Frontiers in Health Informatics ISSN-Online 2676-7104 www.healthinformaticsjournal.com

2024; Vol 13: Issue 3 Open Access

Antioxidant, Anti-Inflammatory and Anticancer Activity of Nigella *Sativa* and *Terminalia Chebula*

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Cite this paper as: Thenmozhi Marudhadurai (2024). Antioxidant, Anti-Inflammatory and Anticancer Activity of Nigella *sativa* and *Terminalia chebula.* Frontiers in Health Informatics 13(3), 10297-10312.

ABSTRACT

Aims: *The selected herbs treat many diseases, as per folkmedicinal data. Nowadays, due to the food habits of this generation, they are easily prone to various diseases and infections. This present study aims to study the anticancer activity (Colon cancer cells) of the selected powdered skin of Terminalia chebula and the powdered Nigella sativa extracts since the selected components are traditionally used to treat many diseases.*

Settings and Design: *The extract prepared separately from the powdered skin of Terminalia chebula and the powdered Nigella sativa. The individual extracts were studied for the anti-cancer activity in the basis of 1:1 ratio in the colon cancer cells.*

Methods and Material: *The following studies were performed, which are as follows: FT-IR, GC-MS, to confirm the presence of chemical constituents present in the prepared extracts. DPPH Assay for Antioxidant activity, Anti-inflammatory Assay, and MTT Assay in colon cancer cell lines performed to study for anticancer activity.*

Results: *The presence of Thymoquinone, cyclohexasiloxanedodecamethyl from the Nigellea sativa, and Terminalia chebularepectively using FT-IR & GC-MS analysis. The prepared extracts exhibit antioxidant activity at the concentration of 80 µg/ml using DPPH assay.The protein denaturation analysis proved the prepared extracts showed anti-inflammatory activity at a concentration of80 µg/ml and MTT assay showed the better results of the prepared extracts in 1:1 ration at 90.90µg/ml.*

Conclusions: *Based on the results the prepared extracts are promising chemotherapeutic agent in treating colon cancer cell. This study helps to develop the promising dosage forms in treating colon cancer cells.*

Keywords: *FT-IR, GC-MS, Anti-inflammatory, Anti-Oxidant, MTT assay.*

Key Messages:

Cancer is a leading cause of death in recent days. This study aims to prove the selected extracts have the medicinal value in treating coloncancer

INTRODUCTION

Cancer is associated with multiple diseases. Uncontrollable cell growth or masking of apoptosis triggering protein observed in cancer patients. There are various factors like environmental and chemical factors plays major role in the development of cancer. Undoubtedly, one of the biggest problems facing humanity today is the Figureht against cancer. The lack of comprehensive early detection methods, the unfavorable prognosis for those diagnosed with the disease later on, and the disease's increasing prevalence worldwide all add to the significance of cancer as a global health issue.

A colon or rectal tumour that may start off as non-cancerous polyps. A proliferation of cells in the lower part of the digestive tract is called colorectal cancer. The majority of these malignancies begin as polyps, which are benign growths. Health care experts encourage tests for persons over 45 or at high risk of cancer since removing polyps can prevent the disease.

Any plant that has chemicals in one or more of its organs that have medical value or that serve as building blocks for the creation of effective medications is considered medicinal. This description enables one to distinguish between plants that are considered medicinal but have not yet undergone a comprehensive scientific investigation, and plants whose therapeutic qualities and ingredients have been scientifically proven.

Nigella sativa seeds are used to cure a number of illnesses, including skin conditions, rheumatism, diarrhoea, asthma, and bronchitis. It has emmenagogue, appetite-stimulating, anti-diarrheal, and liver-tonic properties. It is used to treat digestive issues, boost immune system function, and combat parasite infections by encouraging nursing moms to produce more milk. Due to their very low degree of toxicity, seeds are also employed as flavouring additives in breads and pickles. Seeds can be used to treat skin outbreaks and worms. Oil is applied externally as a local anaesthetic and antiseptic. To stop the vomiting, roasted black seeds are administered internally.

In India and Iran, traditional medicine uses *Terminalia chebula* (family: Combretaceae) extensively to cure a variety of ailments, such as diabetes, constipation, and dementia. The fruit of this tree, known as halileh or halilaj in Iranian traditional medicine (ITM), is used to create remedies. In action, the harar fruit has febrifuge, diuretic, astringent, cardiotonic, and antibacterial properties.It works well as a laxative, alterative, and purgative.It is a key component of "triphala" an Ayurvedic remedy used to cure renal problems, eye conditions, sore throats, constipation, and colic discomfort.Ripe fruits are astringent, whereas unripe ones are more purgative.

In this present study *Nigella sativa* and *Terminalia chebula*are selected to study antioxidant activity, anti-inflammatory and anticancer activities since the selected species have enormous traditional medical values.

Nigella sativa seeds powder extract and *Terminalia chebula*skin powder extract prepared and performed the following analysis which are as follows phytochemical analysis, GC-MS study, FTIR analysis, Antioxidant study using DPPH assay, Anti-inflammatory activity using albumin assay and Anticancer activity using MTT assay. That the prepared extracts were significantly produced positive results.

