***Trachyspermum ammi* seed extract inhibits cell proliferation on A549 lung cancer cell: An in vitro analysis**

ABSTRACT:

The aim of this study was to investigate the in vitro anti proliferative activity of the ethanolic extract of *Trachyspermum ammi* seeds against A549 adenocarcinomic human alveolar basal epithelial cells using MTT assay and morphological analysis by inverted phase contrast microscopy. The MTT assay results showed that 50% of the cell proliferation (IC_{50},µg/ml) has been inhibited upon *T.ammi* seed extract treatment for 24hrs incubation. The Dose-dependent studies revealed cytotoxic dose level IC_{50} of 50µg/ml for *T.ammi* seed extract on lung cancer cells. It clearly shows that the dose dependent treatments significantly ($p < 0.001$) reduces the cell viability thereby inhibiting the cancer cell proliferation when compared and it was further confirmed with morphological evaluation with microscopic study. From this study we concluded that the ethanol extract of *T.ammi* significantly inhibits cancer cell proliferation against A549 cells and it might be a potent antiproliferative value for further evaluation to determine the therapeutic agent for cancer treatment.

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Keywords: Cell proliferation, *Trachyspermum ammi*, Lung cancer, cytotoxic

Running Title: Assessment on effect of *Trachyspermum ammi* on inhibition of cell proliferation in A549 lung cancer cell line.

INTRODUCTION:

Cancer is a multi-stage disorder with physical, environmental, biochemical, chemical, and genetic components (1). Lung cancer is a more malignant form of neoplasm. Both males and females were affected equally worldwide. In 2018, GLOBOCAN survey estimated that more than 1.8 million deaths occurred due to lung cancer (2). Indicators used to predict cancer statistics by incidence, mortality and survival. Tobacco smoking remains a predominant risk factor for lung cancer. Other than smoking, it can be due to exogenous factors like environmental and occupational exposure, chronic lung disease, viral, bacterial infection and lifestyle modification (3). Lung cancer was classified based on the microscopic appearance of tumour cells as small cell lung cancer and non-small cell lung cancer (4). Most aggressive form of lung cancer is non-small cell lung cancer. It has a more metastatic rate, but can be diagnosed only after spreading to the whole body. MicroRNAs (miRNAs) are a large group of small non-coding RNAs that control gene expression and have been shown to function as tumor suppressor genes (oncogenes) as well as influence homeostatic processes including cell proliferation and death (5). Alteration in specific genes like k-ras, p53, and Ink4A/Arf can be the carcinogenesis of lung

cancer. Various early research focuses on distinct chromosomal loci (3p, 9p, 13q, 17p, and others)identification. It implies that sequential genetic events occur during the initiation and development of lung carcinogenesis. Several suppressor genes have been identified and cloned at these chromosomal loci, including Rb (13q), P53 (17p), and P16 (9p) (6). Despite advancements in diagnostic methods and targeted treatments, the 5-year overall survival rate is still just 15%. As a result, understanding the molecular mechanisms of cancer cell proliferation and metastasis in NSCLC is critical for the production of new drugs.

Trachyspermum ammi is commonly known as Ajwain(omum) , the native plant of egypt. Due to their diverse applications, natural resources, especially medicinal plants, have remained excellent sources of phytochemicals for traditional medicines, modern medicines, nutraceuticals, pharmaceutical intermediates, folk medicines, food supplements, and chemical entities for synthetic drugs (7). In India it is cultivated in Madhya pradesh, Rajasthan, West Bengal and Bihar (8). Herb belongs to the family Apiaceae. Most of the members of the family had great medicinal values. Traditional herb Ajwain seems to cure various plants and animal diseases. Alcoholic extract has a high content of hygroscopic saponin. Principal oil constituent has 46% of carvone, 38% of limonene and 9% of dillapiole. *T. ammi* has also been used as a galactagogue in humans in history. *T. ammi* seed had the second highest overall phytoestrogen content of the eight herbs examined, at 473 ppm (total phytoestrogen contents 131-593 ppm) (9). Root extract of the herb possesses good aphrodisiac properties and seeds have good diuretic properties. Oil of the herb is used for treatment of gastrointestinal ailments, for lack of appetite and bronchial disorders. Oil exhibits excellent fungicidal, antimicrobial and anti-inflammatory properties. It can be used as a powerful remedy for flatulence, atonic dyspepsia and diarrhoea. It can be used to cure abdominal tumours, abdominal pain and piles(10) (11) (12) (13).

