Original Article

The Antioxidant and Antiangiogenic Effects of Dietary supplement of *Nigella sativa* Crude Oil on Breast Tumor in BALB/c Mice

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Abstract

Background and Aim: Cancer embraces a vast range of diseases including that of the breast and is triggered by various factors such as reactive species of oxygen. Angiogenesis, then, aids the cancerous mass to meet its needs and develop further. *Nigella sativa L*. (NS), traditionally cultivated and consumed in the Middle East, has long been appreciated for its medicinal benefits. In this study, we aimed to look into its antioxidant and anti-angiogenic effects on breast tumor in BALB/c mice.

Materials and Methods: 3 groups of BALB/c mice, respectively received 1, 2 and 4 ml/kg/day of NS crude oil via gavaging for 4 months before breast tumor transplantation, while the control mice were gavaged with distilled water. Then tumor volume, the activity of antioxidant enzymes, the amounts of VEGF and endostatin were studied to examine NS crude oil's impact against breast tumor.

Results and Conclusions: Our findings revealed that the mice pretreated with 4 ml/kg/day of NS oil had significantly smaller tumor volumes, higher SOD and CAT activity, reduced VEGF and increased endostatin amounts. So, we came to this that Nigella sativa crude oil seems to inhibit breast tumor growth in part by improving the activity of antioxidant enzymes and in part by interfering with angiogenesis. More studies are, yet, required to better illuminate its other mechanisms of involvement.

Keywords: Breast cancer, Antioxidants, Nigella sativa L., Superoxide dismutase, Catalase, Angiogenesis, Endostatin

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Introduction

All cancer cells share a disrupted cell cycle which is, in normal ones, maintained by various genes (1). Any malfunction in these genes ends in anomalies in metabolic reactions, which in turn cause an increase in reactive oxygen species and free radicals (2). In physiological conditions, safe and well-ordered amounts of ROS are produced and contribute to many biological activities such as diverse cell signaling pathways, mitogenic responses and immunity against infections (3). However, in a loss of control, there are excessive amounts of ROS which damage lipids, DNA and proteins (2). These damages result in more chromosomal instability and altered gene expression that lead to a number of disorders including cancer (4). To prevent this process, aerobic organisms use a complex, antioxidant system consisting of enzymes

and nutrients such as superoxide dismutase, catalase, minerals, vitamins and phytochemicals (5, 6). Beating this system, cancer cells develop and proliferate into a tumor mass which is in great need of blood. Cancer cells stimulate the existing vessels to sprout toward the tumor, also known as angiogenesis to meet their needs (7).To do so, they have to turn the "angiogenic switch" on, otherwise the mass remains dormant and unable to grow beyond 1-2 mm^3 (8). Vascular endothelia growth factor (VEGF) is considered the major promoting factor in the process of angiogenesis while endostatin is believed to be the most potent, inhibitory agent and by manipulating these two, tumors are potentially promised to be suppressed (9).

Since the existing therapies have shown considerable side effects and regarding that Mediterranean and Asian populations have lower rates of cancer incidence (10), the impact of nutrition and medicinal plants have recently gained more consideration. Of those plants, *Nigella sativa* L. is traditionally cultivated and consumed in the Middle East to soothe headaches, asthma, gastrointestinal problems, eczema, hypertension, etc. (11). Black seeds of *Nigella sativa* L. Rich in fatty acids, proteins and vitamins. This black seeds are more medically valued for their quinone components such as thymohydroquinone, dithymoquinone, thymol and, specially, thymoquinone (TQ) (12, 13).

Since in 2012, breast cancer had, in Iran, the most victims which came to 14.2% of casualties by cancer (14) and because methanolic, ethanolic and hexane extracts of *Nigella sativa*L., as well as TQ, are widely reported to be antitumor and antioxidant (15-19) while there is barely any work done on its crude oil, thus we decided to examine the antioxidant and antiangiogenic effects of pretreatment with NS crude oil on tumor growth, antioxidant enzymes and angiogenesis in breast adenocarcinoma in BALB/c mice.

Materials and Methods

All the experiments were performed according to the approved protocol of the Ethics Committee of Shahid Beheshti University [Tehran, Iran].

