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Reduced IKK/NF- kB Expression by *Nigella Sativa* Extract in Breast Cancer

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Abstract

Background: Activation of IKK/NF-kB signaling pathway plays a critical role in inflammation-driven tumor progression. Several natural compounds able to inhibit the IKK/NF-kB activation pathway have been shown to either prevent cancer or inhibit cell growth. Extensive studies have been carried out on the *Nigella sativa* (*N. sativa*) by many researchers, and its pharmacological activities including anticancer, analgesic, and anti-inflammatory functions have been explored.

This study investigated the effect of *N. sativa* extract on the mRNA level of NFk (p50, RelB) and IKK (IKKA, IKKB) to determine one of the anti-inflammatory mechanisms of *N. sativa* in breast cancer cells.

Methods: In this experimental study, MCF7 cell line was treated with different concentrations of hydroalcoholic extracts of *N. sativa* (0, 200, 400,600,800 µg/mL) for 24, 48 and 72 h. Effects of the extract on cell viability and NFk (p50, RelB) and IKK (IKKA, IKKB) gene expression were analyzed by MTT assay and real time PCR, respectively.

Results: mRNA expression levels of NFk (p50, RelB) and IKK (IKKA, IKKB) in the treatment group were lower than the untreated (control) group. Fold difference (p50, RelB) of gene expressions in treatment groups were statistically significant (P =0.001 and P =0.003) and the fold difference of IKK (IKKA, IKKB) in the treatment groups was lower than that of the untreated groups (P=0.01 and P=0.001).

Conclusion: One possible anti-inflammatory mechanism of *N. sativa* is associated with the reduction in mRNA levels of NFk (p50, RelB) and IKK (IKKA, IKKB) in breast cancer.

Keywords: IKK, NF-kB, *Nigella sativa*, Breast cancer

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Introduction

The American Cancer Society has announced that every year, nearly 200,000 women are diagnosed with breast cancer, among whom %1-%7 are diagnosed with the most invasive breast cancer type, the inflammatory breast cancer.[1] In Iran, breast cancer is the most prevalent cancer among women, comprising 21.4% of all cancers among females.[1-2]

NF-kB is a nuclear transcription factor regulating the expression of a large number of genes, which is critical for the regulation of apoptosis, survival, tumorigenesis, angiogenesis, and inflammation.

NF-kB activation plays a acritical role in inflammation-driven tumor progression as demonstrated in breast cancer xenograft mouse model and Mdr2-deficient mice, which develop hepatitis followed by hepatocellular carcinoma.[3] Furthermore, constitutive NF-kB activation has been observed in human breast cancer tumors as well as breast cancer cell lines.[4]

NF-kB consists of a number of closely-related protein dimers that bind to a common sequence motif known as the kB site. The identification of the p50 NF-kB subunit, as a member of the Rel family, provides the first link between NF-kB and cancer because v-Rel is the oncoprotein of the reticuloendotheliosis (REV-T) retrovirus.The Rel proteins belong to two classes, one consisting of RelA (p65), RelB, and c-Rel and the other synthesized in their mature forms.[5]

When a cell is stimulated by an appropriate extracellular signal, the IkB kinase (IKK) complex, comprised of the regulatory subunits of IKKA and IKKB catalytic subunit, is activated.[2] This activated complex phosphorylates IkB, which then undergoes proteasomal-mediated degradation and releases NF-kB dimers that travel to the nucleus and bind to DNA at NF-kB response elements to modulate the expression of a wide variety of genes, including inflammatory cytokines, chemokines, immune receptors, and cell adhesion molecules. In contrast to IKKA, which does not play a critical role in NF-kB activation by pro inflammatory cytokines, the disruption of IKKB gene corroborates the essential function of IKKB in NF-k B activation.[6]

A causal relation has been proposed between inflammation and cancer and cancers have been suggested to stem from the regions of chronic inflammation. How exactly the inflammation is linked to tumorigenesis is yet to be fully fathomed. However, NF-kB could well be an important player in this process since it is activated in chronic inflammation and its constitutive activation has been described in cancer, hence the usefulness of inhibiting NF-kB activation for cancer therapy.[7] Owing to their remarkable composition, plants are considered as natural resources of bioactive compounds with specific biological activities, hence employed in complementary medicine in various forms such as tea, powder, tincture, and extract.[8]

