

A network pharmacology approach to explore the mechanism of HuangZhi YiShen Capsule for treatment of diabetic kidney disease

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ABSTRACT

Background and Objective: HuangZhi YiShen Capsule (HZYS) is a Chinese patent herbal drug that protects kidney function in diabetic kidney disease (DKD) patients. However, the pharmacologic mechanisms of HZYS remain unclear. This study would use network pharmacology to explore the pharmacologic mechanisms of HZYS. **Methods:** Chemical constituents of HZYS were obtained through the Traditional Chinese Medicine Systems Pharmacology Database (TCMSP) and literature search. Potential targets of HZYS were identified by using the TCMSP and the SwissTarget Prediction databases. DKD-related target genes were collected by using the Online Mendelian Inheritance in Man, Therapeutic Target Database, GeneCards, DisGeNET, and Drugbank databases. Gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analyses were carried out to further explore the mechanisms of HZYS in treating DKD. Molecular docking was conducted to verify the potential interactions between the prime compounds and the hub genes. **Results:** 179 active compounds and 620 target genes were obtained, and 571 common targets were considered potential therapeutic targets. The top 10 main active compounds of HZYS were heparin, quercetin, kaempferol, luteolin, methyl14-methylpentadecanoate, methyl (Z)-11-hexadecenoate, 17-hydroxycorticosterone, 4-pregnene-17 α , 20 β , 21-triol-3, 11-dione, wogonin, and hydroxyecdysone. Hub signaling pathways by which HZYS treating DKD were PI3K-Akt, MAPK, AGE-RAGE in diabetic complications, TNF, and apoptosis. The top 10 target genes associated with these pathways were *IL6*, *MAPK1*, *AKT1*, *RELA*, *BCL2*, *JUN*, *MAPK3*, *MAP2K1*, *CASP3*, and *TNF*. Quercetin and Luteolin were verified to have good binding capability with the hub potential targets *IL6*, *MAPK1*, *AKT1* through molecular docking. **Conclusion:** HZYS appeared to treat DKD by regulating the inflammatory, oxidative stress, apoptotic, and fibrosis signaling pathways. This study provided a novel perspective for further research of HZYS.

Key words: HuangZhi YiShen Capsule, diabetic kidney disease, network pharmacology, molecular docking

INTRODUCTION

Diabetic kidney disease (DKD) is a severe microvascular complication of diabetes mellitus (DM).^[1] The number of people

with diabetes globally was estimated to be 9.3% (463 million people) in 2019, with this figure anticipated to rise to 10.2% (578 million) in 2030 and 10.9% (700 million) in 2045.^[2] Approximately 40% of DM patients

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deteriorate into DKD, the main cause of end-stage renal disease (ESRD).^[3] Therefore, DKD is indeed a global health concern. However, the mechanism of DKD is not fully understood. Research has shown that hyperglycemia-induced metabolic impairment and hemodynamic disruptions are involved in the progression of DKD.^[4] These changes lead to oxidative stress, inflammation, and generation of advanced glycation end products (AGEs). Currently, DKD treatment methods are mainly tight glucose and blood pressure control, especially to inhibit the renin-angiotensin system by using medications.^[5] However, in the United States, for example, the percentage of DKD patients who progress to ESRD has not dropped significantly.^[6]

Traditional Chinese medicine (TCM) is guided by its long upheld theories and has the unique characteristics of multiple components, multiple targets, and multiple pathways in treating both simple and complex diseases. In China, herbal medicines, including patent medicines, are used to treat DKD^[7,8] and the application scope of many Chinese patent medicines that are effective in treating chronic kidney disease (CKD) has been expanding. One such medicine is HZYS, which was formerly known as **Shen Yan Ning**, has been found to be effective for CKD. In TCM theory, HZYS nourishes *qi* and *yin*, promotes blood circulation and diuresis and can reduce proteinuria and hematuria in chronic nephritis patients who have deficiency of both *qi* and *yin*.^[9] Clinical trials have found that therapeutic outcomes of HZYS combined with conventional drugs are superior to conventional drugs alone.^[10,11] It was reported that HZYS combined with Candesartan Cilexetil tablets had better effect on DKD by reducing proteinuria, improving kidney function than Candesartan Cilexetil tablets.^[12] Moreover, clinicians found that HZYS can improve kidney function in patients with DKD.^[13] Unfortunately, its clinical effective evidences for treating DKD are relatively rare. The pharmacologic mechanisms and the specific ingredients of HZYS as related to DKD remained unclear due to the limited published clinical and experimental research. Therefore, the aim of this study was to explore the potential key pharmacologic mechanism of HZYS on DKD.

Network pharmacology was firstly proposed by Andrew Hopkins based on chemoinformatics, bioinformatics, network biology, and pharmacology.^[14] It can integrate virtual computing technology, public databases, and high-throughput data for revealing the complex network relationship between biologically active ingredients and potential targets and mechanisms of Chinese herbal medicines.^[15] HZYS, as a Chinese patent herbal drug, have multiple compounds as traditional Chinese medicine, however, it has not been explored about the specific

ingredients of the drug and the potential mechanism in the treatment of DKD. Therefore, the network pharmacology based on a compound-target-disease interaction database and biologic analysis was utilized to explore the relationship between the multiple compounds, targets, and disease in this study.^[16] And the key active compounds, important targets and core pathways for HZYS treating DKD were explored by network pharmacology methodology in this study. The study flowchart is shown in Figure 1.

METHODS

Screening Active Compounds and Corresponding Targets

HZYS consists of the following herbs: astragalus root (*Astragalus membranaceus* (Fisch.) Bunge), leech (*Hirudo nipponica* Whitman), scrophularia root (*Scrophularia ningpoensis* Hemsl.), glehnia root (*Glehnia littoralis* F. Schmidtex Miq.), notoginseng root (*Panax notoginseng* (Burkill) F.H.Chen), Chinese motherwort (*Leonurus japonicus* Houtt.), achyranthes root (*Achyranthes bidentata* Blume), goji berry (*Lycium barbarum* L.), Chinese yam (*Dioscorea oppositifolia* L.), coix seed (*Coix lacryma-jobi* L.), eclipta (*Eclipta prostrata* (L.) L.), human placenta, eucommia bark (*Eucommia ulmoides* Oliv.), plantain seed (*Plantaginis Seme*), and cicada molting (*Cryptotympana atrata* Fabr.) The chemical constituents of HZYS were accessed through the Traditional Chinese Medicine System Pharmacology Database (TCMSP; <http://lsp.nwu.edu.cn/tcmsp.php>). This database holds information on more than 13,000 pure compounds isolated from 505 Chinese herbs, corresponding targets and associated drug-target-disease networks. In addition, it provides data on absorption, distribution, metabolism, and excretion (ADME) screening for bioactive compounds.^[17,18] In this study, the bioactive compounds were retrieved under the retrieval filters of oral bioavailability (OB) $\geq 30\%$ and drug-likeness (DL) ≥ 0.18 .^[15,19,20] The active compounds of undetected drugs (such as leech, Chinese motherwort, and cicada molting) were obtained from the published literature. The chemical structures of related compounds were obtained from the PubChem database (<https://pubchem.ncbi.nlm.nih.gov>). Chemical structures that were not available were rendered using ChemDraw software (PerkinElmer; San Jose, CA, USA). All the chemical structure files were in SDF format and uploaded to the Swisstarget prediction server (Swiss Institute of Bioinformatics, <http://www.swisstargetprediction.ch>) for further target prediction and analysis.^[21,22]

Screening Potential Targets for DKD

To find the relevant disease targets more comprehensively, information on DKD-associated target genes were

