

Integrated Network Pharmacology and Experimental Validation Approach to Investigate the Mechanisms of *Stigmasterol* in the Treatment of Rheumatoid Arthritis

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Background: Rheumatoid arthritis (RA) is a chronic inflammatory disease of the joints associated with systemic comorbidities. *Sinomenium acutum* is regarded as an effective traditional Chinese medicine (TCM) for the treatment of RA.

Materials and Methods: Based on network pharmacology and Gene Expression Omnibus (GEO) database, 33 RA-related differentially-expressed genes (DEGs) targeting active compounds of *Sinomenium acutum* were initially screened in our investigation.

Results: Gene Ontology (GO) and Kyoto encyclopaedia of genes and genome (KEGG) analyses found the important involvement of these DEGs in osteoclast differentiation, and finally 5 core DEGs, including NCF4, NFKB1, CYBA, IL-1 β and NCF1 were determined through protein-protein interaction (PPI) network. We also identified the related active component of *Sinomenium acutum* include *Stigmasterol*. Finally, in order to experimentally verify these results, a rat model of collagen-induced arthritis (CIA) was established, and subsequently treated with *Stigmasterol* solution.

Conclusion: Similar to the healing effect of Indomethacin, *Stigmasterol* was observed to reduce the levels of inflammatory factors (IL-6 and IL-1 β) and osteoclast differentiation-related factors (RANKL, ACP5 and Cathepsin K), which can also reduce the arthritis index score and alleviate the degree of pathological injury of rat ankle joints. The predictions and experimental data uncover the involvement of *Stigmasterol*, an active component of *Sinomenium acutum*, in regulation of osteoclast differentiation, exerting great medicinal potential in the treatment of RA.

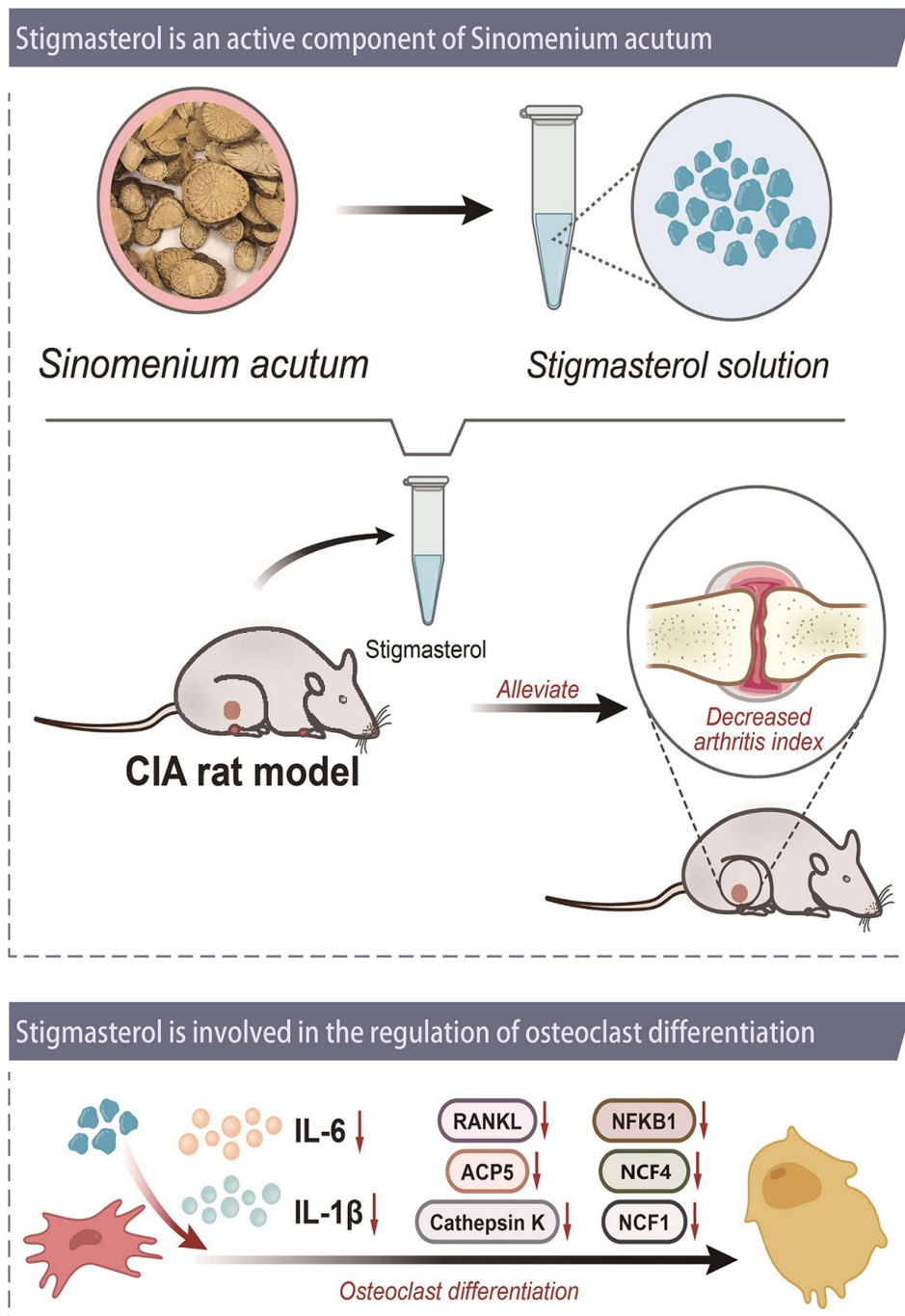
Keywords: rheumatoid arthritis, *Sinomenium acutum*, *stigmasterol*, network pharmacology, osteoclasts

Introduction

Rheumatoid arthritis (RA) is a systemic autoimmune disease that is mainly aggressive, multiple and chronic in nature.^{1,2} The prevalence of RA is 0.5–1% worldwide, with women suffering from the disease approximately four times more often than men.³ The main pathological manifestations of RA are chronic inflammation of the synovial membrane, formation of vascular opacities, and persistent inflammation that damages articular cartilage, ligaments and tendons, resulting in joint deformity and loss of function.^{4,5} These complications can be accompanied by systemic symptoms such as weight loss, hypothermia and fatigue.⁶ These complications seriously affect the work, life and studies of RA patients and reduce the social workforce.

