Could Thymoquinone Protect The Liver Against Indomethacin Toxicity?

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Abstract: Background: The non-steroidal anti-inflammatory drug indomethacin (IND), utilized in the treatment of inflammatory disorders, has been found to induce liver disorders in both animals and humans. Its administration in a high dose causes hepatic toxicities and results in liver cell deaths by activating multiple stress pathways. **Objectives:** This study was conducted to investigate the effects of thymoquinone (TQ) on indomethacin-induced hepatotoxicity in rats. **Study design:** Male adult albino rats were divided into 4 groups: Group I: control non-treated group; the rats were treated orally with normal saline, Group II: TQ-treated (TQ) group; the rats were treated with TQ in the dose of 10 mg/kg/day orally, Group III: single high dose IND- treated (SHDI) group; the rats were treated with IND in the dose of 25 mg/kg once orally at the end of the duration of normal saline treatment, and Group IV: concomitant TQ- and IND- treated (CT/I) group; the rats were treated orally with TQ in the dose of 10 mg/kg/day, and then with IND in the dose of 25 mg/kg once at the end of TQ treatment. The treatment was for 4 weeks for all groups. At the end of the experiment, serum alanine transaminase (ALT), aspartate transaminase (AST), albumin, total antioxidative capacity (TAC) were measured in all rats. Agarose gel electrophoresis for DNA (DNA analysis) for rat's liver tissue was investigated also after sacrificing the rats. These parameters were expressed as the means of the effects ± SEM. **Results:** There were significant ($P < 0.001$) decrease in serum ALT and AST. and significant ($P < 0.001$) increase in serum TAC and DNA analysis between CT/I group and SHDI group. Albumin was not significantly changed ($P > 0.05$) between CT/I group and SHDI and control groups. **Conclusion:** These findings suggested that TQ is strong protective agent against IND-induced hepatotoxicity in rats. The collective data demonstrated that TQ has the potential to scavenge oxidants and decrease apoptotic changes and revealed a possible protective target mechanism for the damaging effects of IND on liver in rats.
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1. Introduction

Drug-induced liver injury is the leading cause of acute liver failure and transplantation in western countries. The frequent involvement of the liver in drug-induced toxicity depends on its anatomical location (as it is the primary port of entry for ingested drugs) and its physiological and biochemical functions because of the abundance of metabolizing enzymes. The detection of new drugs that have protective effects against drug-induced hepatotoxicity is a major challenge in clinical practice (**Grattagliano et al., 2009**).

The adverse effects of the non-steroidal anti-inflammatory drug indomethacin (IND) involve the gastrointestinal tract, liver and CNS in both animals and humans. IND causes a decrease of hepatic microsomal cytochrome P-450 dependent monooxygenase system and prostaglandin depletion (**Falzon et al., 1985, Whiting et al., 1987 & Nancy Perron et al., 2013).**

Grattagliano et al. (2005) stated that, hepatic clearance of drugs depends on the activity of transport proteins that are located on the hepatocyte canalicular membrane. Alterations of these transporters by drugs or genetic polymorphisms increase the susceptibility to cholestasis injury which is one of the most important features of drug-induced hepatotoxicity. Substrates for hepatic transport proteins include IND, statins, and other drugs (**Grattagliano et al., 2009).**

Although the underlying molecular mechanisms of IND-induced hepatotoxicity are not completely determined, mitochondrial dysfunction, altered calcium homeostasis and apoptosis-related proteins have been implicated in producing this hepatotoxicity (**Rudnick et al., 2006**). IND overdose potentially damages the liver by activating multiple stress pathways and causes hepatic disorders (**Rudnick et al., 2006, Maity et al., 2009**).

In the late era, the use of herbal therapies for the prevention and treatment of certain diseases is increased. *Nigella sativa* (*N. sativa*) is used as a food condiment in the Middle East, and its seeds/oil possesses anti-inflammatory, antiviral and antineoplastic activities in various *in vitro* and *in vivo* studies (**Zaher et al., 2008**). **Gali-Muhtasib et al. (2004)** declared that, The black seed herb grows in

India, countries bordering the Mediterranean Sea and Africa, and is used in the Middle East since long to promote health and fight diseases. Also, the crude *N. sativa* oil and its fractions have shown antioxidant and radical scavenging effects **(Burits and Bucar 2000, Ramadan *et al.,* 2003)**. Many reports of *N. sativa* biological activities proved immunopotentiation, antitumor, anti-inflammatory, analgesic, antihypertensive, antidiabetic, respiratory stimulation, antibacterial, antifungal, anticestode and antinematode effects **(Swamy and Tan, 2000, Ali and Blunden 2003, Al-Naggar *et al.,* 2003, Barakat *et al.,* 2013)**.

