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Trachyspermum ammi Seed Ethanolic Extract Inhibits Cell Proliferation on A549 Lung Cancer Cell: An *In-vitro* Analysis

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

Introduction: Lung cancer is the second most common type of cancer, accounting for one in every five male cancers and one in every nine female cancers. Lung cancer treatment is dictated by the cell type of the illness, the amount to which it has spread, and the patient's general condition. It is well understood that tumours confer resistance to chemotherapeutic drugs or radiation in part due to apoptotic pathway malfunction in cancer cells.
Aim: The aim of this study was to investigate the in vitro anti proliferative activity of the ethanolic extract of *Trachyspermum ammi (T.ammi)* seeds against A549 adenocarcinomic human alveolar basal epithelial cells.
Materials and Methods: The cytotoxicity and anti-cancer effects of ethanolic extract of Trachyspermum ammi (T.ammi) seeds against A549 cells were analyzed using MTT assay and morphological analysis by inverted phase contrast microscopy.
Results: The MTT assay results showed that 50% of the cell proliferation (IC_{50}) has been inhibited

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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 showed 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.

Conclusion: 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 anti-proliferative value for further evaluation to determine the therapeutic agent for cancer treatment.

Keywords: Cell proliferation; T. ammi; Lung cancer; cytotoxicity.

1. 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 researches focus 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 Non-small-cell lung carcinoma (NSCLC) is critical for the production of new drugs.

T. 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 [10-13].

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 [17–51]. Our aim of the study is to assess the potential of *T. ammi* seed and its aqueous extract towards inhibition of cell proliferation in lung cancer cell lines.

2. MATERIALS AND METHODS

2.1 Chemicals

Dulbecco's Modified Eagle Medium (DMEM) medium, 0.25 percent Trypsin-EDTA solution, sodium bicarbonate solution, bovine serum albumin (BSA), Trypsin-EDTA and 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT) purchased from Sigma Chemicals Co., St. Louis, USA. Himedia supplied Fetal Bovine serum (FBS), Phosphate Buffered Saline antibiotic/antimycotic solution, and Dimethyl sulfoxide (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.

2.2 Plant Collection

The *T. ammi* whole plant powder was purchased from The Indian Medical Practitioners Co-operative Pharmacy and Stores Ltd (IMPCOPS) (Chennai, India)

2.3 Preparation of the Herbal Extract

The current research used *T. ammi* 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 were used to filter the solution. After rotary evaporation of the fine filtrate, 3 g of the substance was collected. The total ethanol extract was concentrated in a vacuum evaporate and immediately stored at 4℃.

Compound Name	Cell line tested	Concentration of extract used
T.ammi (TA) extract	A549	30, 60, 90, 100, 200, 300 µg/ml

2.4 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.

2.5 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/ml MTT 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 *T. ammi* seed extract 30-300µg/ml of different concentrations for 24 hours. At the end of treatment, the medium from control and *T. ammi* seed extract 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.

2.6 Morphology Study

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

2.7 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 $p<0.05$.

3. RESULTS

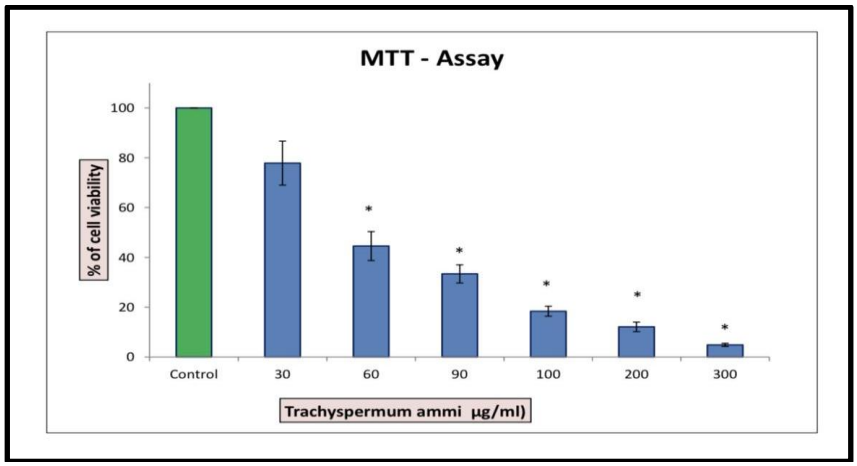

Fig. 1. The bar graph represents the anti-cancer effect of *T. ammi* ethanolic seed extract with control via MTT assay for 24 hours. X-axis represents the different concentration of *T. ammi* seed extract in 30-300µg/ml. and Y-axis represents the % of cell viability. IC50 value was calculated as follow:

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

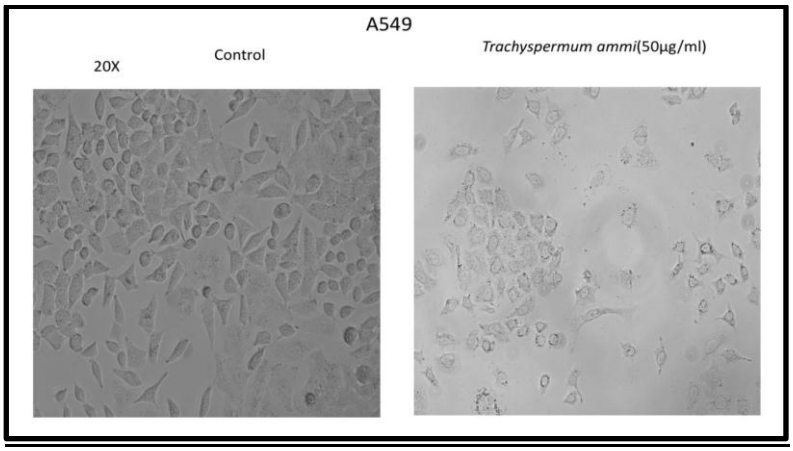

Fig. 2. Shows the different morphological representation of lung cancer cells upon treatment with *T. ammi* seed ethanolic extract compared to control cells. Images were captured with inverted phase contrast microscopy in 20x magnification

4. DISCUSSION

The above results, implies that at 50µg/ml *T. ammi* seed ethanolic extract can kill half of the lung cancer cells. At 50µg/ml *T. ammi* seed ethanolic extract concentration only 50% of the cells were viable. From that we can infer that as concentration of *T. ammi* extract increases, cell viability will decrease dose dependently, both are indirectly correlated. *T. ammi* seed ethanolic extract is toxic to lung cancer cells at specific concentration. During cancer initiation, 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 *T. 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 *T. 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 [58]. It promoted apoptosis by activation of the protein called PUMA [59]. Medicinal plants, curcumin (turmeric) also have a role in inhibition of cell growth through Bcl-2/Bax gene and also have effect on mitochondrial apoptosis pathway [60,61]. In the present study also *T. ammi* seed ethanolic extract treatment significantly decreased the lung cancer cell proliferation. *T. ammi* seed ethanolic extract (50µg/ml) treatment significantly induces the lung cancer cell morphological changes at 24hrs treatment period. 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 [33,62-75]. The future studies should concentrate more on in vivo study to assess the drug interaction of *T. ammi.*

5. CONCLUSION

The *T. ammi* seed ethanolic extract 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.

DISCLAIMER

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.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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