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Research Article

Isolation and evaluation of flavonoids from propolis against triple-negative breast cancer cells: Insights from *in vitro* and *in silico* studies

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ABSTRACT

Bee propolis, a resinous substance synthesized by honeybees, has been widely recognized for its antimicrobial, anti-inflammatory, and antioxidant properties. This study focused on isolating and characterizing the bioactive phytoconstituents from the methanolic extract of bee propolis while simultaneously evaluating propolis' anticancer activity. Three bioactive flavonoids, which are apigenin, pinocembrin, and castillicetin-2, were successively separated by column chromatography and their structures elucidated by liquid chromatography-mass spectrometry (LC-MS) and nuclear magnetic resonance (NMR) spectroscopy. Using a cell viability assay, their cytotoxic activity was evaluated against the triple-negative breast cancer cell line (MDA-MB-231). It was found that castillicetin-2 had a significant cytotoxic effect at EC_{50} of 7.7 ± 0.6 µg/mL, apigenin at 10.82 ± 0.7 µg/mL, and pinocembrin at 25.24 ± 0.8 µg/mL. Further evaluation of the compounds revealed that these compounds, alongside their respective molecular docking, proved to have strong binding ability to benefit important targets to breast cancer, such as HER2 and CDK6, which provided more insight into these compounds being useful as potential therapeutic agents. These findings demonstrated the bioactive efficacy of the flavonoids derived from propolis and how these findings augment concerns associated with the increased attention towards the treatment of breast cancer.

1. Introduction

Bee propolis, also known as "bee glue," is a resinous substance synthesized by honey bees from plant resins, beeswax, pollen, and secretions. This adhesive material protects the hive structurally and provides antimicrobial defenses against pathogens and environmental threats. Propolis has been widespread in traditional medicine and was utilized by Egyptians, Greeks, and Romans to treat microbial infections due to its antimicrobial, anti-inflammatory, and antioxidant properties, and also used in wound healing (Asfaram *et al.*, 2021). Its chemical composition exhibits regional variability based on botanical sources but predominantly contains plant resins (50-60%), beeswax (30-40%), essential oils (5-10%), and pollen (5%) (Salatino *et al.*, 2021). Propolis has diverse pharmacological properties and is rich in active compounds, including flavonoids, phenolic acids, and terpenes. Traditional Chinese and Ayurvedic medicine have long associated propolis with the treatment of respiratory ailments, gastrointestinal disorders, and skin infections (Hossain *et al.*, 2022; Zullkiflee *et al.*, 2022).

Research conducted in the recent past has validated the complex biological and pharmacological functions of propolis, highlighting its therapeutic potential in numerous health applications. It shows strong antimicrobial activity towards bacteria like *Staphylococcus aureus*, *Escherichia coli*, and *Helicobacter pylori*, while also having antifungal and antiviral activities (Pasupuleti *et al.*, 2017; Zulhendri *et al.*, 2021). The anti-inflammatory capability of propolis, attributed to flavonoids and phenolic acids, further helps control inflammatory disorders like arthritis and asthma. In addition, his strong antioxidant activity enables the destruction of free radicals, protecting from oxidative stress illnesses like cardiovascular and neurodegenerative diseases (Magnavacca *et al.*, 2022; Bhatti *et al.*, 2024). Also, new studies show that propolis alters the immune system, balancing immune response with potential use in autoimmune disease treatment (El-Seedi *et al.*, 2022).

This study aimed to isolate and characterize the bioactive phytoconstituents from the methanolic extract associated with the anticancer activity of bee propolis. The compounds pinocembrin, apigenin, and castillicetin-2 were isolated, and their structures were elucidated. They were studied for their cytotoxic activity and molecular docking on breast cancer cells (MDA-MB-231). Importantly, castillicetin-2 was shown to be present in propolis collected from the Kashmiri Himalayan region for the first time.

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2. Materials and Methods

2.1 Material collection and preparation of propolis ethanolic extract (PEE)

The propolis sample was gathered from Rangil, Ganderbal, in Central Kashmir (part of Jammu and Kashmir, India). It was identified and authenticated at the 'Research and Training Center for Pollinator, Pollinizer, and Pollination Management' at Sher-e-Kashmir University of Agricultural Sciences and Technology, Kashmir, India, under the specimen voucher number AU/DR/NAE-II/137. The propolis was packed into a percolation chamber and extracted with 100% EtOH at room temperature for 24 h while the solution was continuously stirred. After multiple extraction cycles, the extract was filtered, and the resultant solution was subjected to reduced pressure. The extract's moisture content was reduced by placing it in a desiccator, and afterward, it was stored in a refrigerator for later use (Wali *et al.*, 2015).

2.2 Chemicals

Silica gel (60-120 mesh) for column chromatography was procured from Rankem Laboratories, Mumbai, India. All solvents (analytical grade) were purchased from Rankem Laboratories. Thin layer chromatography (TLC) was performed on pre-derivatised plastic sheets with Silica F254 gel on the surfaces (20x20cm,200µm,60°A, Merck India Ltd).

2.3 Preparation of PEE for column chromatography

Propolis was extracted with 2 L of EtOH at room temperature for 24 h while being stirred. The residue was subsequently exhaustively extracted with EtOH (500 mL × 3) every 3 h. The filtered extract (PEE) was concentrated under reduced pressure and purified by column chromatography. This extraction sequence facilitated the most efficient isolation of bioactive compounds, enabling the downstream fractionation and analysis of the ethanolic soluble extract.

