Exploring the effect and mechanisms of *Epimedium* on diabetic testicular injury using network pharmacology, molecular docking, and cell experiments

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Abstract

**Background:** Studying the potential targets and mechanisms of *Epimedium* for anti-diabetic testicular injury using network pharmacology, molecular docking, and cell experiments.

**Methods:** Acquisition of major components and targets of *Epimedium* was based on TCMSP, TCMID, and Symmap databases and retrieval of diabetic testicular injury targets by OMIM, GeneCards, Pharmgkb, and Drugbank databases. Intersecting targets were obtained from the Venny 2.1.0 database and input SRTING data to construct a protein-protein interaction (PPI) network, and key targets were screened in Cytoscape 3.8.0 software. Then the Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analyses of intersecting targets were conducted through the DAVID database. Further, AutoDock software was used to verify docking between the main components and the core target proteins. In addition, a Cell Counting Kit-8 (CCK-8) assay was used to determine the survival effect of quercetin, the main component of *Epimedium*, on TM4 sertoli-like cells exposed to palmitic acid (PA).

**Results:** Quercetin, kaempferol and luteolin in *epimedium* were identified as the main components in the treatment of diabetic testicular injury. It has core target proteins including MMP9, AKT1, and TNF. The biological process mainly involves the regulation of the apoptotic signaling pathway. The key pathways of KEGG are the AGE-RAGE signaling pathway in diabetic complications, PI3K-Akt and MAPK signaling pathway. Molecular docking results showed that quercetin had the strongest binding ability to MMP9. Also, PA-challenged cells had a lower survival rate, which was alleviated by the administration of quercetin.

**Conclusion:** Our findings suggest that *Epimedium* attenuates diabetes mellitus (DM)-induced testicular injury through AGE-RAGE, PI3K-Akt and MAPK signaling pathway. These insights offer a potential therapeutic strategy for managing DM-induced testicular injury, will be the basis for future clinical research.

**Keywords:** *Epimedium*; diabetic testicular injury; network pharmacology; molecular docking
Introduction

Diabetes mellitus (DM) is a metabolic disorder characterised by hyperglycaemia. According to the International Diabetes Federation, there will be 783 million diabetes patients by 2045 [1]. Diabetic testicular injury is a very common complication in diabetic men. Approximately 90% of male patients experience various degrees of testicular dysfunction [2]. Diabetic testicular dysfunction includes testicular atrophy, decreased spermatogonium, spermatogenic dysfunction, and sexual hormone disorders [3]. Previous evidence suggests that damage to the reproductive system in diabetic men is closely related to apoptosis, oxidative stress, inflammation, and a variety of factors [4, 5]. However, the underlying mechanisms in the testes of diabetic patients that lead to hypogonadism have not been completely elucidated.

Epimedium is a perennial herb in the Berberidaceae family. In traditional Chinese medicine, Epimedium is commonly used to treat impotence, kidney yang deficiency symptoms, infertility, osteoporosis, cardiovascular disease and other conditions. Epimedium’s modern pharmacology has demonstrated that it contains a variety of flavonoids with multiple biological activities. These include anti-inflammatory, anti-tumor, anti-oxidation, anti-apoptosis and other pharmacological activities [6]. In addition, Icarin has been reported to be effective in protecting mesenchymal stem cell injection (ADSC) from oxidative stress in the combined treatment of diabetes-associated erectile dysfunction (DMEM) with ADSC, which enhances the therapeutic effect on DMEM [7]. Epimedium alleviates diabetic tubulointerstitial fibrosis by restoring autophagy [8]. Epimedium enhances male reproductive health by regulating reproductive and endocrine functions, improving sperm quality and erectile function [9, 10]. However, its potential mechanisms in the treatment of diabetic testicular injury have not been clarified completely.

An investigation of the mechanism of Epimedium in treating diabetic testicular damage is presented in this article using network pharmacology methods, Figure 1 shows cyberpharmacology and drug repurposing will enable precise and effective therapeutic interventions [11]. The basic research process, first of all, through different databases were screened drug and disease effective active ingredients, target genes, take the intersection target using gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis reveals the association between the active components of the components of the pathway. Further, molecular docking verified interactions between effective activities and core genes. Cellular experiments further validate. Finally, we elucidate the key components, core target protein and mechanism of epimedium in treating diabetic testicular injury. Epimedium has few side effects, is safe and non-toxic, and can be used for long-term treatment of diabetes patients, which provides a basis for future treatment of the disease [12].