Subjects and Methods:

Materials and Methods

Sample Preparation

Samples (*Nigella Sativa* and *Terminalia chebula*) were collected and dried using hot air oven to remove the excess moisture.

***Nigella Sativa* extract Preparation**

The powdered sample is measured about 10g using weighing machine and tied by gauze and the hexane as a solvent to prepare extract from the prepared powder with the ratio of sample to solvent 1:10.

***Terminalia Chebula* extract Preparation**

The powdered skin of *Teminaliachebula* is about 10g using weighing machine and tied by gauze and ethanol as solvent to prepare extract from the prepared powder using soxhlet apparatus.

Sample Analysis

FTIR analysis

Fourier transform infrared spectrophotometer (FTIR) is perhaps the most powerful tools for identifying the types of chemical bonds (functional groups) present in compounds. Dried powders of different solvent extracts of each plant material were used for FTIR analysis. 10mg of the dried extract powder was encapsulated in 100 mg of KBr pellet, in order to prepare translucent sample disc. The powdered sample of each plant specimen was loaded in FTIR Spectroscope (Shimadzu, IR Affinity1, Japan), with a scan range from 400 to 4000 cm-1 with a resolution of 4cm-1.

GC-MS analysis

The hexane extract of Nigella sativa and the ethanol extract of Terminalia chebula have undergone Gas Chromatography-mass spectrometry for qualitative analysis. Gas Chromatography-mass spectrometry (GC-MS) is an analytical method that combines the features of Gas Chromatography-mass spectrometry to identify different substance within a test sample. The sample solution is injected into the GC inlet where it is vapourised and swept onto a chromatographic column by the carrier gas(usually helium).The sample flows through the column and the compounds comprising the mixture of interest are separated by virtue of their relative interaction with the coating of the column(stationary phase) and the carrier gas(mobile phase).

The later part of the column passes through a heated transfer line and ends at the entrance to ion source where compounds eluting from the column are converted to ions. The sample was taken 1µl and injected with carrier gas helium into the GCMS inlet. Separation of a compound on GC by capillary column with diameter 0.25mm and length 60m with initial temperature 40oC (temperature raise in 15oC per minute until temperature reaches 290oC and end time of 10mins). The identification of compounds is done by matching the mass spectral data with the database present in the WILEY 9th library and NIST 14th library.

Antioxidant Activity

1, 1 Diphenyl 2- Picryl Hydrazyl is a stable (in powder form) free radical with red color which turns yellow when scavenged. The DPPH assay uses this character to show free radical scavenging activity. The scavenging reaction between (DPPH) and an antioxidant (HA) can be written as, DPPH-H + (A)□(DPPH) + (H-A). Antioxidants react with DPPH and reduce it to DPPH-H and as consequence the absorbance decreases. The degree of discoloration indicates the scavenging potential of the antioxidant compounds or extracts in terms of hydrogen donating ability. Different volumes (2 - 20µl) of plant extracts were made up to 40µl with DMSO and 2.96ml DPPH (0.1mM) solution was added. The reaction mixture was incubated in dark condition at room temperature for 20 min. After 20 min, the absorbance of the mixture was read at 517 nm. 3ml of DPPH was taken as control. The % radical scavenging activity of the plant extracts was calculated using the following formula,

%RSA =[(Abs control –Abs sample)/Abs control]*100

Where, RSA is the Radical Scavenging Activity; Abs control is the absorbance of DPPH radical + ethanol; Abs sample is the absorbance of DPPH radical + plant extract.

Anti-inflammatory Activity

A drug or treatment that decreases inflammation or swelling is said to be anti-inflammatory or antiphlogistic. Approximately half of analgesics are anti-inflammatory medications, also referred to as anti-inflammatories. Protein denaturation inhibition: With a few minor adjustments, the methods of Sakat et al. [12] and Mizushima and Kobayashi [11] were used to assess the inhibition of protein denaturation. 500 µL of 1% bovine serum albumin was added to 100 µL of plant extract. This mixture was kept at room temperature for 10 minutes, followed by heating at 51°C for 20 minutes. The resulting solution was cooled down to room temperature and absorbance was recorded at 660 nm. Acetyl salicylic acid was taken as a positive control. The experiment was carried out in triplicates and percent inhibition for protein denaturation was calculated using:

% Inhibition=100− ((A1 -A2)/A0)*100)

Where A1 is the absorbance of the sample, A2 is the absorbance of the product control and A0 is the absorbance of the positive control.

Anticancer Activity

Anticancer activity of the prepared extracts using was studied with HT-29 (Human colon cancer cells) by MTT assay.

Cell culture

HT-29 (Human colon cancer cells) cell line was purchased from NCCS, Pune and were cultured in liquid medium (DMEM) supplemented 10% Foetal Bovine Serum (FBS), 100 ug/ml penicillin and 100 µg/ml streptomycin, and maintained under an atmosphere of 5% CO2 at 37oC.

