

Unveiling the Mechanisms and Therapeutic Effects of Xiaoyao Sanjie Decoction in Triple-Negative Breast Cancer: A Network Pharmacology and Experimental Validation Approach

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Purpose: Triple-negative breast cancer (TNBC) is a disease associated with high incidence and high mortality, which is a major problem threatening women's health. Xiaoyao Sanjie Decoction (XYSJD) exhibits remarkable therapeutic efficacy on TNBC; however, the underlying mechanism is unclear. This study verified the efficacy of YYSJD and its active component in the treatment of TNBC and explored its potential mechanism.

Methods: Ultra-high performance liquid chromatography-hybrid quadrupole orbitrap mass spectrometry (UHPLC-Q Exactive HFX-MS) was applied to explore the main chemical constituents of YYSJD. The key targets and potential mechanisms of YYSJD in the treatment of TNBC were predicted through network pharmacology, bioinformatics analysis and molecular docking. The effects of YYSJD against TNBC cells were evaluated by CCK-8 assay, EdU assay, wound healing assay, transwell assay, Hoechst-PI staining and flow cytometry. The mechanism of action was validated by Western blot analysis. Finally, the effect and mechanism of YYSJD and Que on TNBC were further verified by the tumor formation model.

Results: UHPLC-Q Exactive HFX-MS identified a total of 9 compounds in YYSJD. Network pharmacological methods identified 206 targets for anti-TNBC. Bioinformatics analysis suggests that the EZH2/AKT1 signaling pathway might play an important role in the effects of YYSJD against TNBC. Gene Ontology enrichment analysis showed that the biological process of YYSJD in TNBC treatment mainly involved apoptosis. YYSJD and Que were observed to have a good anticancer effect in vivo and in vitro. In addition, quercetin could induce the apoptosis of TNBC cells by decreased the expression levels of EZH2/AKT1 signaling pathway. Furthermore, AKT1 overexpression, treatment with the AKT activator (SC79) and EZH2 overexpression could reverse apoptosis induced by quercetin in TNBC cells.

Conclusion: This study revealed the anti-TNBC efficacy of YYSJD. Quercetin, the effective component of YYSJD, promoted apoptosis of TNBC cells via blockade of the EZH2/AKT1 signaling pathway. These findings aim to provide a more reliable basis for the clinical application of YYSJD in the treatment of TNBC.

Keywords: Xiaoyao Sanjie Decoction, triple-negative breast cancer, network pharmacology, quercetin, EZH2/AKT1 signaling pathway

Introduction

Breast cancer (BC) is the most common type of malignant tumor in women worldwide, and it is also the main cause of cancer death.¹ Triple-negative breast cancer (TNBC) accounts for 15–20% of the total incidence of BC.^{2,3} Compared with other subtypes of BC, TNBC exhibits lower differentiation and increased invasiveness, with approximately 46% of TNBC patients experiencing distant metastasis.^{4,5} Due to the lack of estrogen receptor (ER), progesterone receptor and human epidermal growth factor receptor 2 (HER2), TNBC patients are unable to benefit from targeted therapy against ER α or HER2.^{6,7} Currently, surgery and systemic chemotherapy are the main treatment methods for TNBC;^{8,9} however, when dealing with recurrence or metastasis, these conventional treatments do not work well.¹⁰ Therefore, compared with other subtypes of BC, TNBC patients have a shorter overall survival time.¹¹ In summary, the treatment of TNBC remains a considerable clinical challenge. However, as a vital part of complementary medicine, Traditional Chinese Medicine (TCM), which represents testable alternatives for secondary prevention or treatment of TNBC, is attracting more attention.^{12,13}

Xiaoyao Sanjie Decoction (XYSJD), also known as Ruyan Sanjie Decoction, is a prescription treatment for TNBC created by Professor Li Jiageng, a famous TCM doctor. YYSJD is composed of *Radix Bupleuri*, *Radix Paeoniae Rubra*, *Poria cocos*, *Angelica sinensis*, *Hedyotis diffusa*, *Prunella vulgaris* L., *Astragalus membranaceus*, *Curcuma zedoaria* and licorice. Many studies have shown that the basic prescription of Xiaoyao Powder not only inhibits the growth of BC, but improves the clinical symptoms of BC.^{14–17} Furthermore, compounds within YYSJD have been shown to exert anti-TNBC effects; for example, Saikosaponin D from *Radix Bupleuri* can suppress TNBC cell growth by targeting β -catenin signaling.¹⁸ Paeoniflorin, derived from *Radix Paeoniae Rubra*, has been reported to inhibit the active EMT progress in MDA-MB-231 cells induced by hypoxic conditions via suppressing the PI3K/Akt/HIF-1 α pathway.¹⁹ Isoliquiritigenin, extracted from licorice root, has been shown to have anticancer effects on TNBC cells.²⁰ According to the TCM theory, blood stasis is the main causes of tumor occurrence and development.^{21,22} Therefore, on the basis of Xiaoyao Powder, Chinese medicine with blood activating, stasis removing and anti-cancer effects, inducing *Hedyotis diffusa*, *Curcuma zedoaria* and *Prunella vulgaris* L. are added to the prescription. Methylantraquinone, extracted from *Hedyotis diffusa* can inhibit proliferation, destroy mitochondrial integrity and promote apoptosis of MCF-7 cells.²³ It has recently been reported that *Curcuma zedoaria* extract can inhibit the invasion and metastasis of TNBC.²⁴ Studies have also shown that *Prunella vulgaris* L. extract and its compounds may inhibit proliferation and promote apoptosis of BC in vitro and in vivo.²⁵ Quercetin, a flavonol prevalent in fruits, vegetables, and Chinese medicine, has demonstrated promising efficacy in the treatment of TNBC.^{26,27} More and more researches have also confirmed that quercetin is a crucial component of Xiaoyao Powder,^{28,29} and several medicinal materials in YYSJD, such as *Radix Paeoniae Rubra*, *Hedyotis diffusa*, *Prunella vulgaris* L., *Astragalus membranaceus* and licorice, contain the key anticancer ingredient quercetin.^{30–34} Therefore, we speculated that quercetin may be the main component of YYSJD in the treatment of TNBC, which will be further verified by network pharmacology and UHPLC-Q Exactive HFX-MS in subsequent experiments. However, unlike monomer compound, as a kind of TCM prescription, YYSJD has multi-component, multi-target and multi-pathway characteristics. Thus, the main component and potential mechanism of action of YYSJD treatment for TNBC remains unclear.

Network pharmacology establishes a complex network to reveal the relationship between “targets, drugs and diseases”, and helps us clarify the effective compounds of Chinese medicine and predict the potential pharmacological mechanisms from gene distribution, molecular function and signaling pathways.³⁵ In this study, network pharmacology and bioinformatics methods were used to identify the key targets and pathways of YYSJD in the treatment of TNBC, and to clarify the expression differences and clinical significance of key targets in TNBC. Molecular docking was used to judge the binding situation of the effective compound and the core targets. Finally, the anti-TNBC effect and specific mechanism of YYSJD and its active component were verified through in vitro and in vivo experiments. The present study aims to provide evidence to support the application of YYSJD in the treatment of TNBC. The detailed process of this study is shown in Figure 1.

