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Mechanisms of huajiao in alleviating *Shigella flexneri*-induced colitis via NF- κ B/TNF- α pathway

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ABSTRACT: Huajiao, a medicinal and edible spice, is widely recognized for its potential in alleviating intestinal inflammation. This study investigated the antibacterial activity of five plant parts from three huajiao cultivars against *S. flexneri*, identifying the pericarp of the “Dahongpao” variety as the most potent. Untargeted metabolomic profiling combined with chemometric analysis revealed significant differences in metabolite composition among pericarp extracts prepared with solvents of varying polarity ($P < 0.05$). Partial least squares (PLS) modeling highlighted several compounds—nepetin 7-glucoside, quercetin 3-O-malonylglucoside, quercitrin, guaiaiverin, and hyperoside—as likely contributors to the observed antibacterial effects. Network pharmacology and molecular docking further identified TNF, NF- κ B1, BCL2, CASP3, and EGFR as potential molecular targets of the active compounds. Gene Ontology (GO) and KEGG enrichment analyses indicated that the NF- κ B/TNF- α signaling pathway plays a central role in mediating the anti-inflammatory response. In a murine model of *S. flexneri*-induced enteritis, treatment with huajiao extracts alleviated colonic inflammatory responses, increased goblet cell numbers, and significantly reduced colonic mRNA and protein levels of TNF- α , IL-1 β , and IL-6 ($P < 0.01$). Western blot analysis confirmed decreased phosphorylation of I κ B α and NF- κ B p65, indicating inhibition of NF- κ B activation and alleviation of intestinal inflammation. Collectively, these results indicate that huajiao is a functional food source with potential for alleviating colitis.

Keywords: huajiao, colitis, untargeted metabolomics, network pharmacology, in vivo validation

1. Introduction

Huajiao (*Zanthoxylum* spp.), a plant of the *Rutaceae* family, is widely distributed in China and other Asian countries, including Japan, India, and Korea [1,2]. Traditionally used as both a culinary spice and medicinal herb, huajiao (Sichuan pepper) imparts a unique numbing flavor to dishes while holding a long-standing place in Chinese medicine. Ancient texts such as *Ming Yi Bie Lu* (Supplementary Records of Famous Physicians) describe its use in treating abdominal cold, typhoid fever, intermittent chills, anhidrotic wind syndromes, fluid retention, indigestion, and diarrhea caused by intestinal infections. Likewise, the

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Compendium of Materia Medica highlights its effects in dispersing cold and dampness, relieving stagnation, promoting digestion, regulating qi in the triple burner, warming the spleen and stomach, tonifying the kidneys and mingmen (the "gate of life"), expelling intestinal parasites, and stopping diarrhea—emphasizing its traditional role in gastrointestinal therapy [3]. Contemporary studies have validated several of these traditional claims. Kubota [4] showed that Dajianzhong decoction (TU-100), which includes huajiao, alleviates abdominal pain, intestinal obstruction, and erosive gastritis in isolated rat colon and murine colitis models. These effects are attributed to hydroxy- α -sanshool, a key bioactive compound that activates TRPA1 channels in the colon and enteric nervous system. Similarly, Hong [5] demonstrated that huajiao essential oil suppresses *Escherichia coli* growth and enhances beneficial gut microbiota, supporting its role in maintaining intestinal health. Collectively, both historical usage and modern evidence highlight huajiao's therapeutic potential for preventing and managing intestinal disorders, offering a compelling link between traditional medicine and contemporary pharmacology.

In addition to its pericarp, other parts of huajiao—including the seeds, leaves, roots, and stems—are also rich in bioactive compounds. Lu [6] identified more than 150 constituents from the roots and stems of *Zanthoxylum nitidum* (Roxb.) DC., primarily comprising alkaloids, flavonoids, coumarins, lignans, and terpenes. Among them, nitidine chloride (NC) has been recognized as a key pharmacological marker for the quality control of traditional Chinese medicines, due to its antitumor, anti-inflammatory, and antibacterial properties [7]. Similarly, Zhang [8] identified 27 phenolic compounds in huajiao leaves, including abundant levels of isoquercitrin, hyperoside, chlorogenic acid, quercitrin, myricetin, and ferulic acid.

Shigella flexneri-induced colitis is a major global cause of diarrheal disease, marked by bloody diarrhea, fever, and abdominal pain [9]. It accounts for an estimated 270 million cases annually, predominantly in children under five, and causes approximately 210,000 deaths each year—ranking second only to rotavirus in diarrheal mortality [10]. *Shigella flexneri* has been found to infect a variety of foods such as salads, shrimp and sandwiches for a long time [11,12]. Therefore, colitis induced by human infection with *Shigella flexneri* due to food contamination has become a significant hidden danger. Interest in plant-based therapeutics has grown, driven by their safety, low cost, and environmental compatibility. These natural compounds present a promising avenue for treating shigellosis and addressing antibiotic resistance [13,14].

Given this diverse phytochemical profile, huajiao holds considerable promise for the treatment of *Shigella flexneri*-induced diarrhea. This study systematically evaluates its therapeutic potential through four integrated phases: (1) In vitro antibacterial screening of extracts from three Chinese huajiao cultivars, using various plant parts and solvents with different polarities to assess activity against *S. flexneri*. (2) Untargeted metabolomic profiling combined with chemometric analysis to construct PLS models for identifying candidate anti-*S. flexneri* phytochemicals, based on differential antibacterial activity among solvent fractions. (3) Mechanistic prediction using network pharmacology and molecular docking to identify potential in vivo targets of the active compounds. (4) In vivo validation of anti-diarrheal efficacy in a murine model of *S. flexneri* infection.

2. Materials and Methods

2.1 Materials and reagents

2.1.1 Plant material

The ‘Gongjiao’ red huajiao was supplied by the Sichuan Hanyuan Shanyou Gongjiao Planting Cooperative. The ‘Dahongpao’ red huajiao was obtained from the Shaanxi Hancheng Kangjin Dahongpao Huajiao Base. The ‘Jiuyeqing’ green huajiao was provided by Chongqing Kaiyang Agricultural Development Co., Ltd.

2.1.2 Bacterial strain

Shigella flexneri ATCC 12022 was obtained from the China Center of Industrial Culture Collection (Beijing, China).

2.1.3 Chemicals and media

Ethanol, sodium chloride, petroleum ether, trichloromethane, ethyl acetate, and n-butanol, all of analytical grade, were acquired from Chengdu Chuandong Chemical Company. Dimethyl sulfoxide (DMSO), also of analytical grade, was sourced from Shanghai Macklin Co., Ltd. Methanol and formic acid, both of HPLC grade, were purchased from Shanghai Macklin Co., Ltd. Nutrient broth and Mueller-Hinton broth media were obtained from Beijing Aoboxing Co., Ltd.

For the Western blotting experiments, primary antibodies— β -Actin (rabbit-derived, cat. no. GB15003), p65 (mouse-derived, cat. no. GB12142), I κ B α (mouse-derived, cat. no. GB151509), p-I κ B α (rabbit-derived, cat. no. GB15212), p-p65 (rabbit-derived, cat. no. GB113882), and TNF- α (rabbit-derived, cat. no. GB11188-100)—were used, all provided by Servicebio Co., Ltd. (Wuhan, China). Secondary antibodies, including HRP-conjugated goat anti-rabbit IgG (cat. no. GB23303) for β -Actin, p-I κ B α , p-p65, and TNF- α , and HRP-conjugated goat anti-mouse IgG (cat. no. GB23301) for p65 and I κ B α , were also employed.

2.2 Activation of strains

S. flexneri was inoculated into nutrient broth and incubated at 37°C with shaking at 120 rpm for 24 h, as described by Song ^[15]. The culture was then centrifuged at 4°C and 5000 $\times$ g for 10 min. The cell pellet was washed twice with sterile normal saline (0.85%, v/v), and the bacterial suspension was diluted and adjusted to a 0.5 McFarland standard (approximately 1×10^8 CFU/mL) by turbidimetry for further use.

2.3 Preparation of huajiao extract

The pericarps, seeds, leaves, stems, and roots of three huajiao varieties—Jiuyeqing, Dahongpao, and Gongjiao—were dried in a 40°C oven, then ground using a crusher (YF-111B, Xi'an Yongli Pharmaceutical Machinery Co., Ltd., Xi'an, China) and sieved through a 40-mesh filter to obtain coarse powder. The powder was sealed and stored in a -40°C freezer for later use. Following Ren ^[16], 10 g of each powdered sample (pericarps, seeds, leaves, stems, and roots) was weighed and soaked in 60 mL of 75% ethanol. The mixture was shaken at 120 rpm for 27 h at 35°C. The supernatant was filtered through a Buchner funnel, and the remaining residue was subjected to ultrasound-assisted extraction with 50 mL of 75% ethanol at 40 kHz for 30

min. After 10 min of standing, the supernatant was filtered again. This extraction procedure was repeated twice, and the collected clear liquid was evaporated using a rotary evaporator (R-215, BUCHI, Switzerland) at 45°C. Finally, the extract was freeze-dried (Scientz-30F/A, Ningbo Xinzhi Freeze-Drying Equipment Co., Ltd., Zhejiang, China) to remove moisture, yielding the ethanol fraction, which was stored at -20°C for future use.

2.4 Extraction of pericarp fractions by solvent polarity

The ethanol extract of huajiao pericarp, obtained in step 2.3, was suspended in pure water and sequentially extracted with petroleum ether, trichloromethane, ethyl acetate, and n-butanol, each for three cycles at a 1:1 volume ratio. The extract solutions were concentrated under reduced pressure, yielding five fractions: petroleum ether, trichloromethane, ethyl acetate, n-butanol, and water. The fractions were freeze-dried, and their yields were determined by weighing. Finally, the fractions were stored at -20°C for future use.

2.5 Evaluation of huajiao extract's antibacterial activity against *S. flexneri*

2.5.1 Antibacterial activity assay

In accordance with the method of Liu ^[17], 1.2 g of freeze-dried huajiao extract powder was weighed and dissolved in 3 mL of dimethyl sulfoxide to prepare a 400 mg/mL stock solution. A 300 µL aliquot of the stock was transferred to a sterile 10 mL centrifuge tube and combined with 5.7 mL of Mueller–Hinton (MH) agar cooled to approximately 60°C. After thorough mixing, the solution was poured into a Petri dish to produce a drug-containing plate with a final concentration of 20 mg/mL. Control plates were prepared identically, excluding the huajiao extract and including 5% dimethyl sulfoxide. Following solidification, 20 µL of bacterial suspension (5×10^5 CFU/mL) was uniformly spread onto each plate. The plates were then incubated at 37°C for 24 h, and bacterial growth was subsequently evaluated.

