Texila International Journal of Public Health
Special Issue-2024
DOI: 10.21522/TIJPH.2013.SE.24.03.Art012

Molecular Mechanisms to Identify Anticancer Activity of Tomentin in A549 Lung Adeno Carcinoma Cells: Role of p53/Caspase-Mediated Pathways

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Abstract

Cancer, a multifaceted disease with increasing prevalence, remains a significant global health concern. Lung cancer, in particular, presents a formidable challenge due to its high mortality rates. Tomentin, a phytochemical extracted from Sphaeralcea angustifolia, has garnered interest for its potential anti-cancer properties. This study employs both in vitro and in silico methods to elucidate tomentin's efficacy against lung cancer, focusing on the A549 cell line, a model for lung adenocarcinoma. The study begins by exploring the cytotoxic effects of tomentin on A549 cells through viability assays, apoptosis induction, and molecular pathway modulation. Results indicate dose-dependent inhibition of cell proliferation and activation of apoptotic pathways by tomentin treatment. Further analysis reveals tomentin's ability to scavenge DPPH radicals and inhibit protein denaturation, suggesting potent antioxidant and anti-inflammatory properties. Moreover, mRNA expression analysis demonstrates tomentin's regulatory effects on key genes involved in inflammation and apoptosis. Molecular docking studies reveal strong binding affinity between tomentin and critical proteins implicated in cancer progression, including MCL1, p53, Bcl-2, and Caspases.

Keywords: *Anticancer, Antioxidants Capacity, Apoptosis, Caspase, Health and Well-being, Lung Cancer, Molecular Docking, Novel Methods, p53, Tomentin.*

Introduction

Cancer, once a rare disease over a century ago, has become increasingly prevalent in recent decades, likely due to changes in lifestyle, habits, and longer lifespans. Today, it is one of the most feared diseases, continuing to rise in incidence into the 21st century. Approximately one in four people now face a lifelong risk of developing cancer, highlighting the severity of the situation [1]. Cancer is characterized by the uncontrolled growth of cells, which can originate from any organ or part of the body. Detection often occurs incidentally during routine screenings or tests, but generally, a cancerous growth must reach a size of 1 cm or contain about 1 million cells to be diagnosed. Exceptions include leukaemias and lymphomas, which do not typically form a mass [2].

One of the significant challenges in cancer treatment is preventing tumor growth [3]. Lung cancer is the leading cause of cancer-related deaths globally. Despite declining incidence rates in some countries, survival rates remain low. Lung cancer is classified into two main types: small-cell lung cancer (SCLC) and non-small-cell lung cancer (NSCLC), with NSCLC further divided into

Received: 13.06.2024 ***Accepted: 12.08.2024*** ***Published on: 30.08.2024***

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adenocarcinoma, squamous cell carcinoma, and large cell carcinoma. The type of lung cancer influences both prognosis and treatment [4]. Smoking is a major risk factor, significantly increasing the likelihood of developing lung cancer, with a relative risk (RR) of 10 to 30. Smoking is directly linked to 90% of lung cancer cases in women and 79% in men, with second-hand smoke also being a potential risk factor [5].

Most lung cancer patients present with symptoms at diagnosis, though early symptoms are often nonspecific. These symptoms can result from distant metastases, intrathoracic extension, or endobronchial growth. In some cases, symptoms of systemic cachexia or paraneoplastic syndrome may also be observed [6]. For individuals who smoke regularly and experience persistent or new respiratory symptoms, lung cancer should be considered. Diagnosis typically begins with a chest X-ray, followed by a computed tomography (CT) scan if abnormalities are found. The treatment course is determined by the tumor's location. Central tumors are often examined with bronchoscopy, which allows for biopsy and direct imaging, while peripheral tumors may require transthoracic biopsies guided by CT or ultrasound. Other surgical procedures, such as thoracoscopy, mediastinoscopy, and thoracotomy, are reserved for cases where initial diagnostic methods fail to provide a definitive diagnosis [7]. Treatment for lung cancer varies based on the type and stage of the disease. Options include surgery, chemotherapy, radiation therapy, immunotherapy, targeted therapy, or a combination of these approaches. Surgery may be effective for early-stage NSCLC, while more advanced stages may require radiation therapy or chemotherapy, either alone or in combination. Targeted therapies are available for certain NSCLC types, focusing on specific genetic mutations. Immunotherapy, which helps the body's immune system recognize and attack cancer cells, is used in advanced NSCLC and some SCLC cases. Palliative care is also crucial, especially in advanced cases, to manage symptoms and improve quality of life. Treatment decisions are made collaboratively between patients and their healthcare teams, considering personal preferences, tumor characteristics, and overall health [8].

