

## Basic Study

# Potential application of *Nardostachyos Radix et Rhizoma-Rhubarb* for the treatment of diabetic kidney disease based on network pharmacology and cell culture experimental verification

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## Abstract

### BACKGROUND

Diabetic kidney disease (DKD) is one of the serious complications of diabetes mellitus, and the existing treatments cannot meet the needs of today's patients. Traditional Chinese medicine has been validated for its efficacy in DKD after many years of clinical application. However, the specific mechanism by which it works is still unclear. Elucidating the molecular mechanism of the *Nardostachyos Radix et Rhizoma-rhubarb* drug pair (NRDP) for the treatment of DKD will provide a new way of thinking for the research and development of new drugs.

### AIM

To investigate the mechanism of the NRDP in DKD by network pharmacology combined with molecular docking, and then verify the initial findings by *in vitro* experiments.

## METHODS

The Traditional Chinese Medicine Systems Pharmacology (TCMSP) database was used to screen active ingredient targets of NRDP. Targets for DKD were obtained based on the Genecards, OMIM, and TTD databases. The Venny 2.1 database was used to obtain DKD and NRDP intersection targets and their Venn diagram, and Cytoscape 3.9.0 was used to build a "drug-component-target-disease" network. The String database was used to construct protein interaction networks. Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis and Gene Ontology analysis were performed based on the DAVID database. After selecting the targets and the active ingredients, Autodock software was used to perform molecular docking. In experimental validation using renal tubular epithelial cells (TCMK-1), we used the Cell Counting Kit-8 assay to detect the effect of NRDP on cell viability, with glucose solution used to mimic a hyperglycemic environment. Flow cytometry was used to detect the cell cycle progression and apoptosis. Western blot was used to detect the protein expression of STAT3, p-STAT3, BAX, BCL-2, Caspase9, and Caspase3.

## RESULTS

A total of 10 active ingredients and 85 targets with 111 disease-related signaling pathways were obtained for NRDP. Enrichment analysis of KEGG pathways was performed to determine advanced glycation end products (AGEs)-receptor for AGEs (RAGE) signaling as the core pathway. Molecular docking showed good binding between each active ingredient and its core targets. *In vitro* experiments showed that NRDP inhibited the viability of TCMK-1 cells, blocked cell cycle progression in the G0/G1 phase, and reduced apoptosis in a concentration-dependent manner. Based on the results of Western blot analysis, NRDP differentially downregulated p-STAT3, BAX, Caspase3, and Caspase9 protein levels ( $P < 0.01$  or  $P < 0.05$ ). In addition, BAX/BCL-2 and p-STAT3/STAT3 ratios were reduced, while BCL-2 and STAT3 protein expression was upregulated ( $P < 0.01$ ).

## CONCLUSION

NRDP may upregulate BCL-2 and STAT3 protein expression, and downregulate BAX, Caspase3, and Caspase9 protein expression, thus activating the AGE-RAGE signaling pathway, inhibiting the vitality of TCMK-1 cells, reducing their apoptosis, and arresting them in the G0/G1 phase to protect them from damage by high glucose.

**Key Words:** *Nardostachys Radix et Rhizoma*-rhubarb; Diabetic kidney disease; Molecular docking; Network pharmacology; Experimental validation

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**Core Tip:** The *Nardostachys Radix et Rhizoma*-rhubarb drug pair may upregulate BCL-2 and STAT3 protein expression, and downregulate BAX, Caspase3, and Caspase9 protein expression, thus activating the advanced glycation end products (AGEs)-receptor for AGEs signaling pathway, inhibiting the vitality of TCMK-1 cells, reducing their apoptosis, and arresting them in the G0/G1 phase to protect them from damage by high glucose.

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## INTRODUCTION

Diabetes mellitus (DM) is a clinical syndrome characterized mainly by elevated blood sugar caused by genetic factors. Delayed treatment will eventually lead to a series of serious complications, mainly diabetic kidney disease (DKD)[1]. DKD is one of the leading causes of end-stage renal disease, a leading cause of kidney failure. According to the epidemiological survey data released by the International Diabetes Federation, the global incidence of DM is 9.3% [2]; among them, 20%-40% develop DKD [3]. With the increase in the number of DKD patients, the treatment of DKD is imminent. The main treatment for DKD in Western medicine is to control blood sugar and improve renal function [4]. Clinical medications are mostly angiotensin-converting enzyme inhibitors and sulfonyleureas to improve renal blood circulation. Despite this, the effect of these medications in relieving symptoms and reducing the disease's progression is not obvious. Hence, we desperately need to find effective drugs or compounds with minimal side effects to treat DKD [5].

As a traditional Chinese medicine in China, *Nardostachys Radix et Rhizoma* belongs to the dried roots and rhizomes of *Nardostachys jatamansi*, a plant of the Septoria family [6]. Modern pharmacological studies have found that it is effective against brain diseases, heart diseases, spleen diseases, skin diseases, erectile dysfunction, tumors, and other diseases [7]. The chemical components of *Nardostachys Radix et Rhizoma* are mainly terpenoids, coumarin, and lignans [8]. The active

compounds mansonopsin and naringin not only relieve cardiac hypertrophy[9] but also have anti-inflammatory, antibacterial, anti-osteoporosis, myocardium-protective, anti-malaria, liver-protective, anti-apoptosis, anti-tumor, sedative, antihypertensive, and anti-oxidative stress effects[10,11]. Other studies have shown that *Nardostachyos Radix et Rhizoma* can control blood glucose metabolism, regulate the islet function, and protect the kidney[12]. Rhubarb, which belongs to the dried roots and rhizome of *Rheum officinale Baill* in the Polygonum family, is widely utilized to cure diverse diseases. Rhubarb prevents the progression of DKD through a variety of mechanisms[13]. Modern pharmacological studies have found that anthraquinone derivatives contained in rhubarb have purgative effects[14]. Anthracene has an antidiarrheal effect. Emodin and rhein have anti-inflammatory, antibacterial, antiviral, anti-oxidative stress, anti-tumor, anti-fibrosis, lipid-regulating, and hypoglycemic effects[15,16]. Rhubarb tannin improves nitrogen waste metabolism; rhubarb anthraquinone and rhubarb anthraquinone glucoside can inhibit mesangial cell growth, improve renal tubular function, and protect the kidney[17]. Rhein has antitumor effects[18]. In addition, it has hemostatic, antiviral, antibacterial, liver-protecting[19], gallbladder-protecting, stomach-protecting[20], and kidney-protecting properties. In the treatment of DKD, rhubarb can reduce uremic toxin levels, regulate intestinal flora[14], and delay the progression of renal interstitial fibrosis. However, the drug targets and molecular mechanisms of *Nardostachyos Radix et Rhizoma*-rhubarb drug pair (NRDP) in the treatment of DKD have not been clarified. Therefore, we investigated the specific drug targets and molecular mechanisms of NRDP in the treatment of DKD based on network pharmacology combined with pharmacology.

DM and aberrant renal function are the causes of DKD. While the precise etiology remains unknown, certain research has demonstrated that advanced glycation end products (AGEs) formation is essential to the development of DKD. When the receptor for AGEs (RAGE) is activated, other associated pathways are impacted, which increases oxidative stress and inflammation in renal cells, encouraging apoptosis and exacerbating the progression of DKD[21]. Traditional Chinese medicine is often used to treat chronic diseases. Under high-glucose environment, the AGE-RAGE pathway will be activated to increase kidney damage. Studies have shown that traditional Chinese medicine monomers and compounds can regulate PI3K-AKT, NF- $\kappa$ B, JAK/STAT, and other pathways, and reduce oxidative stress, cell apoptosis, and inflammation, thereby improving kidney damage and delaying the course of DKD[22]. Since there have been few reports on the effects and mechanisms of NRDP in treating DKD, we analyzed the active components and targets of NRDP, and explored the mechanism underlying the therapeutic effects of NRDP on DKD in the present study.

Traditional Chinese medicine compounds exhibit multi-target, multi-component, and multi-pathway actions that are consistent with network pharmacology. In this study, we employed network pharmacology and experimental verification to confirm the mechanism of action of the compound on DKD. We searched for drug and disease targets using the TCMSP, Gene Cards, OMIM, and TTD databases, and then identified the core target pathway through the protein-protein interaction (PPI) network. Using NRDP components, we conducted Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analyses, as well as molecular docking. Finally, we performed experimental verification to prove the predictions made on the mechanism of NRDP in DKD (Figure 1), with an aim to provide new ideas and methods for the subsequent treatment of DKD with traditional Chinese medicine.

## MATERIALS AND METHODS

### Acquisition of active ingredients and targets of NRDP

The TCMSP database analysis platform (<https://tcmsp-e.com/>) was searched for the active ingredients and targets of *Nardostachyos Radix et Rhizoma* and rhubarb. The screening criteria for the active ingredients in drugs and their corresponding were oral availability  $\geq 30\%$  and drug likeness  $\geq 0.18$ . Then, the UniProt (<https://www.uniprot.org/>) database was used to translate the targets into gene names.

### Identification of DKD targets

In the GeneCards (<http://www.genecards.org/>), OMIM (<http://omim.org/>), and TTD (<https://db.idrblab.net/ttd/>) databases, "Diabetic Kidney Diseases" and "Diabetic Nephropathy" were searched as keywords, and the DKD targets were obtained.

### Identification of intersection targets for NRDP and DKD

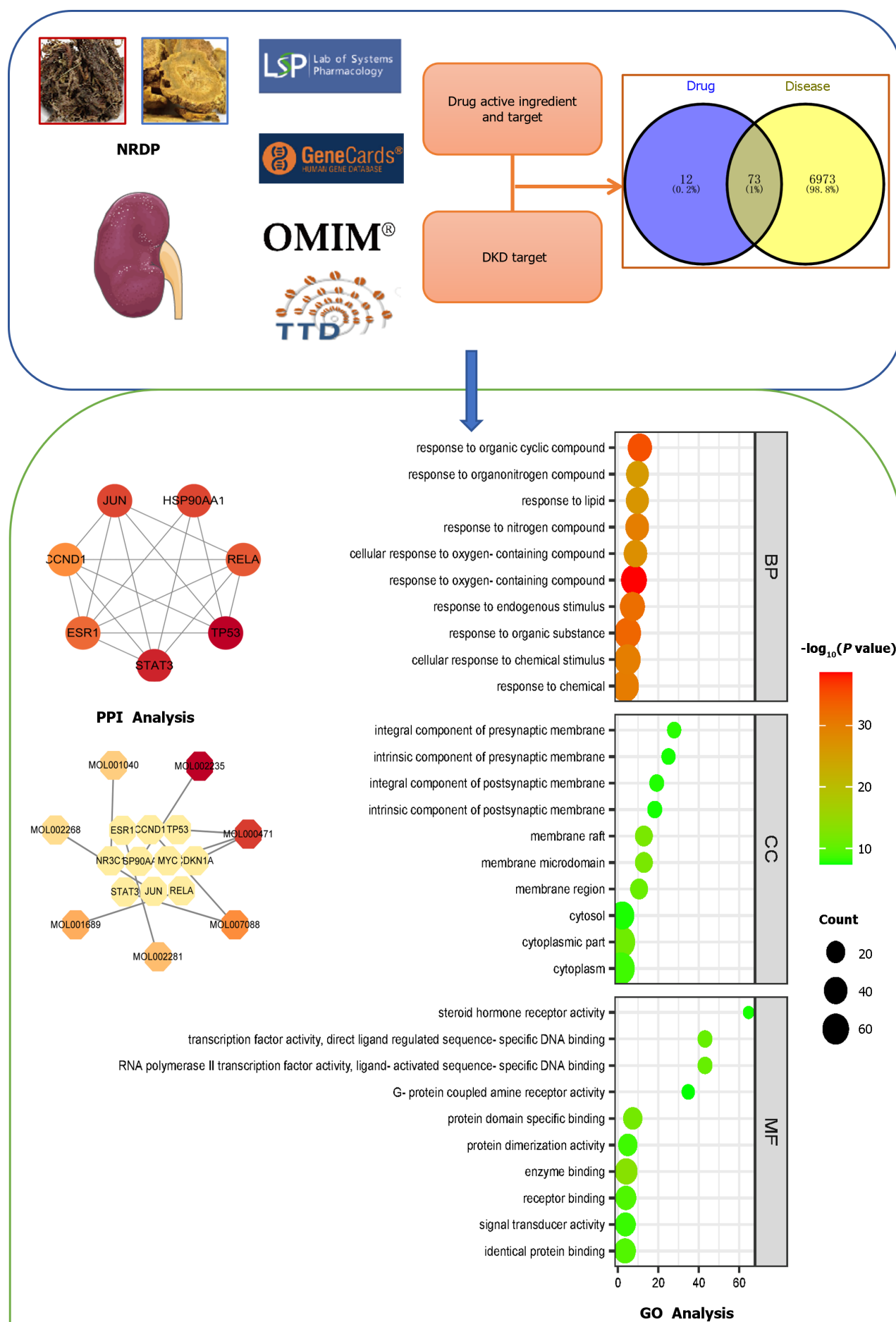
The Venny2.1.0 platform (<https://bioinfogp.cnb.csic.es/tools/venny/>) was used to obtain the common targets between the NRDP and DKD.

