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Original Article

Unraveling the Therapeutic Potential of Apigenin: Targeting CDK6 in Cancer Treatment

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ABSTRACT

Background: The pursuit of effective cancer therapies hinges upon the identification and characterization of molecular targets amenable to precise modulation. Herein, we present a comprehensive investigation into the potential of Apigenin as a therapeutic agent targeting Cyclin-Dependent Kinase 6 (CDK6), a critical regulator of cell cycle progression implicated in tumorigenesis. **Materials and Methods:** Initially, we employed protein structure homology modelling to elucidate the high-resolution structure of CDK6, revealing a model of exceptional quality with a GMQE score of 0.87 and a QMEANDisCo Global score of 0.96±0.05. Subsequent cavity detection analysis unveiled five prominent cavities within CDK6, with Cavity 1 (C1) exhibiting remarkable size and volume, thus warranting further exploration through molecular docking studies. Molecular docking simulations identified. **Results:** Apigenin as the most promising inhibitor of CDK6, demonstrating a strong binding affinity with the target protein. This interaction was validated through redocking, which consistently reaffirmed the robust binding between Apigenin and CDK6. Furthermore, ADMET predictions and SwissTargetPrediction confirmed Apigenin's drug-like properties and its potential to target CDK6 inhibition. Functional assays revealed Apigenin's concentration-dependent cytotoxic effects on HCT-15 (colorectal cancer), HeLa-229 (cervical adenocarcinoma), and PC-3 (prostatic adenocarcinoma) cancer cell lines, accompanied by a significant reduction in CDK6 mRNA expression ($p<0.001$) and kinase activity ($p<0.01$). **Conclusion:** Collectively, these findings underscore Apigenin's potential as a therapeutic candidate for targeting CDK6 in cancer treatment, warranting for further preclinical and clinical investigations.

Keywords: Apigenin, Cancer, Cyclin-Dependent Kinase 6 (CDK6), HCT-15 cancer cells, HeLa-229 cancer cells, PC-3 cancer cells, targeted therapy.

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INTRODUCTION

Cancer treatment confronts numerous challenges, including therapeutic resistance, off-target toxicity, and tumor heterogeneity.[1,2] New approaches, such as immunotherapy, targeted therapy, and precision medicine, offer innovative strategies to overcome these hurdles.[3,4] therapy or targeting specific proteins in cancer therapeutics plays a crucial role in disrupting key pathways essential for tumor growth and survival.[5,6] By selectively inhibiting oncogenic proteins involved in cell proliferation, angiogenesis, and metastasis, targeted therapies can induce tumor regression and improve patient outcomes.[7-9] This approach minimizes systemic toxicity compared to traditional chemotherapy, as it spares normal cells. Moreover, targeting specific proteins allows for personalized treatment based on individual tumor characteristics, optimizing therapeutic efficacy.[2,10,11] As our understanding of cancer biology deepens, targeting specific proteins is becoming an increasingly pivotal strategy, unlocking innovative approaches for precision medicine in cancer treatment.

The cell cycle, a tightly regulated process, ensures the accurate duplication and distribution of genetic material to daughter cells. CDK6, along with its counterpart CDK6, belongs to the family of Cyclin-Dependent Kinases (CDKs), which are key regulators of cell cycle progression.[12,13] CDK6 operates at the G1 phase of the cell cycle, a critical checkpoint where cells decide whether to initiate DNA replication and commit to cell division or enter a state of quiescence.[14-16] CDK6's activity is tightly controlled by its association with cyclin D proteins, specifically cyclin D1, D2, and D3, which act as regulatory subunits.[12] Upon receiving extracellular signals, such as growth factors, cyclin D levels rise, leading to the formation of active CDK6-cyclin D complexes.[17] These complexes phosphorylate and inactivate the Retinoblastoma protein (pRb), a tumor suppressor that inhibits cell cycle progression by sequestering E2F transcription factors.[18] Phosphorylation of pRb liberates E2F, allowing it to activate

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the transcription of genes essential for DNA replication and cell cycle progression.[15] In addition to its role in promoting cell cycle entry, CDK6 also participates in the regulation of other cellular processes, including differentiation, apoptosis, and metabolism.[19] Emerging evidence suggests that CDK6 may exert its effects beyond the cell cycle by phosphorylating non-canonical substrates involved in these diverse biological processes.[20-22]

Despite its indispensable role in normal cellular physiology, dysregulation of CDK6 has been implicated in various human cancers.[23-25] Overexpression or hyperactivation of CDK6 can drive uncontrolled cell proliferation, a hallmark of cancer.[26] Moreover, alterations in genes encoding CDK6 regulators, such as cyclin D1 amplification or loss of the CDK inhibitor p16INK4a, are frequently observed in cancer cells and contribute to CDK6 hyperactivity.[27,28] Numerous studies have demonstrated the oncogenic potential of CDK6 in various cancer types. For instance, in hematological malignancies such as leukemia and lymphoma, CDK6 overexpression promotes aberrant cell cycle progression and enhances the survival and proliferation of malignant cells.[22,29,30] Similarly, in solid tumors like breast, lung, and pancreatic cancer, CDK6 hyperactivity is associated with aggressive tumor growth and poor patient prognosis.[31,32]

The pivotal role of CDK6 in cancer pathogenesis has spurred significant interest in targeting this kinase for therapeutic intervention. Several strategies have been developed to inhibit CDK6 activity, either alone or in combination with other targeted therapies or conventional cytotoxic agents. The role of identification and development of small molecule inhibitors in cancer therapeutics represents a pivotal aspect of modern oncology.[33-35] Targeted therapies, such as small molecule inhibitors, have emerged as potent tools in the fight against cancer by specifically targeting key molecules and pathways involved in tumor progression. One approach involves the use of small molecule inhibitors that selectively block the ATP-binding site of CDK6, thereby preventing its kinase activity.[36] Palbociclib, ribociclib, and abemaciclib are FDA-approved CDK6/6 inhibitors that have shown promising clinical efficacy in the treatment of hormone receptor-positive breast cancer, both as monotherapy and in combination with endocrine therapy.[37,38] These agents have also demonstrated activity in other cancer types, including mantle cell lymphoma and certain subtypes of lung cancer.[39]

The rationale for further research in this area is driven by the proven efficacy of small molecule inhibitors that target specific molecules and pathways in cancer therapeutics. As highlighted in the text, these inhibitors are integral to modern oncology, as they selectively block key molecules involved in tumor progression, offering a promising approach for effective cancer treatment. The focus on small molecule inhibitors that target CDK6 activity provides a compelling basis for investigation. This study aims to identify potential CDK6 inhibitors using both *in silico* and *in vitro* methods, with the goal of advancing cancer therapeutics.

MATERIALS AND METHODS

Protein structure homology modelling and preparation of CDK6 receptor

Homology modelling is a widely used technique to predict the three-dimensional structure of a protein based on its homologous proteins with known structures. SWISS-MODEL is a popular web-based tool for protein structure homology modelling (https://swissmodel.expasy.org/).[40-44] The 3D structure of the target protein, CDK6, was modelled using Swiss-Model. Briefly, the FASTA sequences of CDK6 was obtained from PubMed. The template sequences of homologous protein with known three-dimensional structures was searched using Protein Data Bank (PDB). Thereafter, we selected one suitable template structure for target protein (CDK6: PDB ID - 1BLX) based on sequence similarity, structure quality, and biological relevance. This template structure was used to model the structure of CDK6 using SWISS-MODEL.[40-42,45] High resolution structure of CDK6 was retrieved from the Swiss-MODEL server (Figure 1a) and visualized using PyMOL and Chimera to analyse the structural features and properties. Preparing the receptors for molecular docking is a crucial step, standard receptor preparation protocol was followed to refine the structure of CDK6.[34,35] The structure of CDK6 was inspected for steric clashes, missing atoms, or unusual bond angles using PyMOL. Partial charges and atom types were assigned to the protein atoms using the AMBER force field parameters, which are suitable for molecular docking studies. The structures were then saved in PDBQT format, a format compatible with molecular docking.

