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Thymoquinone induces apoptosis via targeting the Bax/BAD and Bcl-2 pathway in breast cancer cells

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Abstract

Objective: *Nigella sativa*, commonly known as the black seed, has been used since ancient times in folk medicine, and its various therapeutic benefits have been mentioned in some ancient medical sources and confirmed by modern science. Thymoquinone is the major compound and essential active ingredient of *Nigella sativa* seed oil. Thymoquinone has been reported to have high biological activity and broad therapeutic potential through several mechanisms that affect cells including anti-oxidant, anti-inflammatory, and anti-tumor properties. Although it has been widely reported that Thymoquinone inhibits cell growth and proliferation and stimulates apoptosis in various types of cancer cells, the mechanisms and signaling pathways are not fully understood. The aim of this study was to determine the effect of Thymoquinone on apoptosis in both tumor and non-tumor cells.

Methods: In this study, breast cancer cell line (MCF-7) was used as tumor cells and Human Embryonic Kidney Cell line (HEK293) was used as non-tumor cells. Cells were treated with Thymoquinone and viability test performed by MTT assay.Gene expression of apoptosis markers such as *Bax, BAD, Bcl-2*, and *p53* was determined by Real-Time PCR. HEK293 cells were used as non-tumor control.

Results and Conclusion: Results suggest that Thymoquinone has a strong effect on cell proliferation and vitality. Thymoquinone has increased the expression of *BAD*, *Bax* genes which induce apoptosis and decreased the *p53* gene in breast cancer cells. Therefore Thymoquinone promotes apoptosis and enhances anti-cancer efficacy in breast cancer cells.

Keywords: Thymoquinone, breast cancer, MCF-7 cells, HEK293 cells, apoptosis.

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Timokinon meme kanseri hücrelerinde Bax/BAD ve Bcl-2 yolakları aracılığıyla apoptozu uyarmaktadır

Öz

Amaç: Bazı eski tıbbi kaynaklarda çeşitli terapötik yararlarından bahsedilen ve yaygın olarak kara tohum ismiyle bilinen *Nigellasativa*, modern bilim tarafından da onaylanmış ve halk hekimliğinde de kullanılmakta olan bir bitkidir. Timokinon, *Nigellasativa* tohumu yağının ana bileşiği ve temel etken maddesidir. Timokinon'un, anti-oksidan, anti-enflamatuar ve anti-tümör özellikleri dahil olmak üzere hücreleri etkileyen bazı mekanizmalar ile yüksek biyolojik aktiviteye ve geniş terapötik potansiyele sahip olduğu rapor edilmiştir. Her ne kadar Timokinon'un, hücre büyümesini ve proliferasyonunu inhibe ettiği ve çeşitli kanser hücrelerinde apoptozu uyardığı yaygın olarak bildirilmişse de, mekanizmalar ve sinyal yolakları tam olarak anlaşılmamıştır. Bu çalışmadaki amacımız, Timokinon'unapoptoz üzerindeki etkisini hem tümör ve hem de non-tümör hücrelerde ortaya koymaktır.

Yöntemler: Bu çalışmada, tümör hücreleri olarak meme kanseri hücreleri olan MCF-7 hücreleri, non-tümör hücreler olarak da Human Embrionic Kidney Cell (HEK293) hücreleri kullanıldı. Hücrelerin canlılık analizi, Timokinon uygulaması sonrasında MTT analizi ile gerçekleştirildi. *Bax, BAD, Bcl-2* ve *p53* gibi apoptoz belirteçlerinin gen ekspresyonu Real-Time PCR ile belirlendi.

Bulgular ve Sonuç: Sonuçlar, Timokinon'un hücre çoğalması ve canlılığı üzerinde güçlü bir etkisi olduğunu göstermektedir. Timokinon, apoptozu indükleyen *BAD* ve *Bax* genlerinin ekspresyonunu arttırmış ve meme kanseri hücrelerinde *p53* geni ekspresyonunu azaltmıştır. Bu nedenle, Timokinon'unapoptozu destekleyerek meme kanseri hücrelerinde anti-kanser etkinliğini arttırdığı sonucuna varılmıştır.

