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DATABASE ANALYSIS

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A Systematic Pharmacology and *In Vitro* Study to Identify the Role of the Active Compounds of *Achyranthes bidentata* in the Treatment of Osteoarthritis

Authors' Contribution:
Study Design A
Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
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Backgrounds: *Achyranthes bidentata* is a Chinese traditional herbal medicine widely used to treat osteoarthritis (OA). This study aimed to identify active compounds from *Achyranthes bidentata* through systematic pharmacology and *in vitro* experiments to find the targets of *Achyranthes bidentata* in the treatment of OA.

Material/Methods: We screened the active compounds of *Achyranthes bidentata* from the Traditional Chinese Medicine Systems Pharmacology (TCMSP) database. Then, we used STITCH and Open Targets Platform databases to screen the active components and predict the potential targets of *Achyranthes bidentata* in the treatment of OA. Subsequently, we studied the compound-target network and protein interaction network and analyzed the enrichment of potential target proteins. Finally, we used Western blot analysis to verify the therapeutic effect of *Achyranthes bidentata* extract on the expression of OA-related target proteins.

Results: There were 7 active components in *Achyranthes bidentata*, which were strongly related to the 74 targets of OA. *Quercetin, baicalein,* and *berberine* are the critical active compounds of *Achyranthes bidentata* in the treatment of OA. Protein interaction analysis and *in vitro* experiments suggested that TNF, IL-6, and TP53 are the critical targets of *Achyranthes bidentata* in the treatment of OA. Functional enrichment analysis showed that *Achyranthes bidentata* plays a pharmacological role in OA through apoptosis, inflammation, and immune regulation.

Conclusions: Quercetin, baicalein, and berberine are the critical active compounds of *Achyranthes bidentata* in the treatment of OA. TNF, IL-6, and TP53 may be potential targets for the treatment of OA.

MeSH Keywords: **Medicine, Chinese Traditional • Molecular Mechanisms of Pharmacological Action • Osteoarthritis • Computer Communication Networks • Achyranthes**

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Background

Osteoarthritis is a chronic degenerative and disabling arthropathy of complex etiology. Its main clinical manifestations are joint pain and deformity and movement limitation [1]. Most scholars believe that it is the result of an imbalance between degradation and synthesis among articular cartilage cells, extracellular matrix, and sub-cartilage bone [2]. At present, the leading role of drugs in the treatment of osteoarthritis is to relieve symptoms, but they cannot effectively prevent the development of osteoarthritis [3]. Therefore, it is of considerable significance to find a safe and effective drug for treating OA.

Achyranthes bidentata is a natural plant-derived traditional Chinese medicine (TCM), which is called 'NIU XI'' in China. *Achyranthes bidentata* has long been used in TCM to treat "rheumatism arthralgia syndrome" by removing blood stasis and relieving pain, and it is a TCM commonly used in the treatment of osteoarthritis [4,5]. Recent pharmacological studies have demonstrated that *Achyranthes bidentata* can protect cartilage by reducing inflammation and apoptosis and by promoting cartilage proliferation [6,7]. However, the chemical composition of *Achyranthes bidentata* is complex, and the mechanism by which it affects osteoarthritis at the molecular level is unclear.

Systematic pharmacology applies the knowledge of system biology to pharmacology. The purpose of this discipline is to clarify how drugs act on the body through biological systems. Unlike evaluating the effect of a drug by a particular protein–drug reaction, systematic pharmacology suggests that drugs act by forming a network of interactions [8]. Interaction systems may include drug–protein and protein–protein interactions, as well as genetic, signaling, and physiological interactions. Systematic pharmacology uses bioinformatics and statistics to integrate and explain the network of drug interactions, which provides an effective method to study the biological mechanisms of TCM in treating diseases [9,10].

A recent systematic pharmacological study of *Achyranthes bidentata* suggested that PIM1, CYP1B1, and HSPA2 are critical targets in the treatment of OA using *Achyranthes bidentata*, but this has not been verified [11]. In the presewnt study, we combined systematic pharmacology and *in vitro* experiments to screen the active components of *Achyranthes bidentata* in treating OA, to predict and verify its targets, and to provide a theoretical basis for the clinical application of drugs. The study flowchart is presented in Figure 1.

