Antitumor activity of an ethanol extract of *Nigella sativa* seeds

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Effects of ethanol extract of *Nigella sativa* L. on the growth of Ehrlich ascites tumor (EAT), mitotic index, labeling index and on the life span of tumor bearing mice were studied. The results from treated animals showed a decrease in viable cell count, an increase in the life span of EAT bearing mice and an increase in the glutathione peroxidation of heart tissue. Cytological studies have revealed a reduction in the mitosis and DNA synthesis. Treatment with the *N. sativa* extract also resulted in improvements in the morphological features of tumor cells, along with a reduction in intracytoplasmic vacuoles, the appearance of cell membrane blebbing and the staining intensity. The results of the present study suggest that the ethanol extract of *Nigella sativa* seeds can generate antioxidants, possess antitumor activity, and ameliorate and prolong the life span of mice bearing EAT.

Key words: Ehrlich ascites tumor, *Nigella sativa*, mitotic index, labeling index.

Introduction

Nigella sativa L. is an annual dicotyledon of Ranunculaceae family known commonly as "black cumin". It is a herbaceous plant that grows in Middle East countries and have been widely used in Turkey as condiment in bread and other foods. Black cumin seeds have also been used as additive for spice (ABOUTABL et al., 1986) and flavored and aromatic substances (MERFORT et al., 1997). *N. sativa* extract (NSE) can be used in the preservation of food and prevention of food poisoning (HANAFY & HATEM, 1991). Recently, many biological activities of NSE have been reported, including antifungal, antibacterial, antiviral and antihelminthic ones (AKHTAR & JAVED, 1991; MORSI, 2000; SALEM & HOSSAIN, 2000). Others have reported that the seeds are used for treatment of flatulence and abdominal ailments (EL-DAKHAKHNY et al., 2000), to decrease fasting plasma glucose concentration in rabbits (AL-HADER et al., 1993), increase serum total protein (AL-GABY, 1998), as diuretic and hypotensive (ZAOUI et al., 2000). Antitumor activity of NSE

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has been recorded by many authors (SALOMI et al., 1992). Moreover it has been reported to inhibit eicosanoid generation in leucocytes and non-enzymatic peroxidation in ox brain phospholipids liposomes (HOUGHTON et al., 1995) and protect organs against the oxidative damage induced by a variety of free radicals (BADARY et al., 1997; NAGI et al., 2000).

In this study we investigated the effect of *Nigella sativa* extract on the DNA synthesis, cell proliferation and the ability of scavenging superoxide radicals in mice bearing tumor cells. Most studies of *N. sativa* treatments against tumors have been conducted by intraperitoneal injection *in vivo* or direct addition to isolated cells *in vitro*. Humans normally consume *N. sativa* orally. Therefore, a study has been conducted to investigate the antitumor effects of *N. sativa* extract when administered orally to mice bearing the Ehrlich ascites tumor (EAT).

Material and methods

Animals

Adult Balb/C male mice weighing 22 ± 2 g were obtained from DETAM (Experimental Medical Research Center, Capa Medical College, Istanbul, Turkey). The mice were maintained as 6 animals per cage in 12h/12h dark/light cycle under normal laboratory condition of temperature and humidity, fed with rodent pellets and tap water ad libitum.

Tumor cells

Ehrlich ascites tumor (EAT) described in this paper was hyperdiploid subline (obtained from Istanbul University, Department of Biology, Turkey), which has been propagated in our laboratory by weekly intraperitoneal inoculation of about 10^6 cells/mouse. Unless otherwise indicated, the EAT was obtained from donor mice on the sixth day of tumor growth. The ascites tumor cells, obtained from donor mouse, were diluted with buffer solution saline (BSS) and counted with haemocytometer.

Plant material

N. sativa seeds were purchased from the local herbalist in Sanliurfa. The seeds were botanically authenticated by a specialist of plant taxonomy in biology department. A specimen has been preserved at 4 ℃. The seeds were identified, cleaned, dried, mechanically powdered and extracted with 96% ethanol and evaporated with rotary evaporator to render the extract alcohol free. The extract was kept in a domestic refrigerator at 4 ℃.

