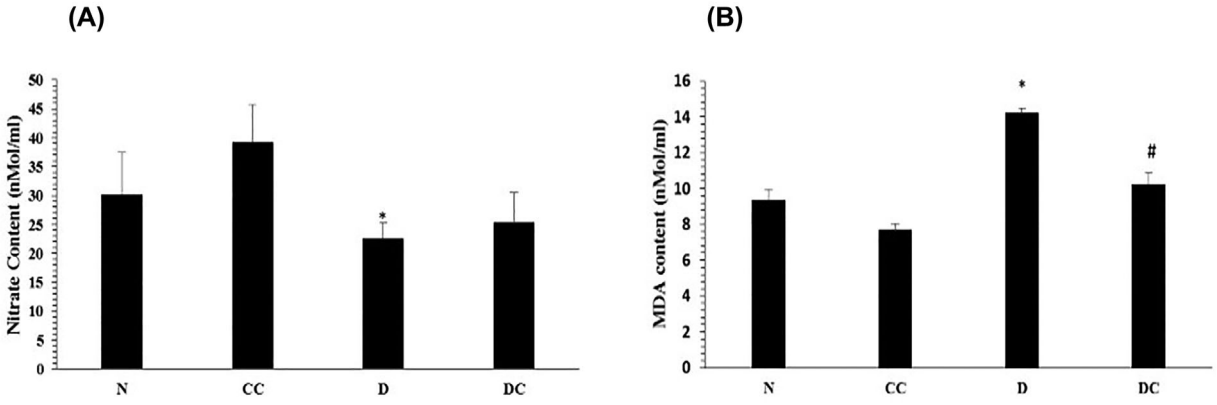

FIGURE 3 Determination of (A) nitrate content (nmol/mL) and (B) MDA levels (nmol/mL) in normal (N), normal + crocin (C), diabetic (D), and diabetic + crocin (DC) groups at the end of study. All values are presented as mean ± SD. In section (A): *($P = 0.01$) shows significant differences compared to control group (N). In section (B): *($P = 0.00$) shows significant differences compared to control group (N). #($P = 0.00$) shows significant differences compared to diabetic group (D)

for 90 min and afterwards moved to a dark room and washed there with PBS four times and DAPI solution was added. Finally, samples were observed by fluorescent microscope for imaging.

2.6.4 | Data analysis

Kolmogorov-Smirnov (KS) test was applied for testing normal distribution of data. Then, one-way ANOVA (with