The A549 human lung adenocarcinoma cell line is commonly used in cancer research, particularly for studying lung cancer and developing therapeutic agents. These cells serve as a model for carcinogenic alveolar type II epithelial cells [9]. Nucleostemin (NS), a guanine nucleotide-binding protein, plays a significant role in the aggressive behavior of cancer cells. NS is highly expressed in the nucleolus of neuroepithelial, embryonic, primitive stem cells, and cancerous cells, where it acts as a catalyst for uncontrolled cell division and tumor development. Knockdown of NS has been shown to significantly inhibit the proliferation of cancer and stem cells, suggesting that NS is a key regulator of cancer cell growth. Recent research has indicated that NS regulates the JAK-STAT signaling pathway in cancer, which is involved in cell proliferation, invasion, survival, and immunosuppression. This pathway also promotes cancer growth through inflammatory signals, stem cell networks, and the pre-metastatic niche [10]. Apoptosis, or programmed cell death, is typically triggered by intrinsic mechanisms when cells detect uncontrolled growth or DNA damage. This pathway activates specific genes that lead to cell death. Apoptosis is essential for maintaining tissue homeostasis, inflammatory responses, and development in multicellular organisms. It can be initiated by either intrinsic or extrinsic signals. Extrinsic signals, such as death ligands or cytokines, bind to cell surface receptors, triggering a cascade that leads to cell death. Intrinsic signals arise from within the cell, often in response to internal stress or DNA damage. A critical step in apoptotic signaling is the activation of

caspases, protease enzymes that carry out the process by cleaving essential cellular components. Caspases can be activated by regulatory proteins like cytochrome c, released from mitochondria in response to intrinsic signals or by proteolytic cleavage by other caspases. This activation results in the breakdown of the cell into apoptotic bodies, which are quickly removed by phagocytic cells to minimize inflammation and protect tissue integrity [11].

Inflammatory signaling is a complex network of biochemical processes used by the immune system to respond to various stimuli, including infection, tissue damage, and stress. This signaling pathway is crucial for initiating and regulating the body's defense mechanisms against harmful agents. Inflammatory signaling begins at the cellular level with the activation of immune cells, particularly mast cells and macrophages, leading to the release of chemokines and cytokines. Key inflammatory signaling molecules include interleukins (ILs), interferons (IFNs), and tumor necrosis factor-alpha (TNF-α). These molecules recruit more immune cells to the site of infection or injury, enhance the inflammatory response, and coordinate the clearance of pathogens or damaged tissue [12]. Flavonoids, a category of polyphenolic compounds found in plants, fruits, vegetables, and leaves, have antiviral, anticancer, antioxidant, and anti-inflammatory properties [13]. Tomentin, a compound extracted from *Sphaeralcea angustifolia*, is of particular interest due to its anti-inflammatory effects. It has been shown to reduce λ-carrageenan-induced footpad edema by 58% and inhibit phorbol ester-induced auricular edema by 57%. Tomentin, with a molecular weight of 222.00 and the formula $C_{11}H_{10}O_5$, is a phenylpropanoid and coumarin compound [14].