Materials and methods

Screening of Epimedium active ingredients

Using the web-based pharmacy database (https://www.tcmsp-e.com/), TCMD (https://www.megabionet.org) and Symmap (https://www.Symmap.org/) database platforms, ‘Epimedium’ was used as a keyword to search for drug active ingredients and combine drug active ingredients to take the intersection. The oral bioavailability (OB) and drug-like (DL) values were determined as ≥30% and 0.18, respectively and correlation screening was performed again. This screening identifies the drug's active ingredients and related targets [13, 14].

Screening of Epimedium related targets and acquisition of target maps of effective active ingredients

The Uniprot corresponding to the screened active ingredients were entered into the Uniprot (https://www.uniprot.org/) database one by one, converted into the corresponding gene names, and the duplicate genes were deleted. Active ingredient target maps were generated using Cytoscape 3.8.0 software.

Acquisition of diabetic testicular injury targets and targets intersecting with Epimedium

The disease name "diabetic testicular injury" was entered as the disease target in four databases: OMIM (https://www.omim.org/), GeneCards (https://www.geneCards.org/), Pharmgkb (https://www.pharmgkb.org/), Drugbank (https://go.durbank.com/). Where GeneCards data is to be calculated for the median correlation score. The corresponding disease targets were then filtered based on relevance scores. Finally, the disease targets of all databases were merged, and the genes presented in both databases were used as disease targets. The intersection targets of disease and drug were obtained from the Venny 2.1.0 database and represented in Venn diagrams.

Figure 1 A flowchart illustrates the overall design of this study

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PPI construction of protein network interaction
A PPI network was constructed by entering the common target of the intersection of *Epimedium* and diabetic testicular injury into STRING (https://string-db.org/) and setting up the species "*Homo sapiens". The plots were imported into Cytoscape 3.8.0 and key targets were filtered out by setting the Degree, Betweenness and Closeness greater than their respective thresholds in the apps plugin centiscape2.2.

Construction of *Epimedium* active ingredient-disease-common target maps using Cytoscape 3.8.0
The target of *Epimedium* and the common target of disease were sorted out, and the corresponding interacting components of the common target were determined. The corresponding targets of effective active ingredients of *Epimedium* and the intersection points of diabetic testicular injury were then converted into attribute files and network files. These files were imported into Cytoscape 3.8.0 to draw an *epimedium* active ingredient and diabetic testicular injury intersection target point network map.

Construction of GO functional enrichment and KEGG enrichment analyses
The Intersection targets of *Epimedium* and diabetic testicular injury were entered into the DAVID (https://david.ncifcrf.gov/) database, with a significance set at $P < 0.05$. The data will be sorted by P-value from smallest to largest, and the top ten and twenty will be taken to the microbiology platform and analyzed for GO and KEGG enriched signaling pathways, respectively.

Molecular docking
The 3D files of the target protein and small molecules were searched for in the PubChem (https://pubchem.ncbi.nlm.nih.gov/) and PDB (http://www.rcsb.org/) databases. The optimal docking method was calculated using AutoDock and visualized with PyMOL.

Reagents and equipment
Normal mouse testicular supporting cells, namely TM4, were sourced from the China Typical Culture Collection Center. PA, Quercetin, DMEM/F12, Trypsin Solution without EDTA, CCK8, mixed solution of penicillin and streptomycin was purchased from Dalian Meilun Biotech Co., Ltd. Fetal bovine serum was sourced from Zhejiang Tianhang Biotechnology Co., Ltd. PBS buffer was purchased from Wuhan Saiweier Biotechnology Co., Ltd. DMSO was purchased from Sigma Company, Anhydrous ethanol was purchased from China National Pharmaceutical Group Chemical Reagent Co., Ltd. CO$_2$ incubator and Automatic Enzyme Labeling Instrument.

