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Efficacy and potential pharmacological mechanism of *Astragalus-Salvia miltiorrhiza* combination in diabetic nephropathy: integrating meta-analysis, network pharmacology, molecular docking, and experimental validation

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ABSTRACT

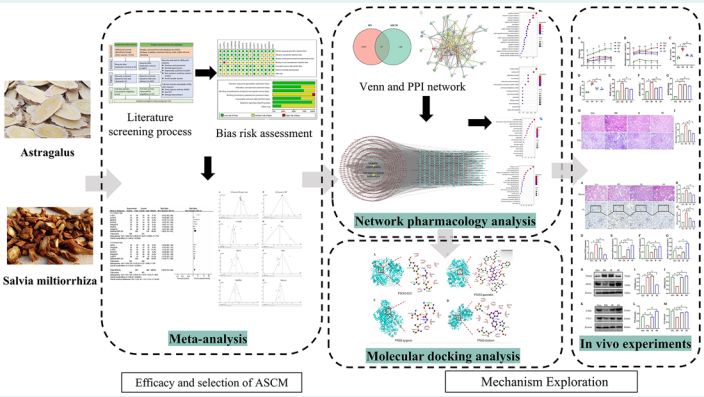
Background: Diabetic nephropathy (DN) is a diabetes mellitus (DM)-induced complication that poses high morbidity and mortality risks. The *Astragalus* and *Salvia miltiorrhiza* couplet medicines (AS) are commonly employed in DN clinical treatment in China, but their clinical efficacy and potential pharmacological mechanisms are yet to be evaluated.

Material and Methods: A meta-analysis of 15 studies involving 1,443 patients was conducted. Furthermore, network pharmacology predicted components and targets, which were verified by molecular docking and *in vivo* validation.

Results: In our meta-analysis, AS notably elevated clinical outcomes and renal function among patients with DN. Meanwhile, when the treatment duration exceeds 12 weeks, AS demonstrated a significant reduction in fasting blood glucose levels, indicating a time-dependent effect. Moreover, based on network pharmacology results, AS likely enhanced clinical outcomes by interacting with vital signaling pathways, including PI3K/Akt, MAPK, and NF-kappa B. Molecular docking studies have confirmed that PTGS2, the key therapeutic target of AS, can be closely combined with bioactive components *GLY*, *quercetin*, *apigenin,* and *daidzein*. Additionally, *in vivo* experiments have corroborated that AS can ameliorate renal function, UACR, and biomarkers associated with iron metabolism, such as GPX4, PTGS2, FTH1, and FTL1.

Conclusion: Through rigorous experimental validation, our study demonstrates AS's significant clinical efficacy in managing DN. Specifically, AS has been shown to enhance renal function, ameliorate renal fibrosis, and positively influence iron metabolism. Despite these promising outcomes, future research with a larger sample size must be conducted to further substantiate these findings.

GRAPHICAL ABSTRACT



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KEYWORDS

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Abbreviations: DN: diabetic nephropathy; DM: diabetes mellitus; AS: Astragalus and Salvia miltiorrhiza couplet medicines; ESRD: end-stage renal disease; TCM: Traditional Chinese Medicine; RCTs: randomized controlled trials; Con, control group; CT: treated with conventional therapy; TG: treatment group; 24-hour UTP: 24-hour urine protein; UAER: 24-hour urinary albumin excretion rate; FPG: fasting plasma glucose; HbA1c: glycosylated hemoglobin; 2hrPPG: 2-hour postprandial plasma glucose; Scr: serum creatinine; BUN: blood urea nitrogen; WMD: weighted mean difference; RR: risk ratio; CI: confidence interval; TT: therapeutic time; TCMSP: Traditional Chinese Medicine Systems Pharmacology Database; MF: molecular function; CC: cellular components; BP: biological process; KEGG: Kyoto Encyclopedia of Genes and Genomes; IB: Irbesartan treatment group; STZ: streptozotocin; ELISA: Enzyme-Linked Immunosorbent Assay; HE: Hematoxylin-eosin; MAPK: mitogen-activated protein kinase

Background

Diabetes nephropathy (DN) accounts for 45% of all global end-stage renal disease (ESRD) cases [1], making it the leading cause of ESRD worldwide [2]. DN is characterized by glomerular hypertrophy, proteinuria, reduced glomerular filtration rate, and renal fibrosis, accompanied by loss of renal function due to high glucose conditions [3]. According to the International Diabetes Federation, approximately 592 million people worldwide are expected to incur diabetes mellitus (DM) by 2035 [4]. As a complication of DM, DN is considered a major threat based on high mortality and morbidity rates [5], affecting more than 30% of patients with DM [6]. Currently, the treatment of DN mainly aims to control blood glucose, lower blood pressure, and reduce renal microvascular damage. Among them, renin-angiotensin system inhibitors are commonly used as first-line therapies [7,8]. However, some unsatisfactory effects remain. For example, long-term medication may lead to adverse reactions, such as allergies, and damage to the liver and kidneys [9]. Therefore, the discovery of a secure and efficient medication to treat DN is crucial.

Recently, Traditional Chinese Medicine (TCM) has received significant attention [10]. DN is known as an "emaciation-thirst disease" in TCM. The condition manifests with both underlying weakness and superficial excess symptoms, reflecting a complex interplay of "Yin and Yang imbalances". Specifically, the deficiency is due to "Qi depletion", and the excess is mainly a result of "blood stasis". Therefore, ancient practitioners believed that drugs for tonifying Qi and promoting blood circulation can be used to treat DN. *Astragalus*, a famous Qi-tonifying drug, has a long history [11]. In addition, *Salvia miltiorrhiza* promotes blood circulation and removes "blood stasis" [12]. Furthermore, Lin et al. [13] analyzed data from 495 TCM in 2,884 articles and found that *Astragalus* and *S. miltiorrhiza* had the highest usage frequencies in DN, accounting for 7.46% and 5.46%, respectively. Particularly, *Astragalus-S. miltiorrhiza* couplet medicines (AS) had the highest usage frequency at 46.88%. Couplet medicines are compatible with two Chinese medicines [14], which are interdependent and mutually restricted and can exert synergistic and cascading effects [15]. Moreover, AS can alleviate the pathological changes of renal tubular swelling and mesangial proliferation by regulating PI3K/Akt and CAMKK/AMPK pathways [16]. Although *Astragalus* and *S. miltiorrhiza* are well-documented in terms of their individual therapeutic effects on DN, the literature lacks comprehensive studies on their combined application in TCM. Additionally, the efficacy of AS has not been fully verified, and there is a lack of high-quality and large-sample studies. Meanwhile, its active ingredients and potential mechanism have not been fully elucidated. Therefore, systematic evaluations and comprehensive explorations of the efficacy and pharmacological mechanisms of AS on DN are urgently warranted.

Fortunately, meta-analysis, network pharmacology, and molecular docking are promising paradigms that address the above-mentioned problems. Specifically, meta-analysis is an objective and quantitative method for analyzing the results of previous studies and provides valuable evidence-based scientific data [17]. Network pharmacology, a discipline rooted in system biology and bioinformatics, can clarify drug action mechanisms at the molecular level. Additionally, it supports new drug design, clinical diagnosis, and treatment guidance [18]. To corroborate the findings from our meta-analysis and network pharmacology, molecular docking assesses the binding affinity of AS compounds with their targets, providing a molecular perspective on therapeutic effects. This is followed by *in vivo* experiments that serve as a critical step in validating the efficacy of the identified targets and AS's pharmacological impact in DN.

Therefore, our study meticulously examines the therapeutic role of AS in DN through a coherent sequence of data synthesis, molecular exploration, and experimental validation. This systematic approach guarantees a thorough and scientifically robust analysis of AS's efficacy in DN treatment.

Material and methods

Protocol & guidance

The analysis was performed following the Preferred Reporting Items for Systematic Reviews and Meta-Analyses criteria, which provides a comprehensive framework to ensure transparency and accuracy in the analysis process [19].

Literature search strategy

The databases employed in this study encompassed Chinese Science and Technology Journal, Wanfang, China National Knowledge Infrastructure, Pubmed, Chinese Biomedical

Literature, Embase, and Cochrane Library. The keywords were as follows: "*Astragalus*," "*Salvia miltiorrhiza*," and "Diabetic nephropathy." The search was modified to meet the needs of each database with MeSH and text terms. The retrieval period extended from the database's inception until March 2, 2023.

Inclusion criteria

Based on the PICO search strategy, the research involved should meet the following conditions: 1) Research must focus on a compound formula that *Astragalus* and *S. miltiorrhiza* serve as the most fundamental and essential components; 2) all studies are randomized controlled trials (RCTs) without the constraint of blinding and allocation concealment; 3) the diagnosis of DM meets the diagnostic criteria of the World Health Organization (1999) or the American Diabetes Association (2010); 4) individuals are not restricted by DN stage, age, gender, course of the disease, and race; 5) the control group (Con) is treated with conventional therapy (CT). The treatment group (TG) comprised the same CT combined with TCM. Notably, both are not limited by dosage form, dose, and intervention time.

Exclusion criteria

The exclusion criteria for this study were as follows: 1) non-clinical RCT literature, including animal experiments and retrospective reviews, among others; 2) repeated publications, similar literature, and studies with incorrect or incomplete data; and 3) literature on the combination of other cardiovascular, kidney, lung, and liver diseases, such as hypertensive nephropathy and nephrotic syndrome.