Employing column chromatography facilitates the separation of phytoconstituents, enhancing the strategies of structural characterization and bioactivity studies of isolated compounds.

2.4 Column chromatography of PEE

The propolis ethanolic extract (150 g) was adsorbed onto silica gel (60–120 mesh) to prepare a slurry. The mixture was then dried and loaded onto the top of a silica gel column pre-packed with petroleum ether. The column chromatography was performed using a gradient elution method with petroleum ether, ethyl acetate, and methanol to increase polarity to facilitate the isolation of pure compounds.

Fractions were collected and analyzed using TLC to monitor separation efficiency. Fraction F27, eluted with 10% ethyl acetate in petroleum ether, yielded compound 1 (15.64 mg). Subsequently, fractions F45 to F49 were pooled (90.34 mg) and subjected to further purification via column chromatography using a solvent gradient ranging from 100% chloroform to 100% MeOH.

From this secondary purification step, fractions F15 and F16 were pooled based on their TLC profiles, leading to the isolation of compound **2** (10.24 mg). Similarly, fractions F39 and F40 were combined to yield compound **3** (9.26 mg).

2.5 Screening bioactive compounds

2.5.1 Cell line and cell culture

The study utilized the MDA-MB-231 cell line, which is a triple-negative human breast cancer cell line (Russo-Abrahao *et al.*, 2018). The cells were grown in a culture medium that consisted of Dulbecco's modified Eagle's medium (DMEM), 10% fetal bovine serum (FBS), 100 U/mL penicillin-streptomycin, and Dulbecco's Phosphate Buffered Saline (DPBS). The incubator was maintained at 37°, 5% CO_2, and 95% humidity. The cells were washed and subcultured in 1X phosphate buffer saline (PBS). Once the cells reached confluence, we removed the confluent medium, washed the sub-cultured cells with 1xPBS, and then added Trypsin to detach the MDA-MB-231 cells from the substrate. Following the incubation and neutralization of trypsin, viable cells were collected and seeded into assay-specific culture vessels for viability testing. Dimethylsulfoxide (DMSO)/EtOH vehicle controls were used at concentrations below 0.1%, and no significant cytotoxic effects were observed compared to untreated controls (Gallardo-Villagrán *et al.*, 2022).

2.5.2 Treatment of MDA-MB-231 with isolated compounds

The MDA-MB-231 cells were seeded into 96-well plates with a seeding density of 5000 cells per well. Cells were incubated overnight and then treated the next day with progressively increasing concentrations of isolated compounds (50, 100, 200, 400, and 600 µg/mL) that were prepared in 70% EtOH, with EtOH serving as a control. Treatment duration of 24, 48, and 72 h was followed by cell viability measurement.

2.5.3 Cell viability assay

At the specified time points, the medium in every well was removed, and 50 µL of fresh medium was added to each. Next, 50 µL of MTT solution was added to each well and mixed. The plate was mixed for 2 min and held at room temperature for an additional 10 min, and subsequently, the luminescence was quantified. The viability data were calculated as a percentage of treated cells to untreated control cells, which were set to have 100% viability.

2.5.4 Molecular docking study

All molecular docking studies were conducted employing Maestro v12.8, a molecular modeling software from Schrödinger Inc. The PDB files corresponding to the breast cancer targets were downloaded from the Protein Data Bank (PDB): Human Epidermal Growth Factor Receptor 2 (HER2: PDB ID: 3PP0) and Cyclin-dependent kinase 6 (CDK6: PDB ID: 5L2S). The Protein Preparation Wizard was employed to prepare the proteins, which included the addition of missing hydrogen atoms, bond order assignments, removal of heteroatoms and solvent molecules in a 5 Å radius sphere, optimization of hydrogen bonds, and formation of zero-order bonds with metal ions. Missing loops and chains were validated and added using Prime in Maestro. The resulting protein structures were thereafter energy-minimized with the OPLS force field (Madhavi Sastry *et al.*, 2013; Yousif *et al.*, 2023).

With the aid of the Receptor Grid Generation tool, the binding sites for receptors were determined based on the crystallized ligand binding sites. The active phytocompounds and reference molecules were prepared with the help of the MacroModel tool, making sure that all ligands had their geometries optimized. For all target proteins, molecular docking was conducted with the Glide XP mode to assess the docking, binding, and interaction possibilities of the ligands within the active sites of the proteins.

2.5.5 Statistical analysis

In all the experiments, the data representing values were given in the form of Mean ± Standard Errors (SE) of triplicate values. It was considered that all values were significant at the 5% level when $p \leq 0.05$ was attained.

3. Results

3.1 Characterization of isolated compounds

The PPE was subjected to column chromatography, which led to the isolation of three compounds, which were characterized with the help of ^{1}H NMR, ^{13}C NMR, and Mass spectroscopic techniques.

Compound 1 was collected as a yellowish, amorphous solid. From high resolution-electrospray ionization-mass spectroscopy (HR-ESI-MS), a molecular ion peak was observed at m/z 255.0623 $[M-H]^-$, which was confirmed to have a molecular formula $C_{15}H_{12}O_4$.

