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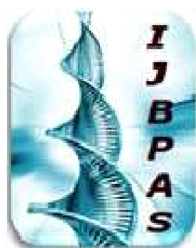

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ASSESSMENT OF ANTICANCER ACTIVITY OF *NIGELLA SATIVA* STEM – AN *IN VITRO* AND *IN VIVO* DESIGN

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ABSTRACT

Herbal medicinal plants have been used for centuries across various cultures as natural remedies for treating diseases and maintaining overall health. These plants contain bioactive compounds such as alkaloids, flavonoids, tannins, and essential oils, which contribute to their therapeutic properties. *Nigella* is a small genus of the family Ranunculaceae, which includes some popular species due to their culinary and medicinal properties, especially in Eastern Europe, Middle East, Western, and Central Asia. The aim of the present study is to investigate the anti-cancer activity of *Nigella sativa* stem ethanolic extract by *in vitro* and *in vivo*. The *in vitro* anti-cancer activity was performed by using MTT assay method. For *in vivo* study, the Institutional Animal Ethics Committee (IAEC) approved the experimental protocol and the conditions in the animal house approved by Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA). The animals were divided into four groups of 6 animals each. In *in vivo* screening performed by DMH induced colorectal changes. Results of *in vivo* pharmacological screening shows that oral administration of *Nigella sativa* stem extract brought a reduction in ACF to a small extent in comparison with normal control and only DMH treated group. There were no significant changes in the relative weight of individual organs among the groups and also in haematological parameters. Nonetheless, the main limitations are that more studies, especially, clinical trials are required to standardize the results, e.g. to establish active molecules, dosage, chemical profile, long-term effects and impact of cooking/incorporation into foods.

Keywords: Traditional system, *Nigella sativa,* anti-cancer, MTT assay, apoptosis, colon cancer

INTRODUCTION

Traditional systems of medicine continue to be widely practised on many accounts. Population rise, inadequate supply of drugs, prohibitive cost of treatments, side effects of several synthetic drugs and development of resistance to currently used drugs for infectious diseases have led to increased emphasis on the use of plant materials as a source of medicines for a wide variety of human ailments **[1, 2]**.

Cancer is most life-threatening diseases which are characterized by un-controlled cellular growth of abnormal cells anywhere in the body. Normally body forms new cells as it need cells to replace old cells which may die. Cancer makes this go wrong. Cancer makes new cells even when body doesn't require it and the old won't die **[3, 4]**.

Nigella, also known as fennel flower, is a small genus belonging to the family Ranunculaceae and includes around 20 species. The plant *Nigella sativa* seed has been an important nutritional flavouring agent and natural remedy for many ailments for centuries in ancient system of medicine including Unani, Ayurveda, Chinese and Arabic medicines. Traditionally, there is a common Islamic belief that blackseed is a panacea for all ailments, but cannot prevent aging or death. It originated from Southeastern Asia and also used in ancient Egypt, Greece, Middle east and Africa. Colon and rectal cancer are one of the common cancers among men and women from the India as per recent reports. It ranks sixth or seventh among all other cancers. The incidence (newly diagnosed cases of cancer in a year) of colon and rectal cancers in India is about 4 patients per 1,00,000 population for both sexes together.

Figure 1: *Nigella sativa* plant

The plant is found wild in Southern Europe, Northern Africa, Asia Minor and in the Mediterranean region, but has been cultivated into other parts of the world including Saudi Arabia, Mediterranean countries, Northern Africa and parts of Asia, in UAE rarely cultivated in private farms. Annual herb 30-50 cm high, pubescent or glandular-hirsute. Leaves much divided, finely pinnate, leaf-segments linear to linear-lanceolate. Flowers solitary at end of branches, blue, star-shaped, sepals 5, petaloid, oval c.7- 14 x 6-8 mm, shortly clawed; petals smaller than the sepals, 5(-8), nectariferous, with a bent claw and two lobes, stamens numerous, carpels 2-10 fused with five free styles. Fruit several follicles, crowned by persistent styles, many-seeded, brownish when ripe; seeds dark grey to black, trigonous, wrinkled, white and oleaginous inside, aromatic. *Nigella sativa* seeds were used against cough, amenorrhoea, thermogenic, carminative, diuretic, emmenagogue, anodyne, anti-bacterial, anti-inflammatory already **[2, 5-9]**.

Figure 2: ***Nigella sativa*** **stem**

The primary objective of the present research is to screen the anti-cancer activity of *Nigella sativa* stem ethanolic extract (NSEE) on cell proliferation of colon carcinoma by using *in vitro* and *in vivo*.

