

## RESEARCH ARTICLE

## Antiproliferative Properties of Methanolic Extract of *Nigella sativa* against the MDA-MB-231 Cancer Cell Line

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### Abstract

Breast cancer is the most commonly diagnosed cancer in women in the world and is one of the leading causes of death due to cancer. Health benefits have been linked to additive and synergistic combinations of phytochemicals in fruits and vegetables. *Nigella sativa* has been shown to possess anti-carcinogenic activity, inhibiting growth of several cancer cell lines in vitro. However, the molecular mechanisms of the anti-cancer properties of *Nigella sativa* phytochemical extracts have not been completely understood. Our data showed that *Nigella sativa* extracts significantly inhibited human breast cancer MDA-MB-231 cell proliferation at doses of 2.5-5 µg/mL (P<0.05). Apoptotic induction in MDA-MB-231 cells was observed in a dose-dependent manner after exposure to *Nigella sativa* extracts for 48 h. Real time PCR and flow cytometry analyses suggested that *Nigella sativa* extracts possess the ability to suppress the proliferation of human breast cancer cells through induction of apoptosis.

**Keywords:** Phytochemicals - MDA-MB-231 - *Nigella sativa*

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### Introduction

Breast cancer is one of the most prevalent cancers throughout the world and especially in the western countries. This is continuously increasing worldwide due to rapid changes in dietary pattern and preferences. Many epidemiological studies indicated that western-style diet, primarily, the consumption of red meat, is positively associated with a high breast cancer incidence (Abeyasinghe et al., 2007), and is blindly followed in Saudi Arabia. Continuous efforts are being made for search of novel source of bioactive compounds to prevent breast carcinoma. In this direction, bioactive compounds of natural origin, particularly from a dietary source, are gaining significance. In the recent years, *Nigella sativa* (NS) seed has gained importance due to their ability to provide multitude health benefits. Several investigations from different laboratories have provided evidences for health-promoting properties of these compounds, such as in vitro antioxidant activity, prevention of cancer, in animal and cell culture studies (Jayaprakasha et al., 2007; 2008, Hasan et al., 2011).

NS is an annual herb belonging to the family Ranunculaceae and is commonly known as black cumin and is a natural food additive in India and many parts of Asia. The seeds of NS are the source of active ingredient of this plant. In the *Unani* system, it is regarded as one of the greatest forms of healing medicine available. NS

is cultivated across the globe mostly in hot subtropical or tropical regions such as Southern Florida in USA, India, Mexico, Egypt, and West Indies (Morton, 1987). Both juice and volatile oils are the major commercial products of NS, and volatile oil is widely used as a flavouring agent in beverages and food products (Chamblee et al., 1997). Pharmaceutical industries also use NS volatile oil as a flavouring agent in syrups and suspensions (Porta et al., 1997). In perfumery, NS volatile oils have been used as a base for many compositions, which have a higher market value per pound than other citrus varieties such as orange, grapefruit, or tangerine volatile oils.

Active principles of plant origin are known to inhibit cancer cells growth. Volatile oil of NS has shown inhibition of 1,2-dimethylhydrazine-induced aberrant crypt foci in rats (Salim and Fukushima, 2003). *Patrinia scabra* Bunge root volatile oil has shown cytotoxic activity in human ovarian carcinoma and hepatoma cells (Hong et al., 2005). Induction of cell death by D-limonene on mammary carcinoma cells (Haag et al., 1992) and apoptosis by chloroform extract of NS on HeLa cells (Shafi et al., 2009) were reported. It is possible that the presence of these major constituents is responsible for the inhibition of breast cancer cell proliferation.

The above studies have demonstrated that NS and their bioactive compounds have a significant role in human disease prevention. However, the antiproliferative activity of NS has never been explored in MDA-MB-231.

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In this context, study was conducted to analyse potential of antiproliferative mechanism on cultured human adenocarcinoma cells and has been studied for the first time.

## Materials and Methods

### Preparation of plant extract

Air dried NS seeds were pulverized using a milling machine and extracted with Methanol using the Soxhlet apparatus. And organic phase was later evaporated under reduced pressure to obtain the residue, using a rotary evaporator to dryness in order to obtain the lyophilized powder/paste, weight and required quantity was dissolved in Dimethyl Sulfoxide (DMSO).

