Original Article

Interpreting the pharmacological mechanisms of Juanbi recipe on rheumatoid arthritis through network pharmacology, molecular docking

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Background: Traditional Chinese medicine (TCM) offers the advantage of effectively relieving rheumatoid arthritis (RA) with minimal side effects. The Juanbi recipe is a commonly utilized TCM treatment for RA, yet its pharmacological mechanism remains unclear. Network pharmacology serves as an effective tool for identifying pharmaceutical ingredients and potential therapeutic targets of TCM, thereby uncovering its mechanisms. This study aimed to identify the core target genes and explore the mechanisms underlying the treatment of RA with the Juanbi recipe.

Methods: This study adopted the method of network pharmacology to filter key gene targets of Juanbi recipe in RA treatment. Single-cell ribonucleic acid (RNA) sequencing data was used to screen the key genes to form the core genes of Juanbi recipe in RA treatment. The molecular docking technique was used to verify the core target genes and explore the mechanisms of Juanbi recipe in RA treatment. The RA model of mice was induced by the collagen-induced arthritis and the effect of Juanbi recipe was evaluated by intragastric administrating of extraction of Juanbi recipe. Enzyme linked immunosorbent assay was used to analysis serum inflammatory factors. Hematoxylin and eosin staining was used to evaluate inflammation and immunohistochemical (IHC) staining was used to evaluate core target genes and pathways in synovium of ankle.

Results: This study screened out 281 active molecules in Juanbi recipe, found 105 key target genes of Juanbi recipe in RA treatment, and drew an "ingredient – molecule – gene" diagram. Juanbi recipe reduced the levels of serum interleukin (IL)-1 and IL-6, the inflammatory infiltration in synovium, demonstration that Juanbi recipe reduced both systemic and synovial inflammatory response. Single cell RNA sequencing data were used to select six core target genes and six core active molecules of Juanbi recipe in RA treatment. The pathways of Juanbi recipe in RA treatment involved in activator protein-1 (*AP-1*) and nuclear factor kappa B (*NF-κB*) pathway. Results of western blot and IHC staining showed that Juanbi recipe decreased the expressions of *c-jun* and *p65*, which demonstrated that Juanbi recipe inhibited the expression of *AP-1* and *NF-κB* pathway in RA.

Conclusions: The core active molecules of Juanbi recipe could inhibit key factors of *AP-1* and *NF-κB* pathway to inhibit the inflammation, which played a protective role in RA.

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Keywords: Rheumatoid arthritis (RA); Juanbi recipe; activator protein-1 (AP-1); nuclear factor kappa B (NF-κB); network pharmacology

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Introduction

Rheumatoid arthritis (RA) is a common autoimmune disease. The number of RA patients in China is about 5 million, the incidence rate is about 0.42%, and the global incidence rate is about 1% (1,2). At present, the mechanism of RA pathogenesis and progression is not clear, and the widely recognized mechanism is the abnormal activation of immune cells including T cells, B cells, monocytes (3), and the inflammatory changes of joint synovial cells (4). Although the development of RA can be controlled through glucocorticoids, disease-modifying antirheumatic drugs (DMARDs), biological agents, the side effects of these drugs are relatively large (5). For instance, glucocorticoids may cause metabolic disorder and necrosis of femoral head; DMARDs and biological agents cause immunosuppression increasing the risk of infection. At present, more and more studies the use of traditional Chinese medicine (TCM) alone or with DMARDs alleviated both symptoms and inflammatory factors in RA patients without major side effects (6,7). Therefore, TCMs have great advantages and prospects in RA treatment.

Highlight box

Key findings

- Juanbi recipe inhibited key factors of activator protein-1 (*AP-1*) and nuclear factor kappa B (*NF-κB*) pathway to inhibit the inflammation in treating rheumatoid arthritis (RA).

What is known and what is new?

- Juanbi recipe which is an ancient prescription of Traditional Chinese Medicine (TCM) has an effect on releasing symptom of RA.
- Juanbi recipe inhibited key factors of *AP-1* and *NF-κB* pathway to reduce inflammation, which played a protective role in RA.

What is the implication, and what should change now?

- The analysis predicted that Juanbi recipe could combine to c-jun and FOS and inhibit *AP-1* and *NF-κB* pathway. A further study should be conducted to verify the effect of Juanbi recipe on treating RA.

Juanbi recipe is an ancient prescription of TCM. Studies showed that Juanbi recipe relieved RA in both animal experiments (8-10) and clinical trial (11). However, the pharmacological mechanism of Juanbi recipe in RA treatment is still unclear. This study intended to explore the core target genes and mechanisms of Juanbi recipe in RA treatment.

There are many kinds of compounds in an ingredient of TCMs, which means that the method of one molecule targeting to one target is not efficient to detect new drugs. However, network pharmacology provides a network model with multiple molecules, multiple targets, and multiple pathways, which is a new method to study the mechanism of drug action. Network pharmacology helped researchers identifying pharmaceutical ingredients and potential therapeutic targets (12). The combination of molecular docking technology and network pharmacology is applied to the design of new Chinese medicine, to reveal the multi-target and multi-pathway therapeutic potential of Chinese medicine (13). Network pharmacology has become a strategy and application for TCM development and biomarkers (14).

In the study of new targets and mechanisms of TCM, network pharmacology was used to select the molecules and target proteins of a TCM, proteins involved in a specific disease were filtered by the disease database, and the analysis of the intersection between the target proteins of TCM molecules and the disease-involved proteins was performed. Although this method has the advantage of high efficiency, the conclusions obtained are not specific. Single-cell ribonucleic acid (RNA) sequencing results could be used to verify the expression of target proteins in the diseases, especially revealing the expression of interested proteins in different types of cells, which was an effective tool to improve the specificity of network pharmacological analysis. This study used technology of network pharmacology and molecular docking, with the help of single cell RNA sequencing data, to explore the core target genes and mechanisms of Juanbi recipe in RA treatment. Firstly, we used the method of network pharmacology to screen all target genes and active molecules of Juanbi recipe, and then used the disease gene database to filter key target genes of

Juanbi recipe in RA treatment. Secondly, we used single cell RNA sequencing data to screen core target genes and core active molecules of Juanbi recipe in RA treatment. Thirdly, the core target genes and the core active molecules were verified by molecular docking technology. Finally, the target proteins and pathways were verified by mice RA model treated by Juanbi recipe.

Methods

Determining the ingredients of Juanbi recipe

Juanbi recipe is a common prescription of TCM, which is recorded in many ancient books of TCM, such as *Yang's Family Recipe* (15), *Yan's Ji Shi Recipe*, *Wei's Family Recipe* (16), *Yi Xue Xin Wu* (17), *Song Ya Zun Sheng* (18) and *Yi Zong Jin Jian* (19). However, the records of Juanbi recipe from these books are slightly different. This study reviewed the ingredients of Juanbi recipe in six ancient Chinese medical books and took the most frequent ingredients for research object.

Component collection and target gene screening of Juanbi recipe

In this study, the TCM systems pharmacology database and analysis platform (TCMSP) were used to search all the ingredients of Juanbi recipe, The criteria of active molecules included drug likeness no less than 0.30 and oral bioavailability no less than 0.18. We use TCMSP to obtain proteins targeted by the active molecule of Juanbi recipe and convert the proteins into the target gene through Uniprot database (www.uniprot.org).

Screening key genes of Juanbi recipe in treating RA by disease database

We searched the Genecard database (http://www.genecards.org) with the keyword "rheumatoid arthritis" to find the genes related to RA. We used bioinformatic database (www.bioinformatics.com.cn) to analyze key genes of Juanbi recipe in RA treatment. We inputted the genes that related to RA and the genes targeted by the active molecules of Juanbi recipe to construct a Venn map and the intersection genes were considered as key genes of Juanbi recipe in RA treatment. We constructed an "ingredient – molecule – gene" diagram for Juanbi recipe in RA treatment to display the relationship among ingredients of Juanbi Recipe, active molecules and targeting genes.

Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis

To determine the important function and pathways that the key genes involved, we introduced the key genes into bioinformatic database (www.bioinformatics.com.cn) to perform GO and KEGG (20). We analyzed three GO catalogs including biological processes, cellular components, and molecular functions. We defined false discovery rate less than 0.05 as the significance standard of enrichment analysis.

