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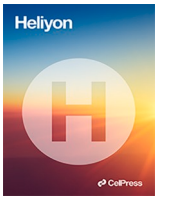

Research article

Mass spectrum oriented metabolomics for evaluating the efficacy and discovering the mechanism of Shaofuzhuyu Decoction for endometriosis of cold coagulation and blood stasis

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ABSTRACT

Shaofuzhuyu Decoction (SFZYD) is a classical formula for treating endometriosis of cold coagulation and blood stasis (ECB). The clinical efficacy is definite, but the potential mechanisms require further exploration. The study aimed to reveal the metabolic mechanisms of SFZYD for treating ECB using mass spectrum oriented metabolomics. Firstly, the study has used metabolomics data to identify biomarkers and to investigate metabolic pathways. Then, the targets of SFZYD for treating ECB were dug by building and analyzing a biological network of biomarkers. Finally, the obtained targets were validated by molecular docking. This study found that SFZYD could significantly improve the biochemical indicators and metabolic abnormalities of ECB. A total of 18 ECB-related biomarkers in 7 pathways were identified. SFZYD was able to regulate the levels of 14 biomarkers that were involved in 5 metabolic pathways. Furthermore, the study yielded 119 SFZYD active ingredients, 1119 target proteins associated with endometriosis, 610 targets associated with biomarkers, 727 GO functions, and 159 KEGG pathways. Biological network analysis constructed a network diagram of herbs-ingredients-targets-biomarkers, and found 6 key active ingredients and 9 core targets. Molecular docking showed high affinities between key ingredients and core targets. This study elucidated that SFZYD plays a role in treating ECB through multi-component, multi-target, and multi-pathway.

1. Introduction

Endometriosis is a gynecological disease in which the endometrial cells grow ectopically in other tissues, manifesting clinically as irregular menstruation, dysmenorrhea, infertility, etc [1]. It is considered an estrogen-dependent, inflammatory disease that affects 5–10 % of reproductive-age women worldwide [2]. Available drug treatments mostly involve inhibiting ovulation and reducing circulating estrogen levels at the cost of affecting the patient's normal menstrual cycle, which fails to meet the patient's fertility needs and limits their long-term use [3]. Currently, surgical treatment is also an important means of treating endometriosis, but it is an invasive procedure and has drawbacks such as leaving sequelae easily and high postoperative recurrence rate [4]. Therefore, looking

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for a safe and effective drug is of great clinical significance.

Guided by the holistic view and treatment with syndrome differentiation, TCM can effectively improve clinical symptoms, with the advantages of good efficacy, minimal adverse reactions, no impact on menstrual physiology, and stable long-term efficacy, thereby improving the quality of life for patients and playing an important role in the clinical treatment of endometriosis [5]. TCM holds that the fundamental pathogenesis of endometriosis lies in cold coagulation and blood stasis [6].

SFZYD was first recorded in "Yilin Gaicuo" by Wang Qingren during the Qing Dynasty, which has the effects of promoting blood circulation, removing blood stasis, warming channels, and relieving pain [7,8]. It is composed of 10 drugs. Among them, Angelicae sinensis radix, Paeoniae radix rubra, and Chuanxiong rhizome can regulate Qi and menstruation, promote blood circulation, and relieve blood stasis. Trogopterori Faeces, Typhae pollen, Corydalis rhizoma, and Myrrha dissolve blood stasis and relieve pain. Foeniculi fructus, Zingiberis rhizoma, and Cinnamomi cortex warm the channels, disperse cold, and help guide the other drugs to the lesions. Pharmacological studies showed that Angelicae sinensis radix can reduce pain response by inhibiting uterine contraction in dysmenorrhea mice. At the same time, it improved blood circulation by increasing the level of NO in uterine tissue, reducing the level of Ca^{2+} [9]. Chuanxiong rhizoma can inhibit platelet activation and prevent thrombosis by preventing Ca^{2+} influx, and at the same time, it can relieve endometrial pain by reducing blood viscosity [10]. Paeoniae radix rubra can increase the level of NO and decrease the level of ET in plasma, thus improving the indexes of hemorheology and promoting blood [11]. Trogopterori Faeces can relieve uterine spasm, regulate blood coagulation function, resist inflammation and scavenge free radicals. Typhae pollen can inhibit platelet adhesion, thrombosis and improve hemorheological parameters [12]. SFZYD is a classic formula for treating ECB, with proven clinical efficacy [7,13,14]. It treats ECB by anti-inflammatory, anticoagulant, spasmolytic and analgesic, and regulating reproductive hormones. However, there is still a need for scientific research strategies and methods to explore the mechanisms of SFZYD in the treatment of ECB.

Metabolic factors are increasingly considered to be closely related to the occurrence of endometriosis, and there is convincing evidence that severe metabolic dysfunction will affect the progress of endometriosis [15]. Metabolomics analysis can comprehensively demonstrate the characteristics of TCM in exerting therapeutic effects through multiple pathways and targets [16]. With its unique advantages of holistic and dynamic expression, metabolomics coincides with the TCM's holistic view and treatment with syndrome differentiation. In recent years, metabolomics technology has become a powerful platform for the comprehensive understanding of TCM theories and has been widely used for the discovery of biomarkers, metabolic mechanisms, and potential target studies of TCM. Mass spectrometry (MS) is the main analytical platform in metabolomics. MS oriented metabolomics has a wide range of metabolite coverage due to its high sensitivity. What's more, MS oriented metabolomics interprets the mechanism of TCM in modern scientific language and builds a bridge from traditional to modern for TCM research [17–19].

Therefore, this study aimed to determine the therapeutic effect of SFZYD on ECB model rats and to reveal the metabolic mechanism of SFZYD treatment of ECB by MS oriented metabolomics(Fig. 1).

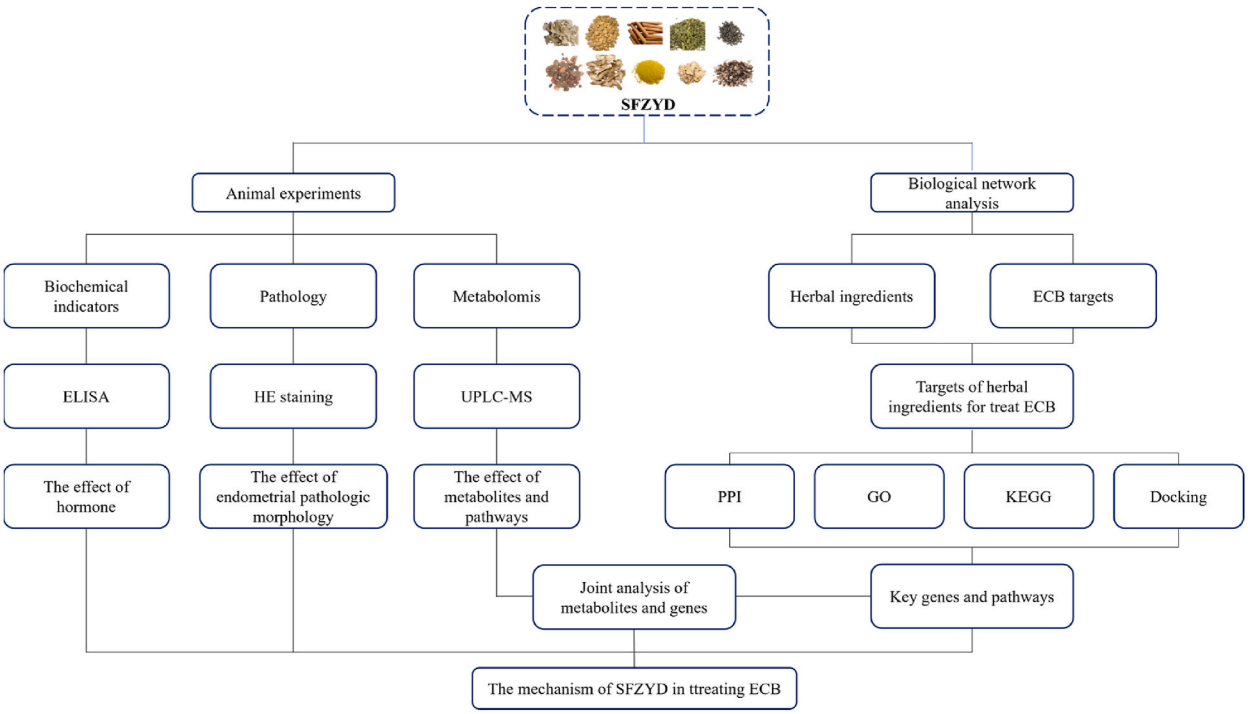

Fig. 1. Workflow for studies the mechanisms of SFZYD for treating ECB.

