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ORIGINAL RESEARCH

Anti-Inflammatory Activity of Sanjie Zhentong Capsule Assessed By Network Pharmacology Analysis of Adenomyosis Treatment

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Background: Sanjie Zhentong capsule (SZC) offers excellent effect in treating adenomyosis (AM), which is a common and difficult gynecological disease in the clinic. However, the systematic analysis of its mechanism has not been carried out yet and further studies are needed to reveal the role of SZC.

Methods: A systematic network pharmacology analysis was conducted by integrating construction of SZC compound database and AM target database, prediction of potential active compounds and targets by molecular docking combined with compound-target prediction graph (CTPG), protein-protein interaction (PPI) analysis, Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis. Then, the anti–inflammation experiments in vitro were performed by investigating SZC and the representative compounds regulating nitric oxide (NO), interleukin-6 (IL-6), and interleukin-10 (IL-10).

Results: Our findings show that SZC mainly treated AM by stimulating 28 core targets through 30 key potential active compounds, and affecting 4 crucial pathways. The treatment was associated with inflammation reaction, hormone regulation, cell adhesion, proliferation, and angiogenesis. Additionally, SZC achieved the anti–inflammatory activity by the cooperation of the compounds through inhibiting NO and IL-6, both promoting and inhibiting IL-10.

Conclusion: This study investigated the anti–inflammatory activity of SZC based on a systematic analysis of SZC remedying AM, which was revealed to be one of the essential mechanisms. These findings will provide valuable guidance for further research of the SZC treatment of AM, and help improve the comprehension of SZC pharmacological basis as well as AM pathogenesis.

Keywords: Sanjie Zhentong capsule, adenomyosis, network pharmacology, molecular docking, compound-target prediction graph, anti-inflammation

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Introduction

Adenomyosis (AM), a common difficult gynecological disease, occurs in 6–10% women of childbearing age, the rate increases to 60% in infertile women. With the discriminative pathological characteristics of benign infiltration and growth of the endometrium into the myometrium, the ectopic endometrium of the AM patients changes the menstrual cycle (includes bleeding, shedding, and invasion) and results in the proliferation of muscle cells, a thickened myometrium, and enlarged uterine volume.[1] Although the pathogenesis is still unclear, uterus basement membrane invagination caused by endometrial tissue damage and repair, and embryonic stem cell residue and differentiation, are the two existing theories of AM at present.[2] The

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former considered that AM is mainly associated with high hormone levels,[3] tissue damage, and repair,[4] the latter believed that AM is caused by an embryonic remnant or adult stem cell differentiation.[5] It has also been revealed that once AM is formed, the critical factors for its evolution and development are invasion and migration.[6] On the other hand, from the perspective of traditional Chinese medicine (TCM), AM belongs to the same category as "dysmenorrhea" and "various syndromes before and after menstruation", with lesions in the uterus and Chong ren, stagnant blood, qi, and body fluid, and dampness accumulated by phlegm as the pathogenesis.[7] Since hormones and nonsteroidal anti–inflammatory drugs are generally applied in the treatment of AM, which put a heavy load on patients due to the high cost, high recurrence rate, and severe side effects, the application of TCM is gradually showing its unique advantages. Definitive curative effects have been achieved through TCM therapeutics, for instance, patients with AM treated with Xuanyu Tongjing decoction achieved a recovery rate of 90.9%, the treatment improving dysmenorrhea symptoms, relieving pain, and causing few adverse reactions.[8] The total effective rate reached 96.8% when the patients had been treated with Jiawei Siwu decoction, and this treatment exhibiting a significant efficacy difference compared with that provided by a chemical medicine group.[9]

Sanjie Zhentong capsule (SZC) is a traditionally prescribed as Chinese medicine that is made by the original powder of four natural drugs: Longxuejie, Sanqi, Zhebeimu, and Yiyiren. It has been used for secondary dysmenorrhea, irregular menstruation, pelvic mass and infertility caused by endometriosis (phlegm and blood stasis mixed with qi stagnation syndrome) in the clinic. SZC is beneficial in treating ovarian cysts,[10] reducing the development of endometriosis[11] and achieving superior inhibition of the focused growth of endometriosis in rats.[12] In addition, SZC has also been used in the clinical application of AM. Researchers have found that SZC could dramatically reduce the operation rate of AM by improving the pregnancy rate and clinical symptoms in AM patients who experience infertility,[13] and by treating dysmenorrhea caused by AM.[14] It has also been presented that SZC treatment of AM might be related to the significant decrease of CA125, TNF-α, and PGF-2α in the serum.[15] Nevertheless, the complex pathogenesis of AM, as well as the multiplex mechanism of SZC remain unclear, the principles and mechanisms by which SZC is effective for treating AM are needed to be uncovered.

In recent years, increasing attention has been paid on the holistic philosophy of TCM, the mode of drug discovery design has shifted from single-target to network-target and multiple-component-therapeutics with a systematic view.[16] With the updates of bioinformatics and computer technology, network pharmacology has been developed rapidly to become a credible modern approach for predicting the potential active compounds of TCM and available targets of disease, and exploring mechanisms. For example, Shi has proposed a systematic network pharmacology approach, identified 29 compounds acting on 16 pathways (regulated by 32 targets), and revealed the blood enriching mechanism of Danggui buxue decoction.[17] Yang applied network pharmacology to investigate the anti-metastasis mechanism of Oldenlandia Diffusa in breast cancer and found that 12 compounds and 85 targets were associated with the treatment.[18] However, the systematic analysis of SZC treatment of AM has not yet been carried out. Herein, we conducted a network pharmacological analysis of SZC treatment of AM by proposing an integrated approach. Furthermore, experiments in vitro were performed to observe the anti–inflammation activity of SZC. The workflow is shown in Supplementary Figure S1.

Materials and Methods

SZC Compound Database Construction

We applied Web crawler technology to automatically pick up compound information from the TCMSP database (http://lsp.nwu.edu.cn/tcmspsearch.php), and then stored it in the SZC compound database. Longxuejie is not in the TCM databases, wide-scale text mining of PubMed (https://www.ncbi.nlm.nih.gov/pubmed/) and CNKI (http://www.cnki.net/) databases was performed. The 3D structure files with SDF format of the compounds were crawled and downloaded from the NCBI compound database according to their retrieved names and PubChem identifiers from the database of chemicals (https://pubchem.ncbi.nlm.nih.gov/). Besides, we used the selenium crawler framework, an automated testing framework that is ideal for crawling dynamic web pages, making it easy to crawl data quickly and precisely on objective sites. Throughout the process, we adopted python language to design crawlers which help realize automatically acquiring and recognizing information of compound data.

Target Database Construction of AM

To prepare the protein information for molecular docking, we acquired information of targets from the NCBI gene database by searching the name of disease "adenomyosis" and stored it into the AM target database. Then, corresponding protein information was searched from UniProt (https://www.uniprot.org/) and stored in the AM target database using the web crawler technology earlier described. Notably, when performing protein screening in UniProt, proteins of homo species with a lower resolution were selected.

Prediction of Potentially Active Compounds and Targets

To predict the potential active compounds and targets, we adopted molecular docking which has been widely used to describe the strength of binding interactions between molecules. The prediction process consists of two phases. First, System Dock Web Site (http://systemsdock.unit.oist.jp/iddp/home), which is a very popular web server in network pharmacology-based prediction, was linked to perform the molecular docking process with the help of automatic link docking algorithms. We have implemented the algorithms to implement the prediction. Generally, compound-target pairs with the score ≥ 4.25 are considered to have particular binding activity. The score ≥ 5.0 indicates a greater binding activity and score ≥ 7.0 indicates a stronger binding interaction between compound and target.[19] Second, a Compound-Target Prediction Graph (CTPG) is proposed to model the predicting of the potential active compounds. The targets was built based on a directed graph with weighted edges, which manifests the cohesion of the network[20] and illustrates the cohesion degree of the elements with the clustering coefficient.[21] In *CTPG = (V, E, W), V* denotes a finite set of vertexes, which represents the compounds or targets. *E* denotes the directed edge including the output edge and the input edge between compounds and targets. *W* denotes the total score of a particular compound or target. After calculating the *W* value and the number of targets linked to a compound as well as the number of compounds linked to a target, we selected the compound or target with superior *W* value and a bigger linked number as the potentially active one. Therefore, the CTPG was effective to explain the degree of strength that they contribute to the mechanism by analyzing the distribution of compounds and targets. The details of adopting CTPG can be found in our experiments.