Material and Methods

Screening for active compounds

The Traditional Chinese Medicine Systems Pharmacology database (TCMSP; *http://tcmspw.com/tcmsp.php*) is a systematic pharmacological database for evaluating the pharmacokinetic properties of traditional Chinese medicine or related compounds [12]. It provides data on the absorption, distribution, metabolism, and excretion (ADME) of traditional Chinese herbs *in vivo*, including oral bioavailability (OB) and drug-like (DL). OB is the most critical index used to evaluate the effectiveness of oral drugs [13]. DL refers to the similarity between components and known drugs. In drug development, DL evaluation helps to identify qualified compounds and improve the success rate of candidate drugs [14]. Based on the published literature and preliminary information in the TCMSP database [15], we entered the drug name "*Achyranthes bidentata*" into the search box in the TCMSP database and screened the ingredients with OB ≥30% and DL ≥18% for further study.

Identification of drug targets

The STITCH (*http://stitch.embl.de/*) database was used to search the potential target proteins of active components in *Achyranthes bidentate* [16]. STITCH is a database used to explore and predict the interaction between compounds and proteins. Through experiments, databases, and evidence in the literature, chemicals are shown to be associated with other chemicals and proteins. In the present study, we input active components into the STITCH database to screen *Achyranthes bidentata* target proteins with confidence >0.4 in the human species for further analysis.

Identification of disease targets

OA targets were collected from the Open Targets Platform (*https://www.targetvalidation.org*) [17], which is a comprehensive and authoritative database for screening and visualizing potential drug targets related to diseases. It brings together a variety of data types designed to help users identify and prioritize targets for further research [18]. We used the keyword "osteoarthritis" in the Open Targets Platform. Then, the proteins overlapping the *Achyranthes bidentata* targets were selected for further study.

Construction of a component–target network

A 'component–target network' diagram was constructed using Cytoscape 3.7.1 (*https://cytoscape.org/*) software to reveal the main active components and regulatory mechanisms of *Achyranthes bidentata* in the treatment of OA [19].

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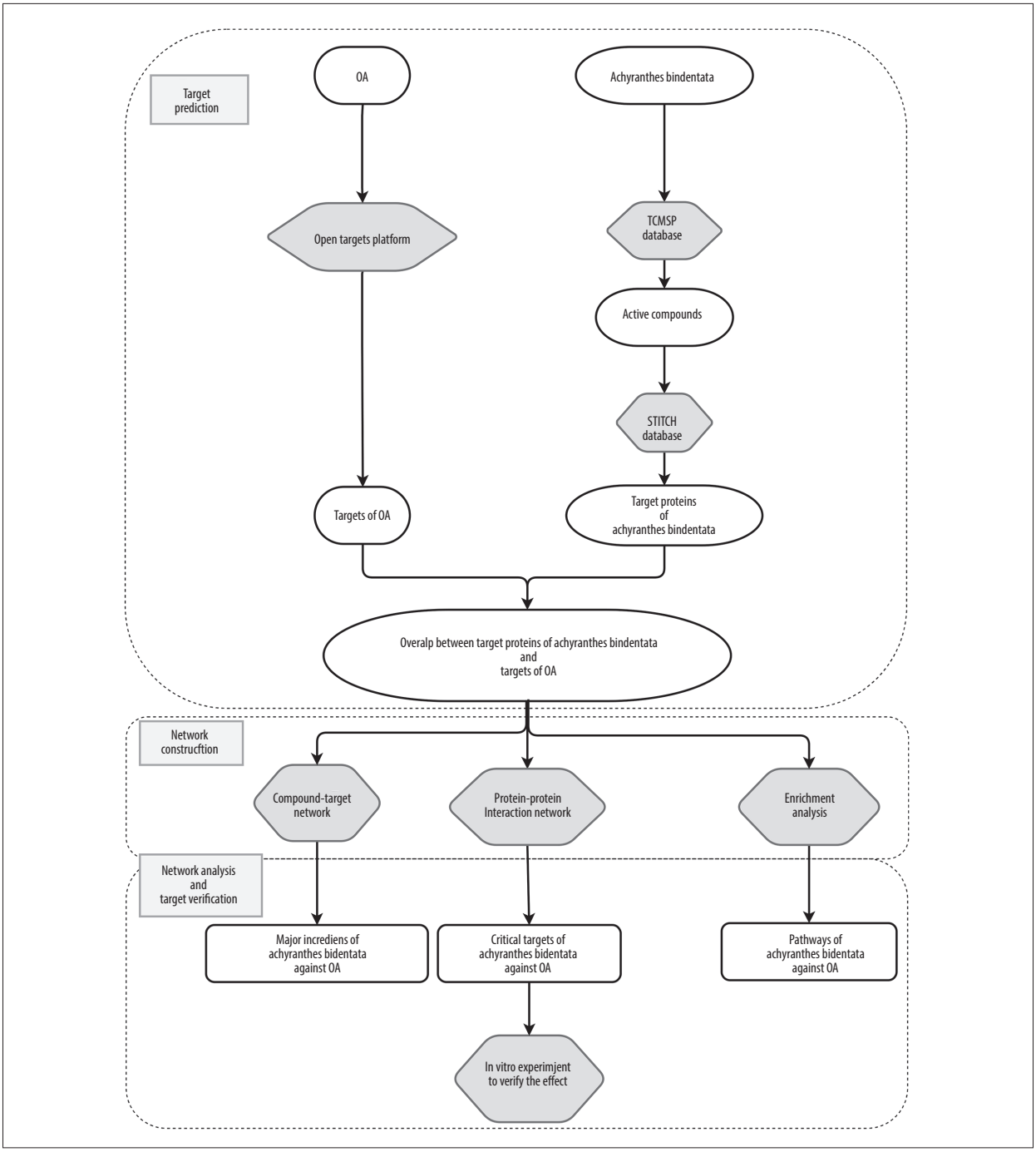

