

Based on network pharmacology method to discovered the targets and therapeutic mechanism of *Paederia scandens* against nonalcoholic fatty liver disease in chicken

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ABSTRACT The aim of the study is to determine the target of *Paederia scandens* in nonalcoholic fatty liver disease (NAFLD). The Chinese herbal medicine pharmacology data and analysis platform were used to search and screen for the effective components of the *Paederia scandens* compounds and to analyze the possible therapeutic targets based on network topology. In addition, various known disease target databases were enrolled, the therapeutic target proteins in NAFLD were screened, and a protein–protein interaction network was constructed. Enrichment analysis was performed on key nodes. Finally, the inhibitory effect of *Paederia scandens* on NAFLD was verified by experiments. We identified 33 major candidate targets of

Paederia scandens and successfully constructed a “drug-compound-target-disease” network. Abovementioned targets revealed by gene enrichment analysis have played a significant role in the cell cycle, apoptosis, and related signal pathways. We demonstrated that *Paederia scandens* downregulated serum triglyceride and lipopolysaccharides levels in NAFLD chickens by feeding with a high-capacity diet and endotoxin of *Salmonella enteritidis* was given by gavage. *Paederia scandens* may regulate the hepatic cell cycle and apoptosis through the *Salmonella* infection pathway, Toll-like receptor signaling pathway, and apoptosis pathway. For NAFLD, *Paederia scandens* may be a promising, long-lasting treatment strategy.

Key words: nonalcoholic fatty liver disease, *Paederia scandens*, cell cycle, apoptosis, network pharmacology

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INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) is the most common nutrition metabolic disease worldwide. Chicken NAFLD is the disease responsible for the morbidity and/or mortality occurring in the poultry industry (Sánchez-Polo et al., 2015). The estimated prevalence of chicken NAFLD is around 5%, but occasional outbreaks occur in which up to 20% of a flock have been lost (Bannister, 1976). Up to now, NAFLD is becoming an increasingly common medical problem in the poultry industry, which still is associated with the lack of any effective treatment (Martín-Castillo et al., 2010; Glass et al., 2019; Huang et al., 2020). For the treatment of NAFLD, losing weight by exercise or diet remains the standard treatment in human, because no effective

pharmacological therapy has yet been developed for NAFLD (Takeda et al., 2016). To apply effective preventive and/or treatment strategies against chicken NAFLD, it is becoming increasingly important to detect the target of drug in NAFLD.

Traditional Chinese medicine (TCM) is a valuable part of Chinese excellent traditional culture. It is a collection of 1000 yr of practical experience and has made tremendous contributions to human disease prevention and treatment (Guohua et al., 2017). In clinical application, compared with the exposure to single-target chemical drugs with substantial toxic side-effects and poor therapeutic effects on complex disease (Zhang et al., 2016), TCM reflects multicomponent, multitarget point, and multipathway synergies (Du et al., 2009), and the advantage of TCM are gradually becoming more prominent. At present, the diseases can cause metabolic syndromes, consisting of obesity and dyslipidemia (Takeda et al., 2016). The TCM has been widely used as an alternative treatment for NAFLD, including reducing injury, inhibition of inflammation, and inhibition of apoptosis (Xu, 2006). *Paederia scandens* belongs to the Medicinal Material in Miao-medicine. A large

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number of studies have revealed that *Paederia scandens* has significant clinical effects, including antibiosis, anti-toxin, and anti-inflammatory (Qiang et al., 2012).

