

ORIGINAL ARTICLE

Demethylzeylasteral reduces the level of proteinuria in diabetic nephropathy: Screening of network pharmacology and verification by animal experiment

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Abstract

This study aimed to use network pharmacology to detail the natural components isolated from *Tripterygium wilfordii* Hook F (TwHF) and examine the effect of the main component (demethylzeylasteral, DEM) on rat models of diabetic nephropathy (DN). In this study, we used network pharmacology to detail the natural components isolated from TwHF, referenced a gene library when screening for components effective in the management of DN, and DEM was confirmed in DN rats. All data were analyzed using the Discovery Studio 4.5 System and the systems Dock online docking method platform. All 24 rats were divided into 4 groups: control, DN, TwHF, and DEM. Blood and urine samples were tested at 0, 8, and 12 weeks. Renal histopathological changes were scored. Network pharmacology indicated that 370 compounds and 46 small molecules (including DEM) were biologically active constituents of TwHF, mainly affecting the inflammatory response through PI3K-Akt and Jak-STAT pathways. Proteinuria in the TwHF and DEM groups was significantly lower than in the DN group ($p \leq .001$), and the decrease in proteinuria in the DEM group was more obvious than in the TwHF group ($p = .004$). The tubular interstitial scores were better in the DEM group than in the TwHF and DN groups. These results indicate that DEM effectively reduced proteinuria and alleviated the tubular interstitial changes in rat models of DN, which may provide a scientific foundation for the development of novel drugs for treatment of DN.

KEYWORDS

analytical pharmacology/toxicology, anti-inflammatory drugs

Abbreviations: ALK, alkaloids; ALT, alanine aminotransferase; AST, aspartate aminotransferase; CKD, chronic kidney disease; DEM, demethylzeylasteral; DIT, diterpenes; DN, diabetic nephropathy; GLU, glucose; GSI, glomerular sclerosis index; sCr, serum creatinine; SES, sesquiterpenes; TRI, triterpenes; TwHF, *Tripterygium wilfordii* Hook F; UREA, urea; VDR, vitamin D receptor.

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1 | INTRODUCTION

Diabetic nephropathy (DN) is a microvascular complication of diabetes mellitus that may progress to chronic kidney disease and is a major cause of end-stage renal disease.^{1,2} Approximately 20%–40% of patients with diabetes develop DN.³ DN is a distinct clinical-pathologic entity with the clinical presentation of microalbuminuria or macroalbuminuria, increased arterial blood pressure, and distinct morphologic pattern of injury on renal biopsy.^{1,2} Risk factors for diabetic kidney disease include genetic factors, smoking, duration of diabetes, early onset of diabetes, poor glycemic control, hypertension, proteinuria, dyslipidemia, and older age.^{1,2,4}

Tripterygium wilfordii Hook F (TwHF), an annual vine of the family Euonymus, is commonly used in traditional Chinese medicine. It has anti-inflammatory, antitumor, antibacterial, immunosuppressive, contraceptive, and analgesic properties. In 1977, Li Leishi first reported that TwHF reduced proteinuria in patients with glomerulonephritis. Chinese randomized controlled trials found that TwHF is equivalent to or better than a renin-angiotensin-aldosterone system inhibitor in treating massive proteinuria due to DN and delayed the deterioration of renal function in DN.⁵ Because inflammation plays an important role in the pathogenesis of DN, TwHF controls the progression of DN through anti-inflammation.

One of the main known constituents of TwHF is demethylzeylasteral (DEM), a carbopolycyclic compound. DEM is known for its immunosuppressive and anti-inflammatory effects,⁶ anti-angiogenic effects,^{7–9} anti-atherosclerotic effects,¹⁰ chemotherapy-enhancement effects in cancer,^{11,12} and anticancer effects through decreased cell proliferation and increased apoptosis.^{9,13} A study in a mouse model indicated that DEM could alleviate lupus nephritis.⁶ DEM can also decrease inflammation in a rat model of unilateral ureteral obstruction.¹⁴ Inflammation is very important in the pathogenesis of DN. Therefore, we want to know whether DEM can treat DN through anti-inflammatory and bring fewer side effects than TwHF.

As an herbal medicine, TwHF formulations are complex and contain a wide variety of compounds. Still, no study, to date, has systematically summarized the composition of TwHF. Therefore, this study aimed to use network pharmacology to detail the natural components isolated from TwHF and examine the effect of the main component (DEM) on rat models of DN.

2 | MATERIALS AND METHODS

2.1 | Network pharmacology

2.1.1 | Data sources

TwHF, used as a title or keyword, combined with search words including “ingredients,” “components,” or “content,” was searched in CNKI, Wanfang, VIP, PubMed, and relevant databases. Data reported from April to June 2017 were retrieved with 410 articles related to the natural constituents of TwHF. Basic information was

imported into the Endnote software; 117 repeated articles were excluded, while data from 236 studies were included in this study.

2.1.2 | Pharmacophore screening

All data analyses were carried out using the Discovery Studio 4.5 System (DS 4.5) and the systemsDock online docking method (<http://systemsdock.unit.oist.jp/iddp/home/index>) platform. Unless specified, the calculations were carried out using default values.

