A Network Pharmacology-based Approach to Explore the Active Ingredients and Molecular Mechanism of Lei-gong-gen Formula Granule on a Spontaneously Hypertensive Rat Model

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Research

Keywords: Centella asiatica (L.) Urb., Eclipta prostrata (L.) L., Smilax glabra Roxb., hypertension, network pharmacology

Posted Date: March 2nd, 2021

DOI: https://doi.org/10.21203/rs.3.rs-253571/v1

Abstract

Background

Lei-gong-gen formula granule (LFG) is a folk prescription derived from Zhuang nationality, which is the largest ethnic minority among the 56 nationalities in China. It consists of three herbs, namely *Eclipta prostrata* (L.) L., *Smilax glabra* Roxb, and *Centella asiatica* (L.) Urb. It has been widely used as health protection tea for hundreds of years to prevent hypertension in Guangxi Zhuang Autonomous Region. The purpose of this study is to validate the antihypertensive effect of LFG on a SHR model and further to identify the active ingredients of LFG and its anti-hypertension molecular mechanism.

Methods

Firstly, a spontaneously hypertensive rat model was used to observe the effects of LFG on blood pressure, body weight, and heart rate. The serum levels of NO, ANG Ⅱ, and ET-1 were measured and H&E staining was applied to observe the pathology of the heart. Secondly, network pharmacology analysis was performed to collect the ingredients of LFG by using the database of traditional Chinese medicine (TCMSP, TCMID, BATMAN-TCM) and to predict the active compounds, their corresponding targets, and hypertension associated pathways. Then the predicted results were verified by molecular biology experiments such as RT-qPCR and western blot. Finally, the potential active compounds were predicted by molecular docking technology, and the LFG chemical constituents were analyzed and identified by UPLC-QTOF/MS technology.

Results

LFG significantly reduced blood pressure and increased serum NO content in SHR rats. LFG ameliorated the pathological changes such as cardiac hypertrophy and interstitial inflammation. Based on the results of public database searching, 53 candidate active compounds from LFG were collected, which link to 765 potential targets. 828 hypertension associated targets were retrieved. 12 overlapped targets both related to candidate active compounds from LFG and hypertension were screened and used as the potential targets of LFG on antihypertensive effect. And the 12 overlapped targets were validated using RT-qPCR, and the results showed that LFG could upregulate the mRNA expression of NOS3 and proto-oncogene tyrosine-protein kinase SRC (SRC) in the thoracic aorta. Pathway enrichment results showed that PI3K-AKT signaling pathway was closely related to the expression of NOS3 and SRC. To verify this result, western blot was used to detect the key proteins (PI3K, AKT, and p-AKT) of the PI3K-AKT signaling pathway. The results showed that LFG significantly increased the protein expression levels of PI3K and phosphorylated AKT in SHR rats, suggesting that LFG may active PI3K-AKT signaling pathway to decrease hypertension. Molecular docking study further supported that p-hydroxybenzoic acid, cedar acid, shikimic acid, salicylic acid, nicotinic acid, linalool, and histidine can be well binding with NOS3, SRC,

PI3K, and AKT. UPLC-QTOF/MS analysis confirmed that p-hydroxybenzoic acid, shikimic acid, salicylic acid, and nicotinic acid existed in LFG.

Conclusion

LFG can reduce the blood pressure of SHR rats, which might be attributed to increasing the serum NO level for promoting vasodilation via upregulating SRC expression level and activating the PI3K-AKT-NOS3 signaling pathway. p-Hydroxybenzoic acid, shikimic acid, salicylic acid, and nicotinic acid might be the underlying compounds for LFG antihypertensive effect.

1. Introduction

Hypertension is one of the most common cardiovascular diseases, which can cause damage to the heart, brain, kidney, and other important organs [1]. It is an important risk factor for the death of the global population and poses a serious threat to the safety and quality of patients' life. China has conducted large-scale population surveys of hypertension, and these surveys showed consistently a rapidly increasing trend in the hypertension prevalence, from 5.1% in 1959 to 27.8% in 2014 [2]. In Europe, Canada, and the United States, the prevalence is reported to vary from 27–55% [3]. Hypertension is a chronic disease, and there is no complete cure for high blood pressure. Currently, drug treatment is mainly used to control the level of blood pressure and bring it down to a normal range in clinical practice. Although the existing antihypertensive drugs can effectively control blood pressure in the short term, it is prone to develop adverse effects for the long term taken, which greatly affects their treatment compliance [4]. Therefore, the development of drugs which can effectively lower the blood pressure with fewer adverse reactions is still the main direction of current research. Traditional Chinese Medicine (TCM) has accumulated much experience in the treatment of hypertension [5]. TCM has the characteristics of multi-component, multi-target, and multi-pathways, especially has a certain effect in improving the complications of hypertension [6]. Thus, screening high-efficiency antihypertensive drugs based on Chinese medicine formula with fewer side effects has attracted considerable attention.

Lei-gong-gen formula granule (LFG) is an effective antihypertensive traditional Chinese folk prescription which is soaked in wine or taken as tea by the local residents in Guangxi Zhuang Autonomous Region for many years [7]. Epidemiological data showed that the prevalence of hypertension in local residents was 13.70%, which was significantly lower than the national average (25.2%) [8]. It is speculated that the lower incidence of hypertension of the local people may be related to their long-term using of this formula. LFG is composed of three herbs, containing *Centella asiatica* (L.) Urb. (Ji Xue Cao, also known as Lei-gong-gen in Chinese medicine), *Eclipta prostrata* (L.) L. (Mo Han Lian), *and Smilax glabra* Roxb. (Tu Fu Ling). *Centella asiatica* (L.) Urb., a tropical medicinal plant belonging to the family *Apiaceae* (Umbelliferae), is widely distributed in many parts of the world especially in Asian countries including India, China, and Nepal. *Centella asiatica* (L.) Urb. was reported to exhibit cardioprotective, antihypertensive, antioxidant, and anti-inflammatory activities [9]. Recent studies showed that asiatic acid

reduced blood pressure, improved vascular function by restoring endothelial NO synthase (eNOS), and p47phox expression, and alleviates cardiovascular remodeling by restoring iNOS/eNOS expression in L-NAME hypertensive rats [10]. *Eclipta prostrata* (L.) L. is an annual herb of the Asteraceae family, native to China, Japan, and India [11]. *Eclipta prostrata* (L.) L. was reported to have significant pharmacological features such as antioxidant, anti-inflammatory, and hypotensive activities [12]. *Smilax glabra* Roxb., is a rhizome of the Liliaceae plant [13]. Pharmacological studies demonstrated that the flavonoids of *Smilax glabra* Roxb. exhibited anti-cardiac hypertrophy and anti-hypertensive effects on renovascular hypertension rats through regulating the level of atrial natriuretic peptide [14].

Chinese herbal formulae containing a large number of compounds are too complex to be analyzed by traditional experimental methods based on the paradigm of “One gene, one drug, one disease” [15]. Accumulating evidence shows that network pharmacology can elucidate the potential mechanisms of multi-component and multi-target agents by analyzing various complex and multi-level interaction networks [16]. Network pharmacology has become a powerful tool to reveal the functions and behaviors of complex biological systems systematically, and it has become a novel and effective way to explore the pharmacological mechanism of TCM [17].

In this study, we investigated the active ingredients, corresponding targets, and pharmacological mechanisms of LFG antihypertensive effect by integrating network pharmacology prediction and molecular biology experimental validations. As shown in Fig. 1, the whole study consists of three steps. Step 1: animal experiments. A spontaneously hypertensive rat (SHR) model was firstly used to observe the antihypertensive effect of LFG. Step 2: network pharmacology analysis. The active ingredients, corresponding targets, and hypertension associated pathways were predicted by public databases. Protein-protein interaction (PPI) networks of LFG-related and hypertension-related targets were constructed, and core targets were identified through topological analysis. Step 3: experimental verification. The predicted results were verified by molecular biology methods such as RT-qPCR and western blot based on SHR model. The potential active ingredients were predicted by molecular docking technology, and the LFG chemical constituents were analyzed and identified by Ultra performance liquid chromatography/quadrupole time-of-flight mass spectrometry (UPLC-QTOF/MS) technology. The findings of this study may contribute to the clarification of active ingredients and underlying mechanisms of LFG and provide an accurate and reasonable reference of LFG clinical application.

2. Materials And Methods

2.1. Drugs and reagents

Lei-gong-gen formula granule (LFG) was provided by Guangxi Institute of Chinese Medicine and Pharmaceutical Science (Guangxi, China, lot No. 20170210). The authentication of three dried herbal drugs (*Centella asiatica* (L.) Urb., *Eclipta prostrata* (L.) L., *Smilax glabra* Roxb.) has been specified, and the preparation of LFG was shown in our previous research [18, 19]. Captopril was obtained from Teyi Pharmaceutical Group Co., Ltd (Guangdong, China, lot No. 20170501).

Nitric oxide (NO) assay kit was obtained from Nanjing Jiancheng Bioengineering Institute (Nanjing, China, Cat. A013-2). Rat angiotensin Ⅱ (ANG Ⅱ, Cat. CSB-E04494r) and rat endothelin 1 (ET-1, Cat. CSB-E06979r) ELISA kits were obtained from CUSABIO (Wuhan, China). Trizol reagent (Cat. 15596026), cDNA Reverse Transcription Kit (Cat. 4368814), SYBR Green Master Mix (Cat. A25742), Pierce Protease and Phosphatase Inhibitor Mini Tablets (Cat. A32961), RIPA buffer (Cat. 89900), Prestained Protein Ladder (Cat. 26616), Tween 20 (Cat. 85113), Chemiluminescent Substrate (Cat. 24580) were purchased from Thermo Fisher Scientific (Waltham, USA). The Bicinchoninic acid (BCA) Protein Assay Kit (No. P0011) was purchased from Beyotime Biotechnology Co., Ltd. (Shanghai, China).

GAPDH (14C10) Rabbit mAb (Cat. 2118), PI3 Kinase p110α (C73F8) Rabbit mAb (Cat. 4249), AKT Antibody (Cat. 9272), Phospho-AKT (Ser473) (D9E) XP Rabbit mAb (Cat. 4060) and Anti-Rabbit IgG HRP-linked antibodies (Cat.7074) were purchased from Cell Signaling Technology (Danvers, USA).

Methanol (HPLC grade) was purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). Formic acid (HPLC grade) was purchased from Aladdin Reagent Co., Ltd. (Shanghai, China). The reference standard of shikimic acid (lot No. C11015240) was purchased from Shanghai Macklin Biochemical Co., Ltd. The reference standard of isoengelitin (lot No. ZW190519-14) was purchased from Stanford Analytical Chemicals Inc. The reference standard of 4-dicaffeoylquinic acid (lot No. 34770010) was purchased from ANPEL Laboratory Technologies (Shanghai) Inc. The reference standard of p-hydroxybenzoic acid (lot No. H1926195) was purchased from Shanghai Aladdin Reagent Co., Ltd. The reference standards of engeletin (lot No. 111906–201102), astilbin (lot No. 111798–201504), ferulic acid (lot No. 110773–201313), luteolin (lot No. 111520–201605), isochlorogenic acid C (lot No. 111894–201102), salicylic acid (lot No. 100106–201104), nicotinic acid (lot No. 100434–201603) were purchased from National Institutes for Food and Drug Control (Beijing, China). The reference standards of neoastilbin (lot No. DST191209-077), isoastilbin (lot No. DST190922-216), neoisoastilbin (lot No. DST191025-078) were purchased from Chengdu Pufei De Biotech Co., Ltd.

2.2. Animals and experimental design

A total of 40 male Spontaneously hypertensive rats (SHR) (180-220g), aged 10 weeks, were provided by Beijing Vital River Laboratory Animal Technologies Co. Ltd (Beijing, China, certificate number: SCXK-JING 2016-0006). All animals were maintained under standard laboratory conditions with a regular 12-hour light/dark cycle, a relative humidity of 50 ± 5%, and temperature of 25 ± 2°C. The experimental procedures were approved by the Institutional Animal Ethics Committee (IAEC), Guangxi Medical University.