Obtaining the core target gene of Juanbi recipe in treating RA through single cell sequencing data

The single cell RNA sequencing data of peripheral blood were obtained from the National Biological Information Center (ngdc.cncb.ac.cn, No. HRA000916) (21), and the single cell sequencing data of rheumatoid synovial tissue were obtained from ImmPort (www.immport.org, No. SDY998) (22). We analyzed the single cell data through R (www.r-project.org, version 4.1) and used Seurat (atijalab.org/seurat, version 4.1) for cell cluster analysis. Peripheral blood mononuclear cells (PBMCs) were divided into five main clusters, namely, B cells, dendritic cells, monocytes, natural killer cells, and T cells. The synovium is divided into four main clusters, namely T cells, B cells, fibroblasts, and monocytes. The differential gene was obtained through differential analysis with the standard of absolute value of fold change larger than 0.5 and P less than 0.05 (23).

We screened the key genes of Juanbi recipe in treating RA through PBMCs and synovium single sell RNA sequencing data to gain core target genes. The core target gene of Juanbi recipe in treating RA was obtained by intersection of the differential genes and the key target gene. We then discovered the core active molecule by mapping the core target genes to the "ingredient – molecule – gene" figure.

Verification by molecular docking

We used molecular docking technology to verify the mechanism and binding affinity between the core molecules and the core target genes of Juanbi recipe in RA treatment. First, we obtained the structure of the core molecules from Pubchem (pubchem.ncbi.nlm.nih.gov), and obtained the crystal structure of the core target proteins from the protein data bank (www.rcsb.org). We deleted the water molecule and ligand, and added hydrogen atoms through

PyMOL 2.5 (www.lfd.uci.edu/~gohlke/pythonlibs, version 2.5) and AutoDock (ccsb.scripps.edu/mgltools/downloads, version 1.5.7). Finally, Autodock and PyMOL are used for molecular docking and visual analysis respectively. We calculated the binding energy of the core active molecules binding to the core target genes. The binding energy less then –5 kcal/mol was the criterion for stable binding.

Preparation of Juanbi recipe

Herbs in Juanbi recipe were prepared by a pharmacognosist of Union Hospital affiliated to Tongji Medical College, Huazhong University of Science and Technology, in accordance with standard protocols. The ingredients of Juanbi recipe were *Radix notopterygiae* (10 g), *Radix Notopterygiae* (10 g), *Radix Gentianae* (10 g), *Radix Angelicae* (3 g), *Ligustrum* (7 g), *Radix glycyrrhizae* (5 g), *Kaifeng* (20 g), *mulberry branch* (3 g), *frankincense* (8 g), *Radix xylocarum* 8 points. In line with standard methods of Chinese Pharmacopoeia, all ingredients were soaked in 12 volumes of water for 40 min and boiled for 40 min. The extracts were filtered, and the filter residue was boiled in eight volumes of water for another 40 min and the solution was filtered again. Both batches of the filtrate of the drugs were mixed and concentrated to 0.557 kg/L, which were prepared for intragastric administration.

Model establishment of collagen-induced arthritis (CIA)

Experiments were performed under a project license (No. 3353) granted by Institutional Animal Care and Use Committee of Huazhong University of Science and Technology, in compliance with the guiding principles for the care and use of laboratory animals approved by the Animal Regulations of National Science and Technology Committee of China. C57BL/6 male mice (4- to 5-week-old) were obtained from Tongji Medical College of Huazhong University of Science and Technology (Wuhan, China). The CIA model was established in accordance with a previously reported protocol (24). Briefly, the mice were immunized intradermally with 100 mg of bovine type II collagen emulsified in complete Freund's adjuvant. To ensure a high incidence of RA induction in the CIA model, a booster immunization of bovine type II collagen emulsified in incomplete Freund's adjuvant was used at 21st day after the primary immunization. Typically, the first signs of arthritis appeared in this model at 21st–28th after immunization. CIA was considered to have successfully developed when swelling was observed in at least one paw.

Experimental groups

Nine C57BL/6 mice were randomly divided into three groups (3 mice/each group): (I) control group; (II) RA model group, mice which suffered CIA; (III) RA model + Juanbi recipe group, mice suffered CIA and treated by Juanbi recipe (12 g/kg/day). After three groups were treated 4 weeks, peripheral blood was taken, and then the mice were sacrificed by neck amputation. The ankle joint was obtained and fixed in 4% paraformaldehyde at room temperature for 48 hours, decalcified in 10% ethylene diamine tetraacetic acid (EDTA) for 20 days, dehydrated by ethanol, and encased in paraffin wax.

Histopathological examination

Sample of ankles were prepared into 4-mm thick consecutive sections in the sagittal position and were collected and mounted on common slides and were divided into three levels. One section from each of the three levels was subjected to hematoxylin and eosin (H&E) staining and immunohistochemical (IHC) staining.

Enzyme linked immunosorbent assay (ELISA)

Serum was collected by centrifugating blood collected from the mice. The serum and reagent were added to the plate according to the steps indicated in the ELISA instructions. Optical density (OD) values were read at 450 nm wavelength. Serum protein concentration was calculated by standard curve. The detail of ELISA kits was listed in Table S1.

Western blot

The protein of ankle synovium was collected, and 20 μg of it from each sample was separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene fluoride membrane. After blocking with 5% bovine serum albumin in Tris buffered saline + Tween (TBST) at room temperature for 1 hour, the membranes were incubated with the corresponding primary antibodies overnight at 4 ℃. After washing with TBST three times, the membranes were incubated with the secondary antibodies. Proteins were scanned and analyzed by the chemiluminescence system and autoradiography. The detail of antibody was listed in Table S1.

IHC staining

Slides of ankle were dewaxed, hydrated and prepared for antigen-retrieval by serum-exposure at room temperature for 45 min. The corresponding primary antibodies were added drop-wise. Samples were incubated overnight at 4 ℃. For the negative control group, phosphate-buffered saline (PBS) was used in place of primary antibody. Samples were washed three times with PBS-tween every 5 min. Biotinylated secondary goat anti-rabbit (1:1,500) and rabbit anti-goat (1:1,500) antibodies were added, respectively, and incubated for 60 min at 37 ℃. Samples were washed three times with PBS-T every 5 min. After 3,3-N-diaminobenzidine tetrahydrochloride color development and dehydration, samples were sealed with transparent neutral balsam. Pale or dark brown granular staining was recorded as positive. The detail of antibody was listed in Table S1.

Statistical analysis

The data were presented as mean ± standard deviation (SD) from at least three separate experiments. Statistical analysis was performed using R (Version 4.2). One-way ANOVA was used to determine the significance of differences between groups. Post hoc two-sided paired *t*-tests were conducted for pairwise comparison among the three groups following statistically significant ANOVA omnibus tests. Significance was set at 0.05.

Results

Determining six most frequently used ingredients of Juanbi recipe

We reviewed Juanbi recipe from *Yang's Family Recipe*, *Yan's Ji Shi Recipe*, *Wei's Family Recipe*, *Yi Xue Xin Wu*, *Song Ya Zun Sheng*, and *Yi Zong Jin Jian*, and then counted their ingredients. The most frequent ingredients were Angelicae Sinensis Radix, Notopterygii Rhizoma Et Radix, Licorice, Saposhnikoviae Radix, Hedysarum Multijugum Maxim, and Curcumaelongae Rhizoma (Table S2). We took these ingredients as Juanbi recipe for further research.

Juanbi Recipe had a significant effect on reducing RA

To investigate the gross articular morphology, we perform H&E staining of ankle. The results of H&E staining indicated that inflammatory cells infiltrated into the synovium in the RA model group, while the inflammatory cell infiltration was significantly decreased in treatment with Juanbi recipe (*Figure 1*).

To investigate the inflammation level in blood, we performed ELISA test for serum *IL-1* and *IL-6* which were typical inflammatory factors in RA. The results of ELISA showed that serum *IL-1* (P=0.0041) and *IL-6* (P=0.0031) had a significant difference among the group of control, RA model and RA model + Juanbi recipe. Results of pairwise comparison are displayed in *Figure 2*, which demonstrated that the RA model caused the increase of systemic inflammatory factors in mice, while the treatment of Juanbi recipe could reduce the levels of these inflammatory factors.