Anahtar kelimler: Timokinon, meme kanseri, MCF-7 hücreleri, HEK293 hücreleri, apoptoz.

INTRODUCTION

Breast cancer is the most common cancer among women and its incidence continues to increase in almost all countries[1]. In the treatment regimens, aggressive surgery with radical mastectomy and the incision of nodules is the first choice before the chemo- and radiotherapy[1,2]. Breast cancer is a challenge for all women and the investigation of the protection strategies may be better than treatment strategies. Like all other cancers, abnormal cell proliferation is the main characteristic of breast cancer[3]. Supplements against to the cell proliferation, especially against to the cancer cells may contribute protection from breast cancer. There are some reports about the anti-proliferative effect of Thymoquinone on cell proliferation. Studies indicate that in synergy with apoptosis, Thymoquinone stimulates autophagy in treated cancer cells and leads mitochondrial-dependent autophagic cell death in cancer cells via MOMP, p38 and JNK activation in some types of tumor cell lines[4]. Cell proliferation is substantially related to cell cycle and evidence suggest that Thymoquinone effectively arresting cancer cell cycle progression at different stages of cell cycle, G_o, G_1, S, G_2, and M[5,6].

Thymoquinone is the basic active substance and major compound of *Nigella sativa* seed oil and characterized by its biological activity and extensive therapeutic potential[7,8]. The anti-oxidant, anti-inflammatory and anti-tumor properties of Thymoquinone have been reported[9,10]. Previous studies have shown the anticancer effects of thymoquinone on various cancer models. The anticancer property of Thymoquinone may be due to the command of cancer cells to programmed cell death or prevent the normal cells from abnormal genetic changes. It also increases the number and activation of the macrophage cells[11]. Thymoquinone shows inhibitory effects on the vital processes of cancer cells including cell proliferation, invasion, migration, and angiogenesis[12,13], while also induces apoptosis

via p53-dependent and p53-independent pathways[14,15].

In this study, we aimed to investigate the effect of Thymoquinone on cell proliferation and vitality in breast cancer cell line and also identify the effects of Thymoquinone on mediators that control the apoptosis of breast cancer cells.

METHODS

Cell culture

Human breast adenocarcinoma cell line (MCF-7) and human embryonic kidney cell line (HEK293) were used in this study. MCF-7 cells were cultured in Dulbecco's modified Eagle's medium with 10% fetal bovine serum (FBS) (Sigma-Aldrich, Germany) and 1% P/S antibiotics in a 5% CO_2 humidified incubator at 37 ^{o}C. HEK293 cells were cultured in RPMI 1640 medium with the same other supplements and conditions for MCF-7 cells.

MTT proliferation assay

The viability and proliferation of MCF-7 and HEK293 cells were quantified by standard Methyl-thiazolyl-tetrazolium (MTT) proliferation assay. MCF-7 and HEK293 cells were seeded in a 96 well plate at a density of about $5x10^3$ cells per well with 100 µl of culture medium and cells were cultured for 48 hr. Then, Thymoquinone applied to wells at 15 µM, 25 µM, and 35 µM concentration for 24, 48 and 72 hours. Thymoquinone treated and untreated cells subjected to 10 µl of 12mM MTT solution and incubated at 37°C for 4 hours (MTT; Sigma Aldrich, Germany). After that, the media were removed and replaced with 50 µl of DMSO and incubated for 20 minutes with gentle shaking to dissolve the crystal blue melts. The absorbance at 540 nm was recorded by using microplate reader and IC50 dosage (represents the concentration of Thymoquinone that exhibited 50% cell viability for MCF-7 and HEK293 cells in vitro) of Thymoquinone was determined as 25 µM.

RNA extraction, cDNA synthesis, and Real-Time quantitative PCR

MCF-7 and HEK293 cells were seeded and treated with 25 µM of Thymoquinone for 48 hours. After that total RNA was isolated by using Trizol reagent (Invitrogen, USA) according to the manufacturers' protocols.cDNA synthesis was performed with reverse transcriptase by using the Transcriptor High Fidelity cDNA Synthesis (Roche) Kit according to the manufacturer's instructions. Relative transcript levels of *Bax, BAD, Bcl-2*, and *p53* genes were quantified by Real-Time PCR reaction with SYBR Green dye(Thermo Scientific, UK). The relative gene expression analysis was then calculated by using the $2^{-\Delta\Delta Ct}$ method.