Figure 1. Study flow chart.

Construction of a target–target interaction network

A target–target interaction network based on STRING11.0 (*http://string-db.org/*) was constructed in Cytoscape3.7.1 to identify the critical proteins among the target proteins listed above [20].

Analysis of gene function and signal pathway enrichment

ClusterProfiler was used to analyze the enrichment of biological processes and signaling pathways. ClusterProfiler is an R package for gene cluster enrichment analysis, which can be used to thoroughly understand the function of genes and the enrichment of pathways [21]. Given the overlapping target gene list, clusterProfiler will use the Gene Ontology (GO) and

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the Kyoto Encyclopedia of Genes and Genomes (KEGG) databases to analyze the biological process and signal pathway enrichment of the given gene list [22–24].

In vitro verification

Animals

We purchased a total of 6 Sprague Dawley (SD) rats (4 weeks old, weight 95–110 g, both sexes) from Shanghai SLAC Laboratory Animal, Inc., China [Laboratory Animal Use Certificate no. SCXK(SH)2017-0005] and raised them in a sterile environment. Experiments involving animals complied with the 2006 edition of the guidelines for the Care and Use of Experimental Animals by the Ministry of Science and Technology, China [2]5.

Preparation of Achyranthes bidentata extract

Achyranthes bidentata was purchased from Guo Yi Tang Pharmaceutical in Fujian Province, China. Dr. Xu Wen, Department of Pharmacology, Fujian University of Traditional Chinese Medicine, identified the original herbs as *Achyranthes bidentata*. Then, *Achyranthes bidentata* (500 g) was crushed into a fine powder and boiled twice with 4 L of 80% ethanol for 1 hour. We collected the ethanol extract and filtered it. The filtrate was concentrated to 500 mL at 50°C under decompression, and the concentration was 2 g/mL.

Chondrocytes culture and identification

Briefly, the rats were anesthetized by intraperitoneal injection of 2% pentobarbital and then sacrificed via rapid decapitation. Then, cartilage tissue of rats was taken under aseptic conditions. The cartilage tissue was cut into pieces and digested with 0.5% type II collagenase (Sigma, Darmstadt, Germany) at 37°C for 5 hours. We filtered the cells with a 70-μm cell filter and washed them 3 times with aseptic phosphate-buffered saline (PBS; HyClone, Logan, UT, USA). After that, the collected cells were inoculated into a cell culture flask containing Dulbecco's modified Eagle's medium (DMEM; HyClone, Logan, UT, USA) with 10% fetal bovine serum (Gibco, New York, USA) and cultured at 37°C and 5% CO_2. We changed the culture medium every 48 hours. We identified primary chondrocytes by type II collagen immunofluorescence staining using a previously described method [26,27]. Only primary or second-generation chondrocytes were used for the experiment.

Establishment of a degenerative chondrocyte model

We used IL-1β to establish a model of degenerative chondrocytes using methods described elsewhere [28,29]. Briefly, we treated secondary chondrocytes with 10 ng/ml IL-1β for 24 hours.

Experimental grouping

We divided the cells into 2 groups: i) the degenerative chondrocyte group and ii) the degenerative chondrocyte with *Achyranthes bidentata* extract group. The intervention time for each group was 24 hours. We treated the chondrocytes with the *Achyranthes bidentata* extract, as described previously [30].

