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Anti-tumor properties of BLACK SEED (*Nigella sativa* L.) extracts

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Abstract

The objective of the present study was to evaluate the *in vitro* and *in vivo* anti-cancer effect of *Nigella sativa* L. seed extracts. The essential oil (IC₅₀ = 0.6%, v/v) and ethyl acetate (IC₅₀ = 0.75%) extracts were more cytotoxic against the P815 cell line than the butanol extract (IC₅₀ = 2%). Similar results were obtained with the Vero cell line. Although all extracts had a comparable cytotoxic effect against the ICO1 cell line, with IC₅₀ values ranging from 0.2 to 0.26% (v/v), tests on the BSR cell line revealed a high cytotoxic effect of the ethyl acetate extract (IC₅₀ = 0.2%) compared to the essential oil (IC₅₀ = 1.2%). These data show that the cytotoxicity of each extract

depends on the tumor cell type. *In vivo*, using the DBA2/P815 (H₂^d) mouse model, our results clearly showed that the injection of the essential oil into the tumor site significantly inhibited solid tumor development. Indeed, on the 30th day of treatment, the tumor volume of the control animals was $2.5 \pm 0.6 \text{ cm}^3$, whereas the tumor volumes of the essential oil-treated animals were 0.22 ± 0.1 and $0.16 \pm 0.1 \text{ cm}^3$ when the animals were injected with 30 μL (28.5 mg)/mouse and 50 μL (47.5 mg)/mouse per 48 h (six times), respectively. Interestingly, the administration of the essential oil into the tumor site inhibited the incidence of liver metastasis development and improved mouse survival.

Key words: *Nigella sativa* L., Extracts, Cytotoxicity, Anti-tumor activity

Introduction

Plants are invaluable sources of new drugs. There is an enormous historical legacy regarding the use of plant preparations in folk medicine (1). Scientific studies on plants used in ethnomedicine have led to the discovery of many valuable drugs such as taxol, navelbine, camptothecin, vincristine, etc.

Nigella sativa L. (Ranunculaceae family) seeds, commonly known as blackseed or black cumin, have been employed for thousands of years as a spice and food preservative, as well as a protective and curative remedy for numerous disorders (2,3). Traditionally, there is a common Islamic belief that blackseed is a panacea for all ailments, but cannot prevent aging or death. Blackseed is also identified as the curative black cumin in the Holy Bible and is described as *Melanthion* by Hippocrates and Dioscorides and as *Gith* by Pliny (4). Many studies have been conducted, particularly during the last two decades, on the effect of *N. sativa* L. seed extracts on various body systems *in vitro* or *in vivo*. The pharmacological investigations of the seed extracts reveal a broad spectrum of activities including immunopotentiality (5) and antihistaminic (6), antidiabetic (7), anti-hypertensive (8), anti-inflammatory (9), and antimicrobial activities (10). Many of these activities have been attributed to the quinone constituents of the seed (11,12).

Furthermore, blackseed preparations may have a cancer chemopreventive potential and may reduce the toxicity of standard antineoplastic drugs (13). In fact, topical application of a blackseed extract inhibited the two-stage initiation-promotion of skin carcinogenesis in mice by dimethylbenz[a]anthracene (croton oil) (13). In addition, others have reported an antitumor activity of some crude and purified components of *N. sativa*. In fact, Salomi et al. (14) have shown that a crude methanol extract of blackseed exhibited a strong cytotoxic action on Erlich ascites carcinoma, Dalton's ascites lymphoma and sarcoma 180 cells, with minimal cytotoxicity to normal lymphocytes. These investigators have also described the cytotoxic property of extracts of the seeds *in vivo*, as shown by inhibition of the growth of Erlich ascites carcinoma in mice receiving 2 mg of the extract per mouse per day for 10 days (14). Furthermore, a new triterpene saponin (α -hederin) recently isolated from blackseed showed significant dose-dependent tumor inhibition when given intraperitoneally for 7 days at 5 and 10 mg/kg to mice with formed tumors (15). Similarly, Worthen and colleagues (4) have tested *in vitro* a crude gum, a fixed oil and two purified components of the seed (thymoquinone (TQ) and dithymoquinone (DTQ)) for cytotoxicity to some parental and multidrug-resistant human tumor cell lines. They found that the gum and the oil (up to 1%, w/v) were devoid of cytotoxicity, while both TQ and DTQ were cytotoxic to all cell lines including parental and multidrug-resistant phenotype-expressing cells (14). In addition, other investigators have recently reported that TQ triggers apoptotic cell death in human colorectal cancer cells, which was correlated with G1 phase arrest of the cell cycle (16). Apoptosis induction by TQ was associated with an increase in mRNA expression of the p53 target gene, p21^{WAF1}, and a significant inhibition of the anti-apoptotic Bcl-2 protein (16).

