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Original Article

Network pharmacology-based approach uncovers the JAK/STAT signaling mechanism underlying *paederia scandens* extract treatment of rheumatoid arthritis

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Abstract: Background: Rheumatoid arthritis (RA) is a common autoimmune disease. *Paederia scandens* (Lour.) Merr is a common folk remedy used in Hainan, China, to dispel the wind and dampness associated with RA. Methods: The active components of *Paederia scandens* were extracted using network pharmacology. The potential targets of active components were used to determine activated pathways, and the *in vitro* effects of *Paederia scandens* extracts were verified in RA fibroblast-like synoviocytes (HFLS-RA). Results: We identified 27 active components using ultra-high-performance liquid chromatography (UHPLC)-quadrupole time-of-flight (QTOF)-mass spectrometry (MS). Among the major target genes with high connectivity, *IL-1β*, *PI3K*, *TNF*, and *JAK2* are known to play key roles in RA development. High-affinity interactions were identified between active compounds in *Paederia scandens* extract and Janus kinase JAK 2, which are key components of the JAK-signal transducer and activator of transcription (STAT) signaling pathway. In HFLS-RA cells, *Paederia scandens* extract treatment reduced the mRNA levels of *IL-6*, *IL-1β*, and *IL-17*. *Paederia scandens* extract treatment also significantly inhibited the phosphorylation of JAK 2 and STAT3, regulating cell proliferation. Conclusions: Based on these results, we confirmed that *Paederia scandens* has potential for application as a therapeutic and preventive food and acts through the modulation and suppression of JAK-STAT pathway activation to control the inflammatory response in RA.

Keywords: Rheumatoid arthritis, *Paederia scandens* (Lour.) Merr, JAK/STAT signaling pathway

Introduction

Rheumatoid arthritis (RA) is an auto-immune disease leading to progressive joint damage, persistent synovial inflammation, pannus formation, cartilage and bone damage in severe cases [1, 2]. If not adequately treated, RA can progress, leading to loss of joint function and an increased risk of mortality. The global prevalence of RA is about 0.5%-1.0%, and RA can occur at any age, with two to three times high prevalence in female [3]. Fibroblasts and macrophages play major pathogenic roles in inflammatory joint diseases and have gained considerable attention as potential therapeutic targets. The inhibition of inflammation is a common therapeutic strategy for RA treatment, and several targeted drugs have been developed that aim to alter innate or adaptive immune responses through inhibition of pro-inflammatory cytokines, B cells, or T cells [4]. Among these developed therapeutic strategies, cytokine-targeting therapies have long been considered the most promising therapies. In clinical treatment, the inhibition of cytokine activity can prevent cytokine binding with surface receptors on target cells, leading to reduced inflammation.

New anti-RA medicines are safe and efficacious, but their use is often limited due to high costs. Traditional Chinese Medicine (TCM), a well-established and key component in China's health care system, can serve as an alternative and complementary approach to modern medi-

cines, reducing the symptoms of various inflammatory diseases in numerous studies [5, 6]. *Paederia scandens (Lour.) Merr.* (family: Rubiaceae) is a notable TCM well-known for its use in regional folk tonics and is known as "JiShiTeng" (JST) in Mandarin. *Paederia scandens* has a long history of medicinal use for its analgesic and anti-inflammatory activities and has been used to treat bruises, uric acid nephropathy, and rheumatism [7, 8]. According to TCM theory, *Paederia scandens* can clear away heat and excrete dampness. In TCM theory, "wind (feng)", "damp (shi)", and "cold (han)" are three factors that influence RA development [9] and jeopardize body homeostasis, also known as Yin and Yang imbalance. *Paederia scandens* has been used in anti-RA TCM formulas, with good curative effects in both clinical and folk medical settings. However, some concerns have been raised regarding the mechanisms through which *Paederia scandens* alleviates RA, and the components found in *Paederia scandens* that exert anti-RA effects have not been fully elucidated. Therefore, further systematic investigations into the activities of *Paederia scandens* components remain necessary. Although *Paederia scandens* has been shown to possess anti-inflammatory properties in both clinical and research settings [10-12], due to its complex composition, the underlying mechanisms require further investigation.

Here, network pharmacology and other experimental methods were applied to explore the relationships among active compounds, their target proteins, and associated signaling pathways to reveal potential mechanisms, so as to evaluate the effects mediated by *Paederia scandens* treatment. Network pharmacology combines systems biology, pharmacology, and computer analytics technologies to investigate the complicated interactions among active components, their targets, and disease states. To explore the multi-component and complex effects of TCM therapies, we can use network pharmacology to construct multi-layered networks showing disease-phenotype-gene-drug interactions, which can facilitate the identification of potential biological processes and pathways through which TCM therapies exert their functions [13, 14]. By analyzing the disease-associated targets of TCM components, critical clues and new avenues of research can be illuminated, contributing to the design and development of new drugs. Molecular docking is a drug design method that simulates ligand-receptor docking using informatics data to predict the potential binding modes between ligands and acceptors. In recent years, molecular docking has been increasingly applied to improve understanding of the relevant mechanisms involved in ligand-receptor binding, contributing to the design of new drugs and the development of effective therapeutic agents [15].

In this study, we investigated the mechanisms through which *Paederia scandens* extract acts on RA using a network pharmacology approach. The current study systematically investigated predicted targets and biological signaling pathways involved in anti-RA activities and used RA fibroblast-like synoviocytes (HFLS-RA) as a cellular model to confirm the predicted results. We found that the Janus kinase (JAK)-signal transducer and activator of transcription (STAT) pathway was a crucial pathway regulated by *Paederia scandens* extract, suggesting that this pathway may represent a novel target for RA treatment, suggesting a potential therapeutic strategy toward treating RA.

Materials and methods

UHPLC-QE-MS non-targeted metabolomics assay

Metabolite profiles were generated by ultra-high-performance liquid chromatography (UHPLC)-Q extractive (QE)-mass spectrometry (MS). These experiments were performed by Shanghai Biotree Biomedical Technology Co., Ltd.

