RESEARCH ARTICLE

Roles of p53 and Caspases in Induction of Apoptosis in MCF-7 Breast Cancer Cells Treated with a Methanolic Extract of Nigella Sativa Seeds

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Abstract

Background: *Nigella Sativa* (NS) is an herb from the Ranunculaceae family that exhibits numerous medicinal properties and has been used as important constituent of many complementary and alternative medicines (CAMs). The ability of NS to kill cancer cells such as PC3, HeLa and hepatoma cells is well established. However, our understanding of the mode of death caused by NS remains nebulous. The objective of this study was to gain further insight into the mode and mechanism of death caused by NS in breast cancer MCF-7 cells. Materials and Methods: Human breast cancer cells (MCF-7) were treated with a methanolic extract of NS, and a dose- and time-dependent study was performed. The IC_{50} was calculated using a Cell Titer Blue® viability assay assay, and evidence for DNA fragmentation was obtained by fluorescence microscopy TUNEL assay. Gene expression was also profiled for a number of apoptosis-related genes (*Caspase-3, -8, -9* and p53 genes) through qPCR. Results: The IC_{50} of MCF-7 cells was 62.8μL/mL. When MCF-7 cells were exposed to 50 μL/mL and 100 μL/mL NS for 24h, 48h and 72h, microscopic examination (TUNEL assay) revealed a dose- and time-dependent increase in apoptosis. Similarly, the expression of the *Caspase-3, -8, -9* and p53 genes increased significantly according to the dose and time. Conclusions: NS induced apoptosis in MCF-7 cells through both the p53 and caspase pathways. NS could potentially represent an alternative source of medicine for breast cancer therapy.

Keywords: *Nigella sativa* seeds - MCF-7 cells - anticancer, apoptosis - p53 - caspases-3, 8, 9

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Introduction

Nigella sativa (NS), a dicotyledon of the Ranunculaceae family, is an amazing herb and a promising medicinal plant with a rich historical and a religious background (Goreja, 2003). NS is commonly known as "black cumin seed" and has been used in many Middle Eastern countries for thousands of years as a spice, food preservative and curative remedy for numerous disorders (Chopra et al, 1956; Nadakarni, 1976; Yesilada et al., 1995). In the past two decades, considerable effort has contributed to the scientific validation of the medicinal properties of NS and its use as a medicine for the treatment of asthma (El-Tahir et al., 1993), bronchitis, headache, rheumatism, fever, influenza and eczema as well as for the treatment of dyslipidemia, hyperglycemia and related abnormalities (Zaoui et al., 2002). In addition, the anti-inflammatory (Houghton et al., 1995; Mutabagani and El-Mahdy, 1997), anti-diabetic (Al-Hader et al., 1993; El-Shabrawy and Nada, 1996), anthelminthic (Mahmoud et al., 2002), analgesic (Khanna et al., 1993), antibacterial (El-Kamali et al., 1998; Mouhajir et al., 1999) and anticancer properties of NS have been tested and validated (Nair et al., 1991; Salomi et al., 1992; El-Daly., 1998).

Methanolic crude extract of NS seed exhibits antitumor effects *in vitro* on carcinoma, lymphoma and sarcoma (Salomi et al., 1991; Salomi et al., 1992) Methanolic extract of NS has been reported to possess *in vitro* cytotoxicity in the PC3 cell line, whereas the chloroform fraction of NS was found to induce apoptosis in HeLa cells and SiHa cells (Shafi et al., 2008; Shafi et al., 2009). Two purified components of the NS seed, thymoquinone (TQ) and dithymoquinone (DTQ), have been established to exhibit cytotoxic activities in some parental and multidrug-resistant human tumor cell lines. TQ triggers apoptotic cell death in human colorectal cancer cells, and apoptosis is associated with the up-regulation of *p53* and $p21^{WAF1}$ and a significant down-regulation of the anti-apoptotic Bcl-2 protein (Gali-Muhtasib et al, 2004). In addition to these direct anti-tumor effects, black seed preparations may exhibit potential in cancer chemoprevention as well as in reducing the toxicity of standard antineoplastic drugs (Nair et al., 1991; Salomi et al., 1991).