2.5.2 Determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

As outlined in Section 2.5.1, drug-containing plates were prepared with final concentrations of 40, 20, 10, 5, 2.5, and 1.25 mg/mL. After solidification, 20 µL of bacterial suspension (5×10^5 CFU/mL) was uniformly spread onto each plate. The plates were incubated at 37°C for 24 h, and bacterial growth was subsequently assessed. The lowest concentration showing no visible bacterial growth was defined as the MIC. Plates containing concentrations at or above the MIC were further incubated for 24 h to evaluate bactericidal activity. The MBC was determined as the lowest concentration at which no bacterial growth was observed after the extended incubation period for *S. flexneri*.

2.5.3 Determination of inhibition rate

Drug-containing plates were prepared at specified concentrations. A 35 µL aliquot of bacterial suspension ($\sim 5 \times 10^3$ CFU/mL) was uniformly applied to each plate. After incubation at 37 °C for 24 h, colony counts were recorded. The inhibition rate was calculated using Equation (1) ^[18]:

$$x = \frac{C_{ck} - C_t}{C_{ck}} \times 100\% \quad (1)$$

where x represents the inhibition rate (%), C_{ck} is the colony count in the control group (CFU), and C_t is the colony count on the drug-containing plate (CFU).

2.6 Untargeted metabolomics analysis

The Untargeted metabolomics was entrusted to Guangzhou Kigenio Biotechnology Co., Ltd. for completion. Ultra-performance liquid chromatography (UPLC) was performed using a 1290 Infinity LC system (Agilent Technologies) coupled with a quadrupole time-of-flight mass spectrometer (Triple-TOF 6600, AB Sciex, Shanghai). Hydrophilic interaction chromatography (HILIC) was carried out using a 2.1 mm $\times$ 100 mm ACQUIY UPLC BEH column (1.7 μ m; Waters, Ireland). Analyses were conducted in both positive and negative electrospray ionization (ESI) modes. The mobile phase consisted of 25 mM ammonium acetate and 25 mM ammonium hydroxide in water (phase A) and acetonitrile (phase B). The gradient began with 85% B for 1 min, decreased linearly to 65% over 11 min, then to 40% in 0.1 min, held for 4 min, and finally increased to 85% in 0.1 min. A re-equilibration period of 5 min was applied. ESI source parameters were set as follows: Ion Source Gas 1 and 2 at 60 psi, curtain gas at 30 psi, source temperature at 600 $^{\circ}$ C, and IonSpray Voltage Floating (ISVF) at $\pm$ 5500 V. For MS-only acquisition, data were collected over an m/z range of 60–1000 Da with a TOF MS accumulation time of 0.20 s per spectrum. For auto MS/MS acquisition, the m/z range was set to 25–1000 Da with a product ion scan accumulation time of 0.05 s per spectrum. Product ions were acquired using information-dependent acquisition (IDA) in high-sensitivity mode. The collision energy (CE) was fixed at 35 V with a spread of $\pm$ 15 eV, and the declustering potential (DP) was set at +60 V (positive mode) and –60 V (negative mode). Isotopes within 4 Da were excluded, and up to 10 candidate ions were selected per cycle. The normalized data was used to predict the molecular formula based on additive ions, molecular ion peaks and fragment ions and then peaks were matched with the company's self-built database to obtain the accurate qualitative and relative quantitative results.

All samples were maintained in a 4 $^{\circ}$ C autosampler. To minimize variability due to instrumental drift, samples were analyzed consecutively in randomized order. Quality control (QC) samples, prepared by pooling equal volumes from all test samples, were interspersed throughout the run to monitor system stability and ensure data reliability.

2.7 Network pharmacology analysis

2.7.1 Identification of metabolite targets

Using the PLS model generated from untargeted metabolomics, the top 50 metabolites associated with the inhibition of *S. flexneri* by huajiao pericarp were identified in both positive and negative ion modes, resulting in a total of 100 metabolites. Their corresponding SMILES structures were retrieved from PubChem (<https://pubchem.ncbi.nlm.nih.gov/>). These SMILES strings were then submitted to SwissTargetPrediction (<http://www.swisstargetprediction.ch/>) and Super-PRED (<https://prediction.charite.de/>). Predicted targets were integrated, and duplicates were removed to generate a final set of potential targets^[19].

2.7.2 Collection of antibacterial targets

Antibacterial targets associated with *Shigella flexneri* were identified using the keyword “*Shigella flexneri*” and extracted from the GeneCards (<https://www.genecards.org/>) and OMIM (<https://www.omim.org/>) databases [20].

2.7.3 Identification of overlapping targets and construction of PPI network

Overlapping targets were identified through Venn diagram analysis using the OmicShare platform (<https://www.omicshare.com/>). These targets were entered into the STRING database (<https://cn.string-db.org/>), with an interaction score threshold of 0.4 applied [21]. Isolated nodes were removed to generate the protein–protein interaction (PPI) network. Network topology was analyzed in Cytoscape 3.8.1 using the cytoNCA plugin. Core target genes were identified based on degree centrality, betweenness centrality, and closeness centrality [22].

2.7.4 KEGG pathway and GO enrichment analysis

The overlapping targets from Section 2.7.3 were submitted to the Metascape database (<http://metascape.org>) [23], with *Homo sapiens* selected as the reference species. KEGG pathway and Gene Ontology (GO) enrichment analyses were conducted to explore potential targets of huajiao pericarp involved in *S. flexneri* inhibition [24]. Visualization and bubble charts of KEGG and GO results were generated using the Weishengxin platform (<https://www.bioinformatics.com.cn/>).

2.8 Molecular docking simulation

Molecular docking between key active compounds from huajiao pericarp and core target proteins was conducted using AutoDock Tools 1.5.7. Ligand structures for 22 active compounds were acquired in MOL format from TCMSP (<https://old.tcmssp-e.com/tcmssp.php>) and PubChem (<https://pubchem.ncbi.nlm.nih.gov/>). Receptor structures for five key target proteins were retrieved from the Protein Data Bank (<https://www.rcsb.org/>), with the following specifications: BCL2 (PDB ID: 8HTR, 1.60 Å), CASP3 (PDB ID: 1NME, 1.60 Å), EGFR (PDB ID: 3POZ, 1.50 Å), TNF (PDB ID: 5M2J, 1.90 Å), and NF-κB1 (PDB ID: 8TQD, 2.02 Å). All protein structures were determined via X-ray crystallography and selected on the basis of high resolution as well as relevance to the pathway under investigation. Prior to molecular docking, protein structure preparation was performed using AutoDock Tools 1.5.7, including water molecule removal, hydrogen atom addition, Gasteiger charge assignment and missing residue repair. For ligand preparation, geometric optimization, charge distribution and rotatable bond definition were carried out sequentially. Both proteins and ligands were converted into PDBQT format for subsequent docking simulations. To verify the reliability of the established method, the root-mean-square deviation (RMSD) values between the docked conformations and the crystal conformations were calculated using PyMOL 3.3.1.1. The binding energy (ΔG , kcal/mol) and interaction modes of the docking results were analyzed by AutoDock Tools, and visualization diagrams were generated with Discovery Studio 4.5 to illustrate the binding patterns and non-covalent interactions.

2.9 Animal experiments

Approval for the animal experiments was granted by the Ethics Committee of the Laboratory Animal Center, Southwest University (IACUC-20250403-02). The 20 g, 6-8-week-old SPF male BALB/c mice used in this experiment were purchased from Beijing Sibeifu Company. The experimental environment was maintained at a temperature of 25°C, a relative humidity of 45-55%, and a 12 h light-dark cycle. Before the experiment began, the mice were freely fed with commercially available sterile mouse chow and sterile water and were acclimated to the environment for 7 d. The concentration of *Shigella flexneri* that induced diarrhea was determined through a pre-experiment. The dose of huajiao extract was determined by the acute oral toxicity test - limit test method in GB 15193.3-2014. The results showed that no deaths or abnormal behaviors occurred in the mice fed at a concentration of 500 mg/kg for 7 d in this study.

2.9.1 Establishment of *Shigella flexneri*-induced mouse enteritis model

A *Shigella flexneri* suspension (3×10^9 CFU/mL, plate count method) was prepared for the induction of enteritis in mice. BALB/c mice, fasted for 6 h, were randomly allocated to control and experimental groups. The control group was administered 0.2 mL of sterile normal saline, while the experimental group was orally gavaged with 0.2 mL of the bacterial suspension. Treatments were performed once daily for 7 consecutive days. Diarrhea severity was evaluated every 24 h. Diarrhea was defined as the occurrence of loose or watery stools, and successful modeling was confirmed in mice displaying persistent symptoms for three or more consecutive days [25,26].

2.9.2 Experimental treatments

Following confirmation of diarrhea, all mice were randomly assigned to five groups (n = 8 per group): Group 1 (normal control) was administered 0.2 mL of sterile normal saline daily; Group 2 (model control) was given 0.2 mL of sterile normal saline daily after diarrhea induction; Group 3 (positive control) was treated with 6 mg/kg norfloxacin dissolved in 0.2 mL of normal saline; Group 4 received 250 mg/kg huajiao extract suspended in 0.2 mL of 0.5% carboxymethyl cellulose (CMC); and Group 5 received 500 mg/kg of the same extract and suspension. All treatments were administered via oral gavage once daily for seven days. Mice were maintained under sterile conditions with autoclaved food and water, and gavage was performed at consistent times each day.

On day 8, following the final treatment, all mice were euthanized by cervical dislocation after blood collection via orbital extraction. Blood samples were left to stand for 1 h and then centrifuged at $3000 \times g$ for 10 min. The resulting serum was collected and stored at -80°C for later analysis. The distal colon was excised and divided into three segments: one was rapidly frozen in liquid nitrogen and stored at -80°C for ELISA, qPCR, and Western blot analyses; another was fixed in 4% paraformaldehyde for hematoxylin and eosin (H&E) staining and stored at 4°C ; and the third was fixed in Carnoy's solution for Alcian Blue–Periodic Acid–Schiff (AB-PAS) staining. All tissues were handled gently to avoid mechanical damage to the colonic epithelium.

