

INVITRO STUDIES ON THE EFFECT OF *NIGELLA SATIVA* IN COLON CANCER HCT-116 CELL LINES

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ABSTRACT

The seeds of *Nigella sativa* L., known as black seed are commonly used in traditional medicine by many Asian and Middle Eastern Countries to treat abdominal pain, headache, diarrhea, coughs, asthma and many other diseases. This seed extracts are found to have antioxidant and anticancer activities. Thymoquinone, is the active principle which is responsible for these beneficial effects. This work studied the effect of *Nigella Sativa* on colon cancer HCT 116 cell lines and found methanolic extracts of *Nigella Sativa* seeds has more phytochemical compounds having maximum inhibitory scavenging and anticancer activities.

KEYWORDS: *Nigella Sativa*, thymoquinone, HCT – 116 cell lines, HPLC.

INTRODUCTION

The human body is made up many living cells. These cells grow, divide, and die in an orderly process which are tightly regulated and controlled by the DNA machinery present within the cell. In adult, most cells divide either to replace dying cells or to repair. When the cells start growing out of control in a particular site, those may become abnormal cells which are then known as cancerous cell. In addition, these cancerous cells can also invade other tissues.^[1] Colorectal cancer is the development of abnormal cells in the colon or rectum. It is due to the abnormal growth of cells which has the ability to invade and spread throughout the body. Signs and symptoms may include blood in stool, bowel movement changes and loss. Colorectal cancer (CRC) is the third most common cancer in the world.^[2] Recently medicinal plants are effectively used for the treatment of colon cancer. Medicinal plants contain chemo preventive molecules. During the last 20 years 45% of drugs used for the colon cancer treatment are directly derived from plants; while the other 35% are derive chemically from natural products. The major advantage of using such compounds for cancer treatment is because of their relatively non-toxic, bio availability and anti-tumor properties.^[3] The main objective of this work was to study the effect of *Nigella Sativa* on colon cancer HCT 116 cell lines

HCT 116 cell line

Organism: *Homo sapiens*, human

Tissue: colon

Product Format: Frozen

Morphology: Epithelial

Culture Properties: Adherent

Disease: Colorectal carcinoma

Age: Adult

Gender: Male

Storage Conditions: Liquid nitrogen vapor phase

Human colorectal carcinoma (HCT116) cell line was obtained from American Tissue Culture Collection (ATCC) and grown in Roswell Park Memorial Institute medium (RPMI-1640) supplemented with 2gm of sodium bicarbonate. The pH 7.4 was maintained and the cells were incubated at 37°C with 5% CO₂ in humidified incubator. HCT116 cells (5.0 X 10⁴) were plated in 96 well plates with Serum free RPMI-1640 media aliquots with plant extracts at 0, 2, 4, 8, 16, 32, 64 and 128 µg/ml concentration in triplicates and incubated for 24 hours at 37°C in a 5% CO₂ incubator.^[4]

HCT 116 cell line Applications

This cell line is a suitable transfection host. This line has a mutation in codon 13 of the ras proto-oncogene and can be used as a positive control for PCR assays of mutation in this codon.^[5]

Nigella Sativa

Medicinal plants are paid special attention due to their low price, bioavailability and better acceptance by

patients in the treatment of human diseases.^[6] *Nigella sativa*, is also known as black cumin and panacea is a member of the Ranunculaceae family. It is approximately 20–30 cm in height and has white, yellow, pink, light blue or red flowers. The plant contains various compounds including nonvolatile oils, alkaloids, saponin, oleic and linoleic acids, thymoquinone (TQ), p-cymene, t-anethole, carvacrol, 4-terpineol and longifoline. Other components include sterols, phospholipids, tannins, resins, hydroxyl ketones, polyphenols, tocopherols and vitamins. TQ is an active component and has several immunoprotective properties effects such as antioxidant, anti-inflammatory, prevents liver and kidney toxicity.^[7]

Traditionally *N. Sativa seeds* are used as aromatic, anthelmintic, diuretic and diaphoretic. They are used as spices in cooking traditional Indian foods especially in vegetables. Tinctures obtained from these seeds are useful in diarrhea, loss of appetite and in the treatment of worms and skin eruptions. This seed oil applied externally is found to have an antiseptic property.^[8]

