

ARTICLE

Anticancer potential, antioxidant activity, chemical content, and enzyme inhibitory properties of *Inula aschersoniana* Janka, supported by an integrated network pharmacology study

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Abstract

This study explores the biological and pharmacological potential of *Inula aschersoniana* Janka (*I. aschersoniana*) by utilizing both in silico computational and in vitro analyses, focusing on anticancer, antioxidant, and enzyme inhibitory activities. *I. aschersoniana* extracts were observed to have effective properties against breast cancer (MCF-7) and human colon adenocarcinoma (HT-29) cell lines compared to normal human umbilical vein endothelial (HUVEC) cell line. Also, effective antioxidant activity of the plant sample was determined by using several in vitro antioxidant methods. Furthermore, inhibitory effects of *I. aschersoniana* extracts against alpha-glucosidase (α -Gly) and glutathione S-transferase (GST) enzymes were evaluated. The IC_{50} value of *I. aschersoniana* ethyl acetate extract was determined as 4.05 $\mu\text{g/mL}$ for α -Gly and 1.67 $\mu\text{g/mL}$ for GST. Similarly, IC_{50} value of the ethanol extract was measured as 3.74 $\mu\text{g/mL}$ for α -Gly and 2.71 $\mu\text{g/mL}$ for GST. Also, main organic compounds of *I. aschersoniana* were detected to be vanillic acid, rutin, and naringin by HPLC technique. Finally, integrated network pharmacology, molecular docking, and molecular dynamics simulations were performed to elucidate the potential interactions between the active components of *I. aschersoniana* and genes associated with breast and colon cancer. To ensure reliability, molecular docking results were validated using re-docking and comparison with reference inhibitors or co-crystallized ligands. RMSD and RMSF analyses revealed that naringin, the major compound of *I. aschersoniana*, exhibited dynamically stable binding within the active sites of AKT1, EGFR, and PPARG proteins, with AKT1@Naringin and PPARG@Naringin complexes displaying a more stable dynamic profile. In this network pharmacology study, forty-five common targets between the major compounds of *I. aschersoniana* with breast and colon cancers were identified.

Keywords Enzyme inhibition · Antioxidant activity, MCF-7 · HT-29 · *Inula aschersoniana* · Network pharmacology



Introduction

Asteraceae (Compositae) is one of the largest plant families worldwide, encompassing numerous genera and species spread across diverse regions. Due to the economic and medicinal importance of many of its members, this family is frequently featured in chemotaxonomic research. Globally, it comprises approximately 25,000 taxa (Bremer 2004). Many taxa within Asteraceae have been studied for their biochemical properties (Kilic and Bagci 2013). The genus *Inula* L., belonging to the *Asteraceae* family, contains over 100 species, many of which have long been used in traditional medicine. Literature reviews indicate that *Inula* species are particularly rich in biologically active metabolites, especially sesquiterpene lactones, flavonoids, and triterpenoids (Seca et al. 2015). Many *Inula* taxa have been traditionally used in the treatment of various ailments such as bronchitis, diabetes, and intestinal ulcers. Furthermore, the most frequently reported components of *Inula* species are sesquiterpenes and monoterpenes (Bokadia et al. 1986).

Inula aschersoniana Janka is a perennial plant that typically sprouts and grows on open limestone slopes and in rocky crevices at altitudes ranging from 100 to 1800 m (Davis 2019). *I. aschersoniana* grows in many Eastern Europe countries including Bulgaria, Greece, Macedonia, and Türkiye. A previous study reported the chemical content of volatile oil of *I. aschersoniana* as fatty acids, alkanes, monoterpenes, sesquiterpenoids, and aromatic compounds (Todorova et al. 2017).

Plant-based sources, including fruits, nuts, seeds, grains, and vegetables are known in maintaining and promoting human health (Lynch et al. 2018). Thus, increasing attention has been given to the impact of natural plant-based diets on human health (Atalar et al. 2021; Bursal et al. 2021; Satija et al. 2017).

Breast cancer is the most frequently diagnosed malignancy among women and continues to represent a major public health challenge. In global cancer statistics, it ranks as the second most common cancer type following lung cancer (Çakır et al. 2016). In many developed countries, the five-year relative survival rate for breast cancer patients exceeds 80%; this is largely attributed to early diagnosis and timely treatment. Breast cancer consists of various stages depending on the pattern of metastasis and the number of tumors (Kosova and Arı 2008). Globally, colorectal cancer ranks as the third most diagnosed malignancy and the fourth leading cause of cancer-related mortality. Each year, 1–2 million new colorectal cancer cases are identified, resulting in approximately 600,000 deaths. The incidence is low in individuals under the age of 50 but increases significantly with age (Ferlay et al. 2010).

Reactive oxygen species (ROS) are molecules that contain a single unpaired electron and are therefore highly reactive (Atalar et al. 2021; Turrens 2003), and are generated as byproducts of cellular metabolism from molecular oxygen. ROS are associated with several health conditions, such as cancers (Moloney and Cotter 2018) cardiovascular diseases, diabetes mellitus, and insulin resistance (Alfadda and Sallam 2012). The human body uses antioxidant defense systems to maintain a balance between antioxidants and reactive oxygen species, thereby protecting cells and tissues from oxidative stress (Bursal et al. 2021).

Glutathione transferase enzyme facilitates the nucleophilic conjugation of glutathione (GSH) with various electrophilic compounds, serving a vital role in the mammalian detoxification system against oxidative stress. However, cancer cells take advantage of this mechanism to boost their survival and develop resistance to drugs (Allocati et al. 2018; Aras et al. 2021). Consequently, in certain cases, its activity needs to be suppressed using targeted inhibitors (Çelik et al. 2021).

In recent years, pharmacological network analyses of plant-based components in cancer therapy have increased (Han et al. 2023; Taha et al. 2020). This study aims to provide insights into the anticancer properties of the *I. aschersoniana* by integrating in silico and in vitro studies, addressing the traditional experimental limitations. In this study, biological activity of the plant on HT29 (colon cancer) and MCF-7 (breast cancer) cell lines was investigated, and the data obtained suggest that the plant affects cancer cell pathways. This effect is likely associated with various biological pathways, as supported by network pharmacology and molecular docking analyses. To ensure the reliability of the docking results, re-docking and comparison with reference inhibitors or

co-crystallized ligands were performed. The integration of network pharmacology and molecular docking studies in this work aims to provide a deeper understanding of the molecular targets and pathways involved in the plant's possible anticancer activity (Yuan et al. 2021). This research is expected to make a significant contribution to the expanding body of knowledge on natural product-based anticancer agents, emphasizing the integration of computational and experimental approaches in drug discovery. The findings may facilitate the development of new, targeted anticancer therapies derived from *I. aschersoniana* and other medicinal plants. Furthermore, KEGG analysis revealed that the plant's active components influence several biological pathways, including Nitrogen metabolism, Bladder cancer, Endocrine resistance, and the AGE-RAGE signaling pathway, which may be involved in cancer-related biological pathways.

The objective of the current study is to sufficiently understand the *I. aschersoniana* extract properties against the MCF7 which is commonly used breast cancer cell line and human colon adenocarcinoma cell line HT29, oxidative stress, GST, and α -Gly. For this purpose, commonly used in vitro assays were applied to determine the enzyme inhibition of *I. aschersoniana* by calculating the IC₅₀ values.

Materials and methods

Plant sample

I. aschersoniana plant sample was collected from northern limestone slopes of Akdağ mountain in Adıyaman, Türkiye, on June 28th 2019, at an altitude of 1500–1600 m, by taxonomist Dr. Omer Kilic. The plants were identified based on The Flora of Turkey and East Aegean Islands, with volume 5 [23]. The voucher specimen is deposited at the Adıyaman University, Pharmacy Faculty, Pharmaceutical Botany, with collected herbarium number ÖK6835 (Davis et al. 1972).

Preparation of extracts

Different parts of a plant contain varying levels of secondary metabolites, each with specific polarity characteristics, requiring the use of solvents with suitable polarity indices for effective extraction (Wakeel et al. 2019). In this study, five solvents which have different polarities such as ethyl acetate (moderately polar), acetonitrile (highly polar), isopropyl alcohol (polar), methanol (highly polar), and ethanol (polar) solvents were used for the extraction of the *I. aschersoniana* plant aerial parts. For this aim, 20 g of the plant sample was dissolved in 200 mL of each solvent and kept for one day with its lid closed. The solution was filtered with Whatman filter paper and transferred to a volumetric flask. The solvent was evaporated in an evaporator at 50 rpm and 50 °C. Then, 279.6 mg of precipitate from the acetate solution, 66.4 mg from the acetonitrile solution, 79 mg from the isopropyl alcohol solution, 106.6 mg from the methanol solution and 61.5 mg from the ethanol solution was obtained. The stock solutions were prepared by taking 1 mg for each extract and dissolving in 10 mL of the same solvent. After the plant extraction, samples were stored in glass tubes at -20 °C for the studies.

Cell culture-cytotoxicity analysis

For the in vitro experiments, HT-29 (ATCC no: HTB-38), MCF-7 (ATCC no: HTB-22), and HUVEC (ATCC no: CRL-1730) cell lines were utilized in the cell culture studies. The HT-29, MCF-7, and HUVEC cell lines were procured from the American Type Culture Collection (ATCC, Manassas, VA, USA). The culture medium was supplemented with 10% fetal bovine serum (FBS), 1% L-glutamine, and 1% penicillin–streptomycin, all of which were sourced from Sigma–Aldrich. The HT-29, MCF-7 and HUVEC cell lines were propagated in a 5% CO₂ in a incubator at 37 °C. After washing the cells with phosphate buffer, trypsin was added and after incubation for 5 min, the cells were separated from the surface. The cells were pelleted by centrifugation at 1300 rpm

for 4 minutes at 4°C. The supernatant was discarded and the pellet was homogenized by adding 2 mL of DMEM (Dulbecco's modified Eagle's medium). After cell counting, 1×10^4 cells were seeded into each well. After the incubation, 10 μ L of the samples with different concentrations (313, 625, 1250, 2500 and 5000 μ g/mL) were seeded into the well plates. Then, MTT was added and incubated for three hours. Finally, the absorbance values were measured at 24, 48 and 72 h (Weyermann et al. 2005). The MTT cytotoxicity assay was performed in triplicate ($n=3$), and the results were expressed as mean $\pm$ standard deviation (SD).

The first wells where only the media were left were the control group and the cell viability was accepted as 100%. The percentage viability of the cells was found with the formula given below;

$$\text{Live cells (\%)} = 100 \times \frac{A_1}{A_0}$$

Herein, A_1 refers to the cell absorbance of samples applied at different concentrations and A_0 refers to the control cell absorbance (Çevik et al. 2022; Kapalczyńska et al. 2018).