2. Methods and materials

2.1. Chemicals and reagents

The ten drugs forming SFZYD were obtained from Harbin Pharmaceutical Group Shiyitang Co., Ltd. (Harbin, China) and were identified by the Teaching and Research Department of Chinese Medicine Identification of Heilongjiang University of Chinese Medicine. The specimens of these ten drugs were stored in National Key Laboratory of Innovation in Integration of Traditional Chinese Medicine and Western Medicine, labeled SFZYD-20190514-001, SFZYD-20190514-002, SFZYD-20190514-003, SFZYD-20190514-004, SFZYD-20190514-005, SFZYD-20190514-006, SFZYD-20190514-007, SFZYD-20190514-008, SFZYD-20190514-009, SFZYD-20190514-010, respectively. ELISA Kits (E2, LH, CA125, IL-6) were obtained from R&D Systems (Minneapolis, MN, USA, batch number: E20190501A). HPLC grade acetonitrile and methanol were purchased from Fisher Scientific Corporation (Waltham, USA, batch number: 196101 and F19M67201), while formic acid and leucine enkephalin were purchased from Sigma–Aldrich (St Louis, MO, USA, batch number: 192271). Pure distilled water used for LC-MS analysis was purchased from Watsons Water (Guangzhou, China, batch number: GB19298).

2.2. Drug decoction methods

According to Danggui, Chishao, Chuanxiong, Wulingzhi, Puhuang, Yanhusuo, Moyao, Xiaohuixiang, Ganjiang, and Rougui in a proportion of 3: 2: 2: 2: 3: 1: 2: 1.5: 3: 3 (Table 1), 984g of drugs for SFZYD were weighed and dissolved in 10-fold water to extract 2 times, for 40 min each time. The decoctions were combined, concentrated, and freeze-dried, to obtain SFZYD freeze-dried powder with a yield of 30.92 %.

To ensure the quality of SFZYD extracts, the fingerprint profiles of SFZYD were established by HPLC, and the contents of gallic acid, oxidized oxypaeoniflora, paeoniflorin, isorhamnetin-3-*O*-neohesperidoside, ferulic acid, typhaneoside, senkyunolide I, and naringenin were determined simultaneously [20].

2.3. Animal administration

SPF grade Wistar rats (female, 200–220 g) (Permit No. SCXK2014-004) were adapted to a constant and humid environment for a week before experimental processing, as supported by the Drug Safety Evaluation Center of Heilongjiang University of Chinese Medicine (Harbin, China). Based on the previous research, a rat model of ECB was established by combining autologous transplantation and ice-water immersion [21] (Fig. 2). The model rats were randomly divided into 5 groups: the endometriosis of cold coagulation and blood stasis (Mod) group, the Danazol (Dan) group (72 mg/kg), the low-dose SFZYD (SFZYD-L) group (0.707 g/kg), the medium-dose SFZYD (SFZYD-M) group (1.413 g/kg), the high-dose SFZYD (SFZYD-H) group (2.826 g/kg). In addition, a sham operation (Con) group was set as the control group. The “low dose” of SFZYD was half the clinical equivalent dose, the “medium dose” of SFZYD was the clinical equivalent dose, and the “high dose” of SFZYD was double the clinical equivalent dose. The administered dose was determined by the weight of the rats (1 mL/100 g), and continuous intragastric administration was performed for 4 weeks. The experiment was authorized by the Ethics Committee of Heilongjiang University of Chinese Medicine under the guidance of the Helsinki Declaration (Approval No.2019042501).

2.4. Evaluation of ECB model and SFZYD efficacy

2.4.1. Detection of estradiol (E2), luteinizing hormone (LH), cancer antigen 125 (CA125) and interleukin-6 (IL-6)

One hour after the last administration, rats were anesthetized with 2 % pentobarbital sodium (0.2 mL/100 g). Blood was collected from the portal vein, and the serum was separated by centrifugation at 3000 rpm for 10 min at 4 °C. The levels of E2, LH, CA125, and IL-6 were measured according to the instructions of the ELISA kit.

Table 1
Drugs in the Shaofuzhuyu Decoction (SFZYD).

English names	Chinese name	Material	Weight %
Angelicae sinensis radix	Danggui	Radix	13.3 %
Paeoniae radix rubra	Chishao	Radix rubra	8.9 %
Chuanxiong rhizoma	Chuanxiong	Rhizoma	8.9 %
Trogopterori Faeces	Wulingzhi	Faeces	8.9 %
Typhae pollen	Puhuang	Pollen	13.3 %
Corydalis rhizoma	Yanhusuo	Rhizoma	4.5 %
Myrrha	Moyao	Resin	8.9 %
Foeniculi fructus	Xiaohuixiang	Fructus	6.7 %
Zingiberis rhizoma	Ganjiang	Rhizoma	13.3 %
Cinnamomi cortex	Rougui	Cortex	13.3 %

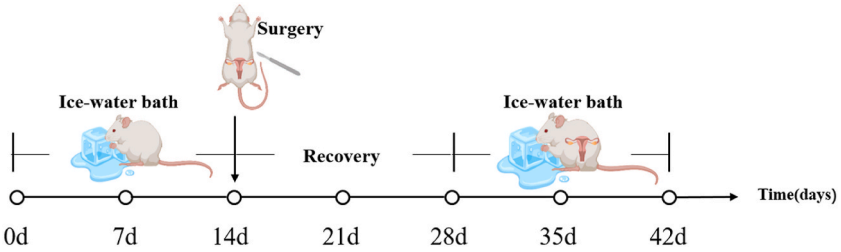

Fig. 2. The process of model establishment. Ice-water immersion (14d) - endometrial autograft surgery - recovery period (14d) - ice-water immersion (14d).

2.4.2. Hematoxylin and eosin (HE) staining

The rats were sacrificed, The ectopic endometrium was fixed in 10 % volume of 4 % formalin. Then, the tissue was stained with hematoxylin and eosin and observed with a light microscope (× 100).

2.5. Metabolomics analysis

2.5.1. Sample collection and preparation

At time points of 0 d, 7 d, 14 d, 21 d, 28 d, 35 d, 42 d, and 70 d, urine samples of rats were collected. The collected urine samples were stored at −80 °C. For urine sample analysis, the urine samples were first thawed, diluted 2-fold with distilled water, and centrifuged at 13000 rpm for 10 min at 4 °C. The supernatant was then taken, passed through a 0.22 μm filter membrane, and placed in a sample bottle for UPLC-MS analysis.

The quality control (QC) sample was used to assess the stability of the UPLC-Q-TOF-MS system. The QC sample was prepared by pooling 10 μL of every sample.

2.5.2. Chromatography and mass spectrometry conditions

A Waters Acquity UPLC System combined with a Waters LCT Premier XE TOF-MS controlled by Masslynx 4.1 with an ACQUITY UPLC™ Phenomenex Column (2.1 mm × 50 mm, 1.7 μm, Waters, USA) was used to acquire data at 40 °C. The mobile phase was optimized, in which 0.1 % formic acid in acetonitrile and 0.1 % formic acid in water were selected as mobile phases A and B, respectively. The mobile phases were used at a flow rate of 0.4 mL/min with a gradient of 0–2 min at 2–12 % A, 2–7 min at 12–24 % A, 7–11 min at 24–100 % A, 11–14 min at 100-100 % A. Electrospray ionization (ESI) source was used to collect data in positive ion mode and negative ion mode. The capillary voltage was 1.0 kV, the cone voltage was 40 V, and the extraction voltage was 4.0 V in positive ion mode. At the same time, the capillary voltage was 1.5 kV, the cone voltage was 50 V, and the extraction voltage was 3.0 V in the negative ion mode. The temperature of ion source was 110 °C. The desolvation temperature was 350 °C and the flow rate was 750 L/h. The mass scan range was *m/z* 100–1500 Da. The low energy was set as 10–30 eV, and the high energywas set as 30–50 eV for MS/MS fragment acquisition in centroid mode.

