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Zhizi-Bopi decoction ameliorates ANIT-induced cholestatic liver injury in mice through IL-17/NF-κB inflammatory pathways

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Author contributions

The authors declared that all data were generated in-house and that no paper mill was used. Cui W, Chen T, Bi XF, and Zhang WJ conducted the experiments and data analysis. Huang JY, Deng LJ, and Hu JY played a significant role in the research design and visualization of the results. Xiao YP conceptualized and designed the study and drafted the manuscript. Liu CH and Fang W revised the final manuscript. All authors read and approved the final manuscript.

Competing interests

The authors declare no conflicts of interest.

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Abbreviations

ALP, alkaline phosphatase; ALT, alanine aminotransferase; ANIT, α-naphthylisothiocyanate; AST, aspartate aminotransferase; CLD, cholestatic liver disease; CLI, cholestatic liver injury; DEGs, differential expression genes; FXR, farnesoid X receptor; GO, gene ontology functional enrichment analysis; H&E, hematoxylin-eosin; KEGG, Kyoto gene and genome encyclopedia enrichment analysis; scRNA-seq, Single-cell RNA sequencing; SEM, standard error of the mean; PBC, primary biliary cholangitis; PPI, protein-protein interactions; TBA, total bile acids; TCM, traditional Chinese medicine; UDCA, ursodeoxycholic acid; ZZBPD, Zhizi-Bopi decoction.

Citation

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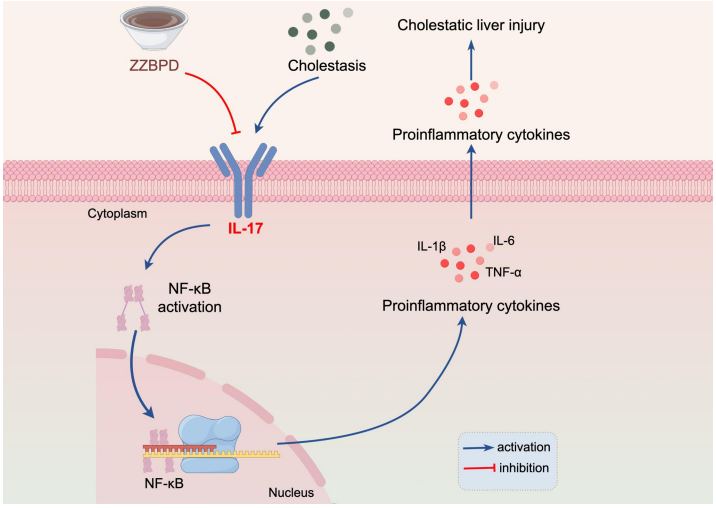
Abstract

Background: ZhiZi-BoPi Decoction (ZZBPD), a traditional prescription for liver and gallbladder protection, has garnered significant clinical interest due to its hepatoprotective properties. Despite its proven efficacy in mitigating intrahepatic cholestasis, the precise mechanisms underlying its therapeutic effects remain inadequately understood. This study aims to comprehensively investigate the pharmacological mechanisms underlying the therapeutic effects of ZZBPD in cholestatic liver injury (CLI). **Methods:** Firstly, we evaluated the hepatoprotective effects of ZZBPD on mice with CLI induced by α-naphthylisothiocyanate (ANIT), by measuring biochemical markers, inflammatory factors, and bile acid levels. Subsequently, we employed network pharmacology and single-cell RNA sequencing (scRNA-seq) to identify key targets and potential signaling pathways for the prevention and treatment of CLI. Finally, we further validated the mechanism of action of ZZBPD on these key targets through molecular docking, western blotting, and immunofluorescence techniques. **Results:** ZZBPD notably improved serum liver function, reduced hepatic inflammation, and restored bile acid balance. Through network pharmacology and scRNA-seq analysis, 48 core targets were identified, including TNF, IL-6, and NFKB1, all of which are linked to the IL-17 and NF-κB signaling pathways, as shown by KEGG enrichment analysis. Molecular docking further confirmed stable interactions between ZZBPD's key active components and molecules such as IL-6, IL-17, and NF-κB. Additionally, western blotting and immunofluorescence validated the downregulation of IL-17 and NF-κB protein expression in liver tissue. **Conclusion:** ZZBPD effectively treats CLI by activating pathways related to the bile acid receptor FXR, while also modulating the IL-17/NF-κB signaling pathway. This dual action enhances bile secretion and alleviates liver inflammation. These findings offer important insights into the pharmacological mechanisms of ZZBPD and underscore its potential as a promising therapeutic for CLI.

Keywords: Zhizi-Bopi decoction; cholestatic liver injury; network pharmacology; single-cell RNA sequencing; IL-17/NF-κB signaling pathway



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Highlights

ZhiZi-BoPi Decoction (ZZBPD) can alleviate liver injury induced by α-naphthyl isothiocyanate (ANIT).

By integrating network pharmacology and single-cell RNA sequencing (scRNA-seq) analysis, we discovered that ZZBPD may exert its hepatoprotective effect through the IL-17/NF-κB signaling pathway.

ZZBPD effectively treats cholestatic liver injury by activating multiple signaling pathways associated with the bile acid receptor FXR and simultaneously modulating the IL-17/NF-κB pathway. This dual action leads to improved bile secretion and reduced liver inflammation.

Medical history of objective

In traditional Chinese medicine (TCM), cholestasis is classified as "jaundice" due to bile overflow resulting from impaired liver function. The renowned physician Zhang Zhongjing introduced a classic formula known as ZZBPD in his treatise, "*Treatise on Febrile Diseases*." The book *Treatise on Febrile Diseases* was written by Zhang Zhongjing during the Eastern Han Dynasty, around 200 C.E. This classical formula is efficacious in clearing heat, promoting urination to eliminate dampness, stimulating bile secretion, and resolving jaundice. Modern pharmacological studies have demonstrated that ZZBPD exerts hepatoprotective effects in mice with intrahepatic cholestasis induced by a 1% cholic acid-containing diet. These protective effects are primarily attributed to its capabilities to reduce the accumulation of bile acids in the liver, inhibit the activation of the NLRP3 inflammasome, and alleviate hepatocyte pyroptosis.

Background

Cholestasis is a pathological condition characterized by impaired bile flow into the duodenum, leading to bile regurgitation into the bloodstream. This disruption may arise from defects in bile production, secretion, or excretion, either intrahepatically or extrahepatically [1, 2]. Cholestasis can result in progressive liver deterioration, encompassing liver fibrosis, cirrhosis, and hepatocellular carcinoma, underscoring the urgent necessity for efficient prevention and management of cholestatic liver injury (CLI) to maintain liver well-being. The pathogenesis of cholestasis is highly complex, and current therapeutic options remain limited. Clinically, medications such as ursodeoxycholic acid (UDCA), obeticholic acid, and fibrates (such as fenofibrate and bezafibrate) are commonly prescribed [3–5]. However, these treatments are often associated with low response rates, significant adverse effects, and risks of hepatotoxicity and nephrotoxicity [6, 7]. Consequently, there exists a pressing demand for the development of more efficacious and less harmful therapies for cholestasis.

Growing evidence highlights the effectiveness of traditional Chinese medicine (TCM) in treating cholestasis [8, 9]. According to TCM theory, cholestasis is classified as "jaundice," which is believed to result from bile overflow due to impaired liver function. The primary symptoms include yellowing of the eyes, skin, and urine, with ocular yellowing being the most prominent. According to the "*Synopsis of Golden Chamber*", jaundice arises from the invasion of dampness and heat pathogens. The core therapeutic principle involves clearing heat, eliminating dampness, and resolving jaundice.

Zhizi-Bopi Decoction (ZZBPD), originating from the "*Treatise on Febrile Diseases*" by the renowned physician Zhongjing Zhang, consists of three herbal components: *Gardeniae Fructus* (Zhizi), *Phellodendri Chinensis Cortex* (Huangbo), and *Glycyrrhizae Radix et Rhizoma* (Gancao) [10]. This traditional formula is known for its ability to clear heat, expel dampness, promote bile secretion, and reduce jaundice. It is commonly used to treat conditions such as jaundice, viral hepatitis, and cholecystitis [11, 12]. Modern pharmacological research has demonstrated that ZZBPD exerts hepatoprotective effects in mice with intrahepatic cholestasis induced by a 1% cholic acid diet. These effects are attributed to its ability to reduce intrahepatic bile acid accumulation, inhibit NLRP3 inflammasome activation, and decrease pyroptosis [13]. However, the precise mechanisms through which ZZBPD alleviates CLI remain incompletely understood.

