RESEARCH ARTICLE

Cytotoxicity of *Nigella Sativa* Seed Oil and Extract Against Human Lung Cancer Cell Line

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Abstract

***Nigella sativa* (*N sativa*), commonly known as black seed, has been used in traditional medicine to treat many diseases. The antioxidant, anti-inflammatory, and antibacterial activities of *N sativa* extracts are well known. Therefore, the present study was designed to investigate the anticancer activity of seed extract (NSE) and seed oil (NSO) of *N sativa* against a human lung cancer cell line. Cells were exposed to 0.01 to 1 mg/ml of NSE and NSO for 24 h, then percent cell viability was assessed by 3-(4, 5-dimethylthiazol-2yl)-2, 5-biphenyl tetrazolium bromide (MTT) and neutral red uptake (NRU) assays, and cellular morphology by phase contrast inverted microscopy. The results showed NSE and NSO significantly reduce the cell viability and alter the cellular morphology of A-549 cells in a concentration dependent manner. The percent cell viability was recorded as 75%, 50%, and 26% at 0.25, 0.5, and 1 mg/ml of NSE by MTT assay and 73%, 48%, and 23% at 0.25, 0.5, and 1 mg/ml of NSE by NRU assay. Exposure to NSO concentrations of 0.1 mg/ml and above for 24 h was also found to be cytotoxic. The decrease in cell viability at 0.1, 0.25, 0.5, and 1 mg/ml of NSO was recorded to be 89%, 52%, 41%, and 13% by MTT assay and 85%, 52%, 38%, and 11% by NRU assay, respectively. A-549 cells exposed to 0.25, 0.5 and 1 mg/ml of NSE and NSO lost their typical morphology and appeared smaller in size. The data revealed that the treatment of seed extract (NSE) and seed oil (NSO) of *Nigella sativa* significantly reduce viability of human lung cancer cells.**

Keywords: *Nigella sativa* - A-549 cells – cytotoxicity - cellular morphology

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Introduction

Nigella sativa (*N sativa*) is an annual herb of the *Ranunculaceae* family, which is used as an important nutritional flavoring agent and natural health remedy in traditional folk medicine for the treatment of numerous disorders in ancient systems of Unani, Ayurveda, Chinese and Arabic medicine for thousands of years (Randhawa and Alghamdi, 2011). The extracts of *N sativa* seeds have anti-inflammatory and antioxidant activities, and being used by patients to suppress coughs, disintegrate renal calculi, retard the carcinogenic process, treat abdominal pain, diarrhea, flatulence and polio (Ahmad et al., 2013; Al-Khalaf and Ramadan, 2013). The seed of this plant, commonly known as black seed, are eaten alone or in combination with honey and in many food preparations and the oil prepared by compressing the seeds of *N sativa* is used for cooking (Al-Khalaf and Ramadan, 2013). The seeds of *N sativa* contain both fixed and essential oils, proteins, alkaloids and saponin (Ali and Blunden, 2003; Khan et al., 2011). Many active ingredients found in the seeds of *N sativa* have beneficial effects against various cancer diseases, including cervical cancer (Effenberger et al., 2010), blood cancer (El-Mahdy et al., 2005), hepatic cancer (Thabrew et al., 2005), colon cancer (Salim and Fukushima, 2003), pancreatic cancer (Chehl et al., 2009), skin cancer (Salomi et al., 1991), fibrosarcoma (Awad, 2005), renal cancer (Khan and Sultana, 2005), prostate cancer (Yi et al., 2008), and breast cancers (Farah and Begum, 2003; Ahmad et al., 2012). Pharmacologically important components of *N sativa* extracts have also been studied against lung cancer as an anticancer agent. In one of the study Swamy and Huat (2003) have shown the antitumor activity of α-hederin from *N sativa* against Lewis lung carcinoma in BDF1 mice. Protective effect of *N sativa* extracts against methylnitrosourea-induced oxidative stress, inflammatory response and carcinogenesis in lung cells has also been shown (Mabrouk et al., 2002). These studies showed that *N sativa* extracts can protect lung cells, but the molecular mechanisms of *N sativa* extracts against lung cancer cells have not been explored till date. Therefore, the present study was designed to investigate the *in vitro* cytotoxic activity of *N sativa* seed extracts against human lung cancer cell line A-549.

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Materials and Methods

Chemicals and consumables

Dulbecco's Modified Eagle's Medium (DMEM) culture medium, antibiotics-atimycotic solution, fetal bovine serum (FBS) and trypsin were purchased from Invitogen, Life Sciences, USA. Consumables and culture wares used in the study were procured from Nunc, Denmark. Ethanol and all other specified reagents and solvents were purchased from Sigma Chemical Company Pvt. Ltd. St. Louis, MO, USA.

