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RESEARCH ARTICLE

PHYTOCHEMISTRY OF METHANOLIC EXTRACTS OF *BRASSICA OLERACEA* AND ITS EFFECT ON ANTI-PROLIFERATIVE ACTIVITY IN HUMAN LUNG ADENOCARCINOMA A549 CELL LINE

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ABSTRACT

This study will decipher the phytochemistry and the antiproliferative activity of *Brassica oleracea* methanolic extract on lung cancer A549 cell line. The effect of methanolic extract was to ascertain its cytotoxic effect and anti-proliferative activity through *in vitro* studies by 3-(4, 5 dimethyl thiazole-2-yl)-2, 5-diphenyl tetrazolium bromide- MTT assay) on Normal VERO cell line and A549 (Human lung adenocarcinoma cell line). Methanolic extracts of *Brassica oleracea* was found to be effective in the prevention of cell proliferation by lung adenocarcinoma cell lines. Phases of cell cycle in the present study reveal the alterations in molecular events associated with the cancerous cells.

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INTRODUCTION

Cancer is still a growing health problem world-wide characterized by the irregular proliferation of the cells, as a cell progresses from normal to cancerous tissue, the biological imperative to survive and perpetuate, drives fundamental changes in cells behaviour. The risk of cancer can be reduced by habitual consumption of cancer protective foods. Lung cancer has been the leading cause of cancer-related deaths for many years and incidence and mortality statistics vary widely worldwide (Barta *et al*., 2019). There are two main subtypes of lung cancer: Non-small cell lung cancer (NSCLC) and small cell lung cancer (SCLC). Around 80% of all lung cancers are determined to be NSCLC (Matsuda and Machii, 2015). Tobacco consumption is a major risk factor for lung cancer. Other factors include genetic susceptibility, diet, alcohol consumption, occupational exposures, and air pollution (Malhotra *et al*., 2016). However, many of these chemotherapeutic drugs have been shown to produce significant toxic side effects and drug resistance. Several anticancer agents have been found in natural products which have been investigated and developed to become effective chemotherapeutic cancer drugs (Rayan *et al*., 2017). Apoptosis, the well-known cell death mechanism, is induced by many chemotherapeutic agents. Membrane blebbing, nuclear condensation, and apoptotic bodies are unique morphology characteristics of apoptotic cells that occur without cell inflammation (Elmore, 2007). There are two main intrinsic and extrinsic pathways in apoptotic signaling. The intrinsic pathway which is induced by intracellular stimuli such as DNA damage or oxidative stress. The extrinsic pathway which is induced by death ligand-receptor binding on the cell membrane (Hongmei, 2012). Natural therapies such as the use of the crude plant extracts or bioactive products with multiple phytochemical properties are being beneficial to combat cancer Thus, continued research needs to be pursued to find more effective natural products that provide fewer negative side effects (Amaral *et al*., 2019). Brassica plants are the rich source of phytochemical compounds of medicinal importance and is been studied for their bioactive phytochemical components and antioxidant potential. The edible parts of these plants show antimicrobial, anti-aging, anti-ulcer, anti-hyperglycemic, anti-hyperlipidemic, anti-proliferative, neuroprotective, anti-genotoxic and antioxidant activities (Podsedek 2007; Miraj, 2016). This study will focus on the phytochemistry and the antiproliferative activity of *Brassica oleracea* methanolic extract on lung cancer A549 cell line. Cell cycle events will be analysed to find out the dynamics of the methanolic extracts on the various phases of cell cycle in human lung adenocarcinoma cancer cell lines.

MATERIALS AND METHODS

Collection and Identification of Plant Material: Inflorescence of *Brassica oleracea* (Plate-1) used for the study were purchased from wholesale market, Chennai, Tamil Nadu during April 2021. Fresh plant specimens collected were authenticated by Dr. P. Jayaraman, Director, Plant Anatomy Research Center, Tambaram, Chennai. Registration No. (PARC/2021/4704).

Plate 1. Fresh inflorescence of *Brassica oleracea*

Processing and Preservation of Plant Materials: The florets from the whole inflorescence (Plate-2) were separated from freshly collected plants, washed in running tap water and rinsed in distilled water. The plant materials were chopped into small pieces and were shade dried for two weeks for complete dryness. The dried plant materials were powdered, using mechanical grinder. They were ground well to fine powder and then transferred into airtight containers until further use.

