RESEARCH ARTICLE

Evaluation of the Anticancer Effects of *Nigella sativa* Stem Extract using Inhibition of Cell Proliferation

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ABSTRACT

The unregulated spread of abnormal cells across the body characterizes cancer, one of the most devastating diseases that humanity has ever encountered. The normal process of cell division occurs when the body needs new cells to replace damaged or dead cells. The study found that *Nigella sativa* stem has anticancer activity, with phenols, flavonoids, saponins, and glycosides present in its phytochemical screening. In an *in-vivo* study, rats supplemented with *N. sativa* showed no significant changes in liver and heart architecture, serum ALT and AST levels, or liver tissue pathology. We still don't know which chemical components in the stem are responsible for its anticancer action, but the study did find that participants' body weights stayed healthy.

Keywords: Cancer, *Nigella sativa*, MTT assay, Serum ALT, AST levels, Anticancer

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INTRODUCTION

Cancer is a malignancy that is distinguished by the unregulated proliferation of aberrant cells across the entire body. The body often generates fresh cells to replace cells that have been damaged or have died. Things go wrong because of cancer.[1] During carcinogenesis, oncogenesis, or tumorigenesis, normally functioning cells undergo a transformation into cancer cells. Abnormal cell division, which can eventually develop into a malignant mass, and alterations at the genetic and epigenetic levels of cells characterize the process. Initiation, promotion, conversion, and progression are the four main steps in the multi-stage process known as carcinogenesis.[1-3]

Many people still rely on older, more conventional methods of healthcare. Factors such as the growing number of infectious diseases, the increasing expense of treatments, the negative effects of many synthetic drugs, and the market's lack of drugs have shifted attention to the possibility of plant materials as a source of medications for a variety of human ailments. As Indians, we should take great pride in our rich history of alternative medicine, which includes the revered Ayurvedic system of treatment. This is a rapidly developing area of medicine where we can discover numerous natural remedies that work. Research into potential plant-based treatments for this deadly disease has thus become both an obligation and a competitive sport. Among the leading causes of death, cancer is right up there with myocardial infarction.[2-4]

Cancer kills millions of people and leaves countless more with its devastating aftereffects and repercussions. Modern medicinal science has come a long way in the last century, allowing for much better illness control. Unfortunately, there is currently no cure for cancer and many other diseases. Even though all of them are moving toward the Allopathic model of medicine, many still practice traditional or folk medicine alongside it. There has been recent success with cancer awareness campaigns and preventative measures. Where herbal remedies are most useful for warding off disease. Ancient medical systems, such as Unani, Ayurveda, Chinese, and Arabic medicine, have used the seeds of the *Nigella sativa* plant for ages as a nutritional flavouring agent and natural cure for various diseases.[3-5]

While black seed is not capable of halting the progression of time or preventing death, it is widely regarded by numerous Muslims as a miraculous remedy for various ailments. It was utilized in ancient Egypt, Greece, the Middle East, and Africa;

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it came from Southeast Asia. Recent data indicate that colon and rectal cancer is among the most frequent cancers among men and women in India. The disease disproportionately affects men and women in India. The medicinal potential of phytochemicals and medications produced from plants has recently attracted a lot of interest in the treatment of cancer and other infectious and non-infectious disorders.[4-6]

There was no scientific assessment of traditional medicine, in contrast to its alternative versions such as Siddha, Ayurveda, and Unnati. The main objective of this study is to investigate the anticancer efficacy of *N. sativa* stem extract on the growth of colon carcinoma cells by *in-vitro* and *in-vivo* studies.

MATERIALS AND METHODS

The Process of Gathering and Verifying Plant Specimens

The *N. sativa* stem was obtained from the Botany Central Council for Research in Ayurveda and Siddha, which is a government institution in India. Subsequently, the research institute at this institution verified its validity.

Extracting the Stems of *N. sativa* (Tables 1 and 2)

After three days of shade drying at room temperature, the stem material was coarsely pulverized. Hexane was used to defeat the powdered plant material. The defatted substance was subsequently dissolved in methanol, which was allowed to sit overnight before being filtered. Rotavapor was used to completely evaporate the filtrate under reduced pressure, yielding 13.6% w/w. We used distilled water to extract the byproducts of the ethanol extraction process. The filtrate was filtered and dried at reduced pressure using a Rotavapor, resulting in a yield of 25.2% w/w. Anthraquinone glycosides, flavonoids, phenols, and carbohydrates were identified in the initial phytochemical examination of the defatted methanol extract.[1-3]

Phytochemical Analysis

Carbohydrates test

It includes Molisch's test and Fehling's test etc.

