THYMOQUINONE SUPPLEMENTATION AMELIORATES ACUTE ENDOTOXEMIA-INDUCED LIVER DYSFUNCTION IN RATS

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ABSTRACT

Endotoxemia caused by lipopolysaccharide (LPS) produced an inflammatory condition contributing to multiple organ failure. This study was carried out to investigate the effects of thymoquinone (TQ), the main constituent of *Nigella sativa* seeds, against LPS-induced hepatotoxicity. The obtained data revealed that LPS markedly depleted liver reduced glutathione (GSH) and significantly increased the level of malondialdehyde (MDA) and the activity of caspase-3 enzyme in the liver. Serum tumour necrosis factor-alpha (TNF-α) and bilirubin levels and the activities of alkaline phosphatase (ALP) and gamma-glutamyl transferase (γ-GT) enzymes were markedly increased in LPS-treated rats. TQ supplementation resulted in normalization of liver GSH and decreases in the levels of MDA and caspase-3 activity in the liver with reduction of serum TNF-α, serum total bilirubin and the actvities of ALP and γ-GTenzymes. Histopathological examination revealed that TQ administration improved LPS-induced pathological abnormalities in liver tissues. The present study conclude that TQ reduced acute endoxemia-induced liver dysfunction at least in part by its anti-inflammatory, antiapoptotic and antioxidant activities.

Keywords: Thymoquinone, endotoxemia, inflammation, TNF-α, oxidative stress, liver dysfunction.

INTRODUCTION

Endotoxemia-induced hepatic failure is implicated in high mortality (Ho *et al*., 2008). LPS is an endotoxin of Gram-negative bacteria implicated in organ damage during sepsis (Su, 2002). The liver is the organ that plays the major role in the defense against LPS-toxicity (Hines and Wheeler, 2004). I was found that LPS binds to proteins with subsequent activation of oxygen free radicals and proinflammatory cytokines (Luster *et al*., 1994). Release of these toxic mediators is the contributing factor to most of LPS toxicity in the liver and in the systemic circulation (Hartung and Wendel, 1991). LPS was found to the kupffer cells in the liver with release of cytotoxic agents, inflammatory mediators and reactive oxygen species (ROS) and subsequent severe hepatic damage (Wang *et al*., 2005). Several studies have described the LPS increased the rate of lipid peroxidation in many tissues (Yoshikawa *et al*., 1994). In addition, LPS was reported to induce depletion of hepatic natural reducing agents such as reduced glutathione (GSH) (Jaeschke *et al*., 1993). This effect may be due to consumption of liver GSH or decreased liver GSH synthesis (Kaur *et al*., 2006). Reduction of natural antioxidant might give a hand to ROS attack vital components in liver cells including cell membrane, lipids, proteins and DNA (McKillop and Schrum, 2005). In addition, implication of LPS-induced TNF-alpha in liver cell apoptosis has been documented (Niet *et al*., 2009).

A great number of plant species contain various chemical substances exhibiting health benefit properties, antioxidative, anti-inflammatory and mainly anti anti-microbial effects, and their preventive and therapeutic use is increasing (Juhás *et al*., 2008).TQ is the major volatile oil of *Nigella sativa* seeds (Aboutabl *et al*., 1986). Although there is no studies intercorrelate the effect of TQ supplementation on endotoxemia-induced liver toxicity, the prophylactic effect of TQ against CCl_4-inducd hepatotoxicity has been documented (Burits and Bucar, 2000; Enomoto *et al*., 2001). In addition, TQ administration was reported to counteract the nephrotic hyperlipidemia and hyperproteinuria; and normalizes the values of oxidative stress markers (Badary *et al*., 2000). Oral administration of TQ in water before and during ifosfamide administration in rats minimized ifosfamide-induced renal damage (Badary, 1999). Furthermore, the protective effect of TQ against genotoxicity produced by infection has been reported (Aboul-Ela, 2002).

