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Mechanisms of *Coreopsis tinctoria* Nutt in the Treatment of Diabetic Nephropathy Based on Network Pharmacyology Analysis of its Active Ingredients

Mecanismos de *Coreopsis tinctoria* Nutt. en el Tratamiento de la Nefropatía Diabética Basado en el Análisis Farmacológico de Red de sus Principios Activos

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SUMMARY: *Coreopsis tinctoria* Nutt. (*C. tinctoria* Nutt.) can protect diabetic kidneys, but the mechanisms are unclear. This work is to investigate the potential mechanisms of *C. tinctoria* Nutt. in the treatment of diabetic nephropathy based on network pharmacology analysis of its active ingredients. Twelve small molecular compounds of *C. tinctoria* Nutt. and targets related to diabetic nephropathy were docked by Discovery Studio 3.0. DAVID database was used for GO enrichment and KEGG pathway analysis. Cytoscape 3.6.1 was used to construct active ingredient-target network. Cell viability was detected with MTT. Glucose consumption was analyzed with glucose oxidase method. Protein expression was measured with Western blot and immunofluorescence. Electron microscopy observed autophagosomes. The core active ingredients of *C. tinctoria* Nutt. included heriguard, flavanomarein, maritimein, and marein. Twenty-one core targets of the 43 potential targets were PYGM, TLR2, RAF1, PRKAA2, GPR119, INS, CSF2, TNF, IAPP, AKR1B1, GSK3B, SYK, NFKB2, ESR2, CDK2, FGFR1, HTRA1, AMY2A, CAMK4, GCK, and ABL2. These 21 core targets were significantly enriched in 50 signaling pathways. Thirty-four signaling pathways were closely related to diabetic nephropathy, of which the top pathways were PI3K/AKT, insulin, and mTOR, and insulin resistance. The enriched GO terms included biological processes of protein phosphorylation, and the positive regulation of PI3K signaling and cytokine secretion; cellular components of cytosol, extracellular region, and extracellular space; and molecular function of protein kinase activity, ATP binding, and non-membrane spanning protein tyrosine kinase activity. In vitro experiments found that marein increased the expression of phosphorylated AKT/AKT in human renal glomerular endothelial cells of an insulin resistance model induced by high glucose, as well as increased and decreased, respectively, the levels of the microtubule-associated proteins, LC3 and P62. *C. tinctoria* Nutt. has many active ingredients, with main ingredients of heriguard, flavanomarein, maritimein, and marein, and may exert anti-diabetic nephropathy effect through various signaling pathways and targets.

KEY WORDS: Network pharmacology; *Coreopsis tinctoria* Nutt; Diabetic nephropathy; Marein.

INTRODUCTION

Coreopsis tinctoria Nutt. (*C. tinctoria* Nutt.) belongs to the Asteraceae genus, the Composite family (Zhao *et al*., 2013; Wang *et al*., 2015). The native species of *C. tinctoria* Nutt. is from North America and *C. tinctoria* Nutt. is now

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widely distributed around the world. *C. tinctoria* Nutt. is cultivated in large amounts in the Tianshan mountains at high altitude with abundant snow water resources in South Xinjiang, China. The dry flowers of *C. tinctoria* Nutt. have traditionally been used as healthcare tea to prevent diabetes. Modern pharmacological studies have shown that it has anti-inflammatory (Yao *et al*., 2015), anti-hyperlipidemia (Li *et al*., 2014), and glycemic regulatory activities (Woo *et al*., 2010; Cai *et al*., 2016), has antidiabetic effects and protects diabetic kidneys (Yu *et al*., 2019). However, the active ingredients of *C. tinctoria* Nutt. and their mechanisms of action in the treatment of diabetic nephropathy need further research and clarification.

Diabetic nephropathy is a common and serious complication of diabetes (Thomas & Karalliedde, 2019). The symptoms of diabetic nephropathy include edema, proteinuria, and hypertension, which can lead to end-stage renal disease. According to data from the International Diabetes Federation, the prevalence of diabetic nephropathy is increasing year by year, along with the complications of diabetes (Cho *et al*., 2018). At present, there is no specific drug for the treatment of diabetic nephropathy. China has the highest number of diabetic nephropathy cases in the world. Therefore, it is of great significance to identify safe and effective drugs from Chinese herbal medicine, and to identify appropriate targets in diabetic nephropathy.

Our previous study found that an ethyl acetate extract of *C. tinctoria* Nutt. could reduce blood glucose and blood lipids, and improve renal function in rats with diabetic nephropathy, suggesting that *C. tinctoria* Nutt. has obvious protective effects on diabetic kidneys (Yao *et al*., 2015). However, the mechanism underlying this protective effect is unclear. The complex activities of multiple ingredients have led to difficulties in the study of the anti-diabetic nephropathy mechanism of *C. tinctoria* Nutt. Network pharmacology uses high-throughput omics data analysis, computer-aided drug design software, network visualization, and network analysis to reveal the complex biological network relationships among drugs, targets, and pathways, providing new ideas for research and development of traditional Chinese medicine (Hopkins, 2007; Zhang *et al*., 2013; Hao Da & Xiao, 2014; Wu & Wu, 2015).

Here, we applied network pharmacology to identify active ingredients and mechanisms of *C. tinctoria* Nutt. Discovery Studio was used to perform molecular docking of active ingredients and their diabetic nephropathy targets. A ingredient-target network was constructed using Cytoscape 3.6.0. The Database for Annotation, Visualization and Integrated Discovery (DAVID) was used for gene ontology (GO) enrichment analysis and Kyoto Encyclopaedia of Genes and Genomes (KEGG) pathway analysis (Tiezzi *et al*., 2015). Subsequently, we validated some targets and pathways of *C. tinctoria* Nutt at the cellular level. Our findings may provide theoretical basis for the further development of therapeutic drugs from *C. tinctoria* Nutt. for treatment of diabetic nephropathy.

MATERIAL AND METHOD

Identification of active ingredients. According to previous studies (Yao *et al*., 2015; Du *et al*., 2018; Zhang *et al*., 2019; Guo *et al*., 2020) on the chemical separation and serum pharmacochemistry of *C. tinctoria* Nutt., a total of 12 small and possibly active molecules were collected. The 3D structures of these small molecules were obtained from ZINC database (http://zinc.docking.org/), and then imported into Discovery Studio 3.0 (BIOVIA) for ligand preparation.

Screening of target proteins. The crystal structure–determined diabetic nephropathy target proteins of *C. tinctoria* Nutt. were obtained from Therapeutic Target database (TTD, http://bidd.nus.edu.sg/group/cjttd/) and DrugBank (http://www.drugbank.ca/). The 3D structures of these proteins were obtained from Protein Data Bank (PDB) database (http://www.wwpdb.org/) and then imported into Discovery Studio 3.0 (BIOVIA) for receptor preparation.

Molecular docking. The processed target proteins and small molecules of *C. tinctoria* Nutt. were imported into CDOCKER of Discovery Studio for molecular docking.

KEGG pathway and GO functional enrichment analysis. KEGG pathway and GO functional enrichment analysis was performed using the DAVID database (https://david.ncifcrf.gov/) (DAVID version 6.8) (Dennis Jr. *et al*., 2003). Official abbreviations of potential drug targets were uploaded to the DAVID database for analysis. $P < 0.05$ in enrichment was considered to be statistically significant.

