Spectrum-Effect Relationship and Component Knock-Out or Knock-In in Total Flavones of Abelmoschus manihot

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Research Article

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Abstract

Background Diabetic kidney disease(DKD) is one of the microvascular complications of diabetes. The total flavones of *Abelmoschus Manihot* (TFA) has been widely used in China to reduce podocyte injury in DKD, however, not each flavone monomer can play the same effect. In a specific disease or pharmacological model, there may also be a group of chemical components with clear composition and content ratio that can play the same role as *Abelmoschus Manihot* extract.

Methods The composition of the seven flavone monomers was investigated by spectrum-effect relationship and component knock-out and knock-in methods. We used HPLC to establish the chemical fingerprints, and assayed the protective effect and anti-inflammatory activity of podocytes in vitro. The protein expression level of synaptopodin and nephrin were measured by immunofluorescence staining, and the release of inflammatory factors of TNF-α and IL-1β were determined by enzyme-linked immunosorbent assay (ELISA) or real-time fluorescence quantitative polymerase chain reaction (RT-qPCR). Partial least squares method and multi-objective optimization were used to examine the spectrum-effect relationships. Then, we also determined the expression of TRPC6 in podocytes by RT-qPCR and western blot.

Results When the compatibility proportion of rutin, hyperoside, isoquercetin, hibifolin, myricetin, quercetin-3'-o-glucoside, and quercetin was 0, 1.2, 0.88, 0.88, 0.48, 2.08, 0.4, the protein expression of synaptopodin and nephrin may be equivalent to TFA. And when the compatibility proportionof 7 active components was 0, 2.08, 1.74, 1.50, 0.12, 0.54, 0.1, which decreased the mRNA expression of TNF-α. Further, we found that B (hibifolin/hyperoside), C (hyperoside/quercetin-3'-o-glucoside), and E (isoquercetin/quercetin-3'-o-glucoside) have significant effects on synaptopodin, nephrin, and TNF- α, IL-1β. Collectively, our data indicated that TFA could ameliorate cytoskeleton rearrangement and inflammatory injury in podocytes under hign glucose conditions, and the proportion of hyperoside, isoquercitrin, hibifolin and quercetin-3'-o-glucoside had the similar efficacy, they may be the main active components of TFA. What's more, the best combination of hyperoside, isoquercetin, hibifolin and quercetin-3'-o-glucoside decreased the expression of TRPC6.

Conclusion The combination of hyperoside, isoquercetin, hibifolin, and quercetin-3'-o-glucoside in TFA was optimized by spectral efficiency relationship, and flavone monomer combination may play a protective role in podocyte cytoskeleton through TRPC6 pathway.

Introduction

Diabetic kidney disease (DKD) is the main cause of end-stage renal disease (ESRD), about 30%~50% of ESRD in the world is caused by DKD [1-4], which seriously endangers the health of Chinese residents. The incidence rate and prevalence of DKD in China have also increased in the past decade, reaching 11.2% from 2015 to 2017, of which type II diabetes was more than 90%[5-6]. Clinically, the treatment of DKD is still focused on the control of blood sugar and blood pressure, so as to delay the progress of DKD. However, it has been proved that this method can only retard the development of DKD, but cannot prevent the occurrence of the disease [7]. The prevalence of DKD is still increasing.

Huangkui capsule is a Chinese patent medicine product jointly developed by Jiangsu Provincial Hospital of Traditional Chinese Medicine and Suzhong Pharmaceutical Group Co. Ltd., which has achieved significant effects in the treatment of proteinuria in various chronic kidney diseases [8-9]. As we all know, traditional Chinese medicine is a whole concept in the process of treating diseases, it is difficult to determine the exact therapeutic components, which greatly restricts the clinical application of traditional Chinese medicine. According to previous research, total flavone of *Abelmoschus Manihot* can also reduce the urine protein in DKD mice. In high glucose induced HK-2 cells, similar to the NADPH oxidase inhibitor, quercetin, hyperoside, isoquercetin, hibifolin and quercetin-3 '- o-glucoside inhibit the expression of α-SMA, p-ERK1/2, NOX1, NOX2 and NOX4[10]. Rutin, hyperoside, isoquercetin, hibifolin, myricetin, quercetin-3'-o-glucoside, and quercetin may be the main pharmacological active components in TFA, however, the proportion relationship among these seven components is currently unclear. Therefore, the "spectrum effect relationship", "component knock-out technology" and "high performance liquid chromatography" were used to study the proportion relationship among seven flavone monomers in the treatment of DKD.

In this study, the differential fingerprint of total flavone in *Abelmoschus Manihot* was established by HPLC, and the pharmacodynamics of 23 differential samples were evaluated in vitro. So as to determine the efficacy components of total flavones of *Abelmoschus Manihot* (TFA) in the treatment of DKD, seven flavone monomers were selected to analyze the correlation between chemical components and efficacy data, and we established a spectrum effect relationship model to screen the best flavone compatibility.

Materials and methods

Materials

Rutin was purchased from National Institutes for Food and Drug Control (Beijing,China), isoquercetin was purchased from Chengdu Chroma-Biotechnology Co., Ltd.(Chengdu,China). Hyperoside, hibifolin, myricetin, quercetin-3 '- o-glucoside, quercetin and TFA were obtained Suzhong Pharmaceutical Group Co. Ltd.,(Taizhou,China).Roswell Park Memorial Institute (RPMI) 1640 medium was purchased from KeyGEN BioTECH.(Nanjing,China), and foetal bovine serum (FBS) was purchased from Gibco(Gibco Company, USA). D-glucose and HEPES were purchased from sigma(Shanghai,China), mannitol was purchased from Aladdin. Antibodies for synoptopdin was purchased from Proteintech(Wuhan,China), nephrin was purchased from Affinity(Jiangsu,China),TRPC6 was purchased from Bioworld(Nanjing,China). Mouse TNF-α and IL-1β ELISA kit was purchased from Elabscience(Wuhan,China). Mouse podocytes (MPC-5) was purchased from Beijing Beina Chuanglian Biotechnology Institute (Beijing China).

