Exploration of Anticancer Activity of Portieria Hornemanni Against Lung Cancer Cellline (A549)

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Abstract
Cancer remains a major global health issue for which there are now no entirely viable treatments. As a result, natural product-based alternative therapies have been suggested. Studies have demonstrated the biological activity of seaweed, particularly its anticancer properties. The review's main subject is lung cancer. It also lists some substances that have been taken out of a variety of seaweeds and demonstrated to either completely prevent cancer or significantly delay its spread.
The main objectives of the study are to find the cytotoxicity effect of Porteria hornimanni in human lung cancer cell line A549 adopting MTT assay and utilizing fluorescent labelling techniques to detect morphological changes in the cells following P. hornimanni therapy.
The present study indicates that P. hornemanni's ethanolic extract exhibits anti-cancer properties in the lung cancer cell line A549.This study shows how cancer cells can employ their anticancer properties. Additional research is necessary to identify the specific chemical in P. hornemanni that has cytotoxic effects on cancer.
Keywords: Portieria Horemanni, Lung Cancer, Cell Line

Introduction

A group of illnesses known as cancers are characterized by aberrant cell proliferation that has the potential to spread to other body areas.

(Lewandowska et al., 2008). Taxonomically, cancers are classified into many forms: quasi-classes, genera and species, each with its origin cells and mutational spectrum. Each cancer is distinct from other (Greaves et al., 2022).

Normal cells have several growth restrictions but are nevertheless able to divide and develop. They only proliferate in response to growth hormones, mutations, and specific environmental circumstances. If they are damaged, a molecular brake and stops them from dividing and become inactive condition until they are repaired (Demers et al., 2000). They surrender to the process of programmed cell death (apoptosis) if they are unable to be repaired. Under certain conditions, they are able to split a finite number of ties. They stay where they belong because they are a component of the tissue structure.

The ability of cancer cells to maintain persistent growth is perhaps their most basic characteristic. In order to maintain homeostasis in cell number and, consequently, normal tissue architecture and function, normal tissues meticulously regulate the synthesis and release of growth-promoting signals that direct entry into progression through

the cell growth and division cycle (Greaves et al., 202). Cancer cells take control of their own fate by manipulating these signals. Growth factors that attach to cell-surface receptors—which usually include intracellular tyrosine kinase domains—play a major role in transmitting the enabling signals.

The latter then go on to release signals through branched intracellular signaling pathways that control both the growth (i.e., increase in size) and progression through the cell cycle. These signals also frequently impact other cell-biological characteristics like energy metabolism and cell survival. There are several potential methods in which cancer cells can get the capacity to maintain proliferative signals. (Hynes Mec Donald et al., 2009

The development of apoptotic resistance in cancer cells is a characteristic shared by all forms of cancer. Resistance of apoptosis occur by loss of pro-apoptotic regulator through mutation of tumor suppressor genes (Lewison et al., 2022). Through mutation accumulation or deletion of the tumor suppressor gene, tumor cells render the gene inactive. Over half of all human cancers have a p53 gene mutation or deletion. (Gutschner et al., 202). The proteins RB pathway causes absence of gatekeeper of cell-cycle progression that allows cell proliferation without apoptosis. The apoptosis-inducing circuitry's sensor is eliminated when the TP53 protein is lost. (Chaffer et al., 2020). Whereas normal cells can only undergo a finite number of cell division cycles, malignant cells exhibit almost infinite multiplication

(Fingeret et al., 204). The unlimited replicative potential of cancer cells required to form macroscopic tumor. Telomeres, which cap the ends of chromosomes and make them eternal, must be actively maintained in cancers (Gentzel, 203). A specific type of DNA polymerase called telomerase lengthens the ends of chromosomes that are shortening during cell division. (Talib et al., 208). Blood vessel production, or angiogenesis, is a crucial step in the initiation and spread of cancer because it supplies the nutrients and oxygen required for continuous cell division (Folkman et al., 995). According to Mitsuhashi et al. (2004), tumor cells promote angiogenesis by activating angiogenic factors and suppressing factors that prevent angiogenesis. Angiogenesis-inducing ligands are encoded by the VEGF-A gene.

