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Study on improving hematopoietic function of rats with blood deficiency syndrome by Shengxuebao mixture

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Author contributions

Shu Y and Yang ZF designed this project, processed the samples, performed the experiments. Shu Y wrote the article. Cao JY and Qiao Y revised the article. Zhao Y and Chen Y analyzed the data. Guo H and Liu HN guided the experiment, Yang ZF provided funding for the research.

Competing interests

The authors declare no conflicts of interest.

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Peer review information

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Abbreviations

SXBM, Shengxuebao mixture; BDS, blood deficiency syndrome; APH, acetylphenylhydrazine; CTX, cyclophosphamide; TCM, traditional Chinese medicine; RBC, red blood cell; WBC, white blood cell; HIF-1, hypoxia-inducible factor 1; PLT, platelet; ASP, angelica sinensis polysaccharide; IL, interleukin; TNF, tumor necrosis factor; DAVID, Database for Annotation, Visualization, and Integrated Discovery; FEJ, Fufang E'jiao Jiang.

Citation

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Abstract

Background: Shengxuebao mixture (SXBM) is a novel herbal drug approved by China State Food and Drug Administration for the treatment of Leukopenia and iron deficiency anemia caused by radiotherapy and chemotherapy**. Methods:** To explore the mechanism of SXBM in treating blood deficiency syndrome (BDS). Firstly, network pharmacology and in vivo experiments were used to screen candidate targets and important signaling pathways of SXBM, GO functional enrichment and KEGG pathway analysis were performed. Secondly, a BDS rat model was established to verify the results of the analysis of network pharmacological enrichment. Histopathology and routine peripheral blood examination were observed. The expressions of tumor necrosis factor-α, interleukin (IL)-6, HIF-1α and NF-κB were detected by Western blot, and the expressions of IL-6, IL-1β were detected by ELISA**. Results:** 62 bioactive components, 66 potential targets and 131 signaling pathways of BDS were successfully identified by network pharmacology. Molecular docking simulation techniques showed that key targets tumor necrosis factor-α, IL-6, IL-1β can dock well with crucial components, and the BDS-related signaling pathways HIF-1 and JAK-STAT play a vital role. The combined model experiment of acetylphenylhydrazine and cyclophosphamide showed that the model group had obvious blood deficiency, and the histopathology and blood routine were effectively restored after administration. Our findings indicate that SXBM's therapeutic effect on BDS primarily involves the mediation of the HIF-1α/NF-κB signaling pathway and the regulation of hematopoietic factor expression. **Conclusion:** This study not only affirmed the protective properties of SXBM against BDS but also provided insights into a potential mechanism for blood replenishment in the treatment of BDS using SXBM.

Keywords: network pharmacology; molecular docking technology; blood deficiency syndrome; hematopoietic factors; HIF-1α/NF-κB

Highlights

1. The main components, targets and signaling pathways of Shengxuebao mixture were screened by network pharmacology for the first time.
2. Shengxuebao mixture's therapeutic effect on blood deficiency syndrome primarily involves the mediation of the HIF-1α/NF-κB signaling pathway and the regulation of hematopoietic factor expression.

Medical history of objective

The main components of SXBM are *Polygoni Multiflori Radix* and *Astragali Radix*, The clinical application of *Polygoni Multiflori Radix* was first recorded in Li Ao's *Polygonum multiflorum*, (*Li Wengong Collection* volume 18, the book was written in 813 C.E.). It has the effect of anti-aging, replenishing blood and anti-atherosclerosis. *Astragali Radix*, was first published in *The Classic of Herbal Medicine* (200 C.E.–250 C.E.). It has the biological activities of enhancing immunity, protecting organs and anti-tumor.

Background

Blood deficiency syndrome (BDS) represents one of the most prevalent symptoms associated with a range of diseases in clinical practice. Its prevalence has increased due to factors such as the aging population, dietary habits, pharmaceutical treatments, and the fast-paced modern lifestyle. In recent years, significant progress has been made in the field of traditional Chinese medicine (TCM) in the development of treatments for BDS. Researchers have conducted comprehensive investigations, employing TCM syndromes and clinical observations to gain a preliminary understanding of disease treatment progress. Animal experiments have been carried out to make corresponding models and explore the targets and signaling pathways of TCM on the disease from the molecular, proteomic and cellular levels TCM theory posits that blood production is intricately linked to the functional activities of the body's five viscera, with the kidney and bone marrow playing pivotal roles in the Zang-fu theory of TCM [1]. Bone marrow serves as the fundamental material for blood generation. Hematopoietic cells and bone marrow microenvironment supporting the growth and development of hematopoietic cells in normal body. Relevant studies have shown that blood deficiency (A pathological condition in which blood does not produce enough blood to nourish the body), will cause pathological state of bone marrow and inhibit the hematopoietic function, manifested as hematopoietic dysfunction, resulting in total blood cell count attenuation, thereby reducing the body's immunity [2]. It is characterized by a combination of pale complexion, pale eyelids and weak veins. According to literature, up to 80% of the patients suffer from anemia nationwide every year, among which the incidence of anemia in developing countries is far higher than that in developed countries [3]. Moreover, the number of individuals succumbing to various diseases attributable to anemia is on the rise. Aside from the adverse health consequences associated with anemia, it also imposes substantial economic burdens, costing billions of dollars to society.

In recent years, drug-induced BDS has garnered attention, with a focus on the effects of acetylphenylhydrazine (APH) and cyclophosphamide (CTX) on the hematopoietic system. APH has been found to induce slow and progressive oxidative damage to red blood cells (RBC), rendering them more susceptible to disintegration and leading to hemolytic anemia. It can destroy the antioxidant system composed of SOD, glutathione and other components during the metabolism of erythrocyte membrane. CTX is a non-specific alkylating agent of cell cycle, causing more serious damage to white blood cells (WBC). Meanwhile, the immune organs of the body are damaged, and the BDS model is constructed from the dual links, which can significantly destroy the hematopoietic microenvironment, and the blood deficiency state lasts relatively long, which is conducive to testing the effect of drugs and exploring the mechanism [4]. Hypoxia-inducible factor 1 (HIF-1) protects function from Hypoxia, especially in the case of hypoxia in vital organs, by regulating target genes to maintain oxygen supply and demand levels in the body, therefore hold oxygen homeostasis in all cells [5]. HIF-1α plays a vital role by participating in angiogenesis, erythropoiesis, and anaerobic metabolism. Some TCM have a good influence on the treatment of BDS, and the price is cheap, few side effects, which has been widely concerned by researchers [6]. There are many classic prescriptions for the clinical treatment of BDS, such as Siwu decoction and Angelica tonifying blood decoction. It is very important to find suitable drugs and clinical regimen for the prevention and treatment of anemia. The mechanism of action and pivotal targets of Shengxuebao mixture (SXBM) in the BDS treatment are foreseen through network pharmacology a novel, distinctive approach for the systematic examination and exploration of the intricate interplay between drugs and diseases, grounded in the principles of systems biology and network analysis [7]. This approach has seen growing application in uncovering the systematic mechanisms of TCM in complex diseases, emphasizing the evolution towards network-targeted multi-component therapy.

SXBM is composed of *Polygoni Multiflori Radix*, *Ligustri Lucidi Fructus*, *Mori Fructus*, *Paeoniae Radix Alba* and other TCM. It possesses the dual functions of addressing liver and kidney deficiencies while also promoting blood production. Currently, researchers have conducted relevant experimental and clinical investigations on SXBM during the prior period and have observed its potential to cincrease the reduced count of WBCs resulting from myelosuppression induced by CTX injection in rats. Moreover, SXBM has demonstrated the capacity to ameliorate leukocytosis subsequent to chemotherapy. Clinical studies have shown that SXBM is effective for iron deficiency anemia, bone marrow suppression after radiotherapy and chemotherapy, and the combination of SXBM with other drugs for the prevention and treatment of related anemia, etc. Nonetheless, the mechanism of action of SXBM remains inadequately explored, and the constituent elements responsible for its efficacy have not been comprehensively elucidated. These knowledge gaps significantly impede the secondary development of SXBM and the modernization of TCM. Therefore, this study aims to conduct inflammation-based experiments utilizing network pharmacology to identify the effective targets and pivotal pathways through which SXBM treats BDS.

