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School of Pharmacy & Pharmaceutical Sciences
Isfahan University of Medical Sciences

Original Article

Ferula gummosa in colorectal cancer: a bioinformatics and experimental validation study

Abbas Alibakhshi[1,2], Shima Gharibi[3], Ali Shojaeian[1], Atefeh Asgari[4], Razieh Amini[1], Mehdi Rahimmalek[5,6], Shahrzad Ahangarzadeh[7,*], and Antoni Szumny[6,*]

[1]Research Center for Molecular Medicine, Institute of Cancer, Avicenna Health Research Institute (AHRI), Hamadan University of Medical Sciences, Hamadan, I.R. Iran. [2]Cancer Research Center, Institute of Cancer, Hamadan University of Medical Sciences, Hamadan, I.R. Iran. [3]Pharmaceutical Sciences Research Center, Isfahan University of Medical Sciences, Isfahan 81746-73461, I.R. Iran. [4]Student Research Committee, Hamadan University of Medical Sciences, Hamadan 65178-38678, I.R. Iran. [5]Department of Horticulture, College of Agriculture, Isfahan University of Technology, Isfahan 84156-83111, I.R. Iran. [6]Department of Food Chemistry and Biocatalysis, Wroclaw University of Life Sciences, 50-375 Wroclaw, Poland. [7]Regenerative Medicine Research Center, Isfahan University of Medical Sciences, Isfahan 81746-73461, I.R. Iran.

Abstract

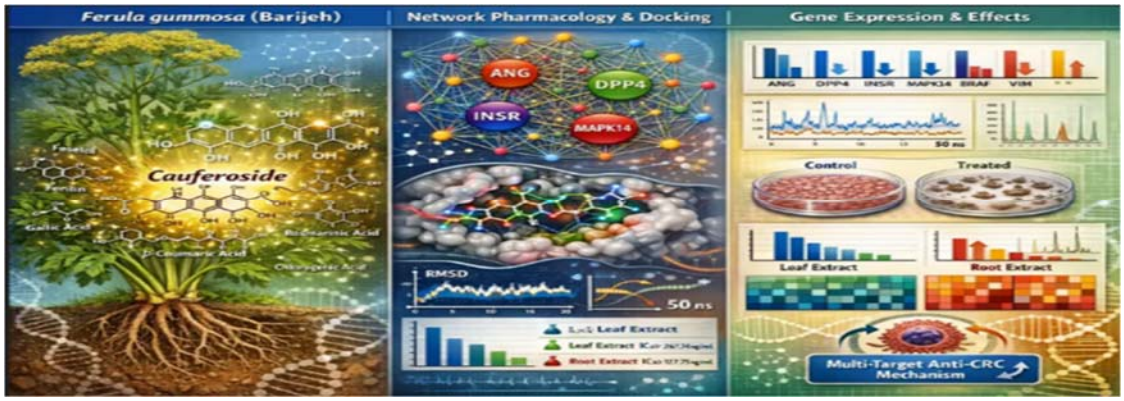
Background and purpose: Colorectal cancer (CRC) is a significant global health challenge, necessitating a comprehensive molecular understanding for personalized treatments. Molecular profiling has elucidated key biomarkers that are essential for prognosis, treatment responsiveness, and targeted therapeutic interventions.

Experimental approach: This study explored the role of indigenous phytochemicals, using bioinformatics and experimental assays to identify potential CRC-specific therapeutic targets.

Findings/Results: A system biology and drug-target network analysis identified four proteins (ANG, DPP4, INSR, and MAPK14) as potential targets for further investigation. Molecular docking studies showed that the cauferoside from *Ferula gummosa* has a strong binding affinity for these proteins. Molecular dynamics simulations confirmed the stability of the compound-protein complexes. *In vitro* assays demonstrated the cytotoxic effects of *F. gummosa* extracts on CRC cells. The leaf extract significantly downregulated the expression of the *ANG*, *DPP4*, *INSR*, and *MAPK14* genes, while the root extract exhibited differential effects on gene expression.

Conclusion and implications: The findings suggest the potential therapeutic efficacy of *F. gummosa* against CRC and emphasize the importance of a dual methodology involving bioinformatics and experimental validation in drug discovery. Further i*n vivo* and clinical studies are warranted to validate these findings and facilitate potential therapeutic applications.

Keywords: Colorectal cancer; *Ferula gummosa*; Molecular docking; Molecular dynamics; Phytochemicals; System biology.



*Corresponding authors:
S. Ahangarzadeh, Tel: +98-3136687087, Fax: +98-3137929076
Email: shahrzadahangar@res.mui.ac.ir
A. Szumny, Tel: +48-69647222
Email: antoni.szumny@upwr.edu.pl

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INTRODUCTION

Colorectal cancer (CRC) is a formidable global health challenge, accounting for a significant portion of cancer-related morbidity and mortality. CRC is a complex and heterogeneous disease, and the integration of molecular biomarkers has become instrumental in the design of treatment strategies. Factors that influence the development of CRC include genetic alterations and mutations in genes, environmental and dietary factors, lifestyle and behavioral factors, inflammatory bowel disease, gut microbiota, and age and sex (1). Molecular profiling has enhanced our understanding of CRC, allowing the identification of specific biomarkers that play crucial roles in prognosis, treatment response, and the development of targeted therapies (2). As our understanding of the molecular intricacies governing this malignancy evolves, researchers are increasingly exploring novel avenues for therapeutic intervention. Studies have shown that factors such as mutations in the rat sarcoma (*RAS*) and B-Raf proto-oncogene, serine/threonine kinase (*BRAF*) genes (3), high epidermal growth factor receptor (EGFR) expression levels (4), mutations associated with the activation of the PI3K-AKT-mTOR pathway (5), and some additional molecular alterations are critical contributors and determinants of the progression of CRC.

The incorporation of molecular biomarkers in the management of CRC signifies a fundamental shift towards precision medicine. Understanding the genetic and molecular characteristics of specific tumors can help healthcare professionals make informed choices, thereby enhancing therapeutic outcomes and reducing adverse effects. The advances in this domain offer hope for further enhancing CRC treatment by disclosing novel targets and fostering versatile personalized treatment strategies in CRC treatment (6). Interfering with specific molecules that play a crucial role in cancer cell growth and survival could inhibit the growth and spread of cancer cells. Natural plant compounds or phytochemicals have been the subject of extensive research for their potential role in cancer prevention and treatment. Many plants produce bioactive compounds with diverse chemical structures that exhibit anticancer properties. They can interact with specific molecules involved in cancer development and progression (7). They may modulate epigenetic processes, inhibit cell proliferation, induce apoptosis, and regulate signaling pathways associated with cancer development. Some plant compounds, such as luteolin, resveratrol, and quercetin, target specific kinases and cell cycle regulators that play crucial roles in cancer cell growth and survival. Myricetin and indole alkaloids can also target molecules involved in apoptosis. Other studies showed that specific molecular targets for plant compounds can vary depending on the compound and the type of cancer studied (8-10).

The integration of network pharmacology with traditional medicine facilitates a better understanding of complex interactions between bioactive compounds, molecular targets, and disease pathways. The combination of traditional medicine and network pharmacology is congruent with the holistic paradigm of health, as it considers the myriad targets and pathways influenced by traditional therapeutic agents. This synthesis has the potential to enhance drug discovery processes, refine therapeutic outcomes, and advance the principles of personalized medicine. Several studies published on this subject cover a broad array of applications pertaining to network pharmacology within the realm of traditional medicine. These studies provide valuable insights into the methodologies and applications associated with network pharmacology, while simultaneously showcasing significant advancements in this innovative and promising area of research (11).

