Indian Journal of Experimental Biology
Vol. 62, May 2024, pp. 317-325
DOI: 10.56042/ijeb.v62i05.3890

Effect of *Nigella sativa* L. extract and thymoquinone on the genes responsible for cell proliferation, migration and NK cell cytotoxicity in breast cancer

[1]Tuğcan Korak, Zeliha Emrence[2], Sema Sırma Ekmekci[2], Neslihan Abacı[2] & Emel Ergül[1]*

[1]Department of Medical Biology, Faculty of Medicine, Kocaeli University, Kocaeli 41001, Türkiye
[2]Department of Genetics, Aziz Sancar Institute of Experimental Medicine, Istanbul University, Istanbul 34093, Türkiye

Received 11 July 2023; *revised 23 October 2023*

The black cumin, *Nigella sativa* L. is known to be effective against various diseases including cancer. Thymoquinone (TQ), active ingredient of *N. sativa* extracts, can inhibit proliferation metastasis and regulating immune system in different cancers as with breast cancer (BC). The mechanisms of action behind TQ are not clearly understood yet. The purpose of the current study is to investigate the effects of TQ, water (WE) and alcohol extracts (AE) of *N. sativa* on BC cells by focusing attention on the following genes*; CDK4*, *MYC*, *NF-κB1*, *VEGFA*, *FGF1*, *N-cadherin*, *ULBP1*, *ULBP2* and *CD155*. Conventional protocols were performed in order to obtain extracts. Cell viability was measured by RTCA and MTT assay, and gene expressions were analyzed by qRT-PCR. Association was significant for *CDK4* (P =0.07), *MYC* (P <0.001), *NF-κB1* (P =0.011), *VEGFA* (P =0.013), *FGF1* (P <0.001), and *ULBP1* (P =0.021) genes. *CDK4* and *MYC* genes may be candidate genes for mechanisms involved in reduced cell proliferation induced by AE and TQ. Increased ULBP1 expression through AE and TQ indicates that *N. sativa* may trigger ULBP1-mediated NK cell cytotoxicity. Our results support the idea that active ingredients in *N. sativa* promise an encouraging therapeutic approach in the future.

Keywords: Anticancer, Black cumin, Kalonji

Breast cancer (BC) is the most common type of cancer among women and is the second leading cause of death among them. The occurrence rates for breast cancer showed an annual increase ranging from 0.6% to 1% between 2015 and 2019. Additionally, nearly 310,720 new cases and 42,250 deaths are predicted in women living United States for 2024[1–3]. According to 2020 Globocan data, breast cancer in India represented 13.5% of all cancer cases and 10.6% of all cancer-related deaths, with a cumulative risk of 2.81. Recent trends indicate a higher occurrence of the disease at younger ages in Indian women compared to the Western population[4]. Conventional strategies including surgery, radiotherapy and chemotherapy have been applied safely over the years, but treatment resistance and effective therapy to all BC subtypes remain a major challenge[5]. Therefore, special attention should be paid to BC treatment and new studies that may support the current therapies will be highly valuable.

Known also as a black seed, *Nigella sativa* (*N. sativa*), has been evaluated as a valuable plant with the broad spectrum of medicinal effects on various diseases including liver, kidney, cardio-vascular and skin disease as well as cancer. It originates from Southern Europe, North Africa, Southwest Asia, and the Middle East, and could be grown in nearly each region of the world. *N. sativa*, a member of the Ranunculaceae family, typically reaches heights of 20 to 90 centimetres and produces fruits in the form of capsules, comprising compound follicles that house numerous seeds. Numerous active compounds have been discovered within *N. sativa* seeds. The most significant among them, along with their respective proportions in the extract compositions, include thymoquinone (TQ) (30-48%), thymohydroquinone, dithymoquinone, p-cymene (7-15%), and 4-terpineol (2-7%). *In vivo* and *in vitro* studies on cancer cells, including breast cancer, provide evidence that *N. sativa* extracts and its active compound, thymoquinone (TQ), have inhibitory effects on proliferation and metastatis[6] . Many studies demonstrated comprehensively that *N. sativa* extracts and TQ had antiproliferative and antimetastatic effects on BC cells[6–11] by regulating Caspase-3, -8, -9[12] in apoptotic pathways and reducing expression of Brca1, Brca2 and P53 genes in breast tissues of female rats[13].

*Correspondence:
E-Mail: ergulemel1641@gmail.com

In addition, *N. sativa* has been shown to have anticancer effects by increasing the cytotoxic activity of Natural killer (NK) cells, which function in tumor surveillance and provide direct cytotoxicity in the cells they target[14,15]. In our previous study, we selected five experimentally verified *N. sativa* gene targets associated with proliferation, apoptosis and angiogenesis and performed pathway analysis in order to show *N. sativa* affected signalling pathways potential. The analysis revealed that there were 18 affected pathways related to *N. sativa* administration. Thus, *N. sativa*, accompanied by other affected genes, may regulate diverse signal transduction pathways in a cell[6]. Many of the mechanisms underlying the effect of *N. sativa* on cancer and BC are still waiting for clarification.

Based on these, here, we conducted this *in vitro* study to investigate the effects of thymoquinone (TQ), water (WE) and alcohol extracts (AE) of *N. sativa* on the genes including *CDK4* (cyclin-dependent kinase 4), *MYC* (c-MYC), *NF-κB1* (nuclear Factor kappa B1), *VEGFA* (vascular endothelial growth factor A), *FGF1* (fibroblast growth factor 1), *N-cadherin* (neural cadherin), *ULBP1*, *ULBP2* (UL16 binding protein 1, 2) and *CD155* (Poliovirus receptor, PVR) in BC cells. While *ULBP1/2* and *CD155* are playing a role in cytotoxic activity of NK cells, others are mainly involved in tumorigenesis and/or metastasis.