Antioxidant activity

Radical scavenging method

DPPH free radical scavenging activity of *I. aschersoniana* was evaluated by Blois method (Blois 1958), with some modifications as described detailed in our previous study (Aras 2022). Absorbance was measured against the 517 nm blank. The absorbance of control solution which contain only ethanol and DPPH radical solutions were adjusted around 1.50 abs. Decreasing absorbance value indicates radical scavenging activity of the plant sample. Similarly, ABTS method was used to evaluate the antioxidant activities of the different extracts of *I. aschersoniana* according to the study of Re et al. (Re et al. 1999), with some changes as described previously (Atalar et al. 2021). Absorbance values were measured and recorded at 734 nm.

Reduction antioxidant power determination methods

CUPRAC and FRAP methods were used to evaluate the reduction of antioxidant properties of *I. aschersoniana* extracts. Cupric ion (Cu^{2+}) reduction capacities of the plant extracts were determined according to the CUPRAC method as described detailed in our previous study (Bursal et al. 2021). Similarly, ferric ion (Fe^{3+}) reduction capacities of the plant extracts were determined according to the FRAP method as described in the same study. Absorbance values were measured at 450 nm and 700 nm for CUPRAC and FRAP methods. The increased absorbance of the reaction extracts indicated high reducing capacity. All antioxidant assays were carried out in triplicate ($n=3$), and the results were expressed as mean $\pm$ standard deviation (SD).

Enzyme inhibition activity

Glutathione S-transferase

Glutathione S-transferase (GST) catalyzes the association of the glutathione molecule with an aromatic electrophile like CDNB (1-chloro-2,4-dinitrobenzene) (Aksoy et al. 2016). Following the enzymatic reaction, dinitrobenzene S-glutathione (DNB-SG) is formed, which exhibits maximum absorbance at 340 nm. Therefore, the enzyme activity can be quantified by monitoring the increase in absorbance at this wavelength. Briefly, 50 μ L of GSH (20 mM), 20 μ L of 95% CDNB (in ethanol), 200 μ L of 0.1 M phosphate buffer (pH: 7.0), 730 μ L pure water, and different concentrations of the samples were added to the cuvettes and their absorbance values for three minutes were recorded (Pabst et al. 1974).

α -Glucosidase activity

The α -glycosidase inhibitory activity was assessed using p-nitrophenyl-D-glycopyranoside (pNPG) as the substrate according to an updated procedure of Tao et al. (Tao et al. 2013). Samples were prepared by dissolving 4 mg of the extract in 4 mL of an ethanol–water solvent. Enzyme inhibition studies were carried out according to details of the former studies (Gulçin et al. 2018; Shen et al. 2010).

Phenolic content analysis with HPLC

Quantitative analysis of organic compounds of *I. aschersoniana* extracts were done by HPLC method (Agilent, 1260 infinity II, Liquid Chromatograph Systems, USA). A reverse phase-HPLC column (Accegenerix5 C18 column, 4.6 × 250 mm, 5 μ m, Agilent 1260 Infinity series) was used for the analyses, as detailed in previous studies (Bozkurt et al. 2025; Türkan et al. 2020). For the HPLC operating conditions of the analysis of phenolic compounds are given below; Device Name: Agilent 1260 infinity series, Mobile phase A: 83% (0.1% phosphoric acid in water), Mobile phase C: 17% (100% Acetonitrile), Flow Rate: 08 mL/min, Injection Volume: 10 μ L, Column temperature: 30 °C, Column Type: C18, Ace Generix (4.6 × 250 mm) 5 micron Detector System: DAD Detector, Wavelength: 300/200 nm REF; 500/100 nm, Working System: Gradient.

Network pharmacology

The major organic compounds of *I. aschersoniana* identified by HPLC were evaluated for network pharmacology. Among these, vanillic acid, rutin, and naringin were selected as representative bioactive compounds. The selection of these three compounds was based not only on their relatively higher abundance in the HPLC chromatogram (peak area %) but also on their reported pharmacological relevance in the literature, particularly their antioxidant and anticancer activities. In addition, these compounds were prioritized as they satisfy basic drug-likeness criteria and are more suitable for in silico target prediction compared to minor constituents. Including all detected compounds could increase noise and reduce the specificity and interpretability of the network pharmacology results.

Target identification

To explore the potential molecular targets of these compounds, target identification was performed as follows. The SMILES notation of vanillic acid, rutin, and naringin were retrieved from the PubChem database (<https://pubchem.ncbi.nlm.nih.gov/>). Potential targets were identified using publicly available tools, including the Swiss Target Prediction and STITCH platforms (Kuhn et al. 2012). Redundant targets were removed from the results to ensure the identification of unique potential targets. Additionally, target genes associated with breast and colon cancer were obtained from the GeneCards (<https://www.genecards.org/>) and OMIM databases (<https://omim.org/search/advanced/>). To improve the specificity and biological relevance of the analysis, genes obtained from GeneCards were filtered based on their relevance scores, and only the top-ranked genes were included in further analysis. This filtering step minimized background noise and enhanced the reliability of the identified targets. To determine the intersection between disease-related genes and the potential targets of the active components, the VENNY 2.1 software package was used to generate a Venn diagram for further analysis (<https://bioinfogp.cnb.csic.es/tools/venny/>).

Protein-Protein interaction construction

Common targets obtained using the Venny 2.1 tool were transferred to the STRING database (Szklarczyk et al. 2023) to construct protein–protein interaction (PPI) networks for *Homo sapiens*. The confidence score was set

to a medium-to-high level (0.400), and the false discovery rate (FDR) threshold was defined as 5% to ensure the reliability of the interactions. The obtained data were directly transferred to Cytoscape 3.10.3 software via the “Send network to Cytoscape” option for more detailed analysis, and these data were visualized (Shannon et al. 2003). The PPI networks obtained in Cytoscape were shaped with various parameters to visually represent the interactions between proteins. Topological analysis of the PPI network identified key hub genes based on degree centrality, with AKT1, PPARG, and EGFR emerging as the most influential targets. These genes are well known to play central roles in cancer-related signaling pathways and were therefore prioritized for further analysis.

Network construction and topological analysis

All the PPI data imported to Cytoscape 3.10.3 were analyzed using the software’s network analysis tool to perform a comprehensive topological parameter analysis. Herein, nodes in the network represent the target proteins, biological pathways, and components, while the edges represent interactions between these nodes (Noor et al. 2023). When examining the topological features of the network, the degree of connectivity of the nodes is a key parameter for determining their centrality and importance in the network. Therefore, a node with a higher number of direct connections is considered more influential and central in the network (Berger and Iyengar 2009). Based on this approach, the top three hub genes (AKT1, PPARG, and EGFR) were selected for subsequent molecular docking studies, as they exhibited the highest degree values and represent the most biologically relevant targets within the network.

Gene ontology and KEGG enrichment analysis

In this part of the study, the functional roles of the targets in the intersection set were examined using the ShinyGO 0.82 tool (Ge et al. 2020). The analysis included categories such as biological processes, cellular components, and molecular functions under Gene Ontology (GO), as well as KEGG pathways (Kanehisa et al. 2021). The analysis was conducted with a false discovery rate (FDR) threshold of 0.05. The obtained analysis results were visualized using dot plots to highlight the significant pathways and processes associated with the intersecting targets. Although a broad range of pathways was identified, the interpretation was focused on biologically relevant cancer-associated pathways to improve specificity. In particular, pathways such as “Pathways in cancer,” “Estrogen signaling pathway,” and “Endocrine resistance” were emphasized due to their direct involvement in breast and colon cancer progression and treatment response. While “Nitrogen metabolism” was among the enriched pathways, it was not prioritized in the discussion due to its relatively limited direct relevance to cancer mechanisms in this context. Instead, pathways with clearer mechanistic links to tumor development and progression were highlighted. Additionally, pathways such as AGE-RAGE signaling were identified. However, these pathways should be interpreted with caution and require further experimental validation.

The network pharmacology analysis provided a systematic framework for identifying potential targets and pathways associated with the bioactive compounds of *I. aschersoniana*. These findings were further supported by molecular docking and molecular dynamics simulations, establishing a multi-step validation strategy in which target prediction, binding affinity, and dynamic stability were evaluated sequentially. This integrative approach enhances the robustness and reliability of the proposed anticancer mechanisms. This multi-level validation strategy reduces the limitations of individual computational approaches and provides more reliable mechanistic insights.

Molecular docking

The 3D structures of vanillic acid and rutin, identified as the major constituents of *I. aschersoniana* extracts, were retrieved in SDF format from the PubChem database to investigate their interaction mechanisms with target proteins via molecular docking studies. The proteins utilized in this study were identified as the top three hub

genes-AKT1, PPARG, and EGFR-based on the results of the network pharmacology analysis, which determined the ten hub genes with the highest degree. The 3D crystal structures of the target proteins were obtained from the Protein Data Bank with the following PDB identification numbers: 3O96 (AKT1), 7AWD (PPARG), and 1M17 (EGFR).

To validate the docking protocol, re-docking analyses were performed using the co-crystallized ligands: IQO444 for AKT1, NQD502 for PPARG, and AQ4999 for EGFR. Ligands were removed from the protein structures, re-docked, and the RMSD values were evaluated using BIOVIA Discovery Studio to confirm docking accuracy.

Finally, ligands, water molecules, heteroatoms, and unnecessary chains present in the protein structures were removed using procedures previously applied in our studies (Bozkurt et al. 2024; Yetek et al. 2024). UCSF Chimera software was performed for the molecular docking evaluation (Pettersen et al. 2004). The prepared proteins and downloaded ligands were imported into the software. Polar hydrogens and charges were added to both the proteins and ligands. The binding scores between ligands and proteins were evaluated using the Autodock Vina platform (Trott and Olson 2009), which works seamlessly with UCSF. Docking results were compared with reference control inhibitors—Mk-2206 for AKT1, Rosiglitazone for PPARG, and Gefitinib for EGFR—to provide a qualitative and quantitative assessment of ligand binding affinities. Blind docking was preferred for the grid box settings in this study to avoid bias and to explore potential alternative binding sites.

The interactions of ligand compounds with the active sites of target proteins were visually analyzed using the BIOVIA Discovery Studio Visualizer tool. The types of interactions between ligands and the active sites of the proteins were presented in both 2D and 3D formats.

Molecular dynamics simulations

Molecular dynamics (MD) simulations were conducted to assess the stability and dynamic behavior of the protein–ligand complex. The simulations were performed using GROMACS 2023, with the protein modeled using the CHARMM27 force field (Abraham et al. 2015). The topology and parameter files for the ligand were prepared using the SwissParam online tool and were made compatible with a CHARMM-based force field (Zoete et al. 2011). The complex was placed in a triclinic water box (TIP3P water model) with an approximate minimum distance of 1.0 nm between the protein and the box edges, and appropriate counterions (Na^+ or Cl^-) were added to neutralize the system (Jorgensen et al. 1983).

Energy minimization was carried out using the steepest descent algorithm until the maximum force fell below a defined threshold. The system was then equilibrated under NVT (constant number of particles, volume, and temperature) conditions, followed by NPT (constant number of particles, pressure, and temperature) conditions for a short period. Temperature and pressure were controlled using the V-rescale thermostat and the Parrinello-Rahman barostat, respectively (Bussi et al. 2007; Parrinello and Rahman 1981). Production MD simulations were subsequently performed with a 2 fs time step for a total of 50 ns, which is widely accepted as sufficient for evaluating the stability of protein–ligand complexes in preliminary studies.