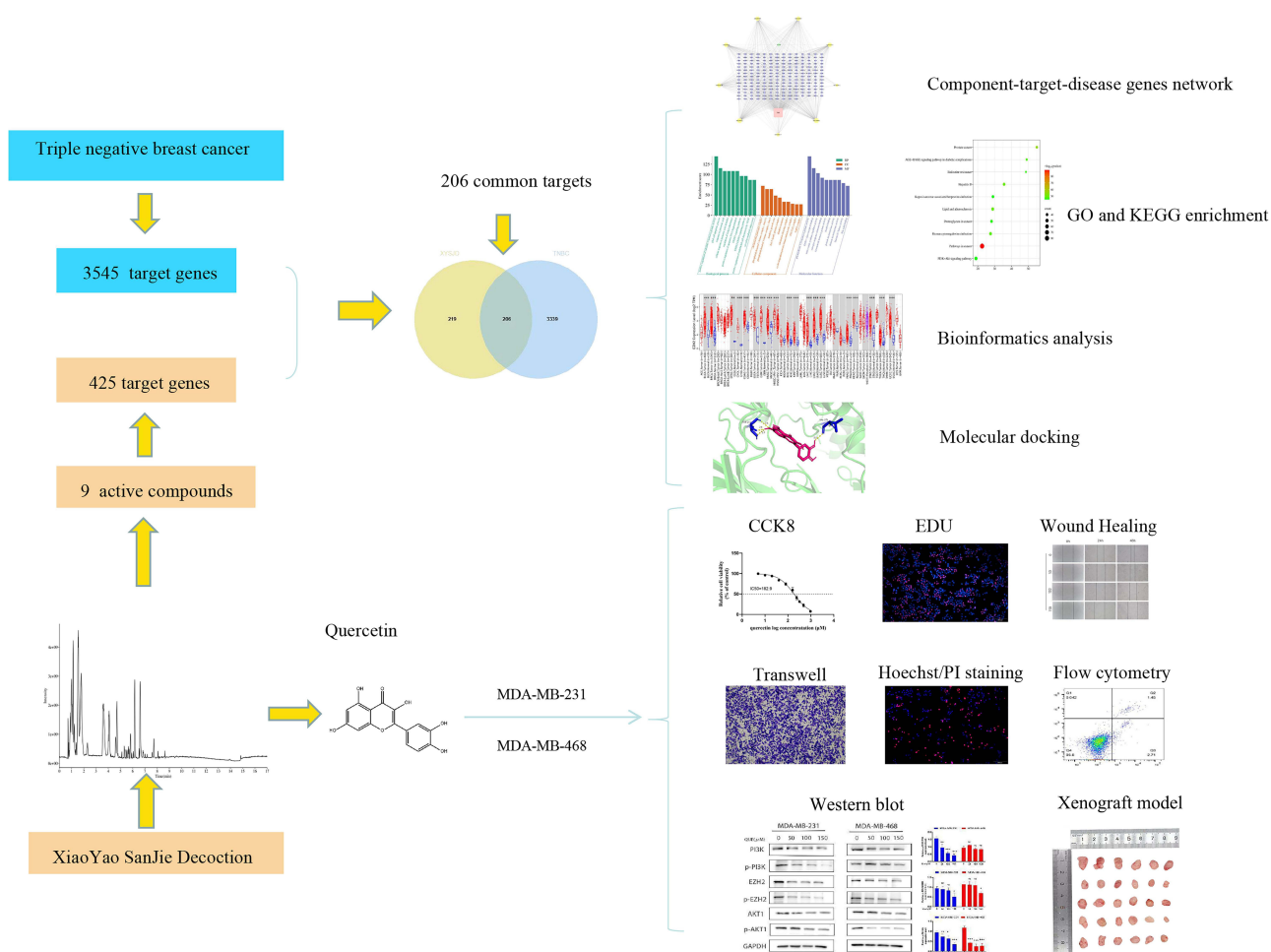


Figure 1 The flow chart of the study.

Materials and Methods

Network Pharmacology and Bioinformatics Analysis of the Active Compounds and Mechanism of XYSJD in the Treatment of TNBC

Constituents Analysis of XYSJD by UHPLC-Q Exactive HFX-MS

The XYSJD Granula (100 mg) was accurately weighed into a centrifuge tube, to which 0.1 mL precooled water was added and homogenized for 60 sec. Subsequently, 0.4 mL extraction solution (acetonitrile-methanol mixed solution, 1:1, v/v) was added, homogenized for 60 sec and extracted for 30 min by two pulses of low-temperature ultrasonication. The solution was then maintained for 1 h to precipitate the protein at -20°C , then centrifuged for 10 min at 12000 rpm and 4°C , after which, the supernatant solution was dried in a vacuum, 200 μL 30% acetonitrile solution was added to re-dissolve it, and it was homogenized and centrifuged for 15 min at 14000 rpm and 4°C ; the supernatant was collected for computer detection. The UPLC analytical conditions were as follows: column, Waters HSS T3 (100x2.1 mm, 1.8 μm); column temperature, 40°C ; flow rate, 0.3 mL/min; injection volume, 2 μL ; solvent system, phase A consisted of Milli-Q water (0.1% formic acid), phase B comprised isopropyl alcohol-acetonitrile mixed solution (0.1% formic acid); gradient program, 0 min phase A/phase B (90:10, v/v), 2 min phase A/phase B (90:10, v/v), 6 min phase A/phase B (40:60, v/v), 15 min phase A/phase B (40:60, v/v), 15.1 min phase A/phase B (90:10, v/v), 17 min phase A/phase B (90:10, v/v). High-Resolution Mass Spectrometry (HRMS) data were recorded on a Q Exactive HFX Hybrid Quadrupole Orbitrap mass spectrometer equipped with a heated ESI source (Thermo Fisher Scientific) utilizing the Full-MS-ddMS2 acquisition method. The ESI source parameters were set as follows: sheath gas pressure, 40 arb; aux gas pressure, 10 arb; spray

voltage, +3000v/-2800v; temperature, 350°C; and ion transport tube temperature, 320°C. The scanning range of the primary mass spectrometry was (scan *m/z* range) 70–1050 Da, with a primary resolution of 70000 and a secondary resolution of 17500. The raw MS data were acquired on the Q Exactive HF-X Hybrid Quadrupole-Orbitrap using Xcalibur 4.1 (Thermo Scientific), and processed by Progenesis QI (Waters Corporation, Milford, USA) for baseline filtering, peak recognition, retention time correction and peak alignment; finally, a data matrix was obtained for retention time, mass charge ratio and peak intensity. Identification of peaks containing MS2 data was performed using the self-built secondary mass spectrometry database of TCM by San Shu Biology. The parameter was set to MS1 with a difference of less than 15 ppm and MS2 fragment similarity score greater than 0.7.

Identification of the Active Ingredients in XYSJD and the Related Targets

In order to identify whether by UHPLC-Q Exactive HFX-MS identification of compounds with biological activity, we set a threshold of OB $\geq 30\%$ ³⁶ and DL ≥ 0.18 ³⁷ through the TCMSP database (<https://tcmssp.com/tcmssp.php>) to screen the active ingredients. In addition, the SMILES structures of each active ingredient were obtained using the PubChem database (<https://pubchem.ncbi.nlm.nih.gov/>). The SMILES structures were then put into the SwissTargetPrediction platform (<https://www.swisstargetprediction.ch/>) for target prediction. The species was set to “Homo sapiens” via the UniProt database (<https://www.uniprot.org/>) and the target names were converted into the standard gene ID names.

Potential Targets for XYSJD in the Treatment of TNBC

“Homo sapiens” was used as the search target, and “Triple-negative breast cancer” was used as the key word to search the GeneCards database (<https://www.genecards.org/>) and OMIM databases (<https://www.omim.org/>). Jvenn (<http://www.bioinformatics.com.cn/static/others/jvenn/example.html>) was used to screen for overlapping XYSJD compound targets and TNBC-related targets. The potential targets for XYSJD treatment of TNBC were thus obtained.

Construction of the XYSJD-Compound-Target-TNBC and Protein-Protein Interaction (PPI) Network

In order to clarify the potential interactions between TNBC target genes and XYSJD compounds, the active compounds and intersection targets were imported into Cytoscape 3.8.0 software to construct the “XYSJD-compound-target-TNBC” network. To further analyze the interaction between targets, the overlapping target proteins of XYSJD and TNBC were imported into the STRING platform (<https://string-db.org/>). After relevant score screening, the TSV document of string-interactions was visualized using Cytoscape. The plug-in cytoHubba was used to further delete and select important nodes in an interactome network using several topological algorithms and to identify central proteins of the network.

Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) Pathway Enrichment Analyses

The common target genes were put into Metascape (<https://metascape.org/gp/index.html#/main/step1>), $P < 0.01$ and FDR < 0.01 were recognized as significant of GO and KEGG Enrichment. The results were analyzed and visualized using the bioinformatics free online platform (www.bioinformatics.com.cn).

Related Gene Expression and Prognostic Analysis

The expression differences of AKT1 and EZH2 in different tumor tissues and adjacent tissues were analyzed by TIMER2.0 (<http://timer.comp-genomics.org/>). The correlation of AKT1 and EZH2 was evaluated using the GEPIA2 database (<http://gepia2.cancer-pku.cn/#index>). The relative expression of AKT1 and EZH2 in BC subtypes, their expression in TNBC, and their relationship with disease stage and distant metastasis were analyzed through the UALCAN (<https://ualcan.path.uab.edu/cgi-bin/ualcan-res.pl>) and GSE65194 dataset. Furthermore, the relationship between gene expression and prognosis was evaluated in the GSE21653 dataset, and a Kaplan-Meier survival curve was drawn, indicating that the survival rate ($P < 0.05$) was statistically significant. The experiment was approved by Institutional Review Board of Hubei University of Science and Technology, and the IACUC approval number is IACUC-202410001.

Molecular Docking Validation

Through the degrees of targets in the PPI network and the results of the KEGG pathway analysis, we selected AKT1 (PDBID:3O96) and EZH2 (PDBID:4MI5) as the key protein receptors, and downloaded the 3D structures from the

Protein Data Bank database (<http://www.rcsb.org>). We used PyMOL3.8 software to dehydrate and deligate the proteins. According to the characteristic parameters of the network analysis, Que was selected as a ligand and its mol2 format was downloaded from TCMSP. Subsequently, we used AutoDock Tools to perform hydrogenation and charge calculation, and used AutoDock Vina (<http://vina.scripps.edu/>) to calculate the binding ability. Finally, we applied PyMOL to visualize their three-dimensional structures.

Experimental Verification

Drug and Rat Serum Preparation

All herbal medicine granules in YYSJD were purchased from Hubei Tianji Pharmaceutical Co., Ltd. (Hubei, China). The concentration of herbs in YYSJD is listed in Table 1. The granules were fully dissolved in an appropriate amount of boiled distilled water and concentrated to 2 g/mL. In this trial, we selected female Sprague Dawley rats (age, 8 weeks; weight, 250±20 g), which were purchased from Hubei Beiente Biotechnology Co., Ltd. (SCXK-2021-0027), and were kept in the institutional animal facility under standard animal room conditions (25°C, 12-h light-dark cycle, 55% humidity, 3 animals/cage). The rats were randomly divided into two groups (YYSJD and control) and fed adaptively for 1 week. According to the body surface area conversion ratio of humans and rats, the YYSJD group received 6.0 g/kg YYSJD by gavage twice a day for 3 days while the control group was treated with an equal volume of distilled water. On the 4th day, 1 h after the last gavage, all of the rats were intraperitoneally anesthetized by 2% pentobarbital sodium (30 mg/kg). Blood was collected from the abdominal aorta and maintained at room temperature for 1 h, and the rats were sacrificed by cervical dislocation. After centrifugation at 3000 rpm for 15 min, the upper serum was extracted and inactivated at 56°C, filtered through a 0.22-μm filter and stored at -80°C. Animal experiment was approved by the formal review of experimental animal ethics at Huazhong university of Science and Technology. And the IACUC approval number is IACUC-2022-3324. Animal experiments strictly abide by the national laboratory animal-related laws, regulations and standards, including but not limited to “Guidelines for Ethical Review of the welfare of laboratory animals GB/T35892”, “International Guiding Principles for Biomedical Research Involving Animals”.