Network pharmacology facilitates the identification of action sites and interrelationships among various components of TCM, thereby providing a more comprehensive understanding of its pharmacological properties [14, 15]. Single-cell RNA sequencing (scRNA-seq) enables high-resolution characterization of gene expression at the transcriptional level, offering a robust foundation for exploring disease mechanisms [16]. This technology has revealed cellular subpopulations involved in disease pathogenesis, providing new insights into the integration of genomics and pathology [17]. Consequently, combining network pharmacology with scRNA-seq to examine dynamic changes in cell types and gene expression during cholestasis induced by TCM is essential for elucidating the underlying mechanisms of TCM in the prevention and treatment of cholestasis.

We employed an integrated systems pharmacology approach to elucidate the therapeutic efficacy and molecular mechanisms of ZZBPD in treating cholestasis. This approach integrated animal experiments, network pharmacology, bioinformatics, scRNA-seq, and molecular biology methods. Our preliminary study aimed to identify the key targets and potential pathways of ZZBPD, providing a scientific basis for its clinical application in anti-cholestasis treatment.

Materials and methods

Chemicals and reagents

Ursodeoxycholic acid (UDCA, purity ≥ 99%, E2306039) was purchased from Shanghai Aladdin Biochemical Technology Co., Ltd. (Shanghai, China), while α-naphthyl isothiocyanate (ANIT, purity ≥ 99%, STBK0295) was obtained from Sigma-Aldrich (St. Louis, MO, USA). Kits for alkaline phosphatase (ALP), alanine aminotransferase (ALT), aspartate aminotransferase (AST), and total bile acid (TBA) were all sourced from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Antibodies for IL-17 (66148–1-Ig) and GAPDH (60004–1-Ig) were acquired from Proteintech Biotechnology Co., Ltd. (Wuhan, China). Antibodies for NF-κB (#8242) were acquired from Cell Signaling Technology (Danvers, MA, USA).

Preparation of ZZBPD

ZZBPD consists of three traditional Chinese herbal medicines: *Gardeniae Fructus*, *Phellodendri Chinensis Cortex*, and *Glycyrrhizae Radix et Rhizoma* (Table 1). According to the Chinese Pharmacopoeia, these herbal medicines were sourced from Chongqing University Three Gorges Hospital and authenticated by chief pharmacist Boqun Li from the same institution. The preparation method involved soaking the herbs in 10 times the volume (v/w) of distilled water for 1 h, followed by boiling for 1 h and filtering to obtain the supernatant. This process was repeated 8 times the volume (v/w) of distilled water for 30 min. The resulting decoctions were combined, filtered, and concentrated to a final concentration of 1 g/mL for subsequent use.

Animal experiment

SPF-grade male C57BL/6J mice (8 weeks old, 18–22 g) were provided by Beijing SPF Biotechnology Co., Ltd. (Certificate No. SCXK (Beijing) 2019-0010). All animals were housed in an SPF-grade environment, and the animal experiments were conducted following the ARRIVE guidelines and approved by the Ethics Committee of Chongqing University Three Gorges Hospital (No. SXYYDW2023-026).

After a 7-day adaptation period, the mice were randomly divided into 5 groups (n = 8 per group): normal control group (NC group), model group (ANIT group), ZZBPD 3 g/kg and 6 g/kg groups (ZZBPD-L and ZZBPD-H, respectively), and ursodeoxycholic acid group (UDCA, 150 mg/kg group). The dosages of ZZBPD were determined based on the conversion coefficient of body surface area

between humans and animals, combined with preliminary experiments. The treatment groups were administered the corresponding drugs via gavage, while the NC and ANIT groups received an equal volume of 0.9% saline daily for 7 consecutive days. As shown in Figure 1, on day 5, the NC group was administered olive oil via gavage, while the other groups were induced with 60 mg/kg ANIT dissolved in olive oil. The modeling dose of ANIT was determined based on literature and preliminary experiments [18]. At the end of the treatment period, mice were euthanized by intraperitoneal injection of an overdose of pentobarbital sodium. Blood samples were collected from the orbital sinus to obtain serum, and a portion of the liver tissue was preserved in 10% formalin or stored at −80 °C for subsequent analysis.

Liver function and bile acid level detection

According to the instructions of the kit, the levels of ALT, AST, ALP, and TBA in mouse serum were detected.

H&E staining

Liver tissue sections were stained with H&E according to the method described previously. Finally, three experienced pathologists assessed the degree of pathological damage in the liver.

Network pharmacology analysis

Screening targets of ZZBPD. We used multiple drug-related databases, including SymMap, HERB, and TCMSP, to search for potential targets of the active components in ZZBPD.

Collection of CLI targets. The GSE79850 dataset, obtained from the GEO database, sequenced the livers of 8 healthy volunteers and 9 patients at high risk of primary biliary cholangitis (PBC). The limma package in R software was used to investigate differentially expressed genes (DEGs) and identify potential disease targets. Additional disease targets related to CLI were collected from the GeneCards, OMIM, and DisGeNET databases using "cholestatic liver injury" as the search term. Finally, the targets from all databases were merged and duplicates were removed.

Target collection and PPI network construction for ZZBPD treatment of CLI. A Venn diagram was employed to identify the overlapping targets between the active ingredients of ZZBPD and those associated with CLI, thereby revealing potential targets for ZZBPD in the treatment of CLI. These potential targets were then input into the String database to obtain protein-protein interaction (PPI). Using Cytoscape software, a visual PPI network was constructed, and the degree value of each node was calculated to identify key targets within the network.

GO and KEGG enrichment analyses. The cluster profile package in R software was employed to perform GO and KEGG enrichment analysis. With a threshold of $P < 0.05$, unrelated pathways were excluded to identify the top-ranked biological processes or pathways, which were then visualized. GO enrichment analysis was primarily aimed at understanding the main functional processes of the targets, while KEGG enrichment analysis helped to elucidate the various pathways, including metabolism and signal transduction, involved in the targets.

Download and analysis of scRNA-seq data

The scRNA-seq gene expression dataset GSE193337 for CLD was retrieved from the GEO database using the search term "cholestasis." From this dataset, seven groups of CLI tissues and two groups of normal tissues were selected. R packages, including "Seurat" and "SingleR," were employed for the analysis of the scRNA-seq data. Cells were clustered into distinct groups using UMAP clustering analysis. The scMayoMap automated annotation package was applied to annotate the cell types of each cluster. The data were categorized according to their source, with normal tissues designated as "HL" and cholestatic liver tissues as "CLD". The FindMarkers function was used to identify DEGs between the same cell types in CLI tissues and normal tissues, with genes having a *P*-value < 0.05 considered as DEGs.

Potential mechanism of ZZBPD in treating CLI revealed by combining network pharmacology with scRNA-seq

By overlapping the potential targets obtained from network pharmacology with the DEGs identified through scRNA-seq, we aim to recognize the potential targets of ZZBPD in treating CLI at the single-cell level. The PPI relationships among the DEGs were acquired through the STRING database, followed by GO and KEGG enrichment analyses. In summary, the DEGs were uploaded to the STRING database, and the results were visualized using Cytoscape software. Furthermore, based on the constructed PPI network, we utilized the CytoHubba plug-in in Cytoscape to search for hub genes, identifying the top 10 hub nodes using the MCC calculation method.