Protein-Protein Interaction (PPI) Analysis

The interaction among proteins of the potential active targets was analyzed furtherly. STRING database (https://STRING-db.org/) was adopted to analyze the PPI. The protein data of potential targets was imported into STRING, the organism was set as homo sapiens, and then the result of PPI was visualized with Cytoscape v3.7.1.

Go and KEGG Enrichment Analysis

To understand the enrichment of the acquired potential target proteins and differential genes in biological functions and pathological pathways, cellular localization, GO annotation analysis and the KEGG pathway enrichment analysis of the achieved targets were performed. The terms with P-value < 0.05 were screened for the main functional annotation and significant pathways clustering. The less correlated ones were removed. ClueGO and CluePedia plugins of Cytoscape v3.7.1 were utilized in the GO enrichment analysis and KEGG pathway enrichment analysis.

Network Construction and Analysis

To intuitively understand the mechanisms of SZC treatment on AM, both the compound-target network and target-pathway network were constructed. The graphs of the networks were generated and visualized using Cytoscape v3.7.1. The formation of the compound-target network was based on the potential active compounds and the corresponding targets, obtained by docking and CTPG analysis. The target-pathway network was built by connecting targets to the signaling pathways. In the network graphs, nodes represent compounds, targets, or signaling pathways, and edges indicate the interactions of compound-target or target-pathway. The degree of a node was determined by the number of edges connected with it.

Anti-Inflammatory Activity of SZC in vitro

Reagents

Ginsenoside Rg1 (HPLC ≥ 98%), ginsenoside Rb1 (HPLC ≥ 98%), notoginsenoside R1 (HPLC ≥ 98%), ginsenoside Rd (HPLC ≥ 98%), resveratrol (HPLC ≥ 98%), pterostilbene (HPLC ≥ 98%), 7.4'-dihydroxyflavone (HPLC ≥ 98%), loureirin A (HPLC ≥ 98%), loureirin B (HPLC ≥ 98%) and peiminine (HPLC ≥ 98%) were obtained from the Standardization Research Center of TCM (Shanghai,

China). The concentration of DMSO was < 0.1% in this study. SZC was obtained from Kanion Pharmaceutical Co., Ltd. (Jiangsu, China). The compounds were stable under the conditions used in the study. All chemical structures are shown in Supplementary Figure S2. Cell Counting Kit-8 (CCK-8) was obtained from Beyotime (Shanghai, China), fetal bovine serum (FBS), dulbecco's modified Eagle's medium (DMEM) and penicillin/streptomycin were obtained from Gibco Life Technologies (Waltham, MA, USA), lipopolysaccharide (LPS), sulfanilamide and N-1-naphthyl ethylenediamine hydrochloride were obtained from Sigma-Aldrich LLC (Darmstadt, Germany).

Cell Culture

RAW264.7 cells (purchased from the Chinese Academy of Sciences, Shanghai, China) were cultured in DMEM containing 10% FBS and 1% penicillin/streptomycin at 37°C in the cell incubator with a humid atmosphere containing 5% CO_2.

Cell Viability Assay

Cell viability test was taken using CCK-8 assay after treatment with the compounds of SZC. In brief, RAW264.7 cells in a logarithmic phase were seeded in a 96-well plate at a density of 1×10^4 cells in the absence or presence of 24 h. After aspirating the medium, the cells were incubated with 10% CCK-8 for 1h and ELISA (MD, USA) was applied to read OD value at 450 nm.

Measurement of Nitric Oxide

Cells were seeded in a 96-well plate at a density of 5×10^4 cells and incubated as above for 24 h, 0.01 μg/mL LPS was added after the cells were incubated with a gradient concentration of SZC for 30 min. To determine the concentration of nitrite in the culture media after 24 h, Griess reagent (1% sulfanilamide, 0.1% N-1-naphthyl ethylenediamine hydrochloride) and supernatant at each treatment condition were mixed with the same volume and OD value was read using ELISA reader at 540 nm.

Cytokine Beads Array Assay

Cells were seeded and treated with SZC and LPS according to the same procedure described above. Supernatant was collected and stored at −80°C until analyzed. Cytokines were measured using the Cytometric Bead Array (CBA) kit (BD Biosciences, CA) according to the manufacturer's protocol. Samples were incubated with capture beads and phycoerythrin detection reagent for 2 h, including standard and test ones, washed with wash buffer and then analyzed using the FACSCalibur flow cytometer with BD CBA software.

Statistical Analysis

All results were expressed as means (± SD). Statistical analysis was performed by Graphpad Prism 7.0, using the one-way analysis of variance (ANOVA) test, followed by Dunnett's multiple comparison post hoc test. $P < 0.05$ was considered to be statistically significant.

Results

Potential Active Compounds and Targets of SZC

With the help of selenium and python, the automatic data mining and docking platform linking were realized; combined docking with the compound-target prediction graph (CTPG), active compounds and targets were identified and classified according to their different binding activities, As a result, 224 compounds and 106 targets were stored in the SZC database and target database, respectively. After docking, 105 candidate molecules with docking score above 4.25 (in which, 95 molecules had a docking score above 5 and 38 molecules had a docking score above 7) were screened as compounds with binding activity. We display four compound-protein pairs with a docking score above 7 in Figure 1. The CTPG exhibited the contribution degree of the compounds and targets with a docking score above 4.25 for SZC treating AM. As there was a sharp decline when the total weighted scores came down to 200, 78 compounds with docking scores above 4.25 and total weighted scores above 200 that displayed a concentrated distribution were taken for the potential active components of SZC in treating AM. We show the distribution of active compounds in Figure 2A. Additionally, 42 proteins with docking scores above 4.25 and a total weighted score above 400 were screened as the potential targets of AM treated by SZC. We show the distribution of potential targets in Figure 2B. The data of docking and distribution are listed in Supplementary Table S1–S2, respectively.

The Interaction of Proteins

The 42 potential targets of AM related to SZC obtained above were introduced into the STRING database. as shown in Figure 3. The network involved a total of 42 nodes and 259 edges, in which, neurogenic locus notch homolog protein 1 (NOTCH1), cyclin-D1 (CCND1), estrogen receptor (ESR1), C-C motif chemokine ligand 2

