



Quercetin through mitigation of inflammatory response and oxidative stress exerts protective effects in rat model of diclofenac-induced liver toxicity

[Efectos protectores de quercetina mediante la mitigación de la respuesta inflamatoria y el estrés oxidativo sobre la toxicidad hepática inducida por diclofenaco en ratas]

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Abstract

Context: Diclofenac (DIC) is known for its anti-inflammatory and analgesic properties but liver toxicity is one of the main targets to use this drug. Previous studies have demonstrated that quercetin may decrease the toxicity of synthetic drugs.

Aims: To assess the protective effect of quercetin against DIC-induced liver toxicity in rats.

Methods: The rats exposed to DIC (50 mg/kg; i.p.) were treated with different doses of quercetin (20, 40 and 80 mg/kg). The levels of glutathione peroxidase (GPx), superoxide dismutase (SOD), intracellular glutathione (GSH) and catalase (CAT) in the liver tissue were assessed.

Results: Results indicated a significant decline in above-mentioned factors in DIC-alone treated group compared to the control group. Also, levels of the triglyceride (TG), total cholesterol (TC), low density lipoprotein cholesterol (LDL-C), very low density lipoprotein cholesterol (VLDL-C), total bilirubin, alkaline phosphatase (ALP), nitrite content, alanine aminotransferase (ALT), malondialdehyde (MDA), serum tumor necrosis factor- α (TNF- α), serum interleukin-1 β (IL-1 β), aspartate aminotransferase (AST), and inflammatory cytokines were evaluated and results indicted remarkable elevation in these factors in DIC-alone treated group compared to the control group. Treatment with quercetin caused a significant elevation in GPx, SOD, GSH, CAT and a significant reduction in levels of TG, TC, LDL-C, VLDL-C, total bilirubin, ALP, nitrite content, ALT, MDA, serum TNF- α , serum IL-1 β , AST and inflammatory cytokines in DIC-alone treated group compared to the control group ($p < 0.05$). Histopathological alterations were also improved following quercetin administration.

Conclusions: Quercetin may exert a protective effect against DIC-induced liver toxicity in rats through mitigation of oxidative stress and inflammatory response.

Keywords: diclofenac; IL-1 β ; liver toxicity; oxidative stress; quercetin; TNF- α .

Resumen

Contexto: El diclofenaco (DIC) posee propiedades antiinflamatorias y analgésicas, pero produce toxicidad hepática. Estudios anteriores han demostrado que la quercetina (Q) puede disminuir la toxicidad de los fármacos sintéticos.

Objetivos: Evaluar el efecto protector de la Q contra la toxicidad hepática inducida por DIC en ratas.

Métodos: Las ratas expuestas a DIC (50 mg/kg; i.p.) se trataron con diferentes dosis de Q (20, 40 y 80 mg/kg). Se evaluaron los niveles de glutatión peroxidasa (GPx), superóxido dismutasa (SOD), glutatión intracelular (GSH) y catalasa (CAT) en el tejido hepático.

Resultados: Se detectó una disminución significativa en los factores mencionados anteriormente en el grupo tratado con DIC solo en comparación con el grupo control. Además, los niveles de triglicéridos (TG), colesterol total (TC), lipoproteínas de baja densidad (LDL-C), lipoproteínas de muy baja densidad (VLDL-C), bilirrubina total, fosfatasa alcalina (ALP), contenido de nitritos, alanina aminotransferasa (ALT), malondialdehído (MDA), factor de necrosis tumoral sérica (TNF- α), interleucina-1 β (IL-1 β) sérica, aspartato aminotransferasa (AST) y citocinas inflamatorias fueron evaluadas y los resultados indicaron una notable elevación en estas factores en el grupo tratado con DIC en comparación con el grupo control. El tratamiento con Q causó una elevación significativa en GPx, SOD, GSH, CAT y una reducción significativa en los niveles de TG, TC, LDL-C, VLDL-C, bilirrubina total, ALP, contenido de nitrito, ALT, MDA, suero TNF- α , IL-1 β en suero, AST y citocinas inflamatorias en el grupo tratado con DIC solo en comparación con el grupo control ($p < 0.05$). Las alteraciones histopatológicas también mejoraron después de la administración de Q.

Conclusiones: La Q ejerce un efecto protector contra la toxicidad hepática inducida por DIC en ratas mediante la mitigación del estrés oxidativo y la respuesta inflamatoria.

Palabras Clave: diclofenaco; estrés oxidativo; IL-1 β ; quercetina; TNF- α ; toxicidad hepática.

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INTRODUCTION

The liver is the most important organ for detoxification of exogenous and endogenous metabolites (Biour et al., 2000). Drug induced liver toxicity is one of the main concerns for patients with high economic burden (Labbe et al., 2008). Non-steroidal anti-inflammatory drugs (NSAIDs) are widely prescribed for their anti-inflammatory and analgesic properties (Masubuchi et al., 1998; Boelsterli, 2013). Diclofenac (DIC) belongs to the NSAID family that leads to liver toxicity at high doses (Todd and Sorkin, 1988). The mechanism of liver toxicity induced by DIC is partly associated with mitochondrial damage (Siu et al., 2008; Adeyemi and Olayaki, 2018), impairment of antioxidant defense system (Galati et al., 2002), change in the integrity of covalent protein by reactive metabolites (Gil et al., 1995), and dysfunction in the immune system (Lim et al., 2006). DIC is in the FDA's black box warning for its liver toxicity (Dykens and Will, 2007). Therefore, introduction of new drugs with potential hepatoprotective effects is a pressing need for physicians.

Antioxidant defense is one of the mechanisms for protection against oxidants. The enzymatic antioxidant systems including superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase (CAT) are more important with respect to antioxidant defense. In addition, reduced form of glutathione (GSH) is the major non-enzymatic antioxidant pathway in the body (Di Luzio, 1967; Jia et al., 2013; Ghatreh-Samani et al., 2016).

