Protective Mechanism of Qi-Dan-Dihuang Decoction in Diabetic Kidney Disease Based on Network pharmacology

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Abstract

Background: Qi-Dan-Dihuang Decoction (QDD) has been used for treating diabetic kidney disease (DKD), but the mechanisms are poorly understood. The aim of this study is to reveal the therapeutic effects and the mechanism of QDD in ameliorating DKD by network pharmacology, in vivo, and in vitro studies.

Methods: The effect of QDD on body weight, fast blood glucose, oral glucose tolerance test (OGTT), 24 h urinary protein (24hU-Pro), serum creatinine (Scr), blood urea nitrogen (BUN), and pathological evaluation in kidney were investigated *in vivo* using C57BLKS/J db/db mice. The main active compounds of QDD, compound-disease interaction targets, and related processes and pathways were discerned by network pharmacology analysis through Chinese Medicine Systems Pharmacology Database (TCMSP) and TCM Database@Taiwan. The protein-protein interaction (PPI) network were established through STRING database. GO and KEGG pathway were used for analysis processes and pathways. Then Western blot was used to verified the predicted results. Finally, cell viability, wound healing and mainly pathway protein expression were detected *in vitro* using renal tubular epithelial cells HK-2 and NRK-52E cells.

Results: Although QDD treatment showed no significant difference in FBG and AUC of OGTT, but had significant reduction in Scr level in C57BLKS/J db/db mice. Histopathologic results showed that QDD ameliorated the expansion of mesangial area, thickened membranes of Bowman's capsules and basement membrane of glomerular capillaries, renal tubular epithelial cells vacuolar degeneration and reversed the glomerular and tubulointerstitial in C57BLKS/J db/db mice. For network pharmacology analysis of QDD, 143 active compounds related to 274 possible targets in QDD obtained and 117 compound-disease interaction targets were screened out combining with Genecards database. 18 key targets was excavated through network topological analysis. GO and KEGG pathway enrichment analysis showed that compound-disease interaction targets were significantly enriched in processes and pathways that are closely related to DKD. Western blot results showed that QDD significantly attenuated EMT-related proteins, p-NF-κb, IL-1β, IL-18, p-p38MAPK/p38MAPK, p-AKT/AKT, and p-mTOR/ mTOR protein expressions. Treatment with QDD could alleviate cell viability damaged, EMT process, p-NF-κb, IL-1β, IL-18, p-p38MAPK/p38MAPK, p-AKT/AKT and p-mTOR/ mTOR protein expressions by high D-gulcose.

Conclusions: This study provides convincing evidence suggest that QDD protects renal fibrosis of DKD, by regulating EMT in RTECs and inflammatory response through p38MAPK and AKT/mTOR signaling pathways.

1. Introduction

Diabetic kidney disease (DKD) is a common complication of diabetes mellitus, which refers to specific pathological structural and functional changes in the kidney of diabetes mellitus (DM) patients. It is clinically characterized by proteinuria, hypertension, and progressive reduction in renal functions[1, 2]. The pathologic changes in diabetic kidney disease include renal hypertrophy and extracellular matrix accumulation, which contribute to glomerular sclerosis, and gradually lead to renal failure through the

tubular interstitial fibrosis. Among 170 million DM patients are suffered renal failure[3], which has been the leading cause of end-stage renal disease (ESRD) worldwide. Despite rennin-angiotensin system inhibitor has good glycemic and blood pressure control, which is the standard treatment for DKD, the risk of ESRD remains high [4]. It not only causes serious health problems, but casts heavy medical burden to individuals and the society. Therefore, there is an urgent need for more effective treatment to prevent the development of DKD.

Traditional Chines Medicine (TCM), being widely used in the management of DKD in China for thousands of years, demonstrated remarkable efficacy in decreasing blood glucose, reducing urinary albumin, protecting renal function, and improving life qualities [5–7]. Increasing numbers of studies have been focusing on how traditional Chinese herbs exert their effects. Qi-Dan-Dihuang Decoction (QDD) is developing based on the etiology and pathogenesis of DKD in TCM, “Qi and Yin deficiency with blood stasis”, and combined with evidence-based data analysis[8]. QDD is composed of *Hedysarum Multijugum Maxim* (HMM, Huangqi), *Radix Salviae* (RS, Danshen), *Rehmanniae Radix* (RR, Dihuang), *Rhizoma Dioscoreae* (RD, Shanyao), and *Licorice* (LC, Gancao), with a weight ratio of 6 : 3 : 3 : 3 : 1. This Chines patent formula has been proven to reduce urinary albumin and protect renal functions both in diabetic patients and STZ-induced diabetic rats[9–11]. However, the active compounds in QDD and its specific molecular mechanisms in the treatment of DKD remain unclear.

Network pharmacology, as an new discipline designed to tackle the increasing failure rate of new-developed medicine clinical trials over the past decades[12], clarifies the synergistic effects of biological network model which combines active molecular screening, target prediction, network construction and analysis, helping to understand the relationships between ingredients, genes, proteins and diseases. Based on high-throughput omics data analysis, virtual computing technology and network database retrieval, it provides a new perspective in the discovery of the underlying mechanisms of TCM. Therefore, this study aims to identify pharmacological mechanism of QDD in ameliorating DKD based on network pharmacology, and verify the prediction *in vivo* and *in vitro* experiments.

2. Materials And Methods

2.1. Preparation of QDD Aqueous Extract

Hedysarum Multijugum Maxim (HMM), *Radix Salviae* (RS), *Radix Rehmanniae* (RR), *Rhizoma Dioscoreae* (RD), and *licorice* (LC), with a weight ratio of 6 : 3 : 3 : 3 : 1, were obtained from TCM dispensary in Southern Hospital. A total of 160 g herbs was soaked in 500 ml distilled water for 30 min, and then were decocted for 45 min twice. The solvent was merged and then lyophilized to powder. The lyophilized powder was re-dissolved in distilled water to 10 mg/ml, filtered with a 0.22 μm pore-size filter and stored at -20°C for future use.

2.2. Animals and experimental design

24 C57BLKS/J db/db mice (25-30 g) and 6 db/m mice (18-20g) were purchased from Model Animal Research Center of Nanjing University (Nanjing, China, license number: SCXK2015-0001). Mice were fed a standard mouse diet and water ad libitum, and housed under the condition of 12 h light-dark cycle with temperature of 23 ± 1℃ and humidity of 53 ± 2%. After 1 week of acclimation, db/db mice were randomly divided into four experimental groups as follows (n = 6 for each group): (1) Model Group, fed with normal diet; (2) Dapagliflozin group, fed with dapagliflozin (50 mg/kg dapagliflozin in diet); (3) QDD-L group, fed with 2% QDD (20 mg/kg QDD in diet); (4) QDD-H group, fed with 4% QDD (40 mg/kg QDD in diet). The age-matched db/m mice were used as a control group and fed with normal diet. All procedures were approved by the Standards for Animal Ethics in Southern Medical University.

2.3. Biochemical Analysis

The body weight was measured weekly. Fast blood glucose and oral glucose tolerance test (OGTT) were assesses every four weeks. After 12 weeks of administration, 24 h urine of mice was collected for 24 h urinary protein (24hU-Pro) detection using Urine protein test kit (Nanjing Jiancheng Bioengineering Institution, China). Serum and kidney were dissected from mice after anesthetized with 1% pentobarbital (40 mg/kg). Serum creatinine (Scr) and blood urea nitrogen (BUN) were measured with serum creatinine and BUN assay kit (Nanjing Jiancheng Bioengineering Institution, China).

2.4. Morphologic Evaluation

Kidney tissues fixed in 10% formalin in phosphate buffer (pH 7.4) were embedded in paraffin and 4 μm thick slices and subsequently stained with the hematoxylin-eosin (HE), periodic acid-Schiff (PAS), and Masson methods. Images under bright light microscope were acquired with a digital camera with magnification ×400 (Nikon, Japan).

2.5. Identification of candidate compounds in QDD

Compounds of four herbs (HMM, RS, RD, and LC) in QDD were obtained from Traditional Chinese Medicines Systems Pharmacology (TCMSP, http://lsp.nwsuaf.edu.cn/tcmsp.php) database. The compounds of RC were obtained from TCM Database@Taiwan (http://tcm.cmu.edu.tw/zh-tw/index.php) because there is no item for RC in TCMSP database.

2.6. Screening for active compounds

The properties of absorption, distribution, metabolism and excretion (ADME) are important indicators for pharmacokinetics of compounds and help to minimize their potential drug-drug interactions in herbs[13]. Oral bioavailability (OB), drug-likeness (DL), half-life (HL), and Caco-2 permeability (Caco-2) are ADME-related parameters are employed to screen bioactive compounds. In this study, OB ≥ 30%, DL ≥ 0.18, Caco-2 ≥ -0.4, and HL ≥ 4 were used to selecte active compounds for further analysis as previous study recommended[14].