Ferula gummosa (*F. gummosa*), belonging to the Apiaceae family, is an endemic species indigenous to the Middle Eastern region, predominantly found in Iran, where it is referred to as Barijeh. This plant is characterized by its aromatic essential oils, and its numerous therapeutic attributes have been elucidated in the literature (12). Notably, research has focused on *F. gummosa's* implications for CRC. For instance, a specific investigation revealed that the gum derived

from this plant induced a reduction in cellular proliferation within the CRC cell line SW-480, suggesting that *F. gummosa* possessed significant cytotoxic effects on neoplastic cells (13). Furthermore, Nosrat *et al*. demonstrated that the nanoemulsions of *F. gummosa* essential oil exhibited anti-cancer properties against CRC through multiple mechanisms (14). Additional research also corroborated the potential therapeutic benefits of compounds isolated from *F. gummosa* concerning other cancers (15,16).

Therefore, to identify appropriate targets and bioactive compounds with significant capacity to impede cancer proliferation and progression, it is essential to employ suitable methodologies to determine the most effective experimental approach. Bioinformatics plays a pivotal role in cancer therapy by utilizing computational methodologies and analyzing large biological datasets to improve our understanding of cancer and formulate more effective treatment strategies. Cancer bioinformatics is a rapidly evolving field that facilitates the prediction of therapeutic responses to various treatment alternatives. By analyzing genomic data, bioinformatics tools can identify genetic alterations and molecular signatures that can influence treatment outcomes. Furthermore, these tools can aid in therapy selection by analyzing genomic data and identifying potential therapeutic vulnerabilities in cancer cells. They can prioritize therapeutic targets and guide the selection of appropriate treatment strategies (17). Bioinformatics techniques also enable virtual screening and docking studies, where large databases of chemical compounds are screened against target proteins to identify potential drug candidates. These methods help prioritize compounds for further experimental testing, saving time and resources. Existing approaches can be used to identify new therapeutic uses for existing drugs. Through the examination of gene expression profiles, protein interaction networks or pharmacology-based methods as drug-target (DT) networks, these methodologies have the potential to propose alternative applications for drugs or natural compounds that have already been approved or are currently being used in clinical settings (18,19).

Bioinformatics analyses can encounter challenges in accurately predicting interactions between phytochemicals and molecular targets, necessitating robust validation through experimental assays. Therefore, this study aimed to identify the most effective indigenous plant compounds and CRC-specific therapeutic targets through a rigorous integration of bioinformatics methodologies and experimental validation. By leveraging bioinformatics tools, we sought to identify promising candidates within the vast array of plant compounds, subsequently subjecting them to experimental evaluation. We enhanced the explanation of the bioinformatic methodologies by emphasizing their role in addressing challenges in CRC treatment, particularly in predicting interactions between plant compounds and molecular targets. Despite the significant advancements achieved through bioinformatic approaches in unraveling the molecular landscape of CRC, translating these computational insights into clinical applications remains complex. While integrative analyses of genomic, transcriptomic, and epigenomic data have facilitated the identification of potential biomarkers and therapeutic targets, their validation and clinical utility often encounter obstacles related to tumor heterogeneity, data reproducibility, and the lack of standardized analytical pipelines. These factors highlighted the need for a rigorous assessment of the limitations inherent in current bioinformatics-driven strategies for cancer treatment such as CRC (20,21).

MATERIALS AND METHODS

Data retrieval of compounds

Identification of medicinal plants native to Iran was performed by searching online databases and reviewing done by online database searching the Internet and reviewing local books (22), and their names were recorded in an Excel file. By downloading all the chemical compounds available in the Chemical Entities of Biological Interest (ChEBI) database (23), and matching their sources with the file of native plant names, the compounds that are present in indigenous medicinal plants were

identified. In addition to their names, the three-dimensional structures of the compounds were recovered as a structural data file (SDF) from the PubChem database (24).

Identification of potential target candidates for compounds

The web-based server PharmMapper was used to identify potential target candidates of extracted compounds from native plants. PharmMapper is a repository of pharmacophore models derived from targets across four drug-related databases: TargetBank, DrugBank, BindingDB, and the Potential Drug Target Database (PDTD). Human protein targets were selected for the target set option, and the maximum number of reserved matching targets was set to 300. The output of each compound was saved as an Excel file, and then, after selecting the top 20 targets for each compound, all possible targets were collected in one file. After merging and removing duplicates, a final file of targets was prepared for the next steps.

Retrieval of CRC targets

Using the National Center for Biotechnology Information (NCBI) database, all differentially expressed genes in CRC were retrieved. The genes were selected based on literature review and previous studies, gene expression databases, and involvement in CRC pathogenesis. Specifically, the genes showing a significant increase in expression were selected. Next, the output of this step was matched with the output obtained from the previous step to select common and identical names. In this way, possible targets for the compounds in CRC were obtained (25).

Construction of drug target network

The DT network produces useful information for the analysis of relationships between drugs, targets, and diseases, as well as for the discovery of new drug targets. In this study, a DT network was constructed using Cytoscape v3.6.0 software. An input file with possible CRC targets for each compound was prepared and imported into the software. After building the network, the NetworkAnalyzer plugin (26) was used to analyze the quantitative characteristics of the undirected network. The degree and betweenness centrality parameters were used to select the nodes (9). Degree centrality is the number of direct connections a node has and betweenness centrality measures how often a node lies on the shortest path between other nodes in the network. Nodes are entities in a biological process or system. Then, the main targets in terms of gene expression were checked by a literature review to select genes with a significant increase in expression compared to normal tissue. The method and results of the work have been designed and obtained in line with the international guidelines for network pharmacology (27). Multiple analytical criteria were evaluated to enhance the reliability of the network pharmacology results.

Retrieval of the three-dimensional structure of proteins

In this study, four proteins were finally selected as potential targets for CRC to retrieve their three-dimensional structure for further study. Using the database Protein Data Bank (PDB, available on http://www.rcsb.org/PDB) (28), the structures of the proteins, including angiogenin (ANG, PDB ID: 1B1I), dipeptidyl peptidase IV (DPP4, PDB ID: 3SWW), insulin receptor (INSR, PDB ID: 5KQV), and mitogen-activated protein kinase (MAPK) 14 (PDB ID: 5XYY), were recovered. The information and selection of the correct structure for each protein were guided with the help of the UniProt database (29). The structures were saved as PDB files.

Molecular docking

The SDF structures of selected compounds (47 compounds) were converted to mol2 files using Open Babel software (30) and then converted to PDBQT using the Raccoon program within AutoDockTools 1.5.7 (31). For the four protein structures in PDB format, the first water molecules and ligands available in the structure were removed using Chimera 1.16 software (32) and then converted to PDBQT using Autodock Tools software. All structures were subsequently imported into PyRx software (33). Using the Autodock Vina program (34) hosted in this software, molecular docking was performed for each of the target

proteins. Depending on the type of target protein, the binding site position of each protein was considered the target box. Nine modes were selected for each compound, and the results were sorted according to the interaction energy. The molecular docking was also performed with paclitaxel as a general anticancer drug and fluorouracil as a CRC-specific anticancer drug to compare the docking results with the studied compounds. The docked protein-compound complexes were then analyzed by PyMOL (https://pymol.org/2/) and Discovery Studio software (https://discover.3ds.com/discovery-studio-visualizer-download) in terms of 2D and 3D structures, amino acids, and molecules involved in binding (35).

