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Quercetin mitigates liver injury in a rat model of liver cholestasis

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Keywords

- Cholestasis
- Quercetin
- oxidative stress
- Inflammation
- Fibrosis

Abstract

Background: cholestasis is a prevalent health problem associated with liver oxidative stress, inflammation, and fibrosis. Quercetin has been shown to afford a beneficial effect in a variety of liver diseases. This study was designed to investigate the potential protective effect of quercetin on liver cholestasis and the possible underlying mechanisms in a rat model of bile duct ligation (BDL). **Methods**: This study was carried out on adult male rats which were randomly divided into: Sham-operated, BDL and BDL- quercetin treated (BDL- Q) groups. Quercetin was given by gavage in a dose of 50 mg/kg/day. **Results**: Bile duct ligation resulted in a significant increase in serum levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), and liver levels of myeloperoxidase (MPO), tumor necrosis factor alpha (TNF-α),and transforming growth factor beta 1(TGF-β1), along with a significant decrease in serum levels of total proteins (TPs) and liver glutathione peroxidase(GPX) in BDL group versus sham-operated controls. Quercetin treatment significantly lowered serum levels of AST, ALT, ALP, and MPO, TNF-α, and TGF-β1 in liver tissues associated with a significant increase in serum TPs and liver GPX in BDL-Q group versus BDL rats. Histological studies revealed enhancement of inflammation and a significant increase in the percentage area of collagen deposition in BDL versus sham-operated group. These changes were attenuated in BDL-Q group compared to BDL rats. **Conclusions:** Quercetin alleviated cholestasis induced liver injury and improved liver function possibly via attenuating liver oxidative stress, inflammation and fibrosis.

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INTRODUCTION

Chronic cholestasis is a prevalent health problem[1] produced by impairment of bile flow and/or secretion followed by accumulation of toxic bile acids in hepatocytes, evoking severe liver injury which may progress to cirrhosis and liver failure[2] . It is caused by various etiologies such as primary biliary cirrhosis, primary sclerosing cholangitis and progressive familial intrahepatic cholestasis [3]**.**

Recent studies emphasized the crucial role of oxidative stress as well as inflammation in the development and progression of liver injury during cholestasis [4]**.** Exposure of hepatocytes to high concentrations of potentially toxic bile acids initiates hepatocellular injury, followed by a leukocytic phase in which activated neutrophils infiltrate and attack the bile acid stressed hepatocytes through reactive oxygen species (ROS) formation[5]**.** Excessive production of ROS has been demonstrated to induce cellular damage[6] and promote inflammation by upregulating TNF-α signaling pathway, and IL-6 mRNA expression in chronic liver diseases [7]**.** TGF-β, a potent profibrotic cytokine, was also activated by oxidative stress [8]and in a consequence stimulates the transformation of hepatic stellate cells into myofibroblasts which increase the extracellular matrix formation, promoting liver fibrosis [9]**.** During the past decades, the mechanisms of liver cholestasis have been investigated, but few therapeutic strategies are available to efficiently interrupt the progression of liver injury.

Quercetin (3,3,4,5,7-pentahydroxyflavone), a natural flavonoid compound found in vegetables and fruits, has a prominent antioxidant, and anti-inflammatory activities[10]. Quercetin was found to downregulate the serum levels of immuno-inflammatory mediators, TNF-α, IL-6 and C-reactive protein, protecting liver in atitanium dioxide nanoparticles induced liver injury rat model [11]. Additionally, quercetin affords antifibrotic effect by preventing TGF-β signaling pathways and fibroblast activation in carbon tetrachloride and thioacetamide induced hepatic toxicity in rats[6]. Therefore, it is highly possible that quercetin may be effective in prohibiting oxidative stress induced liver injury in chronic cholestasis. Thus, the present study was planned to investigate the possible protective effect and the related mechanisms of quercetin against cholestasis induced by bile duct ligation in rats, a well settled experimental model of cholestasis that initiates a complex cascade of pathological events similar to that of human [12]**.**

Materials and Methods

Animals

Sixty five adult male albino rats, weighing 150-250 gwere purchased from Experimental Animal Farm, Giza, Egypt.Rats werekept in animal cages and maintained under suitable ventilation, temperature of 22-25^{o}C, 12 hours day /dark cycle and free access to food and water in the Medical Research Center, Faculty of Medicine, Ain-Shams University. Rats were kept for 7 days for acclimation before starting the experiment. All experimental procedures were carried out according to the guidelines of FMASU, REC (Faculty of Medicine, Ain Shams University, Research Ethics Committee, Cairo, Egypt. FWA000017585).