Molisch's test

Separately dissolve the 300 mg of alcoholic and dried *N. sativa* leaf extract in 4 mL of distilled water. Filter the mixture. Putting the filtrate through Molisch's test is an absolute must.

Test by Fehling

It is recommended to dilute a small amount of NS stem extract in water before adding it to Fehling's solution.

Test for phenols

Apply a portion of NS stem extract onto a piece of filter paper. Apply a small quantity of phosphomolybdic acid reagent over the selected area and subject it to the vapors of ammonia.

Test for flavonoids

In order to prepare 2 to 3 mL of NS extract, it is necessary to introduce a magnesium ribbon and 1-mL of intense hydrochloric acid. Conduct a lead acetate test by adding 1-mL of lead acetate solution to 5 mL of NS stem extract.

Test for tannins

Add a 10% alcoholic ferric chloride solution to 2 to 3 mL of NS stem extract.

Test for terpenoid

Add 1-mL of chloroform to 1-mL of *N. sativa* stem extract; add 2 to 3 mL of acetic anhydride; and add 1 to 2 drops of strong sulfuric acid. If the solution is dark green in color, it means steroids are present, and if it's dark pink or red, it means terpenoids are present.

Assess for the presence of alkaloids

A minute amount of *N. sativa* stem extract was administered onto a pre-coated TLC plate, and then modified Draggendorf's reagent was placed onto the plate.

Hager's test

Apply a small amount of Hager's reagent to the *N. sativa* stem extract.

Tests for glycosides

The *N. sativa* stem extract was dissolved in pyridine, and then sodium nitroprusside solution was added. In order to convert it into an alkaline solution, sodium hydroxide was used.

Saponins

About 20 mL of distilled water was added to 1-mL of *N. sativa* stem extract, and the mixture was agitated for 15 minutes using a calibrated cylinder.

Determination of anthraquinones

Combine 1-mL of strong hydrochloric acid with 10% ferric chloride and about 50 mg of powdered *N. sativa* stem extract. Chilling the extract, filtering it, and agitating the filtrate with diethyl ether are all required steps. Additional extraction of the ether extract is carried out using highly concentrated ammonia.

Determination of amino acids

First, dissolve a tiny amount of *N. sativa* stem extract in a small amount of water. Then, add 1-mL of ninhydrin reagent to make the solution.

In-vitro Anticancer Activity

MTT assay

After filtering the MTT solution, place it in the dark to prepare a 96-well plate with freshly acidified isopropanol. Centrifugation of the micro-plate followed by cell pelleting is suggested as a means to accept non-adherent cells. Ten microliters of the 12 mM MTT stock solution should be added to every well. A suggested negative control would be to add 10 µL of the MTT stock solution to 100 µL of medium without any other additives. For optimal results, incubate the sample at 37°C for 4 hours. The incubation time can be shortened to 2 hours with higher cell densities. Use the pipette to thoroughly mix 100 µL of the SDS-HCl solution into each well. Put the microplate in a humidifier set at 37°C for four hours. The assay's sensitivity will decrease with longer incubation times. Use a pipette to mix the samples again, and then measure their absorbance at 570 nm.

Table 1: Hydrogenation of *N. sativa* stems

Name of plant	*Plant part*	*Technique*	*Used solvents*	*Yield (%)*
Nigella sativa	Stem	Maceration	ethanol	21.0

Acute toxicity study

The animals were starved overnight and their weights were measured. The initial doses of *N. sativa* stem ethanolic extract were calculated according to the body weight of each animal that had fasted, and administered orally through gavage at a dose of 2000 mg/kg. The animals were closely monitored for any abnormal behavior and general indications of toxicity after receiving the dosage for the first 24 hours. Special attention was given to the first 4 hours. Subsequently, the act of observing was consistently carried out on a daily basis for a period of 14 days. There was no mortality. However, I saw alterations in behavior, such as writhing and loose stools, when administering a dosage of 2000 mg/kg. Therefore, a dosage of 200 mg/kg and 400 mg/kg, which is equivalent to one-fifth and one-tenth of the initial dose of 2000 mg/kg, has been administered (Table 3).

Assessment of the in-vivo efficacy against colon cancer

Typically, animals were given unlimited access to food. And measured before doing the study. Within the three groups, group 1 was designated as the normal control and was administered a vehicle. In contrast, group 2 served as the control group and received an intraperitoneal injection of DMH for two weeks in a row. About 30 mg per kg of body weight was the dose given. Patients in group 3 were given *N. sativa* stem extract orally once daily at a dose after the aforementioned surgical induction of colon cancer.

