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Title Page

Hepatoprotective Effects of Curcumin in rats after bile duct ligation via down-regulation of Rac1 and NOX1

Running Title: *Curcumin attenuates hepatic injury*

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ABSTRACT

Objectives: New evidence has proven hepatoprotective activity of curcumin, but its underlying mechanisms remain to be elucidated. We investigated the protective effect of curcumin on hepatic damage via measuring the antioxidant capacity and expression level of Rho-related C3 botulinum toxin substrate (Rac1), Rac1- Guanosine triphosphate (Rac1-GTP) and NADPH oxidase 1(NOX1) in biliary duct ligated (BDL)fibrotic rat model. **Methods:** Wistar rats weighing 200-250gr were divided into four groups (n=8 for each): sham group; sham+Cur group (received curcumin 100 mg\kg\day); BDL+ Cur group and BDL group. Messenger RNA (mRNA) and protein expression levels of Rac1, Rac1-GTP and NOX1 were measured by real-time PCR and Western blotting, respectively. **Results:** Curcumin treatment of BDL rats reduced liver injury, as verified by improvement of hepatic cell histological alterations, and by reduction of hepatic enzymes. Moreover, the increase in the expression of Rac1, Rac1-GTP and NOX1 observed in BDL rats was precluded and reversed back toward normalcy by curcumin treatment ($P<0.05$). We also observed an escalation of protein thiol groups, increased enzyme activity of serum antioxidant markers e.g. superoxide dismutase, while a decrease of carbonylation in curcumin-treated BDL rats compared to BDL rats ($P<0.05$). **Conclusions:** Curcumin attenuated liver damage through down-regulating of Rac1, Rac1-GTP, and NOX1 as well as reducing oxidative stress in serum and liver tissue of BDL rats.

Keywords: Curcumin; Liver damage; Rac1; NOX1

1. Introduction

Bile duct ligation (BDL) is an experimental model of induced cholestasis, which develops hepatocellular damage and fibrogenesis, and ultimately results in liver fibrosis [1]. During liver fibrogenesis, quiescent hepatic stellate cells (HSCs) become active and undergo a transformation that is associated with enhanced cell proliferation and overproduction of Extra cellular matrix (ECM) [2, 3]. Oxidative stress reflects an imbalance between the systemic manifestation of reactive oxygen species (ROS) and a biological system's capability to detoxify the reactive intermediates or to repair the resulting damage. Oxidative stress increases the HSC transformation to activated HSCs named myofibroblasts (MFs), and induces fibrosis [4]. Recent studies revealed that oxidative stress resulting from the metabolism of BDL plays a key role in the progress of hepatic fibrosis [5]. The imbalance can arise from the lack of antioxidant capacity or by an excess of ROS from other factors.

Among the different molecules involved in ROS production during liver damage, a critical role is played via the NADPH oxidase (NOX) complex [6-8]. NOX catalyzes the NADPH-dependent reduction of molecular oxygen to generate superoxide, which can transform to form secondary metabolites including hydrogen peroxide and Hypochlorous acid (HOCl). Among the NOX family, NOX1, NOX2, and NOX4 are expressed on HSCs and may contribute to liver fibrosis [9]. NOX1 knockout HSCs produce less ROS compared to NOX2 knockout HSCs; hence, NOX1 seems to play more crucial role in ROS generation in HSCs than NOX2 [4, 10].

Rac1 (Rho-related C3 botulinum toxin substrate) is one of the central mediators of wound healing by promoting fibrosis. In a hepatic fibrosis model, Rac1 promoted NOX activation, ROS generation, and subsequent liver fibrosis [11, 12].The Rac1 and Rho GTPase have been implicated in various diseases such as atherosclerosis, hypertension, cancers, and fibrosis formation [4, 13], and have several downstream effectors such NOX or cell surface receptor-

mediated signaling pathways that induce inflammatory responses [11, 12]. Numerous stimuli have been identified with the ability to induce activation of Rac1 and formation of Rac1-GTP (biologically activate form of Rac1), including cytokines, growth factors, mechanical stress, and oxidative stress. Rac1-GTP translocation to the membrane-bound cytochrome complex induces the production of enzymatically active NOX1 and NOX2 [4, 6].

Curcumin (diferuloylmethane) is a secondary metabolite of turmeric, derived from Curcuma longa [14, 15]. Extensive research on curcumin over decades provided greater understanding into its medicinal and health benefits. Curcumin has several pharmacologic effects, and can be used as an effective medicine for various disorders such as asthma, and hepatic disease [16]. There are many reports of its anti-oxidant, anti-inflammatory, hepatoprotective, cardioprotective, and anti-fibrosis activity. The molecular mechanisms underlying the targets of curcumin are varied and involve combinations of multiple signaling pathways [4, 17, 18]. However, the molecular mechanism of the curcumin effect on liver fibrosis remains largely unidentified.

According to the evidence that Rac1 and NOX1 expressions are associated with oxidative stress and hepatic fibrogenesis, in the current study we aimed to evaluate the impact of curcumin on Rac1, Rac1-GTP, and NOX1 expression in BDL fibrotic rat model. As the markers of oxidative stress, we also quantified protein carbonylation and total reduced thiols (Glutathiones), and also assessed enzymatic activities of superoxide dismutase (SOD) and catalase.

2. Materials and Methods

2.1. Animals and Experimental Procedures

Adult male Wistar rats (200-250 g, Pasteur Institute, Tehran, Iran) were used in this research. Rats were housed in an air-conditioned room at 25°C with a 12-h darkness/light cycle and

had free access to rat food diet and drinking water. All of the study's protocols fulfilled the current ethical considerations of local ethical committee of animal use.

A total of 32 rats were randomly detached in two groups: sham group and BDL group. Each group was also separated into two subgroups for treatment with either curcumin 100 mg/kg/day (Sigma Chemicals Co. Purity (HPLC)>80%, USA) suspended in 5% carboxymethyl cellulose (CMC) [19, 20] or the same volume/weight of the 5% CMC vehicle by oral gavage once a day from the day after surgery for 28 days [20]. BDL was accomplished as described previously [21]. Concisely, under general anesthesia by ketamine (90 mg/kg) and xylazine (10 mg/kg) intraperitoneally (i.p.), the common bile duct was exposed by a midline abdominal incision under sterile conditions. It was then ligated in two places with a silk thread and sectioned between the ligatures [22] and Cefazolin antibiotic was used to prevent infection.