Selection and preparation of ligands for molecular docking with CDK6

To assemble a diverse set of ligand molecules for virtual screening, a small library was curated from the DrugBank database, accessible at https://go.drugbank.com/. This library comprised 23 entries encompassing US-approved, experimental, and investigational nutraceutical small molecules, selected based on their relevance to the study's objectives. Subsequently, the three-Dimensional (3D) structures of these small molecules were obtained in Structure Data Format (SDF) files from the PubChem database, available at https://pubchem.ncbi.nlm.nih.gov/. Each SDF file underwent a rigorous validation process to ensure structural integrity. Bond lengths, bond angles, and torsional angles were meticulously scrutinized and corrected as necessary to rectify any discrepancies or irregularities. Following the validation and correction process, the structures of the ligand molecules were converted into the PDBQT format. This conversion facilitated compatibility with molecular docking software and ensured accurate representation of the ligand structures during subsequent docking simulations.

Structure and target based cavity detection on CDK6

To identify potential binding sites on CDK6, cavity detection was carried out using CB-Dock2, accessible at http://cadd.labshare.cn/cb-dock2/. CB-Dock2 is a sophisticated protein-ligand docking tool that automates the process of identifying binding sites on proteins. It calculates the center and size of these binding sites and customizes the docking box size accordingly based on the query ligands provided.[46,47] The docking process is executed using the AutoDock Vina software, a widely used molecular docking program. To initiate the cavity detection process, the CDK6 file in Protein Data Bank (PDB) format was uploaded to the CB-Dock2 server. During the setup, various parameters were specified, including the probe radius and cavity size, to optimize the cavity detection process and enhance the accuracy of the results. These parameters allow for the precise identification of potential binding pockets on the CDK6 protein structure.

Screening and molecular docking

A comprehensive screening of 23 compounds was conducted to evaluate their binding affinity against CDK6, a key target in cancer therapeutics. Table 1 presents detailed information on the fitness, binding affinity, and best docked pose with CDK6 for each of the 23 small molecules screened in this study. Based on their fitness scores and binding affinities, "Apigenin" emerged as the top hit and was selected for further molecular docking studies with CDK6.

The molecular docking of Apigenin with CDK6 was performed using CB-Dock2, a sophisticated server renowned for its capabilities in protein-ligand blind docking.[46] CB-Dock2 integrates various functionalities, including cavity detection, docking, and homologous template fitting, enabling accurate prediction of binding sites and affinity between proteins and ligands. This approach allows researchers to gain insights into the potential interactions between Apigenin and CDK6 at the molecular level.

Furthermore, to validate the binding affinity of the protein-ligand complex, the Apigenin-CDK6 complex was subjected to redocking using SeamDock.[48] SeamDock is a reliable tool for revaluating the binding affinity of protein-ligand complexes, providing an additional layer of validation to the docking results obtained from CB-Dock2.

Assessment of ADMET properties of Apigenin

The ADMET (Absorption, Distribution, Metabolism, Excretion, and Toxicity) properties of candidate molecules is paramount to understanding their pharmacokinetic and toxicity profiles. To assess these properties for Apigenin, the current study employed the pkCSM online tool, which is accessible at https://biosig.unimelb.edu.au/pkcsm/. The pkCSM tool is specifically designed to predict pharmacokinetic and toxicity properties of small molecules. It offers a comprehensive analysis of various ADMET parameters, providing insights into how a molecule may be absorbed, distributed, metabolized, excreted, and its potential toxicity profile. To perform the assessment, the chemical structure of Apigenin, represented in SMILES format, was uploaded to the pkCSM online tool. This allowed for the prediction of ADMET properties associated with Apigenin based on its structural features and known structure-activity relationships.

Activity Prediction of Apigenin Using SwissTargetPrediction

In the field of chemoinformatics and drug discovery, predicting the activity spectra for ligands plays a crucial role in identifying potential biological targets and understanding the pharmacological effects of molecules. To explore the biological activity spectrum associated with Apigenin, the current study utilized the SwissTargetPrediction tool, accessible at http://www.swisstargetprediction.ch/. SwissTargetPrediction is an online platform specifically developed to predict potential targets for small molecules or drugs. Its primary objective is to assist researchers in identifying proteins that may interact with a given compound, thus providing valuable insights into the compound's pharmacological profile. The SwissTargetPrediction tool operates by analysing the chemical structure of a compound, typically represented in SMILES notation, and predicting potential protein targets based on structural similarities and known ligand-protein interaction patterns. By inputting the SMILES notation for Apigenin into the SwissTargetPrediction tool, the current study aimed to elucidate the potential biological targets and activity spectrum associated with this compound. This approach enables researchers to gain a deeper understanding of the molecular mechanisms underlying the biological effects of Apigenin, thereby facilitating drug discovery efforts and informing further experimental studies aimed at exploring its therapeutic potential.

Test compound and HEK-293, HCT-15, HeLa-229, and PC-3 cell lines

Apigenin, with a purity of ≥95.0% as determined by high-performance liquid chromatography, was procured from Sigma (CAS No: 520-36-5). To prepare the stock solution, Apigenin was dissolved in Phosphate-Buffered Saline (PBS) to achieve a concentration of 300 µg/mL.

The cell lines utilized in this study, namely HEK-293 (human embryonic kidney), HCT-15 (colorectal cancer), HeLa-229 (cervical adenocarcinoma), and PC-3 (prostatic adenocarcinoma), were obtained from the National Center for Cell Sciences (NCCS)< Pune, India. HEK-293 cells were cultured and maintained in Dulbecco's Modified Eagle Medium (DMEM), while HCT-15 cells were cultured in RPMI medium. HeLa-229 cells were cultured in DMEM, and PC-3 cells were cultured in F-12K medium. All culture media were supplemented with 10% Fetal Bovine Serum (FBS) and 1% antibiotics to support cell growth and maintain sterility. These optimized culture conditions

ensured the robust growth and viability of the respective cell lines throughout the experimental procedures.

Cell culture and cytotoxicity assay

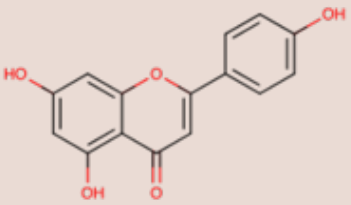
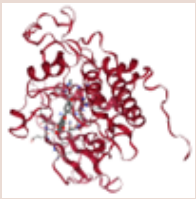
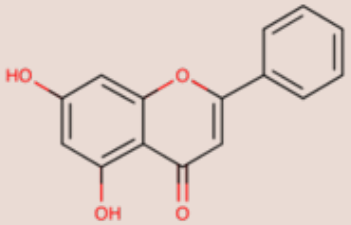
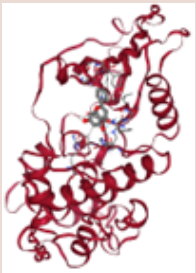
HEK-293 cells were seeded into each well of a 96-well microplate at a density of 5,000 cells per well, with each well containing 100 µL of DMEM medium. Following a 24-hr incubation period at 37 ºC with 5% humidity to allow for cell attachment and growth, the cells were subjected to treatment with various concentrations of Apigenin ranging from 0 to 300 µM. This treatment was administered for a duration of 72 hr to investigate the dose-dependent effects of Apigenin on cell viability. After the 72-hr treatment period, the culture medium was aspirated, and each well was replenished with 100 µL of fresh medium containing 0.5 mg/mL of MTT reagent. The microplate was then incubated for an additional 4 hr to allow for the conversion of MTT to formazan crystals by metabolically active cells. Following this incubation period, the MTT-containing medium was carefully aspirated, and 100 µL of Dimethyl Sulfoxide (DMSO) was added to each well to dissolve the formazan crystals through gentle agitation for 10 min. The absorbance of the formazan solution was measured at 570 nm using a plate reader. To obtain the net absorbance value, readings from blank wells (containing only medium and MTT reagent) were subtracted from the absorbance readings of the test wells. Cell viability was then calculated by dividing the net absorbance of the test wells by that of the control wells (untreated cells) and multiplying the result by 100.

