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Exploring the molecular mechanism of Suoquan pill in the treatment of diabetic kidney disease based on network pharmacology, molecular docking, in vitro experiment

Zi-Jie Yan[1#], Yu Kang[2#], Shu-Man Liu[1], Fang-Yu Wang[3], Man Xiao[4*], Yi-Qiang Xie[1*]

[1]College of Traditional Chinese Medicine, Hainan Medical University, Haikou 571199, China. [2]Graduate School, Heilongjiang Academy of Traditional Chinese Medicine, Harbin 150001, China. [3]Department of Pharmacology, College of Pharmacy, Hainan Medical University, Haikou 571199, China. [4]Key Laboratory of Biochemistry and Molecular Biology, Hainan Medical University, Haikou 571199, China.

[#]These authors contributed equally to this work and are co-first authors for this paper.
[*]**Correspondence to:** Man Xiao, Key Laboratory of Biochemistry and Molecular Biology, Hainan Medical University, No. 4, Xue-Yuan Road, Haikou 571199, China. E-mail: xiaoman_shen2004@163.com. Yi-Qiang Xie, College of Traditional Chinese Medicine, Hainan Medical University, No. 4, Xue-Yuan Road, Haikou 571199, China. E-mail: xieyiqiang@hainmc.edu.cn.

Author contributions
Xie YQ offered funds. Xie YQ and Xiao M designed the study. Yan ZJ, Kang Y, Liu SM, Wang FY participated in the experiment. Yan ZJ wrote the paper. All the authors approved the final edited version of the manuscript.

Competing interests
The authors declare no conflicts of interest.

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Abbreviations
AOF, *Alpiniae Oxyphyliae Fructus*; SQP, Suoquan pill; BP, biological processes; DM, Diabetes mellitus; DKD, diabetic kidney disease; GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; LR, *Linderae Radix*; PDB, Protein Data Bank; PPI, protein-protein interaction; RD, *Rhizoma Dioscoreae*; EGFR, epidermal growth factor receptor; SRC, non-receptor tyrosine kinase; CCK8, cell counting kit-8; HG, high glucose; OD, optical density; TCM, traditional Chinese medicine; TCMSP, Traditional Chinese Medicine Systematic Pharmacology; PAF, platelet-activating factor.

Citation
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Abstract
Background: Diabetic kidney disease (DKD) is a microvascular complication of diabetes mellitus and is the main cause of end-stage renal failure. Suoquan pills (SQP) has a variety of pharmacological activities and multiple therapeutic effects, and it is used clinically as a basic formula for the treatment of DKD. **Methods:** Public databases were used to identify SQP compounds and the potential targets of SQP and DKD. A drug-component-therapeutic target network was constructed. Protein-protein interaction network analysis, Gene Ontology functional analysis, and Kyoto Encyclopedia of Genes and Genomes pathway enrichment analysis were used to analyse the potential molecular mechanisms of SQP based on common targets of drugs and diseases. Molecular docking simulations were conducted to confirm the binding abity of the core compounds to key targets. The efficacy and predicted molecular mechanisms of SQP were validated using cell counting kit-8 assay, flow cytometry, and western blotting with HK-2 cells as a model. **Results:** Network pharmacology analysis showed that 26 compounds and 207 potential targets of SQP were involved in the treatment of DKD; boldine, denudatin B, pinocembrin, kaempferoid, and quercetin were considered core compounds, and epidermal growth factor receptor (EGFR) and proto-oncogene, non-receptor tyrosine kinase (SRC) were considered key targets. Gene Ontology enrichment analysis indicated that protein phosphorylation and negative regulation of apoptotic processes are important biological processes in the treatment of DKD by SQP. Molecular docking confirmed the excellent binding abilities of boldine, denudatin B, kaempferide, and quercetin to EGFR and SRC. The results of in vitro experiments showed that treatment with an ethanolic extract of SQP significantly protected HK-2 cells from high glucose-induced cell damage. In addition, the SQP ethanol extract inhibited the phosphorylation of EGFR and SRC, suppressed the apoptosis rate, and regulated apoptosis-related proteins in HK-2 cells under high glucose stress. **Conclusion:** This study systematically and intuitively illustrated the possible pharmacological mechanisms of SQP against DKD through multiple components, targets, and signalling pathways, especially the inhibition of EGFR and SRC phosphorylation and apoptosis.

Keywords: traditional Chinese medicine; diabetic kidney disease; Suoquan pill; network analysis; molecular docking

Highlights

We explored the targeted therapeutic effect of Suoquan pill on diabetic kidney disease by utilizing a network pharmacology approach, which not only provided a basis for the treatment of diabetic kidney disease by traditional Chinese medicine, but also expanded the scope of clinical application of Suoquan pill.

Medical history of objective

Suoquan pill, from Xue Ji's "*Jiaozhu Furen Liangfang*" (1529 C.E.) is used to treat diabetic kidney disease because it is in line with the characteristics of the Chinese medical mechanism of the disease. Modern research shows that Suoquan pill has the ability to lower blood sugar and improve kidney tissue and function damage.

Background

Diabetes mellitus (DM) is a metabolic disorder characterised by chronic hyperglycaemia caused by disturbances in insulin action, insulin secretion, or both. Diabetic kidney disease (DKD) is one of the most common microvascular complications of DM and is a major cause of end-stage renal disease. DKD poses a great burden to society and the economy, as well as to people's health, owing to its high prevalence and poor prognosis [1]. Current strategies for DKD treatment and management mainly involve reduction in body weight, blood glucose, and blood pressure with the use of renin-angiotensin system inhibitors, including angiotensin-converting enzyme inhibitors or angiotensin receptor blockers. However, treating DKD using conventional methods does not always result in satisfactory outcomes because of the involvement of multiple signalling pathways and targets in its pathogenesis [2, 3].

