Potential molecular mechanism of Yishen capsule in the treatment of diabetic nephropathy based on network pharmacology and molecular docking technology

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Abstract

Background: Using network pharmacology and molecular docking technology to explore the mechanism of Yishen capsules in the treatment of diabetic nephropathy.

Methods: Active components of Yishen Capsules were obtained using database such as TCMSP and TCMID, and diabetic nephropathy targets were obtained from databases such as Gencards, OMIM, DisGeNET. A network of "Yishen Capsule Components-Diabetic Nephropathy Targets-Pathways" was constructed by analyzing data above to screening out core targets for molecular docking verification. Finally, a rat model of diabetic nephropathy was generated, and renal tubular epithelial cells were extracted and cultured under high glucose conditions. Based on these experimental models, the key signal pathway target protein genes screened by network pharmacology were verified both in vitro and in vivo.

Results: The main active components of Yishen Capsule in the treatment of diabetic nephropathy include quercetin, kaempferol, gallic acid, astragaloside IV and so on. Some key targets (such as AR, AKT1, TP53, ESR1, JUN) and important signal pathways (such as AGE-RAGE signal pathway, HIF-1 signal pathway and JAK-STAT signal pathway) were included in the treatment of diabetic nephropathy of Yishen Capsule. Molecular docking assay showed that most of the targets have good binding activity with the components of Yishen Capsules. Based on the results of network pharmacology, key target proteins in HIF-1α and JAK2/STAT3 signaling pathways were selected for experimental verification. Results presented that HIF-1α, JAK2, STAT3, TGF-β and MCP were increased under high glucose environment. With the treatment of Yishen Capsule, the expression of HIF-1α further increased, while the expression of JAK2, STAT3, MCP-1 and TGF-β were decreased.

Conclusions: This study revealed the mechanism of Yishen Capsules in the treatment of diabetic nephropathy, which possesses the characteristics of multi-component, multi-target, and multi-pathway. Further experiments confirmed that Yishen Capsules interfered with HIF-1α and JAK/STAT signaling pathways to reduce inflammation and fibrosis damage in the kidney tissue of rats with diabetic nephropathy.

Key Words: Diabetic Nephropathy(DN); Network pharmacology; Molecular docking;HIF-1α; JAK/STAT

Background

Diabetic Nephropathy (DN) is one of the most common microvascular pathologies of diabetes, and further development of it may progress to the stage of end-stage renal disease, which requires renal replacement therapy and seriously affects the patient's health and quality of life.

The pathogenesis of DN is complex. Reported evidences showed it is related to changes in glomerular hemodynamics, excessive activation of Renin-Angiotensin-Aldosterone System (RAAS), luxuriant generation of reactive oxygen species (ROS), inflammation, mitochondrial dysfunction and so on. However, its exact mechanism is not yet clear [1].

The current clinical treatments for DN mainly include blood sugar control, blood pressure control, correction of metabolic disorders, and improvement of microcirculation, while the clinical treatment has not yet achieved satisfactory results [2]. In recent years, the unique advantages of traditional Chinese medicine for the treatment of DN have been continuously highlighted. The application of special Chinese medicine treatments for DN possesses the advantages of multi-target, multi-level treatment mechanism, fewer adverse reactions and high safety.

The Chinese medicine preparation Yishen Capsule is composed of Astragalus, Angelica, Gordon euryale, Alisma, Schisandra, Rhodiola and other Chinese medicines. Previous studies have indicated that Yishen Capsules can improve kidney function through multiple targets and mechanisms, which effectively delays the progression of DN [3-9].

Network pharmacology is based on bioinformatics and computer technology, integrating a large amount of biological information and data. It systematically and comprehensively reflects the intervention mechanism of drugs on the disease network, which is similar to the mechanism that the complex components of traditional Chinese medicines interfere with diseases through multiple channels and multiple targets. This study intends to explore the targets of Yishen Capsules in the treatment of diabetic nephropathy and to elucidate the therapeutic mechanism of Yishen Capsules on diabetic nephropathy from many aspects. Further, some target proteins of key signaling pathway were verified through experiment both in vitro and in vivo, which may provide evidence for the treatment of DN using Yishen Capsules.

Materials and methods

Network pharmacology analysis

Screening of active chemical components and acquisition of targets

Using the TCMSP database (http://tcmspw.com/) to retrieve all the chemical components of the 6 traditional Chinese medicines (Astragalus, Angelica, Gordon euryale, Alisma, Schisandra and Rhodiola) in Yishen Capsules. According to the two ADME attribute values of oral bioavailability (≥ 30%) and drug-likeness (≥ 0.18) of these ingredients, preliminary screening was performed to obtain the active compounds, as well as their protein targets. According to the TCMID database (http://www.megabionet.org/tcmid/) and published literature reports [10], some

unpredicted active compounds and reported targets have been supplemented. In the Uniprot protein database (https://www.uniprot.org), human-derived targets were screened and standardized.

Target screening of diabetic nephropathy

Searching the GeneCards database (https://www.genecards.org), OMIM database (http://www.omim.org), TTD database (http://bidd.nus.edu.sg/group/cjttd) and DisGeNET database (https://www.disgenet.org) for potential targets of diabetic nephropathy, by using "Diabetic nephropathy" and "Diabetic Kidney Disease" as keywords. And some target sites for disease were also supplemented through searching the DrugBank database (https://www.drugbank.ca). According to experience, the target points with Score ≥30 in the Genecards database and Score ≥0.3 in the DisGeNET database were selected as potential targets for diabetic nephropathy.

Intersection of Chinese Medicine and Disease Target Genes and Construction of PPI Network

The intersection of the drug target of Yishen Capsule and the target of diabetic nephropathy were selected to draw the Venny diagram, using Venny 2.1.0 (https://bioinfogp.cnb.csic.es/tools/venny/index.html). Submiting the intersection target to the STRING11.0 database (https://string-db.org) to construct a protein interaction (PPI) network [11]. The PPI network was visually analyzed by Cytoscape 3.7.2 software, and PPI core network proteins were screened out according to the degree value≥ 2 times the median.

GO and KEGG pathway enrichment analysis

Using Metascape platform (http://metascape.org/gp/index.html) for GO and KEGG pathway enrichment analysis. Setting $P<0.01$ is statistically significant, and the analysis results were visualized.

Construction of Components of Yishen Capsules-Targets of Diabetic Nephropathy Disease-Pathway Network Diagram

Using CytoScape 3.7.2 to construct a network diagram of Yishen Capsule components-diabetic nephropathy disease targets-pathway network. Then, using CytoScape 3.7.2 built-in tools to analyze the network topology parameters of active ingredients and targets, which helps to determine core targets and main active ingredients that exert efficacy. The core targets of Cytoscape network analysis and the core target proteins of PPI network were intersected to obtain the key targets of Yishen Capsule in the treatment of diabetic nephropathy, according to which the Venn diagram was drawn.

Verification by using molecular docking

Downloading the crystal structure of the target protein from the PDB database (https://www.rcsb.org/), and using PyMOL and AutoDock software to remove water, remove the original ligand of the active center, and to hydrogenate the target protein. Downloading the 3D structure file of the core components of traditional Chinese medicine from the PubChem database (https://www.ncbi.nlm.nih.gov/pccompound/). And then loading it to the AutoDock Tools 1.5.6 software to add atomic charges, assign atomic types. All flexible bonds were set as rotatable. Running AutoDock Vina for molecular docking. The conformation with the highest

score is selected as the docking conformation, and the PyMOL program was used to visualize the results.

Experimental verification

Reagents

Yishen Capsule was prepared at Department of Pharmacy of the First Hospital of Shanxi Medical Universit. The full scientific species (Latin binomial nomenclature) names of all ingredients of Yishen Capsule see table1. It was composed of A. membranaceus, A.sinensis, E. ferox, A. orientale, and R. rosea in a ratio of 3:2:3:2:1.Boiling the herbs in water thrice for 1 h. The decoctions were combined and filtered through a 3 M membrane, then concentrated through vacuum evaporation to a density of 1.20-1.24 at 70 ℃. The concentrated decoction was further spray-dried into particles that were subsequently filled into capsules.

Streptozotocin, penicillin, streptomycin, D-anhydrous glucose, horseradish peroxidase labeled Goat anti-rabbit IgG were obtained from Solarbio (Beijing, China). PAS staining solution kit and Masson staining solution kit were bought from Zhuhai Bezo Biotech Co., Ltd. Fetal bovine serum and DMEM medium were purchased from Gibco Thermo Fisher Scientific. 10X Tris-Glycine-SDS electrophoresis buffer, 10X Tris-glycine-SDS transfer buffer, 5X SDS-PAGE protein loading buffer, PVDF membrane, WB transfer filter paper, ultra-sensitive ECL chemiluminescence solution and 4% paraformaldehyde fixative were purchased from Wuhan Boster Co., Ltd. Rabbit anti-HIF-1α antibody, rabbit anti-p-JAK2 antibody, rabbit anti-p-STAT3 antibody, rabbit anti-TGF-β and rabbit anti-MCP-1 antibody were bought from Abcam.