2.9.3 H&E and AB-PAS staining

Fixed colon tissues were trimmed in a fume hood using a scalpel to create smooth surfaces. The trimmed samples were labeled and placed in embedding frames. The cassettes containing the tissues were transferred to a tissue processor for sequential dehydration with graded ethanol. Paraffin infiltration was then performed, followed by embedding in a paraffin station. The embedded blocks were cooled on a -20°C freezing platform. Once solidified, the blocks were removed, trimmed, and sectioned into $4\text{ }\mu\text{m}$ slices using a rotary microtome. The sections were floated on 40°C water to flatten the tissues and then mounted onto glass slides. The slides were baked in a 60°C oven until the wax melted and water fully evaporated, then stored at room temperature for subsequent analysis.

H&E staining: Paraffin sections were sequentially dewaxed and rehydrated using an environment-friendly dewaxing solution, absolute ethanol, and distilled water. A 1-min pretreatment with a high-definition staining enhancer was performed, followed by hematoxylin and eosin staining. The stained sections were dehydrated through graded absolute ethanol, n-butanol, and xylene, then mounted with neutral balsam and examined under a light microscope [27].

AB-PAS staining: After dewaxing and rehydration, sections were stained with AB-PAS Stain C, acidified with Stain B, and counterstained with Stain A. Following staining, dehydration was conducted using absolute ethanol and xylene. The sections were then mounted with neutral balsam and examined microscopically.

2.9.4 Immunofluorescence staining (IF)

Following dewaxing and rehydration, antigen retrieval was achieved by heating the sections for 15 min, followed by passive cooling to room temperature. The slides were then immersed in PBS (pH 7.4) and washed three times on a decolorizing shaker for 5 min each. Residual buffer was removed, and tissue boundaries were outlined using a hydrophobic barrier pen. Blocking was performed with BSA for 30 min at room temperature. The primary antibody against TNF- α (GB115702, Servicebio, Wuhan, China) was applied dropwise, and the sections were incubated overnight at 4°C in a humidified chamber. After three additional PBS washes, the corresponding secondary antibody was applied, followed by a 50-min incubation at room temperature in the dark. Nuclear staining was conducted using DAPI, and autofluorescence was quenched prior to mounting with an anti-fade medium. Fluorescence imaging was conducted under the following parameters: DAPI (excitation 330–380 nm, emission 420 nm); TNF- α (CY3, excitation 510–560 nm, emission 590 nm) [28].

2.9.5 RT-qPCR analysis

qPCR assays were performed by Servicebio (Wuhan, China). Total RNA was extracted from 5–20 mg of mouse colonic tissue using an RNA lysis buffer. RNA concentration and purity were determined using a Nanodrop 2000, following blank calibration. For each sample, $2.5\text{ }\mu\text{L}$ of RNA was applied to the detection pedestal for absorbance measurement. Samples exceeding the optimal concentration were diluted to $200\text{ ng}/\mu\text{L}$. Reverse transcription was carried out using a thermal cycler, followed by real-time PCR amplification.

Relative gene expression was quantified using the $\Delta\Delta C_t$ method with GAPDH as the internal reference. The information on qPCR primers is provided in Supplementary Table A.1

2.9.6 ELISA for Inflammatory cytokines

Inflammatory cytokine levels in mouse colonic tissue were quantified by ELISA. Lesioned tissue was homogenized in pre-chilled PBS until fully dispersed and then centrifuged at $3,000 \times g$ for 15 min. The supernatant was collected for analysis. IL-1 β , TNF- α , and IL-6 concentrations were determined using ELISA kits according to the manufacturer's instructions.

2.9.7 Western blot analysis

Mouse colonic tissues were washed 2–3 times with pre-chilled PBS to remove blood residues, then homogenized in lysis buffer ($10\times$ tissue volume) containing protease inhibitors. The homogenate was kept on ice for 30 min with intermittent shaking every 5 min to facilitate complete lysis, followed by centrifugation at $12,000 \times g$ for 10 min at 4°C . The supernatant was collected as the total protein extract. Protein concentration was measured using a BCA assay. The extract was mixed with $5\times$ reducing loading buffer at a 4:1 ratio, denatured in a boiling water bath for 15 min, and stored at -20°C . Proteins were separated by SDS-PAGE and transferred to an activated PVDF membrane at a constant current of 350 mA for 30 min. After blocking with 5% milk, the membrane was incubated overnight at 4°C with primary antibodies against the target proteins. Following three washes, incubation with HRP-conjugated secondary antibodies was carried out for 30 min at room temperature. Protein bands were visualized using enhanced chemiluminescence (ECL) reagents and detected by exposure.

2.10 Statistical analysis

All measurements were conducted in triplicate, and data were presented as mean $\pm$ standard deviation. Statistical analyses were performed using SPSS 24.0 (SPSS Inc., Chicago, IL, USA). One-way analysis of variance (ANOVA) was applied, followed by Duncan's post hoc test, with statistical significance defined as $P < 0.05$. In untargeted metabolomics, compounds with a confidence score greater than 0.8 were retained for stoichiometric analysis. Principal component analysis (PCA) and partial least squares-discriminant analysis (PLS-DA) were performed using the OmicShare platform (<https://www.omicshare.com/>). Partial least squares (PLS) modeling was carried out using SIMCA 14.1. Goblet cell quantification from AB-PAS staining and immunofluorescence imaging was conducted using ImageJ software. Western blot images in TIFF format were analyzed using AIWBwell™ software.

3. Results and discussion

3.1 In vitro screening of huajiao extracts against *Shigella flexneri*

3.1.1 Antibacterial activity analysis

To determine the most potent huajiao material against *Shigella flexneri*, agar diffusion assays were performed using extracts standardized to 20 mg/mL. Anatomical parts from three huajiao cultivars were evaluated. As shown in Supplementary Table A.2, pericarp extracts from all cultivars demonstrated

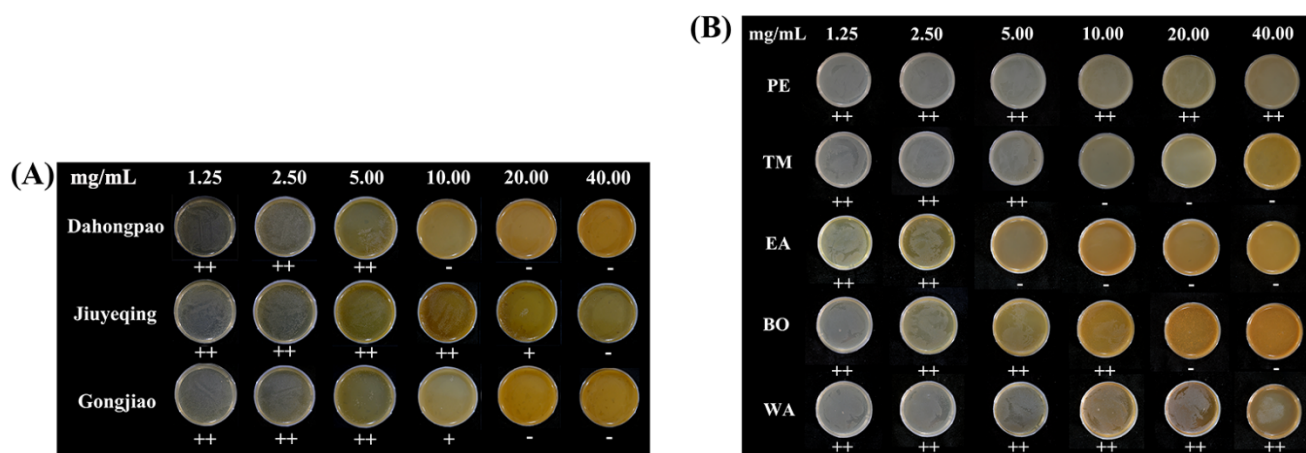
significantly stronger antibacterial activity than other plant parts. Notably, the pericarps of Dahongpao and Gongjiao completely suppressed bacterial growth, with no visible colonies after 24 h. In contrast, the Jiuye Qing pericarp extract exhibited only partial inhibition, permitting sparse colony formation.

3.1.2 MIC and MBC determination

The antibacterial properties of pericarp extracts from three huajiao cultivars were assessed against *Shigella flexneri* by determining their MIC and MBC. Antimicrobial efficacy varied notably among the cultivars: Jiuye Qing exhibited a higher MIC of 20 mg/mL, while both Dahongpao and Gongjiao showed stronger inhibitory effects with lower MICs of 10 mg/mL. As illustrated in Fig. 1A, MBC evaluation after 48 h revealed that Dahongpao maintained bactericidal activity at 10 mg/mL, whereas Gongjiao required a higher concentration for complete bacterial eradication. These findings highlight Dahongpao's superior antibacterial potency, leading to its selection for subsequent mechanistic investigations.

3.2 Antibacterial effects of solvent-partitioned pericarp extracts

Huajiao pericarp was sequentially extracted with solvents of increasing polarity, and the resulting fractions were tested for antibacterial activity against *Shigella flexneri* at concentrations ranging from 1.25 to 40 mg/mL. As shown in Fig. 1B, the petroleum ether and aqueous fractions showed no significant inhibition, even at 40 mg/mL. In contrast, the chloroform, ethyl acetate, and n-butanol fractions exhibited MICs of 10, 5, and 20 mg/mL, respectively. To further distinguish the antibacterial efficacy among fractions, inhibition rates were measured (Fig. 1C). At 1 mg/mL, the ethyl acetate fraction demonstrated significantly higher antibacterial activity than all other groups ($P < 0.05$), achieving complete inhibition (100%). *S. flexneri* infection, a gastrointestinal illness associated with severe intestinal inflammation, may respond particularly well to agents with anti-inflammatory properties. Previous studies by Liu^[29] and Santhanam^[30] reported that the ethyl acetate fraction of huajiao pericarp is rich in bioactive compounds with anti-inflammatory effects. These findings support our observation that the key antibacterial constituents against *S. flexneri* are concentrated in the ethyl acetate fraction.