Although the effect of NS on different biological processes has been established, there are comparatively few studies on its anticancer property against breast

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cancer. This study was therefore designed to determine whether NS induces apoptosis in the MCF-7 breast cancer cell line and, if so, which pathway(s) is/are involved the programmed cell death: the mitochondrial (*caspases*) pathway, the nuclear (*p53*) pathway or both.

Materials and Methods

Nigella sativa seeds were purchased from local markets and authenticated at department of Botany, King Saud University, Riyadh. The seeds were washed with distilled water and dried in the air. Dried seeds were powdered, and 500 grams of grounded seeds were soaked in 1 L of high-performance liquid chromatography (HPLC)-grade methanol in a sterile bottle and kept for 24 hours at room temperature with stirring with a sterile rod twice. Extraction was performed three times as described above. The extract was then filtered using sterile filter paper. The solvent from the extract was removed under reduced pressure. Approximately 8 mL of extract was obtained after concentration. The extract was aliquoted into 1-ml vials and stored at -20°C until used for experiments (Hasan et al., 2007).

Maintenance of MCF-7 cells

The MCF-7 breast cancer cell line was a kind gift from Dr. M. A. Akbarshah at the Mahatma Gandhi-Doerenkamp Center (MGDC) for Alternatives to Use of Animals in Life Science Education, Bharathidasan University, India. Maintenance and culture procedures were exactly similar to that of reported in our earlier study (Alshatwi et al., 2013). Briefly, The cell line was maintained and propagated in 90% Dulbecco's Modified Eagle's Medium (HyClone, Thermo Scientific, MA, USA) containing 10% fetal bovine serum (HyClone, Thermo Scientific) and 1% penicillin/streptomycin (HyClone, Thermo Scientific). Cells were cultured as an adherent monolayer (i.e., cultured at 70% to 80% confluence) and maintained at 37°C in a humidified atmosphere of 5% CO_2. Cells were harvested after brief trypsinization. All chemicals used were of research grade.

Cell viability assays

Cell viability was assayed where and as required, using a trypan blue exclusion test with slight modifications (El Sharkawi et al, 2014). To assess the toxicity of different concentrations of NS on MCF-7 cells, the Cell Titer Blue® viability assay (Promega Madison, WI) was performed. The assay was performed by following the manufacturer's instructions. Briefly, MCF-7 cells (2×10^4 cells/well) were plated in 96-well plates and treated with a range of doses (0, 25, 50, 75, 100 and 125 μL/mL NS) for 24h. Subsequently, 40 μL of the Cell Titer Blue solution was directly added to the wells and incubated at 37°C for 6h. The fluorescence was recorded with a 560 nm/590 nm (excitation/emission) filter set using a Bio-Tek microplate fluorescence reader (FL×800™), and the IC_{50} was calculated. Samples were run in quadruplets for each concentration of NS in three independent experiments.

NS Treatment for a dose- and time-dependent study

For a dose- and time-dependent study, MCF-7 cells were treated with either 50 μL/mL or 100 μg/mL NS for 24, 48 and 72h for the terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) assay. The cells were incubated with the same NS concentrations for 24 and 48h for real-time quantitative PCR analysis.

Terminal deoxynucleotidyl transferase dUTP nick end-labeling (TUNEL) assay

The DeadEnd® TUNEL assay kit (Promega, Madison, WI) was used to study apoptosis in a time- and dose-dependent manner. The manufacturer's instructions were followed with slight modifications. Briefly, MCF-7 cells (1.5×10^6 cells/well) were cultured in 6-well plates to study apoptosis in adherent cells. Cells were treated with 50 μL/mL and 100 μL/mL NS for 24, 48 and 72h. After the incubation period, the culture medium was aspirated, and the cell layers were trypsinized. The trypsinized cells were reattached on 0.01% polylysine-coated slides, fixed with 4% methanol-free formaldehyde solution, and stained according to the DeadEnd fluorometric TUNEL system protocol (Zhang et al, 2014). The stained cells were observed using a Carl-Zeiss (Axiovert) epifluorescence microscope using a triple band-pass filter. To determine the percentage of cells undergoing apoptosis, 1000 cells were counted in each experiment (Shafi et al, 2009).