Thymoquinone (TQ), one of the active ingredients of the volatile oil of black cumin *N. sativa* seeds, has broad pharmacological effects and is used for medicinal purposes **(Das *et al.,* 2012 & Lei *et al.,* 2012)**. The present study was designed to investigate the effect of TQ 'the active ingredient of *N. sativa* seed' on the oxidative and apoptotic changes in IND-induced hepatotoxicity in rats.

2. Material And Methods

Animals: Forty adult male albino rats of local strain, weighing 150-200 grams, bred in the animal house of Faculty of Medicine- Menoufeya University, were used throughout the study. The rats, housed in a temperature-controlled environment at $22 \pm 2°C$ with a 12 hour lighting cycle and caged in fully ventilated cages, were fed on normal laboratory rat chow and free access to water (**Whiting *et al.,* 1987, Ullah *et al.,* 2012**).

Drugs and chemicals: Indomethacin (Sigma-Aldrich Chemie GmbH, CAS-No. 53-86-1, UK) was dissolved in isomolar (11.2 mM) sodium carbonate to give a neutral pH solution **(Whiting *et al.,* 1987)**. Thymoquinone (Sigma-Aldrich Chemie GmbH, CAS-No. 490-91-5) was dissolved in water.

Experimental Design: The rats were allotted into the four groups (n = 10):

Group I: Non-treated control group: The rats received normal saline in the dose of 1 ml /kg body weight/day, as a control to the other treated groups.

Group II: TQ-treated (TQ) group: The rats received TQ in the dose of 10 mg/kg/day **(Helal, 2010)**.

Group III: Single high dose IND-treated (SHDI) group: The rats received the single high dose IND of 25 mg/kg **(Abbas and Sakr, 2013)** at the end of the duration of normal saline treatment.

Group IV: Concomitant TQ- and IND-treated (CT/I) group: The rats received TQ in the dose of 10 mg/kg/day, and then received IND in the dose of 25 mg/kg once at the end of the duration of TQ treatment. The duration of treatment for all rats in different groups was 4 weeks.

The drugs were administered orally with an intragastric tube (using a Portex 4FG cannula, Portex Ltd., Hythe, UK). In all groups, after finishing the experiment, the rats were held in a chamber of isofluran's gas to be anesthetized. The rat's blood was obtained from the retro-orbital plexus (24 hours after the last drug administration) using heparinized microcapillary tubes. Serum is expressed from clotted blood by centrifugation (5000 g, 10 minutes), stored at − 20□C until required. Rats were then sacrificed and their liver were removed and stored frozen in -20□C until required.

The serum was used to measure AST, ALT, albumin, total antioxidative capacity (TAC) and DNA agarose gel electrophoresis (DNA analysis).

DNA fragmentation by agarose gel electrophoresis: The liver cells from the control and treated groups were subjected to lysis in a buffer containing 10 mM Tris (pH 7.4), 150 mM NaCl, 5 mM EDTA and 0.5% Triton X-100 for 45 minutes on ice. Lysates were subjected to vortexing and then centrifugation at 10,000 g for 20 minutes. DNA in the supernatant was extracted with an equal volume of a neutral phenol□chloroform□isoamyl alcohol mixture (25□24□1) and then was examined electrophoretically on 1.8% agarose gels containing 0.1 μg/mL ethidium bromide **(Mandal *et al.,* 2001, Das *et al.,* 2012)**.

The total antioxidant capacity (TAC) assay: Serum samples were assayed by commercially available kits (Randox labs, Grumlin, UK). The assay principle was based on the ability of antioxidants to quench the absorbance of the radical cation that is formed by the reaction of a chromogen with the peroxide and H_2O_2 **(Miller *et al.,* 1993)**.

