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Title: The effect of thymoquinone on cell proliferation, 8-hydroxy-2' -deoxyguanosine level and expression changes of DNA repair and oxidative stress-related genes in MCF-7 breast cancer cells.

Short title: Effects of thymoquinone on MCF-7 cells via DNA repair and oxidative stress genes.

Abstract

Purpose: The aim of this study was to determine the effects of thymoquinone on cell proliferation, 8-hydroxy-2' -deoxyguanosine level and expression changes of oxidative stress and DNA repair-related genes in MCF-7 breast cancer cells.

Material methods: Cell proliferation in MCF-7 cells after thymoquinone exposure was determined by MTT assay. 8-hydroxy-2' -deoxyguanosine protein concentration was measured by ELISA assay. Total RNA isolation from control and thymoquinone treated cell was performed by Trizol and cDNA was synthesized. mRNA expression changes of *OGG1, NEIL-1, CRCC1 SOD2, CAT* and *NRF-2* were also determined in RT-PCR using SYBER Green method.

Results: In this study, the IC50 dose of thymoquinone in MCF-7 cells was determined as 7.867 µM at 24th hour. It was investigated that thymoquinone inhibited 8-hydroxy-2' -deoxyguanosine level in breast cancer cells according to RT-PCR results, thymoquinone increased *XRRC1* expression 4.71-fold and catalase expression 6.68-fold in breast cancer cells.

Conclusion: In conclusion, TQ inhibits MCF-7 cell proliferation under in vitro conditions. It also alters the expression of genes associated with DNA repair and oxidative stress and acts through this oxidative stress mechanism. This study contributes to the existing literature and provides preliminary data for more comprehensive studies.

Keywords: Thymoquinone, MCF-7 cells, breast cancer, DNA repair genes, oxidative stress.

Makale başlığı: Timokinonun MCF-7 meme kanseri hücrelerinde hücre proliferasyonu, 8-hidroksi-2' –deoksiguanozin seviyesi ile DNA tamiri ve oksidatif stres ilişkili genlerin ekspresyon değişimlerine etkisi

Kısa başlık: Timokinonun MCF-7 hücreleri üzerinde DNA onarımı ve oksidatif stres genleri aracılığıyla etkileri

Öz

Amaç: Bu çalışmanın amacı, timokinonun MCF-7 meme kanseri hücrelerinde hücre proliferasyonu, 8-hidroksi-2' -deoksiguanozin düzeyi ve oksidatif stres ve DNA onarımı ile ilişkili genlerin ekspresyon değişiklikleri üzerindeki etkilerini belirlemektir.

Gereç ve yöntem: Timokinon maruziyetinden sonra MCF-7 hücrelerinde hücre proliferasyonu MTT testi ile belirlendi. 8-hidroksi-2' -deoksiguanozin protein konsantrasyonu ELISA testi ile ölçüldü. Kontrol ve timokinon uygulanan hücrelerden Trizol ile total RNA izolasyonu yapıldı ve cDNA sentezlendi. *OGG1, NEIL-1, CRCC1 SOD2, CAT* ve *NRF-2*'nin mRNA ekspresyon değişiklikleri de SYBER Green yöntemi ile RT-PCR'da belirlendi.

Bulgular: Bu çalışmada, MCF-7 hücrelerinde timokinonun IC_{50} dozu 24. saatte 7.867 µM olarak belirlenmiştir. Timokinonun meme kanseri hücrelerinde 8-hidroksi-2' -deoksiguanozin seviyesini inhibe ettiği araştırılmıştır Ayrıca RT-PCR sonuçlarına göre, timokinon meme kanseri hücrelerinde *XRRC1* ekspresyonunu 4,71 kat ve katalaz ekspresyonunu 6,68 kat artırmaktadır.

Sonuç: Sonuç olarak, TQ in vitro koşullar altında MCF-7 hücre proliferasyonunu inhibe eder. Ayrıca DNA onarımı ve oksidatif stres ile ilişkili genlerin ekspresyonunu değiştirir ve bu oksidatif stres mekanizması yoluyla etki eder. Bu çalışma mevcut literatüre katkıda bulunmakta ve daha kapsamlı çalışmalar için ön veri sağlamaktadır.

