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Baohuoside I from Epimedii Folium Exhibits Anti-Triple-Negative Breast Cancer Effects by arresting Cell Cycle Progression

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ABSTRACT: Epimedii Folium has long been used in China as a water-decocted or alcohol-macerated preparation to nourish bodily functions. In 2002, it was officially approved by China's Ministry of Health as a medicinal herb permitted for use in health food products. Baohuoside I (BHS), a characteristic component of Epimedii Folium, exhibits a broad spectrum of anti-cancer activities. This study aims to explore the therapeutic effects and underlying mechanistic basis of BHS against triple-negative breast cancer (TNBC) using integrated pharmacological and bioinformatics approaches. *In vitro* experiments demonstrated that BHS dose-dependently inhibited TNBC cell viability (IC₅₀: MDA-MB-468 = 20.02 ± 3.46 μM; MDA-MB-231 = 41.76 ± 1.77 μM), with also significant suppressing cell proliferation and migration ($P < 0.05$). *In vivo*, BHS exhibited a favorable safety profile in both healthy and TNBC xenograft model mice ($P > 0.05$), and also effectively suppressed TNBC xenograft growth ($P < 0.05$). Transcriptomic analysis combined with machine learning-based bioinformatic analysis identified ten core cell cycle regulatory genes (*MCM2*, *MCM3*, *MCM5*, *EXO1*, *PARP1*, *CDC45*, *POLE2*, *RFC3*, *PCNA*, and *CCNE2*), whose expression was substantially downregulated by BHS, as validated through molecular biological assays. Cellular thermal shift assay and drug affinity responsive target stability assay identified PMS2 as a putative direct binding target of BHS, with molecular docking validating a high-affinity interaction (binding energy: -8 kcal/mol). Collectively, these results support BHS as a promising safe and effective therapeutic candidate for TNBC, with its anti-tumor activity predominantly associated with the regulation of cell cycle progression.

Keywords: Baohuoside I, Triple-negative breast cancer, Cell cycle, Machine learning, Bioinformatics

1. Introduction

Global cancer statistics show that breast cancer (BC) accounts for 11.6% of total cancer incidence and contributes to 6.9% of female cancer mortality globally ^[1]. Triple-negative breast cancer (TNBC), representing 15-20% of all BC cases, is a biologically aggressive subtype defined histopathologically by high-grade nuclear atypia and hyperproliferative tumor cells. The lack of actionable molecular targets for precision medicine, coupled with these inherent biological features, results in the poorest prognosis among all BC subtypes ^[2].

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This multidimensional evidence, which includes demographic trends, tumor biological characteristics, and therapeutic challenges, highlights the need for coordinated efforts to advance molecular-level research, with the goal of developing mechanism-based therapeutic strategies to tackle this critical public health burden. Although surgery and conventional chemotherapy remain the current standard of care, traditional Chinese medicine (TCM) has emerged as a promising candidate therapeutic modality for TNBC and other malignancies, drawing on its unique theoretical framework and centuries of clinical experience [3]. From a TCM perspective, the pathogenesis of TNBC involves two core mechanisms: invasion by exogenous pathogenic factors and depletion of righteous qi (zhengqi, vital energy). This dual perturbation disrupts the homeostasis of meridians, the dynamic balance of qi, blood, and body fluids, and yin-yang equilibrium, ultimately impairing the physiological integrity of the mammary gland [4].

The concept of medicinal and edible homology is deeply rooted in TCM theory, highlighting the intrinsic link and mutual interdependence between nutritional and medicinal constituents [5]. Such substances can be consumed long-term to prevent the onset or progression of disease, or act as adjuvant therapies to improve patients' quality of life [6]. Of particular note is their considerable potential as antitumor agents for cancer therapy. Epimedii Folium has been an integral part of TCM since its classification as a tonic herb in the classic *Shennong Ben Cao Jing* more than 2,000 years ago. Its medicinal value was further recognized in 2002, when China's Ministry of Health approved it for use as a functional health food. In folk medicine, Epimedii Folium is commonly prepared via water decoction or alcoholic maceration for medicinal drinks, or cooked with mutton or pig heart for therapeutic purposes. Research has demonstrated that it possesses yang-tonifying properties and can regulate yin-yang balance in tumor patients [7]. Modern pharmacological researches have identified its primary bioactive components as flavonoids, including baohuoside I (BHS), icariin, icaritin, and epimedins A, B, and C, all of which have well-documented antitumor activities [8]. Notably, BHS exhibits superior intestinal absorption kinetics compared to other major flavonoids in Epimedii Folium and acts as the primary intestinal metabolite of icariin following oral administration [9]. Mechanistic studies have shown that BHS potently suppresses the invasion of MDA-MB-231 and SK-BR-3, which are established TNBC cell lines, and induces apoptosis by suppressing the NF- κ B pathway, EGFR downstream signaling cascades, and regulating apoptosis-related proteins (e.g., Bcl-2, Bax, Caspase-3, and PARP)^[10]. Furthermore, BHS inhibits metastasis by blocking the M2 polarization of tumor-associated macrophages, reducing CXCL1 secretion, suppressing the β -catenin/CSCs pathway, and inhibiting FLOT2/RAB31-dependent EV-Apo release^[11, 12]. Despite these insights into the partial mechanisms underlying BHS-mediated anti-TNBC activity, the candidate direct molecular targets of BHS remain undefined. This critical mechanistic gap highlights the necessity of integrating bioinformatics methodologies with innovative target identification strategies to comprehensively elucidate the molecular mechanisms underlying BHS-mediated anti-TNBC activity.

In this study, the potent anti-TNBC efficacy of BHS was systematically validated using rigorous *in vitro* cellular assays and a xenograft mouse model. To identify and characterize the direct molecular targets of BHS in TNBC cells, an innovative integrative strategy encompassing clinical databases minging, transcriptome

sequencing, machine learning, and drug affinity responsive target stability-mass spectrometry (DARTS-MS). Subsequently, complementary validation assays, including DARTS-western blot (DARTS-WB), cell thermal shift assay-western blot (CETSA-WB), and *in silico* docking simulations were used to validate the binding affinity and molecular interaction models between BHS and its candidate targets. Additionally, to comprehensively assess the safety profile of BHS, we evaluated its potential hepatotoxic and nephrotoxic effects *in vivo* under the same therapeutic dose and administration regimen were evaluated used in the efficacy studies. This study not only elucidates critical mechanistic insights into BHS-mediated anti-TNBC activity by identifying its candidate direct molecular targets but also lays a solid foundation for the translational development of BHS by providing robust preclinical evidence supporting its potential as a promising therapeutic agent for TNBC.

2. Materials and methods

2.1. Chemicals

BHS (Cat# PS011047, purity 98% by HPLC), Icariin (Cat# PS010429, purity 98% by HPLC) and Paclitaxel (Cat# PS0036, purity 98% by HPLC) were purchased from Chengdu Push Bio-technology Co., Ltd. (Sichuan, China). Carboxymethyl cellulose sodium was procured from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China), Pronase E (Cat# S10014) from yuanye Bio-Technology Co., Ltd (Shanghai, China), and Coomassie Blue Fast Staining and No-decoloring Solution (Cat# PS111) from Epizyme Biomedical Technology Co., Ltd (Shanghai, China). Dimethyl sulfoxide (DMSO, Cat# D4540) and 3-(4,5)-Dimethylthiaziazolo(-z-y1)-3,5-di-phenyltetrazolium bromide (*MTT*, Cat# M5655) were sourced from Sigma-Aldrich Corporation (Missouri, USA). PBS (pH 7.4, Cat# BL2215A), 4% paraformaldehyde (Cat# BL539A) and 5× SDS-PAGE loading buffer (Cat# 143189) were purchased from Biosharp (Anhui, China). Beyotime (Shanghai, China) provided crystal violet staining solution (Cat# C0121), while TRIzol reagent (1 mL/well, Cat# 15596026) was acquired from Invitrogen (USA). The Spin Column Animal Total RNA Purification Kit (Cat# B518651) from Sangon Biotech (Shanghai, China), the Revert Aid First Strand cDNA Synthesis Kit (Cat# RK20429) from ABclonal (Hubei, China), and SYBR® Green Realtime PCR Master Mix (Cat# QPK-201) from TOYOBO (Shanghai, China) were also purchased. Elabscience (Hubei, China) was the supplier for the Cell Cycle Assay Kit (Red Fluorescence, Cat# E-CK-A351), RIPA lysis buffer (Cat# E-BC-R327), BCA Quantification Kit (Cat# E-BC-K318-M), Alanine Aminotransferase Activity Assay Kit (ALT/GPT, Cat# E-BC-K235-M) and Aspartate Aminotransferase Activity Assay Kit (AST/GOT, Cat# E-BC-K236-M). The SDS-PAGE Rapid Gel Dispensing Kit (Cat# PMK1017B) and Ultrasensitive ECL Luminescent Substrate Detection Kit (Cat# PMK0448) were purchased from Pumoke Biotechnology Co., Ltd. (Hubei, China).

2.2 *In vitro* pharmacological evaluation of BHS against TNBC

2.2.1 Cell proliferation experiment

MDA-MB-468 (Cat# CL-0290B), MDA-MB-231 (Cat# CL-0150), MCF-7 (Cat# CL-0007) and 4T1

cells (Cat# CL-0149) were obtained from Procell Life Science & Technology Co., Ltd. (Wuhan, Hubei, China). All cell lines were cultured in their respective complete media (procured from Procell, Cat#: CM-0290B for MDA-MB-468, Cat#: CM-0150B for MDA-MB-231, Cat#: CM-0149 for 4T1, and Cat#: CM-0007 for MCF-7) under standard conditions (37 °C, 5% CO₂). For cell proliferation assay, 5.0×10^3 cells were seeded per well of 96-well plates and then treated with serial dilutions (12.5 to 200 µM) of BHS or Icaritin for 24 h. Cellular viability was determined via *MTT* assay, with optical density measurements recorded at 490 nm per well. Dose-response curves generated from triplicate experiments were used to calculate the half-maximal inhibitory concentrations (IC₅₀) of each compound across all cell lines.