Knock-Out Components and Negative Samples

Weighed about 400g TFA, added 10% ethanol for ultrasonic dissolution, and extracted with continuous countercurrent extractor. The ratio of solids and liquid was 1:3, and the time was 30min. Extracted twice, and collected the extraction phase and the raffinate phase respectively. To apply D101 macroporous resin for separation and purification of TFA. Loaded D101 macroporous resin into a column (diameter: 9.6cm, height: 57.6cm, volume: 4167mL/BV),washed with 95% ethanol until no turbidity ,and then wash with distilled water until no alcohol smell. Diluted the concentration of the extract with water to 3.56mg/mL,loaded the sample at a flow rate of 2BV/h, and removed impurities with 6BV pure water and 3BV 5% ethanol. 4BV 60% ethanol was eluted at a flow rate of 2BV/h, and the eluent was collected. Flushed D101 macroporous resin with 2BV 95% ethanol and collect the effluent. Enriched the 60% ethanol eluent until it had no alcohol smell, added water to dilute the concentration to 2.45mg/ml, and collected the residue after preparing the liquid phase to knock out flavonoids. Separation was performed with a Waters C18 chromatographic column (20 × 250mm,10 μm; Waters, Milford, MA, USA) . The mobile phase was a mixture of acetonitrile (A) and 0.1% formic acid-water (B), with an optimized linear gradient elution as follows:0-10min, 18% - 23% (A); 10-30min,23%-25% (A); 30-40min, 25%-30% (A); 40-50min,30%-35% (A); 50-60%,35%-40% (A); 60-70min,40%-18% (A); 70-75min,18%(A). The flow rate was 40.0 mL·min^{-1}. The injection volume was 60 mL. The column temperature was set at 30℃. The collections was evaporated and concentrated at 70 ℃, and then the concentrated solution was placed on a freeze dryer for drying. The powder obtained were target components and negative sample.

Preparation of the working solution

DPS software was used to carry out 7 factors and 21 levels uniform design for the TFA. Differential samples was designed according to the content of each component in TFA. Based on the proportion in the uniform design table, seven flavone monomers were knocked into the negative sample to obtain the S1-S21. S22 (TFA) was the positive control, and S23 was the negative sample control. Took 2 copies of all samples , weighed them accurately, and added 70% ethanol for ultrasonic dissolution, passed 0.45 μm microporous filter membrane to obtain the test solution for HPLC experiment. The other was added DMSO for cell experiment.

Establishment of HPLC fingerprint

Chromatographic Conditions:C18 chromatographic column (4.6×250mm-5 μm); Column temperature: 30 ℃; Detection wavelength: 360nm; Flow rate 1.0mL/min; Injection volume 10 μ L; The mobile phase was acetonitrile (A) - 0.1% phosphoric acid solution (B), gradient elution, 0-20min, 6%-17% (A); 20-30min, 17%(A); 30-50min, 17%-40%(A); 50-55min, 40%-6%(A); 55-60min, 6% (A).

Cell culture

Conditionally immortalized mouse podocytes (MPC5) were obtained from Beijing Beina Chuanglian Biotechnology Institute (Beijing, China). Podocytes were cultured in growth permissive conditions at 37℃ in RPMI 1640 medium containing 10% FBS, 100U/ml penicillin plus, 100μg/ml streptomycin, and 1% HEPES. To induce differentiation, podocytes were cultured at 37℃ without IFN-γ for 2 weeks and were used for experiment.

Cell viability

MTT assay was used to determine cell viability by the manufacturer's instruction. MPC-5 cells were plated in 96-well plates at a density of 5×10^3 cells/well and incubated with normal glucose (NG, 5.5 mM glucose+ 24.5 mM mannitol), hign glucose (HG, 30 mM glucose), osmotic control (24.5 mM Mannitol was added to the NG group) and HG with TFA at different concentrations (5, 10, 20, 40 μg/mL) for 48 h. Then, an MTT solution (5 mg/mL) was added to each well, and the MPC-5 cells were incubated for 2-3 h at 37 °C. The supernatants were then aspirated, and 100 μL of dimethyl sulfoxide (DMSO) was added to solubilize formazan crystals with shaking for 10 min. Absorbance readings of the test and control samples were measured at 490 nm with a microplate reader (TECAN, Switzerland) and cell

viability was expressed in terms of the percentage viability, which was calculated as the ratio of the absorbance of a treated sample to the absorbance of the untreated control sample multiplied by 100.

Determination of inflammatory cytokine levels in podocytes

Commercial ELISA kits were used to determine inflammatory cytokines, tumour necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) levels in podocytes according to the manufacturer's instructions.

Immunofluorescence staining

Cells were washed with PBS, fixed with paraformaldehyde and permeabilized with 0.1% Triton X-100, blocked with 5% BSA for 1 h followed by incubation with primary antibody overnight. After washing with PBS and incubating with Alexa Fluor 488-conjugated goat anti-rabbit (Beyotime, Shanghai, China), Hoechst33324 (Beyotime, Shanghai, China) was used to stain nuclei. Fluorescence microscope (Leica, Germany) was used to acquire images.

RT-qPCR

TRIzol Reagent (Vazyme Biotech Co., Ltd, Nanjing, China) was used to extract total RNA from cells, following reversely transcribed into cDNA and prepared to test by real-time PCR using SYBR Green Master Mix. The results were analyzed by 2-ΔΔCT method. The primers were listed as follows: TRPC6:(forward) 5'-GCAGCTGTTCAGGATGAAAAC -3', (reverse) 5'- TTCAGCCCATATCATGCCT -3'; GAPDH: (forward) 5'-GGTGAAGGTCGGTGTGAACG -3' (reverse) 5'- CTCGCTCCTGGAAGATGGTG-3'. TNF-α: (forward) 5'- CCTGTAGCCCACGTCGTAG -3', (reverse) 5'- GGAGTAGACAAGGTACAACCC -3'.

Western blot

Protein was separated by SDS-PAGE, followed by transferring to PVDF membranes. After blocking in 5% skimmed milk powder, the membranes were incubated overnight with primary antibodies at 4 °C, then incubated with secondary antibodies at 37 °C for 1-2 h[11]. After that, the protein bands were identified by Development of Odyssey two-color infrared laser imaging system.

Spectrum-Effect Relationship

Correlation analysis was conducted between the flavonoid monomers with different combination and pharmacodynamic data of HPLC chromatogram from 23 various groups. Partial Least Squares Regression Analysis and Multi-Objective Optimization were used for processing, and the combination of common components significantly correlated with the pharmacodynamics were found.

Statistical Analysis

GraphPad Prism 8.0.2 software was used for mapping, experimental data were represented as means ± SD. Differences between two groups were assessed by one-way ANOVA. Differences were statistically significant when $p < 0.05$.

Results

HPLC Chromatogram of Knock-Out Components and Negative Samples

The chromatogram knock-out method is commonly used for its simple operation [12], rapid preparation and high precision. It is very suitable for polar and weak polar and non-polar compounds based on liquid-liquid partition chromatography. In Table 1, it showed that the content of seven flavonoid monomers in the knock-out sample was 76.37%, while the content in the negative sample was 15.80%. The knock-out of hyperoside, isoquercetin, hibifolin, quercetin-3'-o-glucoside was relatively thorough. The purity of these target knocked-out components was high and they could not be found in the negative samples on the whole, as shown in Figure 1 (A).

