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Chia Seed (Salvia Hispanica L.)-Derived Linolenic Acid Induces Apoptosis and Inhibits Migration in Human Lung Cancer Cells Via Bax-Mediated Pathways

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ABSTRACT

Lung cancer continues to be among the top forms of cancer death globally. The demand for safer and natural therapeutic agents has made searching for plant anticancer agents more urgent. Chia seeds (Salvia hispanica L.), which are linolenic acid-rich,have demonstrated good bioactive activity, such as pro-apoptotic action.To explore the anticancer action of chia seed extract, specifically its potential to induce apoptosis, suppress cell proliferation and migration, and regulate gene expression in A-549 lung cancer cells.Human lung cancer cells (A-549) were seeded and incubated with different concentrations of Chia extract. Cytotoxicity was assessed with MTT assay. Apoptosis was investigated by DAPI staining. The morphology was determined through phase-contrast microscopy.The expression of apoptotic marker genes was determined through real-time PCR. The cell migration was evaluated with a scratch wound healing assay. Molecular docking was performed to analyze the binding between linolenic acid (from chia seed) and Bax protein (PDB IDs: 1F16 and 4S0O).Chia seed extract significantly decreased A-549 cell viability in a dose-dependent manner with IC_{50} of 40 µg/ml. Treated cells exhibited characteristic apoptotic changes such as cell shrinkage and nuclear fragmentation. Real-time PCR validated upregulation of pro-apoptotic genes. Scratch assay demonstrated reduced cell migration . Molecular docking showed moderate binding of linolenic acid to Bax protein (-4.1 and -3.1 kcal/mol binding energies), implying strengthening of apoptotic pathways.Chia seed-derived linolenic acid shows considerable anticancer activity against A-549 lung cancer cells by inducing apoptosis, suppressing cell proliferation and migration. The results indicate the therapeutic potential of compounds from chia seed as natural anticancer agents, deserving further in vivo confirmation and clinical investigation..

Keywords: *Chia seeds,Anticancer activity,Linolenic acid, A-549 lung cancer cells,Cell viability assay (MTT)..*

1. INTRODUCTION

Cancer is a complex group of diseases characterized by the uncontrolled growth of abnormal cells, which can invade surrounding tissues and metastasize to distant sites. The term "cancer" has historical roots, originating from the Greek word for crab, reflecting the invasive nature of tumorsl [1].Carcinomas, sarcomas, lymphomas, leukemias, adenocarcinomas, and myelomas are the major types of cancer**[2]** Lung cancer, a leading cause of cancer-related mortality globally, is characterized by uncontrolled cell growth in lung tissues, primarily classified into small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC) [3].**Tobacco Smoking,**The primary risk factor, with a strong correlation between smoking rates and lung cancer incidence. Historical data shows a lag time of 20-30 years between increased smoking and lung cancer cases[4][5]**Air Pollution**: Exposure to particulate matter and other pollutants is a significant risk, particularly in urban areas and developing

countries[5][6].**Radon Gas and Asbestos**: These environmental carcinogens are linked to lung cancer, especially in occupational settings[4]**Genetic and Lifestyle Factors**: Low physical activity, poor diet, and genetic predispositions also play a role in increasing susceptibility to lung cancer[6].Chia seeds (Salvia hispanica L.) are increasingly recognized for their rich nutritional profile and health benefits, making them a popular functional food. These small seeds are high in protein, dietary fiber, omega-3 and omega-6 fatty acids, and various micronutrients, contributing to their potential in preventing chronic diseases such as cardiovascular issues, diabetes, and obesity. Their versatility allows for consumption in various forms, including whole seeds, flour, and oil, enhancing their application in the food industry.Chia seeds contain 18-24% protein and 30-34% dietary fiber, promoting satiety and digestive health [7]They are particularly rich in omega-3 fatty acids (54-67%), which are essential for heart health[8] Chia seeds provide essential vitamins and minerals, including calcium and antioxidants like polyphenols[9] Regular consumption is linked to reduced risks of cardiovascular diseases, diabetes, and obesity [10][7]Chia seeds exhibit anti-inflammatory and antioxidant effects, supporting overall health and immunity[11].In this study ,we learn about anticancer activity of CHIA SEED extract in lung cancer cell (A549) line

2. MATERIAL AND METHODS:

2.1 SAMPLE COLLECTION:

The place of purchase of the chia seeds was Reliance Super Market in Chennai, Tamilnadu, India's Anna Nagar.