2.7. Identification of drug targets and compounds-targets network construction

The targets of active compounds were identified from TCMSP database and then transformed into official symbols *via* the UniProt KB search function in the UniProt database (http://www.uniprot.org/) by entering the protein name and defining the species as "homo sapiens". According to the relationship between herbs, active ingredients and corresponding targets, the "herb-compound-target" network were established by Cytoscape 3.6.1. The nodes in the network represent herbs, active compounds or targets, and the edges indicate the relationships between the herbs and components, or compounds and targets. Then the network analyzer plugin in the software were used to analyze the topological properties of the network, such as degree, betwe/enness centrality and closeness centrality.

2.8. Collection of genes related to DKD fibrosis

The GeneCards database (https://www.genecards.org/) provides comprehensive information on all annotated and predicted human genes, which integrates gene-centric data from about 150 web sources including genomic, transcriptomic, proteomic, genetic, clinical and functional information. The related genes of DKD and fibrosis together with scores that ranks genes by how closely they are associated with the disease were obtained from GeneCards by the searching strategy “([disorders] (diabetic AND nephropathy) OR [disorders] (diabetic AND kidney AND disease)) AND [disorders] (fibrosis)”.

2.9. PPI network construction and key targets identification

The compound-disease interaction targets were obtained by intersecting targets in compounds and disease. The PPI network data was imported from the STRING database (https://string-db.org/) by inputting the protein symbols of interaction targets and choosing “Homo Sapiens” as organism. The Cytoscape was used to draw the PPI network, whose topological features, including degree centrality (DC), betweenness centrality (BC), and eigenvector centrality (EC), Closeness centrality (CC), Network centrality (NC) and Local average connectivity (LAC), were subsequently analyzed by cytoNCA[15, 16].

2.10. Gene Ontology and KEGG pathway enrichment analysis for targets

DAVID Bioinformatics Resources 6.8 (https://david.ncifcrf.gov/) was used to subject GO and KEGG pathway enrichment analysis for compound-disease interaction targets, and $p < 0.05$ was considered significant with hypergeometric test.

ClueGO was utilized to assess the enriched KEGG pathways of interaction targets [17], only terms with at least 5 input genes and 5% of term associated genes were enriched, and terms with $p < 0.05$ as well as kappa score > 0.3 were shown.

2.11. Cell culture and treatment

Human and rat renal tubular epithelial cells HK-2 and NRK-52E cells were purchase from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). NRK-52E cells were maintained in Dulbecco’s Modified Eagle Medium (DMEM; Gibco, USA), and HK-2 cells were cultured in DMEM/F-12

medium (Gibco, USA), in a humidified atmosphere with 5% CO_2 at 37°C. Culture medium for all cells was supplemented with 10% fetal bovine serum (Biological Industries, Israel), 100 U/mL penicillin and 0.1 mg/mL streptomycin (Invitrogen, USA). 30 mmol/L D-glucose in medium for 48 h was used to induce epithelial-mesenchymal transition (EMT) process in the cells.

2.12. Cell viability assays

Cells were seeded in 96-well plates in the density of 3×10^4 cells/well. After 24 h treatment with different mediums, cells were incubated with MTT for 4 h and 100 μL DMSO was added to each well. The absorbance was recorded at 570 nm by Multiskan FC Microplate Reader (Thermo Scientific, USA)

2.13. Wound healing assay

Cells were plated onto 6-well plates until confluence. Wounds were scratched by 200 μl pipette tip. The culture medium was replaced with fresh serum-free medium. The pictures were taken by an inverted microscope (Nikon, Japan) every 24 h. The wound areas were measured and the relative wound measured were calculated.

2.14. Western Blotting

The tissues and cells were lysed in RIPA buffer (Beyotime, China) and protein concentration was measured by BCA Protein Assay Kit (Beyotime, China). A total of 30 μg of total protein was separated by 10% SDS-PAGE and then transferred to a polyvinylidene difluoride film (Millipore, MA, USA). Immune complexes were detected using enhanced chemiluminescence (ECL kit, Affinity, USA). Protein expression levels were normalized to β-actin and quantified by Image J (version 1.6)

2.15. Statistical analysis

All data are expressed as the means ± SDs and analyzed by SPSS 20.0 (IBM, Armonk, USA). Tukey's test was used for multiple comparison. The values were considered statistically significant when $p < 0.05$.

3. Results

3.1. QDD alleviates renal dysfunction and fibrosis in db/db mice

Compared to db/m mice, the fast blood glucose, area under the curve (AUC) of OGTT, 24 h U-Pro and Scr were significantly increased in db/db mice at 0, 4, 8, 12 weeks, which was reversed by dapagliflozin at 4, 8, 12 weeks. However, QDD treatment showed no significant difference in FBG and AUC of OGTT

compared to model group. QDD (2% and 4% in diet) and dapagliflozin treatment group mice were significantly decreased 24 h U-Pro and Scr levels (Figure 1A-G). BUN levels shown no significant differences in each group (Figure 1H).

The improvement effects of QDD on pathological changes are shown in Figure 2A-C. H&E staining showed that mesangial area moderately-expanded, membranes of Bowman's capsules and basement membrane of glomerular capillaries thickened, and renal tubular epithelial cell the vacuole denaturalized in kidney of model group. QDD and dapagliflozin could reversed the pathologic changes in different degrees. PAS and MASSON staining results showed that, compared with model group, QDD and dapagliflozin could reverse the kidney glycogen and collagen deposition in glomerular and tubulointerstitial. These results suggested that QDD ameliorates the renal pathological conditions by reducing the damages of kidney in the diabetic db/db mice.

3.2. Screening of active compounds in QDD

A total of 657 compounds were retrieved from TCMSP and TCM Database@Taiwan databases, among them, 87 in HMM, 202 in RS, 17 in RR, 71 in RD, and 280 in LC respectively. By ADME screening, 143 candidate compounds of QDD were identified after removing 7 duplicate ingredients. More than 50% compounds in HMM, RS, RD and LC, possess high OB value (Figure 3A), demonstrated that QDD exhibited high oral absorption and utilization rate. Furthermore, most of ingredients were exacted from LC, which could explain the reconcile function of LC in QDD.

3.3. Targets identification of QDD and DKD

Based on TCMSP database, except 9 compounds without any relevant targets, the potential targets of active ingredients in QDD were obtained. In total, 134 of the 143 compounds in QDD were associated with 273 targets after eliminating overlapping proteins. A compound-target network was constructed in Cytoscape (Figure 3C). In the compound-target relationship, top 10 compounds that connects the most targets were MOL000098 (quercetin), MOL000422 (kaempferol), MOL000006 (luteolin), MOL003896 (7-Methoxy-2-methyl isoflavone), MOL007154 (tanshinone□a), MOL000392 (formononetin), MOL000354 (isorhamnetin), MOL004328 (naringenin), MOL007100 (dihydrotanshinlactone), and MOL002565 (medicarpin), which come from LQ (Figure 3B). The major compounds connected with top ten targets were Prostaglandin-Endoperoxide Synthase 2 (PTGS2), Estrogen Receptor 1 (ESR1), Heat Shock Protein 90 Alpha Family Class B Member 1 (HSP90AB1), Androgen Receptor (AR), Calmodulin 1 (CALM1), Sodium Voltage-Gated Channel Alpha Subunit 5(SCN5A), Nitric Oxide Synthase 2 (NOS2), PPARG Coactivator 1 Alpha (PPARG), Serine Protease 1 (PRSS1), and Prostaglandin-Endoperoxide Synthase 1 (PTGS1) (Figure 3B). With high connectivity, these compounds and targets were probably the crucial molecules involved in the mechanisms underlying the therapeutic effects of QDD.

There were 1837 genes related to DKD and fibrosis from GeneCards, with the most relative genes being Insulin (INS), ACE (Angiotensin I Converting Enzyme), IL6 (Interleukin 6), and VEGFA (Vascular Endothelial Growth Factor A) (Figure 3D).

3.4. Network construction for Compound-Target-Disease

134 compounds and 146 compound-disease interaction targets were used to construct the compound-target-disease (CTD) network based on their relationships shown in Cytoscape (Figure 4A). In the CTD network, compounds or targets with bigger size indicates higher degree, such as quercetin, luteolin, PTGS2, and ESR1, which suggested quercetin and luteolin were probably contribute to the major therapeutic effect through PTGS2 and ESR1. However, some components only had one target, such as denudatin B, doradexanthin, gadelaidic acid, implied that these components may played a less important role or are poorly studied in their therapeutic effects in DKD. The network suggested that the therapeutic effects of QDD for DKD are mediated by multiple compounds *via* multiple targets.