The effects of TQ in endotoxemia-induced liver dysfunction have not been studied till now. The present work was designed to study the role of oral supplementation of TQ in protection against LPS-induced hepatotoxicity in rats.

MATERIALS AND METHODS

Animals

Adult male Wistar albino rats weighing 150–200 g were obtained from the Animal Care Center, College of Pharmacy, King Saud University, Riyadh, Saudi Arabia. Rats were housed separately in metabolic cages under standard conditions (12 h light/ dark cycle at 25 ± 2°C)

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with free access to standard rat pellet food and tap water.

Experimental protocol

Rats were classified into four groups (10 rat/ group): Control group without any treatment. TQ-treated group in which rats administered. TQ orally in water (10 mg/ kg) daily for 7 days. The third group administered a single dose (5 mg/ kg) of LPS at day 6 from the start of the experiment. In the fourth group, rats received TQ orally 10 mg/ kg daily, and one dose of LPS at day 6 from the start of the experiment. Animals in all groups were anaesthetized with ether and sacrificed 24 h after LPS injection. Blood samples were collected separately from each animal and livers were dissected out on ice. Serum was used for evaluation of the level of the inflammatory cytokine tumour necrosis factor-alpha (TNF-α) and liver function parameters including serum total bilirubin and the activities of alkaline phosphatase (ALP) and gamm-glutamyl transferase (γ-GT). The levels of GSH and MDA as well as the activity of caspase-3 enzyme were assessed in liver homogenates. In addition, hispathological examination of liver sections was carried out.

Determination of serum total bilirubin

Total bilirubin in the serum was measured by using the kit supplied by Spinreact, S.A (Ctra. Santa Coloma, Spain**)** where serum samples (100 µl) were mixed with reaction mixture composed of 1.5 ml R1 reagent (sulfanilic acid, 30mmol/L, hydrochloric acid, 50mmol/L, and Dimethyl-suphoxide, 7 mmol/L). The samples were incubated at room temperature for 5 minutes then absorbances were read at 555 nm against blank. The level of total bilirubin was calculated from the following equation:

Total Bilirubin (mg/dL) = Sample (Ab) □ Blank (Ab) × 19.1

Determination of alkaline phosphatase (ALP) activity

ALP activity in the serum was determined by using the commercial kit supplied by Spinreact, S.A (Ctra. Santa Coloma, Spain**)** where 20 µl of serum sample was added to1.2 ml of working buffer composed of 19 volumes of R1 buffer (1mmol/L diethanolamine, pH 10.4 + 0.5 mmol/L magnesium chloride) and 1 volume of substrate solution (10 mmol/l of ρ-nitrophenylphosphate) in1 mL cuvette, mixed gently and incubated for 1 minute at 25^{o}C. Absorbance for each sample was read against blank at 1 minute intervals for 3 minutes in spectrophotometer at 405 nm. The activity of ALP was calculated as follows:

ALP activity (U/L) = (ΔA/min) × 3300**.**

Determination of serum gamma-glutamyl transferase (γ-GT) activity

Serum *γ-GT* activity was measured by using the kit supplied by Spinreact, S.A (Ctra. Santa Coloma, Spain**)** where 100 µl of serum sample was added to 1ml of working buffer composed of 19 volumes of R1 buffer consists of Tris, pH 8.6 (100 mmol/L) and glycylglycine (100 mmol/L) and 1 volume of substrate solution (L-γ-glutamyl-3-carboxy-4-nitroanilide, 3 mmol/L) in 1ml cuvette, mixed gently and incubated for 1 minute at 25^{0}C. Absorbance for each sample was read against blank at 1 minute intervals for 3 minutes in spectrophotometer at 405 nm. The activity of γ-GT was calculated from the following equation:

$$\gamma\text{-GT (U/L)} = (\Delta A/\text{min}) \times 1190.$$

Determination of liver GSH content

Reduced glutathione (GSH) was determined I liver tissue according to the method of Ellman (1959). In brief, in a centrifuge tube 0.5 ml of liver homogenate added to 0.5 ml of 5% trichloroacetic acid then centrifuged at 1000xg for 5 min. 200 µl of the supernatant was added to 1750 µl of 0.1 M potassium phosphate buffer (pH 8) and 50 µl of 5, 5 dithiobis-2-nitrobenzoic acid (DTNB) reagent in a tube and mixed thoroughly. The produced yellow color was measured at 412 nm in a spectrophotometer. GSH in each sample (µmol/g tissue) was calculated from standard curve of reduced glutathione.

