Ind. J. Pharm. Edu. Res., 2025; 59(1):221-229.
https://www.ijper.org

Original Article

Anticancer, Apoptotic, Cell Migration and Invasion Inhibition and *in silico* Molecular Docking Studies of Naringetol in A-549 Human Lung Cancer Cells

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ABSTRACT

Aims: The main objective of this research work was to investigate the anticancer effects of Naringetol in A-549 human lung cancer cells along with evaluating its effects on cell apoptosis, cell migration and cell invasion and decoding the molecular mechanism of action by studying interactions of this molecule with the target protein using *in silico* molecular docking studies. **Materials and Methods:** MTT assay was used to study effects on cell viability while as effects on cell colony was evaluated by clonogenic assay. Acridine orange/ethidium bromide, DAPI staining assays using fluorescence microscopy were used to study effects on cell apoptosis. Cell migration and cell invasion inhibition was evaluated by Transwell assay. AutoDoc Vina software used to carry out docking simulation studies using Naringetol and EGFR (epidermal growth factor receptor) protein. **Results:** Results indicated that Naringetol induced dose and time-dependent inhibition of A-549 cancer cell viability along with inhibiting cell colony formation. Fluorescence microscopy revealed that naringetol molecule induced apoptosis like features in A-549 cells including nuclear and chromatin condensation and deformed cell membrane structures. Naringetol also led to a significant inhibition of cell migration and invasion hinting to its anti-metastatic potency. Molecular docking simulation studies indicated potential binding of naringetol with the key amino acid residues of the EGFR target protein with a binding score of -8.5 kcal/mole. **Conclusion:** In conclusion, these results reveal that naringetol inhibits lung cancer cell proliferation through the induction of cell apoptosis and suppression of cell migration and cell invasion. The target protein involved might be the EGFR protein as revealed by *in silico* molecular docking simulation studies.

Keywords: Naringetol, Lung cancer, Cell apoptosis, Cell migration, Cell invasion.

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Received: 13-06-2024;
Revised: 07-07-2024;
Accepted: 23-08-2024.

INTRODUCTION

Lung cancer continues to pose a serious threat to world health, with rates of both incidence and death rising significantly. China is one of the countries facing this concerning trend, since the country has seen a sharp increase in the incidence of lung cancer in recent years. China, the most populated nation on earth, has particular difficulties in diagnosing and treating lung cancer because of the intricate interactions between genetic, environmental and demographic factors.[1,2] It is essential to comprehend the genetic makeup of lung cancer in the Chinese population in order to develop efficient treatment plans. Chinese lung cancer patients differ from their Western counterparts in the patterns of genetic variations and mutations, such as EGFR and ALK changes. This emphasizes how crucial region-specific research is to improving therapeutic and diagnostic strategies for China's heterogeneous population. Recent developments in diagnostic technology are critical to attaining the objective of improving lung cancer outcomes, because early detection is essential. Liquid biopsy methods and Low-Dose Computed Tomography (LDCT) are two cutting-edge screening modalities that China has been at the forefront of investigating.[3,4] Treatment landscape for lung cancer include the conventional therapies like surgery, chemotherapy and radiotherapy which still remain the integral components of lung cancer treatment. However, recently the use of targeted therapies and immunotherapy has altogether revolutionized the treatment landscape of lung cancer. Various clinical trials conducted in China have shown the promise and efficacy of the targeted

DOI: 10.5530/ijper.20250091

therapies like EGFR and ALK inhibitors in specific lung cancer patients. Moreover, the use of immune checkpoint inhibitors like nivolumab and pembrolizumab have demonstrated promising results in various Chinese patients. This points out to the fact that immunotherapy is likely to become the new cornerstone in the management and treatment of advanced lung cancer.[5-7] However, the use of these targeted and immunotherapies is not without drawbacks especially their very high cost of treatment. Additionally, immunotherapy can cause adverse immune reactions due to an overactive immune system. Therefore, there is a pressing need for an alternative treatment involving the use of natural products especially from plants. Flavanones from plants have been shown to exert various pharmacological effects including their anticancer effects in various human cancer cells both *in vitro* and *in vivo*. These flavanone compounds exert their anticancer effects by reducing oxidative stress, anti-inflammatory effects, apoptosis induction, cell cycle regulation and inhibition of angiogenesis. Naringetol (4',5,7-trihydroxyflavanone-7-rham noglucoside) which is synonymously also known as naringin is classified as a flavanone-7-O-glycosidenaturally present in various fruits like grapefruit and citrus fruits. Naringenin is the aglycone form of naringetol.[8,9] Naringin or naringetol has been reported by various researchers to induce anticancer and apoptosis in various cancer cell lines. The anticancer effects induced by naringin has been demonstrated to occur via initiation of apoptotic cell death, suppression of cellular proliferation, inhibition of metabolic activity, enhanced carcinogen detoxification, antioxidant effects, oncogene inactivation.[10-14] The main objective of the current research work was to investigate the anticancer potential of naringetol in A-549 human lung cancer cells along with evaluating its effects on cell apoptosis, cell migration and cell invasion. We also used *in silico* molecular docking simulation using AutoDoc Vina software to evaluate molecular interactions and binding energy values between naringin (ligand) and the target protein which in case was Epidermal Growth Factor Receptor (EGFR) target protein.