Cells and Reagents

The human TNBC cell lines MDA-MB-231 and MDA-MB-468 were purchased from Wuhan Pricella Biotechnology Co., Ltd. (China). Que (purity ≥95%; SQ8030) was purchased from Beijing Solarbio Science & Technology Co., Ltd. (Beijing, China). SC-79 was purchased from Beyotime (Shanghai, China). The cells were maintained in DMEM (Servicebio, China) containing 10% FBS (MeiSenCTCC, China) and were incubated at 37°C in a humidified incubator containing 5% carbon dioxide. Cell line morphology was assessed periodically.

Cell Viability Assay

MDA-MB-231 and MDA-MB-468 cells were diluted with complete culture medium to 4×10⁴ cells/mL and were inoculated into 96-well plates for 12 h. Different concentrations of YYSJD (0%, 5%, 10%, 15%, 20%) were used on TNBC cells for 48 h and 72h, different concentrations of blank rat serum (20%, 15%, 10%, 5%, 0%) were added to make

Table 1 Herbal Formula of YYSJD

Latin Name of the Medicinal Material	Material	Chinese Name	Dose (g)
Radix bupleuri	root	Chai Hu	10
Radix Paeoniae Rubra	root	Chi Shao	10
Poria cocos	sclerotium	Fu Ling	10
Angelica sinensis	root	Dang Gui	10
Hedyotis Diffusa	herb	Bai Hua She She Cao	15
Prunella vulgaris L	fruiting spikes	Xia Ku Cao	10
Astragalus Membranaceus	rootstalk	Huang Qi	10
Curcuma zedoaria	rootstalk	EZhu	15
Licorice	root and rootstal	Gan Cao	6

the serum concentration to 20% in all groups. Following the same procedure as aforementioned to inoculate the cells into plates and treated with Que (0, 5, 10, 20, 40, 80, 160, 240, 320, 480 and 960 μ M). After 48 h, 10 μ L Cell Counting Kit (CCK)-8 solution (Beyotime, China) was added to each well and cultured in an incubator for 1 h. Absorbance was measured at 450 nm with a microplate reader (PerkinElmer, EnSpire, USA).

EdU Assay

MDA-MB-231 and MDA-MB-468 cells were inoculated into 96-well plates and treated with Que (0, 50, 100 and 150 μ M) for 24 h. According to the instructions of the BeyoClick™ EdU Cell Proliferation Kit with Alexa Fluor 594 (Beyotime, China), the cells were cultured with 10 μ M EDU for 2 h, immobilized with 4% paraformaldehyde fixative, and permeabilized with 0.3% Triton X-100. Then, 70 μ L of click reaction mixture was added to each well and incubated for 30 min at room temperature in the dark. Finally, Hoechst 33258 (Beyotime, China) was used to stain the nuclei of all cells for 10 min at room temperature in the dark. Images of the stained cells were captured by fluorescence microscopy (IX51, Olympus, Japan).

Wound Healing Assay

MDA-MB-231 and MDA-MB-468 cells were added to 12-well plates and were placed overnight in an incubator. We then used a 200- μ L pipette tip to scrape a straight line into the cells, which were treated with different concentrations of Que (0, 50, 100 and 150 μ M) in a medium containing a reduced percentage of serum (5%). The wounds were observed and captured under an inverted fluorescence microscope at 0, 24 and 48 h.

Transwell Assay

MDA-MB-231 and MDA-MB-468 cells were added to 12-well plates, treated with different concentrations of Que (0, 50, 100 and 150 μ M) for 24 h, digested with pancreatic enzyme and counted. For the migration assay, 3×10^4 cells/well of MDA-MB-231 cells were inoculated into the upper chamber in serum-free medium, and complete medium containing 10% FBS was added to the lower chamber. However, since it is relatively difficult to induce migration of MDA-MB-468 cells, we inoculated 4×10^4 cells/well of this cell line into the upper chamber, and increased the serum concentration in the lower chamber to 20%. After culturing in an incubator for 24 h, we cleaned the chamber with PBS, and fixed and stained the cells with 4% paraformaldehyde and 0.1% crystal violet, respectively. Then the remaining cells in the upper chamber were cleaned with cotton swabs. The migrating cells were observed and photographed under a microscope. For the invasion assay, the serum-free medium was diluted Matrix-Gel™ Basement Membrane Matrix (Beyotime, Hubei, CHN) on ice in a ratio of 1:3 was seeded in the upper chamber of the transwell insert and allow it to solidify for 3 hour at 37°C. The subsequent procedure is the same as the transwell cell migration experiment.

Cell Apoptosis Assay

Apoptosis was detected using the Annexin V-FITC apoptosis detection kit (Beyotime, China). After MDA-MB-231 and MDA-MB-468 cells in 6-well plates were treated with different concentrations of Que for 48 h, they were digested with pancreatic enzymes and resuspended in 500 μ L 1X binding buffer. Subsequently, 5 μ L Annexin V-FITC and 5 μ L PI staining solution was added to the cells, which were shielded from the light for 15 min at room temperature. The samples were then collected and detected using a CytoFLEX flow cytometer (FACS Aria™ II; BECTON DICKINSON, USA).

Hoechst/PI Staining Assay

MDA-MB-231 and MDA-MB-468 cells were inoculated in 48-well plates, and were treated with Que at different concentrations for 48 h. Then, the medium was removed, and the cells were washed with PBS, and stained with PI and Hoechst 33342 (Beyotime, China) in the dark at room temperature for 15 min, respectively. Fluorescence images were taken under an inverted microscope.

Plasmid and siRNA Transfection Assay

MDA-MB-231 and MDA-MB-468 cells were transfected with the pcDNA3.1(+)-EZH2 expression vector, the pcDNA3.1 (+)-control vector (Sangon Biotech (Shanghai) Technology Co. Ltd., China) or the AKT1 siRNA (Wuhan Qijinnong Biological Technology Co., Ltd., China) using Lipofectamine 3000 reagent (Invitrogen) and OptiMEM (Gibco),

according to the manufacturers' instructions. After 6 h, the transfection medium was replaced with fresh medium and the cells were incubated for 24 h before drug treatment. The transfection efficiency was verified by reverse transcription-quantitative (RT-qPCR) and Western blotting. The transfected cells were then used for flow cytometry and Western blot analysis.

RNA Extraction and RT-qPCR Analysis

After transfection for 24 h, the total RNA of MDA-MB-231 and MDA-MB-468 cells was extracted using Trizol reagent and the RNA concentration was measured using a NANODROP ONE^c (Thermo, USA). The RT of RNA was performed using Primescript RT master mix (Takara Bio Inc., China) to synthesize cDNA, and qPCR was performed using the StepOnePlus system (Applied Biosystems, USA). According to the manufacturer's protocol, β -actin was used as the internal control gene, and the relative expression levels of EZH2 and AKT1 were calculated using the relative quantification ($2^{-\Delta\Delta C_t}$) method. Each experiment was conducted in triplicate. The primers were synthesized by Shanghai Sangon Biotechnology (Shanghai, China) and the sequences are listed in Table 2.

Western Blot Analysis

After transfection or treatment with various concentrations of Que for 48 h, RIPA lysis buffer (Servicebio, China) and PMSF (Servicebio, China) were added to lyse the TNBC cells, and the supernatants were collected after centrifugation. The protein concentration was measured using a BCA Kit (Beyotime, China). After adding 10 μ g protein sample to each lane, the proteins were separated by SDS-PAGE (Servicebio, China). According to the molecular weight of the proteins and the displayed position, the appropriate size of the NC membrane was cut. Subsequently, the separated proteins were transferred onto the membrane, which was blocked in 5% nonfat skim milk for 1 h, and then incubated with the following primary antibodies: PI3K p110 beta, phosphorylated (p)-PI3K p85 α , AKT1, p-AKT1 (Abways, China, dilution 1:1000), EZH2 and p-EZH2 (ABclonal, China, dilution 1:1000), GAPDH (Abways, China, dilution 1:3500) overnight at 4°C. The next day, the membranes were washed with TBST 3 times, and goat anti-rabbit IgG (Abways, China, dilution 1:10000) was added for 1 h, followed by repeated cleaning with TBST 3 times (10 min each time). Finally, the gray value of each protein band was determined using a gel imaging system (ChemiScope 6200; CLINX, China). GAPDH was used as an internal reference protein and ImageJ software win64.exe was used to analyse the results to compare the relative expression changes of target proteins.