Animal experiment and preparation of plasma containing Yishen components

Forty SPF-grade healthy male SD rats (185-215g, 40) were purchased from the Animal Experimental Center of Shanxi Medical University, with the animal certificate number of SCXK (Jin) 2019-0004. Ten rats were randomly selected to give Yishen Capsules by gavage, with the frequency of once a day for 7 days. Two hours after the last gavage, the rats were anesthetized by intraperitoneal injection of 3% pentobarbital sodium. Blood was collected from the abdominal aorta. Blood was centrifugation and the upper serum was collected and stored after inactivation and sterilization.

The remaining 30 rats were randomly divided into the control group (N group, 10 rats) and the model group (20 rats). After one week of adaptive feeding, the model group was injected intraperitoneally with streptozotocin (50 mg/kg) after left nephrectomy. After 72 hours of injection, the rats with random blood glucose> 16.7 mmol/L and 24-hour urine protein quantification> 30 mg are the successful model rats. The successfully modeled DN rats were randomly divided into diabetic nephropathy group (DN) and Yishen capsule group (YS). The YS group was given Yishen capsule gavage solution at 1.25 g/kg/d by gavage (The previous study of the research has carried out different doses of intervention treatment, and it is confirmed that 1.25 g/kg/d is the effective dose of gavage treatment) [3], and the N group and DN group were given the same amount of normal saline gavage for 8 consecutive weeks. After 8 weeks of treatment, the right kidney specimens of each group of rats were collected for subsequent immunohistochemistry and Western Blotting detection. All animal experiment protocols were reviewed and approved by the Animal Ethics Committee of Shanxi Medical University (batch number: 2019LL242), and the study was conducted in accordance with the guidelines for the care and use of laboratory animals.

Cell culture and treatment

Rat renal tubular epithelial cells (NRK-52E) were purchased from the cell bank of the Chinese Academy of Sciences. And it was cultured in a standard medium (DMEM high glucose medium, containing fetal bovine serum and penicillin- streptomycin antibiotic), and in a 5% CO2 atmosphere at 37 °C. The experimental groups were as follows: normal group (5.5mmol/L glucose), high glucose group (30mmol/L glucose) and Yishen containing-serum treatment group (30 mmol/L glucose + 10% Yishen containing-serum). After each group was cultured for 24 hours, cell proteins were collected for Western Blotting detection.

Pathology examination of kidney

Specimens of right kidney were collected, fixed in 4% paraformaldehyde solution, embedded in paraffin, and sectioned routinely. After section, slides were stained with HE, PAS, and Masson. The pathological changes of the renal tissue were observed under light microscope.

Immunohistochemistry

Kidney tissues were embedded in paraffin, conventionally sectioned, deparaffinized, sealed, and incubated at room temperature. Slides were incubated with rabbit anti-HIF-1α, rabbit anti-p-JAK2, and rabbit anti-p-STAT3 antibody, respectively overnight at 4°C. Then Slides were incubated with biotin Labeled with goat anti-rabbit IgG (secondary antibody) at 37°C for 15 min. After that, Slides were orderly incubated with DAB reagent and hematoxylin (for 2 min). Then, slides were dehydrated and transparent, and mount with neutral gum. The tissue sections were examined under an optical microscope and quantitative analysis was performed using Image-Proplus6.0 medical pathology graphic color analysis software.

Western Blotting

Extracting and collecting total protein from cell and kidney tissue and determining concentration of the total protein. The protein was denatured by loading buffer and then separated by electrophoresis with SDS-PAGE gel (8%). Then, the protein in the gel was transferred to the PVDF membrane using a Bio-Rad electrotransmitter. Subsequently, 5% skimmed milk powder was used to seal the PVDF membrane at room temperature. After blocking, adding the primary antibody and incubating overnight at 4°C. The next day, the washed PVDF membrane was incubated with horseradish peroxidase-labeled secondary antibody at room temperature. After incubating for 1 hour, the strips on the PVDF membrane were developed with ECL chemiluminescence reagent in a dark room, and the strips were scanned and analyzed by Alpha-view software.

Data analysis and statistics

SPSS 22.0 statistical software was used for data analysis. If the measurement data obey a normal distribution, it is expressed as the mean ± SD. One-way analysis of variance was used for comparison between groups. LDS-t test was used for multiple comparisons. For non-normal distribution or uneven variance, a non-parametric test of multiple independent samples is used. Extended t test was used for multiple comparisons. $P<0.05$ indicates that the difference is statistically significant.

Results

Network pharmacology analysis

Screening of active chemical components and acquisition of targets

Through searching the TCMSP and TCMID database and published literature, a total of 53 active ingredients of traditional Chinese medicine were obtained. Among them, there were 20, 7, 1, 7, 8 and 10 ingredients belonging to Astragalus, Angelica, Gordon euryale, Alisma, Schisandra, and Rhodiola respectively (see Table 2). There are 3 repeated active ingredients in each traditional Chinese medicine. 571, 106, 21, 9, 27, and 562 targets of effective active compounds of Astragalus, Angelica, Gordon euryale, Alisma, Schisandra, and Rhodiola were obtained respectively. After merging and deleting duplicate values, a total of 516 targets were obtained.

Acquisition of target genes in diabetic nephropathy

Diabetic nephropathy disease targets were collected through GeneCards, OMIM, TTD, DisGeNET and DrugBank databases. After merging and deleting duplicate values, a total of 1324 disease targets were obtained.

PPI network

Taking the intersection of the diabetic nephropathy targets and the drug action targets of the Yishen capsule, and obtaining a total of 150 intersection targets. And a Venn diagram was drawn based on the above data (see Figure 1A). The intersection targets were submitted to the STRING11.0 platform to obtain the PPI network of the components of the Yishen capsule-diabetic nephropathy disease targets (see Figure 1B). The PPI network was visually analyzed by Cytoscape software (see Figure 1C). Setting the degree value ≥ 2 times the median, and 26 core network proteins were obtained using this as a threshold value to screen out. The core network proteins screened out include STAT3, AGT, TP53, SRC, CAV1, EGFR, CTNNB1, ESR1,

STAT1, PTEN, JAK2, AKT1, MAPK1, JUN, TNF, AR, CCND1, FGF2, TLR4, CDH1, MYC, VEGFA, MAPK8, EGF, FOS, HSPB1, and the above target proteins are the core nodes in the PPI network.

GO and KEGG pathway enrichment analysis

GO and KEGG enrichment analysis were performed on the targets of Yishen Capsules in the treatment of diabetic nephropathy. A total of 255 significantly related biological processes were screened, including MAPK cascade regulation, DNA binding transcription factor activity regulation, lipopolysaccharide response, reactive oxygen metabolism and apoptosis signaling pathway (see Figure 2A). A total of 74 significant related cell components were screened, mainly including various cell structures such as membrane rafts, nucleoli, myelin sheaths, endoplasmic reticulum lumen, and extracellular matrix (see Figure 2B). 96 significant related molecular functions were obtained, mainly related to protein tyrosine kinase activity, receptor regulatory activity, kinase binding, protein homodimerization activity, transcription factor binding and integrin binding (see Figure 2C).

KEGG enrichment screening resulted in 159 main signal pathways, mainly including AGE-RAGE signal pathway, PI3K-Akt signal pathway, HIF-1 signal pathway, JAK-STAT signal pathway, insulin signal pathway and other signal pathways. Pathways ranking the top 20 was visualized (see Figure 2D).

Construction of Components of Yishen Capsules-Targets of Diabetic Nephropathy Disease-Pathway Network Diagram

CytoScape 3.7.2 was used to construct a network diagram of Yishen Capsule components-diabetic nephropathy disease target-pathway network. The network has a total of 586 nodes and 1748 component-target protein-pathway relationships (see Figure 3A). Analyzing network topology parameters through CytoScape's built-in Network Analyzer to obtain core components and core targets. According to analysis and prediction, Quercetin was the main component of Yishen Capsule in the treatment of diabetic nephropathy. Kaempferol (Kaempferol), gallic acid (Epigallocatechin gallate), and astragaloside IV (Astragaloside IV) were minor components (see Table 3-1). It was predicted that PTGS2 was the main target of Yishen Capsule in the treatment of diabetic nephropathy, and PTGS1, AR, AKT1, NOS2, and PPARG were the secondary targets (see Table 3-2). Taking the intersection of the above targets and the core targets of the PPI network and drawing the Venn diagram (see Figure 3B). Ten intersection targets were obtained, namely AR, AKT1, TP53, ESR1, JUN, TNF, MAPK1, EGFR, CCND1, VEGFA.