The rats were provided with standard rat chow and water *ad libitum*. After an acclimatization period for two weeks, the basic value of blood pressure, body weight, and heart rate were measured. SHR rats were randomly assigned to five groups based on the basic value of blood pressure: model group (saline, 10 mL/kg), captopril group (0.03 g/kg), LFG high dose group (20 g/kg), LFG middle dose group (10 g/kg), LFG low dose group (5 g/kg), eight rats per group. Saline served as a negative control and captopril, a proven hypertensive drug [20], as a positive control. LFG and captopril were administered intragastrically twice a day for 3 weeks. The systolic blood pressure, body weight, and heart rate of rats were measured

every three days. The blood pressure and heart rate were measured using ALC-NIBP non-invasive blood pressure system (Shanghai Alcott Biotech Co., Ltd, China) at the tail artery employing tail-cuff method, while body weight was measured using an electronic balance (CP214, Ohaus, USA). When the experimental period was complete, rats were anaesthetized and blood was collected from the abdominal aorta. The serum samples were obtained after the blood samples were centrifuged. The heart and thoracic aorta were collected respectively for hematoxylin-eosin (H&E) staining and stored at -80ºC for real-time PCR and western blot analysis.

2.3. Determination of serum NO, ANG Ⅱ, and ET-1 content

Serum NO level was measured by Nitric oxide (NO) assay kit obtained from Nanjing Jiancheng Bioengineering Institute. Serum ANG Ⅱ and ET-1 content were respectively measured using commercially available immunoassay ELISA kits for rats.

2.4. Heart histopathological analysis

Heart tissue samples were fixed in 4% paraformaldehyde, dehydrated, and washed. The samples were embedded in paraffin and sliced into 5 μm by paraffin slicing machine. The sections were dewaxed in xylene and dehydrated in descending grades of ethanol. The sections were stained by hematoxylin for 5 min. After washing, the sections were treated with eosin solution for 2 min for coloration, followed by ethanol dehydration, and neutral gum sealing. Finally, the specimens were further observed and photographed under a BX53 microscope (Olympus, Takachiho, Japan).

2.5. Network pharmacology analysis

2.5.1. Composite compounds of LFG

LFG consists of three herbs, namely *Centella asiatica* (L.) Urb., *Eclipta prostrata* (L.) L., *Smilax glabra* Roxb. To collect the compounds of LFG, we used the TCM Systems Pharmacology Database [21] (TCMSP, http://tcmspw.com/tcmsp.php) (accessed 18th January 2018), TCM Integrated Database [22] (TCMID, http://www.megabionet.org/tcmid/) (accessed 18th January 2018), and Bioinformatics Analysis Tool for Molecular mechanism of TCM [23] (BATMAN-TCM, http://bionet.ncpsb.org/batman-tcm/) (accessed 19th January 2018). Candidate compounds were screened according to the criteria of oral bioavailability (OB) > 30% of the pharmacokinetic parameters [24].

2.5.2. Targets collection

The SMILES of the candidate compounds in LFG as the inputs of target fishing database were obtained from Pubchem database (https://pubchem.ncbi.nlm.nih.gov/). Subsequently, the predicted targets were retrieved from ChemMapper database [25] (http://lilab.ecust.edu.cn/chemmapper/) (accessed 26th January 2018), an online tool based on 3D similarity for targets prediction. The targets associated with hypertension were collected by Text-mined Hypertension, Obesity and Diabetes candidate gene database [26] (T-HOD, http://bws.iis.sinica.edu.tw/THOD/) (accessed 28th January 2018), a tool developed to help trace existing research on three kinds of cardiovascular disease. Furthermore, the overlapped targets that

were both related to the candidate compounds and hypertension were kept for network construction and analysis.

2.5.3. Network construction

Network construction was performed as follows: (1) Herb-compound network; (2) Compound-target network; (3) Hypertension-target network; (4) The protein-protein interaction (PPI) network of intersection targets between compounds and hypertension targets.

All the networks were created via utilizing Cytoscape (htttps://cytoscape.org/, Version 3.2.1), a general platform for complex network analysis and visualization [27]. The PPI network was constructed by Search Tool for the Retrieval of Interacting Genes/Proteins database [28] (STRING, https://string-db.org/, Version 11.0), and then visualized in Cytoscape. The topological parameters of nodes in PPI network were calculated by Network Analyzer, and the nodes whose degree values were twice the average degree values of the network were selected as the hub targets for further analysis.

2.5.4. Pathway enrichment

The Database for Annotation, Visualization and Integrated Discovery [29] (DAVID, https://david.ncifcrf.gov/, Version 6.8) was applied for Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis of the hub targets. *P*-values were set at 0.05 as the cut-off criterion.

2.6. Real-time RT-PCR analysis

Quantitative PCR analysis was performed to examine the mRNA expression levels of predicted hub targets. In brief, total RNA was extracted from thoracic aorta tissue using Trizol reagent, and the quality and purity of RNA were evaluated by Nanodrop2000 (Thermo Fisher, USA). The RNA (2 μg) was reverse-transcribed using the High Capacity cDNA Reverse Transcription Kit under reaction conditions with 25°C for 10 min, 37°C for 120 min and 85°C for 5 min. Real-time PCR amplification was performed with SYBR Green Master Mix on the 7300 Real time PCR system (Applied Biosystems) following the manufacturer's instructions. The relative RNA expression levels were calculated using the ΔΔ cycle threshold method [30], and β-actin was applied to normalize the expression levels of target genes for each sample. Primer sequences were listed in Table 1.

Table 1
Primer sequence used for PCR analysis

Gene	Forward	Reverse
NOS3	5'-CAGAGATTGGCATGAGGGACC-3'	5'-TCCACAGTGATGAGGTTGTCC-3'
SRC	5'-CAAGATCACTAGACGGGAATCAG-3'	5'-GTTTCACATTTAGGCCCTTGG-3'
PTGS2	5'-ATCAGAACCGCATTGCCTCT-3'	5'-GCCAGCAATCTGTCTGGTGA-3'
NGF	5'-GTCTGGGCCCAATAAAGGCT-3'	5'-TGTACGCCGATCAAAAACGC-3'
VEGFA	5'-CGTCCTGTGTGCCCCTAATG-3'	5'-TGTGCTGGCTTTGGTGAGGT-3'
EGF	5'-CTACTACAGGACTCGGAAGCAG-3'	5'-GTTGGGGACCAGAAGACACC-3'
EGFR	5'-AGCCGTCCTGTCCAACTATG-3'	5'-TTGCTAAATCGCACAGCACC-3'
ERK2	5'-ATTGGTCAGGACAAGGGCTCA-3'	5'-CCACTACGACCAGAACTGCC-3'
CCL2	5'-TGCAGGTCTCTGTCACGCTTC-3'	5'-TTCTCCAGCCGACTCATTGG-3'
NPY	5'-GCCAGATACTACTCCGCTCTG-3'	5'-GTCTCAGGGCTGGATCTCTTG-3'
ESR1	5'-CAGACAGGGAGCTGGTTCATA-3'	5'-GCACACTCCAGAAGGTGAACT-3'
TH	5'-GTCTCAGAGCAGGATGCCAAG-3'	5'-ATCCTCGATGAGACTCTGTCGC-3'
β-actin	5'-GTCAGGTCATCACTATCGGCAAT-3'	5'-AGAGGTCTTTACGGATGTCAACGT-3'

2.7. Western blot analysis

The key protein expression levels of PI3K-AKT signaling pathway were determined by western blot analysis. Total proteins extracted from thoracic aorta by lysis buffer supplemented with phosphatase and protease inhibitors. The protein concentrations were measured by BCA Protein Assay Kit. Protein samples (30 μg) were separated on 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to polyvinylidene difluoride (PVDF) membranes. The membranes were blocked for 1 hour at 4°C with 5% nonfat milk, while the phosphorylated proteins were blocked with 5% bovine serum albumin (BSA), and incubated overnight with anti-PI3K (1:1000) antibody, anti-AKT (1:1000) antibody, anti-p-AKT (1:1000) antibody and anti-GAPDH (1:5000) antibody, respectively. The membranes were washed 3 times and incubated with secondary antibody (horseradish peroxidase-conjugated, 1:5000 dilution) for 1 hour at room temperature. The GAPDH was applied to show equal protein loading for each sample. The bands were visualized by chemiluminescence using MiniChemi 610 Plus (Sage Creation, Beijing), and Image J software (Version 1.43) was used to analysis the intensity of bands.

2.8. Molecular docking

Molecular docking is a computational tool that can predict the interaction energy between receptors and ligands. The binding mode and affinity between targets and compounds was performed using the Surflex-Dock (SFXC) module of Sybyl X2.0 and visualized with PyMOL (Version 1.5.0.3). The structures of

the potential active compounds related to the potential targets were downloaded from the Pubchem database, and converted into mol2 format by Open Babel GUI (Version 2.2.1). All the crystal structures of the targets were downloaded from Protein Data Bank (PDB, https://www.rcsb.org/), including endothelial nitric oxide synthase (NOS3) (PDB ID: 4D1O), SRC (PDB ID: 1A07), PI3K (PDB ID: 4L23), and AKT (PDB ID: 1H1O). Before docking, the proteins were subjected to the necessary preparation steps (remove water molecules, and add hydrogen atoms), and ligands were optimized by Tripos force field. The total score (pKd) calculated by SFXC was used to determine the affinity of receptors and ligands. The total score greater than 6 was regarded as good protein-ligand binding.

2.9. UPLC-QTOF/MS analysis of LFG

LFG extract was dissolved by ultrasonification in 50% methanol (in water) for 1 hour, and filtered through a 0.22 μm syringe filter before analysis.

UPLC analysis conditions were as follows: Separation of the compounds was carried out on a Dionex Ultimate 3000 UPLC system (Thermo fisher, USA) equipped with a SunFire C18 column (250 mm × 4.6 mm, i.d., 4.6μm, Waters, USA). The analytical column was maintained at a temperature of 35°C and the mobile phase was composed of water A (containing 0.1% formic acid) and acetonitrile B. A solvent gradient system was used: 10–10% B for 0-10min, 10–30% B for 10-50min, 30–90% B for 50–58 min, 90–90% B for 58–64 min, 90 – 10% B for 64–65 min and 10–10%B for 65–75 min. The flow rate was 0.8 ml/min. The injection volume was 10 μl. A DAD detector and a mass spectrometer detector were connected in series behind the column. The outlet of the DAD detector was split into the mass spectrometer detector with a split ratio of 4:1.

MS analysis conditions were as follows: MS analysis was performed on the Bruker definition accurate mass quadrupole time-of-flight (Q-TOF) Impact II mass spectrometer (Bruker Daltonics Inc., GER) equipped with electrospray ionization (ESI) source. The capillary voltage was set at 2500 V, the dry temperature was 200°C, the dry gas rate was set at 4.0 L/min, the energies for collision induced dissociation (CID) were 10 eV for the precursor ion and 32.5–97.5 eV for fragmentation information. A full-scan mass range of m/z 50-2000 was scanned. Data were acquired in Line Spectra mode.

2.10. Statistical analysis

Statistical analysis was performed by SPSS software (Version 22.0). The data were expressed as mean ± SEM. The parameters among different groups were compared by one-way analysis of variance (ANOVA) followed by LSD (equal variances) or Dunnett's T3 (unequal variances). *P*-value < 0.05 was considered as statistically significant.

3. Results

3.1. LFG exerts an anti-hypertensive effect in SHR

To assess the anti-hypertensive effect of LFG, SHR were treated with different doses of LFG for 3 weeks. As shown in Fig. 2A, the blood pressure of LFG treatment groups was significantly decreased compared to model group ($P < 0.05$). But there were no differences both in body weight and heart rate between LFG treatment groups and model group ($P > 0.05$) (Fig. 2B-C).

3.2. Effect of LFG on serum NO, ANG Ⅱ and ET-1 levels

Serum NO, ANG Ⅱ and ET-1 levels of animals from various groups were summarized in Fig. 3. The level of serum NO increased significantly in LFG high-dose group and LFG middle-dose group compared to model group ($P < 0.01$ or 0.05) (Fig. 3A). However, there was no significant difference of LFG low-dose group compared to model group, and significant differences in the ANG Ⅱ and ET-1 contents between model group and treatment groups were not observed ($P > 0.05$) (Fig. 3A-C).

