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Aloe emodin Inhibits Proliferation and Promotes Apoptosis in Non-Small Cell Lung Carcinoma (NSCLC) Cells by Deactivating PI3K/Akt/mTOR Signaling Pathways

Keshav Rajesh[1], R Priyadharshini[2*], Selvaraj Jayaraman[3]
[1]*Department of Pathology, Saveetha Dental College and Hospitals, Saveetha Institute of Medical and Technical Sciences, Saveetha University, Chennai, 600 077, Tamil Nadu, India*
[2]*Department of Oral Pathology, Saveetha Dental College and Hospitals, Saveetha Institute of Medical and Technical Sciences, Saveetha University, Chennai, 600 077, Tamil Nadu, India*
[3]*Centre of Molecular Medicine and Diagnostics (COMManD), Department of Biochemistry, Saveetha Dental College & Hospital, Saveetha Institute of Medical & Technical Sciences, Saveetha University, Chennai, 600 077, India*

Abstract

Non-small cell lung cancer (NSCLC) accounts for approximately 85% of lung cancer cases and is often detected at advanced stages, limiting treatment options and adversely affecting patient outcomes. Recognized by the WHO as the leading cause of cancer-related deaths, NSCLC necessitates improved diagnostic and therapeutic strategies. Aloe-emodin (AE), a natural compound with known anticancer properties, has demonstrated efficacy in inducing apoptosis and inhibiting cell proliferation in various cancer types. This study investigates AE's potential in treating NSCLC by targeting the PI3K/Akt/mTOR signalling pathway, crucial for cancer cell survival and proliferation. A549 lung cancer cells were treated with varying concentrations of AE, and cell viability was assessed using the MTT assay. Molecular docking studies were conducted to explore AE's interactions with PI3K, Akt1, and mTOR. Gene expression levels of these proteins were analysed using RT-PCR. Results showed a concentration-dependent decrease in NSCLC cell viability, with significant reductions observed at 72 hours. RT-PCR analysis indicated the downregulation of PI3K, Akt1, and mTOR in AE-treated cells. Docking studies revealed strong binding affinities of AE to PI3K, suggesting its inhibitory potential. These findings highlight AE's promise as a therapeutic agent against NSCLC, warranting further investigation into its mechanisms and clinical applicability.

Keywords: *Aloe emodin, Apoptosis, Gene Expression Analysis, Molecular Docking, Non-Small Cell Lung Carcinoma, Signalling.*

Introduction

Non-small cell lung cancer (NSCLC) is the most common type of lung cancer, accounting for about 85% of all cases. NSCLC is frequently detected in later stages, which restricts the available treatment choices and hurts patient outcomes [1]. Lung cancer is recognized by the World Health Organization (WHO) as the primary cause of cancer-related deaths globally, resulting in approximately 9.7 million deaths in 2023 [2]. Research into cost-effective and accessible treatments for NSCLC is crucial due to the significant burden it places on the healthcare system with a significant number of deaths related to NSCLC highlighting the requirement for enhanced diagnostic and therapeutic approaches [3,4].

Aloe-emodin (AE) is a natural compound that has gained recognition for its promising anticancer properties. It has been extensively studied for its potential therapeutic benefits in

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****Corresponding Author: priyadharshinir.sdc@saveetha.com***

treating different forms of cancer and its ability to trigger apoptosis in cervical cancer cells and boost the antiproliferative activity of tamoxifen in breast cancer cells [5]. It is thought that its mode of action involves triggering apoptosis in cancer cells, a critical process for preventing tumor growth and proliferation. This compound has shown effectiveness in inducing apoptotic cell death in colon cancer cells. It achieves this by increasing the levels of pro-apoptotic proteins and decreasing the levels of anti-apoptotic proteins [6]. In addition, there have been reports of AE specifically targeting neuroectodermal tumour cells, suggesting that it may have a more targeted approach to fighting cancer [7].

AE has been shown to possess numerous pharmacological effects. These include antiviral properties achieved by up-regulating galectin-3, antibacterial actions, antioxidant capabilities, and anti-inflammatory effects via the inhibition of NF- κ B, MAPK, and PI3K pathways [8-11]. It also exhibits antimicrobial activities, neuroprotective effects through the regulation of ERK phosphorylation, and hepatoprotective actions by restoring characteristic morphological changes, reducing DNA synthesis, and inhibiting the production of type I collagen and expression of smooth muscle α-actin [12]. Moreover, AE has garnered significant attention for its notable antineoplastic activity against various tumour cells, such as those found in cervical, lung, gastric, bladder, and other types of cancer [13].