Gene mining of rheumatoid arthritis

Confirmed RA target proteins indexed in the OMIM database (https://omim.org/) were searched, and original data were collected. Proteins with a combined score > 0.8 were selected as RA-specific target genes.

Screening and target prediction of effective Paederia scandens compounds

Effective compounds present in *Paederia scandens* extract were screened by MS analysis. The target predictions for screened compounds were performed using online query platforms

for compounds (PubChem: https://pubchem.ncbi.nlm.nih.gov/; Herb target prediction website: http://herb.ac.cn/).

Protein-protein interaction network analysis

The identified targets of *Paederia scandens* components and RA-specific target genes were compared using a Venn diagram. The overlapping region of the Venn diagram represents common genes that are both targets of *Paederia scandens* compounds and RA-specific target genes. The overlapping genes were imported into the protein-protein interaction (PPI) platform (STRING https://string-db.org/, GeneMANIA http://genemania.org/) to extract interaction relationships, and the files were saved and imported into Cytoscape software for mapping. After Hub analysis, key target genes in the candidate network were identified as intersections among PPI pathways, and the topological features of the PPI network were analyzed to determine the core network.

Gene Ontology (GO) and pathway enrichment analysis

The key target genes were input into the database for annotation, visualization, and integrated discovery (DAVID; https://David.ncifcrf.gov/) for Gene Ontology (GO) enrichment analysis. The Kyoto Encyclopedia of Genes and Genomes (KEGG) was used for pathway analysis, and significantly enriched pathways with $P < 0.05$ are presented. The enrichment results are presented as a bubble diagram.

Compound-target interactions validation

The crystal structures of identified hub protein targets were obtained from the Protein Data Bank (https://www.rcsb.org/). The three-dimensional structures of potential active substances were obtained from PubChem (https://pubchem.ncbi.nlm.nih.gov/), an open repository for chemical data. AutoDockTools (version 1.5.6, http://autodock.scripps.edu/) was used to convert the downloaded active chemicals and hub protein targets into pdbqt formats. AutoDock Vina (http://vina.scripps.edu/) was used to perform molecular docking and calculate binding affinities. PyMOL software (version 2.2, https://pymol.org/2/) was used to visualize the docking results between active chemicals and protein targets.

Cells and cell culture

Primary HFLS-RA cells were brought from BFB Biotechnology Co., Ltd. (Shanghai, China) and cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin antibiotics in cell culture incubator with 5% CO_2 at 37°C.

Preparation of Paederia scandens aqueous extract

Paederia scandens was bought from Tongrentang (Beijing, China) (500 g) and was soaked in 1.5 L water and boiled, followed by filtering through gauze. After adsorption with Mitsubishi adsorption resin DIAION (HP20), the effluent was collected by washing the chromatography column with 60% ethanol, and the collected solution was mixed with anhydrous methanol at a ratio of 1:1 overnight for extraction in a liquid separation funnel. The lower layer was collected in a liquid drying box and allowed to air dry.

Experimental grouping

The normal control group (HFLS-RA) consisted of HFLS-RA cells grown under normal culture conditions. The positive drug control group (HFLS-RA+MTX) consisted of HFLS-RA cells treated with low-dose methotrexate (MTX; 0.01 g/+ for 24 hours). The experimental drug treatment was tested at different concentrations: a high dose (2.4 g/*mL Paederia scandens* extract, HFLS-RA+GJST), a medium dose (1.2 g/*mL Paederia scandens* extract, HFLS-RA+ZJST), and a low dose (0.6 g/*mL Paederia scandens* extract, HFLS-RA+DJST). At the same time, we designed to add 10 μM coumermycin A1 (MCE, China) treatment for 12 h after the addition of *Paederia scandens* in order to complete the JAK2 activator trial.

Cell viability assay

HFLS-RA cells were placed into 96-well cell culture plates with a density of 3.5×10^3 cells per well. After overnight culture, the indicated treatment was applied for 24 hours. Cell counting kit 8 (CCK-8) solution was then added according to the CCK-8 kit instructions (Dojindo. Japan).

Cell apoptosis assay

Certain amount of HFLS-RA cells were plated in 6-well plates and treated for 24 hours after the

cells reached 60%-80% confluence. An annexin V apoptosis detection kit (BD Biosciences) was used to detect apoptotic cells.

Measurement of cytokine concentrations

To detect interleukin (IL)-1β (Sigma-Aldrich, St. Louis, MO), IL-6 (Sigma-Aldrich), and IL-10 (Sigma-Aldrich) concentrations, cell supernatants were analyzed by enzyme-linked *immunosorbent assay, according to the manufacturer's instructions.*

Real-time PCR assay

Total RNA was extracted with a kit by following the manual (Promega) and reverse transcribed into cDNA (YEASEN). The relative quantitation of gene expression was calculated using the $2^{-\Delta\Delta CT}$ method, with normalization of all target threshold cycle (CT) values against those of an internal housekeeping gene (*GAPDH*). Quantitative real-time PCR assays were performed using a q225 Real-Time PCR Detection System.

Western blot assay

Total protein was extracted using radioimmunoprecipitation assay lysis buffer (RIPA), and the protein concentration was determined with BCA assay (Beyotime Biotechnology). Cell lysates were run with PAGE and transferred to PVDF membrane. Immune blot were carried out by incubation with primary antibody overnight at 4°C, after washing with PBST three times, then incubated with secondary antibody (Beyotime Biotechnology) in appropriate dilution ratio. Primary antibodies used were listed as follow: rabbit anti-JAK2 (abcam), anti-JAK2 (phospho Y1007+Y1008) (abcam), anti-STAT3, and anti-STAT3 (phospho Y705) monoclonal antibodies (Beijing Boaosen Biotechnology). The gray value of each imaged protein band was analyzed using the Gel Imaging System. The expression levels of p-JAK2/JAK2, and p-STAT3/STAT3 were analyzed using glyceraldehyde 3-phosphate dehydrogenase (GAPDH, Beyotime Biotechnology) as the internal reference.