Materials and Methods

N. sativa water and alcohol extraction

Extraction process was mainly performed according to conventional protocols with some modifications and improvements. *N. sativa* (Black cumin, black seed) was supplied from the Mediterranean Region of Turkey as a fresh crop black seed form (Aslan Group, Isparta)[9]. Black cumin seeds were thoroughly washed with plenty of cold water before extracting, kept in a cool environment until completely dry, and all further steps were carried out in a cold environment as much as possible. *N. sativa* seeds were completely pulverized by an electric grinder (Sinbo;SCM2934). For WE, 200 mL of sterile de-ionized water (milliQsynthesis-F9JN15604I) was added to 20 g powder form and kept at 4°C for 36 h. For AE, 500 mL of 95% ethanol was added to 100 g of powder form and kept at 4°C for 36 h. Then, supernatants of both extracts were placed into the different sterile wide pan and dried under the separate hood.

For WE, the dried material (nearly after 28 h) was scraped from the surface, 1 g was weighed and resuspended by adding 100 mL of sterile de-ionized water. This solution was poured into two 50 mL falcon tubes and centrifuged at 4500 rpm for 15 min to remove water-insoluble particles. Supernatant was filtered by 0.20 µm filter (Sartorius stedim biotech, Minisart, syringe filter, Germany) and taken into sterile bottles. The extract (10.000 µg/mL) was stored at –20°C for further use.

For AE, the dried gel-like material (nearly after 72 h) was scraped from the surface, 1 g was weighed and resuspended by adding 100 mL of dimethyl sulfoxide (DMSO; BioShop, Canada). This homogeneous solution was filtered by DMSO compatible 0.22 µm filter (Molgen Biotechnology, RC-Syringe Filter, Turkey) and taken into sterile bottles. The extract (10,000 µg/mL) was stored at –20°C for further use.

Thymoquinone solution preparation

Thymoquinone (TQ) (Glentham Life Sciences (GT5953), United Kingdom) in powder form was weighed as 0.1 g and dissolved in 10 mL of DMSO. As a result, 10.000 µg/mL (~60.000 µM) stock solution was obtained.

Cell culture

MCF-7 (Breast cancer cell line) was commercially obtained from American Type Culture Collection (ATCC). MCF-7 cells (passage number:14) were grown in DMEM with L-glutamine (GIBCO, Thermo Fisher Scientific, Waltham, MA, USA) including 10% fetal bovine serum (GIBCO, Thermo Fisher Scientific, Waltham, MA, USA), 1% penicillin/ streptomycin (100 IU/mL penicillin, 0.1 mg/mL streptomycin) (Wisent Bioproducts, Canada). The cell line was incubated at 37°C in 5% CO_2 incubator. Cell viability and counting were performed on a Cell Viability Analyzer (Vi-Cell Beckman Coulter, USA).

xCELLigence real-time cell analysis (RTCA): dose concentration and exposure time optimization

After adding different numbers of cells (from 5000 to 20000) to the 96 well E-plate (Roche, Switzerland), the cells were kept in the cabinet for 30 min at room temperature. Then, E-plate was placed in the xCELLigence device (Roche, Switzerland) and incubated at 37°C for 24 h. Based on the analysis results, optimum cell number for the study was determined as 17.500. Next, different concentrations of WE, AE and TQ was applied for 72 h in order to obtain doses for IC_{50} calculation and to figure out

effective exposure time through xCELLigence system.

MTT cell viability assay

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) test was performed to detect cell viability at determined doses (WE: 600 µg/mL, AE: 197 µg/mL, TQ: 2.5 µg/mL) and time (48 h). For the MTT test, cells were seeded 24 h before on a 96-well plate with 17,500 cells in a volume of 200 µL. It was incubated at 37°C for 24 h. Next, the doses were applied on the cells and the plate was incubated at 37°C for 48 h. Finally, cell viability was analyzed in accordance with the manufacturer's instructions of CyQUANT™ MTT Cell Viability Assay Kit (Thermo-Fisher Scientific, V13154). The MTT test was carried out in four replicates for each dose and controls. Absorbance was measured by Multiskan Spectrum (Thermo Fisher Scientific, USA) at 540 nm.

Total RNA isolation

Total RNA from cells was isolated using the PureLinkTM RNA Mini Kit (Thermo-Fisher Scientific) protocol. RNA concentrations were measured using a spectrophotometer (Nanodrop 2000; Thermo Fisher Scientific, USA).

Real-Time quantitative PCR (qRT-PCR)

Firstly, cDNA synthesis was carried out in accordance with Transcriptor First Strand cDNA Synthesis Kit (Roche Life Science, Germany) protocol using 200 ng/µL total RNA. For gene expression analysis, primers were specifically designed for *CDK4*, *MYC*, *NF-κB*, *VEGFA*, *FGF1*, *N-cadherin*, *ULBP1*, *ULBP2*, *CD155* and housekeeping *TATA binding protein gene* (TBP) genes. Melting temperature was optimized for each gene and qRT-PCR conditions were as following: 95°C for 5 min, 95°C for 10 s, 61°C (*FGF1*, *MYC*, *ULBP1*, *ULBP2*)/62°C (*CDK4*, *NF-κB*, *VEGFA*, *N-cadherin*)/ 65°C (*CD155*) for 20 s, and 72°C for 10 s (45 cycles). Primer sequences were listed in Table 1. qRT-PCR reaction was done by SYBR green (SensiFAST SYBR No-ROX Kit, Bioline) in LightCycler 480 Instrument II (Roche). All experiments were performed in triplicate. Relative standard curve method was used for quantification and *TBP* gene was used in normalization.

Statistical analysis

All statistical analyses were performed by IBM SPSS 20.0 (IBM Corp., Armonk, NY, USA). Comparisons of applied materials (WE, AE, TQ) and control group with respect to gene expressions were carried out by one-way analysis of variance (one-way ANOVA). Tukey's and Dunnett's tests were used for pairwise multiple comparisons. A P <0.05 was considered statistically significant.