Traditional Chinese medicine (TCM) has been shown to have multi-component drug properties that produce synergistic effects on multiple targets, steps, and levels [2]. In China, TCM has been widely used as an independent or adjunctive treatment for DKD and has shown good efficacy in clinical practice [4]. According to TCM, DM is a consumptive thirst disease (Xiaoke in Chinese). DKD belongs to the category of "Xiaoke", and the pathogenesis of its kidney deficiency (Kidney is one of the five organs in traditional Chinese medicine. Kidney deficiency mainly manifests itself in the form of lumbar and knee soreness and weakness, poor mental performance or distraction, and even a decline in sexual function and fertility) persists throughout the pathogenesis of DKD [5]. Suoquan pill (SQP) has a long history. Since SQP was formulated, it has been quoted extensively by medical practitioners of all dynasties, who have different opinions on its composition, proportion, dosage, and main treatment. Therefore, there are many additions and subtractions to SQP [6]. In the *Chinese Pharmacopoeia*, SQP consists of *Alpiniae Oxyphyliae Fructus* (AOF) (dried mature fruit of *Alpinia oxyphylla* Miq., Family Zingiberaceae, Genus *Alpinia*), *Linderae Radix* (LR) (dried tuberous roots of *Lindera aggregata* (Sims) Kos-term. Family Lauraceae), *Dioscoreae Rhizoma* (RD) (dried rhizome of *Dioscorea opposita* Thunb., Family Dioscoreaceae) in equal proportions, which is in line with the TCM pathogenesis of DKD and can be used clinically as a basic formula for the treatment of DKD [7, 8].

Because TCM has complex medicinal components, multiple targets, and a wide range of signalling pathways for exerting therapeutic effects, conducting in-depth research on the mechanism of action of Chinese herbal medicines and promoting their modernisation is a challenging task. Network pharmacology generates complex interaction networks based on compounds, molecular targets, and biological functions of herbal medicines. This provides a systematic view of the mechanism of action of herbal medicines in the treatment of diseases at the molecular level and a new vision for the discovery of bioactive compounds, mechanistic research, quality control, and many other fields [9]. Molecular docking is a computer-aided drug design method that predicts the conformation of small-molecule ligands at the target binding site and their affinity with considerable precision and has been widely used in the material basis of TCM [10]. Therefore, the combination of network pharmacology and molecular docking can provide new insights into the selection and mechanistic exploration of active compounds and accelerate the development of Chinese medicine to some extent.

In this study, a network analysis approach was used to explore the potential active compounds and mechanism of SQP for DKD, and the prediction was validated using molecular docking and in vitro experiments. The flow chart of the study is shown in Figure 1.

Materials and methods

Network analysis

In this study, potential active components of SQP were screened using oral bioavailability ≥ 30% and drug-like properties ≥ 0.18 as the screening criterion using the Traditional Chinese Medicine Systematic Pharmacology (TCMSP) database (http://old.tcmsp-e.com/index.php) [11]. The component targets of SQP were collected from the Swisstarget Prediction database (http://www.swisstargetprediction.ch), and probability > 0.1 was used as the screening criterion [12]. The keywords "DKD" and "diabetic nephropathy" were used to identify the target genes related to DKD from the Online Mendelian Inheritance in Man (http://www.omim.org) and GeneCards (https://www.genecards.org) databases [13, 14]. Venny 2.1.0 (https://bioinfogp.cnb.csic.es/tools/venny/index.html) was used to identify common targets of drugs and diseases. The common targets were imported into the STRING database (https://string-db.org) to obtain protein-protein interaction (PPI) network maps where the screening parameter was "*Homo sapiens*" and a medium confidence level (0.400) was used as the minimum interaction score required [15]. The visualisation and mapping of "drug-component-target" networks and PPI networks was performed by Cytoscape 3.9.1 software. Common targets were imported into the DAVID database (https://david.ncifcrf.gov) for Gene Ontology (GO) enrichment analysis and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis [16]. The enrichment results were exported as bubble plots via the online platform Bioinformatics (https://www.bioinformatics.com.cn).

Molecular docking

The crystal structures of proteins were retrieved from the Protein Data Bank (PDB) (http://www.rcsb.org/pdb/) [17]. Water molecules and small-molecule ligands of the protein were removed using PyMOL software and the protein was subjected to hydrogenation using AutoDock Vina 1.1.2. The epidermal growth factor receptor (EGFR) protein molecule had four identical chains, one of which was retained for subsequent processing. Structures of small-molecule compounds in the mol2 file format were obtained from the TCMSP database. Components and targets were converted to PDBQT format files using AutoDock Vina 1.1.2, and molecular docking simulations were performed to measure the interactions between small-molecule compounds and proteins. After molecular docking, the conformation with the lowest binding energy was selected as the binding conformation between the ligand and target protein. Finally, the ligand-protein complexes were analysed and visualised using PyMOL software.

Reagents and antibodies

AOF, RD and LR were purchased from Tong Ren Tang (Longkun Nan Store, Haikou, China). The origin of AOF was Guangdong, produced by Beijing Ben Cao Fang Yuan Company (Beijing, China), with batch number 20230309; the origin of LR was Zhejiang, produced by Beijing Ben Cao Fang Yuan Company (batch number 20230214, Beijing, China); the origin of RD was Henan, produced by Beijing Tong Ren Tang Health Pharmaceutical Company (batch number Y2208014, Beijing, China). BCA Protein Quantification kit, ECL kit, Annexin V-FITC/PI Apoptosis Detection kit were purchased by Yeasen

(Shanghai, China); cell counting kit-8 (CCK8), RIPA Lysis Buffer, Protease and phosphatase inhibitor cocktail, 5× protein loading buffer, HRP-labeled Goat Anti-Rabbit IgG were purchased by Beyotime (Shanghai, China); SDS-PAGE Preparation kit, PVDF blotting film were purchased by Sangon (Shanghai, China); EGFR antibody (1:10,000), SRC antibody (1:2,000) were purchased by Affinity (Changzhou, China); P-EGFR antibody (1:2,000), Bax antibody (1:2,000), Bcl-2 antibody (1:2,000) were purchased by Abcam (Shanghai, China); P-SRC antibody (1:5,000), Cleaved-caspase 3 antibody (1:5,000) were purchased by Cell Signaling Technology (Boston, MA, USA); β-Actin antibody (1:2,000) was purchased by Servicebio (Wuhan, China).

Preparation of ethanolic extract of SQP

SQP herbs (AOF:RD:LR = 1:1:1) were crushed and soaked in 75% ethanol for 12 h (drug at a drug-to-solvent ratio of 1:8). The filtrate was filtered using a vacuum pump (Xiande, Shanghai, China) to obtain a clarified filtrate, which was then extracted using a rotary evaporator (Xiande, Shanghai, China) to obtain the ethanol extract of the herb. The above process was repeated twice. The two extracts obtained were mixed, concentrated using a rotary evaporator, and dried. Finally, 200 mg of the extract was dissolved in 1 mL dimethyl sulfoxide. Subsequent cell assays were diluted with cell culture medium to the desired concentration. Ultra-high-performance liquid chromatography-quadrupole time-of-flight mass spectrometry (UHPLC-QTOF-MS) (Thermo Fisher Scientific, Waltham, MA, USA) confirmed the quality of the SQP, as shown in Supplementary Figure S1, Table S1.