Materials and methods

Chemicals and reagents

SXBM: Tsing Hua De Ren Xi'an Happiness Pharmaceutical Co., Ltd. (Shaanxi, China). Cyclophosphamide for injection: CTX (20221129), Jiangsu Hengrui Pharmaceutical Co., Ltd. (Jiangsu, China). Acetyl phenylhydrazine: APH (A100226), Sinopod Chemical Reagents Co., Ltd. (Guizhou, China). Compound Ejiao Slurry: Fufang E'jiao Jiang (FEJ), Shandong Dong'e Ejiao Co., Ltd. (Shandong, China). ELISA detection kits for interleukin (IL)-6 (ERC003) (Shenzhen, China), IL-1β (ERC007) (Shenzhen, China). tumor necrosis factor (TNF)-α (EPR19147), NF-κB (ab16502), IL-6 (DF6087), HIF-1α (ab179483) Antibodies were purchased from ABCAM.

Pharmacology of network

Chemical composition collection and active ingredient screening. SXBM is composed of *Polygoni Multiflori Radix, Polygoni Multiflori Radix Praeparata, Paeoniae Radix Alba, Cibotii Rhizoma, Mori Fructus, Astragali Radix, Ligustri Lucidi Fructus, Ecliptae Herba.* Corresponding chemical components were found in the Chinese Medicine System Pharmacology Database and Analysis platform (TCMSP), https://bidd.group/TCMID/. The literature required that the compounds of OB ≥ 30% and DL ≥ 0.18 should be used as the preselected active components, and the active ingredients for the treatment of BDS in the literature were included too.

Forecast and screening of drug and target. All the target proteins

were imported into the Uniprot (https://www.UniProt.org/) database, and the corresponding proteins of "Human" were selected, the target information related to the active ingredient was recorded. GeneCards (https://www.genecards.org/) database was searched for disease targets using the keywords "blood deficiency syndrome".

Construction of protein-protein correlation network. The targets of SXBM and BDS were intersected, and the drug components-common targets of diseases were imported into the STRING (https://STRING-db.org/) database to construct the protein-protein interaction (PPI) network model, the PPI network was obtained by setting the protein species as "*Homo sapiens*" and threshold > 0.9.

Target enrichment analysis of SXBM regulating blood deficiency prediction. Database for Annotation, Visualization, and Integrated Discovery (DAVID) (https://david.ncifcrf.gov/) relies on the GO database for gene annotation functions and accesses information from the KEGG database. Therefore, DAVID's online analytical tools were employed to conduct GO and KEGG enrichment analyses concerning common target proteins associated with pharmaceutical ingredients and diseases. Additionally, it was discovered that SXBM plays a regulatory role in the principal molecular functions and signaling pathways relevant to BDS.

Construct the signal pathway network map of SXBM regulating the related targets of BDS. The Count and *P* were sorted to construct the signal pathway diagram of SXBM regulating related targets of BDS. DAVID import Image GP predicted signal pathway data in the database(http://www.ehbio.com/Cloud_Platform/front/upsetview.html) mapping software.

Molecular simulated docking. The molecular structure of the active compound and the protein structure of the overlapping target were downloaded by ordering OB and DL of each component. Discovery Studio (DS) 20 program was used for compound-target virtual docking, and PyMOL software was used for visual analysis. The specific analysis proceeded as follows: initially, ligands in MOL2 format associated with the active compounds of SXBM were gathered and subjected to preprocessing. Subsequently, water molecules within both the original ligand and protein receptor were eliminated, and the data was stored in PDB format. Following the conversion, the PDB files were processed into PDBQT format. Subsequently, molecular docking was performed by utilizing AutoGrid to establish the mesh size and configure the parameters. After the docking process, molecules within SXBM demonstrating superior binding characteristics, based on their binding energy, different molecules were selected for analysis. A binding energy of ≤ -5.0 kJ/mol served as a suitable criterion to assess the molecule's binding affinity to the target.

Animal experiment verification

BDS model and treatments. The SPF Male SD rats were purchases from Animal Experimental Center of the Fourth Military Medical University (SCXK (Shaan) 2019-001, Shaanxi, China). This animal experiment has been approved by the Animal athics Committee of Laboratory Animal Center, Military Medical University, PLA Air Force (IACUC-20231256). A total of 36 male SD rats (body weight 250 ± 20 g, 8 weeks) were provided. The feeding conditions are strictly controlled: temperature (22 ± 2 °C), humidity (55%–75%) and light (12 h cycle). All experimental steps were performed in strict accordance with the guidelines for the Welfare and Ethics Committee, Laboratory Animal Center, Air Force Medical University. In addition, this study was conducted in accordance with the arrival standard while implementing procedures to minimize test animals potential hazards. The rats were randomly divided into 6 groups with 6 rats in each group, including control group, model group, positive group (FEJ) and SXBM-H, SXBM-M and SXBM-L group. The drug was administered intragastric once a day from day 1 to day 10. According to the equivalent dose coefficient conversion method, SXBM-H (5.24 mL/kg), SXBM-M (2.62 mL/kg), SXBM-L (1.31 mL/kg), FEJ (6 mL/kg) were obtained [8]. The BDS model development was performed by subcutaneous injection of APH and intraperitoneal injection of CTX. The specific methods were as follows: in addition to the control group, the model group, SXBM group and FEJ group were given APH 20 mg/kg subcutaneously on the 1st day, APH 40 mg/kg subcutaneously on the 4th day, and CTX 20 mg/kg intraperitoneally from the 4th day [9]. Replicable model for 4 consecutive days. Animals in blank control group were injected with equal volume normal saline subcutaneously.

Sample collection. Blood was collected from the abdominal aorta within 2 h of the last gavage. Part of the blood sample was collected for detection of WBC, RBC, HGB and platelet (PLT), and remaining part was preserved at −80 °C. Then spleen, thymus organs were collected and weighed, organ index (mg/g) = (organ weight/body weight) × 100%. One femur was sliced to observe the morphology of bone marrow tissue. Spleen specimens were fixed with paraformaldehyde and then embedded with paraffin. The pathological changes of spleen and femur were observed by hematoxylin-eosin staining. The analysis was performed under a light microscope and photographed through a ×200 objective lens. Femoral bone marrow collection: both ends of the femur were cut off with scissors and transferred to a clean table. The femur was rinsed back and forth 5 times with PBS in a centrifuge tube using a 5 mL syringe and then centrifuged at 12,000 rpm for 10 min. After centrifugation, the supernatant was discarded and the femoral bone marrow at the bottom was kept in a 1.5 mL EP tube and stored at −80 °C.

Blood routine test and organs index. After the final administration, blood was collected from the abdominal aorta in a sterile vacuum collection vessel containing ethylenediamine tetra-acetic acid. Blood was analyzed using an animal hematology analyzer (Drew Scientific Group, Dallas, Texas, USA) to quantify RBC, WBC, HGB, and PLT. After blood collection, spleen, thymus are weighed, spleen/thymus index (mg/g) = (organ weight/body weight) × 100%.

Histopathological changes. The rat femur samples were fixed with 4% paraformaldehyde, then trimmed, dehydrated, embedded, sliced, stained, and sealed with standard operation procedure. The tissue sections were observed in detail under different multiples, and the basic pathological changes in the sections, such as congestion, degeneration, necrosis, mechanization, inflammatory changes, etc. The analysis was performed under a light microscope and photographed through a ×200 objective lens.

