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Network pharmacology and experimental verification in vivo reveal the mechanism of Zhushao Granules against ulcerative colitis

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Abstract

Background Zhushao Granules (ZSG) had exhibited beneficial effects in the treatment of ulcerative colitis (UC) as an effective herbal prescription in Traditional Chinese Medicine. However, the underlying anti-inflammatory mechanism of ZSG remains unclear. This study aimed to decipher the mechanism of ZSG against UC combining network pharmacology and animal-based experiments.

Methods Network pharmacology was employed to identify active components and therapeutic targets of ZSG against UC. The protein–protein interaction (PPI) network was constructed among the therapeutic targets using the STRING database, and GO and pathway analyses were carried out using DAVID. Then, the "herb-component-target-pathway" network based on therapeutic targets was established and the topological parameters were subsequently calculated to identify hub active components, targets and pathways by Cytoscape. Finally, the therapeutic function and the special pathway of ZSG against UC were validated using a TNBS-induced UC model in BABL/c mice.

Results Ninety-four active components of ZSG and 460 potential targets were acquired from the Encyclopedia of Traditional Chinese Medicine and Tradition Chinese Medicine Systems Pharmacology Database and Analysis Platform. 884 potential targets of UC were obtained from OMIM and HINT. Sixty-two overlapping potential targets were identified as therapeutic targets of ZSG against UC. PPI network filtered out 61 therapeutic targets. GO and pathway analyses extracted 48, 25, and 98 terms corresponding to biological processes, molecular functions and Reactome pathways, respectively. Enrichment analysis suggested that the therapeutic targets were mainly involved in immune regulation, especially RIP-mediated NF-κB activation via ZBP1. Topological analysis of the "herb-component-target-pathway" network recognized 9 hub components, 20 hub targets and 18 hub pathways. The animal-based experiments revealed that ZSG ameliorated symptoms and histological changes in TNBS-induced colitis by significantly inhibiting the ZBP1/RIP/NF-κB pathway.

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Conclusions ZSG might alleviate the mucosal damage and ameliorate colitis via targeting ZBP1/RIP/NF-κB pathway, which laid the theoretical foundation for the clinical application and further study of ZSG and provided new insights into UC treatment.

Keywords Zhushao granules, Ulcerative colitis, Network pharmacology, ZBP1/RIP/NF-κB pathway

Background

Ulcerative colitis (UC) is a chronic, immune-mediated and non-specific inflammatory disease that is affected by mucosal inflammation in the rectum and colon [1, 2]. UC is characterized by hemorrhagic diarrhea and passage of mucus or pus, which could cause abdominal cramping during bowel movements [3]. UC has a prolonged disease duration and is prone to recurrent attacks due to inflammatory cell infiltration, which raises the risk of colitis-associated cancer (CAC) [4]. The World Health Organization (WHO) has identified UC as a modern refractory disease [5]. The pathogenesis of UC is associated with hereditary abnormalities, dietary factors, intestinal barrier dysfunction, intestinal flora imbalance and immune dysregulation [6]. Currently, clinical treatment mainly includes sulfasalazine, mesalazine, azathioprine, prednisone, methotrexate, Janus kinase blockers and stem cell-based therapy [7]. These therapies are less than satisfactory, mainly because they only remit UC rather than completely cure the disease. UC is prone to relapse after discontinuation of the treatment and long-term medication could produce a series of side effects [8, 9]. It is therefore necessary to explore safer and more effective alternatives for UC therapy. Traditional Chinese Medicine (TCM) is a rich resource for exploring UC therapeutic drugs due to its multi-component, multi-target and multi-channel features, which could enhance body functions and reduce drug toxicity through the synergistic actions of active components during long-term clinical usage.

Zhushao Granules (ZSG) is derived from the addition and subtraction of ancient prescriptions of TCM "Tongxieyaofang" (TXYF, recorded in JingYue Encyclopedia) and has been prescribed for patients with UC in the clinic. ZSG combines fifteen herbs with functions such as enhancing the spleen and stomach functions [e.g., *Atractylodes macrocephala Koidz.* (Bai Zhu, BZ), *Astragalus mongholicus Bunge* (Huang Qi, HQ), *Jujubae Fructus* (Da Zao, DZ), *GlycyrrhizaeRadix et Rhizoma* (Gan Cao, GC)], relieving pain [e.g., *Paeonia lactiflora Pall.* (Bai Shao, BS), *Saposhnikovia divaricata (Turcz.) Schischk.* (Fang Feng, FF), *Cinnamomum cassia Presl* (Gui Zhi, GZ), *LiliiBulbus* (Bai He, BH)], stopping diarrhea [e.g., *Cullen corylifolium (L.) Medik.* (Bu Gu Zhi, BGZ), *Terminalia chebula Retz.* (He Zi, HZ), *Lablab Semen Album* (Bai Bian Dou, BBD), *Zingiberis Rhizoma* (Gan Jiang, GJ), *AmomiFructus* (Sha Ren, SR)], and regulating gastrointestinal motility [e.g., *Cttri reticulatae Pericarpium* (Chen Pi, CP), *LinderaeRadix* (Wu Yao, WY)]. These herbs work synergistically to treat UC by strengthening the spleen, relieving pain, and stopping diarrhea. However, due to the multi-component and multi-target characteristics of ZSG, its pharmacological mechanism is complex and undefined, which limits its extensive clinical application.

Network pharmacology is a modern theory based on multi-directional systematic pharmacology and biology [10–12], and has been used in TCM research in recent years. Network pharmacology emphasizes the multi-component, multi-target and multi-channel characteristics of TCM, so that it can reveal the scientific basis and therapeutic mechanism of TCM formulae more systematically and comprehensively [13–15]. Therefore, this study employed a network pharmacology method to decipher the potential mechanism of ZSG in treatment of UC from multiple perspectives.

UC is a multifactorial autoimmune disease that is characterized by immune dysfunctions in both innate and adaptive immune systems [16]. And immune cells have important influence on the etiology of UC, especially those participating in both adaptive and innate reactions. Antigens could activate the innate immune response via antigen-presenting cells (APCs) and T cells and trigger an inflammatory cascade, which then activate the adaptive immune system [17]. Dendritic cells serve as APCs in the innate immune system and abundantly express Toll-like receptors (TLRs) that recognize pathogen patterns and trigger inflammatory cascades via activating transcription factors such as nuclear factor-κB (NF-κB) [18]. Neutrophils are the primary factors in the inflammatory process of the gastrointestinal tissue and contribute to tissue damage, degradation of epithelial barrier function and the production of inflammatory cytokines in UC [19]. Innate lymphoid cells locate near the intestinal epithelium and produce various cytokines to maintain epithelial integrity and defend against infections, but altered microbiota and excessive antigens might lead to the initiation and perpetuation of UC [20]. Although the adaptive immune response takes longer, it is more precise than the innate immune response. CD8 + T cells could cause intestinal pathological alterations and contribute to epithelial cell destruction and intestinal ulcers developing through producing and releasing proinflammatory

cytokines [20]. CD4 + T cells include T helper (Th) cells and regulatory T (Treg) cells, and the imbalance between Th17 cells and Treg cells is identified to be a crucial factor in the pathogenesis of UC [21]. Th17 cells could induce autoimmunity and contribute to tissue damage, and the opposite is that Treg cells could suppress tissue damage caused by immune and inflammation [22, 23]. Therefore, the involvement of immune regulation in developing UC is highly significant.

