Cancer chemopreventive potential of volatile oil from black cumin seeds, *Nigella sativa* L., in a rat multi-organ carcinogenesis bioassay

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Abstract. *Nigella sativa* (*N. sativa*) is a herbal plant of the *Ranunculaceae* family that has been widely used for various medicinal and nutritional purposes. Volatile oil extracts along with its major constituents, such as thymoquinone, have recently attracted considerable attention for their antioxidant, immunoprotective and antitumor properties. The present study was conducted to assess the chemopreventive potential of crude oils in *N. sativa* on tumor formation using a well-established rat multi-organ carcinogenesis model featuring initial treatment with five different carcinogens. Post-initiation administration of 1000 or 4000 ppm *N. sativa* volatile oil in the diet of male Wistar rats for 30 weeks significantly reduced malignant and benign colon tumor sizes, incidences and multiplicities. The treatment also significantly decreased the incidences and multiplicities of tumors in the lungs and in different parts of the alimentary canal, particularly the esophagus and forestomach. Bromodeoxyuridine labeling indices, reflecting cell proliferation were significantly decreased in various organs and lesions after treatment with the two doses of *N. sativa*. The plasma levels of insulin growth factor, triglycerides and prostaglandin E2 were also altered. The findings show, for the first time, that *N. sativa* administration exerts potent inhibitory effects on rat tumor development and on cellular proliferation in multiple organ sites. In particular, the ability to significantly inhibit murine colon, lung, esophageal and forestomach tumors was demonstrated in the post-initiation phase, with no evidence of clinical side effects. The mechanisms are likely to be related to suppression of cell proliferation.

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Abbreviations: ABC, avidin biotin complex; BrdU, 5-bromo 2'-deoxyuridine; b.w., body weight; DMBDD, combination of the following 5 carcinogens: diethylnitrosamine (DEN), *N*-methyl-*N*-nitrosourea (MNU), *N*-butyl-*N*-(4-hydroxybutyl) nitrosamine (BBN), 2,2'-dihydroxy-di-*N*-propylnitrosamine (DHPN) and 1,2-dimethylhydrazine dihydrochloride (DMH); GST-P, glutathione s transferase-positive foci; HDL-cholesterol, high density cholesterol; I.g, intragastroluminal gavage; IgG, immunoglobulin G; IGF-I, insulin growth factor 1; IHC, immunohistochemistry; i.p., intraperitoneal; LDL-cholesterol, low-density cholesterol; *N. sativa, Nigella sativa*; PGE2, prostaglandin E2; ppm, part per million; P/N, papillary or nodular hyperplasia; s.c., subcutaneously; SCC, squamous cell carcinoma; TBS, Tris buffered solution; TG, triglycerides; v/v, volume per volume

Key words: Nigella sativa, multi-organ carcinogenesis, rat, cancer

Introduction

The search for compounds that prevent cancer has intensified with mounting evidence that many types of cancer are caused or triggered by factors related to lifestyle and environment, particularly agents in foodstuffs. There is a growing body of evidence from *in vitro* and *in vivo* experiments that *N. sativa* seeds (black cumin seeds) or the extracted volatile oils, exert anti-carcinogenic effects (1,2). *N. sativa* is a common herbal plant that has been used since ancient times by the Egyptians and Greeks for various medicinal purposes. It has many common applications by people in the countries surrounding the Mediterranean Sea, the Arab world, Indians and Persians as an antimicrobial, antihelmintic, diuretic, diaphonetic and antiasthmatic medicine. *N. sativa* is also used by the Europeans, Americans and Japanese as a food additive for cheese and bakery products (3).

A large number of *in vitro* and *in vivo* studies have shown significant modulatory effects of *N. sativa* extracts with different pathological, toxicological and cytotoxic protocols. Previously, volatile oils from *N. sativa* seeds were found to possess antitumor effects *in vitro* against Ehrlich ascites carcinoma, Dalton's lymphoma ascites and sarcoma (S-180) cell lines (4). Recently, it was postulated that extracted proteins from *N. sativa* exhibit modulatory effects on the immune system (5) with anti-protein-oxidation activity. This effects may be rheologically useful, particularly in pathological conditions related to free radical stress (6). A previous study showed that extra-virgin crude volatile oils extracted from Egyptian *N. sativa* exert significant inhibitory effects on rat colon carcinogenesis when administered in the post-initiation

phase along with a marked anti-proliferative effect on the colonic epithelium when administered at different stages of colon carcinogenesis (with pre-carcinogen, simultaneous or post-carcinogen administration protocols) (7).

In the present study, a rat medium-term multi-organ carcinogenesis bioassay (8) was used to detect the anti-carcinogenic potential of *N. sativa*. This assay was based on the two-stage concept of carcinogenesis using five different genotoxic carcinogens applied for initiation: diethylnitrosamine (DEN), a well-known liver-specific carcinogen (9); *N*-methyl-*N*-nitrosourea (MNU), which has a multi-site carcinogenic potential particularly targeting the gastrointestinal lumen and lung (10); 2,2'-dihydroxy-di-*N*-propylnitrosamine (DHPN), mainly causing lung tumors (11); 1,2-dimethylhydrazine (DMH), a genotoxic colon cancer initiator (12); and *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine (BBN), which is known to initiate urinary bladder carcinogenesis (13). The rat multi-organ carcinogenesis bioassay is widely used for the detection of carcinogenesis and the evaluation of cancer chemopreventive agents (14,15). This approach is beneficial for assessing the modifying effects of chemicals on wide-range multi-organ carcinogenesis using a single experiment within a relatively short experimental period (16).

In the last few years, numerous research results have shown that *N. sativa* is able to inhibit different types of tumors in experimental animals when administered as ground seeds (17), as a crude extract (volatile oil) (1) or as a single ingredient, thymoquinone, which is the main active constituent of volatile oils of *N. sativa* seeds (18,19). However, there is currently only limited information on the actual mechanism of action of *N. sativa* or its active forms. Previous studies showed that the more significnt components of the Egyptian *N. sativa* are 21% protein, 35.5% fat, 6.6% fibre, 5.5% moisture and 3.7% ash, while the remaining constituents include total carbohydrates, minerals, vitamins and polyphenols (20). The active ingredients are mainly thymoquinone (primarily in essential oil), dithymoquinone (DTQ) (nigellone), thymol (THY) and thymohydroquinone (THQ). Other components include α-hederin (a triterpene saponin), monosaccharides (glucose, rhamnose, xylose and arabinose) and unsaturated fatty acids (linoleic acid and oleic acids) (21,22). The biological activities, particularly the antioxidant or antitumor activities, of thymoquinone and nigellamines identified from the Egyptian *N. sativa* (23) or alkaloids (24) have yet to be adequately determined.