Experimental Design

Rats were divided randomly into 3 groups: (1) Sham-operated control group (SHAM, n=11): Rats were subjected to all surgical procedure without bile duct ligation and received D.W.; (2) Bile duct -ligated rats (BDL, n=9): Rats in this group underwent bile duct ligation and received D.W.; (3) Bile duct ligated quercetin- treated rats (BDL-Q, n=10): Rats in this group underwent bile duct ligation then received quercetin.

Surgical procedure and Quercetintreatment

Common bile duct ligation was performed according to Haddadian et al[13]. Briefly, a midline abdominal incision was made and the peritoneal cavity was opened under complete sterile conditions. The common bile duct was exposed at its entry to the intestine and was double ligated with a 3/0 silk suture. The peritoneal cavity was rinsed with 0.9% NaCl solution, and the abdominal organs were placed to their positions and the incision was closed [13].

Quercetin treatment started on the 4th day of the operation, to minimize the exposure of rats to pain stress, and extended till the 28th day of the operation. Quercetin (3,3',4',5,6-Pentahydroxyflavone, Sigma, St. Louis, Missouri, USA) was dissolved in distilled water (D.W.) and given by gavage in a dose of 50 mg/Kg / day[14].

At the end of the experimental period the overnight fasted rats were weighed (BW),anesthetized, and blood samples were collected from abdominal aorta in plastic tubes. Blood was allowed to coagulate at room temperature then centrifuged at 3000 rpm. for 15 min. and serum was collected and stored at -80^{o}C, till used for determination of aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALK) and total proteins (TPs). Both liver and spleen were excised and used to determine hepatosomatic and splenosomatic indiceswhich were calculated according to the following formula[15]:

Hepatosomatic index= $\frac{LW(g)x100}{BW(g)}$ &Splenosomatic index= $\frac{SW(g)x100}{BW(g)}$

Also, liver tissues wereused for histological examination, and determination of liver levels of myeloperoxidase (MPO), glutathione peroxidase (GPX), tumor necrosis factor alpha (TNF-α),and transforming growth factor beta (TGF-β1).

Biochemical analysis:

Using kits supplied by Biodiagnostic (Giza, Egypt), serum AST and ALT were determined according to the methods described by Reitman and Frankel[16].Serum ALP, and TPs were measuredaccording to the methods reported by Belfield and Goldberg [17]; and Gornal et al. [18], respectively.

Measurement of MPOand GPX in liver tissue

MPOwas measured by using myeloperoxidase ELISA Kit (BioSource International, Inc., California, USA.).GPXwas measured according to the method described by Paglia and Valentine[19], using kits supplied by Biodiagnostic (Giza, Egypt).

Measurement of TNF-α and TGF-β1 in liver tissue

TNF-α was determined using RayBio® Rat TNF-alpha ELISA Kit (RayBiotech, Inc., Norcross, Georgia, USA.) according to the manufacturer's instructions. TGF-β1 was

determined by quantitative sandwich ELISA method according to Sporn et al. [20] , by using Rat TGF-β1 PicoKine ™ ELISA Kits(Pleasanton, USA.).

Histological examination*:*

Liver tissues were fixed in 10% formalin, processed and paraffin sections were prepared.The sections were stained by Hematoxylin&Eosin (H&E) and Masson's trichrome (MTC).All liver tissue sections were evaluated blindly. The liver inflammation was graded into 5 grades according to Scheuer [21] . Inflammation was classified as normal (no inflammation or necrosis), minimal (portal inflammation without necrosis), mild (periportal inflammation with lobular focal or unicellular necrosis), moderate (periportal inflammation with more extensive lobular necrosis), severe (periportal inflammation with lobular bridging necrosis). The percentage area of collagen fibers was measured by Digital Image Analysis System (Carl Zeiss Axiovision Product Suite DVD 30), using Leica Quin 500C Image Analyzer Computer System (Leica Imaging System Ltd., Cambridge, England).