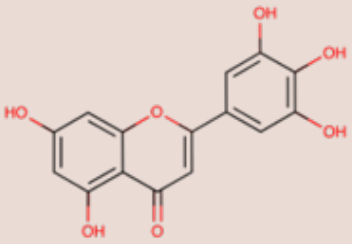
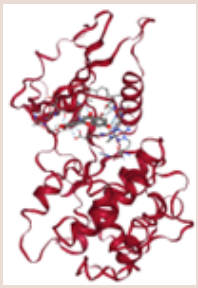
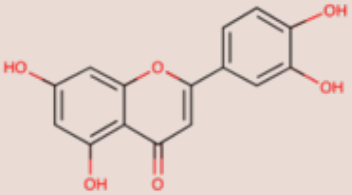
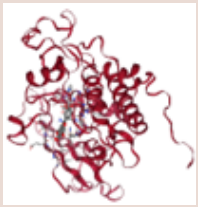
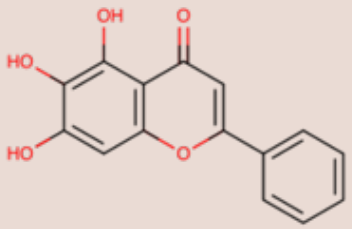
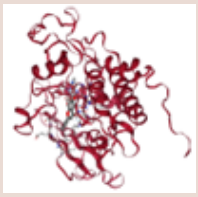
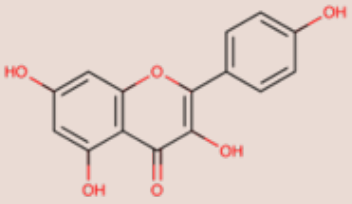
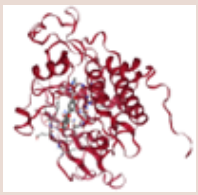
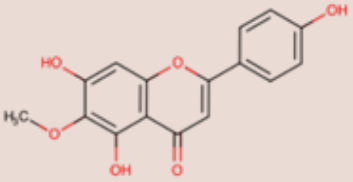
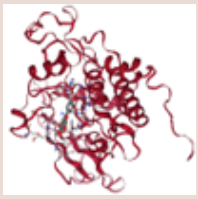
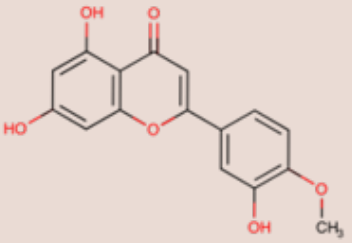
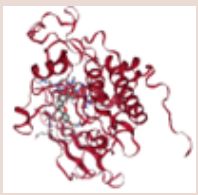
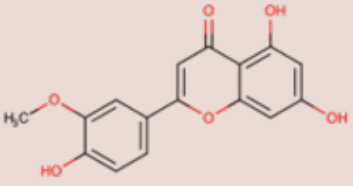
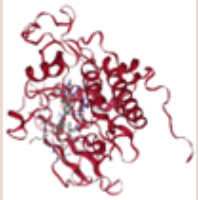
Furthermore, to assess the time-dependent effects of the mean IC_{50} concentrations of Apigenin on HEK-293 cells, a separate time-dependent cell viability assay was conducted. This assay involved treating HEK-293 cells with the mean IC_{50} concentrations of Apigenin for durations of 24, 48, and 72 hr, followed by the MTT assay procedure described above. This approach allowed for the evaluation of both the concentration-dependent and time-dependent effects of Apigenin on the viability of HEK-293 cells, providing comprehensive insights into its potential cytotoxicity over different exposure durations.

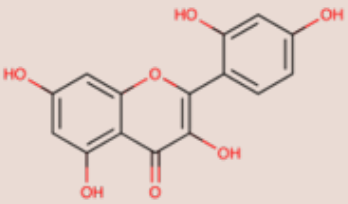
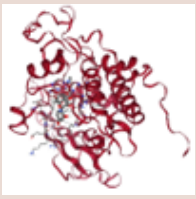
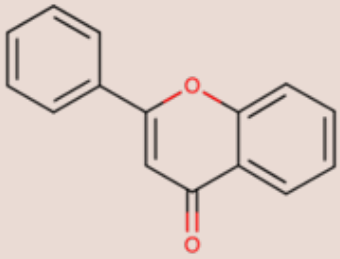
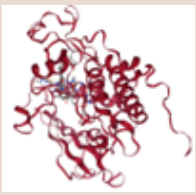
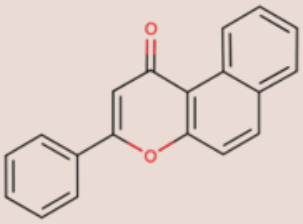
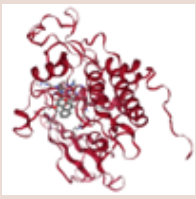
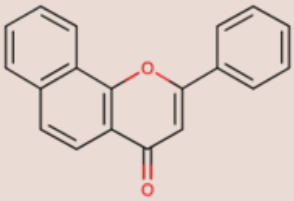
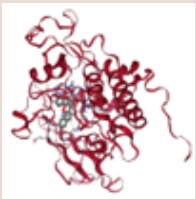
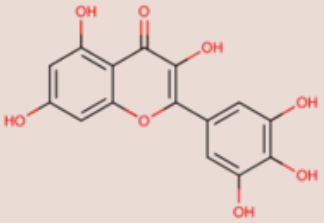
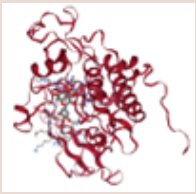
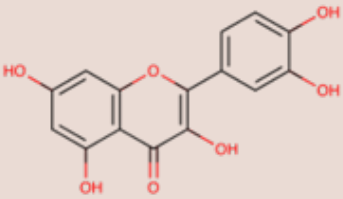
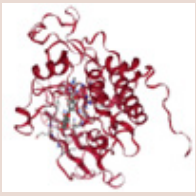
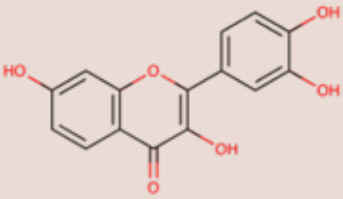
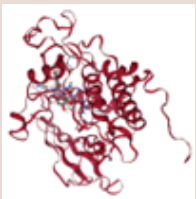
mRNA expression of CDK6 in Apigenin treated HCT-15, HeLa-229, and PC-3 cell lines

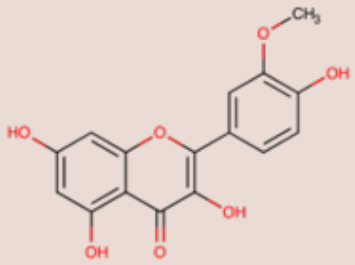
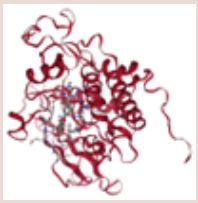
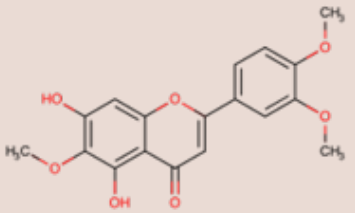
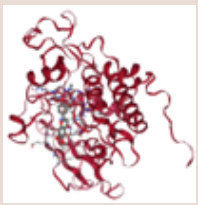
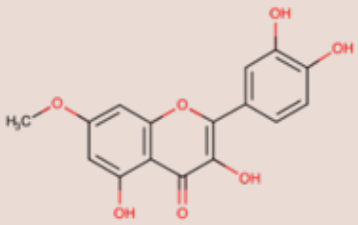
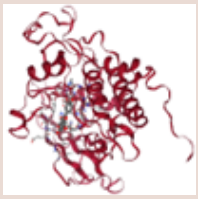
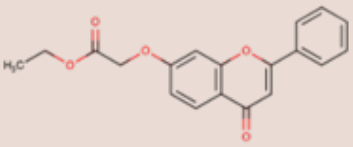
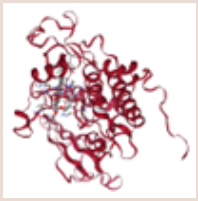
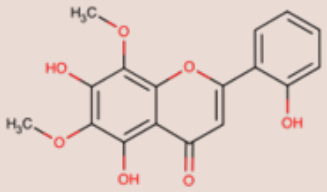
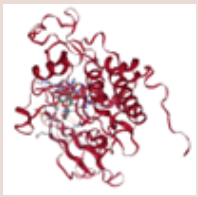
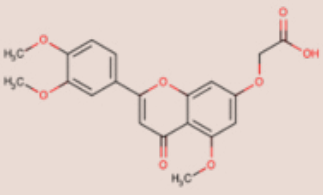
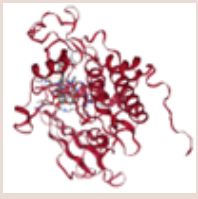
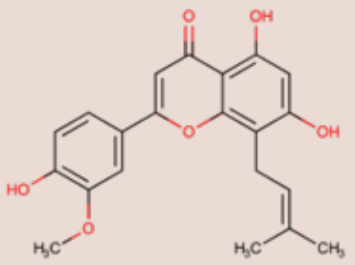
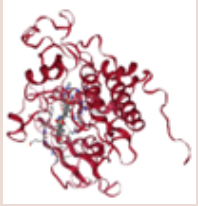
To investigate the effect of Apigenin on CDK6 gene expression, HCT-15, HeLa-229, and PC-3 cell lines were treated with their respective IC_{50} concentrations of Apigenin for a duration of 48 hr. Subsequently, RNA extraction was carried out using TRIzol™ Reagent (Ambion, Carlsbad, CA, USA) according to the manufacturer's instructions. The extracted RNA was then subjected to cDNA synthesis using the Verso cDNA Synthesis Kit (Thermo Fisher Scientific, USA). Quantitative real-time Polymerase Chain Reaction (qRT-PCR) was performed using SYBR™ Green Master Mix (Thermo Fisher Scientific, USA) on an Applied Biosystems RT-PCR System. The expression level of the target gene, CDK6, was assessed and normalized to that of the housekeeping gene GAPDH. The relative fold change in CDK6 expression was calculated using the $2^{-\Delta\Delta CT}$ method. Primers for CDK6 and GAPDH were adapted from previous studies.[19,49] The primer sequences utilized were as follows: CDK6: forward 5'- GAGAAGGACGGCCTGT-3' and reverse 5'-TCAAGTCTTGATCGAC-3', GAPDH: forward 5'-TCCCTGAGCTGAACGGGAAG-3' and reverse 5'-GGAGGAGTGGGTGTCGCTGT-3'. This experimental approach enabled the assessment of the impact of Apigenin treatment on CDK6 expression in the tested cancer cell lines at the molecular level.