3.5. PPI network of drug-disease interaction targets and network analysis

The PPI information of compound-disease interaction targets obtained from STRING were imported into Cytoscape to established the network (Figure 4B). The topological features of the network were calculated by cytoscape plugin CytoNCA. The median value of BC, CC, DC, EC, LAC, NC were 42.530605, 0.56640625, 40, 0.0694326, 28.2749445, and 31.0584795, respectively. Nodes were selected as hubs if the degree of the node is larger than 80, and 18 hubs selected were meet the criteria of key nodes whose BC, CC, EC, NC and LAC values were greater than the median of all nodes. The hubs include AKT1, TP53, MAPK3, VEGFA, IL6, CASP3, EGFR, TNF, MAPK1, EGF, JUN, MYC, STAT3, MAPK8, MMP9, PTGS2, ESR1, FOS (Figure 4C).

3.6. GO enrichment analysis

In GO enrichment analysis, there were enriched 300, 53, and 94 significantly GO terms in biological processes, cell components and molecular functions and listed and ranked by significance in descending order (Figure 5A). These terms are essential structures and functions for the survival of cells and organisms. Furthermore, there are many GO terms in biological processes corresponding to DKD fibrosis development processes, such as positive regulation of fibroblast proliferation, response to insulin, wound healing, inflammatory response, glucose metabolic process, response to leptin, response to oxidative stress, and epithelial to mesenchymal transition (Figure 5B).

3.7. Pathways enrichment analysis for key targets

To examine the genetic pathways that were manipulated by the compounds and influenced the disease development, the pathway network of interaction targets was constructed by KEGG and visualized by Cytoscape (Figure 6A). A total of 128 significant pathways were identified by KEGG enrichment analysis and the top 20 significantly enriched pathways were shown (Figure 6B-C). The potential targets were major distributed in endocrine and metabolic systems, immune and inflammatory systems, and signaling transduction and disease-related pathways. Signaling transduction pathways such as PI3K-Akt signaling pathway, VEGF signaling pathway, MAPK signaling pathway, mTOR signaling pathway, NF-κB signaling pathway, and TGF-beta signaling pathway, are closely related to the pathogenesis of DKD. Our study

found, in 123 enriched pathways, 12 and 15 pathways were classified to endocrine and immune systems, respectively, which constituted the core pathological mechanisms of DKD. Moreover, we also found that apart from type I and II diabetes mellitus, there were many other diseases highlighted, such as cancer and infectious disease. It indicated that QDD may had a potential therapeutic application for them, which matched the TCM theory of “Homotherapy for Heteropathy”. These results suggested that QDD could ameliorated DKD conditions by regulating the endocrine and immune systems through multiple signaling pathways.

3.8. QDD attenuated EMT in the kidney of db/db mice and renal tubular epithelial cells

As indicated by the network pharmacology prediction, many candidate targets of QDD were related to the EMT, which is a proven cause of renal fibrosis. Western blot was used to evaluate the expression levels of EMT-related proteins. As shown in Figure 7A, compared with control group, kidneys in model group displayed higher expression of fibronectin, N-cadherin, α-SMA, and Vimentin, and lower expression of ZO-1, E-cadherin. QDD and dapagliflozin treatments could remarkably reverse the changes in EMT-related proteins. These results suggested that QDD attenuated EMT process, which may underline its therapeutic effects on fibrosis and renal dysfunction in db/db mice.

In HK-2 and NRK-52E cells, high glucose treatment reduced the protein expressions of E-Cadherin, ZO-1, upregulated N-Cadherin, α-SMA, and Vimentin. QDD significantly increased the protein levels of E-Cadherin and ZO-1, decreased the protein levels of N-Cadherin, α-SMA, and Vimentin compared to high glucose group (Figure 7B). Then, we determined the effect of QDD on high glucose cultured tubular epithelial cell migration using wound healing assay. Relative healing areas in the wounded cells were measured at 0, 24, 48, and 72 h after the wound injury. As shown in Figure 7C, the relative healing areas were significantly increased by high glucose compared with normal group, and QDD significantly reduced healing areas compared with high glucose group, indicated that QDD inhibited high glucose-induced cell migration.

3.9. QDD attenuated inflammatory response in the kidney of db/db mice and renal tubular epithelial cells

Compared with control group, the protein expressions of p-NF-κB, IL-1β, and IL-18 in the kidneys were significantly increased in model group, and QDD group showed significant decreasing in p-NF-κb, IL-1β and IL-18 protein expressions (Figures 8A). *In vitro*, QDD suppressed the expressions of p-NF-κb, IL-1β and IL-18 in HK-2 and NRK-52E cells, which were upregulated by high glucose (Figure 8B), indicated that QDD was a promising intervention to resolve inflammation in the kidneys of db/db mice and high glucose-cultured renal tubular epithelial cells.

3.10. Effect of QDD on p38MAPK and AKT-mTOR signaling pathways

Network pharmacology predicted that QDD delayed the progression of DKD by regulating MAPK, PI3K-Akt-mTOR signaling pathways, western blot was used to detected the changes in MAPK and PI3K-Akt-mTOR effector proteins. As shown in Figure 9A, mice in model group possessed higher levels of p-p38MAPK/p38MAPK, p-AKT/AKT, and p-mTOR/mTOR than in control group, and QDD treatment significantly reduced the protein expressions of p-p38MAPK/p38MAPK, p-AKT/AKT, and p-mTOR/mTOR compared with model group. Moreover, QDD reduced the expressions of p-p38MAPK/p38MAPK, p-AKT/AKT, and p-mTOR/mTOR in HK-2 and NRK-52E cells, which were increased by high glucose (Figure 9B). These results suggest that QDD alleviated EMT and resolved inflammation in kidneys of db/db mice and high glucose-cultured RTECs probably through the p38MAPK, AKT-mTOR signaling pathways.

4. Discussion

In this study, we found that QDD treatment could reduced Scr level and ameliorated the histopathological damage in kidney in C57BLKS/J db/db mice. Then, we used network pharmacology to analysis the potential targets of QDD. PPI network analysis revealed that AKT1 and mTOR could be the key targets for renal protective effects of QDD. GO functionally analysis for biological process showed that EMT was significantly enriched, and KEGG pathway analysis elucidated that PI3K/AKT signaling pathway, mTOR signaling pathway and MAPK signaling pathway may be closely related to the therapeutic effects of QDD. Western blot of kidney tissues showed that QDD significantly attenuated EMT-related proteins, p-NF-κb, IL-1β, IL-18, p-p38MAPK/p38MAPK, p-AKT/AKT, and p-mTOR/ mTOR protein expressions. Treatment with QDD could alleviate cell viability damaged, EMT process, p-NF-κb, IL-1β, IL-18, p-p38MAPK/p38MAPK, p-AKT/AKT and p-mTOR/ mTOR protein expressions by high D-gulcose. According to the result, we hypothesize that QDD potentially attenuates renal fibrosis by regulating EMT via the p38MAPK and AKT/mTOR signaling pathways.

TCM has been used for DKD prevention and treatment for centuries. With the proven therapeutic efficacy and few adverse effects, TCM has attracted increasing attention and wide range of applications in DKD [18–20]. However, the complicated ingredients, miscellaneous targets and pathways are still the big challenge for TCM research. Meanwhile, DKD involves variety of physiological and pathological process[21], which makes it more difficult for us to reveal the mechanisms of action of the TCM formula therapeutic effects. Our previous studies demonstrated that QDD attenuated renal fibrosis in DKD by regulating the renin-angiotensin system and the NF-κB pathway [9, 22]. In the present study, we have an in-depth investigation on the therapeutic role of QDD in DKD.

The data in this study suggested that the mechanism of QDD for DKD treatment might have multiple components and multiple targets. Some compounds, which predicted in QDD, have been confirmed to be related to DKD in previous reported studies. For example, quercetin, a compound screened out from HMM and LC, protects renal function in STZ-induced diabetic nephropathy rats, with anti-oxidation, anti-fibrosis, anti-inflammatory effects, and regulates glucose and lipid metabolism[23–26]. Luteolin, tanshinone□a, formononetin, and isorhamnetin, effective components in QDD, also exhibits renal protective and anti-diabetic properties [27–30].