Construction of the ingredient-target network. According to molecular docking results, molecules with docking score ≥30 were extracted and then imported into Cytoscape 3.6.1 (https://cytoscape.org/) to construct a ingredient–target network. Network characteristics including the degree, betweenness, network density, and cluster coefficient were analyzed through a network analyzer in Cytoscape 3.6.1 (Cline *et al*., 2007; Smoot *et al*., 2011). Potentially active molecular groups and potential target protein groups in *C. tinctoria* Nutt. were estimated according to network characteristics.

Cell culture and treatment. The HRGEC (Human glomerular microvascular endothelial cells) were obtained from Beina Biological Company (Wuhan, China), which is an insulin resistance model induced by high glucose. HRGEC were cultured in RPMI 1640 (Hyclone, Logan, UT, USA) supplemented with 10 % heat-inactivated fetal bovine serum (Gibco, Carlsbad, CA, USA) and antibiotics (100 U/mL penicillin and 100 mg/mL streptomycin) at 37 °C under 5 % CO_2. After the intervention with 50 mmol/L high glucose for 24 h, the cells were treated with metformin (100 μmol/L), heriguard (1, 3, and 10 μmol/L), flavanomarein (1, 3, and 10 μmol/L), maritimein (1, 3, and 10 μmol/L) and marein (1, 3, and 10 μmol/L) for 24 h. The compounds of heriguard, flavanomarein, maritimein and marein were obtained from the Extrasynthese (purity 99 %; Lyon, France). Metformin was from MedChemExpress (Monmouth Junction, NJ, USA). For autophagy inhibition, HRGEC were pretreated with the autophagy inhibitor 3-MA (3-methyladenine), at 1 μM for 1 h, and then treated with marein (1, 3, and 10 μmol/L) for 24 h.

MTT assay. Cell viability was measured by a conventional MTT assay. Briefly, cells were plated in 96-well plates and treated with drugs as described above. After 24 h, 10 μL MTT was added and incubated for 4 h followed by oscillation incubation with 150 μL DMSO. OD568 was measured on a microplate reader (Flexstation® 3, Molecular Devices, USA). Cell viability rate relative to control was calculated.

Glucose oxidase method. A glucose oxidase method was used to detect the content of glucose in the medium. HRGECs were inoculated into 96-well plates at 5,000 cells/well. The supernatants of cells after different treatments were collected. The 10 mM glucose standard sample was diluted to 2000, 1000, 500, 250, 125, 62.5, 31.25, and 15.625 μM in distilled water. Then the working liquid was added separately and mixed for 15 min in a water bath at 37 °C. A sample of 200 μL was taken from each tube and placed into 96-well plates. An optical density value was obtained with a wavelength of 505 nm in a microplate reader (Flexstation® 3, Molecular Devices, USA). A standard curve was drawn and the glucose concentration was calculated accordingly.

Immunofluorescence. HRGECs ($1x10^4$ cells per well) were inoculated into a laser confocal dish and cultured overnight. For each well, 1 mL RPMI medium containing 10 % fetal bovine serum, high glucose (50 mM) and marein (10 μM) was added. After culturing for 24 h, cells were fixed with 4 % paraformaldehyde. After fixation, 5 % bovine serum albumin was added and incubated for 30 min for blocking. Then, LC3 antibodies (1:100; Cell Signaling Technology, Danvers, MA, USA) were added for incubation overnight followed by incubation with goat anti-rabbit secondary antibodies (1:100, ab 150080) for 2 h. After washing, cells were stained with DAPI for 5 min. Images were observed under confocal microscopy. The fluorescence intensity of LC3 was analyzed with IPP6.0 software.

Electron microscopy analysis of autophagosome. After treatment, HRGECs were fixed in 2 % glutaraldehyde/0.1 M phosphate buffer (pH 7.4) and 1 % osmium tetroxide/0.1 M phosphate buffer (pH 7.4). Samples were then dehydrated using a graded series of ethanol and embedded in epoxy resin. A transmission electron microscope (H-7500; Hitachi, Tokyo, Japan) was used to visualize ultrathin sections stained with uranyl acetate and lead citrate. The number of autophagosome per cell was calculated.

Western blot analysis. Proteins were extracted with radioimmuno precipitation lysis buffer (Thermo Fisher, Waltham, MA, USA) containing a protease and phosphatase inhibitor cocktail. The protein concentration was measured by BCA Protein Assay Kit (Thermo Fisher). Proteins (20 μg) were separated by 12 % SDS–polyacrylamide gel electrophoresis and transferred onto a polyvinylidene fluoride membrane (Millipore, Billerica, MA, USA). After blocking in 5 % (v/v) skimmed milk for 1 h and washing three times, the membrane was incubated with primary antibodies against p-AKT, P62, mammalian target of rapamycin (mTOR), AKT, insulin receptor substrate 1 (IRS1), phosphatidylinositol 3-kinase (PI3K), LC3 (1:1000; Cell Signaling Technology, Danvers, MA, USA), or GAPDH (1; 2000; Genview, Houston, TX, USA) at 4 °C overnight. Finally, the membrane was washed and incubated for 1 h with secondary horseradish peroxidase-conjugated antibodies (1:2000; Dingguo Changsheng Biotech, Beijing, China) at room temperature. Protein bands were visualized using an ECL system (Amersham Biosciences, Little Chalfont, UK) and an image analyzer (Bio-Rad Laboratories, Hercules, CA, USA). Image J software was used for densitometry measurements of protein bands on western blots.

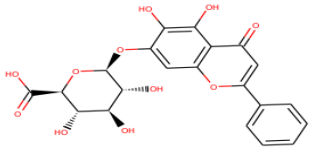
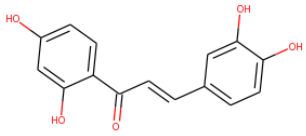
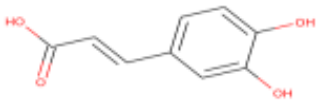
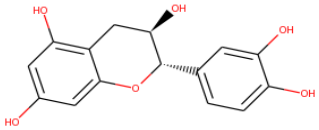
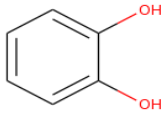
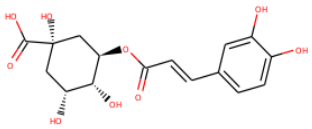
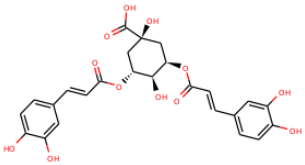
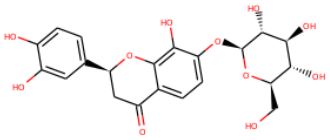
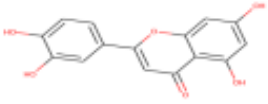
Statistical analysis. All data are presented as the mean ± SD. The significance of difference among groups was determined by using one-way ANOVA and LSD test. Data were considered statistically significant at $P < 0.05$.

RESULTS

Screening of active ingredients and target proteins of *C. tinctoria* Nutt. Based on previous studies (Yao *et al*., 2015; Du *et al*., 2018; Zhang *et al*., 2019; Guo *et al*., 2020) on the chemical separation and serum pharmacochemistry of *C.*

tinctoria Nutt., 12 small and potentially active ingredients in *C. tinctoria* Nutt. were identified (Table I). These ingredients included baicalin, butein, caffeic, catechin, catechol, heriguard, dicaffeoylquinic, flavanomarein, luteolin, marein, maritimein, and meletin. Their 3D structures were obtained from ZINC15 software (http://zinc.docking.org/).

Table I. Possible active small molecules in *C. tinctoria* Nutt.

