RESEARCH ARTICLE

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The Cytotoxic Effect of Thymoquinone Enhance on HepG2 Cell Line due to Induction of Fenton Reaction by Hydrogen Peroxide: An In Vitro and In Silico Study

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Abstract

Objective: Thymoquinone (TQ) is a component derived from the volatile oil of Nigella sativa. Fenton reaction induction is a well-known strategy to prevent the growth of cancer cells which can stimulate by hydrogen peroxide. This study was designed to investigate the TQ effects on hydrogen peroxide-induced cytotoxicity. **Methods:** In this study, HepG2 cell survival, reactive oxygen species (ROS) production, cell membrane integrity, and changes of superoxide dismutase (SOD)/ catalase (CAT) activity were evaluated following incubation of HepG2 cells with 31 µM hydrogen peroxide and different concentrations of TQ (18.5, 37 and 75 µM). In addition, molecular docking studies on the interference of TQ with CAT/SOD enzymes were investigated. **Results:** Our findings showed that TQ low concentration can increase the survival of HepG2 cells when exposed to hydrogen peroxide, and on the contrary, its high concentration can potentiate cytotoxicity induced by hydrogen peroxide. The TQ alongside hydrogen peroxide increased the production of ROS, which was related to increase CAT and SOD activity in the HepG2 cells. Molecular docking findings showed that TQ effects on the formation of free radicals were not related to its chemical interference with the structure of the SOD/CAT molecules. **Conclusion:** Fenton reaction induction may increase the effectiveness of TQ in preventing HepG2 cells proliferation.

Keywords: Thymoquinone- hydrogen peroxide- oxidative stress- superoxide dismutase- catalase

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Introduction

Thymoquinone (TQ; 2-isopropyl-5-methyl-1,4-benzoquinone) is the most significant active constituent in the volatile oil of Nigella sativa seeds (18.4-24%) (Darakhshan et al., 2015; Nili-Ahmadabadi et al., 2018; Chaleshtori et al., 2020). This compound exists in tautomeric forms such as the enol form, keto form, and a mixture (Almajali et al., 2021). Among these, its keto form is responsible for the main pharmacological properties of TQ (Ahmad et al., 2019). Previous studies have reported TQ therapeutic potential against oxidative damage by xenobiotic agents in various tissues (Nili-Ahmadabadi et al., 2018; Bilgiç et al., 2023). In addition, there is evidence of the TQ effects on preventing the growth and proliferation of different types of cancer cells (Fatfat et al., 2019; Almajali et al., 2021). Induction of P27 and P21 proteins (Alhmied et al., 2021), inhibition of cyclins and cell cycle arrest (Homayoonfal et al., 2022), an increase in the activity of caspases 9 and 3, followed by an enhancement in the cellular BAX/bcl2 ratio (Samarghandian et al., 2019), induction of PPAR-γ activity (Woo et al., 2011), and an increase in the formation of free radicals are among the most important known mechanisms of TQ in the occurrence of apoptosis and preventing the proliferation of cancer cells.

The Fenton reaction is the reaction between iron and hydrogen peroxide, generating hydroxyl radical, which is highly reactive and toxic to living cells. This reaction has been challenged for use in directly attacking cancer cells, but it is difficult to treat them because of the low amounts of generated hydroxyl radicals (Abe et al., 2022). In recent years, extensive studies have been reported regarding the induction of Fenton reaction in cancer cells by various compounds (Wang et al., 2018; Zhou et al., 2021).

The superoxide dismutase (SOD)/ catalase (CAT) system plays an important role in the detoxification of reactive oxygen species (ROS) and plays an important role in the production and destruction of hydrogen peroxide (Firozian et al., 2020). Following the activity

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of intracellular SOD enzyme, superoxide anions are converted into oxygen and hydrogen peroxide molecules and conditions for CAT enzyme activity are provided (Ahmed Amar et al., 2019). Then, the CAT enzyme metabolizes hydrogen peroxide into water molecules and molecular oxygen (Nandi et al., 2019). The interaction of natural compounds with SOD/CAT enzymes can play an important role in toxicity of reactive oxygen species (Ghasemi et al., 2022).