The relationships between DKD, biological processes, and the enriched pathways have been reported previously, including the regulation of glucose and lipid metabolism, oxidative stress, and signaling transduction pathways [31–35]. Epithelial cells acquire the stromal cell phenotype is the EMT biological process. Under high glucose, renal tubular epithelial cells could transform into mesenchymal-like cells by advancing glycosylation products, chronic inflammation, oxidative stress, and undergoing a series of signaling cascades, to avoid apoptosis[36]. Therefore, EMT plays important role in the development of DKD[37] and is considered to be one of the initiating factors in the occurrence and development of renal TIF[38].

It has been reported that MAPK activation is related to the secretion of TGF-β1 and ECM protein[39]. P38MAPK is positively correlated with the expression of type 3 and type 4 collagen in type 2 diabetic nephropathy rat model[40]. In vivo studies, renal fibrosis can be improved by blocking MAPK signaling pathway[41]. *In vitro* studies shown that, under high glucose, p38MAPK activation promotes renal tubular epithelial cell EMT progress [42], and p38 inhibitor SB203580 significantly reverses the EMT progress by downregulated the expression of E-Cadherin and type 1 collagen in HK-2 cells [43, 44].

mTOR is a serine/threonine kinase, which belongs to the family of phosphatidylinositol kinase-related kinases. MTOR responds to a variety of extracellular stimuli including growth factors, insulin, and nutrients. It regulates various physiological functions such as cell growth and cell cycle mainly through the PI3K/AKT/mTOR pathway[45, 46]. Animal experiments and clinical studies have reported the roles of mTOR in diabetic nephropathy. The inhibition on mTORC1 alleviates the morphological changes and dysfunction of diabetic kidneys[47]. In addition, mTOR promotes interstitial fibrosis by enhancing the proliferation of fibroblasts, regulation of EMT[48]. In summary, AKT-mTOR plays a role in EMT induced by high glucose and DKD.

5. Conclusion

The study employs network pharmacology and *in vivo* and *in vitro* experiments to demonstrate that QDD delays the progression of DKD by regulating EMT *via* p38MAPK and AKT/mTOR signaling pathways.

Abbreviations

DKD: Diabetic kidney disease

DM: diabetes mellitus

ESRD: end-stage renal disease

TCM: Traditional Chines Medicine

QDD: Qi-Dan-Dihuang Decoction

HMM: Hedysarum Multijugum Maxim

RS: Radix Salviae

RR: Rehmanniae Radix

RD: Rhizoma Dioscoreae

LC: Licorice

OGTT: oral glucose tolerance test

24hU-Pro: 24 h urinary protein

Scr: Serum creatinine

BUN: blood urea nitrogen

HE: hematoxylin-eosin

PAS: periodic acid-Schiff

TCMSP: Traditional Chinese Medicines Systems Pharmacology

ADME: absorption, distribution, metabolism and excretion

OB: Oral bioavailability

DL: drug-likeness

HL: half-life

Caco-2: Caco-2 permeability

DC: degree centrality

BC: betweenness centrality

EC: eigenvector centrality

CC: Closeness centrality

NC: Network centrality

LAC: Local average connectivity

EMT: epithelial-mesenchymal transition

AUC: area under the curve

PTGS2: Prostaglandin-Endoperoxide Synthase 2

ESR1: Estrogen Receptor 1

HSP90AB1: Heat Shock Protein 90 Alpha Family Class B Member 1

AR: Androgen Receptor

CALM1: Calmodulin 1

SCN5A: Sodium Voltage-Gated Channel Alpha Subunit 5

NOS2: Nitric Oxide Synthase 2

PPARG: PPARG Coactivator 1 Alpha

PRSS1: Serine Protease 1

PTGS1: Prostaglandin-Endoperoxide Synthase 1

INS: Insulin

ACE: Angiotensin I Converting Enzyme

IL6: Interleukin 6

VEGFA: Vascular Endothelial Growth Factor A

CTD: compound-target-disease

Declarations

Availability of data and materials

The datasets generated for this study are available on request to the corresponding author.

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Contributions

All the authors contributed sufficiently for their participation in the study, as follows: Xiaoshan Zhao and Ren Luo conceived, designed, and supervised the study; Liuyan Kuang, Yanting You and Jieyu Chen performed network pharmacology, Liuyan Kuang, Yanting You, and Jieying Qi performed cell research and Liuyang Kuang, Jieying Qi, Xinghong Zhou, and Shuai Ji performed the animal research; Liuyan Kuang, Min Wang, and Jingru Cheng did data analysis and interpretation; Liuyan Kuang, and Yanting You wrote the paper, Hiuyee Kwan and Xiaomin Sun modificated the paper. All authors have read and approved the final manuscript.

Ethics declarations

Ethics approval and consent to participate

The animal study was reviewed and approved by Animal Care and Use Committee of South Medical University.

Consent for publication

Not applicable.

Competing interests

The authors declared no potential conflicts of interests with respect to the authorship and/or publication of this article.

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Figures

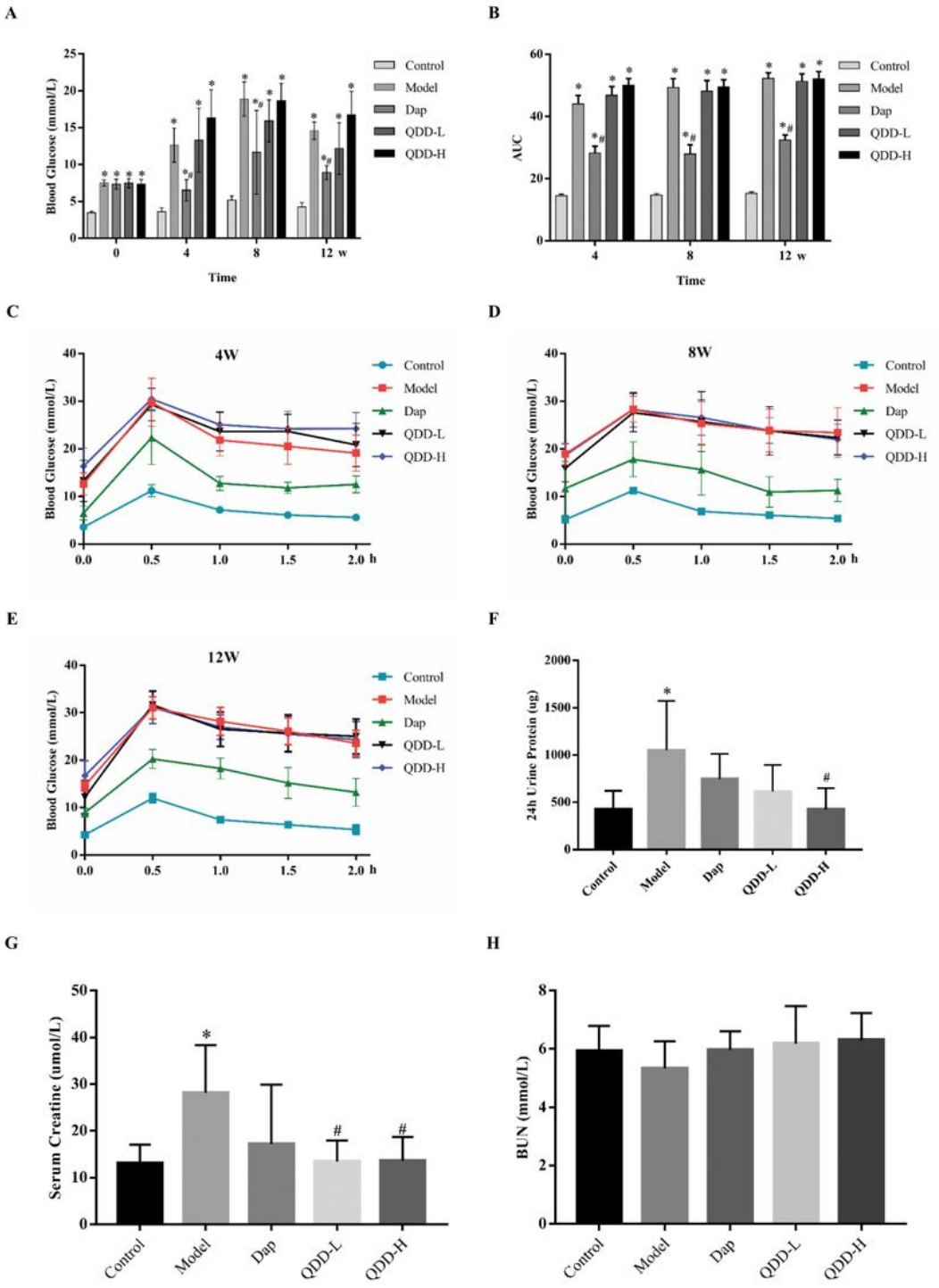

Figure 1

QDD protects renal function in db/db mice. (A) FBG changes in each group after treatment for 4, 8 and 12 weeks. (B) AUC of OGTT changes in each group after treatment for 4, 8 and 12 weeks. (C) OGTT curve in each group after treatment for 4 weeks. (D) OGTT curve in each group after treatment for 8 weeks. (E) OGTT curve in each group after treatment for 12 weeks. (F) 24 h U-pro changes in each group after

treatment for 12 weeks. (G) Scr changes in each group after treatment for 12 weeks. (H) BUN changes in each group after treatment for 12 weeks. (*p < 0.05 vs. control group; # p < 0.05 vs. model group).