Verification with molecular docking

Selecting the key genes AR, AKT1, TP53 obtained in the previous steps, and the core targets HIF-1α, JAK2, and STAT3 in the key signal pathway, and carrying out molecular docking with the main active ingredients of the Yishen capsule. The docking results were shown in Figure 4A. Among the 60 docking results, 57 suggested a better docking activity (binding energy less than -1.2kcal/mol). Among them, β-sitosterol binded best to the core target protein, and TP53 was the target protein with the best binding activity to the key chemical components in the Yishen capsule.

The docking results with better binding energy was shown in Fig. 4. Formononetin formed a hydrogen bond with the AKT1 receptor target protein through the amino acid residue ASN-231

(see Fig. 4B), and formed two hydrogen bonds with the STAT3 receptor target through the amino acid residues GLU-444 and THR-443 (see Figure 4C), formed two hydrogen bonds with the TP53 receptor target through the amino acid residues GLU-221 and THR-224 (see Figure 4D). β-sitosterol formed a hydrogen bond with the AR receptor target protein through the amino acid residue PRO-913 (see Figure 4E), and binded to the TP53 receptor target protein through the amino acid residue GLN-100 (see Figure 4F)). Verbascum isoflavones binded to the HIF-1α receptor target protein through amino acid residues ILE-566 and GLN-96 to form two hydrogen bonds (see Figure 4G), and formed three hydrogen bond through amino acid residues ASN-981, GLY-856, and LEU-855 with the JAK2 receptor target protein (see Figure 4H). The above-mentioned ligand compounds could be well embedded in the active pocket of the receptor target protein.

In vitro and in vivo experimental verification

Pathological changes of rat kidney tissues

The results of HE staining of kidney tissue specimens (see Figure 5A) showed that the glomerulus in group N was normal, and the structure of mesangium and basement membrane was normal. In the DN group, the glomerular capillary loops were hypertrophy, the basement membrane was thickened, the mesangial cells and mesangial matrix were proliferated, and the renal tubular epithelial cells were hypertrophy. However, there was no glomerular sclerosis or interstitial fibrosis. Compared with the DN group, the YS group had increased glomerular volume, mesangial hyperplasia, basement membrane thickening, and renal tubular epithelial cell hypertrophy to varying degrees. The results of PAS staining (see Fig. 5B) showed that the glomeruli and tubules of rats in group N were normal in structure and morphology. PAS-positive

protein deposits were seen in the glomerular mesangial area and renal tubular epithelial cells of rats in the DN group. Compared with the DN group, the YS group had less PAS-positive protein deposition. The results of Masson staining (see Fig. 5C) showed no abnormal changes in the morphology and structure of the kidney tissue in the normal group, and blue staining of collagen tissue was rarely seen in Masson staining. The glomerulus volume of rats in the DN group increased, the renal tubular epithelial cells were obviously edema, and the lumen became narrow. Masson staining showed blue-stained collagen tissue. Compared with the DN group, the YS group had increased glomerular volume, reduced renal tubular epithelial cell edema, and decreased blue-stained collagen tissue.

Immunohistochemical detection of key proteins in the signal pathway in rat kidney tissue

After 8 weeks of rearing, the kidney tissues of rats in each group were collected for immunohistochemical examination. The results showed that HIF-1α was expressed in the nucleus and cytoplasm of renal tubular cells. There was a certain amount of HIF-1α expression in the kidney tissue of rats in the N group. The expression of HIF-1α in the DN group was higher than that in the N group. Compared with the DN group, the expression of HIF-1α in the YS group was further increased ($P<0.05$, see Figure 6A). JAK-2 and STAT3 were expressed in the nucleus and cytoplasm of renal tubules. There was a small amount of JAK-2 and STAT3 expression in the kidney tissue of rats in the N group. The expression of JAK-2 and STAT3 in the DN group was higher than that in the N group, while the expression in the YS group was lower than that in the DN group ($P<0.05$, see Fig. 6B-C). The above results indicated that the expressions of HIF-1α, JAK-2 and STAT3 in diabetic nephropathy were elevated. Yishen

Capsule could interfere with HIF-1α and JAK/STAT signaling pathways and improved the renal tubular interstitial damage in rats with diabetic nephropathy.

Effects of high sugar and Yishen capsule intervention on renal signaling pathway, inflammation and fibrosis index expression

There was a trace amount of HIF-1α expression in kidney tissue and renal tubular epithelial cells of rats in the normal group. After 24 hours of high glucose stimulation, results showed that the expression of HIF-1α, STAT3, TGF-β and MCP-1 protein in renal tubular epithelial cells all increased ($P<0.05$), and the expression of JAK2, STAT3, TGF-β and MCP-1 protein in renal tissues all increased ($P<0.05$). Compared with the normal group, the expression of HIF-1α in kidney tissue increased after the administration of Yishen capsule ($P<0.05$). Compared with the high glucose group, after the intervention of Yishen Capsule, the expression of HIF-1α in renal tubular epithelial cells was increased ($P<0.05$), and the expression of JAK2, STAT3, MCP-1 and TGF-β in renal tissue and renal tubular epithelial cells were all decreased ($P<0.05$). The above results were shown in Fig. 7A-D.

Discussion

DN has become the leading cause of end-stage renal disease worldwide, but there is still a lack of effective intervention methods. Traditional Chinese medicine has certain advantages in treating DN. Yishen Capsule is composed of Astragalus, Angelica, Gordon euryale, Alisma, Schisandra, Rhodiola and other Chinese medicines. It possesses the effect of improving the pathological changes of DN kidney tissue and delaying the progression of the disease, while its specific mechanism of action is still unknown.

This study applied network pharmacology methods to discover the key active ingredients of Yishen Capsules in the treatment of DN. Our results showed that Quercetin, Kaempferol, Epigallocatechin gallate, Astragaloside IV, Luteolin and Calycosin were important components in the treatment of DN, which may act on multiple pharmacology targets of DN treatment. Lei et al. found that quercetin inhibits the proliferation of mouse glomerular mesangial cells induced by high glucose and DN rat kidney glomerular mesangial cells by activating the Hippo pathway [12]. The study of Alshehri confirmed that kaempferol exerts an antioxidant effect by activating the Nrf-2/Ho-1 axis to reduce DN damage [13]. Clinical and animal experiments have confirmed that gallic acid targets the activation of Notch signal by inhibiting the TGFβ/Smad3 pathway in diabetic mice, leading to the improvement of renal fibrosis [14]. Astragaloside IV is a saponin extracted from Astragalus. Astragaloside IV reduces endoplasmic reticulum stress, inhibits mitochondrial division and increases autophagy activity through mechanisms of anti-fibrosis, anti-oxidation, and anti-apoptosis, which is benefit to the improvement of DN symptoms [15]. Recent studies have found that astragaloside IV may inhibit oxidative stress and reduce DN podocyte apoptosis by activating the PPARγ-Klotho-FoxO1 signaling pathway [16]. Luteolin, a natural flavonoid compound, may inhibit the activation of STAT3, inhibit inflammation and oxidative stress, and improve glomerular sclerosis and renal interstitial fibrosis in DN mice [17]. In addition, mullein, β-sitosterol, formononetin may also act on DN disease targets and signal pathways through a variety of biological processes.

Based on network pharmacology, the potential target genes and signal pathways of Yishen capsules in the treatment of DN were predicted. Our results showed that AR, AKT1, TP53, ESR1, JUN, TNF, MAPK1, EGFR, CCND1 and VEGFA were the potential key targets for the treatment of DN. And AGE-RAGE, PI3K-Akt, HIF-1, JAK-STAT and insulin signaling pathway

may be the key signaling pathways for the treatment of DN. Some of the above results have been confirmed in the preliminary research of our group. Yishen capsule gavage treatment can reduce urinary protein excretion in DN rats, improve renal function, reduce foot process fusion, improve podocyte Podocin expression, and reduce DN podocyte damage [4]. Treated with Yishen capsule-containing serum, the expression of p-JAK2 and p-STAT3 in high glucose cultured podocytes were decreased [5]. These results initially suggest that Yishen Capsule can alleviate the pathological damage of the kidney of DN and improve the podocyte damage induced by high glucose. Cytokine suppressor (SOCS) is a classic JAK/STAT signaling pathway inhibitor [18]. Yishen Capsule may reduce the degree of glomerular sclerosis and tubular interstitial fibrosis in DN rats by up-regulating the expression of SOCS-3 [6,7]. In addition, Yishen Capsules can also inhibit the overexpression of TLR4, NF-κB[8] and other inflammatory factors and Vascular Endothelial Growth Factor (VEGF)[9] in the kidney tissue of DN rats. The reduction of inflammatory response can inhibit the damage of vascular epithelial cells caused by proliferative inflammatory response, which leads to the delay of DN progression. Recent research team found that Yishen Capsule has the function of improving diabetic nephropathy by promoting autophagy and inhibiting inflammation [3].