Using the Ligand Profiler module within the DS 4.5 Prepare Ligands subprogram, small molecular ligands were docked with pharmacophores from the PharmaDB database. The BEST parameter was chosen to generate the lowest possible energy conformations. An energy threshold was set at 20 kcal/mol. Over 10000 pharmacophores were connected with small molecules (termed reverse molecular docking). The fit value was used as the evaluation standard. As higher fit values indicate better compound combination with given pharmacophores, we selected small molecules with fit values of more than 0.5 to analyze molecular docking. Relevant protein data were subsequently obtained from high fit value pharmacophores. The systemsDock platform was used to select semi-flexible small molecules to join with primary screening targets in a final step to identify fine targets.

2.1.3 | Screening of components effective in DN management

Using online big data to screen for genetic information relevant to DN and combining the results from five databases, the most relevant genetic information was sorted. The databases included DiGSeE (<http://210.107.182.61/geneSearch/>), TTD (<http://bidd.nus.edu.sg/BIDD-Databases/TTD/TTD.asp>), GeneCards (<http://www.genecards.org/>), NCBI, and GAD. Compared with pharmacophores screened in the previous step, targets related to DN were selected, and molecules acting in DN were analyzed.

2.2 | Animal models of DN

SPF-grade SD rats were purchased from the Beijing Vital River Laboratory Animal Co., Ltd. All animal experiments were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. The experiments were approved by the animal use ethics committee of the Fifth Medical Center of PLA General Hospital.

All 34 rats were male, 6-week-old, with an average weight of 180 ± 20 g. The rats were divided into four groups: control, DN, TwHF (Shanghai DND Pharm-Technology Co., Inc.; 10 mg/tablet), and DEM (Solarbio Science & Technology Co., Ltd.; HPLC-grade $\geq 98\%$). The rats were kept by specialized technicians in the SPF animal center of the Fifth Medical Center of PLA General Hospital at

20–26°C (daily temperature difference $\leq 4^\circ\text{C}$), relative humidity of 40%–70%, ventilation frequency of 15–20 times/h, and a light–dark cycle of 12/12h. The rats were kept in plastic cages, 3–5 animals/cage, with free access to food and water. The litter was changed three times a week, and the experimental equipment was regularly disinfected. The conventional feed (Soybean meal, fish meal, vegetable oil, bran, corn, etc., purchased from Beijing huafukang Technology Co., Ltd.) was used for 1 week, followed by a high-sugar, high-fat diet (10% lard, 20% sucrose, 2.5% cholesterol, 1% cholate, 66.5% conventional feed, purchased from Beijing huafukang Technology Co., Ltd.) for 4 weeks in the DN, TwHF, and DEM groups. After fasting (no water either) for 12h, the tail vein was injected with streptozotocin (30mg/kg, Sigma) diluted with citric acid-sodium citrate buffer. After 72h, the tail vein blood was monitored. Blood glucose (GLU) ≥ 16.7 mmol/L at this time indicated successful modeling. In the TwHF group, the rats were gavaged with TwHF, 12mg/kg/day (Referring to the previous animal experimental studies of TwHF in the treatment of DN rats, the doses were 4, 8, 16 or 5, 10 mg/kg/day, respectively. Therefore, we selected the intermediate dose of 12 mg/kg/day as our study dose.) The rats in the DEM group were gavaged with DEM, 1 mg/kg/day (In view of the fact that there is no research on the application of DEM in the animal model of diabetes for reference, we searched the literature on the treatment of renal transplantation rats with DEM at the doses of 5, 10 and 20mg/kg/day, respectively. Considering that (i) the dose of anti-rejection drugs in clinical renal transplantation is greater than that in the treatment of glomerular disease; (ii) the cost of DEM is high; and (iii) our research is preliminary, therefore, one fifth of the low dose of rejection study, 1 mg/kg/day, was selected as our study dose.) The other rats were gavaged with the same amount of normal saline. Blood and urine samples were taken after 8 and 12 weeks of intervention.

Two, three, and three rats died in the DN, TwHF, and DEM groups during the experiment, respectively. Thus, the final grouping was six rats in the control group, six in the DN group, seven in the TwHF group, and seven in the DEM group.

2.2.1 | Urinalysis and biochemistry

Blood and urine samples of rats at 0, 8, and 12 weeks were taken to detect the following indexes (008AS Automatic biochemical analyzer, Hitachi): serum creatinine (enzymatic method, sCr), urea (UREA), alanine aminotransferase (ALT), aspartate aminotransferase (AST), GLU, high-density lipoprotein cholesterol, low-density lipoprotein cholesterol, triglycerides, total cholesterol, and urinary protein/creatinine.

2.2.2 | Renal pathologic examination and evaluation

At the 12th week of intervention, the rats were killed by cervical dislocation, and bilateral kidneys were collected and fixed with 10%

formalin. After paraffin-embedding, sections were routinely prepared, stained with H&E staining, PAS staining, and Masson staining, and observed using optical microscopy. The glomerular sclerosis score, glomerular sclerosis index (GSI), and renal tubulointerstitial score were determined from 20 randomly selected non-overlapping fields under a low-power microscope, and the number of glomeruli in each field was calculated. The pathological changes of the glomeruli and renal tubules were observed under a high-power microscope, and the results of the low-power field examination were semi-quantitatively analyzed.