3.3. Effect of LFG on myocardial morphological change

As shown in Fig. 4, myocardial samples from model group developed obvious cardiac hypertrophy and fibrosis, as the swelling and sparsely arranged myocardial cells, increased single myocardial cell area, granular content, and karyopyknosis in some myocardial cells (Fig. 4A). These phenomena were improved in captopril group (Fig. 4B). Compared with model group, LFG treatment groups reduced the swelling degree of myocardial cells and decreased the single myocardial cell area, and three doses of LFG administration decreased the area of myocardial fibrosis to some extent (Fig. 4C-E).

3.4. Composite compounds of LFG

A total of 70 compounds were collected according to the ADME threshold of OB≥30%, and the herb-compound network was shown in Fig. 5A, which included 73 nodes and 77 edges. The ellipse nodes represented herbs, and the hexagon nodes were compounds. Among these compounds, yellow parts were the common components of *Centella asiatica* (L.) Urb. and *Smilax glabra Roxb.*, and quercetin (red hexagon) was common compounds of 3 herbs.

3.5. Compound-target network

We predicted the putative targets of compounds based on the similarities in drug structures by ChemMapper database. While 17 herbal compounds' relevant targets were not observed, so they were removed from the list of 70 herbal compounds. A total of 765 targets were predicted to be hit by the chemical constituents containing in LFG, and the detailed information about the targets were shown in our previous study [19]. The compound-target network was shown in Fig. 5B, the V shape nodes represented 53 compounds, and the 765 yellow nodes were targets corresponding to the components.

3.6. Hypertension targets and key targets

After collection, 828 hypertension related genes were obtained from T-HOD database (Additional file 1, Table S1). The hypertension-target network was established, as shown in Fig. 5C, the red node in the center was hypertension, and the green nodes represented gene targets. A total of 101 overlapped genes, which were both related to candidate compounds and hypertension were kept for PPI network construction using STRING database. The PPI network was visualized by Cytoscape software, as shown in Fig. 5D, the size of nodes changes from big to small according to degree values, and the 12 yellow nodes were recognized as the key targets after screening, including NOS3, SRC, prostaglandin G/H synthase 2 (PTGS2), nerve growth factor (NGF), vascular endothelial growth factor A (VEGFA), epidermal growth factor (EGF), epidermal growth factor receptor (EGFR), mitogen-activated protein kinase 1 (ERK2), C-C motif chemokine 2 (CCL2), neuropeptide Y (NPY), estrogen receptor (ESR1), and tyrosine 3-monooxygenase (TH). There were 37 compounds associated with these 12 key genes (Table 2).

Table 2
The information of 12 key targets between compounds and hypertension targets

Gene	Compounds	Gene	Compounds
VEGFA	glucoside	MAPK1	Cedar acid
NPY	Ferulic Acid	MAPK1	Ferulic Acid
EGF	salicylic acid	NOS3	L-Bornyl acetate
EGF	nicotinic acid	NOS3	p-Hydroxybenzoic acid
EGF	p-Hydroxybenzoic acid	NOS3	salicylic acid
EGFR	naringenin	NOS3	Pulegone
TH	(1R,2R,5S)-2,6,6-trimethylnorpinan-3-one	NOS3	Cedar acid
TH	Ferulic Acid	NOS3	nicotinic acid
TH	Histidine	NOS3	Histidine
NGF	(-)-alpha-Pinene	NOS3	(R)-linalool
NGF	p-Hydroxybenzoic acid	NOS3	5-Hydroxymethylfurfural
NGF	(-)-nopinene	ESR1	L-Bornyl acetate
SRC	Shikimic Acid	ESR1	(1R,2R,5S)-2,6,6-trimethylnorpinan-3-one
SRC	nicotinic acid	ESR1	Pratensein
SRC	Histidine	ESR1	alpha-Terthienyl methyl acetate
PTGS2	naringenin	ESR1	diosgenin
PTGS2	Histidine	ESR1	(+)-Isoborneol
PTGS2	demethylwedelolactone	ESR1	sitosterol
PTGS2	acacetin	ESR1	Stigmasterol
PTGS2	Ferulic Acid	ESR1	butin
PTGS2	Xanthanoic acid	ESR1	Baimuxinol
PTGS2	5-Hydroxymethylfurfural	ESR1	(Z)-1-(2,4-dihydroxyphenyl)-3-(3,4-dihydroxyphenyl)prop-2-en-1-one
PTGS2	(L)-alpha-Terpineol	ESR1	(-)-nopinene

Gene	Compounds	Gene	Compounds
PTGS2	(1R,2R,5S)-2,6,6-trimethylnorpinan-3-one	ESR1	1,8-cineole
PTGS2	Pulegone	ESR1	(-)-alpha-Pinene
PTGS2	MENTHOL	ESR1	demethylwedelolactone
PTGS2	L-Limonen	ESR1	3'-O-Methylorobol
PTGS2	salicylic acid	ESR1	wedelolactone
PTGS2	nicotinic acid	ESR1	luteolin
PTGS2	p-Hydroxybenzoic acid	ESR1	acacetin
PTGS2	L-Bornyl acetate	ESR1	Dihydroresveratrol
PTGS2	Cedar acid	ESR1	naringenin
CCL2	Ferulic Acid	ESR1	cis-resveratrol
MAPK1	p-Hydroxybenzoic acid		

3.7. Pathway enrichment of key targets

To elucidate the crucial pathways among the key targets in hypertension treatment, KEGG pathway enrichment analysis was performed. The top 10 pathways were shown in Fig. 6A, including Bladder cancer, VEGF signaling pathway, Rap1 signaling pathway, Estrogen signaling pathway, HIF-1 signaling pathway, Oxytocin signaling pathway, PI3K-AKT signaling pathway, Pancreatic cancer, Proteoglycans in cancer, and Prolactin signaling pathway. The compound-target-pathway network was summarized in Fig. 6B, where compounds were indicated by purple nodes; gene targets were shown as yellow nodes; 34 pathways were labeled with green nodes.

3.8. RT-qPCR verification

To further validate the 12 hub targets predicted from network pharmacology approach, the mRNA expression levels were measured by real-time RT-PCR. As shown in Fig. 7, the results revealed that the mRNA expression levels of NOS3 and SRC in LFG high-dose group increased significantly compared with model group ($P < 0.01$ or 0.05). Compared to model group, the mRNA expression level of NOS3 was significantly upregulated in LFG middle-dose group ($P < 0.05$). For the other predicted genes, there were no significant difference between model groups and LFG treatment groups ($P > 0.05$) (Fig. 7C-L). Therefore, we only focused on NOS3 and SRC in the following study.

3.9. Western blot verification

The results of RT-qPCR revealed that LFG could regulate the expression of NOS3 and SRC which associated with 10 compounds. At the same time, pathway enrichment analysis of these 2 genes was performed, and the result shown that the antihypertensive effect of LFG may be exerted through PI3K-AKT signaling pathway. Then we conducted the compound-target-pathway network of significant genes. As shown in Fig. 8A, compounds were indicated by diamond nodes (p-Hydroxybenzoic acid, Nicotinic acid, Salicylic acid, (R)-Linalool, Cedar acid, L-Bornyl acetate, Pulegone, 5-Hydroxymethylfurfural, Shikimic acid, and Histidine), and pathways were shown as hexagon nodes.

To further verify the role of PI3K-AKT signaling pathway on the antihypertensive effect of LFG, the protein expression levels of PI3K, AKT, and p-AKT in the thoracic aorta tissue were measured by western blot analysis. As shown in Fig. 8B, expression levels of PI3K and p-AKT were increased in LFG high-dose group and LFG middle-dose group compared to model group ($P < 0.01$ or 0.05). There was no significant difference in the level of total AKT expression between model group and treatment groups ($P > 0.05$).

3.10. Molecular docking analysis

To elucidate the interaction between targets (NOS3, SRC, PI3K, and AKT) and the 10 potential active compounds, a molecular docking simulation was performed using Sybyl X2.0 to investigate the binding modes of them. Docking score greater than 6 indicates good protein-ligand binding activity. The docking results of protein-ligand interaction were summarized in Table S2-S5 (Additional file 1). The binding modes of receptors and ligands with good affinity were shown in Fig. 9. In summary, histidine, cedar acid, linalool, and p-hydroxybenzoic acid were successfully docked in NOS3 (Fig. 9A-D). Shikimic acid, salicylic acid, histidine, cedar acid, nicotinic acid, and p-hydroxybenzoic acid were docked in SRC (Fig. 9E-J). Cedar acid, p-hydroxybenzoic acid, and linalool were docked in PI3K (Fig. 9K-M). Cedar acid, shikimic acid, and nicotinic acid were docked in AKT (Fig. 9N-P). Interestingly, cedar acid can be docked in NOS3, SRC, PI3K, and AKT, and p-hydroxybenzoic acid can be docked in NOS3, SRC, and PI3K. Molecular docking results revealed that 7 compounds (Cedar acid, p-hydroxybenzoic acid, histidine, shikimic acid, salicylic acid, linalool, nicotinic acid) exhibited good interaction with NOS3, SRC, PI3K, and AKT.

3.11. LC-MS/MS analysis of LFG

In order to verify whether the 7 components predicted by molecular docking exist in LFG, LC-MS/MS was performed to analysis the chemical constituents of LFG. The representative base peak intensity (BPI) chromatogram in negative ion mode was shown in Fig. 10. After comparing the components with the reference standard and LFG (Additional file 2, Figure S1-S18), eighteen compounds were detected, and the detail information of these compounds was shown in Table 3. Among these compounds, four compounds (p-hydroxybenzoic acid, shikimic acid, salicylic acid, nicotinic acid) well docked in NOS3, SRC, PI3K, and AKT were confirmed to exist in LFG.

Table 3
Identify of the compounds of LFG by UPLC- Q-TOF/MS analysis in negative ion mode

NO.	R-T	m/z	MS/MS	Elemental composition	Ion form	ppm	Compounds
1	3.73	173.0458	93.0354	$C_7H_{10}O_5$	$[M-H]^-$	-1.3	Shikimic acid
2	3.90	124.0394	78.0334/80.0490	$C_6H_5NO_2$	$[M-H]^+$	-0.7	Nicotinic acid
3	18.59	137.0244	93.0354	$C_7H_6O_3$	$[M-H]^-$	0.2	p-Hydroxybenzoic acid
4	19.72	353.0882	191.0565	$C_{16}H_{18}O_9$	$[M-H]^-$	-1.0	Chlorogenic acid
5	21.78	353.0881	191.0563/173.0456	$C_{16}H_{18}O_9$	$[M-H]^-$	-0.9	4-Dicaffeoylquinic acid
6	28.69	515.1196	191.0563/179.0351	$C_{25}H_{24}O_{12}$	$[M-H]^-$	-0.1	Isochlorogenic acid C
7	35.37	193.0509	106.0438/134.0376	$C_{10}H_{10}O_4$	$[M-H]^-$	-1.4	Ferulic acid
8	36.84	449.1089	151.0036/285.0405	$C_{21}H_{22}O_{11}$	$[M-H]^-$	0.1	Neoastilbin
9	38.09	449.1099	151.0040/285.0413	$C_{21}H_{22}O_{11}$	$[M-H]^-$	-2.3	Astilbin
10	40.67	449.1095	151.0037/285.0410	$C_{21}H_{22}O_{11}$	$[M-H]^-$	-1.3	Neoisoastilbin
11	41.50	449.1094	151.0037/285.0408	$C_{21}H_{22}O_{11}$	$[M-H]^-$	-0.9	Isoastilbin
12	43.45	433.1147	180.0068/269.0463	$C_{21}H_{22}O_{10}$	$[M-H]^-$	-1.6	Engeletin
13	47.33	433.1137	180.0059/269.0448	$C_{21}H_{22}O_{10}$	$[M-H]^-$	-0.4	Isoengelitin
14	49.31	137.0245	93.0354	$C_7H_6O_3$	$[M-H]^-$	-0.5	Salicylic acid
15	55.45	285.0409	133.0297/151.0033	$C_{15}H_{10}O_6$	$[M-H]^-$	-1.7	Luteolin

NO.	R-T	m/z	MS/MS	Elemental composition	Ion form	ppm	Compounds
16	56.15	313.0368	298.0126	$C_{16}H_{10}O_7$	$[M-H]^-$	-4.4	Wedelolactone
17	59.00	503.3381	389.2861/437.3066	$C_{30}H_{48}O_6$	$[M-H]^-$	-0.6	Madecassic-acid
18	60.20	487.3431	379.3034/409.3156	$C_{30}H_{48}O_5$	$[M-H]^-$	-0.4	Asiatic acid

4. Discussion

Hypertension is a major risk factor for cardiovascular and cerebrovascular diseases as current complications such as stroke, heart failure, myocardial infarction and renal failure [31]. Blood pressure can be controlled by changing diet and lifestyle, but drug treatment is used when these methods are not effective. Drugs used to treat hypertension include calcium channel blockers, β-blockers, angiotensin converting enzyme inhibitors (ACEI) and angiotensin II receptor blockers. However, various side effects of these drugs have been reported [32, 33]. Long-established Chinese herbal formulas can not only stabilize the blood pressure, but also improve the quality of life, minimize the risk factors associated with high blood pressure, and prevent organ damage to improve patient survival [34]. In this study, we used a SHR model to access the effects of LFG on attenuating blood pressure. Our data showed that compared with model group, LFG significantly reduced blood pressure and increased serum NO content in SHR rats. LFG ameliorated the pathological changes such as cardiac hypertrophy and interstitial inflammation.