Flavonoids are natural compounds widely present in plants (Ahmadipour et al., 2016; Jivad et al., 2016; Parsaei et al., 2016). Flavonoids have various properties including antiallergic, anti-inflammatory, antitumoral, antiviral, and antibacterial (Middleton, 1984; Palma-Tenango et al., 2017). Flavonoids have antioxidant properties due to their ability to neutralize free radicals and stabilize cell membranes (Middleton, 1984; Rouhi-Boroujeni et al., 2016; Palma-Tenango et al., 2017). Quercetin is one of the flavonoids found in vegetables and fruits (Saffari-Chaleshtori et al., 2016). Previous studies have determined antioxidant

properties of quercetin (Ghosh et al., 2009; Renugadevi and Prabu, 2010).

However, the exact molecular mechanisms of DIC-induced liver toxicity as well as beneficial effects of quercetin have not yet been adequately understood. This study was conducted to evaluate the protective effect of quercetin against DIC-induced liver toxicity in rats and evaluate the beneficial effects of quercetin with further focus on its anti-inflammatory and antioxidant properties.

MATERIAL AND METHODS

Chemicals

2-Thiobarbituric acid, sodium acetate, and H₂O₂ were procured from the Merck (Darmstadt, Germany). Quercetin, 5,5-dithiobis-2-nitrobenzoic acid, Griess reagent, GSH, nitro blue tetrazolium and TPTZ (2,4,6-tripyridyl-s-triazine) were purchased from the Sigma (St. Louis, MO). SYBR Green Real Time-PCR Master Mix was provided from the Qiagen Co. (Dusseldorf, Germany). Total bilirubin, ALP (alkaline phosphatase), ALT (alanine aminotransferase), AST (aspartate aminotransferase), TG (triglyceride), TC (total cholesterol) and LDL-C (low-density lipoprotein cholesterol) were evaluated using commercially available kits (Pars Azmuoon Co., Tehran, Iran).

Animals

Forty-eight male Wistar rats weighting 200 ± 20 g (6-8-week-old) obtained from the JondiShapour University (Ahwaz, Iran) were used. The rats were kept in standard laboratory conditions, including 25°C ambient temperature, 50% relative humidity and a 12-h: 12-h dark-light cycle, and were allowed free access to water and standard diet. The whole protocol of the study was confirmed by Shahrekord University of Medical Sciences Ethics Committee, Shahrekord, Iran (ethics approval no.: IR.SKUMS.REC.94.146) based on the ethical standards of experiments designed in accordance with the Code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments in-

volving humans and EU Directive 2010/63/EU for animal experiments.

Study design

The 48 male Wistar rats were randomly assigned into six groups (n=8). Group 1 (control group) received normal saline (1 mL/kg, i.p.), group 2 received 1 mL distilled water containing 0.1% Tween 80 as the solvent of quercetin by gavage for 5 consecutive days (Ma et al., 2015). Group 3 received DIC only (50 mg/kg, i.p.) (Aydin et al., 2003; Giridharan and Sabina, 2017), groups 4, 5 and 6 received DIC (50 mg/kg, i.p.) plus quercetin (20, 40, and 80 mg/kg, p.o, respectively) for 5 consecutive days (Liu et al., 2012; Ma et al., 2015). At the end of study, rats were kept fasted for 8 hours and then killed under anesthesia. Whole blood specimens were gathered by cardiac puncture procedure to obtain serum and plasma. In addition, liver tissues were removed for determining liver CAT, SOD and GPx activities, nitrite content, lipid peroxidation (LPO), gene expression of inflammatory cytokines, and histological studies.

Biochemical analysis

Serum biochemical markers such as TG, TC, LDL-C, total bilirubin, ALP, AST and ALT contents were measured by an enzymatic method (Pars Azmoon kit, Pars Azmoon Co., Tehran, Iran) using autoanalyzer (BT 3000, Biotechnica, Cergy Pontoise Cedex, France).

Determination of ferric reducing/antioxidant power (FRAP)

Ferric reducing/antioxidant power was determined by Heidarian and Soofiniya (2011) procedure. Briefly, FRAP reagent was prepared by mixing 25 mL acetate buffer, 2.5 mL TPTZ solution and 2.5 mL FeCl₃. Then, 25 µL of plasma was mixed with 1.5 mL of FRAP reagent and was incubated 10 min at 37°C. Optical density of blue color was measured by spectrophotometer (Shimadzu Spectrophotometer UV-1800, Japan) at 593 nm. FeSO₄ was used as a standard of FRAP assay at a concentration range between 100 and 1000 µM (equation curve: $Y = 2133 X + 9.8$, $R^2 = 0.996$). The

measurements were done in duplicate and the results were expressed in µmol/L.

Determination of nitrite content

Content of nitrite was evaluated using a Griess reaction (Ahmad et al., 2008). Briefly, 50 µL of liver supernatant was mixed with 200 µL of Griess reagent (0.1% N-(1-naphthyl) ethylene diamine, 1.0% sulfanilamide and 2.5% orthophosphoric acid), and then was incubated at 37°C for 30 min. Optical density was measured by spectrophotometer (Shimadzu Spectrophotometer UV-1800, Japan) at 548 nm. Nitrite content was assessed using standard curve of sodium nitrite (equation curve: $Y = 21.3 X - 0.42$, $R^2 = 0.992$) and was reported as µM/mg tissue.