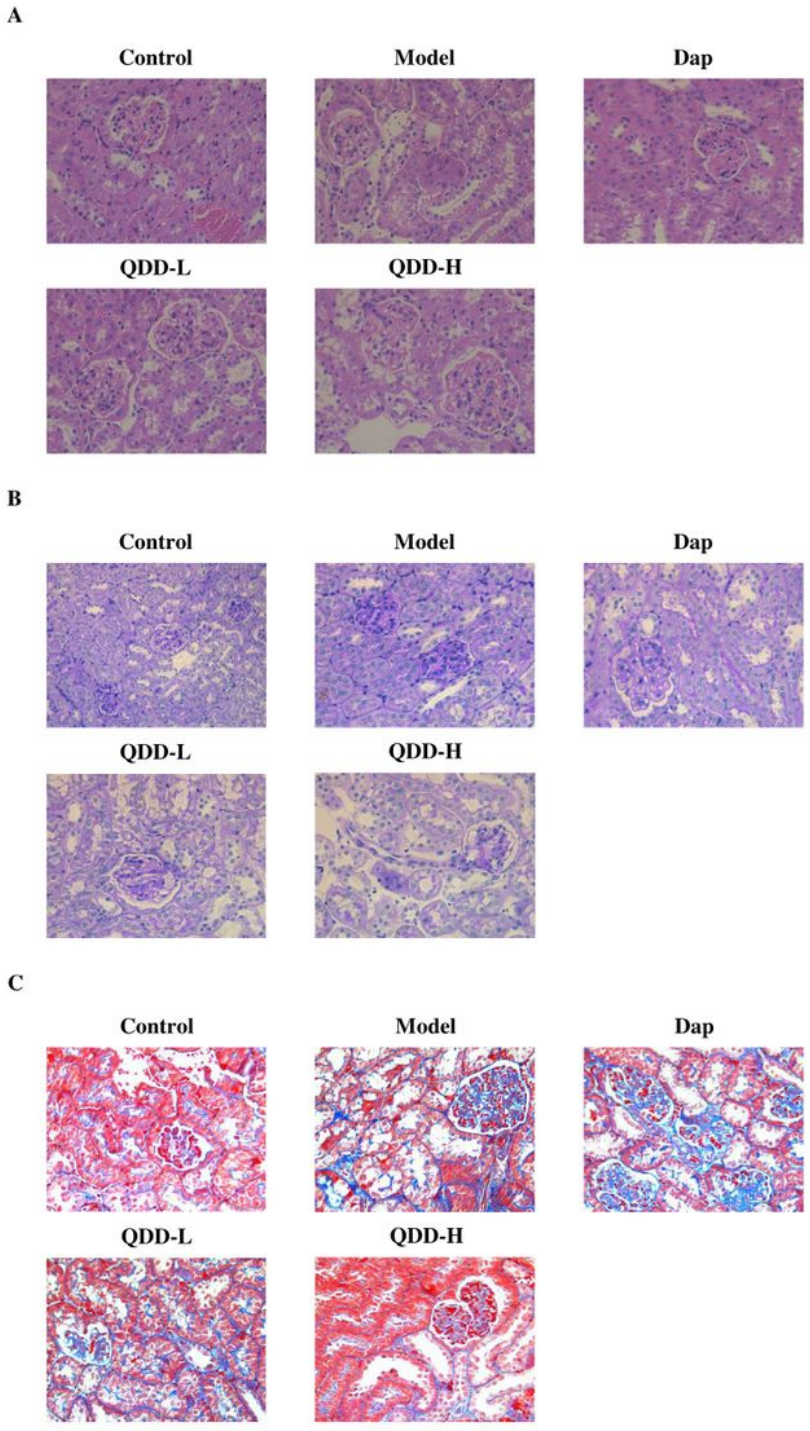

Figure 2

QDD alleviates fibrosis in kidneys of db/db mice. (A) Pathological changes in kidneys of db/db mice by HE staining. (B) Pathological changes in kidneys of db/db mice PAS staining. (C) Pathological changes in kidneys of db/db mice by Masson staining.

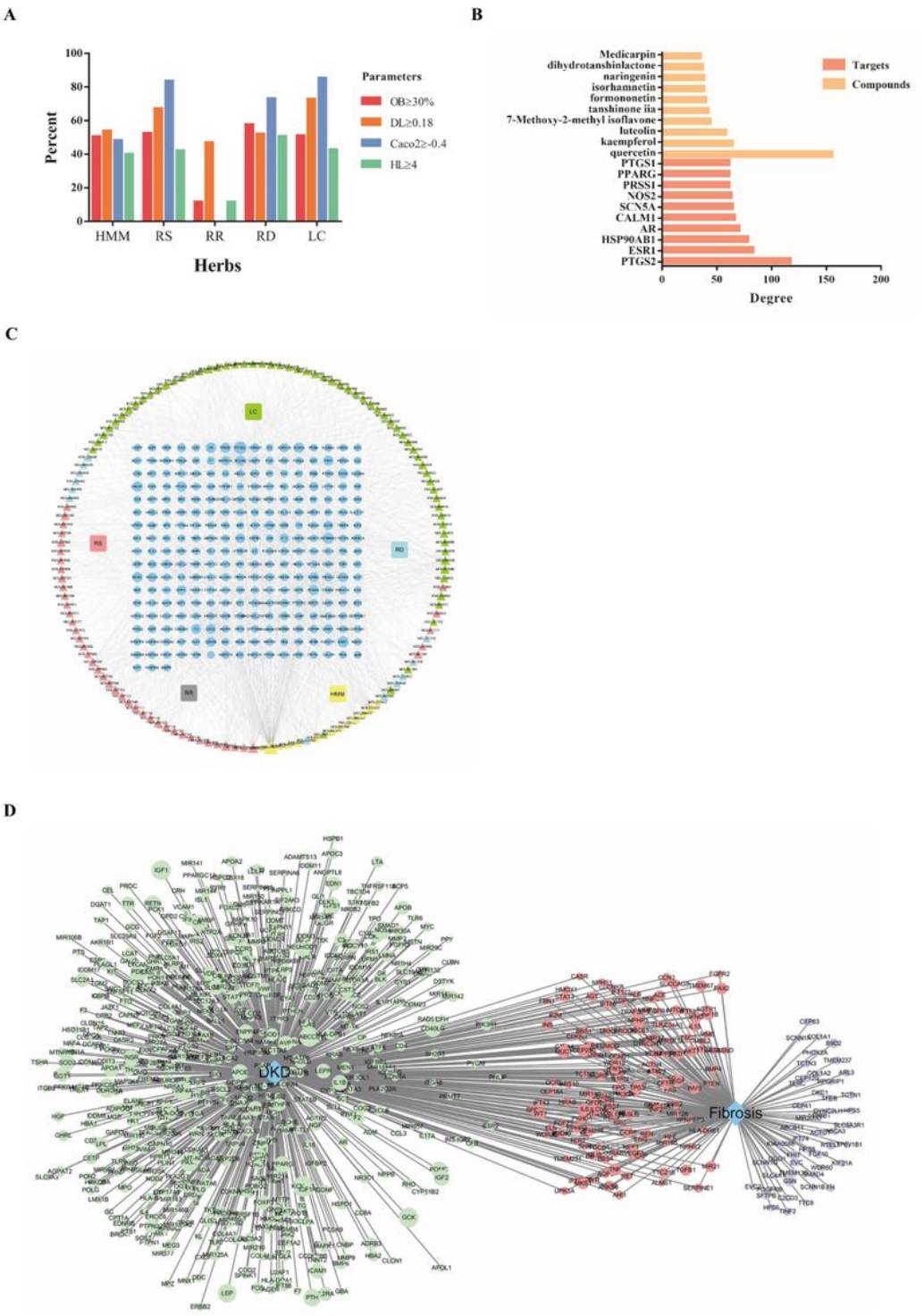

Figure 3

Analysis of active compounds of QDD and targets. (A) ADME parameter distribution for different herbs. Hedysarum Multijugum Maxim: HMM, Radix Salviae: RS, Rehmanniae Radix: RR, Rhizoma Dioscoreae: RD, and Licorice: LC. (B) Top 10 compounds and targets QDD-related compounds and targets. (C) The compound-target(C-T) network. Nodes represent active compounds (different triangle colors represent

compounds identified from different herbs) and targets (blue dots). (D) The disease-target(D-T) network. Nodes represent disease (blue) and targets (green, red, and purple).