Daily monitoring of all rats was conducted for a duration of 4 hours following dose to detect any indications of toxicity. During the remaining 14-day trial period, animals were carefully monitored for any additional signs of toxicity, both in terms of behavior and clinical symptoms. Prior to delivering the drug, the animal's body weight was evaluated, and subsequent assessments were conducted on days 7 and 14. Following the trial, euthanasia was performed on all animals and the LD_{50} value was calculated. An extensive analysis was performed, encompassing clinical observations and meticulous pathological evaluation.

Initially, we obtained a cohort of 18 male Wistar rats that exhibited satisfactory health conditions. Subsequently, we partitioned them into three distinct groups. Over a period of two weeks, the second group was administered intraperitoneal injections of DMH at a dosage of 30 mg/kg body weight. This group was created with the purpose of functioning as the disease control. The technique outlined above was employed in groups 3 and 4 to produce colorectal cancer. During the trial, the subjects were orally administered *N. sativa* stem extract at doses of 200 and 400 mg/kg of body weight once daily. Each of the four polypropylene cages contained the animals, which were fed a pellet diet for seven days. Afterward, six rats per group were placed in controlled environments with regulated humidity, temperature, and a 12-hour light/dark cycle. To make sure the carcinogen would be stable before use, it was dissolved in a 1-mM EDTA solution and the pH was adjusted with NaOH. The second and third groups of rats were given 20 mg/kg of body weight of DMH intraperitoneally injected twice weekly for two weeks.

Body weight changes

Rats given DMH, *N. sativa* stem extract, or a control group were observed to have their body weights tracked throughout the study. Beginning at the beginning of the trial, weekly, and finally just before the sacrifice, the rats' weights were recorded.

Identification of anomalous cryptic foci

A potassium phosphate-buffered saline wash was administered to the rats' colons once the experiment was completed. After being cut longitudinally, the colons were set on strips of filter paper with their luminal surfaces exposed and left open. The luminal surface was further covered with an additional layer of filter paper. A tray containing 10% buffered formalin was used to immobilize and protect the colons for an extended period of time. Each fixed colon had its distal and proximal ends chopped into pieces of uniform length, and those portions were further sliced into 2 cm pieces. Once each segment was placed on a petri dish, it was stained with a 0.2% methylene blue solution and left to sit for 2 minutes. By inspecting the segments at a low magnification using a light microscope, we were able to calculate the total number of ACF and crypt counts per focus. Differentiating ACF from regular crypts were the thicker, darker-stained, elevated walls. Some other characteristics that set them apart were their long, narrow lumens and the significantly greater distance between the cell base and the lamina.

Quantification of apoptosis in the colonic mucosa

Slices of normal colonic mucosa and malignancies stained with hematoxylin-eosin were used to examine apoptosis. The slices were fixed in paraffin. A total of twenty longitudinal crypt slices were obtained from healthy rat mucosa, and each of them exhibited evident indications of cellular demise, including cellular shrinkage, abnormal cell-to-cell adhesion, chromatin condensation, and the formation of round or oval nuclear fragments. Multiple apoptotic structures found within the boundaries of a single cell were identified as fragments of a single apoptotic cell. Based on the previously mentioned methodologies, tumor apoptosis was assessed by setting a minimum threshold of 1000 cells per rat to identify apoptotic cells.

Hematological Parameters

Hematological analysis was conducted on blood samples, which were collected in vacutainer tubes containing 1.5% EDTA. The samples were then differently quantified using a Coulter for WBC, RBC, and platelet counts, as well as hemoglobin determination. Throughout the trial, the animals' hematological parameters were monitored every 5th day. In this study, blood samples were obtained from the caudal vein and transferred into tubes containing heparin. The total white blood cell count and hemoglobin level were subsequently assessed.

Count of WBC

In order to dilute the whole blood, a diluent was used that causes the hemolysis of red blood cells while keeping all of the nucleated cells intact. A counting chamber was used to quantify white blood cells within a specific volume and dilution. Thoroughly blended with 0.38 mL of diluting solution was a volume of 0.02 mL of blood. To charge the diluted blood, a Neubauer counting chamber was utilized.