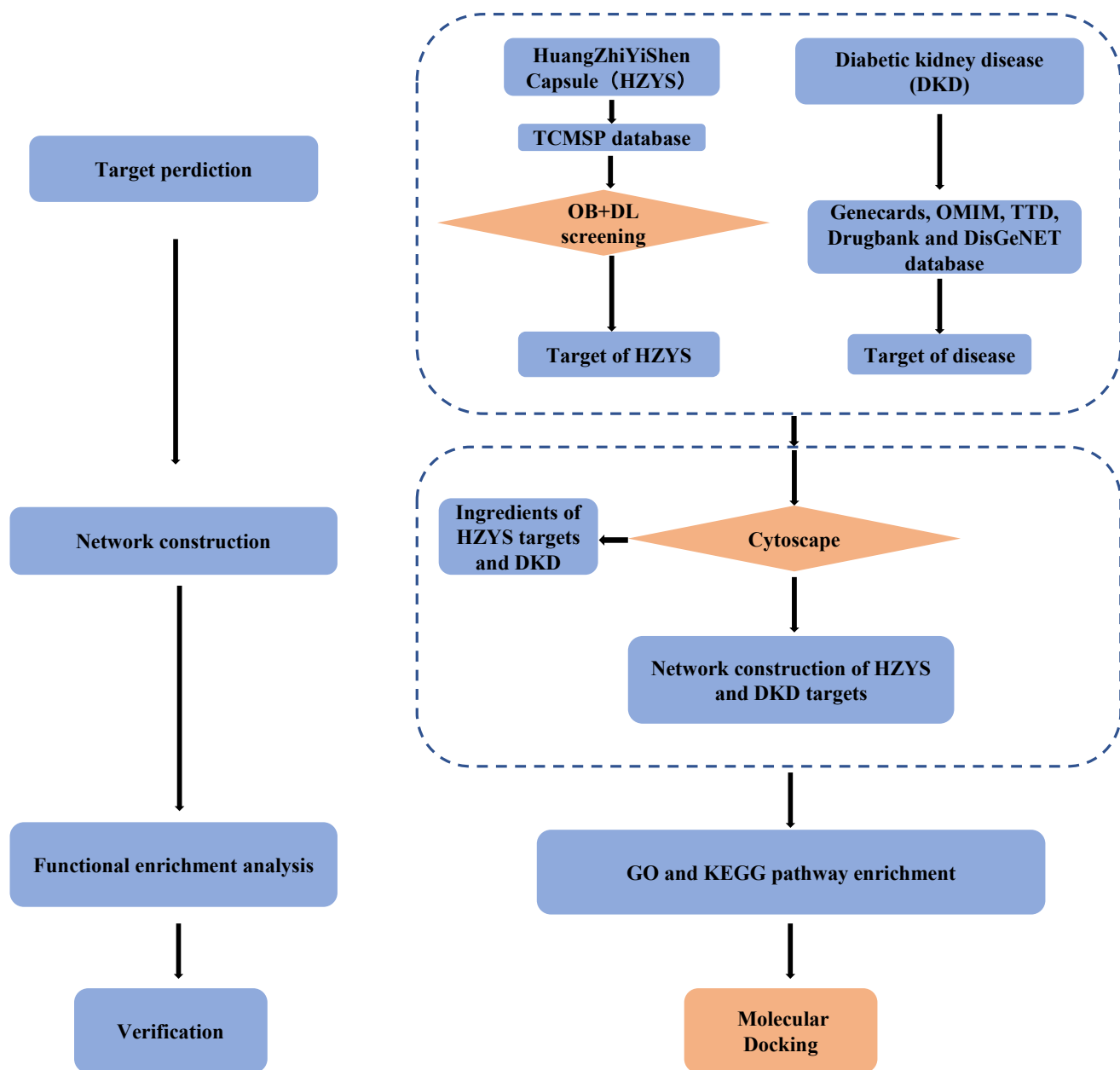


Figure 1: Schema of network pharmacology analysis of HZYS.

gathered from the following 5 databases: Genecards (<https://www.genecards.org>),^[23] DisGeNET(<https://www.disgenet.org/home/>),^[24] Online Mendelian Inheritance in Man (OMIM; <https://omim.org>),^[25] Therapeutic Target Database (TTD; <http://db.idrblab.net/ttd/>),^[26] and Drugbank (<https://www.drugbank.ca>).^[27] Genecards database furnishes extensive information on human genes including genomic, transcriptomic, proteomic, genetic, clinical, and functional statistics.^[23] DisGeNET is a knowledge platform of genes and variants associated with human diseases compiled and integrated from the curated repositories, genome-wide association

study catalogs, animal models, and the scientific literature.^[24] OMIM is a comprehensive inventory of human genes and genetic phenotypes that has information on all the known mendelian disorders and over 15,000 genes, especially the relationship between phenotype and genotype.^[25] TTD has information on therapeutic protein and nucleic acid targets, the targeted disease its matching therapeutic drugs, as well as pathway information.^[26] DrugBank is a knowledge base that combines highly specific drug information with extensive drug target data and the related disease target information.

In this study, the keyword “diabetic kidney disease” was used in the aforementioned databases to screen targets related to DKD. The targets were collected and the duplicates were deleted. And the obtained targets were standardized through the Uniprot Knowledgebase (UniprotKB; <https://www.uniprot.org>), which collects information on functional information on proteins, their sequences, identification, and gene name at each of the targets.^[28]

Screening Compound-Disease Common Targets

The screened HZYS chemical targets and DKD targets were imported into R software for statistical computing (version 4.0.2; <https://www.r-project.org>) and the common targets of compound-disease were obtained as the potential targets for further analysis.^[29]

Constructing the Herb-Compound-Disease-Target Network

To find the core compounds of HZYS for treating DKD, a herb-compound-disease-target network was established by using Cytoscape (version 3.7.2; <https://cytoscape.org>).^[30] A text named “Type,” which consists of the herb, compounds, disease, their common targets, and their logic relationship was imported into the Cytoscape software. Besides, a text named “network” was also imported into the Cytoscape software. The resulting node size was used to reflect the number of combined targets (degree). The nodes represented the herb, compounds, disease, and targets, and the edges indicated the interactions among them.

Mapping the Protein-Protein Interaction Network

To find the core targets of HZYS treating DKD, the protein-protein interaction (PPI) network for compound-disease common targets was established by using STRING (version 11.0; <https://string-db.org>), which collects nearly all the publicly-available sources of PPI information.^[31] The species was defined as “*Homo sapiens*” to obtain the target interaction information. The results were imported into Cytoscape (version 3.7.2; <https://cytoscape.org>) and R for statistical computing, and the core targets of compound-disease were obtained as the potential targets for further analysis. PPI networks and a bar plot was then drawn.

Analysis of Gene Ontology and KEGG Pathway

To obtain key pathways of HZYS treating DKD, the Database for Annotation, Visualization and Integrated Discovery (DAVID; version 6.8; <https://david.ncifcrf.gov>) was used to conduct the gene ontology (GO) and KEGG Pathway Enrichment Analysis.^[32–35] Biologic process (BP), molecular function (MF), and cell component (CC) are the general modules that are included in GO term enrichment. The Kyoto Encyclopedia of Genes and Genomes (KEGG) database (<https://www.kegg.jp>) was

used to conduct pathway enrichment analyses. R software was also used for GO functional annotation and KEGG pathway enrichment analyses. Results with $P < 0.05$ were destined for further analysis.

Molecular Docking

The software AutoDock 4.0 was used to perform molecular docking between the top 4 compounds of HZYS (heparin, quercetin, kaempferol, and luteolin) and the top 3 hub target gene whose crystal structures were screened from the RSCB Protein Data Bank (<https://www.rcsb.org/>) (IL6, MAPK1, and AKT1, [entry:1ALU,6SLG,4ZZN and 1UNQ, respectively]). In all the dockings, the grid boxes of AKT1, IL6, and MAPK1 were set at $50 \times 50 \times 50$ points, $60 \times 50 \times 60$ points, $60 \times 50 \times 70$ points, and $40 \times 40 \times 50$ points, respectively. The ligand binding sites were the centers of the grid maps, and the grid space was 0.375 \AA . Sixteen runs were generated with the Lamarckian genetic algorithm search. Each compound's docked conformation was ranked into clusters according to the binding energy, and the top-ranked conformations whose position with the lowest binding energy (as the most suitable conformation) was selected to be visually analyzed by using Discovery Studio 4.5.

RESULTS

Active Compounds and Corresponding Targets of HZYS

Using the TCMSP database, the active compound targets and corresponding targets of HZYS were searched for each chemical component. A total of 150 compounds were collected with the values of $OB \geq 30\%$ and $DL \geq 0.18$ properties, applied to screen the active compounds from HZYS. A total of 53 compounds were retrieved from literature reports. Finally, 179 candidate ingredients were selected from HZYS compounds (Supplementary Table S1) along with obtaining the common ingredients of herbs in HZYS (Table 1). In total, 620 targets were identified after removing the duplicate data (Supplementary Table S2).

Retrieval of Potential Compound-Disease Common Targets

Through the GeneCards, DisGeNET, OMIM, TTD, and Drugbank databases, the retrieved results were integrated to obtain the DKD-related disease protein targets. The potential target genes in HZYS were mapped to the disease target genes using R, and a Venn diagram was drawn (Figure 2). A total of 571 potential drug-disease co-targets were obtained. These targets were sorted according to the degree value in the compound-target network (Supplementary Figure 1) and the target information was identified (Supplementary Table S3).