N. sativa (black seed) is one of the most utilized plants in folk medicine in the Mediterranean region and West Asia. *N. sativa* contains approximately 900 species reported to have a wide range of biological activities, including anti-inflammatory, antimicrobial, anticholinesterase, antioxidative, and antitumor activities.[9]

The active ingredient of *N. sativa* is Thymoquinone and alpha-Hederin, which have been studied in reducing inflammation, fighting cancer, and antitumor and antioxidant activity.[10-12] *N. sativa* was observed to induce antitumor effects in breast, lung, multiple myeloma, pancreatic, cervical, and colon cancers.[13-18] The aim of this study was to investigate the effects of *N. sativa* extract on the mRNA levels of NF-kB (p50, RelB) and IKK (IKKA, IKKB) to determine one of the anti-inflammatory mechanisms of *N. sativa* in breast cancer.

Materials and Methods

The N. sativa seeds material and extract preparation

In this experimental study, the plant was purchased from a local market and its scientific name was confirmed by the Faculty of Pharmacy, Kerman University of Medical Sciences. Hydro-alcoholic extraction was performed via Soxhlet. 100 g of *N. sativa* L powder was extracted with ethanol 96%. The extract was then shacked, filtered, and evaporated in a rotating evaporator

under reducd pressure until dryness.

Cell culture

The human breast cancer cell line, MCF-7, was purchased from the National Cell Bank of Iran (Pasteur Institute, Tehran, Iran); the cells were then cultured in Dulbecco's Modified Eagle Medium (DMEM) (Gibco Invitrogen, 1846736 Karlsruhe, Germany) with 10% Fetal Bovine Serum (FBS) (Gibco Invitrogen, Karlsruhe, Germany) and 1% penicillin and streptomycin (Sigma-Aldrich, Schnelldorf, Germany). Next, MCF-7 cells were incubated in a humidified incubator (MEMMERT® incubator, Bellevue WA 98005, US) at 37 ºC and 5% CO_2 and employed in the third passages.

Cell viability evaluation after treatment with methyl thiazol tetrazolium (MTT) assay

Cells (1×10^5 /well) were plated in 0.2 mL of medium/well in 96-well plates and separately treated with different concentrations (100, 200, 400, 600, 800 µg/ml) at time interval (24h, 48h,72h) of hydroalcoholic extracts of *N. sativa* and incubated at 37ºC; control cells were untreated. Following the stipulated time, the culture medium was discarded and cells were incubated with 5 mg/mL MTT solution for 4 hours; 100µL of DMSO (DMSO, Sigma, USA) was added to dissolve the formazan crystals. Absorbance was read at 570 nm using an ELISA plate reader, and IC50 was calculated with Prism software.

Real-time RT-PCR

Total RNA was extracted from the treated and untreated cell lines using TRIzol (Invitrogen), and cDNA was generated using a reverse transcription system Revert Aid RT Kit (K1621) (Thermo Fisher Company, Germany – Darmstadt). The relative gene expression was conducted by real-time PCR. Real QPlus 2x Master Mix Green with high ROXô (Ampliqon Company, Denmark) was further used. The primer sequences are presented in table 1. Housekeeping gene GAPDH was used as an internal control, and the relative expression between samples was calculated by the comparative Ct method.

The PCR amplification was accomplished with an initial denaturation at 95ºC for 15 min, followed by 45 cycles at 95ºC for 30 s and 63ºC for 1 min by step One Plus real-time PCR system (Applied Bio system, USA). During the melt cycle, the temperature was increased by increments of 1ºC from 60ºC to 95ºC.

Real-time RT-PCR is a powerful tool for quantifying gene expression. The quantitative endpoint for real-time PCR is the threshold cycle (CT) defined as the PCR cycle at which the fluorescent signal of the reporter dye crosses an arbitrarily placed threshold.[19-20]

Gene expression was normalized with GAPDH by Δ CT formula.