Statistical analysis

The data were analyzed using GraphPad Prism 6.0 and SPSS 20.0 software. The statistical significance was determined by one-way analysis of variance (ANOVA) followed by a Dunnett's test or a Kruskal-Wallis ANOVA on Ranks followed by a Dunn's test for multiple comparisons and expressed as themean ± standard deviation. $*P < 0.05$ was considered a significant difference.

Results

UHPLC-QE-MS non-targeted metabolomics detection in Paederia scandens (JST) extract

First, MS/MS peaks were identified using the two-stage MS database built by Baiqu by applying the fragmentation pattern search method. The chemical composition of *Paederia scandens* extract was obtained by MS screen, using oral availability (OB) > 30% and drug-like (DL) > 0.18 as search criteria. We selected 27 active compounds from the total list of identified agents. The related substances were labeled according to the total ion current diagram of positive and negative ion patterns detected by UHPLC-QTOF-MS, as shown in **Figure 1A**, **1B**.

Gene targets for RA

RA target proteins that have been confirmed and are indexed in disease-based gene databases such as OMIM (https://omim.org/) and DisGeNET (http://www.disgenet.org/) were identified, and the original data were collected. Proteins with a combined score > 0.8 were selected as RA-specific target genes. In total, 832 target genes were identified, and selected part of results are described below and presented in **Table 1**.

Chemical component screening and target prediction for Paederia scandens components

A target gene prediction platform (SWisstar http://swisstarget prediction.ch/prediction) was used to predict screening results, and 510 effective targets were identified using the criteria Combinescore ≥ 0.5, with select part of genes presented in **Table 2**.

Construction and analysis of an interactive network

The *Paederia scandens* component targets and RA-specific target genes were compared using a Venn diagram, revealing 24 key, shared target genes: PIK3CD, PLA2G2A, ALPL, CTSK,

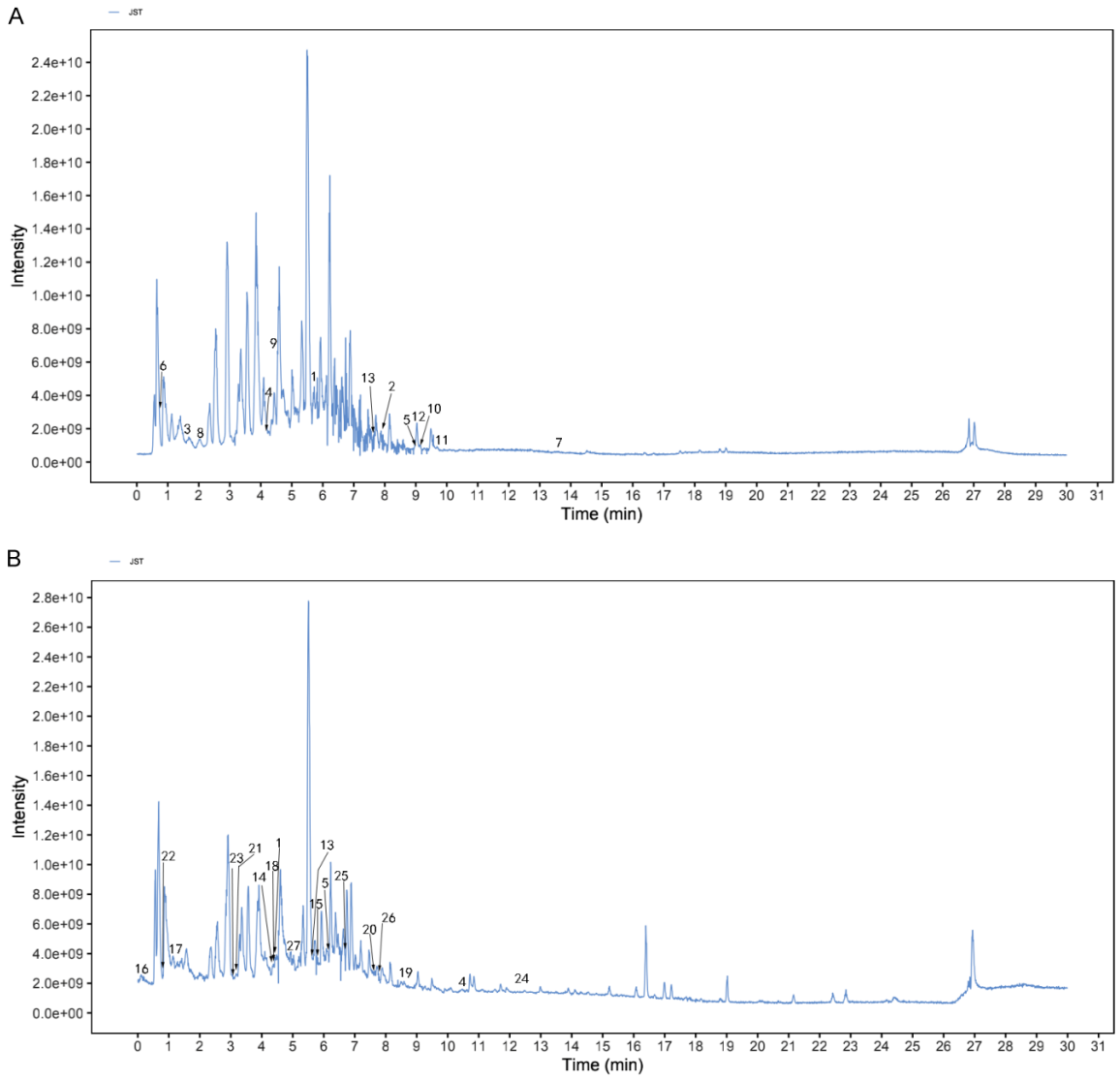


Figure 1. A. Total ion current for negative ions detected by UHPLC-QTOF-MS of *Paederia scandens* samples. B. Total ion current pattern of positive ions detected by UHPLC-QTOF-MS of *Paederia scandens* samples. The image is annotated with effective compounds as follows: 1, ellagic acid; 2, quercetin; 3, 6-o-syringyl-8-o-acetyl shanzhiside methyl ester; 4, eudesmin; 5, isorhamnetin; 6, 5'-uridylic acid; 7, triptophenolide; 8, catechin; 9, coniferin; 10, wedelolactone; 11, dihydroartemisinin; 12, pedunculoside; 13, picroside III; 14, kaempferol; 15, morin; 16, lindenenol; 17, digallic acid; 18, ginkgolide B; 19, isobavachin; 20, curcumenolactone A; 21, epigallocatechin gallate; 22, isobavachin; 23, diosbulbin B; 24, palmatine chloride; 25, remerine; 26, indigo; 27, quinoline.