Although the animal experimental results showed that LFG could exert an antihypertensive effect in SHR, the active compounds and underlying molecular mechanisms are not clear. In the present study, a network pharmacology approach was applied to identify the active compounds, corresponding targets, and pharmacological mechanisms of LFG antihypertensive effect. Based on the results of public databases searching, 53 candidate compounds from LFG were collected, which linked to 765 potential targets. 828 hypertension associated targets were retrieved. The potential targets of antihypertensive effect were found by comparing LFG genes with hypertension genes, and 12 key targets were obtained after screening. After pathway enrichment of 12 key targets, 37 components and 34 pathways were found, among which the top 10 pathways were Bladder cancer, VEGF signaling pathway, Rap1 signaling pathway, Estrogen signaling pathway, HIF-1 signaling pathway, Oxytocin signaling pathway, PI3K-AKT signaling pathway, Pancreatic cancer, Proteoglycans in cancer, and Prolactin signaling pathway were screened.

Twelve key genes were validated by RT-qPCR, and the results showed that LFG could upregulate the mRNA expression of NOS and SRC in thoracic aorta. Endothelial nitric oxide synthase 3 (NOS3) is mainly expressed in endothelial cells. NO produced by NOS3 is a major vascular protective agent because of its vasodilator, antioxidant and antiproliferative properties [35]. It was reported that demethylasterriquinone

b1 induced Akt activation may stimulate the expression and activity of NOS3 and inhibit the expression of p22phox subunit of NADPH oxidase to reduce oxidative stress and subsequently improve vascular endothelial dysfunction [36]. It was showed that AVE3085 can effectively reduce the blood pressure of hypertensive rats. AVE3085 restored the impaired endothelium dependent relaxations in the aortae of SHRs, so the improvement of endothelial function may be related to its antihypertensive effect [37]. SRC tyrosine protein kinase is a non-receptor protein tyrosine kinase. It plays an important role in regulation of cell differentiation, proliferation, and transformation, and also plays an important biological role in diseases such as hypertension and tumor [38, 39]. It was reported that SRC was involved in the production of NO and prostaglini2 (PGI2) induced by VEGF, suggesting that SRC was involved in the process of reducing vascular tension, relaxing blood vessel, and lowering blood pressure of VEGF in the vascular endothelium [40]. Taken together, we demonstrated that the expression of NOS3 and SRC were altered in model group, and LFG attenuated hypertension through restoring their expression to normal levels.

Pathway enrichment results showed that PI3K-AKT signaling pathway was closely related to the expression of NOS3 and SRC. To verify this result, western blot was used to detect the key proteins (PI3K, AKT, and p-AKT) of PI3K-AKT signaling pathway. The results showed that LFG significantly increased the protein expression levels of PI3K and p-AKT in SHR rats, suggesting that LFG may play a role through the PI3K-AKT signaling pathway. The PI3K-AKT signaling pathway is activated by many types of cellular stimuli or toxic insults and regulates fundamental cellular functions such as transcription, translation, proliferation, growth, and survival. PI3K catalyzes the production of phosphatidylinositol-3,4,5-triphosphate (PIP3) at the cell membrane. PIP3 in turn serves as a second messenger that helps to activate AKT. Once activated, AKT can control key cellular processes by phosphorylating substrates involved in apoptosis, protein synthesis, metabolism, and cell cycle. It was reported that the AKT activity of blood vessels characterized by Ser473 phosphorylation is downregulated in a number of hypertensive rat models, including in SHRs [41]. Importantly, phosphorylation of AKT at Ser473 contributes to NOS3 activation [42]. SRC regulates the activity of PI3K-AKT signaling, and contributes to improving endothelial dysfunction [43]. Taken together, the mechanism of LFG exerting antihypertensive effect was showed in Fig. 11. LFG can reduce the blood pressure of SHR rats, which might be attributed to upregulating SRC expression level, activating of PI3K-AKT-NOS3 signaling pathway to further increase the serum NO level for promoting vasodilation.

Molecular docking study further supported that p-hydroxybenzoic acid, cedar acid, shikimic acid, salicylic acid, nicotinic acid, linalool, and histidine can be well binding with NOS3, SRC, PI3K, and AKT. These compounds were potential active ingredients for LFG antihypertensive effects. p-Hydroxybenzoic acid can be isolated from a variety of plants, such as oil palm, Vitex negundo and virgin olive oil [44–46]. It was reported that p-hydroxybenzoic acid possessed a variety of biological properties, including anti-inflammatory, antioxidant cardio protective and vasodilatory effects [47]. p-Hydroxybenzoic acid caused a decrease in blood pressure and dilatation of the aortic rings. The biological principles were to act directly on the vascular smooth muscle to cause vasodilatation, and indirectly by stimulating the release of NO from the vascular endothelium to promote the vasodilator activity of the compound [48]. Cedar

acid, one of the phenolic compounds, is abundantly present in olives, dates, spices, pumpkin, grapes, acai palm and other plants [49]. Also, it exhibits multi-pharmacological properties such as antihypertensive, antioxidant, anti-inflammatory, and hepatoprotective activities [50, 51]. Cedar acid showed antihypertensive activity in hypertensive rats induced by N-nitro-L-arginine methyl ester (L-NAME). It was evidenced that Cedar acid treatment might reduce the blood pressure, lipid peroxides, and increase NO availability and antioxidant levels in blood samples of rats [52]. Shikimic acid originally extracted from Chinese star anise, exerts diverse pharmacological actions such as analgesic, anti-inflammatory, and antioxidant effects [53–55]. A previous study indicated that Shikimic acid inhibited inflammatory events including the production of NO and the expression of pro-inflammatory cytokines [56]. Chemically, Salicylic acid is 2-hydroxybenzoic acid or orthohydrobenzoic acid. Sources of Salicylic acid include willow bark, sweet birch, and wintergreen leaves, and it can also be synthesized artificially [57]. Salicylic acid, the Xinjiang red raspberry fruit extract, moderately reduced SHR blood pressure, the effect may be related to increasing serum NO, SOD activity and T-AOC levels, enhanced NOS3 mRNA expression [58]. Niacin, also known as vitamin B3, illustrates the many functions of water-soluble vitamins. Niacin has been widely used in the treatment of dyslipidemia and atherosclerotic coronary heart disease [59]. In addition to its lipid-lowering effect, Niacin also has powerful antioxidant and anti-inflammatory properties [60]. Cho etc. showed that long-term Niacin administration improves hypertension and proteinuria, and attenuates the accumulation of lipids in the remnant kidneys of animals with CRF induced by subtotal nephrectomy [61]. Linalool is a monoterpene and is generally considered to be the main component of essential oils obtained from aromatic plant species [62]. Linalool showed a wide range of biological activities including anti-inflammatory, anti-cancer, anti-hyperlipidemic, anti-microbial effects [63]. A data showed that sub-chronic treatment with Linalool reduced the development of hypertension in SHR, reducing cardiac hypertrophy, increasing IL-10 anti-inflammatory cytokine, improving vasodilatory function and decreasing vasoconstriction [64]. Histidine is an essential amino acid in humans and other mammals which exhibits antioxidant and anti-inflammatory properties [65]. In a dietary intervention and evaluation experiment, the relationship between the intake of specific amino acids and blood pressure levels was analyzed, and the results demonstrated that intakes of Methionine and Alanine were associated positively with higher blood pressure, while intakes of Threonine and Histidine had inverse associations [66]. Toba etc. have demonstrated the antihypertensive effect of L-histidine after oral administration in SHR. These effects may be mediated by central histamine H3 receptors and appear to be related to an increase in NO in the rostral ventrolateral medulla [67].

After comparing the components with the reference standard and LFG by LC-MS/MS, p-hydroxybenzoic acid, shikimic acid, salicylic acid, and nicotinic acid were confirmed to exist in LFG. In conclusion, p-hydroxybenzoic acid, shikimic acid, salicylic acid, and nicotinic acid might be the underlying compounds of LFG to play an antihypertensive effect.

5. Conclusions

LFG can reduce the blood pressure of SHR rats, which might be attributed to upregulating SRC expression level, activating of PI3K-AKT-NOS3 signaling pathway to further increase the serum NO level for

promoting vasodilation. p-Hydroxybenzoic acid, shikimic acid, salicylic acid, and nicotinic acid might be the underlying active compounds which affects the potential targets for LFG antihypertensive effect.

Declarations

7. **Acknowledgements**

Not applicable

8. **Authors' contributions**

HG, DZ, ZS contributed to designing the work. QL, TL and WC wrote the manuscript, analyzed and interpreted of data. QL, TL, SH, XL, WZ, QZ, XC, SG and YH performed the experiments. HG and DZ provided reagents/materials/analysis tools. All authors read and approved the final version.

9. **Funding**

This work was supported by Guangxi Natural Science Foundation (2020GXNSFAA259030, 2018GXNSFAA138096); Guangxi Science and Technology Projects (GUIKEGONG 1598013-8); Open Project of Guangxi Key Laboratory of Regenerative Medicine (GUIZAIZHONGKAI 201903), Guangxi First-class Discipline Project for Pharmaceutical Sciences (GXFCDP-PS-2018); Natural Science Foundation of Guangxi University of Chinese Medicine (2019QN004).

10. **Competing interests**

The authors declare that they have no competing interests.

11. **Availability of data and materials**

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

12. **Consent for publication**

Not applicable.

13. **Ethics approval and consent to participate**

The experimental procedures were approved by the Institutional Animal Ethics Committee (IAEC), Guangxi Medical University.

14. **Supplementary information**

Additional file 1: Table S1. The 828 significant genes associated with hypertension. **Table S2.** Docking results of 10 active ingredients and NOS3. **Table S3.** Docking results of 10 active ingredients and SRC.

Table S4. Docking results of 10 active ingredients and PI3K. **Table S5.** Docking results of 10 active ingredients and AKT.

Additional file 2: Figure S1-S18. The comparing of components with the reference standard and LFG.

Abbreviations

AKT, Protein kinase B; ANG-Ⅱ, Angiotensin Ⅱ; BATMAN-TCM, Bioinformatics Analysis Tool for Molecular Mechanism of Traditional Chinese Medicine; BCA, Bicinchoninic acid; BSA, Bovine Serum Albumin; CCL2, C-C motif chemokine 2; DAVID, Database for Annotation, Visualization and Integrated Discovery; EGF, Epidermal growth factor; EGFR, Epidermal growth factor receptor; ESR1, Estrogen receptor; ET-1, Endothelin 1; H&E staining, Hematoxylin-eosin staining; KEGG, Kyoto Encyclopedia of Genes and Genomes; LFG, Lei-gong-gen formula granule; ERK2, Mitogen-activated protein kinase 1; NGF, Nerve growth factor; NO, Nitric oxide; NOS3, Endothelial nitric oxide synthase; NPY, neuropeptide Y; OB, Oral bioavailability; PDB, Protein Data Bank; PI3K, Phosphatidylinositol 3 kinase; PTGS2, Prostaglandin G/H synthase 2; SHR, Spontaneously hypertensive rats; SRC, Proto-oncogene tyrosine-protein kinase SRC; STRING, Search Tool for the Retrieval of Interacting Genes/Proteins; TCMID, Traditional Chinese Medicines Integrated Database; TCMSP, Traditional Chinese Medicine Systems Pharmacology Database and Analysis Platform; TH, Tyrosine 3-monooxygenase; T-HOD, Text-mined Hypertension, Obesity and Diabetes candidate gene database; UPLC-QTOF/MS, Ultra performance liquid chromatography/quadrupole time-of-flight mass spectrometry; VEGFA, Vascular endothelial growth factor A.

