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In vivo attenuation of angiogenesis in hepatocellular carcinoma by *Nigella sativa*

Moustafa FATHY[1,2], Toshio NIKAIDO[1,*]

[1]Department of Regenerative Medicine, Graduate School of Medicine and Pharmaceutical Sciences, University of Toyama, Toyama, Japan

[2]Department of Biochemistry, Faculty of Pharmacy, Minia University, Minia, Egypt

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Background/aim: Angiogenesis is imperative in malignant tumor growth. Hepatocellular carcinoma is a typical hypervascular tumor that relies on angiogenesis. The aim of this study is to investigate the in vivo molecular mechanism underlying the antitumor properties of *Nigella sativa* ethanolic extract (NSEE) through its antiangiogenic effect against diethyl nitrosamine (DENA)-induced hepatocarcinogenesis.

Materials and methods: Male Wistar rats were divided into 4 groups: normal, NSEE, DENA, and NSEE-DENA groups. Final body weight, hepatosomatic indices, serum AFP, serum vascular endothelial growth factor (VEGF) levels, and liver tissue hepatocyte growth factor β (HGFβ) protein expression were estimated. Liver sections were stained for histological examination. AFP levels with the histological variations were chosen to confirm cancer development.

Results: DENA significantly increased liver weight, hepatosomatic indices, serum AFP and VEGF levels, and liver HGFβ protein expression and significantly decreased final body weight. These effects were significantly reversed by NSEE. Furthermore, the histopathological changes that appeared in rat livers due to DENA were reduced by NSEE without harmful effects when taken alone.

Conclusion: The results of the present investigation provide evidence that the in vivo antiangiogenic effect of NSEE through downregulation of serum VEGF and liver HGFβ protein could be implicated in its antitumor activity. Its consumption has health benefits that favor liver cancer management. These findings might prove useful and helpful for the progression of therapies for hepatocarcinogenesis treatment.

Key words: *Nigella sativa* ethanolic extract, angiogenesis, hepatocarcinogenesis, vascular endothelial growth factor, hepatocyte growth factor β

1. Introduction

Angiogenesis, which is the growing of new blood vessels from preexisting vessels, is imperative for malignant tumor growth. It permits cancer cells to enter circulation and then metastasize to other distant organs (1). Angiogenesis is now identified as the result of evolving interference between different tumor cells and their stroma (2). With tissue remodeling and stimulating angiogenesis, the proinflammatory response at the tumor stroma can be rerouted in a tumor-promoting direction (3).

Vascular endothelial growth factor (VEGF) represents one of the best validated angiogenesis signaling pathways (4), which was identified as a positive regulator. The VEGF family is a key target of antiangiogenic agents. VEGF, released from the extracellular matrix, primarily targets endothelial cells and is secreted by cancer cells to mediate tumor angiogenesis and metastasis (5,6).

Hepatocyte growth factor (HGF) is a proangiogenic factor that is produced by mesenchymal cells and earmarks endothelial and epithelial cells. It has been suggested that it plays a vital role in the modulation of many processes such as angiogenesis, mitogenesis, and tissue regeneration (7). It is often overexpressed in invasive cancer cells and activated endothelial cells. Its activation has been coupled to the upregulation of different angiogenic stimulators such as VEGF, IL-8, and cyclooxygenase-2. It has been declared that VEGF can interact synergistically with HGFβ to induce endothelial cell migration and proliferation (8).

Hepatocellular carcinoma (HCC), a diverse disease with various etiologies such as alcohol, hepatitis C, hepatitis B, aflatoxin B1, and other hepatotoxic substances, is the fifth most common tumor worldwide and, therefore, the third leading cause of death related to cancer (9–11). It is a typical hypervascular tumor that depends on angiogenesis to receive an adequate supply of nutrients

* Correspondence: tnikaido@med.u-toyama.ac.jp

and oxygen (12). Tumor proliferation requires increased synthesis by tumor-infiltrating inflammatory cells, hepatic stellate cells, or HCC cells of VEGF, HGFβ, and other factors that promote the expansion of the new vessels from nearby existing vessels (13). Recognition of a therapeutic potential for dominating the tumoral neovascularization has led to the search for safe and potent antiangiogenic factors (14,15).