Western blot analysis. Total protein Extraction and Western Blotting analysis bone marrow proteins were extracted using RIPA lysis buffer (Solarbio, Beijing, China) and centrifuged at 4 °C at 12,000 g for 30 min. The protein concentration was determined by BCA protein assay. A large amount of protein was isolated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and then transferred to a 0.45 mm polyvinylidene fluoride membrane (Biosharp, Beijing, China). The nonspecific binding sites on the membrane were blocked with 5% skimmed milk powder and Tween (10 mM Tris, 150 mM NaCl, pH 7.4) saline tris buffered saline (0.1% Tween 20) for 2 h. Subsequently, bands were incubated with primary antibodies (anti-IL6, anti-TNF-α, anti-NF-κB, anti-HIF-1α; Abcam, Cambridge, UK) overnight at 4 °C respectively. After that, tris buffered saline with Tween was washed three times for 10 min and then treated with secondary antibody (Rabbit or mouse resistance 1:10,000) was soaked at room temperature for 2 h, and then washed in tris buffered saline with Tween for 3 times for 10 min each time. Finally, a multifunctional gel imaging system was used to observe the chemiluminescence signals and analyze the protein expression of each group.

Analysis of IL-6, IL-1β in serum. Blood samples were centrifuged at 3,000 rpm to obtain serum. ELISA was used to detect the activities of hematopoietic function factor IL-6, IL-1β in serum.

Statistical analysis. The data were expressed as mean ± standard deviation. The difference between groups was analyzed by One-Way analysis of variance. T-test was used to compare the mean between groups. $P < 0.05$ was set as the standard for the significant difference test, and $P < 0.01$ was the standard for extremely significant differences. $P < 0.05$ was considered statistically significant.

Results

Network construction

Construction of bioactive compound. The chemical components of SXBM were obtained through TCMSP and TCMID databases. Compounds with OB ≥ 30% and DL ≥ 0.18 and those with proven efficacy were screened as required, and the potential active ingredients of SXBM were finally obtained, as shown in Table 1.

Construction of PPI network and screening of the core targets. The targets corresponding to the active components of SXBM were removed and imported into Uniprot database for gene name conversion, with scores greater than 15 were screened out using Genecards database, and 66 SXBM potential targets for BDS treatment were obtained by intersection in Venny.

Gene ontology and pathway enrichment analyses for core targets. The selected drug ingredients and common disease targets were inputted into the STRING database to create a PPI network model. Subsequently, PPI enrichment was performed. Network topology parameters, specifically degree and combined score, were subjected to analysis using Network Analyzer. This analysis revealed that key targets within the network included STAT3, TNF, IL-6, IL1β, among others, as depicted in Figure 1.

Construction of component-core target-pathway network. SXBM and the main ingredients-drug ingredients-disease common targets are summarized into a table and imported into Cytoscape3.7.2 software to construct the drug-compound-disease common target network diagram, as shown in Figure 2.

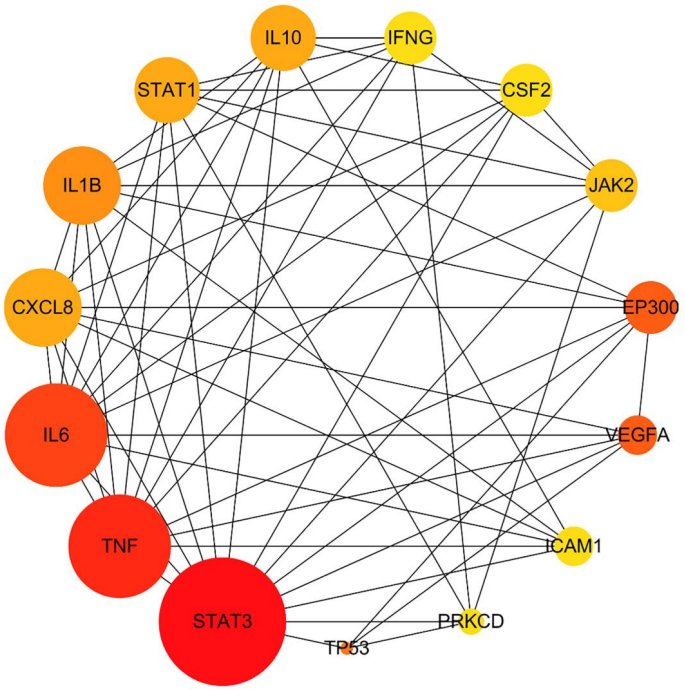

Figure 1 Fifteen key targets. IL, interleukin; TNF, tumor necrosis factor.

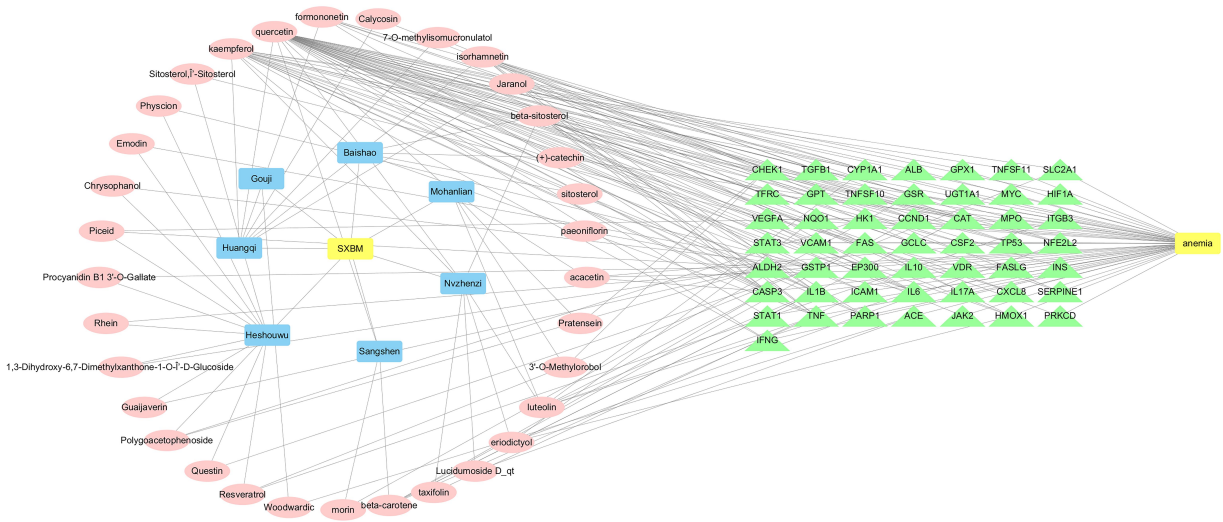

Figure 2 Drug-compound-drug disease common target network diagram. SXBM, Shengxuebao mixture; IL, interleukin; TNF, tumor necrosis factor.