Hepatocellular carcinoma is the sixth most common cancer in the world (Rashed et al., 2020). Several studies regarding the use of therapeutic supplements to improve the efficiency of anticancer drugs are being conducted. For instance, Aslan et al. (2021) showed that TQ could decrease the growth and proliferation of MCF-7 cell lines through the formation of reactive oxygen species. It seems that the interference of free radicals resulting from Fenton reaction and prooxidant drugs can effectively prevent the growth and proliferation of cancer cells. According to this hypothesis, the current study was designed to investigate the interference of TQ and hydrogen peroxide on the proliferation of HepG2 cells and the SOD/CAT intracellular system.

Materials and Methods

Chemicals

All chemicals were purchased from Merck, Darmstate, Germany unless otherwise stated. Thymoquinone (98% purity, CAS no: 490-91-5), 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT; CAS no: 298-93-1), 2',7'-dichorodihydrofluorescein-diacetate (DCFH-DA; CAS no: 4091-99-0), Nicotinamide adenine dinucleotide phosphate (NADPH; CAS no: 24292-60-2) and adenosine 5'-diphosphate (ADP) sodium salt (CAS no: 20398-34-9) were purchased from Sigma-Aldrich Chemical Company (St. Louis, MO, USA).

Molecular docking analysis

The three-dimensional (3D) structures and PDB files of the SOD (PDB ID: 2jlp) and CAT (PDB ID: 1F4J) enzymes were obtained from the www.rcsb.org server. The energy minimization of PDB files was done by Swiss-PdbViewer software. Additionally, the 3D structure of TQ (CID: 10281) was obtained from the Pubchem database and then converted to a PDB file and optimized using Avogadro V:1.2 software.

The AutoDock V.4.2.6 software was utilized for docking TQ to the SOD and CAT enzymes, after minimization the energy of all molecules. The TQ file was prepared as a ligand and the SOD and CAT files were prepared as receptors in PDBQT format using Auto-Dock Tools. All possible binding sites were explored through blind docking with a grid box that covered all dimensions of the receptor. The grid box size for the CAT and SOD enzymes were 90 Å × 90 Å × 90 Å and 120 Å × 120 Å × 130 Å, respectively. A standard docking protocol with the addition of Gasteiger atomic charges and the assignment of default atom-types was used. Also, the Kollman bar was used to attribute the bar to the protein. A genetic algorithm was used to implement the docking and a maximum of 200 runs was considered. The lowest binding energy was the best run of 200 independent runs that were done in the docking procedure. The Discovery Studio Visualizer v.1.6 and LigPlot+ software was used to determine all interactions between the ligand and receptors in each binding site.

Chemical interactions of TQ and hydrogen peroxide

Firstly, a solution of TQ (6 µM) was prepared in 96% ethanol. Then, the changes in the absorption spectrum of TQ in the wavelength range of 220-500 nm were detected using a spectrophotometer (Perkin Elmer Lambda 25 UV/Visible Spectrophotometer) in the presence of different concentrations of hydrogen peroxide (15.6-125 µM).

Cell culture

The HepG2 cell line was obtained from the Iranian Biological Resource Center, Tehran, Iran. The cell line was cultured into a cell culture flasks (25 cm^2), containing HG-DMEM supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin, and maintained in a humidified atmosphere of 5% CO2 at 37 °C.

Cytotoxicity assay

A density of 10,000 live cells were added to wells of a 96-well plate and incubated for 24 h. Afterwards, different concentrations of TQ (4.6-1200 µM) and hydrogen peroxide (7.5-2000 µM) were added to separate wells and incubated for 24 h. The percentage of cell viability was measured using the 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) reagent as explained by Ebrahimifar et al. (2017). Briefly, 5 mg/ml of MTT reagent was added to each well and incubated for 4 hours at 37 °C. Then, 100 µl of dimethyl sulfoxide (DMSO) solvent was added to each well to dissolve the formazan crystals, and the absorbance changes were read at a wavelength of 570 nm using a microplate reader (Synergy HTX, Biotek, USA). It should be noted that cytotoxicity was measured as the concentration that inhibits the growth of 50% of cells (IC_{50}).

