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Effect of Black seeds (*Nigella sativa*) volatile oil on the cervical cancer: *In vitro* study, on *Hela cell* lines

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ABSTRACT

Cervical cancer is the second most common type of gynecological tumors in North America. Most chemotherapeutic agents used by oncologists were found to kill cancer cells as well as healthy ones. Unfortunately, the side effects may overwhelm its beneficial effect. Seeds of *Nigella sativa*, often referred to as black seeds or black cumin, have been used by traditional medicinal practitioners for centuries to treat inflammation, infections, and cancers. In fact, previous studies have shown the herb to have anti-cancer effects on colon and prostate cancers. In the present study, volatile oil was prepared from 2 patches of *N. sativa* seeds (Alqassim and Ethiopia). *HeLa cell* lines (derived from cervical cancer cells) were used. *N. sativa* volatile oil was incubated with the cells at different concentrations (10, 100, 1000) and compared with cisplatin. *Hela cell*s were used to assess the cytotoxic effect of *N. sativa*. Study showed decrease in *Hela cells* survival rate at 100 concentration. A decrease the adhesion protein of *Hela cells* in a dose dependant manner with extract from Alqassim patch was reported. Anti cancer effect was confirmed by morphological study where shrinkage and apoptotic changes were observed in cultured cells. In conclusion, *N. sativa* volatile oil has a similar anti cancer effect to cisplatin and could be used as adjuvant therapy.

Keywords: *Nigella sativa*, *Hela cells*, cytotoxicity.

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INTRODUCTION

Apoptosis is an active physiological process resulting in cellular self-destruction of unwanted cells which was known to be challenged in cancer cells. Apoptosis is characterized by distinct morphologic changes, including cell shrinkage, membrane blubbing, chromatin condensation, DNA fragmentation and the formation of apoptotic bodies (Wyllie, 1997).

The regulation of apoptosis in normal and malignant cells has become an area of intensive study in cancer research (Johnstone et al., 2002). Inducing apoptosis is thus become the target for therapeutic application and development of anti-tumor drugs (Hong et al., 2003). It is therefore essential to identify novel apoptosis-inducing compounds which will act as anti-tumour agents.

Nigella sativa, (Ranunculaceae family) or as traditionally named black seed, is an annual herb that grows in countries bordering Mediterranean area, Pakistan and India (Marsik et al., 2005; Norwood et al., 2006; Padhye et al., 2008).

The seed has been used as a natural remedy for more than 2000 years to promote health and treat diseases. Medicinal properties of black seeds have even been mentioned by the Prophet of Islam, Muhammad (Peace be upon him) and its use was recommended for various ailments (Bhatti et al., 2009).

N. sativa seeds were proved to contain more than 30%

fixed oil and 0.40 to 0.45% volatile oil (Ali and Blunden, 2003). Thymoquinone represents 18.4 to 24% of the *Nigella sativa* volatile oil (Arslan et al., 2005). Recently, several clinical studies were conducted in humans receiving oral *N. sativa* extract or black seeds for up to 8 weeks for evaluating its anti-inflammatory, antihypertensive, or antioxidant effects (Dehkordi and Kamkhah, 2008; Al-Jenoobi et al., 2010). Most of the known biological activities of the seed have been attributed to its active ingredient; Thymoquinone (TQ). TQ has been shown to be safe on a wide variety of normal cells (Gali-Muhtasib et al., 2004).

There is a growing interest in the therapeutic potential of TQ in cancer therapy, with great success in its effect as an inhibitor of many types of human cancer cell proliferation (Gali-Muhtasib et al., 2008; Effenberger-Neidnicht and Schobert, 2010; Ravindran et al., 2010; El-Najjar et al., 2011).

In the present study *Hela cell* line was used to re-evaluate the anti-cancer activity the *N. sativa* seed extract and to investigate the ability of *Nigella sativa* to induce apoptosis.

MATERIALS AND METHODS

The project was conducted between 2011 and 2012 in King Fahad Medical Research Center (KFMRC) in King Abdulaziz University (KAU), Jeddah, Saudi Arabia.

Materials

Nigella sativa seeds (Alqassim and Ethopia), cisplatin (Hospital of King Abdulaziz University), Dulbecco's Modified Eagle Medium (DMEM) was purchased from MP Biomedicals Inc, USA, fetal calf serum (FCS) was obtained from Gibco, dimethyl sulfoxide (DMSO) and sulforhodamine B (SRB) were obtained from (MP Biomedicals, Illkrich, France).