The specific causes and pathogenesis of RA are still unclear, the pathogenesis is complex and involves many factors, and there is still a lack of a systematic theory that can fully explain its pathogenesis. According to available research, the development of RA involves at least genetics, gender, smoking, environment, infections, alcohol consumption, obesity, low educational level and even menopause, and often not just a single factor, but different factors often interact with each other.^{7–9} In terms of pathological mechanisms, osteoclasts are involved in the pathological process of bone destruction in

Graphical Abstract



RA. Osteoclasts are the major mediators of bone destruction, which are regarded as the main cell type of bone destruction in RA under pathological state.^{10,11} In the process of bone resorption and metabolism, osteoclasts are mainly responsible for bone resorption and osteoblasts for bone formation. It has been known that normal bone mass can only be maintained when bone resorption and bone formation are in relative balance in the body.¹² During the development of RA, pro-inflammatory cytokines secreted by synovial fibroblasts can activate the RANKL pathway (Receptor Activator of Nuclear Factor-kappa B Ligand, RANKL), promoting ACP5 and Cathepsin K expression levels and consequently

activating osteoclasts and promoting excessive osteoclast proliferation, leading to an imbalance between bone resorption and osteogenesis, ultimately resulting in bone loss and bone damage.^{13–15} In addition to activation of the RANKL pathway system, anti-CCP antibodies are another risk factor strongly associated with bone erosion, and numerous studies have shown significant specific associations with serum markers of RA osteoclast-mediated bone resorption.^{16,17} The mechanisms of bone destruction in RA are complex and not yet fully understood. However, there is great potential for research into the treatment of RA by adjusting the balance between bone resorption and bone formation.

In the last 30 years, there has been an increasing number of treatments for RA, but there is no complete cure for RA and it has a number of unwanted side effects. For example, Nonsteroidal anti-inflammatory drugs (NSAIDs) achieve analgesic and anti-inflammatory effects by inhibiting cyclooxygenase to reduce the metabolism of prostaglandins. Celecoxib, meloxicam and etoricoxib are commonly used in clinic, and their side effects include adverse reactions to gastrointestinal irritation, cardiovascular system and urinary system.^{18,19} Slow-acting anti-rheumatic drugs (SAARDs) are anti-rheumatic drugs that have a certain control effect on RA, but they work relatively slowly, taking 1–6 months in the clinic. These drugs are toxic to some extent and prolonged use leads to serious adverse reactions in patients;²⁰ Glucocorticoids, which have a rapid onset of action and a significant anti-inflammatory and analgesic effect, are greatly limited in their use by the presence of numerous side effects such as gastrointestinal, osteoporosis and Cushing's syndrome. It is often used in combination with NSAIDs as a transitional bridge drug in the acute phase of RA, requiring reduction and withdrawal within 3–6 months. Due to their limitations, glucocorticoids are not mandatory in the treatment of RA.^{21–23} Adreno cortico hormones have a powerful anti-inflammatory effect and can rapidly relieve joint swelling and pain and systemic inflammation, but can only be used in small doses and for short courses.²⁴ Biologic DMARDs, such as tumour necrosis factor antibody antagonists such as ETANERCEPT and HUMIRA, interleukin-6 inhibitors such as ACTEMRA and JAK pathway inhibitors such as TOFACITINIB, have significant efficacy, but increase the risk of infection and are limited in use in patients with hepatitis B and tuberculosis, and are expensive and difficult for the average patient to support on an ongoing basis.²⁵ Therefore, there is an urgent need to find drugs that have fewer side effects and are more effective. In recent years, traditional Chinese medicines (TCMs) have attracted the attention of the international rheumatology community. The advantages of TCMs in the treatment of RA are mainly reflected in its effectiveness, safety and bi-directionality. TCMs is a natural plant, so there are few adverse effects. TCMs is a two-way treatment, as it can not only support the body's immune function, but also promote microcirculation, anti-inflammation and analgesia, thus achieving the goal of treating both the symptoms and the root cause.^{26,27} Therefore, research on the treatment of RA with TCMs is receiving increasing attention, and as research progresses, the anti-RA active ingredients contained in TCMs will show even broader application prospects and development values.

Sinomenium acutum, also called as *Qingfengteng* in Chinese, is a well-known herbal TCM for the treatment of RA.²⁸ It has been found that *Sinomenium acutum* has good curative effects on anti-inflammatory, sedation, analgesia, anti-hypertension, anti-arrhythmia and immunosuppression, and also play important roles of anti-inflammatory and prevention of bone destruction in RA treatment, so as to alleviate the process of this disease.²⁹ *Sinomenine* has been considered as the main active chemical component of *Sinomenium acutum* involved in the treatment of RA in some previous studies, which can regulate the secretion of multiple inflammatory cytokines and monocyte/macrophage subsets, and inhibit the progression of RA.³⁰ However, with the more and more in-depth research of *Sinomenium acutum* in recent years, the function mechanism of *Sinomenium acutum* has been further explored. It has been reported that, in *Sinomenium acutum*, not only *Sinomenine* but also other similar compounds may play the roles of anti-inflammatory and anti-bone destruction.³¹

Stigmasterol is one of the active compounds of *Sinomenium acutum*, also called as bean steroid or sterol. *Stigmasterol* has strong physiological activity and surface activity, and also has anti-inflammatory, anti-oxidation, anti-cancer and cholesterol-lowering effects.³² Scientists evaluated the knee joint of rabbits with osteoarthritis (OA) by morphology, histology and other methods, and found that stigmasterol can significantly inhibit the expression of matrix metalloproteinases (MMPs) and inhibit the degradation of cartilage, indicating that *stigmasterol* has the potential to treat osteoarthritis.³³ In addition, *stigmasterol* is non-toxic, easy to purify, and has great clinical application value. However, due to the paucity of information about the effect of *stigmasterol* on RA bone balance in vivo, this study will deeply explore the pharmacological value of stigmasterol based on network pharmacology and in vivo experiments.

