


# Network pharmacology explores the mechanisms of *Eucommia ulmoides* cortex against postmenopausal osteoporosis

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## Abstract

Postmenopausal osteoporosis (PMOP) has become one of most frequent chronic disease worldwide with aging population. *Eucommia ulmoides* cortex (EU), a traditional Chinese medicine, has long since been used to treat PMOP. The aim of this study is to explore pharmacological mechanisms of EU against PMOP through using network pharmacology approach.

The active ingredients of EU were obtained from Traditional Chinese Medicine System Pharmacology database, and target fishing was performed on these ingredients in UniProt database for identification of their relative targets. Then, we screened the targets of PMOP using GeneCards database and DisGeNET database. The overlapping genes between PMOP and EU were obtained to performed protein–protein interaction, Gene Ontology analysis, Kyoto encyclopedia of genes, and genomes analysis.

Twenty-eight active ingredients were identified in EU, and corresponded to 207 targets. Also, 292 targets were closely associated with PMOP, and 50 of them matched with the targets of EU were considered as therapeutically relevant. Gene ontology enrichment analysis suggested that EU exerted anti-PMOP effects via modulating multiple biological processes including cell proliferation, angiogenesis, and inflammatory response. Kyoto encyclopedia of genes and genomes enrichment analysis revealed several pathways, such as PI3K-AKT pathway, mitogen-activated protein kinase pathway, hypoxia-inducible factors-1 pathway, tumor necrosis factor pathway, and interleukin-17 pathway that might be involved in regulating the above biological processes.

Through the method of network pharmacology, we systematically investigated the mechanisms of EU against PMOP. The multi-targets and multi-pathways identified here could provide new insights for further determination of more exact mechanisms of EU.

**Abbreviations:** BC = betweenness centrality, BP = biological processes, CC = closeness centrality, DC = degree centrality, DL = drug-likeness, EGF = pro-epidermal growth factor, EU = *Eucommia ulmoides* cortex, GO = Gene ontology, HIF-1 = hypoxia-inducible factors-1, IL-1 = interleukin-1, IL-17 = interleukin-17, IL-6 = interleukin-6, KEGG = Kyoto encyclopedia of genes and genomes, MAPK = mitogen-activated protein kinase, OB = oral bioavailability, PMOP = postmenopausal osteoporosis, PPI = protein–protein interaction, TCM = traditional Chinese medicine, TCMSP = Traditional Chinese Medicine System Pharmacology Database, TNF = tumor necrosis factor, VEGFA = vascular endothelial growth factor A.

**Keywords:** database, *Eucommia ulmoides*, herb medicine, network pharmacology, postmenopausal osteoporosis

## 1. Introduction

Postmenopausal osteoporosis (PMOP) is a metabolic bone disease frequently happened in postmenopausal women. PMOP

patients commonly present low bone mass, abnormal micro-structure, and high risk of fragility fracture.<sup>[1]</sup> About 15% of women >50 age are suffering in PMOP worldwide,<sup>[2]</sup> seriously

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YS and SC contributed equally to this work.

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**Data Availability Statement:** All our main data used to support the findings of this study have been deposited by the corresponding author. The datasets supporting the conclusions of this article are available in public database from TCMSP, UniProt, GeneCards, DisGeNET, String, DAVID, KEGG.

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

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affecting their health and quality of life, and the incidence will continue to rise with aging population. Currently, the treatment strategies for PMOP are consist of calcium, bone resorption inhibitors (e.g., bisphosphonates, estrogen receptor modulators, and calcitonin), and bone formation promoters (e.g., parathyroid hormone, fluorides).<sup>[3]</sup> Although these drugs can alleviate the clinical symptoms of PMOP, they cannot fundamentally improve the bone metabolism and have certain adverse reaction.<sup>[4]</sup> Therefore, exploring safer and more effective therapeutic strategies is largely needed for PMOP treatment.

Natural productions attract an increasing attention because of their relative safety and potential anti-osteoporosis effects. *Eucommia ulmoides* cortex (EU) also called Du-Zhong, a representative Chinese kidney-tonifying herb, is used alone or mixed with other herbs to treat various bone diseases for a long history.<sup>[5]</sup> Massive clinical and animal researches reported that EU has bone protect effects and can prevent the estrogen deficiency-induced bone loss and destruction.<sup>[6–8]</sup> The theory of “kidney governing bone” has well explained the anti-PMOP effects of EU from traditional Chinese medicine (TCM) perspective. PMOP occurs due to kidney deficiency and EU strengthens bone through nourishing kidney.<sup>[9]</sup> Modern composition study revealed EU rich in phytoestrogens including flavonoids, phenolic acids, and lignans.<sup>[10]</sup> Nevertheless, the pharmacological mechanisms of EU against PMOP remain largely unclear.

