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RESEARCH ARTICLE

Traditional Mongolian medicine Wu-Lan thirteen-flavor decoction protects rat from hypertension-induced renal injury via aryl hydrocarbon receptor-mediated pathway

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ABSTRACT

Background: Wu-Lan Thirteen-Flavour decoction (WLTd), a traditional Mongolian medicine, has been used for treating hypertension in clinical practice, but the chemical basis and underlying mechanisms remain unknown.
Methods: The main components of WLTd were identified and quantified using HPLC and UPLC–MS/MS techniques. A compound-target-disease network was constructed using network pharmacology analysis to forecast the potential anti-hypertension targets. *In vivo* animal and *in vitro* cellular experiments were performed to validate the efficacy and molecular mechanisms of renal protection of WLTd and its main active components in spontaneous hypertension.
Results: A total of 136 active compounds in WLTd were collected through relevant databases, and network pharmacology analysis identified that the aryl hydrocarbon receptor (AhR) signaling pathway may serve as a potential anti-hypertension targets. Eight of the active components, including vitexin, kaempferol, toosendanin, ursolic acid, matrine, oxymatrine, gardenoside and quercetin, were identified and quantified by HPLC and UPLC–MS/MS. WLTd effectively lowered the mean blood pressure (159.16 ± 13.91 vs 135 ± 13.37 mmHg), reduced the BUN (391.55 ± 59.96 vs 240.88 ± 51.15 mmol/L) and creatinine (1.78 ± 0.41 vs 0.67 ± 0.34 nmol/L) levels, and reduced hypertension-induced renal damage in SHR. AhR and related key gene expression changes predicted by network pharmacology analysis were validated by immunohistochemistry, RT-qPCR, and Western blot analyses. *In vitro,* studies also showed that WLTd up-regulated AhR expression in angiotensin II-induced HEK293 cell injury.
Conclusions: Wu-Lan Thirteen-Flavour decoction effectively protects hypertension-induced renal injury by regulating the Aryl Hydrocarbon Receptor signaling pathway.

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Traditional Mongolian medicine; Wu-Lan thirteen-flavor decoction; hypertensive renal injury; network pharmacology; aryl hydrocarbon receptor; human embryonic kidney cell HEK293; molecular docking

Introduction

Hypertension is a complex disorder with environmental and genetic factors. It is also the most important risk factor for cardiovascular and cerebrovascular diseases, leading to stroke, acute myocardial infarction, atrial fibrillation, and renal insufficiency [1]. Among these, the progressive loss of renal function is particularly important, not only for the direct consequences of kidney damage but also because the loss of renal function is associated with the amplification of other adverse cardiovascular outcomes [2]. Currently, the clinical landscape boasts an array of over a hundred antihypertensive drugs. Despite this abundance, adherence to hypertension treatment remains suboptimal. A primary contributing factor is attributed to the unidirectional mechanism of action employed by these drugs, where they primarily lower blood pressure without addressing the comprehensive symptoms experienced by a substantial number of patients.

Traditional Mongolian Medicine (TMM) originated in the early thirteenth century. Numerous traditional Mongolian remedies have been successfully employed in clinical practice, demonstrating commendable therapeutic efficacy. Over time, TMM has evolved into a distinctive medical system, garnering a wealth of clinical insights in Inner Mongolia and its surrounding regions [3, 4]. WLTd is a common treatment for 'black pulse disease' in Mongolian medicine. With the effect of clearing blood and heat, it is used to treat headaches, red eyes, and hypertension. WLTd is composed of thirteen herbs, including Inula helenium (Asteraceae), Eriobotrya japonica (Rosaceae), Sophora flavescens Aiton (Leguminosae), Tropaeolum tenuirostre Steud (Tropaeolaceae), Melia azedarach (Meliaceae), Balsamina lacca (Geraniaceae), Rubus insularis (Rosaceae), Gardenia subgen (Rubiaceae), Masdevallia mystica Luer (Orchidaceae), Acorellus laevigatus (Cyperaceae), K. galanga Linnaeus (Zingiberaceae), Rubia argyi (Rubiaceae) and Lithospermum altamiranense (Boraginaceae). Chinese pharmacopoeia (1998 edition) has

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listed WLTd as a was added to the national standard and was included in the drug standards of the Ministry of Health of the People's Republic of China (Mongolian medicine sub-list) [5, 6].

The identification and prediction of drug targets constitute a crucial step in the process of drug discovery. Over the past few years, numerous algorithms and software have been introduced for the prediction of drug-target interactions, including Random Walk, PRINCE, and Cytoscape. By leveraging these computational tools, drug repositioning based on interactions between drugs and disease-gene relationships can be achieved, enabling identification of novel indications for existing drugs and providing guidance for drug repositioning. The field of network pharmacology has further advanced our understanding of the relationship between disease-associated proteins and drug targets in the human protein interactome. These network-based methods have enabled more efficient quantification of these relationships and have facilitated drug repositioning. Additionally, network pharmacology-based approaches can be utilized to study the differences and connections between various Traditional Chinese Medicine (TCM) symptoms, opening up new avenues for the development of combination therapies. The emergence of these computational algorithms, software, and databases has enabled the flourishing of network pharmacology, driving innovations in the field of drug discovery and enhancing our ability to identify promising drug targets. In this article, the "compound-target-pathway" was constructed by systematic network pharmacology. The biological network predicted the main active ingredients, core targets and key pathways of WLTd based on a large-scale analysis of the literature. Network pharmacology conducted a core analysis on the potential targets of WLTd for the treatment of hypertension, among which the aryl hydrocarbon receptor (AhR) signaling pathway ranked the first [7].

The AhR plays an important role in normal development and in many physiological processes including cellular homeostasis, reproduction, immunity, circadian rhythms, skin physiology, and endocrine functions [8, 9]. AhR is a ligand-activated transcription factor found in the cytoplasm of mammalian cells [10, 11]. It is a polyprotein complex including HSP90 and proteins interacting with AhR. The AhR–ligand complex is transferred to the nucleus to form a heterodimer complex with the aromatic nuclear translocator (ARNT) [12]. The complex is consistent with DNA sequences found in promoters of a variety of genes, including those involved in the encoding of enzymes involved in exogenous detoxification (CYP1A1, CYP1A2, and CYP1B1) and AhR repressor (AhRR). AhR is involved in the expression of exogenous metabolic enzymes, inflammatory cytokines, and adhesion molecules, and is the mediator of the toxic reactions of halogenated hydrocarbons and polycyclic aromatic hydrocarbons (TCDD), with carcinogenic and teratogenic effects [13]. In addition to regulating metabolism enzymes, the AhR has roles in regulating immunity, stem cell maintenance, and cellular differentiation. However, accumulated evidence over the past few years suggests that AhR is not only an exogenous receptor but can also be activated by several endogenous ligands. In response to activation by a ligand, AhR translocates from the cytoplasm to the nucleus where it controls the transcription of a wide variety of target genes. Although AhR was initially recognized as a mediator of the toxic effects of dioxins, multiple physiologic ligands are provided by the diet, the commensal flora, and also the host's metabolism [14]. The identification of these natural ligands and the analysis of AhR-deficient mice has revealed important physiological roles for AhR [15–17]. Flavonoids are known phytochemicals that are AhR agonists and antagonists including flavones such as quercetin and luteolin as well as the isoflavones genistein and daizein. The effects of flavonoids are cell type and cell context-dependent [18]. The active ingredients of WLTd are rich in flavonoids, such as gardenia, Szechwan Chinaberry fruit, honeysuckle, loquat leaf, and radix sophorae flavescentis, all of which contain quercetin. Meanwhile, radix sophorae flavescentis is also rich in luteolin. As a new science, network pharmacology, combining with the application of systems biology, multi-disciplinary pharmacology, computational biological network analysis and other multi-disciplinary technical means and contents, constructs a multi-level network, and explores the relationship between drugs and diseases holistically, which has strong integrity and systematicness [19]. In this study, combined with network pharmacological prediction, HPLC, and UPLC-MS/MS detection techniques, the main active components of WLTd were identified, and *in vivo* and *in vitro* experiments were conducted to explore the pharmacodynamic effects and related mechanisms of WLTd on renal injury in hypertensive rats. These results suggest that WLTd has a protective effect on renal injury (fibrosis) in essential hypertensive rats by inhibiting the AhR signaling pathway.

Materials and methods

Drugs and reagents

WLTd was provided by Inner Mongolia Medical College Hospital (Hohhot, China) (Drug approval number: M13010055; Batch number: 20190159). The chemical standard vitexin (DST200325-034), kaempferol (DSTDS005701), toosendanin (DSTDC001801), ursolic acid (DSTDX001901), quercetin (DSTDH002801), matrine (DSTDK000901), oxymatrine (DSTDY004001) and gardenoside (DST200713-032) were purchased from Chengdu DeSiTe Biological Technology Co., Ltd. (Chengdu, China). High-efficiency radioimmunoprecipitation assay (RIPA) lysis buffer (R0010), 2,3,5-Triphenyl-2H-tetrazolium chloride (TTC T8170), bicinchoninic acid (PC0020), and the protein concentration assay kit were purchased from Solarbio (Beijing, China). Anti-AhR polyclonal antibody was purchased from Immunoway (YT0145, Shanghai, China). Assay kits for rat CRE and BUN were purchased from Jiancheng Bioengineering Institute (Nanjing, China).