Anahtar kelimeler: Timokinon, MCF-7 hücreleri, meme kanseri, DNA onarım genleri, oksidatif stres.

Introduction

Cancer is a pathological condition characterised by the excessive and uncontrolled growth of cells that have acquired abnormal properties and have a highly complex molecular genetic structure. These abnormal cells have malignant properties, including the ability to invade and metastasise [1, 2]. The process of carcinogenesis is a gradual integration of biological events in which oncogenic mutations in a normal cell affect cellular mechanisms such as survival, differentiation, growth, and signal transduction. This leads to the acquisition of new capabilities by the cells and provides a clonal advantage [3, 4].

Breast cancer is the second leading cause of cancer-related mortality in women, following lung cancer. In terms of incidence, breast cancer accounts for 32% of all cancer cases and 15% of cancer-related deaths [5]. Breast cancer detected at an early stage can be treated with treatments such as surgery and radiotherapy. However, metastatic breast cancer is the leading cause of death [6]. Although the development of resistance, toxicity in non-target organs and side effects are limitations of chemotherapy and other cancer treatment strategies, studies are underway to develop potential cancer drugs and treatment strategies that overcome these limitations [7-10].

Natural and bioactive compounds isolated from plants have been used for many years in the treatment of many diseases due to their strong pharmacological properties, low side effects, low toxicity, natural abundance and cost-effectiveness. Scientific studies have also highlighted the discovery of the strong anticancer potential of these bioactive molecules [11-14]. *Nigella sativa* is a dicotyledonous plant of the Ranunculaceae family. It has been used for many years as a sweetener, appetizer, spice or food in many regions, particularly in Asia, Europe and Africa. The main bioactive compound of the widely used medicinal plant *Nigella sativa* is thymoquinone (TQ) [15-17]. Both cell culture and animal model studies have demonstrated that TQ exhibits anti-cancer, anti-microbial, anti-parasitic, anti-inflammatory, anti-diabetic, and antioxidant properties. [6, 18]. TQ has been shown to have antiproliferative activity in several types of cancer, including larynx, colon, breast, endometrium, pancreatic and lung cancer. Its effects are mediated through cell cycle inhibition, induction of apoptosis and regulation of gene expressions involved in various signalling pathways [17-22]. Recent studies have reported that the combination of TQ with nanoparticles or nanoparticle-based functionalised nanoformulations enhances its bioavailability and helps to make it more effective in cancer treatment. These properties are believed to make thymoquinone nanoparticles an attractive potential agent for cancer therapy [22, 23]. Although the antiproliferative effects of

thymoquinone in breast cancer have been demonstrated, studies to elucidate its mechanisms of action are still ongoing. In this study, we aimed to determine the molecular biological mechanism underlying the antiproliferative effects of thymoquinone in MCF-7 cells by examining changes in the levels of 8-hydroxy-2′-deoxyguanosine (8-OHdG) and the mRNA expression of genes related to oxidative stress and DNA repair.

Materials and methods

Cell culture

The MCF-7 breast cancer cells were cultured in–Dulbecco's Modified Eagle Medium (DMEM; Gibco, USA) enriched with 10%. Fetal bovine serum (FBS; HyClone, USA) and 1% penicillin (100 U/mL)/streptomycin (0.1 mg/mL; Sigma Aldrich, USA) under standard conditions of 37°C in a humidified incubator with 5% CO2. Subculturing was performed in T-flasks two to three times weekly, with cells being harvested for assays once they reached approximately 90% confluency.

MTT assay

The antiproliferative effect of TQ was analysed by MTT assay (GoldBio, USA) according to kit protocol. The MCF-7 cells (1.0×10^4 cells/well) were cultured in 96-well plates in media for 24 hours. Cells were treated with increasing concentrations of 12.5, 25, and 50 µM of Thymoquinone (Sigma Aldrich, USA) for 24 h. 10 µL of MTT stock solution (5 mg/mL) was added to each well, and the plates were incubated at 37°C for 3 h. After incubation, the media were carefully removed by aspiration. Dimethyl sulfoxide (DMSO, 100 µL/well; Sigma Aldrich, USA) was used to solubilize the formazan crystals, and the plates were incubated at 37°C for 15 minutes. Finally, the absorbance was read at 570 nm with a microplate reader (Biotek-EPOCH2). The half maximum inhibitory concentration (IC_{50}) of TQ was determined via non-linear regression by using GraphPad Prism (version 9.4.1). The percentage of MCF-7 cell viability was determined using the following formula:

Cell Viability (%) = [(Treatment group OD570 - Blank well OD570) / (Untreated group OD570 - Blank well OD570)] ×100%.

