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Research Article

In vitro Cytotoxicity Studies of *Anaphalis neelgherryana* DC. Leaves and Barks against Human Colorectal Cancer Cell Lines (HCT 15)

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Abstract

Medicinal plants have been used for the cure of various diseases all over the world before the invention of clinical drugs. The use of medicinal plants for treatment and control of diseases has been gaining importance worldwide particularly in developing countries. Cancer is the most dreadful and one of the leading causes of death all around the world. Colorectal cancers are the third most common cancer in both men and women. A number of chemical compounds and their derivatives reported from a vast variety of medicinal plants are accepted as alternative medicine for cancer. With this back ground, the present study is focused on evaluating the cytotoxic effects of ethanolic extracts of *Anaphalis neelgherryana* leaves and barks against HCT -15 (Human Colorectal Carcinoma) cell lines using MTT assay. Ethanolic extracts of bark showed the strongest cytotoxic activity against the cell lines. The results revealed that the cytotoxic potential of *A. neelgherryana* increased when the concentration of plant extracts increases. The present study may provide the beacon for further purification to obtain a potentially active and pure compound from *A. neelgherryana* for effective anticancer drugs.

INTRODUCTION

Medicinal plants have been used for centuries as remedies for human diseases they contain components of therapeutic value. Leaves, flowers, stems, roots, seeds, fruit and bark can all be constituents of herbal medicines. The medicinal values of these plants lie in their component phytochemicals, which produce definite physiological actions on the human body. India is bestowed with a very rich wealth of medicinal plants which in turn are a source of genetic diversity. Many of these indigenous medicinal plants are also used for medicinal purposes. Herbal drugs or medicinal plants, their extracts and their isolated compounds have demonstrated spectrum of biological activities. Such natural medicines have been used and continued to be used as medicine in folklore or food supplement for various disorders. Ethnopharmacological studies on such herbs/medicinally important plants continue to interest investigators throughout the world. Many medicinal plants are used in modern medicine where they occupy a very significant place as raw material for important drugs and plants used in traditional system of medicine in pharmaceutical houses are collected from wild sources (Audu *et al*., 2007).

The use of medicinal plant extracts for the treatment of human diseases is an ancient practice; this has greatly increased in recent years. For a long time, plants are being used in the treatment of cancer (Richardson, 2001). Natural compounds have provided many effective anticancer agents in current use. Currently, over 50% of drugs used in clinical trials for anticancer activity were isolated

from natural sources or are related to them (Newman and Gragg, 2007). On the whole, cancer is a disease in which there is uncontrolled multiplication and spread within the body of abnormal forms of the body's own cell, is the second leading cause of more than six million deaths each year in the world.

Cancer is a major public health problem in the world. Colorectal cancer is also a major cause for cancer deaths. Since chemotherapy has its own side effects and developing multidrug resistance, finding natural compounds from medicinal plants has become inevitable. Plant derived drugs are desired for anticancer treatment as they are natural and readily available. They can be readily administered orally as part of patient's dietary intake. Also, being naturally derived compounds from plants they are generally more tolerated and non-toxic to normal human cells (Unnati *et al*., 2013). The members of Asteraceae are a source of many biologically active compounds. Many species of the Asteraceae family are used as remedies for many health problems, including tumor diseases, which are the second most common disease of death (Smolarz *et al*., 2012).

The objective of the study was to examine *in vitro* cytotoxic properties of ethanol extracts from the leaves and barks of *Anaphalis neelgherryana*, DC. an important medicinal plant belonging to the family Asteraceae, rare and endemic to Southern Western Ghats.

MATERIALS AND METHODS

For the present study, *Anaphalis neelgherryana*, DC. plants are collected from Nilgiris, southern Western Ghats, Coimbatore, Tamil Nadu and authenticated by Botanical Survey of India (The voucher number is BSI/SRC/5/23/2018/Tech-1830 dt. 1.80.2015).

In vitro Cytotoxicity Studies

Cell lines and Culture medium

HCT-15 (Human Colorectal carcinoma) cell lines were procured from National Centre for Cell Sciences (NCCS), Pune, India. Stock cells were cultured in DMEM supplemented with 10% inactivated. Fetal Bovine Serum (FBS), Penicillin (100 IU/ml), Streptomycin (100 µg/ml) and Amphotericin B (5 µg/ml) in an humidified atmosphere of 5% CO_2 at 37°C until confluent. The cells were dissociated with TPVG solution (0.2% trypsin, 0.02% EDTA, 0.05% glucose in PBS). The stock cultures were grown in 25cm culture flasks and all experiments were carried out in 96 microtitre plates (Tarsons India Pvt. Ltd., Kolkata, India).

