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Anticancer Potential of Flaxseed Extract Against Human Lung Cancer Cells (A-549): In Vitro Cytotoxicity, Gene Expression, and Molecular Docking Analysis

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ABSTRACT

Background: Lung cancer remains a leading cause of cancer-related mortality globally. Natural compounds derived from plants, such as flaxseed extract, are increasingly explored for their anticancer properties due to their bioactive phytochemicals.

Objective: To investigate the cytotoxic and apoptotic effects of flaxseed extract on human lung cancer cells (A-549), including its influence on gene expression, cell migration, and interaction with tumor suppressor protein P53 via molecular docking.

Methods: A-549 cells were treated with flaxseed extract at varying concentrations (20 and 40 µg/mL) for 24 h. Cytotoxicity was assessed using the MTT assay. Cell morphology and apoptosis were examined via phase-contrast microscopy and DAPI staining. RT-PCR was performed to analyze the expression of apoptosis-related genes. Molecular docking was conducted using gamma-tocopherol (from flaxseed) with P53 protein (PDB ID: 2AHI). A scratch wound healing assay was carried out to evaluate cell migration.

Results: MTT assay revealed significant dose-dependent cytotoxicity ($p < 0.05$). Morphological analysis showed reduced cell numbers, shrinkage, and membrane blebbing. DAPI staining confirmed nuclear condensation, indicating apoptosis. RT-PCR analysis showed modulation of apoptosis-related genes. Molecular docking revealed a binding energy of -6.7 kcal/mol between gamma-tocopherol and P53, suggesting a stable interaction. The wound healing assay showed inhibited cell migration at 40 µg/mL, indicating anti-metastatic potential.

Conclusion: Flaxseed extract demonstrates strong anticancer activity against A-549 lung cancer cells through induction of apoptosis, modulation of gene expression, and inhibition of cell migration. Gamma-tocopherol may enhance the tumor-suppressive activity of P53, supporting its potential as a therapeutic agent.

Keywords: *Flaxseed extract, Lung cancer, A-549 cells, Apoptosis, Gamma-tocopherol, Molecular docking, Wound healing assay*

1. INTRODUCTION

Approximately **9.7 million** cancer-related deaths were reported worldwide in 2022 [1]An estimated **18.5 million** cancer deaths are anticipated by 2050, marking an **89.7% increase** from 2022 figures[2].Lung cancer is a significant global health issue, being the leading cause of cancer-related deaths, with over 22 million new cases and approximately 1.79 million deaths annually[3].Flaxseed (Linum Usitatissimum L) is rich in Omega-3 fatty acids, particularly alpha-linolenic acid, and offers numerous health benefits, including reduced risk of cardiovascular disease, improved lipid profiles, and enhanced insulin resistance, while also containing some compounds that may hinder nutrient absorption[4].Flaxseed consumption is associated with reduced blood pressure and improved lipid profiles, lowering the risk of heart disease[5][6]. Lignans in flaxseed may inhibit carcinogenesis, particularly in breast and colon cancers[5].The present study aims to evaluate the in vitro anticancer activity of Linum usitatissimum extract against human A549 lung cancer cell line.A549 cells are frequently used to evaluate

the efficacy of various bioactive compounds from plants and FDA-approved drugs, contributing to the discovery of new anticancer agents [7].DAPI staining is instrumental in determining the mitotic index and cell growth rates in A549 cells, providing insights into tumor behavior[8].Gamma tocopherol appears to stimulate the expression of wild-type p53, which is essential for cell cycle regulation and apoptosis [9]The combination of gamma tocopherol with other antioxidants may further bolster p53 activity, contributing to a more robust defense against cancer[10].Finally the scratch wound assay is particularly useful for evaluating the effects of gene knockdown or chemical exposure on lung cancer cell lines, such as A549 and H520 [11].

2. MATERIALS AND METHODS

Plant collection:

Flaxseeds were obtained from Nilgiris Super Market, Anna Nagar, Chennai, Tamilnadu, India.