Statistical analysis:

One-way analysis of variance (ANOVA) was used to determine the differences between groups. In the case of a significant F value ($P \leq 0.05$), a least significant difference test was used to find significant intergroup differences. P values ≤0.05 were considered statistically significant. SPSS windows version 20 (SPSS Inc., Chicago, IL, USA) was used in the analysis.

Results

Changes in hepatosomatic and splenosomatic indices:

Hepatosomatic and splenosomatic indices were significantly higher in BDL rats compared to sham-operated group (Hepatosomatic index :SHAM, 2.82±0.22 versus BDL, 4.92±0.53,$P \leq 0.05$; splenosomatic index: SHAM, 0.39± 0.05versus BDL, 0.61± 0.09,$P \leq 0.05$). Quercetin treatment decreased these parameters in BDL-Q rats compared to BDL group, however such decrease was statistically insignificant(Hepatosomatic index :BDL, 4.92±0.53 versus BDL-Q, 4.19±0.39;splenosomatic index: BDL, 0.61± 0.09,versus BDL-Q, 0.58± 0.05).

Changes in serum levels of enzyme markers of liver injury and total proteins (TPs):

AST, ALT and ALP, were significantly elevated in BDL rats versus sham-operated group. All were significantly diminished in BDL-Q group versus BDL rats, achieving levels equivalent to those of the sham-operated group except for AST(Figure 1).

TPs were significantly decreased in BDL group compared to sham-operated group. Quercetin treatment significantly increased the TPs in BDL-Q group versus BDL rats, restoring their levels to match those of the sham-operated group (Figure 1).

Changes in liver tissue levels of oxidative stress parameters:

A significant increase in MPO associated with a significant decrease in GPX were observed in BDL group versus sham-operated group. Quercetin treatment significantly decreased MPO and increased GPX in BDL-Q group compared to BDL rats (Figure 2).

Changes in liver tissue levels of TNF-α, and TGF-β1:

BDL rats presented significantly higher levels of TNF-α, and TGF-β1**,** compared to sham-operated group. Quercetin treatment significantly diminished TNF-α and TGF-β1 in BDL-Q group versus BDL rats (Figure 2).

Histological and morphological changes

Hematoxylin and eosin stained liver sections

Sham-operated group showed normal architecture of classic hepatic lobules (Fig. 3 A and B). BDL group showed moderate periportal inflammation with areas of lobular necrosis (Figure 3 C, D and E). BDL-Q group showed mild periportal inflammation with no lobular inflammation or necrosis. (Fig.3 F and G).

Masson's trichrome stained liver sections

Sham-operated group showed average collagen distribution around central veins and in portal tracts (Fig. 4 A and B). BDL group showed dense deposition of collagen fibers in the portal and peri-portal areas (Fig.4 C and D). In BDL-Q group, evident decrease in the amount of collagen fibers was identified (Fig.4 E and F).The percentage area of collagen deposition was significantly increased in the BDL group compared to sham-operated group. Quercetin treatment resulted in a significant diminishment of the percentage area of collagen deposition in BDL-Q group versus BDL rats, reaching level equivalent to that of sham-operated group (Fig.4 G).

