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Original Article

Thymoquinone mitigates diclofenac-induced hepatorenal toxicity in male Wistar rats by balancing the redox state and modulating Bax/Bcl-2/caspase-3 apoptotic pathways and NF-κB signaling

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Abstract

Background and purpose: Diclofenac (DF), a widely used non-steroidal anti-inflammatory drug, can induce hepatotoxicity and nephrotoxicity. This study investigated the protective effects of thymoquinone (TQ), a bioactive compound from *Nigella sativa*, against DF-induced organ damage in rats.
Experimental approach: Forty-eight male rats were divided into six groups (8 each) and treated orally for seven days as follows: group 1 (control): normal saline; group 2: DF (50 mg/kg); group 3: DF (50 mg/kg) + silymarin (50 mg/kg); groups 4-6: DF (50 mg/kg) + TQ at 10, 20, or 40 mg/kg, respectively. Serum biochemical parameters, hepatorenal oxidative stress markers, pro-inflammatory cytokines, and apoptosis-related genes were assessed. Histopathological examinations of liver and kidney tissues were also performed.
Findings/Results: DF administration induced significant liver and kidney damage, evidenced by elevated serum biochemical markers, increased oxidative stress, inflammation, apoptosis-related gene expression, and histopathological alterations. TQ treatment, particularly at the highest dose (40 mg/kg) effectively attenuated these changes. TQ improved liver and kidney function, reduced oxidative stress markers, suppressed inflammation, modulated apoptosis-related gene expression, and ameliorated histopathological damage.
Conclusion and implication: TQ exerted significant protective effects against DF-induced hepatorenal toxicity in rats, potentially through its antioxidant, anti-inflammatory, and anti-apoptotic properties. These findings suggest that TQ may be a promising therapeutic agent for mitigating DF-induced organ damage. However, further research, including clinical trials, is needed to confirm its efficacy and safety in humans.

Keywords: Apoptosis; Diclofenac; Hepatorenal toxicity; Inflammation; Oxidative stress; Thymoquinone.

INTRODUCTION

Diclofenac (DF), a non-steroidal anti-inflammatory drug (NSAID) is widely used for its analgesic and anti-inflammatory properties in various conditions such as osteoarthritis, rheumatoid arthritis, and dysmenorrhea (1,2). However, its clinical use is limited by significant adverse effects, particularly liver and kidney toxicity (3).

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DF-induced toxicity results from a complex interaction of factors, including the production of reactive oxygen species (ROS), impaired mitochondrial function, and changes in cellular signaling pathways (4). In the liver, DF is metabolized into toxic byproducts through phase I and II reactions. This metabolism increases ROS production and depletes the antioxidant glutathione (5). This oxidative stress can damage mitochondria, deplete ATP, and ultimately lead to the death of liver cells (hepatocytes). Similarly, in the kidneys, DF can induce oxidative stress, inflammation, and cellular damage, potentially leading to acute kidney injury or chronic renal dysfunction (6).

The underlying molecular mechanisms of DF-induced hepatorenal toxicity involve complex interactions between oxidative stress, apoptotic pathways, and inflammatory signaling. The Bax/Bcl-2 ratio and caspase-3 activation are key determinants of cellular apoptosis, while the nuclear factor kappa B (NF-κB) pathway plays a crucial role in modulating inflammation and cell survival (7, 8). Understanding these pathways is essential for developing effective strategies to mitigate DF-induced toxicity.

Recent studies have explored various natural compounds for their potential protective effects against DF-induced organ damage. For instance, Gupta *et al.* demonstrated that *Terminalia bellirica* fruit extract and its bioactive compound ellagic acid can ameliorate diclofenac-induced hepatotoxicity by modulating oxidative stress and inflammatory markers (9). Similarly, naringin and hesperidin have been reported to exhibit hepatoprotective effects against DF-induced liver injury through their antioxidant and anti-inflammatory properties (10).

Thymoquinone (TQ), an active component of *Nigella sativa* seed oil, has gained attention for its diverse pharmacological properties, including antioxidant, anti-inflammatory, and hepatoprotective effects (11). Recent studies have demonstrated TQ's potential in ameliorating drug-induced organ toxicity through various mechanisms, including the modulation of oxidative stress, apoptotic pathways, and inflammatory responses (12,13). For instance, TQ has shown a protective effect against acetaminophen-induced hepatotoxicity by enhancing antioxidant defenses and reducing inflammatory responses (14). In addition, TQ ameliorated methotrexate-induced hepatorenal toxicity by reducing oxidative stress, inflammation, and apoptosis, while enhancing antioxidant defenses (15). Furthermore, Aycan *et al.* reported that TQ alleviated DF-induced gastrointestinal and renal toxicity, while Hashem *et al.* demonstrated TQ's protective effects against DF-induced acute kidney injury by regulating Mfn2 and miR-34a expression (16,17).Given the significant clinical implications of DF-induced hepatorenal toxicity and the promising properties of TQ, this study aimed to investigate the protective effects of TQ against DF-induced liver and kidney damage in male Wistar rats. Specifically, TQ's potential to attenuate hepatorenal toxicity by modulating redox imbalance, Bax/Bcl-2/caspase-3 apoptotic pathways, and NF-κB signaling was evaluated. The mechanisms by which TQ may alleviate DF-induced organ damage were investigated through comprehensive biochemical, histopathological, and molecular analyses. This study sought to elucidate potential therapeutic strategies for alleviating NSAID-associated toxicity. By examining the efficacy of natural compounds, the research aimed to enrich the existing evidence that underscores their role in mitigating drug-induced organ damage. The findings may offer valuable insights for developing safer pharmacological interventions and enhancing patient care.

MATERIAL AND METHODS

Drugs and chemicals

DF was purchased from Raha Pharmaceutical Co. (Isfahan, Iran). Alanine transaminase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), total bilirubin, urea, and creatinine were purchased from Pars Azmoon Co. (Tehran, Iran). 2-Thiobarbituric acid (TBA), hydrogen peroxide (H_2O_2), sodium acetate, ferric chloride ($FeCl_3.6H_2O$), and ethanol were obtained from Merck Co. (Darmstadt, Germany). Silymarin, TQ, 2,4,6-tripyridyl-s-triazine (TPTZ), 2,4-dinitrophenylhydrazine (2,4-DNPH), nitro blue tetrazolium (NBT), and 5,5-dithio-bis-(2-nitrobenzoic acid) (DTNB) were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA).

SYBR® Green PCR Master Mix was obtained from Qiagen Co. (Dusseldorf, Germany).