MATERIALS AND METHODS

Cell culture conditions and MTT cell viability assay

The mouse fibroblast cell line (3T3L1) and the human lung cancer established cell line Cell Culture A549 were obtained from the Institute of Biochemistry and Cell Biology, Shanghai Institute of Life Sciences, Chinese Academy of Sciences, China. The cells were cultivated in RPMI 1640 medium comprising of 10% fetal bovine serum (FBS) (Sigma Aldrich, USA), 100 µg/mL penicillin and 100 µg/mL streptomycin (Gibco, USA) and cultured at 37 and 5% CO_2. MTT (3-(4,5-dimethylthiazol-2-yl)-2, 5 diphenyltetrazolium bromide)-based analysis was conducted in order to examine the effect of naringetol (Cat. No.: N5893; Merck KGaA, Darmstadt, Germany) on cell viability. The cells were seeded in a 96-well plate at a concentration of $1x10^6$ cells/mL for this assay. After 24 hr, the cells were treated with naringetol (0, 5, 25, 75 and 150 µM) and prepared for the MTT assay. After 48 hr of incubation, 20 µL of MTT solution (5 mg/mL) (Cat. No.: M5655; Merck KGaA, Darmstadt, Germany) was added and the cells were left to incubate for an additional 5 hr. The medium was discarded after 5 hr and 0.1% DMSO (100 µL; Cat. No.: 102950; Merck KGaA, Darmstadt, Germany) was added in order to dissolve the formazan crystals formed as a result of the reaction. A microtiter plate reader (FLUOstar Omega, BMG Labtech, Ortenberg, Germany) was used to measure the absorbance at 570 nm. Every experiment was run in triplicate. The IC_{50} for the cell line was determined using the dosage response curve.

Clonogenic assay (Assay for colony formation)

After being plated into 6-well plates, A-549 lung cancer cells were subjected to varying doses (0, 25, 75, 150 µM) of naringetol for 24 hr. After washing with PBS (Sigma, St. Louis, MO, USA), the cells were trypsinized (with 0.30% trypsin (Sigma, St. Louis, MO, USA)). Next, fresh 6-well plates were plated with 15,00 viable cells. After growing cells for 7 days, the cells were stained with crystal violet after being fixed in 4% paraformaldehyde (Beyotime Institute of Biotechnology, Shanghai, China). A MshOt MS60 digital microscope camera (Nikon, Tokyo, Japan) was used to take pictures of the macroscopic colonies (>60 cells) and the total number of colonies that formed was recorded.

Apoptosis assessment by fluorescence microscopy

The A-549 cells were cultured for 24 hr at varying concentrations of naringetol (0, 25, 75 and 150 µM). Then, 20 µL of AO/EB (acridine orange/ethidium bromide, 10 µL each) staining solution (Sigma, St. Louis, MO, USA) was added to the cell suspension and kept in the dark for 20 min. Then, the different morphological types of the cells-live and dead-were analysed immediately using a fluorescent microscope (Nikon, Japan) equipped with ImageJ software to determine the proportion of dead cells.

For DAPI analysis, following procedure was followed:

At a density of 1×10^6 cells/well, A-549 cells were plated in 96-well plates to determine the apoptosis-like morphological features of cells. Following a 24 hr incubation period, naringetol (0, 25, 75 and 150 µM) was added to seed A-549 cells. The naringetol-treated cells were then treated with DAPI solution (Sigma, St. Louis, MO, USA) and the cells were then washed with PBS. Following that, 10% formaldehyde (Sigma, St. Louis, MO, USA) was used to fix DAPI-stained cells. Finally, a fluorescent microscope (Nikon, Japan) was used to analyse cell apoptosis.

Cell migration and invasion assay

The cell migration and cell invasion assays were carried out using 24-well Millicell coated with Matrigel or without Matrigel (Millpore, Billerica, USA). 100 µL of 1x105/mL cells were transferred to the Transwell chambers and then cultured for 24 hr letting the A-549 cells to move through the extracellular matrix