Statistical Analysis

The statistical analyzes were carried out by applying two-tailed student's t-test from at least three independent experiments. Data were discussed as fold degrees. $p<0.05$ was regarded as statistically significant.

RESULTS

Thymoquinone inhibits proliferation of human breast cancer cell lines in vitro

MCF-7 and HEK293 cells (were used as the control) were exposed to different concentrations of Thymoquinone (15 µM, 25 µM, and 35 µM) during 24, 48 and 72 hour periods. The IC50 value of Thymoquinone was determined 25 µM at 48 hours, indicating that Thymoquinone has a strong effect against the proliferation of MCF-7 breast cancer cells in this dose. As shown in Figures 1 and 2, results clearly indicate that Thymoquinone suppresses the growth and proliferation of MCF-7 breast cancer cells, especially after 72 hours of treatment.

Thymoquinone's apoptotic effect on MCF-7 breast cancer cells

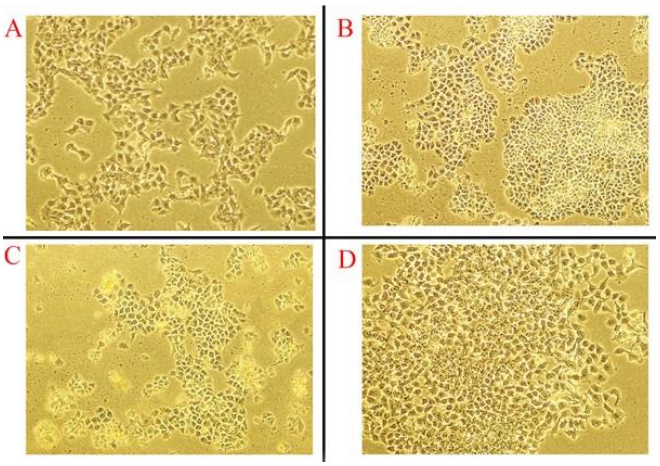


Figure 1.MCF-7 Cell culture, Cells before and after treatment with thymoquinone.(A) MCF-7 Cell before treatment with Thymoquinone, (B) MCF-7 Cell after 24 hrs treatment with Thymoquinone, (C) MCF-7 Cell after 48 hrs treatment with Thymoquinone, and (D)MCF-7 cells after 48 hrs without treatment. Inverted light microscope images (20× magnification).

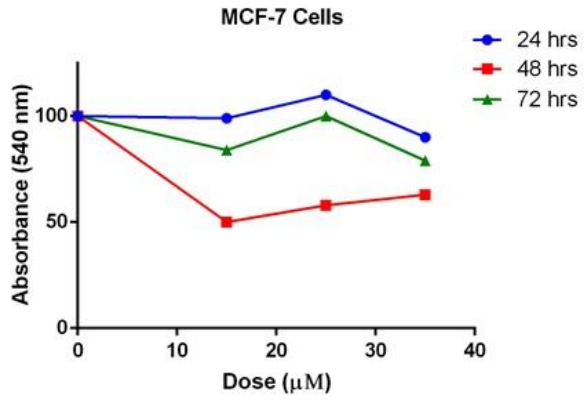


Figure 2. MCF-7 cells proliferation and viability, MTT results. Thymoquinone shows an anti-proliferative effect on breast cancer cells. Dose-response curves of the Thymoquinone after the administration for 12, 24 and 48 hrs treatment. The results were represented as the percentage of viable cells.

The inhibition mechanisms of Thymoquinone on cancer cell growth is still unclear and require many studies. In order to understand the inhibition mechanisms behind cell proliferation, we investigated the gene expression levels of specific apoptotic markers such as *Bax, BAD, Bcl-2*, and *p53*. The results showed significant changes in some apoptotic markers in both cancer cells and control cells (Figure 3).