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Tables

Table 1: Primer sequence used for PCR analysis

Gene	Forward	Reverse
NOS3	5'-CAGAGATTGGCATGAGGGACC-3'	5'-TCCACAGTGATGAGGTTGTCC-3'
SRC	5'-CAAGATCACTAGACGGGAATCAG-3'	5'-GTTTCACATTTAGGCCCTTGG-3'
PTGS2	5'-ATCAGAACCGCATTGCCTCT-3'	5'-GCCAGCAATCTGTCTGGTGA-3'
NGF	5'-GTCTGGGCCCAATAAAGGCT-3'	5'-TGTACGCCGATCAAAAACGC-3'
VEGFA	5'-CGTCCTGTGTGCCCCTAATG-3'	5'-TGTGCTGGCTTTGGTGAGGT-3'
EGF	5'-CTACTACAGGACTCGGAAGCAG-3'	5'-GTTGGGGACCAGAAGACACC-3'
EGFR	5'-AGCCGTCCTGTCCAACTATG-3'	5'-TTGCTAAATCGCACAGCACC-3'
ERK2	5'-ATTGGTCAGGACAAGGGCTCA-3'	5'-CCACTACGACCAGAACTGCC-3'
CCL2	5'-TGCAGGTCTCTGTCACGCTTC-3'	5'-TTCTCCAGCCGACTCATTGG-3'
NPY	5'-GCCAGATACTACTCCGCTCTG-3'	5'-GTCTCAGGGCTGGATCTCTTG-3'
ESR1	5'-CAGACAGGGAGCTGGTTCATA-3'	5'-GCACACTCCAGAAGGTGAACT-3'
TH	5'-GTCTCAGAGCAGGATGCCAAG-3'	5'-ATCCTCGATGAGACTCTGTCGC-3'
β-actin	5'-GTCAGGTCATCACTATCGGCAAT-3'	5'-AGAGGTCTTTACGGATGTCAACGT-3'

Table 2: The information of 12 key targets between compounds and hypertension targets

Gene	Compounds	Gene	Compounds
VEGFA	glucoside	MAPK1	Cedar acid
NPY	Ferulic Acid	MAPK1	Ferulic Acid
EGF	salicylic acid	NOS3	L-Bornyl acetate
EGF	nicotinic acid	NOS3	p-Hydroxybenzoic acid
EGF	p-Hydroxybenzoic acid	NOS3	salicylic acid
EGFR	naringenin	NOS3	Pulegone
TH	(1R,2R,5S)-2,6,6-trimethylnorpinan-3-one	NOS3	Cedar acid
TH	Ferulic Acid	NOS3	nicotinic acid
TH	Histidine	NOS3	Histidine
NGF	(-)-alpha-Pinene	NOS3	(R)-linalool
NGF	p-Hydroxybenzoic acid	NOS3	5-Hydroxymethylfurfural
NGF	(-)-nopinene	ESR1	L-Bornyl acetate
SRC	Shikimic Acid	ESR1	(1R,2R,5S)-2,6,6-trimethylnorpinan-3-one
SRC	nicotinic acid	ESR1	Pratensein
SRC	Histidine	ESR1	alpha-Terthienyl methyl acetate
PTGS2	naringenin	ESR1	diosgenin
PTGS2	Histidine	ESR1	(+)-Isoborneol
PTGS2	demethylwedelolactone	ESR1	sitosterol
PTGS2	acacetin	ESR1	Stigmasterol
PTGS2	Ferulic Acid	ESR1	butin
PTGS2	Xanthanoic acid	ESR1	Baimuxinol
PTGS2	5-Hydroxymethylfurfural	ESR1	(Z)-1-(2,4-dihydroxyphenyl)-3-(3,4-dihydroxyphenyl)prop-2-en-1-one
PTGS2	(L)-alpha-Terpineol	ESR1	(-)-nopinene
PTGS2	(1R,2R,5S)-2,6,6-trimethylnorpinan-3-one	ESR1	1,8-cineole
PTGS2	Pulegone	ESR1	(-)-alpha-Pinene
PTGS2	MENTHOL	ESR1	demethylwedelolactone
PTGS2	L-Limonen	ESR1	3'-O-Methylorobol
PTGS2	salicylic acid	ESR1	wedelolactone
PTGS2	nicotinic acid	ESR1	luteolin
PTGS2	p-Hydroxybenzoic acid	ESR1	acacetin
PTGS2	L-Bornyl acetate	ESR1	Dihydroresveratrol
PTGS2	Cedar acid	ESR1	naringenin
CCL2	Ferulic Acid	ESR1	cis-resveratrol
MAPK1	p-Hydroxybenzoic acid		

Table 3: Identify of the compounds of LFG by UPLC- Q-TOF/MS analysis in negative ion mode

NO.	R-T	m/z	MS/MS	Elemental composition	Ion form	ppm	Compounds
1	3.73	173.0458	93.0354	$C_7H_{10}O_5$	$[M-H]^-$	-1.3	Shikimic acid
2	3.90	124.0394	78.0334/80.0490	$C_6H_5NO_2$	$[M-H]^+$	-0.7	Nicotinic acid
3	18.59	137.0244	93.0354	$C_7H_6O_3$	$[M-H]^-$	0.2	p-Hydroxybenzoic acid
4	19.72	353.0882	191.0565	$C_{16}H_{18}O_9$	$[M-H]^-$	-1.0	Chlorogenic acid
5	21.78	353.0881	191.0563/173.0456	$C_{16}H_{18}O_9$	$[M-H]^-$	-0.9	4-Dicaffeoylquinic acid
6	28.69	515.1196	191.0563/179.0351	$C_{25}H_{24}O_{12}$	$[M-H]^-$	-0.1	Isochlorogenic acid C
7	35.37	193.0509	106.0438/134.0376	$C_{10}H_{10}O_4$	$[M-H]^-$	-1.4	Ferulic acid
8	36.84	449.1089	151.0036/285.0405	$C_{21}H_{22}O_{11}$	$[M-H]^-$	0.1	Neoastilbin
9	38.09	449.1099	151.0040/285.0413	$C_{21}H_{22}O_{11}$	$[M-H]^-$	-2.3	Astilbin
10	40.67	449.1095	151.0037/285.0410	$C_{21}H_{22}O_{11}$	$[M-H]^-$	-1.3	Neoisoastilbin
11	41.50	449.1094	151.0037/285.0408	$C_{21}H_{22}O_{11}$	$[M-H]^-$	-0.9	Isoastilbin
12	43.45	433.1147	180.0068/269.0463	$C_{21}H_{22}O_{10}$	$[M-H]^-$	-1.6	Engeletin
13	47.33	433.1137	180.0059/269.0448	$C_{21}H_{22}O_{10}$	$[M-H]^-$	-0.4	Isoengelitin
14	49.31	137.0245	93.0354	$C_7H_6O_3$	$[M-H]^-$	-0.5	Salicylic acid
15	55.45	285.0409	133.0297/151.0033	$C_{15}H_{10}O_6$	$[M-H]^-$	-1.7	Luteolin
16	56.15	313.0368	298.0126	$C_{16}H_{10}O_7$	$[M-H]^-$	-4.4	Wedelolactone
17	59.00	503.3381	389.2861/437.3066	$C_{30}H_{48}O_6$	$[M-H]^-$	-0.6	Madecassic-acid
18	60.20	487.3431	379.3034/409.3156	$C_{30}H_{48}O_5$	$[M-H]^-$	-0.4	Asiatic acid

Figures

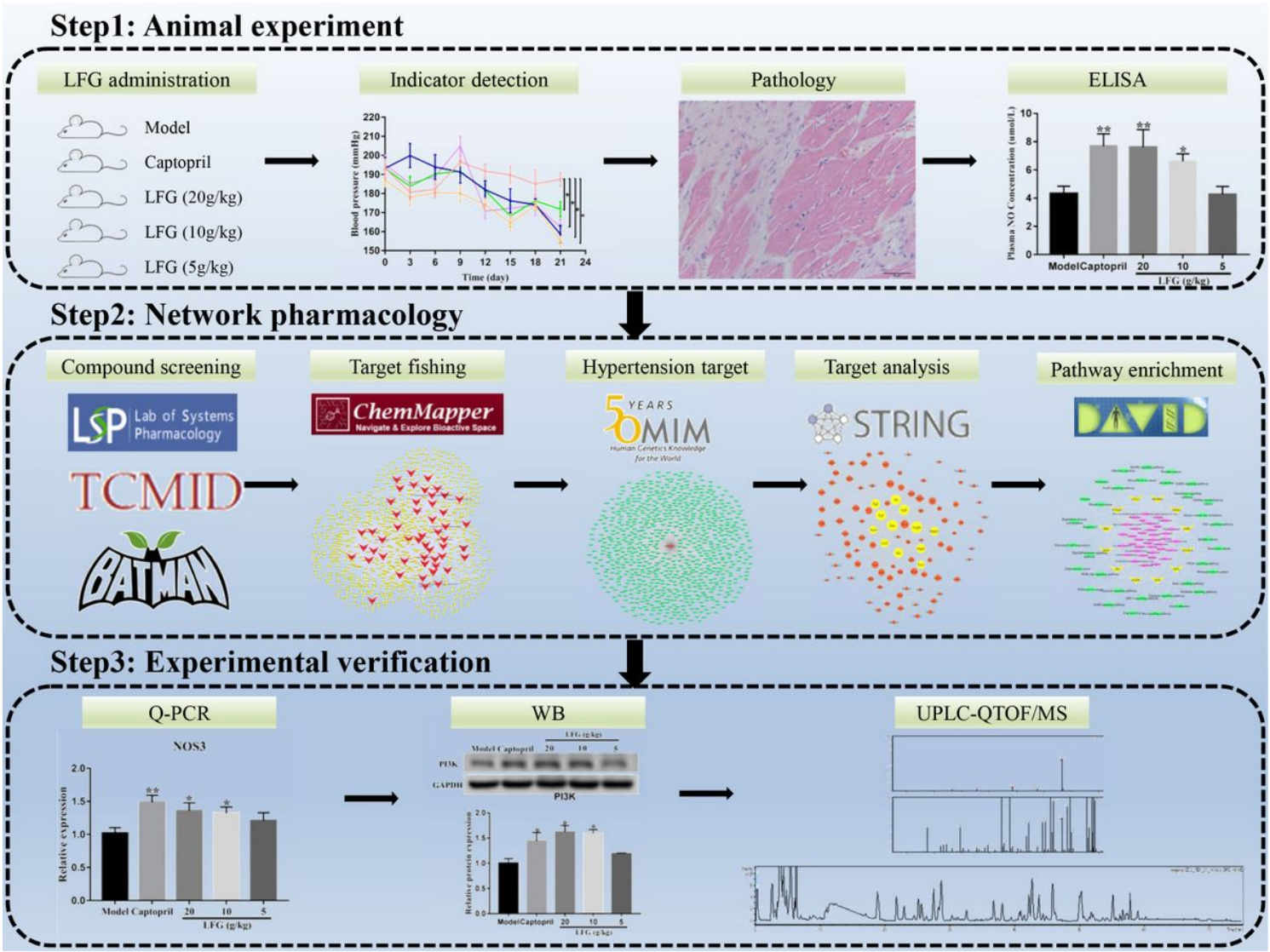

Figure 1

Work flow of the study.