NS crude oil preparation

Nigella sativa seeds were collected from farms of Semirom (Isfahan, Iran) and authenticated at the School of Pharmacy of Shahid Beheshti University of Medical Sciences, were cold-pressed. The oil was then stored at 4 degrees centigrade in dark glass bottles and a little of it was analyzed in Medicinal Plants Research Center (Shahid Beheshti University, Tehran). Fatty acid composition of *Nigella sativa*`s fixed oil includes palmitic acid (11.21%), linoleic acid (63.85%), oleic acid (17.85%), stearic acid (3.48%), and eicosadienoic acid (3.53%). Chemical composition of volatile constituents of *Nigella sativa* seeds contains α-thujene (10.16%), p-Cymene (43.54%), and Thymoquinone (39.25%). When needed, some was moved to a tube and used during the daily pretreatment of the mice.

Mice

Female BALB/c mice were Purchased from Pasture Institute of Iran, and kept in constant, standard conditions [12:12 L/D, 22°C] within the same facility for a week with unlimited access to food and water throughout the experiment.

Induction of the tumor

After a 4-month pretreatment with NS crude oil, all mice were subcutaneously transplanted with spontaneous murine mammary tumor [SMMT], volumes of less than $0.5cm^3$, according to a previously tried protocol (20). SMMT is an invasive carcinoma [ductal] spontaneously developing in female BALB/c mice. The pieces were transplanted, from a syngeneic donor, into the right flank of the mice.

Experimental protocol

The mice were randomly caged into 4 groups of 7 mice and gavaged for 4 months prior to tumor transplantation: The mice in the control group (CTRL) received distilled water daily. Mice within the next three groups (NS1, NS2, and NS4), were gavaged with 1, 2 and 4ml/kg/day of NS crude oil respectively.

Measurement of tumor volumes

When the tumors were palpable, the tumor sizes were recorded using a digital caliper twice a week for about 3 weeks. The tumor volume was calculated as follows (20).

$$V= (LW^2)/2$$

(V=volume, L=the length, W= the width)

On the last day of the experiment, the mice were sacrificed and the tumors were kept frozen in -80°C for further uses. Prior to freezing and over ice-filled plates, the necrosis was rapidly removed and the tumors were rinsed with normal saline to make sure there was no/little blood left within the tissue.

Tumor homogenate and protein extraction

Following the protocol earlier used by Bigdeli *et al*. (21), small pieces of tumors were homogenized using a sonicator (Bandelin Sonopuls; HD 2070) in 1 ml of a homogenizing buffer composed of EDTA (1 mmol/l), sucrose (0.32mol/l) and Tris-Hcl (10 nmol/l; pH=7.4). We centrifuged the blend for 30 min at 13,600×g. Next, the supernatant was taken to measure superoxide dismutase and catalase activity. We applied Bradford method (22) to determine protein concentration.

SOD activity measurement

Through a previously practiced protocol (21), we assessed the total activity of superoxide dismutase as briefly follows: 1ml of our mix had pyrogallol (0.48 mM), sodium phosphate buffer(50mM), enzymatic extract (20µl) and EDTA (0.1mM). At 420 nm, we monitored and recorded the changes in this mixture's absorbance at 25°C for 4 min and compared it to a blank buffer having all the mentioned elements except for the previously-prepared supernatant.

Catalase activity measurement

To measure catalase activity according to the method formerly mentioned (21), we prepared a mix (1ml) having hydrogen peroxide (10mM), sodium phosphate buffer (50mM; pH 7.0) and enzymatic extract (20µl). We monitored and recorded the reduced absorbance of this mixture for 2 min at 240 nm at 25°C against a blank having every ingredient mentioned above, but the tissue homogenized.

Western blotting

The samples were homogenized through sonication. Briefly and through a previously applied method (23), the buffer contained NaCl (150 mM), EDTA (0.03%), Tris-HCl (50 mM, pH 7.0), sodium deoxycholate (0.5%), SDS (0.1%) and one tablet of protease inhibitor cocktail (Roche). A total protein of 80µg was loaded for all samples, with the protein ladder (Thermo Scientific), into an 8% SDS-PAGE gel to be separated by size. The proteins were blotted onto PVDF membranes (Millipore). The blots, for an hour, remained in blocking reagent (GE Health Care, US) at room temperature before incubation with specific primary antibodies to VEGF (Abcam 3109, 1:1000), endostatin (Abcam 64569, 1:500) and β-actin (Cell signaling 4967S, 1:1000) separately. Horseradish peroxide-conjugated secondary anti-rabbit antibody against β-actin (1:1000, Dakocytomation) and HP-conjugated secondary anti-mouse antibody (1:10000, Abcam 6728) against VEGF and endostatin were used then to incubate the blots in room temperature for 1 h. The immune-reactive proteins were detectable using chemoluminescence agents (Amersham Bioscience) while being exposed to films.