Assay of liver MDA content

The level of MDA in the liver was determined according to the method of Uchiyama and Mihara (1978). In brief, 5% homogenate of liver a buffer composed of 50 mmol/L phosphate buffer (pH 7.0) and 0.1 mmol/L EDTA. Then 2.5 ml of 20% acetic acid and 1 ml TBA solution were added to the tissue homogenate in a Teflon-covered test tube, and boiled for 30 min in a water bath then cooled under tap water. For each sample, 4 ml *n*-butanol was added then samples were shaken vigorously and centrifuged at 600 *g* for 5 min. The upper coloured layer was aspirated where the absorbance was measured at 532 nm in a spectrophotometer. MDA level (nmol/mg protein) was calculated from a standard calibration curve.

Determination of liver caspase-3 activity

Cell suspension was first prepared from liver tissue according to the method described by Sameto (1990). Briefly, in a Petri dish, liver from each animal was cut into small pieces and suspended in 10 ml sterile saline solution containing 0.25% v/v trypsin. The suspension was then transferred to screw-capped Erlenmeyer flask using glass pipette and put on a rotary shaker (80 r.p.m. for 15 min at 37^{o}C). 10 ml serum supplemental medium (SSM) containing 10% v/v fetal bovine serum (FBS) was added to stop trypsinization. The suspension was then aspirated and filtered through Nitex 210 then Nitex 130 filters (TetKo, Elmeford, NY, USA) by gravity then centrifuged at 800 *g* for 5 min at room temperature. Viable cells were counted by haemocytometer after addition of trypan blue 0.4% in phosphate-buffered saline containing 1 mmol/L EDTA (1:16).

Liver caspase-3 activity was measured by using Caspase-3/CPP32 activity Colorimetric Assay Kit (Biovision Incorporate, USA) according the procedure supplied by the manufacturer. Briefly, the prepared cell suspension was` centrifuged. The produced cell pellets were resuspended in chilled cell lysis buffer (1-5x10^6 cells/50 µl) and incubated on ice for 10 minutes, centrifuged for 1 min in a microcentrifuge (10,000 x g). Protein concentration was measured in the supernatant. From each sample (cytosolic extract), aliquots equivalent to 50-200 µg protein were diluted in 50 µl cell lysis buffer followed by addition of 50 µl of 2X reaction buffer (containing 10 mM DTT). 5 µl of the 4 mM DEVD-*p*NA substrate solution (200 µM final conc.) was added to each sample and incubated at 37°C for 2 h. Samples were transferred to microtiter plate and the absorbance was read at 405 nm in ELISA reader.

Determination of serum TNF-α

Serum TNF-α, was assayed by ELISA using R&D Systems kit (Minneapolis, MN, USA). Briefly, 50 µl of the assay diluent added to each well in a microplate. Then 50 µl of the diluted serum sample (2-fold dilution in the calibrator diluent) were added to each well and mixed gently for 1 min then the plate was covered and left for 2 h at room temperature. After incubation, each well in the plate was then aspirated and washed 5 times with washing buffer. 100 µl substrate solution was added and placed for 30 min at room temperature in dark. The optical density was then measured at 450 nm using 96-well plate spectrometer (Spectra Max 190, Molecular Devices Corp., Sunnyvale, CA, USA). TNF-α level (pg/ml) was calculated from a standard calibration curve and multiplied by the dilution factor.