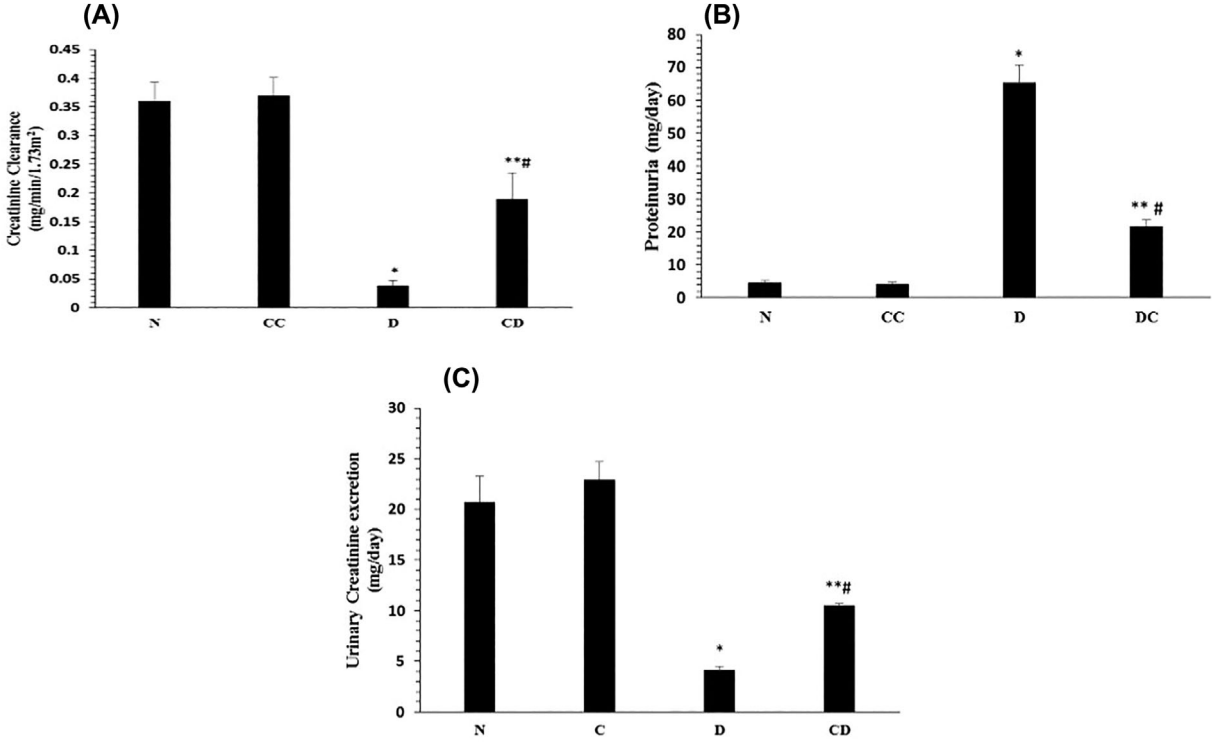

FIGURE 4 Levels of (A) creatinine clearance (mg/min/1.73m^2), (B) proteinuria (mg/day), and (C) urinary creatinine excretion (mg/day) in normal (N), normal + crocin (C), diabetic (D) and diabetic + crocin (DC) groups at the end of study. All values are presented as mean ± SD. In section (A): *($P = 0.000$) and **($P = 0.01$) show significant differences compared to control group (N). #($P = 0.00$) shows significant differences compared to diabetic group (D). In section (B): *($P = 0.000$) and **($P = 0.00$) show significant differences compared to control group (N). #($P = 0.00$) shows significant differences compared to diabetic group (D). In section (C): *($P = 0.000$) and **($P = 0.00$) show significant differences compared to control group (N). #($P = 0.00$) shows significant differences compared to diabetic group (D)

Tukey-Kramer as post hoc test) and *t*-test were applied. Data are expressed as Mean ± SD and a $P < 0.05$ was considered statistically significant.

3 | RESULTS

Changes in blood glucose levels (mg/dl) in all groups are depicted in Figure 1A. In normal animals, mean blood glucose on the first day, was 106 ± 9.4 mg/dl which declined to 96 ± 18 mg/dl, but this change was not statistically significant. STZ significantly increased blood glucose to 422 ± 23 mg/dl ($P = 0.00$) which confirms the induction of a diabetes model. Crocin significantly declined blood glucose to 81 ± 20 mg/dl in normal rats ($P = 0.05$). But, in diabetic animals crocin had no significant effect.

Concerning blood levels of uric acid (mg/dl), normal animals had a mean blood uric acid of 2.79 ± 0.36 mg/dl on the first day which declined to 2.53 ± 0.65 mg/dl on the last day, but this change was not significant. STZ administration significantly raised blood uric acid to 4.94 ± 0.36 mg/dl ($P = 0.00$). Crocin had no significant effect on this parameter in normal crocin-treated rats, but in diabetic animals, crocin declined uric acid to 4.09 ± 0.42 mg/dl ($P = 0.03$) (Figure 1B).

In normal animals, mean blood creatinine on first and on the 56th day were 0.63 ± 0.17 and 0.66 ± 0.14 mg/dl, respectively. STZ administration significantly raised blood creatinine to 4.51 ± 0.45 mg/dl ($P = 0.00$). Crocin did not significantly affect creatinine in normal crocin-treated rats, but in diabetic animals, crocin reduced this parameter to 1.07 ± 0.16 mg/dl ($P = 0.00$) (Figure 1C).

Moreover, mean values of BUN (mg/dl) in normal animals were 56 ± 11 and 57 ± 7.9 mg/dl on the first and the 56th day, respectively which were not significantly different. In normal rats treated with crocin, mean value of BUN in the first and the 56th day were 53 ± 4.9 and 51 ± 4.8 mg/dl, respectively which did not differ significantly. Diabetes significantly increased BUN to 284.87 ± 32 mg/dl ($P = 0.00$). But, crocin significantly diminished BUN to 64 ± 8.6 mg/dl ($P = 0.00$) (Figure 1D).