Animal Experiments

A total of 30 BALB/c-nu mice (4 weeks old, 18–22 g, female) were provided by Shulaibao (Wuhan) Biotechnology Co., Ltd. (Hubei, China). After seven days of adaptive feeding, 5×10^6 MDA-MB-231 cells were suspended in a mixture of 50 μ L PBS and 50 μ L Matrix-Gel™ Basement Membrane Matrix and injected into the right flanks of the mice to establish a xenograft mouse model. Once the tumors reached a 100 mm³, the mice were randomly divided into five groups: Control (saline 0.1 mL/kg), Cisplatin (DDP) 2 mg/kg, XYSJD 0.1 mL/kg, Que 80 mg/kg, Que 40 mg/kg groups. Saline, or XYSJD were given via gavage once a day for 21 days, Cisplatin was given via injected intraperitoneally every two days, and Que was given via intraperitoneal injection once a day for 21 days. Body weight and tumor volume were measured every three days. The mice were sacrificed by cervical dislocation 12 hours after the last dose. The tumors were

Table 2 The List of Primers and siRNA Sequence

Gene	Forward Primer	Reverse Primer
β -ACTIN	CATGTACGTTGCTATCCAGGC	CTCCTTAATGTCACGCACGAT
EZH2	CAGTTCGTGCCCTTGTTGATAG	CTCTCGACAGCCAGGTAGC
AKT1	AGGATGTGGACCAACGTGAGG	GCAGGCAGCGGATGATGAAG
Sequences for siRNA		
AKT1	CUAUGGCGCUGAGAUUGUGUCdTdT	GACACAAUCUCAGCGCCAUAAGdTdT

removed, photographed and weighed. The liver and kidney of nude mice were removed, fixed with 4% paraformaldehyde, and stained with hematoxylin-eosin (H&E) according to standard protocols.

Statistical Analysis

The experimental data are presented as the mean $\pm$ standard deviation. GraphPad Prism 10.1.2 was used to analyze the data; Student's *t*-test was used to compare between the two groups. And one-way ANOVA was used to analyze differences between more than two groups using one-way analysis of variance. The significance levels were set at $P < 0.001$, $P < 0.01$ and $P < 0.05$.

Results

Active Compounds and Potential Mechanisms of XYSJD in the Treatment of TNBC

According to the UHPLC-Q Exactive HFX-MS analysis results (Figure 2) (Supplementary Table), combined with the relevant OB $\geq 30\%$ and DL ≥ 0.18 conditions for screening, we obtained 9 compounds in XYSJD (Table 3) and 425 corresponding targets, and applied them to further analysis. We searched the GeneCards and OMIM platforms to eliminate duplicate targets; a total of 3545 TNBC target genes were identified. XYSJD compounds and potential targets

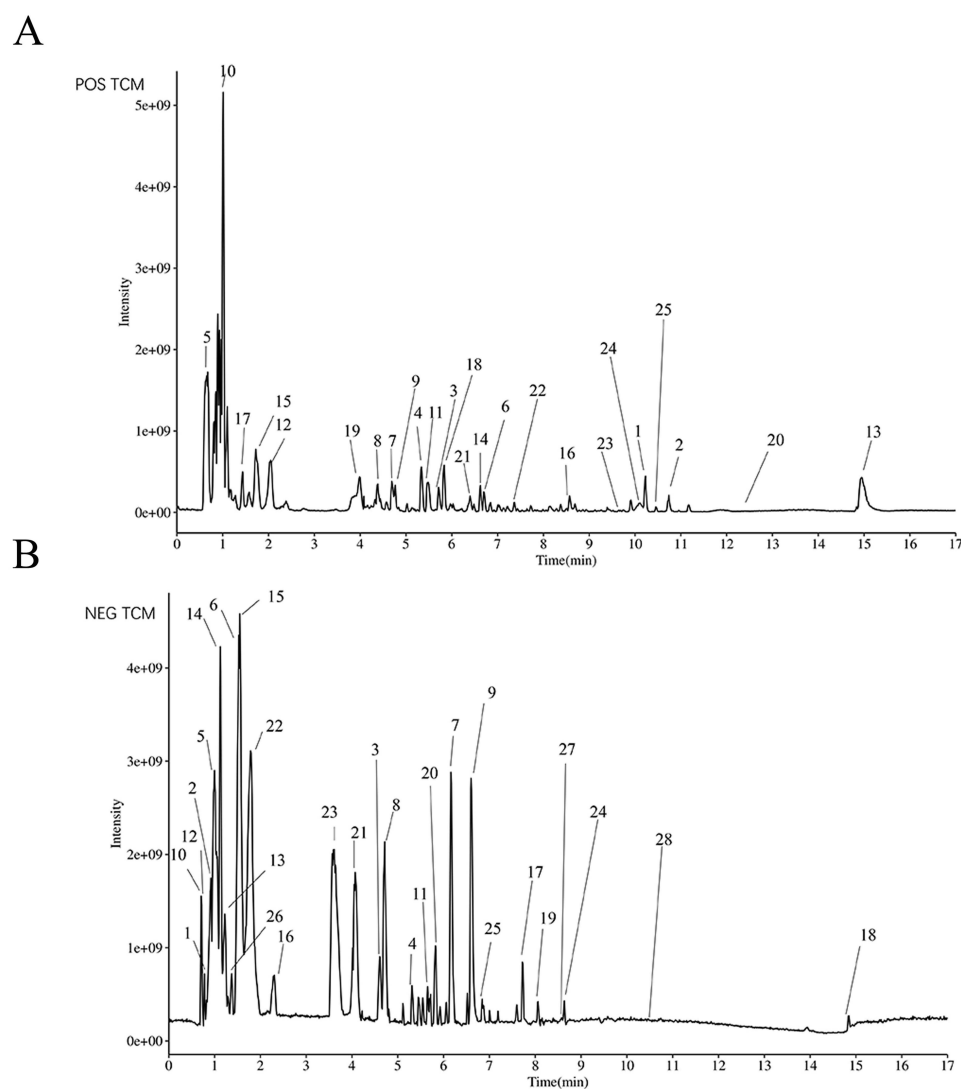


Figure 2 The total ion chromatograms of the XYSJD by UHPLC-Q Exactive HFX-MS. (A) The positive-ion mode; (B) The negative-ion modes.

Table 3 9 Bioactive Compounds of XYSJD

MOL ID	Metabolite	Retention time(min)	OB(%)	DL	Degree
MOL000098	Quercetin	5.614	46.43	0.28	82
MOL000422	Kaempferol	7.970	41.88	0.24	75
MOL002714	Baicalein	6.475	33.52	0.21	57
MOL002565	Medicarpin	8.743	49.22	0.34	55
MOL000006	Luteolin	9.672	36.16	0.25	55
MOL004908	Glabridin	10.386	53.25	0.47	54
MOL000279	Cerevisterol	12.373	37.96	0.77	50
MOL000417	Calycosin	7.323	47.75	0.24	23
MOL004912	Glabrone	10.439	52.51	0.50	6

of TNBC were input into Jvenn, and 206 potential targets of XYSJD for the treatment of TNBC were identified (Figure 3A). We constructed a network to demonstrate the correlations between the compounds and targets (Figure 3B), consisting of 217 nodes and 663 edges. By further calculating the degree value, we determined that Que (degree value=82) was one of the compounds of XYSJD that may play an important role in the treatment of TNBC. We put the overlapping targets into the STRING database to build a PPI network (Figure 3C). After using the Cytoscape plugin cytoHubba, we obtained the top 10 key genes (Figure 3D) as the key targets for TNBC treatment, among which the degrees of TP53 and AKT1 were the highest.

In order to further understand the mechanism of XYSJD against TNBC, we used Metascape for enrichment analysis. The results showed that the enriched biological processes of GO mainly included positive regulation of apoptotic DNA fragmentation, etc. (Figure 3E). Combined with the degree of the top 10 hub genes, we screened out apoptosis-related genes, such as BCL2 and TP53, and we speculated that the effective compounds in XYSJD might play a therapeutic role by inducing TNBC cell apoptosis, which was further verified by relevant experiments. Subsequently, the results of KEGG