In the present study, we report comparative data regarding the *in vitro* cytotoxic effect of the essential oil and the ethyl acetate and butanol extracts of *N. sativa* seeds on a panel of tumor cell lines. We show that the essential oil and ethyl acetate fractions possess a strong cytotoxic effect against tumor cells. Furthermore, we present conclusive evidence that the *in vivo* effect of the essential oil when injected into the tumor site induced the regression of solid tumor volume and the inhibition of metastasis development and delayed mortality.

Material and Methods

Chemicals, plant materials, cell lines, and culture

N. sativa L. seeds were purchased on the local market and authenticated by Pr. A. Ouyahia (Scientific Institute, Rabat, Morocco) and stored as a voucher specimen in the same institute. Adriamycin, dimethylsulfoxide (DMSO), crystal violet, butanol, ethyl acetate, and hexane were purchased from Sigma Chemical Co., Saint Quentin, France. The murine mastocytoma cell line (P815), the kidney carcinoma cell lines of monkeys (Vero) and hamsters (BSR) as well as the sheep heart carcinoma (ICO1) were kindly provided by Professor G. Lemaire, Institute of Biochemistry, University of Paris XI, France. These cell lines are routinely cultured in complete medium (Dulbecco's modified Eagle's medium supplemented with 5% fetal calf serum (Gibco BRL, Cergy Pontoise, France), 1% penicillin-streptomycin-neomycin, and 0.2% sodium bicarbonate (Sigma)).

Preparation of blackseed extracts

Extraction of the essential oil. Steam distillation of the crushed blackseed removes the volatile oil content as an aqueous fraction from which the essential oil is isolated by simple extraction with diethyl ether followed by evaporation of the organic layer under reduced pressure. The essential oil content of *N. sativa* L. was determined to be 0.4-0.5% (w/w). This extract was analyzed by gas chromatography-mass spectrometry and revealed the presence of the following major components (62.17% TQ, 16.84% carvacrol, 8.29% 2-methyl-5-Prop-2-enyldihydroquinone, 6.99% dihydrothymoquinone, 2.07% terpin-4-en-1-ol, 3.11% monoterpenes).

Preparation of ethyl-acetate and butanol extracts. One kilogram *N. sativa* L. seeds was washed, dried, and crushed to a powder with an electric micronizer. The powder was exhaustively extracted with 90% ethanol at room temperature and the extract was centrifuged at 10,000 g for 15 min to remove residual solid debris. The clear supernatant was then concentrated under reduced pressure.

The concentrated extract was partitioned between 10% methanol-water and n-hexane. After removing the n-hexane fraction, the aqueous layer was partitioned again with ethyl acetate, followed by n-butanol. The yields of the butanol and ethyl acetate extracts were 2.1 and 0.23% (w/w), respectively. Analysis by nuclear magnetic resonance showed that the butanol extract contained two saponosides derived from α -amyrin. However, the ethyl acetate extract did not contain polyphenols and did contain monoxideterpenes.

Methyl thiazole tetrazolium cell viability test

The methyl thiazole tetrazolium (MTT) test was used to determine the effect of the essential oil and of the butanol and ethyl acetate extracts, all solubilized in DMSO, on the growth of non-adherent cells. The test was performed as described (17). Briefly, P815 cells were washed twice with PBS and incubated at a density of 7.5×10^4 cells/mL in flat-bottomed 96-well microtiter plates (Bioster, Bastia di Rovolon, Italy) in 100 μ L of complete medium. Several dilutions starting from a concentration of 2% (v/v) of these extracts in 100 μ L of complete medium were then added. After

48-h incubation in a humidified atmosphere at 37°C, 5% CO₂, 100 µL of medium was carefully removed from each well and replaced with 20 µL MTT solution (5 mg/mL PBS). After 4-h incubation under the same conditions, the cleavage of MTT to formazan by metabolically active cells was quantified by scanning the plates at 540 and 630 nm using a multiscan apparatus. Three independent sets of experiments performed in duplicate were evaluated. Controls with DMSO alone were performed for each concentration. The growth inhibition rate was calculated as percentage of parallel negative controls. Adriamycin was used as positive control.

