1. Introduction

Lung cancer is a primary global health concern and a leading cause of cancer-related mortality [1]. Among the various subtypes of lung cancer, non-small cell lung cancer (NSCLC) is a complex and prevalent form of lung cancer. It stands as a significant global health concern characterized by its aggressive nature, limited treatment options, and considerable impact on patient morbidity and mortality, demanding comprehensive research and innovative therapeutic strategies [2]. NSCLC accounts for approximately 85% of all lung cancer cases, making it the most common type of lung cancer [3]. The incidence and prevalence of NSCLC exhibit regional and gender-based variations. Developed countries with a higher prevalence of tobacco smoking tend to have a higher incidence of NSCLC [1]. While lung cancer has historically been more common in men, the gap has been narrowing due to changing smoking patterns among women. In addition to gender and tobacco use, age plays a significant role, with the risk of NSCLC increasing with age [4].

The primary risk factor for NSCLC is tobacco and smoking [5]. Occupational exposures to carcinogens, including asbestos, radon, and various chemicals, can contribute to the development of NSCLC [6]. Additionally, indoor air pollution from sources such as wood-burning stoves and poorly ventilated homes has been linked to an elevated risk of lung cancer in specific populations [7]. Genetic factors also play a role in NSCLC. Genetic mutations play a central role in the initiation and progression of NSCLC [8]. Several key mutations have been identified. Epidermal Growth Factor Receptor (EGFR) mutations are among the most common in NSCLC [9]. These mutations lead to the activation of the EGFR signaling pathway, promoting uncontrolled cell growth. EGFR-targeted therapies

Non-small cell lung cancer (NSCLC) is a global health concern with a significant impact on morbidity and mortality. Small molecule inhibitors targeting genetic mutations like EGFR and ALK have shown promise in NSCLC treatment. This study focuses on Protein Kinase C-alpha (PKCα), implicated in NSCLC pathogenesis. Overexpression of PKCα correlates with advanced disease stages. Preclinical studies suggest its inhibition can suppress NSCLC cell growth. The research employs molecular docking to identify Pulsatillic acid (PA) as a potential PKCα inhibitor. ADMET predictions support PA's candidacy and PASS analysis and Swiss Target Prediction reveal its biological properties. Fluorescence-based binding assays demonstrate PA's inhibitory potency on PKCα, aligning with molecular docking findings. Cytotoxicity assays show PA's minimal impact on HEK-293 cell viability, with an IC50 of 21.03 μM in A549 cells. mRNA expression analysis in A549 cells indicates PA's potential inhibitory effect on PKCα. In conclusion, this study highlights that PA may emerge as a promising therapeutic candidate for NSCLC, emphasizing the need for further research, validation, and exploration of its translational potential. The study contributes valuable insights into NSCLC treatment strategies, emphasizing the significance of targeting PKCα.

Keywords: A549, Cytotoxicity, Inhibitor, PKCα, ADMET, PASS analysis.
have been developed to inhibit this pathway. Anaplastic Lymphoma Kinase (ALK) gene rearrangements are present in a subset of NSCLC cases [10]. ALK fusion proteins lead to abnormal cell growth, and ALK inhibitors have proven effective in treating ALK-positive NSCLC. ROS1 and BRAF mutations are additional molecular alterations in NSCLC [11]. ROS1 fusion proteins and BRAF mutations can be targeted with specific inhibitors to impede cancer cell proliferation.

The treatment of NSCLC is multifaceted and highly individualized, considering factors such as the cancer's stage, the patient's overall health, and specific genetic mutations. Recent developments in cancer therapeutics have led to the identification and development of small molecule inhibitors as a promising approach for cancer treatment. Small molecule inhibitors are designed to target specific molecules or signaling pathways involved in the growth and progression of cancer cells. One of the most significant breakthroughs in NSCLC treatment has been the development of small molecule inhibitors that target specific genetic mutations, such as those in the EGFR and ALK genes [12]. These inhibitors work by blocking the aberrant signaling pathways that drive the growth of cancer cells. The NSCLC stands as a significant global health concern, characterized by its aggressive nature, limited treatment options, and considerable impact on patient morbidity and mortality. As the most prevalent type of lung cancer, NSCLC demands an urgent and focused research effort to identify innovative therapeutic approaches and molecules for the therapeutics of disease.