Recent studies have revealed the interactions of AE with important proteins in cancer pathways, including the PI3K-AKT signalling pathway and the TP53 signalling pathway [14]. The PI3K/Akt/mTOR signalling pathway plays a crucial role in controlling cell growth, proliferation, and survival. The pathway starts with the activation of PI3K, which then phosphorylates and activates Akt. Then, Akt phosphorylates various downstream targets, including mTOR, which is a key player in cellular metabolism and growth by controlling the cell cycle which can impact the growth of cancer cells [15]. The mTOR protein is involved in the formation of two distinct complexes, mTORC1 and mTORC2, which have different functions in the regulation of protein synthesis and actin cytoskeleton organization [16]. This pathway plays a crucial role in the development of cancer by promoting uncontrolled cell growth and survival. As a result, it has become a major focus for therapeutic intervention. Tumor growth can be decreased and promote survival by altering the PI3K/Akt/mTOR pathway [17]. This study seeks to explore the potential of *Aloe-emodin* in inhibiting the growth and inducing programmed cell death in non-small cell lung cancer (NSCLC) cells. Specifically, it aims to investigate the deactivation of the PI3K/Akt/mTOR signalling pathway, which is known to be vital for cancer cell survival and proliferation.

Materials and Methods

Cell Line

A549, lung cancer cell line was procured from the National Centre for Cell Sciences, Pune, India. The cells were maintained and grown in a controlled environment inside a CO_2 incubator set at a temperature of 37°C. DMEM, together with 10% FBS and 1% penicillin-streptomycin antibiotics, was the culture media utilized for cell growth.

Cell Viability Assay

The cytocompatibility of *Aloe-emodin* was assessed against the A549 cell line utilizing the MTT colourimetric assay. Cells were initially seeded into a 96-well plate at a density of $1x10^4$ cells per well and left to attach overnight. Subsequently, the samples were diluted in a mixture of 1% dimethyl sulfoxide (DMSO) and DMEM media to achieve concentrations ranging from 0 to 125µM. The *Aloe-emodin* were then added to the wells and incubated for either 24, 48, or 72 hours in a CO_2 incubator. Following treatment, the supernatant was

aspirated, and the wells were washed with 1xPBS. Next, 10µl of MTT solution was added to each well and incubated for 2 hours. The resulting formazan crystals were dissolved using 100µl of DMSO, and the absorbance was measured at 590 nm. Morphological changes of *Aloe-emodin* in different period were visualized using a Labomed inverted microscope. Percent cell viability was calculated relative to a control, and the data and IC_{50} Concentrations were plotted and measured using GraphPad Prism software.

Molecular Docking Analysis

The molecular docking program AutoDock 4.2 was used to study the binding of *Aloe-emodin* to mTOR regulating targets such as AKT1, PI3K and mTOR. The crystal structures of these proteins such as AKT1 (PDB ID: 1UNQ), PI3K ((PDB ID: 4oVU), and mTOR (PDB ID: 4JSV) were obtained from the Protein Data Bank (https://www.pdb.org/pdb). A grid box (90 Å × 90 Å × 90 Å) with a grid spacing of 0.45 Å was used during the docking process. The docking calculations were performed using the Lamarckian genetic algorithm (LGA) and the number of genetic algorithm runs was 100. The docking results were analyzed to identify the high-pose interactions between β-sitosterol and the apoptosis-regulating targets. The binding affinities of the ligand targeting the receptor were also analyzed to describe the binding mode. The 3D structured outcome complex docking results were visualized using BIOVIA Discovery studio.

Gene Expression by RT-PCR

Reverse Transcription Polymerase Chain Reaction (RT-PCR) is a molecular biology technique widely employed to analyze and quantify gene expression levels in biological samples. The process begins with the extraction of RNA from cells or tissues, followed by the reverse transcription step, where RNA is converted into complementary DNA (cDNA) using the enzyme reverse transcriptase. This cDNA serves as a stable template for subsequent PCR amplification. Target gene expression is then assessed through the amplification of cDNA using specific primers designed to anneal to the gene of interest. Real-time quantitative PCR (qPCR) is a common variation of RT-PCR that enables the simultaneous amplification and quantification of the target gene during each cycle of the reaction. The resulting data can be used to determine the initial amount of mRNA present in the sample, offering insights into the relative expression levels of the gene of interest under different experimental conditions. RT-PCR is highly sensitive and specific, allowing for the detection of even low-abundance transcripts. It serves as a fundamental tool in molecular biology, enabling researchers to investigate gene expression patterns, validate microarray data, and gain a deeper understanding of cellular processes and responses.