The estimations of serum aspartate transaminase (AST), alanine transaminase (ALT) and albumin were done using auto analyzer (Hitachi, Japan). The kits were supplied by Technichon (Germany) **(Meshkibaf *et al.,* 2006).**

Reviewing the available literatures, there was rarity in similar studies. So, the current investigation was conducted with intent to shed light on IND-induced hepatic influences and to evaluate the possible ameliorating role of TQ on such alterations.

Statistical analysis:

The parameters recorded were expressed as means and standard error of means (mean ± SEM) for all groups. Statistical analysis was performed using SPSS (version 16) software, for the one-way analysis of variance (one-way ANOVA) followed by Post Hoc and least significant difference (LSD) tests to determine the significance of differences between the parameters of different groups. In every case, the acceptance level for statistical significance was $P < 0.05$ **(Ghosh, 1971, Aaronson *et al.,* 2006 & Ullah *et al.,* 2012).**

3. Results
The effects of TQ, SHDI and CT/I treatments on the serum AST and ALT in rats (Table 1 and Figure 1):

There were insignificant changes ($P > 0.05$) in s.ALT and s.AST in the TQ-treated group when compared to control group. But there were significant increases ($P < 0.05$) in serum ALT and AST in SHDI-treated group when compared to control group. On the other hand, there were significant decreases in these enzymes in the rats of CT/I-treated group when compared to rats of SHDI-treated group, but there were still significant increases in these enzymes in the rats of CT/I-treated group when compared to control group. (I.e. TQ alone did not affect serum ALT and AST in rats). Single high IND dose increased significantly serum ALT and AST in rats. TQ, when administered before IND, prevented significantly the IND-induced increases in serum ALT and AST in rats).

Table 1: The effects of TQ, SHDI and CT/I treatments on serum AST (s.AST) and ALT (s.ALT) in rats.

	s.AST (U/L)	s.ALT (U/L)
Control group	56.25 ± 0.53	37.5 ± 0.53
TQ-treated group	56 ± 0.65	37.13 ± 0.52
SHDI-treated group	107 ± 0.65 #	59 ± 0.46 #
CT/I-treated group	70 ± 0.46 # $	40.5 ± 0.33 # $

\#: Significant in comparison to control group.
$: Significant in comparison to SHDI-treated group.
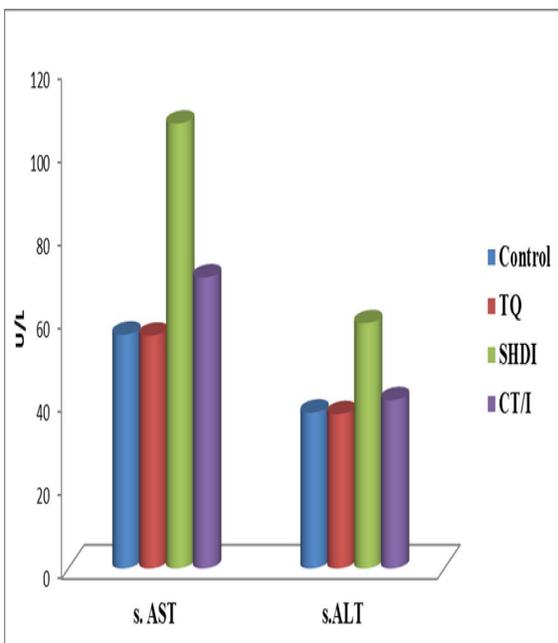
Figure 1: Showing the effects of TQ, SHDI and CT/I treatments on serum AST and ALT in rats.

The effects of TQ, SHDI and CT/I treatments on the serum albumin in rats (Table 2 and Figure 2):

There were insignificant changes in serum albumin in the TQ-treated, SHDI-treated and CT/I-treated groups when compared to control group. In addition, there was insignificant change in serum albumin among rats of CT/I-treated group when compared to rats of SHDI-treated group. (I.e. single high IND dose or TQ, when given either alone or in combination, did not change serum albumin level in rats).