Determination of lipid peroxidation (LPO)

MDA contents in rat liver and serum were determined by the TBA reactive substance test as previously described by Heidarian and Soofiniya (2011). Briefly, 100 µL of plasma or supernatant was mixed with 100 µL of sodium dodecyl sulfate (8.1%) and 2.5 mL of TBA/buffer that included 0.53% thiobarbituric acid in 20% acetic acid and its pH was adjusted to 3.5 by addition of NaOH. Then, the mixture was incubated at 95°C for 60 min. The reaction was stopped by placing tubes on ice followed by centrifugation at 4000 rpm for 10 min. The optical density of pink color was measured by spectrophotometer (Shimadzu Spectrophotometer UV-1800, Japan) at 532 nm. 1,1,3,3-tetraethoxypropane was used as a standard of MDA assay. The measurements were done in duplicate and the results were expressed in µmol/L.

Determination of intracellular GSH levels

Intracellular GSH content was determined using the method of Ellman (1959). Two mL of supernatant was mixed with 4.0 mL of 0.4 M Tris buffer (pH: 8.9), and 0.1 mL of Ellman's reagent [5,5'-dithiobis-2-nitro-benzoic acid (DTNB)] and the resulting sample was shaken. The absorbance was read at 412 nm within 5 minutes after the addition of DTNB against a reagent blank with no homogenate. The tissue GSH level was expressed as µmol/g wet tissue.

Sulphydryl groups (SG)

SG values were determined according to Sedlak and Lindsay (1968). RBC lysates were dissolved in 1 mL potassium phosphate buffer (50 mM, pH = 7.5) and centrifuged. The supernatant was transferred into a vial containing Ellman's reagent and absorbance was determined at 600 nm. The same procedure was used in the case of the nonoxidized control protein sample (egg albumin). All reagents were purchased from JT Baker, USA, except egg albumin from Sigma-Aldrich, Missouri, USA.

Determination of Tissue GPx activity

The activity of GPx was assayed by the method of Rotruck et al. (1973). The reaction mixture consisting of 0.2 mL of ethylene diamine tetraacetate, sodium azide and H₂O₂ with 0.4 mL of phosphate buffer, and 0.1 mL of rat liver homogenate was incubated at 37°C at different time intervals. The reaction was stopped by the addition of 0.5 mL of TCA and the tubes were centrifuged at 2000 rpm. To 0.5 mL of supernatant, 4 mL of disodium hydrogen phosphate and 0.5 mL DTNB were added, and immediately after the color developed, the absorbance was read at 412 nm. The activity of GPx was expressed as U/mg protein.

Determination of tissue CAT and SOD activities

The liver CAT activity of experimental groups was measured as described previously by Heidarian et al. (2014b). Briefly, 995 µL H₂O₂ solution (composed of: 10 mmol H₂O₂ in 50 mM phosphate buffer, pH 7.4), and 5 µL homogenate was pipetted into a cuvette. The reduction of H₂O₂ was followed at a wavelength of 240 nm for 2 min. The activity

of liver SOD was evaluated using the inhibition of the nitro blue tetrazolium (NBT) photochemical reaction at 560 nm (Flohé and Otting, 1984). All total protein samples were assessed using the method of Bradford (1976). Data were reported as U/mg protein.

Determination of serum TNF-α and IL-1β

Serum levels of TNF-α and IL-1β were assessed using the ELISA assay kit (BT-Laboratory, China) according to the manufacturer's instructions. Data were reported as pg/mL.

Real-time RT-PCR analysis for liver inflammatory genes

The mRNA was extracted using the BIOZOL kit reagent (Bioer, China) according to the instructions. The quality and quantity of total RNA were measured by reading absorbance at 260/280 nm using a spectrophotometer (Nanodrop2000, Thermo, USA). cDNA measurement was done by the PrimeScript™ reagent kit (Takara Bio Inc. Japan) in accordance with the manufacturer's instructions. Then, cDNA was amplified according to RT-qPCR using SYBR® Green PCR Master Mix in the presence of specific primers (Table 1). Primers after being designed with Oligo 7.0 software was approved using Blast-Nucleotide (NCBI). PCR carried out in primary denaturation at 95°C for 10 minutes. RT-q PCR was performed in 40 cycles (including secondary denaturation at 95°C for 15 seconds, annealing at 60°C for 20 seconds, and extension at 72°C for 25 seconds). Beta-actin gene was used as internal control gene to control the expression of mRNA. Further, ΔΔCT method was used for analysis of gene expression.

Table 1. Primer sequences.

Gene	Forward sequence	Reverse sequence
TNF-α	5'-CTGGCGTGTTCATCCGTTC-3'	5'-CCGAGACTCCTCATCTGCTATT-3'
IL-1β	5'-CAACAAAAATGCCTCGTGCTG-3'	5'-TCGTTGCTTGTCTCTCCTTGTA-3'
β-actin	5'-CGCAAATTACCCACTCCCGAC-3'	5'-GTAACCTCCCGTTCAGACCAC-3'

Histopathological study

After sacrificing the rats, their livers were fixed in 10% formaldehyde solution and then by microtome (AMR 400, Amos Scientific, Australia) were dissected in 5 μ m pieces, and after paraffin embedding, stained with hematoxylin and eosin (H&E) (Bancroft and Gamble, 2008). Tissue changes were observed with the optical microscope (Nikon Eclipse E400 microscope with digital camera, USA). Histopathology was performed by H&E stained sections of liver at 400X magnification.

Statistical analysis

One-way analysis of variance (ANOVA) was used for data analysis using SPSS software (Statistical Package for the Social Sciences, version 20.0, SPSS Inc, Chicago, IL). All data were expressed as mean \pm SEM. Mean values of the groups were compared using the Tukey's *post hoc* test. $P < 0.05$ was considered significance level.

RESULTS

Effect of quercetin on serum lipid profile

Administration of DIC for 5 days led to significant increase ($p < 0.05$) in the serum TC, TG, LDL-C, and VLDL-C compared to the control group (Table 2). Treatment with quercetin at doses of 40 and 80 mg/kg reduced the above-mentioned factors in a dose dependent manner compared to the group receiving DIC alone. The decrease in lipid profiles in the group receiving 80 mg/kg of quercetin was significant ($p < 0.05$) in comparison to the group receiving 40 mg/kg of quercetin. However, treatment with 20 mg/kg of quercetin did not significantly change lipid profiles.