Animals and experimental design

Male 12-week-old SHR (spontaneous hypertension rat) and age-matched male normotensive WKY rats (240 ± 10 g) were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China, Certificate No.: SCXK Jing 2017-0005). This study was conducted according to the principles of the Basel Declaration and recommendations of the Care and Use of Laboratory Animals issued by the Ministry of Science and Technology of China. The experimental animal ethics committee of Tianjin University of Traditional Chinese Medicine approved the protocol (permit number: TCM-LAEC2014004). The experimental animals were housed under room temperature (25 ± 2 °C) and relative humidity (40% ± 5%) conditions with 12-h light/dark cycles. For the experimental design, animals were randomly divided into six groups with 5–6 rats in each group, including normal control (normal saline), model, positive-control drug (Losartan, 10 mg/kg), and low (0.9 g/kg), medium (1.8 g/kg) and high (3.6 g/kg) doses of WLTd groups. After 4 weeks of continuous drug administration, the levels of CRE and BUN in the urine were measured, and the blood serum was collected post-anaesthesia and then stored at −80 °C. Kidneys were harvested, with one part placed in 10% formalin for

H&E staining and immunohistochemical analysis, and the other part snap-frozen in liquid nitrogen for qRT-PCR and western blot analysis.

Network pharmacology analysis

Using TCMSP, BATMAN-TCM (http://bionet.ncpsb.org/batman-tcm/index.php/) and CNKI databases, and combining with manual literature mining, the main chemical compositions of WLTd were collected. The quality of the data analysis and processing was improved by taking oral bioavailability (OB) ≥30% and drug-likeness (DL) ≥0.18 as filter conditions for active compounds. In the BATMAN-TCM database, the active compounds were screened and extracted by setting a score cutoff of 20 and adjusted the *P*-value cutoff to 0.05. "Hypertension" was used as a keyword in the search for targets associated with disease in the DisGeNET database (http://www.disgenet.org/) and COREMINE database (https://www.coremine.com/medical/). By matching the target of the active ingredient and the target of hypertension, the potential targets of WLTd for the treatment of hypertension were obtained. The gene expression data derived from potential targets were used to generate core analyses and obtain information of disease, functions and pathways by Ingenuity Pathway Analysis (IPA, http://www.ingenuity.com). Furthermore, canonical pathways, diseases, and functions analysis were performed to identify significantly relevant pathways and functions, which were considered for further analysis [20].

HPLC and UPLC–MS/MS for component analysis

Standard solutions were prepared by dissolving an appropriate amount of the compounds in methanol and filtering through a 0.20 µm membrane. For sample solutions, 0.2 g of WLTd sample was accurately weighed and added to 10 ml methanol for ultrasonic treatment for 30 min, then filtered through a 0.20 µm membrane. Determination of methanol-soluble active components in WLTd was carried out using a Thermo U-3000 series HPLC system equipped with a Lpg-3400SD pump, a WPS-3000 automatic sampler, a Hypersil ODS2 column, and a photodiode array detector (or diode array detector). Chromatographic separation was achieved on an Elite reversed-phase C18 column (4.5 × 250 mm, 5 µm) maintained at 30 °C. Acetonitrile (solvent A) and water (solvent B) served as the mobile phases. The gradient programme is as follows: 0–20 min, 0–10% A; 20–50 min, 10–20% A; 50–70 min, 20–35% A; 70–85 min, 35–45% A. The detection wavelength was set at 200 nm.

UPLC-MS/MS analysis was performed with an electrospray ionization (ESI) source. The analysis was conducted using an analytical C18 column (100 mm × 2.1 mm, 1.7 µm). Mobile phase A consisted of 0.05% formic acid in H2O, and mobile phase B consisted of 0.05% methanol formate; the solvent gradient varied from 10% B in the first 2 min to 90% B over 10 min at a flow rate of 0.4 ml/min; the column oven temperature was 40 °C. The acquisition of mass spectra was conducted in positive and negative ion modes at the dry gas flow rate of 15.0 L/min and the gas temperature of 250 °C.

Molecular docking

Small molecule ligand and protein data were downloaded from PubChem (https://pubchem.ncbi.nlm.nih.gov/) and PDB (https://www.rcsb.org/) databases. POCASA 1.1 was used to predict protein binding sites. AutoDock Vina 1.1.2 was used for docking and PyMOL 2.3.0 was used to analyze the interaction mode of docking results [21, 22].

Measurement of rat blood pressure

Before and after the initiation of the drug treatments, noninvasive blood pressure (BP) was measured at the rat caudal artery using an 8-channel CODA acquisition system (Kent Scientific Corporation, Connecticut, USA) as we previously reported [5, 6, 23]. In brief, the rats were first put into size and shape-adjusted insulation sleeves of the BP system, and the sphygmomanometer was preheated for 5–10 min at 40 °C, and then the rat pressure sensor was inserted from the tip to the 2-cm mark of the rat tail to measure the value of the caudal artery BP. Systolic, diastolic, and mean BP, as well as heart rate, were recorded.

Determination of CRE and BUN levels

CRE and BUN levels were measured using commercially available assay kits (JianCheng Biomedical, Nanjing, China) according to the manufacturer's instructions, as we described previously [5, 6].

Hematoxylin and eosin staining

Renal specimens from each animal were fixed in 10% formalin for more than 48 h, paraffin-embedded and sectioned at 5 µm thickness using a manual slicer (HM355S, Thermo, Ltd, United States), deparaffinized and rehydrated [24], then placed into an automatic staining machine (Gemini, Thermo, Ltd, USA) for staining. After the sections were mounted using an automatic sealing machine (ClearVue, Thermo, Ltd, USA), myocardial tissues for pathological changes were observed using an optical microscope (DP71, OLYMPUS, Japan).

Masson staining

A Masson's Trichrome Stain Kit (Solarbio, China, Catalog No. G1340) was used to detect collagen deposition in the kidney. The paraffin-embedded kidney was sectioned to a 5 µm thickness. The renal pathological changes (collagen-stained blue, nuclei-stained dark brown, and muscle fibers stained red) were detected by morphological analysis under an optical microscope (DP71, OLYMPUS, Japan).

Immunohistochemistry

Paraffin sections of kidney tissues (5 µm thick) were deparaffinized, incubated in distilled water, endogenous peroxidase blocked by incubation with 3% H2O2 for 10 min in a humidified box, rinsed (3 × 5 min each) in PBS, and then antigen retrieval in a microwave oven. Following washes in PBS (3 × 5 min each), the sections were incubated for 1 h in a blocking buffer (10% bovine serum albumin). The first antibody AhR (1:175) was added and incubated at 4 °C overnight or at 37 °C for 1 h, and then washed in PBS (3 × 5 min each). Then, the second antibody (Biotinylated Goat anti-rabbit IgG) was added and incubated at 37 °C for 40 min. After cleaning with PBS (3 × 5 min), the image was developed using DAB color developer for 4 min, followed by redyeing with hematoxylin for 40 s, differentiation with 1% hydrochloric acid alcohol, bluish

regurgitating in alkaline water, then subjected to dehydration in the reverse order of 70, 80, 95, and 100% alcohols and finally cleared in xylene. At last, the slide was sealed using an automatic sealing machine. The protein expression in the tissue was observed and captured using an optical microscope (DP71, OLYMPUS, Japan) and the stained area was quantified using Image-Pro Plus 6.0 software (National Institutes of Health, Bethesda, MD, USA) [25].

RNA isolation and quantitative real-time PCR analysis

RNA was isolated from kidney tissues using the RNeasy Mini Kit (QIAGEN, Valencia, CA), according to the manufacturer's instructions. RNA concentrations were measured using an ultraviolet spectrophotometer and denaturing gel electrophoresis. All raw RNA samples were purified, and then the concentration was adjusted to 50 ng/μL [26], and stored at −80 °C for future use. The cDNA was synthesized using the Verso cDNA Synthesis kit (Thermo Fisher, USA), and real-time PCR was performed on a CFX96 (Bio-Rad, CA, USA).

mRNA levels of the target genes AhR, IL-1β and RelA were quantified by real-time quantitative polymerase chain reaction (RT-qPCR) using SYBR Premix Ex Taq (Takara, Dalian, China) [27]. For all reactions, a standard amplification programme was used (2 min for a single cycle of 95 °C, 44 cycles of 95 °C for 30 s, 60 °C for 40 s and 72 °C for 40 s, 1 cycle of 95 °C for 10 s). The data were calculated by melting curve and "Threshold Cycle" (Ct) using CFX Manager software. GAPDH was used as an internal control, and results were determined by $2^{-\Delta\Delta Ct}$, expressed as the fold change over the sham group. The primers are listed in Table 1 and were synthesized by Sangon Biotechnology (Shanghai, China).

Cell culture and treatment

Human embryonic kidney cells, HEK293, were purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). Cells were cultured in DMEM containing 4.5 g/L glucose, supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. All the cultured cells were maintained in a humidified incubator with 95% air and 5% CO2 at 37 °C. HEK293 cells were exposed to Ang II (100 nM) for 24 h, then treated with WLTd for 24 h.

Protein extraction and Western blot analysis

Kidney tissues were collected from rats with different treatments, and total proteins were extracted using lysis buffer. Protein concentrations were measured by a Solarbio bicinchoninic acid assay (BCA) kit [28] and adjusted to 5 μg/μL. Protein samples were subjected to SDS-PAGE and then electrotransferred to polyvinylidene difluoride membranes (Millipore, USA). After blocking in skim milk, the membranes were incubated with primary antibodies: anti-AhR (1:500, Immunoway, Beijing, China) and anti-GAPDH (1:3000, Immunoway, Beijing, China), at 4 °C overnight. Horseradish peroxidase (HRP) AffiniPure Goat Anti-Rabbit IgG (Beyotime, Shanghai, China) was added at a 1:10,000 dilution for 2 h, followed by washes in TBST (3 × 15 min each). Blot reactions were developed using an enhanced chemiluminescence solution and quantified using Image-Pro Plus 6.0 software (National Institutes of Health, Bethesda, MD, USA).