Research studies have shown that the mechanism of action of *N. sativa* may correlate with antioxidant properties of the active ingredients found in its volatile oil such as thymoquinone, or due to its immunosuppressive and cytotoxic effects (25). Extracts of *N. sativa* are known to exert insulin-sensitizing actions and lipid-modulating properties (26). These modulating effects extend to cholesterol, triglyceride and prostaglandin levels in rats (27). Furthermore, the same active constituents of *N. sativa* are known to affect tumor insulin growth factors through the suppression of kinase signaling pathways (28). In the present study, bromodeoxyuridine (BrdU) labeling indices (LIs) were detected immunohistochemically to assess the population of cells actively synthesizing DNA (29). BrdU, a thymidine analog which replaces (^{3}H) thymidine (30), is incorporated into the newly synthesized DNA strands of actively proliferating cells following partial denaturation of double-stranded DNA. Measurement of (^{3}H) thymidine incorporation as cells enter the S phase of the cell cycle is the traditional method for the detection of cell proliferation, particularly in tumors (31). Evaluation of cell cycle progression and cellular proliferation is essential for investigations in many scientific fields, including cancer chemoprevention studies.

Therefore, to further elucidate the effects of crude volatile oils extracted from *N. sativa*, the present experiment was designed as a multi-organ carcinogenesis bioassay in Wistar rats. BrdU LIs as well as plasma levels of insulin growth factor (IGF-I), triglycerides (TG), prostaglandin E2 (PGE2) and cholesterol were evaluated.

Materials and methods

Animals. A total of 76 male Wistar rats were obtained at 5 weeks of age from the Helwan Breeding Facility of Helwan University, Cairo, Egypt. The rats were divided into 5 groups according to their body weight (b.w.) to minimize inter-group standard errors (20 rats each for groups 1-3 and 8 each for groups 4 and 5). The animals were housed 4 or 5 per plastic cage with wood chips for bedding and maintained at a temperature of 22±2˚C, with 45±4% relative humidity with a 12-h light/12-h dark cycle. The experiment commenced after acclimatization for one week to the animal house conditions. Diet and water were available *ad libitum*, and animal growth and food consumption were measured weekly during the experiment to assess toxicity. The Institutional Animal Care Facility of the Zoology Department, Faculty of Science, Tanta University, Egypt approved the experimental design.

Chemicals. DEN, DMH (purity 99%), DHPN and MNU were obtained from Sigma-Aldrich (St. Louis, MO, USA), while BBN was obtained from Tokyo Kasei Kogyo Co., Ltd. (Tokyo, Japan). Crude extra virgin oil extracted from fresh *N. sativa* seeds was purchased from Kahira Pharm. and Chem. Co., Cairo, Egypt. The oil was stored and protected from light exposure in dark glass bottles at room temperature between 15-20˚C. Calculated doses of 1000 and 4000 ppm of the oil were then mixed fresh (v/v) with basal-powdered diet and administered *ad libitum* to the rats. The contents presented in the basal diet were: crude protein, not less than ~18%; crude lipid, not less than ~2.6%; crude fiber, not more than ~11.43%; with an ingestion energy of not more than 284.83 kcal/100 g. The doses of *N. sativa* were equivalent, respectively, to 50 and 200 mg/kg b.w. of the oil calculated according to data for food consumption in our previous experiment (7); the doses were not associated with any signs of toxicity.

Mid-term multi-organ carcinogenesis bioassay. The experimental protocol is shown in Fig. 1. Rats in groups 1-3 were treated sequentially with DEN [100 mg/kg b.w., intraperitoneally (i.p.), single dose] at commencement; MNU, (20 mg/kg b.w., i.p.) on days 2, 5, 8 and 11; and DMH, [40 mg/kg b.w., subcutaneously (s.c.)] on days 14, 17, 20 and 23. Animals were simultaneously given BBN, (0.05% in the drinking water) during weeks 1 and 2 and DHPN (0.1% in the drinking water) during weeks 3 and 4. Following this combination treat-

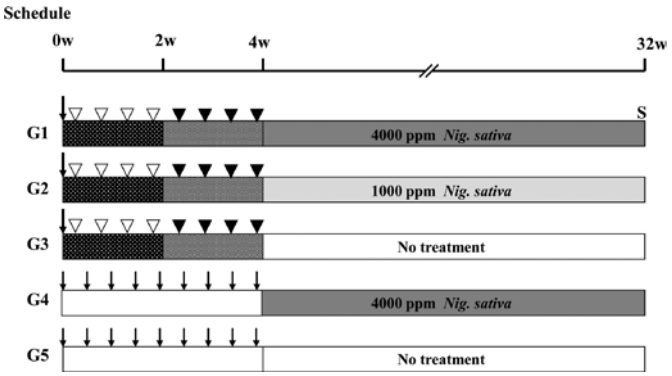

Figure 1. Experimental design. Bold arrows, DEN (diethylnitrosamine) 100 mg/kg i.p. Black arrowheads, MNU (*N*-methylnitrosamine) 20 mg/kg i.p. Black dotted squares, BBN (*N*-butyl-*N*-(4-hydroxybutyl)nitrosamine. Grey dotted squares, DHPN (2,2'-dihydroxy-di-*N*-propylnitrosamine 0.1%) in drinking water. White arrowheads, DMH (dimethylhydrazine), 40 mg/kg s.c. S, sacrifice and blood analysis. Normal arrows, saline 0.9%.

ment with DEN, MNU, BBN, DMH and DHPN (DMBDD), animals in groups 1 and 2 were fed a powdered basal diet containing 1000 or 4000 ppm of *N. sativa* until the end of the experiment, while animals in group 3 received a basal diet and served as a carcinogen-treated control for groups 1 and 2. Animals in groups 4 and 5 did not receive any carcinogen administration. However, they received 0.9% saline injections instead according to the same schedule. Animals in group 4 received *N. sativa* 4000 ppm as a drug treatment control and those of group 5 received a normal basal diet as a negative control. The animals were sacrificed under ether anesthesia at week 32 following commencement of the experiment. A total of 5 rats from each of groups 1-3 received a single injection of BrdU (Sigma Chemicals, St. Louis, MO, USA; 100 mg/kg b.w. i.p.) 1 h prior to sacrifice.

Biochemical analysis of blood. To assess the serum biochemical levels, blood samples were obtained at the time of autopsy from the inferior vena cava in tubes containing no anti-coagulant. The blood was centrifuged at 4000 rpm for 30 min at 4˚C until the serum was obtained. Blood was collected from 10 rats of groups 1-3 and from 6 rats from each of groups 4 and 5, that had not received BrdU injections.