Photochemical constituents of the herb possess good pharmacological activities. On looking into the effect of herb, extract has maximum degradation of aflatoxin G1(AFG1) (14). Herb also induces oxidative stress, toxicity in rats was examined in previous studies (15). Herb showed hepatic free radical scavenging stress, that will cause toxicity. Till date, many articles have been published regarding the *T.ammi* effect towards peptic ulcers, antispasmodic, anthelmintic effects (16). Our team has extensive knowledge and research experience that has translated into high quality publications(17–21),(22),(23),(17),(24),(25),(26),(27)(19,28,29),(30–34), (35) (36) (37) (38) (38,39) (40) (41) (42) (43) (44) (45) (46) (47) (48) (49) (50) (51). Our aim of the study is to assess the potential of *Trachyspermum ammi* seed and its aqueous extract towards inhibition of cell proliferation in lung cancer cell lines.

MATERIALS AND METHODS:

CHEMICALS

DMEM medium, 0.25 percent Trypsin-EDTA solution, sodium bicarbonate solution, bovine serum albumin (BSA) and MTT purchased from Sigma Chemicals Co., St. Louis, USA . Himedia supplied FBS, antibiotic/antimycotic solution, and DMSO. Sodium phosphate monobasic and dibasic, sodium chloride, sodium hydroxide, sodium carbonate, hydrochloric acid, and methanol were purchased separately from Sisco Research Laboratories (SRL) India.

Plant collection:

The *Trachyspermum ammi* whole plant powder was purchased from from IMPCOPS(Chennai, India)

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Preparation of the Herbal Extract:

The current research used *Trachyspermum ammi*(TA) stem powder obtained. In a static state, 50 g of TA powder was soaked in 500 mL of aqueous and held at room temperature for 3 days. Filter paper and whatman paper are used to filter the solution. After rota evaporation of the fine filtrate, 3g of the substance was collected. The total ethanol extract was concentrated in a vacuum evaporate and immediately stored at 4℃.

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Compound Name	Cell line tested	Concentration of extract used
Trachyspermum ammi (TA)extract	A549	30,60,90,100,200,300 µM

CELL CULTURE REAGENTS

DMEM

The sodium bicarbonate solution in commercially available DMEM is 7.5 percent. 5ml penicillin/streptomycin solution and 0.5ml amphotericin B solution were applied to 500ml DMEM. Within the hood, the medium was then sterile filtered (0.22). After that, the medium was poured into sterile containers and held at 4°C.

Growth Medium (DMEM with 10% FBS) was rendered up to 100ml using sterile DMEM and 10ml of FBS. It was stored in a sterile container in cool and aseptic condition.

Phosphate Buffered Saline is a form of saline that has been buffered with (PBS; pH 7.4) In 500 ml of double autoclaved milliQ water, 0.63 g sodium phosphate monobasic (NaH2PO4), 0.17 g sodium phosphate dibasic (Na2HPO4), and 4.5 g sodium chloride (NaCl) were dissolved. After adjusting the pH to 7.4 with 1 N HCl and 1 N NaOH, the solution was sterile filtered (0.22), and placed in a sterile bottle.

Trypsin-EDTA

Trypsin was purchased as 1 x with EDTA (0.5% trypsin, 5.3 mM EDTA sodium salt). (Note: Freeze-thaw process does not affect the enzyme activity. Thawing is done at room temperature).0.89% Physiological Saline, 890 mg of sodium chloride was dissolved in 100 ml of double autoclaved milliQ water.

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CELL LINE

Human lung adenocarcinoma-A549 cell line was procured from the National Centre for Cell Science (NCCS, Pune), India. The cells were grown in T255 culture flasks containing DMEM medium supplemented with 10% FBS. On reaching confluence, the trypsin- EDTA solution was removed from the cells.

TESTING VIABILITY OF CELLS

The viability of A549 cells was assessed by trypan blue exclusion test by *Perry et al., (1997)*. Trypan blue solution: (0.5% trypan blue (w/v) in physiological saline).

100 l of trypan blue solution was mixed with 100 l of cells contained in the medium and incubated for 5 min at 37oC. The cells were then washed thrice with saline and 10 l of this suspension was placed in a haemocytometer and viewed under a microscope. The unstained cells represented the viable cells whereas the damaged cells were stained. The number of stained and unstained cells was counted and the percentage of viable cells was calculated using the formula:

$$\% \text{ of viability} = \frac{\text{No. of unstained cells}}{\text{Total no. of cells}} \times 100$$

The viability of the cells was found to be between 90-95%.

MTT ASSAY

The proliferation of A549 cells was assessed by MTT assay (52)

This is based on action of metabolically active cells which reduces soluble yellow tetrazolium salts to insoluble purple formazan crystals.Only live cells are able to take up the tetrazolium salt.

Mitochondrial dehydrogenase enzyme present in mitochondria helps convert internally present tetrazolium salt to formazan crystals which are purple in colour. Then the cells are lysed using a 20% SDS solution, which releases the formazan crystal. These crystals are solubilized by DMF present in the solubilizer. The colour developed is then determined in an ELISA reader at 570 nm. MTT [3-(4,5-dimethylthiazol-2-yl) 2,5-diphenyl tetrazolium bromide]: 0.5 mg MTT/ml of serum-free DMEM Solubilization solution: 20% w/v SDS in 50% of Dimethyl formamide and Phosphate Buffered Saline (PBS; pH 7.4).