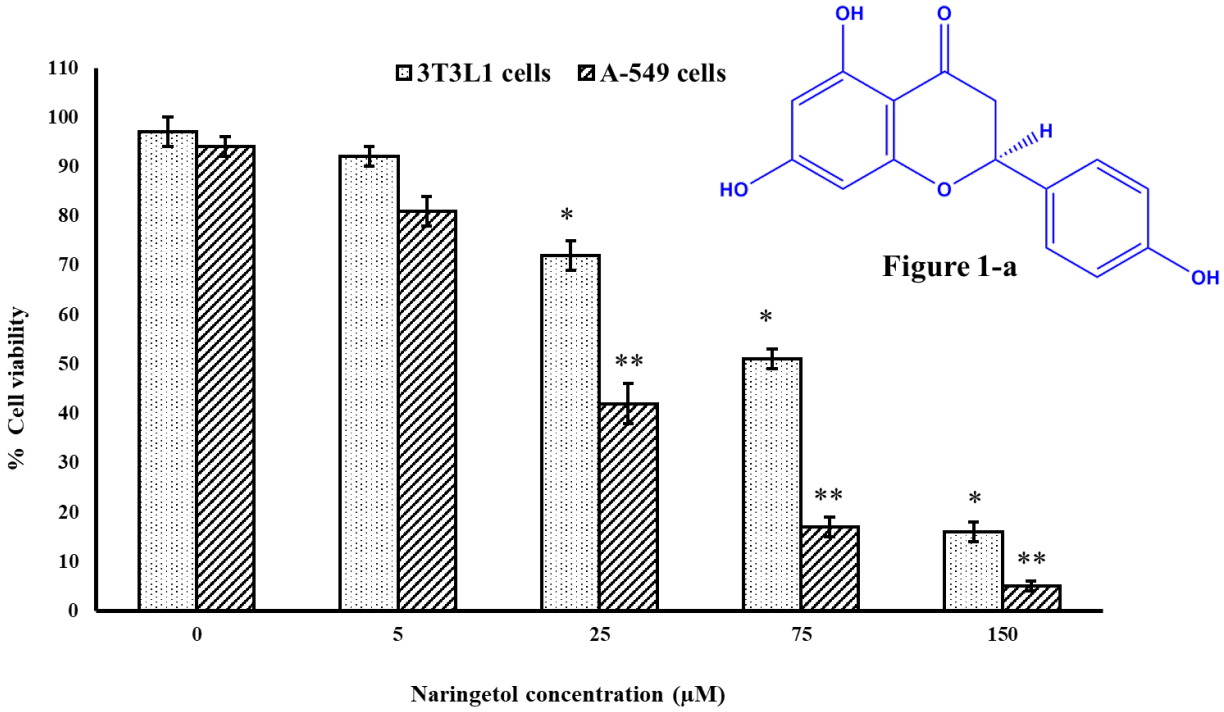

Figure 1-a

Figure 1-b

Figure 1-a: Chemical structure of naringetol. Figure 1-b: MTT assay results indicating the significant, dose-dependent and selective reduction in cell viability induced by naringetol in A-549 human lung cancer cells as compared to the mouse fibroblast cell line (3T3L1) which is a normal cell line. Data of individual triplicate experiments were presented as ±SEM, * $p<0.05$, ** $p<0.01$ as statistically significant with respect to the control.

Figure 2-a

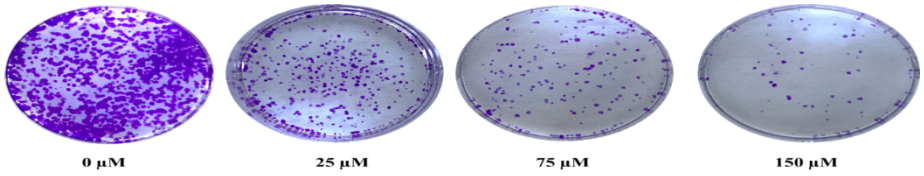

Figure 2-b

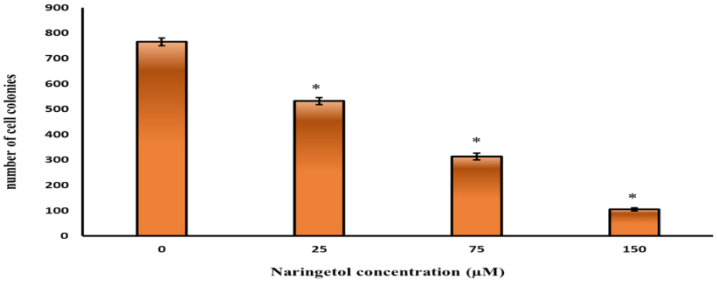

Figure 2-a and Figure 2-b: Colony formation assay for evaluating the effect of various concentrations of naringetol on A-549 lung cancer cell colony formation. Naringetol induced a significant (* $p<0.01$) and concentration-dependent suppression of colony formation as shown graphically as well. All the experiments were repeated three times and data was revealed as mean±SEM, * $p<0.01$ vs control group.

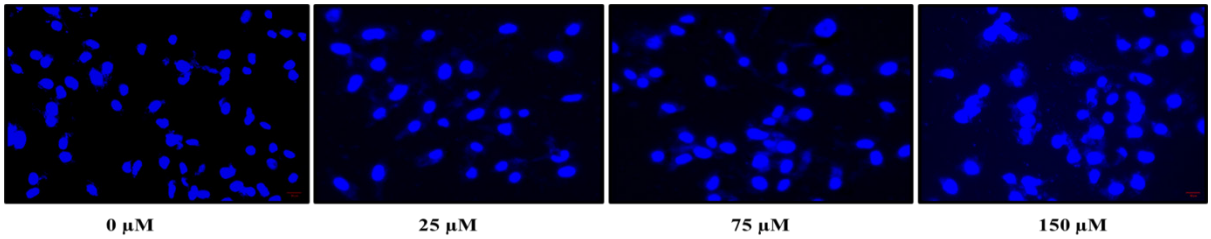

Figure 3-a

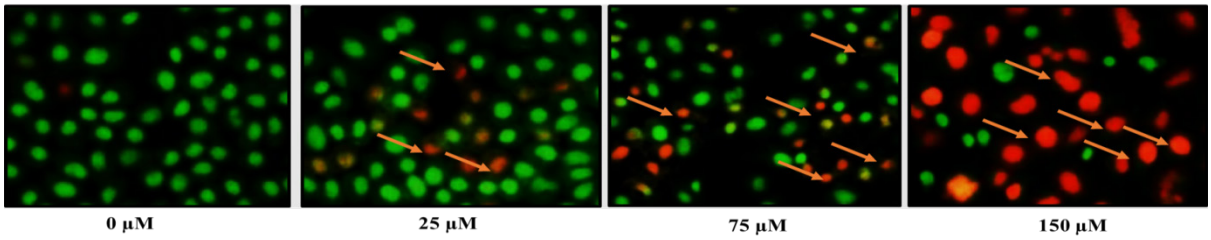

Figure 3-b

Figure 3-a: Apoptosis analysis; the DAPI staining assay using fluorescence microscopy indicated that as compared to the untreated control cells which showed normal cell morphology, the naringetol treated cells showed signs of cellular apoptosis including chromatin condensation and distorted cell morphology in A-549 human lung cancer cells. Figure 3-b: The Acridine Orange (AO)/Ethidium Bromide (EB) assay showing that as compared to the untreated control cells which mostly exhibited green fluorescence, the naringetol-treated cells showed orange/red fluorescence which is a marker of apoptosis. Arrows indicate apoptotic cells which increase with increasing concentration of naringetol.