In normal and normal corcin-treated rats, CAT enzyme activity were 46.73 ± 11.5 and 56.06 ± 3.5 Unit/mL, respectively which showed no significant difference. Diabetes induction significantly declined this value to 9.6 ± 1.3 Unit/mL ($P = 0.00$). However, crocin significantly

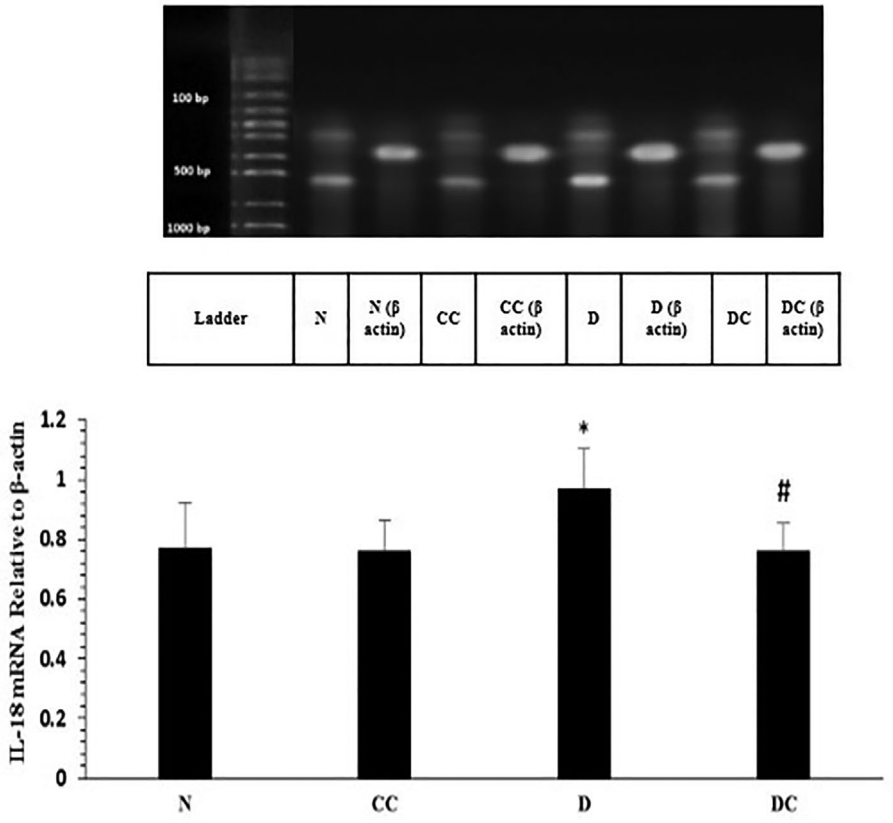

FIGURE 5 *IL-18* expression level normalized againstβ-actin in normal (N), normal treated with crocin (CC), diabetic (D), and diabetic treated with crocin (DC) groups at the end of study. All values are presented as mean ± SD. *($P = 0.01$) shows significant differences compared to control group (N) #($P = 0.05$) shows significant differences compared to diabetic group (D)

increased CAT activity to 28.15 ± 3.76 Unit/mL ($P = 0.01$), which was still lower than that of normal animals ($P = 0.01$) (Figure 2A).

In normal and normal crocin-treated rats, SOD enzyme activity were 0.82 ± 0.05 and 0.88 ± 0.07 Unit/mL, respectively which showed no significant difference. Diabetes induction significantly declined this value to 0.58 ± 0.06 Unit/mL ($P = 0.00$). On the other hand, crocin significantly increased CAT activity to 0.66 ± 0.04 Unit/mL ($P = 0.01$) (Figure 2B).

Based on our results, in normal and normal crocin-treated rats, GLT content were 2704 ± 351 and 2408 ± 357 nmol/mL, respectively which indicated no insignificant difference. Diabetes induction significantly declined this value to 1623 ± 114 Unit/mL ($P = 0.00$) while crocin significantly increased it to 3179 ± 493 Unit/mL ($P = 0.00$) (Figure 2C).

Concerning nitrate content (mmol/mL) in renal tissue, normal and normal crocin-treated animals showed 3.22 ± 7.23 and 39.23 ± 6.56 mmol/mL of nitrate, respectively which shows no significant differences. Diabetes significantly declined this value to 22.65 ± 2.56 mmol/mL ($P \leq 0.01$). But, treatment with crocin had no significant effect on it (Figure 3A).