### Construction of "component-target-disease" network

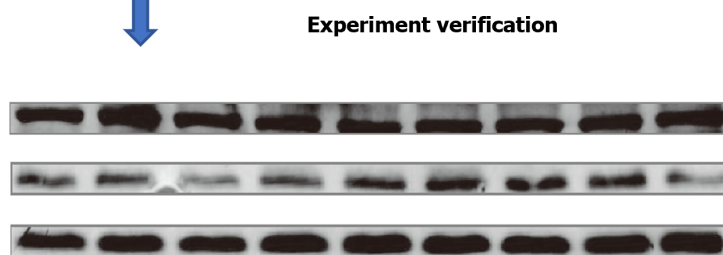
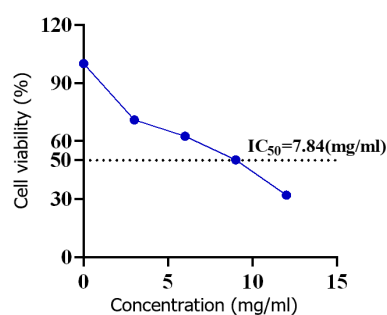
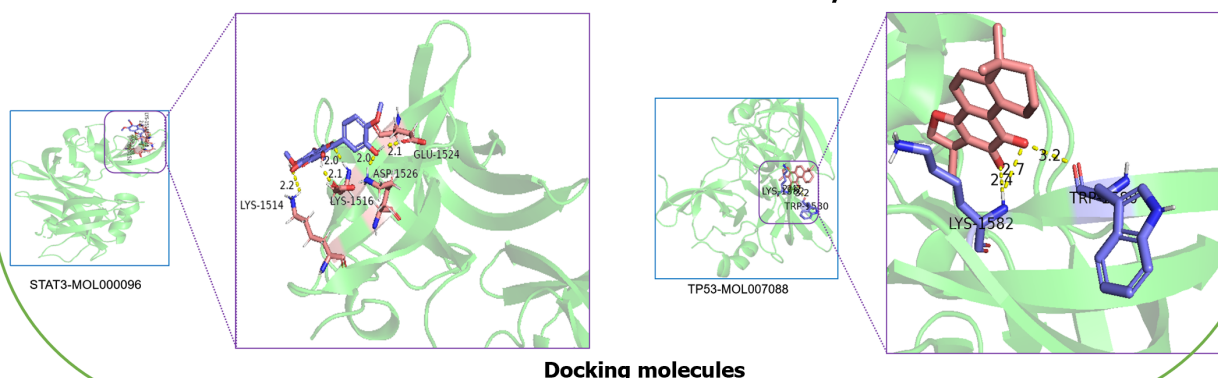
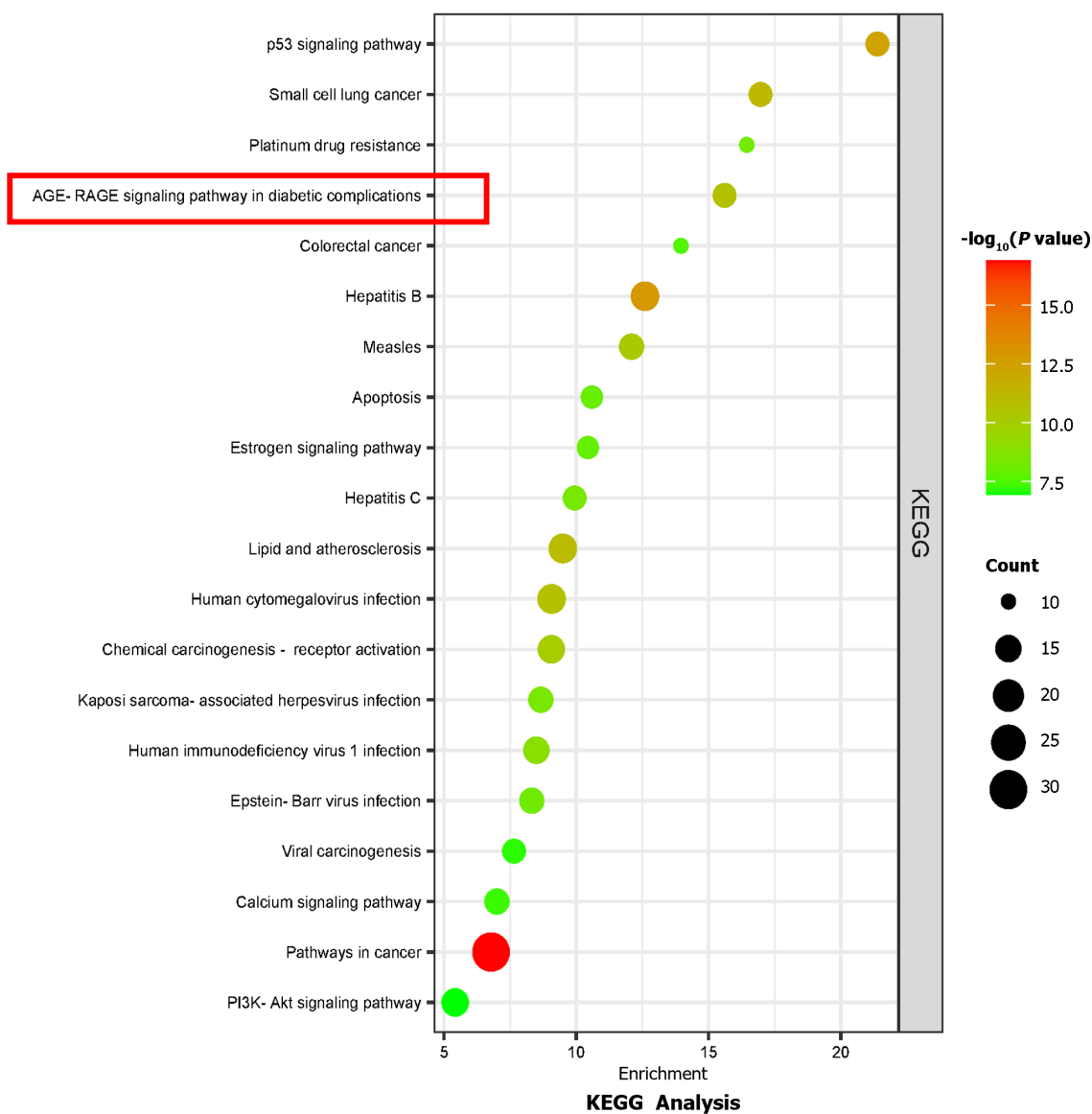
To visualize the results, a "component-target-disease" network was constructed using Cytoscape 3.9.0 with the active ingredients and targets of NRDP and DKD.

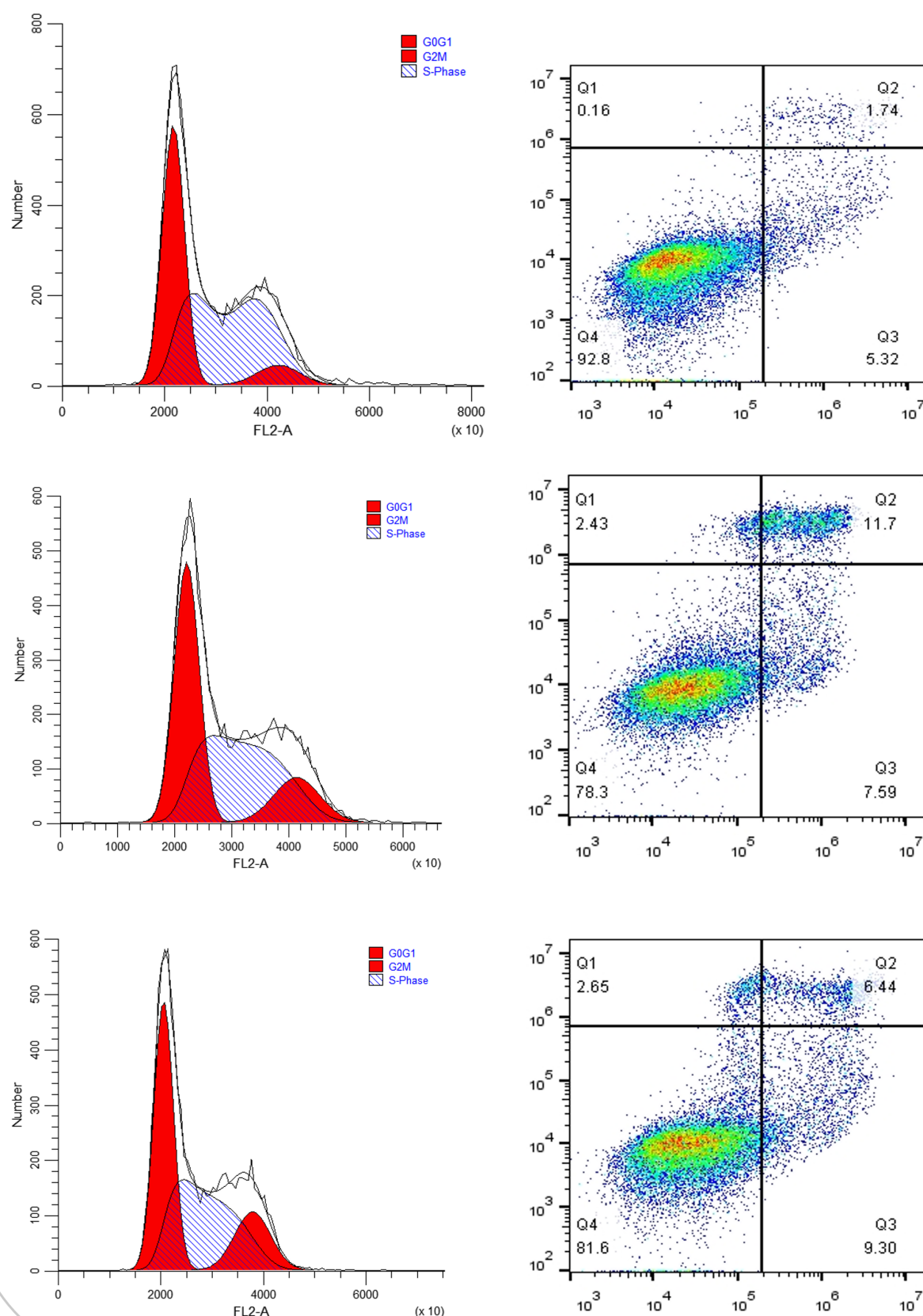
### Construction of PPI network

PPI network diagrams were constructed by importing the common targets of NRDP and DKD into the STRING 11.5 database (<https://cn.string-db.org/>), and the species was set as "*Homo sapiens*". The minimum interaction threshold was set as "highest confidence" ( $> 0.9$ ), to hide isolated nodes, and the rest of the settings were set as default. To obtain protein interaction data, the TSV file was downloaded and imported to Cytoscape software. The Network Analyzer plug-in was used to analyze network characteristics, and screen core targets in the PPI network according to the degree of nodes.