Animals

Forty-eight male Wistar rats weighing 200 ± 50 g (6 to 8 weeks old) were obtained from the Pasteur Institute of Iran (Tehran, Iran). Rats were housed in an animal care facility with a controlled temperature (25 ± 1 °C), a 12/12-h light/dark cycle, and free access to food and water. Before the treatment, rats were left for seven days to acclimatize.

Animals received humane care following the US National Institutes of Health (NIH Publication No. 85.23, revised 1985) guide for the care of laboratory animals. They were approved by the Institutional Animal Care and Use Committee of the Shahrekord University of Medical Sciences (Ethics code: IR.SKUMS.REC.1399.248). Possible efforts were made to decrease animal numbers and distress.

Experimental design

Animals were randomly divided into six different groups (8 rats each). Group 1 (control) received an intraperitoneal (i.p) injection of normal saline and gavage of distilled water/ethanol solution with a ratio of 1:10 at an interval of 1 h per day for seven consecutive days. Group 2, the untreated experimental group, received DF (i.p) at 50 mg/kg of body weight (BW) (18) and gavage of distilled water and ethanol solution with an interval of 1 h per day for seven consecutive days. Group 3, the positive control group, received i.p injection of DF at 50 mg/kg plus silymarin at 50 mg/kg (19) gavage at 1 h interval per day for seven days. For groups 4-6, i.p injection of DF at 50 mg/kg and TQ gavage at 10, 20, and 40 mg/kg of BW (20) at an interval of 1 h per day for seven days was performed.

Samples

Twenty-four hours after the last treatment, rats were anesthetized by i.p injection of a xylazine/ketamine cocktail (10 mg/kg xylazine and 90 mg/kg ketamine), and blood was taken through heart puncture. Serum and plasma were separated by centrifugation for 10 min at 3000 *g* and stored at -20 °C until analysis. Then, the animals were sacrificed by decapitation, liver and kidney samples were taken for examination, and each organ was divided into three parts. One part was fixed in 10% phosphate-buffered formalin for subsequent staining and histological studies, whereas the other part was homogenized (1/10 w/v) in ice-cold Tris-HCl buffer (0.1 M, pH 7.4) and kept at -20 °C for biochemical assays, and the last part was shock-frozen in liquid nitrogen and kept at -80 °C for real-time polymerase chain reaction (RT-PCR) studies.

Protein measurement in liver and kidney homogenates

Protein content in homogenates was measured using Bradford's method, using crystalline bovine serum albumin as standard (21). Briefly, liver and kidney tissue samples were homogenized in ice-cold phosphate-buffered saline (PBS, pH 7.4) using a tissue homogenizer. The homogenates were then centrifuged at 10,000 *g* for 15 min at 4 °C to remove cellular debris. The supernatant was collected and used for protein quantification.

A standard curve using bovine serum albumin (BSA) at concentrations ranging from 0 to 1 mg/L was prepared. Twenty microliters of each standard or sample were added to 180 µL of Bradford reagent (Sigma-Aldrich, St. Louis, MO, USA) in a 96-well microplate. The plate was incubated at room temperature for 5 min, and the absorbance was measured at 595 nm using a spectrophotometer (UV-1800, Shimadzu, Japan).

All measurements were performed in triplicate. Protein concentrations in the liver and kidney homogenates were calculated by comparing the sample absorbance values to the BSA standard curve. Results were expressed as mg of protein per gram of liver and kidney tissues. The same protein measurement procedure was applied to both liver and kidney homogenates.

Biochemical parameters assays

Serum biochemical and oxidative stress assessments

Serum activities of ALT, AST, and ALP, as well as serum levels of TB, urea, and creatinine were measured using an auto-analyzer system (BT-3000, Rome, Italy) with commercially available enzymatic kits according to the manufacturer's instructions.

Lipid peroxidation (LPO) was assessed by measuring malondialdehyde (MDA) levels using the reaction method as described by Ohkawa *et al*. (22). Briefly, serum samples were mixed with TBA reagent and heated, and the resulting pink chromogen was measured spectrophotometrically at 532 nm.

Protein carbonyl, an indicator of protein oxidation, was estimated based on the reaction of DNPH with carbonyl groups according to the method described by Levine *et al*. (23). The protein-hydrazone products were quantified spectrophotometrically at 370 nm.

The total antioxidant capacity of plasma was measured using the ferric reducing ability of plasma (FRAP) method as described by Benzie and Strain (24). This method is based on the ability of antioxidants in the sample to reduce ferric-tripyridyltriazine (Fe^{3+}-TPTZ) to ferrous-tripyridyltriazine (Fe^{2+}-TPTZ). The reaction produces a blue-colored complex with maximum absorbance at 593 nm, which is proportional to the total antioxidant capacity of the sample.

Hepatorenal antioxidant enzymes' activity assessments

Catalase activity in the liver and kidney was determined spectrophotometrically by measuring the rate of H_2O_2 degradation at 240 nm according to the method described previously (25). Hepatic and renal superoxide dismutase (SOD) activity was assayed by measuring the inhibition of NBT reduction at 560 nm following the method of Beauchamp and Fridovich (26). In addition, the reduced glutathione (GSH) content in the liver and kidney tissue was determined based on the formation of a yellow-colored complex with Ellman's reagent (DTNB) (27). GSH peroxidase (GPx) activity was measured with the GPx kit (Randox Labs, Crumlin, UK). This was calculated in a coupled enzyme assay with glutathione reductase by summing the oxidation of NADPH to $NADP^+$, keeping H_2O_2 as the substrate at 340 nm (28).

RT-PCR

Based on the supplier's protocol, total RNA was isolated from the liver tissues using an RNX-plus kit (GMbiolab, China). Total RNA was also extracted from kidney tissues using the same kit. Quality and quantity of total RNA were also measured by the absorbance at 260/280 nm by spectrophotometer (Nanodrop2000, Thermo, USA). One microgram of total RNA and random primers were used for cDNA synthesis, using the PrimeScriptTM reagent kit (Takara Bio Inc. Japan) regarding the kits' protocol. RT-PCR reactions were performed using Power SYBR® Green PCR Master Mix (Qiagen, Germany) and conducted on the Rotor-Gene 3000 real-time thermal cycler (Corbett Research, Australia) in the presence of specific primers for IL-1β, TNF-α, and NF-κB, Bax, caspase-3, and Bcl-2. The typical thermal profile used was 95 °C for 5 min, followed by 45 cycles of 95 °C for 30 s, 56 °C for 30 s, and 72 °C for 30 s. After PCR amplification, the ΔCt was calculated by subtracting the β-actin Ct from each sample Ct. Primers for the genes of interest were designed using Primer 3.0 and Oligo 7.0 software and then confirmed by BLAST Nucleotide (NCBI) (Table 1).