Cell culture

HK-2 cells were acquired from Procell Life Science & Technology Co., Ltd. (Wuhan, China) and grown at 37 °C in humid air with 5% CO_2 in a Minimum Essential Medium containing 10% foetal bovine serum, 1% Penicillin-Streptomycin Solution.

CCK8 assay for cell viability

HK-2 cells were resuspended and seeded into 96-well plates (1,500 cells/well). The normal group was cultured under the original culture conditions. The model group was treated with 60 mM high glucose (HG) condition for 72 h. The SQP group was treated with different concentrations of SQP (10, 25, 50, 100, and 200 μg/mL) for 24 h under the condition of 60 mM HG for 72 h. Finally, 100 μL of basic medium and 10 μL of CCK8 solution were added to each well and incubated for 1 h. The optical density (OD) values were measured at 460 nm. Cell viability was calculated using the following Equation (1):

$$\text{Cell Viability} = [(\text{OD of Experimental Group}) - (\text{OD of Cell-Free Group})]/[(\text{OD of Control Group}) - (\text{OD of Cell-Free Group})] \times 100\% \quad (1)$$

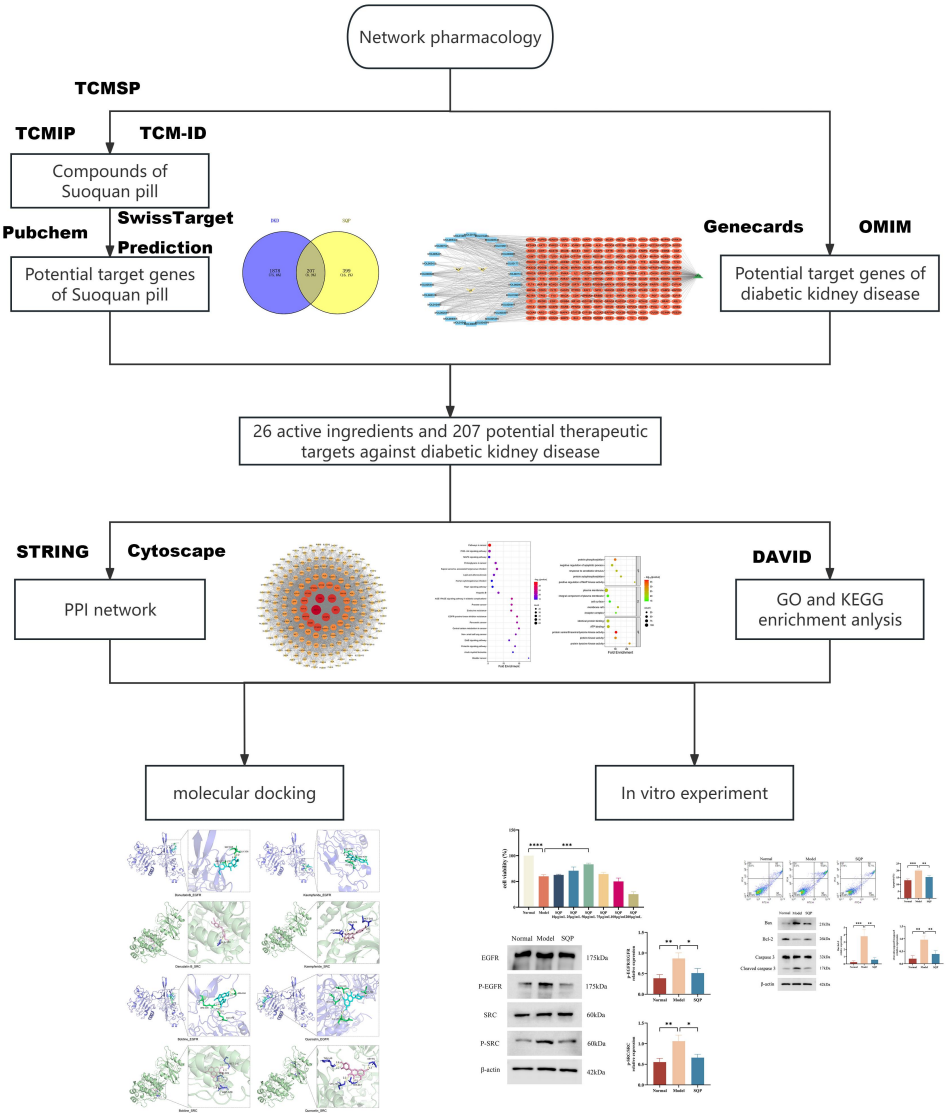

Figure 1 Brief flow chart of this study. PPI, protein-protein interaction; GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes.

Flow cytometry analysis

The Annexin V-FITC/PI apoptosis detection kit was used to detect apoptosis, according to the manufacturer's instructions. Briefly, 1×10^5 cells were suspended in the binding buffer and double-stained with Annexin V-FITC and PI for 15 min in the dark. Stained cells were analysed using a NovoCyte flow cytometer (Agilent, Beijing, China).

Western blot

Proteins were extracted from different groups of HK-2 cells using RIPA lysis buffer containing a 1:50 protease and phosphatase inhibitor cocktail. Proteins were quantified using the BCA kit according to the manufacturer's instructions. After adding 5× protein loading buffer, all samples were denatured by boiling at 100 °C for 10 min and separated by SDS-PAGE. Electrophoresis was initially performed at a constant voltage of 80 V and switched to a constant voltage of 120 V for electrophoresis as bromophenol blue moved to the separating gel. The gel was then blotted onto the PVDF membrane for 60 min at a constant current of 300 mA. The PVDF membranes were closed with 5% skim milk for 2 h at about 26 °C and then incubated with the corresponding primary antibody overnight at 4 °C. The PVDF membranes were washed three times with TBST for 10 min each, followed by further incubation with a secondary antibody for 1 h at room temperature. Finally, the PVDF membranes were photographed using an eBlot Touch Imager (eBlot, Shanghai, China) after using an ECL kit. Quantitative analysis was performed using the Image J.

Statistical analysis

Experimental data are expressed as mean ± standard deviation. Statistical analyses of multiple groups of samples were performed using one-way analysis of variance. Tukey's method was used to compare two groups of data. All statistical data were analysed using GraphPad Prism (version 9.4.1). $P < 0.05$ was considered statistically significant.