Cell culture and treatment
TM4 cells were cultured in 10% FBS supplemented with 1% penicillin–streptomycin solution at 37 °C and 5% CO$_2$. Thereafter, the cells were exposed to PA (400 μM) alone or in combination with quercetin (5, 10, 20 μM) for 24 h.

CCK8 method for detecting cell viability
To evaluate quercetin's cytoprotective properties, cell viability was measured by CCK8 method. TM4 cells were counted after they had been digested, centrifuged and resuspended. Inoculated into 96-well culture plates at a concentration of 5 × 103/well [16]. After the cells adhered to the wall, each group was added to the drug-containing medium and cultured for 24 h. Each well was filled with a 10% CCK-8 solution (avoiding bubbles). Static incubators incubated culture plates for 2 to 4 hours. Using a multi-function enzyme labeling instrument, the absorbance at 490 nm was determined, and the difference was calculated.

Statistical methods
SPSS and Prism were used to analyze the data. Statistical analysis was performed using one-way ANOVA in SPSS software, and results were expressed as mean ± standard error (T ± SEM). All data resulting from three independently repeated experiments were considered statistically significant at $P < 0.05$.

Result
Potential target acquisition and active ingredient target map of *Epimedium*
After entering the keyword "Epimedium" into the TCMSP, TCMD and Symmap databases, each database corresponds to 54, 54, and 107 active ingredients, respectively, resulting in a total of 47 active ingredients across the three databases (Figure 2A). In the second screening, conditions of OB ≥ 30% and DL ≥ 0.18% were met, and 8 active ingredients and 180 potential targets were selected. Sorted by degree value, the three most prominent active ingredients were MOL000098, MOL00006, and MOL000422, corresponding to quercetin, luteolin, and kaempferol, respectively. And it was visualized as a diagram of *Epimedium's* active ingredients-associated targets as in Figure 2B.

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Acquisition of potential targets for diabetic testicular injury and genes intersecting with Epimedium
A total of 1688 target genes associated with diabetic testicular injury were retrieved from the GeneCards database using "diabetic testicular injury" as the keyword. Further, the median relevance score was set to be greater than or equal to 4.206. As a result, 1371 target genes associated with diabetic testicular damage were screened.

Additionally, the Pharmakb, OMIM and Drugbank databases were searched for 151, 142 and 20 target genes, respectively. The genes in the two databases were used as disease-targeted genes, resulting in a final retrieval of 1597 target genes (Figure 3). A Venn diagram of the intersection of Epimedium targets with diabetic testicular injury targets was plotted from the Venny database. As shown in Figure 4, there are a total of 102 intersecting targets.

Acquisition of core targets for the PPI protein network
The cross targets of diabetic testicular injury and Epimedium drugs were entered into the STRING database (Figure 5A). The graph contained a total of 102 number of nodes with 1797 number of edges, and the average node degree value was 35.2. Cytoscape 3.8.0 builds PPI network interactions (Figure 5B). The top 10 core targets, selected based on screening Degree, Betweenness and Closeness, are threonine protein kinase (AKT1), tumor necrosis factor (TNF), cellular tumor antigen p53 (TP53), interleukin-1 beta (IL1β), Matrix metalloproteinase 9 (MMP9), Caspase-3 (CASP3), as shown in Figure 5C.
Epimedium major active ingredient-common target-diabetic testicular injury constructs

In order to examine the therapeutic mechanism of Epimedium in the treatment of diabetic testicular injury, we have constructed a network diagram (Figure 6) of the intersection targets between Epimedium's major active ingredient and diabetic testicular injury. 102 intersecting targets and 7 active ingredients closely related to Epimedium treatment were used to construct a network diagram of Epimedium-diabetic testicular injury intersection targets. From the figure, it can be seen that quercetin (Degree = 151), luteolin (Degree = 57) and kaempferol (Degree = 45) have the most targets for the active ingredients. These data suggest that the active ingredient of Epimedium is likely to be an important mechanism for the treatment of diabetic testicular injury.