Hemoglobin (Hb) content determination

Hydrogen phosphate, potassium ferricyanide, and potassium cyanide were the reagents used to treat hemoglobin. Ferricyanide produces methemoglobin, which is then transformed into cyanmethaemoglobin by the influence of cyanide. The color intensity is measured at 546 nm, relative to a blank sample. The optical density is directly proportional to the concentration of hemoglobin. A solution was prepared by combining 0.02 mL of fresh whole blood with 5 mL of cyanmeth reagent. Following a period of incubation at room temperature for 5 minutes, the optical density was assessed at a wavelength of 546 nm relative to a control sample. With a reagent blank, we were able to test the standard solution's OD at 546 nm; this solution includes 60 mg/dl of hemoglobin.

Statistical analysis

The values of each group are shown as the average mean ± standard deviation. Using a one-way ANOVA, the results were analyzed. There is a level of statistical significance in the gathered data.

RESULTS AND DISCUSSION

N. Sativa Stem Extraction

The cold maceration method resulted in a yield of 10.2% w/w for the *N. sativa* stem extract.

Preliminary Phytochemical Screening (Table 2).

The stem extract of *N. sativa* underwent a series of chemical tests to identify it and the results were presented.

Molisch's test

The observation of a reddish-brown ring formation serves as an indication of the presence of carbohydrates.

Test by Fehling

The brown hue signifies the existence of carbohydrates.

Phenols test

The blue color of the spot indicates that phenols are present. If the medication's solution turns pink or red, it means that flavonoids are in it.

Shinoda test

The development of a white, flocculent precipitate demonstrated the presence of flavonoids.

Braemer's test

Any medicine that has a dark blue or greenish-gray color to it likely contains tannins.

Libermann Buchard test

The solution displays a dark green color, indicating the presence of steroids, while a dark pink or red color indicates the presence of terpenoids.

Dragendroff's test

Since the area was orange in color, it was likely caused by alkaloids.

Acute toxicity study

The animals were continuously and individually observed for changes in behavior and overall signs of toxicity after receiving the treatment over the initial 24-hour period, with specific attention given to the first 4 hours. Subsequently, daily monitoring was conducted for a duration of 14 days. The mortality rate was zero. However, certain behavioral abnormalities, a writhing response, and loose feces were noticed while administering a dosage of 2000 mg/kg (Table 4).

In-vitro Anticancer Activity

MTT assay

The colorimetric technique employed in this study involves the quantification through the action of mitochondrial succinate dehydrogenase. Upon entering the cells, MTT is transported to the mitochondria, where it undergoes reduction to form an insoluble, colored substance known as Formazan.

Weight changes

Compared to groups 1 and 3, the carcinogen-exposed rats showed significantly slower growth and a much lower gain in body weight during the 30-day experiment. Nevertheless, in comparison to DMH alone, the oral administration of 200 mg/kg body weight of *N. sativa* stem extract significantly improved weight gain. Each group's numbers are the mean plus or minus the standard deviation, and there were 6 in the sample. These values show statistical significance when contrasted with the disease control group. Each group has a sample size of 6, and the results represent the mean plus or minus the standard deviation (Table 5).

Scoring of abnormal crypt foci

Signs of ACF development were observed in all groups that were induced with DMH. The distal colon of rats treated with DMH had the highest concentration of ACF. Oral administration of *N. sativa* stem extract at a dose of 200 mg/kg body weight reduced the quantity and generation of abnormal cell formations in rats in comparison to rats injected with DMH alone. But, there was no decline in the number of abnormal crypts. The results show the mean plus or minus the standard deviation, with six people in each category. There is a statistically significant difference between these values and the illness control group. The results show the mean plus or minus the standard deviation, with six people in each category. When compared to the disease-free control group, the alterations are statistically significant (Table 5).

Apoptosis scoring

To estimate the apoptosis index, we counted all the cells in the intestinal crypt and divided them by the number of apoptotic

Table 2: A preliminary phytochemical screening was conducted on the stem extract of *N. sativa*

S. No.	*Compounds*	*Inference*
1.	Carbohydrate	-
2.	Phenol	+
3.	Flavanoid	+
4.	Tannin	-
5.	Alkaloids	-
6.	Glycosides	+
7.	Saponin	+
8.	Sterol	-
9.	Amino Acid	-
10.	Terpenoid	-

Table 3: Animal grouping

S. No.	*Group*	*Dose (mg/kg)*
1.	I	10
2.	II	60
3.	III	400
4.	IV	2000

Table 4: *In-vitro* cytotoxic activity

S. No.	*Sample conc.*	*%viability (MTT assay)*
1.	Control	97.23 ± 0.54
2.	50	85.10 ± 0.32
3.	100	76.22 ± 0.32
4.	150	70.12 ± 0.54
5.	200	59.72 ± 0.30
6.	250	51.62 ± 0.52

cells. The groups treated with DMH+NS extract had a higher apoptotic index in comparison to the group treated just with DMH. The values represent the mean ± standard deviation, with a sample size of 6 in each group. The statistical significance is 0.01 when compared to the disease control group (Table 6).