An angiogenic switch is virtually always triggered and stays on throughout tumor progression, forcing normally quiescent vasculature to continuously sprout new vessels that support the growing neoplastic growths. Different patterns of neovas cularization are seen in tumors when angiogenesis is triggered. (Hanahan et al., 996). As a cancer progresses, cancer cells from the main tumor spread to distant locations, infect nearby normal tissues, and establish new colonies. An estimated 90 % of deaths related to cancer are thought to be caused by metastasis.

(Chaffer et al.,20). There are numerous phases involved in cancer metastasis, such as tissue invasion, loss of cell contact, intravasation, movement throughout the body, extravasation at the secondary site, and formation of a secondary tumor.

(Gupta et al.,2006). Cell adhension molecules in immunoglobulin and calcium dependent cadherin molecules mediate the cell-cell interaction. Integrin is involved in a cell's interaction with substrates of the extracellular matrix. The inactivation of cadherin and integrin molecules and upregulation of protease enzyme activate invasion and angiogenesis (Jorge et al., 2022). By overexpressing anti-apoptosis proteins that impede the apoptotic process, cancer cells can avoid dying. B-cell lymphoma-2 (Bcl-2) family play an important role in apoptosis resistance (Talib et al., 208). It is possible to develop resistance to apoptosis by mutating the p53 tumor suppressor gene, which results in the lack of a proapoptotic regulator. Apoptosis defects are a common occurrence, and aberrant proliferation is crucial to the development of tumors. (Ruizcasado et al, 207).

Seaweeds

An essential marine renewable resource in coastal waters is seaweed.Many nations have long used fresh and dry seaweeds as a marine vegetable, and it is known that they are safe to eat (Manivannan et al., 20). Their nutritional worth is great, and their fat and protein content is superior to that of other vegetables.In addition to being a significant source of anticancer compounds, red and brown algae are primarily used as food for humans. Researchers have conducted several anticancer studies using the crude and refined chemicals derived from seaweeds, which are among the primary biological agents that have been examined for their diverse biological properties.

Antibiotics, anticancer activities, laxatives, anticoagulants, and anti-ulcer medicines are all found in seaweeds. Despite having such a diverse array of bioactive compounds, seaweeds are still a material that is not fully utilized. Seaweeds may be used in the pharmaceutical and neutraceutical sectors with more research on these components. (Pereira et al., 2020).

Porteriahornimannii

The significance of marine algae for the pharmaceutical industry led to the evaluation of their pharmacognostic properties and the preliminary phytochemical, biochemical, and inorganic mineral examination of the red seaweed Portieria hornemannii's sequential extract. (Tripathi et al., 2022). P. hornimannii is a species of red algae in the family Rhizophyllidaceae. The source of carrageenan is found in upper to lower subtidal locations exposed to moderate to vigorous water circulation, where it is stuck to rocks or dead corals.

Figure 1 Portieriahornemanni (Division: Rhodophyta, Class: lorideophyceae, Order: Gigartinales Family: Rhizophyllidaceae, Gene: Portieria, Species: P.hornim

Materials and Methods

Maintenance of Cells

The standard culture media for A549 cells is F2/K (Gibco/Invitrogen). To create the full growth medium, the base medium is supplemented with 0 % fetal bovine serum (FBS). Complete medium, which consists of Dulbecco's MEM (DMEM) adjusted with 0% FBS, can also be used to cultivate the cells.

The National Centre for Cell Science in Pune, India's human long cancer cell line A549 was cultured as a monolayer in DMEM media containing 2 % antibiotics and 0 % FBS. Once the cells were harvested using trypsin EDTA and seeded in tissue culture flasks to keep in the exponential phase, stock cultures were sub-cultured every seven days.

Chemicals

Cell culture media: Himedia provided the DMEM medium (Cat. No. AL007S -6x500ML) that is used to grow cells. It contains high glucose and sodium bicarbonate.

Serum: Fetal Bowine Serum Albumin (FBS) (CatNo.RM9955-500ML) was used for the growth of cells and was obtained from Himedia.