MATERIALS AND METHODS

Collection and authentication of plant

The stem of *Nigella sativa* was procured from the Botany Central Council for Research in Ayurveda and Siddha, Govt. of India, and authenticated by Chelladurai. V, Research Officer, Botany, Central Council for Research in Ayurveda and Siddha, Govt. of India (Certificate No.: UWAN/109/16).

Extraction of *Nigella sativa* stem

Stem material was shade dried in room temperature for 3 days and then coarsely powdered. Powdered plant material (500 g) was defatted using hexane and defatted material was extracted with (2000 ml × 3) of ethanol which was kept overnight (cold maceration) and filtered, filtrate was completely evaporated under reduced pressure using Rotavapor (MeBG) yield 13.6% w/w. The spent material after ethanol extraction was extracted with distilled water (2000 ml × 3) filtered and filtrate was completely dried under reduced pressure using Rotavapor (WtBG) yield 25.2% w/w **[10, 11]**.

Drugs and chemicals

All the drugs, chemicals and reagents were procured from S.D. Fine Chemicals, Mumbai, India. All chemicals and reagents used were of analytical reagent.

Ethical approval

The Institutional Animal Ethics Committee (IAEC) approved the experimental protocol and the conditions in the animal house approved by Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA). The study was conducted in accordance with IAEC guidelines (IAEC approval No: SIP-22XF-1S0-109).

***In vitro* anti-cancer activity [12-14]**

MTT assay:

For adherent cells, remove the medium and replace it with 100 µL of fresh culture medium. For non-adherent cells, centrifuge the micro-plate, pellet the cells, carefully remove as much medium as possible and replace it with 100 µL of fresh medium. Add 10 µL of the 12 mM MTT stock solution (prepared in step 1.1) to each well. Include a negative control of 10 µL of the MTT stock solution added to 100 µL of medium alone. Incubate at 37°C for 4 h. At high cell densities (>100,000 cells per well) the incubation time can be shortened to 2 h. Add 100 µL of the SDS-HCl solution (prepared in step 1.2) to each well and mix thoroughly using the pipette. Incubate the microplate at 37°C for 4 h in a humidified chamber. Longer incubations will decrease the sensitivity of the assay. Mix each sample again using a pipette and read absorbance at 570 nm. Percentage inhibition of the extract against all cell line was calculated using the following formula:

% of Cell inhibition = Absorbance of sample - Absorbance of blank / Absorbance of control X 100

The effects of extracts were expressed by IC_{50} values calculated from dose response curves.

***In vivo* anti-colon cancer activity [15-20]**

Animals (Male Wistar rats of 6-8 weeks old):

The experimental animal room temperature maintained at 22°C±3°C OECD guideline-

425, 2001. These ranges are designed to allow homeotherms to maintain metabolic rate or to be within their thermoneutral zones. Because, temperature below the recommended range leads to increased food intake, increased energy expenditure but decrease in efficiency. In contrast, temperature above the recommended range leads to decreased food. The relative humidity maintained at 40%-60% preferably not exceeds 70% (OECD-425, 2001). The relative humidity below the recommended range can develop lesions such as ring tail and food consumption may be increased take. 12-12 h, light/dark cycle. Appropriate lighting and light cycle play a key role in maintaining the physiology and the behaviour of rat. Light provided for adequate vision and for neuroendocrine regulation of diurnal and circadian cycles (CPCSEA guidelines for laboratory animal facility 2003).

Clinical observations:

All rats were monitored continuously for 4 h after dosing for signs of toxicity. For the remainder of the 14 days study period, animals were monitored and any additional behavioural or clinical signs of toxicity. Animal's body weight was measured prior to dosing and on days 7 and 14. On all animals were killed and at the end of the study LD_{50} value was established. Clinical observations and gross pathological examination were carried out.

DMH induced colon cancer:

Animals:

Healthy adult male Wistar rats 18 numbers are procured and assigned to three groups. In these groups Group 1 served as normal control which received vehicle (*Gum acacia* suspension), Group 2 prepared as disease control were given intra peritoneal injection of DMH (Di Methyl Hydrazine) twice a week for two conseductive weeks at a dose of 30 mg/kg body weight. And the group 3 and 4 where the same aforementioned method used to induce colorectal cancer treated with *Nigella sativa* Stem extract at a dose of 200 mg/kg and 400 mg/kg body weight daily for total 30 days of study period by oral route. The animals were kept in polypropylene cages (4 per cage) and fed standard pel-let diet for 1 week. Thereafter, the animals were randomly divided into three groups each containing 6 rats and maintained under controlled conditions of temperature (24 ± 2 °C), humidity (50 ± 10%), and 12 h light/dark cycle and tap water was provided ad libitum.