Z-DNA binding protein 1 (ZBP1, also known as DAI) has a critical regulatory role in cell death, inflammation and immunity [24]. ZBP1 possesses two well-characterized and a third supposed receptor-interacting protein (RIP) homotypic interaction motifs (RHIMs), which enables it to interact with other proteins containing RHIMs such as RIP1 and RIP3 [25, 26]. ZBP1-mediated NF-κB activation is controlled by interaction with RIP1 and regulated by RIP3 [25]. ZBP1 replies to microbial, cytosolic viral or host DNA via stimulating NF-κB activation, which forces subsequent proinflammatory and antiviral cytokine production in the cell-intrinsic innate immune response [27, 28]. Moreover, NF-κB plays central roles in various inflammatory conditions and regulates the production of inflammatory molecules in colitis, which make it an important therapeutic target [29]. Our study deduced that ZSG might target ZBP1/RIP/NF-κB pathway against UC based on network pharmacology. ZSG originated from TXYF, an efficacious prescription of TCM widely used in easing abdominal pain and diarrhea [30]. TXYF was shown to regulate macrophage polarization to improve DSS-induced colitis via NF-κB/NLRP3 signaling pathway, and suppress inflammation in UC rat model via regulating MAPK/AKT signaling pathway [30, 31]. Though ZSG, the modified formula of TXYF, has been used to treat UC, the comprehending of the specific mechanism of ZSG against UC remains limited and lacks systematic insights. Thus, it's necessary and crucial to employ creative strategies and new perspectives for comprehensive analysis of the anti-inflammatory mechanism of ZSG.

Given the complex and undefined pharmacological mechanisms of ZSG, this study aimed to identify its active components, targets, and pathways involved in UC treatment using network pharmacology, and to validate these findings through animal experiments. This study might provide a theoretical foundation for the clinical application and further study of ZSG in the treatment of UC.

Materials and methods

Screening of active components of ZSG

Thirteen herbal active components of ZSG were collected and filtered with Drug-likeness Weight (DW) ≥ 0.49 from the Encyclopedia of Traditional Chinese Medicine (ETCM), except for HZ and WY. ETCM (http://www.tcmip.cn/ETCM/index.php) [32] provides comprehensive and standardized information, and predicts target genes of TCM ingredients, herbs and formulae. The active components of HZ and WY were supplemented and determined with oral bioavailability (OB) ≥ 30% and drug-likeness (DL) ≥ 0.18 using the Traditional Chinese Medicine Systems Pharmacology Database and Analysis Platform (TCMSP). TCMSP (https://tcmsp.com/tcmsp.php) [33] is a distinct system pharmacology platform of Chinese herbal medicines, which captures the relationships among chemicals, targets and diseases, and pharmacokinetic properties of natural compounds.

Prediction of potential targets of ZSG and UC

The ETCM database can match predicted target genes of TCM components, and the potential targets of ZSG were obtained with a score ≥ 0.8 through ETCM except HZ and WY. The related targets of HZ and WY were supplemented and acquired from TCMSP. The related targets of UC were inquired from the OMIM (https://www.omim.org/) [34], and the interacting proteins were extracted from HINT (http://hint.yulab.org/). The overlapping targets between ZSG and UC were identified as therapeutic targets of ZSG against UC and the corresponding Venn diagram was drawn.

Protein–protein interaction (PPI) network construction

The protein–protein interaction data among therapeutic targets were exported from the STRING database (http://string-db.org) [35], with species limited to "Homo sapiens" and a confidence score of 0.4. The network visualization and analysis were executed by Cytoscape 3.6.1 [36].

Gene ontology (GO) and pathway analysis

GO analysis was performed to further confirm whether the therapeutic targets indeed matched UC in both biological process (BP) and molecular function (MF). Reactome pathway analysis was conducted to comprehensively illuminate the mechanism of ZSG against UC. Both GO and Reactome pathway analyses were conducted using the Database for Annotation, Visualization and Integrated Discovery (DAVID) (https://david-d.ncifcrf.gov/) [37]. Therapeutic targets filtered through the STRING database were inputted into DAVID database and the species was set to "Homo sapiens". The significant enrichment threshold was set as $P < 0.001$.

Construction of the "herb-component-target-pathway" network

Based on the therapeutic targets of ZSG against UC, the corresponding active components and herbs that they

stemmed from were traced. The "herb-component-target-pathway" network was subsequently established by Cytoscape 3.6.1, which exhibited the regulation diagram of ZSG in treatment of UC. Then, the topological parameters of the network were calculated using the "Network-Analyzer" plugin and the hub active components of ZSG related to anti-UC activity were identified.

Drugs and reagents

ZSG, consisting of fifteen herbs: *Atractylodes macrocephala Koidz.*, *Paeonia lactiflora Pall.*, *Astragalus mongholicus Bunge*, *Saposhnikovia divaricata (Turcz.) Schischk.*, *Cullen corylifolium (L.) Medik.*, *Terminalia chebula Retz.*, *Cttri reticulatae Pericarpium*, *Lablab Semen Album*, *Cinnamomum cassia Presl*, *Zingiberis Rhizoma*, *Amomi Fructus*, *Lilii Bulbus*, *Linderae Radix*, *Jujubae Fructus* and *Glycyrrhizae Radix et Rhizoma*, was purchased from Yantai Hospital of Traditional Chinese Medicine. ZSG was developed and formulated as their own medicine of Yantai Hospital of Traditional Chinese Medicine (ZYSL-YAT201600057). Salicylazosulfapyridine (SASP, lot: H31020557) was purchased from Shanghai Xinyi Tianping Pharmaceutical Co., Ltd (Shanghai, China). 2,4,6-Trinitrobenzenesulfonic acid (TNBS) was purchased from Beijing Innochem Technology Co., Ltd (Beijing, China).

Experimental animals

BALB/c mice were purchased from Jinan Pengyue Experimental Animal Breeding Co., Ltd (Jinan, China). They were raised under controlled pathogen-free conditions with free access to food and water at 23 ± 1℃ room temperature with 40–45% relative humidity and a 12 h light/dark cycle, and were acclimatized for 7 days before any treatment. The animal experiment protocols were reviewed and approved by Ethics Committee of Yantai Yuhuangding Hospital (No. 2022–61).

Induction of UC and treatment

All mice accepted adaptive feeding for 7 days. BALB/c mice were randomly divided into six groups: Control group ($n=4$), TNBS model group ($n=4$), ZSG low-dose group (ZSG-L, 1.56 g/kg, $n=7$), ZSG medium-dose group (ZSG-M, 3.12 g/kg, $n=7$), ZSG high-dose group (ZSG-H, 6.24 g/kg, $n=7$), and SASP group (390 mg/kg, $n=7$). The mice were fasted for 24 h and chronic UC was induced using TNBS/50% ethanol dilution enema for 6 weeks according to a reported protocol with modification [38]. TNBS (5%, wt/vol) was prepared in 50% ethanol and TNBS at the dose of 1.5 mg, 2 mg, or 2.5 mg was administrated corresponding to weeks 1 and 2, 3 and 4, 5 and 6, respectively. The 3.5-F catheter was introduced into the colon through the anus for ~ 4 cm. Then, 100 μL of TNBS solution was slowly injected into the lumen of the colon and the mouse was positioned upside down for 1 min to ensure even distribution of the solution in the colon. The mice of the three ZSG groups and the SASP group were administrated with ZSG or SASP for 21 days. The Control and TNBS model mice were subjected to autoclaved water enema instead.

Assessment of disease activity index (DAI)

DAI can be used to assess the health status of UC mice according to a previous description [39]. DAI was scored by monitoring the circumstances of weight loss, stool consistency and fecal occult blood according to the criteria in Table 1 during the 21-day administration period. DAI was calculated as DAI = (Weight loss + Stool consistency + Fecal blood) / 3.