Antibiotics: Pencilin and streptomycin (Cat No. A00-00ML) were obtained from Himedia
Trypsin-EDTA: 25 µgWe purchased trypsin-EDTA (Cat No. TCL45-00ML) from Himedia.

Cell Counting
Reagents and Equipments
0.04 % Tryphan blue in PBS, an inverted microscope, and a hemocytometer.

Technique
After gently mixing the cell suspension, an aliquot was added to the Tryphan blue solution (00µl cell suspension:00µl) and the hemocytometer was used to count the cells.

Compute
Number of viable cells overall = A × B × C × 04
Total dead cell count is equal to A × B × C × 4.
Dead cells plus visible cells equals the total number of cells. %Viability = Dependent cell count ×00 / Total cell counts Whereas
A=Cell Volume
B is the dilution factor.
C= Average quantity of uncolored cells
D=Mean quantity of stained or dead cells
For 0. cu.mm to mL, the conversion factor is 4.

MTT Assay
Materials
Dimethyl sulphate (DMSO)
MTTstocksolution(5mg/mL)
The preparation was done as follows: PBS 0 mL, MTT 50 mg.
A micron filter was used to filter it following a 20-minute vortex. Due of the light sensitivity of MTT, the vial was wrapped in paper or aluminum foil to block out light. The mixture was kept cold, at 4°C.

Method
In 96-well microtitre plates (5 x 03 cells/well), human lung cancer cellline A549 was cultured for a whole day. Following the seeding of the plate, it is incubated for 24 hours both with and without P. hornimannii ethanolic extract at varying doses (from 00µg to 000µg/mL). 20µl of MTT (5 mg/ml) was added after the medium had been refilled. The plates were left in the dark for three to four hours. After solubilizing the formazan crystals with 00µL of DMSO, the plate was left in the dark for a further five minutes.In an ELISA reader, the color generated was measured at 570 nm and compared to a reference wavelength of 630 nm.

Assessment of Cell Morphology
Acridine Orange and Ethidium Bromide: A crucial dye that may stain both living and dead cells is acrylidine orange. Only dead cells will be stained by ethidium bromide.
Stock: Acridine orange - 0 mg/mL in PBS Ethidium Bromide -0 mg/mL in PBS Working solution – AO - µL from stock is diluted with 00 µl of PBS EtBr – µL from stock is dilutedwith00µL of PBSAO/Et Brstain is prepared by mixing the both stains in: ratio.

Method

For twenty-four hours, human lung cancer cell line A549 was cultured in six wellplates (5×03 cells/well). After that, the cells were cultured for 24 hours with the P. hornimannii ethanolic extract IC50. After discarding the medium, PBS was used to wash the cells. After the cells were trypsinized, they were put on a glass plate and stained with ethidium bromide and cadmium orange. Following that, an epifluorescent microscope was used to view the cells.

Gene Expression

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

The following study is performed to analyze the expression of mRNAs including BCL2, BAX, CASPASE9, PI3K and PTEN. The quantity of the above mentioned molecules are interpreted by quantification with RT PCR.

Table Primers'Oligonucleotid

Gene Name	Forward primer	Reverseprimer
β-actin	GCGAGTACAACCTTCTTGCAG	CATACCCACCATCACACCCTG
BCL2	AAACGAAAGCAACAGGAACACT	GTGGCAATTGAAAGGCGTGT
BAX	CATGGGCTGGACATTGGACT	CTTCCAGATGGTGAGCGAGG
CASPASE9	TCCCAATCCAACGGGGATAA	GTCTTCCCCTCTTCACAGGATG
PI3K	GGGAGCCCCAGAAAAGCAGA	AGTTCTCCAGCTCCATGCCC
PTEN	GCGTGCGGATAATGACAAGG	AGCCTCTGGATTTGATGGCTC

RNA Isolation

Using ml of the TRIZOL reagent, the cells were thoroughly homogenized.After that, the homogenate was kept at 4´C for 5 minutes to allow the nucleoproteins to fully associate. After adding 0.2 ml of chloroform/ml of RNA reagent, this was centrifuged for 5 minutes at 4℃ at 2000 rpm. After separating the RNA-containing upper aqueous phase, an equivalent volume of isopropanol was added, and the mixture was centrifuged at 2000 rpm for 0 minutes at 4̄C.