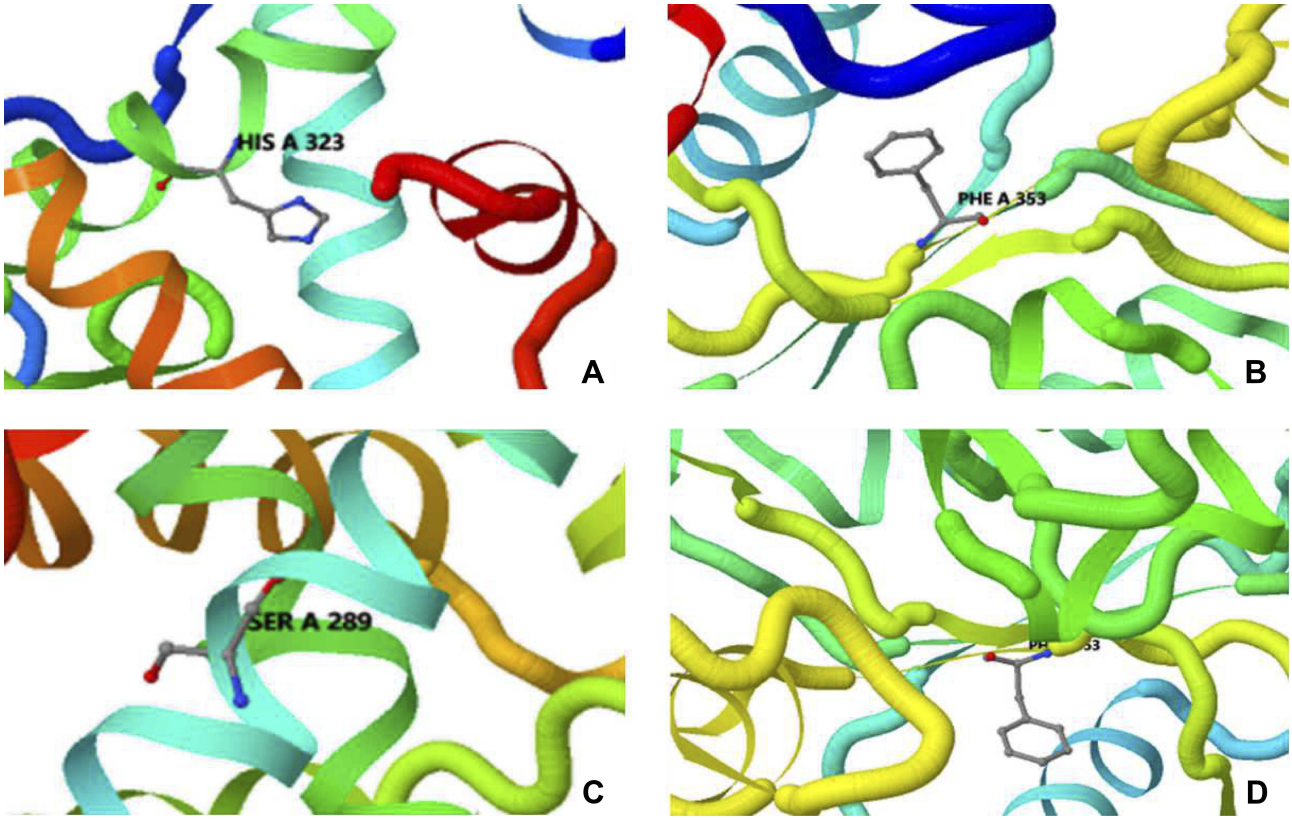

Figure 1 Compound-target pairs with a docking score above 7. (**A**) Sitosterol-ESR2, the score is 8.421, (**B**) Panaxatriol-CYR61, the score is 8.409, (**C**) Sitogluside-PPARG, the score is 8.337, (**D**) Peiminine-NOS3, the score is 8.201.

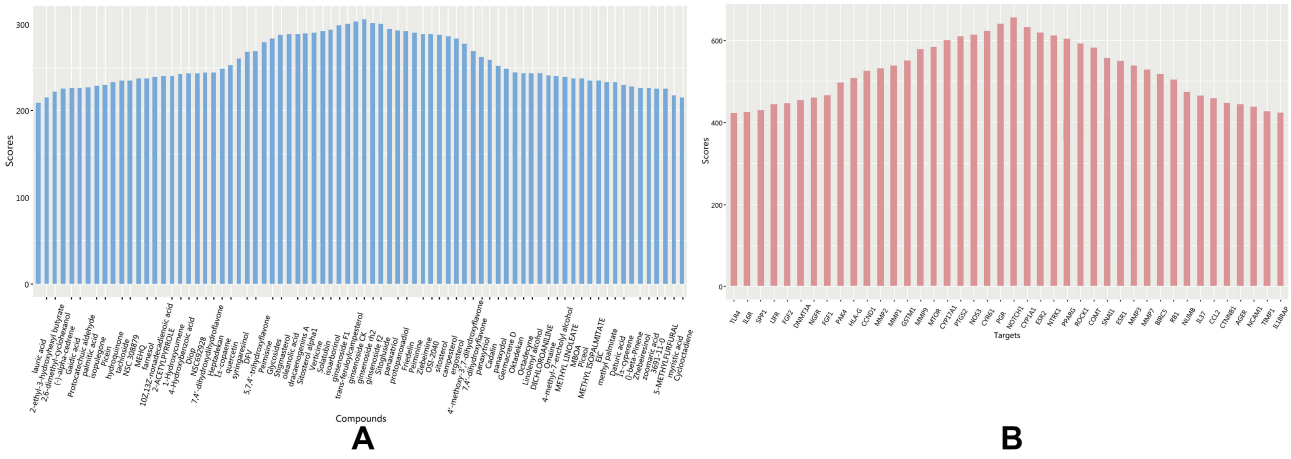

Figure 2 CTPG of SZC treating AM. (**A**) Distribution of potentially active compounds of SZC for treating AM. The y-axis represents total weighted scores of compounds, and the x-axis shows all compounds with total weighted scores higher than 200. (**B**) Distribution of the potential targets of SZC treated by AM. The y-axis represents total weighted scores of targets, and the x-axis shows all targets with total weighted scores higher than 400.

(CCL2), prostaglandin G/H synthase 2 (PTGS2) and matrix metalloproteinase 2/9 (MMP2/9) displayed the biggest size and the darkest color, which interacted with the other targets strongly and predominated the remedy for AM.

GO Annotation and KEGG Enrichment Analysis

The results of the gene ontology biological process (GOBP) annotation analysis revealed that the potential targets we acquired are closely involved in diverse

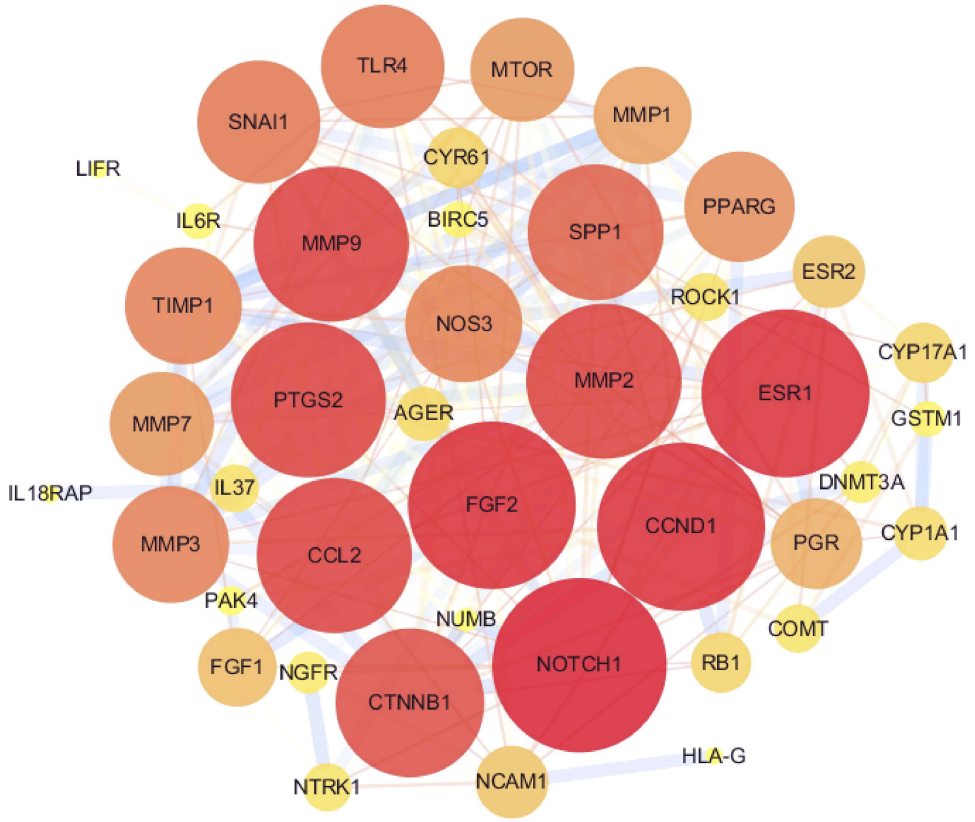

Figure 3 Protein-protein interaction analysis of potential target genes. The nodes represented the proteins, and the edges represented the interrelationships among the proteins. The size and color of the nodes indicate the degree of importance of the targets. The larger the node is, the higher the degree of the importance is, and the degree becomes greater as the color changes from yellow to red. The thickness of the edge indicates the association confidence: the thicker the edge is, the stronger the proteins combined.

biological functions and process concentrated on inflammation, hormones, adhesion, proliferation, and angiogenesis, which are intensely related to the pathogenesis of AM. The top 20 that significantly enriched GOBP terms ($P < 0.05$) are listed in Figure 4.

Network Construction and Analysis

Compound-Target Network Construction and Analysis

After the CTPG prediction, 30 key potential compounds and 28 targets were screened, showing strong binding activity and major contribution in the group with the docking score above 7, the compound total weighted score above 200 and the target total weighted score above 400. The network of key compounds with targets that play the most contributory role in SZC for treating AM is displayed in Figure 5, there were 12 compounds completely from Sanqi; 7 compounds completely from Zhebeimu; 6 compounds completely from Yiyiren; 3 compounds completely from Longxuejie, while, 1 compound from both Sanqi and Yiyiren; 1 compound from Longxuejie, Zhebeimu, and Yiyiren. Besides, ginsenoside Rh2 (from Sanqi), peiminine (from Zhebeimu), trans-feruloylcampesterol (from Yiyiren) and dracaenogenins A (from Longxuejie), interacted with 27, 27, 23 and 17 targets, respectively; for targets, estrogen receptor (ESR) interacted with 52 compounds, progesterone receptor (PGR) with 28 compounds, PTGS2 with 26 compounds and Nitric oxide synthase 3 (NOS3) with 25 compounds. It significantly indicated the multi-component and multi-target characteristic of SZC in the treatment of AM. Detailed information on the key potential compounds and targets is listed in Tables 1 and 2.