Crystal violet test

This test was performed as previously described (18,19). Briefly, BSR, ICO1 and Vero cells were trypsinized and 7.5×10^4 cells per mL were incubated in flat-bottomed 96-well microtiter plates in 100 µL of complete medium. Several dilutions starting from the concentration of 2% (v/v) of these extracts were then added in a final volume of 100 µL of complete medium. All extracts were solubilized in DMSO. Controls with DMSO alone were performed in parallel for each concentration. After 48-h incubation at 37°C and 5% CO₂, the medium was removed and replaced with 100 µL 0.5% crystal violet solution. After 10-min incubation at room temperature, the plates were washed and viable crystal violet-stained cells were lysed with 1% SDS solution. Absorbance at 540 nm was then measured in each well. Adriamycin was used as positive control. The results of this test were also confirmed by the MTT test both qualitatively and quantitatively.

Using this colorimetric procedure, extract-mediated cell lysis and the cytotoxic effect of adriamycin (used as a positive control) were measured and compared to the viability of untreated cells according to the following calculation: % cell killing = $100 \times (1 - A/A_0)$, where A_0 and A are the absorbances obtained from negative control cells and extract (or adriamycin)-treated cells, respectively.

The test compounds were compared using the IC₅₀ value, i.e., the concentration of an individual compound leading to 50% death.

Tumor induction in mice and treatment

DBA2 mice (H₂^d haplotype), purchased from the animal breeding center of Orleans (France), were maintained under specific pathogen-free conditions on a 12-h light-dark cycle. Mice were provided with sterile food and water *ad libitum* and were used at 6-8 weeks of age with an average weight of 20-25 g. All animal experiments were performed according to national regulations which are comparable to the accepted principles for laboratory animal use and care of the European Community guidelines.

A primitive tumor isograft was induced in mice after subcutaneous injection of 5×10^6 P815 cells (H₂^d). When the primitive tumor reached a palpable volume, the donor mouse was sacrificed, and the tumor was mechanically dissociated. Mice were then subcutaneously grafted with tumor fragments of similar size. Mouse treatment was started when a palpable grafted tumor had reached a volume of approximately 100 mm³. Three lots of 6 mice each were assigned to each treatment group. These mice were treated with PBS (group A) as negative control, or essential oil of *N. sativa* L. (30 µL (28.5 mg) per mouse for group B and 50 µL (47.5 mg) per mouse for group C). These quantities were injected into DBA2-tumor-bearing mice into the tumor itself at days 0, 2, 4, 6, 8, and 10 (one injection every 48 h, six times) (20). Thereafter, the tumor volume of each mouse was carefully measured with a calliper every 2 days for a period of 30 days according to the following calculation: tumor volume (cm³) = $D \times d^2/2$, where "D" is the tumor length and "d" the tumor width (21). The weight of each mouse was also determined every 2 days for the same period of time.

Metastasis evaluation

To evaluate the effect of the essential oil of *N. sativa* L. on metastasis development, tumors were induced in DBA2 mice and treated using the protocol described above. After the 30th day, all surviving mice were sacrificed by cervical dislocation and submitted to autopsy. Autopsy was also performed on the animals that died before the 30th day. The incidence of liver metastasis was evaluated by counting the number of nodules in the liver of each mouse in all groups.

Statistical analysis

Data are reported as means \pm SD. Statistical differences between experimental groups were assessed by analysis of variance (ANOVA), with the level of significance set at $P < 0.05$.