Filtering 281 active molecules and target genes for Juanbi recipe and 661 related genes for RA

We used TCMSP database to obtain all molecules of each ingredient from Juanbi recipe and screened the active components with oral bioavailability not less than 0.3 and drug-likeness not less than 0.18 as criteria. We found that Juanbi recipe contained 281 active molecules. We found target genes corresponding to active molecules through TCMSP database and Uniprot database. The active molecules of Juanbi acted on 439 target genes.

We used Genecard database to search for genes related to RA, and found 661 genes that had close relationship to RA.

Obtaining 105 key target genes of Juanbi recipe in RA treatment

We intersected the target genes of Juanbi recipe, and the genes related to RA, and thereby found 105 key target genes of Juanbi recipe in RA treatment (*Figure 3A*). We draw the diagram of "ingredient – molecule – gene" for Juanbi recipe in RA treatment (*Figure 3B*).

GO and KEGG analysis for key target genes of Juanbi recipe in RA treatment

In order to study the function key target genes of Juanbi recipe in RA treatment, we conducted GO analysis and KEGG analysis on these genes. The results of GO were divided into biological processes, cellular components, and molecular functions. The top three items of biological processes contained reactive oxygen species metabolic process, response to lipopolysaccharide, response to molecule of bacterial origin (*Figure 4A*). The top three items of cellular

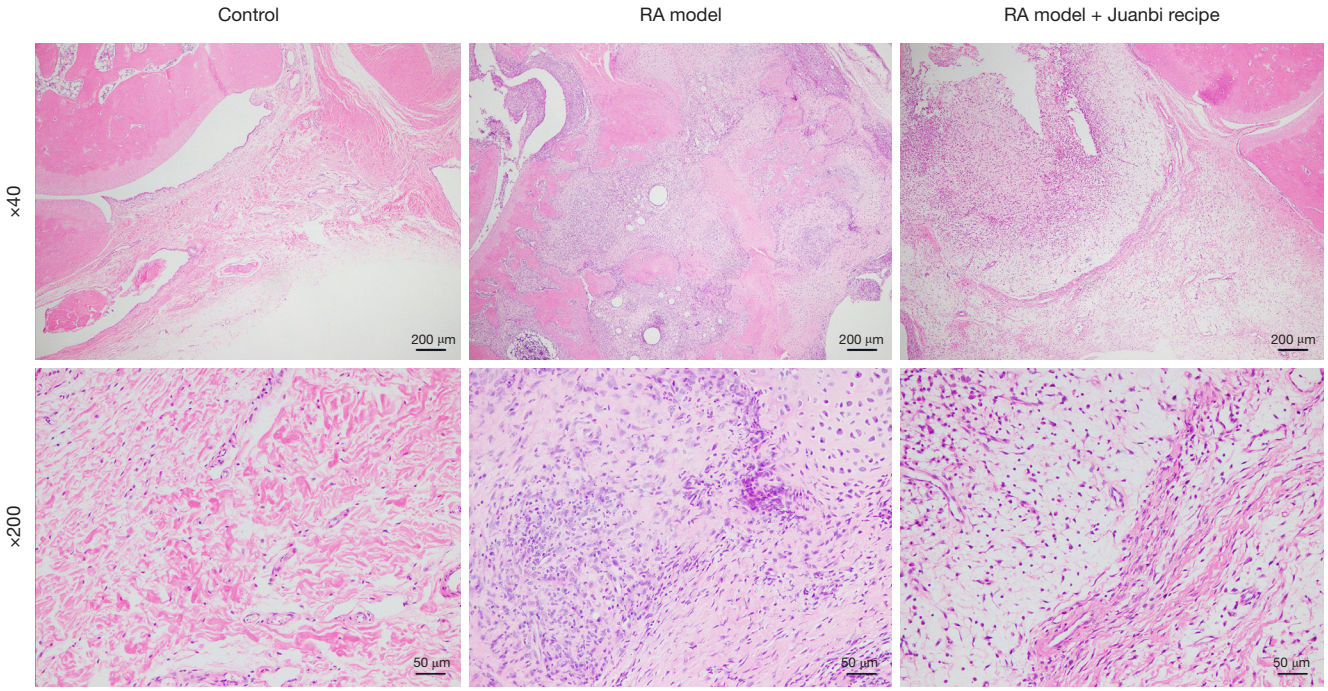


Figure 1 Hematoxylin and eosin staining of ankle joints. RA, rheumatoid arthritis.

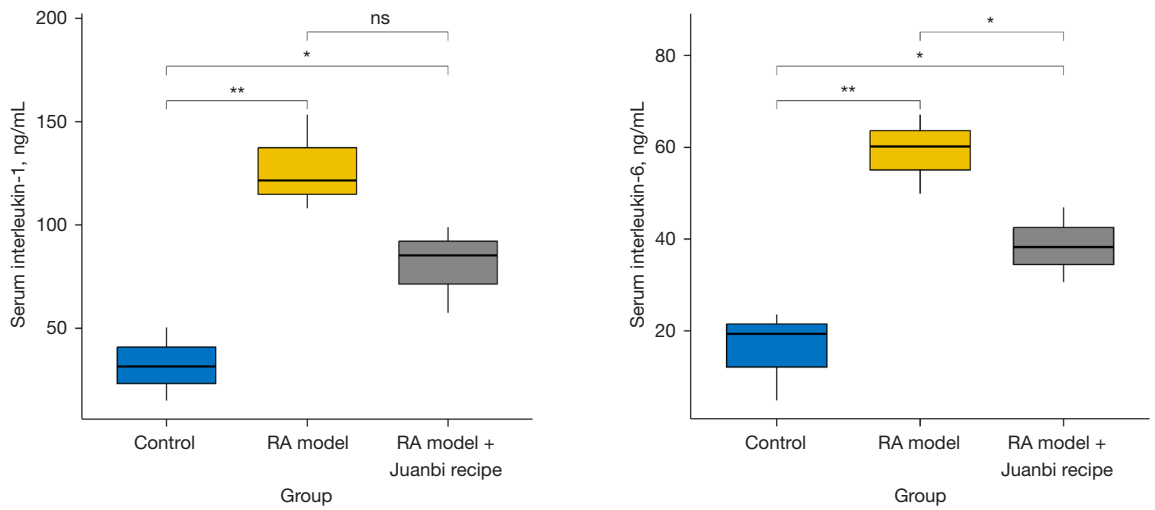


Figure 2 Serum interleukin-1 and -6 levels. *, P<0.05; **, P<0.01; ns, not significant. RA, rheumatoid arthritis.

components included vesicle lumen, cytoplasmic vesicle lumen, secretory granule lumen (*Figure 4B*). The top three items of molecular functions were cytokine receptor binding, cytokine activity, receptor ligand activity (*Figure 4C*). The top three items of KEGG were AGE-RAGE signaling pathway in diabetic complications, lipid and atherosclerosis, fluid shear stress and atherosclerosis (*Figure 4D*).