Serum was collected and preserved frozen at -80˚C before measurement of different biochemical parameters. To detect serum levels of IGF-I protein, 3-ml aliquots of the supernatant were subjected to an immunoradiometric assay with a kit (Somatmedim CII, Bayer) according to a previously described method (32). To determine the serum PGE2 levels, 1 ml of serum supernatant was mixed with an equal volume of 1 M phosphate buffer (pH 6.8) containing methanol and (^{3}H)PGE2 (4000 d.p.m). Following mixing and centrifugation, the samples were processed and measured for PGE2 by radioimmunoassay (using a kit from NEN, Boston, MA, USA). Recovery by this method was satisfactory (~80%). Total cholesterol and TG concentrations in the serum were determined enzymatically using commercially available reagent kits (assay kits for the TDX system; Abbott Laboratory, Irving, TX, USA). Briefly, after the blood was centrifuged to obtain serum, the sample was analyzed for total cholesterol, total TG, HDL- and LDL-cholesterol levels by precipitation techniques and modified enzymatic procedures as previously described by Bucolo and David (33) and Allain *et al* (34). Total-cholesterol levels were calculated using the Friedewald equation (35).

Tissue processing. All of the surviving animals were sacrificed under ether anaesthesia, and all of the major organs were excised and fixed in 10% phosphate-buffered formalin. The fixative was injected into the head, the lumina of the alimentary canal and the urinary bladder. The liver, kidneys, spleen and testes were excised and quickly weighed. The organs were then wet cut into 3-mm sections, and three liver slices (one each from the anterior, right posterior and caudate lobes) were prepared, and fixed in the same fixative. The alimentary tract from the esophagus to the rectum, the urinary bladder and lungs with the trachea and thyroid gland were excised and fixed. All of the alimentary canal areas were cut open along the median axis from the esophagus to the anal end and were further fixed flat between two sheets of filter paper in formalin. Other organs, such as the pituitary glands, adrenal glands, thymus, salivary glands and lymph nodes, were also excised, fixed in formalin and prepared for histopathological examination. Body areas were assessed for the presence of any treatment-related abnormalities or lesions. Sketches were made for all of the tumors found at any site to determine location. Tumor size (length, width and height) was measured using calipers.

Histopathological evaluation. The liver, spleen, kidneys, lungs, testes, as well as selected samples from all of the collected organs, lesions and tumors were routinely embedded in paraffin, sectioned at 3-4 μm, and prepared for histological examination following staining with hematoxylin and eosin. The lungs were inflated with 10% phosphate-buffered formalin. At least two sections from each lobe (>10 sections from each rat), including any visible macroscopic nodular lesions on the surface of the lungs, were embedded in paraffin and routinely prepared for histopathological examination. Microscopically, the lung epithelial lesions were classified into three histological categories: alveolar epithelial hyperplasia, adenoma and carcinoma. Carcinomas were classified into adenocarcinomas and squamous cell carcinomas (SCCs). Histopathological evaluation for the lungs and all other organs was performed according to Boorman *et al* (36).

Immunohistochemical (IHC) staining for BrdU and GST-P-positive foci. The method of Hsu *et al* (37) was used for the IHC staining in the present study. For BrdU, after being deparaffinized and hydrated through a graded ethanol series, 4 μm of the colon, lung, esophagus and forestomach was used. Briefly, the sections were incubated with 0.3% hydrogen peroxide for 30 min to block endogenous peroxidase activity. They were then incubated with 10% normal horse serum for 30 min at room temperature, and overnight at 4˚C with the BrdU antibody (dilution 1:500) in Tris(hydroxymethyl) aminomethane-buffered saline (TBS). Following exposure for 30 min at room temperature to biotinylated horse anti-mouse IgG (Vector Laboratories, Burlingame, CA, USA), sections

Table I. Data for final body (g) and relative organ weights (%).

Group	Treatment	Effective no. of rats	Final body weight (g)[a]	Relative liver weight (%)	Relative kidney weight (%)		Relative spleen weight (%)	Relative testis weight (%)
					R	L		
1	DMBDD + 4000 ppm	19	324.0±13	2.8	0.53	0.36	0.26	0.75
2	DMBDD + 1000 ppm	18	324.6±9	2.9	0.31[b]	0.41	0.26	0.78
3	DMBDD only	19	326.9±12	3.0	0.51	0.42	0.30	0.78
4	4000 ppm only	8	435.4±11	2.6	0.25	0.24	0.18	0.78
5	No treatment	8	429.6±11	2.6	0.23	0.25	0.19	0.79

[a]Means ± SD. Relative weights are the ratio of organ weights/body weight (%). [b]Significant vs. G1, G3 at P<0.05. R, right; L, left kidney.

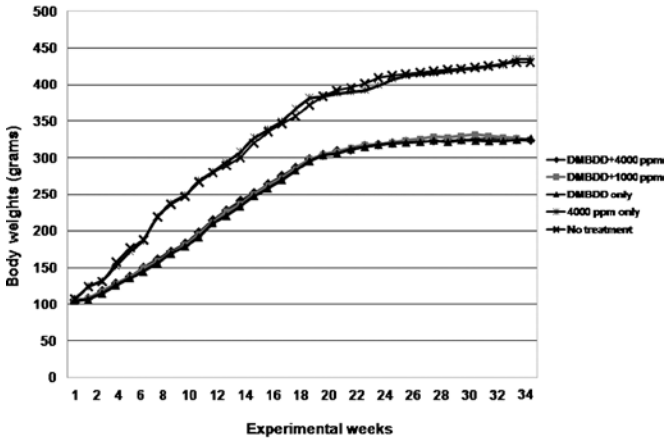

Figure 2. Growth curves of rats in all experimental groups.

were incubated with the avidin-biotin complex (ABC) at a 1:25 dilution. Washing with TBS followed each step. To score cell proliferation, cells stained for BrdU expression were counted in at least 2000 cells from each organ. To generate the BrdU labeling index (LI, %), the total number of proliferating cells was divided by the total number of cells x 100.

Regarding IHC staining of GST-P-positive foci in the liver, after incubation with 0.3% hydrogen peroxide, sections were treated sequentially with 10% normal goat serum for 40 min and incubated with anti-rabbit GST-P polyclonal antibody (Medical & Biological Lab. Co. Ltd., Nagoya, Japan) at a dilution of 1:1000 overnight at 4˚C. The sections were then treated with biotin-labeled goat anti-rabbit IgG and the avidin-biotin-peroxidase complex (ABC kit; Vector Laboratories). For both BrdU- and GST-P-positive foci staining, peroxidase activity was visualized by treatment with a 0.02% solution of diaminobenzidine tetrahydrochloride containing 0.05% hydrogen peroxide. The nuclei were counterstained with hematoxylin. The numbers and areas of GST-P-positive cell foci >0.2 mm in diameter and the total areas of the liver sections were measured with the aid of the color video image analyzer (Qwin Image Analysis System, Leica, Wetzlar, Germany).