System regulation of TCM is similar to the study thought of network pharmacology. Network pharmacology combines the field of systems biology with pharmacokinetic and pharmacodynamic properties to study drugs, protein targets, and their pharmacological activities (Yameng et al., 2018). This field combines “-omic” (genomics, proteomics, transcriptomics, and metabolomics) profiles and metabolites to construct networks to explore drug mechanisms (Liu et al., 2015). Network pharmacology is based on drug–gene–target–disease interaction networks and systematically observes the effects of drugs on some disease. This approach is in line with the theory that TCM emphasizes the diagnosis and treatment of diseases from a holistic perspective and the synergy between Chinese medicine and its compounds (Ru et al., 2014). Therefore, in this study, we simulated the network relationships between bioactive components of *Paederia scandens* and their targets based on the network pharmacology method, which was used to clarify the synergistic effect and mechanism of *Paederia scandens*. In addition, we conducted an experiment to verify the mechanism by which *Paederia scandens* mediates its therapeutic effect on NAFLD.

MATERIALS AND METHODS

Compounds Profiling and Disease Targets Identification

The bioactive compounds of the *Paederia scandens* were collected from the Traditional Chinese Medicine Systems Pharmacology (<https://tcmsp.com/tcmssp.php>) database, Herbal Ingredients Targets Database Introduction (<http://lifecenter.sgst.cn/hit/>), and Traditional Chinese Medicine Integrated Database (<http://www.megabionet.org/tcmid/>). Bioavailability and drug likeness were used for the candidate active ingredients screening, and we set bioavailability $\geq 30\%$ and drug likeness ≥ 0.18 as the threshold for candidate active compounds. Afterward, the chicken target points corresponding to the active compounds screened from the PharmMapper database and PubMed database were standardized in UniProt (<https://www.uniprot.org/>). Disease-related target points were mined from the GeneCards (<https://www.genecards.org/>) and OMIM (<https://omim.org/>) databases. All of disease gene targets were normalized with R software using the Bioconductor package when redundancy was deleted (Yu et al., 2012).

Network Establish

Screening for drug–disease crossover genes was performed. Based on previous steps, 2 sets of target lists were prepared: drug-related target points and disease-related target points. The crossover target points were

filtered with R software using the Venn Diagram package. The Stringdb database (<http://string-db.org/>) was used to analyze the intersecting protein–protein interactions (PPI), and the common target points were counted with R software (Guohua et al., 2017). Finally, Cytoscape 3.6.1 software was used, and a “drug–compound–target–disease” network was constructed.

Bioinformatic Annotation

The proteins with overlapping expression patterns were evaluated by bioinformatic annotation with R software using the Bioconductor package, including a Gallus database, PPI analysis (<https://string-db.org/>), a gene ontology annotation database website (<http://www.geneontology.org/>), and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis (<http://www.genome.jp/kegg/>).

Animals and Experimental Design

Thirty-six, 1-day-old, healthy Ross 305 chicks (33–40 g) were selected from the breeding chicken farm of Hunan Huaihua JinSheng Livestock Co., Ltd. (Huaihua, China). All chickens were housed in wood cages under the recommended environment. Chicks were brooded at 33°C during the first week; the brooding temperature was reduced 3°C/wk to approximately 24°C by week 4 of age. Light was provided continually using incandescent lamps. The environmental humidity was controlled at 60 to 65%.

The chickens were randomly divided into control group, model group, and drug-treated group, 12 individuals per group, and all groups were given a high-capacity diet in the experimental process. Cooked pigs' oil was the main calorie sources of the high-capacity diet. Meanwhile, model group was fed the diet mixed with 5% dose *Paederia scandens*. Endotoxin of *Salmonella enteritidis* 0.3 mL, with 1.0×10^9 cfu/mL and making 60 times by ultrasonic processing at 40 Hz for 20 s, was given into the model group and drug-treated group every week by gavage, respectively, and distilled water was given in control group at the same way. The dose of endotoxin of *S. enteritidis* was calculated by previous experiments, and 0.1 mL per chick was the safe and effective dose to treat NAFLD in chicken (Yu et al., 2012). The high-capacity diet formulations are shown in Table 1. All chickens were given free access to laboratory feed and water for 5 wk, and the chickens had no access to food for 12 h before they were anesthetized and sacrificed. Five chickens were randomly selected and weighted (Table 2), and at each blood sampling, 6 mL of blood were taken by syringe from the wing vein and gently transferred into blood tubes. Afterward, 5 chickens were killed in each group, and the livers were excised and removed promptly for further analysis. All the animal tests were in accordance with the Administration of Affairs Concerning Experimental Animals of the State Council of the People's Republic of China. The experiment protocol was approved by the

Committee on Experimental Animal Management of Tongren Polytechnic College in Guizhou (with protocol No. 2016051407).