Molecular dynamics simulations

Molecular dynamics (MD) simulations were employed to predict the time-dependent physical movements of the atoms and molecules, providing insights into protein-ligand interactions (35). The dynamics of four docked complexes consisting of the ANG, DPP4, INSR, and MAPK14 proteins and cauferoside compound were simulated using GROMACS 5.4.1 software (36). In this method, the 54A7 force field (37) was used on molecules surrounded by simple point charge (SPC) water. The system was neutralized by Na^+ ions, and then energy was minimized for the relaxation of internal constraints. Equilibration in the NVT and NPT ensembles was done under positional restraints for 100 ps. Finally, the MD production run was performed for 50 ns with a time step of 3 fs. The trajectory was then analyzed by root mean square deviation (RMSD) and root mean square fluctuation (RMSF) to characterize the dynamics of the protein-ligand complexes (38).

Plant extract preparation

Plant materials

The leaves and roots *of F. gummosa* were collected in Naghan, Chahar Mahal-e Bakhtiari (31º980 N and 50º680 N) at an altitude of 2026 m and deposited under herbarium number 13342. The samples were collected and identified by Prof. Mehdi Rahimmalek using Flora Iranica, and the samples were deposited in the herbarium of Isfahan University of Technology, Isfahan, Iran. The collection of the samples was permitted by the research institute of forests and rangelands and complied with local and national guidelines and legislation. The collected plants were dried at 25 °C for three days under-shade conditions.

High-performance liquid chromatography analysis

As the study was conducted using whole plant extracts, high-performance liquid chromatography (HPLC) analysis was performed to characterize the major plant compounds. *F. gummosa* aerial parts and root were used for polyphenolic compound determination according to several standards, such as gallic acid, caffeic acid, ferulic acid, *p*-coumaric acid, rutin, rosmarinic acid, chlorogenic acid, and other available standards (98% purity, Phytolab, Germany). In this method, 15 g of dried plant materials were mixed with 300 mL of methanol and shaken at 90 rpm for 24 h. The filtered extract was concentrated and dried using a rotary evaporator under vacuum at 40 °C. The extract was dissolved in 1 mL of HPLC solvent A, filtered with a 0.22 µm membrane filter, and 20 µL of the filtrate was injected into an Agilent 1090 system with a detection range of 260 and 350 nm. In this experiment, a 250 × 4.6 mm, 5 µm, symmetry C18 column (Waters Corp., Milford, MA, USA) was applied. The mobile phase consisted of formic acid in water (99.9:0.1, v/v) as solution (A) and acetonitrile/formic acid (99.9:0.1) as solution (B) with gradient elution at 25 ℃ and a flow rate of 0.8 mL/min. The gradient program was started from A:B (90:10) for 1 min, followed by 10-26% B for 40 min, 26-65% B for 30 min, and finally 65-100% B for 5 min, followed by equilibration with 0-90% A for 4 min. Polyphenolic compounds were determined by comparing UV spectra and retention times with pure standards, and the amount was reported in mg/100 g of dry sample weight (39).

Cell culture

The SW948 cell line (Pasteur Institute, Tehran, Iran) was maintained in Roswell Park Memorial Institute (RPMI) 1640 medium,

supplemented with 10% v/v fetal bovine serum (FBS, Gibco, Parsley, UK). The medium was also enriched with streptomycin (100 µg/mL) and penicillin (100 U/mL) (Gibco, Paisley, UK). The cells were maintained in a humidified atmosphere containing 5% carbon dioxide at a temperature of 37 °C, and then cultured in flasks with a surface area of 25 cm^2. To keep the cells in their exponential growth phase, they were passaged twice a week (40).

Cytotoxicity assay

Cell viability was evaluated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT, Sigma-Aldrich Corp., UK) assay, which is based on the ability of the mitochondrial succinate dehydrogenase to convert the tetrazolium salt into insoluble violet crystals. This reaction is indicative of the number of viable cells. SW948 cells were initially placed in a 96-well plate at a density of 1×10^4 cells/well and left to incubate overnight. A serial concentration from 0 (as control) to 500 µg/mL of both leaf and root extracts was prepared and added to the wells in triplicate (41). The cytotoxicity test was then performed at 24-h intervals. Subsequently, 60 µL of MTT (0.5 mg/mL) was added to each well, which was then incubated at 37 °C for 4 h. To dissolve the formazan crystals, 100 µL of dimethylsulfoxide (DMSO, Merck, Germany) was added to each well. After 30 min, absorbance was measured at 570 nm using an enzyme-linked immunosorbent assay (ELISA) reader (Stat-Fax-2100, USA) (42,43). The growth inhibitory effects of *F. gummosa* (leaf and root extracts) were evaluated by determining the IC_{50} values.

Cell treatment

Three flasks were designated for root extract, leaf extract, and control. After cells reached the desired confluence, 250 µg/mL of leaf extract and 125 µg/mL of root extract were added separately to two flasks. After 24 h, the cells were harvested to be used for the next step.

RNA isolation, cDNA synthesis, and quantitative reverse transcription polymerase chain reaction

Total RNA was isolated from SW948 cells using RNX-Plus Reagent (Sinaclon, Tehran, Iran) according to the manufacturer's protocol and quantified using a NanoDrop™ 2000/2000c Spectrophotometer (Thermo Fisher Scientific, MA, USA). The 260/280 and 260/230 ratios were both greater than 1.9, confirming RNA purity. Furthermore, 1 µg total RNA of each sample was synthesized using a cDNA Synthesis Kit (Yekta-Tajhiz-Azma [YTA], Tehran, Iran) and transferred into the real-time quantitative reverse transcription polymerase chain reaction (RT-qPCR). The transcription levels of *ANG*, *DPP4*, insulin receptor (*INSR*), *MAPK14*, *BRAF*, and vimentin (*VIM*) were also evaluated using SYBR® Green PCR Master Mix (Yekta-Tajhiz-Azma [YTA], Tehran, Iran). Specific cycling parameters in the qRT-PCR included an initial denaturation step at 95 °C for 2 min, denaturation at 95 °C for 10 s, and annealing at 61 °C for 20 s, followed by an extension step at 72 °C for 25 s. The number of cycles was optimized at 40. Before RT-qPCR, primer sets were validated for specificity and efficiency using melt curve analysis and standard curves. A universal annealing temperature of 61 °C was selected based on preliminary tests confirming optimal amplification for all targets. Amplification efficiency (90-110%) and single-peak melt curves validated the suitability of the cycling parameters for comparative gene expression analysis. The primer sequences used in this study are represented in Table 1. In addition, the transcription level of glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was used as an endogenous control. The $2^{-\Delta\Delta Ct}$ method was also utilized to determine the relative expression of each sample at the time points analyzed. RT-qPCR was performed using a Roche LightCycler® 480 instrument (Roche, Germany) (44). The baseline and threshold values for qPCR analysis were determined using the Roche LightCycler® 480 software. The baseline was automatically set during the exponential phase of amplification, while the threshold was manually adjusted to intersect all amplification curves in their linear phase. Amplification efficiency (90-110%) was validated for all primer pairs to ensure accurate quantification using the $2^{-\Delta\Delta Ct}$ method.