Effect of quercetin on serum ALT, AST, ALP and total bilirubin

The results indicated that DIC treatment for 5 days led to significant increase ($p < 0.05$) in total bilirubin, AST, ALP and ALT in rats receiving DIC alone compared to the control group (Table 2). Treatment with quercetin at doses of 20, 40 and 80 mg/kg reduced the serum levels of above-mentioned parameters in a dose-dependent man-

ner when compared to the group receiving DIC alone. Total bilirubin, AST, ALP and ALT levels decreased significantly in the groups receiving the doses of 40 and 80 mg/kg ($p < 0.05$) compared to those receiving DIC alone.

Effect of quercetin on plasma antioxidant capacity, nitrite content and MDA levels

Table 3 shows that injection of DIC in the DIC-alone treated group caused a remarkable decrease ($p < 0.05$) in plasma antioxidant capacity and a noticeable increase in nitrite level, liver MDA and serum MDA contents compared to the control animals (group 1). In addition, treatment with quercetin at 40 and 80 mg/kg caused a significant increase ($p < 0.05$) in plasma antioxidant capacity and a significant decrease ($p < 0.05$) in nitrite level, liver MDA and serum MDA contents compared to the DIC-alone treated group. A significant change ($P < 0.05$) in plasma antioxidant capacity, nitrite content, serum MDA and MDA levels were observed between rats receiving quercetin at 40 and 80 mg/kg.

Effect of quercetin on CAT, SOD, GPx activities and GSH level

Table 4 shows that injection of DIC led to a significant reduction ($p < 0.05$) in hepatic CAT and SOD activities in the DIC-alone treated group compared to the control group. A significant increase ($p < 0.05$) in hepatic SOD and CAT activities was observed after quercetin treatment in groups 4 and 5 compared to the DIC-alone treated group. Furthermore, a pronounced decrease ($p < 0.05$) was observed in liver GPx activity in the DIC-alone treated group when compared to the control animals. However, treatment with quercetin at doses of 40 and 80 mg/kg remarkably elevated liver GPx activity compared to DIC-alone treated group. Besides that, injection of DIC in DIC-alone treated group led to a noticeable reduction ($p < 0.05$) in liver GSH compared to the control animals (Table 4). Nevertheless, in quercetin-treated groups at doses of 20, 40 and 80 mg/kg, liver GSH remarkably elevated ($p < 0.05$) in comparison with DIC-alone treated group.

Table 2. Effect of quercetin on serum biochemical parameters.

Parameters	Group 1	Group 2	Group 3	Group 4	Group 5	Group 6
TC (mg/dL)	73.75 ± 5.97	75.86 ± 6.11	128.37 ± 9.68 ^{ab}	116.21 ± 8.83 ^{ab}	90.62 ± 6.78 ^{abcd}	79.21 ± 5.83 ^{cde}
TG (mg/dL)	68.22 ± 4.71	72.26 ± 5.26	142.37 ± 8.55 ^{ab}	136.32 ± 7.95 ^{ab}	91.87 ± 6.21 ^{abcd}	75.42 ± 5.72 ^{cde}
LDL-C (mg/dL)	9.77 ± 0.63	10.13 ± 0.71	15.61 ± 0.96 ^{ab}	14.18 ± 1.12 ^{ab}	12.5 ± 0.86 ^{abc}	10.43 ± 0.79 ^{cde}
VLDL-C (mg/dL)	13.21 ± 1.16	13.16 ± 1.21	30.61 ± 2.46 ^{ab}	27.1 ± 2.13 ^{ab}	19.5 ± 2.28 ^{abcd}	14.12 ± 1.39 ^{cde}
ALT (U/L)	36.18 ± 5.43	37.49 ± 4.86	92.1 ± 7.84 ^{ab}	86.45 ± 6.44 ^{ab}	50.86 ± 5.86 ^{abcd}	38.09 ± 4.53 ^{cde}
AST (U/L)	61.66 ± 4.28	63.12 ± 3.87	132.7 ± 10.23 ^{ab}	117.65 ± 8.32 ^{ab}	88.76 ± 6.85 ^{abcd}	68.12 ± 3.76 ^{cde}
ALP (U/L)	169.6 ± 6.75	171.27 ± 6.5	428.1 ± 18.25 ^{ab}	369.76 ± 12.5 ^{abc}	252.32 ± 9.8 ^{abcd}	176.2 ± 7.32 ^{cde}
Total bilirubin (mg/dL)	0.85 ± 0.06	0.86 ± 0.07	2.68 ± 0.15 ^{ab}	2.02 ± 0.18 ^{abc}	1.38 ± 0.09 ^{abc}	0.88 ± 0.06 ^{cde}

Data are expressed as mean ± SEM (n=8) and analyzed by one-way ANOVA followed by Tukey post hoc test. Group 1: control group; Group 2: treated by solvent of quercetin (distilled water containing 0.1% Tween 80); Group 3: diclofenac-alone treated group; Groups 4, 5 and 6 were treated by diclofenac plus quercetin (20, 40, 80 mg/kg p.o., respectively). ^ap<0.05 versus control group (Group 1), ^bp<0.05 versus distilled water containing 0.1% Tween 80 treated group (Group 2), ^cp<0.05 versus diclofenac-alone treated group (Group 3), ^dp<0.05 versus group treated with quercetin at dose of 20 mg/kg (Group 4) and ^ep<0.05 versus group treated with quercetin at dose of 40 mg/kg (Group 5).