Compound	Chemical structure	ZINC ID
baicalin		ZINC3943903
butein		ZINC12428433
caffeic		ZINC58172
catechin		ZINC119985
catechol		ZINC13512214
heriguard		ZINC2138728
dicaffeoylquinic		ZINC100037586
flavanomarein		ZINC13827634
luteolin		ZINC8185774

We obtained crystal structure–determined diabetic nephropathy target proteins from TTD database and DrugBank. A total of 43 proteins were obtained (Table II). The 3D structures of these proteins were obtained from PDB. To further verify the relationship between the 12 small ingredients and the 43 target proteins in diabetic nephropathy, we performed molecular docking with the CDOCKER module of Discovery studio 3.0. The results showed that the 43 target proteins were strongly bound to some of the 12 small molecules, with a CDOCKER interaction energy of ≥30 kcal/mol. The docking scores are shown in supplementary Table I.

Pharmacological mechanisms of *C. tinctoria* Nutt. against diabetic nephropathy. The ingredient–target network was constructed using Cytoscape 3.6.1 based on the 12 small ingredients and 43 target proteins of *C. tinctoria* Nutt. (Fig. 1A). The result of network characteristics showed that the average degree of nodes was 25.4 (Supplementary Tabla II), with the degree of eight nodes larger than the average value. The eight core active ingredients of *C. tinctoria* Nutt. were butein, catechin, luteolin, heriguard, meletin, flavanomarein, maritimein, and marein. The average degree of the 43 target proteins in the network was 7.4 (Supplementary Tabla III), with 21 targets having higher degree than the average degree. The 21 target proteins were respectively: PYGM, TLR2, RAF1, PRKAA2, GPR119, INS, CSF2, TNF, IAPP, AKR1B1, GSK3B, SYK, NFKB2, ESR2, CDK2, FGFR1, HTRA1, AMY2A, CAMK4, GCK, and ABL2. These 21 target proteins are speculated to be important targets and to play an extremely important role in the treatment of diabetic nephropathy. In addition, four active ingredients of heriguard, flavanomarein, maritimein, and marein in *C. tinctoria* Nutt. showed strong interactions with all 21 target proteins (Figs. 1D and 2, and supplementary Table IV). Therefore, these four monomer compounds were selected as candidate drugs for subsequent *in vitro* biological experiments.

Table II. Putative protein targets of *C. tinctoria* Nutt. related to diabetic nephropathy.

No.	PDB ID	Gene Name	Protein name	Uniprot ID
1	5ISO	PRKAA2	AMP-activated protein kinase	P54646
2	3G7V	IAPP	Islet amyloid polypeptide	P10997
3	1J1B	GSK3B	Glycogen synthase kinase-3 beta	P49841
4	3F9M	GCK	Hexokinase 4	P3557
5	5LWM	JAK3	Tyrosine-protein kinase JAK3	P52333
6	4N8D	DPP4	Dipeptidyl peptidase 4	P27487
7	5TZR	FFAR1	GPR40 (Free fatty acid receptor 1)	O14842
8	4UDD	NR3C1	Glucocorticoid receptor	P04150
9	1A8M	TNF	Tumor necrosis factor alpha	P01375
10	4IBM	INSR	Insulin receptor	P06213
11	1HTV	INS	Insulin	P01308
12	2R24	AKR1B1	Aldose reductase	P15121
13	1Z8D	PYGM	Glycogen phosphorylase, muscle form	P11217
14	2QKH	GPR119	Glucose-dependent insulinotropic receptor	P09681
15	2DDF	ADAM17	Disintegrin and metalloproteinase domain-containing protein 17	P78536
16	1UMG	FBP	Fructose-1,6-bisphosphatase	F9VMT6
17	1CSG	CSF2	Granulocyte-macrophage colony-stimulating factor	P04141
18	2D9Q	CSF3R	Granulocyte colony-stimulating factor receptor	Q99062
19	3V6O	LEPR	Leptin receptor	P48357
20	1C1Y	RAF1	RAF proto-oncogene serine/threonine-protein kinase	P04049
21	1XBB	SYK	Tyrosine-protein kinase SYK	P43405
22	1DR9	CD80	T-lymphocyte activation antigen CD80	P33681
23	1FYW	TLR2	Toll-like receptor 2	O60603
24	1UU3	PDPK1	3-phosphoinositide dependent protein kinase-1	O15530
25	2W4O	CAMK4	Calcium/calmodulin-dependent protein kinase type IV	Q16566
26	3IJ8	AMY2A	Pancreatic alpha-amylase	P04746
27	2WMS	CHEK1	Serine/threonine-protein kinase Chk1	O14757
28	3HMI	ABL2	Abelson tyrosine-protein kinase 2	P42684
29	1E1X	CDK2	Cyclin-dependent kinase 2	P24941
30	3RHX	FGFR1	Fibroblast growth factor receptor 1	P11362
31	3S74	CA2	Carbonic anhydrase 2	P00918
32	3RI1	FGFR2	Fibroblast growth factor receptor 2	P21802
33	3NZI	HTRA1	Serine protease HTRA1	Q92743
34	1U3R	ESR2	Estrogen receptor beta	Q92731
35	3E64	JAK2	Tyrosine-protein kinase JAK2	O60674
36	1WQJ	IGFBP4	Insulin-like growth factor-binding protein 4	P22692
37	3MJG	PDGFB	Platelet-derived growth factor subunit B	P01127
38	1IMV	SERPINF1	Pigment epithelium derived factor	P36955
39	3QTK	VEGF	Vascular endothelial growth factor-A	P15692
40	3M7P	FN1	Fibronectin	P02751
41	4OIB	ICAM5	Intercellular adhesion molecule-1	Q9UMF0
42	1KLC	TGFB1	Transforming growth factor-_1	P01137
43	1A3Q	NFKB2	Nuclear factor NF-kappa-B p100 subunit	Q00653

KEGG pathway and GO enrichment analyses. In order to elucidate the possible signaling pathways and mechanisms, the 21 genes corresponding to the target proteins were subjected to KEGG pathway and GO enrichment analyses. The results of KEGG pathway enrichment analysis showed that the 21 genes were significantly enriched in 50 signaling pathways ($P < 0.05$). Among them, 34 signaling pathways were directly related to the occurrence and development of diabetic nephropathy, indicating that these signaling pathways might be involved in the effects of *C. tinctoria* Nutt. on diabetic nephropathy. Detailed information on the top 10 pathways is shown in Figure 1B. We found that many pathways were associated with insulin resistance, such as the PI3K/AKT and insulin signaling pathways, and insulin resistance and secretion. The top pathways were the PI3K/AKT signaling pathway, insulin resistance signaling pathway, insulin resistance, and mTOR signaling pathway. Therefore, we speculate that the four monomer compounds in *C. tinctoria*