2.5.3. Multivariate data processing

The raw data obtained from UPLC-Q-TOF-MS was processed using Progenesis QI software (version 2.0, Waters Corp., Milford, MA, USA), which included the alignment of peaks, peak integration, normalization, and peak identification. The process described above was used to get a data matrix. The normalized urine sample data matrix was imported into SIMCA-P (version 14.1, Umetrics, Umea, Sweden) for partial least squares-discrimination analysis (PLS-DA) and orthogonal partial least squares-discriminant analysis (OPLS-DA). Then substances with variable importance in projection (VIP) > 1.0 and $P < 0.05$ were selected as potential biomarkers. The pathways related to ECB Pathway analysis were performed with the MetPA database (https://www.metaboanalyst.ca/).

2.5.4. Building and analyzing a biological network of crucial biomarkers

The active ingredients of the drugs in SFZYD were retrieved from the TCMSP database (https://tcmsp-e.com/) and manually supplemented according to the literature. Then, the target genes corresponding to the active ingredients of the drugs were retrieved using TCMSP. Targets related to "endometriosis" were retrieved from the GeneCards database (https://www.genecards.org). The biomarkers related targets were predicted in SwissTargetPrediction (http://www.swisstargetprediction.ch/). Intersecting targets were obtained by the Venn diagram tool (http://bioinformatics.psb.ugent.be/webtools/Venn/) and PPI analysis was performed using the STRING database (https://string-db.org/). The interacting proteins (confidence value > 0.7) were visualized for mapping by applying Cytoscape software (version 3.8.2, Cytoscape Consortium, USA). Gene Ontology (GO) and KEGG biofunctional enrichment analysis were performed on the obtained targets using the DAVID (https://david.ncifcrf.gov/tools.jsp). The Degree value of each node was calculated and the top 9 targets were filtered as core targets. Cytoscape software was used to construct an "active ingredient-target-biomarker" network to screen out the key active ingredients in each drug. The core targets were docked with the key active ingredients. The 3D structures of the core targets were downloaded from the PDB (https:/www.rcsh.org/). The 3D structures of the key active ingredients (.sdf format) were searched and downloaded from the PubChem database. Both were converted to.pdb format, imported into AutoDock Vina for docking, and visualized using PyMOL software (version 1.7.6, Molecular Graphics System,

Schrödinger).

2.6. Statistical analysis

The study data were expressed as the mean and standard deviation (mean ± standard deviation). The statistical analysis was performed by unpaired *t*-test using Excel (version 2021, Microsoft Corporation, USA), Differences at $p < 0.01$ were considered highly significant, and differences at $p < 0.05$ were considered significant. Graphs were drawn using generated by Graphpad Prism (version 9.0, Graphpad Software, USA).

3. Results

3.1. Therapeutic effects of SFZYD on ECB

3.1.1. Results of E2, LH, CA125 and IL-6

Compared with the Con group, the serum E2, LH, CA125, and IL-6 levels in the Mod group were extremely significantly increased ($p < 0.01$). Compared with the Mod group, the serum E2 level in the Dan, SFZYD-L, SFZYD-M, and SFZYD-H group was extremely significantly decreased ($p < 0.01$) (Fig. 3A). Compared with the Mod group, the serum LH level in the Dan, SFZYD-L, SFZYD-M, and

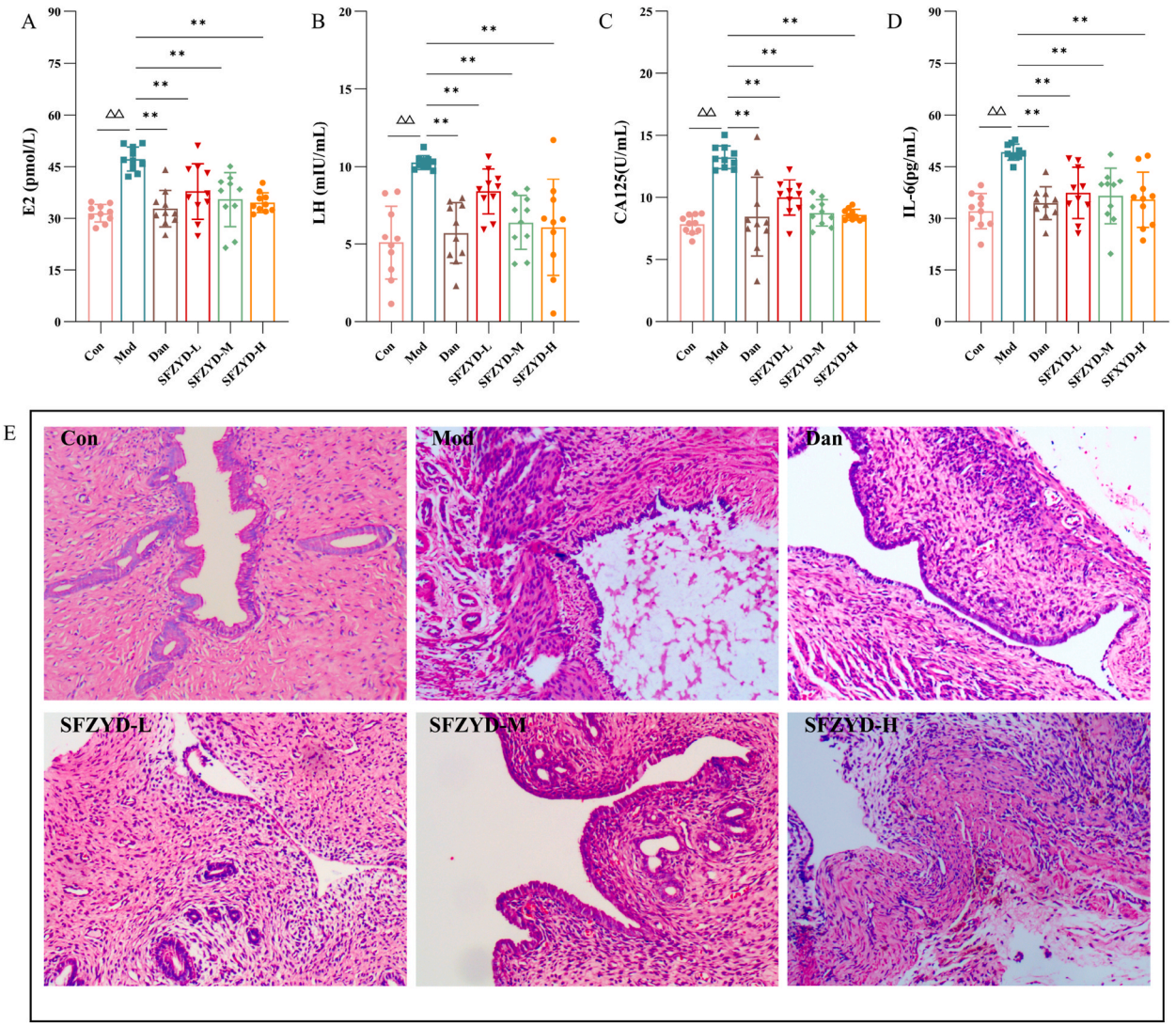

Fig. 3. Results of therapeutic effects for each group. The results showed the levels of E2 (A), LH (B), CA125 (C), and IL-6 (D) in response to treatment with Dan, and SFZYD groups (n = 10). Results of HE staining in response to treatment with Dan, and SFZYD groups (E). Compared to the Con group, $^{\triangle\triangle}p < 0.01$; compared to the Mod group, $^{*}p < 0.05$ and $^{**}p < 0.01$.

