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DECIPHERING THE MOLECULAR MECHANISMS OF EPIMEDIUM IN STEROID-INDUCED AVASCULAR NECROSIS OF THE FEMORAL HEAD: A NETWORK PHARMACOLOGY APPROACH

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Abstract: *Steroid-induced avascular necrosis of the femoral head (SANFH) is a debilitating condition caused by the prolonged and improper use of glucocorticoids. If left untreated, it can lead to femoral head collapse and significant impairment of a patient's quality of life. Currently, there is no effective non-surgical treatment for SANFH. Traditional Chinese medicine, with its holistic approach, is gaining importance in SANFH diagnosis and treatment. Epimedium, a medicinal herb with properties that nourish kidney yang and strengthen bones, is a promising candidate for SANFH therapy. In this study, we employ network pharmacology to elucidate the interactions between Epimedium and SANFH. Our analysis aims to reveal the action targets, as well as the signaling pathways, of Epimedium's active components in the treatment of SANFH, providing a foundation for the development of novel treatments.*

Keywords: *Steroid-induced avascular necrosis of the femoral head, SANFH, Epimedium, network pharmacology, traditional Chinese medicine.*

1. Introduction

Steroid-induced avascular necrosis of femoral head (SANFH) is caused by long-term improper use of glucocorticoids and is characterized by ischemia of the femoral head and damage to cells associated with bone formation, and if left untreated, the ultimate outcome is the collapse of the trabecular structure [1]. In a Chinese epidemiological study, SANFH accounted for approximately 24.1 percent of all cases of osteonecrosis of the femoral head in China [2]. The pathogenesis of SANFH is unclear because it involves a variety of different signaling pathways, and genetic factors and environmental backgrounds also play an important role [3]. The collapse of the femoral head has a great impact on the quality of life of patients, so early detection and timely treatment before collapse is critical, and there is currently no targeted drug treatment, and there is no other ideal non-surgical option [4]. With the increasing influence of the traditional Chinese medicine industry, the field of traditional Chinese medicine has played a significant role in the diagnosis and treatment of SANFH, so the discovery of SANFH with strong targeting of traditional Chinese medicinal materials, and the prediction of their related targets and signaling pathways, hoping to lay a solid foundation for the research and development of new drugs. Epimedium is pungent, bitter, and slightly warm, enters the kidney meridian of Foot Shaoyin, and has the functions of tonifying kidney yang and strengthening muscles and bones [5]. From the perspective of syndrome classification in traditional Chinese medicine,

steroid-induced necrosis of the femoral head is mainly divided into the following four types: qi stagnation and blood stasis type, wind-cold dampness type, liver and kidney deficiency type, and qi-blood deficiency type[6]. Clinically, Epimedium is often used as the king medicine, supplemented by medicines for promoting blood circulation, removing blood stasis, and nourishing qi and blood, so as to nourish the liver and kidney, and strengthen the muscles and bones, so it is of great benefit to the formation of bones. However, its mechanism of action is still unclear. Network pharmacology screens the relationship between drugs and disease gene targets and signaling pathways, and gets rid of the previous research method of "single target, single disease, and single drug". To explore the mechanism of interaction between diseases and drug ingredients at an overall level, which coincides with the holistic view of TCM syndrome differentiation and treatment [7]. In this study, based on network pharmacology, the interaction system network between the traditional Chinese medicine Epimedium and SANFH was constructed, aiming to explore the action targets and signaling pathway mechanisms of the potential active ingredients of Epimedium in the treatment of SANFH.

2. Method

2.1 Collecting the active ingredients and targets of Epimedium

The active ingredients of Epimedium were collected through the Traditional Chinese Medicine Systematic Pharmacological Analysis Database (TCMSP, http://tcmspw.com/tcmsp.php), and screening criteria were set to find out the active ingredients with oral bioavailability (OB) > 30% and drug-like (DL) >0.18, then exported the data information and saved as a ".xslx" format file, and then exported the data information and saved as a ".xslx" format file, and then passed the data through UniProt Database (https://www.uniprot.org/) was converted to the target gene name.