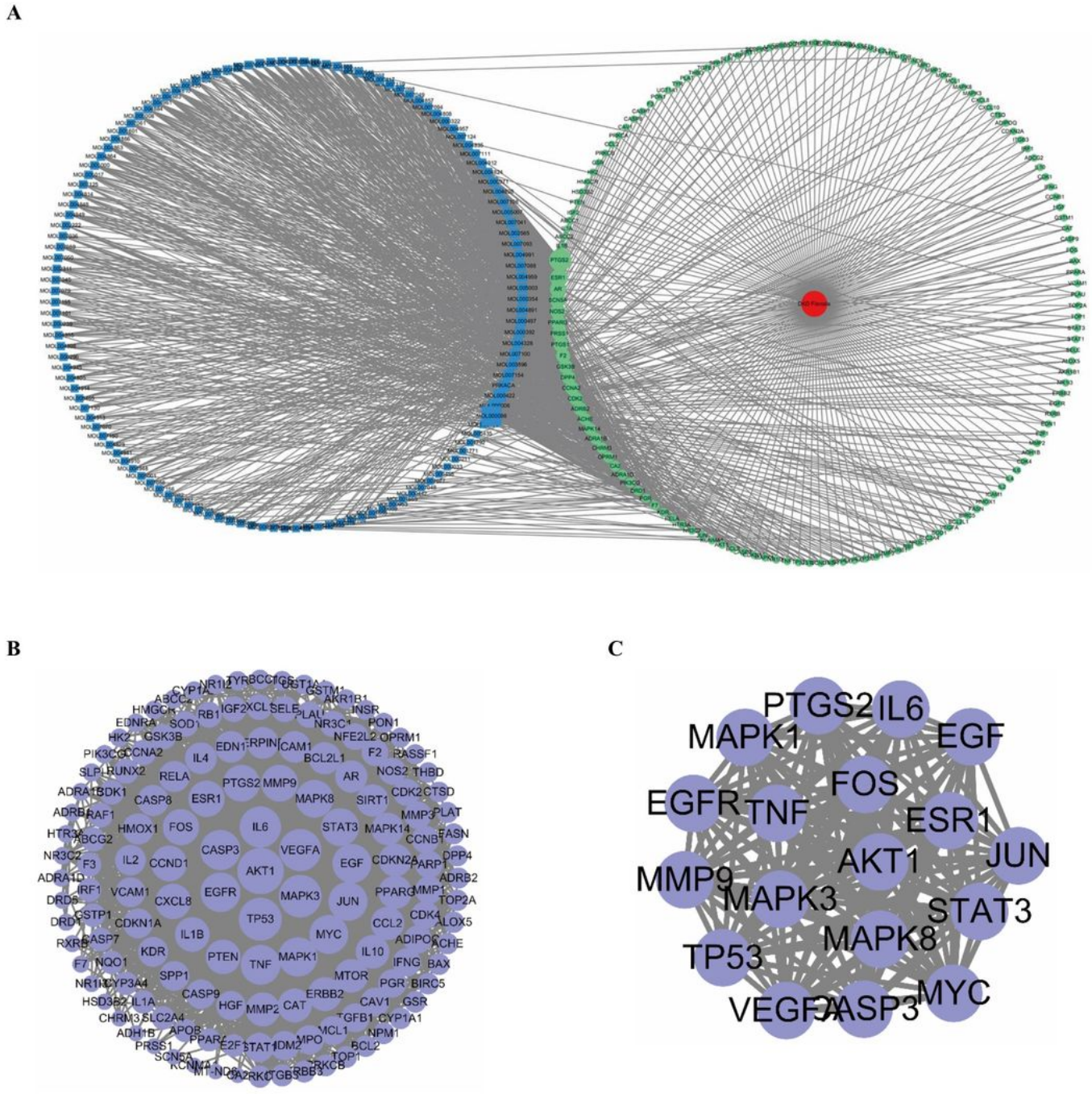

Figure 4

Compound-Target-Disease(CTD) and protein-protein interaction (PPI) network. (A) CTD network of QDD. Nodes represent compounds (Blue square), targets (green dot) and disease (red dot). (B) PPI network of compound-disease interaction targets. (C)PPI network of key targets.

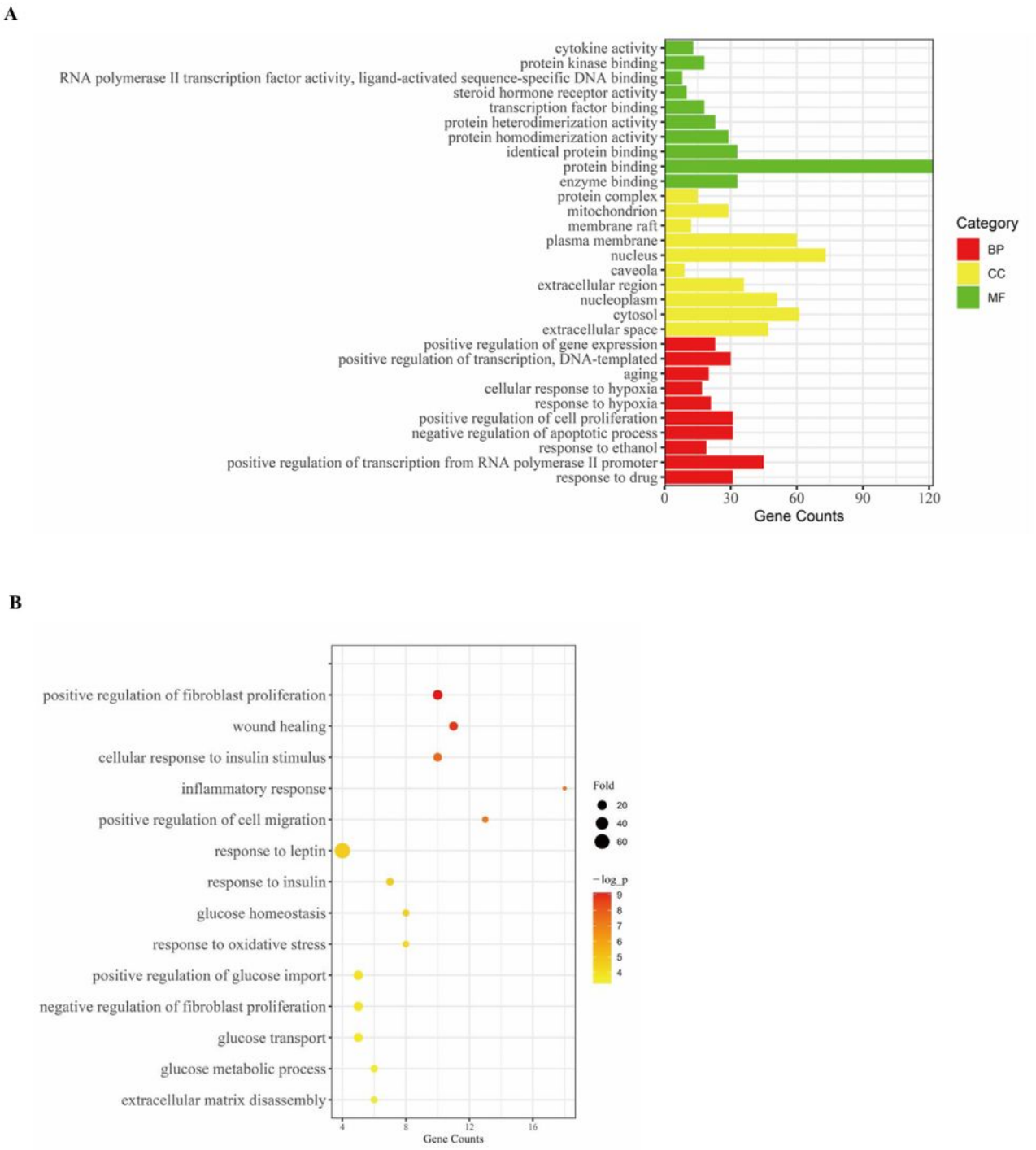

Figure 5

GO enrichment analysis of compound-disease interaction targets. (A) Top 10 enriched GO terms for biological process, cellular component and molecular function of putative targets with $p < 0.05$. (B) GO terms for biological process closely related to DKD.