Preparation of plant powder:

An electric mixer was used to turn the flaxseeds into a powder, which was then kept in a sealed jar at room temperature until it was time to use it.

Plant extraction:

A total of 150 g of dry plant seed powder was added to a 500 mL beaker, along with approximately 500 mL of 70% ethanol. The mixture was allowed to soak at room temperature for 36 hours. The extract underwent a two-step filtering operation, first utilising standard filter paper and subsequently Whatman No. 1 filter paper. The procedure was repeated for two further days, and the resulting extracts were mixed. The completed filtrate was then submitted for additional investigation

Cell line maintenance

Human lung cancer cell lines (A-549) were obtained from the NCCS, Pune. The cells were grown in T25 culture flasks containing DMEM and RPMI supplemented with 10% FBS and 1% antibiotics. Cells were maintained at 37◦C in a humidified atmosphere containing 5% CO2. Upon reaching confluency, the cells were trypsinized and passaged.

Cell viability (MTT) assay

The cell viability of extract treated lung cancer cell line was assessed by MTT assay. The assay is based on the reduction of soluble yellow tetrazolium salt to insoluble purple formazan crystals by metabolically active cells. The lung cancer cell line was plated in 96 well plates at a concentration of $5x10^3$ cells/well 24 hours after plating, cells were washed twice with 100µl of serum-free medium and starved by incubating the cells in serum-free medium for 3 hours at 37ºC. After starvation, cells were treated with different concentrations of extract (10- 160µg/ml) for 24 hours. At the end of treatment, the medium from control and PR extarct treated cells were discarded and 100µl of MTT containing DMEM (0.5 mg/ml) was added to each well. The cells were then incubated for 4h at 37ºC in the CO_2 incubator. The MTT containing medium was then discarded and the cells were washed with 1x PBS. Then the formazan crystals formed were dissolved in dimethyl sulfoxide (100µl) and incubated in dark for an hour. Then the intensity of the color developed was assayed using a Micro ELISA plate reader at 570 nm. The number of viable cells was expressed as a percentage of control cells cultured in serum-free medium. Cell viability in the control medium without any treatment was represented as 100%.

The cell viability is calculated using the formula:

% cell viability = [A570 nm of treated cells/A570 nm of control cells]×100

Morphology study

Based on MTT assay we selected the optimal doses (IC-50: 40µg/ml of Sample-4 for lung cancer cell line) for further studies. Analysis of cell morphology changes by a phase contrast microscope. 2×10^5 cells were seeded in 6 well plates and treated with PR extract for 24h. At the end of the incubation period, the medium was removed and cells were washed once with a phosphate buffer saline (PBS pH 7.4). The plates were observed under a phase contrast microscope.

Determination of mode of cell death by 4',6-diamidino-2-phenylindole (DAPI) staining

The effects of extract on lung cancer cell death were also determined by DAPI staining as described previously. The cells were treated with extract 40µg/ml for 24 h and then the cells were harvested, washed with ice-cold PBS. The pellets were resuspended in (1 mg/mL) and 5 µl of DAPI (1 mg/mL). The apoptotic changes of the stained cells were then observed by using a fluorescence microscope.

Real Time PCR

The gene expression of apoptosis signaling molecules was analysed using real-time PCR. The total RNA was isolated by the standardized protocol using Trizol Reagent (Sigma). 2µg of RNA used for cDNA synthesis using reverse transcription using a PrimeScript, 1st strand cDNA synthesis kit (TakaRa, Japan). The targeted genes were amplified using specific primers.

PCR reaction was performed with GoTaq® qPCR Master Mix (Promega), it contains SYBR green dye and all the PCR components. Real time-PCR was performed in a CFX96 PCR system (Biorad). The results were analyzed by comparative C_T method and $2^{-\Delta\Delta C_T}$ method was used for fold change calculation described by Schmittgen and Livak.