Due to the multi-components and multi-targets of TCM, traditional “one drug, one target” research strategy cannot meet the requirements to analyze numerous herbal compounds as a whole.<sup>[11]</sup> Network pharmacology, an emerging interdisciplinarity integrating bioinformatics, pharmacology, biology and computer science,<sup>[12]</sup> has been proposed as a new method for TCM research in recent years.<sup>[13]</sup> With the aid of database information and “Medicine - Targets - pathways - Disease” research mode, network pharmacology provides a more integrative and systematic research strategy on the mechanisms of TCM.<sup>[14]</sup>

In the present study, network pharmacology was used to comprehensively investigate the mechanisms of EU against PMOP. We screened out the potential targets of EU and PMOP via several databases. Based on their overlapping genes, we built a protein–protein interaction (PPI) network to analyze their interactions, and performed Gene ontology (GO) and Kyoto encyclopedia of genes and genomes (KEGG) analyses to reveal the key biological processes and signaling pathways. The specific protocol is shown in Fig. 1.

## 2. Material and methods

Ethical approval was waived or not necessary, all procedures performed in studies do not involve human participants or animals.

### 2.1. Collection of active ingredients of EU

Traditional Chinese Medicine System Pharmacology Database (TCMSP, <http://lsp.nwu.edu.cn/tcmsp.php>, Version 2.3) is a pharmacological platform of TCM that collected 499 herbs registered in Chinese pharmacopoeia (2015), related chemical ingredients and targets.<sup>[15]</sup> Oral bioavailability (OB) and drug-likeness (DL), 2 pharmacokinetic parameters, are commonly selected to identify the active ingredients. OB is defined as the efficiency of drug delivery to the systemic circulation. DL is a

qualitative concept used to assess drug properties including solubility and chemical stability. In this study, the screening criteria for EU were set as  $OB \geq 30\%$  and  $DL \geq 0.18$ .<sup>[15]</sup>

### 2.2. Targets prediction of active ingredients

The targets related to these active ingredients were predicted using the computer targeting technology developed by the TCMSP. With the filter of “swiss-prot reviewed” and “Homo sapiens,” the information of targets were further input into UniProt database (<https://www.uniprot.org/>) to obtain their gene symbols and ID.

### 2.3. PMOP targets

Two databases were used to select targets associated with PMOP. “postmenopausal osteoporosis” acting as the keyword was input into DisGeNET (<https://www.disgenet.org/>) and GeneCards (<https://www.genecards.org/>). After filtered by score  $>0.1$  in DisGeNET and score  $>10$  in GeneCards,<sup>[14]</sup> we finally obtained a total of 292 PMOP-related targets.

### 2.4. Venn diagram

A Venn analysis was performed on an online website (<https://bioinfogp.cnb.csic.es/tools/venny/index.html>) to identify the overlapping genes between EU and PMOP for further bioinformatic analyses.

### 2.5. Protein–protein interaction

The overlapping genes of EU and PMOP were imported into the STRING 11.0 (<https://string-db.org/>) for PPI analysis with the parameters of “Homo sapiens” and “confidence score  $>0.4$ .” The node–node data exported from STRING were visualized and analyzed their interconnection network using Cytoscape 3.7.1, and plug-in “Network Analysis” was performed to analyze degree centrality (DC), betweenness centrality (BC), and closeness centrality (CC), 3 topological properties of the network.<sup>[15]</sup>