The structural behavior and potential flexibility of the protein–ligand complex were evaluated using Root Mean Square Deviation (RMSD) and Root Mean Square Fluctuation (RMSF) analyses. RMSD was employed to monitor possible deviations of the protein backbone atoms relative to the initial structure, while RMSF was used to examine the fluctuations of individual residues throughout the simulation, providing insight into potentially flexible regions. The trajectories were analyzed and visualized using VMD and the built-in tools of GROMACS.

Binding free energy calculations (MM/GBSA)

Binding free energy calculations were performed using the MM/GBSA method to further evaluate the stability and binding affinity of the protein–ligand complexes obtained from molecular dynamics simulations. The calculations were carried out using the `gmx_MMPBSA` tool integrated with the GROMACS package. Snapshots

extracted from the last 10 ns of the MD trajectories were used for analysis. The total binding free energy (ΔG_{bind}) was calculated as the sum of van der Waals, electrostatic, polar solvation, and non-polar solvation energy components.

Result and discussion

Anticancer activity

Initially, cytotoxicity of the *I. aschersoniana* methanol extract was measured at a relatively high concentration in the HUVEC (Human Umbilical Vein Endothelial Cells), HT-29 (Human Colon Cancer Cell Line), and MCF-7 (Breast Cancer Cell Line). The cytotoxicity of *I. aschersoniana* extracts against cancer cells was proved in 313, 625, 1250, 2500, and 5000 $\mu\text{g/mL}$ concentrations against normal cells to measure IC_{50} values. The IC_{50} values of normal and cancer cells were compared to each other. The Endothelial Cells (HUVEC) that *I. aschersoniana* methanol extract was treated, did not show any significant MTT viability at 24, 48, and 72 h. It was determined that, the *I. aschersoniana* methanol extract did not exhibit cytotoxic effects on the healthy cell line HUVEC. The cytotoxic effect of the cancer drug cisplatin that had applied to the HUVEC cell line was found to be lower at different doses and time points compared to the HT-29, MCF-7 cell lines (Fig. 1).

The cancer drug cisplatin, used as a positive control group, was applied to the HT-29, MCF-7, and HUVEC cell line at different concentrations (313, 625, 1250, 2500, and 5000 $\mu\text{g/mL}$) and time points (24 h, 48 h, and 72 h). Cisplatin applied to the HT-29 and MCF-7 cell lines were found to exhibit a significant cytotoxic effect at different doses and time points (Table 1).

The ethanol extract of the *I. aschersoniana* showed a significant effect of apoptosis at different concentrations compared with the untreated cells. The apoptotic effects of the *I. aschersoniana* methanol extracts were dose-dependent. In the HT-29 cell line, at the highest concentrations (5000 and 2500 $\mu\text{g/mL}$), the 24-hour cell viability rates were 71.6% and 75.3%, respectively; at 48 h, 64.4% and 69.7%; and at 72 h, 51.7% and 67.0%. In the MCF-7 cell line, the 24-hour cell viability rates were 86.2% and 89.0%; at 48 h, 78.8% and 80.4%; and at 72 h, 77.4% and 81.2%, respectively.

The cytotoxic effects of the extract varied among the tested cell lines. In HT-29 cells, cell viability decreased in a dose- and time-dependent manner, reaching $51.7 \pm 4.1\%$ at 5000 $\mu\text{g/mL}$ after 72 h, corresponding to an approximate IC_{50} value of ~ 5200 $\mu\text{g/mL}$. In contrast, MCF-7 cells exhibited lower sensitivity, and the IC_{50} value remained above 5000 $\mu\text{g/mL}$ at all incubation times. Importantly, no cytotoxic effect was observed in HUVEC normal endothelial cells, where cell viability remained above 100% even at the highest concentration tested. Statistical analysis revealed significant differences between HT-29 and HUVEC cells ($p < 0.001$) and between MCF-7 and HUVEC cells ($p < 0.01$) at the highest tested concentration. Furthermore, a significant difference was

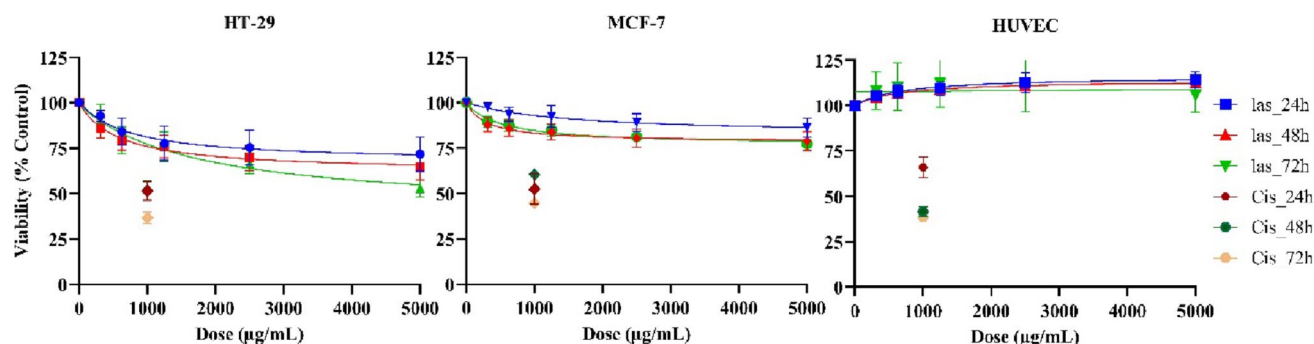


Fig. 1 MTT cell viability activity of *I. aschersoniana* against the MCF-7, HT-29, and HUVEC cell lines at 24, 48 and 72 h

Table 1 Statistical data of HT-29, MCF-7, and HUVEC cells for *I. aschersoniana*

Cell Line	Dose (µg/mL)	24 h Mean±SD	48 h Mean±SD	72 h Mean±SD
HT-29	NT	100±0 ^a	100±0 ^a	100±0 ^a
	313	92.8±3.0 ^b	85.9±5.4 ^b	92.7±6.7 ^b
	625	84.0±7.5 ^c	79.1±5.5 ^c	81.0±7.0 ^c
	1250	77.6±9.4 ^c	75.9±6.2 ^c	77.3±8.3 ^c
	2500	75.3±9.7 ^c	69.7±7.1 ^d	67.0±6.1 ^d
	5000	71.6±9.4 ^d	64.4±7.0 ^c	51.7±4.1 ^{***}
	R ²	0.6719	0.7052	0.9067
MCF-7	NT	100±0 ^a	100±0 ^a	100±0 ^a
	313	97.6±1.1 ^b	87.9±3.9 ^b	89.5±3.0 ^b
	625	93.6±3.8 ^b	85.7±4.3 ^c	87.5±3.2 ^c
	1250	92.5±5.8 ^b	83.7±4.4 ^c	85.0±3.4 ^c
	2500	89.0±4.8 ^b	80.4±4.9 ^c	81.2±2.1 ^c
	5000	86.2±5.2 ^c	78.8±5.3 ^c	77.4±3.5 ^{d**}
	R ²	0.8363	0.5561	0.7068
HUVEC	NT	100±0 ^a	100±0 ^a	100±0 ^a
	313	105.4±2.8 ^a	104.4±1.7 ^a	108.2±10.4 ^a
	625	107.9±3.8 ^a	106.7±2.1 ^a	110.3±13.1 ^a
	1250	109.4±3.9 ^a	109.0±2.1 ^a	112.2±13.1 ^a
	2500	112.6±5.4 ^a	110.8±2.5 ^a	111.0±14.2 ^a
	5000	114.3±4.4 ^a	112.4±2.4 ^a	105.9±9.5 ^a
	R ²	0.7284	0.7104	0.5117
Cis-HT-29	1000	51.5±5.1 ^d	51.8±5.1 ^d	36.7±3.2 ^{§*}
Cis-MCF-7	1000	52.5±8.4 ^d	60.4±2.2 ^c	44.7±1.3 ^{†*}
Cis-HUVEC	1000	65.8±5.9 ^c	41.6±2.6 ^f	38.5±2.3 ^{§*}

Values are expressed as mean±SD (*n*=3). Statistical analysis was performed using one-way ANOVA followed by Tukey's post hoc test. Within the same column, values with different superscript letters (a–g) are significantly different

The level of significance is indicated as follows:

p*<0.05, *p*<0.01, ****p*<0.001

also observed between HT-29 and MCF-7 cells (*p*<0.05), indicating that HT-29 cells were more sensitive to the treatment than MCF-7 cells. Cisplatin significantly reduced cell viability in both HT-29 and MCF-7 cancer cell lines compared to HUVEC normal endothelial cells. Specifically, a significant difference was observed between Cis-HT-29 and Cis-HUVEC (*p*<0.05) and between Cis-MCF-7 and Cis-HUVEC (*p*<0.05), indicating that cisplatin exerts selective cytotoxicity toward cancer cells. No significant difference was detected between Cis-HT-29 and Cis-MCF-7 (*p*>0.05), suggesting that both cancer cell lines exhibited similar sensitivity to cisplatin under these conditions.

