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Thymoquinone upregulates TRAIL/TRAILR2 expression and attenuates hepatocellular carcinoma in vivo model

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ABSTRACT

Aims: Hepatocellular carcinoma (HCC) is the second leading cause of cancer-related mortality worldwide. Indeed, chemotherapeutic drugs-induced systemic toxicity results in suboptimal cancer treatment. Consequently, there is a need for exploring of a safe and effective therapy for cancer patients. This study aimed to evaluate the hepatoprotective effect of thymoquinone (TQ) against thioacetamide (TAA)-induced HCC. Also, we investigated TQ's ability to sensitize cancer cells toward TRAIL/TRAILR2 apoptotic pathway.

Main methods: Forty male Sprague Dawley rats were divided into 4 groups (n = 10) as follows: control group, CMC group, HCC group and HCC + TQ group. Serum levels of liver function biomarkers and Alpha-Fetoprotein (AFP), as well as hepatic levels of glutathione (GSH) and Alpha-Fetoprotein (MDA) were measured. Transforming growth factor-beta 1 (TGF-β1), TRAILR2, TRAIL, caspase-3, caspase-9, caspase-8 and B cell lymphoma-2 (Bcl-2) mRNA levels were assessed by Quantitative, Real-Time PCR. Fibrosis percentage and necroinflammation were quantified by histopathological examination.

Key findings: Our results indicated improvement in liver functions, decrease in AFP level and attenuation of HCC progression in TQ treated rats. TQ upregulated TRAIL/TRAILR2 and subsequently enhanced apoptosis as hinted by caspase-3 upregulation and Bcl-2 downregulation. Also, TQ decreased TGF-β1 gene expression level. Moreover, HCC + TQ group showed significant increase in hepatic GSH level and marked decrease in hepatic MDA level.

Significance: This study proved that TQ is able to suppress HCC development via decreasing oxidative stress, suppression of TGF-β1 and induction of TRAIL-mediated apoptosis.

1. Introduction

Hepatocellular carcinoma (HCC) is considered the second leading cause of cancer deaths worldwide [1]. It is suggested that chronic hepatitis strongly predisposes to development of cirrhosis, dysplastic nodules (low- and high-grade) and subsequent development of malignant tumors [2]. Despite improvement in prevention methods, screening, and advanced techniques in both diagnosis and treatment, HCC-incidence and related death are growing [3]. Unfortunately, apoptosis evasion by cancer cells, via overexpression of anti-apoptotic proteins and/or downregulation of death receptors, is the main obstacle hindering both intrinsic and extrinsic apoptotic pathways execution [4,5].

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) can induce apoptosis through binding to its receptors; TRAILR1 and TRAILR2 [6]. This interaction leads to formation of death inducing signaling complex (DISC) and activation of initiator caspase-8 which trigger caspase cascade activation through extrinsic and/or intrinsic apoptotic pathway [7]. In contrast, apoptosis is suppressed by overexpression of B cell lymphoma-2 (Bcl-2) anti-apoptotic proteins family like Bcl-2 or Bcl-2-related gene long form (Bcl-X_L). Such increase in the levels of anti-apoptotic proteins enables cancer cells to resist

Abbreviations: AFP, Alpha-Fetoprotein; ALT, alanine aminotransferase; AST, aspartate aminotransferase; Bid, Bcl-2 inhibitory BH3-domain interacting protein; BCL-2, B cell lymphoma-2; Bcl-X_L, BCL-2-related gene long form; CMC, Carboxymethyl cellulose; CV, central vein; DD, death domain; DISC, death inducing signaling complex; ECM, extracellular matrix; FADD, Fas-associated protein with death domain; GSH, glutathione; HCC, hepatocellular carcinoma; H&E, hematoxylin and eosin; HPFs, high power fields; Hprt-1, hypoxanthine phosphoribosyl transferase-1; MDA, Malondialdhyde; PT, portal tract; ROS, reactive oxygen species; TAA, thioacetamide; TGF-β, Transforming growth factor-beta; TQ, thymoquinone; TRAIL, Tumor necrosis factor-related apoptosis-inducing ligand; TRAIL-R1, Tumor necrosis factor-related apoptosis-inducing ligand receptor-1; TRAIL-R2, Tumor necrosis factor-related apoptosis-inducing ligand receptor-2

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chemotherapeutic treatment leading to cancer progression [8]. Also, serum level of transforming growth factor-β1 (TGF-β1) was found to be high in HCC patients triggering tumor growth, progression and invasion [9]. TGF-β signaling induces extracellular matrix deposition creating an environment that supports tumor cell growth [10].

Reactive oxygen species (ROS) generation is a natural part of aerobic life that plays a role in the cellular functions manifestation such as signal transduction pathways, protection against foreign microorganisms [11]. However, excessive ROS result in oxidative stress, which seems to be a principal factor in the progression of different diseases such as chronic liver disease (CLD) and HCC [12,13]. Therefore, a balance between ROS production and elimination is essential to preserve redox homeostasis and avoid physiological functions impairment [11].

Nigella sativa (*N. sativa*), also termed as black seed, has attracted the scientific attention as a miracle herb that is useful in prevention and/or treatment of different ailments. The most abundant biologically active constituent in *N. sativa* seeds is thymoquinone (TQ). TQ is characterized by a great number of pharmacological actions including anti-oxidant, anti-inflammatory, anti-fibrotic, and anti-tumor effects. Moreover, it serves to protect gastric system, liver, kidney as well as neurons from injury and degeneration. It plays also a protective role in cardiovascular diseases, diabetes, reproductive ailments, respiratory disorders and bone complications treatment. TQ as a natural product attracts a great scientific attention over conventional drugs as it has minor adverse effects [14,15].

Accordingly, this work aims to assess the hepatoprotective effect of TQ against HCC in rats. Also, the role of TQ in apoptosis induction and oxidative stress attenuation were evaluated.

2. Materials and methods

2.1. Drugs and chemicals

TQ was bought from Santa Cruz Biotechnology Inc. (Dallas TX, USA). Thioacetamide (TAA), 99% purity was supplied by Sigma Aldrich Chemicals Co. (St. Louise, MO, USA). Carboxymethyl cellulose (CMC) was purchased from Elgomhoria Co. (Mansoura, Egypt). The other feeding ingredients were of great analytical grades.

2.2. Animals

Forty Male Sprague Dawley rats weighing 190–250 g were allowed free access to food and water. Rats were kept under standard conditions of temperature 25 ± 2 °C, with 12-light/12-dark cycles. Each animal was weighed weekly throughout experimental period. Animal care and experiments were complied with “Research Ethics Committee” Faculty of Pharmacy, Mansoura University, Egypt that fall in with “laboratory Animal Care Principles” (National Materials Institute of Health publication No. 85-23, revised 1985).

2.3. Experimental design

Male Sprague Dawley rats were divided into four groups as follows:

- Control group (10 rats): received no treatment
- CMC group (10 rats): received the drug vehicle (0.5% CMC) daily by orogastric gavage tube for 16 weeks.
- HCC group (10 rats): received TAA (i.p. injection of 200 mg/kg body weight) twice per week for 16 weeks [16].
- HCC + TQ group (10 rats): received TQ (20 mg/kg body weight suspended in 0.5% CMC), daily by oral gavage tube along with TAA i.p. injection for 16 weeks [17].

2.4. Sample collection

At the end of the experiment, rats were fasted for 12 h and allowed free access to water. Blood samples were withdrawn from rats through retro-orbital puncture and centrifuged for serum separation. Instantly after sacrificing the rats, liver was isolated and divided into three sections. The first liver section was flash frozen in liquid nitrogen for measurement of gene expression by Quantitative, Real-time PCR. A second one of 0.5 g was homogenized in 5 ml ice-cold phosphate buffer saline (pH 7.4) for measurement of glutathione (GSH) and malondialdehyde (MDA) levels. The third section was fixed in neutral formalin, embedded in paraffin blocks and used for histopathological examination.