Plate 2. Dried inflorescence of *Brassica oleracea*

Solvent extraction: About one kg Broccoli was weighed, the inflorescence of was washed under running tap water followed by washing with distilled water to remove the surface debris. The heads were obtained by cutting the main stalk. The florets, together with about 1 cm of the stalk were cut off from the rest of the stalk and used for crude sample extraction. Finally, the above-prepared vegetable samples were chopped into small pieces using a cutter and later minced using a mixer grinder and finely macerated. After homogenization, it was extracted in 70% methanol for 7 days in the dark at normal room temperature with intermittent shaking. After 7 days, the whole extracts are filtered using muslin cloth at first and then through Whatman No. 1 filter paper, and the filtrate is concentrated using rotary evaporator. The yield of crude extracts obtained was noted, stored in desiccators for maximum of 3 days. The methanolic extracts were later preserved in a deep freezer (−20°C) for further use (Jamuna *et al* ., 2015).

Qualitatative analysis of Phytoconstituents: The aqueous extract was evaluated for preliminary phytochemical screening (Harborne, 1984).

Quantitative analysis of Antioxidant Content: β- carotene, total carotenoids, Tocopherol and Ascorbic acid was determined following the standard procedures British Pharmacopoeia, 2018.

Procurement and Maintenance of Cell lines: Normal Vero (African Green Monkey Kidney) and A549 cell lines were procured from National Centre for Cell Sciences (NCCS), Pune, India. The cell lines procured were maintained at Life Teck Research Centre, Arumbakkam, Chennai, Tamilnadu, India. The cells were maintained in Minimal Essential Media (MEM) and was supplemented with 10% Fetal Bovine Serum (FBS), Penicillin (100 IU/ml) and Streptomycin (100µg/ ml) in a humidified atmosphere of 5% CO_2 at 37°C.

Cell Viability Assay: *In vitro* studies: Methanolic extracts of *Brassica oleracea* was analyzed for its cytotoxicity in Normal Vero cell line and for anticancer activity in A549 cell line based on the principle of MTT assay (Mosmann *et al*., 1983) .

Maintenance of Vero and A549 cell lines: Cells (1×10^5 / well) were plated in 24-well plates and incubated in 37°C with 5% CO_2 condition. After the cell reaches the confluence, the prepared sample plant extract was added and incubated for 24 h. After incubation, the sample was removed from the well and washed with phosphate-buffered saline (pH 7.4) 100 µl / well without serum. 5 mg / ml of 0.5% (MTT) was added and incubated for 4 h. After incubation, 1.0 ml of 0.1% DMSO was added to all the wells. The absorbance at 570 nm was measured with UV- spectrophotometer using DMSO as blank in triplicates. Measurements were performed and the concentration required for a 50% inhibition (IC_{50}) was determined. Cell control and sample control is included in each assay to compare the complete the cell viability assessments. The % cell viability was calculated using the following formula:

Morphological studies of Vero and A549 cells after 24 h incubation: The methanolic extract treated cell lines (Normal Vero and A549) were observed and photographed under inverted animal cell culture microscope (LABOVERT-FS) under 40x objective.

Cell Viability Analysis - Trypan Blue Dye Exclusion method: A simple cell count method was performed to assess live and dead cells using hemocytometer and Trypan blue, a vital dye. This assay was based on the assumption that the dead cells will take up the dye and viable cells will not take up dye (Jauregui *et al*., 1981). Cell count was performed for A549 cell lines treated with aqueous stem extract by staining with trypan blue dye. The dead and live cells counted were obtained from the IC_{50} concentration at the end of 24 h incubation. The percentage of viable cells (live cells) and non-viable cells (dead cells) from the aqueous extract treated cell line were calculated. The percentage growth inhibition was calculated as using the following formula

% Growth Inhibition (Dead Cells) = 100 – (Total Cells – Dead Cells / Total Cells) x 100

Cell Cycle Analysis: Flow cytometric measurements of cellular DNA contents were perfomed with ethanol (70%) fixed cells using the intercalating DNA fluorochrome, propidium iodide. Lung cancer cell line (A549) used in the study were seeded (2×10^5 cells/ml) on 6-well plate and incubated for 24 hours of time at 37°C at 5% CO_2. After incubation, the monolayers of cells were treated with IC_{50} concentration of the sample (methanolic extracts of *Brassica oleracea*) and control (maintained in DMEM medium) was incubated for 24 hours. The treated cells were washed with sterile PBS and harvested. 0.2 ml of propidium iodide (10 µg/ml) was added to the cells and incubated for 30 minutes at room temperature and observed at 488 nm of excitation wavelength by flow cytometer. The percentage of each cell cycle phases was established using the cell quest software. Mathematical algorithms and software are used to analyze cell distribution histograms and estimate cell repartition in the various cell cycle phases (Shan *et al*., 2006).