Antioxidant activity

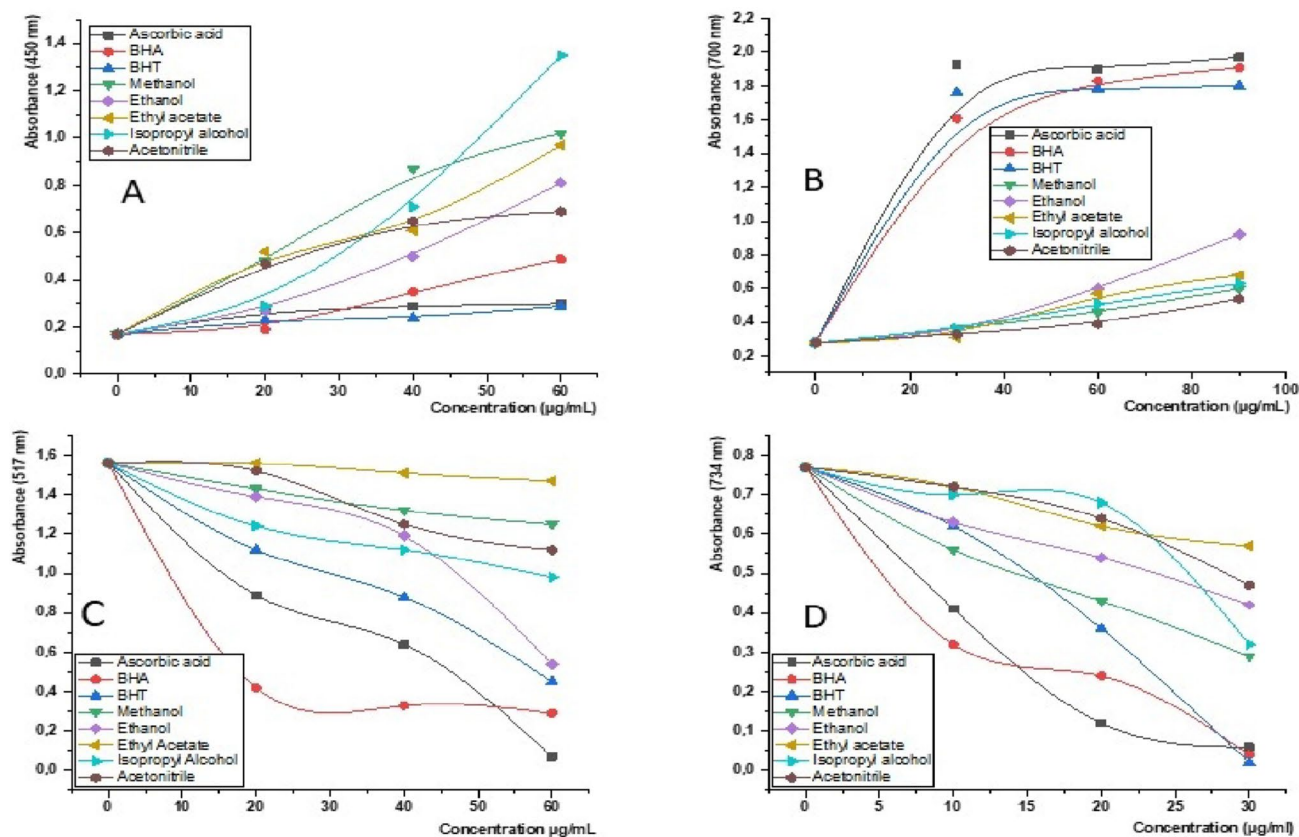
DPPH and ABTS methods are the most frequently applied radical scavenging antioxidant determination methods. For the DPPH and ABTS radical scavenging assays, different concentrations (20–60 µg/mL) of *I. aschersoniana* extracts were prepared. According to the DPPH results, the IC₅₀ values of ascorbic acid, BHA, and BHT standards, as well as methanol, ethanol, ethyl acetate, isopropyl, and acetonitrile extracts were calculated as 11.1 µg/mL, 9.8 µg/mL, 27.4 µg/mL, 60.4 µg/mL, 44.0 µg/mL, 156 µg/mL, 27.4 µg/mL, and 122.7 µg/mL, respectively. It was determined that the acetonitrile, ethyl acetate, and methanol extracts of *I. aschersoniana* had weaker DPPH radical scavenging activity compared to standard antioxidants. Remarkably, isopropyl and ethanol extracts had stronger DPPH radical scavenging activities compared to the acetonitrile, ethyl acetate, and methanol extracts.

Based on the ABTS^{•+} scavenging activity, the IC₅₀ values of ascorbic acid, BHA, and BHT standards, and methanol, ethanol, ethyl acetate, isopropyl, and acetonitrile extracts were calculated as 13.5 µg/mL, 10.1 µg/mL, 20.5 µg/mL, 16.65 µg/mL, 30.25 µg/mL, 67.4 µg/mL, 40.35 µg/mL and 57.75 µg/mL, respectively. Remarkably, it was determined that the methanol extract had stronger ABTS^{•+} radical scavenging activity compared to standard antioxidants as given in the Table 2.

Table 2 Radical scavenging and enzyme inhibitions IC₅₀ values of *I. aschersoniana* extracts

Plant extracts and standards	ABTS ⁺⁺	DPPH [*]	α -Glucosidase		GST	
	IC ₅₀ (μ g/mL)	IC ₅₀ (μ g/mL)	IC ₅₀ (μ g/mL)	R ²	IC ₅₀ (μ g/mL)	R ²
Methanol	16.7	60.4	ND	ND	6.47	0.90
Ethyl acetate	67.4	156	4.05	0.97	1.67	0.88
Ethanol	30.3	44	3.74	0.91	2.71	0.76
Isopropyl alcohol	40.4	27.4	NT	NT	NT	NT
Acetonitrile	57.75	122.7	NT	NT	NT	NT
Ascorbic acid*	13.5	11.1	N/A	N/A	N/A	N/A
BHA*	10.1	9.8	N/A	N/A	N/A	N/A
BHT*	20.5	27.4	N/A	N/A	N/A	N/A

ND: Not detected, NT: Not tested, N/A: Not applicable, *Used as positive control antioxidant standards

**Fig. 2** Antioxidant activities of *I. aschersoniana* extracts and standards by CUPRAC (A), FRAP (B), DPPH (C), and ABTS (D) methods

The cupric ion (Cu^{2+}) reduction capacities of the methanol, ethanol, ethyl acetate, isopropyl, and acetonitrile extracts of *I. aschersoniana* were studied to see the solvent effects for the antioxidant activity. The reduction capacities of the extracts and the standard antioxidants were given in Fig. 2.

The antioxidant powers of the extracts on the reduction of cupric ions (Cu^{2+}) to cuprous ions (Cu^{+}) at 30 $\mu\text{g/mL}$ concentrations was found as follows: isopropyl extract > methanol extract > ethyl acetate extract > ethanol extract > acetonitrile extract > BHA > ascorbic acid > BHT. According to the FRAP assay, the antioxidant capacity of the samples on Fe^{+3} to Fe^{+2} reduction were found as: ascorbic acid > BHA > BHT > ethanol extract > ethyl acetate extract > isopropyl extract > methanol extract > acetonitrile extract. It is accepted that antioxidant effects increase as the concentrations of plant extracts increase for both CUPRAC and FRAP methods as seen in Fig. 2A/B.

Enzyme inhibitory activity

Enzyme inhibition effects of the different extracts of *I. aschersoniana* were examined on α -glucosidase and GST enzymes. IC₅₀ (inhibition concentration that reduces half of the enzyme activity), correlation coefficient (R²), and % activity values were calculated for the quantitative determination.

According to the results, the extracts were determined to be effective for the both enzymes. For the α -glucosidase enzyme; the IC₅₀ values of the ethyl acetate and ethanol extracts were obtained as, 4.05 μ g/mL (R²: 0.9684) and 3.74 μ g/mL (R²: 0.9066). On the other hand, the IC₅₀ values of the ethyl acetate, ethanol, and methanol extracts against the GST enzyme were found as 1.67 μ g/mL (R²: 0.8788), 2.71 μ g/mL (R²: 0.7615), and 6.47 μ g/mL (R²: 0.9024), respectively as given in Table 2.

Phytochemical compounds of *I. aschersoniana* extracts

HPLC analysis was carried out to determine the major organic compounds of the methanol extract of *I. aschersoniana*. The quantitative amounts of identified phenolic and flavonoid compounds are summarized as follows: Ascorbic acid (2.67 μ g/g extract), gallic acid (4.23 μ g/g extract), protocatechuic acid (3.16 μ g/g extract), vanillic acid (5.89 μ g/g extract), rutin (9.6 μ g/g extract), ferulic acid (1.42 μ g/g extract), naringin (14.99 μ g/g extract), o-coumaric acid (4.01 μ g/g extract), neohesperidin (4.28 μ g/g extract), and alizarin (1.62 μ g/g extract). The amount of major constituents of the methanol extract of *I. aschersoniana* can be seen in Fig. 3. The most common phenolic compounds in the methanol extract was determined to be naringin 14.99 μ g/g extract, rutin 9.60 μ g/g extract, and vanillic acid 5.89 μ g/g extract. In addition, ascorbic acid, gallic acid, o-coumaric acid, alizarin, protocatechuic acid, ferulic acid, and neohesperidin compounds were detected in certain amounts.

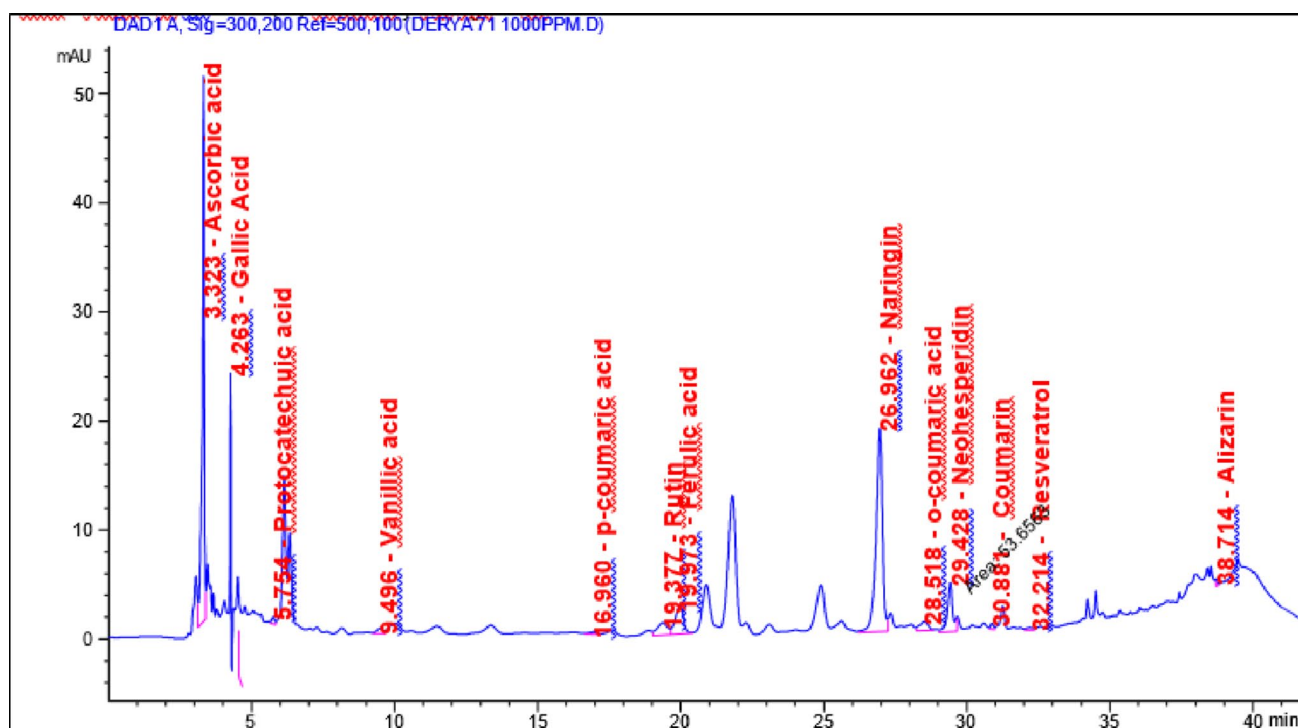


Fig. 3 The HPLC chromatograms of *I. aschersoniana*

Network pharmacology analysis

In the in vitro studies, we focused on the major components of *I. aschersoniana* identified for use in breast and colon cancer, specifically vanillic acid, rutin, and naringin. For this aim the screening of public databases, namely SwissTargetPrediction and Stitch tools, were identified for 46 potential targets. In this study, a total of 26,513 genes were obtained from the OMIM and GeneCards databases to identify genes associated with breast and colon cancer, and these genes were subsequently filtered based on relevance scores to reduce noise and enhance biological significance. The comparison of targets related to breast and colon cancers and genes involved in cancer causation was performed using network pharmacology analysis. The overlap between genes associated with breast and colon cancers and targets linked to the active compounds was determined using a Venn diagram generated by the Venny 2.1 software, as shown in Fig. 4a. As depicted in Figs. 4a and 45 (0.2%) common targets related either directly or indirectly to cancer diseases were identified for vanillic acid, rutin, and naringin compounds. Furthermore, an intersection network between these active compounds and their targets was constructed and presented in Fig. 4b.