RORC, GBA, PTGS2, MAPK, ADAM17, IL1B, STAT1, FN1, CCR5, TLR9, PRKCD, ABCG2, ADH1C, NFKB1, HPGD, PIK3R1, F2RL1, MAPK9, TPMT, and TNF. The results are shown in **Figure 2A**.

Common genetic data were collected and imported into the PPI platform (STRING) to extract interaction relationships. The files were saved and input into Cytoscape software for mapping. After Hub analysis, TNF, IL1B, PTGS2, CCR5, NFKB1, TLR9, STAT1, PRKCD, and FN1 were identified as association hubs and may represent key target genes. The results are shown in **Figure 2B**.

GO and pathway enrichment analysis

The identified target genes were input into DAVID for GO enrichment, using $P < 0.05$ as an indicator of significant biological function. The biological processes identified were cancer-

Table 1. List of RA-specific target genes

Gene Name					
PSORS7	PLA2G2A	IL23R	RFH1	SLAMF1	PRG4
TNFRSF25	PLA2G2F	UOX	CTSK	CD244	PTGS2
TNFRSF9	ALPL	GFI1	PSMB4	ADAMTS4	PLA2G4A
PIK3CD	C1QA	COL11A1	RORC	FCGR2A	CHI3L1
TNFRSF1B	C1QC	PTPN22	S100A9	FCGR3A	MAPK
CLCNKB	C1QB	AMPD1	S100A8	FCGR3B	IL10
PADI2	MATN1	NOTCH2	GBA	FCGR2B	CR1
PADI1	MMACHC	HJV	FCRH3	ILDR2	NLRP3
PADI3	PRDX1	FCGR1A	APCS	FASLG	ADAM17
PADI4	C8B	PSORS4	COPA	RC3H1	MATN3

Table 2. Candidate targets of *Paederia scandens*

Gene Name					
KCNH2	DNMT1	CA3	CA5A	TAS2R31	RAF1
BACE1	DYRK1A	MMP12	MMP9	PGF	BRAF
ST3GAL3	APP	CA2	PTGS1	VEGFA	SNCA
FUT7	MAPK14	CA1	MMP13	CA13	DRD2
FUT4	TERT	CA12	ABCG2	DNM1	HSD17B2
MMP2	MET	HIF1A	CYP1B1	KIT	ADORA3
MMP14	ABCB1	CA9	KLK1	FGFR1	CELA1
PGD	STAT1	CA7	KLK2	MCL1	PREP
BCL2	SQLE	CA6	CA4	SHBG	PDK1

related pathways. Identified cell components included apical junction complexes, destruction complexes, and beta-catenin destruction complex. In addition, 95 molecular functions, primarily involving proteins with serine/threonine/tyrosine kinase activity, protein phosphatase binding, and inhibitory SMAD (I-SMAD) binding, were identified. The analysis results are shown in **Figure 3A** ($P < 0.05$).

Pathway enrichment results were visualized using a bubble map (**Figure 3B**, $P < 0.05$). The following pathways were identified as highly ranked signaling pathways associated with RA: JAK-STAT signaling pathway, Toll-like receptor (TLR) signaling pathway, B cell receptor signaling pathway, and IL-17 signaling pathway.

Compound-target interaction verification

Molecular docking was used to examine interactions between important active drugs and major targets in greater depth. Conformations are considered to have good interactions if their binding affinities are below 5.0 kcal/mol. The molecular docking results revealed that the conformations of active drugs and protein targets displayed good binding interactions that were also dependable. The conformations of important active molecules and significant hub targets are shown in **Figure 4**.

Paederia scandens extract inhibited HFLS-RA cell proliferation

Cell viability was examined in cells treated with MTX or different concentrations of *Paederia scandens* extract using the CCK8 method, as shown in **Figure 5**. The 24-hour inhibitory rates for the HFLS-RA+MTX, HFLS-RA+GJST, HFLS-RA+ZJST, and HFLS-RA+DJST groups were 0.23, 0.43, 0.64, and 0.82, respectively ($P < 0.01$).

Paederia scandens extract promotes HFLS-RA apoptosis

After examining the effects of *Paederia scandens* extract on the proliferation and survival of HFLS-RA cells, HFLS-RA+GJST was used as the experimental group for subsequent experiments. The results showed that *Paederia scandens* extract induced apoptosis at a rate of 79.2%, which was 76.9% higher than that of the control group. The apoptosis rate of MTX was 91.2%, 87.9% higher than control group. These results are shown in **Figure 6**.

Inflammatory factors secretion in the cell supernatant

The IL-1β and IL-6 level in the cell supernatant were significantly lower in the HFLS-RA+GJST and HFLS-RA+MTX groups than in the HFLS-RA group, whereas IL-10 levels were significantly increased. The results are shown in **Figure 7**.

The mRNA levels of several key targets, including IL-1β, IL-6, IL-10 were validated by quantitative PCR. *Paederia scandens* extract downregulated IL-1β, IL-6 gene expression and upregulated IL-10 gene expression **Figure 8**.