Visual Analysis of the Herb-Compound-Disease-Target Network

A total of 571 potential targets of DKD-HZYS were imported into Cytoscape for analysis. A herb-compound-disease-target network was constructed to further

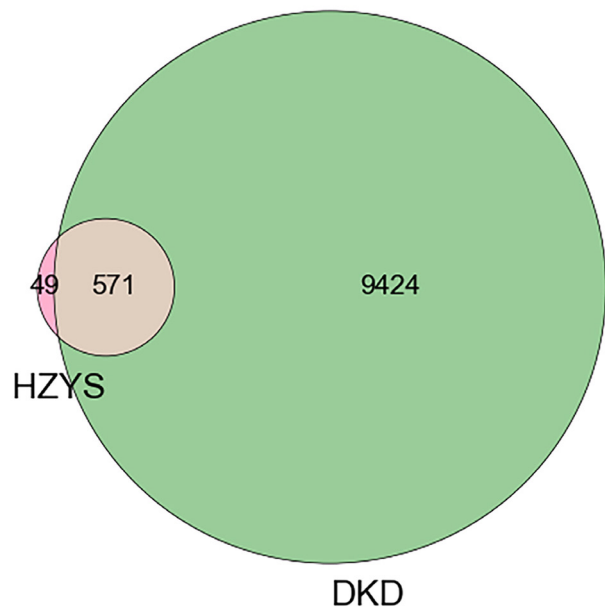


Figure 2: Matching of target genes between DKD and HZYS.

understand the interactions between the drug, compound, disease, and their targets (Figure 3). The network consisted of 747 nodes and 2787 edges. The sizes and colors of the borders were arranged according to the degree value. The top 10 chemical compounds with high degree values and closeness centralities were heparin, quercetin, kaempferol, luteolin, methyl14-methylpentadecanoate, methyl (Z)-11-hexadecenoate, 17-hydroxycorticosterone, 4-pregnene-17 α , 20 β , 21-triol-3, 11-dione, wogonin, and hydroxyecdysone. These ten are potentially the main chemical components of HZYS in the treatment of DKD.

Protein-Protein-Interactions Network

The potential 571 co-target genes associated with DKD were imported into the STRING database for analysis. To establish a high-quality and convincing protein-protein interaction (PPI) network, we selected protein targets with a high confidence score of 0.900 to diagram the network using STRING.^[31] A total of 570 nodes and 4230 edges were acquired, and the average node degree was 14.8 (Supplementary Figure 2). To further analyze the results and explore the core targets that HZYS interacts with DKD, the relevant data is uploaded to Cytoscape, a PPI network was mapped using the common potential targets of HZYS capsule and DKD, consisting of 495 nodes and 4230 interaction edges (Figure 4A). 2X median analysis was used to analyze the PPI network to obtain core clusters,

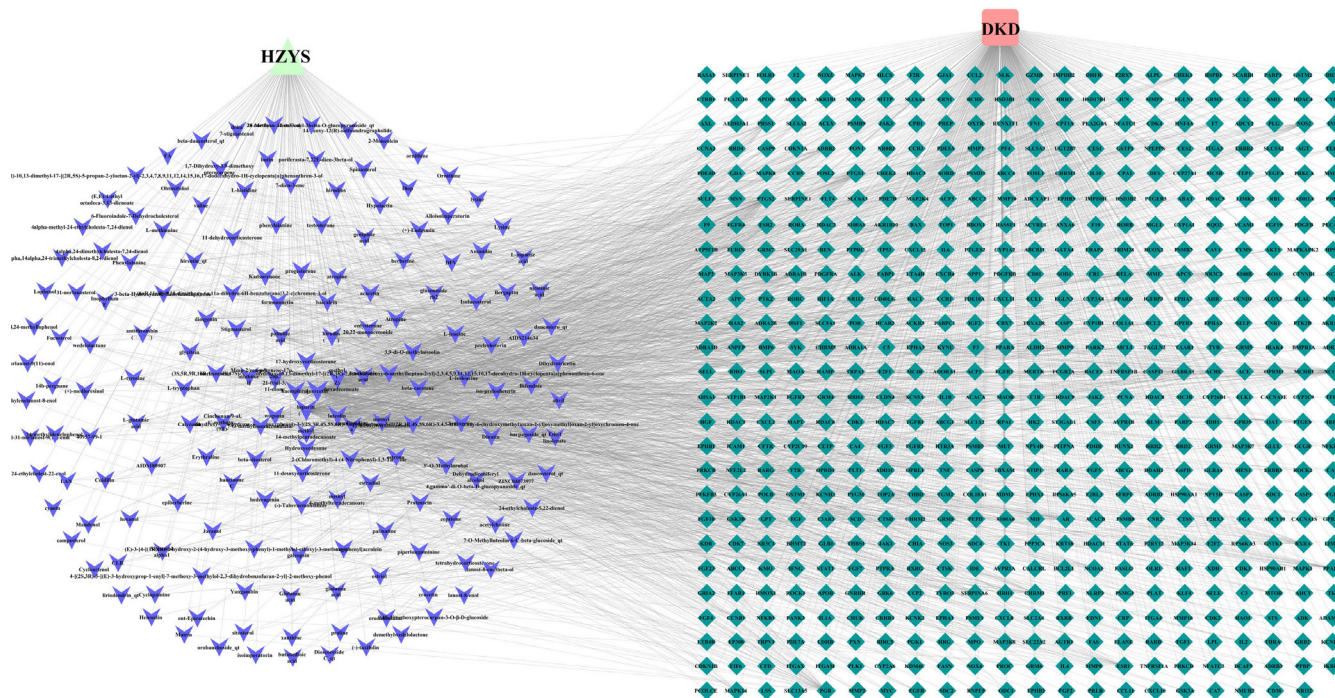


Figure 3: Visual analysis of herb-compound-disease-common target network. Green diamonds represent the targets; purple Vs represent the calculated ingredients; light green triangles represent the calculated drugs. The size and color of the border are arranged according to the degree value. (DKD: diabetic kidney disease; HZYS: Huangzhi Yishen capsule.)

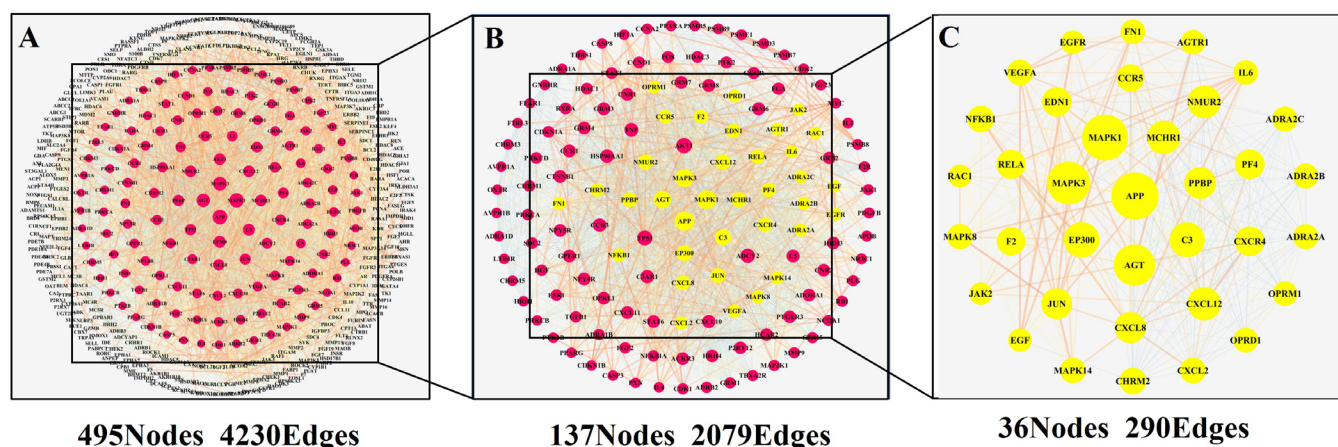


Figure 4: Common target PPI network between DKD and HZYS.