Real-time quantitative PCR analysis

Reverse transcription-polymerase chain reaction (RT-PCR; Applied Biosystems 7500 Fast, Foster City, CA) was used to analyze the expression of apoptotic genes by using a real-time SYBR Green/ROX gene expression assay kit (QIAgen, Germany). The Fastlane® Cell cDNA kit (QIAGEN, Germany) was used to prepare cDNA directly from cultured cells., and the mRNA levels of *Caspase-3, Caspase-8, Caspase-9* and *tp53* as well as the reference gene, GAPDH, were assayed using gene-specific SYBR Green-based QuantiTect® Primer assays (QIAGEN, Germany). Quantitative real-time RT-PCR was performed in a reaction volume of 25 μL according to the manufacturer's instructions. Briefly, 12.5 μL of master mix, 2.5 μL of primer assay (10x) and 10 μL of template cDNA (100 μg) were added to each well. After a brief centrifugation, the PCR plate was subjected to 35 cycles of the following conditions: *(i)* PCR activation at 95°C for 5 min; *(ii)* denaturation at 95°C for 5 sec; *(iii)* annealing/extension at 60°C for 10 sec. All samples and controls were run in triplicates on an ABI 7500 Fast Real-time PCR system. The quantitative RT-PCR data were analyzed by a comparative threshold (Ct) method, and the fold inductions of samples were compared with the untreated samples. GAPDH was used as an internal reference gene to normalize the expression of the apoptotic genes. The Ct cycle was used to determine the expression level in control cells and MCF-7 cells treated with NS for 24 and 48h. The gene expression level was then calculated as described by Yuan et al. (2006). The results were expressed as the ratio of the reference gene to the target gene using the following formula: ΔCt=Ct (apoptotic genes)-Ct (GAPDH). To determine the relative expression levels, the

following formula was used: ΔΔCt=ΔCt (Treated)-ΔCt (Control). Thus, the expression levels were expressed as n-fold differences relative to the calibrator. The value was used to plot the expression of apoptotic genes using the expression $2^{-\Delta\Delta Ct}$.

Results

Effect of NS on MCF-7 breast cancer cell proliferation and apoptosis

To explore the anticancer effect of NS on MCF-7 human breast cancer cells, several *in vitro* experiments were conducted.

Determination of NS toxicity on MCF-7 cells

The cytotoxic effect of 0 to 125 μL/mL NS on MCF-7 cells was examined. A dose-dependent reduction in color was observed after 24h of treatment with NS, and 87.71% of the cells were dead at the highest concentration of NS tested (125 μL/mL). The IC_{50} of NS for MCF-7 cells was determined to be 62.83 μL/mL (Figure 1).

Quantification of apoptosis by a TUNEL assay

To determine whether the inhibition of cell proliferation by NS was due to the induction of apoptosis, a TUNEL assay was used. Figures 2, 3, 4 and 5 summarize the effect of NS on MCF-7 cells. A dose- and time-dependent increase in the induction of apoptosis was observed when MCF-7 cells were treated with NS.

At 24h, 43.65% and 58.32% of the cells treated with 50 μL/mL and 100 μL/mL, respectively, underwent apoptosis when normalized with control. Similarly, 57.42% and 73.11% of the treated cells for 48 hours and 68.27% and 76.81% of the treated cells for 72h were apoptotic when treated with 50 μL/mL and 100 μL/mL, respectively (Figure 5).