**Figure 1** Flow chart of network pharmacological prediction and experimental validation. GO: Gene Ontology; KEGG: Kyoto Encyclopedia of Genes and Genomes; DKD: Diabetic kidney disease; PPI: Protein-protein interaction; BP: Biological process; CC: Cell composition; MF: Molecular function; IC<sub>50</sub>: Half maximal inhibitory concentration.

### Composition-target network diagram construction

Cytoscape software was used to construct a network for common targets and NRDP active components. To analyze the characteristics of the network, the Network Analyzer plug-in was used, and the interaction between NRDP active components and core targets was analyzed according to the degree of nodes.

### GO and KEGG enrichment analysis

Intersection targets were imported to the DAVID database (<https://david.ncifcrf.gov/>) for GO function and KEGG pathway enrichment analysis. *P* value < 0.01 and false discovery rate < 0.01 were used as the conditions for the screening. With the help of online data analysis, the visualization platform - microscopical letter (<http://www.bioinformatics.com.cn/>) resulted in visualization.

### Construction of component-target network diagram on signaling pathway

The key pathway targets obtained following KEGG enrichment were used to generate a full PPI network based on the STRING11.5 database. The TSV file of PPI was downloaded and imported into Cytoscape software. After analysis with the Network Analyzer plug-in, a component-target network diagram about the pathway was reconstructed with the active components of NRDP. According to the degree of the node, the interaction between the active components of NRDP and the pathway targets was analyzed.

### Molecular docking

NRDP medicine mol2 structures were downloaded from the PubChem database (<https://pubchem.ncbi.nlm.nih.gov/>), and the core target protein 3D structure was downloaded from the PDB database (<https://www.rcsb.org/>). Then, the water molecules and small molecular ligands of proteins were removed using Pymol 2.4.0 software, and AutoDock 1.5.7 software was employed for hydrogenation. Molecular docking of receptor and ligand was performed and their binding activity was evaluated.

### Cell experiment verification

**Cells:** TCMK-1 cells (renal tubule epithelial cells) were purchased from BeNa Culture Collection (No. BNCC339820).

**Drugs and reagents:** NRDP was prepared by the Preparation Center, Affiliated Hospital of Traditional Chinese Medicine, Ningxia Medical University.

**Cell culture:** TCMK-1 cells were cultured with complete medium (89% high-glucose DMEM + 10% fetal bovine serum + 1% penicillin-streptomycin mixture) in a 5% CO<sub>2</sub> incubator at 37 °C. A microscope was used to observe cell growth, and cells were passed at 80% confluence.

### Determination of half inhibitory concentration of NRDP by CCK8 method

TCMK-1 cells in the logarithmic growth phase were digested with trypsin for cell suspension preparation. Cells were counted under a 20 times microscope, and  $5 \times 10^3$  cells were inoculated per well into 96-well plates and incubated with complete cell culture medium (control group), 60 mmol/L high glucose culture medium (model group), or high glucose culture medium + different concentrations of NRDP (NRDP groups), with each group having five replicate wells. NRDP was diluted multiple times according to the drug concentration gradient, and then 100 µL of the diluted NRDP solution was added to each well and incubated for 24 h. At the end of the drug intervention, incubation with CCK8 (10 µL/well) was performed for 1 h under no light conditions. Optical density (OD) was then read at 450 nm.

### Determination of effect of NRDP on the cell cycle of TCMK-1 cells treated with high glucose by flow cytometry

TCMK-1 cells in the logarithmic growth phase were divided into five groups: Control group (complete culture medium), model group (60 mmol/L high-glucose culture medium), low-dose group (high-glucose culture medium + 4 mg/mL NRDP), medium-dose group (high-glucose culture medium + 7 mg/mL NRDP), and high-dose group (high-glucose culture medium + 10 mg/mL NRDP). Three replicates were run for each group. Cells were inoculated into 6-well plates at a density of  $1.0 \times 10^5$  cells/well. Following 24 h of culture in an incubator, serum-free medium was added to each group and incubated for 12 h. Cells were then digested, fixed overnight, and pre-cooled by adding 70% ethanol. A commercial cell cycle kit (KeyGEN Biotech, China) was used to detect the cell cycle progression with a CytoFLEX flow cytometer (Beckman Coulter, United States).

### Detection of effect of NRDP on apoptosis of TCMK-1 cells treated with high glucose by flow cytometry

TCMK-1 cells in the logarithmic growth phase were divided into five groups as stated above, and three replicates were run for each group. Cells were inoculated into 6-well plates at a density of  $1.0 \times 10^5$  cells/well. Following 24 h of culture in an incubator, each group of cells were exposed to the corresponding culture medium. After cells were digested and collected, the corresponding reagents were added according to the Annexin V-FITC/propidium iodide apoptosis detection kit (KeyGEN Biotech, China) instructions. The results were detected with a CytoFLEX flow cytometry (Beckman Coulter, United States).

### Protein expression detection by Western blot analysis

TCMK-1 cells in the logarithmic growth phase were divided into three groups: Control group, model group, and medium dose group (7 mg/mL NRDP), and three replicates were run for each group. After cells were digested and collected, 200

μL of RIPA lysis buffer was added according to the total protein extraction kit (KeyGEN Biotech, China) instructions. The extracted proteins were subjected to protein content determination and then resolved by SDS-PAGE. After transfer to a membrane and membrane blockade, the membrane was incubated with the primary antibody at 4 °C overnight, followed by incubation with the secondary antibody for 1 h. Chemidoc (Ge1Doc XR+, BIO-RAD, United States) was used for chemiluminescence detection. Image J software was used to determine gray values for statistical analysis.

### Statistical methods

GraphPad Prism 8.0.2 software was used for statistical analyses and one-way analysis of variance was used to compare the difference among groups. The SNK test was used to test for homogeneity of variances, and the Tamhane's *T* test was used to test for heterogeneity of variances. *P* values < 0.05 were regarded as statistically significant.

## RESULTS

### Chemical constituents and targets of NRDP

A total of 15 active ingredients were obtained from the TCMSP database, including five active ingredients from *Nardostachyos Radix et Rhizoma* and ten from rhubarb. After removing duplicate targets, 43 targets of *Nardostachyos Radix et Rhizoma*, 69 targets of rhubarb, and 85 GRDP targets were obtained (Figure 2A).

### Drug-disease intersection targets and Venn diagram

Based on GeneCards (<https://www.genecards.org/>), OMIM (<https://omim.org/>), DrugBank (<https://go.drug-bank.com/>), and TTD databases (<http://db.idrblab.net/ttd/>), a total of 7046 relevant targets for DKD were screened (Figure 2A). A Venn diagram was drawn for 73 intersection targets between NRDP and DKD (Figure 2B).

### Construction of drug-disease-active ingredient-target network

The NRDP active ingredients and common targets were imported into Cytoscape 3.9.0 software to obtain a visualized regulatory network diagram (Figure 2C). The nodes in the diagram include drug, disease, active ingredient, and target, where the edges indicate that there is an interrelationship between them. Orange represents diseases and drugs. Fuchsia represents the co-interacting active ingredients of NRDP-disease. Light blue represents the active ingredients of *Nardostachyos Radix et Rhizoma*. Light yellow represents the active ingredients of rhubarb. Light purple represents gene targets for NRDP-disease co-action. Pink represents the target genes for NRDP. Light orange represents target genes for rhubarb. This network diagram shows that NRDP works through multiple components and targets in the treatment of DKD.

### Protein interaction network diagram

Using the STRING database, we obtained the protein interaction network diagram of NRDP and DKD (Figure 2D). There are 54 nodes and 310 edges, with the nodes representing proteins.

### PPI network analysis

The TSV file of the above PPI network diagram was downloaded, and the Network Analyzer plug-in in Cytoscape was used to analyze the network characteristics, including 54 nodes and 178 edges. Nodes with a degree median greater than 13 were carded out to obtain the PPI network, including the core targets of NRDP for DKD treatment. The targets were TP53, STAT3, HSP90AA1, JUN, RELA, ESR1, CCND1, MYC, CDKN1A, NR3C1, and CDK1. The results are shown in Figure 2E.

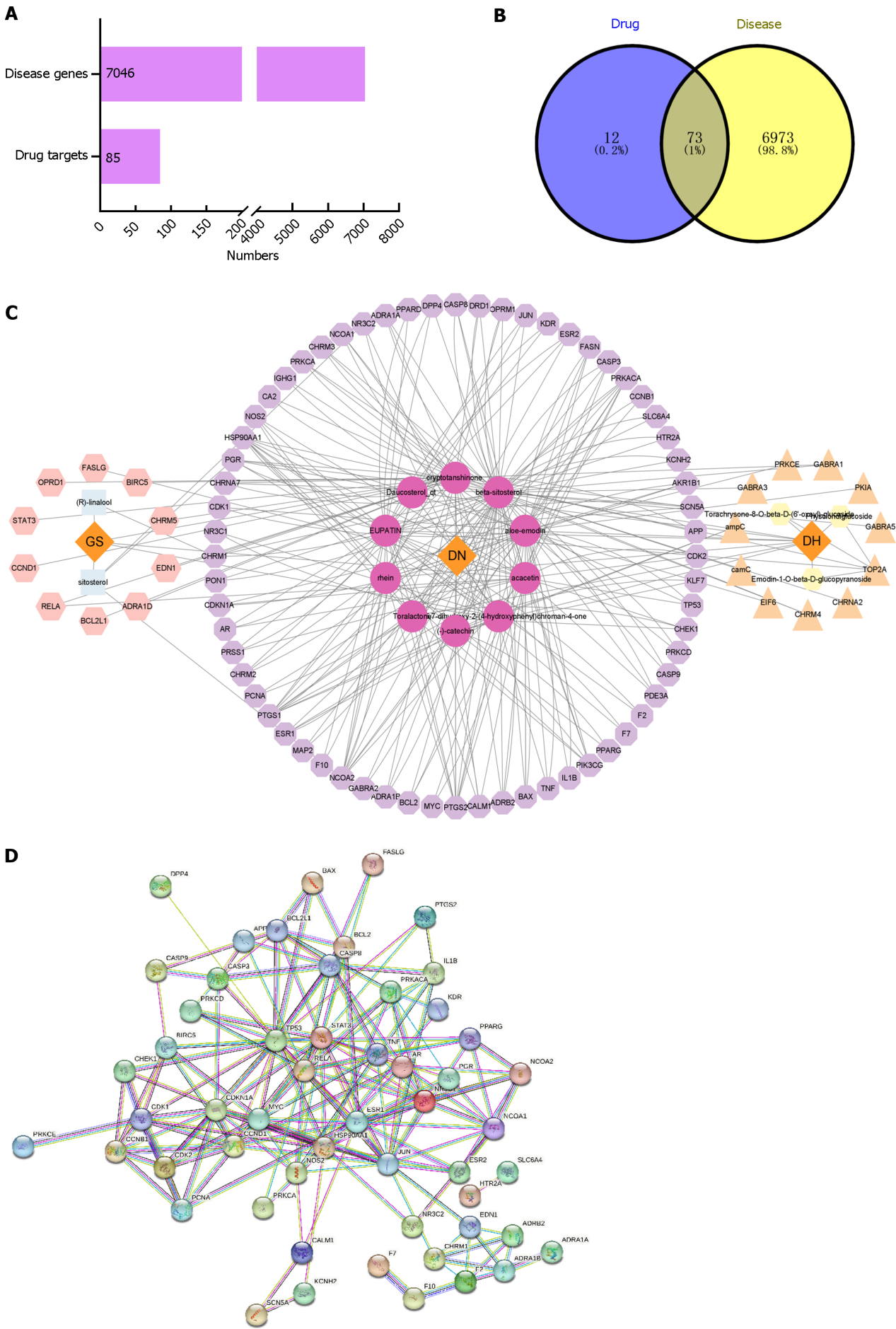
### Composition and target network analysis

A network diagram was constructed between PPI targets and NRDP active components. Through analysis with the Network Analyzer plug-in in Cytoscape software, the interaction between NRDP active components and 54 targets and their degree values were obtained. Nodes with a median of greater than 13 degrees were identified. Seven main components were obtained, including aloe-emodin, cryptotanshinone, EUPATIN, rhein, and acacetin, as shown in Figure 2F.