Materials and Methods

Invitro Analysis

Antioxidant Activity

2, 2-Diphenyl-1-picrylhydrazyl (DPPH) Free Radical Scavenging Activity

Scavenging of DPPH radical was assessed by the method of [15] (Hatano *et al.,* 1989). Briefly, DPPH solution (1.0 ml) was added to 1mg/ml of tomentin at different concentrations (100, 200, 300, 400, and 500 µg/ml). The mixture was kept at room temperature for 50 min and the activity was measured at 517 nm. Ascorbic acid at various concentrations (100, 200, 300, 400, and 500 µg/ml) was used as standard. The capability to scavenge the DPPH radical was calculated using the following formula:

$$Inhibition\ \% = \frac{Absorbance\ of\ Control - Absorbance\ of\ Test\ Sample}{Absorbance\ of\ Control} \times 100$$

Anti-Inflammatory Activity

Inhibition of Protein Denaturation

To evaluate the anti-inflammatory effects of the tomentin the protocol described by [16,17] was used with small modifications. A volume of 1 mg of tomentin (aqueous and ethanolic) or of diclofenac sodium at different concentrations (100, 200, 500, and 1000µg/ml) was homogenized with 1 ml of aqueous solution of bovine serum albumin (5%) and incubated at 27°C for 15 minutes. The mixture of distilled water and BSA constituted the control tube. Denaturation of the proteins was caused by placing the mixture in a water bath for 10 minutes at 70°C. The mixture was cooling inside the ambient room temperature, and the activity of each mixture was measured at 660 nm. Each test was done three times. The following formula was used to calculate the inhibition percentage:

$$Inhibition\ \% = \frac{Absorbance\ of\ Control - Absorbance\ of\ Test\ Sample}{Absorbance\ of\ Control} \times 100$$

Procurement and Culture of Human Lung Cancer Cell Line (A549)

The A549 cell line was acquired from The National Centre for Cell Science (NCCS), located in Pune, India, and cultivated following the prescribed cell culture protocols. In summary, the lung cancer cells were cultured in Minimum Essential Medium (MEM**)** supplemented with 10% fetal bovine serum (FBS) under standard conditions of 37°C temperature and 5% CO_2 atmosphere. Culturing was carried out at 37°C in a humidified atmosphere containing 5% CO2 to provide optimal growth conditions for the A549 cells. This optimal culture environment facilitated the robust growth and maintenance of the A 549 lung cancer cell line, ensuring its viability and suitability for subsequent experimental procedures.

Cell Viability Assay

Lung cancer cells were seeded at a density of 5x10*5 cells/well in 96-well plates and allowed to attach to the well overnight. After incubation, cultured cells were stimulated with various concentrations of tomentin in triplicate and incubated at 37˚C in a 5% humidified CO_2 incubator for 24 h [18]. .Subsequently, 3-(4,5-dimethylthiazol2-yl)-2,5-diphenyltetrazolium bromide (MTT) was added to each well, and incubation was continued for a further 4 h at 37℃. To dissolve the formazan formed from MTT, the cells were resuspended in 200 µl dimethyl sulfoxide (DMSO), and the optical density (OD) of the solution was determined using a spectrometer at a wavelength of 570 nm. The experiments were repeated 3 times, independently. The mean optical density (OD) ± SD for each group of replicates was calculated. The entire procedure was repeated 3 times. The mean optical (OD) ±SD for each group replicates were calculated. The inhibitory rate of cell growth was calculated using Growth inhibition %.

% Growth inhibition = (1 - OD extract treated)/OD negative control x 100

Gene Expression Analysis by REAL TIME PCR

Gene expression levels were examined using real-time PCR. The total RNA was isolated by using TriR Reagent (Sigma). Total RNA (2 µg) from each sample was reverse transcribed using a commercial Superscript III first strand cDNA synthesis kit (Invitrogen, USA) according to the manufacturer's protocol. Real time-PCR was carried out in a MX3000p PCR system (Stratagene, Europe). Reaction was performed using MESA Green PCR master mix (It contains all the PCR components along with SYBR green dye) Eurogentec, USA. The specificity of the amplification product was determined by melting curve analysis for each primer pairs . The data were analyzed by comparative CT method and the fold change is calculated by 2−CT method using CFX Manager Version 2.1 (Bio Rad, USA).