Western blot analysis

The expression level of the target protein in chondrocytes of each group was calculated by Western blot and semi-quantitative analysis, using methods we described previously [31]. The following antibodies were used: TNF-α antibody (1: 1000; Abcam, Cambridge, MA, USA), IL-6 antibody (1: 1000; Abcam, Cambridge, MA, USA), p53 antibody (1: 1000; Cell Signaling Technology, Inc., Danvers, MA, USA), β-actin antibody (1: 2000; Abcam, Cambridge, MA, USA), and secondary antibodies (1: 20 000; Merck Millipore, Darmstadt, Germany).

Results

Screening results of components and potential targets for *Achyranthes bidentata*

As described in the Methods section above, we searched the TCMSP database with the keyword "*Achyranthes bidentata*" for 176 herbal ingredients. According to the threshold described in the Methods section, 20 herbal ingredients were obtained.

We searched the STITCH database for 20 compounds screened from the TCMSP database, of which 7 compounds had 131 target proteins. Among them, *baicalin* has 37 targets, β-*sitosterol* has 11 targets, *kaempferol* has 18 targets, *quercetin* has 46 targets, stigmasterol has 3 targets, *baicalin* has 11 targets, and *berberine* has 49 targets. Table 1 shows the ADME properties of these 7 compounds.

Identification of osteoarthritis-related proteins

By searching the Open Targets Platform database, we identified 74 targets closely related to OA as potential targets for the treatment of OA (Figure 2A). Then, we established a network of compounds–targets (Figure 2B). Among these 7 compounds, *quercetin* (Degree=28), *berberine* (Degree=25), and *baicalein* (Degree=26) connect more targets than the other 4 compounds, which suggests that they play an essential role in OA. All active ingredients of *Achyranthes bidentata* are linked to more than 2 targets, which indicates that *Achyranthes bidentata* showed multi-targeting in the treatment of OA.

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Table 1. The absorption, distribution, metabolism, and excretion (ADME) properties of 7 active compounds from *Achyranthes bidentate*.

Mol ID	Molecule name	OB (%)	DL
MOL001454	Berberine	36.86	0.78
MOL000173	Wogonin	30.68	0.23
MOL002714	Baicalein	33.52	0.21
MOL00035	β-sitosterol	36.91	0.75
MOL000422	Kaempferol	41.88	0.24
MOL000449	Stigmasterol	43.83	0.76
MOL000098	Quercetin	46.43	0.28

Target–target interaction analysis

By using Cytoscape 3.7.1, we constructed a diagram of the "target–target network" based on the results from the STRING database (Figure 3A). We constructed a bar plot according to the degrees of connection between the targets (Figure 3B). The results show that IL-6, TNF, and TP53 are the central targets in the network and connect to the most targets.

Gene function and signal pathway enrichment analysis

GO and KEGG analyses were performed with clusterProfiler to analyze the functional characteristics of the targets, and the threshold was set as P≤0.01. The results of GO analysis showed that 317 GO terms were enriched in 'Biological Process' (BP). The first 20 items with the most significant P-values are shown in Figure 4A. As presented, response to lipopolysaccharide, response to oxidative stress, response to a steroid hormone, and regulation of inflammatory response are involved in supporting the role of *Achyranthes bidentata* in treating OA.

The results of KEGG pathway enrichment showed that there are 34 signal pathways. The first 20 signaling pathways with the most significant P-values are shown in Figure 4B. As presented, Apoptosis, TNF signal pathway, T cell receptor signal pathway, Toll-like receptor signal pathway, Osteoclast differentiation, NF-κB signal pathway, and MAPK signal pathway are involved in supporting the effect of *Achyranthes bidentata* in treating OA.

Western blot analysis

The protein expressions of TNF-α, IL-6, and p53 were calculated to verify the effect of *Achyranthes bidentata* on OA, as shown in Figure 5A–5D. Compared with the degenerative chondrocyte group, the *Achyranthes bidentata* group had lower expressions of TNF-α (P<0.05), IL-6 (P<0.01), and p53 (P<0.05) proteins.