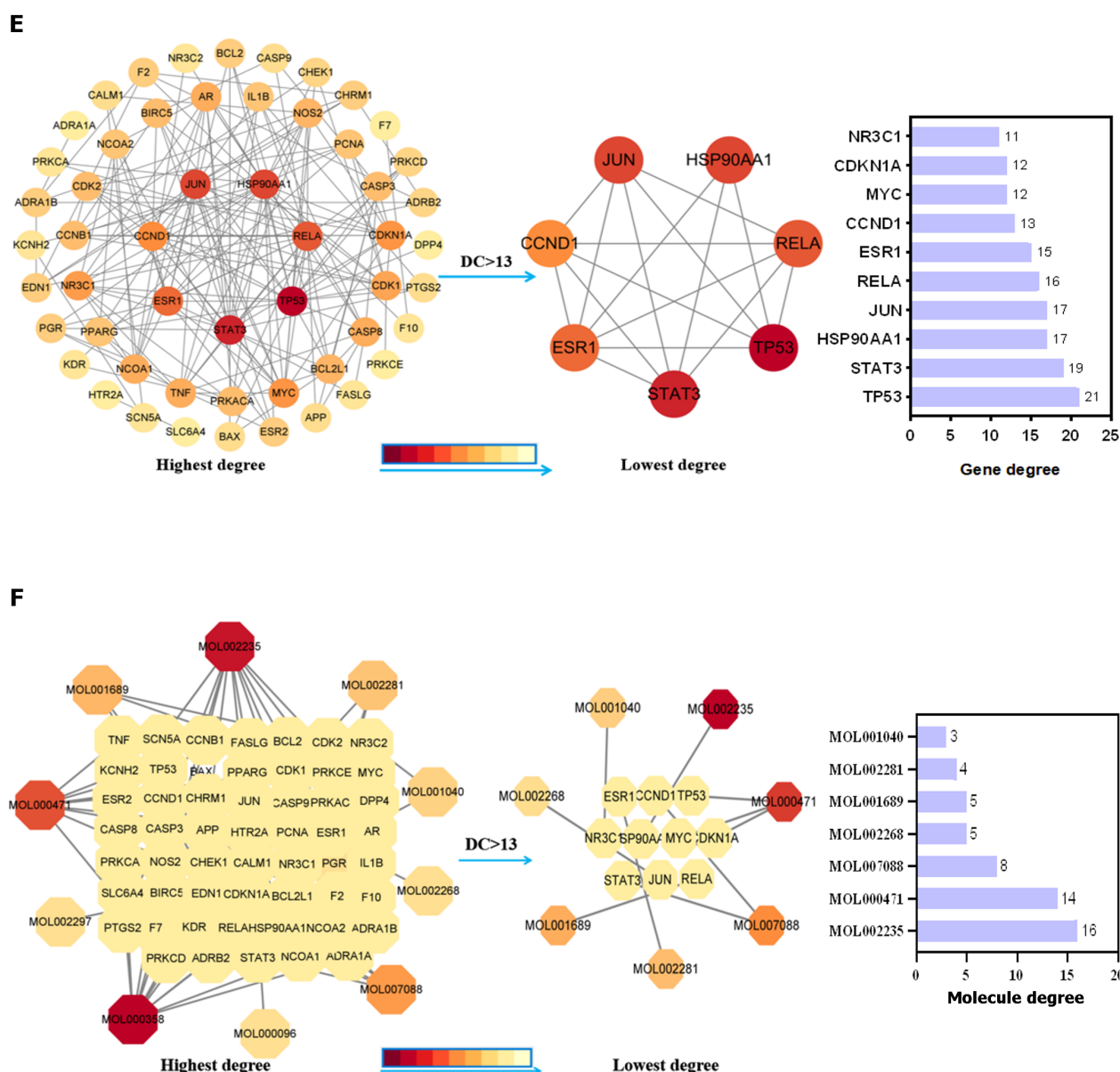
### GO and KEGG analysis

A total of 1528 biological process entries were obtained after GO enrichment analysis, which mainly involves response to oxygen-containing compound and response to organic cyclic compound, cellular response to chemical stimulus, cellular response to oxygen-containing compound, *etc.* There were 107 cell composition items, including the membrane raft, cytoplasmic part, an integral component of the presynaptic membrane and plasma membrane, extracellular exosome, and cytosol. There were 115 molecular function entries, mainly involving enzyme binding, protein domain-specific binding, transcription factor activity, direct ligand regulated sequence-specific DNA binding, *etc.* The top 10 enrichment results of each group are plotted (Figure 3A). The KEGG pathway enrichment analysis identified 111 pathways. The top 20 pathways are plotted in Figure 3B. The important pathways involved in the therapeutic effects of NRDP on DKD include pathways in cancer, PI3K-AKT signaling pathway, p53 signaling pathway, AGE-RAGE signaling pathway in diabetic complications, and apoptosis signaling pathway. The AGE-RAGE signaling pathway is the core pathway. The component-target network diagram in the signal pathway was constructed with NRDP active components using the









**Figure 2 Identification of drug and disease targets and protein-protein interaction enrichment analysis.** A: Drug and disease targets; B: Intersection of drug and disease targets; C: Drug-disease-target network diagram; D: Protein-protein interaction (PPI) network diagram of intersection genes of *Nardostachys Radix et Rhizoma-rhubarb* drug pair (NRDP) and disease; E: PPI network containing 73 intersection targets and 10 core targets constructed using Cytoscape; F: Selected core targets and active components of NRDP were analyzed. Each node's color denotes the degree.

STRING11.5 database and Network Analyzer plug-in of Cytoscape software, as shown in Figure 3C. The signal pathway diagram was also generated (Figure 3D).

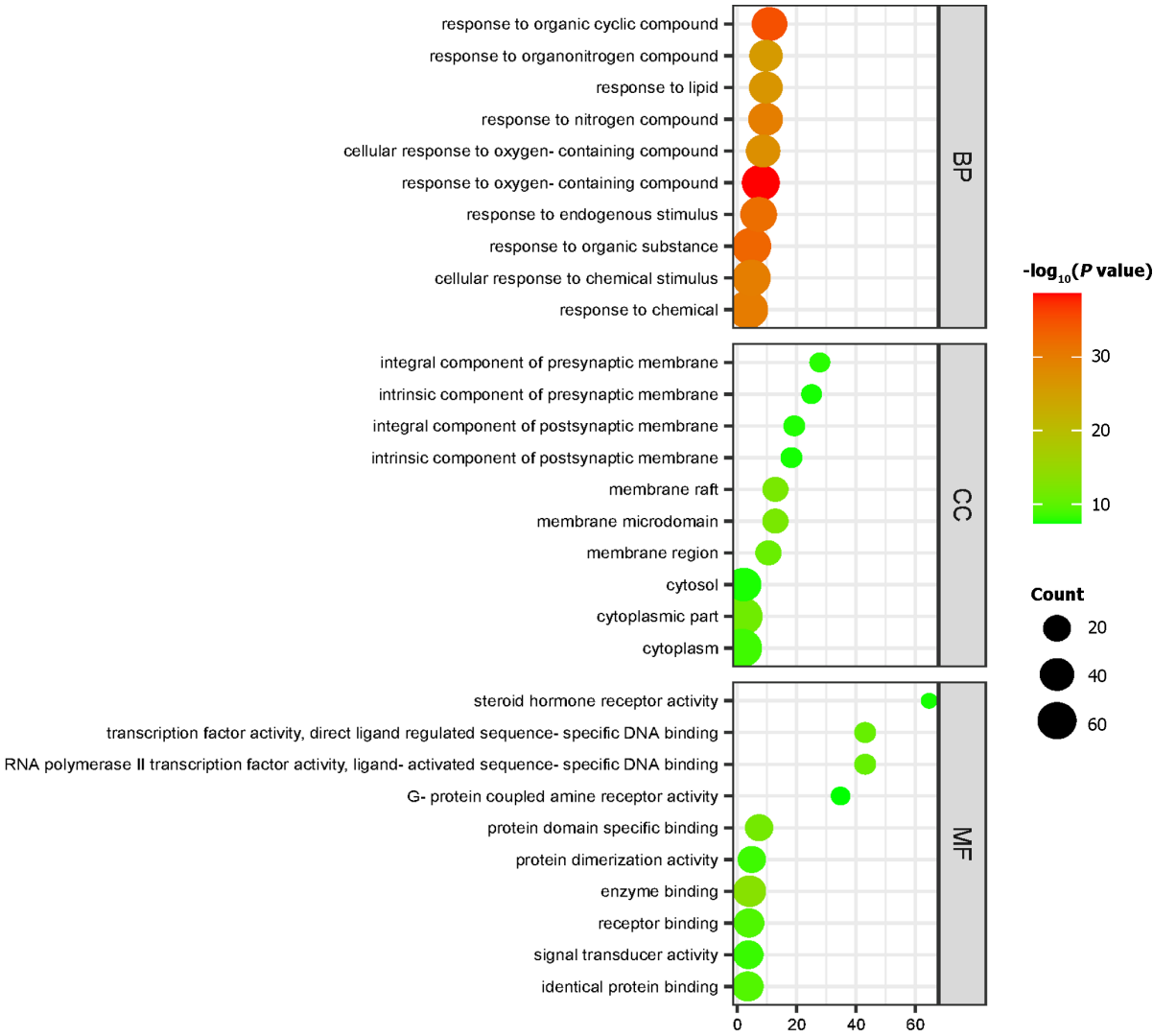
### Molecular docking

To further analyze the feasibility of NRDP for the treatment of DKD, the core proteins TP53, STAT3, HSP90AA1, JUN, RELA, ESR1, and CCND1, which have top seven degree values, were molecularly docked with the active components of NRDP. The PDB ID of ESR1, RELA, TP53, HSP90AA1, JUN, CCND1, and STAT3 is 6CHW, 3CBQ, 3DCY, 1BYQ, 2P33, 2VTH, and 6NJS, respectively. Pymol software was used to visualize the docking results (Figure 4A). Binding activity was evaluated according to docking scores: Scores < -4.25 kcal/mol indicated low binding activity, scores < -5.0 kcal/mol indicated good binding activity, and scores < -7.0 kcal/mol indicated strong binding activity. ChiPlot (<https://www.chiplot.online/#Heatmap>) online tools were used for the visualization output (Figure 4B). The binding energy of the ten active ingredients with the seven core target proteins was all less than -5.0 kcal/mol, among which the binding energy of STAT3 and (-)-Catechin was the smallest at -9.3 kcal/mol.

