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Integration of network pharmacology and biological validation reveals the mechanisms of alkaloids from *Veratrum nigrum* L. in ameliorating hypertension and vascular remodeling

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ABSTRACT: *Introduction:* Hypertension is a major risk factor for vascular diseases. The roots and rhizomes of *Veratrum nigrum* L. have been used to treat hypertension with alkaloids (component A) from these plants showing potential as antihypertensive agents.

Objectives: This study aims to identify the key active ingredients in component A and investigate their mechanisms in treating hypertension and vascular remodeling.

Methods: The active ingredients of component A were predicted through blood-entry compounds analysis and validated its effects on hypertension and vascular remodeling in SHR and HUVECs. Network pharmacology and molecular docking were employed to identify related targets and pathways, with further confirmation from experiments.

Results: The blood-entry compounds of component A consisted of 12 *Veratrum* alkaloids, including Rubijervine, Jervine, Cevine, etc. Component A dose-dependently and effectively lowered blood pressure (BP), improved vascular remodeling and endothelial dysfunction, and regulated the renin-angiotensin system (RAS) in SHR. Network pharmacology identified 10 hub genes associated with 12 blood-entry compounds for the treatment of hypertension. Subsequent experiments validated that component A modulates the expression of key targets such as ADRB2, AGTR1, MMP9, eNOS, TNF- α , and NNMT. When NNMT was knocked down in HUVEC, the levels of P-eNOS, eNOS, and NO were decreased simultaneously. These modulations inhibit renal sympathetic nerve activity and the Smad pathway, while activating vagal nerve activity, NNMT/MNA/eNOS/NO pathway and the cAMP-mediated inhibition of the RhoA/MLC pathway. Moreover, component A, specifically rubijervine, binds directly to ADRB2, AGTR1, MMP9, and eNOS.

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Conclusions: Rubijervine may be the active ingredient of component A. Component A effectively alleviate hypertension and vascular remodeling. The underlying mechanism involves modulating ADRB2, AGTR1, MMP9, eNOS, TNF- α , and NNMT to inhibit TGF- β /Smad pathway, activate NNMT/MNA/ eNOS/NO pathway and cAMP-mediated inhibition of RhoA/MLC pathway, as well as balance RAS and nervous system.

Keyword: *Veratrum nigrum* L.; Component A; Network pharmacology; Blood-entry components; Hypertension and vascular remodeling

1. Introduction

Hypertension significantly increases the risk of developing cardiovascular and cerebrovascular diseases, leading to a higher likelihood of premature death [1]. By 2025, it is estimated that 29.2% of the world's population will suffer from hypertension [2]. This condition can result in vascular damage and remodeling, establishing a structural basis for the maintenance and worsening of hypertension. The damage to the blood vessels encourages further remodeling, which may lead to structural or functional impairments in vital organs. Consequently, this increases the risk of serious complications, including stroke, myocardial infarction, sudden death, heart failure, and peripheral arterial disease. Currently, effective treatments for hypertension include angiotensin-converting enzyme inhibitors, angiotensin receptor blockers, diuretics, calcium channel blockers, and anti-adrenergic drugs. However, hypertension often does not respond well to single-drug therapy, so combination therapy is recommended for patients who do not respond adequately to initial treatment [3]. Additionally, the disease is often difficult to manage due to numerous side effects associated with antihypertensive medications. Therefore, further research and new options are needed.

Veratri Nigri Radix et Rhizoma, derived from the roots and rhizomes of *Veratrum nigrum* L., has a long history of use in traditional Chinese medicine(TCM). As documented in “Ben Cao Gang Mu”, it is employed to treat headaches caused by various winds and to address loss of consciousness resulting from a stroke [4], suggesting that the roots and rhizomes of *Veratrum nigrum* L. is effective in treating strokes. Many studies have proven that the most significant risk factor for strokes is hypertension. Prolonged elevated BP can directly induce ischemic or hemorrhagic stroke by damaging vascular structure and function. Modern medicine employs the Veratrum plant to manage hypertension, particularly in late-stage hypertension and patients experiencing a hypertensive crisis [5,6]. The primary bioactive components of this genus plant are steroidal alkaloids (SD), which possess a range of beneficial effects, including anti-hypertensive, anti-thrombotic, anti-inflammatory, and analgesic properties [7]. Various alkaloids, including veratramine, protoveratrine, isorubijervine, rubijervine, etc, have been shown to decrease both systolic and diastolic blood pressure in SHRs, renal hypertensive rats, hypertensive mice, cats, and macaques[8–12]. Component A, isolated from total SD of Veratri Nigri Radix et Rhizoma, has shown promise as an effective antihypertensive agent for alleviating hypertension [13]. However, the characteristics and mechanisms underlying its antihypertensive and vascular protective effects remain poorly understood.

Network pharmacology is an emerging interdisciplinary field that integrates systematic drug research with big data and artificial intelligence. Researchers have utilized network pharmacology alongside molecular docking to investigate the pharmacodynamic basis and mechanism of TCM [14]. The blood-entry components

usually serve as the material basis for the efficacy of oral TCM. Component A contains at least 35 alkaloid components detected by UPLC-MS/MS [13], and this study specifically examined the blood-entry components of component A, identified using the UPLC/Q-TOF-MS method. The aim of this study is to establish a link between the blood-entry components of component A and its effectiveness in treating hypertension and vascular remodeling, and to elucidate the primary active components and mechanisms of action of component A against hypertension. To achieve this, molecular docking and CETSA were conducted to confirm the specific binding sites and capacities between the active compounds in component A and key targets. The key targets were selected for further experimental validation. Overall, this research provides valuable data for the advanced development of component A in combating hypertension.

2. Material and methods

2.1 Materials and reagents

The roots and rhizomes of *Veratrum nigrum* L. were obtained from Bozhou Sanyitang Biotechnology Co., Ltd, China in September 2020. Prof. Yue Cong of Henan University verified the authenticity of the roots and rhizomes, and a voucher specimen (2,020,009) is on file at the School of Pharmacy, Henan University, China. Rubijervine ($\geq 95\%$) was prepared by our research group as described previously [15]. Perindopril (Perin) was acquired from Servier Pharmaceutical Co., Ltd. (batch number 2,018,501, China). Deionized water was meticulously prepared using a Milli-Q reagent water system from Millipore. Enzyme-linked immunosorbent assay (ELISA) kits for NE, Ang II, ET-1, cAMP, ACE2, Ang-(1-7), ACE, PGI₂, MDA, SOD, TNF- α , IL-6, MNA, and NO kits were all purchased from Elabscience Biotechnology Co., Ltd (China). Total cholesterol (TC), total glyceride (TG), low-density lipoprotein (LDL), and high-density lipoprotein (HDL) kits were bought from Nanjing Jiancheng Bioengineering Institute (China). MMP9 (NO. GB11132) were purchased from Servicebio (China). AGTR1 (NO. 25343-1-AP), Renin (NO.14291-1-AP), NNMT (NO. 15123-1-AP), eNOS (NO. 27120-1-AP), P-eNOS (NO. AP0515), and RhoA (NO.10749-1-AP) were obtained from Proteintech (USA). ADRB2 (NO. Ab182136) was bought from Abcam (USA). P-MLC (NO. AF5443) and MLC (NO. AF5423) were purchased from Affinit (China), β -Actin Antibody (NO. AB0035), Bcl-2 (NO. CY6717), Bax (NO. CY5059), Smad3 (NO. CY5593), Smad2 (NO. CY5013), P-Smad3 (NO. CY5140), P-Smad2 (NO. CY5859), GAPDH antibodies (NO. AB0037), and HRP-goat anti-rabbit IgG (NO. AB0101) were obtained from Abways (China). HUVEC was obtained from Jiangsu Kaiji Biological(China). F12K medium (Cat.No.LA1320), NP-40 lysis buffer (Cat.No.N8032), sterile deionized water (Cat.No.F0020), trypsin-EDTA digestive solution (Cat.No.T1300), penicillin-streptomycin mixture (Cat.No.P1400), DMSO (Cat.No.D8371), PBS buffer (Cat.No.G4250) were purchased from Solarbio, and Fetal Bovine Serum (Cat.No.Z7186FBS-500) was purchased from ZETA life (Australia). Angiotensin II human(Cat.No.4474-91-3) was obtained from AbMole (USA). All the other organic and inorganic reagents (analytical grade) were provided by local suppliers.

All male SHR rats and WKY rats (107 male SHR rats and 18 male WKY) weighing 190–210 g were bought from Beijing Weitong Lihua Experimental Animal Technology Co., Ltd [SCXK(Jing) 2022-0006]. All

male SD rats (20 rats) were purchased from Henan Skobes Biotechnology Co., Ltd [SCXK(Yu) 2020-0005]. Experimental protocols and housing facilities were carried out in complete adherence to the guidelines established by the Animal Care and Use Committee of Henan University (Ethics No. HUSOM2020169).

2.2 Preparation of component A

The dried roots and rhizomes of *Veratrum nigrum* L. (15 kg) were ground into a fine powder. The powder was soaked in 10% (v/v) ammonia water for 2 h, followed by soaking in a solution containing 95% (v/v) ethanol in water for 4 h. The extraction process was carried out using a JHBE-100A homogenizer (Zhijing Biotechnology Co., China) four times for 10 minutes each time. The specific preparation method for component A is described in the literature (Zhou et al., 2023).

2.3 UPLC–MS/MS analysis for blood-absorbed compounds

Five SHR rats were orally administered component A concentrate at a dose of 20 mg/kg. Blood samples were collected from the retro-orbital venous plexus at 0.5, 1, 2, 4, 8, and 12 h and tentatively stored in collection tubes containing heparin sodium. Then, the blood samples were centrifuged at 3000 rpm for 10 min to obtain plasma samples. The plasmas were mixed, and a 1 mL sample was subsequently treated with acetonitrile at a 1:4 ratio to precipitate the protein. The supernatant obtained after centrifugation was dried under nitrogen gas at room temperature. The residue was dissolved in 200 µL of acetonitrile and used for UHPLC–Q-TOF/MS analysis after centrifugation at 12000 rpm for 10 min.

UPLC–MS/MS analysis was conducted on the Q Exactive Plus mass spectrometry (Thermo Fisher Scientific). The UPLC was carried out on a Hypersil GOLD column (2.1 × 100 mm, 1.9 µm). The specific parameters of mass spectrometry and chromatographic conditions were the same as those in the previous report[13].

2.4 Blood Pressure (BP) Measurement

2.4.1 Non-invasive BP measurement

12 WKY rats were selected for a control group. The SHR rats were randomly divided into five groups, each consisting of 12 rats. These groups included a model group, a perin group (positive group), and component A groups with different dosages. The SHR rats in the treatment groups received oral doses of 5, 10, and 20mg/kg of component A once per day for 14 days, referring to our previous study [13]. Rats in the positive group were given perin at 0.4 mg/kg via gavage. The sample was evenly dispersed in 0.5% NaCMC. The control group and the model group were treated with vehicle i.g.. These rats were measured for BP using a BP-100A full-automatic noninvasive blood pressure measurement system (Chengdu Taimeng Software Co., Ltd, China) via the tail cuff method [13].

2.4.2 Invasive BP measurement

6 WKY rats were selected for a control group. The SHR rats were randomly divided into four groups, each consisting of 8 rats. These groups included a model group, a perin group, and component A groups with the doses of 10 and 20mg/kg once per day for 14 days. After intraperitoneal injection of 3% pentobarbital

sodium for anesthesia, the rats were fixed on a surgical plate with a midline opening in the neck. The trachea was cut open, and an endotracheal tube was inserted to maintain smooth breathing. The femoral artery is intubated and connected to a pressure transducer, and the femoral BP is measured through an amplifier. Record various indicators such as systolic arterial pressure (SAP), diastolic arterial pressure (DAP), mean arterial pressure (MAP), and heart rate (HR) 30-60 minutes after the last administration using the BL-420F biological function experiment system[16].

2.5 Recording of the electrocardiogram in unconscious rats

The electrocardiogram (ECG) of SHR rats in all groups administered for 14 days was recorded employing BL-420F biological function experiment system (Chengdu Taimeng Technology Co., Ltd). The recording of ECG was performed according to the previous report [13].

