Texila International Journal of Public Health
Special Issue-2025
DOI: 10.21522/TIJPH.2013.SE.25.01.Art003

Molecular Mechanisms Underlying in the Anticancer Activity of Verbacoside Against Human Lung Adeno Carcinoma (A549) Cells Via Modulating Apoptotic Signalling

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Abstract

Verbascoside (VERB), a phenylethanoid-phenylpropanoid glycoside, has garnered significant interest due to its potential therapeutic effects, particularly its anticancer properties. This study investigates the molecular mechanisms underlying the anticancer activity of VERB against A549 cells, a model of non-small cell lung cancer (NSCLC). Our findings demonstrate that VERB induces apoptosis in A549 cells through the modulation of key apoptotic signaling pathways. Specifically, VERB treatment resulted in the activation of caspases, upregulation of pro-apoptotic proteins, and downregulation of anti-apoptotic proteins. Additionally, VERB was observed to inhibit the NF-kB pathway, thereby reducing inflammation and promoting apoptotic cell death. These results suggest that VERB exerts its anticancer effects by targeting multiple cellular pathways involved in cell survival and apoptosis, providing a promising avenue for the development of novel NSCLC therapies.

Keywords: *A549 Cells, Intrinsic Apoptotic Pathway, Inflammatory Signalling, Novel Methods, NSCLC, Public health, Verbacoside.*

Introduction

Lung cancer comprises several types, such as bronchogenic carcinomas, which are malignant neoplasms arising from the bronchus or bronchioles, and lung carcinoid tumors, which are neuroendocrine tumors. Lung cancer is a leading cause of cancer deaths globally. Histologically, lung cancers can be classified into small cell lung cancer (SCLC), squamous cell carcinoma (SCC), and adenocarcinoma (ADC), each originating from different compartments within the lung. Non-small cell lung cancer (NSCLC) includes adenocarcinoma (and the relatively rare bronchioalveolar carcinoma [BAC]), squamous cell carcinoma, and large cell carcinoma (undifferentiated NSCLC). NSCLC is a major type of cancer affecting both genders worldwide, accounting for 80% of lung cancers and causing more than 1.2 million deaths annually [1]. While smoking is the predominant cause of lung cancer, other environmental factors such as diet and lifestyle may also play a role [2].

Lung cancer remains a significant cause of mortality and morbidity among seriously ill patients [3, 4]. Despite ongoing research, the specific mechanisms underlying the development and progression of lung cancer remain unclear. However, inflammatory responses and apoptosis are recognized as major contributing factors [5]. The nuclear factor-kappa B (NF-kB), a crucial transcription factor, plays a pivotal role in inflammation,

Received: 14.06.2024 ***Accepted: 09.09.2024*** ***Published on: 31.01.2025***
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immunity, cell proliferation, differentiation, and survival. Given its involvement in pathogenic processes like acute lung injury and asthma, NF-kB represents a potential target for lung cancer treatment [6, 7]. Regarding apoptosis, mitochondria, as cellular organelles, are intricately involved in the regulation of cell death. Mitochondrial outer membrane permeabilization allows the release of cell death factors such as cytochrome c into the cytoplasm, which triggers caspase activation and apoptosis. This process is predominantly controlled by the Bcl-2 family, which includes both proapoptotic and antiapoptotic members [8-10].

Increasing studies have indicated that many Chinese medicines can be used to induce cell apoptosis and suppress inflammatory response. Phytochemicals are naturally occurring compounds from plants which serve as vital resources for cancer therapy and production of novel drugs [11]. Polyphenols have been thoroughly investigated for their potential benefits, including antioxidant, anti-angiogenic, antiproliferative, antitumor, hypoglycaemic, and immunomodulatory properties. These attributes have led to an expansion of their applications across functional foods, pharmaceuticals, and cosmetic industries [12]. Phenylethanoid-phenylpropanoid glycosides (PPGs) are compounds widely found throughout the plant kingdom, predominantly isolated from medicinal plants. They are soluble in both water and organic solvents [13]. These glycosides are not confined to specific plant organs; they have been extracted from various parts such as leaves, aerial parts, barks, roots, callus, and cell cultures [14,15]. In recent years, there has been increasing interest in this class of glycosides, particularly verbascoside (VERB), due to the substantial body of literature highlighting its significant role in the prevention and treatment of various disorders and diseases. This study focuses on the unveiling the underlying mechanism of action of verbacoside against lung cancer in A549 cells via apoptotic signalling through *in vitro* cell culture, molecular and in silico approaches.