MTT ASSAY (Francis and Rita, 1986)

MTT assay is based on the ability of viable cells with active mitochondria to produce succinate dehydrogenase enzyme which cleave the tetrazolium rings of MTT (Mosmann, 1983) where the optical density (OD) obtained was proportional to the number of healthy viable cells.

Principle

The reduction of tetrazolium salts is now widely accepted as a reliable way to determine cell proliferation and viability. In metabolically active cells, the yellow, water soluble tetrazolium salt MTT [3, (4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl-tetrazolium bromide] is converted to purple, water insoluble formazan by dehydrogenase enzymes of the mitochondria. The resulting intracellular formazan is directly proportional to the number of metabolically active cells. If the purple colour does not appear, then MTT is not metabolized, indicating that no live cells are present. Percentage of cell viability was determined by spectrophotometric determination of accumulated formazan derivative in treated cells at 570 nm in comparison with the control.

Preparation of Test Solutions

For cytotoxicity studies, each weighed test samples were separately dissolved in distilled DMSO and volume was made up with DMEM supplemented with 2% inactivated FBS to obtain a stock solution of 1mg/ml concentration and sterilized by filtration. Serial dilutions were prepared from this for carrying out cytotoxic studies.

Procedure

The monolayer cell culture was trypsinized and the cell count was adjusted to $1X10^6$cells/ml using DMEM containing 10% FBS. To each well of the 96 well microtitre plate, 0.1ml of the diluted cell suspension (approximately 10,000 cells) was added. After 24h, when a partial monolayer was formed, the supernatant was flicked off, washed the monolayer once with medium and 100 µl of different test concentrations of test drugs were added on to the partial monolayer in microtitre plates. The plates were then incubated at 37°C for 3 days in 5% CO_2 atmosphere and microscopic examination was carried out and observations were noted every 24h interval. After 72h, the drug solutions in the wells were discarded and 50 µl of MTT in PBS was added to each well.

The plates were gently shaken and incubated for 3h at 37°C in 5% CO_2 atmosphere. The supernatant was removed and 100 µl of propanol was added and the plates were gently shaken to solubilize the formed formazan. The absorbance was measured using a microplate reader at a wavelength of 570nm. The percentage of growth inhibition was calculated using the following formula and concentration of test drug needed to inhibit cell growth by 50% (CTC_{50}) values is generated from the dose-response curves for each cell line.

$$\% \text{ of Growth Inhibiton} = \frac{\text{Mean OD of Individual test group}}{\text{Mean OD of Control group}} \times 100$$

RESULTS AND DISCUSSION

In vitro cytotoxicity studies

Cancer is a serious clinical problem that possesses significant social and economic challenges to the health care system. Despite the improved imaging and molecular diagnostic techniques, cancer continues to affect millions of people globally (Guo and Wang, 2009). Thus, lots of research is now being carried out in searching for better chemotherapy drug from naturally occurring compounds which can suppress or prevent the process of carcinogenesis. The ethanolic extracts of *Anaphalis neelgherryana* leaves and barks were evaluated for the cytotoxic properties on HCT – 15 (Human Colorectal Carcinoma) cells by employing MTT assay and the cytotoxicity effects of the extracts against HCT cell lines were analyzed and the results are exhibited in Table 1 & 2 and Figures 1 & 2. The morphological changes occurred during the cytotoxic effects are shown Plates 1 & 2.

For the MTT assay, HCT-15 cell lines were treated with different concentrations (20, 40, 80, 120 and 200 µg/ml) of ethanolic extract of *A. neelgherryana* leaves (*An*-Le) for 48h. The results are shown in Table 1 and Figure 1. Succinate deydrogenase, an active mitochondrial enzyme present only in the viable cells can cleave the tetazolium rings of MTT (Mosemann, 1983) and convert it into insoluble purple formazan. Therefore, the amount of formazan produced is directly proportional to the number of viable cells.

The extract was found to be cytotoxic against the cell lines and exhibited a percentage of cell death 6.29±1.45, 14.05±0.44, 21.53±2.38, 32.05±2.54 and 48.39±2.84 for the different concentrations 20, 40, 80, 120 and 200 µg/ml respectively. IC_{50} value for *An*-Le was found to be 197.10±3.50 (Table 1 and Figure 2).