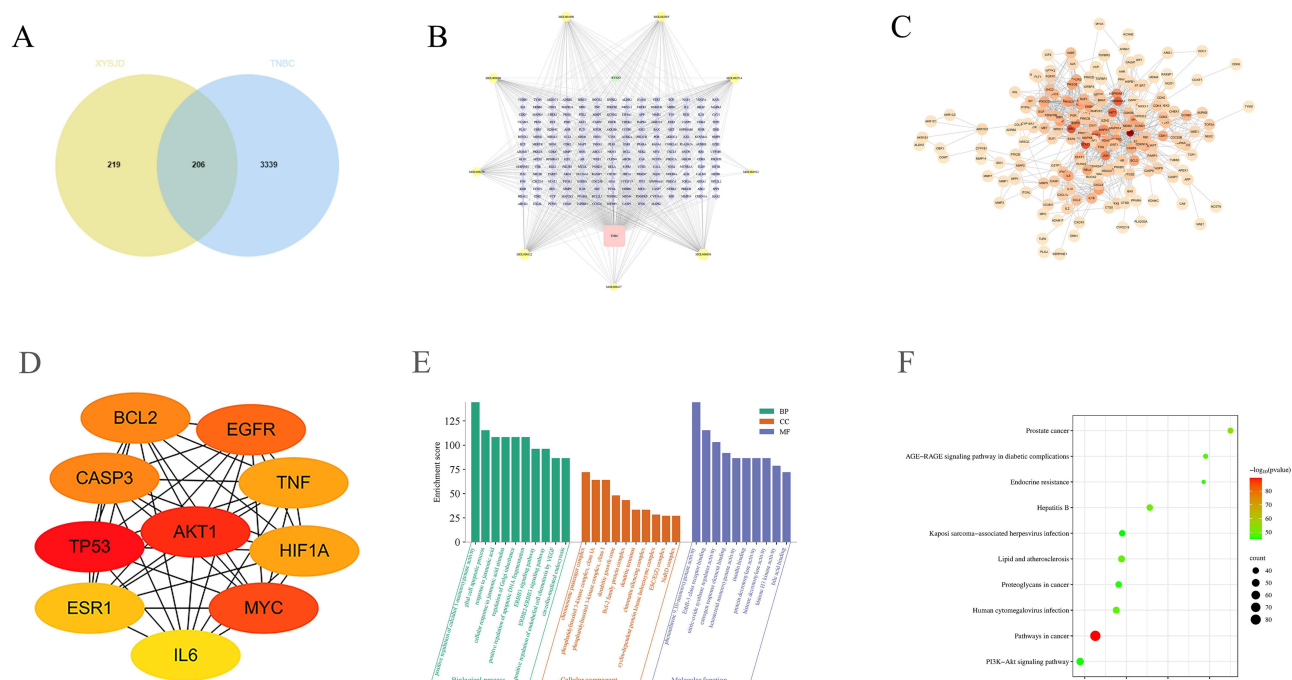


Figure 3 Network pharmacology predicted the possible active components and mechanisms of XYSJD's anti-TNBC effect. (A) Venn analysis of the targets for the treatment of TNBC with XYSJD; (B) compound-target network of XYSJD against TNBC; (C) PPI network. The color of the nodes are set according to the betweenness centrality, the brighter the color, indicating that the target is the core gene. (D) The top 10 hub genes of XYSJD against TNBC; (E) GO enrichment analysis include biological processes (BP), cellular compartments (CC), and molecular functions (MF); (F) KEGG enrichment analysis of the detailed pathways.

pathway analysis showed that TNBC-related pathways included Tumor pathway, PI3K-Akt signaling pathway, etc. (Figure 3F). Combined with the previous cytoHubba results, AKT1 had a high degree value, suggesting that the PI3K/AKT1 signaling pathway may play an important role in the treatment of TNBC with XYSJD.

Analysis of Key Gene Expression and Prognosis

Through the TIMER database, we found that compared with in paracancerous tissues, EZH2 and AKT1 expression levels were elevated in most tumor tissues, especially in BC, thyroid cancer and thymoma (Figure 4A). In the GEPIA database, we found that EZH2 and AKT1 were positively correlated in TNBC (Figure 4B). Through the UALCAN and GSE65194 dataset, compared with in other subtypes of BC and paracancerous tissues, AKT1 and EZH2 expression levels were elevated in TNBC tissues (Figure 4C and D). We also found that TNBC patients with high expression of EZH2, AKT1 were more likely to progress to advanced stages (Figure 4E), and high expression of AKT1 were more likely to have nodal metastasis status (Figure 4F). We evaluated the relationship between gene expression and patient outcomes by analyzing the GSE21653 dataset; the Kaplan-Meier curve clarified that high expression of AKT1 was associated with significantly poorer survival. By contrast, high expression of EZH2 was not significantly correlated with TNBC survival (Figure 4G). Based on these results, both AKT1 and EZH2 are considered important genes in TNBC progression; therefore, we selected AKT1 and EZH2 as key targets to conduct subsequent molecular docking verification.

Molecular Docking Verification

According to the results of the molecular docking analysis, it was revealed that Que had a strong binding affinity with the core target proteins AKT1 and EZH2. The binding energies were -9.6 and -8.3 kcal·mol $^{-1}$; since both were ≤ -7.0 kcal·mol $^{-1}$, this implied that the core targets displayed a strong affinity to Que (Figure 4H).

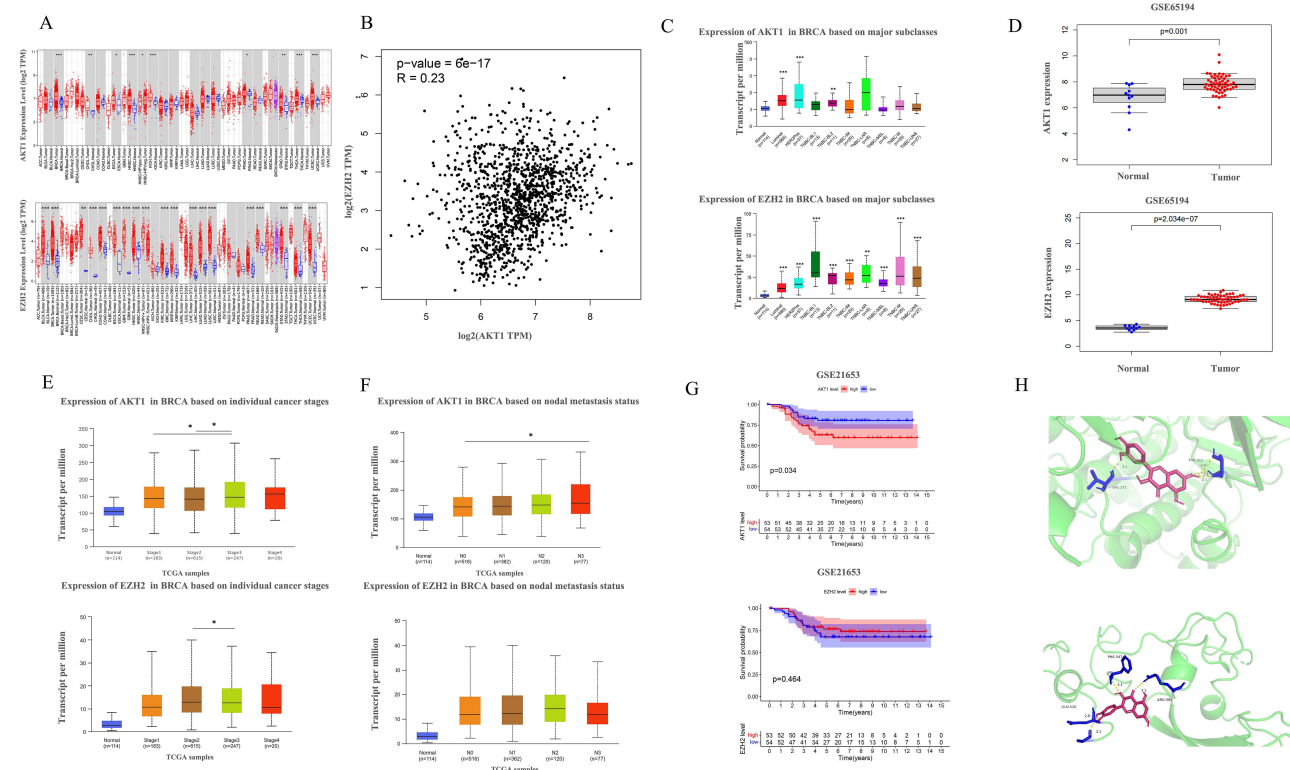


Figure 4 Expression, clinical significance, prognostic analysis of target genes and molecular docking verification. (A)Pan-cancer AKT1 and EZH2 expression, as analyzed with TIMER (* $p < 0.05$, *** $p < 0.001$); (B)Expression of AKT1 and EZH2 in BRCA based on major subclasses (with TNBC types),as analyzed with UALCAN (* $p < 0.05$,** $p < 0.01$, *** $p < 0.001$); (C)AKT1 and EZH2 correlation analysis, as analyzed with GEPIA2; (D)Different expression of AKT1 and EZH2 between TNBC tissues and adjacent normal tissues in GSE65194; (E)Expression of AKT1 and EZH2 in BRCA based on individual cancer stages, with UALCAN (* $p < 0.05$); (F)Expression of AKT1 and EZH2 in BRCA based on nodal metastasis status; (G)KM survival analysis of high- and low-expression groups in GSE21653; (H)Molecular docking of Que with AKT1 and EZH2.

XYSD Drug-Containing Serum Significantly Inhibits the Proliferation of TNBC Cells

After 48 and 72 h of intervention, compared with in the control group, 10, 15 and 20% XYSD drug-containing serum inhibited the proliferation of MDA-MB-231 and MDA-MB-468 cells, and the difference was statistically significant ($P < 0.01$), the inhibitory effect was gradually enhanced with the increase in serum concentration and intervention time. The results showed that 5% drug-containing serum had an inhibitory effect on TNBC cells at 48 h, but the difference was not statistically significant. At 72 h, 5% could effectively inhibit the proliferation of TNBC cells ($P < 0.05$; Figure 5A). These results indicated that the XYSD drug-containing serum could significantly inhibit the proliferation of TNBC cells in a dose-dependent manner.

The Active Ingredient Que Significantly Inhibits the Proliferation of TNBC Cells

After TNBC cells were treated with Que (Figure 5B) for 48 h, the calculated half maximal inhibitory concentration (IC₅₀) values of MDA-MB-231 and MDA-MB-468 cells were 182.9 and 175.9 μ M, respectively (Figure 5C). According to the IC₅₀ values, the subsequent dosing concentrations of Que were set to 0, 50, 100 and 150 μ M. As determined using the EdU assay, we found that, compared with in the blank group, the EdU-labeled bright red fluorescence of the Que group (100 and 150 μ M) was gradually reduced (Figure 5D), which indicated that the proportion of proliferating cells/total cells was decreased with the increase in dosage. These results indicated that Que could significantly inhibit the proliferation of TNBC cells in a concentration-dependent manner.