At the end of the 4-week period, blood samples were collected by puncturing the heart under deep anesthesia and they were centrifuged at 3000g for 15 min. The serum was isolated for further experiments. Liver tissues were divided into three parts, one part was frozen in liquid nitrogen for RNA extraction, the second part was kept at -70˚C to make homogenized tissue for assessment of oxidative stress parameters, Rac1, Rac1-GTP, NOX1 Western blot analyses and the last one fixed with 10% neutral formalin for histology.

2.2. Histopathological Evaluation

The liver specimens were fixed in 10% neutral formalin individually, dehydrated in alcohol and embedded in paraffin and sections were stained with hematoxylin and eosin (H&E). Lobular and portal inflammation, focal hepatocyte necrosis, ductular proliferation, portal and septal fibrosis were examined by a pathologist. The rats in BDL group with no significant histopathological changes were excluded from the study.

2.3. Blood Chemistry

The collected serum was examined for alanine aminotransferase (ALT), aspartate aminotransferase (AST), and alkaline phosphatase (ALP) as indicators of the hepatic function using standard animal diagnostic kits (Pars Azmon Diagnostic Co., Iran) and a Roche BT3000 Auto Analyzer.

2.4. Hepatic hydroxyproline content

An automated procedure for quantitative assay of hydroxyproline in tissue is based on the oxidation of hydroxyproline by chloramine T in aqueous solution. The oxidation product reacts with Ehrlich's reagent, and the chromogen obtained is registered in a recorder connected to the colorimeter. The amount of hydroxyproline is expressed as µg/mg tissue [23].

2.5. Determination of thiol groups in liver tissue

Ellman's reagent 5, 5′ -dithiobis (2-nitrobenzoic acid) (DTNB) was first introduced for the assessment of free thiol groups. The procedure is dependent on the reaction of the thiol with DTNB to give the mixed disulfide and 2-nitro-5-thiobenzoic acid (TNB) which is quantified via the absorbance of the anion (TNB2-) at 412 nm[24]. Free thiol groups were measured by adding: 50 µl Tris, 25 µl DTNB, and420 µl water (495µl initial volume take blank) and +5µl of sample (final volume of 500 µl). The solution was mixed gently using pipette. The cuvette was placed into UV-Vis spectrophotometer and reading was taken at 412 nm. Absorbance was taken for each homogenized tissue sample and then the results were expressed in nmol/mg protein.

2.6. Determination of protein carbonylation in liver tissue

Homogenizedtissue samples were treated with in 2.5 N HCl, 10 Mm 2.4dinitrophenylhydrazine for 1 hour in dark at room temperature. After treatment with 20%

trichloracetic acid and separation via centrifugation, the precipitate was washed three times with a mixture of absolute ethylic alcohol and ethylic acetate 1:1(v/v). Subsequently, protein precipitate was dissolved in 6M guanidine hydrochloride. Protein concentration was quantified in these samples through measuring the absorbance at355nm and the final results were expressed in nmol/ mg protein.

2.7. Catalase activity in liver tissue

Catalase activity was assayed by the method described by Beers et al. andSizer et al. [25, 26]in which the vanishing of peroxide is followed spectrophotometricallyat 240 nm. The incubation mixture contained 0.05 M potassiumphosphate, pH 7.0, 0.020 M hydrogen peroxide and a sample (0.05 ml) of thesupernatant fluid, in a final volume of 3 ml. The decrease in absorbance was recorded at 240 nm for 3 min. The rate of decrease in absorbance per min was calculated from the initial linear portion of the curve. The value of 0.0394 $cm^{-1}M^{-1}$ proposed by Nelson and Kiesow was used as the destructioncoefficient of H_20_2. One unit of catalase was defined as the amount of enzyme which decomposed one mol of H_20_2 per minute at 25°C and pH 7.0 under the specified conditions, and the final results were expressed in IU/mg protein.

2.8. SOD activity in liver tissues

The principle of SOD activity measurement was based on the inhibition of nitrobluetetrazolium (NBT) reduction. Illumination of riboflavin in the presence of O2 and methionine (electron donor) generates superoxide anions and this has been introduced as the basis of the assay of SOD. The reduction of NBT by superoxide radicals to blue coloured formazan was recorded at 560 nm, as previously described by Fridovich and Beauchamp [27]. The reaction mixture contained 1.9 ml of phosphate buffer [pH 7.8], 16.8×10^{-5} M NBT, 1.17×10^{-6} M riboflavin and 10^{-2} M methionine, with suitably diluted homogenized tissue in a total volume of 3 ml. The absorbance was recorded at 560 nm for 5 min. The rate of increase

in absorbance per min was calculated from the initial linear portion of the curve. The value of 0.00436 $cm^{-1}mol^{-1}$ was used as the extinction coefficient. The values were expressed in IU/mg protein.

2.9. Western blot analysis of liver tissue Rac1, Rac1-GTP and NOX1 levels

Liver cell protein was extracted by homogenization of tissue samples (30 mg) using phosphate saline buffer (100 mM Tris–HCl, 150 mM NaCl, 0.1% SDS and 1% NP-40; pH 7.4 with protease-inhibitor cocktail, 1:100; Sigma, St Louis, MO, USA) by incubation on ice for 30 min and subsequent centrifugation at 15000 g (4°C, 30 min). Protein concentrations were determined in the supernatants by the Bradford assay. Protein (100 µg) was separated on a 12% SDS-polyacrylamide gel and transferred onto a nitrocellulose membrane. The membrane was blocked with 3% non-fat dried milk in Tris-buffered saline, pH 7.4, with 0.05% Tween-20 (TBS/T) for 2 h and probed with monoclonal rabbit anti-Rac1 primary antibody (Abcam, Cambridge, UK), polyclonal rabbit anti- NOX1(Abcam, Cambridge, UK), monoclonal mouse anti-Rac1-GTP (Newest biosciences) and polyclonal rabbit anti-β-actin (Abcam, Cambridge, UK) as a reference at 4°C overnight. The membranes were incubated with a goat anti-rabbit and anti-mouse IgG secondary antibody (1:4000) conjugated with horse radish peroxidase (cell signaling, Munich, Germany) for 45 min. The predicted sizes for Rac1 (21KDa), Rac1-GTP (~22KDa), NOX1 (71KDa) and β-actin (42 kDa) were checked using molecular weight markers. Specific bands were visualized by an enhanced chemiluminescence reagent (GE) on a ChemiDoc system (Syngene GBOX, 680X) and quantified densitometrically with the program Quantity GeneTools (SynGene, V4.1).