Paederia scandens extract regulates JAK2 and STAT3 in HFLS-RA cells

Paederia scandens extract inhibited the JAK-STAT pathway. Various target protein levels, including JAK2 and STAT3, were determined by

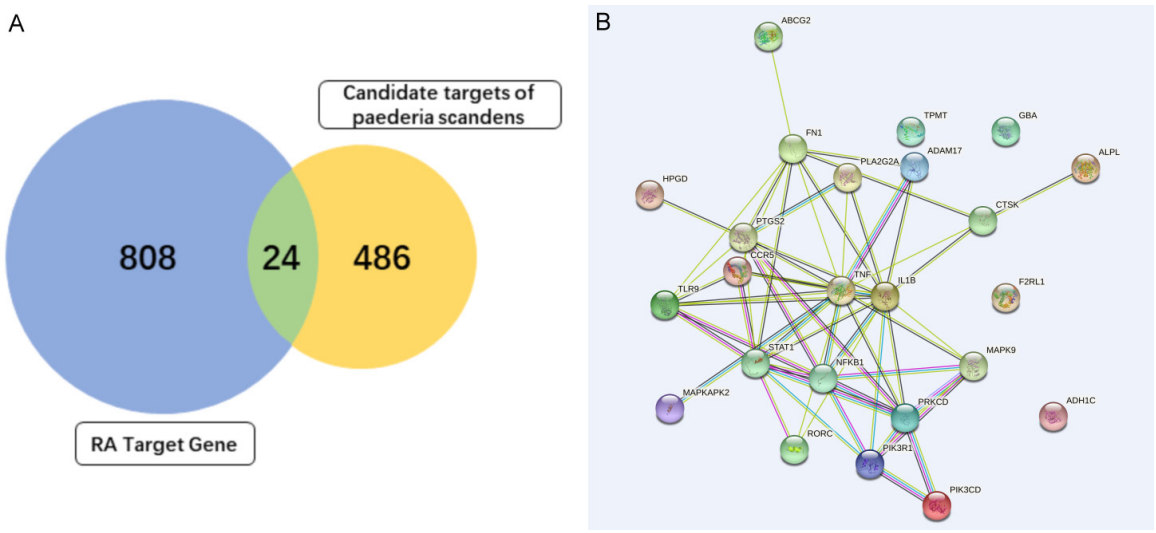


Figure 2. A. Venn diagram showing 24 genes targeted by *Paederia scandens* (JST) and RA. B. The disease targe protein-protein interaction network.

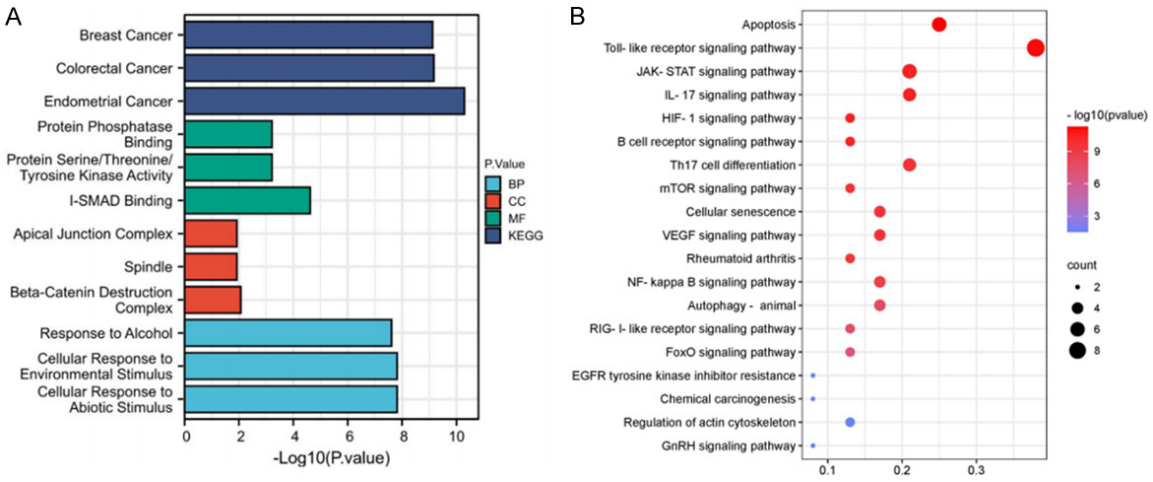


Figure 3. A. KEGG pathway analysis for the major targets of *Paederia scandens*. B. GO analysis for the major targets of *Paederia scandens.*

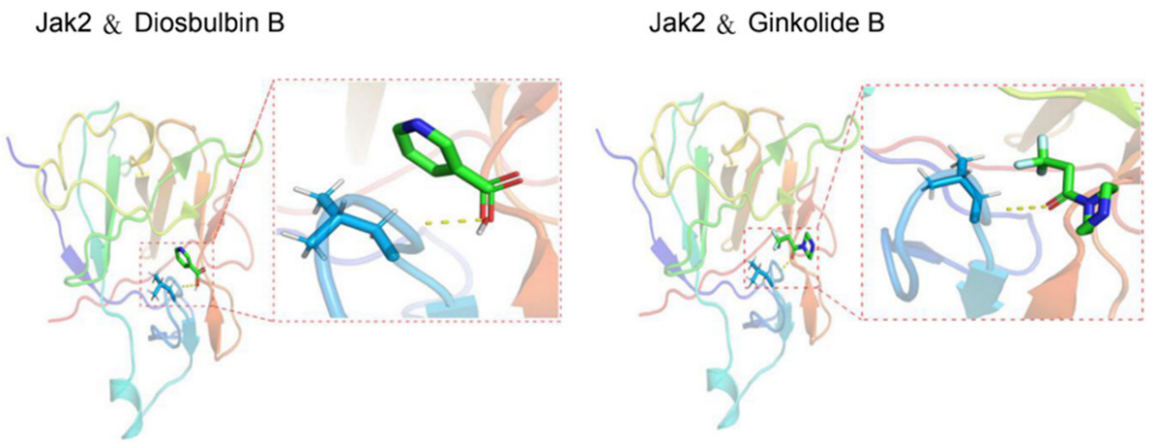


Figure 4. The conformations of the primary active compounds and major hub targets.