Obtaining six core target genes of Juanbi in RA treatment by using single cell RNA sequencing data

We used single cell RNA sequencing data of peripheral blood to analyze the PBMCs of patients with RA and normal controls, and divided the PBMCs into B cells, dendritic cells, monocytes, natural killer cells and T cells (*Table 1* and

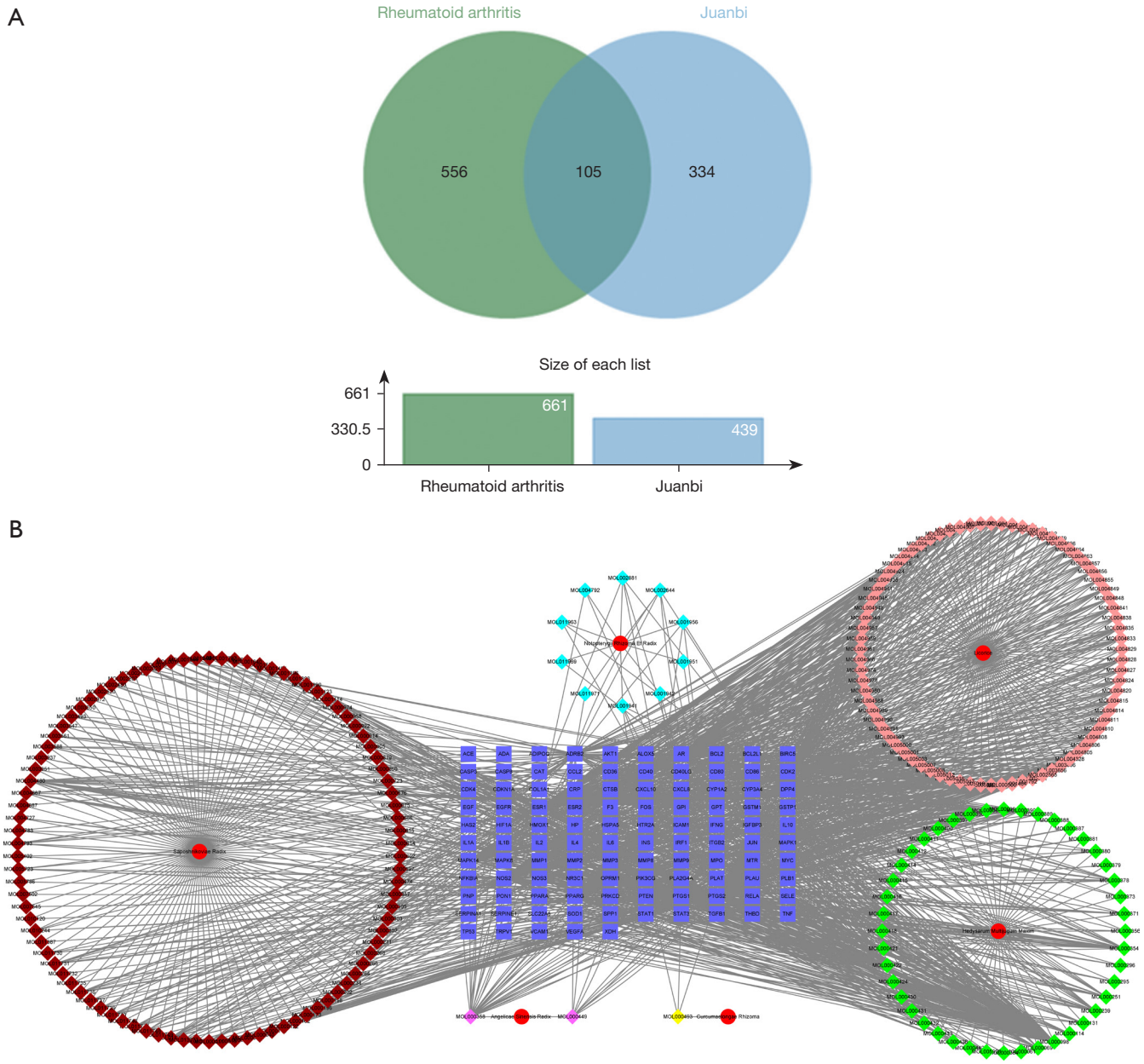
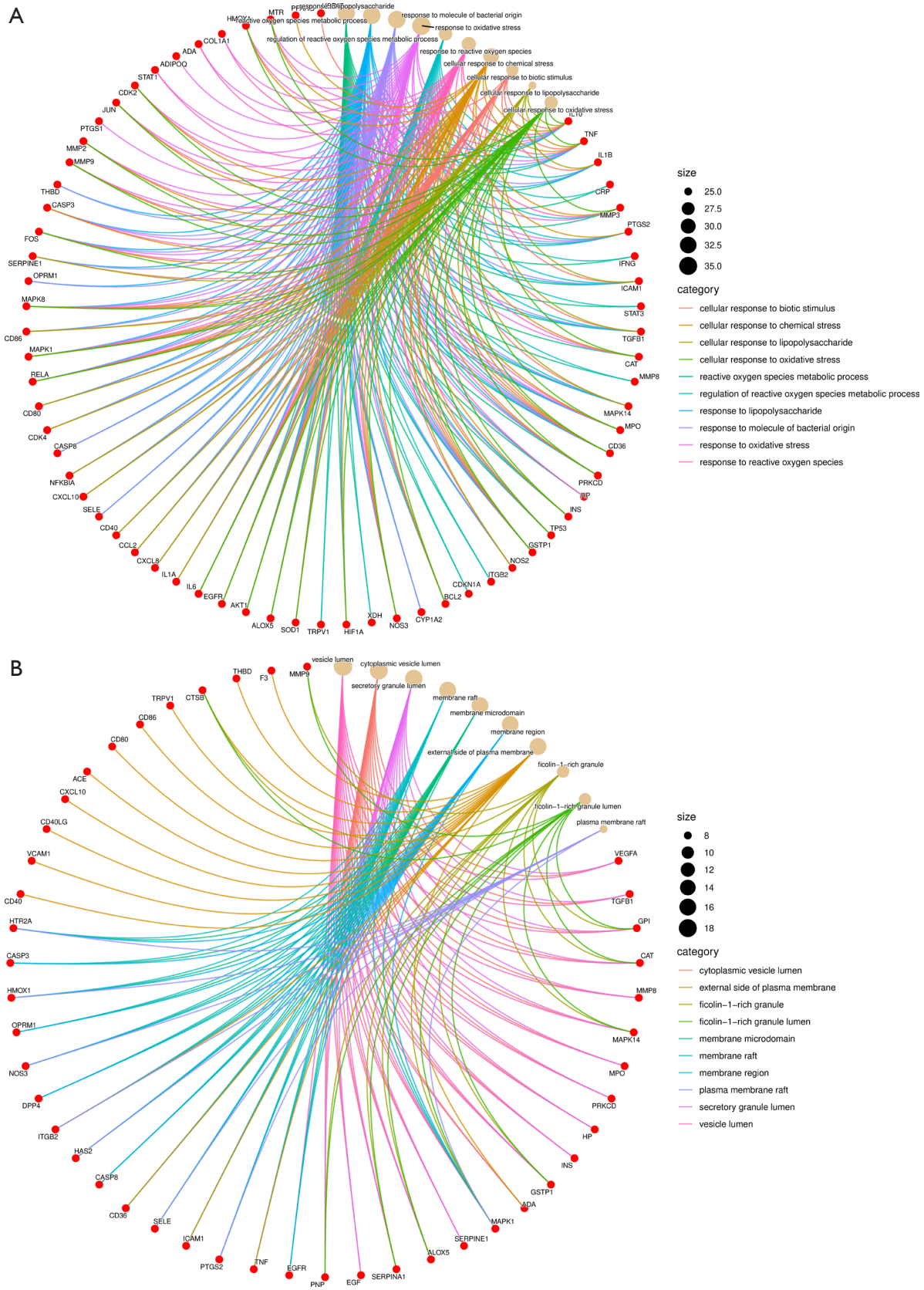


Figure 3 Key target genes of Juanbi recipe in treating rheumatoid arthritis. (A) There are 661 genes related to rheumatoid arthritis, 439 genes targeted by Juanbi recipe, and 105 key target genes of Juanbi recipe in the treatment of rheumatoid arthritis. (B) Diagram of "drug molecule target gene" of Juanbi recipe on the treatment of rheumatoid arthritis.