^{1}H NMR studies showed doublets for H6 and H8 at 6.65 and 6.68 (J = 2.0 Hz). Additional doublets were noted for C2' and C6' at 7.04 (J

= 1.97, 7.48 Hz). Furthermore, H3' and H5' resonated as a doublet of doublets at 7.04 d (J = 1.963, 7.66 Hz). H4' was assigned as a multiplet at 8.48 (J = 194, 7.51 Hz). Importantly, the lack of a prominent H3 proton signal in the flavanone moiety was noted, and an ABX system was evident. The X component was observed as a doublet doublet (d) 4.37, J = 13.20, 2.92 Hz) assigned to H2, while the AB parts were recognized as two d at H3α (3.90, J = 15.96, 2.92 Hz) and H3β (4.33, J = 13.44, 15.97 Hz).

According to the 13C NMR spectrum, 15 signals of carbon were identified, encompassing one methylene, six quaternary, and eight methine carbons. For ring A, the following signals were assigned: C10 (δ 102.00), C5 (δ 163.71), C6 (δ 96.20), C7 (δ 162.94), C8 (δ 95.31), and C9 (δ 166.99). In ring B, C1′ (δ 138.85), C2′ and C6′ (δ 126.83), and C5 (δ 128.81) were assigned. In ring C, a peak was observed for a carbonyl at C4 δ 196.14, while supporting signals were also at C2 (δ 78.63) and C3 (42.30), confirming the flavanone scaffold. Thus, compound 1 was identified as Pinocembrin because its instrumental analysis data, as shown in Supplementary data S1, corresponded with the reference data for Pinocembrin.

Compound **2** was obtained as pale-yellow crystalline solids. The compound with the formula $C_{15}H_{10}O_5$ showed a peak for its molecular ion in the HR-ESI-MS spectrum at m/z 270.1078 $[M-H]^-$. Fragmentation ions of 242.18, 153.09, and 118.07 were observed.

The 1H NMR spectra showed two doublets at δ 7.90 and 7.89, and δ 6.92 and 6.90, respectively, for H3′ & H5′ and H2′ & H6′. Aromatic methine protons H6 and H8 were observed as δ 6.18 and 6.73 meta-oriented broad singlets.

^{13}C NMR spectroscopy corroborated the existence of the flavone component. Notable downfield shifts for C4, indicative of carbonyl carbon at δ 181.73 and C6 at the value of δ 163.86 were noted. The signal found at C5 (δ 163.76) suggested O substitution at C5 and C6, while a peak found at C7 (δ 98.66) downfield strongly reinforced partial oxygen substitution at these sites. Compound **2** data shown in Supplementary data S2 was compared with the reference data for Apigenin(Cvetanović *et al.*, 2017; Kumar *et al.*, 2018).

A yellow powder was isolated as Compound **3**. The molecular formula $_{10}$ was estimated from the ^1H NMR spectrum, m/z 464.07252 $[M-H]^-$, and a measure of 6 hydroxyl groups suggests the presence of the data gained from the HR-ESI-MS showed a deprotonated molecular ion peak.

The ^1H NMR spectrum contained an ABX pattern consistent with the presence of two 1,3,4-trisubstituted benzene rings along with the chemical shifts at δ 7.03 (J = 1.17 Hz, H-2'), δ 6.97 (J = 8.44 Hz, H-5'), δ 7.56 (J = 8.44, 1.17 Hz, H-6'), δ 6.97 (J = 1.17 Hz, H-2''), δ 6.75 (J = 8.44 Hz, H-5''), and δ 7.67 (J = 8.44, 1.17 Hz, H-6''). Signals at δ 6.41 (J = 1.17 Hz, H-8) and δ 6.19 (J = 1.17 Hz, H-6) also supported the presence of the flavonoid nucleus.

The ^{13}C NMR spectrum showed 24 carbon signals, comprising 10 methine and 14 non-active carbons. A prominent downfield shift around δ 176.29 (C4) was ascribed to a carbonyl carbon. Based on the peaks assigned to C5 and C7, as well as C3' and C4', functional group interactions were proposed. An ester bond was indicated by the resonance at δ 168.37 (C-9''). Notable Heteronuclear Multiple Bond Correlation (HMBC) interactions, including C-4' (δ 147.26) with C-2 (δ 161.17), other C-2 with H-2' (δ 7.03), and H-6' confirmed important structural deductions via HMBC. The structure was further confirmed by 1H–1H COSY spectral data together with the HMBC spectra shown in Supplementary data S3 and Supplementary Figure 1 and comparison with literature values, the compound was therefore elucidated Quercetin-3-caffeate or commonly called Castillicetin-2 (Subban *et al.*, 2008; Gray *et al.*, 2018).

3.2 Cytotoxic effect of isolation compounds on triple negative breast cancer cell (MDA-MB-231)

In the current study, the cytotoxicity of apigenin, pinocembrin, and castillicetin-2 compounds was evaluated using a cell viability assay on breast cancer cells (MDA-MB-231), which were exposed to concentrations ranging from 20 to 100 µg/mL for incubation periods of 24, 48, and 72 h.