On account of the high mortality and morbidity of cancers, problems encountered in their treatment, and the toxic effects of cancer chemotherapy, an endeavor has been made to investigate the anticancer effects of comparatively safe edible herbs (16). One such herb is *Nigella sativa*. Several pharmacological effects of *N. sativa* seeds, various extracts, and its oil and active constituents were identified, and few authors have depicted their anticancer effects (17–19). Chronic and acute toxicity studies have recently confirmed the safety of *N. sativa* oil, especially when given orally, and that of thymoquinone, its most abundant active component (17,20–23). Recently, the therapeutic and molecular potential of thymoquinone in lung, pancreatic, gastric, and colorectal cancers showed that it inhibits tumor angiogenesis and tumor growth (19,24–31), but no studies have investigated the molecular effect of *N. sativa* seed extracts on angiogenesis.

It has been shown that *N. sativa* ethanolic extract (NSEE) attenuated the inducible nitric oxide synthase (iNOS) pathway (32) and decreased serum nitric oxide, which can induce proangiogenic factors. The aim of the present work is to investigate the in vivo molecular mechanism underlying the antitumor properties of NSEE through its antiangiogenic effect against diethyl nitrosamine (DENA)-induced hepatocarcinogenesis.

2. Materials and methods

2.1. Animals

Male Wistar rats weighing 160 ± 5 g were used in this experiment. These rats were given an ordinary rodent diet and water ad libitum. They were housed in polyethylene cages in a humid room with a controlled 12-h light and 12-h dark cycle for 2 weeks before the experiment. They were classified into 4 groups (10 rats/group). Animal care and experimental protocols were performed in accordance with the guidelines established by the Research Ethics Committee of the Experimental Animal Center, University of Toyama.

2.2. Chemicals

All chemicals were of analytical grade and were obtained from local suppliers.

2.3. *N. Sativa* ethanolic extract

N. sativa seeds (purchased from Kahira Pharm. and Chem. Co., Cairo, Egypt) were washed, dried, and then powdered with an electric micronizer (Jiangyin Hongke Shredder Machinery Co., Ltd., Jiangsu, China). Powder was extracted with 80% ethanol three times and the solvent, under reduced pressure, was evaporated at 40 °C. The solid and oily phases, present in the produced two-phased extract, were recombined in proportion to their yield (typically 30% and 70%, respectively). The NSEE was protected from humidity and light and conserved at 4 °C.

2.4. Experimental design

The rats in Group 1 (normal control group) were given daily, by gavage, 0.5 mL of normal saline for 5 successive days and simultaneously received a single intraperitoneal (i.p.) injection of normal saline (2.5 mL/kg) on day 3. Animals in Group 2 (NSEE group) were given, orally by gavage, NSEE at 250 mg/kg (in normal saline of a total volume of 0.5 mL) daily for 5 successive days (33). Rats of Group 3 (DENA group) were given, for 5 successive days, the same dose of normal saline and a single dose of DENA (Sigma Chemical Company, St Louis, MO, USA) at 200 mg/2.5 mL per kilogram of body weight in saline, i.p. (34), on the third day. Animals in Group 4 (NSEE-DENA group) received the same dose of NSEE for 5 successive days and the same single dose of DENA (200 mg/kg, i.p.) on the third day. Fourteen weeks after the administration of DENA, animals were fasted overnight, then weighed and killed by exposure to an increasing concentration of CO_2. Blood samples were obtained by cardiac puncture, left to clot for 1 h at 37 °C, centrifuged at 10,000 rpm at 4 °C to separate serum samples, and then kept at –80 °C for additional use.

Liver samples were quickly separated and washed in ice-cold phosphate-buffered saline (PBS), and were then desiccated. Individual liver weight was accurately recorded and the hepatosomatic index for each rat was calculated (liver weight/body weight × 100). The liver was split into two parts. The first part, for histological examination, was kept in 10% buffered formalin. The second part was homogenized in 10 mM Tris, 0.25 mM sucrose, 1% protease inhibitor cocktail (Boehringer Mannheim, Indianapolis, IN, USA), and 1 mM ethylene diamine tetraacetic acid (EDTA) (pH 7.4). Liver homogenates were centrifuged at 4 °C for 15 min at 4000 rpm, the supernatant was gathered, and total protein was determined. Samples were stored as aliquots in Eppendorf tubes and kept at –80 °C for investigation.