2.2 PREPARATION OF SEED POWDER:

Utilising an electric blender, the chia seeds were blended into an extremely fine powder, which was then sealed and kept at ambient temperature until needed.

2.3 CHIA SEED EXTRACTION:

A 500 mL beaker was used to combine approximately 500 mL of ethanol with 150 g of dried chia seed powder. The combination was left to soak for 36 hours at room temperature. A twofold filtering technique was used, first passing the extract through standard filter paper and subsequently through Whatman No. 1 filter paper. The method was repeated for two more days, and the resultant extracts were combined. The finished filtrate was then submitted to additional investigation

2.4 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.

2.5 CELL VIABILITY (MTT) ASSAY

The cell viability of sample 2 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 sample 2(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.

2.6 MORPHOLOGY STUDY

Based on MTT assay we selected the optimal doses (IC-50: 40µg/ml of sample 2 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.

2.7 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) PBS and 5 µl of DAPI (1 mg/mL). The apoptotic changes of the stained cells were then observed by using a fluorescence microscope.

2.8 SCRATCH WOUND HEALING ASSAY

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-2 (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.

2.9 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

STATISTICAL ANALYSIS

All data obtained were analyzed by One way ANOVA flowed by Students-t-test using SPSS, represented as mean ± SD for triplicates. The level of statistical significance was set at $p<0.05$.