Table 3. Effect of quercetin on ferric reducing/antioxidant power (FRAP), nitrite content and malondialdehyde (MDA) levels in the experimental groups.

Parameter	Group 1	Group 2	Group 3	Group 4	Group 5	Group 6
Plasma FRAP (µM)	635.4 ± 23.2	621.2 ± 30.11	384.28 ± 25.41 ^{ab}	428.54 ± 27.64 ^{ab}	524.67 ± 23.4 ^{abcd}	622.25 ± 28.6 ^{cde}
Nitrite content (µM/mg tissue)	6.33 ± 0.31	6.48 ± 0.35	14.32 ± 0.98 ^{ab}	12.88 ± 0.88 ^{ab}	8.85 ± 0.52 ^{abcd}	6.56 ± 0.38 ^{cde}
Serum MDA (nmol/L)	9.45 ± 1.82	9.28 ± 1.74	20.66 ± 4.18 ^{ab}	16.65 ± 3.15 ^{ab}	12.31 ± 2.34 ^{abc}	8.97 ± 1.87 ^{cde}
Liver MDA (nmol/mg protein)	2.51 ± 0.51	2.55 ± 0.50	6.41 ± 1.25 ^{ab}	5.72 ± 1.02 ^{ab}	3.56 ± 0.44 ^{abcd}	2.47 ± 0.54 ^{cde}

Data are expressed as mean ± SEM (n=8) and analyzed by one-way ANOVA followed by Tukey post hoc test. Group 1: control group; Group 2: treated by solvent of quercetin (distilled water containing 0.1% Tween 80); Group 3: diclofenac-alone treated group; Groups 4, 5 and 6 were treated by diclofenac plus quercetin (20, 40, 80 mg/kg p.o. respectively). ^ap<0.05 versus control group (Group 1), ^bp<0.05 versus distilled water containing 0.1% Tween 80 treated group (Group 2), ^cp<0.05 versus diclofenac-alone treated group (Group 3), ^dp<0.05 versus group treated with quercetin at dose of 20 mg/kg (Group 4) and ^ep<0.05 versus group treated with quercetin at dose of 40 mg/kg (Group 5).

Table 4. Effects of quercetin on CAT (catalase) activity, SOD (superoxide dismutase) activity, GPx (glutathione peroxidase) activity and GSH (Intracellular glutathione) level.

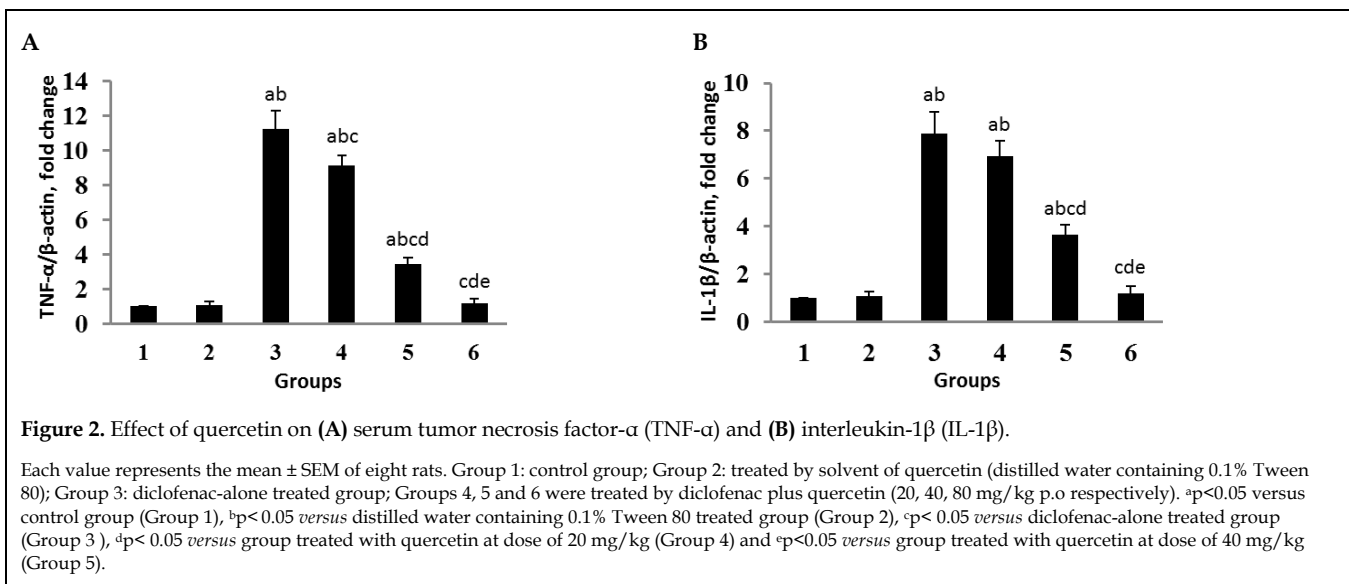
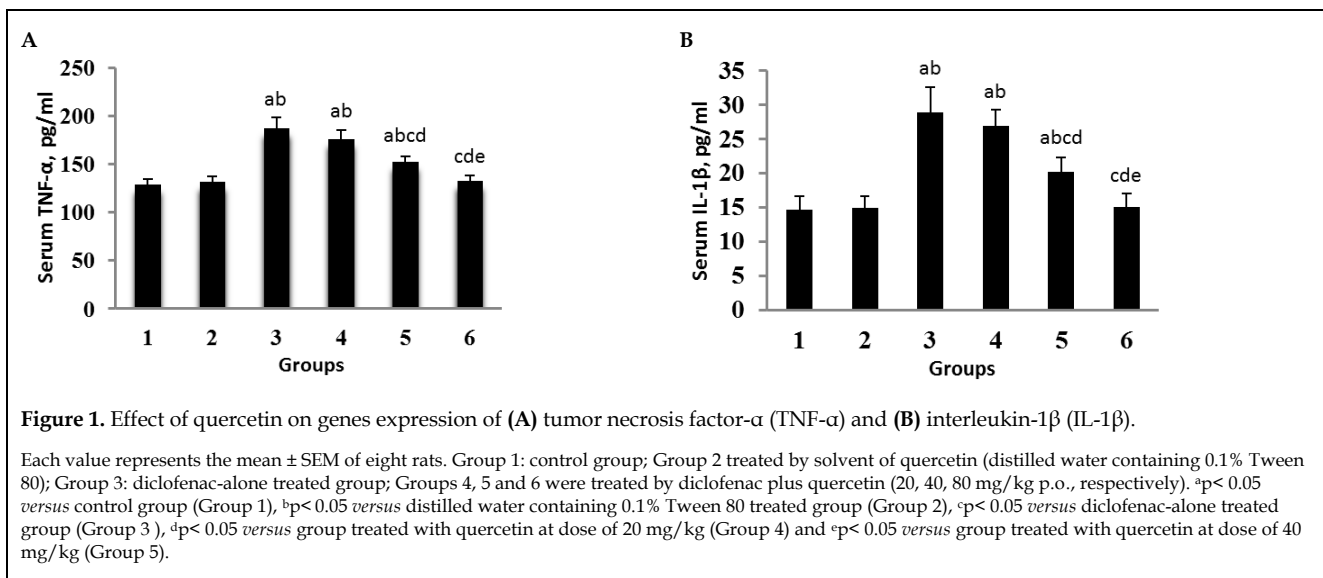
Parameter	Group 1	Group 2	Group 3	Group 4	Group 5	Group 6
CAT (U/mg protein)	172.22 ± 14.2	170.12 ± 13.81	56.67±7.12 ^{ab}	65.43 ± 8.46 ^{ab}	112.87±9.76 ^{abcd}	167±15.13 ^{cde}
SOD (U/mg protein)	48.62 ± 5.12	48.15 ± 4.87	26.71±2.79 ^{ab}	30.11±3.13 ^{ab}	38.34 ± 2.92 ^{abcd}	47.43±3.98 ^{cde}
GPx (U/mg protein)	29.34 ± 1.29	29.04 ± 1.18	18.62 ± 0.86 ^{ab}	20.12 ± 0.91 ^{ab}	24.86 ± 1.07 ^{abcd}	28.42 ± 1.12 ^{cde}
GSH (µmol/g tissue)	13.95 ± 0.19	13.58 ± 0.24	5.61 ± 0.09 ^{ab}	6.86 ± 0.12 ^{abc}	8.67 ± 0.18 ^{abcd}	13.02 ± 0.29 ^{cde}