In normal (N) and normal corcin-treated (CC) animals, MDA content were 9.35 ± 0.62 and 7.69 ± 0.35 nmol/mL, respectively which showed no significant difference. Diabetes induction significantly increased this value to 14.23 ± 0.23 nmol/mL ($P = 0.00$). But, treatment with crocin significantly decreased this value to 10.22 ± 0.65 nmol/mL ($P = 0.00$) (Figure 3B).

As shown in Figure 4A, mean value of creatinine clearance in normal animals (N) was 0.36 ± 0.07 mg/mi/1.73m^2. Uncontrolled diabetes declined this value to 0.038 ± 0.003 mg/min/1.73m^2 which was a significant change ($P = 0.000$). Treatment with crocin had no significant effect on normal crocin-treated rats (CC), but increased this value in diabetic animals (CD) ($P = 0.00$), although it was much lower than normal animals ($P = 0.00$) (Figure 4A).

Regarding the level of proteinuria, normal and normal corcin-treated animals had 4.6 ± 0.82 and 4.3 ± 0.46 mg/day of proteinuria, respectively which were not significantly different from each other. Diabetes significantly increased this value to 65.25 ± 5.36 mg/day ($P = 0.000$), but crocin declined it to 21.79 ± 2.01 mg/day ($P = 0.01$), however, it was still much higher than that of normal animals ($P = 0.00$) (Figure 4B).

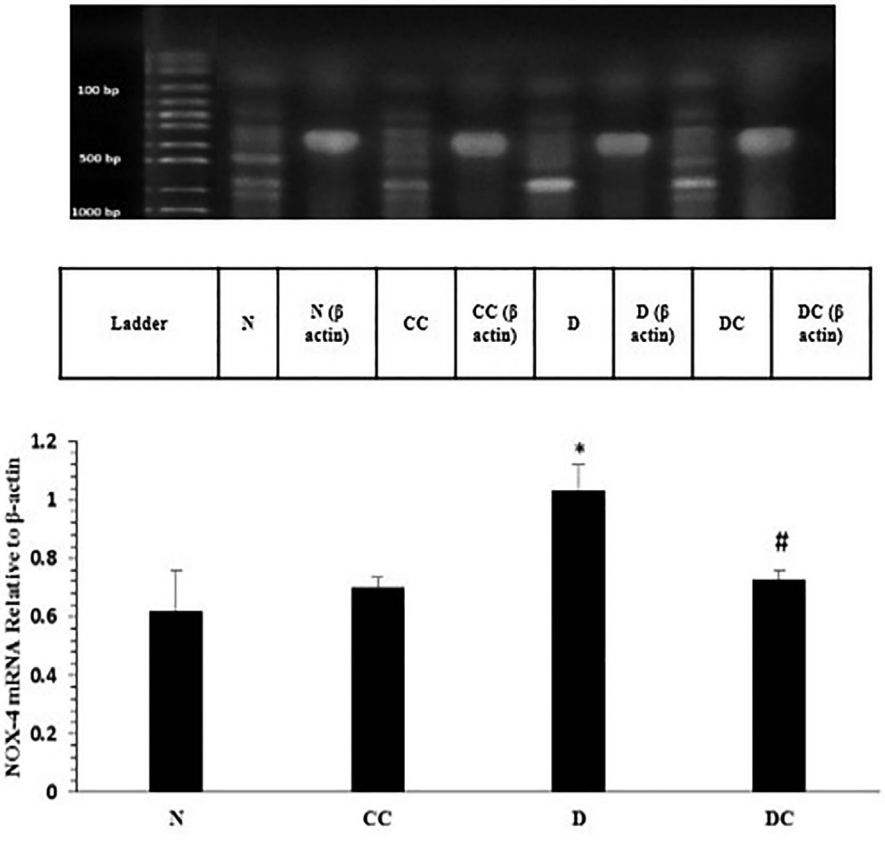

FIGURE 6 *NOX-4* expression level normalized againstβ-actin in normal (N), normal treated with crocin (CC), diabetic (D), and diabetic treated with crocin (DC) groups at the end of study. All values are presented as mean ± SD. *($P = 0.01$) shows significant differences compared to control group (N) #($P = 0.05$) shows significant differences compared to diabetic group (D)