Statistical analyses. The significance of differences between group mean values for body weight, food intake, tumor size, tumor multiplicity and immunohistochemistry data were subjected to SuperANOVA Duncan New Multiple Range analysis (StatView v.4.5; Abacus Concepts Inc., Berkeley, CA, USA). Data for tumor incidence were analyzed according to Fisher's exact probability test or the Chi-squared test (StatView, ver. 5, SAS Institute Inc., Cary, NC, USA). Statistical significance was estimated at P<0.05.

Results

General observations, body and organ weights. In the DMBDD-treated groups (1-3), one rat from group 1, two rats from group 2 and one rat from group 3 were moribund before the end point of the scheduled sacrifice. Therefore, their data were not included in the study. The growth rates of rats in all of the groups (Fig. 2) were constant during the experiment with no differences among the DMBDD-administered groups 1-3 or between groups 4 and 5. The final body weights in groups 1-3 were less than those of groups 4 and 5, while the relative organ weights, except for those of the testes, showed an increase in the DMBDD-treated groups as compared to the rats in groups 4 and 5. The mean relative right kidney weights of the rats in group 2 were essentially similar to those of the non-carcinogen-treated rats. No other significant inter-group variation was observed between the comparable groups (Table I).

Food consumption and N. sativa intake. Food consumption values (Table II) in the DMBDD-initiated groups were lower than those in the groups without initiation (4 and 5), while water intake was similar among the groups. No inter-group variation within the DMBDD-treated groups or between groups 4 and 5 was noted. The total (mg/experimental period) and average intake of *N. Sativa* per rat (mg/rat/day) were found to be consistent with the administration dose among the treated groups (Table II).

Histopathological examination and tumor occurrence. Preneoplastic or neoplastic lesions were observed in the esophagus, stomach, small intestine, colon, lungs, liver, kidneys, urinary bladder, prostate, spleen and skin/subcutis. Significant inter-group differences in the incidences and multiplicities of lesions were found mainly in the colon, lungs, esophagus and forestomach. No tumors were found in any rat from the control

Table II. Average food consumption, water and *N. Sativa* intake.

Group	Treatment	Food consumption (g/rat/day)[a]	Water intake (ml/rat/day)	*N. sativa* intake	
				Total (mg)	Average (mg/rat/day)
1	DMBDD + 4000 ppm	12.1±5.3	17.3±2.3	10,164	48.4±9.2
2	DMBDD + 1000 ppm	12.6±6.8	18.2±3.3	2,646	12.6±7.5
3	DMBDD only	12.4±5.1	17.4±3.9	0	0
4	4000 ppm only	14.1±6.7	18.9±3.4	11,844	56.4±9.5
5	No treatment	13.7±5.2	19.1±3.3	0	0

[a]Means ± SD.

Table III. Incidence (%) and multiplicity of the colon tumors.

Colon tumors	DMBDD + 4000 ppm (G1: n=19)		DMBDD + 1000 ppm (G2: n=18)		DMBDD only (G3: n=19)	
	Incidence[a]	Multiplicity[b]	Incidence[a]	Multiplicity[b]	Incidence[a]	Multiplicity[b]
Adenoma	1 (5.3)[c]	0.05±0.01[c]	1 (5.6)[c]	0.06±0.01[c]	8 (42.1)	0.40±1.68
Adenocarcinoma	5 (26.3)	0.32±0.31[c]	4 (22.2)[c]	0.28±0.40[c]	12 (63.2)	0.40±1.90
Signet-ring cell carcinoma	0	0	0	0	1 (5.3)	0.04±0.01
Mucinous adenocarcinoma	0	0	1 (5.6)	0.06±0.01	1 (5.3)	0.05±0.01
Fibrous histiosarcoma	1 (5.3)	0.05±0.01	0	0	0	0
All tumors	7 (36.8)[c]	0.47±1.2[c]	7 (38.8)[c]	0.44±0.60[c]	15 (79.0)	1.85±0.1.8

[a]Numbers in parentheses are incidence (%): no. of rats bearing tumors (outside parenthesis)/total no. of rats in the test group. [b]Mean no. of tumors per effective rat; means ± SD. [c]Significant vs. control DMBDD group 3 at $P<0.05$.

Table IV. Average colon tumor sizes (mm^3) and BrdU labeling indices.

Group	Treatment	Tumor size[a]	BrdU labeling indices (%)		
			Mucosa	Adenoma	Carcinoma
1	DMBDD + 4000 ppm	12.1±11.85[b]	8.4[b]	9.2[b]	9.4[b]
2	DMBDD + 1000 ppm	24.5±18.24	8.1[b]	9.4[b]	10.1[b]
3	DMBDD only	45.6±47.40	12.2	14.8	14.9

[a]Tumors measured are adenomas and adenocarcinomas >2 mm in diameter; means ± SD. [b]Significant vs. control DMBDD group 3 at $P<0.01$.

groups 4 and 5. In the colon, tumors were mainly adenomas and adenocarcinomas, with a few signet ring cell carcinomas, mucinous adenocarcinomas and fibrous histiosarcomas. *N. sativa* significantly decreased the incidences and multiplicities of the colon tumors (Table III). The effect was similar for both treatment doses. Colon tumor sizes were significantly decreased by the *N. sativa* treatment in a dose-dependent manner (Table IV).

In the lungs, epithelial lesions were mainly alveolar epithelial hyperplasias, alveolar cell adenomas, SCCs and adenocarcinomas. Table V shows data for incidences and multiplicities of the lesions. In groups 1 and 2 treated with *N. sativa* after DMBDD, the incidences and multiplicities of the lung tumors were generally less than those found in the DMBDD only-treated group 3. Significant values were obtained in the multiplicities of adenomas and adenocarcinomas in the 1000-ppm *N. sativa*-treated group and in the incidences and multiplicities of the 4000-ppm-treated group. When the incidences and multiplicities of the tumors (adenoma + carcinomas) were collectively calculated together, a significant decrease was evident for the two *N. sativa* treatment doses as compared to the corresponding data obtained from group 3. The multiplicities of lung cancer (data for the two cancer types together; SCCs + adenocarcinomas) were

Table V. Incidence (%) and multiplicity of the lung neoplasias.