Glomerular sclerosis scoring criteria. Normal glomerular morphology was grade 0. In grade I, the proliferation of glomerular mesangial cells and mesangial area was not obvious, and the degree of fibrosis was small (<25% of the corresponding field view area). In grade II, mesangial cells and mesangial matrix were increased moderately, with moderate fibrosis, and with a small amount of adhesion on the balloon wall (corresponding to 25%–50% of the field of view). In grade III, mesangial cells and mesangial matrix were significantly increased, fibrosis was severe, and with basement homogeneous membrane thickening and obvious adhesion of the balloon wall (>50% of the corresponding visual field area).

The GSI was calculated as $GSI = [(1 \times s1 + 2 \times s2 + 3 \times s3)] / \text{total number of glomeruli in each section}$ ($s1$ = number of grade I glomeruli; $s2$ = number of grade II glomeruli; $s3$ = number of grade III glomeruli).

Tubular interstitial scoring criteria. Normal renal tubule morphology and structure is grade 0 (0 points). Grade I is characterized by a small amount of inflammatory cell infiltration in the renal tubular interstitium, a light degree of epithelial cell degeneration, and not obvious renal tubular dilatation and fibrosis (corresponding visual field area of <25%) (1 point). In grade II, renal tubule interstitial inflammatory cells infiltration was obvious, epithelial cells were mildly diffuse, and renal tubules were moderately dilated and fibrotic (corresponding visual field area of 25%–50%) (2 points). In grade III, renal tubular epithelial cells showed moderately diffuse degeneration, there was a large number of inflammatory cell infiltration in the renal tubule interstitium, and the degree of renal tubule expansion and fibrosis was high (corresponding visual field area >50%) (3 points).

2.3 | Statistical analysis

Data were analyzed using SPSS 22.0 (IBM). A normality test was carried out on the continuous data. Mean \pm standard deviation was used for the statistical description of continuous with a normal or approximately normal distribution, and then inter-group comparison was conducted. In the case of homogeneity of variance, one-way ANOVA was used for inter-group comparison, with the LSD post hoc test for pairwise comparison. Median (interquartile spacing) was used for the statistical description of continuous data that did not meet a normal distribution, and a rank-sum test was used for comparison among groups. Differences were considered statistically significant at $p < .05$.

3 | RESULTS

3.1 | Network pharmacology analysis of the effective components of TwHF and the action way of DEM

3.1.1 | Screening target proteins of TwHF and their possible pathway of action

The chemical constituents of TwHF were identified in the literature, and a total of 370 compounds classed into four main categories were obtained. Among them, there were 36 sesquiterpenes (components numbers: SES 1–36), 93 diterpenes (components numbers: DIT 1–93), 133 triterpenes (components numbers: TRI 1–133), 106 alkaloids (components numbers: ALK 1–106), and two other compound categories.

The 87 primarily screened target proteins in systemsDock could bind with 370 small molecules in a semi-flexible manner. A total of 35 target proteins (from 87 proteins, fit value >8) were selected (Table S1), and 46 small-molecule constituents (Table S2) of TwHF acted on these 35 target proteins. Figure 1 details the interactions between target proteins and TwHF molecules. Among the target proteins, AURKA was found to bind best with TwHF small molecules, while vitamin D receptor (VDR), AR, and RXRA bound with the greatest quantity of small molecules.

We classified these target proteins as belonging to three groups (Figure S1): involved in either inflammation, sex hormone interactions, or VDR associations. Figure 2 shows all signal pathways after target protein enrichment. The inflammatory

pathways, including PI3K-Akt and Jak-STAT, were found to play important roles.

3.1.2 | Screening of effective TwHF small molecules

A total of 46 small molecules were found to act on target proteins with fit values >8. These small molecules almost completely interfered with the three mechanisms mentioned above, including the main role of TwHF in treating kidney diseases and clinical side effects. In order to identify the constituents that play therapeutic roles and reduce side effects to a minimum, we explored target proteins possessing fit values of 7–8 and found that four small molecules (triptonoditerpenic acid, regeol C, DEM, and demethylzeylasterone) mainly affected the inflammatory response, while five small molecules (hinokione, linarionoside A, linarionoside B, alangionoside J, and [-]-wilforonide) mainly acted in the sex hormone-related pathways (and are thus likely responsible for the main gonadal side effects of TwHF). The structures of these molecules are shown in Figure S2.

3.1.3 | TwHF molecules acting on DN

A total of 191 DN-related genes were screened from five databases. Seven target proteins with high correlation scores were selected: VDR, JAK1, JAK2, JAK3, PPARG, MARK14, and TGFB1. Forty-six molecules acted on the above target proteins (Table S2). The interactions between these seven target proteins and molecules are detailed in Figure 3. The most frequent interactions with TwHF