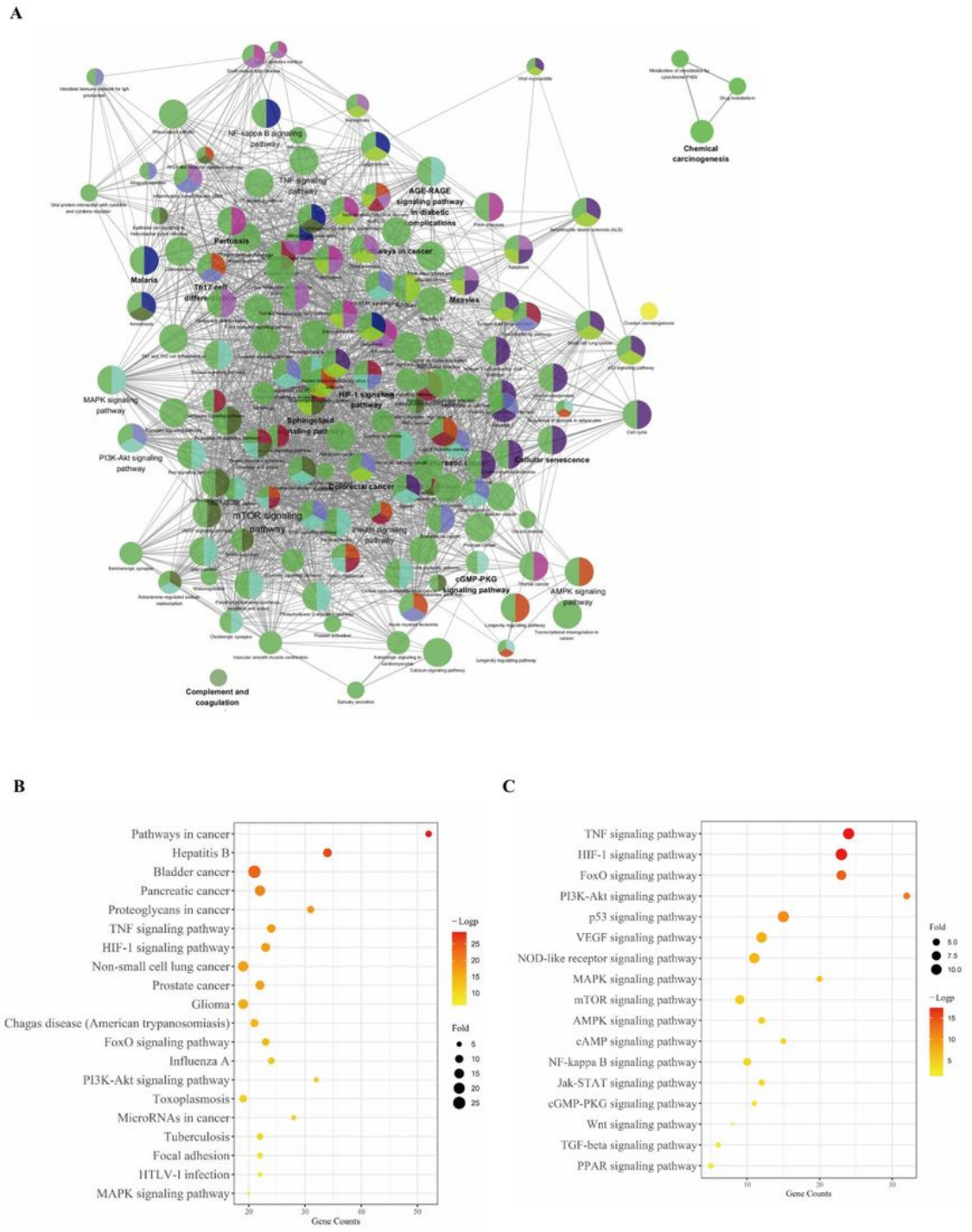

Figure 6

KEGG pathway enrichment analysis of compound-disease interaction targets. (A) A functionally grouped network of enriched KEGG pathways was generated for putative targets. KEGG terms are represented as nodes, and node size represents the term's enrichment significance. Functionally related groups partially overlap. Only the most significant term in the group was labeled. (B) Top 20 enriched KEGG pathways of

putative targets with p < 0.05. (C) Signaling transduction pathways related to DKD in KEGG pathway enrichment analysis.

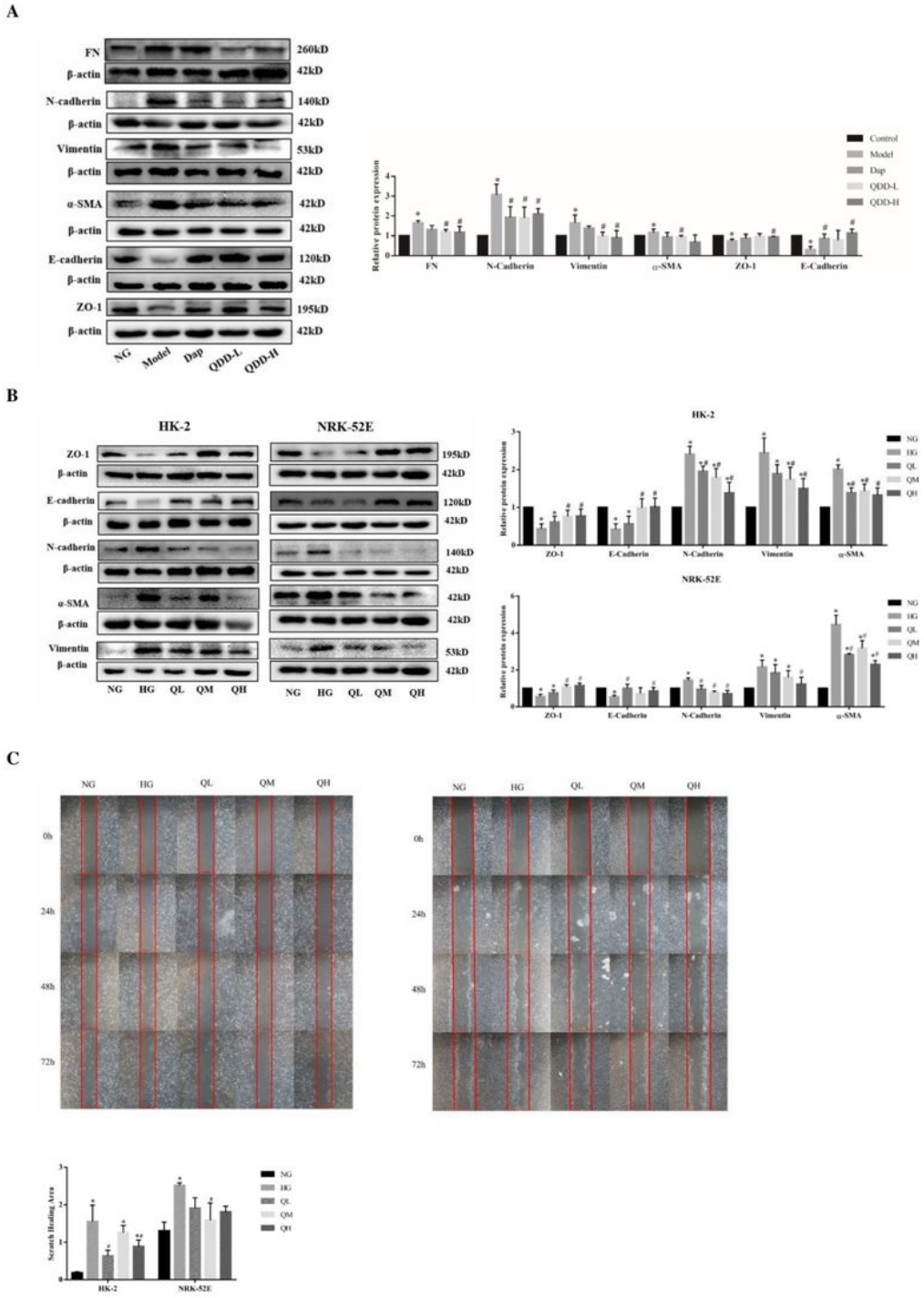

Figure 7

QDD alleviates fibrosis and EMT in kidneys of db/db mice and high glucose-cultured RTECs. (A) QDD alleviates the expression of EMT-related proteins in kidneys of db/db mice. (B) QDD alleviates the expression of EMT-related proteins in in glucose-cultured HK-2 and NRK-52E cells. (C) QDD inhibits

migration capacity in glucose-cultured HK-2 and NRK-52E cells. (* p<0.05 vs. control/normal group; # p<0.05 vs. model/high glucose group).