Figure 5A). Through differential analysis, 263 differential genes were found in PBMC between RA and normal controls (*Figure 5B*). We used single cell RNA sequencing data of synovium to analyze the synovial cells of patients between RA and osteoarthritis (control), and divided the cells into B cells, fibroblasts, monocytes, and T cells (*Table 2* and *Figure 5C*). Through differential analysis, we found 76 differential genes in synovium between RA and control (*Figure 5D*). We intersected the key target genes of Juanbi recipe in RA treatment with the differential genes from single cell sequencing data, and obtained 10 overlap genes, namely, Fos proto-oncogene (*FOS*), glutathione S-transferase P1 (*GSTP1*), interferon gamma (*IFNG*), Jun proto-oncogene (*JUN*), matrix metalloproteinases (*MMP*)-3, nuclear



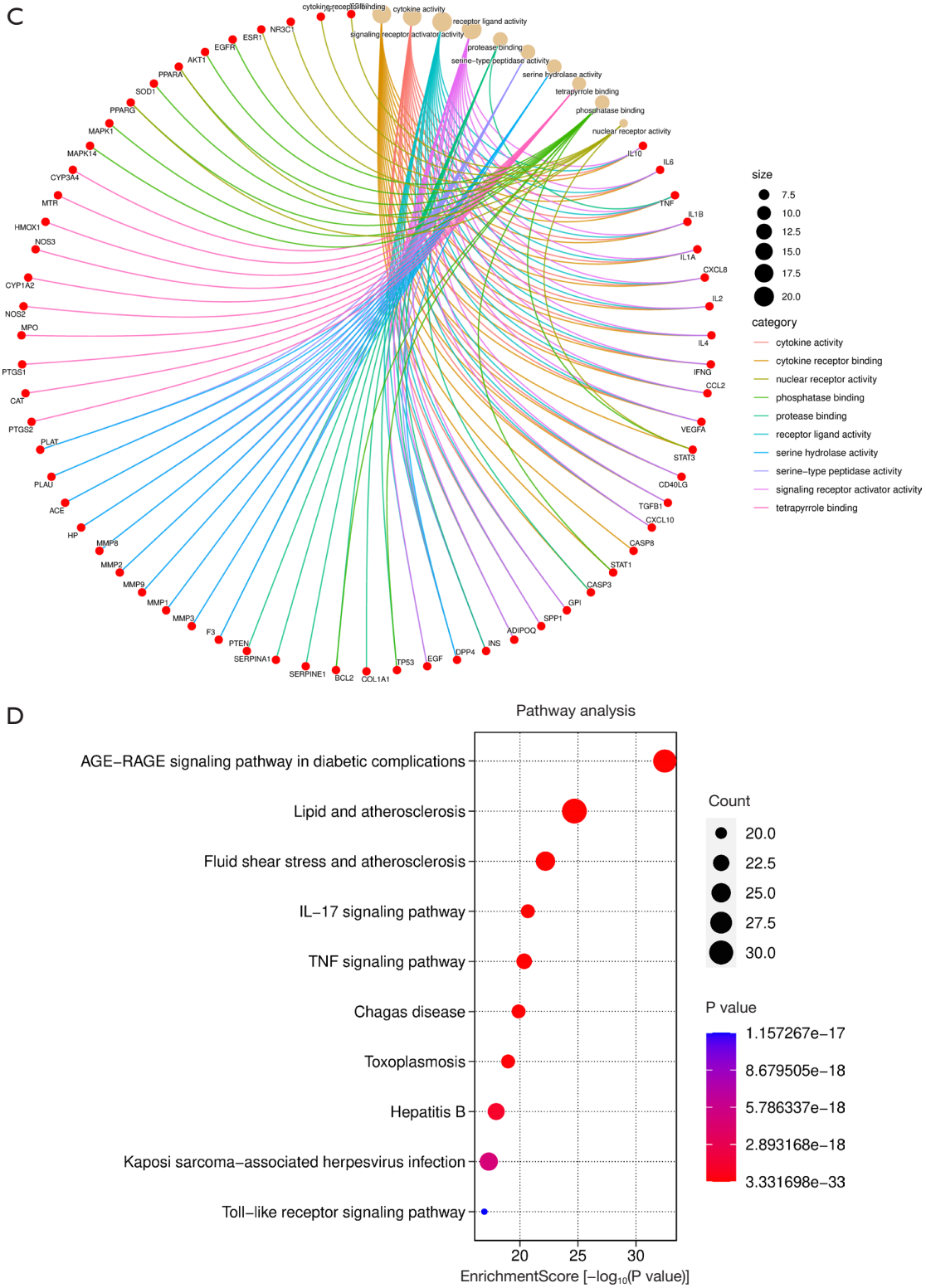
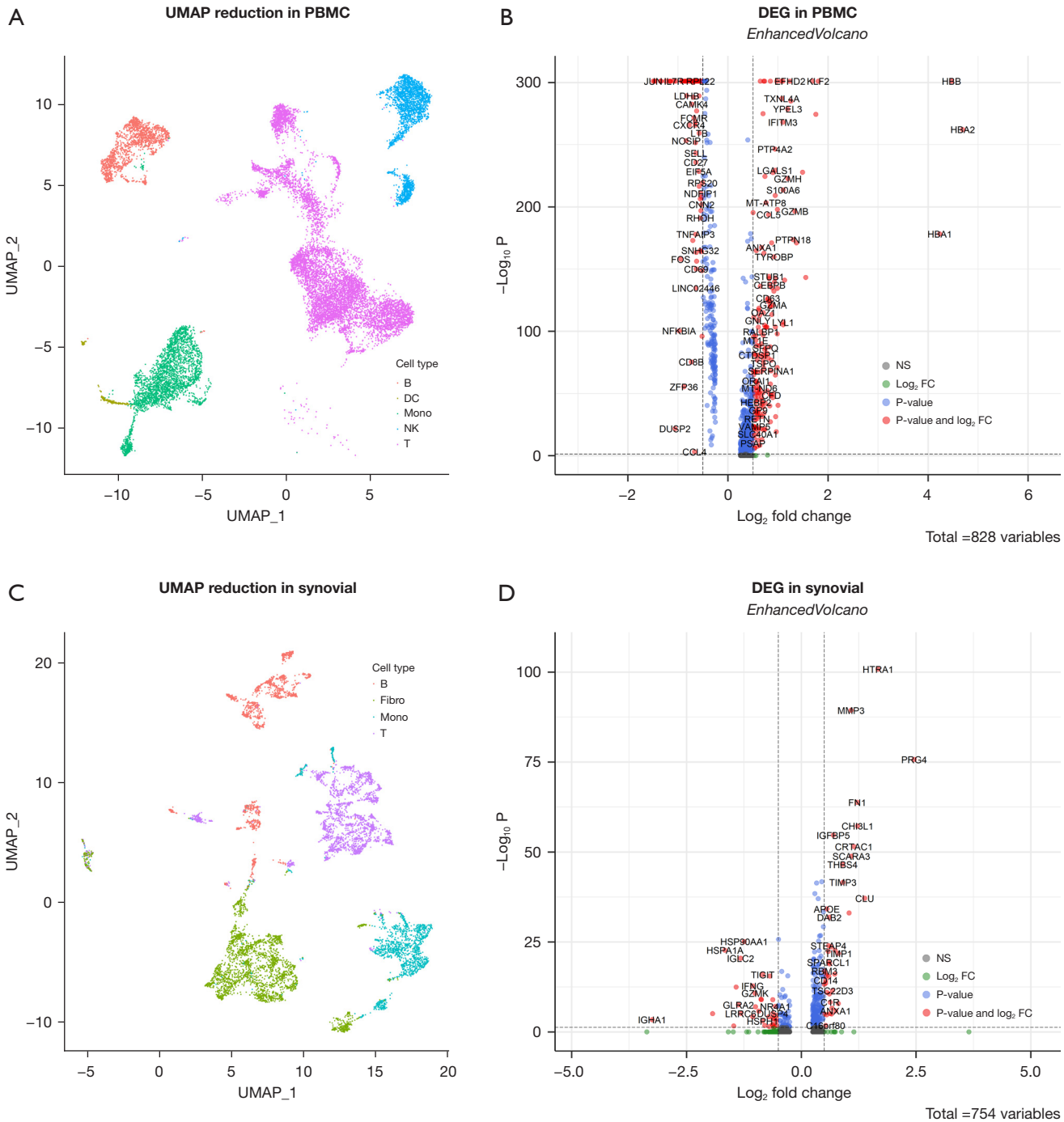


Figure 4 GO and KEGG analysis of key target genes of Juanbi recipe in RA treatment. GO analysis contained biological processes (A), cellular components (B), and molecular functions (C). (D) Pathways involved analyzed by KEGG. GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; RA, rheumatoid arthritis; IL-17, interleukin 17; TNF, tumour necrosis factor.