2.5. Calculation of liver index

The liver index was calculated by the following formula: Liver index = [Liver weight (g) / Body weight (g)] × 100 [18].

2.6. Biochemical analysis

- Sera were used for measurement of albumin, total protein (BioMed Company, Helioplise, Egypt), total bilirubin levels (Diamond Diagnostics Company, Heliopolis, Egypt), aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activities (Spectrum Diagnostics company, Egypt). ALT measurement was based on the fact that the transfer of the amino group from L-alanine to α-ketoglutarate is catalyzed by ALT giving pyruvate and L-glutamate. The formed pyruvate is further reduced to lactate in the presence of lactate dehydrogenase and NADH. While, AST measurement was based on the fact that the transfer of the amino group L-aspartate to α-ketoglutarate is catalyzed by AST giving oxaloacetate and L-glutamate. The formed oxaloacetate is further reduced to malate in the presence of Malate dehydrogenase and NADH. Finally, both ALT and AST activities are determined via measuring the rate of oxidation of NADH at 340 nm [19,20].
- Hepatic homogenates were used for assessment of lipid peroxidation and antioxidant activity via measurement of MDA and GSH levels, respectively (Biodiagnostic Company (Dokki, Giza, Egypt)). The assay of GSH is based on the ability of GSH to reduce 5,5′ dithiobis (2-nitrobenzoic acid) forming a yellow colored compound that is spectrophotometrically measured at 405 nm [21].

2.7. Histopathological examination of hepatic tissues

After being fixed in formalin, liver tissues were embedded in paraffin blocks, dissected into 4 μm-thickness to be stained with hematoxylin and eosin (H&E) and Masson's trichrome for histopathological examination by means of light microscope. H&E stained hepatic sections were used to evaluate necroinflammatory scores guided by Ishak's activity index [22].

Collagen fiber deposition was quantified by morphometric analysis of Masson's trichrome stained liver sections [23]. Images describing histopathological changes were taken via digital camera mounted on a BX51 Olympus optical microscope (Olympus Corporation, Tokyo, Japan). NIH Image software was used to assess collagenous areas and fibrosis percentage.

2.8. Immunohistochemical analysis

For immunohistochemical analysis, primary antibodies against active Caspase-3 (AB3623) (MerckMillipore, Darmstadt, Germany) were used in accordance with the standard protocols. Concisely, 5 μm thick hepatocyte sections were deparaffinized, and then rehydrated. Slides were incubated with horseradish peroxidase (HRP) conjugated secondary antibodies. Visualization of immune reactions were done using

Table 1
Gene specific primers sequence.

Gene of interest	Primer sequence	Reference sequence	Product size
Hprt-1 Forward	5′-CCTAAAAGACAGCGGCAAGT-3′	NM_012583.2	173
Hprt-1 Reverse	5′-AATCAAAAGGGACGCAGCAA-3′		
TRAIL Forward	5′-GCTTCAGTCAGCACTTCACG-3′	NM_145681.2	179
TRAIL Reverse	5′-GTCCCAAAAATCCCCATCTT-3′		
TRAILR2 Forward	5′-CTCACCACAACACGGAACCT-3′	NM_001108873.1	153
TRAILR2 Reverse	5′-TGAGACGGTCCCAGGAGTTA-3′		
Caspase-3 Forward	5′-GGAGCAGTTTTGTGTGTGTGA-3′	NM_012922.2	191
Caspase-3 Reverse	5′-TGTCTCAATACCGCAGTCCA-3′		
Caspase-9 Forward	5′-TGGCATACACCCTGGACTC-3′	NM_031632.1	193
Caspase-9 Reverse	5′-GCCGTGACCATTTTCTTAGC-3′		
Caspase-8 Forward	5′-CCTTTCTCCTCCCTCTGACCTC-3′	NM_022277.1	193
Caspase-8 Reverse	5′-GTAACCTGTCGCCGAGTCCC-3′		
TGF-β1 Forward	5′-CCGCAACAACGCAATCTATGA-3′	NM_021578.2	89
TGF-β1 Reverse	5′-GCACTGCTTCCCGAATGTCT-3′		
Bcl-2 Forward	5′-AGGATAACGGAGGCTGGGATG-3′	NM_016993.1	179
Bcl-2 Reverse	5′-TATTTGTTTGGGGCAGGTCT-3′		

Hprt-1: Hypoxanthine phosphoribosyl transferase-1, TRAIL: Tumor necrosis factor-related apoptosis-inducing ligand, TRAIL-R2: Tumor necrosis factor-related apoptosis-inducing ligand receptor-2, BCL-2: B cell lymphoma-2.

Table 2
Effect of thymoquinone (TQ) on body weight, liver weight and liver index of rats.

Group	n	Body weight (g)	Liver weight (g)	Liver index
Control	10	232.5 ± 4.4	5.2 ± 0.4	2.3 ± 0.2
CMC	10	233.2 ± 3.1	5.3 ± 0.2	2.3 ± 0.1
HCC	10	185.8 ± 8.1[a]	8.9 ± 0.3[a]	4.8 ± 0.2[a]
HCC + TQ	10	225 ± 5.8[b]	7.2 ± 0.4[c]	3.1 ± 0.2[d]

Each value represents the mean ± SEM.
CMC: Carboxymethyl cellulose, HCC: hepatocellular carcinoma.
[a] $P < 0.0001$ compared with control group.
[b] $P < 0.001$ compared with HCC group.
[c] $P < 0.01$ compared with HCC group.
[d] $P < 0.0001$ compared with HCC group.

3,3′-diaminobenzidine tetra hydrochloride reagent (Genemed, Biotechnologies INC., USA) following counter-staining with hematoxylin under light microscope.

2.9. Calculation of apoptotic index

Apoptotic index is measured as number of apoptotic cells per 10 high power fields (HPFs) [24].

2.10. Quantitative, real-time PCR

Direct-zol™ RNA Miniprep extraction kit (ZYMO RESEARCH, USA) was used to extract total RNA from hepatic tissues according to the manufacturer's protocol. Then, 1 μg RNA was used for c-DNA synthesis using SensiFAST™ cDNA Synthesis Kit (Bioline, USA), in accordance with the manufacturer's instructions. RT-PCR was done using SensiFAST™ SYBR® No-ROX Kit (Bioline, USA) in Piko Real-PCR System (Thermo Fisher Scientific Inc., USA) based on the instructions of the manufacturer. The sequence of primers is represented in Table 1. TGF-β1, TRAILR2, TRAIL, caspase-3, caspase-9, caspase-8 and Bcl-2 mRNA levels were normalized to rat hypoxanthine phosphoribosyl transferase-1 (Hprt-1). mRNA levels were quantified using $2^{-\Delta\Delta CT}$ method.

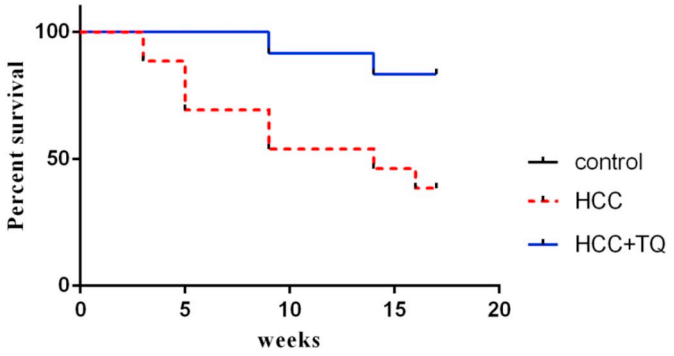

Fig. 1. Kaplan-Meier survival percent curve of rats in control, hepatocellular carcinoma (HCC) and HCC + Thymoquinone (TQ) groups.