Table 1 — Primer sequences of target genes

Genes	Primers	Primer Sequences
CDK4	F	5'-ACACCCGTGGTTGTTACACT-3'
	R	5'-GTCGGCTTCAGAGTTTCCAC-3'
MYC204	F	5'-CCGTCCTCGGATTCTCTGC-3'
	R	5'-TTGTTCCTCCTCAGAGTCGC-3'
NF-κB1	F	5'-CTGGAACCCGTGGTATCAGA-3'
	R	5'-CATCCAGCTGTCCTGTCCATT-3'
VEGFA	F	5'-GGCCTCCGAAACCATGAAC-3'
	R	5'-GCTGCGCTGATAGACATCCA-3'
FGF1	F	5'-CTTTTATACGGCTCACAGACACC-3'
	R	5'-CTCCCATTCTTCTTGAGGCCAA-3'
N-Cadherin	F	5'-TGCCCGGTTTCATTTAGGGG-3'
	R	5'-TGTTCCAGGCTTTGATCCCTCA-3'
ULBP1	F	5'-GGGGATTGTAAGATGTGGCT-3'
	R	5'-GGAAGATGATGAGAAGCCTCC-3'
ULBP2	F	5'-GCCGCTACCAAGATCCTTCTG-3'
	R	5'-CTGGCCTTGAACCGCACAC-3'
CD155	F	5'-CCTGCTGGGGATCGGGATTT-3'
	R	5'-ATTAGCTGAGGCGCTGGCAT-3'
TBP	F	5'-ACTTGACCTAAAGACCATTGCAC-3'
	R	5'-CTTGAAGTCCAAGAACTTAGCTGG-3'

[F: Forward; and R: Reverse]

Results

Extraction times for *N. Sativa* WE and AE

Modifications and enhancements were made to the *N. sativa* extraction process. While optimizing the 36 h incubation period for extract preparation, seed powders were incubated in their respective solvents for 12, 24, 36, 48, and 60 h. The samples from the liquid parts of extracts were collected at these time points for optical density (OD) measurements. Subsequently, their optic densities were measured at 254 and 360 nm (maximum absorbance value for TQ and appropriate absorbance value providing an overview for other substances such as phenolic acids, alpha hederin and kaempferol found in both extracts, respectively[16–18]) to deduce at which time the more concentrated extract was obtained. Finally, it was observed that density of both extracts increases up to 36 hours and then decreases. Therefore, waiting for 36 h were decided as more convenient time for this step (Fig. 1).

Beside the determination of extraction time, optical density (OD) values can give some indication of the presence and concentration of ingredients within plant extract. Spectrophotometric methods are a useful tool for assessing the total concentration of high volumes

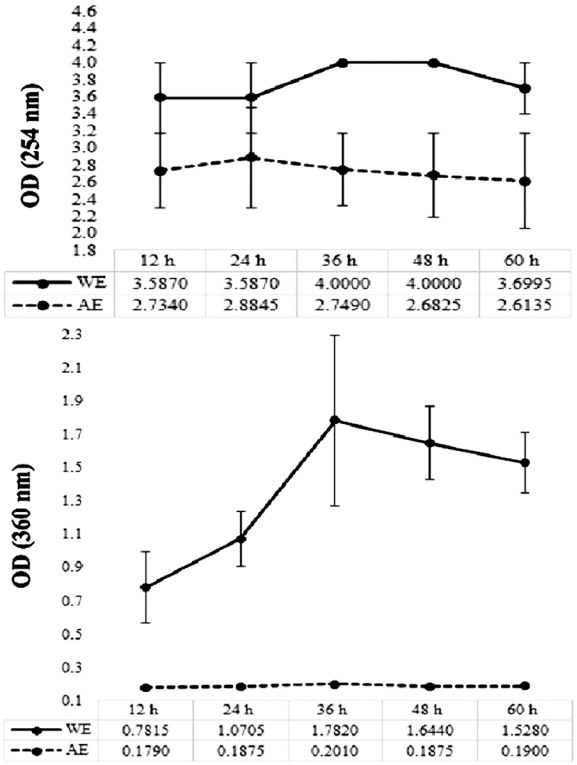

Fig. 1 — Spectrophotometric analysis of WE and AE at 254 nm and 360 nm. [Time-dependent extraction were indicated as OD mean ± standard deviation (SD)]

of plant extracts in a cost-effective and efficient manner, For instance, high linear relationship was found between simple spectrophotometric analysis and HPLC analysis when total quercetin content were compared. In addition, the spectrophotometer has demonstrated its reliability in estimating the combined concentrations of the two primary flavanol conjugates in a related raw plant extract[19]. Although not as descriptive as analytical techniques providing more comprehensive information about the composition of plant extract, our data indicated the presence TQ and other mentioned components in AE and WE (Fig. 1).

Dose determination and the time of administration

After optimum cell number was assigned as 17.500 using xCELLigence system, dose administration time was determined as 48 h. Because the curves in xCELLigence cell index graph did not show complete difference between 24 and 48 h, treatment of the cells for 24 h was eliminated. Besides, the curves at the 72nd h was similar to the 48th h, and the calculated IC_{50} concentrations were also found to be similar in these hours. Therefore, it was determined that 48 h treatment is sufficient to detect applied dose effects.

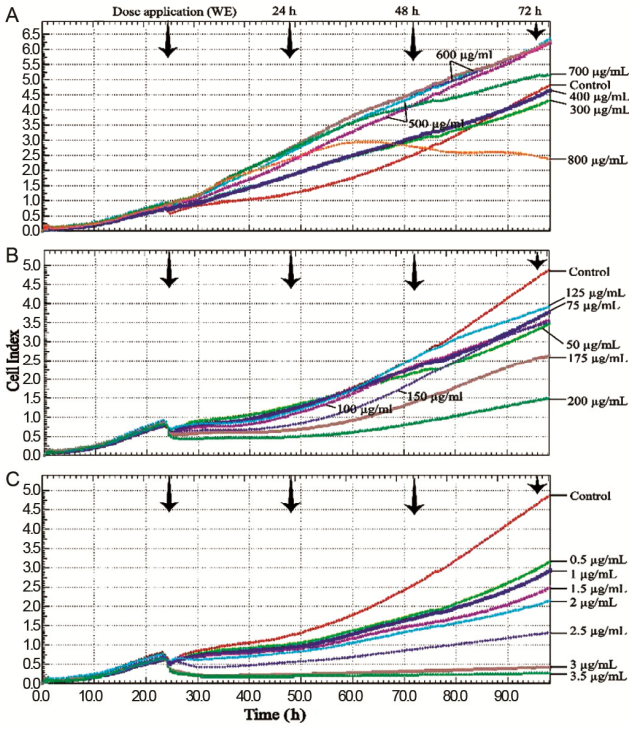

Fig. 2 — Real time cell index of MCF-7 cells after treatment of different concentrations of WE, AE and TQ for 24, 48 and 72 hours. Treatment with different concentrations of (A) WE; (B) AE; and (C) TQ. MCF-7 cells incubated with medium culture only were used as control, represented as red line. The lines of the graphs illustrate the concentrations of WE, AE, and TQ that were administered. These concentrations are displayed on the right side of the graph. The arrows above the graph indicate the timing of dose administration in hour.