2.GRAPHICAL ABSTRACT

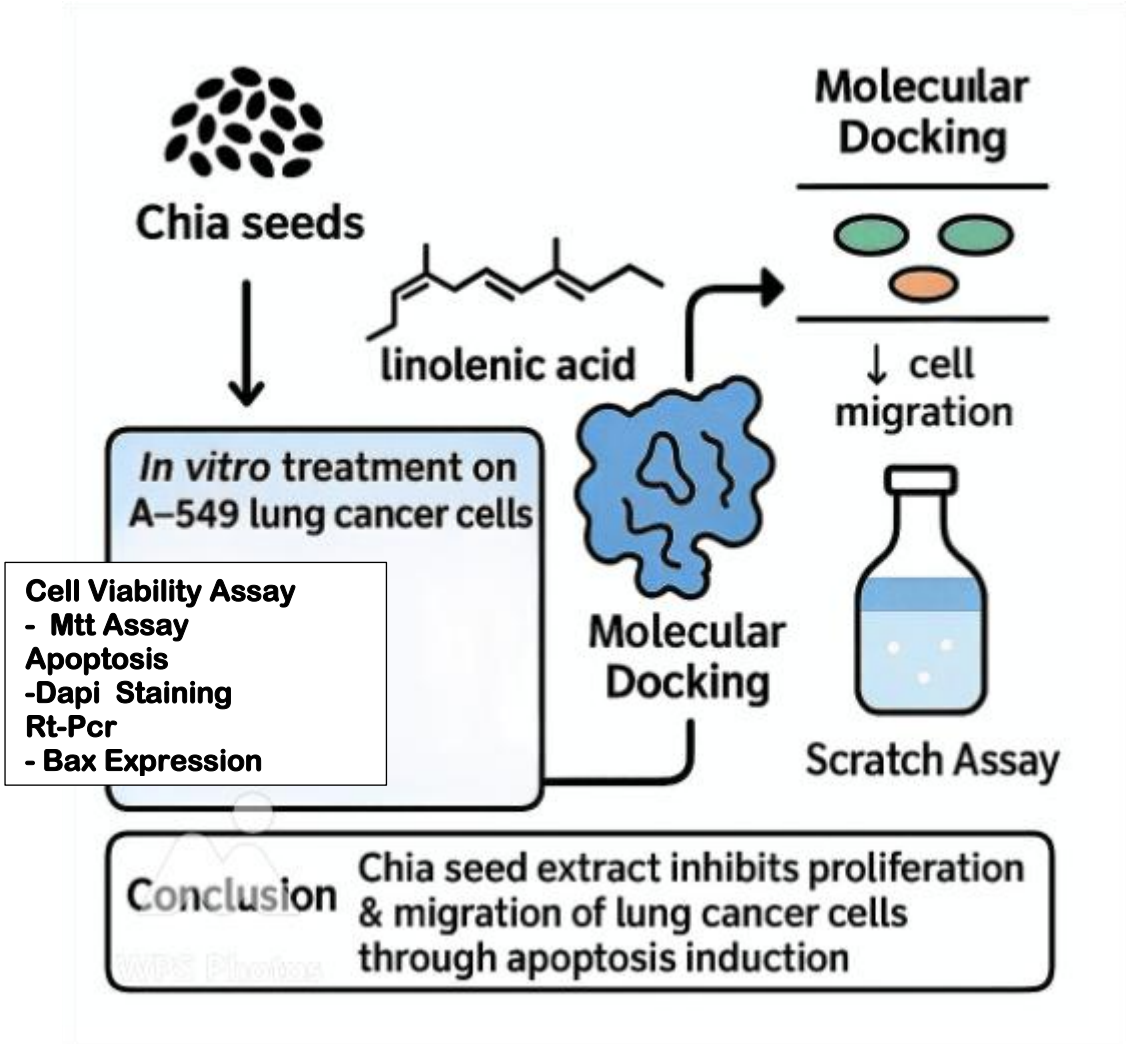

Figure 1. Graphical abstract illustrating the workflow of the study

3. RESULTS

3.1 ANTI CANCER ACTIVITY IN LUNG CANCER CELL LINE

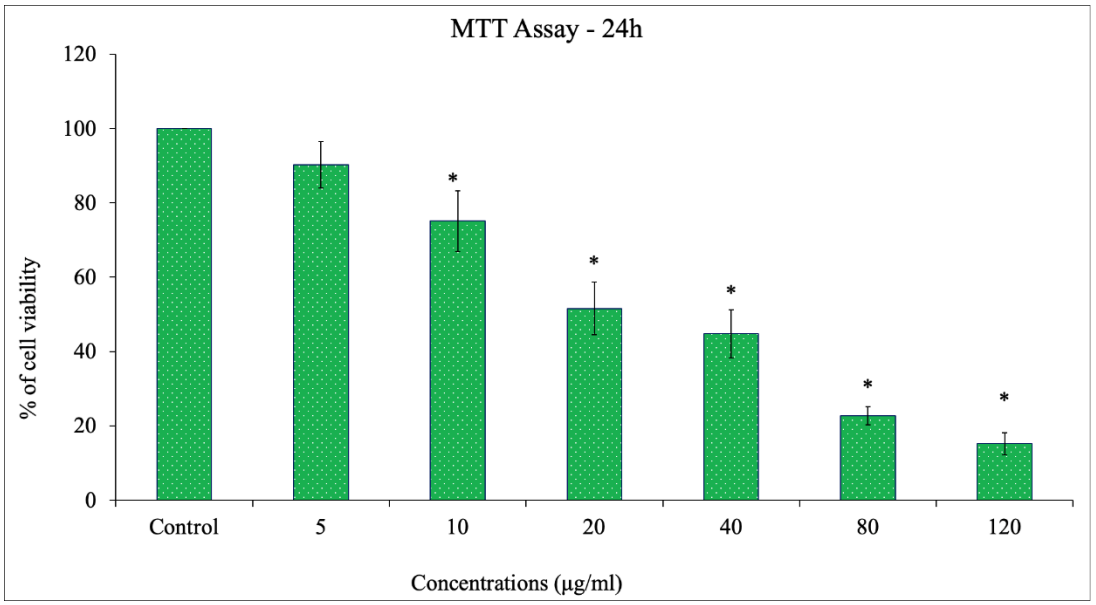

Figure 2. Effect of Sample 2 (chia seed extract) on the viability of A-549 lung cancer cells after 24 h treatment as measured by MTT assay. Data are presented as mean ± SD of three independent experiments ($p < 0.05$ vs. control).

