





# To Investigate the Mechanism of Qinpi Tongfeng Formula in Treating Acute Gouty Arthritis by UHPLC-Q-Orbitrap-MS, Network Pharmacology and Experimental Validation

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**Background:** Acute gouty arthritis (AGA) is characterized by the accumulation of monosodium urate crystals within the joints, leading to inflammation and severe pain. Western medicine treatments have limitations in addressing this condition. Previous studies have shown the efficacy of Qinpi Tongfeng formula (QPTFF) in treating AGA, but further investigation is needed to understand its mechanism of action.

**Methods:** We used ultra-high-performance liquid chromatography tandem Q-Exactive Orbitrap high-resolution mass spectrometry (UHPLC-Q-Orbitrap-MS) to identify compounds in QPTFF. Target proteins regulated by these compounds were obtained from the Traditional Chinese Medicine Systems Pharmacology Database and Analysis Platform, Chemistry Database, and Swiss Target Prediction Database. AGA-related targets were searched and screened from various databases, including Genecards, PharmGKB, Drugbank, etc. Intersection targets of QPTFF and AGA were analyzed for protein-protein interaction networks, GO function enrichment, and KEGG pathway enrichment. We then verified QPTFF's mechanism of action using an AGA rat model, assessing pathological changes via H&E staining and target expression via ELISA, RT-qPCR, and Western blot.

**Results:** UHPLC-Q-Orbitrap-MS identified 207 compounds in QPTFF, with 55 selected through network pharmacology. Of 589 compound-regulated targets and 1204 AGA-related targets, 183 potential targets were implicated in QPTFF's treatment of AGA. Main target proteins included IL-1 $\beta$ , NFKBIA, IL-6, TNF, CXCL8, and MMP9, with the IL-17 signaling pathway primarily regulated by QPTFF. Experimental results showed that medium and high doses of QPTFF significantly reduced serum inflammatory factors and MMP-9 expression, and inhibited IL-17A, IL-6, IKK- $\beta$ , and NF- $\kappa$ B p65 mRNA and protein expression in AGA rats compared to the model group.

**Conclusion:** Key targets of QPTFF include IL-1 $\beta$ , NFKBIA, IL-6, TNF- $\alpha$ , CXCL8, and MMP9. QPTFF effectively alleviates joint inflammation in AGA rats, with high doses demonstrating no liver or kidney toxicity. Its anti-inflammatory mechanism in treating AGA involves the IL-17A/NF- $\kappa$ B p65 signaling pathway.

**Keywords:** acute gouty arthritis, Qinpi Tongfeng formula, network pharmacology, UHPLC-Q-Orbitrap-MS, IL-17 signaling pathway

## Introduction

As a metabolic rheumatic disorder, gout arises from the accumulation of urate in the joints, cartilage, and kidneys when serum uric acid increases to a certain extent due to abnormal purine metabolism in the human body.<sup>1</sup> The presence of acute gouty arthritis (AGA) is indicative of gout during its acute phase, which is characterized by sudden and severe joint pain, often recurrent.<sup>2</sup> Recurrent attacks of AGA can cause urate accumulation in joints, and kidney to cause joint

destruction and chronic kidney disease and even renal failure, respectively.<sup>3</sup> Because gout is often accompanied by hyperuricemia, long-term hyperuricemia may cause an elevated risk of cardiovascular and cerebrovascular diseases.<sup>4</sup> The global prevalence of gout ranges from 1% to 6.8%. As modern living standards continue to improve, the prevalence of gout is steadily rising and affecting individuals at younger ages, largely attributed to shifts in dietary patterns and lifestyle habits.<sup>5</sup>

For the treatment of AGA, the American College of Rheumatology recommends drugs for inflammation and pain symptoms, which mainly include colchicine and non-steroidal anti-inflammatory drugs (NSAIDs).<sup>6</sup> While the administration of colchicine can promptly alleviate the patient's symptoms, varying levels of negative responses such as hepatic, renal, gastrointestinal impairment, and suppression of bone marrow may occur subsequent to treatment, and the disease is prone to rebound after drug withdrawal.<sup>7,8</sup> NSAIDs also have side effects such as gastrointestinal damage, liver and kidney damage and causing cardiovascular disease.<sup>9</sup> Hence, it is imperative to investigate a treatment option that ensures safety and efficacy while minimizing adverse reactions.

Traditional Chinese medicine (TCM) such as acupuncture and moxibustion, oral administration and external application of Chinese medicine, exerts a positive impact on AGA.<sup>10–12</sup> Compared with western medicine treatment alone, the external application of Chinese herbal medicine combined with western medicine treatment has better efficacy in the treatment of joint swelling and pain in patients with AGA.<sup>13</sup> Chinese herbal medicine can regulate macrophage autophagy through AMPK $\alpha$ /mTOR/ULK1 signaling pathway to alleviate MSU-induced arthritis.<sup>14</sup> Meanwhile, Chinese herbal medicine can promote M2 polarization of macrophages through PI3K/Akt signaling pathway, exert anti-inflammatory effects and improve joint symptoms of AGA rats.<sup>15</sup> The Qinpi Tongfeng formula (QPTFF) is optimized and improved from Qinpi powder in the ancient Chinese book *Taiping Shenghui recipe* (AD 992). QPTFF has been successfully utilized for over a decade at the First Affiliated Hospital of Tianjin University of TCM (TUTCM) as an effective compound in treating AGA. Prior research has established that QPTFF can relieve the symptoms of joint pain in AGA patients, which is equivalent to the efficacy of diclofenac sodium sustained-release tablets. At the same time, QPTFF can also improve the symptoms of joint swelling in AGA patients, reduce inflammatory markers and serum uric acid levels, and will not cause adverse effects on the liver and kidney function in AGA patients.<sup>16</sup> However, the primary compounds and mechanisms of QPTFF in treating AGA need to be further explored.

As an emerging research method, the application of network pharmacology has gained significant popularity in investigating single drugs and compounds of TCM in recent years.<sup>17,18</sup> Network pharmacology can predict biological informations such as the active components of TCM, TCM-related targets, TCM-related pathways, to investigate the potential mechanism of TCM at multiple levels, aligning with the distinctive attributes of TCM compounds that target multiple components and exert therapeutic effects. We used Ultra-high-performance liquid chromatography tandem Q-Exactive Orbitrap high-resolution mass spectrometry (UHPLC-Q-Orbitrap-MS) and network pharmacology methods to investigate the compounds and potential mechanisms of our formula. The mechanism of the anti-inflammatory effect of QPTFF in AGA based on IL-17A/NF- $\kappa$ B p65 signaling pathway was investigated through animal experiments, which provided a basis for the treatment of AGA with Chinese medicine. The study's research process is illustrated in [Figure 1](#).

## Materials and Methods

### UHPLC-Q-Orbitrap-MS

#### Main Experimental Instruments

UHPLC-Q-Orbitrap-MS (Thermo, USA); Refrigerated centrifuges (Thermo, USA); KQ-300DV CNC ultrasonic cleaner (Kunshan Ultrasonic Instruments Co., Ltd., China); S0200-230V-EU vortex oscillator (Labnet, USA); N-1000 rotary evaporator (EYELA, Japan); Mill-Q-II ultra-pure water generator (Millipore, USA); FDU-2100 freeze dryer (EYELA, Japan).

#### Main Experimental Reagent

Formic acid was obtained from Tokyo Chemical Industry Co., Ltd. (Lot#: K3JHG.LS, purity  $\geq$  98%); Thermo Fisher Scientific Co., Ltd. was the source of chromatographic-grade methanol and acetonitrile.

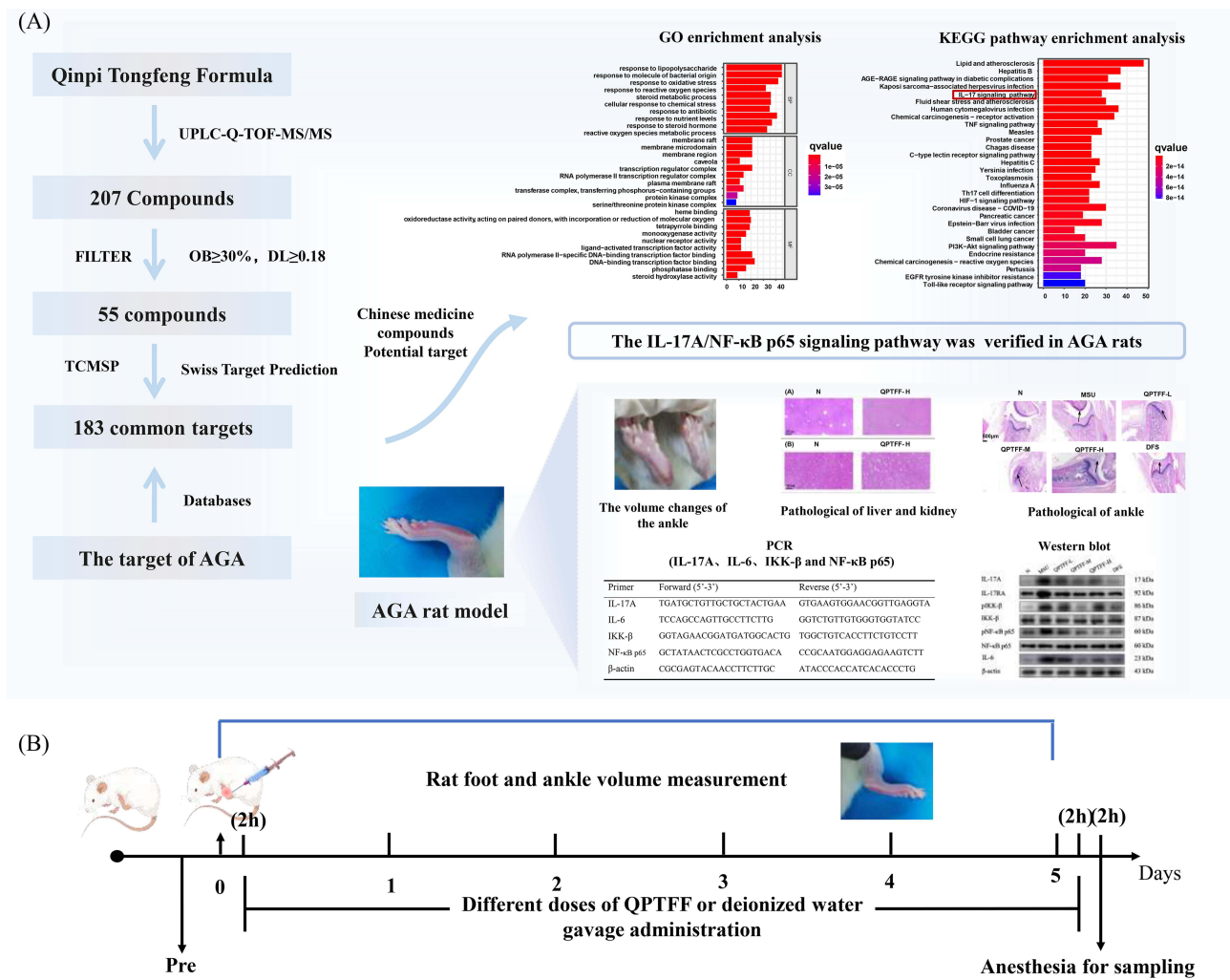


Figure 1 Flow chart of research scheme of Qinpi Tongfeng formula. (A) Flow chart of the entire experimental design; (B) Flow chart of animal experiment.

### Preparation of Qinpi Tongfeng Formula Extract Solution

QPTFF was composed of Qinpi (*Cortex Fraxini*) 30 g, Tufuling (*Rhizoma Smilacis Glabrae*) 80 g, Huanglian (*Rhizoma Coptidis*) 10 g, Xixiancao (*Herba Siegesbeckiae*) 30 g, Bixie (*Rhizome Dioscoreae Hypoglaucae*) 30 g, Cheqianzi (*Semen Plantaginis*) 20 g, Weilingxian (*Radix Clematidis*) 20 g, and Fangfeng (*Radix Saposhnikoviae*) 10 g. The decoction pieces contained in QPTFF were purchased from the pharmacy of the First Teaching Hospital of Tianjin University of Traditional Chinese Medicine. These decoction pieces were identified by Dr Liming Wang of Tianjin University of Traditional Chinese Medicine. One dose of the aforementioned decoction pieces was placed in a round-bottomed flask, followed by the addition of 2000 mL of deionized water. After soaking for 30 min, the mixture was heated and boiled until approximately 200 mL of extractive solution was obtained. The extraction process was repeated twice, and subsequently, the extractive solutions were combined. After the extract was cooled naturally, we transferred it to a freeze-dryer for freeze-drying for 24 h by rotary evaporation concentration, and QPTFF extract powder was finally collected. We added QPTFF extract powder (1.00 g) with deionized water (10 mL) and dissolved by ultrasound for 30 min to obtain a stock solution. A total of 100 μL of stock solution was accurately measured, diluted with deionized water at a ratio of 10-fold, and passed through a membrane filter with a pore size of 0.22 μm to acquire a filtrate for subsequent analysis.