Protein Kinase C-alpha (PKCα) is a serine/threonine kinase that plays a crucial role in cellular signal transduction pathways, regulating fundamental cellular processes such as proliferation, differentiation, and survival [13]. Emerging evidence suggests that dysregulation of PKCα is implicated in the pathogenesis of several cancers, including NSCLC (14). Several studies have reported overexpression and hyperactivation of PKCα in NSCLC tissues, correlating with advanced disease stages and poor clinical outcomes [15]. This observation underscores the significance of PKCα in NSCLC progression and raises the possibility of targeting this kinase for therapeutic intervention. Furthermore, preclinical studies have demonstrated that inhibition of PKCα can suppress NSCLC cell growth, induce apoptosis, and inhibit angiogenesis, suggesting its potential as a promising therapeutic target [16,17].

Identifying and developing small molecule inhibitors for NSCLC offer a promising avenue for improving the treatment landscape of this challenging disease. This study underscores the critical importance of identifying small-molecule inhibitors for NSCLC treatment. This research rationale advocates for a multidisciplinary approach, leveraging the power of molecular docking and in vitro models to unravel the therapeutic potential of PKCα in NSCLC. By elucidating the molecular interactions and dynamics at the atomic level, this study seeks to identify novel compounds that can inhibit PKCα activity for the therapeutics of NSCLC.

2. Material and methods

2.1 Target preparation and molecular docking

The crystallographic configuration of Protein Kinase Cα (PKCα) was obtained from the Protein Data Bank, identified by the PDB accession code 3IW4, as reported by Wagner et al. in 2009 [16]. The resolution of this structure was determined to be 2.80 Å, and it was observed to be in association with NVP-AEB071. Subsequently, the NPACT database, as detailed by Mangal et al. in 2013 [17], comprising 1574 entries, was systematically examined. Pulsatillic acid (PA) emerged as the chosen lead molecule through this screening process. The two-dimensional structure of the selected ligand, PA (Identifier: NPACT00887), was procured from the NPACT database, accessible at [https://webs.iiitd.edu.in/raghava/npact/cmpnd4.php?a=NPACT00887](https://webs.iiitd.edu.in/raghava/npact/cmpnd4.php?a=NPACT00887). The structural depiction of PA is illustrated in Figure 1.

A standard receptor preparation protocol, as outlined [18,19,20,21], was systematically implemented to ascertain the target protein's suitability for docking studies. Docking calculations were executed using Docking Server, following the proposed methodology [22]. Gasteiger partial charges were assigned to the ligand atoms, non-polar hydrogen atoms were consolidated, and rotatable bonds were precisely defined. The docking calculations were conducted on the ligand-protein model. As described, essential hydrogen atoms, Kollman united atom type charges, and solvation parameters were incorporated using AutoDock tools [23]. Affinity (grid) maps, with a spacing of 0.375 Å, were generated through the Auto grid program [24]. The AutoDock parameter set and distance-dependent dielectric functions were employed to compute the van der Waals and electrostatic terms, respectively. Docking simulations used the Lamarckian genetic algorithm (LGA) and the Solis & Wets local search method [25]. The ligand molecules' initial position, orientation, and torsions were set randomly, with all rotatable torsions being released during the docking process. Each docking experiment consisted of two different runs, each terminating after a maximum of 250,000 energy evaluations. The population size was fixed at 150, and during the search, a translational step of 0.2 Å, along with quaternion and torsion steps of 5, was applied.

2.2 ADME & Toxicity properties and Prediction of Activity Spectra for PA using PASS online and Swiss Target Prediction tool

The evaluation of the Absorption, Distribution, Metabolism, Excretion, and Toxicity (ADME and toxicity) attributes of PA was undertaken using the pkCSM online tool. The Simplified Molecular Input Line Entry System (SMILES) identifier associated with PA was obtained from the NPACT database ([https://webs.iiitd.edu.in/raghava/npact/cmpnd4.php?a=NPACT00887](https://webs.iiitd.edu.in/raghava/npact/cmpnd4.php?a=NPACT00887)) and subsequently utilized in the pkCSM server to elucidate its ADME and

![Fig. 1. Structure of Pulsatillic acid.](image-url)
Toxicity properties.