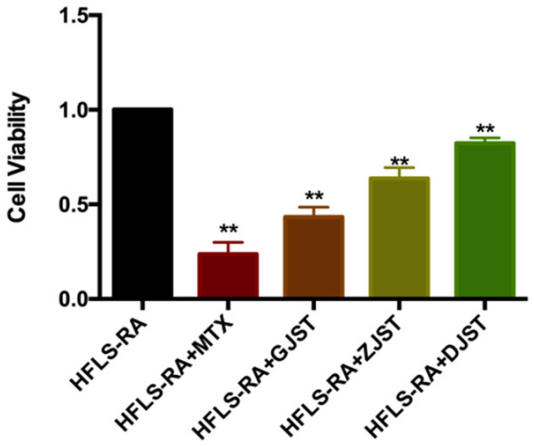


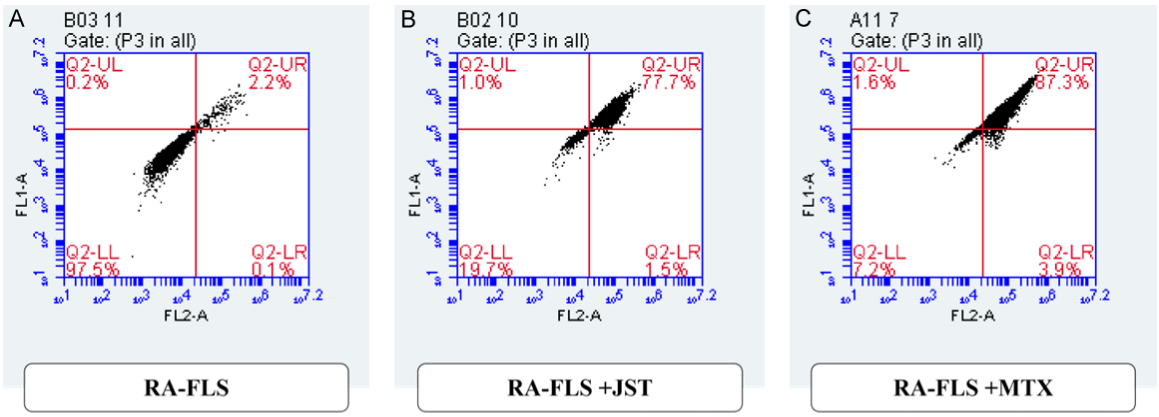
Figure 5. A bar chart showing the results of the cell viability assay. Data are presented as the mean ± SD (n = 3), $^{**}P < 0.01$ vs. HFLS-RA. HLFS-RA, control group; HFLS-RA+MTX, treated with 0.01 g/*mL* methotrexate for 24 hours; HFLS-RA+GJST, treated with 2.4 g/*mL Paederia scandens* extract; HFLS-RA+ZJST, treated with 1.2 g/*mL Paederia scandens* extract; HFLS-RA-DJST, treated with 0.6 g/*mL Paederia scandens* extract.

western blot analysis. As shown in **Figure 9**, *Paederia scandens* extract treatment decreased JAK2 and STAT3 phosphorylation level. The phosphorylation of JAK2 and STAT3 activated by Coumermycin A1 was significantly inhibited by treatment with *Paederia scandens* extracts.

Discussion

According to TCM theory, RA can be classified as a "Bi syndrome" (or "Bi" pattern, in Mandarin), caused by the invasion of heat pathogens, wind, or dampness into the human body [16]. TCM therapies that function to dispel wind and remove dampness have been demonstrated to induce curative effects with relatively good safety profiles and few side effects, leading to their increased popularity for the prevention and treatment of disease. Modern medicine views RA as a systemic autoimmune disease characterized by chronic joint inflammation [17], and many TCM therapies show promising anti-inflammatory and antioxidative activities that can contribute to restoring the balance of immune cells [18]. *Paederia scandens* is used as a TCM herb that is consumed due to its various activities in "dispelling dampness", "relieving pain and swelling", and immunomodulatory and anti-inflammatory effects. *Paederia scandens* is often used in the treatment of autoimmune and inflammatory diseases, such as psoriasis, RA, and asthma [19, 20]. However, the underlying mechanism through which *Paederia scandens* contributes to alleviating RA symptoms remains unknown, hindering its further clinical application.

Unlike Western medicines, TCM therapies possess multi-component drug properties, which can result in multitarget, multi-step, and multi-level synergistic effects [21]. Rapid advances in genomics and bioinformatics analyses have led to the growth of TCM network pharmacology, which aims to clarify the pharmacologic mechanisms underlying the effects of active ingredients in TCM therapies at the molecular level [22]. Network pharmacology integrates pharmacology, omics, systems biology, and computational biology to describe the outcomes of TCM therapies in terms of the biological networks targeted by TCM components [23, 24]. However, multiple approaches can be used to perform network pharmacology analyses of TCM therapies, and each approach has advantages and disadvantages. The compounds present in TCM therapies are typically collected from databases; however, some substances are not detectable in decoctions, resulting in false positive results. Here, we dissected the chemical constituents of *Paederia scandens* extract using LC-MS and employed a network pharmacology analysis to capture the bioactive components and their potential targets to explore the mechanisms underlying the effects of *Paederia scandens* extract in RA. Thus, the compounds identified by LC-MS identification can reduce the scope of potentially active compounds and increase the accuracy of the predicted results, providing important information for further pharmacological investigations. Using UPLC-QTOF-MS (performed by Biotree Biotechnology, Shanghai, China), we identified more than 100 different compounds in *Paederia scandens* extract based on chromatogram matching. After screening these compounds for oral bioavailability (OB ≥ 30%) and drug-likeness (DL ≥ 0.18), we selected 27 active ingredients. Most of the compounds showed favorable absorption properties. We used a virtual docking platform to predict and screen each compound for interactions with target proteins. In the present study, we constructed a Venn diagram to examine the over-



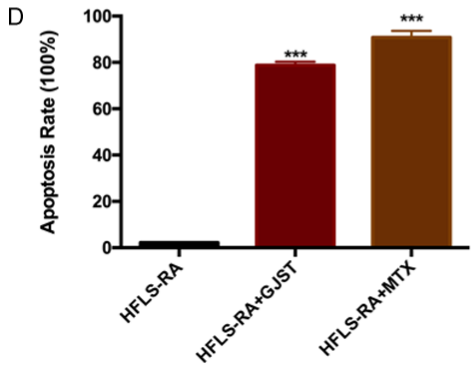


Figure 6. Representative profiles showing apoptosis of HFLS-RA cells treated with vehicle (A), MTX (B), or *Paederia scandens* extract (C) alone, as determined by annexin V-fluorescein isothiocyanate (FITC) and propidium iodide (PI) staining. (D) The relative HFLS-RA cell populations in cell cycle arrest. All data are expressed as the mean ± SD (n = 3); ***P < 0.001 vs. HFLS-RA. HLFS-RA, control group; HFLS-RA+MTX, treated with 0.01 g/*mL* methotrexate for 24 hours; HFLS-RA+GJST, treated with 2.4 g/*mL Paederia scandens* extract.