In Fig. 1, cell viability tests showed that all three compounds suppressed the growth rate of MDA-MB-231 cells in a dose and time-dependent manner. Moreover, the compounds exerted considerable cytotoxicity against MDA-MB-231 cells, with IC_{50} values of 10.82 ± 0.7, 25.24 ± 0.8, and 7.7 ± 0.6 µg/mL for apigenin, pinocembrin, and castillicetin-2 compounds, respectively. Most importantly, apigenin and castillicetin-2 had a greater cytotoxicity effect than pinocembrin because they had lower IC_{50} values at the same dose concentrations.

Among the three compounds, apigenin and castillicetin-2 were the most active in terms of cytotoxicity, with IC_{50} of MDM-B-231 cells being 7.7 µg/mL. Similar results were seen for doxorubicin, which showed an IC_{50} of 6.2 ± 0.5 µM for MDA-MB-231 cells ($p < 0.01$), serving as a standard positive control.

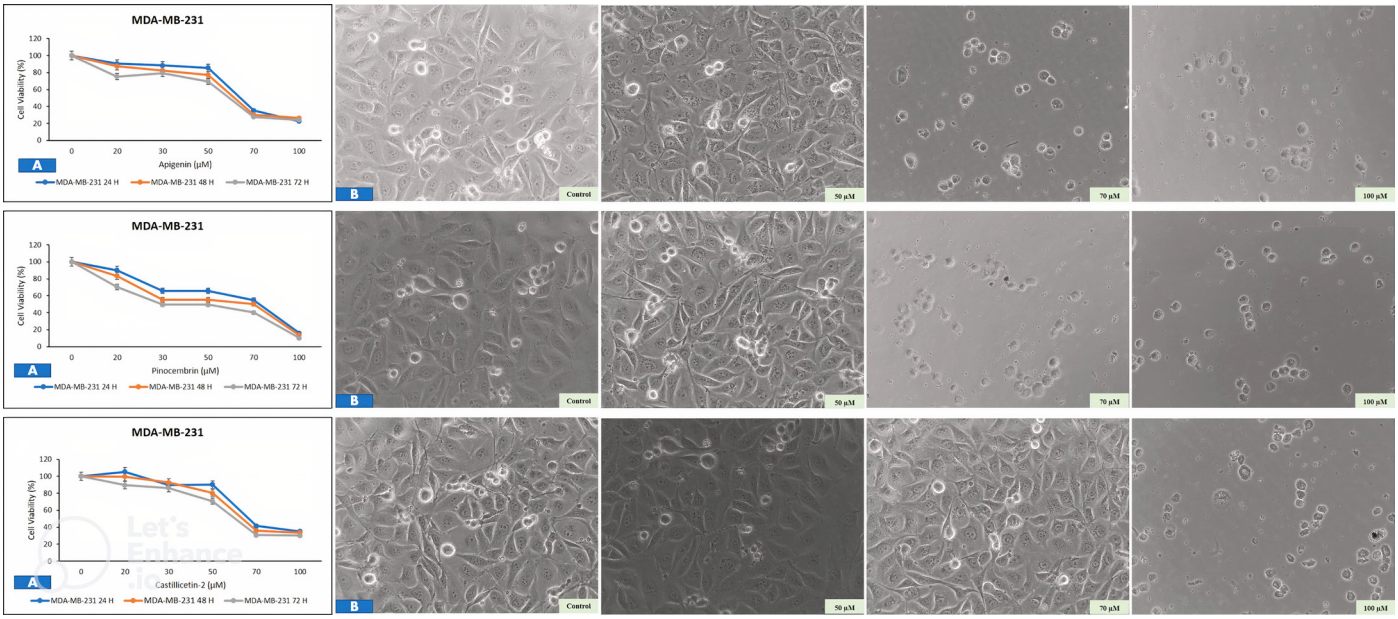

Fig. 1. Effects of Apigenin, Pinocembrin, and Castillicetin-2 on MDA-MB-231 human breast cancer cell line morphological changes and their viability. (a) Changes in viability (%) of Apigenin, Pinocembrin, and Castillicetin-2 treated, control, and untreated exponential MDA-MB-231 cells for 24, 48, and 72 hours. (b) Imaging of MDA-MB-231 cells incubated with different concentrations of Apigenin, Pinocembrin, and Castillicetin-2 for 48 hours. The data shows the mean ± standard error from three independent experiments, each performed in triplicate ($p < 0.05$, $p < 0.01$).

Table 1.
Docking scores and affinity constants for ligand HER2 and CDK6 receptor complexes.

Ligand	G score (Kcal/mol) HER2	Affinity constant (Ka)	G score (Kcal/mol) CDK6	Affinity constant (Ka)
Apigenin	-7.149	2.1 x 10^6 M^-1	-9.139	2.7 x 10^6 M^-1
Pinocembrin	-8.739	1.2 x 10^6 M^-1	-7.558	1.8 x 10^6 M^-1
Castillicetin-2	-11.267	2.5 x 10^6 M^-1	-9.312	3.1 x 10^6 M^-1

Also, we followed the morphological changes in the cells after exposure to apigenin, pinocembrin, and castillicetin-2. A light microscope view showed a drop in the cell number with an increase in the concentration of the compounds, signifying the ability of the compounds to diminish cellular proliferation.

The current findings affirm that apigenin, pinocembrin, and castillicetin-2 are indeed very bioactive and effective as potential chemotherapeutics against the MDA-MB-231 breast cancer cell line.