The amplification of cDNA was performed for 10 min at 95 °C. RT-qPCR was done in three steps and 40 cycles. These steps were as follows: for 15 s at 95 °C for secondary denaturation, for 20 s at 60 °C for annealing, and for 25 s at 72 °C for extension). Besides, β-actin was used as the control gene and normalized gene expression.

Table 1. Primer sequences of the genes used for quantitative real-time polymerase chain reaction.

Gene name	Forward primer (5´-3´)	Reverse primer (5´-3´)
IL-1β	CAACAAAAATGCCTCGTGCTG	TCGTTGCTTGTCTCTCCTTGTA
TNF-α	CTGGCGTGTTCATCCGTTC	GGCTCTGAGGAGTAGACGATAA
NF-κβ	CTGGCCATGGACGATCTGTT	TGATCTTGATGGTGGGGTGC
Bax	TGCTTCAGGGTTTCATCCA	GGCGGCAATCATCCTCTG
Bcl-2	GATGGGATCGTTGCCTTATGC	CAGTCTACTTCCTCTGTGATGTTGT
Caspase-3	ACTCCACAGCACCTGGTTATT	TCTGTTGCCACCTTTCGGTT
β-actin	AGGAGTACGATGAGTCCGGC	CGCAGCTCAGTAACAGTCCG

IL-1β, Interleukin-1 beta; TNF-α, tumor necrosis factor-alpha; NF-κB, nuclear factor kappa B; B**cl**-2, B-cell lymphoma 2.

Histopathological assessments

Each experimental group's liver and kidney tissues were fixed in 10% formalin for at least 24 h. Then, the tissues were dehydrated with a sequence of ethanol solutions, embedded in paraffin, cut into 5 µm sections (Rotary microtome (DS4055)), and stained with hematoxylin & eosin dye (H&E stain). Slides were observed under a light microscope (Nikon Labophot, Japan) in a blind manner, and the following indices were assessed histological changes such as hyperemia, inflammatory cell infiltration, necrosis, and fibrosis. Histopathological studies were conducted using a Dino-Lite digital camera and Dino-capture 2 Software.

Statistical analysis

Results are expressed as mean ± SD. Statistical analyses were performed using one-way analysis of variance (ANOVA) followed by Tukey's post hoc test using SPSS software (Statistical Package for Social Sciences, version 20.0, SPSS Inc, Chicago, IL, USA). *P*-values less than 0.05 were considered statistically significant.

RESULTS

Serum biochemical and oxidative stress markers of liver and kidney function

DF treatment (group 2) significantly increased serum creatinine levels compared to the control group (group 1). Treatment with silymarin (group 3) significantly attenuated this increase, indicating a nephroprotective effect. Further analysis revealed that the creatinine levels in the silymarin group were still significantly higher than the control group, suggesting that while silymarin offered protection, it did not completely prevent DF-induced changes in creatinine. Co-administration of DF with TQ at different doses (groups 4-6) also showed varying degrees of protection against DF-induced creatinine elevation (Table 2).

Table 2. Serum biochemical and oxidative stress markers in the control and experimental groups. Data are expressed as mean ± SD, n = 8; and analyzed by one-way ANOVA followed by Tukey post-hoc test. Group 1 represents the control; group 2: diclofenac only; group 3: treated with diclofenac plus silymarin; groups 4-6: treated with diclofenac plus thymoquinone at 10, 20, and 40 mg/kg, respectively. $^{a}P < 0.05$ Indicates significant differences versus the control group; $^{b}P < 0.05$ versus the diclofenac-alone treated group; and $^{c}P < 0.05$ against the group treated with silymarin.

Parameters	Group 1	Group 2	Group 3	Group 4	Group 5	Group 6
ALT (U/L)	44.91 ± 1.43	99.48 ± 2.51^{a}	65.28 ± 2.30^{b}	92.13 ± 2.13^{a}	76.51 ± 1.79^{a}	46.49 ± 1.94^{bc}
AST (U/L)	84.13 ± 3.39	183.83 ± 2.80^{a}	102.84 ± 3.07^{b}	167.59 ± 1.95^{a}	133.00 ± 3.46^{a}	100.39 ± 3.04^{bc}
ALP (U/L)	104.21 ± 3.70	306.32 ± 1.84^{a}	122.27 ± 2.61^{b}	260.50 ± 2.29^{a}	188.42 ± 2.50^{a}	110.24 ± 1.67^{bc}
TB (mg/dL)	0.51 ± 0.01	1.50 ± 0.01^{a}	0.98 ± 0.01^{a}	1.32 ± 0.01^{a}	0.90 ± 0.01^{b}	0.59 ± 0.01^{b}
Urea (mg/dL)	27.24 ± 0.87	70.48 ± 1.53^{a}	29.44 ± 1.31^{b}	66.66 ± 0.91^{ac}	47.62 ± 0.96^{a}	30.88 ± 1.47^{bc}
Creatinine (mg/dL)	0.67 ± 0.009	1.60 ± 0.1^{a}	0.81 ± 0.01^{a}	1.57 ± 0.01^{a}	0.79 ± 0.01^{b}	0.70 ± 0.01^{b}
Serum MDA (nmol/L)	6.91 ± 0.78	11.04 ± 1.53^{a}	7.37 ± 0.61^{b}	9.35 ± 1.05^{abc}	8.50 ± 0.68^{ab}	6.02 ± 0.41^{b}
Plasma FRAP (µM)	608.82 ± 75.42	549.27 ± 107.28	615.92 ± 86.13	629.22 ± 126.28	709.85 ± 161.64	1048.21 ± 127.28^{abc}
Protein carbonyl (nmol NADPH/mg protein)	4.87 ± 0.24	12.08 ± 0.58^{a}	5.93 ± 0.77^{b}	11.88 ± 0.58^{ac}	7.70 ± 0.40^{a}	5.41 ± 0.44^{b}

ALT, Alanine transaminase; AST, aspartate aminotransferase; ALP, alkaline phosphatase; TB, thiobarbiturate; MDA, malondialdehyde; FRAP, ferric reducing ability of plasma.

Liver and kidney tissue oxidative stress biomarkers

DF administration significantly elevated MDA levels in both liver and kidney tissues compared to the control group. Concurrently, DF treatment significantly decreased the levels of catalase, SOD, GSH, and GPx in both liver and kidney tissues relative to the control group.

Co-administration of silymarin with DF significantly attenuated MDA levels and enhanced catalase, SOD, GSH, and GPx levels in both liver and kidney tissues compared to the DF-alone group. Moreover, groups treated with DF plus TQ at different doses (10, 20, and 40 mg/kg) exhibited dose-dependent improvements in oxidative stress biomarkers. These groups demonstrated significantly lower MDA levels and higher catalase, SOD, GSH, and GPx levels in both liver and kidney tissues compared to the DF-alone group. Notably, the group treated with DF plus the highest dose of TQ (40 mg/kg) showed comparable or even superior improvement in oxidative stress markers compared to the silymarin-treated group (Table 3).

