Utilizing network pharmacology and molecular docking to explore the underlying mechanism of Guizhi Fuling Wan in treating endometriosis

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ABSTRACT

Background. Guizhi Fuling Wan (GZFLW) is a widely used classical Chinese herbal formulae prescribed for the treatment of endometriosis (EMs). This study aimed to predict the key targets and mechanisms of GZFLW in the treatment of EMs by network pharmacology and molecular docking.

Methods. Firstly, related compounds and targets of GZFLW were identified through the TCMSP, BATMAN-TCM and CASC database. Then, the EMs target database was built by GeneCards. The overlapping targets between GZFLW and EMs were screened out, and then data of the PPI network was obtained by the STRING Database to analyze the interrelationship of these targets. Furthermore, a topological analysis was performed to screen the hub targets. After that, molecular docking technology was used to confirm the binding degree of the main active compounds and hub targets. Finally, the DAVID database and Metascape database were used for GO and KEGG enrichment analysis.

Results. A total of 89 GZFLW compounds and 284 targets were collected. One hundred one matching targets were picked out as the correlative targets of GZFLW in treating EMs. Among these, 25 significant hub targets were recognized by the PPI network. Coincidently, molecular docking simulation indicated that the hub targets had a good bonding activity with most active compounds (69.71%). Furthermore, 116 items, including the inflammatory reaction, RNA polymerase, DNA transcription, growth factor activity, and steroid-binding, were selected by GO enrichment analysis. Moreover, the KEGG enrichment analysis results included 100 pathways focused on the AGE-RAGE pathway, HIF pathway, PI3K Akt pathway, MAPK pathway, and TP53 pathway, which exposed the potential mechanisms of GZFLW in treating EMs. Also, the MTT colorimetric assay indicated that the cell proliferation could be inhibited by GZFLW. Compared with the control group, the protein levels of P53, BAX, and caspase3 in the drug groups were all increased in Western blotting results. The results of flow cytometry showed that the percentage of apoptotic cells in the GZFLW group was significantly higher than that in the control group.

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Conclusion. Through the exploration of network pharmacology and molecular docking technology, GZFLW has a therapeutic effect on EMs through multi-target mechanism. This study provided a good foundation for further experimental research.

Subjects Bioinformatics, Computational Biology, Evidence Based Medicine, Translational Medicine, Women's Health
Keywords Herbal medicine, Complementary medicine, Natural product, Endometriosis, Network pharmacology, Molecular docking

INTRODUCTION

Endometriosis (EMs), a common painful gynecological disease, occurs in 10% women of childbearing age (*Reid et al., 2019*). The most frequent symptoms of EMs include dysmenorrhea, chronic pelvic pain, and infertility (*Bruun et al., 2018*; *Lalani et al., 2018*). The incidence of developing many comorbidities, including irritable bowel syndrome, constipation, ovarian cancer, endometrial cancer, is significantly higher among EMs patients comparing to the general population (*Schomacker et al., 2018*). Although current therapies, including surgery, non-steroidal anti-inflammatory drugs (NSAIDs), hormone treatments, and so on, could relief some significant symptoms of EMs, these treatments have also led to some side effects and a high recurrence rate (*Prefumo & Rossi, 2018*; *Rabinerson, Hiersch & Gabbay-Ben-Ziv, 2018*). The patient's quality of life declines, and half of them are not satisfied with the available medical support (*Verket et al., 2018*). Therefore, it is still necessary to find novel and useful treatment methods (*Lukas et al., 2018*).

Historical Chinese medical texts have documented the use of the traditional Chinese herbal formula Guizhi Fuling Wan (GZFLW) for the EMs-like symptoms such as dysmenorrhea since the late Eastern Han Dynasty (200-210AD), and it is still widely used today for the treatment of EMs (*Li et al., 2018*; *Wu et al., 2015*; *Zhao, 2016*) due to excellent therapeutic effect, low side effects (*Wang et al., 2018*), and safety. GZFLW consists of the original powder of five natural plants, including Cinnamon Twig (CT), Poria Cocos (PC), Cortex Moutan (CM), Radix Paeoniae Rubra (RPR), and Peach Kernel (PK). Animal experiments have confirmed that GZFLW could relieve dysmenorrhea effectively (*Lang et al., 2018*; *Yang, 2019*). Nevertheless, the complex pathogenesis of EMs and the multiplex mechanism of GZFLW remains unclear. The principles and mechanisms by which GZFLW is useful for treating EMs need to be uncovered.

The holistic treatment of traditional Chinese medicine (TCM) has attracted more and more attention. The mechanism of drug therapy has shifted from single target to multiple interacting targets mediated by multiple compounds. Nowadays, the extensive application of network pharmacology and molecular docking provides a more effective method for the research and evaluation of the multi-target effect of multi-component drugs on diseases, which can reveal the mechanism of TCM treatment from a holistic perspective (*Hopkins, 2008*). In the progress of bioinformatics and pharmacy, network pharmacology has become a capable vehicle to reveal the compatibility mechanism of TCM prescription (*Fang et al., 2017*; *Ming et al., 2017*; *Zhao et al., 2015*). An increasing number of researches have used

them to analyze the possible molecular mechanisms of TCMs. This systematic research conception is consistent with the holistic theory and the synergistic mechanism of TCM (*Lang et al., 2018*). However, the possible mechanism of GZFLW treating EMs has not been systematically studied by network pharmacology.

Therefore, in this study, we screened multiple databases to find the active components of GZFLW and its possible targets for treating EMs. Target genes of GZFLW and EMs were matched to obtain overlapping results. PPI network data was obtained through the STRING database, and the hub targets were screened through topology analysis. After that, molecular docking technology was used to screen and verify the binding degree of the main active compounds and hub targets. Finally, the DAVID database and Metascape database were used for gene ontology (GO) and Kyoto Encyclopaedia of Genes and Genomes (KEGG) enrichment analysis. GO is generally used to describe the genes function and genes relationships (*Ashburner et al., 2000*), and KEGG is used for the enrichment of functions and signaling pathways (*Kanehis & Goto, 2000*). The vital active ingredients and mechanism of GZFLW in the treatment of EMs were confirmed by molecular docking technology. The workflow is shown in Fig. 1.

MATERIALS & METHODS

Chemical ingredients database building

To collect the chemical ingredients of the five herbs contained in GZFLW, the Traditional Chinese Medicine Systems Pharmacology Database (*Ru et al., 2014*) (TCMSP, https://tcmspw.com/tcmsp.php), the Bioinformatics Analysis Tool for Molecular Mechanism of Traditional Chinese Medicine (*Liu et al., 2016*) (BATMAN-TCM, http://bionet.ncpsb.org/batman-tcm/index.php) and Chinese Academy of Sciences Chemistry Database (*Wang et al., 2015*) (CASC, http://www.organchem.csdb.cn/scdb/default.htm) were used, which are both bioinformatics analysis tools for the main components of TCM. Four hundred ninety-four herbal ingredients were screened out.

Active ingredients screening

According to the characteristics of absorption, distribution, metabolism, and excretion (ADME) of drug, oral bioavailability (OB), and drug-likeness (DL) were used as screening indexes. OB is the percentage of oral drugs absorbed into the bloodstream, which is a frequently-used pharmacokinetic parameter. The OB was obtained using the OBioavail1.1 software, which covers 805 different drug and drug-like molecules (*Liu et al., 2013*). OB is a crucial indicator to judge whether the active ingredient can become a feasible therapeutic molecule (*Xu et al., 2012*). DL is a parameter to measure the ADME of drug molecules, which could help optimize pharmacokinetic and pharmaceutical properties. The DL threshold is 0.18 (*Liu et al., 2013*), which depends on the average of Drugbank (https://www.drugbank.ca/). DL is often used to select the active ingredients with "drug-like" properties in TCM composition (*Tao et al., 2013*). The OB and DL indices of all the related ingredients are presented in the TCMSP. In this process, those ingredients with OB $\geqq$30% and DL $\geqq$0.18 were chosen as the potential effect components for the next step.

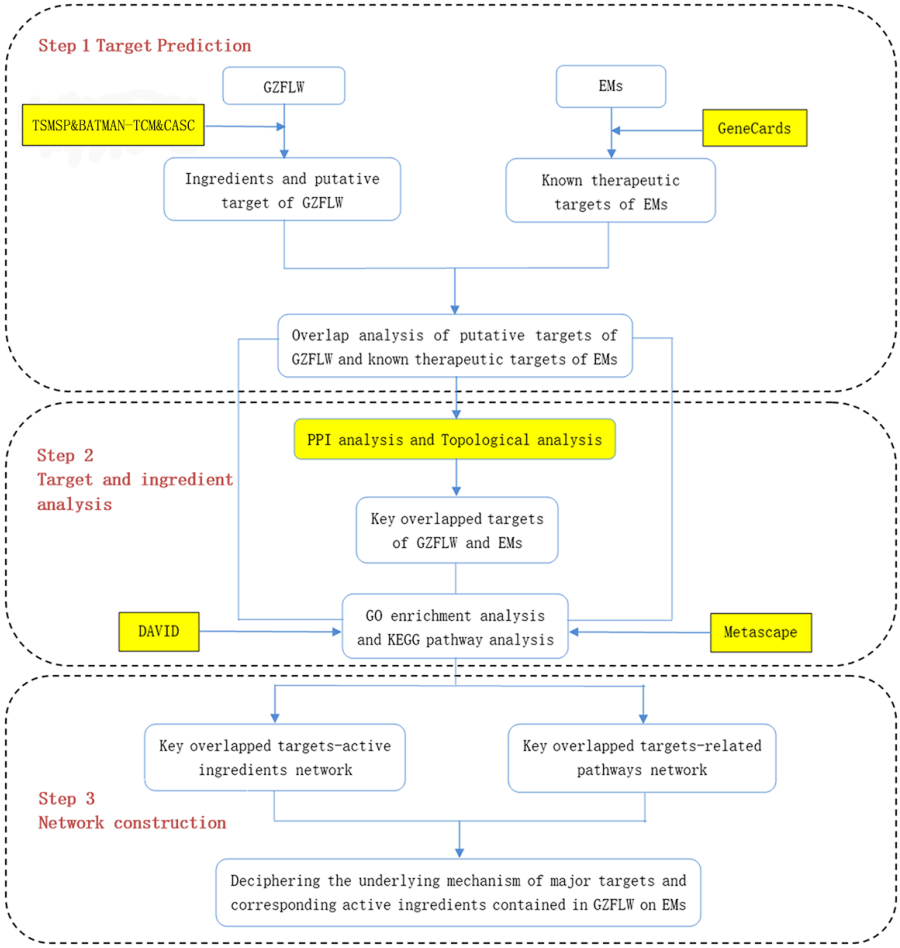

Figure 1 **The whole framework of the research process.**

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GZFLW targets prediction

The active compounds' targets in GZFLW were obtained from TCMSP, BATMAN-TCM, and CASC database, with the species limited as "Homo sapiens".