After determination of IC_{50} for TQ, the HepG2 cells were pretreated with concentrations of 18.5, 37 and 75 µM of TQ (~10-40% of IC_{50}) before being exposed to various concentrations of hydrogen peroxide. After 24 h, the IC_{50} of hydrogen peroxide was determined by the MTT method following exposure to TQ.

Reactive oxygen species

The HepG2 cells were pretreated with concentrations of 18.5, 37 and 75 µM of TQ (~10-40% of IC_{50}) before being exposed to 31.2 µM of hydrogen peroxide (~ 10% of IC_{50}). After 24 h, the culture medium was removed. Then, buffer assay containing 20 mM NaH2PO4, 0.1 mM FeCl3, 130 mM KCl, 1.7 mM ADP, 5 mM MgCl2, 20 mM Tris–HCl, and 0.1 mM NADPH as well as 5 µL of the DCFH-DA (5 µM) was added to each well in 96-well dark plate and incubated at 37 °C for 15 min. The changes of absorption was read by a fluorometer set at a wavelength of EX/EM = 485/528 nm every 15 min for 2 h. Finally, the data were reported as a percentage of the control (Nili-Ahmadabadi et al., 2013).

SOD and CAT assay

The HepG2 cells were pretreated with concentrations of 18.5, 37 and 75 µM of TQ (~10-40% of IC_{50}) before being exposed to 31.2 µM of hydrogen peroxide (~ 10% of IC_{50}). After 24 h, the culture medium was removed and the cells was lysed by PBS buffer containing a protease inhibitor enzyme. The resulting product was used to measure the activity of the SOD and CAT enzymes. Briefly, SOD enzyme activity was measured by inhibiting the production of a blue-colored formazan at 560 nm based on kit brochure purchased from ZellBio GmbH Company, Germany (Cat no: RK03200). Additionally, CAT enzyme activity was determined based on absorption changes of purpald-formaldehyde complex at 560 nm, as explained in kit brochure purchased from Kiazist Company, Iran (Cat no: KCAT96).

Cell membrane integrity

The HepG2 cells were pretreated with concentrations of 18.5, 37 and 75 µM of TQ (~10-40% of IC_{50}) before being exposed to 31.2 µM of hydrogen peroxide (~ 10% of IC_{50}). After 24 h, the culture medium was removed. The lactate dehydrogenase activity (LDH), as an index of cell membrane integrity, was assayed in culture medium at 340 nm, as explained in the kit brochure from Pars Azmun Company, Iran (Cat no: HITACHI917).

Statistical analysis

Data are expressed as Mean ± SD from five independent experiments. Comparisons between groups were analyzed, by the SPSS software version 18, using the one-way ANOVA with Tukey's test. The significance level was set at 0.05.

Results

Molecular docking analysis

The lowest binding energy (BE) from docking the TQ to the SOD enzyme was -5.38 kcal/mol, and the estimated inhibition constant (Ki) was 114.31 µM. There were 11 amino acid residues in the binding site, including Ser217, Ala218, Thr219, Leu220, Arg226, Ser384, Ala385, Leu387, Asp388, Ala389, and Arg393. Additionally, the lowest BE from docking TQ to the CAT enzyme was -5.34 kcal/mol, and the estimated Ki was 121.33 µM. There were 9 amino acid residues in the binding site, including Pro34, Gln53, Val55, Met339, Pro340, Ile343, Glu344, Ala418, and Glu420 (Figure 1 and Table 1).

Chemical interactions of TQ and hydrogen peroxide

In this study, no significant chemical interactions were observed between TQ and different concentrations of hydrogen peroxide in the absorption spectrum obtained from the wavelength range of 220-500 nm (Figure 2).