3.3 Molecular docking

The purpose of molecular docking is mainly relevant in modern drug design and bioactive compounds. It permits an in-depth analysis of the interactions between particular drug candidates and target proteins to ascertain the efficacy and clinical relevance of the interactions. In this study, the binding interactions between the selected drug candidates and target proteins related to cancer were examined through molecular docking analysis.

Table 1 provides the docking results for the interactions of CDK6 and HER2 receptors, for which lapatinib was used as a positive control, given its established binding to the receptor.

The ligands of interest are naturally relevant compounds obtained from certain plants, some of which are already used in managing some diseases. Considering the extensive range of available phytocompounds, this study chose to include compounds of varying structures in investigating of identifying some structure–activity relationships with the receptors of interest.

The docking energy values, given as negative free energy, indicate the strength of interaction; a more negative docking energy value means a higher binding affinity. This is significant since free energy is related to the dissociation constant (Kd); the higher the free energy, the lower the Kd, in this case. This suggests that ligands with higher negative dissociation constants have greater affinities for the receptors and support their candidacy as possible therapeutics.

The dissociation constant (Kd) reflects the interaction between a receptor and a ligand, as it shows the binding affinity of a ligand to its receptor. From the molecular docking studies, it was found that pinocembrin, apigenin, and castillicetin-2 showed competitive docking energies with lapatinib, the control. However, castillicetin-2 was shown to have the most negative docking energies and the lowest Kd values, which indicates that it binds to HER2 more effectively than pinocembrin and apigenin, which means that it would be more therapeutically beneficial. This implies that these ligands have significant therapeutic value in targeting HER2 for cancer treatment.

The results of the molecular docking analysis for the various ligands interacting with the CDK6 are presented in Table 1. In comparison to HER2, pinocembrin, apigenin, and castillicetin-2 exhibited more positive binding energies with CDK6, which suggests selectivity towards HER2. This difference in binding behavior indicates that these ligands are likely to bind more to HER2 than CDK6, indicating that they may be more useful in treating HER2-positive cancers.

Out of all evaluated ligands, castillicetin-2 had the strongest binding affinity for the HER2 receptor since its docking energy values relative to lapatinib, a clinically tested HER2 inhibitor, were much more positive. This means that castillicetin-2 may be more selective for HER2-positive breast cancer, representing a potential targeted therapy for this aggressive cancer subtype.

Besides this, castillicetin-2 had the most positive binding energy with CDK6 among the tested ligands, although it was still less favorable than HER2. On the other hand, apigenin and pinocembrin exhibited binding energies that were near lapatinib but did not exceed it. While most of the ligands had nearly the same binding energies as lapatinib, only castillicetin-2 had a greater affinity than the reference drug. This advantage may render castillicetin-2 a more attractive candidate for advanced studies, considering its high binding energies to both HER2 and CDK6, which may increase its usefulness in treating more conditions.

Docking results from interaction analyses showed the molecular interactions that aid in the stabilization of the ligand in the pockets of HER2 and CDK6 receptors. The sustaining contacts, namely hydrogen bonds, hydrophobic, and π-π interactions, outline the possible efficacy of the ligands as inhibitors.

3.3.1 HER2 receptor interactions

Pinocembrin showed a stable pose in the HER2 receptor pocket where it interacted with GLY and ANS850 through hydrogen bonds. These bonds played an important part in sustaining the ligand. Additionally, hydrophobic interaction with LEU726 and PHE864 increased the stability of the complex, which further increased the binding of pinocembrin to HER2.

Castillicetin-2 showed a strong interaction with HER2 by forming hydrogen bonds with ASP808 and THR862. Complex stability was further increased by the interaction of the ligand with the receptor by means of stacking bonds with PHE864, which means greater binding energy for the complex due to non-destructive forces.

For apigenin, its interaction within the HER2 receptor was also very active as it was binding through hydrogen bonds with LYS753 and ASP808. Besides, hydrophobic interaction with LEU726 also facilitated the binding stability, which is important to keep the ligand in the receptor pocket and thus to keep the receptor in the binding pocket of the receptor.

3.3.2 CDK6 receptor interactions

Pinocembrin's affinity toward CDK6 was noteworthy because it establishes hydrogen bonds with GLN98 and ASP163. Moreover, hydrophobic interactions with ILE19 and PHE93 further anchored the ligand in the binding pocket, corroborating the claim that it may act as a potent CDK6 inhibitor.

Castillicetin-2 was found to have considerable binding stability with CDK6, which resulted from hydrogen bonding interactions with LYS43 and ASP145. Enhanced stability for the complex was provided by stacking interactions with HIS100, which served to consolidate the position of the ligand and confirm its inhibitory efficacy.

Apigenin bound oxygenable regions CDK6 receptor pocket and verified the ligand interaction by hydrogen bonds with ASP145 and GLN98. Other than necked angle of interaction bonds were applied, which are known as Van der Waals interactions, ligands were bolstered in the receptor, stating apigenin's assumption as a CDK6 inhibitor.

The docking results provide insights into the key hydrogen bonds, hydrophobic interactions, and π-π stacking formations that contribute to the binding stability of these molecules within the active sites of the target proteins. The 2D interaction diagrams illustrate the specific amino acid residues involved in ligand stabilization, while the 3D docking conformations confirm the spatial orientation of the molecules within the binding pockets (Fig. 2).