The results showed the progressive increase in the percentage of cell death with the increase in concentration of the extracts. Viability of the HCT-15 cell lines cancer cells was decreased with increasing concentration of *An*-Le (20 to 200µg/ml) (Figure 1 and Plate 1). These findings are in accordance with the reports of Khozaymeh *et al*. (2018) for *Alpinia galanga.*

The ethanolic extracts of *Anaphalis neelgherryana* barks (*An*-Ba) were also treated against HCT-15 cell lines with different concentrations of 20, 40, 80, 120 and 200 µg/ml with the percentage of cell death 8.50±1.65, 15.68±0.10, 33.93±6.01, 42.53±5.04 and 70.87±1.42 respectively. IC_{50} value for *An*-Le was found to be 138.01±8.07 (Table 2 & Figure 2).

Among the two extracts, *An*-Ba was found to have more cyt otoxic effect when compared to *An*-Le as all the tested concentrations had increased percentage of cell death. The strongest anticancer activity (70.87±1.42) was found for the barks with the concentration of 200µg/ml.

The cells with normal morphology are obvious in untreated cells. But the HCT-15 cells showed morphological changes with size and shapes (Plate 1 & 2). Similar results were reported by Narayana moorthi *et al*. (2018) for aqueous and ethanolic extracts of whole plant *Peperomia pellucida.* Similar intensive apoptotic response was also observed by the morphological changes in the stimulated leukemic cells (Smolarz *el al*., 2012). Cell viability significantly decreased after administration of various aqueous concentrations of the extracts of *Portulaca oleracea* against oral cancer cell lines (Azarifar *et al*. 2018). Cytotoxic activity and IC_{50} values were obtained for *Plumbago zeylanica* using MTT assay by Karpaga Raja Sundari *et al*. (2017). It is highly desirable to have compounds that can cause cancer cell death via apoptosis. Apoptosis eliminates malignant or cancer cells without damaging normal cells and surrounding tissues. Apoptosis is characterized by cell morphological changes, chromatin condensation, DNA cleavage and nuclear fragmentation (Elmore, 2007).

Table 1. Effect of ethanolic leaf extract of *Anaphalis neelgherryana* (*An*-Le) on HCT-15 cell lines (MTT Assay)

Conc. (µg/mL)	% Cell Death			Mean	SD
	I	II	III		
0	0.00	0.00	0.00	**0.00**	0.00
20	7.85	5.00	6.02	**6.29**	1.45
40	14.15	13.57	14.44	**14.05**	0.44
80	24.17	19.54	20.88	**21.53**	2.38
120	30.36	30.82	34.96	**32.05**	2.54
200	46.83	51.66	46.68	**48.39**	2.84
IC_{50}	200.40	193.42	197.47	**197.10**	3.50

Figure 1. Effect of ethanolic leaf extract of *Anaphalis neelgherryana* (*An*-Le) on HCT-15 cell lines (MTT Assay)

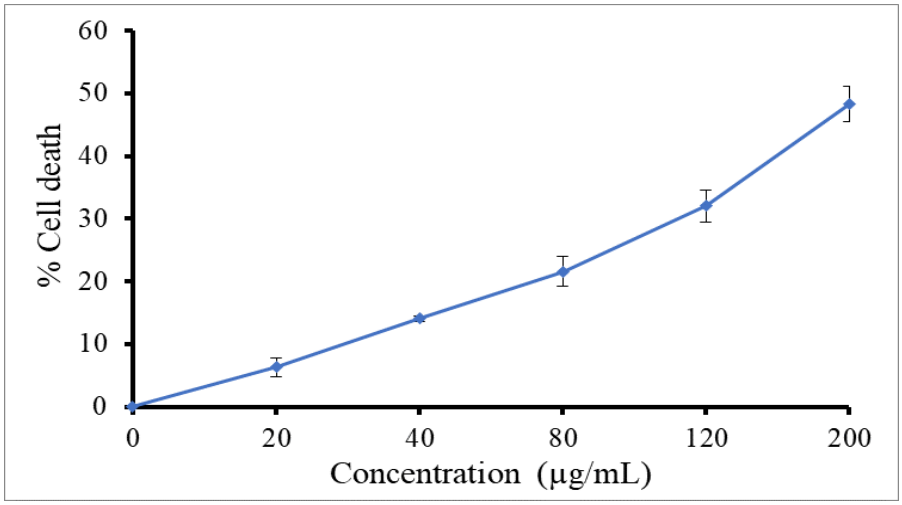


Table 2. Effect of ethanolic bark extract of *Anaphalis neelgherryana* (*An*-Ba) on HCT-15 cell lines (MTT Assay)

Conc. (µg/mL)	% Cell Death			Mean	SD
	I	II	III		
0	0.00	0.00	0.00	**0.00**	0.00
20	7.33	9.67	4.49	**8.50**	1.65
40	15.61	15.75	9.51	**15.68**	0.10
80	38.18	29.68	16.54	**33.93**	6.01
120	46.10	38.96	22.83	**42.53**	5.04
200	71.87	69.86	35.16	**70.87**	1.42
IC_{50}	132.31	143.72	135.76	**138.01**	8.07

Many polyphenols and flavonoids have been shown to inhibit proliferation and angiogenesis of tumor cells *in vitro* (Fotsis *et al.*, 1997). Phenolic compounds were also reported to possess the biological properties such as antiapoptosis, anticarcinogen, the inhibition of angiogenesis and cell proliferation activity (Hans *et al.*, 2 007).