Table 1 Clustering of peripheral mononuclear blood cells from single cell RNA sequencing data

Groups	B cells	Dendritic cells	Monocytes	Natural killer cells	T cells
Normal control	1,112	97	1,153	1039	5881
Rheumatoid arthritis	696	127	2,192	1447	4258



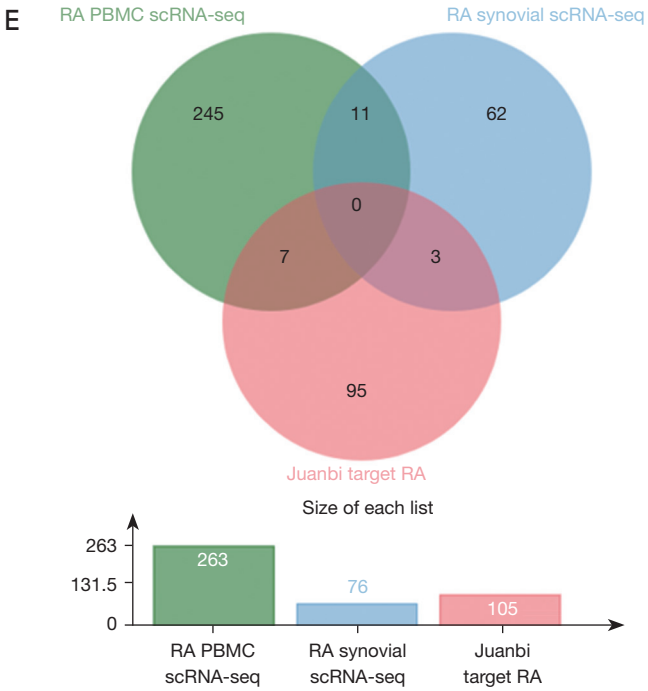


Figure 5 Clustering and differential analysis of PBMCs and synovium from single cell RNA sequencing data. PBMCs were classified into 5 groups of cells (A) and 263 differential genes were found (B). Synovial cells were classified into 4 groups of cells (C) and 76 differential genes were found (D). The key target genes of Juanbi recipe were intersected with differential genes from the two single cell RNA sequencing data (E). UMAP, uniform manifold approximation and projection; DEG, differential expression gene; FC, fold change; NS, not significant; RA, rheumatoid arthritis; scRNA-seq, single-cell RNA sequencing; PBMCs, peripheral blood mononuclear cells; DC, dendritic cells; NK, natural killer cells.

Table 2 Clustering of synovial cells from single cell RNA sequencing data

Groups	B cells	Fibroblasts	Monocytes	T cells
Osteoarthritis (control)	139	266	211	270
Rheumatoid arthritis	1,210	2,341	1314	1,963

factor-kappa-B-inhibitor alpha (*NFKBIA*), prostaglandin-endoperoxide synthase 1 (*PTGS1*), serpin family A member 1 (*SERPINA1*), secreted phosphoprotein (*SPP1*), and transforming growth factor beta (*TGFB*)-1 (*Figure 5E*).

In order to understand the expression of the 10 genes in each cell cluster, we analyzed the expression of these 10 genes in both PBMCs including B cells, dendritic cells, monocytes, natural killer cells, and T cells, and synovium including B cells, fibroblasts, monocytes, and T cells. The criteria for screening genes were genes expressed in all clusters in either PBMCs or synovium (*Table 2*). *FOS*, *JUN*, and *NFKBIA* were significantly expressed in all clusters of PBMCs and *IFNG*, *MMP3*, and *SPP1* were significantly expressed in all clusters of synovial cells (Figure S1). These genes were treated as core target genes Juanbi recipe in treating RA. By reviewing the diagram of "ingredient – molecule – gene" for Juanbi recipe in RA treatment, we found the core active molecules of Juanbi recipe in treating RA, including beta-sitosterol, daidzein, formononetin, kaempferol, quercetin, and wogonin.

Core active molecules of Juanbi recipe verified by molecular docking

We calculated the binding energy between the core active molecules and the core target proteins (*Table 3*). The

Table 3 The docking analysis of core active molecules and core target genes of Juanbi recipe in rheumatoid arthritis treatment

Molecule ID	Molecule name	Traditional Chinese medicine	Target gene	Binding energy
MOL000358	Beta-sitosterol	Notopterygii Rhizoma Et Radix	*JUN*	–5.24
MOL000390	Daidzein	Hedysarum Multijugum Maxim	*FOS*	–6.17
MOL000390	Daidzein	Hedysarum Multijugum Maxim	*JUN*	–4.42
MOL000392	Formononetin	Hedysarum Multijugum Maxim	*JUN*	–4.36
MOL000422	Kaempferol	Hedysarum Multijugum Maxim	*JUN*	–3.9
MOL000098	Quercetin	Hedysarum Multijugum Maxim	*MMP3*	–7.42
MOL000098	Quercetin	Hedysarum Multijugum Maxim	*NFKBIA*	–6.72
MOL000098	Quercetin	Hedysarum Multijugum Maxim	*FOS*	–6.04
MOL000098	Quercetin	Hedysarum Multijugum Maxim	*SPP1*	–5.73
MOL000098	Quercetin	Hedysarum Multijugum Maxim	*IFNG*	–4.58
MOL000098	Quercetin	Hedysarum Multijugum Maxim	*JUN*	–3.92
MOL000173	Wogonin	Saposhnikoviae Radix	*JUN*	–3.89

standard of a stable binding was the binding energy less than –5 kcal/mol. We visualized the conformations of the stable binding the core active molecules and the core target proteins (*Figure 6*).

Juanbi recipe inhibited the expression of AP-1 and NF-κB pathway in RA

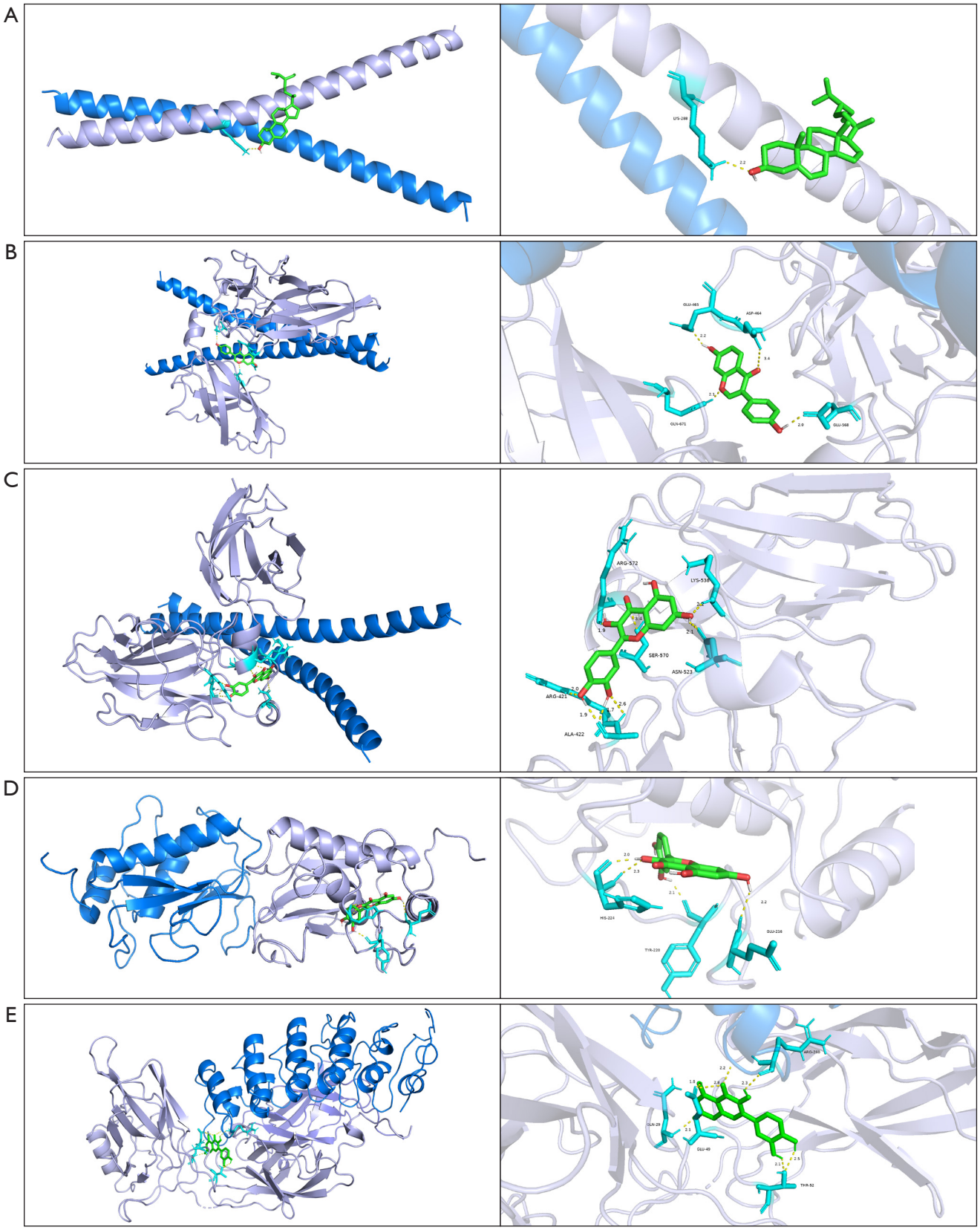
To investigate the expression of *AP-1* and *NF-κB* in ankle, we performed Western blot and IHC staining for both c-jun and P65. c-jun was the signature protein of AP-1 pathway, and p65 was the signature protein of *NF-κB* pathway. *c-jun* highly expressed in RA model while the expression was suppressed by the treatment of Juanbi recipe (*Figures 7,8*). p65 highly expressed in RA model while the expression was suppressed by the treatment of Juanbi recipe (*Figures 7,9*). These results indicated that Juanbi recipe inhibited the expression of *AP-1* and *NF-κB* pathway in RA.