4. Discussion

The present study successfully isolated and characterized three bioactive flavonoid compounds, namely apigenin, pinocembrin, and castillicetin-2, from PPE using column chromatography. The cytotoxic potential of these compounds was further evaluated against MDA-MB-231 breast cancer cells, revealing significant antiproliferative activity.

Pinocembrin was identified based on its HR-ESI-MS and NMR spectra. The former spectrum showed a deprotonated molecular ion peak at m/z 255.0623, corresponding to a molecular formula of $C_{15}H_{12}O_4$.

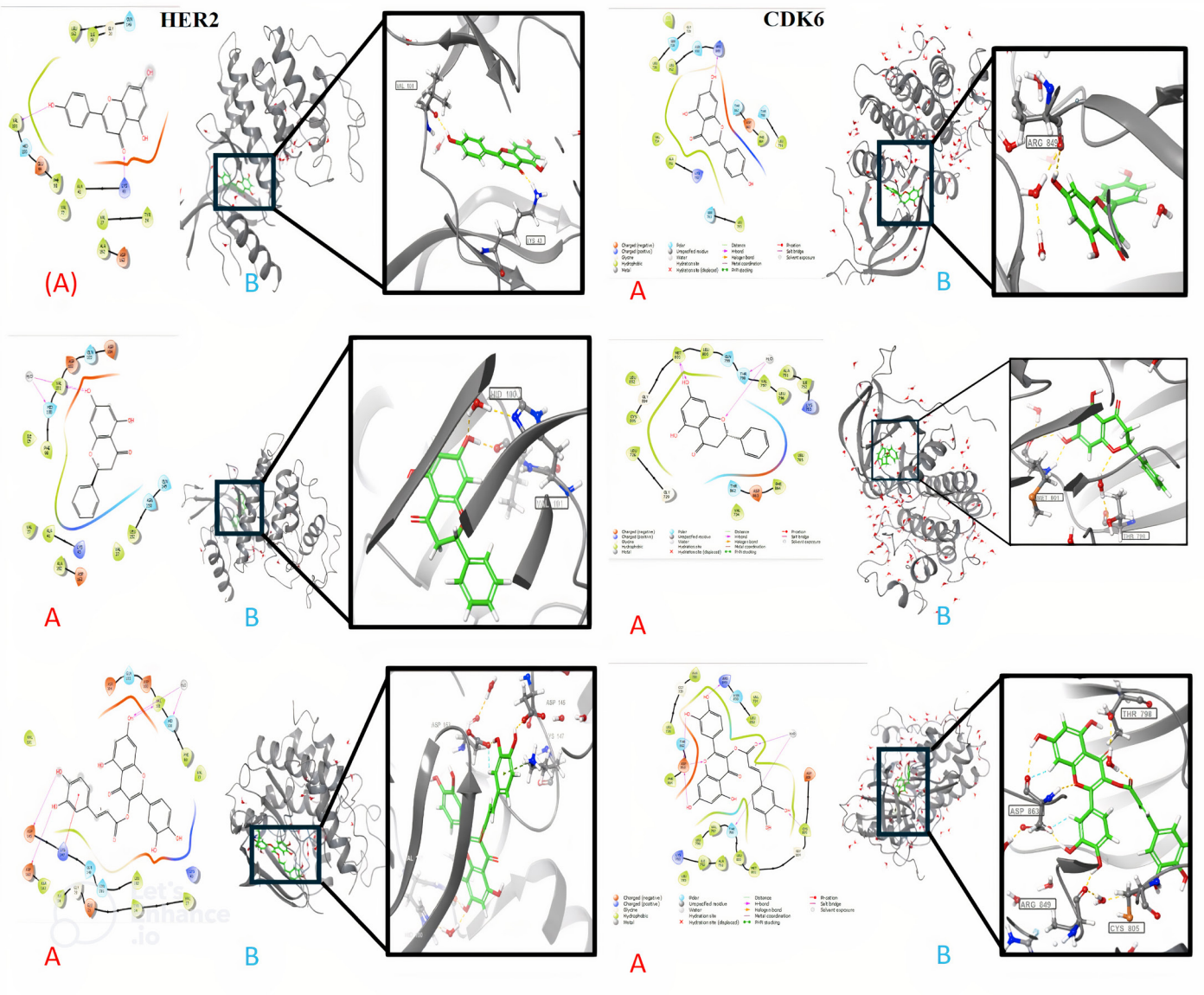

Fig. 2. 2D and 3D representations of binding interactions between isolated compounds and amino acid residues within 4 Å of the active sites of HER2 and CDK6. The figure also shows the orientation of the compounds within the binding pockets. Hydrogen bonds, aromatic–hydrogen interactions, and ?–? stacking interactions are indicated by yellow, blue, and green dotted lines, respectively.

The ^{1}H NMR spectrum exhibited a characteristic ABX system, with two doublets at δ 6.65 and δ 6.68 (H6 and H8), suggesting a typical flavanone framework. Further, doublets at δ 7.04 (H2′ and H6′) and δ 7.04 (H3′ and H5′) were identified, confirming a para-substituted benzene ring. The ^{13}C NMR spectrum showed 15 carbon signals, including a deshielded carbonyl at δ 196.14 (C4) and hydroxylated carbons at δ 163.71 (C5) and δ 162.94 (C7). These findings are in agreement with previous reports on pinocembrin (Rasul *et al.*, 2013; Wang *et al.*, 2023).