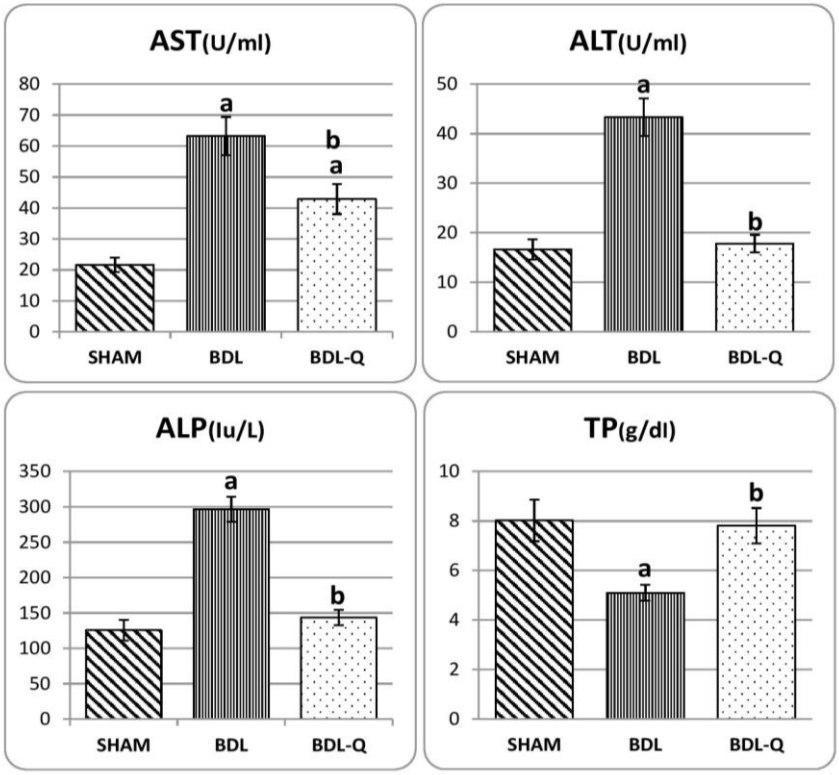

Figure (1): Changes in serum levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), and total proteins (TPs) in the three study groups. a: significance of difference from SHAM group.b: significance of difference from BDL group. SHAM, sham-operated group (n=11); BDL, bile duct-ligated group (n=9); BDL-Q, bile duct ligated quercetin-treated group (n=10).

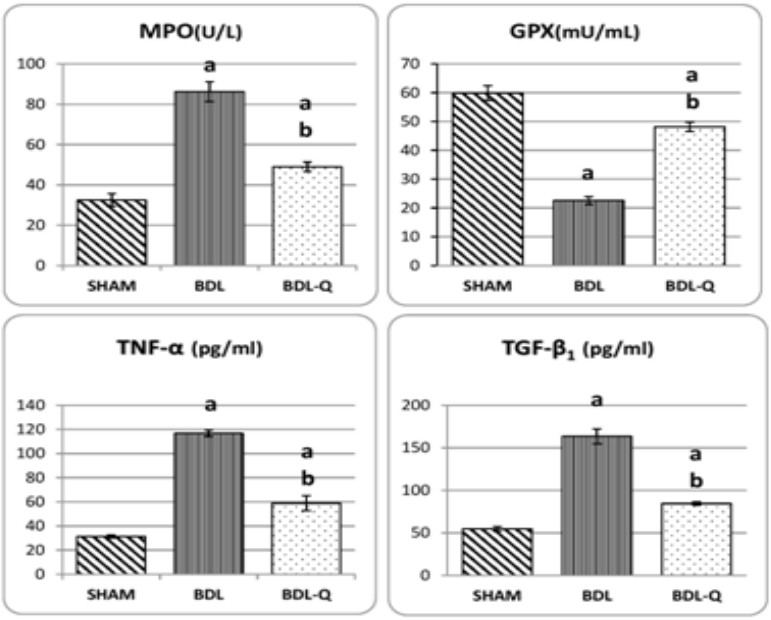

Figure (2): Changes in liver tissue levels of myeloperoxidase (MPO), Glutahione peroxidase (GPX), tumor necrosis factor alpha (TNF-α), and transforming growth factor beta 1 (TGF-β1) in the three study groups. a: significance of difference from SHAM group. b: significance of difference from BDL group. SHAM, sham-operated group (n=11); BDL, bile duct-ligated group (n=9); BDL-Q, bile duct ligated quercetin-treated group (n=10).