Band images were quantified via Image J software and densitometry analysis was performed after having normalized them with β-actin.

Statistical analysis

Tumor volume was analyzed using factorial ANOVA and the enzymatic activity, as well as western blotting data were analyzed by one-way ANOVA. The post-hock test we used was LSD and statistical significance was reported as $P<0.05$. Data were presented as mean±SD.

Results

The effect of NS crude oil pretreatment on tumor volume

Analysis of the data on tumor volume showed that a 4-month pretreatment with NS crude oil at the dose of 4 ml/kg/day results in significantly reduced volumes ($550.46mm^3$±48.05; n=7) when compared to the control group ($875.46.5mm^3$±80.17).

NS1 tumor volumes ($1322.83mm^3$±128.8; n=7) were significantly larger than the control volumes (Figure 1). There were no significant differences in mean volume of tumor tissue between NS2 and control group.

The effect of NS crude oil pretreatment on antioxidant enzymes activity

Both SOD and CAT activities, at the dose of 4 ml/kg/day of NS crude oil, were significantly higher than those of the control group with $P<0.05$ (n=4) (Figure 2, 3).

In comparison with controls, no significant differences in SOD and CAT activities were observed in 1 and 2 ml/kg/day groups.

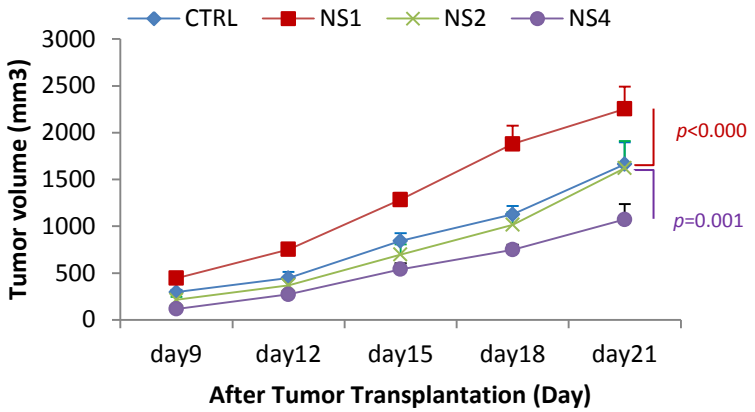

Fig. 1. Breast tumor volume changes in BALB/C mice after a 4-month pretreatment with *Nigella sativa* crude oil at doses of 1, 2 and 4 m/kg/day [n=7].

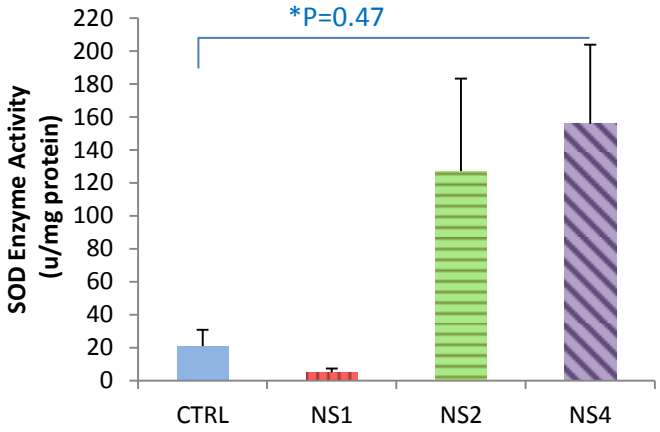

Fig. 2. Changes in SOD activity after 4 months of pretreatment with 1, 2 and 4 ml/kg/day of NS crude oil [n=4].

The effect of NS crude oil pretreatment on VEGF levels

VEGF was significantly reduced at the dose of 4 ml/kg/day (n=4) while it was significantly higher than that of control at the dose of 1 ml/kg/day (n=4). The differences in VEGF levels between NS2 and Control groups did not reach to statistical significance (Figure 4).

