Journal of
Pharmacology and Drug Metabolism

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Review Article Open Access

Molecular Mechanism of Erzhi Pill in the Treatment of Premature Ovarian Insufficiency Based on Network Pharmacology

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Received Date: August 07, 2021 **Accepted Date:** September 07, 2021 **Published Date:** September 09, 2021

Citation: Haixia Huang (2021) Molecular Mechanism of Erzhi Pill in the Treatment of Premature Ovarian Insufficiency Based on Network Pharmacology. J Pharmacol Drug Metab 4: 1-14.

Abstract

To explore the molecular mechanism of Erzhi Pill (EZP) in the treatment of premature ovarian insufficiency (POI) by network pharmacology. Methods. the main chemical components and their targets of EZP were obtained through the Traditional Chinese Medicine Systems Pharmacology Database and Analysis Platform (TCMSP), and the main active components were screened according to ADME. GeneCard, OMIM, DisGeNET, DrugBank, and PharmGkb were used to establish target databases for POI. The visualization network diagram of "EZP active ingredient -Target- POI" was constructed by using Cytoscape software. The STRING database was used to construct the protein interaction network and Cytoscape software was used to screen out the key targets. Gene Ontology (GO) biological function analysis and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis for key targets were performed. Finally, Discovery Studio software was used to verify the molecular docking of active ingredients and key targets. Results: 17 active components of EZP were screened, including 117 common targets. EZP can increase the number of follicles in patients with POI through PI3K / Akt signaling pathway, p53 signaling pathway, apoptosis, and MAPK signaling pathway, and can prevent long-term complications including osteoporosis and cardiovascular issues. The molecular docking results showed that the active components of EZP had a good match with the targets of AKT1, TP53, MAPK1, JUN, RB1, TNF , and MYC. Conclusion. EZP has the characteristics of multisystem, multicomponent, and multitarget in the treatment of POI. Its possible mechanisms include anti-apoptosis, inhibition of oxidative stress, and inflammatory response to control the occurrence and development of POI. Quercetin, luteolin, and kaempferol may be the material basis of EZP in the treatment of POI.

Keywords: Premature Ovarian Insufficiency; Erzhi Pill; Network Pharmacology

List of Abbreviations: POI: Premature ovarian insufficiency; POF: Premature ovarian failure CHM: Chinese herbal medicine; EZP: Erzhi pill; HRT: Hormone replacement therapy; OB: Oral bioavailability; DL: Drug-likeness; GO: Gene Ontology; KEGG: Kyoto Encyclopedia of Genes and Genomes; PPI network: Protein-protein interaction network; LLF: Ligustri Lucidi Fructus; EH: Ecliptae Herba; TCMSP: Traditional Chinese Medicine Systems Pharmacology Database and Analysis Platform; DC: Degree centrality; BC: Betweenness centrality; CC: Closeness centrality; EC:Eigenvector centrality; NC: Network centrality; LAC: Local average connectivity

Introduction

Primary ovarian insufficiency refers to the severe decline of ovarian function in women before the age of 40, with menstrual disorders, infertility, raised gonadotrophins, and low estradiol as the main clinical manifestations[1]. It was once called premature ovarian failure (POF). The ovarian function of patients with POF has declined seriously, and some of them have cardiovascular diseases [2] and osteoporosis[3]. Therefore, POF should be changed to POI, FSH>40 U/L should be reduced to FSH>25 U/L, to achieve the purpose of early diagnosis and early treatment to reduce complications.

The prevalence of POI is on the rise, and it is younger. At present, the global prevalence of POI is 3.7% [4], and the lower development status is closely associated with increased risk for POI [5]. Although dormant follicles in vitro activation (IVA) [6,7] and stem cell therapy[8] have been used in the treatment of POI, it is controversial because of its high technical level, high treatment cost, and unclear clinical efficacy and safety. At present, hormone replacement therapy (HRT) is still the main treatment. Considering that HRT increases the risk of malignant tumors including cervical cancer and breast cancer, some patients have poor compliance.

Chinese herbal medicine (CHMs) have certain advantages in the treatment of POI, which can improve the ovarian function of patients with POI without increasing the risk of malignant tumor [9].

EZP first appeared in Wu Minji's "Fu Shou Jing Fang" in the Ming Dynasty. It is composed of two kinds of medicine, Ligustri Lucidi Fructus (LLF) and Ecliptae Herba (EH). Because LLF is best picked on the winter solstice and EH is best picked on the summer solstice, it is named EZP. It is widely used in the field of female reproductive endocrine. Due to its simple formula, EZP is often used as a basic drug in clinical practice. For example, EZP combined with Er Xian Decoction can significantly reduce the serum FSH level, increase the serum E2 level, and improve the ovarian function of POF compared with HRT [10]. It also can combine with Dingjing Decoction to improve the menstruation of POF [11]. Modified EZP can improve the E2 level of perimenopausal women, improve perimenopausal symptoms and female sexual dysfunction [12]. Although clinical studies have confirmed that EZP can improve the clinical symptoms of POI, its molecular mechanism is not clear.