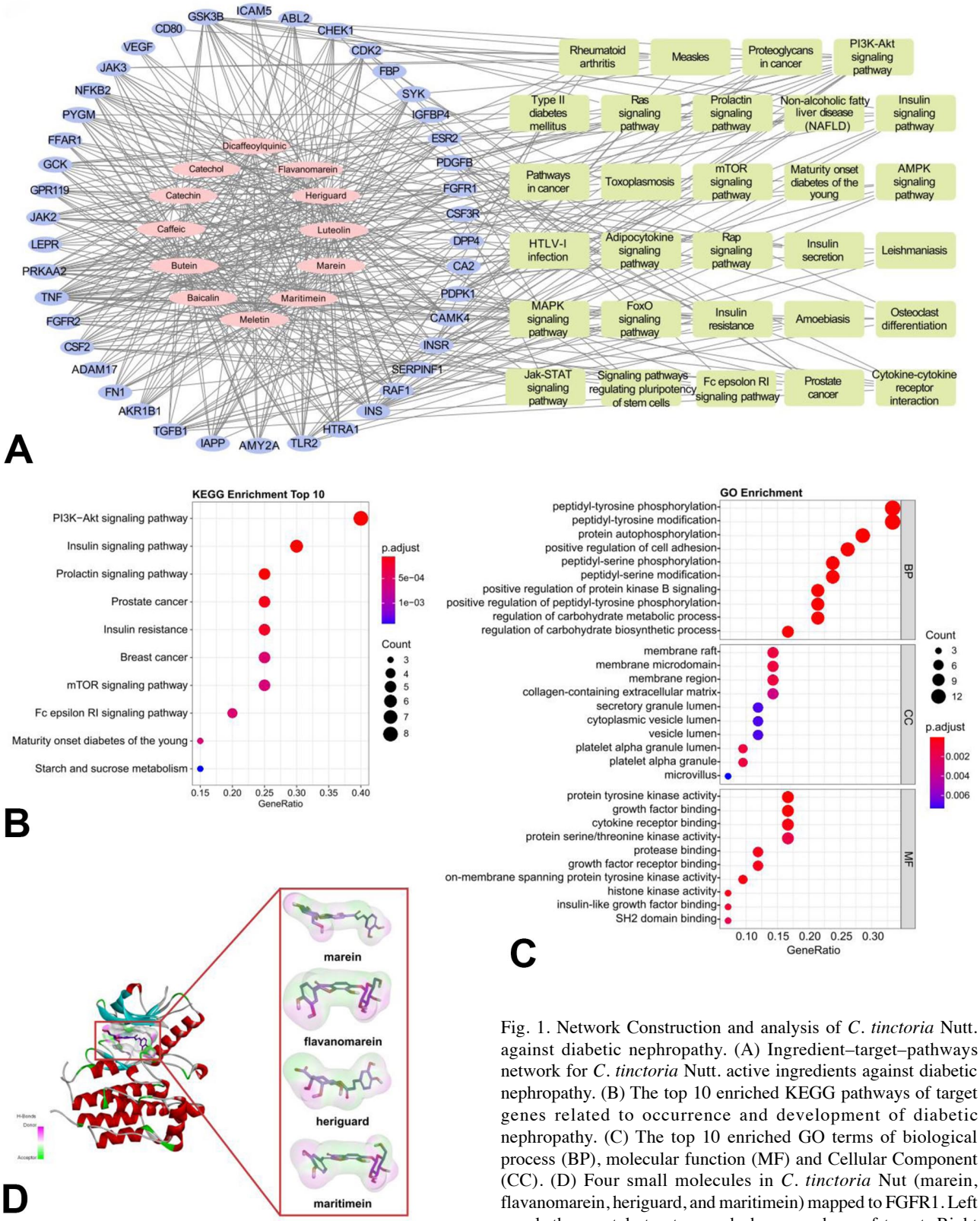


Fig. 1. Network Construction and analysis of *C. tinctoria* Nutt. against diabetic nephropathy. (A) Ingredient–target–pathways network for *C. tinctoria* Nutt. active ingredients against diabetic nephropathy. (B) The top 10 enriched KEGG pathways of target genes related to occurrence and development of diabetic nephropathy. (C) The top 10 enriched GO terms of biological process (BP), molecular function (MF) and Cellular Component (CC). (D) Four small molecules in *C. tinctoria* Nut (marein, flavanomarein, heriguard, and maritimein) mapped to FGFR1. Left panel: the crystal structure and pharmacophore of target; Right panel: small molecules in *C. tinctoria* Nut fitted to the target.

Nutt. may improve insulin resistance by inducing autophagy, thus achieving the effect of protecting the kidneys.

The results of GO enrichment involved 44 terms in biological process, seven terms in molecular function and three terms in cellular component (Fig. 1C). The top three terms in biological process were protein phosphorylation, positive regulation of PI3K signaling and cytokine secretion. The main terms in cellular component were the cytosol, extracellular region, and extracellular space, while those in molecular function involved mainly acting on protein kinase activity, ATP binding\non-membrane spanning, and protein tyrosine kinase activity. Glucose consumption of different drug concentrations of *C. tinctoria* Nutt. in high glucose–treated human renal glomerular endothelial cells.

Effects of heriguard, flavanomarein, maritimein, and marein of *C. tinctoria* Nutt. on cell viability and glucose consumption. We used an insulin resistance model of HRGEC to examine the effects of four candidate monomer compounds (heriguard, flavanomarein, maritimein, and marein) of *C. tinctoria* Nutt. on cell viability and glucose consumption *in vitro* . MTT assay found that these compounds had no significant effect on cell viability at the corresponding concentrations (Fig. 3A). As shown in Figure 3B, after HRGEC were treated with high glucose (50 mmol/L) for 24 h, the glucose consumption of the cells was significantly reduced compared with the control group ($P < 0.05$). Compared with high glucose group, the treatment of heriguard, flavanomarein, maritimein, and marein at 1, 3, and 10 mmol/L significantly increased glucose consumption in a dose dependent manner, suggesting that they may promote glucose absorption and improve the insulin sensitivity of HRGEC (Fig. 3B). Moreover, marein showed the most obvious effects among the four drugs and used in the subsequent experiments.