2.2 Collection of targets related to SANFH

Through the OMIM (https://www.omim.org/) and GeneCards (https://www.genecards.org/) Databases, enter "steroid-induced avascular necrosis of femoral head", export and save the data results as a ".xslx" format file, and then deduplicate the relevant targets through Excel software. Then the union of the two sets of data was taken to obtain the relevant targets of SANFH.

2.3 Construction of TCM regulation network diagram

The target related to SANFH and the target of the active ingredient of Epimedium were intersected through the Venn diagram online platform (https://bioinfogp.cnb.csic.es/tools/venny/index.html), and the Venn diagram was drawn with the help of the Venn diagram online platform. After obtaining the intersection target, Cytoscape3.8.2 software was used to make a TCM regulatory network diagram of "Epimedium - Active Ingredient - Target - SANFH" with the intersection target, active ingredient, epimedium and disease data as input files.

2.4 The GO enrichment analysis and KEGG pathway enrichment analysis

The Matascape database website was used to perform gene ontolog (GO) enrichment analysis and genome encyclopedia (KEGG) metabolic pathway enrichment analysis for intersection targets, and then the microbiotic information mapping platform (http://www.bioinformatics.com.cn) was used to visualize the exported data results, and the enrichment analysis results were selected according to the significance of the p-value to draw a bubble chart. According to the results of KEGG pathway enrichment analysis, the relevant pathway map was made, and then the three GO enrichment analyses were represented in the same column chart through the microbiotic mapping platform, and detailed data were exported.

2.5 Building a protein interaction network diagram (PPI)

Draw a preliminary protein-protein interaction (PPI) network diagram by clicking STRING (https://string-db.org/) online website on the above intersection targets, and then set the filter through the website. Taking the protein interaction comprehensive score ≥ 0.9 as the standard, and removing the free unrelated nodes, reset the PPI network construction diagram, download and save the picture, and then export the data as "PPI.cys" for further image modification visualization in Cytoscape 3.82 software, and finally export the picture to obtain the protein interaction network diagram.

2.6 Screening of key targets

By analyzing the above protein interaction network (PPI) data, the top 5 target genes can be obtained according to the Degree value, and if the more nodes in the PPI are connected to other nodes, that is, the larger the Degree value, the more critical this node is, so as to obtain the key targets in the protein interaction network.

2.7 Molecular docking verification

Through the above screened key targets, and then with the help of the correlation between the active ingredients and intersection targets derived from the TCM regulatory network, the active ingredients connected by these key targets can be found, and then the 3D chemical structure of "ingredients.mol2" can be obtained by searching the TCMSP database, and the RCSB PDB database (http:// www.rcsb.org) Search key targets, consult relevant literature, determine the protein crystal structure that meets the standard, remove water molecules and remove excess ligands through PyMOL software and save it to PDB format, and then use AutoDock Tools software to hydrogenate the structure, calculate the charge and a series of operations, set the Grid Box size according to all the protein coverage, and perform molecular docking through the Lamarckiam GA algorithm. The docking effect between ligand and acceptor was evaluated by binding energy, the file format was converted by OpenBabel software, and the molecular docking results were visualized by PyMOL software.

3. Results

3.1 Active ingredients of Epimedium and their targets

With the help of TCMSP website, a total of 130 ingredients were retrieved, after filtering and screening, a total of 23 active ingredients of Epimedium were obtained, and the specific information of active ingredients of Epimedium is shown in Table 1, and then 479 targets related to the active ingredients of Epimedium were retrieved with the help of the TCMSP website (there were duplicate targets), and a total of 209 targets of active ingredients of Epimedium were obtained after deduplication.

Table 1: Epimedium active ingredient information table.