### Chromatography and MS Parameters

The liquid chromatography was performed utilizing the UltiMate 3000 UHPLC system, which was linked to a column of ACQUITY UPLC HSS T3 column (2.1 mm × 100 mm, 1.8 μm). Mobile phase: 0.1% formic acid aqueous solution

(A)- acetonitrile (B); the flow rate was set 0.4 mL/min; elution procedure: 0 ~ 2.0 min, 1% B; 2 ~ 2.5 min, 1% ~ 10% B; 2.5 ~ 3.5 min, 10% ~ 20% B; 3.5 ~ 5.5 min, 20% ~ 30% B; 5.5 ~ 7.5 min, 30% ~ 46% B; 7.5 ~ 8.5 min, 46% ~ 100% B; 8.5 ~ 9.0 min, 100% B; 9.0 ~ 9.5 min, 100% ~ 1%B; 9.5 ~ 12.0 min, 1% B; adjust the temperature to 35 degree Celsius, while the injection volume was precisely measured at 2 microliters.

### Mass Spectrum Condition

The mass spectrometric data were obtained in Thermo Fisher Q-Orbitrap MS (Q-Exactive) system with high-energy electrospray ion source (HESI source), using Full-*ms/dd-*ms**<sup>2</sup> scanning mode in both ionization polarities, namely positive and negative. The voltage applied to the source for positive ions was 3.5 kV, while for negative ions it was 2.8 kV. The capillary temperature during analysis was set at 320°C, and both the auxiliary gas and sheath gas (N<sub>2</sub>) were maintained at temperatures of 350°C and a flow rate of 35 units, respectively. Auxiliary gas (N<sub>2</sub>) was 10, S-lens level is 50 V, collision energy was set 20 V, 40 V, 60 V, scan range *m/z* 100 ~ 1500 Da.

## Analysis of Network Pharmacology

### Construction of Compounds Contained in Qinpi Tongfeng Formula and Their Targets

UHPLC-Q-Orbitrap-MS identified the screening of compounds of QPTFF. Oral Bioavailability (OB) and Drug likeness (DL) of them were obtained in the TCM Systems Pharmacology Database and Analysis Platform (TCMSP, <https://old.tcm-sp-e.com/tcm-sp.php>), and then screened (OB ≥ 30% and DL ≥ 0.18), and the targets corresponding to the compounds were obtained. If the compounds were not included in this platform, the OB values of the compounds of QPTFF were obtained through the Chemistry Database (<http://www.organchem.csdb.cn/scdb/default.htm?nCount=196260370>), and based on OB ≥ 30% for preliminary screening, the SMILES of the compounds were then downloaded using the PubChem database (<https://pubchem.ncbi.nlm.nih.gov/>), and we imported the SMILES into SwissADME database (<http://www.swissadme.ch/>) for prediction of drug-like properties,<sup>19</sup> opted components conforming to Lipinski's rule of 5.<sup>20</sup> Finally, the SMILES obtained were utilized in the Swiss Target Prediction database (<http://www.swisstargetprediction.ch/>) for drug target prediction and screened according to Probability > 0<sup>21</sup> to obtain the targets corresponding to the compounds of QPTFF.

### Search for Genes Related to Acute Gouty Arthritis

We conducted a comprehensive search for AGA-related genes across multiple databases, including the Human Gene Database (Genecards, <https://www.genecards.org/>), Online Mendelian Inheritance in Man (OMIM, <https://www.omim.org/>), the pharmacogenomics knowledgebase database (PharmGKB, <https://www.pharmgkb.org/>), the Drugbank database (<https://www.drugbank.ca/>), DisGeNET database (<https://www.disgenet.org/>), the therapeutic target database (TTD, <http://db.idrblab.net/ttd/>), Comparative Toxicogenomics Database (CTD, <http://ctdbase.org/>) using the keywords “Acute gouty arthritis” or “Arthritis, Gouty”. By merging the results from these databases and eliminating any duplicate target genes, we identified the relevant genes associated with AGA.

### Predict Potential Targets of Qinpi Tongfeng Formula in Treating Acute Gouty Arthritis

We intersected the drug targets regulated by the compounds in QPTFF with the AGA-related targets to obtain common targets, ie potential targets of QPTFF in treating AGA. The Venn diagram of drug targets of QPTFF-AGA related targets was drawn.

### Construct the “Chinese Medicine - Compounds - Potential Target” (CMCPT) Network

We utilized the Cytoscape 3.8.0 software to construct a network entitled “Chinese Medicine-Compounds-Potential Targets” that incorporates the potential targets of QPTFF for AGA treatment, along with their corresponding compounds, thereby facilitating visualization and analysis.

### Construct the Protein-Protein Interaction (PPI) Networks and Screen Key Targets

Import potential QPTFF targets for AGA into STRING database (<https://string-db.org/>), selecting “Homo sapiens” as species and setting the minimum required interaction score to medium confidence (0.400), to obtain the PPI information and save it in “TSV” format, then “TSV” files were imported into Cytoscape 3.8.0 software to construct the PPI network.



The network's nodes were subjected to topological analysis using the CytoNCA plug-in. The topological parameters were Degree Centrality (DC), Betweenness Centrality (BC), Closeness Centrality (CC), Local Average Connectivity (LAC), and Eigenvector Centrality (EC). DC reflected frequency of node interaction with other nodes,<sup>22</sup> BC reflected the node's capacity to regulate the flow of information among other nodes, with greater BC indicating higher influence.<sup>23</sup> Compute DC for every node and eliminate nodes with  $DC \geq$  the mean value as part of the initial screening process. Reconfigure a sub-network and employ the CytoNCA plug-in once more to compute the DC, BC, CC, LAC and EC values for the nodes within the sub-network. Subsequently, identify and filter out the nodes which are greater than the average value of each parameter to complete the second screening.

### GO Functional and KEGG Pathway Enrichment Analyses

We utilized R 4.2.0 software along with the "org.Hs.eg.db" package to retrieve the IDs of the intersection targets, and then used "clusterProfiler". Subsequently, we employed various packages including "clusterProfiler" to conduct GO function enrichment analysis on them. The top 10 functional categories in Biological Process (BP), Cellular Component (CC) and Molecular function (MF) were selected. The KEGG enrichment analysis was performed on the intersection targets, and a demonstration was conducted by selecting the top 20 KEGG pathways.

### Molecular Docking

The 2D structures of quercetin, eburicoic acid and polyporenic acid C were retrieved from the PubChem database (<http://zinc.docking.org/>). The 2D structures of the compounds were imported into Chem3D software to calculate the minimum free energy (.mol2) of the compounds. The 3D structures of the core targets were retrieved from the PDB protein structure database (<http://www.rcsb.org/>), and the water molecules and small molecular ligands (.PDB) were removed by PyMol software. The obtained macromolecular proteins and small molecular ligands were hydrotreated and converted into formats (.pdbqt and.gpf) by AutoDockTools software. Finally, the molecular docking was performed by AutoDock Vina program to calculate the minimum binding energy. The lower the binding energy, the stronger the binding force between the compound and the target. The binding energy was less than 0, indicating that the compound had a certain binding ability with the target. The binding energy was less than  $-5.0\text{kJ/mol}$ , indicating that the binding activity was good, and less than  $-7.0\text{kJ/mol}$ , indicating that the binding was stable.<sup>24</sup>

## Experimental Verification

### Experimental Animals

Six-week-old Sprague-Dawley male rats (SPF grade) weighing between 180 and 200 g were obtained from Beijing Huafukang Biotechnology Co., Ltd, animal license number: SCXK (Beijing) 2019-0008, housed at 20–25°C and 40–60% RH, and the experiments were carried out after 1 week of acclimatization. The Animal Ethics Committee of TUTCM granted approval for the animal experiment protocol (TCM-LAEC2021137).

### Experimental Drug

The QPTFF dry paste powder made in the State Key Laboratory of Component TCM, TUTCM from the QPTFF decoction (see 2.1.3 for preparation method). Each dose of QPTFF could be converted into 13.99 g of dry paste powder. Diclofenac sodium (DFS) (0.1 g/tablet) was from Hunan Huana Pharmaceutical Co. (H20067776).

### Experimental Instruments

Toe volume measurement instrument was from Jinan Benefit Technology Development Co., Ltd (YLS-7C); fully automatic dewatering machine was from Leica, Germany (ASP200S); paraffin sectioning machine was from Leica, Germany (RM2235); baking table was from Leica, Germany (HI1220); water bath was from Leica, Germany (HI1220); inverted phase contrast microscope was from Leica, Germany (DM3000); tabletop high-speed frozen centrifuge was from Beijing Beili Technology Co., Ltd (GTR16-2); fluorescence quantitative PCR instrument was from Thermo Fisher Scientific, USA (QuantStudio 1); electrophoresis instrument was from Beijing Liuyi Biotechnology Co., Ltd (DYY-6C); gel imager was from Beijing Saizhi Technology Co., Ltd (Champchemi 610 plus); spectrophotometer was from Shanghai Jeanqi Instrument Technology Co., Ltd (D8PCS).

## Experimental Reagents

Monosodium urate (MSU) came from Shanghai Maclean Biochemical Technology Co., Ltd (U886060); IL-1 $\beta$ ELISA kit was from Beijing Solabao Technology Co., Ltd (SEKR-0002); IL-8 ELISA kit was from Beijing Solaibao Technology Co., Ltd (SEKM-0071); IL-17A ELISA kit was from Wuhan Huamei Biological Engineering Co., Ltd (CSB-E07451r); IL-6 ELISA kit was from Wuhan PhD Biological Engineering Co., Ltd (EK0412); TNF- $\alpha$  ELISA kit was from Wuhan PhD Bioengineering Co., Ltd (EK0526); MMP-9 ELISA kit was from Wuhan PhD Bioengineering Co., Ltd (EK1463); Mouse Anti- $\beta$ actin mAb (43 kDa) was from Beijing Zhongshan Jinqiao Biotechnology Co., Ltd (TA-09); IKK- $\beta$  (87 kDa) antibody was from Wuhan PhD Biological Engineering Co., Ltd (PB9221); pIKK- $\beta$  (86 kDa) antibody was from Bioworld (BS4101); NF- $\kappa$ B p65 (60 kDa) antibody was from Bioworld (BS3157); pNF- $\kappa$ B p65 (60 kDa) antibody was from Bioworld (BS4137); IL-6 (23 kDa) antibody was from Bioworld (BS6419); IL-17RA (92 kDa) antibody was from Beijing Bioss Company (bs-2606R); IL-17A (17 kDa) antibody was from Wuhan PhD Biological Engineering Co., Ltd (BA12416).

## Establishment of AGA Rat Model and Experimental Grouping

Referring to the classical modeling method of Coderre et al<sup>25</sup> rats were anesthetized with a 1% sodium pentobarbital solution (5 mL/kg) administered intraperitoneally,<sup>26</sup> the selected injection orientation was the lateral posterior aspect of the right ankle joint followed by local disinfection. A 0.2 mL suspension of sodium urate crystals at a concentration of 50 mg/mL was administered into the ankle joint cavity using a 4.5-gauge needle, following the contralateral expansion of the joint capsule as the injection criterion. In contrast to the control group, the rats in the AGA model exhibited inflammation and edema in their right ankle joint, deepening of the color of the bottom of the foot in a reddish-purple color, slow walking, paw curling, and even hind limb prostration lameness, suggesting successful modeling.<sup>27</sup> After 7 days of acclimatization, divide 36 rats into normal control group (N), model group (MSU), Qinpi Tongfeng formula Low Dose Group (QPTFF-L), Qinpi Tongfeng formula Medium Dose Group (QPTFF-M), Qinpi Tongfeng formula High Dose Group (QPTFF-H), and Diclofenac Sodium Group (DFS) using the randomized zone group method. Six rats in each group, except for group N, were constructed as rat AGA model. 0.2 mL of saline was injected into the right ankle joint of group N. All rats were gavaged 2 h after modeling, and calculated a daily gavage dose of 10.5 mg/kg per rat for DFS group based on an equivalent dose ratio of 6.3 between rats and adults.<sup>28</sup> According to the ratio of 0.5:1:2 for low, medium and high doses, the gavage doses of QPTFF for rats in QPTFF-L, QPTFF-M, QPTFF-H groups were 0.74 g/kg, 1.47 g/kg and 2.94 g/kg, respectively. Rats in the group N and MSU group were gavaged with an equal volume of deionized water, and the remaining rats received daily gavage administration for a duration of 5 consecutive days.