2.11. Statistical analysis

Results were evaluated by one-way ANOVA then Tukey's post-hoc. Rats' survival was assessed using the Kaplan-Meier method. The results of necro-inflammatory and immunohistochemical scoring were analyzed by the non-parametric Kruskal-Wallis test followed by Dunn's method. Statistical tests were carried out using Graph Pad Prism 6.01 (Graph Pad Software, San Diego, CA, USA). Data were expressed as

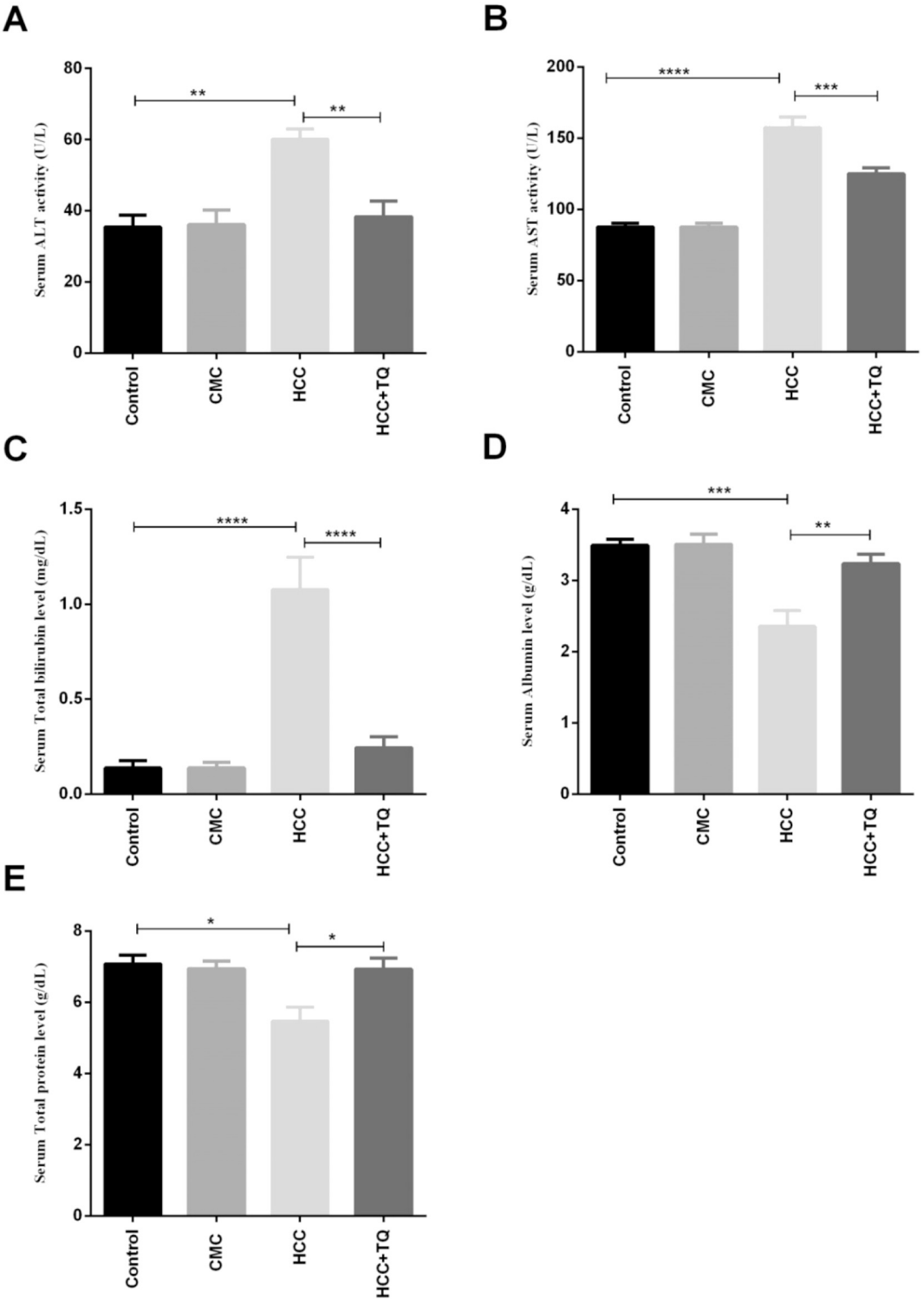

Fig. 2. Thymoquinone (TQ) improved hepatocyte functions.

Animals were divided into control, Carboxymethyl cellulose (CMC) (received the drug vehicle (0.5% CMC)), hepatocellular carcinoma (HCC) (treated with thioacetamide (TAA) 200 mg/kg twice weekly for 16 weeks) and HCC + TQ (treated with TQ 20 mg/kg daily along with TAA injection for 16 weeks) groups. Liver functions were assessed in different groups by measuring serum alanine aminotransferase (ALT) (A), aspartate aminotransferase (AST) (B) activities, total bilirubin (C), albumin (D) and total protein (E) levels. Bars represent Mean ± SEM. (*$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$, ****$P \leq 0.0001$).

