



Effect of Thymoquinone on P53 Gene Expression and Consequence Apoptosis in Breast Cancer Cell Line

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ABSTRACT

Background: *Nigella sativa* has been a nutritional flavoring factor and natural treatment for many ailments for so many years in medical science. Earlier studies have been reported that thymoquinone (TQ), an active compound of its seed, contains anticancer properties. Previous studies have shown that TQ induces apoptosis in breast cancer cells but it is unclear the role of P53 in the apoptotic pathway. Hereby, this study reports the potency of TQ on expression of tumor suppressor gene P53 and apoptosis induction in breast cancer cell line Michigan Cancer Foundation-7 (MCF-7).

Methods: MCF-7 cell line was cultured and treated with TQ, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was carried out for evaluating the half-maximal inhibitory concentration (IC₅₀) values after 24 h of treatment. The percentage of apoptotic cells was measured by flow cytometry. Real-time polymerase chain reaction (PCR) was performed to estimate the messenger RNA expression of P53 in MCF-7 cell line at different times.

Results: The IC₅₀ value for the TQ in MCF-7 cells was 25 μM that determined using MTT assay. The flow cytometry and real-time PCR results showed that TQ could induce apoptosis in MCF-7 cells, and the P53 gene expression was dramatically up-regulated by ascending time, respectively. Hence, there was significant difference in 48 and 72 h.

Conclusions: Our results demonstrated that TQ could induce apoptosis in MCF-7 cells through up-regulation of P53 expression in breast cancer cell line (MCF-7) by time-dependent manner.

Keywords: Apoptosis, genes P53, Michigan Cancer Foundation-7 cells, thymoquinone

INTRODUCTION

Breast cancer is the most common cancer among women worldwide and it has been reported that each year about 502,000 die from the disease.^[1,2] Breast cancer originates

in the breast but metastasizes to other distant organs such as bone, lungs, and brain.^[3] Global statistics showed that by the year 2020, there will be twenty million new cancer patients every year.^[4] Herbal medications are the source of many chemotherapeutic agents and it has also been demonstrated to have anti-neoplastic potential (*Nigella sativa*, paclitaxel, vincristine, etc.).^[5] Thymoquinone (TQ) is as a potential chemo preventive

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and chemotherapeutic compound of oil of the black seed.^[4] Many investigations have shown that TQ has anti-inflammation, anti-hypertensive, anti-asthmatic, anti-microbial, and anti-oxidant effects.^[5-7] In addition, anticancer properties of TQ have also been reviewed and has been found that TQ applies anticancer effects (both *in vitro* and *in vivo*) in various patterns of carcinogenesis.^[8,9] In earlier works, it was found that TQ prevents the growth of different types of cancer, including breast adenocarcinoma (multi-drug-resistant Michigan Cancer Foundation-7 [MCF-7]/TOPO, MCF-7, MDA-MB-231, and BT-474),^[8,10] lung cancer (NCI-H460 and A549),^[11] pancreatic cancer (MIA PaCa-2, HPAC, and BxPC-3),^[11] and prostate cancer (LNCaP, C4-2B, and DU145).^[12-14] Several lines of studies have determined that TQ exhibits antimetastatic effects, and it can inhibit angiogenesis, in a way that, this compound was found to prevent C26 colon cancer cell invasion.^[9] Moreover, some other studies have reported that TQ was able to suppress tumor growth in PC-3 prostate cancer cells, probably through angiogenesis inhibition and it can cause to significant reduction in the number of blood vessels in the tumor.^[15] TQ has shown little effect on noncancerous cells such as mouse fibroblasts,^[16] prostate epithelial cells,^[12] human normal intestinal cells,^[17] and human normal lung fibroblasts.^[18] This result shows that TQ may have beneficial effects in different types of malignancy while has little effect on normal human cells. Shoieb *et al.* found that TQ induces apoptosis in osteosarcoma cells and also decrease the number of cells in S-phase. Hence, it induces the cell cycle arrest at G1-phase.^[19] Other investigations have demonstrated that TQ can induce apoptosis via p53-dependent/independent pathways.^[20-23] In spite of TQ good anti-neoplastic activities, the molecular mechanism of its pharmacologic effects is not fully understood. Cali-Muhtasib *et al.* investigated the effects of TQ against HCT-116 human colon cancer cells. They found that TQ induced apoptosis by up-regulating p53 and the downstream p53 target gene, p21WAF1.^[20] Roepke *et al.* have shown that TQ induce apoptosis by p53-independent pathway.^[23] Regarding increasing incidence of breast cancer in developing countries like Iran, many investigations have done on different cell line of breast cancer and many different ways have supposed for TQ induced apoptosis. Hence, in the present study, we hypothesized that TQ may up-regulate activity of P53 gene in breast cancer cell line MCF-7 that has a wild-type P53, and inhibit tumor cell growth.