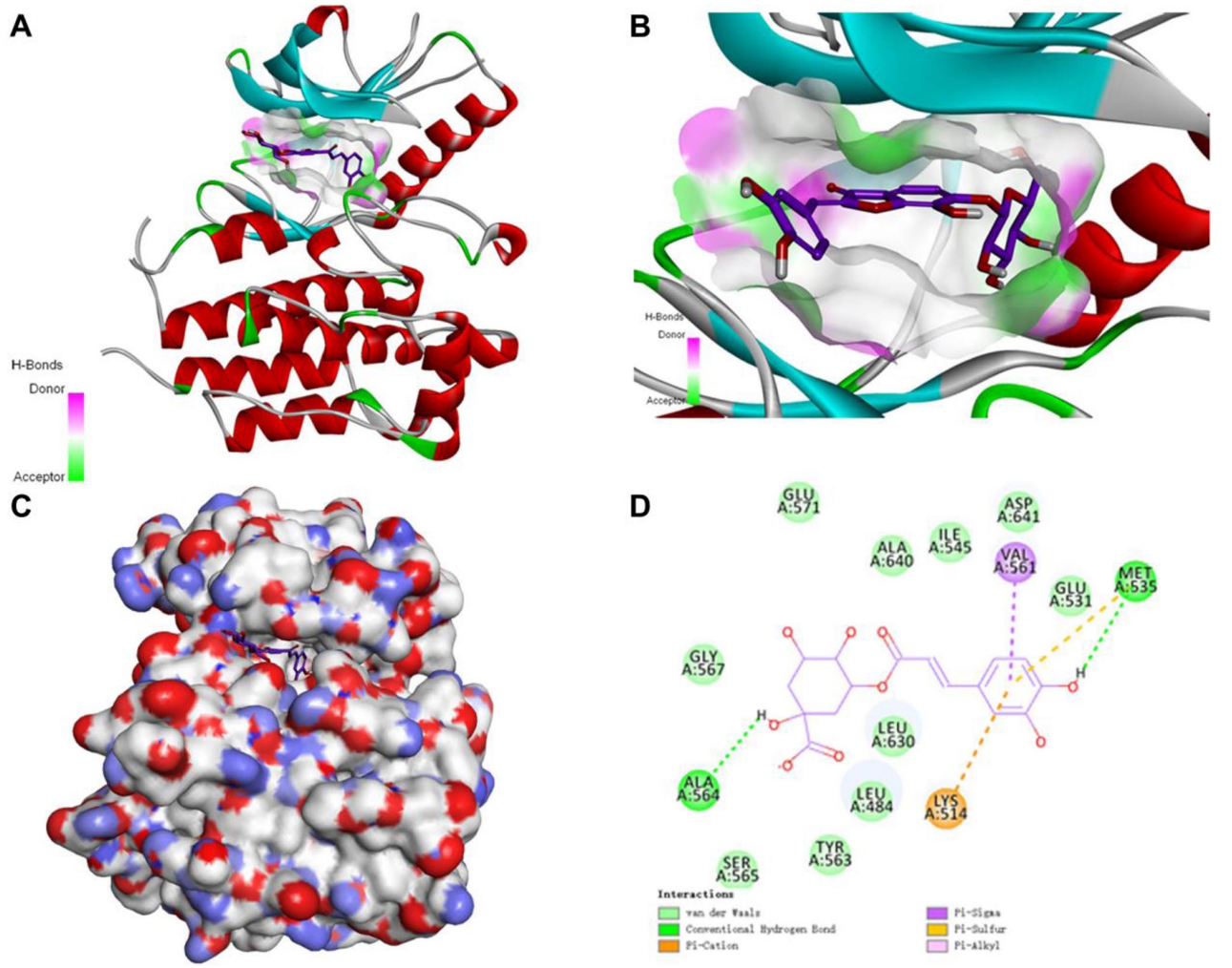


Fig. 2. Protein-ligand interaction model of FGFR1 and marein. (A and B) Interaction models of FGFR1 and marein in the optimal docking pose. The cdocker interaction energy score was -54.5275 kcal/mol. (C) External connection interaction models of FGFR1 and marein. (D) Detailed interaction modes of FGFR1 and marein in the optimal docking pose.

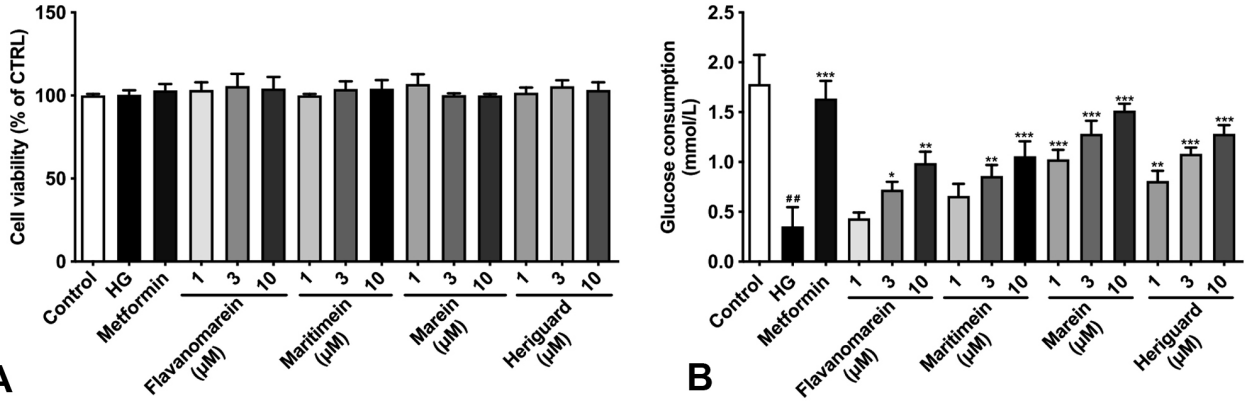


Fig. 3. Effects of marein, flavanomarein, heriguard, and maritimein on cell viability and glucose consumption. (A) Cell viability after treatment with different drug concentrations of *C. tinctoria* Nutt. in HG–treated HRGEC after 24 h. (B) Glucose consumption after treatment with different drug concentrations of *C. tinctoria* Nutt. in high glucose (HG, 50mM)–treated human glomerular endothelial cells (HRGEC) for 24 h. Values are presented as mean ± SD, n = 3. ##$P < 0.01$ vs. control (CTRL); *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ *vs.* HG.

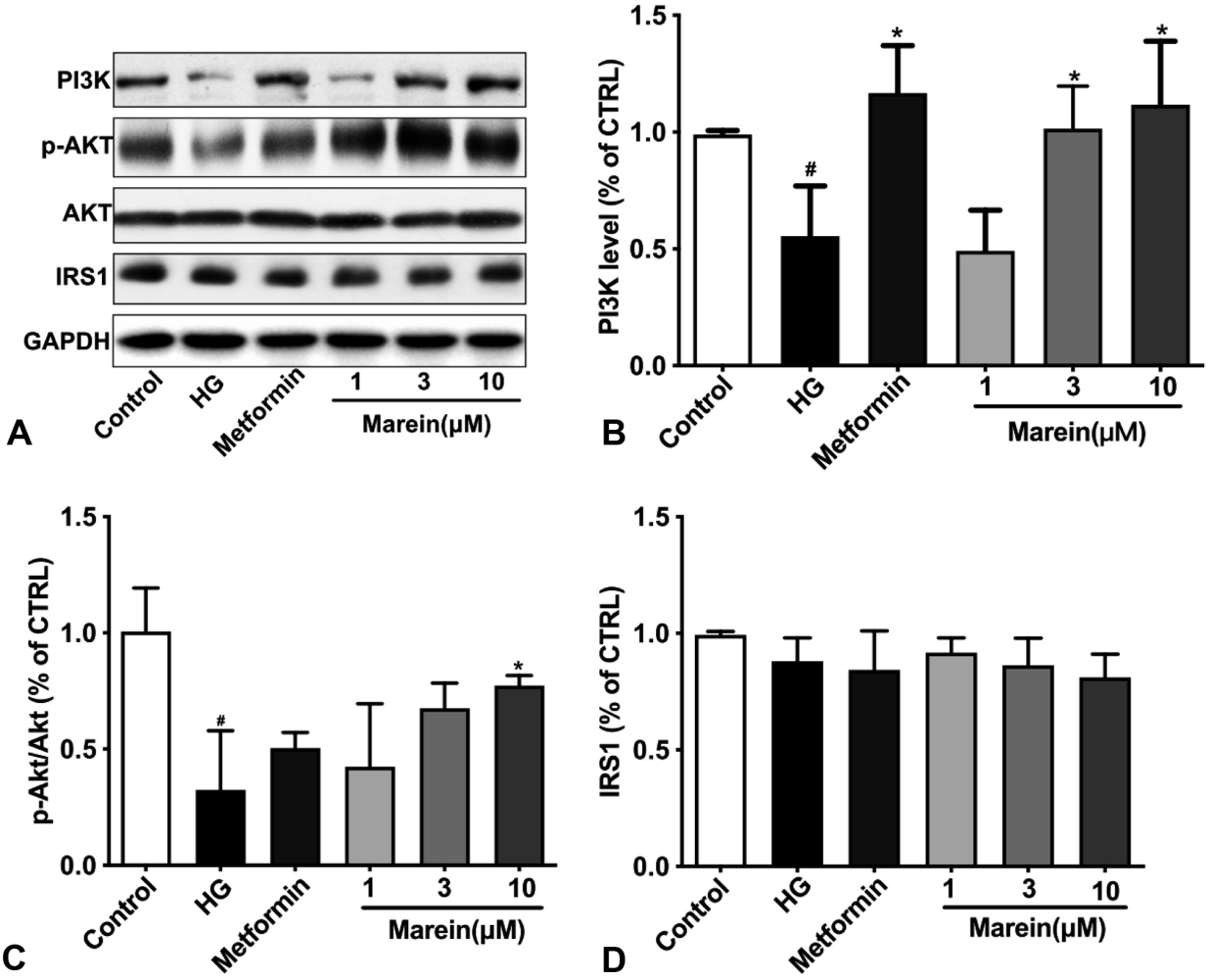


Fig. 4. Effects of marein on protein levels of PI3K, p-AKT (Ser473)/AKT, and IRS1. Human renal glomerular endothelial cells (HRGEC) were treated with different concentrations (1, 3, and 10 mM) of marein and metformin (100mM) for 24 h. (A) The expression of the indicated proteins was detected by Western blot. Bar graphs show the relative protein levels of PI3K (B), p-AKT (Ser473)/AKT (C), and IRS1 (D). Results are expressed relative to GAPDH. HG, high glucose; All values are expressed as mean ± SD (n=3). #$P < 0.05$ vs. control (CTRL); *$P < 0.05$ vs. HG.