Outcome measure

The outcome indicators included in these studies were as follows: clinical efficiency rate; 24-h urine protein (24-h UTP); 24-h urinary albumin excretion rate (UAER); fasting plasma glucose (FPG); glycosylated hemoglobin (HbA1c); 2-h postprandial plasma glucose (2hrPPG); serum creatinine (Scr); and blood urea nitrogen (BUN).

Data extraction and management

Two authors independently extracted data, cross-checked, and corrected each other based on the inclusion and exclusion criteria. Specifically, the data extracted included the first author, year of publication, number of patients, interventions, treatment cycles, and outcome indicators. Any differences were discussed with the third author.

Risk of bias and quality assessment

Cochrane's bias risk assessment method [20] evaluated the quality of literature as either "low risk," "high risk," or "unclear." Meanwhile, funnel plots were used to analyze publication risk and bias. If the funnel plot displays asymmetry, it may indicate bias (i.e. tests with negative results may not be published).

Meta statistical analysis

Statistical analyses were performed using RevMan5.3 software. Continuous and binary classification results were analyzed using weighted mean difference (WMD) and risk ratio (RR), respectively; both measures were accompanied by a 95% confidence interval (CI). In addition, the χ^2 test was performed to evaluate heterogeneity. The random effect model was used in case of statistical heterogeneity (If I^2>50%). Conversely, the fixed effect model was adopted. Notably, subgroup analysis was used to determine whether the effect of AS on DN was time-dependent. Specifically, the subgroup analysis was based on therapeutic time (TT), which was categorized into TT <12 weeks and TT ≥12 weeks groups.

Components and targets in AS

The active components of AS were obtained using the Traditional Chinese Medicine Systems Pharmacology Database (TCMSP, https://tcmspw.com). The TCMSP parameters were set as bioavailability ≥30% and drug-likeness ≥0.18. Accordingly, the targets corresponding to the ingredients were fetched from the Swiss Target Prediction Database (http://swisstargetprediction.ch/) and then standardized in the UniProt (https://www.uniprot.org) database.

DN-related targets

The search term "Diabetic nephropathy" was used to retrieve relevant information from GeneCards (https://www.genecards.org/) and DisGeNET (https://www.disgenet.org/) databases. Subsequently, we removed the duplicate genes obtained in the above databases. Finally, the Venn diagram web tool (http://bioinformatics.psb.ugent.be/webtools/Venn/) was used to analyze the targets of active components in AS, as well as DN-related targets.

Construction of a "component-target" interaction network

The sorted information was input into Cytoscape 3.9.1 [21] to create a "component-target" interaction network, in which edges connected nodes of components and related targets to indicate their correlation. Subsequently, the intersection of chemical composition targets and disease targets was screened.

Protein–protein interaction (PPI) and enrichment analysis

The obtained targets were inputted into the STRING database (https://string-db.org) for PPI analysis. The species was set as "*Homo sapiens*" with a confidence score of 0.4. CytoHubba plug-in in Cytoscape 3.9.1 software was used to

screen key targets. The overlapping targets were enriched through the Metascape database (https://metascape.org/gp/index.html#/main/step1), which mainly included molecular function (MF), cellular components (CC), biological process (BP), and Kyoto Encyclopedia of Genes and Genomes (KEGG). Subsequently, the results are available for visualization through biological online tools.

Molecular docking

Effective selection of key targets and components is a prerequisite for molecular docking. Utilizing insights derived from network pharmacology, we have meticulously chosen the foremost four constituents implicated in the treatment of DN with AS to undergo docking analysis with the pivotal target PTGS2. Specifically, the PDB database (https://www.rcsb.org/) was utilized to obtain the core target structure, while the Pub Chem database (https://pubchem.ncbi.nlm.nih.gov/) was used for the key component structure. Moreover, the PYMOL software was utilized to eliminate both water molecules and small molecule ligands. Subsequently, we adopted Autodock Vina [22] for molecular docking and used Pymol and Ligplus to select the best four docking combinations for visualization.

Animals and treatment

Twenty-six-week-old male C57BL/6JGpt mice were obtained from GemPharmatech (Nanjing, China) and housed in a specific pathogen-free environment. Conditions included a temperature of 21 ± 3 °C, 50% humidity, and a 12-h light/dark cycle. Mice were acclimated to these conditions for one week before experimentation. Animal care and use adhered to the U.S. guidelines (NIH publication #85-23, revised 1985), and the study was approved by the Animal Experiment Ethics Committee of Jinan University (Approval No. 20231102-18).

The mice were randomly assigned into four groups, each comprising five animals: (1) the control group (Con); (2) the diabetic nephropathy model group (DN); (3) the Irbesartan treatment group (IB); and (4) the *Astragalus* and *S. miltiorrhiza* treatment group (AS). The control group received no treatment. The other groups were given streptozotocin (STZ, Sigma Aldrich, St. Louis, USA) at 50 mg/kg, dissolved in a 1% citric acid buffer at pH 4.5. STZ was administered intraperitoneally daily for 5 days. Diabetes was confirmed one week later by blood glucose levels, >13.0 mmol/L indicated the establishment of a successful model.

For 8 weeks, the IB group was treated with irbesartan at 50 mg/kg *via* gavage, and the AS group was administered a combination of *Astragalus* and *S. miltiorrhiza* at 6 g/kg/d. The dosage of irbesartan was determined based on the previous studies [23,24]. Furthermore, the drug dosage of AS was calculated according to the clinical dose, which was 45 g (30 g *Astragalus* and 15 g *S. miltiorrhiza*) for a person who weighs 70 kg a day. Then, according to the ratio of human and mouse body surface area, the equivalent dose for mice is 5.85 g/kg. Therefore, we took the integer and used the dosage of 6 g/kg for the AS treatment group. The medication and TCM granules were obtained from the First Affiliated Hospital of Jinan University. After 8 weeks, mice were anesthetized with pentobarbital sodium (60 mg/kg, intraperitoneal). Blood was taken from the posterior orbital plexus for biochemical analysis, and kidney tissues were collected for further study. Body weight and blood glucose were monitored biweekly throughout the study. Blood glucose levels were assessed using a glucometer (Roche Diabetes Care Ltd., UK).

Serum and urine biochemistry assays

BUN, Scr, ALB, and UCR were measured using the corresponding commercial Enzyme-Linked Immunosorbent Assay (ELISA) kits (Jiangsu Meimian Industrial Co., Ltd, MM-0692M1, China; Jiangsu Meimian Industrial Co., Ltd, MM-44455M1, China; Jiangsu Meimian Industrial Co., Ltd, MM-43983M1, China; Jiangsu Meimian Industrial Co., Ltd, MM-44289M1, China).

Histopathological analysis

Mouse kidney tissues were subjected to histopathological examination. Tissues were fixed in 4% buffered formalin, followed by paraffin embedding and sectioning. Sections were stained using Hematoxylin-eosin (HE), periodic acid-Schiff, and Masson's trichrome. Observations were made with an Eclipse Ci-L microscope (Nikon, Japan). Image-Pro Plus 6.0 software (Media Cybernetics, USA) facilitated image acquisition and analysis, providing a comprehensive histopathological evaluation.

Immunohistochemistry

To observe the degree of renal fibrosis, we conducted the following experimental steps: (1) dewax the sections through a graded ethanol series to water; (2) repair antigens using citric acid and microwave treatment, then wash with PBS; (3) block endogenous peroxidase with 3% hydrogen peroxide, followed by serum blocking with 3% BSA; (4) apply primary antibody (Fn:GB13091;1:200) overnight at 4 °C; (5) after washing, apply secondary antibody (HRP labeled) for 50 min; (6) develop color with DAB, then counterstain nuclei with hematoxylin; (7) dehydrate through alcohols and xylene, and seal with resin; and (8) examine and analyze the slides under a microscope.

Quantitative real-time polymerase chain reaction

Total RNA was isolated from renal tissues utilizing the TRIzol reagent (TAKARA, Japan). Quantification of RNA was conducted employing a fluorometric assay. Complementary DNA (cDNA) was synthesized employing the PrimeScript RT Reagent Kit (TAKARA, Japan). Moreover, quantitative real-time polymerase chain reaction (qPCR) was executed utilizing the TB Green Premix Ex Taq II (RNaseH Plus) (TAKARA, Japan).

The qPCR reactions were conducted on a CFX96 Touch Real-Time PCR Detection System (Bio-Rad, USA). *Glyceraldehyde-3-phosphate dehydrogenase* served as an endogenous reference gene. The relative quantification of gene expression was calculated using the comparative Ct ($2^{-\Delta\Delta Ct}$) method. The gene-specific primer sequences utilized in the study are as follows:

PTGS2: forward, 5′-CCTTCACCTCGATGCCTTGT-3′, and reverse, 5′- GGGTCTGTGTGGCCTGTATC −3′;
GPX4:forward, 5′ - TGTGCATCCCGCGATGATT-3′, and reverse, 5′-CCCTGTACTTATCCAGGCAGA −3′;
FIH1:forward, 5′-GGCTGAATGCAATGGAGTGTG-3′, and reverse, 5′ -GTGGTCACCCAGTTCTTTAATGG-3′;
FTL1::forward, 5′-CGTCAGAATTATTCCACCGAGG-3′, and reverse, 5′ -GCCACGTCATCCCGATCAAA-3′.