Table 1 Composition of ZZBPD

Chinese name	Family name	Latin name	Quantity (g)
Zhizi	Rubiaceae	*Gardeniae Fructus*	10 g
Huangbo	Rutaceae	*Phellodendri Chinensis Cortex*	6 g
Gancao	Leguminosae	*Glycyrrhizae Radix et Rhizoma*	3 g

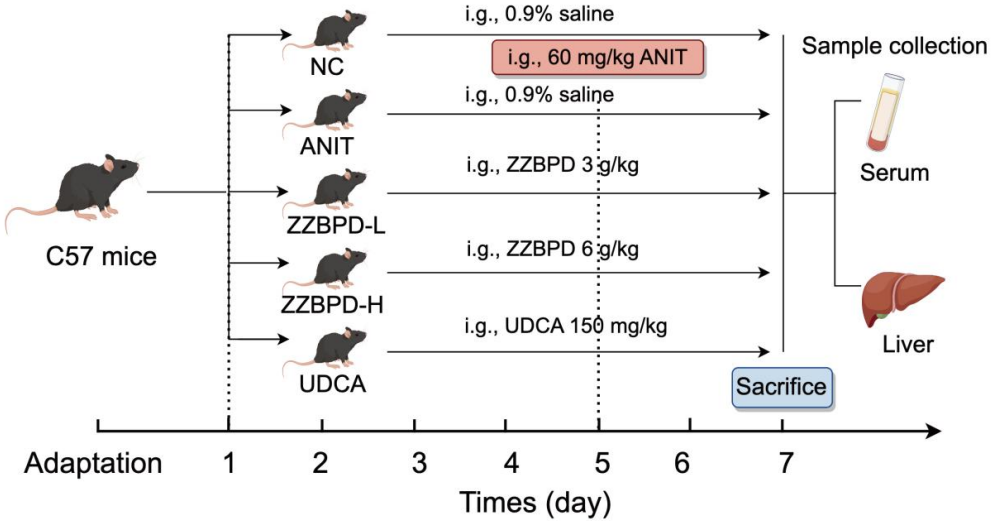

Figure 1 Flow chart of the ZZBPD experiment for treating cholestatic mice

Real-time quantitative PCR

Total RNA was extracted from mouse liver tissue following the manufacturer's instructions. Reverse transcription and Real-time quantitative PCR were subsequently performed as described in previous studies [19]. The primer sequences used are provided in Supplementary Table S1.

Western blotting

Standard procedures were followed for western blotting analysis. Antibodies used were anti-IL-17 and anti-NF-κB (both diluted at 1:1,000), as well as GAPDH antibody (diluted at 1:2,000) and corresponding secondary antibodies (diluted at 1:5,000). Finally, ECL color imaging was performed, and the grayscale of the protein bands was analyzed using Image J software.

Immunofluorescence

Standard procedures were followed for immunofluorescence analysis. Antibodies used were anti-IL-17 and anti-NF-κB (both diluted at 1:200), and corresponding secondary antibodies (diluted at 1:500). Following DAPI nuclear staining, the sections were mounted with anti-fluorescence quenching mounting medium and observed under an inverted fluorescence microscope to examine the expression of IL-17 and NF-κB in the liver tissue.

Statistical analysis

One-way ANOVA was performed on multiple groups of data using SPSS 26.0 statistical software, and all results were expressed as mean ± standard error of the mean (mean ± SEM). Histograms were drawn using GraphPad Prism 9.0 software. When $P < 0.05$, the statistical results were considered significant.

Results

Effects of ZZBPD on ANIT-induced CLI in mice

ALT and AST are widely recognized as biochemical markers for assessing liver function. Cholestasis is typically accompanied by an increased release of ALT and AST, which are indicative of liver injury and inflammation. Consistent with previous findings, this study identified marked liver damage and hyperbilirubinemia in mice with ANIT-induced CLI [20]. The serum ALT and AST levels in the ANIT group were significantly higher than those in the NC group. However, treatment with ZZBPD significantly reduced serum ALT and AST levels, suggesting a protective effect of ZZBPD against ANIT-induced liver injury (Figure 2A, 2B). Elevated ALP and TBA levels are indicative of liver or biliary disorders, such as hepatitis, gallstones, or cirrhosis. In this study, ALP and TBA levels were markedly elevated in the ANIT group compared to the NC group (Figure 2C, 2D). Treatment with ZZBPD attenuated these increases. Histopathological analysis corroborated these findings. Previous studies have shown that ANIT induces pathological alterations in liver tissue, including disrupted cellular arrangement, extensive necrosis in the portal area, and inflammatory cell infiltration [21]. As demonstrated in Figure 2F, the livers of NC group mice appeared pink, with smooth surfaces and no visible cholestasis. Conversely, livers from the ANIT group were enlarged, yellowish, darker in color, and exhibited clear signs of cholestasis. Following ZZBPD treatment, cholestasis symptoms were notably reduced. H&E staining revealed intact hepatic cell structures, organized lobules, uniform cell sizes, and clear nuclei in the NC group, with no evidence of necrosis or inflammatory infiltration. In contrast, the ANIT group displayed disorganized hepatocytes of varying sizes, cytoplasmic loosening, focal necrosis, increased eosinophilia, spotty necrosis, and inflammatory cell infiltration (highlighted by black circles). Treatment with ZZBPD or UDCA substantially mitigated liver tissue damage compared to the ANIT group (Figure 2E, 2G). These results collectively indicate that ZZBPD exerts a protective effect against ANIT-induced liver injury by alleviating biochemical and histopathological damage.

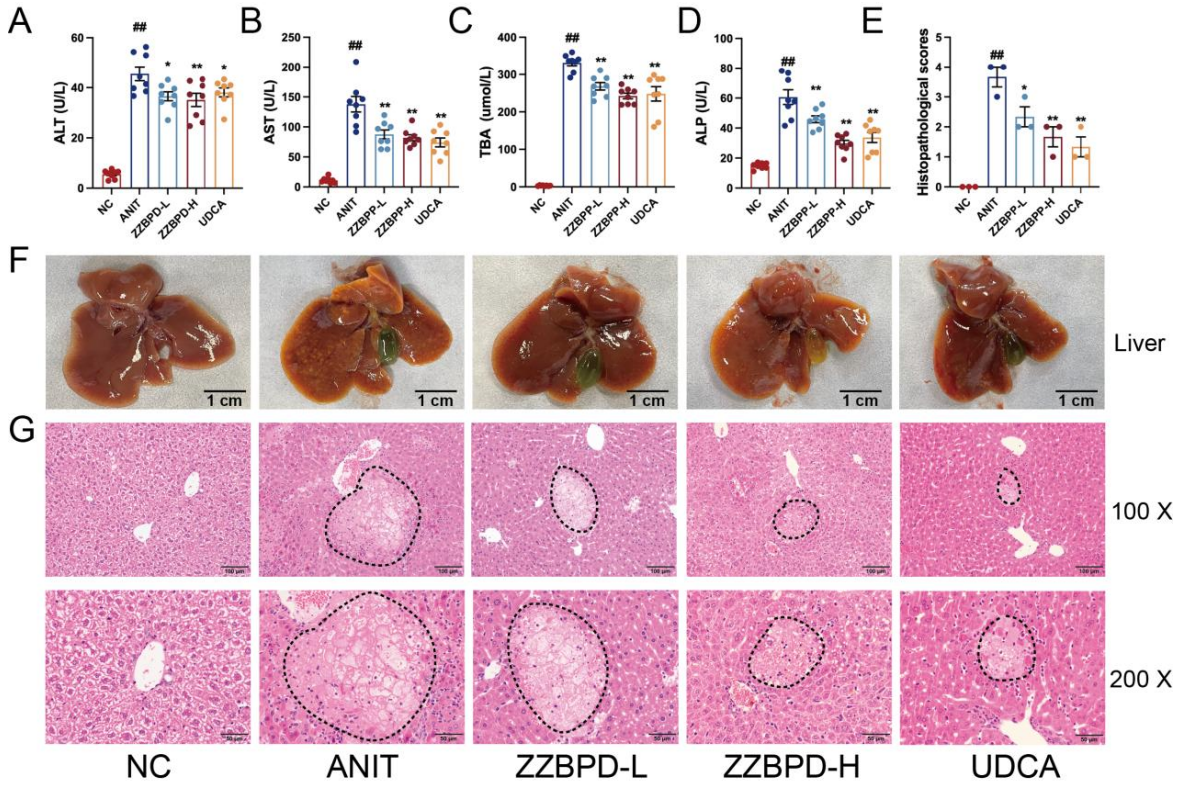

Figure 2 Effects of ZZBPD on liver injury in ANIT-induced cholestatic mice. (A–D) Levels of ALT, AST, TBA, and ALP in serum (n = 8 per group). (E) Pathological damage scores of the liver in each group of mice (n = 3 per group). (F) Gross morphology of the liver in each group of mice. (G) H&E staining showing the effect on pathological liver damage in cholestatic mice. Note: Black circles indicate inflammatory cell infiltration and necrosis. Data were expressed as mean ± SEM. $^{\#\#}P < 0.01$ compared with the NC group; $^{*}P < 0.05$, $^{**}P < 0.01$ compared with ANIT group.