Drug	Mol ID	Ingredients name	OB (%)	DL
Epimedium	MOL000622	Magnograndiolide	63.71	0.19
Epimedium	MOL004367	olivil	62.23	0.41
Epimedium	MOL004388	6-hydroxy-11,12-dimethoxy-2,2-dimethyl-1,8-dioxo-2,3,4,8-tetrahydro-1Hisochromeno[3,4-h]isoquinolin-2-ium	60.64	0.66
Epimedium	MOL004382	Yinyanghuo A	56.96	0.77
Epimedium	MOL004396	1,2-bis(4-hydroxy-3-methoxyphenyl)propan-1,3-diol	52.31	0.22
Epimedium	MOL004386	Yinyanghuo E	51.63	0.55
Epimedium	MOL004391	8-(3-methylbut-2-enyl)-2-phenyl-chromone	48.54	0.25
Epimedium	MOL000098	quercetin	46.43	0.28
Epimedium	MOL004384	Yinyanghuo C	45.67	0.5
Epimedium	MOL004373	Anhydroicaritin	45.41	0.44
Epimedium	MOL001645	Linoleyl acetate	42.1	0.2
Epimedium	MOL000422	kaempferol	41.88	0.24
Epimedium	MOL004394	Anhydroicaritin-3-O-alpha-L-rhamnoside	41.58	0.61
Epimedium	MOL004425	Icariin	41.58	0.61
Epimedium	MOL004380	C-Homoerythrinan, 1,6-didehydro-3,15,16-trimethoxy-, (3.beta.)	39.14	0.49

Epimedium	MOL003542	8-Isopentenyl-kaempferol	38.04	0.39
Epimedium	MOL001510	24-epicampesterol	37.58	0.71
Epimedium	MOL001771	poriferast-5-en-3beta-ol	36.91	0.75
Epimedium	MOL000359	sitosterol	36.91	0.75
Epimedium	MOL000006	luteolin	36.16	0.25
Epimedium	MOL003044	Chryseriol	35.85	0.27
Epimedium	MOL001792	DFV	32.76	0.18
Epimedium	MOL004427	Icariside A7	31.91	0.86

3.2 The targets related to SANFH

The 257 known targets related to SANFH were retrieved through the GeneCards database, the 2090 known targets related to SANFH were retrieved through the OMIM database, and after deduplication treatment, a total of 1550 related targets were obtained for inclusion in the study.

3.3 Diagram of the regulatory network of Traditional Chinese Medicine

Through the Wayne diagram online platform, 1550 SANFH related targets and 479 epimedium active ingredient targets were imported into the website, and then the Wayne diagram was made by means of intersection, and the results showed that there were a total of 128 intersection targets for the treatment of SANFH of epimedium in the treatment of SANFH, and they are shown in Figure 1. According to the active ingredients and intersection targets obtained by the above search, the working file was imported, and the TCM regulatory network diagram was made and analyzed by Cytoscape software, and they are shown in Figure 2. This network contains a total of 153 nodes and 454 edges, and among the active ingredients of epimedium, the largest number of quercetin targets is 101, followed by luteolin. In terms of core targets, prostaglandin G/H synthetase 2 (PTGS2) can be linked to 17 active ingredients, and steroid receptor activating protein 2 (NCOA2) can be linked to 16 active ingredients.

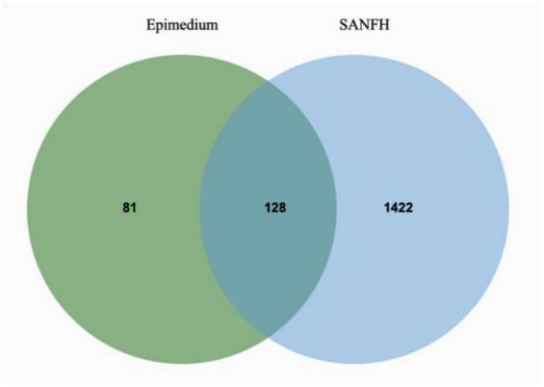

Figure 1: Venn diagram.