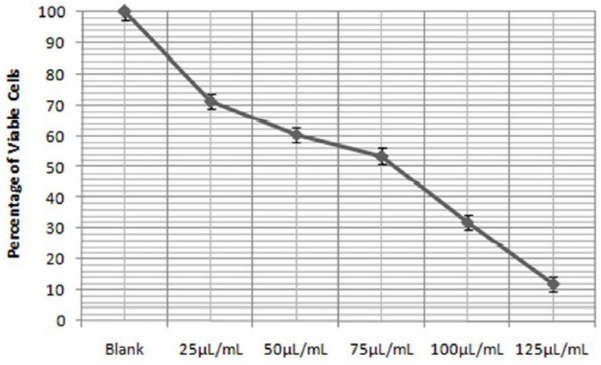

Figure 1. A graphical Representation of Viability at Different Doses of NS. The IC_{50} of Nigella sativa seed methanolic extract for human breast cancer (MCF-7) cells was calculated to be 62.83 μL/mL

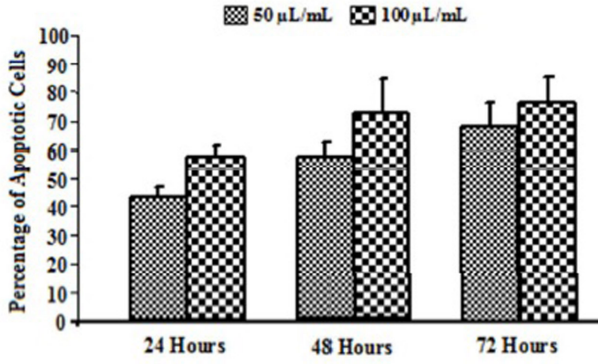

Figure 2. Comparative Representation of TUNEL-Positive (Apoptotic) Cells. The percentage of apoptotic MCF-7 cells after 24, 48 and 72h of exposure to two concentrations (50 μL/mL and 100 μL/mL) of Nigella sativa methanolic extract after normalization with the value of untreated control

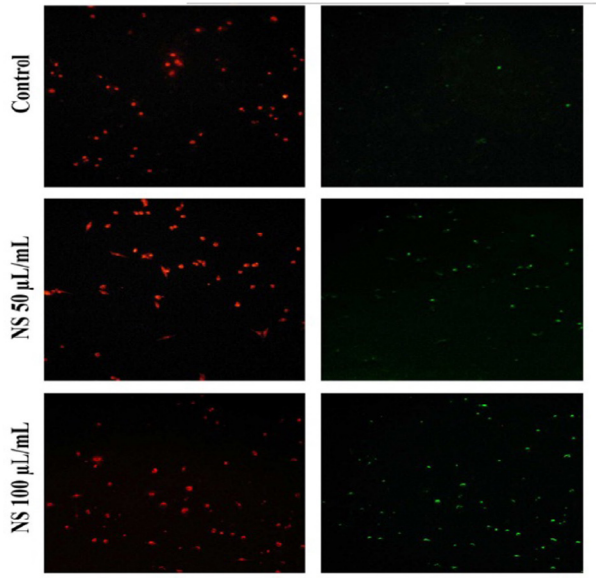

Figure 3. Microscopic TUNEL Assay for 24 hrs. TUNEL assay (magnification=200 X) after a 24-h incubation of MCF-7 cells with NS treatment. The red fluorescence is due to propidium iodide staining with observations made under a green filter. The green fluorescence is due to FITC staining with observations made under a blue filter

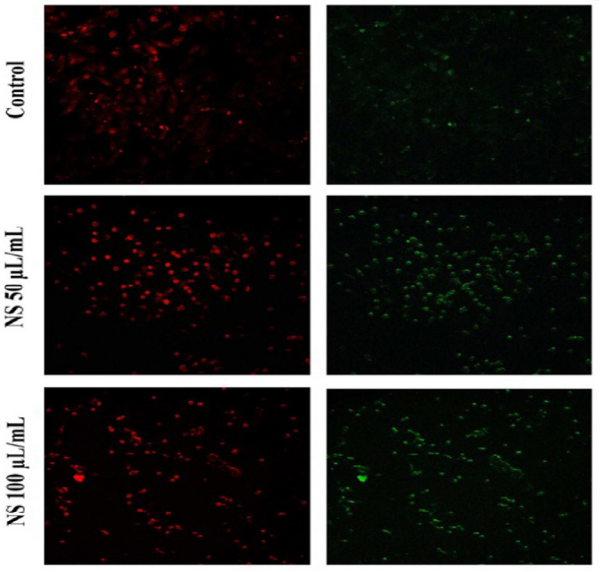

Figure 4. Microscopic TUNEL Assay for 48 hrs. TUNEL assay (microscopic, Resolution=200 X) after a 48-h incubation of MCF-7 cells with NS treatment. The red fluorescence is due to propidium iodide staining with observations made under a green filter. The green fluorescence is due to FITC staining with observations made under a blue filter