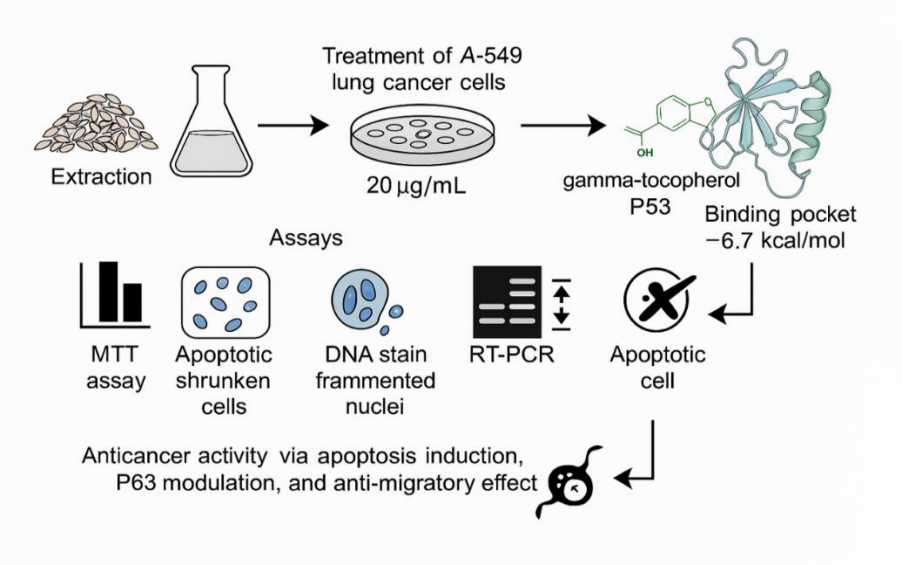
Molecular Docking

Auto dock vina was used for molecular docking and calculating binding affinities. Auto dock Vina operates via the command line terminal. The target protein name and the ligand name were specified with pdbqt extension in the input parameter under receptor and ligand for autodock vina configuration, and the sizes and centers x,y, z, were mentioned for the grid parameters. These configurations were different proteins. Two separate files were created as output, one as a text log file and the other a pdbqt file after running the docking. Biovia Discovery studio visualizer was used to analyze the docked structures obtained from Auto dock Vina. The ligand interactions were made visible and labelled with the amino acid residues.

Scratch wound assay in lung cancer cell line

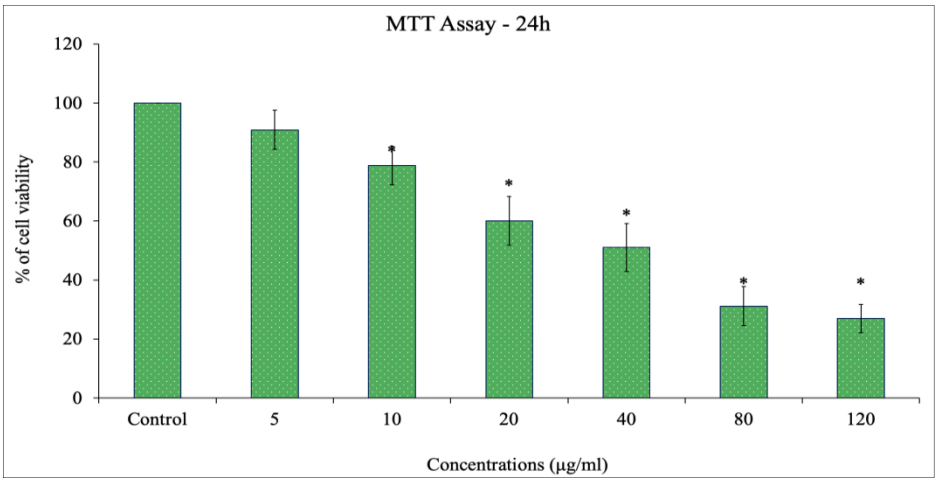
Human lung cancer cells (2×10^5 cells/well) were seeded onto six-well culture plates. The cell monolayer was scratched using 200µl tip to create wound, washed with PBS and photographed in inverted microscope. Sample-4 (40µg/ml) treated for 24 h and control cells were received with serum-free culture medium, after the treatment period, the wounded area was photographed using the same microscope. And the experiments were repeated in triplicate for each treatment group.