METHODS

Cell lines, drug, culture, and treatment

Human breast cancer cell line (MCF-7) was purchased from the national cell bank of Iran-Pasteur Institute. TQ was purchased from Sigma (C6499, USA). Cell line used in the present study was cultured in Dulbecco's modified

Eagle medium-F12 medium (Sigma) supplemented with 10% fetal bovine serum (Sigma) and 1% penicillin-streptomycin (Sigma), and incubated at 37°C and in humidified atmosphere containing 5% CO₂. TQ was dissolved in minimum volume of dimethyl sulfoxide (DMSO) (2%) as working solution were prepared by adding of adequate water to reach the appropriate concentrations according to reported procedures.^[24,25] After the cells were >80% confluent and growing exponentially in flask 75, 10⁵ cells (MCF-7) were counted and plated in flask 25 and kept in culture medium for 24 h which were then incubated with certain concentrations of TQ, based on half-maximal inhibitory concentration (IC₅₀) index, at different times (24, 48, and 72 h).

Half-maximal inhibitory concentration assay

The IC₅₀ values for the TQ in MCF-7 cells were acquired after 24 h of treatment. Briefly, 10⁴ cells (MCF-7) were counted and placed into each well of a 12well microplate and were treated with various drug concentrations (0, 10, 20, 30, 40, 50, 60, 70, and 80 μM doses) for 24 h, and the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) survival assay was done according to the protocol, for evaluating the cell viability in all cells.^[26,27] A graph of viability versus drug concentration was used to calculate IC₅₀ values for MCF-7 cell line.^[28]

Flow-cytometric analysis

Percentage of apoptotic cells was measured by flow cytometry following Annexin V flow cytometry 1-histogram (FL1-H) and propidium iodide (FL2-H) labeling. A minimum of 5 × 10⁵ cells/ml were analyzed for each sample. Cells were treated with TQ (25 μmol/L) for 24, 48, and 72 h and so washed in phosphate-buffered saline and resuspended in binding buffer (10X; 5 μl). Annexin V-FITC was added to 195 μl cell suspensions, and so analysis was done according to the manufacturer's protocol (BMS500F1/100CE Annexin V-FITC Apoptosis Detection Kit - eBioscience, USA). Eventually, the apoptotic cells were counted by FACScan flow cytometry (Becton Dickinson, Heidelberg, Germany). These experiments were done in triplicate and were, independently, repeated at least 3 times.^[25,26]

Real-time polymerase chain reaction analysis

Real-time polymerase chain reaction (PCR) was performed to quantitatively assessment the messenger RNA (mRNA) expression of P53 gene in MCF-7 cells and after treatment with TQ at different times. Total RNA was isolated by RNeasy mini kit treated by RNase-Free DNase set (Qiagen) to remove the genomic DNA. The RNA concentration was defined using a BioPhotometer (Eppendorf BioPhotometer® D30). Total RNA (100 ng) was reverse-transcribed to complementary DNA (cDNA) using the RevertAid™ First Strand cDNA Synthesis Kit (Fermentas) according to the manufacturer's protocol. The maxima SYBR Green ROX

qPCR Master Mix kit (Fermentas) was used for real-time PCR. Real-time PCR reactions were performed with using Step One Plus (Step One™ Real-Time PCR Systems - Applied Biosystems). The PCR amplification conditions consisted of 10 min at 95°C followed by 40 cycles of denaturation step at 95°C for 15 s and annealing and extension for 1 min at 60°C. This data were analyzed using the comparative Ct ($\Delta\Delta C_t$) method. The relative expression level of P53 was calculated by determining a ratio between the amount of P53 and that of endogenous control (glyceraldehyde-3-phosphate dehydrogenase [GAPDH]). Melting curve analysis (60°C → 95°C increment of 0.3°C) was used to determine the melting temperature of special amplification products and primer dimers. These experiments were done in triplicate and were independently repeated at least 3 times.^[27] Primer sequences for P53 and GAPDH were as follow:^[29]

- PF GAPDH: CACCACCATGGAGAAGGCTGG
- PR GAPDH: CCAAAGTTGTCATGGATGACC
- PF P53: CGAGTCCCGCGGTAATTCTT
- PR P53: TGCAGAAGAGGTGCAAGACC.

