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## **Effect of Thymoquinone on Ethanol-Induced Hepatotoxicity in Wistar Rats**

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The aim of the present study is to investigate the protective effects of thymoquinone on ethanol induced hepatotoxicity in Wistar rats. The rats were pretreated orally (using rats feeding needle) with different doses (5, 10 and 20 mg kg<sup>-1</sup> body weight) of TQ for 7 days and then ethanol (7.5 g kg<sup>-1</sup>) was injected. Silymarin was used as standard hepato-protective agent for comparison. The activities of Aspartate Aminotransferase (AST), Alanine Aminotransferase (ALT), Acid Phosphatase (ACP) and Alkaline Phosphatase (ALP) and levels of proinflammatory cytokines (IL-6 and TNF- $\alpha$ ) were estimated in serum. Malondialdehyde (MDA), Glutathione (GSH), Superoxide Dismutase (SOD), Catalase (CAT), nucleic acids (DNA and RNA) and Total Proteins (TP) levels were estimated in liver. It has been observed that pretreatment with TQ at 10 and 20 mg kg<sup>-1</sup> of body weight respectively could able to minimize the toxicity in compare to ethanol treated group as revealed by the different enzymatic assay in serum. Serum cytokines, IL-6 and TNF- $\alpha$  levels also significantly decreased in TQ treated groups compared to ethanol group. SOD, CAT, GSH, DNA, RNA, TP levels were decreased and MDA was increased significantly in liver of ethanol treated rats. Pretreatment with TQ protected dose dependently the damage-induced in rats liver by the ethanol injection. In conclusion, TQ protects against the liver injury caused by ethanol administration. In view of its antioxidative nature, it may be developed as an effective therapeutic agent for alcohol-induced liver disease by its antioxidative stress and anti-inflammatory features.

**Key words:** Thymoquinone, ethanol, oxidative stress, cytokines, nucleic acids

## INTRODUCTION

Alcohol abuse and alcoholism are serious current health and socioeconomic problems throughout the world. Despite great progress made in the field, the development of suitable medications for the treatment of alcoholism remains a challenging goal for alcohol research. Alcoholic Liver Disease (ALD) is the common consequence of prolonged and heavy alcohol intake. The fatal changes in the liver include fatty liver, hepatitis and hepatic cirrhosis (Seitz *et al.*, 2005). Ethanol is a fat-soluble non-electrolyte, which is readily absorbed from the gastrointestinal tract, diffuses rapidly into circulation and is distributed uniformly throughout the body (McDonough, 2003). Ethanol is almost exclusively metabolized in the body by enzyme catalyzed oxidative processes. The acetaldehyde formed further oxidized to acetate, which is then converted to carbon dioxide via the citric acid cycle (Albano, 2006). Ethanol can also cause auto-oxidation of the hepatic cells either by acting as a pro-oxidation of the hepatic cells or by reducing the antioxidant levels resulting in marked hepatotoxicity (Crawford and Balakenhoan, 1991). Hepatic oxidative stress of the resulting lipid peroxidation has been identified as playing a pathogenic role in alcoholic liver disease (Bunout, 1999).

Thymoquinone active constituent of *Nigella sativa* is pharmacologically active quinine, which possesses several properties including analgesic and anti-inflammatory actions (Abdel-Fattah *et al.*, 2000; Houghton *et al.*, 1995), protection against chemical induced carcinogenesis (Worthen *et al.*, 1998) and the inhibition of eicosanoids generation (Houghton *et al.*, 1995). Furthermore, it has been reported that TQ prevents oxidative injury in hepatocytes induced by CCl<sub>4</sub> or tert-butyl hydroperoxide in various *in vitro* (Daba and Abdel-Rahman, 1998) and *in vivo* (Mansour *et al.*, 2001) hepatotoxicity models, as well as acetic acid-induced colitis in rats (Mahgoub, 2003). It has been prevented the membrane lipid peroxidation in hepatocytes (Mansour *et al.*, 2002). In a recent El Gazzar *et al.* (2006) showed that, TQ attenuated pulmonary inflammation in a mouse model of allergic asthma by decreasing Th2 cytokines and inflammatory cell infiltration in the lung. In another recent study, El Gazzar *et al.* (2007) reported that, TQ attenuates the proinflammatory response in LPS-stimulated mast cells by modulating nuclear transactivation of NF- $\kappa$ B and TNF- $\alpha$  production.