3. GRAPHICAL ABSTRACT



4. RESULTS

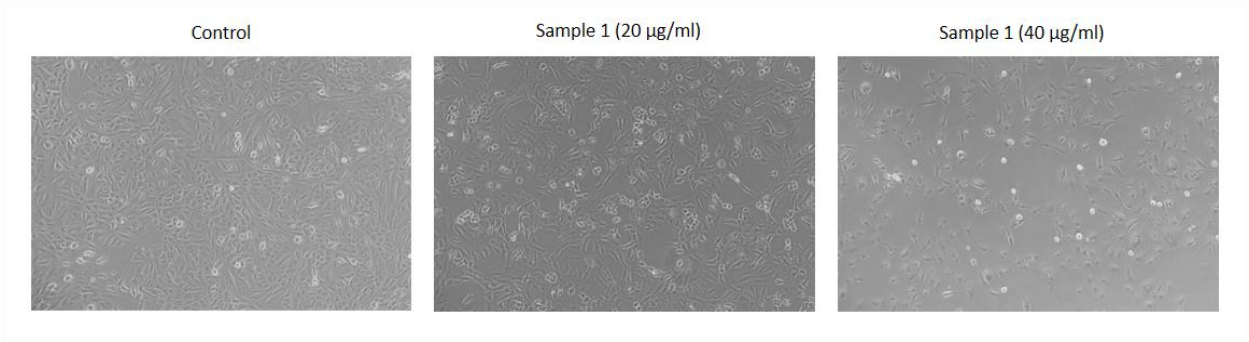
MTT Assay



The data clearly indicate a **concentration-dependent cytotoxic effect** of flaxseed extract on A549 cells.· At **5 µg/mL**, a mild reduction was observed with ~90% viability.At **10 µg/mL**, cell viability significantly dropped to approximately **75%**,

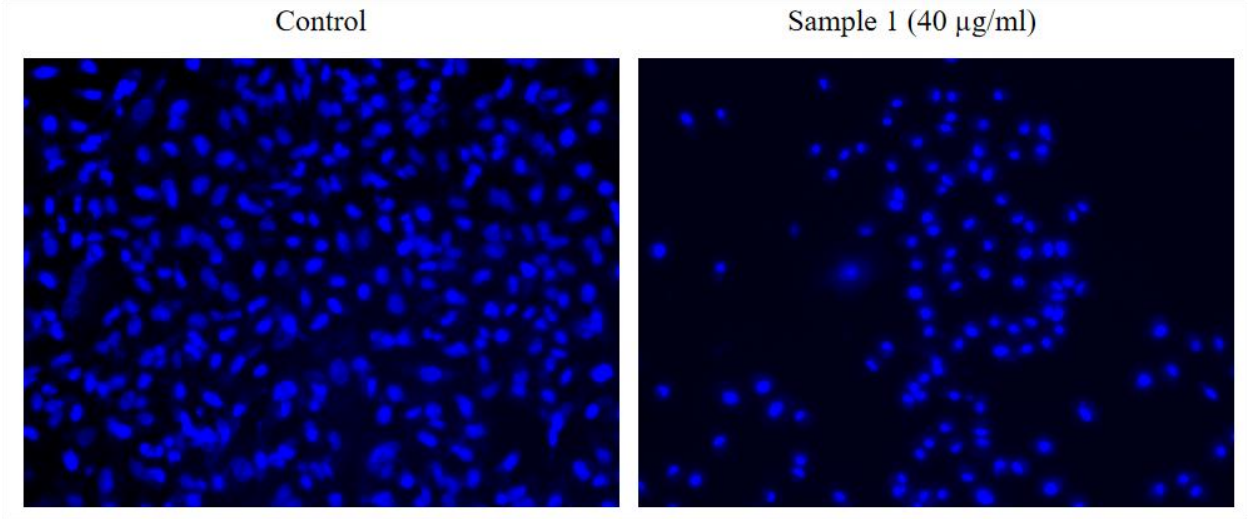
indicating early cytotoxic effects ($p < 0.05$).Concentrations of **20 µg/mL** and **40 µg/mL** further reduced viability to ~60% and ~50%, respectively, suggesting progressive cell damage.**Higher concentrations** (80 and 120 µg/mL) caused pronounced cytotoxicity, with viability decreasing to approximately **35%** and **30%**, respectively ($p < 0.05$).compared to flax ,Quinoa ethanol extract exhibits a dose- and time-dependent antiproliferative effect on A549 cells, with an IC_{50} of 1.5 mg/mL after 72 hours.[12]