2.6 Body weight and organ index

Weigh once a week at a fixed time. Organ index was calculated according to the following formula: organ index = organ (g)/rat body weight (g) * 100% [17].

2.7 Histological analysis

Kidney, left ventricular, aorta, and mesenteric artery in each group were used to prepare pathological sections by hematoxylin-eosin (HE) or masson trichrome staining, as previously described [18].

2.8 Biochemical analysis

Left ventricular, vessel, and kidney tissues were homogenized in chilled physiological saline for 25 minutes, followed by centrifugation at 5000g for 10 minutes at 4°C to obtain the supernatant. Blood samples were collected into heparinized tubes and immediately centrifuged at 1160 g for 10 min at 4°C to harvest the plasma. The supernatant and plasma were then tested with NE, ET-1, Ang II, ACE, ACE2, Ang (1-7), cAMP, NO, PGI₂, MDA, SOD, TNF- α , IL-6, MNA, TC, TG, LDL, and HDL assays using commercial kits. All the procedures complied with the manufacturer's instructions.

2.9 Immunohistochemistry examination

The aorta, mesenteric artery, and left ventricular tissue samples were embedded in paraffin and sliced into 4 μ m-thick sections. After deparaffinization and rehydration, antigen retrieval was performed using 10 μ M sodium citrate. The samples were treated with hydrogen peroxide for 10 min to inactivate endogenous peroxidase. The sections were then incubated overnight at 4°C with the primary antibodies MMP9, ADRB2, AGTR1, or RhoA at a dilution ratio of 1:200. After washing with PBS, the sections were sequentially incubated with the secondary antibody at 37°C for 15 min and then washed with PBS. Streptavidin PoD solution was added to the tissue surface and incubated for 30 minutes at room temperature, followed by four 5-minute washes with PBS. The sections were stained with diaminobenzidine and hematoxylin. The total and positively stained cells in 6 random fields of each section were counted at 200 $\times$ magnification. The integral optical density (IOD) of positive cells was analyzed by Image-Pro plus 6.0 software [19].

For immunofluorescence, after permeabilization with 0.5% Triton X-100 and blocking with 5% goat serum, the cells or mesenteric artery sections were incubated overnight at 4°C with eNOS or NNMT antibody (1:500). They were then stained for 1 h at 37°C with an Alexa Fluor 488-conjugated secondary antibody and counterstained with DAPI for 10 min at room temperature, before imaging with a AXR laser scanning confocal microscope (Nikon, Japan).

2.10 Network pharmacology analysis

2.10.1 Target prediction of blood-absorbed compounds of component A

According to LC-MS/MS data, compounds detected in blood were considered to be the main therapeutic agents of component A for treating hypertension. PubChem was applied to search for information on the compounds. The targets of these compounds were predicted using SwissTargetPrediction ([http://www. swiss targetprediction.ch/](http://www.swiss-targetprediction.ch/)) and SEA (<http://sea.bkslab.org/>). Next, UniProt ([http://www. Uniprot.org/](http://www.uniprot.org/)) was used to convert protein names to standard gene names.

2.10.2 Screening of hypertension-related genes

“Hypertension” was designated as the keyword in TTD ([http://db.idrblab. net/ttd/](http://db.idrblab.net/ttd/)), Drug Bank (<https://www.drugbank.ca/>), and GAD ([https://geneticassociationdb.nih. gov/](https://geneticassociationdb.nih.gov/)) for screening [20]. All results were combined to obtain a database of hypertension disease targets. Next, the hypertension disease targets were input into the UniProt database for standardization. Using Venny 2.1 website tool, a Venn diagram was construct, and the intersection of component A targets and hypertension-related genes was considered to be the targets of component A against hypertension.

2.10.3 Construction of compounds-targets-pathways network.

The STRING database (<https://www.string-db.org/>) was applied to identify interactions between targets of component A for the therapy of hypertension. Cytoscape software (version 3.8.2) and the Network Analyzer plug-in were used to analyze the degree of target protein interaction and construct the protein-protein interaction (PPI) network. GO and KEGG pathway enrichment analyses were performed to predict pathways of hub genes in the PPI network. Finally, the “compounds-targets-pathways” network was created using Cytoscape.

2.11 Molecular docking

The crystal structures of key targets of Homo sapiens were obtained from the Protein Data Bank (PDB ID: see Table S1). Molecular docking was carried out using Autodock Tools 1.5.6 software [21]. The proteins were used as the receptor, and the binding sites were defined using the PDB site record based on the ligand’s location. Rubijervine was selected to dock into ADRB2, AGTR1, MMP9, and NOS3 using the Semiflexible Protein-Ligand DOCKING method. CB-DOCK2 software was used for mapping.

2.12 Regulation of nervous system

2.12.1 Effects of unilateral RVLM microinjection of component A on BP in SHR

After being anesthetized, 15 rats were connected to the BL-420F biological function experiment system for measuring arterial BP, as described in section 2.4.2. to stereotaxic coordinates, a unilateral microsyringe was lowered into the rostral ventrolateral medulla (RVLM) (3mm backward to the lambda, 2 mm to the left of the midline, and 10 mm below the surface level of the skull) according to the fifth edition of the rat brain atlas [22]. The microsyringe was filled with L-glutamate (Glu), component A, or artificial cerebrospinal fluid (aCSF, in mM: 133.3 NaCl, 3.4 KCl, 1.3 CaCl₂, 1.2 MgCl₂, 0.5 NaH₂PO₄, 32.0 NaHCO₃, 3.4 glucose, with pH 7.4). Glu and component A (0.03 mg/mL, 0.06 mg/mL, 1.0 mg/mL, 1.5 mg/mL) were dissolved in aCSF. aCSF served as the control. All drugs were slowly injected into the RVLM in a volume of 100nl by a syringe. Functional identification of the RVLM depended on the depressor response produced by prior injection of 50 mmol/L Glu. At the end of the experiments, 10 nl of 2% Pontamine Sky Blue was microinjected into the drug site to confirm the marked location at the histological construction. Intracardiac perfusion was performed with 0.9% NaCl (150 mL) and subsequently with a 4% paraformaldehyde solution (200 mL). After perfusion, the brain was quickly removed and placed in paraformaldehyde solution, and then transferred to both 5% and 30% sucrose until it sank. Subsequently, the frozen brain was sectioned in coronal view[22].

2.12.2 Effects of component A on vagal nerve activity and renal sympathetic nerve activity in SD rats

10 Rats were anesthetized via intraperitoneal injection of 2% sodium pentobarbital, fixed in a supine position, and a midline incision approximately 2–3 cm in length was made on the neck to expose the right vagal nerve and major cervical blood vessels. The vagal nerve was carefully isolated and placed onto a recording electrode. An additional reference electrode was clamped onto the fold of the rat's skin to monitor vagal nerve electrical signals using the BL-420F biological function experiment system[23]. After approximately 30 minutes of stable baseline recording, component A was administered in doses of 25µg/mL, 50µg/mL, and 100µg/mL by intravenous injection, with normal saline used as a control.

After anesthesia, an abdominal incision was made to expose the left kidney of 10 rats. Subsequently, under an operating microscope, a renal nerve bundle was isolated and placed on a bipolar platinum electrode. The electrical changes in renal sympathetic nerve activity (RSNA) were amplified and monitored using the BL-420F biological function experiment system[14]. The dosage and method of administration were the same as above.

2.13 Western blotting analysis

The kidney, vessel, left ventricular tissues, liver, and collected HUVEC cells were extracted for total proteins using ice-cold RIPA lysis buffer (Solarbio, China). The protein concentration was tested with a Bio-Rad protein assay kit. Western blotting analysis was performed using a previously reported method [24]. The proteins were visualized using an enhanced chemiluminescence kit (Codonx Life Science, China) and then assayed with a chemiluminescence imaging analysis system (Beijing Sage Creation Science, China). Densitometric analysis was conducted by Image J software. The antibodies used were as follows: ADRB2, Renin, AGTR1, MMP9, RHOA, MLC/P-MLC, NNMT, eNOS/P-eNOS, NNMT, α -SMA, Bcl-2, Bax,

Smad2/P-Smad2, Smad3/P-Smad3(rabbit, 1: 1000), β -Actin (rabbit, 1: 10000), GAPDH (rabbit, 1: 15000), and Goat Anti-Rabbit IgG(H+L) HRP (1: 20000).

2.14 RT-qPCR analysis

The artery was used to extract total RNA using the TRIzol method following the manufacturer's protocol. The isolated RNA was then reverse-transcribed into cDNA using the HiScript III All-in-one RT SuperMix Perfect for qPCR kit (Vazyme, China) following the standard protocol. The qPCR assay was performed using ChamQ Universal SYBR qPCR Master Mix (Vazyme, China) on the CFX96TM Real-Time System (BIO-RAD, United States). The primers used for the target genes are listed in Table S1 with β -actin serving as the amplification control. The level of gene expression was calculated using the $2^{-\Delta\Delta C_t}$ method [25].

2.15 The effects of component A on AngII-induced HUVECs injury

HUVEC were cultured in FA2K complete medium, with 5% fetal bovine serum and 1% penicillin/streptomycin at 37°C in 5% CO₂ incubator. The cytotoxicity of component A and AngII was assessed using the CCK-8 assay. The cells were plated at 1×10^4 cells per well in a 96-well plate and treated with component A (1.25~40 μ g/mL) for 24 h in serum-containing DMEM.

HUVEC cells were seeded at a concentration of 1×10^4 cells per well into 96-well plates for CCK-8 assay and cultured at 37 °C for 24 h. The cells were randomly divided into five groups and incubated with the corresponding drugs for 24h. The five groups included: a control group, an Ang II (10 μ M) group, and three Ang II (10 μ M) + A groups with concentrations of 2.5 μ g/mL, 5 μ g/mL, and 10 μ g/mL[26]. HUVEC cells were seeded at a concentration of 1×10^6 cells per well into 6-well plates, cultured at 37 °C for 24 h, and then collected after each treatment for Western blot and immunofluorescence.

HUVEC cells were plated at 1×10^6 cells per well in 6-well plates. Collect and centrifuge cell culture supernatants at 1300 rpm for 10 minutes to remove debris. NO, SOD, MDA, and MNA levels were then tested using kits following the manufacturer's instructions.

2.16 Cellular Thermal Shift Assay (CETSA)

Each group of 2×10^7 HUVEC cells was seeded in a 10 cm culture dish. Following a 24-hour period, the cells were treated with 10 μ g/ml component A or 10 μ M rubijervine (dissolved in sterile water, DMSO < 0.1 %, V/V) for 24 h, while the control group received the same volume of solvent. After 24 hours of culture, the cells were treated with trypsin and centrifuged at 140 g for 5 min to obtain cell samples. The samples were added to the cell lysate (containing 1% PMSF and 1% phosphoprotease inhibitor) and subjected to three freeze-thaw cycles in a heating instrument. During each cycle, the sample was heated at specified temperatures (37°C, 40°C, 43°C, 46°C, 49°C, 52°C, 55°C) for 20 min, and then frozen in liquid nitrogen for 5 min. The supernatant was centrifuged at 13680 g for 20 min at 4°C for Western blotting [27].

2.17 Cell transfection

To knock down NNMT, HUVEC cells were transfected with small interfering RNA (siRNA) targeting NNMT (si-NNMT) or control siRNA (si-Con), which were purchased from Genepharma (China).

NNMT-siRNA target sequences were listed in Table S2. The transfection was performed using Lipofectamine 8000 reagent (Genepharma) according to the manufacturer's protocols.

2.18 Statistical analysis

All data are presented as mean $\pm$ SD. Statistical comparisons were conducted using one-way ANOVA, followed by Tukey's Multiple Comparison Test to analyze variations between multiple groups. Statistical procedures were carried out using GraphPad Prism 8.0.2.

3. Results

3.1 Blood-entry compounds of component A detect by UPLC-MS/MS

UPLC-MS/MS was used to analyze the steroidal alkaloids in plasma. The Xcalibur 3.2 and Compound Discoverer 3.2 software packages were used to process the obtained chromatographic information. Each compound was identified in conjunction with the mzCloud database, reference substances, and the chemical composition of component A [13]. A total of 12 steroidal alkaloids were identified from plasma samples, as shown in Figure 1 and Table S3.