2.5. Enzyme-linked immunosorbent assay determinations

High-sensitivity kits were used for determinations. Standards were analyzed in triplicate. Alpha-fetoprotein (AFP) serum levels were determined using an ELISA UBI AFP quantitative CM-101 kit (MagiWells, Ladera Ranch, CA, USA) according to the instructions of the manufacturer. VEGF serum levels were estimated using an ELISA kit (R & S Systems, Minneapolis, MN, USA) according to the manufacturer's instructions.

2.6. Western blot assay of HGFβ protein expression

Fifty micrograms of protein, present in samples, was separated using sodium dodecyl sulfate–polyacrylamide gel electrophoresis (12%–14% acrylamide) and then transferred onto polyvinylidene fluoride membranes (Millipore, Bedford, MA, USA). To block the nonspecific binding, we performed preincubation of the membranes at 37 °C in PBS containing nonfat dried milk (3%–5%) for 30 min. Membranes were then incubated overnight at 4 °C with polyclonal anti-HGFβ antibody (1:1000) (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Bound primary antibody was detected using a horseradish peroxidase-conjugated antigoat secondary antibody (Dako, Denmark) by chemiluminescence, using an enhanced chemiluminescence kit (ECL, GE Healthcare, Chicago, IL, USA), according to the manufacturer's instructions. Immunoreactive proteins were detected using an analyzer for luminescent images (LAS-4000, Fujifilm Co., Tokyo, Japan). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH, Santa Cruz Biotechnology) was used (1:1000) as a loading control to confirm equal loading and transfer to the membrane from the gel through the entire gel. Electrophoresis and electroblotting were accomplished using a discontinuous buffer system in a Bio-Rad Trans-Blot SD Cell apparatus (Bio-Rad Laboratories, Hercules, CA, USA). Resulting bands of HGFβ protein of the different groups were analyzed densitometrically using Image J software, relative to the normal control group. Data were equalized to GAPDH levels.

2.7. Histological examination

Liver pieces were fixed for histological examination with 24-h immersion in 10% buffered formalin. In a hierarchic series of ethyl alcohol, the blocks were dehydrated and then immersed into paraffin wax. Sections were stained using hematoxylin and eosin (H&E) stain.

2.8. Statistical analysis

Data were expressed as mean ± standard deviation (SD), n = 10 rats. Statistical significance was determined by one-way analysis of variance (ANOVA), using Excel and GraphPad Prism 5. $P < 0.05$ was presumed to be statistically significant.

3. Results

3.1. Body weight, liver weight, and hepatosomatic index

Regarding the general conditions of the rats during the experiment, we observed that final body weight differed significantly among groups, although the initial body weight was similar in all groups. The rats of the cancer group (DENA group) showed significant decrease in final body weight ($P < 0.001$) and significant increase in hepatosomatic indices ($P < 0.001$) when compared to normal group rats, whereas NSEE-treated animals showed no significant changes compared to normal group rats. In Group 4 (NSEE-DENA group), the rats' final body weight was significantly increased ($P < 0.001$) and hepatosomatic indices were significantly reduced ($P < 0.001$) when compared to DENA group rats (as shown in the Table).

3.2. Serum AFP level

As an indicator of cancer incidence, AFP serum levels were measured. DENA administration significantly ($P < 0.001$) elevated AFP serum levels compared to those of the normal group animals. This elevation was significantly reversed ($P < 0.001$) by NSEE in the animals of the NSEE-DENA group. Serum AFP levels in the rats of the NSEE group showed no significant changes when compared to those of the rats in the normal group, as shown in Figure 1.

3.3. Serum VEGF levels

The level of VEGF (a potent angiogenesis stimulator) in the serum was determined by ELISA assay according to the manufacturer's instructions. Figure 2 shows that VEGF levels in serum were significantly increased ($P < 0.001$) by DENA (in DENA group rats), whereas NSEE group rats showed no significant changes when compared to normal group rats. Additionally, VEGF levels were significantly reduced ($P < 0.001$) in the rats of the NSEE-DENA group when compared to DENA group rats.

Table. Body weight, liver weight, and hepatosomatic indices for all groups.