Target-Pathway Network Construction and Analysis

Pathways of AM treated by SZC were enriched in KEGG, excluding the less relevant ones, 41 pathways ($P < 0.05$) directly related to AM were obtained, and classified into five main regulation modules, including inflammation reaction, hormone regulation, cell adhesion, proliferation,

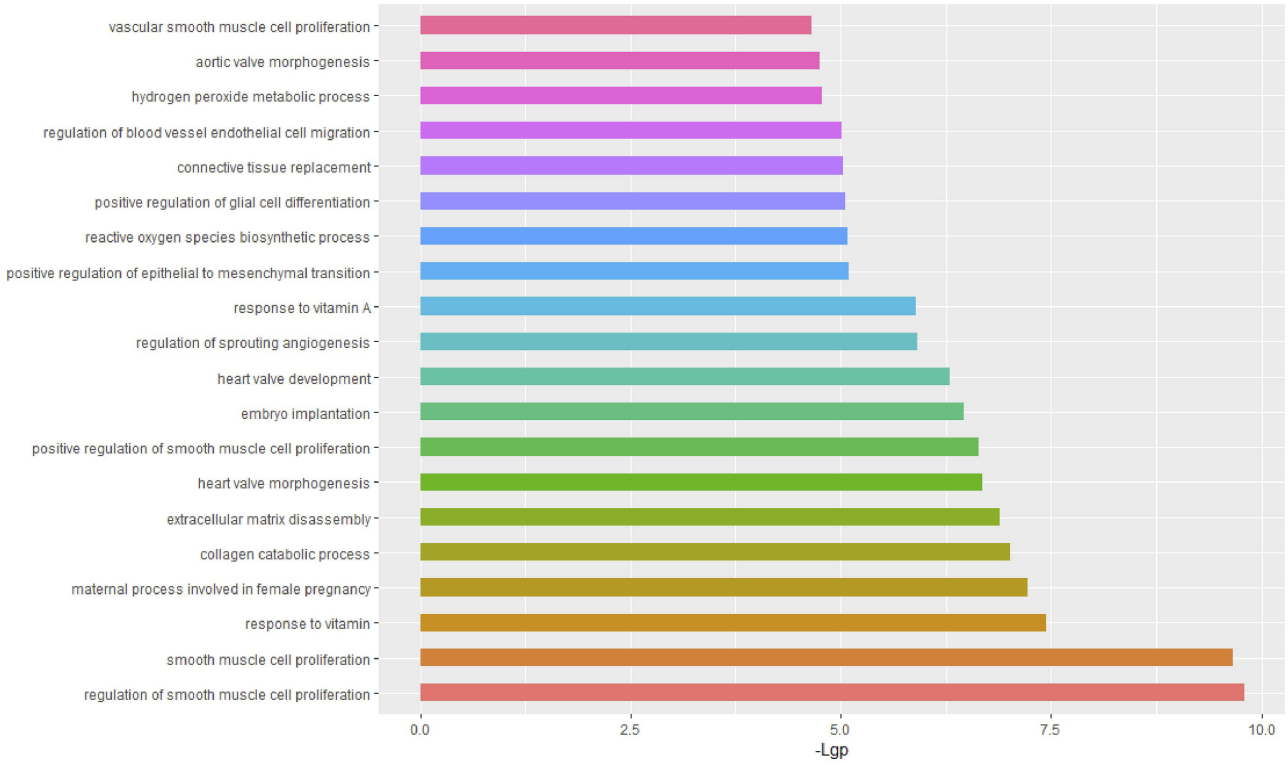

Figure 4 Gene ontology analysis of the potential target genes, the y-axis shows the top 20 significantly relevant biological processes enriched with these genes, and the x-axis shows the enrichment scores of biological process terms ($p < 0.05$).

and angiogenesis. The target-pathway network graph is presented in Figure 6 including 81 nodes (5 modules, 35 targets, 41 pathways) and 367 edges. We further emphasized on the most representative pathways (endocrine resistance, the PI3K-Akt signaling pathway, focal adhesion, and the NF-κB signaling pathway) and merged them into a confluence map that the PI3K-Akt signaling pathway and the NF-κB signaling pathway intersected at TLR4; endocrine resistance, the PI3K-Akt signaling pathway, and focal adhesion crossed at mTOR; and CCND1 is the common target protein in the four pathways, as shown in Figure 7. The data of Go terms, pathways and function modules is listed in Supplementary Table S3-S4.

Anti-Inflammatory Activity of SZC in vitro

Since the inflammatory regulation of SZC was identified to be one of the essential regulation modules in treating AM, we examined the anti–inflammatory activity of SZC as well as ten active compounds of SZC with less cytotoxicity that had been quantified in the previous study.[22,23] The cytotoxicity test showed that resveratrol, pterostilbene and 7,4'-dihydroxyflavone were cytotoxic to LPS-treated RAW264.7 cells at a concentration exceeding 20 μM, while other compounds were safe at concentration even higher than 100 μM. Then, we set the concentration of pterostilbene and 7,4'-dihydroxyflavone at 20 μM, loureirin A, loureirin B, notoginsenoside R1, ginsenoside Rg1, ginsenoside Rb1, ginsenoside Rd, peiminine at 50 μM, dexamethasone (DXM) at 1 μM, and SZC at 50 μg/mL as the test concentrations for the next experiments.

After being treated by the selected compounds at certain concentrations, the cell viability was not inhibited compared with the untreated group, which indicated that the compounds at the concentrations were all noncytotoxic to the LPS-treated RAW264.7 cells. The result of the cell viability is presented in Figure 8A.

The NO Synthesis Inhibition of SZC

As shown in Figure 8B, pterostilbene and SZC exhibited significantly stronger inhibition of NO than DXM ($P < 0.001$); resveratrol and ginsenoside Rd inhibited NO with no significant difference versus DXM, loureirin A and loureirin B exhibited weaker inhibition of NO with a significant difference versus DXM ($P < 0.05$); while 7,4'-dihydroxyflavone, notoginsenoside R1, ginsenoside Rg1, ginsenoside Rb1, and peiminine exhibited significantly