The study investigated the cytotoxic action of Sample 2 against A-549 lung cancer cells using an MTT assay. The results showed a dose-dependent reduction in cell viability, with high viability at 5 µg/ml, moderate loss at 10 µg/ml, and clear cytotoxic effect at 20 µg/ml. The study suggests that Sample 2 has bioactive substances that can cause cytotoxicity in lung cancer cells, possibly through inhibition of mitochondrial activity or apoptosis. Further studies are needed to determine the pathway.Piperine showed a similar dose-dependent inhibition of A549 viability with mitochondrial-dependent apoptosis.Like piperine, chia extract reduces metabolic activity in cancer cells, indicating potential anticancer activity[12].

3.2 CELL MORPHOLOGY OF HUMAN LUNG CANCER CELLS (A-549)

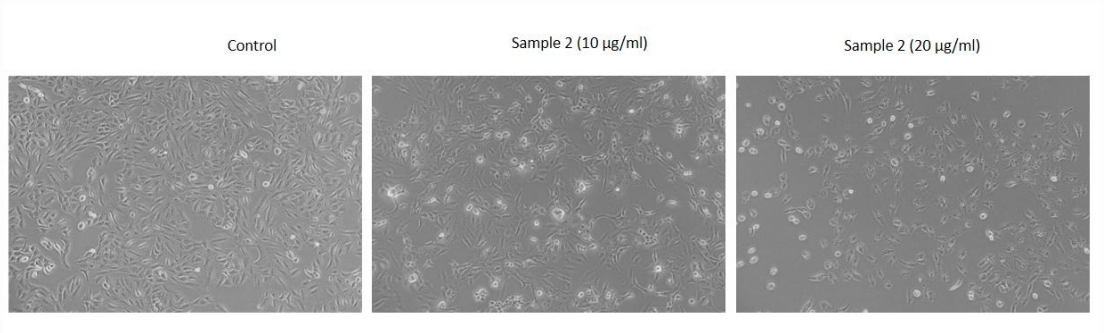

Figure 3. Phase-contrast microscopic images showing morphological changes in A-549 cells after 24 h treatment with Sample 2. Treated cells show characteristics of apoptosis such as shrinkage, detachment, and membrane blebbing.

The research finds that Sample 2 extract, which is administered in a dose-dependent manner, triggers cytotoxicity, which includes the hallmark of apoptosis, such as membrane blebbing, cell shrinkage, and detachment, and is more evident at elevated concentrations. These results lend credence to the hypothesis that the sample may initiate apoptotic pathways, resulting in cell death among A-549 lung cancer cells. The

morphological alterations seen in the cells exposed to Sample 2 indicate that the extract possesses great anti-cancer activity through cytotoxic as well as apoptosis induction action.Gypenosides induced similar apoptotic features in A549 cells such as cell rounding and blebbing, indicative of mitochondrial apoptosis.[13]