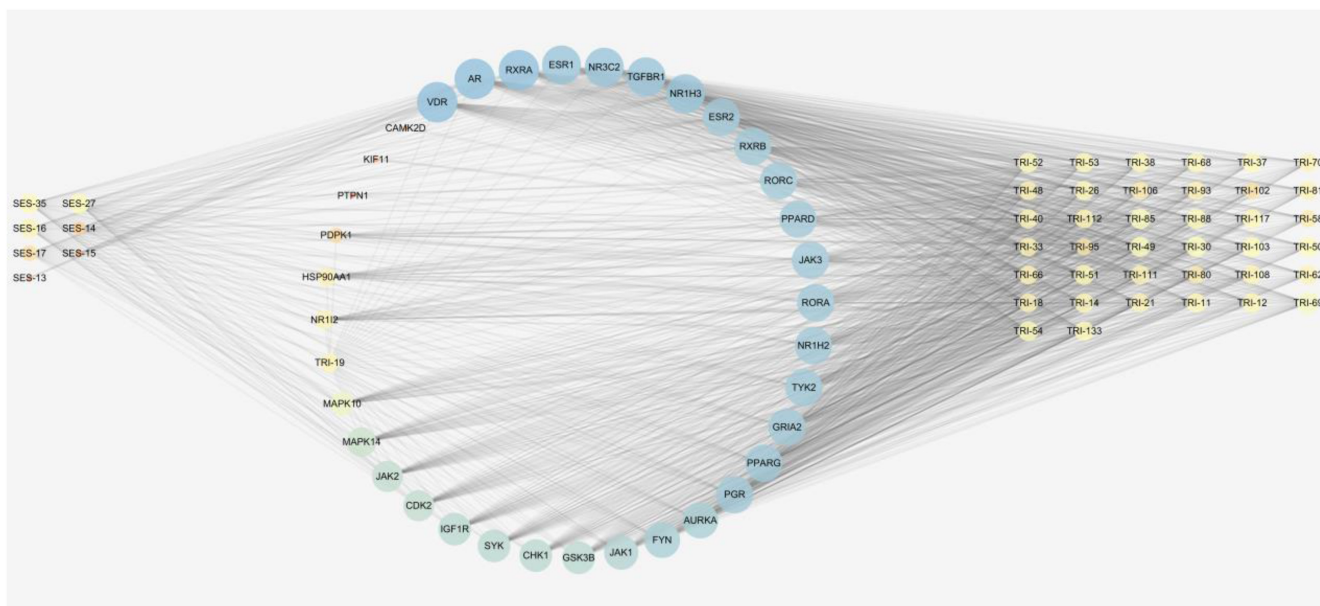


FIGURE 1 Interactions between the 35 target proteins and 46 TwHF molecules. Among the target proteins, AURKA was found to bind best with TwHF small molecules, while VDR, AR, and RXRA bound with the greatest quantity of small molecules. Solid lines represent interactions between molecules and proteins. The more molecules/proteins affected, the larger the circle and the darker the blue/yellow. For example, all 46 small molecules acted on VDR, AR, and RXRA, but only one small molecule acted on CAMK2D. TwHF, *Tripterygium wilfordii* Hook F; VDR, vitamin D receptor.