Groups	Initial body weight (gm)	Final body weight (gm)	Liver weight (gm)	Hepatosomatic index (%)
Group 1	160.2 ± 3.23	235.1 ± 3.51	5.7 ± 0.11	2.4 ± 0.05
Group 2	155.8 ± 2.78	245.9 ± 4.38^{ns}	5.2 ± 0.33^{ns}	2.1 ± 0.13^{ns}
Group 3	158.3 ± 3.81	$201.7 \pm 6.63^{*}$	$8.4 \pm 0.40^{*}$	$4.2 \pm 0.32^{*}$
Group 4	164.7 ± 3.72	$231.3 \pm 4.61^{\S}$	$6.2 \pm 0.28^{\S}$	$2.7 \pm 0.15^{\S}$

Data are expressed as mean ± SD (n = 10). Significant difference between groups is analyzed by one-way ANOVA test, where: *: $P < 0.001$ compared to Group 1 (control group), §: $P < 0.001$ compared to Group 3 (DENA group), ^{ns}: nonsignificant compared to Group 1 (control group). DENA: Diethyl nitrosamine.

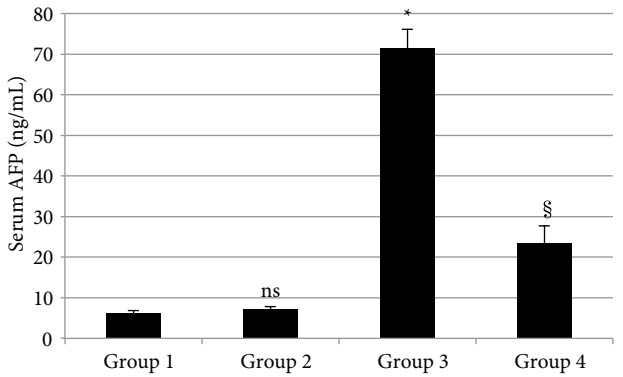

Figure 1. Serum AFP levels. Bars represent mean ± SD, n = 10. Significant difference between groups is analyzed by one-way ANOVA test, where: *: P < 0.001 compared to Group 1 (control group), §: P < 0.001 compared to Group 3 (DENA group), ns: nonsignificant compared to Group 1 (control group). AFP: Alpha-fetoprotein; DENA: diethyl nitrosamine.

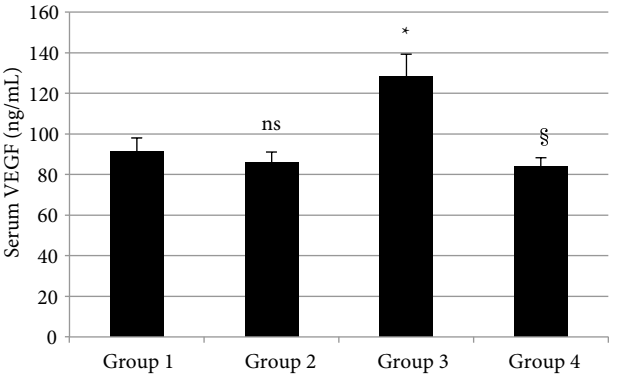

Figure 2. Serum VEGF levels. Bars represent mean ± SD, n = 10. Significant difference between groups is analyzed by one-way ANOVA test, where: *: P < 0.001 compared to Group 1 (control group), §: P < 0.001 compared to Group 3 (DENA group), ns: nonsignificant compared to Group 1 (control group). VEGF: Vascular endothelial growth factor; DENA: diethyl nitrosamine.

3.4. Liver HGFβ protein expression

The inhibitory effect of NSEE on the expression of liver HGFβ protein (another potent angiogenesis stimulator) was examined by western blot. This demonstrated a significant increase (P < 0.001) in the expression of DENA group rats, whereas NSEE group rats showed nonsignificant changes when compared to normal group rats. Furthermore, NSEE significantly reduced (P < 0.001) HGFβ protein expression in NSEE-DENA group rats when compared to those treated with DENA, as shown in Figure 3.

3.5. Histological examination of liver tissues

Histological examination of liver tissue, together with serum AFP level, was used as an indication of cancer incidence. Liver sections of normal control animals revealed normal cells with small, uniform nuclei and granulated cytoplasm, as well as normal architecture and normal sinusoids (Figure 4A), whereas no apparent changes were found in the livers of NSEE animals (Figure 4B). DENA-treated rats' liver sections showed loss of normal architecture with irregular or oval-shaped neoplastic hepatocytes. The nuclei were found to be polymorphic and hyperchromatic; furthermore, cancerous tissue growing outside the dysplastic nodules, with formation of neovessels and enlargement of sinusoids, was observed (Figure 4C). Liver sections of NSEE-DENA group animals showed these changes still present as neoplastically transformed cells, but generally less than in DENA group rats, indicating certain improvement (Figure 4D).