Data are expressed as mean ± SEM (n=8) and analyzed by one-way ANOVA followed by Tukey post hoc test. Group 1: control group; Group 2: treated by solvent of quercetin (distilled water containing 0.1% Tween 80); Group 3: diclofenac-alone treated group; Groups 4, 5 and 6 were treated by diclofenac plus quercetin (20, 40, 80 mg/kg p.o., respectively). ^ap < 0.05 versus control group (Group 1), ^bp<0.05 versus distilled water containing 0.1% Tween 80 treated group (group 2), ^cp<0.05 versus diclofenac-alone treated group (Group 3), ^dp<0.05 versus group treated with quercetin at dose of 20 mg/kg (Group 4) and ^ep<0.05 versus group treated with quercetin at dose of 40 mg/kg (Group 5).

Effect of quercetin on serum and gene expression of inflammatory cytokines

Injection of DIC in the DIC-alone treated group resulted in a significant increase ($p < 0.05$) in serum levels of TNF- α and IL-1 β in comparison with the control group (Fig. 1). However, in groups treated with quercetin at doses of 40 and 80 mg/kg, the serum level of these proteins significantly ($p < 0.05$) decreased in comparison to the DIC-alone treated group. There was no significant difference between the group treated with 80 mg/kg quercetin and the control group.

In addition, Fig. 2 illustrates changes in the expression of TNF- α and IL-1 β in the studied groups. The results of this study demonstrated that expression of TNF- α and IL-1 β genes in the DIC-alone treated group increased in comparison with the control group ($p < 0.05$). However, in the groups receiving quercetin at 40 and 80 mg/kg, the expression of these genes decreased significantly ($p < 0.05$) compared to the DIC-alone treated group. Quercetin was able to reduce the TNF- α and IL-1 β levels at 80 mg/kg more than two other doses.