Figure 2. Effect of ethanolic bark extract of *Anaphalis neelgherryana* (*An*-Ba) on HCT-15 cell lines (MTT Assay)

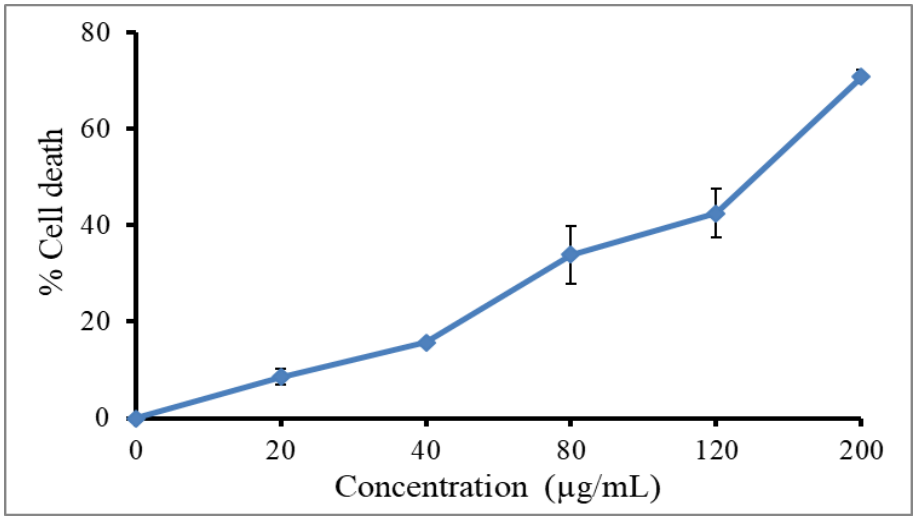
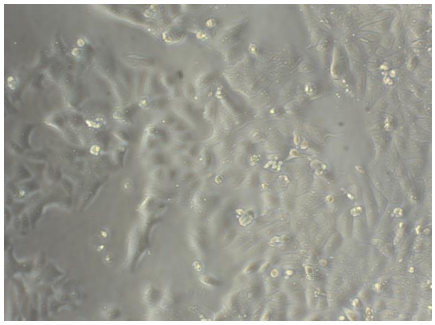
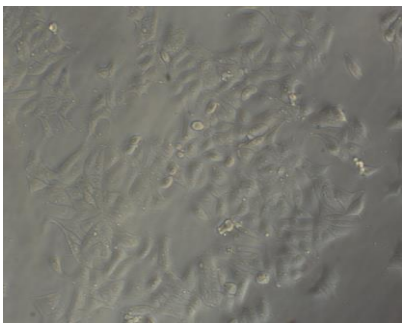


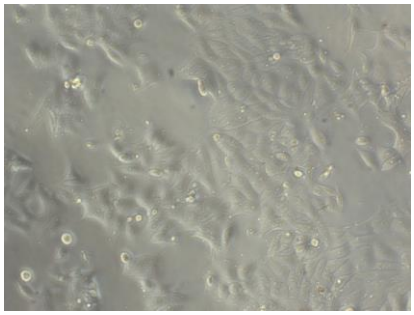
Plate 1.