DMH had to be dissolved in 1mM EDTA just before use and pH was adjusted with NaOH to confirm the stability of carcinogen. Rats of Group 2 and 3 are given with intraperitoneal injections (i.p.) of DMH twice a week for 2 weeks at a dose of 20 mg/kg of body weight.

Group 1: Normal control which receive vehicle

Group 2: Disease control where 30 m/kg of i.p. injection of DMH given twice for 2 consecutive weeks.

Group 3: Treated group where DMH induced colon cancer symptoms were treated with prepared extract at a dose of 200 mg/kg body weight for 30 days.

Group 4: Treated group Where DMH induced colon cancer symptoms were treated with prepared extract at a dose of 400 mg/kg body weight.

Body weight changes:

The body weight changes of the control, DMH and *Nigella sativa* stem extract treated rats were measured throughout the study. The rats were weighed at the beginning of the experiment and then subsequently once a week and finally before sacrifice.

Determination of aberrant cryptic foci:

At the end of the study, rat colons were removed and flushed with potassium phosphate buffered saline (0.1 M, pH 7.2). Colons were split open longitudinally and placed on strips of filter paper with their luminal surfaces open and exposed. Another strip of filter paper was placed on top of the luminal surface. The colons were then secured and fixed in a tray containing 10% buffered formalin overnight. Each of the fixed colons was cut into proximal and distal portions of equal lengths and each portion was further cut into 2 cm long segments. Each segment was placed in a petri dish and stained with 0.2% methylene blue solution for 2 min. The segments were examined using a light microscope at low magnification to score the total number of aberrant crypt foci (ACF) as well as the number of crypts per focus. ACF were distinguished from normal crypts by their thicker, darker-stained, raised walls with elongated slit-like lumens and significantly increased distance from the lamina to basal surface of cells.

Apoptosis measurement in colonic mucosa:

Apoptosis evaluation was carried out in paraffin-embedded sections of normal colonic mucosa and tumours stained with haematoxylin-eosin. At least 20 full longitudinal crypt sections of normal mucosa/rat were scored at the microscope, determining the presence of cells in each crypt with the following characteristics of apoptosis: cell shrinkage, loss of normal contact with the adjacent cells of the crypt, chromatin condensation or formation of round or oval nuclear fragments (apoptotic bodies). When clusters of more than one apoptotic body were seen within the diameter of one cell, these bodies were considered as fragments of one apoptotic cell. Tumour apoptosis was determined by scoring at least 1000 cells/rat for the presence of apoptotic cells that were coded as described above.

Apoptotic index (AI) = Number of apoptotic cells/cells scored × 100

Haematological parameters:

Blood samples were collected for haematological analysis in Vacutainer tubes with 1.5% EDTA and differentially quantified through a Coulter T890 for the following: WBC, RBC and platelet counts and hemoglobin determination. During the course of the study, the haematological parameters of the animals were tracked on every 5th day. For this, blood was collected from the caudal vein into heparinised tubes and total WBC count and haemoglobin level were checked.

WBC count:

The whole blood was diluted using a diluent which haemolyses red cells, leaving all the nucleated cells intact. The number of white cells in a known volume and known dilution were counted using a counting chamber. 0.02 ml of blood was added to 0.38 ml of diluting fluid and mixed well. The diluted blood was charged into a Neubauer counting chamber. After 3-4 min, the total number of white blood cells in the four large corner square chambers was counted.

Total WBC = (Number of cells counted x 50) / mm^3

Determination of haemoglobin (Hb) content (Cyanmethemoglobin method):

Haemoglobin was treated with a reagent containing potassium ferricyanide, potassium cyanide and potassium dihydrogen phosphate. The ferricyanide forms methaemoglobin, which is converted to cyanmethemoglobin by cyanide. The intensity of colour formed is measured at 546 nm against blank. The optical density is directly proportional amount of hemoglobin.

Procedure: 0.02 ml of fresh whole blood was mixed with 5 ml of the cyan meth reagent. The optical density was measured at 546 nm against blank after 5 min incubation at room temperature. The OD of standard solution corresponding to 60 mg/dl haemoglobin at 546 nm was also read against reagent blank.

Haemoglobin (g/dL) = (OD of treated x 60 x 0.251) / OD of standard

During necropsy, the liver, kidneys, colon, spleen, heart, pancreas, stomach, lungs were removed, and weighed.