Inflammation-associated factors

To assess the involvement of inflammation following the DF challenge and to evaluate TQ efficacy, gene expression IL-1β, TNF-α, NF-kB, and kidney IL-1β, TNF-α, and NF-kB in liver tissue was measured (Fig. 1A-F). DF treatment significantly elevated hepatic gene expression of IL-1β, TNF-α, and NF-κB compared to the control group. All doses of TQ significantly decreased IL-1β, TNF-α, and NF-κB gene expression in the liver compared to the DF group, except for the 10 mg/kg dose which did not considerably alter NF-κB expression relative to the DF-treated group. Similarly, the DF challenge significantly increased renal gene expression of IL-1β, TNF-α, and NF-κB. Administration of TQ at all tested doses notably reduced renal expression levels of IL-1β, TNF-α, and NF-κB compared to the DF group. However, it is important to note that the 10 mg/kg dosage of TQ did not yield a considerable effect on the expression of IL-1β and NF-κB once compared to the DF.

The lower dose of TQ (group 3) showed less pronounced effects on renal inflammatory markers. In both liver and kidney tissues, group 4 showed intermediate levels of inflammatory marker expression between the DF-challenged group and the higher-dose TQ treatment groups.

Table 3. Liver and kidney tissues oxidative stress biomarkers in control and experimental groups. Data are expressed as mean ± SD, n = 8; and analyzed by one-way ANOVA followed by Tukey post-hoc test. Group 1 represents the control; group 2: diclofenac only; group 3: treated with diclofenac plus silymarin; groups 4-6: treated with diclofenac plus thymoquinone at 10, 20, and 40 mg/kg, respectively. $^{a}P < 0.05$ Indicates significant differences versus the control group; $^{b}P < 0.05$ versus the diclofenac-alone treated group; and $^{c}P < 0.05$ against the group treated with silymarin.

Parameters	Group 1	Group 2	Group 3	Group 4	Group 5	Group 6
MDA (nmol/mg tissue)						
Liver	1.58 ± 0.15	10.09 ± 0.64^{a}	5.33 ± 1.14^{ab}	9.98 ± 0.96^{ac}	8.57 ± 1.44^{abc}	4.98 ± 0.86^{ab}
Kidney	2.13 ± 0.58	10.62 ± 0.87^{a}	6.15 ± 0.68^{ab}	9.36 ± 1.00^{ac}	7.95 ± 0.76^{abc}	5.94 ± 0.94^{ab}
CAT (U/mg tissue)						
Liver	170.65 ± 3.76	52.96 ± 3.96^{a}	140.11 ± 4.21^{b}	74.94 ± 6.78^{a}	133.79 ± 9.11	173.05 ± 14.27^{b}
Kidney	169.02 ± 5.68	64.39 ± 5.70^{a}	151.11 ± 6.04^{b}	82.26 ± 4.52^{a}	143.23 ± 4.09	170.52 ± 3.75^{b}
SOD (U/mg tissue)						
Liver	40.99 ± 3.39	21.38 ± 2.71^{a}	33.37 ± 2.90^{ab}	20.89 ± 2.56^{ac}	27.65 ± 2.37^{abc}	35.75 ± 1.75^{ab}
Kidney	33.25 ± 4.18	17.13 ± 3.11^{a}	22.40 ± 2.81^{ab}	18.04 ± 1.77^{a}	25.24 ± 2.73^{ab}	33.78 ± 2.42^{bc}
GSH (µmol/g tissue)						
Liver	13.21 ± 0.35	5.12 ± 0.18^{a}	11.28 ± 0.40	7.56 ± 0.34^{a}	11.86 ± 0.45^{b}	13.47 ± 0.30^{b}
Kidney	13.59 ± 0.33	7.22 ± 0.31^{a}	12.07 ± 0.21	7.30 ± 0.20^{a}	11.98 ± 0.32	13.48 ± 0.13^{b}
GPx (U/mg protein)						
Liver	26.39 ± 0.83	19.50 ± 0.48^{a}	25.09 ± 1.03^{b}	19.91 ± 0.32^{ac}	21.26 ± 0.35^{a}	25.61 ± 0.42^{b}
Kidney	26.09 ± 0.19	15.23 ± 0.21^{a}	24.56 ± 0.20^{b}	17.55 ± 0.18^{a}	22.85 ± 0.29	26.15 ± 0.20^{b}

MDA, Malondialdehyde; CAT, catalase; SOD, superoxide dismutase; GSH, glutathione; GPx, glutathione peroxidase.