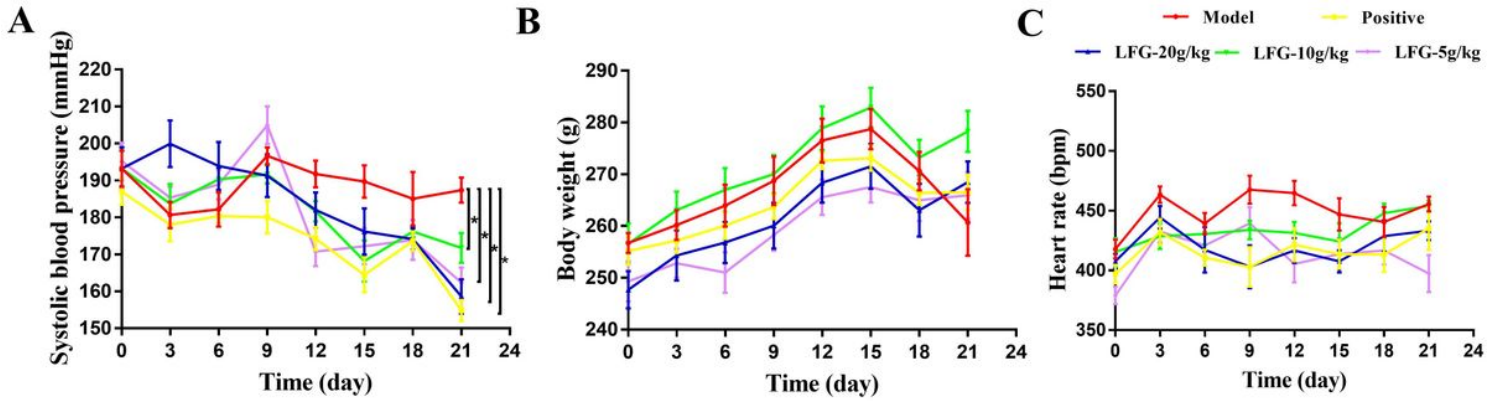

Figure 2

Effect of LFG on blood pressure, body weight and heart rate of SHR. (A) Blood pressure changes; (B) body weight changes; (C) heart rate changes. Compared with model group, *P<0.05.

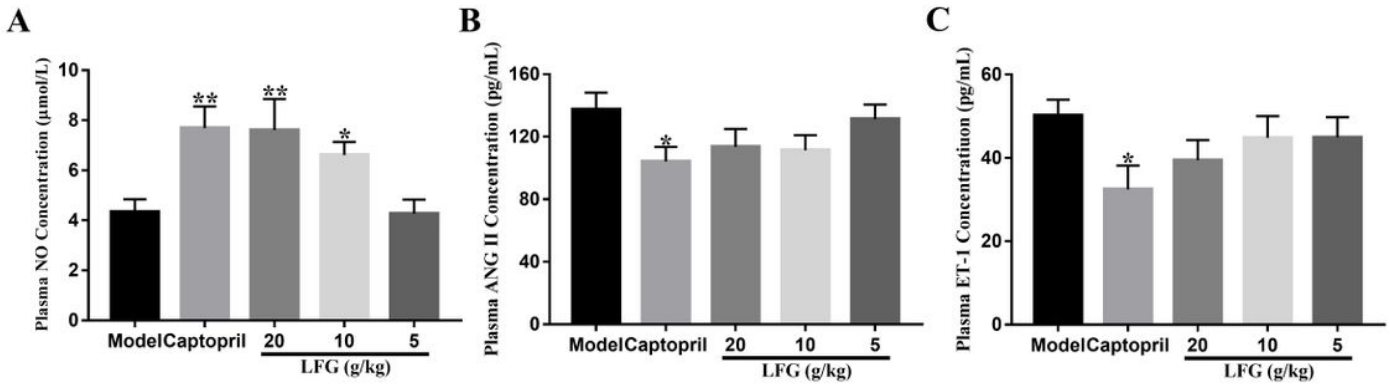

Figure 3

Effect of LFG on (A) NO, (B) Ang Ⅱ and (C) ET-1 levels in serum of SHR. Compared with model group, * P <0.05, ** P <0.01.

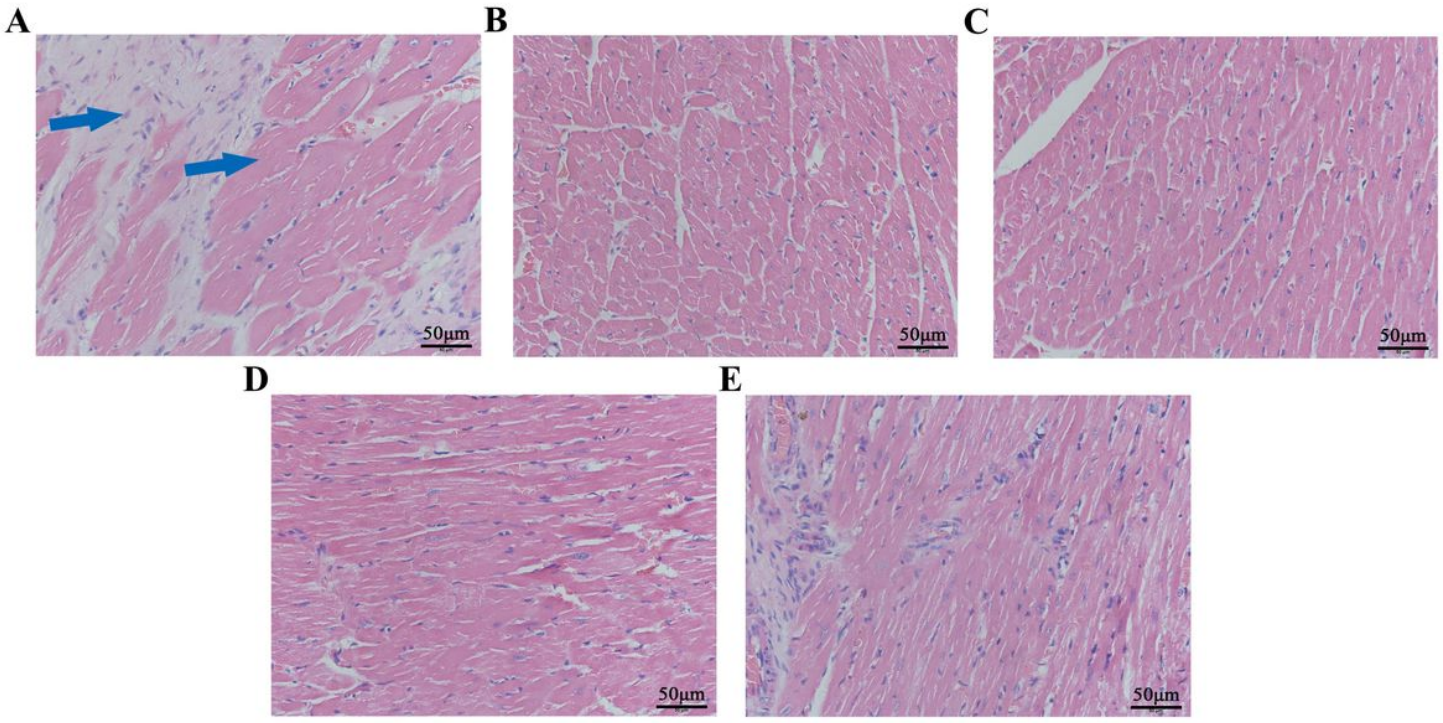

Figure 4

H&E staining results of heart tissue. (A) Model group; (B) Captopril group; (C) High-dose group; (D) Middle-dose group; (E) Low-dose group. Magnification 400×, scale bar = 50μm. The blue arrows indicate cardiac hypertrophy and fibrosis.

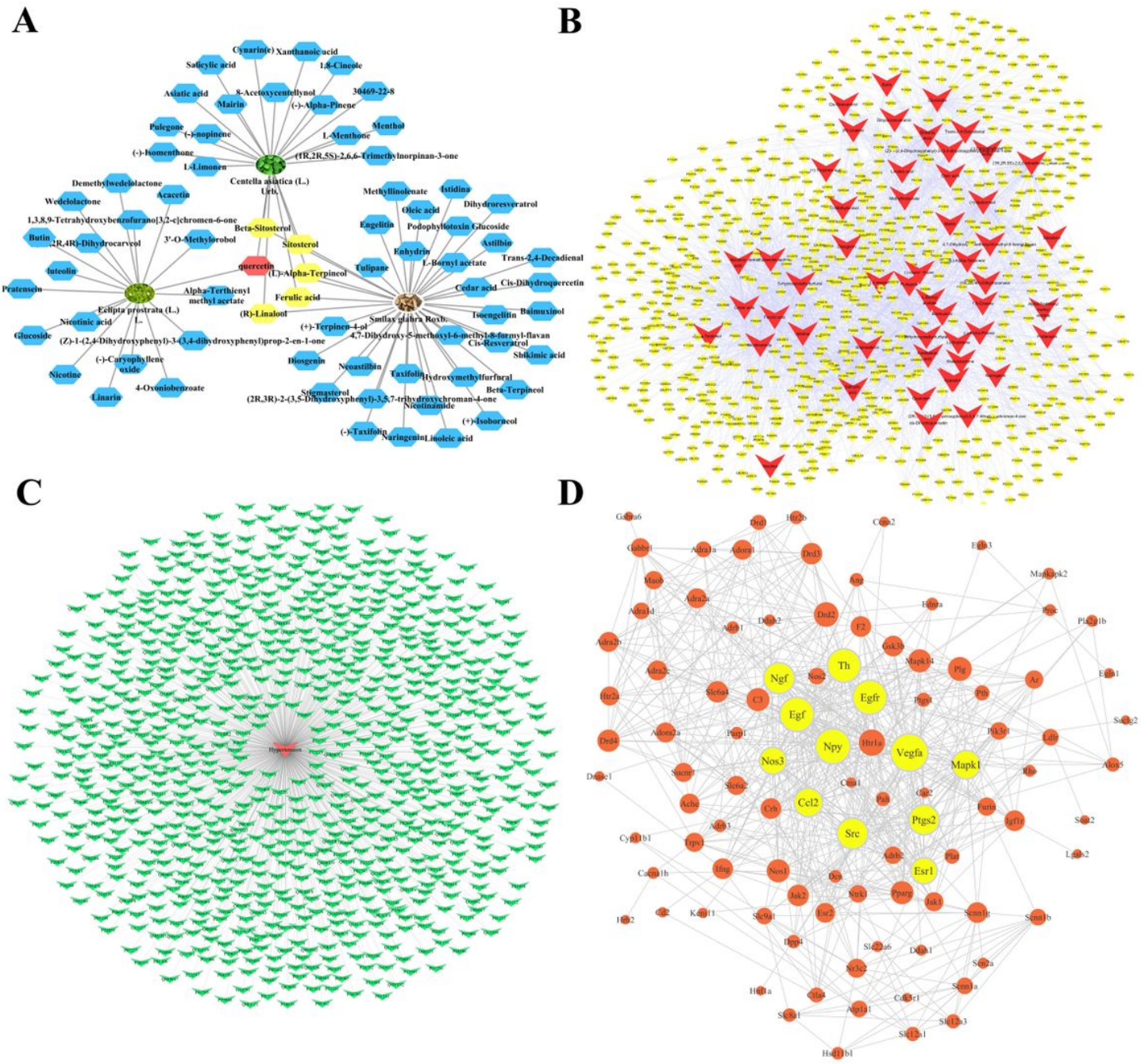

Figure 5

The results of network pharmacology prediction. (A) The herb-compound network. The ellipse nodes represent herbs, and the hexagon nodes are compounds. Among these compounds, yellow parts are the common components of Centella asiatica (L.) Urb. and Smilax glabra Roxb., and quercetin (red hexagon) is common compounds of 3 herbs. (B) The compound-target network. The V shape nodes represent 53 compounds, and the 765 yellow nodes are targets corresponding to the compounds. (C) The hypertension-target network. In this figure, the red node in the center is hypertension, and the green nodes represent gene targets. (D) The protein-protein interaction network within the common targets. It shows

the relationship between 101 common targets. The size of nodes changes from big to small according to degree values, and the 12 yellow nodes are key targets after screening.

