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Running Title: Thymoquinone Effects on Oxidative Stress in Lung Cancer
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Evaluation of the Effects of Thymoquinone on Oxidative Stress in A549 Lung Cancer Cell Line

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Abstract

Background: Thymoquinone (TQ), an active part of Nigella sativa, has been reported as an anti-cancer agent. This study aimed to evaluate different anti-cancer effects of TQ on oxidative stress markers and Peroxiredoxin4 (P4) in lung cancer A549 cell line.
Method: In this experimental study, we used TQ concentrations to treat lung A549 cells and determined cell viability by the 3-(4,5-dimethylthiazol-2-yl)-2,5- diphenyltetrazolium bromide (MTT) assay at 12h, 24h, and 48h times. The IC50 concentration of TQ was found with MTT assay. We studied the total antioxidant capacity (TAC) and total oxidant status (TOS) using the manual assay, and analyzed catalase (CAT), superoxide dismutases (SOD), and glutathione peroxidase (GPx) activity using the enzyme-linked immunoassay (ELISA) kits. Moreover, the concentration of peroxiredoxin-4 (PRXD4) was measured with the ELISA Kit.
Results: The IC50 of TQ for A549 cells was calculated to be 40 µM concentration for 24 h of incubation. TAC index significantly decreased in the treated cells compared to the controls (P = 0.05) whereas TOS and PRXD4 levels showed a significant increase (P = 0.05). Additionally, the results showed that the CAT, SOD, and GPX activity enzymes significantly decreased in 20, 40, and 60 µM TQ in comparison with the control cells (P = 0.05).
Conclusion: TQ has significant inhibitory effects on A549 cells and could be utilized in novel therapy not only for lung cancer, but also for other tumors.
Keywords: Thymoquinone, Oxidative stress, Peroxiredoxin 4, Lung neoplasms

Introduction

According to the World Health Organization in 2018, lung cancer is the most prevalent type of cancer worldwide and has the highest mortality rate (1.76 million deaths) compared to other malignant tumors. Lung adenocarcinoma is more common in people over the age of 50, and smoking is the most effective risk factor for this disease.[1] Lung cancer is classified into two main groups: small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC); the latter accounts for 85% of cases. A549 Lung cancer cell line is commonly used as an NSCLC cell line in drug discovery and basic research.[2] These cancerous epithelial cells were obtained from a 58-year-old Caucasian male patient.[3] Oxidative stress is a major feature of tissue and cell damage. Several studies have shown the importance of anti-oxidant and pro-oxidant markers in various cancers.[4] Furthermore, the beneficial role of antioxidant-rich diets in the risk of carcinoma diseases has been proven. Oxidative stress occurs when there is an imbalance between the antioxidant /oxidant systems and the cell is unable to neutralize pro-oxidants and free radicals.[1] Reactive oxygen species (ROS) are the main class of radicals produced in cells, which include the hydroxyl, perhydroxyl, peroxyl, and superoxide radicals along with non-free radical species, such as singlet oxygen and hydrogen peroxide.[5] Recent studies have shown conflicting results regarding the role of ROS in cancers. On one hand, increase in ROS induces apoptosis[6, 7]by activating caspases and suppressing cancer by inactivating the STAT3 / JAK2 pathway in several types of cancer.[7-10] On the other hand numerous research findings have indicated that elevated ROS levels may lead to mutagenesis through DNA damage, especially if the accumulation of DNA damage is combined with an incomplete repair system or a deficient apoptotic pathway.[11] The pro-oxidants are constantly produced in living cells; accordingly, certain antioxidant defense systems are needed to counteract them and prevent oxidative stress. These defense systems contain antioxidants, such as vitamin E, vitamin C, and glutathione (GSH) (reduction form) in addition to (antioxidant) enzymes, such as catalase (CAT), glutathione peroxidase (GPx), superoxide dismutase (SOD), and peroxiredoxin-4 (PRXD4).[12, 13]SOD enzyme modulates cellular oxidative stress by scavenging superoxide radicals and converting them to hydrogen peroxide. Afterwards, Gpx and CAT eliminate the hydrogen peroxide through oxidation of GSH and producing water, respectively.[14] In addition to CAT and GPX, the Prxs protein family converts H_2O_2 to water and harmless metabolites; among these PRXDs, PRXD4 plays an important role in tumor metastasis and progression in lung adenocarcinoma.[13]