Table 1. Forward and reverse primer sequences. The temperature for all primers was 60°C.

Gene	Primer sequences	
ANG	Forward	TAGCAGCTCTGGTTCCGTTT
	Reverse	CTCCTGGGTGTGTTTCCTGT
DPP4	Forward	CTGCTTGCTCCAATTTAGCC
	Reverse	ACACTTGCTAGAGCCCAGGA
INSR	Forward	GAAGCTCTGTGCCAAGAACC
	Reverse	CCGTTGCTACAAGGGTCATT
MAPK14	Forward	CCAGAGGCAGTTTTCTCCTG
	Reverse	TGCTCACCCACATGTTTTGT
BRAF	Forward	CTTCATGAAGACCTCCAGT
	Reverse	CATCCACAAAATGGATCCAG
VIM	Forward	GAAGAGAACTTTGCCGTTGAAG
	Reverse	TGAGCAGGTCTTGGTATTCAC
GAPDH	Forward	GAGTCCACTGGCGTCTTCAC
	Reverse	ATGACGAACATGGGGGCA

ANG, Angiogenin; *DPP4*, dipeptidyl peptidase IV; *INSR*, insulin receptor; *MAPK14*, mitogen-activated protein kinase 14; *VIM*, vimentin; *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase.

Table 2. Major phenolic and flavonoid compounds of *Ferula gummosa*. Data were presented as mean ± SD.

Compound	RT	Root (mg/100 g DW)	Leaf (mg/100 g DW)
Gallic acid	5.09	11.18 ± 0.3	25.05 ± 0.5
Chlorogenic acid	13.57	3.23 ± 0.2	3.38 ± 0.3
Caffeic acid	14.49	0.02 ± 0.01	1.29 ± 0.1
***p*-Coumaric acid**	26.85	4.2 ± 0.1	4.9 ± 0.2
Rutin	28.41	2.94 ± 0.08	1.26 ± 0.1
Ferulic acid	29.20	0.06 ± 0.04	0.01 ± 0.02
Rosmarinic acid	39.10	0.07 ± 0.02	5.64 ± 0.23

SD, Standard deviation; RT, retention time; DW, dry weight.

Statistical analysis

Data were presented as mean ± standard deviation (SD). The RT-qPCR data were statistically analyzed using the GraphPad Prism software (version 8, GraphPad Software, CA, USA). A one-way analysis of variance (ANOVA) followed by Tukey's post hoc test was used to compare the differences between the experimental groups and the control. *P*-values < 0.05 were considered statistically significant (45).

Supplementary materials

The supplementary materials for this article can be found online at: https://github.com/shahangarzadeh63-web/Supp.table.RPS-.git

RESULTS

Investigation of compound and protein libraries

Our search identified 315 approved native medicinal plants, from which we extracted 53 known compounds based on information available in the ChEBI database. By cross-referencing the final targets obtained from PharmMapper with those associated with CRC, we identified 14 target proteins for further investigation. Detailed lists of the medicinal plants, compounds, and proteins were provided in the Table S1.

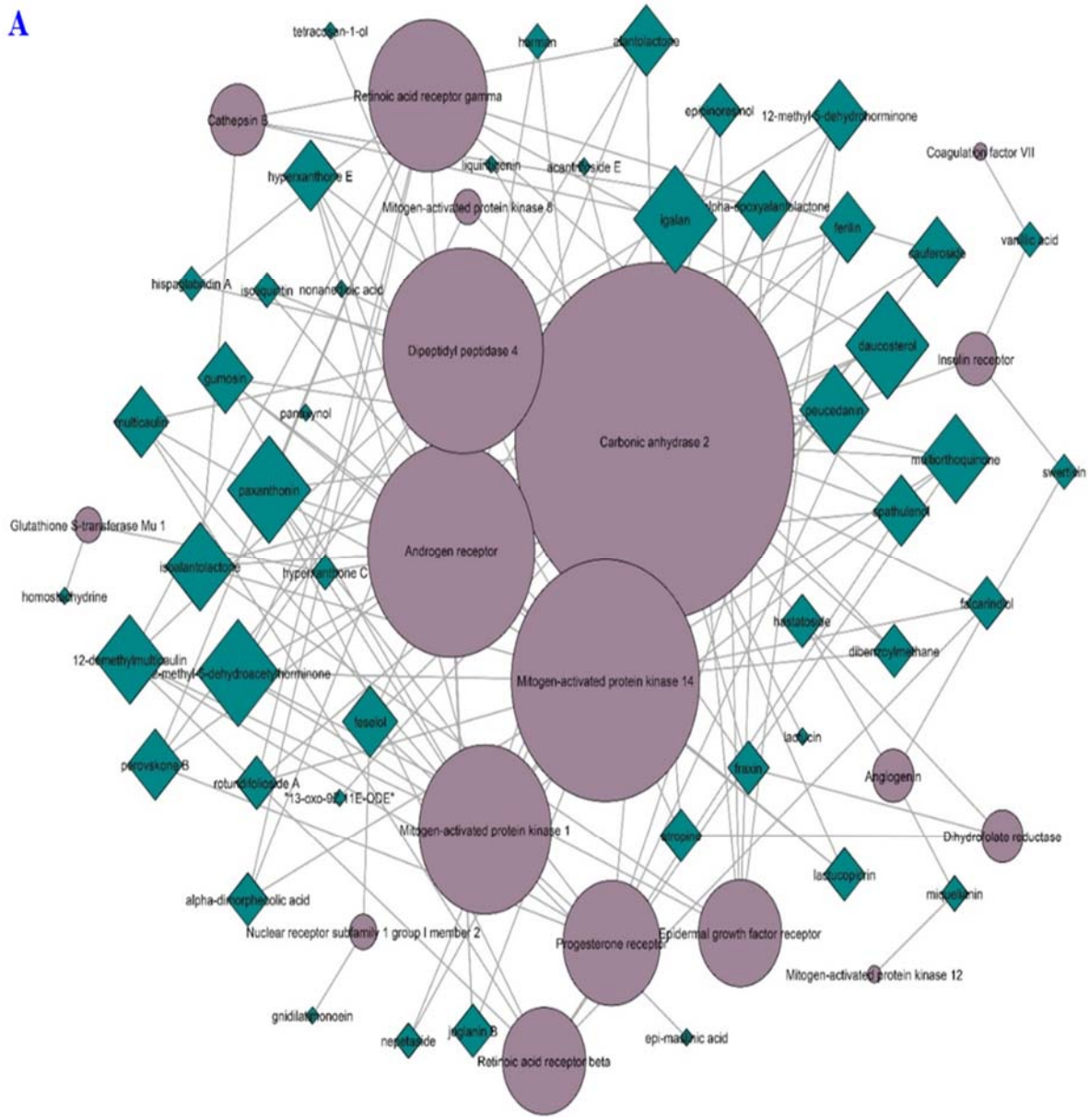
HPLC analysis

The results of the HPLC analyses revealed the presence of some major phenolic compounds (Table 2). The roots and leaves showed a different pattern of phenolic accumulation. The phenolic compound concentration was much higher in the leaves than in other parts of *F. gummosa*. The most abundant phenolic acids in leaves were gallic

acid and rosmarinic acid, while in the roots, gallic acid, *p*-coumaric acid, and chlorogenic acid were the most frequent ones (Table 2).