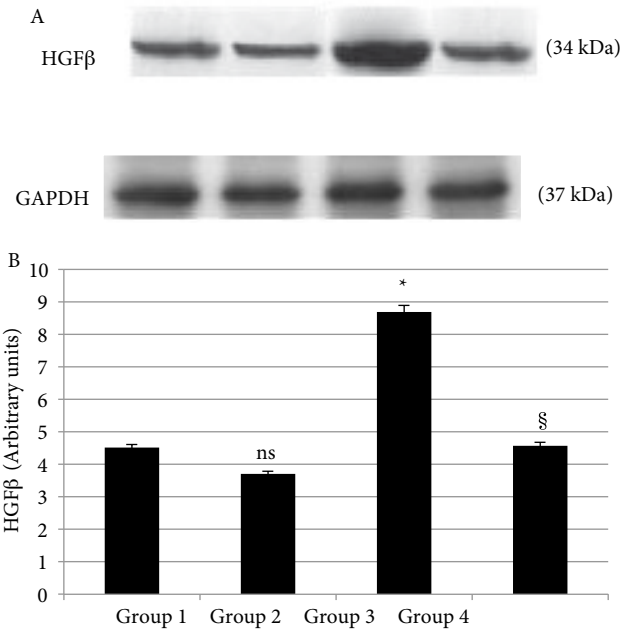

Figure 3. Liver HGFβ protein expression. A) Representative immunoprecipitation blots of the expression of liver HGFβ protein for the different groups. B) Bars represent mean ± SD, n = 10. Significant difference between groups is analyzed by one-way ANOVA test, where: *: P < 0.001 compared to Group 1 (control group), §: P < 0.001 compared to Group 3 (DENA group), ns: nonsignificant compared to Group 1 (control group). HGFβ: Hepatocyte growth factor β; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; DENA: diethyl nitrosamine.

4. Discussion

Angiogenesis is a ubiquitous process that is needed for tumor growth. Angiogenesis and proangiogenic factors are logical objects for pharmacological manipulation, proving the vital role they play in cancer formation, growth, and proliferation, using a number of distinct mechanisms (1,35). It is thought that tumor stroma endothelial cells