Molecular Docking

The binding interactions of a compound with specific target proteins were investigated using the molecular docking software PyRx. Crystal structures of these target proteins were retrieved from the Protein Data Bank (PDB). Throughout the docking process, a grid box measuring 90 Å × 90 Å × 90 Å, with a grid spacing of 0.45 Å, was employed to facilitate accurate ligand-protein interaction prediction. Docking calculations were carried out utilizing the Lamarckian genetic algorithm (LGA), with 100 genetic algorithm runs executed to ensure comprehensive exploration of the conformational space. Post-docking analysis was conducted to identify and characterize high-pose interactions between the compound of interest (tomentin) and the apoptosis-regulating target proteins. Additionally, the binding affinities of the ligand towards the receptors were meticulously evaluated to elucidate the binding mode. The resulting 3D structures of the ligand-protein complexes from the docking simulations were visualized

and analyzed using BIOVIA Discovery Studio, enabling a detailed examination of the molecular interactions and the binding conformations. This comprehensive approach provides insights into the potential mechanisms of action and therapeutic relevance of the compound in modulating apoptotic pathways through its interactions with specific protein targets.

Statistical Analysis

The data presented are represented as the means ± standard deviation (SD) derived from three independent experiments conducted in triplicate. Statistical analysis was carried out using one-way ANOVA, with a significance level set at $p<0.05$ to indicate statistically significant results.

Results

Effect of Tomentin on DPPH Radical Scavenging Activity

The DPPH (α, α-diphenyl-β-picrylhydrazyl) radical scavenging activity is a method performed to assess the antioxidant capacity of a substance, extract, or further biological origins. This widely used spectrophotometric methodology can be used to assess substance's or food's antioxidant content as well as the antioxidant effectiveness of herbal extracts.In this study tomentin significantly increased the dpph radical formation in a dose dependent manner and the percentage of inhibition was found to be 26,37,48,62 and 71 respectively and the inhibition percentage was near to that of standard drug vitamin c whose inhibition was found to be (Figure 1 and table 1).

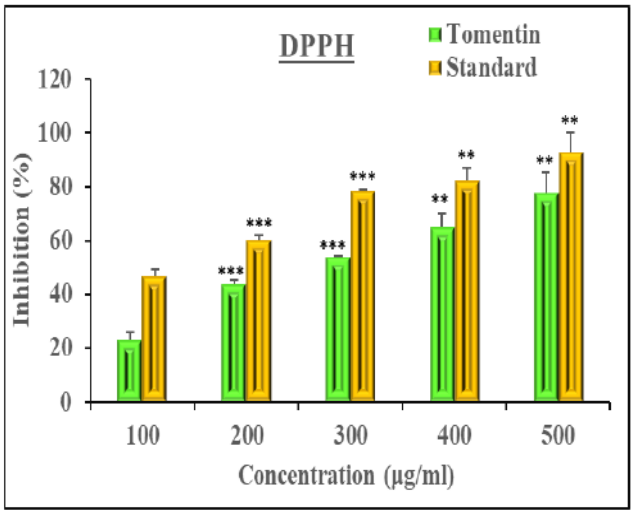

Figure 1. Represents the DPPH Activity (% of Inhibition). Values are Expressed in Mean ± SEM of 3 Replication. * Denotes Statistical Significance.

Table 1. Represents the DPPH Activity (% of Inhibition). Values are Expressed in Mean ± SEM of 3 replication. * Denotes Statistical Significance.