The RNA precipitates as a white pellet at the bottom of the tube and is centrifuged at 7500 rpm for 5 minutes at 4℃ after being twice cleaned with 75% ethanol. To achieve total solubility of the resultant RNA pellet, it was dissolved in 30µL of 0.2% sterile water and maintained in a waterbath at 60´C for 0 minutes. After that, the RNA sample was vortexed for 0 minutes and kept at -80℃.

Total RNA Quantification

The total RNA that was extracted was measured using a nanodrop device. On the separated RNA, three quality checks were carried out. RT-PCR sequences Three things needed to be determined: first, the amount of RNA; second, its purity; and third, the integrity of the extracted RNA. The nanodrop equipment 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, protein and nucleic acid extraction processes have both used the ratio of absorbance at certain wavelengths as a purity indicator.

DEPC water was used to calibrate the Nano drop. When RNA purity is compared to impurities that absorb in the UV, like protein, it can be calculated using the ratio between the absorbance values at 260 and 280 nm. 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, µg of total RNA was utilized. After autoclaving a.5 mL vial, the following chemicals were added to create the RT reaction mixture. Ultimately, the extracted cDNA was kept frozen at -20°C until it was needed for real-time PCR.

Real Time PCR form RNA 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.

Statisticalana

Excel was used for data processing, and students' t-test was used to analyze the data for two group comparisons in Graph Pad Prism. The fold change difference between the control and experimental groups was computed using the 2-ΔΔCt technique (Xing et al., 209) Value of probability ≤0.05. The data are presented as the mean standard deviation from at least three separate, duplicate experiments.

Result and Discussion

Ethanolic Extract of Porteria Hornimanni's cytotoxic impact on the lung cancer cell line A549. The cytotoxic impact of P. hornimanni's ethanolic extract was investigated on the human lung cancer cell line a549. The cells were exposed to concentrations ranging from 50µg to 250µg for a duration of 24 hours. After dissolving the decreased MTT formazan in DMSO, the absorbance was measured using a 96-well plate reader. The figures were plotted against the drug concentration (x-axis) as a percentage of inhibition (absorbance at y-axis). The medication concentration needed to cut the absorbance in half compared to the control was found to be the IC50 concentration. Figure 4 displayed the IC50 value for the ethanolic extract of P. hornemanni on the cell line. The cancer cells were killed by the ethanolic extract of P. hornimanni in a way that was dependent on both dose and duration.

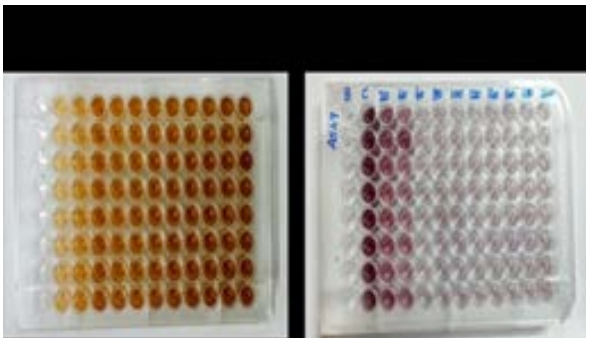


Figure 2 Image of MTT Assay 96 Wellplates

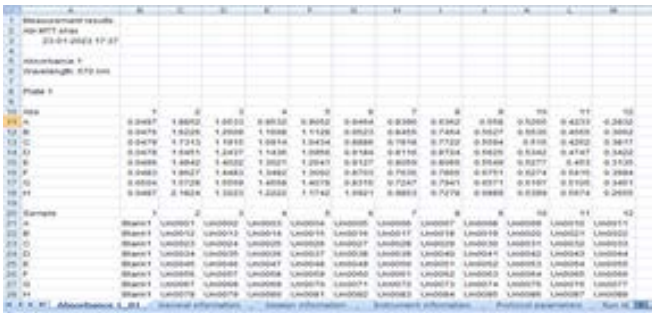


Figure 3 Measured Absorbance of an MTT-assayed Plate by using a Multi Skan Sky High Microplate Reader