Endometriosis therapeutic targets database building

Related targets of EMs were screened out by the GeneCards Database (*Stelzer et al., 2016*) (https://www.genecards.org/). The GeneCards database is often used to predict genetic information related to human diseases. We searched the database with the keyword "endometriosis" to obtain targets. Finally, the targets of GZFLW active ingredients were matched with the therapeutic targets of EMs. The overlapping targets were chosen as the potential targets of GZFLW in the treatment of EMs. These targets were then uploaded to STRING Database (*Szklarczyk et al., 2017*) (http://string-db.org/) to obtain the interactions of the screened targets with the confidence $\geqq$0.7 and the result of protein-protein interaction (PPI).

Network topological feature set definitions

We chose two parameters to evaluate the topological characteristics: "Degree" reflects the number of other nodes interacting with this node; "Betweenness Centrality" (BC) is measured by the percentage of all shortest paths. The nodes with high betweenness can significantly impact the net by controlling the information transmitted between other nodes. The node parameters are positively correlated with their topology importance in the network.

Molecular docking of hub targets and active ingredients

Molecular docking is a computational tool to predict the binding ability and connection type of proteins and ligands. It can calculate and predict the conformation and direction of ligands at active protein sites. AutoDock 1.5.6 (http://autodock.scripps.edu/) was a molecular docking software that could be used to dock the hub targets and active ingredients based on network pharmacology. In this docking process, the 3D structure of thirteen hub targets were retrieved from RCSB Protein Data Bank (PDB) (http://www.rcsb.org/): AKT1 (PDB ID: 4EKL, 6S9W), TNF (PDB ID: 2E7A, 2ZJC, 2AZ5), TP53 (PDB ID: 4MZI, 6FF9), VEGFA (PDB ID: 4WPB, 3QTK, 4QAF), MAPK1 (PDB ID: 4G6N, 1WZY), MMP9 (PDB ID: 6ESM, 4WZV), JUN (PDB ID: 5T01, 1T2K), MAPK8 (PDB ID: 3VUK, 4L7F), INS (PDB ID: 4AJX, 4CY7), EGF (PDB ID: 1JL9,), IL6 (PDB ID: 4O9H, 1ALU), PTGS2 (PDB ID: 5F19), and FOS (PDB ID: 1FOS); the 3D shapes of active compounds were provided from ZINC Database (http://zinc.docking.org/) and PubChem Database (https://pubchem.ncbi.nlm.nih.gov/). Binding energy was used as docking score to evaluate the protein-ligand binding potential of molecular docking. Among them, those results with value $\leq$ -5 were selected and considered to have moderate binding potential and tight combination.

Enrichment analysis

We used the DAVID (https://david.ncifcrf.gov/) (*Huang, Sherman & Lempicki, 2008*) for GO enrichment analysis, including biological process (BP), molecular function (MF) and cellular component (CC). KEGG enrichment analysis was performed using the Metascape Database to obtain potential target-pathways (*Zhou et al., 2019*) (http://metascape.org/).

Network construction

Networks, including the hub target-compound and target-pathway, were then constructed. All networks were built using Cytoscape 3.7.2 (http://www.cytoscape.org/) (*Shannon et al., 2003*), an open-source software platform for visualization and data analysis of complex networks.

Primary endometrial stromal cells culture

HEM15a cells 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. After that, the vimentin staining of cells was identified by immunofluorescence.

MTT colorimetric assay

The hEM15a cells were seeded in a 96-well plate and divided into the control group and the drug group. After incubation at 37 °C for 48 h and reacted with GZFLW of different concentrations, hEM15a cells were reacted with 10 μL MTT solution. Finally, 150 μL dimethylsulfoxide (DMSO) was added after reacting for 4 h. The optical absorbance was detected at 568 nm by a plate reader.

Western blotting

The hEM15a cells were splitted in RIPA buffer (Beyotime) added with 10 μl PMSF (Aladdin) and phosphatase inhibitors (Beyotime) to extract total proteins. After that, equivalent amounts of proteins were resolved by poly acrylamide gel electrophoresis (PAGE) and transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, Massachusetts, USA). Then, TBST with 5% skim milk was adopted for blocking. Afterward, the membranes were reacted with primary antibodies against GAPDH (1:1000, AB-P-R 001, Xianzhi, Hangzhou, China), BAX (1:1000, Ab32503, Abcam, Britain), P53 (1:1000, 10442-1-AP, Sanying, Wuhan, China), and caspase3 (1:2000, Ab184787, Abcam), at 4 °C overnight. Subsequently, the matched secondary antibody was added to the membranes.

Apoptosis analysis by flow cytometry

The apoptosis of hEM15a cells was evaluated by Annexin V-FITC/PI apoptosis kit (KaiJi, Nanjing, China). The collected hEM15a cells, divided into normal group and GZFLW group, were resuspended in 500 ul binding buffer and added with Annexin V-PI solution. Then, the hEM15a cells were incubated for 5∼15 min without light. The percentage of apoptotic cells was then immediately detected on a flow cytometry (Beckmancoulter).

RESULTS

Active compounds of GZFLW

By retrieving from the TCMSP, BATMAN-TCM, and CASC database, there were 494 related ingredients of GZFLW in total, and there were 220 (44.5%) of CT, 34 (6.9%) of PC, 55 (11.1%) of CM, 119 (24.1%) of RPR, 66 (13.3%) of PK. With OB ≧30% and DL ≧ 0.18 as indexes, 89 active ingredients were screened out (Table 1), and the Herbs-Compounds network was constructed as Fig. 2.

Target prediction analysis

In this process, we collected 284 targets of 89 active ingredients, and there were 58 in CT, 48 in PC, 185 in CM, 104 in RPR, and 62 in PK. Via the keyword of "endometriosis," the therapeutic targets of EMs were obtained from GeneCard Database with a total of 1350. One hundred one overlapping targets were then obtained as the related targets of GZFLW in the treatment of EMs (Fig. 3).

The data of the PPI network of those 101 targets were subsequently obtained in STRING Database. With confidence ≧0.7, there were 95 nodes and 751 edges in total. Taking two essential parameters of "degree" and "betweenness" as screening indexes, the topological analysis of targets mentioned above was performed. Targets more significant than or equal

to the median are used as hub targets for GZFLW against EMs. The screened thresholds were degree ≧11.5 and betweenness ≧0.007, and the results were 25 hub nodes with 234 edges, including IL6, JUN, TNF, MAPK1, TP53, EGF, MAPK8, MMP9, VEGFA, AKT1, INS, FOS, ICAM1, PTGS2, CCL2, CCND1, EGFR, IL1B, MYC, IL10, PTEN, ESR1, PPARG, RELA and MMP2 (Table 2). When the 25 significant hub nodes and other 70 nodes were distributed with "degree" and "betweenness", the network of 95 nodes was built as Fig. 4.

Based on the 25 key targets and related 29 active ingredients, we further established the network of Hub nodes—Compounds (Fig. 5). Among them, quercetin (MOL000098) is associated with 21 hub targets, and kaempferol (MOL000422) interrelates to 7 key targets. Besides, baicalein (MOL002714) is related to 6 key targets, while pachymic acid (MOL000289), ellagic acid (MOL001002), and taxifolin (MOL004576) act on 3 key targets respectively.

Molecular Docking

Thirteen hub targets with top degrees of GZFLW were identified with seven active compounds by AutoDock. There were 122 results (69.71%) of them had a moderate binding potential, which indicated that active ingredients of GZFLW could well bind to the targets for the treatment of EMs (Table 3). The ligands are mainly linked with corresponding proteins and critical amino acids around them in the form of hydrogen bonds (Fig. 6).

GO and KEGG pathway enrichment analysis

With the database of DAVID and Metascape, the enrichment analysis on 101 targets was performed and resulted in 116 GO items and 100 KEGG pathways.

GO enrichment analysis

After 116 items were sorted in descending order based on *P*-value, the first eight items of three parts, BP, MF, and CC, were selected (Fig. 7). In the aspect of BP, we mainly had: inflammatory response (GO:0006954), positive regulation of transcription from RNA polymerase II promoter (GO:0045944), transcription DNA-templated (GO:0006351), lipopolysaccharide-mediated signaling pathway (GO:0031663), positive regulation of cell division (GO:0051781), negative regulation of growth of symbiont in host (GO:0044130), response to toxic substance (GO:0009636) and positive regulation of cytokine secretion (GO:0050715); in the part of MF, we obtained heme binding (GO:0020037), sequence-specific DNA binding (GO:0043565), transcription factor activity sequence-specific DNA binding (GO:0003700), growth factor activity (GO:0008083), identical protein binding (GO:0042802), protein homodimerization activity (GO:0042803), cytokine activity (GO:0005125) and steroid binding (GO:0005496); in the aspect of CC, there were extracellular space (GO:0005615), nucleus (GO:00056340), cytosol (GO:0005829), membrane raft (GO:0045121), apical plasma (GO:0016324), external side of plasma membrane (GO:0009897), endoplasmic reticulum membrane (GO:0005789) and extrinsic component of external side of plasma membrane (GO:0031232). Based on the above three aspects, it is possible that the mechanism of GZFLW in treating EMs was the result of multi-pathway synergy.

Table 1 Information for 89 active ingredients.

Herb name	Mol ID	compound	Code name	OB/%	DL/%
Cinnamon Twig	MOL001736	(-)-taxifolin	CT-1	60.51	0.27
	MOL004576	taxifolin	CT-2	57.84	0.27
	MOL000492	(+)-catechin	CT-3	54.83	0.24
	MOL000073	ent-Epicatechin	CT-4	48.96	0.24
	MOL000358	beta-sitosterol	CT-5	36.91	0.75
	MOL000359	sitosterol	CT-6	36.91	0.75
	MOL000991	cinnamaldehyde	CT-7	31.99	0.02
Paria cocos	MOL000282	ergosta-7,22E-dien-3beta-ol	PC-1	43.51	0.72
	MOL000283	Ergosterol peroxide	PC-2	40.36	0.81
	MOL000275	trametenolic acid	PC-3	38.71	0.8
	MOL000296	hederagenin	PC-4	36.91	0.75
	MOL000289	Pachymic Acid	PC-5	33.63	0.81
	MOL000273	(2R)-2-[(3S,5R,10S,13R,14R,16R,17R)-3, 16-dihydroxy-4,4,10,13,14-pentamethyl-2,3, 5,6,12,15,16,17-octahydro-1H-cyclopenta[a]phenanthren-17-yl] -6-methylhept-5-enoic acid	PC-6	30.93	0.81
Cortex Moutan	MOL000211	Mairin	CM-1	55.38	0.78
	MOL000492	(+)-catechin	CM-2	54.83	0.24
	MOL000098	quercetin	CM-3	46.43	0.28
	MOL000422	kaempferol	CM-4	41.88	0.24
	MOL000359	sitosterol	CM-5	36.91	0.75
	MOL000874	Paeonol	CM-6	28.79	0.04
Radix Paeoniae Rubra	MOL001918	paeoniflorgenone	RPR-1	87.59	0.37
	MOL006992	(2R,3R)-4-methoxyl-distylin	RPR-2	59.98	0.3
	MOL000492	(+)-catechin	RPR-3	54.83	0.24
	MOL001924	paeoniflorin	RPR-4	53.87	0.79
	MOL000449	Stigmasterol	RPR-5	43.83	0.76
	MOL001002	ellagic acid	RPR-6	43.06	0.43
	MOL004355	Spinasterol	RPR-7	42.98	0.76
	MOL002776	Baicalin	RPR-8	40.12	0.75
	MOL005043	campest-5-en-3beta-ol	RPR-9	37.58	0.71
	MOL006999	stigmast-7-en-3-ol	RPR-10	37.42	0.75
	MOL000358	beta-sitosterol	RPR-11	36.91	0.75
	MOL000359	sitosterol	RPR-12	36.91	0.75
	MOL002714	baicalein	RPR-13	33.52	0.21
	MOL002883	Ethyl oleate (NF)	RPR-14	32.4	0.19
Peach kenel	MOL001351	Gibberellin A44	PK-1	101.61	0.54
	MOL001353	GA60	PK-2	93.17	0.53
	MOL001349	4a-formyl-7alpha-hydroxy-1-methyl-8-methylidene-4aalpha, 4bbeta-gibbane-1alpha,10beta-dicarboxylic acid	PK-3	88.6	0.46