The protective action of antioxidant is usually due to the inhibition of free radical-induced chain reaction and the resultant prevention of peroxidative deterioration of structural lipids in membranous organelles. Circulating enzymic and non-enzymic antioxidants such as

superoxide dismutase, catalase, cytokines and reduced glutathione play an important role in alleviating tissue damage due to the formation of free radicals (Thampi *et al.*, 1991). The present study aims to investigate the protective effect of TQ on toxic damage induced by ethanol in Wistar male rats.

## MATERIALS AND METHODS

**Animals:** The present study was designed and studied during May 2007. Male albino rats, weighing 160-180 g were obtained from Experimental Animal Care Center, College of Pharmacy, King Saud University, Riyadh. They were housed in a temperature controlled room (25 $\pm$ 2°C), humidity 50-55% and with 12 h light/dark cycles for at least 1 week. After the stabilization period, rats were randomly divided into 6 groups (six rats in each group): (1) Control (vehicle), (2) Ethanol (7.5 g kg<sup>-1</sup>), (3) TQ (5 mg kg<sup>-1</sup>) + Ethanol, (4) TQ (10 mg kg<sup>-1</sup>) + Ethanol (5) TQ (20 mg kg<sup>-1</sup>) + Ethanol and Silymarin (10 mg kg<sup>-1</sup>) + Ethanol. TQ and silymarin were suspended in 0.8% (v/v) Tween 80 and administered orally by using rat feeding needle on every alternate day for 7 days period of study. Animals in groups 1 and 2 were received the same amount of vehicle. The rats in groups 2 to 6 were injected 25% ethanol (7.5 g kg<sup>-1</sup> of body weight) intraperitoneally (ip.) 30 min after pretreatment with different doses of TQ, single dose of Silymarin (SL) and vehicle. All rats were kept on free access to standard rat pellet and drinking water.

At the end of the study time, all animals were anaesthetized using ether, blood samples were collected through cardiac puncture. Immediately liver was dissected, dipped in liquid nitrogen for a minute and kept them in deep freezer at -70°C till analysis. Blood samples were centrifuged at 3000 rpm for 10 min, the serum samples were kept in freezer at -20°C till analysis.

**Enzymatic assays for serum:** Aspartate transaminase (AST), Alanine Transaminase (ALT), Acid Phosphatase (ACP) and Alkaline Phosphatase (ALP) concentrations were estimated in serum by using commercially available diagnostic kits (Randox diagnostic reagents, Randox Laboratories, USA).

**Estimation of TNF- $\alpha$  and interleukin-6 (IL-6) in serum:** Serum TNF- $\alpha$  concentration was estimated by using the CYTELISA rat TNF- $\alpha$  obtained from CYTIMMUNE Sciences Inc., Maryland, USA. All samples were assayed in duplicate. The intra assay variation was 6.7%. To avoid inter assay variation all samples were run at one time. Optical density of each well was determined by using a microplate reader (Thermo Labsystems, Finland).

Serum IL-6 was estimated by using the ELISA Quantikine rat IL-6 immunoassay kit obtained from R and D Systems Inc., Minneapolis, MN, USA. The samples were assayed in duplicate. The intra assay variation was 5.5%. To avoid inter assay variation all samples were run at one time. Optical density of each well was determined by using a microplate reader (Thermo Labsystems, Finland).

**Estimation of MDA in liver:** The method described by Ohkawa *et al.* (1979) was used. Malondialdehyde (MDA) was measured as an indicator of lipid peroxidation. Liver tissues were homogenized in KCl solution and incubated with thiobarbituric acid. After centrifugation the pink clear layer was read at 532 nm. Malondialdehyde bis (dimethyl acetal) was used as an external standard.