Δ CT= CT target gene – CT housekeeping gene, where p50, RelB, IKKA, IKKB are the target genes and GAPDH is the housekeeping gene. The lower the difference between the target gene CT and the housekeeping gene CT, the higher the gene expression; hence, the inverse relationship between gene expression and Δ CT.[20]

Fold change or a fold-difference of gene expression in treatment group vs. untreated group $= 2^{-\Delta\Delta CT}$.[19]

Statistical analysis

Data analysis was performed by SPSS version 17.0 with significant $P<0.05$. Data were expressed as the mean±standard error of the mean of observations. Significant differences between individual treatment and untreated groups were analyzed by an unpaired student's t-test. Differences between the expression of genes in the treatment group (p50-RelB, IKKA, IKKB) were analyzed by one way analysis of variance test (ANOVA) followed by Tukey-Kramer.

Result

Mcf-7 cells were cultured by different doses of *N. sativa* (0,100, 200,400,600,800 µg/mL) at time intervals of 24, 48, and 72 hours. The percentage of cell viability was also calculated. The results showed that *N. sativa* inhibited the

Table 1. Oligonucleotide primers used for quantitative RT-PCR

Gene name	Primer sequence 5-3
P50	
Sense	AAAGAGCTAATCCGCCAAGCA
anti-sense	AGCTGTAAAACATGAGCCGCAC
RELB	
Sense	TGTGGTGAGGATCTGCTTCCAG
anti-sense	TCGGCAAATCCGCAGCTCTGAT
IKKA	
Sense	ACAGAGTTCTGCCCGGTCCCT
anti-sense	CTGCTGAAGTCGGGGGCAGC
IKKB	
Sense	CGCCCAATGACCTGCCCCTG
anti-sense	GGCACCTTCCCGCAGACCAC
GAPDH	
Sense	CCCTCTGGAAAGCTGTGG
anti-sense	AGTGGATGCAGGGA TGATG

growth of cancer cells in a dose- and time-dependent manner (Figure 1). Also, IC50 was calculated with Prism software. IC50 *N. sativa* at 24 hours (3297 µg/ml) and 48 hours (1153 µg/ml) was not significantly different from the controls; however, *N. sativa* had a significant effect at 72 hours (440.9 µg/ml), a time considered for other tests.

RT-PCR

The effect of N. sativa extracts on mRNA expression levels of IKK/NF-kB

The mRNA expression levels of NFk (p50, RelB) and IKK (IKKA, IKKB) family in treated and untreated MCF-7 cell line were analyzed by qRT-PCR. Treatment group had a lower p50 gene expression (-3.5 fold decreases) compared with untreated group, (P=0.001). Moreover, the treatment group had a significantly lower RelB gene expression (-5fold decreases) compared with the untreated group (P=0.03, Table 2, Figure 2).

The mRNA expression levels of IKKA and IKKB were lower in the treatment group. The difference in IKKA and IKKB gene expressions