Network pharmacology is recognized as a new research mode to investigate the complex network relationship between multi-targets and multiple-diseases on the basis of the database information of genes, proteins, diseases and drugs.^{34,35} In the current research, we plan to initially predict the key active compounds and potential targets of *Sinomenium acutum* in the treatment of RA through the network pharmacology website and Gene Expression Omnibus (GEO) database. Then, we will further explore the regulation mechanism of the regulatory functional genes related to *Sinomenium acutum* in RA. Finally, these investigational findings will be subsequently validated by constructing a rat model of RA, so as to provide experimental data and theoretical basis for the further exploration of the active compounds and targets of *Sinomenium acutum* in the treatment of RA. The study analysis process is presented in Figure 1.

Materials and Methods

Active Compounds and Targets of *Sinomenium Acutum*

Bioinformatics Analysis Tool for Molecular mechanism of TCM (BATMAN-TCM) (<http://bionet.ncpsb.org/batman-tcm/>) was applied to screen the active compounds of *Sinomenium acutum* with Score cut-off ≥ 20 and the corresponding target genes by using “Qingfengteng” as the key word.

Gene Expression Omnibus (GEO) Data Analysis

RA-related gene expression profile datasets (GSE10500, GSE29746, GSE56649, GSE77289, and GSE97779) were obtained from the GEO database (<https://www.ncbi.nlm.nih.gov/gds/>), which were collected from the joint tissues of RA patients and normal joint tissues.

Subsequently, differential expression analysis was conducted by the “limma” package in R language (<https://www.ncbi.nlm.nih.gov/gds/>) to screen differentially-expressed mRNAs, and p value is calculated by empirical Bayesian method, with $|\log \text{ fold change (FC)}| > 1$ and $p < 0.05$ as cut-off criteria. Then, volcano plot and heat

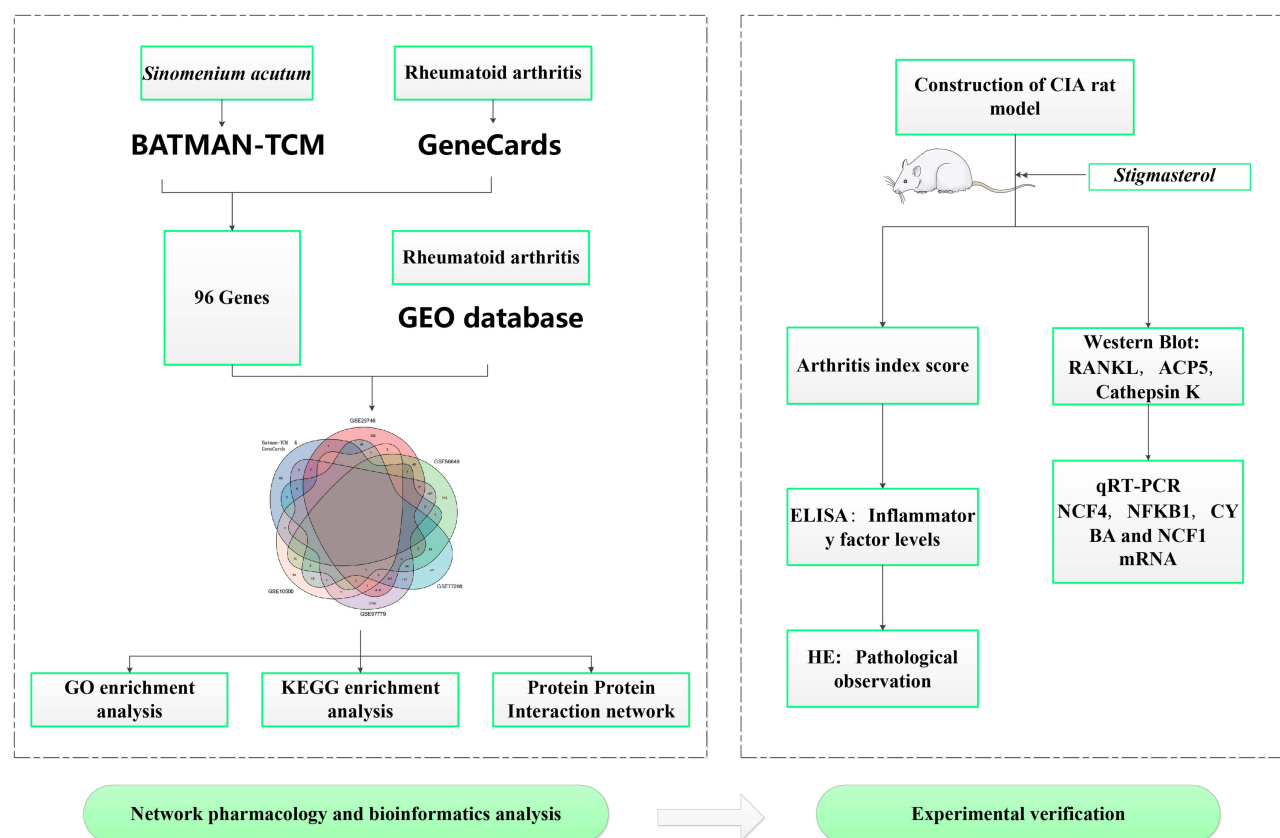


Figure 1 Flow chart of the study analysis process.

map of differential gene expression were drawn by the “ggplot2” package and “heatmap” package of R language, respectively.