Role of Thymoquinone in colon cancer treatment

Natural anticancer drugs are of four classes that includes methyltransferase inhibitors, DNA damaging/pro-oxidant drugs, HDAC inhibitors (HDACi) and mitotic disrupters. Many medicinal plants and their composition have been analysed effectively in the identification and characterization of DNA methylation inhibitors. Chromatin acetylation is another major epigenetic modification that is regulated by the balanced action of histone acetyl transferases (HAT) and deacetylases (HDAC). HDAC inhibitors (HDACi) reactivate epigenetically-silenced genes in cancer cells, triggering cell cycle arrest and apoptosis. Recent evidence suggests that dietary constituents, such as the iso thiocyanates found in cruciferous vegetables, can act as HDACi. Broccoli sprouts are a rich source of sulforaphane, an isothiocyanate that inhibits HDAC activity in human colon, prostate and breast cancer cells.^[9] The main bioactive component of the volatile oil of the black seed (*Nigella sativa*) is Thymoquinone (TQ) which is a pleiotropic agent targeting multiple signaling pathways in many patho-physiological conditions. The mechanisms in which thymoquinone prevents cells from cancer are antioxidant, antiinflammatory and by inducing apoptosis.^[10] Thymoquinone causes lysosome membrane permeability and lead to leakage of lysosomal proteases, such as cathepsin B and D that induce apoptotic cell death.^[11]

Cytotoxicity studies using HCT116 cell line by MTT assay

Traditionally, the in vitro determinations of toxic effects of unknown compounds have been performed by counting viable cells after staining with a vital dye. Alternative methods used are measurement of radioisotope incorporation as a measure of DNA synthesis, counting by automated counters and others

which rely on dyes and cellular activity. The MTT system is a means of measuring the activity of living cells via mitochondrial dehydrogenases. The MTT method is simple, accurate and yields reproducible results. The key component is (3-[4, 5- dimethylthiazol-2-yl]-2, 5-diphenyl).^[12]

MATERIALS AND METHODS

Sample

Nigella Sativa seeds were bought from University of Agricultural sciences, Bengaluru. The seeds were air dried at room temperature and grinded in to powder using electric grinder and stored for experimental analysis.

Extract Preparation

Eight different solvent extracts of *N. Sativa* seeds were prepared to screen the anti-bacterial activity. Finely grinded *N. Sativa* seed powder was subjected to aqueous and seven organic solvents. The dry extract then stored at 4°C for further use.^[13]

1. Phytochemical analysis

Phytochemical analysis of *Nigella Sativa* linn seed extracts were done using the protocols described by Aisha Kamal and Iffat Zareen Ahmad^[14] for the following.

Test for Sterols - Salkowaski reaction

Tests for Alkaloids - Hager's reagent and Wagner's reagent (Iodine-potassium iodine)

Tests for Tannins - Ferric chloride reagent test and Lead acetate test

Tests for Saponins - Foam test

Tests for Phenols - Ferric chloride reagent test and Lead acetate test

Tests for Flavonoids

Test for Terpenoids

Test for cardiac glycosides

2. DPPH antioxidant assay

Principle

DPPH [1,1-diphenyl-2-picryl hydrazyl] is a stable free radical with purple colour. Antioxidants reduce DPPH to 1, 1-diphenyl-2-picryl hydrazine, colourless compound which is measured at an absorbance of 510 nm.

Procedure

In brief, 75 µl of DPPH solution; various concentration of test solution and quantity sufficient to 3 ml with HPLC grade methanol. The different concentrations tested for reference standard are 0.5, 1.0, 1.5, 2.0, 2.5 mcg/ml. The reaction mixture is mixed and incubated at 25°C for 15 minutes. The absorbance is measured at 510 nm using semi-autoanalyzer. A control reaction is carried out without the test sample.^[15]

3. HPLC analysis

Plant Extraction

10gms plant powder was extracted with 50ml methanol at 50°C for 4 hours. The methanolic extracts were filtered through Whatmann No. 1 filter paper and filtrate was evaporated to dryness. Methanolic extract (10mg/ml) was used for HPLC analysis.

Quercetin Standard: 100ug/ml prepared in methanol.

HPLC Condition

Instrument: Shimadzu LC- Prominence 20AT

Column: C18 column 250 mm x 4.6 mm, 5u particle

Mobile Phase: Linear

HPLC grade Acetonitrile (60%), HPLC grade water (40%)

Flow Rate: 1.0 ml/min

Injection volume: 10ul

Quantification of Quercetin in plant extracts:

Concentration of Standard injected: 100µg/ml

Sample concentration: 10mg/ml

Formula used for quantification of quercetin in plant extract

Quercetin (Microgram/gram) = Sample area / Standard area x Standard concentration injected x Standard concentration injected.