MTT Assay

The Thymoquinone & Chebulagic acid sample was tested for in vitro cytotoxicity, usingHT-29 cells by MTT assay. Briefly, the cultured HT-29 cells were harvested by trypsinization, pooled in a 15 ml tube. Then, the cells were plated at a density of 1×105 cells/ml cells/well (200 µL) into the 96-well tissue culture plate in DMEM medium containing 10 % FBS and 1% antibiotic solution for 24-48 hour at 37°C. The wells were washed with sterile PBS and treated with various concentrations of the Thymoquinone & Chebulagic acid sample in a serum free DMEM medium. Each sample was replicated three times and the cells were incubated at 37°C in a humidified 5% CO2 incubator for 24 h. After the incubation period, MTT (20 µL of 5 mg/ml) was added into each well and the cells incubated for another 2-4 h until purple precipitates were clearly visible under an inverted microscope. Finally, the medium together with MTT (220 µL) were aspirated off the wells and washed with 1X PBS (200 µl). Furthermore, to dissolve formazan crystals, DMSO (100 µL) was added and the plate was shaken for 5 min. The absorbance for each well was measured at 570 nm using a micro plate reader (Thermo Fisher Scientific, USA) and the percentage cell viability and IC50 value was calculated using Graph Pad Prism 8.0 software (USA).