Statistical Analysis

Each measurement was obtained from three parallel tests. All experimental data are presented as the mean ± standard deviation (SD). Statistical analyses were conducted using GraphPad Prism 7 (GraphPad Software, USA). One-way analysis of variance (ANOVA) followed by Tukey's test was used for multiple-group comparisons, and Student's t-test was used for two-group comparisons. p-value of less than 0.05 was considered statistically significant.

Results

Cell viability was observed to decrease in a concentration-dependent manner following treatment.

With *Aloe emodin* as seen in Figure 1. Cell viability at 24 hours was approximately 80% at the highest concentration (125 µM), but it decreased to around 40% at 72 hours (Figure 1).

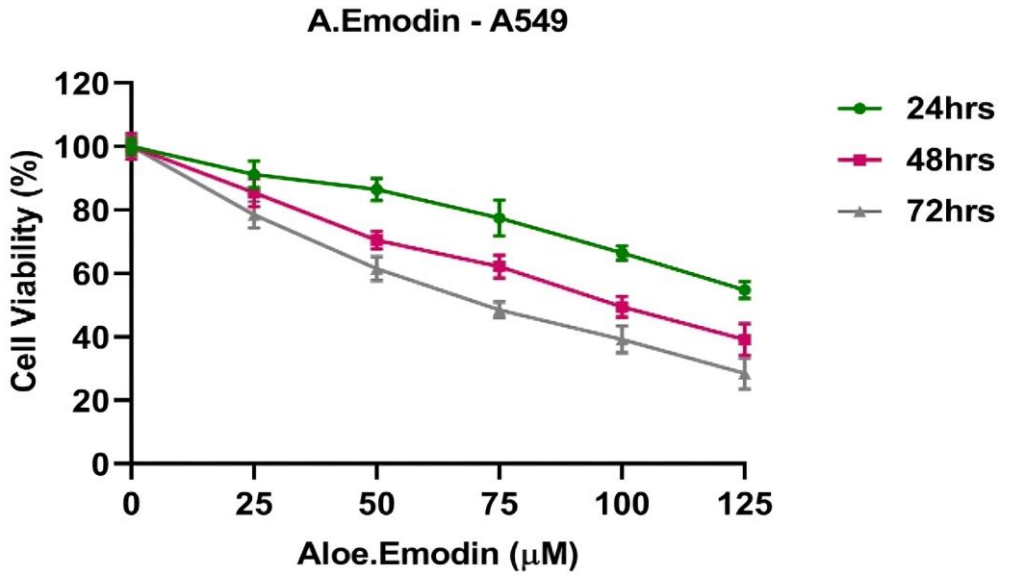

Figure 1. Treatment with *Aloe-emodin* inhibited the cell viability of A549 cells. (A) Cell viability of A549 cells was detected after *Aloe emodin* treatment at concentrations of 0, 25, 50, 75, 100,125 µM; showed a decrease in cell viability (%) with rise in concentration of *Aloe emodin* (µM).

Aloe-emodin efficacy was evaluated through an A549 cell line assay to investigate its impact. After 72 hours of treatment, the effectiveness of *Aloe emodin* exhibited a significant reduction in NSCLC cell viability caused by the treatment (Figure 2). These findings showed the potent influence of *Aloe-emodin* on NSCLC cells.

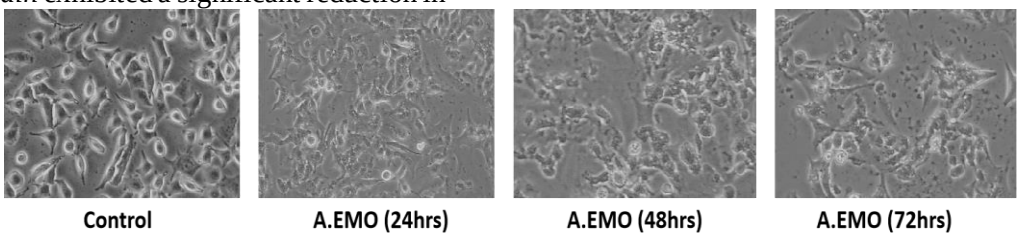

Figure 2. *Aloe-emodin* was treated with A549 cells (0 to 125 µM), and morphological changes in cells were observed under an Inverted light microscope at 10x magnification.