Experimental design

For this study, forty adult healthy male mice were used and randomly divided into two equal groups. EAT cells collected from peritoneum of tumor-bearing mice were diluted with BSS, and counted using Neubauer haemocytometer. The final cell number was 3×10^5/cells/mL. Each animal in the groups was inoculated with 3×10^5 cells intraperitoneally on 0 day and treated as follows:

Group I: Each mouse received orally 1 g/kg/day NSE suspended in 10 mL of 5% gum arabica in normal saline by gavage for 7 days (the final concentration was 10 mg/0.1 mL). The optimum oral dose of NSE was 0.88 g/kg body weight. This corresponded to the optimum effective dose of NSE in treatment of stomach ulcers (EL-DAKHAKHNY et al., 2000).

Group II: Each mouse received orally 0.2 mL/day vehicle (5% gum arabica in normal saline) suspension, for 7 days and served as control.

At 2, 4, 6 and 8 day intervals, 5 animals from each group were injected intraperitoneally with 20 μCi tritiated thymidine (^{3}H-TdR) (Perkin Elmer Life Science Inc., Boston, MA 02118 USA) 30 minutes before sacrificing the mice. Immediately after death, cells were flushed from the peritoneum of each animal into a known volume (20 or 50 mL) of heparinized normal saline by repeated injection and withdrawal of 3-5 mL volumes. Cancer cells were recognized and counted using Neubauer haemocytometer. The mean cell number for each group of 5 animals is shown in Figure 1. Ascitic fluid with EAT was smeared on clean slides, air dried and fixed in Clarke's fixative (PEACOCK, 1973). Three slides were processed for autoradiography according to the method described by ROSE & ROSE (1965), and three slides processed for evaluation of mitotic index. For autoradiography, 3 slides were prepared by the dip-coating method using Kodak emulsion (Eastman Kodak Company, N.Y., USA). The exposure time was 7 days and developed in Kodak D 19 b developer. The uptake of thymidine by EAT cells was expressed as a percentage of labeled tumor cells, determined, for each animal, on 1000 ± 10 cells/smear at random. The limits of accuracy of the cell count were obtained by determining the percentage of labeled tumor cells in autoradiographs of smears prepared from the same ascitic fluid sample. Because the total number of cells counted was the same in each case, statistical analysis (t-test) was made between the means of the number of labeled cells. It was assumed that EAT cells labeled with ^{3}H-TdR had synthesized DNA (PAINTER et al., 1960).

Survival time

Thirty adult male mice randomly divided into three groups (A, B, and C; $n = 10$) were used for this study. Each mouse was inoculated with 3×10^5 EAT cells suspended in BSS on day 0. Each mouse in the groups A and B was orally treated with 1 g and 2 g NSE/kg/day, respectively, while that in the group C (control) was orally administered 0.2 mL/day gum arabica vehicle. Treatment with NSE (1g/kg and 2g/kg body weight)

and vehicle orally using catheter fixed to 1 mL standart syringe was started 24 hours after EAT inoculation. Daily treatment continued until the last dead mouse in the control group was recorded (this period was found to be 15 days). Median survival time for the three groups was evaluated. The animals surviving 35 days were considered to be cured. All surviving animals were sacrificed at the end of the experiment, to compare the tumor efficacy of NSE (1g/kg and 2g/kg body weight) and the histopathological changes. The viable tumor cell counts (trypan blue test) were carried out with Neubauer haemocytometer.

Lipid peroxidation (LPO)
Lipid peroxidation was determined by measuring the thiobarbituric acid reactive substances (TBARs) formed by the thiobarbutiric acid (TBA) reaction according to Jo & Ahn (1998), using spectrofluorometer with 520 nm excitation and 550 nm emission.

Glutathione level (GSH)
The total GSH was determined according to the method of Browne & Armstrong (1998), using a spectrofluorometric method with 350 nm excitation and 420 nm emission.

Statistical analysis
All data are expressed as means ± SD. The data were analyzed using the Student t-test; differences were considered significant when $p < 0.05$.

Results

Tumor growth
Studies on the effect of ethanol extract of *Nigella sativa* on tumor growth were monitored. It was found that 1g/kg body weight of extract orally administration by gavage could inhibit the cell proliferation at 2, 4, 6, and 8 days, respectively, compared with the corresponding cell numbers in the control group (Fig. 1). The viable tumor cell number was found significantly inhibited ($p < 0.001$) in treated groups. The percentage of trypan blue-positive dead tumor cells were also increased in the treated groups compared with the controls; especially more pronounced effects were observed at 6th and 8th days.