SFZYD-H group was extremely significantly decreased ($p < 0.01$) (Fig. 3B). Compared with the Mod group, the serum CA125 level in the Dan, SFZYD-L, SFZYD-M, and SFZYD-H group was extremely significantly decreased ($p < 0.01$) (Fig. 3C). Compared with the Mod group, the serum IL-6 levels in the Dan, SFZYD-L, SFZYD-M, and SFZYD-H groups were all extremely significantly decreased ($p < 0.01$). The results showed that SFZYD had a significant therapeutic effect on ECB in a dosage-dependent manner.

3.1.2. Results of HE staining

The endometrium of Con group rats was structurally intact. The glandular epithelial cells were high columnar, neatly and tightly arranged. The cytoplasm was abundant. The mesenchymal cells were spindle-shaped, well arranged and evenly distributed. The epithelial cells of the ectopic endothelium of Mod group rats were proliferated in a short columnar shape, and some of them had a pseudo-complex structure with incomplete cell morphology. The number of mesenchymal cells and glands was significantly increased. And blood vessels were abundant. Compared with the model group, there were different degrees of improvement in the above pathology in each drug administration group, such as the ectopic endothelial tissue had different degrees of atrophy, the cystic lumen was reduced, the glandular epithelial cells were short columnar or flat, some of them degenerated, necrotic or detached, loosely arranged, with irregular nuclei in irregular positions. The glands were reduced in number and atrophic, with some areas containing ferredoxin. The mesenchymal cells are reduced in number, small and sparse, with reduced vascularity, etc (Fig. 3E). The results showed that SFZYD could inhibit the proliferation of ectopic endometrial tissue, so that the lesion could be controlled and treated.

SFZYD-M is the clinical dosage and its therapeutic effects have been proven to be significant. SFZYD-M was carried out further metabolomics research.

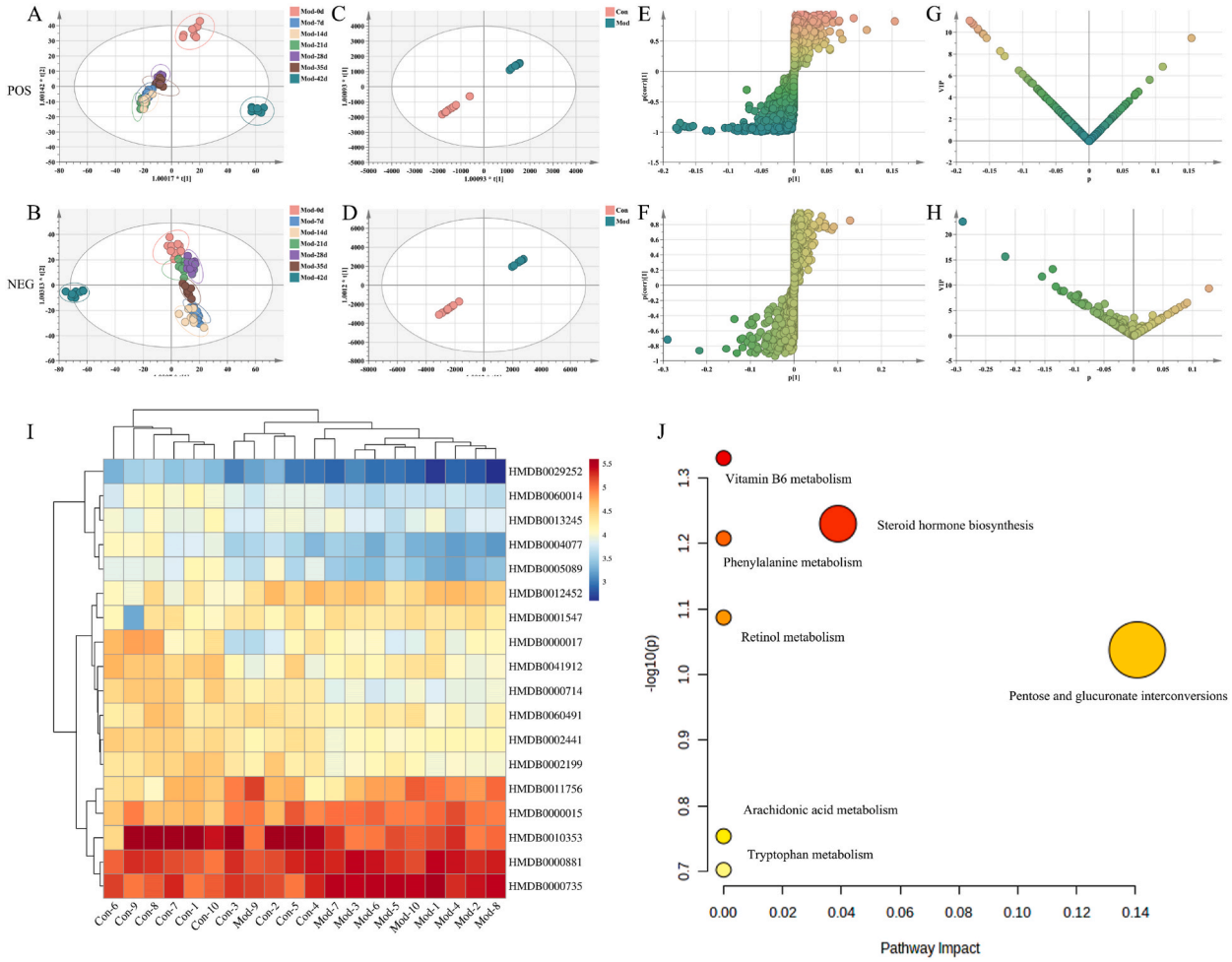

Fig. 4. Urine metabolic profiles and multivariate statistical analysis for finding biomarkers. Metabolic profile during ECB modeling in positive ion mode (A). Metabolic profile during ECB modeling in negative ion mode (B). OPLS-DA plot between Con group and Mod group in positive ion mode (C). OPLS-DA plot between Con group and Mod group in negative ion mode (D). S-plot between Con group and Mod group in positive ion mode (E). S-plot between Con group and Mod group in negative ion mode (F). VIP between Con group and Mod group in positive ion mode (G). VIP between Con group and Mod group in negative ion mode (H). Heatmap of changes in levels of 18 potential biomarkers in Con and Mod groups (I). Metabolism pathway analysis of 7 potential biomarkers with MetPA (J).