2.2.2 Wound healing experiment

Cell monolayers of MDA-MB-468, MDA-MB-231, MCF-7, and 4T1 cells were established in 6-well plates, followed by generation of a standardized linear wound using a scratch assay. Control group was cultured in standard medium, while treatment groups were treated with BHS at cell type-specific concentrations: 20 and 40 µM for MDA-MB-468, 30 and 60 µM for MDA-MB-231 and MCF-7, and 50 and 100 µM for 4T1. Phase-contrast microscopy was used to capture images of the wound area immediately after scratching (0 h) and 24 h post-treatment. Subsequently, wound closure was quantified using ImageJ software (v1.54), and the migration rate of each group was calculated with the formula: Migration rate (%) = [(initial width - final width)/initial width] × 100%. This experimental setup facilitated the precise assessment of BHS concentration-dependent effects on cellular motility across distinct BC subtypes. Standardized scratch dimensions and temporal monitoring were implemented to ensure experimental consistency.

2.2.3 Colony formation experiment

MDA-MB-468, MDA-MB-231, MCF-7 and 4T1 cells were seeded into 6-well plates at a density of 2.0×10^3 cells/well. After cell adhesion, the cells were divided into the control group and experimental groups, and the latter were treated with BHS at predetermined concentrations for 24 h. Following treatment, the BHS-containing medium was substituted with fresh BHS-free medium, and the cells were cultured under standard conditions for 14 days. The resulting colonies were fixed with 4% paraformaldehyde solution for 20 min, stained with 1 mL crystal violet solution (30 min), and then two rinsed twice with phosphate-buffered saline (PBS, pH 7.4). Documentation was achieved by photographing air-dried plates.

2.3 In vivo safety assessment and anti-TNBC efficacy of BHS

2.3.1 In vivo safety assessment of BHS in mice

To assess the *in vivo* safety of BHS in mice, 30 specific pathogen-free (SPF) female BALB/c mice were acclimatized under standard laboratory conditions for 7 days. Then, the mice were randomly assigned to three groups (n = 10 per group): control (oral administration of 0.5% carboxymethylcellulose sodium), low-dose BHS (10 mg/kg, oral gavage), and high-dose BHS (40 mg/kg, oral gavage). BHS or the vehicle (0.5% sodium carboxymethylcellulose) was administered daily by oral gavage at 0.02 mL/g body weight for 16 consecutive

days. Throughout the treatment period, the general health status of the mice was monitored daily, and body weights were measured and recorded using an electronic balance. One day after the final administration, the mice were euthanized. Blood samples, heart, liver, spleen, lungs, and kidneys were collected and weighed immediately. Liver and kidney tissue sections were placed in specimen vials, fixed in 4% paraformaldehyde solution, and subsequently processed for embedding, sectioning, and hematoxylin and eosin staining (HE, Cat# S191003, Pinuofei, Wuhan, China).

2.3.2 Serum alanine transaminase and aspartate transaminase activity assays

Alanine transaminase (ALT) and aspartate transaminase (AST) are primarily localized in hepatocytes and participate in amino acid metabolism. When hepatocytes are damaged, the levels of ALT and AST in the serum increase significantly [13]. Therefore, quantification of serum ALT and AST levels can serve as an initial indicator for assessing liver injury. Serum samples from mice in different groups were dispensed into a 96-well plate. Reagents were then added sequentially per the manufacturer's protocol, followed by incubation at 37 °C. Subsequently, 200 µL of Reagent 5 working solution was added to each well, vortex-mixed for 10 s, and incubated at room temperature for 15 min. Finally, the optical density of each well was measured at 510 nm using a microplate reader.

2.3.3 Analysis of the *in vivo* anti-TNBC efficacy of BHS

To establish TNBC xenografts, 4T1 cells (2×10^6) were subcutaneously inoculated into the right posterior flanks of SPF female BALB/c mice (18–20 g). When the tumor volume approached approximately 80 mm³, 30 tumor-bearing mice were randomly assigned to five experimental groups (n = 6 per group): 1. model group (oral administration of 0.5% carboxymethylcellulose sodium); 2. Low-dose BHS (10 mg/kg, oral gavage); 3. Middle-dose BHS (20 mg/kg, oral gavage); 4. High-dose BHS (40 mg/kg, oral gavage); 5. paclitaxel (positive group, 15 mg/kg, intraperitoneal injection every 72 h) [14, 15]. During the 16-day treatment period, the general health status of the mice was monitored daily, and body weights were measured and recorded. Tumor volumes were calculated using the formula: $V = (\text{length} \times \text{width}^2)/2$, where tumor length and width were measured with digital calipers. After euthanasia, the excised tumor tissues were fixed in 4% paraformaldehyde solution, embedded in paraffin, and sectioned using a LEICA RM2016 microtome. These tissue sections were subjected to HE staining for histological evaluation. Stained sections were mounted, imaged under a microscope, and analyzed for histological parameters. All animal experimental procedures were approved by the Animal Ethics Committee of Hubei University of Chinese Medicine.

2.4 Identification of core targets of BHS against TNBC

2.4.1 Transcriptome sequencing and analysis

Transcriptomic analysis was conducted on both tumor tissues (model group and high-dose BHS group (40 mg/mL)) and MDA-MB-468 cells cultured in 6-well plates (BHS-treated group (25 µM) and untreated control group). Total RNA was isolated using TRIzol reagent (1 mL/well), followed by complementary DNA

(cDNA) library construction. Prior to Illumina NovaSeq 6000 sequencing, RNA integrity was assessed using an Agilent Bioanalyzer 2100 (Agilent Technologies, USA). Following quality control of raw sequencing reads, alignment to the reference genome was carried out with HISAT2 (v2.2.1) and STAR (v2.7.10a). Transcript abundance quantification was then performed with RSEM (v1.3.1). Principal component analysis (PCA) was implemented using the ggplot2 package (v3.4.2) to reduce data dimensionality and verify inter-group separation. Differentially expressed genes (DEGs) were identified using DESeq2 (v1.38.3) in R (v4.2.2), with significance thresholds set at $|\log_2 \text{Fold Change}| \geq 0.263$ (tumor tissues), $|\log_2 \text{Fold Change}| \geq 1$ (cellular samples) and $P < 0.05$, respectively.

2.4.2 Clinical transcriptome data mining

The Assistant for Clinical Bioinformatics platform (<https://www.aclbi.com>) was used to retrieve transcriptomic data from breast tissues of both TNBC patients and healthy controls [16, 17]. Using the "Basal-like" molecular subtype classification, 16 human TNBC samples and 572 healthy controls were extracted from The Cancer Genome Atlas (TCGA) database. Additionally, 55 TNBC cases and 11 healthy controls were acquired from the Gene Expression Omnibus (GEO) dataset GSE65212. DEGs between TNBC and control groups were screened using DESeq2 (R v4.2.2) with thresholds of $|\log_2 \text{Fold Change}| \geq 1.5$ and $\text{adj-}P < 0.05$ (Benjamini-Hochberg method).

2.4.3 Topological algorithms and machine learning analysis

To elucidate potential targets of BHS, an integrative analysis was performed by cross-referencing the TNBC cellular transcriptome data with TCGA and GEO clinical datasets, based on the hypothesis that genes upregulated in TNBC would be downregulated following BHS treatment. Overlapping genes were visualized via the Venny tool (v2.1). Functional enrichment analysis was performed using the Database for Annotation, Visualization and Integrated Discovery (DAVID (v2023q2), <https://david.ncifcrf.gov/>) [18], covering Gene Ontology (GO) terms and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways. A protein-protein interaction (PPI) network was constructed using the STRING (<https://string-db.org/>) database and visualized within Cytoscape (v3.10.1, <https://cytoscape.org/download.html>) software [19, 20]. Candidate core targets of BHS against TNBC were identified by integrating results from six topological algorithms (MCC, Closeness, DMNC, Degree, MNC, and EPC) implemented using the cytoHubba plugin in Cytoscape, and six machine learning models (Random Forest (RF), Generalized Linear Model (GLM), k -Nearest Neighbors (KNN), Decision Tree (DT), Neural Network (NN), Support Vector Machine (SVM)).

2.5 The impact of BHS on the expression levels of candidate core targets

2.5.1 Cell cycle analysis

BHS exposure concentrations were cell line-specific: MDA-MB-468 cells were treated with 0, 20, or 40 μM BHS, while MDA-MB-231 cells were exposed with 0, 30, or 60 μM BHS. Cell cycle distribution was analyzed using the Cell Cycle Assay Kit following the manufacturer's instructions. Specifically, after

collection, cells underwent PBS washing followed by fixation in pre-chilled 70% ethanol at -20 °C for 4 h. After fixation, cells were rewashed with PBS, incubated with RNase A to remove RNA contamination, followed by staining with propidium iodide solution. After 30 min of incubation on ice in the dark, nuclear DNA content was analyzed by a flow cytometer (NovoCyte D2060R, Agilent Technologies, USA). Data were acquired, and cell cycle phase distributions were quantified using NovoExpress software (v1.6.1, Agilent Technologies Inc., USA), with gating parameters set to exclude cellular debris and aggregates.

2.5.2 Quantitative real-time polymerase chain reaction (qPCR) analysis

Total RNA was isolated from cells in each experimental groups using a Spin Column Animal Total RNA Purification Kit, followed by reverse transcription into cDNA with the RevertAid First Strand cDNA Synthesis Kit. qPCR reactions were performed in triplicate using SYBR® Green Realtime PCR Master Mix according to manufacturer's protocols. Thermal cycling parameters initiated with an initial denaturation phase: 37 °C for 2 min and 95 °C for 3 min, followed by 40 amplification cycles (each cycle: 95 °C for 5 s and 60 °C for 30 s). Target gene expression levels were normalized to the reference genes β -actin (for MDA-MB-468 cells) and *GAPDH* (for MDA-MB-231 cells), with relative quantification via the comparative $\Delta\Delta C_t$ method. Primer sequences are detailed in Supplementary Table 1.

2.5.3 Western blot analysis

After BHS treatment, cellular proteins were extracted using RIPA buffer (containing protease inhibitors), and the protein concentration of the cell lysates was measured using a BCA Quantification Kit. Equal amounts of total protein were separated by SDS-PAGE gels (10%) and then transferred onto polyvinylidene difluoride (PVDF) membranes (Cat# BS-PVDF-45-S, Biosharp, China). Subsequently, the membranes were blocked with 3% skim milk (Cat# GC310001-100g, Servicebio, China) in TBST at 25 °C, then incubated overnight at 4 °C with the following primary antibodies against: MCM2 (1:1000, Cat# CY5200, Abways, China), MCM3 (1:2000, Cat# 15597-1-AP, Protrintech Group, China), MCM5 (1:2000, Cat# CY7185, Abways, China), EXO1 (1:10000, Cat# 16253-1-AP, Protrintech Group, China), PARP1 (1:5000, Cat# 13371-1-AP, Protrintech Group, China), CDC45 (1:2000, Cat# CY5936, Abways, China), RFC3 (1:500, Cat# BD-PE-1758, Biodragon, China), POLE2 (1:1000, Cat# DF9446, Affinity, USA), PCNA (1:10000, Cat# 10205-2-AP, Protrintech Group, China), and CCNE2 (1:1000, Cat# CY5821, Abways, China). β -actin (1:3000, Cat# AF7018, Affinity, USA) and *GAPDH* (1:10000, Cat# AB0037, Abways, China) were used as internal loading controls. Protein signals were visualized using an Ultrasensitive ECL Luminescent Substrate Detection Kit, and images were acquired using a Tanon 4600 system. Quantitative densitometric analysis of the protein bands was conducted using ImageJ software (v1.54g) with background normalization.