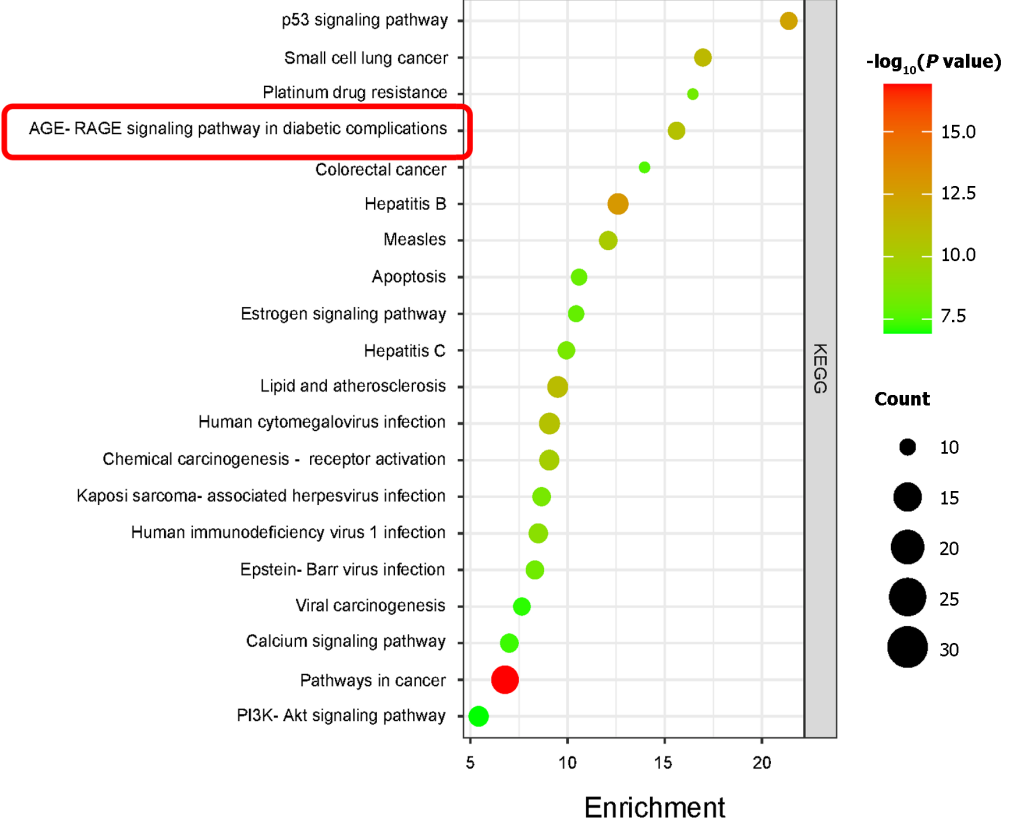
### Median inhibitory concentration of NRDP

To determine the inhibition rate of NRDP in each group, the OD values from the 24-h experiment were used for inhibition rate calculation. With GraphPad Prism 8.0.2, half-inhibitory doses for TCMK-1 cells were fitted (Figure 5A). According to

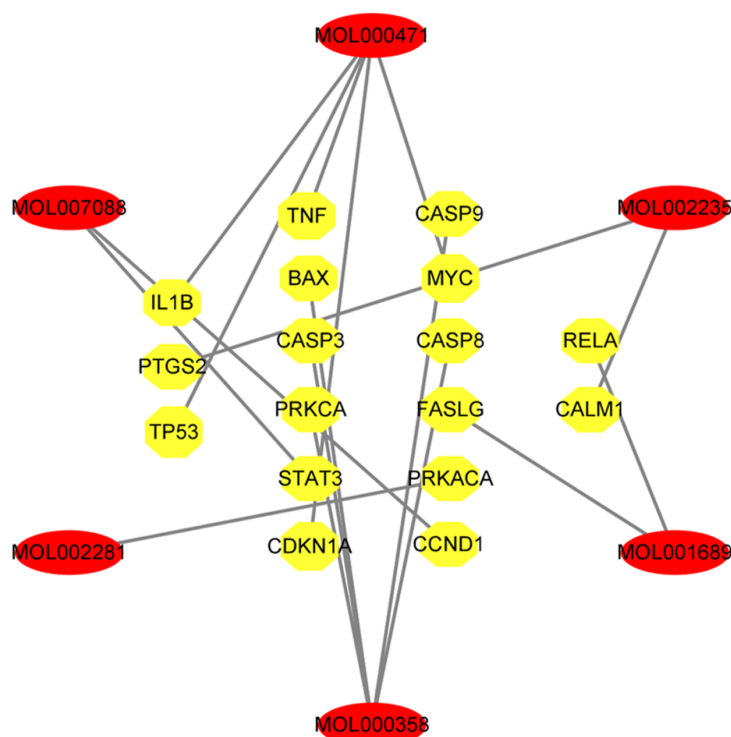
**A**



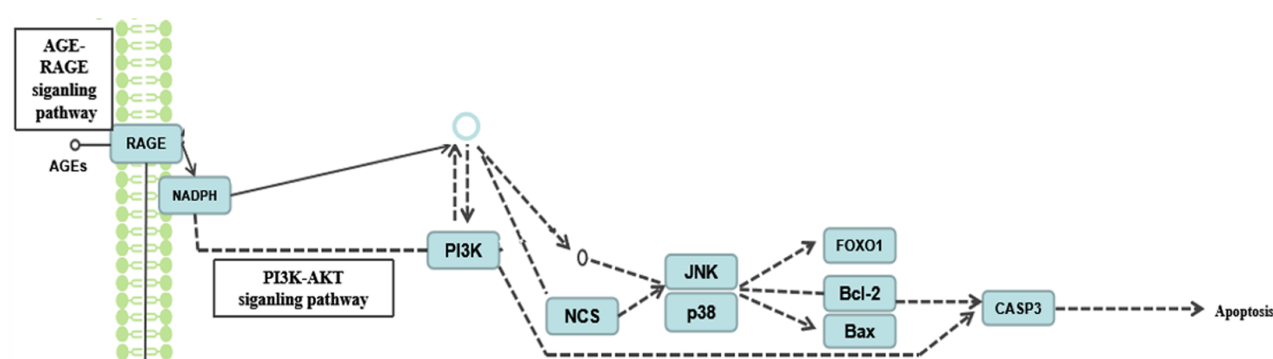
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D



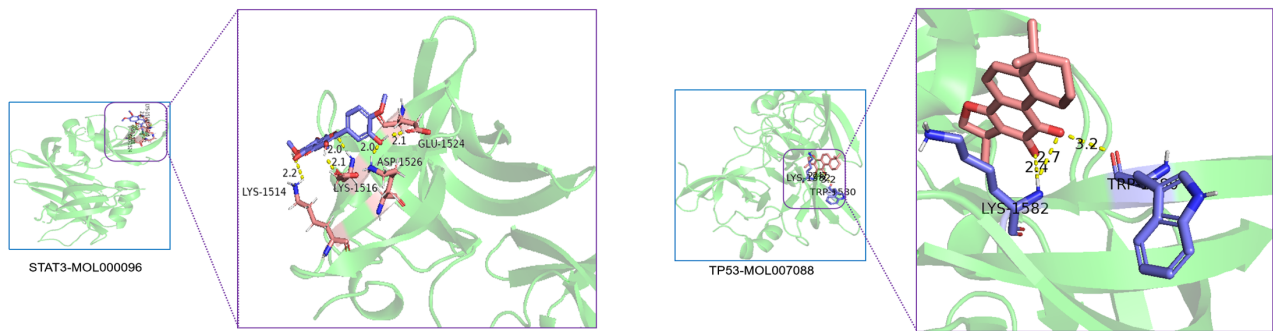
**Figure 3 Gene Ontology and Kyoto Encyclopedia of Genes and Genomes analysis.** A: Gene Ontology enrichment analysis; B: Kyoto Encyclopedia of Genes and Genomes pathway enrichment analysis; C: Enriched core genes in the AGE-RAGE signaling pathway; D: Correlation between AGE-RAGE signaling pathway and apoptosis.

the experimental results, the  $IC_{50}$  (half maximal inhibitory concentration) value of NRDP was 7.84 mg/mL. We determined that the half inhibitory concentration was 7 mg/mL, and the optimal low-, medium-, and high-doses administration concentration was 4 mg/m, 7 mg/mL, and 10 mg/mL, respectively. Cell Counting Kit-8 assay showed that compared with that of the control group, the cell viability of the model group was significantly increased ( $P < 0.01$ ). Compared with the model group, TCMK-1 cell viability decreased after NRDP intervention ( $P < 0.01$ ) in a dose-dependent manner. The higher the NRDP dose, the more obvious the decline in TCMK-1 cell viability (Figure 5B).

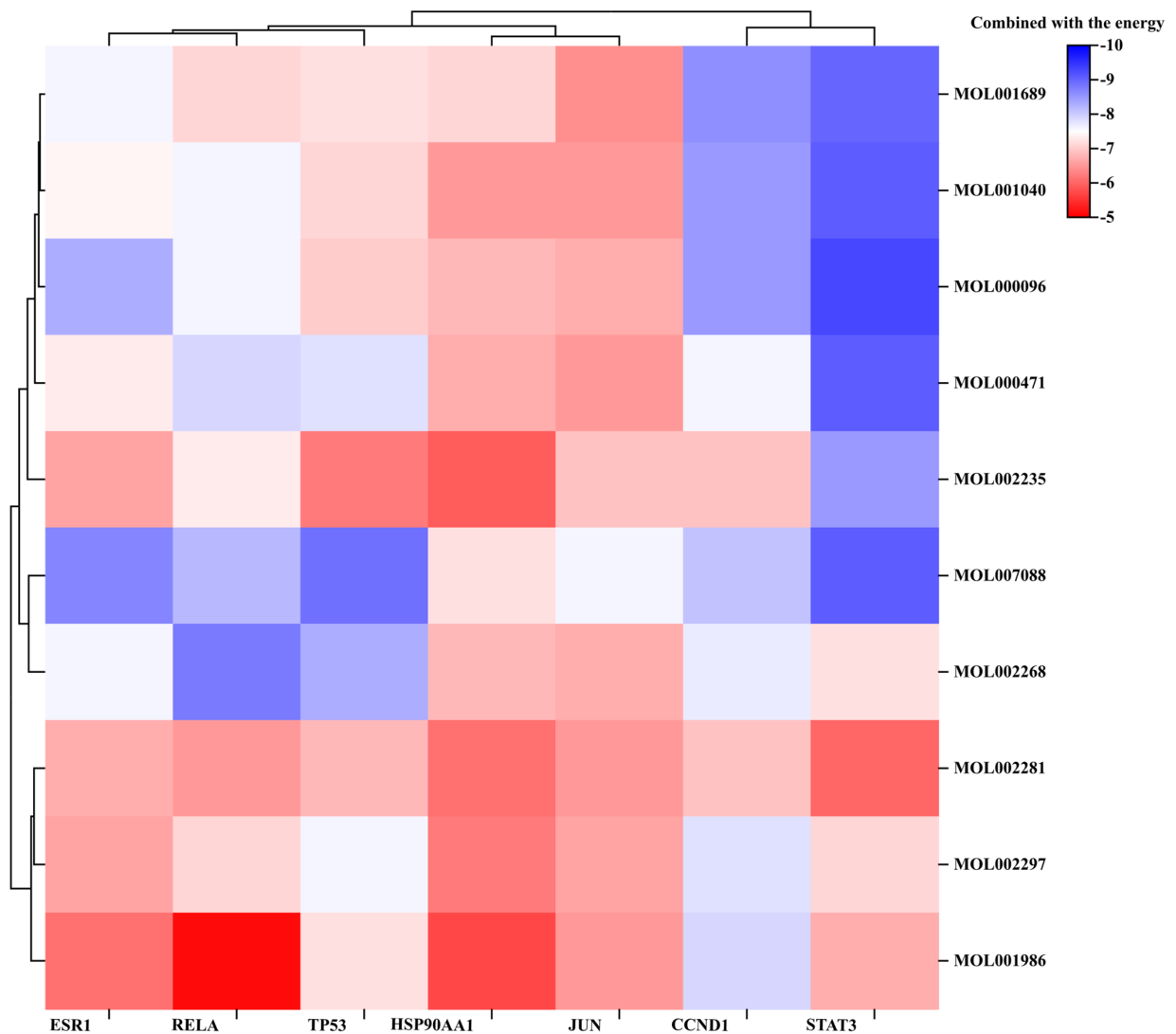
#### Flow cytometry determination of effect of NRDP on the cell cycle of TCMK-1 cells induced by high glucose

After 24 h of NRDP intervention, flow cytometry was used to determine the cell cycle of TCMK-1 cells. Compared to cells in the control group ( $60.1 \pm 0.70$ ), cells of the model group showed an increase in the percentage of cells in the G0/G1 phase ( $40.23 \pm 1.07$ ;  $P < 0.01$ ). Compared with the model group, the percentage of cells in the G0/G1 phase increased and that of the cells in the S phase decreased after NRDP intervention (NRDP-L:  $45.55 \pm 1.23$ , NRDP-M:  $48.93 \pm 0.75$ , NRDP-H:  $61.04 \pm 1.66$ ;  $P < 0.01$ ). Thus, NRDP could block TCMK-1 cells in the G0/G1 phase (Figure 5C).