The effect of NS crude oil pretreatment on endostatin levels

Endostatin amount was significantly higher than the control group at the dose of 4 ml/kg/day (n=4) but at the dose of 1 ml/kg/day of NS crude oil there was a significant reduction in endostatin levels (n=4) (Figure 5). The differences in endostatin levels between 2 ml/kg/day and control groups did not reach to statistical significance.

Discussion

In the present study, we demonstrated that the mice pretreated with 4 ml/kg/day of NS oil had significantly smaller tumor volumes, higher SOD and CAT activity, reduced VEGF and increased endostatin amounts. We believe that Nigella sativa crude oil, hence, seems to inhibit breast tumor growth in part by improving the activity of antioxidant enzymes and in part by interfering with angiogenesis, although more studies are, yet, required to better illuminate its other mechanisms of involvement.

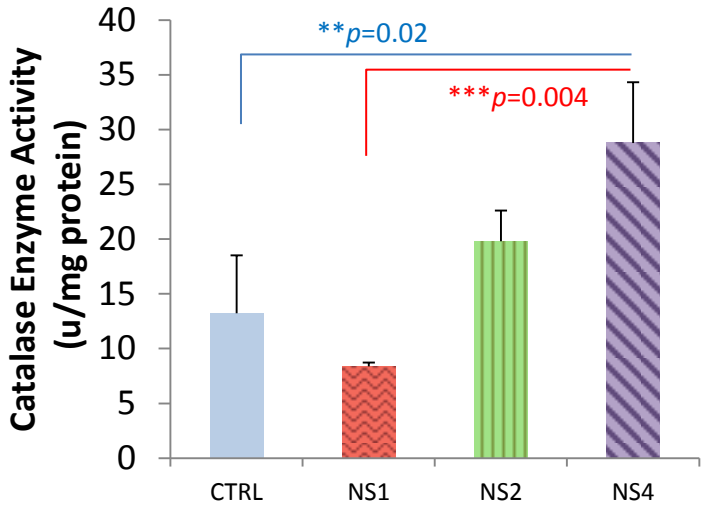

Fig. 3. Changes in CAT activity after pretreatment with 1, 2 and 4 ml/kg/day of NS crude oil for 4 months [n=4].

Carcinogenesis results from a variety of events leading to vast molecular shifts which change the fundamental features of normal cells (24). Amongst these alterations was an increase in ROS production by cancer cells. These cells are shown to suffer an imbalanced activity of antioxidant enzymes (25).

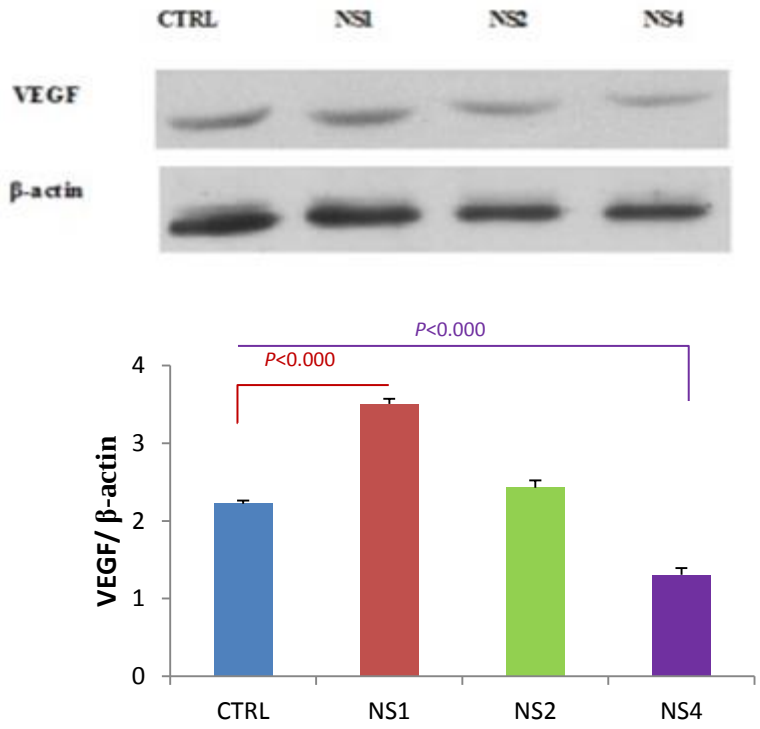

Fig. 4. The effect of pretreatment with 1, 2 and 4 ml/kg/day of NS crude oil on VEGF levels. Top: VEGF band from western blotting. Bottom: the VEGF/β-actin ratio in each group shown as mean±SD [n=4].