TG and LPS Assays

Standing for 5 min, the serum of blood sampling were collected, the centrifuge condition of which is rotation speed 4,000 r/min and time 5 min, and preserved at -20°C for triglyceride (TG) and lipopolysaccharides (LPS). The levels of TG and LPS were measured by assays kits from the Nanjing Jiancheng Bioengineering Research Institute (Nanjing, China).

Detection of Hepatic Apoptosis and Cell Cycle

Freshly excised liver tissue was washed with normal saline on ice, dried it with filter paper. Approximately, 1 g of the liver was transferred to 10 mL of ice-cold PBS solution containing 1.25 mg/mL Type IV collagenase (Biochrom, Berlin, Germany), minced using a scalpel blade, and teased apart into a single cell suspensions which were incubated at 37°C for 30 min with occasional gentle mixing by inversion, followed by triturating by gentle pipetting, filtering first through 100 μm and then 40 μm cell strainers (Becton Dickinson, Franklin Lakes, NJ) and adjusting cell density to 5×10^6 cell/mL in the incubation buffer, and resuspended in PBS to a final concentration of 1×10^6 cells/mL. 1×10^6 cells were incubated with 5 μL Annexin V-FITC and 5 μL PI (propidium iodide) for 30 min at 4°C according to the manufacturer's instructions. The cells were diluted with the incubation buffer to a final volume of 600 μL , and stained cells were analyzed by FACSCalibur (BD Biosciences) using 488 nm excitation and 515 nm band-pass filter for fluorescein detection (FL1) and a filter > 600 nm for PI detection (FL2). Data were collected for 10,000 cells, and the number of the cells in each stage was calculated with the ModFit LT cell cycle analysis program (Verity Software House), and apoptosis was calculated using CellQuest software from BD Biosciences according to the manufacturer's instructions. Annexin V-FITC and PI were provided by Sigma.

Statistical Analysis

Statistical analysis was performed using PASW statistics 19.0 software package (ICM, Armonk, NY). The data were presented as means and standard deviation (means \pm SD), and group comparison was done by independent-sample *t* test.

RESULTS

The Assumed Targets of *Paederia scandens* and NAFLD

In the present study, we collected a total of 11 compounds from *Paederia scandens* (Supplementary Table 1) according to the screening conditions. The

Table 1. Composition and nutrient levels of the diets.

Ingredients	Proportion (%)	Nutritional level	
Corn	53.00	ME	16.07 (MJ/kg)
Fish meal	1.00	CP	160.0 (g/kg)
Fried meal	29.30	Ca	9.0 (g/kg)
Cooked pigs' oil	12.95	AP	3.5 (g/kg)
Additives	0.75	Lys	10.0 (g/kg)
Lys	0.12	Met	3.8 (g/kg)
Met	0.12		
CaHPO ₄	1.11		
Limestone meal	1.52		
Salt	0.13		

The additives contains 0.50% of microelements, per kg dietary contains Fe 80 mg, Zn 40 mg, Cu 8 mg, Mn 60 mg, I 0.35 mg, Se 0.15 mg, 0.20% of choline, Va 1,500 IU, VD₃ 200 IU, VE 10 IU, VK₃ 0.05 mg, VB₁ 1.80 mg, VB₂ 3.60 mg, VB₁₂ 0.01 mg, VB₇ 0.15 mg, VB₉ 0.55 mg, VB₅ 35 mg, VB₃ 10 mg, VB₆ 3.50 mg.

number of targets of each compound is different, the targets overlap significantly among, therefore a network is utilized in treatment with the compounds of *Paederia scandens*. We found 97 target proteins (Supplementary Table 2) which were added symbol by chicken target points database from 357 target points with these compounds.