Sample concentration	% of concentration (Tomentin)	% of concentration (Standard – Vit C)
100 µg	26±2.1	46.5±1.547
200 µg	37.755±4	60.12±1.854
300 µg	48.75±1.5	78.54±0.54

400 µg	62.44±2.6	82.654±3.54
500 µg	71.9±3.5	92.8±3.245

Effect of Tomentin on Protein Denaturation Activity

In this part of the research, tomentin shows decrease in their protein denaturation action in a dose-dependent manner and the percentage of inhibition was found to be 15, 25, 40 and 55 respectively And the inhibition percentage was found to be less than the compared drug.

Protein denaturation assay: A protein loses its function when both its tertiary and secondary structures undergo modification, a process known as protein denaturation (Figure 2 & table 2).

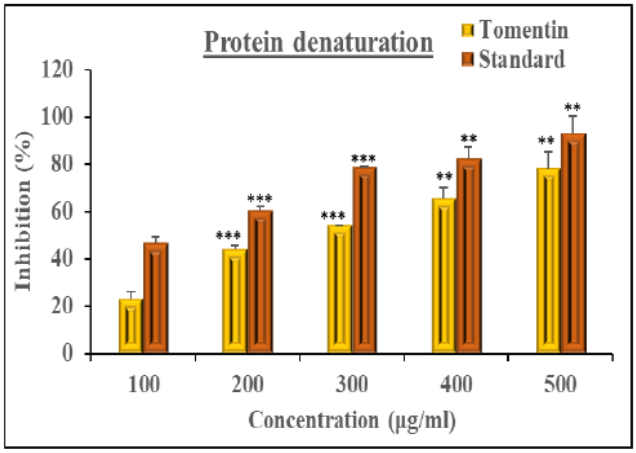

Figure 2. Represents the Protein Denaturation (% of Inhibition). Values are Expressed in Mean ± SEM of 3 Replication. * Denotes Statistical Significance.

Table 2. Represents the Protein Denaturation (% of Inhibition). Values are Expressed in Mean ± SEM of 3 Replication.

Sample concentration	**% of concentration (Tomentin)**	**% of concentration (Standard – Dichlopenac)**
10 µg	15.5±11	43.75±2.14
20 µg	25.9±3	58.41±2.541
30 µg	40.8±3.7	74.15±0.54
40 µg	55.65±10.6	81.65±3.41
50 µg	71±11	95.74±4.74

Effect of Tomentin on Cell Viability in A549 Cells

The loss of cellular or intercellular structure and/or functions can be detected by cell cytotoxicity examinations. They may show signs of causing destruction to cells and systems. Tomentin has the ability to cause tissue injury in the lung cancer cells compared to the other drug. the cell viability of tomentin was found to be effective and the 50% was attained at the concentration of 100 µM (Figure 3 a & b).