LLF is the mature fruit of Ligustrum lucidum. Modern pharmacological studies have found that the chemical constituents of LLF include triterpenoids, flavonoids, phenylethanoid glycosides, polysaccharides, volatile components, etc, and the main compounds include salidroside, oleanolic acid, hydroxytyrosol, ursolic acid, teligustrin, ligustrin G13, ligustrin, etc [13]. It was found that the aqueous extract of LLF could inhibit oxidative stress in ovariectomized rats by the Nox4-ROS-NF-κB pathway [14]. Salidroside and hydroxytyrosol can play the role of anti-oxidation and anti-aging by scavenging free radicals and reducing intracellular peroxide [15]. Salidroside can not only reduce the levels of fatty acids, cholesterol, and triglycerides in the serum and liver of atherosclerotic mice by inhibiting the expression of srebf1 and srebf2, but also promote the degradation of fatty acids and cholesterol metabolism in vivo in atherosclerotic mice, to alleviate atherosclerosis in mice [16]. Besides, LLF can also regulate the diversity of intestinal flora, reduce the level of trimethylamine-N-oxide (TMAO), and increase the level of sirtuin 6, to maintain the bone content of aged mice [17].

EH is the whole plant of ophiocephalus Argus Compositae. Traditional Chinese medicine (TCM) thinks that the main function of EH is to nourish the liver and kidneys, cool blood, and stanch bleeding, so it is often used to treat tooth loosening, dizziness, tinnitus, whitening of hair, bleeding, and other related diseases. EH contains a variety of chemical constituents, including triterpenoids, flavonoids, thiophenes, coumarins, lipids, sterols, and so on [18]. Its extract has anti-aging, hypolipidemic, anti osteoporosis, and hepatoprotective effects[19]. The results showed that EH extract could enhance the antioxidant capacity of D-galactose-induced aging in rats by increasing the levels of da, NE, and 5-HT and reducing the levels of iNOS and NO, thus improving the spatial learning and memory impairment of aging rats [20]. The aqueous extract of EH can inhibit bone loss and increase the bone mass of ovariectomized rats by downregulating RANKL, which proves that EH can be used to treat postmenopausal osteoporosis [21]. Besides, EH has hypolipidemic effects, the ethanol extract of EH can improve the blood lipid level of hyperlipidemic hamsters by increasing the mRNA expression of low-density lipoprotein receptor (LDLR), lecithin-cholesterol transferase (LCAT), peroxisome proliferator-activated receptor α (PPARα), and scavenger receptor class B type I receptor (SR-BI), and reducing the mRNA expression of 3-hydroxy-3-methyl-glutaryl-CoA reductase (HMGR) in the liver [22].

Network pharmacology is the integration of medicine, biology, computer science, and other disciplines to study the interaction of "disease-gene-target-drug", which

is used to reflect the intervention mechanism of drugs on the disease network [23]. Our research is based on the network pharmacology research method to clarify the key target and molecular signal transduction pathway of EZP in the treatment of POI. A flowchart of this study is depicted in Figure 1.

Query and Prediction of POI-Related Targets. "Primary Ovarian Insufficient" and "Premature Ovarian Failure" were used as keywords to search the GeneCards database (https://www.genecards.org), DrugBank database [25] (https://www.drugbank.ca), DisGeNET database (https://www.disgenet.org), PharmGkb database (https://www.pharmgkb.org), and