Based on the preliminary research foundation of our group and the results of this study, HIF-1α and JAK2/STAT3 signaling pathway were selected for further experimental verification. JAK/STAT signaling pathway is involved in the signal transduction of IL-10, IL-6, tumor necrosis factor-α and other cytokines and activates the expression of genes related to inflammation and cell proliferation [19], which promotes the progression of DN. This study suggests that Yishen Capsules may inhibit the downstream inflammation cascade by inhibiting the JAK/STAT signaling pathway, and improve DN kidney injury.

HIF-1α is a regulatory protein that monitors the body's sensitivity to oxygen [20]. Hypoxia exits in the subclinical stage of DN, and hypoxic environment may induce the expression of HIF-1α [21]. In addition to hypoxia, factors such as inflammation and stress can also up-regulate the expression of HIF-1α [22,23]. Studies have suggested that enhanced HIF-1α activity is one of the reasons for the dysfunction of podocytes, glomerular mesangial cells, and tubular epithelial cells and for the activation of pro-inflammatory pathways [24,25]. Thus, HIF-1α may be related to glomerulosclerosis and renal interstitial fibrosis. Diabetes hyperglycemia environment and advanced glycation end products directly affect the transcription of HIF-1α and lead to HIF-1α activation [26]. Inhibiting the HIF-1α/VEGF signaling pathway in DN renal tubular epithelial cells can reduce the expression of extracellular matrix (ECM) markers [26,27]. However, studies have also found that the diabetic high glucose environment activates the atypical proteasome-dependent pathway of HIF-1α degradation in human renal tubular epithelial cells (HK-2), and reduces the basic expression of HIF-1α [28]. HIF-1α can promote autophagy, which reduces kidney damage caused by oxidative stress, ischemia and diabetes [29,30]. Conditional knockout of HIF-1α aggravates DN renal tubular damage, promotes mitochondrial rupture in HK-2 cells cultured under hypoxia, promotes ROS generation and promotes mitochondrial membrane potential loss and apoptosis. However, overexpression of HIF-1α or HO- 1 agonist treatment can reverse the above changes [31]. The hypoglycemic drug SGLT2 inhibitor empagliflozin can reduce the proximal tubular epithelial cell damage induced by high glucose, through up-regulation of HIF-1α [32]. The controversial results may be due to the intensity of the HIF response in diabetic nephropathy that regulates the progression of kidney injury in a different subtype and time-dependent manner. The results of this study showed that the expression of HIF-1α in renal tubular epithelial cells increased after the intervention of high glucose. And the

expression of HIF-1α further increased after the intervention of Yishen Capsules, suggesting that Yishen Capsules may improve diabetes caused by inducing the expression of HIF-1α Kidney damage.

Molecular docking is mainly used to study the interaction between molecules. The more stable the binding conformation of the ligand and the receptor, the stronger the binding and the lower the energy [33]. The screened active ingredients and key targets were verified by molecular docking technology.β-sitosterol binds best to the core target protein, suggesting that it plays the greatest role in the treatment of diabetic nephropathy by Yishen capsules. Among the core targets obtained by network analysis, the top-ranked targets are mainly related to cell proliferation, apoptosis, inflammation, immunity and differentiation. According to the optimal composite structure of key targets and active ingredients, it is found that formononetin has better binding ability with AKT1, STAT3 and TP53. In addition, β-sitosterol has good binding ability with AR and TP53, and mullein has good binding ability with HIF-1α and JAK2. The above data suggest that the key chemical components of Yishen Capsule have good binding activity with key targets of diseases.

Conclusion

This study used network pharmacology and molecular docking technology to initially analyze the mechanism of action of Yishen capsules in the treatment of DN, and selected key pathways for experimental verification both in vitro and in vivo. Results showed that the identical compound of Yishen Capsule may regulate different targets, and the identical target may interfere with different biological processes and signal pathways. It reflects the combined effect characteristics of multi-pathway and multi-target of Yishen Capsule. This study provides a

scientific basis for the clinical application of Yishen Capsules in the treatment of diabetic nephropathy, and also provides a new direction for exploring the potential mechanism of Yishen Capsules.

Abbreviations

hypoxia-Inducible factor 1-alpha, HIF-1α; janus kinase 2, JAK2; signal transducer and activator of transcription, STAT3; monocyte chemo-attractant protein-1, MCP-1; transforming growth factor-β, TGF-β.

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Authors' contributions

YLH and SL conducted bioinformatics data analysis. YLH, ZYZ, and SFL conducted in vivo experiments. YXL and DLS conducted in vitro experiments. YLH, ZYZ, and YXL conducted molecular biological analysis. YLH and SL prepared the manuscript. WYL, GZ and JAF conceptualized this research and finalized this manuscript.

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Availability of data and materials

The datasets used or analyzed during the study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

The animal experiment schemes involved in this study were approved by the Animal Ethics Committee of Shanxi Medical University (2019LL242), in accordance with the Guideline for the Care and Use of Laboratory Animals.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Table

Chinese name	Full scientific species name	Identifier No.	Medicinal part	Ratio
Huang-Qi	Astragalus membranaceus (Fisch.) Bunge	ILDIS 32156	Root	3
Dang-Gui	Angelica omeiensisC.Q.Yuan&R.H.Shan	IPNI 905661-1	Root	2
Qian-Shi	Euryale indica Planch.	IPNI 605351-1	Kernel	2
Ze-Xie	Alisma orientale (Sam.) Juz.	IPNI 58233-1	Tuber	2
Hong-Jing-Tian	Rhodiola crenulata (Hook.f.&Thomson) H.Ohba	IPNI 274793-1	Root and rhizome	1
Dai-Huang	Rheum hotaoense C.Y.Cheng & T.C.Kao	IPNI 696743-1	Root	2

Table 1: Ingredients of Yishen Decoction. This table shows the full scientific species (Latin binomial nomenclature) names of all ingredients of Yishen Decoction.

Drugs	MOL ID	Compounds	OB	DL
Astragalus	MOL000033	(3S,8S,9S,10R,13R,14S,17R)-10,13-dimethyl-17-[(2R,5S)-5-propan-2-yloctan-2-yl]-2,3,4,7,8,9,11,12,14,15,16,17-dodecahydro-1H-cyclopenta[a]phenanthren-3-ol	36.23	0.78
	MOL000098	quercetin	46.43	0.28
	MOL000211	Mairin	55.38	0.78
	MOL000239	Jaranol	50.83	0.29
	MOL000296	hederagenin	36.91	0.75
	MOL000354	isorhamnetin	49.6	0.31
	MOL000359	β-sitosterol	36.91	0.75
	MOL000371	3,9-di-O-methylnissolin	53.74	0.48
	MOL000378	7-O-methylisomucronulatol	74.69	0.3
	MOL000379	9,10-dimethoxypterocarpan-3-O-β-D-glucoside	36.74	0.92
	MOL000380	(6aR,11aR)-9,10-dimethoxy-6a,11a-dihydro-6H-benzofurano[3,2-c]chromen-3-ol	64.26	0.42
	MOL000387	Bifendate	31.1	0.67
	MOL000392	formononetin	69.67	0.21
	MOL000409	AstragalosideIV	17.74	0.15
	MOL000417	Calycosin	47.75	0.24
	MOL000422	kaempferol	41.88	0.24
	MOL000433	FA	68.96	0.71
	MOL000439	isomucronulatol-7,2'-di-O-glucosiole	49.28	0.62
	MOL000442	1,7-Dihydroxy-3,9-dimethoxy pterocarpene	39.05	0.48
	MOL002565	Medicarpin	49.22	0.34
Angelica	MOL000358	beta-sitosterol	36.91	0.75
	MOL000449	Stigmasterol	43.83	0.76
	MOL001771	angelicin/poriferast-5-en-3beta-ol	36.91	0.75
	MOL001942	isoimperatorin	45.46	0.23
	MOL001956	Cnidilin	32.69	0.28
	MOL003957	1-methyl-2-pentadecyl-4-quinolone	44.52	0.46
	MOL009722	folinicacid/L-Folinic acid	31.79	0.74
gordon euryale	MOL002773	beta-carotene	37.18	0.58
Alisma	MOL000359	sitosterol	36.91	0.75
	MOL000831	Alisol B monoacetate	35.58	0.81
	MOL000849	16β-methoxyalisol B monoacetate	32.43	0.77
	MOL000853	alisol B	36.76	0.82
	MOL000856	alisol C monoacetate	33.06	0.83
	MOL000862	[(1S,3R)-1-[(2R)-3,3-dimethyloxiran-2-yl]-3-[(5R,8S,9S,10S,11S,14R)-11-hydroxy-4,4,8,10,14-pentamethyl-3-oxo-1,2,5,6,7,9,11,12,15,16-decahydrocyclopenta[a]phenanthren-17-yl]butyl] acetate	35.58	0.81
	MOL002464	1-Monolinolein	37.18	0.3
Schisandra	MOL004624	Longikaurin A	47.72	0.53