The effectiveness of *Paederia scandens* in preventing and controlling NAFLD depends on the synergy between multiple compounds and their target points. For the disease target identification, we collected NAFLD-related target points from the GeneCards and OMIM databases. And topological analysis of protein interaction network nodes, including screening key nodes to remove duplicates, revealed a total of 1,309 target points.

Network Analysis of Targets

In an R software analysis, all the drug target points and disease-related target points were listed as 2 independent sets. The closed-loop form of the fixed position was used to represent the set and its relationship to obtain a Venn diagram, as shown in Supplementary Figure 1. We obtained a total of 33 interaction target points, and we used String-db (<https://string-db.org/>) to obtain the PPI among 132 simulated target points, as shown in Supplementary Table 3.

At the same time, we constructed an interactive drug-compound-target-disease network (Figure 1). Quercetin and kaempferol are used as ingredients in *Paederia scandens*, which is the main therapeutic candidates for NAFLD. Their potency was crucial to *Paederia scandens*. As shown in Supplementary Table 3, the targets of *Paederia scandens* include 33 of the 132 intersecting targets screened previously. These results further

Table 2. The mortality and weight gain in the fifth wk.

Groups	Numbers	Mortality %	Mean mass gain (kg)
Control group	12	0	2.190 \pm 0.86
Model group	12	17	1.904 \pm 0.66**
Drug group	12	0	2.015 \pm 0.72

**Means *P* < 0.01 vs. control group.

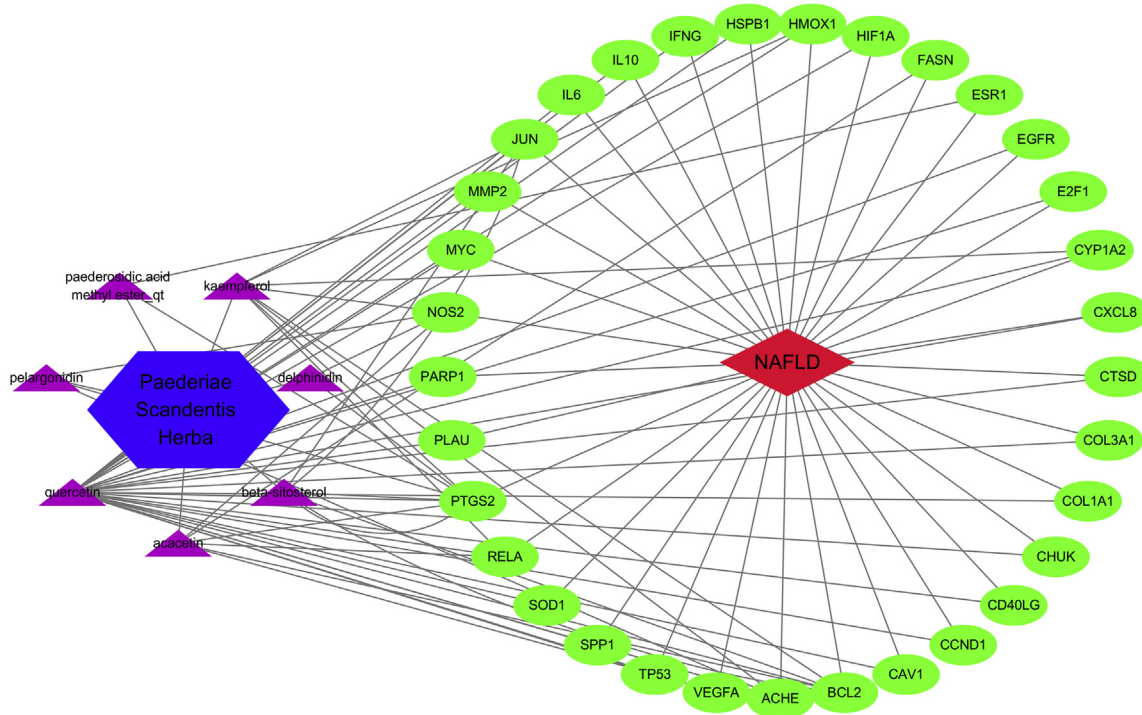


Figure 1. Network analysis of targets. “drug–compound–target–disease” network. Blue indicates drug–disease intersection targets. Red indicates NAFLD disease, and other colors indicate active compounds. Abbreviation: NAFLD, nonalcoholic fatty liver disease.