### Maintenance of MDA-MB-231 cell line

MDA-MB-231 cells were procured from the ATCC, USA. Cell lines were maintained and propagated in 90% Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. Cells were cultured as adherent monolayer and maintained at 37°C in a humidified atmosphere of 5% CO<sub>2</sub>. Cells were harvested after subjecting them to brief trypsinization. All Chemicals were research grade.

### Cell viability assay

Cell viability was assayed where and as required by trypan blue exclusion test (James and Warburton, 1999) with slight modifications. Briefly, MDA-MB-231 cells were treated and cultured as described. They were harvested and Trypan blue dye solution was added to the cell suspensions. Total cell counts and viable cell number (survival rate) were determined by a standard hemocytometer procedure. Live-viable cells were seen as colourless (impermeable to the dye due to intact cell membrane) and dead cells were seen as blue (permeable to dye due to disruption of cell membrane). The viability of cells was found to fall between 90-95%. % of Live Cells = (Live Cells Counted X Total Cells Counted)/100.

### Gene expression by quantitative RT-PCR analysis

MDA-MB-231 were treated with two different conc. of methanolic extract of NS seed and the procedures of quantitative RT-PCR analysis were carried out according to the manufacturer's instructions. Briefly, cDNA was directly prepared from cultured cells by using Cell-to-cDNA synthesis reagent kit (Promega) and the levels of Bax, Caspase-3 and Bcl-2 mRNA as well as the reference gene GAPDH was assayed by the gene-specific SYBR Green gene expression assays (QIAGEN). All samples and controls were run in triplicates on an ABI 7,900 Real-Time PCR system. The quantitative RT-PCR data was analyzed by the comparative cycle number threshold method and the fold inductions of samples were compared with the untreated samples. The amount of gene expression was then calculated as the difference cycle threshold ( $\Delta$ CT) between the CT value of the target gene and GAPDH.  $\Delta\Delta$ CT is the difference between the  $\Delta$ CT values of the test sample and the control. Relative expression of target

genes was calculated as  $2^{-\Delta\Delta$ CT}.

### Analysis of cell cycle and DNA fragmentation

For DNA fragmentation detection, the terminal deoxynucleotide transferase-mediated dUTP nick end-labeling (TUNEL) assay was performed using an Apo-Direct kit (Phoenix Flow Systems, San Diego, CA). Briefly, cells (approximately  $1 \times 10^6$ ) were washed in phosphate-buffered saline, fixed in 1% paraformaldehyde for 15 minutes on ice, washed with phosphate-buffered saline and then fixed in 70% ethanol. The cells were incubated in DNA-labeling solution that contained TdT Enzyme, TdT reaction buffer and fluorescein-dUTP. After washing the cells with buffer, they were incubated with propidium iodide/RNase A solution for 30 minutes prior to flow cytometric analysis. Propidium iodide staining was used to assess the DNA cell cycle.

For cell cycle analysis, 10,000 cells were analyzed for cell cycle distribution by a Becton Dickinson FACScan flow cytometer equipped with a 488-nm argon laser and Cell QUEST acquisition and analysis software (Becton Dickinson Immunocytometry Systems, San Jose, CA).

### Statistical analysis

The results of each series of experiments (performed in triplicates) are expressed as the mean values  $\pm$  standard deviation of the mean (SD). Cell cycle distribution was analyzed by Mod-fit software and statistical analysis was performed using Student's t test. Data were analyzed using events gated for doublet discrimination on a dot plot of area versus width of propidium iodide fluorescence. Each experiment was repeated a minimum of three times.

## Results

NS has previously shown significant cytotoxicity against several cancer cell lines such as human cervical adenocarcinoma (HeLa) cells (Latifah et al., 2009), canine osteosarcoma (COS31), its cisplatin-resistant variant (COS31/rCDDP), human breast adenocarcinoma (MCF7), and human ovarian adenocarcinoma (BG-1) cells (Pignatelli et al., 2003).