### 2.6. GO enrichment analysis

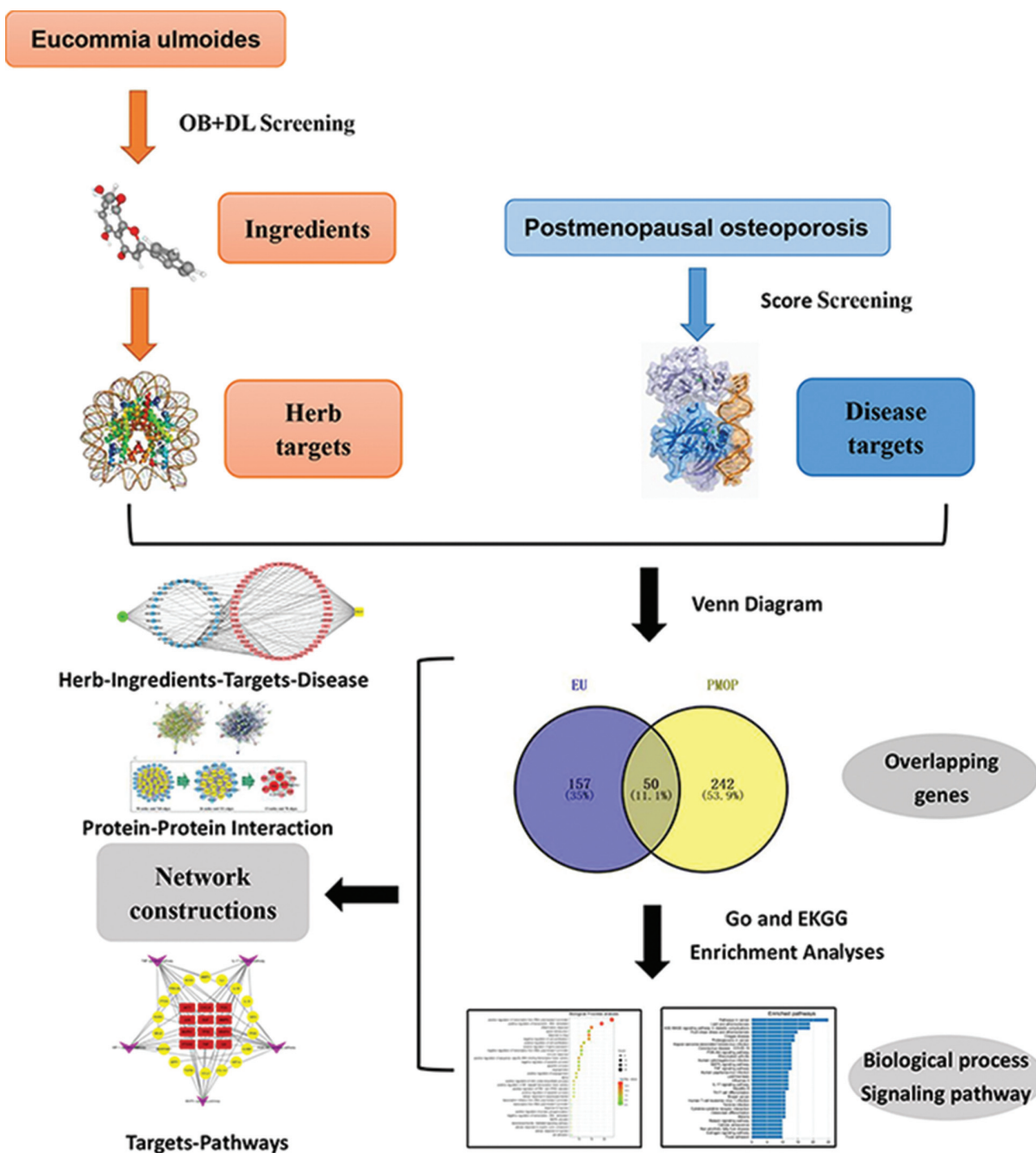
The overlapping genes of EU and PMOP were imported into the Annotation, Visualization, and Integrated Discovery (DAVID, <https://david.ncifcrf.gov/home.jsp>, version 6.8) for GO enrichment analysis. Three sub-items including molecular function, cellular component, and biological process (BP) were all analyzed. After filtered by False Discovery Rate  $<0.05$ ,<sup>[16]</sup> the information of top 30 BP terms were listed in a bubble diagram based on the ascending order of  $\log P$ -value.

### 2.7. KEGG enrichment analysis

The ID of overlapping genes were imported into KEGG (<https://www.kegg.jp/>) for pathway enrichment analysis. According to the descending order of gene number enriched in each pathway, we listed the top 30 signaling pathways in a bar diagram.

### 2.8. Network construction

We constructed networks as follows: Herb-Ingredients-Targets-Disease network. Targets-Pathways network. All visualized



**Figure 1.** The flow chart of network pharmacology based study for investigating underlying mechanisms of EU against PMOP. EU = *Eucommia ulmoides* cortex, PMOP = postmenopausal osteoporosis.

graphs of above networks were established by Cytoscape 3.7.1 (<https://cytoscape.org/>).

### 3. Results

#### 3.1. Active ingredients and target prediction of EU

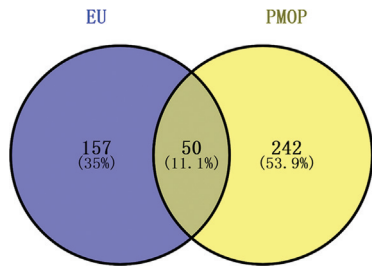
Through searching “duzhong” or “Eucommiae Cortex” in the TCMSP database, we obtained 147 related ingredients of EU. After screening with  $DL \geq 0.18$  and  $OB \geq 30\%$ , 28 active ingredients were identified in EU. These active ingredients were

further used to fish target genes using the Uniprot database, and 207 target genes were obtained.