All experiments were performed using IC50 dose of TQ obtained from cell proliferation assays for MCF-7 cells.

8-hydroxy-2′-deoxyguanosine (8-OHdG) ELISA assay

The protein level of 8-OHdG in MCF-7 cells was investigated by an enzyme linked immunosorbent assay (BT LAB, Catalog No: EA0048Hu, China ELISA) kit according to kit protocol. Control and dose group MCF-7 cells were homogenized in lysis buffer including protease inhibitors cocktail and centrifuged at 12,000 g for 15 min at 4°C. The

supernatant was harvested and utilized for measuring 8-OHdG levels. The concentration of 8-OHdG was determined by comparing the sample readings to a pre-established standard curve and was expressed in ng/mL.

Gene expression assay

MCF-7 cells were treated with TQ at IC_{50} dose detected through MTT assay or media only (control). Total RNA was isolated using TRIzol (Hibrizol, Hibrigen, Türkiye), following the protocol provided by the manufacturer. NanoDrop was used to measure the amount and quality of RNA (BioSpec-nano, Shimadzu, Japan). The cDNA for each sample was then synthesized using the OneScript Plus cDNA Synthesis Kit (Abm, Cat No: G236, Canada), following the manufacturer's protocol. Relative RNA levels were detected using the Rotor Gene 6000 Real-time PCR Thermocycler (Corbett, USA) with NucleoGene qPCR SYBER-Green Master Mix (NucleoGene, Türkiye). The primer sequences for amplification of human *NEIL1, OGG1*, *XRCC1, NRF2, SOD2,* and *CAT* are presented in Table 1. The cycle conditions were as follows: 95°C for 15 min, 95°C for 15 s, and 60°C for 1 min, for a total of 40 cycles. The expression fold-change for each target was calculated by the $2^{-\Delta\Delta CT}$ method using *ACTB* as a normalization control.

Table 1. Reverse and Forward sequences of the primers

Gene symbol	Gene name	Primers
NEIL1	Nei like DNA glycosylase 1	Forward:GACAGAGGCAAGTGGCAAAGCA Reverse:GCCTCATTCACAAACTGGCTGG
OGG1	8-oxoguanine DNA glycosylase	Forward: GGCTCAACTGTATCACCACTGG Reverse:GGCGATGTTGTTGTTGGAGGAAC
XRCC1	X-ray repair cross complementing 1	Forward: CGGATGAGAACACGGACAGTGA Reverse: GAAGGCTGTGACGTATCGGATG
SOD2	Superoxide dismutase 2	Forward: CTGGACAAACCTCAGCCCTAAC ReverseAACCTGAGCCTTGGACACCAAC
NRF2	NFE2 like bZIP transcription factor 2	Forward:CACATCCAGTCAGAAACCAGTGG Reverse: GGAATGTCTGCGCCAAAAGCTG
CAT	Catalase	Forward: GTGCGGAGATTCAACACTGCCA Reverse: CGGCAATGTTCTCACACAGACG
ACTB	Actin beta	Forward: CACCATTGGCAATGAGCGGT Reverse: AGGTCTTTGCGGATGTCCAC

Statistical analyses

The Real-time PCR data were quantified using the ΔΔCT method, with analysis facilitated by the Gene Globe RT-PCR Analysis RT2 Profile PCR Array Data Analysis tool (Qiagen). Statistical evaluations were conducted using GraphPad Prism 9.4.1 software. All results are presented as mean ± standard deviation (S.D.) derived from at least three

independent biological replicates. Mean comparisons were performed using either an Unpaired t-test or one-way analysis of variance (ANOVA), with Dunnett's test applied for post hoc analysis.

Results

Antiproliferative effect of thymoquinone treatment on MCF-7 cells

The MCF-7 cell viability after TQ treatment (12.5, 25, and 50 µM) for 24 hours was assessed by the MTT assay. The cell viability results and IC_{50} values are presented in Figure 1. As depicted in Figure 1A, 12.5, 25, and 50 µM of TQ significantly inhibited cell viability in MCF-7 cells, with an IC50 value of 7.867 µM (95% CI: 4.64-11.74 µM) (Figure 1B).