Table 1. Primers for real-time PCR.

Primer	Sequence(5′–3′)
IL-1B	Forward:CCAGGATGAGGACCCAAGCA
	Reverse:TCCCGACCATTGCTGTTTCC
RELA	Forward:GCCTCATCCACATGACTTG
	Reverse:TTACTCGGCAGATCTTGAGC
GAPDH	Forward:GGCCTTCCGTGTTCCTACC
	Reverse:CGCCTGCTTCACCACCTTC

Immunofluorescence

After treating with different concentrations of WLTd for 24 h, the cells in a 96-well plate were fixed with hexanol at room temperature for 30 min, treated with blocking solution (5% bovine serum albumin in phosphate-buffered saline) for 30 min, incubated with the primary antibody AhR (1:300) and refrigerated at 4 °C overnight. The next day, after washing with PBST, the cells were incubated with an Alexa Fluor 488 dye-labelled secondary anti-IgG antibody (1:1000) and Hoechst (1:300) for 2 hours, and then quantitatively analyzed by a high-content fluorescence imaging system (Operetta, PerkinElmer, MA, USA).

Statistical analysis

All the results were expressed as means ± SEM. Statistical calculations were performed using GraphPad Prism 8.0.1 software. To determine the statistical significance, a one-way analysis of variance followed by Tukey's multiple comparison test was performed. The Kruskal–Wallis test was applied to compare the categorical data. Statistically significant differences between groups were defined as *P*-values less than 0.05.

Results

Establishment of the molecular pharmacological network of WLTd

A flow chart of the network pharmacology approach used in the study is shown in Figure 1(A). From relevant herbal and natural product databases, a total of 136 WLTd compounds were collected, including 32 flavonoids, 21 terpenoids, 18 alkaloids, 17 aliphatics, 13 quinonoids, 12 phenolic acids, 11 steroids, 8 glycosides, 2 phenylpropanoids, and 2 coumarins (Figure 1(B)). Among 5,821 hypertension-related molecular targets, 247 were found to interact with one or more WLTd active ingredients (Figure 1(C)). Ranking the pathways of WLTd targets placed the aryl hydrocarbon receptor (AhR) signaling as the most involved pathway (Figure 2). The main active compounds potentially involved in the AhR receptor signaling pathway include quercetin, luteolin, kaempferol, and oxymatrine, while their action targets include AhR, RELA, CYP1A1, IL6, IL1B, JUN, TP53, and CYP1A2 in the AhR signaling pathway (Figures 3 and 4).

Identification of bioactive components in WLTd

The presence of the phytochemical components collected from herbal databases in a WLTd preparation was identified by HPLC and UPLC–MS/MS (Figure 5). At signal acquisition, set at an absorption wavelength of 200 nm, a HPLC profile of WLTd demonstrated multiple peaks (Figure 5(A)). Five compounds were identified (Figure 5(C)) and quantitatively analyzed (Figure 5(D)) by comparing with standard substances, namely vitexin, quercetin, kaempferol, toosendanin, and ursolic acid. The ion chromatogram (Figure 5(B)), the

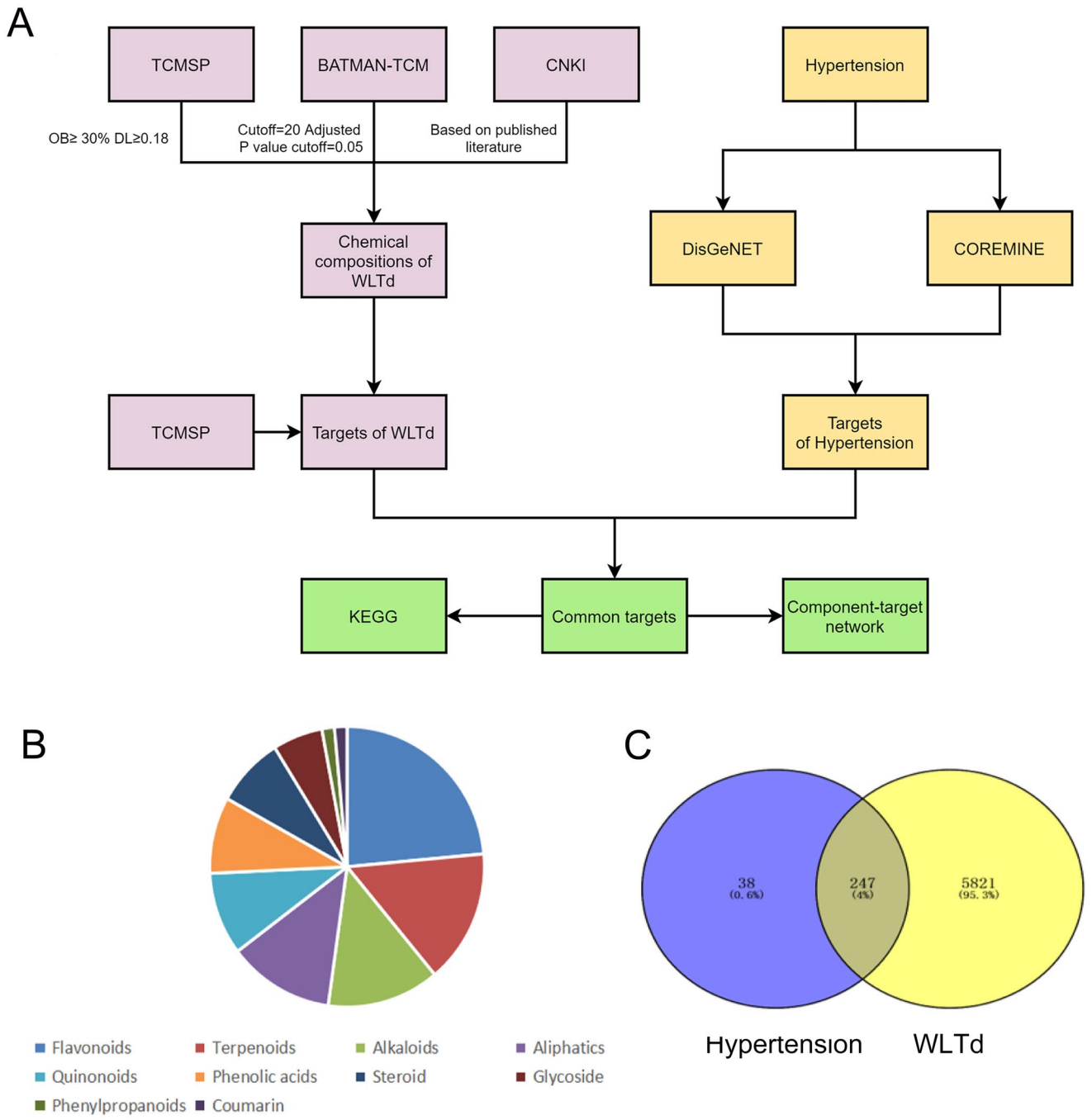


Figure 1. Network pharmacology analysis of WLTd in the treatment of hypertension. (A) Pictorial form of the network pharmacology approach used in the study. (B) The classification of the WLTd active ingredients for in silico analysis. Numbers are the compounds in the class. (C) Venn diagram showing the hypertension targets of WLTd active components.

structure (Figure 5(C)), and the content of the compounds (Figure 5(E)) are shown. Four chromatographic peaks were identified, which were matrine, oxymatrine, gardenoside, and quercetin. The top eight compounds (vitexin, quercetin, kaempferol, toosendanin, ursolic acid, matrine, oxymatrine, and gadenoside) were subjected to molecular docking analysis to investigate their binding potential to AhR. The binding energy ranged from −6.3 kcal/mol (kaempferol), to −7.9 kcal/mol (oxymatrine). Compared to the AhR agonist 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) (−4.9 ± 1.5 kcal/mol) [29], all the key compounds within the network exhibited robust binding affinity toward the AhR protein, as evidenced by the results obtained (Figure 6).

WLTd lowered blood pressure in SHR

The effect of WLTd on BP was first evaluated in SHR and age-/gender-matched normotensive WKY rats, using Losartan (LST) as a positive control drug [23]. As expected, Losartan had a significant antihypertensive effect (Figure 7). Similar to Losartan, a moderate dose of WLTd (1.8 g/kg) also significantly reduced the diastolic, systolic, and mean BP compared to those of saline controls (Figure 7(A–C)). As shown in Figure 7(D), quantification of the mean BP 25 days after drug administration showed that WLTd was effective in reducing blood pressure (135 ± 13.37 mmHg) compared to that of the SHR (159.16 ± 13.91 mmHg).