Table 1: Common compounds of herbs in HZYS

Term	Mol Id	Mol name (compound)	Herb compound is found in common
A1	MOL000098	Quercetin	Astragalus root, glehnia root, notoginseng root, Chinese motherwort, achyranthes root, goji berry, eclipta, eucommia bark, plantain seed
A2	MOL000354	Isorhamnetin	Astragalus root, Chinese motherwort
A3	MOL000422	Kaempferol	Astragalus mongholicus, motherwort, Radix Achyranthis Bidentatae, Eucommia
A4	MOL000211	Mairin	Astragalus root, Chinese motherwort, achyranthes root, eucommia bark
B1	MOL000358	Beta-sitosterol	Glehnia root, notoginseng, achyranthes root, goji berry, eucommia bark, scrophularia root
B2	MOL000449	Stigmasterol	Glehnia root, notoginseng, achyranthes root, goji berry, Chinese yam, coix seed
C	MOL001494	Mandenol	Notoginseng, goji berry, coix seed
D1	MOL000953	CLR	Goji berry, Chinese yam, coix seed
D2	MOL005438	Campesterol	Goji berry, Chinese yam, coix seed
D3	MOL001323	Sitosterol alpha1	Goji berry, coix seed
E	MOL000359	Sitosterol	Coix seed, plantain seed, scrophularia root

consisting of 137 nodes and 2079 interaction edges (Figure 4B). And then 36 hub-targets were screened after being analyzed by Betweenness (BC), Closeness (CC), and Degree (DC) filtering (Figure 4C). The results were then uploaded into R to diagram the bar plot. This led to the acquisition of 10 core targets that have the most connection nodes (Figure 5). Thus, the top 10 targets with the highest number of nodes are the genes *APP*, *MAPK1*, *MAPK3*, *AGT*, *EP300*, *C3*, *AKT1*, *CXCL12*, *MCHR1*, and *NMUR2*, which correspond to 98, 95, 88, 84, 70, 69, 65, 65, 64 connection nodes respectively, indicating that the targets in the PPI network are significant.

Gene Ontology Enrichment Analysis

The potential 571 co-target genes were uploaded into the DAVID system for gene ontology (GO) analysis, which uncovers the potential co-target's function that are related

to biologic processes, molecular functions, and cellular components.

The 1104 biologic processes were then enriched. The top 30 terms that correspond with the criterion of $P < 0.05$ were selected for further analyses, such as drug response, inflammatory response, positive regulation of cell proliferation, mitogen-activated protein kinase (MAPK) cascade, lipopolysaccharide response, and negative regulation of apoptotic process (Figure 6). Results indicated that HZYS treatment of DKD involves numerous biologic processes, and understanding these processes can help elucidate the mechanisms by which HZYS treats DKD.

The 145 molecular functions were enriched in using GO and the top 30 terms were selected with the criterion of $P < 0.05$ for further analysis (Figure 7). The potential 571

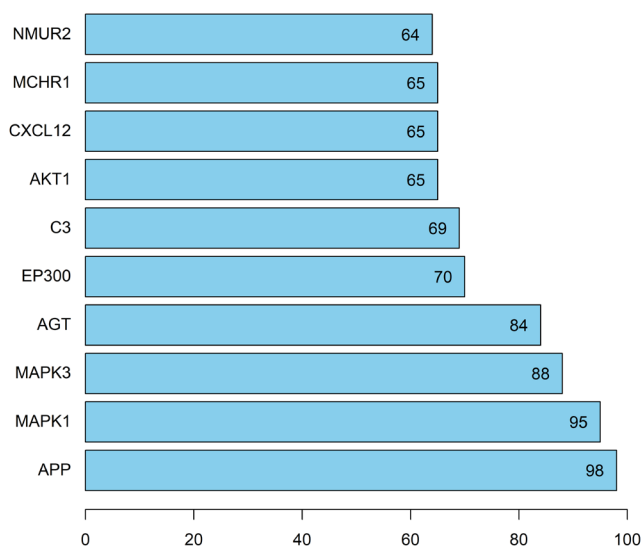


Figure 5: Bar plot of 30 core targets that have more connection nodes based on the PPI network. The number of each bar represents the number of connection nodes associated with the target.

co-target genes are involved in molecular functions related to enzyme binding, protein tyrosine kinase activity, drug binding, protein binding, steroid hormone receptor activity, among other functions.

A total of 120 cell components were obtained from GO analysis and the top 30 terms with $P < 0.05$ underwent further analysis (Figure 8). The resulting potential co-target genes are related to the plasma membrane, extracellular space, cell surface, cytosol, membrane raft, and extracellular exosome. However, the potential mechanisms associated with HZYS treatment of DKD need further exploration.

Finally, the data underwent comprehensive analysis using R to obtain the core functions of the co-targets. The top 20 terms with $P < 0.05$ were selected (Figure 9). The resulting targets are mainly involved with the protein tyrosine kinase activity, transmembrane receptor protein tyrosine kinase activity, transmembrane receptor protein kinase activity, steroid hormone receptor activity, nuclear receptor activity, ligand-

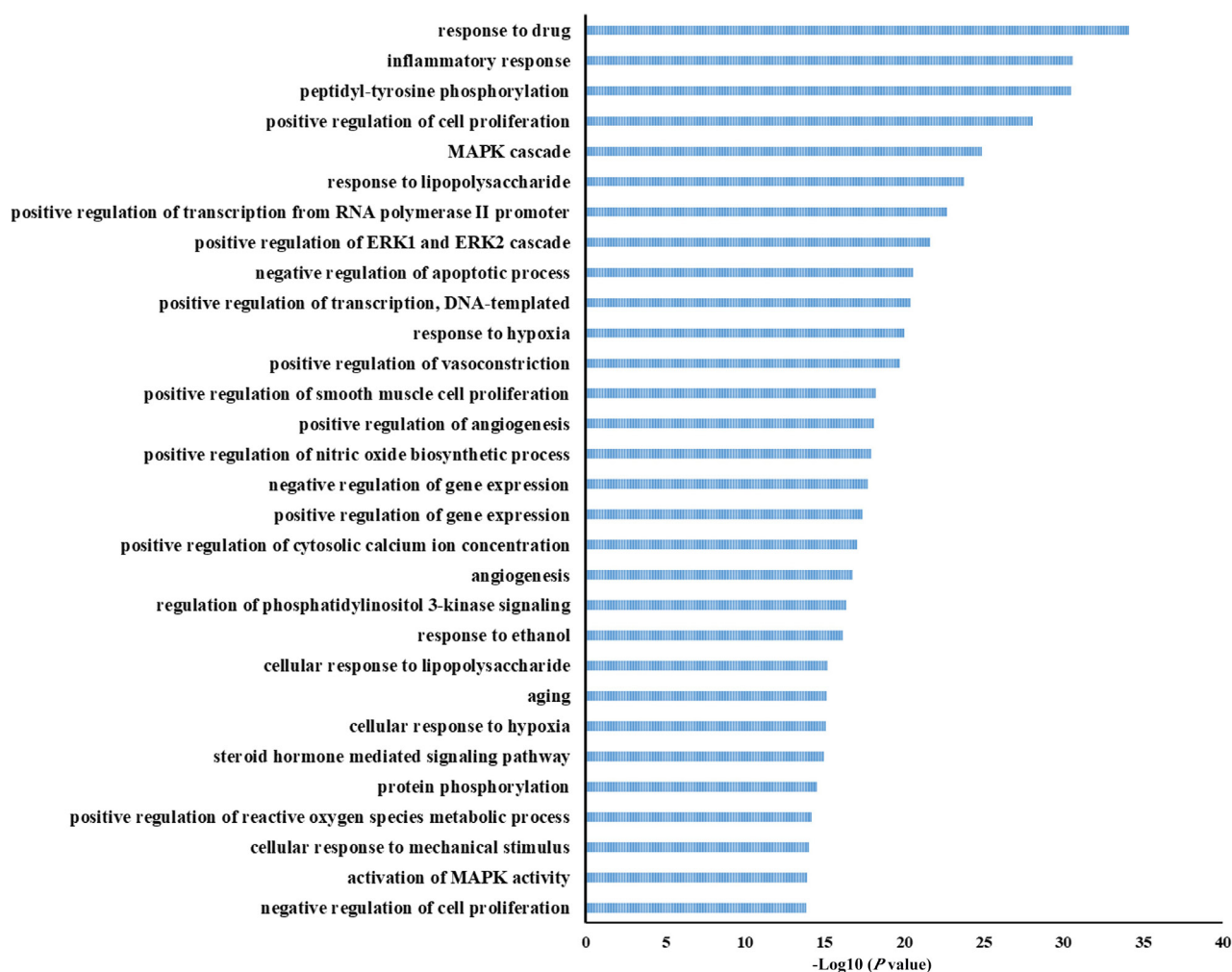


Figure 6: GO enrichment of biologic process (BP) of potential targets of the main active ingredients in HZYS. The top 30 biologic process are shown in this figure. The abscissa is the adjusted P -value, the larger the value, the smaller the P -value.