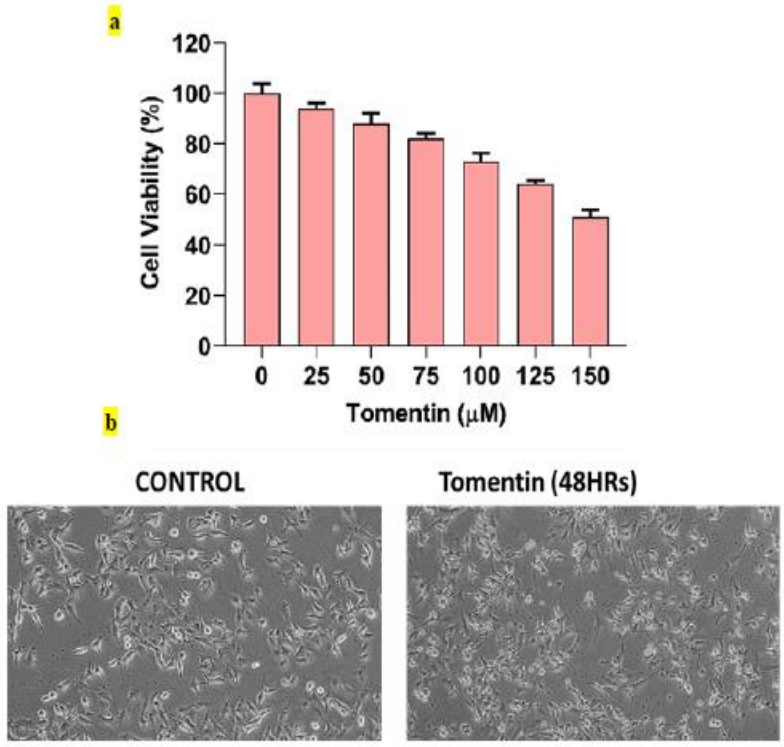

Figure 3a & b. Cytotoxicity Assay of Tomentin in A549 Cells on 48 hours' Time Intervals and Cell Morphology.

Effect of Tomentin on the mRNA Expression of Apoptotic Signaling Molecules (Bax, Bcl2, p53, MCL1, Caaspase-3, and Caspase-9) in A549 Cells

In this study, A549 cells treated with tomentin significantly reduced the antiapoptotic proteins (Bcl2, Mcl-1) while it improved the mRNA expression of proapoptotic molecules (Bax, Caspase-3 and 9) indicating that tomentin has a potential role controlling apoptotic signaling in A549 cells (Figure 4a-f).

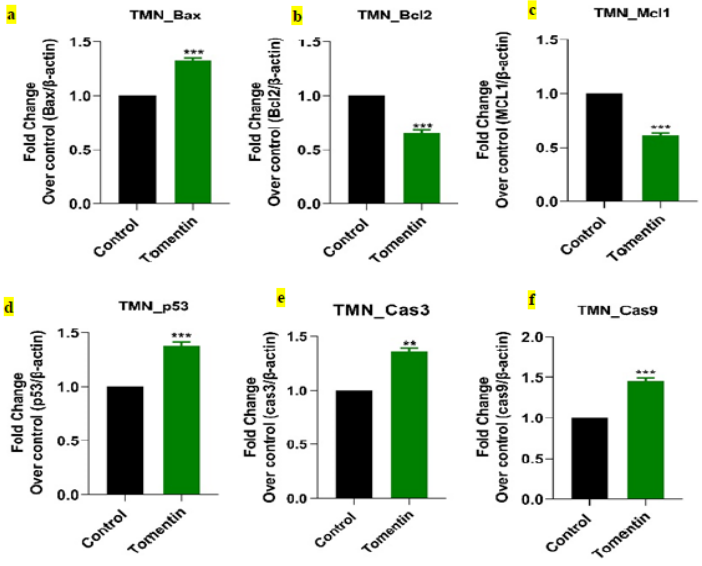

Figure 4a-f. Effect of Tomentin on Bax, Bcl2, p53, MCL1, Cas3, and Cas9 mRNA Expression in A549 Cells.

Molecular Interaction of Tomentin with Apoptotic Signaling Molecules

A computational method called molecular docking is used to predict a ligand-receptor complex's structure. It is a necessary instrument for computer-assisted drug design and structural molecular biology. In silico findings clearly indicates that tomentin has a strong binding affinity with apoptotic targets which shows that tomentin has a definite role in controlling lung cancer growth (Figure 5a-f & Table 3).