Table 1: Detailed information of the compounds screened against CDK6. Binding affinity and top docking pose are also represented.

Sl. No.	Name of the compound	Structure of the compound	Target protein	Binding affinity (Kcal/mol)	Docking pose
1.	Apigenin [$C_{15}H_{10}O_5$] DB ID: DB07352 Experimental		CDK6	-8.4	
2.	Chrysin [$C_{15}H_{10}O_4$] DB ID: DB15581 Experimental			-6.7	

Sl. No.	Name of the compound	Structure of the compound	Target protein	Binding affinity (Kcal/mol)	Docking pose
3.	Tricetin [$C_{15}H_{10}O_7$] DB ID: DB08230 Experimental			-6.9	
4.	Luteolin [$C_{15}H_{10}O_6$] DB ID: DB15584 Experimental			-7.2	
5.	Baicalein [$C_{15}H_{10}O_5$] DB ID: DB16101 Investigational			-7.1	
6.	Kaempherol [$C_{15}H_{10}O_6$] DB ID: DB01852 Experimental			-6.7	
7	Hispidulin [$C_{16}H_{12}O_6$] DB ID: DB14008 Experimental			-6.9	
8	Diosmetin [$C_{16}H_{12}O_6$] DB ID: DB11259 Experimental			-6.8	
9	Chrysoeriol [$C_{16}H_{12}O_6$] DB ID: DB17283 Investigational			-7.3	

Sl. No.	Name of the compound	Structure of the compound	Target protein	Binding affinity (Kcal/mol)	Docking pose
10	Morin [$C_{15}H_{10}O_7$] DB ID: DB16770 Experimental			-7.0	
11	Flavone [$C_{15}H_{10}O_2$] DB ID: DB07776 Approved, Experimental			-6.8	
12	Beta-Naphthoflavone [$C_{19}H_{12}O_2$] DB ID: DB06723 Experimental			-7.8	
13	Alpha-Naphthoflavone [$C_{19}H_{12}O_2$] DB ID: DB07453 Experimental			-7.3	
14	Myricetin [$C_{15}H_{10}O_8$] DB ID: DB02375 Experimental			-7.0	
15	Quercetin [$C_{15}H_{10}O_7$] DB ID: DB04216 Experimental, Investigational			-7.2	
16	Fisetin [$C_{15}H_{10}O_6$] DB ID: DB07795 Experimental			-7.1	

Sl. No.	Name of the compound	Structure of the compound	Target protein	Binding affinity (Kcal/mol)	Docking pose
17	Isorhamnetin [$C_{16}H_{12}O_7$] DB ID: DB16767 Experimental			-7.4	
18	Eupatilin [$C_{18}H_{16}O_7$] DB ID: DB16885 Experimental			-6.7	
19	Rhamnetin [$C_{16}H_{12}O_7$] DB ID: DB16772 Experimental			-7.4	
20	Efloxate [$C_{19}H_{16}O_5$] DB ID: DB13333 Experimental			-7.1	
21	5,7,2′-trihydroxy-6,8-dimethoxyflavone [$C_{17}H_{14}O_7$] DB ID: DB13983 Experimental			-6.8	
22	Recoflavone [$C_{20}H_{18}O_8$] DB ID: DB12058 Investigational			-7.0	
23	Caflanone [$C_{21}H_{20}O_6$] DB ID: DB17263 Investigational			-7.4	

Enzyme inhibition assay

To explore the influence of Apigenin on CDK6 kinase activity, a series of experiments were conducted. Initially, various concentrations of Apigenin ranging from 0 to 44 µM were added to CDK6 at a fixed concentration of 2 µM, within wells of a 96-well plate. Following this, the plate was subjected to a 1-hr incubation period at 25°C to allow for the interaction between Apigenin and CDK6. Subsequently, a reaction mixture containing ATP at a concentration of 100 µM and $MgCl_2$ at 10 mM was introduced into the wells. This reaction mixture underwent further incubation for 30 min, after which it was terminated by the addition of BIOMOL® reagent. The formation of a green-coloured complex was then monitored at 620 nm using a plate reader. The detection of inorganic phosphate released during ATP hydrolysis was facilitated by the presence of Malachite green reagent. The intensity of the green coloration corresponded to the level of inorganic phosphate generated, which directly reflects the activity of CDK6 kinase. Notably, the activity of native CDK6 in the absence of Apigenin was considered as 100%, serving as a baseline reference for comparison. This experimental setup allowed for the systematic evaluation of Apigenin's impact on CDK6 kinase activity across a range of concentrations, providing valuable insights into its inhibitory effects on this enzyme.

RESULTS

Protein structure homology modelling and preparation of CDK6

Figure 1a shows the high-resolution structure of the CDK6 model obtained through Swiss-MODEL. The model boasts a remarkable sequence Identity and coverage of 100%, indicating a precise alignment with the target sequence. Notably, the quality assessment of the model reveals excellence, as evidenced by a GMQE (Global Model Quality Estimation) score of 0.87 and a QMEANDisCo (Qualitative Model Energy Analysis and DisCo) Global score of 0.96±0.05. These scores, ranging between 0 and 1, provide an overall evaluation of model quality, with higher values reflecting superior expected quality. It's essential to note that while GMQE is coverage-dependent, meaning it considers the alignment coverage of the model, QMEANDisCo evaluates model quality independently of explicit coverage dependency. To offer a more granular assessment, Figure 1b presents a graphical representation of the QMEANDisCo local quality estimate, providing insights into the local quality of the model. Additionally, Figure 1c depicts the Ramachandran Plot of the modelled structure, offering a visualization of energetically favored regions for the backbone dihedral angles of amino acid residues within the protein structure. By delineating the contours of favored regions, Figure 1c provides valuable information about the overall structural integrity and reliability of the CDK6 model.

Cavity detection on CDK6

Table 2 elucidates the principal findings regarding the detection of cavities within CDK6, providing a detailed overview of the number and characteristics of these identified structural features through CB-Dock2 analysis. Notably, five prominent cavities labelled as C1 to C5 were successfully identified, with Table 2 presenting comprehensive information regarding their size, volume, and other relevant attributes. Among these cavities, Cavity 1 (C1) stood out due to its maximum Cavity Size and Cavity Volume, making it particularly noteworthy for further investigation through molecular docking experiments. To provide a visual representation, the sequence-based depiction of the identified cavities (C1, C2, C3, C4, and C5) is presented in Figure 2A, while Figure 2B offers a structural visualization of all the identified structure-based cavities (C1, C2, C3, C4, and C5) within the CDK6 protein. These detailed analyses offer valuable insights into the structural characteristics of CDK6, providing a foundation for subsequent molecular docking studies aimed at understanding its interaction with ligands such as Apigenin.