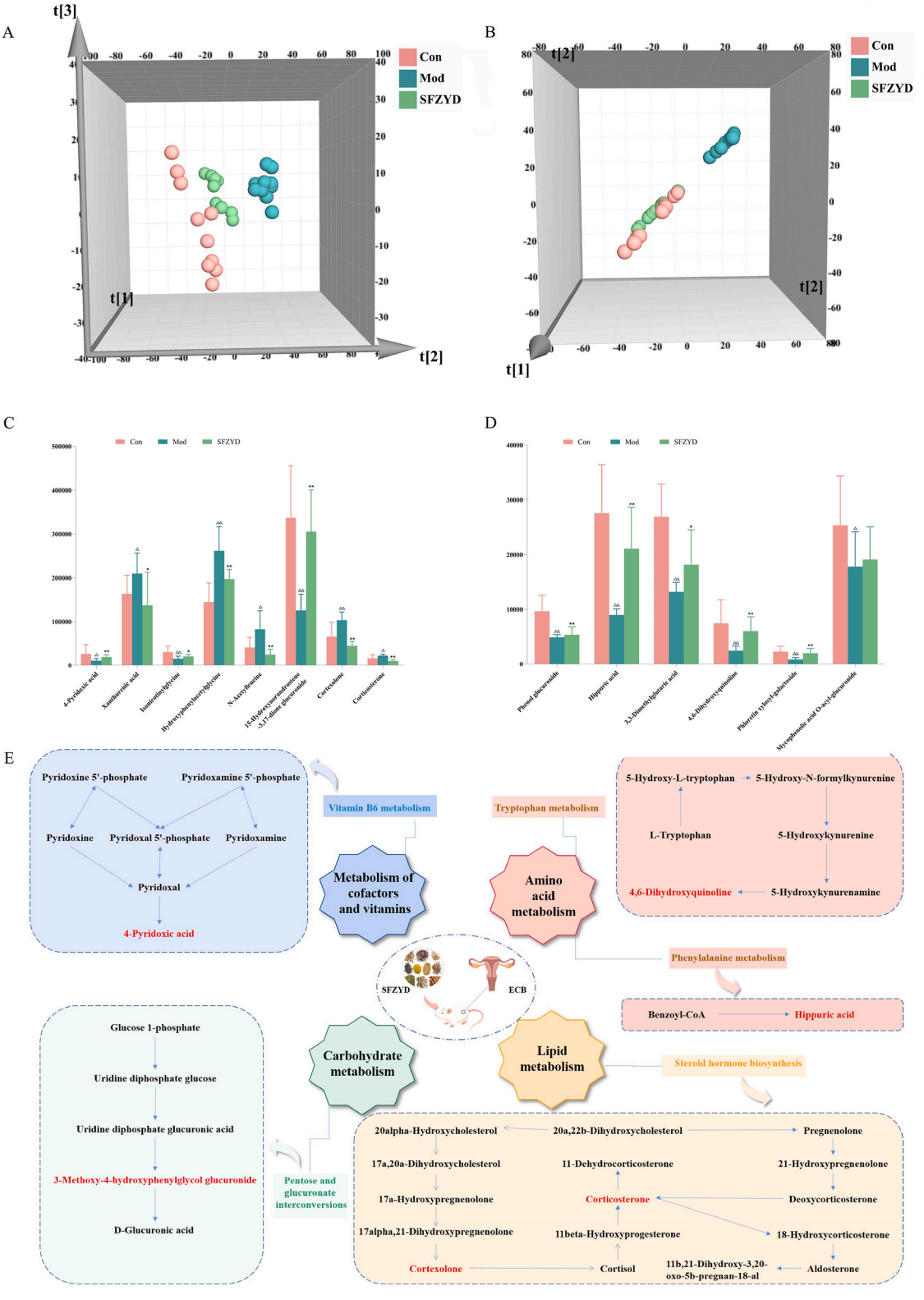

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Fig. 5. Effects of SFZYD on urine biomarkers. PLS-DA analysis of the Con, Mod, and SFZYD groups in positive ion mode (A). PLS-DA analysis of the Con, Mod, and SFZYD groups in negative ion mode (B). The SFZYD group regulated biomarkers related to the ECB model in ion mode (C). The SFZYD group regulated biomarkers related to the ECB model (D). Association networks of biomarkers in response to the therapeutic effects of SFZYD on ECB. Borderless rectangle: pathway, dashed rectangle: metabolite, solid frame polygon: type of metabolic pathway, red font: biomarkers of SFZYD callbacks (E).

3.2. Metabolomics analysis

3.2.1. Analysis of urine metabolic profiles during the ECB model preparation

Information on urine ESI-MS metabolic profile data of rats at time points (0 d, 7 d, 14 d, 21 d, 28 d, 35 d, 42 d) during the ECB model preparation was analyzed. Starting from the 7th day of model preparation, there was some grouping between the Mod group and the Con group rats, but there was still some overlap. On 42 d of model preparation, there was a significant difference between the Mod group and the Con group. From the metabolic perspective, the results elucidated the transformation of normal rats into model rats induced by modeling factors (Fig. 4A and B) (Supplementary Fig. S1).

3.2.2. Found and identified potential biomarkers in the urine of ECB model rats

Based on the analysis of biochemical indicators and metabolic profiling, the 42 d after model preparation was determined to be the successful modeling time. Using the analysis methods mentioned earlier, OPLS-DA analysis (ESI^+: R^2Y-0.970, Q^2-0.958; ESI^-: R^2Y-0.997, Q^2-0.938 (Supplementary Fig. S2) was performed on the urine ESI-MS metabolic profile data of Con group and Mod group on the 42 d, which obtained the Score plot (Fig. 4C and D). Further analysis obtained the S-plot (Fig. 4E and F) and VIP plot (Fig. 4G and H) that could intuitively reflect the contribution rate of inter-group differences. A total of 18 potential biomarkers related to the ECB rat model were identified. In positive ion mode, 9 substances were identified, including 4-Pyridoxic acid (HMDB0000017), Xanthurenic acid (HMDB0000881), Isonicotinylglycine (HMDB0041912), Hydroxyphenylacetylglycine (HMDB0000735), N-Acetylleucine (HMDB0011756), 15-Hydroxynorandrostene-3,17-dione glucuronide (HMDB0010353), Cortexolone (HMDB0000015), all-trans-18-Hydroxyretinoic acid (HMDB0012452), Corticosterone (HMDB0001547). In negative ion mode, 9 substances were identified, including Phenol glucuronide (HMDB0060014), Hippuric acid (HMDB0000714), 3,3-Dimethylglutaric acid (HMDB0002441), *m*-Methylhippuric acid (HMDB0013245), 4,6-Dihydroxyquinoline (HMDB0004077), Phloretin xylosyl-galactoside (HMDB0029252), Mycophenolic acid-*O*-acyl-glucuronide (HMDB0060491), (12S)-Leukotriene B4 (HMDB0005089), Desaminotyrosine (HMDB0002199) (Fig. 3I) (Supplementary Table S1). The results indicated that the occurrence and development of ECB could lead to significant changes in the above metabolites in the body.

3.2.3. Analysis of ECB-related metabolic pathways

7 metabolic pathways related to the ECB model were found by the 18 biomarkers of ECB model, including Vitamin B6 metabolism, Steroid hormone biosynthesis, Phenylalanine metabolism, Retinol metabolism, Pentose and glucuronate interconversions, Arachidonic acid metabolism, and Tryptophan metabolism. The results indicated that these endogenous substances underwent significant perturbations that affected the metabolic pathway when the body underwent pathological changes (Fig. 4J) (Supplementary Table S2).

Table 2
Trend of ECM urine potential biomarkers called back after oral administration of Shaofu Zhuyu Decoction.

No.	Rt min	Adducts	Proposed Composition	Postulated Identity	Trend (Model/Control)	SFZYD
1	1.66	M + H	$C_8H_9NO_4$	4-Pyridoxic acid	$\downarrow^{\triangle\triangle}$	✓**
2	2.38	M-H	$C_{12}H_{14}O_7$	Phenol glucuronide	$\uparrow^{\triangle\triangle}$	✓**
3	2.69	M-H	$C_9H_9NO_3$	Hippuric acid	$\downarrow^{\triangle}$	✓
4	2.74	M-H	$C_7H_{12}O_4$	3,3-Dimethylglutaric acid	$\downarrow^{\triangle\triangle}$	✓*
5	2.75	M + H	$C_{10}H_7NO_4$	Xanthurenic acid	$\downarrow^{\triangle\triangle}$	✓*
6	2.77	M + H	$C_8H_8N_2O_3$	Isonicotinylglycine	$\downarrow^{\triangle\triangle}$	✓*
7	3.16	M-H	$C_{10}H_{11}NO_3$	*m*-Methylhippuric acid	↓	–
8	3.38	M-H	$C_9H_7NO_2$	4,6-Dihydroxyquinoline	$\uparrow^{\triangle\triangle}$	✓**
9	3.63	M-H	$C_{26}H_{32}O_{14}$	Phloretin xylosyl-galactoside	$\uparrow^{\triangle\triangle}$	✓**
10	3.95	M-H	$C_9H_{10}O_3$	Desaminotyrosine	$\uparrow^{\triangle\triangle}$	–
11	4.08	M + H	$C_{10}H_{11}NO_4$	Hydroxyphenylacetylglycine	↓	✓**
12	4.80	M + H	$C_8H_{15}NO_3$	N-Acetylleucine	$\downarrow^{\triangle\triangle}$	✓**
13	6.39	M + H	$C_{24}H_{32}O_9$	15-Hydroxynorandrostene-3,17-dione glucuronide	$\downarrow^{\triangle\triangle}$	✓**
14	7.16	M-H	$C_{23}H_{28}O_{12}$	Mycophenolic acid O-acyl-glucuronide	↑	✓
15	9.03	M-H	$C_{20}H_{32}O_4$	12(S)-Leukotriene B4	$\downarrow^{\triangle}$	–
16	9.13	M + H	$C_{21}H_{30}O_4$	Cortexolone	↑	✓**
17	9.35	M + H	$C_{20}H_{28}O_3$	all-trans-18-Hydroxyretinoic acid	$\downarrow^{\triangle}$	–
18	9.87	M + H	$C_{21}H_{30}O_4$	Corticosterone	↑	✓**

Note: ↑ ↓ indicates that the level of biomarkers in the urine of rats in the model group increased or decreased.
✓&- indicates that the drug has or does not have a callback effect on the marker; compared with the blank control group, $^{\triangle}P < 0.05$, $^{\triangle\triangle}P < 0.01$; compared with the model group, $^{*}P < 0.05$, $^{**}P < 0.01$.