	MOL005317	Deoxyharringtonine	39.27	0.81
	MOL008956	Angeloylgomisin O	31.97	0.85
	MOL008957	Schizandrer B	30.71	0.83
	MOL008968	Gomisin-A	30.69	0.78
	MOL008978	Gomisin R	34.84	0.86
	MOL008992	Wuweizisu C	46.27	0.84
		gomisin n		
Rhodiola	MOL000006	Luteolin	36.16	0.25
	MOL000098	Quercetin	46.43	0.28
	MOL000422	Kaempferol	41.88	0.24
	MOL001002	Ellagic acid	43.06	0.43
	MOL002309	indirubin	48.59	0.26
	MOL002514	Sexangularetin	62.86	0.3
	MOL002823	Herbacetin	36.07	0.27
	MOL004020	Gossypetin	35	0.31
	MOL005190	Eriodictyol	71.79	0.24
	MOL006821	Epigallocatechin gallate	55.09	0.77

Table 2: Main ingredients of Yishen Capsules. OB：oral bioavailability；DL：drug-likeness

MOL ID	Chemical Name	Degree	Betweenness Centrality	Closeness Centrality
MOL000098	Quercetin	144	0.20224427	0.40873855
MOL000422	Kaempferol	139	0.26004743	0.41399001
MOL006821	Epigallocatechin gallate	136	0.23064706	0.40531097
MOL000409	AstragalosideIV	117	0.29637036	03734707
MOL000006	Luteolin	55	0.04071746	0.3627267
MOL000417	Calycosin	49	0.09173082	0.35780382
MOL000378	7-O-methylisomucronulatol	40	0.03573611	0.35430666
MOL000358	beta-sitosterol	35	0.02520255	0.31504617
MOL000392	formononetin	34	0.03465001	0.35130224
MOL000354	isorhamnetin	32	0.01995551	0.35130224

Table 3-1 Network node characteristic parameters of the main active ingredients of Yishen Capsules.

Target	Degree	Betweenness Centrality	Closeness Centrality
PTGS2	33	0.05520299	0.42028986
PTGS1	21	0.01998458	0.37516171
AR	20	0.02058265	0.37419355
AKT1	17	0.04015246	0.42274052
NOS2	16	0.01531578	0.37760417
PPARG	16	0.01758892	0.39835165
IL6	16	0.00356237	0.34077556
BAX	16	0.01003944	0.37227214
RELA	16	0.00720066	0.37371134
PRSS1	16	0.01121045	0.35846724
NCOA2	16	0.01523082	0.36069652
TP53	15	0.03585158	0.41786744
CASP3	15	0.01468196	0.37809648
CDKN1A	15	0.0118844	0.38359788
BCL2	15	0.00936918	0.37323037
ESR1	14	0.01481544	0.37809648
NOS3	14	0.00924872	0.35452323
JUN	14	0.0098228	0.38057743
TNF	14	0.00615412	0.36942675
MAPK1	14	0.00327309	0.33957845
EGFR	14	0.00274312	0.33918129
CCND1	13	0.01120024	0.33878505
VEGFA	13	0.0030311	0.34198113
GABRA1	13	0.00689756	0.34238489
ADRB2	13	0.00777459	0.33838973
DPP4	13	0.00848346	0.35409035

Table 3-2 The characteristic parameters of the target network node of the main active ingredients of Yishen Capsules.

Figure legends

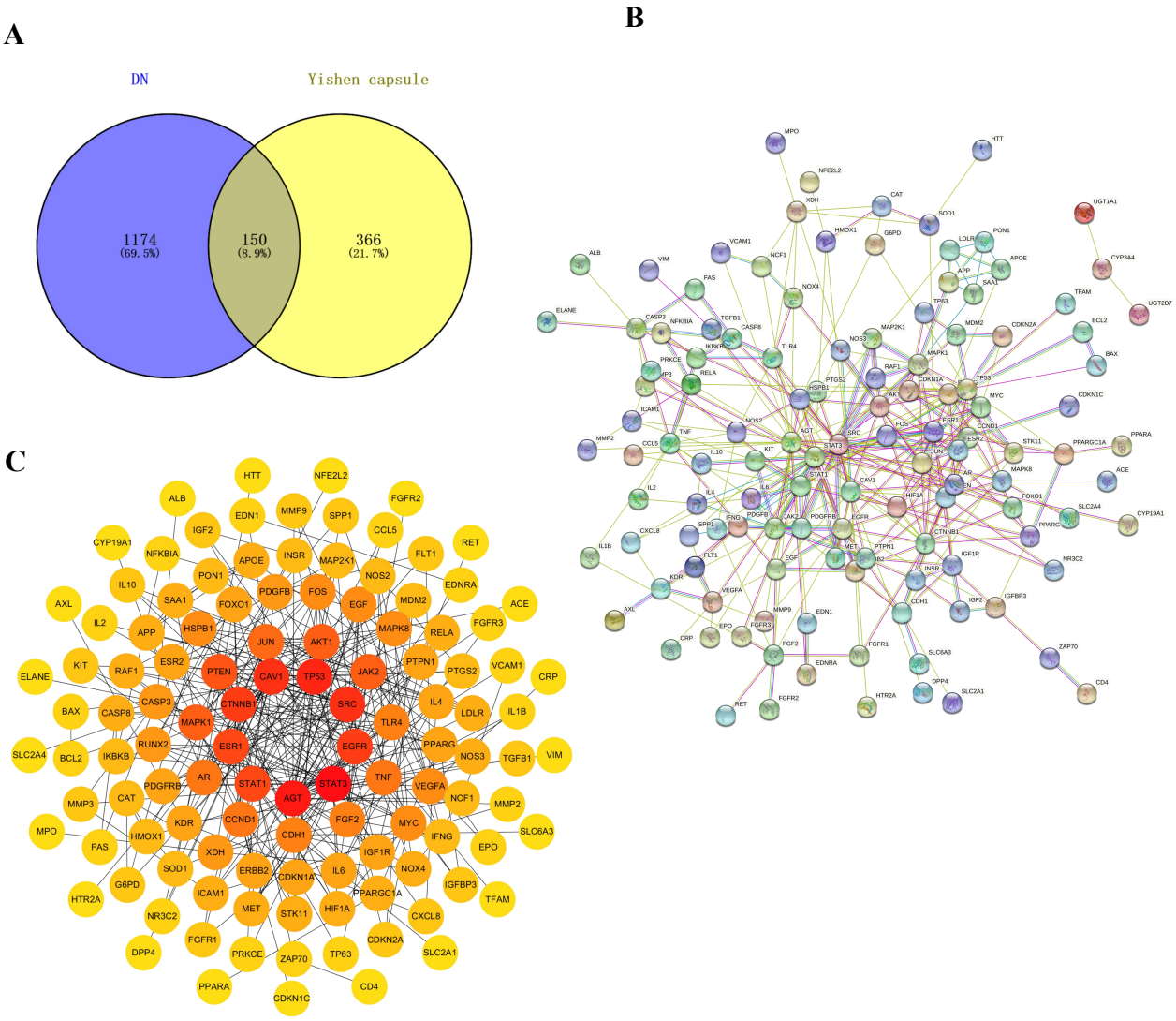


Figure 1: Common targets of Yishen capsule ingredients and diabetic nephropathy. (A) Venny diagram. Venny diagram shows the intersection of Yishen Capsule target genes and DN disease target genes, and the overlapping part is the number of intersection target genes. (B) PPI network of the common targets of Yishen Capsule and diabetic nephropathy. (C) PPI protein interaction network diagram. The nodes in the picture represent the common target proteins, and the color depth is positively correlated with the degree value of the node.