The HR-ESI-MS spectrum confirmed the molecular formula $C_{15}H_{10}O_5$, with a molecular ion peak at m/z 270.1078.

In the ^{1}H NMR spectrum, the aromatic protons H3′ and H5′, along with H2′ and H6′, appeared as doublets at δ 7.90 and δ 7.89, respectively. The H6 and H8 signals resonated as broad singlets at δ 6.18 and δ 6.73, indicative of meta-coupled protons in a flavone nucleus. The ^{13}C NMR spectrum showed characteristic signals at δ 181.73 (C4, carbonyl), δ 163.86 (C6), and δ 163.76 (C5), which confirmed the flavone structure. These spectral data matched with literature values for apigenin (Kumar *et al.*, 2018; Tavakoli *et al.*, 2022).

Castillicetin-2, isolated as a yellow powder, was identified as castillicetin based on its mass spectrometric and NMR spectral data. The HR-ESI-MS spectrum exhibited a deprotonated molecular ion peak at m/z 464.07252, consistent with a molecular formula of $C_{24}H_{16}O_{10}$. The ^{1}H NMR spectrum displayed characteristic ABX patterns, with proton shifts at δ 7.03 (H-2’), δ 6.97 (H-5’), and δ 7.56 (H-6’). These findings strongly indicated a flavonol structure.

The ^{13}C NMR spectrum showed a deshielded carbonyl resonance at δ 176.29 (C4), and downfield shifts at δ 168.37 (C9”) suggested the presence of an ester bond. The HMBC spectrum confirmed key correlations, supporting the structure assignment. The obtained spectral data were consistent with literature reports on Castillicetin-2 (Subban *et al.*, 2008).

The cytotoxic effects of pinocembrin, apigenin, and castillicetin-2 on MDA-MB-231 breast cancer cells were assessed using a cell viability assay, revealing a dose- and time-dependent inhibition of cell proliferation. The IC_{50} values indicated that apigenin (10.82 ± 0.7 μg/mL) and castillicetin-2 (7.7 ± 0.6 μg/mL) exhibited stronger cytotoxicity compared to pinocembrin (25.24 ± 0.8 μg/mL), with castillicetin-2 demonstrating the most potent activity. These findings suggest that apigenin and castillicetin-2 could serve as promising chemotherapeutic agents due to their superior efficacy. A comparison with other naturally derived flavonoids, such as quercetin and luteolin, reveals that apigenin

and castillicetin-2 exhibit cytotoxic properties comparable to these well-studied compounds. Previous studies have shown that quercetin has an IC_{50} of approximately 10 μM against MDA-MB-231 cells (Alzahrani *et al.*, 2024), which aligns with the observed cytotoxicity of apigenin. Similarly, luteolin has demonstrated IC_{50} values in the range of 5-10 μM (Tian *et al.*, 2021), further supporting the potent anticancer potential of castillicetin-2. These similarities reinforce the importance of flavonoids in breast cancer therapy and highlight castillicetin-2 as a particularly strong candidate due to its lower IC_{50} value. A comparison with other naturally derived flavonoids, such as quercetin and luteolin, reveals that apigenin and castillicetin-2 exhibit cytotoxic properties comparable to these well-studied compounds. These similarities reinforce the importance of flavonoids in breast cancer therapy and highlight castillicetin-2 as a particularly strong candidate due to its lower IC_{50} value. These results corroborate prior studies underscoring the anticancer activities of apigenin and pinocembrin. As a dietary flavonoid, apigenin was shown to affect several major signaling pathways responsible for cancer cell proliferation, invasion, and metastasis, which include JAK/STAT, PI3K/Akt/mTOR, MAPK/ERK, NF-□B, and Wnt/β-catenin pathways (Singh *et al.*, 2019; Javed *et al.*, 2021; Zhu *et al.*, 2021). Moreover, apigenin has also been shown to inhibit the TNFα/IL-1α-induced CCL_2 release in MDA-MB-231 cells, which could play a role in the inhibition of macrophage infiltration into the tumor (Bauer *et al.*, 2017). Pinocembrin, another flavonoid, has displayed significant anti-proliferative and anti-metastatic properties against breast cancer cells by downregulating the PI3K/AKT signaling pathway. It has been demonstrated that pinocembrin suppresses MCF-7 and MDA-MB-231 cell proliferation and induces apoptosis via cell cycle arrest *in vitro*. Besides, several *in vivo* studies using murine models of breast cancer tumors have validated the suppressive impact of Pinocembrin on tumor growth (Márquez-Garbán *et al.*, 2024). While the anticancer properties of apigenin and pinocembrin are well-documented, there is limited information on castillicetin-2. The present study's finding that castillicetin-2 exhibits the most potent cytotoxic effect among the three compounds suggests that it may be a promising candidate for further investigation as a potential chemotherapeutic agent.

Morphological assessments of MDA-MB-231 cells following treatment with the three compounds further substantiated their cytotoxic effects. Light microscopy revealed a significant reduction in cell number and changes in cellular morphology, including cell shrinkage and detachment, suggesting apoptotic induction. These observations align with previous reports on flavonoids inducing apoptosis in breast cancer cells by modulating key pathways such as PI3K/Akt and MAPK(Nordin *et al.*, 2019). Also, the distinct morphological features of MDA-MB-231 cells, such as the decline in cell count along with certain changes that suggest apoptosis, bolster the claim of the compounds' antiproliferative properties. These findings are consistent with prior studies indicating that flavonoids such as Apigenin can induce apoptosis and\ inhibit metastasis in breast cancer cells (Zhang *et al.*, 2024).

