

Identification of Extracts from “one Steaming and one Sun-drying” Black *Panax quinquefolius* and Mechanism of Majoroside F₆ in Inhibiting Breast Cancer Cell

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Abstract Many reports universal followed on common saponins of ginseng research field, few about functional identification of rare saponins. This study mainly researched new types of processed “one Steaming and one Sun-drying” Black *Panax quinquefolius* rare saponins (BPQRS) which extracted from Black *Panax quinquefolius* and its mechanism of inhibiting breast cancer cells. In our work, the extracts of Black *Panax quinquefolius* total saponins were analyzed used UPLC-Q/TOF-MS and 33 components were identified included 11 rare saponins. To further explored the inhibitory effect of BPQRS on disease pathways, network pharmacology and molecular docking simulation were carried out among 11 rare saponins, the rare saponins majoroside F₆ and ginsenoside Rk1 expressed high correlation on inhibition pathway of breast cancer. Lots of reported discussed the function of ginsenoside Rk1, finally chose majoroside F₆ of BPQRS to separated and purified by method of HSCCC. For identifying the function of majoroside F₆, breast cancer cell MCF7 in vitro experiment confirmed that majoroside F₆ exerted an significantly inhibiting cell proliferation by used MTT, flow cytometry and ELISA methods which upregulated caspase-3, Bax, inhibiting PI3K, AKT and Bcl-2 expression. This study laid a theoretical basis for developing and utilizing the medicinal value of BPQ.

Keywords: One Steaming and one Sun-drying Black *Panax quinquefolius*, Inhibit breast cancer cell, Majoroside F₆, Network Pharmacology, UPLC-Q/TOF-MS

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1. Introduction

Panax quinquefolius was a perennial herbaceous plant in the genus *Panax ginseng* of the family Pencosaceae [1]. Traditional ginseng could no longer satisfied people's daily needs and most ginseng was deeply processed to obtain some rare saponins in *Panax ginseng* research, however little reports was relatively on *Panax quinquefolius* processing. Traditional black ginseng was processed used the “Nine Steaming and Nine Sun-drying” method [2]. This method consumes more resources and time which was not conducive to the development of the economy and environment. The material in this work was black *Panax quinquefolius* (BPQ) used the “one Steaming and one Sun-drying” method which was a new type of ginseng product that enables the conversion of rare saponins more fully and also effectively controlled the harmful substance benzo (a) pyrene produced during the processing [3]. BPQ had strong pharmacological effects and rare saponins content were also higher than that of

fresh unprocessed ginseng. Therefore, it was necessary to develop and utilize the BPQ. On the one hand, it improved the efficiency of resource utilization, it drove local economic development in *Panax quinquefolius* planting area on the other hand.

At present, there were more than hundred kinds of triterpenoid saponins identified in *Panax ginseng* and *Panax quinquefolius*, the majority of research remains focused on structural studies about functional research primarily concentrated on common saponins and several rare saponins. Such as the report of Peng, D. et al [4]. showed that ginsenoside Rg₃, R₀, Rb₁, Rc, Rd others had anti-fatigue effect and Fan, W. et al. [5] found that ginsenoside Rg₁, Rh₂, Rk₁, Rs₂ could inhibit the production of pro-inflammatory cytokines that thus have anti-inflammatory effects. However, there were few reports on the triterpenoid components and functional studies of black ginseng that *Panax ginseng*, *Panax quinquefolius* processed products and functional characterisation of rare saponins, simply identified rare saponin structures. Therefore, this article selected “one Steaming and one Sun-drying” *Panax quinquefolius* as the material to identified the rare saponins in its components

and screens out monomers through network pharmacology for functional studies.

Therefore, this article conducted relevant research on BPQ which main component was triterpenoid saponins. The UPLC-Q/TOF-MS was used to identify and analyze the structure of BPQ. In addition, utilizing network pharmacology virtual screening to identify monomeric saponins. Molecular docking technology was used to clarify the mechanism of action of monomeric saponins on breast cancer through the binding degree of ligands and receptors. Finally, the hypothesis of network pharmacology was validated through MTT assay, cell apoptosis assay and changes in protein expression levels which upregulated caspase-3, Bax, inhibiting PI3K, AKT and Bcl-2 expression. This study provided a reference for further research, development and utilization of BPQ.

2. Materials and Methods

2.1. Chemicals and Reagents

Black *Panax quinquefolius* and *Panax quinquefolius* produced by Jilin Ginseng Research Institute, Fusong, Jilin Province; FC 500 flow cytometer (Beckman Coulter, Brea, CA, USA); ACQUITY UPLC binary pump and sample manager, Xevo G2-S Q-TOF quadrupole time-of-flight mass spectrometer (Waters Corporation, USA); OLYMPUS IX71; RT-6100 Enzyme Microplate Reader; ginsenoside Rg₁, Re, Rg₅, Rg₄, Rk₃ and soyasaponin Bb reference standard (purity ≥ 98%) were purchased from Shanghai Yuanye Biotechnology Co., Ltd.; The Human breast cancer cells MCF-7 was purchased from Wuhan Pricella Biotechnology Co., Ltd.; The Human PI3K, AKT, Caspase3, Bax and Bcl-2 Elisa kit were purchased from MULTISCIENCES LIANKE Biotech, Co., Hangzhou, China. All medicinal materials were identified according to the 2020 edition of the Chinese Pharmacopoeia. Other reagents were analytical pure.

2.2. Structural Identification

2.2.1. Sample Preparation

The extraction of BPQ and PQ were conducted according to our previous experiment. The BPQ and PQ was stored in a refrigerator at -80°C and then crushed to obtain powder. Weigh 10g of BPQ and PQ powder and placed it in a 50mL centrifuge tube. Add 210mL of 84% ethanol, mixed, sonicated at 60°C for 20 minutes (400W), centrifuge at 1500 r/min for 15 minutes, repeated extraction twice and combined the extracts. Extracted the crude extract from ethanol used a vacuum rotary evaporator and freeze-dry it to obtain the natural extract powder of BPQ [6].

2.2.2. Preparation of a Sample Solution

Weigh 29.9 mg of BPQ coarse powder, add 0.1 mg of soyasaponin Bb as an internal reference, dissolve with 80% methanol to prepare a test solution with a concentration of approximately 3 mg/mL, filter through a

0.22 μm microporous membrane and transfer to a sample bottle for injection.

2.2.3. Preparation of a Standard Solution

Weigh accurately the appropriate amount of reference substances such as ginsenoside Rg₁, Re, Rg₅, Rg₄ and Rk₃ etc. dissolve them in methanol and prepare a mixed reference solution containing approximately 0.1-0.3 mg/mL of each reference substance [7].

The standard reference materials of ginsenoside Re, Rc, Rd, soyasaponin, etc. were weighed separately at 2.00 mg. Each sample was placed in a 10 mL volumetric flask and a certain amount of ethanol was added for ultrasonic treatment. After dissolution, a certain volume of ethanol was added to obtain the stock solution of each monomer standard. The standard solution was diluted tenfold with ethanol before injection and filtered through a 0.22 μm microporous membrane to obtain the filtrate for testing [8].

2.2.4. UPLC Condition

The chromatographic column was an ACQUITY UPLC BEH C18 column (100 mm × 2.1 mm, 1.7 μm), the column temperature box was set at 30 °C, the sample manager temperature was set at 15 °C, the mobile phase A was a 0.1% formic acid aqueous solution and the mobile phase B was a 0.1% formic acid acetonitrile solution (v/v). The gradient elution conditions were as follows: 0-2 min, 10% B; 2-26 min, 10% B → 90% B; 26-30 min, 90% B; flow rate: 0.4 mL/min; injection volume: 5 μL; strong wash solution was a 90% acetonitrile aqueous solution and weak wash was a 10% acetonitrile aqueous solution.

2.2.5. ESI-MS/MS Conditions

Xevo G2-S Q-TOF; Electric spray ion source (ESI); Source temperature: 150 °C; Desolvation temperature: 400°C; Capillary voltage: 2.2 kV (ESI-); Cone hole voltage: 40 V; Cone hole air flow: 50 L/h; Solvent removal gas flow rate: 800.0 L/h; In MSE mode, the energy of the low-energy channel was 6 V; High-energy channel energy: 20 V~40 V; Calibrate the mass spectrometer with Sodium formate (100~1500 Da); Leucine-enkephalin (m/z 556.2771 [M+H]⁺, m/z 554.2615 [M-H]⁻) (Lock Spray TM, 100 ng/mL, 15 μL/min) standard solution was used as a calibration solution. In MSE continuum mode, Masslynx™ V4.1 workstation collects data.

2.2.6. Chemical Composition Database Analysis

The UNIFI natural product analysis platform was used to simplify the workflow for data processing, analyze the mass spectrometric fragmentation behavior, structural characteristics and characteristic fragment ions of compounds and rapidly identify compounds that match self-built and built-in databases. The main parameters for data processing method settings were: minimum peak area for 2D peak detection: 200; peak intensity for 3D peak detection with high and low energies set to more than 200 and 1000, respectively. Leucine-enkephalin (m/z : [M+H]⁺ 556.2766, [M-H]⁻ 554.2620) correction. Positive adducts select + H and + Na, while negative adducts select + COOH and - H. Screening parameters were as follows: mass error within ± 5 ppm; response

value greater than 5000. Finally, the structure of the compound was determined based on literature and the retention time (t_R), relative mass and typical fragment ions of the reference substance.

