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Naringin from *Citrus maxima* peel: a potential therapeutic agent for breast cancer via PKM2 inhibition

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Abstract

Introduction An extensive array of medicinal plants has undergone investigation, underscoring the imperative for the continued screening of natural inhibitors with the potential to target cancer metabolism. The current research endeavor was directed toward evaluating the chemotherapeutic efficacy of peel extracts of *Citrus maxima* and its constituent flavonoid, Naringin (NA), in the context of breast cancer, specifically targeting the pyruvate kinase isozyme M2 (PKM2).

Materials and methods Extracts from the peel of *Citrus maxima* were prepared and analyzed using liquid chromatography–mass spectrometry (LC–MS) to detect the presence of the bioactive compound, NA. The potential anti-proliferative effects of these peel extracts of *Citrus maxima* and NA were examined against human breast cancer cell lines utilizing an MTT assay. To investigate the distribution of the cell cycle, cell cycle analysis was conducted. The induction of apoptosis was ascertained using Annexin V-FITC through flow cytometry. The protein expression of PKM2 was analyzed using Western blotting. Molecular docking and dynamics simulations analysis were employed.

Results Liquid chromatography–mass spectrometry (LC–MS) analysis confirmed the existence of NA within the extracts of *Citrus maxima*. Both the crude extracts and NA demonstrated a dose-dependent inhibition of breast cancer cell proliferation. Our findings indicate that these crude extracts and NA instigate both early and late apoptosis, in addition to causing cell cycle arrest in the G2/M phase. Immunoblotting studies further revealed that the expression of PKM2 protein was suppressed by both the crude extracts and NA. Computational analysis demonstrated stable binding affinity with Ser77, His78, and Lys207 of PKM2.

Conclusion This investigation unveils the presence of NA within *Citrus maxima* extracts, exhibiting robust affinity for PKM2 via molecular docking and dynamics simulations. Extracts and NA dose-dependently inhibit breast cancer cell proliferation. Notably, PKM2 regulates cancer cell glycolysis, promising intricate therapeutic prospects for breast cancer.

Keywords *Citrus maxima* peel extract, PKM2, Naringin, Breast cancer, LC–MS, Molecular docking, Dynamics simulations

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1 Introduction

Breast cancer (BC) represents the most frequently diagnosed cancer in the female population, accounting for over one-tenth of all new cancer cases annually. As per the Globocan data for the year 2020, BC was responsible for 10.6% of total mortality and constituted 13.5% of all cancer incidences in India [1]. While existing therapeutic strategies exhibit efficacy, they often compromise the patient's quality of life due to their toxic nature, thereby necessitating the exploration for safer, naturally-derived pharmaceuticals. The strategy of targeting cancer cell metabolism has recently emerged as a promising approach for the development of novel adjuvant therapies in oncology. Tumor cells predominantly rely on aerobic glycolysis in order to meet the high energy demands of the cells producing lactate even in the presence of oxygen a phenomenon termed as Warburg effect. Pyruvate kinase (PK), a key enzyme in the glycolytic metabolic pathway, converts phosphoenol pyruvate to pyruvate. One of the isoforms of PK is pyruvate kinase M2 (PKM2) that plays a vital role in regulating the Warburg effect, that exists in dimer (less active) and tetramer (highly active) forms. The glycolytic role of the PKM2 dimer is predominantly observed in proliferative cells, such as embryonic and cancer cells. Here, it facilitates the generation of glycolytic intermediates essential for macromolecular biosynthesis. Additionally, the translocation of PKM2 into the nucleus, where it associates with transcription factors, instigates the transcription of a multitude of genes implicated in the progression of cancer cells, a function that is non-glycolytic in nature. PKM2 is implicated in a pivotal capacity in the pathogenesis of tumors [2]. Consequently, it has been elucidated that the strategy of targeting enzymes involved in glucose metabolism presents a feasible methodology for the synthesis of anti-cancer therapeutics. Several studies have also unveiled that specific natural compounds possess the capacity to decelerate the proliferation of cancer cells by modulating the enzymatic processes associated with glucose metabolism [3, 4], whereas only a limited number of PKM2 inhibitors have undergone comprehensive scrutiny. Shikonin has emerged as a potent PKM2 inhibitor; however, its pharmaceutical exploration and clinical trial parameters remain incompletely delineated [5]. Consequently, the pursuit for innovative agents exhibiting minimal toxicity toward both normal and cancer cells is currently underway. *Citrus maxima* (*C. maxima*) or *Citrus grandis* (L.), colloquially referred to as pommelo, is the largest citrus fruit belonging to the Rutaceae family and remains a subject for further exploration. Prior scientific investigations have identified the presence of active polyphenolic compounds predominantly in the peel fraction of *C. maxima* extract, as compared to other

components of the fruit. Notably, these peel extracts of *C. maxima* have demonstrated significant antioxidant activity [6]. Low molecular weight entities such as flavonoids represent significant bioactive constituents in citrus peel combating cancer and other diseases. Their ability to inhibit the function of intracellular signaling molecules, including topoisomerases, phosphodiesterases, kinases, and other regulatory enzymes, has been associated with their pharmacological effectiveness [7]. High-performance liquid chromatography (HPLC) analysis of *C. maxima* peel, identified Naringin (NA) and Naringenin as the most abundant flavonoids [8] serving as therapeutic agents due to their bioactive properties. These flavonoids have demonstrated anti-cancer properties in both in vitro and in vivo studies, inhibiting cellular proliferation, inducing cell cycle disruption, and triggering apoptosis, thereby mitigating the risk of cancer [8]. Nevertheless, only a limited number of investigations have explored the impact of *Citrus maxima* peel extracts on BC and its metabolic processes, specifically targeting PKM2.

The current research endeavor is centered on evaluating the anti-cancer efficacy of both aqueous and ethanol extracts derived from the peel of *Citrus maxima*, in addition to the flavonoid NA, against the breast cancer cell lines MDA-MB-231 and MCF-7. Furthermore, the study seeks to elucidate their mechanism of action against PKM2 through the application of in silico molecular docking and dynamic simulations. This approach could potentially provide valuable insights into the therapeutic potential of these compounds in the context of BC management.

2 Materials and method

2.1 Plant material and extraction

Citrus maxima fruits were collected from Shakleshpur, Karnataka, India. The fruits were washed and cleaned in running tap water and peels were separated from the fruit. *C. maxima* peel fractions were then dried for seven days at 40–50 °C in a hot air oven. The dried peel was powdered. **Extract preparation—For Aqueous extract**—The finely ground powder was boiled for 20 min in distilled water, which it was filtered with Whatman No 1 filter paper. **For ethanolic extract**—The dried material had been extracted with ethanol (99%) in Soxhlet apparatus at 55 °C for 24 h. Both extracts were eventually concentrated by evaporating the solvent with a rotary flash evaporator and stored at 4 °C until use [9].

2.2 LC–MS analysis

The peel extracts of *C. maxima* (aqueous and ethanolic) and standard NA was obtained from Santa Cruz Biotechnology, TX, USA, were subjected to LC–MS analysis in

a Shimadzu LCMS-8030 Triple quadrupole, Japan system. The LC–MS instrument outfitted with autosampler, Shimadzu Nexera uHPLC/HPLC front end, UV detector was used for mass detection. 1 mg of the dried aqueous and ethanolic extracts and standard NA were dissolved in one milliliter of LC-grade methanol for LC–MS analysis, which was then, filtered using a syringe filter (0.2 μ m). The injection volume was 0.2–1 μ L/min; sample flow rate was 1.00 mL/min and nebulizing gas flow: 3.00 L/min. Interface temperature was maintained at 350 °C and DL temperature at 250 °C. In order to identify the presence of NA with the molecular weight m/z 580.4, the standard NA (10 ng/mL in LC-grade methanol) and the sample were separately injected into the mass spectrometer (MS). The detection was carried out through electrospray ionization (ESI) probe at positive and negative modes with mass range from 165 to 1000 m/z . As a mobile phase, 0.1% formic acid and methanol solvents were used [10].

2.3 Cell culture condition and treatment

The human breast cancer cell lines MDA-MB 231 and MCF-7 were obtained from NCCS Pune. Using Dulbecco's Modified Eagles' medium (DMEM, Gibco) supplemented with FBS (10%) (Gibco), penicillin/streptomycin (100 U/mL) (ABAM, Himedia), these cells were cultured. Both the cells were maintained at 5%CO₂ at 37 °C in a humidified incubator. *Citrus maxima* aqueous extract 1 mg dissolved in 1 mL of DMEM without FBS and ethanolic extract (200 mg and 1000 mg) and NA (100 mM and 500 mM, Sigma Aldrich) stock solutions were prepared in dimethylsulfoxide (DMSO) and stored in –20 °C for further use [11].