Table1 HPLC determination results								
	Rutin(%)	hyperoside (%)	isoquercetin (%)	hibifolin (%)	myricetin (%)	quercetin-3'-o-glucoside (%)	quercetin (%)	Total content (%)
knock-out sample	0.06	25.21	20.71	14.84	0.96	13.27	1.34	76.37
negative sample	1.25	1.40	1.22	4.13	2.22	3.77	1.82	15.80

Establishment of HPLC fingerprint

23 different samples were injected into HPLC chromatography, recorded chromatogram, and then imported into the Similarity Evaluation System for Chromatographic Fingerprints of Traditional Chinese Medicine to generate HPLC fingerprints of TFA. TFA was used as the reference spectrum. The chromatographic peaks with relatively high content and good separation in 23 batches of samples were selected for multipoint correction, and the average method was used to generate the control map by the software"Evaluation system of similarity of chromatographic fingerprint of traditional Chinese Medicine version 2010". Seven common peaks were selected from 23 characteristic maps, as shown in Figure 1 (B), and the similarity data were shown in Table 2.

The Cell Viability of high glucose-induced Podocytes

In Figure 2 A-B, MTT was used to detect the effect of TFA on normal or high glucose-induced podocytes, and the half lethal rate (IC50) of TFA on podocytes was 28.68 μg/mL. After podocytes were incubated with normal glucose(5.5 mM glucose), mannitol (MA, 5.5 mM glucose+ 24.5 mM mannitol), hign glucose (HG, 30 mM glucose) and HG with TFA at different concentrations (5, 10, 20, 40μg/mL) for 48 h, the results showed that compared with NG group (5.5 mM glucose), the podocytes viability in MA group (5.5 Mm+24.5 mM mannitol) had no significant difference, and in HG group (30 mM glucose) decreased ($P<0.01$). TFA at concentration of 5, 10, 20 μg/mL increased the viability of podocytes, 40 μg/mL decreased the viability of podocytes.

TFA mitigates HG-induced podocyte injury

In Figure 3, immunofluorescence method was used to detect the effect of different flavonoid monomer compatibility groups on podocyte cytoskeletal markers induced by high glucose. Compared with the normal glucose group and mannitol control group, 30 mM glucose was sufficient to down-regulate the expressions of synaptopodin and nephrin, and other samples could protect the skeleton of podocytes in varying degrees.

The anti-inflammatory effect of TFA against HG-induced podocyte injury

Previous studies showed that HG could lead to inflammation [13].In addition, DKD is widely considered as a kind of inflammatory disease. So we detected the secretion of TNF-α and IL-1β in MPC-5 after treating with high glucose. As shown in Figure 4 (A-B), HG also induced an increase of TNF-α and IL-1β secretion in MPC-5. And we further explored if the combination of flavonoid monomers could inhibit the secretion of inflammatory factors in MPC-5. HG-induced podocytes were treated with various combination of flavonoid monomers for 48h. The results showed that combination of flavonoid monomers was sufficient to inhibit the secretion of TNF-α and IL-1β in MPC-5.

Partial Least Squares(PLS) Regression Analysis

The VIP value represents the explanatory importance of the independent variable X to the dependent variable. Based on the VIP value and the number of principal components, the optimal number of principal components was determined to be 1. PLS regression could also display the regression relationship expression between the dependent variable Y and the independent variable X. In Figure 5, the results showed that B (hibifolin/hyperoside), C (hyperoside/quercetin-3'-o-glucoside), and E (isoquercetin/quercetin-3'-o-glucoside) had significant effects on synaptophodin, nephrin, and TNF- α IL-1β. And P value < 0.15, as shown in Table 3.

Table 3 regression coefficient

Y	X	regression coefficient	standard erro	*t* value	*p* value
synaptopodin	A	0.010	0.265	0.038	0.970
	B	-1.873	1.227	-1.526	0.143
	C	3.918	2.257	1.736	0.099
	D	-0.162	1.043	-0.155	0.878
	E	1.968	1.265	1.556	0.136
	F	1.255	0.815	1.540	0.140
	G	0.557	0.636	0.875	0.393
	I	-3.247	7.624	-0.426	0.675
	H	-0.238	0.293	-0.810	0.428
nephrin	A	-0.022	0.570	-0.039	0.969
	B	4.095	2.330	1.758	0.095
	C	-8.565	4.862	-1.762	0.094
	D	0.354	2.321	0.153	0.880
	E	-4.302	2.197	-1.958	0.065
	F	-2.744	1.963	-1.398	0.178
	G	-1.217	1.255	-0.969	0.345
	I	7.099	15.911	0.446	0.661
	H	0.520	0.561	0.927	0.365
TNF-α	A	0.011	0.275	0.039	0.970
	B	-1.966	0.995	-1.976	0.063
	C	4.111	2.529	1.626	0.121
	D	-0.170	1.181	-0.144	0.887
	E	2.065	1.206	1.712	0.103
	F	1.317	1.113	1.184	0.251
	G	0.584	0.606	0.964	0.347
	I	-3.408	7.778	-0.438	0.666
	H	-0.250	0.259	-0.962	0.348
IL-1β	A	0.006	0.152	0.038	0.970
	B	-1.077	0.332	-3.242	0.004
	C	2.252	0.650	3.464	0.003
	D	-0.093	0.695	-0.134	0.895
	E	1.131	0.297	3.813	0.001
	F	0.721	0.529	1.365	0.188
	G	0.320	0.230	1.391	0.180
	I	-1.866	3.826	-0.488	0.631
	H	-0.137	0.142	-0.964	0.347

Multi-objective optimization

In our study, the different proportion of flavonoid monomers were set as the independent variable(X), activation rate of synaptopodin and nephrin, inhibition rate of TNF-α and IL-1β were taken as the dependent variable (Y). Then, minitab 18 statistical software was used for the establishment of fitting regression model. The results showed that when performing stepwise regression analysis, R-sq reached maximum and four regression equations were obtained, as follows:

Y1=1.754 - 0.473 x4 - 0.2109 x4*x6 - 1.92 x5*x7 + 2.153 x4*x5*x7 - 0.538 x2*x3*x5*x6 R-sq=72.55%