(*continued on next page*)

Table 1 (*continued*)

Herb name	Mol ID	compound	Code name	OB/%	DL/%
	MOL001344	GA122-isolactone	PK-4	88.11	0.54
	MOL001329	2,3-didehydro GA77	PK-5	88.08	0.53
	MOL001360	GA77	PK-6	87.89	0.53
	MOL001340	GA120	PK-7	84.85	0.45
	MOL001339	GA119	PK-8	76.36	0.49
	MOL001358	gibberellin 7	PK-9	73.8	0.5
	MOL001342	GA121-isolactone	PK-10	72.7	0.54
	MOL001361	GA87	PK-11	68.85	0.57
	MOL001355	GA63	PK-12	65.54	0.54
	MOL001352	GA54	PK-13	64.21	0.53
	MOL001328	2,3-didehydro GA70	PK-14	63.29	0.5
	MOL001323	Sitosterol alpha1	PK-15	43.28	0.78
	MOL001368	3-O-p-coumaroylquinic acid	PK-16	37.63	0.29
	MOL000493	campesterol	PK-17	37.58	0.71
	MOL000296	hederagenin	PK-18	36.91	0.75
	MOL000358	beta-sitosterol	PK-19	36.91	0.75
	MOL001320	Amygdalin	PK-20	4.42	0.61

KEGG pathway enrichment analysis

To further illustrate the potential mechanism of GZFLW in the treatment of EMs, we performed the KEGG pathway enrichment analysis on 101 targets. We selected the top 20 pathways based on the *P*-value, such as the AGE-RAGE signaling pathway(hsa04933), HIF-1 signaling pathway (hsa04066), PI3K-Akt signaling pathway (hsa04151), MAPK signaling pathway (hsa04010), EGFR tyrosine kinase inhibitor resistance (hsa01521) (Fig. 8). Then, we constructed the Target-Pathway Network to intuitively reveal the relationship between the hub targets and pathways (Fig. 9).

Primary endometrial stromal cells culture

The primary endometrial stromal cells were spindle-shaped or star-shaped with large and round nuclei, and proliferative fibrous tissue could be seen. After that, the immunofluorescence was used to identified the vimentin staining of cells in different groups (Fig. 10).

MTT colorimetric assay

In comparison with the control group, in addition to 0.5 mg/ml, the other different doses of treatment groups (1 mg/ml, 3 mg/ml, 5 mg/ml, 10 mg/ml, 20 mg/ml) had significant inhibitory effect on the proliferation of endometrial stromal cells ($P < 0.05$, 0.01 or 0.001; Fig. 11).

Western blotting

Western blot analysis revealed that compared to the control groups, drug treatment increased the protein levels of P53, BAX, and caspase3 ($P < 0.05$ or 0.01) in endometriotic lesions (Fig. 12).

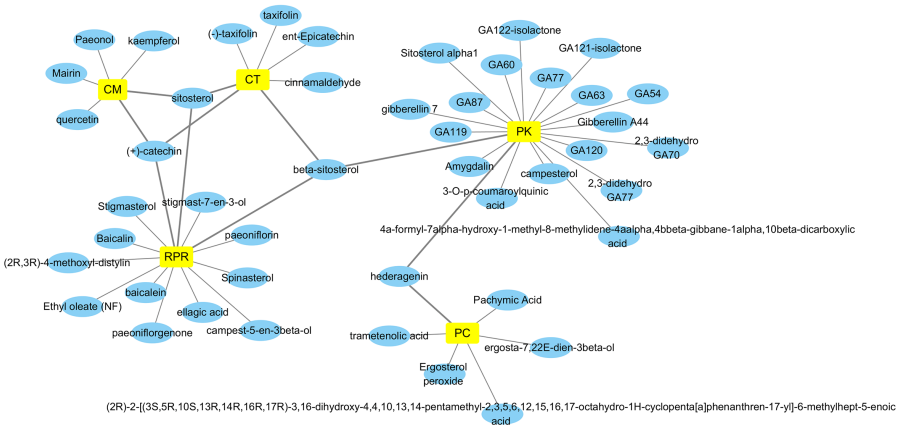

Figure 2 **Herbs-Compounds Network.** The yellow nodes represent herbs in GZFLW, and the blue nodes represent active compounds. CT represents Cinnamon Twig, PC represents Paria cocos, CM represents Cortex Moutan, RPR represents Radix Paeoniae Rubra, and PK represents Peach kenel. The edges represent the relationship between them.

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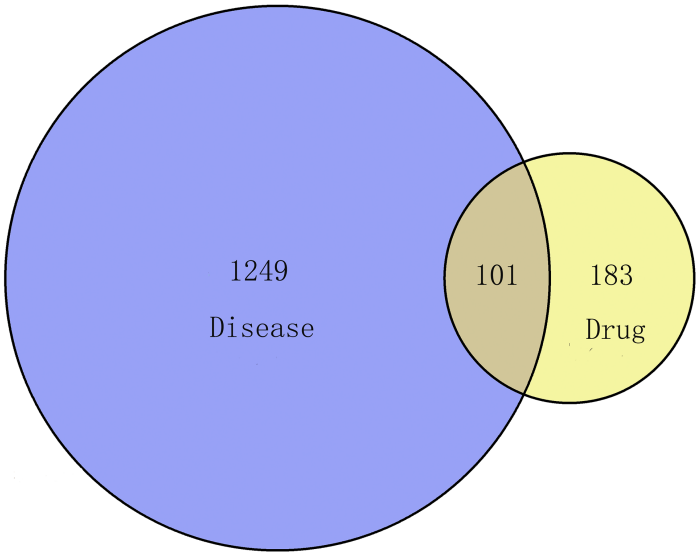

Figure 3 **The Venn diagram of the targets both in endometriosis targets and GZFLW targets.**

Full-size DOI: 10.7717/peerj.11087/fig-3

Apoptosis analysis by flow cytometry

Compared with the control group, the percentage of apoptotic cells in the GZFLW group with the dose of 4 mg increased significantly (Fig. 13).

DISCUSSION

As we all know, TCM is characterized by multiple components, multiple targets, and multiple pathways in the treatment of diseases (*Lang et al., 2018*). Because of the complex composition, TCM's clinical and pharmacological research is often difficult to carry out.

Table 2 Information for 25 hub targets.

NO.	Gene	Degree	BC	NO.	Gene	Degree	BC	NO.	Gene	Degree	BC
1	IL6	23	9.1459	10	AKT1	20	6.5181	19	MYC	17	3.4007
2	JUN	23	8.9097	11	INS	20	5.9093	20	IL10	16	2.4666
3	TNF	23	8.8208	12	FOS	19	6.1117	21	PTEN	16	3.0134
4	MAPK1	22	8.6040	13	ICAM1	18	3.4111	22	ESR1	15	1.5358
5	TP53	22	7.9533	14	PTGS2	18	5.1667	23	PPARG	15	2.2070
6	EGF	21	6.3421	15	CCL2	17	2.6724	24	RELA	15	2.6052
7	MAPK8	21	7.5937	16	CCND1	17	3.3755	25	MMP2	14	2.0489
8	MMP9	21	6.9767	17	EGFR	17	3.4135				
9	VEGFA	21	8.0120	18	IL1B	17	3.2828				

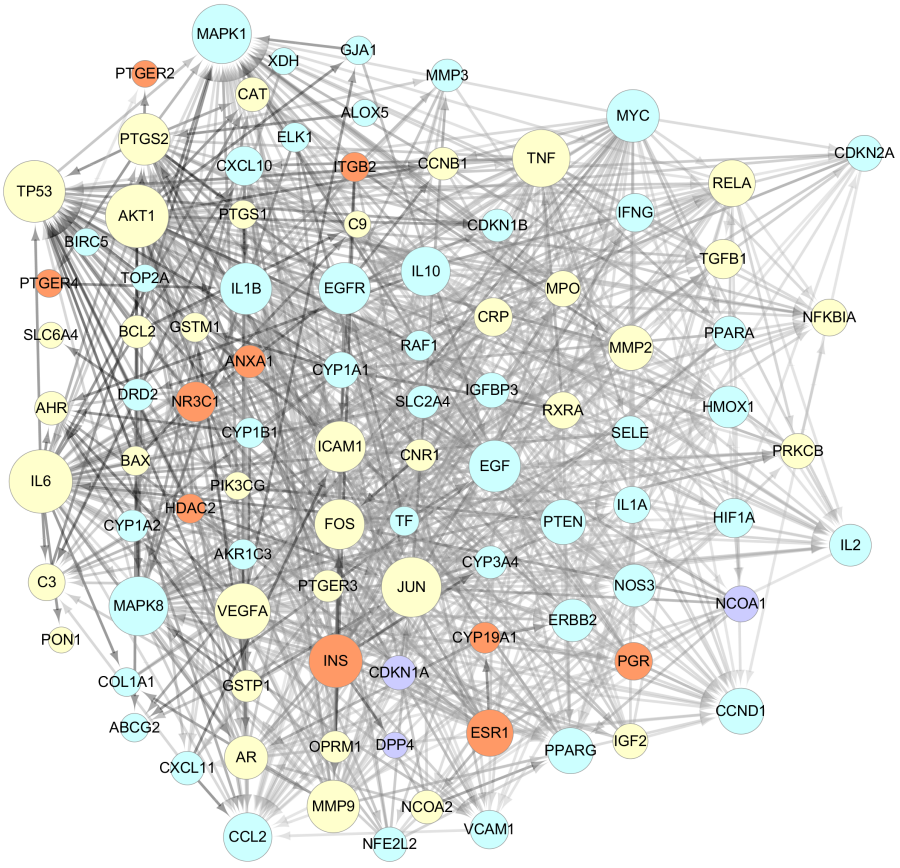

Figure 4 **The network of 95 nodes.** The different color represents different nodes from each ingredients: The blue nodes represent the targets from CM, the orange nodes represent the targets from PC, the purple nodes represent the targets from RPR, and the yellow nodes represent the targets that are targeted by more than one ingredient. The node size is proportional to the target degree in the network. The edge color changes from light to dark reflect the betweenness value changes from low to high in the network.
Full-size DOI: 10.7717/peerj.11087/fig-4