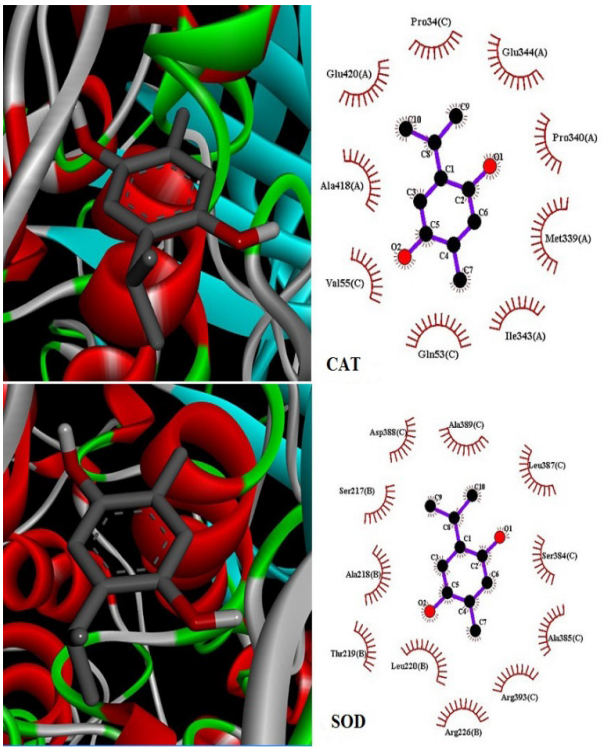

Figure 1. The 3-Dimensional Structures of Thymoquinone in the Binding Sites of Catalase (CAT) and Superoxide Dismutase (SOD) Enzymes. The amino acid residues that interacted with thymoquinone in the binding sites have been shown.

Cytotoxicity assay

As shown in Figure 3, the IC_{50} for TQ (Figure 3-A) and hydrogen peroxide (Figure 3-B) were determined to be 192.3 µM and 309.1 µM, respectively. Following the interaction effect of TQ and hydrogen peroxide, the IC_{50} obtained from the dose-response curve of hydrogen peroxide after exposure to concentrations of 18.5 and 37

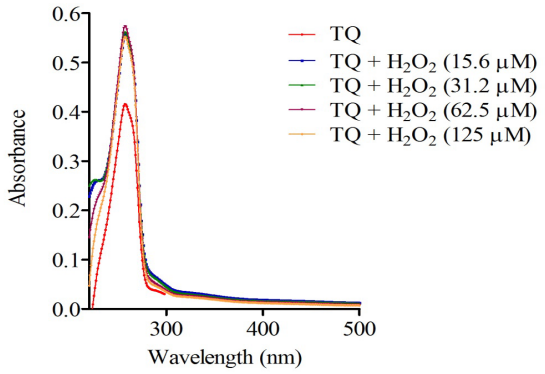

Figure 2. Chemical Interactions of Hydrogen Peroxide (H_2O_2) and Thymoquinone (TQ) at 220–500 nm.

Table 1. Docking Parameters of Thymoquinone into the Binding Sites

Ligand-protein	ESI (Ki) µM	BE kcal/mol	Amino acid residues in binding site
TQ-SOD	114.31	-5.38	Ser217, Ala218, Thr219, Leu220, Arg226, Ser384, Ala385, Leu387, Asp388, Ala389, Arg393
TQ-CAT	121.33	-5.34	Pro34, Gln53, Val55 , Met339, Pro340, Ile343, Glu344, Ala418, Glu420

ESI, Estimated inhibition constant; BE, Binding energy; CAT, Catalase; SOD, Superoxide dismutase; TQ, Thymoquinone.