Plant material and extractions

The *Nigella sativa* (*N sativa*) seeds used in this study were obtained from the local market of Riyadh, Saudi Arabia. The seeds were screened manually to remove bad ones. The oil from *N sativa* seeds was extracted by continuous extraction in Soxhlet apparatus for 12 h using petroleum ether (60-80°C boiling range) as a solvent according to the method described by AOCS (Horwitz, 1980). At the end of the extraction the solvent was evaporated. The oil thus obtained was dried over anhydrous sodium sulphate and stored -4°C for further analysis. For the preparation of alcoholic extract, the seeds were macerated in alcohol and then filtered. The procedure was repeated several times. The solvent was then evaporated using a rotary evaporator and the residue so obtained was called as the alcoholic extract.

Cell culture

A-549, Human lung cancer cells were cultured in DMEM, supplemented with 10% FBS, 0.2% sodium bicarbonate and antibiotic/antimycotic solution (100x, 1ml/100 ml of medium). Cells were grown in 5% CO_2 at 37°C in high humid atmosphere. Before the experiments, viability of cells was assessed following the protocol of (Siddiqui et al., 2008). A-549 cells showing more than 95% cell viability and passage number between 6 and 8 were used in the present study.

Experimental design

A-549 cells were exposed to various concentrations of seed extract (NSE) and seed oil (NSO) of *N sativa* (0.01-1 mg/ml) for a period of 24 h. Following the exposures of NSE and NSO, A-549 cells were subjected to assess the cytotoxic responses using 3-(4,5-dimethylthiazol-2yl)-2, 5-biphenyl tetrazolium bromide (MTT), neutral red uptake (NRU) assays, and cellular morphology by phase contrast inverted microscope.

Drug solutions

The *N sativa* seed extracts were not completely soluble in aqueous medium solution, therefore the stock solutions of all the extracts were prepared in dimethylsulphoxide (DMSO) and diluted in culture medium to reach the desired concentrations. The concentration of DMSO in culture medium was not more that 0.1% and this medium was used as control.

Cytotoxicity screening

MTT assay: percent cell viability was assessed using the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) assay as described (Siddiqui et al., 2008). Briefly, A-549 cells (1×10^4) were allowed to adhere for 24 h CO_2 incubator at 37°C in 96 well culture plates. After the respective exposure, MTT (5 mg/ml of stock in PBS) was added (10 µl/well in 100 µl of cell suspension), and plates were incubated for 4 h. Then, supernatants were discarded and 200 µl of DMSO were added to each well and mixed gently. The developed color was read at 550 nm using Multiwell Microplate Reader (Thermo Scientific, USA). Untreated sets were also run under identical conditions and served as control.

Neutral red uptake (NRU) assay: neutral red uptake (NRU) assay was carried out following the protocol described by (Siddiqui et al., 2010). Briefly, after the exposure, the medium was aspirated and cells were washed twice with PBS, and incubated for 3 h in a medium supplemented with neutral red (50 µg/ml). Medium was washed off rapidly with a solution containing 0.5% formaldehyde and 1% calcium chloride. Cells were subjected to further incubation of 20 min at 370C in a mixture of acetic acid (1%) and ethanol (50%) to extract the dye. The plates were read at 540 nm using multiwell microplate reader (Thermo Scientific, USA). The values were compared with the control sets run under identical conditions.

Morphological analysis: morphological changes in A-549 cells exposed to increasing concentrations (0.01-1 mg/ml) of NSE and NSO were taken using an inverted phase contrast microscope (OLYMPUS CKX 41) at 20 X magnification.

Statistical analysis

The results were expressed as mean and standard error of means (SEM). One way ANOVA was employed to detect differences between the groups of treated and control. The values showing $p<0.05$ were considered as statistically significant.

Results

MTT and NRU assays

The cytotoxicity of seed extracts (NSE) and seed oil (NSO) of *N sativa* was assessed using MTT and NRU assays, after exposing the A-549 cells at 0.01-1 mg/ml concentrations for 24 h. The percent cell viability of A-549 cells against NSE as observed by MTT and NRU assays are presented in Figure 1. Result shows that NSE induced statistically significant ($p<0.001$) decrease in cell viability of A-549 cells in a concentration dependent manner (Figure 1). The A-549 cells exposed to NSE at 0.25 mg/ml and above concentrations for 24 h were found to be cytotoxic. The percent cell viability was recorded 75%, 50%, and 26% at 0.25, 0.5, and 1 mg/ml of NSE respectively by MTT assay (Figure 1) and 73%, 48%, and 23% at 0.25, 0.5, and 1 mg/ml of NSE respectively by NRU assay (Figure 1). NSE at 0.1 mg/ml and lower concentrations did not show any decrease in the cell viability of A-549 cells.