to the lower chamber. The cells in the underside were fixed with 4% paraformaldehyde (Sigma, St. Louis, MO, USA) for 20 min and then stained with 0.2% crystal violet. Each experiment was replicated three times in an independent manner. Three randomly selected fields on the fixed Transwell chambers were counted with three repetitions and then photographed.

In silico molecular docking analysis

The structural file information for naringetol (PubChem CID: 442428) was downloaded from the PubChem database. ChemBio3D Ultra Version 11.0 structure generator was used to generate 3-dimensional structure of naringetol. The details about the target protein (PDB ID: 6LUD) and the three-dimensional crystal structure was downloaded from RSC-PDB database (https://www.rcsb.org/). CHARMM force field implementation and removal of water molecules and HETATM from original 3D structures were carried out utilising Discovery Studio visualizer. We screened naringetol according to its inclination to bind to a specific receptor target protein EGFR (epidermal growth factor receptor) (PDB ID: 6LUD) using the AutoDock software tool. AutoDock4 generates fast and effective interaction analysis results. The docking mechanism was implemented in compliance with previously published studies. Docking assisted virtual screening was performed on the active site following the configuration of the AutoDock default parameters. The Lamarckian Genetic Algorithm (LGA) was utilised in computations pertaining to flexible receptor-compound docking. Discovery Studio Visualizer was used to analyse the interactions between the docked structure and the receptor that AutoDock created. Discovery Studio Visualizer was used to create 2D and 3D models of the docked molecule and protein based on data acquired from the interaction of the target protein with naringetol.

Statistical analysis

The data values from individual triplicate experiments were presented as mean±SEM and analyzed using SPSS software (V-21; Jingke Crop, Shanghai, China). Student t-test was used to determine significant differences and p values were regarded as significant at $^{*}p<0.05$ and $^{**}p<0.01$.

RESULTS

Antiproliferative effects of naringetol

Figure 1-a show the chemical structure of naringetol while as Figure 1-b shows the inhibition of cell viability induced by naringetol in A-549 human lung cancer cells and mouse fibroblast cell line (3T3L1). The cell viability was tested by using MTT assay using 0, 5, 25, 75 and 150 µM concentrations of naringetol. The results reveal that naringetol molecule not only induced concentration-dependent inhibition of cell viability but also selective cell viability inhibition showing higher inhibitory effects against cancer cells as compared to the normal 3T3L1 cells. As compared to the untreated control cells which showed least cell viability inhibition, the naringetol-treated cancer cells showed significant growth inhibition ($^{**}p<0.01$), same trend was seen with mouse fibroblast cells (3T3L1) but the impact was much lesser ($^{*}p<0.05$).