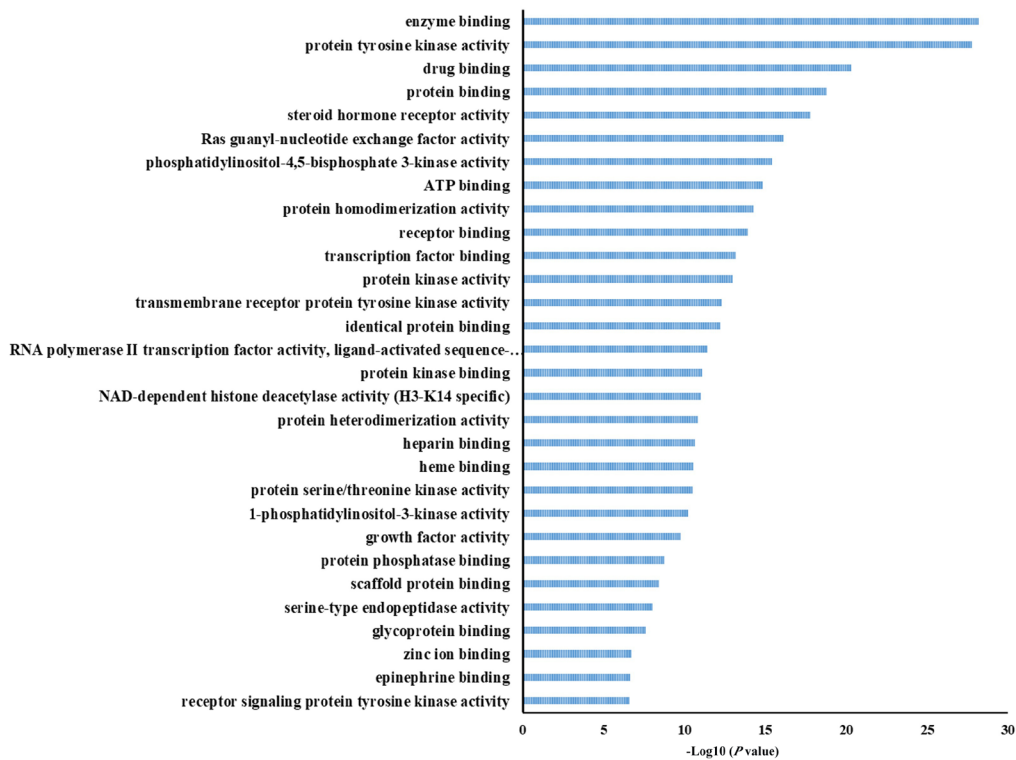


Figure 7: GO enrichment of molecular function (MF) of potential targets of the main active ingredients in HZYS. The top 30 terms were shown in this figure. The abscissa is the adjusted *P*-value, the larger the value, the smaller the *P*-value.

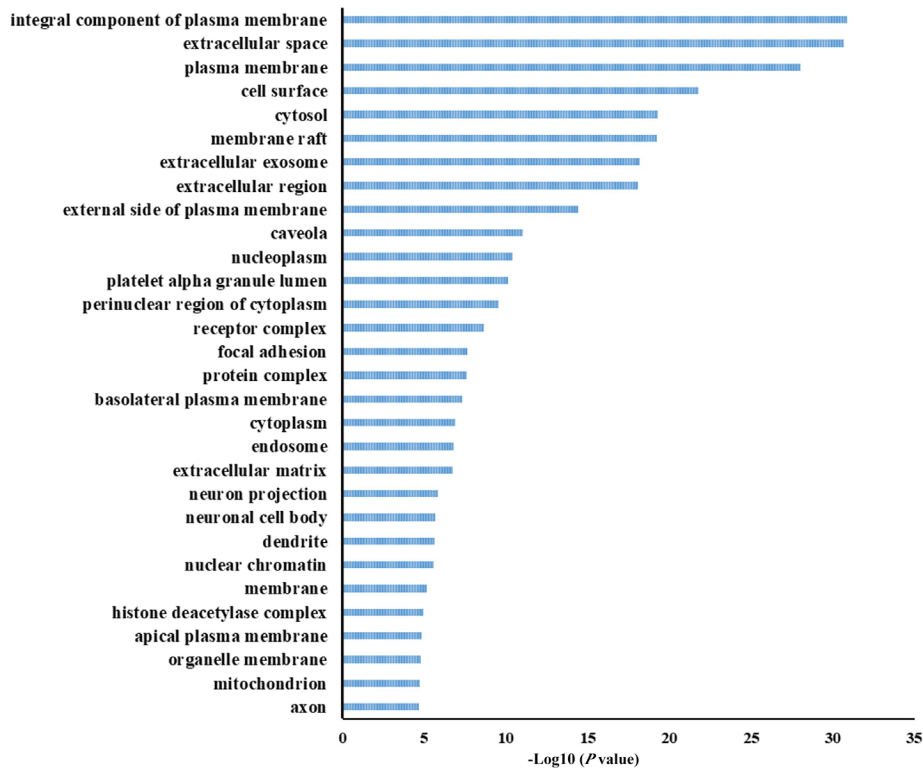


Figure 8: GO enrichment of the cell component (CC) of potential targets of the main active ingredients in HZYS. The top 30 terms were shown in this figure. The abscissa is the adjusted *P*-value, the larger the value, the smaller the *P*-value.

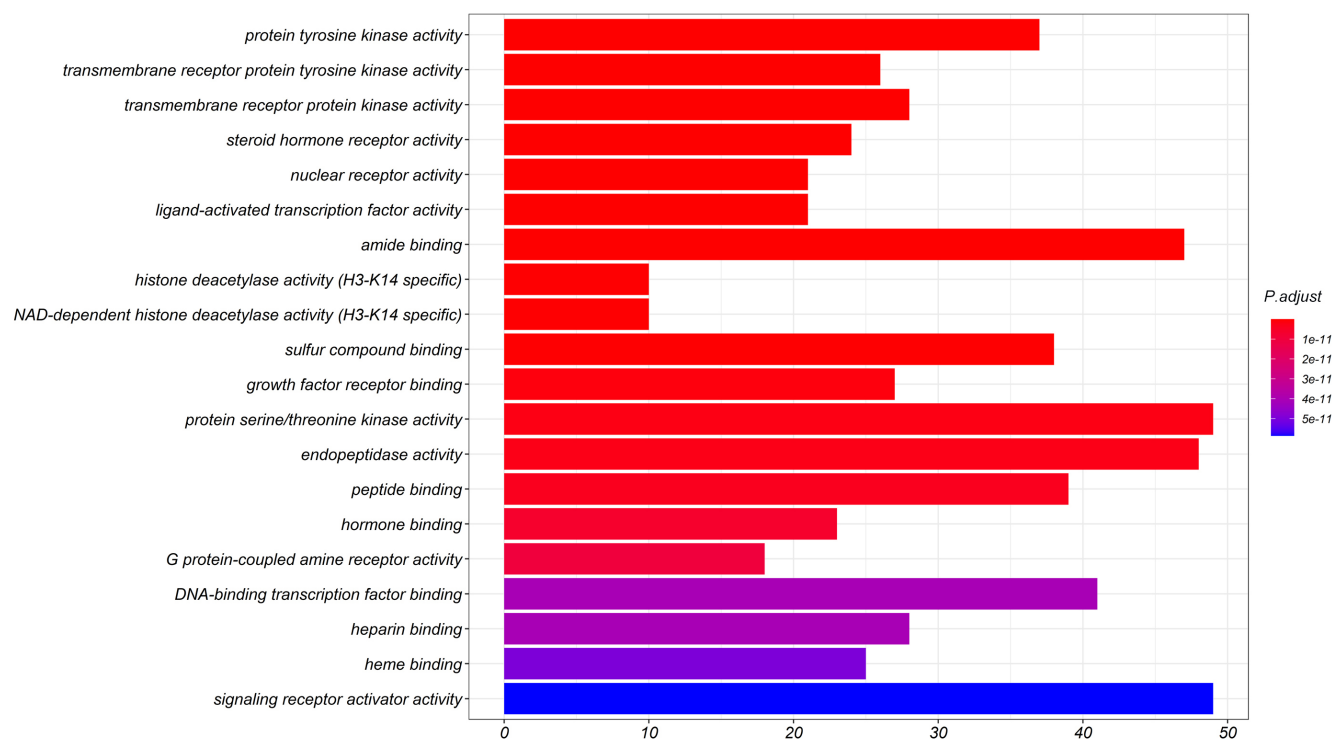


Figure 9: GO enrichment (from R) of potential targets of the main active ingredients in HZYS. Xs represent the number of genes, Ys represent the adjusted P-value. Color of the bar is shown in a gradient from red to blue based on ascending P-value. The top 20 terms are shown in this figure.

activated transcription factor activity, amide binding, sulfur compound binding, and growth factor receptor binding.