#### 3.2. Overlapping targets between EU and PMOP

Though searching “postmenopausal osteoporosis” in the GeneCards database and DisGeNet database with the thresholds of given scores, a total of 292 molecular targets obtained. Then, 50 overlapping genes were identified by collecting the co-part of EU and PMOP in the Venn diagram (Fig. 2). These overlapping genes were regarded as the therapeutic targets through which EU





**Figure 2.** The Venn diagram of the targets both in EU and PMOP. The overlap part indicates the potential therapeutic targets by which EU treats PMOP. EU=*Eucommia ulmoides* cortex, PMOP=postmenopausal osteoporosis.

might exact anti-PMOP effects. Furthermore, a Herb-Ingredients-Targets-Disease network was constructed (Fig. 3). According to the number of edge between ingredient nodes and target nodes, we identified the top 3 ingredients, MOL000098 (quercetin, OB=46.43, DL=0.28), MOL000422 (kaempferol, OB=41.88, DL=0.24), and MOL002773 (beta-carotene, OB=37.18, DL=0.58), which were connected with 42, 15, and 8 overlapping genes, respectively. These 3 ingredients with high edge number played a crucial role when EU treats PMOP.

**3.3. PPI network and hub therapeutic targets**

Next, these 50 overlapping genes were imported into String database to establish a PPI network, obtaining 50 nodes and 740 edges. We plotted a PPI network according to the strength of evidence and confidence level (Fig. 4A), respectively. Moreover, 3 parameters including DC, BC, and CC were used to select the pivot nodes. Through the first screening round with the thresholds of  $DC \geq 20$ ,  $BC \geq 0.003$ , and  $CC \geq 0.720$ , we obtained 311 edges and 26 nodes. After screening with the thresholds of  $DC \geq 30$ ,  $BC \geq 0.008$ , and  $CC \geq 0.800$ , only 78 edges and 13 nodes were left (Fig. 4B). Therefore, these remained genes, serum albumin, interleukin-6 (IL-6), vascular endothelial growth factor A (VEGFA), matrix metalloproteinase-9, AKT, pro-epidermal growth factor (EGF), tumor necrosis factor (TNF), JUN,

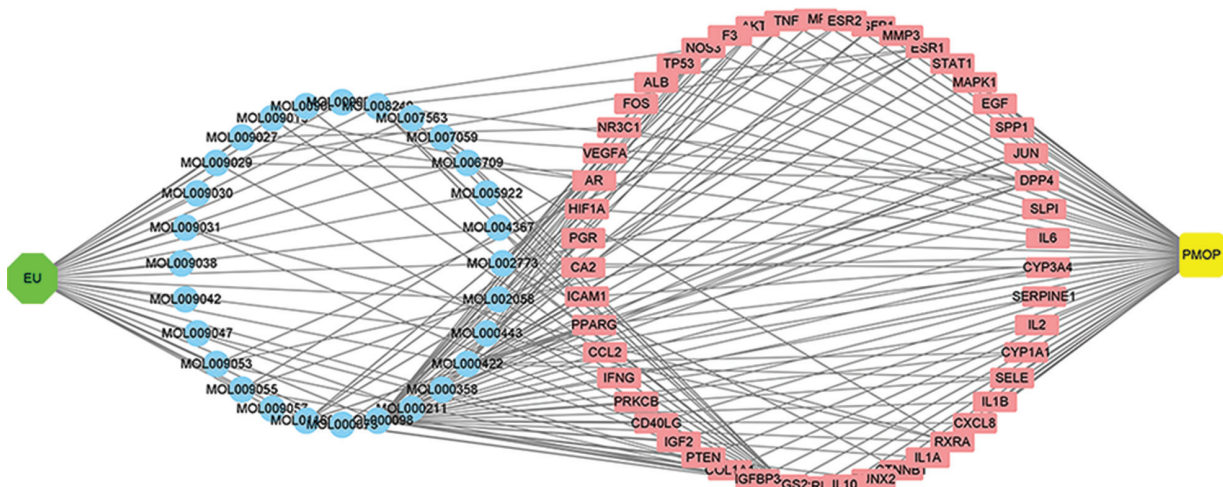
interleukin-8, TP53, prostaglandin G/H synthase 2, MAPK1, and FOS were considered as the hub therapeutic targets of EU. Their specific information were listed in Table 1.