Y2=2.90816 - 6.01647 x7 - 0.785652 x2*x5 - 0.129003 x3*x4 - 0.956731 x4*x5 + 0.617381 x5*x6 - 0.641880 x2*x3*x6 + 3.28706 x2*x3*x7 + 0.499171 x2*x4*x6 - 1.13338 x2*x4*x7 + 0.391850 x3*x4*x5 + 0.052890 x3*x6*x7 - 0.772530 x2*x3*x4*x6 - 0.644397 x3*x4*x5*x6 + 7.30141 x3*x4*x5*x6*x7

R-sq=100.00%

Y3= 0.2586 + 0.4692 x2*x3 - 5.01 x1*x2*x3 - 1.571 x1*x5*x6 - 1.155 x3*x4*x7 + 11.02 x1*x3*x4*x6*x7 + 13.97 x1*x2*x3*x4*x5*x6 R-sq=93.34%

Y4=0.885769 + 0.056260 x2 - 0.625680 x5 - 0.271157 x6 - 1.69680 x7 -0.813207 x1*x4 + 0.029018 x4*x5 + 0.370236 x5*x6 + 1.82650 x5*x7 + 0.948984 x6*x7 - 0.005471 x2*x3*x5 + 0.006847 x3*x4*x5 - 1.52599 x5*x6*x7 + 0.741533 x1*x2*x4*x5- 0.001706x2*x4*x5*x6*x7+2.49608x1*x2*x3*x4*x5*x7 R-sq=100.00%

Comprehensive optimization was carried out according to the four fitted objective functions. Based on Pareto sorting, genetic algorithm was used for optimization, the multi-objective function was gradually optimized, pareto optimal solution set under the fixed total dose was obtained. The proportion of rutin, hyperoside, isoquercitrin, hibifolin, myricetin, quercetin-3'-o- glucoside, and quercetin was 0.03, 1.55, 1.31, 1.67, 0.51, 1.25, 0.21; 0.03, 0.12, 1.74, 2.21, 0.67, 1.66, 0.28; 0, 1.2, 0.88, 0.88, 0.48, 2.08, 0.4 and 0, 0.9, 0.66, 0.66, 0.54, 3.30, 0.30, which can significantly improve the exspression of synaptopodin and nephrin; When the proportion of rutin, hyperoside, isoquercitrin, hibifolin, quercetin-3'-o-glucoside, myricetin, quercetin were 0.05,1.04,1.72,2.19, 0.22, 0.85, 0.09;0, 2.08, 1.74, 1.50, 0.12, 0.54, 0.1 and 0.08, 1.56, 2.61, 0.98, 0.33, 1.28, 0.14, which had a good effect on the inhibition rate of TNF-α and IL-1β.

Pharmacodynamic validation of flavonoids monomer compatibility

In order to further prove the reliability of the previous results of the spectral efficiency relationship, we selected the cell model in vitro for validation. The effects of seven flavonoid monomers on the cytoskeletal markers and inflammatory factors of podocytes induced by high glucose were determined according to the predicted compatibility ratio. By comparing their efficacy values, we found the chemical composition related to the efficacy, and further verified the effect of the ratio of flavonoid monomers on the efficacy.

As shown in Figure 6 (A-C) , the optimized flavonoid monomer was tested to determine its effect on the podocyte cytoskeletal markers of synaptopodin and nephrin. The results showed that compared with NG group (5.5mM glucose), MA group (5.5mM glucose+24.5mM mannitol) had no significant difference; In HG group (30mM glucose), the protein expression of synaptopodin and nephrin was down-regulated, and the flavonoid monomer compatibility group and TFA could up-regulate the expression of synaptopodin and nephrin to different degrees, and S3 was the most effective than other groups. It was speculated that the ratio of rutin, hyperoside, isoquercitrin, hibifolin, myricetin, quercetin-3'-o-glucoside, quercetin was 0, 1.2,0.88, 0.88, 0.48, 2.08 and 0.4, the protective effect on podocytes cytoskeletal markers reached the best. In Figure 6 (D), RT-qPCR method was used to detect the inflammatory factors of podocyte induced by high glucose in flavone monomer compatibility groups after multi-objective optimization. The results showed that compared with NG group (5.5mM glucose), MA group (5.5mM glucose+24.5mM mannitol) had no significant difference. The mRNA expression of the TNF- α was increased in HG group. Compared with HG group, TFA and N1, N2, N3 could make the mRNA expression of TNF-α decrease. It was speculated that when the ratio of rutin, Hyperoside, isoquercetin, hibifolin, myricetin, quercetin-3'-o-glucoside and quercetin is 0.08, 1.56, 2.61, 0.98, 0.33, 1.28 and 0.14, the anti-inflammatory effect on podocytes was the best.

Determination of TRPC6 expression in podocytes

In Figure 6 (E-F), we detected the expression level of TRPC6 mRNA and protein in podocytes induced by high glucose. Compared with NG group, TRPC6 in MA group did not change significantly, while in HG group ,increased from 1.00 ± 0.04 to 1.72 ± 022; Compared with HG group, TRPC6 in TFA and HIHQ (hyperoside: isoquercetin: hibifolin: quercetin-3'-o-glucoside=1.2:0.88:0.88:2.08) group decreased from 1.72 ± 022 to 1.17 ± 0.27 and 1.00 ± 0.17.

Discussion

In recent years, the research on the progress mechanism and intervention measures of chronic kidney disease (CKD) has become the focus of nephrology at home and abroad. The traditional Chinese medicine preparations have also achieved a certain effect on the treatment of CKD, and Huangkui capsules is one of them[14-16].The main components of Huangkui capsules are the total flavonoids extracted from *Abelmoschus manihot*. They have been widely used to treat CKD, DKD and IgA nephropathy[17-23]. However, it is rarely reported whether the proportion relationship among seven flavonoid monomers has significant effect in the treatment of DKD.

Pharmacological and clinical studies have shown that TFA can inhibit myocardial and cerebral ischemia-reperfusion injury, intestinal inflammation in IBD model mice, and reduce micro albuminuria and glomerular cell apoptosis in early DKD[24-27]. Hyperoside, isoquercetin, and quercetin also have anti-oxidative, anti-inflammatory, and anti- hyperglycemic effects. Among them, hyperoside can reduce podocyte damage, pancreatic beta cell apoptosis, inhibit glomerular mesangial cell proliferation, isoquercetin improve glucose tolerance by increasing the secretion of GLP-1 , quercetin inhibits LPO and AGEs, pentosidine, and lipid peroxides in renal tissue[28-31].