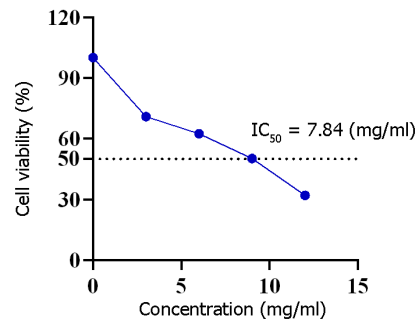
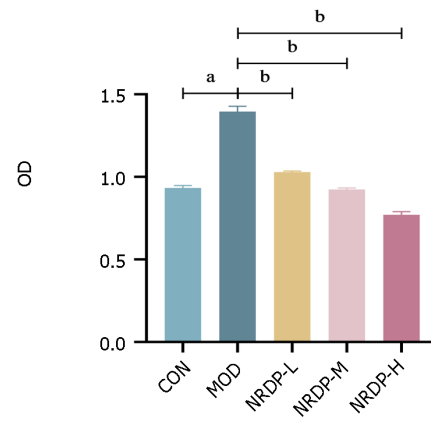
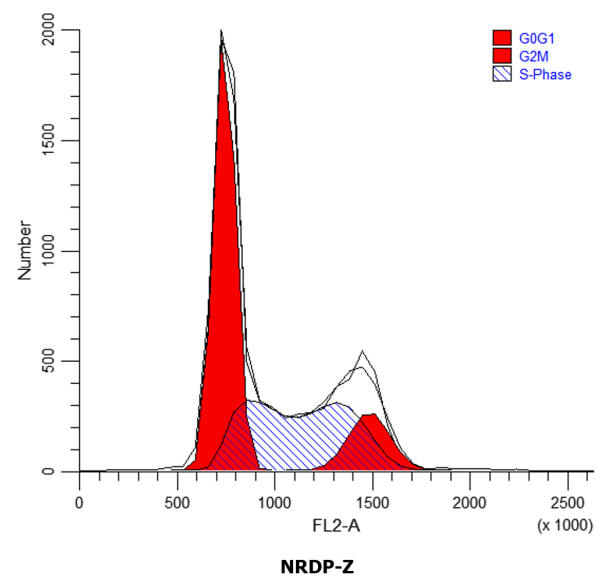
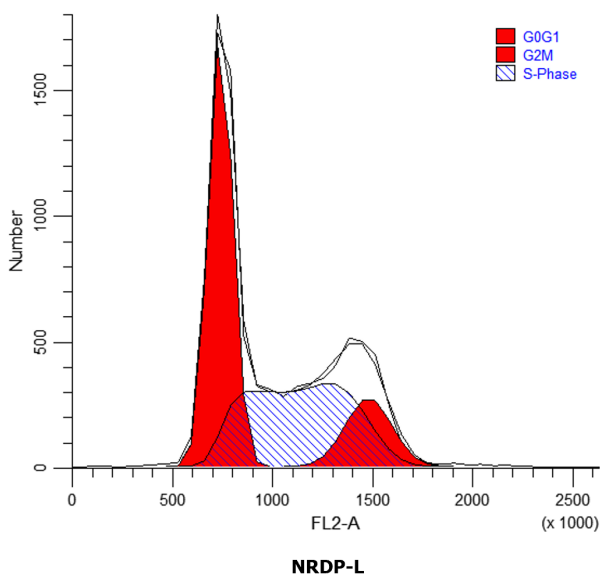
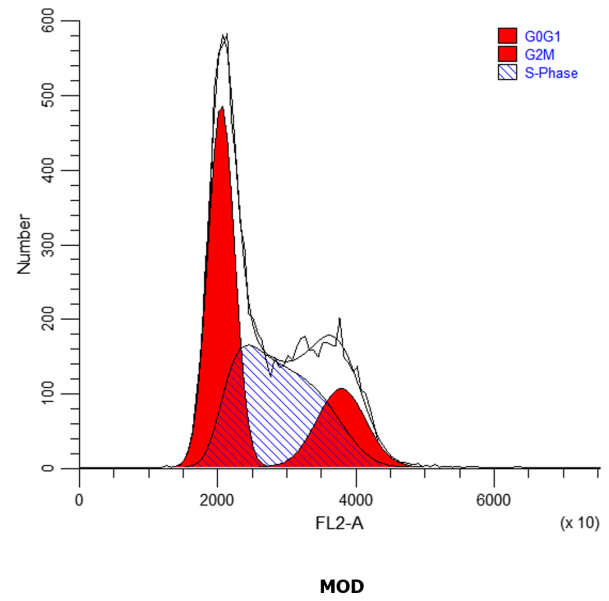
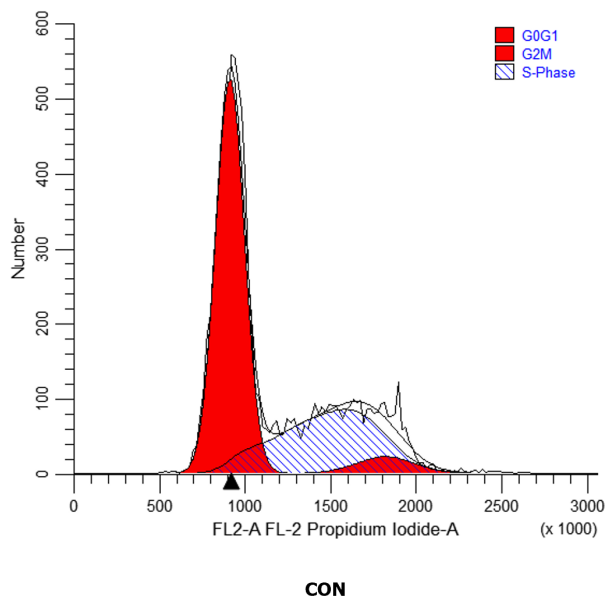
**A**



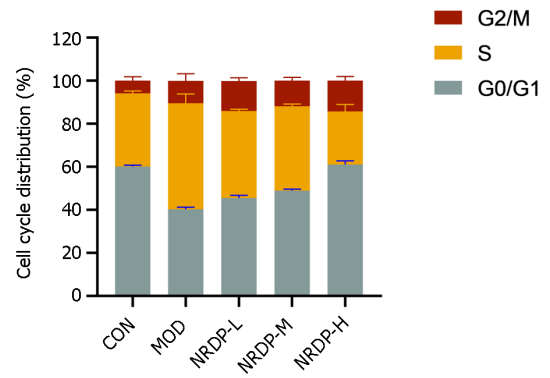
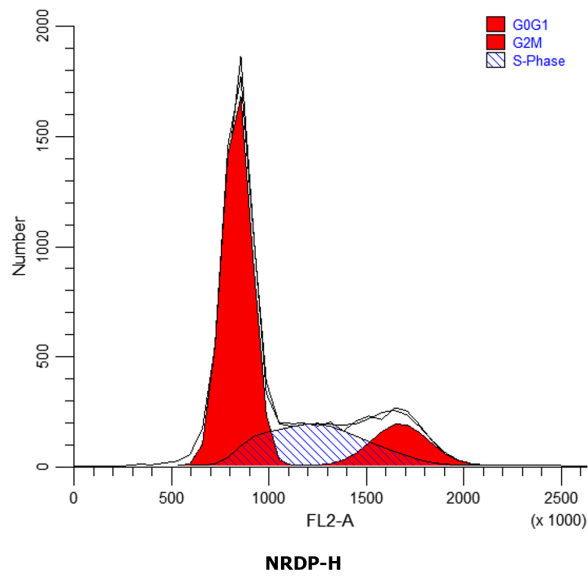
**B**



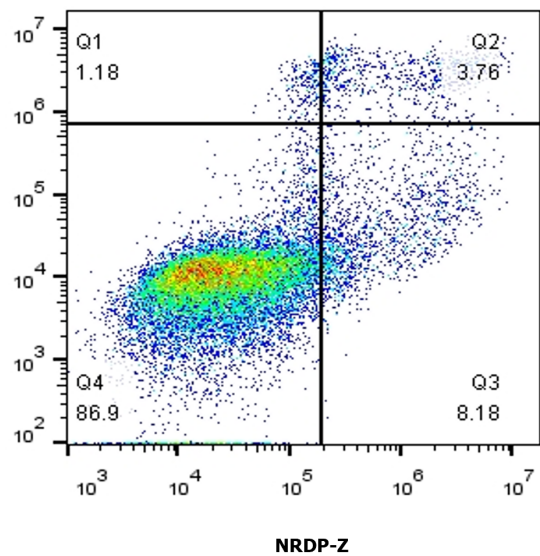
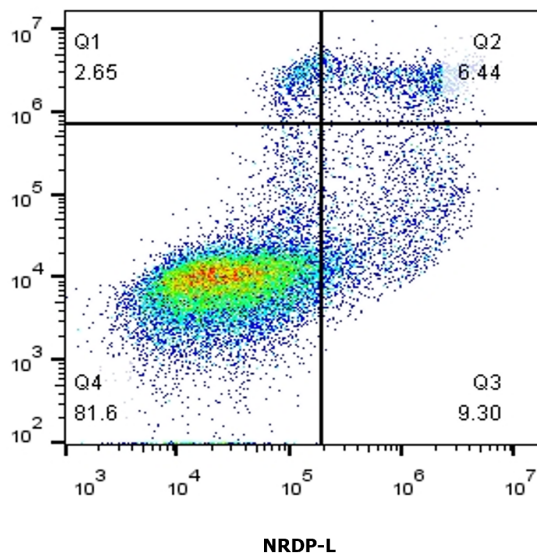
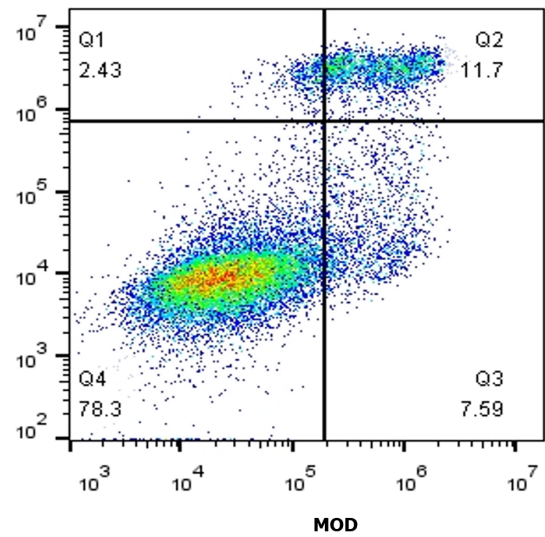
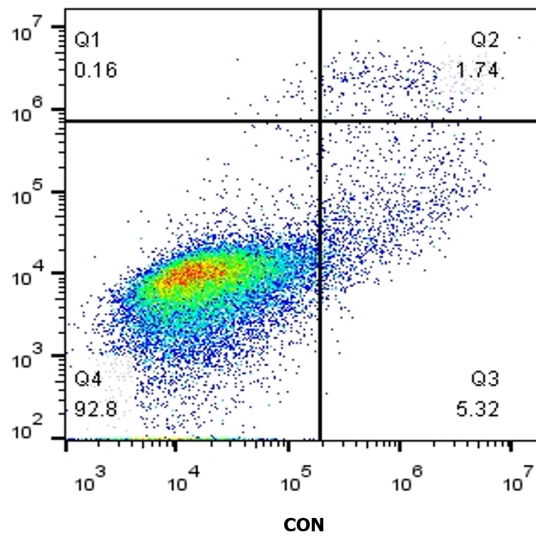
**Figure 4 Molecules docking.** A: Docking results between *Nardostachyos Radix et Rhizoma-rhubarb* drug pair components and core target molecules; B: Heat map of molecular docking showing the binding energy of core target molecules.