Materials and Methods

Chemicals and Reagents

All chemicals and reagents utilized in this study were procured from reputable sources.

Antioxidant Activity

DPPH Radical Scavenging Activity

Assessment of antioxidant potential of verbacoside using DPPH radical scavenging assay followed by the protocol of Hatano et al (1989) [16]. Ascorbic acid is used as a standard at different concentrations. Absorbance was measured at 517nm and the capability of verbacoside to scavenge the free radical was measured using the formula:

% of free radicals scavenged = [(Control absorbance – Sample absorbance)/Control absorbance] × 100.

Anti-Inflammatory Activity

Protein Denaturation Inhibition Activity

To evaluate the anti-inflammatory potential of verbacoside the method determined by Padbanabham and Jangle (2012) [17] & Elias and Rao (1988) [18] was used with some modifications. The activity of protein denaturation inhibition was measured at 660 nm. Verbacoside's anti-inflammatory potential was calculated using the % inhibition formula:

% inhibition of BSA denaturation = [(Absorbance of Control – Absorbance of Sample OD)/Absorbance of Control] × 100.

Cell Lines

The A549 cell were obtained from National Centre for Cell Science, Pune, India and grown in culture flasks, separately containing DMEM medium, respectively supplemented with 10% FBS under 5% CO_2, 95% air at 37°C. Upon reaching confluence, the cells were trypsinized and passaged.

Cell vZ by Real Time PCR

mRNA expression levels were examined using real-time PCR. The total RNA was isolated by using Tri 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 an MX3000p PCR system (Stratagene, Europe). The 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 pair. The data were analyzed by comparative CT method and the fold change was calculated by the 2−CT method described by Schmittgen and Livak (2008) [20] using CFX Manager Version 2.1 (Bio-Rad, USA).

Molecular Docking Analysis

It is a computational procedure employed for forecasting the manner in which a small molecule (known as a ligand) binds to a target protein or receptor, as well as estimating their binding strength. Docking results are analyzed and visually inspected to select promising ligands for further experimental validation. The docking analysis was carried out in PyRx software and the Visualisation of 2D and 3D was done by Biovia Discovery studio.

Statistical Analysis

Data were expressed as the means ± SD of 3 individual experiments performed in triplicate. Statistical analysis was performed using the one-way ANOVA and $p<0.05$ was considered to indicate a statistically significant result.

Results

In Vitro Assays

Effect of Verbacoside on DPPH Radical Formation Inhibition

The study's results revealed a dose-dependent increase in inhibition percentage against DPPH radicals, encompassing concentrations of 12.5 to 200 µg/ml, for both the extract and the standard substance. Notably, the most pronounced antioxidant activity was observed at the concentrations of 400 and 500µg/ml, as depicted in Figure 1 and detailed in Table 1.