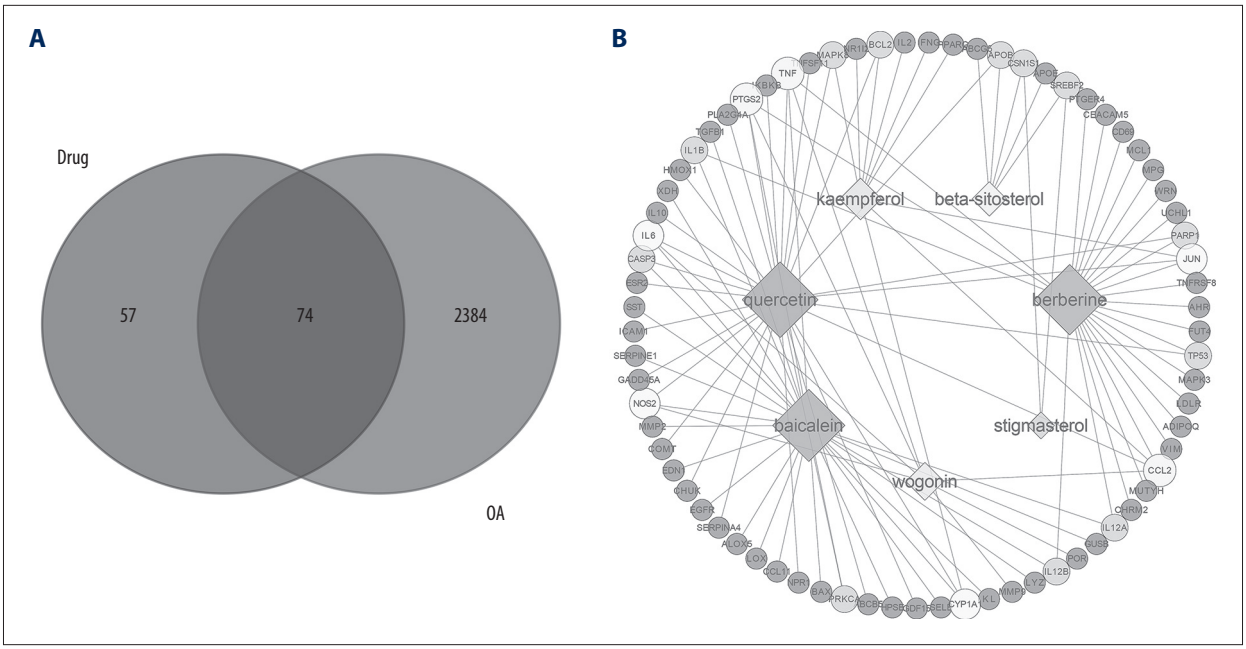

Figure 2. Venn analysis and network analysis of active compounds and potential targets of *Achyranthes bidentata* on OA. (**A**) The Venn diagram of target proteins of *Achyranthes bidentata* and OA-related targets based on the Open Targets Platform. (**B**) The compound-targets network diagram of *Achyranthes bidentata* to treat OA, the square nodes represent compounds. The circular nodes represent target proteins.

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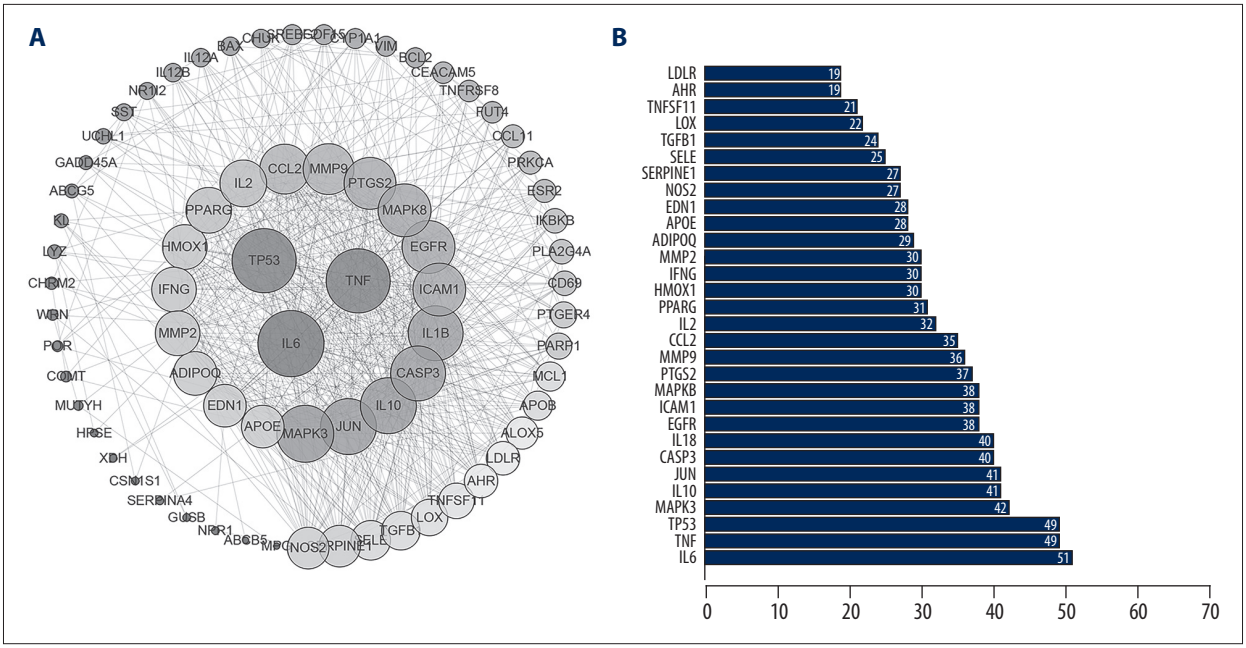