2.4 Cell viability assay

Breast cancer cells (BCC) (0.05×10^6) were cultured in 24 well plates and cultured in CO₂ incubator at 37 °C with appropriate growth culture media. Upon reaching confluence (70%) and cells underwent serum starvation for 24 h, following which they were treated with different concentrations of *Citrus maxima* extracts and NA with control and DMSO for 48 h. The cells were washed with phosphate buffer saline (PBS) and replaced with fresh media. The plates were incubated at 37 °C for 4 h after adding 40 μ L of MTT (SRL) 5 mg per mL of PBS. After removing the media, 300 μ L of DMSO was added. After solubilization of the formazan crystals, the absorbance was assessed at 570 nm with a microplate reader [11].

2.5 Cell cycle analysis

0.3×10^6 cells were seeded in 6 well plates treated with *Citrus maxima* peel extracts and NA concentrations with IC₅₀ and control and DMSO control for 48 h. The

cells were washed with PBS and harvested, then fixed with 70% ethanol at 20 °C overnight. The fixed cells were stained with 50 μ g/mL propidium iodide (Himedia) and 1 mg/mL Rnase A (Himedia) in PBS and incubated at 37 °C for 15 min to determine the cell cycle distribution, which was performed using BD Accuri™ C6 Plus flow cytometer (BD Biosciences, San Diego, CA, USA). The data was analyzed using Flow Jo software [12].

2.6 Apoptosis analysis by annexin V-FITC binding assay

0.3×10^6 cells were seeded in 6 well plates and treated with *Citrus maxima* extract and NA concentrations for 48 h. Apoptosis was analyzed using eBioscience Annexin V Apoptosis Detection kit. The cells were washed with 1X PBS and 1X binding buffer and resuspended with 1×binding buffer to which 5 μ L of Annexin V and 5 μ L of propidium iodide staining solution was added, incubated for 10–15 min at room temperature for each stain. BD Accuri C6 plus flow cytometer (BD Biosciences) was used to analyze the stained samples and, data were analyzed using the Flow Jo software [13, 14].

2.7 Western blot analysis

MDA-MB –231 and MCF-7 cells were harvested after 48 h treatment with *Citrus maxima* extracts and NA. Cells were lysed with lysis buffer, and total proteins were estimated using bicinchoninic acid assay [15]. Protein components were suspended in SDS-loading buffer and heated at 100 °C for 5 min. Proteins were loaded and separated on sodium dodecyl sulfate–polyacrylamide gels and transferred onto nitrocellulose membrane (Himedia). After blocking the membrane with 3% BSA (Himedia) in 1×TBST for 1 h, followed by incubation overnight at 4 °C with primary antibody (1:1000 dilution) anti-PKM2 (R&D Systems Minneapolis, MN) and then incubated with HRP conjugate anti-mouse secondary antibody (1:2000 dilutions). GAPDH (Santa Cruz, CA) used as internal control. Using a ChemiDoc system, protein bands were visualized [16].

2.8 In silico approach

2.8.1 Protein preparation

The co-crystal structures of PKM2 (PDB ID: 6B6U) complexed with 2-[3-(2-hydroxy-1,1-dihydroxymethyl-ethylamino)-propylamino]–2-hydroxymethyl-propane-1,3-diol (B3P) were obtained from the protein data bank (PDB) (<https://www.rcsb.org/>). Subsequently, this structure explored processing using the protein preparation wizard, which is a component of the Schrodinger suite 2023–2 [17]. During the preparatory phase, the structural integrity was assessed by verifying the presence of all residues. Additionally, the Prime module was utilized to incorporate loop segments in close proximity to the

active site. Furthermore, the hydrogen atoms were introduced subsequent to the removal of the co-crystal complex. Subsequently, bond orders for amino acid residues were adjusted accordingly. To optimize the enzyme under neutral pH conditions, the protein structure endured minimization using the optimized potentials for liquid simulations (OPLS3) force field, encompassing all atoms.

During molecular docking, PKM2 and B3P established six hydrogen bonds that functioned as active site residues. The residues involved were Asp177, Asp178, Gln329, Glu332 (two), and Gly363, with respective bond lengths of 2.83, 3.12, 3.23, 2.89, 2.93, and 2.87 Å, within a 4 Å region from B3P [17] (Fig. 6B). Additionally, in the context of the structure 6B6U, the residues Leu180 and Lys207 were observed to form van der Waals interactions (vdW) with B3P. These interactions involve the close proximity and attractive forces between the atoms of Leu180 and Lys207 and those of B3P. The vdW interactions contribute to the stabilization and binding of the molecules, further influencing the overall conformation and function of the system. To investigate the active site residues of the protein structure with the PDB ID 6B6U, a grid of dimensions 10 Å × 10 Å × 10 Å was created around it. B3P was used to create the grid using a distance cutoff of 4 Å. This process was carried out using Glide module (Schrödinger, LLC, New York, NY, United States, 2023) [18, 19]. In addition to the grid-based docking performed with Glide, the SiteMap module was employed to verify the presence of active site residues within the 4 Å radius of the B3P. SiteMap is a module within the Schrödinger software suite that aids in the identification and characterization of active/binding sites in protein structures [20].

2.8.2 Ligand preparation

The Naringin (PubChem: 442428) 3-dimensional coordinates was obtained from the PubChem database (<https://pubchem.ncbi.nlm.nih.gov/>). The chemical was processed utilizing the LigPrep module, which treats it as a ligand and applies the OPLS3 force field, in order to get it ready for additional analysis. In order to explore different protonation states and ionization, the Epik tool was employed. This involved conducting energy minimization and considering all possible protomers (protonation states) for Naringin at a neutral pH of 7.0 ± 2.0 [21–23]. Stereo-isomers were created for structure of ligand in order to take into consideration the existence of unassigned stereogenic centers. For each ligand, a maximum of 32 stereo-isomers were examined. Each produced ligand stereoisomer's output was used for the ensuing molecular docking analysis, which aims to understand the interactions between the ligand (Naringin) and 6B6U [24].

2.8.3 Molecular docking

The molecular docking analysis of the prepared protein and ligand was performed using the Glide tool within the maestro software, specifically employing the rigid receptor docking (RRD) approach. Each ligand was docked into the protein's improved low-energy conformation in this analysis, which was produced by previous stages of the investigation using a high-through virtual screening and conventional precision techniques. The binding affinity and possible interactions of the resultant docked complexes were assessed using the Glide score (XPG Score). To visualize the interactions between the ligand and the active site residues of the proteins, the ligand interaction tool was utilized. Furthermore, molecular mechanics/generalized born surface area (MM/GBSA) computations were used to further analyze the docked complexes [19]. By predicting the binding free energies of complexes, this method offers important information on the strength and stability of ligand–protein interactions [24].

2.8.4 Molecular dynamics (MD) simulations analysis

Molecular dynamics (MD) simulations were used in an exquisite conformational stability analysis to further evaluate the MM/GBSA docked complex of Naringin with PKM2 binding interactions with minimal binding energy. Using the Desmond version 2022.4 program, the MD simulations were carefully carried out for up to 100 ns (ns). Determining the conformational stability of the docked complex and the complexes inside a solvated model system that integrated with ordered water molecules were the goals of this investigation. These molecules, organized in their arrangement, potentially participate within the protein binding sites, hold the potential to significantly influence the binding of PKM2 and Naringin, effectively bridging the interactions of the complex. The binding affinity may be significantly influenced by this bridging action. The particle mesh-based Ewald algorithms were used to calculate long-range electrostatic energy and forces. The orthorhombic simple point charge (SPC) water model was skillfully used to solve the PKM2 and Naringin docked complexes. This solvated system underwent process of neutralization through counter ions, ensuring a physiological salt limited no more than 0.15 M.

Utilizing the optimal potentials for liquid simulations-AA (OPLS-AA) 2005 force field, the complete docked complex system was parameterized [19]. The particle mesh Ewald (PME) method was skillfully used to handle the electrostatic interactions, and the system was established within periodic boundary conditions. 10 Å was chosen as the cutoff for Lennard–Jones interactions. The SHAKE algorithm was skillfully used to limit the motion of covalent bonds comprising hydrogen atoms in

order to better preserve structural integrity. Before starting the demanding MD simulations, the solvated model system was subjected to a thorough six-step relaxation technique for relaxation and energy minimization. Following that, the reduced system was carefully examined in the NVT ensemble in a 12-picosecond (ps) simulation at 10 K, where non-hydrogen solute atoms were expertly constrained at 300 K for 24 ps. Subsequently, the system underwent a 24-ps simulation within the NPT ensemble at 300 K, now devoid of restraints [22–24]. Having achieved this equilibrium, the unencumbered minimized system was then subjected to a comprehensive 100 ns of NPT simulations for production, effectively enabled the exploration of the dynamic behavior of the system. During the first simulations, Berendsen thermostats and barostats were used to control temperature and pressure. For these intricate MD simulations, the Desmond software was adeptly harnessed, utilizing constraints enforced through a specialized variant of the SHAKE algorithm [24, 25].