Que Significantly Inhibits the Migration of TNBC Cells

Compared with in the blank group, the wound healing area of cells treated with Que (50, 100 and 150 μ M) for 24 and 48 h tended to decrease, and the migratory ability of cells decreased (Figure 5E). As determined by the transwell migration and invasion assays, we also found that compared with in the blank group, infiltration of the lower chamber were reduced in the cells treated with different concentrations of Que (Figure 5F). These results indicated that the migration and invasion ability of TNBC cells were significantly decreased after treatment with Que in a concentration-dependent manner.

Que Significantly Induces the Apoptosis of TNBC Cells

In the Hoechst/PI staining assay, more fragmented, brighter blue fluorescence (Hoechst) was observed in the Que group (100 and 150 μ M) compared with in the blank group. In addition, the number of cells stained with red (PI) fluorescence gradually increased, and accrual of both dyes was demonstrated in the cells (Figure 5G). These results indicated that the level of TNBC cell apoptosis was increased after Que treatment. As determined by flow cytometry, we also observed that the apoptotic rate of TNBC cells was significantly increased in response to the increase in Que concentration compared with in the blank group (Figure 5H). These results indicated that Que could induce the apoptosis of TNBC cells in a concentration-dependent manner.

Que Blocks the EZH2/AKT1 Signaling Pathway in TNBC Cells

Compared with in the control group, the protein expression levels of p-EZH2/EZH2 and p-AKT1/AKT1 in TNBC cells were significantly reduced in a dose-dependent manner after treatment with Que. However, the protein expression levels of p-PI3K/PI3K were decreased in MDA-MB-231 cells, but were not significantly changed in MDA-MB-468 (Figure 5I). Furthermore, following incubation with the pan-Akt activator SC79, phosphorylation of PI3K and AKT1 was upregulated, while in the Que + SC79 group, the inhibition of p-PI3K/PI3K and p-AKT1/AKT1 in MDA-MB-231 cells induced by Que was reversed, and the inhibition of p-AKT1/AKT1 in MDA-MB-468 cells by Que could also be reversed (Figure 6B). The transfection efficiency of AKT1 and EZH2 were determined by RT-qPCR and Western blotting analysis. The expression of AKT1 and EZH2 were increased in the overexpression group (Figure 6A). Notably, through Western blotting experiments, we also found that the expression levels of p-AKT1/AKT1 in TNBC cells were increased in the EZH2 overexpression group compared with those in the control group. Overexpression of EZH2 could activate p-PI3K/PI3K protein expression in MDA-MB-231 cells, but not in MDA-MB-468 cells, suggesting that overexpression of EZH2 could activate the AKT1 signaling pathway of TNBC cells, but maybe not through PI3K in MDA-MB-468

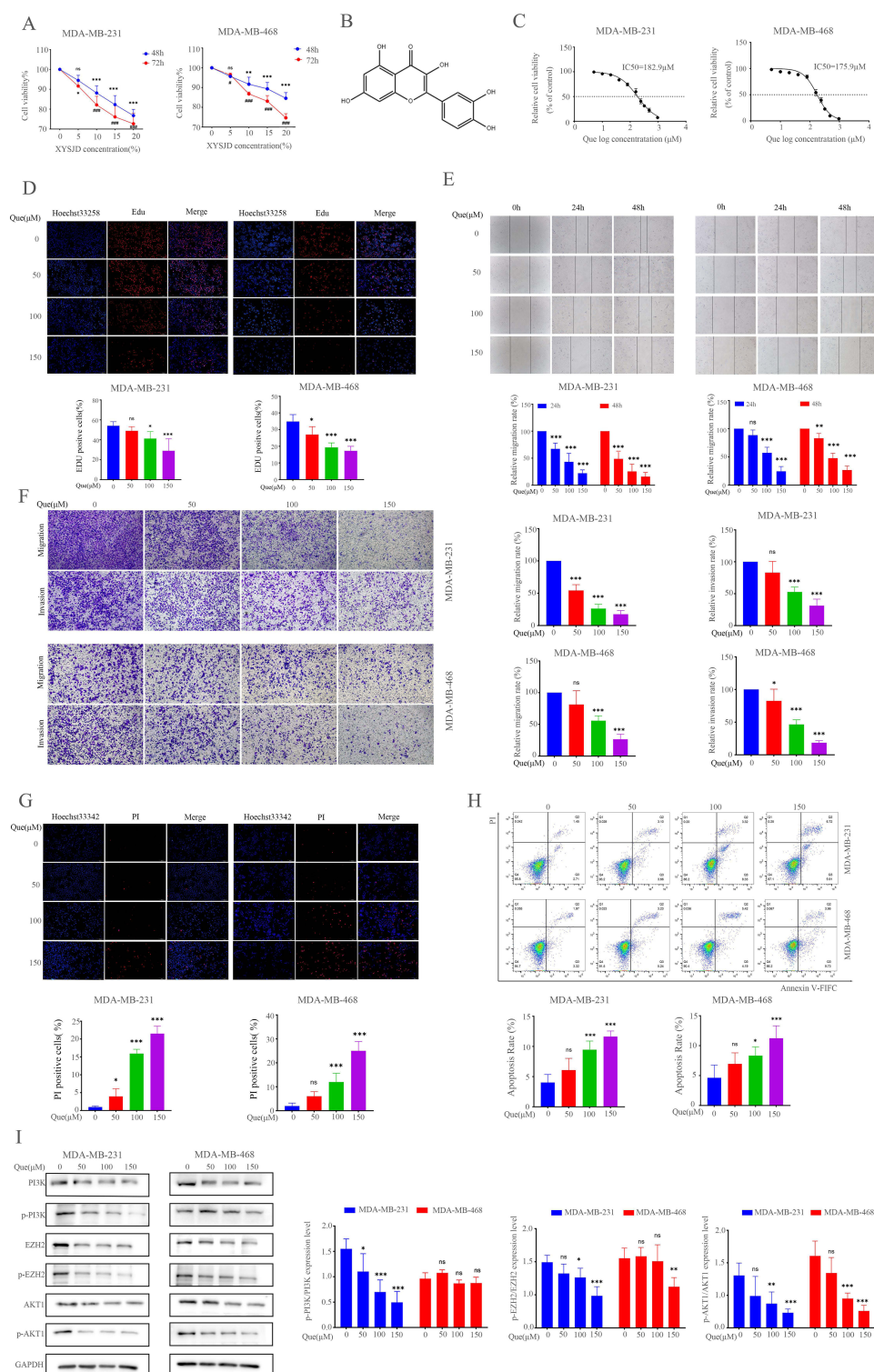


Figure 5 Que inhibited TNBC cells proliferation and migration, induced apoptosis and reduced the expression of EZH2/AKT pathway. **(A)** Different concentrations of XYSD (0%, 5%, 10%, 15%, 20%) treated TNBC cells for 48h and 72h, CCK-8 assay was used to detect cell viability; **(B)** A Molecular structure of Que; **(C)** CCK-8 examination of the viability of Que-treated TNBC cells. Data were normalized by the mean value of the 0μM group; **(D)** Edu staining assay was performed to detect the DNA replication ability of TNBC cells after Que (0, 50, 100, and 150 μM) treatment for 48 h. Red fluorescence indicated DNA replication in the cells. Blue, nuclei (Hoechst 33258); Red, EdU-positive cells; **(E)** Wound healing assay **(F)** and Transwell assay **(F)** were performed to assessed with the migration abilities of TNBC cells after Que treatment for 24 h and 48h; Apoptosis assays of TNBC cells after Que treatment for 48h were detected via Hoechst/PI staining assay **(G)** and flow cytometry in annexin V-FITC/PI stained **(H)**; **(I)** Western blot analyses were performed for PI3K, p-PI3K, EZH2, p-EZH2, AKT1 and p-AKT1 protein expressions in TNBC cells. The data were analyzed with GraphPad Prism 10.1.2 and are presented as the means ± SD. n=5. **P* < 0.05, ***P* < 0.01, and ****P* < 0.001 vs the non-treated group.