Figure 3. 'Target–Target' interaction network analysis. (**A**) The target–target interaction network is constructed by using Cytoscape 3.7.1. The size of the nodes is proportional to degrees. (**B**) The bar plot on degrees of connection among the targets.

Discussion

The systematic pharmacology method was developed for use with the molecular biological network analysis, which is used to discover new therapeutic drugs derived from natural products. Therefore, it provides a systematic means to expand the application of available drug compounds in TCM to treat a variety of complex diseases. OA is a common type of chronic arthritis with painful symptoms that affect patient quality of life. Although *Achyranthes bidentata* is an effective Chinese traditional herbal medicine used in the treatment of OA for several centuries, the molecular mechanism underlying its effects have been unclear.

In this study, we evaluated the active components and target proteins of *Achyranthes bidentata* in treatment of OA using systematic pharmacology, including ADME system evaluation, drug targeting, mechanism and pathway research, and molecular docking. Seven active components were screened and interacted with 73 different targets related to OA. According to the analysis of the "component–target network" model, *quercetin* showed the most target connections (Degree=28), followed by *baicalein* (Degree=26) and *berberine* (Degree=25). *Quercetin* is one of the most abundant flavonoids in plants and is well known for its antioxidant [32] and anti-inflammatory activities [33]. It plays an active role in anti-OA by promoting the apoptosis of fibroblasts-like synoviocytes in arthritis and protecting cartilage cells from oxidative stress [34,35]. *Baicalein* is a natural compound with potent antioxidant and anti-inflammatory activities [35,36]. Studies have shown that *baicalein* reduces the pro-inflammatory mediators in human OA chondrocytes stimulated by inhibiting the NF-κB pathway [35,37]. *Berberine* has a wide range of biological functions *in vivo*, including antioxidant, anti-inflammatory, hypolipidemic, and hypoglycemic effects [38]. A previous study showed that *berberine* can inhibit the inflammatory response in rat articular cartilage cells [39]. Combined with the results of this study, the evidence suggests that *quercetin*, *baicalein*, and *berberine* are 3 critical active components of *Achyranthes bidentata* in the treatment of OA. A previous systematic pharmacological study of *Achyranthes bidentata* showed that *quercetin* is the main active component of *Achyranthes bidentata* in the treatment of OA [11], which agrees with our results. Moreover, as presented in the 'component–target' network analysis, *baicalein* and *berberine* also appear to play essential roles in OA.

TNF-α and IL-6 are 2 essential targets of OA anti-inflammatory therapy and are central inflammatory cytokines involved in articular cartilage degradation. TNF-α has a variety of biological effects that can directly cause peroxidation of chondrocytes and promote degradation of the cartilage matrix. It can also induce the release of other inflammatory cytokines such as IL-1 and IL-6 by activating the NF-κB pathway and MAPK pathway [40]. IL-6 has a synergistic effect with TNF in the pathogenesis of OA and can induce osteoclast differentiation and accelerate the destruction of articular cartilage [41]. In this study, TNF and IL-6 were the found to be critical elements of the protein interaction network, and Western blot analysis confirmed that *Achyranthes bidentata* extract can inhibit the expression of TNF-α and IL-6 in rat articular degenerative chondrocytes