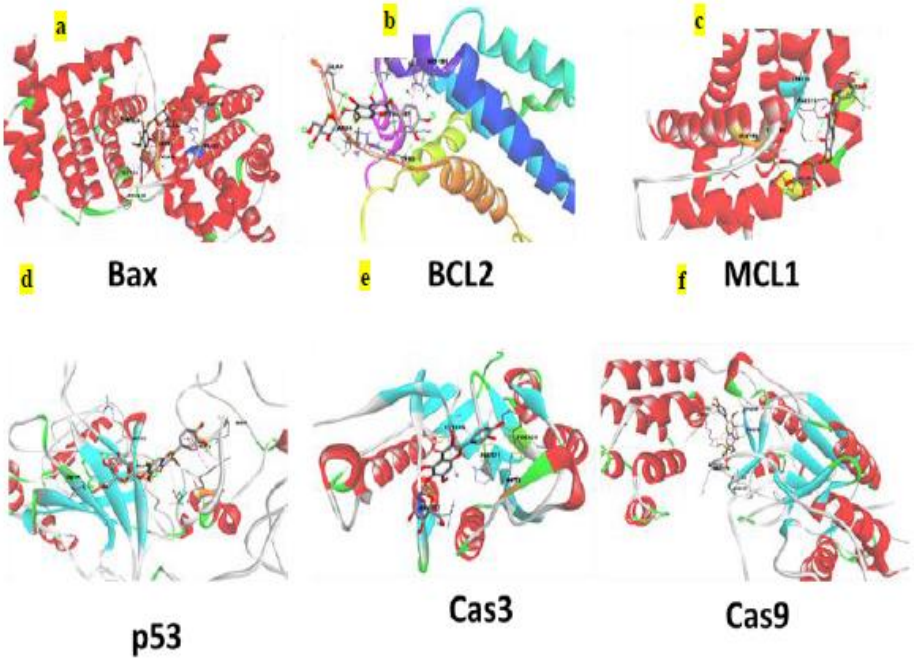

Figure 5a-f. Molecular Docking Analysis of Selected Targets (Bax, Bcl2, p53, Mcl1, Cas3, and Cas9) with Tomentin

Table 3. Binding Affinity Details of Selected Targets Bax, Bcl2, p53, Mcl1, Cas3, and Cas9 with Tomentin.

Compound	Proteins	Binding score (Kcal/mol)	Amino acids with H bonds
Tomentin (CID ID: 44259810)	Bax	-8.1	ASP48, GLN32, GLN28, PRO130
	Bcl-2	-7.6	ARG6, ALA4, GLN190, TYR9
	MCL-1	-6.5	GLN229, GLU188, GLN189, ASP218
	P53	-7.3	VAL203, ARG156, ARG202, ASN30
	Cas-3	-7.4	HIS121, ARG207
	Cas-9	-7.2	ARG7, GLY322, ARG324

Discussion

Cancer is a widespread non-communicable disease characterized by carcinogenesis, a process involving genetic mutations that disrupt normal biological processes like cell differentiation and death, leading to invasive tumors [19]. Oral carcinoma, a rare type of head and neck cancer, originates from squamous cells lining the mouth. Traditional treatments include surgery, radiation, and chemotherapy, but there is growing interest in natural compounds derived from plants as potential therapeutic agents. These compounds are being explored for their ability to inhibit cancer cell growth, induce apoptosis, and

mitigate the adverse effects of conventional therapies [20]. Lung cancer, like oral cancer, arises from carcinogenesis involving multiple molecular pathways. Understanding these pathways is crucial for developing targeted treatments [20]. Apoptosis, a form of programmed cell death, is a key mechanism by which the body eliminates damaged cells. Significant physiological changes, such as DNA fragmentation and chromatin condensation, indicate apoptosis [21].

The 2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenging activity assay is commonly used to assess the antioxidant potential of compounds. Tomentin, a compound found in various plants, has been studied using the DPPH assay for its antioxidant and anti-inflammatory properties. Tomentin exhibits strong antioxidant activity, which is concentration-dependent, meaning higher concentrations result in greater efficiency in neutralizing DPPH radicals [22]. This antioxidant capacity is due to tomentin's ability to donate hydrogen atoms or electrons to stabilize the DPPH radical, converting it into a non-radical form. This action highlights tomentin's potential in combating oxidative stress-related diseases such as cancer, cardiovascular disease, and neurological disorders [23]. Tomentin's antioxidant properties could lead to new strategies for preventing and treating oxidative stress-related conditions [24].