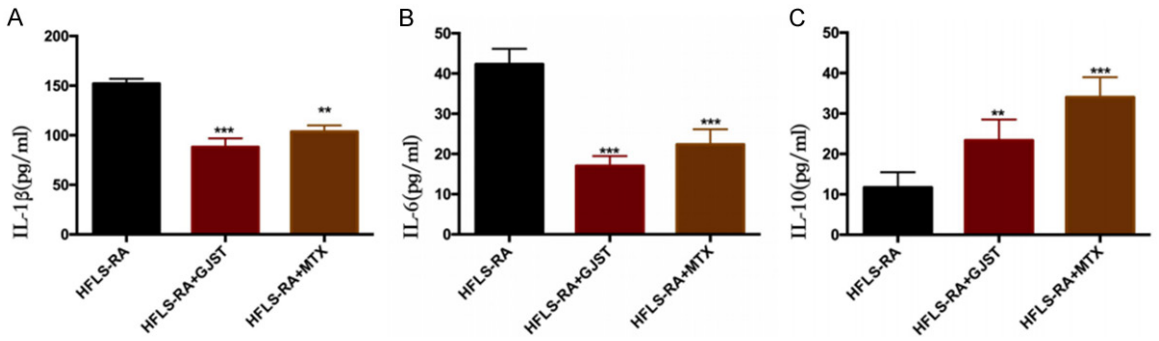


Figure 7. The expression levels of IL-1β (A), IL-6 (B), and IL-10 (C) in cell supernatants as assessed by ELISA. All data were are expressed as the mean ± SD (n = 3); **P < 0.01; ***P < 0.001 vs. HFLS-RA. HLFS-RA, control group; HFLS-RA+MTX, treated with 0.01 g/*mL* methotrexate for 24 hours; HFLS-RA+GJST, treated with 2.4 g/*mL Paederia scandens* extract.

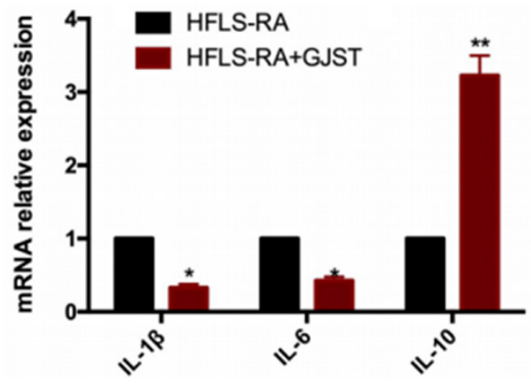


Figure 8. The mRNA expression levels of IL-1β, IL-6, and IL-10. The mRNA levels were examined by real-time PCR. Data are expressed as the mean ± SD (n = 3), P < 0.05; **P < 0.01 vs. HFLS-RA. HLFS-RA, control group; HFLS-RA+MTX, treated with 0.01 g/*mL* methotrexate for 24 hours; HFLS-RA+GJST, treated with 2.4 g/*mL Paederia scandens* extract.

lap between the 832 predicted targets of active components and 527 RA-related targets genes, which revealed 24 shared target genes. Therefore, *Paederia scandens* extract was hypothesized to mediate anti-RA effects through

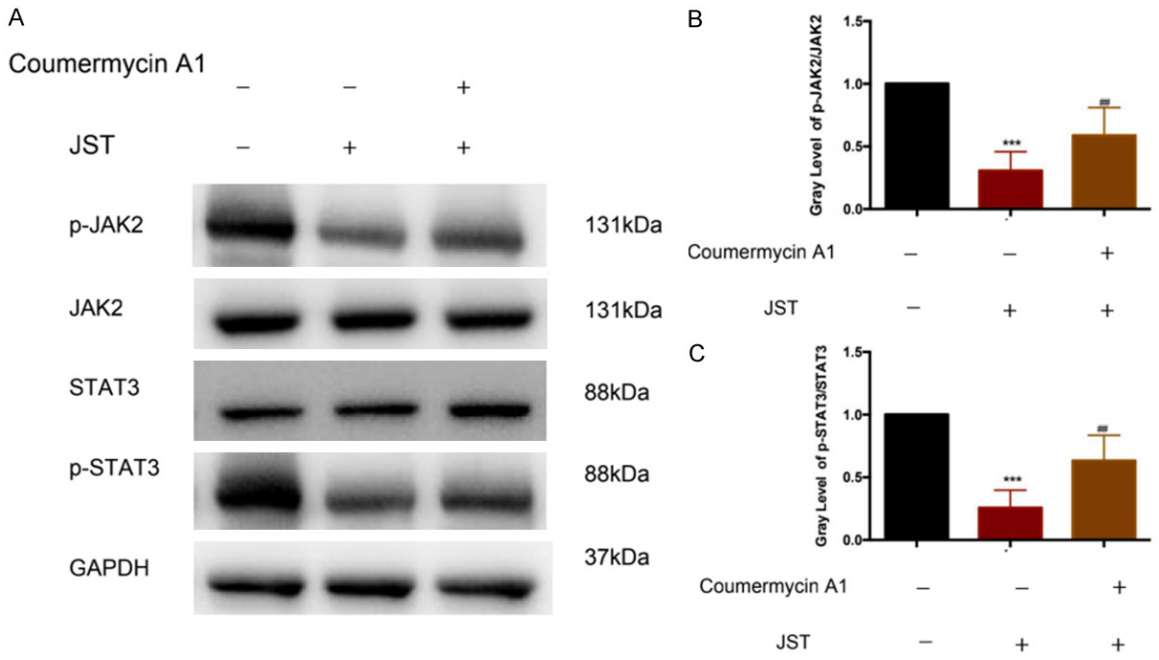


Figure 9. A. The total protein expression levels of JAK2, p-JAK2, STAT3, and p-STAT3. B and C. Quantitative analysis of protein expression levels. Data are presented as the mean ± SD (n = 3). ***$P < 0.001$ vs. control group; ##$P < 0.01$ vs. JST+Coumermycin A1; JST treated with 2.4 g/*mL Paederia scandens* extract, treated with 10 μM Coumermycin A1.

multi-component and multitarget mechanisms.