Effect of ZZBPD on the levels of inflammatory cytokines in liver tissue

The distinct structural features of ANIT make it susceptible to bile duct obstruction, thereby hindering the excretion of intrahepatic bile acids and resulting in intrahepatic cholestasis. This condition subsequently induces an inflammatory response in the liver [22]. To evaluate the impact of ZZBPD on the inflammatory response in CLI mice, we measured the mRNA levels of proinflammatory cytokines (such as *Tnf-α*, *Il-6*, and *Il-1β*) and the anti-inflammatory cytokine *Il-10* in the Liver. The results indicated that ANIT stimulation significantly increased the mRNA levels of *Tnf-α, Il-6*, and *Il-1β* in the liver while decreasing the mRNA level of *Il-10*, confirming that ANIT can induce the secretion of inflammatory cytokines by hepatocytes. Furthermore, ZZBPD treatment significantly inhibited the ANIT-induced increase in *Tnf-α, Il-6*, and *Il-1β* levels and the decrease in *Il-10* level, suggesting that ZZBPD can suppress the elevation of inflammatory cytokines induced by ANIT (Figure 3).

Effect of ZZBPD on primary bile acid synthesis-related genes in cholestatic liver

Under conditions of cholestasis, FXR is activated and recognized as the primary regulator of bile acid homeostasis. It has been established that abnormalities in FXR function can trigger disease manifestations in patients with cholestasis [23, 24]. Therefore, activating the FXR signaling pathway represents a promising approach for the treatment of CLI. To further investigate the impact of ZZBPD on bile acid homeostasis in the livers of mice with cholestasis, we examined its effects on the expression of genes related to bile acid synthesis and transport, specifically FXR and its downstream targets, in the livers of ANIT-induced cholestatic mice. As illustrated in Figure 4, compared to the NC group, the model group showed significant downregulation inthe expression levels of key bile acid nuclear receptors (*Fxr*, *Shp*), apical efflux transporters (*Bsep*, *Mrp2*), the rate-limiting enzyme for bile acid synthesis (*Cyp7a1*), and the bile acid reabsorption transporter (*Ntcp*) in liver tissue. In comparison to the ANIT group, the

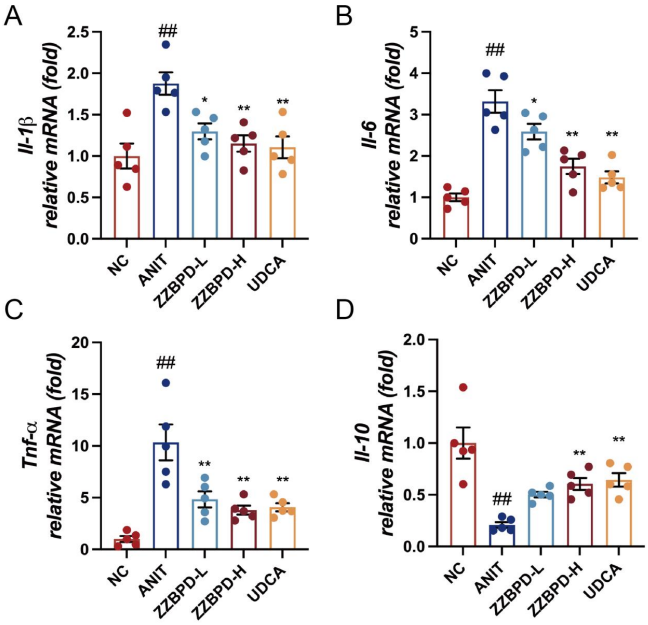

Figure 3 The effect of ZZBPD on inflammatory cytokines in the liver of ANIT-induced mice. (A–D) The expression level of *Il-1β, Il-6, Tnf-α,* and *Il-10* mRNA (n = 5 per group). Data were expressed as mean ± SEM. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ compared with the NC group; $^{*}P < 0.05$, $^{**}P < 0.01$ compared with ANIT group.

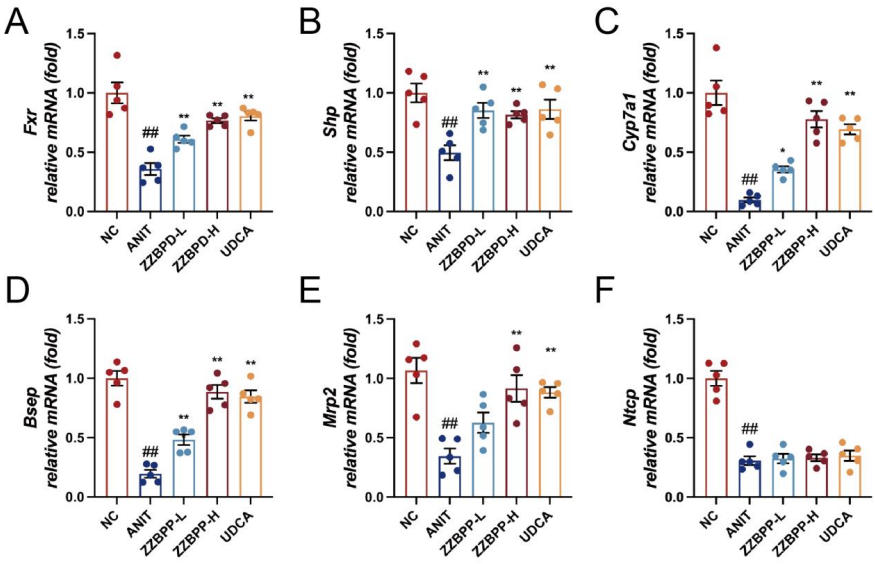

Figure 4 The effect of ZZBPD on bile acid metabolism-related genes in the liver of ANIT-induced mice. (A–F) The expression level of *Fxr*, *Shp*, *Cyp7a1*, *Besp*, *Mrp2*, and *Ntcp* mRNA (n = 5 per group). Data were expressed as mean ± SEM. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ compared with the NC group; $^{*}P < 0.05$, $^{**}P < 0.01$ compared with ANIT group.

high-dose ZZBPD group demonstrated significant upregulation in the mRNA expression levels of *Fxr*, *Shp*, *Cyp7a1*, *Bsep*, and *Mrp2*, while the difference in *Ntcp* mRNA expression was not statistically significant. These findings suggest that ZZBPD may alleviate liver injury in ANIT-induced cholestatic mice by activating *Fxr* expression, upregulating the expression of the bile acid transporter *Bsep*, and enhancing bile acid efflux from the liver, thereby reducing bile acid accumulation in the liver.

Results of network pharmacology analysis of ZZBPD in the treatment of CLI

We utilized network pharmacology to preliminarily explore the potential mechanisms by which ZZBPD ameliorates liver injury in cholestatic mice. The active components of ZZBPD were collected according to the screening criteria of the TCMSP database, as shown in Supplementary Table S2. Potential targets of ZZBPD were gathered from literature and the TCMSP, HERB, and SymMap databases, resulting in 555 unique targets after merging and deduplication. Using $P < 0.05$ and $|\log FC| \geq 2$ as screening criteria, R language analysis identified 763 DEGs in the GSE14841 dataset. Volcano and heatmap plots revealed 392 DEGs, with 308 upregulated and 84 downregulated genes, as illustrated in Figure 5A, 5B. Subsequently, potential targets for treating CLI were compiled from the Genecards, OMIM, Disgenet, and GEO databases, resulting in 1,084 unique targets after merging and deduplication.