Screening of Intersection Genes

Draw Venn Diagram tool (<http://bioinformatics.psb.ugent.be/webtools/Venn/>) was performed to take an intersection of GEO databases, RA-related genes, and inflammation-related genes to obtain the candidate differentially-expressed genes (DEGs).

Functional Enrichment Analysis

The functional enrichment analysis of mRNAs was employed by “ClusterProfiler” package in R language. Fisher test was conducted to identify the significantly enriched Gene Ontology (GO) terms and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways, with $p < 0.05$ considered as statistically significant.

Screening of Osteoclasts-Related Genes

GeneCards (<https://www.genecards.org/>) was conducted to screen the genes related to “Rheumatoid arthritis” and “Osteoclasts”. A PPI network of human species of these screened genes was constructed using STRING database (<https://string-db.org/>), and the network diagram was drawn by Cytoscape software (v3.6.0).

Establishment of Collagen-Induced Arthritis (CIA) Rat Model

Sixty male Sprague Dawley (SD) rats (aging 6–8 weeks, weighing 150–170g) were commercially purchased from the Hunan SJA Laboratory Animal Co., Ltd (Changsha, Hunan, China) (License No.: SCXK (Hunan) 2020–0010). The rats were grouped into 6 groups (10 rats per group), including normal (NOR) group, model (MOD) group, Indomethacin (IND) group, *Stigmasterol*-Low (STG-L) group, *Stigmasterol*-Middle (STG-M) group, and *Stigmasterol*-High (STG-H) group. All rats were housed under specific pathogen-free (SPF) conditions in the First Affiliated Hospital of Hunan University of Traditional Chinese Medicine, and acclimated for 3 days. The experimental processes are shown in the flowchart Figure 2.

For CIA rat model establishment, 2 mg/mL of bovine collagen II (CII; Chondrex, Redmond, WA, USA) were emulsified in equal amounts of incomplete Freund’s adjuvant (Difco Laboratories, Detroit, MI, USA) on ice to prepare 1 mg/mL of emulsion. Next, 200 μ L of emulsion were injected subcutaneously into the rats via the base of tail for the first immunization. After 7 days, another 200 μ L of emulsion were injected subcutaneously into the rats via the back of tail for the second immunization. The research was conducted in accordance with the US guidelines (NIH publication #85-23, revised in 1985) for laboratory animal use and care, all experiments involving animals were evaluated and approved by the Animal Ethics Committee of our unit (Approval No.: ZYFY20211016).

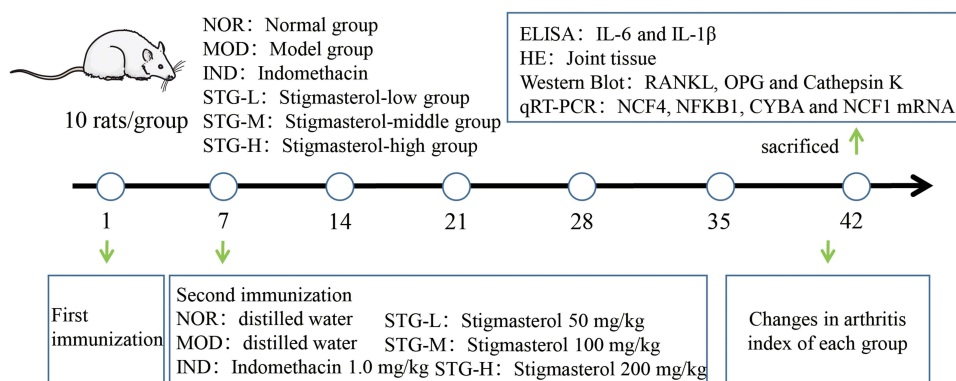


Figure 2 Flow chart of animal experiments.

Drug Administration

The rats were prophylactically administrated with the drugs by gavage once daily from the day of the second immunization once to the day of tissue collection. Specifically, the rats in the NOR and MOD groups were administrated with 2 mL of distilled water, while the rats in the IND, STG-L, STG-M and STG-H groups were administrated with 1.0 mg/kg of *Indomethacin* (#F17100; Shanxi Yunpeng Pharmaceutical Co., Ltd., Shanxi, China), 50 mg/kg, 100 mg/kg of and 200 mg/kg of *Stigmasterol* (#SS8710; Beijing Solarbio Science & Technology Co., Ltd., Beijing, China) for 21 days, respectively.

Evaluation of Arthritis Index (AI)

The AI of rats was evaluated by observing the pathological changes of rat joints in a double-blind manner. The score criteria were as follows: 0 = normal; 1 = erythema and slight swelling in the toe joint; 2 = swelling in the toe joint and toe; 3 = swelling in the paw below the ankle joint; 4 = swelling in the whole paw including the ankle joint. AI refers to the total score of four limbs of each rat (range from 0 to 16).³⁶

Hematoxylin-Eosin (H&E) Staining

After 42 days, the rats were anesthetized and euthanized by cervical dislocation. The joint from the ankle to the most swollen part was amputated from each rat, followed by 4% formaldehyde fixation for 30–50 min, wash it with water, dehydrate it, make it transparent, soak it with wax, embed it, and slice it. Flatten the tissue sections and paste them on the glass slides, dry them in a 45 °C incubator, dewax them, and then wash them with distilled water for 5 minutes through high to low concentration alcohol. Hematoxylin semen was stained for 5 minutes, then the slices were washed in running water for 3 seconds, differentiated in 1% hydrochloric acid ethanol for 3 seconds, and stained in 5% eosin solution for about 3 minutes, then dehydrated, transparent, and sealed. The histopathological changes were evaluated by observing the tissue sections under the microscope.