Evaluation of histological damage

The colon tissues were fixed with 4% formalin solution and sequentially embedded in paraffin. Then, the samples were subjected to sectioning, deparaffinization, hydration, and staining with hematoxylin–eosin (H&E). Light microscopy was employed to observe colonic lesions, such as severity and extent of inflammation, crypt damage, and range of lesions. Histological damage scores were calculated in a blinded fashion according to the following criteria: (1) inflammation severity (0, none; 1, slight; 2, severe); (2) lesion depth (0, none; 1, submucosa; 2, muscularis; 3, serosa); (3) crypt damage: (0, none; 1, basal 1/3 damaged; 2, basal 2/3 damaged; 3, only surface epithelium intact; 4, entire crypt and epithelium lost); (4) range of lesions (1, 1–25%; 2, 26–50%; 3, 51–75%; 4, 76–100%) [40]. The total histological scores were the summary of the individual scores.

Western blot analysis

Total proteins were extracted from colon tissues using RIPA lysis buffer containing protease and phosphorylase inhibitor cocktail (Beyotime, China). The protein concentration in the supernatant was detected using the BCA protein assay kit (Beyotime). Equal amounts of protein from each sample were separated by 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE)

Table 1 Criteria for DAI score

Scoring	Weight loss	Stool consistency	Fecal blood
0	< 1%	well-formed pellets	normal(-)
1	1–5%	loose stools	occult bleeding (+)
2	5–10%	loose stools	occult bleeding (++)
3	10–15%	diarrhea	visible bleeding (+)
4	> 15%	diarrhea	visible bleeding (++)

and transferred to nitrocellulose membranes. Then, the membranes were blocked with 5% non-fat milk with TBST buffer for 1 h at room temperature, followed by incubation with primary antibodies overnight at 4 ℃. The primary antibodies included rabbit anti-ZBP1 antibody (1:500, Abcam, Cat#ab81526), rabbit anti-RIP3 antibody (1:500, Affinity, Cat#AF7942), rabbit anti-NF-κB p65 antibody (1:500, Affinity, Cat#AF5006), rabbit anti-phospho-NF-κB p65 antibody (1:500, Affinity, Cat#AF2006), rabbit anti-GAPDH antibody (1:2000, Affinity, Cat#AF7021). The membranes were washed with TBST three times and incubated with HRP-conjugated secondary antibodies for 1 h at room temperature. Finally, the protein bands were visualized on an enhanced chemiluminescence (ECL) system, and the density of blots was analyzed with optical density analysis software.

Statistical analysis

All data were processed using GraphPad Prism 6.0 and expressed as mean ± SEM. One-way analysis of variance (ANOVA) and unpaired two-tailed *t*-test were used to assess the significance of differences between groups. A value of $P < 0.05$ was considered statistically significant.

Results

Active components of ZSG

There were 94 active components in ZSG that were extracted from ETCM for 13 herbs with DW ≥ 0.49, supplemented with HZ and WY extracted from TCMSP with OB ≥ 30% and DL ≥ 0.18 (Supplementary Table 1). Among them, 6 compounds were from BZ, 5 compounds from BS, 1 from HQ, 10 from FF, 7 from BGZ, 7 from HZ, 10 from CP, 1 from BBD, 3 from GZ, 5 from GJ, 3 from SR, 2 from BH, 9 from WY, 9 from DZ, and 23 from GC. Duplicated components from different herbs were deleted from the pool.

Therapeutic targets of ZSG against UC

According to the target prediction of the ETCM platform, we identified 87 targets associated with BZ, 111 targets with BS, 65 with HQ, 12 with FF, 103with BGZ, 21 with CP, 7 with BBD, 12 with GZ, 6 with GJ, 30 with SR, 20 with BH, 170 with DZ, and 169 with GC. According to the target prediction of the TCMSP platform, 72 targets were associated with HZ, and 179 targets were associated with WY. A total of 460 targets were retained after deleting duplicates (Supplementary Table 2).

A total of 63 related targets of UC were identified from the OMIM database, and 821 interacting proteins were excavated from the HINT database, all of which were recognized as potential targets of UC. There were 884 potential targets of UC in total (Supplementary Table 3). As shown in Fig. 1A, 62 therapeutic targets of ZSG against UC were identified by overlapping the targets of ZSG active components and the potential targets of UC (Supplementary Table 4). Among them, 61 targets interacted with others with a confidence score ≥ 0.4 (Fig. 1B). TP53 was the most key target, followed by HSP90AA1, ESR1, HIF1A, and CCND1. The top 30 targets in action frequency were shown in Fig. 1C.

GO and pathway analyses

GO and Reactome pathway enrichment analyses were performed to gain insights into the possible mechanisms of ZSG against UC. The GO enrichment results were classified into two categories: BP and MF. At the BP level, ZSG had a great influence on positive regulation of DNA-templated transcription, positive regulation of transcription from RNA polymerase II promoter, protein phosphorylation, positive regulation of gene expression, peptidyl-serine phosphorylation, etc. (Fig. 2A, Supplementary Table 5). At the MF level, ZSG was mainly involved in enzyme binding, protein kinase binding, ubiquitin protein ligase binding, transcription factor binding, identical protein binding, etc. (Fig. 2B, Supplementary Table 6). The Reactome pathway enrichment analysis revealed that ZSG achieved therapeutic effects for UC through multiple pathways, and the top 10 most significantly enriched pathways were mainly involved in Signal Transduction, Signaling by Interleukins, Interleukin-4 and Interleukin-13 signaling, Cytokine Signaling in Immune system, Generic Transcription Pathway, RNA Polymerase II Transcription, Gene expression (Transcription), Disease, Cellular Senescence, and Cellular responses to stress (Fig. 2C, Supplementary Table 7).

Construction of the "herb-component-target-pathway" network

Using the therapeutic targets as sources, the corresponding active components and the herbs acting on the targets were traced and the "herb-component-target-pathway" network was constructed and visualized using Cytoscape software. As shown in Fig. 3, the network was composed of 219 nodes (12 herbs, 48 components, 61 targets and 98 pathways) and 1374 edges. Each component corresponded to multiple targets and herbs, and vice versa. The network was consistent with the characteristics of multi-compound, multi-target and multi-pathway of TCM. The topological parameters of the network were calculated and the nodes with degree, betweenness centrality and closeness centrality above the corresponding average value were identified as the hub components, targets and pathways of ZSG against UC (Fig. 4). The hub components included quercetin, Catechin, Kumatakenin, Neobavachalcone, Isoneobavachalcone, Corylinal,