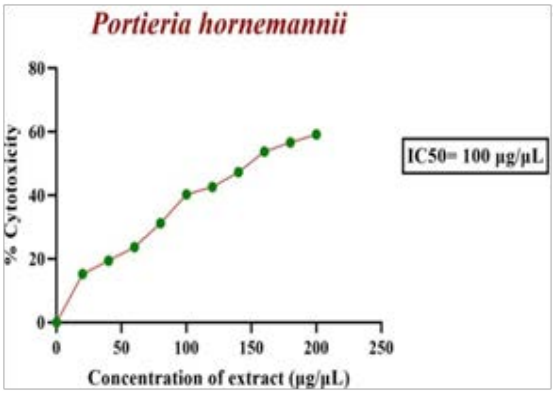


Figure 4 P.Hornimannie Thanolic Extract's Effect on Lung Cancer Cell (A549)

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

Lung cancer cell line A549 was treated with an ethanolic extract at an IC50 concentration for 24 and 48 hours, following which Acridine orange (AO) and Ethidium bromide (EtBr) staining was performed.

The control cell appeared green,where as treated cells showed abnormalities.Apoptotic death was higher in lung cancer cell line treated with P.hornimanni but necrotic death was lower. cell staining revealed membrane blebbing , cytoplasmic vacuolation, the presence of apoptotic aggregates, chromatin marginalization , late apoptotic signal of dot-like chromatin, and numerous micro nuclei in cells with apoptotic characteristics.

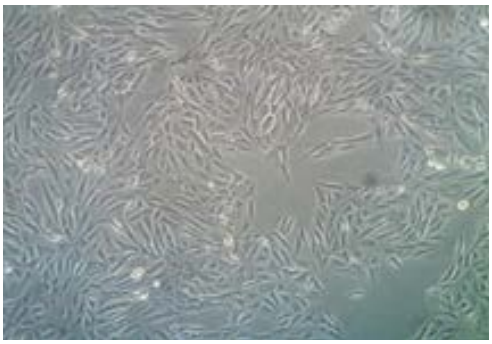


Figure 5 Phase Contrast Image of A549 Cell Line

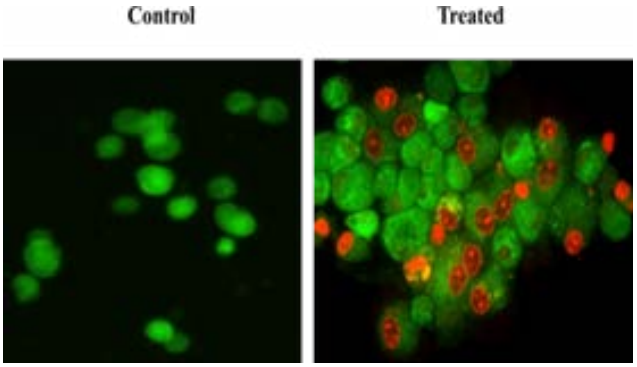


Figure 6 A549 Cells on Treatment with P.thornemanni Stained using AO/EB Pointed Arrow – Apoptotic Cells; Solid Arrow –Necrotic Cells m RNA Expression Analysis by Real Time PCR

In the expression analysis, mRNA levels of BCL2, PI3K, and PTEN were down regulated in treated condition.

In the treated group, there was an upregulation of the mRNA expression levels of BAX and CASPASE 9.The mRNA expression of BCL2(A), BAX(B), CASPASE9 (C), PI3K (D), and PTEN (E) was evaluated using RT-PCR to standardize the results between the control and treated groups. Data are shown as mean ±SD, (n=3), *p<0.05, **p < 0.0, and ***p < 0.00.