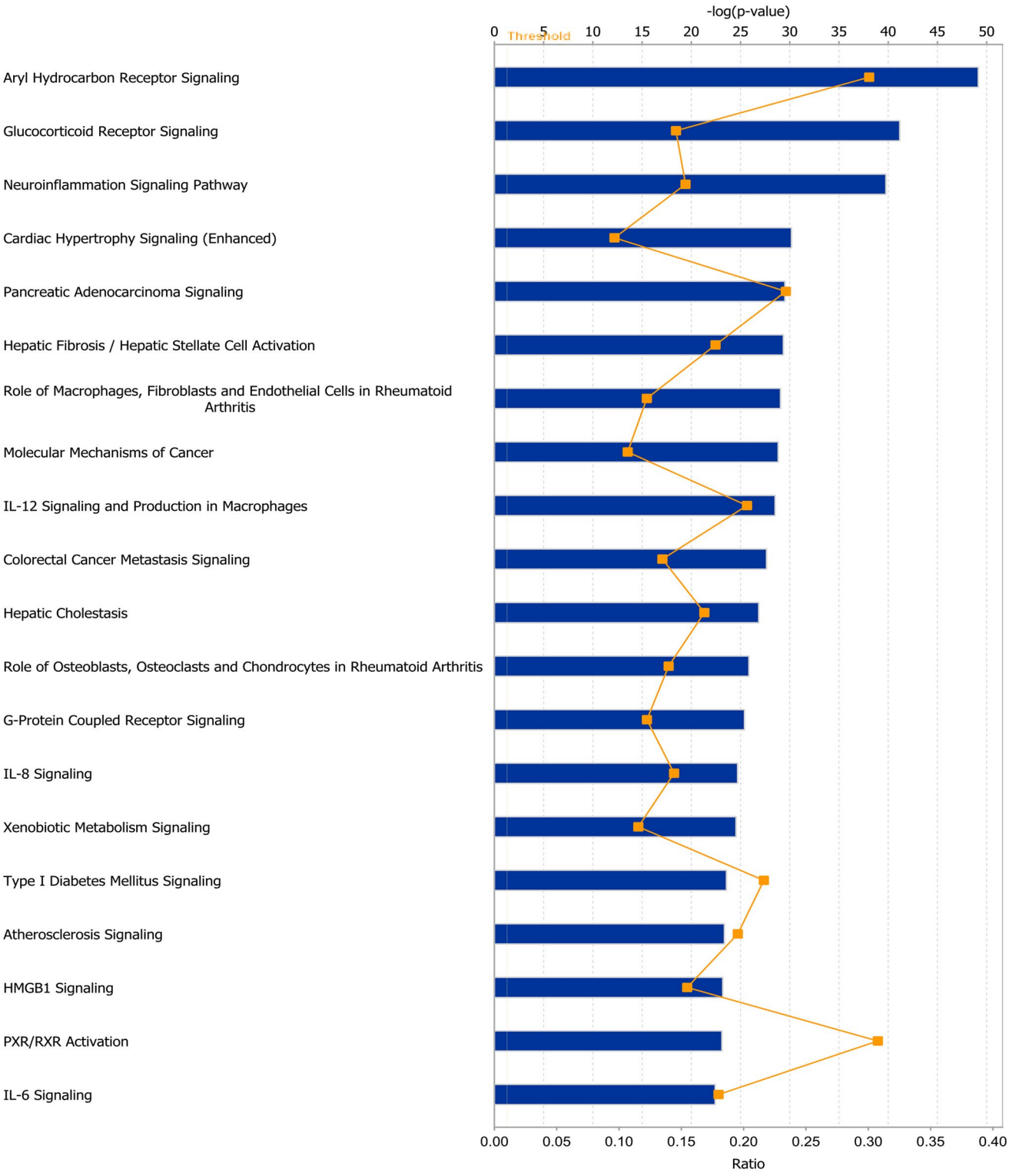


Figure 2. KEGG Pathway analysis. Top 19 pathways potentially regulated by WLTd, ranked in an order from highest to lowest by –log(p-value), revealed by IPA core analysis, aryl hydrocarbon receptor (red box) ranked number one on the list.

Effect of WLTd on hypertension-induced renal injury in SHR

At the organ level, H&E staining revealed histopathological changes in the renal cortex in different groups. Compared with those of WKY controls, SHR kidneys showed obvious dilated renal tubules and ischemic changes, such as glomerular shrinkage and thickening of the basement membrane (Figure 8(A), blue arrows). Twenty-five days of WLTd treatment significantly ameliorated renal injury and improved the histoarchitecture of the kidneys as shown by reduction in tubular dilation, cyst formation, and glomerular damage compared to Model group.

Masson's staining showed that the degree of renal interstitial fibrosis in the SHR model group was significantly increased (Figure 8(A),

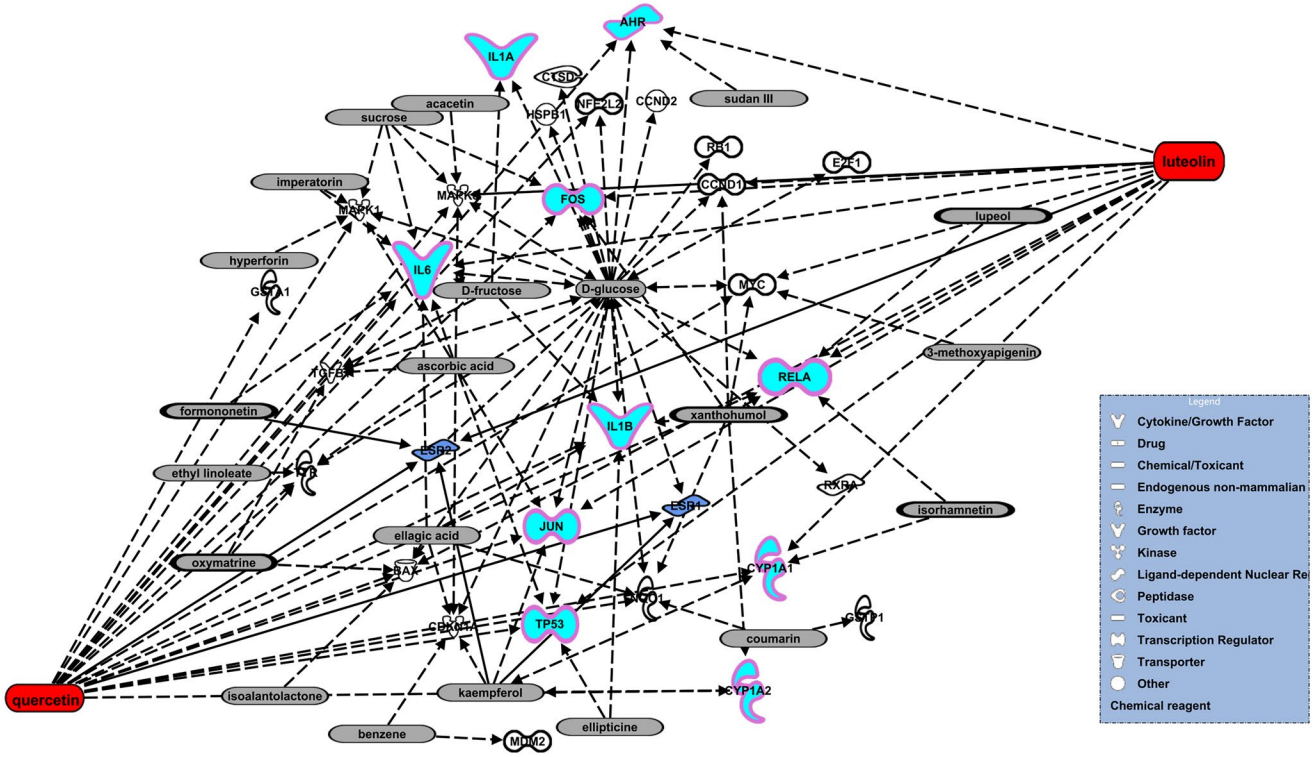


Figure 3. Compound-target network. Interaction between WLTd active components and related targets in the aryl hydrocarbon receptor signaling pathway. Green: WLTd active components; red: interacting protein targets. Some of the components are multi-targeting, as indicated by multiple dotted lines.

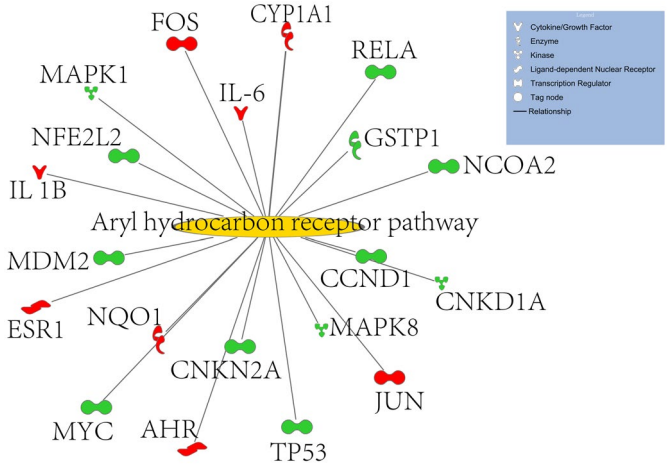


Figure 4. AhR Receptor signaling pathway and its action targets. AhR receptor signaling pathway and its action targets include AhR, RELA, CYP1A1, IL6, IL1B, JUN, TP53, and CYP1A2 in the AhR signaling pathway.

yellow arrows) compared with the WKY control group. This pathological abnormality was reversed by the WLTd treatment (Figure 8(B)).

CRE and BUN are two important indices in the endogenous system, which can be used to evaluate renal damage indirectly by reflecting the filtration ability of the glomerulus[30]. Compared with that of saline-treated SHR, the CRE levels in LST- and WLTd-treated SHR urines were significantly decreased (1.78 ± 0.41 nmol/L for the model group and 0.67 ± 0.34 nmol/L for the WLTd group, Figure 8(C)). Similarly, the BUN level in the WLTd-treated SHR urine was significantly decreased, while losartan-treated SHR urine showed no significant change (391.55 ± 59.96 mmol/L for the model group and 240.88 ± 51.15 mmol/L for the WLTd group, Figure 8(D)), indicating that WLTd was effective in reducing CRE and BUN in SHR.