A-549 cells exposed to NSO for 24 h also show the statistically significant ($p<0.001$) decrease in the

cell viability in a concentration dependent manner (Figure 3). A-459 cells exposed to 0.1 mg/ml and above concentrations of NSO for 24 h were found to be cytotoxic. The cell viability at 0.1 mg/ml was recorded to be 89 % by MTT (Figure 3) and 85% by NRU assay (Figure 3). The decrease in the cell viability at 0.25, 0.5, and 1 mg/ml of NSO was recorded to be 52%, 41%, and 13% by MTT assay (Figure 3) and 52%, 38%, and 11% by NRU assay (Figure 3), respectively. The concentrations of NSO at 0.05 mg/ml and lower did not show decrease in the cell viability of A-549 cells as shown by MTT and NRU assays. The NSO was found to be more cytotoxic to A-549 cells as compared to NSE.

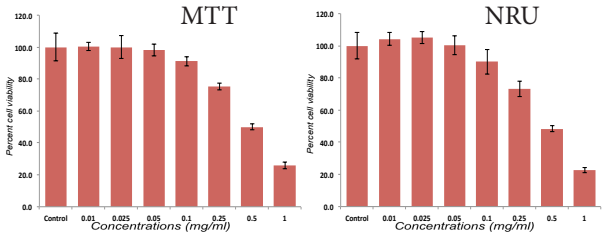

Figure 1. Cytotoxicity Assessments by MTT and NRU Assay in A-549 Cells Following the Exposure of Various Concentrations of Seed Extract of Nigella Sativa (NSE) for 24 h. Values are mean±SD of three independent experiments. (*p<0.05, **p<0.01, ***p<0.001 vs Control)

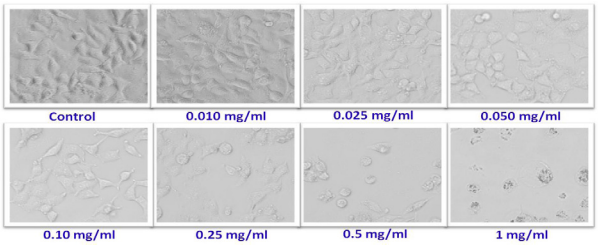

Figure 2. Morphological Changes in A-549 Cells Exposed to Various Concentrations of Seed Extract of Nigella Sativa (NSE) for 24 h. Images were taken using an inverted phase contrast microscope (OLYMPUS CKX 41) at 20× magnification

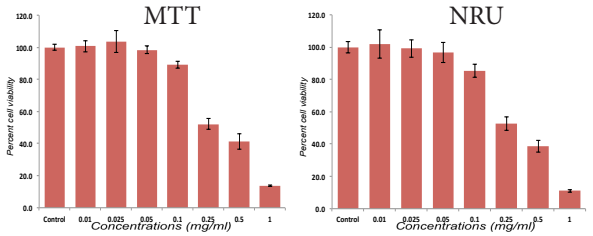

Figure 3. Cytotoxicity Assessments by MTT and NRU Assay in A-549 Cells Following the Exposure of Various Concentrations of Seed Oil of Nigella Sativa (NSO) for 24 h. Values are mean±SD of three independent experiments. (*p<0.05, **p<0.01, ***p<0.001 vs Control)

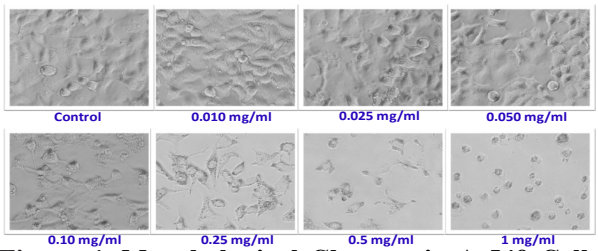

Figure 4. Morphological Changes in A-549 Cells Exposed to Various Concentrations of Seed Oil of Nigella Sativa (NSO) for 24 h. Images were taken using an inverted phase contrast microscope (OLYMPUS CKX 41) at 20× magnification

Morphological changes

Alterations in the morphology of A-549 cells exposed to NSE and NSO were found to be in a concentration dependent manner. The morphological changes observed in A-549 cells by NSE and NSO are shown in Figures 2 and 4 respectively. Cells exposed to 0.25 mg/ml and above concentrations of NSE for 24 h reduced the normal morphology and cell adhesion capacity of A-549 cells as compared to control (Figure 2). In case of NSO, the morphology of A-549 cells at 0.1 mg/ml concentrations started to reduce the normal shape and cell adhesion capacity as compared to control (Figure 4). As shown most of the cells exposed to 0.25, 0.5 and 1mg/ml of NSE and NSO lost their typical morphology and appeared smaller in size.