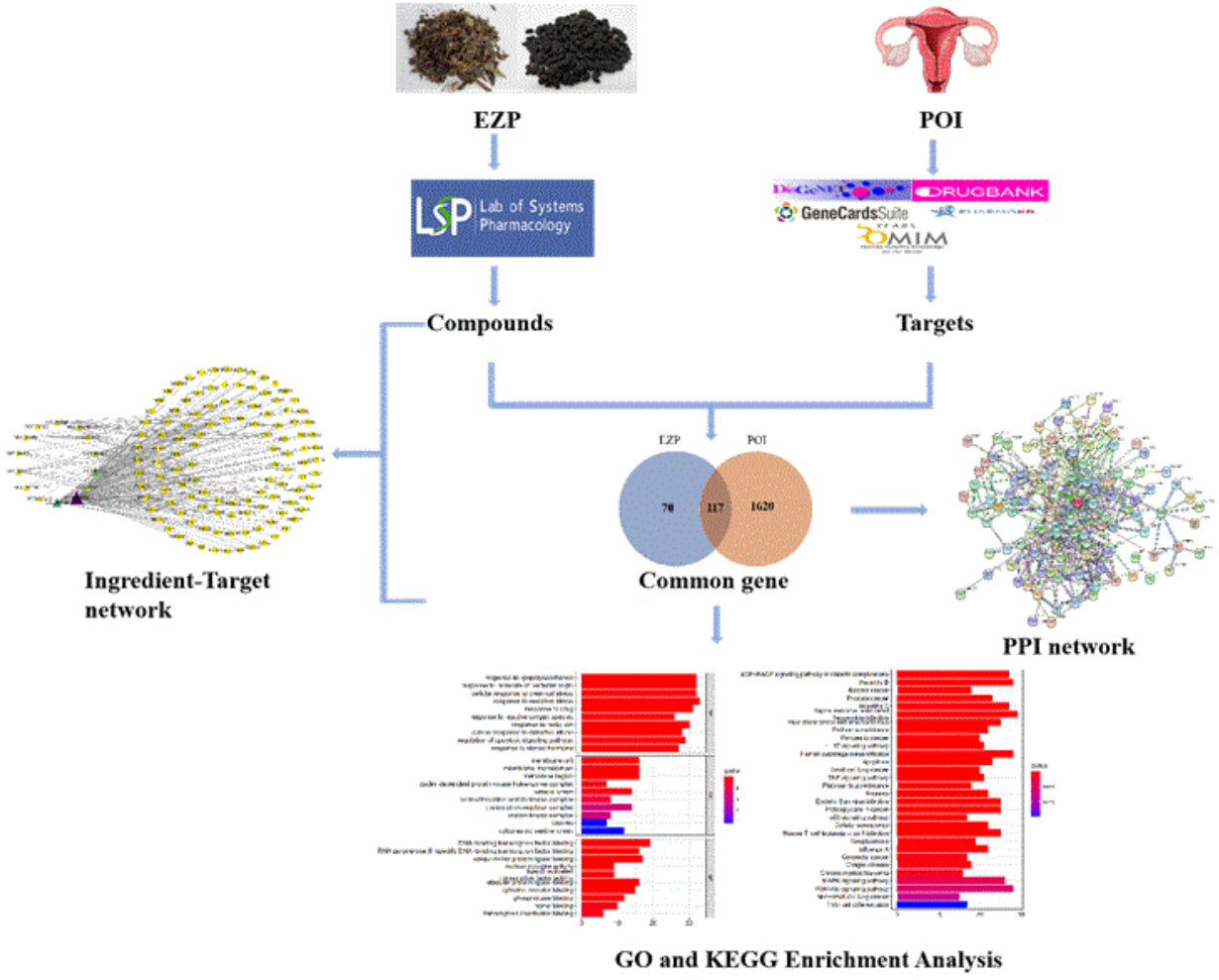

Figure 1: Whole framework based on network pharmacology

Materials and Methods

Screening of Gene Targets of Chemical Components of EZP. TCMSP database [24] (http://tcmspw.com) was used to find the chemical components of LLF and EH, and then the active components and potential protein targets were screened out according to ADME (oral bioavailability (OB)≥30% and drug–likeness (DL)≥0.18), and the target was supplemented with relevant literature. Finally, the UniProt database (https://www.uniprot.org) was used to standardize the screened protein targets.

OMIM database (http://www.omim.org). After merging the five disease database targets, the duplicate value was deleted to get all targets of POI. In Genecards database, the target with a score greater than the median is set as the potential target of POI.

Obtain the Common Target of EZP and POI and Construct the Network. The intersection of the active components of EZP and the retrieval results of POI-related targets is determined by R software and the Venn diagrams are drawn, and the common target is the potential target of EZP in the treatment of POI. The active components of

EZP and the potential target were imported into Cytoscape 3.7.1 software[26], and the network of active components and POI target network of EZP was constructed for network visualization.

Protein-Protein Interaction (PPI) Network Construction and Core Gene Screening. The potential targets of EZP in the treatment of POI were imported into STRING database[27](https://string-db.org), the organism was set as "Homo sapiens", the minimum interaction threshold were set to "highest confidence" (> 0.9), and the free nodes were hidden. Download the TSV format file, use the CytoNCA plug-in in Cytoscape 3.7.1 to analyze the topological properties of the PPI network. The targets above the median of " degree centrality (DC)", "betweenness centrality (BC)", "closeness centrality (CC)", "eigenvector centrality (EC)", "network centrality (NC)" and "local average connectivity (LAC)" were selected as the main targets, and the hub gene was obtained after twice screening.

GO and KEGG enrichment analysis. First of all, the potential target of EZP in the treatment of POI was transformed into Enter Z ID, and the data were analyzed by GO database (http://geneontology.org) and KEGG database (https://www.kegg.jp). The enrichment analysis of go function included three aspects including molecular function (MF), biological process (BP), and cellular components (CC), setting, a p value cutoff of 0.05, and a q value cutoff of 0.05, the top 10 terms with the highest GO enrichment and the top 30 terms of KEGG enrichment were shown in the bar chart.

Molecular Docking. In this study, Discovery Studio 2016 3.0 software was used to verify all hub genes and the main active ingredients of EZP. If the molecules successfully dock to the target protein, the LibDock score will be generated, and the higher the LibDock score, the higher the predicted target binding activity will be.

Firstly, the mol2 structure of the pharmaceutical active ingredient of EZP was obtained from the Chemical Book database (https://www.chemicalbook.com/). Then the protein crystal structure of POI-related target proteins was obtained from the RCSB PDB Database (https://www.rcsb.org). Finally, the above results were imported into Discovery Studio software for analysis.