Lung lesions	DMBDD + 4000 ppm (Group 1: n=19)		DMBDD + 1000 ppm (Group 2: n=18)		DMBDD only (Group 3: n=19)	
	Incidence[a]	Multiplicity[b]	Incidence[a]	Multiplicity[b]	Incidence[a]	Multiplicity[b]
Adenoma (alveolar cell type)	8 (42.1)	0.61±0.92[d]	6 (33.3)	0.44±1.54[d]	12 (63.2)	1.10±2.90
Adenocarcinoma	1 (5.3)[d]	0.05±0.01[d]	2 (11.1)	0.11±0.24[d]	5 (26.3)	0.40±2.00
Squamous cell carcinoma	1 (5.3)	0.11±0.20	0	0	1 (5.3)	0.05±0.01
Adenoma + carcinoma	8 (42.1)[d]	0.47±1.20[d]	8 (44.4)[d]	0.56±0.60[d]	16 (84.2)	1.45±0.18
Cancer incidence[c]	2 (10.5)	0.16±0.28[d]	2 (11.1)	0.17±0.24[d]	5 (26.3)	0.45±2.20

[a]Numbers in parentheses are incidence (%): no. of rats bearing tumors (outside parenthesis)/total no. of rats in the test group. [b]Mean no. of tumors per effective rat; [c]Adenocarcinomas + SCCs; means ± SD. [d]Significant vs. control DMBDD group 3 at P<0.05.

Table VI. Incidence (%) and multiplicity of neoplastic lesions in the esophagus and forestomach.

	DMBDD + 4000 ppm (Group 1: n=19)		DMBDD + 1000 ppm (Group 2: n=18)		DMBDD only (Group 3: n=19)	
	Incidence[a]	Multiplicity[b]	Incidence[a]	Multiplicity[b]	Incidence[a]	Multiplicity[b]
Esophagus						
P/N hyperplasia	5 (26.3)	0.37±0.6	5 (27.8)	0.28±0.50	9 (47.4)	0.45±0.5
Papilloma	5 (26.3)	0.26±0.5[c]	4 (22.2)	0.28±0.58	9 (47.4)	0.60±0.8
Squamous cell carcinoma	3 (15.8)	0.16±0.4[c]	4 (22.2)	0.22±0.40	7 (36.8)	0.40±0.6
All lesions	7 (36.8)[c]	0.42±0.9[c]	8 (44.4)[c]	0.51±1.1[c]	16 (84.2)	1.00±0.9
Forestomach						
P/N hyperplasia	2 (10.5)[c]	0.16±0.4[c]	8 (44.4)	0.28±0.5	8 (42.1)	0.45±0.5
Papilloma	2 (10.5)[c]	0.32±0.1[c]	5 (27.8)	0.28±0.6[c]	9 (47.4)	0.60±0.8
Squamous cell carcinoma	2 (10.5)[c]	0.26±0.5	4 (22.2)	0.22±0.4	8 (42.1)	0.45±0.6
All lesions	4 (21.1)[c]	0.50±0.7[c]	9 (50.0)	0.20±0.7	15 (79.0)	1.10±0.6

[a]Numbers in parentheses are incidence (%): no. of rats bearing tumors (outside parenthesis)/total no. of rats in the test group. [b]No. of tumors per effective rat; means ± SD. [c]Significant vs. control DMBDD group 3 at P<0.05. P/N, papillary or nodular.

significantly decreased by the two treatment doses of *N. sativa* while the incidences also decreased, although this did not reach statistical significance (Table V).

In the esophagus, the neoplastic lesions found [papillary or nodular (P/N) hyperplasias, squamous cell papillomas and SCCs] had lower incidences and decreased numbers in the *N. sativa*-treated groups 1 and 2 as compared to the data obtained for group 3. The incidences and multiplicities of the sum of the lesions calculated together were significantly decreased by the two *N. sativa* treatment doses. Additionally, the multiplicities of the papillomas and SCCs of the 4000 ppm-treated group were also significantly inhibited (Table VI).

In the forestomach, a dose-dependent inhibition of tumor incidences and multiplicities was observed. The majority of the lesions were significantly inhibited by the 4000 ppm dose, while for the 1000 ppm-treated dose, significance was only noted for the multiplicity of papillomas (Table VI). Data for the incidences and multiplicities of the tumors found in the other organs of groups 1, 2 and 3 are shown in Table VII. None of the data showed any significant inter-group differences.

BrdU LIs. The IHC examination of the lesions in the colons, lungs, esophagus and forestomach showed lower BrdU LIs in the *N. sativa*-treated groups when compared to the DMBDD only-treated group 3. In the colon, (Table IV) administration of *N. sativa* (groups 1 and 2) significantly reduced the BrdU LIs in the mucosal crypts, adenomas and adenocarcinomas as compared with the group 3 values (P<0.01). The inhibition was similar for the two doses of *N. sativa*. In the lungs, esophagus and forestomach, the values for the BrdU LIs were significantly reduced in lesions of the rats treated with 4000 ppm when compared to the values of the DMBDD only-treated group 3 (Table VIII). However, for the 1000 ppm-treated groups, a significant decrease in the BrdU LIs was more evident in the forestomach as compared to the other two organs (Table VIII).

GST-P-positive foci in the liver. Results of the quantitative evaluation of GST-P-positive foci in the livers of the DMBDD-initiated groups are shown in Fig. 3. In the *N. sativa*-treated groups (1 and 2), the numbers and areas of GST-P-positive

Table VII. Tumor incidences in other organs.

	DMBDD + 4000 ppm (G1: n=19)	DMBDD + 1000 ppm (G2: n=18)	DMBDD only (G3: n=19)
Spleen			
Malig. lymphoma/leukemia	1 (5.3)	1 (5.6)	3 (15.8)
Hemangiosarcoma	0	0	1 (5.3)
Metastatic fibrosarcoma	0	1 (5.6)	4 (21.0)
All tumor incidences	1 (5.3)	2 (11.1)	4 (21.0)
Kidney(s)			
Nephroblastoma	13 (68.4)	9 (50.0)	14 (73.7)
Renal cystic adenoma	1 (5.3)	0	0
Renal cell adenoma	0	0	5 (26.3)
Transitional cell adenoma	1 (5.3)	0	0
Renal cell carcinoma	2 (10.5)	3 (16.7)	6 (31.6)
Malignant fibrosarcoma	1 (5.3)	0	1 (5.3)
All tumor incidences	17 (89.5)	12 (66.6)	17 (89.5)
Urinary bladder			
P/N hyperplasia	8 (42.1)	11 (61.1)	9 (47.4)
Paoilloma	0	4 (22.2)	3 (15.8)
TCC	1 (5.3)	0	2 (10.5)
Squamous cell carcinoma	1 (5.3)	1 (5.6)	0
All tumor incidences	2 (10.5)	5 (27.8)	5 (26.3)
Prostate			
Adenoma	0	1 (5.6)	1 (5.3)
Skin/subcutis			
Squamous cell carcinoma	0	1 (5.6)	0
Small intestine			
Adenoma	2 (10.5)	1 (5.6)	1 (5.3)
Adenocarcinoma	5 (26.3)	3 (16.7)	7 (36.8)

None of the data presented in this table was significantly different from the other corresponding groups; Malig. lymphoma, malignant lymphoma; P/N, papillary or nodular; TCC, transitional cell carcinoma.