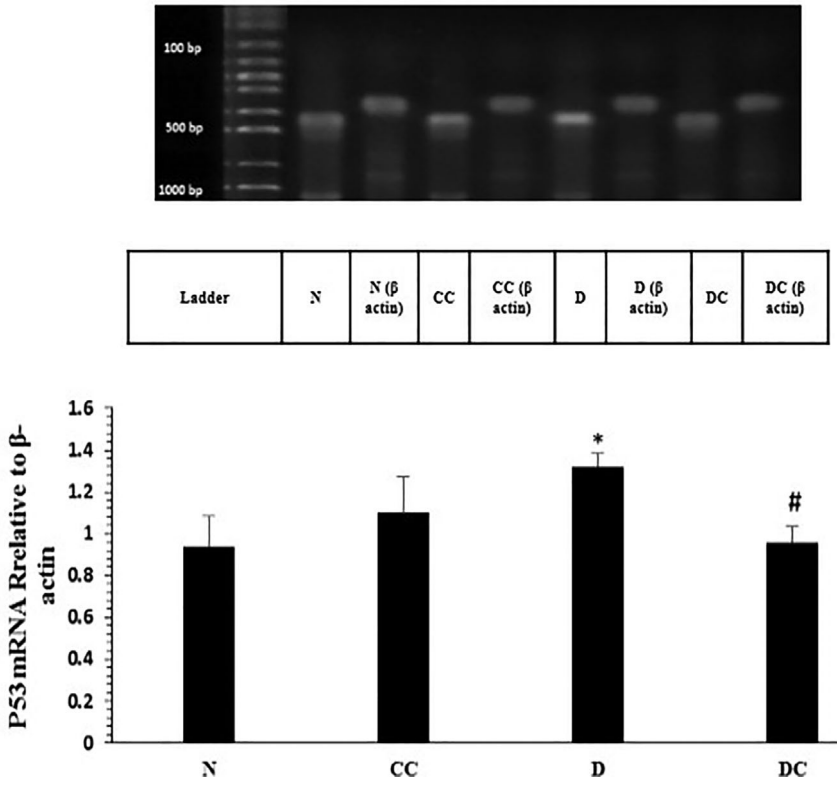

FIGURE 7 *p53* expression level normalized against β-actin in normal (N), normal treated with crocin (CC), diabetic (D), and diabetic treated with crocin (DC) groups at the end of study. All values are presented as mean ± SD.*($P = 0.01$) shows significant differences compared to control group (N) #($P = 0.05$) shows significant differences compared to diabetic group (D)

Figure 4C presents changes in urinary creatinine excretion (mg/day) in all experimental groups. Normal and normal crocin-treated rats had urinary creatinine excretion of 20.75 ± 2.55 and 22.76 ± 1.96 mg/day, respectively which were not significantly different from each other. Diabetes declined this value to 4.13 ± 0.347 mg/day ($P = 0.000$). Treatment with crocin increased this value to 10.49 ± 0.18 mg/day ($P = 0.00$) in diabetic rats, but it was much lower normal animals ($P = 0.00$).

Figure 5 shows *IL-18* expression level normalized againstβ-actin in all experimental groups. Crocin had no significant effect on *IL-18* expression. Diabetes increased this value ($P = 0.01$), but crocin declined it in diabetic rats ($P = 0.05$). Figure 6 shows *NOX-4* expression level normalized againstβ-actin in all experimental groups. Crocin had no significant effect on *NOX-4* expression. Diabetes increased this value ($P = 0.01$), but crocin declined it in diabetic rats ($P = 0.05$). Figure 7 shows *p53* expression level normalized against β-actin in all experimental groups. Crocin had no significant effect on *p53* expression. Diabetes increased this value ($P = 0.01$), but crocin declined it in diabetic rats ($P = 0.05$).

3.1 | Immunohistochemical findings

As shown in Figure 8, renal tissue of normal rats and also crocin-treated normal group did not show immunoreaction towards IL-18 (Figure 8A), p53 (Figure 8B), and NOX-4 (Figure 8C). Diabetes resulted in a severe elevation in IL-18, p53, and NOX-4 immunoreactivity in the renal tissue of diabetic rats compared to normal rats. These elevations were reduced by crocin administration in diabetic animals (Figure 8).

3.2 | Histopathological findings

According to the histopathological assessments of normal renal tissue, the general architecture including glomerular and tubular structure showed normal appearance in normal rats whereas diabetes induced several renal tissue damages in diabetic rats after 8 weeks. Several tissue damages including glomerulosclerosis, expansion of Bowman capsule space, tubular impairment, interstitial fibrosis, and vacuolization were clearly observed in diabetic rats. Treatment with crocin considerably decreased these damages in the renal tissue of treated diabetic rats, whereas crocin did not change the renal structure of treated normal rats (Figure 9).