In this study, using compound-target-pathway network analysis, 10 nodes, including FN1, PLA2G2A, PTGS2, CCR5, TLR9, PI3K, IL1β, TNF, NFKB1, PRKCD, and STAT1, were identified as highly connected nodes in the relationships among *Paederia scandens* component targets and RA-related genes. Phospholipase A2 group IIA (PLA2G2A) is an inflammatory biomarker that is positively correlated with disease indices, such as joint pain, joint swelling, and the erythrocyte sedimentation rate (ESR) [25]. Prostaglandin-endoperoxide synthase 2 (PTGS2, also known as cyclooxygenase 2 [COX-2]) produces the inflammatory mediator prostaglandin E2, which can promote fibroblast-like synoviocyte (FLS) proliferation in RA [26]. In the pathogenesis of RA, C-C motif chemokine receptor type 5 (CCR5) is abundantly expressed in T cells, monocytes, and macrophages, and its ligand, C-C motif chemokine ligand 5 (CCL5), is found in the synovial fluid of patients with RA; therefore, CCR5 is thought to play a pro-inflammatory role in RA [27]. Synoviocyte and monocyte subsets isolated from RA patients show increased expression levels of TLR2 and TLR9 [28]. Increased TLR9 is associated with the increased production of inflammatory cytokines [29]. IL-1β and tumor necrosis factor-α (TNFα) are well-known pro-inflammatory cytokines that play pivotal roles in tissue destruction and overall RA pathogenesis. In addition, TNFα promotes FLS migration and invasion in RA via the nuclear factor kappa B (NF-κB) pathway [30]. As previously described, pro-inflammatory cytokines (TNF-α, interferon-γ, IL-1β, IL-17, and IL-6) result in increased expression of STAT1, STAT3, and STAT5, in addition to the upregulation of STAT protein phosphorylation [31]. Therefore, we speculate that the effects of *Paederia scandens* extract in RA may be related to these biological processes.

Of the 10 leading target genes with high connectivity in the PPI network, IL1β, PI3K, TNF, and STAT1 play critical roles in RA development. The GO analysis demonstrated that *Paederia scandens* compounds are associated with components of major biological processes, including the positive regulation of cytokine production, the response to lipopolysaccharide, regulation of IL-6 production, stress-activated mitogen-activated protein kinase (MAPK)

cascade, chemokine production, response to TNF, stress-activated protein kinase signaling cascade, and the regulation of inflammatory responses. The KEGG pathway analysis revealed that *Paederia scandens* extract produced therapeutic effects against RA by regulating various signaling pathways, such as mammalian target of rapamycin (mTOR), forkhead box O (FoxO), hypoxia-induced factor 1 (HIF-1), RA, NF-κB, vascular endothelial growth factor (VEGF), Th17 cell differentiation, IL-17, TLR, and JAK-STAT. Based on the GO functional enrichment analysis and KEGG pathway enrichment analyses of all active component targets, *Paederia scandens* extract may exert anti-RA effects through multiple pathways and targets, resulting in synergistic effects on the anti-inflammatory response, cell differentiation, cell apoptosis, and cell invasiveness. The experimental results in this study confirm that the active compounds found in *Paederia scandens* extract inhibit proliferation and induce apoptosis in HFLS-RA cells, delaying RA progression.

Recent studies have shown that JAK-STAT signaling pathway inhibitors are often used to treat RA [32]. Using the molecular docking analysis, we found high affinities between active compounds and JAK2, which are key components in the JAK-STAT signaling pathway. The anti-RA therapeutic effects of *Paederia scandens* extract are likely mediated by the modulation of several targets involved in the JAK-STAT signaling pathway, indicating that *Paederia scandens* extract has targeted therapeutic effects in RA. To further validate these findings, we conducted an experiment examining the effects of *Paederia scandens* extract treatment on protein expression levels of p-JAK2 and p-STAT3, which were significantly decreased in HFLS-RA cells treated with *Paederia scandens* extract. In addition, the mRNA levels of IL-6, IL-1β, and IL-17 were significantly decreased. These results show that *Paederia scandens* extract demonstrated anti-inflammatory activity mediated by the modulation of JAK-STAT signaling in an in vitro model of RA inflammation. We propose that *Paederia scandens* extract may serve as a potential therapeutic or preventive agent for the selective suppression of JAK 2 and the regulation of the inflammatory response.

Conclusions

In conclusion, *Paederia scandens* extract demonstrated its therapeutic effect to RA in HELS-ra cells in this study. We also employed a network pharmacology approach to explore its molecular mechanisms. The performance of LC-MS/MS analyses followed by a network pharmacology investigation revealed that β-sitosterol, baicalein, stigmasterol, cavidine, and coniferin in *Paederia scandens* extract are likely involved in the treatment effects against RA. PPI network analysis and KEGG pathway enrichment analysis identified the potential mechanism is consist of inhibition of key gene expression, including FN1, PLA2G2A, PTGS2, CCR5, TLR9, PI3K, IL1β, TNF, NFKB1, PRKCD, and STAT1, and regulation of inflammation-related pathways, such as TLR, JAK-STAT, NF-κB, VEGF, Th17 differentiation, IL-17, and others. In summary, these results show that *Paederia scandens* exerts anti-inflammatory effects in RA. We experimentally corroborated these network pharmacology predictions, supporting the anti-inflammatory mechanism through which *Paederia scandens* inhibits the phosphorylation of JAK2 and STAT3 in the JAK-STAT signaling pathway.

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Disclosure of conflict of interest

None.

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