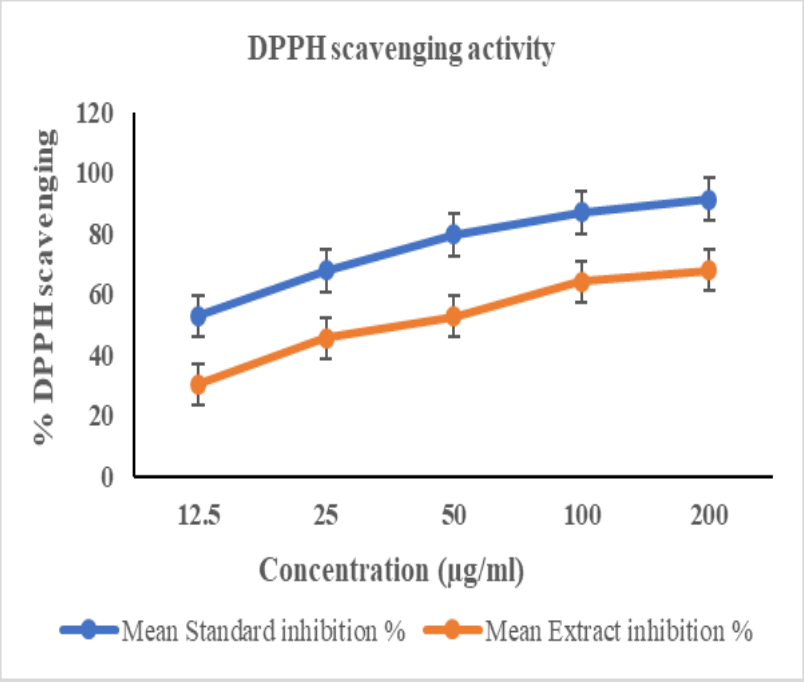

Figure 1. DPPH Radical Scavenging Activity of the Verbacoside at Various Concentrations

Table 1. Ddepicts the % Inhibition of Verbacoside Against dpph Radical Formation

Concentration (µg/ml)	Mean Standard inhibition %	Mean Extract inhibition %
12.5	52.87	30.45
25	67.89	45.72
50	79.56	52.67
100	86.9	64.31
200	91.23	67.87

Effect of Verbacoside Albumin Protein Denaturation Inhibition

Similarly, the study revealed a consistent pattern in the suppression of inflammatory effects, with inhibition rates increasing proportionally with higher concentrations (ranging from 12.5 to 200 µg/ml) of both the verbacoside and the reference compound. The highest level of anti-inflammatory potency for both the verbacoside and the standard substance was observed at a concentration of 200µg/ml. This is represented in Figure 2 and further detailed in Table 2.

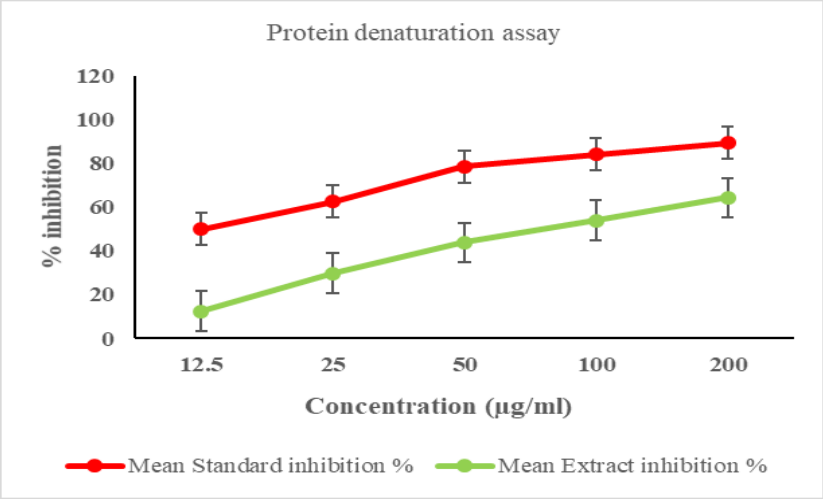

Figure 2. Represents the Protein Denaturation Inhibition Activity of the Verbacoside

Concentration (µg/ml)	Mean Standard Inhibition %	Mean Extract Inhibition %
12.5	50.02	12.45
25	62.48	29.67
50	78.45	43.76
100	83.98	53.98
200	89.34	64.23

Table 2. Depicts the Anti-Inflammatory Activity of Verbacoside

Effect of Verbacoside Compound on Cell Viability in Human A549 Cells

The cell viability of A549 cells was assessed following treatment with verbacoside at varying concentrations (0, 12.5, 25, 50, 100 µg/ml). The viability of the cells was determined using the MTT assay and expressed as a percentage relative to the untreated control cells. The results demonstrated that cell

viability decreased in a dose-dependent manner (figure 3). Specifically, at the lowest concentration of 12.5µg/ml, the cell viability was 98% compared to the control, whereas at the highest concentration of 100µg/ml, the viability dropped to 48%. The verbacoside treatment exhibited significant cytotoxic effects at concentrations above 12.5µg/ml ($p< 0.05$).