**Estimation of GSH in liver:** Glutathione concentration was assayed using the method of Sedlak and Lindsay (1968). A cross sectional piece of liver tissues (200 mg) were dissected and homogenized in ice-cold 0.02 M ethylenediaminetetraacetic acid (EDTA). Aliquots of 0.5 mL of the tissue homogenates were mixed with 0.2 M Tris buffer, pH 8.2 and 0.1 mL of 0.01 M Ellman's reagent, [5,5'-dithiobis-(2-nitro-benzoic acid)] (DTNB). Tubes were centrifuged at 3000 g at room temperature for 15 min. The absorbance of the clear supernatants was read in a spectrophotometer at 412 m $\mu$  in 1 cm quartz cells. The concentrations were estimated by using the standard curve.

**Estimation of SOD activity in liver:** Superoxide dismutase activity in liver was assayed spectrophotometrically (560 nm) by the method described by Kakkar *et al.* (1984) using nitroblue tetrazolium as the indicator reagent.

**Assay procedure for catalase (CAT) activity in liver:** The catalase activity was measured by the method of Abei (1988) using hydrogen peroxide as substrate. The disappearance of H<sub>2</sub>O<sub>2</sub> was followed at 240 nm. The activity was expressed as  $\mu\text{mole}^{-1} \text{min}^{-1} \text{mg}^{-1}$  protein using the extension coefficient of 0.0436 mM<sup>-1</sup> mg<sup>-1</sup>.

**Estimation of total proteins and nucleic acids:** Total proteins were estimated by the modified Lowry method of Schacterle and Pollack (1973). Bovine serum albumin was used as standard. The method described by Bregman (1983) was used to determine the levels of nucleic acids. Tissues were homogenized and the homogenate was suspended in ice-cold Trichloroacetic Acid (TCA). After centrifugation, the pellet was extracted with ethanol. DNA was determined by treating the nucleic acid extract with diphenylamine reagent and reading the intensity of blue color at 600 nm. For quantification of RNA, the nucleic acid extract was treated with orcinol and the green colour was read at 660 nm. Standard curves were used to determine the amounts of nucleic acids present.

**Statistical analysis:** All data are presented as the mean $\pm$ SE. The data were evaluated by a one-way ANOVA using SPSS program and the differences between the means assessed using Student's t-test.

## RESULTS

Ethanol treatment to control rats caused significant increase in AST, ALT, ALP and ACP activities compared to the control group of rats. Pretreatment of rats with TQ showed significant protective activity in AST, ALT and ACP at higher doses (10 and 20 mg kg<sup>-1</sup>) and in ALP only at high (20 mg kg<sup>-1</sup>) compared to the ethanol supplemented rats. SL treatment (10 mg kg<sup>-1</sup>) also showed protection against the enzymatic activities induced by ethanol treatment (Table 1).

Both the cytokines, TNF- $\alpha$  and IL-6 levels in serum (pg mL<sup>-1</sup>) were found significantly higher (p<0.001) in ethanol supplemented rats compared to the experimental control rats. Such increased levels of TNF- $\alpha$  and IL-6 were significantly restored dose dependent manner in rats treated with TQ. Silymarin (10 mg kg<sup>-1</sup>) treatment showed significant protection against the elevated TNF- $\alpha$  and IL-6 levels induced by ethanol challenge (Table 2).

**Table 1: Effect of THQ on different biochemical parameters in serum of rats**

Treatments (mg kg <sup>-1</sup> day <sup>-1</sup> )	AST (U L <sup>-1</sup> )	ALT (U L <sup>-1</sup> )	ALP (U L <sup>-1</sup> )	ACP (U L <sup>-1</sup> )
Control (Vehicle)	23.62 $\pm$ 1.32	20.61 $\pm$ 1.13	318.34 $\pm$ 11.06	91.15 $\pm$ 2.77
Ethanol	46.90 $\pm$ 3.38***	41.47 $\pm$ 3.31***	390.20 $\pm$ 17.36**	132.71 $\pm$ 6.40***
THQ (5)+Ethanol	40.85 $\pm$ 2.30	34.98 $\pm$ 1.56	384.95 $\pm$ 12.41	124.82 $\pm$ 6.45
THQ (10)+Ethanol	38.36 $\pm$ 1.87*	31.93 $\pm$ 1.46*	367.00 $\pm$ 13.69	110.34 $\pm$ 3.86*
THQ (20)+Ethanol	33.40 $\pm$ 1.37***	27.96 $\pm$ 2.03***	330.65 $\pm$ 11.01*	96.45 $\pm$ 5.67**
SL (10)+Ethanol	29.64 $\pm$ 1.08***	28.86 $\pm$ 2.67***	316.19 $\pm$ 8.84**	91.48 $\pm$ 5.21***