The association of PI3K/β-actin with *Aloe-emodin* in NSCLC cells was analyzed after 24 and 48 hours using Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR). The results indicated a decrease in the fold change of PI3K/β-actin (Figure 3) in NSCLC cells compared to control samples. In RT-PCR, the amplification of PI3K/β-actin increased between 20 to 40 cycles, with RFU values ranging from 580 to 600 (Figure 4). Similarly, there was a decrease in the fold change of AKT1/β-actin associated with *Aloe-emodin* in NSCLC cells after 24 and 48 hours (Figure 3). RT-PCR amplification of AKT1/β-actin also increased between 20 to 40 cycles, with RFU values between 580 and 600 (Figure 4). Additionally, mTOR showed a decreased fold change in association with *Aloe emodin* after 24 and 48 hours in NSCLC cells (Figure 3), with RT-PCR amplification of mTOR/β-actin increasing between 20 to 40 cycles, with RFU values ranging from 350 to 400 (Figure 4).

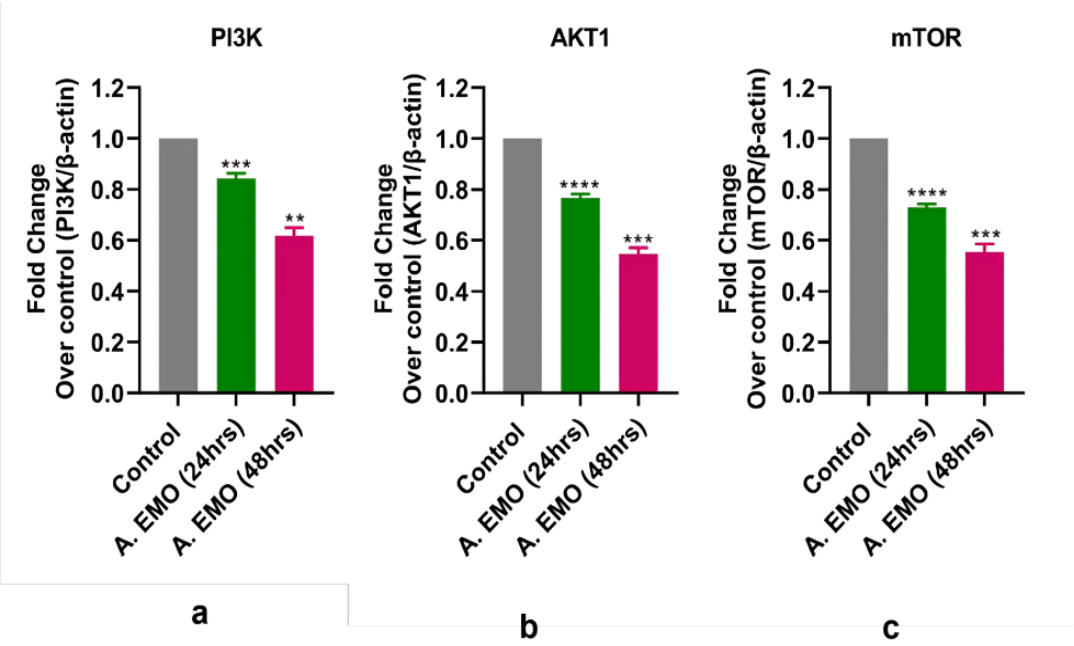

Figure 3. Effect of *Aloe-emodin* on PI3K/Akt/mTOR expression in NSCLC cells.

Real-time RT-PCR amplification of PI3K/Akt/mTOR expression treated with *Aloe emodin* (24 hours and 48 hours) in A549 cells. The 2-ΔΔ Ct method of relative quantification was used to determine the fold change in expression with β-actin. "*" denotes statistical significance at the level of $p \leq 0.001$ when compared with control.