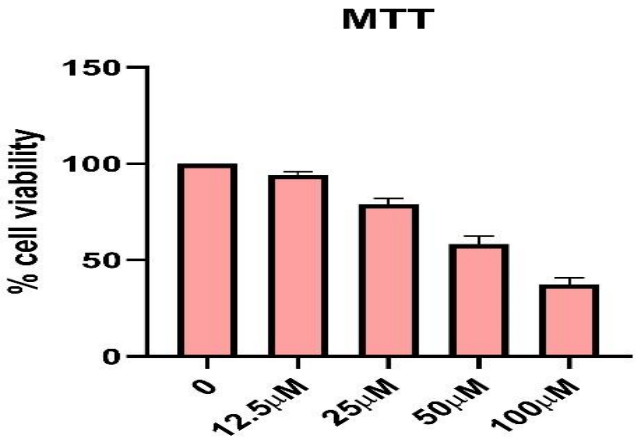

Figure 3. Effect of Verbacoside Compound on Cell Viability in Human A549 Cells

Effect of Verbacoside on the mRNA Expression Analysis Proinflammatory and Apoptotic Signalling Molecules

The mRNA expression levels of genes including NFκB, NEMO, TNFα, BAX, BCL2 were analyzed in A549 cells treated with verbacoside at various concentrations (10, 50, 200 µg/ml). The expression levels were quantified using quantitative real-time PCR (qRT-PCR) with β-actin used as an internal control. Figure 4a-e shows the mRNA expression of key signalling molecules involved in proinflammatory and intrinsic apoptotic signalling pathway. The results showed that treatment with verbacoside led to significant changes in the expression of several key genes involved in inflammatory and apoptotic signalling pathway.

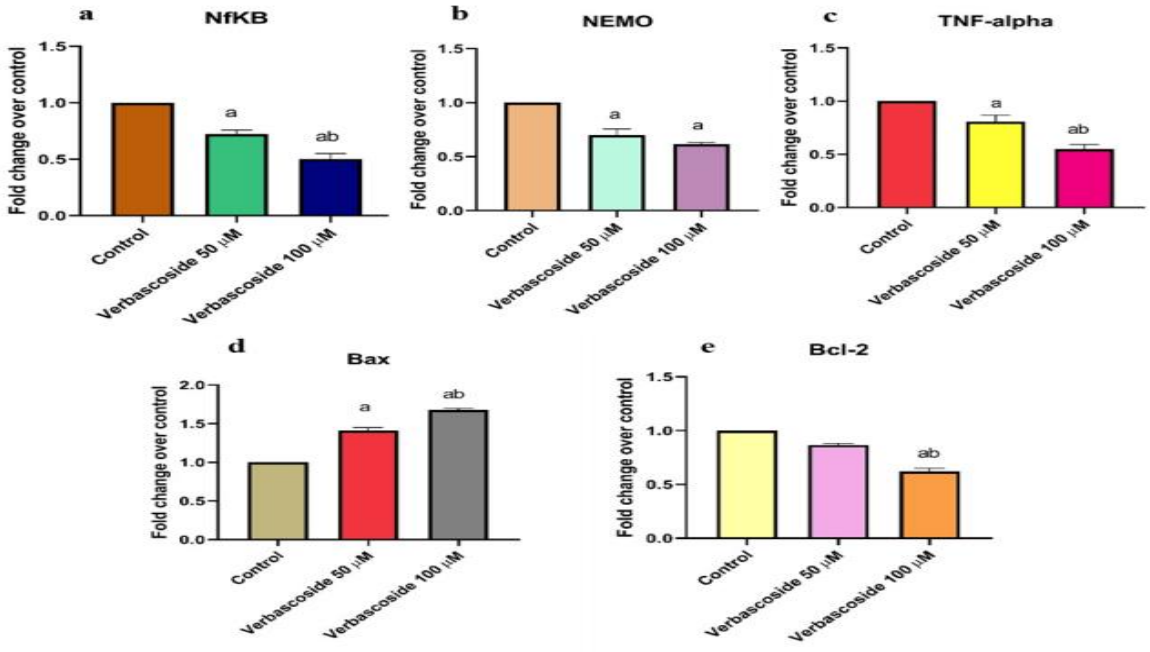

Figure 4a-e. Effect of Verbacoside NFκB, NEMO, TNFα, Bax, and Bcl2 mRNA Expression

Molecular Interaction of Verbacoside on Inflammatory and Apoptotic Signalling Targets

Additionally, to further validate our findings, we performed molecular docking analysis of gallic acid ligands with key inflammatory targets, including Akt, PPAR-γ, and IR. This analysis was conducted using PyRx software and the 3D structures generated by Biovia Discovery Studio (Figure 5a-e). The docking simulations revealed a notably high binding affinity of the phytochemical compound with the inflammatory targets, as shown in Table 3. This computational approach provides additional support to our investigation, reinforcing the potential anti-inflammatory interactions of gallic acid with the specified inflammatory markers, as depicted below.

