In Vitro Anticancer Activity of Amphiroa Fragilissima on Lung Cancer Cell Line (A549)

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Abstract
The primary cause of cancer-related deaths worldwide is lung cancer. Still smoking is the biggest risk factor. Adenocarcinoma, squamous cell carcinoma, large cell carcinoma, and other types of lung cancer are examples of non-small cell carcinomas. The prognosis and available treatments are determined using these classifications. This study illustrates the application of Amphiroa fragilissima in cancer therapy. A. fragilissima inhibits the growth of the lung cancer cell line A549. It is remarkable that A. fragilissima has a more cytotoxic effect on the lung cancer cell line A549, although other cell lines are needed to confirm this conclusion. To fully comprehend how anti-apoptotic agents stimulate the production of the apoptotic pathway proteins, more investigation is required gene deprivation.

Introduction

A collection of aberrant cells that develop out of control and avoid apoptosis is known as cancer (Macheret, 2015). The three main characteristics of cancer are invasion, metastasis, and aberrant cell proliferation. Lung, breast, colon, and prostate cancers are the most prevalent types of cancer. Cancerous growths can arise in nearly every part of the body. The body's cells start to proliferate and grow out of control. These cells have the ability to infiltrate and damage healthy tissues (Halazinetis, 2015). The cells develop into malignant or cancerous due to damage to DNA. Metastasis is the term for when cancer cells spread to other areas of the body and start to proliferate and form new tumors. Globally, the cancer burden is still rising, placing a great deal of physical, psychological, and financial stress on people, families, and healthcare systems (Kaarthic et al., 2018).

Lung Cancer

Globally, lung cancer is the greatest cause of mortality (Inamura, 2017). Genetic and epigenetic changes, the activation of growth-promoting pathways, and the inhibition of tumor suppressor pathways are some of the multistep processes involved in the development of cancer (Cooper et al., 2013). According to Peto et al. (1994), cigarette smoking is the primary cause of lung cancer, accounting for 80 % to 90 % of cases in nations with high cigarette smoking prevalence.

Anticancer Agents

Chemotherapy, hormone therapy, and immune therapy are the three categories of traditional anti-cancer medications (Espinosa et al., 2003). Agents that obstruct cell division have long been employed in the treatment of cancer (Donald, 2004). The growth signals that propel the survival and multiplication of cancer cells as well as the tumor vasculature have been the focus of more recent studies and innovative treatments. Clinically used DNA interaction medicines are among the most significant pharmacological classes in cancer treatment (Grella, 2013).

The Need for New Anti-Cancer Drugs

Before, the mainstay of cancer treatment was systemic, non-specific, high-dosage chemotherapy; today, the objective is to discover a medication that strikes a compromise between the lowest possible side effects and the highest possible anti-cancer efficacy. For patients with advanced metastatic cancer, for whom surgery and radiation therapy are no longer curative, improved systemic medication therapy is especially crucial (Krishnamoorthy et al., 2020). The development of novel targeted therapies and cancer vaccines has been made easier by advances in technology and our growing understanding of the genetic alterations that turn a normal cell into one that is not subject to the regular feedback system (Hoang et al., 2018).

Materials and Methods

Maintenance of Cells

The monolayer human lung cancer cell line A549 was cultured in DMEM media containing 10 % FBS and 2 % antibiotics. After collecting the cells with trypsin EDTA and seeding them in tissue culture flasks to keep in the exponential phase, stock cultures were subcultured every seventh day.

Tumor Cell Line

The National Centre for Cell Science in Pune, India provided the human lung cancer cell line A549 used in this investigation.

Cell Culture Media

The cells are grown in DMEM medium with high glucose and sodium bicarbonate, which was purchased from Himedia (Cat No. AL007S -6x500ML).

Serum

Fetal bovine serum (FBS), available from Himedia (Cat No. RM9955 -500ML), was utilized to cultivate the cells.

Antibiotics

Penicillin and streptomycin were obtained from Himedia (Cat No. A001-100ML).

Trypsin – EDTA

0.25 % Trypsin –EDTA was obtained from Himedia (Cat No. TCL145-100ML).

Cell Counting

0.04 % Trypan Blue in PBS, Haemocytometer and Inverted Microscope

The cell suspension was mixed gently and aliquot was added to the Trypan blue solution (100 µl cell suspension, 100µl dye) and then it was counted in hemocytometer.