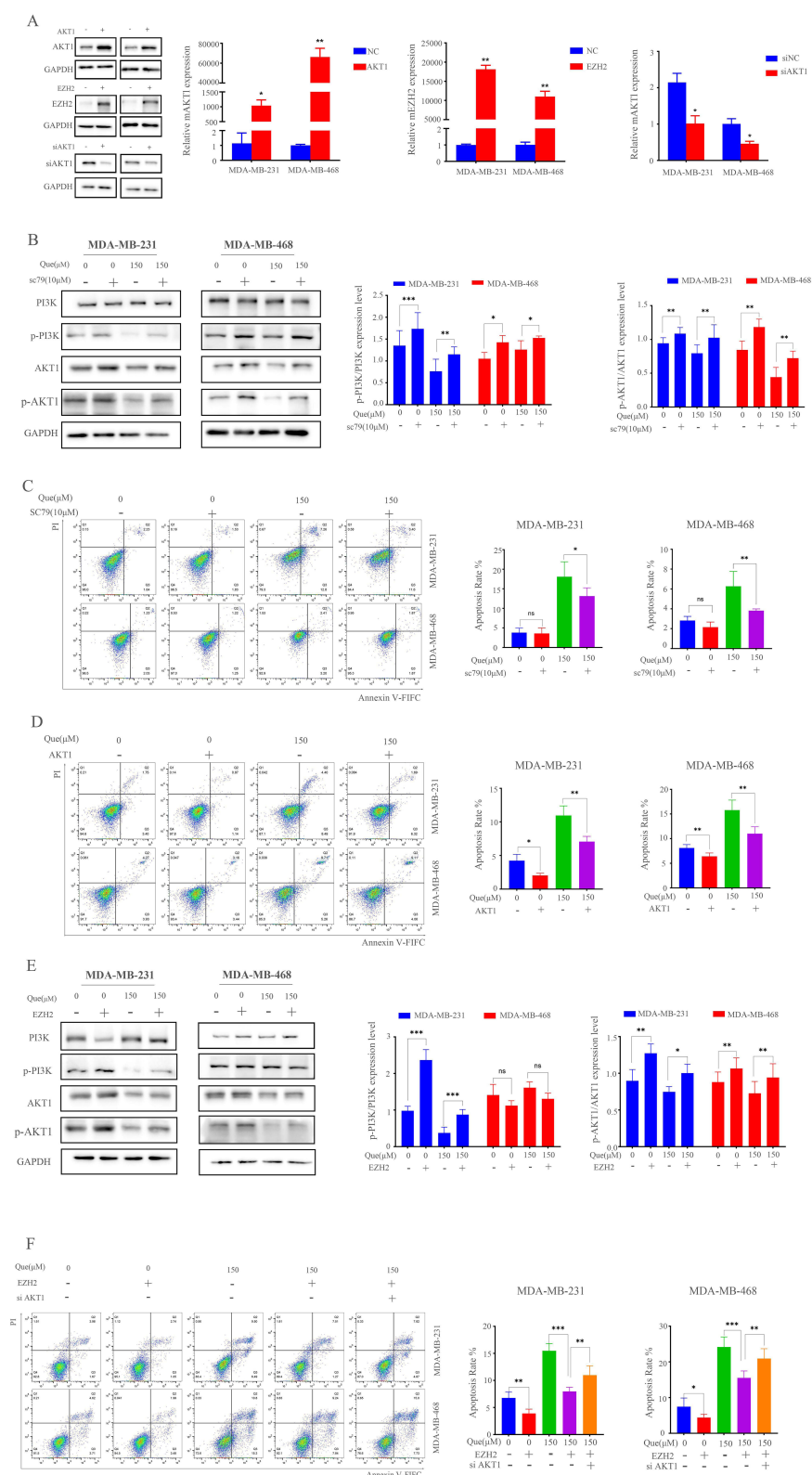


Figure 6 Que induced apoptosis of TNBC cell by blocking EZH2/AKT1 signaling pathway. **(A)** Western blot and qPCR assays were applied to confirm that gene transfection efficiency; **(B)** Effects of Que and SC79 on PI3K/AKT1 pathway proteins expression; **(C)** Effects of Que and SC79 on apoptosis of TNBC cells; **(D)** Effects of Que and overexpression AKT1 on apoptosis of TNBC cells; **(E)** Effects of Que and EZH2 on PI3K/AKT1 pathway proteins expression; **(F)** Effects of Que, overexpression EZH2 and siAKT1 on apoptosis of TNBC cells. The data were analyzed with GraphPad Prism 10.1.2 and are presented as the means $\pm$ SD, $n=5$. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ vs the control group.

cells. Compared with in the Que group, the protein expression levels of p-AKT1/AKT1 were increased in the EZH2 + Que group (Figure 6E), indicating that overexpression of EZH2 could reverse the inhibitory effect of Que on AKT1 in TNBC cells. These results illustrated that Que could block the EZH2/AKT1 signaling pathway in TNBC cells.

Que Induces Apoptosis of TNBC Cells by Suppressing AKT1 Expression

In order to further verify that Que promotes TNBC cell apoptosis by blocking the AKT1 signaling pathway, we treated TNBC cells with Que (150 μ M), while inducing the upregulation of AKT1 with SC79. Compared with in the blank group, the apoptotic rate of the SC79 group was decreased, but the difference was not significant. Compared with in the Que group, the apoptotic rate of the Que + SC79 group was decreased ($P < 0.05$; Figure 6C). Moreover, as determined by flow cytometry, we found that overexpression of AKT1 could inhibit the apoptosis of TNBC cells and could also reverse the inducing effect of Que on TNBC cell apoptosis ($P < 0.05$; Figure 6D). These results confirmed that AKT1 activation can inhibit the apoptosis of TNBC cells, and Que induces apoptosis by suppressing AKT1 expression.

Que Induces the Apoptosis of TNBC Cells by Blocking the EZH2/AKT1 Signaling Pathway

The silencing efficiency of AKT1 was determined by RT-qPCR and Western blotting analysis. The expression of AKT1 was downregulated in the silencing group (Figure 6A). Compared with in the control group, the apoptotic rate of TNBC cells in the EZH2 overexpression group were decreased ($P < 0.05$). Meanwhile, the apoptotic rate of the EZH2 + Que group were lower than that of the Que group ($P < 0.01$), and were also lower than that of the EZH2 + siAKT1 + Que group ($P < 0.05$; Figure 6F). These results suggested that overexpression of EZH2 could reduce the apoptotic rate of TNBC cells, and Que induced the apoptosis of TNBC cells by blocking the EZH2/AKT1 signaling pathway.

XYSD and Que Can Suppress the Growth of Breast Cancer in vivo

In order to confirm whether XYSD and its active component Que could inhibit tumor growth in vivo, we evaluated their efficacy in a human TNBC xenograft mouse model. Compared with the blank group, the volume and weight of xenograft tumors in the Cisplatin group, Que 80mg/kg group and XYSD group were significantly reduced after treatment (Figure 7A–C). Different from the Cisplatin group, the body weight of nude mice in the Que and XYSD groups did not decrease significantly after 30 days of treatment (Figure 7D). HE staining of liver and kidney showed no significant damage in Que and XYSD groups (Figure 7E). In addition, Western blot analysis of tumor tissues in nude mice indicated that the mechanism of tumor inhibition by XYSD and Que may be related to inhibition of PI3K/EZH2/AKT1 pathway (Figure 7F).

Discussion

TCM has a long history and exhibits promising potential in the treatment of TNBC. In the theory of TCM, TNBC belongs to the category of “Ru Yan”, and its formation is closely related to blood stasis. In view of this pathogenesis, XYSD, with blood activating and anti-cancer effects, has been developed and applied in clinical practice. The present study revealed that XYSD drug-containing serum could effectively inhibit the proliferation of TNBC cells. Due to the multi-component, multi-target and multi-pathway characteristics of TCM prescription, the method of combining UHPLC-Q Exactive HFX-MS with network pharmacology were used to predict the main compound and mechanism of action of XYSD in the treatment of TNBC.

After screening the results of UHPLC-Q Exactive HFX-MS according to the relevant criteria, and combining with the degree value calculated in the “XYSD-compound-target-TNBC” network, we believe that Que is the most effective compound in XYSD and may play an important role in the treatment of TNBC. As one of the most common flavonoids, Que is a promising anticancer drug. It has been reported that Que can regulate inflammatory mechanisms, induce cell cycle arrest, reduce development of blood vessels, inhibit the proliferation and migration of colorectal, liver, lung and prostate cancers.^{38,39} It has also been shown that quercetin can affect the apoptotic pathway and induce tumor cell death.⁴⁰ Although with a satisfactory therapeutic effect on BC treatment,⁴¹ the application research of Que to particular