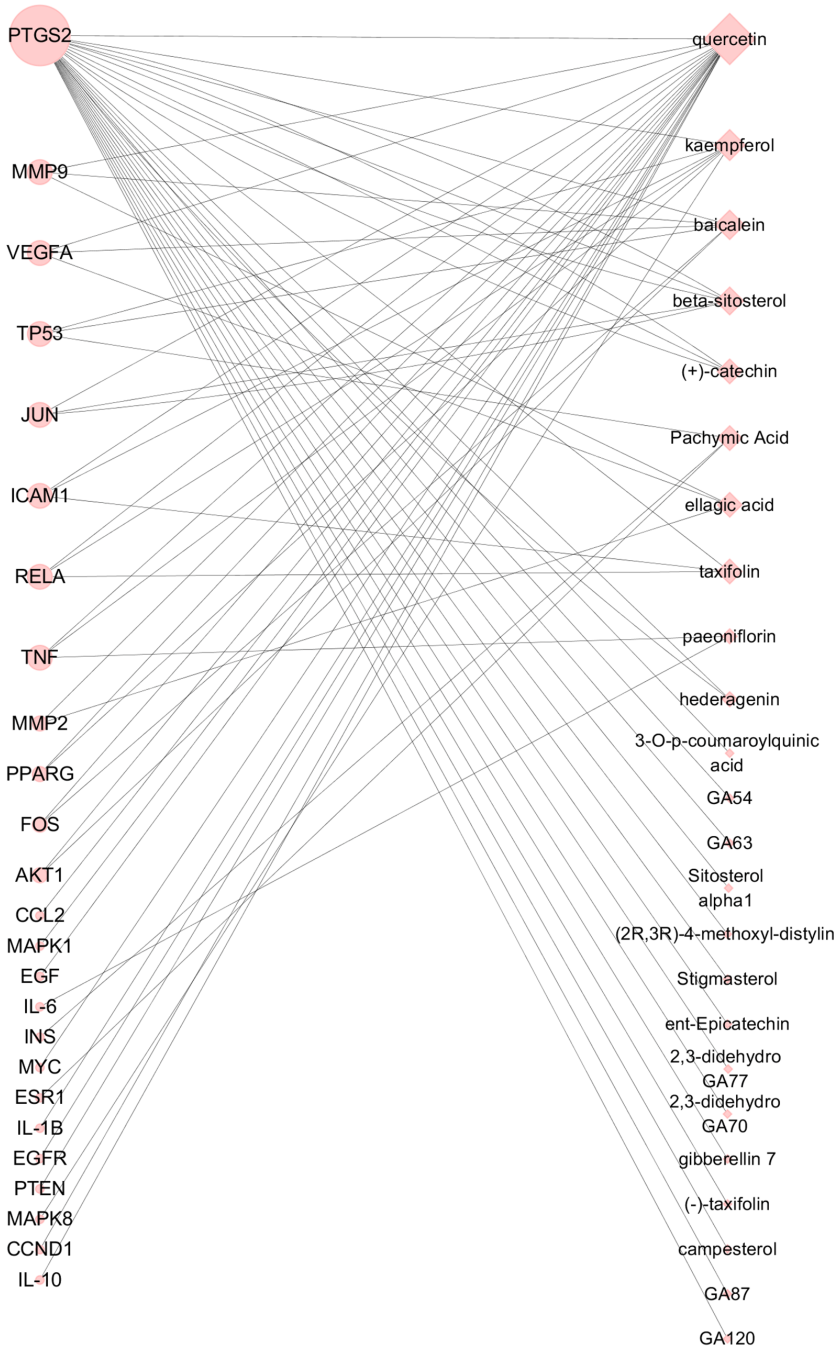

Figure 5 **Hub nodes-Compunds Network.** The circle nodes represent the significant hub nodes, and the diamond nodes represent the compounds. The node size is proportional to the target degree in the network.

Full-size DOI: 10.7717/peerj.11087/fig-5

Network pharmacology is a systematic research method, suitable for the "multi-component, multi-target, multi-pathway" synergistic characteristics of TCM. In this study, we are the first to explore the possible therapeutic mechanism of GZFLW on EMs through network

Table 3 The docking information of 13 targets with the corresponding compounds. The values in this table are docking scores that between targets ans compounds.

	Pachymic Acid	taxifolin	quercetin	paeoniflorin	beta-sitosterol	campesterol	hederagenin
AKT1-4EKL	−5.02	−7.65	−5.58	−4.19	−8.56	−8.27	−7.61
AKT1-6S9W	−7.85	−6.88	−5.08	−4.21	−8.59	−9.99	−8.79
MAPK1-6RQ4	−7.25	−6.22	−5.26	−3.61	−6.25	−7.16	−8.16
MAPK1-1WZY	−6.62	−6.51	−4.3	−3.7	−8.09	−6.64	−8.02
TNF-2E7A	−8.68	−6.61	−7.22	−4.2	−9.17	−10.31	−10.69
TNF-2ZJC	−4.25	−5.06	−3.45	−2.55	−4.9	−5.44	−4.98
TNF-2AZ5	−6.07	−4.57	−4	−3.55	−6.08	−7.16	−6.79
TP53-4MZI	−7.28	−7.11	−5.71	−5.12	−7.65	−6.27	−7.84
TP53-6FF9	−5.44	−4.19	−4.07	−2.93	−5.62	−5.14	−6.28
VEGFA-4WPB	−6.7	−5.81	−5.36	−4.09	−7.48	−7.01	−7
VEGFA-3QTK	−4.31	−3.99	−3.15	−2.58	−4.47	−6.18	−5.61
VEGFA-4QAF	−4.76	−5.41	−3.96	−2.4	−5.93	−5.64	−5.58
MMP9-6ESM	−7.83	−9.32	−7.55	−6.24	−9.03	−9.36	−8.83
MMP9-4WZV	−6.09	−7.53	−7.53	−4.45	−10.01	−10.5	−6.84
JUN-5T01	−5.46	−4.15	−4.63	−1.96	−5.51	−4.85	−7.6
JUN-1T2K	−6.28	−3.07	−3.3	−1.81	−5.17	−5.03	−6.43
MAPK8-3VUK	−6.25	−5.87	−6.17	−4.31	−7.05	−8.9	−6.99
MAPK8-4L7F	−4.96	−5.94	−4	−3.12	−5.5	−5.34	−6.96
INS-4AJX	−8.6	−6.48	−6.66	−6.32	−8.72	−9.18	−7.92
INS-4CY7	−6.62	−4.84	−5.6	−4.13	−6.56	−6.08	−7.58
EGF-IJL9	−7.42	−7.12	−7.29	−3.89	−7.94	−7.58	−9.14
IL6-4O9H	−6.49	−5.34	−4.66	−3.81	−5.96	−5.95	−6.48
IL6-1ALU	−5.23	−3.94	−3.74	−2.5	−4.58	−5.09	−6.45
PTGS2	−7.44	−5.77	−4.24	−2.86	−5.85	−5.87	−9.63
FOS	−5.71	−3.17	−3.57	−1.46	−3.63	−6.04	−6.1

pharmacology and molecular docking methods, to provide directions and ideas for further experimental research.

The major active ingredients of GZFLW

Based on the Hub nodes-Compound Network, we found a few hub compounds: quercetin and kaempferol contained in CM, taxifolin contained in CT, pachymic acid contained in PC, ellagic acid, baicalein, and paeoniflorin contained in RPR, campesterol, hederagenin contained in PK, β-sitosterol, contained in CM, RPR and PK. Among them, quercetin, kaempferol, taxifolin, and baicalein are flavonoids that have shown potent anti-EMs activity by effectively relieving symptoms and inhibiting levels of CA-125 (*Signorile, Viceconte & Baldi, 2018*). Quercetin and kaempferol can significantly reduce the ailing area of the endometrium via anti-proliferation and anti-inflammatory effects (*Park et al., 2019*) in EMs mice (*Ilhan et al., 2020*). Previous studies have demonstrated that phytosterols, including β-sitosterol and campesterol, contributed to the regression of EMs (*Ilhan et al., 2019*). Baicalin has also been reported to treat EMs by reducing the activity of endometrial stromal cell(ESC) (*Jin, Huang & Zhu, 2017*), which provided evidence for our results. In

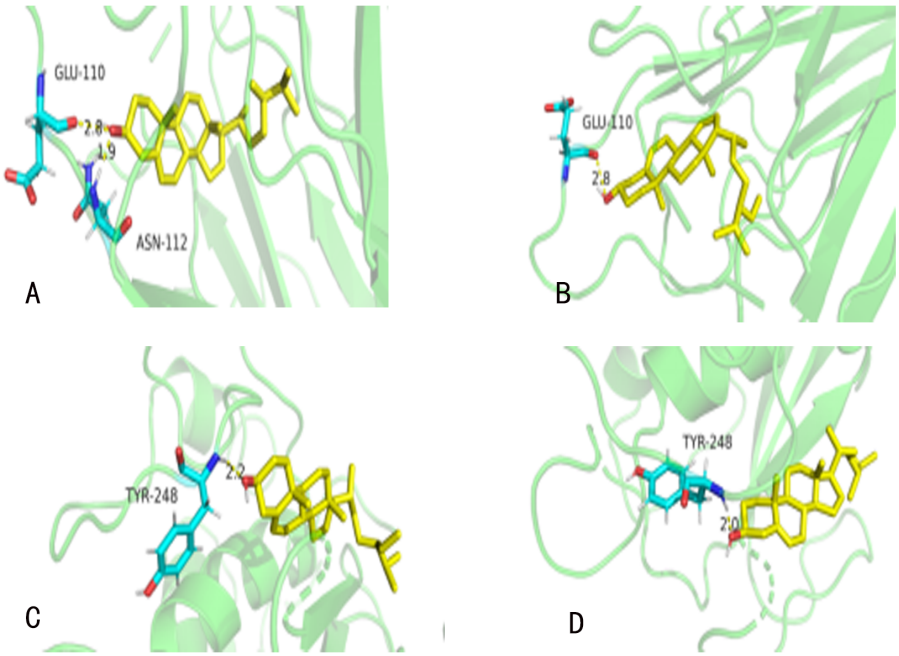

Figure 6 **The molecular docking.** (A) TNF (PDBID: 2E7A): campesterol molecular docking. (B) TNF (PDBID: 2E7A): beta-sitosterol molecular docking. (C) MMP9 (PDBID: 6ESM): campesterol molecular docking. (D) MMP9 (PDBID: 6ESM): beta-sitosterol molecular docking. The yellow sticks represent TNF and VEGFA. The blue sticks represent active compounds.