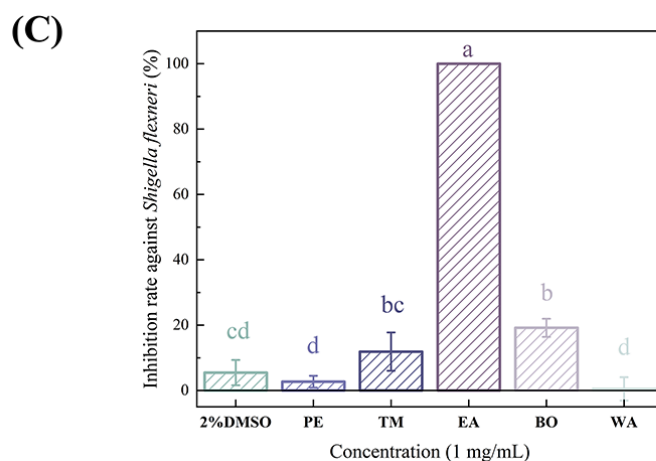


Fig. 1 Antibacterial effects of huajiao pericarp extracts from different varieties and with different solvents at various concentrations against *Shigella flexneri* (1.25–40 mg/mL). (A) Antibacterial effects of pericarp extracts from different huajiao varieties; (B) Antibacterial effects of pericarp extracts with different solvents; (C) Antibacterial rates (%) of huajiao extracts with different solvents at the concentration of 1 mg/mL. PE: petroleum ether, TM: chloroform, EA: ethyl acetate, BO: n-butanol; WA: water.

3.3 Identification of antibacterial components in huajiao pericarp by metabolomics and chemometrics

3.3.1 PCA and PLS-DA analysis

Untargeted metabolomic profiling was conducted on solvent-partitioned fractions of huajiao pericarp. A total of 565 and 406 metabolites with match scores above 0.8 were identified in ESI (+) and ESI (−) modes, respectively. Chemometric analyses were employed to compare the metabolite compositions across fractions and to predict potential antibacterial compounds (Fig. 2A) [31,32]. PCA and PLS-DA were used to assess sample variation under unsupervised and supervised conditions, respectively. The PCA models explained 65.1% of the variance in ESI (+) and 68.3% in ESI (−) (Fig. 2B, 2C), while the PLS-DA models accounted for 76% and 81%, respectively (Fig. 2D, 2E), indicating robust separation and strong model performance. Both PCA and PLS-DA clearly distinguished the five solvent fractions in ESI (+) and (−) modes, confirming significant compositional differences ($P < 0.05$). The petroleum ether and chloroform fractions clustered in the same quadrant, suggesting similar metabolite profiles, while the remaining fractions were distinctly separated, reflecting broader chemical divergence. Differential metabolites were identified using $VIP > 1$ and $P < 0.05$, with the top 20 presented in Fig. 2F and 2G. Given the pronounced antibacterial activity of the ethyl acetate fraction (Fig. 1B), it is likely enriched in key bioactive compounds. Notably, this fraction contained high levels of flavonoids and their derivatives—such as nepetin 7-glucoside, hyperoside, quercitrin, and guaiaverin—which may play a central role in the antibacterial efficacy of huajiao.

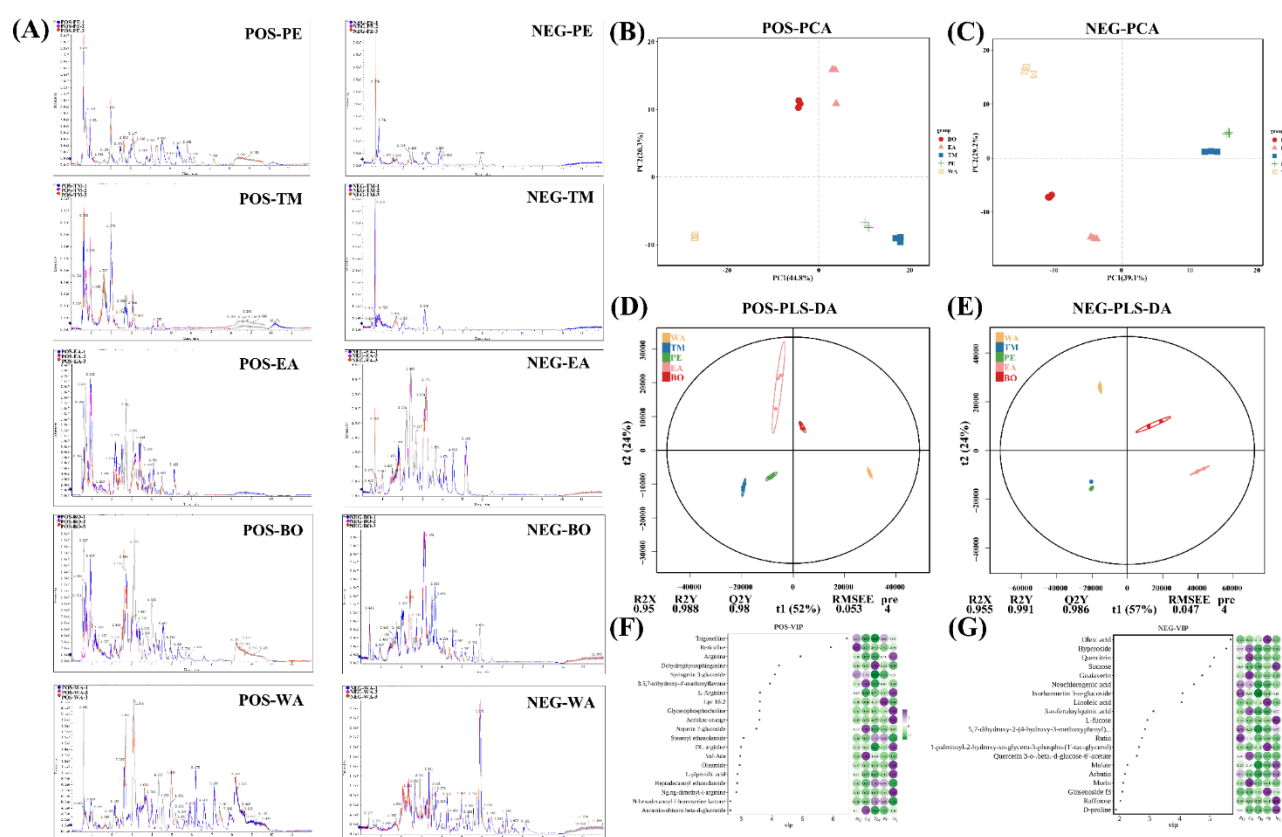


Fig. 2 Multivariate analysis of non-targeted metabolomics based on different solvent extracts of huajiao. (A) Signal plots of huajiao pericarp extracts with different solvents in ESI (+) and ESI (−) modes; (B) and (C) principal component analysis (PCA) for ESI (+) and ESI (−) modes, respectively; (D) and (E) partial least squares discriminant analysis (PLS-DA) for ESI (+) and ESI (−) modes, respectively; (F) and (G) differential metabolites in ESI (+) and ESI (−) modes, respectively. PE: petroleum ether, TM: chloroform, EA: ethyl acetate, BO: n-butanol; WA: water.

3.3.2 PLS regression analysis

PLS regression is a multivariate analysis technique used to model relationships between predictor (X) and response (Y) variables. By selecting features with strong correlations to the response, PLS enhances both model interpretability and predictive accuracy [33]. In this study, PLS was applied to identify antibacterial compounds in huajiao pericarp associated with *Shigella flexneri* inhibition, using antibacterial activity from different solvent fractions as the basis for prediction. Antibacterial rates at 1 mg/mL (Fig. 1C) were assigned as Y variables, while metabolite profiles of the respective solvent fractions served as X variables in the PLS model. As shown in Fig. 3A and 3B, both ESI (+) and ESI (−) modes yielded robust models, with R^2Y values nearing 1 and Q^2 values above 0.5, indicating strong explanatory and predictive performance.

Score plots (Fig. 3C, 3D) revealed tight clustering of the petroleum ether (PE) and trichloromethane (TM) groups, while the other three fractions were clearly separated—consistent with PCA and PLS-DA findings. In the loading plots (Fig. 3E, 3F), compounds located farther from the origin were identified as major contributors. Vectors extending from the origin toward the Y-axis further distinguished positive and negative contributors (Fig. 3G, 3H). Key metabolites identified in ESI (+) included nepetin 7-glucoside, val-asn, quercetin 3-O-malonylglucoside, aurantio-obtusin β -D-glucoside, jaceosidin, syringetin 3-glucoside, and luteolin. In ESI (−), top contributors included quercitrin, guaiaverin, hyperoside, quercetin

3-O- β -D-glucose-6"-acetate, and morin. Based on their contribution values, the top 100 metabolites from both ionization modes (Supplementary Table A.3) were selected for subsequent network pharmacology analysis to further explore their roles in *S. flexneri* inhibition.