Results

In vitro cytotoxic effect of essential oil and the ethyl acetate and butanol extracts on different cell lines

The effect of essential oil and of the ethyl acetate and butanol extracts on different tumor cell lines is summarized in [Table 1](#). The table shows that the *N. sativa* L. extracts have a differential cytotoxic activity on the various cell lines tested. The cytotoxic effect of the butanol extract was low on most of the cell lines tested, while the effect of the ethyl acetate and the essential oil extracts were variable, as indicated by the IC_{50} values. The essential oil and ethyl acetate extract were more cytotoxic than the butanol extract against the P815 cell line (IC_{50} (%v/v) = 0.6, 0.75, and 2%, respectively). This classification of extracts in terms of cytotoxicity was also observed when the Vero cell line was used as target (IC_{50} values: 0.2, 0.22, and 2% for the essential oil, ethyl acetate and butanol extracts, respectively). Interestingly, against the BSR cell line, the ethyl acetate extract was more cytotoxic than the essential oil (IC_{50} : 0.2 and 1.2%, respectively), while the butanol extract showed a low cytotoxic activity. However, using the ICO1 cell line as target, the butanol extract exhibited a greater cytotoxic effect (IC_{50} = 0.26%) than that observed against the P815 (IC_{50} = 2%) and Vero (IC_{50} = 2%) cell lines. Taken together, these results show that the cytotoxicity of each extract depends on the tumor cell type tested.

Effect of the essential oil on solid tumor in mice

Since the essential oil was the most cytotoxic extract against the P815 cell line, it was used to validate our *in vitro* observations in the DBA2/P815 (H_2^d) mouse model. [Figure 1](#) shows that at day zero of treatment the tumor volume was about 0.35 ± 0.06 cm³ for all groups tested ($P > 0.05$). The *in situ* administration of the essential oil significantly reduced solid tumor development in the treated groups compared to the PBS-treated control ($P < 0.05$). On the 30th day, the tumor volume of the control animals was 2.5 cm³ (group A), whereas that of the treated animals were 0.22 ± 0.1 and 0.16 ± 0.1 cm³ for groups B and C, respectively. This difference was found to be significant between the control and treated groups ($P < 0.05$). However, no significant difference was observed between the treated groups (B and C) on day 30. It is noteworthy that the tumor volume decreased between day "0" and day 12 for the treated groups (B and C). This difference was significant ($P < 0.05$) at day 12, suggesting a cytotoxic activity of the extract. These tumor volumes remained constant between the 12th and 30th days, suggesting an inhibition of cell growth during this period.

Effect of essential oil on metastasis development and mouse survival

To determine whether the observed anti-tumor activity of the *N. sativa* essential oil was correlated with the anti-metastatic effect, tumor-bearing mice were sacrificed and autopsied for the evaluation of the incidence of liver metastasis. A comparative

study between the three groups (Table 2) showed that on the 30th day of treatment, the number of nodules in the control animals (group A) was 14 ± 3 , whereas in the treated animals (groups B and C) it was 2 ± 1 and 0, respectively. These results demonstrate either that the essential oil has an anti-metastatic activity in mice or that it inhibits or delays metastasis by rapid reduction of primary tumor volume at the site of induction.

In order to establish the relationship between the *in vivo* cytotoxic activity of the essential oil and mouse survival, the death rate was determined for each group of tumor-bearing mice (Table 2). No mortality was observed in group C on the 30th day of treatment. However, on the same day, 83 and 16.6% mortality rates were noted for groups A and B, respectively, indicating that the essential oil can reduce tumor-bearing mice mortality in a dose-dependent manner.

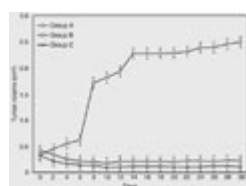


Figure 1. Effect of *Nigella sativa* essential oil on tumor volume evolution in mice. Tumors were induced in DBA2 mice and treated using the protocol described in Material and Methods. Three groups of 6 mice each including controls were used. These groups were treated with PBS as negative control (group A) or essential oil of *N. sativa* L. (30 μ L (28.5 mg) or 50 μ L (47.5 mg) per mouse for groups B and C, respectively). After serial injections of essential oil into the tumor itself at days 0, 2, 4, 6, 8, and 10, tumor volume was carefully measured with a calliper in each mouse every two days as described in Material and Methods. Data are reported as the mean \pm SD of three independent experiments. $P \leq 0.05$ compared to group A (negative control) for all times from 6 to 30 days (ANOVA).

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TABLE				

Table 1. *In vitro* cytotoxic effect of *Nigella sativa* extracts on tumor cells.

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TABLE				

Table 2. Effect of essential oil on survival and metastasis development in tumor-bearing mice.