## Measurement of Foot and Ankle Volume in Rats

Right-sided ankle volumes were measured in all rats before (0 h), 2, 6, 12, 24, 48, 72 and 96 h after modeling using a pedicle volumetric instrument. The ankle volume of each rat was measured thrice the average value was utilized for statistical analysis.

## Specimen Collection

Serum: After 2 h of gavage administration on day 5, rats were administered anesthesia through an intraperitoneal injection of a solution containing 1% sodium pentobarbital (5 mL/kg),<sup>26</sup> then collected whole blood from the abdominal aorta with a negative pressure blood collection tube and then centrifuged and stored the supernatant in a -80°C refrigerator. Ankle joint tissue: rats' skin was peeled off with the ankle joint as the center, and the upper and lower ends of the ankle joint were cut using a sterile scalpel, rinsed with physiological saline and then quickly placed in liquid nitrogen for preservation or fixed in 4% paraformaldehyde.

## Histopathological Observation of the Ankle Joint

The ankle tissue was initially preserved in a solution containing 4% paraformaldehyde and subsequently subjected to decalcification using a 10% EDTA decalcifying solution. Successful decalcification was followed by torrefaction, transparency, wax immersion, embedding and sectioning. Hematoxylin and eosin staining were performed after dewaxing and hydration, and the ankle joint underwent histopathological alterations when examined using light microscopy.

## Determination of Inflammatory Factors and MMP-9 in Rat Serum

The serum stored at  $-80^{\circ}\text{C}$  was taken out, and relevant guidelines from the ELISA kit instructions were followed to assess IL-17A, IL-1 $\beta$ , IL-6, IL-8, TNF- $\alpha$  and MMP-9 levels in the serum.

## mRNA Expression of Relevant Targets in Ankle Joint Tissues

We measured IL-17A, IL-6, IKK- $\beta$  and NF- $\kappa$ B p65 mRNA expression in the test ankle tissue by Real-time PCR. Each sample was repeated 3 times. Total RNA was first extracted from 50 mg AGA rat ankle tissue samples containing 1 mL Trizol lysate, and the RNA purity and concentration were detected by spectrophotometer, where the purity was judged by the ratio of OD260/OD280. Two  $\mu\text{g}$  of total RNA was taken for reverse transcription, using reverse transcription reaction conditions ( $37^{\circ}\text{C}$ , 15 min;  $85^{\circ}\text{C}$ , 5 s). Then the cDNA obtained from the completed reverse transcription was diluted 10 times and stored at  $-80^{\circ}\text{C}$  for backup. Prepare 50  $\mu\text{L}$  qRT-PCR system, place the octet in Step One Plus TM Real-Time PCR System, with 1 cycle of pre-denature ( $95^{\circ}\text{C}$ , 10 min), 40 cycles of denature and anneal/extend. Calculate gene expression levels with the  $2^{-\Delta\Delta\text{Ct}}$  method. The primer sequences of the gene were synthesized by Shanghai Biotech Bioengineering Co., Ltd (Table 1).

## Detection of Relevant Protein Expression in Ankle Joint Tissues Using Western Blot

IL-17A, IL-17RA, IKK- $\beta$ , pIKK- $\beta$ , NF- $\kappa$ B p65, pNF- $\kappa$ B p65 and IL-6 protein levels were measured with Western blot. Fifty milligrams of tissue from the ankle joint were collected and mixed with 500  $\mu\text{L}$  of RIPA lysate, which contained a protease inhibitor (1% PMSF) and a phosphatase inhibitor (1%). The ankle tissue was subsequently subjected to ice-cold lysis for a duration of 30 min, followed by centrifugation at 13,000 g for 13 min at a temperature of  $4^{\circ}\text{C}$ . Subsequently, the supernatant was carefully transferred into a centrifuge tube with a capacity of 1.5 mL. Protein concentrations were detected by the BCA method. Based on the protein concentration values, the concentration of each group of proteins was diluted to 4.34  $\mu\text{g}/\mu\text{L}$  with cell lysate, and then  $5 \times$  loading buffer was added at a ratio of 4:1 to make a protein loading solution concentration of 3.47  $\mu\text{g}/\mu\text{L}$ . The proteins were denatured by cooking in a  $95^{\circ}\text{C}$  water bath for 10 min, and the samples were dispensed and stored in a  $-20^{\circ}\text{C}$  refrigerator. The electrophoretic technique was employed to separate the proteins, followed by their subsequent transfer onto PVDF membranes and then it was closed in 5% BSA solution for 2 h. Dilute the primary antibody (1:500 for IKK- $\beta$  antibody and 1:1000 for the rest of the primary antibody) with reference to the dilution ratio recommended in the instruction manual and previous experimental experience, and wash the membrane with TBST three times at room temperature. Then, secondary antibody was added, where the dilution ratio of secondary antibody was 1:5000, and incubated at 55 rpm for 90 min at room temperature; Use TBST was to wash the membrane 3 times. Observe protein bands according to the ECL chemiluminescence detection method, and use Image-J software to analyze them and calculate the grayscale values.

## Observation of Rat Liver and Kidney Tissues by H&E Staining

In order to investigate whether QPTFF high dose would cause liver and kidney damage to normal blank rats, randomly divide 6 male rats into N group and 3 rats each in QPTFF-H group after 7 days of adaptive feeding, and no rat AGA model was constructed for both groups, and QPTFF-H group was administered continuously by gavage for 5 days (the administered dose was 2.94 g/kg). The rats were executed after administration on day 5, and the livers and kidneys of both groups underwent PBS washing, followed by fixation using 4% paraformaldehyde and subsequent preservation for the generation of pathological sections, which were stained with H&E and observed under light microscopy for specific morphology.

**Table 1** qRT-PCR Primer Design

Primer	Forward (5'-3')	Reverse (5'-3')
IL-17A	TGATGCTGTTGCTGCTACTGAA	GTGAAGTGGAAACGGTTGAGGTA
IL-6	TCCAGCCAGTTGCCCTTCTTG	GGTCTGTTGTGGGTGGTATCC
IKK- $\beta$	GGTAGAACGGATGATGGCACTG	TGGCTGTCACCTTCTGTCCTT
NF- $\kappa$ B p65	GCTATAACTCGCCTGGTGACA	CCGCAATGGAGGAGAAGTCTT
$\beta$ -actin	CGCGAGTACAACCTTCTTGC	ATACCCACCATCACACCCTG

## Statistical Methods

The SPSS 22.0 was employed to complete a thorough statistical analysis of the data obtained, with the results presented as (Mean  $\pm$  SD), and repeated measures ANOVA was used for multiple time point comparisons if the measures satisfied normality and chi-squaredness. Use One-way ANOVA to compare groups at a single time point, and select LSD test to compare groups, and a significance level of  $P < 0.05$  to determine statistically significant distinction. Perform the plotting of all data with GraphPad Prism 8.

## Outcomes

### Compounds in Qinpi Tongfeng Formula

We analyzed the extracts of QPTFF by UHPLC-Q-Orbitrap-MS to obtain the total ion flow diagrams in positive and negative ion modes (Figure 2). Combined with the literature,<sup>29–35</sup> based on the chromatographic information and mass

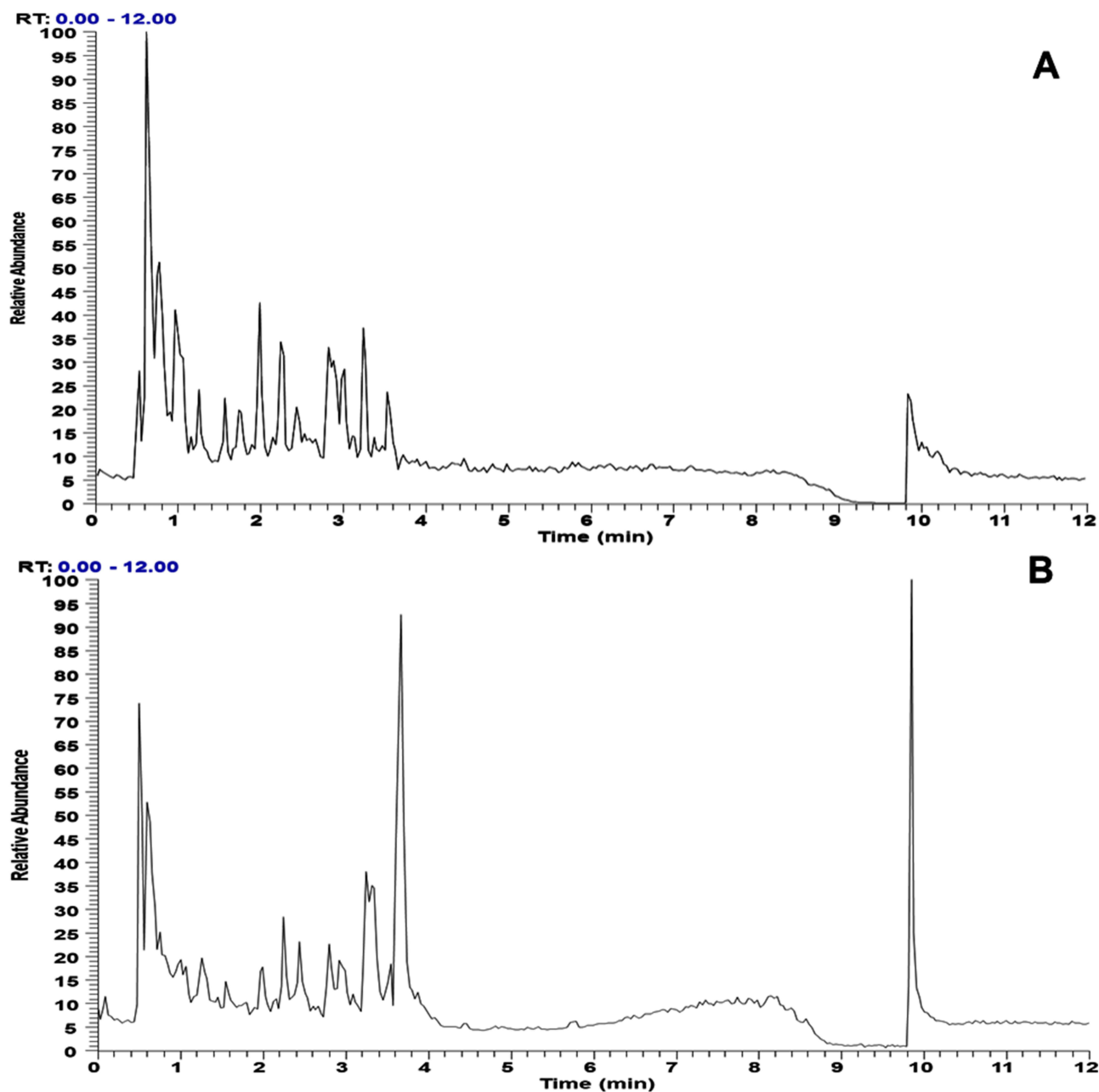


Figure 2 Total ion flow diagram of extract of Qinpi Tongfeng formula under positive (A) and negative ion (B) detection modes.

spectrometry information of the compounds, Qinpi (*Cortex Fraxini*) contained 30 compounds, Tufuling (*Rhizoma Smilacis Glabrae*) contained 36 compounds, Huanglian (*Rhizoma Coptidis*) contained 33 compounds, Bixie (*Rhizome Dioscoreae Hypoglaucae*) contained 10 compounds, Xixiancao (*Herba Siegesbeckiae*) contained 24 compounds, Cheqianzi (*Semen Plantaginis*) contained 47 compounds, Weilingxian (*Radix Clematidis*) contained 25 compounds, and Fangfeng (*Radix Saposhnikoviae*) contained 31 compounds, for a total of 236 compounds, and 207 compounds were finally obtained by removing the duplicates ([Supplementary material Table S1](#)).