Results

Sample Preparation

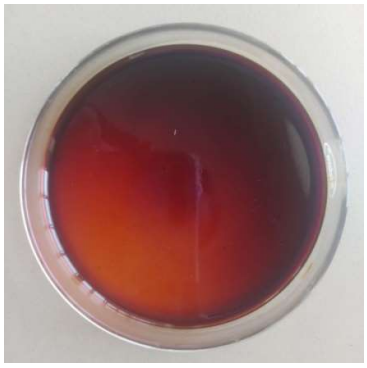

Figure 3:Extract of Nigella sativa

Figure 4:Extract of Terminalia chebula

Sample Analysis

FTIR Analysis

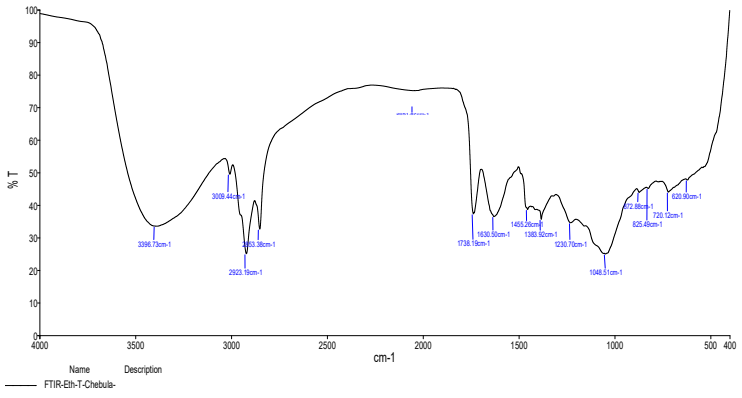

Figure 5: FT-IR Analysis for *Terminalia Chebula*

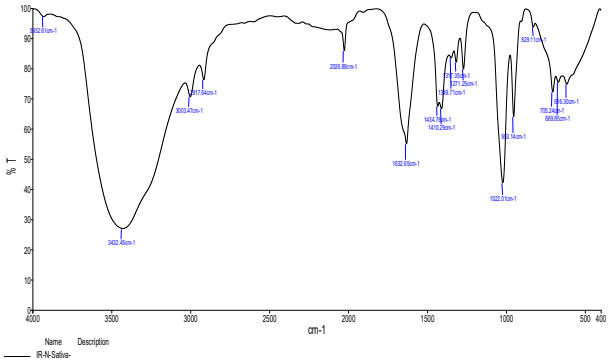

Figure 6: FT-IR Analysis for *Nigella Sativa*

GC-MS Analysis

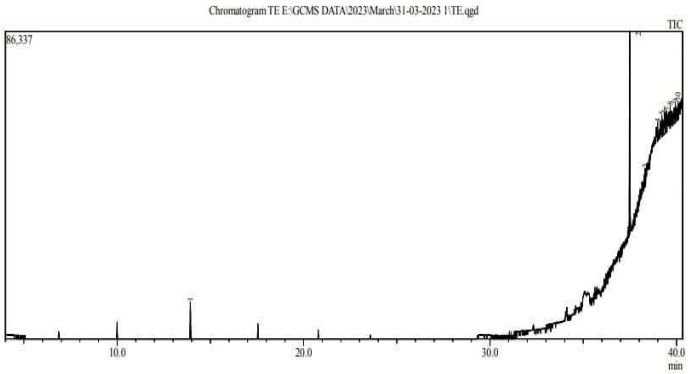

Figure 7: GC-MS Analysis of Prepared extract of *Terminaliachebula*

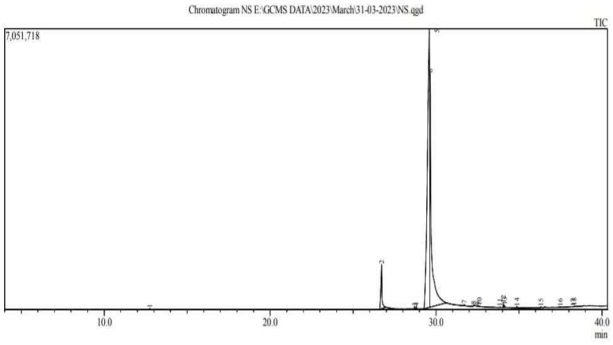

Figure 8:GC-MS analysis of prepared extract of Nigella sativa

Antioxidant Analysis

Table 8: Anti-oxidant Activity of *Terminalia chebula*

S.NO	CONCENTRATION OF EXTRACT(µl)	ABSORBANCE	%RSA
1	20	0.288	80.19
2	40	0.261	82.04
3	60	0.236	83.76
4	80	0.203	86.03
5	100	0.194	86.65

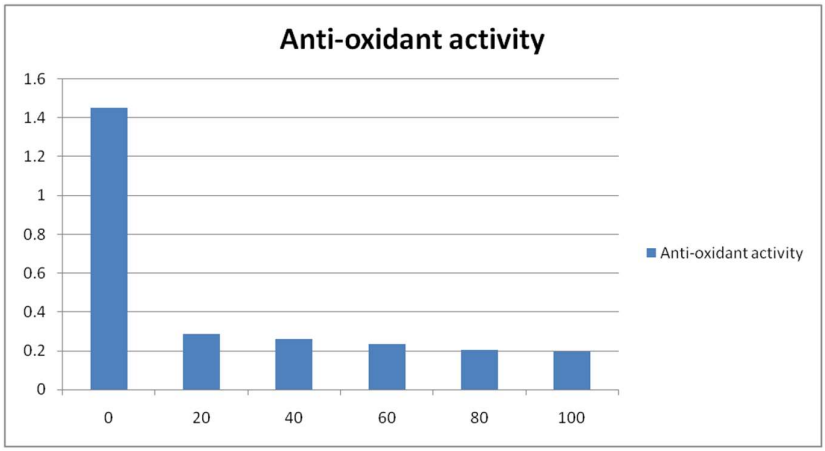

Figure 9: Anti-oxidant analysis(graph) for *Terminalia chebula*

Table 7: Anti-oxidant activity of Nigella sativa

S.NO	CONCENTRATION OF EXTRACT(µl)	ABSORBANCE	%RSA
1	20	0.192	87.91
2	40	0.177	88.86
3	60	0.158	90.05
4	80	0.134	91.56
5	100	0.122	92.32

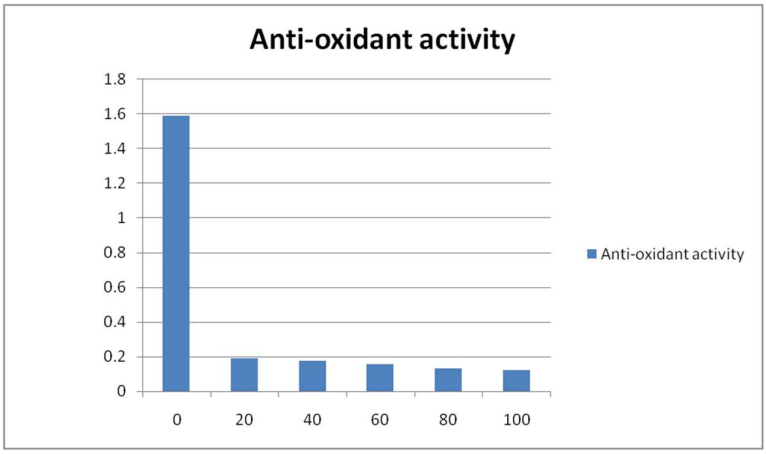

Figure 10: Anti-oxidant analysis(graph) for *Nigella sativa*

Anti-inflammatory activity

Table 10 : Anti-inflammatory activity for *Terminalia chebula*

S.NO	CONCENTRATION OF EXTRACT(µl)	ABSORBANCE	% INHIBITION OF DENATURATION
1	20	0.172	92.4
2	40	0.418	80.1
3	60	0.793	61.35
4	80	1.265	37.75
5	100	1.696	16.2