Table 2: The effects of TQ, SHDI and CT/I treatments on serum albumin (s.Albumin) in rats.

	s. Albumin (g/dL)
Control Group	4.36 ± 0.07
TQ-treated group	4.36 ± 0.05
SHDI-treated group	4.17 ± 0.05
CT/I-treated group	4.23 ± 0.02

- No significant differences.
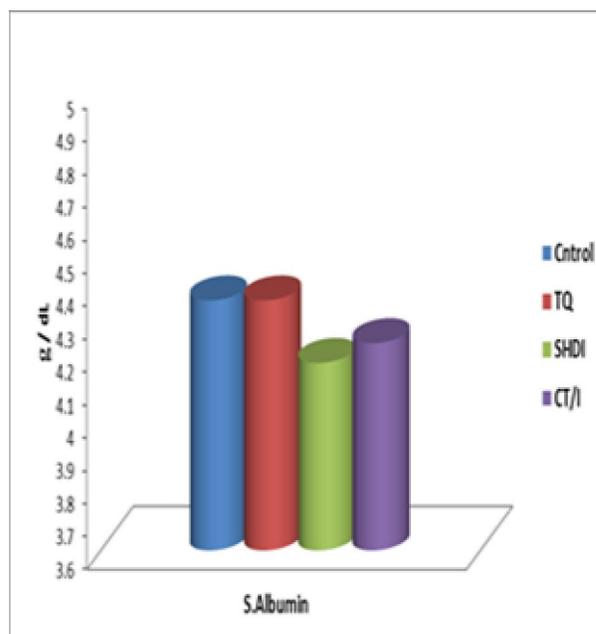
Figure 2: Showing the effects of TQ, SHDI and CT/I treatments on serum albumin in rats.

The effects of TQ, SHDI and CT/I treatments on the serum TAC in rats (Table 3 and Figure 3):

There was an insignificant change in serum TAC in TQ-treated group when compared to control group. But there was a significant decrease in serum TAC in SHDI-treated group when compared to control. On the other hand, there was a significant increase in this parameter in CT/I-treated group when compared SHDI-treated group. At the same time, there was an insignificant change in serum TAC in CT/I-treated group when compared to control group. (I.e. TQ alone

did not affect serum TAC in rats. Single high IND dose decreased significantly serum TAC in rats. TQ, when administered before IND, prevented significantly the IND-induced decrease in serum TAC in rats).

Table 3: The effects of TQ, SHDI and CT/I treatments on serum TAC (s.TAC) in rats.

	s. TAC (µmol /L)
Control Group	2.2 ± 0.05
TQ-treated group	2.18 ± 0.05
SHDI-treated group	1.35 ± 0.03 #
CT/I-treated group	2.01 ± 0.03 $

#: Significant in comparison to control group.
$: Significant in comparison to SHDI-treated group.
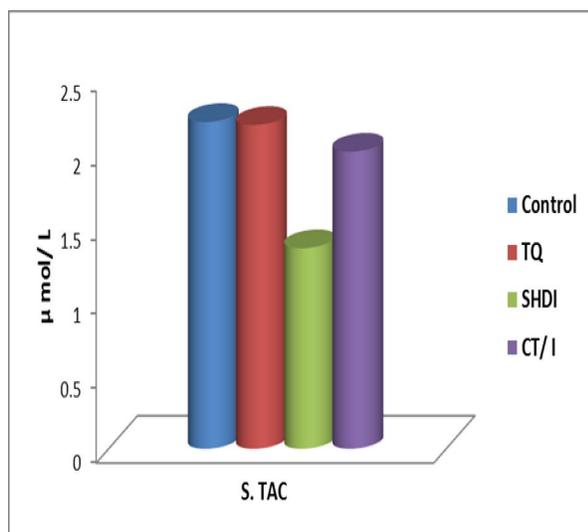
Figure 3: Showing the effects of TQ, SHDI and CT/I treatments on serum TAC in rats.

The effects of TQ, SHDI and CT/I treatments on DNA analysis in rats (Table 4 and Figures 4 and 5):

There was an insignificant change in hepatocytes DNA analysis in TQ-treated group when compared to control group. But there was a significant decrease in DNA analysis (i.e. increased apoptotic changes) in SHDI-treated group when compared to controls. On the other hand, there was a significant increase in DNA analysis in rats of CT/I-treated group when compared to rats of SHDI-treated group, and but still there was a significant decrease in DNA analysis in rats of CT/I-treated group when compared control group. (I.e. TQ alone did not affect in hepatocytes DNA analysis in rats. Single high IND dose decreased significantly hepatocytes DNA analysis (i.e. increased apoptotic changes) in rats. TQ, when administered before IND, prevented significantly the IND-induced decrease hepatocytes DNA analysis in rats, i.e. TQ prevented significantly the IND-induced increased apoptotic changes).