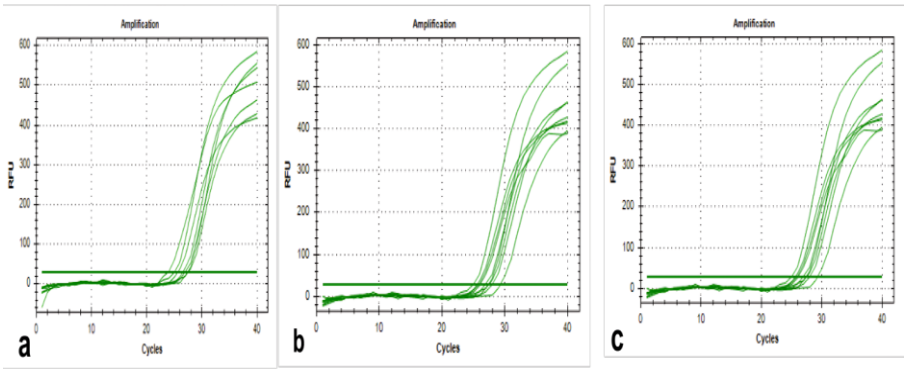

Figure 4. The graph above illustrates the amplification cycle of RT-PCR for PI3K/β-actin, AKT1, and mTOR. In this graph, the X-axis represents the number of cycles in the RT-PCR process, which typically ranges from 0 to 40 cycles. The Y-axis denotes the Relative Fluorescence Units (RFU), a measure of the fluorescence intensity emitted during the PCR amplification. This intensity reflects the amount of amplified product, with higher RFU values indicating greater levels of gene expression. The curves in the graph show how the fluorescence changes across the cycles for each gene, allowing for the comparison of their expression levels under different experimental conditions.

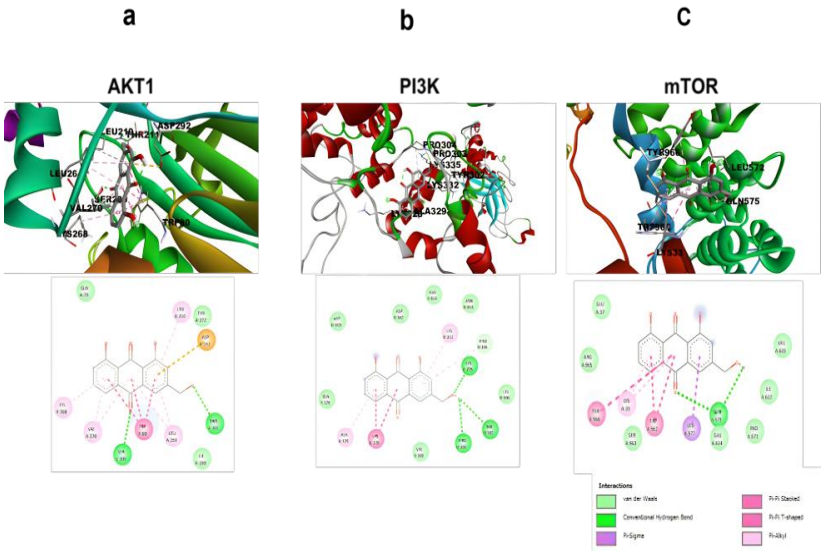

Figure 5. The binding poses of (A) AKT1, (B) PI3K, and (C) mTOR with *Aloe emodin* are illustrated in the cartoon model above. The amino acids interacting with the *Aloe-emodin* compound are labelled with the corresponding interaction colours. Hydrogen bonds and hydrophobic contacts are indicated by green and pink dotted lines, respectively, in the model below. In the molecular modelling tools, the default radius was set to 5 Å.

The study investigates the significant inhibitory effect of *Aloe-emodin* on AKT1/PI3K/mTOR protein signalling. Among the extracts, PI3K demonstrates the highest impact, which is statistically significant compared to the standard drug reference, metformin. Molecular docking is a valuable tool for examining a protein's active site and understanding the binding interactions between ligands and the target protein. For this research, we selected three key proteins AKT1, PI3K, and mTOR to study the impact of *Aloe emodin* on NSCLC cell proliferation. By analyzing individual chemical interactions with *Aloe-emodin*, we conducted a comparative study. The results revealed that the PI3K compounds had the highest docking scores, nearly matching those of the standard reference drug, metformin (Table 1) (Figure 5).