2.5.4 Immunohistochemical analysis

Formalin-fixed, paraffin-embedded tumor sections were subjected to sequential deparaffinization via gradient xylene immersion. Antigen retrieval was conducted using citrate buffer (Cat# P0064, Pinuofei,

Wuhan, China), followed by inactivation of endogenous peroxidase with 3% (v/v) H₂O₂. After blocking nonspecific binding sites using normal serum, the sections were incubated with primary antibodies overnight at 4 °C, and the antibody specifications were consistent with those used in Western blot (WB) experiments. After 1 h incubation at 37°C with horseradish peroxidase-conjugated IgG secondary antibodies (Cat# PN0046, Pinuofei, Wuhan, China), signals were developed using 3,3'-diaminobenzidine substrate. Sections underwent hematoxylin counterstaining^[21], graded ethanol dehydration, xylene clearing, and mounting for microscopic assessment.

2.6 DARTS-MS analysis

DARTS-MS assay was used to identify the candidate targets of BHS, based on the principle that drug-bound target proteins exhibit reduced proteolytic susceptibility. Cellular proteins were extracted from MDA-MB-468 cells using RIPA lysis buffer supplemented with protease and phosphatase inhibitors, and protein concentrations were quantified using BCA assay. For the assay, 100 µg of total protein was incubated with an equal volume of BHS (300 µM) or DMSO (control) at 4 °C for 2 h, and then digested with Pronase E at gradient concentrations (1:1600, 1:800, 1:400, and 3:1000) for 30 min at 40 °C. Reactions were terminated by the addition of 5× loading buffer followed by 70 °C incubation for 10 min. Protein separation was achieved through SDS-PAGE, and subsequent gels were visualized using Coomassie Blue Fast Stain and No-decoloring Solution. Protein bands showing differential stability between BHS-treated and control samples were excised for MS analysis to identify candidate binding targets. Two stringent selection criteria were applied to MS data for candidate target identification: (1) significantly higher peak intensity in the BHS-treated group compared to the untreated control group; (2) molecular weights (kDa) within the 55-100 kDa range, matching the expected size of the differential target bands.

PPI network analysis was performed for the identified candidate targets and bioinformatically predicted core targets using the STRING database. Subsequent visualization and refinement of the PPI network were conducted in Cytoscape software (v3.10.1), which revealed interacting partners of these targets. Special emphasis was placed on functionally relevant interactions between DARTS-MS-identified candidate targets and bioinformatically predicted core targets.

2.7 Validation of BHS's target protein

2.7.1 DARTS-WB analysis

Sample preparation followed the protocol detailed in section 2.6. Briefly, protein aliquots (20 µg) underwent 10% SDS-PAGE separation, electrophoretic transfer to PVDF membranes, blocking with skimmed milk at room temperature, and TBST washes. Primary antibodies targeting PMS2 (1:2000 dilution, cat# CY5114, Abways, China) and β-actin were incubated overnight at 4 °C. After additional TBST washes, the membrane was incubated with suitable secondary antibodies at room temperature. Following a final round of TBST washes, protein signals were detected using a visualization system after adding substrate solution. Band intensities were quantified using ImageJ software with background subtraction. All experimental parameters

were consistent with those described in section 2.5.3. To assess BHS-PMS2 binding interactions, grayscale values of protein bands were compared between BHS-treated and untreated samples at the same enzyme concentrations.

2.7.2 CETSA-WB analysis

CETSA was used to assess drug-target interactions by quantifying ligand-induced changes in protein thermal stability. For this assay, 1 mL of MDA-MB-468 cell protein lysate was incubated with an equal volume of BHS (300 μ M) or DMSO (vehicle control) at 4 °C for 2 h. Samples were then aliquoted into 12 tubes and subjected to a temperature gradient (37-70 °C) for 3 min. After heat exposure, samples were centrifuged at $20,000 \times g$ for 20 min at 4 °C. 5 $\times$ SDS loading buffer was added to the supernatants, followed by denaturation at 100 °C for 10 min, and analyzed by WB assay as described in section 2.5.3. To evaluate BHS-PMS2 binding, grayscale values of protein bands were compared between BHS-treated and control samples across the entire temperature gradient.

2.7.3 Molecular docking

AutoDock vina (v1.2.x) software was used to assess the binding affinity and potential interaction sites between BHS and PMS2. The structures of BHS and PMS2 were retrieved from the PubChem database (<https://pubchem.ncbi.nlm.nih.gov/>)^[22] and RCSB Protein Data Bank database (<https://www.rcsb.org/>), respectively. Potential binding pockets were predicted using the DeepSite database (<https://www.playmolecule.com/deepsite/>), with the top-ranked sites selected for subsequent molecular docking simulations. Structural preprocessing included format conversion to PDB, followed by molecular docking performed using AutoDock Vina (v1.5.7). The optimal binding posture was selected from the docking outputs based on the lowest binding energy (ΔG , kcal/mol). Protein-Ligand Interaction Profiler was used to characterize BHS-PMS2 interaction modes (e.g., hydrogen bonds and hydrophobic contacts), and the results were visualized using PyMOL software (v2.6.0).

2.8 Statistical analysis

All experiments were independently replicated three times. All data were analyzed using Graphpad Prism software (v9.5.1) and presented as mean $\pm$ SD. Unpaired t-tests were used to compare differences between two groups, unless otherwise specified in the figure legends. Statistical significance was defined as $P < 0.05$.

3. Results

3.1 BHS inhibited the proliferation and migration of TNBC cells in vitro

The chemical structures of BHS and Icariin are shown in Figure 1A and Figure 1C, respectively. Pharmacological evaluation using *MTT* assays demonstrated that BHS exerted anti-proliferative effects on four BC cell lines, with IC_{50} values of $20.02 \pm 3.46 \mu$ M (MDA-MB-468), $41.76 \pm 1.77 \mu$ M (MDA-MB-231), $45.00 \pm 1.91 \mu$ M (MCF-7), and $67.48 \pm 1.70 \mu$ M (4T1) (Figure 1B). Comparative analysis revealed

substantially reduced potency of icariin, which required concentrations exceeding 100 μM to induce detectable proliferation inhibition at 24 h (Figure 1D). These results are consistent with our hypothesis that icariin is metabolized into BHS following oral administration, and that BHS exhibits enhanced anti-BC efficacy.

Functional assessment through wound healing assays showed dose-dependent suppression of cell migration by BHS across all tested cell lines: MDA-MB-468 migration decreased from $16.99 \pm 0.88\%$ (0 μM) to $13.39 \pm 1.08\%$ (20 μM , $*P < 0.05$) and $1.84 \pm 1.17\%$ (40 μM , $***P < 0.001$), MDA-MB-231 from $75.52 \pm 3.58\%$ (0 μM) to $62.31 \pm 5.45\%$ (30 μM , $*P < 0.05$) and $31.21 \pm 3.96\%$ (60 μM , $**P < 0.01$), MCF-7 from $22.23 \pm 4.38\%$ (0 μM) to $19.59 \pm 1.46\%$ (30 μM , $P > 0.05$) and $6.52 \pm 2.23\%$ (60 μM , $*P < 0.05$), and 4T1 from $12.23 \pm 3.24\%$ (0 μM) to $2.37 \pm 0.74\%$ (50 μM , $*P < 0.05$) and $0.55 \pm 0.16\%$ (100 μM , $**P < 0.01$, Figure 1E-H). Complementary colony formation assays confirmed impaired clonogenic capacity in all tested cell lines (Figure 1I-L). Collectively, these findings demonstrate that BHS exhibits enhanced cytotoxic efficacy against TNBC subtypes (MDA-MB-231 and MDA-MB-468) compared to hormone receptor-positive MCF-7 cells, as evidenced by differential IC_{50} values and inhibition effects on cell migration and clonogenicity.

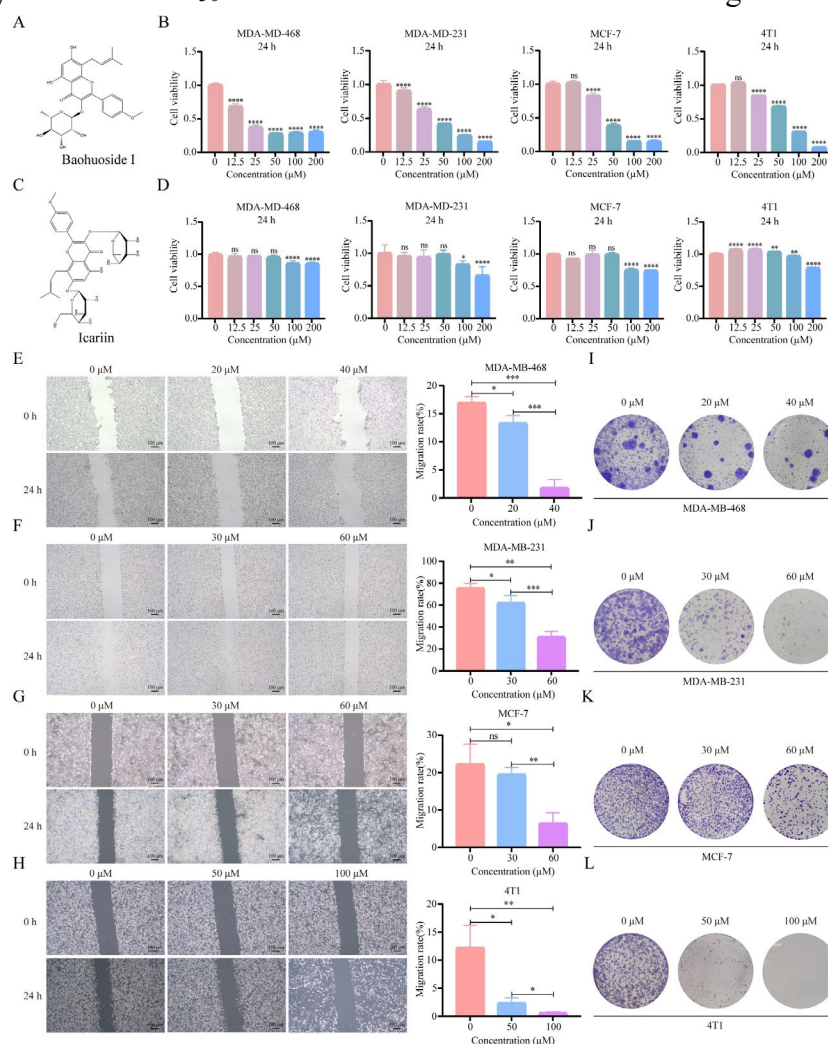


Figure 1: Inhibitory effects of BHS on MDA-MB-468, MDA-MB-231, MCF-7 and 4T1 cells in vitro. (A) Molecular structure of BHS. (B) BHS inhibits viability of breast cancer cell lines (MDA-MB-231, MDA-MB-468, MCF-7 and 4T1). (C) Molecular structure of Icariin. (D) Icariin inhibits viability of breast cancer cell lines (MDA-MB-231, MDA-MB-468, MCF-7 and 4T1). (E-H) Suppressive effects of BHS on the migration formation of MDA-MB-231, MDA-MB-468, MCF-7 and 4T1 cells. (I-L) BHS inhibits colony formation in MDA-MB-231, MDA-MB-468, MCF-7 and 4T1 cells. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, $****P < 0.0001$.