4. Cytotoxicity studies using HCT116 cell line by MTT assay

HCT 116 cell line was obtained from American Type Culture Collection (ATCC) (Rockville, MD USA)

(ATCC Number: CCL-247™). The steps and procedure for cell culture, Thawing, Revival and Propagation of Cells were followed as described by D. F. Basri *et al.*^[16]

Procedure

- Collected the cells when they reach about 70-80% confluency.
- Check for the viability and centrifuge the cells.
- About 50,000 cells / well were seeded in a 96 well plate and incubate for 24 hrs at 37°C, 5% CO₂ incubator.
- Add samples to be tested from 0-320µg/ml (2 fold variation) concentration in RPMI without FBS & are incubated for 24 hr.
- After incubation with test samples, add 100µl/well (50 µg /well) of the MTT (5 mg/10ml of MTT in 1X PBS) was added to the respective wells and incubated for 3 to 4 hours.
- After incubation with MTT reagent, discard the MTT reagent by pipetting without disturbing cells and add 100 µl of DMSO to rapidly solubilize the formazan.
- Measure the Absorbance at 590 nm.

Calculating Inhibition

% Inhibition = 100 – (OD of sample/OD of Control) x 100.

RESULTS AND DISCUSSION

1. Phytochemical analysis of *Nigella Sativa* linn seed extracts

Table.1: Phytochemical Analysis of *Nigella Sativa* linn. Seed Extracts

S.No	Test	Observation	Inference
1	Salkowaski reaction	Red colour was formed.	the presence of Sterols was confirmed
2	Hager's reagent	Orange colour precipitate was formed	The presence of alkaloids was confirmed
3	Wagner's reagent (Iodine-potassium iodine)	Brown flocculent precipitate was formed	The presence of alkaloids was confirmed
4	Ferric chloride reagent	Dark green colour was formed	Presence of tannins and phenols was confirmed.
5	Lead acetate test	Precipitation was obtained	Presence of tannins and phenols was confirmed.
6	Foam test	The formation of froth was observed	Presence of saponins was confirmed.
7	Tests for Flavonoids	The formation of yellow colour was observed	Presence of flavonoids was confirmed.
8	Test for Terpenoids	The formation of reddish brown colour was observed	Presence of terpenoids was confirmed.
9	Test for cardiac glycosides	A brown ring of the interface indicates a deoxysugar characteristic of cardenolides. A violet ring may appear below the brown ring, while in the acetic acid layer, a greenish ring may form just gradually throughout thin layer.	Presence of cardiac glycosides was confirmed

From Table.1 the qualitative analysis of *Nigella Sativa* seed extracts showed the presence of different phytochemical compounds such as Alkaloids, Terpenoids, tannins, flavanoids, Reducing sugar, quinines, proteins, steroids, tannins, flavanoids, saponins and Diterpens. However Terpenoids and Anthocyanins were found absent.

2. DPPH antioxidant assay

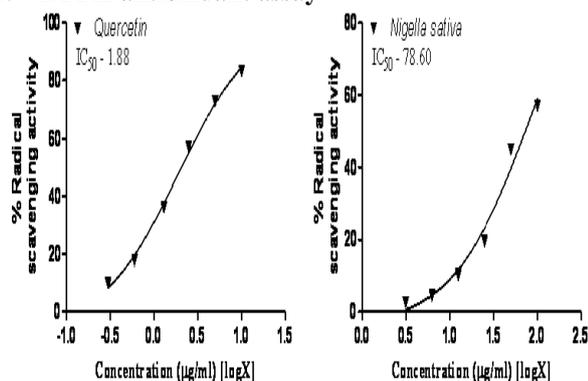


Fig.1: Radical scavenging activity of Quercetin and *Nigella sativa*

It is well known that flavanoids have the capacity to quench free radicals. The antioxidants such as flavanoids reduce DPPH to 1, 1-diphenyl-2-picryl hydrazine. Hence

the presence of phytochemical components show radical scavenging assay. Saponins also a class of natural products which react against leukaemia, lymphoma and other cancer. From Fig.1 The comparative study of DPPH scavenging assay between Quercetin and *Nigella Sativa* using IC_{50} values was found to be 78.60. It may be due to the presence of anticancer activity such as colon cancer.

3. HPLC analysis of Quercetin and *Nigella sativa*

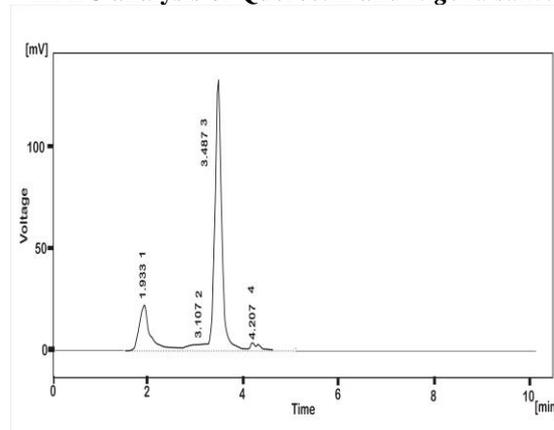


Fig.2: HPLC analysis of Quercetin

Table.2: HPLC analysis of Quercetin

	Retention time (Min)	Area (mV.s)	Height (mv)	Area (%)	Height (%)	W05 (min)
1	1.933	373.177	22.881	20.5	14.0	0.20
2	3.107	92.433	3.352	5.1	2.0	0.49
3	3.487	1296.195	133.916	71.3	81.6	0.14
4	4.207	55.054	3.869	3.0	2.4	0.22
	Total	1816.859	164.018	100.0	100.0	