IC_{50} values of AE and TQ was found as 197 µg/mL and 2.5 µg/mL, respectively. Since the toxic effect was not obtained for WE, the dose (600 µg/mL) leading to best proliferation was determined for treatment (Fig. 2).

Effects of extracts and TQ on cell viability

MTT assay was performed to test cell viability at specified doses and to confirm xCELLigence results[20]. The results showed that WE led to cell proliferation compared to the control in xCELLigence system, while it caused statistically significant decreased cell proliferation in MTT assay ($p=0.001$) (Fig. 2 A and Fig. 3). The cell viability obtained for AE and TQ were consistent for both methods, and it was found to be effective on reducing the viability of the cells at the doses determined in the MCF-7 BC cell line (Fig. 2 B & C and Fig. 3).

Effects of extracts and TQ on gene expressions

According to the results of one-way variance analysis, while significant difference was obtained

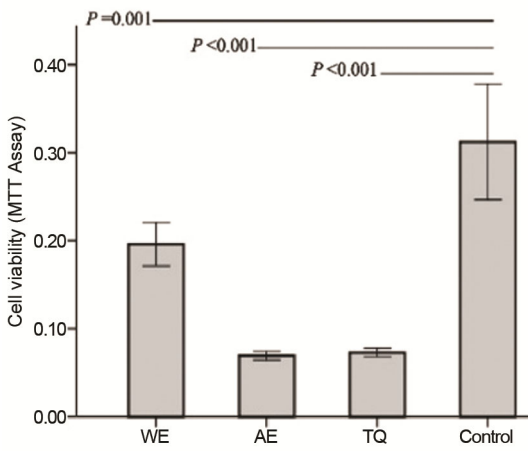

Fig. 3 — Evaluation of MCF-7 cell proliferation via MTT assay after treatment of WE (600 µg/mL), AE (197 µg/mL), TQ (2.5 µg/mL) for 48 hours. Cell viability levels were indicated as mean ± SD. Statistical significance among materials applied (WE, AE, TQ) and Control were represented with *P* values.

between groups (AE, WE, TQ and control) for *CDK4* (*P* =0.07), *MYC* (*P* <0.001), *NF-κB1* (*P* =0.011), *VEGFA* (*P* =0.013), *FGF1* (*P* <0.001), and *ULBP1* (*P* =0.021) genes, there was no significant difference for *N-cadherin* (*P* =1.84), *ULBP2* (*P* =0.131) and *CD155* (*P* =0.071).

For *CDK4* gene expression; when AE and TQ were administered to MCF-7 breast cancer cells, 1.25 and 1.20-fold statistically lower expression was obtained for AE (*P* =0.012) and for TQ (*P* =0.027) compared to control, respectively. When the differences between the administered substances on CDK4 expression were examined, a significant association was found between WE and AE (*P* =0.020) and TQ (*P* =0.043), but no statistical significance was observed between AE and TQ (*P* =0.943) (Fig. 4A). For *MYC* gene expression; AE (*P* =0.001) and TQ (*P* =0.001) led to 2.65 and 2.54-fold statistically lower expression of MYC compared to the control, respectively. There was no significant relationship between WE and control in our study (*P* =0.450). Significant difference was obtained between WE and AE (*P* <0.001) and TQ (*P* <0.001), while no significant effect was observed between AE and TQ (p=0.997) (Fig. 4B). For *NF-κB1* gene expression; AE (*P* =0.238) and TQ (*P* =0.830) did not significantly affect the *NF-κB1* gene expression compared to control, while WE 1.59-fold increased the expression (*P* =0.019) (Fig. 4C). For *VEGFA* gene expression; when the cells were treated with TQ, 2.13-fold higher expression of *VEGFA* compared to the control and it was the only statistically significant data on the

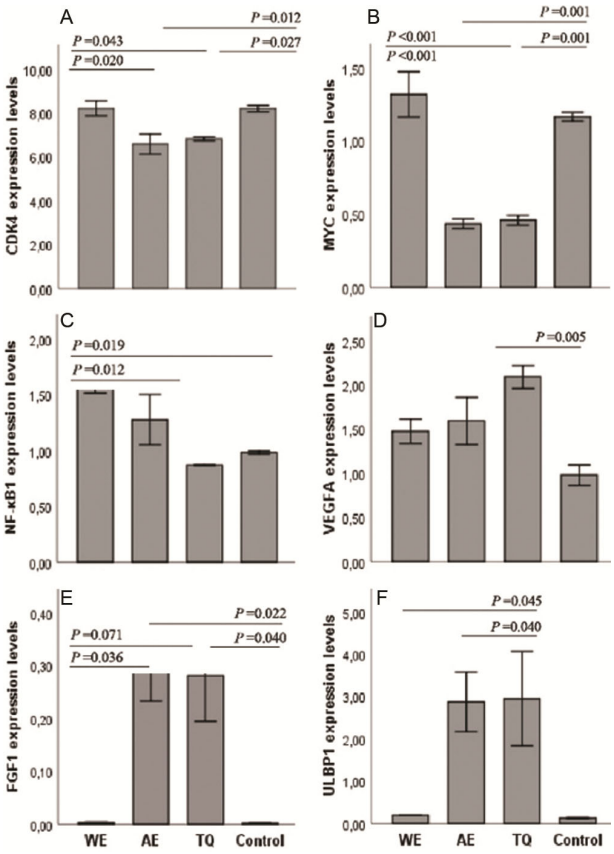

Fig. 4 — Expression of six genes by quantitative PCR after MCF-7 cells were treated with WE (600 µg/mL), AE (197 µg/mL), TQ (2.5 µg/mL) for 48 hours. Expression levels were indicated as mean ± SD and statistically significant differences were represented with *P* values on each graph. (A) *CDK4*; (B) *MYC*; (C) *NF-κB*; (D) *VEGFA*; (E) *FGF1*; and (F) *ULBP1* expression levels.