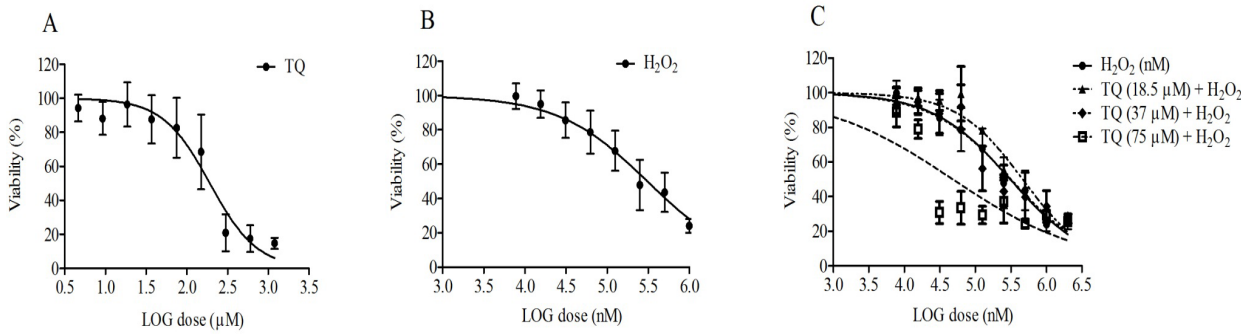

Figure 3. Dose-Response Curves of the MTT Assay in the HepG2 Cell Line Using Thymoquinone (A), hydrogen peroxide (B), and their combination (C). Data are expressed as Mean ± Standard deviation (SD) from five independent experiments. TQ, Thymoquinone; H_2O_2, hydrogen peroxide.

µM of TQ increased to 462.3 and 315.7 µM, respectively. On the other hand, TQ (75 µM) was able to decrease the IC_{50} of hydrogen peroxide by 48.2 µM (Figure 1-C).

Reactive oxygen species

The ROS production after exposing HepG2 cells to the dose of TQ (75 µM) and hydrogen peroxide (31.2 µM) increased significantly compared to the control group ($p < 0.05$ and < 0.001, respectively). The simultaneous treatment of cells with hydrogen peroxide and concentrations of 75, 37, and 18.5 µM of TQ caused a significant increase in the formation of reactive oxygen species compared to the control group and groups receiving equivalent doses of TQ (Figure 4-A).

CAT enzyme activity

As shown in Figure 4-B, the CAT activity in HepG2 cells after exposure to concentrations of 18.5, 37, and 75 µM of TQ increased significantly compared to the control group ($p < 0.01$, $p < 0.001$, and $p < 0.001$, respectively). Furthermore, hydrogen peroxide alone was able to significantly increase CAT activity in HepG2 cells. The simultaneous treatment of cells with different concentrations of TQ and hydrogen peroxide caused the