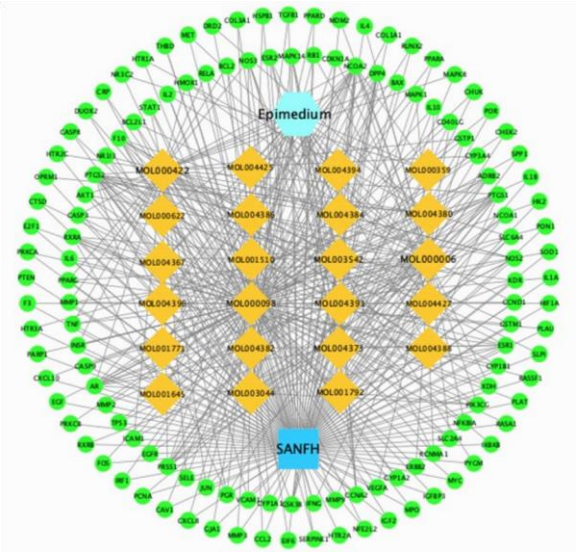

Figure 2: Diagram of regulation network of Traditional Chinese Medicine.

3.4 KEGG pathway enrichment and GO enrichment analysis

In this study, the intersection target of epimedium active ingredient-SANFH of Epimedium was enriched and analyzed through the Matascape website, and the pathway with significant enrichment was inferred by including relevant targets. The species was selected as human, the threshold standard was P<0.01, and the KEGG pathway enrichment analysis data were obtained, and 196 related items could be known after the KEGG pathway enrichment analysis results, with a maximum of 59 intersection targets and a minimum of 3 intersection targets. By exporting the data, the top 20 bits are selected according to the significance of the p-value, and the bubble chart can be obtained by plotting on the microbiotic mapping platform, and they are shown in Figure 3. GO enrichment analysis showed that 1995 bioprocess (BP)-related items were obtained, with a maximum of 48 intersection targets and a minimum of 3 intersection targets. A total of 149 molecular function (MF) related items were obtained, with a maximum of 28 intersection targets and a minimum of 3 intersection targets. 84 cell composition (CC) related entries were obtained, with the maximum effect on 22 intersection targets and the least effect on 3 intersection targets, and the top 10 were selected according to the significance of p-value on the microbiotic information platform for GO enrichment analysis histogram, and they are shown in Figure 4. According to the results of GO and KEGG pathway enrichment analysis, the molecular functions (MF) associated with the treatment of SANFH of Epimedium include cytokine receptor binding, protein kinase activity, protease binding, and protein domain-specific binding. Associated biological processes (BP): response of steroids, response of cells to lipids, oxygen levels, response to cytokines, etc.; Relevant cellular composition (CC) includes membrane rafts, extracellular

matrices, transcription regulator complexes, etc. Signaling pathways include lipid and atherosclerotic pathways, necrotic pathways, MAPK pathways, PI3K-AKT signaling pathways, etc. Figure 5 was obtained by plotting the results of KEGG pathway enrichment analysis, which showed that a total of 32 targets were involved in lipid and atherosclerotic pathways, and the redder targets, the closer the connection with this pathway.

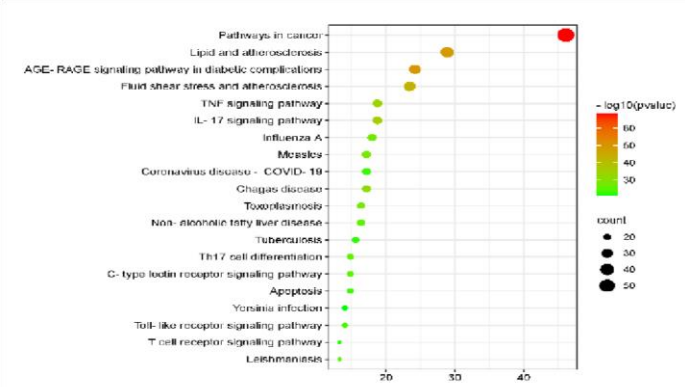

Figure 3: Bubble chart of KEGG pathway enrichment analysis.