3.3 DAPI STAINING TO ASSESS NUCLEAR MORPHOLOGY AND APOPTOSIS

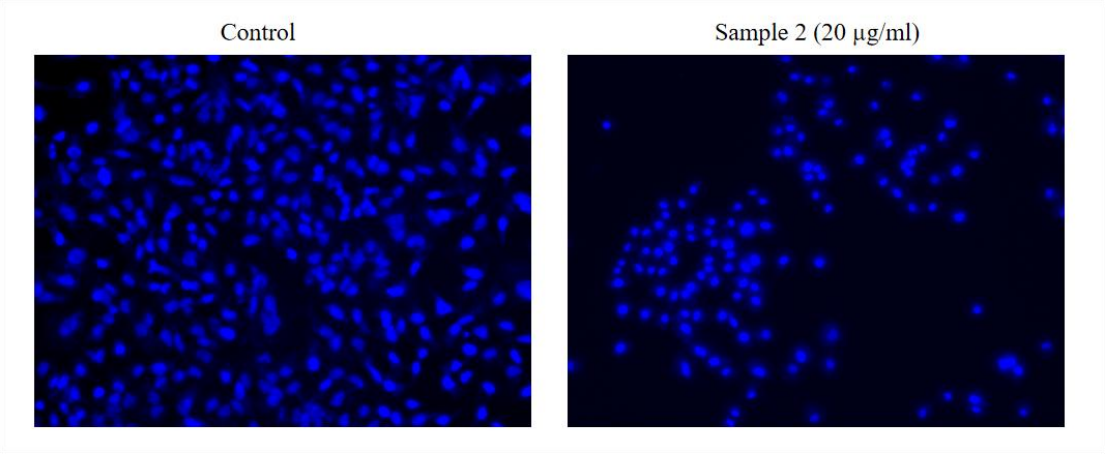

Figure 4.DAPI staining of A-549 lung cancer cells treated with 20 µg/ml of Sample 2 showing nuclear condensation and fragmentation compared to untreated control.

Human lung cancer cells were treated with extract (20µg/ml) for 24 h along with the control group. After the treatment, the cells were incubated with DAPI staining. Images were obtained using an Inverted Fluorescence Phase contrast microscope.

Chia seed extract, was found to induce significant apoptosis in A549 human lung cancer cells, as confirmed by DAPI staining on untreated cells. The cells showed reduced cell number, nuclear condensation, fragmentation, and formation of apoptotic bodies, indicating the extract's cytotoxic and pro-apoptotic potential.Silver nanoparticles (AgNPs) also caused DAPI-visible changes such as nuclear fragmentation and chromatin condensation in A549 cells .[14]The nuclear changes observed in this study confirm apoptosis, consistent with other apoptotic agents.This finding supports the cell viability results obtained in the MTT assay.

3.4 GENE EXPRESSION

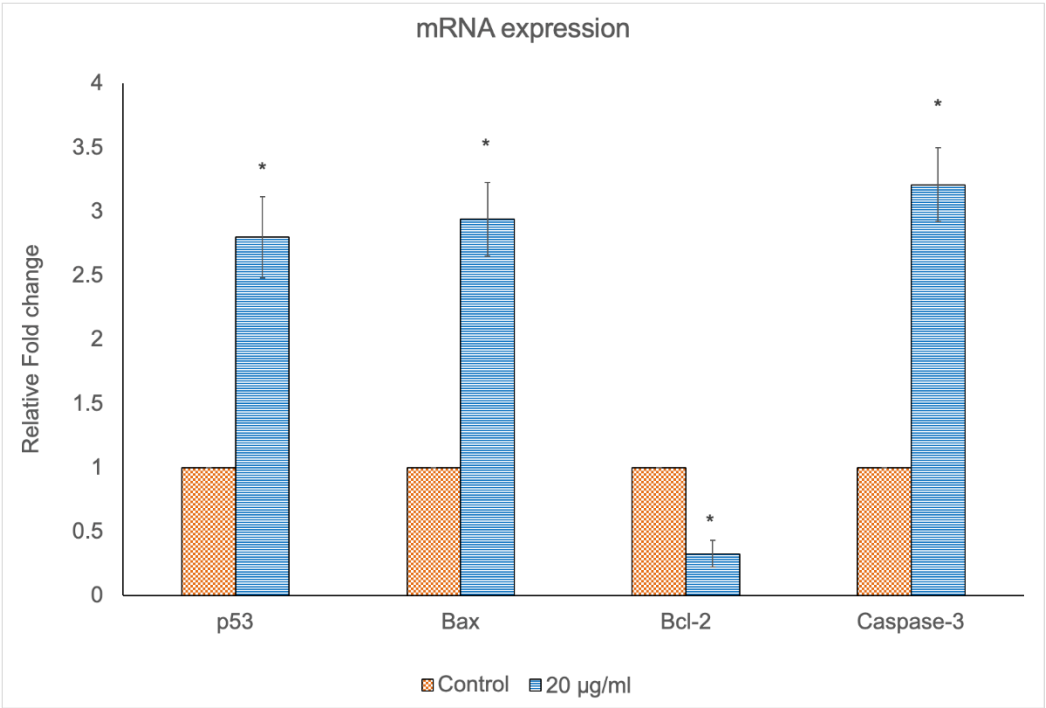

Figure 5. Relative mRNA expression levels of apoptosis-related genes (p53, Bax, Bcl-2, and Caspase-3) after 24 h treatment with Sample 2 in A-549 cells. Upregulation of pro-apoptotic markers and downregulation of anti-apoptotic gene Bcl-2 confirm activation of intrinsic apoptotic pathways.