3.2 BHS exhibited a favorable safety profile in vivo

The safety profile of BHS in mice is presented in Figure 2A. Body weight of all mice was continuously monitored throughout the administration period, and no statistically significant differences were observed between BHS-treated and control groups ($P > 0.05$, Figure 2B). Further evaluation included the determination of organ coefficients for major organs (heart, liver, spleen, lungs, and kidneys), a sensitive indicator for detecting abnormal morphological changes such as hyperplasia, atrophy, or edema. All BHS-treated groups maintained organ coefficients within the normal physiological range, with no significant deviations from the control group ($P > 0.05$, Figure 2C-G), indicating that absence of BHS-induced pathological effects in these organs. To further assess potential hepatotoxicity and nephrotoxicity, serum biochemical parameters and histopathological examinations were performed. Serum biochemical results showed no significant increase in ALT or AST levels in BHS-treated mice compared with the control group ($P > 0.05$, Figure 2H and I), suggesting that BHS did not compromise hepatocyte membrane integrity or cause parenchymal liver cell damage. H&E-stained sections of liver and kidney tissues from all BHS-treated groups exhibited intact histological structures and regular cellular arrangements, with no notable pathological alterations (e.g. edema, degeneration, necrosis, or inflammatory infiltration) (Figure 2J and K). Collectively, these results demonstrate that BHS did not induce significant organ toxicity in healthy mice within the tested dose range and study duration, confirming a favorable in vivo safety profile.

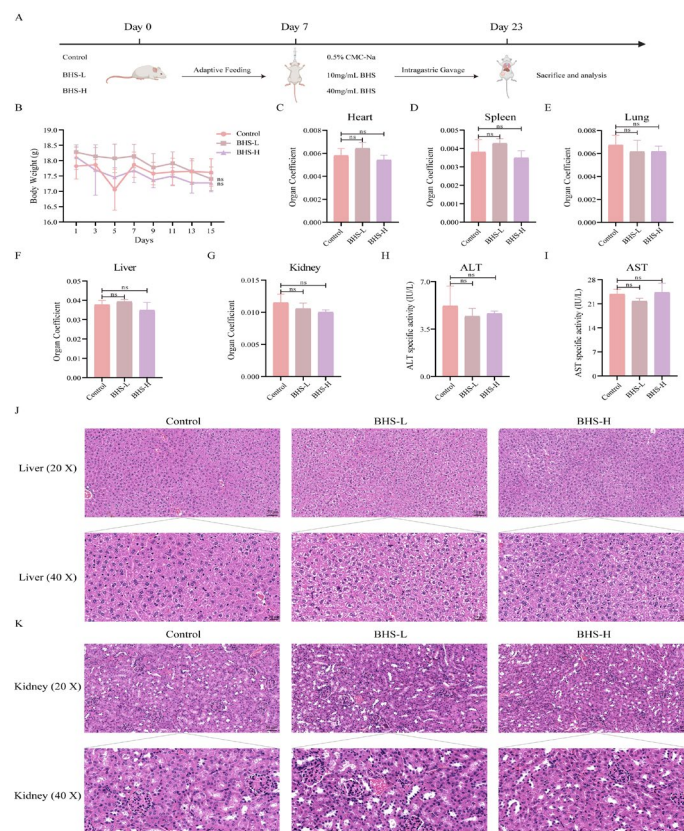


Figure 2: Safety evaluation of BHS in vivo. (A) Flowchart of the in vivo safety evaluation protocol in mice. (B) Body weight changes in mice of each group over the experimental period. (C) Heart index of mice in each group. (D) Spleen index of mice in each group. (E) Lung index of mice in each group. (F) Liver index of mice in each group. (G) Kidney index of mice in each group. (H) Analysis of serum ALT in each group. (I) Analysis of serum AST in each group. (J) H&E staining of liver tissues from each group. (K) H&E staining of kidney tissues from each group.

3.3 BHS exhibited anti-TNBC efficacy *in vivo*

To comprehensively evaluate the therapeutic potential of BHS against TNBC *in vivo*, a subcutaneous xenograft tumor model was established in BALB/c mice using 4T1 cells (Figure 3A). Notably, body weight monitoring throughout the treatment period showed no significant differences between the control group and either the BHS-treated or paclitaxel-treated groups ($P > 0.05$, Figure 3B). Additionally, compared with the control group, mice in the BHS-treated group showed no significant changes in organ coefficients ($P > 0.05$, Figure 3C and D) or histopathological abnormalities in liver and kidney tissues ($P > 0.05$, Figure 3E). These findings confirm that BHS exhibited a favorable safety profile at the tested concentrations over the 16-day treatment period.

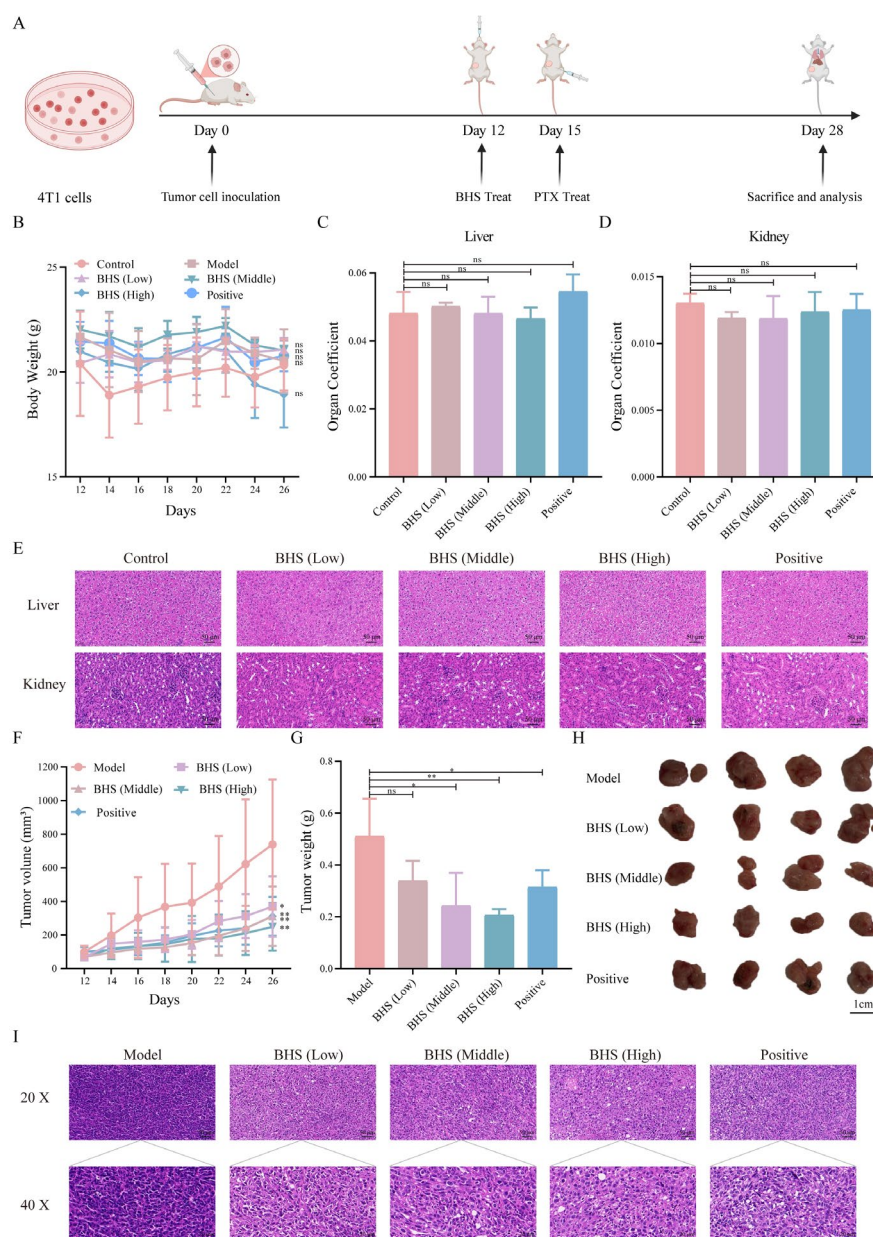


Figure 3: Anti-tumor effects of BHS on TNBC *in vivo*. (A) Schematic diagram of the breast cancer xenograft mouse model experiment workflow. (B) Body weight changes of mice in each group over the experimental period. (C) Liver index of mice in each experimental group. (D) Kidney index of mice in each experimental group. (E) H&E staining of liver and kidney tissues from each group. (F) Tumor volume dynamics in each group over the experimental period. (G) Comparative tumor weight across experimental groups. (H) Tumor morphology of mice in each group. (I) H&E staining of tumor tissues from each group. * $P < 0.05$, ** $P < 0.01$.