Statistical analysis

All the quantitative data were presented as the mean \pm standard deviation. Repeated measure analysis of variance with *post hoc* test was performed to determine the statistical significance among different groups using the SPSS software package 20.0 IBM modeler (2009). Significance was accepted at a level of $P < 0.05$.

RESULTS

Half-maximal inhibitory concentration analysis

After the treatment of MCF-7 cells with MTT solution in this study, the dark blue formazan crystals were seen in cells, which indicated their metabolic activity. The reduction in the number of cells was dependent on the cell type as shown by the IC₅₀ index. The IC₅₀ value for the TQ was established. The results showed that the essential TQ concentration to achieve the IC₅₀ in MCF-7 cells at 24 h was 25 $\mu\text{mol/L}$ [Figure 1].

Flow cytometry

To establish the anti-apoptosis potential of the TQ, we first investigated the effects of TQ on the proliferation in MCF-7 cells. The flow cytometry results showed that TQ at different time points (24, 48 and 72 h) could induce apoptosis in MCF-7 cells, and it was increased with ascending time. TQ arrested MCF-7 cells proliferation (40% of inhibition) in 72 h and (approximately 30% of inhibition) in 48 h [Figure 2a and b, $P < 0.05$]. DMSO was used in the control sample (vehicle drugs). Using Dunnett test, average of apoptotic cells at different times were compared with the control group. Groups 48 h and 72 h had significant difference to the control group ($*P < 0.05$).

Real-time polymerase chain reaction

It was suggested that apoptotic induction in cancer cells by TQ requires the activation of P53 gene expression. To examine this hypothesis, we used MCF-7 cell line, as cancerous cell line. We examined the inhibitory effects of 25 $\mu\text{mol/L}$ TQ (based on IC₅₀ index) at different times on the mRNA expression of P53 in MCF-7 cell line. The P53 gene expression was dramatically up-regulated by ascending time, in particular, at 72 h treatment its expression was increased significantly [Figure 3, $P < 0.05$]. Using Dunnett test, average of P53 gene expression at different times was compared with the control group. Groups 48 h and 72 h had significant difference to the control group ($*P < 0.05$).

DISCUSSION

Breast cancer is the most common cancer among women worldwide and it is one of the most common cancers that lead to death.^[30,31] Therefore, many continuous searches have done for usage of new chemotherapeutic drugs in this type of cancer.^[32] Over the centuries, different plant species have been used for chemotherapy.^[33,34] Several lines of studies have shown cytotoxicity effects of *N. sativa* against various carcinomas.^[35-37] TQ as an active ingredient isolated from *N. sativa* has tested for its efficacy against cancer.^[19] To date, many studies have demonstrated that this compound can inhibit the growth of many different types of cancer, including glioma/glioblastoma (U87 MG),^[18,38] breast adenocarcinoma (multi-drug-resistant MCF-7/TOPO,

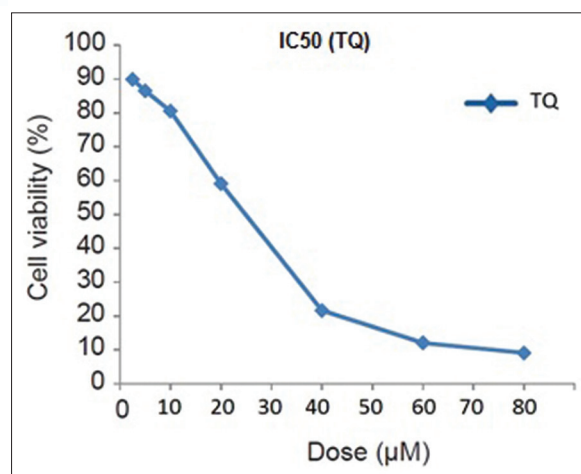


Figure 1: Half-maximal inhibitory concentration assay for half-maximal inhibitory concentration analysis of thymoquinone in Michigan Cancer Foundation-7 cancer cell lines after 24 h of treatment. Cells were incubated with or without the thymoquinone using 25 μM dose. The relative amount of viable cells was estimated by measuring the absorbance of the cell suspension after incubation with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. A graph of viability versus drug concentration was used to calculate half-maximal inhibitory concentration values for Michigan Cancer Foundation-7 cell line