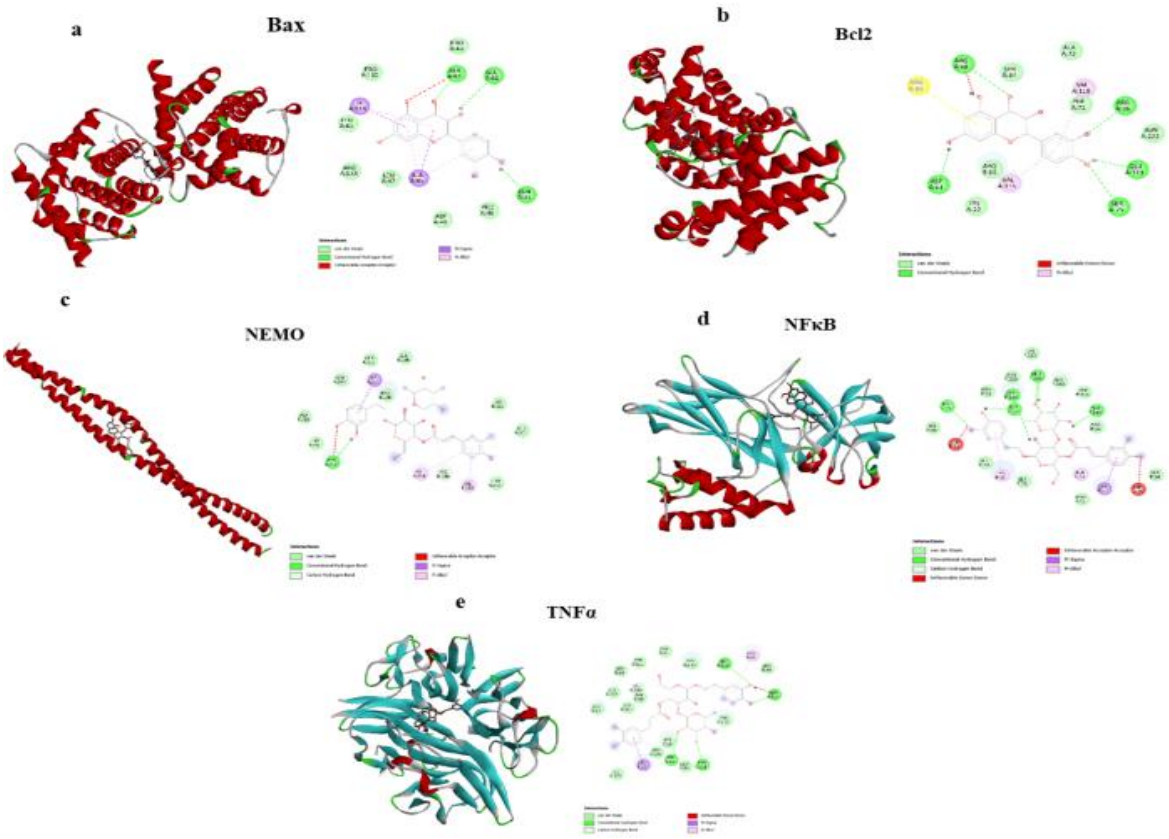

Figure 5a-e. Interaction of Verbacoside Ligand with Bax, Bcl2, NEMO, NFκB, TMFα

Table 3. Binding Affinity of Verbacoside with the Targets Bax, Bcl-2, NEMO, NFκB, TNFα