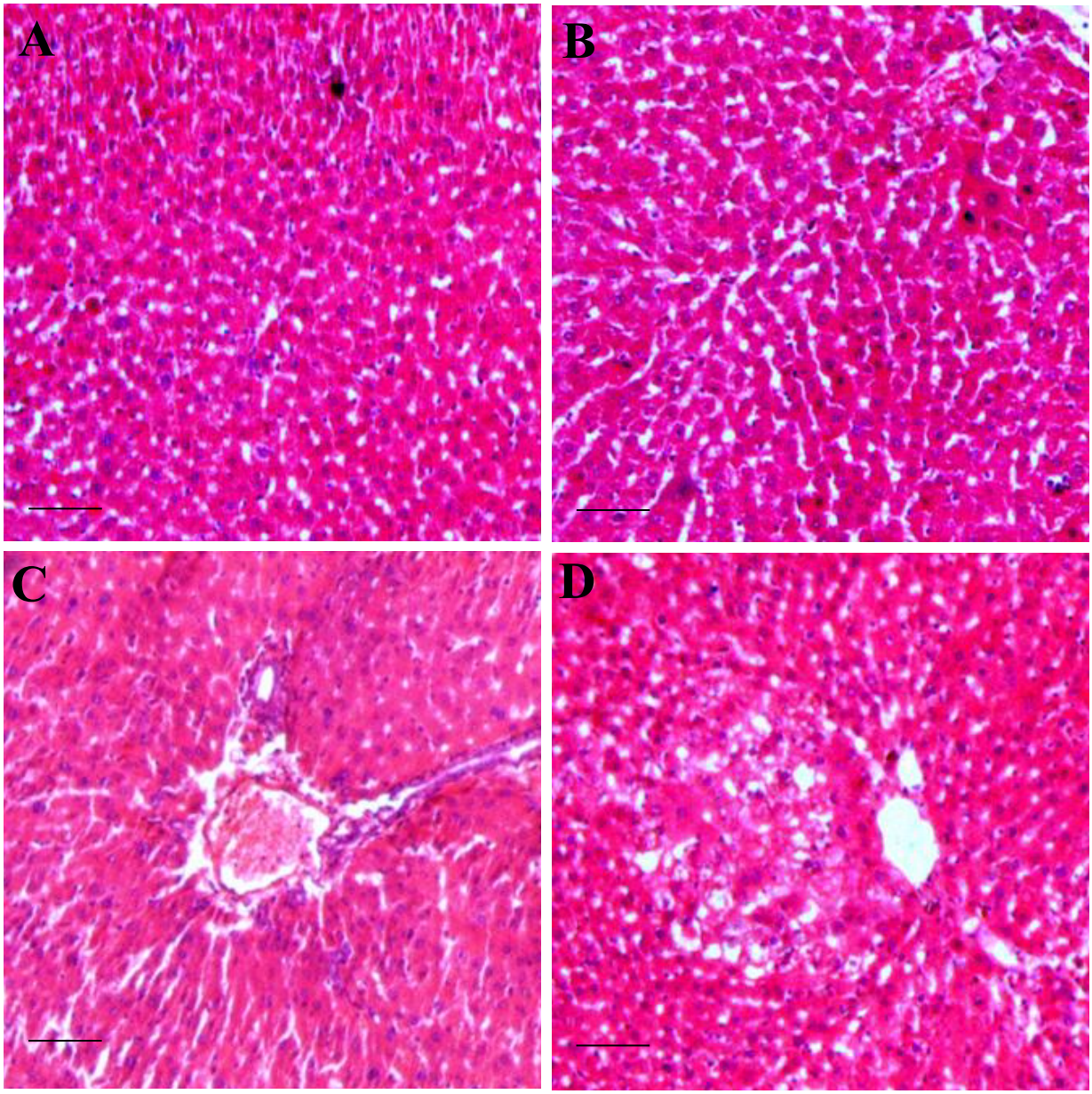

Figure 4. Histological examination for animal livers of all groups. H&E-stained sections of livers of the control group rats (A) show normal architecture and granulated cytoplasm with uniform nuclei. Similarly, no obvious changes were observed in sections of the livers of NSEE group rats (B). In DENA group rats (C), neoplastic cells were irregular with hyperchromatic nuclei, including cancerous tissue growing outside the dysplastic nodules, with formation of neovessels and enlargement of sinusoids. These changes were reduced in the livers of NSEE-DENA group rats (D) with few neoplastically transformed cells. Scale bars represent 100 µm. NSEE: *Nigella sativa* ethanolic extract; DENA: diethyl nitrosamine.

are genetically stable and will not become resistant to drugs in response to antivascular therapy. However, it has been shown that these endothelial cells express neoplastic markers and are aneuploid (36). Furthermore, angiogenic processes are indirectly involved in tumor metastasis and invasion through matrix-degrading proteinases, secreted by vascular endothelial cells, and the tumor cells' ability to travel to other sites via the vascular network (37,38). Signals from different stromal cells are shown to modify tumor growth and their reactivity to therapies in different models, raising the possibility that drugs intrusive upon these pathways may offer further therapeutic strategies (15,39). Future research regarding the role of critical mediators in altering tumor microenvironment concerned in tumor angiogenesis may result in novel therapeutic applications.

HCC tumors are generally hypervascularized (40), suggesting that they might be particularly prone to angiogenesis inhibition. Several endogenous proangiogenic mediators are expressed in HCC (41), and the evidence indicates that they play a role in the HCC pathological process. For example, in advancing HCC stages, VEGF levels increase in serum and become highest in metastatic disease patients (42,43).

This study investigated the antiangiogenic effect of NSEE in vivo and its participation as an antitumor agent against hepatocarcinogenesis induced by DENA. Our data revealed that NSEE administration did not significantly affect all the studied biochemical parameters or alter the liver histological examination, suggesting the safety of this extract. This is in accordance with other researchers (44,45), who found that thymoquinone, p-cymene, and alpha-pinene (*N. sativa* constituents) are safe compounds, especially when given orally, and did not alter the studied biochemical parameters in animals treated with them, suggesting the great safety of this herb.