VEGFA in our study (*P* =0.005) (Fig. 4D). For FGF1 gene expression; the expression was found to be elevated 156.5-fold by AE (*P* <0.001), 140.5-fold by TQ (*P* <0.001) compared to control. Significant difference was obtained between WE and AE (*P* <0.001) and TQ (*P* <0.001), while no significant effect was observed between AE and TQ (*P* =0.870) (Fig. 4E). For *ULBP1* gene expression; when AE (*P* =0.045) and TQ (*P* =0.040) were applied to MCF-7 cells, 23.9 and 24.6-fold statistically higher *ULBP1* expression was obtained for AE and TQ compared to the control, respectively. There was no significant difference between applied materials (WE and AE (*P* =0.080), WE and TQ (*P* =0.071), AE and TQ (*P* =1.000)) (Fig. 4F).

Discussion

In the last decade, there are scientifically confirmed accumulating evidence that indicate effects of

N. sativa extracts and its major ingredient TQ on cancer including BC via involvement in cell proliferation, metastasis and immune system regulation[6,21]. Considering the extensive impacts that *N. sativa* and TQ perform in the cells, the effects of TQ as well as WE and AE of *N. sativa* on the target genes were investigated.

As a first step, we modified the conventional extraction method to improve its quality. For this purpose, *N. sativa* extraction time was clarified and centrifugation steps were added to remove insoluble particles. Following the administration of extracts and TQ to MCF-7 cells, it was observed that WE enhanced the cell proliferation compared to the control in the xCELLigence system. Conversely, a statistically significant decrease in cell proliferation was obtained in the MTT assay. It can be explained by the differences, advantages and disadvantages of the working principles of both systems. The xCELLigence system basically measures the net cell adhesion in the well. Therefore, any cell response that changes cell morphology including cell size, volume, shape, and spread, can affect the measurement value[22]. Based on this logic, the changes in cell morphology (such as cell swelling) caused by WE may lead to the cell index to be observed higher than the control. On the other hand, in tests based on tetrazolium salt, such as the MTT assay, the formazan product may in some cases be reduced by various chemicals or materials such as antioxidants, flavonoids, vitamins and plant extracts in the medium, causing false-positive results[20]. There may be more such phytocomponents in the WE than in AE and it may have led to conflicting results. There is no consensus results in studies involving WE and MCF-7 cells whether WE leads to cell proliferation or not[11,23]. Although different results were obtained for WE on cell death/proliferation with these techniques, WE did not have as much lethality or a drastic effect as AE and TQ in comparison to the control, which is in a similar manner to some other studies[8,9]. On the other hand, decreased cell viability obtained for AE and TQ were consistent for both methods. This data have been supported by previous studies in terms of decrease in cell viability of different cell lines after alcohol extracts of *N. sativa* administration[8,9,12,24,25].

The basis of same directionality for AE and TQ are as follows: TQ is found in the highest ratio (30-48%) in *N. sativa* as compared to other active ingredients and TQ solubility in alcohol is much higher than in water[26,27]. Different cell cytotoxicity between AE and WE of our study may arise from different soluble phytocomponents, variability in their amounts, antioxidant activities of these components and stability of active ingredients[16,28]. These differences may also explain the discrete effects of extracts on the expression of the genes, discussed below.

The CDK4 protein encoded by the *CDK4* gene (12q14.1) is involved in the regulation of the G1-S phase transition in the cell cycle[29] and the product of the gene is usually upregulated in human cancer types[30]. Cyclin D1 is overexpressed in all types of breast cancer, and the cyclin D1-CDK4 complex has been shown to be essential for breast cancer cells to maintain their tumor potential. Cyclin D1 is overexpressed as a result of activation of CDK4, which can alter the cell cycle process and lead to malignancy[31–33]. In the current study, when AE and TQ were administered to MCF-7 breast cancer cells, statistically lower expression was obtained for AE and for TQ compared to control, respectively. Therefore, *N. sativa* AE and TQ may exert their anticancer effect through the regulation of the *CDK4* gene. These results are directly or indirectly consistent with the studies in literature[34,35]. In contrast to AE and TQ, there was no significant difference in CDK4 expression between WE and control. It seems that WE is not as effective as AE and TQ in influencing CDK4 gene expression.

MYC protein encoded by the *MYC* gene (8q24.21) is responsible for the regulation of cell cycle, proliferation, differentiation and apoptosis processes[29]. In the breast cancer tumorigenesis, the tumor suppressor BRCA1 reduces the transcriptional activity of *MYC*, and consequently the absence of BRCA1 and overexpression of MYC triggers the development of breast cancer. MYC overexpression has been demonstrated in “basal-like subtype” breast cancer type which has a poor prognosis and does not have a therapeutic target. In a study conducted on the MCF-7 cell line, it was revealed that *MYC* expression was inhibited by siRNA, resulting in inhibition of tumor growth *in vitro* and *in vivo*. The targeting of overexpressed oncogenes such as *MYC* in various cancer types and the therapeutic strategies developed based on the inhibition of oncogenic pathways are pivotal for cancer research[36,37]. In our study, AE and TQ led to statistically lower expression of MYC

compared to the control. Therefore, cytotoxicity of AE and TQ can be explained through suppression of *MYC* gene and other related pathways. These results are in accordance with the previous studies[35,38–41]. On the other hand, there was no significant relationship between WE and control. Thus, WE has less impact on *MYC* gene expression similar to CDK4.