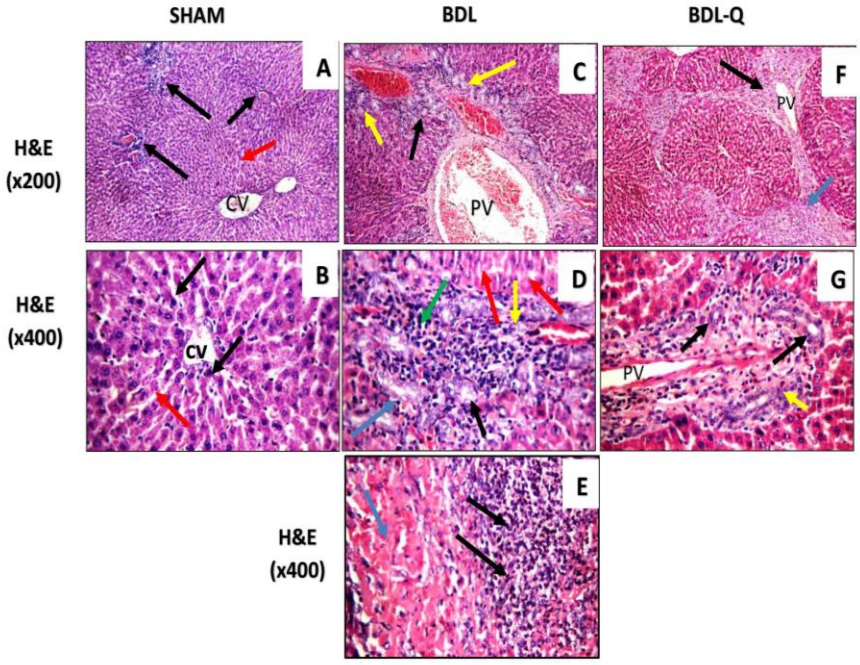

Figure (3): Photomicrographs of H & E stained liver sections of sham-operated controls (SHAM) show: [A] Normal central veins (CV), portal tracts (black arrows), and hepatocytes (red arrow) (X 200). [B] Average central vein (CV), and hepatocytes arranged in single-cell cords (black arrows) with intervening blood sinusoids (red arrow) (H&E X 400). Bile duct ligated (BDL) liver sections show: [C] Markedly dilated congested portal vein (PV) with proliferating bile ducts (black arrows), and inflammatory infiltrate (yellow arrows) (H&E X 200. [D] Proliferating bile ducts with edematous epithelial lining (black arrows), moderate portal and peri-portal inflammatory infiltrate composed mainly of neutrophils (yellow arrow) and macrophages (green arrow), bile stasis (blue arrow), and apoptotic hepatocytes in the interface area (red arrows) (H&E X 400). [E] Area of marked neutrophilic inflammatory infiltrate (black arrows) and area of necrosis (blue arrow) (H&E X 400). Bile duct ligated quercetin treated (BDL-Q) liver sections show: [F] Mildly expanded edematous portal tracts (black arrows) with mild portal inflammatory infiltrate (blue arrow) (H&E X 200). [G] Mildly expanded portal tract showing bile ducts with average epithelial lining (black arrows), mild portal and peri-portal inflammatory infiltrate composed mainly of eosinophils (yellow arrow), and average portal vein (PV) (H&E X 400).