MTT Assay

For a whole day, the human lung cancer cell line A549 was cultured on 96-well micro titer plates (5 × 103 cells/well). Following seeding, the plates are incubated for 24 and 48 hours, respectively, with and without an ethanolic extract of A.fragilissima at concentrations ranging from 100µg to 1000µg/ml. After refreshing the medium, 20µl of MTT (5 mg/ml) was added. The plates were left in the dark for three to four hours. After solubilizing the formazan crystals with 100µl of DMSO, the plate was left for a further five to ten minutes. An ELISA reader (Bio Rad, USA) was used to measure the color generated at 570 nm and 630 nm as the reference wavelength.

Acridine Orange & Ethidium Bromide Staining

Acridine Orange – 10 mg/ml in PBS Ethidium Bromide – 10 mg/ml in PBS

AO - 1µl from stock is diluted with 100µl of PBS EtBr – 1µl from stock is diluted with 100µl of PBS AO/EB stain is prepared by mixing the both stains in 1:1 ratio.

Quantitative Real-Time PCR (RT PCR) on the Expression of mRNA

The following study is performed to analyze the expression of mRNAs including BCL2, BAX, CASPASE 9, PI3K, and PTEN. The quantity of the above mentioned molecules are interpreted by quantification with RT PCR.

Table 1 Oligonucleotide Sequence of Specified Primers for RT-PCR

Gene Name	Forward Primer	Reverse Primer
β-actin	GCGAGTACAACCTTCTTGCAG	CATACCCACCATCACACCCTG
BCL2	AAACGAAAGCAACAGGAACACT	GTGGCAATTGAAAGGCGTGT
BAX	CATGGGCTGGACATTGGACT	CTTCCAGATGGTGAGCGAGG
CASPASE 9	TCCCAATCCAACGGGGATAA	GTCTTCCCCTCTTCACAGGATG
PI3K	GGGAGCCCCAGAAAAGCAGA	AGTTCTCCAGCTCCATGCCC
PTEN	GCGTGCGGATAATGACAAGG	AGCCTCTGGATTTGATGGCTC

RNA Isolation

With 1 milliliter of TRIZOL reagent, the cell line was thoroughly homogenized. After that, the homogenate was kept at 4´C for 5 minutes to allow the nucleoproteins to fully dissociate. After adding 0.2 ml of chloroform/ml of RNA reagent, this was centrifuged for 15 minutes at 4´C at 12000 rpm. After separating the RNA-containing upper aqueous phase, an equivalent volume of isopropanol was added, and the mixture was centrifuged for 10 minutes at 4´C at 12000 rpm. The RNA precipitates as a white pellet at the bottom of the tube and is centrifuged at 7500 rpm for 15 minutes at 4´C after being twice cleaned with 75 % ethanol.

The resulting RNA pellet was then completely soluble in 30 µl of 0.2 % sterile water and incubated for 10 minutes at 60´C in a water bath. After that, the RNA sample was kept at -80´C and vortexed for ten minutes.

Total RNA Quantification

The Nano Drop instrument was used to quantify the total RNA that was extracted. On the separated RNA, three quality checks were carried out. Three things needed to be determined: first, the amount of RNA; second, its purity; and third, the integrity of the extracted RNA. The Multiskan Sky High nano drop device was used to do the measurement. The absorbance maxima of proteins and nucleic acids are located at 260 and 280 nm, respectively. In the past, the ratio of absorbance

at these wavelengths has been utilized to gauge the purity of protein and nucleic acid extraction processes. DEPC water was used to calibrate the Nano drop.

The ratio of the absorbance values at 260 and 280 nm provides an assessment of the purity of RNA with regard to UV-absorbing impurities like protein. For RNA, a ratio of about 2.0 is regarded as pure.

Reverse Transcription of mRNA

Using a Revert Aid First Strand cDNA Synthesis Kit (Thermo Scientific) and the manufacturer's instructions, extracted total RNA was reverse transcribed. Total RNA extraction was done, and the kit's ingredients were defrosted and put on ice. For a 20µl reaction, 1µg of total RNA was utilized. The RT reaction mixture was created by filling a 1.5 ml autoclaved vial with the following components. Ultimately, the extracted cDNA was kept frozen at -20°C until it was needed for real-time PCR.

Real Time PCR for mRNA Expression of Molecular Markers

PCR was performed in real time using a Roche Light Cycler 96 apparatus. Roche SYBR® green fast PCR master mix PCR kit (which includes SYBR® green dye and all the PCR components) was used to accomplish the reaction. After being thawed, the template, primers, SYBR® Green dye, and RNase-free water were put on ice. By combining the ingredients as indicated by the primer sequences employed, the reaction mixture was created. Roche software was used to analyze the acquired data.