Marein regulates key proteins in PI3K/AKT signaling pathway. After 50 mmol/L glucose stimulation of HRGEC for 24 h, we detected the expression of IRS1, PI3K, p-AKT (Ser473), AKT, and GAPDH in HRGEC by Western blot. We found that in HRGEC stimulated by high glucose (50 mmol/L) for 24 h, reduced levels of PI3K and p-AKT (Ser473)/AKT were observed (Fig. 4). Treatment with marein (3 and 10 μM) add metformin (100 μM) for 24 h significantly increased the expression of PI3K and p-AKT (Ser473)/AKT. The levels of IRS1 in each group showed no significant change. The results suggest that marein may regulate the PI3K/AKT signaling pathway to improve insulin sensitivity.

Marein induces autophagy in HRGEC. The effect of marein on autophagy-related proteins (LC3 and P62) in HRGEC was examined by Western blot. Compared with the control group, expression of LC3 in the high glucose group was reduced, and that of P62 was increased (Fig. 5A). However, treatment with marein (1, 3, and 10 μM) significantly increased the expression of LC3 and reduced expression of P62, indicating that marein may promote autophagy in endothelial cells.

To further verify this, HRGEC were pretreated with 3-MA and then with marein. Marein significantly increased the expression of p-AKT/AKT under high glucose ($P < 0.01$) (Fig. 5B). However, pretreatment with 3-MA significantly reduced p-AKT/AKT ($P < 0.01$). After pretreatment with 3-MA, the expression of LC3 in HRGEC of

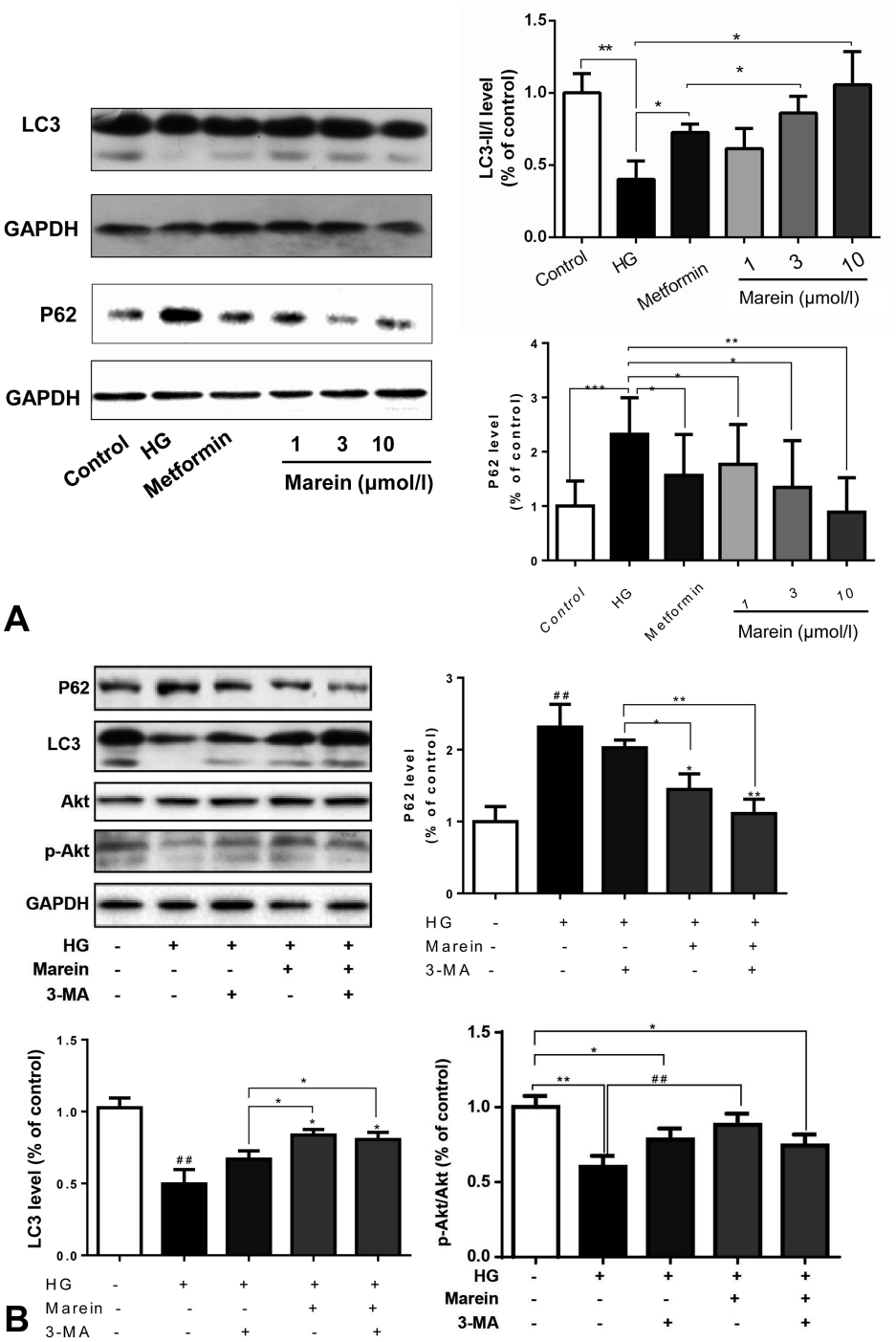


Fig. 5. Effects of marein on protein levels of LC3, P62 and p-AKT (Ser473)/AKT. (A) Western blot analyzed the protein levels of LC3 and P62 after treatment by marein (1, 3, and 10 mM) and metformin (100mM). Bar graphs show the quantification of the indicated proteins. (B) Western blot analyzed the protein levels of LC3, P62 and p-AKT (Ser473)/AKT after treatment by marein and 3-MA. Bar graphs show the quantification of the indicated proteins. Values were expressed as mean ± SD (n=3).

insulin resistance mode was significantly reduced ($P < 0.05$), while the expression of P62 was significantly decreased ($P < 0.001$). Compared with the autophagy inhibitor 3-MA group, the expression of autophagy related protein LC3 was increased in the marein-treated group and the autophagy inhibitor combined with the marein-treated group, while the expression of P62 was significantly decreased.

Furthermore, the expression level of LC3 in each group was detected by immunofluorescence assay. Compared with the control group, LC3 expression in the high glucose group was significantly decreased ($P < 0.05$) (Fig. 6A). After marein treatment for 24 h, the expression of LC3 was significantly increased ($P < 0.05$).