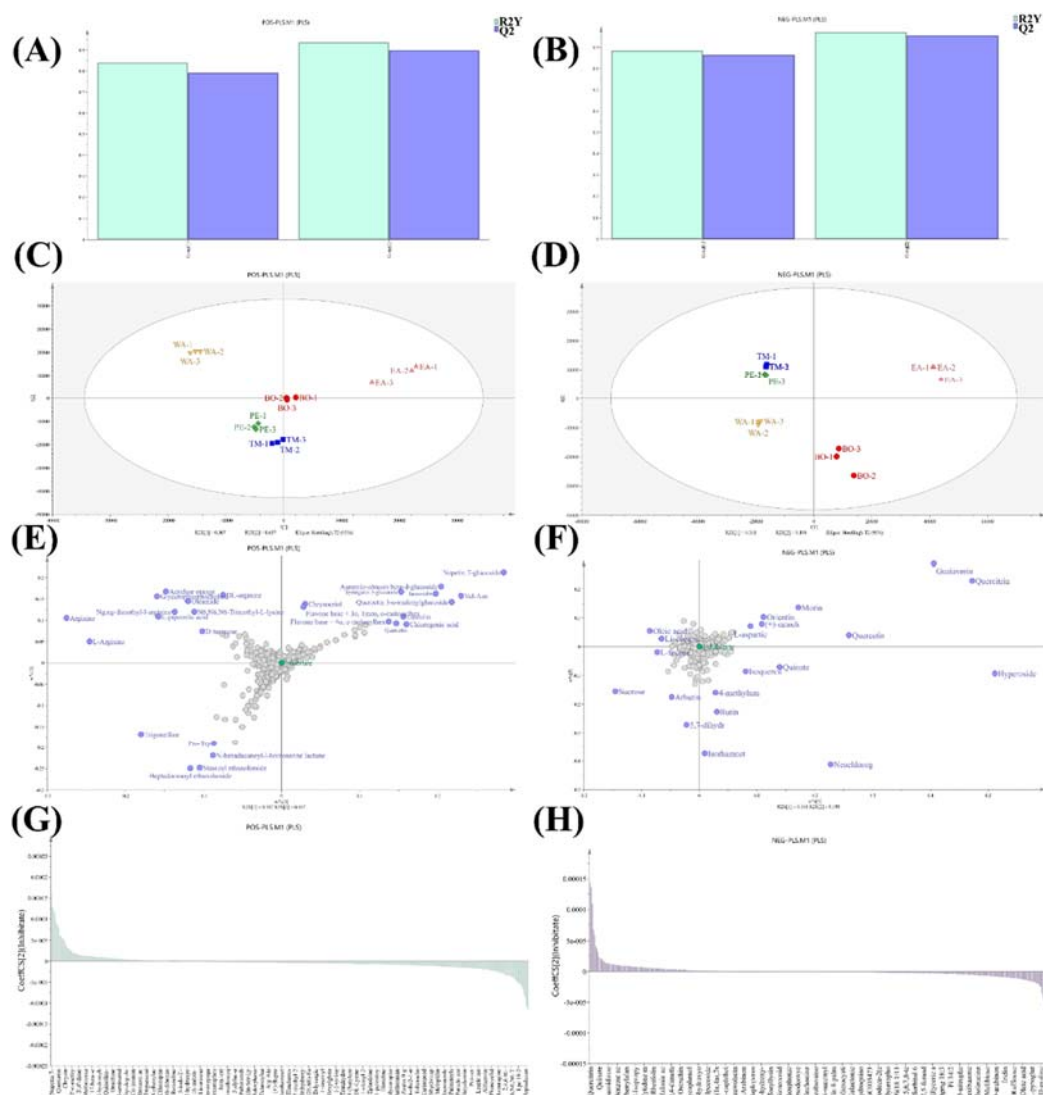


Fig. 3 PLS model analysis of components of huajiao peel extracts in different solvents and their antibacterial effects in vitro. (A) and (B) R^2Y and Q^2 of the PLS models; (C) and (D) Score plots of the PLS models; (E) and (F) Loading plots of the PLS models; (G) and (H) Contributing substances in the PLS models.

3.4 Network pharmacology analysis

3.4.1 Identification of targets for active compounds

Network pharmacology integrates pharmacology, bioinformatics, and computational approaches to systematically explore the mechanisms of bioactive compounds. By combining bioinformatics tools with network analysis, it provides a comprehensive view of compound–target interactions and associated biological pathways [34]. In this study, network pharmacology was employed to predict the molecular targets and signaling pathways through which key metabolites from huajiao exert therapeutic effects against *Shigella flexneri*-induced intestinal infection.

The SMILES structures of the 100 metabolites identified in Section 3.3.2 were submitted to SwissTargetPrediction. After deduplication, 1,013 potential targets were identified.

3.4.2 Collection of targets for *Shigella flexneri*-induced colitis

A total of 329 non-redundant antibacterial targets associated with *Shigella flexneri* were identified from GeneCards and OMIM using the keyword “*Shigella flexneri*.”

3.4.3 PPI network construction for anti-*S. flexneri* targets

Venn diagram analysis revealed 38 overlapping targets between *S. flexneri*-induced diarrhea-associated genes and those linked to huajiao metabolites (Fig. 4A), indicating potential therapeutic targets. These targets were uploaded to the STRING database to construct a PPI network, with the interaction score threshold set at 0.4. After excluding unconnected nodes, the final network comprised 38 nodes and 538 edges (Fig. 4B). To identify key regulatory targets, topological analysis was conducted using CytoNCA in Cytoscape 3.8.1. Median values for degree, betweenness, and closeness centrality were 49, 37.89, and 0.75, respectively (Supplementary file Table A.4). Nodes exceeding all three medians were classified as core targets, resulting in the identification of TNF, NF- κ B1, BCL2, CASP3, and EGFR. During colonic invasion by *S. flexneri*, macrophages regulate inflammation through both transcriptional and post-transcriptional mechanisms. EGFR, a membrane-bound tyrosine kinase receptor, promotes the recruitment, activation, and proliferation of inflammatory cells, as well as the release of pro-inflammatory cytokines [35]. TNF- α , primarily secreted by macrophages, is a key inflammatory mediator, while NF- κ B1 serves as a central transcription factor in the inflammatory response [36,37]. These findings underscore the role of inflammation-related genes as crucial therapeutic targets. In addition, BCL2 and CASP3 are central to the apoptotic pathway. BCL2 inhibits apoptosis by preventing mitochondrial cytochrome c release, thereby suppressing CASP3 activation, a key executioner of apoptosis [38,39]. Collectively, these results suggest that genes involved in inflammation and apoptosis represent essential targets for the treatment of *S. flexneri*-induced diarrhea.

3.4.4 KEGG and GO analyses of overlapping targets

The 38 overlapping targets were analyzed using the Metascape database for pathway enrichment. KEGG analysis identified 157 pathways, ranked by the number of associated genes. Pathways meeting the criteria of $P < 0.01$, gene count ≥ 3 , and enrichment factor > 1.5 were visualized (Fig. 4D). To enhance statistical reliability, q-values—adjusted p-values controlling for the false discovery rate (FDR)—were used. In the resulting bubble plot, deeper purple-red colors represent lower q-values, while larger bubbles denote pathways with more enriched genes. As illustrated in Fig. 4D, huajiao pericarp may mitigate *S. flexneri*-induced diarrhea through multiple pathways, including those associated with Salmonella infection, Shigellosis, and cancer. Notably, all five core targets were involved in the Shigellosis pathway, and four in the Salmonella infection pathway, suggesting a potential shared mechanism of action.

GO enrichment analysis yielded 636 significant terms: 522 for biological processes (BP), 43 for cellular components (CC), and 71 for molecular functions (MF), based on the same selection thresholds. Key BP terms (Fig. 4E) included response to lipopolysaccharide, positive regulation of canonical NF- κ B signaling, and inflammatory response. Enriched CC terms (Fig. 4F) involved membrane rafts, mitochondrial outer

membrane, and cell leading edge. Prominent MF terms (Fig. 4G) included kinase binding, phosphotransferase activity (alcohol group as acceptor), and cysteine-type endopeptidase regulator activity involved in apoptosis.

Collectively, KEGG and GO analyses suggest that huajiao pericarp exerts its therapeutic effect against *S. flexneri*-induced diarrhea by targeting key inflammation- and apoptosis-related pathways, biological processes, and molecular functions.

3.4.5 key bioactive compounds in huajiao against *Shigella flexneri*-induced colitis

To identify the key bioactive compounds in huajiao involved in mitigating *S. flexneri*-induced diarrhea, a compound–target network was constructed based on the five core targets identified in Section 3.4.3. As shown in Fig. 4C, the primary active constituents were flavonoids and their derivatives. The network comprised 28 nodes—1 sample, 5 gene targets, and 22 compounds—and 63 edges. Among the targets, TNF and EGFR were most frequently associated. Notably, jaceosidin, luteolin, quercitrin, and hyperoside interacted with multiple core targets, highlighting their potential therapeutic relevance.

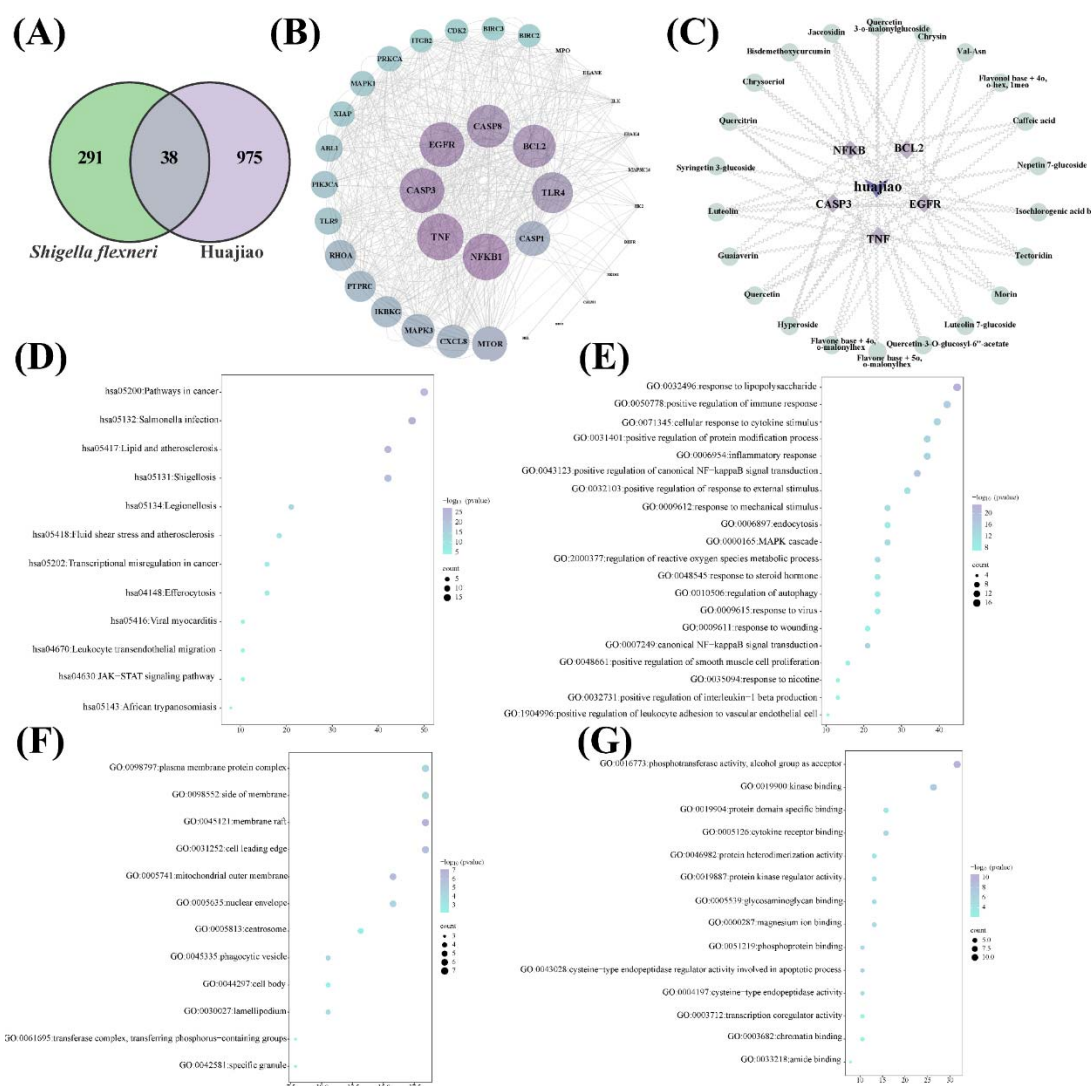


Fig. 4 Network pharmacology analysis of huajiao in alleviating *Shigella flexneri*-induced colitis. (A) Common target proteins between *Shigella flexneri*-induced mouse colitis and huajiao; (B) Network interaction diagram of intersecting targets; (C) Active components corresponding to key targets; (D) KEGG pathways; (E) GO molecular functions; (F) GO biological processes; (G) GO cellular components.