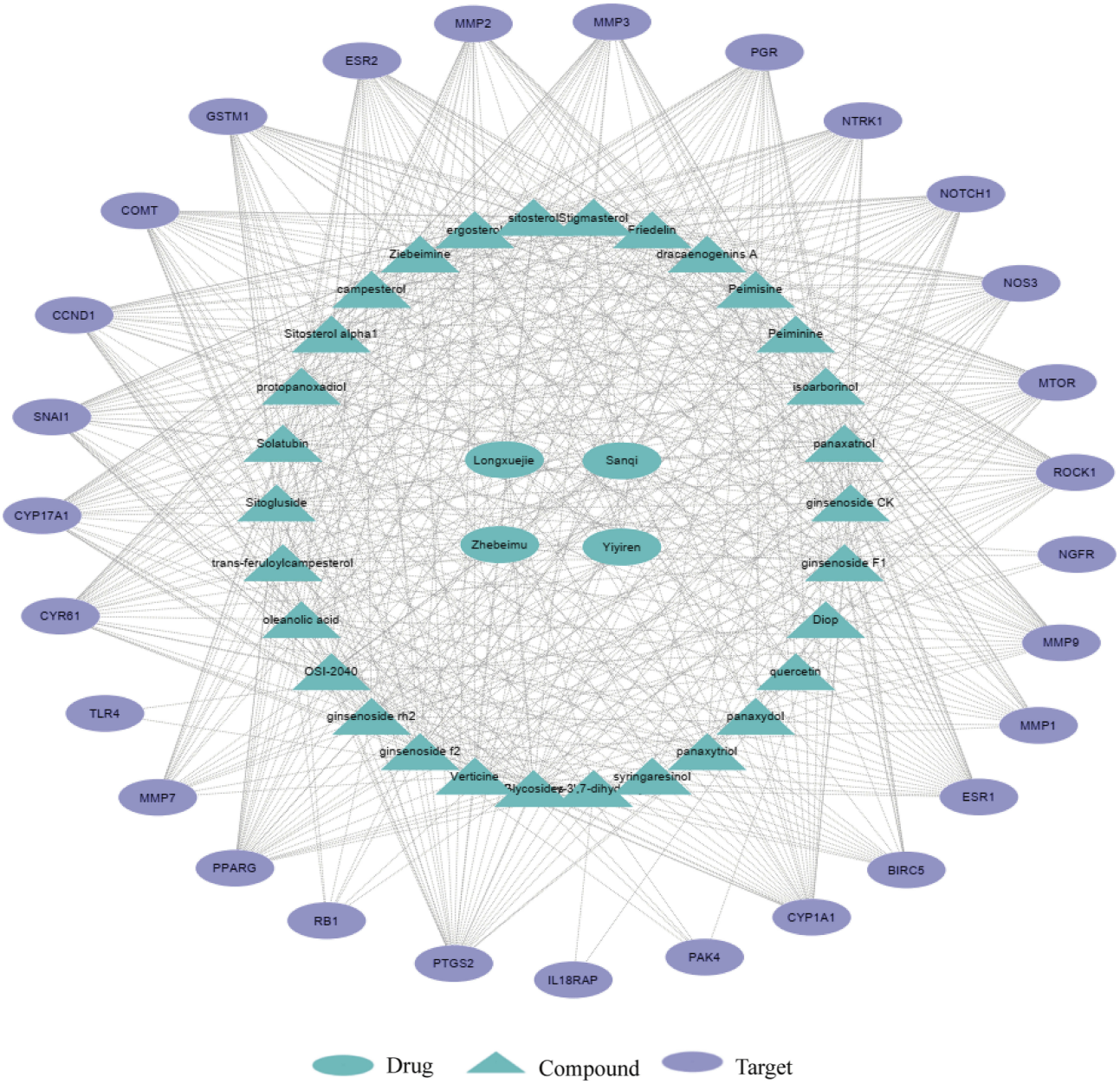

Figure 5 Network of compound-target with strong binding activity and major distribution for SZC treating AM. It was constructed with 62 nodes (4 drugs, 30 compounds, and 28 targets) and 578 edges The blue triangles and purple ellipses represent crucial bioactive compounds of SZC and core corresponding targets of AM, respectively. The blue ellipses represent drugs in SZC. The gray edges represent the mutual relationships between targets and compounds.

weaker inhibition of NO than DXM ($P < 0.001$), of which, notoginsenoside R1 got the lowest inhibition rate.

The Cytokine Regulation of SZC

The results shown in Figure 9A suggested that SZC inhibited IL-6 with no significant difference versus DXM and the compounds exhibited different strength, pterostilbene, 7,4'-dihydroxyflavone, and ginsenoside Rd inhibited IL-6 significantly stronger than DXM ($P < 0.01$), of which, pterostilbene exhibited the strongest inhibition; notoginsenoside R1, ginsenoside Rg1, and ginsenoside Rb1 exhibited a significantly weaker inhibition than DXM ($P < 0.05$) but were still more effective than resveratrol, loureirin A, loureirin B, and peiminine.

SZC promoted the production of IL-10, but the compounds regulated IL-10 in two opposite directions. As shown in Figure 9B, resveratrol, pterostilbene, 7,4'-dihydroxyflavone, loureirin A, loureirin B, and peiminine promoted the production of IL-10, while notoginsenoside R1, ginsenoside Rg1, ginsenoside Rb1, and ginsenoside Rd

Table 1 The 30 Key Potential Active Compounds of SZC in Treating AM

PubChem CID	Compound	Drug
3083151	Ziebeimine	Zhebeimu
131900	Verticine	Zhebeimu
443023	Syringaresinol	Zhebeimu
65727	Solatubin	Zhebeimu
222284	Sitosterol	Zhebeimu/Yiyiren/Sanqi
161294	Peimisine	Zhebeimu
167691	Peiminine	Zhebeimu
6918328	OSI-2040	Zhebeimu
13786591	Trans-feruloylcampesterol	Yiyiren
5280794	Stigmasterol	Yiyiren/Sanqi
9548595	Sitosterol α1	Yiyiren
12305182	Isoarborinol	Yiyiren
91472	Friedelin	Yiyiren
444679	Ergosterol	Yiyiren
173183	Campesterol	Yiyiren
5742590	Sitogluside	Sanqi
5280804	Quercetin	Sanqi
11213350	Protopanoxadiol	Sanqi
93484	Panaxytriol	Sanqi
5283280	Panaxydol	Sanqi
73599	Panaxatriol	Sanqi
10494	Oleanolic acid	Sanqi
119307	Ginsenoside Rh2	Sanqi
9852086	Ginsenoside F2	Sanqi
9809542	Ginsenoside F1	Sanqi
9852086	Ginsenoside CK	Sanqi
395120	Diop	Sanqi
101389811	Dracaenogenins A	Longxuejie
44259961	4'-methoxy -3',7-dihydroxyflavone	Longxuejie
637579	Glycosides	Longxuejie

Table 2 Information of 28 Core Potential Targets in SZC Treatment of AM

UniProt CID	Protein Name	Gene Name
P06401	Progesterone receptor	PGR
P46531	Neurogenic locus notch homolog protein 1	NOTCH1
Q92731	Estrogen receptor β	ESR2
P37231	Peroxisome proliferator-activated receptor gamma	PPARG
P04798	Cytochrome P450 1A1	CYP1A1
O00622	Protein CYR61	CYR61
P35354	Prostaglandin G/H synthase 2	PTGS2
Q13464	Rho-associated protein kinase 1	ROCK1
P29474	Nitric oxide synthase, endothelial	NOS3
P05093	Steroid 17-α-hydroxylase/17,20	CYP17A1
P42345	Serine/threonine-protein kinase mTOR	MTOR
P04629	High-affinity nerve growth factor receptor	NTRK1
P21964	Catechol O-methyltransferase	COMT
P09488	Glutathione S-transferase Mu 1	GSTM1
P14780	Matrix metalloproteinase-9	MMP9
O95863	Zinc finger protein SNAI1	SNAI1
P03372	Estrogen receptor	ESR1
P24385	G1/S-specific cyclin-D1	CCND1
P08254	Stromelysin-1	MMP3
P08253	72 kDa type IV collagenase	MMP2
P09237	Matrilysin	MMP7
P03956	Interstitial collagenase	MMP1
O15392	Baculoviral IAP repeat-containing protein 5	BIRC5
P06400	Retinoblastoma-associated protein	RB1
O96013	Serine/threonine-protein kinase PAK 4	PAK4
O95256	Interleukin-18 receptor accessory protein	IL18RAP
P08138	Tumor necrosis factor receptor superfamily member 16	NGFR
O00206	Toll-like receptor 4	TLR4

discouraged the production of IL-10. SZC exhibited the most extremely significant promotion of IL-10 versus LPS ($P < 0.001$), followed by resveratrol, peiminine, and pterostilbene; nevertheless, notoginsenoside R1, ginsenoside Rg1, and ginsenoside Rd inhibited IL-10 with no significant difference from DXM. The raw array data of cell viability, NO, IL-6 and IL-10 is listed in Supplementary Table S5.

Discussion

Active Compounds of SZC in Treating AM

SZC is known to be composed of four TCMs, each medicine contains numerous compounds while the resin medicine Longxuejie could not be searched from any of the existing TCM databases, the information of absorption, distribution, metabolism, and excretion (ADME) is insufficient, therefore 3D molecular structure docking was applied to completely screening of active compounds from the SZC compound database. It is worth noting that our finding of potentially active compounds were following the previous results, for instance, ginsenoside Rh2 has been proved to be active in preventing the growth of human ovarian cancer cells in vitro and in vivo,[24] inhibiting the proliferation of MCF-7 cells through down-regulation of the expression level of cyclin D,[25] and inhibiting angiogenesis in prostate cancer cells by decreasing VEGF and CNNM1.[26] Elsewhere, peiminine has been reported to be a potential component that exhibited the ability of anti–inflammation. Further study has identified that