**3.4. Biological processes of EU for PMOP treatment**

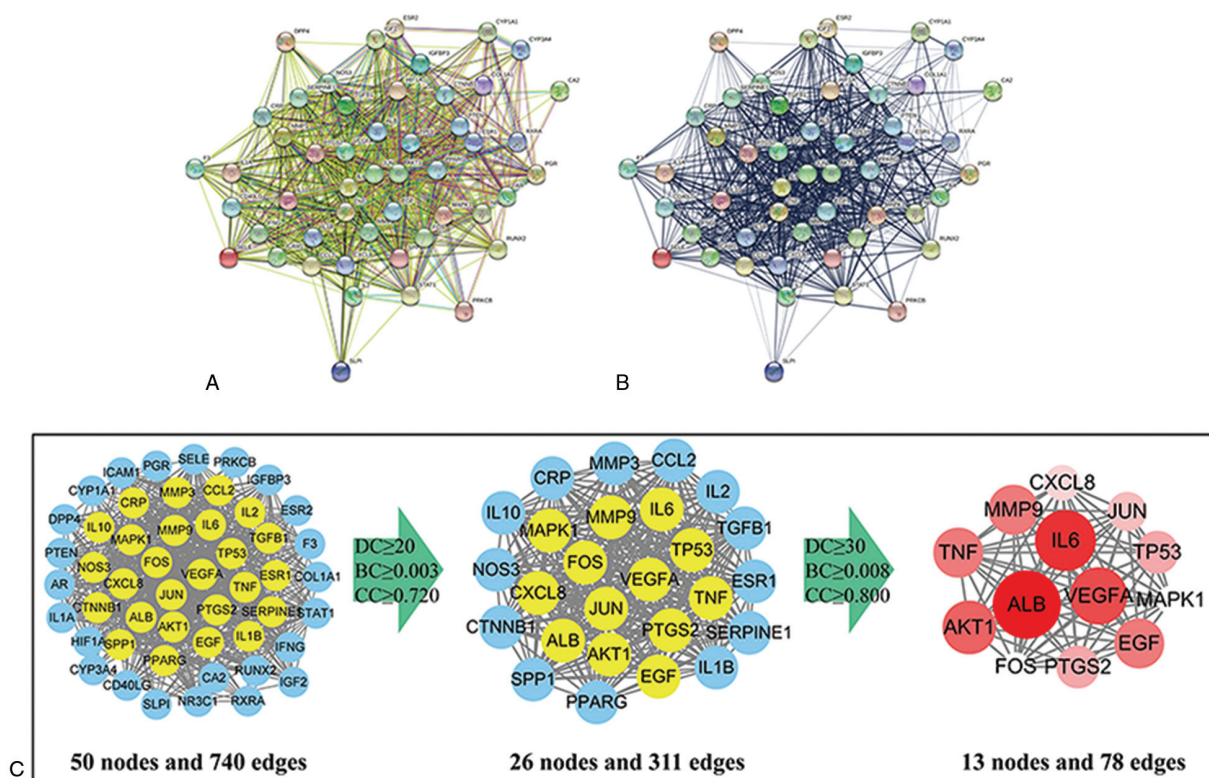
After screening GO items according to  $FDR < 0.05$  in the Annotation, Visualization and Integrated Discovery database, we obtained a total of 159 GO items and found that BP subset played a major role (Fig. 5A). We further build a bubble diagram to list the contents of top 30 BP terms (Fig. 5B). As the diagram shown, these 30 BP terms could be summarized into 3 categories including cell proliferation, angiogenesis, and inflammatory response. In the aspect of cell proliferation, negative regulation of transcription from RNA polymerase II promoter (GO:0000122), transcription from RNA polymerase II promoter (GO:0006366), transcription initiation from RNA polymerase II promoter (GO:0006367), positive regulation of cell proliferation (GO:0008284), negative regulation of cell proliferation (GO:0008285), negative regulation of transcription, DNA-templated (GO:0045892), positive regulation of DNA templated transcription (GO:0045893), positive regulation of transcription from RNA polymerase II promoter (GO:0045944), and positive regulation of sequence-specific DNA binding transcription factor activity (GO:0051091) were involved. In the aspect of angiogenesis, we found angiogenesis (GO:0001525), response to hypoxia (GO:0001666), positive regulation of angiogenesis (GO:0045766), and cellular response to hypoxia (GO:0071456). In the aspect of inflammatory response, lipopolysaccharide-mediated signaling pathway (GO:0031663), inflammatory response (GO:0006954), and cellular response to lipopolysaccharide (GO:0071222) were involved. These findings indicated that EU exacts therapeutic effects on PMOP possibly through a multi-biological process synergetic way.

**3.5. Signaling pathways of EU for PMOP treatment**

To further reveal the potential signaling pathways of EU for PMOP treatment, these 50 overlapping genes were performed



**Figure 3.** Herb-Ingredients-Targets-Disease network. The green hexagon node represents EU, the blue round nodes represent the ingredients, the pink oblong nodes represent targets, and the yellow quadrangle node represents PMOP. EU=*Eucommia ulmoides* cortex, PMOP=postmenopausal osteoporosis.