Since ROS damage cellular components and, eventually, end in more chromosomal instability and alteration in gene expression, antioxidant enzymes and nutrients are required and vital in protection against them (2.4).Superoxide dismutase family, in oxygen-exposed cells, played a key role by scavenging superoxide anions and deficiency in this family is reported to associate with various cancers (26). SOD-null mice failed to live beyond a few days after birth (27). Lack of the cytosolic kind of SOD may cause no obvious defects in mice, when infants and young, but the lifespan was reported to be shortened and larger rates of liver carcinoma appeared to happen in the later phase of their lives (28). Patients suffering from brain tumor were reported to have lower levels of SOD (29). One SOD-derived substance prohibited tumor progression by scavenging superoxide anions(30). SOD facilitated tumor suppression in cell lines of human prostate and breast cancer (31). TEMPOL, which mimics SOD, had a wide range of actions against oxidative stress (32).

Catalase, found in all organisms, was not fully understood. While CAT-lacking mice showed a normal phenotype (33), a deficiency in CAT seems to increase the risk of developing type II diabetes(34, 35). In cancer patients, as well its activity was lower than in normal people as much as 22% (36) and liver CAT was, later, indicated to be less active in patients with malignancies (37). This lowered activity is suggested to associate with higher intracellular amounts of hydrogen peroxide which leads to DNA damage and tumor progression (38). In breast carcinoma, high activity of CAT was linked to a lowered risk (39) while its reduced activity is suggested to support oxidative damage (40).

Cancerous mass fails to grow any bigger than 1-2 mm^3 unless its requirements are met through a private blood supply (41). Angiogenesis dependence of tumors was stunningly indicated in a 1971 experiment. When suspended in the fluid of rabbit eye's anterior chamber, tumors remained dormant.

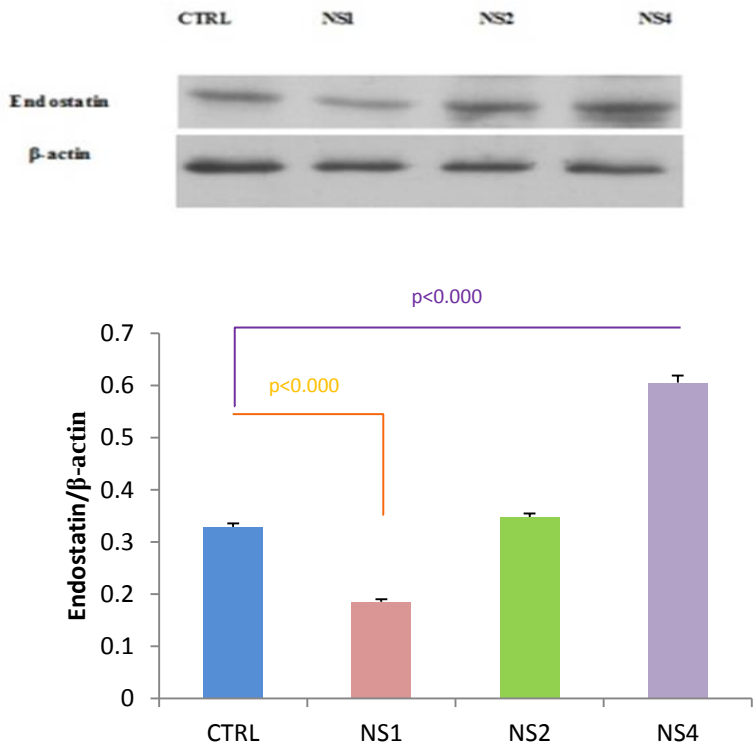

Fig. 5. The effect of pretreatment with 1, 2 and 4 ml/kg/day of NS crude oil for 4 months on endostatin levels. Top: endostatin bands from western blotting. Bottom: the endostatin/β-actin ratio in each group is shown as mean ±SD [n=4].

These tumors induced iris to neovascularize but the newly-formed vessels were out of reach of the floating tumors. As soon as a tumor mass was juxtaposed to the well-vascularized iris, it enlarged to sizes much bigger than its original volume in just 2 weeks (42). Angiogenesis is so cautiously controlled by a variety of factors, of which VEGF is believed to be the major pro-angiogenic one (43).