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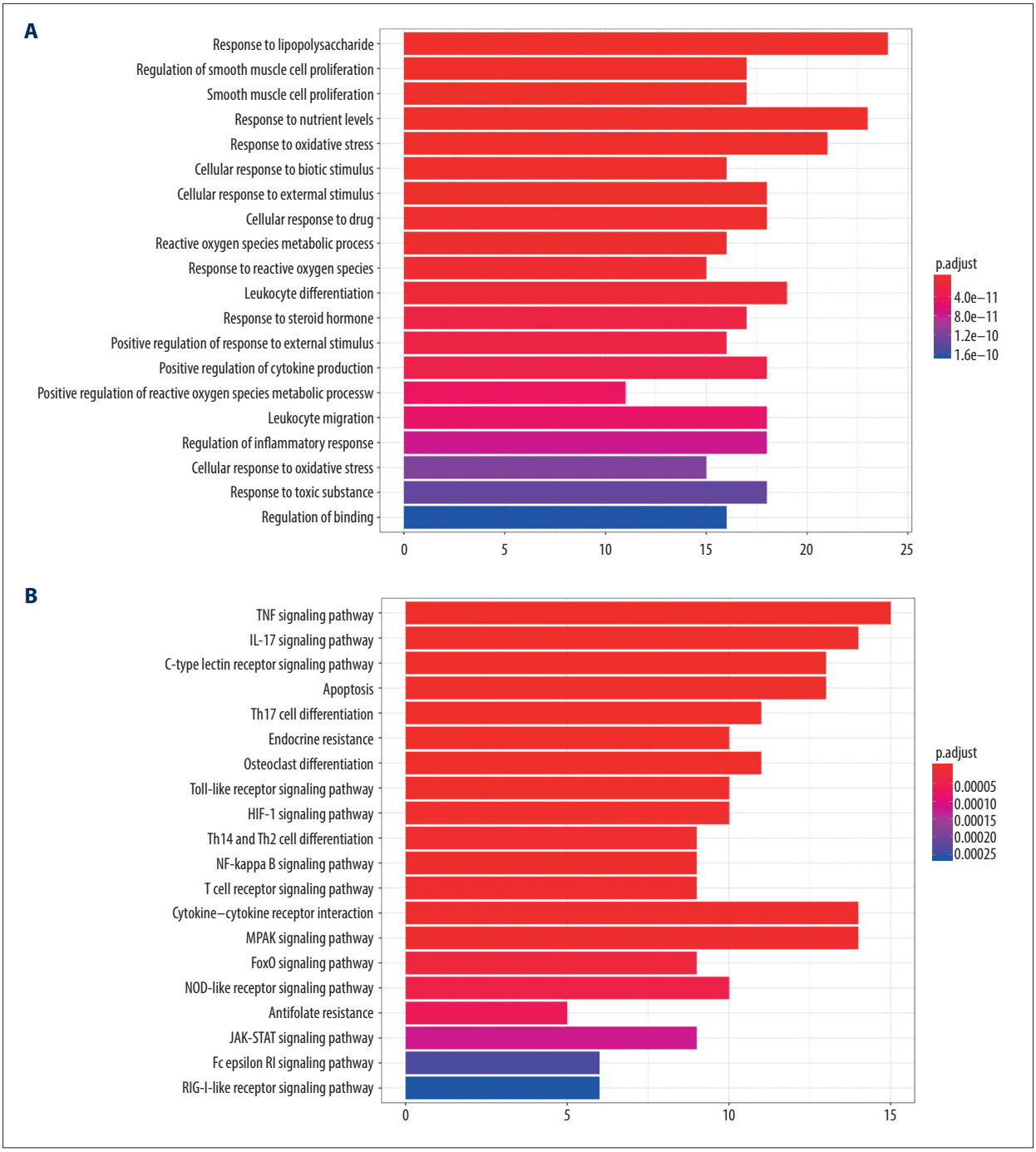

Figure 4. The bar plot of Gene Ontology (GO) and the Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis. (**A**) The first 20 biological processes of GO analysis. (**B**) The first 20 signal pathways of KEGG enrichment analysis.

in vitro. This result suggests that some of the active components in *Achyranthes bidentata* could be used as inhibitors of TNF-α and IL-6.

The TP53-dependent apoptosis pathway is an essential mechanism of OA induced by chondrocyte apoptosis [42]. In osteoarthritis, TP53 can inhibit DNA replication, block cell cycle, lead to apoptosis, and accelerate cartilage degradation [43]. TP53 is also a vital element of the protein interaction network. In this study, Western blot analysis confirmed that *Achyranthes bidentata* extract can inhibit the expression of p53 in rat degenerative chondrocytes *in vitro*, which indicates that some active components of *Achyranthes bidentata* are potential inhibitors of TP53.