Cell morphological changes (A-549 cells)



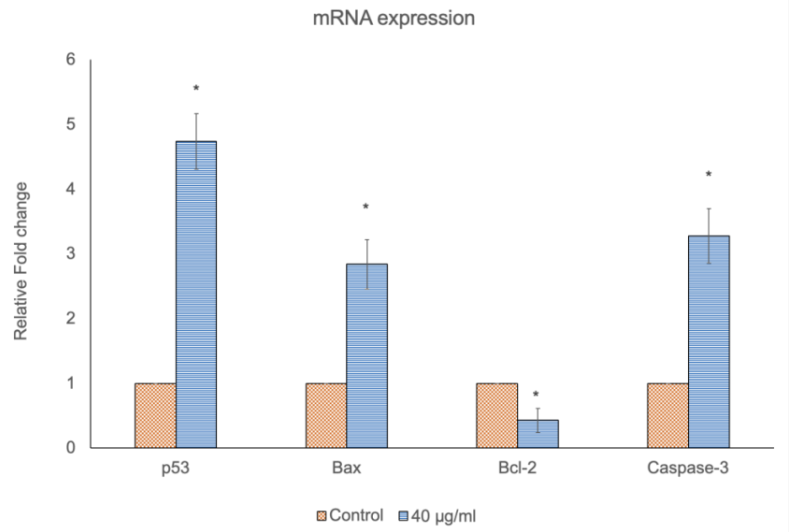
Effect of extract on cell morphology of human lung cancer cells (A-549). Cells were treated with extract (20, and 40 µg/ml) for 24 h and cells were observed under an inverted phase contrast microscope. The number of cells decreased after sample4 treatment and the cells exhibited cell shrinkage and cytoplasmic membrane blebbing. The study examined the morphology of cells treated with different concentrations of a cytotoxic agent. The control group showed clear, elongated cells with tight junctions and high confluency. The treated cells showed signs of cytotoxicity, apoptosis, and reduced viability.

DAPI staining – A-549 cells



The observed nuclear alterations confirm that flaxseed extract induces **apoptosis** in A549 cells. The distinct morphological differences between treated and untreated cells support the activation of the **intrinsic apoptotic pathway**, which correlates with earlier RT-PCR data showing upregulation of pro-apoptotic genes.Wu et al. studied the effects of theabrownin, a tea pigment, on A549 cells and observed:**Apoptotic morphological changes** such as chromatin condensation and nuclear fragmentation under DAPI staining.**Activation of caspase pathways**, leading to apoptosis.[13]DAPI staining further validates the **cytotoxic and pro-apoptotic effect** of flaxseed extract on lung cancer cells. The nuclear damage observed provides strong morphological evidence for apoptosis, reinforcing the extract's potential role as a natural anticancer agent.