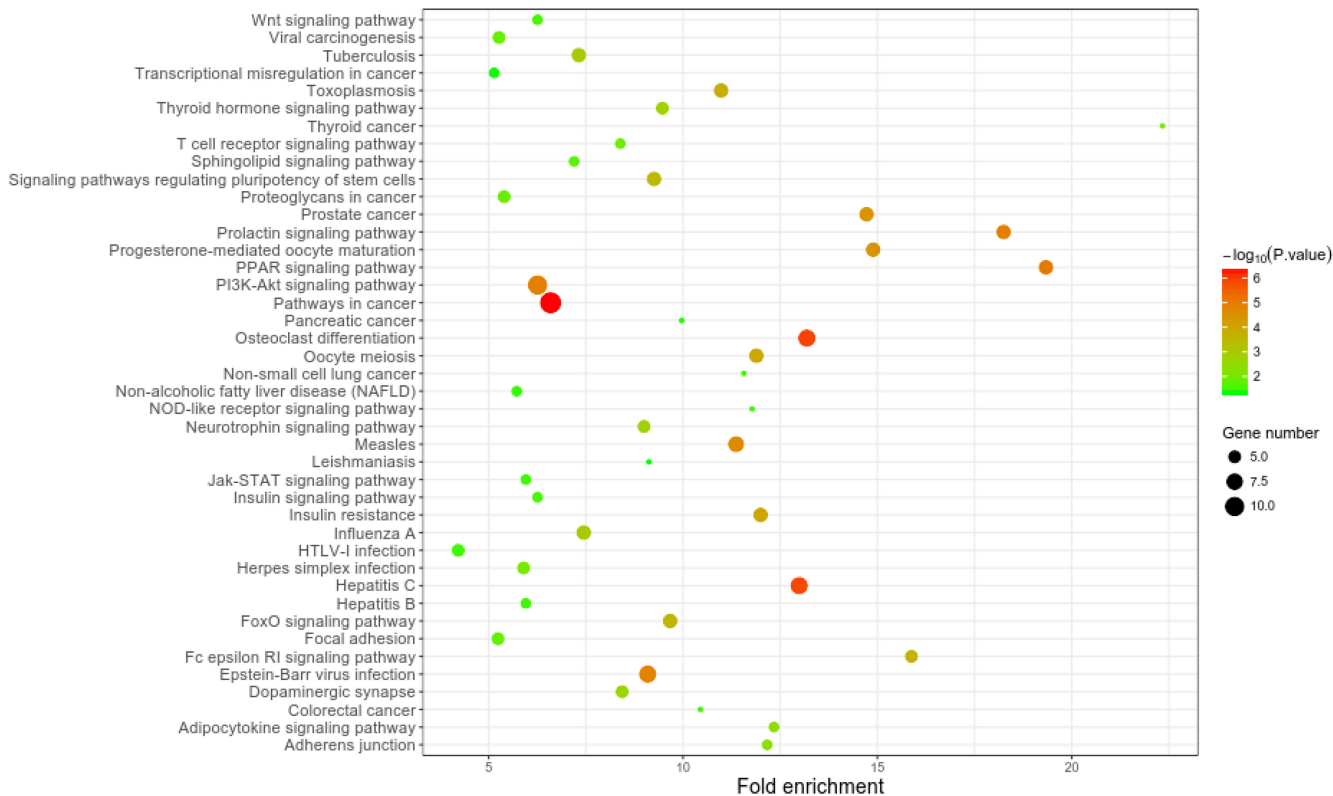


FIGURE 2 A detailing of signal pathways after target protein enrichment. The inflammatory pathways, including PI3K-Akt and Jak-STAT, were found to play important roles. An increased circle size, red color, and larger abscissa indicate pathways of greater importance.

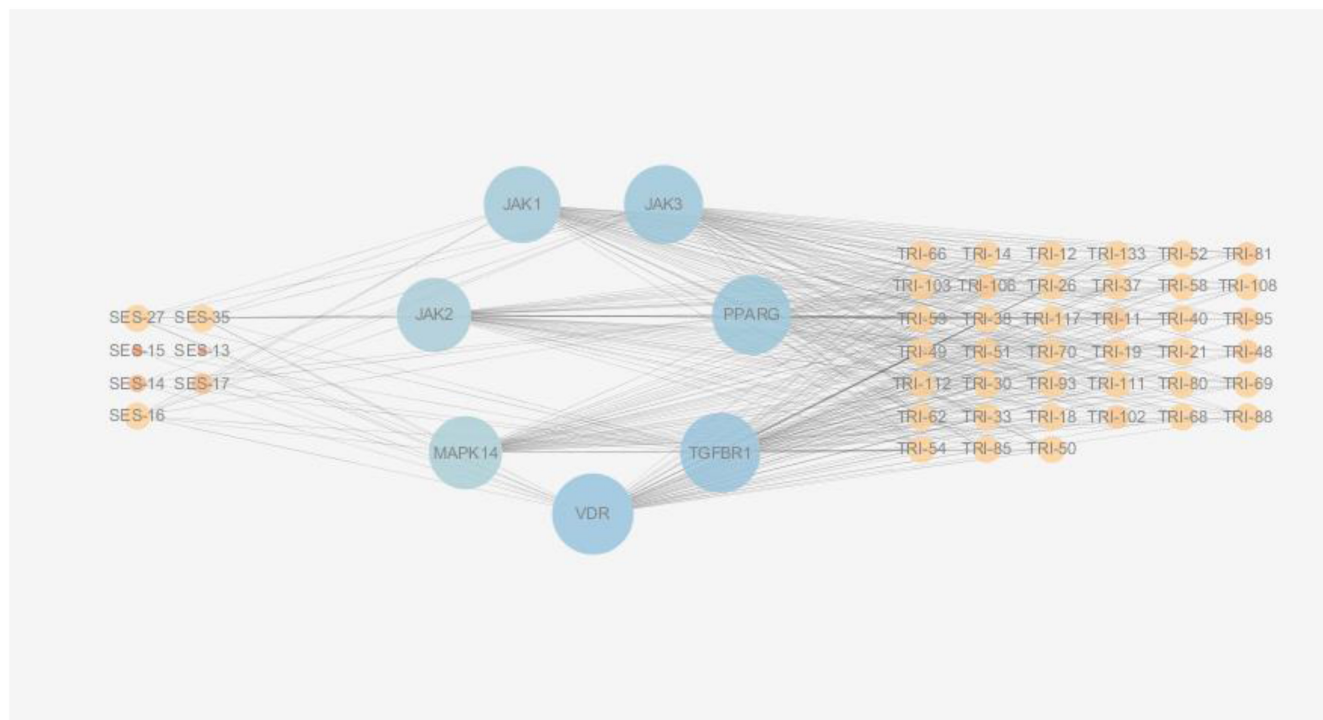


FIGURE 3 Interactions between seven target proteins and TwHF molecules. The most frequent interactions with TwHF molecules (in decreasing frequency) occurred with VDR, TGFBR1, and MARK14. Solid lines represent interactions between molecules and proteins. The more molecules/proteins affected, the larger the circle and the darker the blue / yellow. TwHF, *Tripterygium wilfordii* Hook F; VDR, vitamin D receptor.

molecules (in decreasing frequency) occurred with VDR, TGFBR1, and MARK14.

3.1.4 | Urinary protein and biochemistry changes in DN rats treated with DEM

There were no differences among the four groups in terms of proteinuria at baseline. At 8 weeks, proteinuria in the DN, TwHF, and DEM groups was significantly increased compared with baseline (all $p \leq .001$). At the 12 weeks, proteinuria in the TwHF and DEM groups was significantly lower than in the DN group (both $p \leq .001$), and the decrease in the DEM group was more obvious than that in the TwHF group ($p = .004$) (Figure 4).

As can be seen from Table 1, except for blood GLU, the other indexes had no significant changes after 12 weeks among the four groups. At 8 weeks, ALT and AST in the TwHF group were significantly higher than in the three other groups ($p < .05$). The ALT and AST levels in the DEM group were not different from the control and DN groups.