2.3. Network pharmacology analysis

2.3.1. Identification of Targets for BPQRS Therapy in Breast Cancer

The experiment searched the Traditional Chinese Medicine System Pharmacology (TCMSP) database [9] and SwissTargetPrediction database used the identified rare saponins components of Black *Panax quinquefolius* total saponins (BPQTS) as keywords and obtained targets related to the components of BPQTS. Then, used "breast cancer" as the keyword to search the Mendelian online genetic database [10] and GeneCards database [11] obtained targets related to breast cancer. Venny 2.1 software was used to determine the shared BPQRS and breast cancer related targets. Finally, a visual Venn diagram was generated to illustrate shared goals.

2.3.2. Construction of Disease-Drug-Pathway-Target Network

Used Cytoscape 3.9.1 software, a visual interactive network of "disease-drug-pathway-target" was constructed [12]. Used the CytoNCA plugin, the experiment calculated the average centrality metrics for each node in the network, including the average degree centrality (DC) which quantifies the average degree to that nodes were directly connected, the average closeness centrality (CC) which represents the speed of communication between nodes and the average betweenness centrality (BC) that measures the extent to which nodes act as bridges or key intermediaries along the shortest path between nodes. BPQRS components were sorted in descending order based on their DC value as network nodes. Compounds with higher than average degrees were considered as key compounds for BPQRS treatment of breast cancer.

2.3.3. Construction of Protein-Protein Interaction (PPI) Network and Screening of Key Targets

In order to explore the relationship between cross targets involved in the treatment of breast cancer with BPQRS and identify key target genes, a PPI network was constructed used STRING database. The network was constructed using "homologous species" as species and the confidence score for stronger protein binding interactions was set to >0.700. Visualize the PPI network obtained from the STRING database used Cytoscape 3.9.1. Used the CentiScaPe 2.2 Menu plugin, evaluate the degree, betweenness and closeness of nodes in the network.

2.3.4. Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) Enrichment Analysis

To further understand the interaction between target genes and diseases, as well as the potential mechanisms of BPQRS, GO [13] functional enrichment analysis and KEGG analysis [14] were performed using the Database for Annotation, Visualization and Integrated Discovery

(DAVID) [15]. The list of target genes was imported into David database and the genes were classified and annotated using the "Functional Annotation Tool" analysis tool with a significance threshold of $p < 0.05$. In GO analysis, these genes were compared with known biological processes (BP), cellular components (CC) and molecular functions (MF). In KEGG analysis, these genes were mapped to pathways and functional annotation and pathway analysis were performed. Used the analysis tools provided by bioinformatics platforms, the obtained data were visualized used bar charts and bubble charts.

2.3.5. Molecular Docking Verification

The ligand was selected from the rare saponins in BPQ and the receptor was selected from the core targets. Molecular docking predicts the binding mode of receptors and ligands by analyzing their properties and interactions. The protein database (PDB) was used to determine the 3D structure of the protein and the PubChem database was used to identify the chemical compounds in BPQRS. AutoDock 4 [16] and AutoDock Vina were used to prepare the protein and ligand and perform docking calculations. Based on energy evaluation, the binding affinity was determined with a lower value indicating a stronger binding. The best docking result was selected and visualized using PyMOL software.

2.4. Separation and Purification of rare saponins from BPQ

2.4.1. Preparation of a Sample Solution

Taken BPQ 40g, add 20 times 70% ethanol, reflux extraction 3.5 hours, decompression recovery of ethanol, evaporation, the total crude saponin and then add the crude aqueous solution in AB-8 large pore adsorbent resin (crude mass - resin volume 1:35), washed to colourless, 80% ethanol elution to the absence of saponins, collection of 80% ethanol eluate, decompression recovery of solvent, evaporation to obtain BPQTS. Ethyl acetate was 400ml, 100ml of tanlol, 300 ml of water and 1.6 ml of acetic acid, placed 1000 ml of liquid liquid funnel and shakes well. Repeat the operation once and the upper and lower solutions were merged. Used 0.22cm filter membrane to filter the upper and lower solutions, respectively and ultrasonic illegal exhalation was 30 min. The upper solution was fixed and the lower solution was a flowing phase, spare. Accurately referred to as 50 mg of BPQTS powder with 4 ml fixed phase and 4 ml flowing phase to dissolve as a sample solution.

2.4.2. HSCCC Condition

The HSCCC solvent system was: ethyl acetate-butyl alcohol-water (4: 1: 3, v: v: v), the upper phase was a fixed phase, the lower phase was a flowing phase, the detection wavelength was 254 nm, the speed was 800r/min, the flow rate was 1.5 ml/min, the pillar temperature was 25 °C and the operation was 230 min. Used 20 ml/min to recharge the HSCCC chromatographic column, rotate the color spectrum, run 30 min, import the sample solution until the volume flow of 1.5 ml/min to the fixed phase flow. Pick up each segment according to the HSCCC chromatography peak.

2.4.3. UPLC and ESI-MS/MS Condition

Consistent with 2.2.4 and 2.2.5, change the UPLC eluting gradient to: 0-20 min, 25%~40% B; 20-33 min, 40%~46% B; 33-37 min, 46% B; 37-45 min, 46%~50% B; 45-55 min, 50%~70% B; flow rate: 1 mL/min; injection volume: 10 μ L.

2.5. Vitro Cell Experiments

2.5.1. Cell Culture

The cell lines (MCF7 human breast cancer cells and HEK293 human embryonic kidney cells) were cultured in DMEM medium supplemented with 10% heat inactivated fetal bovine serum (FBS) and 1% antibiotic antimycin 100 X solution and incubated in CO₂ incubator maintained at 37 °C, 5% CO₂ and 95% humidity. Passage once every 48h and take logarithmic growth phase cells for experiments.

2.5.2. MTT Method

The MTT method was used to evaluate the effect of monomeric saponins on the proliferation of MCF7 human breast cancer cells and normal HEK 293 cells. Cells were seeded at a density of 5×10^4 cells well⁻¹ and incubated with the drug. After 24, 48 and 72 hours, the cells were washed with PBS and 0.2 mL of growth medium and 50 μ L of MTT reagent (2 mg/mL) were added to each well. The cells were allowed to grow for another 4 hours until a purple precipitate was visible. Then, the medium was removed and 150 μ L of dimethylsulfoxide was added. The wells were shaken for 10 minutes to fully release the crystals. Finally, cell viability was measured at 570 nm.

2.5.3. Cell morphological observation

MCF7 cells were cultured in 6-well plates for 24 hours and then incubated with different concentrations of majoroside F₆ for 48 hours. The morphology of the cells was observed using an inverted microscope.

2.5.4. Cell apoptosis assay

Apoptosis was evaluated used annexin V-FITC assay which selectively binds to phosphatidylserine on the cell membrane, thereby labeling early apoptotic cells. Propidium iodide (PI) was used to stain late apoptotic or

dead cells. MCF7 cells were treated with different concentrations of majoroside F₆ in 6-well plates for 48 hours. Samples were analyzed using flow cytometry and data were analyzed using Beckman Coulter High Throughput CXP software.

2.5.5. Measurement of protein expressions

MCF7 cells in the logarithmic growth phase were taken and the cell concentration was adjusted to 4×10^5 /mL and inoculated into 96-well plates for culture, 200 μ L per well. After 24 h of culture at 37°C and 5% CO₂, the drug concentrations in the total saponins group were 40 μ g/mL, 60 μ g/mL and 80 μ g/mL. The final dosing concentration for the everolimus group was 1 μ g/mL. Medication intervention for 4 hours. The contents of PI3K, AKT, Caspase3, Bax and Bcl-2 were detected according to the instructions of the ELISA kit.

2.6. Statistical Analyses

GraphPad Prism 9.0 was used to analyze the results of the experiment. The data consisted of five replicates per group. One-way ANOVA was performed to analyze the statistical significance among multiple groups. The experimental data were presented as the mean $\pm$ standard deviation (Mean $\pm$ SD), where $P < 0.05$ indicates a significant difference and $P < 0.01$ indicates a highly significant difference [17].

3. Results and Discussion

3.1. Analysis by UPLC-Q/TOF-MS

The chemical composition characteristics of BPQ were analyzed used UPLC-ESI-Orbitrap-MS in negative ion mode (Figure 1) that a total of 33 compounds were identified through data processing on UNIFI platform, comparing relevant literature and controls. Triterpenoid saponins had characteristic MS fragmentation patterns which were prone to loss of glycosyl groups, resulted in the production of aglycone ions. The main constituents with the basic structure of triterpenoid saponins were listed in Table 1 and ginsenoside Rk₁, majoroside F₆, ginsenoside F₅, ginsenoside Rk₃, notoginsenoside N, isoginsenoside Rh₃, quinquenoside R₁, quinquenoside I, vinaginsenoside R₁, quinquenoside L₁, notoginsenoside G were selected as 11 rare saponins for network pharmacology experiments.