2.10. Gene expression analysis by quantative real-time PCR (qPCR)

Total cellular RNA was isolated from liver samples by using a Fast Pure RNA Kit from TakaRa, Japan) according to the manufacturer's protocol. By measuring the absorbance at 260 nm, concentrations of RNA were identified and its purity was evaluated by 260/280 nm

absorbance ratio (Eppendorf, Hamburg, Germany). One microgram of the total RNA was reverse transcribed to cDNA using MuLV RT enzyme (Fermentas), random hexamers and dNTP in a total volume of 20 µL. The cDNA samples were diluted 1/10, and aliquots were frozen at −70°C until the PCRs were carried out. qPCR was performed triplicate using SYBR-green in the Rotor Gene system (Corbett Research 2004, Australia). Normalization was achieved against β-actin and relative quantity of gene expression was analyzed based on ΔCt method and the results were calculated as $2^{-\Delta\Delta Ct}$.

Oligonucleotide primer sequences used for qPCR were: alpha smooth muscle Actin (α-SMA): forward 5′- GCTCCATCCTGGCTTCTCTATC-3′ and reverse 5′-GGGCCAGCTTCGTCATACTC-3′, Collagen I: forward 5′-ATCAGCCCAAACCCCAAGGAG-3′ and reverse 5′-CGCAGGAAGGTCAGCTGGATAG-3′, Transforming growth factor beta 1 (TGF-β1) forward 5′-AAGAAGTCACCCGCGTGCTA-3′ and reverse 5′- TGTGTGATGTCTTTGGTTTTGTC-3′,

Rac1: forward 5′- GTAAAACCTGCCTGCTCATC-3′ and reverse 5′-GCTTCATCAAACACTGTCTTG-3′, NoX1: forward 5′-TACGAAGTGGCTGTACTGGTTG-3′ and reverse 5′-CTCCCAAAGGAGGTTTTCTG-3′, β-actin: Forward: 5′-CGTTGACATCCGTAAAGACCTC-3′ and reverse: 5′-AGCCACCGATCCACACAGA-3′.

2.11. Statistical Analyses

Differences between obtained values (mean ± SEM) were carried out by one-way analysis of variance (ANOVA) followed by Tukey-Kramer multiple comparison using Graphpad Prism 5 software. The differences below 0.05 were considered statistically significant.

3. Results

3.1 Curcumin attenuated BDL-induced histopathological changes

The histological examination of liver by Hematoxylin and eosin (H&E) staining revealed that sham-operated control rats had normal liver morphology with intact hepatocytes and portal tracts (Fig. 1A, 1B). Parenchymal necrosis and an abundance of newly-formed bile ducts, including bridging of the portal tracts, were found in BDL rats (Fig. 1C). Curcumin supplementation prominently amended all of the histopathological changes observed in BDL group (Fig. 1D).

3.2 Effect of curcumin on serum biochemistry of BDL rats

Table 1 represents the results of the curcumin on serum hepatic enzymes. The serum levels of AST, ALT, and ALP were significantly elevated in BDL rats ($p<0.05$). The high levels of these three hepatic enzymes in BDL rats were significantly decreased after their treatment with curcumin (100mg\kg\day) ($p<0.05$).

3.3. Hydroxyproline quantification

Table 2 represents the results of the curcumin on liver tissue hydroxyprolin level. The hepatic level of hydroxyprolin was significantly elevated in BDL rats ($p<0.05$). The high level of hydroxyproline in BDL rats was significantly decreased after their treatment with curcumin (100mg\kg\day) ($p<0.05$).

3.4. MRNA expression of α-SMA , collagen I and TGF-β1

We measured the level of mRNA expression α-SMA, collagen I and TGF-β1 in liver tissue of four groups (sham, sham+Cur, BDL and BDL+Cur) by qPCR method (Table 2). We observed that α-SMA, collagen I and TGF-β1gene expressions were significantly increased in liver tissue of BDL rats compared with sham group ($p<0.05$). However, curcumin treatment reduced gene expression of α-SMA, collagen I and TGFβ1 in the liver tissue of BDL+Cur group compared with BDL group.

3.5. Curcumin attenuated BDL-induced oxidative stress

We measured thiol groups and carbonyl as two indicators of damage to liver tissue proteins and also determined the activity of catalase and SOD enzymes, two important enzymes in the antioxidant defense. We found that in BDL group in comparison with sham group, the activity of antioxidant enzymes (SOD & Catalase) (Figure 2C & 2D) as well as the concentration of thiol groups (Figure 2A) significantly decreased, whereas the concentration of carbonyl groups (Figure 2B) increased intensely ($P<0.05$). These observations confirmed the presence of oxidative stress in liver tissue of BDL rats. In contrast, curcumin treatment of BDL rats resulted in an increase in the level of free thiol groups, and the enzyme activity of catalase and SOD in the liver tissue (Figure 2A, 2C&2D), whereas the carbonyl level of proteins was reduced (Figure 2B).

3.6. mRNA expression of Rac1, Rac1-GTP and NOX1 was decreased in curcumin administered group

After 28 days from BDL surgery and then treatment with curcumin, we measured the level of mRNA expression Rac1 and NOX1 in liver tissue of four groups (sham, sham+Cur, BDL and BDL+Cur) by qPCR method. We observed that both Rac1 and NOX1 gene expressions significantly increased in liver tissue of BDL rats compared with sham group. However, treatment with curcumin reduced gene expression of Rac1 and NOX1 in the liver tissue of BDL+Cur group compared with BDL group as shown in Fig 3A and 3B.

3.7. Protein Expression of Rac1, Rac1-GTP, and NOX1 was reduced in curcumin administered group

We measured the level of protein expression Rac1,Rac1-GTP and NOX1in liver tissue of four groups(sham,sham+Cur, BDL and BDL+Cur) by Western immunoblottingin order to confirm their mRNA expression profile. We observed that the relative density of Rac1,Rac1-

GTP and NOX1 significantly increased in liver tissue of BDL rats compared with sham group ($P<0.05$).The expression level of Rac1,Rac1-GTP and NOX1 proteins in liver tissue of BDL+Cur rats decreased once compared to BDL rats($P<0.05$) as shown in Fig 4.