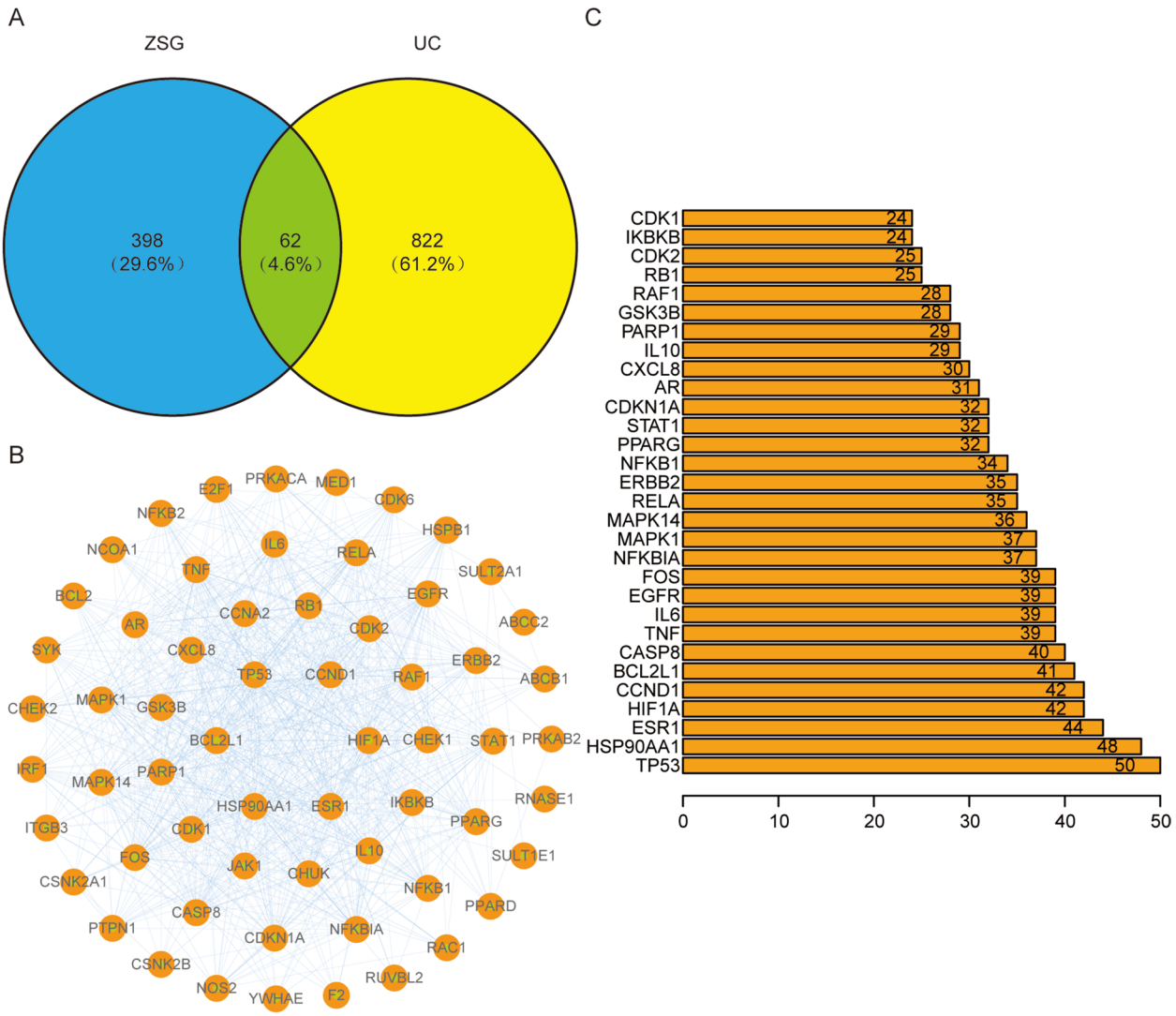

Fig. 1 The therapeutic targets of ZSG against UC. **A** The Venn diagram. **B** Sixty-one interacting nodes in the PPI network. **C** The top 30 targets in action frequency

ellagic acid, 6,7-dimethoxy-2-(2-phenylethyl)chromone and DMPEC. The hub targets were ESR1, AR, RELA, PRKACA, NFKB1, MAPK1, CDK6, TP53, CDKN1A, CDK2, CSNK2A1, MAPK14, NCOA1, CHUK, NFKBIA, HSP90AA1, FOS, IKBKB, CCND1, NFKB2. The hub pathways involved in Signal Transduction, Disease, Gene expression (Transcription), RNA Polymerase II Transcription, Generic Transcription Pathway, Immune System, Cellular responses to stimuli, Cellular responses to stress, Cytokine Signaling in Immune system, Signaling by Interleukins, Developmental Biology, Cell Cycle, Infectious disease, Innate Immune System, Signaling by Receptor Tyrosine Kinases, Intracellular signaling by second messengers, Cell Cycle, Mitotic and Cellular Senescence.

Establishment of the UC mouse model

To verify the mechanism of ZSG against UC identified by Reactome pathway analysis, we established a BALB/c mouse model of UC induced by TNBS enema (TNBS model group, $n=4$), and treated the mice with low/medium/high dosage of ZSG (ZSG-L/M/H group, $n=7$ each). UC mice treated with SASP were used as positive control (SASP group, $n=7$), and uninduced mice were used as negative control (Control group, $n=4$). The schematic diagram of the experiment was showed in Fig. 5A.

Assessment of DAI

DAI was used to assess the health status of UC mice as indicated by weight loss, stool consistency and fecal blood. As shown in Fig. 5B, the TNBS model group exhibited a higher DAI score than the Control group