Assay of total protein in the liver

Tissue content of protein was measured according to the method of Lowery *et al* (1951). In brief, alkaline copper solution (1.0 ml) prepared by mixing 50 ml of 2 % Na_2CO_3 in 0.10 N NaOH with 1.0 ml of 0.50% $CuSO_4$. 5 H_2O in 1% sodium tartrate was added to 0.20 ml of 10% tissue homogenate (w/v) to a test tube. 10 min after mixing, at room temperature, Folin's reagent (phospho-molybdic-phosphotungstic reagent diluted 1:1 in H_2O) was prepared and 0.10 ml of this reagent was added to each tubes and mixed well. The optical density was measured After 30 min at 500 nm against blank. Protein concentration was calculated from a standard calibration curve by using bovine albumin.

Histological examination of liver sections

Liver sections fixed in10% formalin saline solution were placed in paraffin and cut into 5 µm sections. The sections were stained on glass slides by Hx & E and examined under light microscope.

Statistical analysis

Data are presented as mean values ± SEM (n=10).Statistical analysis carried out by Graph Pad Prism4 software using one way ANOVA and Tukey Kramer post-ANOVA test at $P<0.05$.

RESULTS

Liver function parameters

The present data show that bilirubin serum level and the activities of alkaline phosphatase (ALP) and gamma-glutamyl transferase (γ-GT) enzymes as markers of liver function were assessed. Data in table 1 explain that administration of LPS (5mg/kg, i.p, once) resulted in marked increases in serum total bilirubin as well as the activities of serum ALP and γ-GT enzymes amounted to extents of 366%, 182% and 274%, respectively, of the corresponding control values. Administration of TQ daily dose orally (10 mg/ kg) in water for 7 consecutive days for the group administered LPS (5mg/kg, i.p, once at day 6) resulted in significant reductions in serum bilirubin level and the activities of ALP and γ-GT if compared with LPS-treated animals to be 262%, 131% and 178% respectively of the corresponding control values.

Liver reduced glutathione (GSH) content

In the present study, LPS administration (5mg/kg, i.p, once) resulted in significant depletion of liver GSH (54%) compared to the corresponding control value. Administration of 10 mg/ kg TQ as an oral daily dose in water for 7 days as well as LPS (5 mg/kg, i.p, once at the 6th day) markedly increased liver GSH content as it reached normal levels (fig. 1).

Liver malondialdehyde (MDA) content

By evaluation of MDA (as indicator of lipid peroxides formation) in rat liver , the obtained data (fig. 2) explain that LPS administration (5mg/kg, i.p, once) into rats markedly increased the level of liver MDA (161%) compared to the corresponding control animals. However animals administered both TQ (10 mg/ kg, p.o daily) in drinking water for 7 consecutive days and injected with a single dose of LPS (5mg/kg, i.p) at the 6th day showed marked reduction in liver MDA content in comparison with only LPS-administered animals but still at a higher level of significance (122%.) if compared to the control group.

Liver caspase-3 activity

The activity ofcaspase-3 enzyme was `measured in the present study as an apoptotic marker. Fig. 3 shows that caspase-3 enzyme activity in the liver was increased by one fold in rats administered LPS (5mg/kg, i.p, once) when compared to the control values. TQ administration (10 mg/ kg, p.o daily) in water for 7 consecutive days to LPS – treated animals (5mg/kg, i.p, once at day 6) showed markedly reduced level of caspase-3 enzyme