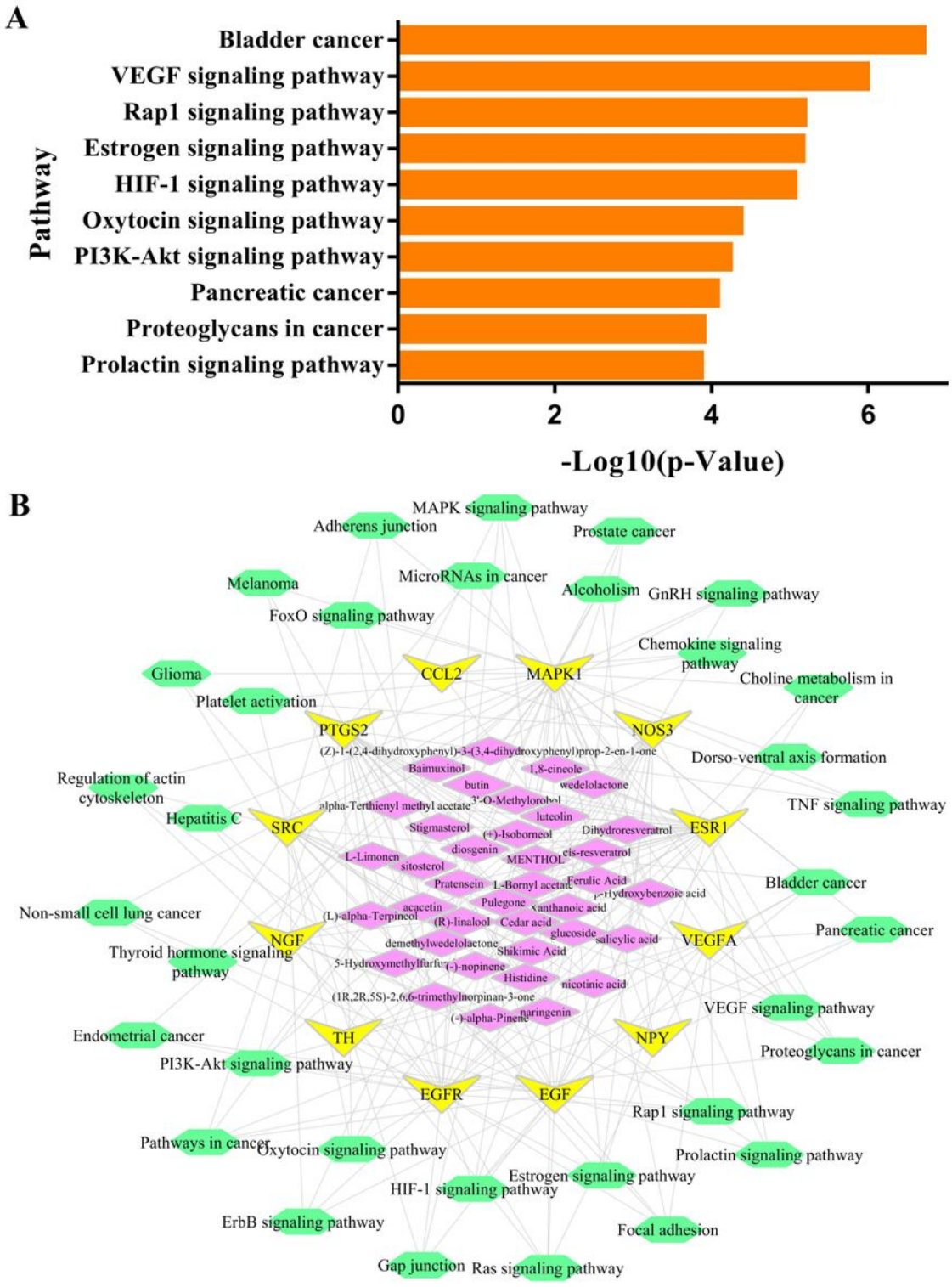

Figure 6

(A) The relationship between TOP10 pathways and their -log10 (P). (B) The compound-target-pathway network. Compounds are indicated by 37 purple nodes; gene targets are shown as 12 yellow nodes; pathways are labeled with 34 green nodes.

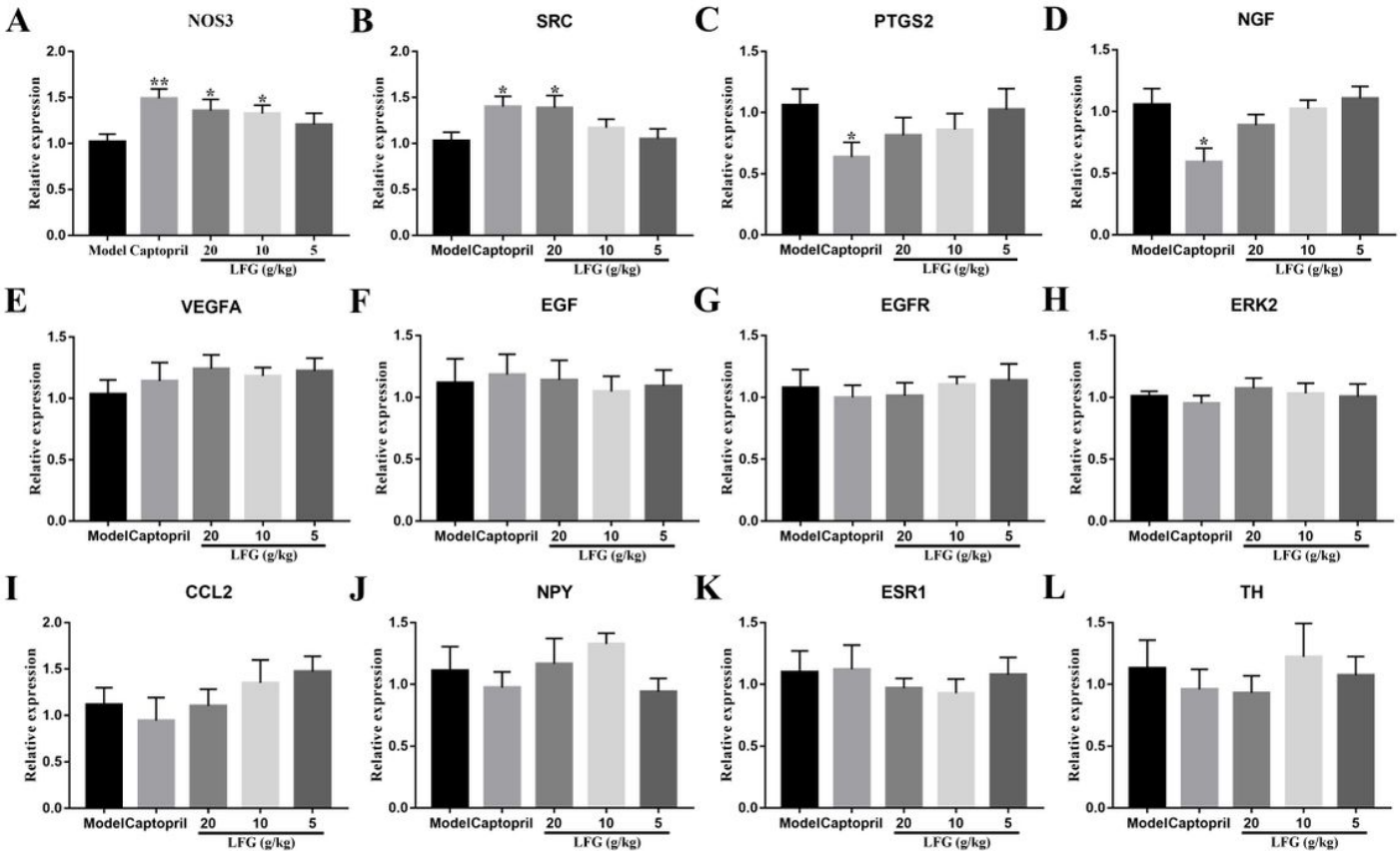

Figure 7

Effect of LFG on mRNA expression levels of key targets. (A) NOS3; (B) SRC; (C) PTGS2; (D) NGF; (E) VEGFA; (F) EGF; (G) EGFR; (H) ERK2; (I) CCL2; (J) NPY; (K) ESR1; (L) TH. Compared with model group, *P<0.05，**P<0.01.

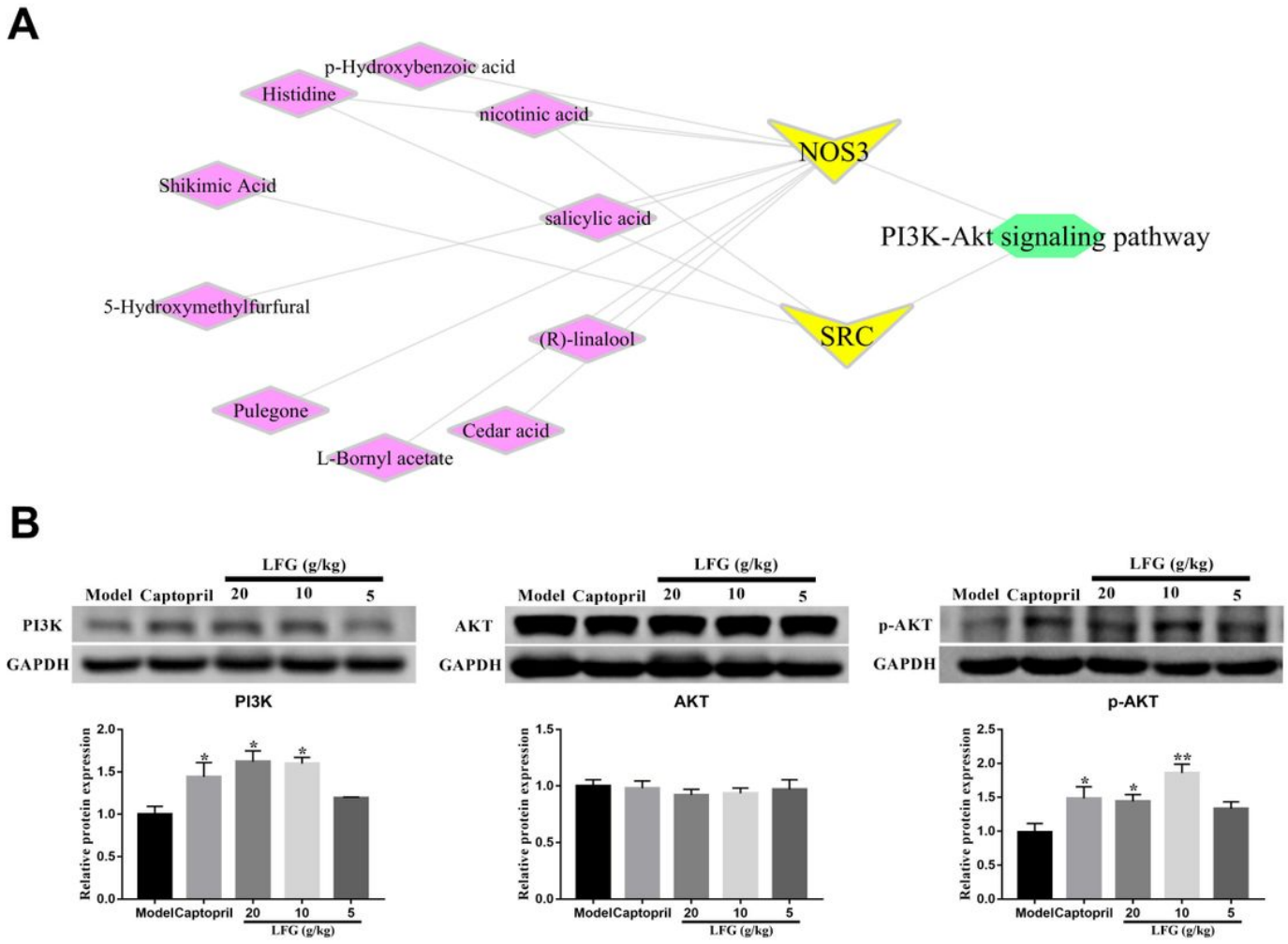

Figure 8

(A) The compound-target-pathway network of significant genes (V nodes). Compounds are indicated by 10 diamond nodes; pathways are shown as hexagon nodes. (B) PI3K, AKT and p-AKT protein expression levels among different groups. Compared with model group, * P <0.05, ** P <0.01.