## Compounds of Qinpi Tongfeng Formula and QPTFF-Related Targets

With the criteria of OB  $\geq$ 30% and DL  $\geq$ 0.18, Qinpi (*Cortex Fraxini*) contained 4 compounds, Huanglian (*Rhizoma Coptidis*) contained 8 compounds; Fangfeng (*Radix Saposhnikoviae*) contained 8 compounds; Cheqianzi (*Semen Plantaginis*) contained 11 compounds; Tufuling (*Rhizoma Smilacis Glabrae*) contained 17 compounds; Bixie (*Rhizome Dioscoreae Hypoglaucae*) contained 2 compounds; Weilingxian (*Radix Clematidis*) contained 5 compounds; and Xixiancao (*Herba Siegesbeckiae*) contained 4 compounds. After removing the duplicate compounds, 55 compounds of QPTFF were obtained. Totally, 1659 compound-related targets were searched on the TCMSP and Swiss Target Prediction platforms, and 589 drug targets of QPTFF were obtained after deleting duplicate targets. [Table 2](#) shows the compounds contained in QPTFF and the number of targets regulated.

**Table 2** Compounds and the Number of Regulatory Targets of Qinpi Tongfeng Formula

Number	Source	Compounds	Number of Targets
1	Cheqianzi( <i>Semen Plantaginis</i> ), Xixiancao( <i>Herba Siegesbeckiae</i> ), Tufuling( <i>Rhizoma Smilacis Glabrae</i> )	quercetin	133
2	Tufuling( <i>Rhizoma Smilacis Glabrae</i> )	polyporenic acid C	100
3	Fangfeng( <i>Radix Saposhnikoviae</i> )	decursin	99
4	Huanglian( <i>Rhizoma Coptidis</i> )	moupinamide	99
5	Fangfeng( <i>Radix Saposhnikoviae</i> )	decursinol angelate	96
6	Tufuling( <i>Rhizoma Smilacis Glabrae</i> )	eburicoic acid	91
7	Tufuling( <i>Rhizoma Smilacis Glabrae</i> )	16 $\alpha$ -hydroxytrametenolic acid	83
8	Tufuling( <i>Rhizoma Smilacis Glabrae</i> )	poricoic acid G	73
9	Tufuling( <i>Rhizoma Smilacis Glabrae</i> )	dehydroeburicoic acid	71
10	Tufuling( <i>Rhizoma Smilacis Glabrae</i> )	dehydropachymic acid	60
11	Cheqianzi( <i>Semen Plantaginis</i> )	ixoroside	56
12	Cheqianzi( <i>Semen Plantaginis</i> )	luteolin	53
13	Qinpi( <i>Cortex Fraxini</i> )	caffeic acid	52
14	Tufuling( <i>Rhizoma Smilacis Glabrae</i> )	pachymic acid	44
15	Cheqianzi( <i>Semen Plantaginis</i> )	campenoside	38
16	Tufuling( <i>Rhizoma Smilacis Glabrae</i> )	poricoic acid BM	38
17	Xixiancao( <i>Herba Siegesbeckiae</i> )	monopalmitin	35
18	Cheqianzi( <i>Semen Plantaginis</i> )	baicalein	34
19	Weilingxian( <i>Radix Clematidis</i> )	nobiletin	34
20	Cheqianzi( <i>Semen Plantaginis</i> ), Xixiancao( <i>Herba Siegesbeckiae</i> )	stigmaterol	27
21	Weilingxian( <i>Radix Clematidis</i> )	clematomandshurica saponin B	25
22	Huanglian( <i>Rhizoma Coptidis</i> )	berberrubine	22
23	Cheqianzi( <i>Semen Plantaginis</i> )	monomelittoside	20
24	Huanglian( <i>Rhizoma Coptidis</i> )	berlambine	18
25	Bixie( <i>Rhizome Dioscoreae Hypoglaucae</i> )	ginsenoside Rd	18
26	Fangfeng( <i>Radix Saposhnikoviae</i> )	marmesin	18

(Continued)



Table 2 (Continued).

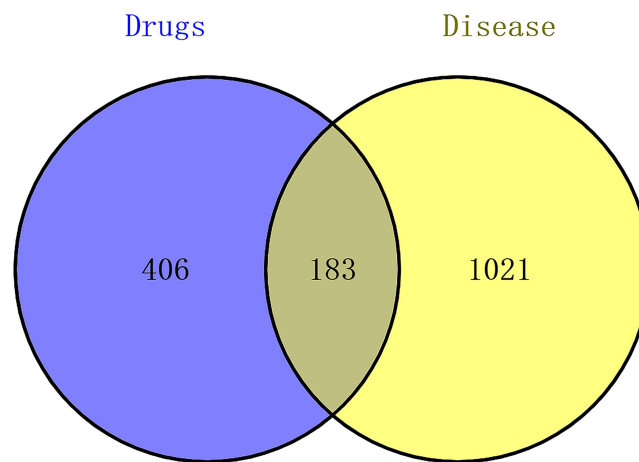
Number	Source	Compounds	Number of Targets
27	Fangfeng( <i>Radix Saposhnikoviae</i> )	nodakenetin	18
28	Huanglian( <i>Rhizoma Coptidis</i> )	palmatine	17
29	Huanglian( <i>Rhizoma Coptidis</i> )	berberine	16
30	Qinpi( <i>Cortex Fraxini</i> )	acetoxypinoresinol	15
31	Tufuling( <i>Rhizoma Smilacis Glabrae</i> ), Bixie( <i>Rhizome Dioscoreae Hypoglaucae</i> )	diosgenin	14
32	Cheqianzi( <i>Semen Plantaginis</i> )	hispidulin	14
33	Cheqianzi( <i>Semen Plantaginis</i> )	hypolaetin	11
34	Weilingxian( <i>Radix Clematidis</i> )	liquiritigenin	11
35	Huanglian( <i>Rhizoma Coptidis</i> )	epiberberine	10
36	Qinpi( <i>Cortex Fraxini</i> )	medioresinol	10
37	Tufuling( <i>Rhizoma Smilacis Glabrae</i> )	taxifolin	10
38	Huanglian( <i>Rhizoma Coptidis</i> )	coptisine	8
39	Fangfeng( <i>Radix Saposhnikoviae</i> )	anomalin	7
40	Fangfeng( <i>Radix Saposhnikoviae</i> )	$\alpha$ -pinene	7
41	Huanglian( <i>Rhizoma Coptidis</i> )	worenine	6
42	Cheqianzi( <i>Semen Plantaginis</i> )	6-hydroxyluteolin	5
43	Tufuling( <i>Rhizoma Smilacis Glabrae</i> )	astilbin	5
44	Tufuling( <i>Rhizoma Smilacis Glabrae</i> )	cis-Dihydroquercetin	5
45	Weilingxian( <i>Radix Clematidis</i> )	embinin	5
46	Qinpi( <i>Cortex Fraxini</i> )	fraxin	5
47	Tufuling( <i>Rhizoma Smilacis Glabrae</i> )	isoastilbin	5
48	Fangfeng( <i>Radix Saposhnikoviae</i> )	phelloptorin	5
49	Tufuling( <i>Rhizoma Smilacis Glabrae</i> )	(-)-taxifolin	3
50	Fangfeng( <i>Radix Saposhnikoviae</i> )	pentanol	3
51	Cheqianzi( <i>Semen Plantaginis</i> )	baicalin	2
52	Xixiancao( <i>Herba Siegesbeckiae</i> )	darutigenol	2
53	Tufuling( <i>Rhizoma Smilacis Glabrae</i> )	$\beta$ -sitosterol	1
54	Weilingxian( <i>Radix Clematidis</i> )	linarin	1
55	Tufuling( <i>Rhizoma Smilacis Glabrae</i> )	trametenolic acid	1

## Collection of AGA-Related Genes and Potential Targets Prediction

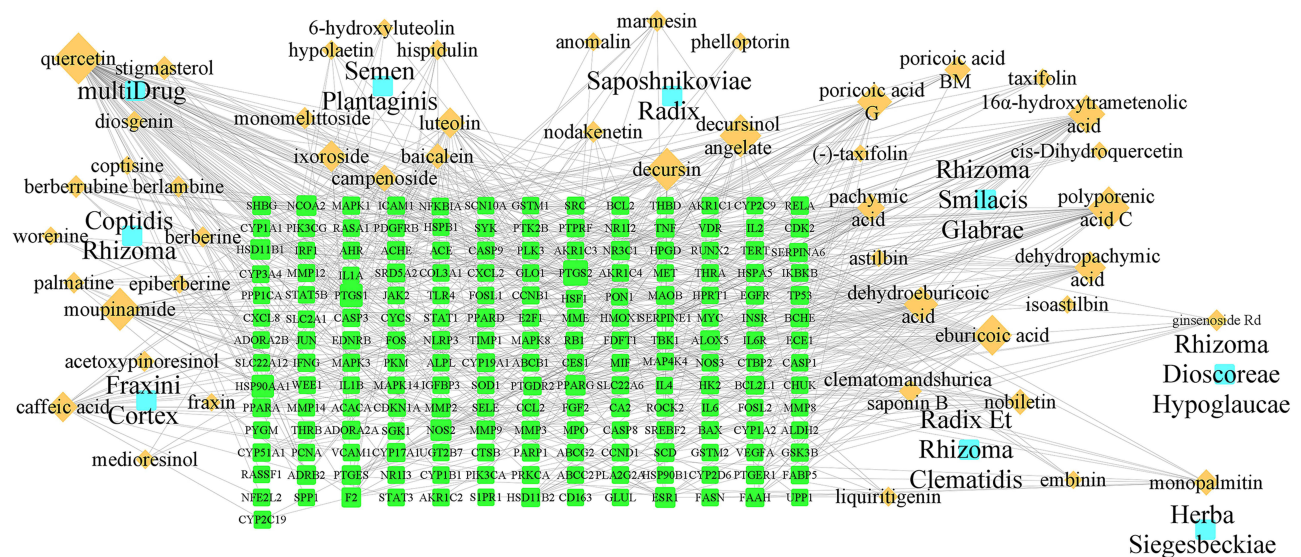
In total, we obtained 1204 AGA-related targets by searching in Genecards, OMIM, PharmGKB, Drugbank, DisGeNET, TTD and CTD databases. We intersected the QPTFF-regulated targets with the AGA-related targets and gained 183 intersected targets that were potential QPTFF-targets in treating AGA (Figure 3).

## Construct the “Chinese Medicine - Compounds - Potential Target” Network

We used Cytoscape 3.8.0 to construct the “Chinese medicine - Compounds - Potential target” network (Figure 4). The graph had a total of 240 nodes (48 compounds, 183 potential targets, 8 drug names, and 1 multidrug). The green square nodes represented potential targets for the regulation of QPTFF, the blue square nodes represented the Chinese herbs in QPTFF, and the yellow diamond nodes represented the compounds in QPTFF. The relationship between nodes suggested a targeted association between drug compounds and potential targets, with larger node degrees and larger node area corresponding to greater significance. Quercetin, eburicoic acid and polyporenic acid C were the top three compounds in Degree value. Among them, quercetin acted on 77 potential targets, which came from Cheqianzi (*Semen Plantaginis*), Tufuling (*Rhizoma Smilacis Glabrae*) and Xixiancao (*Herba Siegesbeckiae*); eburicoic acid was found to act on 37 potential targets from Tufuling (*Rhizoma Smilacis Glabrae*); polyporenic acid C was found to act on 35 potential targets and was derived from Tufuling (*Rhizoma Smilacis Glabrae*).