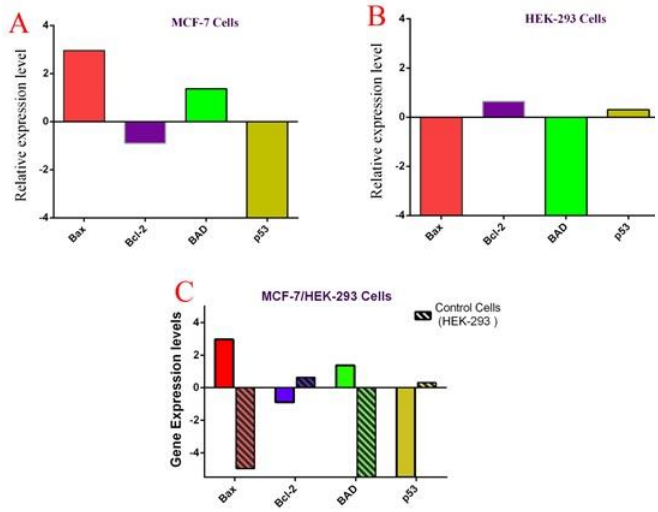


Figure 3. mRNA expression levels of *Bax*, *BAD*, *Bcl-2*, *p53* genes in MCF-7 and HEK-293 cells by Quantitative Real-Time PCR.Results were given as fold. (A) Relative expression levels of genes in MCF-7 cells, (B) Relative expression levels of genes in HEK-293 cells,(C) Comparison of gene expression levels between MCF-7 and HEK-293 cells.

Results showed a significant difference between MCF-7 cells and HEK293 for *Bax, BAD*, and *p53* gene expressions. We found an increase in gene expression of *Bax* gene in MCF-7 cells while a decrease in HEK293 cells. In contrast to *Bax* gene expression, *p53* gene expression was decreased in MCF-7 cells but slightly increased in HEK293 cells. Results also showed an increase in *BAD* gene expression in MCF-7 cells and a decrease in HEK293 cells. This contrast in the results was shown for the *Bcl-2* gene too. We found a slight decrease in *Bcl-2* gene expression in MCF-7 cells and an increase in HEK293 cells.

When we evaluate all the results that we have obtained from this study, we can summarize that Thymoquinone administration was increased *Bax* and *BAD* gene expression in MCF-7 cells but decreased in HEK293 cells.

Also, Thymoquinone administration decreased *p53* and *Bcl-2* gene expression in MCF-7 cells but increased in HEK293 cells.

DISCUSSION

Cancer is one of the most serious diseases for the human being in the present time. Cancer rates are spreading further with continuance and the problem is getting worse for every day. Breast cancer is the most prominent dilemma threating the lives of women and one of the highest causes of death among other cancers[16]. Treatment protocols are being investigated for all type of cancers including breast cancer[1,2]. Chemo- and radiotherapy and also surgery are the main solutions for breast cancer therapy[2]. There is widespread interest in natural products and phytochemicals to complete conventional medicines[17]. Pharmacological ingredients of natural products are tested to detect and determine their effectiveness against malignant tumors. There is a broad consensus in cancer research on the promising activity of Thymoquinone against cancer[3-6,12].

Anti-proliferative effect of Thymoquinone depends on several mechanisms including induction of apoptosis, autophagy, and cell cycle arrest. Several studies based on in-vitro cell experiments document that Thymoquinone-mediates apoptosis by regulating multiple pathways such as DNA fragmentation, chromatin condensation and translocation of phosphatidylserine through the plasma membrane[6] via affecting the*Bcl-xL, PTEN, p53, cyclin D1, ERK1/2, survivin, Mcl-1, PPAR, VEGF, caspase-9*,and *STAT3* genes[13,14].