Results and Discussion

Cytotoxic Effect of Amphiroa Fragilissima on Lung Cancer Cell Line A549

The lung cancer cell line was treated with Amphiroa fragilissima by exposing the cells for two to four days. The absorbance of decreased MTT formazan that had been dissolved in DMSO was measured in a 96-well plate reader. The percentage of inhibition (absorbance at the y-axis) was plotted against the drug concentration (x-axis) in the plots. The IC50 concentration is the amount of medication required to cut the absorbance in half compared to the control (IC50 24HRS–37 & IC50 48hrs µM.). The cell line's amphiroa IC50 value was shown. The amphiroa destroyed cancer cells in a time- and dose-dependent way. Cell viability was significantly decreased by A. fragilissima in a concentration-dependent manner.

The triggering of cell death may be the reason behind the extract's inhibition of cell proliferation. Thus, proof of the in-vitro cytotoxicity is provided by A. fragilissima's inhibitory action.

Drug Treated Plate MTT **Assay Drug Treated Plate**

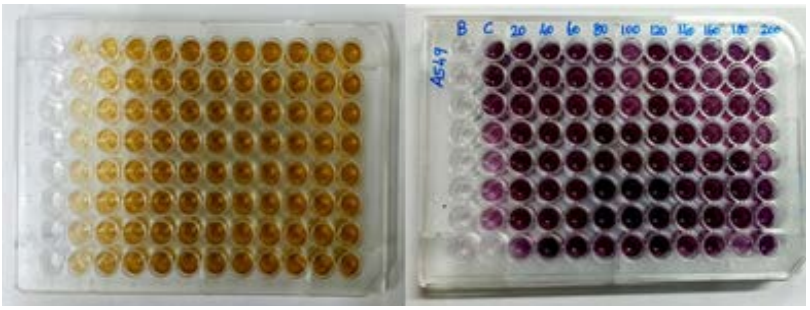

Figure 1 Image of 96 Well Plates of MTT Assay

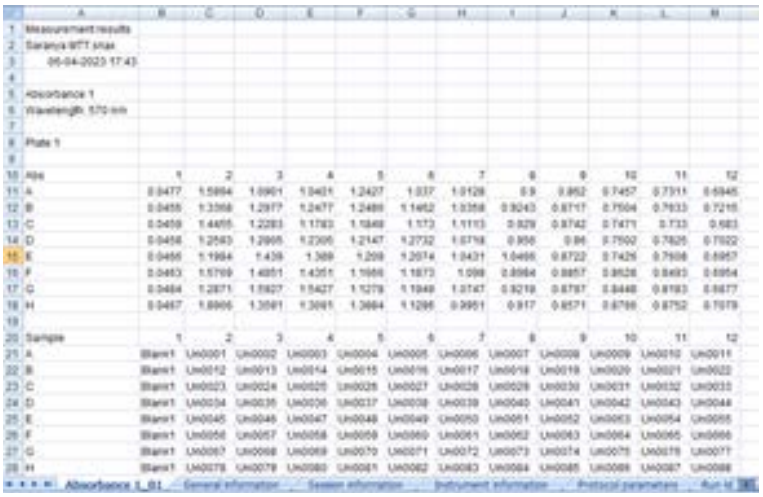

Figure 2 Absorbance Values Measured Values using Multiskan Skyhigh Contrast Image of MTT Assayed Plate

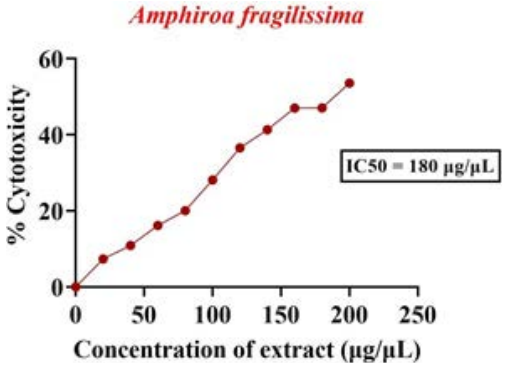

Figure 3 Effect of Ethanolic Extract of A.fragilissima on Lung Cancer Cell (A549)

Morphological Changes in Cells, as Indicators of the Nature of Cell Death

After being exposed for 24 hours to the ethanolic extract at the IC50 concentration, the lung cancer cell line (A549) was stained with AO-EB. The treated cells displayed abnormalities in contrast to the control cells, which fluoresced green and red. In lung cancer cell lines treated with amphiroa, necrotic death was less common but apoptotic death was higher. In cells with apoptotic characteristics, cell staining showed membrane blebbing, cytoplasmic vacuolation, the presence of apoptotic aggregates, chromatin marginalization, late apoptosis signal of dot-like chromatin, and numerous micro nuclei.