Discussion

Lung cancer is the most common cancer worldwide, accounting for 1.3 million deaths annually. Cancer accounted for 13 percent of the 58 million total worldwide deaths in 2004 (WHO, 2009). Herbal medicines have long been viewed as a source of curative remedy based on religious and cultural traditions (Ghazanfer, 1994; Pal and Shukla, 2003; Saetung et al., 2005). Many investigators now believe that traditional medicine is a promising source of new therapeutics against cancer (Pan and Ho, 2008; Al-Oqail et al., 2013; Farshori et al., 2013). *Nigella sativa* (*N sativa*) of *Ranunculaceae* family is widely used medicinal plant throughout the world. It is very popular in various traditional systems of medicine like Unani and Tibb, Ayurveda and Siddha, and the seeds and oil of *N sativa* have a long history of folklore usage in various systems of medicines and food (Ahmad et al., 2013).

Recent studies indicate that *N sativa* extracts has cytotoxic effects against different types of cancer cell lines *in vitro* (Jafri et al., 2010; Bourgou et al., 2012; VSPK et al., 2013). Therefore, the present study was undertaken to provide comparative data on the *in vitro* cytotoxic activity of different extracts of *N sativa* seeds against A-549, a human lung cancer cell lines. The cytotoxic responses of the extracts were determined by the 3-(4, 5-dimethylthiazol-2yl)-2, 5-biphenyl tetrazolium bromide (MTT) and neutral red uptake (NRU) assays. The results indicate that both N. Sativa seed extract (NSE) and oil (NSO) has significant *in vitro* cytotoxic effect on A-549 cells. The results showed that both NSE and NSO of *N sativa* decreased the cell viability of A-549 cells in a concentration-dependent manner. Our results are well in ordinance with the previous studies showing *in vitro* anticancer activity of *N sativa* against the various cell lines (Ivankovic1et al., 2006; Bourgou et al., 2010; Raval et al., 2010), and in *in vivo* setup (Salomi and Panikkar, 1989; Mbarek et al., 2007). An *in vitro* study showed the significant cytotoxic activity (p<0.01) against L929

fibroblast cells in a concentration dependent manner (Ivankovic et al., 2006).The various studies also showed that extract of *N sativa* plant exhibits the inhibition of cancerous cell growth against HL-60 and U- 937 cell lines (Raval et al., 2010). The seed extracts from *N sativa* have also been found active against DLD-1 colon carcinoma and *Staphylococcus aureus* and *Escherichia coli* bacterial strains (Bourgou et al., 2010). The administrations of the essential oil into the tumor site have also been shown to inhibit liver metastasis development and improved the survival of mouse/mice (Salomi and Panikkar, 1989; Mbarek et al., 2007).

The active principles in NSO have been found to exert antineoplastic effects both *in vitro* and *in vivo* using various models of carcinogenesis. The active principles of *N sativa* showed cytotoxicity against number of cancer cells, such as Ehrlich ascites carcinoma, Dalton's lymphoma ascites and Sarcoma-180 cells in a concentration dependent manner (Salomi et al., 1992; El-Najjar et al., 2010). We found that the NSO extracts, even at lower concentrations exhibit the inhibition of A-549 cells, and the cytotoxicity was found more than the NSE extract. The growth inhibitory activity of various plant extracts has been reported (Kaneshiro et al., 2005; Kumar et al., 2011) due to the ability of the extracts to inhibit the DNA synthesis as measured by the incorporation of tritiated thymidine into cells (Worthen et. al., 1998). In another study, ethanolic extract of *N sativa* was shown to possess cytotoxic effects against different classes of cancer cell lines, such as, P388, HepG2, Molt4 and Lewis lung carcinoma cells (Swamy and Tan, 2000). Our results from present study suggest that the anticancer activity of the seeds might be linked to the presence of active compounds (Huang and Zou, 2011; Kma, 2013), which exhibit anticancer potential against human lung cancer cells, A-549.

In conclusion, our results demonstrate that seed extract (NSE) and seed oil (NSO) of *Nigella sativa* significantly reduced the cell viability of human lung cancer cell line, and altered the cellular morphology in a concentration dependent manner. The data also revealed that A-549 cells were more sensitive towards NSO than NSE and the cytotoxicity was found even at lower concentrations of NSO. Further studies are required to understand the mechanism(s) of action of these extracts on A-549 cells.

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