DT network analysis

A DT network with 47 compounds and 14 protein targets was constructed, comprising 65 nodes and 144 edges. Using NetworkAnalyser, the number of node values and betweenness centrality were determined. Results were ranked by both degree and betweenness centrality. Figure 1 shows the bipartite network for DT based on degrees, where the size of the nodes was depicted according to the number of degrees. Also, a circle layout of the DT network was made based on betweenness centrality. Compounds showed degrees in the range of 6 to 1 and proteins 29 to 1. Based on the analysis of this network and the results of the literature review, four protein targets were selected for the molecular docking step. The output of the network based on degree and betweenness centrality is provided in the Table S2.



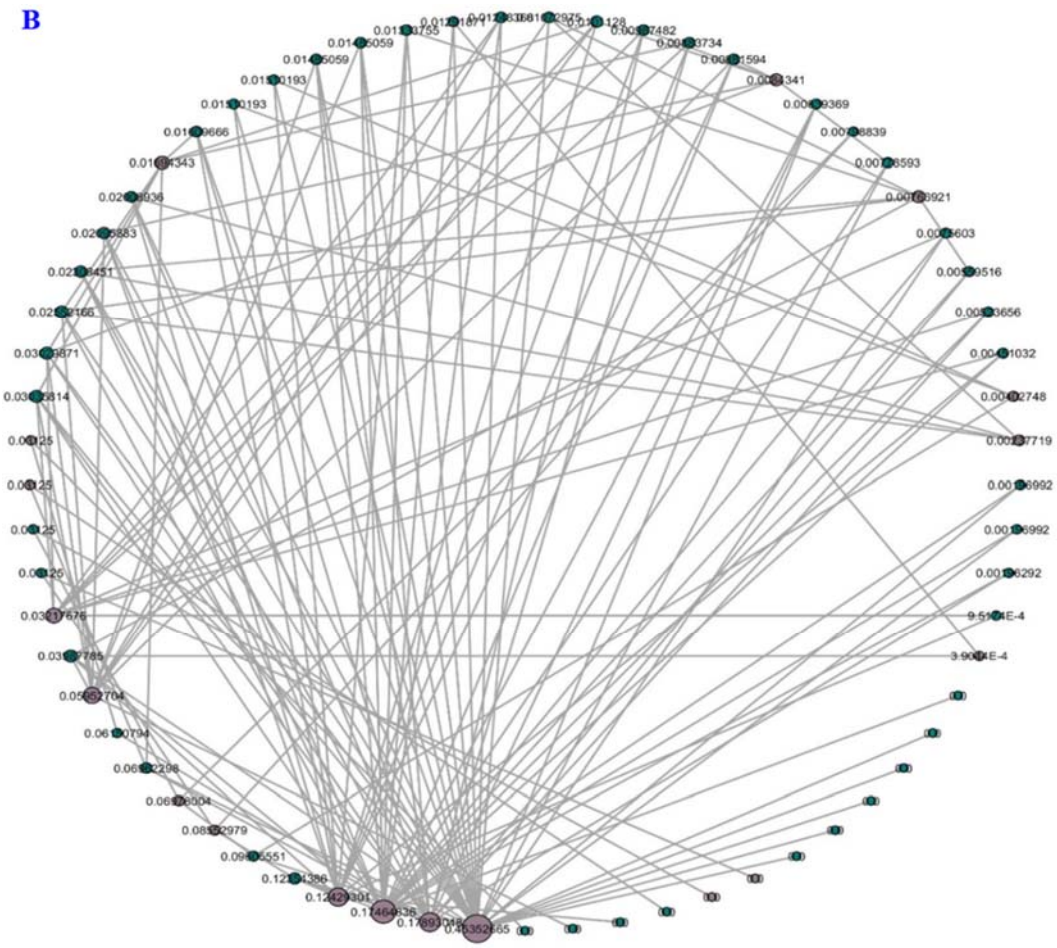


Fig. 1. The DT network. (A) The bipartite DT network and (B) the circle layout of the DT network. The purple circular nodes represent the target proteins, and the green triangular nodes represent the plant compounds. The size of the nodes is based on the number of their degrees. DT, Drug-target.

Molecular interaction analysis

To estimate the binding affinity of the compounds to the protein targets, molecular docking was performed and then results binding affinities were recorded as interaction energies. Table 3 shows the results of the ten compound molecules with the highest binding affinity to each protein. The protein-ligand docked structures were studied in terms of 2D and 3D models by Discovery Studio software, so that the involved amino acids and the manner of their involvement were shown. Based on the amount of interaction energy and the type of interactions, finally, 10 compound molecules were selected for further study along with their botanical sources (Table 4). The top three molecules (cauferoside, feselol, and ferilin) in this list have been observed in the *F. gummosa* plant. Therefore, this plant was selected for investigation in CRC cells *in vitro*. Additionally, four protein targets, including ANG, DPP4, INSR, and MAPK14, were selected for MD simulation and experimental validation. Given the structural similarities among the top three compounds, cauferoside was selected as the representative compound for MD simulation. Figure 2 shows the 2D and 3D structures of the interaction of cauferoside with the 4 mentioned proteins.

Paclitaxel and fluorouracil, two established anticancer drugs, were used as reference compounds and docked against all four targets for comparison. The binding affinity results of two standard compounds against 4 targets were represented in Table 5. The binding affinities of the reference drugs were comparable to, or lower than, those of the best-performing studied compounds.

Table 3. The docking binding energy (kcal/mol) and calculated affinity of the highest scores for 4 target proteins and plant compounds.

ANG	Binding affinity	DPP4	Binding affinity	INSR	Binding affinity	MAPK14	Binding affinity
Cauferoside	-7.4	*epi*-maslinicacid	-8.8	Rotundifolioside A	-7.2	Ferilin	-9.5
Paxanthonin	-6.9	Daucosterol	-8.8	Gnidilatimonoein	-7.2	Gumosin	-9.2
Miquelianin	-6.9	Rotundifolioside A	-8.5	*epi*-Naslinic acid	-7	Cauferoside	-9.2
Lactucopicrin	-6.9	Feselol	-8.5	Ferilin	-6.8	Feselol	-9.1
Rotundifolioside A	-6.8	Cauferoside	-8.4	Perovskone B	-6.7	*epi*-Maslinic acid	-9.1
Feselol	-6.8	Perovskone B	-8.3	Feselol	-6.7	Lactucopicrin	-8.8
Ferilin	-6.7	Ferilin	-8.3	Cauferoside	-6.7	Multiorthoquinone	-8.6
Hispaglabridina	-6.6	Multiorthoquinone	-8.2	12-demethylmulticaulin	-6.6	Multicaulin	-8.5
Gnidilatimonoein	-6.5	Isoliquiritin	-8.2	Multiorthoquinone	-6.5	Hyperxanthone E	-8.4
epi-Pinoresinol	-6.5	Hispaglabridin A	-8	Daucosterol	-6.5	Liquiritigenin	-8.3

ANG, Angiogenin; DPP4, dipeptidyl peptidase IV; INSR, insulin receptor; MAPK14, mitogen-activated protein kinase 14.