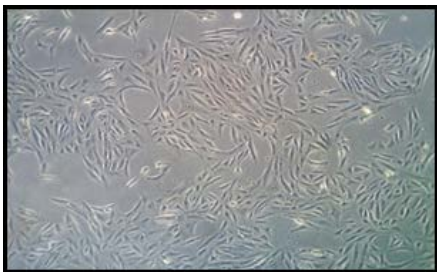

Figure 4 Phase Contrast Image of A549 Cell Line

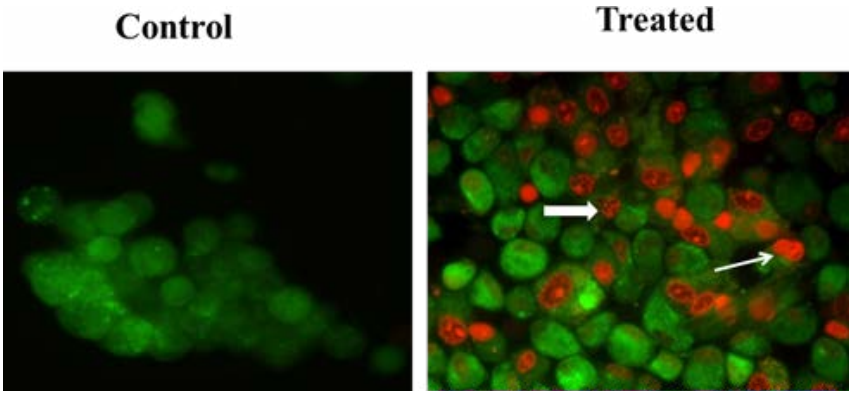

Figure 5 A549 Cells on Treatment with A.fragilissima Stained using AO/EB. Pointed Arrow – Apoptotic Cells; Solid Arrow – Necrotic Cells

mRNA Expression Analysis by Real Time PCR

In the expression analysis, mRNA levels of BCL2, PI3K, and PTEN were downregulated in treated condition. While BAX and CASPASE 9 of mRNA expression levels was upregulated in treated group. Histogram depicts a mRNA expression of BCL2 (A), BAX (B), CASPASE 9 (C), PI3K (D), and PTEN (E) were analyzed between control and treated groups and normalized to β-actin using RT-PCR. The data is shown as mean ± SD, with $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ for the three subjects.