Table 1. Molecular Docking Analysis was Performed using PyRx Software, and the 3D Structures were Visualized with Biovia Discovery Studio

Compound	Protein(s)	Binding energy (Kcal/mol)	No. of amino acid residues	Amino acid residues
Aloe emodin	AKT1	-10.1	2	SER205, THR211
	P13K	-7.4	3	LYS335, TYR302, PRO303
	mTOR	-8.4	1	GLN575

Discussion

Our results show that *Aloe-emodin* reduces cell viability in a concentration-dependent manner, with the highest concentration (125 µM) resulting in approximately 80% viability at 24 hours, which decreased to around 40% at 72 hours. This demonstrates the potent cytotoxic effect of *Aloe-emodin* on NSCLC cells over time. Previous studies have similarly reported the efficacy of *Aloe-emodin* in reducing cell viability across various cancer cell lines, including NSCLC. For instance, a study by Karatoprak et al. (2022) demonstrated that *Aloe-emodin* significantly inhibited the

proliferation of A549 cells, which aligns with our observations of reduced cell viability over 72 hours [13]. In addition, Zhu et al. (2023) found that Aloe-emodin induced apoptosis and inhibited cell proliferation in hepatocellular carcinoma cells by modulating the expression of apoptosis-related genes and proteins. This supports our findings of Aloe emodin's broad-spectrum anti-cancer properties [18].

Our qRT-PCR analysis revealed a decrease in the fold change of PI3K/β-actin, AKT1/β-actin, and mTOR/β-actin in NSCLC cells treated with *Aloe-emodin* for 24 and 48 hours compared to control samples. This indicates that *Aloe-emodin* effectively downregulates these key signalling pathways. These results are consistent with the literature, where inhibition of the PI3K/AKT/mTOR pathway has been identified as a crucial mechanism through which *Aloe-emodin* exerts its anti-cancer effects. A study by Lin et al. (2022) also reported that *Aloe-emodin* inhibited the PI3K/AKT pathway, leading to apoptosis in lung cancer cells [19]. Similarly, Tseng et al. (2017) showed that Aloe-emodin triggered cell cycle arrest and apoptosis in breast cancer cells through the suppression of the PI3K/AKT/mTOR pathway, further corroborating our findings [20].

The molecular docking analysis highlighted that PI3K had the highest docking scores when interacting with *Aloe-emodin*, nearly matching those of the standard reference drug, metformin. This suggests that *Aloe-emodin* has a strong binding affinity to PI3K, which might contribute to its substantial inhibitory effect on NSCLC cell proliferation. Previous docking studies have similarly shown *Aloe emodin's* strong binding affinity to various protein targets involved in cancer cell survival and proliferation [21]. Our findings are in line with earlier research that demonstrated the anti-cancer properties of *Aloe-emodin*. Notably, the observed decrease in cell viability and downregulation of the PI3K/AKT/mTOR signalling pathway highlight the compound's potential as a therapeutic agent against NSCLC. The comparative effectiveness of *Aloe-emodin*, especially its significant impact on PI3K compared to a standard drug like metformin, underscores its potential for further development in cancer treatment protocols.

Limitations

The study primarily used A549 cell lines to evaluate the efficacy of *Aloe-emodin* on NSCLC cells. While this provides valuable insights, the results may not fully translate to in vivo models or human clinical settings due to the complexity of the tumour microenvironment and the body's metabolic responses. The study observed changes in cell viability at different concentrations and over different time points. However, a more detailed time-course study with multiple intermediate time points and concentrations could provide the effects of *Aloe-emodin*. While the study demonstrated a decrease in the fold change of key proteins (PI3K, AKT1, mTOR) associated with *Aloe-emodin* treatment, it did not explore the underlying mechanisms in depth. Further studies are needed to elucidate the precise molecular pathways through which *Aloe-emodin* exerts its effects.

Conclusion

Aloe emodin's ability to disrupt key molecular pathways involved in NSCLC cell proliferation, combined with its significant cytotoxic effects and high binding affinity, highlights its therapeutic potential. These findings support the continued investigation of *Aloe-emodin* as a potential anti-cancer agent, with the possibility of developing it into a new treatment option for patients with NSCLC. Further research, including in vivo studies and clinical trials, will be essential to fully understand its efficacy and safety in a clinical setting.

Conflict of Interest

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

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Author Contribution

1. Keshav - contributed to designing the study, execution of the project, statistical analysis, and manuscript drafting.
2. Dr. Priyadharshini R - contributed to study design, guiding the research work, and manuscript drafting.
3. Dr. Selvaraj - contributed in designing the study, execution of the project, statistical analysis, manuscript correction.

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