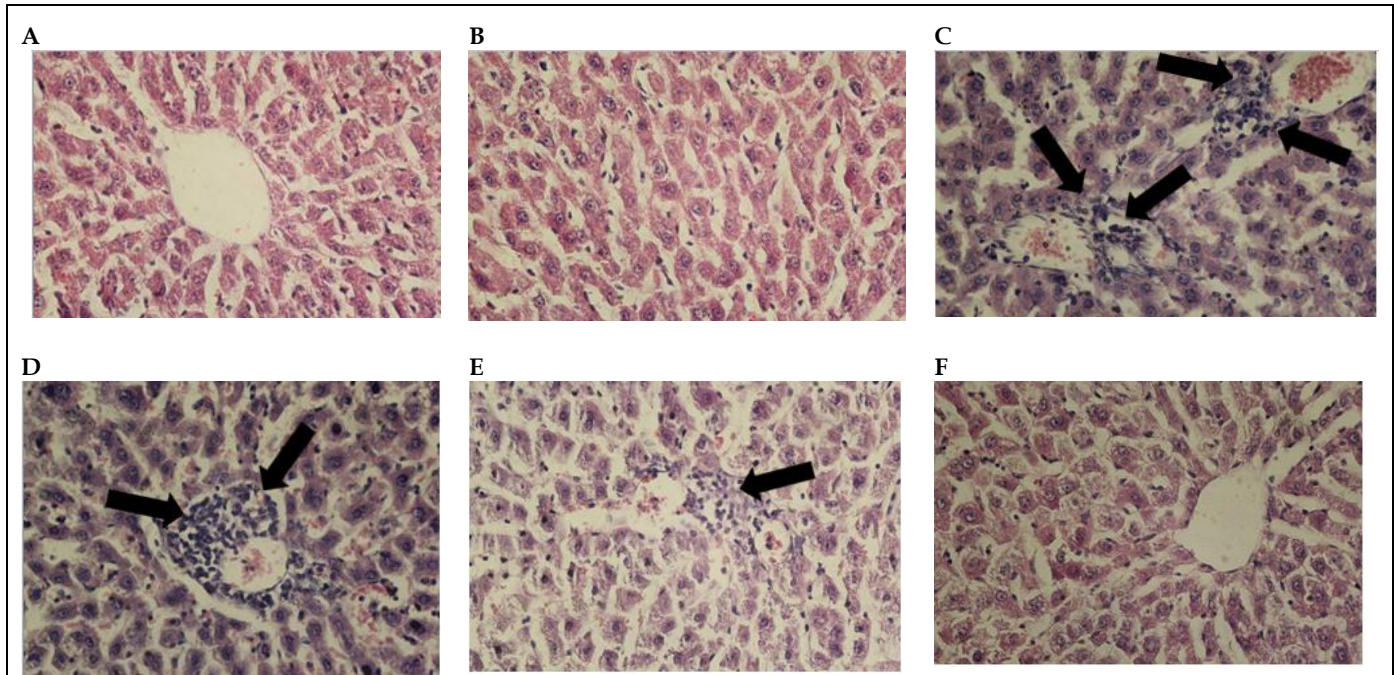


Figure 3. Effects of quercetin on the liver histopathology of experimental groups.

(A) Control group; (B) the rats treated by solvent of quercetin (distilled water containing 0.1% Tween 80); (C) diclofenac-alone treated rats; (D) diclofenac-injected rats supplemented with quercetin (20 mg/kg); (E) diclofenac-injected rats supplemented with quercetin (40 mg/kg); (F) diclofenac-injected rats supplemented with quercetin (800 mg/kg). The black arrows show lymphocyte infiltration. Histopathology was performed by H&E stained sections of liver at 400X magnification.

Histopathological findings

Fig. 3 illustrates the histopathological studies in the experimental groups. As shown, normal hepatocytes are observed in control group treated with distilled water containing 0.1% Tween 80 (Fig. 3A-B). Injection of DIC in the DIC-alone treated group led to infiltration of lymphocyte cells in comparison to the control group (Fig. 3C). Treatment with quercetin at 20 mg/kg could not reduce cell infiltration in comparison with the DIC-alone treated group (Fig. 3D). Liver degeneration and lymphocytic cell infiltration were significantly reduced in groups treated with quercetin at doses of 40 and 80 mg/kg compared to the DIC-alone treated group (Fig. 3E-F).

DISCUSSION

Findings of the present study showed that injection of DIC led to liver toxicity and oxidative stress in male rats. It was observed that the expression of

inflammatory genes increased in the liver of rats exposed to DIC. Evaluation of the hepatic tissue demonstrated that injection of DIC led to the histopathologic changes and also increased the expression of inflammatory genes. Moreover, it was found that quercetin mediated the negative effects of DIC injection. In this regard, the data demonstrated that administration of quercetin could modulate the harmful effects of DIC on antioxidant defense system and liver tissue.

In the present study, DIC led to hyperlipidemia, (elevation of TC, TG, LDL-C, and VLDL-C), which is in agreement with previous investigations (Shalaby and Hammouda, 2014; Abdulmajeed et al., 2015). It has been reported that hyperlipidemia is due to the effect of drugs on acetyl-CoA carboxylase 1 and fatty acid synthesis gene expression that are related to lipogenesis (Cheng et al., 2007). Several studies have indicated that natural compounds can reduce hyperlipidemia (Tung et al.,

2009; Heidarian et al., 2014a; Karimi-Khouzani et al., 2017). The results of this study showed that the reduction in the serum lipid levels might be due to the antioxidant properties of quercetin.

The release of specific enzymes such as AST, ALT, and ALP into the circulatory system is one of the most sensitive signs of liver damage. In the present study, a noticeable elevation was seen in the contents of total bilirubin, AST, ALP and ALT in DIC-alone treated group in comparison with the control group, which approves the hepatotoxic potential of DIC. This is in line with conclusions in previous investigations (Alabi et al., 2017; Giridharan and Sabina, 2017; Adeyemi and Olayaki, 2018). Darbar et al. (2010) demonstrated that the DIC injection resulted in a notable increase in serum AST and ALT, indicating liver toxicity (Nouri et al., 2017). In this study, treatment with quercetin at 40 or 80 mg/kg reversed DIC-induced transformation in the contents of ALT and AST. This indicates that quercetin protected the structural integrity of liver cell membranes and eventually inhibited the leakage of these enzymes into the circulatory system. The elevation of the contents of total bilirubin and ALP observed in the DIC-alone treated animals is a marker of cholestasis in obstructive jaundice (Uemura et al., 2002). Cysteinyl leukotrienes play an important role in inflammatory responses and the escalation of cholestasis in obstructive jaundice (Uemura et al., 2002). The anti-inflammatory and antioxidant properties of quercetin have been reported in previous studies (Liu et al., 2012; Ma et al., 2015; Çelik et al., 2017). In the present study, reduction in the levels of total bilirubin and ALP in quercetin-treated groups can be linked to its anti-inflammatory and antioxidant properties. There seems to be difference in the effect of quercetin at doses of 20, 40 and 80 mg/kg on these markers. Therefore, it can be argued that the effect of quercetin on these biochemical markers is dose dependent.