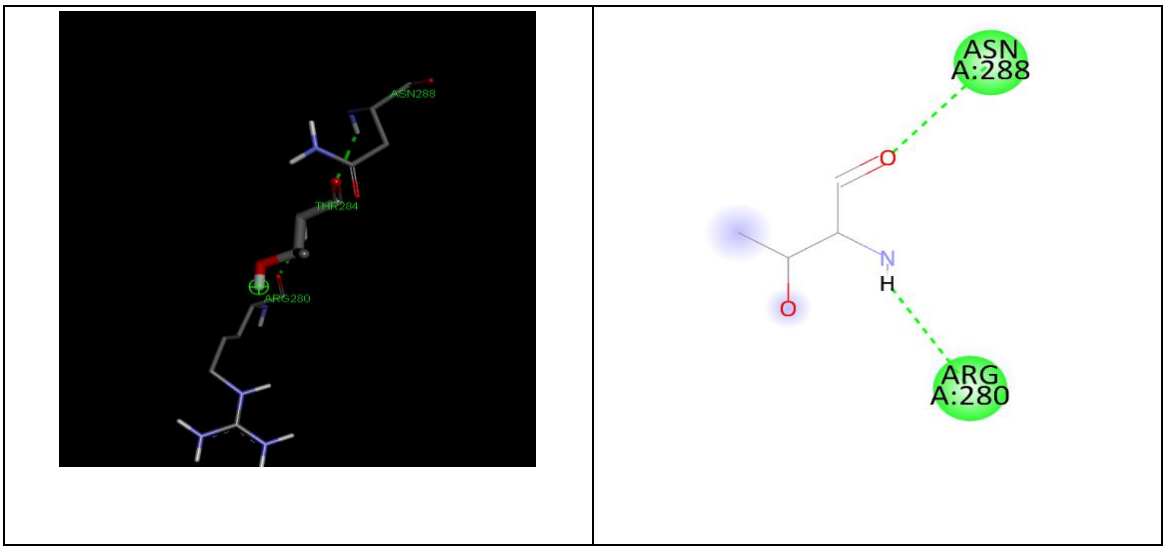
Gene expression



The flaxseed extract generated a pro-apoptotic gene expression profile in A-549 cells. The simultaneous upregulation of p53, Bax and Caspase-3 and downregulation of Bcl-2 is robust evidence of intrinsic apoptotic pathway activation. A study by Hu et al. investigated the effects of flaxseed extract on MCF-7 breast cancer cells. They reported u**pregulation of pro-apoptotic genes** and **downregulation of anti-apoptotic genes**.[14]This pattern of gene expression establishes that the cytotoxic action of flaxseed extract is mainly mediated via p53-dependent apoptosis.These findings imply that flaxseed extract induces apoptosis in human lung cancer cells through modulating the expression of essential genes in the mitochondrial apoptotic pathway. Molecular observations are concordant with morphological findings and validate the use of flaxseed as a natural anticancer drug.