RESULTS

Phytochemical analysis of the methanolic extracts of *Brassica oleracea* revealed the presence of various specific phytoconstituents such as flavonoids, alkaloids, phenols, saponins, anthraquinones, anthocyanins, tannins, proteins and reducing sugars (Table -1). Phytoantioxidants like total carotenoids, β-carotene and ascorbic acid was in considerable quantities when compared to Vitamin E (Table -2).

Table 1. Phytochemical screening of methanolic extracts of *Brassica oleracea*

S.No	Phytochemical Compounds	Unripe fruit extract
1	Flavanoids	+
2	Alkaloids	+
3	Phenols	+
4	Coumarin	-
5	Triterpenes	-
6	Saponins	+
7	Steroids	+
8	Proteins	+
9	Reducing Sugars	+
10	Anthraquinones	+
11	Anthocyanins	+
12	Tannins	+

Presence of the compound (+) and absence of the compound (-)

Table 2. Antioxidants in methanolic extracts of *Brassica oleracea* (Values of Total carotenoids and β-carotene are expressed as µg/100mg, Vitamin E and Vitamin C are expressed mg/gm)

Antioxidants	Unripe fruit
Total carotenoids	141.17 ± 0.84
β-carotene	31.77 ± 0.56
Vitamin E	1.36 ± 0.16
Vitamin C	48.10 ± 0.71

Cytotoxicity and Anti-proliferative activity of methanolic extracts of *Brassica oleracea* on normal Vero Cell line and A549 cell line: The percentage cell viability shown by Vero cell line treated with methanolic extracts of *Brassica oleracea* was 86.75 % at 7.8 µg/ml and 51.42 % at 1000 µg/ml. The IC_{50} was also recorded at 1000 µg/ml during 24 h of incubation with cell viability of 51.42%. The maximum cell viability 69.21% (minimum cell death 30.79%) was observed in 7.8 µg /ml of aqueous fruit extract at 24 hrs. The minimum cell viability of 2.07% (maximum cell death 97.93 %) was also observed in 1000 µg /ml concentration of aqueous fruit extract at 24 hrs. The IC_{50} was 31.2 µg /ml at 24 hrs of incubation with a cell viability of 49.60% (Figure 1).

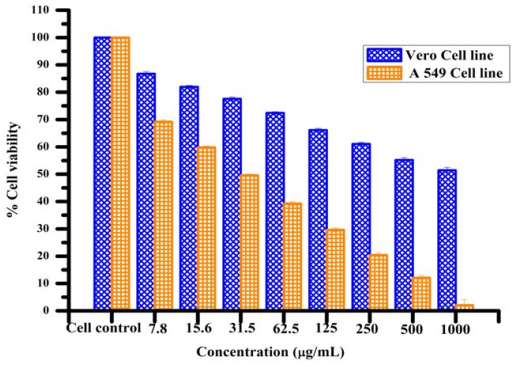


Figure 1. Cytotoxic and anti-proliferative effect on cell viability of A549 lung cancer cell lines treated with methanolic extracts of *Brassica oleracea*

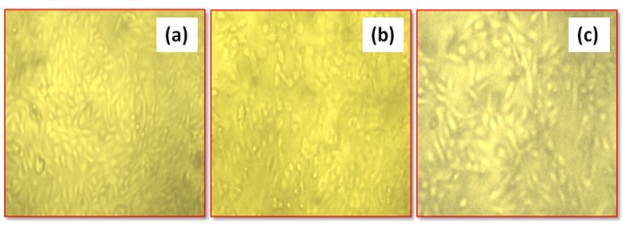


Figure 2. Morphology of Normal Vero cell line treated with methanolic extracts of *Brassica oleracea* at 24 hours of incubation

- Morphological appearance Normal Vero cell line (a)
- Vero cell line treated with extract and appearance of cells at concentration of 7.8 µg/ml (b)
- Vero cell line treated with extract and appearance of cells at concentration of 1000 µg/ml (c)