Molecular docking revealed Apigenin as potential inhibitor of CDK6

Following the screening of a ligand library against CDK6, Apigenin emerged as the most promising candidate, demonstrating a remarkable fitness of 98.97% and the highest predicted binding affinity. Following its identification as the top hit, Apigenin underwent molecular docking against the target protein CDK6. The results of the molecular docking process, specifically involving the formation of the CDK6: Apigenin complex, are visually represented in Figure 3A(i) and 3(ii). Through this docking analysis, affinity scores and docked poses were generated, revealing a notable binding affinity score of -8.4 kcal/mol between Apigenin and CDK6. Figure 3A(i) provides a cartoon presentation

Table 2: Detailed information on the size, volume, or other relevant characteristics of the structure-based cavities (C1-C5) detected on CDK6.

CurPocket ID	Cavity volume (Å3)	Center (x, y, z)	Cavity size (x, y, z)
C1	3249	29, -11, 70	14, 28, 17
C2	821	10, -18, 59	13, 17, 15
C3	642	13, -11, 45	20, 13, 9
C4	375	31, -12, 90	19, 8, 11
C5	311	36, -9, 83	9, 10, 9

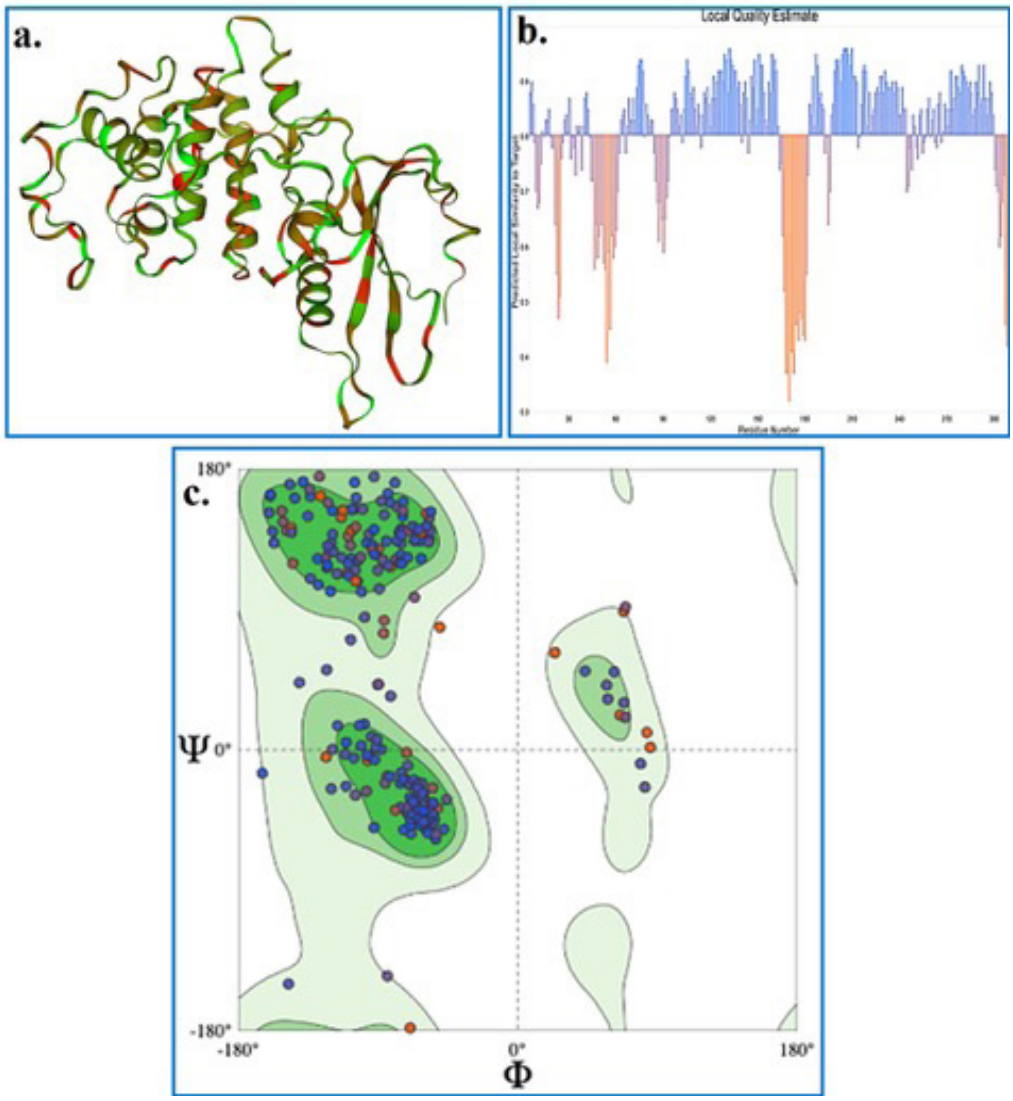


Figure 1: (a) Three-dimensional structure of CDK6 (b) QMEANDisCo local quality estimate graphically represents the model's local quality; (c) Ramachandran Plot visualizes energetically favored regions for backbone dihedral angles, with contours delineating these regions in the modelled structure.

of the CDK6: Apigenin complex, offering insights into the spatial arrangement of the molecules, while Figure 3A(ii) presents a surface view of the complex, offering additional perspectives on their interaction. Evidence of the successful docking of Apigenin into the deep binding cavity (C1) of CDK6 is apparent from the docking pose. This interaction formed a variety of interactions including polar, hydrophobic, and others with multiple amino acid residues of CDK6. Specifically, Apigenin established interactions with ILE19, VAL27, ALA41, LYS43, VAL77, PHE98, GLU99, HIS100, VAL101, ASP102, GLN103, ASP104, LEU152, LEU161, ALA162, ASP163, and PHE164 within the protein structure. These interactions highlight the comprehensive nature of the binding between Apigenin and CDK6, suggesting a strong and multi-faceted affinity between the two molecules.

Furthermore, to validate the binding affinity of the CDK6-Apigenin complex, redocking was conducted using SeamDock (https://bioserv.rpbs.univ-paris-diderot.fr/services/SeamDock/). The outcomes of the top five redocking poses are depicted in Figure 3B (i-v). In these poses, Apigenin exhibited consistent interactions with the binding site of CDK6. The binding affinities for the top five poses were determined as -7.9, -7.3, -7.2, -6.8, and -6.7 kcal/mol, respectively. This consistent binding affinity across multiple poses further substantiates the potential of Apigenin as a significant therapeutic candidate for targeting CDK6.

ADMET predictions and SwissTargetPrediction

The validation of Apigenin's drug-like properties involved comprehensive analyses through ADMET predictions and SwissTargetPrediction. Table 3 presents a detailed summary of ADMET predictions for Apigenin, showing its compliance with critical pharmacokinetic criteria essential for drug development. Additionally, the SwissTargetPrediction analysis not only confirms but also elaborates on a wide range of biological properties linked

to Apigenin, as illustrated in Figure 4. These findings collectively suggest that Apigenin holds promise as a potential therapeutic candidate, especially in its ability to target CDK6 inhibition for cancer treatment.

Cytotoxic effect of Apigenin on HEK-293 normal lung cells & Calculation of IC_{50} conc. of Apigenin for HCT-15, HeLa-229, and PC-3 cancer cells

The outcomes of the concentration-dependent effect of Apigenin on the viability of HEK-293 cells are illustrated in Figure 5a. Upon analysis, it was observed that Apigenin did not induce any significant concentration-dependent effects on the viability of HEK-293 cells. This suggests that within the range of concentrations tested, Apigenin did not exert a notable impact on the viability of these cells.

HCT-15, HeLa-229, and PC-3 cancer cells were treated in concentration dependent manner for 72 hrs. Cells were harvested for MTT assay and absorbance was recorded to determine the respective IC_{50} concentrations. The IC_{50} value for Apigenin was found to be 44.12 µM for HCT-15 cancer cells, 59.24 µM for HeLa-229 cancer cells, and 81.43 µM for PC-3 cancer cells.