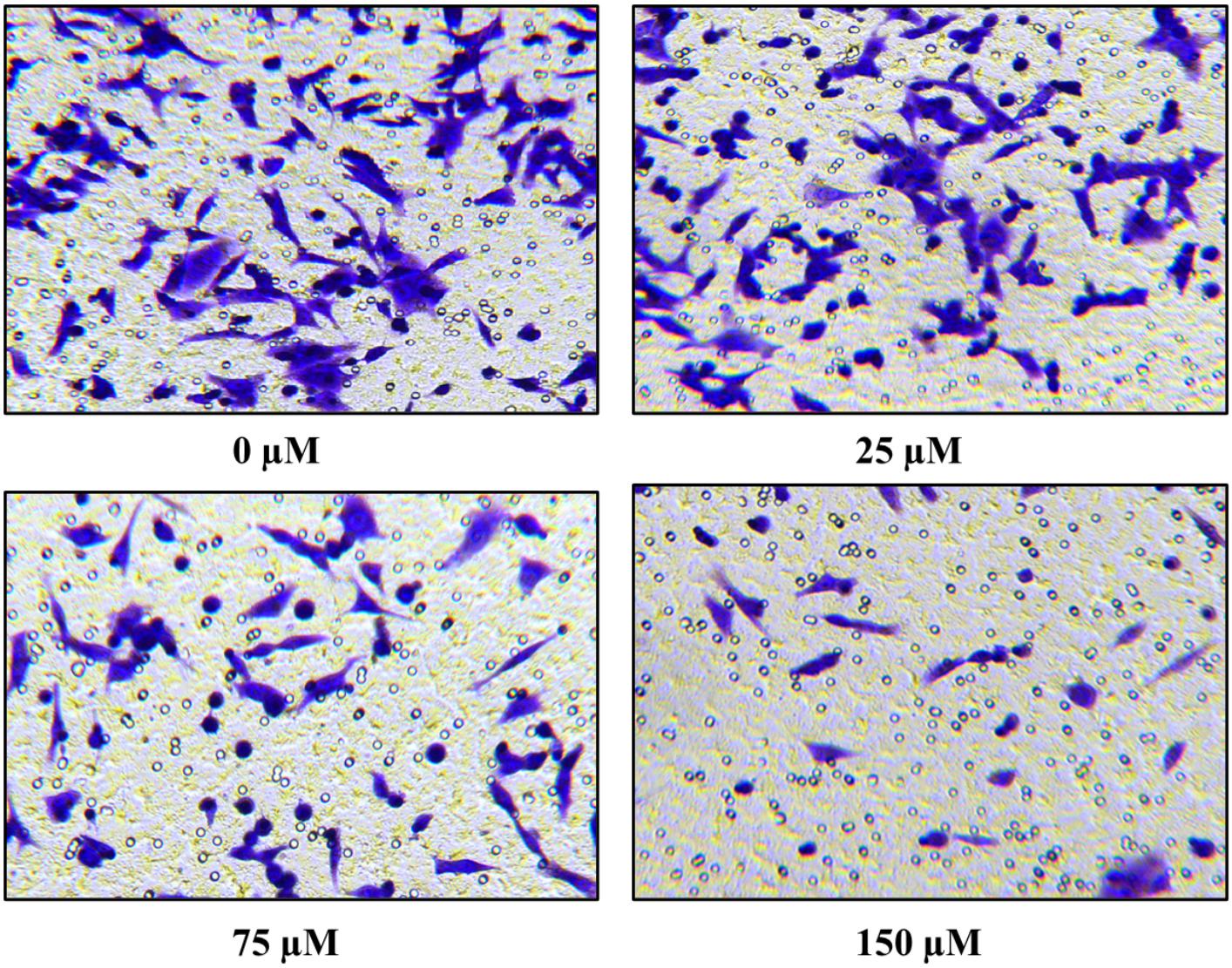

Figure 4: Effect of naringetol on the cell migration suppression in A-549 human lung cancer cells using a Transwell chambers assay. The cell migration evaluation was tested at various concentrations including 0, 25, 75 and 150 µM and it was observed that there was a concentration dependent suppression of cell migration. The picture is shown as representative of three parallel and independent experiments, mean±SEM.

Naringetol also led to cell colony inhibition

Next, we examined effects of naringetol on the cell colony formation using clonogenic assay. The results of this assay are shown in Figures 2-a and 2-b and reveal that naringetol led to a significant (*p<0.01) and concentration dependent inhibition of cell colony formation in A-549 human lung cancer cells. This hints towards the anti-clonogenic effects of this molecule against these cancer cells and can be an effective anticancer drug candidate.

Naringetol induced apoptotic cell in A-549 human lung cancer cells

Figures 3-a and 3-b show the results of the apoptosis assay involving fluorescence microscopy using DAPI and Acridine Orange/Ethidium Bromide (AO/EB) staining agents respectively. These assay point to the fact whether the tested molecule has the tendency to induce programmed cell death in cancer cells. Blue luminescent DNA labeling dye, DAPI, provided evidence of the breakdown of nuclear integrity and the induction of nuclear fragmentation. Figure 3-a show fluorescent microscopy picture of naringetol (0, 25, 75 and 150 µM) and DMSO-treated cells (control). Figure 3-a makes clear that, in comparison to the control condition, the blue fluorescence of the naringetol-treated cells was enhanced. Following treatment, the cells display the morphological changes linked to apoptosis, such as nuclear disintegration and chromatin condensation. Further, AO/EB assay also indicated that increased concentrations of naringetol led to red/orange fluorescence which is indicative of apoptotic cell death induced by naringetol, the arrows indicate the apoptotic cells. The untreated control cells mostly exhibited green fluorescence with signs of apoptosis (Figure 3-b). Thus, both of these assays indicate that naringetol induces apoptotic cell death