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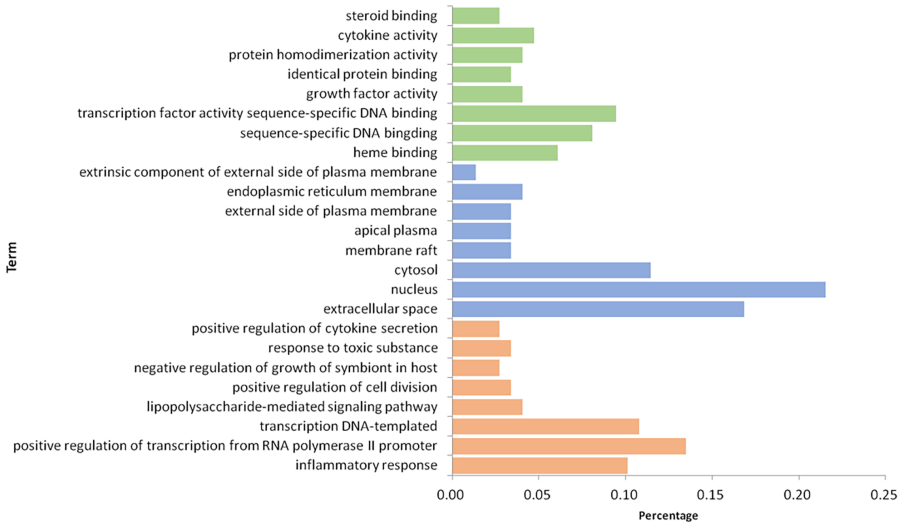

Figure 7 **The GO enrichment analysis of 101 nodes.** The orange part represents the Biological process. The blue part represents the Cellular component. The green part represents the Molecular function.

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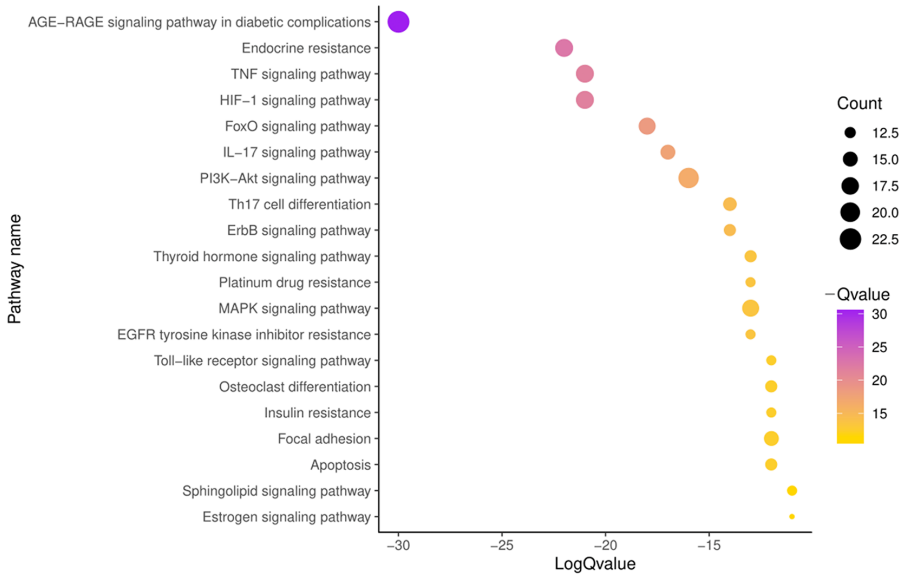

Figure 8 **The top 20 pathways of KEGG enrichment.**

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conclusion, we speculate that these above ingredients are the potential material basis of GZFLW in treating EMs.

It is mainly indicated that the pathways may be associated with treating EMs of GZFLW were regarding the immune response, apoptosis and proliferation, oxidative stress, and angiogenesis.

Inflammatory response

The differentially expressed genes are related to inflammation in EMs (*Ahn et al., 2016*), which suggest its role in the course of EMs. Prostaglandin-endoperoxide synthase (PTGS2), also known as COX-2, is related to the pain and infertility of EMs and the PTGS2/ PGE2 axis is considered as the critical target during EMs (*Li et al., 2020*). TNF, IL1- β are the other hub targets in this study. The immunoreactivity of which induces inflammation in the peritoneal fluid of EMs through activating NF-kappa B and mitogen-activated protein kinase (MAPKs) signaling pathways (*Kralickova et al., 2018*). To some extents, components such as hederagenin, campesterol, β-sitosterol, pachymic acid, quercetin, and taxifolin in GZFLW inhibit the expression of inflammatory factors to influence the whole inflammatory response. Previous studies have demonstrated that hederagenin inhibits the expression of inflammatory factors, including TNF, IL1, IL6, COX-2 via MAPKs and NF-kappa B pathway (*Akhtar et al., 2019*; *Kim et al., 2017*; *Lu et al., 2015*). Campesterol, β-sitosterol, known as sterols, can reduce the levels of TNF-α and IL of peritoneal fluid in the EMs mice (*Ilhan et al., 2019*). Flavonoids have a similar effect. Studies have experimentally proved that quercetin has an anti-inflammatory effect on the EMs (*Park et al., 2019*). Coincidentally, the docking result confirmed all the above, which also provides certain credibility for our results that GZFLW possibly worked against inflammatory in a multi-ingredient way.

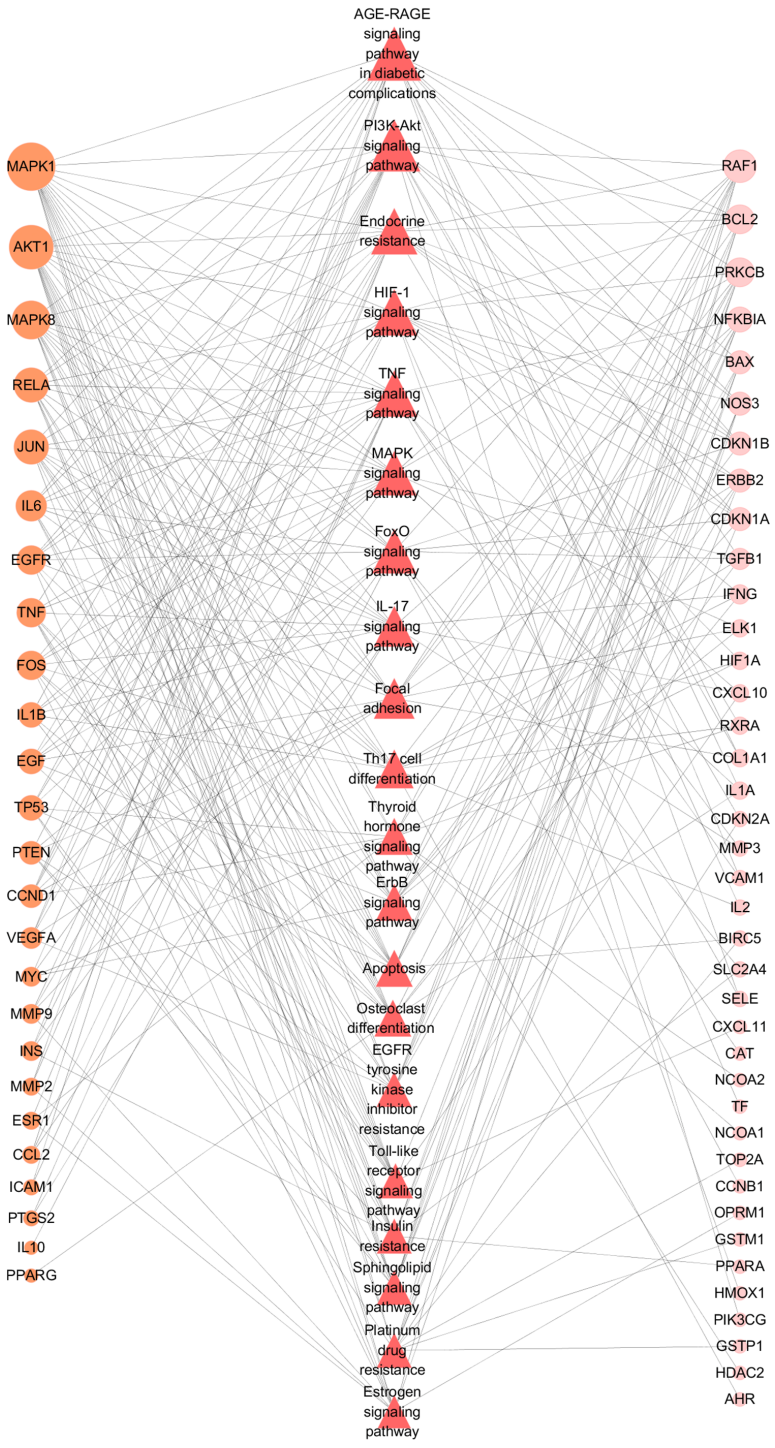

Figure 9 **Target-Pathway Network.** The orange circle nodes represent the hub nodes, and the pink nodes represent the other nodes. The red diamond nodes represent the related pathways. The node size is proportional to the target degree in the network.

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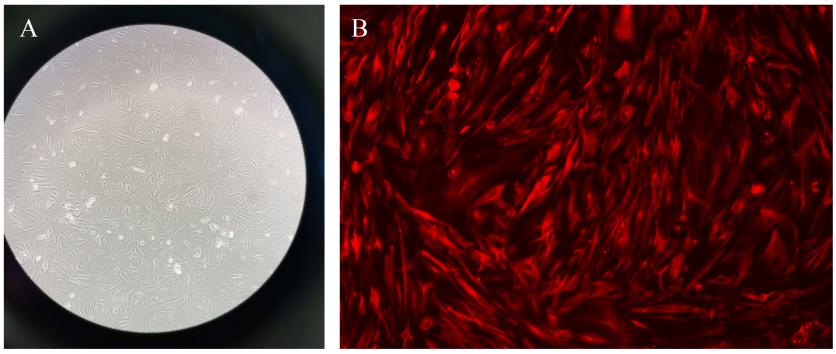

Figure 10 Primary endometrial stromal cells and vimentin staining. (A) Primary endometrial stromal cells. (B) The vimentin staining of cells.