Table 4: The effects of TQ, SHDI and CT/I treatments on DNA analysis in rats.

	DNA analysis (m.w. in bp)
Control group	1875 ± 65
TQ-treated group	1887 ± 72
SHDI-treated group	279 ± 32 #
CT/I-treated group	1582 ± 46 # $

#: Significant in comparison to control group.
$: Significant in comparison to SHDI-treated group.
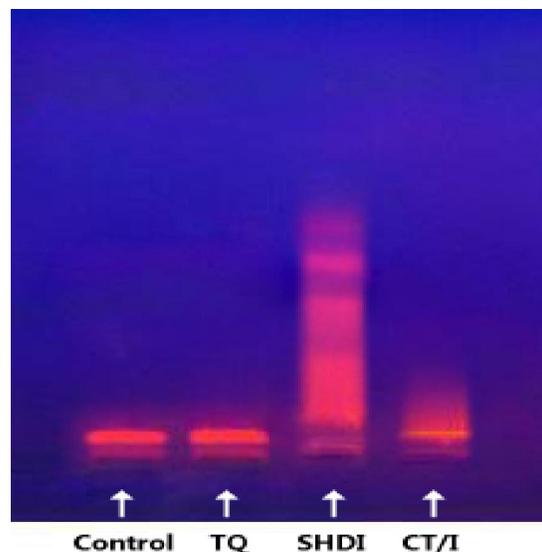
Figure 4: Ultraviolet illuminated photo shows the effects of TQ, SHDI and CT/I treatments on hepatocytes DNA analysis in rats by agarose gel electrophoresis.
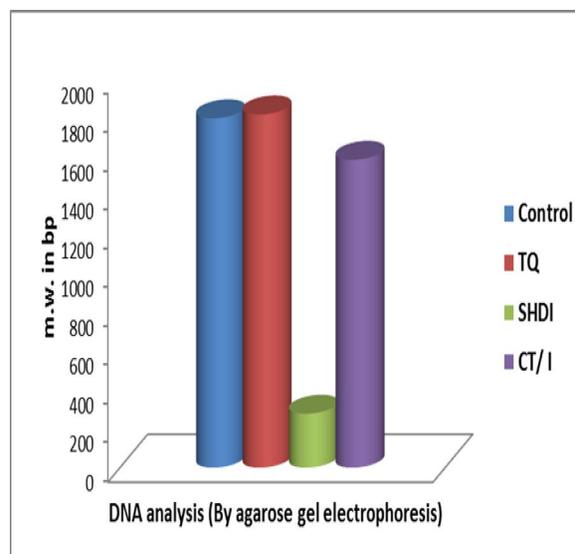
Figure 5: Showing the effects of TQ, SHDI and CT/I treatments on hepatocytes DNA analysis in rats.

4. Discussion

TQ has broad pharmacological effects and is used for medicinal purposes (**Lei** *et al.*, **2012).** The present study showed that single high indomethacin (IND) dose 25 mg/kg orally once caused elevations of the serum ALT and AST liver enzymes, but did not change serum albumin level in rats. In addition, single high IND dose decreased the total antioxidant capacity (TAC) and decreased hepatocytes DNA analysis (i.e. it increased the apoptotic changes) in rats. Thus, IND exposure causes a wide range of functional liver toxicity.

The present results could be due to the deleterious effects of IND on hepatic cell vitality and degenerative changes on normal histological architecture of liver. **Falzon** *et al.* **(1985)** & **Tarek Rahmy** *et al.* **(2013)** in agreement with this study claimed that high doses of IND in rats (three daily doses, 8.5 mg/kg intraperitoneally) caused degenerative changes histologically and cell necrosis in cells surrounding central veins with glycogen depletion and produced decreases in aminopyrine N-demethylase and glucuronyl transferase. However, the present results regarding the IND effects, in some aspects, are in disagreement with previous reports in that, high doses of IND in rats, (three daily doses, 8.5 mg/kg intraperitoneally), decreases serum total protein and albumin concentrations; while serum level of aspartate aminotransferase (a marker of cellular damage) remained unchanged (because of the only minor hepatic damage) in ratsas declared by **Whiting** *et al.* **(1987).**. Furthermore, IND decreased hepatic cytochrome P-450-dependent mono-oxygenase system which was not accompanied by severe damage to hepatocyte structure (**Falzon** *et al.*, **1985).** Also, high dose indomethacin (5 mg/kg body weight i.p. / 24 h over a period of 3 days) caused reductions in serum total protein and albumin concentrations (**Whiting** *et al.*, **1987)**.