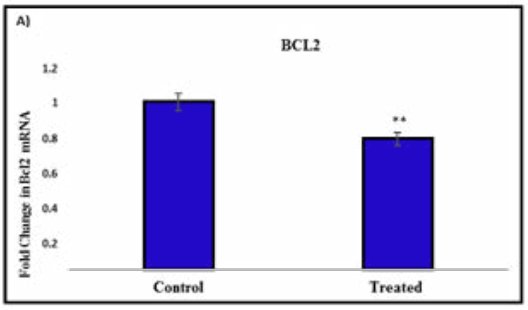

Figure 6 RT-PCR Analysis of mRNA Expression of BCL2

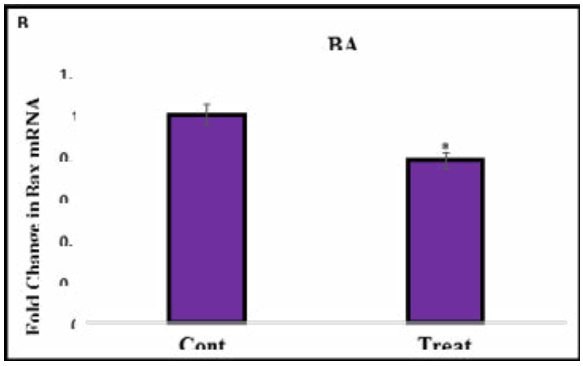

Figure 7 RT-PCR Examination of BAX mRNA Expression

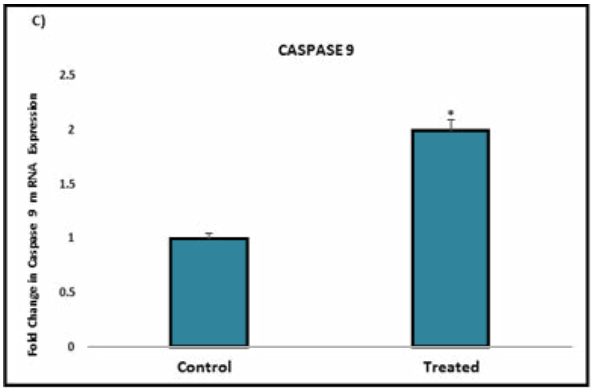

Figure 8 RT-PCR Examination of CASPASE 9 mRNA Expression

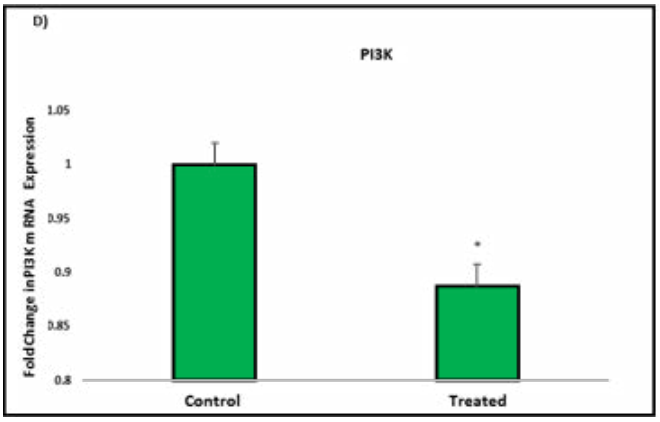

Figure 9 RT-PCR Examination of PI3K mRNA Expression

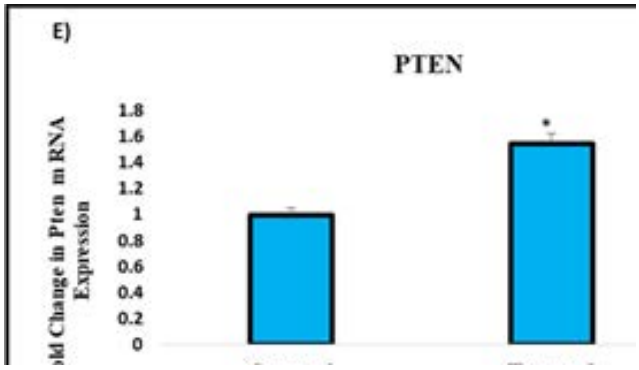

Figure 10 RT-PCR Examination of PTEN mRNA Expression

One important type of cell death that is necessary for both homeostasis maintenance and appropriate development is apoptosis (Du, 2002). Numerous natural substances seem to have strong cytotoxic and chemopreventive effects (Nasim et al., 2019). The two main members of the Bcl-2 family, Bax and Bcl-2, have been the subject of several studies over the past 10 years due to their possible roles in tumor progression and the prognosis of different human malignancies. Bax causes the outer membrane of the mitochondria to permeate in response to a variety of biological stresses, which encourages cell death. Patients with colorectal cancer had their tumoral and adjacent non-tumorous tissues examined for the Bax/Bcl-2 ratio and the degree of mRNA expression of the Bax and Bcl-2 genes.

Therefore, tumor cells can undergo apoptosis once more by inhibiting Bcl2. Bcl2 inhibitor chemicals, therefore, might represent a whole new class of therapeutic medications for the treatment of cancer (Zhou et al., 2019). The cysteine-aspartic protease Caspase-9 regulates both pathological tissue aging and programmed cell death. Its role as an inherent activator of apoptosis is widely acknowledged. Caspase-9 can prevent the growth of malignancies by triggering intrinsic apoptosis in response to cellular damage such as oxidative stress, genomic instability, and aberrant proliferation. The PI3K/Akt/mTOR pathway not only inhibits the growth and death of cancer cells, but it also promotes angiogenesis, both malignant and benign (Rascio et al., 2021). In a considerable proportion of cancers in humans,

Gene silencing, epigenetic modifications, and somatic mutations frequently result in decreased PTEN function. A partial loss of PTEN function has been linked to the development of certain human malignancies (Milella et al., 2015). The efficacy of P. tetrasromatica as a phytotherapeutic in the management of cancer was supported by this investigation.

Summary and Conclusion

Current research indicates that A. fragilissima suppresses the growth of the lung cancer cell line A549. This work demonstrates the use of A. fragilissima in cancer treatment. To find the component in Amphiroa that has the cytotoxic effect on cancer, more research is needed. The observation that A. fragilissima had a larger cytotoxic effect on the lung cancer cell line A549 is noteworthy, but more cell lines are needed to corroborate this conclusion. Additional research is needed to understand how anti-apoptotic gene deprivation activates the apoptotic pathway protein production.

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