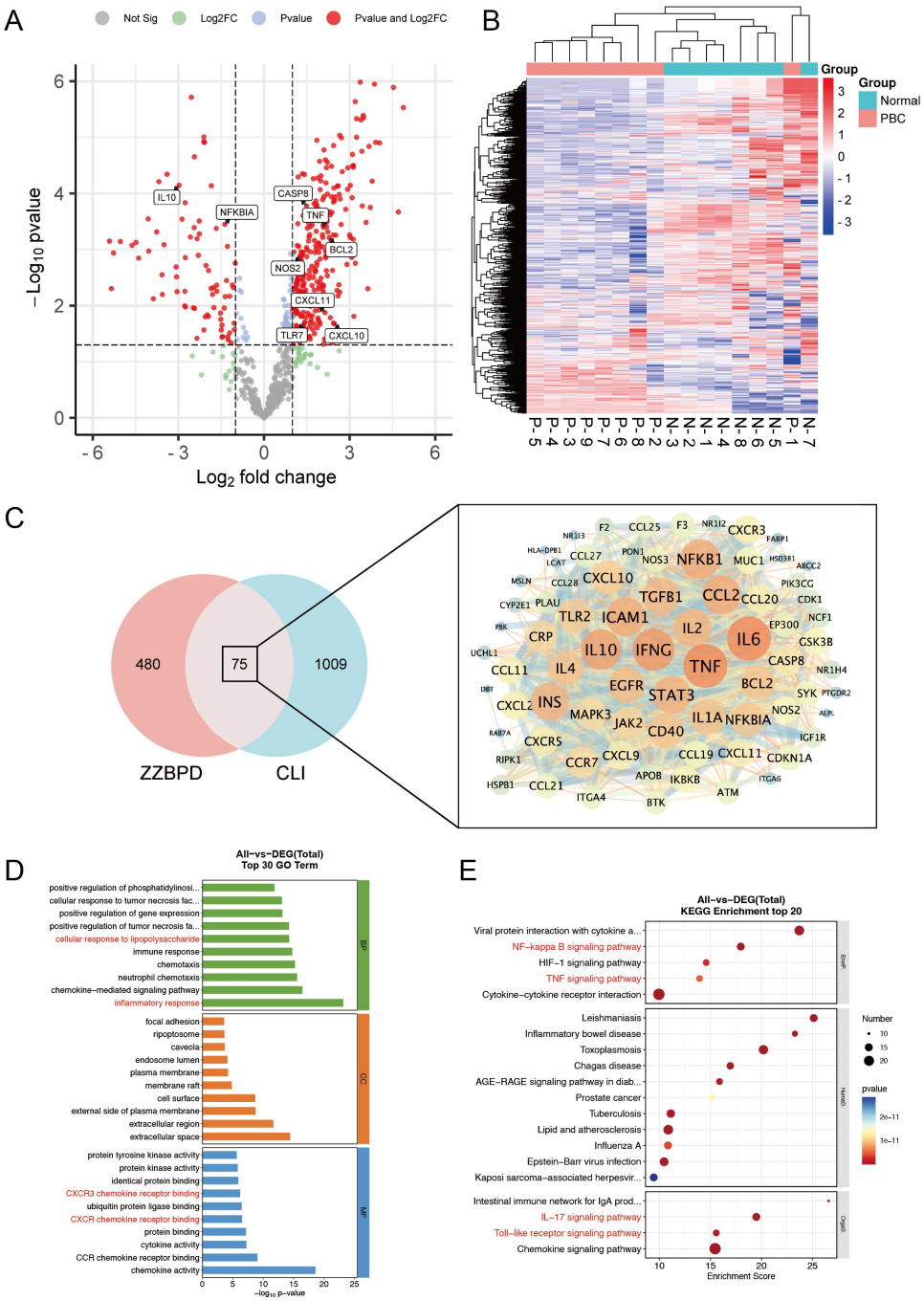

Figure 5 Results of network pharmacology analysis of ZZBPD in treating CLI. (A) Volcano map of DEGs in PBC-related microarrays. (B) Heat map of DEGs clustered in PBC-related microarrays. (C) Venn diagram showing the common targets of ZZBPD and CLI and their PPI interaction network. (D, E) GO and KEGG enrichment analysis of the common targets of ZZBPD and CLI.

As shown in Figure 5C, there are 75 overlapping targets between the active components of ZZBPD and CLI, which are considered potential targets for ZZBPD in treating CLI. A PPI network was constructed for these overlapping targets, and the node size and color reflected their degree values. According to these values, TNF, NFKB1, IL-6, IL-10, and NR1H4 (FXR) are essential targets of ZZBPD in alleviating cholestasis. The above results suggest that ZZBPD may reduce liver injury by alleviating the inflammatory response in patients with cholestasis. GO and KEGG pathway analyses were performed on these 75 potential therapeutic targets. The GO enrichment analysis indicates that ZZBPD treatment for cholestasis is primarily associated with biological processes such as inflammatory response, positive regulation of tumor necrosis factor production, and CXCR chemokine receptor binding (Figure 5D). The KEGG pathway enrichment analysis suggests that ZZBPD may alleviate cholestasis by modulating the IL-17 signaling pathway, NF-kappa B signaling pathway, and Toll-like receptor signaling pathway, primarily involving inflammatory response-related biological processes, as shown in Figure 5E.

Analysis of DEGs in CLI at the scRNA-seq level

We performed a standardized analysis on scRNA-seq data (GSE193337) from patients with cholestasis. As shown in Figure 6A, the box plot presents the distribution of RNA feature counts (nFeature_RNA), total RNA counts (nCount_RNA), and the percentage of mitochondrial genes (percent.mt), with no significant outliers observed. Using t-SNE and UMAP dimensionality reduction, we identified nine subpopulations: Monocytes, T cells, B cells, NK cells, Hepatocytes, Smooth muscle cells, Epithelial cells, Endothelial cells, and Macrophages. The cell distribution is illustrated in Figure 6B. Figure 6C shows a volcano plot of DEGs across these cell populations. Significant expression changes were observed in genes involved in immune responses, such as CXCL13, LCN2, and CCL14, which exhibited differential expression across the different cell types. Finally, we compared gene expression profiles between cholestatic liver tissue from patients and normal liver tissue to identify DEGs at the single-cell level. After removing duplicates and synthesizing the data, a total of 8,389 DEGs were identified.

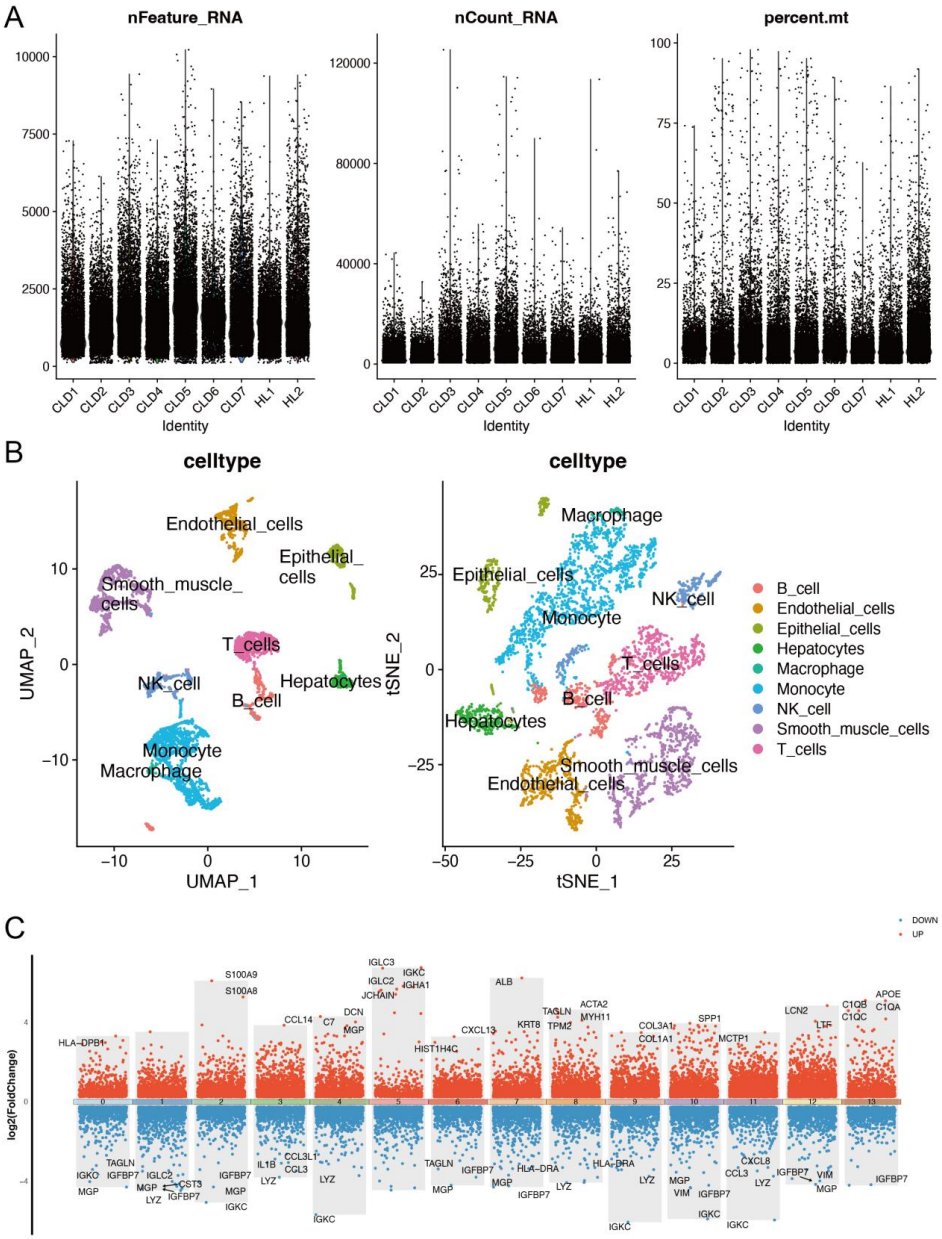

Figure 6 DEGs in cholestasis patient livers at the scRNA-seq level. (A) The box plot illustrates the nFeature_RNA, nCount_RNA, and percent.mt in cholestasis at the single-cell level. HL denotes normal liver tissue; CLD denotes liver tissue from cholestasis patients. (B) Visualization of each cell type in the scRNA-seq data using dimensionality reduction techniques such as t-SNE and UMAP. (C) Display of DEGs in each cluster.