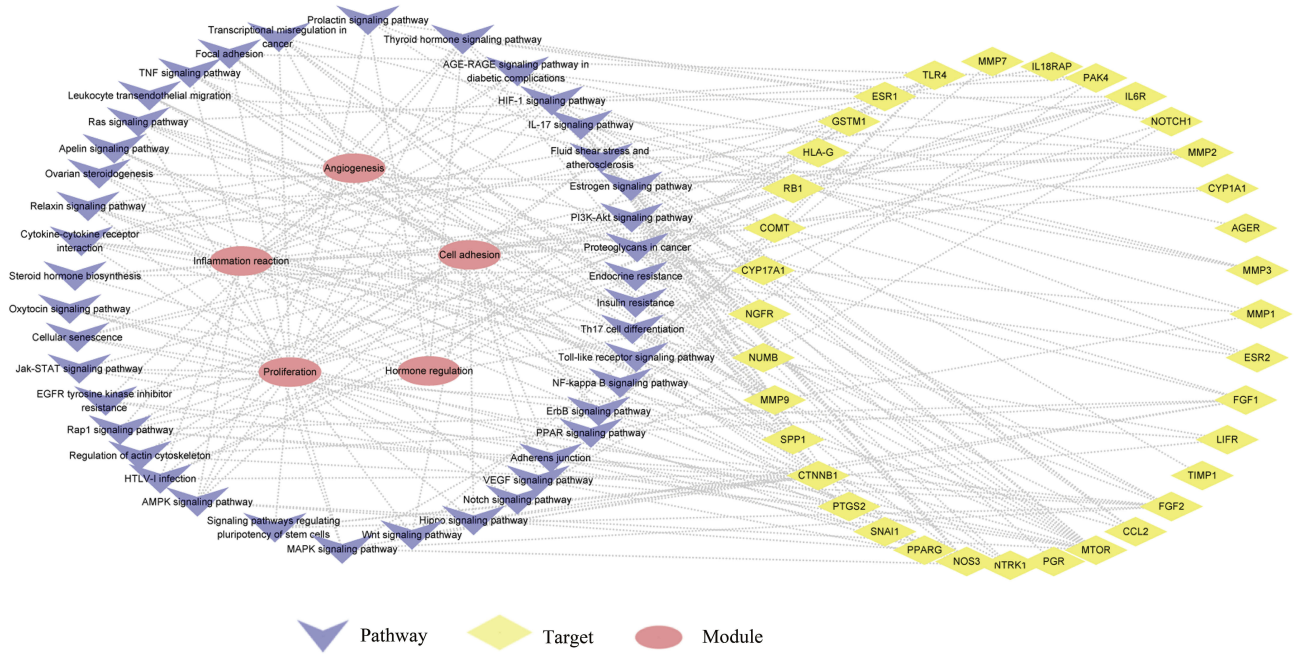

Figure 6 Target-pathway network of SZC treating AM. The purple arrows represent pathways connected with AM, the yellow rhombuses represent potential targets related to AM, and the pink ellipses represent regulatory modules of the pathways. The gray edges represent the mutual relationships between the targets and pathways.

peiminine regulated AKT/NF-κB p65, ERK1/2, and p38 signaling pathways in treating inflammatory mastitis induced by LPS in mice.[27] It has also been found that peiminine inhibited the proliferation of colorectal cancer cells by regulating the PI3K/Akt/mTOR pathway and oxidative stress.[28] Besides, estrogenic activities of 7,4'-dihydroxyflavone were discovered to be related to the properties of its structure.[29] Stigmasterol can be found in three herbs of SZC exhibited diverse bioactivities in estrogenic effect, inflammation, antiproliferation, and angiogenesis. For instance, oxidation products of stigmasterol interfered with the female sex hormone 17β-estradiol in human breast and endometrium cells.[30] Stigmasterol itself decreased inflammation by modulating the MAPK/NF-κB ROCK1 pathway,[31] combined stigmasterols achieved more obvious anti-proliferation in Caco-2 cells than a single stigmasterol,[32] and reduced TNF-α and VEGFR-2 signaling by affecting phosphorylated Src, Akt, PCL, and FAK.[33] It can be inferred that our approach is reliable and the screened active compounds act synergistically in the treatment of AM.

Potential Targets in SZC Treatment of AM

The multi-target therapeutic characteristics of TCM have become increasingly valued in revealing its mechanism, this could be expected to highlight the synergistic effects of TCM with the help of network techniques.[34] From the enrichment of potential targets, it can be concluded that SZC mainly achieved the therapeutic efficacy to AM through regulating hormone regulation (ESR and PGR), inflammation reaction (PTGS2, NOS3, TLR4, and IL6R) and cell adhesion (Rho-associated coiled-coil containing protein kinase 1 [ROCK1], MMP), affecting cell proliferation (NOTCH1, peroxisome-proliferator activated receptor gamma [PPARG], snail family transcriptional repressor 1 [SNAL1]), and angiogenesis (cysteine-rich angiogenic inducer 61 [CYR61]).

Specifically, PTGS2 (COX-2) plays a multifunctional role and has a close connection with various diseases involving inflammation, immunization, cancer, and reproduction.[35] For AM, it was explained that NF-κB mediated PTGS2 and VEGF expression in endometrial stromal cells derived from the tissue of AM patients.[36] Further study of gene polymorphism revealed that those who carried two A alleles were more likely suffer from AM, Samodelkin has explained it to be a genetic variation of G to A at the −1195 locus in the promoter region of the PTGS2 gene that increases the risk of AM.[37] ROCK1 was demonstrated to play a major role in the progression of various types of cancer. Most significantly, the expression level was positively correlated with MMP9, which was associated with invasion and

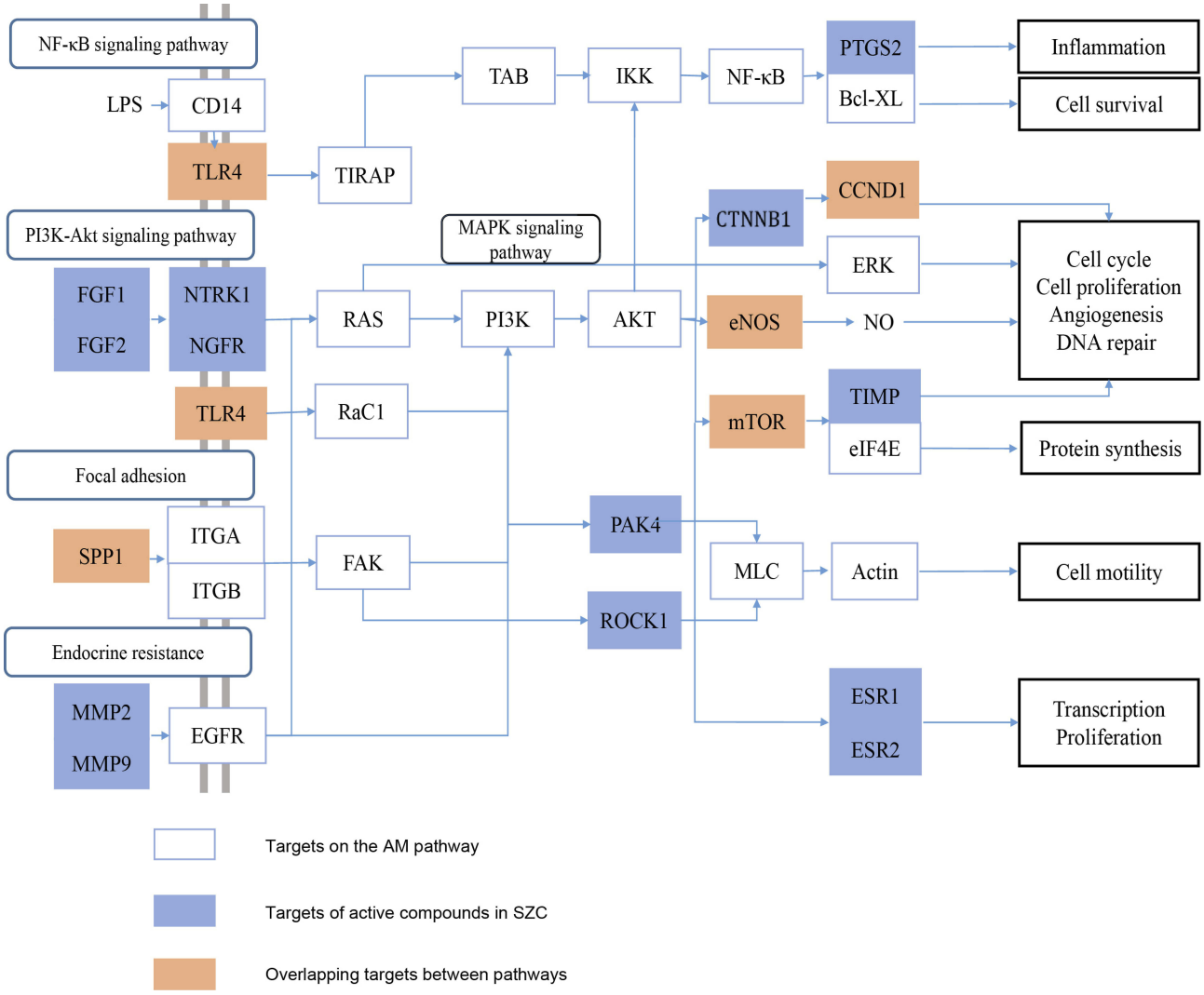

Figure 7 Confluence map of representative pathways. The white rectangles represent targets on the AM pathway, the orange rectangles represent overlapping targets of active compounds between pathways, and the blue rectangles represent targets of active compounds in SZC.