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Discussion

The present study was undertaken to provide comparative data on the *in vitro* cytotoxic activity of different extracts of *N. sativa* L. seeds against various tumor cell lines. The ethyl acetate and essential oil fractions exhibited a significant cytotoxic effect against the majority of tumor cell lines used, while the butanol extract showed only little cytotoxic activity. Interestingly, we report here that the differential cytotoxic effect of these extracts was related not only to their chemical composition but also to the nature of the tumor cell lines. In fact, although all the extracts studied showed a comparable cytotoxic effect against the ICO1 cell line, the effect of the essential oil was higher against the P815 cell line as compared to that of the ethyl acetate, which was more cytotoxic against the BSR cell line than the essential oil. Our results agree

with previous research in which the ethyl acetate fraction was found to exhibit a strong growth inhibitory effect on all malignant cells tested (22). The differential cytotoxicity of these extracts against the same cell line is related to the differential composition of such extracts. These observations agree with previous study (4) in which the crude gum, the fixed oil and two purified components of the essential oil (TQ and DTQ) were tested for *in vitro* cytotoxicity to some parental and multidrug-resistant human tumor cell lines. It was found that the gum and fixed oil were devoid of a cytotoxic effect. However, TQ and DTQ, the major constituents of the essential oil, were both cytotoxic to all cell lines and their action was not related to the generation of free radicals (4). Furthermore, the presence of terpenes as major components in the ethyl acetate extract may explain its high cytotoxic activity. In fact, the anti-cancer potential of these products has been recently reported (23-26). The molecular mechanisms of these purified components have not been well characterized. However, it has been recently reported that TQ triggers apoptotic cell death in human colorectal cancer cells which was correlated with G1 phase arrest of the cell cycle (16). Apoptosis induction by TQ was associated with an increase in mRNA expression of the p53 target gene, p21^{WAF1}, and a significant inhibition of anti-apoptotic Bcl-2 protein (16).

On the other hand, to evaluate the side effects of these extracts, we tested their cytotoxicity toward normal human peripheral blood mononuclear cells. Interestingly, only minimal cytotoxicity was observed for all extracts (data not shown).

Since the essential oil was the extract with the highest *in vitro* cytotoxic activity against P815 cell line compared to the ethyl acetate and butanol extracts, it was used to validate this observation in the DBA2/P815 (H₂^d) mouse model. When we injected the essential oil into the tumor site of tumor-bearing mice a very clear regression curve was observed for tumor volume in relation to time in the treated groups, while the control group exhibited an increasing tumor volume ([Figure 1](#)). These results are consistent with previous findings that demonstrated the *in vivo* cytotoxic property of the seeds by inhibiting the growth of Erlich ascites carcinoma in mice receiving 2 mg of the methanol extract per mouse per day for 10 days (14). They also agree with those of Badary's group (27), which reported an inhibitory effect of the main component of the essential oil (TQ) against 20-methylchloranthrene-induced fibrosarcoma and against benzo (a) pyrene-induced forestomach carcinogenesis (28).

In the present study, the regression of tumor volume was more pronounced in the treated mice (up to 95% for group C; [Figure 1](#)), suggesting that other components present in the essential oil contribute to the observed *in vivo* regression of established malignancy. Furthermore, the incidence of mortality in tumor-bearing mice was delayed by essential oil treatment, with no mortality for group C and only 16.6 ± 2% mortality for group B as compared to 83 ± 4% for group A (PBS-treated group) at day 30 of the study ([Table 2](#)). It is noteworthy that the body weight of treated mice remained stable in group C (data not shown).

Our results indicate for the first time that intra-tumor treatment of tumor-bearing mice with essential oil may have led to the inhibition of metastasis development ([Table 2](#)). These results demonstrate either that the essential oil has an anti-metastatic activity in mice or that it inhibits or delays metastasis by rapid reduction of primary tumor volume at the site of induction. The active principles and their metastasis-inhibiting mechanism still need to be identified.

The present study demonstrates that the cytotoxic activity of blackseed extracts is a complex phenomenon depending not only on the nature of the extract and its components, but also on the tumor cell type. We report here a very high efficacy of the essential oil in reducing tumor volume, inhibiting metastasis development and delaying mortality of P815 tumor-bearing mice. Additional research is now necessary in order to determine the agents responsible for these *in vitro* and *in vivo* anti-cancer activities as well as the molecular mechanisms involved in their effects.

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