4 | DISCUSSION

Current study investigated oxidative stress, inflammation and apoptosis as the main molecular mechanisms underlying DN

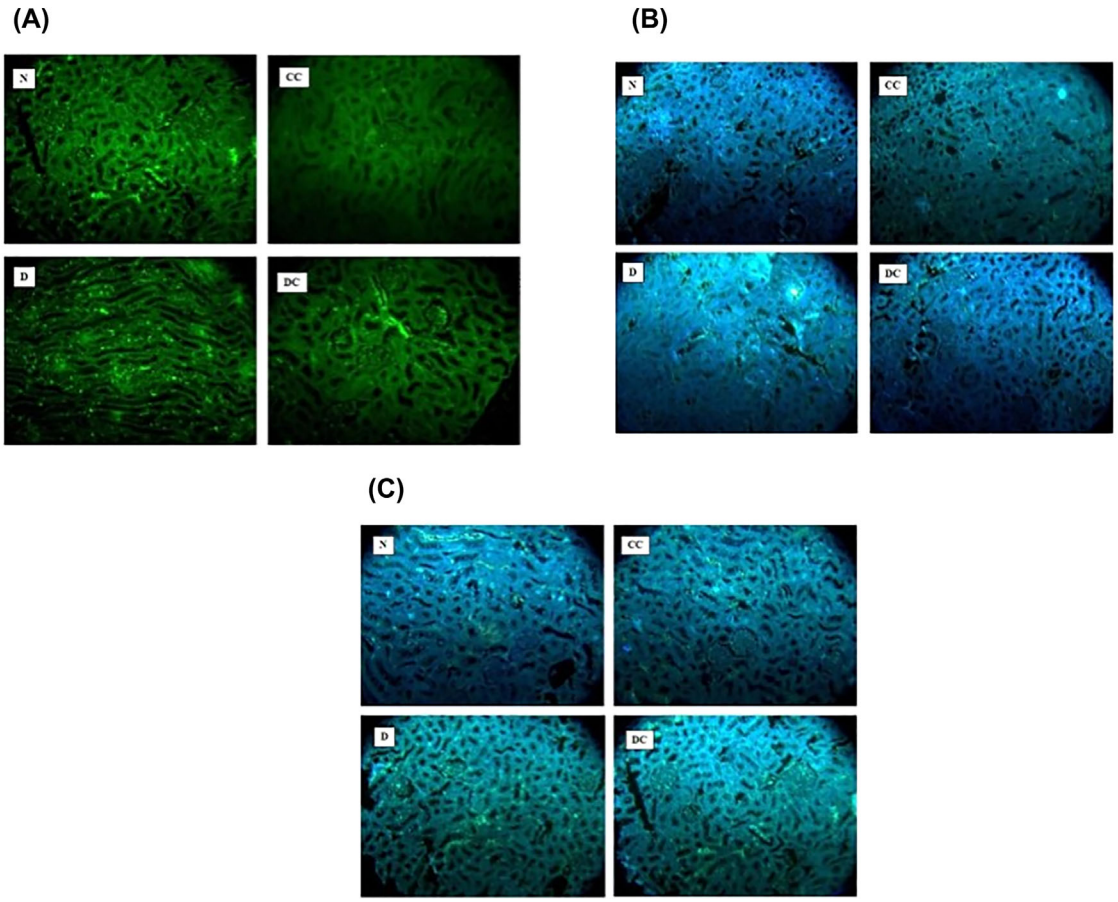

FIGURE 8 IHC images of (A) IL-18, (B) p53, and (C) NOX-4 expression in normal (N), normal treated with crocin (CC), diabetic (D), and diabetic treated with crocin (DC) groups

and evaluated the effect of crocin on these pathological features. In this study, STZ—induced hyperglycemia increased BUN, Cr, and uric acid in plasma, caused histological insults including mesangial expansion, glomerular hypertrophy, and glomerulosclerosis, declined Cr clearance and increased proteinuria which lowered renal function and induced DN. Treatment with crocin declined BUN, Cr and uric acid in plasma, while improved Cr clearance and histological injuries in diabetic rats; thus, based on our results, crocin ameliorated renal function in hyperglycemic rats. It seems that these beneficial effects of crocin are associated with improving oxidative stress, lowering inflammatory responses, and reducing apoptosis rate in renal cells. These findings strongly suggest crocin as a natural agent that can ameliorate some pathological mechanisms involved in DN while having lower side effects compared to conventional synthetic drugs.