Results

Acquisition of compounds and targets of SQP

A total of 32 SQP compounds were screened in the TCMSP database, including 8 compounds in AOF, 9 compounds in LR, and 18 compounds in RD. Basic information on the compounds is shown in Table 1. Target prediction was carried out using the Swisstarget Prediction database, and targets with probability > 0.1 were selected as relevant targets for the ingredients and compounds without predicted targets were removed. In total, 26 compounds and their corresponding 606 potential targets were identified. Specific information regarding the compounds and their corresponding targets is provided in Supplementary Table S2.

Potential targets of SQP for the treatment of DKD

A total of 2,085 disease target genes related to DKD were collected from GeneCards and Online Mendelian Inheritance in Man databases; detailed information is shown in Supplementary Table S3. A total of 207 potential therapeutic targets of SQP in DKD were identified by constructing Venny plots of DKD and SQP (Figure 2A).

Table 1 Basic information about the active compounds of SQP

Mol ID	Molecule name	Oral bioavailability (%)	Drug-like properties	Related herbs
MOL001525	Daucosterol	36.91	0.75	AOF
MOL000359	Sitosterol	36.91	0.75	AOF, LR
MOL000449	Stigmasterol	43.83	0.76	AOF, RD
MOL000358	Beta-sitosterol	36.91	0.75	AOF, LR
MOL009355	Sitosterol palmitate	30.91	0.4	AOF
MOL002844	Pinocembrin	64.72	0.18	AOF
MOL004564	Kaempferide	73.41	0.27	AOF
MOL010485	Eicosapentaenoic acid	45.66	0.21	AOF
MOL001559	Piperlonguminine	30.71	0.18	RD
MOL001736	(-)-taxifolin	60.51	0.27	RD
MOL000310	Denudatin B	61.47	0.38	RD
MOL000322	Kadsurenone	54.72	0.38	RD
MOL005429	Hancinol	64.01	0.37	RD
MOL005430	Hancinone C	59.05	0.39	RD
MOL005435	24-Methylcholest-5-enyl-3belta-O-glucopyranoside_qt	37.58	0.72	RD
MOL005438	Campesterol	37.58	0.71	RD
MOL005440	Isofucosterol	43.78	0.76	RD
MOL005458	Dioscoreside C_qt	36.38	0.87	RD
MOL000546	Diosgenin	80.88	0.81	RD
MOL005461	Doradexanthin	38.16	0.54	RD
MOL005463	Methylcimicifugoside_qt	31.69	0.24	RD
MOL005465	Garcinone B	45.33	0.77	RD
MOL000953	Cholesterol	37.87	0.68	RD
MOL001510	24-epicampesterol	37.58	0.71	RD
MOL001771	Poriferast-5-en-3beta-ol	36.91	0.75	RD
MOL010495	6,7-dimethoxy-2-(2-phenylethyl)chromone	31.93	0.3	LR
MOL010496	6,7-dimethoxy-2-(2-(4-methoxyphenyl)ethyl)chromone	32.38	0.39	LR
MOL010907	Norboldine	40.92	0.46	LR
MOL010913	Linderane	77.09	0.25	LR
MOL010916	Nubigenol	42.55	0.19	LR
MOL010917	Boldine	31.18	0.51	LR
MOL000098	Quercetin	46.43	0.28	LR

SQP, Suoquan pill; AOF, *Alpiniae Oxyphyliae Fructus*; LR, *Linderae Radix*; RD, *Rhizoma Dioscoreae*.

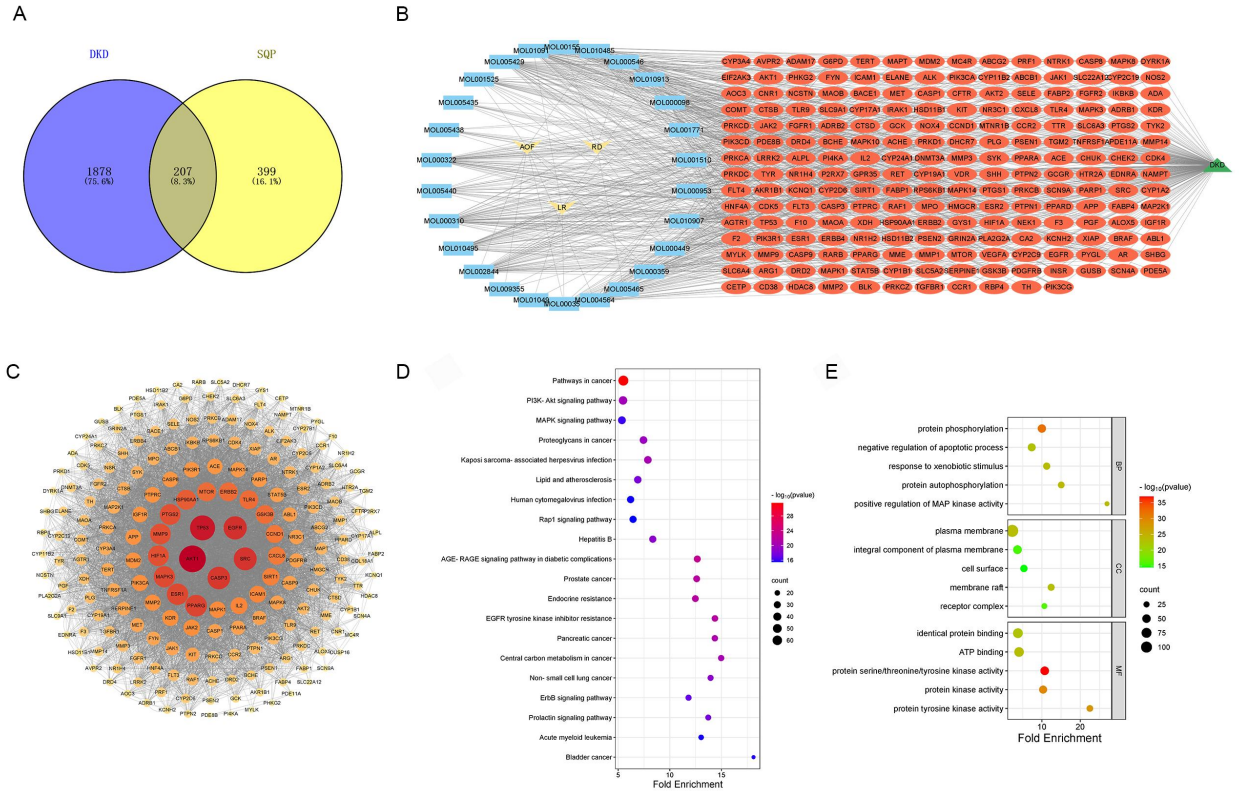

Figure 2 Network pharmacology analysis. Venn diagram of SQP and DKD (A), active ingredient-therapeutic target-DKD network diagram (B), PPI network diagram of the common target of SQP and DKD (C) (Nodes with a larger area and darker colour have a larger degree value), the main pathways in KEGG (D) and GO (E) enrichment analysis. DKD, diabetic kidney disease; SQP, Suoquan pill; AOF, *Alpiniae Oxyphyliae Fructus*; LR, *Linderae Radix*; RD, *Rhizoma Dioscoreae*..