Enzyme Linked Immunosorbent Assay (ELISA)

The orbital blood was sampled from the rats after euthanasia. According to the manufacturer's instruction of ELISA kits (Shanghai Jingkang Bioengineering Co., Ltd, Shanghai, China), the samples and standards were incubated on 96 well plates coated with antibody capture overnight for 2h, and the detection antibody was added. After 2h, wash and incubate with streptavidin peroxidase for 1h. Wash the plate and add TMB solution (provided by the kit) at room temperature for 3 min. Add 2 N sulfuric acid to terminate the reaction, and measure the inflammatory factor IL-1 in the sample and standard through 490 nm absorbance β And IL-6 levels, the serum levels of inflammatory factors IL-1 β (Art.No.: JLC1704) and IL-6 (Art.No.: JLC1721) and were detected.

Western Blot Analysis

The tissues were lysed using radio-immunoprecipitation assay (RIPA) cell lysis buffer containing phenylmethanesulfonyl fluoride (PMSF) and incubated on ice for 30 min. Then, the cells were centrifuged at 12,000 g at 4°C for 10 min to extract the total protein, followed by protein concentration determination. Next, 50 μ g of protein were dissolved in 2 \times sodium dodecyl sulfate (SDS) loading buffer and boiled at 100°C for 5 min, and then were separated using 10% SDS-polyacrylamide gel electrophoresis (PAGE). After being transferred into the polyvinylidene fluoride (PVDF) membrane, the protein samples were sealed with 5% skimmed milk powder at room temperature for 1 h. Subsequently, the PVDF membrane was incubated with diluted primary antibodies against RANKL (2 μ g/mL, ab239607), ACP5 (1/1000, ab235448), and Cathepsin K (2 μ g/mL, ab239506), with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (1/1000, ab8245) as internal control. After overnight incubation at 4°C, the membrane was further probed with immunoglobulin G (IgG) secondary antibodies (1:2000; ab6721 and ab6789, Abcam) for 1 h. All above-mentioned antibodies were purchased from Abcam (Cambridge, UK). Afterwards, the membrane was visualized using enhanced chemiluminescence (ECL) liquid (ECL808-25; Biomiga, San Diego, CA, USA) at room temperature for 1 min. Finally, the

membrane was observed using X-ray film to analyze the gray values of the proteins. The relative protein expression was calculated as the gray value of target protein/the gray value of GAPDH. The experiments were repeated three times.

RNA Isolation and Quantification

Total RNA was extracted using TRIZOL (Invitrogen Inc., Carlsbad, CA, USA). Primers of NCF4, NFKB1, CYBA, and NCF1 were designed and synthesized by Shanghai Sangon Biological Engineering Technology and Services Co., Ltd. (Shanghai, China) ([Supplementary Table 1](#)). Total RNA was reversely transcribed into cDNA using PrimeScript RT kit (RR036A, Takara, Tokyo, Japan) as per the kit instruction. Reverse transcription quantitative polymerase chain reaction (RT-qPCR) experiments were conducted using SYBR® Premix Ex Taq™ II kit (RR820A, Takara) on an ABI7500 real-time fluorescent qPCR instrument (Applied Biosystems, Foster City, CA, USA). The final data were analyzed using $2^{-\Delta\Delta C_t}$ method by using GAPDH as the internal control. The experiments were repeated three times.

Statistical Analysis

All data in this study were analyzed using GraphPad Prism 8.0.1. The R software package used in this study includes “limma” package, “ggplot2” package, “heatmap” package and “ClusterProfiler” package. The measurement data are expressed as mean \pm standard deviation. First, D’Agostino&Pearson test is used for normality test and Bartlett’s test is used for homogeneity test of variance. The results show that the test conforms to normal distribution and the variance is homogeneous. Single factor analysis of variance or analysis of variance of repeated measurement data are used for comparison between multiple groups. Tukey’s test is used for comparison between two groups within the group. Data between groups at different time points are compared, repeated measurement ANOVA was used, and Bonferroni was used for post test. Empirical Bayesian method was used for difference analysis, and Fisher’s exact test was used for GO and KEGG enrichment analysis. A value of $p < 0.05$ was statistically significant.

Results

Screening of Active Compounds and Targets of *Sinomenium Acutum*

Fifteen active compounds of *Sinomenium acutum* were screened from the Batman-TCM website, with Score cut-off ≥ 20 , as listed in [Supplementary Table 2](#). Then, the target genes corresponding to the 15 active compounds were collected and imported into Cytoscape software, followed by construction of the regulation network of the active compounds and their targets ([Figure 3A](#)). Furthermore, 4792 genes related to RA were screened by GeneCards database, which are intersected with the target genes of the active compounds. Finally, 96 common genes were screened ([Figure 3B](#)).

Screening of Genes Related to the Occurrence of RA

Differential analysis of RA-related gene dataset GSE10500 was initially conducted, which screened 136 differential genes, including 73 poorly-expressed genes and 63 highly-expressed genes ([Figure 4A](#)). Then, 827 differential genes were screened in GSE29746 dataset, including 341 poorly-expressed genes and 486 highly-expressed genes ([Figure 4B](#)). In GSE56649 dataset, 1036 differential genes were screened, among which 469 were downregulated and 567 were upregulated ([Figure 4C](#)). Through differential analysis of GSE77289 dataset, 771 differential genes were screened, including 367 downregulated genes and 404 upregulated genes ([Figure 4D](#)). Finally, a total of 3146 differential genes were screened from GSE9779 dataset, which included 1463 genes with low expression and 1683 with high expression ([Figure 4E](#)).