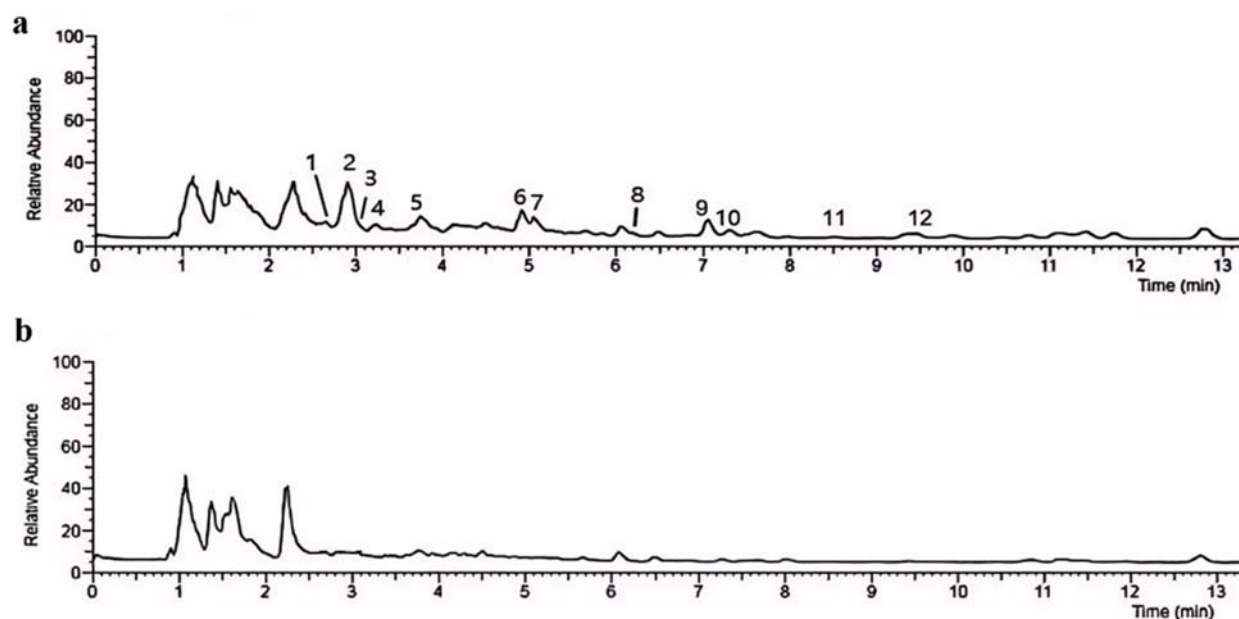


Figure 1. The total ion chromatograms of plasma in positive ion mode. (a: blank group; b: component A group)

3.2 Antihypertensive activity

The BP values showed a dose-dependent decrease after administering component A at doses of 5, 10, 20mg/kg over a period of 14 days. The effect began 0.5hr after administration, with BP reaching its lowest level at 1 h and returning to the premedication level by 5h after treatment (see Figure S1). The high dose of component A led to the most significant reduction in SDP and DBP by approximately 30 mmHg after 14 days of treatment. This outcome is consistent with the BP change observed in SHR after administering component A for 1, 7, and 30 days [13]. Additionally, the BP-lowering effect was further confirmed using a direct BP measurement method (Table S4).

The Electrocardiogram (ECG) of SHR in all groups unequivocally displayed a normal ECG waveform at 85 min after administering component A for 14 days (Figure S2). The recorded heart rates were as follows: 298 (WKY group), 377 (SHR group), 372 (Perin group), 368 (A-5 group), 356 (A-10 group), and 353 (A-20 group) bpm.

3.3 Body weight and organ index

The weight of rats reflected their growth status and changes. Before the treatment, the body weights of the rats in all groups are similar. However, after 14 days of treatment, the Perin group exhibited a significant decrease in body weight ($P < 0.05$, $P < 0.01$) compared to the model group (Figure S3). Throughout the experiment, there were no significant differences in body weight among the component A groups. This suggests that 14 days of treatment with component A did not affect the basal growth rate of the rats when compared to the control and the model groups.

The organ index is a crucial indicator of rat health, and normal rats typically show the relatively constant indices for various organs. The results (see Figure S4) showed a significant increase in the indices of the heart, kidney, liver, and lung in the SHR group compared to the WKY group ($P < 0.05$, $P < 0.001$), indicating notable organ lesions. In contrast, the renal and liver indices in SHRs receiving component A showed substantial decreases ($P < 0.05$, $P < 0.01$, and $P < 0.001$) compared to the model group. A decreasing trend was also observed in the heart index, after 14 days of treatment with component A. These findings suggest that component A has a beneficial impact on organ health.

3.4 Biochemical analysis

In comparison to the WKY group (Figure S5), significant increases ($P < 0.05$; $P < 0.001$) in TG and LDL levels, along with a significant decrease ($P < 0.05$) in HDL levels, were observed in the model group. T-CHO and TG levels significantly decreased in response to the treatment in component A groups (Figure S5). Component A may alleviate the abnormalities in blood lipids of SHRs after 14 days of treatment.

As illustrated in Figure 2, the levels of NE, AngII, ET-1, and ACE were significantly elevated in both plasma and left heart. In contrast, levels of NO, ACE2, Ang-(1-7), and PGI₂ in plasma, vessel, and left heart were reduced in the SHR group compared to the WKY group (Figure 2). After 14 days of treatment with component A, NE, Ang II, and ET-1 levels decreased by 7% to 26% in plasma and by 19% to 59% in left heart compared to the SHR group. Additionally, levels of NO and PGI₂ in plasma and vessels were significantly increased by 6% to 69% and 31% to 73%, respectively, after treatment with component A. A similar trend was also observed in NO levels in the left heart of SHRs. These findings indicate an improvement in endothelial cell function throughout the body. Notably, after treatment with component A, the plasma ACE2 levels increased markedly by 38% to 94%, and Ang-(1-7) levels rose by 22% to 28%. Meanwhile, ACE levels showed a downward trend in plasma compared to the SHR group. In summary, the biochemical analyses suggest that component A alleviates high BP by regulating RAS and restoring vascular endothelial function.

In comparison to the control group, the model group exhibited significantly reduced SOD and MNA levels and elevated TNF- α , IL-6, and MDA levels. Treatment with component A effectively reversed these

alterations. This indicates that component A can reduce inflammatory and oxidative stress responses, ultimately leading to improvements in hypertension-induced vascular injury and remodeling (Figure 2).

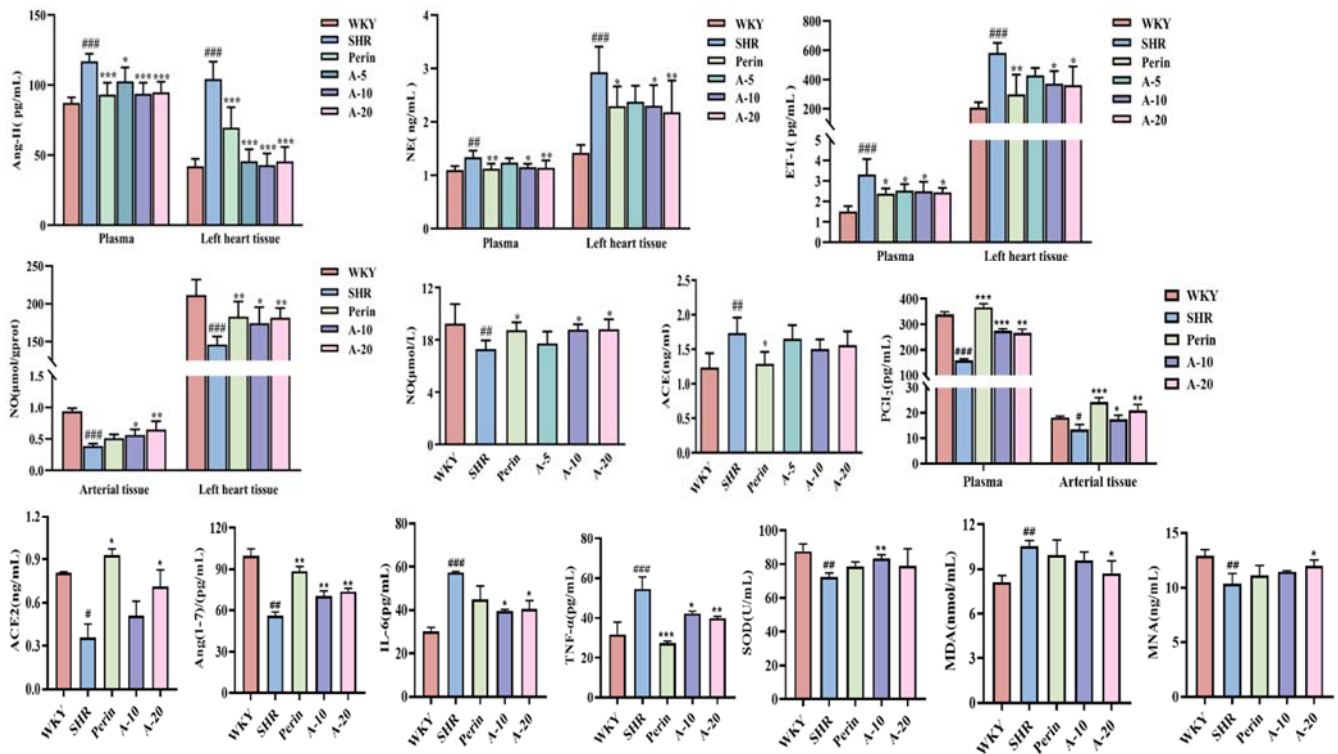


Figure 2. The levels of NE, AngII, ET-1, NO, ACE, ACE2, Ang(1-7), PGI₂, IL-6, TNF- α , SOD, MDA, and MNA in all groups *in vivo*. # $P < 0.05$, ## $P < 0.01$, ### $P < 0.001$, versus the WKY group. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, versus the SHR group. (NE, AngII, and ET-1: plasma, left heart tissue; NO: plasma, left heart tissue, arterial tissue; PGI₂: plasma, arterial tissue; ACE, ACE2, Ang(1-7), IL-6, TNF- α , SOD, MDA, and MNA: plasma)

3.5 Morphological observation

The thoracic aorta and mesenteric artery of the model group show swelling of endothelial cells, increased intima-media thickness (IMT), and thickening of muscle fibers when compared to the WKY group (Figure 3a, 3c). After a 14-day treatment, component A dose-dependently improved the morphology of the thoracic aorta and mesenteric artery. The thickness of the vascular wall and the diameter of the lumen were measured using Image-Pro Plus 6 software. When comparing the model group to the WKY group, significant increases in vascular wall thickness and the ratio of wall thickness to lumen diameter was evident ($P < 0.001$). These changes were efficaciously reversed by treatment with component A at doses of 10 and 20 mg/kg compared to the model group (Figure 3a, 3c). In addition, hypertension can lead to characteristic vascular fibrosis, collagen fiber hyperplasia, and collagen deposition around the arteries, all of which were observed in the SHR group. Following a 14-day treatment, collagen fiber hyperplasia in the thoracic aorta and mesenteric artery was effectively suppressed in the component A groups ($P < 0.001$) compared to the SHR group (Figure 3b, 3d), indicating that component A can effectively prevent vascular remodeling in SHRs.