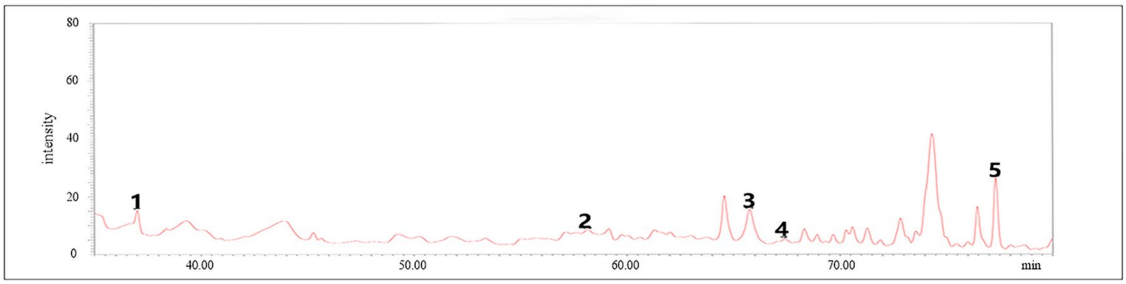
WLTd protected rat from hypertension-induced kidney injury via the AhR-dependent pathway

Immunohistochemical (IHC) analysis showed that the expression of the AhR receptor was reduced in SHR compared to WKY rats (Figure 9(A)). WLTd treatment significantly restored AhR expression in SHR. Moreover, western blot results also revealed a reduction in the expression of the AhR protein in SHR, which was restored by WLTd treatment (Figure 9(B)). The downstream genes of the AhR signaling pathway, IL1B and RelA (Figure 9(D)), were also examined by RT-qPCR. Compared to that of WKY rats, the expression of IL1B and Rela mRNAs was increased in SHR. In contrast, WLTd treatment for 25 days markedly reduced their expressions in SHR (Figure 9(C)).

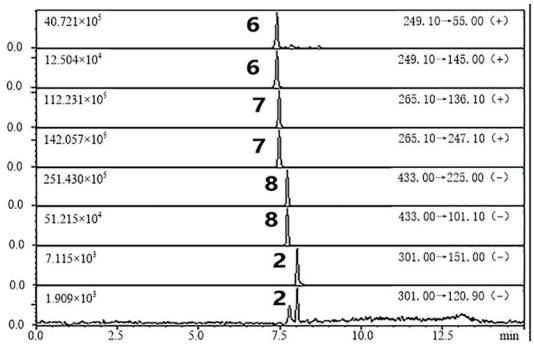
WLTd restored AhR expression in AngII-treated HEK293 renal cells

To determine the effects of WLTd on renal cell expression of AhR *in vitro*, HEK293 cells were treated with Ang II with or without testing drugs. The Immunofluorescence (IF) analysis showed that, compared with the control, AhR was significantly decreased by Ang II in HEK293 cells. As expected, Losartan (1 μmol/L) restored AhR expression. Similarly, WLTd at both low (0.0104 mg/mL) and medium (0.0415 mg/mL) doses also increased AhR expression in Ang II-treated cells (Figure 10(A and B)).

A



B



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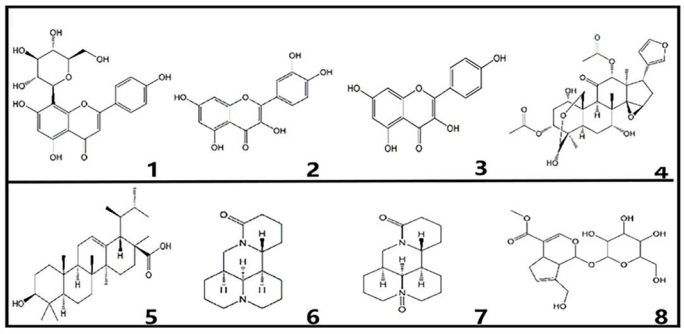

D

	Compounds	CAS	Rt_S/Rt_{TDC}	A_S/A_{TDC}	C(mg/g)
1	vitexin	3681-93-4	36.833/37.010	5601732/146422	0.13
2	quercetin	117-39-5	58.569/58.160	10307923/25972	0.01
3	kaempferol	520-18-3	65.566/65.770	9336635/331668	0.18
4	toosendanin	58812-37-6	67.839/67.404	458328/26551	0.29
5	ursolic acid	77-52-1	77.469/77.322	532788/347887	3.26

E

	Compounds	CAS	Ion pair	Content(mean±SD;μg/g)	RSD(%)
6	matrine	519-02-8	249.10/55.00* 249.10/148.00	173.68±1.53	1.25
7	oxymatrine	16837-52-8	265.10/247.10* 265.10/136.10	1035.45±9.69	1.32
8	gadenoside	24512-62-7	433.00/225.00* 433.00/101.10	5274.31±103.85	0.17
2	quercetin	482-35-9	301.00/151.00* 301.00/120.90	137.80±8.11	1.10

Figure 5. Identification of major chemical components in WLTd. (A) HPLC profile of methanol-soluble components of WLTd. The acetonitrile-deionized aqueous solution was selected as the mobile phase. (B) LC–MS/MS chromatograms of matrine (6), oxymatrine (7), gardenoside (8) and quercetin (2). Top and bottom panel: standard. (C) Chemical structures of the 8 identified components in WLTd. (D) Retention time and quantitation of the identified compounds in WLTd. RtS and RtWLTd represent the retention time of the standard component and WLTd component respectively. AS and AWLTd represent the peak area of the standard component and WLTd component respectively. C (mg/g): concentration in mg per g of total input. (E) Quantitation of the four compounds in WLTd. C (μg/g): concentration in μg per g of total input.

Discussion

In this study, we identified the main chemical ingredients of Wu-Lan thirteen-flavour decoction and applied *in vivo*, *in vitro*, and network pharmacology techniques to demonstrate that WLTd lowers blood pressure and reduces hypertension-induced renal damage in SHR. Mechanistically, WLTd regulates the aryl hydrocarbon receptor (AhR) signaling pathway in SHR kidneys and human embryonic kidney cell lines to reduce renal injury.

Mongolian medicine is an important part of traditional medicine in China. With a long history of more than 2,800 years, it absorbs and draws on the essence of the theories of Indian medicine, Tibetan medicine, and traditional Chinese medicine. Through the long-term medical practice of the Mongolian people, it has gradually formed ethnic medicine with a unique theoretical system, as well as national, regional, and clinical characteristics. Compound Mongolian medicines are the main clinical application forms of Mongolian medicine in the treatment of diseases.

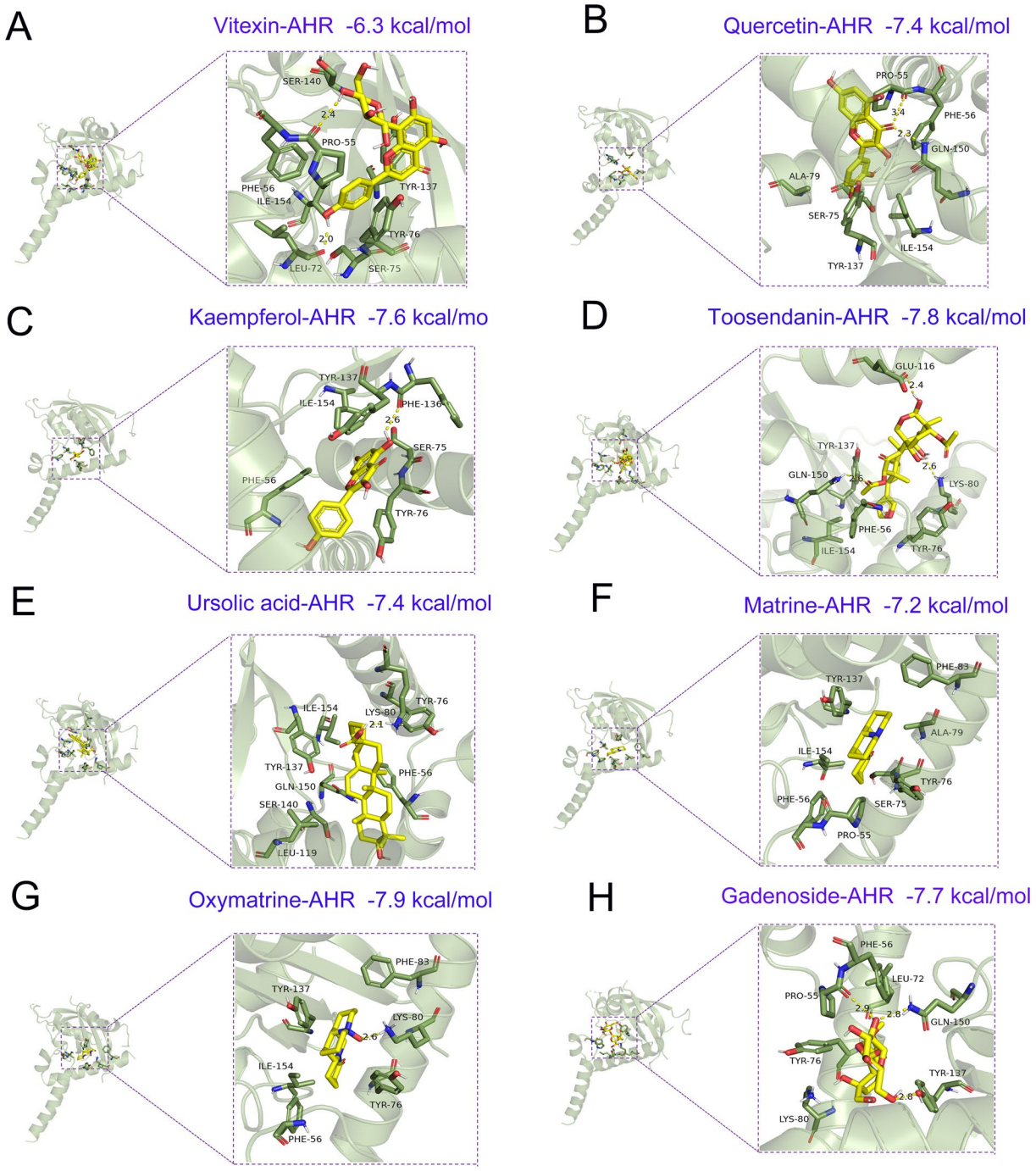

Figure 6. The top eight compounds (A) vitexin, (B) quercetin, (C) kaempferol, (D) toosendanin, (E) ursolic acid, (F) matrine, (G) oxymatrine, and (H) gardenoside were subjected to molecular docking analysis to investigate their potential regulatory role on AhR.