Cytotoxic (Time dependent manner) effect of Apigenin on HEK-293 cells

Figure 5b illustrates the outcomes of investigating the impact of Apigenin on the viability of HEK-293 cells over varying durations in a time-dependent manner. The experimental design involved subjecting HEK-293 cells to increasing exposure durations of Apigenin, specifically at Mean IC_{50} concentrations derived from three IC_{50} values obtained from different cancer cell lines (HCT-15, HeLa-229, and PC-3). These exposure durations

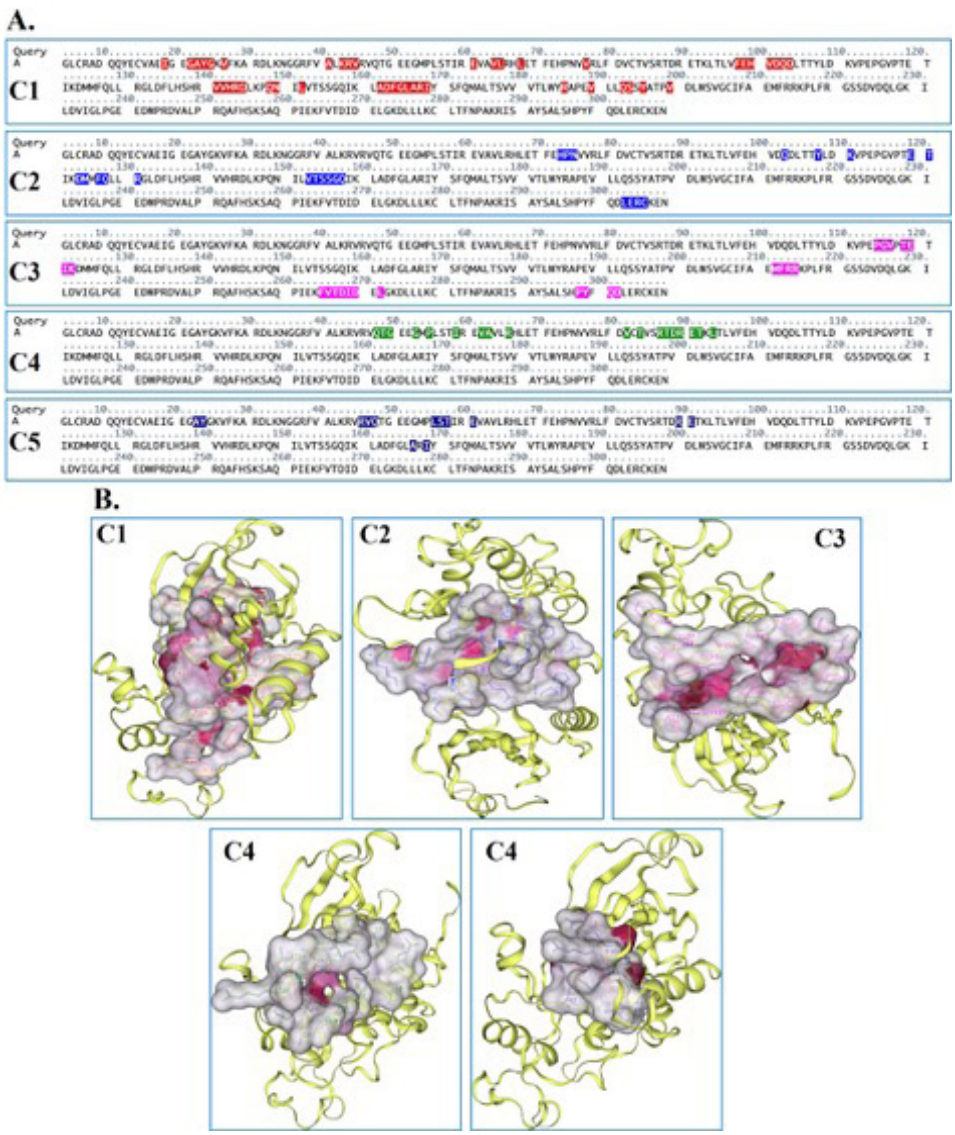


Figure 2: (A) Structure-based cavity detection, sequences representing cavities C1, C2, C3, C4, and C5 of CDK6. (B) Structural visualization of all 5 structure-based identified cavities (C1, C2, C3, C4, and C5) within CDK6.

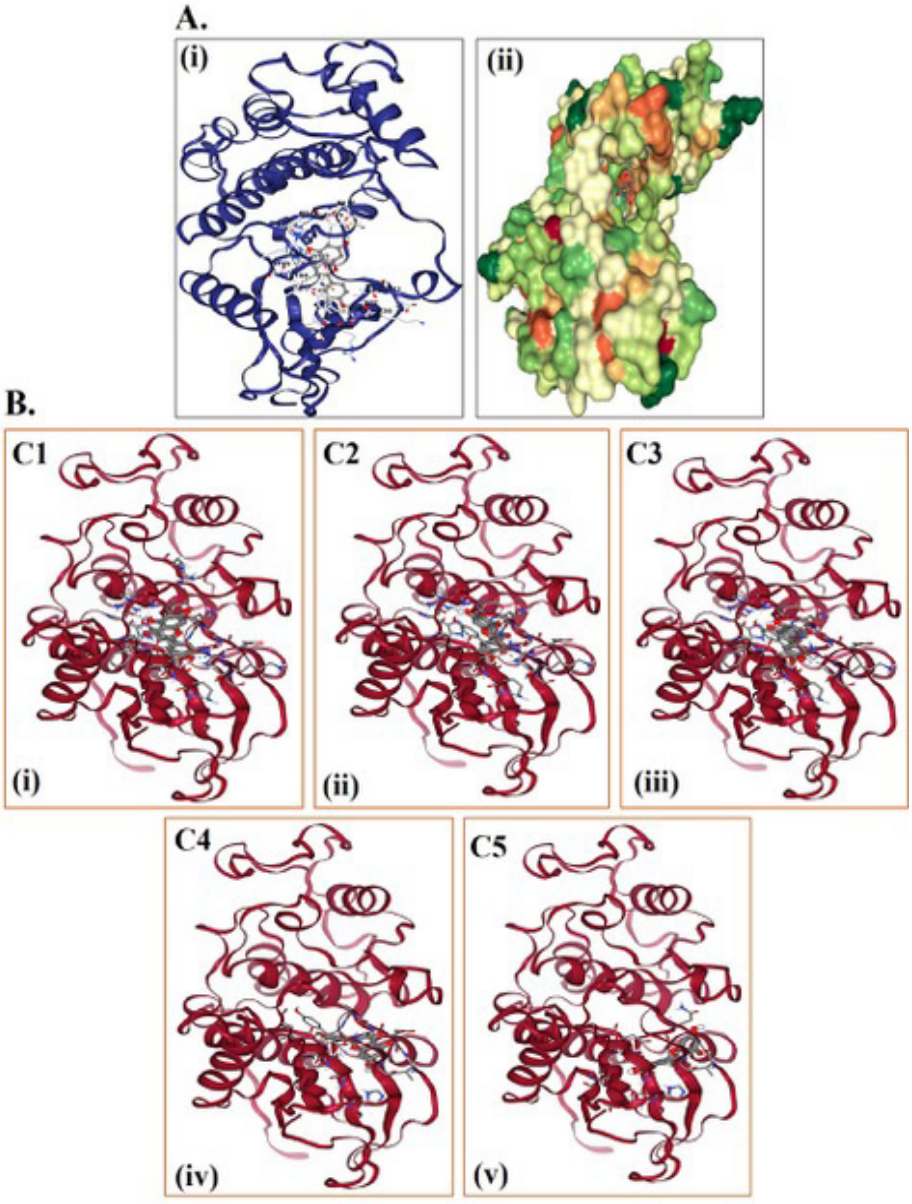


Figure 3: Visual representation of the CDK6: Apigenin molecular docking complex. 3A (i) cartoon presentation of the CDK6: Apigenin complex. 3A (ii) Surface view of the CDK6: Apigenin complex. (3B) Validation of CDK6-Apigenin binding affinity through redocking. The top five redocking poses (i, ii, iii, iv, and v), with corresponding binding affinities of -7.9, -7.3, -7.2, -6.8, and -6.7 kcal/mol, respectively.

included 24 hr, 48 hr, and 72 hr. Upon analysis, it was observed that regardless of the duration of exposure to Apigenin at these Mean IC_{50} concentrations, there was no discernible effect on the viability of HEK-293 cells. This suggests that the compound, even at concentrations effective against cancer cell lines, did not adversely impact the viability of non-cancerous HEK-293 cells over the specified time periods.