metastasis, and had become the key point of adhesion.[38] Also, the overexpression of ROCK1 was proved to take part in dysmenorrhea and menstrual process in AM.[39] As one of the single-pass transmembrane receptor protein family members, NOTCH1 was thought to play a critical role in the epithelial-mesenchymal transition affecting the proliferation of endometrial cells in the pathogenesis of AM for a higher expression of NOTCH1 in the proliferative phase.[40] CYR61 has been identified to be one of the targets of the anti-angiogenic histone deacetylase 5 and was associated with a broad range of pathological processes such as impaired angiogenesis, fibrosis, and cancer on account of its ability to bind different combinations of co-receptors. The higher expression of CYR61 in ectopic endometrium than in eutopic endometrium might be involved in the pathogenesis of AM.[41]

Pathways in SZC Treatment of AM

Close connection and intricate crosstalk were revealed by mapping the four pathways. It can be found that the PI3K-Akt signaling pathway might be the major pathway as it involves the most predicted targets and the other three pathways. The PI3K-Akt signaling pathway is well known to play crucial roles in various cellular processes including proliferation, invasion, metastasis, angiogenesis, and apoptosis which are associated with inflammation regulation. Activation of the PI3K-Akt-eNOS signaling pathway helps to regulate angiogenesis through mediating cell proliferation and migration and leads to NO level and

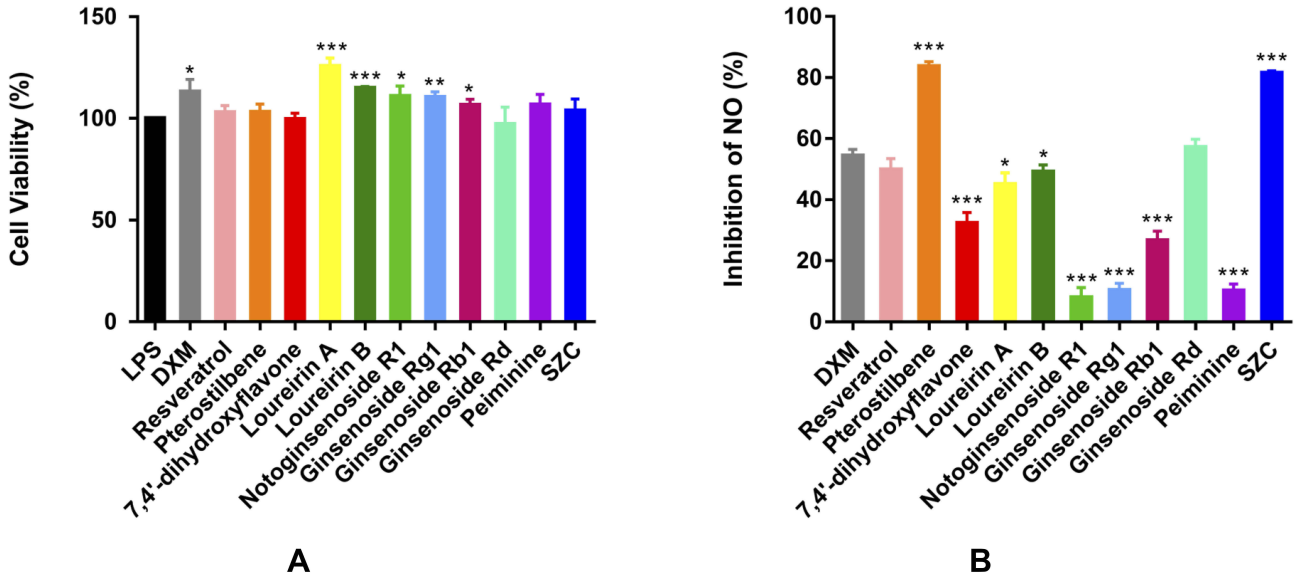

Figure 8 (**A**) Viability of LPS stimulated RAW264.7 cells after being treated by active compounds of SZC and (**B**) inhibition of synthesis of nitric oxide by active compounds of SZC at concentrations of 20 μM (resveratrol, pterostilbene, 7,4'-dihydroxyflavone), 50 μM (loureirin A, loureirin B, notoginsenoside R1, ginsenoside Rg1, ginsenoside Rb1, ginsenoside Rd, and peiminine), 50 μg/mL (SZC), and 1 μM (DXM) as positive control. Data were obtained from triplicate independent experiments and are presented as means (± SD). *P < 0.05, **P < 0.01, ***P < 0.001.

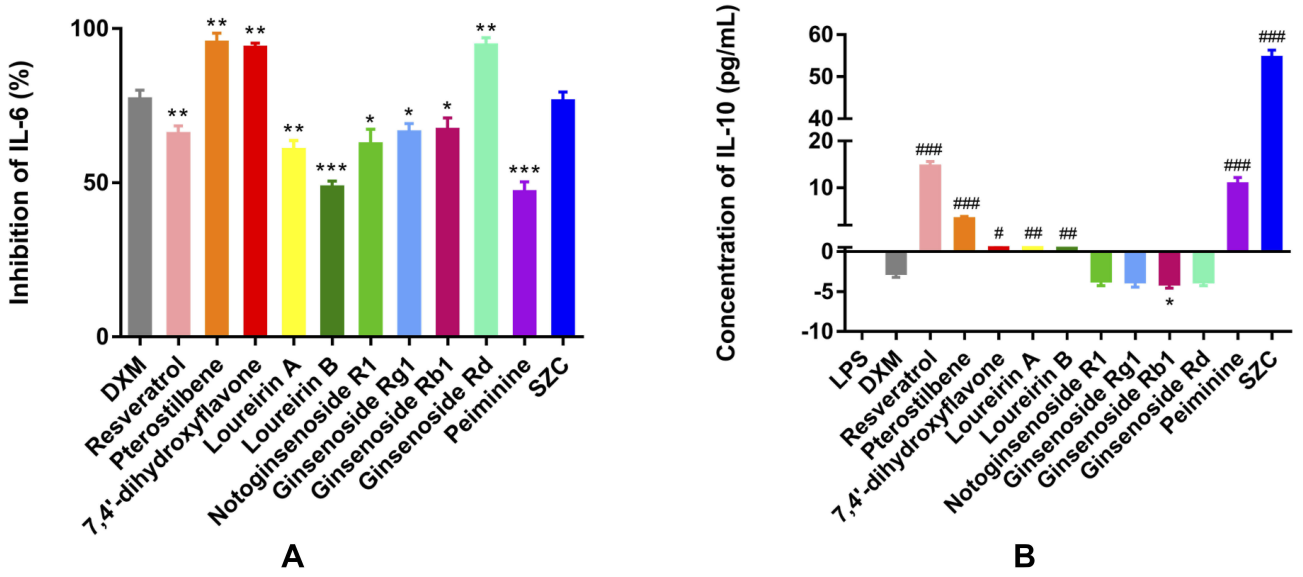

Figure 9 (**A**) Inhibition of releasing IL-6 by active compounds of SZC and (**B**) production of IL-10 after being treated by active compounds of SZC at concentrations of 20 μM (resveratrol, pterostilbene, 7.4'-dihydroxyflavone), 50 μM (loureirin A, loureirin B, notoginsenoside R1, ginsenoside Rg1, ginsenoside Rb1, ginsenoside Rd, and peiminine), 50 μg/mL (SZC), and 1 μM (DXM) as positive control. Data were obtained from triplicate independent experiments and presented as means (± SD). *P < 0.05, **P < 0.01, ***P < 0.001 versus DXM, #P < 0.05, ##P < 0.01, ###P < 0.001 versus LPS.

eNOS activity regulation.[42] It has been demonstrated that PI3K-Akt is essential in decreasing inflammatory cytokines induced with LPS (IL-6, TNF-α, and IL-10),[43,44] accompanied by a decrease in cell proliferation and migration.[45] Additionally, the PI3K-Akt-mTOR (mammalian target of rapamycin) signaling pathway regulates cellular processes through the co-regulated targets with different functions. For example, cytoskeletal changes are associated with PI3K-AKT-mTOR signaling mediated alterations in focal adhesion kinase (FAK). Activated mTORC1 initiates pro-metastatic actin cytoskeleton remodeling by activating ROCK.[46] Moreover, it has been validated that the expression of phosphorylated mTOR was significantly higher in the ectopic endometrial

than that in the eutopic endometria of AM patients, indicated that modulating the PI3K-Akt-mTOR signaling pathway to regulate inflammation might provide a promising therapeutic approach for AM.[47]