Table 4. Top plant compounds according to the amount of interaction energy and their sources.

Compound	Plant	Part
Cauferoside	*Ferula qumosa*	Root
Feselol	*Ferula gumosa*	Root
Ferilin	*Ferula gumosa*	Root
Miquelianin	*Hypericum perforatum*	-
RotundifoliosideA	*Bupleurum rotundifolium*	Fruit
Gnidilatimonoein	*Daphne mucronata*	Leaf
Epi-maslinic acid	*Prunella vulgaris*	Leaf and stem
Multiorthoquinone	*Salvia multicaulis*	Root
Multicaulin	*Salvia multicaulis*	Root
Daucosterol	*Ferula gumosa*	Root

Table 5. The docking binding energy (kcal/mol) and calculated affinity of two standard compounds against 4 targets.

Compound	ANG	DPP4	INSR	MAPK14
Paclitaxel	-7.4	-9.7	-7.8	-8.2
Fluoracil	-4.7	-5	-5.2	-4.8

ANG, Angiogenin; DPP4, dipeptidyl peptidase IV; INSR, insulin receptor; MAPK14, mitogen-activated protein kinase 14.

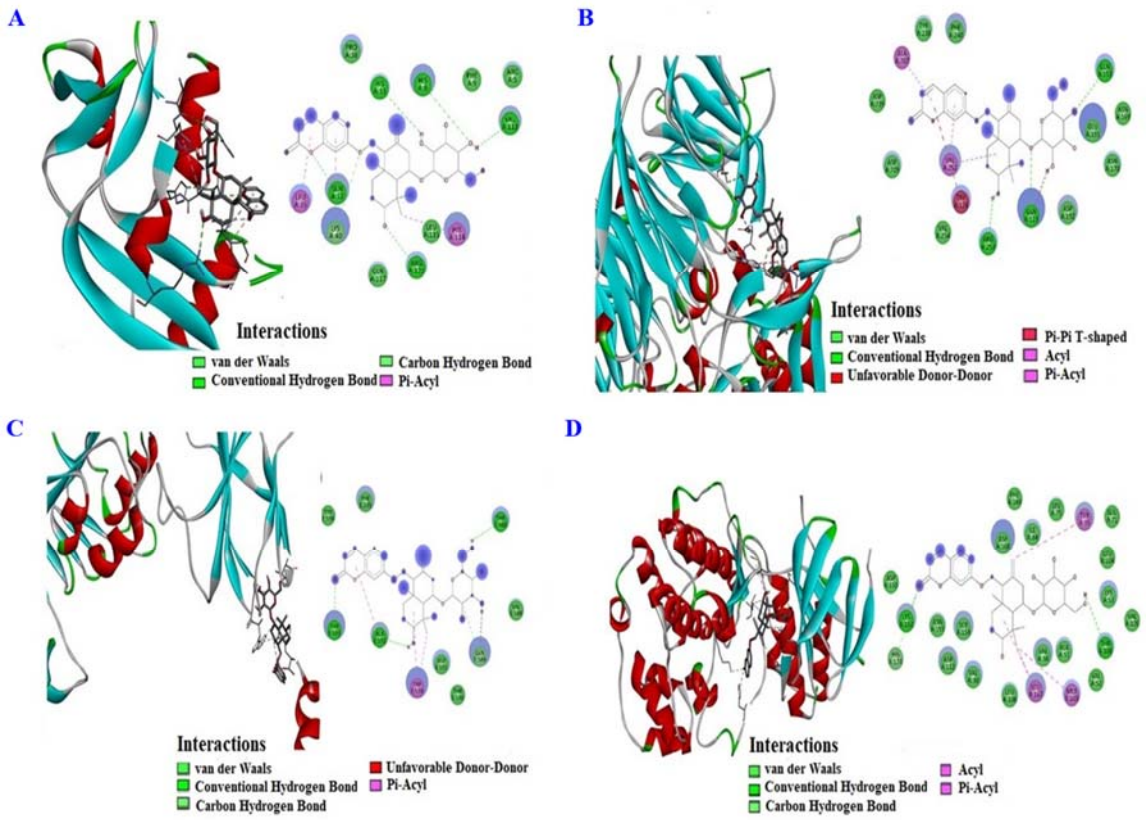


Fig. 2. 2D and 3D structures of complexes formed between 4 important protein targets and an active plant compound, cauferoside. (A) ANG-cauferoside; (B) DPP4-cauferoside; (C) INSR-cauferoside; (D) MAPK14-cauferoside.

MD simulation analysis

After 50 ns of simulation for each of the four docked compound-protein complexes, their structures were checked by RMSD and RMSF (Fig. 3). The average RMSD values were 0.22 ± 0.03, 0.18 ± 0.009, 0.4 ± 0.063, and 0.23 ± 0.024 for ANG-cauferoside, DPP4-cauferoside, INSR-cauferoside, and MAPK14-cauferoside, respectively (Fig. 3A). All of the complexes revealed little fluctuations and change. The INSR-cauferoside complex showed slightly greater structural deviation compared to the other complexes (Fig. 3B).

Evaluation of cytotoxicity of F. gummosa extract via the MTT assay

IC_{50} was evaluated for the SW948 cell line exposed to the plant extract. The IC_{50} for leaf and root extracts was determined to be 267.74 µg/mL and 127.75 µg/mL, respectively (Fig. 4). MTT assay results demonstrated a concentration-dependent decrease in SW948 cell viability for both extracts compared to the control (Fig. 4). When evaluating the cytotoxic effects of compounds used in cancer treatment, it is crucial that the drug selectively inhibits cancer cell lines while minimally affecting normal cell lines.

Therefore, the lower the effective concentration of the compound, the greater its potential for clinical application.

Gene expression analysis via RT-qPCR

The relative expression of the *ANG*, *DPP4*, *INSR*, *MAPK14*, *BRAF*, and *VIM* genes was assessed using RT-qPCR in cells treated with leaf/root extract (Fig. 5). Treatment with leaf extract led to a significant reduction in the expression of all genes compared to the control group. In cells treated with root extract, the expression of the *INSR* and *BRAF* genes significantly decreased in comparison to the control group, whereas no significant changes were observed in the remaining genes. The most pronounced down-regulation was observed in *INSR* mRNA levels after leaf extract (Fig. 5C). Notably, root extract treatment not only failed to suppress *ANG* expression but significantly upregulated it (Fig. 5A). Furthermore, in this study, two genes, *VIM* and *BRAF*, were investigated due to their roles in cancer cell behavior and their position downstream of the primary target genes, to determine whether the decrease in target gene expression exerts direct or indirect effects on oncogenic signaling. RT-qPCR results showed that the effect of plant extracts partially inhibited the expression of these (Fig. 5).