Table 1 The potential active ingredients of SXBM

Mol ID	Molecule name	OB (%)	DL	source
MOL010300	dIDP	41.08	0.57	*Mori Fructus*
MOL002372	(6Z,10E,14E,18E)-2,6,10,15,19,23-hexamethyltetracosa-2,6,10,14,18,22-hexaene	33.55	0.42	*Mori Fructus*
MOL006209	Cyanin	47.42	0.76	*Mori Fructus*
MOL000737	Morin	46.23	0.27	*Mori Fructus*
MOL002773	Beta-carotene	37.18	0.58	*Mori Fructus*
MOL000098	Quercetin	46.43	0.28	*Mori Fructus*
MOL000358	Beta-sitosterol	36.91	0.75	*Ligustri Lucidi Fructus*
MOL000422	Kaempferol	41.88	0.24	*Ligustri Lucidi Fructus*
MOL004576	Taxifolin	57.84	0.27	*Ligustri Lucidi Fructus*
MOL005146	Lucidumoside D	48.87	0.71	*Ligustri Lucidi Fructus*
MOL005147	Lucidumoside D_qt	54.41	0.47	*Ligustri Lucidi Fructus*
MOL005169	(20S)-24-ene-3β,20-diol-3-acetate	40.23	0.82	*Ligustri Lucidi Fructus*
MOL005190	Eriodictyol	71.79	0.24	*Ligustri Lucidi Fructus*
MOL005195	Syringaresinol diglucoside_qt	83.12	0.80	*Ligustri Lucidi Fructus*
MOL005209	Lucidusculine	30.11	0.75	*Ligustri Lucidi Fructus*
MOL005211	Olitoriside	65.45	0.23	*Ligustri Lucidi Fructus*
MOL005212	Olitoriside_qt	103.23	0.78	*Ligustri Lucidi Fructus*
MOL000006	Luteolin	36.16	0.25	*Ligustri Lucidi Fructus*
MOL000098	Quercetin	46.43	0.28	*Ecliptae Herba*
MOL001790	Linarin	39.84	0.71	*Ecliptae Herba*
MOL001689	Acacetin	34.97	0.24	*Ecliptae Herba*
MOL002975	Butin	69.94	0.21	*Ecliptae Herba*
MOL003378	1,3,8,9-tetrahydroxybenzofurano[3,2-c]chromen-6-one	33.94	0.43	*Ecliptae Herba*
MOL003389	3'-O-Methylorobol	57.41	0.27	*Ecliptae Herba*
MOL003398	Pratensein	39.06	0.28	*Ecliptae Herba*
MOL003402	Demethylwedelolactone	72.13	0.43	*Ecliptae Herba*
MOL003404	Wedelolactone	49.6	0.48	*Ecliptae Herba*
MOL000006	Luteolin	36.16	0.25	*Ecliptae Herba*
MOL000098	Quercetin	46.43	0.28	*Ecliptae Herba*
MOL001910	11alpha,12alpha-epoxy-3beta-23-dihydroxy-30-norolean-20-en-28,12beta-olide	64.77	0.38	*Paeoniae Radix Alba*
MOL001918	Paeoniflorgenone	87.59	0.37	*Paeoniae Radix Alba*
MOL001919	(3S,5R,8R,9R,10S,14S)-3,17-dihydroxy-4,4,8,10,14-pentamethyl-2,3,5,6,7,9-hexa hydro-1H-cyclopenta[a]phenanthrene-15,16-dione	43.56	0.53	*Paeoniae Radix Alba*
MOL001921	Lactiflorin	49.12	0.80	*Paeoniae Radix Alba*
MOL001924	Paeoniflorin	53.87	0.79	*Paeoniae Radix Alba*
MOL001925	Paeoniflorin_qt	68.18	0.40	*Paeoniae Radix Alba*
MOL001928	Albiflorin_qt	66.64	0.33	*Paeoniae Radix Alba*
MOL001930	Benzoyl paeoniflorin	31.27	0.75	*Paeoniae Radix Alba*
MOL000211	Mairin	55.38	0.78	*Paeoniae Radix Alba*
MOL000358	Beta-sitosterol	36.91	0.75	*Paeoniae Radix Alba*
MOL000359	Sitosterol	36.91	0.75	*Paeoniae Radix Alba*
MOL000422	Kaempferol	41.88	0.24	*Paeoniae Radix Alba*
MOL000492	(+)-catechin	54.83	0.24	*Paeoniae Radix Alba*
MOL000211	Mairin	55.38	0.78	*Astragali Radix*
MOL000239	Jaranol	50.83	0.29	*Astragali Radix*
MOL000296	Hederagenin	36.91	0.75	*Astragali Radix*
MOL000033	(3S,8S,9S,10R,13R,14S,17R)-10,13-dimethyl-17-[(2R,5S)-5-propan-2-yloctan-2-yl]-2,3,4,7,8,9,11,12,14,15,16,17-dodecahydro-1H-cyclopenta[a]phenanthren-3-ol	36.23	0.78	*Astragali Radix*
MOL000354	Isorhamnetin	49.6	0.31	*Astragali Radix*
MOL000371	3,9-di-O-methylnissolin	53.74	0.48	*Astragali Radix*
MOL000374	5'-hydroxyiso-muronulatol-2',5'-di-O-glucoside	41.72	0.69	*Astragali Radix*
MOL000378	7-O-methylisomucronulatol	74.69	0.30	*Astragali Radix*
MOL000379	9,10-dimethoxypterocarpan-3-O-β-D-glucoside	36.74	0.92	*Astragali Radix*
MOL000380	(6aR,11aR)-9,10-dimethoxy-6a,11a-dihydro-6H-benzofurano[3,2-c]chromen-3-ol	64.26	0.42	*Astragali Radix*
MOL000387	Bifendate	31.1	0.67	*Astragali Radix*
MOL000392	Formononetin	69.67	0.21	*Astragali Radix*
MOL000398	Isoflavanone	109.99	0.3	*Astragali Radix*
MOL000417	Calycosin	47.75	0.24	*Astragali Radix*
MOL000422	Kaempferol	41.88	0.24	*Astragali Radix*
MOL000433	FA	68.96	0.71	*Astragali Radix*
MOL000438	(3R)-3-(2-hydroxy-3,4-dimethoxyphenyl)chroman-7-ol	67.67	0.26	*Astragali Radix*
MOL000439	Isomucronulatol-7,2'-di-O-glucosiole	49.28	0.62	*Astragali Radix*
MOL000442	1,7-Dihydroxy-3,9-dimethoxy pterocarpene	39.05	0.48	*Astragali Radix*
MOL000098	Quercetin	46.43	0.28	*Astragali Radix*

SXBM, Shengxuebao mixture.

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GO and KEGG analysis. The DAVID online analysis tool was used for GO enrichment analysis of the common target proteins of SXBM drug ingredients and BDS, including biological process, molecular function and cellular component. The results from the biological process analysis indicated that the primary processes involved apoptotic processes, drug responses, immune responses, and more, as illustrated in Figure 3. The Image GP online analysis tool was employed to conduct KEGG enrichment analysis of the common target proteins associated with SXBM components and BDS. The results showed that the mechanism of SXBM was mainly related to HIF-1, JAK-STAT, FoxO and other signaling pathways. The KEGG bubble maps of the first 20 channels were plotted according to $P < 0.01$, as shown in Figure 4.

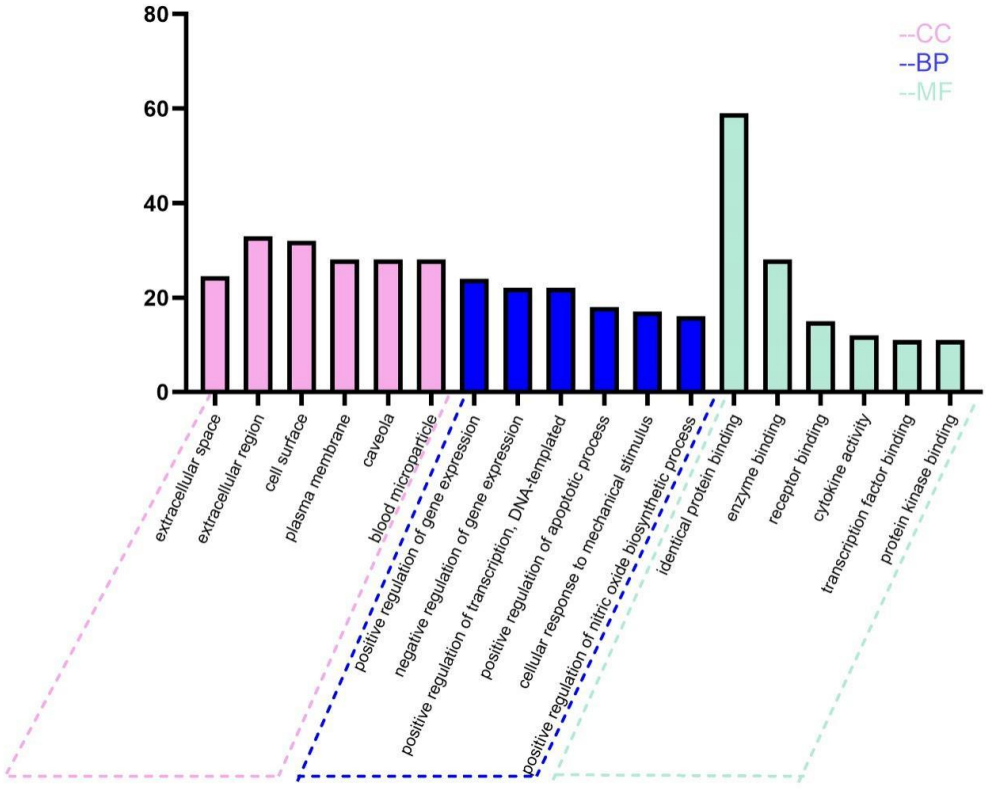

Figure 3 GO enrichment analysis result graph. BP, biological process; MF, molecular function; CC, cellular component.