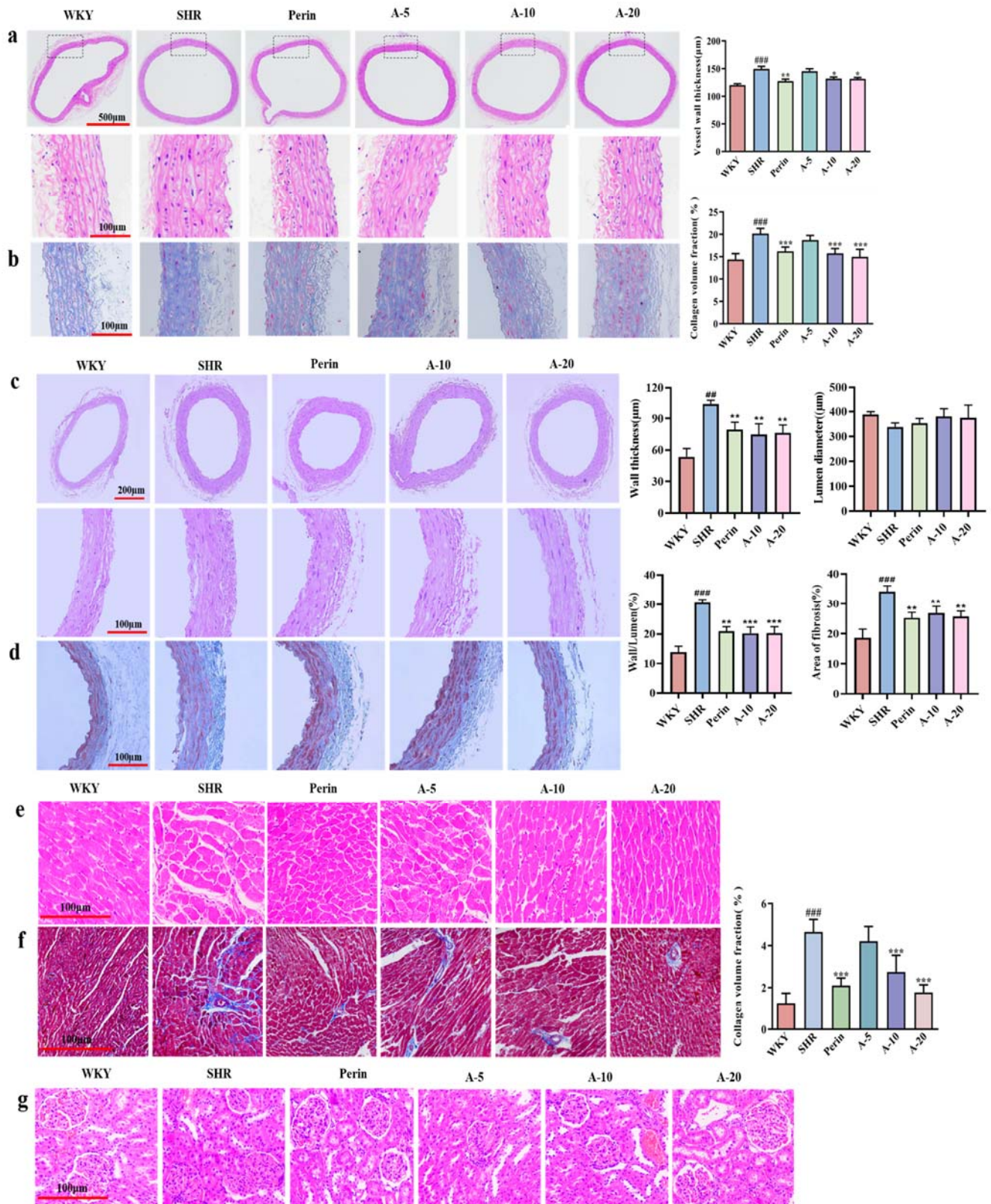


Figure 3. Histological analysis of the aorta, mesenteric vessels, left ventricular, and kidney in all groups *in vivo*. **a.** Representative aorta histological observations (40×, 200×, H&E) and quantitative analysis of vessel wall thickness (n=4); **b.** Representative histological observations and fibrotic area (200×, Masson) (n=4); **c.** Representative histological observations of mesenteric vessels (100×, 400×, H&E) and quantitative analysis of vascular wall thickness and lumen diameter (n=3); **d.** Representative histological observations of the mesenteric vessels (400×, Masson) and quantitative analysis of fibrotic area (n=3); **e.** Representative left ventricular histological changes (×200, H&E) (n=4); **f.** Representative left ventricular histological changes (×200, Masson) and quantitative analysis of fibrotic area (n=4); **g.** Representative kidney histological changes (×200, H&E) (n=4).^{##} $P < 0.01$,^{###} $P < 0.001$, versus the WKY group; $P < 0.05$, $P < 0.01$, $P < 0.001$, versus the SHR group.

High BP typically leads to an increase in the weight index of the heart and stimulates the growth of fibroblasts, both of which play crucial roles in heart remodeling. After 14 days of treatment, the excessive growth of collagen fiber and morphological changes in the left heart showed marked improvement in a dose-dependent manner ($p < 0.001$) compared to the SHR group (Figure 3e, 3f). In comparison to the glomeruli of the WKY group, those in the SHR group displayed structural changes, including abnormal proliferation of mesangial cells, matrix deposition, and reduced Bowman's spaces. These alterations indicated that the SHRs group experienced renal injury due to hypertension. After 14 days of treatment, component A exhibited strong renoprotective effects in SHRs by repairing renal injury (Figure 3g) and reducing the kidney weight index (Figure S4).

3.6 The cytotoxicity of Ang II and component A on HUVECs

The experimental results on cytotoxicity showed (Figure S6) that exposure to Ang II at concentrations of 10 to 20 μM markedly inhibited cell proliferation and caused considerable endothelial cell injury. In contrast, treatment with component A for 24 h exhibited no significant cytotoxicity at concentrations ranging from 1 to 10 $\mu\text{g/mL}$. Based on these findings, 10 μM Ang II was selected to establish the endothelial cell injury model after 24 h of treatment. At the same time, component A was set at doses of 2.5, 5, and 10 $\mu\text{g/mL}$ in subsequent experiments.

3.7 Protective effect of component A on Ang II-Induced HUVECs injury

The impact of component A on the viability of HUVEC induced by Ang II was evaluated using the CCK-8 assay after 24 h of treatment (Figure 4a). Compared to the control group, HUVEC viability in the model group (treated with 10 μM AngII) significantly decreased to 80.35%, indicating substantial endothelial injury. However, treatment with various concentrations of component A significantly restored cell viability compared to the model group. Furthermore, the model group exhibited significantly lower levels of MNA, NO, PGI₂, and SOD, while showing significantly higher levels of MDA compared to the control group. In contrast, all tested concentrations of component A significantly elevated MNA, NO, PGI₂, and SOD levels, while reducing the MDA levels compared to the model group (Figure 4a). These results indicate that component A alleviate oxidative stress and ameliorates AngII-induced endothelial dysfunction and injury in HUVECs.

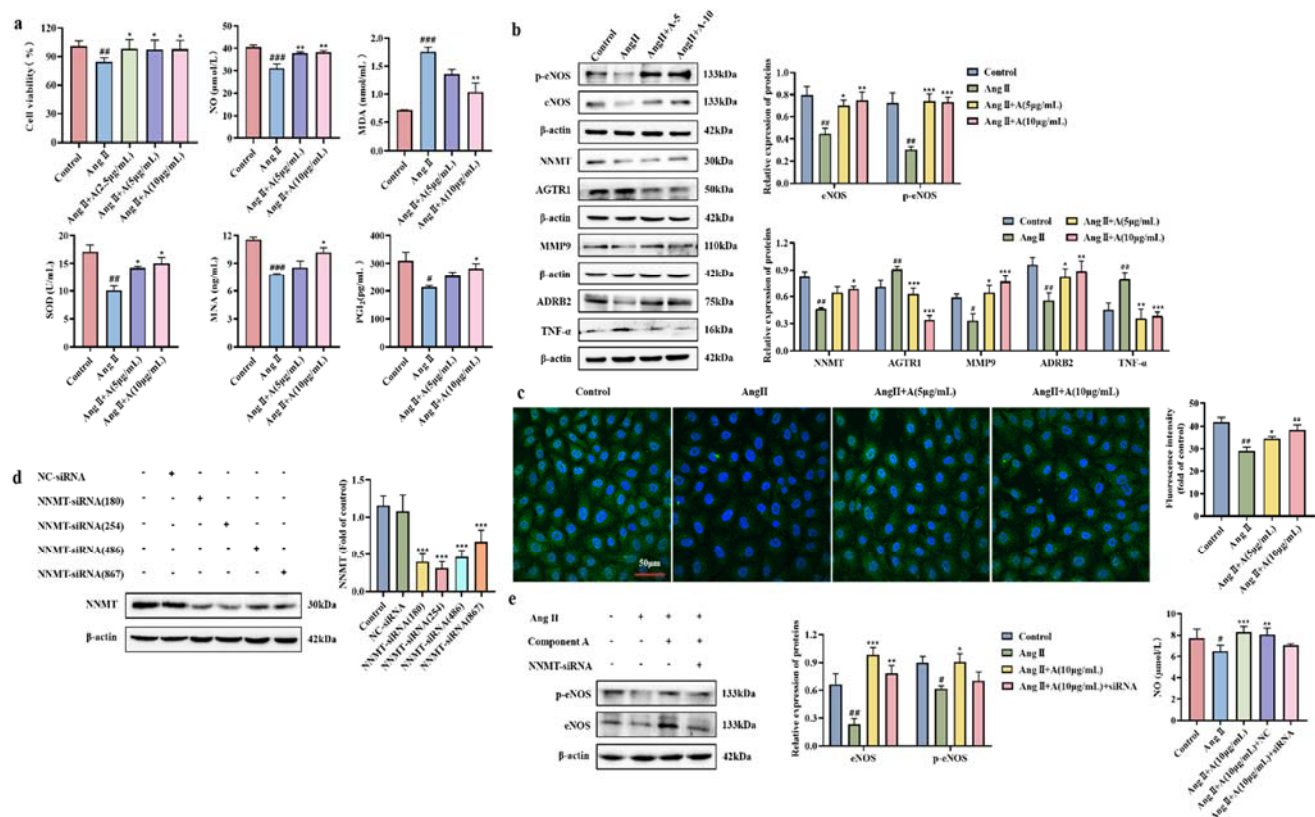


Figure 4. The effects of component A on AngII-induced HUVECs damage *in vitro*. **a.** Effects of component A on the viability of HUVECs and the levels of NO, PGI₂, SOD, MDA, and MNA in all groups after treatment with AngII; **b.** Protein expression of P-eNOS/eNOS, MMP9, AGTR1, ADRB2, TNF-α, and NNMT by Western blot; **c.** Immunofluorescence staining of eNOS (green) in HUVECs (100×); **d.** Knockdown of NNMT expression in HUVECs by siRNA; **e.** The levels of eNOS, P-eNOS, and NO in transfected cells. β-actin was used as a housekeeping protein. Data were presented as mean ± SD, n = 3 per group. # $P < 0.05$, ## $P < 0.01$, ### $P < 0.001$ versus the control group; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ versus the model group.

3.8 Network pharmacology analysis of blood-entry constituents from component A

The analysis focuses on 12 blood-entry compounds, as detailed in Table S3. Initially, we predicted 466 pharmacological targets for these compounds using the SwissTargetPrediction and SEA databases. Targets associated with hypertension were screened from the TTD, DrugBank, and GAD. After merging the data, removing duplicates, and filtering out low-scoring values, we established a database containing 725 hypertension target. By intersecting the two target databases, we identified 80 potential therapeutic targets for component A (Figure 5a). The PPI network for these 80 target proteins is shown in Figure 5b, where the hub proteins include MMP9, ADRB2, AGTR1, ACE, AGTR2, ACE2, PPARG, MAOA, TNF, and NOS3.