3.1.5 | Kidney pathological changes

At 12 weeks, kidney tissue samples were taken for optical microscopy. Compared with the control group, PAS staining showed that the glomerular mesangial matrix was significantly increased in the DN group; the glomerular lesions were improved after treatment, compared with the DN group. The increase of mesangial stroma in the TwHF group and the DEM group was improved, and the improvement in the DEM group was more obvious than in the TwHF group (Figure 5).

3.1.6 | GSI and tubular interstitial score

Compared with the control group, the GSI of the DN group was higher ($p < .05$). Compared with the DN group, the GSI values of the

TwHF and DEM groups were numerically lower, but the difference was not statistically significant (both $p > .05$). Compared with the DN group, the tubular interstitial score of the DEM group was lower ($p < .05$). Compared with the TwHF group, the tubular interstitial score of the DEM group was lower ($p = .003$) (Table 2).

4 | DISCUSSION

In recent years, due to the rapid development of detection technology and the application of new analytical methods such as network pharmacology, the research on the mechanism of Chinese herbal medicine is more and more in-depth and extensive. In this study, we identified 370 separate constituents of TwHF and investigated how to minimize adverse effects as much as possible while not diminishing any therapeutic actions. The targets and active components of TwHF were predicted by computer simulations based on molecular docking and mass data analyses. We found 35 target proteins of TwHF in the human body. These proteins play major roles in signaling pathways involving inflammation, sex hormones, and VDR functions. Anti-inflammatory effects of TwHF were therapeutic, while gonadal suppression remained a side effect. A total of 46 small molecules were found to act on target proteins, and four of them mainly affected the inflammatory response.

Network pharmacology is a novel method of drug research based on complex network construction, technological analysis, systems biology, and multi-directional pharmacology.¹⁵ Mechanisms of pharmacologic intervention and effects on the disease network, as well as synergistic effects between drugs and the human body, can thus be uncovered. Network pharmacology is especially useful in analyzing complex traditional Chinese medicines and the interactions of their components with human effector proteins. In the present study, network pharmacology allowed the identification of active components of TwHF and their potential targets.

To date, no effective treatments for DN exist.¹⁶ There is evidence for the action of TwHF in the treatment of DN,⁵ but the composition

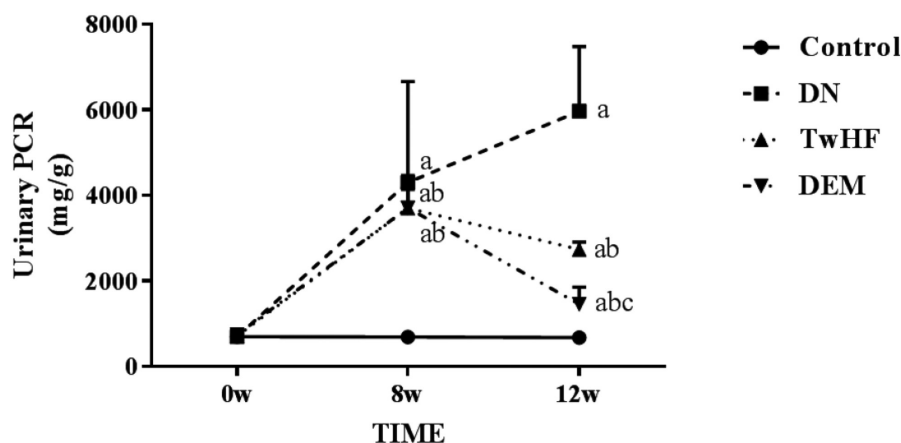


FIGURE 4 Changes of urinary protein in the control, DN, TwHF, and DEM groups of rats. At 8 weeks, proteinuria in the DN, TwHF, and DEM groups was significantly increased compared with baseline. At the 12 weeks, proteinuria in the TwHF and DEM groups was significantly lower than in the DN group, and the decrease in the DEM group was more obvious than that in the TwHF group. a: $p < .001$ vs. the control group; b: $p < .001$ vs. the DN group; c: $p < .05$ vs. the TwHF group. DEM, demethylzeylasteral; DN, diabetic nephropathy; TwHF, *Tripterygium wilfordii* Hook F.