As for the NF-κB signaling pathway, it is the canonical pathway broadly involved in regulating various physiological activities including immunity, inflammation, cell survival, and cell proliferation. The NF-κB protein can be stimulated by a variety of agents like cytokines, chemokines, microbial and viral products, and DNA damage. The genes regulated by NF-κB are highly associated with many other signaling pathways, and the outcome of NF-κB followed the diverse inductions of cells and special regulations of its target genes.[48] For example, NF-κB signaling can be deregulated by microbial products and genetic alterations in NF-κB and other signaling pathway components in many human diseases, including cancers and chronic inflammation.[49] In inflammatory responses, NF-κB plays an essential role in modulating the expression of pro-inflammatory cytokines. It was reported that the TLR4-mediated inflammatory response induced by LPS could be suppressed through the inhibition of iNOS and COX-2 expression and the decrease of NO and pro-inflammatory cytokines (including IL-1β and IL-6) by inhibiting the Akt-NF-κB pathway.[50] Besides, the NF-κB signaling pathway was examined to explain how the anti–inflammatory effect was processed by reducing the production of NO, the expression of iNOS and COX-2, and pro-inflammatory cytokines (including TNF-α, IL-1β, IL-6, and MCP-1).[51] It was also revealed that the anti–inflammatory activity could be exerted through the NF-κB and PI3K/Akt pathways by influencing IL-10, IL-6, and TNF-α release, regulating iNOS, COX-2, NF-κB, Akt, and p-Akt expression.[52] Most of all, NF-κB plays a crucial role in the NF-κB signaling pathway, which was strongly suggested to be the potential target in the treatment of AM, at least in terms of relieving dysmenorrhea and menorrhagia. Not only does it regulate COX-2 but also the genes and their receptors encoding pro-inflammatory cytokines and chemokines, the expression of which has been reported to be related to inflammation in AM.[53–55]

Focal adhesion refers to the specialized structures formed at the cell-extracellular level, which are mediated by membrane receptors, actin-cytoskeleton, protein kinases, and phosphatases, playing an essential role in important biological processes including cell motility and proliferation. It was found focal adhesion took part in epithelial-mesenchymal transition associated with the pathogenesis of AM by regulating FAK, and in a laboratory investigation, results suggested the process might be associated with the PI3K-Akt pathway.[56] Besides, the research revealed that secreted phosphoprotein 1 (SPP1) was the key protein expressed in specific phenotypes with different levels.[57] Furthermore, the abnormal expression of SPP1 might cause infertility in some patients with AM.[58]

Endocrine resistance is regarded as pathogenesis of hormone-dependent diseases. This pathway can be activated by an estrogen signaling pathway to express ER and is closely related to the expression of growth factors, especially the growth factors on the PI3K-Akt-mTOR pathway. It has been proved that upregulating growth factor receptor pathways might help cells escape estrogen or ER dependence through an alternative mechanism.[59] Furthermore, experiments on the inhibition of the apoptotic effects of estrogen stimulation on ER+ cells suggested that a combined blockade of ER and PI3K might be the most effective.[60] On the other hand, MMP-2 and MMP-9 mediated by EGFR are crucial for modulating invasion and metastasis because the regulation occurs through promoting PI3K.[61] Additionally, Miller has discussed that activation of the Cyclin D-associated pathway could promote endocrine resistance, and treatment with the inhibitors could abrogate the proliferation of endocrine-resistant cells.[62]

The Anti-Inflammatory Activity of SZC in vitro

SZC exhibited the anti–inflammatory activity by the cooperation of multi-compound through inhibiting the release of NO and IL-6, and promoting the release of IL-10 in vitro. The compounds contributed differently to the SZC anti–inflammatory activity of SZC, for instance, the flavonoids (resveratrol, pterostilbene, 7,4'-dihydroxyflavone, loureirin A, loureirin B) and the alkaloid (peiminine) exhibited the same trend in regulating NO, IL-6 and IL-10 regulation with SZC. While the saponins (notoginsenoside R1, ginsenoside Rg1, ginsenoside Rb1, ginsenoside Rd) exhibited the same trend in NO, IL-6, but the opposite trend with IL-10 versus SZC, eventually the IL-10 release promotion of flavonoids outweighed the inhibition of the saponins. As the anti–inflammatory cytokine, the production of IL-10 counteracts the expression of IL-6, which is the pro-inflammatory cytokine during inflammation.[63] Besides, the mechanism of the inflammatory response caused by overactivation of macrophages involves phenotypic changes of M1/M2-type macrophages, the pro-inflammatory cytokines such as IL-6, TNF-α, MCP-1, iNOS are produced by M1-type macrophages, and

the anti–inflammatory cytokine including IL-4, IL-10, TGF-β1 are produced by M2-type macrophages.[64,65] Our results revealed that both SZC and compounds could inhibit IL-6 production, while the flavonoids, alkaloid, and SZC promoted IL-10 production. It can be inferred that SZC performed the anti–inflammatory activity by inducing the M1 pro-inflammatory phenotype to polarize to the M2 anti–inflammatory phenotype, and that might be contributed by the flavonoids and alkaloid of SZC. However, the M2 activation might be restrained by saponins downregulating the expression of M2 markers, such as CD206, IL-10, and YM-1 during the inflammation.[66] As the saponins of SZC was found to reduce IL-10 production in our study, we deduced that the activation of macrophages toward M1 and M2 phenotype might coexist during the anti–inflammatory activity of SZC, more M1/M2 subtypes should be examined extensively in further studies.

In clinical research, Li has validated that IL-6 and IL-8 were over-expressed in the eutopic and ectopic endometrium of patients with AM.[67] On the other hand, the expression of IL-10 was decreased in uteri of AM patients, that was supposed to be associated with impaired endometrium receptivity in AM patients.[68] It also has been reported that NO, IL-10, IL-17A, IL-7, and MCP-1 were all found to possibly contribute to the pathogenesis of AM.[69,70] As mentioned above, the NO level and inflammatory cytokines including IL-6, and IL-10 were closely related to the anti–inflammatory activity, might be decreased by suppressing the PI3K-Akt signaling pathway and NF-κB signaling pathway.[71,72] Therefore, it can be concluded that the anti–inflammatory activity of SZC is achieved by the synergetic action of the active compounds inhibiting the NO synthesis, IL-6 expression and the regulation of IL-10 expression through influencing the PI3K-Akt signaling pathway and NF-κB signaling pathway.

Conclusion

In this study, a systematic network pharmacology analysis approach for exploring the mechanism of SZC in the treatment of AM was established and the anti–inflammatory activity of SZC was observed in vitro. The integrated approach automatically acquired the information and linked the docking platform, identified the potential active compounds and targets based on molecular docking combining with CTPG prediction, then implemented the PPI analysis, network construction and KEGG analysis, which provide insights into the biological activity of SZC. The results were as follows. First, 30 major compounds and 28 core targets were identified from the potential active ones. Second, five main therapeutic modules involving inflammation reaction, hormone regulation, cell adhesion, proliferation, and angiogenesis were collected. Third, four crucial intersected pathways including PI3K-Akt signaling pathway, NF-κB signaling pathway, focal adhesion, and endocrine resistance were mapped, among them, the PI3K-Akt signaling pathway and NF-κB signaling pathway were mainly related to the inflammation reaction module. Four, the anti–inflammation activity of SZC was the act by the cooperation of its multiple components regulating NO, IL-6, and IL-10, which was consistent with the analysis result. Overall, our findings indicated that SZC achieved the treatment of AM by influencing inflammation reaction, hormone regulation, cell adhesion, proliferation, and angiogenesis, furthermore, its anti–inflammatory activity was conducted by the multi-compound through inhibiting NO and IL-6, both inhibiting and promoting IL-10, which will provide rich clues for the further research on SZC in the treatment of AM. However, the ADME information and the content of compounds in SZC should be investigated in future research.

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Author Contributions

All authors contributed to data analysis, drafting and revising the article, gave final approval of the version to be published, and agree to be accountable for all aspects of the work.

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Disclosure

Wei Xiao is employed by Jiangsu Kanion Pharmaceutical Co., Ltd, The authors report no other conflicts of interest in this work.

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