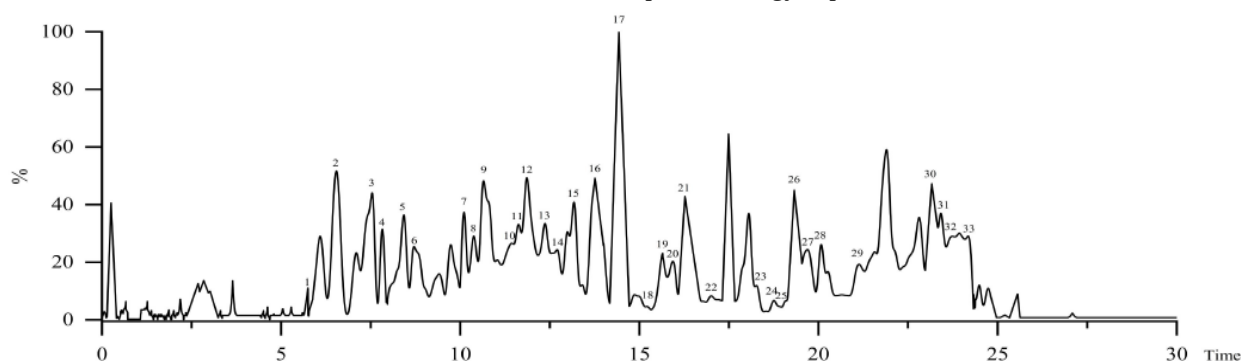


Figure 1. Base peak ion chromatography of BPQ in and negative modes

Table 1. The structures of compounds in BPQTS were identified by HPLC-ESI-MS/MS method

Peak no.	T_R (min)	BPQ Content (%)	Molecular formula	MS ^E Fragments	Identification
1	5.748	0.106	C ₄₈ H ₈₂ O ₁₉	1007.5315[M+HCOO] ⁺ ; 961.5316[M-H] ⁻ ; 799.4168[M-H-Glu] ⁻ ; 653.4635[M-H-Rha-Glu] ⁻ ; 491.3492[M-H-2Glu-Rha] ⁻	Majoroside F ₆
2	6.534	0.211	C ₄₈ H ₈₂ O ₁₉	1007.5405[M+HCOO] ⁺ ; 961.5423[M-H] ⁻ ; 799.4816[M-H-Glu] ⁻ ; 637.4299[M-H-2Glu] ⁻	Notoginsenoside N
3	7.541	0.196	C ₄₂ H ₇₂ O ₁₄	845.4895[M+HCOO] ⁺ ; 799.4886[M-H] ⁻ ; 653.4566[M-H-Rha] ⁻ ; 491.3962[M-H-Glu-Rha] ⁻	(24S)-Pseudo ginsenoside F ₁₁
4	7.826	0.117	C ₄₈ H ₈₂ O ₁₉	1007.5992[M+HCOO] ⁺ ; 961.5124[M-H] ⁻ ; 799.3791[M-H-Glu] ⁻ ; 637.4129[M-H-2Glu] ⁻ ; 475.3698[M-H-3Glu] ⁻	Notoginsenoside R ₆
5	8.424	0.161	C ₄₂ H ₇₂ O ₁₄	845.4618[M+HCOO] ⁺ ; 637.4307[M-H-Glu] ⁻ ; 475.3863[M-H-2Glu] ⁻	Ginsenoside Rg ₁
6	8.709	0.189	C ₄₈ H ₈₂ O ₁₈	991.5499[M+HCOO] ⁺ ; 783.4867[M-H-Glu] ⁻ ; 475.4036[M-H-2Glu-Rha] ⁻	Ginsenoside Re
7	10.105	0.175	C ₄₄ H ₇₄ O ₁₅	887.5001[M+HCOO] ⁺ ; 799.4884[M-H-Ac] ⁻ ; 695.4596[M-H-Glu] ⁻ ; 491.4396[M-H-Rha-Glu-Ac] ⁻	Vinaginsenoside R ₁
8	10.378	0.216	C ₄₈ H ₈₀ O ₁₈	989.5398[M+HCOO] ⁺ ; 943.5436[M-H] ⁻ ; 763.4796[M-H-Glu-H ₂ O] ⁻ ; 619.4159[M-H-2Glu] ⁻	Quinquenoside L ₁
9	10.651	0.246	C ₄₁ H ₇₀ O ₁₃	815.4786[M+HCOO] ⁺ ; 637.4326[M-H-Ara] ⁻ ; 475.3574[M-H-Glu-Ara] ⁻	Ginsenoside F ₅
10	11.435	0.154	C ₅₉ H ₁₀₀ O ₂₇	1285.6219[M-H] ⁻ ; 1077.5797[M-H-Xyl] ⁻ ; 945.5017[M-H-Xyl-Glu] ⁻ ; 783.4694[M-H-Xyl-2Glu] ⁻ ; 459.3731[M-H-Xyl-4Glu] ⁻	Notoginsenoside Fa
11	11.625	0.148	C ₄₈ H ₈₀ O ₁₉	1005.5289[M+HCOO] ⁺ ; 959.5299[M-H] ⁻ ; 797.4714[M-H-Glu] ⁻ ; 635.4159[M-H-2Glu] ⁻	Notoginsenoside G
12	11.861	0.189	C ₄₂ H ₇₂ O ₁₄	845.4521[M+HCOO] ⁺ ; 653.4249[M-H-Rha] ⁻ ; 491.3719[M-H-Rha-Glu] ⁻ ; 1123.6015[M+HCOO] ⁺ ; 1077.5917[M-H] ⁻	Pseudoginsenoside F ₁₁
13	12.365	0.162	C ₅₃ H ₉₀ O ₂₂	621.4268[M-H-2Glu-Araf] ⁻ ; 459.3837[M-H-3Glu-Araf] ⁻	Ginsenoside Rc
14	12.703	0.153	C ₅₆ H ₉₂ O ₂₅	1163.5906[M-H] ⁻ ; 1117.5961[M-H-CO ₂] ⁻ ; 945.5366[M-H-Araf-Mal] ⁻ ; 621.4742[M-H-2Glu-Araf-Mal] ⁻	Ginsenoside F ₃
15	13.174	0.177	C ₄₈ H ₇₆ O ₁₉	955.5107[M-H] ⁻ ; 793.4401[M-H-Glu] ⁻ ; 455.3524[M-H-2Glu-GluA] ⁻	Ginsenoside Ro
16	13.760	0.213	C ₅₆ H ₉₄ O ₂₄	1195.6145[M+HCOO] ⁺ ; 1107.5071[M-H-Ac] ⁻ ; 987.5524[M-H-Glu] ⁻ ; 783.4918[M-H-2Glu-Ac] ⁻ ; 459.3791[M-H-4Glu-Ac] ⁻	Quinquenoside R ₁
17	14.425	0.385	C ₄₈ H ₈₂ O ₁₈	991.5561[M+HCOO] ⁺ ; 783.4957[M-H-Glu] ⁻ ; 621.4432[M-H-2Glu] ⁻	Ginsenoside Rd
18	15.210	0.029	C ₅₅ H ₉₂ O ₂₃	1165.6042[M+HCOO] ⁺ ; 1077.5914[M-H-Ac] ⁻ ; 915.5347[M-H-Ac-Glu] ⁻ ; 459.3829[M-H-Ac-3Glu-Ara] ⁻	Ginsenoside Rs ₂
19	15.648	0.148	C ₅₄ H ₉₂ O ₂₃	1153.6045[M+HCOO] ⁺ ; 783.4813[M-H-2Glu] ⁻ ; 459.3820[M-H-4Glu] ⁻	Ginsenoside Rb ₁
20	15.958	0.167	C ₄₈ H ₈₂ O ₁₈	991.5493[M+HCOO] ⁺ ; 783.4927[M-H-Glu] ⁻ ; 621.4342[M-H-2Glu] ⁻	Gypenoside XVII
21	16.272	0.186	C ₄₇ H ₈₀ O ₁₇	961.5419[M+HCOO] ⁺ ; 915.5411[M-H] ⁻ ; 621.4413[M-H-Glu-Ara] ⁻	Notoginsenoside Fe
22	17.007	0.054	C ₄₇ H ₈₀ O ₁₇	961.5384[M+HCOO] ⁺ ; 783.4948[M-H-Ara] ⁻ ; 621.4375[M-H-Ara-Glu] ⁻ ; 459.3861[M-H-Ara-2Glu] ⁻	Chikusetsu saponin III
23	18.266	0.119	C ₅₂ H ₈₆ O ₁₉	1059.5741[M+HCOO] ⁺ ; 1013.5753[M-H] ⁻ ; 851.4508[M-H-Glu] ⁻	Quinquenoside I
24	18.754	0.066	C ₄₂ H ₇₀ O ₁₂	811.4846[M+HCOO] ⁺ ; 519.4251[M-H-Man] ⁻ ; 457.3894[M-H-Glu-Man] ⁻	Ginsenoside Rg ₄
25	19.129	0.071	C ₃₆ H ₆₀ O ₈	665.4253[M+HCOO] ⁺ ; 457.3865[M-H-Glu] ⁻	Ginsenoside Rk ₃
26	19.324	0.194	C ₄₂ H ₇₂ O ₁₃	829.5012[M+HCOO] ⁺ ; 621.4420[M-H-Glu] ⁻ ; 459.3850[M-H-2Glu] ⁻	Ginsenoside F ₂
27	19.691	0.175	C ₃₆ H ₆₀ O ₇	649.3954[M+HCOO] ⁺ ; 603.4319[M-H] ⁻ ; 441.3859[M-H-Glu] ⁻	Isoginsenoside Rh ₃