For a comprehensive analysis of the effects of the active compounds on breast and colon cancers, a Protein-Protein Interaction (PPI) network was constructed, and the overlapping 45 targets were analyzed in the publicly

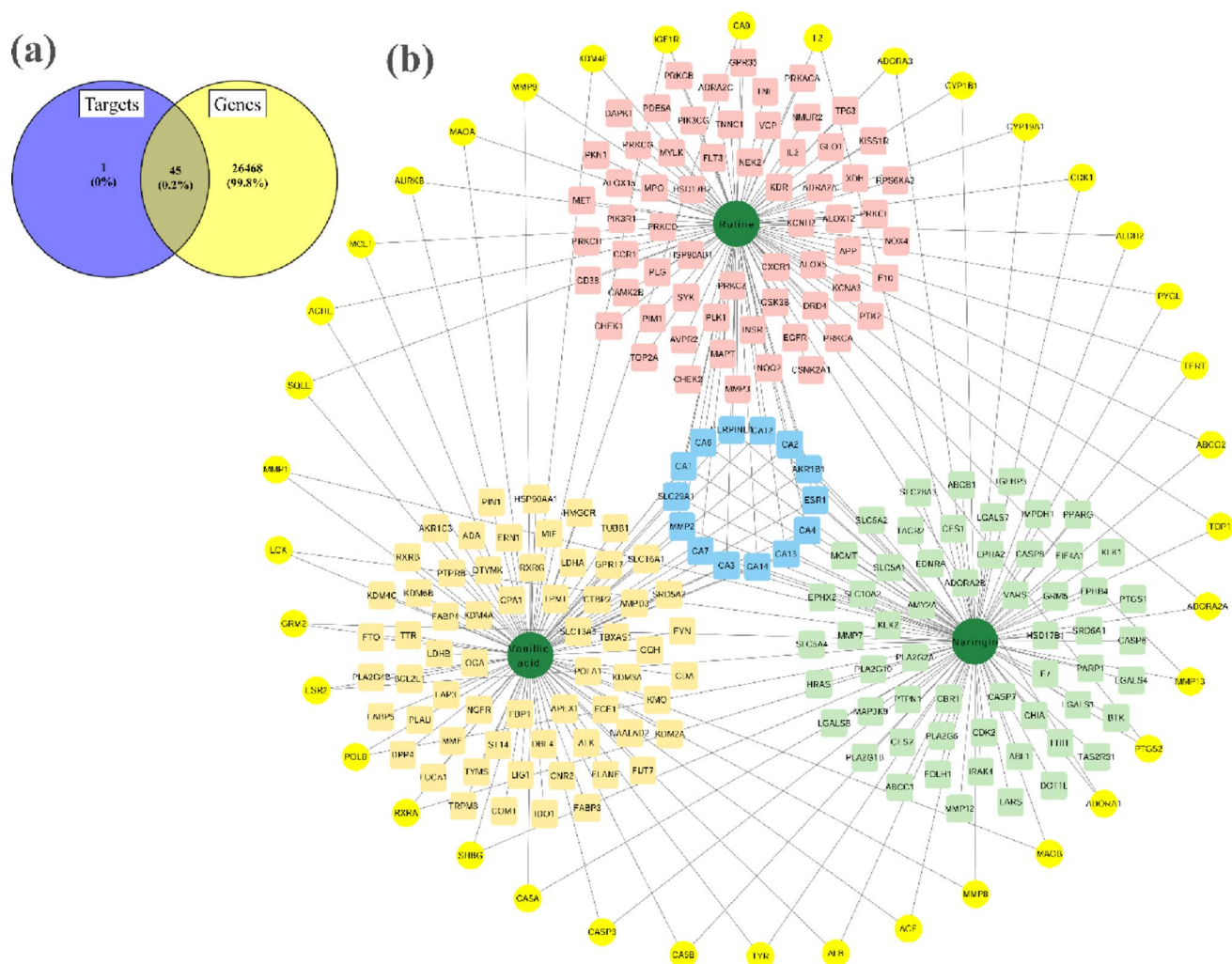


Fig. 4 Compound-target intersection analysis: (a) Venn diagram (compound and cancer intersection targets); (b) Network of compound-intersection targets

available STRING database using their protein IDs. As shown in Fig. 5a, the results of the PPI analysis revealed 45 nodes and 148 edges in the network. The PPI network was used to analyze the interactions between the targets of the active compounds and associated diseases, thereby aiding in a better understanding of the potential therapeutic effects of the compounds and clarifying their roles in biological processes. For more detailed analysis of the PPI network, the network data was transferred from the STRING database to Cytoscape 3.10.3 software for further analysis (Fig. 5b). Cluster analysis of the PPI network was performed by removing targets with centrality degree (DC) values greater than one or two times the median. The PPI network cluster analysis was then carried out using the CytoHubba plugin of Cytoscape 3.10.3, which identified the top 10 hub nodes based on the highest degree of centrality (Chin et al. 2014).

The critical targets playing a role in cancer types were highlighted, and the top 10 hub nodes identified were AKT1 (21), PPARG (18), EGFR (16), MMP9 (14), SERPINE1 (13), PPARA (12), NOX4 (11), XDH (10), MMP2 (10), and CA1 (10) (Fig. 5c). These ten genes with high interaction degrees are likely to play pivotal roles in the biological processes related to the investigated cancers.

Gene ontology and KEGG enrichment analysis

GO enrichment analysis and KEGG pathway enrichment were performed for the 45 common candidate targets using the ShinyGO 0.82 database and visually represented in Fig. 6. GO enrichment analysis is typically used to understand the biological processes in which genes are involved, whereas KEGG pathway enrichment analysis is used to understand the pathways through which the genes exhibit activity. In this study, analysis results with $P \leq 0.05$ were considered statistically significant.

In this study, the 45 potential targets identified were subjected to Gene Ontology (GO) enrichment analysis to understand the biological processes (BP), cellular components (CC), and molecular functions (MF) related to breast and colon cancer in connection with *I. aschersoniana*.

In the BP enrichment analysis, the top 10 target genes were identified as: Response to UV-A (5), One-carbon metabolic process (11), Histone lysine demethylation (6), Histone demethylation (6), Protein demethylation (6), Protein dealkylation (6), Demethylation (7), Cellular response to chemical stress (11), Small molecule metabolic process (24), and Response to chemical (30) (Fig. 6a).

In the CC enrichment analysis, the target genes were identified as: Anchored component of external side of plasma membrane (2), Membrane raft (5), Side of membrane (7), Extracellular exosome (15), Extracellular

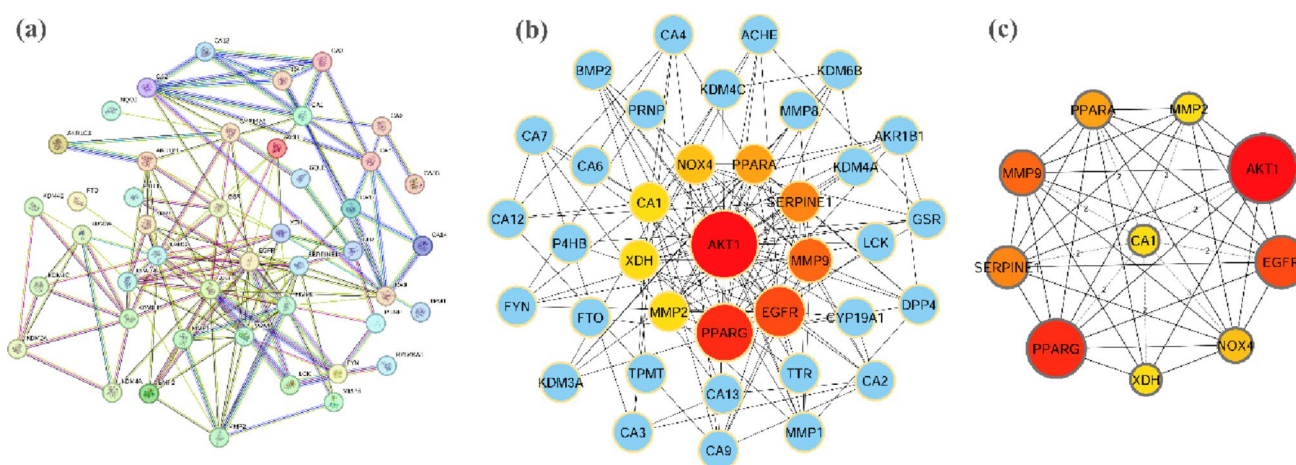


Fig. 5 PPI network of potential target genes: **(a)** Interactions between proteins (nodes) and proteins (edges), **(b)** The core PPI network is constructed based on the degree value of each target, with darker node colors indicating higher degree values, and **(c)** the top 10 hub genes network

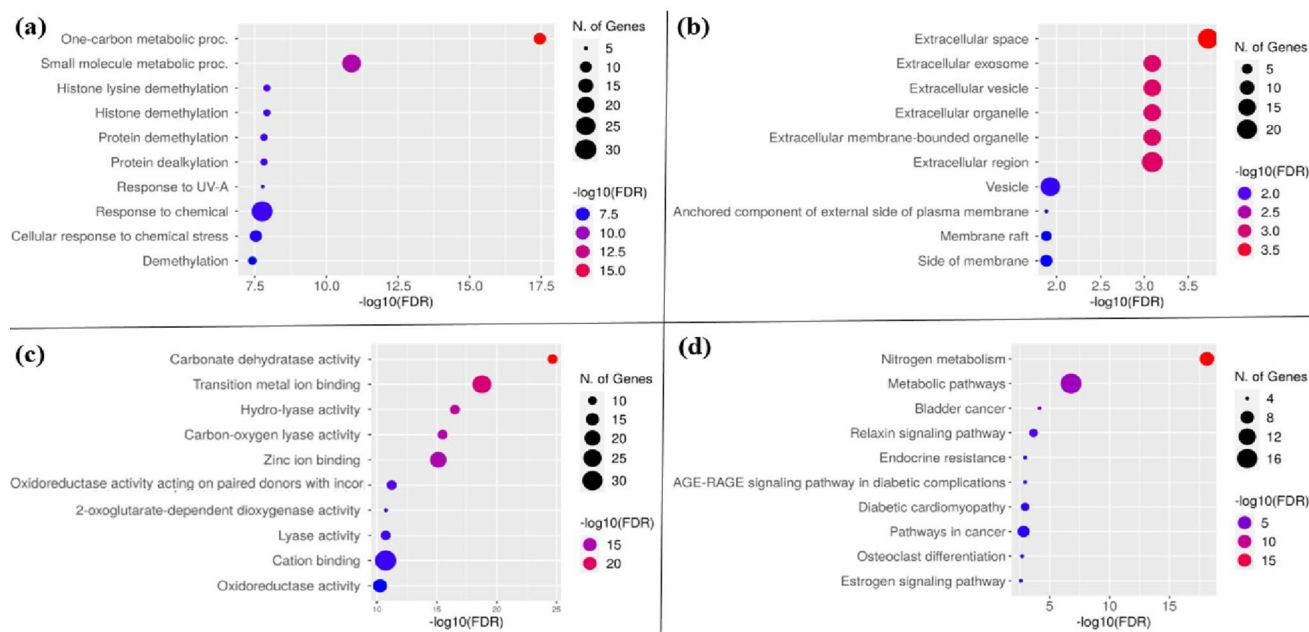


Fig. 6 GO and KEGG enrichment analyses of 45 key targets, (a) GO enrichment analysis of key targets in biological process (BP), (b) cellular component (CC), and (c) molecular function (MF) (d) KEGG pathway enrichment analysis of key targets. The color gradient of the bubbles, from red to blue, indicates the $-\log_{10}(\text{FDR})$ values of the pathways, with red representing the highest and blue the lowest values. Moreover, the circle size corresponds to the number of target genes connected to each pathway

vesicle (15), Extracellular organelle (15), Extracellular membrane-bounded organelle (15), Extracellular space (21), Extracellular region (22), and Vesicle (19) (Fig. 6b).