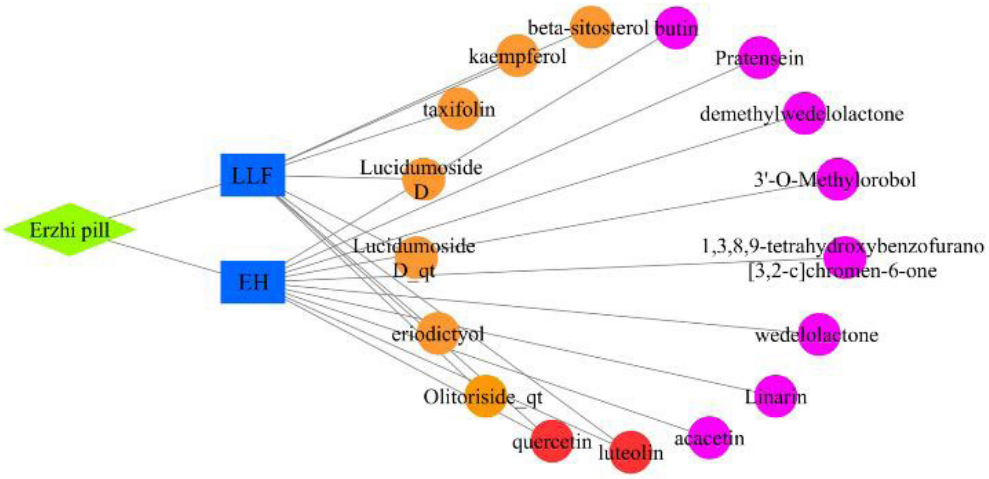

Figure 2: Active Ingredients of EZP: orange represents LLF, pink represents EH, and red represents the same active ingredients

Result

Screening the Active Ingredients and Targets of EZP. Through the TCMSP database, we initially obtained 119 chemical components of LLF and 48 chemical components of EH. 13 active components of LLF and 10 active components of EH were screened by ADME, and 17 active components of EZP were obtained by combining them and deleting duplicate values (Figure 2). Besides, 179 targets

were obtained from LLF, 165 targets were obtained from EH, and 187 targets were obtained from EZP by deleting duplicate values after data combination.

Identification of Targets Related to POI. There were 1137 targets from the GeneCard database, 147 targets from the PharmGkb database, 299 targets from the DisGeNET database, 53 targets from the DrugBank database, and 456 targets from OMIM. Finally, 1737 POI targets were obtained by combining 5 databases and a literature search, among which ESR1 and ESR2 were common targets (Figure 3). ESR1 is the gene code of estrogen receptor - α (ER - α) in the hypothalamus–hypophysis–ovarian (HPO) axis, which is involved in follicle formation; ESR2 is the gene code of estrogen receptor - β (ER - β) in the ovary, which is involved in follicle growth[28, 29].

Construction of Drug Active Ingredient and Disease Target Network. The 187 targets of EZP and 1737 targets of POI were intersected by R software, and the Venn diagrams were drawn (Figure 4). A total of 117 common targets of EZP and POI were obtained, which are therapeutic targets of EZP in the treatment of POI. We introduced 117 therapeutic targets and corresponding active components of EZP into Cytoscape to construct the network (Figure 5). There are 131 nodes and 253 edges, among which the triangle node represents the active component of EZP and the circle node represents the therapeutic target. The size and color of the points reflect the degree of freedom. The greater the degree of freedom, the more biological functions they participate in.

Protein-protein interaction (PPI) network construction and topological analysis. We introduced 117 com-