**Figure 3** Venn diagram of Qinpi Tongfeng formula-related targets and AGA-related targets.



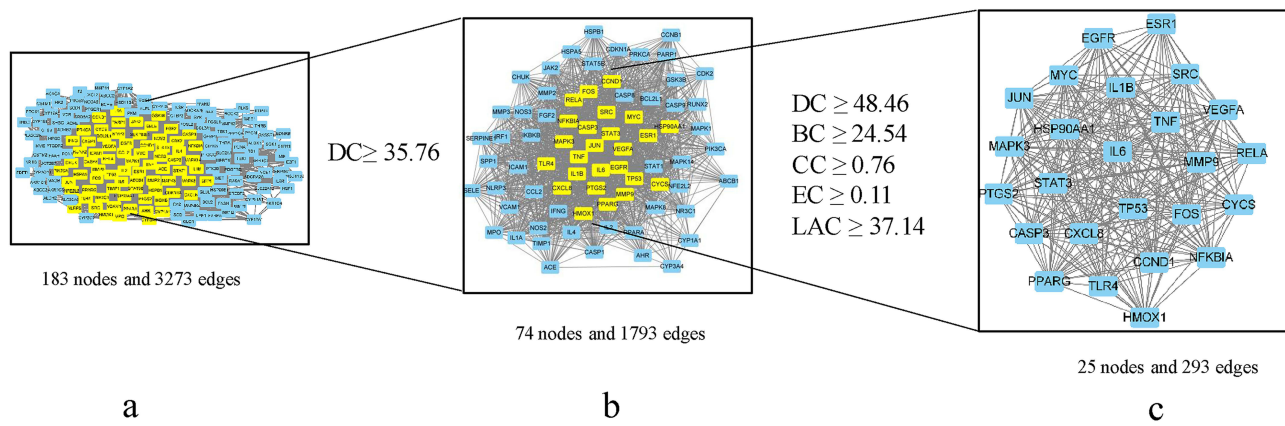
**Figure 4** Chinese medicine - Compounds - Potential target network.

## Construction of PPI Network and Determination of Key Targets

Import the protein interaction data of intersection targets into Cytoscape 3.8.0 to build a PPI network (Figure 5a) (including 182 nodes, 3273 edges). Calculate DCs of 182 target proteins using the plug-in CytoNCA, and subnetworks of 74 target proteins were screened according to  $DC \geq 35.76$  (mean) (Figure 5b). Then calculate DC, BC, CC, EC and LAC of 74 target proteins in the sub-network again as above. According to  $DC \geq 48.46$  (mean),  $BC \geq 24.54$  (mean),  $CC \geq 0.76$  (mean),  $EC \geq 0.11$  (mean),  $LAC \geq 37.14$  (mean), 25 target proteins were screened out (Figure 5c). The key target proteins included IL-1 $\beta$ , NFKBIA, IL-6, TNF, CXCL8, MMP9, etc.

## GO Functional and KEGG Pathway Enrichment Analyses

The 2733 entries, including 2509 BP, 38 CC and 186 MF were from GO functional enrichment analysis of 183 intersection targets. The main focus of BP lies in the reaction to lipopolysaccharide and the response to bacterial-derived molecules; CC primarily occurred in membrane raft and membrane microdomain; MF was associated with heme binding and oxidoreductase activity. Based on  $P < 0.05$ , Figure 6 illustrates the visual representation of the top 10 distinct biological process items that were chosen. One hundred and seventy-two pathways were from KEGG functional enrichment analysis, and according to the  $P$  value,

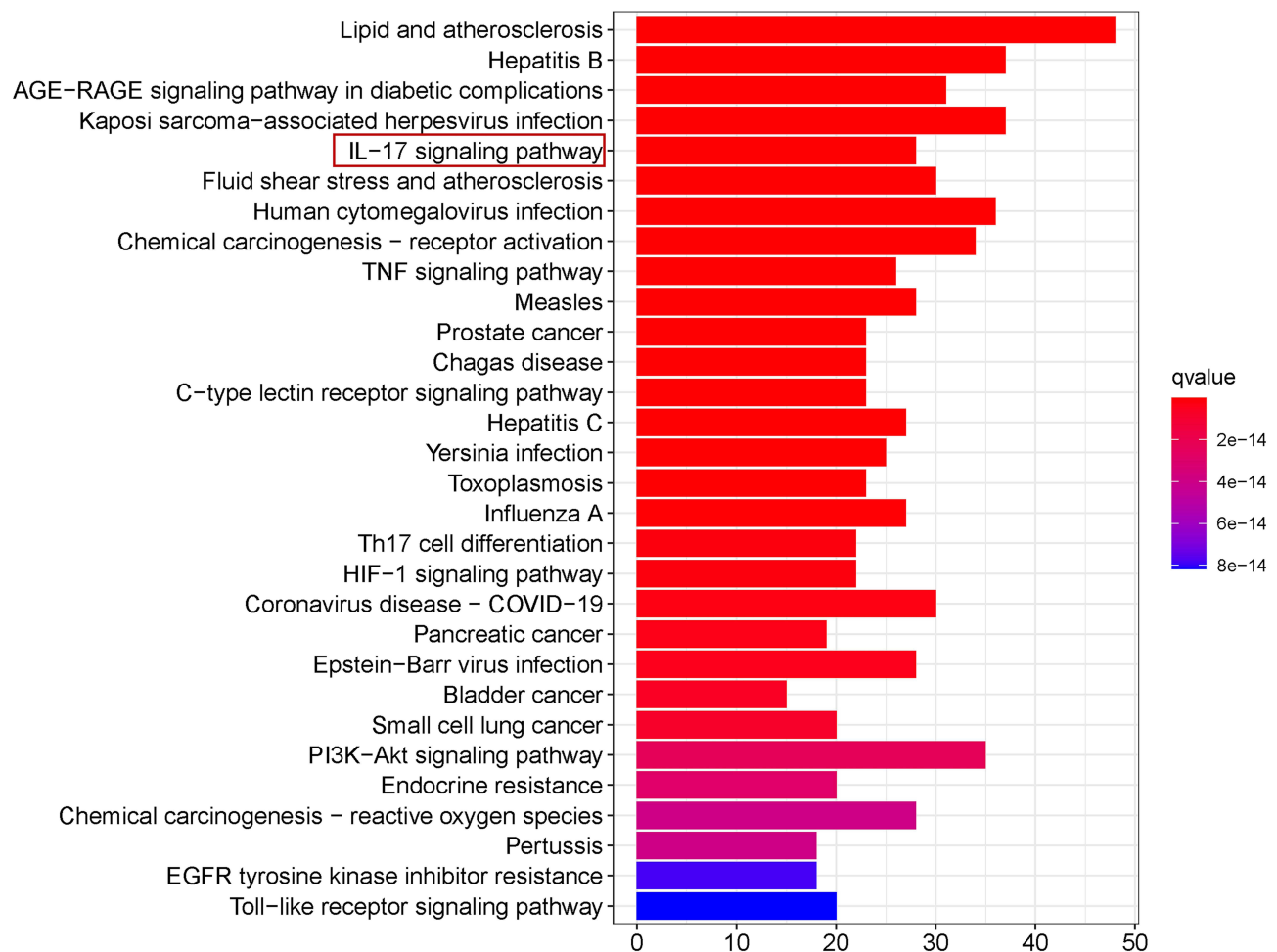


**Figure 5** The PPI network filtering process; Note: (a) The intersection targets of Qinpi Tongfeng formula and AGA; (b) Targets after DC screening; (c) : Potential core targets of Qinpi Tongfeng formula in regulating AGA.



**Figure 6** GO functional enrichment analysis; Notes: The horizontal coordinate represents the number of selected targets contained in each item and the corresponding percentage of the total number of targets. The vertical coordinate is the name of the biological processes (BP), cellular components (CC) and molecular functions (MF). The top 10 significant GO analyses are listed. Q value is the p value after multiple hypothesis test correction. When the q value smaller, the more significant enrichment of GO function.

the visual representation included a selection of the top 30 (Figure 7). In Figures 6 and 7, colors represented enrichment significance, with darker colors representing more significant enrichment of targets in this pathway and smaller *P*-values. The top 4 pathways were Lipid and atherosclerosis, Hepatitis B, AGE-RAGE signaling pathway in diabetic complications, and Kaposi sarcoma-associated herpesvirus infection, which had little correlation with AGA, while IL-17 was the most enriched inflammatory pathway.



**Figure 7** KEGG pathway enrichment analysis. Notes: The horizontal coordinate represents the number of selected targets contained in each item and the corresponding percentage of the total number of targets. The vertical coordinate is the name of the signaling pathway and disease. The top 30 significant KEGG analyses are listed. Q value is the p value after multiple hypothesis test correction. When the q value smaller, the more significant enrichment of signaling pathway.

## Molecular Docking of the Potential Active Compounds of the Qinpi Tongfeng Formula and Core Proteins

Through molecular docking, this study found that the binding energy of quercetin, eburicoic acid and polyporenic acid C with 25 core targets were all less than 0. In addition, the binding energy of 73 pairs of components and core target molecular docking was  $\leq -5.0$ kJ/mol, and that of 59 pairs of components and core target molecular docking was  $\leq -7.0$ kJ/mol. See [Table 3](#) for specific results.

## Experimental Results

### General Observation of Rats

Before modeling, the rats in each group were in good mental state and diet state, and their fur was shiny. After modeling, the right paw of the N group was slightly curled but there was no redness or swelling. The rats in the other 5 groups showed varying degrees of decreased activity, poor mental state, reduced water and food intake, weight loss, and inflammation and edema and claudication of their ankle joints. The right foot performance of the rats in the MSU group and the N group after modeling is shown in [Figure 8](#).

**Table 3** The Binding Energy of the Main Potential Active Compounds in the Qinpi Tongfeng Formula

Core Targets	Quercetin (kcal/mol)	Eburicoic Acid (kcal/mol)	Polyporenic Acid C (kcal/mol)
CASP3	-8.0	-7.3	-7.5
CCND1	-7.5	-8.0	-9.0
CXCL8	-6.2	-5.8	-5.6
CYCS	-7.2	-7.6	-7.7
EGFR	-7.9	-8.2	-8.2
ESR1	-8.4	-8.3	-7.5
FOS	-7.2	-7.2	-7.5
HMOX1	-4.9	-4.8	-5.1
HSP90AA1	-7.5	-6.2	-7.0
IL1B	-7.1	-6.1	-7.2
IL6	-6.9	-7.2	-7.1
JUN	-5.5	-6.4	-5.4
MAPK3	-8.3	-7.7	-8.6
MMP9	-8.4	-7.8	-7.6
MYC	-6.5	-7.0	-7.0
NFKBIA	-7.7	-8.0	-7.6
PPARG	-8.1	-7.9	-8.4
PTGS2	-9.7	-9.2	-9.3
RELA	-7.5	-7.1	-8.0
SRC	-8.8	-8.8	-9.2
STAT3	-7.4	-7.3	-7.5
TLR4	-6.9	-6.2	-6.2
TNF	-10.4	-9.1	-9.8
TP53	-7.2	-7.2	-7.3
VEGFA	-7.0	-6.2	-7.2

### Effect of Qinpi Tongfeng Formula on the Swelling Volume of Foot and Ankle in AGA Rats

The swelling volume of their right ankles in MSU group increased considerably at each time point ( $P < 0.01$ ) vs N group, reached the peak at 12 h, and could be maintained until 96 h after modeling, indicating successful establishment and good stability of models. The swelling volume of their right ankles in QPTFF-M group, QPTFF-H group and MSU group considerably differed at different time points after 6 h ( $P < 0.05$ ,  $P < 0.01$ ) vs MSU group. The swelling volume of their right ankles in MSU group considerably differed at 48 h after ( $P < 0.05$ ) vs QPTFF-L group. The smaller swelling volume of their right ankles was considerably seen in the QPTFF-M and QPTFF-H groups vs QPTFF-L group ( $P < 0.01$ ) ([Supplementary material Table S2](#) and [Figure 9](#)).