Network pharmacology combined with scRNA-seq revealed that ZZBPD exerts anti-CLI effects through the IL-17/NF-κB signaling pathway

To further explore the potential mechanisms of ZZBPD in treating CLI, network pharmacology analysis identified 75 potential targets. Intersection with scRNA-seq DEGs resulted in 48 overlapping genes. As shown in Figure 7A, PPI analysis of these targets revealed that TNF, NFKB1, and IL-6 are potential key targets for ZZBPD in alleviating cholestasis. Subsequently, using the MCC calculation method in Cytoscape, we identified 10 hub genes: TNF, IL6, NFKB1, IFNG, STAT3, ICAM1, CCL2, IL10, TLR2, and CD40 (Figure 7B, 7C). GO enrichment analysis of the 48 overlapping genes indicated involvement in biological processes such as inflammatory response, response to IL-17, positive regulation of IL-1β production, positive regulation of tumor necrosis factor production, and NF-κB binding (Figure 7D). KEGG analysis showed enrichment in pathways such as the NF-κB signaling pathway, IL-17 signaling pathway, and cytokine-cytokine receptor interaction (Figure 7E). The combined network pharmacology and KEGG enrichment analyses suggest that the IL-17/NF-κB signaling pathway may be the key mechanism by which ZZBPD treats CLI.

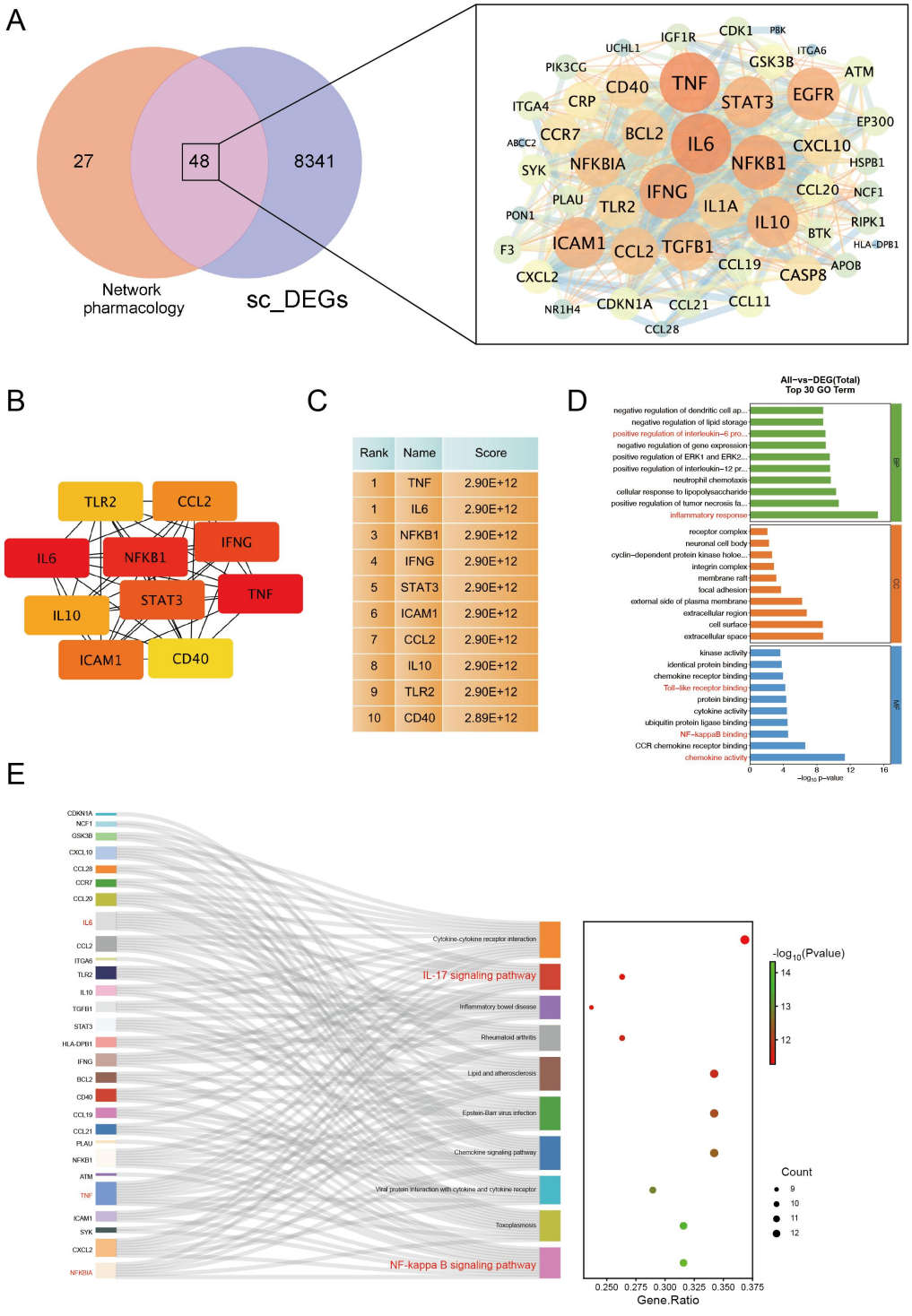

Figure 7 Analysis results of ZZBPD treatment for CLI by network pharmacology combined with scRNA-seq. (A) Venn diagram of common targets and their PPI interaction network of network pharmacology and scRNA-seq. (B) 10 hub genes were obtained using the MCC algorithm. (C) Ranking and scoring of 10 hub genes. (D, E) GO enrichment analysis and Sankey diagram of KEGG enrichment analysis of common targets.

Molecular docking

To preliminarily verify whether ZZBPD alleviates ANIT-induced cholestasis and exerts hepatoprotective effects through the IL-17/NF-κB signaling pathway, we selected the primary active components of ZZBPD and performed molecular docking with key proteins in the IL-17/NF-κB signaling pathway. Based on literature references, three primary active components of ZZBPD-geniposide, berberine, and glycyrrhizic acid were selected for molecular docking [11]. Based on MCC calculations, the top four protein targets, TNF, IL6, NFKB1, and IL17 in the IL17 signaling pathway, were selected as key targets for ZZBPD in treating CLI. Molecular docking was employed to validate the binding energies between these active components and key targets, with the results listed in Table 2, Supplementary Figure S1–S3. Lower binding energies indicate more stable conformations, and the main active components' binding energies with key targets were below −5.0, suggesting binding solid affinity and stable conformations. The structures with the lowest binding energies for each active component were then visualized using Pymol 2.6.0, as shown in Figure 8A–8C.

Effect of ZZBPD on IL-17/NF-κB signaling pathway-related proteins in the liver tissue of cholestatic mic

To explore the potential of ZZBPD in alleviating CLI through the IL-17/NF-κB signaling pathway, we assessed the protein expression levels of IL-17 and NF-κB via Western blotting and immunofluorescence. Western blot analysis revealed a notable increase in IL-17 and NF-κB expression in the ANIT group compared to the NC group, underscoring the activation of the IL-17/NF-κB signaling pathway following ANIT-induced cholestasis. Importantly, following ZZBPD intervention, the expression levels of both IL-17 and NF-κB were markedly reduced compared to the model group (Figure 9A–9C). Consistent with these findings, immunofluorescence analysis further confirmed the downregulation of IL-17 and NF-κB expression by ZZBPD (Figure 9D, 9E). Together, these results highlight the potential of ZZBPD in alleviating CLI, with a particular emphasis on its ability to suppress the IL-17/NF-κB signaling pathway.