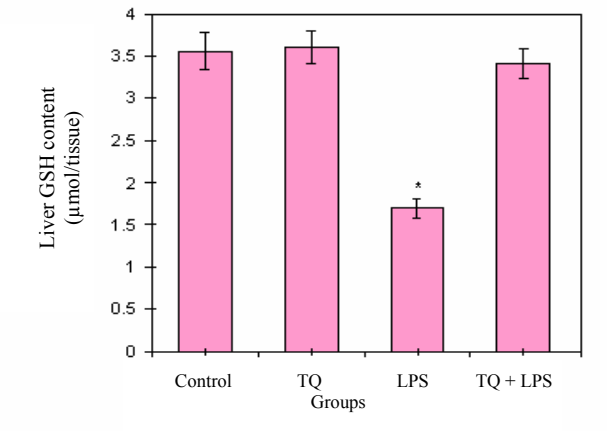

Fig. 1: Effect of lipopolysaccharide (LPS) administration (5mg/kg, i.p, once) with or without thymoquinone (TQ) administration (10 mg/ kg daily, p.o) on the level of reduced glutathione (GSH) in rats.
Data are presented as mean ± SEM (n = 10) and analyzed using Graph Pad Instat software by one way ANOVA followed by Tukey Kramer post-test.
*: Indicate significant difference from control group at $p<0.05$.

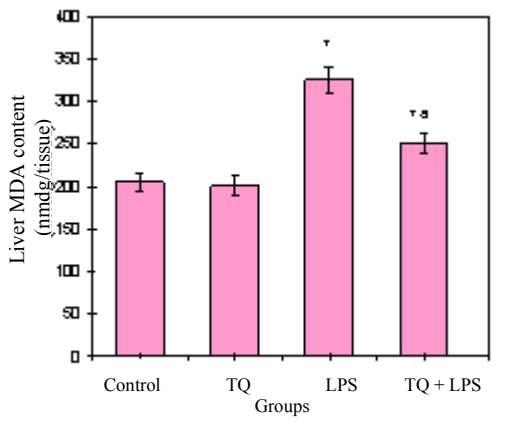

Fig. 2: Effect of lipopolysaccharide (LPS) administration (5mg/kg, i.p, once) with or without thymoquinone (TQ) administration (10 mg/ kg daily, p.o) on the level of malondialdehyde MDA) in rats.
Data are presented as mean ± SEM (n = 10) and analyzed using Graph Pad Instat software by one way ANOVA followed by Tukey Kramer post-test.
*, [a]: Indicate significant differences from control and LPS-treated animals respectively at $p<0.05$.

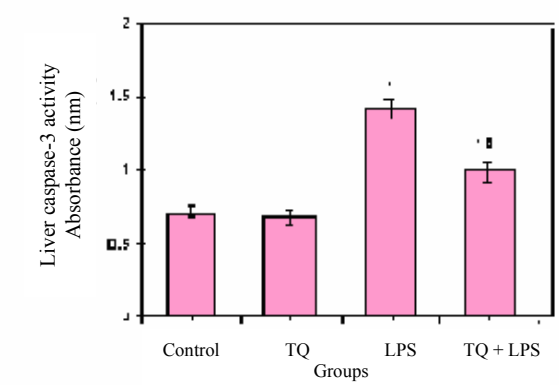

Fig. 3: Effect of lipopolysaccharide (LPS) administration (5 mg/kg, i.p, once) with or without thymoquinone (TQ) administration (10 mg/ kg daily, p.o) on the activity of caspase-3 enzyme in the liver of rats.
Data are presented as mean ± SEM (n = 10) and analyzed using Graph Pad Instat software by one way ANOVA followed by Tukey Kramer post-test.
*, [a]: Indicate significant differences from control and LPS-treated animals respectively at $p < 0.05$.

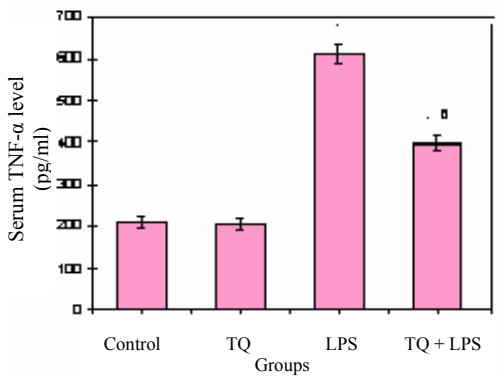

Fig. 4: Effect of lipopolysaccharide (LPS) administration (5 mg/kg, i.p, once) with or without thymoquinone (TQ) administration (10 mg/ kg daily, p.o) on the level of serum tumour necrosis factor (TNF-α) in rats.
Data are presented as mean ± SEM (n = 10) and analyzed using Graph Pad Instat software by one way ANOVA followed by Tukey Kramer post-test.
*, [a]: Indicate significant differences from control and LPS-treated animals respectively at $p < 0.05$.

activity in comparison with LPS-treated group although it was still at a higher level (140%) in comparison with the corresponding control group.