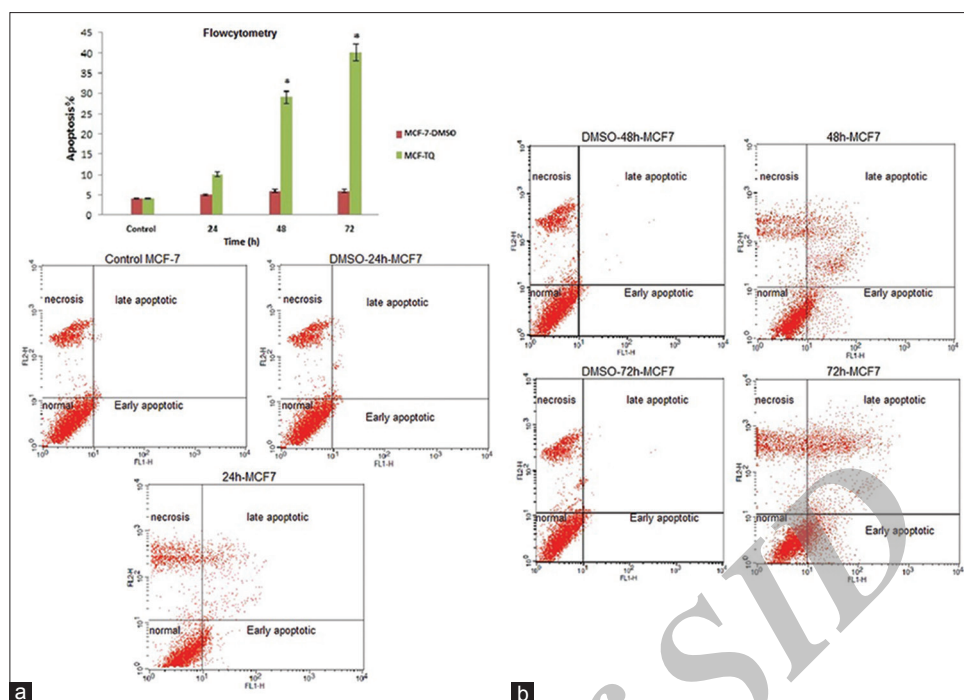


Figure 2: (a) Relative levels of apoptotic cells in Michigan Cancer Foundation-7 treated with thymoquinone for different times. Cells incubated with the vehicle dimethyl sulfoxide were used as a control. The percentage of apoptotic cells was measured using the Annexin V-FITC flow cytometry 1-histogram and propidium iodide flow cytometry 2-histogram. Dunnett's test was used for considered comparisons (* $P < 0.05$) (in any figure, right upper quarter show late apoptotic cells, left upper quarter show necrosis cells, right lower quarter show early apoptotic cells and left lower quarter show normal cells). (b) Cells that are Annexin V-positive and propidium iodide negative are in early apoptosis as phosphatidyl serine translocation has occurred; although, the plasma membrane remains intact. Cells that are positive for both Annexin V and propidium iodide either are in the late stages of apoptosis or are already dead as phosphatidyl serine translocation has occurred and the loss of plasma membrane integrity is visible. * $P < 0.05$ compared to controls. Dunnett's test was used for considered comparisons (* $P < 0.05$)

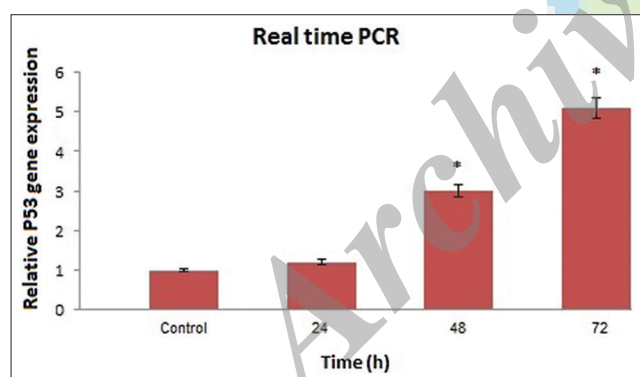


Figure 3: Results of real-time quantitative polymerase chain reaction before and after thymoquinone treatment at different times on the P53 messenger RNA expression in Michigan Cancer Foundation-7 cell. Relative expression levels were obtained using the comparative Ct ($\Delta\Delta C_t$) method (* $P < 0.05$)