Discussion

Our study predicted the key target genes of Juanbi recipe in RA treatment through the network pharmacology and disease database and presented the diagram of "ingredient – molecule – gene" for Juanbi recipe in RA treatment. The core target genes (*IFNG*, *MMP3*, *SPP1*, *FOS*, *JUN*, and *NFKBIA*) and core active molecules (beta-sitosterol, daidzein, formononetin, kaempferol, quercetin, and wogonin) were screened by single cell RNA sequencing data. The molecular docking technique was used to explore the mechanism of the core active molecules binding to the core target genes. The results of molecular docking showed that beta-sitosterol could stably bind to *JUN*, daidzein could stably bind to *FOS*, and quercetin could stably bind to *IFNG*, *MMP3*, and *SPP1*.

Compared with bulk RNA sequencing data, single cell RNA sequencing data provided not only overall differentially expressed genes but also the differentially expressed genes in each cell cluster, which was more advantageous. In this study, we found that *FOS*, *JUN* and *NFKBIA* genes were differentially expressed in all clusters of PBMCs including B cells, dendritic cells, monocytes, natural killer cells, and T cells, and that *IFNG*, *MMP3* and *SPP1* genes were differentially expressed in all clusters of synovial cells including B cells, fibroblasts, monocytes, and T cells. RA was caused by abnormal gene expression in variety kinds of cells. If a gene was abnormally expressed in more clusters of cells, the drugs targeting the gene probably had a better therapeutic effect. We believed that *FOS*, *JUN*, *NFKBIA*, *IFNG*, *MMP3* and *SPP1* were the core target genes of Juanbi recipe in RA treatment.

The core target gene of Juanbi recipe in RA treatment played an important regulatory role in the pathogenesis of RA. As reported, *AP-1* was an important regulatory protein, which was composed of *Fos* and *Jun*. It promoted the activation of MMP family, tumor necrosis factor



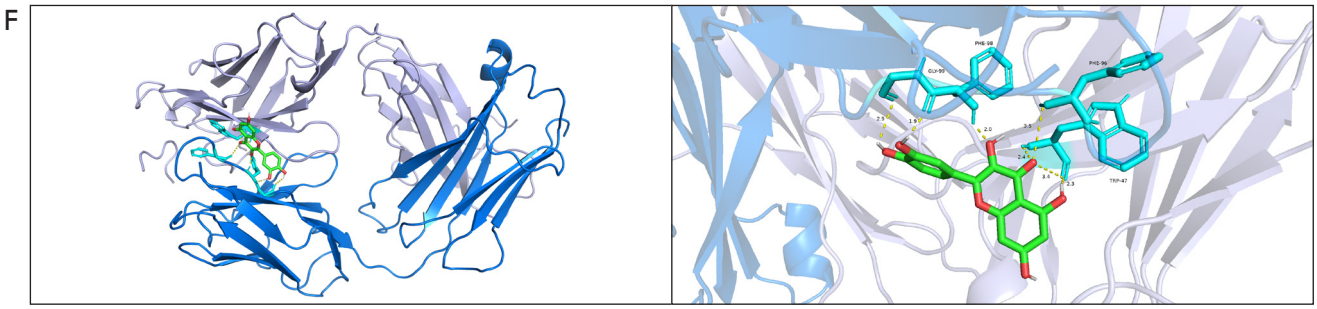


Figure 6 The conformations of the stable binding of the core active molecules and the core target proteins. (A) Beta-sitosterol binding to *JUN*; (B) daidzein binding to *FOS*; (C) quercetin binding to *FOS*; (D) quercetin binding to *MMP3*; (E) quercetin binding to *NFKBIA*; (F) quercetin binding to *SPP1*.

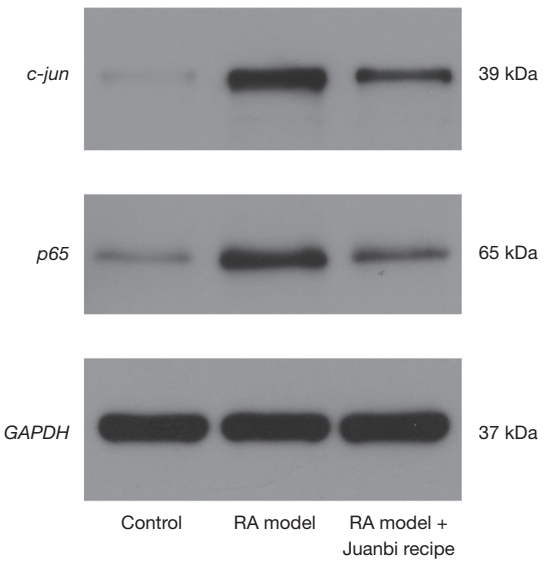


Figure 7 The expression of *c-jun* and *p65* in synovium examined by Western blot. RA, rheumatoid arthritis.

family and nuclear factor of activated T cells, leading to the expression of inflammatory factors in RA. *SPP-1*, also known as *osteopontin*, was an independent risk factor for RA. The MMP families play a role in the degradation and reconstruction of cartilage. *MMP-3* is an important biomarker in the early development of RA. JUN pathway promoted the metamorphosis of articular cartilage through *MMP-3* and played an important role in RA. *NF-κB* pathway was an important inflammatory pathway, which promotes the inflammatory response of RA through receptor activator of nuclear factor-κB ligand (*RANKL*) and C-X-C motif chemokine ligand (*CXCL*) 10 pathways (25). On the contrary, *NFKBIA* was an inhibitor of *NF-κB* pathway, and the variation and abnormal expression of NFKBIA lead to the occurrence of RA (25). *IFNG* was the gene encoding interferon-gamma. Interferons played an important role in RA and the abnormal level of interferon-gamma in the peripheral blood indicated the risk of RA. Interferon-gamma also presented the signal of Toll-like receptor to Jun pathway inducing abnormal immune response. The level of interferon in RA patients affects the efficacy of anti-TNF-alpha biological agents (26).

The core active molecules of Juanbi recipe including beta-sitosterol, daidzein and quercetin, stably combined with the core target gene of Juanbi recipe in RA treatment. Previous research reported that quercetin combined to *AP-1* to inhibit its activity (27), inhibited the expression of downstream *TNF-alpha* pathway, and thus ameliorated inflammation. Quercetin also inhibited the activity of MMP families to protect cartilage. At present, no studies have reported quercetin relieving RA through *SPP-1*. The results of this study showed that quercetin had the ability to combine to osteopontin and probably was a potential target in RA treatment. Beta-sitosterol inhibited the expression of *JUN* through *JNK* pathway, thus inhibiting the expression of inflammatory factors (28). Daidzein was one kind of soybean isoflavones with antioxidant and anti-inflammatory effects, which reduced the secretion of inflammatory factors such as *TNF-alpha* in RA. The mechanism of daidzein alleviating RA is still not clear. This study predicted that daidzein probably combined with FOS, which was a potential target for the RA treatment.