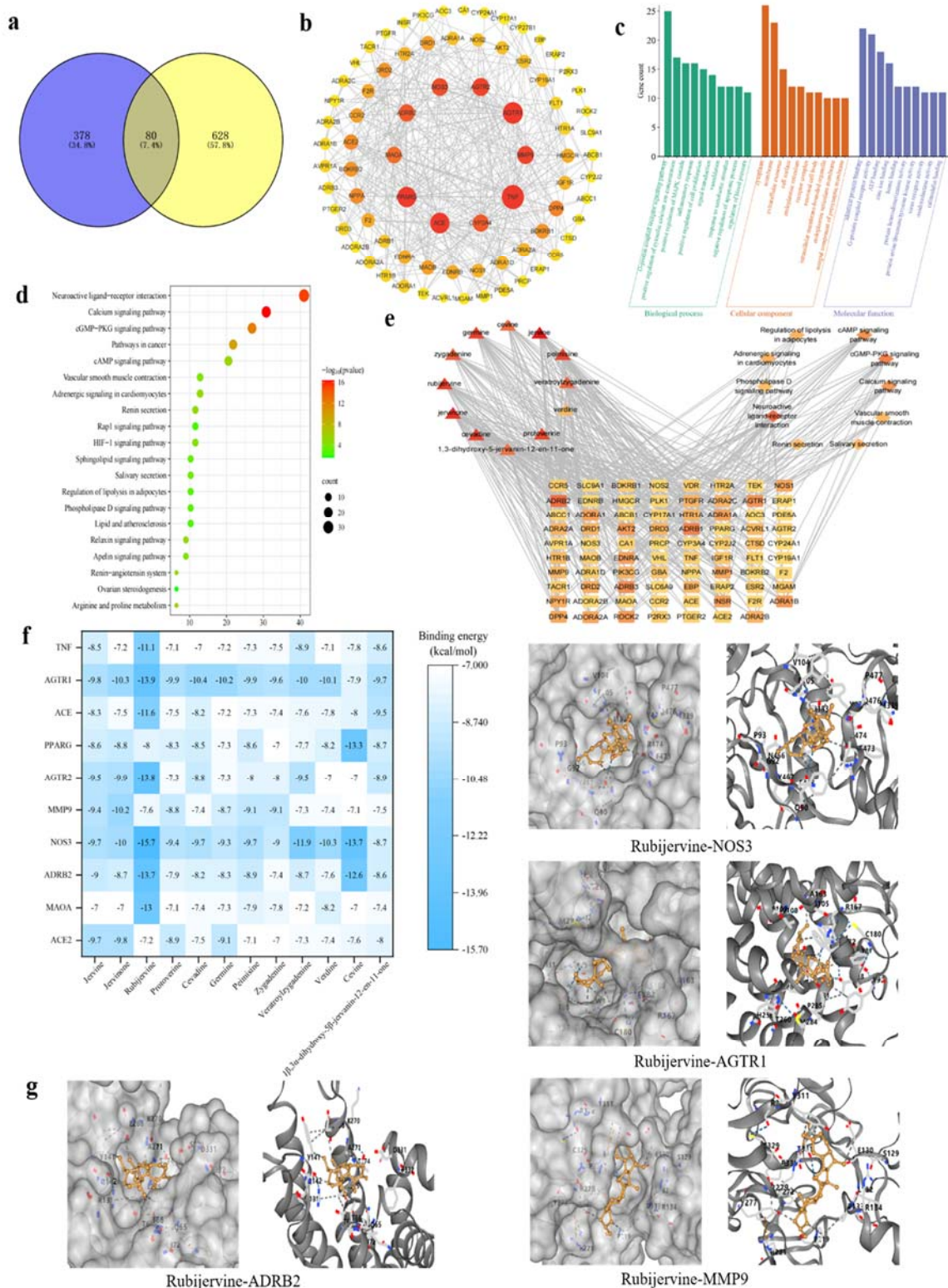


Figure 5. Network pharmacology and molecular docking analyses of the antihypertensive effects of component A. **a.** Venn diagram for intersection targets of component A and hypertension; **b.** PPI network of 80 overlapping targets, the nodes represent proteins, and the edges represent protein–protein interactions; **c.** The biological processes, cellular components and molecular functions identified in GO enrichment analysis of intersection targets; **d.** KEGG pathways identified in the enrichment analysis of intersection targets; **e.** Compounds–targets–pathways network; the triangle nodes represent blood-absorbed compounds, the rectangle nodes represent targets, and the diamond nodes represent pathways; **f.** Heat map of the binding energy for molecular docking between 12 key targets and the blood-absorbed compounds of component A, with binding energy levels represented in white and blue; **g.** The molecular docking conformations between rubijervine and ADRB2/AGTR1/NOS3/ MMP9. Yellow structure represented rubijervine; gray structures represented amino acid residues; blue dotted line represented hydrogen bond; light blue dotted line represented weak hydrogen bond; black dotted line represented hydrophobic interaction.

The GO function analysis revealed that the top enriched Biological Processes was the G-protein coupled receptor signaling pathway, the top enriched Cellular Components was cytoplasm, and the top enriched Molecular Functions was identical protein binding (Figure 5c). The KEGG analysis highlighted that the neuroactive ligand-receptor interaction was the most significantly enriched pathway (Figure 5d). Additionally, the “Compounds-targets-pathways” network analysis, in conjunction with the KEGG pathway findings, indicated that the targets of component A in relation to hypertension were primarily enriched in neuroactive ligand-receptor interaction, cAMP signaling pathway, renin secretion pathway, and vascular smooth muscle contraction (Figure 5e).

3.9 Molecular docking

Individual molecular docking analyses were performed for the 10 targets using Autodock Vina. The molecular docking results for the 12 main compounds indicated that the binding affinities of all models were lower than -7.0 kcal/mol. Among these, the optimal model exhibited an affinity of -15.7 kcal/mol (Fig. 5f), indicating that the 12 compounds and 10 proteins formed stable complexes. Visual representations of the binding interactions between rubijervine and the proteins ADRB2, AGTR1, NOS3, and MMP9 were generated using CB-DOCK2 and are presented in Figure 5g. Rubijervine primarily interacts with the amino acid residues of AGTR1, ADRB2, NOS3, and MMP9 through hydrogen bonds, weak hydrogen bonds, and hydrophobic interaction. These create suitable spatial complementarity with the protein binding pocket, thereby facilitating the formation of more stable complexes.

3.10 The effect of component A on the expression of AGTR, MMP9, ADRB2, and REN

The expression levels of key target proteins in the thoracic aorta was shown in Figure 6a-1. In the model group, there was a markedly lower level of ADRB2 and distinctly higher levels of AGTR and MMP9 compared to the control group ($P < 0.05$, $P < 0.01$). In the treated group, the expression of AGTR1 and MMP9 was significantly reduced, while the expression of ADRB2 was notably increased in the thoracic aorta compared to the model group ($P < 0.05$, $P < 0.01$). These results were further confirmed by immunohistochemical analysis (Figure 6b). Additionally, the levels of ADRB2, AGTR1, and MMP9 in the left heart and kidneys exhibited similar patterns to those in the aortas of SHRs in the treated groups when compared to the SHR group (Figure 6a-2, 6a-3). Moreover, the high dose of component A remarkably decreased the level of REN in the kidneys of rats compared to the model group (Figure 6a-3). *In vitro* experiments, the expression levels of ADRB2 and AGTR1 exhibit the same trends in the AngII-treated HUVECs after treatment with component A (Figure 4b), while the expression of MMP9 displays an opposite trend. AngII promotes the upregulation of MMP9 expression [28]. *In vitro* experiments use AngII to create a damage model in HUVECs, resulting in elevated MMP9 levels, even with treatment using component A, due to high exposure to AngII. *In vivo* experiments demonstrated that the AngII level returned to normal in SHRs after 14 days of administration, leading to a downregulation of MMP9 expression.

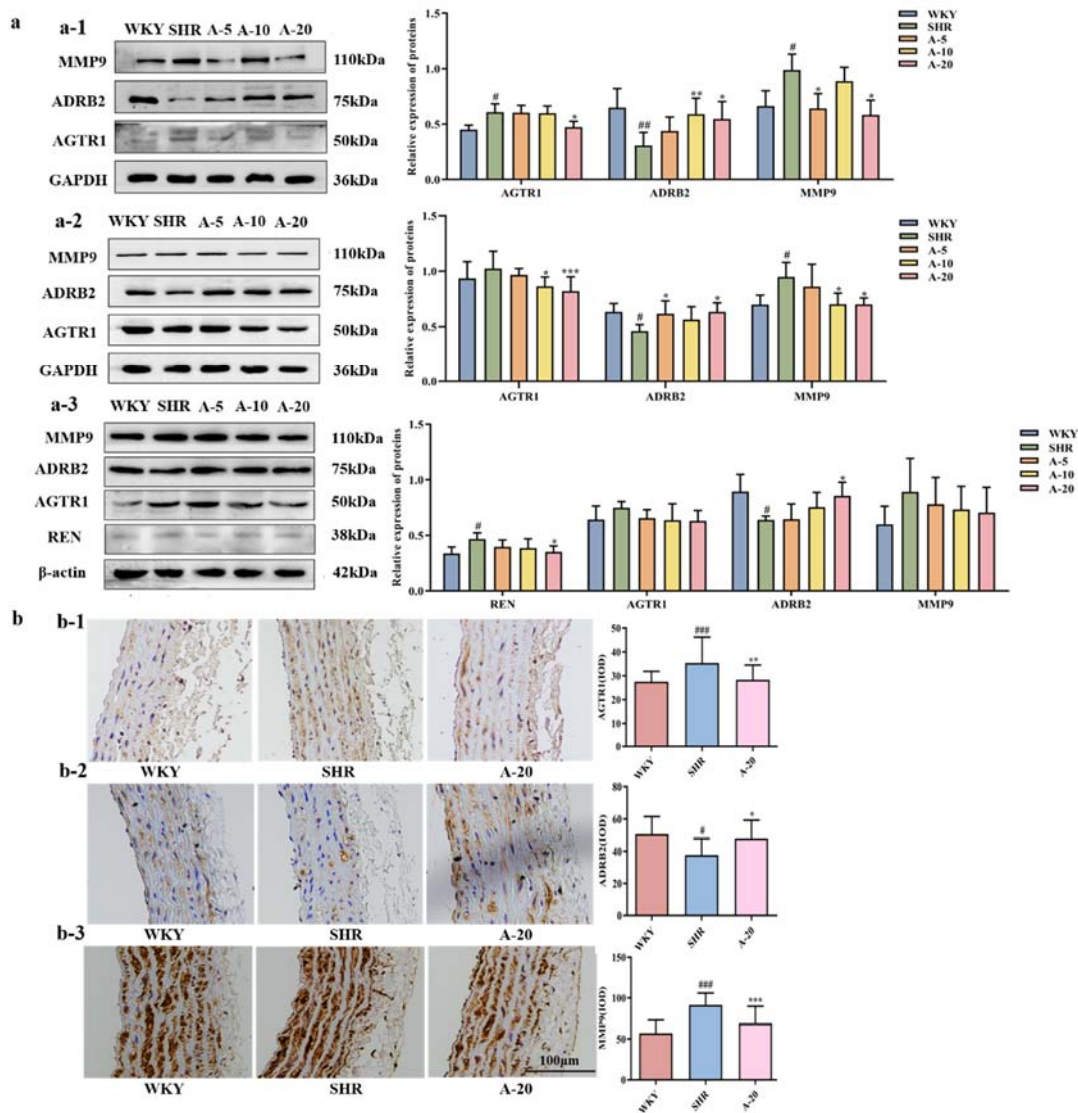


Figure 6. The expression of ADRB2, MMP9, AGTR1, and REN in the aorta, left heart, and kidney *in vivo*. **a.** Western blot; **b.** Immunohistochemical analysis in the aorta. GAPDH and β -actin were used as housekeeping proteins. Data were presented as mean $\pm$ SD, $n = 3$ per group. # $P < 0.05$, ## $P < 0.01$, ### $P < 0.001$ versus the WKY group; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ versus the SHR group. (a-1: aorta; a-2: left heart; a-3: kidney; b-1: AGTR1; b-2: ADRB2; b-3: MMP9)

3.11 The inhibition of component A on RHOA/MLC via the cAMP signaling pathway

In vascular smooth muscle cells, ADRB2 induces vasodilation in the blood vessels through the cAMP/RHOA/MLC signaling pathway. The levels of cAMP, RHOA, MLC, and P-MLC in the aorta were significantly different between the control group and the model group (Figure 7). After treatment, the level of cAMP was significantly upregulated ($P < 0.05$), while the levels of RHOA, P-MLC, and MLC were downregulated as compared to the model group. Similarly, the levels of cAMP, RHOA, MLC, and P-MLC in the left heart exhibited trends similar to those observed in the aorta of the component A group when compared to the SHR group. Immuno- histochemical analysis further confirmed the changes in RHOA levels in both the aorta and the left heart. Overall, these findings suggest that the increase in cAMP levels activates its downstream signaling pathway, leading to the suppression of MLC phosphorylation and the subsequent relaxation of vascular smooth muscles.