28	20.075	0.159	C ₃₆ H ₆₀ O ₈	665.4327[M+HCOO] ⁺ ; 619.4332[M-H] ⁺ ; 457.3926[M-H-Glu] ⁺	Ginsenoside Rh ₄
29	21.129	0.146	C ₄₂ H ₆₆ O ₁₄	793.4537[M-H] ⁺ ; 757.4957[M-H- HCOOH] ⁺ ; 631.3791[M-H-Glu] ⁺	Chikusetsu saponin Iva
30	21.897	0.188	C ₄₇ H ₇₄ O ₁₈	925.4844[M-H] ⁺ ; 793.4863[M-H-Rha] ⁺ ; 731.4375[M-H-Glu] ⁺	Chikusetsu saponin Ib
31	23.412	0.165	C ₄₂ H ₇₂ O ₁₃	455.4958[M-H-Ara-Glu-GluA] ⁺ 829.4984[M+HCOO] ⁺ ; 783.4871[M-H] ⁺ ; 621.4405[M-H-Glu] ⁺ ; 459.3844[M-H- 2Glu] ⁺	(20S)-Ginsenoside Rg ₃
32	23.734	0.154	C ₄₂ H ₇₀ O ₁₂	811.4592[M+HCOO] ⁺ ; 765.4824[M-H] ⁺ ; 603.4241[M-H-Glu] ⁺ ; 441.3469[M-H- 2Glu] ⁺	Ginsenoside Rk ₁
33	24.176	0.152	C ₃₆ H ₆₂ O ₈	667.4322[M+HCOO] ⁺ ; 621.4258[M-H] ⁺ ; 459.3721[M-H-Glu] ⁺	Ginsenoside Rh ₂

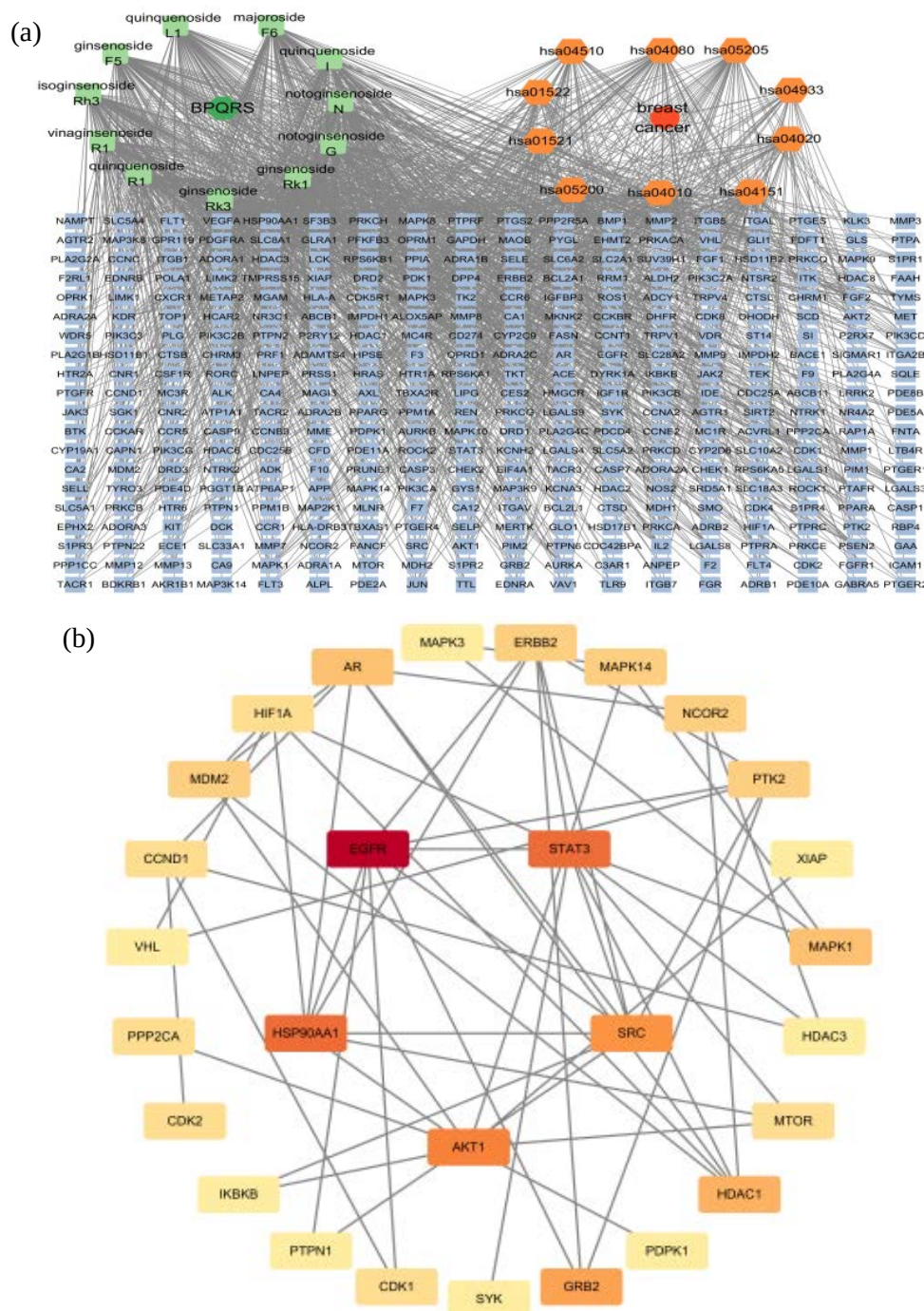


Figure 2. The Disease-Drug-Pathway-Targets Network (a) and Visualize the PPI network through Cytoscape 3.9.1 (b)

Typical components were analyzed as follows, compound 5 was a PPT-type ginsenoside with a retention time (t_R) of 8.424 min which was basically consistent with the t_R of the standard ginsenoside Rg1. Under ESI-, the molecular ion peak [M+HCOO]⁻ m/z : 845.4618 (calcd for C₄₃H₇₃O₁₆, 845.4627), evidenced its molecular formula was C₄₂H₇₂O₁₄. In the high-energy channel of MS², two characteristic fragment ions were generated: m/z 637.4307 was the [M-H-Glc]⁻ peak of the parent ion peak lost one molecule of glucose residue (Glc, 162u), followed by the loss of one molecule of Glc residue to generate the m/z 475.3863 [M-H-2Glc]⁻ peak. The m/z 475.3863 peak was a characteristic ion peak of PPT-type saponins which was basically consistent with the fragment ion peak information of the control sample. Therefore, it was identified as ginsenoside Rg₁ [18]. All these results demonstrated that most of the ionic fragments of common saponins in BPQ were formed by dropping glucose residues and the parent peaks were characteristic ionic peaks of typical PPD or PPT type. The content of common saponins was high and accounts for a large proportion of the saponin species.

Compound 1 with a t_R of 5.748 min. Under ESI-, the molecular ion peak [M+HCOO]⁻ m/z : 1007.5313 (calculated for C₄₉H₈₃O₂₁, 1007.5305) and the molecular ion peak [M-H]⁻ m/z : 961.5314 (calculated for C₄₈H₈₁O₁₉, 961.5322), suggested that its molecular formula was C₄₈H₈₂O₂₀. In the high-energy channel of MS₂, three characteristic ion fragments demonstrated: m/z 799.4166 was the [M-H-Glu]⁻ peak generated by the loss of one molecule of glucose residue (Glu, 162u) from the parent ion peak, followed by the loss of one molecule of rhamnose residue (Rha, 146u) to generate the m/z 653.4635 [M-H-Rha-Glu]⁻ peak, continue to loss 1 molecule of Glu residue to produce a peak of [M-H-2Glu-Rha]⁻ at m/z 491.3493. Therefore, the component was identified as majoroside F₆ [19]. The results indicated that the ionic fragments of rare saponins in BPQ were formed by dropping the rhamnose residue, etc. and the mother peak was only partly PPD or PPT type characteristic ion peak and the rest was oleanolane characteristic ion peak. Rare saponin content was low, in the saponin species accounted for less than had a higher research value.

3.2. Network Pharmacology Analysis

3.2.1. Potential Targets of BPQRS for Breast Cancer Treatment

11 rare saponins of BPQ were used as keywords, it provided that 383 targets related to them in the TCMSP database and Swiss Target Prediction database (Table S1). In addition, 18196 breast cancer related targets were obtained using the keyword "breast cancer" in OMIM and GeneCards databases (Table S2). Used Venny 2.1 software to identify common targets, 360 common targets were obtained (Figure S1) (Table S3). These proteins were potential targets for the treatment of breast cancer with triterpene saponins of BPQRS.

3.2.2. Construction of Disease-Drug-Pathway-Target Network

Used network pharmacology-based analysis to

predicted potential molecular targets. Used Cytoscape 3.9.1, the experiment systematically constructed a "disease-drug-pathway-target" network and presented it in a visualized manner. The network includes 360 colon cancer-related targets, 11 active BPQRS components and 10 core participants in related pathways (Figure 2. a). There were 383 nodes and 1537 lines in the network. Used the CytoNCA plugin to calculate centrality metrics (degree, betweenness and closeness) to evaluate the association between interactions within the disease-drug-pathway-target network. "Degree" measures the strength of association, while "betweenness" identified key mediating agents that facilitated information flow and "closeness" measured communication efficiency. Nodes were ranked according to their average "degree" value which indicates the degree of correlation between compounds and targets. A higher "degree" value indicated a stronger connection between the active component and its target. The top 4 active components were ginsenoside Rk₁, majoroside F₆, ginsenoside F₅, ginsenoside Rk₃ (Table 2). These compounds were potential active components for BPQRS in the treatment of breast cancer.