The compound Mongolian medicine Wu-Lan thirteen-flavour decoction (WLTd) has been used clinically in the treatment of hypertension in China [5, 6]. Studies have shown that Gardeniae Fructus, a fruit of Gardenia jasminoides Ellis, reduces inflammation and headaches, and treats hepatic disorders, hypertension, and icterus [31]. Oxymatrine in Sophora flavescens prevents ventricular remodeling in SHR [32]. Eriobotrya japonica ameliorates cardiac hypertrophy in H9c2 cardiomyoblasts and in spontaneously hypertensive rats [33]. At the same time, it may have a cardioprotective effect against apoptosis and fibrosis in SHR [34]. Nasturtium has long been used in Iranian folk medicine to treat hypertension, hyperglycemia, and renal colic [35].

Within Mongolian medicine, a diverse array of flavonoids is present. Emerging evidence underscores the myriad pharmacological activities inherent in flavonoids, encompassing antithrombotic, hypolidaemic, and anti-inflammatory properties. The clinical significance of these compounds is notably high [36, 37]. Our quantitative study results showed higher contents of ursolic acid, oxymatrine, and gypenoside in WLTd. However, due to the limitations of instrument sensitivity and the lack of suitable reference substances, many components remain to be identified. Therefore, this initial experiment cannot fully define the chemical composition of WLTd. Nevertheless, many of the eight compounds identified in our chemical analysis have been shown to have

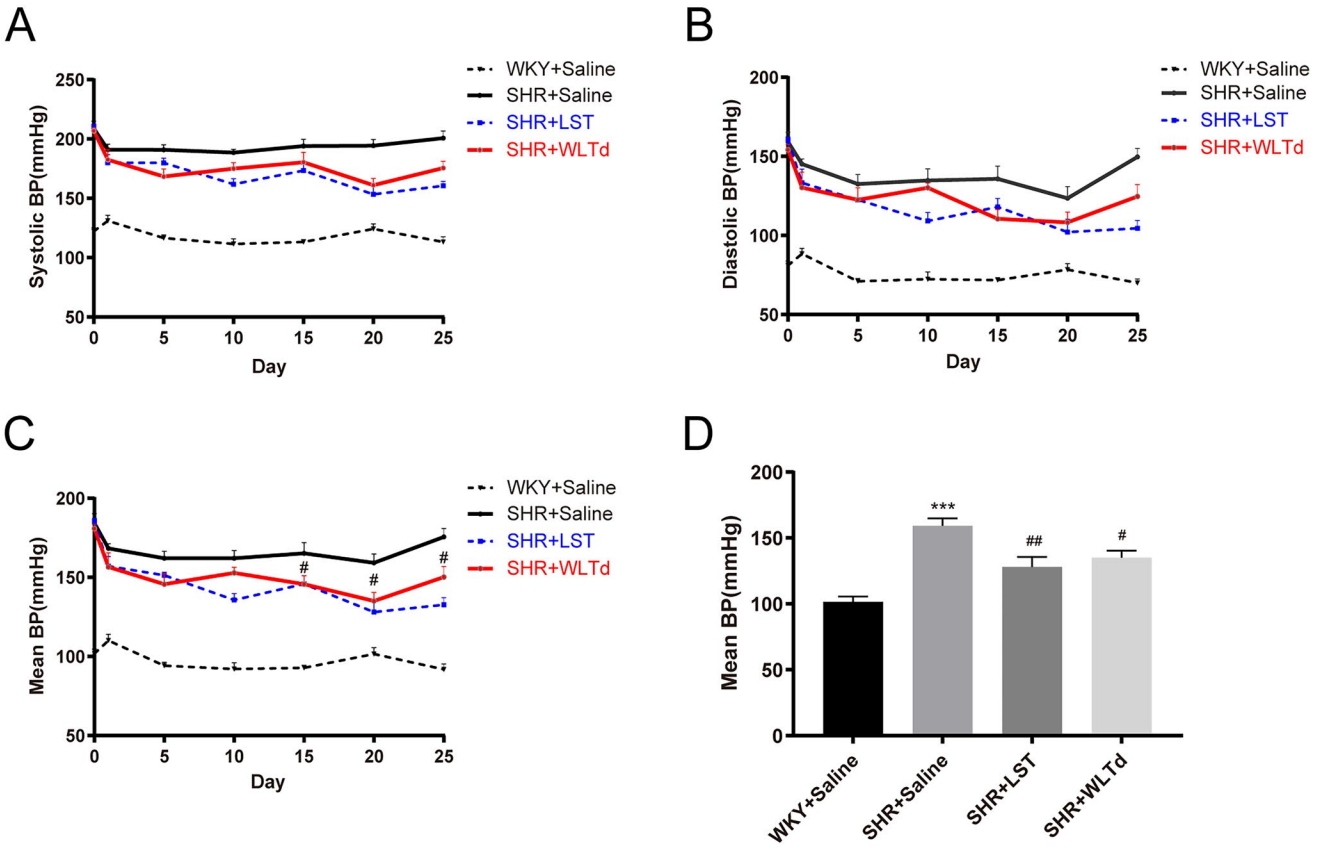

Figure 7. Effects of WLTd on noninvasive blood pressure in SHR. (A) Recording of systolic BP of different treatment groups over 25 days. (B) Recording of diastolic BP of different treatment groups over 25 days. (C) Recording of MBP of different treatment groups in 25 days. (D) Summary plot of the MBP on day 25. Data are expressed as mean ± SEM, $n = 5–6$. $^{****}p < 0.0001$ vs WKY rats; $^{\#}p < 0.05$, $^{\#\#\#\#}p < 0.0001$ vs SHR (saline).

antihypertensive activities, to improve right Ventricular remodeling, and to protect against renal damage caused by hypertension in various animal models, including vitexin [38, 39], ursolic acid [40, 41], matrine [42], oxymatrine [43, 44], kaempferol, and quercetin [45, 46]. This study reveals that the antihypertensive and target organ protective effects of WLTd could be the result of any single compound, other unknown compounds, or a combination of them. Therefore, future research is needed to provide direct evidence of these possibilities.

AhR, when activated by various endogenous and exogenous ligands, can upregulate cytochrome P450 enzymes (e.g. CYP1A1, CYP1B1). These enzymes increase the production of reactive oxygen species (ROS), contributing to oxidative stress within renal cells. Oxidative stress exacerbates hypertension-induced renal injury by damaging the endothelial cells of renal vasculature, which can lead to endothelial dysfunction, increased vascular resistance, and fibrosis. [47]. AhR ligands come from a wide range of sources, including endogenous ligands (e.g. a series of structurally different compounds produced by host metabolism) and exogenous ligands (e.g. environment, microorganisms, natural products, etc) [48, 49]. Previous reports showed that flavonoids from natural products constitute the largest category of AhR ligands [50, 51]. Flavonoids, such as kaempferol and luteolin, are mostly AhR antagonists, but some of them, including baicalein and quercetin, are AhR agonists [52, 53]. Quercetin and kaempferol are known AhR ligands that can have dual roles. While they activate AhR, they also exert antioxidant effects by directly scavenging free radicals and upregulating antioxidant defence enzymes like superoxide dismutase (SOD) and catalase. This dual action is particularly beneficial in hypertensive conditions where oxidative stress significantly contributes to renal injury [52, 53]. The effects of the flavonoids are cell-type and cell-context dependent [18]. Flavonoids such as quercetin, kaempferol, and luteolin interfere with membrane lipid peroxidation by binding to membrane lipid bilayers, thus improving renal tubular cell injury induced by cold storage and playing a protective role in the kidney [54]. Numerous studies have shown that the AhR pathway is associated with multiple physiological functions and disease processes, such as regulating oxidative stress and inflammatory responses [55–57]. AhR has a diverse ligand-binding site, activated by compounds from the environment, microbiome, natural products, and host metabolism [58]. Recent studies suggest that AhR is also activated by endogenous ligands, regulating gene expression and contributing to physiological and pathological processes such as cell proliferation, differentiation, apoptosis, adhesion, and migration. AhR's involvement in cardiovascular disease (CVD), chronic kidney disease (CKD), and renal cell carcinoma (RCC) has been explored in studies by Zhao et al. [59, 60]. AhR activity in patients with CKD, CVD, diabetic nephropathy, and RCC has the potential to serve as a diagnostic and prognostic approach for renal damage. Since natural products have been identified as AhR agonists or antagonists, understanding the diverse structures and biological activities of polyphenols may lead to the discovery of potential therapeutic strategies targeting AhR activation [61, 62]. Our network pharmacological analysis suggested that kaempferol and quercetin may directly act on AhR, IL6, IL1B, RELA, and CYP1A to protect rats from hypertension-induced kidney injury. The results further highlight flavonoids from natural products as AhR agonists or