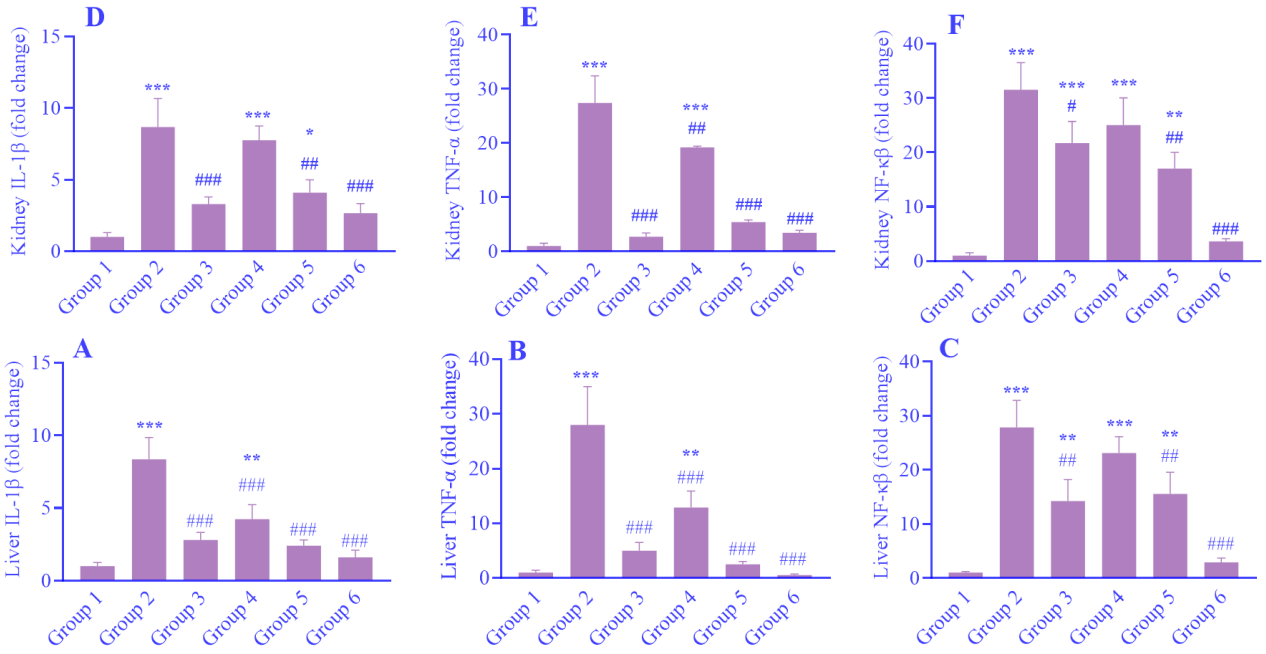

Fig. 1. Gene expression of inflammation-associated factors in the liver and kidney in the control and experimental groups. Data are expressed as mean ± SD, n = 8; and analyzed by one-way ANOVA followed by Tukey post-hoc test. Group 1 represents the control; group 2: diclofenac only; group 3: treated with diclofenac plus silymarin; groups 4-6: treated with diclofenac plus thymoquinone at 10, 20, and 40 mg/kg, respectively. **P* < 0.05, ***P* < 0.01, and ****P* < 0.001 indicate significant differences versus the control group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, and $^{\#\#\#}P < 0.001$ versus the diclofenac-alone treated group. IL-1β, Interleukin-1 beta; TNF-α, tumor necrosis factor-alpha; NF-κB, nuclear factor kappa B.

Tissues cell apoptosis

To evaluate the extent of apoptosis in liver and kidney tissues following DF administration and to assess the anti-apoptotic activity of TQ, the expression levels of three key genes, Bax, Bcl-2, and Caspase-3, involved in the intrinsic apoptosis pathway were measured (Fig. 2A-F). The results are presented as fold changes in gene expression across six different groups.

In liver tissues, Bax expression (Fig. 2A) significantly increased in group 2 treated with DF compared to group 1 (control) while decreased after administration of TQ at 40 mg/kg (group 6) compared to the DF-treated group. Bcl-2 expression in the liver (Fig. 2B) was significantly reduced in group 2 compared to group 1, and groups 5 and 6 showed a significant increase compared to group 2. Liver caspase-3 expression (Fig. 2C) increased significantly in group 2 compared to group 1. At the same time, treatment with TQ at 20 and 40 mg/kg diminished the expression of the gene of interest compared to group 2 which received DF.

In kidney tissues, DF substantially upregulated the expression of Bax and caspase-3 relative to their respective control groups. Conversely, this treatment resulted in a significant downregulation of Bcl-2 expression. TQ, dose-dependently, decreased the expression of Bax and caspase-3 and increased Bcl-2 expression compared to the DF-treated group.

Histopathological results

Liver

In the examination of liver tissue sections, the control group in terms of the structure of liver lobules, hepatocytes, liver sinusoids, distribution of Kupffer cells, and the central vein was normal (Fig. 3A). Tissue changes were evident in the group receiving DF; severe bile duct hyperplasia, necrosis, fibrosis, and scattered lobular inflammation were observed (Fig. 3B). Hyperplasia and mild inflammation were observed in the DF + silymarin group (Fig. 3C). In the DF + TQ (10 mg/kg) group, tissue changes such as hyperplasia of bile ducts, inflammation, necrosis, and relatively severe fibrosis were still visible (Fig. 3D). In the DF + TQ (20 mg/kg) group, the severity of the mentioned side effects was reduced, although visible (Fig. 3E). However, in the DF + TQ group (40 mg/kg), the severity of tissue complications was significantly reduced; except for very slight accumulations of inflammatory cells, no other notable complications were observed (Fig. 3F).

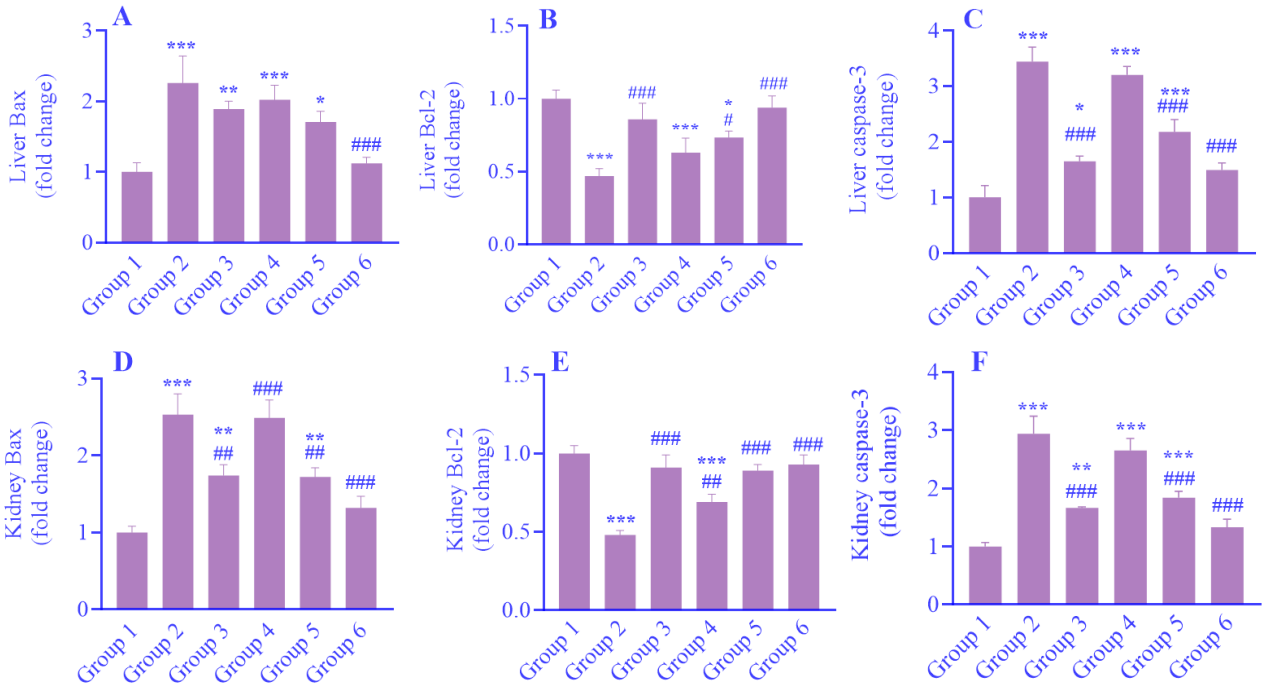

Fig. 2. Gene expression of inflammation-associated factors in the liver and kidney in the control and experimental groups. Data are expressed as mean ± SD, n = 8; and analyzed by one-way ANOVA followed by Tukey post-hoc test. Group 1 represents the control; group 2: diclofenac only; group 3: treated with diclofenac plus silymarin; groups 4-6: treated with diclofenac plus thymoquinone at 10, 20, and 40 mg/kg, respectively. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ indicate significant differences versus the control group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, and $^{\#\#\#}P < 0.001$ versus the diclofenac-alone treated group. Bcl-2, B-cell lymphoma 2.