Table 2. The degree, betweenness and closeness values BPQRS components in the disease-drug-pathway-targets network

Component name	Degree	Betweenness	Closeness
ginsenoside Rk ₁	99	19943.21	0.41075268
majoroside F ₆	99	13864.385	0.41163793
ginsenoside F ₅	98	13647.128	0.41075268
ginsenoside Rk ₃	98	23002.309	0.41075268
notoginsenoside N	98	14187.765	0.40899357
isoginsenoside Rh ₃	97	21619.098	0.40987125
quinquenoside R ₁	96	13837.528	0.40899357
quinquenoside I	95	15848.966	0.40724948
vinaginsenoside R ₁	94	12771.642	0.40638298
quinquenoside L ₁	93	11840.339	0.40638298
notoginsenoside G	91	13191.592	0.40466103

3.2.3. Construction of Protein-Protein Interaction (PPI) Network and Screening of Key Targets

To analyze the interaction between BPQRS compounds and breast cancer-related targets, the experiment used the STRING database to analyze 360 common targets and constructed a PPI network with an interaction score ≥ 0.700 (Figure S2). Cytoscape 3.9.1 was used to visualize the network and the Centiscape plugin was used to organize the lines in the PPI network based on their degree values which reflect the strength of protein interactions. Larger nodes with darker colors and closer to the center indicate higher degree values that means stronger interactions between the targets (Figure 2.b). The top 5 genes in the network were EGFR, STAT3, HSP90AA1, AKT1 and SRC which were important in the "cancer pathway", PI3K-Akt signaling pathway, MAPK signaling pathway and calcium signaling pathway. It was speculated that BPQRS probably exerts its anti-breast cancer effect by influencing the apoptosis and cell cycle of cancer cells.

3.2.4. GO and KEGG Enrichment Analysis

A total of 1110 significant GO term entries were identified by enrichment analysis ($p < 0.05$), included 801 biological processes (BP), 122 cellular components (CC)

and 187 molecular functions (MF) entries. The top 10 enriched terms in each category were visually displayed in a bar chart. The BP enrichment analysis were phosphorylation, protein phosphorylation, positive regulation of MAPK cascade, peptidyl-serine phosphorylation and protein autophosphorylation. The CC enrichment analysis were plasma membrane, receptor complex, membrane raft, cell surface, external side of plasma membrane [20]. The MF enrichment analysis were protein kinase activity, ATP binding, protein serine / threonine kinase activity, protein tyrosine kinase activity, transmembrane receptor protein tyrosine kinase activity and kinase activity [21] (Figure 3.a). These results suggested that BPQRS potentially played its anti breast cancer role by regulated the protein and enzyme activities in the signal pathway.

The analysis of KEGG pathway enrichment revealed 180 significantly enriched pathways ($p < 0.05$) (Figure 3.b). The results in Figure 3. b showed that the top 10 most enriched pathways included "Pathways in cancer", "Proteoglycans in cancer", "Neuroactive ligand-receptor interaction", "PI3K-Akt signaling pathway", "Endocrine resistance", "EGFR tyrosine kinase inhibitor resistance", "Focal adhesion", "AGE-RAGE signaling pathway in diabetic complications", "MAPK signaling pathway", "Calcium signaling pathway". Of the common targets, 49.1% were related to the above-mentioned pathways, particularly those involved apoptosis and cell cycle. These results indicated that BPQRS possible affect the apoptosis and cell cycle of cancer cells, thus contributed to its anti breast cancer effect.

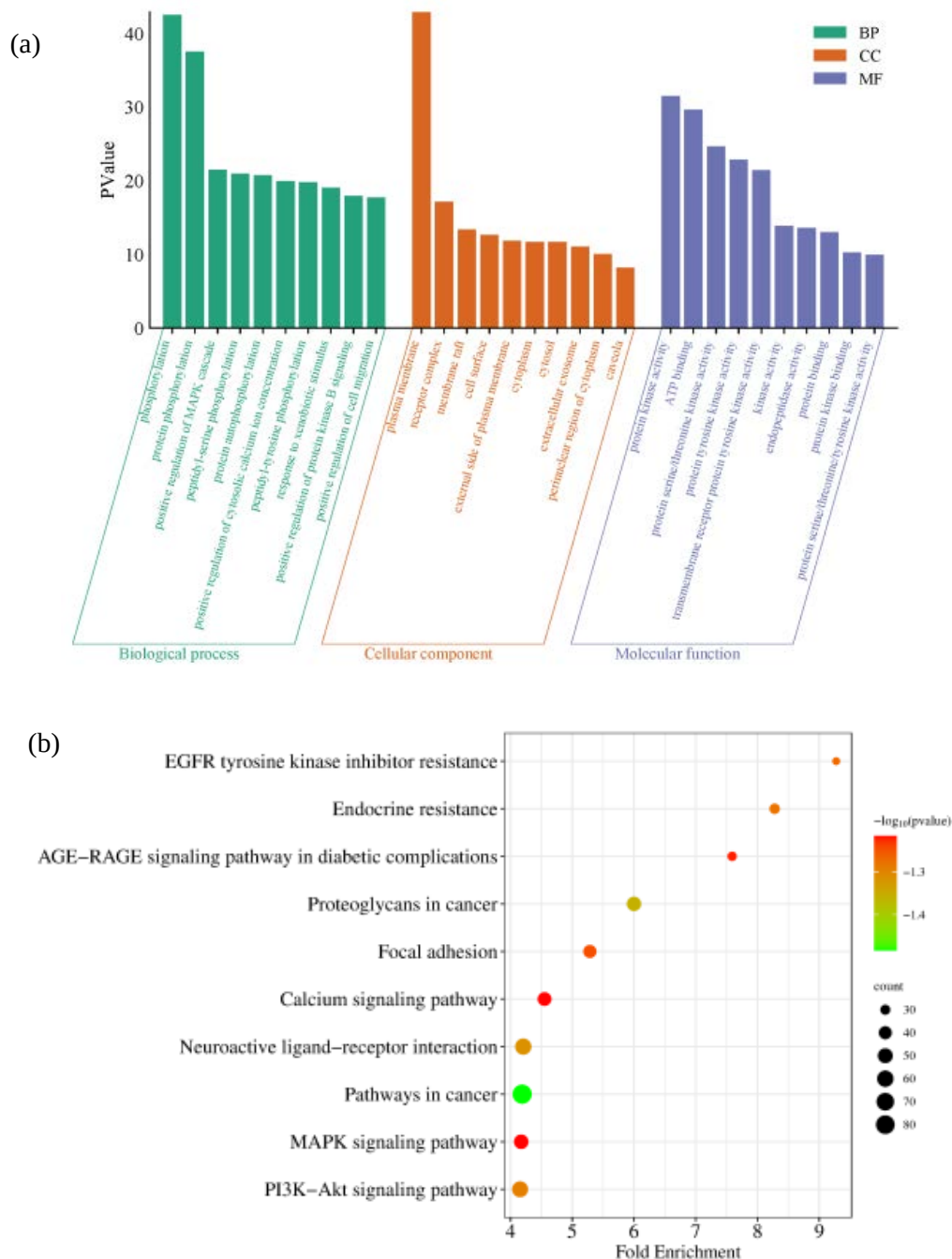


Figure 3. Analysis of GO and KEGG enrichment. (a) Top 10 GO terms ($p < 0.05$) in the biological process (BP), cellular component (CC) and molecular function (MF) categories. (b) Top 10 KEGG pathways ($p < 0.05$)

3.2.5. Molecular Docking Verification

In order to explore the mechanism of the active ingredients of BPQRS in the treatment of breast cancer, AutoDock was used for molecular docking to simulate the binding conformation between these active ingredients and their key targets. The binding energy obtained through molecular docking reflects the complementary binding between the active ingredient and its target. The higher the absolute value of the binding energy, the better the binding stability and the stronger the binding affinity. Based on the degree ranking in the "Potential Target Interaction (PPI) Network" category, the core targets EGFR, STAT3, HSP90AA1, AKT1 and SRC were used as visualization ligands. The docking mode of the key active ingredient with core targets SRC, PIK3CA, PIK3CB, AKT1, PIK3CD, MAPK1, GRB2, STAT3,

MAPK3 and EGFR were displayed in Figure 4. Finally, visualization was performed using PyMol software.

3.3. Separation and Purification of Rare Saponins from BPQ

3.3.1. HSCCC Separation

According to the steps described in 2.5.1, acetate-ethyl-positive butanol-water (4: 1: 3, v: v: v) as the solvent system, separate the extraction of ginsenosides, fixed phases and fixed phase phase that the retaining was 54.3%, the separation time was 180 min. The manual segmented collection, according to the shown in the Figure 5.a., the income flow was collected when each peak was about to reach the peak and 2 peaks were obtained and the numbers were numbered 1 to 2, respectively.