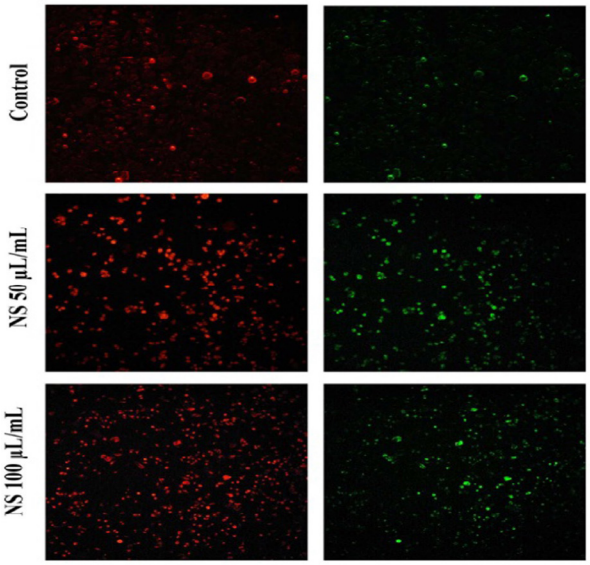

Figure 5. Microscopic TUNEL Assay for 72 hrs. TUNEL assay (microscopic, Resolution=200 X) after a 72-h incubation of MCF-7 cells with NS treatment. The red fluorescence is due to propidium iodide staining with observations made under a green filter. The green fluorescence is due to FITC staining with observations made under a blue filter

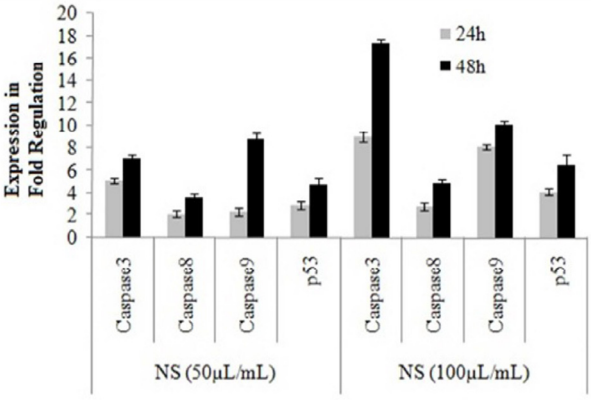

Figure 6. Change in the Fold Regulation of Apoptosis-Related Genes. Comparison of the expression levels of apoptosis-related genes as fold change in MCF-7 cells after 24h and 48h of exposure to 50 µL/mL and 100 µL/mL of NS

Quantification of mRNA levels of apoptotic-related genes

To investigate the molecular mechanism of NS-induced apoptosis in MCF-7 cells, the expression levels of several apoptosis-related genes were examined. Figures 6 and 7 summarize the gene expression changes of *Caspase-3, -8,* and *-9* and *p53*. NS increased the transcripts of *Caspase-3, -8,* and *-9,* and *p53* by several fold. The expression levels of these genes in MCF-7 cells treated with 50 µL/mL NS for 24h increased by 5.15-, 2.10-, 2.3-, and 2.81-fold, respectively, compared with the levels in untreated control cells (Figure 6). Similarly, the expression levels of *Caspase-3, -8,* and *-9* and *p53* in MCF-7 cells treated with 100 µL/mL NS for 24h increased by 7.00, 3.62, 8.78, and 4.72-fold, respectively, compared with levels in untreated control cells (Figure 6). In a time-dependent manner, the expression levels of the apoptosis-related genes in MCF-7 cells treated with 50 or 100 µL/mL NS for 48 h increased when compared with the levels in untreated control cells (Figure 6). However, the expression levels of *Caspase-3, -8,* and *-9* and *p53* in MCF-7 cells treated with 100 µL/mL NS for 48 h markedly increased by 17.37, 4.82, 10.06 and 6.48-fold compared with control untreated cells (Figure 6). Together, these data suggest that the expression of caspases and *p53* was induced by NS in a dose- and time-dependent manner.