Effect of this extract in apoptosis gene expression in lung cancer cells. Total RNA was prepared for reverse transcriptase PCR (RT-PCR) analysis of apoptosis signalling molecules gene expression in lung cancer cells. The experiment was repeated three times. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were used as internal controls for the RT-PCR

analyses.Chia seed extract was determined to cause cytotoxicity in A549 human lung cancer cells by increasing the expression levels of p53, Bax, and Caspase-3. This implies that Sample 2 induces mitochondrial-mediated apoptosis in lung cancer cells by initiating apoptotic signaling pathways. The enhanced expression of pro-apoptotic genes and suppressed expression of anti-apoptotic genes, as evidenced by qRT-PCR and other molecular data, further support the apoptotic activity of Sample 2 in A549 cells.Eudesmin treatment upregulated p53, Bax, and Caspase-3 while downregulating Bcl-2 in A549 cells[15]The modulation of apoptotic markers in your study mirrors mechanisms in several phytochemical-based treatments.

3.5 MOLECULAR DOCKING

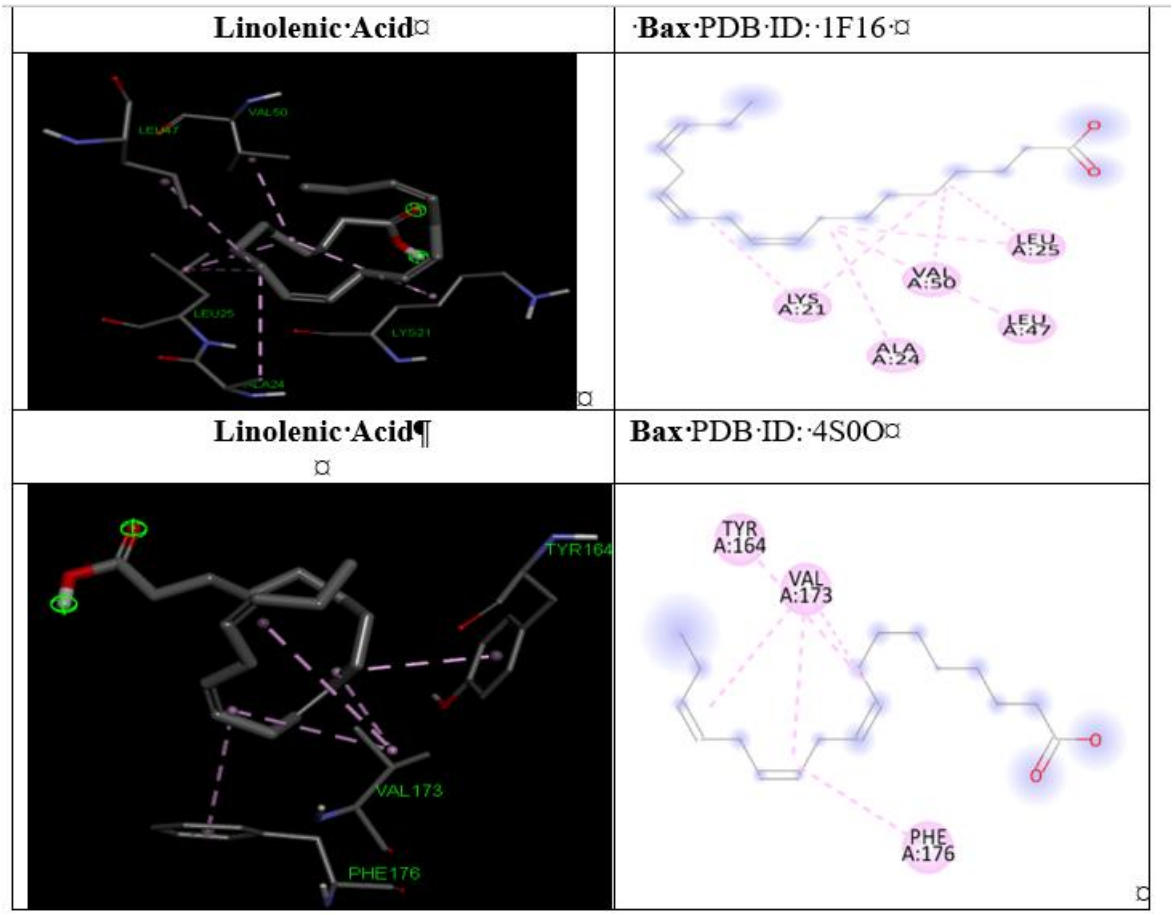

Figure 6. Molecular docking of linolenic acid (from chia seed) with pro-apoptotic protein Bax (PDB ID: 1F16 and 4S0O). Binding energies of -4.1 and -3.1 kcal/mol suggest moderate binding and potential pro-apoptotic activity.

Binding energies for the interaction between Bax and linolenic acid were calculated as -4.1 kcal/mol based on PDB ID 1F16 and -3.1 kcal/mol based on PDB ID 4S0O.These values reflect a moderate affinity, pointing towards an inhibitory interaction that may increase the pro-apoptotic capability of Bax.The molecular interaction implies that linolenic acid can stabilize Bax in its active form, thus facilitating mitochondrial