Next, all these differential genes from GEO datasets and the 96 RA-related target genes of active compounds of *Sinomenium acutum* were subjected to Venn analysis, screening 33 RA-related DEGs targeting active compounds of *Sinomenium acutum* ([Figure 4F](#)). Our subsequent analysis was carried out on this basis.

GO Functional and KEGG Enrichment Analyses on the 33 Putative DEGs

The functional and enrichment analysis of the 33 putative targets of *Sinomenium acutum* active compounds closely related to RA were conducted by GO and KEGG. [Figure 5A–C](#) depict the results of GO functional analysis. In biological

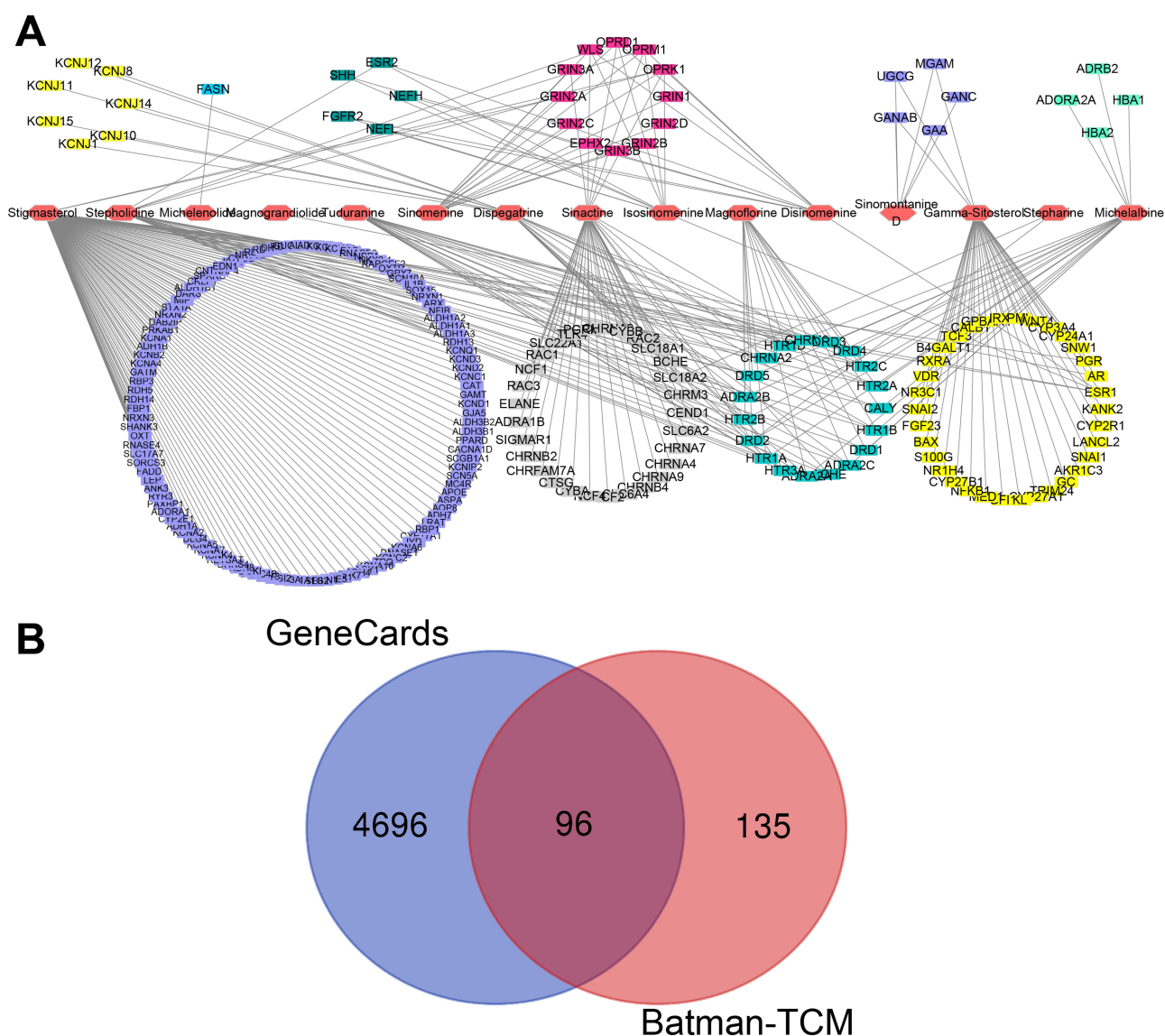


Figure 3 Screening of 15 active compounds and target genes of *Sinomenium acutum*. (A) The regulation network diagram of the 15 active compounds of *Sinomenium acutum* and their targets drawn by Cytoscape software. (B) Venn diagram of the target genes of the 15 active compounds and the screened genes in GeneCards database.

processes (BP), DEGs were found to be mainly enriched in vitamin D biosynthetic process, regulation of vitamin metal process, cellular calcium ion homeostasis, ossification, and calcium ion homeostasis (Figure 5A). In cell compositions (CC), DEGs are mainly enriched in endocytic vesicle, plasma membrane protein complex, cytoplasmic vesicle lumen, vesicle lumen, and glutamatergic synapse (Figure 5B). Besides, in molecular function (MF), it was found that DEGs are mainly enriched in steroid hormone receptor activity, steroid binding, neurotransmitter receptor activity, neurotransmitter binding, and nuclear receptor activity (Figure 5C). The above results suggest that DEGs contribute to the inflammatory response, vitamin D biosynthetic process and cellular calcium ion homeostasis, which are mainly enriched in mitochondria, cell membrane, and cell exosomes. Moreover, the molecular function of DEGs is mainly involved in the regulation of cell-related hormone activity and receptor activity.