Western blotting

Renal tissue proteins were extracted using the RIPA method and quantified with a BCA kit. Proteins were resolved by SDS-PAGE and transferred to nitrocellulose membranes. Membranes were probed with primary antibodies: anti-PTGS2 (1:1000), anti-GPX4 (1:2000), anti-FTH1 (1:3000), and anti-FTL1 (1:3000), followed by HRP-conjugated secondary antibodies. Gel-pro analyzer software was utilized for image analysis.

Statistical analysis

Data analysis was conducted using GraphPad Prism 9 software, with results presented as the mean value accompanied by the standard error of the mean. For comparing multiple groups, a one-way analysis of variance was employed, followed by *post hoc* testing using either Fisher's least significant difference test or the Turkish *post hoc* test, depending on the data distribution and assumptions. Statistical significance was determined by a P-value threshold of less than 0.05. Additionally, the ImageJ software was utilized to quantitatively analyze protein band images.

Results

Study selection and quality assessment

Overall, 1,023 articles were retrieved. Notably, 897 articles were retained after deduplication. After reading the abstract and title, those that did not meet the inclusion criteria were removed, and 493 studies were retained. After further comprehensive reading of the full text, 15 relevant articles were identified, with 478 others excluded based on predetermined criteria [25–39] (Figure 1A).

Overall, 1,443 patients with DN were enrolled in this study, of whom 733 and 710 were in the TG group and Con group, respectively. The included studies were conducted in China, with no significant differences in age and disease duration ($p > 0.05$). All studies evaluated the effect of AS on the efficacy, renal function, or blood glucose level of DN. Specifically, 13, 10, 9, 6, 6, 9, 4, and 5 studies assessed the clinical efficiency rate, 24-h UTP, UAER, Scr, BUN, FPG, 2hrPPG, and HbA1c, respectively (Table 1). Seventeen included studies involved 15, 10, and 11 randomized groupings, covert groupings, and double-blind methods, respectively. Most of the results were fully documented, and no study was withdrawn or lost (Figure 1B-C).

Forest plot results of clinical effective rate, 24-h UTP, UAER, and scr

In a series of studies assessing AS, 13 examined clinical efficacy in 647 TG and 624 Con patients, 10 measured 24-h UTP in 515 TG and 499 Con patients, nine determined UAER in 402 TG and 389 Con patients, and six evaluated Scr in 276 TG and 272 Con patients. Due to heterogeneity, a random-effects model was used for analysis.

For clinical efficacy, a significant benefit was found with AS (RR = 1.24, 95% CI 1.15-1.34, $p < 0.00001$) (Figure 2A). Furthermore, studies on 24-h UTP showed AS significantly reduced protein levels (WMD=−1.17, 95% CI −1.66 to −0.68, $p < 0.00001$) (Figure 2B). UAER was also significantly lowered by AS, especially in treatments lasting 12 weeks or more (WMD

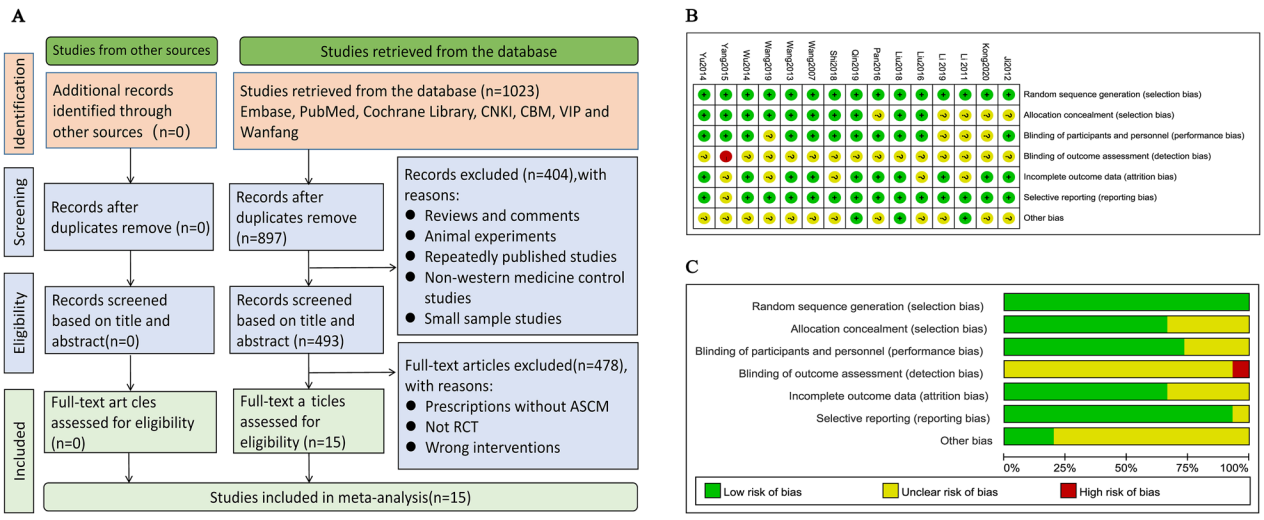

Figure 1. (A) Flow diagram of the study selection process, (B) Detailed risk and (C) overall risk of bias in the included studies.

Table 1. Characteristics of the included studies.

First author, Year	Country	Sample size (T/ C)	Intervention measures		Duration /w	Outcome measures
			Treatment group	Control group		
Kong 2020 [25]	China	35/33	HuangqiXiaoke Decoction + CT	CT	12	①②③④⑤⑥
Li 2019 [26]	China	72/72	DanqiYishen Decoction + CT	CT	12	①②④⑤
Qin 2019 [27]	China	23/22	Huangqi Zhimu Shenqi Decoction + CT	CT	4	①④⑤⑥⑧
Wang 2019 [28]	China	39/39	ShenqiDihuang Decoction + CT	CT	8	①⑤
Liu 2018 [29]	China	26/26	Yiqi Yangyin Huoxue prescription + Telmisartan Tablets + CT	Telmisartan Tablets + CT	4	③④⑤
Shi 2018 [30]	China	33/33	BushenHuoxue Decoction (self-made)+Bailing capsule)+CT	Bailing capsule)+CT	8	①②③
Liu 2016 [31]	China	85/83	HuangqiYishen Decoction (self-made))+CT	CT	8	①②③④⑥⑦⑧
Pan 2016 [32]	China	30/30	HuangqiXiaoke Decoction)+CT	CT	12	①②⑥⑧
Yang 2015 [33]	China	58/58	HuangqiYishen Decoction + CT	CT	8	①②④⑤⑥
Wu 2014 [34]	China	34/34	HuangqiXiaoke Decoction (self-made))+CT	CT	6	①③⑥⑦⑧
Yu 2014 [35]	China	70/70	Chinese herbal compound)+CT	CT	12	①②⑥⑦
Wang 2013 [36]	China	60/60	QiJiBuShen Decoction)+CT	CT	12	③
Ji 2012 [37]	China	40/40	BushenHuoxue Recipe + CT	CT	12	①②③
Li 2011 [38]	China	50/50	BushenHuoxue Decoction + CT	CT	6	①②③⑥⑦
Wang 2007 [39]	China	78/60	HuangqiXiaoke Decoction (self-made))+CT	CT	12	①②③⑥⑧

T, Treatment group; C, Control group; CT, conventional therapy; W, weeks. ① Clinical effifficacy rate; ② 24-h urine protein (24 h UTP); ③ 24-h urinary albumin excretion rate (UAER); ④ serum creatinine (Scr); ⑤ blood urea nitrogen (BUN); ⑥ fasting plasma glucose (FPG); ⑦ 2-h postprandial plasma glucose (2hrPPG); ⑧ glycosylated hemoglobin (HbA1c).

−28.77, 95% CI −47.50 to −10.05, $p=0.003$) (Figure 2C). Regarding Scr levels, an overall reduction was observed with AS (WMD=−7.02, 95% CI −13.69 to 0.35, $p=0.04$) (Figure 2D). Collectively, AS exhibited effectiveness in enhancing clinical outcomes and decreasing levels of proteinuria and Scr, with no clear time-dependent effect.

Forest plot results of BUN and blood glucose levels

In addition to investigating the effects of AS on renal function indices, we comprehensively analyzed its impact on hematological parameters, leading to the following significant findings. Six studies, with 253 patients in the TG and 250 in the Con, reported a significant reduction in BUN levels, indicating an improvement in renal function [WMD= −1.07, 95% CI (−1.71,-0.43), $p=0.0010$] (Figure 3A). Nine studies with 424 TG and 410 Con patients reported on FPG, where AS significantly lowered FPG after 12 weeks or more [WMD=−1.00, 95% CI (−1.25,−0.76), $p<0.00001$], but not in treatments shorter than 12 weeks (Figure 3B). Four studies, encompassing 239 TG and 237 Con patients, demonstrated a significant decrease in 2hrPPG levels across all treatment durations (WMD=−0.56, 95% CI −0.79 to −0.32, $p<0.00001$) (Figure 3C). Lastly, five studies with 211 TG and 199 Con patients reported a significant reduction in HbA1c levels (WMD=−0.72, 95% CI −1.19 to −0.26, $p=0.002$), suggesting effective blood sugar control with AS (Figure 3D). These findings collectively indicate that AS effectively lowers BUN and blood glucose levels, with a time-dependent effect observed specifically for FPG.