Constructing ingredient-therapeutic target network

Cytoscape 3.9.1 software was used to construct the herbal medicine-ingredient-therapeutic target-disease network and perform network topology analysis. The network was mainly constructed by 26 ingredients and 207 potential therapeutic targets (Figure 2B). In the network, the higher the topological parameter, the more critical the substance is. Data regarding the topological parameters of the active compounds are shown in Supplementary Table S4. Among all ingredients, the top five substances were screened out based on the dgree value, Betweenness Centrality value, and Closeness Centrality value, which were boldine, denudatin B, pinocembrin, kaempferide, quercetin. Therefore, we considered these five substances as core compounds.

Construction of PPI network

The 190 common targets of SQP and DKD were imported into the STRING database to construct a PPI network. The network consisted of 207 nodes and 3,756 edges. Cytoscape version 3.9.1 was used to visualise a protein network relationship graph and perform network topology analysis, and detailed information is shown in Supplementary Table S5 (Figure 2C). Based on the degree values, the top five key targets of SQP in DKD were AKT serine/threonine kinase 1 (AKT1), tumor protein p53 (TP53), EGFR, proto-oncogene, SRC, and caspase 3 (CASP3). Among these key nodes, only EGFR and SRC have good binding abilities to the four core components of SQP simultaneously, and EGFR and SRC can be regulated by each other and are closely related [18]. Therefore, we focused on these two targets for the subsequent experimental validation.

GO function and KEGG pathway analysis

Elucidating the biological functions of SQP in DKD. The identified genes were entered into DAVID for GO and KEGG enrichment analyses, using $P < 0.01$ as an indicator of significant biological function. Detailed information is provided in Supplementary Table S6. KEGG pathway enrichment analysis yielded 153 signalling pathways for SQP treatment of DKD. The top 20 enriched KEGG terms were mapped, mainly involving the AGE-RAGE signalling pathway in diabetic complications, endocrine resistance; EGFR tyrosine kinase inhibitor resistance; PI3K-Akt signalling pathway; ErbB signalling pathway; MAPK signalling pathway; and Rap1 signalling pathway (Figure 2D). GO enrichment analysis showed that 467 biological processes (BP), 65 cellular components, and 109 molecular functions were enriched. We selected the top five ranked GO terms (Figure 2E). In the GO_BP category, the terms were mainly involved in responses to protein phosphorylation, negative regulation of the apoptotic process, and positive regulation of MAP activity. In the GO_cellular components category, the terms mainly included plasma membrane, membrane raft, and receptor complex. In the GO_molecular functions category, the terms mainly included protein serine/threonine/tyrosine kinase activity, identical protein binding, and ATP binding.

Molecular docking

The key targets of SQP in DKD include SRC and EGFR, and their ability to bind to the core components of SQP was predicted using molecular docking (Figure 3). In addition, because EGFR and SRC were not identified as targets of pinocembrin simultaneously during the screening of targets of the ingredient, pinocembrin was not subjected to molecular docking. It was generally accepted that the tightness of the protein-ligand association can be expressed in terms of the binding energy of docking binding. A lower binding energy leads to a more stable binding conformation and more likely interactions. Molecular docking showed that the binding energies of both the key-acting protein and the core component of SQP were below -5 kcal/mol, indicating that the component had good affinity for the target protein. Table 2 showed the magnitude of the binding energy between the receptor and ligand.

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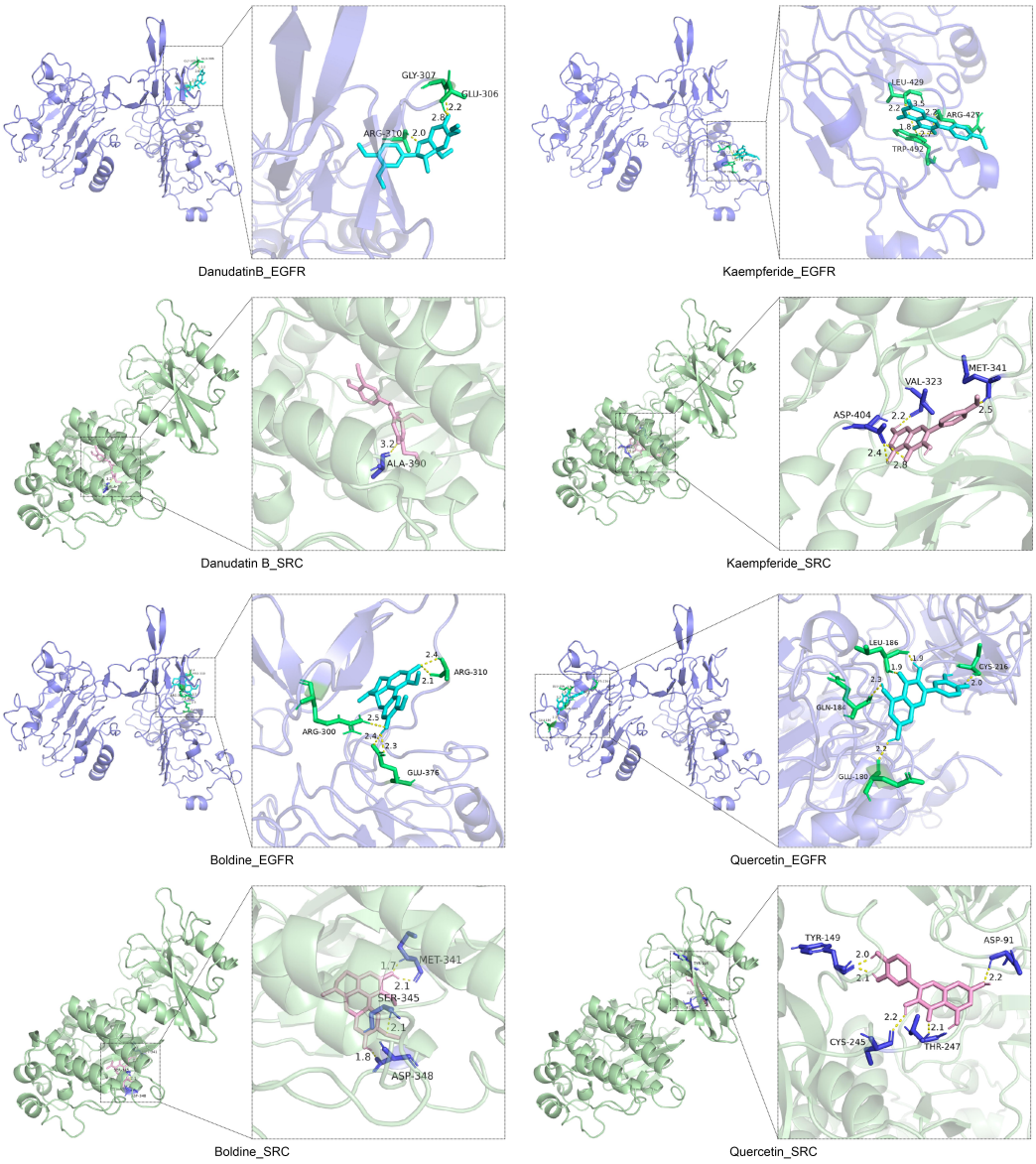