KEGG Pathway Enrichment Analysis

KEGG pathway enrichment analysis was performed using R to further uncover the potential mechanism of HZYS in treating DKD. A total of 174 signaling pathways of the potential co-target genes were enriched (Figure 10), including MAPK, AGE-RAGE in diabetic complications, as well as PI3K-Akt, TNF, IL-17, and HIF-1. These pathways are closely associated with cell proliferation, apoptosis, oxidative stress, and inflammation, and are involved in the development of DKD. Thus, we infer that HZYS may treat DKD by intervening in these pathways. We identified the top 20 pathways that may be associated with the DKD process for further analysis (Table 2). Of interest, signaling pathways correlated with other diseases were revealed, including cancers such as prostate, bladder, melanoma, non-small cell, and gastric as evidenced by the presence of the HIF-1 pathway. In addition, other pathways indicated that HZYS may be a candidate for treatment of other diseases, including hepatitis B, Kaposi sarcoma-associated herpesvirus infection, human cytomegalovirus infection, and yersiniosis.

To further explore the relationships between the predicted pathways, the potential co-target genes, and major

compounds, we selected the top 10 pathways that have the highest likelihood to be associated with DKD. A compound-pathway-target network was then established (Figure 11). The top 5 pathways that have the highest degree values are PI3K-Akt, MAPK, AGE-RAGE in diabetic complications, TNF, and apoptosis. These may be the primary pathways by which HZYS treats DKD. Accordingly, the top 10 gene targets were obtained, including *IL6*, *MAPK1*, *AKT1*, *RELA*, *BCL2*, *JUN*, *MAPK3*, *MAP2K1*, *CASP3*, *TNF*.

Molecular Docking Verification

The interactions between the top 4 compounds and IL6, MAPK1, and AKT1 were investigated by molecular docking analysis. We found that all four compounds interacted differently with IL6, MAPK1, and AKT1 (Table 3). Among them, four interactions with lower K_i values and binding energy, which means that they have good bonding ability, were selected out to be further analyzed (Figure 12).

DISCUSSION

Proper treatment is crucial for patients with DKD. HZYS, a traditional Chinese patent medicine, has long been used with success to treat CKD, including DKD.^[2,36,37] Molecular drug targets were developed by the network

Table 2: Top 20 pathways associated with DKD

ID	Pathway description	Gene Ratio	P value	α value
hsa04010	MAPK signaling pathway	78/514	4.11E-29	5.01E-27
hsa04933	AGE-RAGE signaling pathway in diabetic complications	43/514	1.00E-25	3.07E-24
hsa04151	PI3K-Akt signaling pathway	81/514	1.78E-25	3.78E-24
hsa04668	TNF signaling pathway	40/514	2.03E-20	2.26E-19
hsa04657	IL-17 signaling pathway	35/514	1.11E-18	9.45E-18
hsa01521	EGFR tyrosine kinase inhibitor resistance	32/514	1.81E-18	1.38E-17
hsa04659	Th17 cell differentiation	36/514	1.65E-17	1.06E-16
hsa04066	HIF-1 signaling pathway	36/514	3.29E-17	2.01E-16
hsa04210	Apoptosis	40/514	5.52E-17	3.06E-16
hsa01522	Endocrine resistance	33/514	3.70E-16	1.88E-15
hsa04014	Ras signaling pathway	50/514	8.04E-15	3.50E-14
hsa04625	C-type lectin receptor signaling pathway	31/514	1.33E-13	4.79E-13
hsa04660	T cell receptor signaling pathway	31/514	1.33E-13	4.79E-13
hsa04810	Regulation of actin cytoskeleton	45/514	4.88E-13	1.53E-12
hsa04015	Rap1 signaling pathway	44/514	1.03E-12	3.13E-12
hsa04919	Thyroid hormone signaling pathway	32/514	2.04E-12	5.93E-12
hsa04620	Toll-like receptor signaling pathway	29/514	5.39E-12	1.46E-11
hsa04218	Cellular senescence	36/514	6.71E-12	1.74E-11
hsa04062	Chemokine signaling pathway	40/514	8.30E-12	2.07E-11
hsa04020	Calcium signaling pathway	41/514	1.57149E-11	3.76249E-11

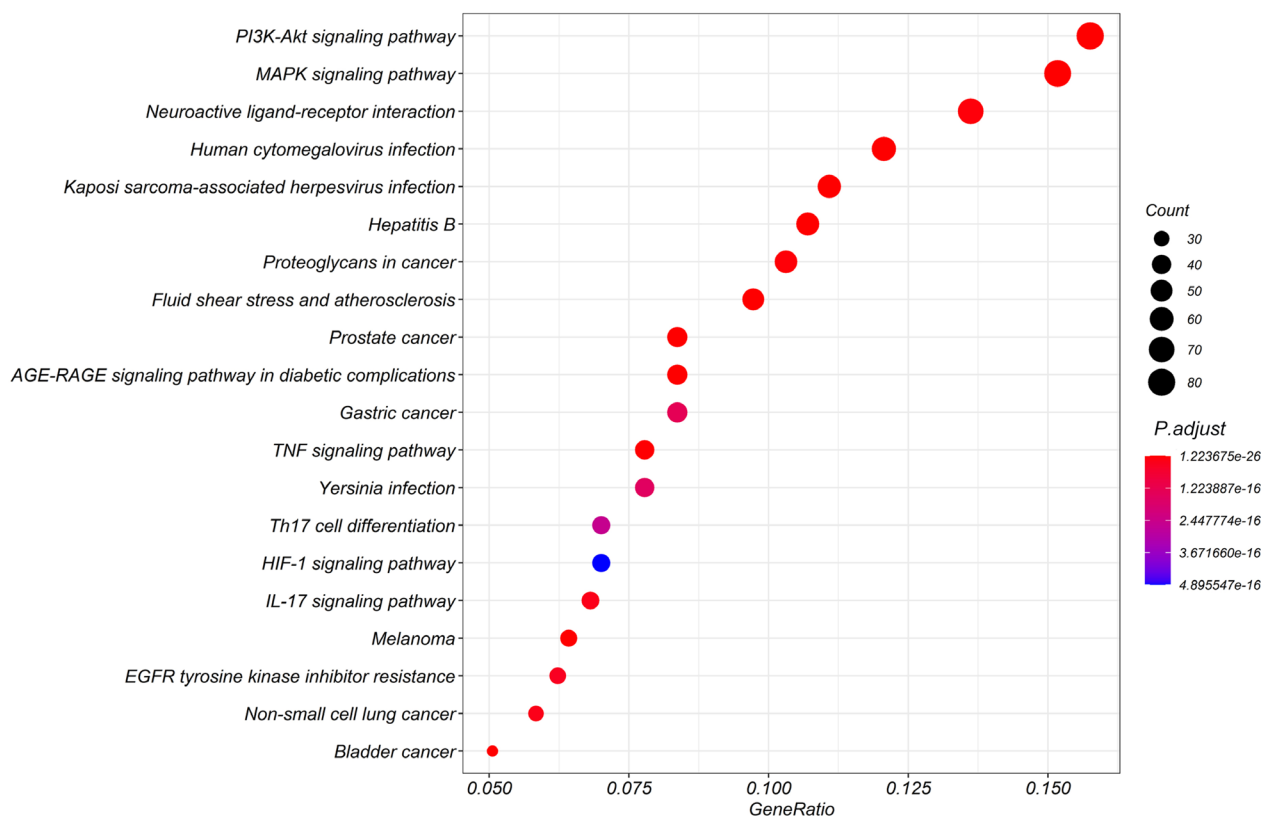


Figure 10: KEGG enrichment (from R) of potential targets of the main active ingredients in HZYS. Xs represent the gene ratios, Ys represent the adjusted P-value. The color of the bubble is shown in a gradient from red to blue based on ascending P-value. Size of the bubble is based on the number of genes. The top 20 terms are shown in this figure.