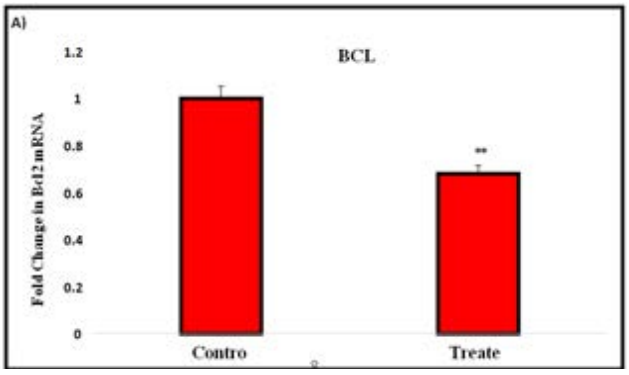
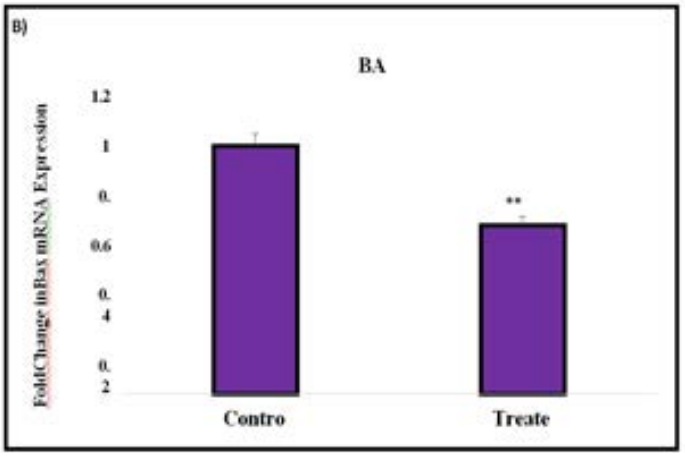
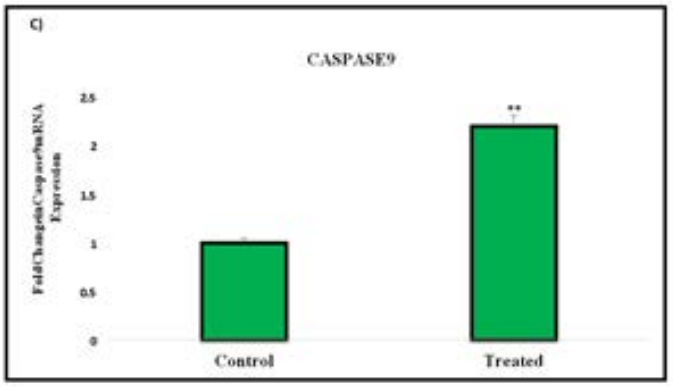


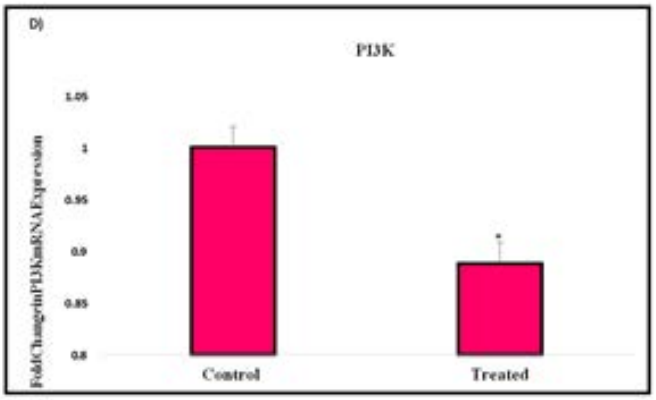
Figure 9 (A) RT-PCR Analysis of mRNA Expression of BCL2



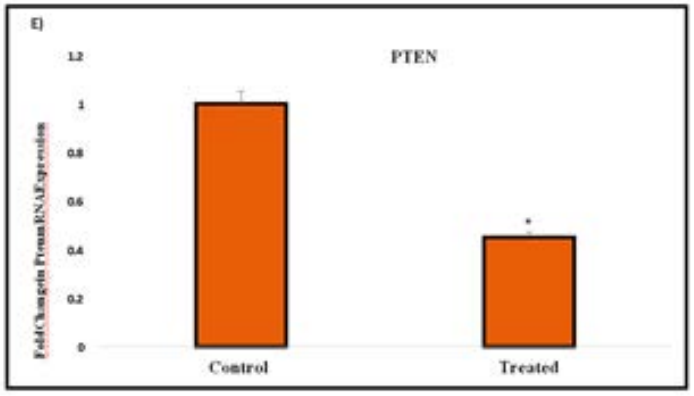
(B) RT-PCR Analysis of mRNA Expression of BAX