The NF-κB1 encoded by the *NF-kB1* gene (4q24) is a subunit of NF-κB that mainly plays a role in cell cycle and inhibition of apoptosis and cell adhesion[29,42]. NF-κB is overexpressed in several cancer types[43]. In this study, AE and TQ did not significantly affect the *NF-κB1* gene expression, while WE increased the expression. WE may increase the expression only at mRNA level but at protein synthesis level *NF-kB1* gene expression may be suppressed by further translation and modification steps, as a result decreased cell survival in the MTT assay may have occurred[44]. The studies previously conducted indicate that AE and/or TQ decrease the expression of NF-κB[38,39,43,45,46]. It seems that our AE and TQ dose concentration was not sufficient to suppress *NF-κB1* gene.

Metastasis related *VEGFA* (6p21.1), *FGF1* (5q31.3) and *N-cadherin* (18q12.1) genes are primarily involved in cell migration and upregulated in many cancer types[29,47]. When the cells were treated with TQ, higher expression of *VEGFA* compared to the control was obtained. Dissolved TQ in AE might be insufficient to affect expression. Six studies in literature are inconsistent with our results and demonstrated the suppressive effect of TQ and AE on *VEGFA* expression in the different cancer types[38,46]. The TQ-based increase in *VEGFA* mRNA may be eliminated by some translational or post-translational modifications and finally we may have obtained triggered cell death[44]. On the other hand, *FGF1* expression was found to be elevated 156.5-fold by AE and 140.5-fold by TQ compared to control. The increase observed here is quite pronounced. This mRNA-level data indicates that AE and TQ may exert their cytotoxic effect independently of *FGF1*. AE and TQ may have a neutralizing effect on FGF1 proteins. Also, they may have triggered the deregulation of the FGF receptors and prevented the binding of FGF1. Furthermore, degradation pathway of the FGF receptors could be activated by AE and TQ, then the receptors might be transported to lysosomes for degradation[48,49]. In addition to potential translational modifications on FGF1, these mechanisms may underlie the inconsistency between our decreased cell proliferation results and increased FGF1 expression after AE and TQ administration.

Besides many others, there are two important NK cell receptors, Natural killer group 2D (NKG2D) and DNAX accessory molecule-1 (DNAM-1), that play a role in the regulation of cellular stress based on NK cells. They induce NK cell-mediated cytotoxicity in target cells after interacting with their ligands. ULBP1 and ULBP2 are the ligands for NKG2D receptors and generally upregulated in stress conditions such as malignant transformation. One of the studies showed that *ULBP1/2* genes (6q25.1) upregulated in lung cancer cells resulting in NK cell cytotoxicity. *CD155* gene (19q13.31) encoded ligands interact with DNAM1 receptors and overexpressed in malignancy[14,29]. During the study planning, we evaluated whether *N. sativa* was responsible for directing NK cytotoxicity in cancer cells by its effect on these ligands, ULBP1, ULBP2, CD155. A study demonstrated that the presence of thymoquinone led to an enhanced NK cells cytotoxicity on BC cells, accompanied by increased secretion of perforin, granzyme B, and IFN-α[50]. In our study, AE and TQ were found to increase *ULBP1* expression. Only *ULBP1* had significant association and thus cytotoxic activity of NK cells could be increased by upregulated ULBP1 ligand as a result of AE and TQ treatment in BC.

To the best our knowledge, this is the first study exhibiting the effect of AE, WE, TQ one the *FGF1*, *ULBP1*, *ULBP2*, *N-Cadherin*, *CD155* gene expressions in MCF-7 cell line. Also, WE effect on the expressions of remaining genes, *CDK4*, *MYC*, *NF-κB1*, *VEGFA* did not studied previously. The significant expression results for *CDK4*, *MYC* and *ULBP1* were consistent with literature and our hypothesis in terms of AE and TQ effects. This finding highlights a novel mechanism through which *N. sativa* may enhance the immune response against cancer cells. In this context, the research possesses unique significance and contribute to expands our understanding of the potential mechanisms associated with *N. sativa* and breast cancer, offering valuable insights into potential therapeutic pathways and future therapeutic approaches for the management of this complex disease. These genes probably play an active role in pathways regulated by *N. sativa.* Only *FGF1*

has strikingly contradictory expression results which is worth further investigation. In future, protein-level or *in vivo* studies may elucidate the therapeutic potential of *N. sativa* by focusing on the pathways associated with *CDK4*, *MYC* and *ULBP1* genes. Consequently, underlying anticancer mechanisms of this medicinal herb, which is highlighted for its broad impact[51, 52], will be demonstrated more clearly.

In future studies, the cytotoxicity differences between WE and AE can be demonstrated more significantly with using high-performance liquid chromatography (HPLC) by revealing the components and their percentages in the *N. sativa*. This data in different studies may also explain the variation in the effects on gene expression. The analyzing of the proteins particularly for the genes obtained statistical significance will be useful to support our transcription-level results and to clarify conflicting results. Additionally, NK cells and extract-treated cells could be co-cultured to demonstrate the targeting of NK cells and the presence of NK cell-based cytotoxicity.

Conclusion

To date, numerous scientifically based research has reported the effect of *Nigella sativa* extracts and thymoquinone (TQ), an active ingredient of the extract, on cell proliferation, migration and beneficial regulation of immune response. The data presented in this study provided improvement of conventional extraction process and the significant effects of both alcohol and water extracts, and TQ on the expression of some targeted genes. In broaden perspective, after standardized extraction process, metabolomics studies may further illustrate *N. sativa* potential. All in all, along with the comprehensive praiseworthy results of *N. sativa* in preclinical studies, it can be evaluated as additional supportive therapeutic agent against breast cancer and other cancers as well.

Conflict of interest

Authors declare no competing interests.

References

1 Dizon DS & Kamal AH, Cancer statistics 2024: All hands on deck. *CA Cancer J Clin*, 74 (2024) 8.

2 Siegel RL, Giaquinto AN & Jemal A, Cancer statistics, 2024. *CA Cancer J Clin*, 74 (2024) 12

3 Shruthi S, Vijayalaxmi K, Rao BS & Shenoy KB, The modulatory effect of septilin on cytotoxicity of cisplatin in a human breast adenocarcinoma cell line *Indian J Tradit Knowl*, 19 (2020) 435.

4 Mehrotra R & Yadav K, Breast cancer in India: Present scenario and the challenges ahead. *World J Clin Oncol*, 13, (2022) 209.