Statistical analysis

Values are given as means ± SD of each group. Data were analyzed by one-way ANOVA. The results are considered statistically significant at $P < 0.05$.

RESULTS AND DISCUSSION

Extraction of *Nigella sativa* stem:

Percentage yield of *Nigella sativa* stem extract by cold maceration was found to be 10.2% w/w.

***In vitro* anti-cancer activity**

MTT assay:

This is a colorimetric assay where we measure the reduction of yellow 3-(4,5-dimethyl thiazole-2-yl-)-2,5-diphenyl tetrazolium bromide (MTT) by mitochondrial succinate dehydrogenase.

When MTT get inside the cells and passes to mitochondria, where it is reduced to an insoluble coloured Formazan product. Reduction of MTT only occurs when cells are metabolically active the level of activity is measured by presence of formazan spectrophotometrically.

Table 1: ***In vitro*** **cytotoxic activity (MTT ASSAY)**

Concentration (µg/ml)	MTT assay % viability
Control	98.50±0.35
50	84.30±0.21
100	75.45±0.26
150	69.98±0.24
200	58.80±0.29
250	50.20±0.30

Body weight changes

During the experimental period 30 days, the carcinogen-exposed rats (Group 2) exhibited a significantly (P < 0.05) low gain in body weight and a low growth rate throughout the experimental period as compared to Groups 1 and 3 oral administration of *Nigella sativa* stem extract at a dose of 200 mg/kg b.w. resulted in a significant improvement in weight gain relative to treatment with DMH alone. Values are mean ± SD, n=6 in each group, statistically significant. **P < 0.01,*P < 0.05 when compared with disease control (DMH treated) group. Values are mean ± SD, n=6 in each group, statistically significant a**P < 0.01, b*P < 0.05 when compared with disease control (DMH treated) group.

Table 2: Body weight changes on treatment with ethanolic extract of ***Nigella sativa*** **stem**

Group	Initial weight (average) in g	Final weight (average) in g	Weight gain
Control	137.83 ± 15.68	158.33±9.66	20.5±10.634**
DMH treated	166.16±18.17	175.33±21.64	9.66±4.082
DMH + NSEE (200 mg/kg)	169.16±26.91	186.3±27.688	17.16±7.08*
DMH + NSEE (400 mg/kg)	149±25.16	167±14.56	18.5±5.08*

Aberrant crypt foci scoring:

ACF formation was observed in all DMH induced groups. The majority of ACF appeared in the distal colon of the rats injected with DMH. Oral administration of *Nigella sativa* stem extract at 200 mg/kg b.w. could slightly inhibit the formation as well as the total number of ACF, as compared to rats injected with DMH alone, but there was no reduction in number of aberrant crypts. Values are mean ±SD, n=6 in each group, statistically significant. **P < 0.01,*P < 0.05 when compared with disease control (DMH treated) group. Values are mean ± SD, n=6 in each group, statistically significant a**P < 0.01 when compared with disease control (DMH treated) group.

Table 3: Distribution of altered ACF category in proximal, distal and total colon of rats exposed to DMH and *Nigella sativa* stem extract

Group	No. of animals	Drug	Dose (mg/kg) & route of administration	Total no. of ACF
Proximal colon				
Control	6	Vehicle	Oral	0
DMH	6	DMH	30 mg/kg i.p.	14.66±2.33
DMH + NSEE (200 mg/kg)	6	DMH + NSEE	30 mg/kg i.p. + 200 mg/kg oral	9± 3.1**
DMH + NSEE (400 mg/kg)	6	DMH + NSEE	30mg/kg i.p. + 400 mg/kg oral	6±1.53**
Distal colon				
Control	6	Vehicle	Oral	0
DMH	6	DMH	30 mg/kg i.p.	25.83±4.3
DMH + NSEE (200 mg/kg)	6	DMH + NSEE	30 mg/kg i.p. + 200 mg/kg oral	18.5±3.08**
DMH + NSEE (400 mg/kg)	6	DMH + NSEE	30mg/kg i.p. + 400 mg/kg oral	15±2.33**
Total colon				
Control	6	Vehicle	Oral	0
DMH	6	DMH	30 mg/kg i.p.	40.49±6.3
DMH + NSEE (200 mg/kg)	6	DMH + NSEE	30 mg/kg i.p. + 200 mg/kg oral	27.5±6.18**
DMH + NSEE (400 mg/kg)	6	DMH + NSEE	30mg/kg i.p. + 400 mg/kg oral	20±5.13**

Apoptosis scoring

The apoptosis index (AI %) was estimated as the percentage of apoptotic cells among the total number of counted cells in a whole colonic crypt. The apoptotic index was increased in groups treated with DMH + NSEE compared to only DMH treated group. Values are mean ± SD, n=6 in each group, statistically significant. **P < 0.01when compared with disease control (DMH treated) group.