2.9 Statistical analysis

Statistical analysis was performed using SPSS 22.0 version and Graph Pad PRISM 8.0 software. Experiments were performed in triplicate. Values were expressed as mean $\pm$ SD. Comparisons between control and treated groups were performed by one way ANOVA by applying Bonferroni's multiple comparisons test. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ were accepted as statistically significant.

3 Results

3.1 LC–MS profiling for screening the target compound

Naringin in different fractions of *C. maxima* peel

An investigation utilizing liquid chromatography–mass spectrometry (LC–MS) was conducted to identify the primary bioactive compound, Naringin. The LC–MS analysis of the peel extracts from *Citrus maxima* confirmed the presence of Naringin (Fig. 1). The Naringin has a retention time of 0.615, with a molecular formula of $C_{27}H_{32}O_{14}$, and a presented identified mass/charge of 579.30.

3.2 *Citrus maxima* peel extracts and flavonoid Naringin decreases viability of BCC

The cytotoxicity effect of peel extracts of *Citrus maxima* and NA were estimated by MTT assay for 48 h. The results of MTT assay shown in Fig. 2(A–F) revealed the anti-cancer activity of the extracts against the breast cancer cell lines. After treatment, the peel extracts and NA inhibited the viability of breast cancer cell lines in a dose-dependent manner. The half maximal inhibitory concentration (IC_{50}) of CMPAE, CMPEE extract and NA against

MDA-MB-231 cells were found to be 370 μ g/ml, 940 μ g/ml, and 150 μ M and against MCF-7 cell line were found to be 415 μ g/ml, 2.05 mg/ml and 920 μ M at 48 h, respectively. The extracts showed the most effective cytotoxicity activity against MDA-MB-231 cells.

3.3 *Citrus maxima* peel extracts and flavonoid Naringin mediated cell cycle arrest of BCC

Citrus maxima peel extracts and NA impact on the cell cycle profile of MDA-MB-231 and MCF-7 cells was studied using flow cytometry. The cells were treated for 48 h with their respective IC_{50} obtained from the MTT assay. The result in Fig. 3(A–D) clearly illustrates the alterations in the relative distribution of cells. Following treatment with the investigated extracts and NA, it was seen that the cell population was significantly increased during the G2/M phase of cell cycle in all the treated extracts and NA. The percentage of accumulated MDA-MB-231 cells in G2/M phase after treatment with CMPAE, CMPEE, and NA was $45.13 \pm 2.83\%$, $48.97 \pm 0.70\%$ and $58.57 \pm 0.91\%$ when compared to control 11.37 ± 2.54 and $8.65 \pm 1.50\%$, respectively, while the percentage of accumulated MCF-7 cells was $48.83 \pm 0.38\%$, $40.87 \pm 2.42\%$, and $54.96 \pm 0.06\%$ when compared to control 10.30 ± 0.99 and $10.74 \pm 4.20\%$, respectively.

3.4 *Citrus maxima* peel extracts and flavonoid Naringin induces apoptotic cell death in BCC

In order to investigate the mechanism by which CMP extracts and NA inhibits cell proliferation by inducing apoptosis, Annexin V-FITC/PI staining and flow cytometry analysis was performed. The distribution of MDA-MB-231 and MCF-7 cells in the four quadrants is depicted in Fig. 4A and C.

After 48 h of treatment with CMP extracts and NA at their respective IC_{50} , there was a decrease in the percentage of live cells and accumulation of early and late apoptotic cells and necrotic cells compared with control in MDA-MB-231 and MCF-7 cell lines. The percentage of MDA-MB-231 and MCF-7 cells that were total live cells before treatment was $95.37 \pm 1.01\%$, $94.83 \pm 1.05\%$, $95.7 \pm 1.31\%$, and 95.77 ± 1.60 , while early apoptotic cells were $1.37 \pm 0.50\%$, $1.10 \pm 0.66\%$, $0.63 \pm 0.21\%$, and $9.17 \pm 0.64\%$ and late apoptotic cells were $0.67 \pm 0.32\%$, $0.63 \pm 0.68\%$, $0.80 \pm 0.10\%$, and $0.80 \pm 0.20\%$. Upon treatment with CMPAE, CMPEE, and NA the percentage of live cells were $58.13 \pm 1.01\%$, $39.90 \pm 0.96\%$, $33.33 \pm 0.67\%$, $72.27 \pm 1.05\%$, $34.77 \pm 0.90\%$, and $31.30 \pm 0.72\%$. There was significant increase in the early apoptotic $4.50 \pm 0.62\%$, $11.50 \pm 1.21\%$, $9.83 \pm 20.85\%$, $9.17 \pm 0.64\%$, $15.27 \pm 0.58\%$, and $10.20 \pm 0.40\%$, and late apoptotic cell population $31.63 \pm 0.96\%$, $34.30 \pm 1.01\%$, $41.97 \pm 0.90\%$, $13.93 \pm 1.53\%$, 36.57 ± 0.65 ,

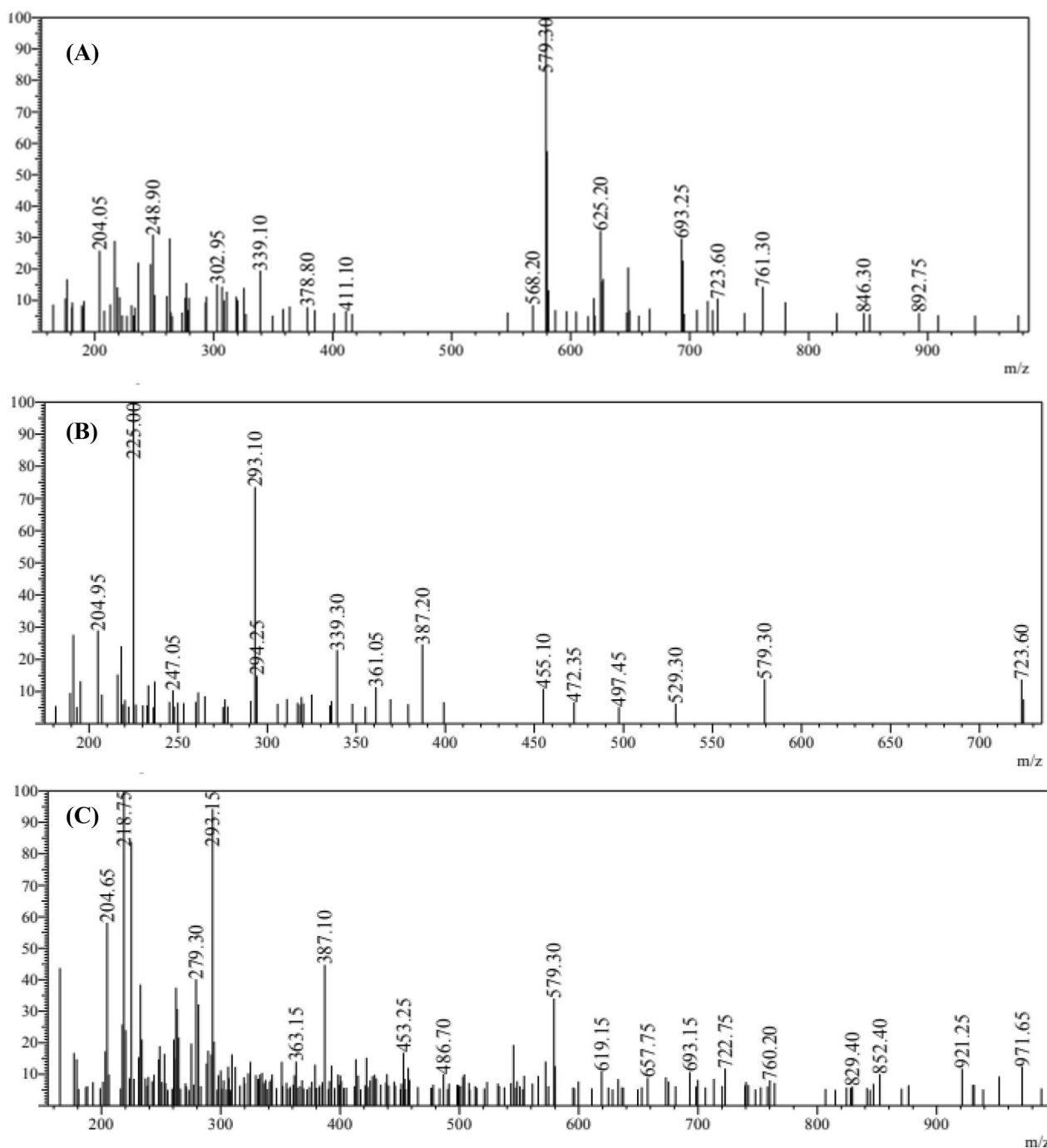


Fig. 1 LC–MS analysis chromatograms (TIC) of **A** NA standard **b** Crude aqueous *Citrus maxima* peel extract and **C** Crude ethanol *Citrus maxima* peel extract (m/z.579.30)

and $45.60 \pm 0.82\%$. A considerable increase in the necrotic cells was observed after treatment with CMPAE, CMPEE, and NA $5.67 \pm 0.68\%$, $13.60 \pm 1.08\%$, and 16.63 ± 0.67 , $4.43 \pm 0.50\%$, $13.30 \pm 1.37\%$, and 12.13 ± 0.25 , respectively, when compared to control $2.33 \pm 0.60\%$, 3.43 ± 0.50 , $2.77 \pm 0.85\%$, and $2.67 \pm 0.78\%$.