3.5 Molecular docking

Molecular docking is a simulation technique that investigates binding mechanisms, offering critical insights into ligand-receptor interactions. Based on the 22 key active components and their respective core targets identified in section 3.4.5, 41 docking experiments were conducted (Supplementary file Table A.6 and Fig. 5). The RMSD value of molecular docking is less than 2 Å, indicating that the difference between the docking conformation and the crystal conformation is within the acceptable range, and the rationality of the docking parameters is good. Binding energies < 0 kcal/mol suggest natural docking, < -1.2 kcal/mol (-5 kJ/mol) indicates good binding, and < -5 kcal/mol reflects strong binding activity^[40]. In this study, the binding energies for 41 protein-ligand interactions ranged from -8.24 to -3.12 kcal/mol, all below -1.2 kcal/mol, confirming favorable binding. Heatmap analysis revealed optimal interactions (Fig. 5A): BCL2 showed the lowest binding energy with luteolin (-5.04 kcal/mol), indicating the strongest affinity; CASP3 bound most stably with morin (-5.63 kcal/mol); EGFR interacted most favorably with bisdemethoxycurcumin (-8.24 kcal/mol); NF-κB had optimal docking with chrysin (-5.94 kcal/mol); and TNF exhibited the highest affinity for luteolin 7-glucoside (-6.59 kcal/mol).

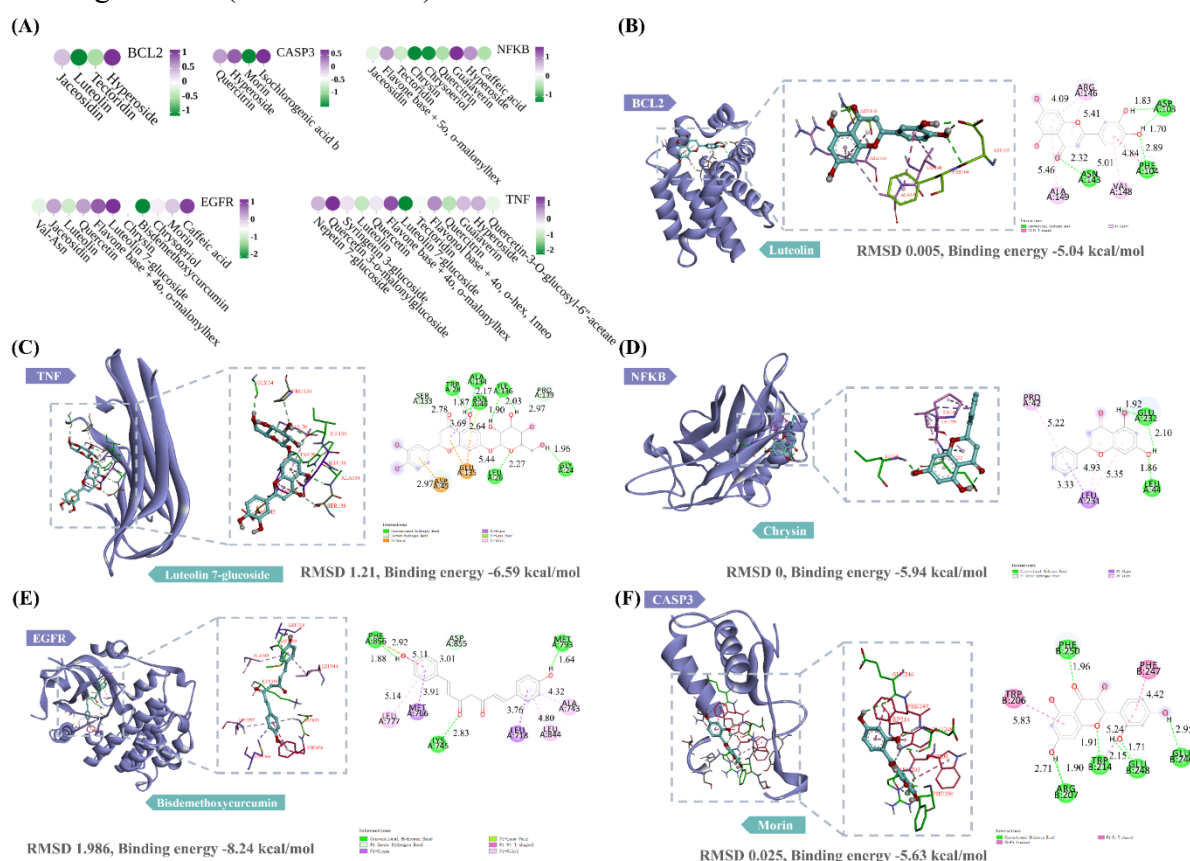


Fig. 5 Molecular docking of key targets and their corresponding components of huajiao pericarp in alleviating *Shigella flexneri*-induced colitis. (A) Heatmap analysis of binding energies between five key targets and the corresponding components in huajiao pericarp; (B–F) Analysis of molecular docking conformations and interaction forces of five key targets with their optimally bound small-molecule compounds.

Fig. 5B–F presents the docking diagrams of five protein–ligand complexes. Each type of interaction is indicated by a distinct line color: green for hydrogen bonds, purple for π –sigma interactions, light pink for π –alkyl, light green for π –donor hydrogen bonds, dark pink for π – π T-shaped stacking, orange for π –anion,

and grass green for π -lone pair interactions. In the luteolin–BCL2 complex, luteolin formed hydrogen bonds with ASP103 (1.83 Å), PHE104 (2.89 Å), and ASN143 (2.32 Å); π -alkyl interactions with ARG146 (4.09 Å), VAL148 (5.01 Å), and ALA149 (5.46 Å); and a π - π T-shaped interaction with PHE104 (4.84 Å). In the luteolin 7-glucoside–TNF complex, hydrogen bonds were observed with TRP28 (1.87 Å), GLY24 (1.96 Å), ALA134 (2.17 Å), and ILE136 (1.90 Å). LEU26 simultaneously engaged in a hydrogen bond (2.27 Å) and a π -alkyl interaction (5.44 Å). ASN46 formed both π -lone pair (2.64 Å) and π -sigma (3.69 Å) interactions, while PRO139 contributed a carbon–hydrogen bond (2.97 Å) and ASP45 a π -anion interaction (2.97 Å). In the chrysin–NF- κ B complex, chrysin established two hydrogen bonds with GLU232 (1.92 and 2.10 Å), a single hydrogen bond with LEU44 (1.86 Å), a π -sigma interaction with LEU231 (3.33 Å), and two π -alkyl interactions with the same residue (4.93 and 5.35 Å). The bisdemethoxycurcumin–EGFR complex featured multiple interactions with PHE856, including a hydrogen bond (1.88 Å), π - π T-shaped stacking (2.92 Å), and a π -lone pair interaction (5.11 Å). Additional hydrogen bonds were formed with MET793 (1.64 Å) and LYS745 (2.83 Å). π -Alkyl interactions occurred with LEU777 (5.14 Å), LEU844 (4.80 Å), and ALA743 (4.32 Å); π -sigma interactions with MET766 (3.91 Å) and LEU718 (3.76 Å); and a π -donor hydrogen bond with ASP855 (3.01 Å). In the morin–CASP3 complex, morin formed hydrogen bonds with ARG207 (1.90 Å), TRP214 (1.91 Å), GLU246 (2.95 Å), GLU248 (1.71 Å), and PHE250 (1.96 Å), as well as π - π T-shaped stacking interactions with TRP206 (5.83 Å), PHE247 (4.42 Å), and TRP214 (5.24 Å).

Key interactions between target proteins and small-molecule ligands are mainly governed by hydrogen bonds and non-covalent π -system forces. Hydrogen bonding is the most specific interaction in protein-ligand binding. The hydroxyl group on the flavonoid ligand binds to the $-\text{COO}^-$ of amino acids (e.g., ASP and GLU) in the protein receptor to form hydrogen bonds, while the carbonyl group forms hydrogen bonds with either the $-\text{NH}_2$ (side chain) of ASN or the $-\text{OH}$ of SER. Non-covalent π -system forces are dominated by electrostatic interactions, among which π -lone pair and π -anion interactions are the two strongest. For example, the benzene ring on the small-molecule ligand forms π -lone pair interactions with the oxygen atom (containing lone pairs) of ASN. As typical acidic amino acids in proteins, the core feature of ASP and GLU is that their side chains contain carboxyl groups ($-\text{COOH}$), which deprotonate to form $-\text{COO}^-$ under physiological pH. The local positive charge (δ^+ region) induced on the benzene ring forms heterocharged attraction with the negative charge of $-\text{COO}^-$, thereby forming π -anion interactions. Together, these forces determine the binding conformation of active compounds at protein targets.

3.6 In vivo validation of anti-colitis effects

Network pharmacology identified NFKB1 and TNF as the key targets of huajiao in alleviating *Shigella flexneri*-induced colitis. The KEGG analysis showed that the pathways with high scores, such as hsa05132 Salmonella infection, hsa05131 Shigellosis, and hsa05134 Legionellosis, were all bacterial infection pathways. The pathogenic components, such as lipopolysaccharide, would activate the NF- κ B pathway through TLR4-like receptor pattern recognition receptors, thereby inducing the release of inflammatory factors such as TNF- α and IL-6. This is the core inflammatory response mechanism of the body against

bacterial infection. Moreover, the GO molecular function enrichment analysis directly indicated that the metabolites of huajiao would affect the signal transduction of the NF- κ B pathway at the molecular level. Therefore, animal experiments were conducted to verify whether the extract of huajiao alleviated *Shigella flexneri*-induced colitis by influencing the signal transduction of the NF- κ B pathway.

3.6.1 H&E and AB-PAS analysis

H&E staining of the distal colon (Fig. 6A) showed no pathological changes in the CK group. In contrast, *S. flexneri*-induced enteritis in mice caused epithelial layer damage, crypt injury, atrophy and necrosis of intestinal glands, moderate inflammatory cell infiltration with necrosis, and ulceration. Treatment with norfloxacin significantly reduced inflammatory infiltration and alleviated gland atrophy, leading to marked improvement. In the low-dose huajiao extract group, modest improvement was observed, but epithelial damage, moderate inflammatory infiltration, and necrosis persisted. In contrast, the high-dose huajiao extract group showed minimal inflammatory cells and considerable recovery of intestinal gland structure.