In this study, liver carcinogenesis was chemically induced by DENA, a well-known model for hepatic cancer, in which AFP level is highly increased in serum and hepatocarcinogenesis can be histopathologically identified (34,46,47). Data indicated that animals treated with DENA alone showed elevated serum AFP and definite histological changes that reflected severe hepatocellular damage. AFP is always used to follow and assess HCC prognostically and diagnostically (48).

Our observations showed that the hepatosomatic index was higher in DENA-treated rats compared to normal group rats. This elevation was significantly attenuated by the NSEE treatment. Furthermore, NSEE significantly downregulated the elevation of serum AFP levels in rats treated with NSEE and DENA. These antineoplastic effects were confirmed in different models by other researchers (49–51).

The extracts of *N. sativa* and thymoquinone have been widely examined and are reported to show anticancer activity. It has been reported that its aqueous extract significantly promotes the cytotoxic effect of NK against YAC-1 cancer cells (52). Furthermore, the ethanolic extract has cytotoxic activity on human hepatoma (HepG2) cell lines (53). Moreover, it has been reported that NSEE shows an in vivo anticancer effect via the attenuation of the iNOS pathway, which is activated in hepatocarcinogenesis, and the suppression of the inflammatory response mediated by tumor necrosis factor-α and interleukin-6 (32). Recently, studies have shown that thymoquinone targets breast cancer in vivo and in vitro with apoptosis induction and angiogenesis inhibition (54,55). However, nothing has been reported about the in vivo antiangiogenic effect of NSEE.

VEGF is a potent angiogenesis stimulator induced by many cancers. It has been reported that circulating VEGF is accompanied by metastasis (56). Furthermore, serum VEGF is closely related to HCC tissue VEGF expression levels, which supports the utilization of serum VEGF to indirectly assess tissue VEGF expression (57,58). It has been suggested that additional proangiogenic mediators contribute to the stimulation of angiogenesis in HCC. One of these mediators, HGFβ, has been suggested to play a vital role in the regulation of angiogenesis, migration, and invasion for various types of cells (59). Its activation was linked to the upregulation of other angiogenic stimulators such as VEGF2 (40), and it can interact synergistically with VEGF to induce cell proliferation and migration. Moreover, HGFβ can induce the expression of VEGF in vivo and in different cell types (60,61). The increase in serum VEGF levels and liver HGFβ protein expression observed in this study and its prevention by NSEE confirm the role of VEGF and HGFβ in the development of tumoral neovascularization in this HCC model, additionally confirming the antiangiogenic role of NSEE.

In this study, biochemical investigations were correlated with the histological examination of liver rats from the different groups. Neoplastic and cancerous findings in the rats of the DENA group were reduced by the NSEE treatment.

This work investigated in vivo the antitumor activity and the possible mechanism of action of *N. sativa* through its antiangiogenic effect. NSEE (containing natural constituents such as thymoquinone, alpha-hederin, quercetin, and kaempferol) downregulated the serum levels of VEGF and liver HGFβ protein expression in rats treated with DENA, as well as their AFP serum levels, suggesting that *N. sativa* exerts its antitumor effect through suppression of the vascularization of the tumor. This leads to decreasing metastasis by decreasing the circulating cancer cells. Different studies reported that inhibition of angiogenesis may result in antineoplastic effects (62). For instance, a new derivative of benzothiazole-2-thiol inhibited breast cancer growth and pulmonary metastasis in vivo through angiogenesis inhibition (14). Additionally, nintedanib, which is a new angiokinase inhibitor that blocks the vascular endothelial growth factor receptors-mediated proangiogenic pathways, is effective in the treatment of advanced nonsmall-cell lung cancer patients (15). Furthermore, apatinib, a highly selective vascular endothelial growth factor receptor-2 inhibitor, inhibited gastric cancer cell propagation and retarded xenograft tumor growth (39).

The use of *N. sativa* introduces supplementary treatment in cancer management, instead of the cytotoxicity and cytodestruction induced by chemotherapeutic

agents. Consequently, it can be concluded that *N. sativa* consumption has health benefits for liver cancer management. Its consumption may be suggested as a dietary supplement throughout the management of liver cancer and after the stoppage of treatment protocols to avoid recurrence.

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