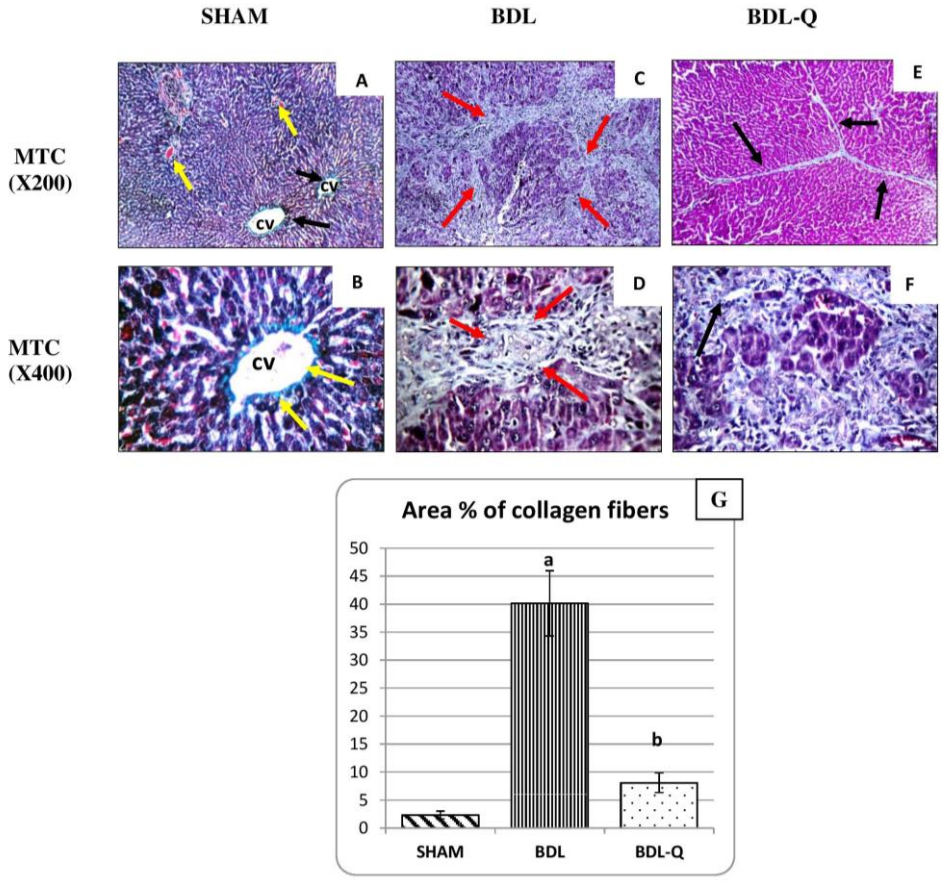

Figure (4): Photomicrograph of Masson trichrome (MTC) stained liver sections of sham-operated controls (SHAM) show: [A] Average collagen distribution around central veins (black arrows) and in portal tracts (yellow arrows) (MTC X 200). [B] High power view showing average collagen distribution around central veins (yellow arrows) (MTC X 400). Bile duct ligated (BDL) liver sections show: [C] Marked deposition of collagen fibers with nodular formation (red arrows) (MTC X 200). [D] high power view showing collagen fibers (early fibrosis) (red arrows) (MTC X 400). Bile duct ligated quercetin treated (BDL-Q) liver sections show: [E] Mild deposition of intra-lobular fibrous bands (black arrows) with preserved architecture (MTC X 200). [F] No collagen in portal tracts (black arrow) (MTC X 200). [G] Changes in the percentage area of collagen fibers in the three study groups. a: significance of difference from SHAM group. b: significance of difference from BDL group.

Discussion

The main finding of the present study is that quercetin treatment in bile duct ligated rats provides protection against cholestasis induced liver injury and attenuates the upregulation of liver oxidative stress, inflammation, and fibrosis, improving clinical liver functions as well as histological structure.

In the current work cholestasis significantly elevated serum levels of AST, ALT, and ALP, well known biochemical markers of hepatocellular damage [22], in BDL rats compared to sham-operated group. These indicate loss of hepatocyte membrane integrity and altered cell membrane permeability, leading to leakage of liver enzymes, a prominent sign of liver injury [23]. Moreover, the significant decrease in serum levels of TPs in BDL rats versus sham-operated group, notifying decreased protein synthesis and asserted impaired liver functions in this group. Such changes were paralleled with enhancement of liver oxidative stress manifested as a significant increase in liver levels of MPO, along with a significant depletion of GPX compared to sham-operated group,

reflecting a role of oxidative stress in cholestasis induced liver injury. In agreement with this assumption**Li et al.**[4] stated that oxidative stress is a conjoint pathological mechanism in the initiation and progression of hepatic injury during cholestasis.

Earlier, accumulation of hydrophobic toxic bile acids in the hepatocytes was reported to kill hepatocytes via their detergent cytolytic actions, leading to membrane damage, and also promote ROS production from hepatic mitochondria which oxidatively damages lipids, proteins, and nucleic acids, ultimately resulting in hepatocyte apoptosis [24].

Quercetin treatment, in the present study, significantly ameliorated liver oxidative stress and upregulated the antioxidant capacity as indicated by the significant decrease in liver levels of MPO associated with significant elevation of GPX in BDL-Q group versus BDL rats. Such antioxidant impact attenuated cholestasis induced liver injury , maintained hepatocyte membrane integrity, and improved liver function as manifested by the significant decrease in ALT, AST and ALP, and the significant increase in TPs in BDL-Q group versus BDL rats, achieving levels comparable to those of the sham-operated group except for AST.These observations agree with the findings of Casas-Grajalesa et al.[6]. The hepatoprotective effect of quercetin against liver diseases was reported by previous studies and was assumed to be mediated by its antioxidant property which depends primarily on its chemical structure [25].

Also, BDL rats in the present study exhibited significantly higher liver levels of TNF-α, a key cytokine that initiates and promotes various proinflammatory signaling pathways [26], compared to sham operated controls, notifying activation of local inflammatory response. This was further confirmed by the histological studies which showed moderate inflammatory changes with a prominent increase in edema, congestion, cellular infiltration, and necrosis.