Thymoquinone (TQ), the bioactive compound of Nigella sativa (black seed oil), was first extracted by El-Dakhakhany and is mostly known as an antioxidant, anti-neoplastic, and anti-inflammatory agent in both vitro and vivo xenograft models.[15] The anti-tumor effects of TQ on decreasing cell viability, inducing apoptosis, inhibiting cell proliferation, and generation of ROS have been proven by numerous papers, yet its central mechanism of action has not been fully understood.[16-18] In addition, there are contradictory reports about the dual effect of TQ on oxidative stress in different cancers. This led us to design a (novel) study to investigate a group of oxidative stress markers in order to attain insights into the effect of TQ on oxidative stress on lung cancer cells and possibly elucidate the antitumor mechanism of TQ. We assessed oxidative stress by measuring the total oxidative status (TOS), total antioxidant capacity (TAC), PRXD4 levels, CAT, GPx, and SOD enzymes activities.

Material and Methods

Cell culture

This work was designed as an experimental study. We purchased human A549 lung epithelial (human non-small lung cancer cell line) cancer cells from Pasteur Institute cell culture collection (Tehran, Iran). A549 cells have a high proliferation rate and long lifespan and maintain their phenotype in culture. RPMI1640 culture media, FBS, 0.25% Trypsin-EDTA solution, and phosphate-buffered saline (PBS) tablets were obtained from Life Technologies (Life Technologies Ltd., Paisley-UK) and Sigma-Aldrich Company (Sigma-Aldrich Co., Steinheim, Germany) supplied DMSO, penicillin, streptomycin, and TQ. A549 cells were cultured in 25-cm^2 flasks in RPMI1640 medium containing 10% fetal bovine serum and 1% penicillin-streptomycin (100 units/ml penicillin and 100 µg/ml streptomycin). The cultured cells were grown at 37°C in 5% CO2 humidified air.

MTT assay

The cell viability was evaluated with MTT (3-[4, 5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay. Briefly, the cells were seeded at a 1×10^4/well density in 96 well plates and incubated at 37°C with 5% CO_2. We carried out the treatment with different concentrations of TQ (0, 20, 40, 60, 80, 100 µM) and incubated the cells for 24 and 48h. Subsequently, 10µl MTT (5mg/ml) was added to each well and the plate was incubated for 4h at 37°C in the dark so that formazan could be formed by mitochondrial succinate dehydrogenase. The content of wells was replaced with 100µL DMSO and the absorbance was measured at 570nm employing ELISA plate reader (Sunrise Tecan, Switzerland). The inhibitory rates were calculated via the following formula:

Inhibitory rates (IR) = $(1-(OD_{treatment}/OD_{control})\times100$

The IC50 was calculated using the following formula according to the inhibitory rate:

$IC_{50} = (50\%-Low_{inh}\ \%)/(High_{inh}\ \%-Low_{inh}\ \%) \times (High_{conc}-Low_{conc}) + Low_{conc}$

Low_{conc} and $High_{conc}$ were the lowest and highest concentrations of TQ, respectively, and Low_{IR} and $High_{IR}$ were respectively defined as the lowest and highest inhibitory rates.

Lysate preparation

The cells were trypsinized and after centrifugation, the cells were washed with cold PBS twice. A PBS-containing protease inhibitor cocktail was used to prepare cell suspension. The cell lysates were then obtained by repeated freeze-thaw in triplicate. The suspension was centrifuged at 12000 rpm for 15 minutes and the supernatant was collected in the tubes that were stored at -20^{oC} until further analysis.

Peroxiredoxin 4

Peroxiredoxin 4 was measured with the ZellBio-GmbH ELISA Kit (Cat. ZB-12927C-H9648, Ulm Germany) according to the manufacturer's protocol. It was based on colorimetric method and the basis of the measurement was a non-competitive sandwich assay. The standard range for Prx4 concentration was 1.25-20 ng/ml.