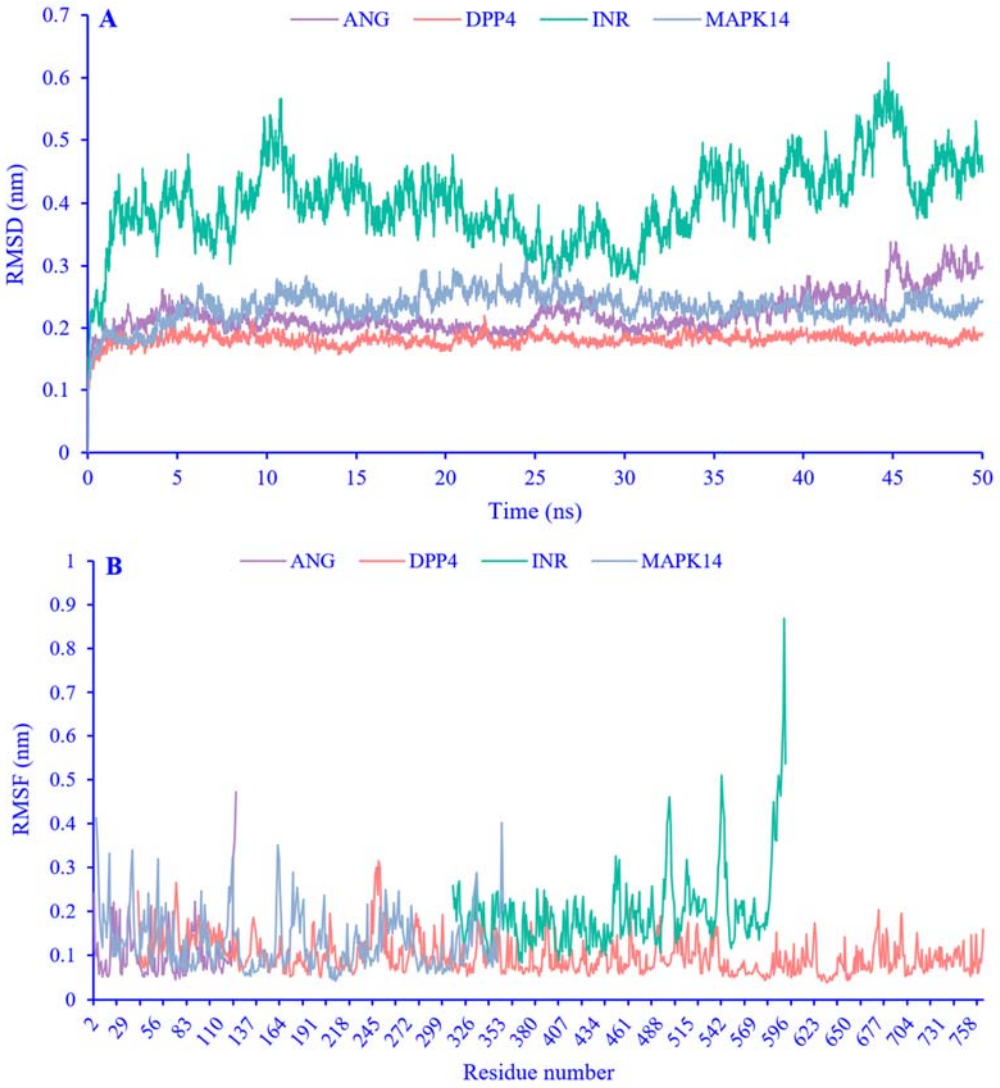


Fig. 3. (A) RMSD and (B) RMSF plots of proteins in complex with cauferoside after MD simulation. RMSD, Root mean square deviation; RMSF, root mean square fluctuation.

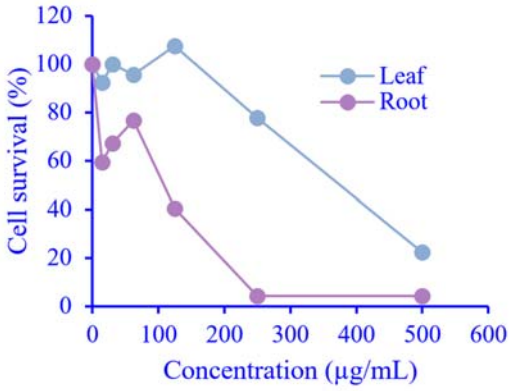


Fig. 4. MTT assay graph as cell survival percent of the living cells versus concentration of plant extract after 24 h.

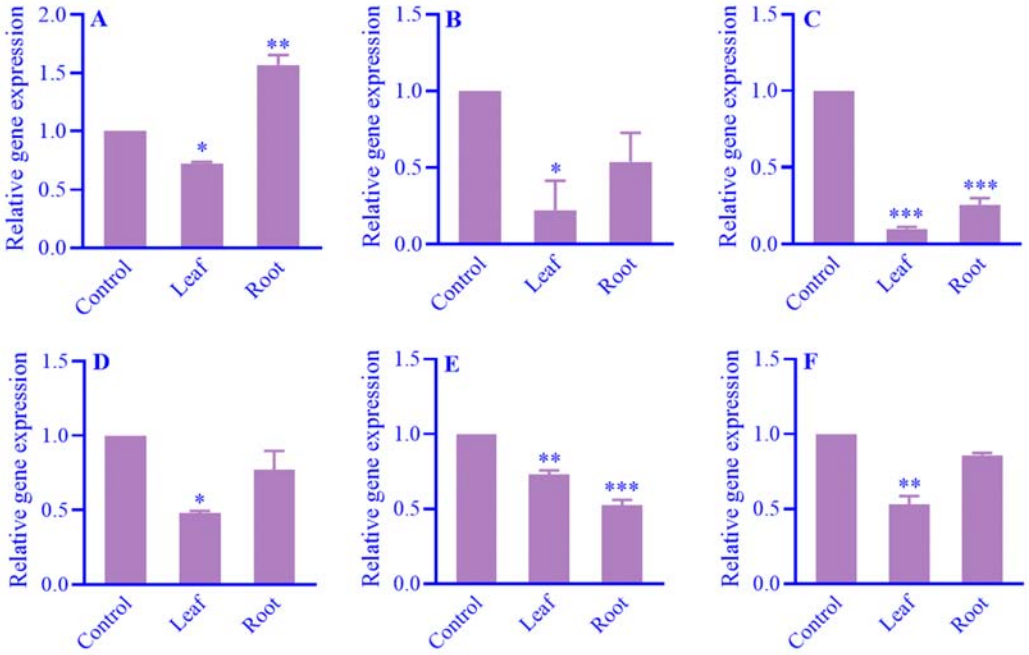


Fig. 5. qRT-PCR analysis for mRNA expression of genes after treatment. (A) *ANG*; (B) *DPP4*; (C) *INSR*; (D) *MAPK14*; (E) *BRAF*; (F) *VIM*. Data were presented as mean ± SD. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ demonstrate significant differences compared to the control group. *ANG*, Angiogenin; *DPP4*, dipeptidyl peptidase IV; *INSR*, insulin receptor; *MAPK14*, mitogen-activated protein kinase 14; *BRAF*, B-Raf proto-oncogene, serine/threonine kinase; *VIM*, vimentin.

DISCUSSION

Colorectal cancer (CRC) is a complex disease influenced by multiple genetic and environmental factors. Given the multicomponent and multitarget nature of traditional herbal medicines, network pharmacology provides a powerful approach to systematically elucidate the mechanisms underlying their therapeutic effects. This study integrated network pharmacology and experimental validation to explore the anti-CRC potential of *F. gummosa*, focusing on the signaling pathways and molecular targets involved. The precise mechanisms through which *F. gummosa* manifests its anti-cancer properties are not entirely elucidated, necessitating further research to delineate the molecular pathways involved. However, some mechanisms are suggested, including that the ethanolic extract of *F. gummosa* has been shown to induce apoptosis and cell cycle arrest in cancer cells, probably *via* the reactive oxygen species (ROS) mechanism (46,47).