In the MF enrichment analysis, the target genes were found to be: Carbonate dehydratase activity (11), Hydro-lyase activity (11), Carbon-oxygen lyase activity (11), 2-oxoglutarate-dependent dioxygenase activity (8), Oxidoreductase activity acting on paired donors with incorporation or reduction of molecules (11), Lyase activity (11), and Zinc ion binding (21) (Fig. 6c).

Additionally, KEGG pathway enrichment analysis was performed on the 45 common target genes associated with the active components of *I. aschersoniana* for breast and colon cancer using the ShinyGO 0.82 tool. KEGG analysis revealed 100 different signaling pathways through which biochemical signals are transmitted within cells. To ensure the reliability of the enrichment analysis, a fold enrichment (FDR) value ≤ 0.05 was chosen. The top 10 signaling pathways with the highest enrichment ratios are shown in Fig. 6d. These results suggest that the active components may exert their effects on breast and colon cancer through pathways such as Nitrogen metabolism (9), Bladder cancer (4), Endocrine resistance (4), AGE-RAGE signaling pathway in diabetic complications (4), Relaxin signaling pathway (5), Osteoclast differentiation (4), Estrogen signaling pathway (4), Diabetic cardiomyopathy (5), Pathways in cancer (7), and Metabolic pathways (17).

The KEGG analysis highlighted that the active components of the plant are particularly effective in significant biological pathways such as Nitrogen metabolism, Bladder cancer, and Estrogen signaling. Nitrogen metabolism pathways are associated with cancer cell growth and metastasis, suggesting that compounds affecting this pathway could have the potential to slow down cancer progression (Yousf et al. 2023). Furthermore, Endocrine resistance and Estrogen signaling pathways play a key role, especially in the treatment of breast cancer.

Previous studies have reported that, despite estrogen receptor (ER)-targeted therapy in breast cancer, escape pathways may develop that promote the survival of cancer cells, and these escape pathways could lead to resistance by limiting the effectiveness of the treatment (AlFakeeh and Brezden-Masley 2018; Rani et al. 2019). In this context, the plant's effect on Estrogen signaling pathways provides a mechanism that may assist in overcoming

endocrine resistance. The AGE-RAGE signaling pathway is an important pathway showing the relationship between diabetic complications and cancer. Modulating this pathway could indicate metabolic and inflammatory regulations that may assist in cancer treatment. These findings demonstrate that the plant's active components are involved in a broad range of biological effects and hold potential as a novel therapeutic strategy for cancer treatment.

Molecular docking study

Molecular docking is a widely employed computational method used to predict the binding orientation and affinity of small molecules within the active sites of target proteins. In the present study, docking analysis was conducted to evaluate the interaction potential of the major bioactive compounds of *I. aschersoniana*—vanillic acid, naringin, and rutin—with key cancer-related hub proteins. This approach was utilized not only to estimate binding affinities but also to provide insights into the potential molecular mechanisms underlying their biological activities.

To validate the reliability of the docking protocol, a re-docking procedure was conducted using the co-crystallized ligands of the selected proteins. The native ligands were removed and re-docked into their respective binding sites. The resulting RMSD values were 1.3975 Å for AKT1, 1.6495 Å for EGFR, and 1.0924 Å for PPARG, all of which are below the acceptable threshold of 2 Å. These findings confirm that the docking protocol is capable of accurately reproducing experimentally observed binding conformations and is therefore suitable for subsequent analyses. Following validation, molecular docking simulations were performed for vanillic acid, rutin, and naringin against AKT1 (PDB ID: 3O96), PPARG (PDB ID: 7AWD), and EGFR (PDB ID: 1M17). In addition, reference inhibitors—Mk-2206 (AKT1), Rosiglitazone (PPARG), and Gefitinib (EGFR)—were included to enable a comparative assessment of binding affinities (Table 3).

The docking results revealed that naringin (−11.7 kcal/mol) and rutin (−11.5 kcal/mol) exhibited stronger binding affinities toward AKT1 compared to the reference inhibitor Mk-2206 (−10.4 kcal/mol). This suggests that these flavonoid compounds may possess significant inhibitory potential against AKT1.

At the molecular level, both compounds formed multiple stabilizing interactions within the active site. Naringin established hydrogen bonds with residues such as Asn54, Thr82, Thr211, and Asp292, along with hydrophobic contacts involving Leu210 and Val270. Similarly, rutin demonstrated extensive hydrogen bonding with key residues including Asn54, Arg273, and Asp274, as well as hydrophobic interactions with Trp80 and Val270. These residues are known to play essential roles in ligand recognition and catalytic activity (Fig. 7).

The stronger binding affinity of these compounds compared to the reference inhibitor may be attributed to their larger molecular size and higher number of functional groups, which allow for more extensive interactions within the binding pocket. This observation is consistent with previous reports indicating that flavonoid derivatives can effectively target kinase domains.

For PPARG, naringin demonstrated the highest binding affinity (−9.4 kcal/mol), surpassing the reference agonist Rosiglitazone (−8.2 kcal/mol), while rutin showed a comparable docking score (−8.4 kcal/mol). In contrast, vanillic acid exhibited a considerably weaker interaction, highlighting the importance of molecular complexity in binding efficiency.

Naringin formed multiple hydrogen bonds with critical residues such as Phe264, Gln273, Arg280, Arg285, and Arg288, along with hydrophobic interactions within the ligand-binding domain. These residues are crucial for ligand stabilization and receptor activation. The presence of both hydrogen bonding and hydrophobic interactions suggests a strong and stable ligand–protein complex. From a functional perspective, these findings indicate that naringin may act as a potential modulator of PPARG activity. The ability to form interactions with key residues involved in receptor activation further supports its possible biological relevance. The detailed interaction profiles are presented in Fig. 8.

Table 3 Molecular docking results of vanillic acid, rutin, and naringin with AKT1, PPARG, and EGFR compared with reference ligands (MK-2206, Rosiglitazone, and Gefitinib)

Name/PDB ID	Active compounds	Docking Score	Interacting amino acid residues
AKT1/3O96	Rutin	-11.5 kcal/mol	Hydrogen Bond: Asn54(2), Arg273, Gln79, Val271, Asp274, Cys296, Tyr272, Hydrophobic: Gln279, Val270(2), Trp80(4), Gln79, Ile89, Val270, Leu264, Electrostatic: Asp292
	Vanillic acid	-5.8 kcal/mol	Hydrophobic: Trp80(2), Leu264, Lys268, Val270
	Naringin	-11.7 kcal/mol	Hydrogen Bond: Asn54, Thr82, Thr211, Asp292 Hydrophobic: Leu210, Val270 Electrostatic: Asp292
PPARG/7AWD	Control ligand (Mk-2206)	-10.4 kcal/mol	Hydrogen Bond: Arg273, Asp274 Hydrophobic: Val270, Trp80(2), Lys268, Leu264
	Rutin	-8.4 kcal/mol	Hydrogen Bond: Gln273 Hydrophobic: Phe287(2), Arg288
	Vanillic Acid	-5.4 kcal/mol	Hydrogen Bond: Glu295, Hydrophobic: Ala292, Ile326, Leu330
EGFR/1M17	Naringin	-9.4 kcal/mol	Hydrogen Bond: Phe264, Gln273, Arg280, Arg285, Arg288 Hydrophobic: Arg280, Arg288(2), Leu333, Ile341 Electrostatic: Arg288
	Control ligand (Rosiglitazone)	-8.2 kcal/mol	Hydrogen Bond: Arg288,Ser342 Hydrophobic: Cys285, Ile341
	Rutin	-9.0 kcal/mol	Hydrogen Bond: Thr830, Gly772, Thr766(2), Hydrophobic: Val702(3), Ala719(2), Lys721(2), Leu694, Leu820
	Vanillic Acid	-6.2 kcal/mol	Hydrogen Bond: Thr766(3), Thr830(2),Leu764, Hydrophobic: Lys721 Electrostatic: Lys721
	Naringin	-8.9 kcal/mol	Hydrogen Bond: Met769(2), Asp831 Hydrophobic: Leu694, Phe699, Val702(2), Lys721, Leu820
	Control ligand (Gefitinib)	-7.8 kcal/mol	Hydrogen Bond: Met769, Gln767, Asp831 Hydrophobic: Leu694, Val702(2), Lys721(2), Leu820(2), Leu764, Ala719 Halogen: Glu738

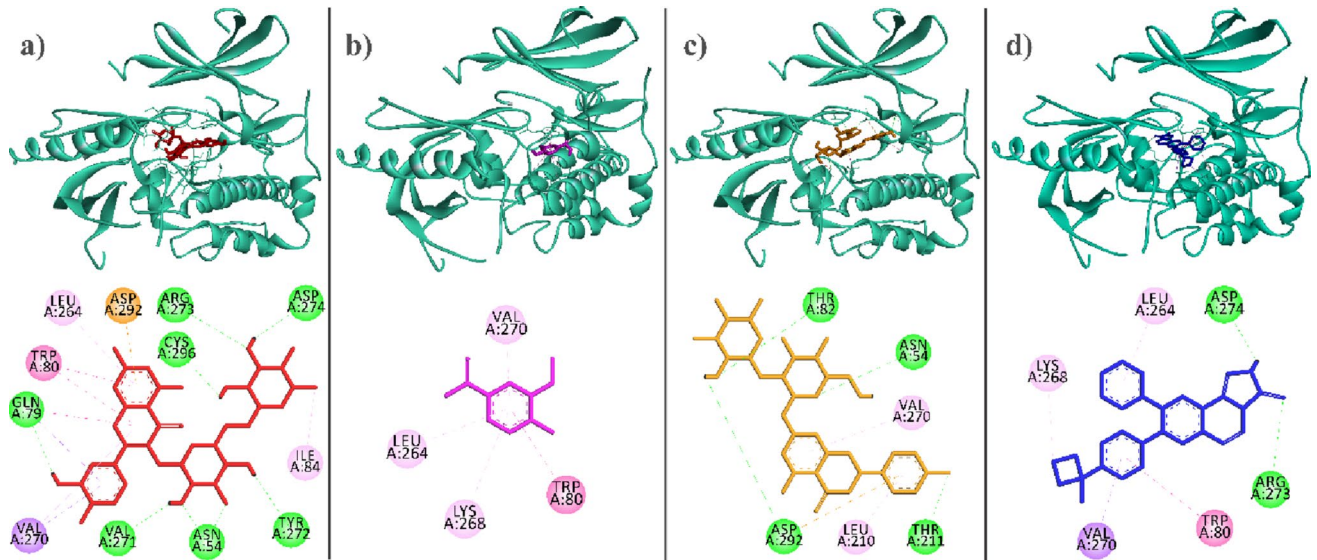


Fig. 7 The complexes formed by AKT1 (PDB: 3O96) with active compounds; (a) rutin, (b) vanillic acid, (c) naringin and (d) control ligand (Mk-2206)