(C) RT-PCR Analysis of mrna Expression of CASPASE9



(D) RT-PCR Analysis of mRNA Expression of PI3K



(E) RT-PCR Analysis of mRNA Expression of PTEN

According to Travis (20), lung cancer is the leading cause of major cancer incidence and mortality in males worldwide, while it is the second most common cause of cancer incidence and death in women. Many methods can be employed for staging and diagnosis. The type and stage of the cancer will determine the course of treatment; a number of personality drugs that were not widely accessible a few years ago are now available. Taking care of someone with lung cancer is difficult. (Nasim, et al., 209).

Based on earlier investigations, the plant Porteria hornimanni was utilized in this study as a medication against cancer cell lines. We investigated the anticancer activity of P. hornimanni in a lung cancer model. P. hornimanni's anticancer effectiveness has been studied in a lung cancer cell line (A549), where the drug markedly reduced cell growth and encouraged cell death. P. hornimanni caused cancer cells to undergo a specified amount of apoptosis, and viable and non-viable cells were distinguished using the staining techniques of Acridine orange and Ethidium bromide.P. hornimanni prevents colonization and causes morphological changes that may be seen under fluorescence microscopy in lung cancer cell lines. These morphologically shrunken cells are thought to be apoptotic cells. 40X cell counting magnification is used to count all apoptotic cells.

BAX is an essential component of apoptosis and a member of the Bcl-2 protein family. The first known oncogene that modifies the regulation of programmed cell death instead of promoting cell division is the Bcl-2 gene. The two main members of the Bcl-2 family, Bax and Bcl-2, are important players in the development of tumors or in the suppression of intrinsic apoptotic pathways that are set off by malfunctioning mitochondria. Thus, the balance between this family's pro- and anti-apoptotic members can dictate the fate of a cell (Khodapasand et al., 205). Caspase-9 is an initiator caspase that causes apoptosis and activates other caspases to cause death signals.

Caspase-9 has a pivotal role in apoptosis in multiple cancer cells types. The regulation of caspase-9 could be phosphorylation at different sites, recruitment of caspase-9 to apoptosome or inhibition of caspase-9 expression (Kim et al., 205).

The phosphorylation of the 3-idroxyl group of phosphatidylinositol on the plasma membrane initiates the phosphoinositide 3-kinases (PI3Ks), a significant family of lipid enzymes (Frumanetal.,204). The primary mutations causing the deregulation of this system in human tumors are found in oncogenes, like the PI3KCA gene that codes for the component p0, and in tumour suppressor genes, like phosphatase and tensin homolog (PTEN) (Sugiyama et al., 209). P. hornimanni can lessen the survival and metastasis of lung cancer cells. It was discovered that P. hornimannican also has molecular effects on cells. P. hornimanni can dramatically lower the growth, proliferation, and death of cancer cells, according to the results of its therapy of lung cancer cell lines.

Conclusion

The current investigation demonstrates that P. hornemanni's ethanolic extract possesses anti-cancer properties against the lung cancer cell line A549.

This work signifies the use of anticancer activity of the cancer cells. P. hornemanni has to be further studied to unravel the particular compound which has the cytotoxic effect on cancer. Although findings of higher cytotoxic effect of ethanolic extract of P. hornemanni has been demonstrated in lung cancer cell line A549 is promising yet this result need to investigated in a different cell lines. To investigate the elevation of apoptotic pathway protein expression upon deprivation of anti-apoptotic genes, more research must be done.

A western blot assay should be conducted to measure the protein expression. The main question needs to be answered in future studies as to how a particular compound from P. hornemanni can be isolated, studied and utilized as a phyto therapeutic medicine for treating cancer has to be carried out.

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