Next, the Prediction of Activity Spectra for Substances (PASS) is a computational tool for predicting the biological activities associated with chemical compounds. To conduct the PASS analysis, the chemical structure of PA, represented in the standardized “SMILES” format, was submitted to the online server available at http://www.pharmaexpert.ru/passonline/. This submission was performed to determine the potential biological activities associated with PA through the PASS methodology.

Moreover, the SwissTargetPrediction online tool, available at http://www.swisstargetprediction.ch/, was employed to investigate potential associations of PA with various biological properties. This web-based server facilitates the estimation of a bioactive small molecule’s most probable macromolecular targets. The prediction methodology is grounded in 2D and 3D similarity assessments with a comprehensive library comprising 370,000 known actives associated with over 3,000 proteins from three distinct species.

2.3 In vitro inhibition assay

In its active form, the recombinant human Protein Kinase Cα (PKCα) protein was procured from Abcam (Product ID: AB55672). This protein, characterized as a Baculovirus-infected Sf9 Full-Length protein spanning the range of 1 to 672 amino acids, boasts a purity exceeding 95%. Fluorescence binding studies investigating the interaction between PKCα and PA were conducted using a JASCO 6300 spectrofluorometer equipped with a 1 cm quartz cell. The generation of fluorescence spectra for both PKCα and the protein-ligand complex involved the following sequential steps: (i) Initiation of protein excitation at a wavelength of 280 nm; (ii) Recording emission spectra from the protein across the range of 300-500 nm. Parameters for excitation and emission slit widths were set at 10 nm, and the response setting was configured to medium sensitivity. Following data acquisition, the fluorescence data from both the protein and the protein-ligand complex underwent comprehensive analysis. This analytical process was conducted with the primary objective of extracting various binding parameters relevant to the protein-ligand complex, thereby elucidating the inhibitory effects of PA on PKCα.

2.3 Preparation of test compound and cell line

PA was dissolved in dimethyl sulfoxide (DMSO) obtained from Sigma, USA, to formulate a stock solution with a 100 µg/mL concentration. The HEK-293 and A549 cell lines were acquired from the National Centre for Cell Science (NCCS), Pune. They were cultured in Dulbecco’s Modified Eagle Medium (DMEM) and Ham’s F12k media supplemented with 10% fetal bovine serum (FBS) and 1% antibiotics.

2.4 Cell culture and cytotoxicity assay

HEK-293 cells were cultivated in a 96-well microplate at a seeding density of 5,000 cells per well, containing 100 µL of DMEM. The microplate was placed within a CO2 incubator at 37°C, maintaining controlled humidity at 5% for 24 hours. Following this initial incubation period, the cells underwent treatment involving various concentrations of PA for an additional 24 hours.

The subsequent step involved replacing the culture medium in each well with 100 µL of fresh medium containing the MTT reagent, added to achieve a final concentration of 0.5 mg/mL. The cells were then subjected to an additional incubation period lasting 4 hours at 37°C. Following this 4-hour incubation, the MTT reagent medium was carefully aspirated from the wells, and 100 µL of DMSO was introduced to each well. DMSO was essential to facilitate the dissolution of the purple formazan crystals generated by the MTT reagent. To ensure complete dissolution, the microplate underwent gentle agitation for 10 minutes.

For quantification, the absorbance of each well was measured at 570 nm using a dedicated plate reader. The calculation of net absorbance involved subtracting the absorbance values of blank wells, which contained only culture medium and MTT reagent, from the absorbance values of the test wells. Finally, to determine the percentage of cell viability, the net absorbance of the test wells was divided by the net absorbance of the control wells (comprising cells without any treatment). The resulting quotient was then multiplied by 100. The IC50 of PA was also calculated as part of this analysis.