3.5 *Citrus maxima* peel extracts and flavonoid Naringin down regulated PKM2 in BCC

We examined the *Citrus maxima* peel extracts and NA potential targets for inhibiting aerobic glycolysis. The crucial glycolytic gene and regulator of Warburg effect PKM2 was studied. The protein expression of PKM2 in

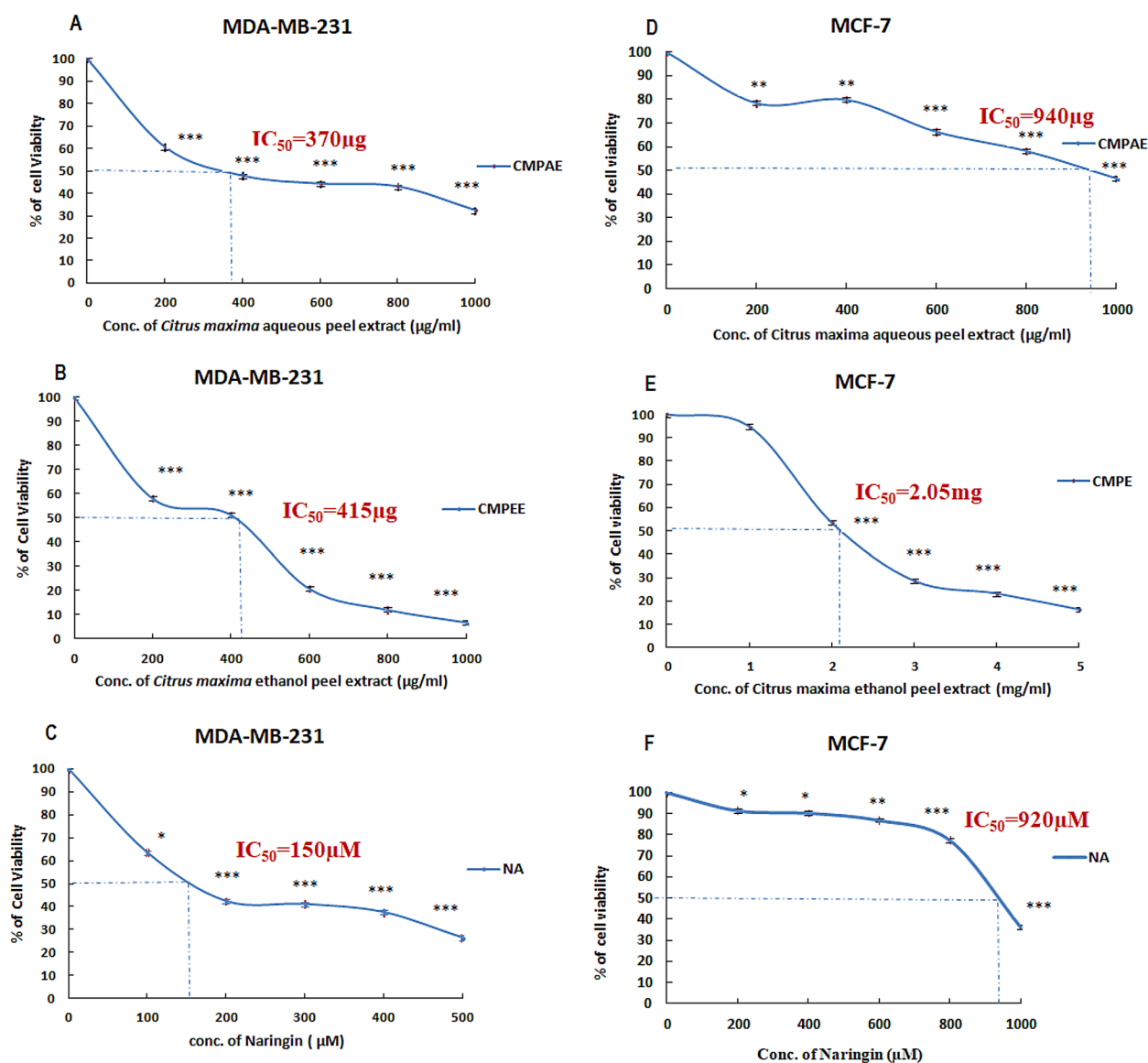


Fig. 2 The cytotoxicity effect of peel extracts of *Citrus maxima* and Naringin on breast cancer cell lines **A–C** on MDA-MB-231 and **D–F** on MCF-7 using MTT assay. The data represent the mean $\pm$ SD, ($n=3$) (ANOVA, Tukey's Test). *indicates a statistically significant difference from the control, and *** $P < 0.001$, ** $P < 0.001$, or * $P < 0.05$ were deemed statistically significant. CMPAE-Citrus maxima peel aqueous extract; CMPEE-Citrus maxima peel ethanolic extract; NA-Naringin

MDA-MB-231 and MCF-7 after treatment with respective concentrations was analyzed by immunoblotting and shown in Fig. 5: A and B. There was a significant decrease in PKM2 protein levels in the treated group compared to the control.

3.6 Molecular interaction analysis

To complement the LC/MS-based identification of NA an in silico ADMET and toxicity profiling was conducted using SwissADME and ADMETLab platforms.

The compound exhibited a molecular weight of 580.53 g/mol and a total of 14 hydrogen bond acceptors and 8 hydrogen bond donors. NA was predicted to have low gastrointestinal (GI) absorption and was not permeable to the blood–brain barrier (BBB). It was identified as a substrate of P-glycoprotein (P-gp), which may influence its efflux and bioavailability. Importantly, NA was not predicted to inhibit any of the major cytochrome P450 enzymes (CYP1A2, CYP2C19, CYP2C9, CYP2D6, or CYP3A4), suggesting a low risk of metabolic drug–drug interactions.