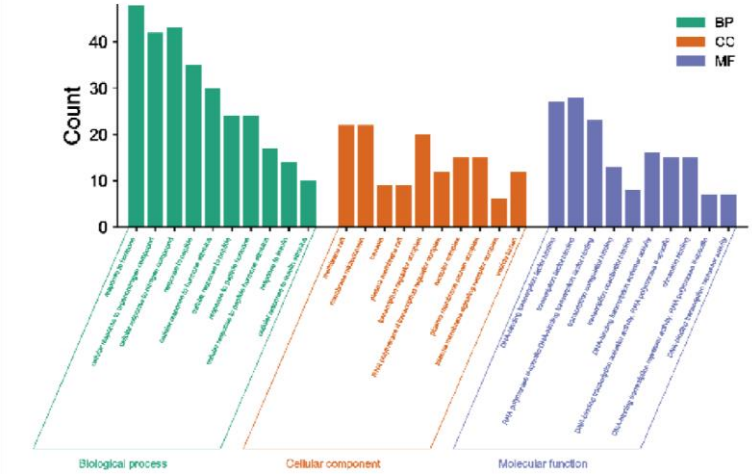

Figure 4: GO enrichment analysis three-in-one histogram.

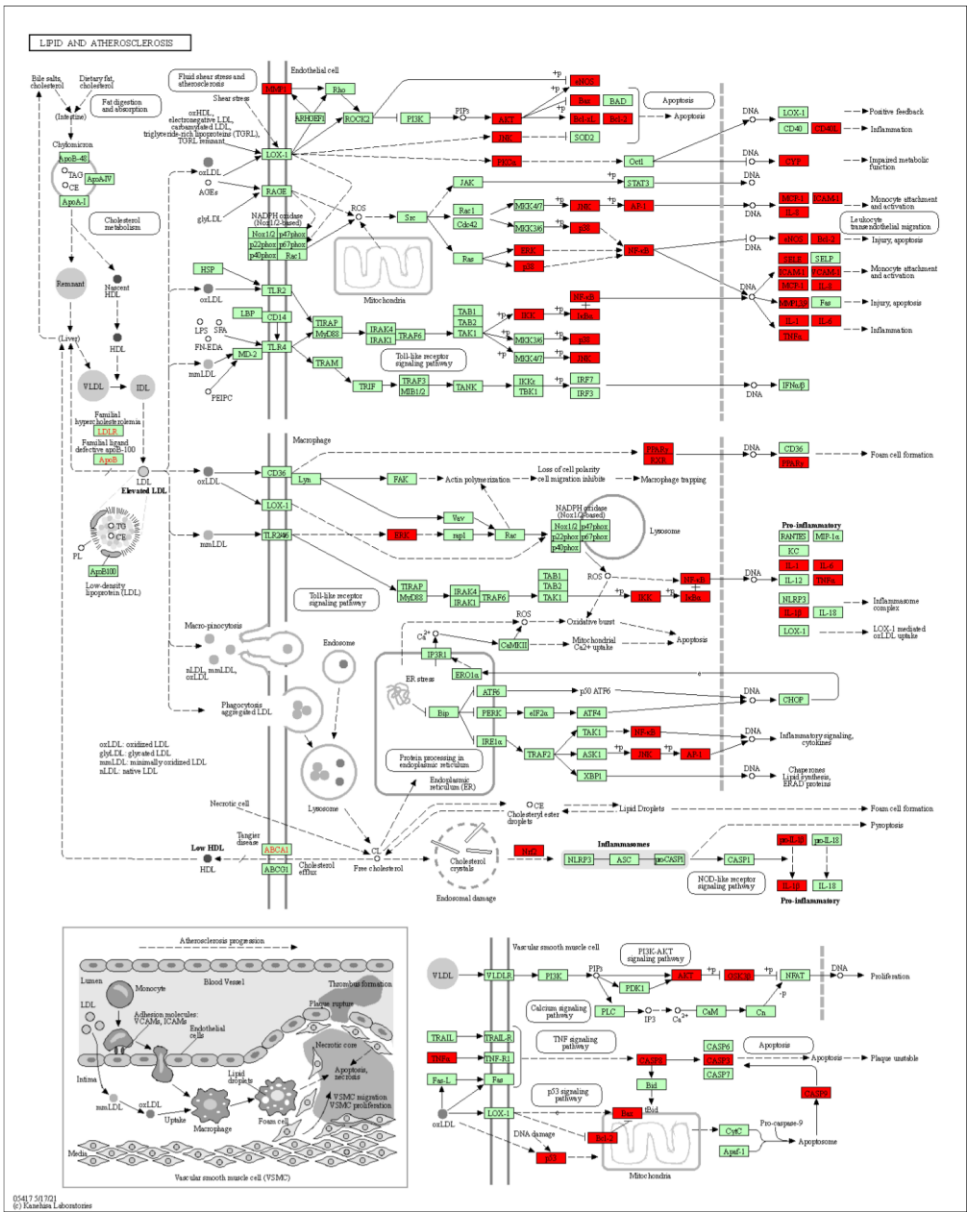

Figure 5: Lipid and atherosclerotic pathways

3.5 Epimedium and SANFH protein network interaction

Through the STRING online website, enter the intersection target of the active ingredient and the disease, obtain the relevant PPI network diagram, and then import the Cytoscape3.82 software for image retouching operations, the redder the color, the larger the Degree value, and they are shown in Figure 6. The network diagram has a total of 128 nodes and 537 edges(Different colors represent different evidence of association[8]). According to data analysis, the top 5 targets of Degree value are JUN, AKT1, TP53, MAPK1, and RELA, which are key targets in protein interaction networks, and the important components of Epimedium closely related to these targets are quercetin, luteolin, and kaempferol.

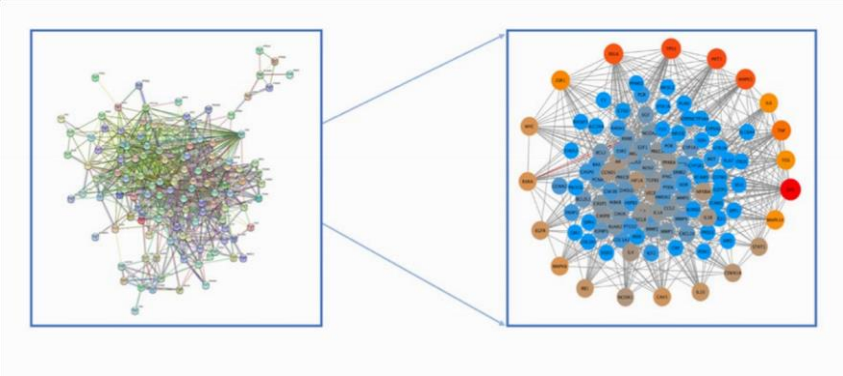

Figure 6: Protein-protein interaction network diagram

3.6 *Molecular docking results*

A molecular binding energy less than 0 indicates that the ligand can bind spontaneously to the receptor, and if the binding energy is less than -5 kcal/mol (1 cal≈ 4.186 J), it indicates strong binding capacity [9]. The key targets obtained above can be seen from the literature query, JUN targets are related to oncogenes, so removal is not included in the analysis, so the binding energy of molecular docking between 4 key targets and 3 active ingredients can be obtained, and they are shown in Table 2. It can be seen from Table 2 that the binding energy of the active ingredient and the key target docking is less than -5 kcal/mol, indicating that the active ingredient of Epimedium and the key target can be well bound. Finally, the PyMOL visualization software was used to visualize the minimum binding energy of each group of four pairs of target receptors and constituents, as shown in Figure 7.

Table 2: Binding energy of key targets and active ingredients

	quercetin	luteolin	kaempferol	Anhydroicaritin
AKT1(1UNQ)	-6.31	-6.77	-6.14	-6.41
TP53(6GGC)	-7.31	-7.15	-8.1	-7.78
MAPK1(6SLG)	-5.88	-6.7	-5.38	-5.08
RELA(6QHL)	-5.01	-5.61	-5.14	-4.51