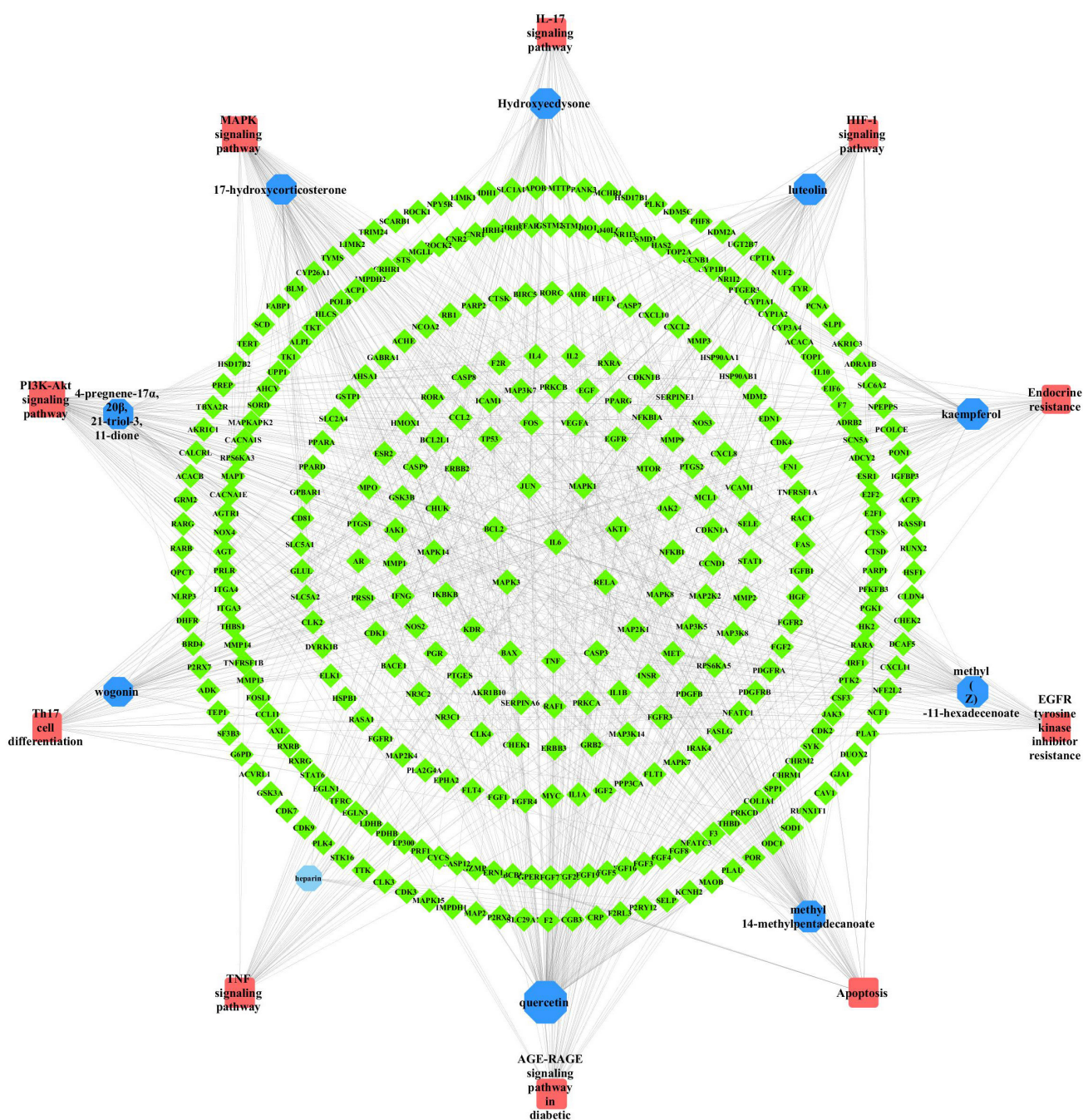


Figure 11: Visual analysis of compound-pathway-target network. Green diamonds represent the targets; blue octagons represent the calculated compounds; red rectangles represent the calculated pathways. The size and color of the border are arranged according to the degree value.

pharmacology, which aims at biological networks and the links among disease, drug, and targets in those networks.^[16,38] Therefore, network pharmacology, as a new approach to study the complex relationship among targets, drugs, diseases, and pathways for drug discovery.^[39] In this study, systematic analysis of HZYS was conducted at the molecular level using network pharmacology. Ten potential hub ingredients, 571 HZYS-DKD-common-targets, 10 hub targets, and 10 pathways were acquired, suggesting

that they have potential value for HZYS in treatment of DKD.

In this study, some of the active compounds in HZYS appeared to be capable of attenuating proteinuria and oxidative stress, and improving renal function. One such compound was heparin, which was found in leech. Heparin is well known as an anticoagulant; it inactivates the thrombin generating ability of plasma.^[40] In DKD

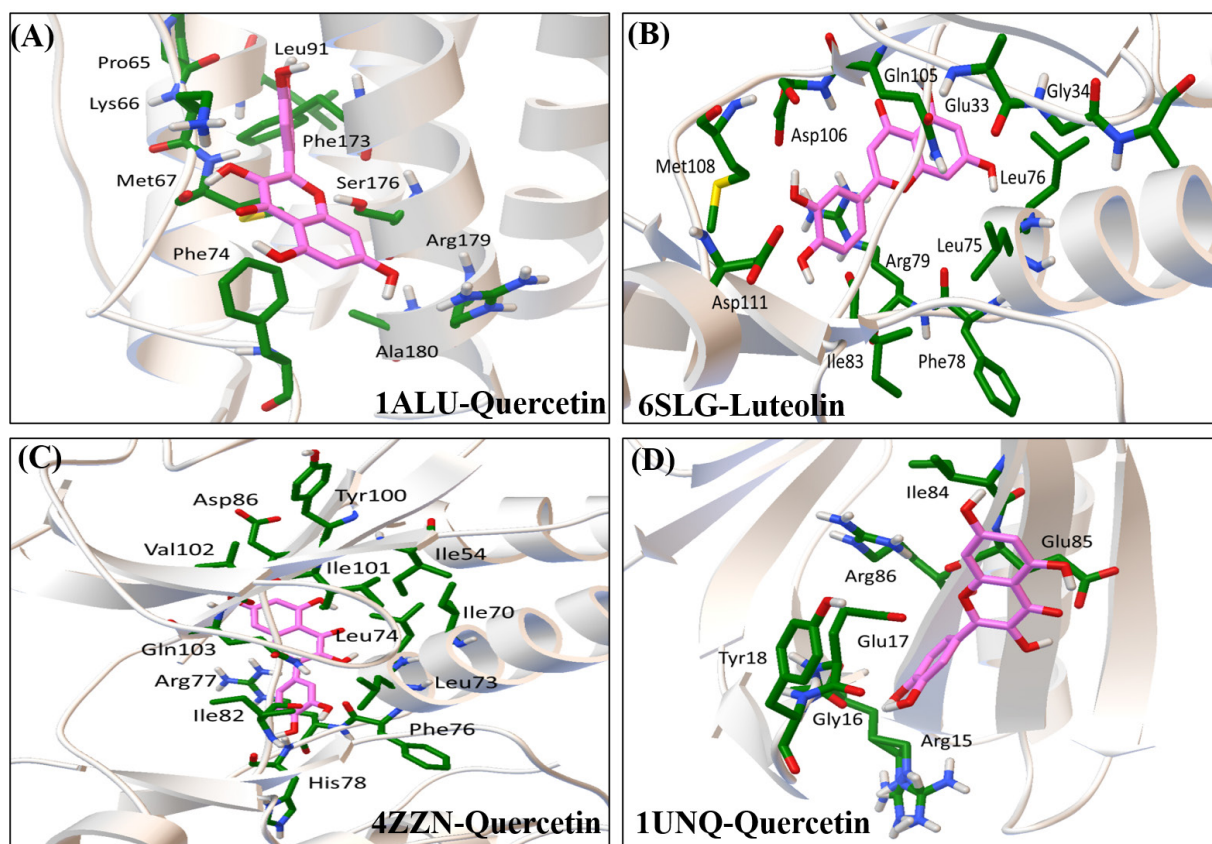


Figure 12: Molecular docking of compound-hub genes. (A) 1ALU to Quercetin (Binding energy = -7.68, Ki = 2.33 μmol/L); (B) 6SLG to Luteolin (Binding energy = -6.32, Ki = 23.31 μmol/L); (C) 4ZZN to Quercetin (Binding energy = -6.78, Ki = 10.74 μmol/L); (D) 1UNQ to Quercetin (Binding energy = -5.82, Ki = 54.16 μmol/L); (1ALU is a crystal structure of IL6; 6SLG is a crystal structure of MAPK1; 4ZZN is a crystal structure of MAPK1; 1UNQ is a crystal structure of AKT1. Interacting amino acids and compound structures are shown in lines, in which amino acids are shown in green, compound structures are shown in pink.)