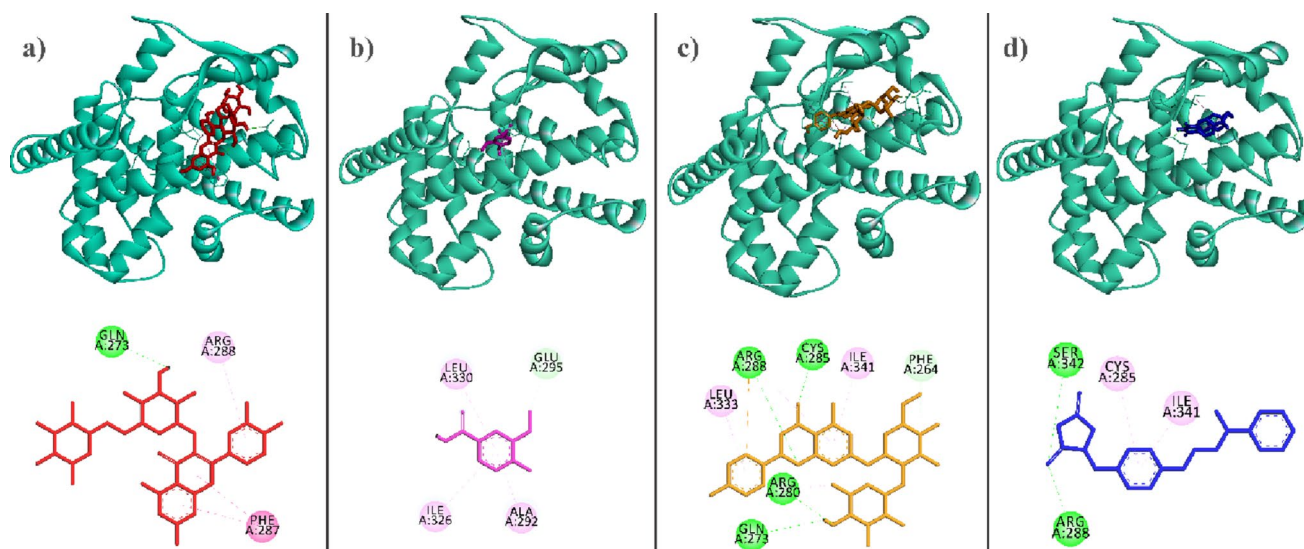


Fig. 8 The complexes formed by PPARG (PDB: 7AWD) with active compounds; **(a)** rutin, **(b)** vanillic acid, **(c)** naringin and **(d)** control ligand (Rosiglitazone)

In the EGFR docking analysis, rutin (−9.0 kcal/mol) and naringin (−8.9 kcal/mol) exhibited higher binding affinities than the reference inhibitor Gefitinib (−7.8 kcal/mol), suggesting their potential as effective inhibitors of EGFR.

Both compounds interacted with key residues located in the ATP-binding pocket, including Met769, Lys721, Val702, and Leu820, which are essential for kinase activity. Rutin formed multiple hydrogen bonds with residues such as Thr766, Thr830, and Gly772, whereas naringin interacted with Met769 and Asp831. Additionally, hydrophobic interactions with residues like Leu694 and Phe699 contributed to the stabilization of the complexes.

The ability of these compounds to occupy the ATP-binding site and interact with critical residues suggests that they may interfere with EGFR signaling pathways. This is particularly important given the central role of EGFR in cancer progression. The interaction maps of these complexes are shown in Fig. 9.

Collectively, the docking results demonstrate that flavonoid compounds, particularly naringin and rutin, exhibit stronger binding affinities toward all studied targets compared to vanillic acid. This can be attributed to their larger molecular frameworks and the presence of multiple hydroxyl groups, which facilitate the formation of extensive hydrogen bonding and hydrophobic interactions.

These findings suggest that structural complexity plays a crucial role in enhancing ligand–protein interactions and may be a key determinant of biological activity. However, it should be noted that molecular docking provides a static representation of binding interactions and does not fully account for protein flexibility and solvent effects.

Therefore, to obtain a more comprehensive understanding of ligand–protein stability and binding energetics, further validation was carried out using molecular dynamics-based MM/GBSA binding free energy calculations.

Molecular dynamics simulations

Molecular dynamics (MD) simulations are widely employed to investigate the time-dependent atomic behavior of protein–ligand complexes, providing a means to validate and complement molecular docking results. MD simulations allow the assessment of ligand-induced effects on protein structure, conformational changes, and the persistence of molecular interactions under near-physiological conditions. In this context, MD simulations provide comprehensive insights into the dynamic stability of complexes beyond static docking predictions. In the present study, 50 ns MD simulations were performed for the AKT1@Nar, EGFR@Nar, and PPARG@Nar complexes, and the resulting trajectories were analyzed using RMSD and RMSF metrics.

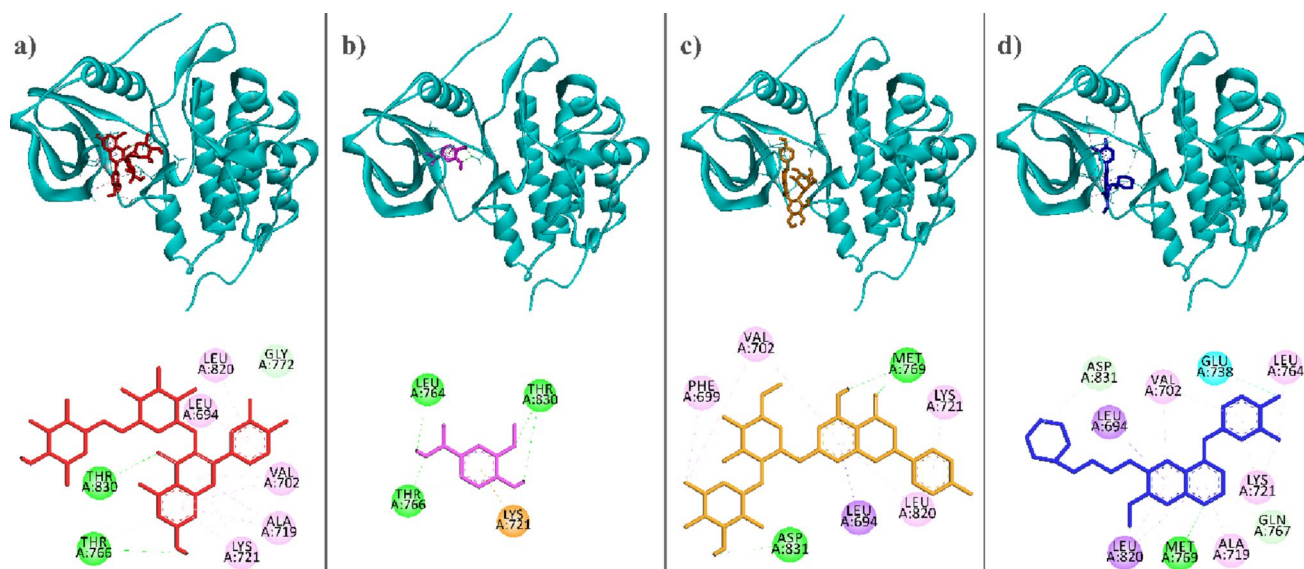
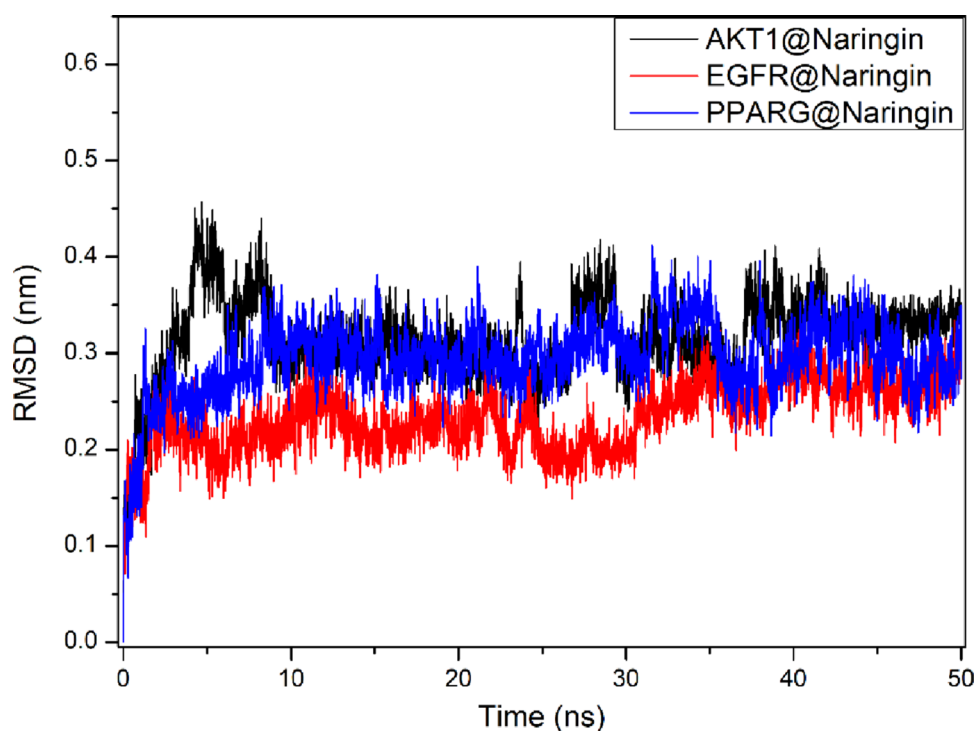


Fig. 9 The complexes formed by EGFR (PDB: 1M17) with active compounds; (a) rutin, (b) vanillic acid, (c) naringin and (d) control ligand (Gefitinib)

Fig. 10 RMSD plots of AKT1@Naringin (black), EGFR@Naringin (red), and PPARG@Naringin (blue) complexes obtained from 50 ns molecular dynamics simulations



RMSD analysis is commonly used in MD simulations to evaluate the structural stability and conformational changes of protein–ligand complexes over time. The RMSD profiles of AKT1@Nar, EGFR@Nar, and PPARG@Nar complexes over 50 ns are presented in Fig. 10, with AKT1@Nar shown as a black line, EGFR@Nar as a red line, and PPARG@Nar as a blue line.