Our study found that the core active molecules in Juanbi recipe, beta-sitosterol and daidzein, and quercetin, stably combined to the core target genes *FOS*, *JUN*, *NFKBIA*, *IFNG*, *MMP3* and *SPP1*, which played a key role in

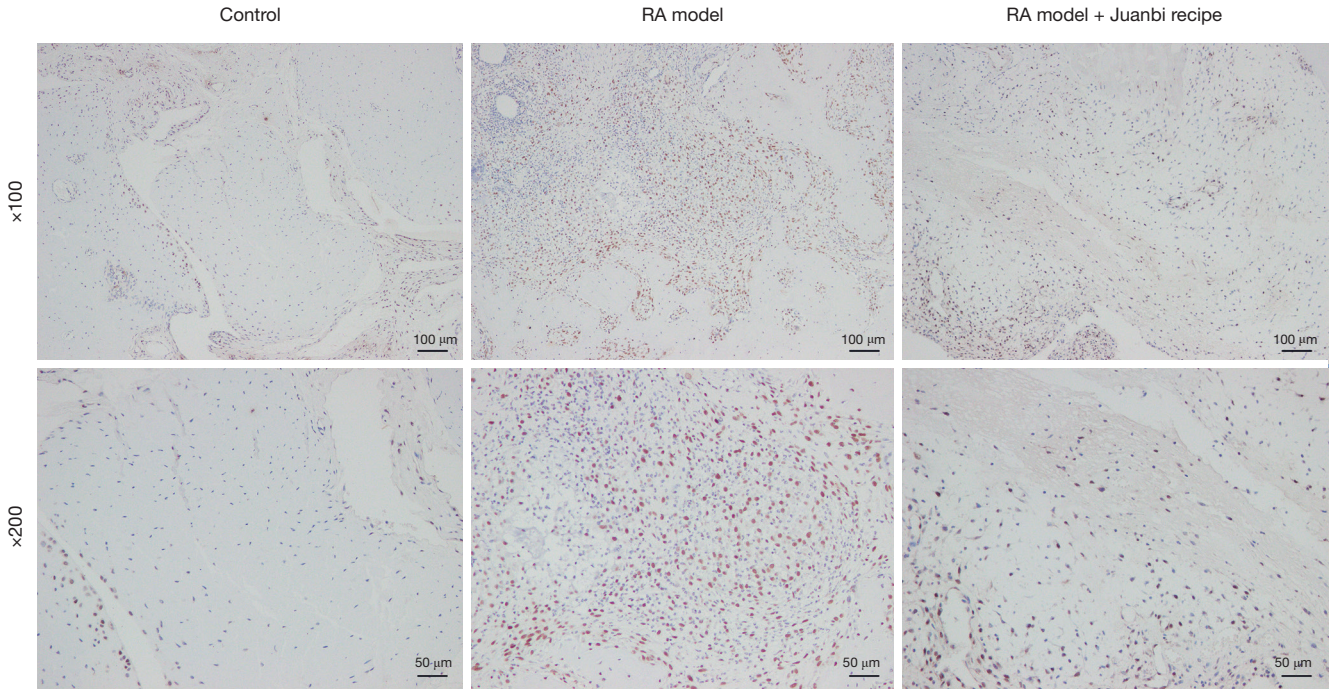


Figure 8 The expression of *c-jun* in ankle joint examined by immunohistochemical staining. RA, rheumatoid arthritis.

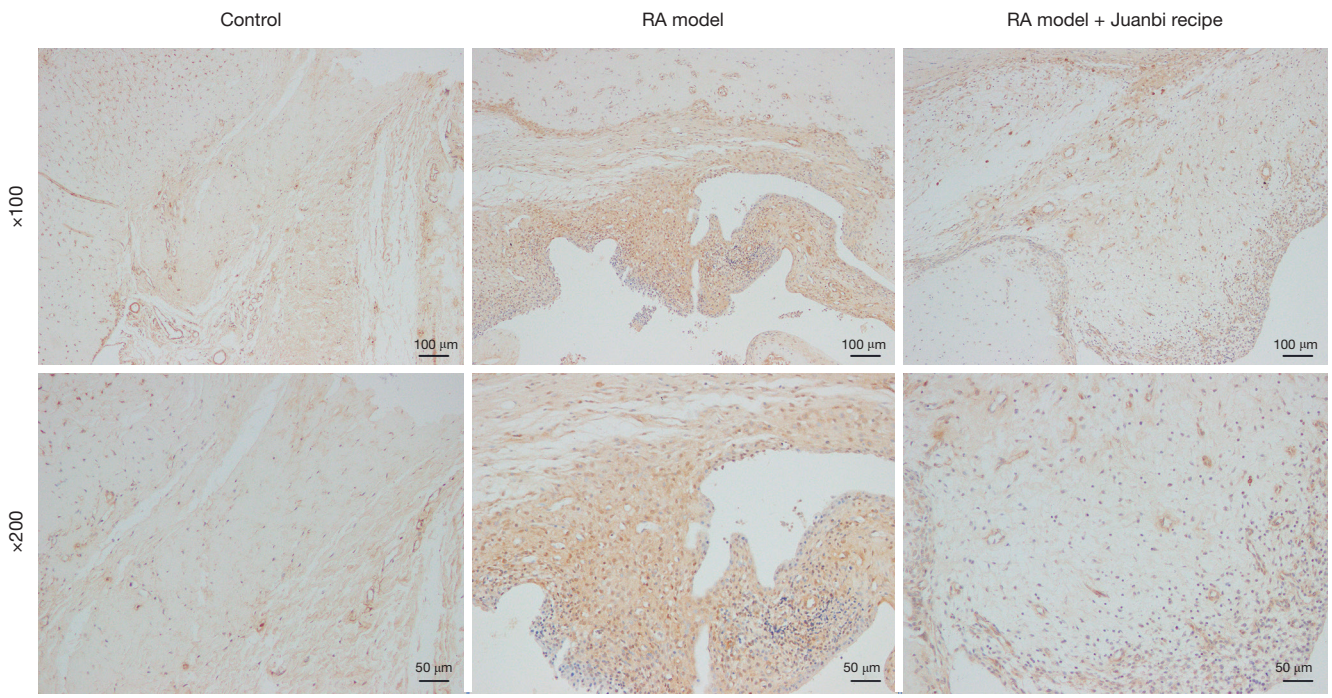


Figure 9 The expression of *p65* in ankle joint examined by immunohistochemical staining. RA, rheumatoid arthritis.

the treatment of RA. Several studies have invested the mechanism of Juanbi recipe on animal models. Li *et al.* used quadrupole time-of-flight mass spectrometry to discover that Juanbi recipe could fight against RA through the *MAPK* pathway (9). Wang *et al.* and Liu *et al.* both reported on animal models that Juanbi recipe had a protective effect on RA by reducing the expression of *NF-κB* pathway (8,10). Huang *et al.* reported aryl hydrocarbon receptor pathway inhibited inflammation of RA through *NF-κB* pathway (29).

There are some limitations in this study. Firstly, we did not evaluate the function of core active molecules (beta-sitosterol, daidzein and quercetin) in treating RA. Secondly, we did not perform metabolite analysis of Juanbi recipe in RA, which is very important for the study of TCMs. We will focus on the metabolism of Juanbi recipe and the role of core active molecules in treating RA in our further study.

Conclusions

This study used network pharmacology, single cell RNA sequencing data and molecular docking analysis to show that the core active molecules of Juanbi recipe could inhibit key factors of *AP-1* and *NF-κB* pathway to inhibit the inflammation, which play a protective role in RA.

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Footnote

Data Sharing Statement: Available at https://aoj.amegroups.com/article/view/10.21037/aoj-23-72/dss

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Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at https://aoj.amegroups.com/article/view/10.21037/aoj-23-72/coif). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. Experiments were performed under a project license (No. 3353) granted by Institutional Animal Care and Use Committee of Huazhong University of Science and Technology, in compliance with the guiding principles for the care and use of laboratory animals approved by the Animal Regulations of National Science and Technology Committee of China.

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