Discussion

The mechanism of action of many anticancer drugs is based on their ability to induce apoptosis (Motomura et al, 2008; Florea and Busselberg, 2011). However, unfavorable side effects and the resistance to many anticancer agents have become serious problems (Sonis et al, 2011). Thus, there is a growing interest in the use of plant-based compounds or dietetic products to develop safe and more effective therapeutic agents for cancer treatment (Kma, 2013; Mangal et al., 2013). In due course our group already has demonstrated the anti-preoperative potential of organic extracts from root bark of *Juglans Regia* against MDA-MB-231 human breast cancer cells (Hasan et al., 2011). In addition of that, our group also demonstrated that methanolic extract of fenugreek whole plant (without root) has potential to induce apoptosis in MCF-7 human breast cancer cells through the involvement of Fas receptor and Caspase-8 (Alshatwi et al., 2013). In present study we are focusing on the anti cancer and apotosis inducing ability of NS seed extract in MCF-7 human breast cancer cells. NS seeds have been well known for their medicinal properties for thousands of years (Goreja, 2003). No side effects or health hazards have been reported for the use of NS as a medicine (elsewhere). In the past decade, considerable research effort has been focused on elucidating the anti-tumor and cytotoxic effects of NS so that the medicinal property of NS against cancer could be validated scientifically. NS reportedly exhibits cytotoxic activity against hepatoma cells (Thabrew et al., 2005), PC3 prostate cancer cells (Shafi et al., 2008) and HeLa cervical cancer cells (Shafi et al., 2009) and SiHa cervix cancer cells (Hasan et al., 2013). Alkaloids from NS seeds (thymoquinone and thymohydroquinone) exhibit anti-tumor properties in many tumor models and *in vivo* (Ivankovic et al., 2006).

There are many mechanisms through which apoptosis can be enhanced in cells. Agents suppressing the proliferation of malignant cells by enhancing apoptosis may constitute a useful mechanistic approach to both cancer chemoprevention and chemotherapy. Most dietetic products with anticancer activity act as strong antioxidants and/or modify the activity of one or more protein kinases involved in cell cycle control; protein kinase A, protein kinase B, protein kinase C, JNK-1, CDK-2, and CDK-4 are either activated or deactivated by these antioxidants. This effect may be mediated through the activation of some tumor suppressor genes, such as p21WAF1/CIP1 and p27KIP1, in a *p53*-independent pathway (Thabrew et al, 2005). In our earlier studies, we observed that HeLa cervical cancer cells underwent apoptosis mediated by the

p53 and *caspase* pathways (Shafi et al., 2009).

Therefore, we hypothesized that apoptosis could be the mode of cell death in MCF-7 breast cancer cell through either the nuclear (*p53*) or mitochondrial *(Caspase)* pathway or a combination of both pathways. To test this hypothesis, we sought to determine the role of NS in inhibiting cell growth and modulating the expression of caspases-3, -8, and -9 and *p53*. The data presented in this paper demonstrate a time- and dose-dependent inhibition by NS of MCF-7 human breast cancer cell proliferation. Recently, we observed that NS treatment resulted in an 88.3% inhibition of proliferation of SiHa human cervical cancer cells at a concentration of 125 μL/mL of methanolic extract at 24 h and a 50% inhibition of proliferation at a concentration of 93.2 μL/mL (Hasan et al., 2013).