Risk and bias assessment

Additionally, we conducted an evaluation of the risks and potential biases associated with the study. The funnel plots for BUN and 2hrPPG exhibited no significant asymmetry, indicating low risks of bias and increased reliability of the meta-analysis outcomes. However, the meta-analysis showed significant asymmetry in the clinical efficiency rate, 24-h UTP, UAER, Scr, FPG, and HbA1c results, suggesting a potential publication bias (Figure 4A–H).

Components-targets network, PPI, and enrichment analysis

In addition to conducting a meta-analysis, we also performed a network pharmacological analysis to further elucidate the mechanisms by which *Astragalus* affects DN. Initially, we identified a total of 213 active components and 191 associated targets after removing duplicates. Furthermore, we retrieved 3,614 DN-related disease targets obtained from the GeneCards and DisGeNET databases. Subsequently, the AS and DN targets were intersected using Excel, yielding 57 intersecting core targets (Figure 5A). Subsequently, we employed the STRING database to construct a PPI network centered on these core targets. The PPI network contained 57 nodes and 319 edges. The average node degree was 11.2, and the p-value of PPI clustering was below 1.0e-16 (Figure 5B). In this network, the degrees of the core targets were ranked as ACTB, JUN, PTGS2, PPARG, CAT, and NOS3, among others. Accordingly, these targets may be the key targets of AS in treating DN (Supplementary Table 1, Figure 5C).

After importing correlation data from Cytoscape software, a "component-target" network comprising 948 nodes and 3,714 edges was constructed (Figure 5D). The key active ingredients were screened using cytoNCA. Accordingly, the 24 active ingredients are listed according to the degree, including glycine (GLY), quercetin, apigenin, daidzein, and kaempferol (Supplementary Table 2). Enrichment analysis was

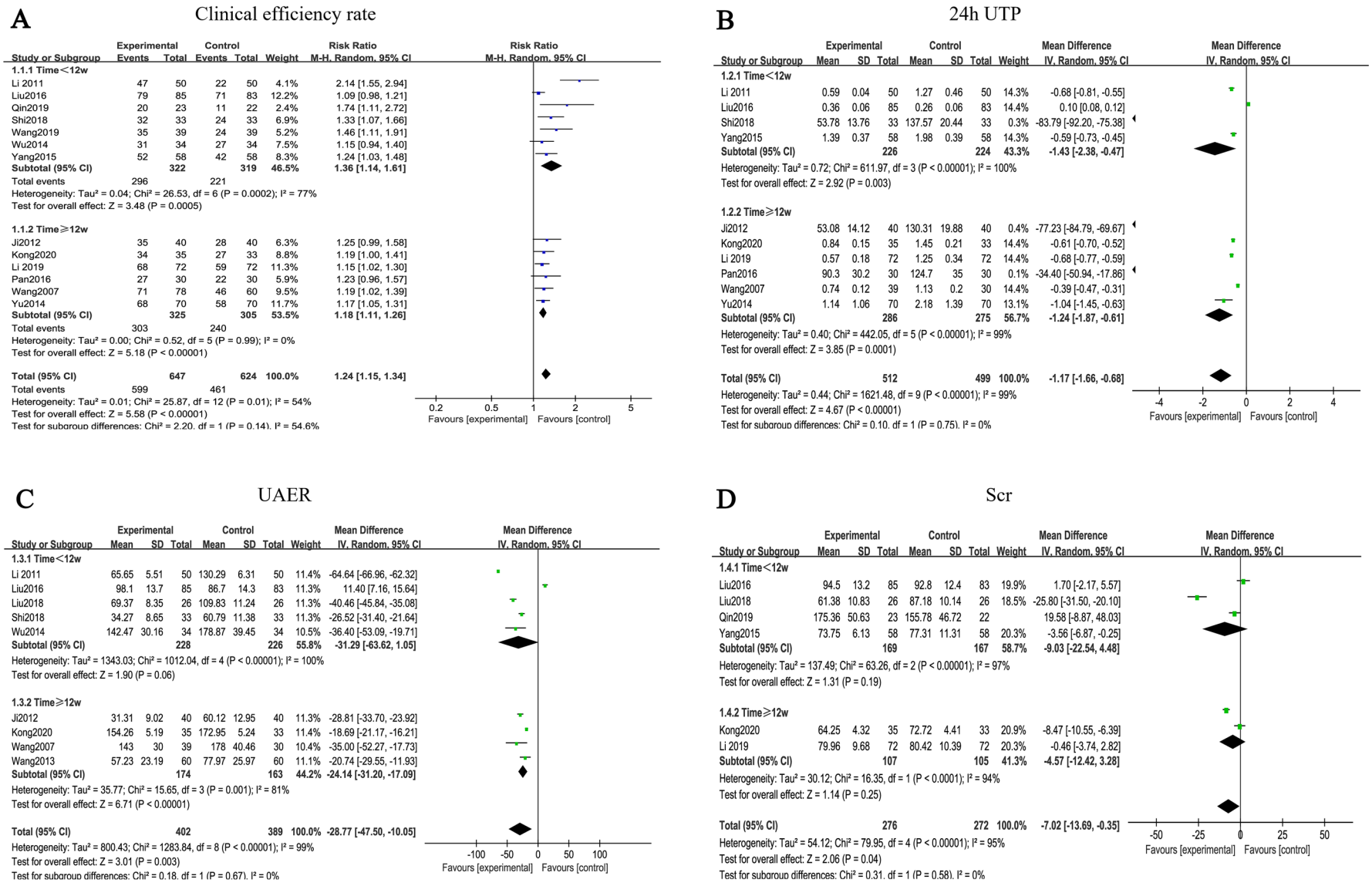

Figure 2. Forest plot of (A) clinical efficiency rate, (B) 24 h UTP, (C) UAER and (D) Scr.

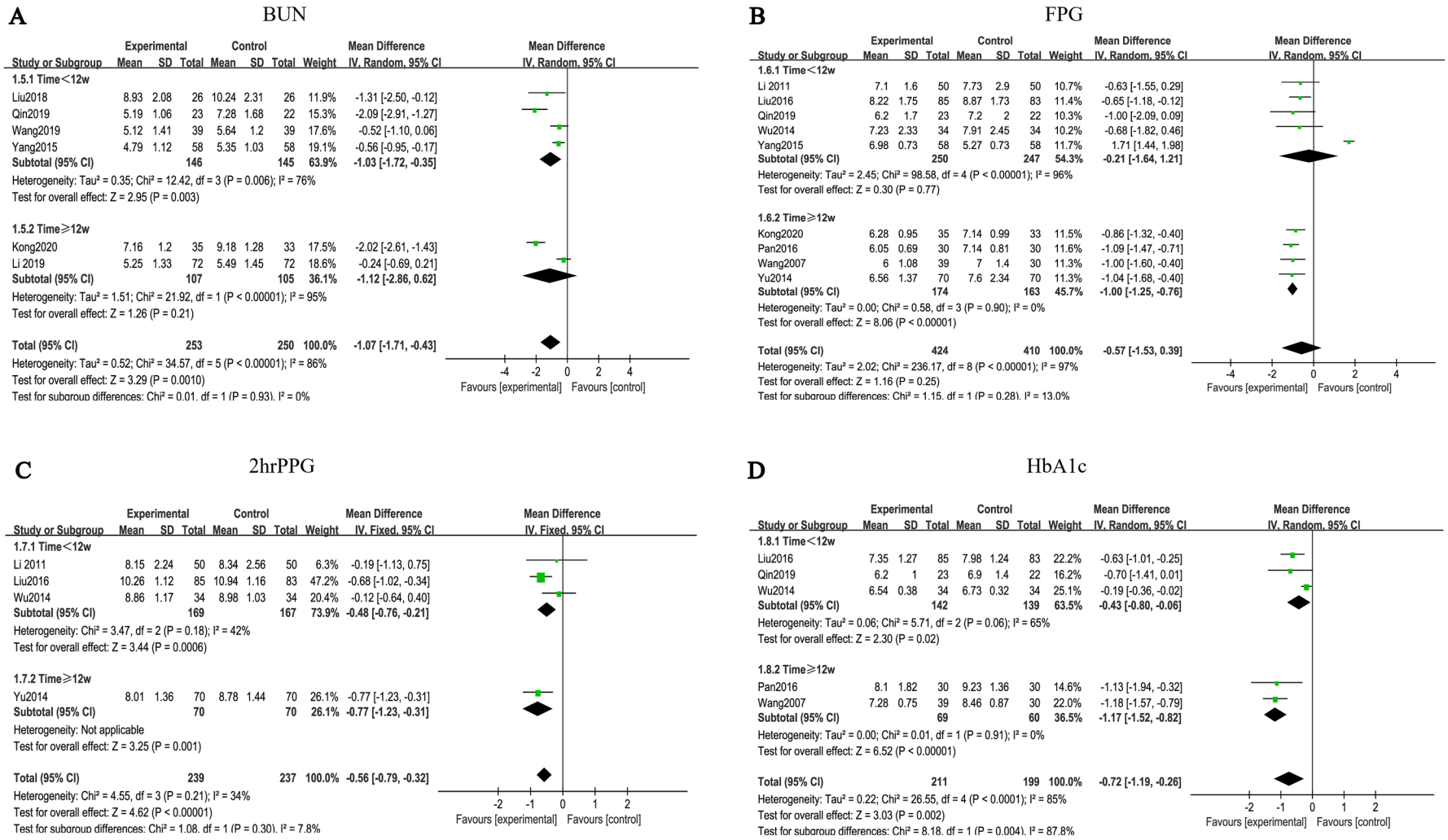

Figure 3. Forest plot of (A) BUN, (B) FPG, (C) 2hrPPG and (D) HbA1c.