Values are expressed in Mean $\pm$ SE of six animals in each group. Statistical analysis was done by ANOVA, \*: p<0.05, \*\*: p<0.01 and \*\*\*: p<0.001 Student's t-test, Ethanol alone group was compared with control group, Drugs pretreated groups were compared with ethanol alone group

**Table 2: Effect of THQ on serum TNF-alpha and IL-6 levels of rats**

Treatments (mg kg <sup>-1</sup> day <sup>-1</sup> )	Serum TNF-alpha (pg mL <sup>-1</sup> )	Serum IL-6 (pg mL <sup>-1</sup> )
Control	58.58±3.65	156.21±5.32
Ethanol	83.47±2.42***	289.31±9.84***
THQ (5)+Ethanol	80.89±2.35	256.24±10.18
THQ (10)+Ethanol	68.66±3.76*	240.19±7.46**
THQ (20)+Ethanol	62.49±3.86**	226.23±6.84***
SL (10)+Ethanol	56.26±3.41***	207.37±7.45***

Values are expressed in Mean±SE of 6 animals in each group. Statistical analysis was done by ANOVA; \*, p<0.05, \*\*, p<0.01 and \*\*\*, p<0.001 Student's t-test, Ethanol alone group was compared with control group. Drugs pretreated groups were compared with ethanol alone group

**Table 3: Effect of THQ on hepatic GSH and MDA concentrations of rats**

Treatments (mg kg <sup>-1</sup> day <sup>-1</sup> )	Liver GSH (mmol/100 mg wet tissue)	Liver MDA (mmol g <sup>-1</sup> wet tissue)
Control	176.53±6.25	263.09±6.25
Alcohol	121.53±4.08***	336.54±10.48***
THQ (5)+Alcohol	130.18±2.49	318.97±6.90
THQ (10)+Alcohol	146.81±4.56*	295.95±7.62**
THQ (20)+Alcohol	160.28±7.60***	287.40±4.48***
SL (10)+Alcohol	164.23±6.98***	256.45±5.78***

Values are expressed in Mean±SE of 6 animals in each group. Statistical analysis was done by ANOVA; \*, p<0.05, \*\*, p<0.01 and \*\*\*, p<0.001 Student's t-test, Ethanol alone group was compared with control group. Drugs pretreated groups were compared with ethanol alone group

**Table 4: Effect of THQ on hepatic SOD and CAT concentrations of rats**

Treatments (mg kg <sup>-1</sup> day <sup>-1</sup> )	Liver SOD (U mg <sup>-1</sup> of proteins)	Liver CAT (µmole min <sup>-1</sup> mg <sup>-1</sup> of proteins)
Control	4.94±0.38	514.30±13.26
Ethanol	3.47±0.17**	413.21±17.16***
THQ (5)+Ethanol	3.74±0.17	402.18±12.74
THQ (10)+Ethanol	4.17±0.26*	464.64±5.67**
THQ (20)+Ethanol	4.72±0.21***	483.94±12.27**
SL (10)+Ethanol	4.66±0.24***	507.44±11.84***

Values are expressed in Mean±SE of 6 animals in each group. Statistical analysis was done by ANOVA; \*, p<0.05, \*\*, p<0.01 and \*\*\*, p<0.001 Student's t-test, Ethanol alone group was compared with control group. Drugs pretreated groups were compared with ethanol alone group

Ethanol-induced significant decrease the hepatic GSH and increase hepatic MDA concentrations as compared to experimental controls. Pretreatment with TQ protected the GSH decrease and MDA increase induced by ethanol as compared to ethanol alone group in dose dependent manner. Silymarin taken as reference drug also showed protection against the changes in induced by ethanol in hepatic GSH and MDA levels (Table 3).