3.2.4. Effects of SFZYD on urine biomarkers

Metabolic analysis was performed in the 4th week of treatment. The PLS-DA (ESI^+: R^2Y-0.954, Q^2-0.880; ESI^-: R^2Y-0.885, Q^2-0.760 (Supplementary Fig. S3)) showed significant metabolic differences among the three experimental groups. From the position on the loading plot, the SFZYD group was more similar to the Con group. The results indicated that SFZYD had a significantly regulative effect on the abnormal urine metabolic profile of rats with ECB (Fig. 5A and B).

The SFZYD group could regulate 14 of the 18 biomarkers related to the ECB model, and had a significant effect on 13 of them (Fig. 5C and D). The 13 biomarkers were 4-Pyridoxic acid, Phenol glucuronide, Hippuric acid, 3,3-Dimethylglutaric acid, Xanthurenic acid, Isonicotinylglycine, 4,6-Dihydroxyquinoline, Phloretin xylosyl-galactoside, Hydroxyphenylacetylglycine, N-Acetylleucine, 15-Hydroxynorandrostene-3,17-dione glucuronide, Cortexolone, and Corticosterone(Table 2). 14 biomarkers were involved in a total of 5 metabolic pathways. 5 metabolic pathways were Vitamin B6 metabolism, Tryptophan metabolism, Phenylalanine metabolism,

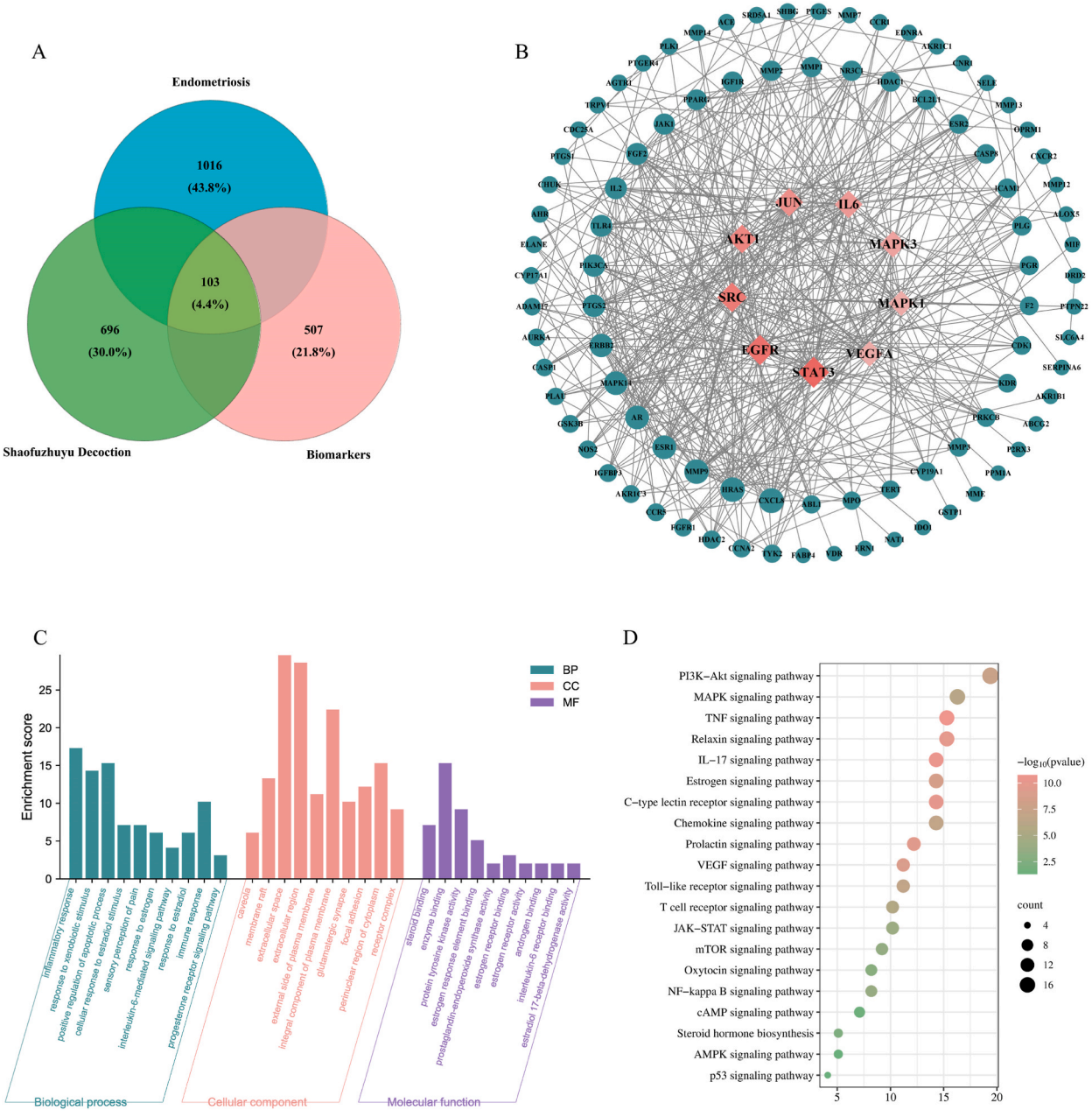

Fig. 6. Biological network analysis of biomarkers. Venn analysis revealed 103 intersecting targets for SFZYD's active ingredient, back-regulated biomarkers, and ECB (A). PPI network of protein targets (confidence >0.7) and the top 9 target genes in the innermost circle are considered core targets (B). GO enrichment analysis (C). KEGG enrichment analysis (D).

Pentose and Glucuronate interconversions, and Steroid hormone biosynthesis (Fig. 5E). The results showed that SFZYD treated ECB by regulating the above metabolic pathways.

3.2.5. *Building and analyzing a biological network of biomarkers*

A total of 119 active ingredients of SFZYD were retrieved from the TCMSP database and literature, and a total of 799 active ingredient-related targets were obtained. By searching the GeneCard database, 1119 target proteins related to endometriosis with a relevance score >1 were identified. SwissTargetPrediction predicted 610 biomarker-related targets. 103 targets were intersected (Fig. 6A). The PPI network constructed by STRING online database included 98 nodes and 484 edges. And the core targets were STAT3, EGFR, SRC, AKT1, JUN, MAPK3, IL6, MAPK1, VEGFA (Fig. 6B). GO enrichment yielded a total of 727 entries, biological process (BP) involved inflammatory response, response to xenobiotic stimulus, cellular response to estradiol stimulus, sensory perception of pain, etc.; cellular component (CC) involved membrane raft, extracellular space, extracellular region, cytosol, etc, molecular function (MF) involved steroid binding, enzyme binding, protein tyrosine kinase activity, estrogen response element binding, etc (Fig. 6C). A total of