**Figure 4.** PPI network construction and screening. (A) PPI networks constructed using String database. Line colors indicate the type of interactive evidence. Line thickness indicates the confidence level of the supporting data. (B) The topological screening with degree centrality (DC), betweenness centrality (BC), and closeness centrality (CC) on PPI network. In the third image, the node with higher DC value represents more brilliant color and bigger size. PPI=protein-protein interaction.

with KEGG enrichment analysis. According to the descending order of gene counts contained in each pathway, we listed the top 30 pathways (Fig. 6A), among which mitogen-activated protein kinase (MAPK) pathway (hsa04010), PI3K-Akt pathway (hsa04151), hypoxia-inducible factors-1 (HIF-1) pathway (hsa04066), TNF pathway (hsa04668), and interleukin-17 (IL-17) pathway (hsa04657) were involved in the above 4 biological processes. Furthermore, a Targets-Pathways network was conducted to present the therapeutic targets enriched in the above pathways (Fig. 6B).

#### 4. Discussion

With aging progression, PMOP has become a public health disease worldwide. The side effects caused by the long-term use of anti-osteoporosis drugs are calling for a more effective and safer treatment strategy.<sup>[17]</sup> EU, a Chinese herbal medicine, has shown anti-PMOP effects both in clinic and animals.<sup>[6-8]</sup> In this study, we comprehensive explored the potential mechanisms of EU through network pharmacology approach.

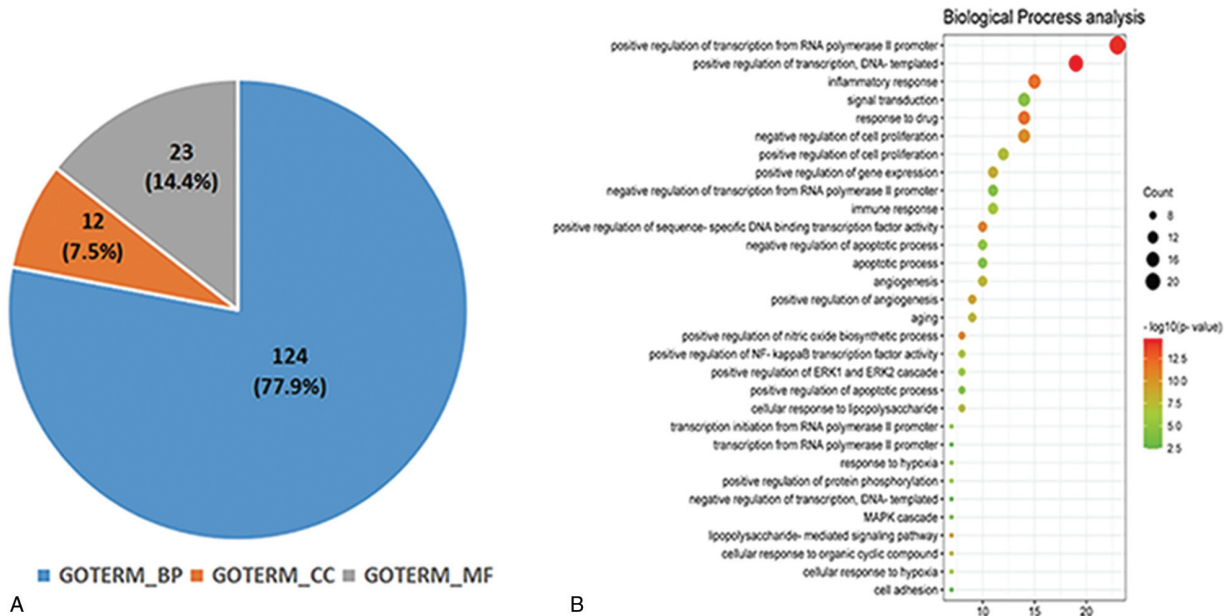
Based on the Herb-Ingredients-Targets-Disease network, we found 3 major indigents, quercetin, kaempferol, and beta-carotene. Through literature search, quercetin is shown to alleviate ovariectomy-induced osteoporosis through inhibiting osteoblast apoptosis and promoting angiogenesis; kaempferol can regulate osteogenic proliferation of bone marrow mesenchymal stem cells to improve bone mass<sup>[18]</sup>; beta-carotene has an inhibition effect on osteoclastogenesis.<sup>[19]</sup> These active ingredients are the important material foundation of EU against PMOP.

After collecting the overlapping genes between EU and PMOP in the Venn diagram, we identified a total of 50 potential therapeutic targets. PPI topological screening with DC, BC, and CC further revealed 13 hub targets that were serum albumin, IL-6, VEGFA, matrix metalloproteinase-9, AKT1, EGF, TNF, JUN, interleukin-8, TP53, prostaglandin G/H synthase 2, MAPK1, and FOS. Based on the findings in GO and KEGG enrichment