TAC

TAC measurement was performed utilizing FRAP manual method. It assessed the ability of antioxidant compounds of sample to reduce Fe^{3+}-TPTZ complex to Fe^{2+}-TPTZ. The reaction mixture comprised 300µL FARP reagent (acetate buffer 300 mM, pH3.6, TPTZ 10mM and ferric chloride 20 mM) and 10µl of the samples was pipetted into 96 well plates and incubated for 10min in the dark at 37°C. The concentrations of standards (1M FeSO4) and the samples were measured with Spectrophotometer (Bell-Italy) at a wavelength of 593 nm. The concentration of each sample was then determined according to the standard curve.[19]

TOS

TOS was evaluated with ferric-xylenol orange (FOX) assay based on the oxidation

of ferrous ions to ferric ions, which results in a colored ferric-xylenol orange complex. The reaction mixture included 10µl of the sample and 190µl of the FOX reagent (250 µM ferrous ion, 150 µM xylenol orange, 100 mM sorbitol and 25 mM H2SO4, pH was 1.8), which was inserted into 96 well plates. H2O2 and PBS were applied as standards and blank, respectively. The absorbance at 560nm was read after 10min of incubation in the room temperature.[20] Finally, the oxidative stress index (OSI) was defined as the percentage ratio of TOS to TAC.

GPx enzyme activity

GPx activity was determined with a ready-to-use kiazist kit (Tehran, Iran) based on the reduction in H_2O_2 by glutathione oxidation, which acts as an electron donor. GSH enzyme was converted to GSSG, and the remaining GSH in the reaction medium could regenerate DTNB and produce a yellow color absorbed at 412 nanometers. Color production was inversely related to enzyme activity. Finally, the GPx activity was reported as U / mg protein.

SOD enzyme activity

SOD activity was assayed with a ready-to-use kiazist kit (Tehran, Iran). In this method, superoxide anion was utilized to convert resazurin to resorufin and to produce chromogen. Ultimately, the chromogen was measured in terms of calorific value at 420 nm and the results were reported as U/mg protein.

CAT enzyme activity

Catalase activity was measured using the previously described spectrophotometric method by Hadwan and Abed.[21] Briefly, 100µl of the samples was added and incubated with 1ml reagent (20 mM H2O2 in 50 mM sodium phosphate buffer, pH 7.4) at 37 °C for 3 min. Afterwards, 4µL of ammonium molybdate (32.4 mM) was added to stop the reaction. The absorbance of the obtained yellowish mixture could be measured at 374 nm. Catalase activity was calculated via the following formula:

Catalase Activity kU = $2.303/t * [\log S^0/S\text{-}M] * V_t/V_s$

Data analysis

Data was analyzed employing the statistical software SPSS 16 (SPSS Inc. Chicago, USA) and t-test was performed to compare the differences between the groups. One-way ANOVA, followed by Tukey-Kramer multiple comparisons test, was utilized to analyze the differences between the groups. The values are given as mean ± SD. The significance level of all the statistical tests was considered to be at 0.05.

Results

TQ inhibited viability and changed the morphology of A549 lung cancer cells

As shown in figure 1, A549 lung cancer cell viability decreased significantly after 24 h of incubation with TQ in a dose-dependent manner ($P < 0.05$). IC50 of TQ for A549 cells was calculated as 40µM concentration; 20 and 60µM concentrations were also used in further experiments as low and high doses, respectively. TQ treatment changed the morphology of the cells. The morphology of A549 adherent epithelial cells changed. They became round in shape and were suspended in their media dose-dependently.

PRXD4

Figure 2 represents a significant dose-dependent decrease in A549 lung cancer cells in PRXD4 concentration after exposure of the cells to 20, 40, and 60 mM. The most PRXD4 decrease was observed in 40 µM TQ dose ($P < 0.05$). Moreover, the cells treated by 40 and 60 µM TQ showed a significant difference with those treated via a 20-µM dose ($P < 0.05$).