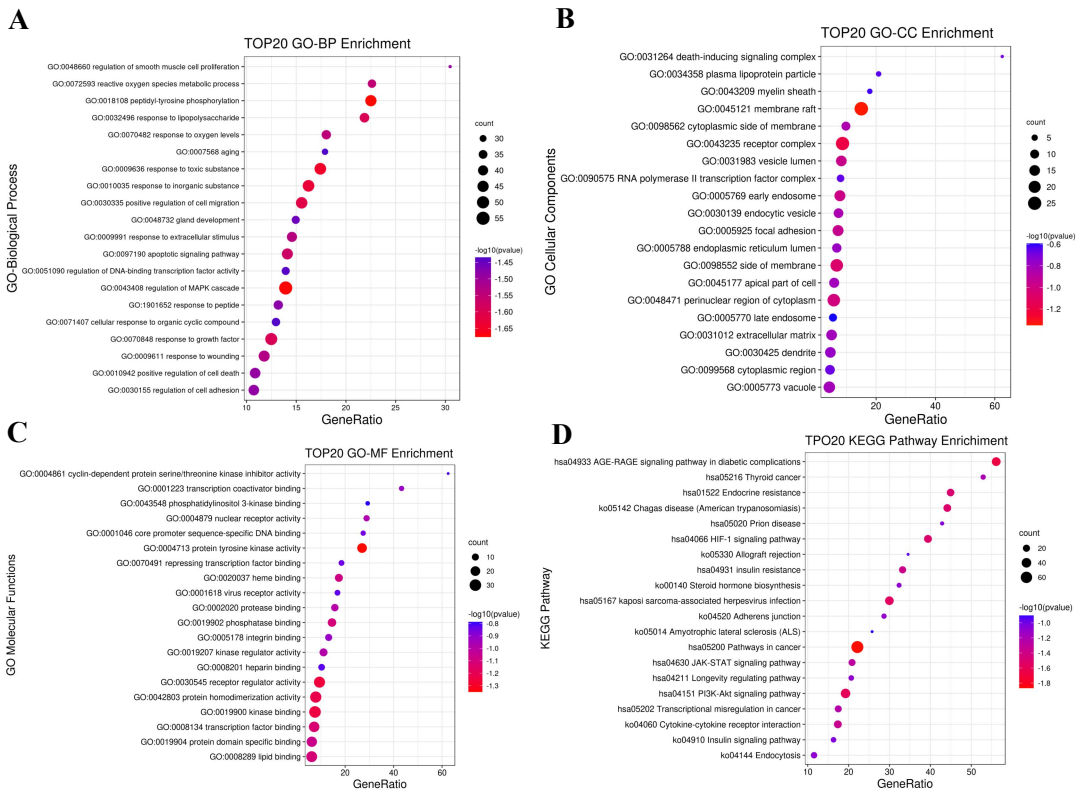
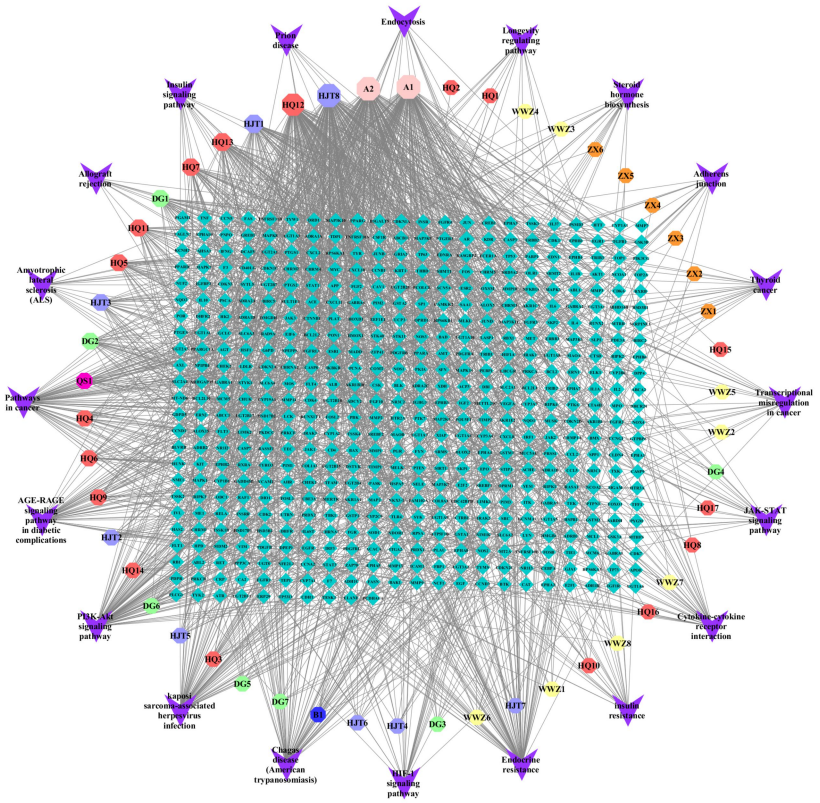


Figure 2: GO and KEGG pathway enrichment analysis. (A-C) The BP, CC and MF of GO enrichment analysis, respectively. (D) The KEGG signal pathway analysis. The size of the dot indicates the number of enriched genes. The color from blue to red indicates significance. The smaller the p.value, the more significant the difference.

A



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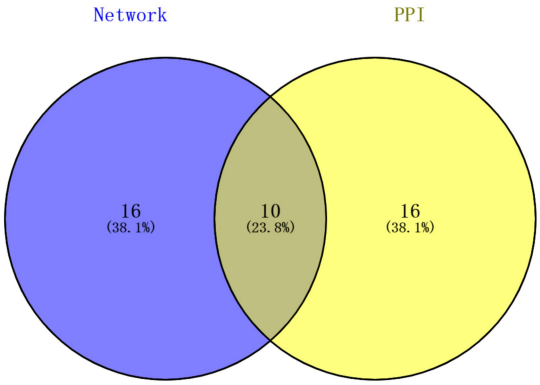
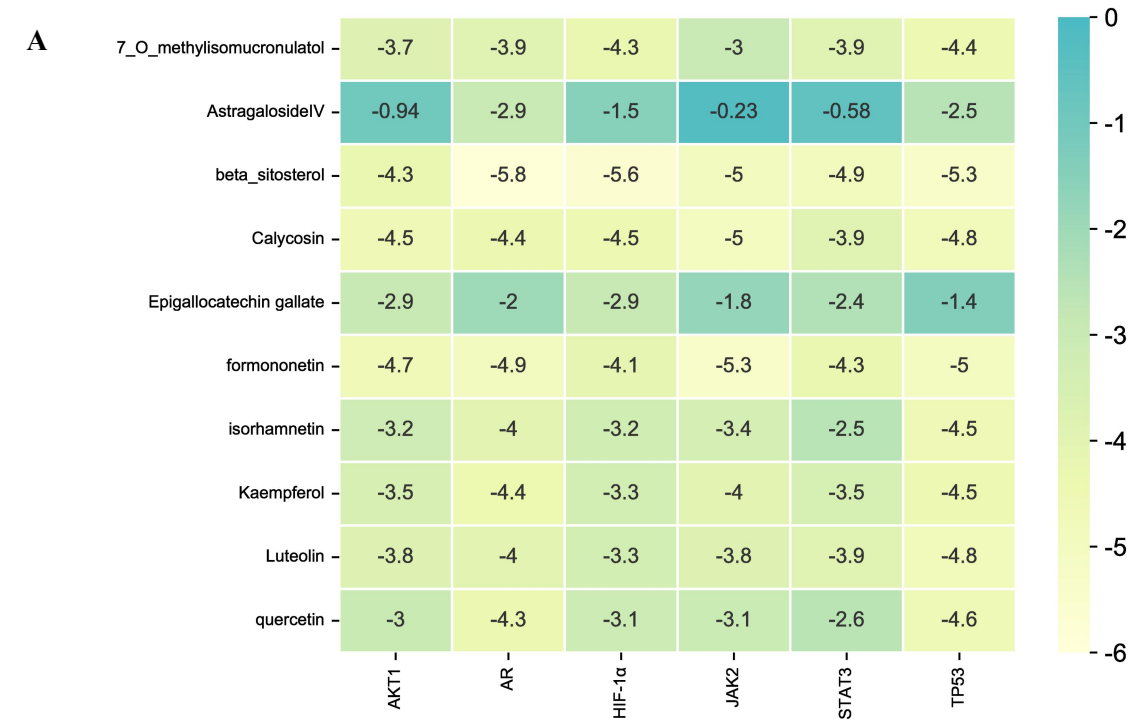


Figure 3: Network diagram and Venn diagram. (A) the inverted triangle node represents the name of the pathway, the octagonal node represents the active ingredient of traditional Chinese medicine, and the diamond node represents the gene. The larger the node area of the traditional Chinese medicine component, the more the number of genes enriched by this component, or the

more this component is enriched in the pathway. (B) Venn diagram shows the intersection of the key target genes screened by the drug-disease-pathway network diagram and the key target genes screened by the PPI network.