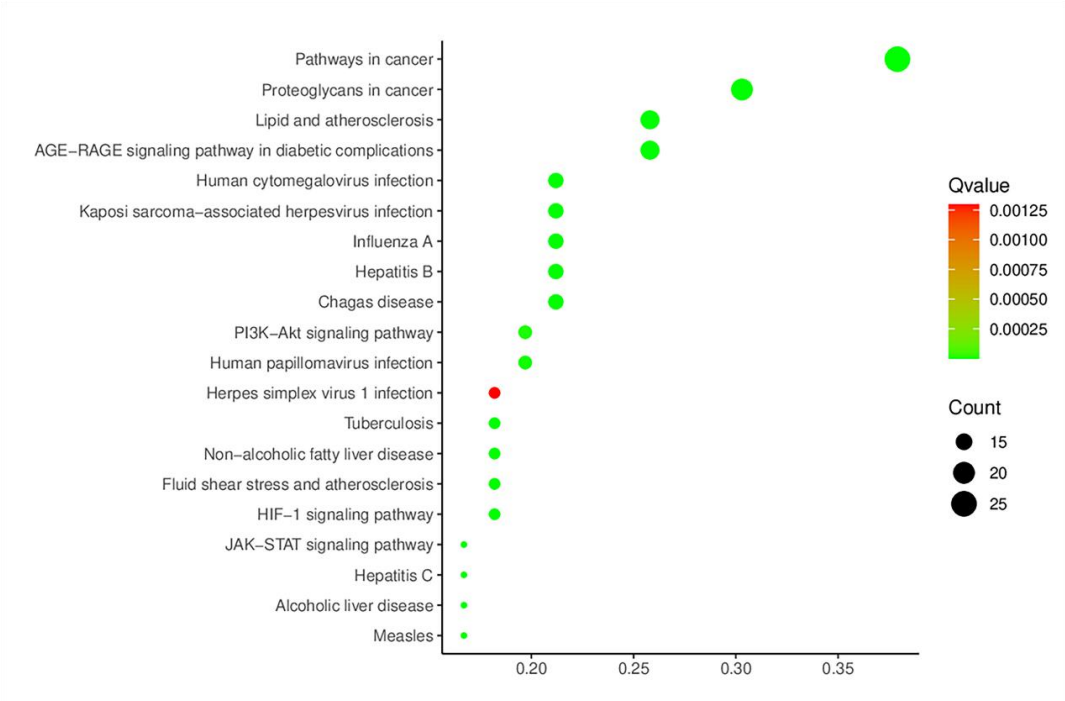

Figure 4 KEGG enrichment analysis result graph

Molecular docking simulation verification. Twelve active compounds, including Olitoriside_qt, isoflavanone, cyanin, morin and demethylwedelolactone, which were ranked top in the "Compounds-Targets-Pathways" network, were selected for molecular simulation and docking with target genes such as STAT3, IL-1β, IL-6 and VEGFA. Molecular docking simulations were performed, and the interactions between compounds and proteins were visualized and analyzed using PyMOL software (Figure 5). A lower binding energy signifies a more stable conformation, and a binding energy less than −7.0 kcal/mol indicates that the molecule exhibits strong binding affinity and a certain degree of stability to the target. IL-1β, IL-6, VEGFA, and IL10 are closely related to hematopoiesis, and the Supplementary Table S1 showed that the docking scores of Olitoriside_qt and IL-1β were −5.7, paeniflorgenone and IL-6 were −6, isoflavanone and VEGFA were −8.9, and cyanin and IL-10 were −8.9, respectively. The docking score of cyanin with IL-10 was −5.8. Other targets such as STAT3, JAK1, JAK2, EP300 have been widely studied in anemia diseases, and the molecular docking results demonstrated that the above targets have a strong association with BDS, proving that the above components and targets may play a major role in SXBM.

Animal experiment verification

Peripheral blood and organs index results. The contents of WBC, RBC, HGB and PLT in peripheral blood of rats were determined (Figure 6). Compared with the control group, the contents of WBC, RBC, HGB and PLT in the model group decreased significantly ($P < 0.05$), the levels of WBC, RBC, HGB and PLT in FEJ Group and SXBM group were significantly increased ($P < 0.05$). Compared with the control group, the indexes of spleen and thymus in the model group decreased ($P < 0.05$), and the indexes of spleen increased after the administration of FEJ and SXBM, but the thymus index was not statistically significant. The results presented above provide strong confirmation of the successful construction of the BDS model through the combined drug modeling method. Furthermore, these findings are in alignment with the pertinent literature on the subject [10].

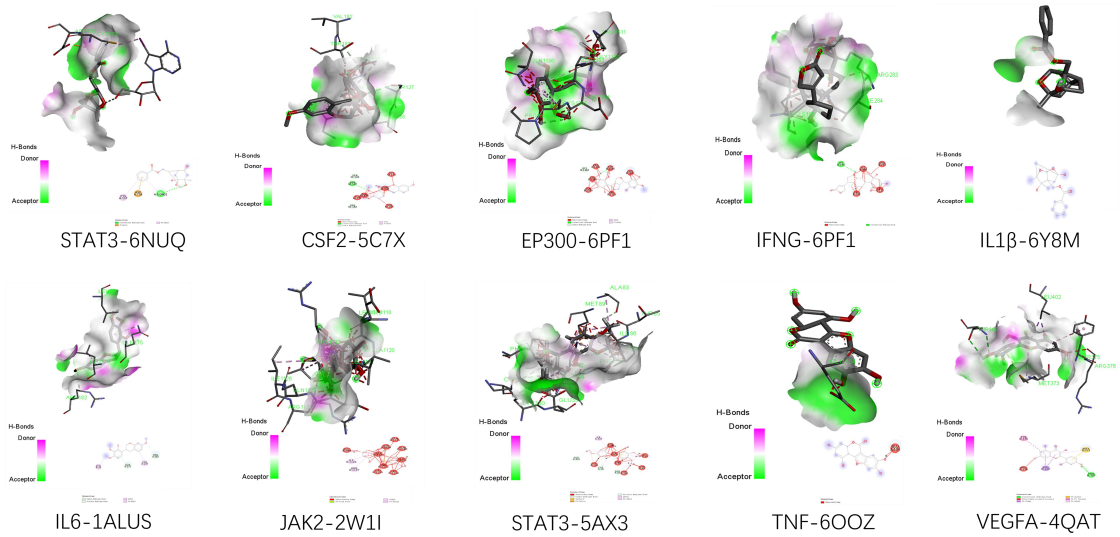

Figure 5 Molecular docking shows results in 3D. IL, interleukin; TNF, tumor necrosis factor.