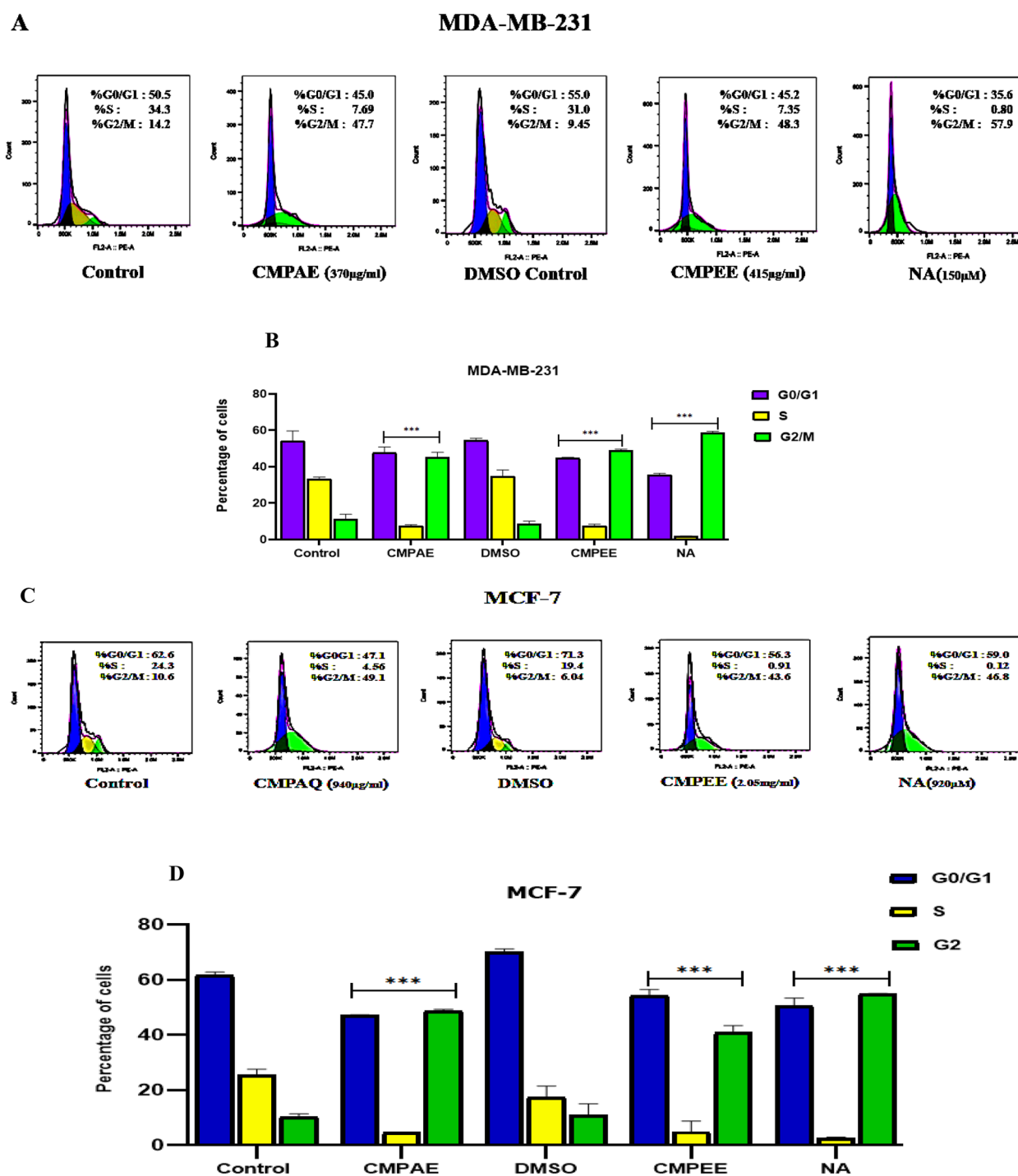


Fig. 3 Cell Cycle analysis on MDA-MB-231 (A, B) and MCF-7 (C, D) by flow cytometry after 48 h treatment with *Citrus maxima* peel extracts and flavonoid NA with their respective IC_{50} in comparison to control. Results were expressed as percentage of cells with the data represent the mean $\pm$ SD, ($n = 3$) (ANOVA, Tukey's Test). *indicates a statistically significant difference from the control, and *** $P < 0.001$, ** $P < 0.001$, or * $P < 0.05$ were deemed statistically significant. CMPAE-Citrus maxima peel aqueous extract; CMPEE-Citrus maxima peel ethanolic extract; NA-Naringin

Solubility predictions across three models—ESOL, Ali, and Silicos-IT—consistently classified NA as soluble, with log S values of -2.98 , -3.82 , and -0.49 , respectively.

The compound showed no structural alerts for toxicity, as indicated by the absence of PAINS and Brenk filters. However, it violated multiple drug-likeness rules: three

violations of Lipinski's rule, four of Ghose, and one each of Veber, Egan, and Muegge filters. The bioavailability score was calculated to be 0.17, and the synthetic accessibility score was 6.16, indicating moderate complexity in synthesis (Supplementary Table 1).

3.6.1 Validation of docking protocol

The co-crystal structure of B3P with PKM2 was assessed in this investigation using two distinct docking strategies: molecular mechanics/generalized born surface area (MM/GBSA by ΔG measured in kcal/mol) and rigid receptor docking (RRD by Glide XP calculated in kcal/mol). To validate the docking protocol, the native complex of PKM2 with B3P was superimposed with the redocked complexes obtained from both RRD (-5.24 kcal/mol) and MM/GBSA (-15.27 kcal/mol) docking score strategies. The eminence of the docking was assessed by measuring the root-mean-square deviation (RMSD) between the native and redocked complexes. The results exhibited that the redocked complexes obtained from RRD and MM/GBSA had RMSD values of 1.99 Å and 0.32 Å, respectively, when compared to the native complex (Fig. 6). This indicated that both docking strategies were able to reproduce the overall conformation of the native complex, with MM/GBSA exhibiting a closer replication than RRD. Furthermore, the molecular interactions observed in the redocked complexes were found to overlap with the interactions observed in the native co-crystal structure. The MM/GBSA strategy demonstrated a better replication of these interactions compared to RRD. To analyze the molecular docking results, the docking scores obtained from both RRD and MM/GBSA were utilized. The MM/GBSA ΔG score was found to exhibit the best correlation among the multiple docking strategies studied. Previous studies have also demonstrated the efficacy of MM/GBSA in predicting binding free energies (ΔG) by performing rescoring of the docked complexes [18, 19, 23, 24]. These ΔG values have been shown to be in good agreement with experimental binding affinities, further supporting the reliability and accuracy of the MM/GBSA approach. Overall, the evaluation of the RRD and MM/GBSA docking strategies using the co-crystal structure of B3P with PKM2 revealed

that both approaches were able to reproduce the native complex. However, MM/GBSA demonstrated a higher level of accuracy in replicating the molecular interactions and exhibited a stronger correlation with experimental binding affinities. These findings highlight the advantages of using MM/GBSA for molecular docking studies and its potential for accurate prediction of binding free energies.

In this context, NA has shown promising interactions with PKM2. The binding affinity between a NA and PKM2 can be quantified by calculating the RRD and Gibbs free energy (ΔG) binding scores. The higher RRD and ΔG score indicate a stronger binding interaction. In this case, NA demonstrated the highest RRD (-7.54 kcal/mol) and ΔG (-28.10 kcal/mol) score compared to a published positive control (B3P) (Fig. 7). In the RRD strategy (Fig. 7B), NA has a strong binding affinity with PKM2, formed five interactions with PKM2, contributing to its strong binding affinity. Three of these interactions were hydrogen bonds formed between NA and the amino acid residues of Ser77, Asp178, and Glu332. Hydrogen bonds are strong intermolecular forces that form between a hydrogen atom and an electronegative atom, such as oxygen or nitrogen. These interactions stabilize the docked complex and contribute to the overall binding affinity. Furthermore, NA formed a π - π interaction with the amino acid residue His78. A π - π interaction of two aromatic systems aligns in a parallel or stacked configuration, allowing for attractive interactions between the delocalized π -electron clouds. This interaction is known to be important for ligand binding and can significantly contribute to the stability of the ligand-protein complex.

Additionally, NA formed π -cation interactions with the amino acid residue Lys207. A π -cation interaction involves the attractive forces between a positively charged π -system and an aromatic or negatively charged species. This interaction can enhance the binding affinity by providing additional stabilization to the docked complex. In addition to these specific interactions, NA also formed van der Waals (vdW) interactions with several other amino acid residues in PKM2. The residues involved in these vdW interactions included Gly79, Arg120, Glu131, Asp177, Ser205, Lys206, Ile335, Ser362, Gly363, Ala366, and Lys367 (Fig. 7B). These vdW interactions contribute

(See figure on next page.)

Fig. 4 The Annexin V-FITC and PI for the detection of apoptosis after 48 h treatment of breast cancer cells with *Citrus maxima* peel extracts and flavonoid NA in comparison with control. The flow cytometry analysis results are represented in the histogram as percentage of cells from each of four different quadrants (Q1-LL = Live cells, Q1-LR = early apoptotic cells, Q1-UR = late apoptotic cells, Q1-UL = necrotic cells as mean $\pm$ SD for MDA-MB-231 (A) and MCF-7 (C) cells for control and treated groups. B, D Bar graph representing percentages of values for each cell, represented as mean $\pm$ standard deviation (SD) of three independent experiments. Results were analyzed using one way-analysis of variance (ANNOVA) at a significance level $**P < 0.01$, $***P < 0.001$ compared to control. CMPAE-Citrus maxima peel aqueous extract; CMPEE-Citrus maxima peel ethanolic extract; NA-Naringin