4. Discussion

The present study investigated hepatoprotective effect of curcumin through evaluation of serum antioxidant markers, hepatic enzymes, hepatic fibrosis markers and gene/protein expression profile of Rac1, Rac1-GTP and NOX1in BDLfibrotic rat model. To the best of our knowledge, this is the first study to determine the hepatoprotective effect of curcumin by evaluating the expression level of NOX1, Rac1 and Rac1-GTP in BDL rats. Our findings revealed that curcumin treatment attenuated liver injury through down-regulation of either mRNA or protein expression of Rac1-GTP, Rac1, and Nox1.To assess the fibrosis process of hepatic tissue during BDL, we measured mRNA expression of three fibrotic markers including α-SMA, collagen I and TGF-β1 in liver tissue of four study groups and also hepatic level of hydroxyproline. Compared to sham group, BDL rats demonstrated a significant increase in the fibrosis markers, but treatment of BDL rats with curcumin led to a reduction in these markers.

Curcumin (diferuloylmethane) is a polyphenol derived from the yellowish pigments of turmeric plant. It has beneficial properties such as anti-cancer, antioxidant, anti-inflammatory, hepatoprotective, and antifibrosis effects [14, 15].Nevertheless, the effect of curcumin on the prevention of liver fibrosis and its molecular mechanism has not been fully understood.

BDL is a representative model of biliary cholestasis in animals which results in oxidative damage. It stimulates the HSCs to secrete more collagen fibrosis and induces the accumulation of ECM leading to liver fibrogenesis [21]. We used the BDL method to induce hepatic fibrosis and confirmed the fibrosis through assessment of the levels of serum hydroxyprolin, the mRNA expression of α-SMA, collagen I and TGF-β1, and by measuring hepatic enzymes including ALT, AST, and ALP as well as H&E staining of hepatic sections

of BDL rats. Based on our observation, curcumin treatment of BDL rats significantly improved fibrosis of the liver tissue and also increased the activity of hepatic enzymes.

Liver is a dynamic organ with fundamental contributions in metabolism, detoxification, and elimination of endogenous and exogenous substances [28]. Huge amount of evidence has demonstrated the implication of oxidative stress in liver damage. Oxidative stress is a disruption in the oxidant-antioxidant balance resulting in potential cellular damage. The imbalance can arise from an absence of antioxidant capacity caused by disturbances in production and distribution, or by an excess of ROS from other factors [29, 30]. Recent studies have revealed that oxidative stress resulting from the metabolism of BDL plays a significant role in the hepatic fibrosis development [5]. Among all antioxidants that are available in the body, thiols constitute the major portion of the total body antioxidants and are responsible for the defense against ROS. Amongst principal plasma antioxidants available in our body fluids, thiols are organic compounds with essential reducing groups that fight against oxidants like ROS [31, 32]. In contrast, carbonylation of proteins which is an irreversible oxidative damage frequently impairs proteins function. It is considered a prevalent indicator of severe oxidative damage and disease-derived protein dysfunction that can be promoted by ROS [33]. Our findings have shown that the thiol group, as the sensitive indicator of rat oxidative stress, was reduced significantly when exposed to oxidative stress caused by BDL. BDL-induced oxidative stress also led to increased production of carbonyl group on proteins' peripheral chains. In agreement with our findings, Dalle-Donne et al. have reported an increase in carbonyl protein levels and a decrease in thiol group in BDL rats compared to the control sham group [34]. Our experiments demonstrated that treatment of BDL rats by curcumin increases the amount of amino acid free thiol groups, whereas it reduces proteins carbonylation.

Under oxidative stress conditions, the antioxidant enzymes such as SOD and catalase remove the extra ROS to maintain homeostasis. Recent studies have shown a significant reduction in the activity of catalase and SOD in BDL rats compared to the control group [35-37].

Likewise, we observed reduced levels of these enzymes in BDL group compared to the sham group. However, the enzyme activity of catalase and SOD were increased in BDL rats after they were treated by curcumin, hence resulting in a protection against hepatic fibrosis through elevation of the antioxidant capacity.

Hepatic fibrosis is a pathologic process characterized by synthesis, secretion and accumulation of ECM derived from increased MF-HSC in the liver [2]. The required event for accumulation of MF-HSC and induction of hepatic injury is the transportation of HSC from the passive phenotype to the MF form. Rac1 has been identified as the key mediator for accumulation of MF-HSC, associated with progression of hepatic fibrosis [38, 39]. Rac1 is a subunit of cytosolic NOX1. The activated Rac1 stimulates NOX1, and the subsequent activity of NOX1 induces conversion of HSCs to MF forms, hence promoting hepatic fibrosis [39]. NOX1 is a multi-protein complex which produces ROS in response to various stimuli [40]. Activated Rac1 (Rac1-GTP) directly binds to NOX1 through TPR site and leads to the regulation of ROS-producer NOX1 [41, 42]. Recent observations have demonstrated that in rats with carbon tetrachloride-induced fibrosis, the Rac1 activity led to overproduction of NOX1-mediated ROS as well as increased activity of HSCs which ultimately aggravated hepatic fibrosis [39]. However, Rac1 deficiency resulted in suppression of NOX1 as well as reduction in oxidative stress [43]. Our experiments indicated that in BDL fibrotic rats the rate of NOX1 expression was significantly increased, and this increase was in parallel to the escalation in the expression rate of Rac1, and Rac1-GTPonce compared to the sham group. However, these rates were reduced upon treatment of BDL fibrotic rats with curcumin (BDL+Cur compared to BDL group).

5. Conclusion

In summary, our results demonstrated that the BDL fibrotic rats expressed higher levels of Rac1, Rac1-GTP, and NOX1 but they have produced a reduced level of antioxidant markers. Curcumin treatment of BDL rats led to down-regulation of Rac1, Rac1-GTP and NOX1, and elevation of antioxidant(thiols, SOD and catalase), hepatic enzymes (ALP, AST, ALT) and

hepatic fibrosis marker (α-SMA, collagen I ,TGFβ1 and hydroxyproline). Therefore, our study suggests that curcumin exerts its anti-hepatofibrotic effects via reduction of oxidative stress as well as down-regulation of Rac1-GTP, Rac1, and NOX1.