A
7_O_methylisomucronulatol
AstragalosideIV
beta_sitosterol
Calycosin
Epigallocatechin gallate
formononetin
isorhamnetin
Kaempferol
Luteolin
quercetin
-3.7 -3.9 -4.3 -3 -3.9 -4.4
-0.94 -2.9 -1.5 -0.23 -0.58 -2.5
-4.3 -5.8 -5.6 -5 -4.9 -5.3
-4.5 -4.4 -4.5 -5 -3.9 -4.8
-2.9 -2 -2.9 -1.8 -2.4 -1.4
-4.7 -4.9 -4.1 -5.3 -4.3 -5
-3.2 -4 -3.2 -3.4 -2.5 -4.5
-3.5 -4.4 -3.3 -4 -3.5 -4.5
-3.8 -4 -3.3 -3.8 -3.9 -4.8
-3 -4.3 -3.1 -3.1 -2.6 -4.6
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AR
HIF-1α
JAK2
STAT3
TP53
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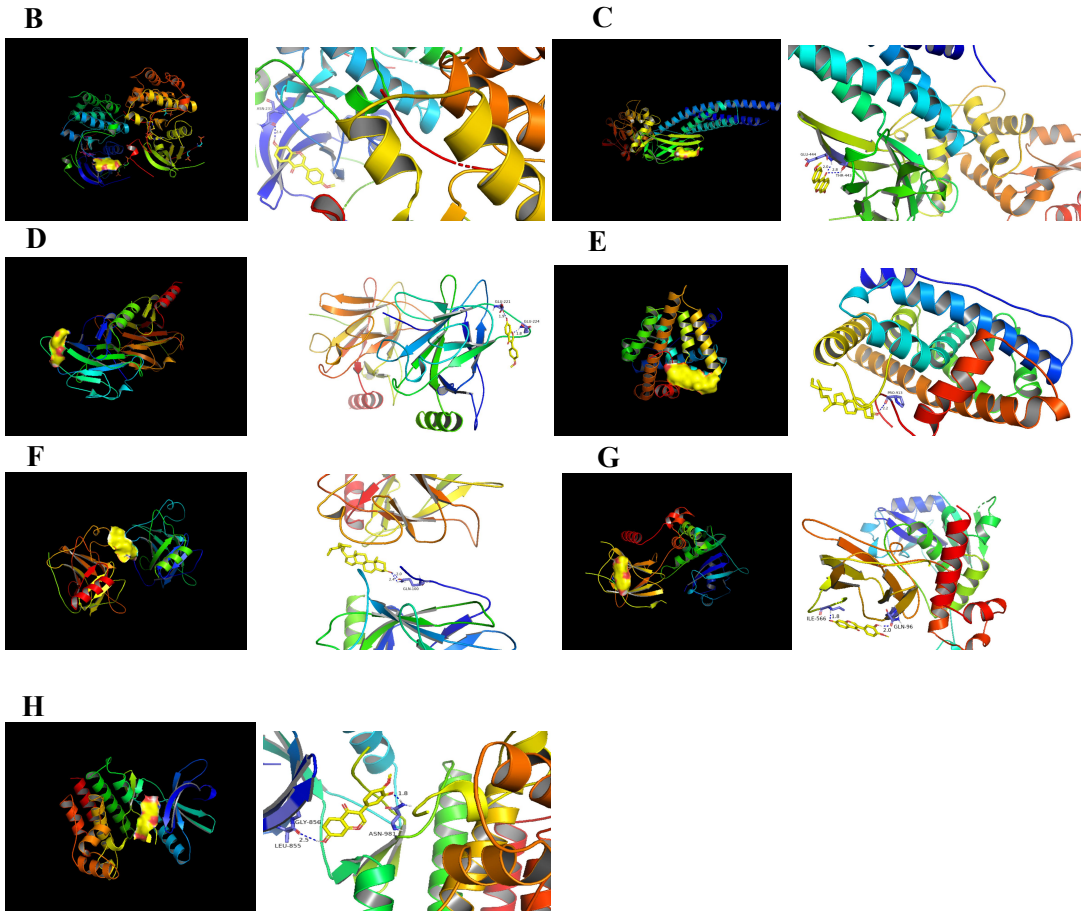
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Figure 4: The molecule docking heat map of the key active compounds in the Yishen capsule and the core target proteins.(A) The vertical axis represents the effective active ingredient of Yishen capsule and the horizontal axis represents the core target protein receptor. Each value represents the total score of molecular docking, and the darker the color, the higher the score. (B) Docking analysis of formononetin with AKT1 receptor. (C) Docking analysis of formononetin with STAT3 receptor. (D) Docking analysis of formononetin with TP53 receptor. (E) Docking analysis of β-sitosterol with AR receptor. (F) Docking analysis of β-sitosterol with TP53 receptor. (G) Docking analysis of verbasin with HIF-1αreceptor. (H) Docking analysis of mullein with JAK2 receptor. For all the docking analysis results, the left panel shows the global diagram of the simulated docking of each ligand molecule and the receptor protein. The colored ribbon model, the protein structure. The yellow sac-like structure, the ligand molecule. The right panel is a partial three-dimensional image of simulated docking. Yellow, small ligand molecules. Purple, amino acid residues that have hydrogen bonds with active molecules. The blue dashed line, interacting hydrogen bonds.

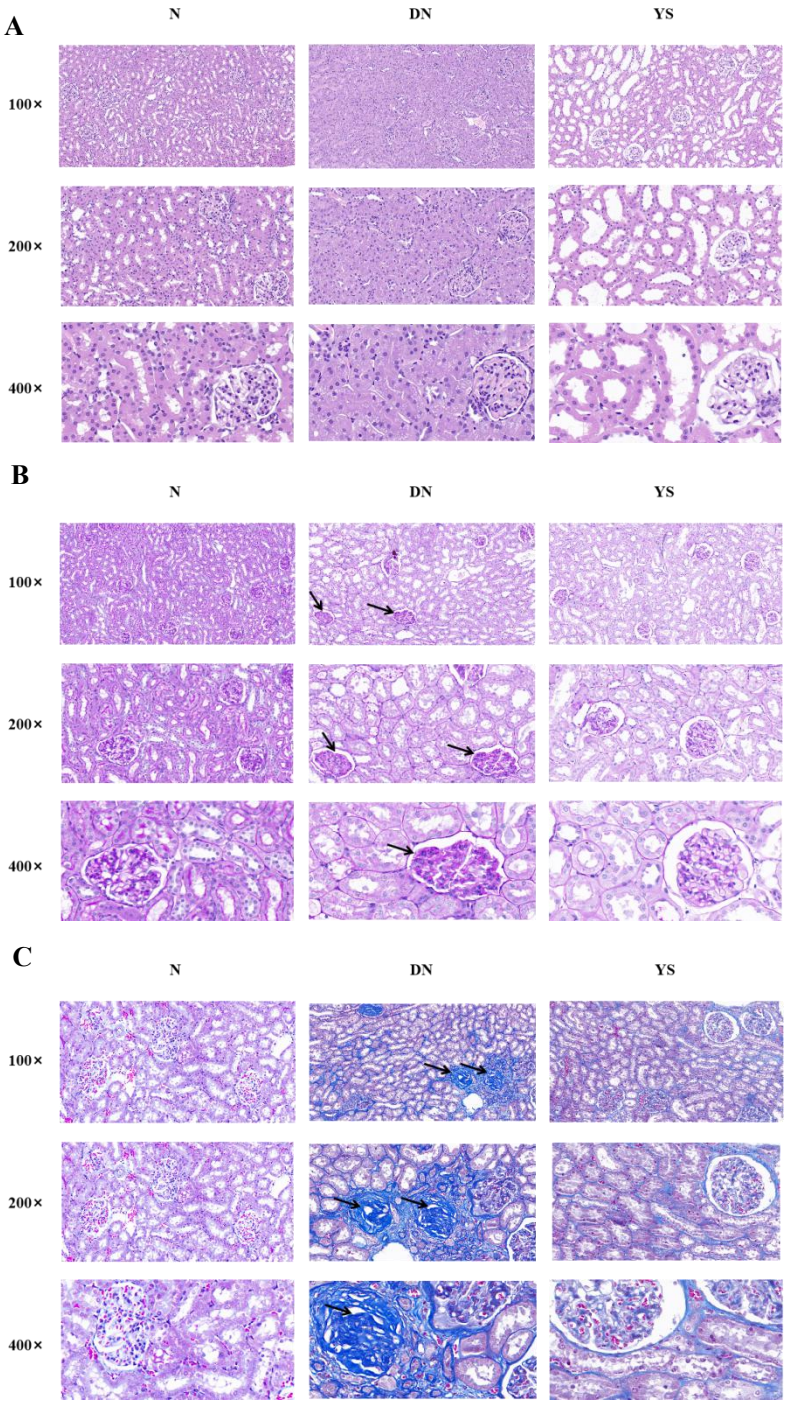

Figure 5: Pathological analysis of rat kidney tissues in each group (×100,×200,×400). (A) HE staining of rat kidney tissue. In the DN group, the glomerular capillary loops were hypertrophy, the basement membrane was thickened, the mesangial cells and mesangial matrix were proliferated, and the renal tubular epithelial cells were hypertrophy, while there was no

glomerular sclerosis or interstitial fibrosis. Compared with the DN group, the above-mentioned lesions in the YS group were reduced to varying degrees. (B) PAS staining. PAS-positive protein deposits can be seen in the glomerular mesangial area and renal tubular epithelial cells of rats in the DN group (Arrows in the figure). Compared with the DN group, the YS group had less PAS-positive protein deposition. (C) Masson staining. There were no abnormal changes in the morphology and structure of the kidney tissue in the normal group. Masson staining rarely showed blue staining of collagen tissue. The glomerulus volume of rats in the DN group increased, the renal tubular epithelial cells were obviously edema, and the lumen became narrow. Masson staining showed blue-stained collagen tissue (Arrow in the figure). Compared with the DN group, the YS group had increased glomerular volume, reduced renal tubular epithelial cell edema, and decreased blue-stained collagen tissue.