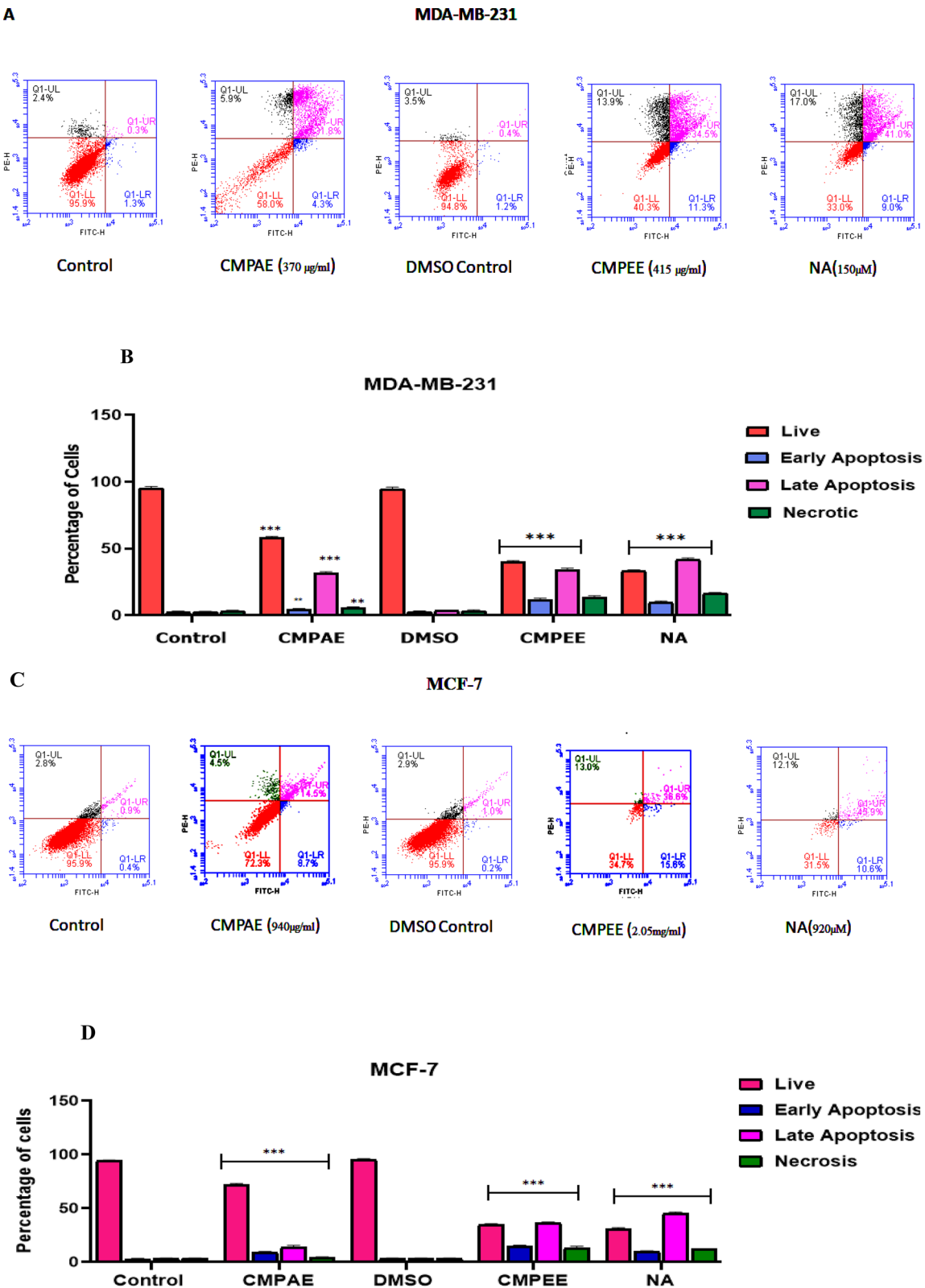


Fig. 4 (See legend on previous page.)

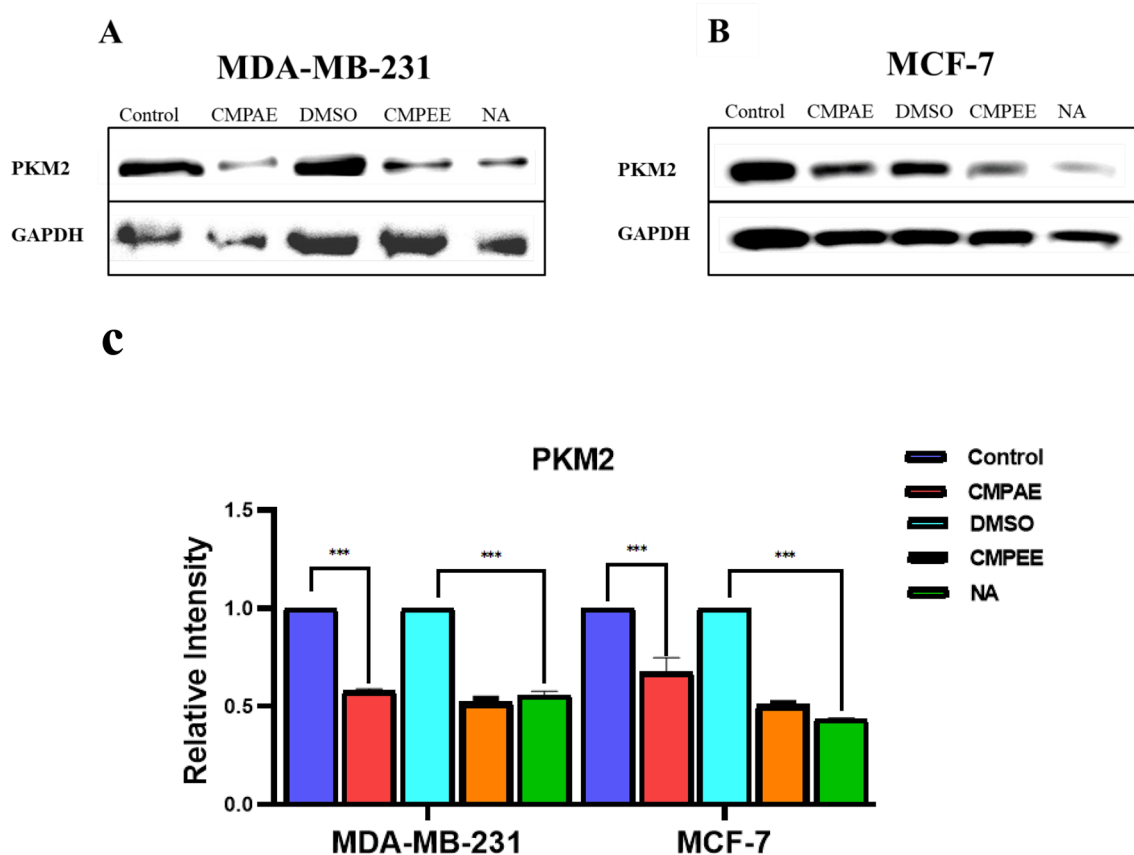


Fig. 5 Western blot analysis of the impact of crude peels extracts of *Citrus maxima* and NA on the expression of PKM2 after 48 h treatment on **A** MDA-MB-231 and **B** MCF-7 cells. **C** Quantification of protein expressions. GAPDH was used as internal control. CMPAE-*Citrus maxima* peel aqueous extract; CMPEE-*Citrus maxima* peel ethanolic extract; NA-Naringin. Data represent mean $\pm$ SD ($n=3$), *** $p < 0.0001$, ** $p < 0.001$, * $p < 0$ (ANOVA, Tukey's test)

to the overall binding affinity by promoting complementary molecular shapes and providing additional stabilization through attractive forces.

During the application of the MMGB-SA, observed that within the interaction interface of PKM2 and NA, specific molecular interactions. Remarkably, the aromatic ring of His78 from PKM2 engaged in a π - π stacking interaction with NA. Simultaneously, one of the hydroxyl (OH) group of NA participated in hydrogen bonding (H-bonding) with Ser77 of PKM2. Further analysis revealed that NA formed two distinct H-bonds with Asp178 of PKM2 (Fig. 7B). The formation of these interactions was facilitated by the presence of a water molecule, which acted as an enabled between NA and PKM2, leading to stable bonds. The complex's overall stability was aided by the water-mediated interaction's notable strength. Additionally, the NA molecule exhibited two π -cation interactions within 4 Å region with Lys206 and Lys207. This π -cation interaction involves the positively charged amino acid side chains of these residues and the electron-rich π system of NA's aromatic ring.

In a broader context, several other amino acid residues were identified to engage in vdW interactions within the 4 Å distance range. These residues include Arg73, Asn75, Ser77, His78, Gly79, Lys115, Arg120, Glu131, Asp177, Asp178, Lue180, Ser205, Lys206, Lys207, Ser328, Glu332, Ile335, Ser362, Kys363, Ala366, and Lys367 (Fig. 7B). These vdW interactions further contributed to the stabilization of the complex formed between NA and PKM2. Overall, the analysis of the molecular interactions between NA and PKM2 using the RRD and MMGB-SA methodology revealed a complex network of π - π stacking, hydrogen bonding, water-mediated interactions, and vdW interactions. These interactions collectively play a crucial role in the stability and binding affinity of the NA-PKM2 complex.

3.6.2 Dynamics simulations analysis

In the analysis of MD simulations, the NA-PKM2 complex exhibited an average potential energy of -121,356.16 kcal/mol, revealed the remarkable stability of the complex. The PKM2 C α and NA atoms