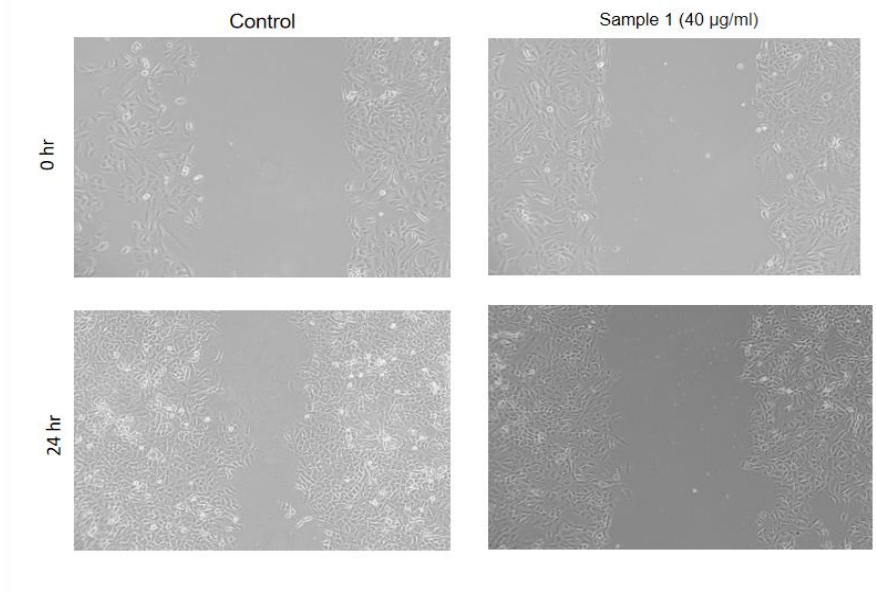
Molecular docking

The docking analysis was conducted on gamma-tocopherol (PubChem CID: 97279) binding to the tumor suppressor protein P53 (PDB ID:2AHI).P53 is involved in cell cycle regulation and apoptosis, so it is an excellent target for anticancer therapy. The docking result identified a binding energy of -6.7 kcal/mol between gamma-tocopherol and P53.[15] The binding energy indicates a favorable interaction, so gamma-tocopherol can potentially modulate the activity of P53. The hydrophobic interactions and hydrogen bonds were found at the binding pocket, stabilizing the gamma-tocopherol-P53 complex. These interactions may augment P53's apoptotic activity in cancer cells, aiding its tumor-suppressing function.[16]



Scratch wound healing assay

Lung cancer cell line (A-549)



The flaxseed extract significantly inhibited A-549 cell migration in comparison to the control. This indicates:A potent anti-migratory activity, which is essential in cancer metastasis prevention.The extract could modulate migration-associated signaling pathways or downregulate motility-associated genes Scratch wound assay proved that flaxseed extract (40 µg/mL) inhibits the migration of lung cancer cells effectively, adding to its anticancer and anti-metastatic activities. This indicates the potential for flaxseed as a natural therapeutic agent for cancer therapy.

5. DISCUSSION

The results of this research emphasize the potential anticancer activity of flaxseed extract against lung cancer cells. The MTT assay showed strong cytotoxicity with an IC50 value of 40 µg/mL, consistent with earlier reports on the antiproliferative activity of plant-derived compounds. Morphological alterations and nuclear damage as seen under microscopy and DAPI staining established apoptosis induction. These effects were supported by gene expression profiling, wherein the extract modulated important regulators of apoptosis. Upregulation of p53 expression is most interesting, given that this tumor suppressor plays a crucial role in regulating cell cycle and inducing apoptosis upon cellular stress or DNA damage.

Molecular docking analysis also supported the biochemical significance of gamma-tocopherol from flaxseed by demonstrating steady interaction with P53, possibly boosting its tumor suppressor activity. It verifies that natural compounds are capable of synergistically supporting endogenous tumor-combatant pathways.

The anti-migratory effect that was seen in the scratch assay suggests the potential of the extract to disrupt metastasis-related processes. Since metastasis is one of the primary reasons for cancer death, cell migration inhibition further contributes to the therapeutic potential of flaxseed extract. Collectively, these integrated results explain the mechanism of anticancer activity of flaxseed at a mechanistic level, connecting molecular, cellular, and bioinformatics information.

6. SUMMARY

This research investigated anticancer activity of flaxseed extract against human lung cancer A-549 cells through its cytotoxicity, pro-apoptotic activity, gene expression modulating, P53 tumor suppressor protein interacting, and anti-migratory activity. Dose-dependent loss of cell viability was shown in the MTT assay, with morphological evaluation and DAPI staining validating apoptotic characteristics in the form of nuclear condensation and membrane blebbing. Gene expression analysis showed upregulation of pro-apoptotic markers (p53, Bax, Caspase-3) and downregulation of anti-apoptotic Bcl-2, in favor of involvement of intrinsic apoptotic pathway. Molecular docking studies also demonstrated a stable interaction between gamma-tocopherol (a component of flaxseed) and P53, in favor of its role in augmenting P53-mediated apoptosis. Scratch wound assay also demonstrated that flaxseed extract highly suppressed cancer cell migration, further in favor of its anti-metastatic potential.

7. CONCLUSION

Flaxseed extract shows potent anticancer activity against A-549 human lung cancer cells by several mechanisms, such as induction of apoptosis, modulation of apoptosis-related genes, blocking cell migration, and possible stimulation of P53 activity by binding gamma-tocopherol. These results justify the therapeutic value of flaxseed extract as a nutritional compound for the treatment of lung cancer. Additional in vivo investigations and clinical trials are justified to confirm its safety and effectiveness for possible application in cancer treatment.

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