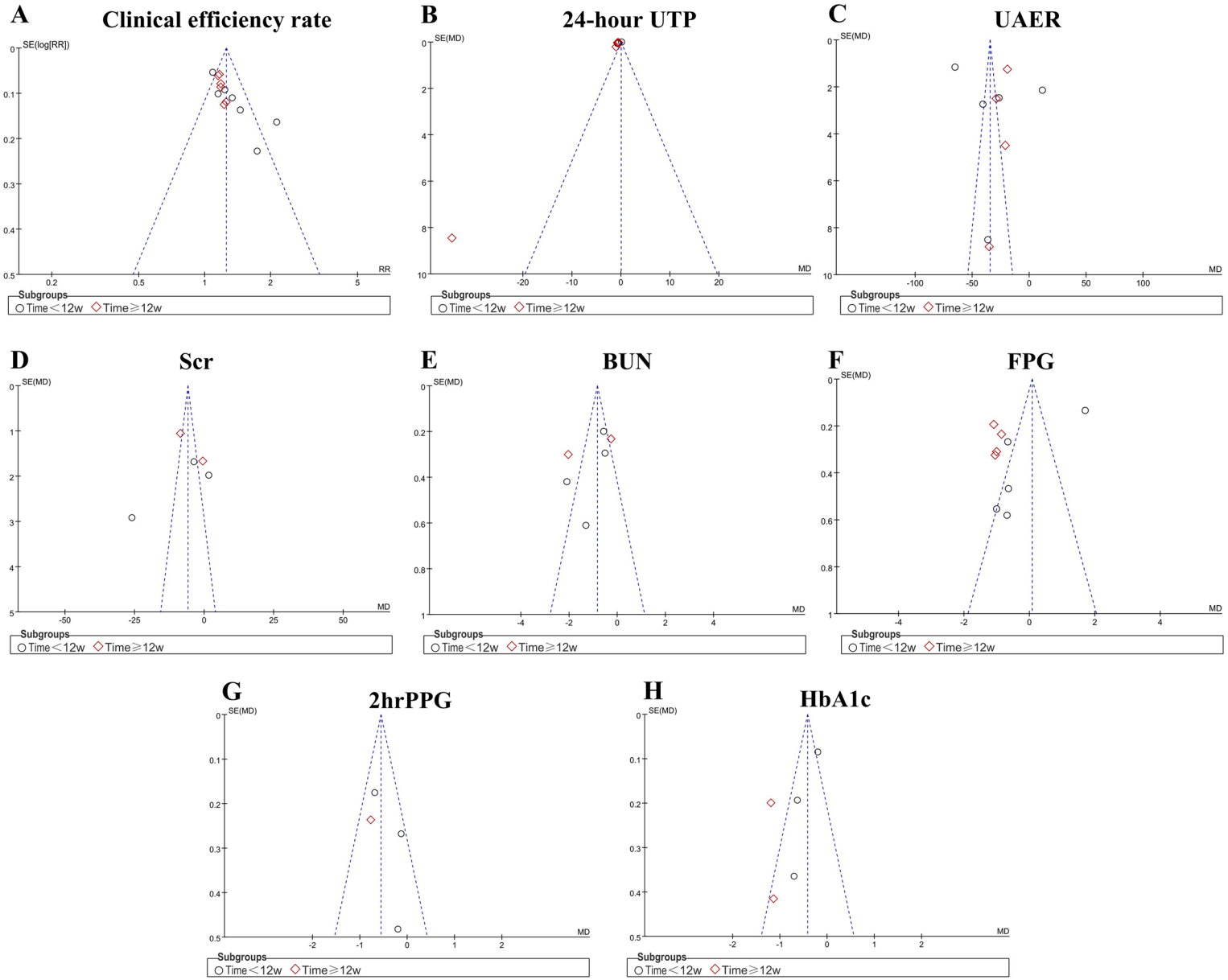

Figure 4. Funnel plot. (A) Clinical efficiency rate; (B) 24h UTP; (C) UAER; (D) Scr; (E) BUN; (F) FPG; (G) 2hrPPG; (H) HbA1c.

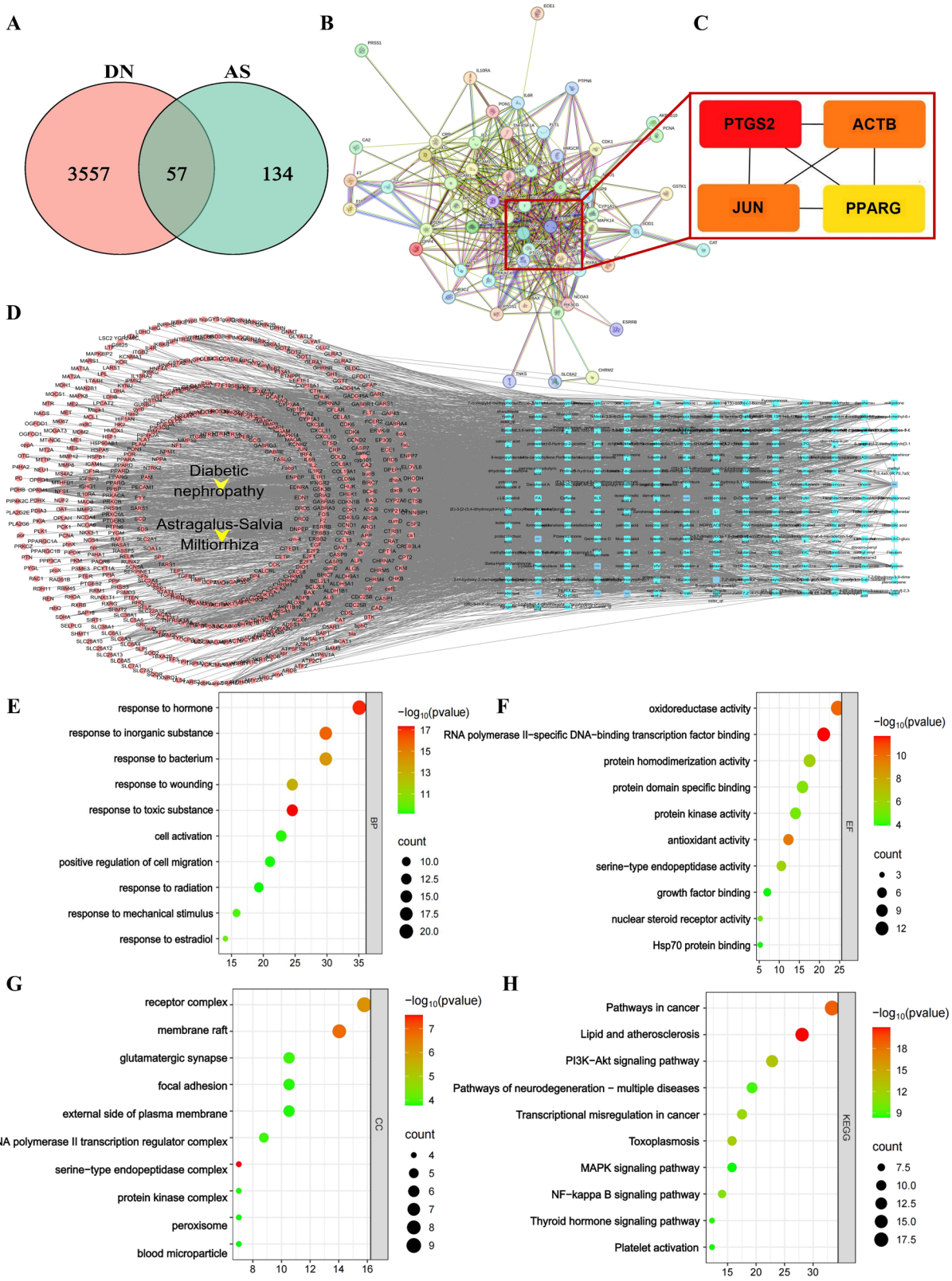

Figure 5. Network pharmacology of AS and DN. (A) VENN of AS-DN intersection targets; (B) PPI of the key target; (C) PPI network of hub genes by screening with CytoNCA; (D) "ingredients-targets" network; (E) Biological process; (F) Molecular function; (G) Cellular components; (H) KEGG.

performed in Metascape by importing the intersection targets. The available Gene Ontology (GO) BPs included inflammatory, wound, bacterium, and inorganic substance response (Figure 5E). The GO MFs contained antioxidant activity, oxidoreductase activity, protein homodimerization activity, and protein domain-specific binding (Figure 5F). The GO CCs included receptor complex, membrane raft, glutamatergic synapse, focal adhesion, and external side of the plasma