A549 cells were plated in 24 well plates at a concentration of 5 x 10^4 cells/well 24 hours after plating, cells were washed twice with 500 µl of serum-free medium and starved by incubating the cells in serum-free medium for an hour at 37℃. After starvation, cells were treated with ION of different concentrations for 24 hours. At the end of treatment, the medium from control and ION treated cells were discarded and 500 µl of MTT containing DMEM (0.5 mg/ml) was added to each well. The cells were then incubated for 4 h at 37℃ in the CO2 incubator.

The MTT containing medium was then discarded and the cells were washed with 1x PBS (1 ml). The crystals were then dissolved by adding 500 µl of solubilization solution and this was mixed properly by pipetting up and down. Spectrophotometric absorbance of the purple blue formazan dye was measured in a microplate reader at 570 nm. The OD of each sample was then compared with the control OD and the graph was plotted.

Morphology Study:

Based on MTT assay we selected the low and high doses of *Trachyspermum ammi* for further studies. The characterisation of morphological changes in lung cancer cells treated with (*Trachyspermum ammi* with low and high doses) compared to their respective controls were observed under phase contrast microscope.

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STATISTICAL ANALYSIS

All data obtained were analyzed by Student's t-test using MS-Excel, represented as mean ± SD for six animals in each group. The results were computed statistically (SPSS/10 Software Package; SPSS Inc., Chicago, IL, USA) using one-way ANOVA. Post-hoc testing was performed for inter comparisons using the LSD. In all tests, the level of statistical significance was set at $p<0.05$.

RESULTS:

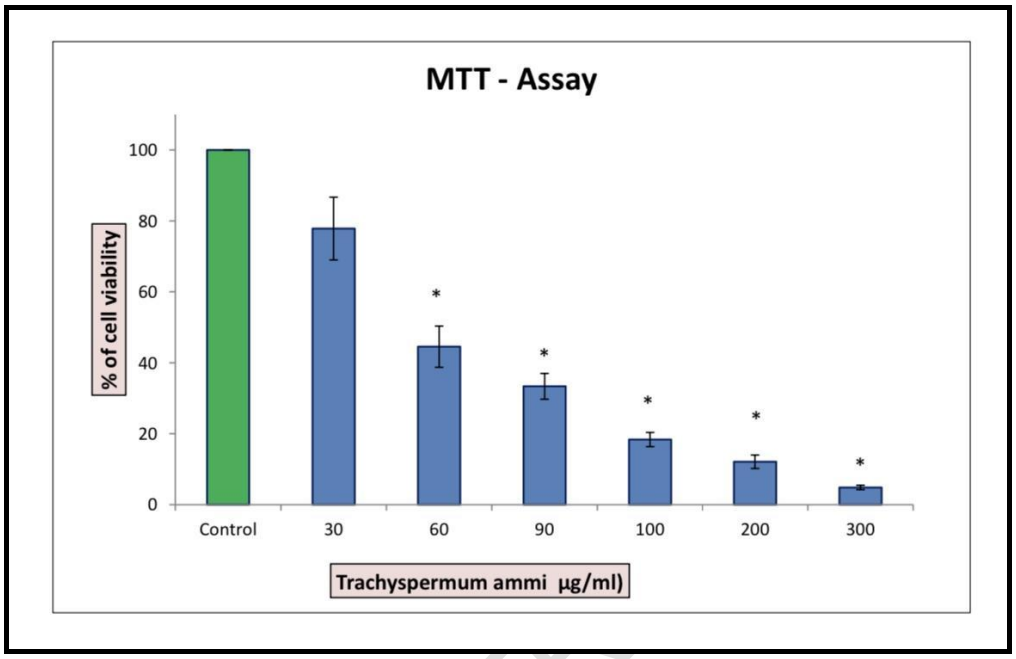

Figure:1 The bar graph represents the anti-cancer effect of *Trachyspermum ammi* with control via MTT assay for 24 hours. X-axis represents the different concentration /dose of *Trachyspermum ammi* extract in µg/ml. and Y-axis represents the % of cell viability. IC50 value was calculated as follow:

S.NO	Concentration of extract(TA) in µg/ml	% of viable cell
1	Control(DMSO)	100
2	30	80
3	60	50
4	90	37
5	100	20
6	200	17
7	300	10