In diabetic milieu, oxidative stress is induced as a result of overproduction of free radicals and/or a weakened of antioxidative defense system.[39] Free-radicals as key oxidants are produced by various molecular pathways, but most of these free radicals are generated by NADPH oxidase enzyme activity[22,40] Sedeek et al. declared that hyperglycemia-induced NOX-4 up-regulation is a major source of free radicals in renal tissues which stimulates redox-sensitive signaling pathways involved in ROS production. They also demonstrated that inhibition of NOX-4 activity by siRNA or GKT-136901 (a specific inhibitor of NOX), significantly declined glucose-induced O_{2^-} and H_2O_2 production.[22] This up-regulation mainly occurs in renal cortex, medullary collecting ducts and also in papillary epithelium and as mentioned before, is strongly associated with oxidative stress.[22]

In the current study, we observed higher *NOX-4* mRNA expression in diabetic rats which was consistent with previous findings.[10,22,41] This was accompanied by lower activity of antioxidative system, higher ROS production (as reflected by increased MDA content) and consequently more histological damages, higher levels of proteinuria, and lower renal efficiency which were all observed in this study. Treatment with crocin improved oxidative damages and enhanced renal efficiency in diabetic animals. Cinsiderable body of evidence indicated that crocin can potentiate antioxidant defense system in different tissues such as the liver and brain,[30]

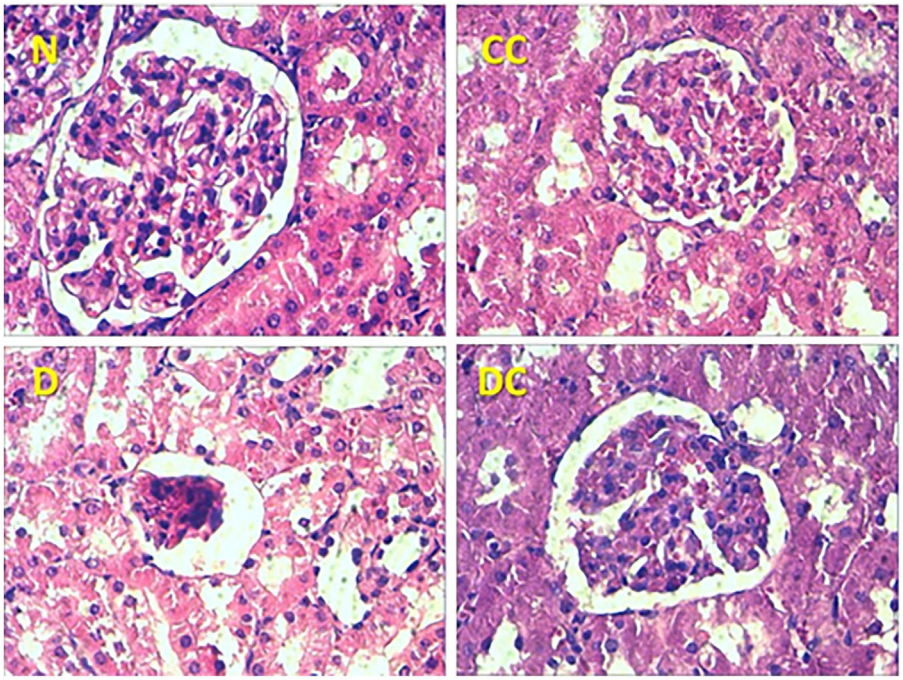

FIGURE 9 Histopathological assessments of renal tissue in normal (N), normal treated with crocin (CC), diabetic (D), and diabetic treated with crocin (DC) groups. Glomerular and tubular damages were clearly detected in diabetic animals, but crocin improved these damages. (Magnification: ×100)

myocardium,[42] and hippocampus.[43] In this study, Crocin enhanced antioxidative system enzymes activity both in normal and diabetic states. In this regard, Hesham et al[30] demonstrated that crocin enhanced gene expression of catalase (CAT) and superoxide dismutase (SOD) enzymes in rats liver and brain tissues. In the current study, crocin increased CAT and SOD enzymes activity and GLT content; so, crocin potentiates antioxidative defenses system. On the other hand, crocin decreased *NOX-4* mRNA expression in diabetic animals. These were accompanied by lower MDA production and indicated that crocin ameliorates oxidative stress in renal tissue. Moreover, crocin diminished histological injuries and proteinuria confirming the therapeutic effect of crocin on DN as it also improved renal function by potentiating antioxidative system defense and decreasing free radical production.