Furthermore, we could explain these differences between the present and previous studies depending on the differences in IND dose, route of its administration and frequency of dosing. In this concept, we are in accordance with the reports exhibited by **Kaplowitz, (2002)** & **Grattagliano** *et al.* **(2009)** who found that the drug-induced hepatotoxicity is dose-dependent and the hepatocytes, and other cells in the liver, including the intracellular organelles and their functions are often the primary targets of hepatotoxicity. Some drugs can induce cholestasis by impairing bile secretion or by causing obstruction of extrahepatic bile ducts. The mechanisms of drug-induced liver injury include necrosis (**Kass, 2006**).

The apoptotic changes that occurred in liver cells due to IND in this study could be due to stimulation of apoptosis pathways, reactive metabolite formation,

antioxidant depletion, and protein alkylation. In agreement with this explanation, **Farber, (1994)** & **Jaeschke and Bajt, (2006)** reported that, apoptosis and necrosis initially may follow common metabolic pathways. Apoptosis occurs when hepatocyte injury affects the maintenance of functional cell programs. Necrosis generally begins at the cytoplasm and involves mitochondria [through reactive oxygen species 'ROS' delivery, with induction of mitochondrial membrane permeability transition and decrease in mitochondrial membrane potential (which favors superoxide generation, thioredoxin oxidation and activation of the kinase-1-dependent apoptosis signaling pathway), with decrease of energy production and release of nucleases) and the nucleus (promoting chromatin condensation and DNA fragmentation) and loss of plasma membrane integrity (**Grattagliano** *et al.*, **2009**). **Kaufmann** *et al.* **(2005)** added that, drug-induced hepatotoxicity decreases state-3 oxidation and respiratory control ratio, uncouples oxidative phosphorylation, and inhibits β oxidation. It increases the production of ROS, and the leakage of cytochrome C, with release of reactive metabolites, with promotion of lipid and protein oxidation and depletion of GSH as announced by **Grattagliano** *et al.* **(2009)**. Also, several mechanisms have been elucidated, including TNF-α-induced apoptosis, inhibition of mitochondrial function, and new antigen formation (**Maciá** *et al.*, **2002** & **Casanelles** *et al.*, **2013**). Activation of Kupffer cells and recruitment of macrophages and immune cells as stated by **Szabo** *et al.* **(2007)** result in inflammation and injury caused by cytokines release. These events are major factors in initiating and maintaining drug-induced liver injury.

The present study exhibited that TQ alone did not change the serum ALT, AST enzymes and albumin levels, and TAC and hepatocytes DNA analysis in rats. Also, TQ, when administered before the single high IND dose, did not change serum albumin level in rats. On the other hand, thymoquinone, when administered before single high IND dose, protected against the IND-induced increases in serum ALT and AST. Also, it protected against the IND-induced decrease in serum TAC and apoptotic changes in rats. These results could be due to the pharmacologic antitoxic effects of this active ingredient of N sativa, as it may scavenge oxidants and preserve cell vitality.

In agreement with the present results regarding the protective effects of TQ, different studies stressed on the concept that TQ, the natural active component of Nigella sativa seed and oil and its fractions have different beneficial pharmacological effects against various cytotoxic insults (**Ramadan** *et al.*, **2003, Mousavi** *et al.*, **2010). Fortunately, Martin** *et al.*, **(2006)** & **Kanter, (2008)** refered these effects to its strong antioxidant activity against free radical-

generating agents by reducing reactive oxygen species (ROS). Also, **Houghton *et al.* (1995) & Nagi and Mansour, (2000)** declared that TQ inhibition of drug-induced cell death in rats might occur via its antioxidant mechanism that maintains mitochondrial integrity. **Mousavi *et al.*(2010)** found that mitochondria play an important role in apoptosis under a variety of pro-apoptotic conditions, such as oxidative stress and cytochrome-c release. The latter is a key event in the activation of caspase-3, a pivotal downstream step in apoptosis initiation. TQ has been used as a protective agent in multiple toxicity models **(Badary *et al.*, 1997, Al-Majed *et al.*, 2006 & Ullah *et al.*, 2012)**.