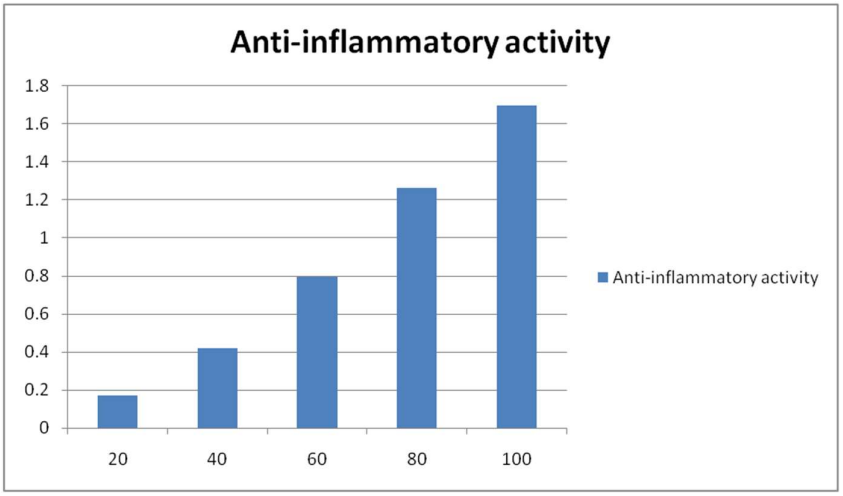

Figure 11: Anti-inflammatory analysis(graph) for *Terminalia chebula*

Table 9: Anti-inflammatory activity for *Nigella sativa*

S.NO	CONCENTRATION OF EXTRACT(µl)	ABSORBANCE	% INHIBITION OF DENATURATION
1	20	0.336	84.2
2	40	0.542	73.9
3	60	0.612	70.4
4	80	0.785	61.75
5	100	1.674	17.3

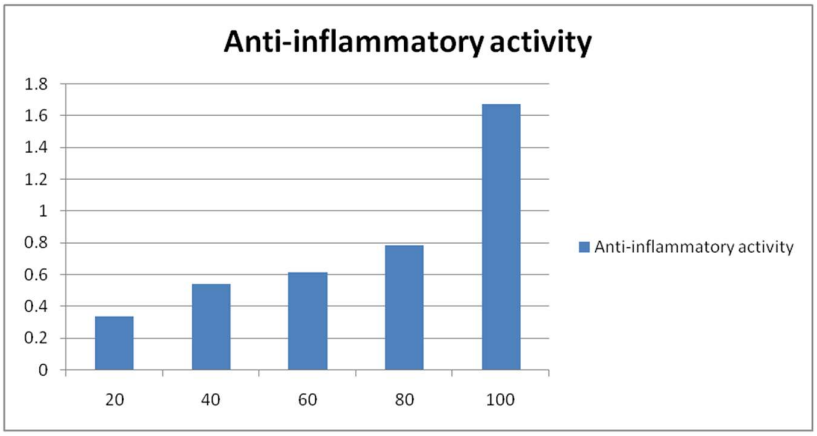

Figure 23: Anti-inflammatory analysis(graph) for *Nigella sativa*

5.5.MTT ASSAY FOR CELL CYTOTOXICITY

A.OD Value at 570 nm

Table 11: OD value at 570nm in MTT Assay in cytotoxicity test Control Mean OD value:0.952

S. No	Tested sample concentration (µg/ml)	OD Value at 570 nm (in triplicates)		
1	Control	0.869	0.998	0.991
2	1 µg/ml	0.712	0.42	0.483
3	5 µg/ml	0.424	0.854	0.336
4	10 µg/ml	0.404	0.672	0.425
5	15 µg/ml	0.234	0.547	0.541
6	50 µg/ml	0.524	0.462	0.333
7	100 µg/ml	0.318	0.44	0.409
8	200 µg/ml	0.273	0.224	0.229
9	300 µg/ml	0.215	0.194	0.216
10	400 µg/ml	0.184	0.162	0.216
11	500 µg/ml	0.164	0.172	0.18

B. Cell Viability (%)

Table 12: Cell viability in MTT Assay

S. No	Tested sample concentration (µg/ml)	Cell viability (%) (in triplicates)			Mean Value (%)
1.	Control	100	100	100	100
2.	1 µg/ml	74.78	44.11	50.73	56.54
3.	5 µg/ml	44.53	89.70	35.29	56.50
4.	10 µg/ml	42.43	70.58	44.64	52.55
5.	15 µg/ml	24.57	57.45	56.82	46.28
6.	50 µg/ml	55.04	48.52	34.97	46.17
7.	100 µg/ml	33.40	46.21	42.96	40.85
8.	200 µg/ml	28.67	23.52	24.05	25.41
9.	300 µg/ml	22.58	20.37	22.68	21.87
10.	400 µg/ml	19.32	17.01	22.68	19.67
11.	500 µg/ml	17.22	18.06	18.90	18.06

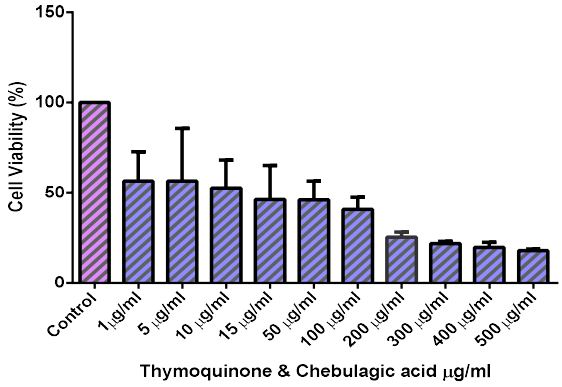

Figure 12: Cell viability(graph) for extracts of *Nigella sativa & Terminalia chebula*