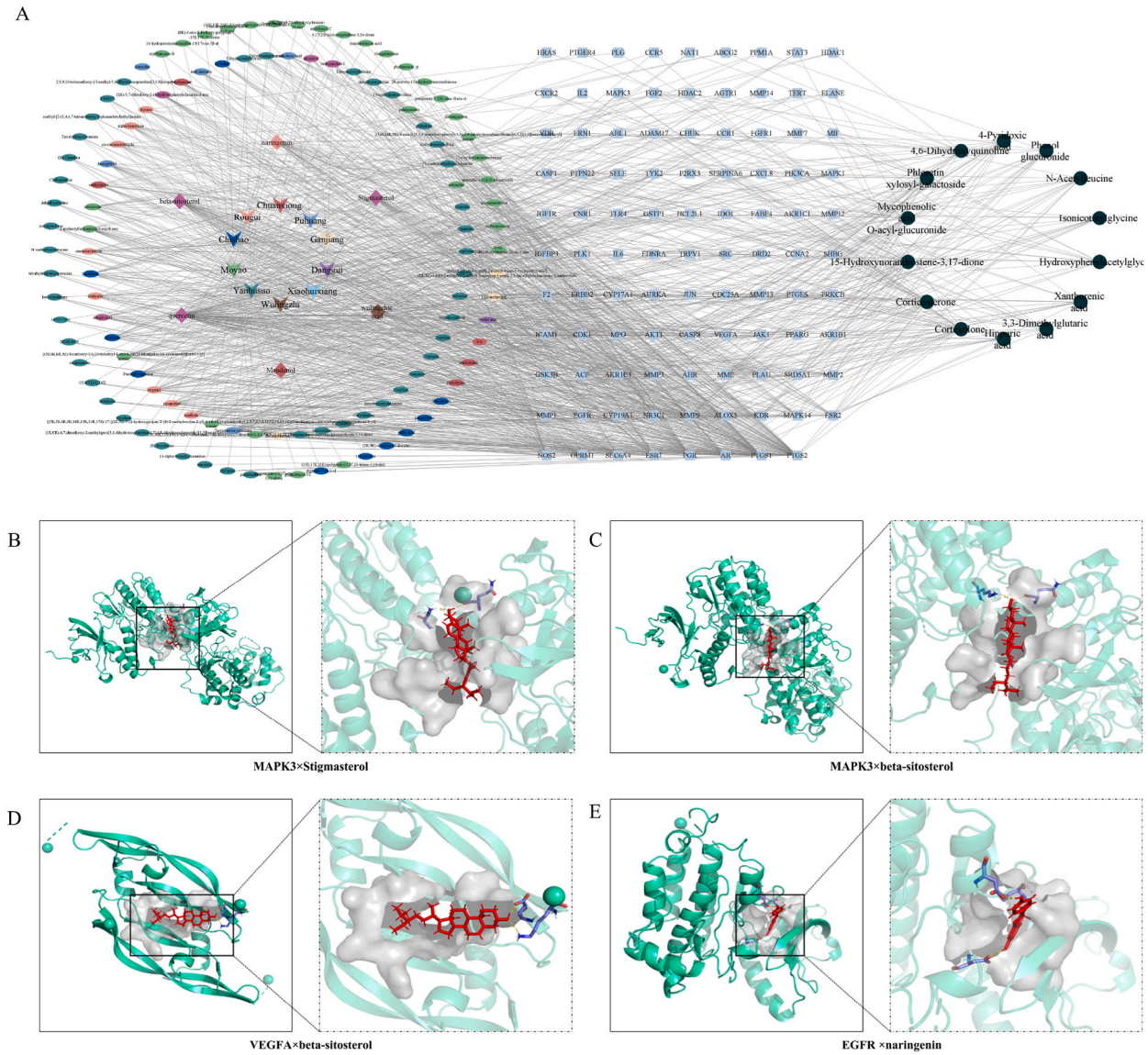

Fig. 7. "Herbs-ingredients-targets-biomarkers": The circle represents the biomarker, the square represents the target, the oval represents the active ingredient, the diamond represents the key active ingredient, and the arrow represents the drug (A). Molecular docking: Interaction between MAPK3 and stigmasterol (B). Interaction between MAPK3 and beta-sitosterol (C). Interaction between VEGFA and beta-sitosterol (D). Interaction between EGFR and naringenin (E).
Note: The smaller the binding energy, the better the docking results. Blue color represents high binding energy and red color represents low binding energy.

159 signaling pathways were enriched by KEGG, and the results showed that they mainly involved TNF signaling pathway, IL-17 signaling pathway, VEGF signaling pathway, Estrogen signaling pathway, PI3K-Akt signaling pathway, etc (Fig. 6D). The results suggested that SFZYD affected biomarkers and related metabolic pathways by affecting the core targets and related signal pathways.

A network diagram of "herbs-ingredients-targets-biomarkers" was constructed. The key active ingredients in SFZYD were beta-sitosterol (Danggui, Chishao, Ganjiang, Puhuang, Xiaohuixiang, Moyao), stigmasterol (Danggui, Chishao, Xiaohuixiang, Yanhusuo, Moyao), mandenol (Chuanxiong), wulingzhic acid (Wulingzhic acid), naringenin (Rougui), quercetin (Puhuang, Yanhusuo, Moyao) (Fig. 7A). The key active ingredients had good binding ability to the core targets (Fig. 7B–E and Table 3). The results verified the therapeutic effects of the key active ingredients in SFZYD on the core targets at the theoretical level.

4. Discussion

In this study, SFZYD significantly suppressed estrogen abnormalities (E2 and LH), inflammation (IL-6) and CA125 levels in ECB rats. Endometriosis is an estrogen-dependent disease, and the estrogen levels in the body reflect the growth of the endometrium, and the serum levels of E2 and LH in the model group were significantly higher than those in the control group, and this hormonal imbalance can lead to increased inflammation [22,23]. IL-6 is a pleiotropic cytokine that plays an important role in reproductive physiology and endometrial regeneration [24]. The serum level of IL-6 was significantly higher in the model group than in sham operation group. The result indicated that the ectopic endometrium was recognized as the "foreigner", activating the immune system, producing antigen-antibody reactions, increasing cytokines, and further promoting the proliferation and growth of ectopic endometrial tissue. CA125 is a protein antigen on the surface of epithelial cell membranes in the body cavity, which is highly expressed in the serum of endometriosis patients, and further promotes endometrial proliferation by affecting progesterone secretion [25]. In the Mod group, CA125 was significantly increased, revealing the successful establishment of the ECB model. The levels of E2, LH, CA125, and IL-6 of rats in the Dan group, and SFZYD group were lower than those in the model group. The results showed that SFZYD could inhibit the occurrence and development of ECB with varying degrees, control and treat the lesions.

On this foundation, metabolomics studies were performed. We found 18 ECB-related biomarkers and further locked 7 pathways, including vitamin B6 metabolism, steroid hormone biosynthesis, phenylalanine metabolism, retinol metabolism, pentose and glucuronate interconversions, arachidonic acid metabolism, and tryptophan metabolism. In addition, SFZYD was able to regulate the levels of 14 biomarkers.

Vitamin B6, as a pain regulator [26], can significantly reduce the intensity and duration of ECB dysmenorrhea, plays an important role in moderating estrogen metabolism, and can provide good relief for female hormone imbalance [27]. The active form of vitamin B6, pyridoxal 5′-phosphate (PLP), is negatively associated with various inflammatory diseases [28,29]. 4-Pyridoxic acid, a metabolite of vitamin B6, is involved in the metabolism of vitamin B6. to be negatively associated with a variety of inflammatory diseases. In this study, 4-Pyridoxic acid was found to be decreased in the urine of ECB model rats, which was consistent with the characteristics of chronic inflammation, hormone dependence, and dysmenorrhea of ECB.

The abnormality of retinol metabolism may be a key factor in the development of ECB [30]. The biomarker related to retinol metabolism is all-trans-18-Hydroxyretinoic acid. All-trans-18-Hydroxyretinoic acid is a metabolite of retinol, which can inhibit the development of ECB [31]. In this study, hydroxyretinoic acid was elevated after SFZYD treatment.

The biomarkers related to steroid hormone biosynthesis include cortexolone and corticosterone. Cortexolone is involved in inflammatory response. Corticosterone is involved in regulating energy, immune response, and stress response, and it has been shown to have multiple effects on metabolism and immune function [32]. Corticosterone is a precursor molecule of the mineralocorticoid hormone aldosterone, which is closely related to the female menstrual cycle [33]. In this study, the levels of cortexolone and corticosterone in the urine of the ECB model rats were increased, which was closely related to the inflammatory response, immune response, and stress response of ECB.