In the process of DKD, the changes of podocyte structure and function are closely related to the occurrence of proteinuria , especially the down-regulation of podocyte cytoskeleton markers such as synaptopodin and nephrin, and the inflammatory injury induced by high glucose. To study the efficacy of flavonoid components compatibility in the treatment of DKD, we detected synaptophodin, nephrin and TNF-α, IL-1β in 23 groups of different samples by establishing a high glucose-induced podocyte injury model. Based on the cell experiment, our results illustrated that the decreased cytoskeleton markers and increased release of inflammatory factors in high glucose-induced podocytes. However, after administration with fermented TFA, the inflammatory injury of podocytes induced by HG was alleviated, accompanied by up-regulated synaptophodin and nephrin, confirming the above-mentioned effect of TFA in reducing cytoskeleton rearrangement and inflammatory injury of podocytes.

Moreover, we found that B (hibifolin/ hyperoside), C (hyperoside /quercetin-3'-o-glucoside), and E (isoquercetin/quercetin-3'-o-glucoside) have significant effects in the treatment of DKD. Considering the content of the four most critical flavonoid monomers in TFA, we further investigated the mechanism of the optimized combination including hyperoside, isoquercitrin, quercetin-3'-o-glucoside and hibifolin.

Transient receptor potential channel 6 (TRPC6) is a non-selective calcium channel protein. In 2005, the gene mutation of TRPC6 in podocytes was first reported in patients with focal segmental glomerulosclerosis (FSGS), indicating the potential importance of Ca^{2+}dynamics mediated by TRPC6 for podocyte function [32]. The abnormal expression of TRPC6 in podocytes is closely related to the occurrence of proteinuria in various DKD. It can participate in the rearrangement of podocyte cytoskeleton by regulating F-actin and eventually lead to proteinuria. DKD is one of the most common and serious complications of diabetes characterized by proteinuria, and is the main cause of end-stage renal disease (ESRD) in the world [33]. Many studies had shown that the abnormal changes of TRPC6 in podocytes plays an important role in the occurrence of proteinuria and the progression of DKD, and its mechanism may involve the rearrangement of podocyte cytoskeleton[34-36]. Since hyperoside, isoquercetin, hibifolin and quercetin-3 '- o-glucoside account for a large proportion of TFA, the following experiments mainly study the effect of four flavonoid monomers on TRPC6 channel. The results showed that the optimized combination of four flavonoid monomers could significantly reduce the mRNA and protein expression level of TRPC6 in podocytes. Based on the overall activity differences between each component and total flavonoid extract, evaluate the contribution of "part" to "whole", and discover the "equivalent component group" that can characterize the efficacy of total flavonoid extract or Huangkui capsule, thereby promoting the quality control research of Huangkui new drugs.

Conclusions

TFA increased the expression of synaptopodin and nephrin in podocytes induced by high glucose and inhibit the release of TNF-α and IL-β. The proportion of hyperoside, isoquercetin, hibifolin and quercetin-3'-o-glucoside have a huge impact on synaptopodin, nephrin, TNF- α and IL-β , which may be equivalent to TFA. And TFA may protect podocyte cytoskeleton through TRPC6 pathway. Our research provides a new perspective for quality control of traditional Chinese medicine.

Abbreviations

DKD	diabetic kidney disease
ESRD	End-stage renal disease
GAPDH	Gyceraldehyde-3-Phosphate dehydrogenase
HG	High glucose
MPC5	Mouse podocyte 5
MA	mannitol
NG	Normal glucose
PAGE	Polyacrylamide gel electrophoresis
PVDF	Polyvinylidene fluoride
RT-qPCR	Real-Time Reverse Transcriptase-Polymerase Chain Reaction
SDS	Sodium dodecyl sulfate
TNF-α	Tumor Necrosis Factor-α
TFA	Total flavone from *Abelmoschus manihot*
TRPC6	Increased transient receptor potential canonical channel type 6

Declarations

Acknowledgments

Not applicable.

Authors' contributions

Xiulan Wu conducted the experiment, wrote and revised this manuscript. Fujiang Wang helped to collect and analyze the data. Haitao Ge designed and supervised the experiments and contributed to the final draft of the paper. The authors read and approved the final manuscript.

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Data Availability

The datasets used and/or analyzed during the current study are available from the first author upon reasonable request.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors have declared no conflict of interest.

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Table

Table 2 is available in the Supplementary Files section

Figures

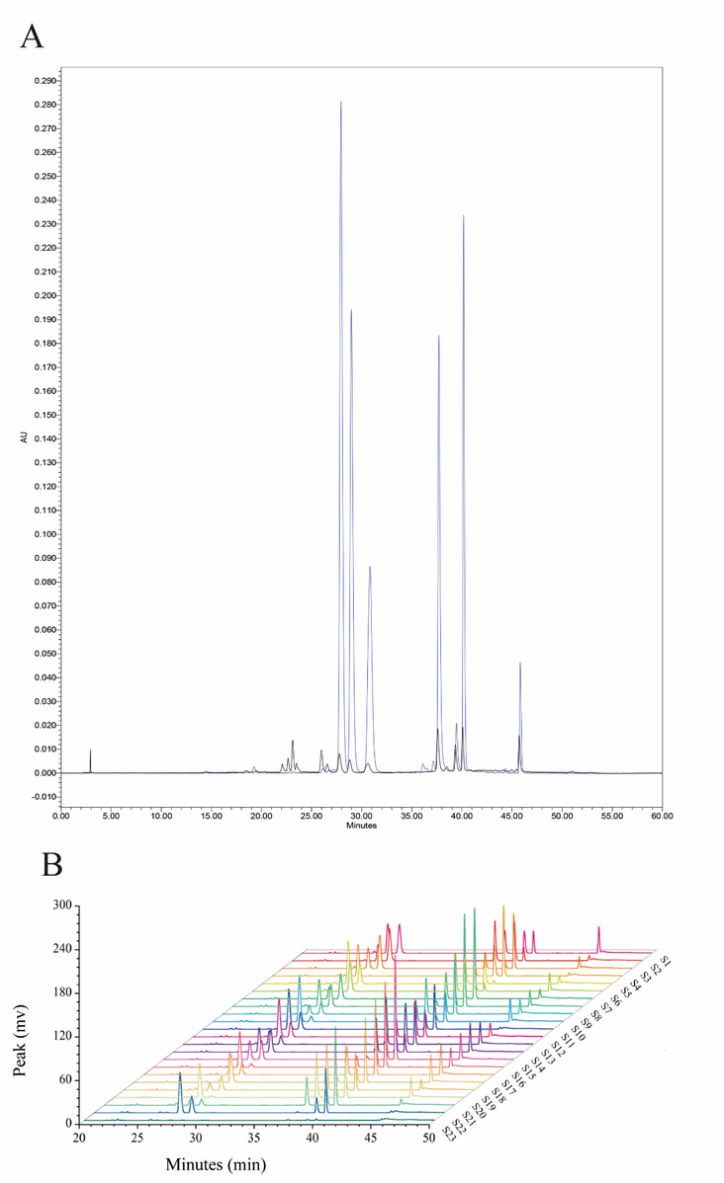

Figure 1

(A) HPLC chromatograms of several peak knocked-out component and negative sample.