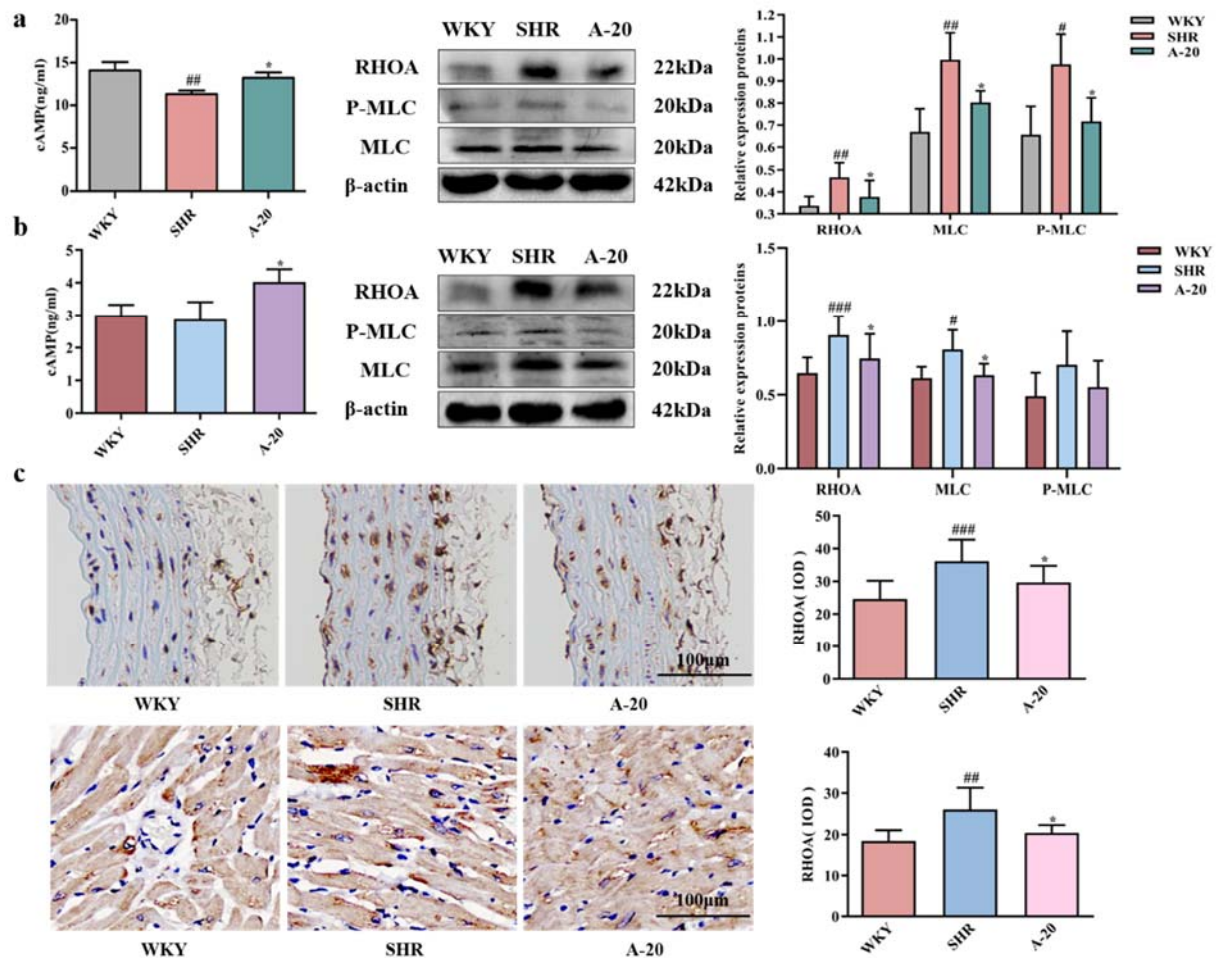


Figure 7. The expression of cAMP/RHOA/MLC in the aorta and left heart *in vivo*. β -actin was used as a housekeeping protein. **a** and **b**. The levels of cAMP in the aorta (**a**) and left heart (**b**) analyzed by ELISA; the levels of RHOA, MLC, and P-MLC in the aorta (**a**) and left heart (**b**) assayed by Western blotting. **c**. Expression of RHOA in the thoracic aorta and left heart determined by immunohistochemistry (200 $\times$). Data were presented as mean $\pm$ SD, $n = 3$ per group. $\# P < 0.05$, $\#\# P < 0.01$, $\### P < 0.001$, versus the WKY group, $* P < 0.05$, versus the SHR group ($n = 3$ per group).

3.12 Promotion of NNMT/MNA/eNOS/NO signaling pathway

The data presented in Figures 2 and 8 indicate that the levels of NNMT, MNA, eNOS, p-eNOS, and NO were significantly lower in the model group compared to the control group. However, after 14 days of treatment with component A, there was a noticeable increase in the levels of NNMT, MNA, eNOS, p-eNOS, and NO in comparison to the model group. Both immunofluorescence analysis and RT-QPCR further verified that component A significantly upregulated NNMT and eNOS levels in the blood vessels of SHRs in Figure 8b and 8c.

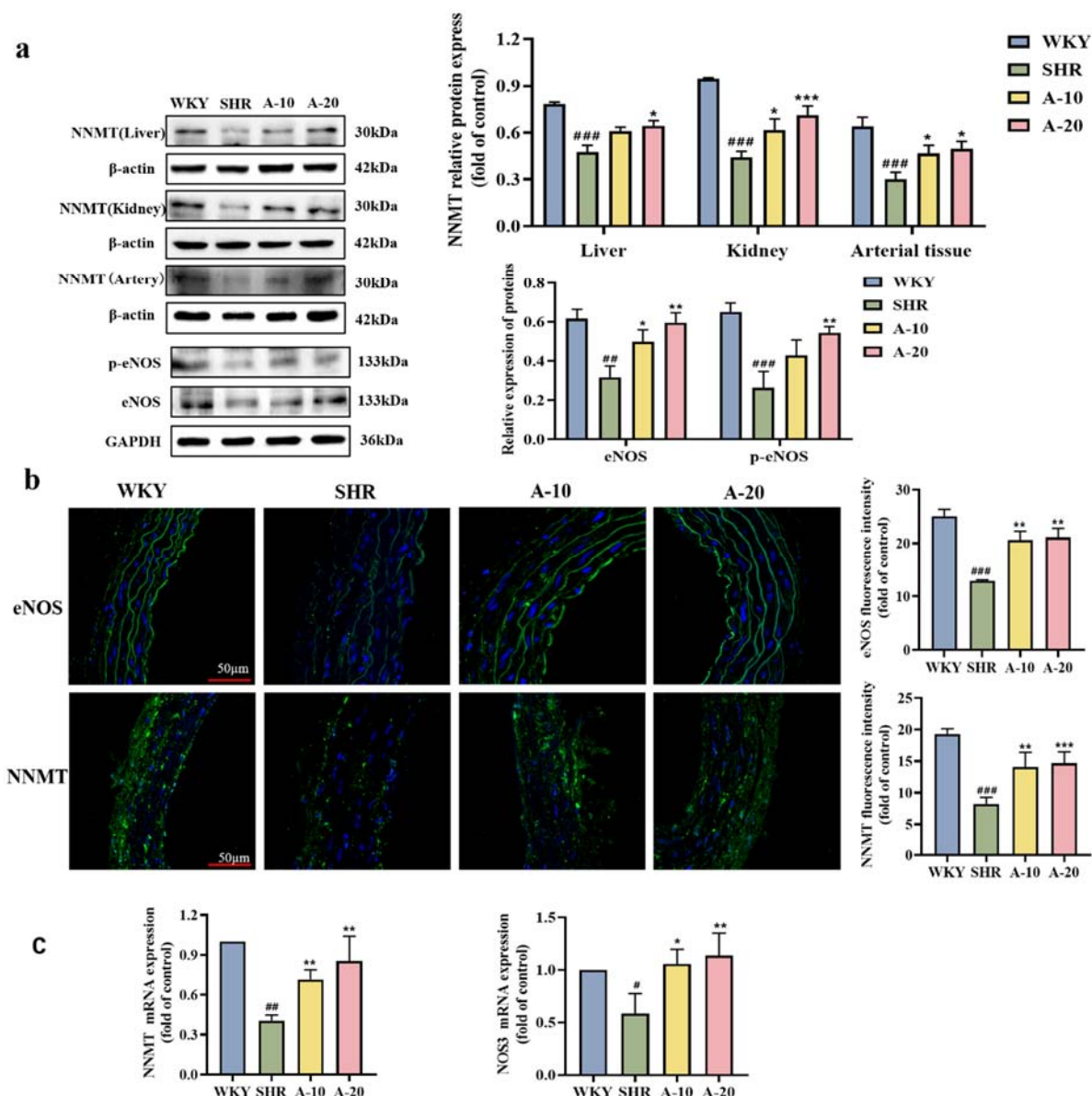


Figure 8. The effects of component A on the expression of eNOS and NNMT in the blood vessel *in vivo*. **a.** Western blot for P-eNOS and eNOS in the aorta and for NNMT in the aorta, liver, and kidney; **b.** Immunofluorescence staining of eNOS(green) and NNMT (green) in the mesenteric vessel (400 $\times$). **c.** RT-qPCR for eNOS and NNMT. GAPDH and β -actin were used as housekeeping proteins. Data were presented as mean $\pm$ SD, $n = 3$ per group. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$, versus the WKY group; $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, versus the SHR group.

Additionally, we investigated the effects of component A on the expression levels of these indicators and proteins in AngII-treated HUVECs. As shown in Figure 4a-4c, AngII significantly downregulated the expression of NNMT, MNA, eNOS, p-eNOS, and NO. In contrast, treatment with component A reversed this trend, leading to an upregulation of these levels. Therefore, we conclude that component A enhances the NNMT/MNA/eNOS/NO signaling pathway, thereby improving vascular function.

To further investigate the role of the NNMT/MNA pathway, NNMT was silenced by transfecting cells with si-NNMT. As shown in Figure 4d, NNMT expression was significantly reduced in cells transfected with si-NNMT (254) compared to the NC group, which was selected for subsequent experiments. In the presence of component A, levels of eNOS, p-eNOS, and NO were elevated compared to the model group. However, si-NNMT transfection substantially inhibited the increase in p-eNOS, NOS, and NO levels induced by

component A (Figure 4e). The knockdown of NNMT suppressed the effect of component A on NO production in Ang II-treated HUVECs. These results demonstrate that component A primarily activates the eNOS/NO pathway through an NNMT-dependent mechanism.

3.13 Suppression of vascular fibrosis and proliferation via the TGF- β /Smads pathway

Hypertension is frequently accompanied by hyperplasia and fibrosis of vascular smooth muscle. α -SMA, COL1A1, and TNF- α are recognized as key markers of vascular fibrosis and hyperplasia. Component A largely reduced the overexpression of α -SMA, COL1A1, and TNF- α induced by hypertension, as demonstrated by Western blotting (Figure 9). As illustrated in Figure 9a, hypertension notably elevated the protein levels of P-smad2, P-smad3, and Bcl-2, while decreasing the protein level of Bax in vascular tissue. These alterations were significantly reversed by component A. We also simultaneously observed elevated mRNA expression levels of Bax and reduced mRNA expression levels of Bcl-2, α -SMA, and COL1A1 after treatment with component A (Figure 9b). Overall, these results indicate that component A efficiently blocked vascular fibrosis and proliferation.