membrane permeabilization and subsequent cytochrome c release. This cascade results in the activation of caspases, essentially triggering apoptosis in cancer cells. The results highlight therapeutic value of linolenic acid derived from chia seeds as an adjuvant in cancer therapy. By augmenting apoptosis mediated by Bax, linolenic acid might act synergistically with current chemotherapeutic agents to offer a natural, less toxic means for cancer control.Guava-derived compounds showed binding energies of -3.5 to -5.2 kcal/mol with cancer-related proteins, including Bax, supporting their apoptotic role [16].

3.6 SCRATCH WOUND HEALING ASSAY

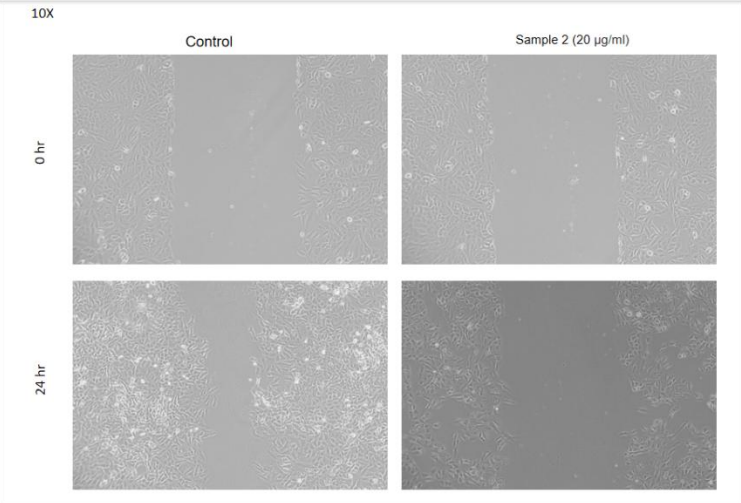

Figure 7.Scratch wound healing assay showing inhibition of A-549 cell migration after 24 h treatment with 20 µg/ml of Sample 2. Quantitative data indicates 85–90% closure in control vs. 20–30% in treated group ($p < 0.05$), suggesting anti-migratory potential.

A study conducted on A-549 lung cancer cells found that CHIA SEED extract significantly inhibited cell migration, a key process in cancer metastasis. The extract, at its IC_{50} level (20 µg/ml), showed no significant difference in the initial wound width. The cells treated with the extract showed fewer cell migration into the gap, indicating its potential anti-metastatic activity. This suggests that CHIA SEED extract could have bioactive molecules that inhibit pathways related to cell motility and cytoskeletal reorganization, potentially playing a role in inhibiting the metastatic spread of lung cancer cells.Curcumin significantly inhibited A549 cell migration in wound healing assays, indicating anti-metastatic potential.[17].CHIA SEED extract's ability to prevent migration is in line with other natural agents that inhibit metastasis.