C.IC50 Value of tested sample: 90.90 µg/ml

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Table 13: IC50 value of tested samples in MTT Assay

log(inhibitor) vs. normalized response -- Variable slope	
Best-fit values	
LogIC50	1.959
HillSlope	-1.513
IC50	90.90
Std. Error	
LogIC50	0.1316
HillSlope	0.5843
95% Confidence Intervals	
LogIC50	1.689 to 2.228
HillSlope	-2.710 to -0.3166
IC50	48.88 to 169.0
Goodness of Fit	
Degrees of Freedom	28
R square	0.6045
Absolute Sum of Squares	27577
Sy.x	31.38
Number of points	
Analyzed	30

D. **Images of control cells and Extracts of *Nigella sativa & Terminalia chebula*treated cells**

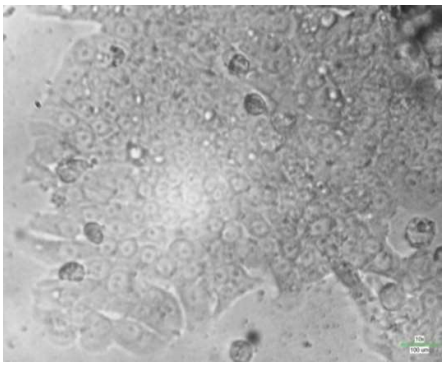

Figure 13:Control cell

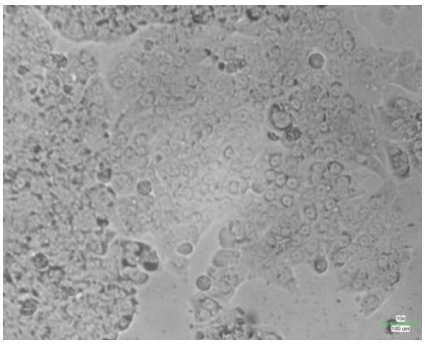

Figure 14:Extracts of *Nigella sativa & Terminalia chebula*1 µg/ml

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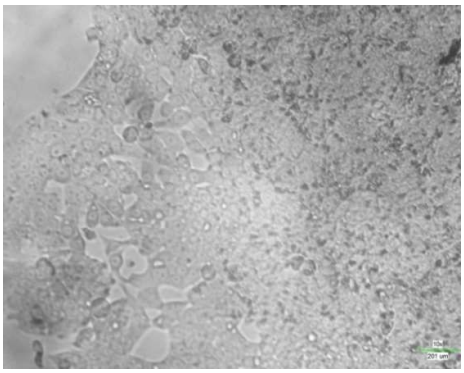

Figure 15:Extracts of *Nigella sativa & Terminalia chebula*10 µg/ml

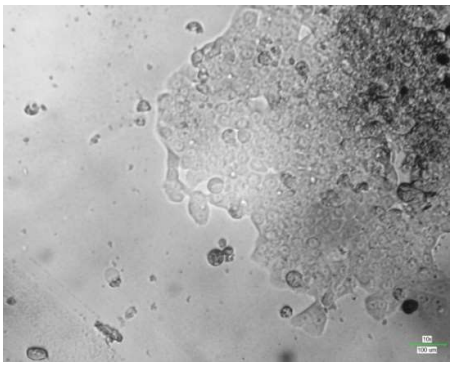

Figure 16: Extracts of *Nigella sativa & Terminalia chebula*50 µg/ml

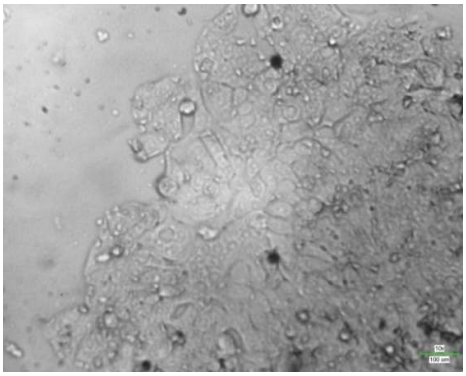

Figure 17:Extracts of *Nigella sativa & Terminalia chebula*100 µg/ml

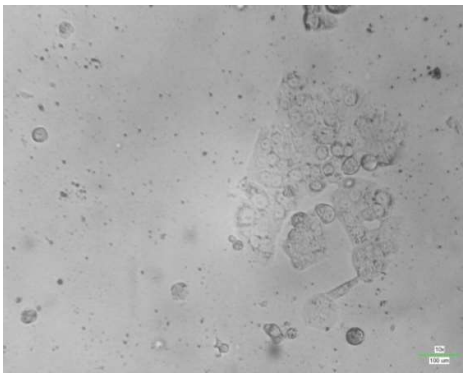

Figure 18:Extracts of *Nigella sativa & Terminalia chebula*300 µg/ml

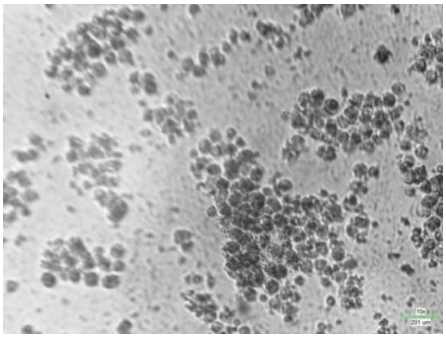

Figure 19: Extracts of *Nigella sativa & Terminalia chebula*500 µg/ml

S.NO	CONCENTRATION OF EXTRACT(µl)	ABSORBANCE	% INHIBITION OF DENATURATION
1	20	0.336	84.2
2	40	0.542	73.9
3	60	0.612	70.4
4	80	0.785	61.75
5	100	1.674	17.3