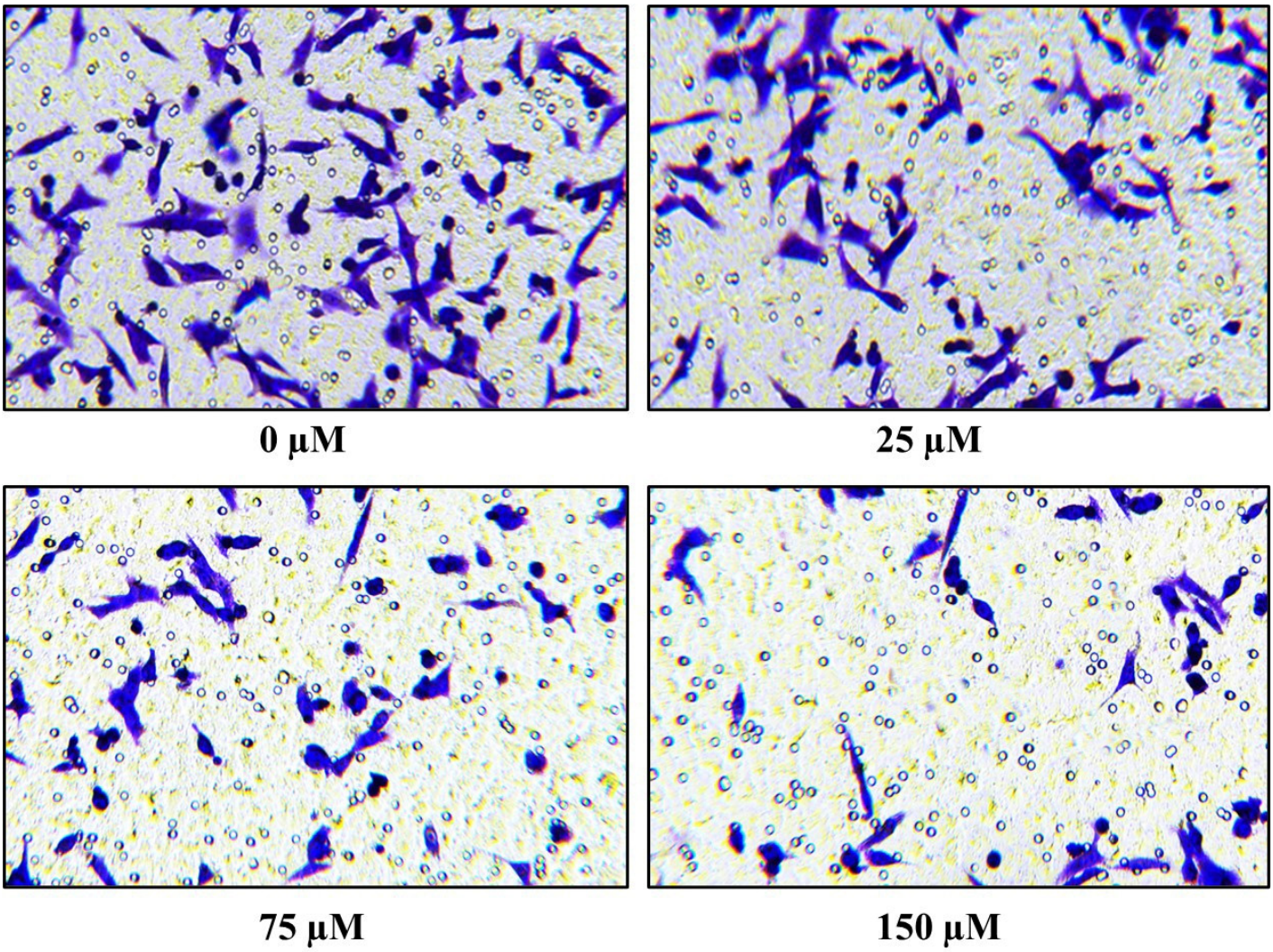

Figure 5: Effect of naringetol on the cell invasion suppression in A-549 human lung cancer cells using a Transwell chambers assay. The cell invasion assessment was established at various concentrations including 0, 25, 75 and 150 µM and it was witnessed that there was a concentration dependent suppression of cell invasion. The picture is shown as representative of three parallel and independent experiments, mean±SEM.

in A-549 human lung cancer cells and that this effect correlates with increased concentration of naringetol.

Naringetol led to the inhibition of cell migration and cell invasion

The anti-metastatic potential of naringetol was evaluated using Transwell Matrigel assay for examining its effects on inhibition of cellular migration and invasion. The results which are shown in Figures 4 and 5 indicate that naringetol could suppress cell migration and cell invasion significantly. The efficacy of naringetol was tested at 0, 25, 75 and 150 µM concentrations and it was observed that as compared to the untreated control cells which showed significant cell migration and cell invasion, naringetol-treated cells exhibited considerable suppression of cellular migration and invasion.

In silico molecular docking results

In silico molecular docking analysis was carried out in order to examine the molecular interactions involved and hence the mode of action as well as binding efficiency of naringetol with the EGFR target protein. The 2-D and the 3-D structures of the ligand (naringetol) are shown in Figures 6-a and 6-b respectively. Figure 6-c shows the three-dimensional crystal structure as well as the active catalytic centre of the EGFR (epidermal growth factor receptor) (PDB ID: 6LUD) target protein with which docking was carried out. Figures 6d, 6e and 6f represent the cartoon surface representations of the protein alone as well as the bound naringetol molecule showing the key binding interactions with the active site along with the involved amino acid residues. A cartoon surface representation, as used in molecular docking, is a simplified graphic representation of a protein's structure that emphasizes its backbone. Figure 7-a represents the two-dimensional representation of the interactions between the target protein and the target ligand in which different colored