Figure 3 Visualisation of the molecular docking results between potential active ingredients and core targets

Table 2 Binding energy between ligands and core target receptors

PDB ID	Gene symbol	Binding energy (kcal/mol)			
		Denudatin B	Kaempferide	Boldine	Quercetin
5WB7	EGFR	−6.58	−5.5	−6.35	−5.27
2SRC	SRC	−7.39	−7.24	−6.52	−7.31

PDB, Protein Data Bank; EGFR, epidermal growth factor receptor; SRC, proto-oncogene, non-receptor tyrosine kinase.

Cell viability assay

To determine the ameliorative effect of the SQP ethanol extract on the damage of HK-2 cells in a HG environment, HK-2 cells were exposed to different concentrations of the drug, and the CCK8 assay was performed to detect cell viability. The viability of HK-2 cells under the intervention of ethanolic extract of SQP at different concentrations gradually increased, and the highest degree of recovery of cell viability was observed when the concentration of SQP was increased to 50 μg/mL. With increasing drug concentration, the cell viability gradually decreased when the drug concentration was 75, 100, and 200 μg/mL. Therefore, 50 μg/mL of SQP was considered a safe and effective concentration and was further used in our study (Figure 4).

Determination of apoptosis

Western blotting and flow cytometry analyses were performed to determine the inhibition of apoptosis in HK-2 cells under HG conditions using the SQP ethanol extract. The results of flow cytometry showed that the apoptosis rate increased under HG intervention compared with that in the normal group, while the SQP ethanol extract significantly reduced the apoptosis rate (Figure 5A). Western blotting showed that the levels of Bax/Bcl-2, cleaved-caspase-3 protein increased significantly in the model group compared with those in the normal group, while treatment with the ethanol extract of SQP reduced the levels of Bax/Bcl-2, cleaved-caspase-3 protein (Figure 5B).

In vitro validation of the predicted core targets

To validate the potential therapeutic targets of the SQP ethanol extract

in HG-induced injury in HK-2 cells, western blotting was used to detect the relative expression levels of the predicted targets. Compared to the normal group, P-EGFR and P-SRC protein levels were significantly increased in the model group, and SQP ethanol extract treatment decreased the protein expression levels of P-EGFR and P-SRC (Figure 6).

Discussion

SQP can be used to treat patients with DKD with significant clinical efficacy and mild side effects [19]. SQP consists of AOF, RD, and LR, and may be suitable for DKD patients with spleen and kidney deficiency syndromes. In addition, the three herbs in SQP can be used alone or in combination for the treatment of DKD, and various herbal formulations based on SQP have been used in the clinical treatment of DKD [5, 8, 20, 21]. Modern research has shown that AOF has a variety of pharmacological effects, including anti-inflammatory, analgesic, antioxidant, antidiuretic, and anti-diabetic activity [22]. Our previous studies have shown that AOF can reduce blood glucose and urinary microalbumin levels in db/db mice, improve kidney function, and reduce kidney pathological tissue damage [23, 24]. LR exerts anti-inflammatory, analgesic, antioxidant, and antitumor [25]. The LR extract improves serum creatinine levels, creatinine clearance, and renal pathological tissue damage in DKD mice. In addition, LR extract was found to reduce the apoptotic rate of glomerular cells and slow DKD progression [26]. RD has pharmacological effects such as a reduction in anti-inflammatory, antioxidant, hypolipidaemic, hypoglycaemic, and insulin resistance. RD alone or herbal combinations containing RD are also used for the treatment of DM and DKD [27, 28]. However, only a few studies have explored the effects of these herbs on HK-2 cells under HG stress. SQP is used to treat DKD; however, its mechanism of action remains unclear. Therefore, we conducted an in vitro experiment to demonstrate that an ethanolic extract of SQP could improve the damage and viability of HK-2 cells under HG stress. Thus, this study provides a theoretical basis for the treatment of DKD using SQP.

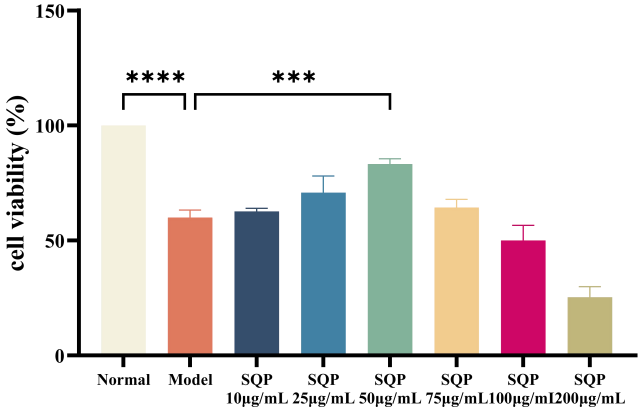

Figure 4 Effect of SQP ethanol extract solution on the viability of HK-2 cells under HG stress. Data are expressed as means ± SD (n = 3 per group) of the representative data from three independent experiments. $^{***}P < 0.001$, $^{****}P < 0.0001$.