2.5 Relative expression of PKCα in A549 cells

The cancer cells, reaching a confluence of 70 to 75%, were treated with the IC50 dose of PA over 24 hours. Subsequently, the cells were harvested and underwent 2X washing with phosphate-buffered saline. RNA extraction was performed using TRIzol™ Reagent (Ambion, Carlsbad, CA, USA), and cDNA synthesis was accomplished using the Verso cDNA Synthesis Kit (Thermo Fisher Scientific, USA). The expression levels of PKCα were evaluated through RT-PCR utilizing SYBR™ Green Master Mix (Thermo Fisher Scientific, USA) and an RT-PCR System from Applied Biosystems. To normalize the target gene, GAPDH was employed as the endogenous control. The relative fold change in the expression of PKCα was determined using the comparative threshold cycle (CT) method, commonly known as the 2^−ΔΔCT method.

The primer sequences utilized for PKCα were as follows: Forward: 5'-ATGGCTGACGTTTTTCCCGG-3' and Reverse: 5'-AGGTGGGCTGCTTGAAGA-3' (26). For GAPDH, the primer sequences were as follows: Forward: 5'-TGACTTCAACAGCGACACCCA-3' and Reverse: 5'-CACCC TGTTGCTGTAGCCAAA-3' (27).

2.6 Statistical analysis

Data was analysed in SPSS (ver. 22). The data for cytotoxicity assay and mRNA expression was pooled from three experiments. The data is plotted as mean ± SD. The t-test was employed to compare the mean values between the two groups. The p<0.05 was considered statistically significant.

3. Results

3.1 Molecular docking analysis of PKCα:PA complex

The molecular docking study identified PA as a potential Protein Kinase Cα (PKCα) inhibitor—table 1 and Figures 2a and 2b present the PKCα:PA complex molecular docking analysis results. The affinity scores and docked poses revealed a significant binding affinity of PA with PKCα, as evidenced by an affinity score of -6.98 kcal/mol (Table 1). PA’s preferential hydrogen bond interaction with the ARG412 amino acid residue of PKCα is highlighted, along with various polar, hydrophobic, and other interac-
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3.2 ADMET predictions and PASS Analysis

Table 3 comprehensively presents the outcomes of ADMET predictions, affirming that PA meets essential pharmacokinetic criteria. This positions PA as a promising candidate for validation and subsequent therapeutic development targeting the enzymatic activity of PKCα in Non-Small Cell Lung Cancer (NSCLC). Additionally, Table 4 outlines the favorable biological properties predicted by PASS analysis, emphasizing PA's association with significant biological processes. The Swiss Target Prediction analysis further corroborates and elucidates the manifold biological properties associated with PA, as depicted in Figure 4.

3.3 Fluorescence-Based Binding Assay

The fluorescence emission spectra of PKCα, recorded at varying PA concentrations (0 to 17 µM), are depicted in Figure 5. The observed decline in fluorescence with increasing PA concentration supports the conclusion that PA functions as an inhibitor of PKCα, hindering its enzymatic activity. This finding underscores the inhibitory potency of PA on PKCα.

3.4 Cytotoxicity Assay

Figure 6 presents the results of the cell toxicity assay, revealing that increasing concentrations of PA did not significantly impact the viability of HEK-293 cells. Furthermore, the IC₅₀ for PA in A549 cells was calculated to be 21.03 µM.

3.5 mRNA Expression of PKCα in A549 Cells

The relative expression of PKCα in A549 cancer cells is illustrated in Figure 7. The results demonstrate a statistically significant reduction in the expression of PKCα in PA-treated A549 cells compared to untreated cells. This

Fig. 4. Swiss Target Prediction analysis reflecting manifold biological properties linked with PA.

Fig. 5. Fluorescence emission spectra of PKCα, acquired at different PA concentrations spanning from 0 to 17 µM.

Fig. 6. Results of cell toxicity assay (MTT assay results). Effect of increasing concentrations of PA on the viability of HEK-293 cells.
Table 1. Predicted binding affinity and estimated inhibition constant of PKCα:PA complex.

<table>
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<tr>
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<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Pulsatillic acid</td>
<td>Protein kinase Ca</td>
<td>-6.98 kcal/mol</td>
<td>225.30 uM</td>
<td>-4.21 kcal/mol</td>
<td>-1.31 kcal/mol</td>
<td>-5.52 kcal/mol</td>
<td>50%</td>
<td>677.762</td>
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Table 2. Decomposed Interaction Energies in kcal/mol.