Functional enrichment analysis of GO biology

The data from the 102 key targets that were screened have been entered into the DAVID database. The top 10 are ranked based on the size of the P-value, from small to large. The size of each bubble indicates the number of genes, and bubble size is positively correlated with genes. The colour of the bubbles indicates the degree of importance. In general, the darker and redder the colour, the stronger the enrichment effect. Furthermore, 6711 biological processes (BP) were used, including the regulation of the apoptotic signaling pathway, response to oxygen levels and response to decreased oxygen levels, etc. Additionally, 90 cellular components (CC) are mainly membrane raft, membrane microdomain, secretory granule lumen, etc. The molecular function (MF) 264 are mainly DNA-binding transcription factor binding, RNA polymerase II-specific DNA-binding transcription factor binding and signaling receptor activator activity, etc. Epimedium may exert anti-diabetic testicular injury effects by regulating these biological processes (Figure 7).

Figure 6 Epimedium major active ingredient-common target-diabetic testicular injury map. The blue circle node graph represents epimedium, the yellow square represents diabetic testicular injury, the active components of epimedium are shown by a red triangle, and its 102 cross genes are shown by an orange diamond.

Figure 7 GO biofunctional enrichment bubble map. Top 10 BP CC MF of GO at the intersection of Epimedium and diabetic testicular injury.

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Enrichment analysis of KEGG pathways
After data processing, the top 20 pathways of Epimedium and diabetic testicular injury were mainly enriched in Fluid shear stress, atherosclerosis, Hepatitis B, Prostate cancer pathways, AGE-RAGE signaling pathway and Lipid and atherosclerosis in diabetic complications, etc (Figure 8A). We found that diabetic testicular injury involves the AGE-RAGE signaling pathway in diabetic complications, the PI3K-Akt signaling pathway, and the MAPK signaling pathway. To demonstrate a clearer and more intuitive connection between the main KEGG pathways and the intersection targets. The pathway-target diagram was constructed and summarized in Cytoscape software, as shown in Figure 8B, which indicates that Epimedium regulates these pathways to treat diabetic testicular injury.

Molecular docking verification
According to the degree value in the analysis of FPI and KEGG pathway enrichment results, the top 5 key targets AKT1, TNF (TNF target selected TNF-α), TP53, IL1β, MMP9 and the top 3 main components quercetin, luteolin and kaempferol were used for molecular docking (Figure 9A–9C). The molecular docking results indicate that the strength of binding between the two molecules increases with higher affinity. This is inversely proportional to the binding energy value. As shown in Table 1, their binding energies were −6.72 (kcal/mol), among which quercetin had the strongest binding ability with MMP9 at −8.9 (kcal/mol) binding ability. The binding energy of luteolin, another active ingredient, to MMP9 is −8.7 (kcal/mol), and kaempferol had a high binding activity of −8.6 (kcal/mol) to MMP9. Therefore, the above three pairs are visualised. Epimedium's active ingredient acts on the core target of diabetic testicular injury to restore tissue and organ function.

Quercetin on the survival rate of PA induced TM4 cells
TM4 cells were damaged by PA, and cell survival increased to a certain extent after the administration of quercetin. When the solubility of quercetin is 10 μM and 20 μM, there was a significant difference in the cell survival rate at that time (Figure 10).

Figure 8 KEGG enrichment bar chart and pathway-target map. (A) The top 20 pathways of KEGG, the intersection targets of Epimedium and diabetic testicular injury. (B) Cytoscape software constructs the top 20 KEGG pathway-target maps.

Figure 9 Docking diagram of the main active components of Epimedium and MMP9 molecules. (A) A docking diagram illustrating the interaction between quercetin and MMP9. (B) A docking diagram illustrating the interaction between luteolin and MMP9. (C) A docking diagram illustrating the interaction between Kaempferol and MMP9 target.
**Discussion**

DM has a high incidence in the world and has become one of the main causes of reproductive dysfunction [17, 18]. Therefore, so male diabetic patients have received great attention. Furthermore, the mechanism of diabetic testicular injury is very complex. *Epimedium* may be a potential drug for diabetic testicular injury. Multitarget research can provide a better understanding of diabetic testicular injury's occurrence and progression. Using network pharmacology and in vitro experiments, the main components and target mechanism of *Epimedium* were examined in the treatment of diabetic testicular injury. According to the degree values, the top 5 targets are AKT1, TNF, TP53, IL1β, and MMP9. This suggests that these targets may play significant roles in the treatment of diabetic testicular injury with *Epimedium*. In addition the active ingredients quercetin, luteolin, and kaempferol had a high-hitting docking and binding activity with AKT1, TNF, TP53, IL1β, and MMP9 molecules, while quercetin had the strongest binding ability with MMP9. Quercetin's significant role was further verified by in vitro experiments.