Serum tumour necrosis factor-alpha (TNF-α)

Data in fig. 4 explain that LPS (5mg/kg, i.p) as a single dose significantly increased serum TNF-α level (up to 3 folds) compared to the corresponding control values. In rats Co-administered TQ (10 mg/ kg, p.o daily) in water for 7 consecutive days and LPS (5mg/kg, i.p, once at day 6) significantly reduced serum TNF-α level to be less than 2 folds of the control values.

Histopathological examination of liver sections

Hx & E staining and microscopic examination (40X) of liver sections from different groups (fig. 5) explains that liver sections from control and thymoquinone (TQ)-treated animals showed normal morphological and histological features without inflammation or necrosis. Liver sections from rats treated with LPS showed obvious

infiltration of inflammatory cells along with necrotic damages. However, livers from the group of rats treated with TQ+LPS appeared with markedly improved histological features with reduced infiltration of inflammatory cells and necrotic damage.

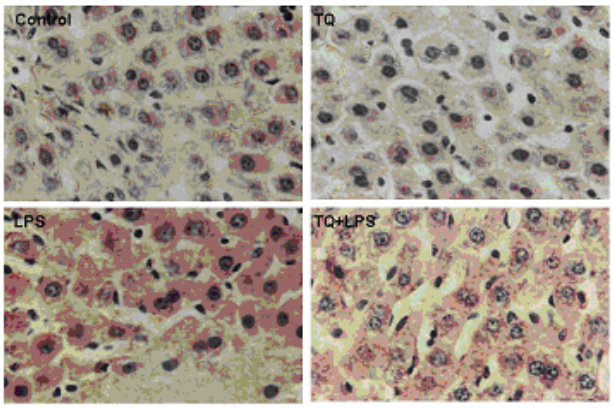

Fig. 5: A photomicrograph of liver sections stained with Hx & E (X 40) from control rats, thymoquinone-treated rats (TQ), LPS-treated rats (LPS) and TQ-treated-LPS-treated rats (TQ+LPS) explains that administration of LPS resulted in histological alterations in liver sections manifested as increased infiltration of inflammatory cells with necrotic compared with either control or TQ-treated animals (TQ). Animals treated with TQ before and during LPS administration show obviously improved histological features with decreased level of inflammation and necrosis.

DISCUSSION

The current study aimed to find whether TQ can provide protection against LPS-induced hepatic toxicity in rats. The obtained data show that LPS administration resulted in marked decrease in liver GSH content along with increased hepatic level of MDA and the activity of caspase-3 enzyme. Also, serum levels of TNF-α and total bilirubin as well as the activities of ALP, γ-GT enzymes were significantly increased by LPS administration. The toxic effect of LPS was further proved by histo-pathological changes in liver sections in the form of increased number of inflammatory cells and necrotic damage. The obtained findings are in harmony with previous studies showed that LPS caused severe damage in different tissues, including liver, because of the increased reactive oxygen intermediates and increased lipid peroxidation (Kono *et al*., 2003; Matsuda *et al*., 1998). The present data are also supported by the work of Kuar *et al*. (2006) who reported that LPS elevated serum levels of the activities of liver function enzymes and bilirubin. The authors explained that elevation of liver enzymes was accompanied with a marked increase in MDA level but decreases in GSH and SOD. The increased serum level of TNF-α and hepatic caspase-3 activity in the present study in response to LPS administration is supported by the fact that inflammatory cytokines were reported to be released by macrophages and neutrophils due to stimulation by endotoxin, and some Gram-positive bacteria via activation of Kupffer cells (Beutler and Cerami, 1988). In addition, it was explained that depletion of GSH lead to activation of TNF-α-induced cell death in mouse hepatocytes in the absence of transcription inhibitor. This can suggest the role of reactive oxygen species (ROS) in TNF-α-induced liver injury (Nagai *et al*., 2002). Recently, it has been explained that LPS induced TNF-α and activated NF-κB-mediated cell death in the murine macrophage cells (Liu *et al*., 2008). Moreover, the very recent study of Ni *et al*. (2009) documented that LPS induced liver cell apoptosis by TNF-α-activated mechanism.