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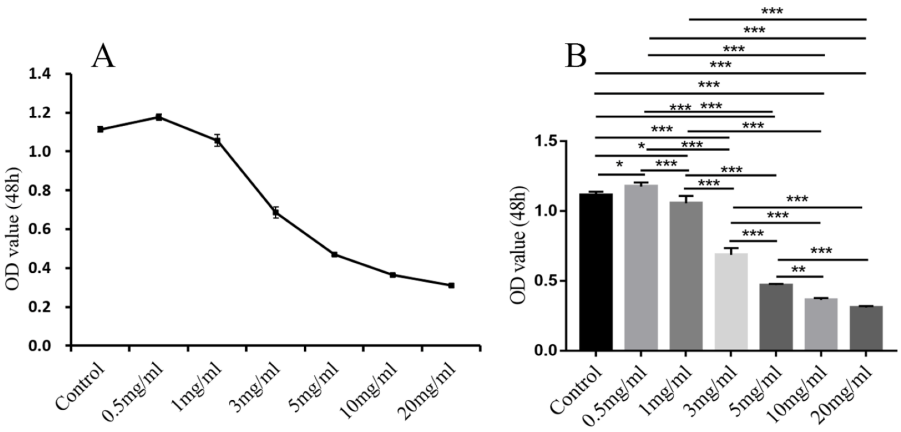

Figure 11 The results of MTT colorimetric assay. The cell proliferation of hEM15a cells was assessed using MTT assay. All results were shown as mean ± standard deviation. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ versus designated group.

Full-size DOI: 10.7717/peerj.11087/fig-11

Apoptosis and proliferation

The cell apoptosis's inhibition and concomitant cell excessive proliferation play some important roles in the development of EMs. We think that the expression and regulation of the Jun and Fos genes are among the key factors in cell proliferation and apoptosis. It is found that high expression of c-Jun related to the proliferation of EMs cells via the JNK / c-Jun signaling pathway (*Yu et al., 2018*). TP53 is another critical tumor suppressor gene in our study; its regulative effect on the cell cycle plays a crucial role in obtaining proliferation activity for ESC of EMs (*Hirakawa et al., 2016*). It is investigated that the polymorphisms of TP53 may be involved in high risk of the generation of EMs (*Hussain et al., 2018*). Epidermal growth factor (EGF) also plays a vital role in regulating cell growth, proliferation, and differentiation. Previous studies have found that the EGF expression in patients with severe EMs significantly increased (*Chatterjee et al., 2018*). Furthermore, there are researches shown that COX-2 expression regulated by the PTGS2 gene can activate

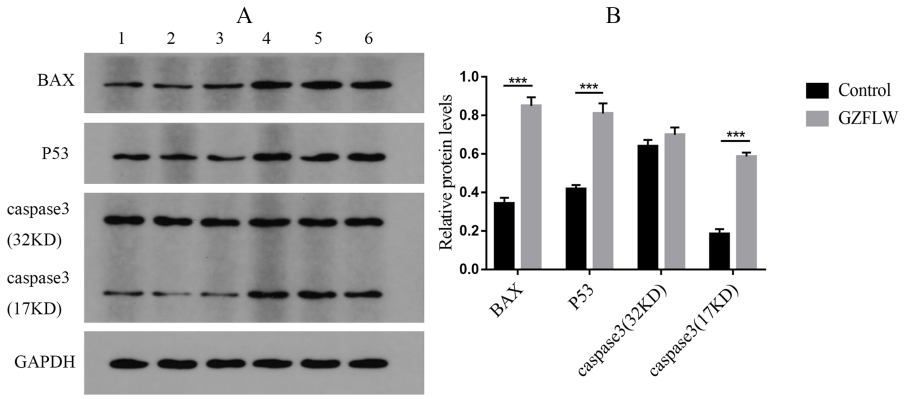

Figure 12 The results of Western blotting about different targets. The group 1–3 represented control group, and the group 4–6 represented GZFLW group. All results were shown as mean ± standard deviation. $^{***}P < 0.001$ versus designated group.

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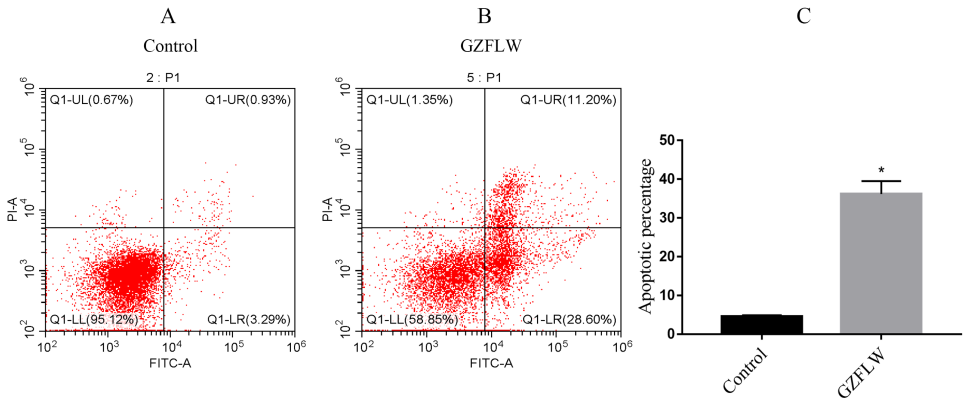

Figure 13 The apoptosis analysis of flow cytometry. The analysis of control group and GZFLW group was assessed by AnnexinV/PI staining using flow cytometry. The result was shown as mean ± standard deviation. $^{*}P < 0.05$ versus designated group.

Full-size DOI: 10.7717/peerj.11087/fig-13

MAPKs, which then subsequently activate some transcription factors and protein kinases, ultimately promoting the proliferation of cells.

GZFLW's regulative effect on cell proliferation and apoptosis in EMs is possibly achieved by regulating flavonoids on the expression of PTGS2 (*Da Silva et al., 2020*), which further regulates the COX-2/PGE2 axis (*Takaoka et al., 2018*). Besides, components such as flavonoids, quercetin in GZFLW affect the proliferation and apoptosis in EMs by inhibiting the ERK1/2, P38 MAPK, and AKT signaling pathway (*Park et al., 2019*). Also, baicalin can affect the activity of ESC by apoptosis inhibition via the NF-kappa B signaling pathway (*Jin, Huang & Zhu, 2017*). Furthermore, the direct or indirect inhibition of natural triterpenoids on proliferation is possibly another essential component of the regulation of cell apoptosis (*Chen et al., 2019*).

It is mentioning that all the primary active ingredients except paeoniflorin had a high docking score with EGF in our docking results. Besides, three active ingredients (hederagenin, phytosterol, and pachymic acid) had tight combinations with Jun, FOS, and PTGS2, and the other three active ingredients (pachymic acid, taxifolin, and hederagenin) had strong putative interaction with P53, which demonstrates that GZFLW is likely to affect on proliferation and apoptosis through a multi-ingredient synergistic way.

To verify the effect of GZFLW on EMs, we conducted in vitro experiments. The results of MTT and flow cytometry confirmed that GZFLW can effectively promote the apoptosis of ectopic stromal cells in EMs. In the treatment group, the expression levels of P53, caspase3 and BAX were significantly up-regulated, suggesting that the therapeutic effect of GZFLW may be related to the regulation of P53 pathway and apoptosis pathway.

Oxidative stress

More and more evidence has shown that the pathophysiological mechanism of EMs is complicated, and hypoxia plays an essential role in EMs damage (*Wu, Hsiao & Tsai, 2019*). The AGE/RAGE and HIF-1alpha signaling pathway were speculated as probable mechanisms of GZFLW against EMs from KEGG results. Previous studies have demonstrated that high expression of RAGE under hypoxia environment increased the accumulation of ROS in peritoneal fluid (*Polak et al., 2018*). Eventually, contribute to the cell invasion ability in EMs (*Seguella et al., 2019*). It is also found that high expression of HIF-1α in the serum of EMs aggravated the severity of dysmenorrheal (*Zhang et al., 2018*).

Fortunately, triterpenoids and flavonoids including hederagenin, β-sitosterol (*Adebiyi et al., 2019*; *Ponnulakshmi et al., 2019*), campesterol (*Alvarez-Sala et al., 2018*; *Eom et al., 2017*; *Ho, Liu & Loke, 2016*; *Ogunlaja et al., 2016*; *Shamloo et al., 2017*; *Toiu et al., 2019*; *Weingartner et al., 2017*), taxifolin, and pachymic acid were reported to be the natural antioxidants, which could reduce oxidative stress by inhibiting RAGE expression to a certain extent (*Song et al., 2017*) via MAPK, and PI3K/Akt pathways (*Wen et al., 2018*). The molecular docking results showed that triterpenoids and flavonoids have tight combinations with the hub targets AKT1, MAPK1, and MAPK8. However, further experiments are needed to confirm whether GZFLW worked against EMs by affecting AGE/RAGE and HIF-1α via MAPK and PI3K/Akt pathways.

Angiogenesis

Hypoxia and inflammatory, as an essential characteristic of EMs microenvironment, can stimulate the transcription of hypoxia-inducible factor HIF (*Lin et al., 2018*) and inflammatory-inducible factor COX-2 gene, consequently activate the angiogenesis through the PI3K/mTOR and MAPK pathways. HIF-1α/ VEGF expression in serum and endometrium has been reported to have a relationship with the stage of EMs and the severity of dysmenorrheal (*Zhang et al., 2018*). Studies confirmed that some plant inhibitors of COX-2 were able to treat EMs and inhibited ESC's angiogenesis by regulating HIF-1α/VEGF in mice models (*Li et al., 2020*).

It is worth mentioning that triterpenoids (β-sitosterol, campesterol, hederagenin) and flavonoids (taxifolin) in GZFLW were detected in our study, they could have a modest effect

on inhibiting VEGF in EMs, which have been confirmed by experiments and in line with our molecular docking results (*Ilhan et al., 2019*). Previous studies have also demonstrated that paeoniflorin could inhibit angiogenesis via the HIF-1 α/ VEGF pathway (*Song et al., 2017*; *Zhou, Ding & Hardiman, 2018*).

CONCLUSIONS

In this study, network pharmacology and molecular docking were utilized for exploring the potential effects of GZFLW in anti-EMs, mainly focusing on four aspects: the suppression of inflammatory response, the regulating of apoptosis and proliferation, the reduction of the oxidative stress, and the inhibition of angiogenesis. Among them, the pro-apoptotic effect was confirmed by our in vitro experiments. In these crucial biological functions, triterpenoids and flavonoids contained in the GZFLW were considered that those might be the critical active ingredients involved in the potential therapy mechanisms.

Our study had several limitations. Firstly, to some extent, network pharmacologic analysis can help to predict the possible molecular mechanism of GZFLW in treating EMs. However, we can only speculate but not confirm if such mechanisms have an impact because, in evidence-based medicine, the conclusion can not be determined until subsequent biological experiments. Secondly, the data set of the GZFLW related compounds and targets were identified through the TCMSP, BATMAN-TCM, and CASC database, and GeneCards built the EMs target database. Choosing different databases may lead to the study biases. Moreover, because all the databases we use are based on existing research results, this may limit the discovery of new targets related to EMs treatment.

In conclusion, our study reveals that GZFLW may have a multiple effect on EMs. Although the mechanism remains confirmed by further experiments, the relevant targets and signaling pathways for GZFLW against EMs have been preliminarily studied and systematically summarized.

ADDITIONAL INFORMATION AND DECLARATIONS

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Competing Interests

The authors declare there are no competing interests.