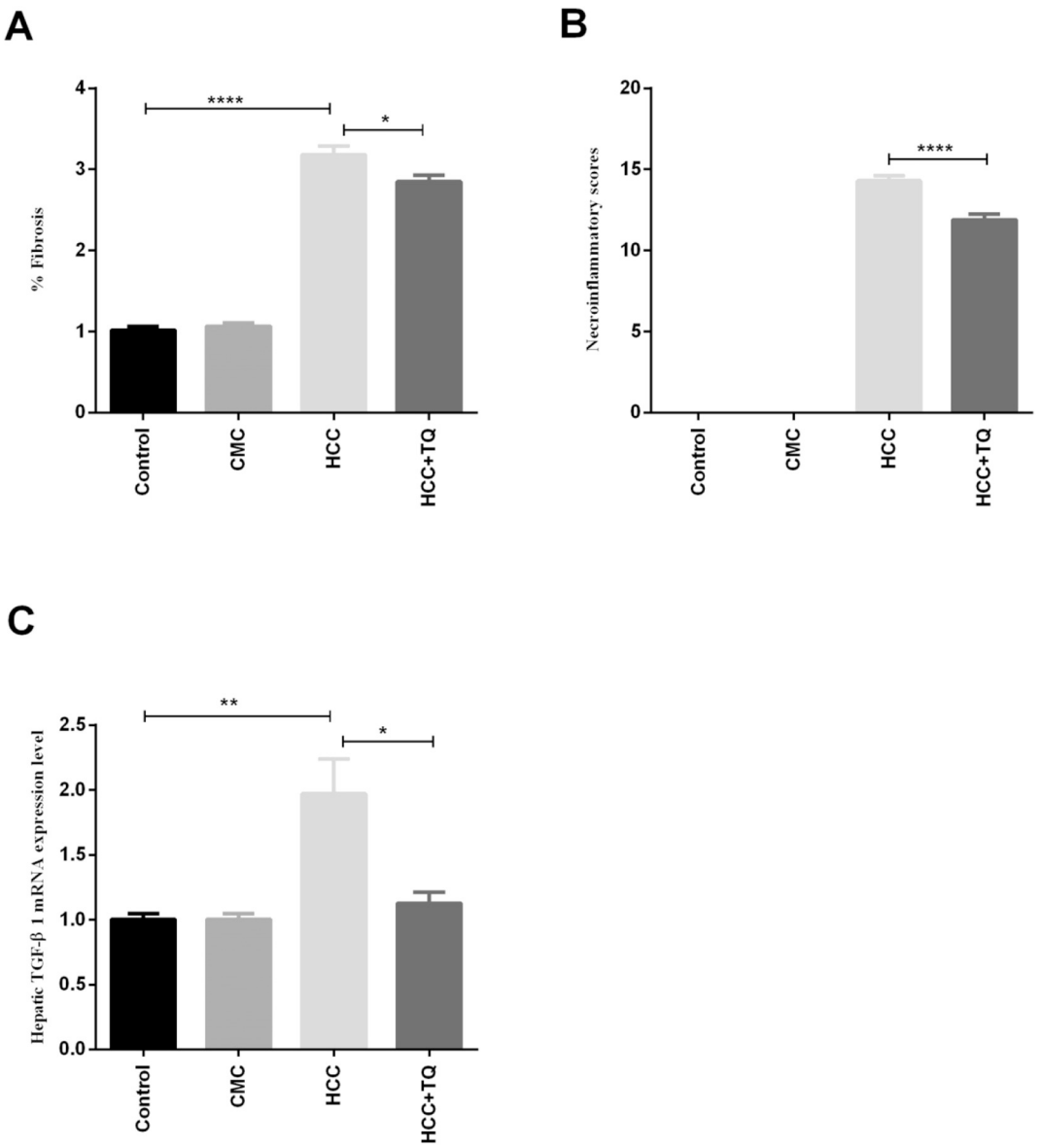

Fig. 3. Thymoquinone (TQ) decreased fibrosis percentage, necroinflammatory scores and mRNA level of Transforming growth factor-beta 1 (TGF-β1). Fibrosis and necrosis were quantified in the different groups using % fibrosis (A) and necroinflammatory scores (B). TGF-β1 mRNA expression level was assessed by PCR technique and represented in the different groups as indicated in panel (C). Bars represent Mean ± SEM. (*$P \leq 0.05$, **$P \leq 0.01$, ****$P \leq 0.0001$).

mean ± standard error of mean (SEM). Statistical significance was considered at P values of < 0.05.

3. Results

3.1. TQ reduced liver injury and increased survival percent

We firstly assessed the effect of TQ on body weight, liver weight and liver index of rats. Body weight was significantly decreased ($P < 0.0001$) in HCC group as compared to control group, while it was significantly increased ($P < 0.001$) in HCC + TQ group as compared to HCC group. Liver weight and index were significantly increased in HCC group as compared to control group, while they were significantly decreased in HCC + TQ group as compared to HCC group (Table 2). In addition, rats' survival percent was 38.5% in HCC group, while HCC + TQ group showed an increase in survival percent to 83.3% (Fig. 1).

3.2. TQ improved hepatocyte functions

HCC group showed significant increase in both serum ALT and AST activities as well as serum bilirubin level compared to control group (Fig. 2A, B and C). On the contrary, TQ significantly decreased both serum ALT and AST activities as well as serum bilirubin level by 1.6, 1.3 and 4.4 folds, respectively compared to HCC group.

TAA significantly decreased albumin and total protein level, while, TQ significantly increased serum albumin and total protein level by 1.4 and 1.3 folds, respectively as compared to HCC group (Fig. 2D and E). It is noteworthy that HCC + TQ group showed non-significant difference in serum ALT activities, bilirubin, albumin and total protein levels as compared to both control group and CMC group. There was non-significant difference between CMC group and control group regarding serum ALT, AST activities, bilirubin, albumin and total protein levels.

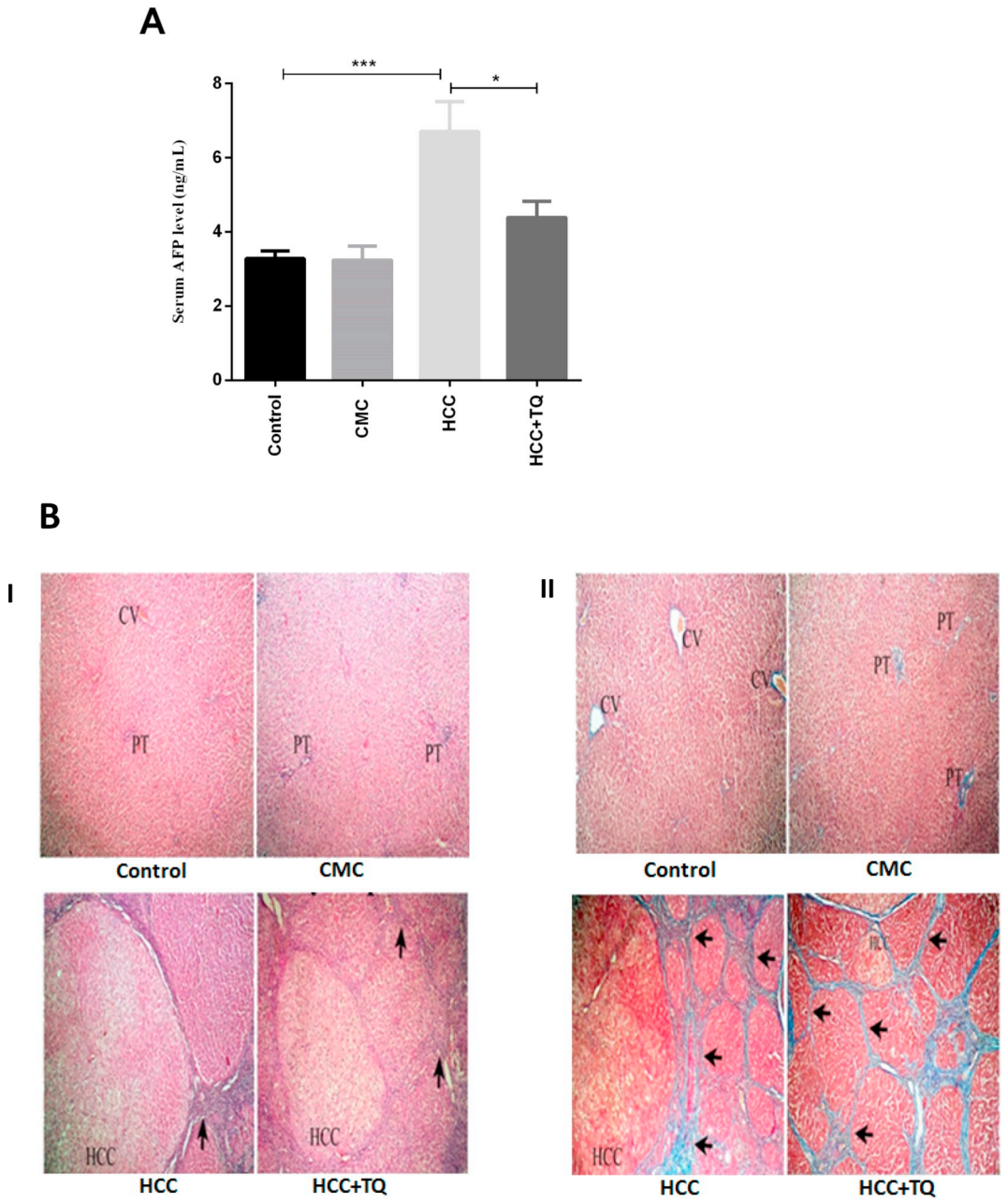

Fig. 4. Thymoquinone (TQ) protected against hepatocellular carcinoma (HCC). Antitumor effect of TQ was assessed in the different groups by; (A) measuring serum Alpha-Fetoprotein (AFP) level. Bars represent Mean ± SEM (*$P \leq 0.05$, ***$P \leq 0.001$). (B) Histopathological examination of liver tissue. Arrows represent fibrous septa and necroinflammatory changes. H&E are used in panel (I) and Masson trichrome stain is used in panel (II), ×100.
CV: central vein, PT: portal tract.