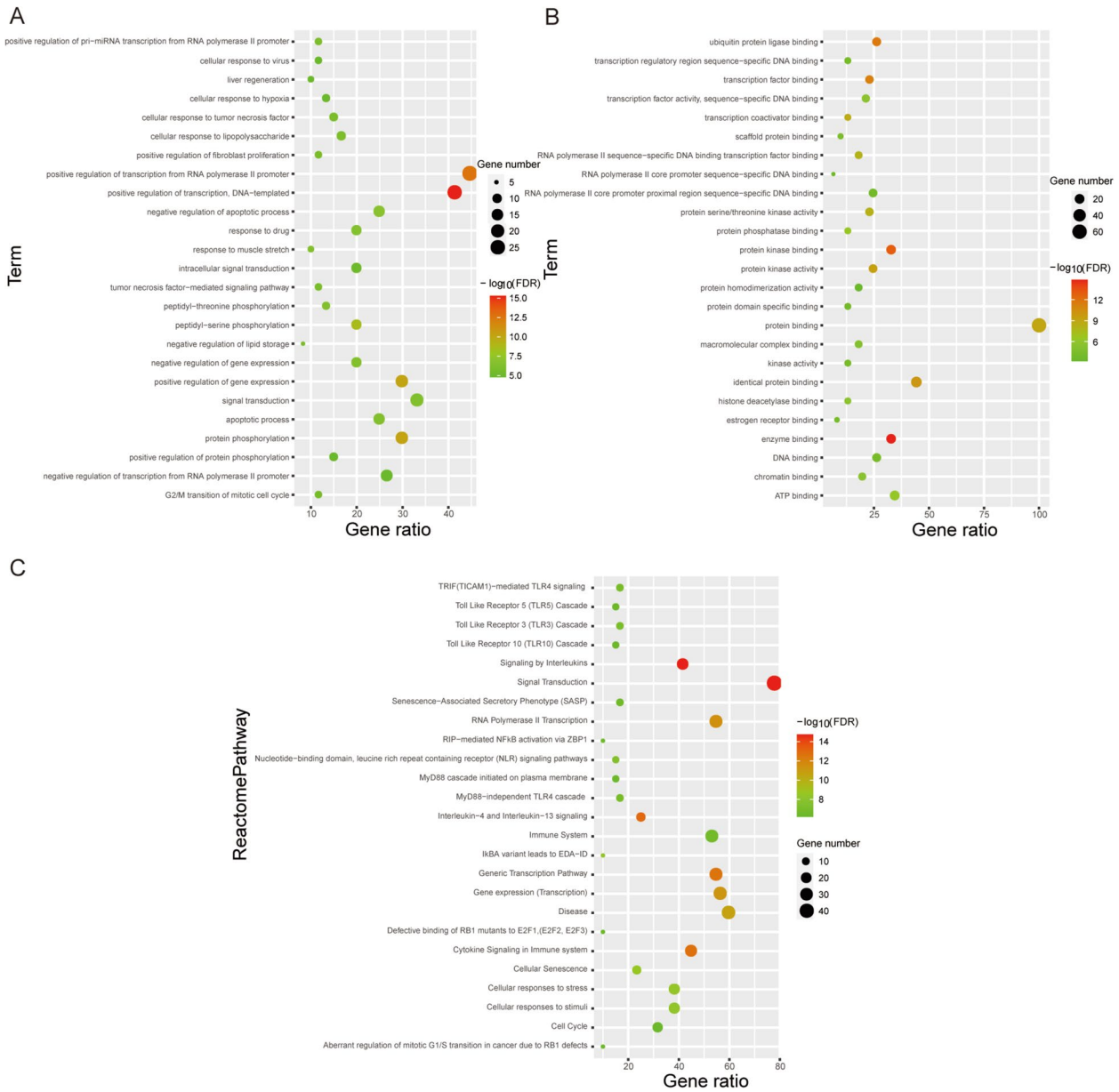

Fig. 2 GO and pathway analysis enriched for therapeutic targets of ZSG against UC. **A** The top 25 BP terms enriched for therapeutic targets of ZSG against UC. **B** The top 25 MF terms enriched for therapeutic targets of ZSG against UC. **C** The top 25 terms of the Reactome pathway enriched for therapeutic targets of ZSG against UC

($P < 0.0001$), which suggested that the TNBS-induced UC model was successfully established. Compared to the TNBS model group, ZSG and SARP administration ameliorated the symptoms of colitis, especially in the high-dose group ($P < 0.001$). However, there were no significant improvements in the low- or medium-dose groups. Furthermore, Two-way analysis of ANOVA showed that the ZSG-H and SARP groups exhibited obvious protective effects on UC starting on day 16 of administration.

Evaluation of histological damage

The histopathological analysis of mouse colon tissues was shown in Fig. 5C-D. The colon tissues in Control group exhibited well-structured and smooth mucosa, with no obvious ulcer or inflammatory cell infiltration. In contrast, the TNBS model group showed extensive mucosal damage, inflammatory infiltration, mucosal edema and crypt loss. The TNBS-induced pathological deterioration in the TNBS model group caused a significant increase in histological scores compared with the Control group

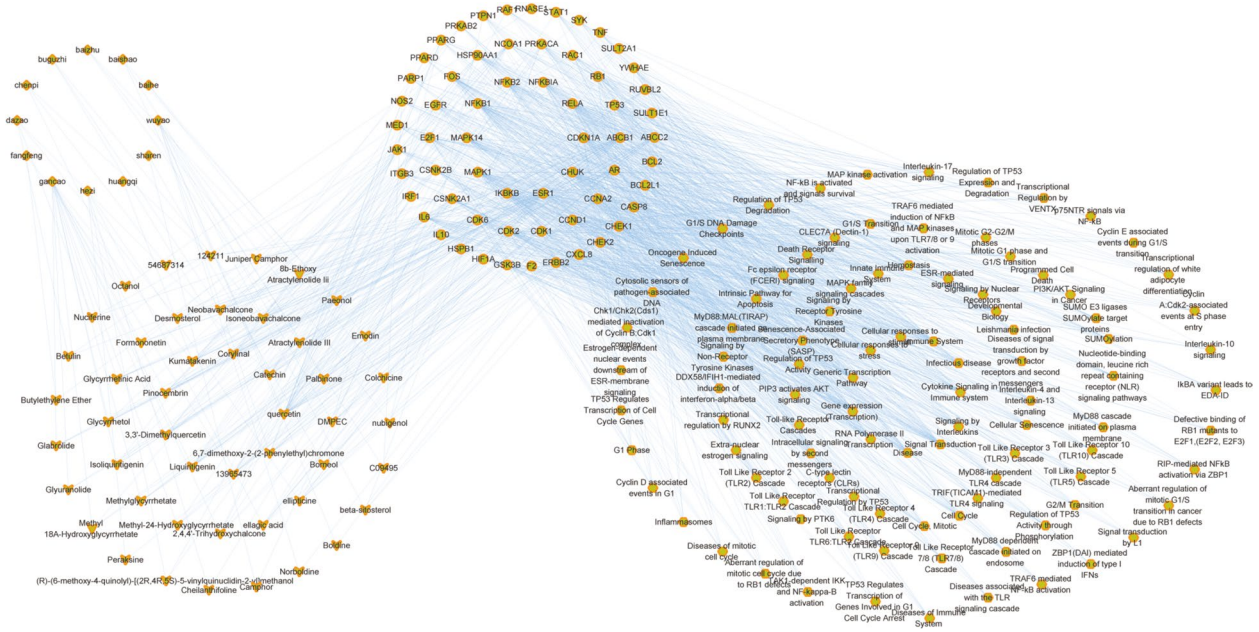

Fig. 3 "Herb-component-target-pathway" network. The diamond, triangular arrowheads, ellipse and hexagon nodes represented the herb, component, target and pathway, respectively. The edges represented the interactions between nodes