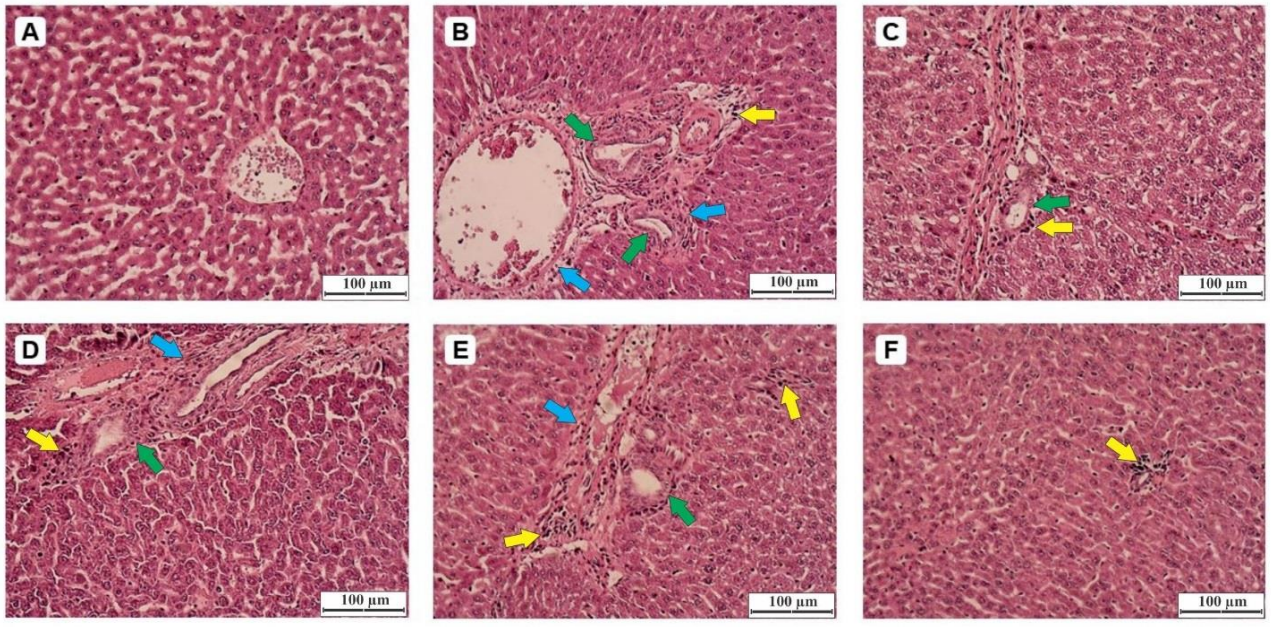

Fig. 3. Effects of diclofenac and thymoquinone on the morphology feature of liver tissue. (A) control, (B) diclofenac only, (C) treated with diclofenac plus silymarin, (D-F) treated with diclofenac plus thymoquinone at 10, 20, and 40 mg/kg, respectively. H&E staining, magnification: 100×. Green arrows represent bile duct hyperplasia; yellow arrows represent lobular inflammation; and blue arrows represent necrosis and fibrosis.

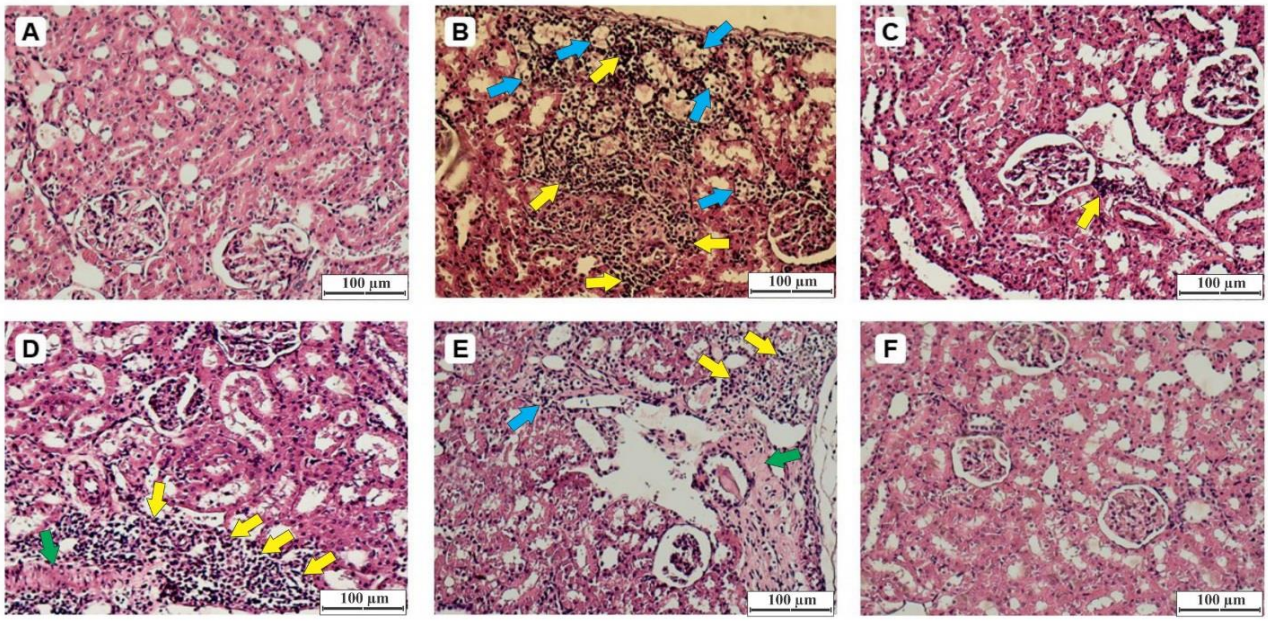

Fig. 4. Effects of diclofenac and thymoquinone on the morphology feature of kidney tissue. (A) control, (B) diclofenac only, (C) treated with diclofenac plus silymarin, (D-F) treated with diclofenac plus thymoquinone at 10, 20, and 40 mg/kg, respectively. H&E staining, magnification: 100×. Green arrows represent fibrosis; yellow arrows represent inflammation; and blue arrows represent necrosis.