demonstrate that quercetin and kaempferol played a critical role in the biological activity of *Paederia scandens*.

Predicting Functional Enrichment Analysis for *Paederia scandens*

Gene ontology annotation revealed that the expressed drug–disease crossover targets were mainly associated with cofactor binding, heme binding, oxidoreductase activity, tetrapyrrole binding, and cytokine activity (Figure 2A). Moreover, KEGG enrichment analysis showed that many target genes were strongly associated with AGE-RAGE signaling pathway in diabetic complications, focal adhesion, mitogen-activated protein kinase signaling pathway, C-type lectin receptor signaling pathway, apoptosis, Salmonella infection, and toll-like receptor signaling pathway (Figure 2B). Intestinal factors are known to be one of the major causes of NAFLD. Our analysis showed that the important target genes were mainly distributed in the Salmonella infection pathway (Figure 3A), Toll-like receptor signaling pathway (Figure 3B), and apoptosis pathway (Figure 3C), which belong to endotoxin-induced hepatitis or intestinal inflammation. These findings explain the molecular mechanism underlying the results that most of the active molecules were associated with intestinal infection and NAFLD, suggesting that *Paederia scandens* may treat NAFLD through antitoxin and anti-inflammatory effects. Based on the molecular mechanisms of these predictions and the results of the network analysis, we designed experiments to verify our hypothesis at the cellular level.

Clinical Symptoms

The clinical manifestations were weight gain, unstable standing, mouth breathing, lethargy, unilateral lying, increased body temperature, and feces rot in most of the NAFLD chickens treated with endotoxin of *Salmonella enteritidis*. Even a small number of chickens died in model group. There was no chicken death in control group and drug-treated group, and no obvious clinical features in drug-treated group.

Serum TG and LPS Levels

The results showed that serum TG levels were significantly higher in model group than control group ($P < 0.01$), and serum LPS levels were significantly higher in model group than control group ($P < 0.01$). Serum TG and LPS levels were lower in drug-treated group than in model group. Endotoxin of *Salmonella enteritidis* significantly upregulated the TG and LPS levels in NAFLD, and *Paederia scandens* downregulated their levels. The results are listed in Table 3.

The Hepatic Cell Cycle and Apoptosis

The present study showed that the proportion of hepatocytes at Gap 0/1 (G0/1) phase were higher in model group than in control group ($P < 0.05$), and *Paederia scandens* downregulated it. The proportion of hepatocytes at S phase (S) and Gap 2/M (G2M) phase were upregulated by *Paederia scandens* significantly when compared with control group and model