To determine the mechanism of NS-induced apoptosis, the expression of Bax, Caspase-3 and Bcl-2 following NS exposure were tested using SYBR Green based Real Time PCR. It was clear from Real Time PCR results that the expression of these genes in MDA-MB-231 was higher in NS methanolic extract in dose dependent ( $2.5 \mu\text{g/mL}$  or  $5 \mu\text{g/mL}$ ) treated cells compared to respective controls. However, the expression of anti apoptotic genes were down regulated in NS methanolic extract treated cells compared to untreated cells in dose dependant manner ( $2.5 \mu\text{g/mL}$  or  $5 \mu\text{g/mL}$ ). This suggested that methanolic extract of NS seed contains some compound(s) which down regulate the expression of anti apoptotic genes and up regulate the expression of apoptotic genes.

Expression of mRNA is considered as a confirmation regarding the execution of molecular actions and it is measured through Real Time PCR. Results of the current study have revealed an elevated expression of both Bax and caspase-3 in cells pretreated with NS for 48 h in a

dose dependant manner (Figure 1). Further, decrease in the expression levels of antiapoptotic-Bcl-2 was observed upon pretreatment of NS for 48 h (Figure 1). The expression ratio of Bax/Bcl-2 is considered as one of the major hallmark of apoptosis, results of our study have shown that treatment of MDA-MB-231 cells with NS elevated ratio by 2 and 4.3 folds in comparison to untreated control cells after 48 h (Figure 1).

Flow cytometric analysis was used to determine apoptotic indices in cells stained by the TUNEL method. The proportion of NS-treated cells that exhibited DNase-mediated DNA cleavage increased in a dose dependant manner, and the apoptotic index for cells treated with 5 µg/L was approximately 7-fold greater than that of cells treated with 2.5 µg/L NS (Figure 2). DNA cell cycle analysis showed an accumulation of a sub-G0/G1 peak in both 5 µg/L and 2.5 µg/L NS-treated cell populations (data not shown). The sub-G0/G1 peak represents cells with lower DNA content than that of G0/G1 cells and includes apoptotic and necrotic cells. There were no changes in cell cycle distribution following exposure to any concentration of NS tested (Table 1).

The inhibition of MDA-MB-231 cell growth by NS was accompanied by a significant loss of cell number, suggesting that apoptosis was involved in the action of NS. Apoptotic programmed cell death contributes to the maintenance of cell number in many tissues. Apoptosis is a highly conserved evolutionary process that removes either undesired but otherwise healthy cells (e.g., tissue restructuring during development) or removes damaged single cells. Cells from various human malignancies have a decreased ability to undergo apoptosis in response to various physiologic stimuli. For this reason, the induction of apoptosis has become a target in cancer chemoprevention and chemotherapy. In the present study, the occurrence and the mode of MDA-MB-231 cell death was dependent on the concentration of NS. Gene

**Table 1. Effect of NS on the Cell Cycle Distribution of MDA-MB-231 Cells**

Cycle Phase	2.5 mg/L	5 mg/L
%G0/G1	49.8±6.9	54.8±9.1
%G2/M	36.6±3.8	33.7±7.6
%S	13.6±4.5	12.3±3.0

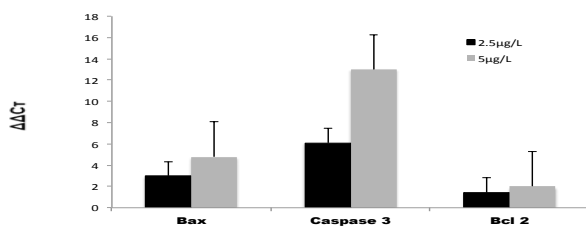
expression and flow cytometric data were consistent in establishing that the cell death occurred in response to NS treatment via induction of apoptosis, caused gene expression changes of apoptotic genes and flow cytometric analysis revealed DNA fragmentation, a characteristic of apoptosis.

There is a possibility that NS could cause cell cycle arrest. In many instances of apoptosis, a period of cell cycle stasis precedes the onset of apoptosis. Many antiproliferative drugs block cell cycle phase transitions. At the two different concentrations tested in the present study, NS did not induce changes in cell cycle distribution, thus it was not apparent at which phase of the cell cycle the MDA-MB-231 cells entered apoptosis. These findings are consistent with the understanding that apoptosis can be initiated and can occur in any phase of cell cycle.