The role of inflammation as another pathological mechanism in DN development, has been verified by several experiments.[7,12] In the current study, hyperglycemia enhanced *IL-18* mRNA expression which was also observed in previous studies[13,44] and confirms the role of inflammatory responses in DN. IL-18 is a potent cytokine which acts as an upstream regulator and triggers a cascade of inflammatory responses; an increase in IL-18 expression is considered a main marker of glucose-induced renal damages and an indicator of DN onset.[10] Therefore, declining its production can be an attractive achievement for suppressing inflammatory responses thus, preventing DN development. In diabetic animals treated with crocin, the *IL-18* mRNA expression level declined and this was accompanied by improvements of all functional and histological changes in diabetic kidneys. We found no direct evidence regarding the effect of crocin on *IL-18* mRNA expression; however, some documents demonstrated that crocin suppresses the inflammation by decreasing the production of other cytokines such as IL-1β, TNF-α, NF-κb, and IL-6.[45–47] Probably, crocin directly decreased *IL-18* gene expression[30] or reduced inflammation by suppressing oxidative stress.[12]

Several DN-associated tissue injuries are due to activated apoptotic pathways.[25] In the current study, apoptotic processes were evaluated via monitoring *p53* mRNA expression level. Our results demonstrated that uncontrolled hyperglycemia increases *p53* mRNA expression which was associated with more histological damages, lower Cr clearance and impaired renal activity. These findings approved the potential importance of p53-related apoptosis in DN development. Supriya et al[48] showed that elevation of p53 expression as a key marker of several kidney disorders (eg DN), is strongly associated with renal fibrosis, glomerulosclerosis, hypertrophy, and proteinuria. This may be mediated via different molecular mechanisms. Fabio et al[49] declared that angiotensin II and aldosterone directly activate p53 protein expression. Assaad et al[50] demonstrated that hyperglycemia inactivates AMP-activated protein kinase

which in turn, phosphorylates and up-regulates p53 and induces podocytes apoptosis. Free-radicals over-load and oxidative stress development,[51] phosphorylation of p53 at serine 18,[49] and production of inflammatory cytokines[52] are other possible underlying mechanisms.

Reports on the interaction between crocin and p53 protein function are not consistent as some researchers introduced crocin as an activator of p53[53] while others suggested that crocin inhibits p53.[54] In our study, crocin significantly declined p53 expression in diabetic rats. As mentioned before, crocin attenuated oxidative stress and inflammation which are both the main pathophysiologic processes that stimulate p53 and apoptosis.[51,52] So, we suggest that inhibitory effects of crocin on p53 expression are probably mediated via suppressing oxidative stress and inflammatory responses. Also, Bahem et al[55] demonstrated that crocin attenuates the renin-angiotensin-aldosterone axis activity which may be another possible mechanism through which, crocin suppresses p53 protein up-regulation.

5 | CONCLUSION

Our results, for first time, showed that crocin can prevent and improve DN through three distinct mechanisms. First, crocin potentiates antioxidative defense system and scavenges free radical so attenuates oxidative stress. Second, crocin inhibits inflammatory reactions by lowering IL-18 cytokine expression, an upstream inflammatory agent that plays an important role in the onset of inflammatory cascade in DN; so, crocin can prevent inflammation-induced DN damages. Third, apoptotic processes which are crucial for DN-induced tissue injuries are weakend by crocin. Therefore, we conclude that crocin can prevent structural and cellular damages observed in uncontrolled diabetes in kidneys and improve renal function.

CONFLICTS OF INTEREST

The authors declare that there is no conflict of interest in this research.

ENDNOTES

[1] Protein kinase C

[2] Transforming growth factor-β

[3] Ethylenediaminetetraacetic acid

[4] 5,5′-Dithiobis(2-nitrobenzoic acid)

[5] Reverse transcriptase- polymerase chain reaction

[6] Immunohistochemistry

[7] Phosphate buffered saline

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