3.6.1 QUANTIFICATION OF WOUND CLOSURE:

The percentage of wound closure was calculated using ImageJ software. Control cells exhibited approximately 85–90% wound closure, while Sample 2(chia seed extract)-treated cells showed only 20–30% closure, confirming the significant inhibition of cell migration ($p < 0.05$).

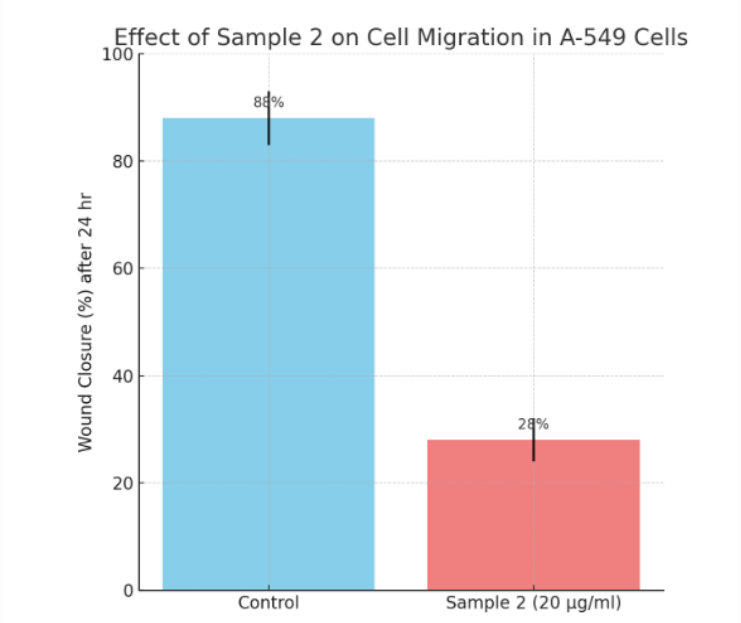

Figure 8.Effect of Sample 2 on Cell Migration in A-549 Cells

This clearly demonstrates that chia seed extract(sample -2) significantly inhibits A-549 cell migration, supporting its anti-metastatic potential.

4. DISCUSSION

The research investigates the anticancer activity of Salvia hispanica L. extract, specifically its content of linolenic acid, on A549 human lung cancer cells. In vitro tests, including MTT cytotoxicity assay, morphological observation, DAPI staining, scratch wound healing assay, gene expression profile, and molecular docking, were used to determine the extract's effect and mode of action. The results showed that the chia seed extract caused a significant dose-dependent decrease in A549 cell viability, with an IC_{50} value of 40 µg/ml. Morphological analysis revealed evidence of apoptosis in treated cells, such as membrane blebbing, cell shrinkage, and detachment. Real-time PCR analysis confirmed these findings, and molecular docking analyses confirmed that linolenic acid binds well to the pro-apoptotic protein Bax, leading to permeabilization of the mitochondrial membrane and apoptosis.

SUMMARY

The research examined the anticancer activity of chia seed extract against human lung cancer cell lines (A-549). The extract significantly inhibited cancer cell viability in a dose-dependent manner with an IC-50 of 40 µg/ml.Apoptosis was established by morphological alterations and nuclear fragmentation using phase contrast and fluorescence microscopy, respectively.The extract suppressed cancer cell migration in wound healing assays, indicating anti-metastatic activity.Gene expression analysis indicated upregulation of pro-apoptotic pathways, corroborating the apoptotic effects.

5. CONCLUSION

This study highlights the therapeutic value of chia seed-derived linolenic acid against lung cancer cells. Molecular docking study showed that linolenic acid binds efficiently to Bax protein, enhancing apoptotic activity. Experimental assays assured that the extract induces dose-dependent cytotoxicity, apoptotic morphology, and remarkable upregulation of genes related to apoptosis. The scratch wound healing assay also demonstrated the compound's function in inhibiting the migration of cancer cells, an important determinant in metastasis. Together, these results support chia seed-derived compounds as potential natural anticancer agents. Future research should emphasize in vivo confirmation and mechanistic information to allow translation of these observations into the clinic

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