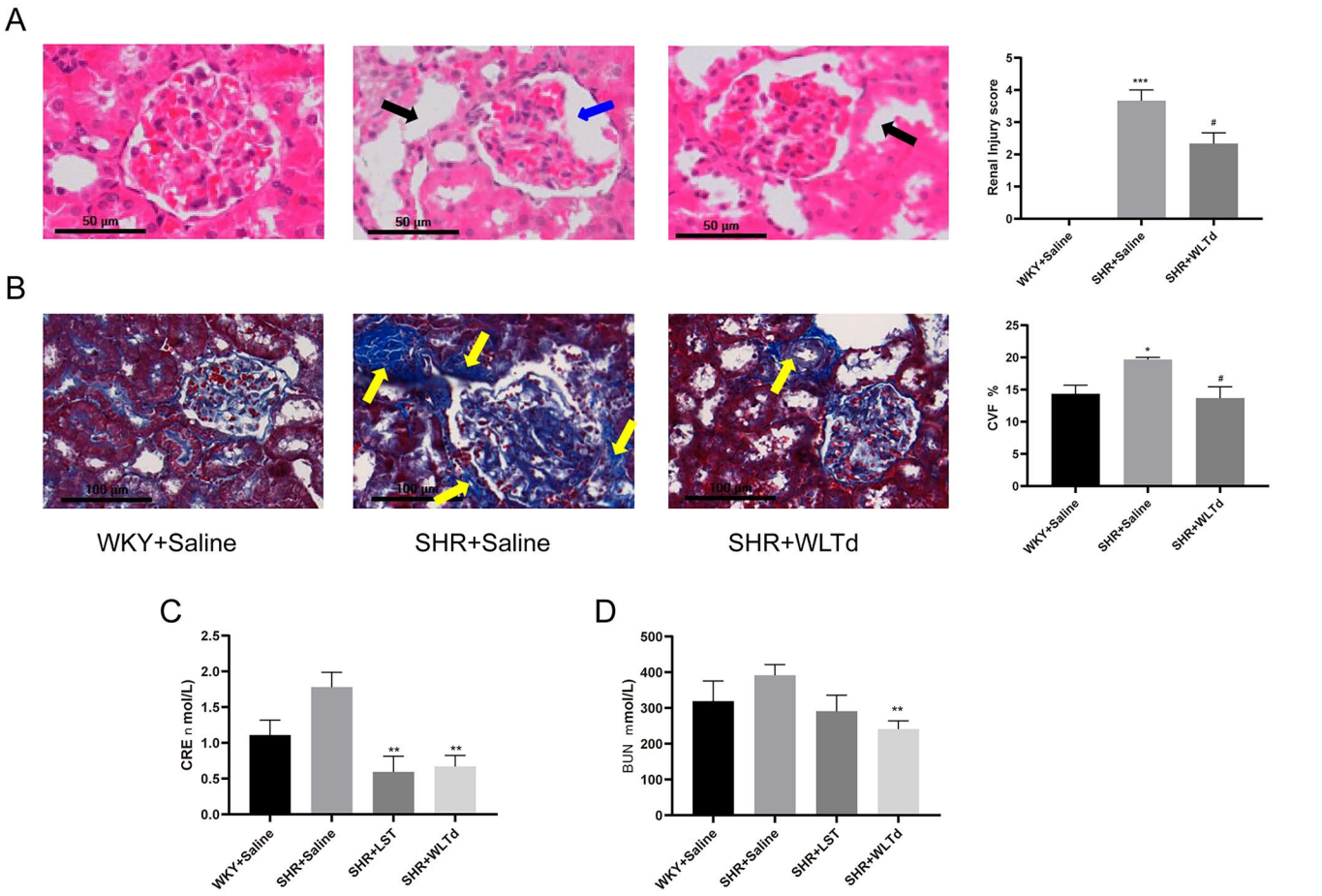

Figure 8. Effect of WLTd on hypertension-induced renal injury in SHR. (A) Representative H&E staining photomicrographs of histopathological changes in renal cortex among different rat groups (200 × magnification). Saline-treated SHR showing glomerular shrinkage and thickening of the basement membrane (blue arrows). Left to right: WKY+saline, SHR+saline, SHR + WLTd, and bar graph summary. Data are expressed as mean ± SEM, $n = 3$. ***$p < 0.001$ vs WKY rats; #$p < 0.05$ vs SHR (saline). (B) Representative masson staining photomicrographs of histopathological changes in renal cortex among different groups (100 × magnification). Saline-treated SHR showing a significant increase in collagen fibers (yellow arrows). Left to right: WKY+saline, SHR+saline, SHR + WLTd, and bar graph summary. Data are expressed as mean ± SEM, $n = 3$. *$p < 0.05$ vs WKY rats; #$p < 0.05$ vs. SHR (saline). (C) Bar graph comparison of the levels of CRE in different rat groups (D) Bar graph comparison of the levels of BUN in different rat groups. Data are expressed as mean ± SEM, $n = 4$–5. **$p < 0.01$ vs WKY rats; ##$p < 0.01$ vs. SHR (saline).

antagonists that regulate AhR activity. It is worth further verifying the roles of kaempferol and quercetin in WLTd and exploring their interactions with the AhR pathway and inflammatory responses.

The multi-target and multi-pathway characteristics of TMM suggest that WLTd components other than luteolin and quercetin may also act on other important targets and pathways to alleviate hypertension or hypertension-induced kidney injury. Natural products are a rich source of new potential drugs [63, 64]. To date, more than 15,000 flavonoids have been identified from natural products [65]. Continued exploration of the pharmacological effects of flavonoids may provide guidance and a reference for the clinical application of WLTd and contribute to a further understanding its pharmacological mechanisms. Although the recently reported AhR ligands have expanded greatly, many related studies on AhR are still rigorously challenging, and a better understanding of structurally diverse flavonoids and AhR biological activities would allow us to elucidate their molecular mechanisms and discover potential therapeutic strategies targeting AhR activation in the future.

Despite the promising translational and clinical application prospects of AhR, most of the current knowledge on its physiological and pathological functions has been uncovered using animal models, limiting the direct transfer of experimental findings to patients [66]. Subsequent research endeavors are poised to concentrate on translating animal experimental findings into clinically applicable insights. The exploration of AhR is anticipated to be significantly influenced by systems biology, incorporating genomics, transcriptomics, proteomics, metabolomics, and lipidomics. This integrative approach is poised to play a pivotal role in advancing the understanding of AhR.

Network pharmacology, an emerging field that combines biology, pharmacology, computational biology, and network analysis, enables comprehensive analysis of drug-drug interactions through the characteristics of multiple targets. This holistic and systematic approach aligns with the overall concept and dialectical treatment of Chinese medicine, making network pharmacology a valuable tool for screening potential active ingredients of Chinese medicine and predicting Chinese medicine-disease targets. Additionally, the use of network pharmacology can aid in the interpreting the mechanism of action of Chinese medicine, leading to new insights in the field of Chinese medicine research. These findings hold great promise for the advancement of Chinese medicine and the development of innovative therapeutic approaches. Using network pharmacology in this study, we reveal for the first time the various advantages of WLTd in protecting rats from hypertension-induced kidney injury. Moreover, WLTd may protect against renal injury caused by hypertension by up-regulating AhR and down-regulating inflammatory cytokines in the aryl hydrocarbon receptor signaling pathway. Overall, the study further