Nigella sativa extract

N. sativa seeds volatile oil was prepared in the Lab of pharmaceutical preparation College of Pharmacy, KAU, Jeddah. The powdered seeds were extracted with MeOH (5 × 1000 ml) by using Ultraturrax T25 homogeniser (Janke and Kunkel, IKA Labortechnik, Stauten, Germany) at room temperature. The combined extracts were evaporated under reduced pressure and, lyophilized and protected from light at 4°C until use. Oil was dissolved in DMSO immediate before use.

Cell lines

Cervical cancer cell (*Hela* cell); American Type Cultural Collection (ATCC) were obtained from cell bank of Virology Unit, King Abdulaziz University Hospital, Jeddah.

Cell culture

Cell lines were grown in DMEM media supplemented with 10% FCS, penicillin (100 units/ml) and streptomycin (100 μg/ml) in culture flasks at 37°C in 5% humidified CO_2 incubator. Cells were fed until confluence.

Evaluation of *Nigella sativa* extract

The cytotoxicity of the *N. sativa* was tested using the method of Skehan et al. (1990). Cells were cultured in 96 plates (10^4 cells/well) for 24 h in a humidified CO_2 incubator at 37°C. Different concentrations of the *N. sativa* (Qassim, Ethiopia) (2.5, 7.5, 10, 50 and 100 μg/ml) were added to the cells and reincubated for 48 h at 37°C in a humidified 5% CO_2 incubator. Cells were fixed with 50 μl of cold 50% trichloroacetic acid (TCA) on the top of growth media in each well. The cultures were incubated at 4°C for 1 h and then washed five times with tap water to remove TCA. TCA-fixed cells were stained for 30 min with 0.4% (wt/vol) sulforhodamine B (SRB) dissolved in 1% acetic acid. At the end of the staining period, SRB was removed and cultures were quickly rinsed four times with 1% acetic acid to remove unbound dye. Bound dye was solubilized with 10 mM unbuffered Tris EDTA (100 μl /well) for 5 min on a gyratory shaker. Color intensity was measured immediately in an ELISA reader at wave length 490 to 530.

Cell viability assay

The numbers of viable cells were counted using a cell viability analyzer which provides an automatic and effective ways to perform the trypan blue dye exclusion method. Cells suspended in original DMEM containing 10% FBS (1×10^5 cell/ml) were cultivated onto 24-well plate. After 24 h the original media was replaced by several concentrations of *Nigella sativa* Seeds extracts and Cisplatin (universally known anticancer drug) (1:10, 1:100, 1:1000). The controls extract contain 0.1% DMSO**.**

Evaluation of cell morphology

Each group of cells were plated onto Petri dishes in DMEM for 24 h, then the media changed with tested media (NS oil concentration of 1:100) and control media, then incubated at 37°C for 24 h. Cells were fixed in 4% formaldehyde for 5 min at room temperature after double washing with 1X PBS each for 5 min. Then cells were stained with Coomassie blue for 5 to 10 min followed by repeated washing with tap water (Khorshid et al., 2005). Treated and untreated (control) cells were viewed and photographed using an inverted phase-contrast microscope connected to digital camera.

Data analysis

All statistical tests were carried out using MS Office Excel 2010 (Microsoft) and Statistica 8.0 software.

RESULTS

Cytotoxic effect against human cancer cell line

The SRB assay provided a sensitive method for measuring the drug-induced cytotoxicity attached cultures in 96-well microtiter plates. *In vitro* cytotoxicity of the two patches of *Nigella sativa* seeds extracts (Alqassim and Ethopia) were evaluated at 2.5, 10 and 50 *μ*g/ml for 48 h against *Hela* cancer cell line (Figure 1). Growth inhibition was observed in the cell line treated with the two extracts.

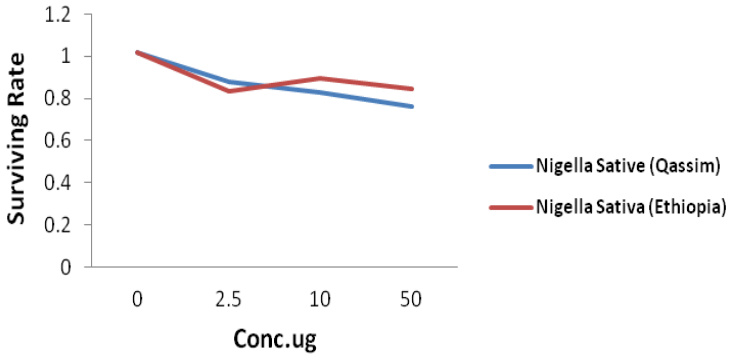

Figure 1. Cytotoxicity of *Nigella sativa (*Alqassim and Ethiopia) on *Hela* cancer cells in 96-well microtiter plates using the SRB assay. Notice: the more effective activity of Alqassim at higher concentration.