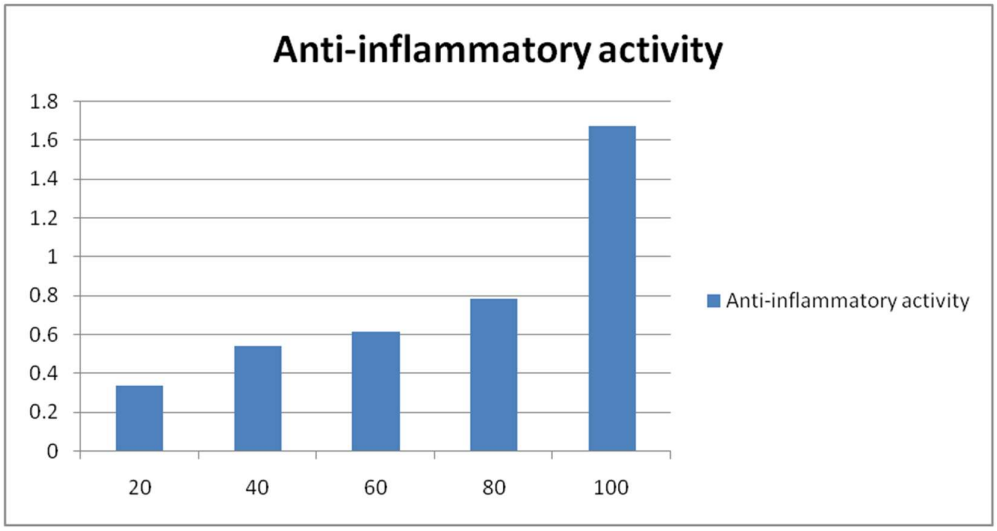

Figure 20: Anti-inflammatory Analysis(graph) for *Nigella sativa*

5.5.MTT ASSAY FOR CELL CYTOTOXICITY

A.OD Value at 570 nm

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S. No	Tested sample concentration (µg/ml)	OD Value at 570 nm (in triplicates)		
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22	500 µg/ml	0.164	0.172	0.18

B. Cell Viability (%)

Table 12: Cell viability in MTT Assay

S. No	Tested sample concentration (µg/ml)	Cell viability (%) (in triplicates)			Mean Value (%)
12	Control	100	100	100	100
13	1 µg/ml	74.78	44.11	50.73	56.54
14	5 µg/ml	44.53	89.70	35.29	56.50
15	10 µg/ml	42.43	70.58	44.64	52.55
16	15 µg/ml	24.57	57.45	56.82	46.28
17	50 µg/ml	55.04	48.52	34.97	46.17
18	100 µg/ml	33.40	46.21	42.96	40.85
19	200 µg/ml	28.67	23.52	24.05	25.41
20	300 µg/ml	22.58	20.37	22.68	21.87
21	400 µg/ml	19.32	17.01	22.68	19.67
22	500 µg/ml	17.22	18.06	18.90	18.06

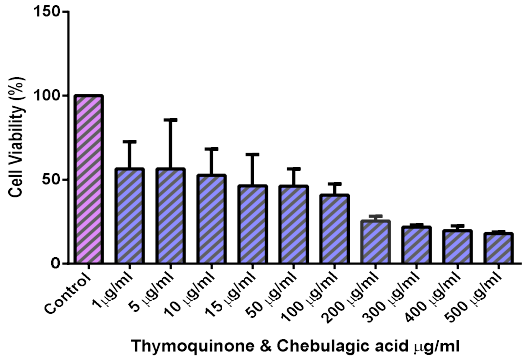

Figure 21: Cell viability(graph) for extracts of *Nigella sativa & Terminalia chebula*

C.IC50 Value of tested sample: 90.90 µg/ml

Table 13: IC50 value of tested samples in MTT Assay

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LogIC50	1.689 to 2.228
HillSlope	-2.710 to -0.3166
IC50	48.88 to 169.0
Goodness of Fit	
Degrees of Freedom	28
R square	0.6045
Absolute Sum of Squares	27577
Sy.x	31.38
Number of points	
Analyzed	30