Protein denaturation, a process where proteins lose their biological activity and functionality, can be triggered by factors like heat, pH extremes, and chemicals. Research indicates that tomentin can inhibit protein denaturation, stabilizing proteins under stressful conditions [25]. Tomentin's ability to prevent denaturation could be due to its direct interaction with proteins, forming stabilizing complexes, or its antioxidant properties that protect against oxidative damage during denaturation [26]. This protective effect has significant implications for pharmaceuticals and food science, where maintaining protein stability is essential [27]. The cytotoxic effects of tomentin are crucial in assessing its safety and therapeutic potential. Studies show that tomentin exhibits dose-dependent cytotoxicity, meaning higher concentrations lead to more cell death. This effect suggests that tomentin may compromise cellular integrity to exert its cytotoxic effects, potentially by inducing apoptosis or disrupting vital cellular processes [28]. Notably, tomentin's selective cytotoxicity towards certain cancer cells while sparing healthy cells is particularly relevant for cancer therapy, where the goal is to target and eliminate malignant cells with minimal harm to healthy tissue [29]. Tomentin's cytotoxicity makes it a promising candidate for anticancer therapies, but further research is needed to understand its mechanisms and safety [30].

mRNA expression analysis is a powerful tool for understanding gene regulation and the synthesis of bioactive compounds like tomentin. Studies show that tomentin's mRNA expression is dynamic, influenced by factors such as stress, environmental conditions, and developmental stages. This suggests that tomentin's biosynthesis in plants is regulated by complex transcriptional networks [31]. Identifying key transcription factors and regulatory genes involved in tomentin production could lead to methods for enhancing its synthesis for medicinal or agricultural purposes [32]. mRNA expression analysis also sheds light on tomentin's physiological roles in plants, such as its potential involvement in defense mechanisms or environmental adaptation [33]. Molecular docking studies provide insights into tomentin's potential interactions with critical proteins involved in apoptosis and metastasis, such as MCL1, P53, BAX, BCL-2, Cas-3, and Cas-9. These studies suggest that tomentin may interfere with these proteins, potentially disrupting signaling pathways that lead to cancer cell death and metastasis [34]. Molecular docking predicts how tomentin binds to target proteins, offering clues to its

therapeutic potential. For instance, tomentin may bind to receptors involved in oxidative stress, inflammation, and cancer cell proliferation, indicating possible therapeutic applications [35]. Docking studies can also guide rational drug design by identifying key binding sites and interactions, allowing for the modification of tomentin's chemical structure to enhance its pharmacological effects [36]. However, predictions from molecular docking need experimental validation to confirm their accuracy and pharmacological relevance [37].

The search for biomarkers in oral cancer is critical for improving diagnosis, prognosis, and treatment. Molecular technologies are increasingly used in diagnostics, but oral cancer lesions may go undetected for long periods, reducing survival rates. Finding a biomarker that aids in diagnosis and prognosis while guiding treatment is crucial [38]. Tomentin's validated cytotoxicity, regulatory effects on key molecular targets, and strong binding interactions with inflammatory proteins make it a promising candidate for further research and clinical trials. Its potential as an anticancer agent, especially for lung cancer, offers hope for developing more effective therapies in the future [39].

Conclusion

The methodology outlined above will enable comprehensive evaluation of tomentin's anti-cancer potential using both *in vitro* and in silico approaches. By employing a combination of viability assays, apoptosis assays, protein denaturation assays, mRNA expression analysis, and molecular docking studies, a thorough understanding of tomentin's mechanisms of action will be achieved. Rigorous data analysis and interpretation will provide insights into tomentin's efficacy and potential as a novel therapeutic agent for lung cancer treatment

Conflict of Interest

The authors hereby declare that there is no conflict of interest in this study.

Acknowledgement

Authors would like to thank Saveetha Dental College and Hospitals, Saveetha Institute of Medical and Technical Sciences, Chennai, India for providing research facilities to carry out this work.

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