<table>
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<th>Hydrogen bonds</th>
<th>Polar</th>
<th>Hydrophobic</th>
<th>Other</th>
</tr>
</thead>
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<tr>
<td>ARG412 (0)</td>
<td>LYS371 (0)</td>
<td>PRO635 (0)</td>
<td>LYS347 (0)</td>
</tr>
<tr>
<td>PRO639 (0)</td>
<td>LYS352 (0)</td>
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</tr>
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</table>

Table 3. In silico prediction of ADME & Toxicity properties for Pulsatillic acid (PA). These properties represent pharmacokinetics and safety profile of a potential drug candidate. The results reflect that PA qualifies the ADME & Toxicity properties test.

<table>
<thead>
<tr>
<th>Properties</th>
<th>Absorption</th>
<th>Distribution</th>
<th>Metabolism</th>
<th>Excretion</th>
<th>Toxicity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Models</td>
<td>Intestinal absorption (human)</td>
<td>VDss (human)</td>
<td>BBB permeability</td>
<td>CNS permeability</td>
<td>CYP Substrate</td>
</tr>
<tr>
<td>Unity</td>
<td>Numeric (% absorbed)</td>
<td>Numeric (log L/kg)</td>
<td>Numeric (log BB)</td>
<td>Numeric (log PS)</td>
<td>Categorical (yes/no)</td>
</tr>
<tr>
<td>Predicted values</td>
<td>PA</td>
<td>-1.5</td>
<td>0.0</td>
<td>-1.6</td>
<td>NO</td>
</tr>
</tbody>
</table>
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highlights the potential of PA in modulating the mRNA expression of PKCα in A549 cells.

4. Discussion

Non-Small Cell Lung Cancer (NSCLC) constitutes the predominant form of lung cancer, accounting for approximately 85% of all cases [3]. It is a complex and heterogeneous disease, often diagnosed at advanced stages, posing significant challenges to effective treatment. Recent years have witnessed a transformative shift in NSCLC management, with targeted therapies addressing specific genetic mutations and immunotherapies harnessing the body’s immune system to combat cancer cells [28]. Precision medicine approaches, tailoring treatment to individual genetic profiles, hold promise in enhancing therapeutic efficacy [29]. Ongoing research explores novel biomarkers, liquid biopsies, and emerging technologies to refine early detection and treatment strategies, aiming to improve outcomes and quality of life for those affected by NSCLC [30,31,32]. Despite challenges, these efforts signify a dynamic and evolving landscape in the quest for more effective interventions against this pervasive and life-threatening disease.

Research indicates that PKCα dysregulation is linked to various cancers, including NSCLC [33-40]. Increased expression and hyperactivation of PKCα are notable in NSCLC tissues, especially in advanced disease stages, aligning with unfavorable clinical outcomes [41]. This emphasizes the significance of PKCα in NSCLC progression and underscores its potential as a therapeutic target. Preliminary studies have demonstrated that inhibiting PKCα can hinder NSCLC cell growth, trigger apoptosis, and inhibit angiogenesis, indicating its potential for therapeutic intervention.

The investigation into PA as a potential inhibitor of PKCα in NSCLC revealed intriguing insights into the molecular interactions, pharmacokinetic properties, biological activities, and potential therapeutic effects of this natural compound. The molecular docking study serves as a pivotal starting point in understanding the potential inhibitory role of PA on PKCα [18,19]. The significant binding affinity, as denoted by the affinity score of -6.98 kcal/mol underscores the strength of the PA-PKCα interaction. The preferential hydrogen bond interaction with the ARG412 amino acid residue, along with other polar and hydrophobic interactions signifies a specific and multifaceted binding mode. However, the validity of these findings largely depends on the accuracy of the docking algorithm and the chosen parameters. A comprehensive validation with experimental data, such as X-ray crystallography or NMR studies, would fortify the reliability of the predicted binding modes. The ADMET predictions play a crucial role in evaluating the drug-likeness and pharmacokinetic characteristics of PA. The affirmative outcomes position PA as a promising candidate for further development. Nevertheless, the predictability of ADMET models may be

![Fig. 7. Relative expression of PKCα in vehicle control and treated cells.](image)