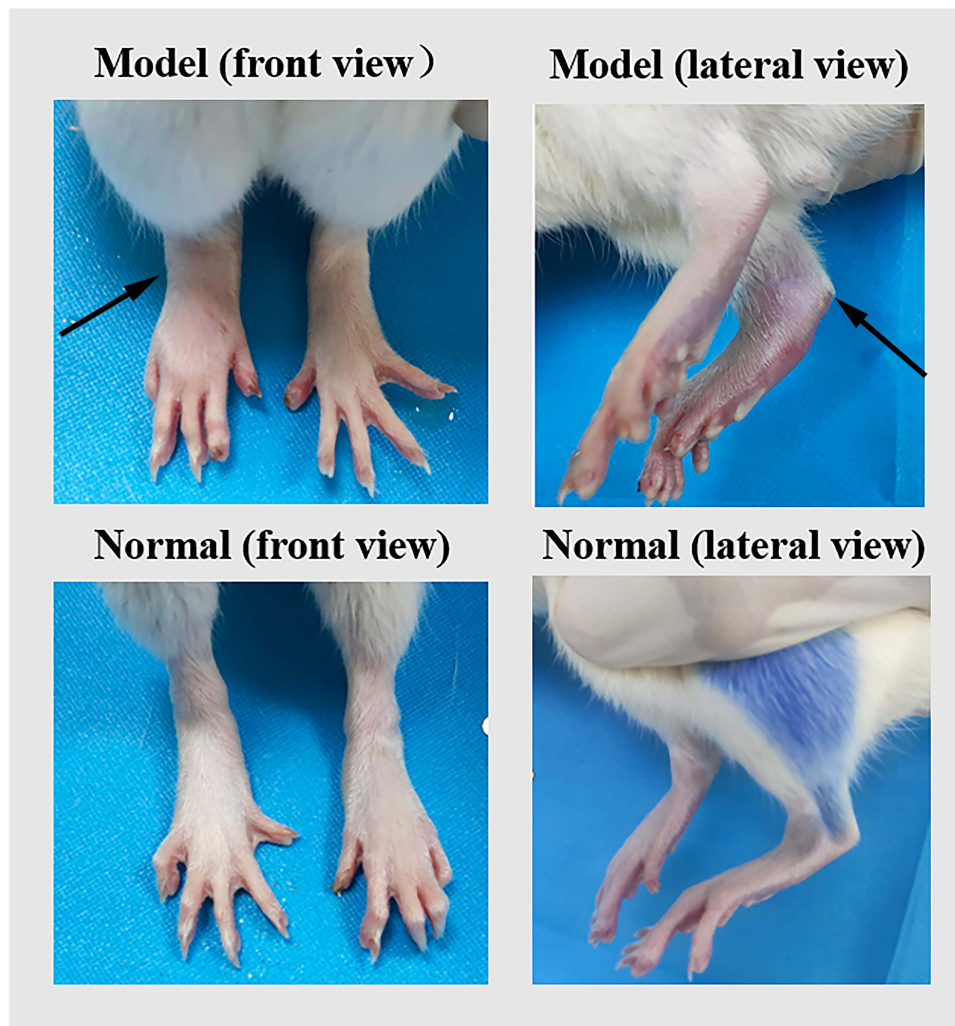
### Effect of Qinpi Tongfeng Formula on Ankle Histopathology in AGA Rats

H&E staining results ([Figure 10](#)) showed no inflammatory cells infiltration such as monocytes and neutrophils of their ankle joint tissues of N group, and cells in the synovial layer were evenly arranged. Compared with the N group, synovial hyperplasia, capillary congestion and edema were obvious in the MSU group, and the infiltration of inflammatory cells such as monocytes and neutrophils was obvious. The above pathological conditions in the QPTFF-L, QPTFF-M, QPTFF-H and DFS groups were significantly improved vs MSU group, that is, inflammatory cell infiltration, synovial layer cell proliferation, capillary congestion and edema were significantly reduced.

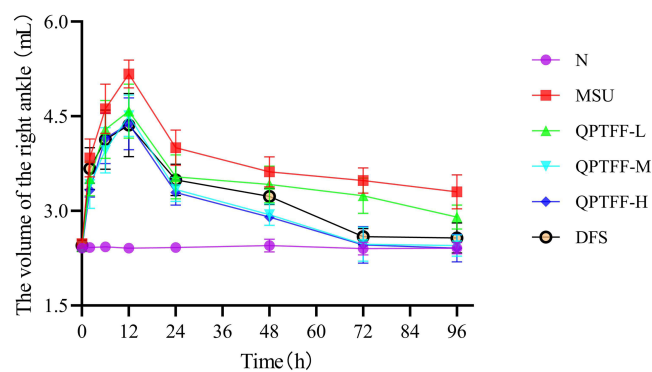
### Effect of Qinpi Tongfeng Formula on Serum Inflammatory Factors and MMP-9 in AGA Rats

The increased IL-17A, IL-1 $\beta$ , IL-6, IL-8, TNF- $\alpha$  and MMP-9 expression levels in the serum of MSU group were considerably seen ( $P < 0.01$ ) vs N group. The decreased inflammatory factors and MMP-9 expression levels were considerably seen in QPTFF-L, QPTFF-M, QPTFF-H and DFS groups ( $P < 0.05$ ,  $P < 0.01$ ) vs MSU group. The lower



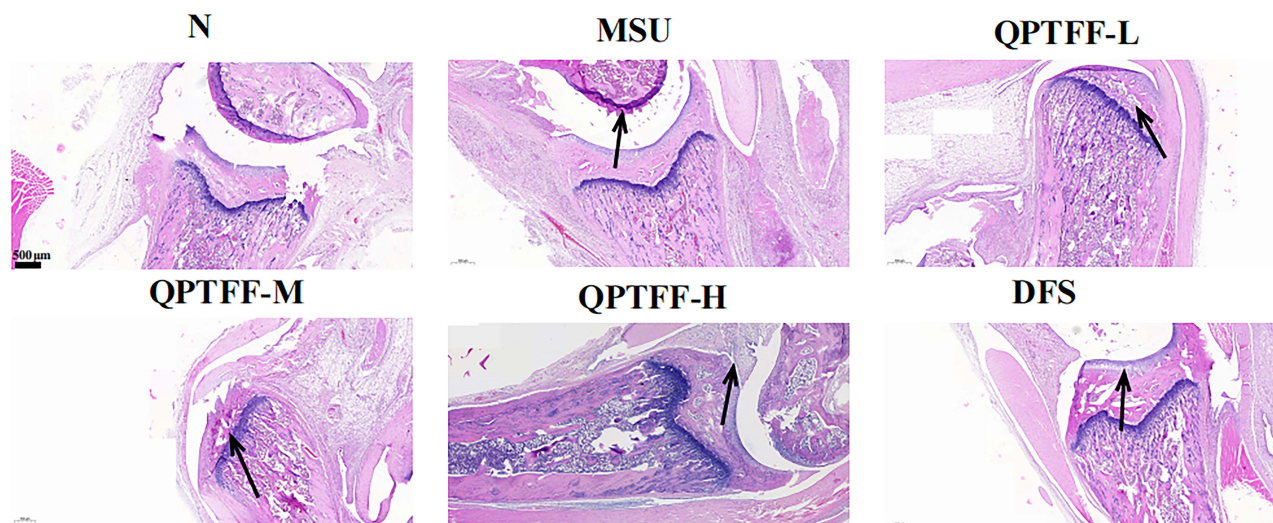


**Figure 8** Right foot performance of rats in the MSU group and N group.



**Figure 9** The volume changes of the right ankle in rats with AGA.

IL-6, IL-8, TNF- $\alpha$  and MMP-9 expression levels were considerably seen in the QPTFF-M group ( $P < 0.05$ ,  $P < 0.01$ ) vs QPTFF-L group. However, no notable variations observed in the serum inflammatory factors and MMP-9 levels were seen among the QPTFF-M, QPTFF-H and DFS groups ( $P > 0.05$ ) (Figure 11).



**Figure 10** Pathological changes in rats with AGA (H&E staining, 25 $\times$ , Scale 500  $\mu$ m).

### Effect of Qinpi Tongfeng Formula on mRNA of Related Targets in Ankle Joints

Measure IL-17A, IKK- $\beta$ , NF- $\kappa$ B p65 and IL-6 mRNA expression levels in rats' ankle joints by qRT-PCR. The considerably increased IL-17A, IKK- $\beta$ , NF- $\kappa$ B p65 and IL-6 mRNA expression levels of MSU group were seen ( $P < 0.01$ ) vs N group. The considerably decreased IL-17A, IKK- $\beta$ , NF- $\kappa$ B p65 and IL-6 mRNA expression levels in QPTFF-M, QPTFF-H and DFS groups were seen ( $P < 0.05$ ,  $P < 0.01$ ) vs MSU group. The lower IL-17A and IL-6 mRNA expression levels were considerably seen in the QPTFF-M group ( $P < 0.05$ ) vs QPTFF-L group. However, no notable variations observed in the IL-17A, IKK- $\beta$ , NF- $\kappa$ B p65 and IL-6 mRNA expression levels among the QPTFF-M, QPTFF-H and DFS groups ( $P > 0.05$ ) (Figure 12).

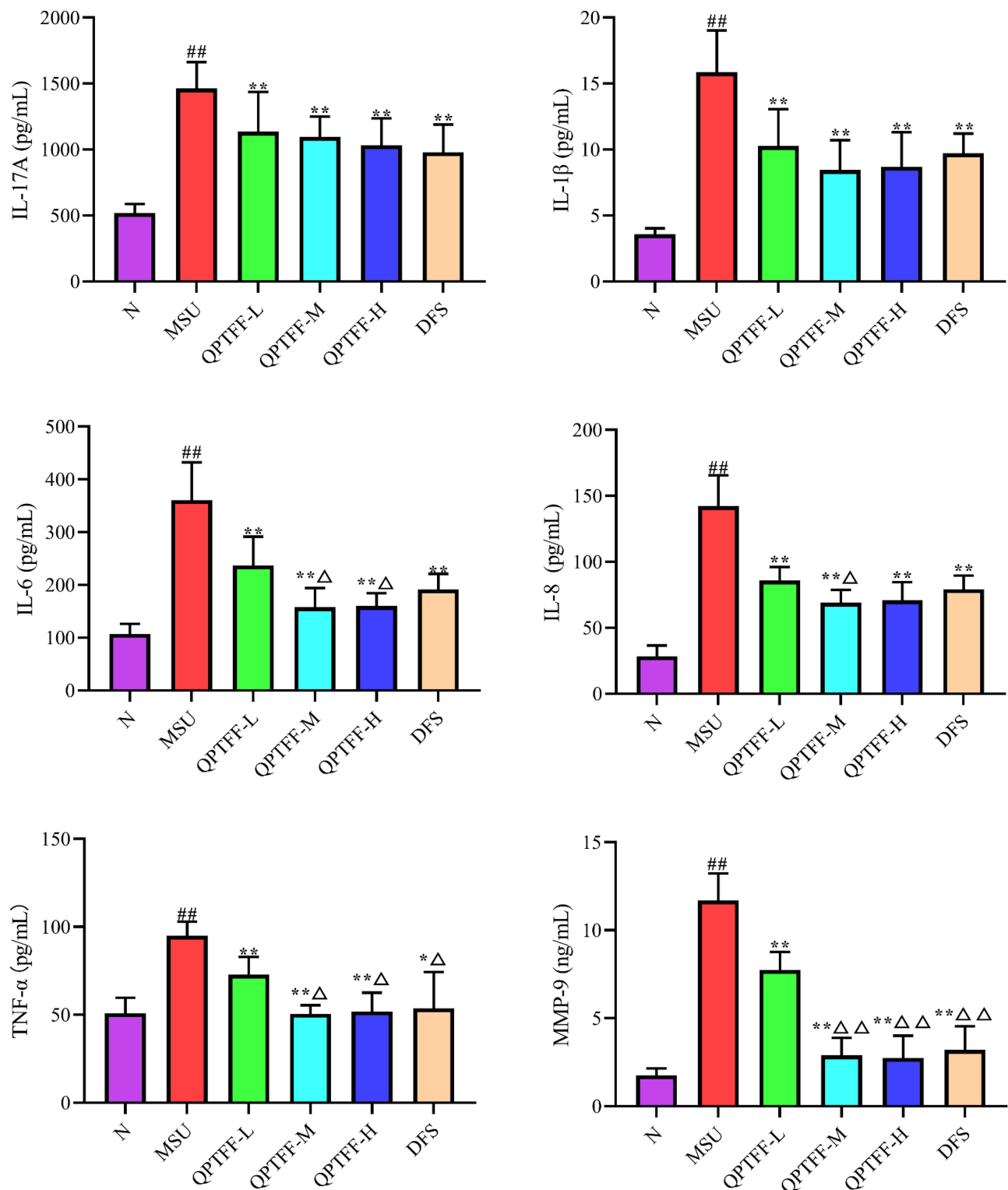
### Expression of Related Proteins Detected by Western Blot

Western blot analysis revealed expression level of IL-17A, IL-17RA, IKK- $\beta$ , NF- $\kappa$ B p65, IL-6 protein and phosphorylation level of IKK- $\beta$  and NF- $\kappa$ B p65 of rats with AGA. The considerably increased protein expression levels of IL-17A, IL-17RA and IL-6 and the levels of pNF- $\kappa$ B p65/NF- $\kappa$ B p65 and pIKK- $\beta$ /IKK- $\beta$  of MSU group were seen ( $P < 0.01$ ) vs N group. The considerably decreased IL-17A, IL-17RA, IL-6 protein expression levels and the ratios of pNF- $\kappa$ B p65/NF- $\kappa$ B p65 and pIKK- $\beta$ /IKK- $\beta$  were seen in QPTFF-M, QPTFF-H and DFS groups ( $P < 0.01$ ) vs MSU group; The considerably decreased IL-17A protein expression and ratio of pNF- $\kappa$ B p65/NF- $\kappa$ B p65 in QPTFF-L group were seen ( $P < 0.05$ ,  $P < 0.01$ ). The considerably lower IL-17A, IL-17RA, and IL-6 protein expression levels of QPTFF-M group were seen ( $P < 0.05$ ) vs QPTFF-L group, however, no notable variations observed in the IL-17A, IL-17RA, IL-6 protein expression levels and the levels of pNF- $\kappa$ B p65/NF- $\kappa$ B p65 and pIKK- $\beta$ /IKK- $\beta$  among the QPTFF-M, QPTFF-H and DFS groups ( $P > 0.05$ ) (Figure 13).