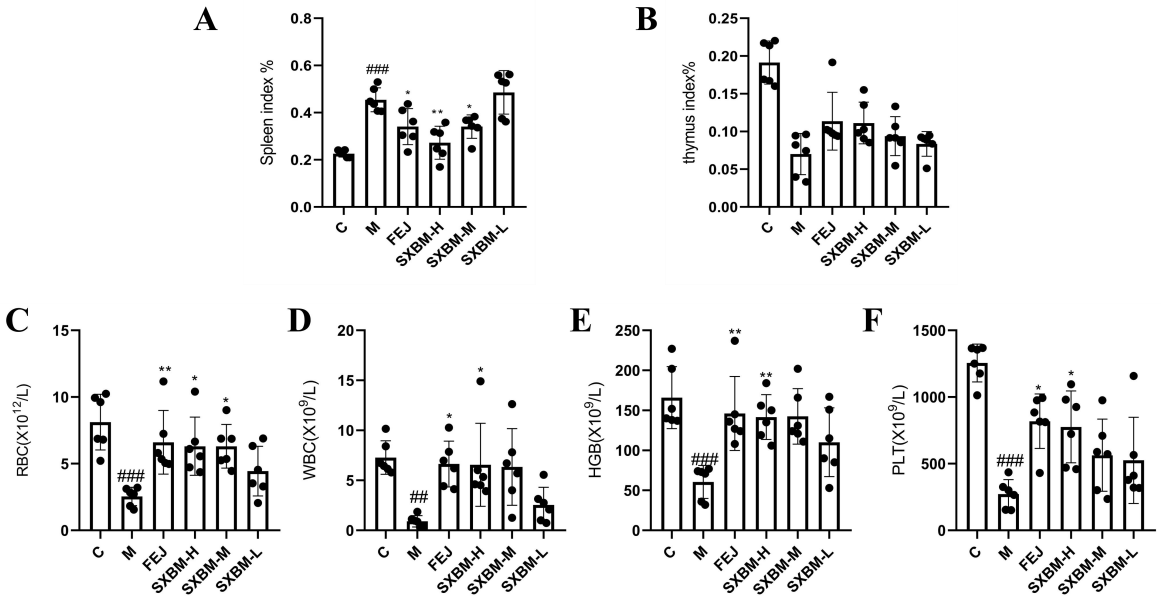

Figure 6 Organ index (mean ± SD, n = 6). (A) Spleen index. (B) Thymus index (C) Red blood cell. (D) White blood cell. (E) Hemoglobin (F) Platelet. $^{###}P < 0.001$, $^{##}P < 0.01$, vs. control group; $^{**}P < 0.01$, $^{*}P < 0.05$, vs. model group. C, control group; M, model group; SXBM, Shengxuebao mixture; FEJ, Fufang E'jiao Jiang (positive group); SXBM-H, SXBM high group; SXBM-M, SXBM middle group; SXBM-L, SXBM low group.

Pathological morphology of femur. The results of H&E staining of femur in each experimental group were shown in Figure 7. In the femoral tissue, when compared to the blank group, the model group exhibited a higher concentration of erythroid cell lines, granulocyte lines, and other hematopoietic cells within the bone marrow cavity. Conversely, there was a decrease in these hematopoietic cells, accompanied by increased proliferation of adipose tissue, which nearly filled the entire bone marrow cavity. Consequently, the hematopoietic area was reduced. However, following administration, there was an increase in both the number of hematopoietic cells and the area dedicated to hematopoiesis.

Western blot analysis of pathways and targets. The expression of IL-6, TNF-α, NF-κB, HIF-1α were shown in Figure 8. The results showed that there were significant differences in protein expression among the groups ($P < 0.05$). Compared with the control group, the expression levels of NF-κB, HIF-1α, TNF-α in the model group were significantly higher than those in the control group ($P < 0.05$) , after treatment with FEJ, SXBM-H, SXBM-M, SXBM-L, the expression levels of the above two proteins were significantly decreased ($P < 0.05$). The expression levels of IL-6 in model group were significantly decreased ($P < 0.05$), the expression levels of FEJ, SXBM-H, SXBM-M, SXBM-L were increased ($P < 0.05$), and the differences were statistically significant ($P < 0.05$). The SXBM-H group exhibited a statistically significant better impact compared to SXBM-M and SXBM-L. This suggests that the high dosage of SXBM-H possesses a notable reversal effect on BDS.

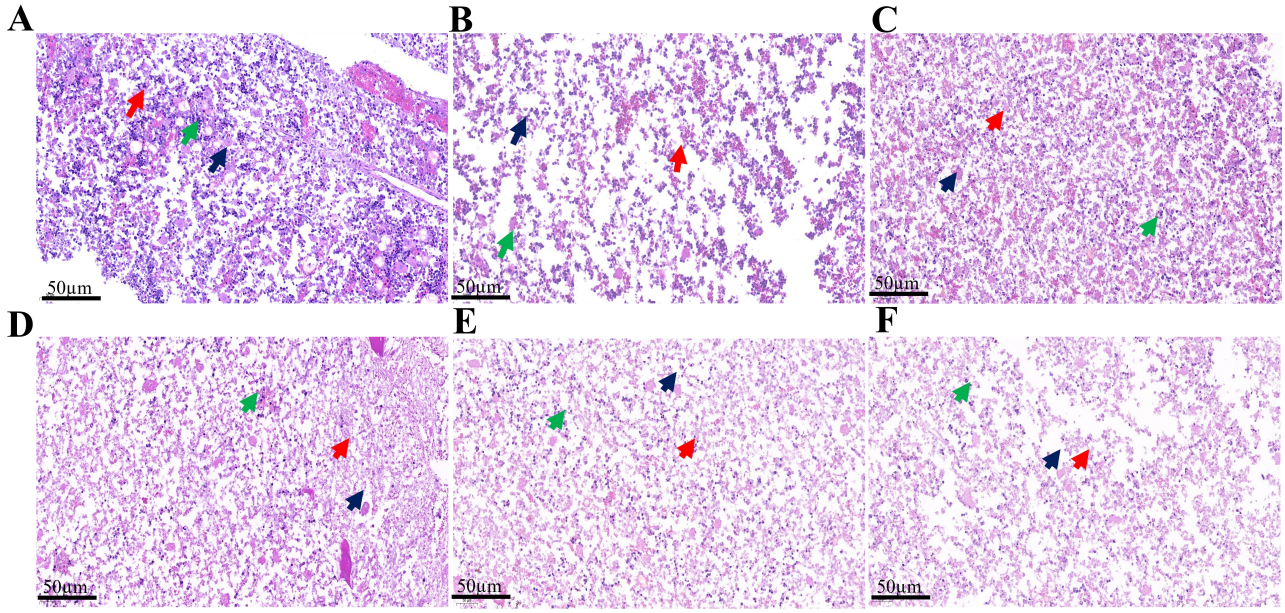

Figure 7 Bone marrow pathology. (A) Control. (B) Model. (C) Fufang E'jiao Jiang. (D) Shengxuebao mixture-high. (E) Shengxuebao mixture-middle. (F) Shengxuebao mixture-low. The green arrow represents the granulation system, the red arrow represents the red system, and the black arrow represents the megakaryon system.