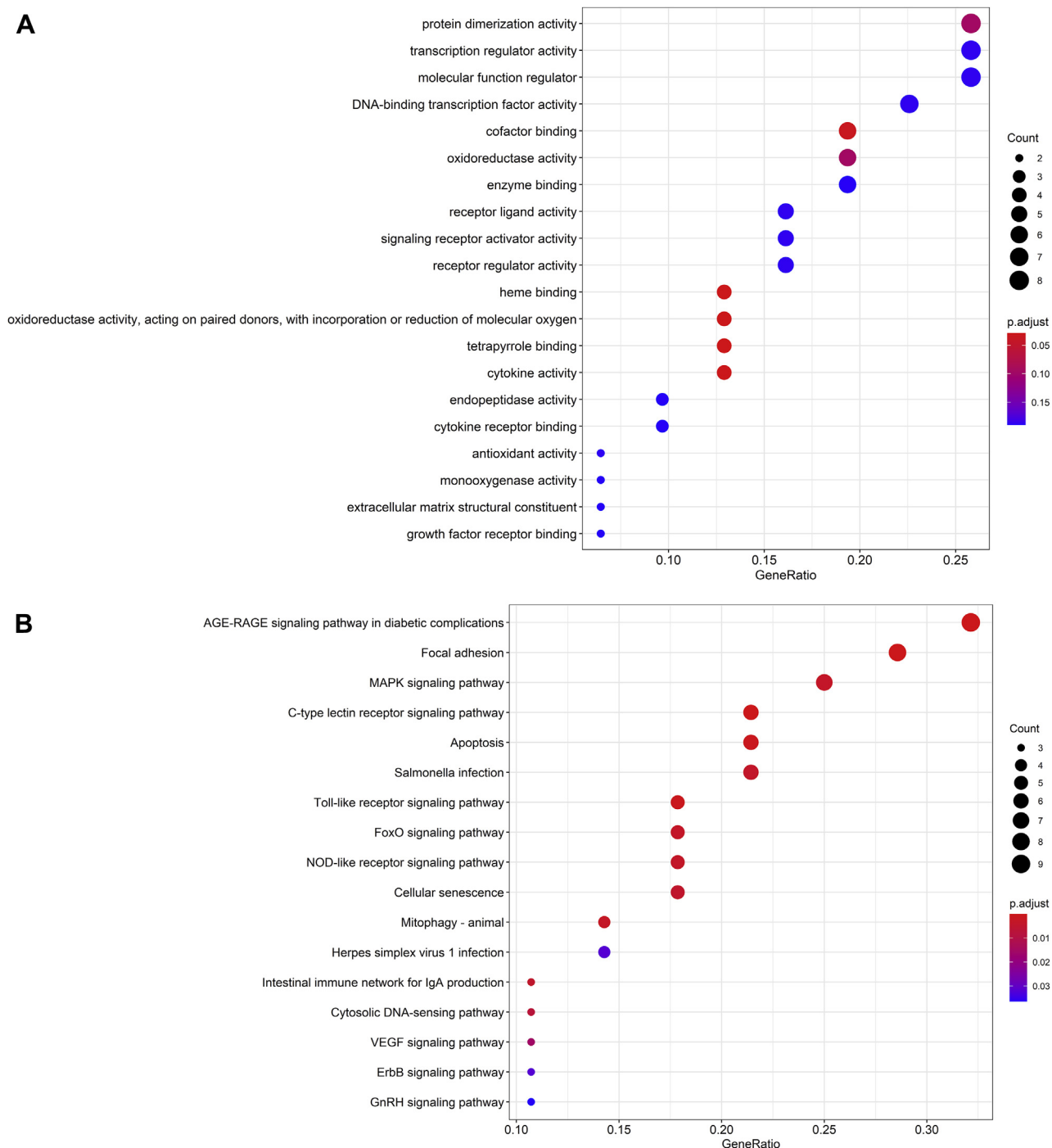


Figure 2. Bioinformatic analyses of drug-disease intersection proteins. (A) Gene ontology annotations; (B) KEGG annotation. Abbreviation: KEGG, Kyoto Encyclopedia of Genes and Genomes.

group ($P < 0.01$ or $P < 0.05$). The proportion of apoptosis was higher in drug group than in control group ($P < 0.01$), and the proportion of apoptosis was smaller in model group than in control group (Table 4). Proliferation index (PI) and S-phase fraction (SPF) were lower in model group than in control group, and *Paederia scandens* significantly upregulated them in drug group than in control group ($P < 0.05$ or $P < 0.01$). The PI and SPF were calculated as follow: $PI = \frac{S+G2M}{G0/1+S+G2M} \times 100\%$, $SPF = \frac{S}{G0/1+S+G2M} \times 100\%$.