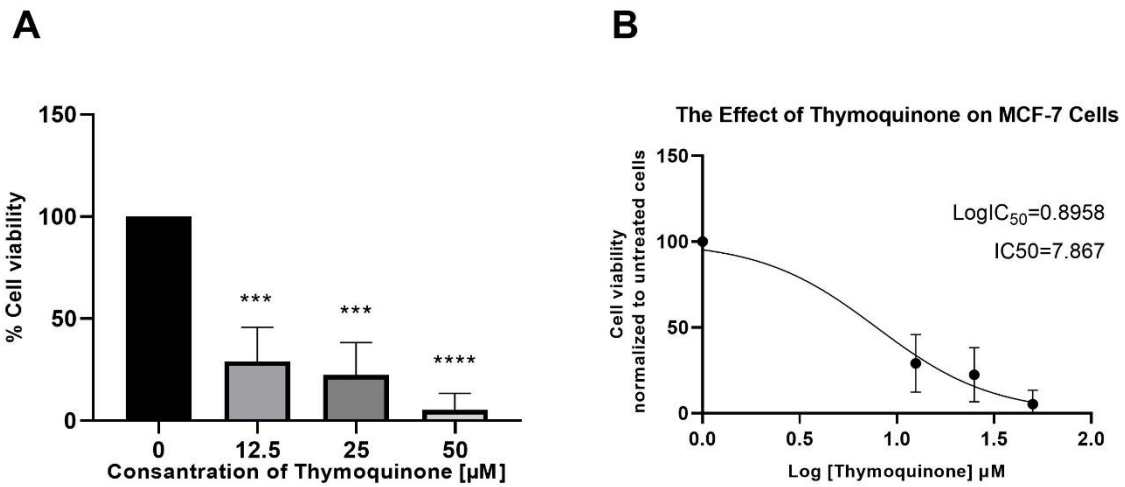

Figure 1. *In vitro* antiproliferative effect of TQ on MCF-7 cells at 24 h post-stimulation. (A) The effect of TQ on cell viability, and (B) Dose-response curve of cell viability with respect to treatment with different concentrations of TQ

The results are reported as cell viability percentage (%) normalized to untreated MCF-7 cells. Comparisons between means were performed using one-way ANOVA and Dunnett's test was utilized for post hoc analysis (ns $p>0.05$, * $p\leq0.05$, ** $p\leq0.01$, *** $p\leq0.001$, and **** $p\leq0.0001$).

The effect of thymoquinone treatment on 8-hydroxy-2′ deoxyguanosine level

Treatment with TQ significantly decreased 8OHdG level in MCF-7 cells from 0.245±0.04 to 0.056±0.01 ng/mL compared with untreated control samples (Figure 2; p=0.0016).

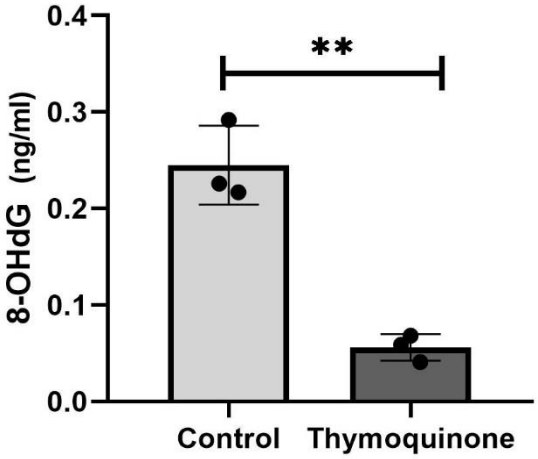

Figure 2. Effect of TQ on the content of 8-OHdG in MCF-7 cells. MCF-7 cells were treated with TQ at IC_{50} dose.

The data are presented as the mean ± S.D. from at least three independent biological replicates. Comparisons between means were performed using Unpaired t-test (ns p>0.05, * p≤0.05, ** p≤0.01, *** p≤0.001, and **** p≤0.0001).