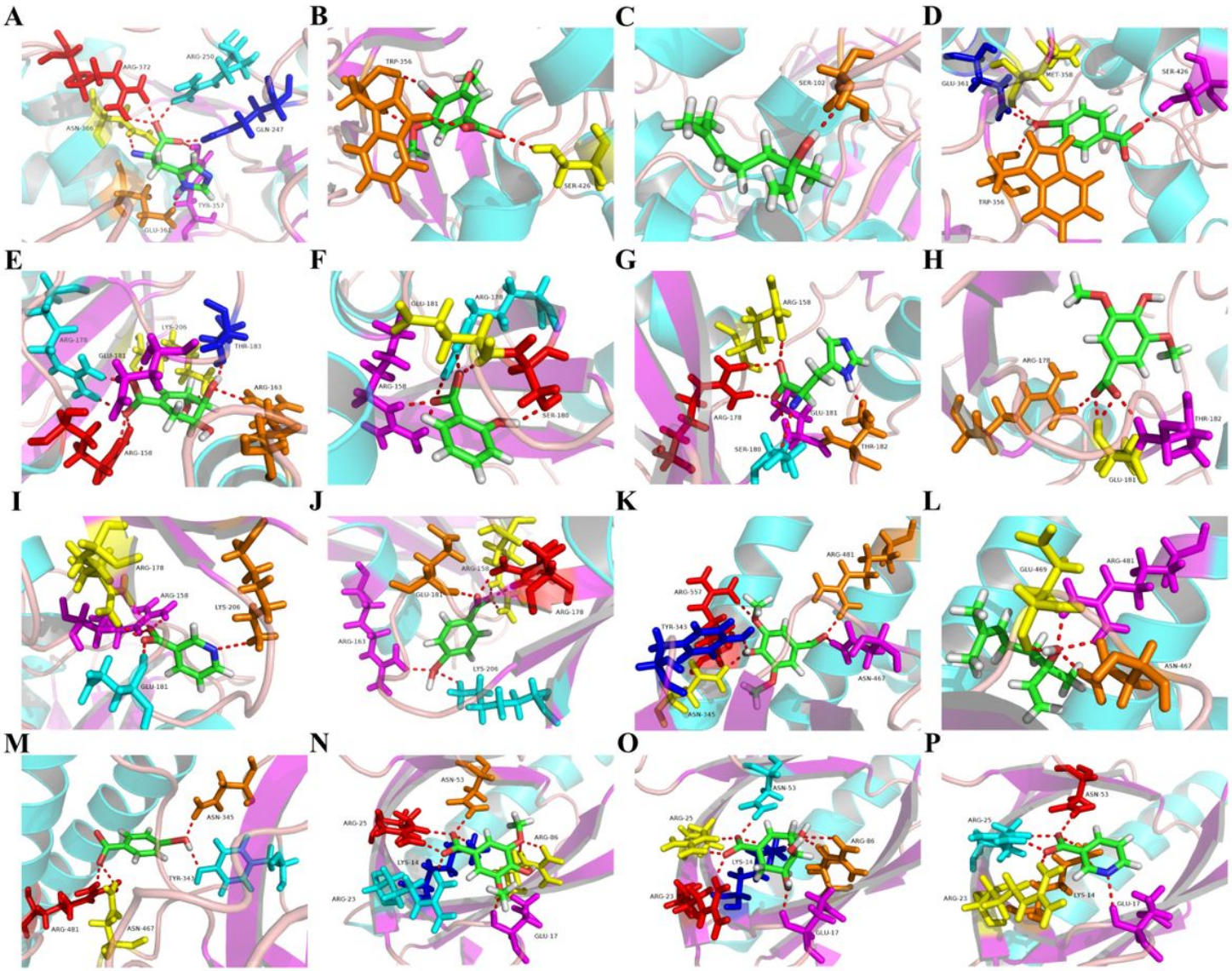

Figure 9

The binding modes of compounds and NOS3: (A) Histidine, (B) Cedar acid, (C) Linalool, (D) p-Hydroxybenzoic acid. The binding modes of compounds and SRC: (E) Shikimic acid, (F) Salicylic acid, (G) Histidine, (H) Cedar acid, (I) Nicotinic acid, (J) p-Hydroxybenzoic acid. The binding modes of compounds and PI3K: (K) Cedar acid, (L) p-Hydroxybenzoic acid, (M) Linalool. The binding modes of compounds and AKT: (N) Cedar acid, (O) Shikimic acid, (P) Nicotinic acid.

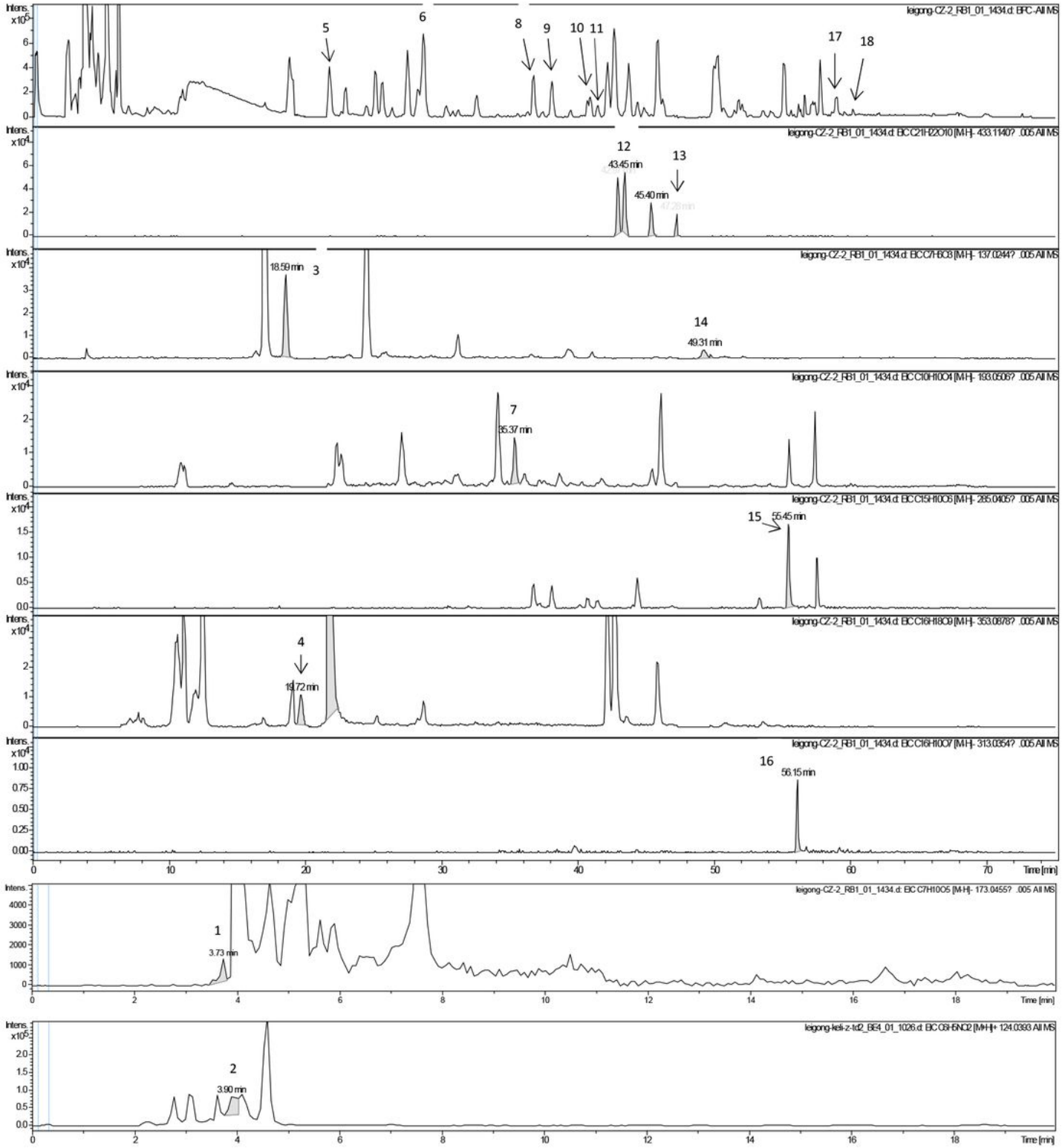

Figure 10

UPLC-QTOF/MS BPI chromatograms of LFG.

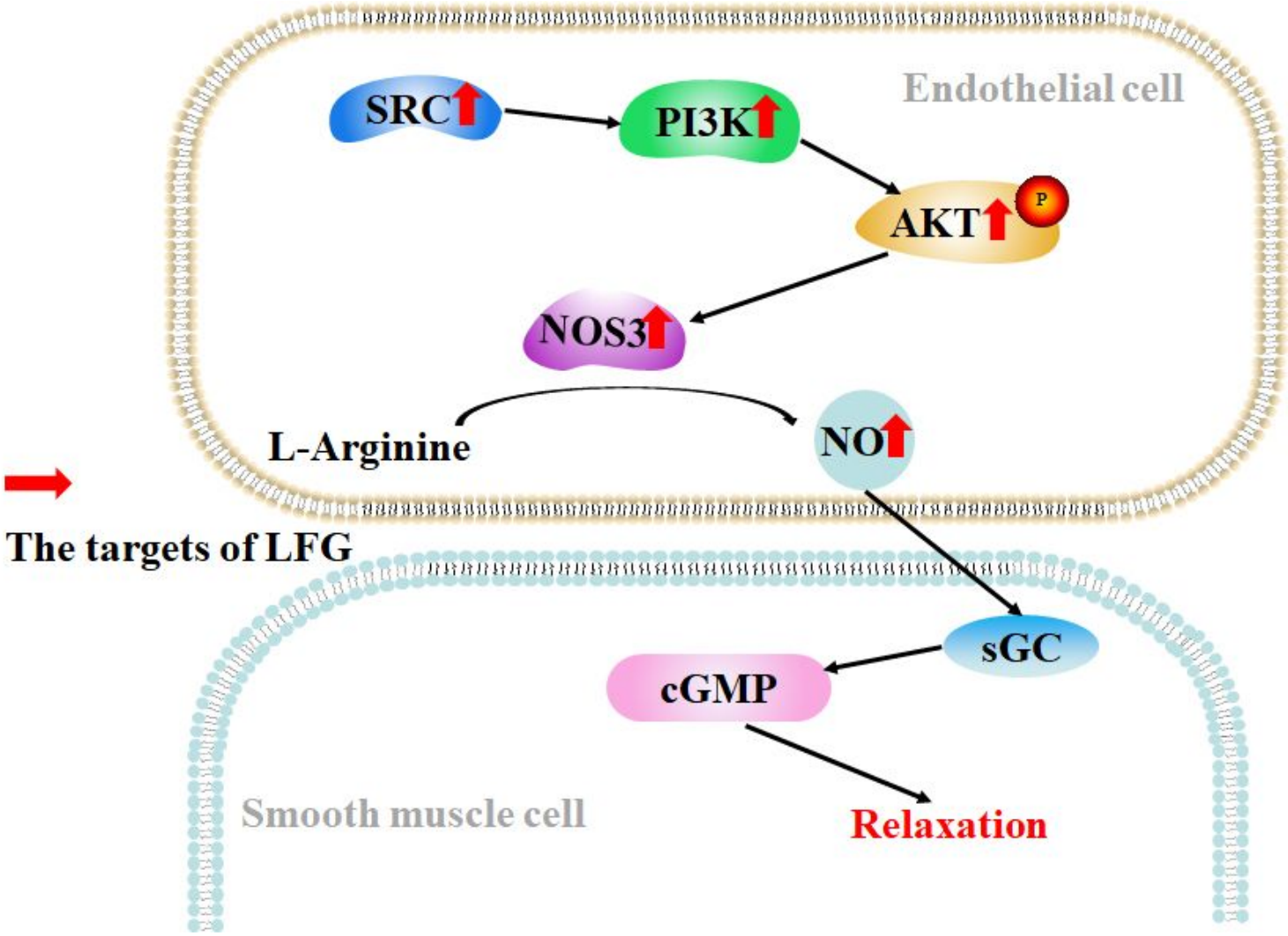

Figure 11

LFG antihypertensive mechanism.

Supplementary Files

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