3.3. TQ decreased fibrosis percentage, necroinflammatory scores and mRNA level of TGF-β1

TAA induced a significant increase in fibrosis percentage, while TQ significantly decreased fibrosis percentage by 1.1 fold ($P < 0.05$) as compared to HCC group (Fig. 3A). Also, there was non-significant difference in fibrosis percentage between CMC group and control group. HCC group showed high necroinflammatory scores (Fig. 3B). On the other hand, HCC + TQ group showed significant decrease in necroinflammatory scores ($P < 0.0001$) in comparison with HCC group.

As shown in Fig. 3C, there was 2 fold significant increase in hepatic TGF-β1 mRNA level in HCC group compared to control group, while, HCC + TQ group showed 1.8 fold significant decrease ($P < 0.05$) in hepatic TGF-β1 mRNA level as compared to HCC group. There was non-significant difference in hepatic TGF-β1 mRNA level between HCC + TQ and both control group and CMC group.

3.4. TQ protected against HCC

AFP level was 2.1 fold significantly increased in HCC group as compared to control group, while HCC + TQ group showed 1.5 fold ($P < 0.05$) significant decrease in serum AFP compared to HCC group. Moreover, HCC + TQ group showed non-significant difference in serum AFP level as compared to both control group and CMC group (Fig. 4A).

Hematoxylin and eosin (H&E) stained liver sections taken from control group and CMC group revealed normal liver structure as well as Masson trichrome stained liver sections taken from the same groups showed minimal collagen fibers. There were great tumor nodules,

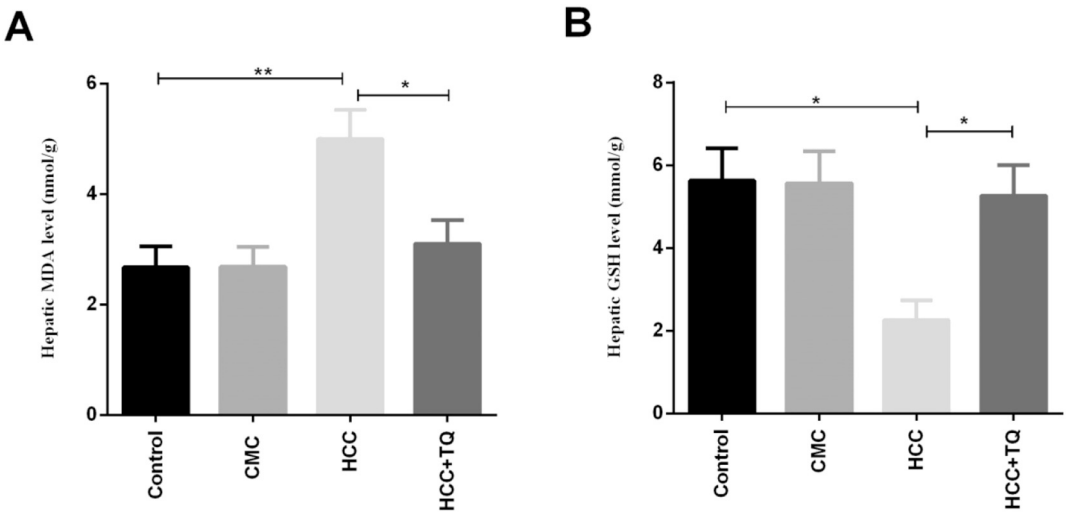

Fig. 5. Thymoquinone (TQ) improved the antioxidant capacity in rats.
Oxidative stress was assessed by measuring the levels of hepatic Malondialdhyde (MDA) (A) and Glutathione (GSH) (B). Bars represent Mean ± SEM. (*$P \leq 0.05$, **$P \leq 0.01$).

significant necroinflammatory changes and rise in the amount and thickness of fibrous septa in HCC group. Little tumor nodules associated with thinner fibrous septa and less necroinflammatory changes were found in HCC + TQ group (Fig. 4B).

3.5. TQ improved the antioxidant capacity in rats

Hepatic MDA level was significantly increased by 85.2%, while hepatic GSH level was significantly decreased by 59.7% in HCC group as compared to control group (Fig. 5A and B). TQ significantly decreased hepatic MDA level by 1.7 fold ($P < 0.05$) and increased hepatic GSH level by 2.3 fold ($P < 0.05$) as compared to HCC group. HCC + TQ group also showed non-significant difference in hepatic MDA and GSH levels as compared to both CMC group and control group. Moreover, there was non-significant difference in hepatic MDA and GSH levels between CMC group and control group.

3.6. TQ upregulated hepatic TRAIL/TRAILR2 and downregulated Bcl-2 mRNA levels

HCC group showed significant downregulation in hepatic TRAIL ($P < 0.01$) and TRAILR2 ($P < 0.0001$) mRNA levels as compared to control group. HCC + TQ group showed significant upregulation of hepatic TRAIL and TRAILR2 mRNA levels, compared to HCC, CMC and control groups (Fig. 6A and B).

Regarding hepatic Bcl-2 mRNA level (Fig. 6C), it was 10 fold significantly increased in HCC group when compared to control group. On the contrary, it was 3.2 fold significantly decreased in HCC + TQ group as compared to HCC group. There was non-significant difference in hepatic Bcl-2 mRNA level between HCC + TQ group and both CMC and control group. Furthermore, CMC group showed non-significant difference in hepatic TRAIL, TRAILR2 and Bcl-2 mRNA levels as compared to control group.

3.7. TQ induced caspase activation

As shown in Fig. 7A, B and C, the hepatic caspase-8, -9 and -3 mRNA levels were significantly decreased in HCC group by 38.3 ($P < 0.0001$), 8.1 ($P < 0.0001$) and 26 ($P < 0.001$) folds, respectively, in comparision with control group. TQ significantly increased hepatic caspase-8, -9 and -3 mRNA levels as compared to HCC, CMC and control groups. Also, there was non-significant difference in hepatic caspase-8, -9 and -3 mRNA levels between CMC and control groups.

In order to make sure that tumor regression was due to apoptosis induction, we assessed active caspase-3 expression and observed that TQ significantly increased active caspase-3 expression when compared to HCC group (Fig. 8A, B and C).

4. Discussion

HCC is considered one of the major causes of cancer-related death globally and its incidence increases rapidly [1]. Such great incidence and grave prognosis of HCC make prevention a realistic approach for decreasing mortality rates [25]. Unfortunately, HCC is poorly responsive to available chemotherapeutic approaches [26]. Thus, several efforts have been made to discover effective and safe treatment approaches for liver cancer patients [27–29].

A great attention has been paid for natural compounds to assess their anti-tumor effect that deemed to be non-toxic to healthy cells [30]. TQ, the major bioactive constituent of black seeds, has shown promising pharmacological and therapeutic effects against in-vitro and in-vivo disease models [31]. There is an increasing research interest to evaluate the anti-tumor effect of TQ [31,32]. Thus, we were motivated to assess its hepatoprotective and anti-tumor effects against TAA-induced HCC.

TAA has been known since 1948 as a potent hepatotoxin that causes centrolobular necrosis associated with an increase in plasma transaminases and bilirubin [33] which comes in agreement with the significant increase in ALT, AST enzymes activity and bilirubin level in HCC group. The chronic application of TAA results in cirrhosis that progress leading to HCC in experimental animal models [29,34].