(B) The matching chromatograms characterized by HPLC.

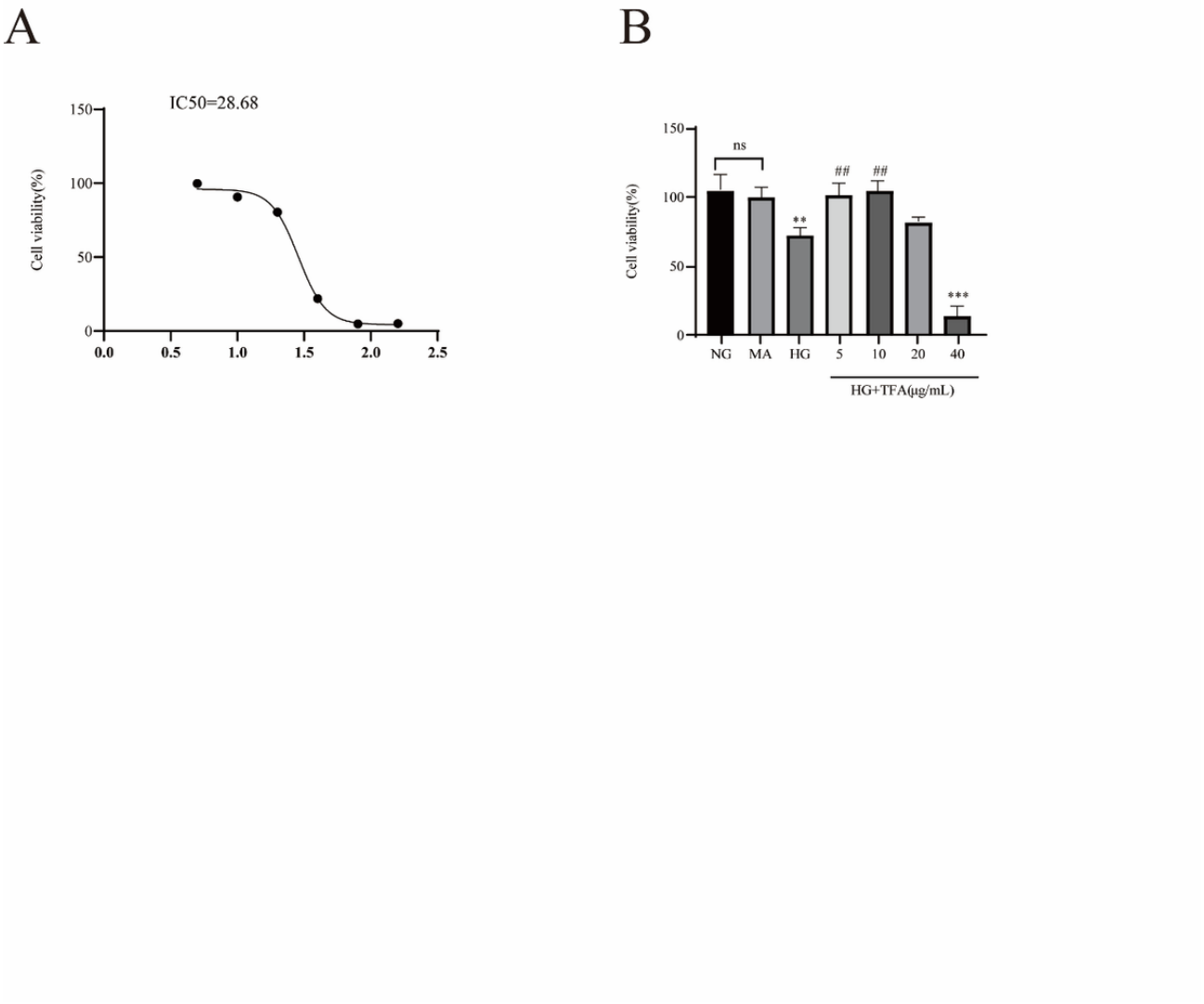

Figure 2

(A-B) The cell viability of normal or high glucose-induced podocytes, podocytes (MPC5) were treated with normal glucose (NG, 5.5 mmol/L) or high glucose (HG, 30 mmol/L) for 48 h.