In 1996, VEGF deficient mice died about the 9^{th} day of gestation for hindered and impaired angiogenesis (44, 45). VEGF is also reported to be expressed in 60% of breast cancer patients (43). It stimulates endothelial cells to secrete proteases which mobilize many of the mitogens and antiapoptotic factors stored in extracellular matrix (46). Treatment of vascularized tumors in mice with antiVEGF antibodies dramatically reduced tumor vessels' diameter and permeability (47).

Of endogenous antiangiogenic factors, endostatin is considered the most potent one (48) and is reported to suppress a variety of genes involved in cell cycle, antiapoptosis and mitosis to inhibit endothelial cell proliferation and migration (49). Endostatin inhibited tumor growth and caused tumor regression in SCID mice with pancreatic carcinoma (50).

In an experiment, gene-therapied mouse liver tumor cells containing the murine endostatin gene were injected either subcutaneously or intraperitoneally and the resulting tumors were 80 times smaller in size compared to the control tumors. Besides, the survival rate of the injected mice was noticeably greater than that of the control group (51).The main mechanisms of endostatin include inhibition of VEGF receptor, down-regulation of VEGF, prohibition of VEGF-triggered endothelial adhesion loss, inhibition of matrix metalloproteinases, G1 arrest of endothelial cells and stabilization of vessels as well as suppression of HIF-1α expression (52, 53).

Different extracts and components obtained from *Nigella sativa* L. were shown, broadly, to affect antioxidant activities in different tissues (54) and are believed to be able to alter the redox state and to scavenge free radicals (16, 55). Enhanced enzymatic antioxidant activity and regulated lipid peroxidation was reported in myocardium (56). NS physiologically protected neurons by preventing the inhibition of SOD, CAT and glutathione peroxidase in CNS (57). In brain ischemia, NS extract and TQ could manage neurodegeneration by reducing ROS and improving the activity of antioxidant enzymes (58).

NS oil consumption prior to γ-irradiation elevated the antioxidant activity to normal levels and indicated radioprotectivity of this medicinal plant (59). In liver, NS and orally consumed TQ enhanced tissue antioxidant capacity, reduced the index of oxidative stress and increased the activity of glutathione transferase and quinone reductase (60, 61). Nephropathy was prevented in kidney by TQ and NS extracts through SOD, CAT and GPx increase (62, 63). Similar results were observed in stomach (64) and ileum (65).

ROS are reported to activate VEGFR2 and overexpress VEGF, which consequently leads to angiogenesis (66).

Hence, by its antioxidant effect, NS indirectly exerts antiangiogenic effects. However other reports indicate that TQ and NS extracts directly inhibit angiogenesis as well. TQ inactivates STAT-3 and down-regulates FAK expression, which cause VEGF reduction and metastasis inhibition (67). Akt and Erk pathways, 2 major signaling pathways toward angiogenesis, are also reported to be suppressed by the NS-derived component. In the same report, endothelial cells were shown to be more sensitive to TQ that tumor cells (18). In osteosarcoma, TQ affected NF-κB pathway and inhibited tumor angiogenesis and growth (68). In another experiment, zebrafish embryos were exposed to TQ for 48 hours. VEGF mRNA amounts were reported to be dose-dependently reduced (69).

Our experiment indicated that NS crude oil can exert antitumor effects at a high dose of 4 ml/kg/day as it improved antioxidant enzymes, enhanced endostatin production and inhibited VEGF. At a low dose of 1 ml/kg/day, it surprisingly acted in benefit of tumor growth, probably because of being so rich in fatty acids and dietary fat is shown to increase the risk of cancer development (70).

We suggest that, at low doses, the TQ within the oil fails to counteract the effects of fatty acids but the bioactive components of the oil can very well challenge the impact of fatty acids at higher doses.

There has been no experiment, so far, regarding NS or TQ effect an endostatin to the best of our knowledge and this study is the first to ever report such aspect of

NS usage. Our study indicated that pretreatment with a high dose of NS crude oil for 4 months led to significantly reduced tumor volumes perhaps through an increase in SOD and CAT activity as well as decreased VEGF levels and elevated endostatin amounts.

Conclusion

Based on our findings and other studies done on *Nigella sativa* L. and antioxidants, we suggest that, possibly, by improving enzymatic antioxidant activity and applying antiangiogenic effects, NS crude oil augments protective systems against cancer, yet more studies are required to elucidate the mechanisms further.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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