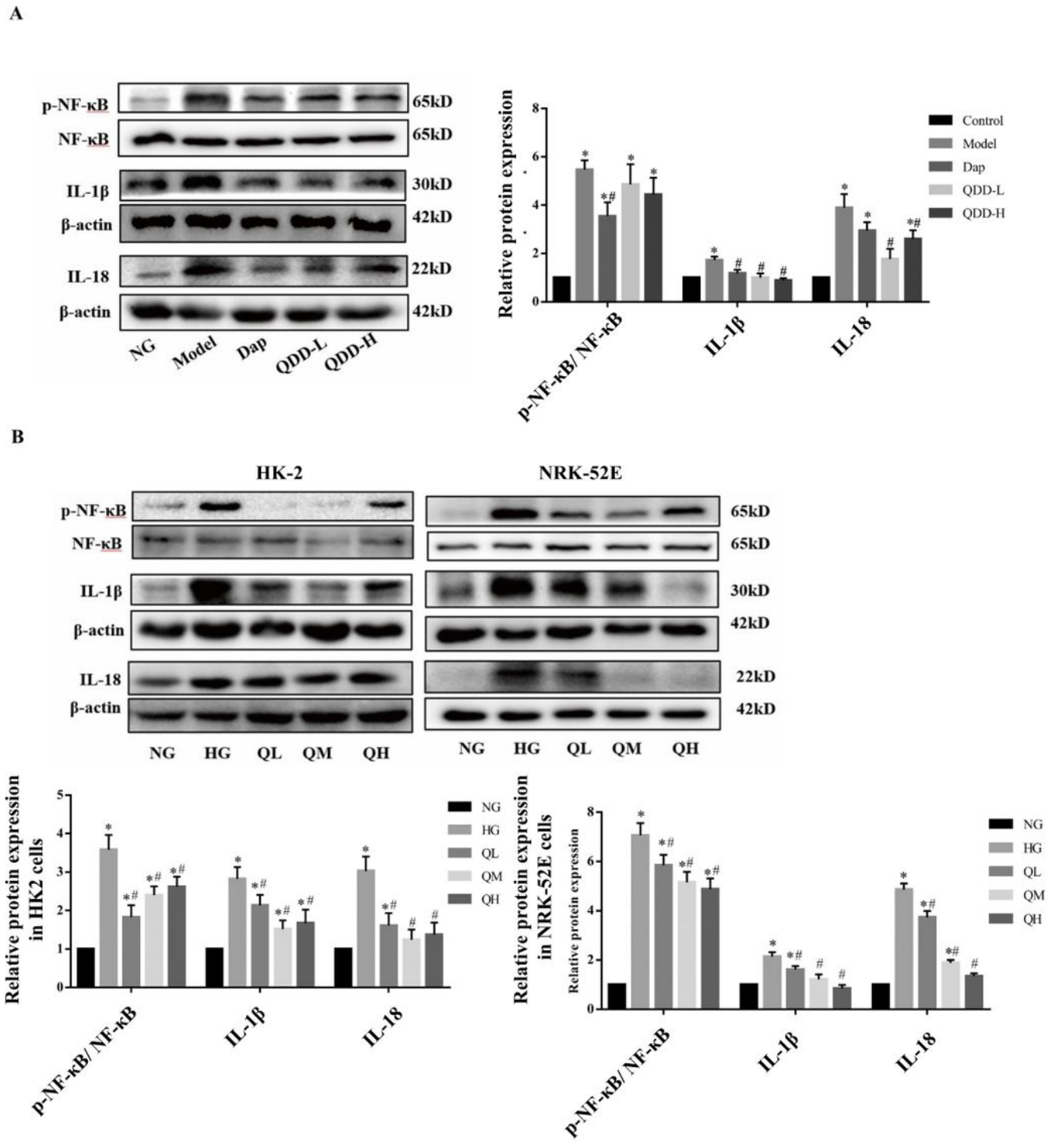

Figure 8

QDD alleviates inflammation in kidneys of db/db mice and high glucose-cultured RTECs. (A) QDD alleviates the expression of inflammatory proteins in kidneys of db/db mice (B) QDD alleviates the

expression of inflammatory proteins in glucose-cultured HK-2 and NRK-52E cells. (* p<0.05 vs. control/normal group; # p<0.05 vs. model/high glucose group)

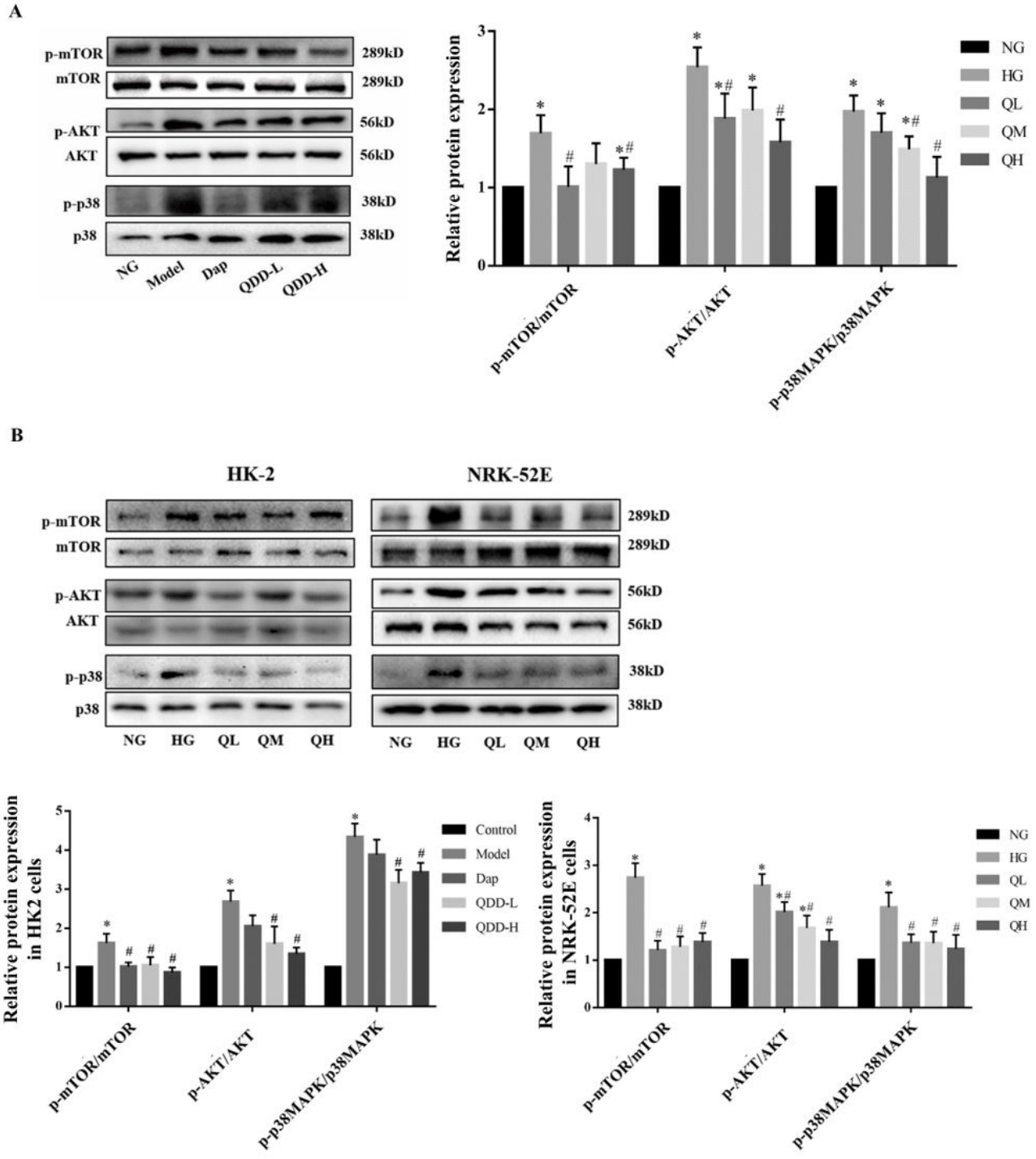

Figure 9

QDD regulates p38MAPK and AKT/mTOR signaling pathways in kidneys of db/db mice and high glucose-cultured RTECs. (A) QDD regulates p38MAPK and AKT/mTOR signaling pathways in kidneys of

db/db mice (B) QDD regulates p38MAPK and AKT/mTOR signaling pathways in glucose-cultured HK-2 and NRK-52E cells. (* $p<0.05$ vs. control/normal group; # $p<0.05$ vs. model/high glucose group)