The molecular docking analysis provided further insights into the interaction of these compounds with key breast cancer-related targets, namely HER2 and CDK6 (Yousif *et al.*, 2023; Millan-Casarrubias *et al.*, 2025). HER2 is a critical oncogenic driver in aggressive breast cancers, and targeting it has led to significant clinical advances, as seen with trastuzumab and lapatinib. Among the tested compounds, castillicetin-2 exhibited the highest binding affinity to HER2, with docking energy values surpassing those of pinocembrin and apigenin (Sharma *et al.*, 2022). This suggests that castillicetin-2 could serve as a more selective HER2-targeted agent, comparable to lapatinib, a clinically approved HER2 inhibitor.

Similarly, CDK6, a regulator of cell cycle progression, has emerged as a therapeutic target in breast cancer, particularly for hormone receptor-positive subtypes. While all three compounds showed moderate binding affinities for CDK6, castillicetin-2 demonstrated the highest stability within the receptor pocket, forming key hydrogen bonds with LYS43 and ASP145 (Finn *et al.*, 2016). This interaction is consistent with previously reported CDK6 inhibitors such as palbociclib, which also engages in hydrogen bonding with similar residues (Susanti and Tjahjono, 2021). Although castillicetin-2 exhibited stronger binding to HER2 than CDK6, its dual-targeting potential may offer a broader therapeutic application.

Pinocembrin, while less cytotoxic than apigenin and castillicetin-2, displayed significant interactions with both HER2 and CDK6, forming hydrogen bonds with GLY102 and ASN850 in HER2 and GLN98 and ASP163 in CDK6. This suggests that pinocembrin may still have therapeutic relevance, particularly as a combination therapy to enhance the efficacy of standard treatments. Prior studies have demonstrated that flavonoids like chrysin and fisetin can sensitize cancer cells to chemotherapy (Lan *et al.*, 2016; Elbatreek *et al.*, 2023), and pinocembrin might serve a similar role in overcoming resistance mechanisms in breast cancer treatment.

Further analysis of the molecular interactions revealed that castillicetin-2 formed additional stacking interactions with PHE864 in HER2, a feature absent in apigenin and pinocembrin. This interaction likely contributes to its superior binding affinity, emphasizing its potential as a lead compound for HER2-targeted drug development. In comparison, apigenin interacted with LYS753 and ASP808, forming stable hydrogen bonds but with slightly lower binding energy, indicating a slightly weaker yet still significant inhibitory effect (Koumarianou *et al.*, 2015).

Taken together, the results from both cytotoxicity assays and molecular docking studies strongly support the potential of castillicetin-2 as a potent therapeutic agent against HER2-positive breast cancer. While apigenin also exhibited strong cytotoxic effects, its slightly lower binding affinity to HER2 suggests that it may function through additional mechanisms, such as modulation of inflammatory pathways (Chen *et al.*, 2023). Pinocembrin, although less effective in direct cytotoxicity, displayed notable interactions with both HER2 and CDK6, suggesting a potential role in combination therapies.

Future studies should focus on *in vivo* evaluations of these compounds to determine their pharmacokinetic profiles and potential synergistic effects with existing chemotherapeutic agents (Carneiro and El-Deiry 2020). Additionally, mechanistic studies exploring their effects on apoptosis-related pathways, such as caspase activation and BCL-2 regulation, would provide further insights into their anticancer mechanisms (Sailo *et al.*, 2025). Overall, the current study highlights the therapeutic promise of pinocembrin, apigenin, and castillicetin-2, with castillicetin-2 emerging as a particularly strong candidate for further drug development in breast cancer treatment.

5. Conclusion

This study described the successful isolation and characterization of three bioactive flavonoids from bee propolis with notable anticancer activity. The cytotoxicity assays show that MDA-MB-231 breast cancer cells were the most inhibited by castillicetin-2, followed by apigenin and pinocembrin. Their molecular docking analysis further corroborated these findings, where the flavonoids were predicted to act as targeted inhibitors of HER2 and CDK6, which are key processes undergoing breast cancer treatment. It appears that propolis-derived flavonoids may serve as promising candidates for the treatment of breast cancer, which deserve additional studies in vivo and mechanistic studies to fully understand their therapeutic capabilities. Future work should concentrate on pharmacokinetic profiling along with combinatorial approaches to currently available chemotherapeutics to improve clinical efficacy. The anticancer impacts of apigenin and Pinocembrin are well documented, yet there is little information regarding castillicetin-2. The finding of its greater cytotoxicity compared to the other two flavonoids demonstrates that further research should be conducted in the area of chemotherapeutic development.

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Declaration of competing interest

The authors declare that they have no competing financial interests or personal relationships that could have influenced the work presented in this paper.

Declaration of Generative AI and AI-assisted technologies in the writing process

The authors confirm that there was no use of artificial intelligence (AI)-assisted technology for assisting in the writing or editing of the manuscript and no images were manipulated using AI.

Supplementary data

Supplementary information are provided in a separate file "Supplementary" https://dx.doi.org/10.25259/JKSUS_735_2025.

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