TAA-induced HCC was confirmed by two evidences: the first one was the marked increase of AFP level in HCC group which has been used in many studies for diagnosis of liver cancer [35,36]. The second evidence was the histopathological examination which revealed a marked increase in necroinflammatory changes, a prevalent thick fibrous septa and large tumor nodules in the liver of rats receiving TAA. Moreover, there were high percentage of fibrosis and necroinflammatory scores in HCC group.

Upon TQ administration, liver functions were improved. These results were in agreement with a previous study which proved that TQ has a hepatoprotective effect which was evident by the decreased leakage of ALT and AST from isolated rat hepatocytes [37]. Also, our results proved that CMC, in which TQ was suspended, did not alter liver functions by improving or worsening as evident through the non-significant difference between CMC and control groups in ALT, AST

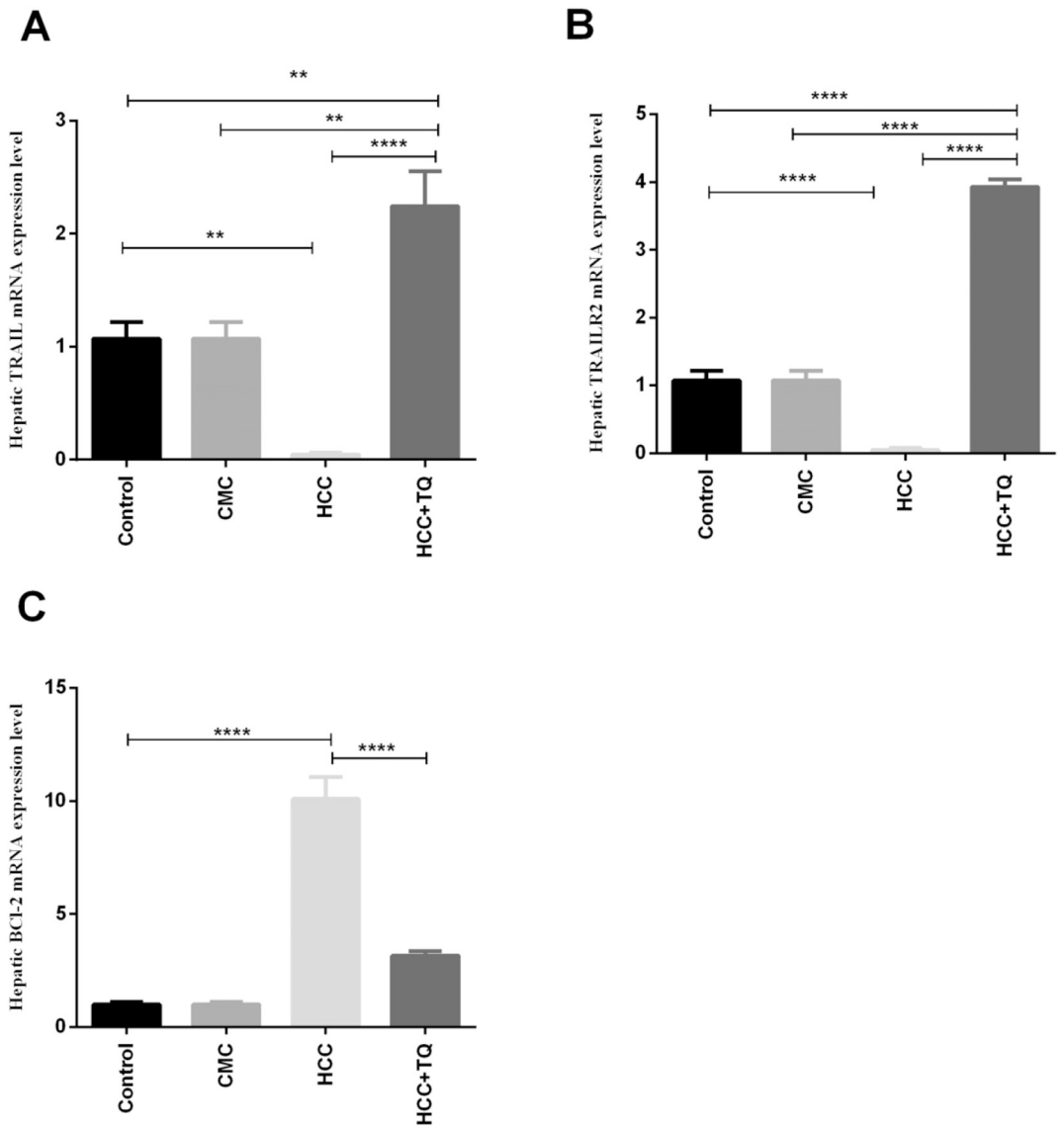

Fig. 6. Thymoquinone (TQ) upregulated hepatic TRAIL/TRAILR2 and downregulated B cell lymphoma-2 (Bcl-2) mRNA levels.
Effect of TQ on mRNA expression levels of TRAIL, TRAILR2 and anti-apoptotic Bcl-2 were assessed by PCR technique and represented in the different groups as indicated in panels (A), (B) and (C), respectively. Bars symbolize Mean ± SEM. (**$P \leq 0.01$, ****$P \leq 0.0001$).

activities, bilirubin, albumin and total protein levels.

TQ significantly decreased AFP level, percentage of fibrosis and necroinflammatory scores, compared to HCC group. In addition, HCC + TQ group showed reduction in tumor nodules and fibrous septa. Histopathological examination of liver tissue separated from HCC + TQ group reflected a promising anti-tumor effect of TQ. This comes in agreement with Sayed-Ahmed, et al. who reported that histopathological examination of liver sections isolated from rats receiving TQ reinforced the anti-tumor effect of TQ [38].

High level of Malondialdhyde (MDA) was seen as a marker for oxidative stress in end stage liver diseases [39]. Thus, its level was investigated as an index of lipid peroxidation. Among the various defense systems against cellular injury, the anti-oxidant Glutathione (GSH) was assessed [40].

This study showed that hepatocytes, separated from HCC group, were under oxidative stress as indicated by marked increase in MDA level. Furthermore, the anti-oxidant defense mechanism was failed as hinted by the decreased level of GSH in HCC group. This can be explained by the fact that TAA metabolism was associated with ROS generation which was found to be implicated in liver fibrosis, cirrhosis and subsequent HCC development and progression [41,42]. Upon TQ administration, MDA level was significantly decreased, while GSH level was significantly elevated which come in agreement with Sayed-Ahmed, et al. who reported that TQ has an anti-oxidant effect. This anti-oxidant effect represents a possible mechanism by which TQ can attenuate TAA-induced HCC [38].

Transforming growth factor-beta (TGF-β) pathway plays a crucial role in all stages of liver disease progression from initial liver injury to cirrhosis and HCC. TGF-β induces fibroblast activation, myofibroblast generation and extracellular matrix (ECM) deposition that may lead to cirrhosis, dysplastic nodules and HCC. Being recognized as a major profibrogenic cytokine, targeting TGF-β signaling pathway has been explored with respect to the inhibition of liver disease progression [10,43,44].