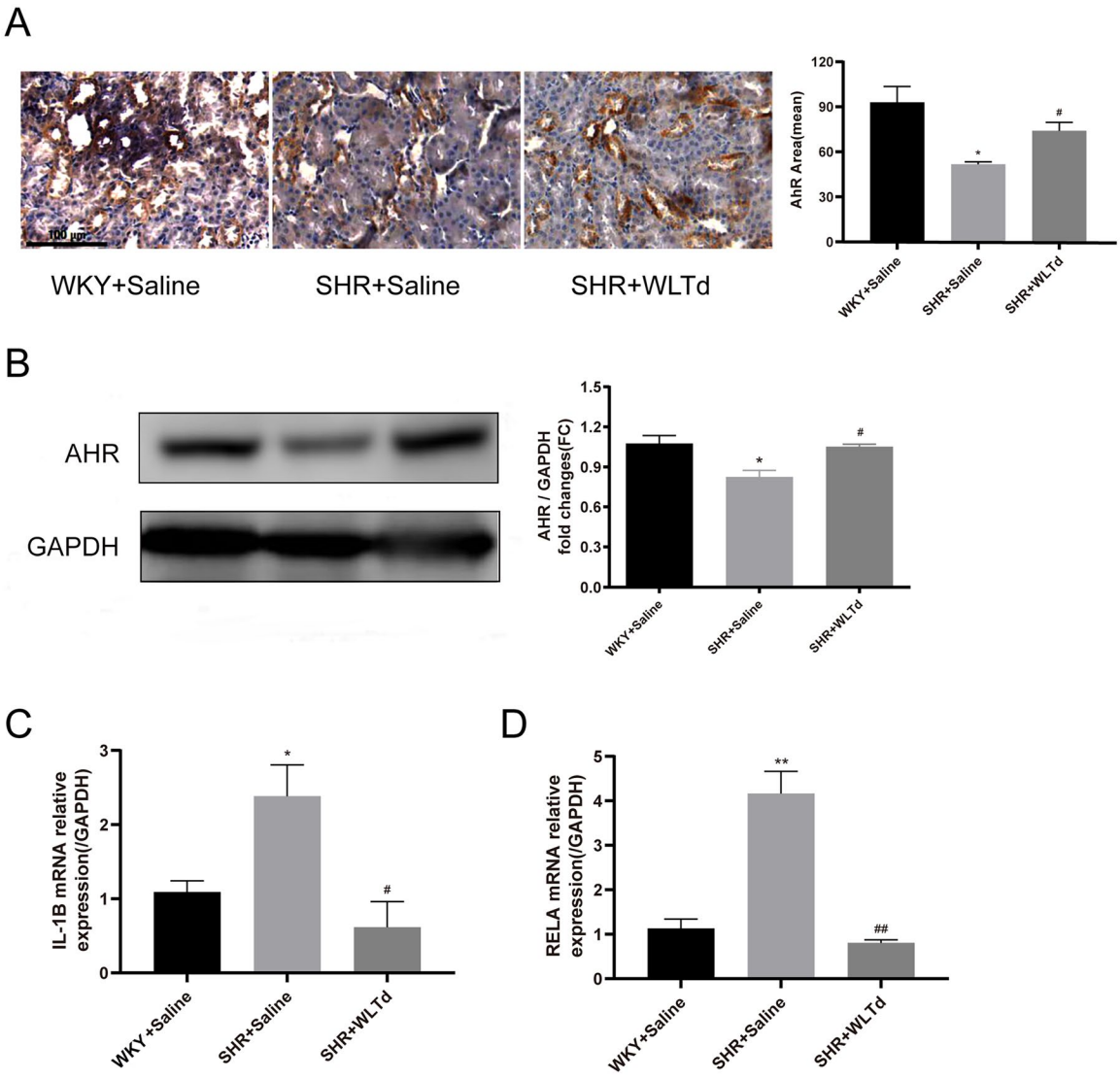

Figure 9. Effect of WLTd on the expression of AHR signaling pathway-related genes in rat kidney. (A) Immunohistochemical staining of AHR protein (in brown) in the kidney tissue section. Left to right: Representative images (100×magnification) of WKY+saline, SHR+saline, SHR+WLTd, and bar graph summary. Data are expressed as mean ± s.e.m., $n = 3$. $^{*}p < 0.05$ vs WKY rats; $^{\#}p < 0.05$ vs SHR. (B) Western blot analysis of AHR protein expression in kidney tissues. Left: Representative WB images of WKY+saline, SHR+saline, SHR+WLTd. Right: bar graph summary. Data are expressed as mean ± s.e.m., $n = 3$. (C) Real-time qPCR quantitation of mRNA expressions of IL-1B. (D) Real-time qPCR quantitation of mRNA expression of RELA. Data are normalized to that of GAPDH and expressed as mean ± SEM, $n = 3$. $^{*}p < 0.05$, $^{**}p < 0.01$ vs WKY rats; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ vs SHR.

elaborates on the material basis and mechanism of WLTd in the treatment of hypertension and hypertension-induced kidney injury. With the continued advancement of traditional Chinese medicine (TCM) and the incorporation of various omics technologies, coupled with advancements in computer technology and experimental methodologies, network pharmacology is poised to provide greater insight into the material basis and mechanisms of action. In the future, a more scientifically rigorous integration of network pharmacology technology with the study of "Chinese medicine theory," "Chinese medicine processing and preparation," and "Chinese medicine compounding" is essential to foster innovative research ideas and facilitate the development of artificial intelligence. Ultimately, this will enable a deeper understanding of the traditional therapeutic effects of Chinese medicine and leverage the unique advantages of TCM, warranting further exploration and contemplation.

The biological and chemical properties of herbal medicines depend heavily on the source of their raw materials. Variability can arise from differences in plant species, geographic location, and cultivation practices. Environmental factors, such as soil quality, climate, and harvest timing, can also affect the concentration of bioactive compounds. For instance, variations in sunlight and moisture levels during growth can lead to differences in the accumulation of flavonoids and other key phytochemicals. The variability in formulations highlights the need for rigorous clinical trials that assess not only the efficacy of herbal treatments but also the impact of different formulations and dosing regimens. Clinical trials are essential for integrating herbal medicine into mainstream healthcare. Further research is needed to explore the mechanisms by which variations in herbal formulations affect clinical outcomes. Investigating the relationships between specific bioactive compounds and their therapeutic effects can provide insights into optimizing herbal treatments for specific patient populations.

Moving forward, as a new drug development, we will continue to promote the use of best methods to address WLTd's future research, which are appropriate, sound, and efficient. (1) Conducting Clinical Trials: Future research should focus on well-designed clinical trials to evaluate the efficacy and safety of the identified herbal compounds in diverse patient populations.

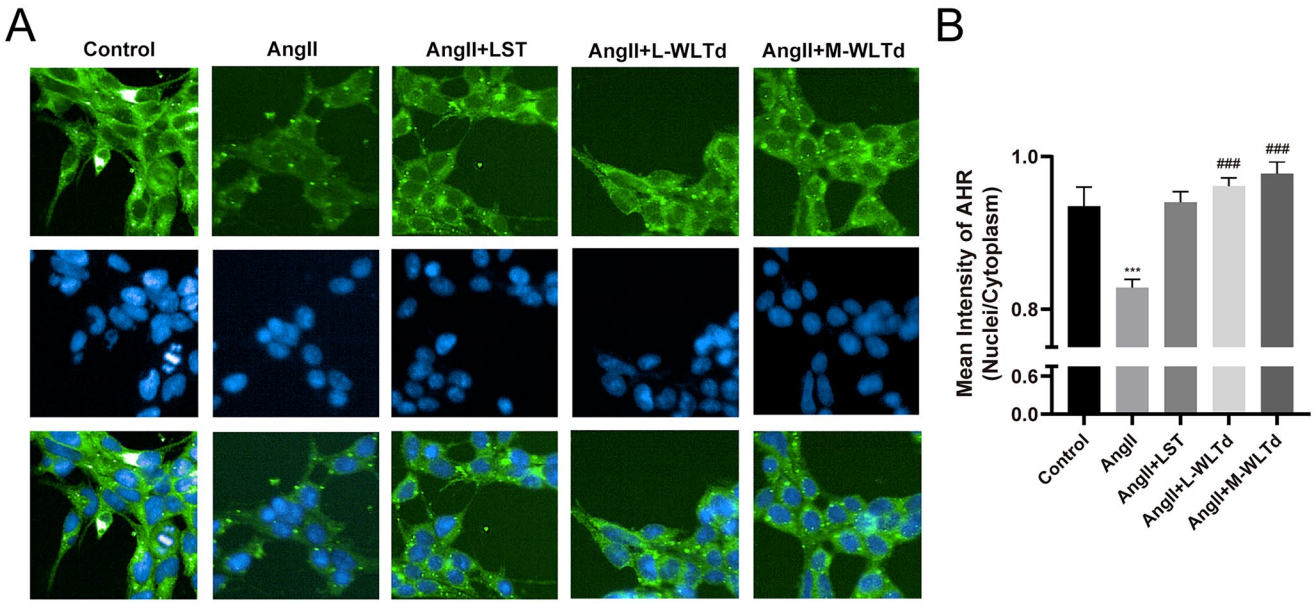

Figure 10. Effect of WLTd on AHR expression in angiotensin II-treated HEK293 cells. (A) Representative immunofluorescence staining images (200×magnification) of anti-AHR antibody (top panels, alexa fluor 488 in green), nucleus (Middle panels, hoechst in blue), and merge (bottom panels). LST: positive-control drug losartan (1 μmol/L); L-WLTd: low dose of WLTd (0.0104 mg/mL); M-WLTd: medium dose of WLTd (0.0415 mg/mL). (B) Quantitation of relative fluorescent intensities in nucleus vs cytoplasm. Data are expressed as mean ± s.e.m., $n = 3$. ***$p < 0.001$ vs control; ###$p < 0.001$ vs ang II-induced HEK293 cells.

These trials should aim to standardize dosages and formulations, taking into account the inherent variability in herbal medicines. (2) Exploring Additional Pathways: Further investigations could assess the interactions of these compounds with other relevant biological pathways, such as the NF-κB or MAPK pathways. This could involve *in vitro* studies using renal cell lines or *in vivo* models to better understand the mechanisms of action and potential synergistic effects. (3) Longitudinal Studies: Implementing longitudinal studies to track the long-term effects of herbal treatments on hypertension and renal health in human subjects would provide valuable insights into their therapeutic potential and sustainability. (4) Pharmacokinetic and Pharmacodynamic Studies: Conducting pharmacokinetic and pharmacodynamic studies would help elucidate the absorption, distribution, metabolism, and excretion of the herbal compounds in humans. Understanding these factors is crucial for optimizing dosing strategies and ensuring therapeutic efficacy. By detailing these concrete steps, I aim to provide a clearer direction for future research in this area. Thank you again for your valuable suggestions, which have helped strengthen the manuscript.

Conclusions

Traditional Mongolian medicine: Wu-Lan thirteen-flavor decoction lowers blood pressure and reduces hypertension-induced renal damage in SHR by regulating the aryl hydrocarbon receptor signaling pathway.

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Ethics approval and consent to participate

The experimental animal ethics committee of Tianjin University of Traditional Chinese Medicine approved the protocol (Permit Number: TCM-LAEC2014004).

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Investigation: Xiaodong Cao.
Methodology: Yu Dong, Gang Li.
Project administration: Yan Zhu.
Supervision: Yan Zhu.
Visualization: Qianqian Tao.
Writing – original draft: Xiaoli Du, Yan Zhu.
Writing – review & editing: Xiaoli Du, Yan Zhu.

Disclosure statement

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

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