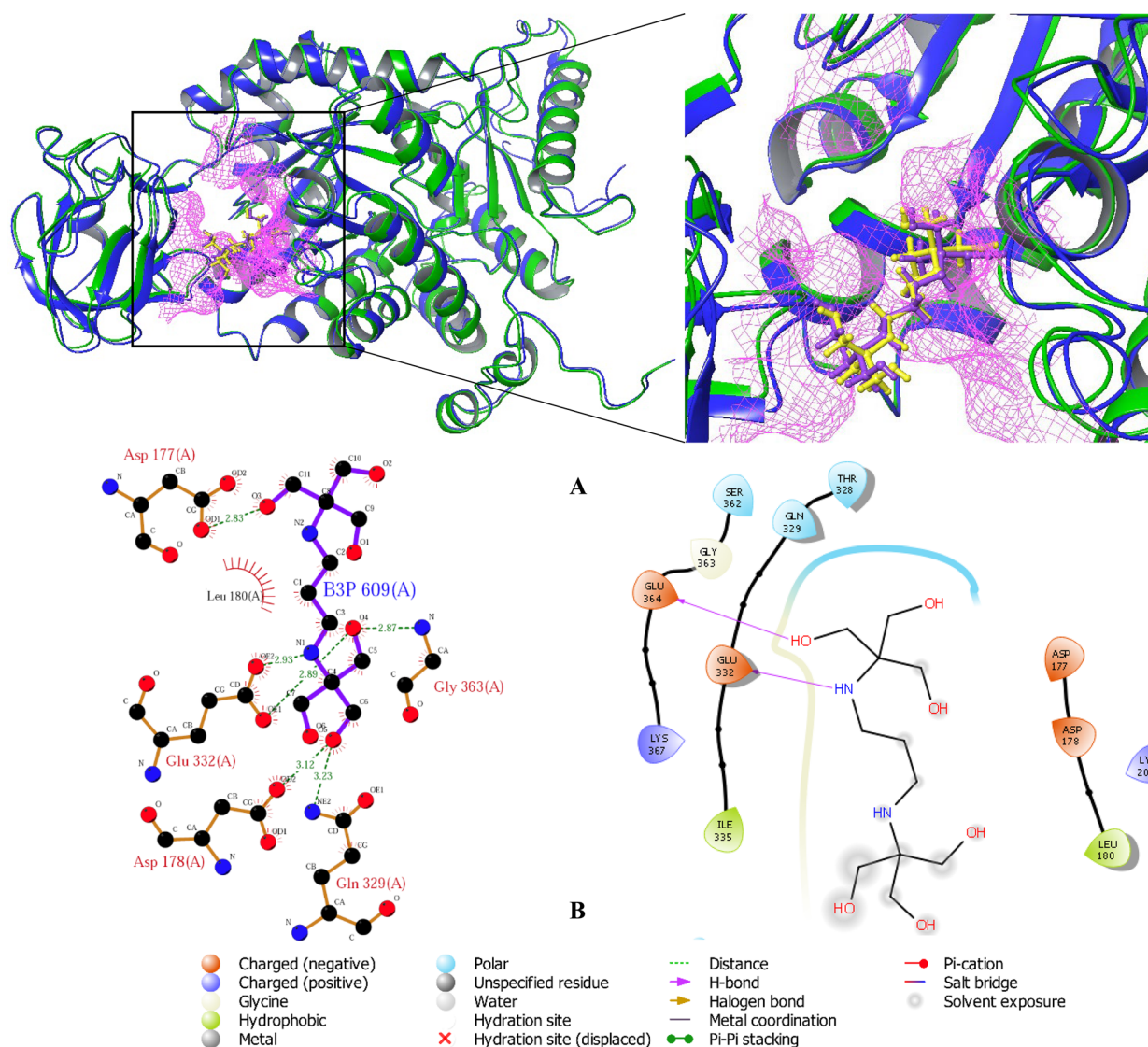


Fig. 6 Validation of the molecular docking technique using superimposition and RMSD. **A** In RRD, the RMSD between the native PKM2 complex (blue) and B3P (violet) and the docked PKM2 complex (green) with B3P (yellow) was 1.99 Å. **B** Molecular interactions of native (left) and docked complex of PKM2 with B3P.

displayed average RMSD of 2.35 Å and 1.45 Å, respectively (Fig. 8A). Furthermore, the PKM2 Cα and side chain exhibited average root-mean-square fluctuations (RMSF) of 1.54 Å and 1.72 Å, respectively, when interacting with NA (Fig. 8B). The NA-PKM2 complex demonstrated a total of 15 interactions involving negative, positive, polar, and water-mediated interactions over the course of 100 ns. Notably, specific interactions were observed during each trajectory of the MD simulations, includes hydrogen bonds with Ser77, Lys206, Lys207, Lys367, and His78, with percentages of 74%, 67%, 34%, 72%, and 52%, respectively. Interactions involving water mediation were also significant. The residues Glu118

and Glu131 exhibited water-mediated interactions with NA at percentages of 51% and 40%, respectively (Fig. 8D). Moreover, the PKM2 residue Arg73 engaged in two water-mediated interactions with NA, with percentages of 32% and 57%, respectively. Furthermore, an intriguing observation was made for Glu118, which was found to mediate with two HOH ions. The percentages of these interactions were 56%, 56%, and 57%, respectively (Fig. 8D). These findings collectively highlight the intricate nature of interactions within the NA-PKM2 complex, shedding light on its structural stability and dynamic behavior during the simulation. The NA formed three residues of Ser77, His78, and Lys207 of PKM2 were

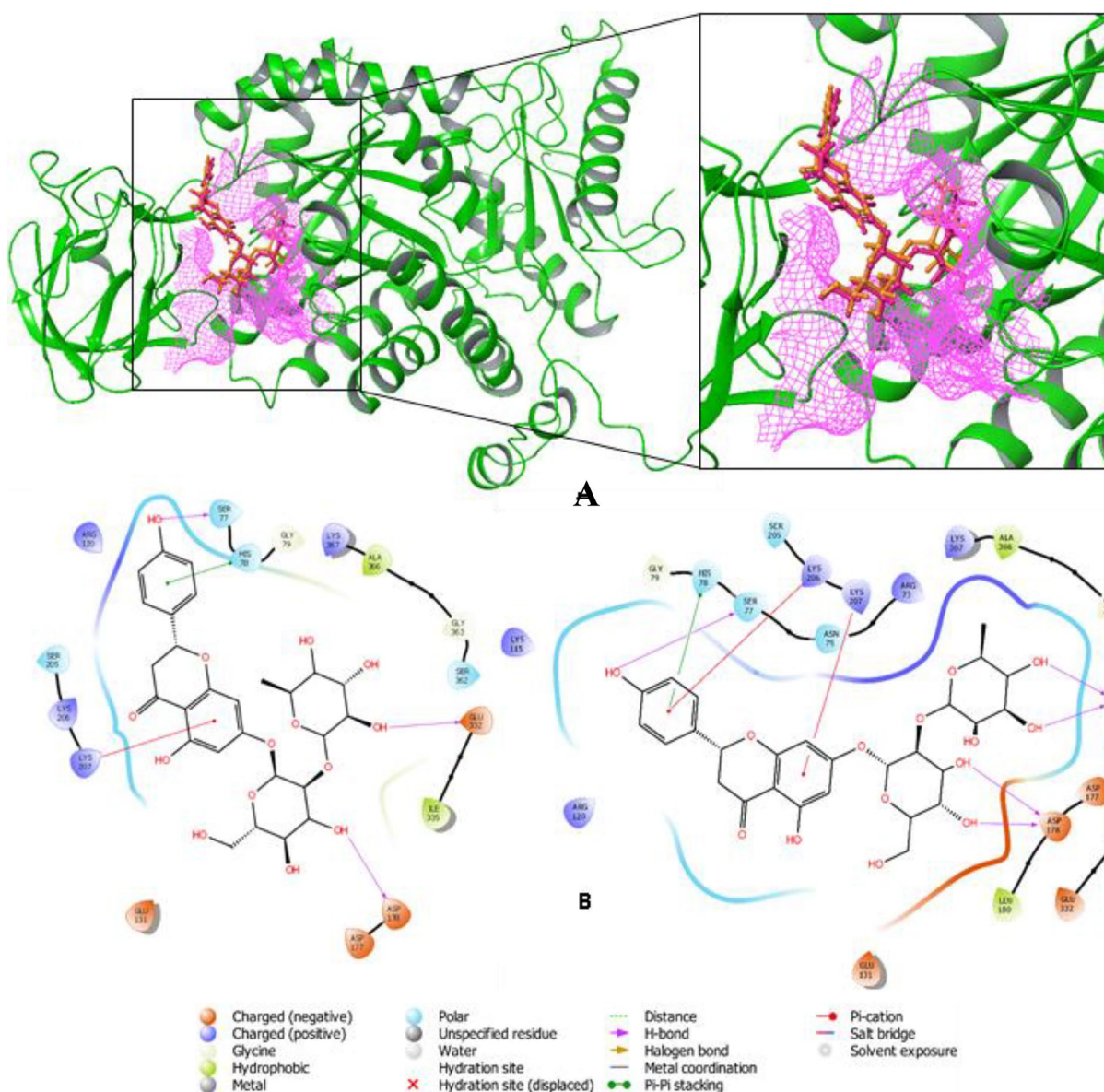


Fig. 7 Molecular docking and interactions of PKM2 and NA. **A** RRD and MMGB-SA docked complexes of PKM2 (green) and Naringin (Pink and orange). **B** Molecular interactions of PKM2 and NA of different docking strategies of RRD (right) and MMGB-SA (left)

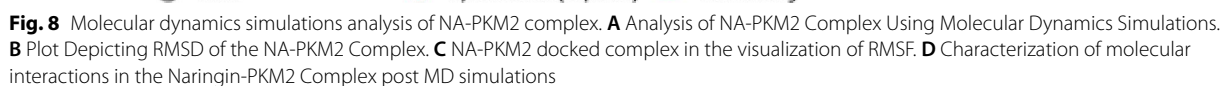
found in the post docking and dynamics simulations interactions observed to remain stable in all the trajectories during MD simulations run (Fig. 8C).