In our work, TGF-β1 mRNA level was significantly increased in HCC group which comes in agreement with Idobe et al. who reported that TGF-β1 was found to be overexpressed in HCC cells [45]. On the contrary, TQ significantly decrease TGF-β1 mRNA level as compared to HCC group. Consistent with our results, Ammar et al. reported that TQ has an anti-inflammatory effect [46]. Such suppression in TGF-β1

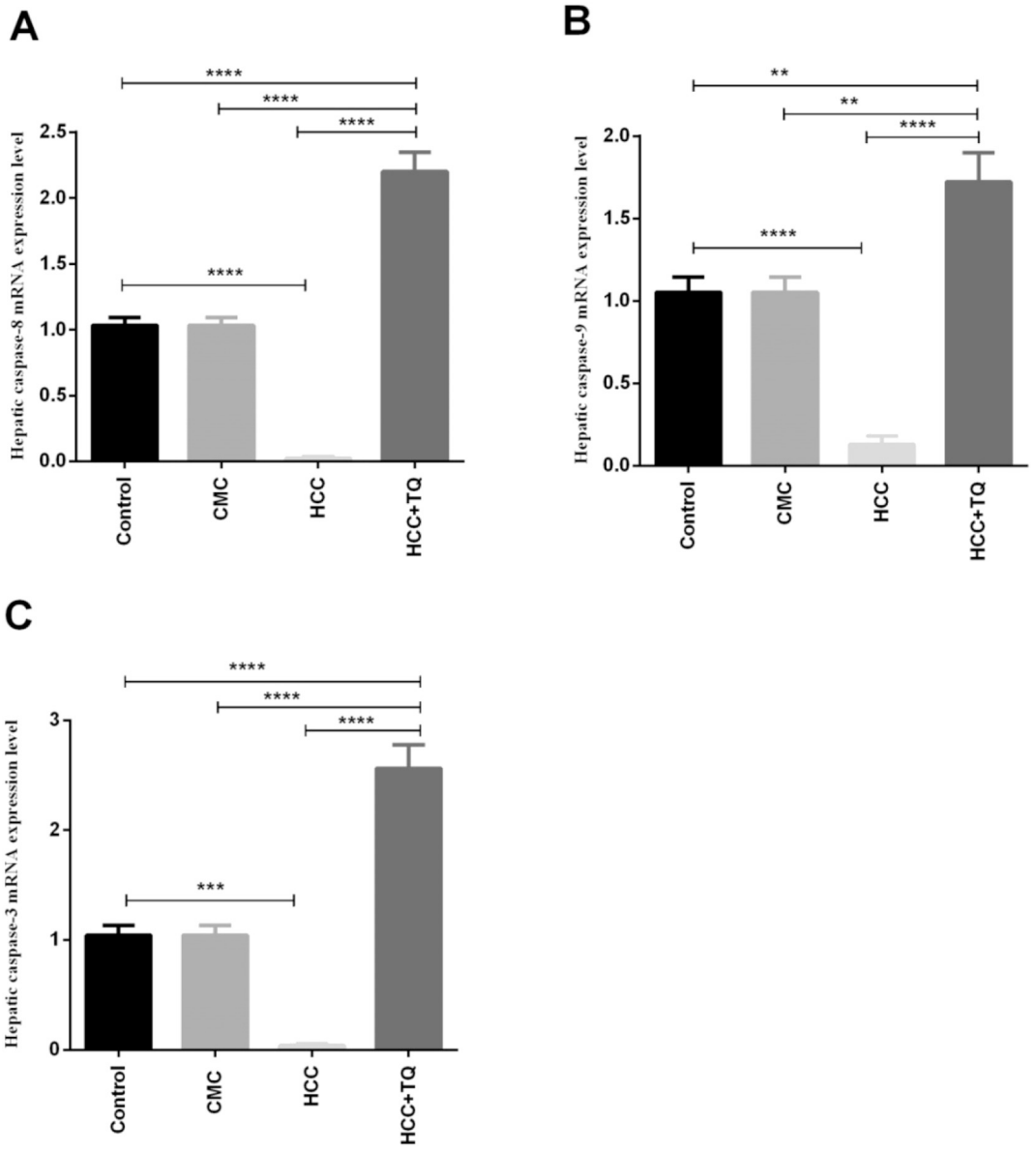

Fig. 7. Thymoquinone (TQ) increased caspases gene expression level.
Caspase cascade activation was appraised by measuring hepatic expression levels of caspase-8 (A), caspase-9 (B) and caspase-3 (C). Bars symbolize Mean ± SEM. (**$P \leq 0.01$, ***$P \leq 0.001$, ****$P \leq 0.0001$).

mRNA level indicated the hepatoprotective effect of TQ and represents a proposed mechanism by which it can suppress HCC progression.

Apoptosis induction is the basic principle in cancer treatment [47]. There is a growing recognition of the importance of restoring TRAIL sensitivity to provide a safe, selective and effective therapy for HCC [5]. Indeed, downregulation of TRAIL-R2 and overexpression of Bcl-2 make cancer cells resist chemotherapeutic treatment [8,48,49]. Therefore, we were motivated to investigate the molecular mechanism behind sensitization of tumor cells toward apoptosis via measuring TRAIL, TRAILR2 and Bcl-2 mRNA levels.

Our results revealed apoptosis evasion by cancer cells which was evident by the marked decrease in TRAIL and TRAILR2, as well as, the notable increase in Bcl-2 mRNA levels in HCC group. This comes in agreement with previous studies reporting that many HCC cells showed marked decrease in TRAIL with increase in anti-apoptotic proteins expression levels [50,51]. Also, it was stated that hepatitis B virus decreases TRAIL-R2 leading to the development of TRAIL resistant HCC cells [48,49].

TQ upregulated TRAIL and TRAILR2 and downregulated Bcl-2 mRNA levels. These results revealed the ability of TQ to sensitize TRAIL resistant cancer cells toward TRAIL-induced apoptosis. Also, the expression of TRAILR2 in HCC + TQ group surpassed control group that reflects a potent antitumor effect that is needed not only to achieve tumor regression, but also to decrease risks of development of more aggressive phenotypes which are implicated in tumor regrowth and treatment failure.

Once TRAIL binds to its receptor, DISC is formed and Fas-associated protein with death domain (FADD) is recruited to interact with the death domain (DD) resulting in procaspase-8/10 activation [7,52]. This could explain the high expression level of caspase-8 found in HCC + TQ group indicating TQ's ability to trigger extrinsic apoptotic pathway.

Besides, caspase-8 activation induces the cleavage of pro-apoptotic BH3 interacting domain (Bid) into truncated Bid (tBid) [53]. It is important to emphasize that the anti-apoptotic Bcl-2 protein plays a crucial role in the suppression and prevention of inner mitochondrial membrane potential disruption as well as subsequent release of