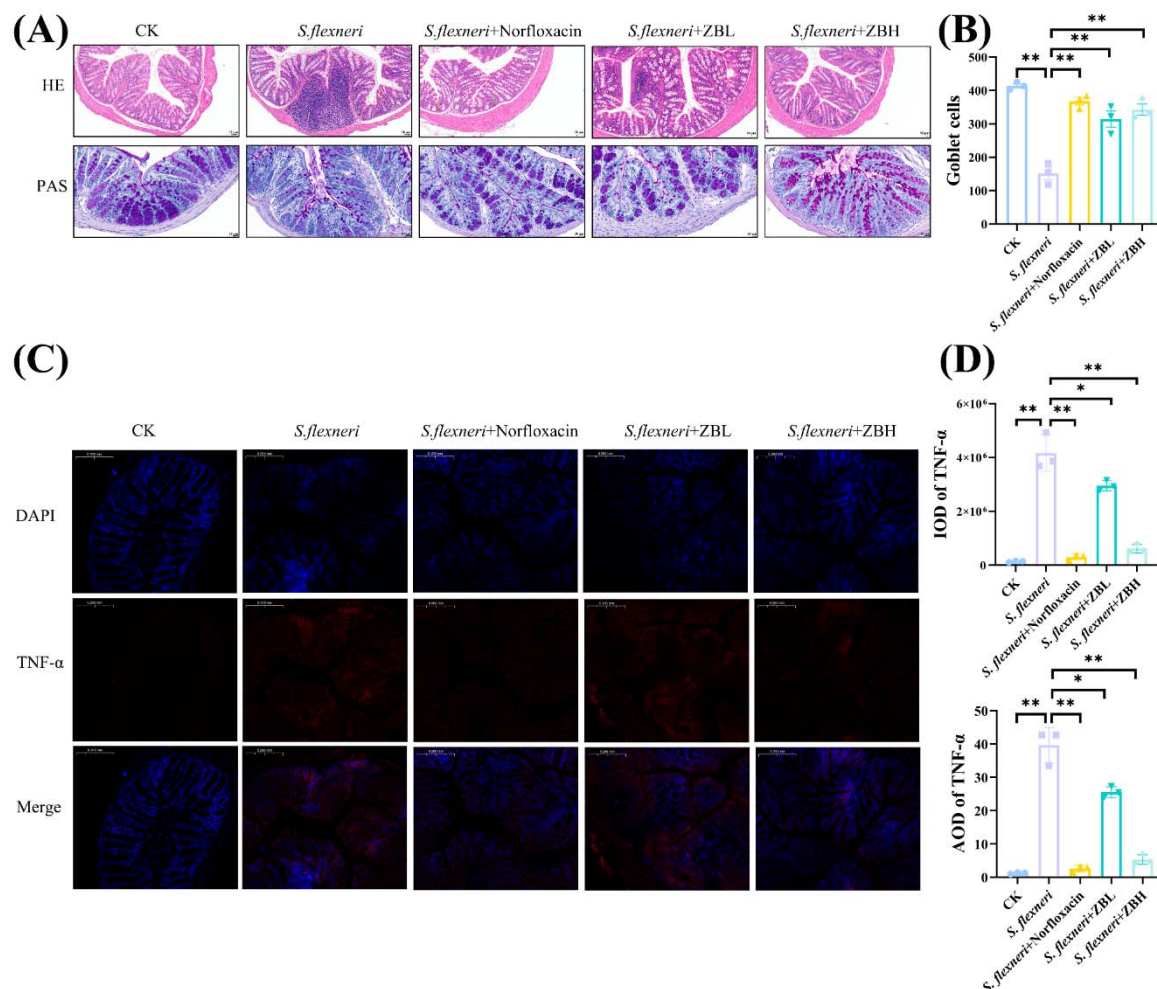


Fig. 6 Pathological analysis of huajiao pericarp in alleviating *Shigella flexneri*-induced colitis in mice. (A) H&E and AB-PAS staining of mouse colon tissues; (B) Number of goblet cells in AB-PAS staining of mouse colon tissues; (C) Immunofluorescence staining of TNF- α in mouse colon tissues; (D) Integrated optical density (IOD) of TNF- α in (C); (E) Average optical density (AOD) of TNF- α in (C) (n=3).

AB-PAS staining was used to visualize goblet cells by targeting mucin-containing polysaccharides and glycoproteins, which are oxidized by periodic acid to aldehydes and subsequently react with Schiff's reagent

to produce a magenta signal (Fig. 6B). Goblet cell numbers were significantly reduced in the model group compared to CK ($P < 0.01$), indicating epithelial barrier disruption and inflammation. In contrast, goblet cell counts were significantly elevated in the norfloxacin, low-dose, and high-dose huajiao groups ($P < 0.01$ vs. model), suggesting attenuation of colonic inflammation and improved mucosal integrity [27].

3.6.2 Immunofluorescence analysis

Immunofluorescence was used to localize TNF- α in mouse colonic tissue through antigen–antibody interactions. As shown in Fig. 6C, *Shigella flexneri*-infected mice displayed significantly increased TNF- α fluorescence intensity, along with elevated integrated optical density (IOD) and average optical density (AOD) compared to the control group ($P < 0.01$), indicating active colonic inflammation. TNF- α expression was significantly reduced in the norfloxacin ($P < 0.01$), low-dose huajiao ($P < 0.05$), and high-dose huajiao ($P < 0.01$) groups, reflecting effective suppression of inflammatory responses.

3.6.3 Inflammatory cytokine profiling

mRNA and protein levels of TNF- α , IL-1 β , and IL-6 in mouse colonic tissue were quantified by qPCR and ELISA, respectively. As shown in Fig. 7A–B, *Shigella flexneri* infection markedly upregulated these proinflammatory cytokines compared to controls ($P < 0.01$). Treatment with norfloxacin or huajiao extract significantly suppressed their expression ($P < 0.01$), indicating mitigation of the inflammatory response and colonic inflammation.

3.6.4 Western blot analysis

Western blotting was used to evaluate key proteins in the canonical NF- κ B signaling pathway. Compared with the control (CK) group, the model group showed no significant changes in total I κ B α or NF- κ B p65 levels; however, levels of their phosphorylated forms—p-I κ B α and p-NF- κ B p65—were significantly increased ($P < 0.01$), as were the p-I κ B α /I κ B α and p-NF- κ B p65/NF- κ B p65 ratios Fig. 7C–F. These findings indicate activation of the NF- κ B pathway through enhanced phosphorylation, reflecting an inflammatory response in the colon. TNF- α , a known activator and downstream target of this pathway, was also significantly elevated in the model group. Treatment with norfloxacin or huajiao extract significantly reduced the expression of p-I κ B α , p-NF- κ B p65, and TNF- α ($P < 0.01$), indicating effective suppression of NF- κ B–driven inflammation. These results are consistent with those of Wei [41], who demonstrated that quercetin alleviates colonic inflammation by inhibiting NF- κ B p65 phosphorylation and promoting intestinal barrier repair.

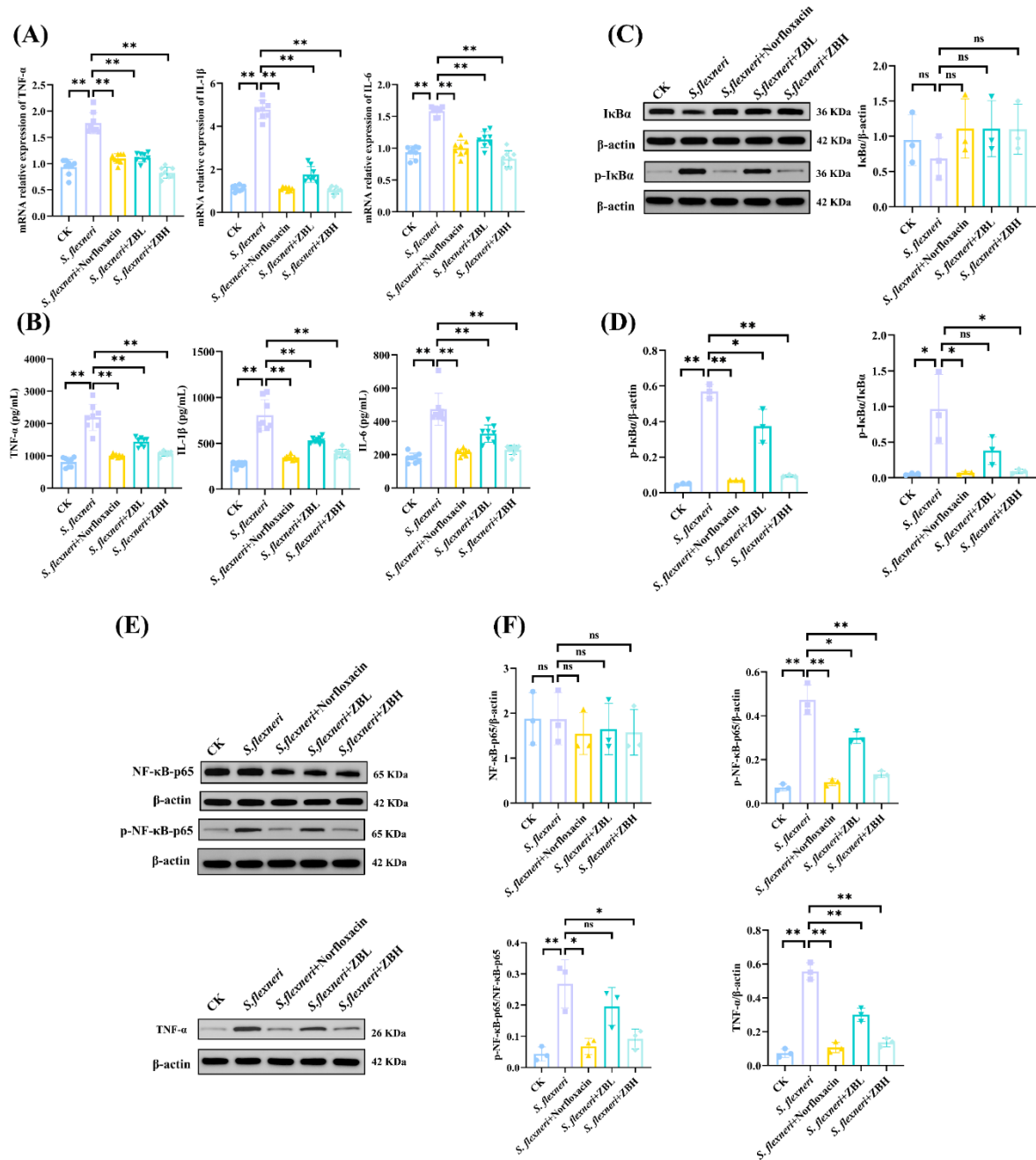


Fig. 7 mRNA and protein expression levels of inflammatory factors, and protein expression levels related to the NF-κB pathway in huajiao pericarp alleviating *Shigella flexneri*-induced colitis in mice. (A) mRNA expression levels of TNF-α, IL-1β, and IL-6 (n=8); (B) Elisa analysis of TNF-α, IL-1β, and IL-6 (n=8); (C) and (D) Protein expression levels of IκBα and phosphorylated IκBα (n=3); (E) and (F) Protein expression levels of NFκB-p65, phosphorylated NFκB-p65, and TNF-α (n=3).