### Pathological Results of Rat Liver and Kidney Tissues

The liver capsule remained undamaged and the hepatic lobular architecture was present in the N group, the hepatic cords were arranged smoothly, there was no necrosis of hepatocytes, a small amount of hepatocyte cytoplasm was loose and lightly stained and balloon-like lesions, and no obvious inflammatory cell infiltration in hepatocytes and hepatic sinusoids be seen. The structure of portal area was relatively complete without fibrous tissue proliferation. No pathological changes were also observed in the QPTFF-H group, similar to the findings in the N group (Figure 14A).

In the N group, the renal capsule was intact, the boundary between cortex and medulla was clear, and scattered glomeruli were seen. The glomerular structure was complete and clear, and no glomerular basal thickening and inflammatory exudation were observed. Some renal tubular epithelial cells showed degeneration. There was no fibrous tissue proliferation in the stroma. No pathological changes were also observed in the QPTFF-H group, and similar to the findings in the N group (Figure 14B).

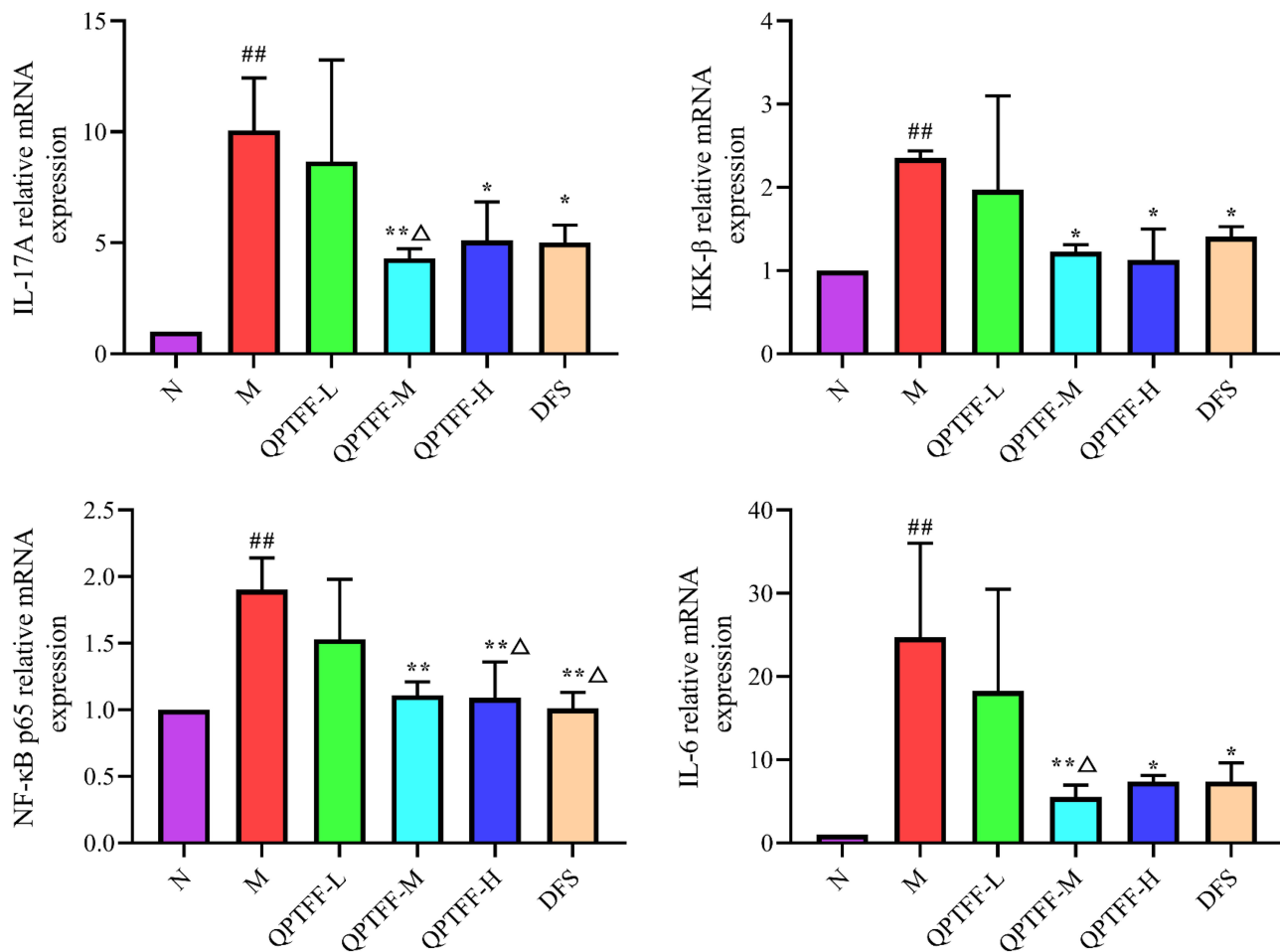


**Figure 11** Inflammatory factors and MMP-9 expression in serum of rats with AGA (Mean  $\pm$ SD,  $n = 6$ ).  
**Notes:** <sup>##</sup> $P < 0.01$  vs N group; <sup>\*</sup> $P < 0.05$ , <sup>\*\*</sup> $P < 0.01$  vs MSU group; <sup>Δ</sup> $P < 0.05$ , <sup>ΔΔ</sup> $P < 0.01$  vs QPTFF-L group.

## Discussion

### Potential Mechanism of QPTFF in Treating AGA

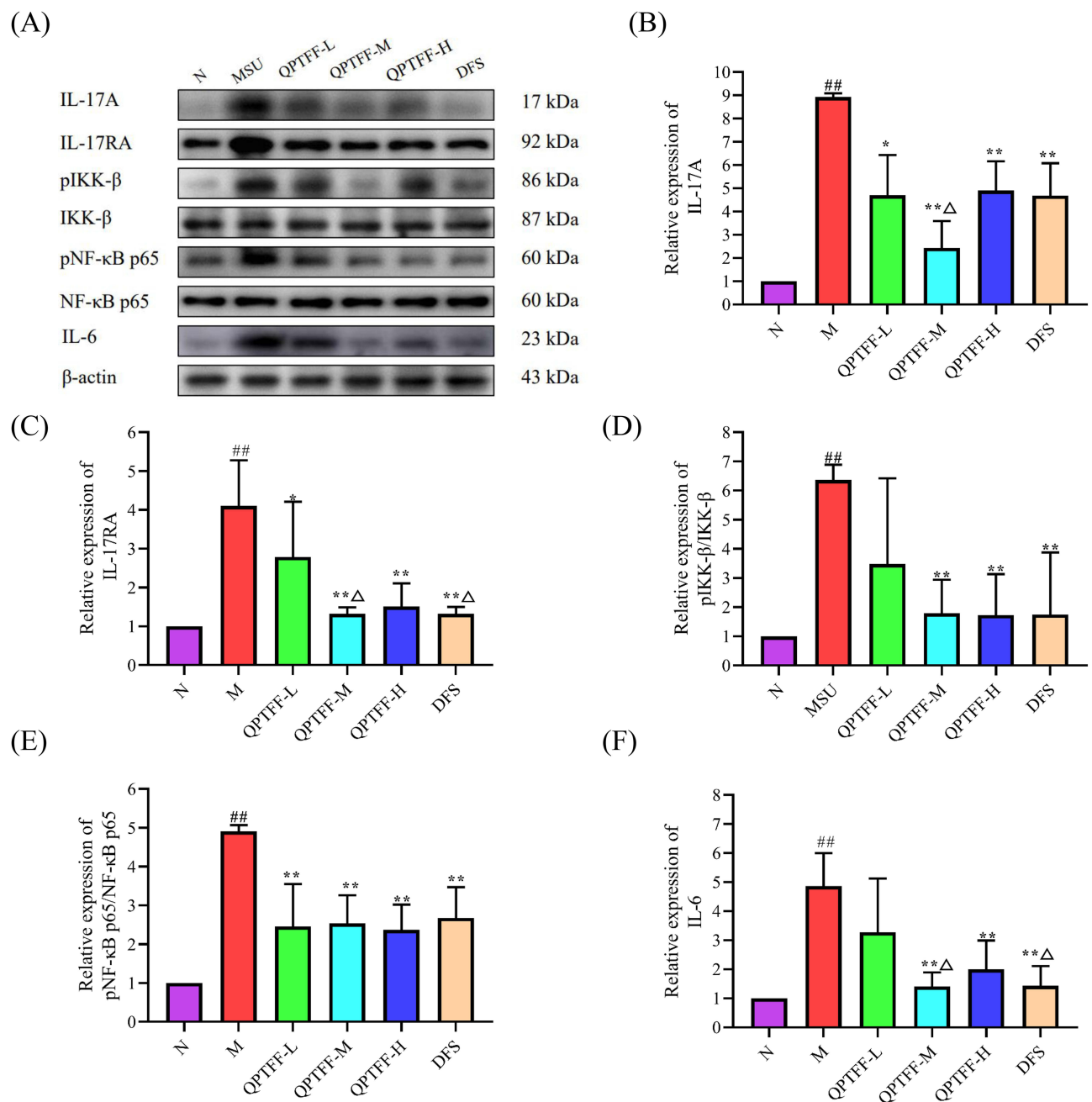
Qinpi Tongfeng formula possesses the abilities to eliminate heat and purify toxins, and specialize in dehumidifying turbidity, alleviating arthralgia and stopping pain, and has a good curative effect on AGA.<sup>36</sup> Fifty-five compounds were



**Figure 12** mRNA relative expression levels of IL-17A, IL-6, IKK- $\beta$  and NF- $\kappa$ B p65 in ankle tissues of rats with AGA (Mean  $\pm$ SD, n = 3).  
**Notes:** ## $P$ <0.01 vs N group; \* $P$ <0.05, \*\* $P$ <0.01 vs MSU group;  $\Delta$  $P$ <0.05 vs QPTFF-L group.

obtained from QPTFF, with 183 potential targets in treating AGA. The network map of “Chinese medicine-Compounds-Potential target” was constructed by network pharmacology, and it was found that different drugs had common compounds, and different compounds also had common targets. Tufuling (*Rhizoma Smilacis Glabrae*), Cheqianzi (*Semen Plantaginis*) and Xixiancao (*Herba Siegesbeckiae*) had common compounds, such as quercetin, and most of the regulated targets, indicating the synergistic effect between Chinese medicines. In addition, Tufuling (*Rhizoma Smilacis Glabrae*) and Bixie (*Rhizome Dioscoreae Hypoglaucae*) also had a common compound, diosgenin, which regulated 14 targets, showing its important role in treating AGA. Based on the degree measurement, the top 3 were quercetin, eburicoic acid and polyporenic acid C, being the possible primary QPTFF-related compounds in treating AGA. Among them, quercetin acted on 77 potential targets, which came from Cheqianzi (*Semen Plantaginis*), Tufuling (*Rhizoma Smilacis Glabrae*) and Xixiancao (*Herba Siegesbeckiae*); Eburicoic acid was found to act on 37 potential targets from Tufuling (*Rhizoma Smilacis Glabrae*); Polyporenic acid C was found to act on 35 potential targets and was derived from Tufuling (*Rhizoma Smilacis Glabrae*). All of these compounds were found in Tufuling (*Rhizoma Smilacis Glabrae*), so the important role of Tufuling (*Rhizoma Smilacis Glabrae*) can be seen. Studies have found that quercetin can improve the acute inflammatory manifestations of urate crystal-induced AGA model rats, inhibit the recruitment of white blood cells, reduce the levels of chemokines and malondialdehyde, the end product of lipid peroxidation, and increase the activities of antioxidant enzymes.<sup>37</sup> Although our study selected quercetin as a potential target for QPTFF, due to its low solubility in water, its bioavailability is influenced by the type of glycosides present in different food sources.<sup>38</sup> Studies have indicated that quercetin is soluble in alcohol and lipids, suggesting that quercetin may need to be