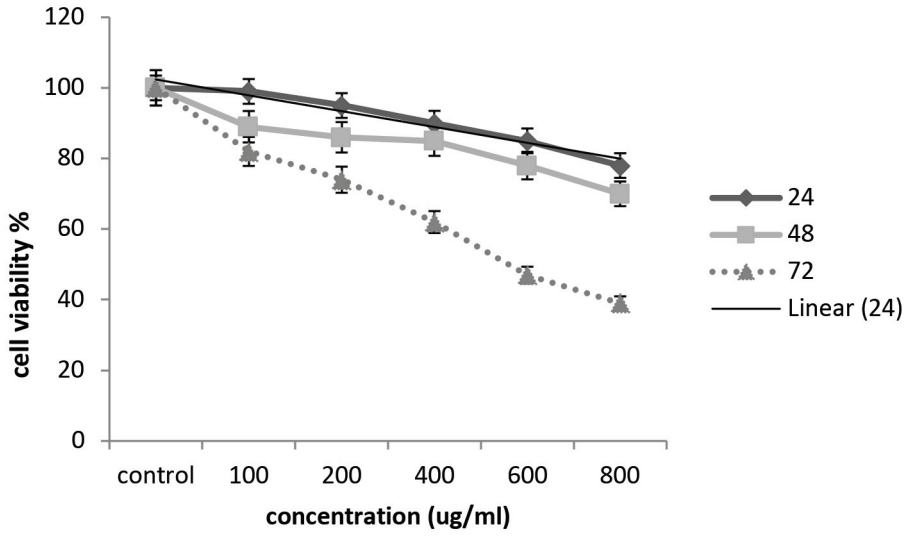

Figure 1. *N. sativa* treated with different concentrations of 400 µg/mL had a significant effect after 72 h. Cell viability was determined by MTT assay and expressed as a percentage of viable cells in the total number of counted cells. Data are presented as the mean ± standard error of five experiments.

was significant (-15 fold decrease *P*=0.01,-42fold decreases, *P*=0.001) (Table 2, Figure 2). The results also showed that IKKB had the most gene expression reduction in the treatment group (Fold change = -42), while P50 had the lowest reduction in the treatment group (Fold change = -3.5) in comparison with the other genes in this study (Figure 3).

Discussion

Many researchers have focused on medicinal plants, particularly *N. sativa*, a widely used medicinal plant throughout the world. This study investigated the effect of *N. sativa* extract on the mRNA levels of NFk (p50, RelB) and IKK (IKKA, IKKB) to determine one of the anti-inflammatory mechanisms of *N. sativa* in breast cancer. Our results showed that *N. sativa* inhibited the growth of breast cancer cells line (MCF7) in a dose- and time-dependent manner. Furthermore, mRNA expression levels of NFk (p50, RelB) and IKK (IKKA, IKKB) in the treatment group were lower than the untreated (control) group. Fold difference of genes expression between the treatment and untreated groups was statistically significant.

As a herbal medicine with a rich historical background, *N. sativa* has been traditionally used in the treatment of several diseases, including fertility, fever, bronchitis, asthma, migraine, dizziness, chest congestion, paralysis, dysmenorrhea, obesity, diabetes, infection and inflammation, rheumatism, hypertension, and gastrointestinal and anti-tumor activities.[21-22]

In 2003, Shoieb et al. reported that the oil of *N. sativa* (TQ) killed cancer cells through inhibiting the cell cycle progression and inducing apoptosis.[23]

Ashour et al. (2014) showed that TQ inhibited the human hepatocellular carcinoma (HCC) cell growth and IL-8 expression.[24] Zhang et al. revealed that the oil of *N. sativa* might result in cell death in colon cancer cells, which is a positive alternative for adjuvant chemotherapy in the treatment of colon cancer.[11]

The results of previous studies have shown that *N. sativa* alone or in combination with oxidative stress inactivates MCF-7 breast cancer cells.[25-27] Similarly, we found that *N. sativa* inhibited breast cancer cell growth. In the present study, MCF7 cell line was treated with different concentrations of hydroalcoholic extracts of *N.*