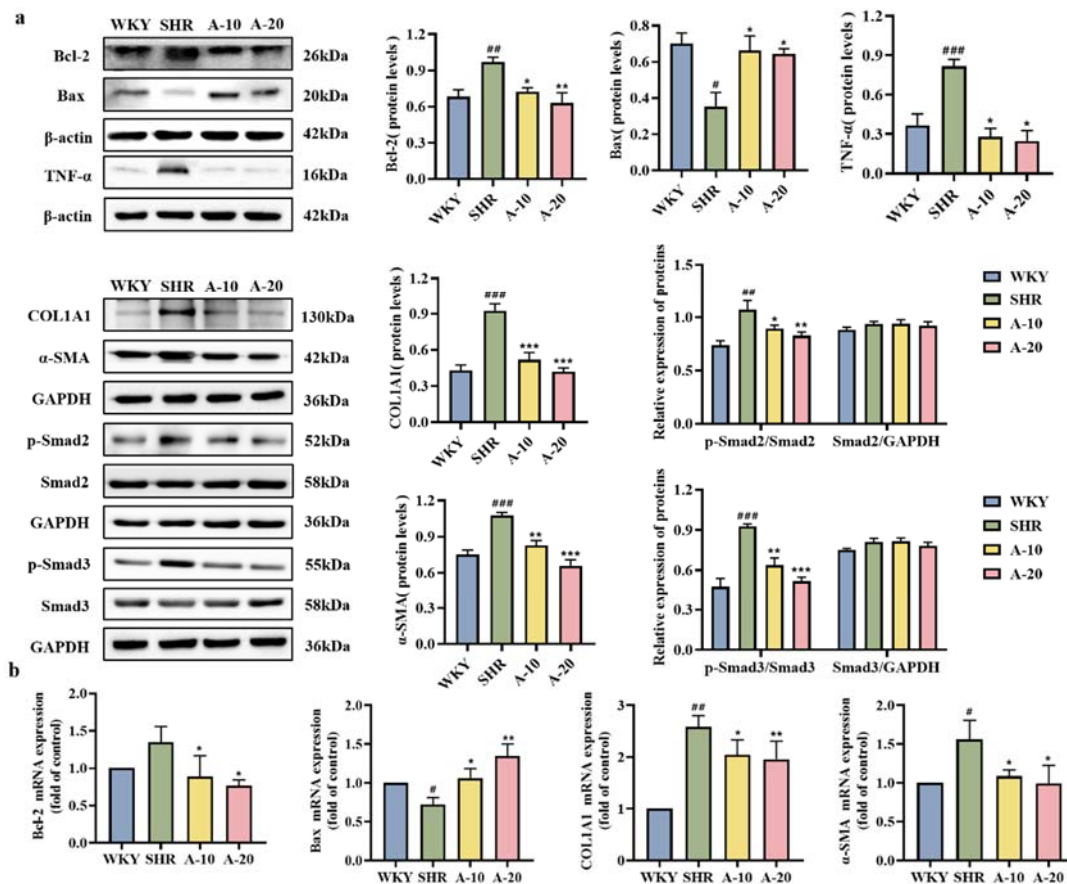


Figure 9. Suppression of vascular fibrosis and proliferation via the TGF- β /Smads pathway *in vivo*. **a.** Western blot; **b.** RT-qPCR for α -SMA, COL1A1, Bcl-2, and Bax. GAPDH and β -actin were used as housekeeping proteins. Data were presented as mean $\pm$ SD, n = 3 per group. [#] P < 0.05, ^{##} P < 0.01, ^{###} P < 0.001, versus the WKY group; ^{*} P < 0.05, ^{**} P < 0.01, ^{***} P < 0.001, versus the SHR group.

3.14 Effects of unilateral RVLM microinjection of component A on BP in SHRs

Histological verification of the microinjection site was performed, as shown in Figure S7. The injection location was identified by using 2% pontamine sky blue, and subsequent analysis of frozen section

confirmed that the site corresponded anatomically with the RVLM coordinates defined in the fifth edition of the rat brain atlas. As illustrated in Figure S7, microinjection of 50 mmol/L L-Glu into the RVLM of SHR produced immediate pressor responses ranging from 20 to 50 mmHg. This finding validates both the role of the RVLM in BP regulation and the accuracy of our anatomical targeting. The subsequent administration of component A at concentrations ranging from 0.03 to 1.5 mg/mL did not induce significant changes in BP, indicating that the antihypertensive effects of component A are not mediated through central RVLM.

3.15 Effects of component A on vagal nerve activity and renal sympathetic nerve activity in SD rats

As shown in Figure 10a, component A at concentrations of 50 $\mu\text{g/mL}$ and 100 $\mu\text{g/mL}$ significantly reduced renal sympathetic nerve activity in a dose-dependent manner compared to the control group. In contrast, both 50 $\mu\text{g/mL}$ and 100 $\mu\text{g/mL}$ component A markedly enhanced vagus nerve activity (Figure 10b), indicated by an increase in discharge frequency and peak value compared to the control group. These results suggest that component A can inhibit renal sympathetic nerve activity while enhancing vagal nerve activity, which may contribute to its hypotensive effect.

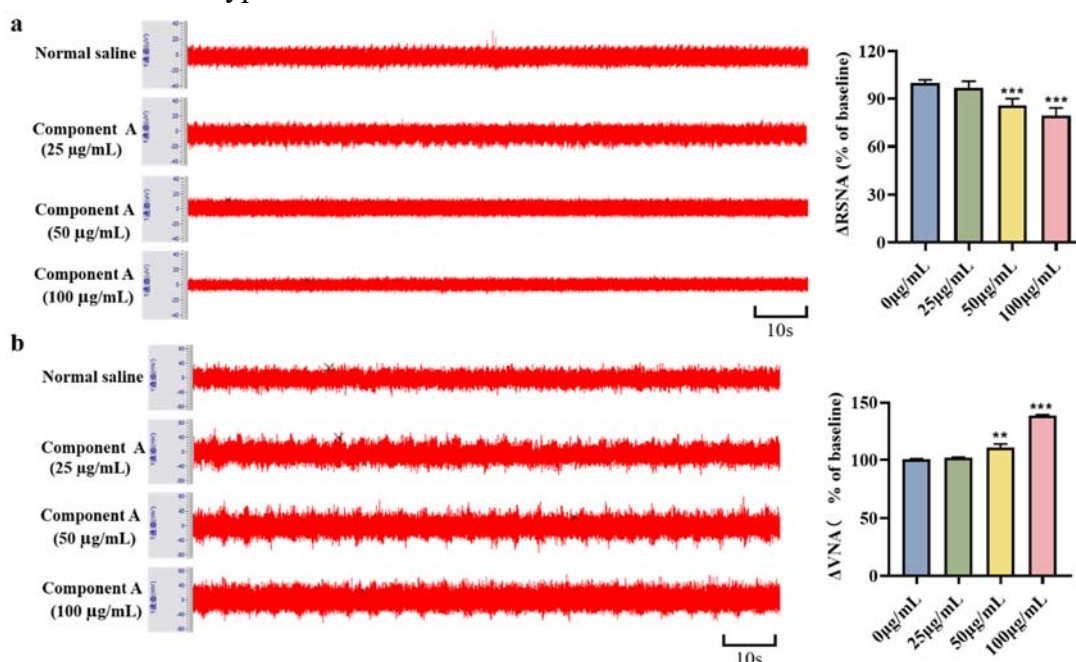


Figure 10. Effect of component A on renal sympathetic nerve activity (RSNA) and vagal nerve activity (VNA) (n=6). **a.** RSNA in SD rats; **b.** VNA in SD rats. The results were presented as mean $\pm$ SD. $\Delta\text{RSNA} \% = [(\text{post-treatment RSNA}/\text{pre-treatment RSNA})] \times 100\%$; $\Delta\text{VNA} \% = [(\text{post-treatment VNA}/\text{pre-treatment VNA})] \times 100\%$. ** $P < 0.01$, *** $P < 0.001$, versus the control group.

3.16 Binding interactions between component A/rubijervine and key proteins

CETSA was employed for label-free target verification, relying on ligand binding to enhance the thermal stability of the target protein. Based on the results of molecular docking, rubijervine was chosen for the CETSA analysis due to its strong binding affinity with the key proteins (ADRB2, AGTR1, MMP9, and eNOS). As illustrated in Figure 11, both component A and rubijervine significantly increased the levels of undegraded ADRB2, AGTR1, MMP9, and eNOS proteins. In the heat melting curve analysis (Figure 11a-2, 11b-2), the curves for HUVEC cells treated with either component A or rubijervine shifted notably to the right at temperatures ranging from 40°C to 55°C when compared to the control curve. This suggests that ADRB2,

AGTR1, MMP9, and eNOS exhibited significant thermal stabilization in the groups treated with component A or rubijervine (Figure 11), with the exception of NNMT. Overall, these findings suggest that component A may directly bind to ADRB2, AGTR1, MMP9, and eNOS to generate biological effects, which is consistent with the results of the molecular docking analysis.

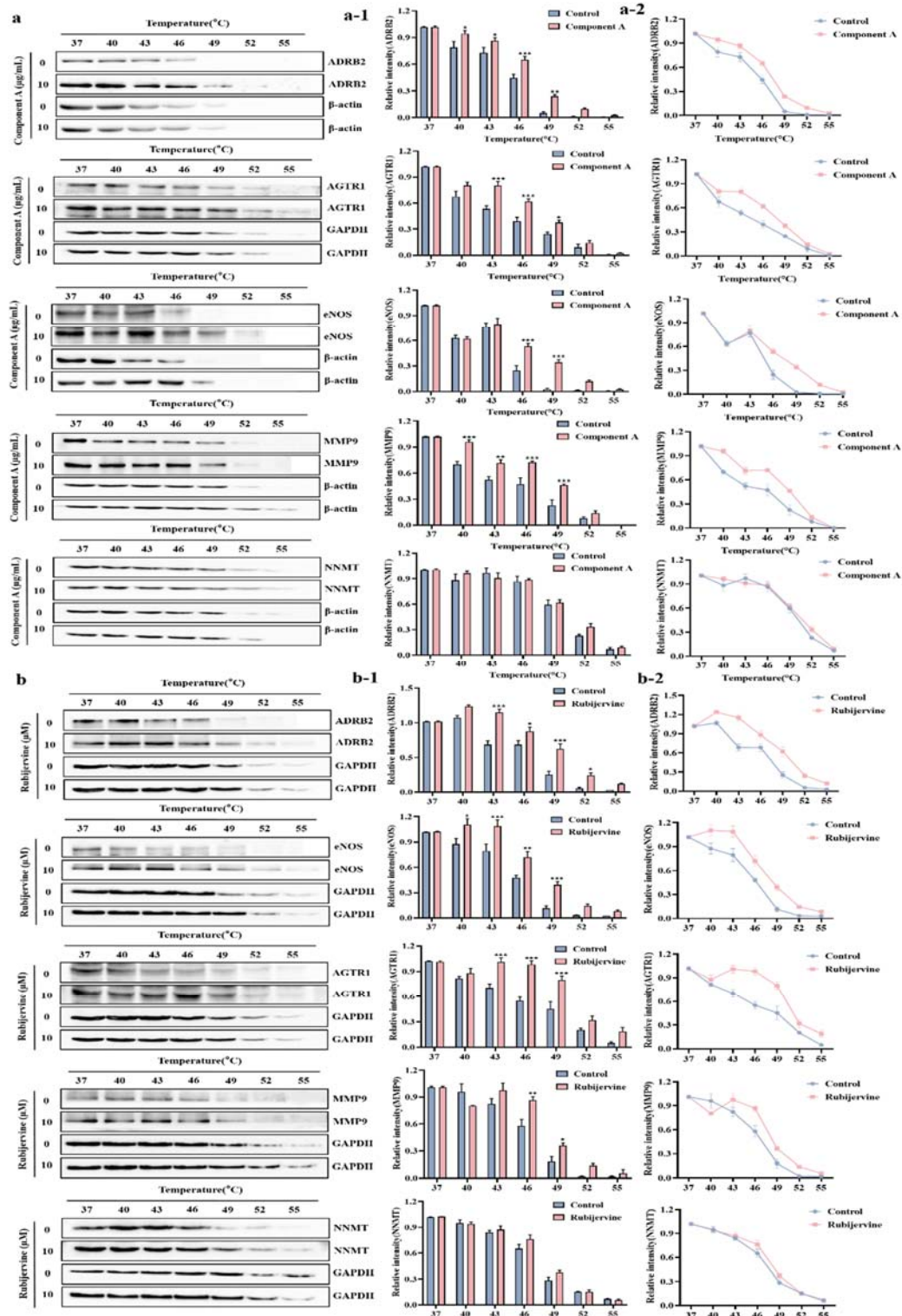


Figure 11. CETSA-based determination of binding between component A/rubijervine and AGTR1, ADRB2, MMP9, eNOS, and NNMT in HUVECs *in vitro*. **a.** component A; **b.** rubijervine. (**a-1** and **b-1**: The quantification of relative intensity of AGTR1, ADRB2, MMP9, eNOS, and NNMT protein versus increased temperature from three independent experiments, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, versus the control group; **a-2** and **b-2**: CETSA curves of AGTR1, ADRB2, MMP9, eNOS, and NNMT in HUVEC cells were determined in the absence and presence of component A/rubijervine. Each band intensity of proteins was normalized with respect to that obtained at 37 $^{\circ}$ C.)