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References

[1] Zhen YZ, Li NR, He HW, Zhao SS, Zhang GL, Hao XF, et al. Protective effect of bicyclol against bile duct ligation-induced hepatic fibrosis in rats. World journal of gastroenterology : WJG. 2015;21:7155-64.

[2] George J. Ascorbic acid concentrations in dimethylnitrosamine-induced hepatic fibrosis in rats. Clinica chimica acta; international journal of clinical chemistry. 2003;335:39-47.

[3] Bataller R, Brenner DA. Liver fibrosis. The Journal of clinical investigation. 2005;115:209-18.

[4] Aoyama T, Paik YH, Watanabe S, Laleu B, Gaggini F, Fioraso-Cartier L, et al. Nicotinamide adenine dinucleotide phosphate oxidase in experimental liver fibrosis: GKT137831 as a novel potential therapeutic agent. Hepatology. 2012;56:2316-27.

[5] Poli G. Pathogenesis of liver fibrosis: role of oxidative stress. Molecular aspects of medicine. 2000;21:49-98.

[6] Bataller R, Schwabe RF, Choi YH, Yang L, Paik YH, Lindquist J, et al. NADPH oxidase signal transduces angiotensin II in hepatic stellate cells and is critical in hepatic fibrosis. The Journal of clinical investigation. 2003;112:1383-94.

[7] Chuong Nguyen MV, Lardy B, Paclet MH, Rousset F, Berthier S, Baillet A, et al. [NADPH oxidases, Nox: new isoenzymes family]. Medecine sciences : M/S. 2015;31:43-52.

[8] Manea SA, Constantin A, Manda G, Sasson S, Manea A. Regulation of Nox enzymes expression in vascular pathophysiology: Focusing on transcription factors and epigenetic mechanisms. Redox biology. 2015;5:358-66.

[9] Brandes RP, Weissmann N, Schroder K. Nox family NADPH oxidases: Molecular mechanisms of activation. Free radical biology & medicine. 2014;76:208-26.

[10] Bopp A, Wartlick F, Henninger C, Kaina B, Fritz G. Rac1 modulates acute and subacute genotoxin-induced hepatic stress responses, fibrosis and liver aging. Cell death & disease. 2013;4:e558.

[11] Fu Y, Zheng S, Lin J, Ryerse J, Chen A. Curcumin protects the rat liver from CCl4-caused injury and fibrogenesis by attenuating oxidative stress and suppressing inflammation. Molecular pharmacology. 2008;73:399-409.

[12] Boureux A, Vignal E, Faure S, Fort P. Evolution of the Rho family of ras-like GTPases in eukaryotes. Molecular biology and evolution. 2007;24:203-16.

[13] Jiang JX, Venugopal S, Serizawa N, Chen X, Scott F, Li Y, et al. Reduced nicotinamide adenine dinucleotide phosphate oxidase 2 plays a key role in stellate cell activation and liver fibrogenesis in vivo. Gastroenterology. 2010;139:1375-84.

[14] Oliveira A, Sousa E, Vasconcelos MH, Pinto MM. Curcumin: a natural lead for potential new drug candidates. Current medicinal chemistry. 2015.

[15] Ghosh S, Banerjee S, Sil PC. The beneficial role of curcumin on inflammation, diabetes and neurodegenerative disease: A recent update. Food and chemical toxicology : an international journal published for the British Industrial Biological Research Association. 2015;83:111-24.

[16] Zheng J, Wu C, Lin Z, Guo Y, Shi L, Dong P, et al. Curcumin up-regulates phosphatase and tensin homologue deleted on chromosome 10 through microRNA-mediated control of DNA methylation--a novel mechanism suppressing liver fibrosis. The FEBS journal. 2014;281:88-103.

[17] He Y, Yue Y, Zheng X, Zhang K, Chen S, Du Z. Curcumin, Inflammation, and Chronic Diseases: How Are They Linked? Molecules (Basel, Switzerland). 2015;20:9183-213.

[18] Alok A, Singh ID, Singh S, Kishore M, Jha PC. Curcumin - Pharmacological Actions And its Role in Oral Submucous Fibrosis: A Review. Journal of clinical and diagnostic research : JCDR. 2015;9:ZE01-3.

[19] Park EJ, Jeon CH, Ko G, Kim J, Sohn DH. Protective effect of curcumin in rat liver injury induced by carbon tetrachloride. The Journal of pharmacy and pharmacology. 2000;52:437-40.

[20] Reyes-Gordillo K, Segovia J, Shibayama M, Tsutsumi V, Vergara P, Moreno MG, et al. Curcumin prevents and reverses cirrhosis induced by bile duct obstruction or CCl4 in rats: role of TGF-beta modulation and oxidative stress. Fundamental & clinical pharmacology. 2008;22:417-27.

[21] Haddadian Z, Eftekhari G, Mazloom R, Jazaeri F, Dehpour AR, Mani AR. Effect of endotoxin on heart rate dynamics in rats with cirrhosis. Autonomic neuroscience : basic & clinical. 2013;177:104-13.