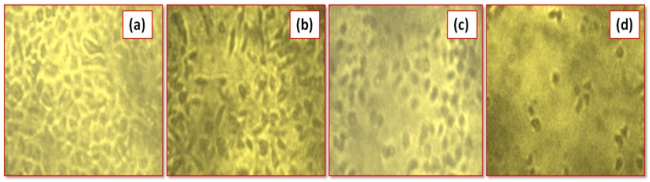


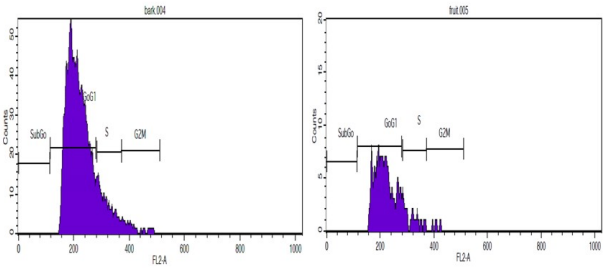
Figure 3. Morphology of A549 lung cancer cell line treated with methanolic extracts of *Brassica oleracea* at 24 hours of incubation

- Normal morphological appearance of A549 cell line (a)
- A549 cell line treated with extract and appearance of cells at concentration of 7.8 µg/ml (b)
- A549 cell line treated with extract and appearance of cells at concentration of 31.2 µg /ml (c)
- A549 cell line treated with extract and appearance of cells at concentration of 1000 µg/ml (d)

A549 lung cancer cell line treated with methanolic extracts of *Brassica oleracea* show differences in the number of live and dead cells. The viability of the cells were 53.3% at IC_{50} concentration of 31.2 µg /ml (Table 3).

Table 3. Cell count to observe live and dead cells of A549 cell line treated with methanolic extracts of *Brassica oleracea* by Trypan blue dye at IC_{50} concentration

No of live cells	161
No of dead cells	142
% Cell viability	53.3%
% Cell death	46.7%



A **B**

Figure 4. Cell cycle phases of A549 lung cancer cell line without (A) and with (B) methanolic extracts of *Brassica oleracea* treatment at 24 hr of incubation

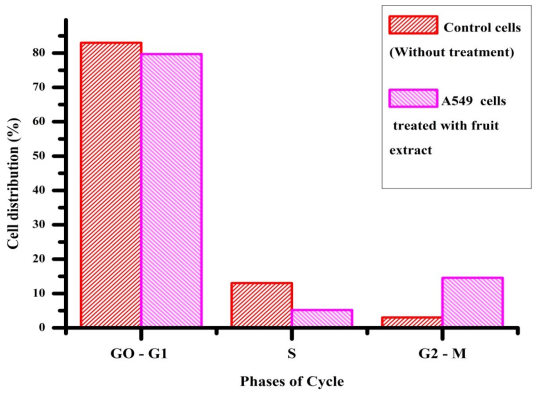


Figure 5. Cell cycle distribution of A549 lung cancer cell line after treatment with methanolic extracts of *Brassica oleracea*

The results showed that lung adenocarcinoma A549 cells without the treatment methanolic extracts of *Brassica oleracea* were distributed at all stages of the cell cycle, whereas, in the treatment groups, the cells were arrested in the G0/G1 phase of the cell cycle. It caused a significant portion of the cells to shift to the G2/M phase.

DISCUSSION

An alarming increase in occurrence of cancer is due to the production of intracellular free radicals which causes progressive effects on cellular DNA, protein, lipids, histone methylation leading to genomic instability and tumour development (Shi *et al*., 2012 ; Jayashree *et al*., 2015). Lung cancer cell line A549 is made up of adenocarcinoma from human alveolar basal epithelial cells which are commonly used as a model for the study of lung cancer and for the development of drug therapies (Kaplan *et al* ., 2017). Recent developments in the field cancer research show promising results at the molecular level by targeting plant phytocompounds which is also exerting lesser side effects. *Brassica oleracea* is a rich source of antioxidants like β-catotene, ascorbic acid and tocopherol. Apart from these antioxidants the unripe fruit also possess flavonoids, alkaloids, phenols and saponins (Sosnowska *et al.,* 2006; Anitha 2014; Thaipratum *et al*., 2014). Phytochemical and antioxidant content analysis revealed that the plant has a wide range of presence of these compounds and are the primary resources for antioxidants (Yang *et al*., 2007). Free radicals formation in the cells play a predominant role in cancer progression. These free radicals are scavenged by antioxidants. These antioxidant sources is an alternative to treat cancer cells (Jaiswal *et al*., 2012; Sharma *et al*., 2015). These compounds contribute to the cell antiproliferative activity, intitate apoptosis by activating signal transduction cascade pathways.