Single dose of ethanol administration to rats induced significant decrease in hepatic SOD and CAT activities as compared to control group of rats. Pretreatment with TQ to ethanol administered rats showed significant increase dose dependent manner in hepatic SOD and CAT activities as compared to the rats only treated with alcohol (Table 4).

Ethanol supplementation induced significant decrease in liver RNA, DNA and protein concentrations as compared to the control group of rats. Pretreatment with TQ showed protection against the decreased levels of RNA, DNA and total proteins by alcohol administration (Table 5).

**Table 5: Effect of THQ on hepatic RNA, DAN and total protein concentrations of rats**

Treatments (mg kg <sup>-1</sup> day <sup>-1</sup> )	RNA (µg/100 mg wet tissue)	DNA (µg/100 mg wet tissue)	Total protein (mg/100 g wet tissue)
Control (Vehicle)	630.94±14.04	154.33±7.19	14.16±0.43
Ethanol (7.5 g kg <sup>-1</sup> b.wt.)	459.26±13.70***	114.80±3.66***	10.80±0.55***
THQ (5)+Ethanol	473.86±18.62	126.41±2.52*	12.39±0.52
THQ (10)+Ethanol	525.34±16.45*	131.88±4.49**	13.54±0.41**
THQ (20)+Ethanol	540.88±16.61**	142.92±5.63***	14.45±0.34***
SL (10)+Ethanol	582.37±18.62***	145.21±6.75***	15.04±0.46***

Values are expressed in Mean±SE of 6 animals in each group. Statistical analysis was done by ANOVA; \*, p<0.05, \*\*, p<0.01 and \*\*\*, p<0.001 Student's t-test, Ethanol alone group was compared with control group. Drugs pretreated groups were compared with ethanol alone group

## DISCUSSION

Oxidative stress is one major factor in etiology of ethanol injury, mainly by Kupffer cell derived Reactive Oxygen Species (ROS) (Vrba and Modriansky, 2002; Wheeler, 2003). Ethanol activates Kupffer cells primarily through the action of a substance called endotoxin, which is released by certain gram-negative bacteria present in the intestine (Keshavarzian *et al.*, 2001). Kupffer cell activation generates ROS and pro-inflammatory TNF-α, it can lead to liver damage (Hoek and Pastorino, 2002; Zhou *et al.*, 2003).

Ethanol causes injury to the liver cells and the activities of AST and ALT are the most sensitive test employed in the diagnosis of hepatic disease (Chenoweth and Hake, 1962) and were increased in ethanol treated rats. Pretreatment with TQ to ethanol treated rats decreased AST and ALT activities. Present results are in agreement with Daba and Abdel-Rahman (1998) showed TQ protection against tert-butyl hydroperoxide-induced hepatotoxicity in rats isolated hepatocytes evidenced by decreased leakage of ALT and AST. This shows TQ can preserve the structural integrity of the liver from the adverse effects of ethanol. Administration of a single dose of ethanol significantly elevated the serum ACP and ALP activities compared to the normal animals. This indicated necrosis of hepatocytes that results in the elevation of serum ACP and ALP from a possible cholestasis (Ajith *et al.*, 2007). Pretreatment with TQ showed significant decrease in ACP and ALP levels as compared the animals administered ethanol only.

Oxidative stress has been implicated in the process of alcohol-induced TNF-α production (Zhou *et al.*, 2003, 2004). In the present study, ethanol administration significantly raised both serum IL-6 and TNF-α levels together this confirmed that local inflammatory changes and release of pro-inflammatory cytokines did occur in our experimental model. Pretreatment with TQ showed significant decrease in ethanol-induced elevation of serum

IL-6 and TNF- $\alpha$  level. El-Mahmoundy *et al.* (2005) reported that TQ administered intraperitoneally in diabetic rats at 3 mg kg<sup>-1</sup> or added to cultured macrophages at 10  $\mu$ M normalized the elevated levels of the proinflammatory cytokines IL-1 $\beta$  and TNF- $\alpha$ . TQ has been shown to inhibit generation of the inflammatory mediators, thromboxane B2 and leukotriene B4, by targeting the cyclooxygenase and 5-lipoxygenase pathways of Arachidonic acid metabolism (Houghton *et al.*, 1995).