TAC and TOS

The findings showed a significant change in TAC index in the cells treated with 20, 40, and 60 mM TQ compared to that of the controls ($P = 0.05$), as shown in figure 3A.

Additionally, the cells treated with 20 and 40 µM TQ had a significant difference with those treated with a 60-µM dose ($P < 0.05$). The result concerning TOS levels indicated a difference between 20, 40, and 60 µM concentrations of TQ with the controls ($P = 0.05$), as demonstrated in figure 3B. The cells treated with 20 and 40 µM TQ had a significant difference with those treated with a 60-µM dose ($P < 0.05$).

CAT, SOD, and GPx

The results of the study of CAT enzyme activity implied that the activity of this enzyme significantly decreased dose-dependently in 20, 40, and 60 µM TQ compared to that of the controls ($P = 0.05$) (Figure 4A). The results regarding SOD enzyme activity revealed that the activity decreased dose-dependently in 20, 40, and 60 µM TQ compared to that of the controls ($P = 0.05$) (Figure 4B). As shown in figure 4C, there was a significant reduction in the amount of GPx enzyme activity in the treated cells compared to the controls. Moreover, there was a significant difference in the cells treated with 20 and 40 µM compared with those treated with 60 µM TQ concentration ($P < 0.05$).

Discussion

Even though there are a number of reports on oxidant/anti-oxidant properties of TQ on lung cancer, this is the first study to investigate the potential effects of TQ on oxidative stress parameters in A549 adenocarcinoma lung cancer cell line. Our findings demonstrated that TQ exposure induced oxidative stress by increasing TOS and decreasing PRDX4, the activity of GPx, SOD, CAT, and TAC in A549 cells. We revealed that the most pro-oxidant effects of TQ were seen in low concentrations. The imbalance between oxidants and anti-oxidant systems leads to oxidative stress. TOS and TAC are two reliable and precise indicators, which are mostly used to determine oxidative status.[22, 23] Our results indicated that TQ induced oxidative stress by elevating TOS and declining TAC significantly in A549 cells. Yih-Farng Liou et al. suggested the anti-proliferative and anticancer properties of TQ due to reactive oxygen species/superoxide generation. Their results showed the production of ROS in 786-O-SI3 renal cancer cells and BFTC-909 transitional cancer cells.[24] In addition, Ji Eun Park et al. conducted a study to provide a mechanism for inducing apoptosis by TQ in A431 epidermoid carcinoma cells. They indicated that exposure to the TQ prompts cells into the oxidative status and induces the generation of ROS.[25] Furthermore, the role of TQ in ROS induction in MDA-MB-231 and MCF-7 breast cancer cell lines and xenograft mouse model has been reported.[25] EM Dergarabetian et al. showed that TQ induced apoptosis in malignant T-cells through ROS generation.[26] Despite the presented data and mentioned studies, there are reports considering TQ as an anti-oxidant agent. The anti-oxidant property of TQ is supported by the decrease in TOS and increase in TAC in Ehrlich acid mouse solid tumor models and mouse mesenchymal stem cells, respectively.[26, 27]SOD, GPX, and CAT are the main antioxidant enzymes that play a crucial role in reducing oxygen radicals and cell oxidative status. SOD converts superoxide and single-chain radicals to an oxygen molecule and hydrogen peroxide. Finally, GPX and CAT remove the produced H_2O_2 to avoid converting it to harmful substances, such as hydroxyl radicals by Fenton reaction.[22] Our findings showed that SOD, GPX, and CAT activities significantly reduced in the treated cells compared with those in the untreated ones. Certain investigations have reported that TQ results in an increase in superoxide anion.[24, 28] Increased superoxide anion in those studies is a result of the reduction in SOD activity, which leads to a decrease in H_2O_2 production.