DISCUSSION

The study of the pharmacodynamics of Chinese medicine compound substances and their mechanisms of action is one of the key scientific issues in the modernization of TCM (Shu et al., 2018). Network pharmacology and systems biology can explain the effects of drugs on bio-net from the perspective of macroscopic or overall regulation and provide new research ideas and technical means for the study of the mechanisms of action of Chinese medicine compound (Chen et al., 2014). In this

Table 3. Contents of TG (mmol/L) and LPS(EU/mL) in serum.

Groups	Control group	Model group	Drug group
TG	0.52 ± 0.15	0.99 ± 0.20**	0.60 ± 0.33
LPS	0.06 ± 0.00	0.09 ± 0.01**	0.07 ± 0.01

**means $P < 0.01$ vs. control group.

in diabetic complications, Focal adhesion, mitogen-activated protein kinase signaling pathway, C-type lectin receptor signaling pathway, apoptosis, Salmonella infection, and Toll-like receptor signaling pathway. In addition, intestinal factors are known to be one of the major causes of NAFLD, and the Salmonella infection pathway, Toll-like receptor signaling pathway, and apoptosis pathway belong to endotoxin-induced hepatitis or intestinal inflammation. These findings might explain the molecular mechanism underlying the results that most of the active molecules were associated with intestinal infection and NAFLD, suggesting that *Paederia scandens* may treat NAFLD-related intestinal factor.

For our further study, we confirmed that the protection of *Paederia scandens* in NAFLD model concomitant with diseases caused by endotoxin of *S. enteritidis*. The results showed *Paederia scandens* can reduce death rate, promote living quality, and downregulate serum TG and LPS levels in NAFLD chickens. These finding demonstrated *Paederia scandens* has an important treatment value for NAFLD.

The SPF is an index of liver regeneration, and PI is a proportion of during proliferation of hepatocytes (Qiang et al., 2008), and increasing PI and SPF showed that hepatocytes grew up actively. *Paederia scandens* can also upregulated PI and SPF of hepatocyte and reduce the proportion of apoptosis in NALFD chicken. The NAFLD research had shown that pathogenesis of NAFLD is the 2 hit hypotheses (Byrne, 2010). This observation also provides us with new direction and possibilities for further exploration for the mechanism for inhibiting NAFLD by *Paederia scandens*. Our enrichment analysis indicates that the regulation of apoptosis, Salmonella infection, and Toll-like receptor signaling pathway may be the key mechanism for inhibiting NAFLD by *Paederia scandens*, and quercetin and kaempferol are used as ingredients in *Paederia scandens*, which is the main therapeutic candidates for NAFLD.

Table 4. The cell cycles and apoptosis of the liver cell (%).

Item	Control group	Model group	Drug group
G0/1	70.67 ± 2.76	74.92 ± 2.28*	72.83 ± 4.89
S	7.51 ± 3.29	6.32 ± 2.84	11.46 ± 2.08**
G2M	7.84 ± 0.88	7.34 ± 1.64	9.53 ± 2.16*
apoptosis	11.82 ± 2.68	11.42 ± 2.69	8.34 ± 3.25**
PI	17.41 ± 3.65	15.42 ± 2.97	22.90 ± 3.42*
SPF	8.52 ± 3.42	7.13 ± 2.66	12.50 ± 2.78**

Abbreviations: PI, proliferation index; SPF, S-phase fraction.

* $P < 0.05$

** $P < 0.01$ vs. control group.

In conclusion, quercetin and kaempferol of *Paederia scandens* are used as ingredients and the main therapeutic candidates for NAFLD and targeting the Salmonella infection pathway, Toll-like receptor signaling pathway, and apoptosis pathway may be a potential therapeutic approach for NAFLD by *Paederia scandens*.

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DISCLOSURE

The authors declare no conflict of interest.

SUPPLEMENTARY DATA

Supplementary data associated with this article can be found in the online version at <https://doi.org/10.1016/j.psj.2020.09.087>.

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