TABLE 1 Comparison of the biochemical indexes in the four groups of rats

Item	Time (weeks)	Control	DN	TwHF	DEM
CRE ($\mu\text{mol/L}$)	0	35.25 \pm 3.77	33.25 \pm 2.50	31.00 \pm 2.16	32.75 \pm 3.30
	8	28.25 \pm 2.22	26.25 \pm 4.43	25.00 \pm 1.41	23.75 \pm 2.36
	12	28.00 \pm 1.41	24.50 \pm 1.73	25.50 \pm 4.73	25.75 \pm 4.50
UREA (mmol/L)	0	4.97 \pm 0.37	3.90 \pm 0.85	4.94 \pm 1.18	3.72 \pm 0.43
	8	4.81 \pm 0.43	7.37 \pm 1.91	10.81 \pm 1.50	6.27 \pm 1.18
	12	4.31 \pm 0.60	7.17 \pm 3.11	9.30 \pm 1.86	8.77 \pm 4.59
ALT (U/L)	0	60.00 \pm 5.60	61.75 \pm 11.32	64.25 \pm 90.00	49.25 \pm 14.36
	8	51.75 \pm 6.50	59.50 \pm 20.40	160.00 \pm 90.52	62.25 \pm 21.00
	12	62.50 \pm 8.35	71.75 \pm 27.69	104.50 \pm 35.29	70.00 \pm 30.14
AST (U/L)	0	147.75 \pm 11.32	123.25 \pm 22.68	122.50 \pm 14.71	106.75 \pm 22.41
	8	107.25 \pm 11.15	114.00 \pm 23.12	205.00 \pm 121.77	98.75 \pm 22.63
	12	121.00 \pm 7.79	106.75 \pm 16.64	130.25 \pm 39.78	94.50 \pm 38.90
GLU (mmol/L)	0	5.98 \pm 0.26	24.53 \pm 5.84	25.13 \pm 5.40	23.33 \pm 4.15
	8	5.28 \pm 0.85	28.03 \pm 9.44	36.13 \pm 2.36	25.60 \pm 6.84
	12	5.68 \pm 0.33	29.68 \pm 10.22	30.90 \pm 2.82	31.10 \pm 9.46
HDL-C (mmol/L)	0	0.68 \pm 0.12	0.73 \pm 0.11	0.74 \pm 0.19	0.75 \pm 0.11
	8	0.65 \pm 0.11	0.87 \pm 0.09	0.80 \pm 0.12	0.84 \pm 0.06
	12	0.77 \pm 0.11	1.01 \pm 0.06	1.00 \pm 0.22	0.96 \pm 0.14
LDL-C (mmol/L)	0	0.28 \pm 0.07	2.44 \pm 0.42	2.15 \pm 0.43	2.07 \pm 0.66
	8	0.36 \pm 0.09	0.30 \pm 0.03	0.30 \pm 0.03	0.35 \pm 0.09
	12	0.31 \pm 0.08	0.25 \pm 0.03	0.30 \pm 0.06	0.29 \pm 0.06
TC (mmol/L)	0	1.55 \pm 0.34	6.45 \pm 1.45	6.16 \pm 1.17	5.52 \pm 1.61
	8	1.85 \pm 0.41	1.94 \pm 0.27	1.56 \pm 0.17	2.09 \pm 0.16
	12	2.05 \pm 0.52	2.16 \pm 0.27	1.90 \pm 0.31	2.11 \pm 0.24
TG (mmol/L)	0	1.27 \pm 0.31	3.88 \pm 2.19	6.43 \pm 3.53	3.27 \pm 1.30
	8	1.30 \pm 0.31	2.86 \pm 1.16	1.93 \pm 0.79	2.21 \pm 0.58
	12	1.21 \pm 0.24	2.87 \pm 0.94	1.10 \pm 0.34	2.51 \pm 0.89

Abbreviations: ALT, alanine transaminase; AST, aspartate transaminase; CRE, creatinine; DEM, demethylzeylasteral; DN, diabetic nephropathy; GLU, glucose; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; TC, total cholesterol; TG, triglycerides; TwHF, *Tripterygium wilfordii* Hook F; UREA, urea.

of TwHF is complex, and it is unknown which compound(s) affected DN. TwHF has significant side effects, like bone marrow suppression, liver damage, and gonadal suppression. Therefore, identifying the compounds active against DN and those responsible for the side effects could allow better treatment of DN.

Inflammation plays a crucial role in the pathogenesis of DN. In the setting of continuous hyperglycemia, marked changes in inflammatory factors and vasoactive substances lead to tissue damage.^{17,18} A previous study by the authors' group found that CD3-, CD15-, CD11b-, and CD11c-positive cells infiltrated the glomeruli and renal interstitium of DN patients,¹⁹ contributing to a significant deposition of advanced glycation end products in the kidney.^{20,21} We confirmed that JAK1, JAK2, JAK3, PPAR, MARK14, and TGFBR1 are involved in the inflammatory response and PI3K-Akt, JAK-STAT, and PPAR signaling pathways. This finding is consistent with the currently accepted knowledge of signal pathways related to DN.²²

DEM is one of the four active components (with fewer side effects) of TwHF. More attention has been paid to the role of DEM in anti-inflammation, suppressing immunity, and tumor therapy. DEM possesses immunosuppressive and anti-inflammatory effects,⁶ anti-angiogenic effects,⁷⁻⁹ and decreased cell proliferation and increased apoptosis effects that can be of use against cancer.^{9,13} DEM could alleviate lupus nephritis in mice⁶ and decrease kidney inflammation after ureteral obstruction in rats.¹⁴ Still, no previous study of DEM on DN was available. The anti-inflammatory effect of DEM was very important. An animal experiment on lupus nephritis showed that DEM could significantly improve the quantity of 24-h urinary protein and the level of serum anti-dsDNA in mice. In addition, DEM decreased the secretion of pro-inflammatory mediators TNF- α , COX-2 and ICAM-1, and reduced the infiltration of macrophages in renal tissue. This study suggested that DEM may have a renal protective effect on lupus nephritis by inhibiting the activation of NF- κ B and reducing the downstream pro-inflammatory mediators.⁶