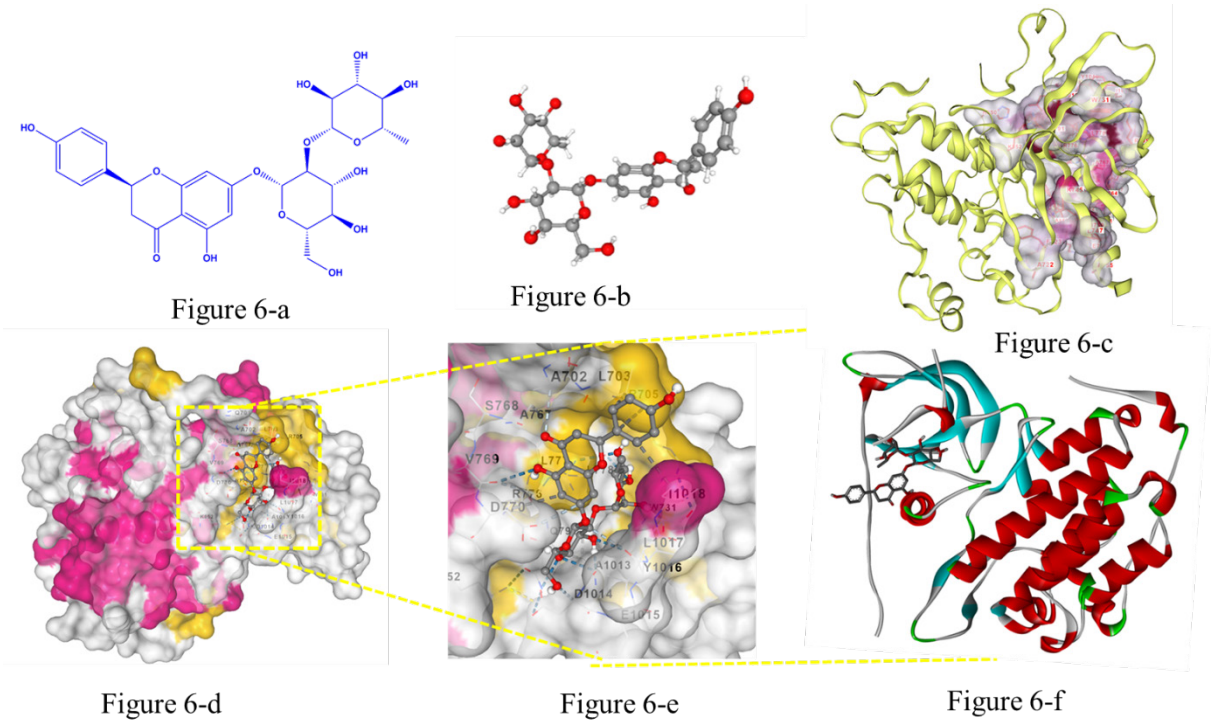

Figure 6: Two- and Three-dimensional structures of naringetol (ligand) (PubChem CID: 442428) and the active catalytic site of the target protein EGFR (epidermal growth factor receptor) (PDB ID: 6LUD) used in this docking study. The cartoon surface representation between the ligand the target protein is also shown.

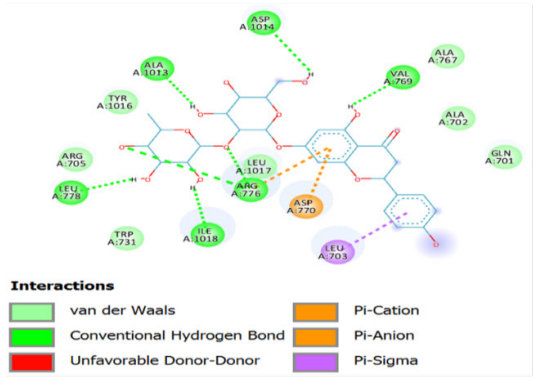

Figure 7-a

Figure 7-b

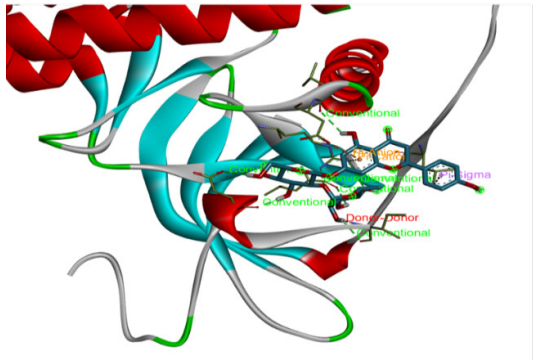

Figure 7-c

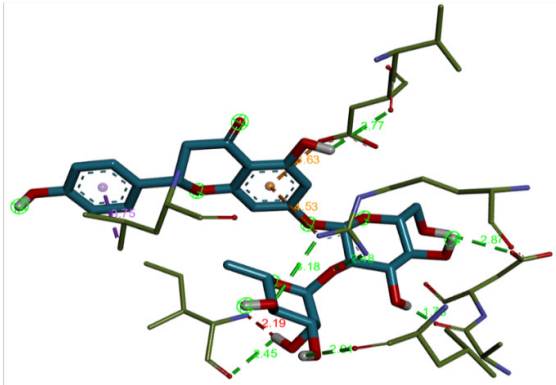

Figure 7-d

Figure 7: 2-dimensional and 3- dimensional representations of the interactions between the ligand (naringetol) and the target protein (EGFR); The 3-D and 2-D interactions show various types of non-bonded interactions between the ligand the target protein including conventional hydrogen bond, Van der Waals, pi-sigma, pi-cation and pi-anion interactions.

dotted lines indicate different molecular interactions between the two. These interactions may involve conventional hydrogen bond, Van der Waals, pi-sigma, pi-cation and pi-anion, pi-alkyl, alkyl-alkyl interactions. This 2-D diagram also depicts the directly involved amino acid residues present within the active catalytic site of the target protein. Figures 7b, 7c and 7d indicate the three-dimensional representation of the interaction. In this case, naringetol mostly uses hydrogen bond and van der Waals interactions of bonding pointing out to the strong binding efficacy between the ligand and the target protein with a binding energy value of -9.0 kcal/mole. The amino acids which were involved in hydrogen bonding with the ligand involve ALA chain A: 1013, ASP Chain A: 1014, VAL chain A: 769, ARG chain A: 776, ILE chain A: 1018, LEU chain A: 778.