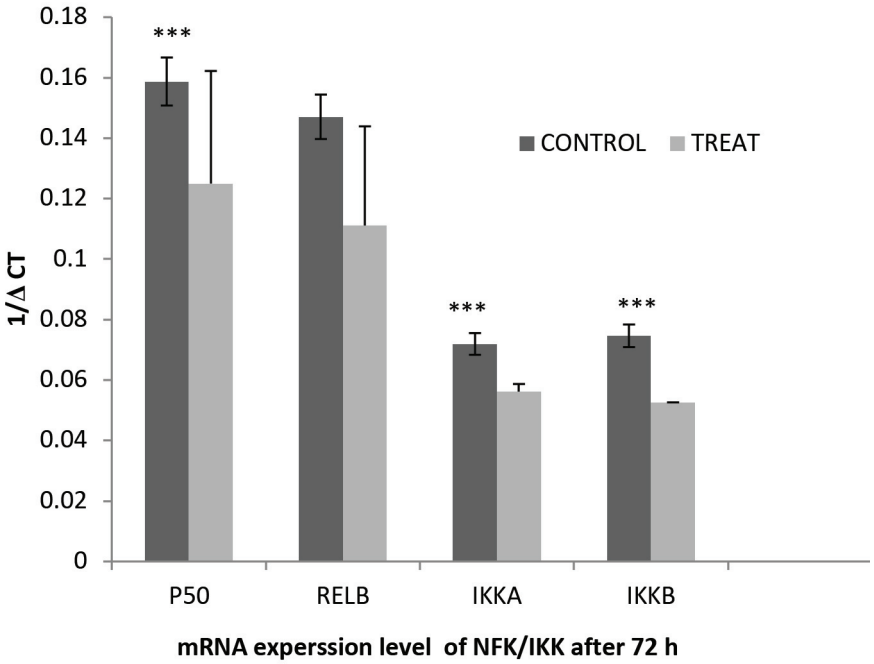

Figure 2. Gene expression was analyzed by qRT-PCR technique. The mRNA expression levels of NFk (p50, RelB) (*P*=0.001, 0.003) and IKK (IKKA, IKKB) (*P*=0.001, 0.00) were lower in the treatment group compared with the control group. *** *P*< 0.001

Table 2. NFk (p50, RelB) and IKK (IKKA, IKKB) expressions in treated and untreated groups

Gene	Expression (treated*[a])	Expression (untreated*[a])	Fold change[b]	*P* value**
P50	0.124±0.0068	0.164±0.006	-3.5	0.001
RELB	0.110±0.004	0.142±0.014	-5	0.003
IKKA	0.050±0.0074	0.070±0.007	-15	0.001
IKKB	0.05±0.055	0.074±0.045	-42	0.00

*(mean ±SD); [a]1/ΔCT values are presented in this table to represent the gene expression. Δ Ct = target gene Ct - housekeeping gene Ct; [b] Fold change = $2^{-\Delta\Delta CT;}$ **P Value is significant at 0.05 levels; Ct: Cycle threshold

sativa (0, 200, 400, 600, 800 µg/mL), among which the concentrations of 600 and 800 were tested for the first time. However, the main purpose of this study was to investigate the anti-inflammatory mechanisms of *N. sativa* in breast cancer cell.

According to several preclinical studies, *N. sativa* is able to suppress inflammatory mediators and oxidative stress.[24-26] Inflammation is one of the main pathophysiological characteristics of many chronic and acute diseases. Al-Ghamdi and coworkers demonstrated that the aqueous extract of *N. sativa* possessed an anti-inflammatory function in carrageenan-induced paw edema similar to 100mg/kg aspirin.[28] According to the study of Sethi et al. on human myeloid KBM-5 cells, it is probable that many anti-inflammatory activities assigned to *N. sativa* and its main constituent thymoquinone, including the effects on enzymes COX, iNOS, and 5-LPO as well as proinflammatory cytokines such as TNF-α, IL-1, and IL-6 are mediated, in part, through the suppression of NF-κB activation. NF-kB activation plays a critical role in inflammation-driven tumor progression and cancers.[29-30]

NF-kB is a ubiquitous transcription factor consisting of p50, RelA, RelB, and nuclear factor-kB inhibitor α (IkBα), which is present in the cytoplasm and is activated in response to certain inflammatory stimuli, environmental pollutants, prooxidants, carcinogens, stress, and growth factors. Composed of p50 and RelB subunits, NF-kB is only active following translocation into the nucleus and dimerization. IkB inactivates NF-kB by retaining it in the cytoplasm. In response to proinflammatory mediators, IkB gets