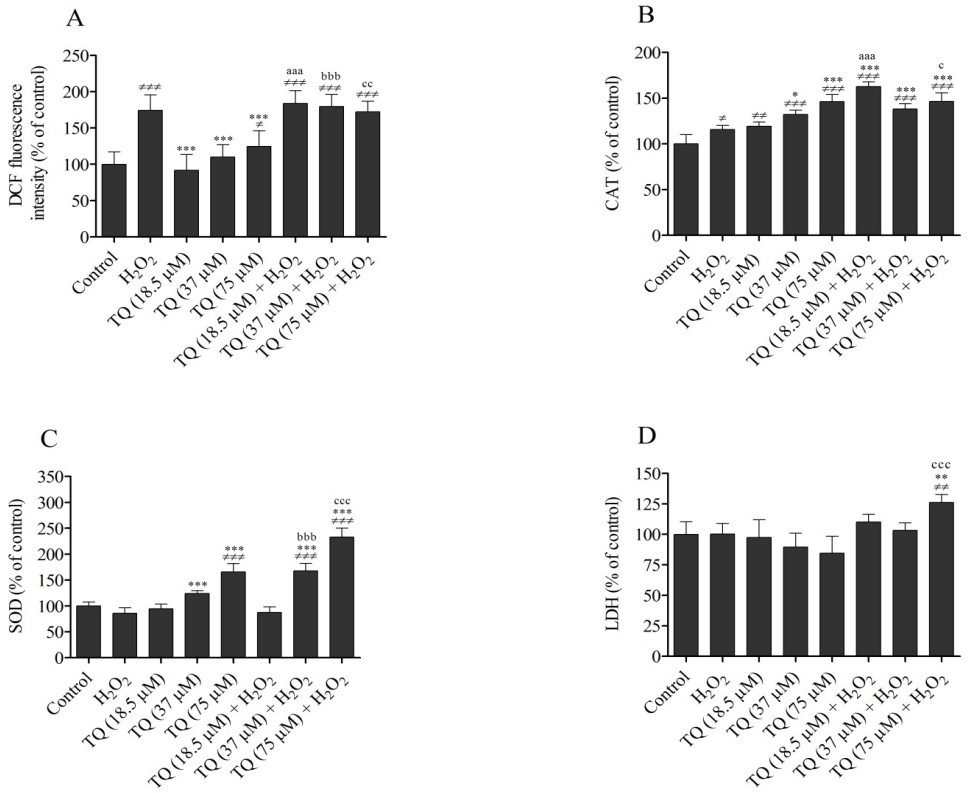

Figure 4. Effect of Thymoquinone on Oxidant/Antioxidant Status in Hydrogen Peroxide–Exposed HepG2 Cells. Statistical analysis used one-way ANOVA with Tukey's test. Data are expressed as Mean ± Standard deviation (SD), n=5 for each group. ##P<0.05, ##P<0.01 and ###P<0.001 vs. control group; *P<0.05, *P<0.01 and ***P<0.001 vs. hydrogen peroxide group. aaaP<0.001 vs. TQ (18.5 µM) group. bbbP<0.001 vs. TQ (37 µM) group. bP<0.05, bbP<0.01 and bbbP<0.001 vs. TQ (75 µM) group. ROS, Reactive oxygen Species (A); CAT, Catalase (B); SOD, Superoxide dismutase (C); LDH, Lactate dehydrogenase (D); TQ, Thymoquinone; H_2O_2, Hydrogen peroxide.

activity of this enzyme to increase significantly compared to the hydrogen peroxide alone ($p < 0.001$).

SOD enzyme activity

The SOD enzyme activity in HepG2 cells after exposure to a concentration of 75 µM of TQ significantly increased compared to the control group ($p < 0.001$) and the cells exposed to hydrogen peroxide alone ($p < 0.001$). The simultaneous treatment of cells with concentrations of 37 and 75 µM of TQ and hydrogen peroxide caused the activity of this enzyme to increase significantly compared to the cells treated with hydrogen peroxide alone and equivalent doses of TQ (Figure 4-C).

Cell membrane integrity

As shown in Figure 4-D, no significant changes were observed following the exposure of HepG2 cells to different doses of TQ and hydrogen peroxide alone. However, TQ (75 µM) along with hydrogen peroxide can increase LDH activity in the culture medium compared to the control ($p < 0.01$), TQ (75 µM; $p < 0.001$) and hydrogen peroxide groups ($p < 0.01$).

Discussion

In recent years, many studies have been conducted to investigate the anticancer effects of TQ, with a particular focus on its oxidant/antioxidant properties (Edris, 2009; Almatroodi et al., 2020). This study examined the interaction effect of TQ on hydrogen peroxide cytotoxicity as an inducer of the Fenton reaction in the HepG2 cell line. In addition, the TQ effects on the spatial structure and activity of SOD/CAT enzymes were investigated due to the role of these antioxidant enzymes in the prooxidant/antioxidant properties of TQ.

In the current study, IC_{50} of hydrogen peroxide was determined approximately 310 µM, which confirms the findings of Tan et al. (2020). In addition, The IC_{50} of TQ was reported around 192 µM, which confirms the report of Celebioğlu et al. (2022) and contradicts the findings of Abdualmjid and Sergi, (2022) and Zhang et al. (2021). This discrepancy may be associated to the role of cell culture condition, such as the type of culture medium and the number of cell passage.