Author Contributions

- Haoxian Wang and Gang Zhou performed the experiments, prepared figures and/or tables, and approved the final draft.
- Gang Zhou and Xianyun Fu conceived and designed the experiments, authored or reviewed drafts of the paper, and approved the final draft.
- Wei Wang conceived and designed the experiments, analyzed the data, prepared figures and/or tables, and approved the final draft.

Data Availability

The following information was supplied regarding data availability:

Raw data are available in the Supplemental Files.

Supplemental Information

Supplemental information for this article can be found online at http://dx.doi.org/10.7717/peerj.11087#supplemental-information.

REFERENCES

Adebiyi OE, Olayemi FO, Olopade JO, Tan NH. 2019. Beta-sitosterol enhances motor coordination, attenuates memory loss and demyelination in a vanadium-induced model of experimental neurotoxicity. *Pathophysiology* **26**:21–29 DOI 10.1016/j.pathophys.2018.12.002.

Ahn SH, Khalaj K, Young SL, Lessey BA, Koti M, Tayade C. 2016. Immune-inflammation gene signatures in endometriosis patients. *Fertility and Sterility* **106**:1420–1431 DOI 10.1016/j.fertnstert.2016.07.005.

Akhtar M, Shaukat A, Zahoor A, Chen Y, Wang Y, Yang M, Umar T, Guo M, Deng G. 2019. Anti-inflammatory effects of Hederacoside-C on Staphylococcus aureus induced inflammation via TLRs and their downstream signal pathway in vivo and in vitro. *Microbial Pathogenesis* **137**:103767 DOI 10.1016/j.micpath.2019.103767.

Alvarez-Sala A, Lopez-Garcia G, Attanzio A, Tesoriere L, Cilla A, Barbera R, Alegria A. 2018. Effects of plant sterols or beta-cryptoxanthin at physiological serum concentrations on suicidal erythrocyte death. *Journal of Agricultural & Food Chemistry* **66**:1157–1166 DOI 10.1021/acs.jafc.7b05575.

Ashburner M, Ball CA, Blake JA, Botein D, Butler H, Cherry JM, Davis AP, Dolinski K, Dwight SS, Eppig JT, Harris MA, Hill DP, Issel-Tarver L, Kasarskis A, Lewis S, Matese JC, Richardson JE, Ringwald M, Rubin GM, Sherlock G. 2000. Gene Ontology: tool for the unification of biology. *Nature Genetics* **25**:25–29 DOI 10.1038/75556.

Bruun MR, Arendt LH, Forman A, Ramlau-Hansen CH. 2018. Endometriosis and adenomyosis are associated with increased risk of preterm delivery and a small-for-gestational-age child: a systematic review and meta-analysis. *Acta Obstetricia et Gynecologica Scandinavica* **97**:1073–1090 DOI 10.1111/aogs.13364.

Chatterjee K, Jana S, DasMahapatra P, Swarnakar S. 2018. EGFR-mediated matrix metalloproteinase-7 up-regulation promotes epithelial-mesenchymal transition via ERK1-AP1 axis during ovarian endometriosis progression. *FASEB Journal* **32**:4560–4572 DOI 10.1096/fj.201701382RR.

Chen Z, Huang KY, Ling Y, Goto M, Duan HQ, Tong XH, Liu YL, Cheng YY, Morris-Natschke SL, Yang PC, Yang SL, Lee KH. 2019. Discovery of an oleanolic acid/hederagenin-nitric oxide donor hybrid as an EGFR tyrosine kinase inhibitor for non-small-cell lung cancer. *Journal of Natural Products* **88**:3065–3073.

Da Silva AB, Cerqueira Coelho PL, das Neves Oliveira M, Oliveira JL, Oliveira Amparo JA, Da Silva KC, Soares JRP, Pitanga BPS, Dos Santos Souza C, de Faria Lopes GP, Da Silva VDA, de Fatima Dias Costa M, Junier MP, Chneiweiss H, Moura-Neto V, Costa SL. 2020. The flavonoid rutin and its aglycone quercetin modulate the microglia inflammatory profile improving antiglioma activity. *Brain, Behavior, and Immunity* **85**:170–185 DOI 10.1016/j.bbi.2019.05.003.

Eom MR, Weon JB, Jung YS, Ryu GH, Yang WS, Ma CJ. 2017. Neuroprotective compounds from Reynoutria sachalinensis. *Archives of Pharmacal Research* **40**:704–712 DOI 10.1007/s12272-017-0918-x.

Fang HY, Zeng HW, Lin LM, Chen X, Shen XN, Fu P, Lv C, Liu Q, Liu RH, Zhang WD, Zhao J. 2017. A network-based method for mechanistic investigation of Shexiang Baoxin Pill's treatment of cardiovascular diseases. *Scientific Reports* **7**:43632 DOI 10.1038/srep43632.

Hirakawa T, Nasu K, Abe W, Aoyagi Y, Okamoto M, Kai K, Takebayashi K, Narahara H. 2016. miR-503, a microRNA epigenetically repressed in endometriosis, induces apoptosis and cell-cycle arrest and inhibits cell proliferation, angiogenesis, and contractility of human ovarian endometriotic stromal cells. *Human Reproduction* **31**:2587–2597 DOI 10.1093/humrep/dew217.

Ho XL, Liu JJ, Loke WM. 2016. Plant sterol-enriched soy milk consumption modulates 5-lipoxygenase, 12-lipoxygenase, and myeloperoxidase activities in healthy adults—a randomized-controlled trial. *Free Radical Research Communications* **50**:1396–1407 DOI 10.1080/10715762.2016.1252839.

Hopkins AL. 2008. Network pharmacology: the next paradigm in drug discovery. *Nature Chemical Biology* **4**:682–690 DOI 10.1038/nchembio.118.

Huang DW, Sherman BT, Lempicki RA. 2008. Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. *Nature Protocols* **4**:44–57.

Hussain R, Khaliq S, Raza SM, Khaliq S, Lone K. 2018. Association of TP53 codon 72 polymorphism in women suffering from endometriosis from Lahore, Pakistan. *Journal of Pakistan Medical Association* **68**:224–230.

Ilhan M, Ali Z, Khan IA, Tastan H. 2020. The regression of endometriosis with glycosylated flavonoids isolated from Melilotus officinalis (L.) Pall. in an endometriosis rat model. *Taiwanese Journal of Obstetrics and Gynecology* **59**:211–219 DOI 10.1016/j.tjog.2020.01.008.

Ilhan M, Ali Z, Khan IA, Tastan H, Akkol EK. 2019. Promising activity of Anthemis austriaca Jacq. on the endometriosis rat model and isolation of its active constituents. *Saudi Pharmaceutical Journal* **27**:889–899 DOI 10.1016/j.jsps.2019.06.002.

Jin Z, Huang J, Zhu Z. 2017. Baicalein reduces endometriosis by suppressing the viability of human endometrial stromal cells through the nuclear factor-kappaB pathway in vitro. *Experimental and Therapeutic Medicine* **14**:2992–2998 DOI 10.3892/etm.2017.4860.

Kanehis M, Goto S. 2000. KEGG: kyoto encyclopedia of genes and genomes. *Nucleic Acids Research* **28**:27–30 DOI 10.1093/nar/28.1.27.

Kim GJ, Song DH, Yoo HS, Chung KH, Lee KJ, An JH. 2017. Hederagenin supplementation alleviates the pro-inflammatory and apoptotic response to alcohol in rats. *Nutrients* **9**:41 DOI 10.3390/nu9010041.

Kralickova M, Fiala L, Losan P, Tomes P, Vetvicka V. 2018. Altered immunity in endometriosis: what came first? *Immunological Investigations* **47**:569–582 DOI 10.1080/08820139.2018.1467926.

Lalani S, Choudhry AJ, Firth B, Bacal V, Walker M, Wen SW, Singh S, Amath A, Hodge M, Chen I. 2018. Endometriosis and adverse maternal, fetal and neonatal outcomes, a systematic review and meta-analysis. *Human Reproduction* **33**:1854–1865 DOI 10.1093/humrep/dey269.

Lang L, Meng Z, Sun L, Xiao W, Zhao L, Xiong Z. 2018. Intergrated metabonomic study of the effects of Guizhi Fuling capsule intervention on primary dysmenorrheal using RP-UPLC-MS complementary with HILIC-UPLC-MS technique. *Biomedical Chromatography* **32(2)**:e4093 DOI 10.1002/bmc.4093.

Li J, Zeng Z, Chang Y, Li M, Wu Q, Chen P, Liang X. 2020. Suppressive effects of ursolic acid on human endometriotic stromal cells survival. *Gynecologic and Obstetric Investigation* **85**:72–81 DOI 10.1159/000502258.

Li X, Xu X, Ma H, Xu F, Tian C, Hou W. 2018. Clinical and experimental research progress on Guizhi Fuling Pill of classical herbal formulae. *Drug Evaluation and Research* **41**:1724–1729.

Lin X, Dai Y, Xu W, Shi L, Jin X, Li C, Zhou F, Pan Y, Zhang Y, Lin X, Zhang X. 2018. Hypoxia promotes ectopic adhesion ability of endometrial stromal cells via TGF-beta1/Smad signaling in endometriosis. *Endocrinology* **159**:1630–1641 DOI 10.1210/en.2017-03227.

Liu H, Wang J, Zhou W, Wang Y, Yang L. 2013. Systems approaches and polypharmacology for drug discovery from herbal medicines: an example using licorice. *Journal of Ethnopharmacology* **146**:773–793 DOI 10.1016/j.jep.2013.02.004.

Liu Z, Guo F, Wang Y, Li C, Zhang X, Li H, Diao L, Gu J, Wang W, Li D, He F. 2016. BATMAN-TCM: a bioinformatics analysis tool for molecular mechanism of traditional chinese medicine. *Scientific Reports* **6**:21146 DOI 10.1038/srep21146.

Lu SH, Guan JH, Huang YL, Pan YW, Yang W, Lan H, Huang S, Hu J, Zhao GP. 2015. Experimental study of antiatherosclerosis effects with hederagenin in rats. *Evidence-based Complementary and Alternative Medicine* **2015**:456354 DOI 10.1155/2015/456354.

Lukas I, Kohl-Schwartz A, Geraedts K, Rauchfuss M, Wolfler MM, Haberlin F, von Orelli S, Eberhard M, Imthurn B, Imesch P, Leeners B. 2018. Satisfaction with medical support in women with endometriosis. *PLOS ONE* **13**:e0208023 DOI 10.1371/journal.pone.0208023.

Ming L, Yan CL, Liu HX, Wang TY, Shi XH, Liu JP, Orgah J, Fan JW, Han JH, Wang XJ, Zhu Y. 2017. Network pharmacology exploration reveals endothelial inflammation as a common mechanism for stroke and coronary artery disease treatment of Danhong injection. *Scientific Reports* **7**:15427 DOI 10.1038/s41598-017-14692-3.