TNF-α has been recognized as a strong mediator of liver failure in several animal models of liver injury [7, 27]. TNF-α knockout in mice has been shown to inhibit liver damage induced by hepatotoxin carbon tetrachloride [28]. Quercetin treatment in the present study attenuated cholestasis induced liver inflammation as manifested by the significant decrease in liver levels of TNF-α and alleviation of all histological markers of inflammation in BDL-Q rats versus BDL group. These results are consistent with the findings ofLi et al.[29].

Quercetin had been reported to control the inflammatory process by modulating the proinflammatory signaling pathways involved in T-cell proliferation, B-lymphocyte stimulation [30] and by restraining the inflammatory reaction through inhibiting TNF-α [31, 32]. Hence, the present anti-inflammatory effect of quercetin might be produced directly via suppressing the release of TNF-α locally in liver tissues.

Additionally, the antioxidant effect of quercetin, observed herein, could be another possible indirect anti-inflammatory mechanism. Oxidative stress is believed to activate Kupffer cells which further produce TNF-α [26]. Also, blocking of the liver prooxidant markers in concomitance with anti-inflammatory markers by quercetin treatment has been reported widely in different animal models of liver injury [11, 33], suggesting that quercetin might exert anti-inflammatory effects notably

through radical scavenging activities. Accordingly, quercetin in the present study could ameliorate cholestatic induced liver inflammation directly via inhibiting the proinflammatory marker, TNF-α, and/or indirectly by reversing the oxidant/antioxidant imbalance.

Furthermore, the present study, demonstrated significant higher liver levels of TGF-β1, along with significant increase in the percentage area of collagen fibers in livers of BDL group versus sham-operated group, announcing the development of liver fibrosis.

It is noteworthy that oxidative stress and its constant companion inflammation, findings recorded in BDL group in the present study, are principle contributors to liver fibrosis [34]. They have been implicated in activation of hepatic stellate cell (HSC) [35], the principle cell-type that mediates hepatic collagen formation [36]. Activation of HSCs was found to trigger their differentiation into myofibroblast-like cells that release TGF-β1 [23].

Earlier, Derynck and Zhang[37] reported that TGF-β1 is a potent profibrotic cytokine that increases collagen production as well as tissue inhibitors of metalloproteinases, leading to decreased extracellular matrix degradation and increased extracellular matrix accumulation.

Quercetin treatment, in the current work, significantly diminished liver levels of TGF-β1, and the percentage area of collagen fibers in BDL-Q group versus BDL group, implying an evident antifibrotic effect. These results agree with the findings of previous studies[6, 23]. It seems likely that controlling of the redox status and the inflammatory response by quercetin treatment, herein, reduced liver levels of TGF-β1 which could partly contribute to the amelioration of liver fibrosis, and improvement of liver function.

In the present study, hepatosomatic and splenosomatic indices were significantly increased in BDL rats versus sham-operated group, and both were insignificantly lowered by quercetin treatment in BDL-Q group versus BDL rats. Cholestasis induced liver congestion, edema, and cellular infiltration; as well as increased collagen deposition, in the present study, might explain hepatomegaly in BDL group. Also, splenomegaly is a sign of portal hypertension [38]and a known complication of liver fibrosis [36]. The anti-inflammatory and the antifibrotic effect of quercetin might partly alleviate the hepatosplenomegaly in the present study.

Failure of quercetin, in the present study, to totally prevent cholestasis induced hepatosplenomegaly, increment of serum AST, and liver oxidative stress, inflammation, and fibrosis might reflect a small dose and/ or short duration of treatment.

Conclusions

In conclusion, quercetin treatment ameliorates cholestatic liver injury induced experimentally by bile duct ligation, and significantly corrects clinical and histological features of liver damage. The main action mechanism depends fundamentally on the ability of quercetin to attenuate liver oxidative stress at least in part via diminishing liver myeloperoxidase and upregulating liver GPX, thereby suppressing the release of the proinflammatory cytokine, TNF-α, as well as the profibrotic marker, TGF-β, alleviating liver inflammation and fibrosis. Taken together, these findings suggest that quercetin may be a promising adjuvant therapy in case of extrahepatic cholestatic liver injury.

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Conflict of interest

The authors declare that there is no conflict of interest associated with this work.

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