Table VIII. Mean BrdU labeling indices (%) in the lung, esophagus and forestomach neoplasia.

Treatment group	DMBDD + 4000 ppm[a]	DMBDD + 1000 ppm	DMBDD only
Lung			
Adenoma[b]	4.00±3.0[d]	5.70±3.7	8.90±6.5
Adenocarcinoma	6.20±5.1[d]	8.10±6.6	10.3±7.0
Esophagus			
Mucosa[c]	7.30±3.0[d]	7.10±4.3[d]	10.5±6.0
Papilloma	7.10 ± 5.1[d]	9.00±4.3	11.7±7.1
Squamous cell carcinoma	9.80±5.1	11.9±4.3	14.2±7.1
Forestomach			
Mucosa	6.40±4.1[d]	9.82±3.5	11.7±8.9
Papilloma	10.6±7.2[d]	9.80±6.2[d]	16.0±6.5
Squamous cell carcinoma	12.2±6.8[d]	11.9±9.0[d]	18.7±7.0

[a]Means ± SD. [b]Alveolar cell type. [c]Squamous cell carcinoma. [d]Significant vs. control DMBDD group 3 at P<0.05.

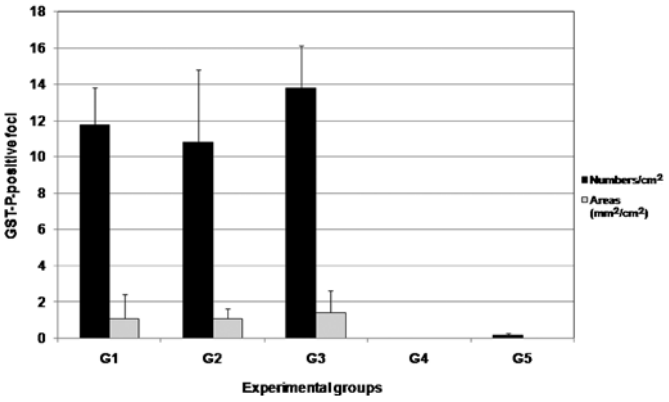

Figure 3. IHC data for the numbers (numbers/cm^2) and areas (mm^2/cm^2) of GST-P-positive foci in the rat livers in the test groups.

foci were fewer than those found in the DMBDD only-treated group 3, albeit without statistical significance. In group 5, IHC staining revealed a positive GST-P focus in one rat.

Serum biochemistry. Fig. 4 shows the serum biochemical levels of IGF-I, PGE2, TG and cholesterol in the studied groups. IGF-I levels of groups 1-3 were generally lower than those of control groups 4 and 5. *N. sativa* treatment in groups 1 and 2 significantly decreased IGF-I levels to below the control levels. No differences in IGF-I levels were found between animals treated with *N. sativa* in group 4 and those of group 5. PGE2 levels were evaluated in groups 1, 3, 4 and 5. The *N. sativa* administration significantly lowered the PGE2 levels close to the normal control levels. Additionally, serum TG levels were significantly restored close to the normal control levels, particularly in the rats of group 1 treated with 4000 ppm *N. sativa* following carcinogen administration. Serum cholesterol levels did not vary significantly among the studied groups.

Discussion

The present investigation found that two doses of *N. sativa* volatile oil, 1000 ppm and 4000 ppm in the diet, significantly inhibited tumor development in multiple sites of the rat body. To the best of our knowledge, this is the first study to simultaneously show the multi-potent effects of the *N. sativa* crude extract in various organs. Benign and malignant colon tumor incidences, multiplicities and sizes, and tumor incidences and multiplicities in the lungs as well as in different parts of the alimentary canal, particularly the esophagus and forestomach, were all significantly reduced by the two doses of *N. sativa*.

Previous and recent *in vitro* and *in vivo* studies suggest that constituents of *N. sativa* seeds have clear anticancer properties (4,19). *N. sativa* volatile oil was found to suppress ferric nitrilotriacetate-induced two-stage renal carcinogenesis in rats (1) and reduce tumors and markers of apoptosis

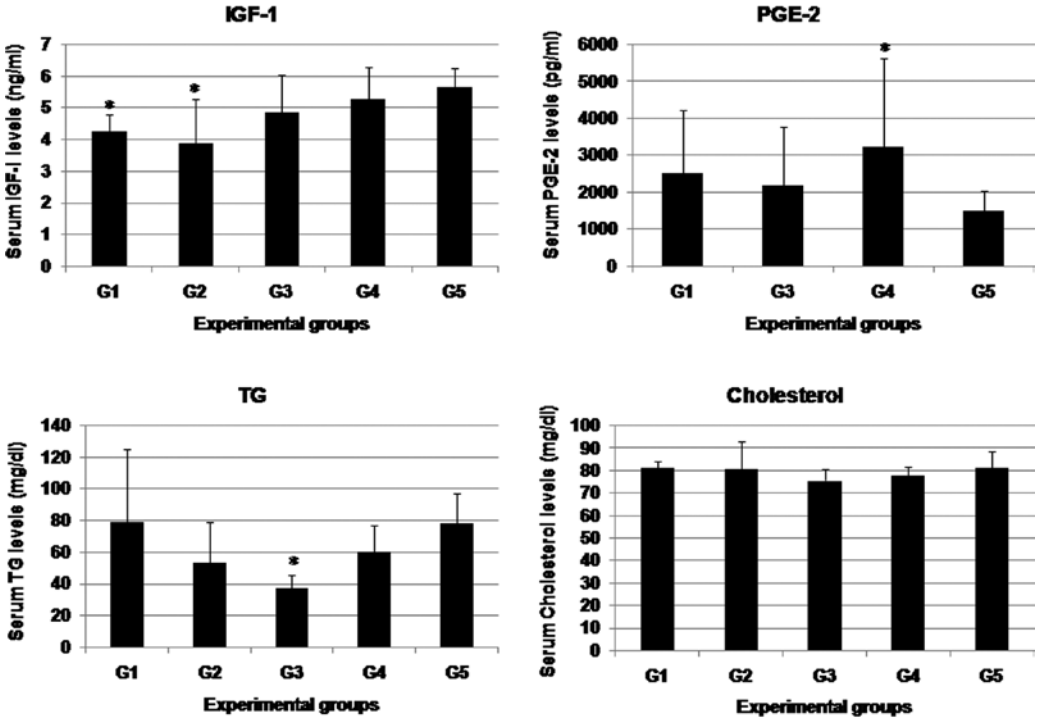

Figure 4. Serum levels of IGF-I, PGE-2, TG and cholesterol of rats in the test groups. *Significantly different vs. the other investigated groups at P<0.05.