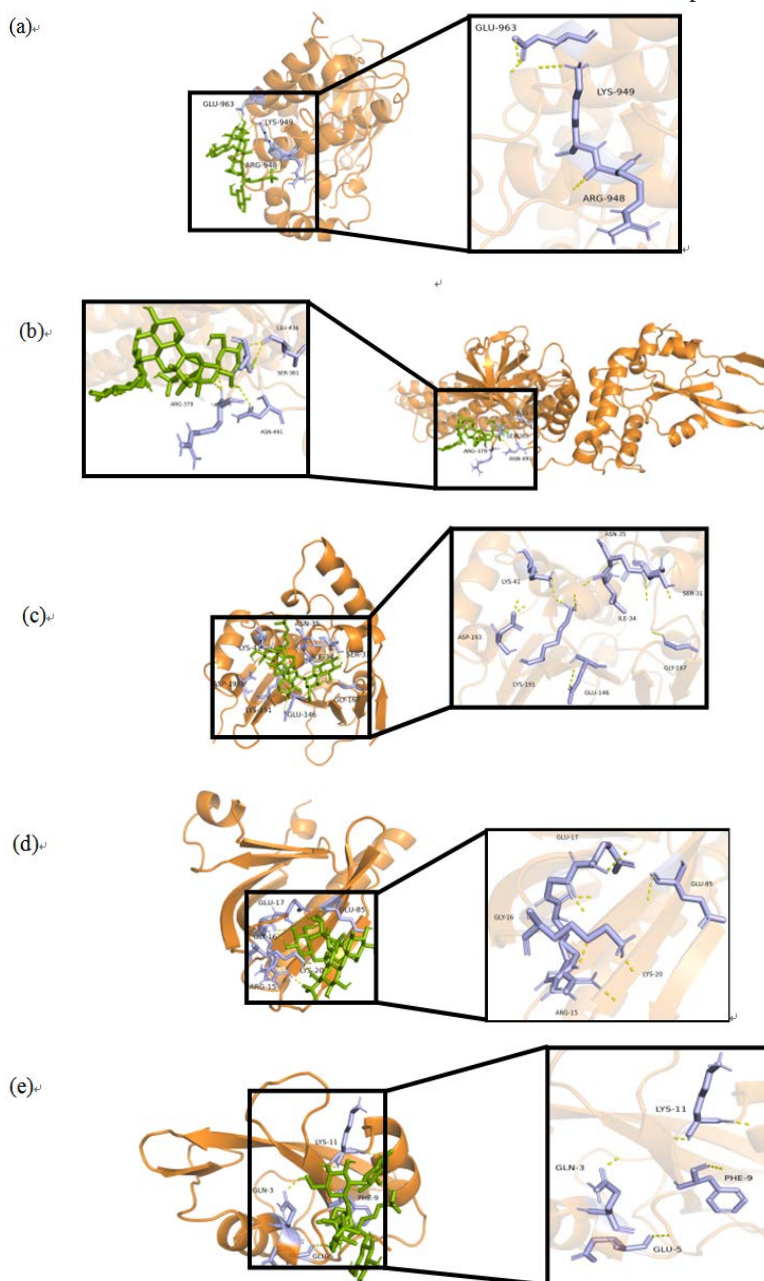


Figure 4. The molecular docking modes of majoroside F6 and (a)EGFR, (b)STAT3, (c)HSP90AA1, (d)AKT1, (e) SRC

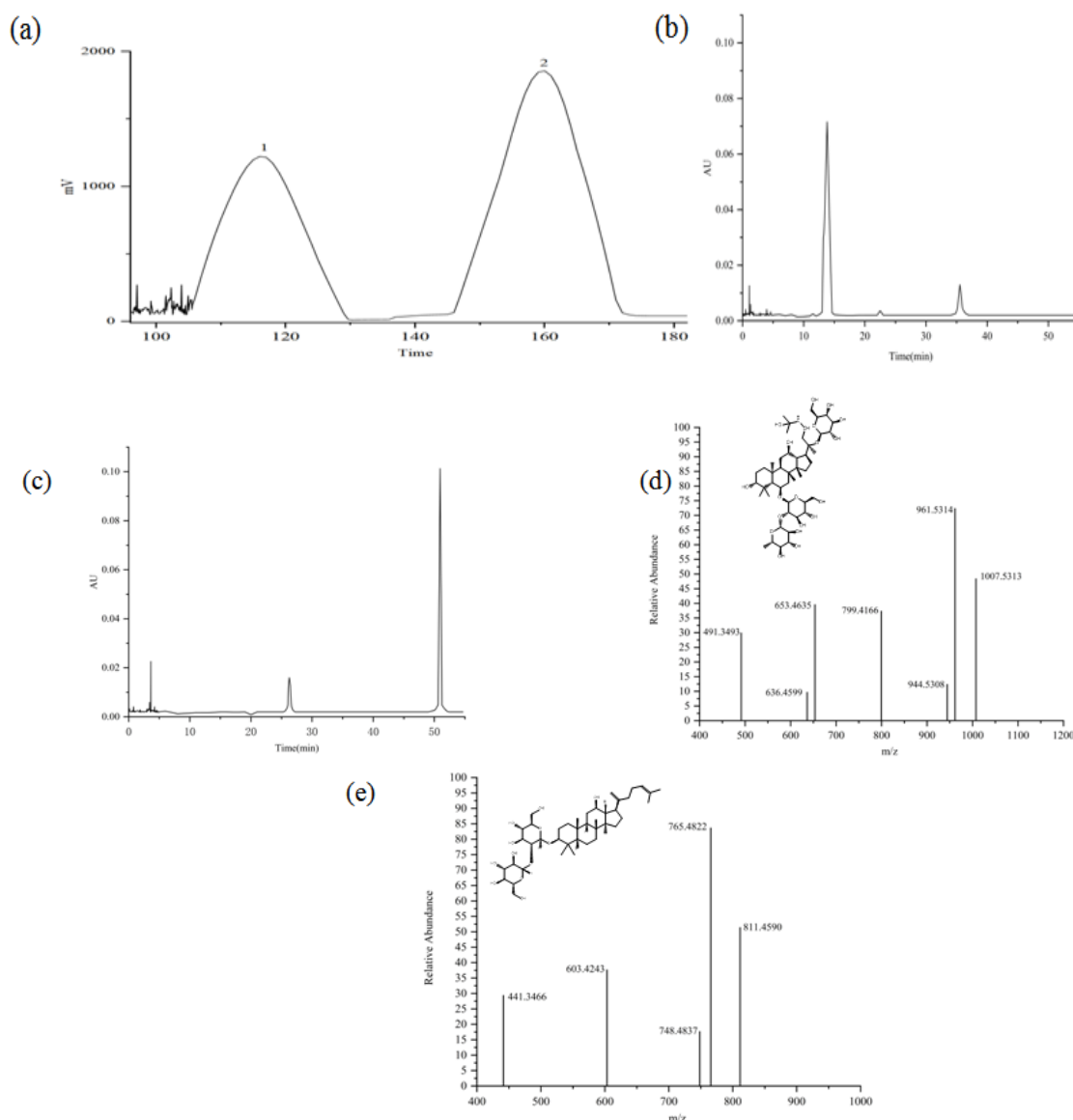


Figure 5. HSCCC of the BPQRS fraction (a), HPLC analysis of HSCCC peak fraction: Peak 1 (b), Peak 2 (c) and ESI-MS/MS spectra of the separated peak fractions on HSCCC: Peak 1, majoroside F₆ (d); Peak 2, ginsenoside Rk1 (e)

3.3.2. UPLC and ESI-MS/MS Analyse Compound in HSCCC

The content of the compounds was calculated according to the peak area of the HPLC chromatogram [22]. Figure 5.b shows the first fraction of HSCCC which was majoroside F₆ with the content of 94.9%. Figure 5.c shows the second stream of HSCCC that was ginsenoside Rk1 with the content of 95.1%. The identified results were compared with 3.1 and it could be judged that peak1 was majoroside F₆ and peak2 was ginsenoside Rk1. The results show that the results of HSCCC isolation and purification were better which indicates a direction for the isolation and purification of rare saponins monomers in the future [23].