4. Discussion

Huajiao, a widely used spice with both medicinal and culinary properties, possesses rich contents of alkaloids, coumarins, flavonoids, and terpenoids, which endow it with physiological functions such as antibacterial, anti-inflammatory, and anti-tumor activities [42]. For example, bergapten, a component of huajiao, has been shown to impair the survival of various pathogenic bacteria, including *Escherichia coli*, *Staphylococcus aureus*, and *Helicobacter pylori* [43]. Yang et al. [44] further demonstrated that the contents of flavonoids (e.g., avicularin, rutin, and narcissoside) in huajiao significantly influence its anti-inflammatory

activity. In the present study, non-targeted metabolomics was utilized to investigate compositional differences among huajiao extracts obtained with solvents of varying polarities, while partial least squares (PLS) analysis was performed in conjunction with in vitro antibacterial assays. The results revealed that nepetin 7-glucoside, quercetin 3-O-malonylglucoside, quercetin, guaiaverin, hyperoside, and other flavonoids are potential antibacterial compounds in huajiao peel that exert in vitro inhibitory effects against *Shigella flexneri*. This suggests that flavonoids are the primary bioactive components of huajiao responsible for its activity against *S. flexneri*.

Network pharmacology enables systematic analysis of molecular association patterns between drugs and diseases by adopting a multi-component-multi-target strategy, making it well-suited for investigating complex diseases and this is fully supported by previous studies [24,40,45]. Based on the exploration of huajiao's dual effects of reducing the intestinal *S. flexneri* load and alleviating the inflammation caused by *S. flexneri* invasion of the intestine, after obtaining the main components of huajiao with in vitro anti-*S. flexneri* activity, these components were further used to identify the key targets (NFκB and TNF) of huajiao in alleviating *S. flexneri*-induced colitis through network pharmacology. Kyoto Encyclopedia of Genes and Genomes (KEGG) and Gene Ontology (GO) analyses also indicated that the metabolites of huajiao affect the signal transduction of the NF-κB pathway at the molecular level. Following *S. flexneri*'s successful intestinal invasion via epithelial M cells, released bacterial peptidoglycan fragments activate nuclear factor κB (NFκB), which in turn triggers intestinal secretion of the inflammatory cytokines TNF-α, IL-1β, and IL-6 [46], leading to further amplification of inflammation. Notably, these inflammatory cytokines also act as NFκB activators, which disrupts the intestinal epithelial barrier and facilitates more severe *S. flexneri* invasion [47]. Molecular docking results demonstrated that huajiao's compound, including chrysin and luteolin 7-glucoside, exhibit strong binding affinities to NFκB (-5.94 kcal/mol) and TNF (-6.59 kcal/mol), suggesting that these components may interfere with the biological functions of these target proteins through direct binding. Consistent with previous reports, chrysin inhibits oxidative stress [48] and mitigates inflammatory damage by suppressing the NF-κB pathway [49], while luteolin 7-glucoside exerts anti-inflammatory effects associated with the JAK/STAT3 and NF-κB pathways [50].

To validate that huajiao extract alleviates *S. flexneri*-induced colitis by regulating the NF-κB pathway, animal experiments were performed. Mice in the model group exhibited colonic gland atrophy, ulcer formation, and reduced goblet cell numbers, which are typical manifestations of intestinal mucosal barrier disruption following *S. flexneri* invasion [25,26]. In contrast, the high-dose huajiao group showed significant intestinal structural repair and reduced inflammatory infiltration ($P < 0.01$), indicating reversal of intestinal damage (Fig. 6A-B). Upregulation of TNF-α, IL-6, and IL-1β in the model group, which are downstream markers of NF-κB pathway activation, was significantly attenuated in the huajiao extract group ($P < 0.01$) (Fig. 7A-B). Notably, as these inflammatory cytokines also act as NF-κB activators, immunofluorescence assays confirmed TNF-α inhibition ($P < 0.01$) (Fig. 6C-D), which directly interrupts the positive feedback loop linking NF-κB activation to inflammatory amplification [51]. IκBα degradation and p65 phosphorylation

are key steps in NF- κ B pathway activation. The reduced p-I κ B α /I κ B α ($P < 0.05$) and p-p65/p65 ($P < 0.01$) ratios in the huajiao extract group indicate that huajiao extract simultaneously suppresses upstream I κ B α degradation and downstream p65 activation—a regulatory pattern consistent with previous reports [41,52]. Furthermore, the anti-inflammatory effect of huajiao extract was dose-dependent, which further confirms a causal relationship between NF- κ B pathway inhibition and colitis alleviation.

By integrating non-targeted metabolomics, network pharmacology, and molecular docking, this study is the first to directly link huajiao components to NFKB/TNF targets, delineate the complete action chain among components, targets, and pathways, and provide a novel direction for the development of safer plant-derived drugs. However, this study focused solely on the NF- κ B pathway and did not investigate its crosstalk with other pathways, such as the MAPK pathway and NLRP3 inflammasome. Future studies should conduct additional in vitro antibacterial and in vivo anti-inflammatory experiments to elucidate these synergistic mechanisms, explore the regulatory effects of huajiao components on the intestinal microbiota, and thereby provide data support for the clinical application of huajiao extract.

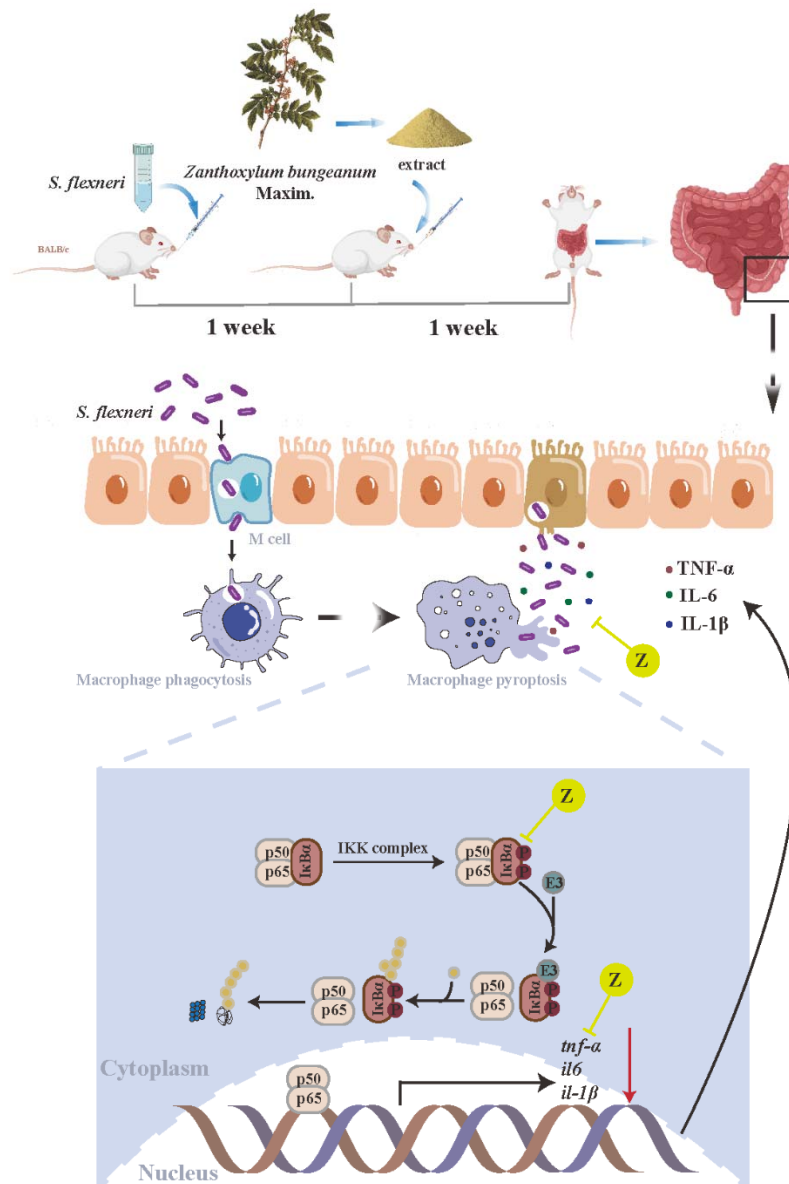


Fig. 8 Mechanism of huajiao pericarp in alleviating *Shigella flexneri*-induced colitis in mice.

5. Conclusion

This study combined untargeted metabolomics and in vitro antibacterial assays with PCA, PLS-DA, and PLS modeling to identify potential anti-*Shigella flexneri* compounds in huajiao pericarp. Network pharmacology and molecular docking pinpointed NF- κ B and TNF as key targets, while KEGG and GO enrichment analyses highlighted the classical NF- κ B pathway as the principal signaling axis. These findings were validated in a mouse model of *S. flexneri*-induced colitis, where huajiao extract significantly mitigated colonic damage by downregulating proinflammatory cytokines (TNF- α , IL-1 β , IL-6) and suppressing phosphorylation of NF- κ B p65 and I κ B α , thereby attenuating intestinal inflammation.

Ethical statement

Approval for the animal experiments was granted by the Ethics Committee of the Laboratory Animal Center, Southwest University (IACUC-20250403-02).

Declaration of Competing Interest

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

Acknowledgments

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