Table 2 Molecular docking results of major active components in ZZBPD with key core targets

Active ingredients	Binding energy (kJ/mol)			
	TNF	IL-6	NFκB1	IL17
Geniposide	−5.7151	−7.1334	−5.3412	−6.8041
Berberine	−5.4851	−6.2077	−5.3586	−6.464
Glycyrrhizic acid	−7.3032	−7.5094	−7.6824	−6.9781

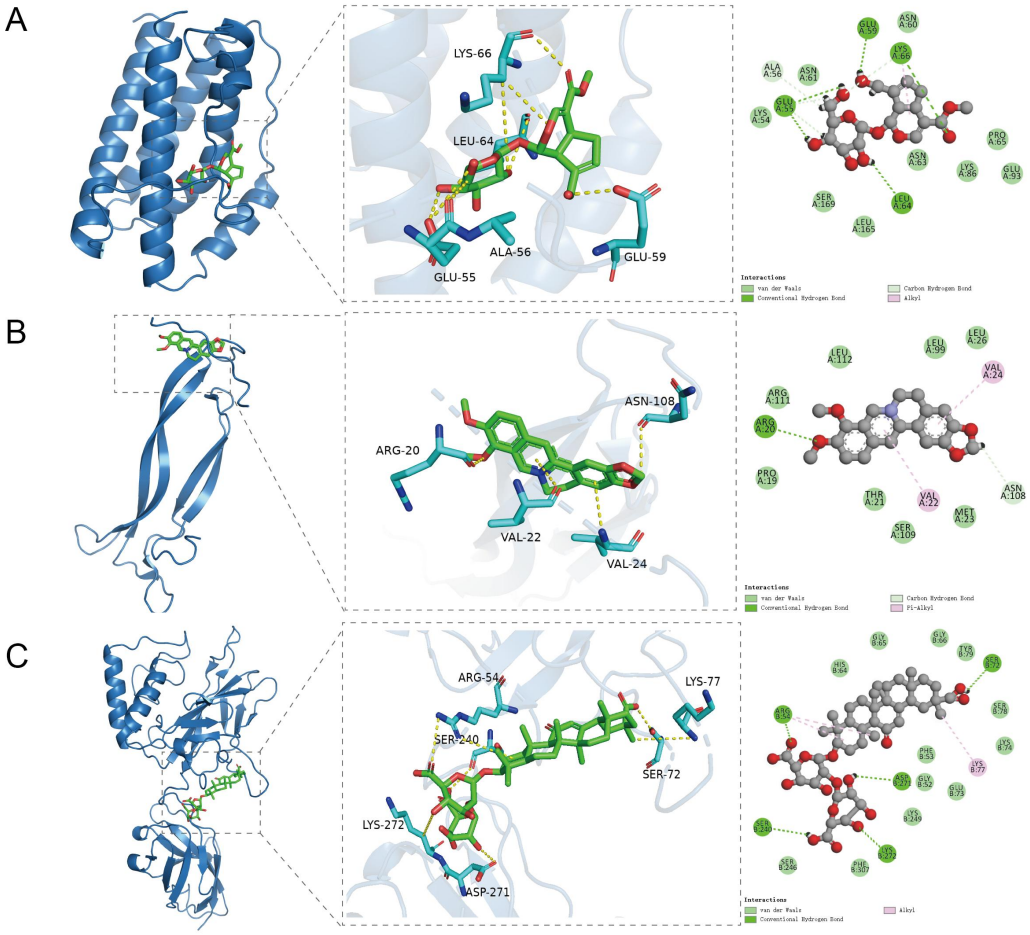

Figure 8 Visualization of interactions between the three active components in ZZBPD with the lowest binding free energies and their respective protein receptors. (A) Geniposide with IL-6. (B) Berberine with IL-17. (C) Glycyrrhizic acid with NFKB1. The left panels depict 3D molecular docking models and the docking ligands within the binding pockets. The right panels show 2D molecular docking models. In the left panels, green structures represent the different ligands. In the right panels, circles represent amino acid residues, and green lines represent conventional hydrogen bonds.

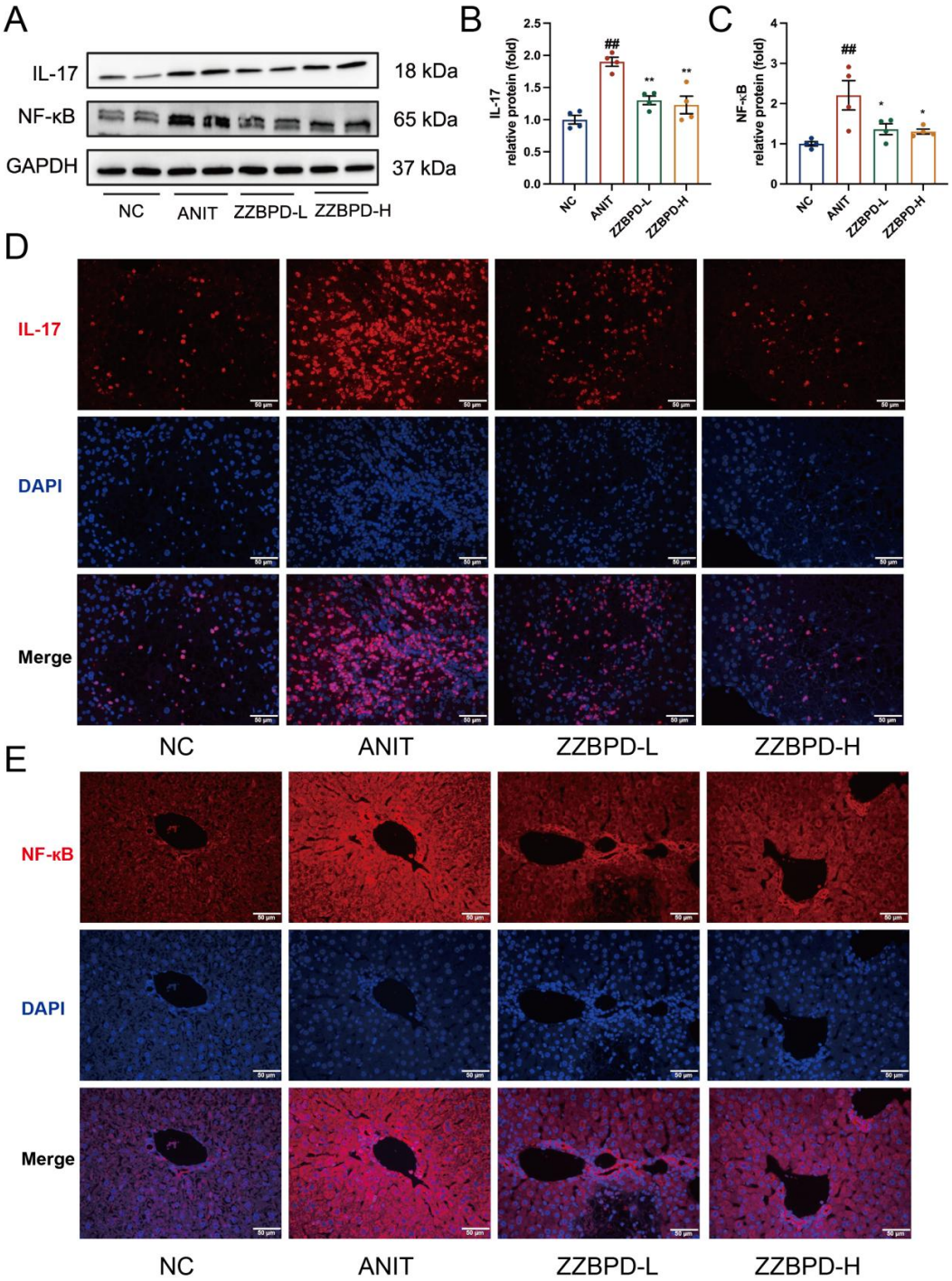

Figure 9 ZZBPD inhibited IL-17/NF-κB expression in ANIT-induced mouse livers. (A) Representative Western blotting bands showing the expression of IL-17 and NF-κB in liver tissue. (B, C) Quantification of Western blotting results for IL-17 and NF-κB expression in liver tissue (n = 4 per group). (D, E) Representative images of immunofluorescence staining showing IL-17 and NF-κB expression in liver tissue. Magnification: 200×, Scale bar = 50 μm. Data were expressed as mean ± SEM. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ compared with the NC group; $^{*}P < 0.05$, $^{**}P < 0.01$ compared with ANIT group.

Discussion

Cholestasis can be caused by various factors, including hepatobiliary diseases, biliary structural abnormalities, drugs, and infections. Recent studies have also reported that components found in food additives, dietary supplements, and parenteral nutrition can induce CLI [25–27]. CLI can lead to jaundice, biliary colic, indigestion, nausea, vomiting, pruritus, and abdominal distension, among other symptoms and complications [28]. The pathological mechanisms of CLI are complex and involve multiple processes. The single drug component or target often only improves certain pathological indicators and may not prevent further disease progression [29]. In contrast, TCM is characterized by multi-drug formulas, multi-component remedies, and multi-pathway, multi-target actions, which can collaboratively improve liver injury and fibrosis caused by cholestasis. This study evaluated the efficacy of ZZBPD through in vivo experiments, identified potential targets for treating cholestasis using network pharmacology combined with scRNA-seq data, and explored the potential mechanisms of ZZBPD in treating CLI through molecular biology techniques.