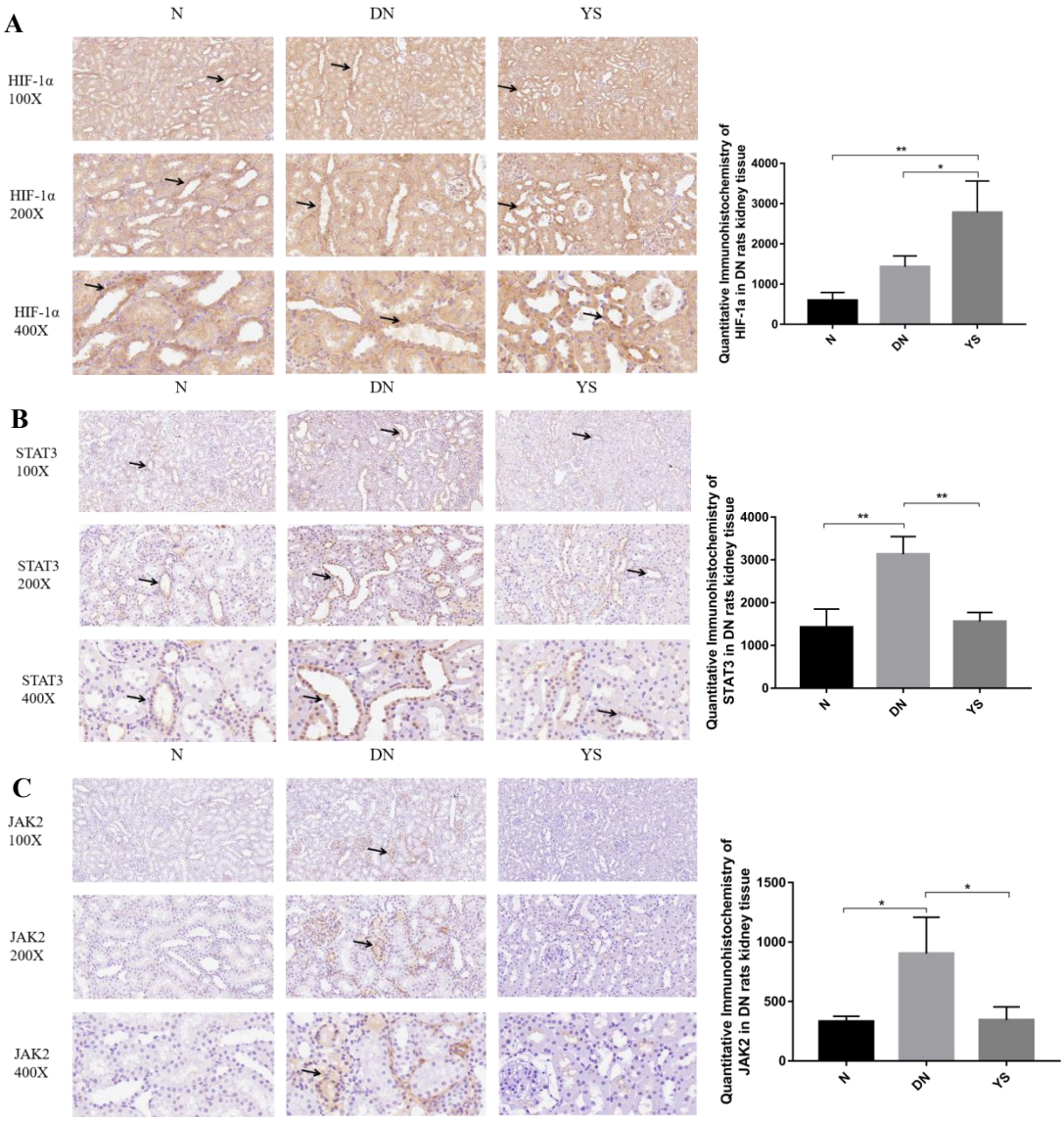

Figure 6: Immunohistochemical detection and quantitative analysis of HIF-1α, STAT3 and JAK2 in kidney tissues of rats in each group. (A) The expression of HIF-1α in the DN group was higher than that in the N group, which was further up-regulated in the YS group ($P<0.05$). (B) The expression of JAK-2 in DN group was higher than that in the N group, while its expression in the YS group was lower than that in the DN group ($P<0.05$). (C) The expression of STAT3 in DN group was higher than that in the N group, while its expression in the YS group was lower than that in the DN group ($P<0.05$). *$P<0.05$, **$P<0.01$.

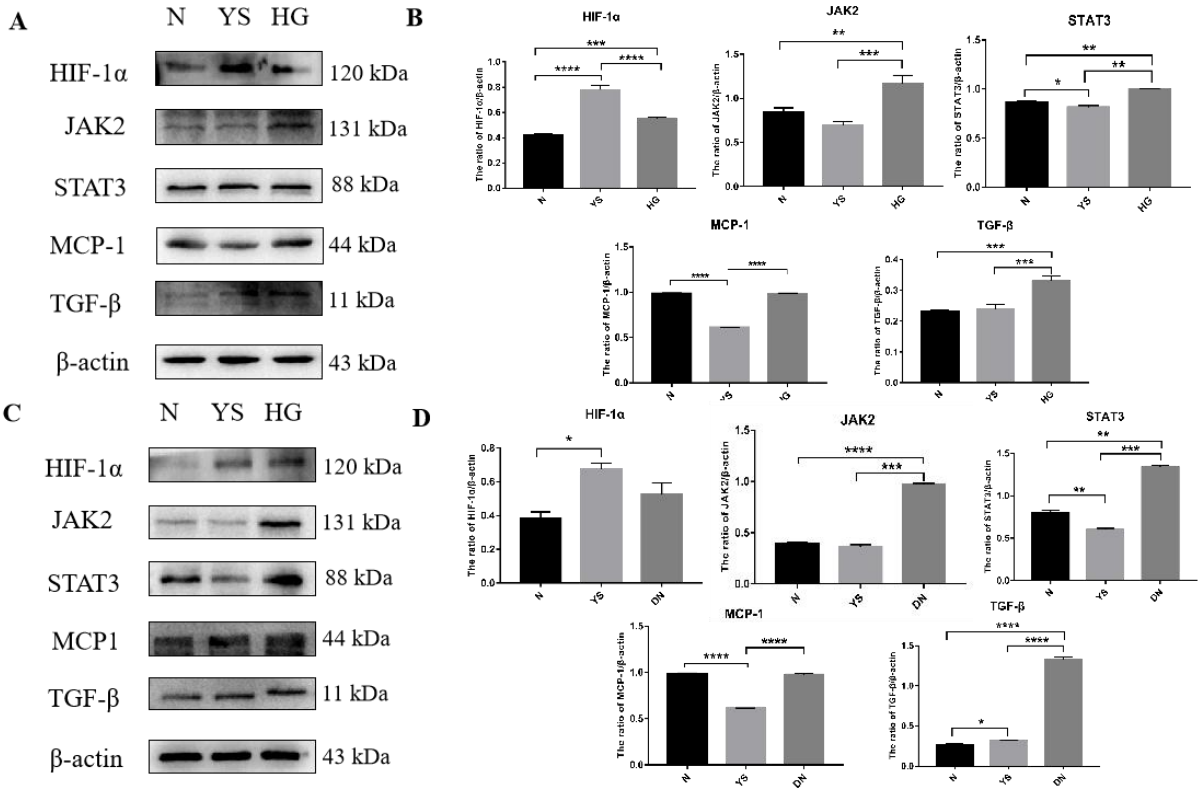

Figure 7: Western blot to detect the expression of key proteins in cells and tissues. (A-B) the expression and quantitative analysis of key proteins in renal tubular epithelial cells. After high glucose (HG) stimulation, the expression of HIF-1α, JAK-2, STAT3 and TGF-β protein increased in renal tubular epithelial cells. After YS treatment, compared with the HG group, the expression of HIF-1α in renal tubular epithelial cells further increased, while the expression of JAK2, STAT3, MCP-1 and TGF-β decreased. (C-D) the expression and quantitative analysis of key proteins in kidney tissue. Compared with the normal group (N), the expression of JAK2, STAT3 and TGF-β protein in the kidney tissue of the DN group increased. After treatment with Yishen Capsule, the expression of HIF-1α in kidney tissue increased compared with that in the the normal group. Compared with the DN group, the expressions of JAK2, STAT3, MCP-1 and TGF-β in kidney tissues all decreased after treatment with Yishen capsule. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.