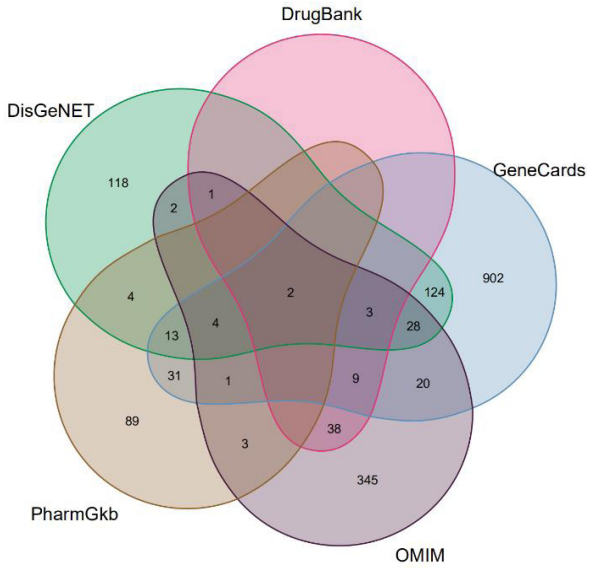

Figure 3: POI gene prediction analysis

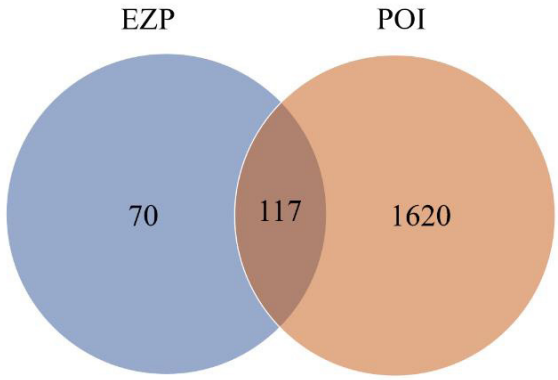

Figure 4: the common targets of EZP and POI

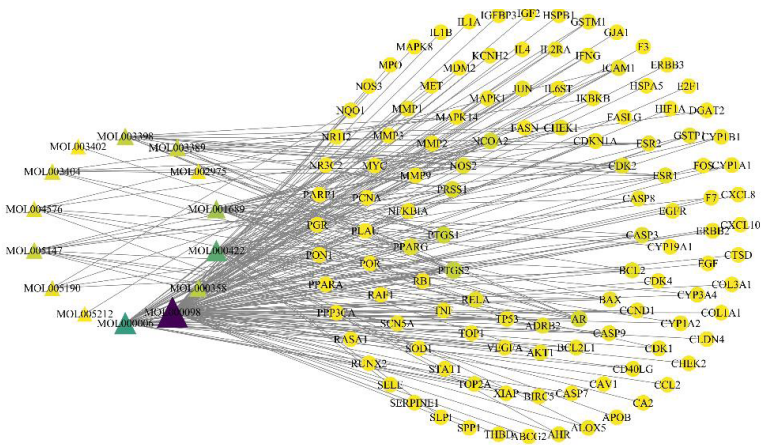

Figure 5: Drug active ingredients and disease target network

mon genes into the STRING database to construct the PPI network to obtain their interactions, and the network consists of 109 nodes and 439 edges (Figure 6). We download the TSV file from the STRING database and analyze the network topology by using the CytoNCA plug-in in Cytoscape 3.7.1. In the first screening, there were 33 nodes and 175 edges were obtained (BC > 41.39939932, CC > 0.151472651, DC > 7, EC > 0.046951622, LAC > 2.285714286 and NC > 3), in the second screening, there were 12 nodes and 46 edges were obtained (BC > 16.10041024, CC > 0.571428571, DC > 9, EC > 0.128233254, LAC > 5.5 and NC > 6.436868687). The results suggest that TP53, JUN, MAPK14, MAPK1, AKT1, ESR1, MYC, TNF, RELA, CCND1, CDKN1A, and RB1 are the core targets of EZP in the treatment of POI (Figure 7).