Initial tumor volumes were comparable among all groups at the start of treatment. After 16 days of treatment, the model group exhibited rapid tumor progression, while BHS treatment significantly suppressed tumor growth in a dose-dependent manner. Compared with the model group, the high-dose BHS group showed a $66.33 \pm 17\%$ reduction ($**P < 0.01$), the middle-dose BHS group exhibited a $57.75 \pm 21\%$ reduction ($**P < 0.01$), and the low-dose BHS group exhibited a $50.05 \pm 21\%$ reduction ($*P < 0.05$) in tumor volume (Figure 3F). Tumor weight quantification revealed significant reductions in the high-dose BHS (0.21 ± 0.02 g, $**P < 0.01$), medium-dose BHS (0.24 ± 0.11 g, $*P < 0.05$), and positive control (0.31 ± 0.06 g, $*P < 0.05$) groups, compared with the model group (0.51 ± 0.13 g) (Figure 3G and H). Histopathological analysis revealed that tumors in the model group maintained intact cellular architecture, whereas tumors from the positive control and BHS-treated groups exhibited dose-dependent cellular disorganization, structural damage, and necrosis, most notably in the high-dose BHS group (Figure 3I). Collectively, these findings demonstrate that BHS effectively inhibited TNBC progression *in vivo* with an acceptable safety profile at therapeutic doses.

3.4 Cell cycle-related genes were core mediators of BHS-mediated anti-TNBC activity

To investigate the underlying mechanisms of BHS against TNBC, transcriptome sequencing data from tumor tissues and MDA-MB-468 cells (both pre- and post-BHS intervention) were analyzed. PCA results of tumor tissues revealed clear separation between the model group and the BHS-H group, with PC1 accounting for 39.08% of the variance and PC2 explaining 21.85% (Figure 4A). Differential expression analysis identified 763 significantly upregulated and 1,161 downregulated genes in the BHS-H group compared to the model group ($P < 0.05$, Figure 4B). KEGG enrichment analysis demonstrated that these DEGs were notably enriched in pathways including DNA replication and the cell cycle ($P < 0.05$, Figure 4C).

Similarly, PCA results of MDA-MB-468 cells revealed distinct clustering between pre- and post-BHS intervention groups, with PC1 explaining 47% of the variance and PC2 contributing to 14.3% (Figure 4D). Differential expression analysis revealed that, relative to the control group, BHS significantly upregulated 1,345 genes and downregulated 1,359 genes ($P < 0.05$, Figure 4E). KEGG pathway enrichment analysis showed that these DEGs were significantly enriched in BC-related pathways, DNA replication, and lipid metabolism/atherosclerosis pathways ($P < 0.05$, Figure 4F). GO enrichment analysis identified 60 significantly enriched cellular component terms, 245 biological process terms, and 63 molecular function terms ($P < 0.05$, Supplementary Figure 1A).

To further validate the robustness of these findings, transcriptome sequencing data from 160 TNBC tissues and 572 healthy breast tissues were analyzed, and a total of 4,354 upregulated and 3,918 downregulated genes were identified in TNBC tissues compared with healthy tissues ($P < 0.05$, Figure 4G). Comparative analysis between these tissue-derived DEGs and those from the cellular transcriptome identified 544 overlapping candidate genes (254 upregulated, 290 downregulated), which may mediate the anti-TNBC effect of BHS (Figure 4H). KEGG enrichment analysis revealed that these candidate genes were significantly associated with 18 signaling pathways, with the most prominent involvement in cell cycle regulation and DNA

replication (Figure 4I). GO analysis further confirmed that these candidate genes were involved in fundamental biological processes, including DNA replication initiation, cell cycle control, and meiotic progression (Supplementary Figure 1B). Collectively, these findings identify a core set of molecular targets that may mediate the anti-TNBC effects of BHS, with a particular focus on cell cycle regulation pathway.

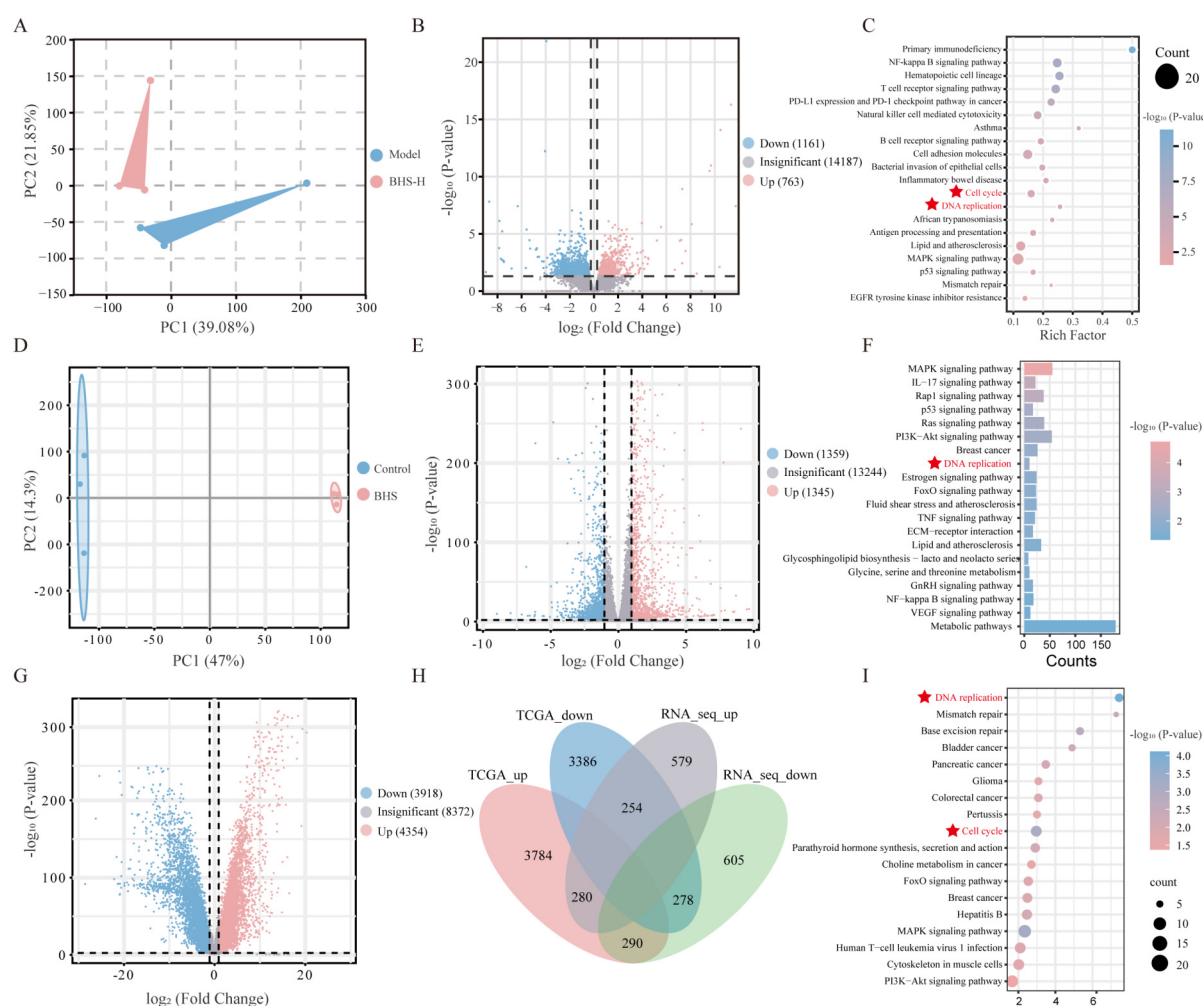


Figure 4: Integrated transcriptome sequencing and TCGA data analysis of BHS-mediated anti-TNBC activity. (A) PCA plot of transcriptome sequencing data from tumor tissues. (B) Volcano plot of differentially expressed genes (DEGs) identified by tumor tissue transcriptome sequencing. (C) Top 20 KEGG pathways enriched with DEGs from tumor tissue transcriptome sequencing. (D) PCA plot of transcriptome sequencing data from TNBC cells. (E) Volcano plot of DEGs identified by cell transcriptome sequencing. (F) Top 20 KEGG pathways enriched with DEGs from cell transcriptome sequencing. (G) Volcano plot of DEGs in TNBC tissues identified from the TCGA database. (H) Overlapping targets between transcriptome sequencing-derived DEGs and TCGA dataset-derived DEGs. (I) KEGG pathways enriched with the overlapping target genes.

3.5 BHS induced cell cycle arrest in both MDA-MB-231 and MDA-MB-468 cells

To further characterize the molecular mechanisms underlying BHS-mediated anti-TNBC activity, we examined its regulatory effects on the cell cycle of TNBC cells. Quantitative flow cytometric analysis revealed distinct phase-specific responses: MDA-MB-468 cells exhibited dose-dependent accumulation in the G2 phase, with the G2-phase proportion increasing from $18.45 \pm 0.34\%$ (untreated) to $20.68 \pm 0.33\%$ (20 μM BHS, $^*P < 0.05$) and $27.27 \pm 1.93\%$ (40 μM BHS, $^{**}P < 0.01$, Figure 5A and B). MDA-MB-231 cells exhibited dose-dependent G1-phase accumulation, rising from $31.00 \pm 7.15\%$ (untreated) to $47.35 \pm 2.67\%$ (30 μM

BHS, $^*P < 0.05$) and $54.38 \pm 0.73\%$ (60 μM BHS, $^*P < 0.05$, Figure 5C and D). To elucidate the molecular basis of these effects, the mRNA expression levels of cyclin dependent kinase 1 (*CDK1*), cell cycle protein D1 (*CCND1*), and cell cycle protein D3 (*CCND3*) were quantified [23]. qPCR analysis demonstrated that BHS treatment significantly downregulated the mRNA expression of all three genes compared with the untreated control group. Specifically, *CDK1* mRNA levels were reduced by 0.56 ± 0.13 - to 0.42 ± 0.10 -fold ($^{**}P < 0.01$), *CCND1* mRNA levels were reduced by 0.19 ± 0.06 - to 0.46 ± 0.13 -fold ($^*P < 0.05$ or $^{**}P < 0.01$), and *CCND3* mRNA levels were reduced by 0.42 ± 0.02 - to 0.41 ± 0.07 -fold ($^{***}P < 0.0001$ or $^{**}P < 0.01$, Figure 5E and F).