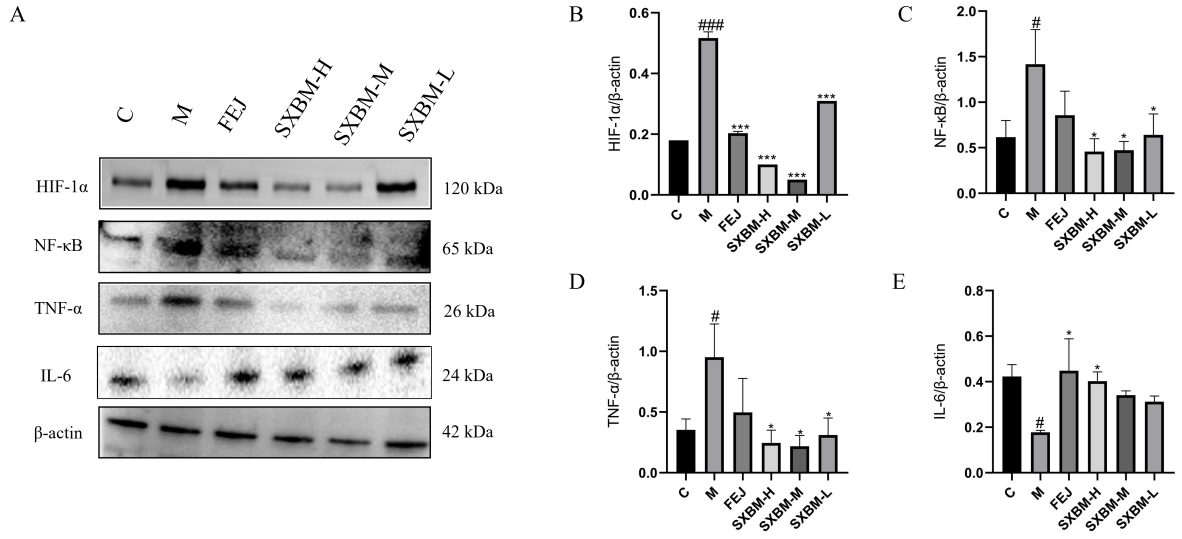

Figure 8 The expression of target protein by Western blot (mean ± SD, n = 3). (A) Protein banding of each group. (B) HIF-1α expression. (C) NF-κB expression. (D) TNF-α expression. (E) IL-6 expression. $^{###}P < 0.001$, $^{#}P < 0.05$, vs. control group; $^{***}P < 0.001$, $^{*}P < 0.05$, vs. model group. C, control group; M, model group; SXBM, Shengxuebao mixture; FEJ, Fufang E'jiao Jiang (positive group); SXBM-H, SXBM high group; SXBM-M, SXBM middle group; SXBM-L, SXBM low group; HIF-1, hypoxia-inducible factor 1; IL, interleukin; TNF, tumor necrosis factor.

The results of ELISA. To validate the molecular mechanism through which SXBM acts against BDS, IL-6 and IL-1β were chosen for further analysis using ELISA, based on the outcomes of previous network pharmacology investigations. As illustrated in Figure 9, SXBM demonstrated the ability to enhance the expression of IL-6 and IL-1β ($P < 0.05$). Importantly, the impact of SXBM exhibited a dose-dependent pattern, with higher doses yielding more significant effects.

Discussion

Blood deficiency manifests in various types of anemic conditions, and current clinical treatments predominantly focus on symptom management. These treatments often entail blood transfusions and the supplementation of hematopoietic materials. However, these pharmacological interventions accompany with a range of safety concerns and can frequently lead to adverse reactions, including gastrointestinal discomfort and allergic responses, among others. In addition, long duration of administration and high doses can lead to drug resistance, and reduced clinical efficacy [11]. BDS can arise from factors such as significant blood loss, insufficient biochemical resources for blood production, or impaired blood nutritional function. Clinical manifestations often include facial pallor, hair thinning or loss, palpitations, and forgetfulness [12]. The diagnosis of BDS follows a similar approach in Western Medicine. In recent years, some TCMs have gained attention from researchers due to their affordability and limited side effects [13].

SXBM was approved and introduced in China on December 1st, 2005. Its primary indications include the treatment of leukopenia and fatigue induced by radiotherapy and chemotherapy in malignant tumor patients. This often manifests as symptoms like weakness in the waist and knees, dizziness, and tinnitus. There have been reports suggesting that SXBM can alleviate leukopenia resulting from CTX and is effective in managing patients with iron deficiency anemia. However, despite its known therapeutic effects, the precise mechanism of SXBM remains unclear, hampering its further development. Consequently, this paper represents the pioneering effort to investigate the mechanism by which SXBM treats BDS through a combination of network pharmacology and experimental validation. Via network pharmacological findings and the results of molecular docking simulations, demethylwedelolactone, 7-O-methylisomucronulatol, isoflavanone, resveratrol and morin are the main active components in SXBM, which have good docking effect on the Resveratrol. Demethylwedelolactone wedelolactone was further studied as a clinically useful Trypsin inhibitor with good liver protection and anti-inflammatory activity [14]. 7-O-methylisomucronol is an active ingredient in *Astragali Radix* that belongs to the flavonoid group, and its pharmacological effects mainly include the regulation of the immune system [15]. As well as demonstrating protective effects against cellular hypoxia, ischemia and radiation damage. Isoflavones are a subclass of flavonoids, known for their diverse biological properties. They possess antioxidant, anti-inflammatory, antimutagenic, antibacterial, and anti-tumor activities. Additionally, isoflavones are capable of regulating various cellular enzyme activities, making them valuable compounds in both traditional medicine and scientific research [16]. Resveratrol is a polyphenol hexene compound, which is widely found in natural plants. It has been found that resveratrol inhibits the production of inflammatory factors by activating SIRT1 [17]. It also exhibit anti-inflammatory effects in macrophages, while resveratrol modulates immune responses by disrupting immune cell regulation, inhibiting the synthesis of proinflammatory cytokines, and influencing gene expression [18]. Morin is a naturally occurring bioflavonoid present in various species of Moraceae [19]. Several studies have suggested that it can elevate antioxidant levels, suppress lipid peroxidation, and modulate apoptosis and autophagy processes by regulating the PI3K/AKT/mTOR signaling pathway.

The GO and KEGG enrichment analyses have unveiled that the HIF-1, JAK-STAT, and PI3K-AKT signaling pathways play pivotal roles in the treatment of BDS with the use of raw blood combination. Firstly, HIF-1 is a central transcription factor responsible for regulating intracellular oxygen homeostasis. It consists of two subunits, α and β. Elevated expression of HIF-1α can exacerbate kidney damage. The HIF-mediated hypoxia-induced pathway is an effective mechanism for modulating iron metabolism and addressing anemia. Within this pathway, the PHD enzyme serves as a key component Among these findings, it has been demonstrated that HIF has the capacity to directly regulate the transcriptional expression of the erythropoietin gene, thereby promoting erythropoiesis [20]. Importantly, the stability of HIF is contingent upon its hydroxylation status, which is influenced by oxygen sensitivity [21]. Indeed, HIF-1 is a pivotal pathway, and HIF-1α is expressed in various adult tissues

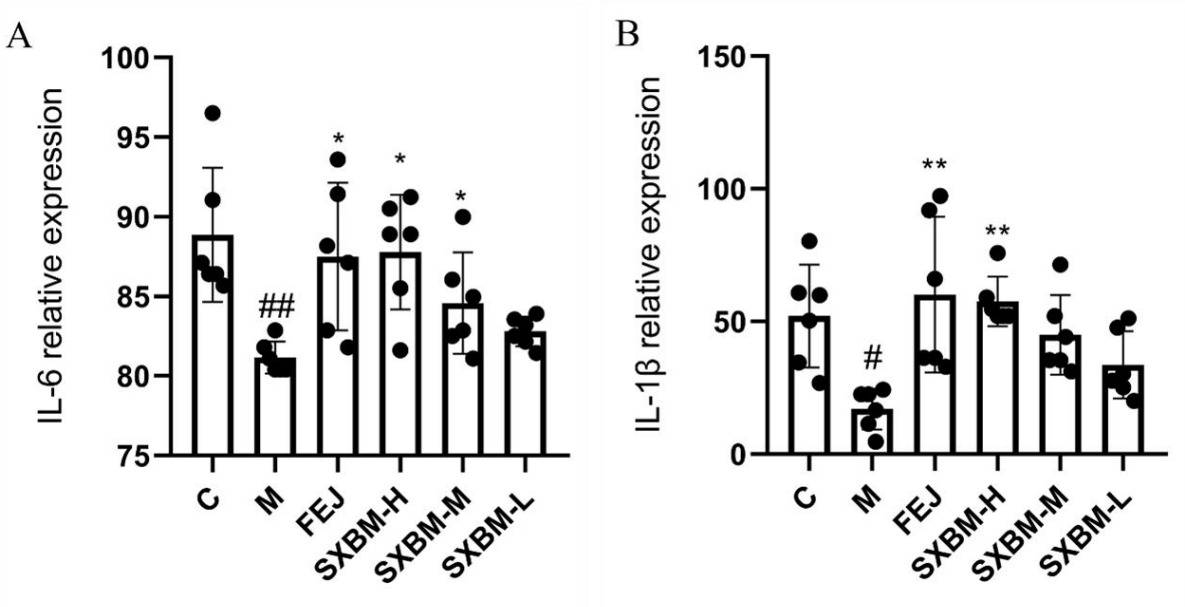

Figure 9 ELISA results of rat serum (mean ± SD, n = 6). (A) IL-6 expression. (B) IL-1β expression. $^{\#\#}P < 0.01$, $^{\#}P < 0.05$, vs. control group; $^{**}P < 0.01$, $^{*}P < 0.05$, vs. model group. C, control group; M, model group; SXBM, Shengxuebao mixture; FEJ, Fufang E'jiao Jiang (positive group); SXBM-H, SXBM high group; SXBM-M, SXBM middle group; SXBM-L, SXBM low group; IL, interleukin.