*Epimedium* has 8 active ingredients, including quercetin, luteolin, kaempferol, etc. It has been shown that quercetin, the active ingredient of the drug, has the greatest number of targets for diabetic testicular injury. This is with 151 core targets. According to the relevant literature, quercetin, a flavonoid, exhibits antioxidant and anti-inflammatory effects, protecting against testicular injury in rats by reducing oxidative stress [19]. Additionally, it is involved in lipid metabolism disorders through the PI3K-Akt signaling pathway, thus reversing reproductive and metabolic disorders [20]. Several studies have suggested that luteolin may contribute to PbAc-induced testicular injury by inhibiting oxidation and inflammation and reducing apoptosis [21]. Treatment of poisoned rats with kaempferol normalizes reproductive hormones, improving sperm function [22]. The above experimental results effectively demonstrate the therapeutic efficacy of quercetin, the main active ingredient in *Epimedium*. However, its specific mechanism for the treatment of diabetic testicular injury has not been shown. Based on the molecular docking results, quercetin has the strongest binding energy to MMP9, AKT1, and TNF. In addition to supporting the rat testicular barrier, MMP9 is a member of the MMP family. It is involved in embryonic development, reproduction, and tissue remodeling in rats [23]. However, the absence of MMP9 can lead to spermatogenic dysfunction [24]. Deletion of the AKT1 has been found to promote early apoptosis in germ cells [25]. AKT acts by participating in the PI3K-Akt signaling pathway to regulate germ cells and reduce reproductive damage [26]. Apoptosis is induced by TNF binding to TNFR receptors, promoting male infertility [27, 28]. *Epimedium* may prevent diabetic testicular injury by inhibiting inflammation and oxidative stress [29].

The results of the GO and KEGG analyses indicate that GO-enriched biological processes primarily involve positive regulation of gene expression, and regulation of the apoptotic signaling pathway. According to the KEGG results, AKT1, TNF were found to mainly enrich the AGE-RAGE signaling pathway in diabetic complications, PI3K-Akt signaling pathway and MAPK signaling pathway. It has been reported that advanced glycation end products (AGEs) play a significant role in DM pathogenesis through a multifactorial mechanism among DM complications. Additionally, studies have shown that blood levels of AGEs and receptors for advanced glycation...
end products (RAGE) are elevated in individuals with DM [30]. DM-induced AGEs and oxidative stress are risk factors for testicular injury. The inhibition of AGE accumulation or binding between RAGE and AGEs, as well as the activation of downstream key regulatory molecules, may thus prevent diabetic testicular injury. Thus, resulting in a protective effect through hormone regulation and resistance to oxidative stress and apoptosis [31]. In addition, the PI3K/AKT/mTOR signaling pathway can activate autophagy under the regulation of high glucose, thus inducing diabetic testicular injury [32]. The PI3K-Akt signaling pathway plays a vital role in spermatogenic cell proliferation and differentiation [33]. By analyzing GO biological process enrichment and KEGG signaling pathway analysis of targets related to Epimedium's anti-diabetic testicular injury, it was shown that Epimedium addresses diabetic testicular injury through multi-gene target proteins and multiple pathways.

Conclusion

A pharmacological study was conducted to determine the key components, core targets, and pathways of epimedium in treating diabetic testicular injury. Based on this evidence, the active ingredients quercetin, luteolin, kaempferol, and others were identified. Furthermore, experiments have shown that quercetin can counteract PA-induced damage to TM4 cell, and may act on MMP9, AKT1, TNF targets in diabetic complications such as AGE-RAGE signaling pathway, PI3K-Akt and MAPK signaling pathway to treat diabetic testicular injury. An investigation of the key mechanism of Epimedium in treating diabetic testicular injury will provide a theoretical basis for future research.

References


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