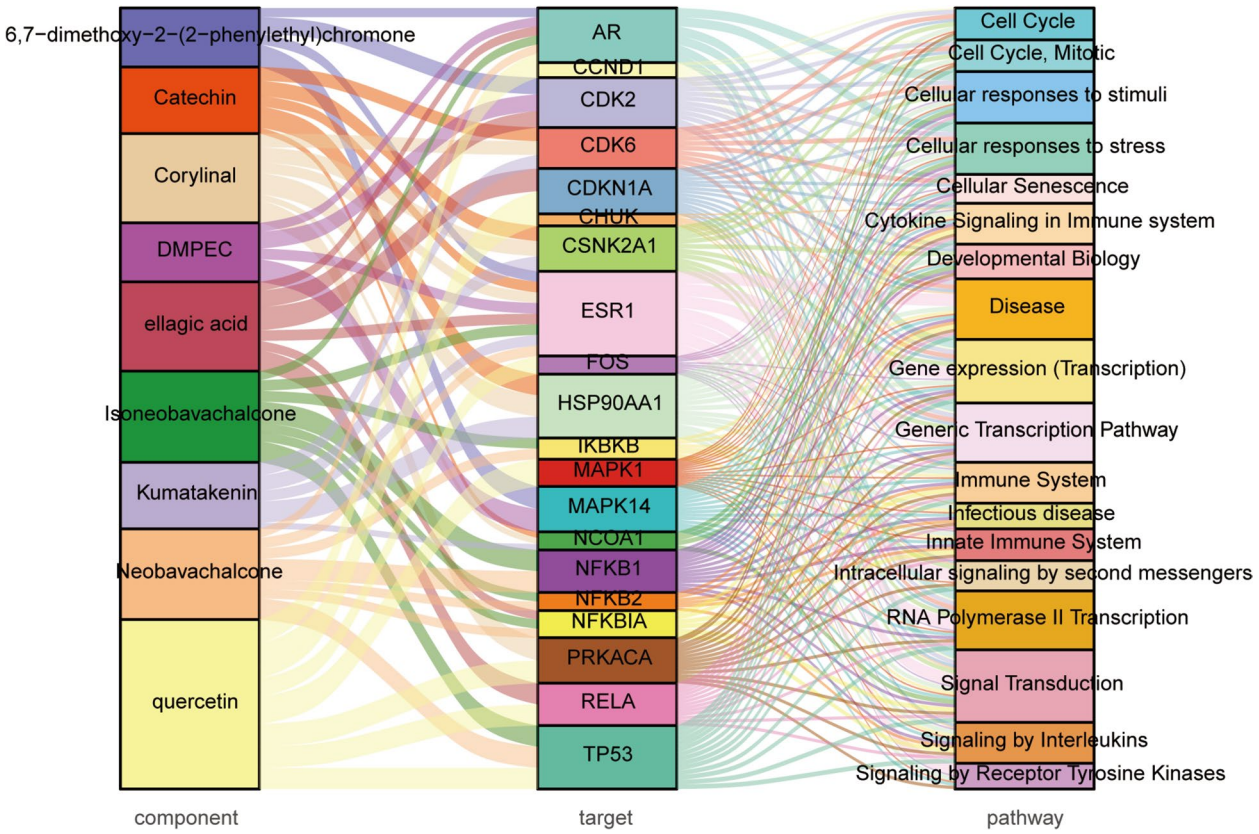

Fig. 4 The hub nodes of the "herb-component-target-pathway" network

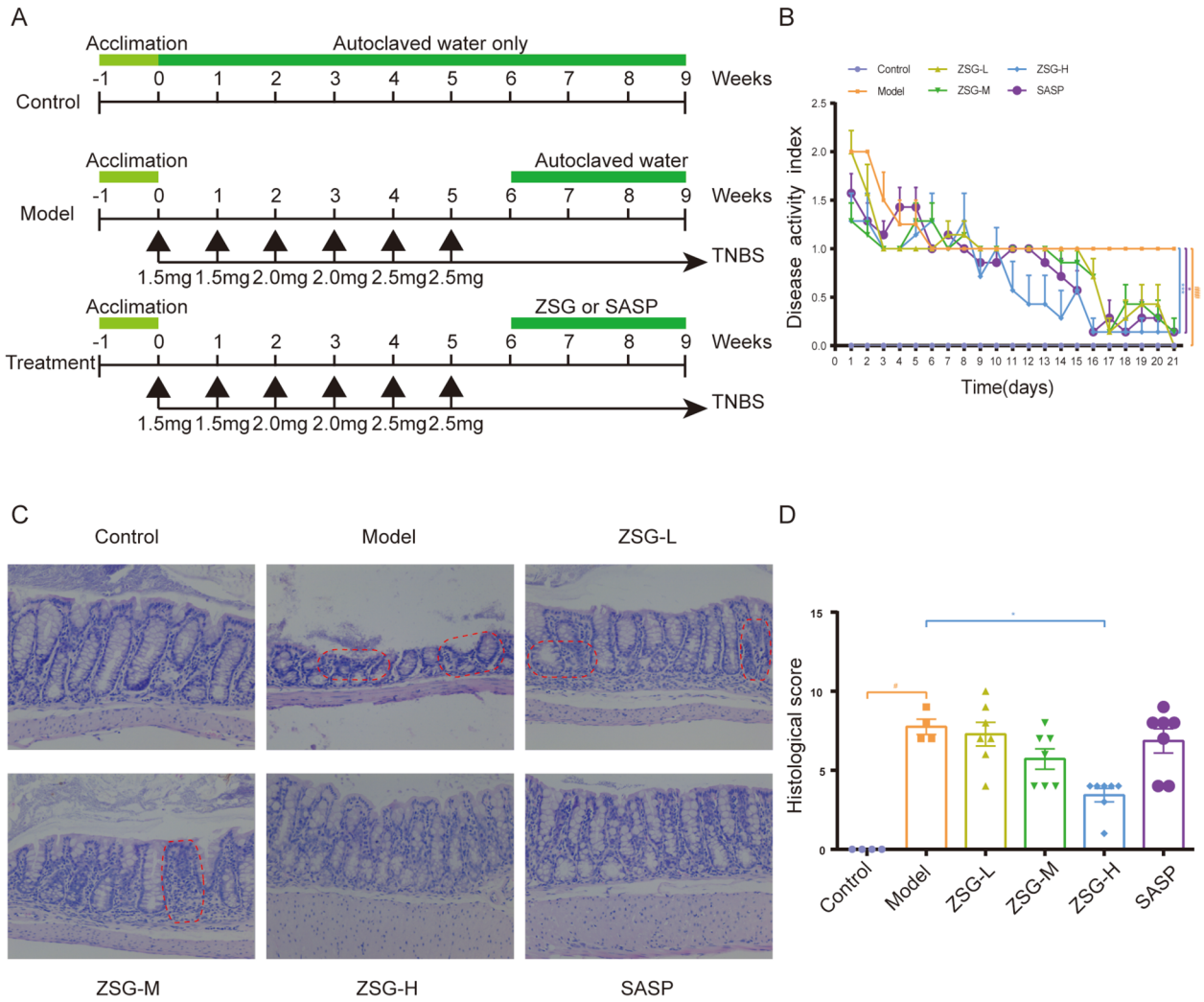

Fig. 5 ZSG administration protected against TNBS-induced colitis. **A** Schematic diagram of the experiment. **B** DAI scores. **C** Representative images of H&E staining in the colon. **D** Histological scores. $^{\#}P<0.05$, $^{\#\#\#\#}P<0.0001$ vs. Control group; $^{*}P<0.05$, $^{***}P<0.001$ vs. TNBS model group

($P<0.05$). ZSG or SARP administration restored intestinal mucosal integrity and resulted in well-preserved crypt structures, and different degrees of alleviation of the histological scores were observed. Specifically, mucosal damage, inflammatory infiltration, mucosal edema and crypt loss in the ZSG-H group were significantly alleviated compared to those in TNBS model group ($P<0.05$), which demonstrated that ZSG possessed significant therapeutic effects on TNBS-induced colon damage in UC mice.

ZSG inhibited the ZBP1/RIP/NF-κB pathway

Through bioinformatics analysis we found that the therapeutic targets of ZSG against UC were mainly responsible for the regulation of the immune system, including RIP-mediated NF-κB activation via ZBP1, which might play a crucial role during ZSG in treatment of UC. To investigate whether ZSG improved colitis via the ZBP1/RIP/NF-κB pathway, the expression of corresponding proteins in the pathway was detected by western blotting. As shown in Fig. 6, the ZBP1/RIP/NF-κB pathway was significantly activated in the TNBS-induced group, which manifested significantly enhanced expression of RIP3 and p-p65 and decreased expression of p65, compared with that in the Control group ($P<0.05$). A significant up-regulation of ZBP1 expression was observed in the ZSG-H and SARP groups ($P<0.05$), but the remission effect of the ZSG-L/M groups on ZBP1 was not obvious as compared with that of the TNBS model group. RIP3 was significantly inhibited in the ZSG-M/H groups ($P<0.05$), with no obvious decrease in the ZSG-L and SARP groups compared with that of the TNBS model group. However, the expression of p65 was markedly facilitated only in the ZSG-M group ($P<0.05$), and had limited alleviation