Additionally, the autophagosomes were detected by electron microscopy. As shown in Figure 6B, in the high glucose group, we found a number of balloon-like structures suggesting damaged lysosomes. These results confirmed that autophagy was impaired in a high glucose environment, leading to the accumulation of damaged lysosomes. On the other hand, marein significantly increased the number of autophagosomes.

Together, these results indicate that marein may induce autophagy in HRGEC under high glucose.

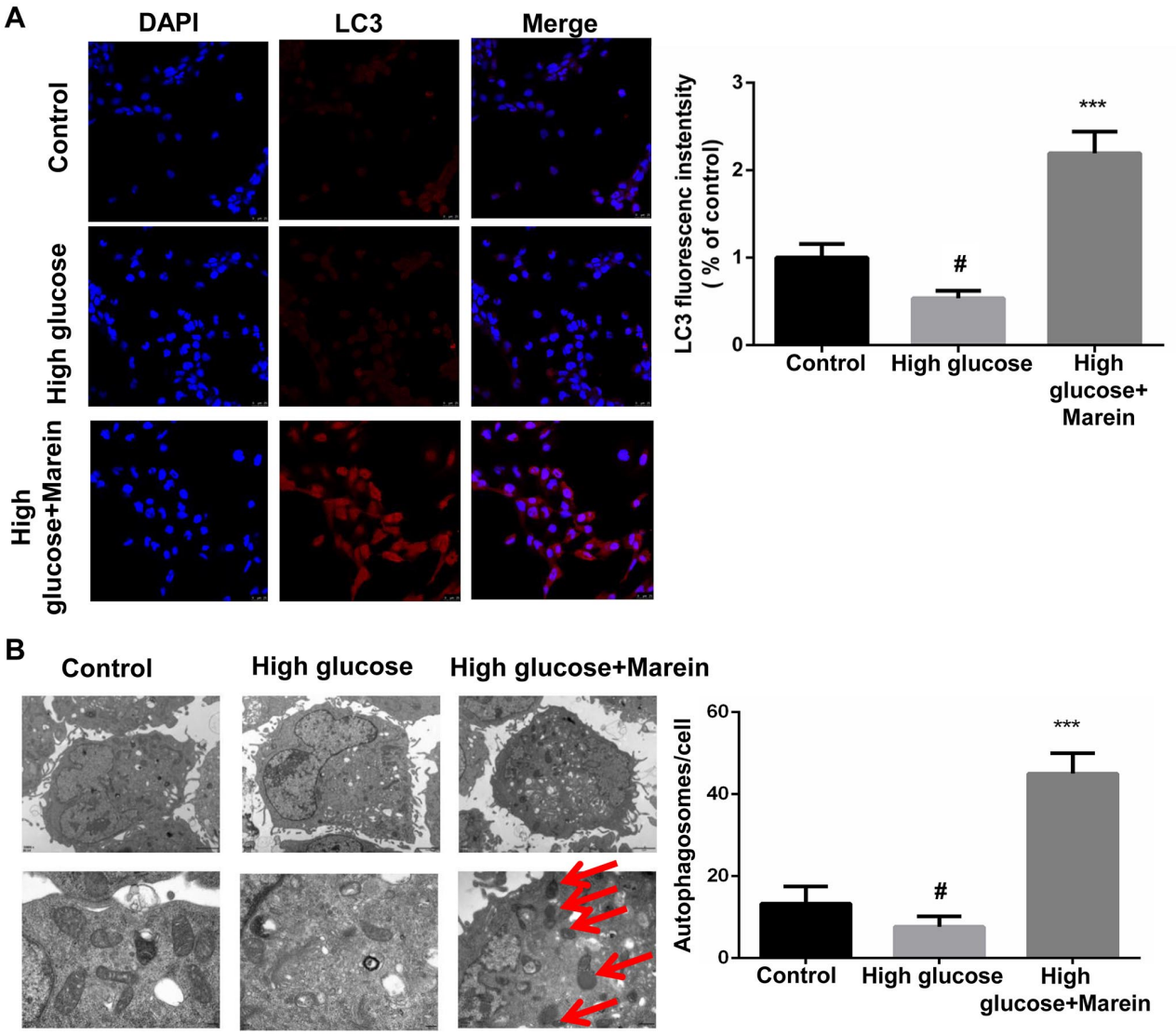


Fig. 6. Effects of marein on LC3 protein level and autophagosomes. Human renal glomerular endothelial cells (HRGECs) were treated with high glucose (50 mM) followed by incubation with or without marein (10 mM) for 24 h. (A) Effects of marein on LC3 fluorescence (immunofluorescence staining ´400). (B) Representative transmission electron microscopy images depicting the ultrastructure of HRGECs. Arrows indicate autophagosomes. Statistical quantities of autophagosomes are shown on the lower panel. Note: Values were expressed as mean ± SD (n=3). #$P < 0.05$ vs. control; ***$P < 0.001$ vs. high glucose.

DISCUSSION

C. tinctoria Nutt., also known as snow chrysanthemum, has glycemic regulatory, anti-hyperlipidemia, and anti-inflammatory activities (Hwang *et al*., 2010). It also has anti-diabetic effects and can protect diabetic kidneys (Yu *et al*., 2019). It has traditionally been used as a healthcare tea to prevent diabetes and cardiovascular diseases (Dias *et al*., 2010; Sun *et al*., 2013). However, the mechanism of the anti-diabetic nephropathy effects of *C. tinctoria* Nutt. is still unclear and needs further investigation. Network pharmacology can be used to study drug mechanisms in a systematic and comprehensive manner based on systems biology, which is considered a very promising new method for the research and development of traditional Chinese medicine.

In this study, we applied network pharmacological approach to identify active ingredients in *C. tinctoria* Nutt. and their potential mechanisms. A total of 12 candidate active ingredients of *C. tinctoria* Nutt. and 43 target proteins of these 12 candidates related to diabetic nephropathy were identified. Based on the degree value of each ingredient and target in the ingredient–target network, we found that the important ingredients of *C. tinctoria* Nutt., such as chlorogenic acid, flavanomarein, maritimein, and marein, acted on 21 core targets, including CDK2, FGFR1, GSK3B, etc.. The degree, which refers to the number of connections between a node and other nodes, was used to evaluate the essentiality of each target and ingredient in the network. Thus, targets and ingredients that play a key role in the mechanisms of *C. tinctoria* Nutt. were revealed. Different ingredients may act on the same target protein, and one ingredient may act on multiple target proteins. This indicates that *C. tinctoria* Nutt. may have a multi-target effect in the treatment of diabetes, with multi-ingredients and multi-targets in many pathways. Through GO and KEGG pathway enrichments, the mechanisms of *C. tinctoria* Nutt. against diabetic nephropathy were related to 34 signaling pathways. These were involved in anti-diabetic nephropathy and were associated with PI3K/AKT signaling pathways, insulin resistance, and insulin signaling and mTOR signaling pathways. Therefore, the key mechanism of *C. tinctoria* Nutt. against diabetes might be to induce autophagy and improve insulin resistance by activating the PI3K–AKT signaling pathway.

Diabetic nephropathy is one of the main complications of type 2 diabetes. Insulin resistance, a key feature in the pathogenesis of diabetic nephropathy, often manifests early in the development of diabetic nephropathy (Winkler & Cseh, 2009; King *et al*., 2016; Wang & Lu, 2018). Glomerular endothelial cells are the first layer of the renal filtration barrier whose function directly determines the filtering function of the kidney. Thus, glomerular endothelial cell injury is one of the main reasons for the development of diabetic nephropathy (Muniyappa & Sowers, 2013; Muris *et al*., 2013). Vascular endothelial cells have a high density of insulin receptors that can promote the occurrence of diabetic complications through complicated mechanisms (Barac *et al*., 2007; Qian *et al*., 2018). We used HRGEC, which is an insulin resistance model induced by high glucose, to verify the biological effects of selected ingredients (heriguard, flavanomarein, maritimein, and marein) of *C. tinctoria* Nutt. *in vitro* . Our results showed that heriguard, flavanomarein, maritimin, and marrein significantly increased glucose consumption in a dose-dependent manner. Among them, marein showed the most significant effect in promoting glucose consumption. Thus, marein was used in subsequent experiments.