Our findings showed that TQ low concentration can increase the survival of HepG2 cells when exposed to hydrogen peroxide, and on the contrary, its high concentration can potentiate cytotoxicity induced by hydrogen peroxide. A part of the dual effects of TQ on hydrogen peroxide-induced cytotoxicity may be related to the antioxidant/pro-oxidant properties of TQ (Darakhshan et al., 2015; Martinovich et al., 2016; Pal et al., 2021). In the current study, although TQ alone at concentrations of 18.5 and 37 µM did not have a significant effect on the formation of reactive oxygen species, its high concentration led to a significant enhancement in ROS production. Interestingly, the different concentrations of TQ alongside hydrogen peroxide increased ROS production when compared to equivalent concentrations of TQ. It seems that the induction of the Fenton reaction following hydrogen peroxide exposure in HepG2 cells can alter TQ metabolism towards enhancing the formation of free radicals.

The SOD and CAT are two critical antioxidant enzymes in the cells, which can remove excess reactive oxygen species and protect cells from stress-induced oxidative injury (Firozian et al., 2020). The SOD enzyme catalytically converts superoxide radicals to oxygen and hydrogen peroxide, which is consequently reduced by the CAT enzyme (Ahmed Amar et al., 2019). In the present study, TQ alone could significantly increase the activity of CAT and SOD in HepG2 cells, which is in the line with the findings of Woo et al. (2013). In this regard, the activity of CAT significantly increased following the interaction of hydrogen peroxide with different concentrations of TQ in the HepG2 cells. A part of this effect may be due to the addition of hydrogen peroxide (as a substrate) into the culture medium (Trawczyńska, 2020), and the other due to the increase SOD activity to inhibit the superoxide radicals caused by TQ (Martinovich et al., 2016). Following the biotransformation of TQ to toxic quinone and then to thymohydroquinone, significant amounts of superoxide radicals are formed, which increases the SOD activity towards converting these radicals into hydrogen peroxide molecules (Darakhshan et al., 2015; Martinovich et al., 2016).

Based on spectroscopic findings, there was no significant chemical interaction between TQ and hydrogen peroxide in the absorption spectrum at wavelengths 220-500 nm. In addition, the molecular docking findings showed that the minimum concentration of TQ needed to interfere with the enzymes SOD and CAT were 114.31 and 121.33 µM, respectively, which is higher than the maximum TQ concentration used in this study. Therefore, it seems unlikely that the strengthening effects of TQ on hydrogen peroxide toxicity are related to its interference with the structure of CAT and SOD enzymes.

LDH is a stable cytoplasmic enzyme that is released into the cell culture medium due to the loss of membrane integrity (Kumar et al., 2018). In the present study, the TQ high concentration along with hydrogen peroxide were able to increase the LDH release into the culture medium, which may be related to the excessive formation of free radicals and the saturation of the SOD/CAT enzyme system.

In conclusion, our findings indicate the dual effects of TQ on hydrogen peroxide-induced cytotoxicity. Molecular docking findings showed that TQ effects on the formation of free radicals were not related to its chemical interference with the structure of the SOD/CAT molecules. It seems that the induction of the Fenton reaction by hydrogen peroxide led to alter TQ metabolism towards the formation of toxic metabolites and the decrease in the biological activity of HepG2 cells. In future studies, it is recommended to investigate other pathways leading to cell death after exposure to TQ.

Author Contribution Statement

Sara Ghelichkhani helped in bibliography and design of the study and did the work and drafted the manuscript as her Pharm D thesis. Javad Saffari-Chaleshtori served as the

adviser to the thesis. Fatemeh Ghaffari assisted in doing cell culture experiments and biochemical analyses. Amir Nili-Ahmadabadi conceived and supervised the study and edited the article and has been the main supervisor of the thesis..

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Ethical approval

The study protocol was approved by the Ethics Committee of Hamadan University of Medical Science with the ethical number: IR.UMSHA.REC.1401.486.

Availability of supporting data

Data will be made available on request.

Conflict of interest

The authors have no conflict of interest.

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