As suggested by KEGG pathway enrichment analysis, DEGs were mainly enriched in osteoclast differentiation, p53 signaling pathway, MAPK signaling pathway, TNF signaling pathway, cAMP signaling pathway, and AMPK signaling pathway (Figure 5D). It indicates the involvement of DEGs in the regulation of multiple signal pathways (Supplementary Table 3). It is noteworthy that DEGs may participate in the regulation of osteoclast differentiation mechanism. Osteoclast

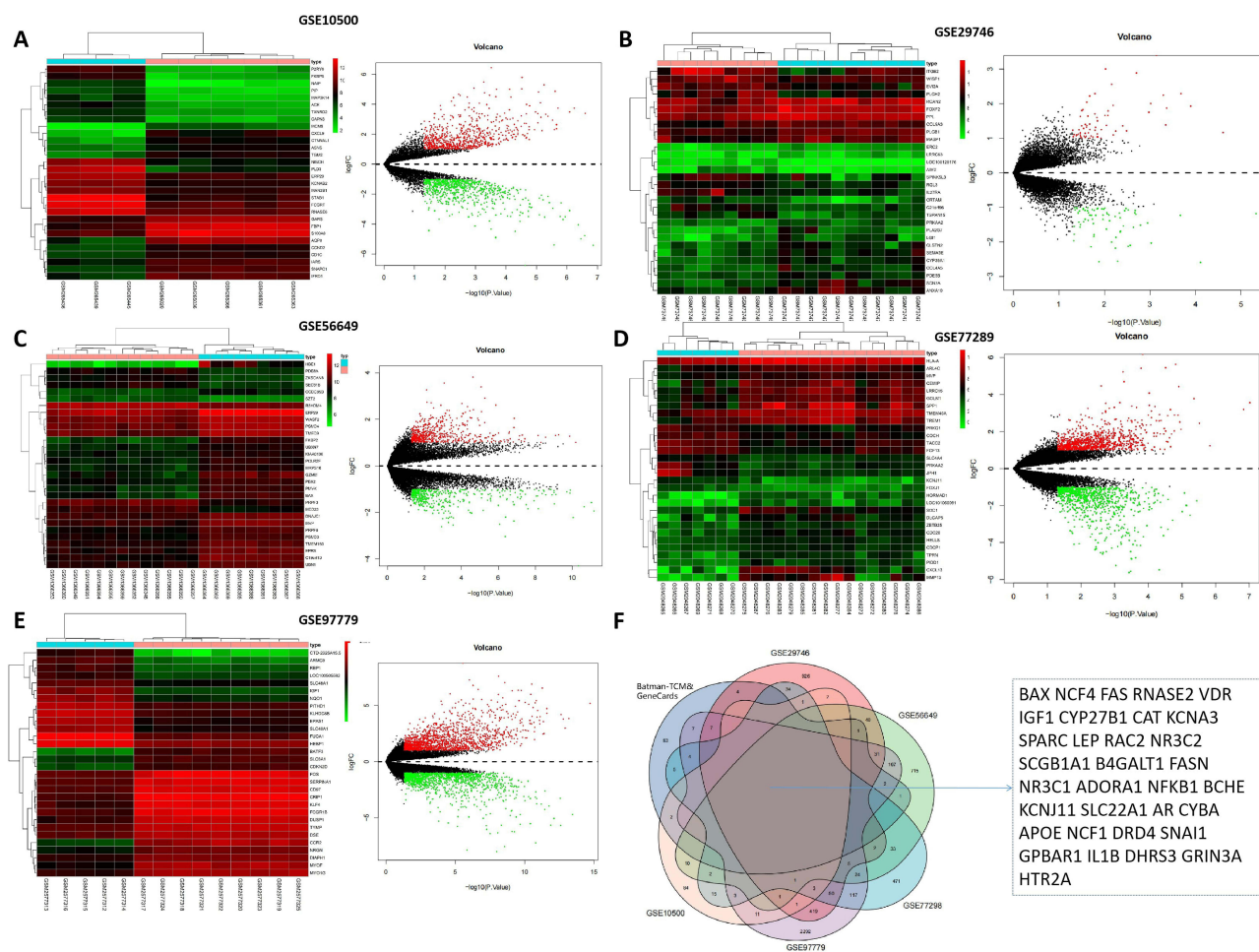


Figure 4 Screening of *Sinomenium acutum* active component target genes related to RA. (A) Heatmap and volcano map of differential analysis of GSE10500 dataset. (B) Heatmap and volcano map of differential analysis of GSE29746 dataset. (C) Heatmap and volcano map of differential analysis of GSE56649 dataset. (D) Heatmap and volcano map of differential analysis of GSE77289 dataset. (E) Heatmap and volcano map of differential analysis of GSE97779 dataset. In volcano maps, the green dots represent the downregulated genes, the red dots represent the upregulated genes, and the gray dots refer to genes with no significance difference. (F) Venn diagram of the GEGs related to RA in GEO datasets and the 96 RA-related target genes of *Sinomenium acutum* active compounds.

is the key link of bone erosion in RA, and its abnormal differentiation is realized through cell-cell interaction and regulation of multiple cytokines. It is of great significance to explore the involved mechanisms.

Screening of Core DEGs Involving in the Regulation of Osteoclast Differentiation in RA

Next, the PPI network of the 33 putative targets of *Sinomenium acutum* active compounds related to RA was constructed, as shown in Figure 6A. Combined with the results of KEGG analysis, the core genes of DEGs regulating osteoclast differentiation in RA were NCF4, NFKB1, CYBA, IL-1 β and NCF1. Then, based on the network pharmacology analysis, it was identified the target genes of the active compounds of *Sinomenium acutum*, including *Stigmasterol*, *Sinactine*, and *Gamma-Sitosterol*. *Stigmasterol* targets IL-1 β , *Sinactine* targets NCF4, NCF1, and CYBA, and *Gamma-Sitosterol* targets NFKB1 (Figure 6B). Next, we further screened the genes related to osteoclast differentiation through GeneCards database to screen the top 50 genes according to the relevance score. These genes were imported into String database together with NCF4, NFKB1, CYBA, IL-1 β and NCF1 to obtain the PPI relationship, followed by construction of the PPI network of RA osteoclast differentiation (Figure 6C). The degree value of the genes in the PPI network was subsequently analyzed, demonstrating that IL-1 β ranked first in the degree value of osteoclast differentiation-related core genes (Figure 6D). These findings suggest that NCF4, NFKB1, CYBA, IL-1 β and NCF1 are recognized as osteoclast differentiation-related genes.