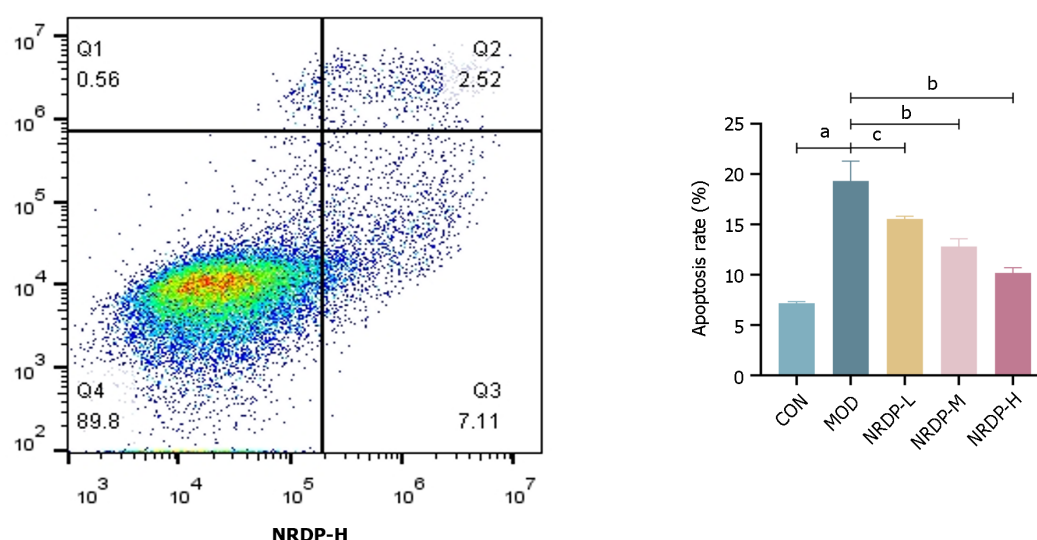
**A**

**B**

**C**






D





**Figure 5** Effect of *Nardostachyos Radix et Rhizoma-rhubarb* drug pair on cellular phenotype of TCMK-1 cells. A and B: *Nardostachyos Radix et Rhizoma-rhubarb* drug pair (NRDP) reduces the activity of TCMK-1 cells; C: Effect of NRDP on the cell cycle of TCMK-1 cells; D: Effect of NRDP on apoptosis of TCMK-1 cells. CON: Control group; MOD: Model group; NRDP: *Nardostachyos Radix et Rhizoma-rhubarb* drug pair; IC<sub>50</sub>: Half maximal inhibitory concentration. <sup>a</sup>*P* < 0.001, <sup>b</sup>*P* < 0.001, <sup>c</sup>*P* < 0.01.

### Flow cytometry detection of effect of NRDP on apoptosis of TCMK-1 cells induced by high glucose

Apoptosis of TCMK-1 cells was detected by AV-PI double staining and flow cytometry after 24 h of NRDP intervention. Compared with that of the control group ( $7.17 \pm 0.168$ ), the apoptosis rate of TCMK-1 cells in the model group was significantly increased ( $19.32 \pm 1.975$ ; *P* < 0.01). Compared with that of the model group, the apoptosis rate in the NRDP groups was decreased (*P* < 0.01), and with the increase of NRDP concentration, the apoptosis rate of TCMK-1 cells decreased more significantly (NRDP-L:  $15.55 \pm 0.257$ , NRDP-M:  $12.80 \pm 0.773$ , NRDP-H:  $10.18 \pm 0.523$ ; Figure 5D).

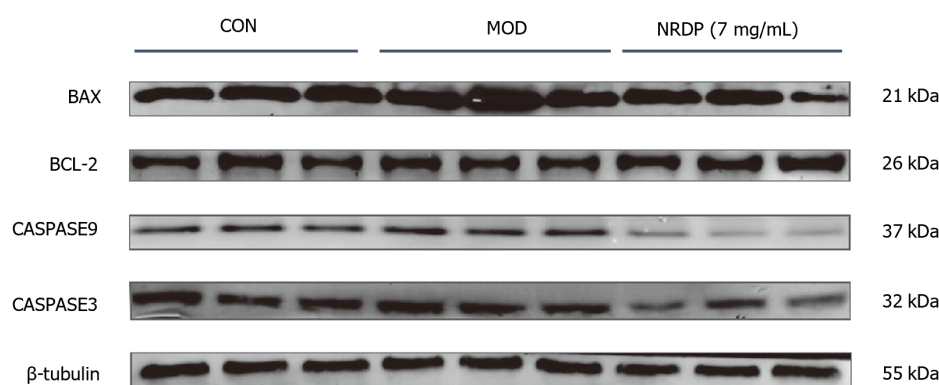
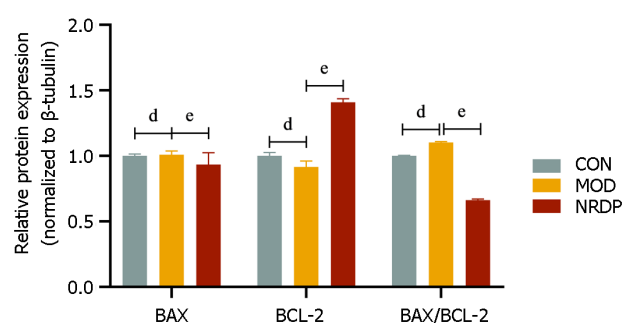
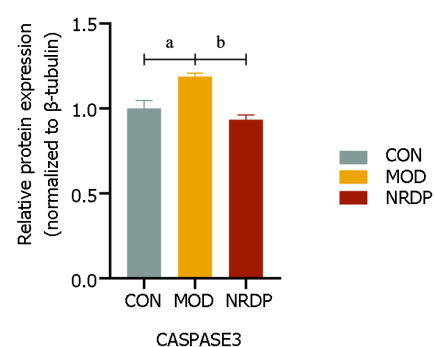
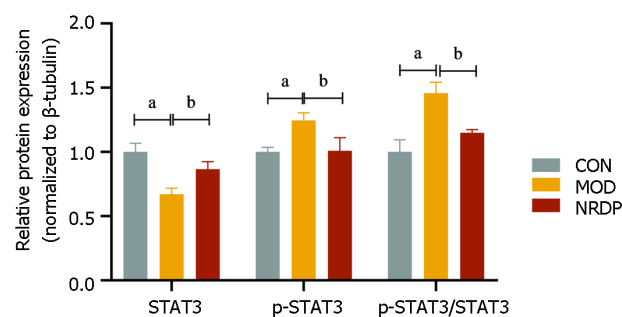
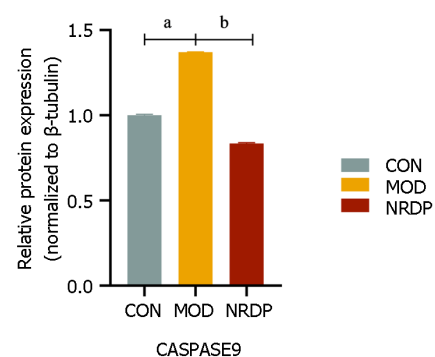
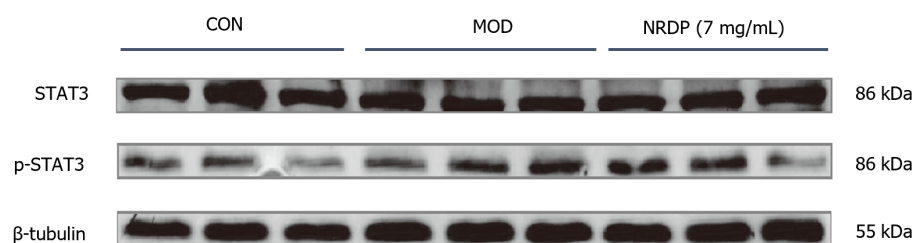
### Western blot analysis of expression of core proteins

Western blot was used to detect the expression of core proteins in TCMK-1 cells after NRDP intervention. The expression of p-STAT3, BAX, Caspase3, and Caspase9, as well as BAX/BCL-2 and p-STAT3/STAT3 ratios, was increased in the model group compared with the control group (*P* < 0.01), while the expression of BCL-2 and STAT3 proteins was decreased (*P* < 0.01). After intervention with NRDP, the expression of p-STAT3, BAX, Caspase3, and Caspase9, as well as BAX/BCL-2 and p-STAT3/STAT3 ratios, was decreased (*P* < 0.01 or *P* < 0.05), and the expression of BCL-2 and STAT3 proteins was increased (*P* < 0.01) (Figure 6).