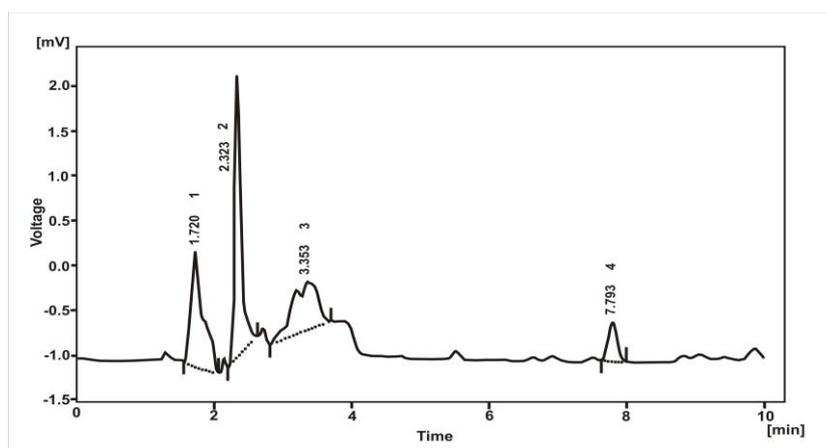


Fig.3: HPLC analysis of Quercetin content in *Nigella Sativa*

Table.3: HPLC analysis of Quercetin content in *Nigella Sativa*

	Retention time (Min)	Area (mV.s)	Height (mv)	Area (%)	Height (%)	W05 (min)
1	1.720	15.036	1.270	28.6	23.7	0.14
2	2.323	20.105	3.138	38.3	58.5	0.10

3	3.353	13.679	0.535	26.0	10.0	0.43
4	7.793	3.741	0.422	7.1	7.9	0.14
	Total	52.561	5.366	100.0	100.0	

Table.4: Quercetin content in plant extract

S.No	Sample	Area	Quercetin (mg/gm)	Quercetin (mg/gm)
1	Quercetin	1296.19		
2	<i>N.Sativa</i>	13.67	105.53	0.10

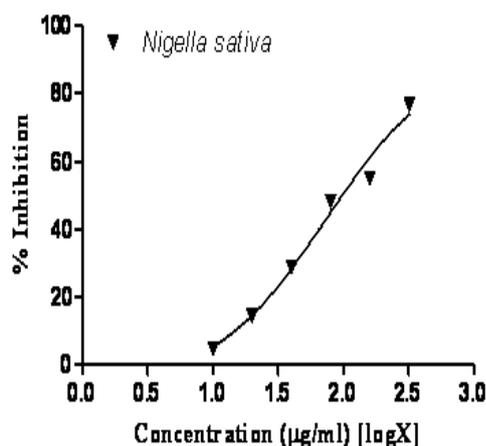
From Fig. 2, Fig.3, Table.2, Table.3 and Table. 4 the HPLC analysis of *Nigella Sativa* using Quercetin as

standard, the minimal concentration of *Nigella Sativa* was found to be 0.10mg/g.

4. Cytotoxicity studies using HCT116 cell line by MTT assay

Table: 5 Cytotoxicity study of *Nigella sativa*

Plants name	Conc. $\mu\text{g/ml}$	OD at 590 nm	% Inhibition	IC ₅₀
	Control	0.5911	0.00	
<i>Nigella Sativa</i>	10	0.5625	4.84	73.86
	20	0.5052	14.53	
	40	0.4198	28.98	
	80	0.3061	48.22	
	160	0.2651	55.15	
	320	0.1372	76.79	

**Fig.4: % Inhibition of *Nigella sativa***

From the Table.5 and Fig.4 the cytotoxicity studies using HCT116 cell line by MTT assay showed that *Nigella Sativa* has significant dose-dependent inhibition of growth of HCT116 cells at IC₅₀ values of 73.86 $\mu\text{g/ml}$. Hence the result shows that the plant extract has significant level of inhibition growth of HCT116 cells.

CONCLUSION

From this study we conclude methanolic extracts of *Nigella Sativa* seed has more phytochemical compounds and also has maximum inhibitory scavenging assay with respect to DPPH when compared to quercetin which shows these phytochemical compounds has anticancer property. Cytotoxicity studies using HCT116 cell line showed maximum IC₅₀ values of *Nigella Sativa*. Hence *Nigella Sativa* may contain better phytochemical components which may be excellent anticancer agents which can be explored by further studies.

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