D. **Images of control cells and Extracts of *Nigella sativa & Terminalia chebula*treated cells**

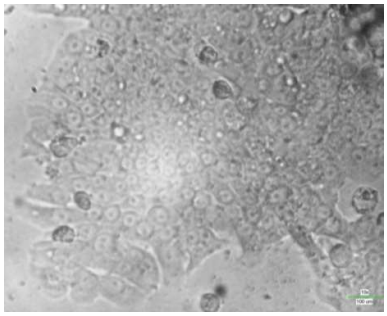

Figure 22:Control Cell

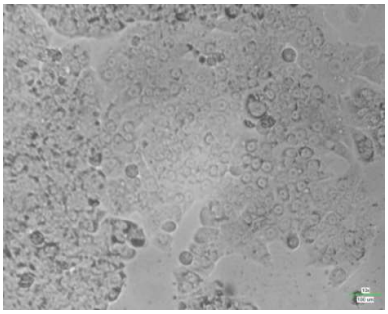

Figure 23:Extracts of *Nigella sativa & Terminalia chebula*1 µg/ml

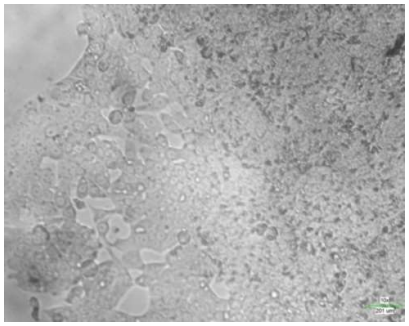

Figure 24:Extracts of *Nigella sativa & Terminalia chebula*10 µg/ml

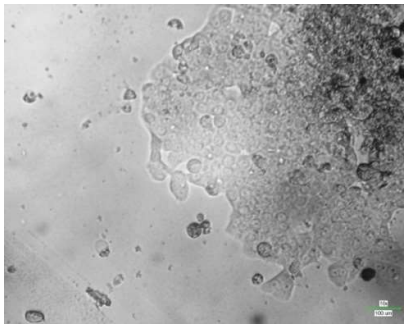

Figure 25: Extracts of *Nigella sativa & Terminalia chebula*50 µg/ml

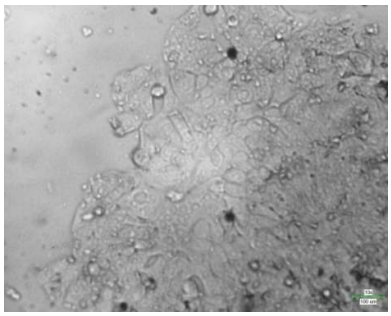

Figure 26:Extracts of *Nigella sativa & Terminalia chebula*100 µg/ml

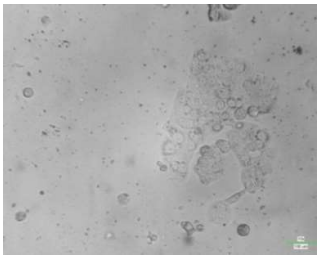

Figure 27:Extracts of *Nigella sativa & Terminalia chebula*300 µg/ml

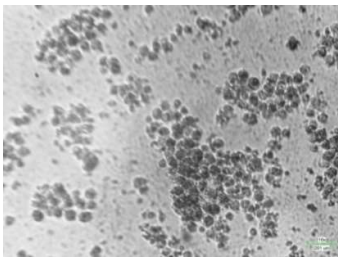

Figure 28:Extracts of Nigella*sativa & Terminalia chebula*500 µg/ml

Discussion

The extraction process was successfully carried out by Soxhlet extractor. The crude extracts are extracted from *Nigellasativa &Terminalia chebula.*

FTIR Analysis

Based on the FTIR results of *Nigella Sativa* seeds extractsconfirms the presence of functional groups like amines, alkanes, acids, ester, alkyl, andalkenes because the peaks are observed in the range from 3300 cm^{-1} to 800 cm^{-1}.

Based on the FTIR results of *Terminalia chebula* skin extract confirms the presence of hydroxyl stretch, C-H stretch, carboxylic stretch, aromatic stretch, -C-O- stretch because of the peaks observed 3200 cm^{-1} to 1000 cm^{-1}.

GC-MS analysis

GC-MS analysis helps to identify the components presence in the selected herbal components of *Nigella sativa* and *Terminalia chebula*. Based on the results confirms the presence of thymoquinone in the *Nigella sativa* extract as well as cyclohexasiloxane presence in the prepared extracts on *Terminalia chebula*skin extract.

Antioxidant and Anti-inflammatory

Based on the antioxidant and anti-inflammatory results shows the IC_{50} value of 50µl of the prepared *nigella sativa* and *terminalia chebula*shows better antioxidant as well as anti-inflammatory activity.

MTT assay

Based on the cytotoxicity analysis we performed the combination of the pure extracts given to the colon cancer cell lines and the extracts showed the significant anticancer activity against the colon cancer cell lines.

In this present aims to identify the potential of the combination of the herbal components activity in colon cancer cell lines since there are various studies performed in the single herbal extract in various cancer cell line study. But present investigation helps to understand the combination of two extracts in controlling cancer progression. Our results suggest that these sampleshave promising anticancer potential and could be considered as a promising chemotherapeutic agent to treat the cancer.

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