Table 3: The binding free energy (ΔG) and inhibition constant (Ki) value of the interactions between compounds and target proteins

	IL6		MAPK1		AKT1	
	1ALU	6SLG	4ZZN	1UNQ	1ALU	6SLG
	ΔG [kcal mol ⁻¹]	Ki(μmol/L)	ΔG [kcal mol ⁻¹]	Ki(μmol/L)	ΔG [kcal mol ⁻¹]	Ki(μmol/L)
kaempferol	-5.89	48.41	-5.21	151.77	-5.39	112.34
luteolin	-7.13	5.99	-6.32	23.31	-6.70	12.27
methyl	-4.16	895.29	-2.62	-	-3.80	-
quercetin	-7.68	2.33	-6.06	36.43	-6.78	10.74

model rats, low-molecular weight heparin has been shown to prevent glomerular basement membrane thickening in the kidney, thus reducing proteinuria.^[41, 42] In clinical trials, heparin has been found to attenuate proteinuria in both type 1 and type 2 diabetes.^[43, 44] Another active compound is quercetin, which is found in several herbs in HZYS, including astragalus root, glehnia root, notoginseng root, Chinese motherwort, achyranthes root, goji berry, eclipta, eucommia bark, and plantain seed. Quercetin appears to decrease insulin resistance by reducing endoplasmic reticulum (ER) stress, oxidative stress, and β-cell death.^[45] In streptozotocin-induced DKD rats, quercetin has been shown to improve renal function by downregulating

the expression of transforming growth factor beta and connective tissue growth factor.^[46] Further studies have discovered that quercetin exhibits several molecular activities. For example, Lu *et al.* found that quercetin reverses high glucose-induced epithelial-mesenchymal transition of HK-2 and NRK-52E cells by inhibiting mTORC1/p70S6K activation in the diabetic renal cortex.^[47] Another research on quercetin has shown that it inhibits the proliferation of high glucose-induced mesangial cells by reactivating the Hippo signaling pathway and suppressing the NF-κB signaling pathway.^[48-51] Another active compound in HZYS is kaempferol. It is found in astragalus root, Chinese motherwort, achyranthes root,

and eucommia bark which were reported to lower blood sugar level and blood lipid levels.^[52,53] In the DKD mouse model, kaempferol has been shown to reduce renal injury and fibrosis by upregulating glucagon-like peptide 1 and insulin.^[54] Kaempferol may also reduce the occurrence of complications of diabetes, such as DKD and diabetic retinopathy.^[55,56] In addition to the aforementioned active compounds, other compounds in HZYS have also been found in this study to have anti-inflammatory, antioxidant, and antifibrotic activities. Two examples are luteolin and wogonin, which in our study are hub components of HZYS. Zhang *et al.* found that luteolin suppresses the inflammatory and oxidative responses by suppressing STAT3 activation,^[57] Meng *et al.* discovered that wogonin exerts an antifibrotic effect on renal tubular epithelial cells through smad3-dependent mechanisms.^[58] In summary, our network pharmacology results and those of experimental studies indicate that active compounds in HZYS have potential therapeutic value in DKD.

To further evaluate the potential key mechanism of HZYS in the treatment of DKD, 507 compound-disease common targets (chosen from 620 compound targets) underwent GO enrichment analyses. Results indicated that HZYS can potentially treat DKD by regulating the inflammatory response, cell proliferation, apoptotic process, MAPK cascade, and serum lipopolysaccharide activity. In addition, these mechanisms were involved with key targets such as MAPK1, MAPK3, AKT1, AGT, PTGS2.

In this study, KEGG pathway enrichment analysis demonstrated that the potential key mechanism by which HZYS treating DKD was mainly involved with several signaling pathways, including AGE-RAGE in diabetic complications, PI3K-Akt, MAPK, TNF, and apoptosis. The top 10 genes we analyzed from the hub pathways were IL6, MAPK1, AKT1, RELA, BCL2, JUN, MAPK3, MAP2K1, CASP3, and TNF. IL6 is a classic pro-inflammatory cytokine involved in DKD.^[59] Human and experimental studies have validated the involvement of the IL-6 signaling pathway in DKD progression. For instance, IL-6 is critically involved in the inflammatory processes of tubular and mesangial cells, and podocytes, which are the resident cells of the kidney.^[59] In our study, IL-6 was found to be associated with the AGE-RAGE, PI3K/Akt, HIF-1, and IL17 signaling pathways, which are closely involved with inflammation, apoptosis, and oxidative stress in DKD.^[60,61] The PI3K/Akt pathway is also a significant player in autophagy regulation of resident kidney cells.^[62] BCL2, CASP3, and TNF are important components of apoptosis and are closely associated with the progression of DKD.^[63–65] In addition, the AGE-RAGE signaling pathway in diabetic complications is highly involved in the DKD formation process. Previous

investigation found that sustained hyperglycemia due to diabetes facilitates increased production of AGEs and stimulates their cell surface receptors, RAGEs,^[66] therefore, inducing endoplasmic reticulum (ER) stress and increasing intracellular calcium level and apoptosis rate,^[67] thus worsening diabetic kidney disease. In contrast, in RAGE-deficient diabetic mice, development of DKD is delayed, with less inflammatory and fibrosis expressed in renal tissues.^[68] Additionally, inflammation and oxidative stress can also induce production of AGEs.^[68] Thus, HZYS may treat DKD by alleviating inflammation and oxidative stress to regulate the AGE-RAGE signaling pathway. Moreover, since the PI3K/Akt signaling pathway may be activated by RAGEs,^[69] HZYS may suppress RAGEs to regulate the PI3K/Akt pathway to attenuate DKD.^[66]

The molecular docking verification presents that the prime compounds of HZYS Quercetin and Luteolin have good binding capability with the hub potential targets IL6, MAPK1, AKT1. Quercetin exhibits a significantly anti-inflammatory effect in kidney disease, among which lessened renal pathologies, and inhibited IL-6 production in mice with LPS-induced AKI.^[70] Quercetin alleviated LPS-stimulated HK-2 cell inflammatory injury by regulating miR-124 and reduce IL6 and TNF- α .^[71] Quercetin has also been reported to have anti-oxidant effects in kidney through modulating the MAPK and NF- κ B signaling pathway.^[72] And it has also been verified in our study that it has a good binding ability with the crystal structures of MAPK1. Additionally, Quercetin was also reported to regulate the Akt signaling pathway to improve glucose and lipid metabolism of DKD rats.^[73] In our study, it was verified to have interaction with AKT1. Luteolin is another prime compound of HZYS. In the past experimental research, luteolin has been proved to have remarkable anti-inflammatory, anti-apoptosis, and anti-oxidant stress effects, which were mainly by regulating MAPK signaling pathway.^[74–82] Surely, Luteolin did have a good interaction with MAPK in this research.

The results indicated that the screened compounds, targets, and pathways are consistent with current research findings, confirming that HZYS treats DKD by mediating inflammation, oxidative stress, apoptosis, fibrosis, and other signaling pathways. However, the results of our network pharmacology investigation need further verification through *in vitro* and *in vivo* experiments to demonstrate the exact mechanism by which HZYS treats DKD.

CONCLUSION

This study provides new insight into the pharmacology of HZYS and the mechanism by which this Chinese herbal formula treating DKD. Our study using network

pharmacology revealed that active compounds in HZYS were potentially involved in regulating the inflammatory, oxidative stress, apoptotic, and fibrosis signaling pathways in DKD.

Data Availability

Data that support the findings of this study are available from the corresponding author upon request.

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Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

Xue-Feng Zhou: conceptualization, design, methodology, software, formal analysis, original draft preparation, visualization, molecular docking; Wei-E Zhou: conceptualization, design, methodology, formal analysis, review and editing, visualization; Wen-Jing Liu: methodology, software, resources; Min-Jing Luo: methodology, resources; Xia-Qing Wu: molecular docking; Ying Wang: methodology, resources; Peng Liu: validation, investigation, review and editing, supervision, project administration, funding acquisition; Yu-Min Wen: validation, funding acquisition; Jia-Lin Li: resources; Ting-Ting Zhao: validation; Hao-Jun Zhang: validation; Hai-Ling Zhao: validation; Ping Li: investigation, review and editing, supervision, project administration, funding acquisition

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