Cytological studies using Feulgen and Giemsa staining methods have revealed a significant ($p < 0.001$) decrease in the number of mitotic cells at 2, 4, 6 and 8 days, in tumor cell smears following NSE treatment, when compared with that of control smears (Fig. 2). Degenerative cells changes in tumor cells (changes in cell morphology, vacuolated cytoplasm, changes in staining intensity, cell membrane blebbing and smaller cell size) were also much reduced in treated mice when compared to those from control animals.
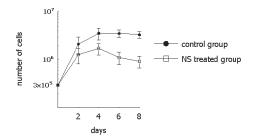

Fig. 1. Growth curve of EAT. Each mouse inoculated with 3×10^5 cells, each point on the chart is the mean of 5 animals sacrificed at each interval. Values are significant ($p < 0.001$).
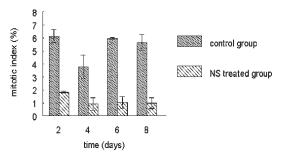

Fig. 2. Percentage of EAT cells in mitosis after treatment with 1g/kg/day NSE. Each point on the chart is 1000 cell counted from each animal. All values are significant ($p < 0.001$).
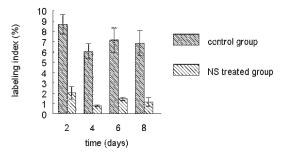

Fig. 3. Uptake of ^{3}H-TdR by EAT cells growing in the peritoneal cavity of mice in control and experimental groups. 20 μCi of tritiated thymidine was administered to each mouse intraperitoneally before 30 minutes of sacrificing the mouse. All values are significant ($p < 0.001$).

Inhibition of DNA synthesis
The results of studies on the effect of an ethanol extract of *N. sativa* on labeling index are shown in

Table 1. Mean survival time of mice treated with different doses of NSE.

Regimen	No. of mice survived after tumor inoculation				Median survival days	Life span (%)	ILS(%)[a]
	10 days	15 days	25 days	35 days			
Control	6/10	0/10	0/10	0/10	13.4±0.6	100	–
EAT+NSE (1g/kg bw)	10/10	10/10	7/10	2/10	34.3±0.3	170.6±0.6	70.6
EAT+NSE (2 g/kg bw)	10/10	10/10	8/10	1/10	32.8±0.8	174.8±0.8	74.8

[a] ILS, increase of life span; ILS = (T − C)/C × 100. T = mean survival time of treated mice, C = mean survival time of control group. Values are mean ± SD from experiment using 10 mice per group. All values in the experiment are significant ($p < 0.001$) when compared with control.
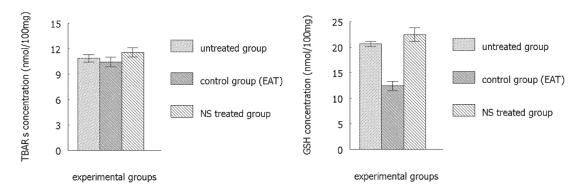

Fig. 4. Thiobarbituric acid reactive substances (TBARs) concentration (mean ± SD). Each point is the median of 8 mice. Untreated group mice treated with water only. Control group mice bearing EAT treated with gum arabica in physiologic water. NS treated group mice bearing EAT treated with *Nigella sativa* extract 2g/kg prepared in gum arabica.

Fig. 5. Glutathion (GSH) concentration (mean±SD). Each point is the median of 8 mice. Untreated group mice treated with water only. Control group mice bearing EAT treated with gum arabica in physiologic water. NS treated group mice bearing EAT treated with *Nigella sativa* extract 2g/kg prepared in gum arabica. Values are significant ($p < 0.05$).

Figure 3. Thymidine incorporation assay demonstrated that DNA synthesis in tumor bearing mice was significantly inhibited ($p < 0.001$) after daily treatment with NSE (1g/kg/day). Decrease in DNA synthesis was more pronounced on days 4, 6 and 8, than on day 2. The percentage of labeled cells on day 2 in the control group was found to be 8.675±0.962; this number decreased to 2.056±0.605 in the experimental group. This value is four times higher in the control group. The decrease in labeled cells on days 4, 6 and 8 are higher than on day 2 of treatment.

Lipid peroxidation (LPO)
TBARs in the heart increased significantly in mice treated with NSE as compared with those in the control group (Fig. 4). Data obtained from heart tissue of the control (normal) group was 10.85±0.43 while in the EAT bearing control group, it was 10.42 ± 0.58. In the group bearing

EAT treated with NSE it increased to 11.575 ± 0.53 nmol/100 mg.