Malondialdehyde (MDA) level is a marker of lipid peroxidation (LPO) (Pirinccioglu et al., 2010). In the present study, the levels of MDA were significantly increased in the DIC-alone injected rats compared to the control group, which is in agreement with previous studies (Giridharan and

Sabina, 2017; Oseni et al., 2018). In addition, administration of quercetin not only led to an increase in FRAP content in treated groups compared to the DIC-alone treated group, but also a decrease in MDA level in the serum and hepatic tissues. The elevated FRAP and decreased LPO content in quercetin-treated groups might be due to its free radical scavenging property.

Increase in the level of nitrite in DIC-alone treated rats indicated the effect of NO in the liver toxicity induced by DIC. The results of present study are in line with the previous studies that have shown NO plays an important role in DIC-induced hepatotoxicity (Guan et al., 2014; Ma et al., 2015). Remarkable reduction of nitrite content in the groups treated with quercetin suggests that this antioxidant produced hepatoprotective effects in DIC exposed rats by decreasing NO content and nitrosative stress.

Intracellular glutathione (GSH), an important non-enzymatic antioxidant, plays a critical role in defense of cell on damage induced by oxidative stress. Both *in vivo* and *in vitro* investigations have shown the effect of GSH depletion on oxidative stress induced by DIC in various model systems (Guan et al., 2014; Alabi et al., 2017). Based on the present study, DIC-induced liver toxicity led to a noticeable elevation in the content of GSH in liver tissues compared to the control group. Moreover, a noticeable decline in GSH level was observed in quercetin treated groups. Quercetin-induced recovery of GSH might be either due to decreased oxidative stress or due to direct increased GSH content.

In order to study, the role of quercetin on metabolism of GSH, we investigated its effect on GPx as GSH metabolizing enzyme. GPx simplifies peroxides neutralization through reacting with GSH, leading to increased content of GSSG (oxidized glutathione), which is then reduced by GR return to the GSH (sulfhydryl form), thereby maintaining the antioxidant content. DIC-induced changes in the activity of GPx in kidney and hepatic tissues have previously been indicated (Yeh, 2016; Giridharan and Sabina, 2017). In our study, DIC-induced increase in GPx activity may be due to the

defense mechanism against elevated LPO and noticeable reduction in GPx activity in the groups treated with quercetin can be due to the decreased levels of the LPO or oxidative stress.

CAT and SOD are main enzymes of antioxidant defense system. The SOD combines two superoxide radicals ($O_2\bullet^-$) and produces H_2O_2 . H_2O_2 is finally converted to oxygen molecule and H_2O by CAT in the peroxisomes (Droge, 2002; Liu et al., 2010). Various investigations have demonstrated that DIC could decline the activities of antioxidant enzymes in liver. Quercetin as an antioxidant agent could remarkably elevate the activity of antioxidant enzymes return to their normal contents (Cui et al., 2014; Krishnappa et al., 2014). It confirms the previous results that disclosed antioxidative potential of quercetin. The reduction of CAT and SOD activities induced by DIC injection was evident by elevated oxidative stress. However, quercetin treatment eventually reduced the MDA and elevated the activities of antioxidant defense system enzymes in hepatic tissue, reflecting its antioxidant potential mediated by hepatoprotective activity.

Several investigations have also shown that DIC can induce circulation of macrophages and monocytes, which leads to the synthesis and release of a variety of proinflammatory cytokines including $TNF-\alpha$ and $IL-1\beta$. Many studies also demonstrated that $IL-1\beta$ and $TNF-\alpha$ play a key role in the development and maintenance of inflammation, and cytokines elevation is associated with liver injury (Carrero et al., 2009; Zyga et al., 2011). The results of the present study indicated that DIC treatment significantly up-regulated the $TNF-\alpha$ and $IL-1\beta$ expressions in the liver tissue. However, quercetin markedly decreased this up-regulation. The findings of this study suggested that quercetin could improve the liver damage caused by DIC by suppressing inflammatory response. When tissue injury occurs, leucocytes quickly migrate to sites of damage and initiate an inflammatory response. Accordingly, infiltration of leucocyte was considered a sign of inflammatory response (Laskin and Laskin, 2001; Pang et al., 2001; Nouri and Heidarian, 2019). As already demonstrated in histopathological findings, leuco-

cyte infiltration markedly increased in the liver of DIC-treated rats. However, treatment with quercetin efficaciously reduced leucocyte infiltration in the liver of the rats receiving received DIC. Therefore, histopathological findings indicated that quercetin could decrease the DIC-induced inflammatory response in the liver tissue.

CONCLUSIONS

The results of the present study indicated that treatment with quercetin led to a significant elevation in GPx, SOD, GSH, and CAT and a remarkable reduction in levels of TG, TC, LDL-C, VLDL-C, total bilirubin, ALP, nitrite content, ALT, MDA, serum $TNF-\alpha$, serum $IL-1\beta$, AST and inflammatory cytokines compared to the DIC-alone treated group. The results of this study indicate that quercetin exerts protective effects against diclofenac-induced liver toxicity in rat model through mitigation of oxidative stress and inflammatory response. Quercetin can therefore be considered a candidate to be evaluated in clinical trials for decreasing liver toxicity of drugs such as DIC.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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AUTHOR CONTRIBUTION:

Contribution	Nouri A	Heidarian E	Amini-Khoei H	Abbaszadeh S	Basati G
Concepts or ideas		x	x		x
Design		x	x		x
Definition of intellectual content		x			
Literature search	x			x	
Experimental studies	x			x	
Data acquisition	x			x	
Data analysis			x		
Statistical analysis			x		x
Manuscript preparation	x		x	x	
Manuscript editing			x		x
Manuscript review	x	x	x	x	x

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