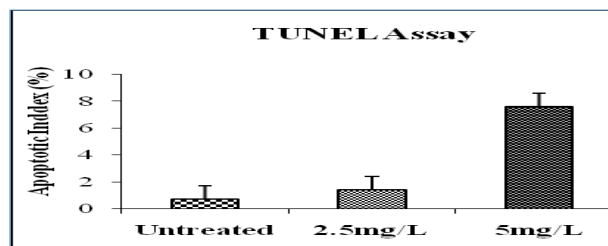
Bax, a member of the Bcl-2 protein family, plays a vital role in the induction of apoptosis (Zhang et al., 2000). It is an integral membrane protein associated with organelles or bound to organelles by Bcl-2 or a soluble protein found in the cytosol and has shown mobility from cytosol to mitochondria during apoptosis (Wolter et al., 1997). Expression levels of Bax (pro-apoptotic) and Bcl (antiapoptotic) gene level provide further information regarding apoptosis. Some of the natural compounds such as flavonoids and polyphenols have shown a significant induction of Bax and depletion in Bcl-2 for an induction of apoptosis-mediated cell death (Mohan et al., 2007). Thymoquinone, a volatile active principle of NS, has shown an induction of apoptosis in colon cancer cells (Gali-Muhtasib et al., 2006) and HeLa cells (Shafi et al., 2009).

Tumor suppressor gene p53 has been reported to be able to regulate the expression of a number of downstream proteins, such as Bax and Bcl-2 in response to DNA damage (Coutts and La Thangue, 2006) and (Roos and Kaina, 2006). High ratio of Bax to Bcl-2 can cause the permeabilization of the outer mitochondrial membrane, resulting in the release of cytochrome c. Cytochrome c forms an apoptosome that is composed of Apaf-1 and procaspase-9, resulting in the activation of caspase-9. Caspase-9 activates the effector procaspases including caspase-3, -6, and -7 to carry out the process of apoptosis (Han and Park, 2009). Therefore, it is presumed that NS induces DNA damage in MDA-MB-231 cells, leading to the increase in the level of p53 expression, and resulting in the regulation of the Bax to Bcl-2 ratio.

The ratio of Bax/Bcl-2 is considered as one of the major biochemical markers for the evaluation of cancer inhibition, i.e. increase in the ratio is considered to be anticarcinogenic and vice versa (Zhang et al., 2003). Only few natural compounds have shown to alter the ratio of Bcl-2 and Bax and those which have an effect are considered to be the effective candidates for the prevention



**Figure 1. Expression of anti-apoptotic genes.** Bcl-2 and pro-apoptotic genes; Bax and caspase -3, in MDA-MB-231 cells treated with methanolic extract of NS



**Figure 2. NS-induced apoptosis of MDA-MB-231 cells.** The percentage of apoptotic events (apoptotic index) was determined by the TUNEL assay using flow cytometry. The cells were treated for 48 hours with 2.5 mg/L or 2.5 mg/L NS

of different types of cancer (Corsetti et al., 2008). NS has shown 4.3 fold enhancements in the ratio of Bax/Bcl-2, after 48 h of treatment.

The present study focused on the effect of NS seed that have shown anticancer activity on MDA-MB-231. The results obtained from the study indicated that methanolic extraction of NS seed exerted an anticancer effect on MDA-MB-231 with a dosage-dependent relationship by regulating apoptotic genes, caspase-3 and bax. In contrast, methanolic extraction of NS seed showed a strong activity in down regulating anti apoptotic gene, Bcl-2 in MDA-MB-231. This bi-functional activity of NS seed suggested the different active components in methanolic extract with anticancer activity.

In summary, the present study revealed anti cancer activity of NS methanolic extract regarding both apoptotic and anti apoptotic responses. Although, the exact mechanism of this effect is not clear, it may be mediated by interactions between active components of extracts and cell surface molecules or growth factors involved in regulation of apoptosis. Another possible action of extracts may be interference with cell signaling. Although further investigations are warranted, the data available to date suggest that NS may have chemo preventive or anticancer actions through apoptosis activity.

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