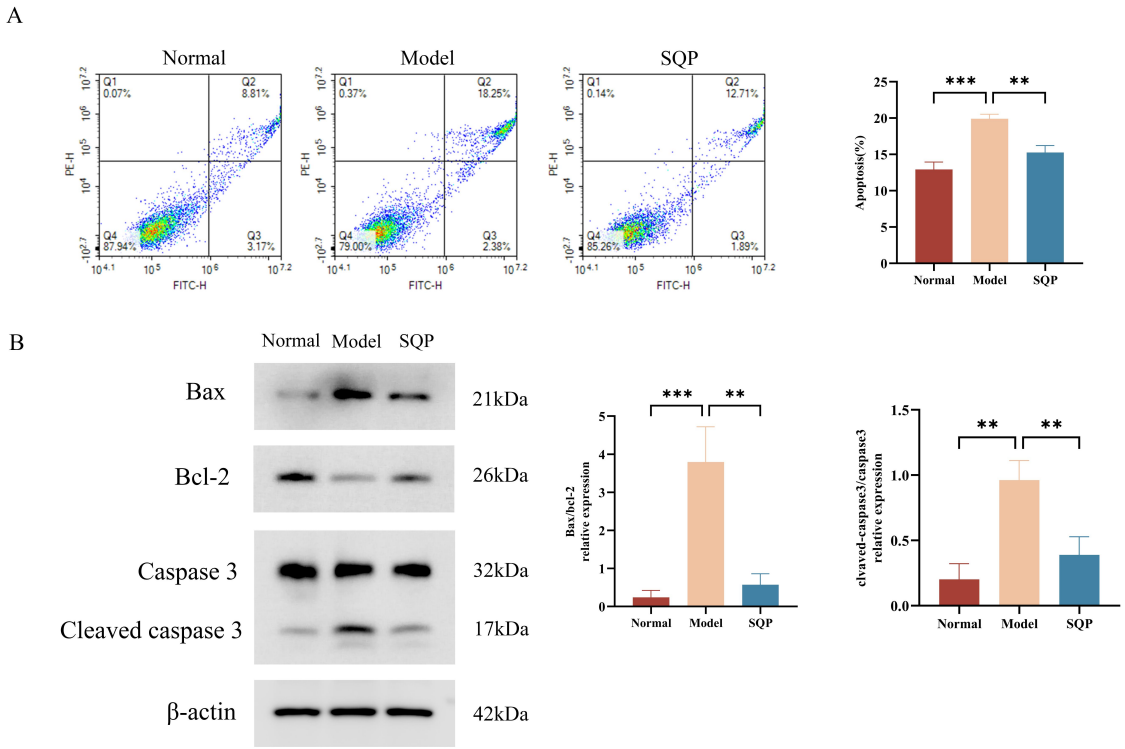

Figure 5 Effect of SQP on apoptosis of HK-2 cells under HG stress detected using flow cytometry (A) and western blot (B). Data are expressed as means ± SD (n = 3). $^{**}P < 0.01$, $^{***}P < 0.001$.

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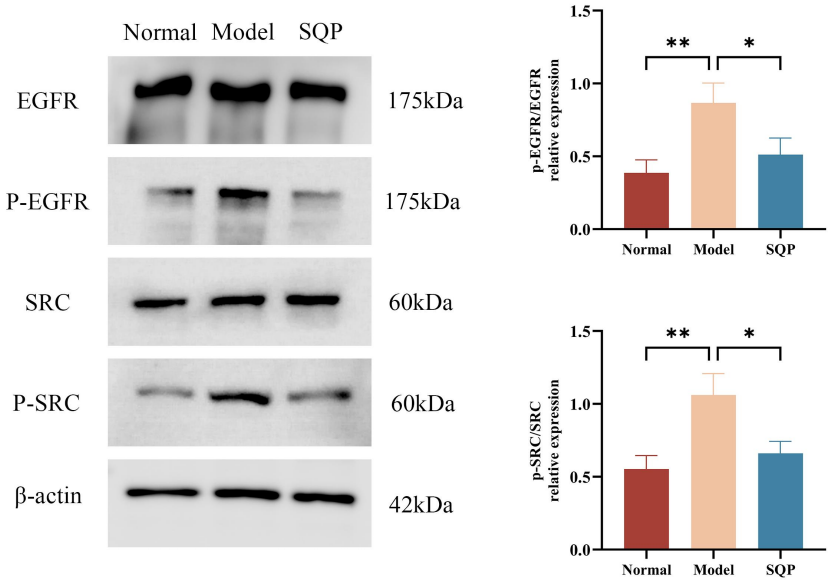

Figure 6 Western blot analyses showed the effect of SQP on the expression levels of EGFR/SRC in HK-2 cells under HG stress conditions. The results are presented as the means ± SD (n = 3). $^{*}P < 0.05$, $^{**}P < 0.01$.

We used network pharmacological analysis combined with molecular docking to reveal the molecular mechanism of action of SQP in DKD treatment. First, we identified five core components of SQP by screening the TCMSP, TCM-ID, and TCMIP databases and combining them using a network analysis approach. Quercetin, the core component of SQP, is a natural flavonoid widely found in fruits, vegetables, and herbs. Quercetin, used in a range of doses, has a high safety profile and possesses a variety of pharmacological activities such as anti-inflammatory, antioxidant, anti-apoptotic, and anti-diabetic [29, 30]. Quercetin improves renal impairment in diabetic mice and attenuates foot cell apoptosis under HG stress by regulating EGFR signalling [31]. In addition, quercetin inhibits ferroptosis in HK-2 cells under HG conditions by modulating the Nrf2/HO-1 signalling pathway, and ameliorates inflammation, oxidative stress and reduces epithelial–mesenchymal transition that occurs in HK-2 cells under the intervention of TGF-β1 [32, 33]. Kaempferide has various pharmacological effects, such as anti-inflammatory, antioxidant, anticancer, and antihypertensive, and has been found to be effective in improving disorders of glucose and lipid metabolism, including lowering blood glucose, blood lipids, serum insulin, and other indicators. Thus, kaempferide may play an important role in the effective treatment of metabolic diseases, such as obesity, diabetes, and nonalcoholic hepatitis [34]. In addition, a recent study found that kaempferide inhibited oxidative stress and induced autophagy to ameliorate cisplatin-induced acute kidney injury in mice and HK-2 cells [35]. These studies suggest that kaempferide is an important natural drug for DKD treatment. Boldine is an aporphine alkaloid with various pharmacological activities, including anti-apoptotic, antioxidative, antidiabetic and cytoprotective [36]. Alterations such as hyperglycaemia, hypertension, and kidney damage in diabetic rats were reduced by boldine treatment, which prevented lipid peroxidation in mesangial cells grown in HG and pro-inflammatory cytokines [37]. Denudatin B is a specific receptor antagonist of platelet-activating factor (PAF) [38]. PAF is a pro-inflammatory phospholipid with multiple pathological and physiological effects and is involved in various processes such as inflammation, apoptosis, and angiogenesis [39]. This study showed that the upregulation of PAF receptors in type 2 diabetes is associated with albuminuria and vascular dysfunction [40]. A dual antagonist of histamine and PAF receptors reduced streptozotocin-induced renal tissue and renal function damage in rats with DKD [41]. Pinocembrin is widely found in various plants and exhibits neuroprotective, anti-inflammatory, hepatoprotective, antihyperlipidaemic, and vasorelaxant properties [42]. One study found that pinocembrin had little effect on body weight and blood glucose but was effective in ameliorating DKD kidney damage [43]. Extensive mechanistic studies on these components could further demonstrate the promising efficacy of SQP in the treatment of DKD and elucidate that its therapeutic effects may be achieved through multiple components, targets, and pharmacological activities.