Expression of CDK6 in Apigenin treated HCT-15, HeLa-229, and PC-3 cancer cells

Figures 5c, 5d, and 5e provide a visual representation of the impact of Apigenin's respective IC_{50} concentrations on the mRNA expression of CDK6 in three distinct cancer cell lines: HCT-15, HeLa-229, and PC-3. The experimental setup involved treating each cell line with Apigenin at its specific IC_{50} concentration. Upon analysis, it was observed that treatment with Apigenin led to a significant decrease ($p<0.001$) in the mRNA expression of CDK6 within each of these cancer cell lines when compared to untreated cells. This significant reduction in CDK6 mRNA expression underscores the inhibitory effect exerted by Apigenin on the activity of CDK6, highlighting its potential as a therapeutic agent for targeting CDK6 in cancer treatment.

Enzyme inhibition assay showed inhibition of CDK6 by Apigenin

The results obtained from the kinase assay profile revealed a significant decrease ($p<0.001$) in the activity of CDK6 kinase concurrent with an increase in the concentration of Apigenin, as depicted in Figure 5f. This observation suggests a potential inhibitory effect of Apigenin on the kinase activity of CDK6. The significant reduction in CDK6 kinase activity upon exposure to higher concentrations of Apigenin indicates a dose-dependent response, further supporting the notion that Apigenin may indeed modulate the activity of CDK6, potentially through direct interaction or downstream regulatory mechanisms.

DISCUSSION

In the realm of cancer research, identifying novel therapeutic agents that selectively target key proteins involved in tumorigenesis is of paramount importance.[50-52] CDK6, a crucial regulator of cell cycle progression, has emerged as a promising target for cancer therapy. In this study, the structural characteristics of CDK6 were elucidated through protein structure homology modelling, cavity detection analysis, and molecular docking studies. Subsequently, the potential of Apigenin, a natural compound, as a CDK6 inhibitor was explored through *in silico* predictions, cytotoxicity assays, and molecular analyses.

The role of modelling the 3D structure of target proteins is instrumental in cancer therapeutics. Understanding the precise 3D structure of target proteins involved in cancer, such as CDK6, enables the rational design of small molecules or biologics that can interact with these proteins.[40,42] By employing computational techniques like homology modelling, researchers can generate accurate representations of protein structures even in cases where experimental structures are unavailable, facilitating the identification of potential drug candidates.[53,54] The study commenced with the generation of a high-resolution model of CDK6 using Swiss-MODEL, demonstrating a remarkable sequence identity and coverage with the target protein. Quality assessment metrics, including GMQE and QMEANDisCo scores, affirmed the excellence of the model, providing a robust foundation for further investigations.

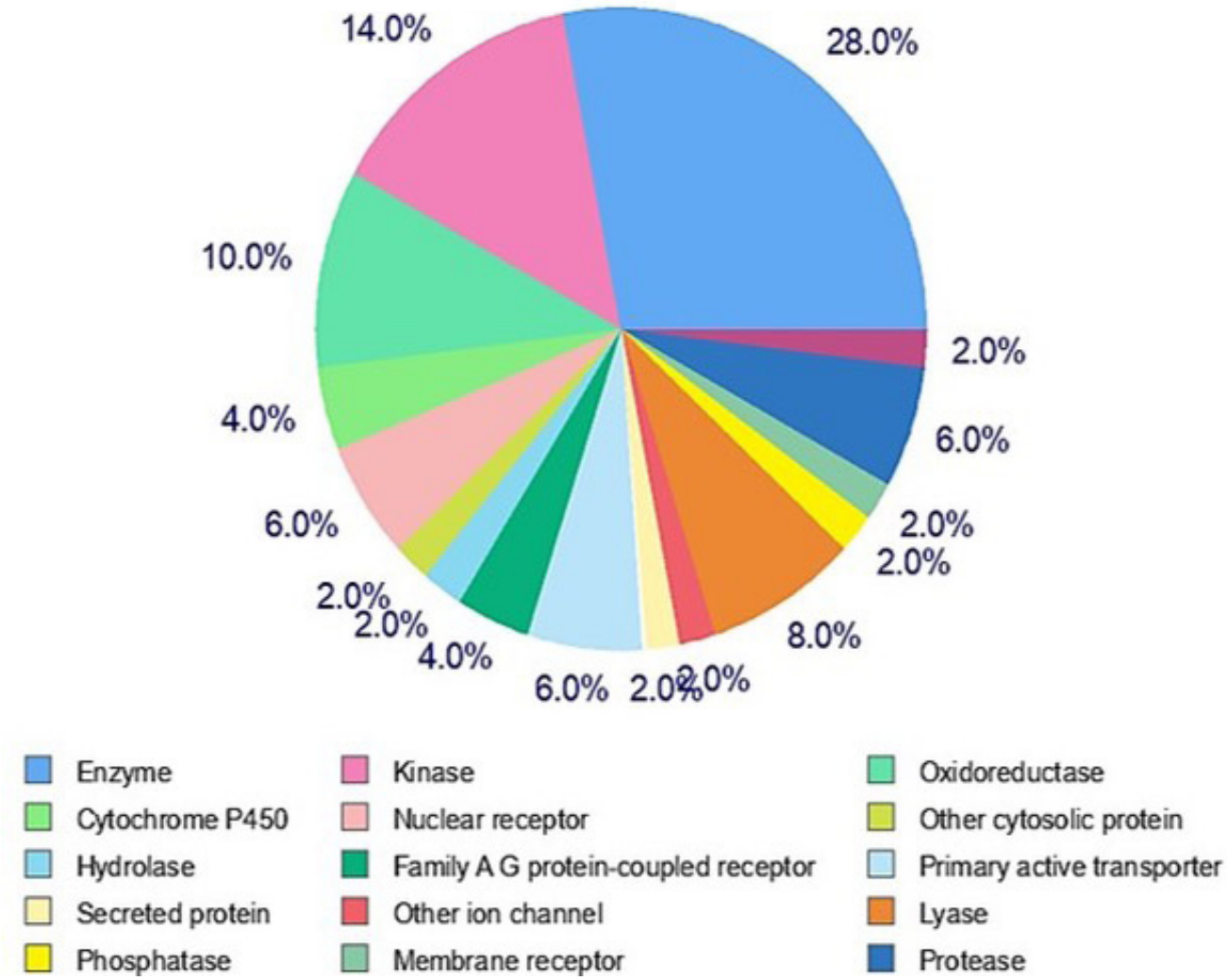


Figure 4: SwissTargetPrediction analysis showing diverse biological properties linked to Apigenin.

Table 3: ***In silico*** **prediction of ADME & Toxicity properties for Apigenin.**

	Properties												
	Absorption	**Distribution**			**Metabolism**							**Excretion**	**Toxicity**
Models	Intestinal absorption (human)	VDss (human)	BBB permeability	CNS permeability	CYP							Total clearance	AMES toxicity/ hepatotoxicity
					Substrate		Inhibitor						
					2D6	3A4	1A2	2C19	2C9	2D6	3A4		
Unity	Numeric (% absorbed)	Numeric (log L/ kg)	Numeric (Log BB)	Numeric(Log PS)	Categorical (yes/no)							Numeric (log mL/ min / kg)	Categorical (yes/ no)
Predicted values													
Apigenin	93.25	0.822	-0.734	-2.061	NO	No	Yes	Yes	No	NO	NO	0.566	NO/No