and oxidative stress in 7,12-di-methylbenz(a)anthracene (DMBA)-induced rat mammary carcinomas (38). *N. sativa* volatile oil constituents such as thymoquinone inhibited 20-methylcholanthrene-induced fibrosarcoma tumorigenesis in male Swiss albino mice, most likely through antioxidant activity and interference with DNA synthesis, coupled with the enhancement of detoxification processes (18). Previously, the post-initiation application of *N. sativa* oil was shown to exert potent chemopreventive potential on DMBA-induced skin carcinogenesis (39) and on methylcholanthrene-induced soft tissue sarcoma development in female BALB/c mice (40). More recently, pre-exposure to the *N. sativa* extract and thymoquinone, before gemcitabine or oxaliplatin *in vitro*, resulted in a 60-80% growth inhibition of pancreatic cancer cells compared with 15-25% when gemcitabine or oxaliplatin were used alone (19).

The results of the present study confirmed previous results on the chemopreventive potential of *N. sativa* volatile oil on DMH-induced colonic aberrant crypt foci (7) and on its modulatory effects on AOM-induced colon carcinogenesis in a short-term period in rats (41). Notably, tumor sizes were decreased dose-dependently by the two doses of *N. sativa*. Previously, a combination of bee honey and *N. sativa* ground seeds was found to inhibit MNU-induced colon carcinogenesis and suppress oxidative stress markers such as nitric oxide and malondialdehyde *in vivo* (17). The majority of available data on the chemopreventive effects of *N. sativa*, and/or its bioactive extracts, were reported for colon cancer cells *in vitro*. *N. sativa* extracts had significant inhibitory effects on human colon cancer cells such as HT-29 (42) and HCT-116 (43). When its anticancer effect was compared with that of 5-fluorouracil (5-FU), the chemotherapeutic gold-standard for the treatment of colon cancer, the *N. sativa* extract had the same effects on colon cancer SW-626 cells as 5-FU but with significantly fewer side effects (44). Gali-Muhtasib *et al* (43) showed that extracts from *N. sativa* affected human colorectal cancer cells by triggering apoptotic cell death via a p53-dependent mechanism.

In the present study, marked inhibitory effects on the lung tumors were noted. To the best of our knowledge, this is the first report on the effect of *the* chemopreventive potential of *N. sativa* on lung tumors *in vivo*. Even when subgroup data did not reach significant statistical values (perhaps due to the relatively small number of tumors studied in each case), the number of tumors decreased when compared to the control group 3. Previously, Kumara and Huat (45) showed the anti-tumor activity of a column fraction 5 (CC-5) of an ethanolic extract of the *N. sativa* seeds against s.c.-implanted LL/2 Lewis lung carcinoma cells and i.p.-implanted murine P388 leukemia in BDF1 mice at doses of 200 and 400 mg/kg b.w. The extract yielded significant tumor inhibition rate values of 70% (P<0.001) and 60% (P<0.001), respectively. In another study (46), α-hederin and thymoquinone, the two principal bioactive constituents of *N. sativa* volatile oil, separately demonstrated dose- and time-dependent cytotoxic and apoptotic/necrotic effects on implanted tumors from four human cancer cell lines [A549 (lung carcinoma), HEp-2 (larynx epidermoid carcinoma), HT-29 (colon adenocarcinoma) and MIA PaCa-2m (pancreatic carcinoma)].

Numerous studies have demonstrated various modulatory effects of *N. sativa* volatile oils on respiratory diseases in experimental animals (47-53). *N. sativa* reduced the peripheral blood eosinophil count, IgG1 and IgG2a levels, cytokine profiles and inflammatory cells in lung tissues in a mouse model of allergic airway inflammation (47). Thymoquinone affected the cyclooxygenase expression and PGE2 production

of airway inflammation in mice (48), and protected against lung injury from exposure to sulfur mustard in Guinea pigs (49). Furthermore, a preventive effect of thymoquinone was detected on tracheal responsiveness and inflammatory cells in the lung lavage of sensitized Guinea pigs (50). The seed extract ameliorated lung tissue damage in rats after experimental pulmonary aspiration (51), and attenuated airway inflammation in a mouse model of allergic asthma by downregulating leukotriene biosynthesis (52). In addition, the *N. sativa* seed aqueous extract showed prophylactic effects on respiratory symptoms and pulmonary function tests in chemical war victims (53). Therefore, the effects of *N. sativa* crude oil and its bioactive constituents on lung cancer warrant further study.

N. sativa was found to exert inhibitory effects on the tumors that appeared in the epithelia of the esophagus and forestomach. After a search of the available data in PubMed, the present study is the first to show a chemopreventive potential of *N. sativa* volatile oil on esophageal tumors *in vivo*. Notably, in the forestomach, the incidences and multiplicities of P/N hyperplasias, papillomas, SCCs separately, and the sum of the lesions were significantly inhibited by *N. sativa* treatment. The results are in accordance with those of Badary *et al* (54) who found that thymoquinone inhibited mice forestomach tumor development, most likely through its antioxidant and anti-inflammatory activities, coupled with the enhancement of detoxification processes. The modulatory effects of *N. sativa* extracts on gastropathies and ulcers were noted. An aqueous suspension of black seed significantly prevented gastric ulcer formation induced by necrotizing agents in pylorus-ligated Shay rats (55). This effect of *N. sativa* was shown to be prostaglandin-mediated and/or through its antioxidant activities. In other studies (56,57), *N. sativa* volatile oil and thymoquinone, particularly the oil, protected gastric mucosa from acute alcohol-induced mucosal injury through antiperoxidative, antioxidant and antihistaminic effects. Another study (58) showed that treatment with *N. sativa* oil and thymoquinone normalized the level of gastric lesions induced in male Wistar rats by ischaemia/reperfusion, which is known to be linked to free radical formation. On the other hand, *N. sativa* oil alone tended to normalize the levels of lactate dehydrogenase, reduced glutathione and superoxide dismutase in the stomach mucosa which may be related to the conservation of the gastric mucosal redox state.