The effect of TQ treatment on gene expression

To determine the effect of TQ to influence the expressions of genes associated with DNA repair and oxidative stress response, MCF-7 cells were treated with TQ at IC_{50} dose, for 24 h. Gene expressions were analyzed using RT-qPCR and all expression levels are normalized to *ACTB* expression levels. Figure 3 and Table 2 showed that treatment of MCF-7 with TQ at IC50 dose significantly increased the mRNA expression of *XRCC1* by 4.71-fold in comparison to untreated cells. Similarly, MCF-7 treated with TQ at IC_{50} dose enhanced *CAT* mRNA expression by 6.68-fold in comparison to untreated cells. MCF-7 treated with TQ at IC_{50} dose increased the expressions of *NEIL1, OGG1*, and *NRF2*, while decreased the mRNA expression level of *SOD2*, although not quite significantly (p>0.05). Fold changes in genes where no significant changes were observed were determined to be 5.72 for *NEIL-1,* 7.36 for *OGG1*, 1.43 for *NRF-2* and 0.70 for SOD2.

Table 2. Gene expressions including fold-change and *P* value of genes associated with DNA repair and oxidative stress response, MCF-7 cells were treated with TQ

Gene symbol	Gene name	Fold-change	*p* value
NEIL1	Nei like DNA glycosylase 1	5.72	0.31
OGG1	8-oxoguanine DNA glycosylase	7.36	0.10
XRCC1	X-ray repair cross complementing 1	4.71	0.005**
SOD2	Superoxide dismutase 2	0.70	0.38
NRF2	NFE2 like bZIP transcription factor 2	1.43	0.087
CAT	Catalase	6.68	0.023*

*represents a significant difference ($p \leq 0.05$); ** represents a significant different ($p \leq 0.01$)