DISCUSSION

Lung cancer is still a major worldwide health concern that requires novel ways to both prevention and treatment. The potential therapeutic advantages of natural products and plant extracts in the treatment of lung cancer have attracted increasing attention in recent years. These plant-derived bioactive chemicals have a variety of modes of action and can target different pathways implicated in the advancement of cancer. For example, green tea polyphenols, such Epigallocatechin Gallate (EGCG), have been shown to have anti-cancer effects through apoptosis induction, suppression of angiogenesis and inhibition of cell proliferation.[15-17] Furthermore, the polyphenol found in turmeric, curcumin, has been the subject of much research due to its anti-inflammatory and anti-cancer properties, including its capacity to alter signalling pathways such as STAT3 and NF-κB. Moreover, resveratrol, a substance found in red grapes, has demonstrated potential in preventing the growth and spread of lung cancer cells via a number of pathways, including the control of the cell cycle and apoptosis.[18-21] Naringin or naringetol belongs to flavanone class of natural products and this class has been explored for their anticancer potential against various human cancers including the lung cancer. Naringin exhibits anticancer properties by being effective against oxidative stress and neutralizing free radicals and reducing cell damage. Another mechanism through which it shows anticancer effects is its anti-inflammatory action thus contributing to cancer prevention and treatment.[22,23]

In the current study, naringetol exhibited selective anticancer properties against A-549 human cancer cells and showed less cytotoxicity against the mouse fibroblast cell line (3T3L1). Naringetol was also shown to inhibit cancer colony formation tendency of these lung cancer cells as revealed by clonogenic assay. Fluorescence microscopy using DAPI and AO/EB staining agents revealed that naringetol induced apoptotic cell death significantly and in a concentration-dependent manner. Thus, indicating that the main mechanism of anticancer action of naringetol may be through apoptosis induction. Naringetol has previously been reported to induce apoptosis in various cancer cell lines.[24] Further, we also examined its effects on cell migration and cell invasion which showed that naringetol significantly inhibited cell migration and cell invasion in A-549 human lung cancer cells. This property can be exploited in controlling cancer metastasis.

Understanding how bioactive compounds interact with their protein targets is a key part of drug development and *in silico* molecular docking is a powerful tool for this process.[25] The binding mechanism, affinity and important interactions, including hydrophobic contacts and hydrogen bonds, may be predicted using this computational technique, which involves predicting the conformation of a ligand (compound) into a protein's binding site. Researchers may better understand how compounds regulate biological processes when they use docking to simulate these molecular interactions and so provide information regarding their mode of action.[26] With high-throughput virtual screening, this is especially helpful in evaluating huge libraries of compounds to find the ones with the best chance of being effective.[27] In addition, molecular docking is useful for Structure-Activity Relationship (SAR) investigations, which improve compound optimization by linking structural properties to biological activity. Furthermore, by anticipating off-target effects and consequently reducing negative consequences, it helps evaluate the selectivity and safety profile of drugs.[28] Molecular docking is a powerful tool for the study of cancer and other conditions by revealing the protein targets that are important for the disease and the manner in which compounds from different sources interact with these targets to alter cell proliferation, apoptosis and metastatic pathways.[29] Furthermore, docking findings may guide experiment design and reinforce experimental data, offering a strong view of the compound's medicinal possibilities. Researchers are making great strides in the area of cancer by effectively exploring and developing novel medicines via the integration of molecular docking with bioinformatics and experimental techniques.[30] Herein, *in silico* molecular docking analysis was carried out which showed the molecular mode of action between naringetol and the target protein which in case was EGFR protein involved in lung cancer progression and development. Naringetol mostly uses hydrogen bond and van der Waals interactions of bonding pointing out to the strong binding efficacy between the ligand and the target protein with a binding energy value of -9.0 kcal/mole. The amino acids which were involved in hydrogen bonding with the ligand involve ALA chain A: 1013, ASP Chain A: 1014, VAL chain A: 769, ARG chain A: 776, ILE chain A: 1018, LEU chain A: 778.

CONCLUSION

In conclusion, the results of the present study provide conclusive evidence about the anticancer properties of naringetol and its mode of action which indicates that naringetol induces apoptotic

cell death and inhibits cell migration and cell invasion. *In silico* molecular docking analysis further indicate that naringetol could inhibit EGFR target protein through which it exerts its anticancer potential.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; **EGFR:** Epidermal growth factor receptor; **ALK:** Anaplastic lymphoma kinase; **LDCT:** Low-dose computed tomography; **FBS:** Fetal bovine serum; **PBS:** Phosphate buffered saline; **DMSO:** Dimethyl sulfoxide; **AO/EB:** Acridine orange/ethidium bromide; **DAPI:** 4′,6-Diamidino-2-phenylindole; **LGA:** Lamarckian Genetic Algorithm.

SUMMARY

With a limited number of treatment options available for lung cancer treatment combined with high cost of immunotherapy as well as the side-effects of the chemotherapy, there is a pressing need for alternative anticancer agents with low unwanted side-effects and cost-effective. The current research work identifies a natural flavonoid namely naringetol to be a potent agent in inducing cancer cell viability inhibition in lung cancer cells *in vitro* and these effects were modulated via apoptosis induction, inhibition of cell migration and cell invasion hinting at its anti-metastatic effects in lung cancer. Further, molecular mechanism of action was determined by studying *in silico* molecular docking studies to identify which type of molecular interactions occur between naringetol and EGFR target protein.

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Cite this article: Li J, Wu B, Huang X, Tang W. Anticancer, Apoptotic, Cell Migration and Invasion Inhibition and *in silico* Molecular Docking Studies of Naringetol in A-549 Human Lung Cancer Cells. Indian J of Pharmaceutical Education and Research. 2025;59(1):221-9.