5 Burguin A, Diorio C & Durocher F, Breast Cancer Treatments: Updates and New Challenges. *J Pers Med*, 11 (2021) 808.

6 Korak T, Ergül E & Sazci A, *Nigella sativa* and Cancer: A Review Focusing on Breast Cancer, Inhibition of Metastasis and Enhancement of Natural Killer Cell Cytotoxicity. *Curr Pharm Biotechnol*, 21 (2020) 1176.

7 Dehghani H, Hashemi M, Entezari M & Mohsenifar A, The comparison of anticancer activity of thymoquinone and nanothymoquinone on human breast adenocarcinoma. *Iran J Pharm Res*, 14 (2015) 539.

8 Farah IO & Begum RA, Effect of *Nigella sativa* (*N. sativa L.*) and oxidative stress on the survival pattern of MCF-7 breast cancer cells. *Biomed Sci Instrum*, 39 (2003) 359.

9 Farah IO, Assessment of cellular responses to oxidative stress using MCF-7 breast cancer cells, black seed (*N. sativa L.*) extracts and H2O2. *Int J Environ Res Public Health*, 2 (2005) 411.

10 Odeh F, Ismail SI, Abu-Dahab R, Mahmoud IS & Al Bawab A, Thymoquinone in liposomes: a study of loading efficiency and biological activity towards breast cancer. *Drug Deliv*, 19 (2012) 371.

11 Mahmoud SS & Torchilin VP, Hormetic/cytotoxic effects of *Nigella sativa* seed alcoholic and aqueous extracts on MCF-7 breast cancer cells alone or in combination with doxorubicin. *Cell Biochem Biophys*, 66 (2013) 451.

12 Alhazmi MI, Hasan TN, Shafi G, Al-Assaf AH, Alfawaz MA & Alshatwi AA, Roles of p53 and caspases in induction of apoptosis in MCF- 7 breast cancer cells treated with a methanolic extract of *Nigella sativa* seeds. *Asian Pac J Cancer Prev*, 15 (2014) 9655.

13 Linjawi SA, Khalil WK, Hassanane MM & Ahmed ES, Evaluation of the protective effect of *Nigella sativa* extract and its primary active component thymoquinone against DMBA-induced breast cancer in female rats. *Arch Med Sci*, 11 (2015) 220.

14 Chan CJ, Smyth MJ & Martinet L, Molecular mechanisms of natural killer cell activation in response to cellular stress. *Cell Death Differ*, 21 (2014) 5.

15 Majdalawieh AF & Fayyad MW, Immunomodulatory and anti-inflammatory action of *Nigella sativa* and thymoquinone: A comprehensive review. *Int Immunopharmacol*, 28 (2015) 295.

16 Iqbal MS, Ahmad A & Pandey B, Solvent based optimization for extraction and stability of thymoquinone from *Nigella sativa* Linn. and its quantification using RP-HPLC. *Physiol Mol Biol Plants*, 24 (2018) 1209.

17 Topcagic A, Cavar Zeljkovic S, Karalija E, Galijasevic S & Sofic E, Evaluation of phenolic profile, enzyme inhibitory and antimicrobial activities of *Nigella sativa* L. seed extracts. *Bosn J Basic Med Sci*, 17 (2017) 286.

18 Altemimi A, Lakhssassi N, Baharlouei A, Watson DG & Lightfoot DA, Phytochemicals: Extraction, Isolation, and Identification of Bioactive Compounds from Plant Extracts. *Plants* (Basel), 6 (2017) 42.

19 Lombard KA, Geoffriau E & Peffley E, Flavonoid Quantification in Onion by Spectrophotometric and High Performance Liquid Chromatography Analysis. *HortScience*, 37 (2002) 682.

20 Stefanowicz-Hajduk J & Ochocka JR, Real-time cell analysis system in cytotoxicity applications: Usefulness and comparison with tetrazolium salt assays. *Toxicol Rep*, 7 (2020) 335.

21 Shabani H, Karami, MH, Kolour J, Sayyahi Z, Parvin MA, Soghala S, Baghini SS, Mardasi M, Chopani A, Moulavi P, Farkhondeh T, Darroudi M, Kabiri M & Samarghandian S, Anticancer activity of thymoquinone against breast cancer cells: Mechanisms of action and delivery approaches. *Biomed Pharmacother*, 165, (2023) 114972.

22 Kho D, MacDonald C, Johnson R, Unsworth CP, O'Carroll SJ, du Mez E, Angel CE & Graham ES, Application of xCELLigence RTCA Biosensor Technology for Revealing the Profile and Window of Drug Responsiveness in Real Time. *Biosensors* (Basel), 5 (2015) 199.

23 Bumidin MS, Johari FA, Risan NF & Mohd Nasir MH, The Effect Of Aqueous Extracts Of *Nigella Sativa* On Breast Cancer Cell Line Mcf-7: An *In Vitro* Study. *Sci Herit J*, 2 (2018) 13.

24 Shafi G, Hasan TN & Syed NA, Methanolic Extract of *Nigella sativa* Seeds is Potent Clonogenic Inhibitor of PC3 Cells. *Int J Pharm*, 4 (2008) 477.

25 Ait Mbarek L, Ait Mouse H, Elabbadi N, Bensalah M, Gamouh A, Aboufatima R, Benharref A, Chait A, Kamal M, Dalal A & Zyad A, Anti-tumor properties of blackseed (*Nigella sativa L.*) extracts. *Braz J Med Biol Res*, 40 (2007) 839.

26 Ahmad A, Husain A, Mujeeb M, Khan SA, Najmi AK, Siddique NA, Damanhouri ZA & Anwar F, A review on therapeutic potential of *Nigella sativa*: A miracle herb. *Asian Pac J Trop Biomed*, 3 (2013) 337.

27 Ahmad A, Raish M, Alkharfy KM, Alsarra IA, Khan A, Ahad A, Jan BL & Shakeel F, Solubility, solubility parameters and solution thermodynamics of thymoquinone in different mono solvents. *J Mol Liq*, 272 (2018) 912.