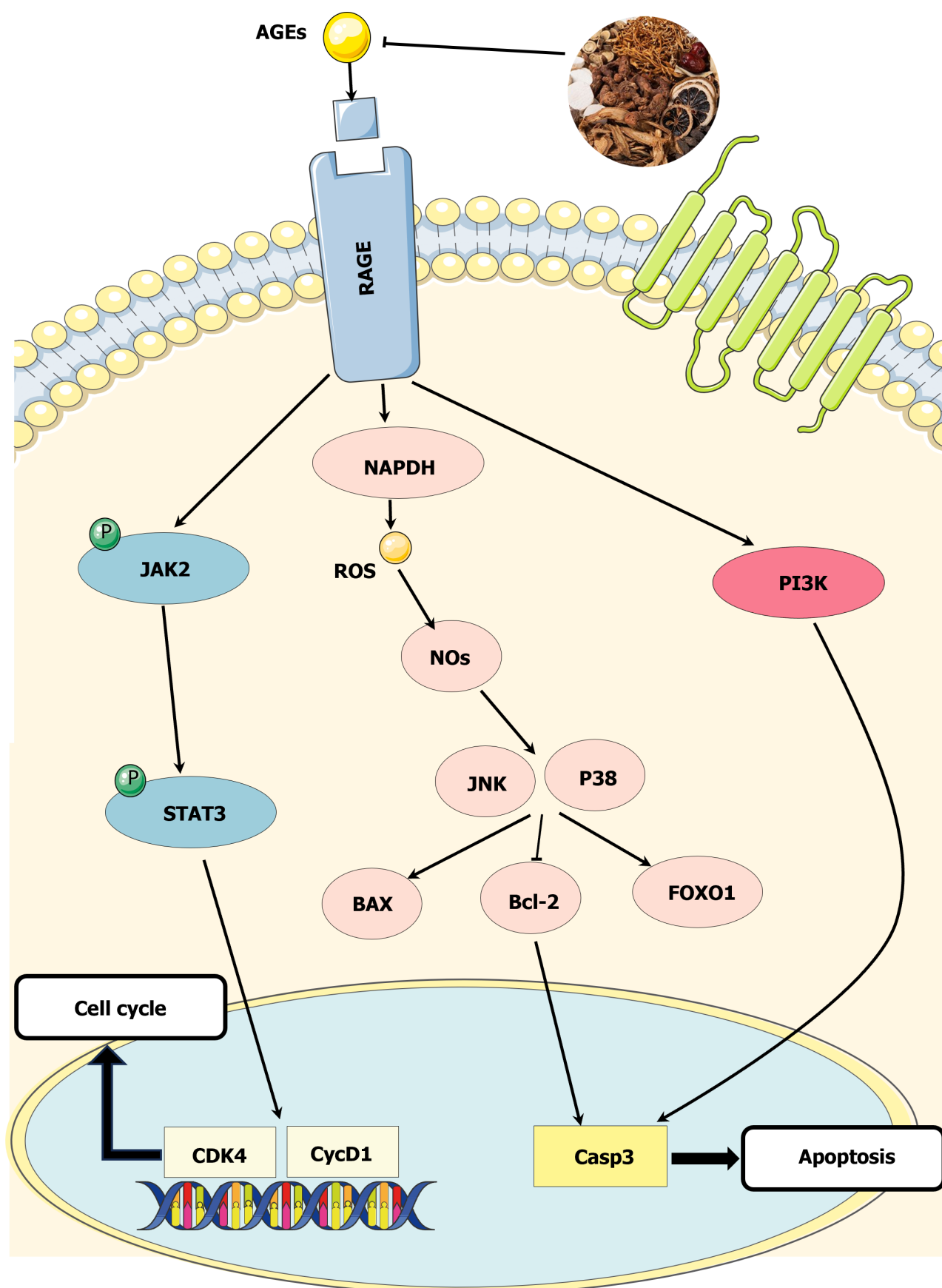
## DISCUSSION

This study investigated the mechanism of action of NRDP on DKD using network pharmacology. The results showed that NRDP had a therapeutic effect on DKD, with 10 active ingredients involving 85 targets. The GO, KEGG, and network interaction analyses revealed that NRDP may act on DKD through the AGE-RAGE signaling pathway (Figure 7). Our *in vitro* cell experiments confirmed that NRDP significantly inhibited TCMK-1 proliferation, promoted cell cycle arrest at the G0/G1 phase, and reduced the apoptosis of TCMK-1 cells in a dose-dependent manner. The results of the Western blot analysis indicated that NRDP intervention led to up-regulation of BCL-2 and STAT3 protein expression, and down-regulation of p-STAT3, BAX, Caspase3, and Caspase9 protein expression. Additionally, the BAX/BCL-2 and p-STAT3/STAT3 ratios were reduced. These findings suggest that NRDP is an effective treatment for DKD. NRDP protects renal tubular epithelial cells from high glucose-induced damage by regulating the AGE-RAGE signaling pathway.

AGEs are a group of complex molecules that form through non-enzymatic reactions between proteins or lipids and glucose or other carbohydrate derivatives. Their receptor RAGE is a multi-ligand receptor belonging to the immunoglobulin superfamily and is expressed in a wide range of tissues, including the vascular system, lung, heart, endothelial, and nervous tissues[23]. Their binding forms a key pathophysiological process associated with the occurrence and progression of many diseases, especially diabetes complications. AGEs from hyperglycemia interact with RAGE to activate many downstream effectors, including the JAK/STAT pathway, which in turn activates transcription factors like STAT3 over time[24]. This increases the inflammatory response and further exacerbates DKD[25]. Tang et al[26] showed through network pharmacology that the AGE-RAGE pathway is the most important pathway for *Coptis Jiedu* decoction to treat DKD, and *in vivo* experiments verified that *Coptis Jiedu* decoction can improve glucose and lipid metabolism disorder and kidney injury by regulating the AGEs-RAGE-AKT-Nrf2 pathway in db/db mice, thus playing a protective role in DKD. Hou et al[27] showed that salvianolic acid A inhibited AGEs-induced actin cytoskeletal rearrangement

**A**

**B**

**C**

**D**

**E**

**F**


**Figure 6** Effect of *Nardostachyos Radix et Rhizoma-rhubarb* drug pair on AGE-RAGE signaling pathway in TCMK-1 cells. A, B, C, and E: Protein expression of BAX, BCL-2, Caspase9, and Caspase3; D and F: Expression of STAT3 and p-STAT3 protein. CON: Control group; MOD: Model group; NRDP: *Nardostachyos Radix et Rhizoma-rhubarb* drug pair. <sup>a</sup>*P* < 0.001, <sup>b</sup>*P* < 0.001, <sup>c</sup>*P* < 0.01, <sup>d</sup>*P* < 0.05, <sup>e</sup>*P* < 0.05.



**Figure 7 Mechanism of *Nardostachys Radix et Rhizoma-rhubarb* drug pair in treatment of diabetic kidney disease.** NAGE: Receptor for advanced glycation end products; ROS: Reactive oxygen species.

through the AGEs-RAGE-Rhoa-Rock pathway, restored glomerular endothelial permeability, weakened AGEs-induced oxidative stress, restored glomerular endothelial function, alleviated renal structural deterioration, and effectively improved early DKD. The changes in the expression of relevant proteins after NRDP intervention in this study showed that the drug alleviated DKD symptoms to some extent.

Numerous studies have demonstrated that the production of AGEs linked to hyperglycemia is a key factor in the pathophysiology of DKD. The RAGE binds to its ligands, inducing oxidative stress and chronic inflammation in renal tissue, ultimately resulting in renal dysfunction. AGEs can alter the extracellular matrix by involving cell surface receptors and producing proinflammatory cytokines. RAGE and its ligands promote angiogenesis, cell migration, proliferation, invasion, and metastasis by limiting apoptotic cell death[28]. Studies have shown that AGEs and their receptor RAGE can induce apoptosis in different cell types. The propagation of apoptosis through the AGE-RAGE signaling pathway involves the cascade reaction of the pro-apoptotic factor, which prompts the apoptotic signal to activate the apoptotic factor Caspase3[29] and initiates the occurrence of apoptosis. Under the influence of certain receptors and factors, the endogenous apoptotic pathway is activated and regulated by the BCL-2 protein, which directly activates Caspase9. The Caspase cascade can activate Caspase3 during apoptosis induced by death receptors and DNA damage, producing intracellular signals that act on cellular targets, ultimately leading to programmed cell death[30]. Previous studies have demonstrated that RAGE expression regulates apoptotic death receptors and mitochondrial pathways by controlling the expression of pro-apoptotic Caspase3, Caspase9, and anti-apoptotic BCL-2. BAX, a proapoptotic protein, and BCL-2, a regulatory protein of apoptosis, can form Bax-Bcl-2 heterodimers when BCL-2 binds to active BAX protein in the cytoplasm, thus playing a role in reducing apoptosis. Reducing the activity of the BAX protein can also negatively regulate apoptosis. The amount of apoptosis can be determined by the degree of binding between BAX and BCL-2. Reducing the activity of BAX and promoting the binding of BCL-2 to BAX protein can reduce apoptosis[31]. Our study found that the expression of apoptosis-related proteins in TCMK-1 cells was detected after the intervention of NRDP. The expression of BAX, Caspase3, and Caspase9 proteins was downregulated, while the expression of BCL-2 protein was upregulated. This may be due to the induction of BCL-2 expression by NRDP. The inhibition of BAX protein activity resulted in a weakened Caspase family cascade and reduced apoptosis of renal tubular epithelial cells. This illustrates the pharmacological effect of NRDP in treating DKD.

In summary, NRDP may prevent TCMK-1 cells from proliferating and reduce cell death by controlling the relevant proteins of the AGE-RAGE signaling pathway, thereby protecting the function of intrinsic kidney cells during high glucose levels. Currently, there are numerous studies on the pathogenesis of DKD, which can be summarized as the result of a combination of metabolic, inflammatory, hemodynamic, and fibrotic factors. Many scholars have explored the treatment of DKD. Some treatments targeting specific pathogenic mechanisms are often used in clinical and experimental studies. Combination therapies involving two or more drugs have been found to have the potential to treat DKD. For instance, combining ERA with SGLT2 inhibitors has shown promise[32]. The present study also validated the efficacy of a herbal combination for treating DKD, providing a preliminary possibility for future exploration of new combinations of traditional Chinese medicine combined with other inhibitors and drugs for treating DKD. However, this study was only limited to *in vitro* cellular experiments due to funding constraints. Our group's research on treating DKD with traditional Chinese medicine is ongoing, and we plan to incorporate high-throughput histological methods for further validation in the future. We will use high-throughput genomics methods for the validation and identification of a safe and effective clinical treatment for DKD, which will improve the prognosis and quality of life of such patients.

## CONCLUSION

In this study, TCMK-1 cells were treated with varying concentrations of NRDP in a hyperglycemic environment. The results indicated that NRDP can regulate the cell cycle of TCMK-1 cells by blocking them in the G0/G1 phase, affecting the process from the late stage of DNA synthesis to the completion of mitosis and reducing apoptosis in a dose-dependent manner. Additionally, NRDP may upregulate the expression of BCL-2 and STAT3. The expression of p-STAT3, BAX, Caspase3, and Caspase9 proteins was downregulated, as well as the BAX/BCL-2 and p-STAT3/STAT3 ratios. Consequently, the impaired AGE-RAGE signal axis has a greater impact on the body during high glucose conditions, and the high glucose environment has a protective effect on renal tubular epithelial cells. This lays the foundation for the search for safe and effective drugs to treat DKD.

## ARTICLE HIGHLIGHTS

### Research background

Diabetic kidney disease (DKD) is one of the serious complications of diabetes mellitus. It has a poor prognosis and is one of the causes of end-stage renal disease. Existing treatments can improve the symptoms of DKD to some extent. However, they have the disadvantages of side effects and high price.

### Research motivation

We performed *in vitro* cellular experiments to validate the effectiveness of the *Nardostachyos Radix et Rhizoma*-rhubarb drug pair (NRDP) and to provide new ideas for clinical treatment of DKD.



## Research objectives

In this study, we used network pharmacology and molecular docking to predict the targets of NRDP for the treatment of DKD and validated the prediction findings using cellular experiments.

## Research methods

Targets for NRDP and DKD were obtained using databases such as TCMSP, Genecards, OMIM, and TTD. Drug-disease intersection targets were obtained based on the Venny 2.1 database and "drug-component-target-disease" network was constructed. Afterward, Kyoto Encyclopedia of Genes and Genomes pathway and Gene Ontology enrichment analyses were performed to further observe the relationship between targets and pathways. Finally, molecular docking was performed on the active ingredients of NRDP. Experiments such as the CCK-8 method, flow cytometry, and Western Blot were used to verify the molecular mechanism of NRDP for DKD.

## Research results

NRDP may inhibit the viability of high glucose-induced TCMK-1 cells by modulating the advanced glycation end products (AGEs)-receptor for AGEs (RAGE) signaling pathway, thereby blocking cell cycle progression in the G0/G1 phase and reducing apoptosis. It also downregulated the protein expression of p-STAT3, BAX, Caspase3, and Caspase9, and up-regulated the protein levels of BCL-2 and STAT3. These findings verified that NRDP could reduce high glucose-induced TCMK-1 cell injury, thereby restoring their function.

## Research conclusions

NRDP may achieve its therapeutic effect on DKD by modulating the AGE-RAGE signaling pathway. NRDP arrests the cell cycle progression at the G0/G1 phase by inhibiting the proliferation of high glucose-induced TCMK-1 cells and reducing their apoptosis. NRDP inhibits the expression of proteins related to the AGE-RAGE signaling pathway in high glucose environment, which delays the progression of DKD.

## Research perspectives

We next plan to conduct *in vivo* animal and omics experiments. To determine the specific components of NRDP in the blood for the treatment of DKD, gene detection will be performed by high-throughput validation methods such as transcriptomics, in order to provide a safe and effective method for clinical treatment of DKD.

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## FOOTNOTES

**Author contributions:** Che MY conducted most of the experiments, analyzed the data, completed the figure production, and wrote the manuscript; Min J carried out a portion of the experiments and participated in the production of the figures and the composition of the manuscript; Xu DJ and Liu WJ carried out part of the experiments and participated in the statistical analysis of the data; Lu DD and Wang KL performed the network pharmacology prediction; Yuan L and Wang YY designed the study; Nan Y revised and improved the manuscript; all authors approved the final version of the article.

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