The effect was found to be dose-dependent especially with extract from Alqassim patch. The results also showed that at high concentration, *N. sativa* seeds extract (from Alqassim) was more effective compared to that from Ethiopia.

Cell viability

Hela cells viability was decreased with all concentration regarding alqassim seeds. The same was observed in Ethiopia seeds except at higher concentration where less effect was observed. Cells were more sensitive to both extracts at the concentration 1:100 of *Nigella sativa* compared to 1:10 and 1:1000 (Figure 2).

Cell morphology

Cells in the control group were seen to form continuous sheet. They have uniform shape. On the other hand, cells treated with *N. sativa* extract showed morphological changes at concentration of 1:100 after 24 h treatment. The changes were in the form of loss of adhesion, rounding. Signs of apoptosis in the form of cell shrinkage, nuclear degeneration and increase staining density were observed. The later results were more evident with *N. sativa* (Ethiopia) (Figure 3).

DISCUSSION

Apoptosis is an integral process in the pathogenesis of cancer (Kfir-Erenfeld et al., 2010). It determines the progress of cell proliferation or degeneration (Elmore, 2007; Gruber et al., 2012). Many factors were known to induce apoptotic changes in cancer such as hypoxia (Chipurupalli et al., 2019; Cosse et al., 2007) or drug induced (Lee et al., 2004; Pfeffer, and Singh, 2018).

Medicinal herbs provide new insights into the molecular mechanisms underlying the apoptotic process in cancer progress (Willenbacher et al., 2019; Powell et al., 2003; Kwon et al., 2007; Kaur et al., 2008). *N. sativa* was proved to contain valuable antioxidant constituents that using *in vitro* (El-Beshbishy et al., 2009; Shafi et al., 2009) or *in vivo* (Ashraf et al., 2011) studies to be protective or curative against cancer development.

In this paper, the influence of different concentration of *N. sativa* seed (Alqassim and Ethopia) was investigated on cellular viability and morphology using two extract fractions. SRB assay was found to be sensitive test for cell viability *in vitro* studies (Aslantürk, 2018).

Alcoholic extract used in this study was found to induce regression of *Hela cells* proliferation most probably via apoptotic affect as indicated by cell shrinkage and nuclear pyknosis. The effect was found to be dose–dependant with Alqassim seeds. Similar results were reported by Shafi et al. (2009) who found that chloroform fraction of *Nigella sativa* (IC_{50}, 0.41 ng/ml) had a much potent effect compared to methanolic or n-hexan extracts. In the present study, the concentration of 1: 100 was the most effective. It contradict with the previous results modification of methanolic extract (Alqassim) concentration found to have dose – response effect on cultured *Hela cells*.

Jafri et al. (2010) demonstrated that TQ; an active ingredient of *N. sativa* was able to inhibit lung cancer cells proliferation, reduce cell viability and induce apoptosis. Norsharina et al. (2011) showed that thymoquinone rich fraction (TQRF) extracted from *N. sativa* seeds and TQ were cytotoxic to colorectal HT29, promyelocytic leukemia (HL60) and lymphoblastic leukemia (CEMSS) cells lines. Also, Al-Naqeeb and Ismail (2009) showed that TQRF and TQ exhibit cytotoxic effects against HepG2 cells with IC50 at 100 μg/ml for TQRF and 3 μg/ml of TQ.

The mechanism/s by which *N. sativa* or its derivatives determine tumor cell proliferation were the focus of interest of many investigators. Ahuja and Murphy (1996) stated that TQ appears affect tumor microenvironment

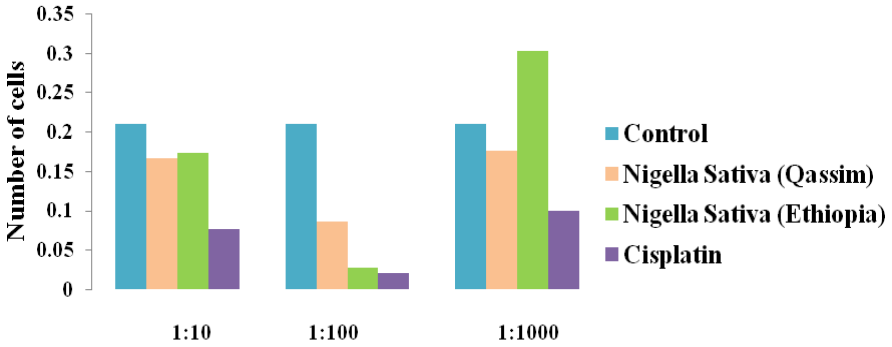

Figure 2. The proliferation differences of *Hela cells* incubated in different concentration of *Nigella sativa* compared with control *Hela cells* incubated in ordinary medium.