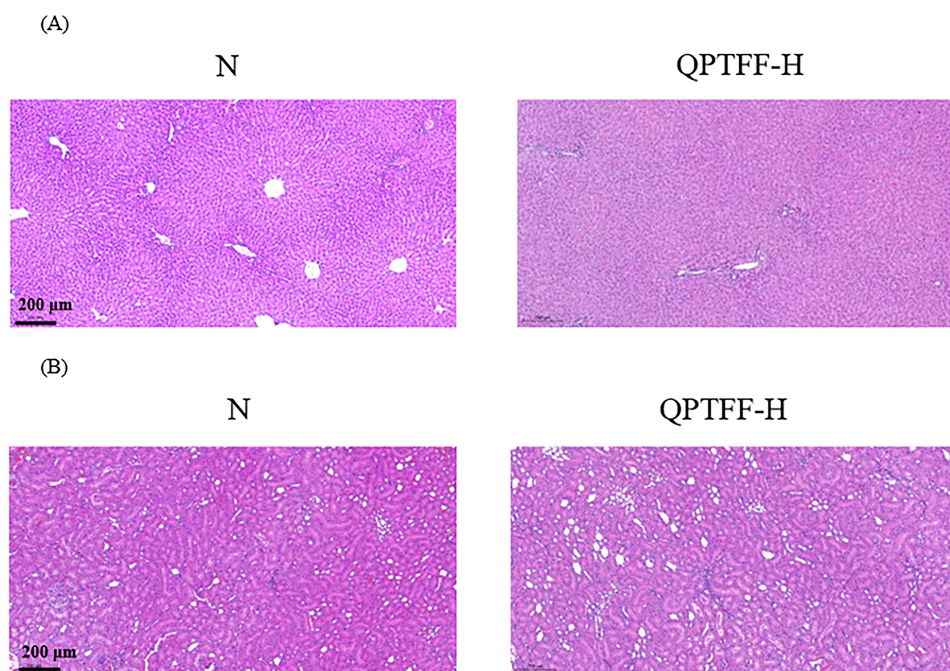
**Figure 13** Relative expression levels of related proteins in tissues of rats ankle joints with AGA (Mean±SD, n = 3).

**Notes:** (A): Western blot strip of IL-17A, IL-17RA, pIKK-β, IKK-β, pNF-κB p65, NF-κB p65 and IL-6 protein; (B-F) histogram of statistical results of IL-17A, IL-17RA, pIKK-β/IKK-β, pNF-κB p65/NF-κB p65 and IL-6 proteins, respectively; ###*P*<0.01 vs N group; \**P*<0.05, \*\**P*<0.01 vs MSU group, Δ*P*<0.05 vs QPTFF-L group.

administered through modified dosage forms, such as the use of nanocarrier systems, to improve its effectiveness and bioavailability in the treatment of arthritis and gouty arthritis.<sup>39</sup> Research findings indicate that eburicoic acid exhibits properties of an antioxidant and possesses anti-inflammatory effects.<sup>40</sup> Eburicoic acid has an obvious anti-inflammatory effect by inhibiting the activation of PI3K/Akt/NF-κB signaling pathway,<sup>41</sup> furthermore, it possesses the ability to suppress xanthine oxidase activity, leading to low uric acid levels.<sup>42</sup> Polyporenic acid C inhibits the inducible NO synthase expression and reduces the level of NO to achieve anti-inflammatory effect.<sup>43,44</sup>

After two topological screenings through the PPI network, 25 core target proteins were finally obtained, among which IL-1β, NFKBIA, IL-6, TNF, CXCL8 and MMP9 being the possible key QPTFF-target proteins in treating AGA. The molecular





**Figure 14** Pathological changes of liver and kidney tissues of rats (H&E staining, 200 $\times$ , Scale 50  $\mu$ m). (A): liver tissue; (B): kidney tissue.

docking analysis revealed that quercetin, eburicoic acid, and polyporenic acid C exhibited robust binding affinity towards the 25 core target proteins. IL-1 $\beta$  is an important inflammatory factor mediating GA.<sup>45</sup> As a key cytokine, IL-1 $\beta$  initiates inflammation and is significantly helpful in progression of AGA. The expression of IL-1 $\beta$  can be suppressed to mitigate the inflammatory response in AGA.<sup>46</sup> *NFKB1A*-encoded NF- $\kappa$ B I $\kappa$ B $\alpha$  is an inhibitor that regulates the movement of transcription factor NF- $\kappa$ B into and out of the nucleus, and NF- $\kappa$ B specializes in the regulation of crucial cytokine expression in AGA.<sup>47</sup> IL-1 $\beta$  released in AGA patients can induce an inflammatory reaction and subsequently enhance the NF- $\kappa$ B activation, and the reciprocal nature of the activity is evident as NF- $\kappa$ B signaling induces an upregulation in pro-IL-1 $\beta$ .<sup>48</sup> Studies have found the considerably higher IL-1 $\beta$ , IL-6, IL-8 and TNF- $\alpha$  serum levels in GA patients,<sup>49</sup> and IL-6 is related to the onset of GA and tophus formation.<sup>50</sup> IL-6 exhibits a cooperative impact with IL-1 $\beta$  and TNF- $\alpha$ , resulting in the production of vascular endothelial growth factor. This factor influences vascular endothelial cells, causing an elevation in their permeability. Consequently, it contributes to the escalation of joint swelling.<sup>51</sup> TNF- $\alpha$  belongs to the family of tumor necrosis factors, and in human neutrophils stimulated with TNF- $\alpha$ , MSU stimulation leads to significant secretion of IL-1 $\beta$ .<sup>52</sup> CXCL8, named IL-8, promotes development of inflammation in the pathogenesis of GA.<sup>53</sup> MMP-9 can degrade the extracellular matrix, cause synovial inflammation of the joint, and destroy the joint.<sup>54</sup>

KEGG enrichment analysis results indicates IL-17 signaling pathway as the most enriched inflammatory pathway. Anti-inflammatory and cartilage protective effects of QPTFF could potentially contribute to the treatment of AGA. Studies have shown that inhibition of IL-17 expression can reduce joint erosion in animal models of arthritis.<sup>55</sup> In the early stages of gout symptoms in AGA patients, serum IL-17 levels were significantly increased.<sup>56</sup> NF- $\kappa$ B p65 signaling pathway is the subsequent pathway of IL-17 signaling pathway. Among the members of the IL-17 family, IL-17A stands out as both the inaugural and extensively researched member to date. It can promote T cells, epithelial cells and other cells to synthesize, secrete lots of cytokines (ig.IL-6, IL-8), thereby aggravating the inflammatory response and leading to tissue damage.<sup>57-59</sup> Studies have confirmed the existence of IL-17A in both the serum and synovial tissue among individuals diagnosed with different forms of arthritis, strong correlation between this cytokine and the development of the disease.<sup>60,61</sup> Hence, we opted to investigate the IL-17A/NF- $\kappa$ B p65 signaling pathway a means of elucidating the mode of action of QPTFF in treating AGA rats.

## Effect of Qinpi Tongfeng Formula on IL-17A/NF- $\kappa$ B p65 Signaling Pathway

IL-17A activates the classical NF- $\kappa$ B pathway. In response to an external stimulus, IL-17A binds to its receptor to form a receptor complex, transmitting signals and recruiting NF- $\kappa$ B activator 1 (Act1) to the receptor complex, meanwhile, TNF receptor-associated factor 6 (TRAF6) is also recruited to the receptor complex and ubiquitinated; Ubiquitinated TRAF6 induces TAK1 phosphorylation and activation; The activated TAK1 can further phosphorylate IKK $\beta$ , thereby activating IKK complex and ultimately activating NF- $\kappa$ B; Activated NF- $\kappa$ B enters the nucleus, enhances the transcription level of target genes, regulates the expression level of downstream genes, and facilitates the release of pro-inflammatory cytokines such as IL-1 $\beta$ , TNF- $\alpha$ , IL-6, IL-8 and MMP9,<sup>61,62</sup> stimulation of IL-1 $\beta$  and TNF- $\alpha$  in turn enhances the phosphorylation of IKK $\beta$ . Thus, the vicious cycle aggravates inflammation;<sup>63</sup> Repeated inflammatory stimulation can cause joint swelling and pain and bone destruction of painful joints in GA patients, which greatly influences the emergence and progression of GA.<sup>64</sup> Therefore, the IL-1 $\beta$ , IL-6, IL-8, TNF- $\alpha$  and MMP9' serum contents can reflect the degree of inflammation in rats to a certain extent, and illustrate the significant help of intervention of IL-17A/ NF- $\kappa$ B p65 signaling pathway in treating AGA.

The anti-inflammatory effect of QPTFF was confirmed in the rat experiment conducted in this study, providing validation for the network pharmacology findings. In the rat experiment, in addition to the intervention of AGA rats with QPTFF, diclofenac sodium, a commonly used drug for clinical treatment of AGA, was added. The IL-17A, IL-1 $\beta$ , IL-6, IL-8, TNF- $\alpha$  and MMP-9 expression levels in serum were significantly reduced by medium and high doses of QPTFF and diclofenac sodium. Compared with the diclofenac sodium group, no notable disparities were observed in the serum levels of inflammatory factors and MMP-9 between the QPTFF-M and QPTFF-H groups, suggesting that QPTFF and diclofenac sodium have similar effects in suppressing the manifestation of IL-17A, IL-1 $\beta$ , IL-6, IL-8, TNF- $\alpha$  and MMP-9. The considerably increased IL-17A, IL-6, IKK- $\beta$  and NF- $\kappa$ B p65 mRNA expression levels of the MSU group were seen. The IL-17A, IL-17RA, IKK- $\beta$ , NF- $\kappa$ B p65 and IL-6 protein expression levels, as well as the pIKK- $\beta$  and pNF- $\kappa$ B p65 levels were also significantly increased, suggesting that IL-17A/NF- $\kappa$ B p65 signaling pathway was closely related to AGA. The considerably decreased IL-17A, IL-6, IKK- $\beta$  and NF- $\kappa$ B p65 mRNA expression levels of the QPTFF-M and QPTFF-H groups were seen vs the MSU group. The considerably decreased IL-17A, IL-17RA, IKK- $\beta$ , NF- $\kappa$ B p65, IL-6 protein expression levels and IKK- $\beta$  and NF- $\kappa$ B p65 phosphorylation levels were also seen. These findings indicated that the involvement of QPTFF in the-17A/NF- $\kappa$ B65 signaling pathway could potentially contribute to the treatment of AGA. There are still some shortcomings in our study. We did not carry out experimental verification on all 25 core targets. Only relevant targets on the IL-17A/NF- $\kappa$ B p65 signaling pathway were selected for verification. In addition, in our experiment, we did not use the blocker of the pathway for experimental verification. In future research, we will further explore the mechanism of action of Qinpi Tongfeng Formula.

## Conclusion

The main targets of QPTFF are IL-1 $\beta$ , NFKBIA, IL-6, TNF- $\alpha$ , CXCL8, MMP9, etc. QPTFF can greatly alleviate the joint inflammation of AGA rats, and high dose of QPTFF has no liver or kidney toxicity. The IL-17A/NF- $\kappa$ B p65 signaling pathway is implicated in the anti-inflammatory effects of QPTFF for treating AGA.

## Data Sharing Statement

All drug compounds and related disease targets data were available from public databases and can also be available from the corresponding author upon receiving a reasonable request. (Wei Liu: fengshiliuwei@163.com).

## Ethical Approval

All experiments were conducted in accordance with the ARRIVE (Animal in Research: In Vivo Experiments) guidelines and followed the principles set by the International Association for the Study of Pain (IASP) Research and Ethics. The animal experiment was approved by the Animal Ethics Committee of Tianjin University of TCM (TCM-LAEC2021137). And this study was approved by the Ethics Committee of First Teaching Hospital of Tianjin University of Traditional Chinese Medicine (TYLL2021[Z]017).

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## Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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## Disclosure

The authors report no conflicts of interest in this work.

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