The sensitivity of cells to any of these stimuli may vary depending on factors such as the expression of pro- and anti-apoptotic proteins. The mitochondrial apoptotic pathways and death receptor pathways are the two major pathways that have been characterized in mammalian cells. The mitochondria have a central role in regulating the *caspase* cascade and apoptosis (Hengartner, 2000). Caspases have a central role in the apoptotic process in that they trigger a cascade of apoptotic pathways. The release of cytochrome-c from mitochondria leads to the activation of procaspase-9 and then caspase-3 (Hengartner, 2000). The activation of caspase-3 is an important downstream step in the apoptotic pathway(Hengartner, 2000). In addition, the effector caspase, caspase-3, and the initiator caspases, caspase-8 and -9, are the main executors of apoptosis (Earnshaw et al., 1999). Caspase-8 is in the death receptor pathway, whereas caspase-9 is in the mitochondrial pathway, and both pathways share caspase-3 (Czabotar et al., 2014).

In a downstream pathway of apoptosis, once caspase-8 is activated, it directly processes the downstream effectors, caspase-3, -6, and -7. In an alternative pathway, caspase-8 activates crosstalk between the death receptor pathway and the mitochondrial pathway by the cleavage of Bid to Bid, a pro-apoptotic member of the Bcl2 family. The activation of caspase-8 has a central role in Fas-mediated apoptosis. Moreover, the cleavage of Bid has been shown to be associated with caspase-8 activation (Pommier et al., 2004). Taken together, the data presented in this study suggest that apoptosis induced by NS treatment may be involved in the mitochondrial pathways, as demonstrated by the increased expression levels of *caspase-3, -8* and *-9* after NS treatment. In addition, this study suggests the involvement of the extrinsic death pathway in MCF-7 breast cancer cell after treatment with NS, as demonstrated by increased expression levels of *caspase-8*. These data are in agreement with our recent published work in which NS exposure increased the expression of *caspase-3, -8* and *-9* several fold. This effect of NS can be attributed to the active compounds present in NS. The anticancer activity of thymoquinone (TQ), an active ingredient isolated from *Nigella sativa*, has been studied. TQ significantly inhibited proliferation, induced apoptosis and activated caspase-3, -7 and -9 in the colon cancer cell line HCT116 (Kundu et al., 2014).

p53, the most commonly mutated gene associated with cancer, helps to regulate the cell cycle and has a key role in ensuring that damaged cells are destroyed by apoptosis. The data presented in this study indicate that the expression levels of *p53* and *caspase-3, -8* and *-9* were markedly increased after NS treatment in a concentration-dependent manner. These data suggest that NS induced apoptosis by regulating pro-apoptotic genes.

The possibility that *p53*-mediated apoptosis may be associated with the activation of *caspase-3, -8* and *-9* is suggested by the ability of *p53* to activate both the extrinsic and intrinsic apoptotic pathways (Salim et al., 2013). *p53* enhances cancer cell apoptosis and prevents cell replication by stopping the cell cycle at G1 or interphase (Fridman and Lowe, 2003). By inducing the release of mitochondrial cytochrome-c, *p53* might be able to activate effector caspases, including caspase-3. *Caspase-3, -8,* and *-9* may be the apoptotic effector machinery engaged by *p53* to mediate teratogen-induced apoptotic pathways (Takaoka et al., 2003; Pekar et al., 2007).

In conclusion, to our knowledge, the results presented in this study show for the first time that NS exerts its anticancer effects by blocking the proliferation of MCF-7 cells and inducing apoptosis in part by modulating the expression levels of *caspase-3, -8,* and *-9* and *p53*. NS induces, at least in part, *p53* and caspase-mediated apoptosis in MCF-7 cells. Therefore, the present study demonstrates that NS significantly inhibits the growth of MCF-7 human breast cancer cells *in vitro* and provides the underlying mechanism for the anticancer activity. NS could potentially represent an alternative source of medicine for Breast cancer therapy. The role of both *p53* and caspases may represent the synergistic effect of different components of NS seeds. This study supports the development of dietetic plant product-based drugs for the treatment of breast cancer. Further research is required to characterize and analyze different components of NS seeds before developing a promising chemotherapeutic agent for breast cancer treatment from NS.

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