Therefore, the lack of enough H_2O_2 as the substrate of GPX and CAT possibly decreases their activity. It has been also suggested that reduction in SOD activity and H_2O_2 for the reason behind increased superoxide anion. It is predicted that CAT and GPX activity decrease in response to H_2O_2 reduction. In contrast with our observations, it has been demonstrated that TQ can attenuate oxidative stress by increasing SOD, GPX, and CAT after partial hepatectomy on Ischemic reperfusion liver of rats. TQ antioxidant effects were explained by its ability to upregulate antioxidant genes and scavenge free radicals.[29]

Peroxiredoxin 4 is a member of peroxiredoxin family proteins, which converts alkyl hydroperoxides and H_2O_2 to alcohol and water through the use of thiol-containing donor molecules, such as glutathione (GSH) and thioredoxine (Trx).[30] Our observations revealed a decrease in PRDX4 after 24h of TQ exposure. It has been reported that TQ consumption results in GSH depletion in mice tissues, like the liver, kidneys, and heart.[31] In addition, it has been shown that GSH declines in HEp-2 and rat C6 glioma cells following TQ treatment.[28, 32]According to these findings, it could be predicted that decreased PRDX4 might be associated with GSH level.

The obtained data herein revealed that the pro-oxidant effects of TQ declined in a dose-dependent manner. Recently, published papers have provided some evidence indicating that TQ induces oxidative stress and ROS generation, specifically at low doses (20-50 μM).[28] They have also reported that 40 μM TQ can increase ROS in various cell lines.[33] The ROS production has been attenuated in the high concentration of TQ.[34] According to these pieces of evidence, it could be suggested that the oxidant/antioxidant effects of TQ depend on its concentration. It seems as though the conflicting effect of TQ on oxidative status possibly depends on its concentration. Contradictory reports concerning pro-oxidant/antioxidant ability of TQ are attributed to its reduction or oxidation (reduction-oxidation) form. Quinones have been known as a redox-cycling compound that can undergo one- or two-electron reductions by cellular reductases. One-electron reduction yields semiquinone that can act as a pro-oxidant and react with molecular oxygen (O2) to form hydrogen peroxide and superoxide.[28, 33] This reaction catalyzes NADH-cytochrome b5 reductase, NADPH-cytochrome P450 reductase, NADH: ubiquinone oxidoreductase. Two-electron reduction yields hydroquinone which is catalyzed by NADH-Quinone oxidoreductase 1 (NQO1) and acts as an antioxidant.[28, 34] According to the induced oxidative stress by TQ in our study, it seems as if in A549 cell lines, the predominant form of quinones is semiquinone. However, further research is recommended to confirm this result. Investigating cellular reductases, such as NQO1 and NADPH-cytochrome P450 reductase involved in TQ reduction, may be conducive to finding the mechanism of TQ in inducing oxidative stress in A549 cells. In addition, using PCR to investigate the related genes (GPx, CAT, SOD) and signaling pathways (STAT3 / JAK2) could provide valuable information about the effects of TQ on oxidative stress.

Conclusion

In conclusion, our study shed light on the fact that TQ induced oxidative stress in A549 lung cancer cells. TQ treatment increased TOS and decreased TAC, PRDX4, SOD, GPX, and CAT activity and the most pro-oxidant effects belonged to low concentrations. These results suggested that the mechanism of TQ as a potential anti-cancer agent may be owing to its pro-oxidant ability in A549 lung cancer cells. Furthermore, using the PCR array method

was shown to provide valuable information about the effects of TQ on oxidative stress and other signaling pathways involved in lung cancer.

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Conflict of Interest
None declared.