Marein is one of the main active ingredient and one of the important flavonoid compounds in *C. tinctoria* Nutt. Marein can significantly up-regulate the insulin signaling pathway related to the PI3K and p-AKT in endothelial cells under insulin resistance caused by high glucose (Li *et al*., 2020). Low PI3K/AKT signaling pathway activity is considered to be an upstream mechanism of glomerular endothelial cell injury (Sun *et al*., 2019). Extracellular insulin signals are transmitted to cells through the insulin receptor subunit, IRS1, which activates PI3K, causes phosphorylation, and then activates downstream AKT so as to maintain the structure and function of endothelial cells (Cleasby *et al*., 2007; Gao *et al*., 2019). If the above signal transduction pathway is blocked, especially if the phosphorylation level of key signaling molecules is reduced, the activity of the IRS1/PI3K/AKT signaling pathway is reduced, resulting in endothelial cell injury, dysfunction or damaged components in cells (Laight *et al*., 1999; Varma *et al*., 2005; Huang *et al*., 2018). The PI3K/AKT signaling pathway maintains the balance of the blood glucose concentration in the body. An activated PI3K/AKT signaling pathway has been shown to improve insulin sensitivity, regulate glucose and lipid metabolism, and also protect vascular endothelial cells (Varma *et al*., 2005). Our results showed that under high glucose, the levels of PI3K and p-AKT (Ser473)/AKT decreased. After marein treatment, their levels increased significantly. There was no significant change in IRS1 levels. These data indicate that marein may act through PI3K/AKT, but not IRS1, to improve insulin sensitivity.

Autophagy can remove a large number of dysfunctional or damaged components under insulin resistance, which leads to intracellular stress responses and

inflammation (Levine & Kroemer, 2008; Yang *et al*., 2010; Ding & Choi, 2015; Zhang *et al*., 2017; Lim *et al*., 2018). Marein can significantly increase the expression of LC3 and lower the expression of P62, which indicates that it can promote autophagy in endothelial cells (Haidar *et al*., 2019). The regulation of autophagy might improve insulin resistance, thereby preventing and treating diabetic nephropathy (Ding & Choi, 2015; Liu *et al*., 2017). Autophagy enables cells to reuse of damaged organelles to maintain cellular homeostasis. Oxidative stress, endoplasmic reticulum stress, and inflammation are closely related to insulin resistance, which causes cellular dysfunction and the accumulation of damaged organelles (Mizushima, 2007; Yang *et al*., 2010; Shi *et al*., 2015). Autophagy plays a very important role in the process of removing such organelles. In a state of insulin resistance, autophagy of various tissues is significantly inhibited. Thus, regulation of autophagy may improve insulin resistance so as to prevent and treat diabetic nephropathy. Maralin can enhance insulin signal transduction of human glomerular vascular endothelial cells and improve insulin resistance. Inhibiting autophagy activity will increase insulin resistance of human glomerular vascular endothelial cells, and the effect of Maralin on enhancing insulin signal transduction and improving insulin resistance will be weakened. Maralin can improve insulin resistance of endothelial cells through a variety of mechanisms, in which the reduction of insulin resistance by increasing autophagy activity may be one of the mechanisms.

In summary, predicting the mechanism action of *C. tinctoria* Nutt. by network pharmacological methods is innovative and can greatly reduce the experimental workload. *C. tinctoria* Nutt. has many active ingredients, with main ingredients of heriguard, flavanomarein, maritimein, and marein, and may exert antidiabetic nephropathy effect through various signaling pathways and targets. *In vitro* experimental studies suggest that the *C. tinctoria* Nutt. monomer compound, marein, could regulate autophagy to improve insulin resistance. These results provide us with a novel direction in studying the mechanism of action of *C. tinctoria* Nutt. and provide experimental evidence for developing new treatments for diabetic nephropathy.

LI, T.; LIU, S.; ABULA, Z.; KADIER, K.; GUO, Y.; GU, S.; WANG, L.; ZHANG, F.; MAO, X. & LI, X. Mecanismos de *Coreopsis tinctoria* Nutt. en el tratamiento de la nefropatía diabética basado en el análisis farmacológico de red de sus principios activos. *Int. J. Morphol., 40(5)*:1152-1164, 2022.

RESUMEN: *Coreopsis tinctoria Nutt. (C. tinctoria Nutt.)* puede proteger riñones diabéticos, sin embargo los mecanismos son desconocidos. Este trabajo se realizó para investigar los potenciales mecanismos de *C. tinctoria* Nutt. en el tratamien-to de la nefropatía diabética basado en el análisis de farmacología en red de sus principios activos. Doce compuestos moleculares pequeños de *C. tinctoria* Nutt. y los objetivos relacionados con la nefropatía diabética fueron acoplados por Discovery Studio 3.0. La base de datos DAVID se utilizó para el enriquecimiento GO y el análisis de la vía KEGG. Se usó Cytoscape 3.6.1 para construir una red de ingrediente-objetivo activa. La viabilidad celular se detectó mediante MTT. El consumo de glucosa se analizó con el método de glucosa oxidasa. La expresión proteica fue determinada mediante Western blot e inmunofluorescencia. En la microscopía electrónica se observó autofagosomas. Los principales ingredientes activos de *C. tinctoria* Nutt. incluyeron heriguard, flavanomarein, maritimin y marein. Veintiún de los 43 objetivos potenciales fueron PYGM, TLR2, RAF1, PRKAA2, GPR119, INS, CSF2, TNF, IAPP, AKR1B1, GSK3B, SYK, NFKB2, ESR2, CDK2, FGFR1, HTRA1, AMY2A, CAMK4, GCK y ABL2. Estos 21 objetivos principales se enriquecieron significativamente en 50 vías de señalización. Treinta y cuatro vías de señalización estuvieron estrechamente relacionadas con la nefropatía diabética, de las cuales las principales vías fueron PI3K/AKT, insulina y mTOR, y resistencia a la insulina. Los términos GO enriquecidos incluyeron procesos biológicos de fosforilación proteica, la regulación positiva de la señalización de PI3K y la secreción de citoquinas; componentes celulares del citosol, región extracelular y espacio extracelular; y la función molecular de la actividad de la proteína quinasa, la unión de ATP y la actividad de la proteína tirosina quinasa que no se extiende por la membrana. Los experimentos *in vitro* encontraron que la mareína aumentaba la expresión de AKT/AKT fosforilada en células endoteliales glomerulares renales humanas en un modelo de resistencia a la insulina inducida por niveles elevados de glucosa, así como aumentaron y disminuyeron respectivamente, los niveles de las proteínas asociadas a los microtúbulos, LC3 y P62. *C. tinctoria* Nutt. tiene muchos principios activos, con ingredientes principales de heriguard, flavanomarein, maritimain y marein, y puede ejercer un efecto de nefropatía antidiabética a través de distintass vías de señalización y objetivos.

PALABRAS CLAVE: Farmacología en red; *Coreopsis tinctoria* Nutt; Nefropatía diabética; Marin.

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