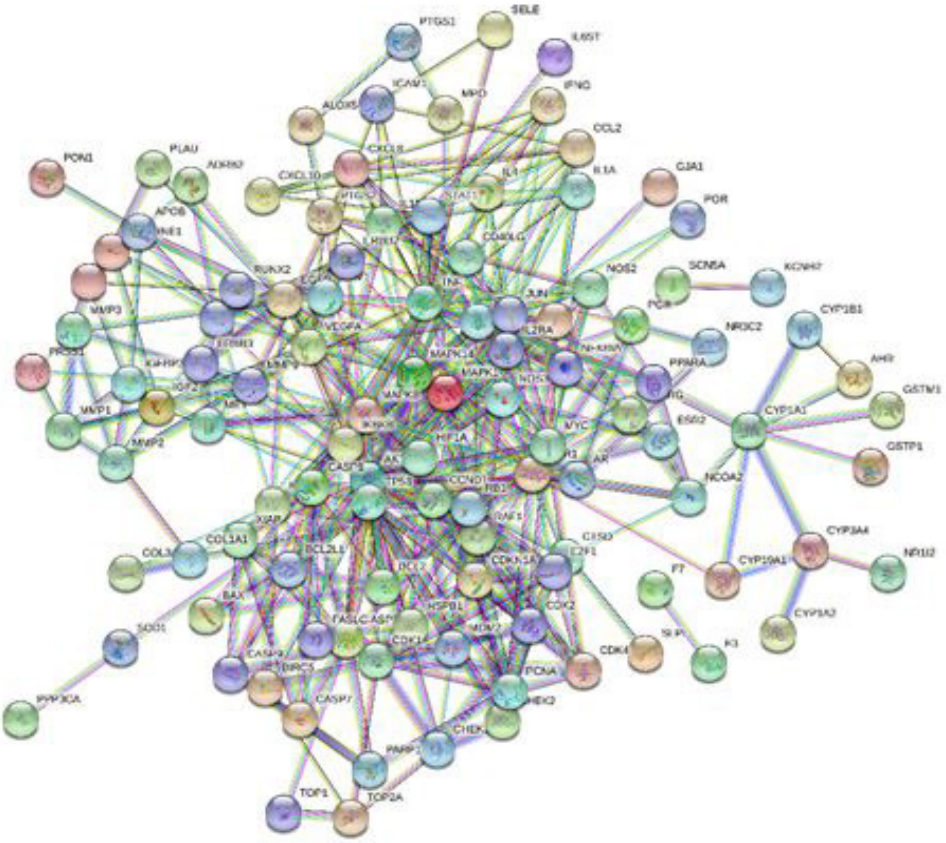

Figure 6: Protein-Protein interaction network

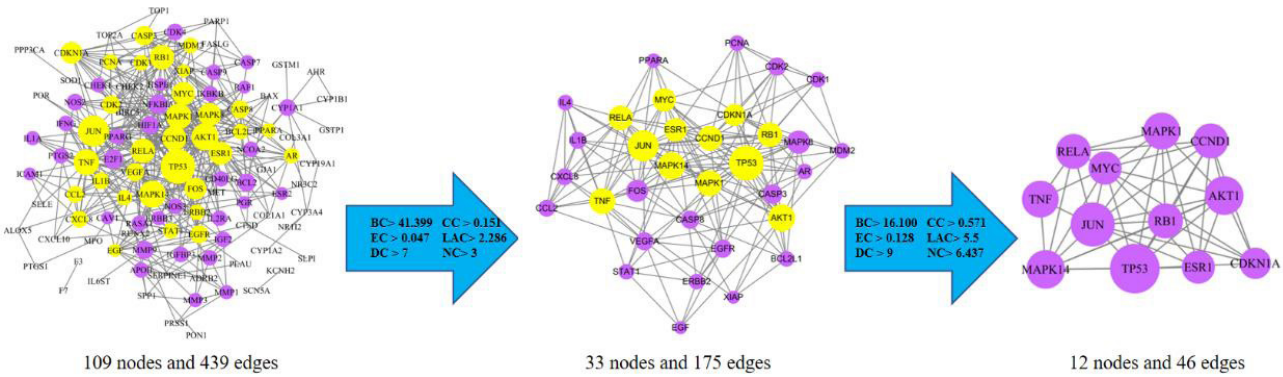

Figure 7: Process of topological screening for the PPI network

Go enrichment analysis and KEGG pathway analysis. GO enrichment analysis: ClusterProfiler was used to analyze GO, and the 2012 biological process, 57 cell components, and 47 molecular functions were obtained. We used P < 0:05 as the threshold to screen the top 10 analysis results of each item (Figure 8). KEGG pathway analysis: we also used ClusterProfiler to analyze the KEGG pathway of EZP in the treatment of POI. A total of 150 pathways were enriched, and the top 30 pathways are shown in Figure 9. POI is a disease of the early decline of human function, and these pathways are closely related to aging, indicating that EZP can improve the ovarian function of patients with POI in a variety of ways.

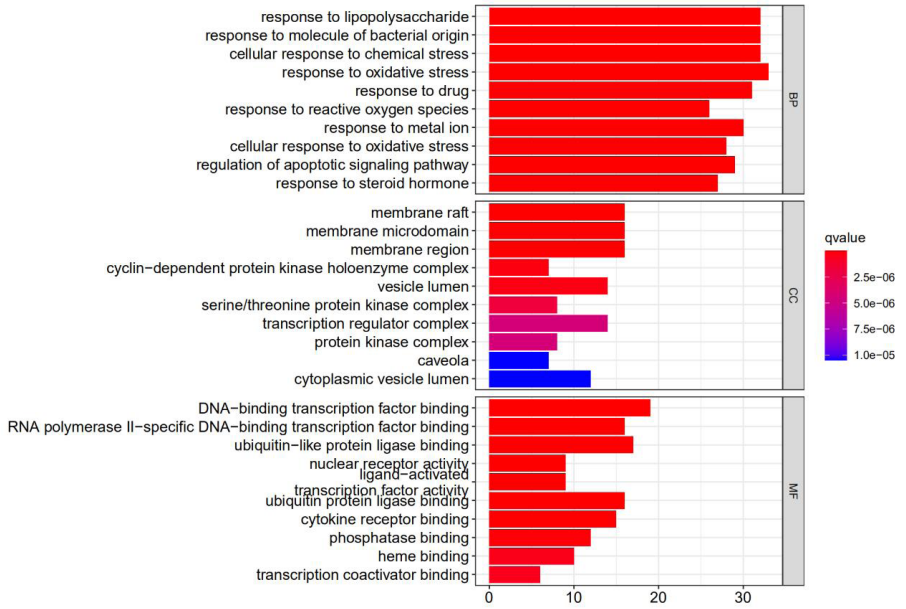

Figure 8: GO enrichment of EZP active components in the treatment of common targets of POI

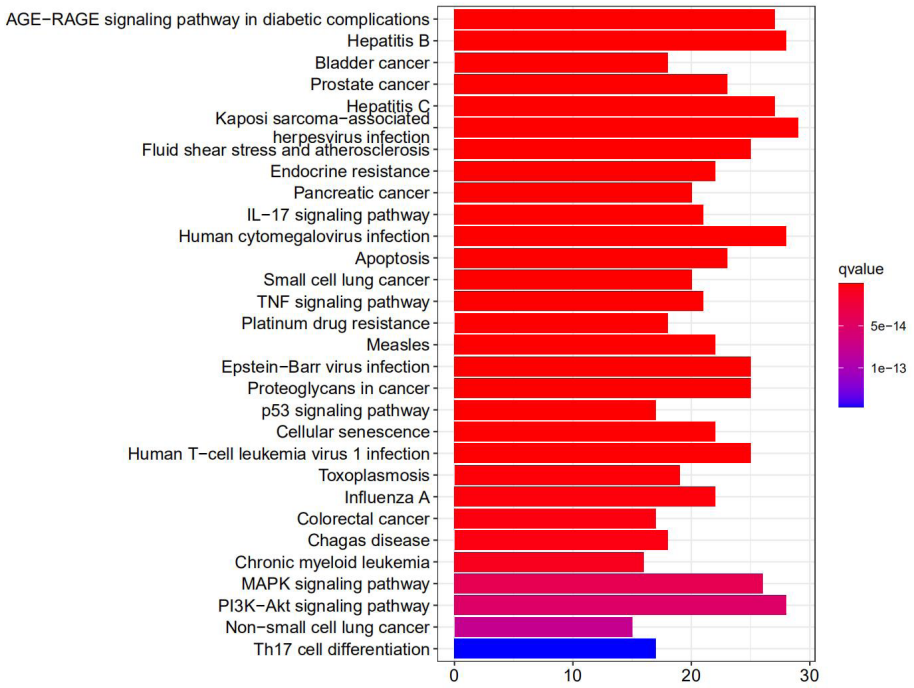

Figure 9: Enriched KEGG pathways of potential targets for treating POI from the main active ingredients of EZP

Molecular Docking Analysis. The molecular docking results show that the active ingredients of EZP match well with the hub genes. EZP targeted regulation of AKT1, TP53, MAPK1, TNF, JUN, RB1, and MYC is part of its mechanism in the treatment of POI (Figure 10). This indicates that the results of molecular docking are consistent with the screening results of network pharmacology, and molecular docking verifies the reliability of network pharmacology.