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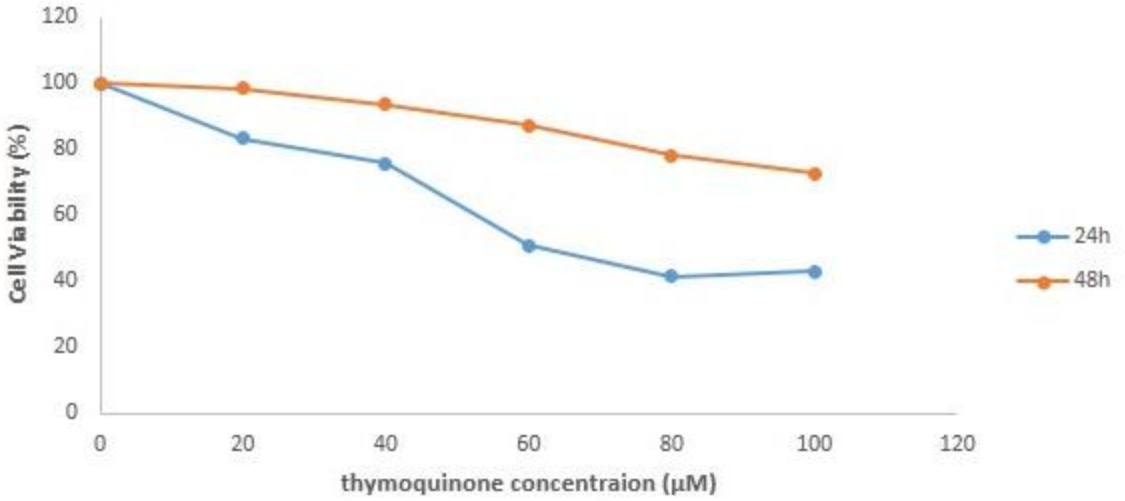

Figure 1. The effect of thymoquinone on A549 cell viability. Exposure of A549 cells to different concentrations of thymoquinone for 24 and 48 h decreased cell viability in a dose-dependent manner. The incubation of the cells with thymoquinone for 24 h exhibited a greater decline in the cell viability compared with longer incubation times.

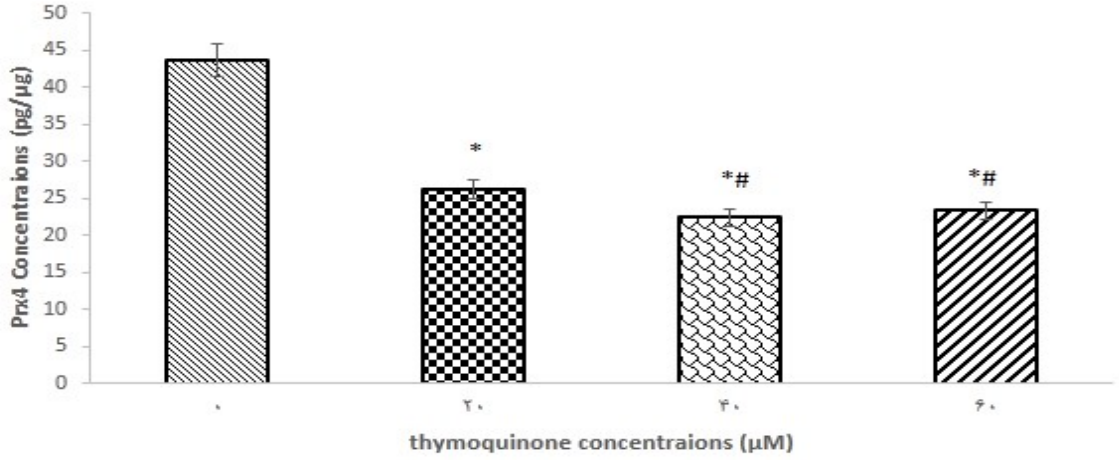

Figure 2. The effects of thymoquinone on the Prx4 concentration in A549 cells. The cells were treated with 0, 20, 40, and 60 mM of thymoquinone; the lowest level of Prx4 concentration was observed in the cells treated with 40 µM thymoquinone. ***** is a significant sign with the control. **#** is a significant sign with the 20 µM thymoquinone. Mean ± SD is considered in all the diagrams. $P < 0.05$ was considered as the level of significance.