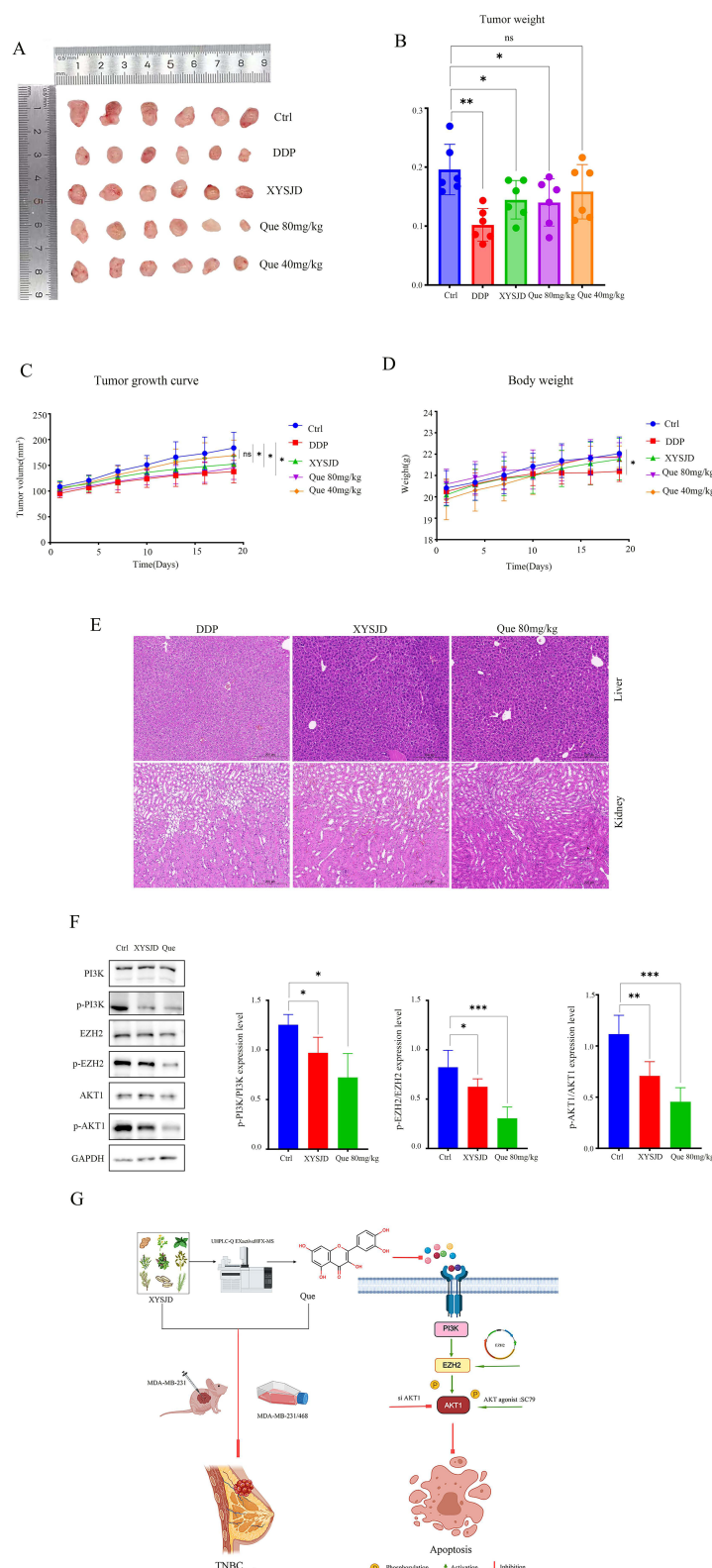


Figure 7 XYSJD and Que inhibited TNBC growth of nude mice. **(A)** Photograph of subcutaneous tumors in the control, cisplatin, XYSJD and Que groups. **(B)** Change in tumor volume in nude mice in each group. **(C)** Tumor tissues were harvested and weighed. **(D)** Weights of nude mice in the five groups during the trial. **(E)** Representative images of H&E staining of liver and kidney tissues of nude mice (scale bar, 200 μ M). **(F)** The expression levels of p-PI3K/PI3K, p-EZH2/EZH2, p-AKT1/AKT1 in tumor tissues were detected by Western blotting. **(G)** Possible active component and molecular mechanism of XYSJD in the treatment of TNBC. The data were analyzed with GraphPad Prism 10.1.2 and are presented as the means $\pm$ SD, $n=5$. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ vs the control group.

subtypes (such as TNBC) is still in the initial stage. In this study, we proved that Que could effectively inhibit the proliferation of TNBC cells through CCK-8 and EdU assays, inhibit TNBC migration and invasion through wound healing and transwell assays, and induce the apoptosis of TNBC cells through Hoechst/PI staining and flow cytometry. Que could effectively inhibit the growth of TNBC *in vivo*, and its effect was similar to Cisplatin without significant hepatorenal toxicity. These results indicated that Que is effective in treating TNBC both *in vitro* and *in vivo*.

Based on the KEGG analysis of network pharmacology, we screened out the PI3K/AKT signaling pathway. The erroneous regulation of this pathway is among the most common genomic abnormalities in various subtypes of BC.⁴² The PI3K/AKT pathway is involved in the regulation of cell growth, proliferation, survival, motility, metabolism and immune response.^{43,44} Mutations in the PI3K/AKT signaling pathway are very common in TNBC.⁴⁵ Related studies have reported that approximately 25% of primary TNBC cases have pathway mutations/alterations, and the frequency of metastatic TNBC may be higher.⁴⁶ AKT is a major downstream mediator of PI3K carcinogenic signaling. There are three subtypes in the AKT family, namely AKT1 (PKB α), AKT2 (PKB β) and AKT3 (PKB γ),⁴⁷ all of which are involved in the occurrence and progression of TNBC.⁴⁸ Combined with the PPI and cytoHubba results, we selected AKT1 as one of the main targets of YYSJD for TNBC treatment. Studies have shown that AKT1 promotes the proliferation and induces apoptosis of TNBC cells by regulating cyclin D1 expression.⁴⁹ It is also involved in important biological processes of TNBC, such as glucose metabolism and autophagy.^{50,51} In terms of invasion and metastasis, relevant experiments have confirmed that AKT1 may promote lung metastasis by preventing the apoptosis of TNBC cells.⁵² In a series of clinical observation experiments, we found great potential in the research and development of inhibitors targeting AKT1.⁵³ According to the results of the PPI analysis, we found that EZH2 is also one of the important targets of YYSJD in the treatment of TNBC. EZH2 is a polycomb group protein involved in the regulation of cellular memory. The high expression levels of EZH2 *in vivo* have been shown to be associated with the occurrence and progression of various types of cancer.⁵⁴ This was also confirmed by pan-cancer analysis through the TIMER database. Furthermore, we found that AKT1 and EZH2 were significant oncogenes in TNBC, and their expression levels were significantly higher in TNBC tissues than those in adjacent tissues. The two genes had a certain correlation, and were involved in the occurrence and progression of TNBC. Studies have shown that EZH2 can activate the PI3K/Akt signaling pathway of BC, and induce the proliferation and invasion of BC cells by specifically activating AKT1.^{55,56} However, in the TNBC subtype of BC, the relationship between EZH2 and the PI3K/AKT1 signaling pathway remains unclear. Therefore, identifying a drug that can both reduce the expression of EZH2 and block the AKT1 signaling pathway may be a new hotspot and could be used in the treatment of TNBC.

Through the results of molecular docking, we found that Que, the main active ingredient in YYSJD, had a good binding ability to AKT1 and EZH2. Western blotting experiments showed that with the increase of Que concentration, the protein expression levels of p-EZH2/EZH2 and p-AKT1/AKT1 in TNBC cells significantly decreased, which is consistent with the results predicted by network pharmacology. We believed that inducing apoptosis may be one of the main biological processes of YYSJD in the treatment of TNBC through GO analysis. Combined with Hoechst/PI staining and flow cytometry assays, it was found that Que, the active component of YYSJD, can effectively induce the apoptosis of TNBC cells. Therefore, we further verified that the mechanism of action of Que in the induction of TNBC cell apoptosis was related to blocking of the EZH2/AKT1 signaling pathway, as determined by rescue experiments.

Flow cytometry confirmed that the apoptotic rate of TNBC cells decreased after the addition of the AKT1 activator SC79 or upon AKT1 overexpression compared with in the control group. The apoptotic rates of TNBC cells in the Que + SC79 group and Que + AKT1 overexpression group decreased compared with in the Que group (Figure 6C and D). These findings indicated that activating AKT1 expression could inhibit the apoptosis of TNBC cells and reverse the apoptosis of TNBC cells induced by Que. These results confirmed that Que induced apoptosis of TNBC cells by suppressing AKT1 expression. Based on the findings in the literature with the PPI results, we speculated that EZH2 may be upstream of AKT1. Western blotting experiments also demonstrated that the increased expression of EZH2 not only activated the AKT1 signaling pathway, but also reversed the inhibitory effect of Que on AKT1 in TNBC cells (Figure 6E). We found that overexpression of EZH2 could inhibit the apoptosis of TNBC cells and significantly reverse the induced apoptosis of TNBC cells by Que. Meanwhile, after knocking down AKT1, the apoptosis induced by

overexpression of EZH2 and combined with Que was further reversed (Figure 6F). Therefore, based on these results, we could conclude that Que induces the apoptosis of TNBC cells by blocking the EZH2/AKT1 signaling pathway.

In summary, the present study highlight the efficacy and safety of XYSJD, providing further evidence for the clinical application of XYSJD in the treatment of TNBC. However, there are still some limitations to this study. It is well known that the components and targets of Chinese medicine are diverse and complex; Therefore, the effect and mechanism of a single Que cannot fully represent the anticancer effect of XYSJD. It is worth noting that XYSJD drug-containing serum can effectively inhibit the cell proliferation of TNBC. In the future, we will integrate a serum pharmacochimistry and network pharmacology approach to explore the effective components of XYSJD in blood. In addition, we will combine transcriptomics, metabolomics, etc. to further screen the potential mechanism of XYSJD against TNBC, truly leveraging the multi-compound, multi-target and multi-pathway characteristics of TCM, and providing guidance for further research.

Conclusions

Combined with network pharmacology, in vivo and in vitro experimental verification, this study revealed the anti-TNBC effects, active compound and mechanisms of XYSJD, providing a new experimental basis and research direction for TCM treatment of TNBC.

Abbreviations

TNBC, Triple-negative breast cancer; XYSJD, XiaoYao SanJie Decoction; UHPLC-Q Exactive HFX-MS, Ultra-high performance liquid chromatography-hybrid quadrupole orbitrap mass spectrometry; Que, quercetin; BC, Breast cancer; TCM, Traditional Chinese Medicine; OB, oral bioavailability; DL, drug-likeness; GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; PPI, Protein-Protein Interaction; BP, biological processes; MF, molecular functions; CC, cellular component; CCK-8, Cell Counting Kit-8; RT-PCR, Quantitative RealTime Polymerase Chain Reaction; WB, Western Blot; SD, standard deviation; PI3K, Phosphatidylinositol 3-kinases; AKT1, serine/threonine-protein kinase 1; EZH2, enhancer of zeste 2 polycomb repressive complex 2 subunit.

Data Sharing Statement

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding authors.

Ethics Statement

Ethical approval was not required for the studies on humans in accordance with the local legislation and institutional requirements because only commercially available established cell lines were used. Animal experiment was approved by the formal review of experimental animal ethics at Huazhong university of Science and Technology. The IACUC approval number is IACUC-2022-3324. As for the bioinformatics experiment, the experiment was approved by Institutional Review Board of Hubei University of Science and Technology, and the IACUC approval number is IACUC-202410001.

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Disclosure

These authors declared that there are no known conflicts of interest.

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