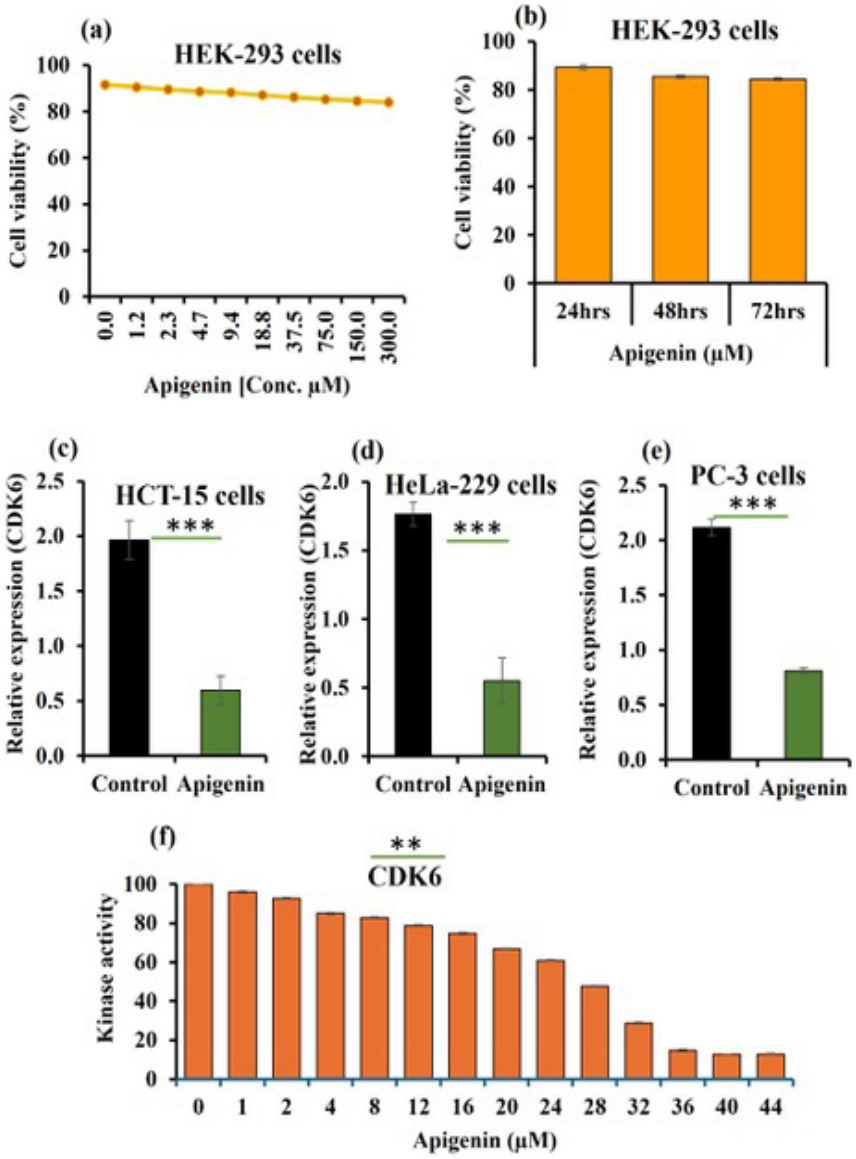


Figure 5: (a) Concentration dependent effect of Apigenin on cell viability of HEK-293 cells; (b) Time-dependent effect of Apigenin on cell viability of HEK-293 cells. (c) mRNA expression of CDK6 in Apigenin treated HCT-15 cancer cells. (d) mRNA expression of CDK6 in Apigenin treated HeLa-229 cancer cells. (e) mRNA expression of CDK6 in Apigenin treated PC-3 cancer cells. Cancer cells were treated with their IC_{50} concentrations. (f) Kinase activity of CDK6 with respect to increasing concentration of Apigenin.

Cavity detection analysis of target proteins plays a significant role in cancer therapeutics by providing insights into potential binding sites for small molecule inhibitors or drug candidates.[55-57] In cancer therapeutics, proteins involved in signalling pathways or cellular processes critical for tumor growth and survival, such as kinases or transcription factors, are often targeted.[50,58-60] Cavity detection helps pinpoint druggable sites within these proteins where inhibitors can bind and modulate their activity.[61] Cavity detection analysis revealed the presence of five prominent cavities within the CDK6 structure, with Cavity 1 (C1) exhibiting notable size and volume. Molecular docking studies subsequently focused on exploring the interaction between CDK6 and potential ligands, with Apigenin emerging as the top candidate due to its favourable binding affinity.

Molecular docking analysis of target proteins and ligands is a vital component of cancer therapeutics research, enabling the identification, characterization, and optimization of potential drug candidates.[34,35] By predicting the binding affinity and orientation of each ligand within the protein's binding site, docking analysis identifies potential drug candidates that have a high likelihood of effectively modulating the target protein's activity.[33] In cancer therapeutics, this approach enables the rapid identification of compounds that selectively inhibit oncogenic proteins or pathways implicated in tumor growth and progression. Molecular docking simulations unveiled a strong binding interaction between Apigenin and CDK6, with the docking pose indicating deep penetration of Apigenin into Cavity 1 of the protein. Multiple interactions, including polar and hydrophobic contacts, underscored the comprehensive nature of the binding.

ADME-Tox (Absorption, Distribution, Metabolism, Excretion, and Toxicity) analysis of ligands is a crucial step in cancer therapeutics to evaluate the pharmacokinetic and toxicological properties of potential drug candidates.[62] Validation of Apigenin's drug-like properties through ADMET predictions and SwissTargetPrediction further supported its potential as a therapeutic agent. The analyses highlighted Apigenin's favourable pharmacokinetic profile and its ability to target CDK6, reinforcing its candidacy for cancer treatment.

Cytotoxicity assays, such as the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay, play a crucial role in cancer therapeutics by evaluating the efficacy of potential anticancer agents in inhibiting the growth and viability of cancer cells.[63,64] Experimental evaluation of Apigenin's cytotoxic effects on cancer cell lines revealed concentration-dependent inhibition, with IC_{50} values determined for HCT-15, HeLa-229, and PC-3 cells. Importantly, Apigenin exhibited minimal cytotoxicity towards normal HEK-293 cells, emphasizing its selectivity towards cancer cells.

Determining mRNA expression under treatment in cancer therapeutics serves several crucial roles in understanding the molecular mechanisms of cancer progression and response to treatment.[65,66] Treatment with Apigenin led to a significant reduction in CDK6 mRNA expression across multiple cancer cell lines, indicative of its inhibitory effect on CDK6 activity. The kinase enzyme inhibition assays play a critical role in the development of targeted therapies for cancer, enabling the identification of novel drug targets, the development of more effective drugs, and the personalization of treatment strategies based on the molecular profile of individual tumors.[50,67,68] Moreover, enzyme inhibition assays demonstrated a dose-dependent decrease in CDK6 kinase activity upon Apigenin exposure, further corroborating its role as a CDK6 inhibitor.

CONCLUSION

In conclusion, the integration of computational techniques with experimental assays has provided valuable insights into the potential of Apigenin as a promising therapeutic agent for cancer treatment, targeting the pivotal protein CDK6. Molecular docking simulations revealed the strong binding affinity between Apigenin and CDK6, highlighting the potential for Apigenin to effectively modulate CDK6 activity. Furthermore, ADMET predictions and cytotoxicity assays supported Apigenin's candidacy as a therapeutic agent, demonstrating its favourable pharmacokinetic profile and selective cytotoxicity towards cancer cells while sparing normal cells. Importantly, Apigenin treatment resulted in a significant reduction in CDK6 mRNA expression and kinase activity, indicating its inhibitory effect on CDK6 function. Overall, the findings of the study underscore the importance and potential of natural compounds like Apigenin in targeting key proteins implicated in tumorigenesis. Moving forward, further preclinical and clinical studies are warranted to validate the efficacy and safety of Apigenin as a potential therapeutic intervention for cancer.

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ABBREVIATIONS

CDK6: Cyclin-Dependent Kinase 6; **C1:** Cavity 1; **CDKs:** Cyclin-dependent kinases; **pRb:** Retinoblastoma protein; **PDB:** Protein Data Bank; **SDF:** Structure Data Format; **DMEM:** Dulbecco's Modified Eagle Medium; **FBS:** Fetal bovine serum; **DMSO:** Dimethyl sulfoxide.

CONFLICT OF INTEREST

The author declares no conflict of interest.

SUMMARY

The study suggests that Apigenin, a natural compound, has the potential to target the key protein CDK6 in cancer treatment. Molecular docking simulations and ADMET predictions show strong binding affinity, and Apigenin's cytotoxicity is selective towards cancer cells. Treatment reduces CDK6 mRNA expression and kinase activity, indicating its inhibitory effect on CDK6 function.

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