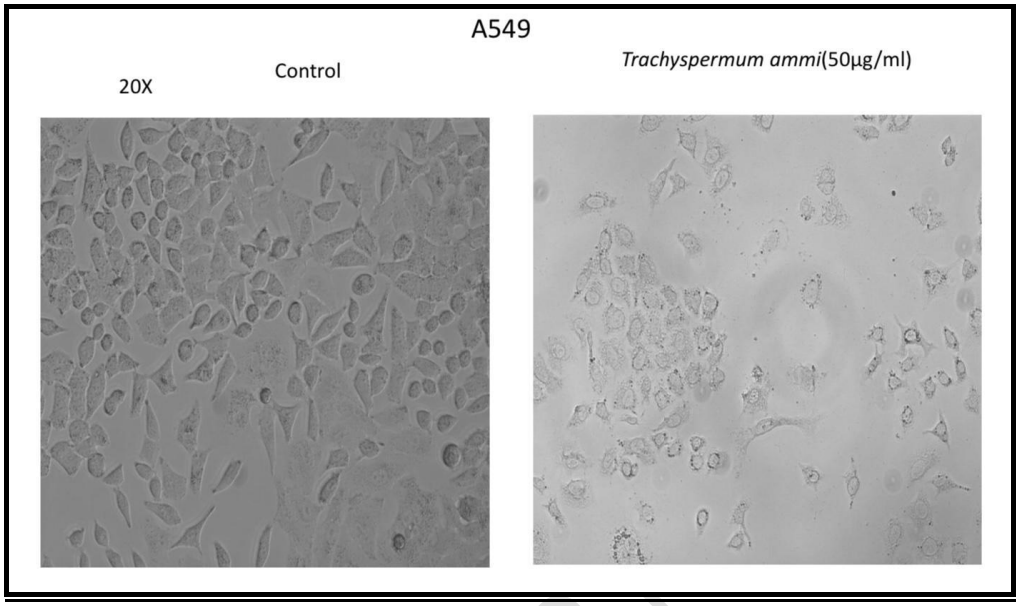

Figure:2 Shows the different morphological representation of lung cancer cells upon treatment with *Trachyspermum ammi* extract compared to control cells. Images were captured with inverted phase contrast microscopy in 20x magnification.

DISCUSSION:

The above findings, implies that at 50µg/ml extract can kill half of the lung cancer cells. At 50µg/ml concentration only 50% of the cells were viable. From that we can infer that as concentration of extract increases, cell viability will decrease. Both are indirectly correlated. Extract is toxic to lung cancer cells at specific concentration.

Cell proliferation is an exponential increase in number and size of the cells. Cell proliferation serves as an important risk factor for cancer. Controlling cell proliferation is necessary for cancer prevention because cell proliferation plays a key role in carcinogenesis, including the initiation and progression of cancer. Previous articles suggest various cell proliferation inhibition techniques by suppressing the signalling molecules like tea catechins significantly suppress cell growth by upregulating let-7 and downregulating the C-MYC/LIN-28 signaling pathway in lung cancer cells (53). And also by pre-treating tumor cells with the hMSCs-conditioned medium(human mesenchymal stem cells medium), soluble factors from hMSCs could suppress tumorigenesis and tumor angiogenesis (54).

Development of lung cancer cells targeting drugs without causing any harm to normal cells is one of the challenging tasks in the field of cancer drug discovery. In recent times, usage of herbal compounds in medicine has enormously increased. This is due to various inventions regarding the therapeutic use of individual plants. The plant *Trachyspermum ammi* serves not only as antioxidant, antispasmodic effects, Its anti-cancer effect also evidenced in previous articles (55). Thymol, one of the constituents of ajwain, induces apoptosis in MCF-7 and affects the gene expression in p53 gene in breast cancer cell line (56). In (57), flow cytometry values show that expression of Plzf and ID-4 genes has been increased when treated with oil of *Trachyspermum ammi*. So the oil extract has increased the cell viability in the spermatogonia cells. An amino acid named taurine was analyzed for its effect towards lung cancer cell lines. It showed good inhibition in cell proliferation. It promoted apoptosis by activation of the protein called PUMA (58). Medicinal plants, cucurmin(turmeric) also have a role in inhibition of cell growth through Bcl2-Bax gene and also have effect on mitochondrial apoptosis pathway (59)(60). Limitations in this study, Since it is in vitro analysis only laboratory and therapeutic use of the herb was analysed, but not about side effects and their interaction inside the body(33) (61) (62) (63) (64) (65) (66) (67)(68) (69) (70) (71) (72) (73) (74). The future studies should concentrate more on in vivo study to assess the drug interaction of *T. ammi*.

CONCLUSION:

The *Trachyspermum ammi* seeds have been widely used in Indian medicine for many years. The Ethanolic extract of *T.ammi* was cytotoxic, an anti-proliferative effect to lung cancer cells. Future studies should assess the drug formulation and interactions.

COMPETING INTERESTS DISCLAIMER:

Authors have declared that no competing interests exist. The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

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