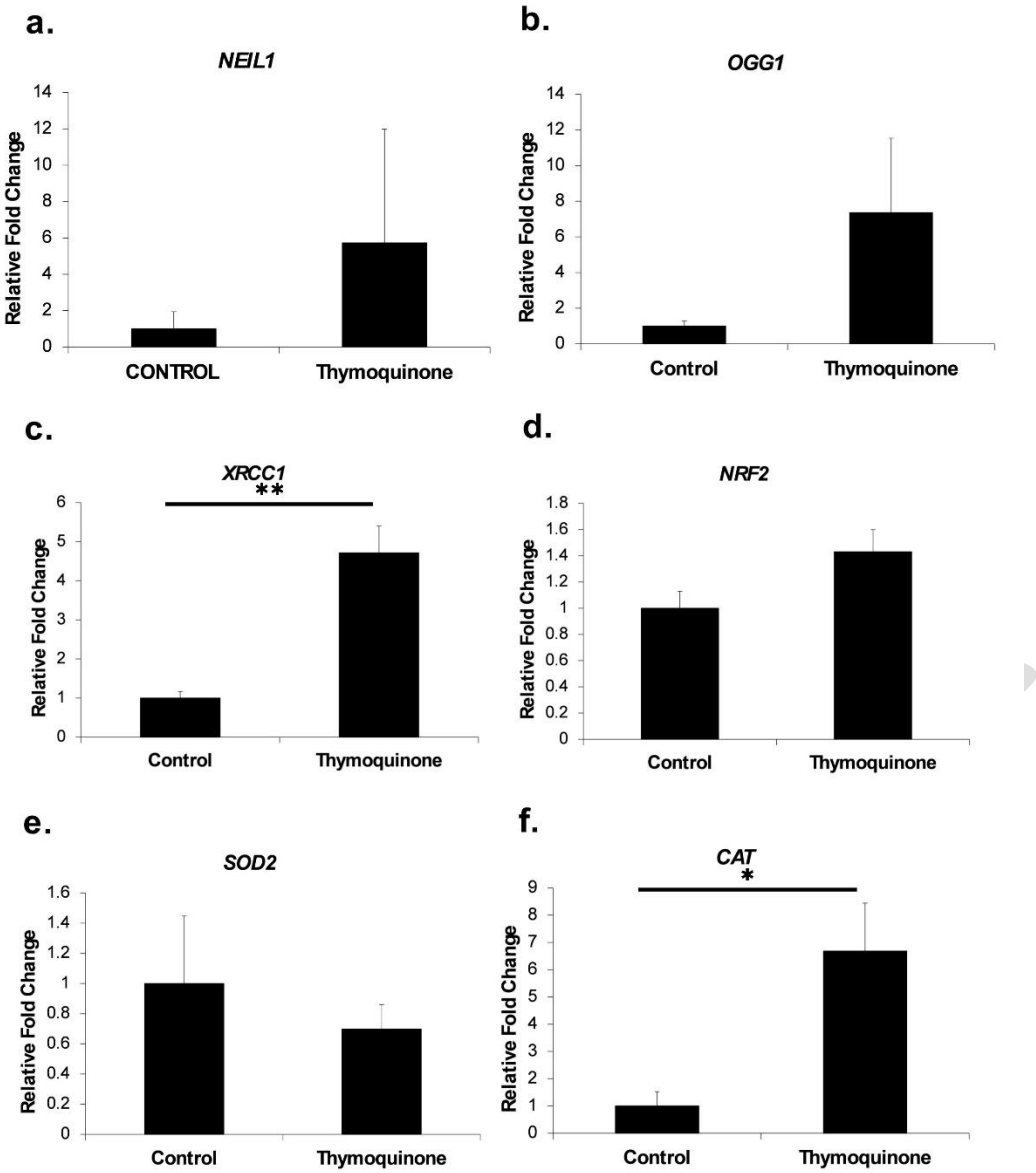

Figure 3. The effect of TQ treatment on the expression levels of genes related to DNA repair and oxidative stress response in breast cancer cells. XRCC1 and CAT show significantly different expression in MCF-7 compared to untreated cells. Data are expressed as fold change

Discussion

Natural compounds isolated from plants, fungi and other organisms have been widely used from ancient times to the present as pharmaceutical agents for various human diseases, especially cancer [12-14]. One of the most important reasons for their use is their strong antioxidant potential, which allows them to exert effects by modulating oxidative stress [12, 24]. One of the most popular phyto-natural products studied for its antineoplastic activities is TQ, a bioactive compound from Nigella sativa, which has been

shown to exhibit anticancer activities by inhibiting cell proliferation, migration, angiogenesis and invasion in cancer cells. [15, 16]. Cancer types in which the effects and anti-cancer activity of TQ have been demonstrated include lung, breast, colon, leukaemia and cervical cancer [17-24].

In this study, by determining the dose-dependent antiproliferative activity of TQ in the range of 12.5-50 μM, it was found that TQ decreased cell viability depending on the increasing dose. The IC_{50} value was determined to be 7.867 μM in MCF-7 cells at the end of the 24th hour. One study found that TQ exhibited cytotoxic activity in T47D and MCF-7 cells under in vitro conditions. It was observed that TQ showed a sudden cytotoxic effect at concentrations higher than 100 μM in T47D cells, while TQ showed a cytotoxic effect in MCF-7 cells. The IC_{50} values were 80.1±9.8, 32.7±1.1 and 64.9±14.5 for 24, 48 and 72 h, respectively [25]. In another study using the MTS assay, the cytotoxic effect of TQ exposure in the dose range of 25-300 μM on breast cancer cells was demonstrated and the IC_{50} value was reported to be 25 μM. In addition to the antiproliferative effect of TQ, it was also reported that TQ induced apoptosis in breast cancer cells and exhibited anticancer activity through cell cycle arrest [26].

Cancer is a multifaceted disease that involves alterations in the regulation of gene expression [3,9]. Several studies have indicated that TQ possesses therapeutic properties and lowers the risk of cancer by modifying genetic pathways [9, 25, 26]. A recent study reported that TQ suppressed VEGF-mediated angiogenesis and stimulated apoptosis in breast cancer [27]. Another study on the molecular biological mechanism of action of TQ in triple negative breast cancer reported that TQ arrests the cell cycle in the G1/S phase via cyclin E, cyclin D1 and p27, suppresses histone deacetylase (HDAC) activity, induces apoptosis by upregulating Bax and downregulating Bcl-2, and affects cell survival by increasing PTEN expression [28].The protective role of TQ in breast tumour xenograft mouse models and breast cancer cells has been demonstrated through molecular mechanisms mediated by induction of p38 phosphorylation, reactive oxygen species (ROS) production, decrease in Ki67, increase in apoptotic cell density and increase in catalase, glutathione and superoxide dismutase concentrations [29].