28 Salmani JM, Asghar S, Lv H & Zhou J, Aqueous solubility and degradation kinetics of the phytochemical anticancer thymoquinone; probing the effects of solvents, pH and light. *Molecules*, 19 (2014) 5925.

29 National Library of Medicine. 2023. Available online: https://www.ncbi.nlm.nih.gov/gene/ (Accessed on 10 May 2023)

30 de Cárcer G, Pérez de Castro I & Malumbres M, Targeting cell cycle kinases for cancer therapy. *Curr Med Chem*, 14 (2007) 969.

31 Alle KM, Henshall SM, Field AS & Sutherland RL, Cyclin D1 protein is overexpressed in hyperplasia and intraductal carcinoma of the breast. *Clin Cancer Res*, 4 (1998) 847.

32 Buckley MF, Sweeney KJ, Hamilton JA, Sini RL, Manning DL, Nicholson RI, deFazio A, Watts CK, Musgrove EA & Sutherland RL, Expression and amplification of cyclin genes in human breast cancer. *Oncogene*, 8 (1993) 2127.

33 Haron AS, Syed Alwi SS, Saiful Yazan L, Abd Razak R, Ong YS, Zakarial Ansar FH & Roshini Alexander H, Cytotoxic Effect of Thymoquinone-Loaded Nanostructured Lipid Carrier (TQ-NLC) on Liver Cancer Cell Integrated with Hepatitis B Genome, Hep3B. *Evid Based Complement Alternat Med*, 2018 (2018) 1549805.

34 Motaghed M, Al-Hassan FM & Hamid SS, Cellular responses with thymoquinone treatment in human breast cancer cell line MCF-7. *Pharmacogn Res*, 5 (2013) 200.

35 Elkady AI, Crude extract of *Nigella sativa* inhibits proliferation and induces apoptosis in human cervical carcinoma HeLa cells. *Afr J Biotech*, 11 (2012) 12710.

36 Xu J, Chen Y & Olopade OI, MYC and Breast Cancer. *Genes Cancer*, 1 (2010) 629.

37 Wang YH, Liu S, Zhang G, Zhou CQ, Zhu HX, Zhou XB, Quan LP, Bai JF & Xu NZ, Knockdown of c-Myc expression by RNAi inhibits MCF-7 breast tumor cells growth *in vitro* and *in vivo*. *Breast Cancer Res*, 7 (2005) R220.

38 Hannan MA, Rahman MA, Sohag AAM, Uddin MJ, Dash R, Sikder MH, Rahman MS, Timalsina B, Munni YA, Sarker PP, Alam M, Mohibbullah M, Haque MN, Jahan I, Hossain MT, Afrin T, Rahman MM, Tahjib-Ul-Arif M, Mitra S, Oktaviani DF, Khan MK, Choi HJ, Moon IS & Kim B, Black Cumin (*Nigella sativa L.*): A Comprehensive Review on Phytochemistry, Health Benefits, Molecular Pharmacology, and Safety. *Nutrients*, 13 (2021) 1784.

39 Sethi G, Ahn KS & Aggarwal BB, Targeting nuclear factor-kappa B activation pathway by thymoquinone: role in suppression of antiapoptotic gene products and enhancement of apoptosis. *Mol Cancer Res*, 6 (2008) 1059.

40 Zhang L, Bai Y & Yang Y, Thymoquinone chemosensitizes colon cancer cells through inhibition of NF-κB. *Oncol Lett*, 12 (2016) 2840.

41 Elkady AI, Hussein RA & El-Assouli SM, Mechanism of Action of *Nigella sativa* on Human Colon Cancer Cells: the Suppression of AP-1 and NF-κB Transcription Factors and the Induction of Cytoprotective Genes. *Asian Pac J Cancer Prev*, 16 (2015) 7943.

42 Okamoto T, Sanda T & Asamitsu K, NF-κB Signaling and Carcinogenesis. *Curr Pharm Des*, 13 (2007) 447.

43 Aslan M, Afşar E, Kırımlıoglu E, Çeker T & Yılmaz Ç, Antiproliferative Effects of Thymoquinone in MCF-7 Breast and HepG2 Liver Cancer Cells: Possible Role of Ceramide and ER Stress. *Nutr Cancer*, 73 (2021) 460.

44 Abreu R de S, Penalva LO, Marcotte EM & Vogel C, Global signatures of protein and mRNA expression levels. *Mol Biosyst*, 5 (2009) 1512.

45 Kordestani Z, Shahrokhi-Farjah M, Yazdi Rouholamini SE & Saberi A, Reduced IKK/NF- kB Expression by *Nigella Sativa* Extract in Breast Cancer. *Middle East J Cancer*, 11 (2020) 150.

46 Imran M, Rauf A, Khan IA, Shahbaz M, Qaisrani TB, Fatmawati S, Abu-Izneid T, Imran A, Rahman KU & Gondal TA, Thymoquinone: A novel strategy to combat cancer: A review. *Biomed Pharmacother*, 106 (2018) 390.

47 Korc M & Friesel RE, The role of fibroblast growth factors in tumor growth. *Curr Cancer Drug Targets*, 9 (2009) 639.

48 Wesche J, Haglund K & Haugsten EM, Fibroblast growth factors and their receptors in cancer. *Biochem J*, 437 (2011) 199.

49 Babina IS & Turner NC, Advances and challenges in targeting FGFR signalling in cancer. *Nat Rev Cancer*, 17 (2017) 318.

50 Alshaibi HF, Aldarmahi, NA, Alkhattabi NA, Alsufiani HM. & Tarbiah NI, Studying the Anticancer Effects of Thymoquinone on Breast Cancer Cells through Natural Killer Cell Activity. *BioMed Res Int*, 2022 (2022) 9218640.

51 Chung KX, Wei PLY & Akowuah GA, Different extraction methods for Thymoquinone from *Nigella sativa* L. seeds and antioxidant activity. *Indian J Nat Prod Resour*, 14 (2023) 75.

52 Wei PLY, Xin CK & Akowuah GA, Effect of different extraction solvents on the content of Thymoquinone content of *Nigella sativa* L. seeds using UV-Visible spectroscopy and evaluation of the free radical scavenging activity. *Indian J Nat Prod Resour*, 14 (2023) 22.