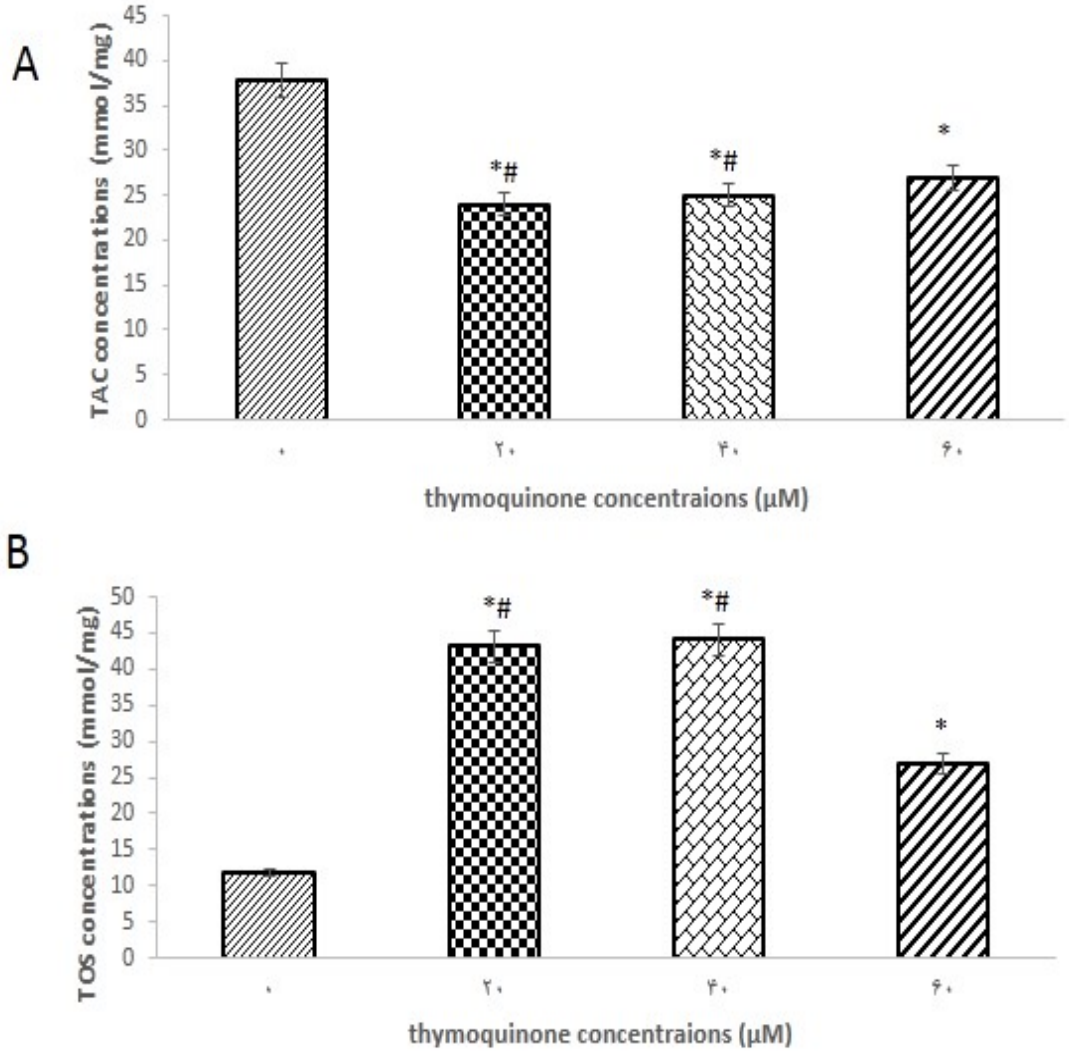

Figure 3. The effects of thymoquinone on the TAC and TOS concentrations in A549 cells. The cells were treated with 0, 20, 40, and 60 mM of thymoquinone; in TAC (A) and TOS (B) levels, there was a significant difference in the cells treated with 20, 40, and 60 µM thymoquinone compared to the controls. * is a significant sign with the control group. # is a significant sign with the 60 µM thymoquinone. Mean ± SD is considered in all the diagrams. $P < 0.05$ was considered as the level of significance.