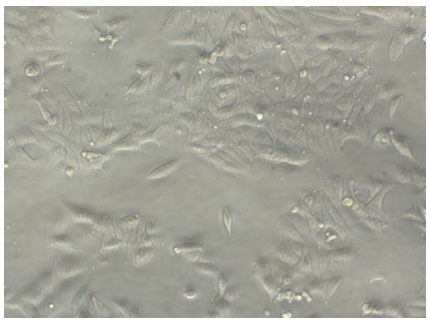
Untreated cells



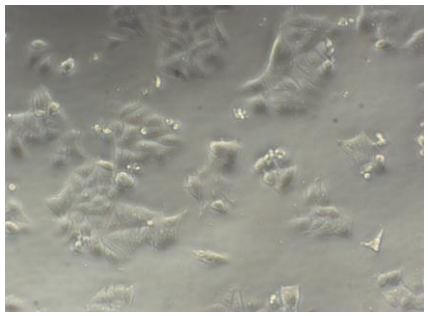
20µg/ml



40 µg/ml

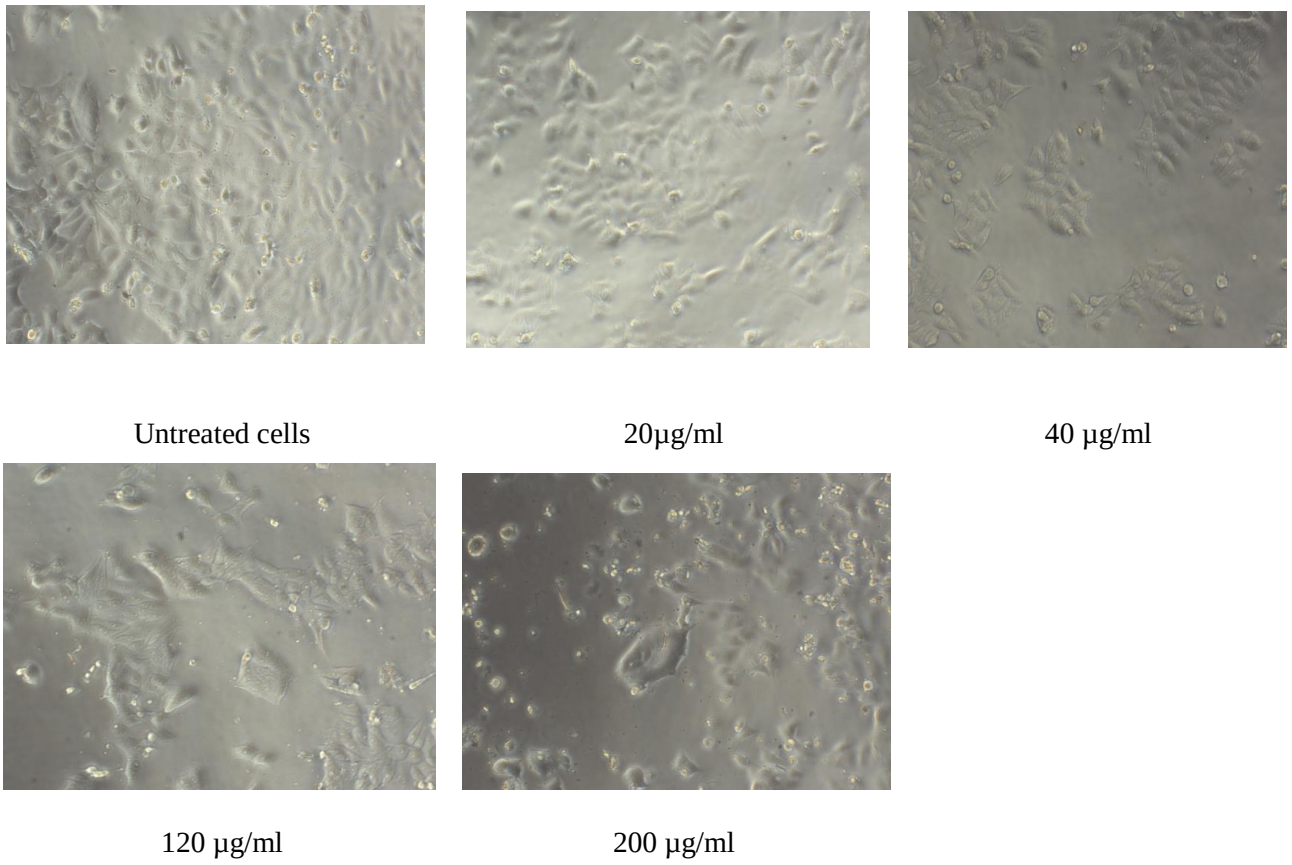


120 µg/ml



200 µg/ml

Cytotoxic effect of *Anaphalis neelgherryana* leaves on HCT -15 cell lines



Untreated cells 20µg/ml 40 µg/ml

120 µg/ml 200 µg/ml

Plate 2. Cytotoxic effect of *Anaphalis neelgherryana* bark on HCT -15 cell lines

CONSLUSION

Cytotoxic assays are an important approach for drug designing from natural compounds. Hence it suggests that extensive work on more number of tumor cell lines, selective toxicity studies and *in vivo* experiments could help to bring out the application of *Anaphalis neelgherryana* extracts for *anticarcino*

genic treatments and further purification to obtain a potentially active and pure compound will be undertaken in the future.

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