Overall, the strong binding affinity of NA to PKM2, as indicated high ΔG score, attributed to the multiple interactions formed docked complex. The hydrogen bonds, π - π interaction, and π -cation interaction provide specific and strong attractive forces, while the vdW interactions contribute to the overall stability of the complex. Understanding the molecular interactions between PKM2 and NA can have important implications for cancer research and the development of therapeutic

strategies. Targeting PKM2 with NA could potentially modulate its activity, leading to altered cancer cell metabolism and potentially inhibiting tumor growth. Further studies exploring the structural details and functional consequences of these interactions will help elucidate the potential of NA as an inhibitor of PKM2 and its potential applications in breast cancer therapy.

4 Discussion

Preclinical and clinical research have shown that targeting the metabolism of cancer cells, particularly aerobic glycolysis, is a promising treatment approach [26]. A



novel approach of treating cancer is provided by emphasizing on metabolic enzymes and transporters. Due to their broad activity, multi-target effects, and ability to increase efficacy while reducing adverse effects, plant-based chemicals are becoming more and more popular.

Citrus maxima peel extracts and their main flavonoid, NA, were evaluated for their anti-cancer properties on MDA-MB-231 and MCF-7 cells. Secondary metabolites were identified by preliminary screening, and bioactive substances with therapeutic potential were validated by GC-MS [6]. The polyphenol NA was additionally identified by LC-MS analysis, implying that it plays a significant role in the extract's beneficial properties (Fig. 1).

The primary flavanones in *Citrus maxima* peel extract, according to HPLC analysis, were NA and Naringenin [8]. Because of their intricate structures that affect numerous cellular and molecular pathways, citrus flavonoids are well known for their anti-cancer properties [7]. According to our research, NA and *C. maxima* peel extracts suppressed the proliferation of breast cancer cells in a concentration-dependent manner (Fig. 2). The compounds triggered apoptosis (Fig. 4) and cell cycle arrest (Fig. 3). These results have been supported by earlier research: Li et al. (2013) demonstrated that Naringin inhibited breast cancer cell proliferation via the β -catenin pathway, while Muristi et al. (2021) reported decreased viability in triple-negative breast cancer cells treated with *C. maxima* peel extract, linked to ROS generation [27]. Citrus peel's flavonoid content, which has shown substantial anti-proliferative effects in a variety of in vitro and in vivo models by upsetting the cell cycle and inducing apoptosis, is primarily responsible for its anti-cancer potential [7, 28]. These results demonstrate the therapeutic potential of bioactives obtained from citrus in the treatment of cancer.

PKM2 is abundantly expressed in tumors and is essential for the metabolic reprogramming that promotes the growth of cancer cells [29, 30]. Its inhibition has been demonstrated to increase the chemosensitivity of tumors. Few research, nevertheless, have investigated plant extracts and the bioactive substances they contain as PKM2 inhibitors [31–35]. Some seem promising, but it is still unclear how they work. A potential path in oncology is marked by the need for more study to fully comprehend these interactions and create potent plant-based cancer treatments. In cancer treatment, finding PKM2 inhibitors that are safe, efficacious, and bioavailable continues to be a top focus. We looked at how *Citrus maxima* peel extracts and its main component, NA, affected PKM2 in MDA-MB-231 and MCF-7 cells. PKM2 expression was decreased in all treated groups (Fig. 5). Citrus peel flavonoids have the potential to prevent and treat cancer since they have been shown to suppress the

proliferation of cancer cells by downregulating oncoproteins and altering cellular proteins [7].

The ADMET and toxicity profiling of NA provides valuable insights into its pharmacokinetic behavior and safety profile. The absence of PAINS and Brenk alerts suggests that NA is structurally clean and unlikely to produce false positives in biological assays. Its non-inhibitory profile against major CYP enzymes further supports its metabolic safety, reducing the likelihood of adverse drug–drug interactions. However, the compound's low GI absorption and classification as a P-gp substrate may limit its oral bioavailability, a common challenge associated with many natural flavonoids.

Despite multiple violations of drug-likeness rules, which are primarily attributed to its high molecular weight and extensive hydrogen bonding capacity, NA demonstrated favorable solubility across all predictive models. This characteristic may partially offset its absorption limitations and supports its potential for formulation development. The moderate synthetic accessibility score also suggests that while the compound is not trivial to synthesize, it remains within a feasible range for chemical modification or optimization. Overall, these findings highlight Naringin's promise as a bioactive compound with a favorable safety profile, warranting further investigation through experimental pharmacokinetic and toxicity studies.

To validate the docking protocol employed in this study, two distinct docking strategies were assessed: RRD and MM/GBSA. The co-crystal structure of PKM2 with B3P was utilized as a reference for validation. The validation process involved comparing the native PKM2-B3P complex with the redocked complexes generated by both RRD and MM/GBSA (Fig. 6). This suggests that both strategies were able to replicate the overall conformation of the native complex, with MM/GBSA showing a closer resemblance. Furthermore, the molecular interactions observed in the redocked complexes were consistent with those in the native co-crystal structure, with MM/GBSA showing a better replication of these interactions. Docking scores from both RRD (Glide XP) and MM/GBSA (ΔG) were utilized for analysis. Markedly, the MM/GBSA ΔG score exhibited the strongest correlation, which is supported by previous studies showcasing the effectiveness of MM/GBSA in predicting binding free energies (ΔG) and binding affinities. This underscores the reliability and accuracy of the MM/GBSA approach. In summary, the assessment of RRD and MM/GBSA using the PKM2-B3P co-crystal structure demonstrated their capability to reproduce the native complex to a certain extent. MM/GBSA, in particular, exhibited a higher accuracy in replicating molecular interactions and showed a stronger correlation with experimental binding affinities.

These findings highlight the advantages of employing MM/GBSA for molecular docking studies and suggest its potential for precise prediction of binding free energies. Figures 6 and 7 represent the validation process and provide insights into the potential interactions between PKM2 and Naringin. These findings have implications for cancer therapy, potentially allowing for the development of targeted treatments that modulate PKM2 activity through NA.

Furthermore, the MD simulations of the NA-PKM2 docked complex revealed nuanced insights into its structural dynamics and stability. The docked complex exhibited a remarkable equilibrium, as indicated by the average potential energy. The RMSD analyses disclosed the complex's propensity for modest deviations from initial positions, with the PKM2 C α and NA atoms displaying. The RMSF analysis illuminated localized fluctuations in PKM2 regions interacting with NA, underscoring the adaptability of their conformational states. The intricate molecular interactions within the complex were explored comprehensively. An array of interactions, including hydrogen bonds, polar interactions, and water-mediated engagements, contributed to its dynamic behavior. The significant hydrogen bonds involving residues Ser77, Lys206, Lys207, Lys367, and His78 played substantial roles in maintaining complex stability. Additionally, water-mediated interactions, exemplified by Glu118 and Glu131, unveiled the involvement of water molecules in sustaining the complex's integrity. The MD simulations discerned a consistent pattern of interactions within the NA-PKM2 complex, highlighting the vital roles of Ser77, His78, and Lys207 residues. This consistency underscores their significance in the complex's stability and behavior. These findings collectively shed light on the intricate nature of interactions, portraying a dynamic yet structurally steadfast complex during the simulations.

This study is one of the first to investigate the anti-cancer potential of *Citrus maxima* peel extracts and its flavonoid NA against PKM2 in breast cancer, both in vitro and in silico. The results demonstrate their potential as metabolically targeted cancer treatments by highlighting their capacity to suppress cell division, trigger cell cycle arrest, encourage apoptosis, and interact with PKM2. These findings lend credence to additional research on their processes and their therapeutic uses.

5 Conclusion

This study assessed the cytotoxic effects on breast cancer cells of NA and peel extracts from *C.maxima*. NA was identified as the primary flavonoid by LC–MS analysis. Strong binding affinity between NA and PKM2 was demonstrated by molecular docking and MD simulations, which were backed by good MM/GBSA scores. In

MDA-MB-231 and MCF-7 cells, the extracts and NA both markedly reduced cell proliferation, caused G2/M cell cycle arrest, and encouraged early and late apoptosis. Notably, PKM2 expression decreased with therapy, pointing to a potential mechanism through modification of metabolic pathways. All things considered, these results demonstrate the therapeutic potential of *C. maxima* and NA in addressing cancer metabolism, providing encouraging treatment options for breast cancer.

Supplementary Information

The online version contains supplementary material available at <https://doi.org/10.1186/s43088-025-00668-0>.

Additional file 1.

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Author contributions

F.M.—investigation, formal analysis, validation, writing original draft, review and editing. V.V.S.—visualization, review and editing R.A.—investigation, review and editing S.N.—review and editing M.M.—methodology, validation, formal analysis, investigation, data curation, writing original draft review and editing P.N.—data curation, software, validation S.S.S.—review *S.K.N.—conceptualization, methodology, supervision, review and editing.

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Data availability

No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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