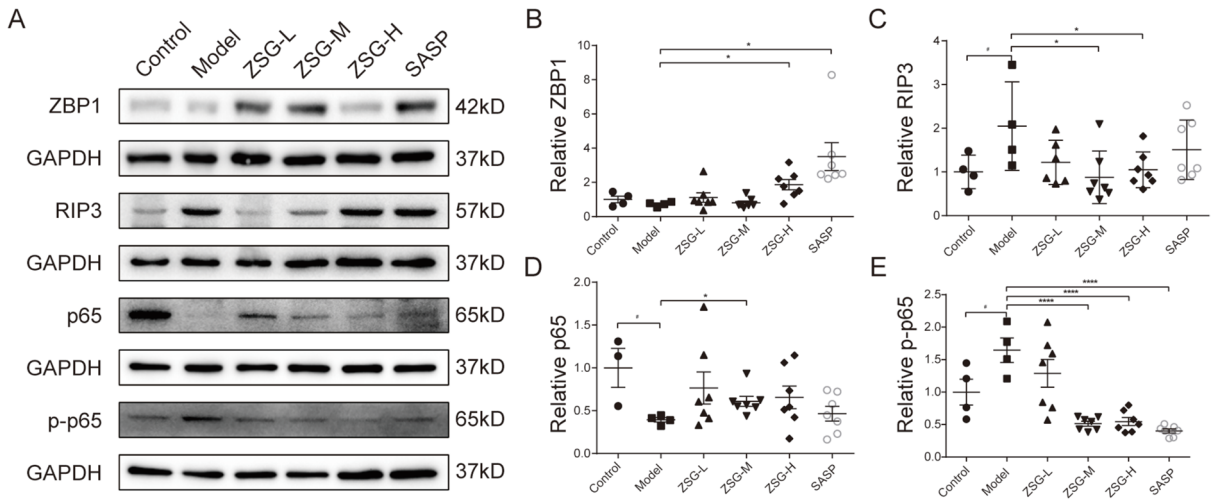

Fig. 6 ZSG administration significantly inhibited the ZBP1/RIP/NF-κB pathway in TNBS-induced mice. **A** Representative protein bands by western blot analysis. **B**-**E** Quantitative analysis of the protein expression levels of ZBP1, RIP3, p65, and p-p65 based on western blot images. $^{\#}P < 0.05$ vs. Control group; $^{*}P < 0.05$, $^{****}P < 0.0001$ vs. TNBS model group

in the ZSG-L/H and SARP groups compared with that of the TNBS model group. The foremost was that the phosphorylation of p65 was evidently inhibited in the ZSG-M/H and SARP groups but not in the ZSG-L group when compared to the TNBS model group ($P < 0.0001$). These results suggested that ZSG possibly relieved UC in mice by targeting the ZBP1/RIP/NF-κB pathway.

Discussion

UC is a serious public health concern with an increasing incidence globally over the recent decade, especially in developing nations [41]. ZSG, a classic prescription of hospital preparations, has exhibited beneficial effects on the treatment of UC as an effective and safe TCM. However, there is a lack of modern experimental evidence and no literature report is available on its active compounds and pharmacological mechanism. The synergistic effects of multi-components and multi-targets of herbs raise new challenges to the mechanistic research of TCM. Therefore, this study employed network pharmacology to illuminate the active components, potential targets and pathway mechanism of ZSG in treatment of UC. Animal-based studies were performed to evaluate the therapeutic effects and verify the specific pathway of ZSG in treating UC.

In this study, network pharmacology was employed to pool 94 active components and 460 potential targets of ZSG. A total of 844 potential targets of UC were also identified. Cross-comparison of the potential targets of ZSG and UC resulted in 62 common targets, which were identified as therapeutic targets of ZSG against UC. The PPI network was constructed among the therapeutic targets, and 61 targets were filtered out. GO and Reactome pathway analyses were carried out to elucidate the therapeutic mechanism of ZSG against UC. Then, the corresponding active components and herbs based on therapeutic targets of ZSG against UC were traced and the "herb-component-target-pathway" network was subsequently established. The topological parameters of the network that followed were calculated to identify the hub active components, targets and pathways of ZSG against UC. The topological analysis showed that 9 hub components, 20 hub targets and 18 hub pathways possessed higher values than the average degree, closeness centrality and betweenness centrality.

In this study, the enrichment analysis results suggested that the therapeutic targets were mainly responsible for the regulation of the immune system, such as Interleukin-4 and Interleukin-13 signaling, Nucleotide-binding domain, leucine rich repeat containing receptor (NLR) signaling pathways, Toll Like Receptor 3 (TLR3) Cascade, TRIF(TICAM1)-mediated TLR4 signaling, MyD88 cascade initiated on plasma membrane, Toll Like Receptor 10 (TLR10) Cascade, and RIP-mediated NF-κB activation via ZBP1. Literature had indicated that IL13, NLR, TLR3, TLR4, MyD88, and TLR10 could directly or indirectly transmit signals to NF-κB, leading to its activation or suppression [42–46]. This highlighted the significance of the NF-κB pathway and underscored the multi-faceted regulatory mechanisms during ZSG treatment for UC. Among them, NF-κB pathway was also the best researched inflammation pathway related to UC. Therefore, RIP-mediated NF-κB activation via ZBP1 might play a crucial role in ZSG against UC. Other pathways were

also reported to be involved in UC. For instance, SIRPα enhanced the alternative activation of macrophages by promoting Interleukin-4 and Interleukin-13 signaling in DSS-induced colitis [47]. Targeting the Nucleotide-binding domain-like receptor family pyrin domain was identified as a potent agent for treatment of colitis [48]. NLRP3 inflammasome activation was inhibited by Sanguinarine in DSS induced ulcerative colitis [49]. TLR3, a key molecule of the inflammatory cascade, was activated during rhinovirus infection and in turn activated transcription factors such as NF-κB and IFN regulatory factor (IRF)-3/7 leading to the production of pro-inflammatory cytokines and chemokines [50]. The immune responses mediated by TRIF(TICAM1)-mediated TLR4 signaling were inhibited by ADAM15 [51]. Total C-21 steroidal glycosides (TCSG) from Baishouwu was reported to regulate inflammatory response through the MyD88 cascade initiated on the plasma membrane against hepatic and renal fibrosis [52]. TLR family members are key players in the inflammation and TLR10 is poorly studied in chronic inflammatory disorders, but the allelic variant of TLR10 impairs NF-κB inhibitory activity and is highly associated with disease severity and low response to infliximab in patients with rheumatoid arthritis [53].