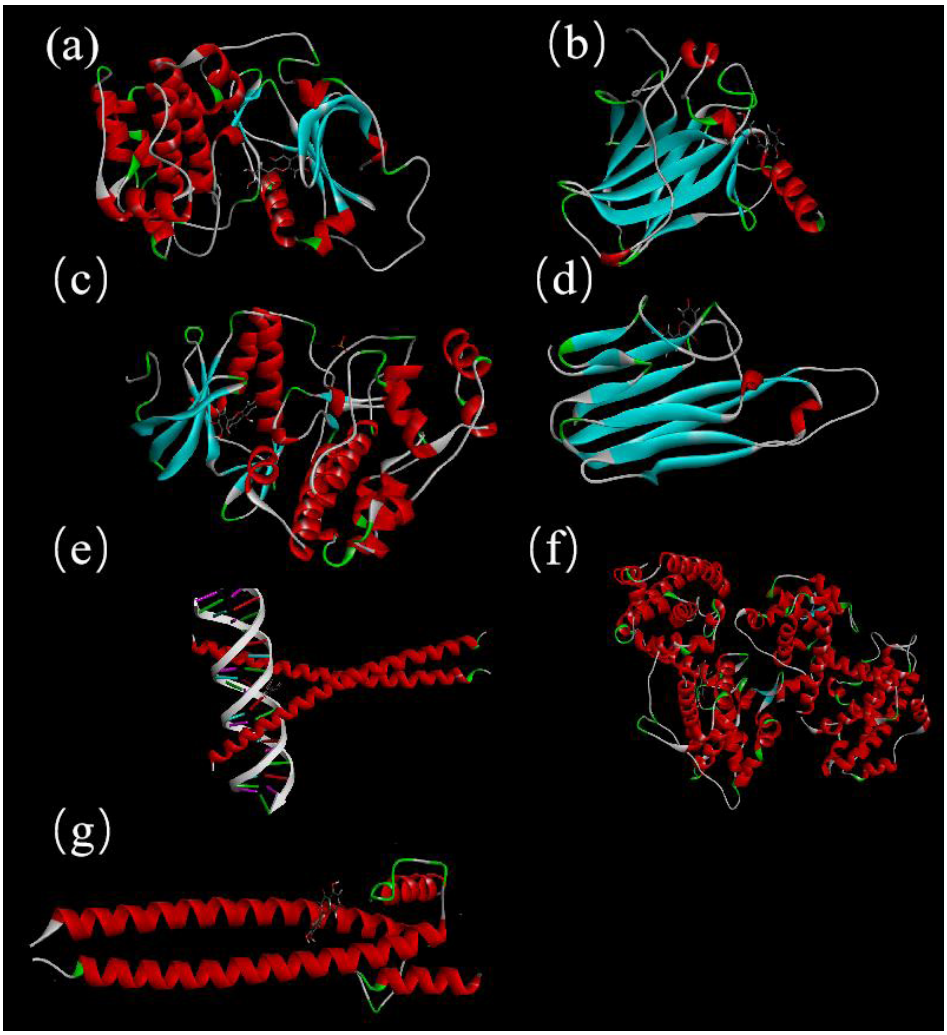

Figure 10: Molecular docking of compounds with core targets. (a) Docking process of quercetin with AKT1; (b) Docking process of quercetin with TP53; (c) Docking process of quercetin with MAPK1; (d) Docking process of quercetin with TNF; (e) Docking process of quercetin with JUN; (f) Docking process of quercetin with RB1; (g) Docking process of quercetin with MYC

Discussion

It is described in Fu Shou Jing Fang written by Wu Min in Ming Dynasty that EZP can "turn the white hair into blackness, strengthen the waist and knees, and strengthen Yin". The study found that EZP extract can regulate the reproductive - endocrine - immune network by stimulating the secretion of E2 by ovarian granulosa cells of female mice and promoting the expression of Er α in splenic cells, to improve the symptoms of low estrogen in the perimenopausal period [30]. EZP can reduce the content of D-Glucose 6-phosphate in aging renal cells of rats induced by D-galactose, and play an anti-aging role [31]. It was found that EZP could significantly induce the expression of luciferase driven by estrogen sensitive elements in the expression vector of pERE-Luc, which confirmed that EZP is an effective and safe estrogenic herbal extract [32]. This study aims to reveal the molecular mechanism of EZP in the treatment of POI and to provide ideas for further experiments and drug research and development.