MCF-7),^[8,10] leukemia (HL-60 and Junket),^[8] lung cancer (NCI-H460),^[11] colorectal carcinoma (HT-29),^[11,17] and pancreatic cancer (HPAC and BxPC-3).^[11] In the present study, we investigated the effect of TQ on P53 gene expression and consequence apoptosis in breast cancer cell line. TQ molecular mechanisms are not fully understood. However, the assumptions indicated that the anti-tumor effect of TQ may happen by one or more of the following mechanisms, antioxidant activity

and cytotoxicity.^[39] The results of the present study showed that TQ inhibits the viability and proliferation of breast cancer cell line by a time-dependent manner. Many studies demonstrated that TQ induces apoptosis in p53-dependent or p53-independent pathway.^[20-23] Mansour and Tornhamre and Badary *et al.* have shown TQ property as a radical scavenger with a potential role in the prevention and treatment of oxidative stress.^[40,41] A number of studies have shown that TQ could prevent from chemical effects of cancer and inhibit tumorigenesis through different molecular mechanisms.^[42] Apoptosis is one form of physiological or a key pathway for regulating homeostasis and morphogenesis of cells and is related to cancer.^[43] Numerous *in vitro* and *in vivo* studies have indicated that TQ has anti-proliferative effects and can induce cell cycle arrest and also induces pro-apoptotic effects in cancer cells.^[23,44] Woo CC *et al.* have reported that the apoptotic effect of TQ is p53-independent in p53-null myeloblastic leukemia HL-60 cells.^[10] They also confirmed that the apoptotic effect of TQ in leukemia cells is caspase-dependent. In some other studies, TQ induces apoptosis through the expression of p53 gene in HCT116 human colorectal carcinoma cells.^[20] Thus, the results of different studies show that the anticancer activity of TQ is dependent on cell type. The potencies and functional mechanisms of the TQ were studied

at the concentration that was confirmed by IC₅₀ on the MCF-7 as breast cancer cell line. In recent years, a few researchers have described the therapeutic effect of TQ by p53 up-regulation in different cell line;^[45] here, we report for the first time that TQ was able to up-regulate the p53 expression in MCF-7 cell line. To understand the inhibitory effect of TQ in breast cancer cells, we used different molecular techniques. In the present study, we have shown that TQ induce apoptosis in MCF-7 cells via time-dependent manner. In addition, real-time PCR results on MCF-7 cells, showed apoptotic induction in cancer cells by TQ requires the activation of P53 gene expression as it was dramatically up-regulated by ascending time, in particular, at 72 h treatment its expression was increased significantly ($P < 0.001$). In this study, treatment by TQ at various time showed a time-dependent increase in apoptotic cell count of the cancerous cells, as measured by flow-cytometric assay. We have found that TQ caused a significant up-regulation p53 in a time-dependent manner. Our results were supported by the findings of Gali-Muhtasib *et al.*, which indicated that TQ is anti-neoplastic and pro-apoptotic against colon cancer cell line HCT116, and the apoptotic effects of TQ are dependent on p53.^[20] Our results were matched with the findings of Woo *et al.* which stated that TQ exerted strong anti-proliferative effect in breast cancer cells.^[10] In addition, they found that TQ activated caspases 8, 9, and 7 in a dose-dependent manner (possible involvement of peroxisome proliferator activated receptor- γ [PPAR- γ] pathway). Besides, El-Mahdy *et al.* have reported that TQ exhibits antiproliferative effect, induces apoptosis and triggers the activation of caspases 8, 9, and 3 in myeloblastic leukemia HL-60 cells (p53-independent).^[10]

Taken together, our results indicated that TQ inhibited breast cancer cell proliferation in a time-dependent manner. Also, for the first time, we have shown that TQ induce apoptosis through activation of the P53 pathway in MCF-7 cell line. Further, it is required to evaluate the effect of TQ on prevention and treatment of breast cancer.

CONCLUSIONS

The results showed that the drug's effect was significant at 48 and 72 hours. Finally, TQ treatment for 72 hours would have to make the most of apoptosis by expression of P53 gene and it's Resumption of activities apoptotic. Considering the valuable properties of the drug, can be hope to the emergence of a new strategy in the fight against cancer.

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Conflicts of interest

There are no conflicts of interest.

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