The RMSD profile of the AKT1@Nar complex (Fig. 10, black) exhibited a rapid increase during the initial 5 ns, reaching approximately 0.40–0.45 nm. This initial fluctuation reflects the system’s equilibration following energy minimization. Subsequently, RMSD values stabilized between 0.28 and 0.32 nm from ~10 ns until the

end of the simulation, with minor fluctuations representing local conformational adjustments, indicating overall structural integrity of the complex.

The EGFR@Nar complex (Fig. 10, red) showed an increase to ~ 0.20 – 0.25 nm within the first 2–3 ns, followed by moderate fluctuations. RMSD values largely remained within 0.20–0.30 nm, stabilizing after ~ 10 ns. Minor increases observed at later stages were considered transient and did not indicate structural destabilization.

For the PPARG@Nar complex (Fig. 10, blue), RMSD initially reached 0.25–0.30 nm, reflecting equilibration. After ~ 5 ns, RMSD values fluctuated mainly between 0.28 and 0.33 nm, with occasional short-term increases up to ~ 0.38 – 0.40 nm. The absence of significant long-term deviations indicates that the PPARG@Nar complex maintained structural stability throughout the simulation.

RMSF analysis was performed to evaluate residue-level flexibility of the protein–ligand complexes and the effect of ligand binding on active site dynamics. RMSF profiles for AKT1@Nar, EGFR@Nar, and PPARG@Nar are shown in Figs. 11a–c, respectively.

In the AKT1@Nar complex, RMSF values were generally low (~ 0.05 – 0.15 nm) across most residues, with notable increases mainly at the N- and C-terminal regions. Consistent with docking results, residues involved in Nar binding (Leu210, Thr211, and Asp292) exhibited low fluctuations, indicating that ligand binding restricted local mobility and stabilized the catalytic site.

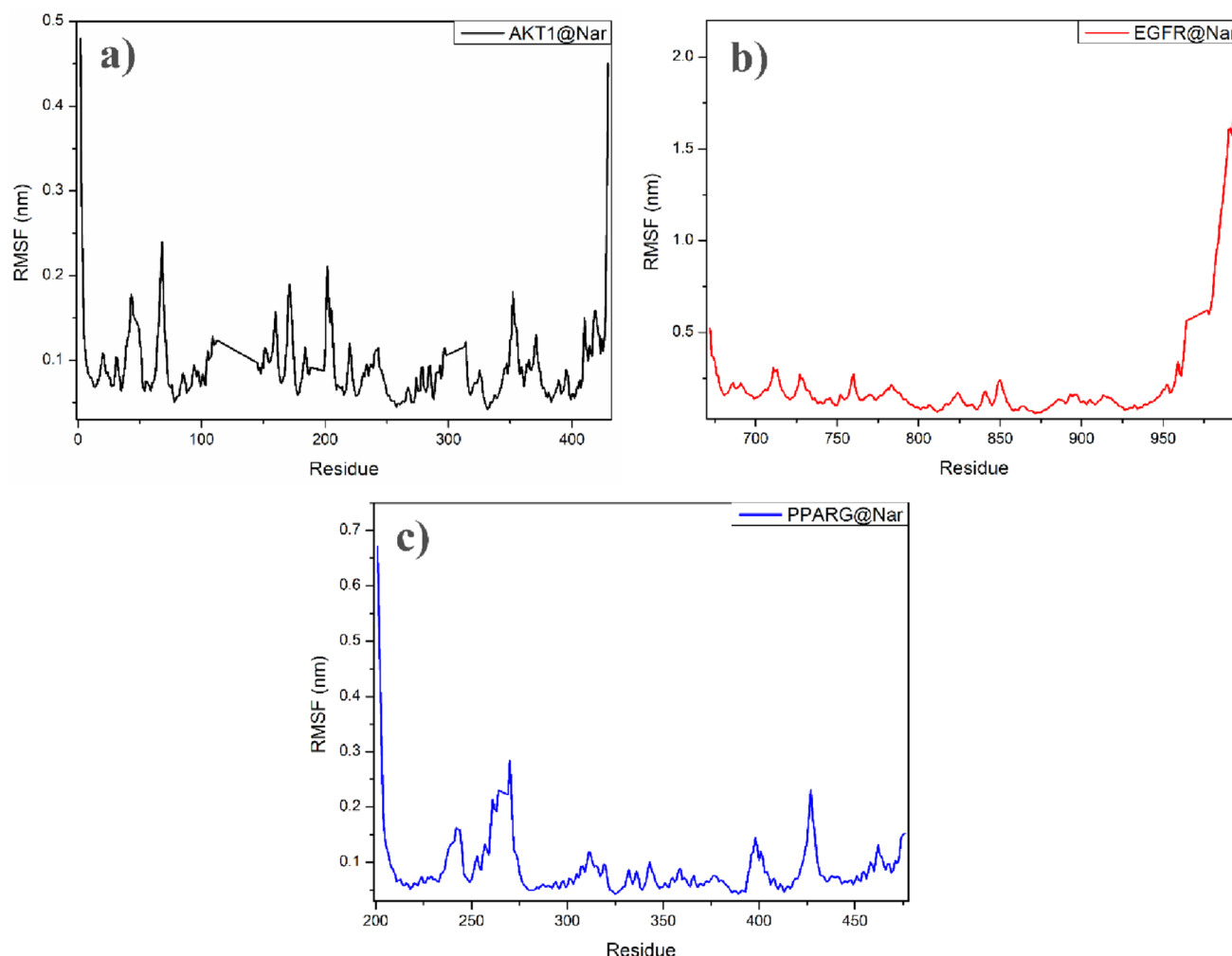


Fig. 11 RMSF plots. **(a)** AKT1@Naringin (black), **(b)** EGFR@Naringin (red) and **(c)** PPARG@Naringin (blue) complexes obtained from 50 ns molecular dynamics simulations

Table 4 MM/GBSA binding free energies (ΔG_{bind}) and energy decomposition of naringin complexes with AKT1, PPARG, and EGFR

Complex	ΔG_{bind} (kcal/mol)	ΔG_{vdW}	ΔG_{elec}	ΔG_{polar}	$\Delta G_{\text{nonpolar}}$
AKT1@Naringin	-72.51 ± 5.47	-57.87 ± 4.09	-62.28 ± 8.98	56.59 ± 4.55	-8.95 ± 0.26
PPARG@Naringin	-42.08 ± 4.61	-46.29 ± 3.56	-64.75 ± 11.35	76.58 ± 7.78	-7.63 ± 0.33
EGFR@Naringin	-32.91 ± 3.90	-40.85 ± 3.42	-65.41 ± 8.65	80.02 ± 7.10	-6.67 ± 0.33

The EGFR@Nar complex displayed moderate fluctuations throughout the protein, with a marked increase at the C-terminal region. Importantly, residues interacting with Nar in the active site (Leu294, Met769, Leu820, and Asp831) showed low RMSF values, suggesting local stabilization of the kinase active site despite overall protein flexibility.

The PPARG@Nar complex exhibited a more uniform and low fluctuation profile compared to the other complexes. Residues involved in ligand binding (Phe264, Gln273, Arg280, Cys285, Arg288, Leu333, and Ile341) maintained very low RMSF values, indicating effective stabilization of the binding pocket. The extensive interactions with key active site residues contribute to the high dynamic stability of the PPARG@Nar complex.

Binding free energy analysis (MM/GBSA)

To complement the static molecular docking results, MM/GBSA binding free energy calculations were performed to provide a more dynamic and reliable estimation of protein–ligand interactions. The binding free energies (ΔG_{bind}) of the naringin complexes with AKT1, PPARG, and EGFR were calculated and decomposed into van der Waals (ΔG_{vdW}), electrostatic (ΔG_{elec}), polar solvation (ΔG_{polar}), and nonpolar solvation ($\Delta G_{\text{nonpolar}}$) contributions, as summarized in Table 4.

The results revealed that AKT1@naringin exhibited the most favorable binding energy ($\Delta G_{\text{bind}} = -72.51 \pm 5.47$ kcal/mol), followed by PPARG@naringin (-42.08 ± 4.61 kcal/mol) and EGFR@naringin (-32.91 ± 3.90 kcal/mol). The dominant contributors to binding were van der Waals (ΔG_{vdW}) and electrostatic (ΔG_{elec}) interactions, partially counteracted by polar solvation energies (ΔG_{polar}), while nonpolar solvation ($\Delta G_{\text{nonpolar}}$) provided modest stabilization.

These findings indicate that naringin forms highly stable complexes with AKT1 through a combination of hydrophobic and electrostatic interactions, consistent with the docking results. For PPARG and EGFR, although ΔG_{bind} values were comparatively lower, the interactions were still stabilized primarily by van der Waals and electrostatic contributions. Overall, MM/GBSA analysis confirms that naringin, as a flavonoid-type compound, exhibits strong and favorable binding with the selected cancer-related targets, supporting its potential therapeutic relevance.

Conclusion

In this study, the biological and pharmacological potential of *I. aschersoniana* Janka was comprehensively evaluated by utilizing both in vitro methods and in silico computational analyses. The findings indicated that the plant extracts exhibited selective anticancer activity, demonstrating a more pronounced cytotoxic effect against the HT-29 colon adenocarcinoma cell line. The results demonstrated that the *I. aschersoniana* methanol extract exhibited notable inhibitory effects on both HT-29 and MCF-7 cells, with a more pronounced inhibition observed in the HT-29 cell line. However, *I. aschersoniana* extracts did not exhibit notable cytotoxicity on the HUVEC endothelial cell line, which was used as the control. According to the results, *I. aschersoniana* extracts have good antioxidant activities that can scavenge radical oxygen species. Also, *I. aschersoniana* extracts have significant α -glucosidase and GST enzymes inhibition effects, as well. Furthermore, HPLC analysis was implemented to confirm the presence of bioactive secondary metabolites, supporting the therapeutic potential of *I. aschersoniana*. The network pharmacology analysis suggests that *I. aschersoniana* may modulate breast and colon cancer pathways via key targets, primarily AKT1, PPARG, and EGFR. Molecular docking indicated that rutin and naringin

bind effectively to these targets, and MM/GBSA calculations further confirmed energetically favorable interactions for naringin KEGG analysis revealed that the plant's active components influence pathways relevant to cancer, including Nitrogen metabolism, Bladder cancer, Endocrine resistance, and Estrogen signaling. RMSD and RMSF analyses showed stable binding of naringin to all three proteins, with AKT1@Nar and PPARG@Nar complexes exhibiting the most balanced dynamics. These findings support the potential of *I. aschersoniana* as a plant-based therapeutic strategy, warranting further in vivo and clinical validation.

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Data availability No datasets were generated or analysed during the current study.

Declarations

Competing interests The authors declare no competing interests.

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