Kidney

In examining kidney tissue sections, the control group was normal regarding the structure of the renal corpuscle, proximal and distal convoluted tubules, collecting tubules, Henle's loop, and mesangial cells (Fig. 4A). In the group receiving DF, necrosis, fibrosis, and severe inflammation were observed (Fig. 4B). DF + silymarin administration led to mild changes (Fig. 4C). In the DF + TQ (10 mg/kg) group, tissue changes such as necrosis and fibrosis were relatively severe (Fig. 4D). In the DF + TQ (20 mg/kg) group, cases were still visible, but compared to the DF + TQ (10 mg/kg) group, the severity of symptoms was reduced (Fig. 4E). While in the DF + TQ (40 mg/kg) group, no side effects were observed (Fig. 4F).

DISCUSSION

The present study demonstrated the protective effects of TQ against DF-induced hepatotoxicity and nephrotoxicity in rats. Our findings revealed multiple mechanisms through which TQ exerts its protective action, including antioxidant, anti-inflammatory, and anti-apoptotic effects.

DF administration induced significant liver injury as evidenced by a marked elevation in serum ALT, AST, ALP, TB, urea, and creatinine levels compared to the control group (Table 2). This is consistent with previous studies demonstrating the hepatotoxic potential of DF (29). The increased serum MDA and protein carbonyl levels followed by decreased plasma FRAP and NADPH/protein levels in the DF-treated group, further indicate oxidative stress and damage induced by DF.

Treatment with silymarin alongside DF significantly attenuated the DF-induced increases in liver function markers (ALT, AST, ALP, TB, urea, and creatinine) and improved markers of oxidative stress (MDA, protein carbonyl, FRAP, and NADPH/protein). These findings indicate that silymarin may confer a protective effect against hepatotoxicity induced by DF, presumably attributed to its potent antioxidant and hepatoprotective properties.

Interestingly, the co-administration of TQ with DF also demonstrated a dose-dependent protective effect against DF-induced liver injury. TQ at all doses (10, 20, and 40 mg/kg) significantly reduced the elevated liver enzymes and improved oxidative stress markers compared to the DF-received group. Notably, the higher doses of TQ (20 and 40 mg/kg) exhibited a greater protective effect, with some parameters (*e.g.* ALT, AST, and protein carbonyl) showing comparable or even better

improvement than the silymarin-treated group. Therefore, TQ is suggested to exert its hepatoprotective effects through mechanisms similar to silymarin, potentially involving antioxidant and anti-inflammatory actions.

Furthermore, it is worth noting that in some parameters, such as ALP and protein carbonyl, the highest dose of TQ (40 mg/kg) provided significantly better protection than even the silymarin treatment. This indicates that TQ could be a potent therapeutic agent for mitigating DF-induced hepatotoxicity at optimal doses. TQ treatment, particularly at higher doses, significantly attenuated these increases. The underlying mechanism behind this hepatoprotective effect can be attributed to TQ's ability to preserve mitochondrial function and reduce oxidative stress. Noorbakhsh *et al*. demonstrated that TQ protects hepatocytes by maintaining mitochondrial membrane potential and reducing ROS production (30). TQ also demonstrated protective effects on the kidneys, as shown by the significant decrease in serum urea and creatinine levels, which were elevated in DF-treated rats (Table 2). Shaterzadeh-Yazdi *et al.* demonstrated that TQ protects against renal injury by modulating the AMPK/mTOR signaling pathway, leading to enhanced autophagy and reduced apoptosis (31).

Our results showed that DF administration induced significant oxidative stress in both liver and kidney tissues, as evidenced by a marked increase in MDA levels and a substantial decrease in the antioxidant enzymes CAT, SOD, GSH, and GPx compared to the control group. This finding aligns with previous studies demonstrating diclofenac's potential to induce oxidative damage in various organs (4).

Co-treatment with silymarin effectively attenuated DF-induced oxidative stress, indicated by a significant reduction in MDA levels and a restoration of CAT, SOD, and GSH levels closer to control values. This protective effect of silymarin can be attributed to its potent antioxidant and free radical scavenging properties.

Interestingly, the administration of TQ alongside DF also exhibited a dose-dependent protective effect against oxidative stress. All three doses of TQ (10, 20, and 40 mg/kg) significantly decreased MDA levels and improved antioxidant enzyme activities compared to the group that merely received DF. TQ at higher doses (20 and 40 mg/kg) demonstrated a more pronounced effect, particularly in restoring CAT and SOD levels in both liver and kidney tissues. This suggests that TQ, similar to silymarin, possesses strong antioxidant properties that can counteract DF-induced oxidative damage.

Furthermore, the highest dose of TQ (40 mg/kg) exhibited comparable or even superior efficacy to silymarin in mitigating oxidative stress markers, particularly in restoring CAT and GSH levels. The antioxidant action of TQ may involve both direct scavenging of free radicals and increased production of antioxidant enzymes, possibly by activating the Nrf2/ARE pathway. Darakhshan *et al.* reported that TQ activates the Nrf2/ARE pathway, leading to increased expression of antioxidant enzymes (11). Kundu *et al*. demonstrated that TQ induces the nuclear translocation of Nrf2, leading to increased transcription of antioxidant enzymes (32). In addition, the administration of TQ effectively induces increases in glutathione transferase and quinone reductase activity, even in the absence of toxic agents (33).

DF administration significantly induced the expression of inflammatory markers in both the liver and kidneys. As depicted in Fig. 1, the DF-only group exhibited a marked increase in the mRNA levels of IL-1β, TNF-α, and NF-κB in the liver (Fig. 1) and IL-1β and TNF-α in the kidneys (Fig. 1) compared to the control group. This upregulation indicates a robust inflammatory response triggered by DF.

The concurrent administration of silymarin and TQ attenuated the inflammation induced by DF, albeit with differing degrees of efficacy. The group treated with DF plus silymarin showed a significant reduction in the expression of all measured inflammatory markers in both liver and kidney tissues compared to the DF-only group. This suggests that silymarin exerts a protective effect against DF-induced inflammation, potentially by modulating the expression of key inflammatory mediators.

Groups treated with DF plus TQ also demonstrated a dose-dependent reduction in the expression of these inflammatory markers in both liver and kidney tissues compared to the DF-only group. This indicates that TQ, like silymarin, possesses anti-inflammatory

properties that can counteract the detrimental effects of DF.