Table 4. PASS (Prediction of Activity Spectra for Substances) analysis of Pulsatillic acid. Probability “to be active” was set at Pa>0.7.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Structure</th>
<th>Pa</th>
<th>Pi</th>
<th>Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pulsatillic acid</td>
<td><img src="image" alt="Chemical Structure" /></td>
<td>0.929</td>
<td>0.005</td>
<td>Antineoplastic</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.813</td>
<td>0.003</td>
<td>Antineoplastic (melanoma)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.767</td>
<td>0.005</td>
<td>Antineoplastic (lung cancer)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.751</td>
<td>0.005</td>
<td>Antineoplastic (colorectal cancer)</td>
</tr>
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<td></td>
<td></td>
<td>0.749</td>
<td>0.005</td>
<td>Antineoplastic (colon cancer)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.729</td>
<td>0.005</td>
<td>Chemopreventive</td>
</tr>
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<td></td>
<td></td>
<td>0.725</td>
<td>0.005</td>
<td>Antileukemic</td>
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<td></td>
<td></td>
<td>0.683</td>
<td>0.006</td>
<td>Antineoplastic (breast cancer)</td>
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<tr>
<td></td>
<td></td>
<td>0.634</td>
<td>0.004</td>
<td>Myc inhibitor</td>
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<tr>
<td></td>
<td></td>
<td>0.630</td>
<td>0.004</td>
<td>Antineoplastic (cervical cancer)</td>
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<td>0.619</td>
<td>0.005</td>
<td>Antineoplastic (ovarian cancer)</td>
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<td></td>
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<td>0.597</td>
<td>0.001</td>
<td>Antineoplastic (thyroid cancer)</td>
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<td></td>
<td></td>
<td>0.565</td>
<td>0.002</td>
<td>Antineoplastic (endocrine cancer)</td>
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<tr>
<td></td>
<td></td>
<td>0.528</td>
<td>0.014</td>
<td>Antimetastatic</td>
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<td></td>
<td></td>
<td>0.458</td>
<td>0.005</td>
<td>Antineoplastic (carcinoma)</td>
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<td></td>
<td></td>
<td>0.426</td>
<td>0.018</td>
<td>Prostate cancer treatment</td>
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<td></td>
<td></td>
<td>0.349</td>
<td>0.042</td>
<td>Anticarcinogenic</td>
</tr>
</tbody>
</table>

Pa - Probability "to be active"; Pi - Probability "to be inactive"
Potential inhibitor of protein kinase C-alpha in NSCLC.

In conclusion, the study on PA as a potential PKCa inhibitor in NSCLC yields insights into its molecular interactions, pharmacokinetics, and biological activities. Molecular docking highlights a strong PA-PKCa binding affinity, emphasizing specificity. While computational findings are promising, experimental validation is crucial. ADMET predictions support PA's drug-likeness, positioning it for further experimental validation. PASS analysis and Swiss Target Prediction hint at biological properties, necessitating experimental validation for a comprehensive understanding. Experimental evidence, including fluorescence decline and cell toxicity results, supports PA's inhibitory effect on PKCa, but further assays and safety evaluations are crucial. Reduction in PKCa mRNA aligns with the proposed inhibitory role, but protein expression analysis and functional assays are needed for validation. PA holds promise as a therapeutic agent for NSCLC, but its translational potential requires rigorous validation, detailed pharmacokinetic studies, and exploration of in vivo efficacy, safety, and off-target effects. In summary, while PA is promising, further research and validation are essential for successful development in NSCLC therapy.

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Conflict of interest
The authors declare no conflict of interest.

Consent for publications
The author approved the final manuscript for publication.

Ethics approval and consent to participate
The present research is not used human or animals.

Informed consent
The authors declare not used any patients in this research.

Author's contribution
BEG: Helps to write the final draft and design the experiment. TAE: Helped to draft manuscript primary, MAE participated in the manuscript, AAP read the manuscript and made suitable editing, RAE support in assay, MA and AKM help in revision and ASA and MA finalised final draft after revision.

Abbreviations
NSCLC (Non-Small Cell Lung Cancer), PKCa (Protein Kinase C-alpha), CT (Threshold cycle), DMSO (Dimethyl Sulfoxide), PASS (Prediction of Activity Spectra for Substances) and LGA (Lamarckian genetic algorithm).

References
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