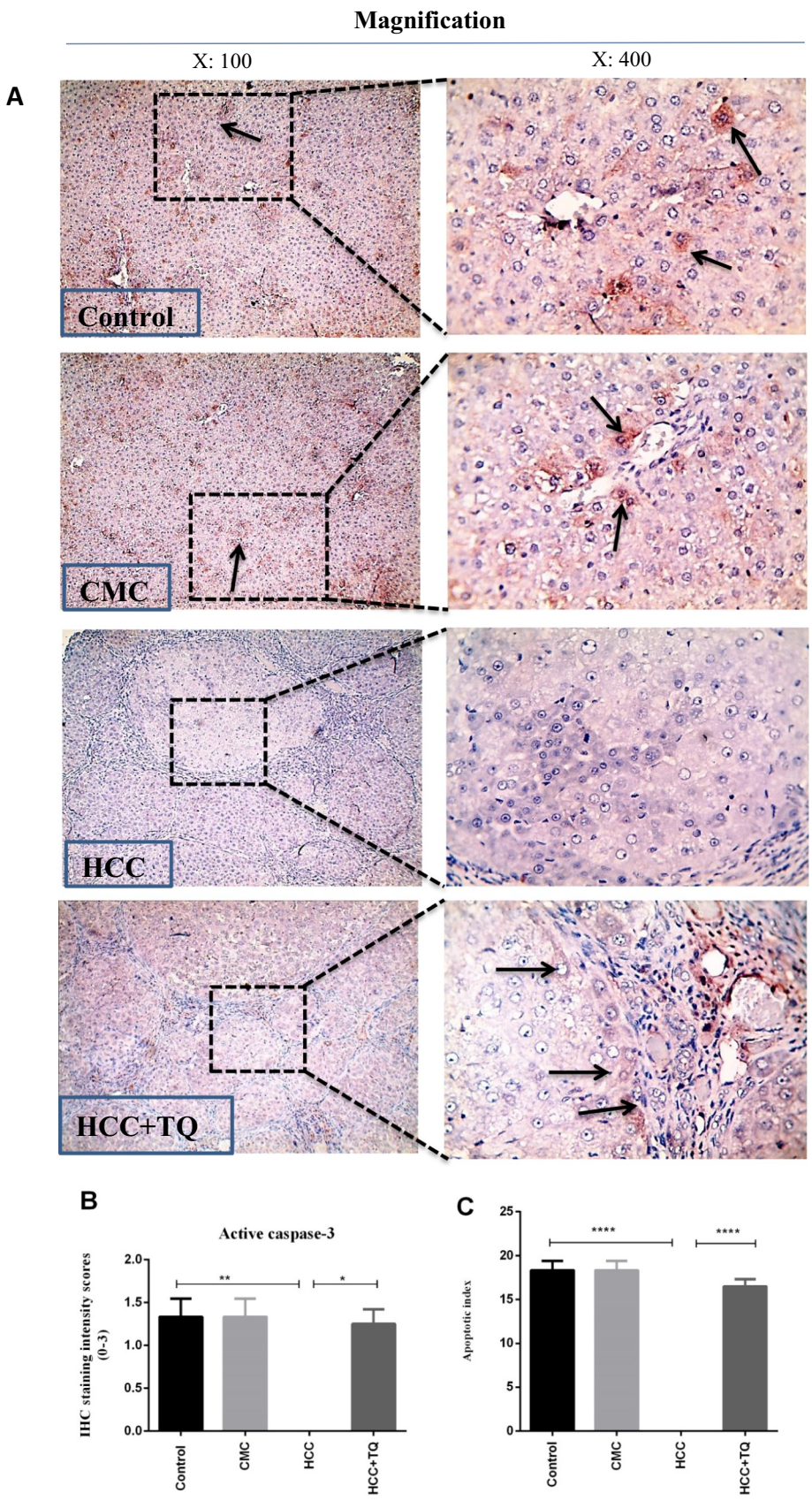

Fig. 8. Thymoquinone (TQ) increased active caspase-3 expression level.

Apoptosis induction was confirmed by assessment of active caspase-3 expression level; (A) microscopic pictures of liver sections immunohistochemical (IHC)-stained against caspase-3 showing mild positive staining as indicated by brown color in few hepatocytes score 1 in control group and CMC group, negative staining score 0 in HCC group, very mild positive staining in hepatocytes particularly at the periphery of cirrhotic nodules score ± 1 in HCC + TQ group. Black arrows point to positive staining. IHC counterstained with Mayer's hematoxylin. X: 100 bar 100 (left panel) and X: 400 bar 50 (right panel). (B) Statistical analysis of IHC staining intensity in four experimental groups showing significant increase in caspase-3 staining scores in HCC + TQ group when compared with HCC group. (C) Statistical analysis of apoptotic index in four experimental groups showing significant increase in number of apoptotic cells in group HCC + TQ group when compared with HCC group.

(B, C) Bars symbolize Mean ± SEM. (*$P \leq 0.05$, **$P \leq 0.01$, ****$P \leq 0.0001$).

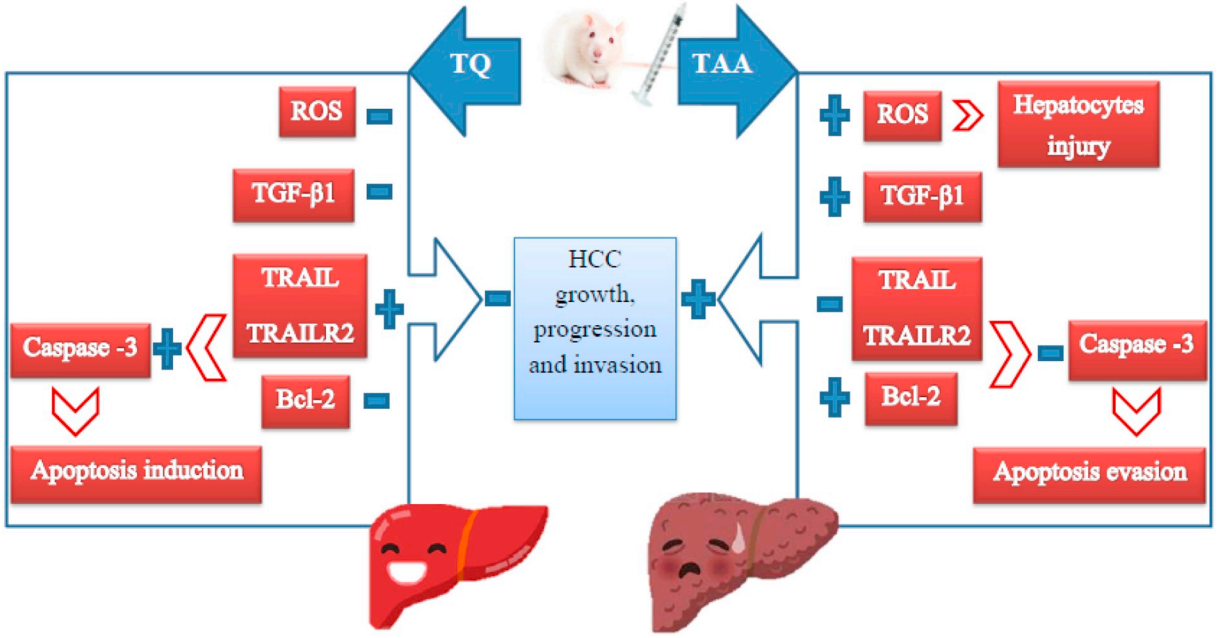

Fig. 9. The proposed mechanism by which thymoquinone (TQ) can suppress thioacetamide (TAA) induced hepatocellular carcinoma (HCC).
(+: increase, −: decrease).
(Bcl-2: B-cell lymphoma-2, ROS: reactive oxygen species, TGF-β1: transforming growth factor-β1, TRAIL: tumor necrosis factor-related apoptosis inducing-ligand, TRAILR2: tumor necrosis factor-related apoptosis inducing-ligand receptor 2).

cytochrome *c* [54,55]. Therefore, both decrease in Bcl-2 gene expression level and activation of Bcl-2-associated X protein (Bax) by tBid trigger the formation of pore in mitochondrial outer membrane and the release of cytochrome *c*. After that, apoptosome is formed and triggers caspase-9 release [56–58]. Thus, in our work, it was not surprising to find caspase-9 expression level elevated in HCC + TQ group reflecting TQ's ability of trigger intrinsic apoptotic pathway.

Caspase-3 is known to be the effector caspase in apoptosis execution [59]. Therefore, apoptosis induction was further reinforced through measuring both gene and protein expression level of caspase-3. Caspase-3 gene and protein expression levels were found to be significantly elevated in HCC + TQ group. Such increased caspase-3 expression level was an expected outcome of the increased caspase-8 and caspase-9 expression levels and the decreased Bcl-2 expression level.

5. Conclusion

This study clearly demonstrated the ability of TQ to suppress HCC development and progression via decreasing oxidative stress, suppression of TGF-β1 and induction of TRAIL-mediated apoptosis (Fig. 9). However, further researches may be required to evaluate TQ antitumor activity in other tumor models and further clinical studies.

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