Solvent extracts of plant materials are screened on cell culture, to determine their efficiency as a potential alternate drug and also to check their efficacy in oncogenic cells (Sharma and Kapoor, 2015) The cell viability of the lung cancer cell line decreased with increase in concentration of the methanolic extract of *Brassica oleracea* and it was found to be the highest in 1000 µg/ml concentration. The decrease in cell viability with increased concentration of the plant extract of suggests the ability of the extract which could suggest a profound effect on lung cancer cell anti-proliferation. Antioxidant and free radical scavenging activity of the extract may be the reason behind its anti-cancer property. Similar anticancer effect on cell viability has been reported in A549 cell lines treated with *Piper betel* leaf extract (Azhagumeena *et al* ., 2021), whole plant extracts of *Naravelia zeylanica* (Umarani *et al*., 2020), *Coleus ambionicus* (Ramalakshmi *et al* ., 2014) and *Solanum torvum* (Saravanan and Raja, 2021). The lung cancer cells have undergone certain morphological changes, like cellular shrinkage and blebbing, which are characteristic features of apoptosis. These morphological changes are observed in treated cells with changes increasing with increase in concentration of the extract. At 1000 µg/ml the morphological changes are higher, followed by 80µg/ml concentration. Presence of phenolic compounds and have shown to induce a cascade based apoptosis in cancer cells, thus inducing cytotoxicity [26]. Similar results on the morphological alterations were observed when methanolic extracts of *Tecoma stans* were treated on A549 cell lines (Robinson *et al*., 2017). Based on our results of cytotoxicty studies, cell viability was performed by staining the cells treated with tryphan blue. The dead and live cells counted were at IC_{50} concentration. Tryphan blue is water soluble dye and it is insoluble in membrane lipids. Chromophores is negatively charged and does not interact with cells unless the membrane is damaged. This could have been the possible mechanism in the present study when dead or non-viable cells show membrane damage and take up the dye whereas the viable cells do not take up the dye and are transparent as revealed from the cell count of the study (Chung *et al*., 2015). Similar results on Trypan blue is reported in A549 cell lines treated with *Tridax procumbens* acetone and ethanol leaf extracts (Vishnu Priya *et al* ., 2015). The cell cycle arrest at G2/M phase is regulated by cell cycle check points which arrests the entry of the cells in the M phase when DNA is damaged.

In the present study accumulation of the cells at G2/M phase leads to accumulation of the cells in the G0/G1 phase which indicates the induction of early apoptosis. This could be due to upregulation of p53 gene, inhibition of cyclins B involved in cell cycle regulation and transcription of genes involved in apoptosis (Hanasakal *et al* ., 2014). Cell cycle arrest and cell death has been caused by the methanolic extract of *Brassica oleracea*. This is a potential target for cancer therapy and the cells are prevented to enter mitotic phase (Juthathip Poofery *et al*., 2020). Phytochemicals from cruciferous vegetables induce detoxification enzymes, scavenge free radicals, alleviate inflammation, stimulate immune functions, decrease the risk for cancers, inhibit malignant transformation and regulate the growth of cancer cells (Herr and Buchler, 2010). Changes in cell cycle regulatory gene expression is frequently found in human lung tumor tissues or cancer cell lines and these cell cycle regulators may represent a new set of potential targets for anticancer drugs. Investigation of the underlying pharmacokinetic mechanisms through which phyto-chemicals evoke their anti-cancer effect introduces a panel of molecular targets. This panel includes apoptotic proteins (caspases, bax etc), protein kinases (PKA, PKC, MAPK, TYK2 etc), anti-apoptotic proteins (bcl2, TRAF1, survivin etc), growth factors (TNF, EGF, FGF, PDGF etc), transcription factors (Apl, NF-KB, Nrf2, p53 etc), cell adhesion molecules (ICAM-1, VCAM etc) and cell cycle proteins (Cyclin D, CDK1, CDK2, p27, p21 etc). Moreover, phytochemicals interfere with multiple cell-signaling pathways (Agarwal and Sishodia, 2006). Advances in our understanding of the biology of lung cancer made over the past several decades using lung cancer cell lines have been tremendous. It suggests strategies that could improve the treatment modalities for lung cancer patients undergoing rigorous and strenuous therapy.

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