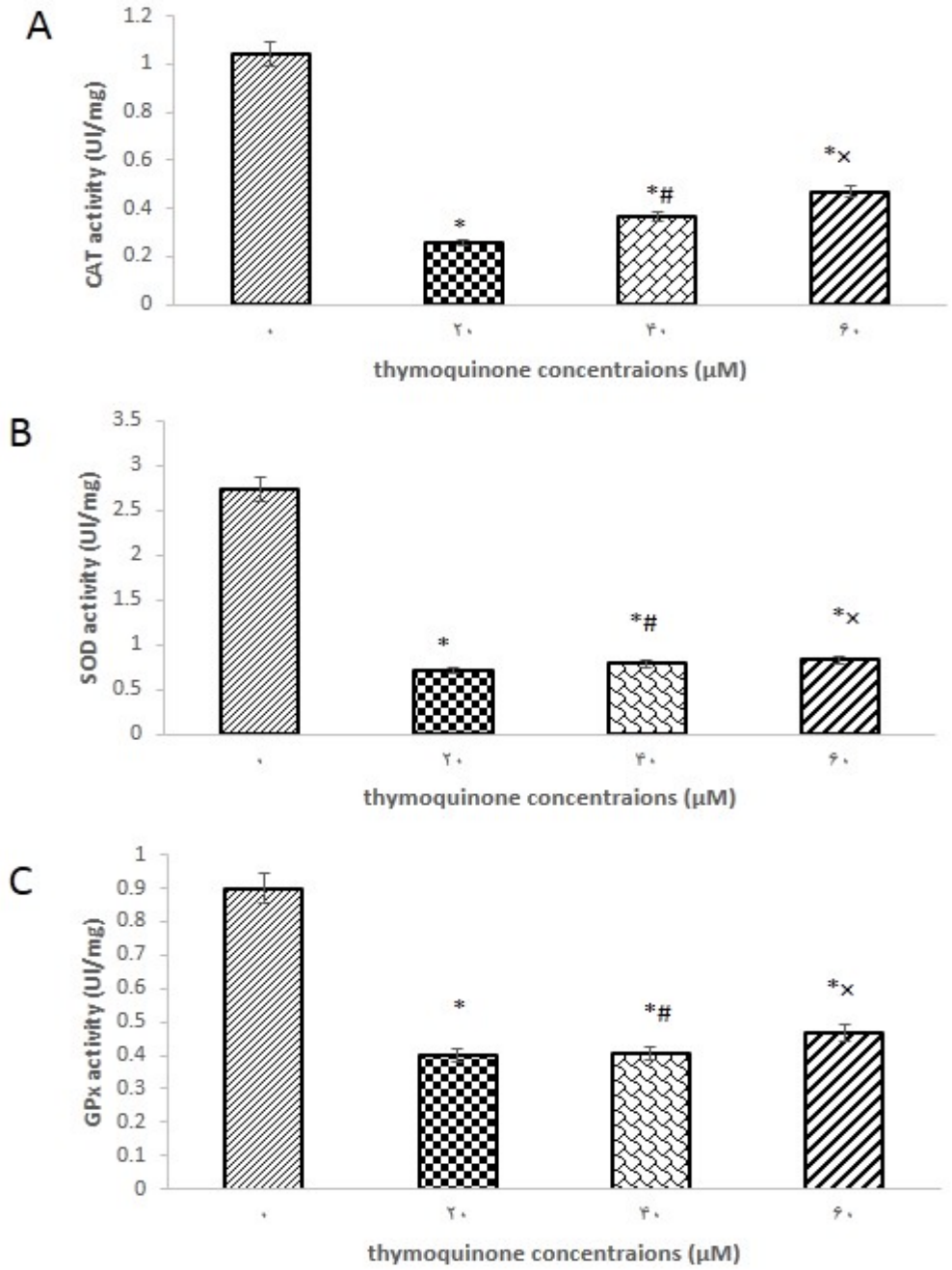

Figure 4. The effects of thymoquinone on the CAT, SOD, and GPx activities in A549 cells. The cells were treated with 0, 20, 40, and 60 mM of thymoquinone; in CAT (A), SOD (B), and GPx (C) activities, there was a significant difference between the cells treated with 20, 40, and 60 µM thymoquinone compared to the controls. * is a significant sign with the control. # is a significant sign with the 60 µM thymoquinone. × is a significant sign with the 20 and 40 µM thymoquinone. Mean ± SD is considered in all the diagrams. $P < 0.05$ was considered as the level of significance.