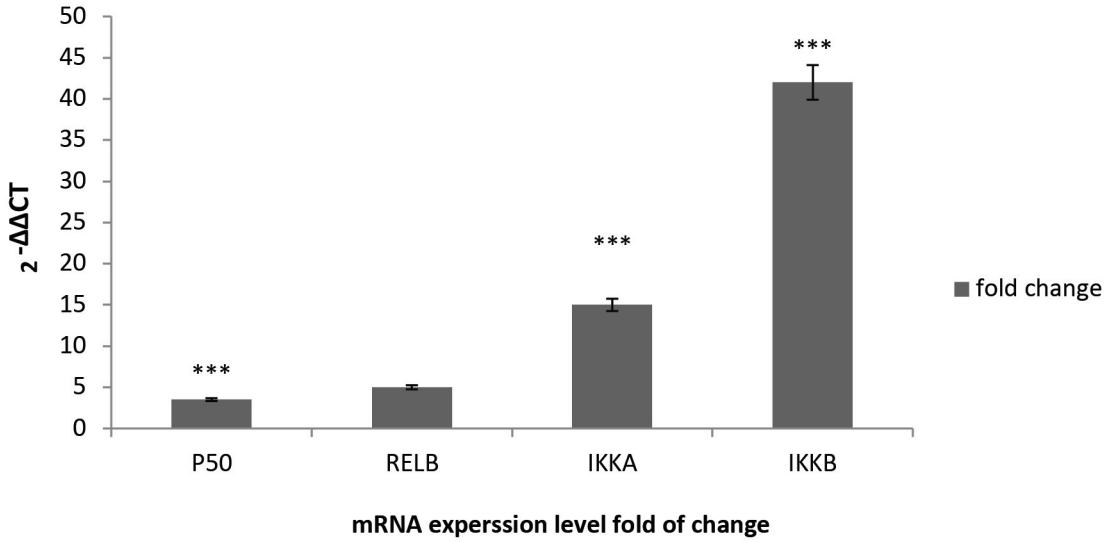

Figure 3. Different expressions of IKK/NF-kB were significant in the treatment group. IKKB had the most gene expression reduction in the treatment group (Fold change = -42 P=0.000), and P50 had the lowest reduction in the treatment group (Fold change = -3.5) compared with other genes in this study. *** P<0.001

phosphorylated by IKK, entailing its degradation and the subsequent activation of NF-kB.[31]

In 2018, Tobias Neumann showed that NF-kB signaling in myeloid cells promoted a permissive lung microenvironment preventing the breast from the development of lung metastasis. NF-kB activation has also been reported in human breast cancer specimens in both tumor cells and tumor stroma.[32]

ZHANG et al. reported that in colon cancer, TQ treatment significantly reduced the level of phosphorylated p65 in the nucleus, indicating the inhibition of NF-kB activation by TQ treatment. Overall, the results of the present study suggested that *N. sativa* induced cell death and chemosensitized colon cancer cells via inhibiting NF-kB signaling. Similarly, it was observed that treatment with *N. sativa* reduced the expression levels of NF-kB (p50, RelB) and IKK (IKKA, IKKB) in breast cancer cell. Michael Karin et al.; however, reported that of the two catalytic subunits, only IKKB is essential for NF-k B activation in response to proinflammatory stimuli. The second catalytic subunit, IKKA, plays a critical role in developmental processes, especially the formation and differentiation of the epidermis.[6] Our findings suggest that IKKB gene expression was reduced more than p50, RelB and IKKA gene expression by *N. sativa* extract (Figure 3). Our results confirm the fact that *N. sativa* apply proinflammatory effect through inhibiting IKKB subunit in complex IKK/NF-kB.

As a positive regulator of cell cycle progression, NF-kB activates target genes such as cyclin D1 c-myc and c-myb or growth factors that stimulate the proliferation of lymphoid and myeloid cells in an autocrine manner (IL-2, GM-CSF, CD40L). Cyclin D1 is probably one of the most important NF-kB target genes in normal mammary gland development and mammary carcinogenesis. It has further been shown that cyclin D1 expression requires a functioning NF-kB activation pathway during mammary gland development.[33]

ZHANG et al. showed that treatment with TQ decreased the expression levels of VEGF and c-Myc in colon cancer,[11] hence the necessity of other investigations to examine the changes in the expression of cyclin D1 and c Myc as target genes of NF with regards to the data of the same group. We hope to investigate the changes in the expression of Cyclin D1 and c- Myc as the target genes of NF-kB[7] (in the data of the same group).

Conclusion

One possible anti-inflammatory mechanism of *N. sativa* is related to the reduction in the mRNA levels of NFk (p50, RelB) and IKK (IKKA, IKKB) in breast cancer.

Acknowledgments

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Conflicts of Interest

None declared.

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