4. Discussion

Hypertension is a major cardiovascular condition that can damage vital organs, increase the risk of other cardiovascular diseases, and pose a serious threat to human health. *Veratrum nigrum* L. had been used in the clinical treatment of hypertension, but few studies have investigated the mechanisms behind its antihypertensive effects. By combining network pharmacology and molecular docking analyses, in vivo and in vitro experiments were conducted to elucidate its characteristics and mechanisms of action.

Hypertensive patients often experience varying degrees of vascular injury, remodeling, and oxidative stress. Vascular endothelial dysfunction and endothelial abnormalities in the endothelium are recognized as the primary molecular mechanisms underlying vascular injury in hypertension [29]. Oxidative stress and inflammation can further worsen endothelial dysfunction during hypertension. In a healthy state, a balance between vasodilating and vasoconstricting factors is crucial for maintaining fundamental vascular tension. However, during hypertension, there is an excessive release of AngII, ET-1, NE, MDA, IL-6, and TNF- α coupled with a decreased production of NO, PGI₂, and SOD. This imbalance can lead to endothelial cell injury, vascular remodeling, and oxidative stress, all of which can exacerbate the condition [30]. Our research has validated that treatment with component A can effectively restore endothelial function while reducing oxidative stress and inflammation in both in vivo and in vitro settings. These findings align with the results from morphological examinations of the aorta and peripheral blood vessels. This effect may be attributed to the re-establishment of balance between vasodilators and vasoconstrictors, as well as the suppression of oxidative stress and inflammation, which helps ameliorate vascular injury and remodeling. However, the impact of component A on vascular tension remains unclear. Future experiments involving isolated rat aorta rings are necessary to evaluate the direct effect of component A on vascular relaxation, which could further dissect the contribution of the endothelium, specifically eNOS-derived NO, and the direct effects on vascular smooth muscle through cAMP/RhoA in the vasorelaxant response.

The network pharmacology results indicated that the biological processes of component A are linked to BP regulation, particularly involving the RAS. Renin, an enzyme that activates angiotensinogen, facilitates the production of angiotensin I, which is subsequently converted to Ang II by ACE. Ang II is the primary peptide responsible for the actions of RAS. Most physiological and pathological effects of Angiotensin II occur through its action on the AGTR1. This interaction induces vasoconstriction, stimulates the release of vasopressin, and promotes the proliferation of VSMCs, resulting in vascular remodeling [31,32]. Component A downregulates the levels of AGTR1 and Ang II in SHR and HUVECs (Figures 4 and 6). Consequently, the biological effects of Ang II can be restrained by component A. Additionally, Ang-(1-7), which is produced from AngII by ACE2, provides protective effects against inflammation and oxidative damage, counteracting the harmful influences of ACE/AngII/AGTR1 pathway [33]. In the cardiovascular systems of SHRs, an overactivation of ACE/AngII/AGTR1 pathway and a weakened ACE2/Ang-(1-7) pathway are observed, both of which are effectively normalized by component A (Figures 2 and 6). Our findings demonstrate that component A can restore balance to the RAS, helping to normalize BP and alleviate vascular injuries.

Matrix metalloproteinases (MMPs) are a group of proteases involved in breaking down the extracellular matrix (ECM), which influences endothelial cell integrity and tissue remodeling [34]. Among MMPs, MMP9, as a key target for the hypotensive effect of component A, is particularly significant due to its role in vascular, myocardial, and renal tissues remodeling. The absence of MMP-9 in VSMCs can prevent the production of active TGF- β , which is crucial for the development of tissue fibrosis [35,36]. Consequently, overexpression of MMP9 can activate the TGF- β /SMAD pathway [37], which inhibits cell apoptosis and promotes the production of ECM proteins, such as collagen, fibronectin, and α -SMA [38]. As expected, component A effectively reduces the expression of MMP9, P-smad2, P-smad3, α -SMA, COL1A1, and TNF- α in SHR (Figures 6 and 9). These reduction aids in preventing vascular remodeling and fibrosis. The effectiveness of component A was confirmed through observations of morphological changes in the aorta and peripheral vessels of SHR (Figure 3). Significant transformations were observed, including a reduction in the thickness of vessel walls, a decline in collagen fiber deposition, and a notable decrease in inflammation. Bcl-2 and Bax are key regulatory factors in apoptosis, playing essential roles in mitochondria-dependent apoptotic signaling. Treatment with component A induces apoptosis and prevents hyperplasia of vascular smooth muscle by via the Bax/Bcl-2 pathway. Since component A and rubijervine have good affinity for MMP9, we infer that component A directly targets MMP9 to suppress the MMP9-mediated TGF- β /Smad pathway.

Adrenoceptors play a crucial role in regulating BP and heart rate by responding to neurotransmitters released from the sympathetic nervous system [39]. When activated, ADRB2 influences sodium excretion in the kidneys, the release of REN, and causes vasodilation by directly activating VSMCs, which helps regulate BP. These mechanisms are essential for both short-term and long-term BP regulation [40,41]. The current study demonstrates that component A and rubijervine directly interact with ADRB2, resulting in increased ADRB2 protein expression in HUVEC cells and SHR (Figures 4, 6, 11). Additionally, component A reduced the expression of REN protein in SHR, suggesting that the biological effects of the ADRB2 protein are enhanced by component A. Furthermore, NE, an alpha-adrenoceptor agonist, can elevate BP. After 14 days of treatment, component A restored ADRB2 and NE levels to normal, indicating that it may help regulate BP by influencing sympathetic activity. Our findings also confirm that component A can modulate both the sympathetic and parasympathetic nervous systems by reducing renal sympathetic nerve activity and enhancing vagal nerve activity, which aligns with predictions from network pharmacology.

The formation of cyclic adenosine 3', 5'-monophosphate (cAMP) in vascular smooth muscle are the prototypical signaling pathway of ADRB [42]. cAMP, as a second messenger, plays an essential role in inhibiting VSMCs, helping to maintain their quiescence, inhibit their proliferation, promote healing after vessel injury, and reduce intimal formation [43]. The relaxation of blood vessels mediated by ADRB2 relies on the cAMP/PKA signaling pathway, which negatively regulates the RhoA/ROCK signaling pathway. RhoA influences MLC phosphorylation through its targets, including ROCK and the myosin-binding subunit (MBS) of myosin phosphatase [44]. This process leads to smooth muscle contraction and stress fiber formation [45]. Additionally, Ang II also directly activates the RhoA/ROCK pathway, promoting VSMC proliferation and

migration by regulating MLC phosphorylation[46]. Our findings imply that component A activates the cAMP pathway, which negatively regulates the RhoA/MLC pathway in the thoracic aorta and heart muscle tissue of SHR. This effect occurs through the upregulation of ADRB2 and cAMP levels, along with the downregulation of AngII levels (Figures 2, 6, 7). Consequently, this cascade not only inhibits the proliferation and migration of VSMCs but also significantly reduces vasoconstriction, suggesting that the activated cAMP signaling pathway may play a critical role in the antihypertensive effects of component A.

Previous research has identified that the nicotinic acid and nicotinamide metabolic pathway is involved in the antihypertension effect of component A[13]. Within this pathway, MNA (a key differential metabolite) is closely associated with vascular remodeling. NNMT is a crucial enzyme that converts nicotinamide into its methylated form, MNA. MNA promotes vasorelaxation by increasing endothelial PGI₂ and NO levels. The vasoprotective, anti-inflammatory, and anti-thrombotic properties of MNA are well-documented, leading to its recognition as a guardian of the cardiovascular system[47]. Experimental results from ELISA, WB, and immunofluorescence studies have demonstrated that both the NNMT/MNA pathway and the eNOS/NO pathway were significantly inhibited *in vivo* and *in vitro* under hypertensive conditions. Notably, intervention with component A effectively reversed this suppression (Figures 2, 4, 8). Furthermore, the NNMT/MNA pathway has been shown to alleviate endothelial dysfunction in human blood vessels by modulating the eNOS/NO pathway[47,48]. Knockdown of NNMT in HUVCEs led to a significant reduction in the component A-induced upregulation of P-eNOS, eNOS, and NO levels (Figure 4e), confirming that NNMT is a vital regulator of the eNOS/NO pathway in the component A-treated HUVCEs. These findings indicate that component A can activate the NNMT/MNA/eNOS/NO pathway. It is also noteworthy that component A can bind directly to eNOS, but not to NNMT. The specific targets of component A that regulate NNMT are currently unclear and will be investigated in future studies.

PPARG is involved in lipid metabolism and inflammation, while MAOA catalyzes the oxidative decomposition of monoamine neurotransmitters. Both proteins emerged as key network hubs with moderate docking scores. The current experimental focus is on the top-ranked targets that are most directly linked to the primary observed phenotypes, such as blood pressure, vascular remodeling, and endothelial function. As a result, the roles of these two proteins have not yet been fully validated and will be explored in future research.

5. Conclusion

In this study, we thoroughly evaluate the therapeutic effectiveness of component A in alleviating hypertension and vascular remodeling after 14 days of administration. Our integrated network pharmacology and biochemical experiments indicate that component A's mechanism for treating hypertension and vascular remodeling involves the modulation of key targets such as ADRB2, AGTR1, MMP9, eNOS, TNF- α , and NNMT. These modulations inhibit the Smad pathway, activate the NNMT/MNA/eNOS/NO pathway and the cAMP-mediated inhibition of the RhoA/MLC pathway, as well as balance the RAS and the nervous system (Figure 12), showcasing the multi-target and multi-pathway nature of the mechanism. Our findings provide a theoretical basis for the development of drugs based on component A.

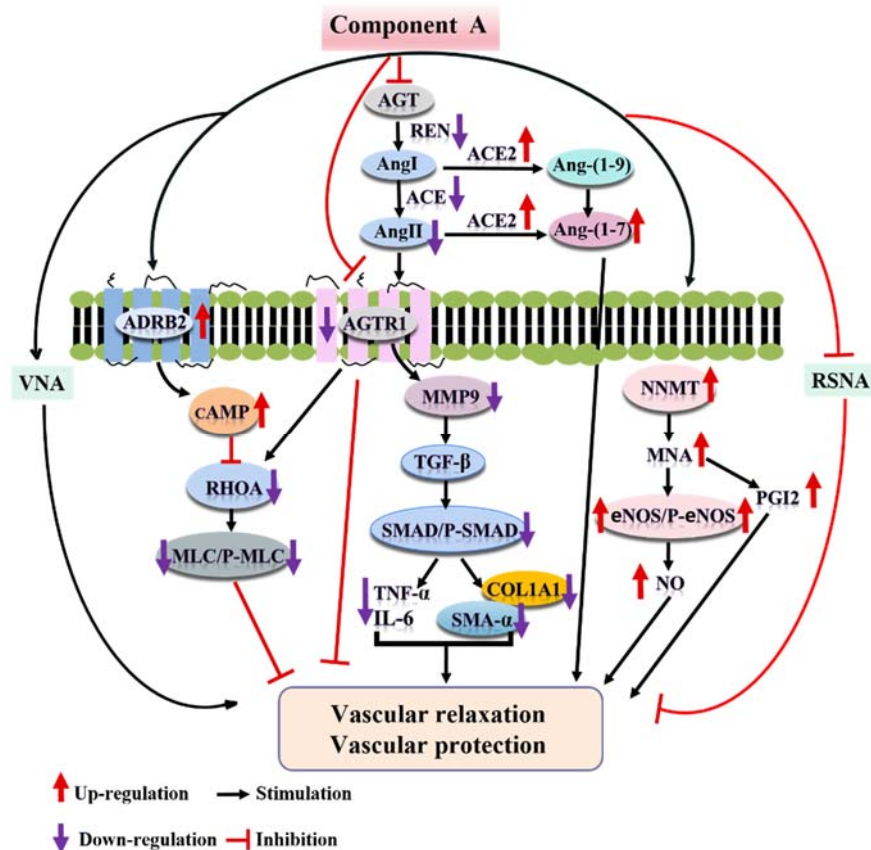


Figure 12. Schematic diagram of the mechanistic role of component A in the treatment of hypertension.

Acknowledgments

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Conflict of Interest

The authors declare that they do not have any conflict of interest.

Data availability statement

Data will be made available on reasonable request.

Supplementary materials

Supplementary material associated with this article can be found in the online version.

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