3.4. Vitro Cell Experiments

3.4.1. Inhibition of MCF7 Cell and HEK293 Cell Proliferation

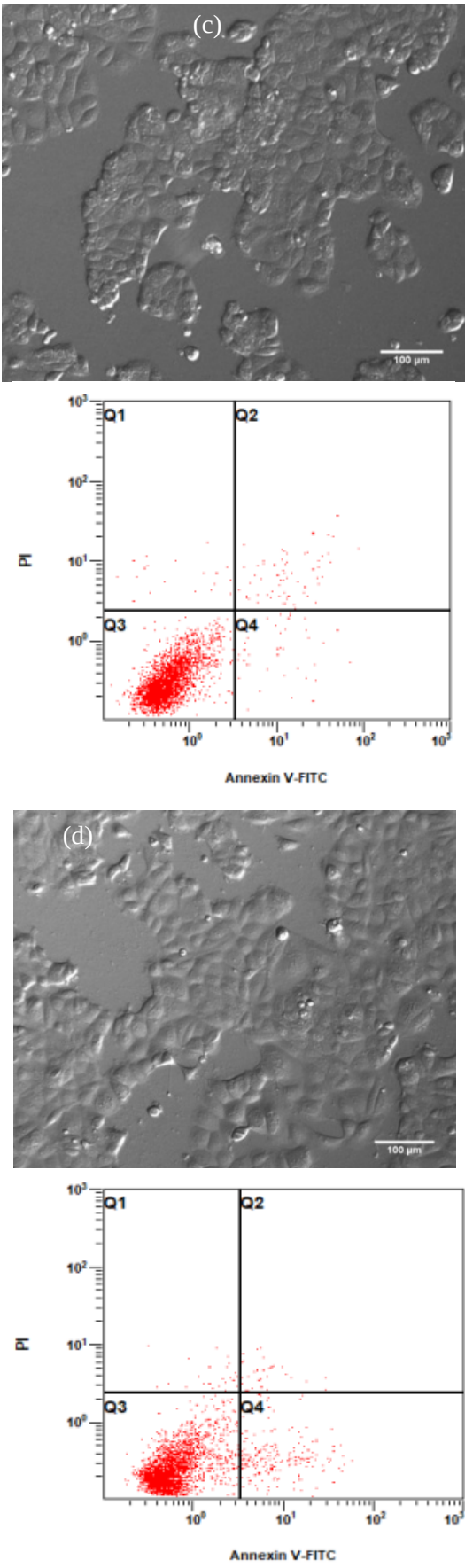
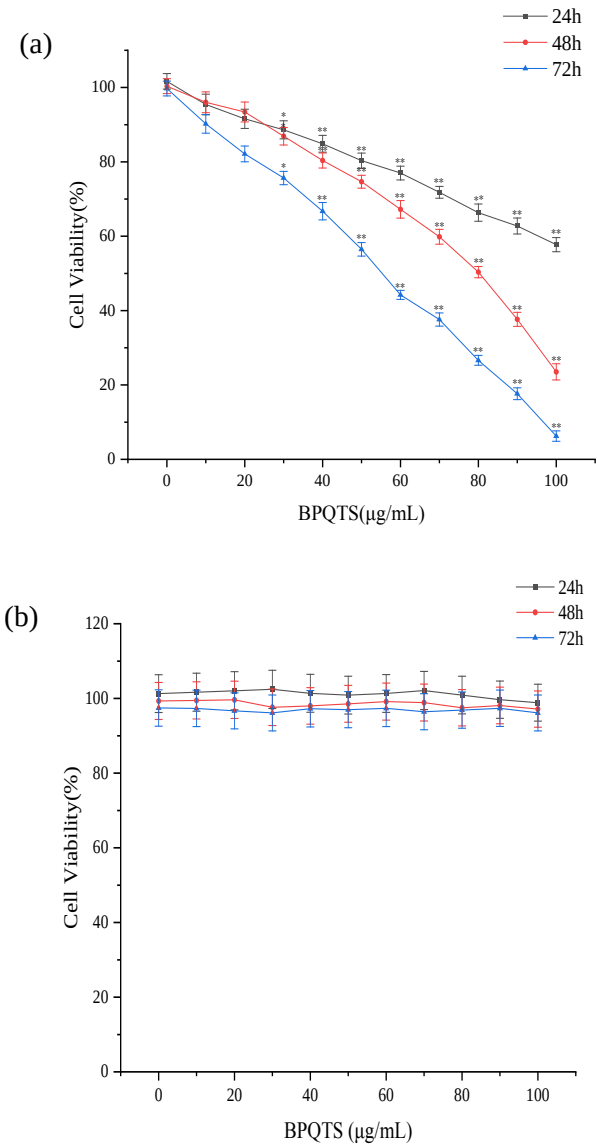
Due to numerous research reports on ginsenoside Rk₁,

majoroside F₆ was chosen as the monomer saponin for validation experiments. The Figure 6 indicated that different concentrations of majoroside F₆ were administered to human breast cancer cells MCF7 and human normal embryonic kidney cells HEK 293. All these results demonstrated that the proliferation of MCF7 human breast cancer cells was significantly inhibited in a concentration dependent manner. Moreover, experimental results were observed for the treatment time increased, the cell viability gradually decreased, indicated a time-dependent effect (Figure 6.a). The IC₅₀ value of majoroside F₆ for 48 hours was $56.82 \pm 0.7 \mu\text{g/mL}$.

In addition, the experimental results showed that the proliferative activity of human normal embryonic kidney cells HEK 293 was not significantly affected by majoroside F₆ in the concentration range of 10-100 $\mu\text{g/mL}$ (Fig.6.b). After 72 hours of treatment, the viability of the cells may decrease due to the senescence stage, continuous depletion of nutrients and accumulation of metabolic wastes [24].

3.4.2. Cell Morphological Observation

The morphology of MCF7 cells before and after treatment with majoroside F₆ was observed using an inverted microscope to investigate the effect of majoroside F₆ on the morphology of MCF7 cells and the results were shown in Figure 6. Under normal conditions, the cultured cells exhibited a polygonal or spindle-shaped morphology, adhered well to the substrate, showed uniform density and had clear cell boundaries (control, Figure 6.c). After treatment with majoroside F₆, the cells exhibited typical apoptotic characteristics, such as shedding, shrinkage and rounding. Higher concentrations of majoroside F₆ resulted in a significant reduction in cell count, loose attachment and dispersed spherical cells. Cell boundaries became unclear and individual cell characteristics were lost (Figure 6.d-e). At a concentration of 80 µg/mL (Figure 6.f), most MCF7 cells rounded up and separated with no intercellular connections. These results were consistent that majoroside F₆ induces dose-dependent morphological changes associated with apoptosis in MCF7 cells [25].



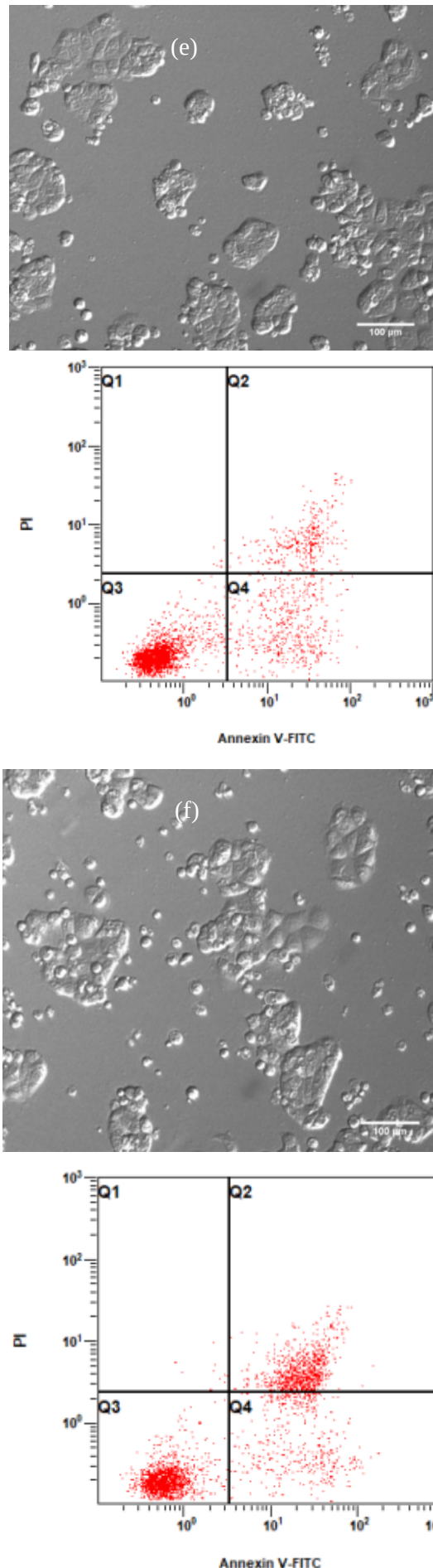


Figure 6. MTT assay for determining cytotoxicity of MCF7 cells (a) and HEK-293 cells (b). Significance levels: ** $p < 0.01$, * $p < 0.05$ compared to the control (0 µg/mL PBP). Typical morphological changes in MCF7 cells and flow cytometry analysis and annexin V-FITC staining were used to evaluate apoptosis in HCT 116 cells. Control (c), 20 µg/mL (d), 60 µg/mL (e) and 80 µg/mL (f) ($n = 3$ per group, repeated 5 times). Histograms of the apoptotic rates (g). Significance levels: ** $p < 0.01$, * $p < 0.05$ compared to the control (0 µg/mL majoroside F₆)

3.4.3. Cell Apoptosis Assay

To evaluate the apoptotic status of MCF7 cells treated with different concentrations of majoroside F₆, flow cytometry was used in combination with the Annexin V-FITC apoptosis detection kit. The cells were classified into four quadrants in the flow cytometry scattergram (Figure 6) based on the levels of red and green fluorescence [26]. In the flow cytometry scattergram (Figure 6), early apoptotic cells (Annexin V+/PI-) were located in the fourth quadrant, late apoptotic cells (Annexin V+/PI+) were located in the first quadrant, normal viable cells (Annexin V-/PI-) were located in the third quadrant and non-specific stained cells (Annexin V-/PI+) were located in the second quadrant. The total apoptotic cells were calculated as the sum of early apoptotic cells and late apoptotic cells. Flow cytometry was used for quantitative analysis of the ratio of total apoptotic cells to total cells [27]. The resulting histogram suggested that the apoptosis rate of cells was observed in Figure 6.g. Compared with the control, treatment with 40, 60 and 80 µg/mL of majoroside F₆ resulted in a higher percentage of total apoptotic MCF7 cells ($7.66 \pm 0.09\%$, $28.13 \pm 1.02\%$ and $51.14 \pm 0.57\%$). The percentage of early apoptotic cells increased from $1.34 \pm 0.79\%$ to $2.32 \pm 0.25\%$, $11.16 \pm 1.07\%$ and $35.84 \pm 1.16\%$ and the percentage of late apoptotic cells increased from $1.58 \pm 0.83\%$ to $5.34 \pm 0.57\%$, $16.97 \pm 0.23\%$ and $15.30 \pm 0.79\%$ (Figure 6) which was similar to the results of Niu, X. et al. [28]. The results indicated that majoroside F₆ had a concentration-dependent inhibitory effect on the proliferation of MCF7 cells and induces apoptosis.