**Table 1**  
Information of 13 hub targets.

Uniprot ID	Gene symbol	Protein name
B7WNRO	ALB	Serum albumin
P05231	IL-6	Interleukin-6
P15692	VEGFA	Vascular endothelial growth factor A
P14780	MMP9	Matrix metalloproteinase-9
P31749	AKT1	RAC-alpha serine/threonine-protein kinase
P01133	EGF	Pro-epidermal growth factor
P01375	TNF	Tumor necrosis factor
P05412	JUN	Transcription factor AP-1
P10145	CXCL8	Interleukin-8
P04637	TP53	Cellular tumor antigen p53
P35354	PTGS2	Prostaglandin G/H synthase 2
P28482	MAPK1	Mitogen-activated protein kinase 1
P01100	FOS	Proto-oncogene c-Fos

ALB=serum albumin, CXCL8=interleukin-8, EGF=pro-epidermal growth factor, IL-6=interleukin-6, MAPK1=mitogen-activated protein kinase 1, MMP9=matrix metalloproteinase-9, PTGS2=prostaglandin G/H synthase 2, TNF=tumor necrosis factor, VEGFA=vascular endothelial growth factor A.



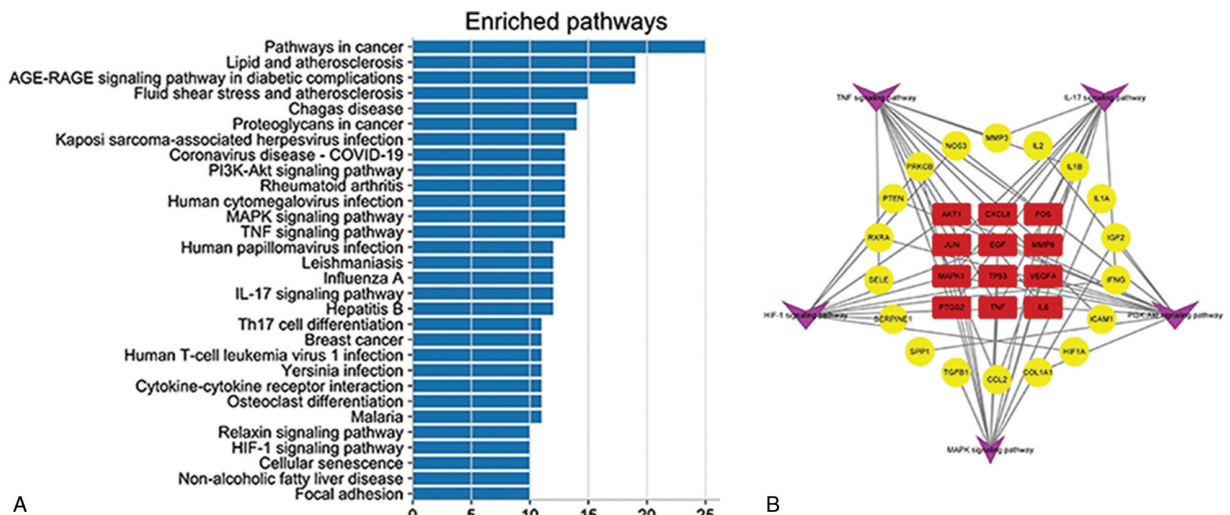
**Figure 5.** GO enrichment analysis on 50 overlapping genes. (A) Three types of GO items in DAVID database (GOTERM\_BP: biological process; GOTERM\_MF: molecular function; GOTERM\_CC: cellular component). (B) The bubble diagram of the top 30 BP items according to descending order of *P*-value. BP = biological processes, DAVID = the Annotation, Visualization, and Integrated Discovery, GO = gene ontology.

analyses, we speculated that EU treats PMOP possibly associated with the regulation of several biological processes and signaling pathways.

**4.1. Cell proliferation**

Bone health depends on a dynamic balance of osteoblastic bone formation and bone resorption mediated by osteoclasts.<sup>[20]</sup> The abnormal proliferation of osteoblast and osteoclast breaks their balance and contributes to pathogenesis of PMOP.<sup>[21]</sup> Both MAPK pathway and PI3K-Akt pathway play a crucial role in regulating cell proliferation.<sup>[22,23]</sup> The Targets-Pathways net-

work showed 6 hub targets involved in PI3K-Akt pathway and 8 hub targets in MAPK pathway. Among these targets, EGF acting as the earliest identified growth factor can activate both PI3K-AKT pathway and MAPK pathway.<sup>[24,25]</sup> Evidence from animal studies revealed that PI3K-AKT pathway promotes osteoblast proliferation<sup>[26]</sup> and is the crucial targets for PMOP treatment.<sup>[27]</sup> While down-regulation of MAPK pathway attenuate postmenopausal bone loss mainly through inhibiting osteoclast proliferation.<sup>[23]</sup> Therefore, we have reasons to believe, cell proliferation regulated by PI3K-AKT pathway and MAPK pathway are an important part of mechanism of EU against PMOP.