Rat GST-P, which is related to human GST-π in enzymatic and immunological properties, is used by many investigators as a reliable marker for preneoplastic lesions, since it is strongly and specifically expressed in the very early phase of chemically induced hepatocarcinogenesis, but not in normal hepatocytes (59). The degree of induction of GST-P-positive foci and nodules in the present bioassay system for hepatocarcinogenesis has been proven to correspond with the incidence of hepatocellular carcinomas observed in long-term *in vivo* assays (60,61). In the present study, the mean number of GST-P-positive foci per cm^2 and the average areas (mm^2) per cm^2 were decreased by the two *N. sativa*-treated doses, although without statistical significance. Iddamaldeniya *et al* (62) previously demonstrated that the combined effect of *N. sativa* seeds, *Hemidesmus indicus* root bark and *Smilax glabra* rhizome resulted in complete inhibition of overt liver tumors or histopathological changes leading to tumor development such as GST-P-positive foci in Wistar rats treated with DEN after long-term (16 months) post-carcinogen treatment. In addition, *N. sativa* and thymoquinone were found to protect against hepatocellular toxicity *in vitro* through induction of detoxifying agents including quinone reductase and glutathione transferase (63). *N. sativa* protected against liver toxicity induced by various agents, such as carbon tetrachloride, by downregulating hepatic cytochrome P450 isozyme mRNA and protein expression, partly due to the downregulation of NO production and the upregulation of anti-inflammatory IL-10 in rats (64). The difference between the present data on the liver and that of Iddamaldeniya *et al* (62) is due to the difference in the combination of the chemopreventive agents and the different protocols used for the two studies. The data of this study showed no clear inhibitory effects on liver carcinogenesis by *N. sativa* that may be explained, in part, by the interference of different active metabolites and the challenge of different biochemical functions in the livers of rats in this experiment. This process thus requires confirmation.

A large number of studies have investigated the precise mechanism of tumor inhibition by *N. sativa* volatile oil. For instance, thymoquinone was found to have antioxidant effects in animal models (54,58,65). These antioxidant effects were also thought to protect tissues from radiation injury (66). It has been found that polyunsaturated fatty acids constitute 83.7% of the total fat in black seeds (67), with linoleic acid (cis-C18:2 ω-6) as the major component (60.8%) and oleic acid (cis-C18:1 ω-9) constituting 21.9% of total fats. It is known that ω-3 polyunsaturated fatty acids exhibit inhibitory effects on various types of tumors in animal experiments, and epidemiological studies have shown that these fatty acids may exert a protective influence in humans (68). Tocopherols, phospholipids, phytosterols and phenols are important natural antioxidants in crude oils. Processing of vegetable oils may induce the formation of antioxidants. Previously, *N. sativa* crude seed oil and oil fractions showed a strong radical scavenging activity, and this activity correlated well with the total content of polyunsaturated fatty acids, unsaponifiables and phospholipids, as well as the initial peroxide values of crude oil (69,70). The presence of antioxidants such as tocopherols and polyphenols in oil extracted from *N. sativa* seeds may be of significance. The total tocopherol (α-, β- and γ-tocopherols) content in *N. sativa* seeds was found to be ~340 μg/g, while total polyphenols are present at 1744 μg/g (67). Naturally occurring plant phenols are widely distributed and a number of these phenols have been found to protect against chemical carcinogenesis (71). Certain plant phenols inhibit the activity of cytochrome P-450-dependent enzymes that metabolize drugs and carcinogens (72). Therefore, the possibility that polyphenols in *N. sativa* oil (67) are involved in the present chemopreventive findings deserves further investigation.

N. sativa markedly reduced the BrdU LIs in colonic, lung, esophagus and forestomach lesions. Modulation of cell proliferation by cancer chemopreventive agents in colon epithelium is widely considered to be a mechanism underlying protective actions (73). Thus, the present inhibitory effects of *N. sativa* oil on BrdU LIs may be explained by the antioxidant and antiproliferative effects of the constituents found in the volatile oil of *N. sativa*, particularly polyphenols, polyunsaturated fatty acids and thymoquinone. The antioxidant effects of

N. sativa extracts ameliorate the hyperproliferative response of cells in rat two-stage renal carcinogenesis (1). Eleostearic acid (α-ESA), a conjugated linolenic acid, blocked ER-negative MDA-MB-231 (MDA-wt) and ER-positive MDA-ERα7 human breast cancer cell proliferation and induced apoptosis through a mechanism that may be oxidation-dependent (74). Carotenoids with antioxidant properties such as astaxanthin decreased colon carcinogenesis, cellular proliferation and increased levels of lipid peroxidation (75). Thymoquinone administered to mice reduced the incidence of stomach tumors (54) via mechanisms that included inhibition of DNA synthesis and the promotion of apoptosis by inhibiting cell growth in the G1 phase. It also inhibited cellular proliferation and induced apoptosis in colon cancer cells by triggering a p53-dependent mechanism (43). Further investigation of the relevance of other genetic or biochemical factors, particularly those related to induction of apoptosis, may be of great significance.

Blood analysis data in the present study showed that *N. sativa* restored IGF-I, PGE2 and TG levels in the rats of groups 1 and 2 to almost control levels. Insulin growth factor I (IGF-I) is mainly related to kinase signaling and tumor progression (28). Prostaglandins are potent proinflammatory mediators generated through arachidonic acid metabolism by cyclooxygenase (COX)-1 and -2 in response to different stimuli, and play important roles in modulating inflammatory responses under a number of conditions. They are highly correlated to the progression of different types of tumors (76). The active constituents of *N. sativa* have been found to exhibit anti-inflammatory effects throughout the allergic response in the lung by inhibition of PGE2 synthesis and attenuation of immune responses (48). LDL and HDL were not measured separately in the present study but triglyceride levels were lowest in the DMBDD-only group and *N. sativa* restored these levels almost to control values. Lower LDL cholesterol levels and total TG are known to correlate well with favorable survival of cancer patients (77). Time-dependent measurements of TG, LDL and HDL should be performed in a similar multi-organ carcinogenesis protocol in the future. Malignant cells maximize their ability to acquire and metabolize nutrients necessary for their rapid growth and progression by fine-tuning a double (lipogenic/lipolytic)-edged metabolic sword in a manner conducive to proliferation as opposed to efficient ATP production (78). Thus, the present inhibitory effects of *N. sativa* on various tumors may correlate partly or indirectly to the three studied hematological factors. Studies on the effects of *N. sativa* on the genes controlling lipid metabolism, kinase signaling, COX-1 and -2, lipid peroxidation, glutathione metabolism, growth, cell cycle regulation and apoptosis are warranted.

Although the exact mechanisms of the chemopreventive effects of *N. sativa* oil and its constituents should be determined more precisely, particularly at the genetic and molecular levels, the present data indicate a promising cancer chemopreventive potential. In particular, it should be stressed that the doses used in the present study exerted no unfavorable side effects.

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