The effective hub components included quercetin, Catechin, Kumatakenin, Neobavachalcone, Isoneobavachalcone, Corylinal, ellagic acid (EA), 6,7-dimethoxy-2-(2-phenylethyl)chromone and DMPEC. Quercetin was rich in vegetables, fruits, red wine and tea, and widely existed in various TCM including *Sophora jaonica L.*, *Artemisia scoparia Waldst.et Kit.*, *Panax notoginseng (Burk.) F. H. Chen*, *Illicium verum Hook.f.*, *Artemisia argyi Lévl.et Vant.*, etc., which had been demonstrated to product beneficial influences on the expression of injury repair molecules, pro-inflammatory cytokines, and NF-κB inhibitory molecules in DSS-induced UC [54]. Higher dietary quercetin intake was found to be beneficial for lowering risk of adverse outcomes among individuals with UC in a prospective cohort study, which provided novel evidence of quercetin protecting UC patients [55]. Catechin was a main plyphenol compound in green tea and was widely found in many TCM such as *Paeonia lactiflora Pall.*, *Ziziphus jujuba Mill.*, *Prunus persica (L.) Batsch*, *Ginkgo biloba L.* and *Areca catechu L* [56]. Catechin was reported to suppress inflammation via NF-κB-mediated pathway and inhibit iNOS enzyme activity in DSS-induced UC mice, which was also predicted to fit well within the safety profile range for humans and have LD_{50} values in the range of 3919–10000 mg/kg body weight [57–59]. Kumatakenin was the main component of TCM *Eugenia caryophyllataThunb.* and *Alpinia purpurata*, and was also isolated from HQ and GC, which was reported to alleviate DSS-induced colitis largely and lack of significant toxicity in normal mice/cell lines [60]. EA was a polyphenolic compound present in many fruits (e.g., pomegranates, peaches, plums, persimmons, raspberries, wild strawberries), seeds (walnuts, almonds), and vegetables, and was also isolated from TCM such as HZ, *Radix Paeoniae Rubra*, *Rubi Fructus*, *Geranium Wilfordii Maxim.* and *Agrimonia Eupatoria* [61]. EA could only be partially absorbed into the blood due to a low solubility and unabsorbed EA were then processed by the gut bacteria [62]. Seeram reported the highest concentration of EA in human plasma was only 31.9 ng/mL after volunteers drank 180 mL pomegranate juice containing 25 mg EA for 1 h and it vanished after 4 h [63]. EA prevented DSS-induced colitis by suppressing the activation of NF-κB pathway and intestinal barrier dysfunction [64]. 6,7-dimethoxy-2-(2-phenylethyl)chromone and DMPEC, mainly in WY and *Linderae Radix*, could significantly inhibit NF-κB activation and did not show cytotoxicity in LPS-stimulated RAW 264.7 macrophages after 24 h treatment, and 6,7-dimethoxy-2-(2-phenylethyl)chromone showed the most effective inhibition of LPS-induced NF-κB activation among the isolated compounds from agarwood [65]. Neobavachalcone, Isoneobavachalcone and Corylinal were all isolated from BGZ, and their research was rarely reported. The above description indicated that the hub active components of ZSG played protective roles during UC progression. They were generally abundant and feasible for therapeutic applications, and any potential side effects had not yet been documented. The main purpose of Chinese medicine compatibility was to enhance efficacy and minimize toxicity. And scientific studies on the mechanisms of Chinese herbal components on human health had mostly been conducted at animal or cellular level. However, the combined use of these hub components had not been thoroughly evaluated, therefore, their overall safety would be assessed at animal level. We aimed to determine the optimal compatibility ratio of these hub components and compare their efficacy with ZSG through randomized controlled trials. ZSG was determined to exert anti-inflammatory effects mainly by targeting ESR1, AR, RELA, PRKACA, NFKB1, MAPK1, CDK6, TP53, CDKN1A, CDK2, CSNK2A1, MAPK14, NCOA1, CHUK, NFKBIA, HSP90AA1, FOS, IKBKB, CCND1, and NFKB2. A quarter of the current hub targets emerged in the pathway of RIP-mediated NF-κB activation via ZBP1, which also implied the vital function of the ZBP1/RIP/NF-κB pathway during ZSG treatment of UC.

ZBP1 recruited RIP1 and RIP3 through RHIM-RHIM homotypic interactions to induce NF-κB activation and drive inflammation and programmed cell death in a context-dependent manner [66]. ZBP1 was pointed to involve in forcing intestinal inflammation in human

patients with CASP8 mutations, who developed severe forms of very early onset Inflammatory Bowel Disease, and RIP1 kinase activity facilitated ZBP1-mediated necroptosis when caspase-8 was inhibited [67]. It was reported that intestinal inflammation was triggered by RIP3-dependent death of FADD-deficient intestinal epithelial cells [68]. Our study firstly identified ZSG targeted the pathway of RIP-mediated NF-κB activation via ZBP1 for the treatment of UC. It had been discovered that ZBP1 or RIP3 alone attracted 20- or 50-fold NF-κB-dependent expression and ZBP1 and RIP3 synergized to activate NF-κB to 200-fold, but RHIM mutant RIP3 or a kinase-deficient RIP3 down regulated pNF-κB expression by a large margin [25]. Our results showed that ZBP1 was not down-regulated with ZSG. For comparison, the expression of pNF-κB decreased along with the decrease of RIP3 with the increase of ZSG dose, which suggested that ZSG might target RIP3 to inhibit ZBP1-dependent activation of NF-κB in treatment of UC. SASP, as a 5-aminosalicylic acid preparation, was a first-line drug used for the treatment of UC and could promote UC remission, which had the superiorities of a definite curative effect and low cost [69, 70]. Our results showed that after SASP treatment, ZBP1 was up-regulated and RIP3 was not significantly down-regulated, but the expression of pNF-κB was significantly inhibited, which implied that ZSG might regulate NF-κB pathway via other targets than ZBP1 or RIP3. However, few therapeutic studies targeting ZBP1/RIP/NF-κB pathway have been reported. In this study, ZSG was revealed to exert its anti-inflammatory effects on TNBS-induced UC by inhibiting phosphorylation of NF-κB acting as an inhibitor of RIP3. Morphologically, ZSG administration effectively alleviated the symptoms of mucosal damage, inflammatory infiltration, mucosal edema and crypt loss, which verified that ZSG displayed significant therapeutic effects on the damaged colon of TNBS-induced UC.

The ZSG-L group did not exhibit significant improvements across several parameters. In contrast, the ZSG-M group demonstrated a marked inhibition of inflammation through the significant suppression of p65 phosphorylation, thereby validating the clinical dosage corresponding to the ZSG-M group. Comprehensive improvements in evaluation parameters were observed in the high-dose group, indicating a dose-dependent efficacy. Although our experiment was meticulously designed and executed to ensure result validity, the absence of multiple replicates limited the generalizability of our findings. Future studies would aim to enhance the level of evidence regarding the effect of ZSG on UC by conducting additional experiments with larger sample sizes, which would further strengthen our conclusions. It should be emphasized that ZSG, particularly at high doses, had comparable or slightly better effects than SASP. And SASP was possible to cause many common adverse reactions referring to its instructions, such as anorexia, headache, nausea, vomiting, stomach upset and apparently reversible oligospermia, which could be avoided with ZSG. Thus, ZSG might be expected to be an alternative therapy due to better efficacy and fewer adverse reactions.

Although this study identified the anti-inflammatory effect of ZSG against TNBS-induced UC through the ZBP1/RIP/NF-κB pathway, further confirmation is still needed. In future work, we aim to systematically identify and validate the hub active components, focusing on elucidating their efficacy and mechanisms in the treatment of UC. Additionally, we will investigate the correlation between these hub targets and UC, and evaluate the underlying mechanisms by refining relevant signaling pathways through knockdown or knockout models during the treatment process. Furthermore, we plan to expand the scale of experimental validation and assess the correlation between phenotypic changes and clinical indicators following application. We believe that our study provides valuable insights into the mechanisms by which ZSG exerts its effects against UC. Moreover, revealing the direct regulatory mechanism of ZSG against UC through the ZBP1/RIP/NF-κB pathway could provide a better understanding of this meaningful pathway in the future.

Conclusions

This study combined network pharmacology and experimental verification to demonstrate the therapeutic effects and pharmacological mechanisms of a complex herbal prescription, ZSG, in treatment of UC. We identified 9 hub active components and 20 hub targets, which were mainly involved in immune regulation. Animal experiment data showed that ZSG significantly ameliorated colitis by inhibiting the ZBP1/RIP/NF-κB pathway. In summary, this work provided a theory basis for the clinical application and pharmaceutical development of ZSG.

Supplementary Information

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Supplementary Material 1.
Supplementary Material 2.
Supplementary Material 3.
Supplementary Material 4.
Supplementary Material 5.
Supplementary Material 6.
Supplementary Material 7.

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Authors' contributions
BG, CZ and SH performed data interpretation and drafted the manuscript. XZ, HZ and JL provided data acquisition and analysis. JW, YK and FL designed and supervised the study, and revised the manuscript. All authors reviewed the manuscript.

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Data availability
Data is provided within supplementary information files.

Declarations

Ethics approval and consent to participate
This study was approved by Ethics Committee of Yantai Yuhuangding Hospital (No. 2022–61), and all operations followed the guidelines on animal research.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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