**Figure 6.** KEGG enrichment analysis and Targets-Pathways network. (A) Details of the top 20 pathways in KEGG database. (B) Network between 5 key pathways and 30 therapeutic targets (outer ring represents 5 KEGG pathways, middle ring represents 18 common targets, inner rectangle represents 12 hub targets). KEGG = Kyoto encyclopedia of genes and genomes.



## 4.2. Angiogenesis

Bone formation both in development and regeneration requires a temporal and spatial involvement of angiogenesis, which is called angiogenesis-osteogenesis coupling.<sup>[28,29]</sup> Spatially, vascular invasion brings essential oxygen, nutrients as well as various bone cells from neighbor tissues.<sup>[30]</sup> Temporally, angiogenesis is a prerequisite for the subsequent mineralization and modeling of bone template.<sup>[31]</sup> Recent studies have identified a new subtype of blood vessel with high expansions of platelet/endothelial cell adhesion molecule 1 and endomucin that contribute to form the niche of osteoprogenitors.<sup>[32,33]</sup> In contrast, reduction of angiogenesis is one of most critical pathogenesis to various bone diseases, such as femoral head necrosis, bone nonunion as well as PMOP. VEGFA, one of the hub targets, can drive proliferation and migration of endothelial cells, vessel pruning, and anastomosis for vascularization.<sup>[34]</sup> VEGFA is a downstream target of HIF-1 pathway, and its transcription and translation are activated after HIF-1 $\alpha$  binding to VEGF gene promoter.<sup>[35]</sup> KEGG enrichment analysis and Targets-Pathways network revealed that VEGFA and HIF-1 pathway were both identified in the anti-PMOP event of EU, and VEGFA was contained in HIF-1 pathway. Thus, promotion of angiogenesis regulated by HIF-1 pathway might be a potential mechanism of EU for PMOP treatment.

## 4.3. Inflammatory response

Inflammatory response plays an important role in the occurrence and development of PMOP.<sup>[36]</sup> Several pro-inflammatory cytokines such as TNF, Interleukin-1 (IL-1), and IL-6, have shown close association with osteoclast bone resorption.<sup>[37]</sup> TNF synthesized by T-lymphocytes can not only promote formation and activation of osteoclasts, but also has potent antiapoptotic effects on osteoclasts, prolonging their lifespan.<sup>[38]</sup> IL-1 is considered as “osteoclast activating factor” that signals on osteoclast lineage cells to stimulate osteoclastogenesis and resorptive capacity.<sup>[39]</sup> IL-6 is highly increased post-menopause and can stimulate osteoclast bone resorption, leading to low bone mineral density in postmenopausal women.<sup>[40]</sup> According to the results of Target-Pathway network, both hub targets (TNF and IL-6) and common targets (IL1A and IL1B) were all enriched in TNF pathway and IL-17 pathway. Therefore, the anti-PMOP effects of EU partly depends on inhibiting inflammatory response induced bone resorption.

There are some limitations in the present study. As these active ingredients of EU are predicted by computer arithmetics, its exact pharmacokinetic profile in human bodies still needs to be determined by using liquid chromatography/tandem mass spectrometry. Bioenrichment analyses reveal several potential targets, biological processes, and signaling pathways of EU against PMOP, while subsequent animal and cellular validations need be performed to determine its exact mechanisms.

## 5. Conclusions

By utilizing network pharmacology, the present study predicts the potential therapeutic targets of EU and reveals that EU treats PMOP possibly through acting on 3 aspects: regulation of bone cell proliferation, promotion of angiogenesis, and inhibition of inflammatory response. KEGG pathway analysis revealed that MAPK pathway, PI3K-Akt pathway, HIF-1 pathway, TNF pathway, and IL-17 pathway are involved in anti-PMOP event of

EU. Thus, it can be concluded that the pharmacological mechanism of EU against PMOP is a direct or indirect synergy way of multi-targets and multi-pathways. Despite it still needs a mount of experimental validations to determine the exact mechanism of EU, we provide promising directions for future research.

## Author contributions

**Conceptualization:** Chenjie Xia, Yan Shao.

**Data curation:** Chenjie Xia, Yan Shao.

**Formal analysis:** Chenjie Xia, Jin Li.

**Funding acquisition:** Chenjie Xia, Jin Li.

**Investigation:** Yan Shao.

**Methodology:** Ke Zhou, Yan Shao.

**Project administration:** Kaifeng Gan, Ke Zhou, Song Chen, Yan Shao.

**Resources:** Song Chen, Yan Shao.

**Software:** Jin Li, Ke Zhou, Song Chen.

**Supervision:** Kaifeng Gan, Song Chen.

**Validation:** Jin Li, Song Chen.

**Writing – original draft:** Chenjie Xia.

**Writing – review & editing:** Chenjie Xia, Ke Zhou.

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