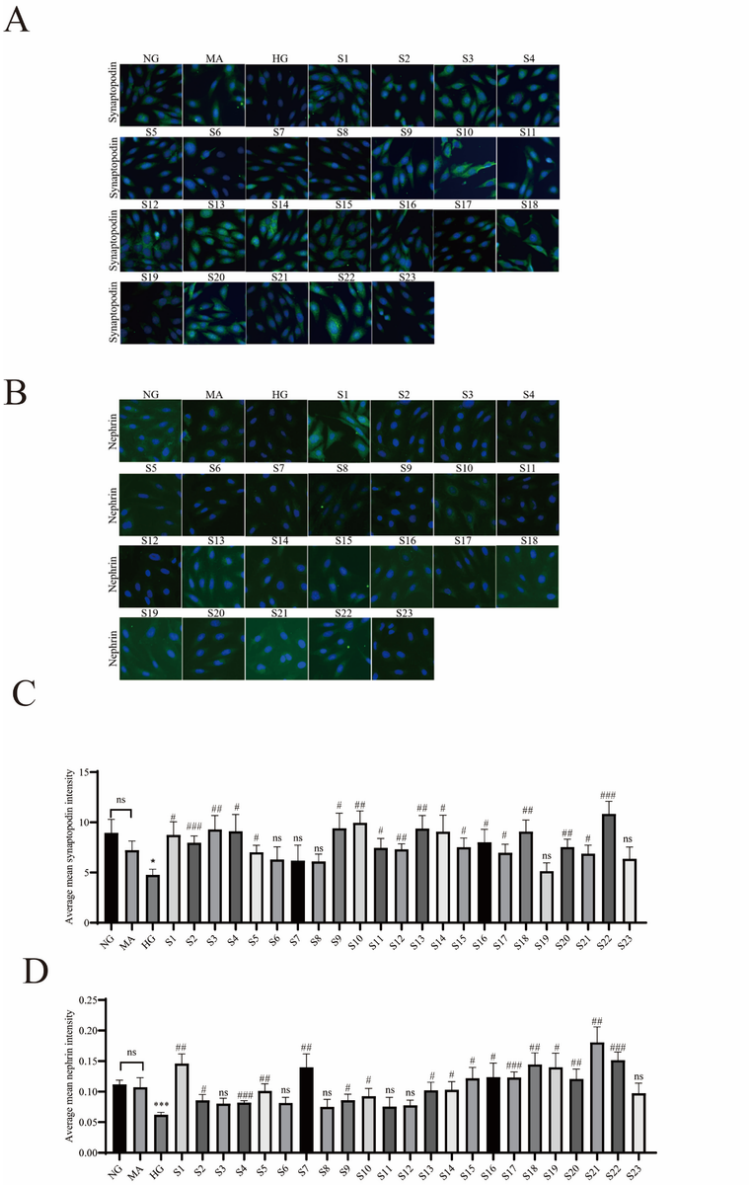

Figure 3

(A-B) The expression of synaptopodin and nephrin were determined by immunofluorescence staining (n=3). (C-D) Summary plots of fluorescence intensity of podocytes. The data (mean ± SD) were representative of three independent experiments. $^{***}P < 0.001$ compared with control group, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ compared with HG group, ns was not statistically significant.

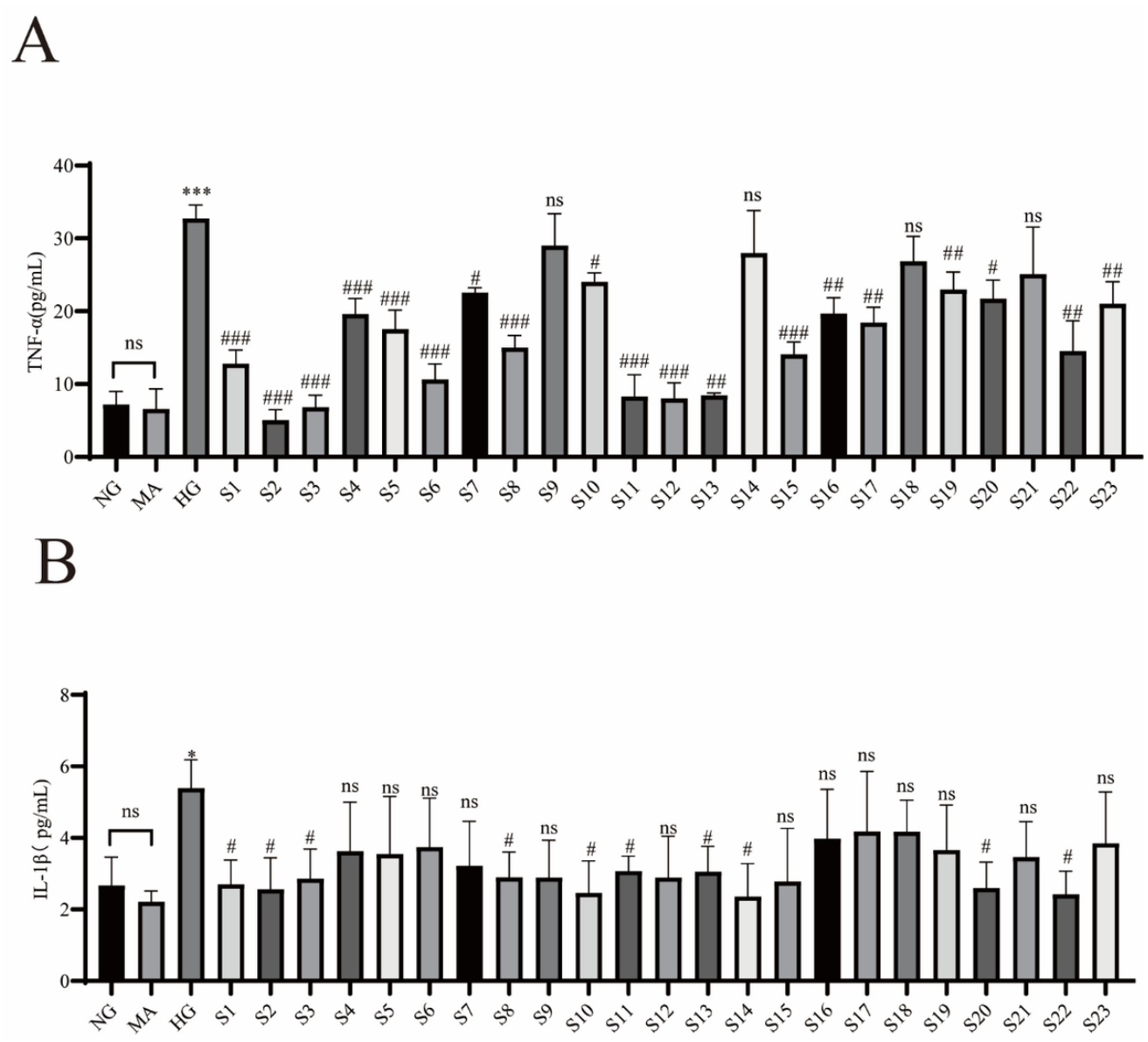

Figure 4

(A-B) The release of TNF-α IL-1β was detected by ELISA. The data (mean ± SD) were representative of three independent experiments. ****P* < 0.001 compared with control group, *#P* < 0.05, *##P* < 0.01, *###P* < 0.001 compared with HG group, ns was not statistically significant.