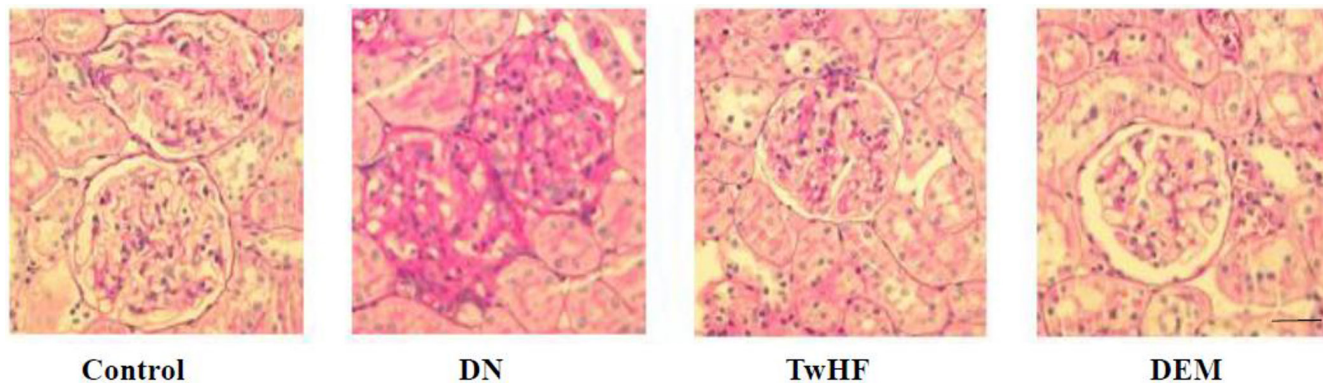


FIGURE 5 Comparison of kidney pathological changes in the four groups of rats (PAS, $\times 400$). Compared with the control group, PAS staining showed that the glomerular mesangial matrix was significantly increased in the DN group; the glomerular lesions were improved after treatment, compared with the DN group, The increase of mesangial stroma in the TwHF group and the DEM group was improved, and the improvement in the DEM group was more obvious than in the TwHF group. DEM, demethylzeylasteral; DN, diabetic nephropathy; TwHF, *Triptergium wilfordii* Hook F.

	Control (n = 6)	DN (n = 6)	TwHF (n = 7)	DEM (n = 7)
Glomerular sclerosis index	1.12 \pm 0.11	2.15 \pm 0.86 ^a	1.81 \pm 0.39	1.54 \pm 0.35
Tubular interstitial score	1.00 \pm 0.00	1.60 \pm 0.41	1.66 \pm 0.23	1.10 \pm 0.17 ^{b,c}

TABLE 2 Comparison of glomerular sclerosis index and tubular interstitial score among the four groups

Abbreviations: DEM, demethylzeylasteral; DN, diabetic nephropathy; TwHF, *Triptergium wilfordii* Hook F.

^a $p < .05$ vs. the control group.

^b $p < .05$ vs. the DN group.

^c $p < .05$ vs. the TwHF groups.

The present study supports that DEM can reduce the proteinuria of DN rats, probably through its anti-inflammatory effects, more effectively than when using whole TwHF. These results support the therapeutic effect of TwHF on DN.⁵ Furthermore, DEM can inhibit the activation of the NF- κ B pathway,⁶ which is involved in the pathogenesis of DN.²³ In addition, the PI3K-Akt,²⁴ JAK-STAT,²⁵ and PPAR²⁶ signaling pathways are also involved in DN. Therefore, the use of pure DEM could circumvent the side effects of TwHF, as suggested by the biochemistry indexes assessed in the present study. Of course, preclinical studies are still necessary before clinical trials of DEM. Nevertheless, DEM could ultimately be a treatment option for patients with DN.

We began to treat from week 0, and there was no obvious effect after 8 weeks of observation; urinary protein decreased significantly at 12 weeks. By studying the literature, we found that the onset time of other drugs on DN rats ranged from 4 weeks to 8 weeks,²⁷⁻²⁹ so we designed two observation points, 8 and 12 weeks respectively. Our study observed that there was no significant effect until 12 weeks, which may be related to the slow onset and accumulation of TwHF and DEM.

This study has some limitations. For example, the number of experimental animals is small, and the observation time is short. In the follow-up study, we will increase the numbers of experimental animals and prolong observation time to further prove the efficacy and

observe the side effects. There are few reports on the mechanism of DEM on DN. More research is needed to explore the mechanism of DEM in DN.

In summary, 46 small molecules were found to be biologically active constituents of TwHF in DN setting using network pharmacology in this study. The findings provide a scientific foundation for subsequent experimental validation and novel drug development against DN. The emergence of network pharmacology has completely altered the research approach towards novel drug development and has greatly compensated for the lack of experimental capabilities. DEM can effectively reduce proteinuria in animal models of DN.

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CONFLICT OF INTEREST

No.

ETHICS STATEMENT

Animal studies were carried out under the approval of the Animal Research Ethics Committee of PLA General Hospital.

AUTHOR CONTRIBUTIONS

Conceptualization, MYH and YLP.; methodology, XLN; software, DXY and YX; validation, ZB; formal analysis, DXY; investigation, ZB; data curation, XLN; writing—original draft preparation, XLN; writing—review and editing, MYH; visualization, DXY; supervision, ZB; project administration, MYH; funding acquisition, MYH. All authors have read and agreed to the published version of the manuscript.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author, MYH, upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

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