The key mechanism underlying the development of cholestasis involves disruptions in the synthesis, absorption, transport, and metabolism of bile acids within hepatocytes [30]. The homeostasis of bile acid levels in the liver is primarily regulated by multiple nuclear receptor transcription factors within hepatocytes, including farnesoid X receptor (FXR), SHP, PXR, PPAR, and CAR [31–33]. These factors regulate downstream molecules involved in bile acid synthesis (such as CYP7A1, CYP8B1, CYP27A1), uptake/transport (NTCP, OATPs), transport (BSEP, MRP2, MRP3, MRP4, OSTα/β), and metabolism (CYP3A4, CYP2B6, UGT1A1) [34, 35]. Any functional abnormalities in these key molecules during bile acid synthesis, uptake/transport, and metabolism can disrupt bile acid homeostasis in the liver, leading to excessive accumulation of bile acids and inducing CLI. The FXR is the most crucial receptor for bile acids, playing a key role in maintaining bile acid homeostasis. FXR primarily functions through the FXR-SHP-BSEP/MRP2 signaling pathway to positively regulate bile acid excretion and the FXR-FGF19-CYP7A1/NTCP signaling pathway to regulate bile acid synthesis and reabsorption negatively [36, 37]. Therefore, maintaining normal FXR expression in the liver is essential for preventing and treating CLI, providing important references for clinical treatment and drug development.

This study established a mouse intrahepatic cholestasis model by gavage of 60 mg/kg of ANIT. The results of this study demonstrate that ANIT-induced cholestasis significantly downregulated the expression of FXR in the liver of model mice. This suppression, in turn, inhibited the expression of the efflux transporter BSEP, thereby promoting the excessive accumulation of bile acids in the liver. As a result, this accumulation triggered chronic hepatocellular inflammation, which progressively advanced to CLI. Consequently, this accumulation induced chronic hepatocellular inflammation, progressing to CLI. Compared to the NC group, the ANIT group showed significantly elevated levels of jaundice indicators such as ALT, AST, ALP, and TBA, alongside marked inflammation and apoptosis in liver tissues. Additionally, the expression of pro-inflammatory-related genes (*Il-6*, *Il-1β*, and *Tnf-α*) was significantly upregulated, while the anti-inflammatory-related gene *Il-10* was significantly downregulated. Upon intervention with ZZBPD, the jaundice indicators and pathological liver damage in ANIT-induced cholestatic mice were notably ameliorated. ZZBPD reversed the downregulation of hepatic FXR expression induced by ANIT. By upregulating the expression of bile acid efflux proteins MRP2 and BSEP, ZZBPD accelerated the excretion of bile acids from the liver, thereby reducing their toxic effects on the liver and improving liver injury. The serum's decreased AST, ALT, TBA, and ALP levels evidenced this.

As the rate-limiting enzyme in the primary pathway of bile acid synthesis in hepatocytes, CYP7A1 is one of the critical downstream targets regulated by FXR. Interestingly, it has been reported in the literature that when ANIT induces cholestasis in the liver, it leads to enhanced CYP7A1 activity, thereby increasing bile acid synthesis and further exacerbating the accumulation of bile acids within hepatocytes, intensifying the hepatotoxicity caused by high concentrations of bile acids [38, 39]. However, in the ANIT-induced cholestasis model of this study, the expression level of CYP7A1 was downregulated, and upon ZZBPD intervention, the expression level of CYP7A1 was upregulated. Additionally, there are reports of downregulation of both mRNA and protein expression levels of CYP7A1 in the ANIT-induced cholestasis model [40–42], which are consistent with the findings of this study. We propose that the downregulation of CYP7A1 in response to cholestasis may not simply be a passive consequence, but rather an active adaptive mechanism aimed at mitigating bile acid toxicity. This adaptive feedback mechanism likely serves as a protective strategy to limit the excessive accumulation of bile acids within hepatocytes, thus inhibiting further bile acid synthesis. Based on our findings, we suggest that ZZBPD may mitigate ANIT-induced liver injury through modulation of the FXR-regulated bile acid metabolic pathway, potentially providing a therapeutic approach for managing cholestasis-associated hepatotoxicity.

ZZBPD is a classical formula for treating hepatobiliary diseases, composed of *Gardeniae Fructus*, *Phellodendri Chinensis Cortex*, and *Glycyrrhizae Radix et Rhizoma* [11]. Network pharmacology results indicate that ZZBPD contains 139 essential active compounds that protect the liver from cholestatic injury, including geniposide, berberine, and glycyrrhizic acid. Geniposide, an iridoid ether terpenoid, can alleviate ANIT-induced hepatotoxicity and cholestasis in rats by regulating enzymes and transporters responsible for bile acid homeostasis [43]. Berberine, an alkaloid, exhibits anti-inflammatory, anti-obesity, and hepatoprotective properties. It modulates bile acid metabolism and restores bile acid homeostasis by altering the expression of key genes in the liver and intestine, thereby mitigating CLI in *Mdr2*$^{-/-}$ mice [44]. Using a combination of network pharmacology and scRNA-seq analyses, the potential targets of ZZBPD in treating CLI were investigated. Forty-eight targets associated with ZZBPD treatment of CLI were identified, and a PPI network was constructed. GO and KEGG analyses of these targets revealed pathways related to cholestasis, including the IL-17 and NF-κB signaling pathways. Both IL-17 and NF-κB signaling pathways are classic inflammatory response pathways that specifically regulate the expression of interleukins during the transcription process, playing crucial roles in various inflammatory and autoimmune diseases [45].

Network pharmacology analysis indicates a close relationship between ZZBPD and the IL-17 and NF-κB signaling pathways. IL-17, an essential proinflammatory cytokine, plays a role in the development of various inflammatory and immune diseases [46]. In cholestatic hepatitis, IL-17 levels are significantly elevated, activating the NF-κB pathway. This activation recruits immune cells, releases inflammatory cytokines such as IL-6, IL-1β, and TNF-α, intensifies the inflammatory response, and ultimately leads to hepatocellular damage [47]. NF-κB serves as a pivotal target in the IL-17 signaling pathway, performing a critical function. Research has revealed that certain active components within ZZBPD can modulate the IL-17 pathway. Specifically, geniposide, in a rat model of rheumatoid arthritis, attenuates inflammatory damage by reducing IL-1β and IL-17 levels. In CLI, NF-κB is activated in response to bile acid accumulation, further amplifying the inflammatory response [48]. The interplay between IL-17 and NF-κB potentiates the inflammatory reaction, resulting in liver damage and dysfunction [49]. Molecular docking analysis demonstrates that geniposide, berberine, and glycyrrhizic acid from ZZBPD exhibit strong binding affinity to IL-17, NFKB1, and IL-6. Based on these findings, IL-17 and NF-κB were selected as key proteins for further investigation into their mechanistic roles. Western blotting and immunofluorescence results suggest that ZZBPD may alleviate CLI by modulating the IL-17/NF-κB pathway.

Although this study provides valuable insights into the protective effects of ZZBPD on cholestasis, there are several limitations to consider. First, the animal models in this study primarily utilized mice. While mouse models are commonly used in cholestasis research, they may not fully replicate the human pathological process due to species differences. Therefore, future studies should explore a broader range of animal models or even clinical samples to validate the efficacy of ZZBPD across different populations. Finally, this study mainly concentrated on the regulatory effects of ZZBPD on FXR pathway-related gene expression and the IL-17/NF-κB pathway, without exploring other potential pathways or molecular mechanisms through which ZZBPD may exert its effects. Future research could further investigate the mechanisms of ZZBPD using multi-omics approaches.

Conclusion

In conclusion, this study demonstrates that ZZBPD enhances liver function in mice with cholestasis. The protective effects of ZZBPD are suggested to involve the activation of the FXR signaling pathway, upregulation of BSEP expression, promotion of bile acid excretion, and inhibition of the IL-17/NF-κB signaling pathway, thereby decreasing the release of inflammatory factors. These findings offer novel insights and experimental evidence that support the potential clinical

application of ZZBPD in treating CLI, thereby providing renewed hope for patients.

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