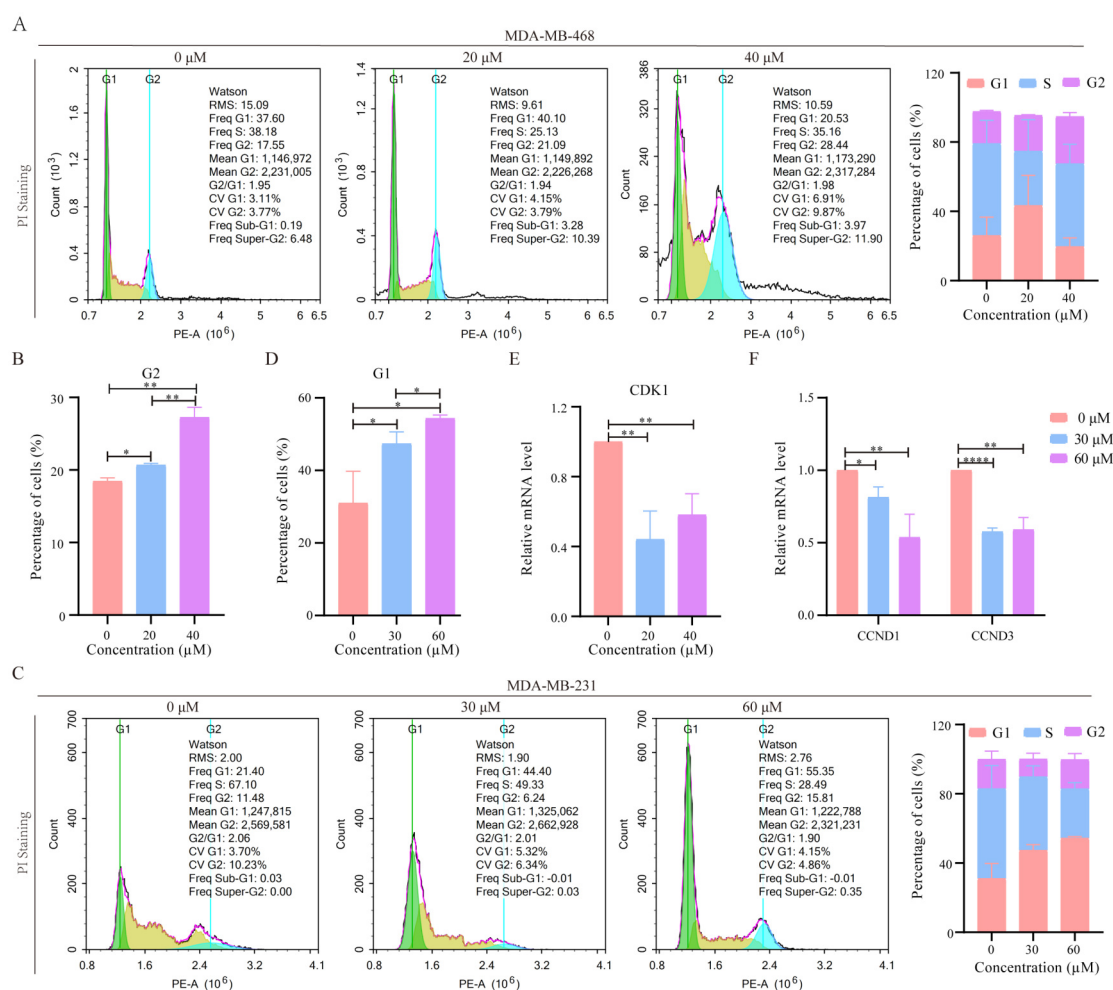


Figure 5: BHS induces cell cycle arrest in MDA-MB-468 and MDA-MB-231 cells. (A-B) Cell cycle distribution in MDA-MB-468 cells pre- and post-BHS treatment. (C-D) Cell cycle distribution in MDA-MB-231 cells pre- and post-BHS treatment. (E) mRNA expression of *CDK1* in MDA-MB-468 cells pre- and post-BHS treatment. (F) mRNA expression of *CCND1* and *CCND3* in MDA-MB-231 cells pre- and post-BHS treatment. $^*P < 0.01$, $^{**}P < 0.01$ or $^{***}P < 0.0001$.

3.6 Identification of core targets of BHS against TNBC

To identify the core targets of BHS against TNBC, the GEO database was integrated with topological and machine learning algorithms (Figure 6A). RNA-seq analysis of 55 TNBC and 11 healthy samples from the GSE65212 dataset identified 3,415 significantly upregulated and 2,721 significantly downregulated genes in TNBC tissues ($P < 0.05$, Figure 6B). Comparative analysis of these GEO-derived genes with the 87 previously identified DEGs yielded 42 candidate DEGs (Figure 6C). A PPI network was constructed via co-expression

analysis using Cytoscape software, comparing 35 target genes and 103 interaction edges. The importance of these targets was evaluated using six topological algorithms, including MCC, DMNC, MNC, Degree, EPC and Closeness. Results showed that *EXO1*, *PCNA*, *MCM2*, *MCM3*, *MCM5*, *CDC45*, *CCNE2*, *RFC3*, *POLE2*, *LMNB1*, *FGF2* and *PARP1* were consistently prioritized across all algorithms, indicating their high relevance to the anti-TNBC effects of BHS (Figure 6D). Six distinct machine learning algorithms (DT, GLM, KNN, NNET, RF, and SVM) were further employed to systematically analyze these 42 targets within the cellular transcriptome-TCGA-GEO network. This analysis further confirmed these 12 genes as core targets associated with BHS's therapeutic effects in TNBC, supported by their consistent prioritization across at least two independent analytical frameworks (Figure 6E).

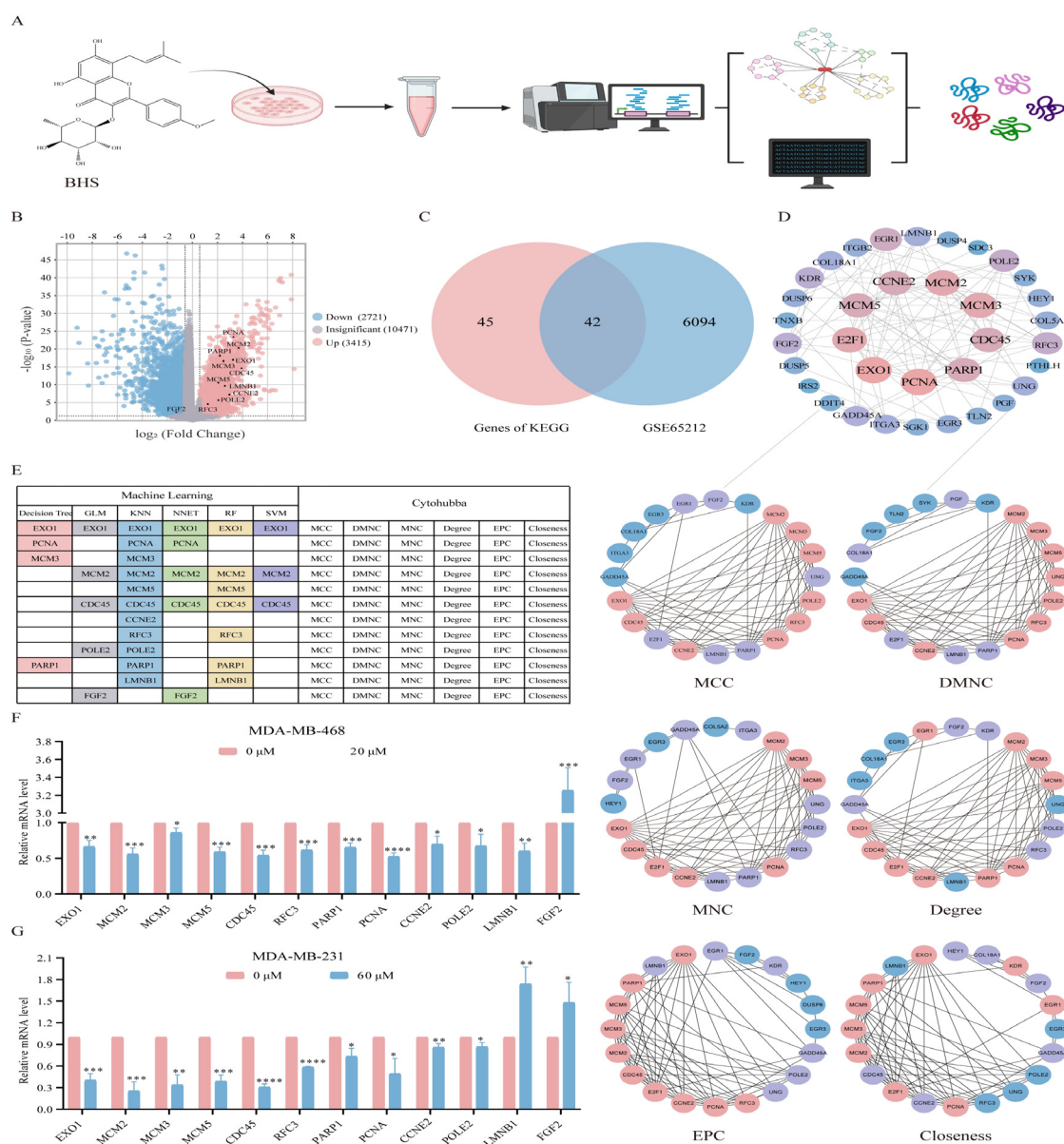


Figure 6: Integrative analysis of GEO data and machine learning to identify core targets of BHS against TNBC. (A) Flowchart of the core gene screening process. (B) Volcano plot of DEGs identified through GEO database. (C) Overlapping targets between KEGG pathway-enriched DEGs and GEO dataset-derived DEGs. (D) PPI network constructed by integrating the 42 overlapping genes and top 20 genes ranked by six cytoHubba algorithms. (E) Twelve core genes identified by combining six machine learning and cytoHubba algorithms. (F) mRNA expression of twelve core targets in MDA-MB-468 cells pre- and post-BHS treatment. (G) mRNA expression of twelve core targets in MDA-MB-231 cells pre- and post-BHS treatment. * $P < 0.01$, ** $P < 0.01$, *** $P < 0.001$ or **** $P < 0.0001$.