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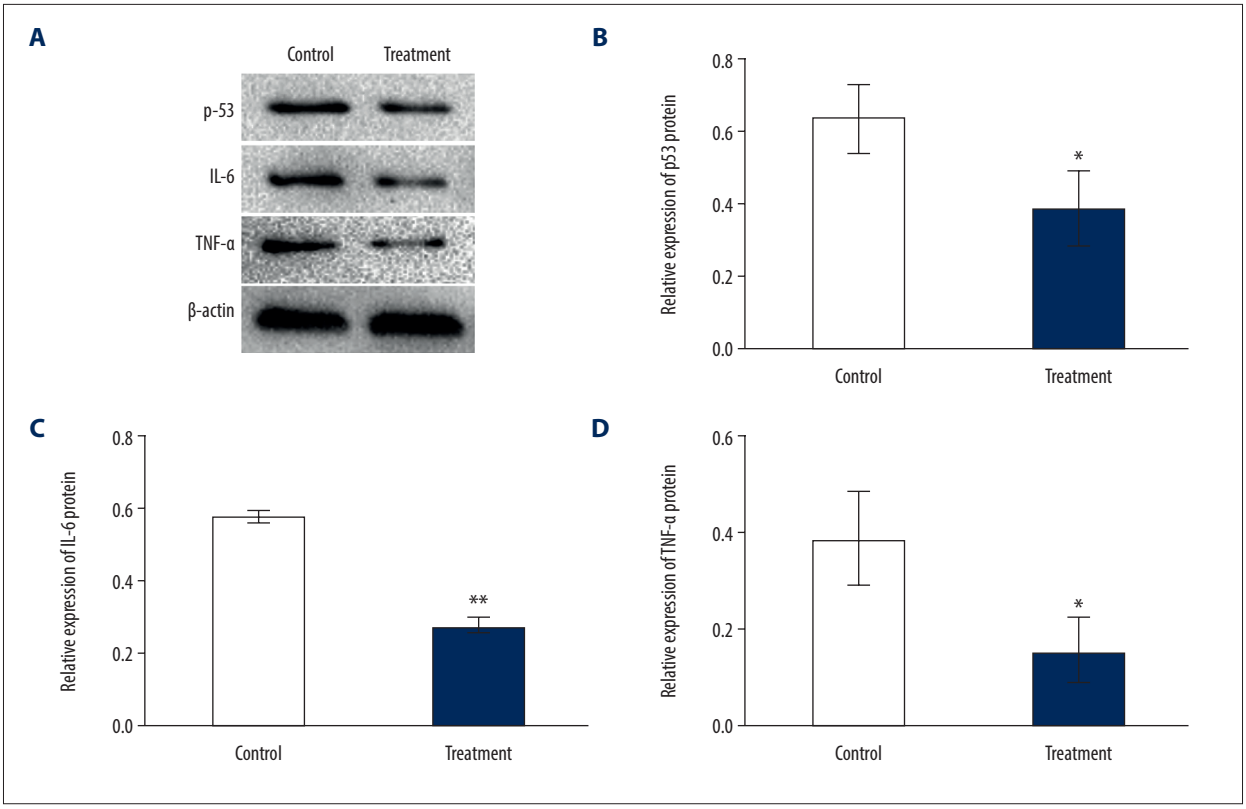

Figure 5. Expression of proteins in chondrocytes exposed to IL-1β and/or *Achyranthes bidentata* extract (**A–D**). * P<0.05 and ** P<0.01 *vs.* IL-1β-treated chondrocytes.

The results of GO and KEGG pathway analyses indicated that *Achyranthes bidentata* exerted its pharmacological effects in OA by modulating multiple pathways, including cell apoptosis, drug metabolism, inflammation, and immune modulation.

There are some limitations to this study. Systematic pharmacology is still in the early stage of development. At present, the data on drugs, compounds, proteins, and genes in the TCM database are incomplete, and the availability of the data obtained is limited [44]. In addition, there are differences between rat chondrocytes and human chondrocytes, and the present results need to be further verified in human chondrocytes and by *in vivo* experiments.

Conclusions

In conclusion, *quercetin, baicalein, and berberine* are 3 important active components in the treatment of OA using *Achyranthes bidentata. Achyranthes bidentata* extract reduced the expression of TNF-α, IL-6, and p53 in degenerative chondrocytes. This study also showed that *Achyranthes bidentata* exerted its pharmacological effects in OA by modulating multiple pathways, including cell apoptosis, drug metabolism, inflammation, and immune modulation. However, this study is based on data mining analysis and *in vitro* experiments using rat chondrocytes, and our results need to be further verified in human chondrocytes and *in vivo* experiments.

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