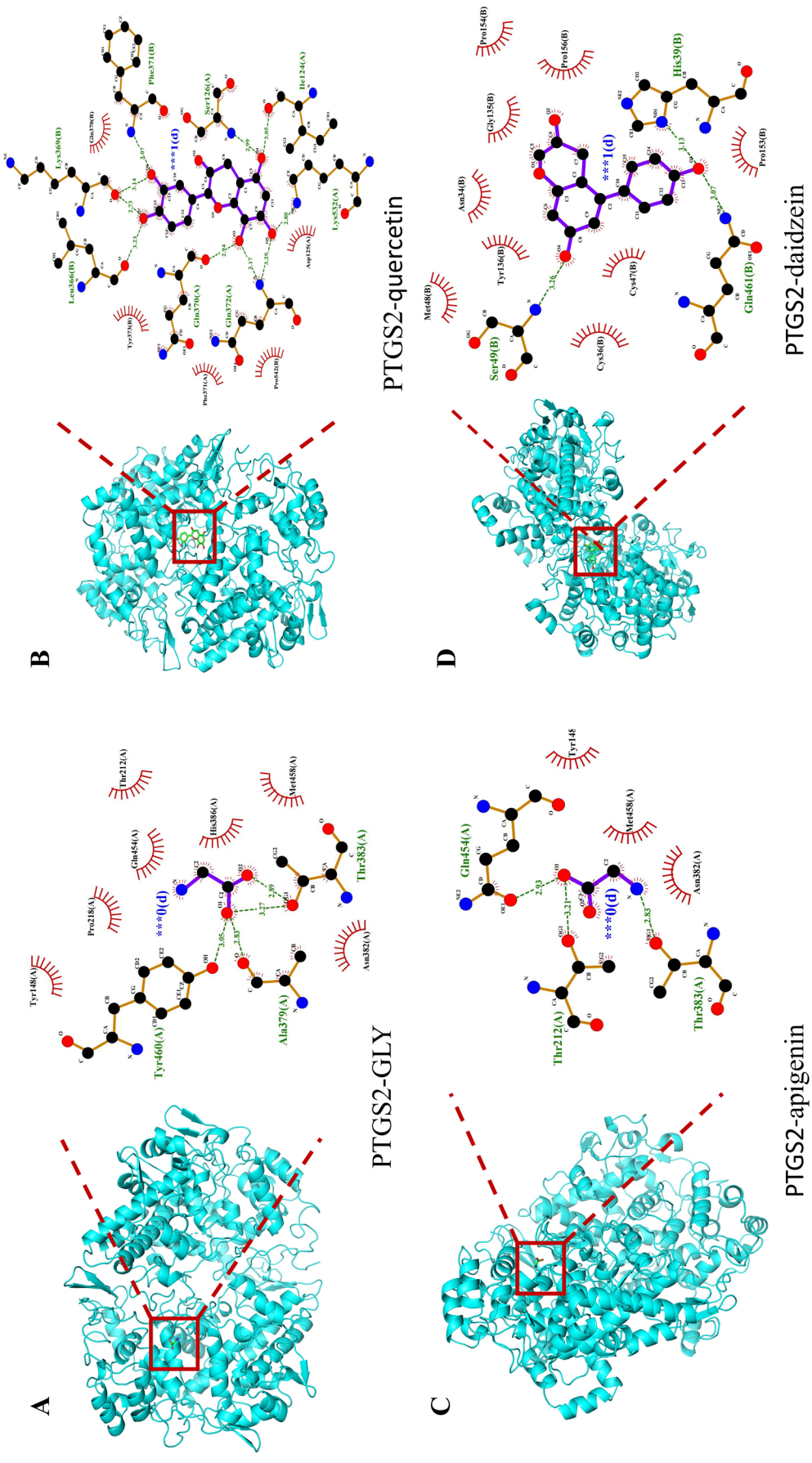

Figure 6. Molecular docking of key targets and key ingredients. (A) PTGS2 and GLY; (B) PTGS2 and quercetin; (C) PTGS2 and apigenin; (D) PTGS2 and daidzein.

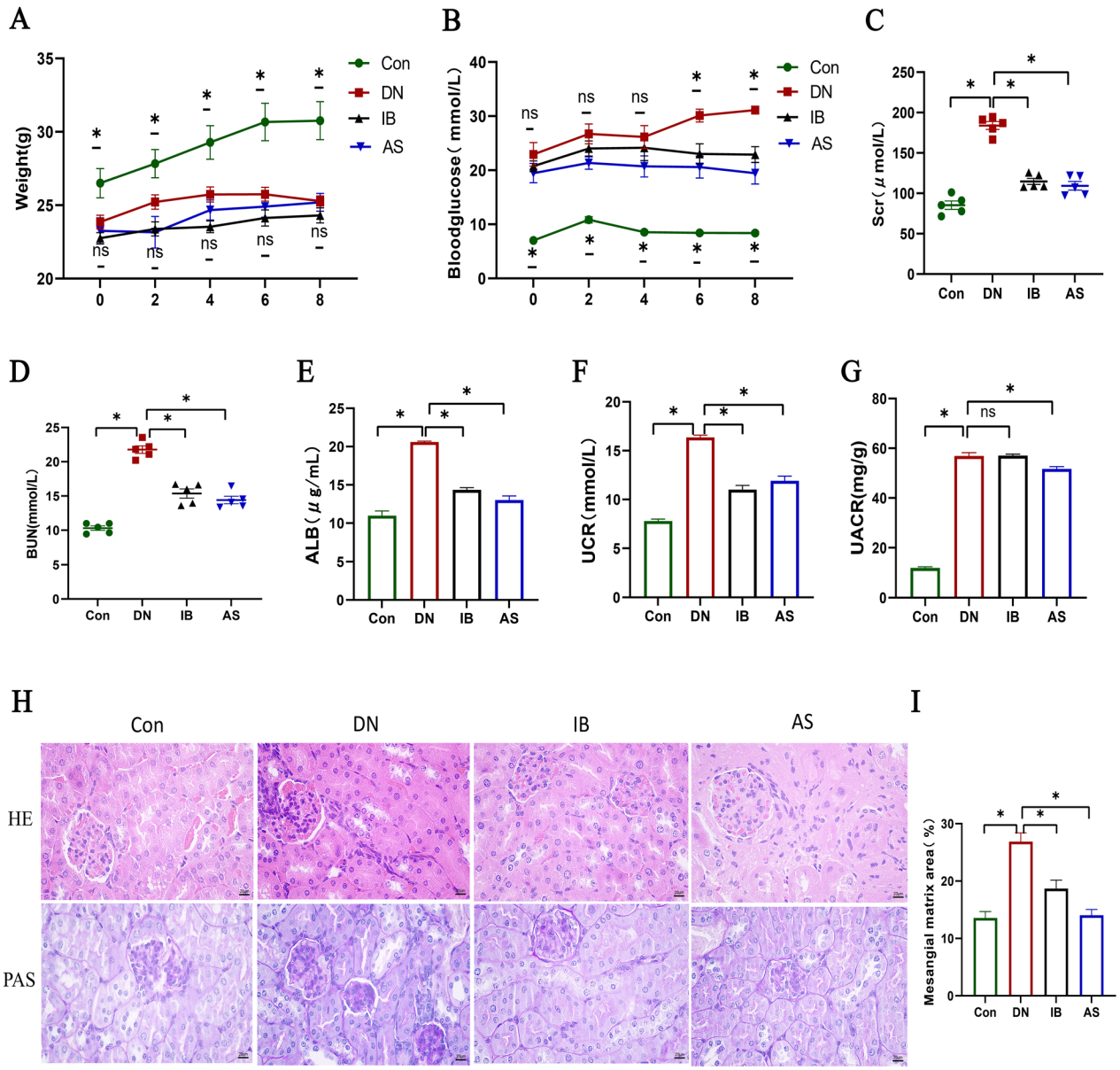

Figure 7. AS ameliorated renal injury in DN mouse model. (A)Body weight in Con, DN, IB, AS group ($n = 5$); (B) Fasting blood glucose in each group ($n = 5$); (C-F) Scr (C), BUN (D), ALB (E), UCR (F) and UACR (G) levels in each group ($n = 5$); (H) HE staining and PAS staining of kidney tissues in mice, scale bar = 400μm; (I) Quantifications of PAS's trichrome staining($n = 3$). Data are presented as the mean ± SEM. *$p < 0.05$; **$p < 0.001$. Con, control group; DN, diabetic nephropathy group; IB, diabetic nephropathy + irbesartan group; AS, diabetic nephropathy+*Astragalus-Salvia miltiorrhiza* couplet medicines group.

membrane (Figure 5G). Furthermore, the visualization of the top 10 KEGG pathways involved PI3K/Akt, mitogen-activated protein kinase (MAPK), and NF-kappa B signaling pathways (Figure 5H).

Molecular docking study

Among the key core targets for AS treatment of DN screened by our network pharmacology, ACTB, JUN, and PTGS2 are ranked in the top three (Supplementary Table 1). Because PTGS2 plays a key role in cell damage and fibrosis in DN and is closely related to ferroptosis, we chose PTGS2 for molecular docking research. We targeted PTGS2 (PDBID: 5F19) for molecular docking with ligands GLY, quercetin, apigenin, and daidzein (Supplementary Table 2). The docking analysis showed an interaction between PTGS2 and GLY. This association involves Tyr-148, Pro-218, and Ghn-454, with a docking energy of −3.9 kcal/mol (Figure 6A). Furthermore, quercetin formed hydrogen bonding interactions with specific residues (Ghn-370, Tyr-373, and Phe-371) on PTGS2, resulting in a docking energy of −10.0 kcal/mol (Figure 6B). PTGS2 is observed to associate with apigenin through hydrogen bonding, involving various residues such as THR-148, Met-458, and ASN-382. This interaction results in a docking energy of −3.8 kcal/mol (Figure 6C). Finally, PTGS2 exhibits a robust binding affinity of −7.8 kcal/mol when interacting with daidzein residues, such as Tyr-136, Gly-135, and Pro-154, through hydrogen bonding (Figure 6D).

