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 anti-diabetic 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 % CO₂. 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, 500, 250, 125, 62.5, 31.25, and 15.625 µmol/L 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 (1x10⁴ 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 autophagosomes 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 (1:100; Cell Signaling Technology, Danvers, MA, USA), or GAPDH (1; 200; 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 pharmacology analysis of its active ingredients. *Int. J. Morphol.*, 40(3):1152-1164, 2022.
**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/).

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

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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.](image)
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, 50 mM)–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 (100 mM) 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) and 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, maritimin, 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, maritimin, and marein) of C. tinctoria Nutt. in vitro. Our results showed that heriguard, flavanomarein, maritimin, and marein 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, maritimain, and marein, and may exert anti diabetic 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.


REFERENCES