3.4.4. Measurement of protein expressions

A key factor in inducing apoptosis was the regulation of the PI3K/AKT signaling pathway in tumor cells. The PIK3 gene family was crucial in the molecular mechanism of PI3K/AKT signaling which functioned through phosphorylation (activation) of downstream proteins to

controlled the apoptosis of cancer cells[29]. To investigate the mechanism of majoroside F₆-induced apoptosis, ELISA was used to analyze the expression of related proteins. As shown in Figure 7, majoroside F₆ treatment significantly upregulated caspase-3 and Bax in MCF7 cells while simultaneously inhibiting PI3K, AKT and Bcl-2 expression. Bcl-2 plays an anti-apoptotic role by preventing the release of cytochrome C, while Bax promotes apoptosis by counteracting Bcl-2 [30-31].

Therefore, majoroside F₆ could induce MCF7 cell apoptosis by upregulated downstream gene Bax and downregulated Bcl-2, leading to MMP depolarization, cytochrome C release, followed by binding with apoptotic factors, activating the caspase cascade reaction and ultimately inducing PARP cleavage and unrepaired DNA damage [32]. In summary, majoroside F₆ treatment could activate key apoptotic pathways and promote apoptosis in MCF cells.

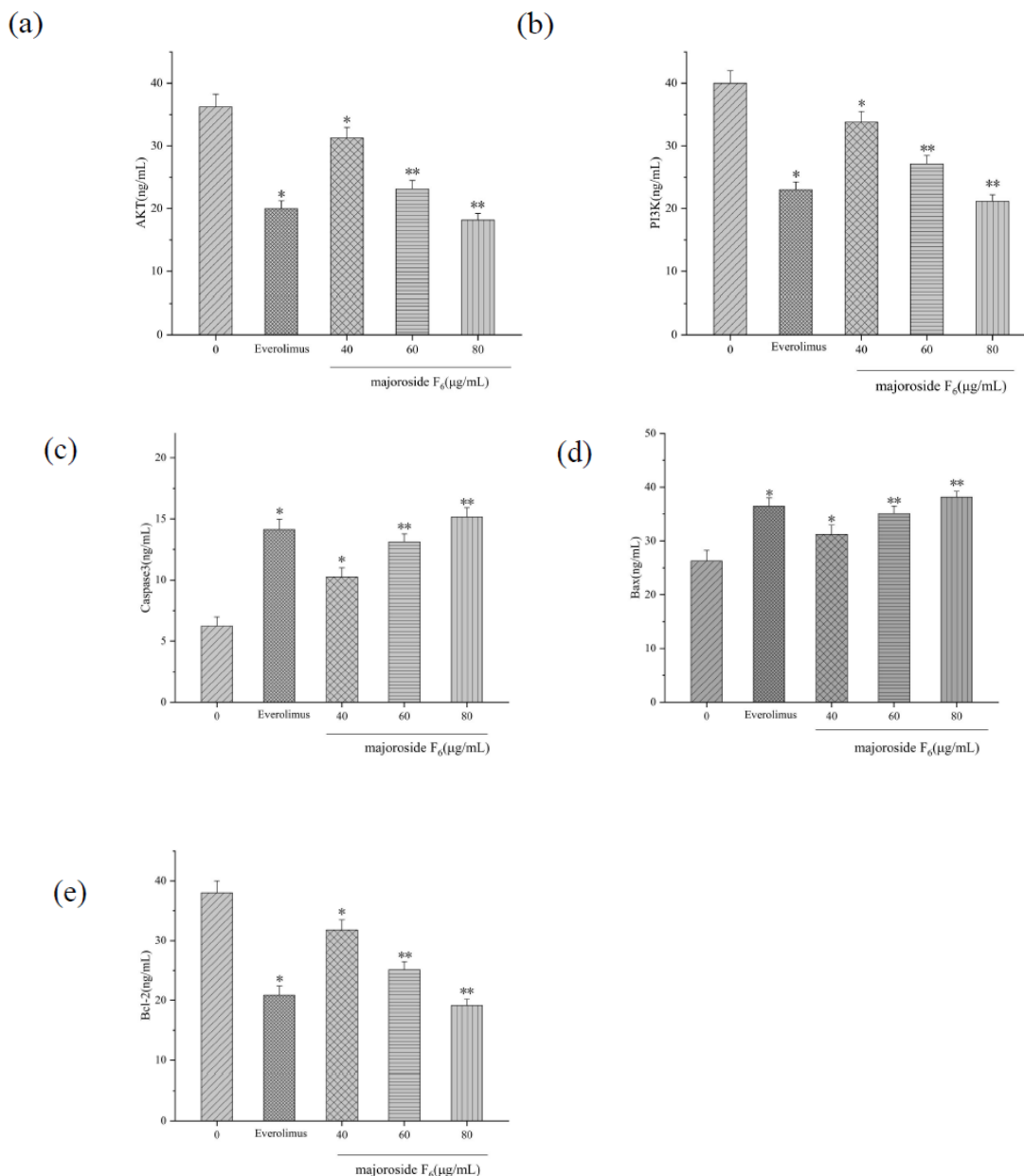


Figure 7. The effect of majoroside F₆ on the content of (a)PI3K, (b)AKT, (c)Caspase3, (d)Bax and (e)Bcl-2 in everolimus-induced MCF7 cells. Significance levels:** $p < 0.01$, * $p < 0.05$ compared to the control (0 μ g/mL majoroside F₆)

4. Conclusion

Traditional *Panax ginseng* was expensive and had a cumbersome concoction process that requires a high level of manpower and resources while *Panax quinquefolius* was affordable and had a higher content of rare saponins after a new type of concoction, it could be fully used as a substitute for ginseng. This study utilized network pharmacology to constructed a comprehensive network

consisting of Disease Drug Pathway Targets and PPI network interactions. The binding affinity and conformation of the main active ingredients of BPQRS with core targets were evaluated through molecular docking. The cell experiment further verified the above findings and predictions. Could observed that BPQRS could significantly inhibited the proliferation of human breast cancer MCF-7 cells and induced typical apoptotic cell morphological characteristics had no obvious toxicity to human normal embryonic kidney cell HEK 293. These

results confirmed that the potential therapeutic effect of BPQRS on human breast cancer. The combination of network pharmacology and cellular experiments was key to understanding the mechanisms by which BPQ treats breast cancer. First, network pharmacology provided the necessary information to screen majoroside F₆ and how it affects breast cancer cells. Second, cellular experiments validated these predictions, including the interaction between majoroside F₆ and key targets. These results suggested that majoroside F₆ was a promising potential drug for the treatment of breast cancer and was worthy of further development and research.

However, this study also had inevitable limitations that needed further exploration and improvement in future experiments. Firstly, in our laboratory, we previously identified and confirmed the presence of 33 different saponins in BPQTS through UPLC-ESI-QTOF-MS. Nevertheless, the precise quantitative distribution of other components in BPQ still needed to be elucidated. Future researchers could focus on addressing this issue by using liquid chromatography-mass spectrometry combined with reference standards to conduct comprehensive quantitative analysis of other components in BPQ. Secondly, our study only involves mechanisms related to cell death and apoptosis. Future research should further investigate whether the anti-cancer effects of black ginseng involve other biological mechanisms, such as autophagy and necrosis, as well as immune regulatory effects. Moreover, the study had not yet conducted in vivo experiments to verify the anti-tumor effect of majoroside F₆ which was another limitation. The anti-tumor effect of BPQTS on mouse breast cancer model and its possible mechanism in vivo need further study.

In conclusion, our research represented a pioneering exploration of the inhibitory effect of majoroside F₆ on breast cancer through the combination of network pharmacology and experimental verification. This study had clarified the inhibitory mechanism of majoroside F₆ on breast cancer cells. These findings provided great hope for advancing our understanding of the therapeutic potential of majoroside F₆ in the treatment of breast cancer. This study provided a solid foundation for further in-depth research on the components and in vivo experiments of BPQ. These future efforts were likely to make significant contributions to the early prevention and suppression of breast cancer, highlighting the significant prospects of natural therapy in clinical oncology.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

List of Abbreviations

Black Panax quinquefolius L.(BPQ)
Protein-protein interaction(PPI)
Electric spray ion source(ESI)
Traditional Chinese Medicine System Pharmacology (TCMSP)
Gene Ontology Consortium(GO)
Biological processes(BP)
Cellular components(CC)
Molecular functions(MF)
The protein database(PDB)
Fetal bovine serum(FBS)
Propidium iodide(PI)
Glucose residue(Glu)
Glucuronic acid residue(GlcA)
Arabinose residue(Araf)

Data availability

Data will be made available on request.

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Supporting Information

In the Supplemental files.

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