Investigation on other plants has shown that plant extracts or phytochemicals usually exert their therapeutic effects, especially anticancer properties, through multiple mechanisms of action (48). Therefore, this study aimed to identify a potential native plant for the treatment of CRC using bioinformatics and *in vitro* methods. The study involved the investigation of compound and protein libraries, DT network analysis, molecular interaction analysis, cytotoxicity MD simulation analysis, assessment of plant extract, and gene expression analysis. We searched and identified 315 approved native medicinal plants. From these plants, 53 known compounds were found based on the information available in the ChEBI database. Furthermore, sharing proteins were obtained using PharmMapper and CRC data, including its differentially expressed genes and protein interaction networks.

Similar to other herbal medicines, *F. gummosa* operates through a multi-component, multi-target mechanism, enhancing its therapeutic efficacy against CRC. The system biology provides a promising framework for understanding the mechanisms of herbal compounds and the multi-targeted nature of herbal formulations (9). In this study, the construction of a DT network provided a promising path, incorporating 47 compounds and 14 protein targets. Through a network-based approach incorporating PharmMapper and CRC-associated proteins, we constructed a DT network comprising 47 compounds and 14 key protein targets. Network topology analysis, particularly node degree and betweenness centrality, prioritized four major targets, including ANG, DPP4, INSR, and MAPK14. Molecular docking studies were conducted to estimate binding affinities between the selected compounds and protein targets. The top 10 compounds exhibiting the highest binding affinity for each protein were identified. Notably, cauferoside, sourced from *F. gummosa*, displayed significant binding affinity to all four proteins. This observation prompted further investigation of the anti-CRC potential of *F. gummosa*. MD simulations were used to scrutinize the stability of compound-protein complexes over a 50 ns timeframe. RMSD and RMSF analyses revealed overall structural stability, with slight fluctuations in the INSR-cauferoside complex. Average RMSD values provided a quantitative measure of stability across the complexes. Subsequently, *in vitro* assays validated the findings. Cytotoxicity assessments using the MTT assay revealed that both leaf and root extracts of *F. gummosa* inhibited CRC cell proliferation in a concentration-dependent manner. The determined IC_{50} values suggested significant cytotoxic activity at relatively low concentrations, emphasizing the clinical potential of the extracts. Gene expression analysis by qRT-PCR further elucidated the molecular mechanisms underlying the observed cytotoxic effects. Treatment with *F. gummosa* leaf extract significantly down-regulated the expression of the *ANG*, *DPP4*, *INSR,* and *MAPK14* genes. In particular, *INSR* mRNA levels exhibited the most pronounced reduction. The root extract showed different effects, with increased expression of *ANG* and minimal impact on other genes.

ANG is a protein that plays a crucial role in angiogenesis, the process of forming new blood vessels. In the context of cancer, ANG has been extensively studied due to its involvement in tumor growth, invasion, and metastasis (49). ANG is overexpressed in various types of cancer, including breast, lung, and CRC. In addition, ANG has angiogenesis-independent effects in cancer, such as promoting cancer cell survival and resistance to therapy. The findings highlighted the importance of ANG as a potential therapeutic target and a biomarker for cancer diagnosis and treatment (50).

DPP4, also known as CD26, is an enzyme that plays a crucial role in various physiological processes, including immune regulation and glucose metabolism (51). In recent years, there has been a growing interest in understanding the involvement of DPP4 in cancer. Literature has revealed that DPP4 expression is dysregulated in several types of cancers, including breast, prostate, colorectal, and pancreatic cancer (52). DPP4 has been implicated in cancer progression and metastasis through its effects on tumor cell invasion, migration, and angiogenesis. Furthermore, DPP4 has been explored as a potential biomarker for the prognosis of cancer and as a target for therapeutic intervention (51).

The INSR is a cell surface receptor that plays a crucial role in mediating the effects of insulin. In recent years, there has been increasing evidence suggesting a link between INSR and cancer. It has been shown that the dysregulation of INSR signaling is associated with tumor growth, progression, and resistance to therapy in various types of cancer, including breast, colorectal, and lung cancer. Aberrant INSR activation can promote cancer cell survival, proliferation, and metastasis by stimulating downstream signaling pathways involved in cell growth and survival (53).

MAPK14, also known as p38 MAPK, is a member of the MAPK family that plays a crucial role in cellular signaling pathways involved in inflammation, stress responses, and cell proliferation. Over the years, there has been increasing evidence linking MAPK14 to cancer development and progression. The dysregulation of MAPK14 signaling has been observed in various cancer types, including breast, lung, colorectal, and pancreatic cancer. MAPK14 activation has been shown to promote tumor cell survival, proliferation, invasion, and metastasis. The different findings underscore the significance of MAPK14 in cancer and its potential as a therapeutic target (54).

The qRT-PCR results also showed partial inhibition of the expression of *VIM* and *BRAF* genes by plant extracts. *BRAF* is a proto-oncogene that is usually involved in cancer cell signaling, and *VIM* participates in cancer cell migration and cell adhesion structures. *VIM* is often associated with the epithelial-mesenchymal transition (EMT), a process in which cells lose their epithelial characteristics and gain mesenchymal properties. EMT is involved in cancer progression and metastasis (55,56).

CONCLUSION

In summary, this study utilized bioinformatics and *in vitro* methods to identify a potential plant for the treatment of CRC. The results provided valuable insights into the potential of *F. gummosa* extract and its compounds for further investigation in the treatment of CRC. In conclusion, the study findings collectively suggested that *F. gummosa*, specifically its cauferoside compound, holds promise as a potential therapeutic agent against CRC. The approach, which integrates system biology, molecular docking, MD simulations, and *in vitro* analyses, strengthens the credibility of the results. While bioinformatics offers powerful tools, it faces limitations in accurately modeling these interactions, making experimental validation essential. This integrated approach is especially crucial in CRC, where tumor molecular heterogeneity complicates the identification of effective treatments. This dual methodology seeks to bridge the gap between computational predictions and empirical validation, offering a smart understanding of the potential benefits and challenges associated with native plant extractions in the context of CRC treatment. Further studies, including *in vivo* experiments and clinical trials, are warranted to validate and translate the findings into practical therapeutic applications, paving the way for the development of novel therapeutic interventions against CRC.

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Conflict of interest statement

The authors declared no conflict of interest in this study. The funders had no role in the design of the study, the collection, analyses, or interpretation of data, the writing of the manuscript, or the decision to publish the results.

Author's contributions

A. Alibakhshi, A. Shojaeian, and A. Asgari contributed to the conceptualization; A. Alibakhshi, M. Rahimmalek, R. Amini, and S. Gharibi conducted methodology and validation; S. Ahangarzadeh provided formal analysis and investigation; A. Alibakhshi, S. Gharibi, and S. Ahangarzadeh wrote the original draft of the manuscript;

M. Rahimmalek and A. Szumny supervised the project and revised the manuscript. All authors have read and approved the finalized article. Each author has fulfilled the authorship criteria and affirmed that this article represents honest and original work.

Data availability statement

The data presented in this study are available upon request from the corresponding author.

AI declaration

The authors did not use any AI-assisted technologies in the preparation of this manuscript.

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