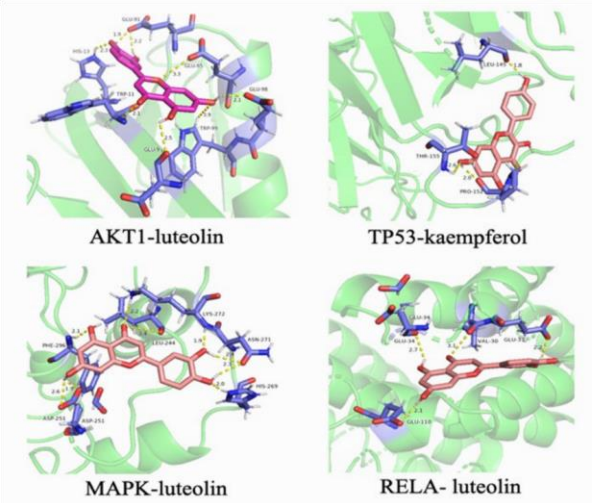

Figure 7: Receptor and ligand molecule docking

4. Discussion

In this study, a total of 23 active ingredients of Epimedium were retrieved, 209 effective targets and 1550 disease targets were predicted, and 128 targets of Epimedium active ingredients for the treatment of SANFH were obtained after the intersection of the two, with the help of "Epimedium - Active Ingredient - Intersection Target - Disease Network" analysis showed that molecular compounds such as quercetin, luteolin and kaempferol can act on multiple targets, protein interaction analysis (PPI)

suggests JUN, AKT1, TP53, MAPK1, RELA, TNF, Targets such as IL6 and ESR1 can also interact with multiple molecular compounds in Epimedium, and the molecular docking results show a good degree of binding, indicating that Epimedium can treat SANFH through multi-target and multi-component.
The GO enrichment analysis showed that Epimedium mainly exerts its therapeutic effect on SANFH through cell proliferation and apoptosis, cytokine expression, and reactive oxygen reaction. The specific mechanism of SANFH remains unclear, but it is certain that the apoptosis process of osteoblasts and osteoclasts and the survival status of osteoclasts are affected by glucocorticoids [10–12], and SANFH may also be associated with the hypoxia-inducible factor-1α pathway[13]. Thus, promoting angiogenesis, osteoblast differentiation, and inhibiting osteoclast proliferation through certain components can slow the onset of SANFH [14].
The enrichment results of KEGG pathway showed that lipid and atherosclerosis, PI3K-AKT signaling pathway and MAPK-related pathway were closely associated with SANFH. (1) Lipid and atherosclerotic pathways: Studies have shown that the application of glucocorticoids can lead to lipid metabolism disorders and hyperlipidemia, resulting in blood supply disorders of the femoral head inducing SANFH [15]. (2) Mitogen-activated protein kinase (MAPK) signaling pathway: a series of biological processes such as growth, development, differentiation, and apoptosis can be regulated through a variety of cellular mechanisms, and the activation of this pathway upregulates the expression of MMP1 and MMP3 in chondrocytes[18]. (3) PI3K-AKT signaling pathway: glucocorticoids activate autophagy through the PI3K/AKT/mTOR pathway, leading to apoptosis of osteocytes, while a variety of biological factors can inhibit autophagy and prevent the process of apoptosis of osteocytes, thereby preventing early SANFH
[19,20].
The results of protein interaction network and molecular docking showed that quercetin, luteolin and kaempferol were the more important components of Epimedium and played a significant role in the treatment of SANFH targets. Quercetin inhibits LPS-induced osteoblast apoptosis, promotes BMSC proliferation, enhances osteoblast differentiation through the MAPK signaling pathway [21–23]. Studies have shown that luteolin can exert anti-apoptotic effects through mitochondrial pathways in osteoblasts [24], and luteolin has been found to have strong anti-inflammatory effects and help inhibit bone resorption induced by mature osteoclasts [25–27]. Kaempferol inhibits osteoclasts and promotes osteoblast differentiation [28]. These results suggest that the active ingredient of Epimedium may play a positive role in preventing SANFH.
In summary, Epimedium can be used by quercetin, kaempferol, luteolin and other active ingredients to anti-inflammatory, repair osteoblasts and other gene targets such as AKT1, TP53, RELA, MAPK1, thereby slowing down the process of SANFH. Follow-up studies can conduct in-depth research on the relevant mechanism of epimedium efficacy according to the signaling target pathway of this screening.

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