[22] Ma Z, Zhang Y, Huet PM, Lee SS. Differential effects of jaundice and cirrhosis on beta-adrenoceptor signaling in three rat models of cirrhotic cardiomyopathy. Journal of hepatology. 1999;30:485-91.
[23] Prockop DJ, Udenfriend S. A specific method for the analysis of hydroxyproline in tissues and urine. Analytical biochemistry. 1960;1:228-39.
[24] Hu ML. Measurement of protein thiol groups and glutathione in plasma. Methods in enzymology. 1994;233:380-5.
[25] Beers RF, Jr., Sizer IW. A spectrophotometric method for measuring the breakdown of hydrogen peroxide by catalase. The Journal of biological chemistry. 1952;195:133-40.
[26] Nelson DP, Kiesow LA. Enthalpy of decomposition of hydrogen peroxide by catalase at 25 degrees C (with molar extinction coefficients of H 2 O 2 solutions in the UV). Analytical biochemistry. 1972;49:474-8.
[27] Rukmini MS, D'Souza B, D'Souza V. Superoxide dismutase and catalase activities and their correlation with malondialdehyde in schizophrenic patients. Indian journal of clinical biochemistry : IJCB. 2004;19:114-8.
[28] Rutherford A, Chung RT. Acute liver failure: mechanisms of hepatocyte injury and regeneration. Seminars in liver disease. 2008;28:167-74.
[29] Tseilikman VE, Pankov NE, Pankova NA, Filimonova TA, Sinitskii AI, Kozochkin DA, et al. Correlation between circulating corticosterone and protein carbonylation in the liver after short-term hypokinesia. Bulletin of experimental biology and medicine. 2013;156:188-90.
[30] De Waal EM, Liang H, Pierce A, Hamilton RT, Buffenstein R, Chaudhuri AR. Elevated protein carbonylation and oxidative stress do not affect protein structure and function in the long-living naked-mole rat: a proteomic approach. Biochemical and biophysical research communications. 2013;434:815-9.
[31] Balcerczyk A, Grzelak A, Janaszewska A, Jakubowski W, Koziol S, Marszalek M, et al. Thiols as major determinants of the total antioxidant capacity. BioFactors. 2003;17:75-82.
[32] Przemyslaw W, Piotr K, Grazyna C, Danuta KP, Malgorzata I, Bernadeta M, et al. Total, free, and protein-bound thiols in plasma of peritoneal dialysis and predialysis patients. International urology and nephrology. 2011;43:1201-9.
[33] Fedorova M, Bollineni RC, Hoffmann R. Protein carbonylation as a major hallmark of oxidative damage: update of analytical strategies. Mass spectrometry reviews. 2014;33:79-97.
[34] Dalle-Donne I, Giustarini D, Colombo R, Rossi R, Milzani A. Protein carbonylation in human diseases. Trends in molecular medicine. 2003;9:169-76.
[35] Trebicka J, Hennenberg M, Odenthal M, Shir K, Klein S, Granzow M, et al. Atorvastatin attenuates hepatic fibrosis in rats after bile duct ligation via decreased turnover of hepatic stellate cells. Journal of hepatology. 2010;53:702-12.
[36] Sallie R, Tredger JM, Williams R. Drugs and the liver. Part 1: Testing liver function. Biopharmaceutics & drug disposition. 1991;12:251-9.
[37] Eskandari-Nasab E, Kharazi-Nejad E, Nakhaee A, Afzali M, Tabatabaei SP, Tirgar-Fakheri K, et al. 50-bp Ins/Del polymorphism of SOD1 is associated with increased risk of cardiovascular disease. Acta medica Iranica. 2014;52:591-5.
[38] Yang L, Wang Y, Mao H, Fleig S, Omenetti A, Brown KD, et al. Sonic hedgehog is an autocrine viability factor for myofibroblastic hepatic stellate cells. Journal of hepatology. 2008;48:98-106.
[39] Choi SS, Sicklick JK, Ma Q, Yang L, Huang J, Qi Y, et al. Sustained activation of Rac1 in hepatic stellate cells promotes liver injury and fibrosis in mice. Hepatology. 2006;44:1267-77.
[40] Choi SS, Witek RP, Yang L, Omenetti A, Syn WK, Moylan CA, et al. Activation of Rac1 promotes hedgehog-mediated acquisition of the myofibroblastic phenotype in rat and human hepatic stellate cells. Hepatology. 2010;52:278-90.
[41] Paik YH, Kim J, Aoyama T, De Minicis S, Bataller R, Brenner DA. Role of NADPH oxidases in liver fibrosis. Antioxidants & redox signaling. 2014;20:2854-72.
[42] Paik YH, Brenner DA. NADPH oxidase mediated oxidative stress in hepatic fibrogenesis. The Korean journal of hepatology. 2011;17:251-7.

[43] Li J, Zhu H, Shen E, Wan L, Arnold JM, Peng T. Deficiency of rac1 blocks NADPH oxidase activation, inhibits endoplasmic reticulum stress, and reduces myocardial remodeling in a mouse model of type 1 diabetes. Diabetes. 2010;59:2033-42.

Figures Caption:

Fig 1: Curcumin attenuated BDL-induced liver injury as shown by histological examination. Representative photomicrographs of H&E staining are (A) Sham, (B) Sham+Cur, (C) BDL and (D) BDL+Cur

Fig 2: Effects of curcumin on the levels of thiol group (A), carbonyl group (B), SOD activity (C) and catalase activity (D) in the liver tissue of the four groups (Sham, Sham + Cur, BDL, BDL+ Cur) ($^{\#}$P value < 0.05 compared with the Sham group; *P value< 0.05 compared with the BDL group).

Fig 3: Gene expression of NOX1 (Fig. 3A) and Rac1 (Fig.3B) in four studied groups (Sham, Sham + Cur, BDL, BDL+ Cur) ($^{\#}$P value < 0.05 compared with the Sham group. *P value< 0.05 compared with the BDL group).

Fig 4: Western blotting pattern of Rac1, Rac1-GTP and NOX1 proteins expression (4A); The relative density of protein expression levels of NOX1 (Fig. 4B) and Rac1, Rac1-GTP (Fig. 4C) in four studied groups (Sham, Sham + Cur, BDL, BDL+ Cur) ($^{\#}$P value < 0.05 compared with the Sham group. *P value< 0.05 compared with the BDL group).

Table 1: Effect of curcumin on hepatic enzymes (AST, ALT, and ALP) in four groups of rats

	Sham	Sham + Cur	BDL	BDL + Cur
AST (U/dl)	171±13.38	142±6.48	488.4±27.59#	310.5±35.68*
ALT (U/dl)	61.5±6.49	58.5±3.44	150.2±14.27#	138.5±5.95*
ALP (U/dl)	263.5±14.5	271±8.11	958±45.05#	559±30.34*