We investigated the changes in the expressions of the DNA repair-related genes *OGG1, NEIL-1* and *XRCC1* and the oxidative stress-related genes NRF2, SOD2 and CAT. According to the real-time PCR results we obtained, the DNA repair-related genes showed the following fold increases *OGG1* 7.36-fold, *NEIL-1* 5.72-fold and *XRCC1* 4.71-fold. Among these increases, the upregulation of *XRCC1* was found to be statistically significant. The results demonstrate that TQ significantly increases the expression of DNA repair genes. In addition, in our study, the changes in the protein concentration of

the important oxidative stress marker 8-OHdG were detected using the ELISA method. After TQ exposure, the 8-OHdG level in breast cancer cells showed a significant decrease. In addition, in this study, it was observed that NRF-2 and Catalase expressions increased as a result of TQ exposure. Catalase gene expression increase was found to be statistically significant. SOD expression was partially downregulated after TQ exposure.

8-OHdG generated as a result of E2-induced oxidative stress, is regarded as a cellular indicator of oxidative stress. 8-OHdG is generated as a result of oxidative stress triggered by E2 and is regarded as a cellular indicator of oxidative DNA damage [30-31]. Decreased levels of OGG1 have been shown to have a significant effect on tumour progression and growth, promoting cancer development [30-33]. The mechanisms of action of substances with antioxidant potential are known to suppress oxidative stress by acting as free radical scavengers through the induction of NRF2-dependent antioxidant enzymes. NRF2 is a transcription factor sensitive to redox changes, playing a crucial role in shielding cells from oxidative harm [31, 34]. It has been suggested that NRF2 may be an important distinguishing feature in cancer development stages associated with breast cancer growth and cell invasion [35]. Mutations or oxidative damage have been implicated in the pathogenesis of several diseases, particularly cancer. Base excision repair (BER) deficiency contributes to genomic instability and promotes carcinogenesis. NEIL-1 also fulfils important repair processes in the DNA repair mechanism [36]. In a study on TQ and its mechanisms of action in cancer treatment, TQ was found to induce apoptosis through ROS production in primary effusion lymphoma [37]. It has recently been reported that the combined application of TQ with piperine reduced oxidative damage in liver tissue and reduced malondialdehyde (MDA) and nitric oxide (NO) levels and induced glutathione peroxidase, SOD and catalase [38]. Evaluating the data obtained in this study, TQ was shown to reduce proliferation in MCF-7 breast cancer cells. In addition, it is thought that TQ attempts to reduce the increased oxidative damage in breast cancer by upregulating the expression of DNA repair genes. Limitations of this study include the use of a single cell line, the lack of confirmation of gene expression changes at the protein level, and the fact that other pathways were not included in the study. Another limitation of this study is that the cytotoxic effect of TQ on normal, non-cancerous cells was not demonstrated using healthy breast cells. The fact that DNA repair genes were also examined in this study as a contribution to similar studies in the literature is an important originality that distinguishes this study from other studies.

In conclusion, this study shows that thymoquinone has significant effects on cell proliferation, 8-OHdG levels and expression of genes associated with DNA repair and

oxidative stress in MCF-7 breast cancer cells. TQ treatment resulted in a decrease in cell proliferation, demonstrating its potential as an anticancer agent. TQ also modulated the expression of key genes involved in DNA repair and oxidative stress responses, further supporting its role in disrupting cellular homeostasis in cancer cells. These findings highlight the potential of TQ to target oxidative stress and DNA repair mechanisms that may contribute to its anticancer effects. A comprehensive understanding of the cellular mechanisms underlying the anti-cancer effects of TQ would further contribute to the therapeutic application of this extensively studied molecule and support strategies for the development of new therapeutic agents. In conclusion, this study will contribute to studies to better understand the molecular mechanisms of action of TQ and the molecular pathways through which it acts, and to evaluate its pharmacological potential in the treatment of breast cancer.

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Author contributions: M.S., S.D.A., G.G. contributed to the conceptualization and design. Y.D. supervised the study. All authors conducted all experiments and wrote the first draft of the manuscript. M.S., S.D.A., G.G. collected, analyzed and interpreted the data. All authors contributed to the critical revision of the manuscript and have read and approved the final version.

Conflict of interest: The authors have no conflicts of interest.

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