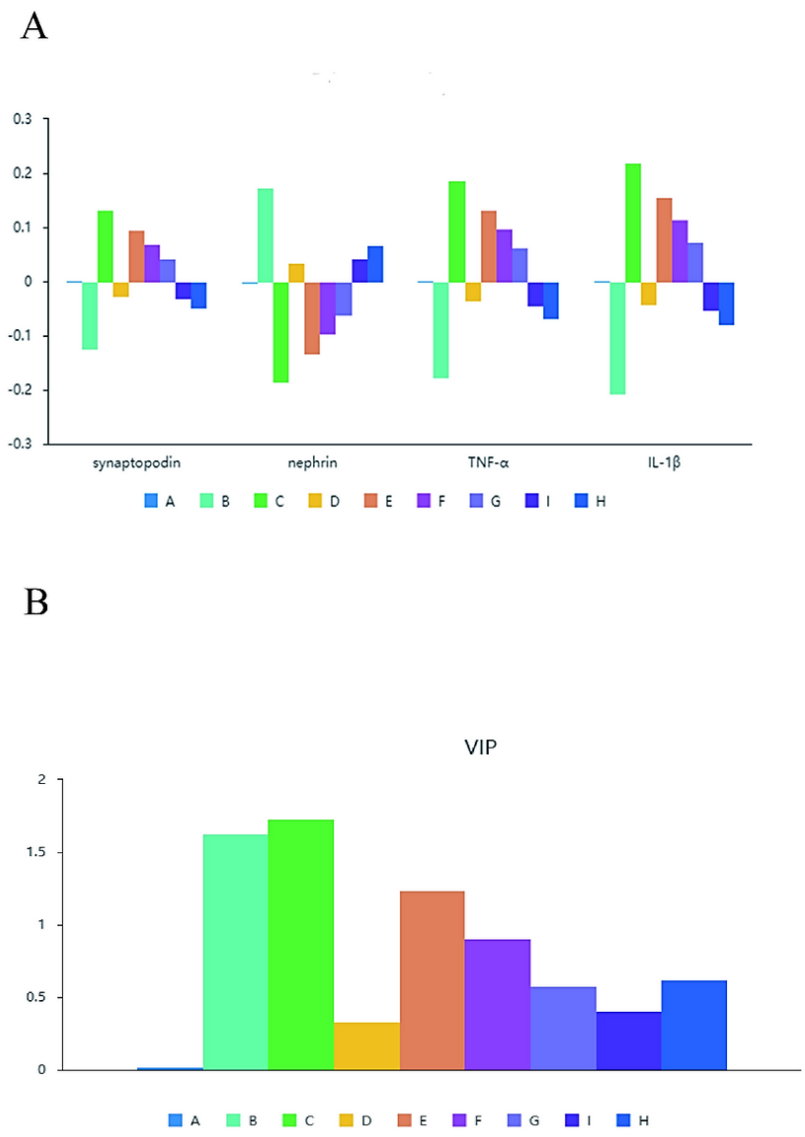

Figure 5

(A) Regression coefficients between different flavonoid combinations and efficacy.

(B) Projection Value VIP. In the legend, A represents hyperoside/ isoquercetin; B represents hibifolin/ hyperoside; C represents hyperoside/quercetin-3'-o-glucoside;D represents hibifolin/ isoquercetin; E represents isoquercetin/quercetin-3'-O-glucoside; F represents hibifolin/ quercetin-3'-o-glucoside; G represents flavonoids with quercetin as the mother nucleus/others; I represents hibifolin/ flavonoids with quercetin as the mother nucleus ; H represents glycosides/aglycones.

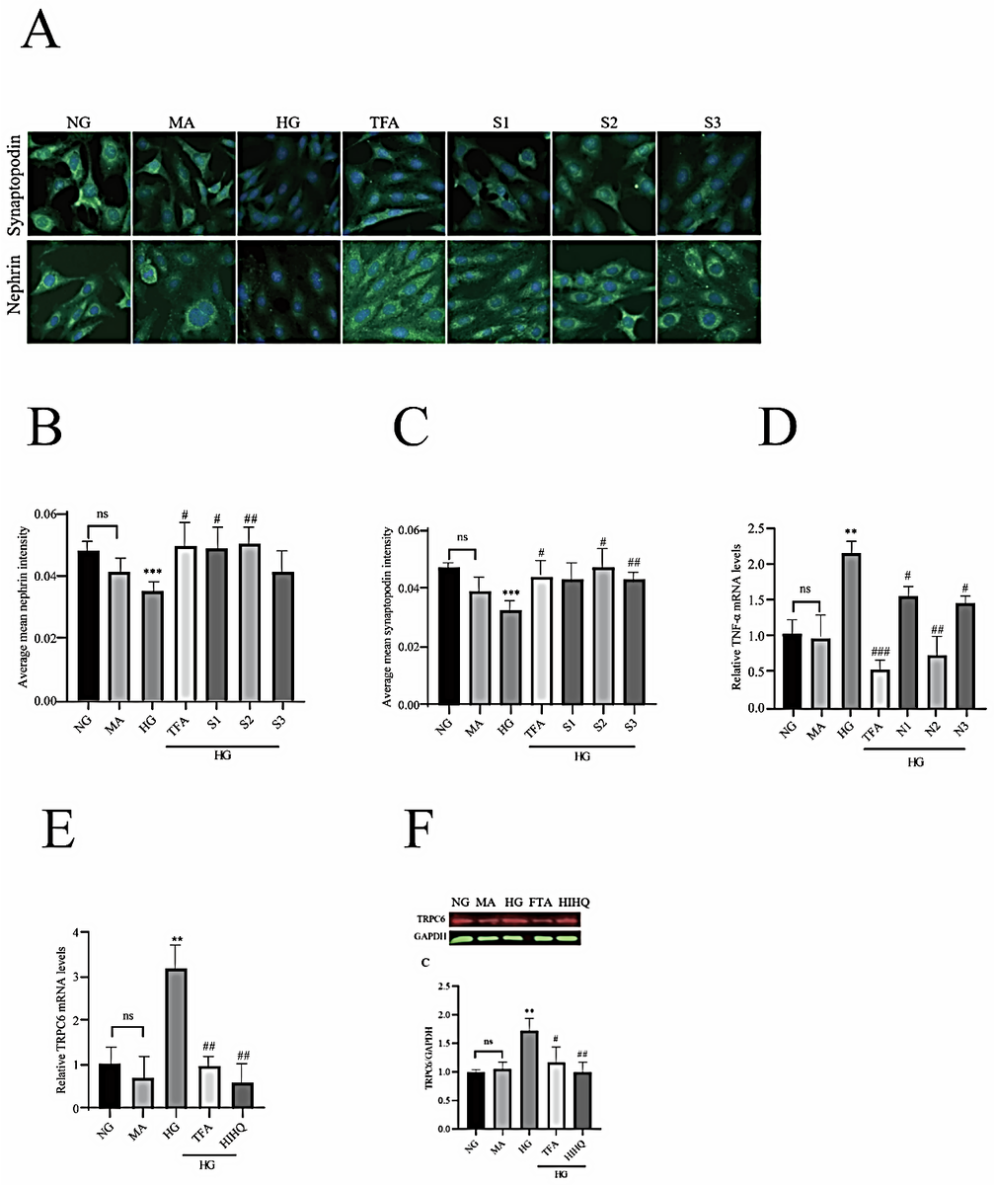

Figure 6

(A) The expression of synaptopodin and nephrin were determined by immunofluorescence staining. (B-C) Summary plots of fluorescence intensity of podocytes. (D) The level of mRNA expression of TNF-α. (E-F) The expression of TRPC6 mRNA and protein on podocytes induced by high glucose. $^{**}P < 0.01$, $^{***}P < 0.001$ compared with control group, $^{\#}P < .05$, $^{\#\#}P < .001$, $^{\#\#\#}P < .0001$ compared with HG group.ns was not statistically significant.

Supplementary Files

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- Table2.docx