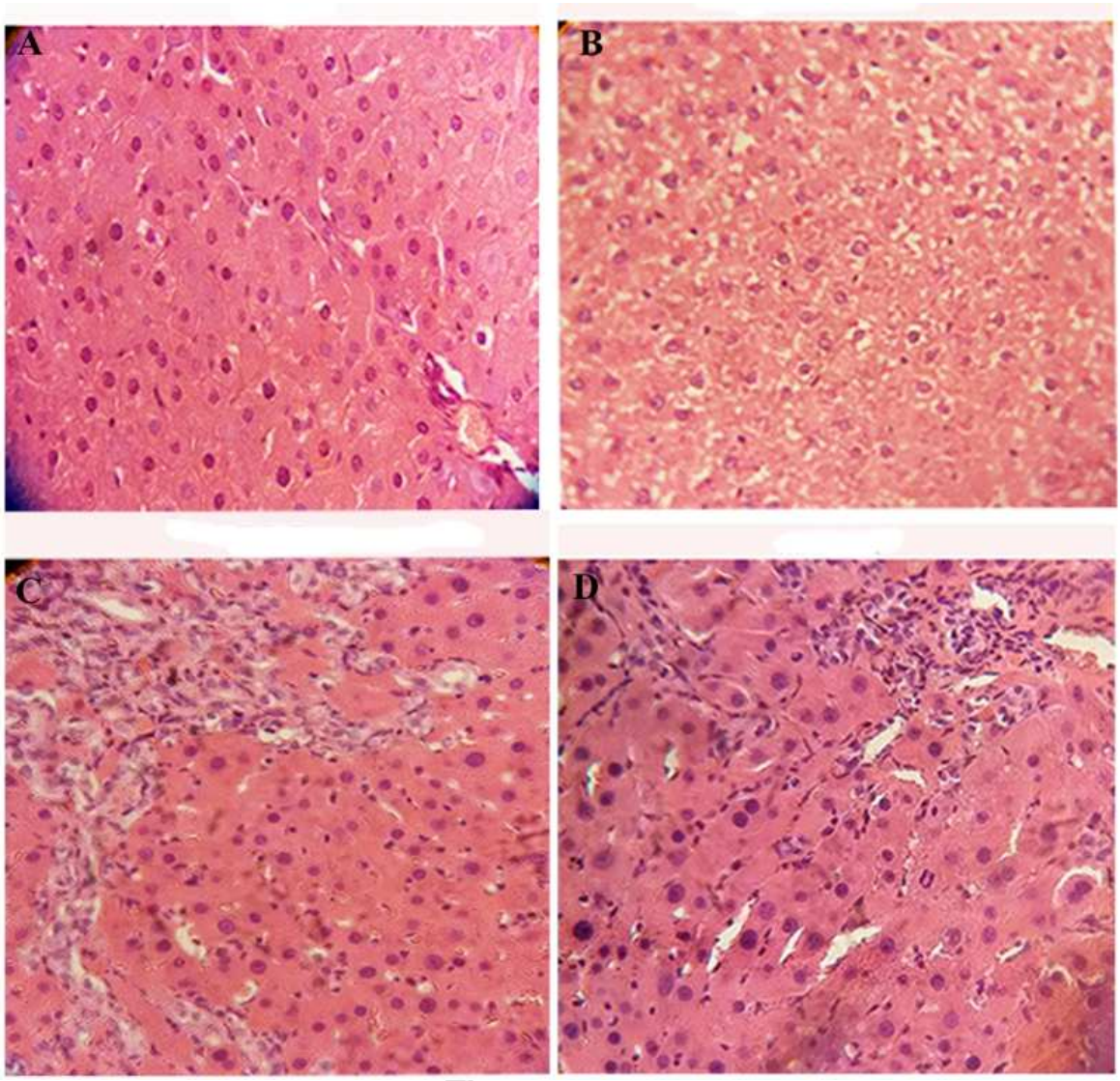
Activity of three liver enzymes (AST, ALT, ALP) in BDL group were increased compared with sham groups (#P <0.05). * Enzymes activity in BDL+Cur group compared with BDL group (*P <0.05). The results are given as mean±S.E.M

Table 2: Comparison of hydroxyproline content and the expression of α-SMA, TGF-β1, and COL 1 in liver tissue of four groups (Sham, sham + Cur, BDL, BDL+ Cur)

	Sham	Sham +Cur	BDL	BDL + Cur
Hydroxyproline (ug/mg tissue)	0.91±0.1	0.84±0.21	#4.24±0.59	*2.54±0.57
α-SMA	3.23±0.09	4.34±0.35	#6.17±0.19	*3.66±0.09
Collagen 1	2.3±0.28	2.61±0.43	#4.06±0.21	*2.22±0.03
TGF-β1	3.98±0.24	3.88±1.04	#6.44±0.04	*3.76±0.51

#P value < 0.05 compared with the Sham group.
*P value< 0.05 compared with the BDL group
The results are given as mean±SD

Fig 1:



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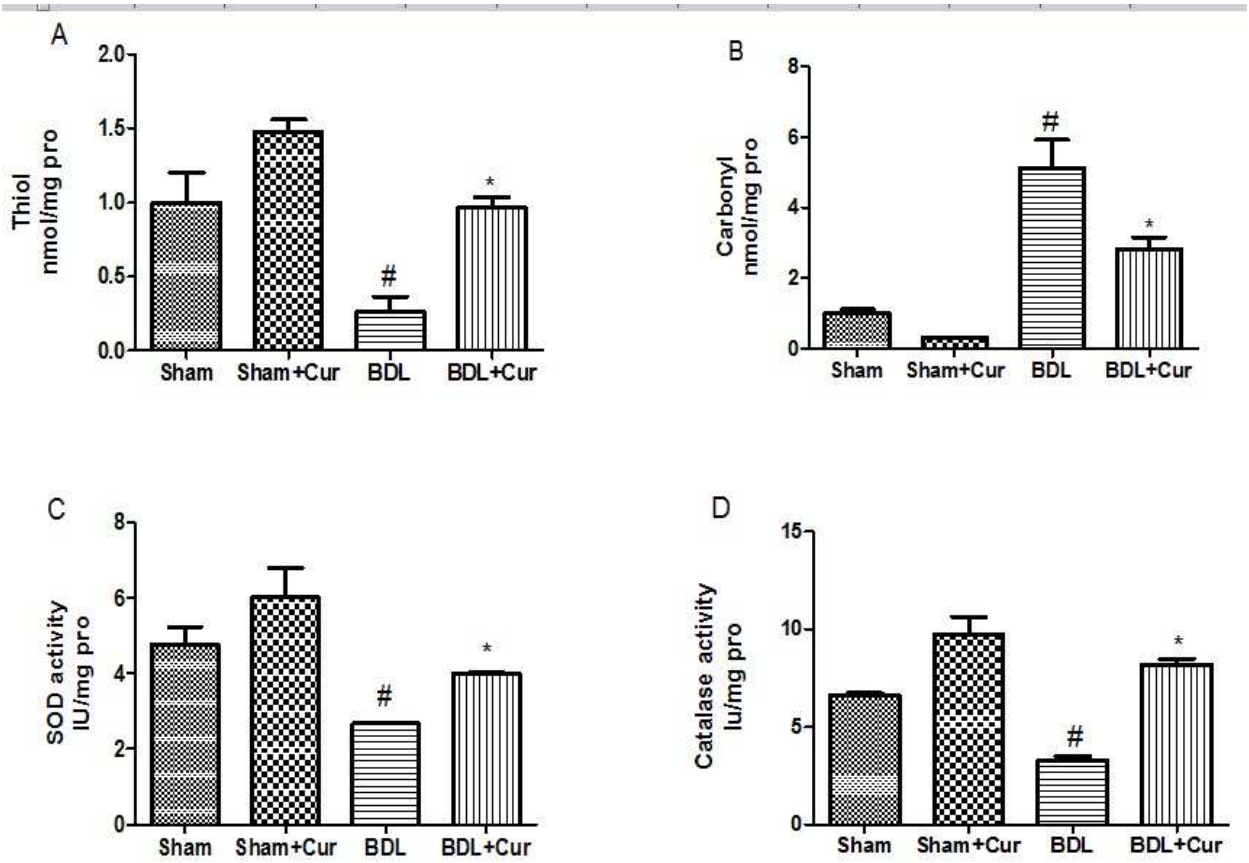
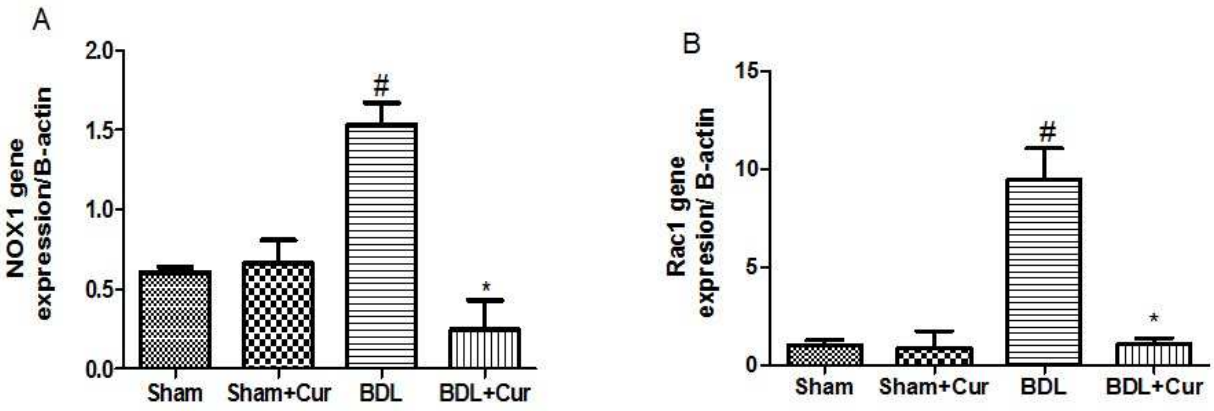
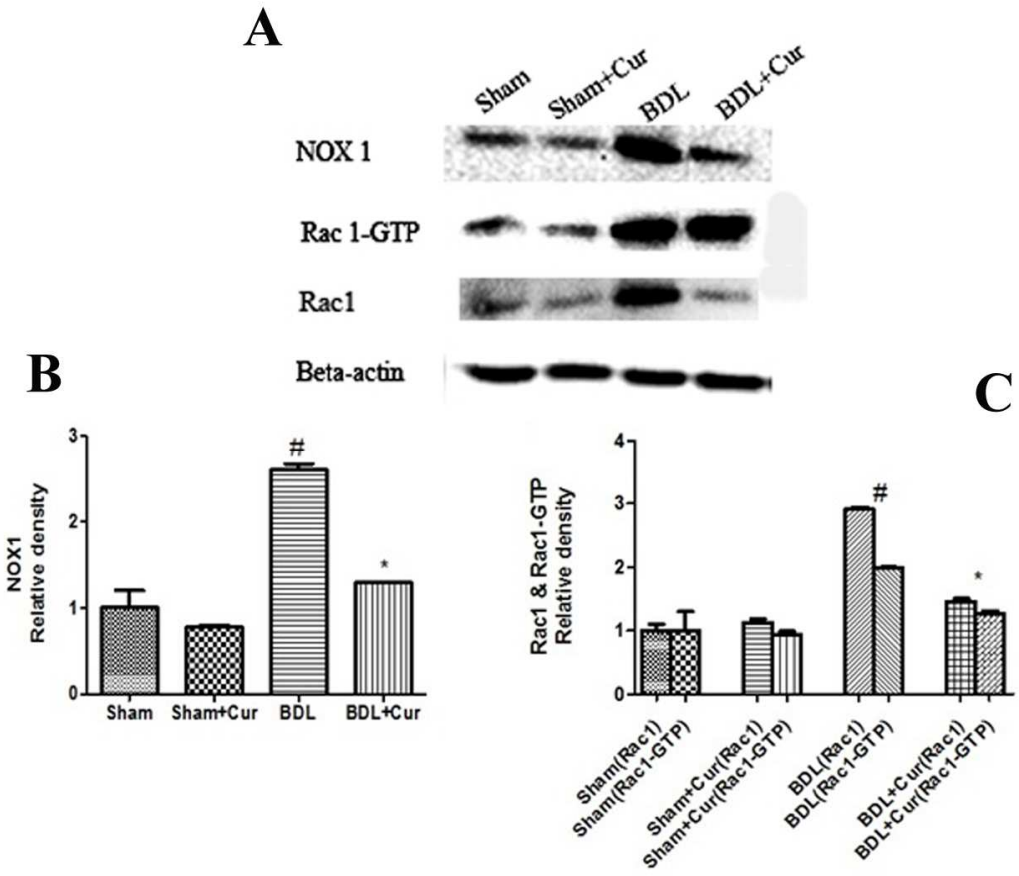


Fig 3:





2

Highlights:

- Hepatoprotective properties of quercetin assessed in BDL rats
- Quercetin treatment of BDL rats attenuated the increased liver injury
- Quercetin treatment of BDL rats reduced enzyme markers of hepatic injury
- Levels of Rac1, Rac1-GTP and NOX1 reduced after quercetin treatment of BDL rats
- Enzyme activity of superoxide dismutase was elevated in quercetin-treated BDL rats
- Enzyme activity of catalase was elevated in quercetin-treated BDL rats
- Quercetin showed hepatoprotective activity against BDL-induced hepatic fibrosis