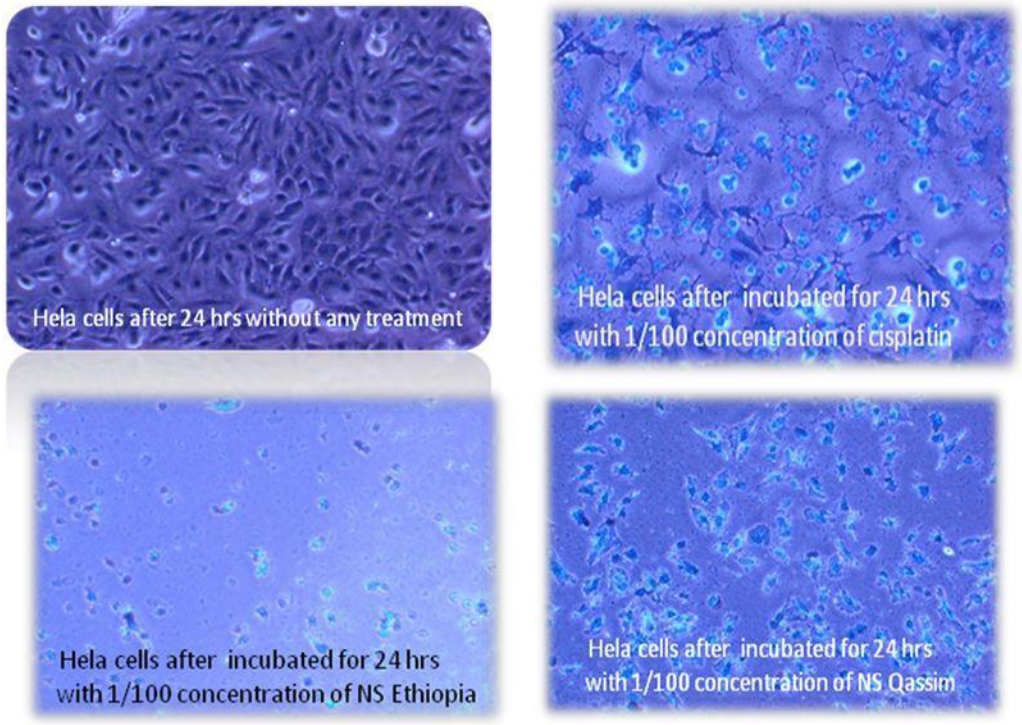

Figure 3. Effect of *Nigella sativa extracts* on cell morphology of *Hela cells* 24 hours after incubation with 1:100 concentration. Notice that the most apoptotic effect was observed with *Nigella sativa* (Ethiopia).

and hence growth and proliferation. The same authors suggested that thymoquinone reduced the release of cytokines which are involved in inflammation and angiogenesis. Another mechanism was reported by Yazan et al. (2009) that thymoquinone induced apoptosis in *Hela cells* by the upregulation of p53 expression dependent pathway. Involvement of oxidative stress was reported to induce apoptosis (Zhu et al., 2012). Koka et al. (2010) reported that TQ-induced growth inhibition in PC-3 and C4-2B prostate cancer cells through generation of reactive oxygen species (ROS).

The interpretation of the effect of concentration of *Nigella sativa* extracts on response of *Hela cells* was questionable. Do low concentration (1:10) was less effective in inducing apoptotic pathway? Regarding higher concentration (1:1000) could have relative pro-oxidant effect? No explanation could be given at the present except in view of some literature that reported that anti oxidant in high dose could less protective activity or even pro-oxidant effect (Tian et al., 2007).

Reviewing available literature showed that a number of recent publications support the current report on the pro-

oxidant effects of TQ at higher concentrations (Gali-Muhtasib et al., 2006; Adams and Cory, 2007; Yi et al., 2008). The shift between pro-oxidant and anti-oxidant activity of TQ can be attributed to variations in semiquinone (single electron transfer) and hydroquinone (two electron transfer) formation in a given cell type (Bolton et al., 2000).

Conclusion

It could be emphasized that methanolic extract, at concentration of 1:100 was the most effective against *Hela cells* especially that of Alqassim patch. Future biochemical analysis of both seed types was needed to explain such differences. The mechanism of anti-tumor effect must also be evaluated using specific genetic. Cytokine and antioxidants analysis of cultured tested cells. As cancer cervix forms the most challenging female neoplasia next to cancer breast. Animal model of cancer cervix will provide valuable evaluation of anti-neoplastic activity of *N. sativa* extract prior to its recommended use in clinical trials.

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