We used the network pharmacology to analyze the "Component - Target - Pathway - Disease" of EZP. We found that EZP mainly contained 17 compounds, such as quercetin,

luteolin, and kaempferol, among which quercetin had the highest correlation. Quercetin can improve the AMH level and the number of primordial follicles of POF mice induced by cyclophosphamide and restore the ovarian function of POF mice [33]. Quercetin combined with vitamin E can increase the trabecular bone volume, bone cells, osteoblasts, and bone weight of ovariectomized mice and prevent osteoporosis after ovariectomy [34]. Quercetin can also inhibit oxidative stress and inflammation through AMPK/SIRT1/NF-κ B signaling pathway, and improve carotid atherosclerosis in diabetic rats [35]. In conclusion, quercetin can not only improve the ovarian function of POI patients but also alleviate the long-term complications such as osteoporosis and cardiovascular disease caused by low estrogen. Luteolin is a flavonoid with estrogen like effect [36], which can promote the secretion of estradiol by rat ovarian granulosa cells [37]. Kaempferol has a therapeutic effect on primary osteoporosis in ovariectomized rats by regulating the balance of Ca2 + metabolism, promoting the generation of bone collagen, and reducing the loss of bone trabecula [38]. In addition, kaempferol can inhibit the apoptosis induced by Brefeldin A by inhibiting the activity of caspase [39].

The occurrence of POI mainly involves two mechanisms: (1) abnormal activation of primordial follicles; (2) increased rates of apoptosis of oocytes [40]. The primordial follicle is the only form of female oocyte reserve, which is the key to maintain ovarian function. If the primordial follicle reserve in the ovary is insufficient or depleted prematurely, it will lead to the premature decline of female ovarian function. Most primordial follicles exist in the primordial follicle pool in the form of resting follicles, only a few follicles are activated and enter the growing follicle pool to mature or degenerate. If the maintenance and activation of the resting state of primordial follicles are out of balance, it will lead to excessive consumption of primordial follicles, which is regulated by many signaling pathways [41], such as the PI3K signaling pathway [42]. The lack of PTEN, a negative regulator of PI3K in mouse oocytes, will lead to the activation of the whole primordial follicle pool and premature depletion of primordial follicles, leading to premature ovarian failure [43].

Threonine kinase (Akt) is an important downstream target kinase of the PI3K signaling pathway, PI3K/Akt signaling pathway can regulate the growth and apoptosis of ovarian granulosa cells, participate in follicular growth and development, and affect female ovarian function[44, 45]. Although no experiment has confirmed that EZP can improve the ovarian function of patients with POI through PI3K/Akt signaling pathway, it has been confirmed that EZP can reduce the production of proinflammatory cytokines and transforming growth factor-β1, inhibit hepatocyte apoptosis and restore liver function by inhibiting PI3K/Akt/Raptor/Rictor signaling pathway [46]. KEGG enrichment analysis showed that EZP had 28 active components enriched in PI3K/Akt signaling pathway, such as AKT1, CDKN1A, CCND1, MAPK1, TP53, RELA, and MYC.

Apoptosis is a programmed cell death, and premature apoptosis of ovarian granulosa cells and oocytes leads to accelerated follicular atresia. The P53 signaling pathway is an important pathway of apoptosis, which is closely related to the occurrence of POI [47]. Activation of caspase family proteins can induce DNA degradation, mediate DNA damage, and eventually lead to apoptosis [48], and P53 is the upstream regulator of caspase family proteins. 17 active components in EZP, such as TP53, CDKN1A, and CCND1, participate in the P53 signaling pathway.

ESR1 is closely related to menarche age[49], menopause age[50], and osteoporosis in women. Studies have found that PvuII and XbaI polymorphisms in the ESR1 gene increase the risk of idiopathic POF in Chinese women [51]. MAPK signaling pathway is involved in cell proliferation, apoptosis, and inflammation [52-54]. Resveratrol has antioxidative stress and anti-inflammatory effects and is commonly used in the treatment of POI to improve ovarian function [55]. In addition, resveratrol can play an antioxidant role through the MAPK signaling pathway [56]. We found that 26 active components in EZP, such as RELA, TP53, MAPK14, MAPK1, TNF, JUN, and MYC, were enriched in the MAPK signaling pathway. Therefore, EZP can also improve ovarian function in patients with POI through the MAPK signaling pathway.

Through GO enrichment analysis, KEGG pathway analysis, and related literature search, we found that EZP Treatment of POI signal pathway mainly includes PI3K/Akt signal pathway, P53 signal pathway, apoptosis, cell aging, and MAPK signal pathway.

Conclusion

The results show that the same compound of EZP can treat POI in different ways, and the same target can interfere with different biological processes and signal pathways of POI. It is confirmed that EZP can improve the ovarian function of patients with POI through multiple pathways and targets, which provides evidence support for the clinical application of EZP in the treat-

ment of POI and provides new ideas for drug research on the treatment of POI. Due to the limitations of network pharmacology, this study only provides a preliminary prediction, and further experimental verification is needed in the future.

Data Availability

The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Disclosure

The funders had no role in the study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Conflict of Interest

All authors declare there are no competing interests.

Authors' Contributions

HHX designed the research and wrote the manuscript. Experimental work and data collection were conducted by HHX and SXQ.WRX searched the database.XGC and TY provided critical comments and revised the manuscript.

Funding Acknowledgments

This present study was supported by National Natural Science Foundation of China (grant no. 81873333) and Jiangsu Provincial Health Planning Commission Science, Education, and Health Strengthening Project: Jiangsu Provincial Clinical Medical Center of Gynecology of Traditional Chinese Medicine (YXZXB2016006).

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