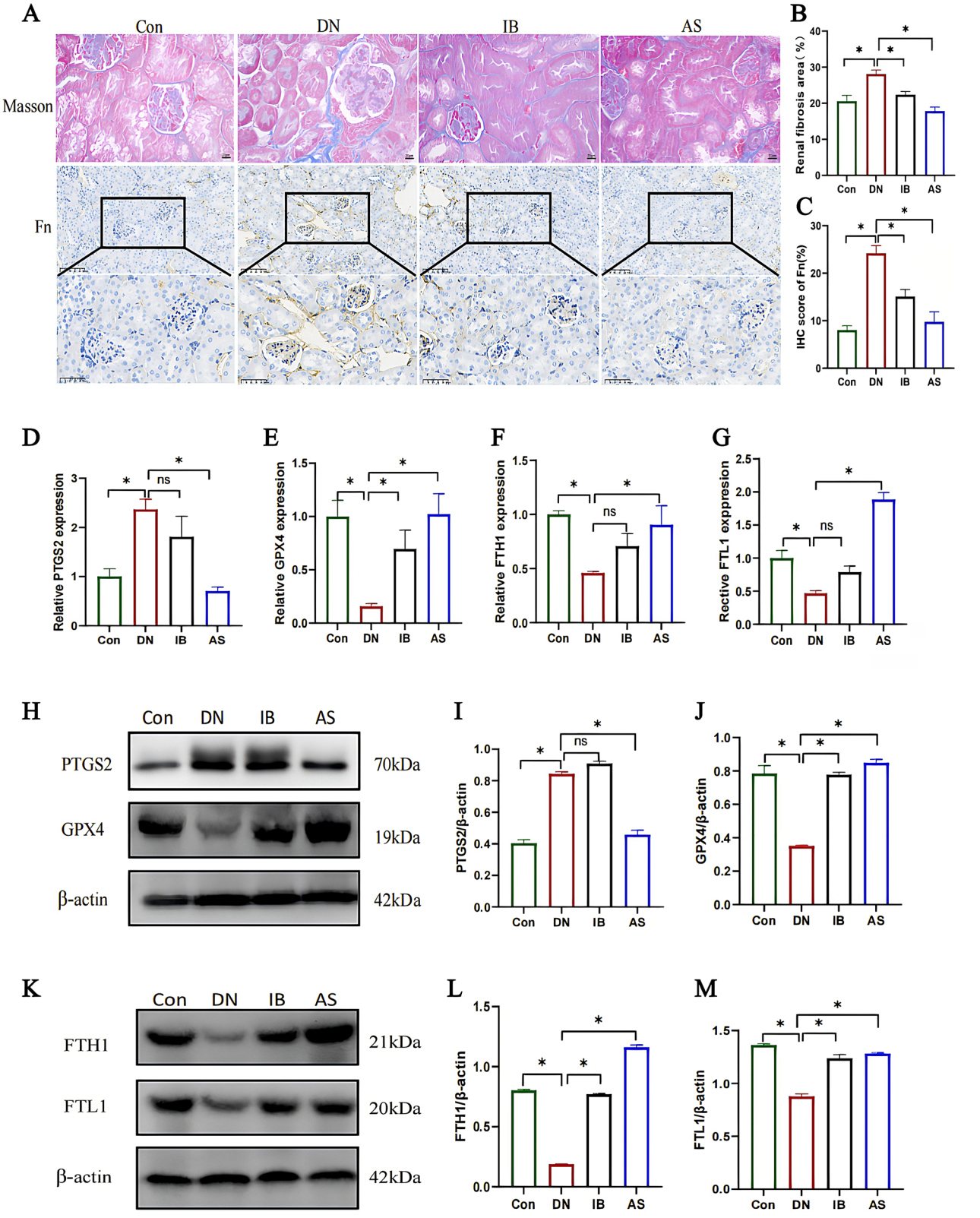

Figure 8. AS can reduce the degree of renal fibrosis and improve iron metabolism. (A)Masson and immunohistochemical detection in mouse kidney tissue. Scale bar = 200 μm, 400 μm; (B-C) Quantitative analysis of masson and immunohistochemistry ($n = 3$); (D-G) PTGS2, GPX4, FTH1, and FTL1 mRNA expression in the kidneys; (H, K) Western blotting results of PTGS2, GPX4, FTH1, and FTL1 in each group; (I-J, L-M) the quantitative analysis for Western blotting results of PTGS2, GPX4, FTH1, and FTL1 ($n = 3$). All data was presented as mean ± SEM. *$p < 0.05$. Con, control group; DN, diabetic nephropathy group; IB, diabetic nephropathy + irbesartan group; AS, diabetic nephropathy+*Astragalus-Salvia miltiorrhiza* couplet medicines group.

AS ameliorated renal injury in DN mouse model

To further verify the therapeutic mechanism of AS, we conducted *in vivo* experiments. Body weight, fasting blood glucose, Scr, BUN, ALB, UCR, and UACR were used to evaluate the role of AS in the DN mouse model. Compared to the control group, a statistically significant reduction in body weight was observed in the other experimental groups ($p < 0.05$). However, the weight loss in the IB and AS treatment groups did not differ significantly from that of the DN group, as depicted in Figure 7A. After the end of treatment, elevated FBG, Scr, BUN, ALB, UCR, and UACR levels in DN were significantly reduced by both IB and AS compared to the control group, with AS showing greater efficacy ($p < 0.05$) (Figures 7B-G). Histopathological analysis showed that renal inflammation, glomerular hypertrophy, mesangial expansion, and basement membrane thickening in the DN group were partially reversed by IB and AS, with AS being more effective (Figure 7H). Quantitative data supported these observations (Figure 7I).

AS can reduce the degree of renal fibrosis and improve iron metabolism

Previous research has indicated the therapeutic efficacy of AS in DN, yet the underlying mechanisms remain elusive. To elucidate these mechanisms, we conducted Masson and immunohistochemistry to assess renal fibrosis in murine kidney tissues. Utilizing PCR and Western blot techniques, we examined renal iron metabolism biomarkers. Our findings revealed a substantial increase in renal fibrosis in the DN group, which was significantly mitigated by both IB and AS treatments, with AS demonstrating the most pronounced reduction (Figure 8A-C). PCR analysis showed that DN upregulated PTGS2 mRNA expression ($p < 0.05$), a change that was effectively reversed by IB and AS (Figure 8D). Conversely, DN downregulated the mRNA levels of GPX4, FTH1, and FTL1, an effect that was counteracted by IB and AS treatments ($p < 0.05$) (Figure 8E-G). Protein levels of these biomarkers mirrored the mRNA trends, further validating the observed effects ($p < 0.05$) (Figure 8H-M).

Discussion

Currently, the primary approach for treating DN has not achieved satisfactory results, and there is still no definitive therapy available to cure DN [40]. AS exhibits desired therapeutic effects in the treatment of DN. Pharmacological studies have demonstrated the beneficial effects of *Astragalus* in treating DN, including the reduction of 24-h UTP, blood glucose levels, and the expression of inflammatory factors [41,42]. Furthermore, *S. miltiorrhiza* has been shown to improve renal blood flow by regulating abnormal glucose and lipid metabolism, suppressing oxidative stress, and reducing inflammation [43]. Despite the availability of individual studies investigating the therapeutic potential of *Astragalus* and *S. miltiorrhiza* in DN, there is a significant gap in research regarding the combined use of AS in the treatment of DN.

The combination of *Astragalus* and *S. miltiorrhiza* exerts more significant effects compared to using either herb alone for treating DN. Shen et al. [44] utilized 16S rDNA, liquid chromatography-tandem mass spectrometry, and RNA seq to identify the target bacteria, target metabolites, and target genes of AS in the treatment of DN. Notably, their study provides evidence that AS ameliorates DN by modulating the gut microbiome *via* the gut-kidney axis [44]. Furthermore, AS increases the abundance of beneficial bacteria, such as *Akkermansia muciniphila* and *Lactobacillus murinus*, which enhance the production of metabolites involved in sphingolipid and glycerophospholipid metabolism. These metabolic pathways play a pivotal role in regulating glycolipid metabolism, a critical factor in DN progression. Elevated levels of metabolites like sphingosine contribute to improved renal function. Furthermore, AS regulates the expression of target mRNAs within these pathways, offering additional support for its role in mitigating DN [44,45]. To the best of our knowledge, our study pioneers a holistic investigation into the clinical efficacy and pharmacological underpinnings of AS therapy for DN. By integrating meta-analysis, network pharmacology, molecular docking, and experimental validation, we aim to deepen our comprehension of AS's therapeutic potential in DN management. This multifaceted approach is anticipated to foster the development of novel and efficacious treatment paradigms.