TQ stimulated resistance to oxidative stress by decreasing the elevated levels of malondialdehyde, glutathione (GSH) contents, catalase and superoxide dismutase (SOD) **(Al-Majed *et al.*, 2006, Radad *et al.*, 2009)**. TQ attenuated the elevation of cytosolic free calcium $[Ca^{2+}]c$ and elevated the lowering of mitochondrial transmembrane potential ($\Delta\psi M$), both caused by drug toxicity and decreased the expression of an anti-apoptotic protein (Bcl-2), increased expression of Bax, and stimulated the release of cytochrome-c from mitochondria **(Merry Korsmeyer, 1997 & Kim *et al.*, 2005)**. In this occasion, TQ reduced fragmentation of PARP-1 which functions in apoptosis by acting as a survival factor and a death promoter, depending on the severity of DNA damage. TQ decreased DNA damage **(Cherian *et al.*, 2008)**. So, TQ stabilizes mitochondrial membrane potential and inhibits apoptotic cascade **(Heaton *et al.*, 2011).**

Furthermore, previous reports stated that TQ exhibits antioxidant, anti-inflammatory and anticancer activities **(Lei *et al.*, 2012)** (mediated via peroxisome proliferator-activated receptor gamma, p53-dependent and p53-independent pathways), against many types of malignancy, with minimal toxicity in normal cells **(Woo *et al.*, 2011, Das *et al.*, 2012)**.

In particular, reactive metabolite formation, GSH depletion and protein alkylation are associated with mitochondrial dysfunction, and represent initiating events for drug-induced toxicity **(Jaeschke and Bajt, 2006 & Miller *et al.*, 2008)**. In this context, the maintenance of the mitochondrial GSH pool is important to detoxify ROS and maintain the reduced status of membrane protein sulfhydryls, including the ATP synthase complex and the Ca^{2+}-dependent ATPase. A fall of total cellular GSH below 15% (< 1 μmol/g) inevitably is associated with lethal cell damage by involving the mitochondrial stores **(Colell *et al.*, 1998 & Haouzi *et al.*, 2001)**. The events that lead to apoptosis and necrosis act through mitochondrial permeabilization and dysfunction, according to ATP availability or deficiency **(Pessayre *et al.*, 1999)**. Some drugs exert toxic effects on mitochondria only after

their metabolic activation at the microsomal level (isoniazid/rifampicin), after inducing endoplasmic reticulum stress (paracetamol) or even lysosomal dysfunction **(Macanas-Pirard *et al.*, 2005 & Chowdhury *et al.*, 2006)**.

Han *et al.* (2010) concluded that drugs can also cause redox changes that inhibit important prosurvival pathways such as NF-κB. The inhibition of NF-κB by subtoxic doses of drug sensitizes hepatocyte to the cytotoxic actions of tumor necrosis factor (TNF). Many drugs will induce liver injury if simultaneously treated with LPS, which promotes inflammation and cytokine release. Drugs may be sensitizing hepatocytes to the cytotoxic effects of cytokines such as TNF, or vice versa. Overall many signaling pathways are important in regulating DILI, and represent potential therapeutic targets to reduce liver injury caused by drugs.

In Conclusion: The current investigation indicated that single high IND dose caused a wide range of functional liver toxicity; elevating the serum ALT and AST, without change in serum albumin level and with decreasing the total antioxidant capacity (TAC) and decreasing the hepatocytes DNA analysis (i.e. increasing the apoptotic changes) in rats.

But fortunately, The same study showed that TQ has a protective effect against indomethacin-induced hepatotoxicity; regarding the IND-induced altered parameters. This provides strong evidence for a protective role of TQ to liver and that TQ has the potential to scavenge oxidants and decrease apoptotic changes and revealed a possible protective target mechanism for the damaging effects of IND on rat liver.

However, the effects of the TQ on the drug-induced hepatotoxicity need further studies on larger scale with different regimens utilizing wide sample of animals for longer durations to determine the molecular mechanisms underlying TQ actions.

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