GO enrichment analysis results suggest that the regulatory role of apoptosis may be one of the BP involved in the treatment of DKD by SQP. Apoptosis, also known as programmed cell death, plays a role in maintaining the stability of the body's internal environment under physiological conditions and is controlled by several genes, such as the Bcl-2 and caspase families [44]. Under the stimulation of various pathological factors, normal cells undergo apoptosis, leading to the development of diseases such as DKD [45, 46]. Flow cytometry analysis showed that SQP treatment significantly reduced the rate of HG-induced apoptosis. The ability of SQP to reduce the Bax/Bcl-2 and cleaved-caspase-3 protein levels was demonstrated using western blotting. These results are consistent with our predictions and suggest that the modulatory effect of SQP on apoptosis plays an important role in DKD treatment.

Through KEGG enrichment analysis and PPI network binding topology analysis, we primarily focused on EGFR and SRC as key nodes in the network and verified the binding ability of these two key node proteins to the four core compounds by molecular docking simulation. Molecular docking showed that denudatin B, kaempferide, boldine, and quercetin had good binding abilities to EGFR and SRC, and GO enrichment analysis showed that the regulation of protein phosphorylation is a biological process that exerts therapeutic effects. The reversible introduction of phosphate groups has a significant effect on proteins; therefore, aberrant phosphorylation is closely associated with the development of many diseases [47]. Therefore, we speculate that SQP intervention in the development of DKD may be achieved through the synergistic regulation of the phosphorylation levels of EGFR and SRC proteins and the anti-apoptotic effects of these four substances.

EGFR, a member of the ErbB family of receptor tyrosine kinases, plays an important role in cell proliferation, differentiation and apoptosis [48]. EGFR is widely expressed in the mammalian kidney. Studies have shown that phosphorylation of EGFR is consistently increased in the kidney of a DKD mouse model and that the use of specific EGFR receptor tyrosine kinase inhibitors significantly reduces structural and functional abnormalities in progressive DKD [49]. Podocyte-specific EGFR deletion attenuated albuminuria and podocyte injury in DKD mice [50]. Persistent activation of EGFR

occurs in HG-treated HK-2 cells [51]. The activation of EGFR as a receptor leads to the phosphorylation of specific tyrosine residues within the cytoplasmic tail. These phosphorylated residues act as docking sites for multiple signalling molecules, and their recruitment leads to the activation of multiple intracellular pathways, including SRC kinase [49]. SRC is a member of the SRC family of membrane-associated non-receptor tyrosine kinases (SFKs), which can be activated by receptor tyrosine kinases, such as EGFR, and various other stimuli altered in the diabetic environment to control cell proliferation, differentiation, and apoptosis [52, 53]. The phosphorylation level of SRC increases in mesangial cells under HG stress and in streptozotocin-induced diabetic rats [54, 55]. In vitro inhibition of SRC activation significantly prevents HG-induced apoptosis in HK-2 cells [56]. In addition, there is a complex relationship between SRC and EGFR, and SRC can act upstream of EGFR; that is, SRC can induce the transactivation of EGFR. HG activation of SRC can mediate EGFR transactivation, leading to the activation of mitogen-activated protein kinase (MAPK) and the synthesis of collagen IV. In type 2 diabetic mice, SRC inhibitor intervention or SRC knockdown inhibits EGFR expression and prevents albuminuria, glomerular matrix protein accumulation, glomerular basement membrane thickening, and podocyte depletion [52]. EGFR and SRC phosphorylation levels are closely associated with the development of DKD. Western blotting showed that treatment with the SQP ethanol extract reduced the phosphorylation levels of EGFR and SRC proteins in HK-2 cells under HG stress.

In this study, we investigated the potential therapeutic mechanisms of the herbal compound SQP in DKD based on network pharmacology analysis. Our study suggests that the components and mechanism of SQP in treating DKD may be the synergistic inhibition of the phosphorylation of EGFR and SRC and the inhibition of apoptosis by the four core compounds, namely, denudatin B, kaempferide, boldine, and quercetin. These four substances may interfere with the development of other diseases by regulating the expression of EGFR and SRC. These results revealed the active compounds and molecular mechanisms of SQP in the treatment of DKD, which may help in the development of new therapeutic strategies and the design of novel anti-DKD drugs. However, our study was only an in vitro experiment, and further in vivo experiments are required to elucidate the efficacy and mechanism of action.

Conclusion

In summary, to decipher the molecular mechanism of action of SQP in the treatment of DKD, a combination of network pharmacology, molecular docking, and in vitro experiments was used. Network pharmacology and molecular docking studies showed that SQP exerted its therapeutic effects on DKD through multiple components, targets, and pathways. Among them, boldine, denudatin B, pinocembrin, kaempferide, and quercetin are considered the key compounds for the therapeutic effect, whereas EGFR and SRC are considered the core targets. The results of in vitro experiments showed that the ethanol extract of SQP increased HK-2 cell viability, regulated the phosphorylation of EGFR and SRC, reduced apoptosis, and regulated apoptosis-related proteins under HG intervention. The present study provides an optimal method for elucidating the pharmacological mechanism of SQP and identifying new drug candidates for the treatment of DKD.

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