To assess the clinical impact of AS in treating DN, we conducted a comprehensive meta-analysis encompassing 15 clinical studies. The results indicated that AS could significantly improve renal function, reduce urinary protein, and lower blood glucose levels, thereby achieving notable therapeutic outcomes compared to conventional treatment. To further explore the effectiveness of AS, we conducted a detailed subgroup analysis to investigate whether the treatment of DN with AS exhibits temporal dependency. Our results revealed a crucial time-dependent relationship between AS administration and FPG levels in DN. Specifically, when the therapeutic time was less than 12 weeks, the impact of AS on FPG levels was relatively minor. However, as the therapeutic time increased to 12 weeks or longer, the effect of AS on reducing FPG levels became significantly pronounced. Based on our comprehensive analysis, it is evident that extending the duration of AS treatment results in a significant reduction in FPG levels among patients with DN. This finding holds substantial implications for the management of this chronic condition. Importantly, a prior study has established that temporal changes in FPG serve as a strong predictor of cardiovascular disease-related mortality in patients with type 2 diabetes [46]. Given this correlation, our discovery that prolonged AS treatment effectively lowered FPG levels suggests a potential means to mitigate the risk of cardiovascular complications.

However, there were some limitations in our meta-analysis. The review was not registered, and a protocol was not prepared. In addition, we faced challenges with data

interpretation and reliability. The presence of publication bias, indicated by asymmetrical funnel plots, and significant heterogeneity (high I^2values >50%), complicate the results' interpretation and may skew the conclusions. Lastly, we attribute these issues to several factors: differences in drug prescriptions, varying DN stages and severities, and discrepancies in conventional treatment protocols across the 15 included studies. Additionally, the suboptimal quality of RCTs and small sample sizes impact the findings' reliability and generalizability. The short follow-up period and incomplete outcome reporting limit the assessment of long-term effectiveness. To address these challenges and improve the validity of our findings, future research with larger cohorts, standardized protocols, and extended follow-up is necessary.

As a metabolic disease, DN is known to involve intricate pathological mechanisms. These mechanisms include the activation of inflammatory mediators, alterations in renal hemodynamics, oxidative stress, and imbalances in glucose and lipid metabolism [47]. Given the complexity of these processes, it is necessary to use network pharmacology methods to comprehensively analyze the pathological and pharmacological mechanisms of AS's impact on DN.

Of note, we conducted an enrichment analysis to predict the signaling pathways and molecular mechanisms involved in the effects of *Astragalus* on diabetic nephropathy. GO analysis revealed a strong association between AS treatment and antioxidant/oxidoreductase activity in DN. This aligns with prior research indicating that excessive oxidative stress triggers glomerulosclerosis and renal fibrosis, promoting DN pathogenesis [48,49]. Notably, oxidative stress is recognized as a critical initiator of iron metabolism. Iron plays an essential role in numerous cellular processes, such as DNA synthesis, respiration, and energy metabolism. However, an imbalance in iron levels, particularly when excessive or unstable, can lead to cellular and organ damage due to the generation of reactive oxygen species, thereby disrupting iron homeostasis [50].

Moreover, the KEGG analysis has revealed that AS's therapeutic effect on DN is primarily mediated through the PI3K/Akt, NF-kappa B, and MAPK signaling pathways. Firstly, the PI3K/Akt signaling pathway is intricately linked to glomerular hypertrophy and extracellular matrix accumulation, two crucial processes in the development of DN [51]. Previous studies have shown that AS can inhibit the expression of p-PI3K, p-Akt, and p-mTOR, thereby alleviating acute lung injury in rats [52]. Accumulating evidence from prior research indicates that overactivation of the PI3K/Akt/mTOR signaling axis can suppress ferroptotic cell death through the upregulation of sterol regulatory element-binding protein 1, which in turn promotes adipogenesis [53]. Furthermore, elevated blood glucose levels are known to initiate renal inflammation, a process that involves multiple signaling pathways, including the MAPK and NF-κB. Activation of these pathways can exacerbate the fibrosis associated with DN [54]. Numerous studies have further corroborated the synergistic effects of AS on modulating the MAPK and NF-κB pathways [55–58]. Additionally, a strong correlation between lipids and atherosclerosis has been found in the KEGG results. Previous studies have confirmed that inhibiting the PI3K/Akt and NF-κB signaling pathways can improve atherosclerosis caused by high-fat diets and vitamin D3 [59]. Furthermore, research shows that activating autophagy and suppressing the MAPK and PI3K/Akt/mTOR pathways can help inhibit the progression of atherosclerosis [60]. In summary, AS demonstrates a significant connection with the signaling cascades of PI3K/Akt, NF-κB, and the MAPK pathway, all of which contribute to its therapeutic impact on DN and atherosclerosis. By regulating these crucial signaling processes, AS effectively reduces inflammation and renal fibrosis, presenting a promising approach for treating DN.

In the aforementioned study, we identified 24 key bioactive components of AS that are implicated in the treatment of diabetic nephropathy. Among them, GLY, quercetin, apigenin, and daidzein are the top four components most closely related to DN. Among the compounds investigated, GLY and apigenin were derived from *S. miltiorrhica*, while quercetin and daidzein were isolated from *Astragalus*. Quercetin is a flavonoid with various biological activities [61], which has been demonstrated to improve renal function in DN animal models by anti-inflammatory, inhibiting renal oxidative stress, and regulating lipid metabolism [62,63]. Mainly, quercetin inhibits DN mesangial cell proliferation and epithelial-mesenchymal transition by activating Hippo and inhibiting TGF-β/PI3K/Akt signaling, respectively [64]. This preclinical evidence suggests that quercetin has potential value in preventing or treating DN. Apigenin, a natural flavonoid, has been found to improve kidney damage induced by DN [65,66]. This is achieved through its inhibitory effect on oxidative stress and fibrosis, which is related to the MAPK pathway [67]. Literature has shown that daidzein exhibits cardiovascular protection and hypoglycemic activity in mice. Its ingredients have positive effects on the treatment of DN [68]. However, there have been no previous reports on GLY for DN. Therefore, this study is the first to identify GLY as a potential core component in the AS treatment of DN, providing a theoretical foundation for future experimental research.

Additionally, we pinpointed four top targets for AS treatment in DN: PTGS2, ACTB, JUN, and PPARG. Among them, PTGS2, alternatively denoted as prostaglandin-endoperoxide synthase 2, functions as a crucial enzyme in the catalytic pathway that leads to prostaglandin synthesis [69]. Notably, the gene encoding PTGS2 has been identified as a significant contributor to the pathogenesis of ferroptosis [70]. To elucidate the potential of active ingredients in alleviating DN by affecting iron metabolism, we conducted a molecular docking study on PTGS2 with GLY, quercetin, apigenin, and daidzein. Our study presents evidence that the enzyme PTGS2 demonstrates significant binding affinity for the flavonoids quercetin and daidzein, as indicated by their respective binding energies of −10.0 kcal/mol and −7.8 kcal/mol. Quercetin has been reported to mitigate neuronal ferroptosis in a mouse model of breast cancer-related depression by modulating PTGS2, thereby enhancing immune response [71]. This evidence supports our hypothesis that quercetin may confer

therapeutic benefits in DN through its efficient interaction with genes central to iron metabolism. However, its precise pharmacological mechanisms in DN require further investigation.

To substantiate the precise therapeutic mechanism of AS in mitigating iron metabolism and treating DN, we conducted *in vivo* experiments utilizing a murine model. The findings indicate that AS treatment significantly ameliorated renal injury in DN mice, as evidenced by a statistically significant reduction in FBG, Scr, BUN, ALB, UCR, and UACR compared to the DN group. Histopathological analysis further confirmed the renoprotective effects of AS, showing a marked reversal of key DN features such as nephritis, glomerular hypertrophy, mesangial dilation, basement membrane thickening, and renal fibrosis. Further comprehensive analyses, encompassing immunohistochemistry, qPCR, and WB, demonstrated that AS treatment notably attenuated renal fibrosis and modulated the expression of pivotal biomarkers implicated in iron metabolism, specifically PTGS2, GPX4, FTH1, and FTL1. These collective alterations in key indicators underscore the capacity of AS to significantly enhance iron homeostasis in DN.

Conclusion

Our research harnessed meta-analysis and network pharmacology, along with molecular docking and experimental validation, to investigate the therapeutic efficacy and mechanisms of AS in DN. Notably, the combination of AS with conventional therapy has emerged as a clinically safe and efficacious treatment for DN. AS's wide-ranging action on multiple pathways, such as PI3K/Akt, MAPK, and NF-kB, likely drives its therapeutic benefits. Molecular docking showed AS's active ingredients—GLY, quercetin, apigenin, and daidzein—have a strong affinity for the PTGS2 target. *In vivo* studies confirmed AS can reverse changes in iron metabolism biomarkers linked to DN, including PTGS2, GPX4, FTH1, and FTL1. Although our findings offer promising insights into DN treatment, we acknowledge the study's limitations and emphasize the need for further comprehensive research with larger sample sizes to corroborate these preliminary results.

Institutional review board statement

Animal experiments were approved by the Animal Experiment Ethics Committee of Jinan University (approval number: IACUC-20231102-18).

Author contributions

YX and XZ conceived and designed the study; HL, ZC, and MZ conducted the experiments and obtained the data; JZ, SL, JC, JY and PJ analyzed and collated the data; YX and HL drafted and written the final version of the manuscript. HL, ZC, and MZ contributed equally to this work. All authors approved the final version of the manuscript.

Disclosure statement

No potential conflict of interest was reported by the author(s).

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Data availability statement

The data sets used to support the findings of this study are available from the corresponding author upon request.

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