Ogunlaja OO, Moodley R, Baijnath H, Jonnalagadda SB. 2016. Chemical constituents and in vitro antioxidant activity of crude extracts and compounds from leaves and stem bark of ficus burtt-davyi. *Acta Poloniae Pharmaceutica* **73**:1593–1600.

Park S, Lim W, Bazer FW, Whang KY, Song G. 2019. Quercetin inhibits proliferation of endometriosis regulating cyclin D1 and its target microRNAs in vitro and in vivo. *Journal of Nutritional Biochemistry* **63**:87–100 DOI 10.1016/j.jnutbio.2018.09.024.

Polak G, Barczynski B, Wertel I, Kwaniewski W, Bednarek W, Derewianka-Polak M, Frszczak K, Olajossy M, Kotarski J. 2018. Disrupted iron metabolism in peritoneal fluid may induce oxidative stress in the peritoneal cavity of women with endometriosis. *Annals of Agricultural and Environmental Medicine* **25**:587–592 DOI 10.26444/aaem/75802.

Ponnulakshmi R, Shyamaladevi B, Vijayalakshmi P, Selvaraj J. 2019. In silico and in vivo analysis to identify the antidiabetic activity of beta sitosterol in adipose tissue of high fat diet and sucrose induced type-2 diabetic experimental rats. *Toxicology Mechanisms and Methods* **29**:276–290 DOI 10.1080/15376516.2018.1545815.

Prefumo F, Rossi AC. 2018. Endometriosis. *Best Practice & Research: Clinical Obstetrics & Gynaeco* **51**:34–40 DOI 10.1016/j.bpobgyn.2018.01.019.

Rabinerson D, Hiersch L, Gabbay-Ben-Ziv R. 2018. [Dysmenorrhea - its prevalence, causes, influence on the affected women and possible treatments]. *Harefuah* **157**:91–94.

Reid R, Steel A, Wardle J, McIntyre E, Harnett J, Foley H, Adams J. 2019. The prevalence of self-reported diagnosed endometriosis in the Australian population: results from a nationally-representative survey. *BMC Research Notes* **12**:88 DOI 10.1186/s13104-019-4114-6.

Ru J, Li P, Wang J, Zhou W, Li B, Huang C, Li P, Guo Z, Tao W, Yang Y, Xu X, Li Y, Wang Y, Yang L. 2014. TCMSP: a database of systems pharmacology

for drug discovery from herbal medicines. *Journal of Cheminformatics* **6**:13 DOI 10.1186/1758-2946-6-13.

Schomacker ML, Hansen KE, Ramlau-Hansen CH, Forman A. 2018. Is endometriosis associated with irritable bowel syndrome? A cross-sectional study. *European Journal of Obstetrics & Gynecology and Reproductive Biology* **231**:65–69 DOI 10.1016/j.ejogrb.2018.10.023.

Seguella L, Capuano R, Pesce M, Annunziata G, Pesce M, Conno BD, Sarnelli G, Aurino L, Esposito G. 2019. S100B protein stimulates proliferation and angiogenic mediators release through RAGE/pAkt/mTOR pathway in human colon adenocarcinoma Caco-2 Cells. *International Journal of Molecular Sciences* **20**:3240 DOI 10.3390/ijms20133240.

Shamloo M, Babawale EA, Furtado A, Henry RJ, Eck PK, Jones PJH. 2017. Effects of genotype and temperature on accumulation of plant secondary metabolites in Canadian and Australian wheat grown under controlled environments. *Scientific Reports* **7**:9133 DOI 10.1038/s41598-017-09681-5.

Shannon P, Markiel A, Ozier O, Baliga NS, Wang JT, Ramage D, Amin N, Schwikowski B, Ideker T. 2003. Cytoscape: a software environment for integrated models of biomolecular interaction networks. *Genome Research* **13**:2498–2504 DOI 10.1101/gr.1239303.

Signorile PG, Viceconte R, Baldi A. 2018. Novel dietary supplement association reduces symptoms in endometriosis patients. *Journal of Cellular Physiology* **233**:5920–5925 DOI 10.1002/jcp.26401.

Song S, Xiao X, Guo D, Mo L, Bu C, Ye W, Den Q, Liu S, Yang X. 2017. Protective effects of Paeoniflorin against AOPP-induced oxidative injury in HUVECs by blocking the ROS-HIF-1alpha/VEGF pathway. *Phytomedicine* **34**:115–126 DOI 10.1016/j.phymed.2017.08.010.

Stelzer G, Rosen N, Plaschkes I, Zimmerman S, Twik M, Fishilevich S, Stein TI, Nudel R, Lieder I, Mazor Y, Kaplan S, Dahary D, Warshawsky D, Guan-Golan Y, Kohn A, Rappaport N, Safran M, Lancet D. 2016. The genecards suite: from gene data mining to disease genome sequence analyses. *Current Protocols in Bioinformatics* **54**:1 30 31–31 30 33.

Szklarczyk D, Morris JH, Cook H, Kuhn M, Wyder S, Simonovic M, Santos A, Doncheva NT, Roth A, Bork P, Jensen JJ, Mering C. 2017. The STRING database in 2017: quality-controlled protein–protein association networks, made broadly accessible. *Nucleic Acids Research* **45**:D362–D368 DOI 10.1093/nar/gkw937.

Takaoka O, Mori T, Ito F, Okimura H, Kataoka H, Tanaka Y, Koshiba A, Kusuki I, Shigehiro S, Amami T, Kitawaki J. 2018. Daidzein-rich isoflavone aglycones inhibit cell growth and inflammation in endometriosis. *Journal of Steroid Biochemistry and Molecular Biology* **181**:125–132 DOI 10.1016/j.jsbmb.2018.04.004.

Tao W, Xu X, Wang X, Li B, Wang Y, Li Y, Yang L. 2013. Network pharmacology-based prediction of the active ingredients and potential targets of Chinese herbal

Radix Curcumae formula for application to cardiovascular disease. *Journal of Ethnopharmacology* **145**:1–10 DOI 10.1016/j.jep.2012.09.051.

Toiu A, Mocan A, Vlase L, Parvu AE, Vodnar DC, Gheldiu AM, Moldovan C, Oniga I. 2019. Comparative phytochemical profile, antioxidant, antimicrobial and in vivo anti-inflammatory activity of different extracts of traditionally used romanian ajuga genevensis L. and A. reptans L. (Lamiaceae). *Molecules* **24**:1957 DOI 10.3390/molecules24081597.

Verket NJ, Uhlig T, Sandvik L, Andersen MH, Tanbo TG, Qvigstad E. 2018. Health-related quality of life in women with endometriosis, compared with the general population and women with rheumatoid arthritis. *Acta Obstetricia et Gynecologica Scandinavica* **97**:1339–1348 DOI 10.1111/aogs.13427.

Wang SS, Xu HY, Yan M, Wang XG, Shi Y, Huang B, Tang SH, Zhang Y, Li DF, Liang RX, Yang HJ. 2015. Characterization and rapid identification of chemical constituents of NaoXinTong capsules by UHPLC-linear ion trap/Orbitrap mass spectrometry. *Journal of Pharmaceutical and Biomedical Analysis* **111**:104–118 DOI 10.1016/j.jpba.2015.01.020.

Wang W, Gao Y, Liu F, Wei R, Xie Y. 2018. Post-marketing surveillance on Guizhi Fuling Jiaonang based on literature review. *China Journal of Chinese Materia Medica* **43**:820–832.

Weingartner O, Bogeski I, Kummerow C, Schirmer SH, Vanmierlo T, Wagenpfeil G, Hoth M, Bohm M, Lutjohann D, Laufs U. 2017. Plant sterol ester diet supplementation increases serum plant sterols and markers of cholesterol synthesis, but has no effect on total cholesterol levels. *The Journal of steroid biochemistry and molecular biology* **169**:219–225 DOI 10.1016/j.jsbmb.2016.07.016.

Wen Z, Hou W, Wu W, Zhao Y, Dong X, Bai X, Peng L, Song L. 2018. 6'-O-Galloylpaeoniflorin attenuates cerebral ischemia reperfusion-induced neuroinflammation and oxidative stress via PI3K/Akt/Nrf2 Activation. *Oxidative Medicine and Cellular Longevity* **2018**:8678267 DOI 10.1155/2018/8678267.

Wu MH, Hsiao KY, Tsai SJ. 2019. Hypoxia: The force of endometriosis. *Journal of Obstetrics and Gynaecology Research* **45**:532–541 DOI 10.1111/jog.13900.

Wu X, Yang E, Sun Z, Piao C, He L, Zhang S. 2015. Experimental research of Guizhi Fuling Pillsfor the treatment of dysmenorrhea of bloodstasis type. *Shanghai J Tradit Chin Med* **49**:102–105.

Xu X, Zhang W, Huang C, Li HY, Wang Y, Duan J, Ling Y. 2012. A novel chemometric method for the prediction of human oral bioavailability. *International Journal of Molecular Sciences* **13**:6964–6982 DOI 10.3390/ijms13066964.

Yang Y. 2019. Systematic evaluation of Chinese patent medicine in the treatment of secondary dysmenorrhea (endometriosis, adenomyosis). Master's thesis, Beijing University of Traditional Chinese Medicine, Beijing, China, 1–89.

Yu J, Francisco AMC, Patel BG, Cline JM, Zou E, Berga SL, Taylor RN. 2018. IL-1beta stimulates brain-derived neurotrophic factor production in eutopic endometriosis

stromal cell cultures: a model for cytokine regulation of neuroangiogenesis. *American Journal of Pathology* **188**:2281–2292 DOI 10.1016/j.ajpath.2018.06.011.

Zhang F, Liu XL, Wang W, Dong HL, Xia YF, Ruan LP, Liu LP. 2018. Expression of MMIF, HIF-1alpha and VEGF in serum and endometrial tissues of patients with endometriosis. *Current Medical Science* **38**:499–504 DOI 10.1007/s11596-018-1906-1.

Zhao F, Guochun L, Yang Y, Shi L, Xu L, Yin L. 2015. A network pharmacology approach to determine active ingredients and rationality of herb combinations of modified-Simiaowan for treatment of gout. *Journal of Ethnopharmacology* **168**:1–16 DOI 10.1016/j.jep.2015.03.035.

Zhao Y. 2016. Clinical study on Guizhi Fuling Pill in the treatment of gynecological diseases. *Journal of Hebei Traditional Chinese Medicine and Pharmacology* **31**:30–31.

Zhou J, Ding ZM, Hardiman PJ. 2018. Understanding the Role of Gui-Zhi-Fu-Ling-Capsules (Chinese Medicine) for treatment of endometriosis in the rat model: using NMR based metabolomics. *Evidence-Based Complementary and Alternative Medicine* **2018**:9864963 DOI 10.1155/2018/9864963.

Zhou Y, Zhou B, Pache L, Chang M, Khodabakhshi AH, Tanaseichuk O, Benner C, Chanda SK. 2019. Metascape provides a biologist-oriented resource for the analysis of systems-level datasets. *Nature Communications* **10**:1523 DOI 10.1038/s41467-019-09234-6.