In this study, we aimed to investigate the effect of Thymoquinone on MCF-7 breast cancer cell line. According to the MTT assay results, Thymoquinone decreased the proliferation of MCF-7 cells. After this finding, we focused on the apoptotic gene markers to detect the effect of Thymoquinone on proliferation via apoptosis. We also included the HEK293 cells in this study to evaluate the effects of Thymoquinone on non-tumor cells and we analyzed the gene expression levels of *Bax, BAD, Bcl-2* and *p53* genes which are the marker of apoptosis. *Bax* is an important mediator of the apoptotic pathway that induces apoptosis[18]. In this study, we found a nearly three-fold increase in *Bax* gene expression when we calculate the relative expression of the *Bax* gene, between treated and untreated MCF-7 cells. In accordance with these results, we found a decrease in the expression of this gene in non-tumor HEK293 cells. The product of *BAD* gene induces apoptosis via reversing the death repressor activity of *Bcl-xL* and *Bcl-2*[19]. In this study, like as *Bax* gene expression, we found a relative increase in the expression of this gene in MCF-7 cells and a decrease in HEK293 cells. Phosphorylation of BAD protein is important in its activity and AKT, MAP kinase and calcineurin are involved in its regulation[20], determine the changes of these mediators may contribute to the understanding of the effect of Thymoquinone.

p53 is the guardian of the genome and a key mediator of apoptosis. It shows its effects in two ways; one way is it can arrest the cell cycle for repair mechanisms and the second is it activates the expression of downstream genes that lead to the programmed cell death[21]. In our study, we found a decrease nearly four times fold in the *p53* gene expression after the Thymoquinone administration. This is compatible with the other results obtained for *Bax* and *BAD* genes. These results suggest that Thymoquinone probably affects via p53 or with the pathways that are related to p53 in the apoptotic process. It has been reported that thymoquinone induces cell cycle arrest in cancer cells in stages of G_0, G_1, S, G_2, and M by cyclin and cyclin-dependent kinases[6,5].

Marjaneh et al. reported the 64 upregulated and 78 down-regulated pathways after the Thymoquinone administration on estrogen receptor-positive breast cancer cells. They observed an increase in the expression of p53

and a decrease in Bcl2 genes due to the inhibition of the P38-MAPK pathway. In this study, they also showed that Thymoquinone administration down-regulated the GPCR and EDF-EGFR signaling pathways and therefore Thymoquinone has antiproliferative property in hormone-dependent cancer[22]. In a study conducted by Arafa et al., it has been shown that Thymoquinone induced apoptosis by increasing the PTEN expression in doxorubicin-resistant human breast cancer cells[23]. Woo et al. reported that Thymoquinone inhibited the expression of anti-apoptotic genes such as *XIAP, survivin, Bcl-xL*, and *Bcl-2* and increased the catalase, superoxide dismutase, and glutathione levels. In this study, they concluded that Thymoquinone induces the phosphorylation of p38 and increases the free oxygen radicals in breast cancer cells and this may be one of the reasons for apoptosis induction[24]. In another study conducted on the breast cancer cell, it has been reported that Thymoquinone inhibited NF-κB-regulated CXCR4 expression and inhibited the metastasis of tumor cells by suppressing the NF- κB pathway[25].

Thymoquinone has also been used in combination with the chemotherapeutic drugs used to treat cancer and the anti-cancer properties of Thymoquinone have been reported[26]. According to their study on breast cancer Kabel et al., concluded that administration of Thymoquinone in combination with Tamoxifen is a 'new therapeutic modality for management of breast cancer'[27]. In a study performed by Bashmail et al., it was shown that Thymoquinone increased the effect of Gemcitabine on the treatment of breast cancer due to the apoptotic and autophagic effects of Thymoquinone[28]. In another study performed on breast cancer cells, the effects of thymoquinone and another active molecule, resveratrol, were studied and the combination of Thymoquinone/resveratrol was shown to induce apoptosis, inhibit angiogenesis and provide immune modulation[29].

In conclusion, our results suggest that Thymoquinone significantly increases the expression of *BAD* and *Bax* genes in MCF-7 breast cancer cells when compared to normal cells. Thus, according to our results, Bax and BAD promote apoptosis. Bax and BAD stimulate apoptosis by binding to the Bcl-2 proteins. The transient combination between p53 and BAD activates pro-apoptotic proteins and releases cytochrome C from mitochondria to the cytoplasm in these cells and this may activate the caspase cascade. Further studies that are related to the upstream of the Bax, BAD, Bcl-2, and p53 may contribute to understanding better the effect mechanism of Thymoquinone.

Conflicts of interest: The authors have no conflict of interests to declare.

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