1	Verbascoside (5281800)	Bax	-7.8
2		Bcl-2	-7.4
3		NEMO	-6.4
4		NFκB	-7.7
5		TNF-alpha	-8.8

Discussion

Despite therapeutic advances in cancer treatment, adverse events and chemoresistance continue to pose significant challenges. Over the past two decades, extensive research has focused on medicinal plants and their phytoconstituents as potential chemo-preventive and anticancer agents [21]. Currently, more than 60% of anticancer drugs in use are derived from natural sources, including plants, marine organisms, and microorganisms. Traditional medicine, including herbal-based drugs, has been used worldwide for a long time to treat various chronic ailments, including cancer [22]. Numerous scientific studies, including ours, have highlighted the potential of medicinal plants as anticancer drug candidates. Ayurveda, the primary traditional medical practice in India, focuses on disease prevention through healthy food habits and lifestyle. Since ancient times, it has effectively utilized natural drugs to prevent or suppress various tumors through different treatment approaches. The Charak Samhita (1500–2000 BC), considered the first text of Ayurveda, describes various formulations in the form of kashya (decoctions), each containing different herbs aimed at specific actions [23, 24]. In this study, we investigated the molecular mechanisms underlying the anticancer activity of verbascoside (VERB) against A549 cells, focusing on its effects on apoptotic signalling pathways. Our comprehensive approach included DPPH and protein denaturation inhibition assays, MTT assay, mRNA expression analysis, and molecular docking studies to elucidate the multifaceted role of verbacoside in inducing apoptosis and inhibiting cancer cell proliferation.

The DPPH assay demonstrated that verbacoside possesses significant antioxidant activity, which is crucial in mitigating oxidative stress associated with cancer progression. The ability of verbacoside to scavenge free radicals may contribute to its protective effects against cellular damage and its potential to inhibit tumour growth. Additionally, the protein denaturation inhibition assay indicated that verbacoside has substantial anti-inflammatory properties. Since chronic inflammation is a known risk factor for cancer development, the anti-inflammatory effects of verbacoside further support its potential as an anticancer agent. The MTT assay revealed that verbacoside significantly inhibits the proliferation of A549 cells in a dose-dependent manner. This cytotoxic effect is indicative of its potential to suppress tumour growth. The reduction in cell viability upon verbacoside treatment suggests that it effectively induces cell death, which we hypothesized to occur through apoptotic mechanisms. mRNA expression analysis provided insights into the molecular mechanisms by which VERB induces apoptosis. We observed upregulation of pro-apoptotic genes such as Bax and downregulation of anti-apoptotic genes such as Bcl-2. This shift in the Bax/Bcl-2 ratio is a critical indicator of apoptosis initiation through the intrinsic pathway. Furthermore, verbacoside treatment resulted in decreased expression of NF-kB and its downstream targets, including TNF-alpha and NEMO, suggesting that verbacoside disrupts the NF-kB signalling pathway, which is known to promote cell survival and proliferation in cancer cells.

Molecular docking studies provided additional confirmation of verbacoside's interaction with key apoptotic and inflammatory proteins. Verbacoside exhibited strong binding affinities to Bax, Bcl-2, NF-kB, TNF-alpha, and NEMO, supporting the observed changes in gene expression. The binding of verbacoside to Bax likely promotes its pro-apoptotic activity, while its interaction with Bcl-2 inhibits the anti-apoptotic function, thus facilitating apoptosis. Inhibition of NF-kB, TNF-alpha, and NEMO by verbacoside underscores its role in attenuating inflammatory and survival signals, further promoting apoptotic cell death. The combined results from

antioxidant assays, cytotoxicity tests, gene expression analyses, and molecular docking studies present a cohesive picture of how verbacoside exerts its anticancer effects. By scavenging free radicals, inhibiting protein denaturation, and modulating key apoptotic and inflammatory pathways, verbacoside effectively induces apoptosis and reduces cancer cell viability. The dual targeting of oxidative stress and signalling pathways critical for cell survival highlights the multifaceted nature of Verbacoside's anticancer activity.

Conclusion

Our study elucidates the complex molecular mechanisms by which verbascoside exerts its anticancer effects on A549 cells. Verbascoside induces apoptosis through the activation of pro-apoptotic genes and inhibition of anti-apoptotic and inflammatory signalling pathways. These findings, supported by antioxidant and anti-inflammatory assays, as well as molecular docking studies, underscore the potential of verbascoside as a therapeutic agent for non-small cell lung cancer. Further research should focus on validating these in vitro results through in vivo studies and exploring the clinical potential of verbascoside in treating NSCLC.

Conflict of Interest

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

Acknowledgement

The 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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