3.7 BHS significantly downregulated the expression of core targets in both MDA-MB-231 and MDA-MB-468 cell lines

Integrated analysis of cellular transcriptome analyses and publicly available clinical transcriptome data showed that BHS significantly downregulated 11 core genes and upregulated one core genes (Figure 4E and Figure 6B). qPCR validation demonstrated that compared with the untreated group, BHS significantly reduced the mRNA expression levels of ten genes (*EXO1*, *PCNA*, *MCM2*, *MCM3*, *MCM5*, *CDC45*, *CCNE2*, *RFC3*, *POLE2*, and *PARP1*) while increasing the expression of *FGF2* and *LMNB1* in both MDA-MB-468 and MDA-MB-231 cell lines ($^*P < 0.01$, $^{**}P < 0.01$, $^{***}P < 0.001$, or $^{****}P < 0.0001$, Figure 6F and G). Notably, due to the discrepancies in *LMNB1* expression between qPCR and transcriptome data, coupled with *FGF2*'s weak correlation with cell cycle regulation, the remaining ten core genes were selected for subsequent analysis. WB analysis further demonstrated significant decreases in the protein levels of these targets in both MDA-MB-468 and MDA-MB-231 cell lines following 24-hour BHS treatment, compared with the untreated group ($^*P < 0.05$, $^{**}P < 0.01$, or $^{***}P < 0.001$, Figure 7A-D). Consistent with *in vitro* findings, immunohistochemical analysis of *in vivo* tumor tissues demonstrated that, compared with the model group, BHS significantly reduced the protein levels of these ten targets, with the exception of *CCNE2* ($^*P < 0.05$, $^{**}P < 0.01$, or $^{***}P < 0.001$, Figure 8A-J). Additionally, Ki-67, a cell proliferation marker associated with TNBC prognosis^[24], exhibited reduced expression in BHS-treated tumors compared with the model group ($^{**}P < 0.01$ or $^{***}P < 0.001$, Figure 8K).

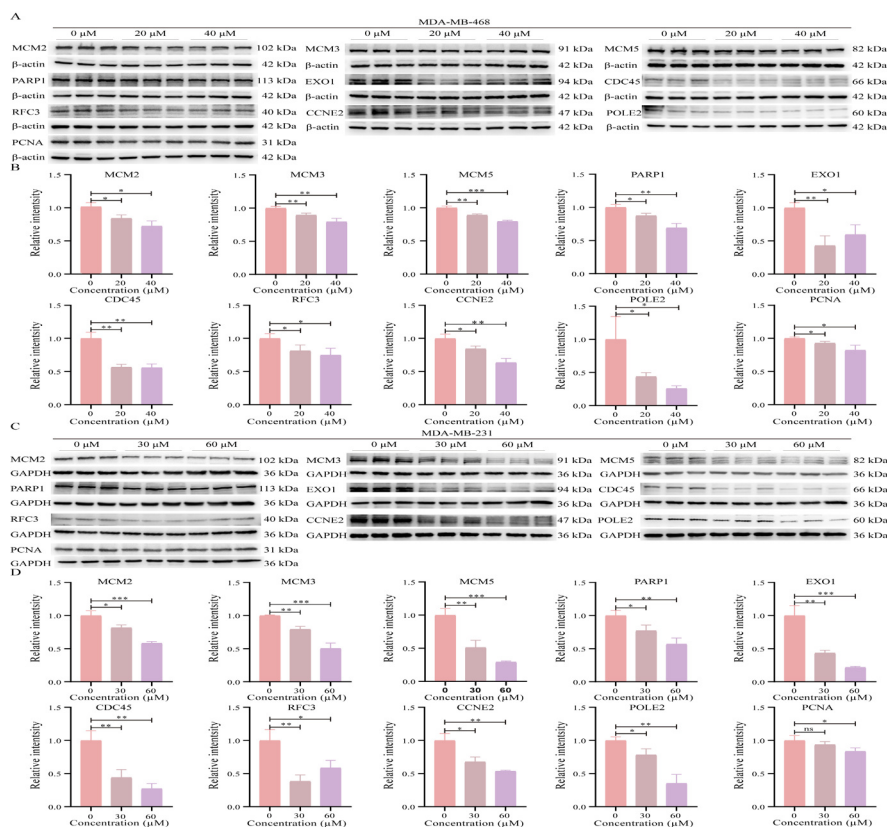


Figure 7: Protein expression of ten core proteins in MDA-MB-468 and MDA-MB-231 cells before and after BHS treatment. (A-B) Western blot analysis and quantitative results of ten core proteins in MDA-MB-468 cells pre- and post-BHS treatment. (C-D) Western blot analysis and quantitative results of ten core proteins in MDA-MB-231 cells pre- and post-BHS treatment. $^*P < 0.01$, $^{**}P < 0.01$ or $^{***}P < 0.001$.

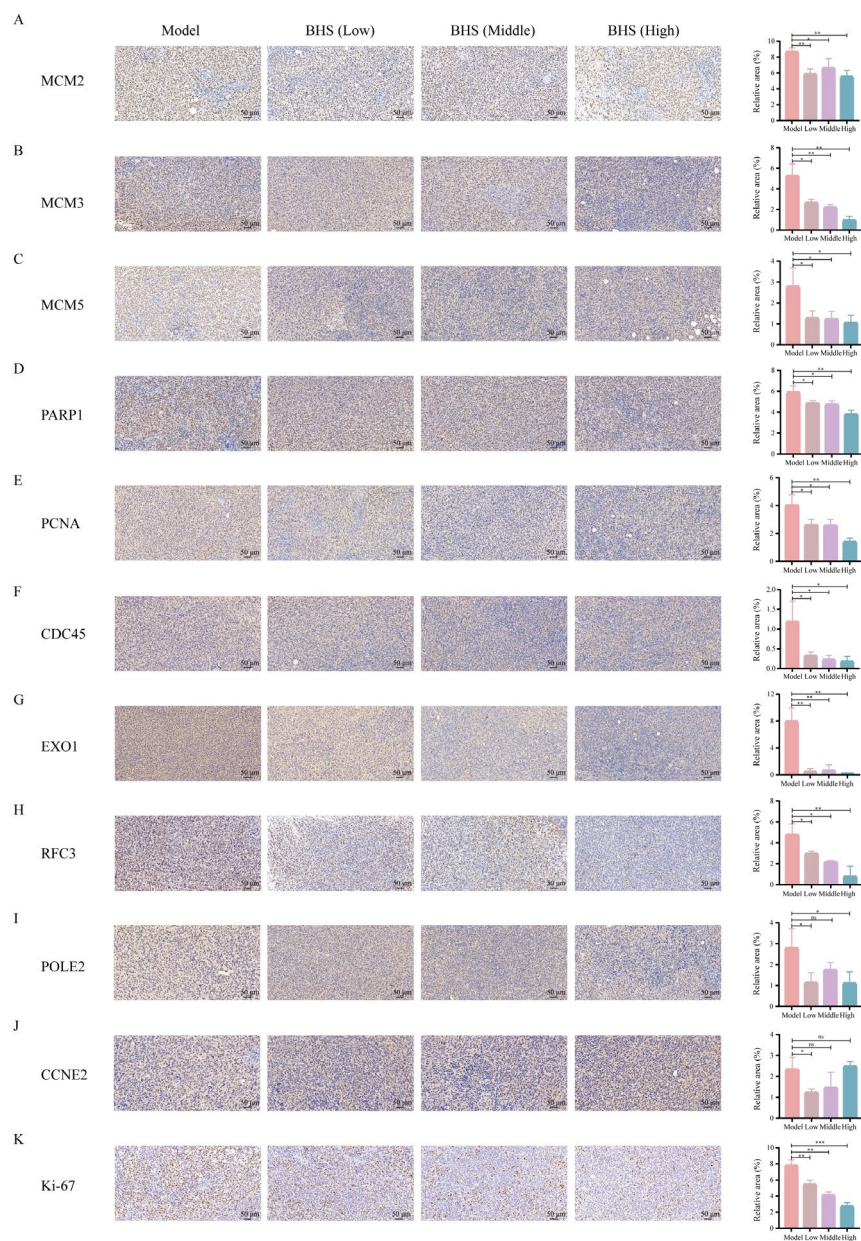


Figure 8: Immunohistochemical analysis of core protein expression across groups (n = 3, magnification, ×200). * $P < 0.05$, ** $P < 0.01$ or *** $P < 0.001$.

3.8 PMS2 was a candidate direct target of BHS against TNBC

To identify the direct targets of BHS against TNBC, a DARTS-MS assay using protein extracts from MDA-MB-468 cells was performed (Figure 9A). SDS-PAGE analysis revealed a distinct band within the 55–100 kDa range when comparing the BHS and DMSO groups at 0.25% Pronase E concentration (Figure 9B). These bands were excised for unbiased, high-throughput protein identification via mass spectrometry, which identified 580 proteins (55–100 kDa) with significantly higher abundance in the BHS-treated group than in the DMSO group ($P < 0.05$). These 580 proteins were integrated with the 10 previously identified core targets for PPI network analysis, yielding 85 proteins associated with these core targets. Of these 85 proteins, 23 were involved in cell cycle regulation and DNA replication. Notably, only PMS2 showed a kurtosis value of 0 in the DMSO group, with a significant increase observed in the BHS-coincubated group. Thus, PMS2 was identified as a candidate direct target of BHS (Figure 9C).