TQ supplementation in the present work could alleviate hepatic toxicity induced by LPS in the form of normalization of GSH hepatic level and reduction of liver function parameters. Also, LPS-induced hepatic lipid peroxides formation (in the form of MDA) and apoptosis (indicated by hepatic caspase-3 activity) were markedly reduced in rats received TQ along with LPS. In addition serum TNF-α and inflammatory changes in the liver section were markedly reduced in rats co-administered

Table 1: Effect of lipopolysaccharide (LPS) administration (5mg/kg, i.p, once) with or without thymoquinone (TQ) administration (10 mg/ kg daily, p.o) on serum bilirubin level and the activities of alkaline phosphatase (ALP) and gamma-glutamyl transferase (γ-GT) enzymes in rats.

Groups	Serum bilirubin level (mg/dL) Mean ± SEM	Serum ALP activity (U/L) Mean ± SEM	Serum γ-GT activity (U/L) Mean ± SEM
Control	0.48 ± 0.02	153.18 ± 4.26	6.68 ± 0.39
TQ	0.51 ± 0.03	148.12 ± 7.63	7.10 ± 0.525
LPS	1.76 ± 0.09*	279.15 ±6.50*	18.32 ± 0.85*
TQ + LPS	1.26 ± 0.08*[a]	201.30±4.18*[a]	11.92 ± 0.67*[a]

Data are presented as mean ± SEM (n = 10) and analyzed using Graph Pad Instat software by one way ANOVA followed by Tukey Kramer post-test.
*, [a]: Indicate significant differences from control and LPS-treated animals respectively at $p < 0.05$.

TQ and LPS in comparison with LPS-treated animals. Although previous studies did not explain the role of TQ in sepsis-induced liver toxicity, the obtained effects of TQ could be explained by the previous findings showed that *N. sativa* seed extract provides protection for erythrocytes from H_2O_2 toxicity (Suboh *et al*., 2004). TQ also significantly protected isolated hepatocytes from tert-butyl hydroperoxide induced toxicity and restores the levels of ALT and ALP (Daba and Abdel-Rahman, 1998). In addition, it has been reported that mice treated with TQ then 1 h later with CCl_4 showed reduced hepatotoxicity from CCl_4 indicated by significant reduction in the levels of serum enzymes along with a significant increase in GSH content (Burits and Bucar, 2000). Moreover, TQ was found to counteract cisplatin-induced nephrotoxicity (Badary, 1997), ifosfamide Fanconi syndrome (Badary, 1999), doxorubicin cardiotoxicity (Al-Shabanah *et al*., 1998) and histamine release (Chakravarty, 1993) and normalized the oxidative stress parameters (Badary *et al*., 2000). The reduced level of serum TNF-α and inflammation in liver sections in rats co-treated with TQ and LPS in the present work agrees the previous study of Tekeoglu *et al*. (2006) who reported the TQ anti-inflammatory effects on experimentally-induced arthritis in rats where decreased levels of TNF-α and IL-1β in circulation were observed. Similarly, Mohamed *et al*. (2005) showed that treatment of rats with thymoquinone at 1 mg/kg/day resulted in prevention of experimental autoimmune encephalomyelitis due to its antioxidant and anti-inflammatory properties (Salem, 2005).

In conclusion, the present data suggest that TQ supplementation could provide hepatoprotection against LPS-induced liver toxicity mediated at least in part by its antioxid and anti-inflammatory mechanisms which may be crucial in reduction of sendotoxemia-induced liver dysfunction.

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