Ethanol is capable of generating oxygen radicals, inhibiting GSH synthesis, producing GSH loss from the tissue, increasing MDA levels and impairing antioxidative defense systems (Genc *et al.*, 1998; Pramyothin *et al.*, 2006). It has been shown that marked decreases in the glutathione pool occur in liver after acute ethanol intoxications (Guerra and Grisolia, 1980), which is partly due to the binding of acetaldehyde with cysteine and/or glutathione (Lieber, 1994). Furthermore, severe GSH depletion is known to associate with lipid peroxidation (Videla *et al.*, 1980). Present results are consisted by acute ethanol treatment (Speisky *et al.*, 1985; Chen *et al.*, 1992) and the administering TQ to alcohol treated rats helped to restore the GSH levels to near those of the experimental control rats. In the present study, MDA formation, the index of lipid peroxidation, was significantly increased after ethanol treatment and TQ supplementation was potentially effective in reducing lipid peroxidation, conform its antioxidant nature.

Natural or synthetic compounds with antioxidant properties may help to alleviate the liver damage totally or partially. Superoxide ion (O<sub>2</sub><sup>-</sup>) and hydroxyl radical are known to cause marked injuries to the surrounding tissues and organs (Reedy and Lokesh, 1992). Removing superoxide ion and hydroxyl radical is probably one of the most effective defense mechanisms against a variety of diseases (Sheela and Angusti, 1995). Activities of SOD and CAT help to scavenge superoxide ions and hydroxyl ions, respectively, were significantly lower in alcohol-treated rats as compared with those of control rats. Lowered activities of SOD and CAT will result in the accumulation of these highly reactive free radicals leading to deleterious effects such as loss of cell membrane integrity and membrane function (Reedy and Lokesh, 1992). This decrease in the SOD activity may be associated with the elevation of the intracellular concentration of H<sub>2</sub>O<sub>2</sub> as a result of CAT inactivation (Antonekov and Panchenko, 1988). CAT has been reported to be responsible for the detoxification of H<sub>2</sub>O<sub>2</sub>, which is an effective inhibitor of SOD (De duve and Baudhuin, 1996). The observed restoration of the SOD and CAT activities in the present study on treatment with

TQ may be due to the direct stimulatory effect of TQ on SOD and CAT. Furthermore, the decrease in the tissue lipid peroxidation on TQ treatment can also be correlated with the elevated SOD and CAT activities.

In present study, hepatic nucleic acids levels depleted significantly in ethanol treated rats. Pretreatment with TQ and SL showed significant protection against decreased hepatic nucleic acids levels by the ethanol administrations. Several studies reported that, toxic agents such as chemotherapy drugs, carcinogens, reactive oxygen and nitrogen species cause damages in DNA and RNA molecules (Bohr, 2002; Moreira *et al.*, 2005; Ungvari *et al.*, 2005; Kang and Hamasaki, 2005). Wieland and Lauterburg (1995) reported that oxidation of DNA and mitochondrial proteins following ethanol administration resulted in permanent damage of the liver in alcoholic subjects. In animal studies, Abou-Seif *et al.* (2003) and El-Missiry *et al.* (2007) reported that, radiation-induced oxidative stress caused significant damage in hepatic RNA concentrations. It is well documented that green tea is an efficient radical scavenger, activates enzymes important for detoxification and prevent *in vivo* DNA damage and lipid peroxidation (Cheng *et al.*, 1991). Present results indeed support such notion because TQ supplementation could effectively reduce ethanol-induced increase in DNA, RNA and total protein damage.

The results of the present study concluded that TQ significantly and dose dependently prevented the ethanol-induced acute hepatotoxicity by enhancing the hepatic antioxidant activity. However, further detailed studies are required to establish its clinical application.

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