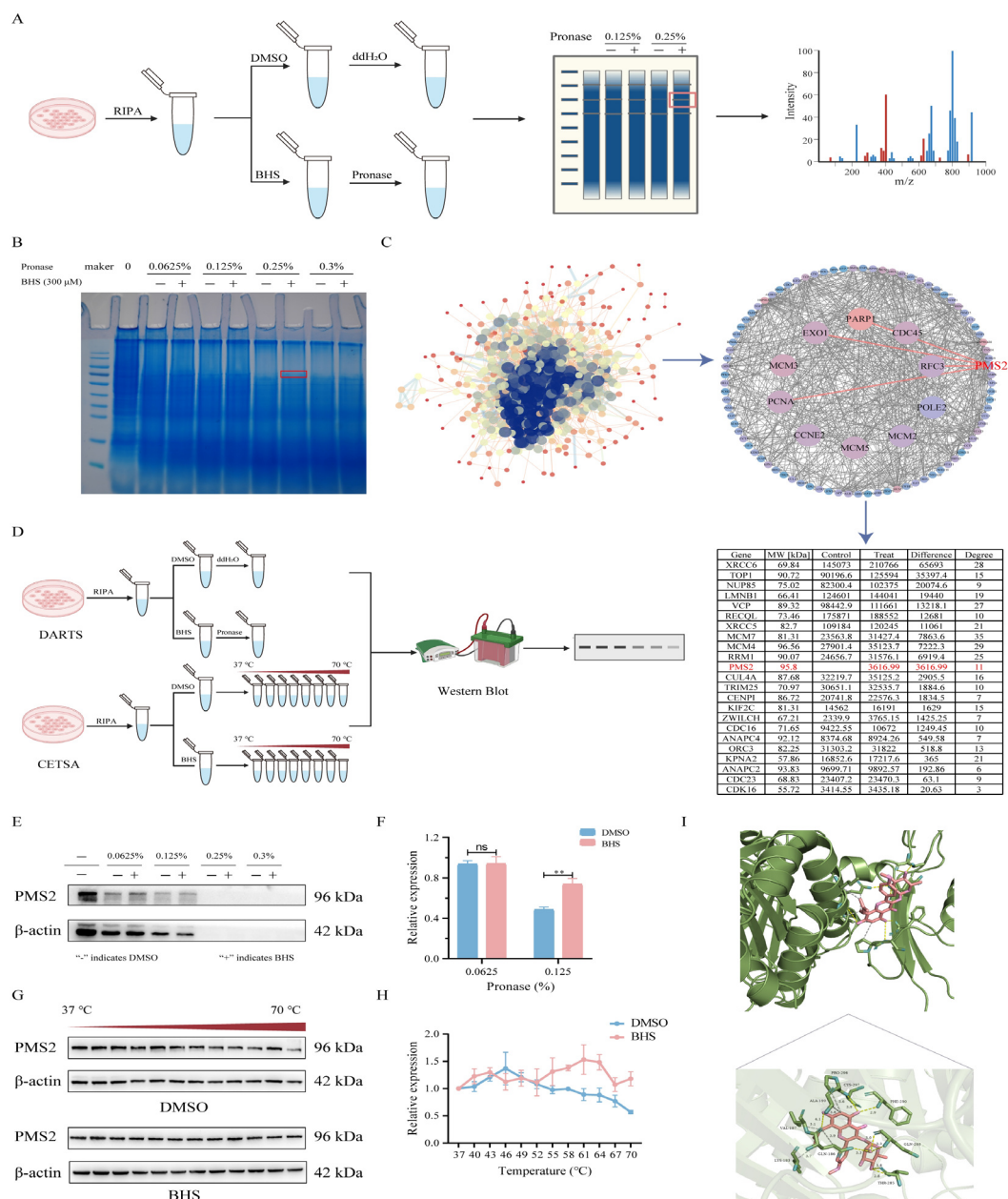


Figure 9: PMS2 was a candidate direct target of BHS against TNBC. (A) Schematic diagram of the DARTS-MS workflow. (B) Coomassie blue staining of SDS-PAGE gels from the DARTS assay. (C) PPI network of 55-100 kDa proteins identified by DARTS-MS. (D) Schematic workflow of DARTS-WB and CETSA-WB. (E) Western blot results of the DARTS assay. (F) Quantitative analysis of band intensities from DARTS-WB assay. (G) Western blot results of the CETSA assay. (H) Quantitative analysis of band intensities from CETSA-WB assay. (I) Molecular docking of the BHS-PMS2 complex, showing details of the binding site and key interaction modes (e.g., hydrogen bonds). ** $P < 0.01$.

To further validate this finding, DARTS-WB and CETSA-WB assays were performed using proteins extracts from MDA-MB-468 cells (Figure 9D). DARTS-WB quantitative analysis demonstrated that BHS bound strongly to PMS2 at 0.125% Pronase E, thereby reducing PMS2 degradation (** $P < 0.01$) (Figure 9E and F). CETSA-WB quantitative analysis demonstrated that BHS significantly increased PMS2 accumulation at 58-70°C compared with the DMSO group, indicating its ability to enhance PMS2 thermal stability (** $P < 0.01$ or * $P < 0.05$) (Figure 9G and H). The BHS-PMS2 interaction was further investigated via molecular docking, which predicted binding affinity and identify critical amino acids involved in the binding interface. The analysis revealed a binding affinity of -8 kcal/mol between BHS and PMS2, with complex stabilization

mediated by eight hydrogen bonds and five hydrophobic interactions. Structural analysis further identified the O3, C27, C23, and C9 moieties of BHS as key contributors to the binding interaction (Figure 9I). Collectively, these molecular docking results corroborated the findings from CETSA-WB and DARTS-WB assays, confirming that PMS2 is a putative direct binding target of BHS. Therefore, we hypothesize that BHS regulates the 10 genes associated with DNA repair and cell cycle processes through PMS2, thereby inhibiting cell cycle progression in triple-negative breast cancer cells.

4. Discussion

Despite significant advancements in early detection and therapeutic strategies, TNBC remains a major global health challenge. Its complex pathogenesis and heterogeneous clinical manifestations hinder the development of effective treatment and prevention strategies [25]. TCM has a long-standing history of application in cancer treatment in China, with thousands of years of clinical practice [26]. Notably, accumulating evidence clearly demonstrates that TCM can improve survival outcomes and reduce mortality risk in TNBC patients, highlighting its substantial potential for TNBC treatment [27, 28].

The cell cycle is a vital biological process, whose precise regulation is essential for normal cell growth, division, and accurate segregation of genetic material. Studies have demonstrated that cell cycle dysregulation is a central driver of TNBC development and progression. In TNBC cells, the ordered progression through the G1, S, G2, and M phases is frequently disrupted, leading to uncontrolled proliferation [29]. Overactivation of key regulators such as cyclins and cyclin-dependent kinases, enables cancer cells to evade critical checkpoints, allowing them to continue dividing despite inhibitory signals, DNA damage, or defective spindle assembly. This loss of cell cycle control accelerates tumor growth, promotes genetic instability accumulation, and drives tumor evolution and metastasis. In TNBC, inducing G1 phase blockade prevents cells from entering the DNA synthesis phase, thereby inhibiting tumor cell proliferation. This provides a time window for DNA repair and may prompt irreparably damaged cells to undergo apoptosis, thereby suppressing tumor growth. Conversely, inducing G2 phase blockade prevents cells that have completed DNA replication from entering mitosis, halting the division of cells with damaged DNA or chromosomal abnormalities. This helps maintain genomic stability and may lead to apoptosis or senescence in cells with failed repair [30]. Therefore, targeted induction of G1 or G2 phase arrest represents a key therapeutic strategy for TNBC, aimed at inhibiting uncontrolled proliferation and promoting cancer cell death.

Our present study corroborates previous findings while providing new insights into the effects of BHS on TNBC cells. BHS significantly inhibits cell cycle progression in MDA-MB-468 and MDA-MB-231 cells, exerting phase-specific regulatory effects on both the G1 and G2 phases. However, despite considerable research on BHS, a systematic bibliometric analysis integrating transcriptomic profiling, bioinformatics, and off-label drug target screening has remained lacking, limiting a comprehensive understanding of its anti-TNBC mechanisms. In this study, we integrated these approaches and identified MCM2, MCM3, MCM5, EXO1, PARP1, CDC45, POLE2, RFC3, PCNA, and CCNE2 as core candidate targets of BHS against TNBC. Notably, the association between these targets and BHS has not been previously reported, underscoring the

utility of the cytoHubba algorithm, machine learning, and DARTS-MS based label-free chemical proteomics in uncovering critical therapeutic targets. Furthermore, publicly available external clinical sequencing data demonstrated significant upregulation of these ten core targets in both TNBC and invasive breast carcinoma tissues relative to healthy controls. qPCR and WB analyses demonstrated that BHS significantly downregulated the expression of these targets at both the mRNA and protein levels, thereby regulating the cell cycle of TNBC cell lines and inducing cancer cell death.

The initial step of DNA replication involves the unwinding of double-stranded DNA by the CMG complex. Composed of CDC45, Go-Ichi-Ni-San (GINS) and the MCM complex hexamer, the CMG complex is the key regulator of DNA replication initiation in eukaryotic cells. Loss-of-function of CDC45 inhibits cell proliferation and leads to cell death due to inhibition of DNA replication and G1-phase arrest^[31]. The MCM complex, a core component of the pre-replication machinery, is essential for various cellular processes, such as DNA replication initiation, damage repair, and checkpoint regulation, in which it plays a critical role^[32]. The MCM2-7 subunits assemble into a heterohexameric complex that serves as the final component loaded onto DNA replication origins. The association of CDC45 with MCM is a crucial step for forming the pre-initiation complex, which eventually leads to the unwinding of the DNA duplex and the assembly of replicative DNA polymerases onto unwound origins^[33]. The replication factor C (RFC) complex, comprising five subunits including RFC3, participates in DNA replication, damage repair, and cell cycle regulation^[34–36]. Overexpression of RFC3 in TNBC promotes cell proliferation and migration, while its suppression attenuates these processes and xenograft progression^[37]. PCNA, which is abundant in proliferating and cancer cells, serves as both a marker for cell proliferation and an indicator of tumor differentiation, stage, and prognosis^[38]. By exploiting the functions of both proteins, it is possible to distinguish the newly synthesized DNA strand from the template strand. EXO1 is overexpressed in TNBC and regulates cell cycle checkpoints, with the capacity to induce double-strand breaks, cell cycle arrest, or cell death^[39, 40]. It excises segments of single-stranded DNA containing mismatched bases that are recognized by PCNA and RFC3. POLE2 is overexpressed in BC, colorectal, and cervical carcinomas, where it influences DNA replication, repair, and cell cycle regulation^[41]. PARP1, a key member of the PARP family, mediates DNA repair and cell survival^[42]. Its knockdown disrupts tumor-associated gene expression, impairs DNA damage repair, and reduces tumor sphere formation in BC cells^[43]. CCNE2, a member of the cyclin E family, functions as a key regulator of cell cycle progression by controlling the G1/S phase transition. Its upregulation has been reported in multiple malignancies, such as BC and lung cancer. Importantly, in lymph node-negative BC patients, high CCNE2 correlate with significantly poorer clinical outcomes^[44]. CETSA and DARTS analyses identified PMS2 as a putative direct binding target of BHS in TNBC treatment, a finding that has not been reported in previous studies. The PPI network demonstrated robust interactions between PMS2 and four core proteins (EXO1, PCNA, RFC3, and PARP1), suggesting a potential mechanism by which BHS may downregulate the expression of EXO1, PCNA, RFC3, PARP1, and their respective downstream target proteins via potential binding to PMS2, thereby inducing cell cycle arrest in TNBC cells (Supplementary Figure 2).

In summary, these findings demonstrate that BHS effectively induces the cell cycle arrest, inhibits proliferation, and suppresses migration in TNBC cells. These anti-tumor effects are mediated by BHS's potential binding to PMS2, which in turn downregulates the expression of EXO1, PCNA, RFC3, PARP1, and their respective downstream target proteins. Importantly, in our *in vivo* experimental settings, BHS exhibited no significant hepatorenal toxicity at the administered dose. Collectively, these results not only support the potential therapeutic application of BHS but also provide a methodological framework for investigating bioactive natural compounds in TCM.

Declaration of interests

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.

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