Table 4: Apoptosis scoring (Effect of *Nigella sativa* and DMH on apoptotic indexes)

Group	Apoptosis index
Control	3.53±0.46***
DMH	0.9±0.37
DMH + NSEE (200 mg/kg)	2±0.63**
DMH + NSEE (400 mg/kg)	2.41±0.52**

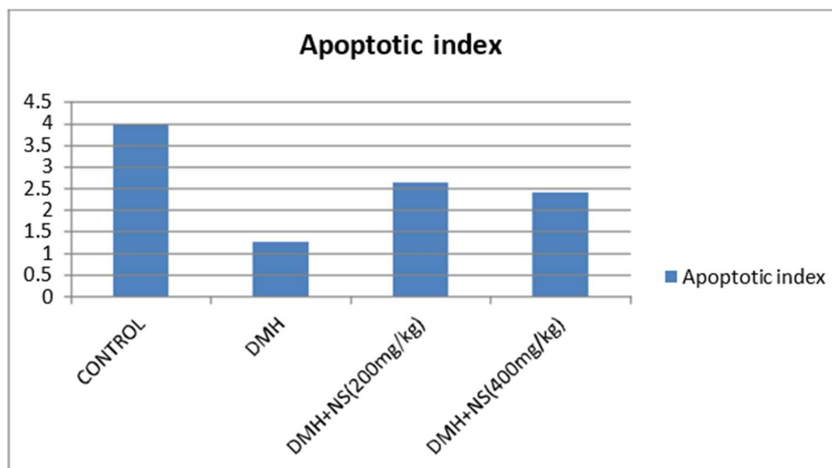

Figure 3: Effect of *Nigella sativa* and DMH on apoptotic indexes

This study performed the evaluation of anti-cancer effect of a *Nigella sativa* stem extract both *in vivo* and *in vitro* for colon cancer. *In vitro* cytotoxic study of *Nigella sativa* stem extract performed by MTT assay. Which indicated a decrease in cell viability with increased dose of extract of *Nigella sativa* stem. It is specific to colonic epithelium as a result of incomplete repair of DNA in the colon compared to other organ tissues.

In *in vivo* screening performed by DMH induced colorectal changes. DMH is a colon carcinogen. Results of *in vivo* pharmacological screening shows that oral administration of *Nigella sativa* stem extract brought a reduction in ACF to a small extent in comparison with normal control and only DMH treated group. There were no significant changes in the relative weight of individual organs among the groups and also in haematological parameters.

A common feature of GI tumours is weight loss which shows aggressiveness of the disease. Similarly, DMH treated groups shown reduction in weight gain compared to normal control rats. In this study colon were examined for ACF 30 days after the first injection of DMH i.p. Crypts were observed at proximal and distal colon. Herb based drug industry is growing day by day. The destruction of natural habitats over exploitation and unsustainable harvest has led to a severe scarcity of raw material. These problems have adversely affected the quantity of herbal drugs. In this study we used DMH as colon cancer inducer and ethanolic extract of stem of *Nigella sativa* as the test drug. The purpose of this investigation, which is a part of a large-scale study to prove the inhibitory effect of *Nigella sativa* on colon cancer using established colon cancer model.

CONCLUSION

The current work revealed that the *Nigella sativa* stem shows anti-cancer activity compared to normal. *In vivo* study of *Nigella sativa* stem extract was performed in DMH induced colon carcinogenesis. The results of present study showed that the supplementation of *Nigella sativa* to the diets of rats for 28 days did not change the haemetological parameters which illustrated normal architecture of liver and heart after treatment. It's proved by no significant changes of serum ALT and AST level in treatment group compare to the control group. Absence of pathological condition of liver tissue in histological evaluation confirmed the result. This study also found that body weights of the rats in all groups are maintained during the experiment which indicating healthy status of animals. Invitro evaluation performed by MTT assay shows decreased cell viability with increasing concentrations of extract being cytotoxic. The identification of chemical compounds presents in the stem of *Nigella sativa* stem

which are responsible for the specific activity is remaining.

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Declarations

Author contributions

All authors contributed to experimental work, data collection, drafting or revising the article, gave final approval of the version to be published, and agreed to be accountable for all aspects of the work.

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Competing interest statement

All authors declare that there is no conflict of interests regarding publication of this paper.

Ethical approval

Approved.

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