Benzoyl-coenzyme A (Benzoyl-CoA) is an intermediate in the metabolism of phenylalanine. Hippuric acid is produced by Benzoyl-CoA, which is an important metabolite in phenylalanine metabolism. Previous studies have shown that the level of phenylalanine in ECB patients was significantly reduced [34]. This is consistent with the trend of decreased hippuric acid levels in the urine of model rats in this study.

The relationship between tryptophan metabolism and the female reproductive system has been widely studied [35]. The effects of

Table 3
The binding energy of the key ingredients and core targets of SFZYD in the treatment of ECB.

Name	PBDB ID	Affinity(kcal/mol)					
		beta-sitosterol	Stigmasterol	Mandenol	wulingzhic acid	naringenin	quercetin
STAT3	6NJS	−7.72	−7.928	−8.008	−6.852	−7.495	−6.717
EGFR	8A2D	−7.799	−7.827	−8.159	−7.269	−8.309	−7.654
SRC	1O4R	−8.17	−8.083	−7.044	−7.248	−7.247	−6.776
AKT1	1UNR	−7.586	−7.627	−7.691	−7.34	−6.494	−7.175
JUN	5FV8	−7.499	−7.507	−8.033	−7.63	−6.687	−7.302
MAPK3	2ZOQ	−8.451	−8.763	−7.382	−6.956	−7.201	−6.464
IL6	1ALU	−7.847	−7.711	−7.34	−7.336	−7.179	−7.286
MAPK1	4ZZN	−8.064	−7.607	−8.199	−7.277	−7.951	−7.289
VEGFA	4GLN	−8.34	−8.172	−8.196	−7.828	−7.219	−7.428

tryptophan metabolites on the reproductive system mainly include regulating the synthesis and secretion of sex hormones, ovarian function, and germ cell apoptosis. 4,6-Dihydroxyquinoline is a biomarker related to tryptophan metabolism, which is a necessary metabolite that is directly involved in the growth, development, or reproduction of the organism. Studies have found that abnormal tryptophan metabolism is an important factor that allows for the immune tolerance of endometriosis implants [36]. In this study, the level of 4,6-Dihydroxyquinoline in the urine of the SFZYD group was significantly higher than that in the Mod group, and abnormal tryptophan metabolism was alleviated.

The biomarker involved in the conversion between pentose and glucuronic acid is 15-Hydroxynorandrostene-3,17-dione glucuronide. It has been shown to have anti-inflammatory effects [37]. In this study, the SFZYD group had elevated levels of 15-Hydroxynorandrostene-3,17-dione glucuronide, exerting an anti-inflammatory effect.

Arachidonic acid is involved in the synthesis of twenty-carbon derivatives such as prostaglandins, thromboxanes, and leukotrienes, which play an important role in regulating platelet aggregation, blood clotting, smooth muscle contraction, leukocyte chemotaxis, production of inflammatory cytokines, and immune function [38]. 12(S)-Leukotriene B4 is a metabolite of arachidonic acid, whose level in the urine of the Mod group was significantly reduced compared to the Con group. This has corresponded to the cold coagulation and blood stasis evidence in the model rats.

To further investigate the mechanism of SFZYD in ameliorating the abnormal biochemical indexes and metabolic abnormalities in ECB model rats, 6 key active ingredients were screened in SFZYD by building and analyzing a biological network of biomarkers. Since different herbs jointly contributed to one ingredient, these 6 key active ingredients were attributed to 10 herbs in SFZYD. 9 core targets that were predicted to be associated with both ECB, SFZYD, and biomarkers. They were STAT3, EGFR, SRC, AKT1, JUN, MAPK3, IL6, MAPK1, and VEGFA. Molecular docking showed high affinity between the key active ingredients and the core targets, and it is speculated that SFZYD plays a role by affecting the above genes. They are all important molecules involved in cell signaling, which can regulate cell growth, differentiation, apoptosis, inflammation and many other physiological and pathological processes through different pathways.

SRC, a non-receptor tyrosine kinase, can directly or indirectly phosphorylate STAT3, thereby promoting its dimerization and nuclear translocation [39]. SRC can also affect the function of STAT3 by activating the Ras/Raf/MEK/ERK pathway and the PI3K/Akt/mTOR pathway [40]. STAT3, as a transcription factor, can be activated by a variety of cytokines and growth factors, such as IL-6 can significantly increase the phosphorylation of STAT3. The dimer formed after STAT3 phosphorylation activation enters the nucleus from the cytoplasm, and further promotes cell growth, resistance to apoptosis, angiogenesis, and invasion through the regulation of the target genes, vascular endothelial growth factor (VEGF). In addition, activation of STAT3 is also key to the inflammatory phenotype of the endometrium [41]. It was found that the level of phosphorylated STAT3 was significantly higher in the ectopic endometrium of women with endometriosis [42]. Therefore, reducing STAT3 phosphorylation levels is a viable treatment for endometriosis [43].

EGFR, a receptor tyrosine kinase, is a receptor for epithelial growth factor cell proliferation and signaling [44] and can promote endometrial cell proliferation [45]. Research has shown that EGFR is overexpressed in ectopic endometrial tissues of patients, which in turn activates the downstream Ras/Raf/MEK/ERK and PI3K/Akt/mTOR pathways to aggravate proliferation, migration, and anti-apoptosis of ectopic endometrium [46]. MAPK1 and MAPK3, the downstream molecules of Ras/Raf/MEK/ERK pathway, and AKT1, another core member of PI3K/Akt/mTOR pathway, are serine/threonine kinases, which can participate in the occurrence and development of endometriosis.

VEGFA is a vascular endothelial growth factor that promotes endothelial cell growth as well as alters vascular permeability and induces inflammation [47], which can stimulate angiogenesis and dilation. Danastas et al. found that the expression level of VEGFA was higher in ectopic endometrial stromal cells than in eutopic endometrial stromal cells, suggesting that VEGFA can participate in ectopic tissue angiogenesis [48]. Moreover, another research showed that VEGFA gene polymorphisms were associated with the susceptibility and severity of endometriosis [49]. JUN gene is a gene encoding c-Jun protein that regulates cell proliferation, differentiation, apoptosis, and stress response [50,51]. The IL-6 gene encodes the IL-6 protein, which is a cytokine involved in inflammatory response and immunomodulation [52]. In the study, SFZYD reduced the level of IL-6 in ECB model rats.

5. Conclusions

In this study, SFZYD significantly improved the abnormal biochemical indexes and metabolic abnormalities in ECB model rats. The specific results are as follows: (1) SFZYD significantly suppressed estrogen abnormalities, inflammation, and CA125 levels in ECB rats. (2) A total of 18 ECB-related biomarkers in 7 pathways were identified by metabolomics. SFZYD was able to regulate the levels of 14 biomarkers that were involved in 5 metabolic pathways. (3) Six key active ingredients were screened out in SFZYD, and some of the six key ingredients were common ingredients of different herbs. (4) Nine core targets associated with ECB, SFZYD, and biomarkers were predicted, namely STAT3, EGFR, SRC, AKT1, JUN, MAPK3, IL6, MAPK1, and VEGFA. (5) Molecular docking showed high affinity between the active ingredients and the core targets, and it was hypothesized that SFZYD played a role by affecting the above-mentioned gene role. In the next study, the results will be verified in detail by experiments. Additionally, MS oriented metabolomics has laid the foundation for establishing a research model and methodological platform for formula's mechanisms.

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Data availability

All data generated or analyzed during this study are available from the corresponding author on reasonable request.

Ethical approval

All processes involving rat were authorized by the Ethics Committee of Heilongjiang University of Chinese Medicine under the guidance of the Helsinki Declaration. Approval No.2019042501 was the approval number for this process.

CRediT authorship contribution statement

Jing Liu: Writing – original draft, Visualization, Validation. **Dongxia Yang:** Visualization, Supervision. **Xiaolan Sun:** Investigation. **Saisai Yang:** Writing – review & editing. **Yao Zhang:** Writing – review & editing. **Qiyao Li:** Investigation. **Siyao Deng:** Investigation. **Haoran Dai:** Supervision. **Xiuhong Wu:** Writing – review & editing, Supervision, Funding acquisition, Data curation.

Declaration of competing interest

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

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e33806.

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