Specifically, in the liver, all doses of TQ significantly reduced the expression of IL-1β, TNF-α, and NF-κB compared to the DF group, with the higher doses (20 and 40 mg/kg) generally exhibiting a greater reduction. Similarly, in the kidneys, TQ treatment significantly decreased the expression of IL-1β and TNF-α in a dose-dependent manner.

Comparing the efficacy of silymarin and TQ, silymarin generally demonstrated a more pronounced reduction in inflammatory markers than the lower doses of TQ (10 and 20 mg/kg). However, the highest dose of TQ achieved a comparable or even greater reduction in some markers than silymarin. This suggests that while both silymarin and TQ mitigate DF-induced inflammation, their optimal doses and relative efficacy may vary depending on the specific marker and tissue being examined. TQ treatment significantly decreased the expression of these inflammatory markers, potentially by inhibiting NF-κB activation. Hossen *et a*l. demonstrated that TQ suppresses NF-κB activation by inhibiting the phosphorylation and degradation of IκBα (34). This mechanism explains the reduction in expression of NF-κB-dependent pro-inflammatory cytokines observed in our study.

Our study also demonstrated that DF administration significantly increased the expression of liver Bax and caspase-3, while decreasing the expression of liver Bcl-2 compared to the control group (Fig. 2). Similar effects were observed in the kidney, with DF increasing kidney Bax and caspase-3 expression and decreasing kidney Bcl-2 expression (Fig. 2). These findings indicate that DF induced apoptosis and inflammation in both the liver and kidney.

Treatment with DF plus silymarin significantly attenuated the DF-induced increase in liver Bax and caspase-3 expression and restored liver Bcl-2 expression compared to the group that received only DF (Fig. 2). This suggests that silymarin exerts a protective effect against DF-induced liver injury by inhibiting apoptosis. Furthermore, co-treatment with DF and TQ at all doses (10, 20, and 40 mg/kg) significantly reduced the DF-induced increase in liver Bax and caspase-3 expression and restored liver Bcl-2 levels compared to the DF-only group (Fig. 2). A similar trend was observed in the kidney, with TQ significantly attenuating the DF-induced changes in Bax, Bcl-2, and caspase-3 expression (Fig. 2). These results demonstrate that TQ, in a dose-dependent fashion, also protects against DF-induced liver and kidney injury by mitigating apoptosis and inflammation.

It is noteworthy that the higher doses of TQ (20 and 40 mg/kg) appeared to be more effective than the 10 mg/kg dose in reducing the expression of pro-apoptotic markers and increasing the expression of the anti-apoptotic marker Bcl-2 in both the liver and kidney. This suggests a potential dose-dependent effect of TQ in its protective action against DF-induced organ damage. The anti-apoptotic effects of TQ observed in our study, characterized by decreased expression of Bax and caspase-3 and increased expression of Bcl-2, can be attributed to its modulatory effects on the intrinsic apoptotic pathway. This is consistent with previous studies demonstrating that TQ can prevent the release of cytochrome c from mitochondria, block caspase-3 activation, and ultimately inhibit apoptosis (35). Nassar *et al.* reported that TQ inhibits apoptosis by decreasing the Bax/Bcl-2 ratio and suppressing caspase-3 expression in the liver (36). This aligns with our observations of increased Bcl-2 and decreased Bax expression in TQ-treated groups.

Our study revealed that TQ treatment significantly lowered protein carbonyl levels, which were increased in DF-treated rats. This suggests that TQ protects against protein oxidation, supporting the findings of Farooq et al. who demonstrated that TQ protects against protein oxidation by enhancing the activity of glutathione peroxidase and catalase (37). The reduction in protein carbonyl levels indicates that TQ inhibits the production of LPO and safeguards cellular proteins against oxidative damage.

Figures 3 and 4 illustrate the effects of DF, with and without protective agents, on liver and kidney tissue morphology, respectively. DF alone (Fig. 3) induced significant damage in both organs, evidenced by increased bile duct

hyperplasia, lobular inflammation, necrosis, and fibrosis in the liver, and fibrosis, inflammation, and necrosis in the kidney.

Treatment with DF plus silymarin provided some degree of protection, particularly in the liver, with a noticeable reduction in damage markers compared to DF alone. However, some degree of injury was still observed.

The addition of TQ to DF treatment demonstrated a dose-dependent protective effect. In both liver and kidney tissues, increasing doses of TQ correlated with a progressive decrease in the indicators of tissue damage. At the highest dose of TQ (40 mg/kg), the liver and kidney tissue morphology appeared closer to the control group, suggesting a substantial mitigation of DF-induced damage. These findings indicate that both silymarin and TQ offer protection against DF-induced organ damage, with TQ demonstrating a potentially more potent and dose-dependent protective effect, which aligns with previous findings of dose-dependent protective effects of TQ against various agents that cause liver and kidney damage (38).

The dose-dependent effects of TQ observed in our study are particularly interesting. At the highest dose (40 mg/kg), TQ demonstrated protective effects comparable to or even superior to silymarin, a well-established hepatoprotective agent. This finding is supported by the work of Bilgic *et al*. who reported that TQ at 100 mg/kg provided superior protection against the side effects of olanzapine compared to lower doses (39). This might be due to greater bioavailability and better tissue penetration of TQ at higher doses.

While our study provided strong evidence for TQ's protective effects against DF-induced hepatorenal toxicity, some limitations should be considered. While changes in apoptosis-related gene expression strongly indicate alterations in apoptotic activity, direct measurements of apoptosis (*e.g.* TUNEL assay or caspase activity assay) would provide stronger evidence.

CONCLUSION

In conclusion, TQ effectively mitigated DF-induced liver and kidney damage by strengthening the body's natural antioxidant defenses, reducing LPO and ROS production, suppressing inflammation, and influencing the gene expression involved in apoptosis. These findings suggest that TQ may help as a valuable protective agent for patients undergoing DF treatment. Nevertheless, comprehensive research, encompassing investigations in diverse animal models and well-designed clinical trials in human subjects, is imperative to ascertain the TQ safety, effectiveness, optimal dosage, and long-term consequences. Such thorough evaluation is essential before the prescription of TQ for patients.

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Conflict of interest statements

The authors declared no conflict of interest in this study.

Authors' contributions

A. Safi contributed to the formal analysis, investigation, visualization, writing the original draft, and reviewing and editing the article. Sh. Mohammadi contributed to the investigation and writing of the original draft. A. Kalantari-Hesari contributed to the investigation, methodology, validation, writing, reviewing, and article editing. M. Emami and A. Radaei contributed to the formal analysis, software utilization, visualization, and writing of the original draft. A. Nouri contributed to the methodology and validation. R. Ahmadi contributed to conceptualization, funding acquisition, methodology, project administration, resources, and supervision. All authors read and approved the finalized article.

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