and organs, including the bone marrow, kidney, and brain. HIF plays a central role in promoting erythropoiesis and orchestrates hypoxia responses tailored to specific cell types. This encompasses various processes, such as erythropoietin synthesis in the kidney and liver, the maturation and proliferation of erythropoietic progenitor cells, and alterations in the hematopoietic microenvironment within the bone marrow [20].

There is a significant association between HIF-1α and inflammation. Research has demonstrated that inflammatory factors, including IL-1β, TNF-α, can stimulate the transcriptional activity of HIF-1α [21, 22]. This underscores the interplay between the HIF-1α pathway and the inflammatory response. HIF-1α indeed assumes a critical role in the synthesis of the positive regulator of bone marrow hematopoietic factor IL-1β, as established by research [23]. Additionally, nuclear factor-kappa B (NF-κB) is a transcription factor found in the cytoplasm of cells in the form of a p50/p65 heterodimer.

The overactivation of NF-κB is intricately linked to the development of various diseases, including tumors and T or B lymphocytic leukemia. Wang's research has revealed that Acidic Polysaccharide from angelica sinensis polysaccharide effectively inhibits the activation of NF-κB p65 via the IKKs-Iκ Bα pathway. This inhibition, in turn, reduces the secretion of IL-6 and TNF-α, both of which are known to impede RBC production [24].

Moreover, an increasing body of research indicates that there is a connection between HIF-1α and NF-κB, with HIF-1α capable of mediating the activation of NF-κB in hypoxic cells [25]. Rachida S has demonstrated that HIF-1α mRNA plays a pivotal role in regulating the hypoxia response following the activation of NF-κB under hypoxic conditions [26]. Additionally, NF-κB target genes are involved in host immune responses, including the regulation of chemokines and cytokines. These target genes can be stimulated and activated by various factors, including cytokines, growth factors, and bacterial agents [27].

The activation and transduction of HIF-1α/NF-κB signaling pathway can produce a variety of cytokines, including inflammatory factors, which play an important role in the occurrence and treatment of diseases. As a cytokine secreted by bone marrow stromal cells, IL-6 is a cytokine that regulates immune response, acute phase reaction and hematopoietic cells, and can induce the death of progenitor cells at very low concentration IL-1β, as a cofactor, can regulate hematopoietic cells and the proliferation and differentiation of hematopoietic stem cells, abnormal hematopoietic regulatory factors in vivo can directly affect the proliferation and differentiation of hematopoietic stem cells [1, 28]. In this research, BDS was induced using a combination of APH and CTX to ensure a more comprehensive representation of pathological changes in blood cells in the animal models. The study's findings suggest that the BDS induced through this combined approach of APH and CTX closely mimics the internal environment associated with blood deficiency, offering a more holistic representation of the condition Following the modeling of the rats, noticeable alterations in their appearance became evident [29]. These changes encompassed disheveled fur, weight loss, reduced appetite, and other related indicators. Furthermore, a significant decline in peripheral blood parameters was observed, mirroring the clinical manifestations commonly associated with blood deficiency [30]. In a confirmatory experiment utilizing evidence of blood deficiency, a rat model was successfully established. The experimental findings indicated several noteworthy outcomes. Firstly, BDS led to a declining trend in the spleen and thymus indices, signifying that both central and peripheral immune organs were affected. Moreover, since organ indices indirectly reflect the etiology of blood deficiency evidence, the histopathological morphology of the spleen and bone marrow was examined. In comparison to the normal control group, the BDS group exhibited spleen abnormalities characterized by hemorrhaging, blurred distinctions between red and white marrow regions, and extensive RBC exudation. Additionally, within the bone marrow cavity, hematopoietic cells such as erythrocytes and granulocytes significantly decreased, and there was marked proliferation of adipose tissue.

Some studies have shown that the organs affected by blood deficiency are mainly the immune (hematopoietic) organs such as bone marrow and spleen The administration of SXBM resulted in an improvement in the pathological condition of the spleen and bone marrow [31]. This suggests that SXBM has the potential to enhance extramedullary hematopoietic function to some degree. These observations indicate that both central and peripheral immune organs were influenced by the treatment. The findings of this study demonstrate that SXBM has the capacity to modulate the body's immune function and facilitate the restoration of hematopoietic function. Secondly, SXBM exhibited the ability to suppress the expression of NF-κ B and HIF-1α while reducing TNF-α levels, and it simultaneously increased the expression of IL-6 and IL-1β ($P < 0.05$). These findings provide confirmation that SXBM can enhance hematopoietic function by modulating the expression of hematopoietic factors and mitigating damage to hematopoietic organs.

In summary, the predicted results from network pharmacology have unveiled that SXBM's treatment of BDS is linked to its anti-inflammatory, antioxidant, and pro-immune effects, achieved through a holistic, multi-target, and multi-pathway approach. Molecular docking techniques have further elucidated the connections between key components and targets in SXBM. Notably, IL-6 has exhibited enhanced binding activities with morin and paeoniflorin. Therefore, it is hypothesized that the binding interactions between these major active components of SXBM and key targets may hold the key to its effectiveness in treating BDS. SXBM exerts its hematopoietic effects by improving histopathological morphology and enhancing the body's hematopoietic function. However, the precise mechanism through which SXBM exerts its hematopoietic effects warrants further validation. Future research endeavors will involve characterizing the chemical composition of SXBM, utilizing techniques such as HPLC and LC-MS, to explore the specific pharmacodynamic constituents of SXBM. Additionally, in vitro validation and methods such as metabolomics will be employed to delve deeper into the potential hematopoietic mechanism of SXBM.

Conclusion

In this study, a comprehensive and holistic examination of SXBM's therapeutic effects against BDS was conducted. The research entailed an analysis of SXBM's mechanism for treating BDS through a network pharmacology approach. Additionally, the key targets identified were validated using molecular docking techniques and in vivo experiments. This multifaceted approach provided a thorough understanding of how SXBM operates in the treatment of BDS. Firstly, it was predicted that SXBM mainly acts on key targets such as IL-6 and IL-1β via active ingredients such as resveratrol, morin, soflavone which regulate the effects of oxidative stress, inflammatory response, apoptosis and immune response, and then modulate HIF-1 pathway. The second experiment effectively confirmed the therapeutic impact of SXBM on BDS. This confirmation was primarily reflected in the restoration of peripheral blood parameters and the improvement of the pathological condition of the spleen, thymus, and bone marrow. In vivo findings indicated that SXBM may inhibit the expression of HIF-1α and NF-κ B in the HIF-1α/NF-κ B pathway. Simultaneously, it promoted the secretion of IL-6 and IL-1β, thereby enhancing the restoration of immune homeostasis and hematopoietic function. Consequently, SXBM exhibited a positive therapeutic effect on BDS.

The results of this study offer an experimental foundation for understanding the mechanism of action of SXBM in the treatment of BDS. They also serve as a valuable reference for comprehending the drug's mechanism and its potential for further development. Future investigations will delve into the pathway mechanisms at molecular, cellular, and systemic levels, leveraging a combination of omics technologies such as metabolomics, transcriptomics, and others.

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