Hematological parameters

The values are shown as mean ± SD, with n = 6 in each group, and there is no statistical significance when compared to the disease control group that received DMH treatment. The hematological parameters were not significantly changed before and after treatment.

Relative weight of organs

The results show the mean ± standard deviation, with 6 participants per group. The relative weights of the organs were unchanged both before and after the therapy. A conclusion on the influence of changes in dietary patterns and the adoption of Western lifestyles on the development of colorectal cancer has been drawn in response to the increasing incidence of

Table 5: Effects of extract on body weight

Group	*Weight initial (in gms)*	*Weight (Final) (Avg)*	*Weight (gain)*
Control	138.12 ± 16.12	159.12 ± 8.33	21.5 ± 10.634**
DMH treated	165.12 ± 19.15	176.12 ± 22.25	10.66 ± 4.082
DMH+NS stem extract treated (200 mg/kg)	170.17 ± 27.92	187.4 ± 28.589	17.16 ± 8.01*
DMH+NS stemextract treated (400 mg/kg)	150 ± 26.17	168 ± 15.62	19.4 ± 5.07*

Table 6: Effect of DMH and *N. sativa* on apoptotic indexes for apoptosis scoring

Sr. No.	*Group*	*Apoptosis index*
1.	Control	4.31 ± 0.54***
2.	DMH	0.8 ± 0.41
3.	*N. sativa* + DMH	3 ± 0.54**
4.	NSSE + DMH	3.32 ± 0.60**

this disease. Colorectal cancer-related fatalities have been documented as a result of delayed diagnosis and treatment. As previously mentioned, the principle of "prevention is better than cure" has prompted me to concentrate my efforts on chemoprevention, a field that seeks to mitigate the occurrence of cancer by utilizing herbal medicines.

Hydrazine and its derivatives, such as DMH, have been demonstrated to possess carcinogenic and mutagenic properties. The presence of abnormal crypts in the colon is the earliest detectable sign of colon cancer. The observed lesions in the colon are hypothesized to be neoplastic in nature. Crypt fission refers to the process of crypts undergoing budding and branching, resulting in the formation of larger foci over time. Single crypts that undergo crypt branching or multiplication emerge within a fortnight following the injection of carcinogens. It is widely recognized that the presence of four or more crypts in an ACF is indicative of the promotion stage of colon carcinogenesis.

An *in-vitro* cytotoxic investigation was conducted on the stem extract of *N. sativa* using the MTT test. The results demonstrated a decline in cell viability as the dosage of *N. sativa* stem extract increased. The colorectal alterations generated by DMH were assessed using *in-vivo* screening. DMH is a carcinogen that can cause cancer in the colon.

The findings from the *in-vivo* pharmacological screening indicate that the oral administration of NS stem extract resulted in a modest decrease in ACF when compared to both the normal control group and the group treated solely with DMH. The groups did not exhibit any notable alterations in the relative weight of various organs or in hematological markers. Weight loss is a prevalent characteristic observed in gastrointestinal

tumors, indicating the disease's aggressive nature. This investigation involved the examination of the colon for ACF 30 days after the initial intraperitoneal injection of DMH. The herbal medicines sector is experiencing continuous growth. The extreme scarcity of raw materials has been a consequence of the degradation of natural habitats due to overexploitation and unethical harvesting practices. The aforementioned issues have had a negative impact on the production of herbal medications. Similar findings were observed with many medicinal plants and other biomolecules in the past few year research to combat cancer and related other consesequences.[7-16]

CONCLUSION

This study found that, compared to its ordinary counterpart, the stem of *N. sativa* had anticancer characteristics. Using methanol for extraction and then screening for phytochemicals is the primary emphasis of this investigation. Phytochemical research has uncovered glycosides, saponins, flavonoids, and polyphenols. The results of this study show that rats' hematological parameters and histological tests were unaffected by including *N. sativa* in their diets for 28 days. After the treatment, these tests showed that the heart and liver looked normal. When comparing the treatment and control groups' serum ALT and AST levels, no statistically significant differences were found. Thanks to histological analysis, we know the liver tissue is completely normal. Furthermore, the rats' steady weights across all groups showed that they were healthy throughout the research, which is consistent with previous findings. The specific chemical components of *N. sativa's* stem that provide its distinctive effect are the subject of ongoing research.

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