Glutathione level (GSH)
The antioxidant glutathione in heart decreased markedly in *N. sativa* treated group when compared with control group (Fig. 5). The glutathione level in heart tissue increased significantly from 12.42 ± 0.92 in EAT control group to 22.48 ± 1.31 nmol/100 mg in treated group.

Survival time
The effect of the two doses of NSE (1 and 2 g/kg/day) orally on survival time of EAT bearing mice is shown in Table 1. The median survival time of animals in the control group was 13.9 ± 0.6 days. This number increased to 34.3 ± 0.3 when animals were treated with 1 g NSE /kg/day orally. This increase in life span is 70.6%. The median survival time of animals treated with 2 g/kg/day

orally, increased to 32.8 ± 0.8 days, i.e. the 74.8% increase in life span.

Discussion

N. sativa seeds are orally ingested by people as a condiment or additive in food dishes. Patients with gastrointestinal disorders ingest seeds mixed with honey. Recent studies indicate that NSE has cytotoxic effects against different types of cancer cell lines *in vitro* and *in vivo*. SALOMI et al. (1992) reported that a number of cell lines, such as Dalton lymphoma, Ehrlich ascites carcinoma and Sarcoma-180 exhibited coordination or sensitivity to *N. sativa* extract when administered intraperitoneally. Our study was undertaken to demonstrate the effects of NSE against EAT propagated in Balb/C mice and to determine whether the activity of extract acts against tumor cells when administered orally at a dose of 1 g/kg/day. The study reveals that the ethanol extract of NSE could inhibit the tumor growth and showed retardation in cell count and tumor development. The inhibition is more pronounced on day 6 and 8 (Fig. 1). The curve was obtained by determining the viability of cells in mice after daily treatments. Tritiated thymidine (^{3}H-TdR) incorporation assay revealed that DNA synthesis was inhibited after treatment with NSE. Autoradiography results showed a decrease in the percentage of labeled cells (Fig. 2). Also the mitotic index decreased significantly ($p < 0.001$) after 2, 4, 6 and 8 days of EAT cells transplantation. Decrease in mitotic index is more pronounced at 4th, 6th and 8th day when compared with control (Fig. 3). Moreover the life span of mice bearing EAT was increased after treatment with NSE by oral administration of 1 g and 2 g/kg/mouse at the end of fifteen days. The median survival time was 34.3 ± 0.3 and 32.8 ± 0.8 days. The percentage increase in life span was found to be 70.6% and 74.8% when treatment with 1 g and 2 g/kg body weight, respectively (Table 1).

Treatment with *N. sativa* extract bearing EAT mice was studied. The results show that the TBARs level was not significantly effected ($p > 0.05$), but the GSH was increased significantly ($p < 0.001$) when compared with the control group. We suggest that the increase in glutathione activity in treated group was due to lowering the toxicity of free radicals in animal tissues and prolonged the life span of the animal.

Results obtained in the present study demonstrate that oral treatment of mice with NSE can inhibit the proliferation of tumor cells and reduce the mitotic index in the treated group compared with the control group. These results confirm the results obtained by SALOMI et al. (1992) on administration of the drug intraperitoneally. They are also in agreement with the results reported by other researchers (ABOUTABL et al., 1986; AKHTAR & JAVED, 1991; HANAFY & HATEM, 1991) when treatment was conducted on tissue culture specimens. Cytological studies of EAT smears from mice used in the current investigation and stained with Feulgen or Giemsa methods showed degenerative changes in treated tumor cells. Also morphological changes were observed, such as the vacuolated cytoplasm, membrane blebbing and reduction in the staining intensity.

Plants that contain essential oil, flavanoids and polyphenols are reported to have many biological properties; they possess powerful antioxidative components (BURITS & BUCAR, 2000; SHEYLESH & PADIKKALA, 2000). The main compound of *N. sativa* oil is 85% fatty acid, which can inhibit the membrane lipid peroxidation (HOUGHTON et al., 1995). Furthermore flavanoids have prevention role in cancer therapy via the effect on signal transduction in cell proliferation (DE AZEVEDO et al., 1996; FOTIS et al., 1997). It has also been reported that antioxidant can inhibit proliferation of cancer cells (REBECCA et al., 1998).

In conclusion, the results of the present study are encouraging, as ethanol extract of NSE has shown significant reduction in cell proliferation, DNA synthesis, mitotic percentage and prolongation of life span of the mice bearing the EAT.

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