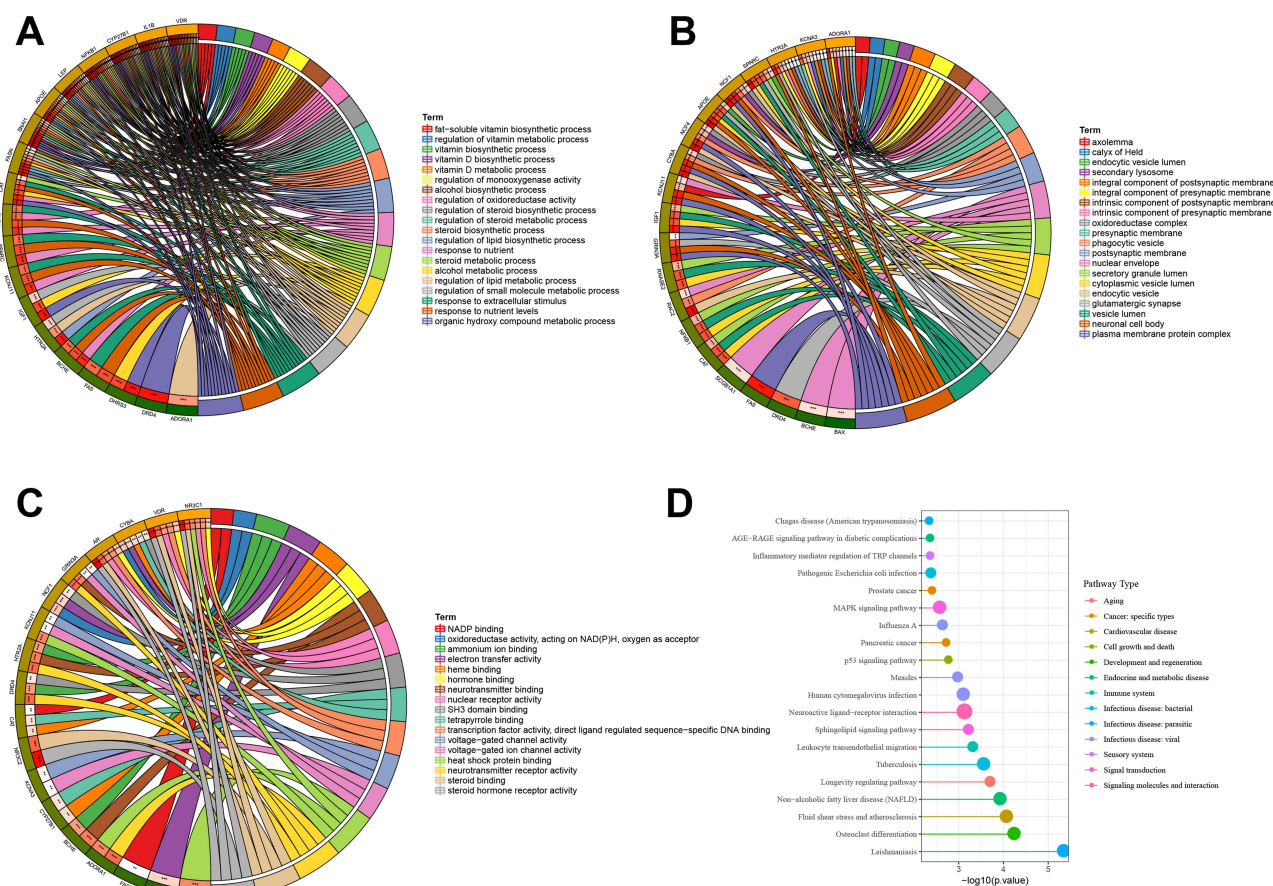


Figure 5 The possible involvement of DEGs in osteoclast differentiation. **(A)** GO functional analysis of DEGs in BP. **(B)** GO functional analysis of DEGs in CC. **(C)** GO functional analysis of DEGs in MF. **(D)** KEGG pathway enrichment analysis of DEGs; the node size represents the number of genes, and the node color refers to the p value of the enrichment analysis.

Stigmasterol Alleviates the Process of RA

As previously documented, Stigmasterol is capable of suppressing the expression of pro-inflammatory factors in RA, thus alleviating RA.³⁷ Here, we constructed a rat model for validating the effect of Stigmasterol on RA. The rats were visually observed during the process of CIA rats subjected to the treatment (**Figure 7A**), with AI scores recorded. It was observed that, relative to the rats in the MOD group, the AI score presented with a decreased trend in the IND and STG-H group, while the STG-L and STG-M group had no obvious decreased trend (**Figure 7B**, $P < 0.001$). In addition, as depicted in **Figure 7C**, H&E staining showed that the ankle joints and tissues of the NOR group were normal and complete. The joints of the rats in the MOD group were swollen, with a large number of fibrous tissues and proliferative synovial cells observed, and the bone tissues were obviously damaged. While, the joints were swollen in the rats in the IND and STG-H group, with narrow cavities, small number of fibrous tissues and proliferative synovial cells observed, and the bone tissues were relative slightly damaged. These experimental findings suggest that the treatment of *Stigmasterol* can alleviate the inflammatory reaction and pathological damage of RA rats.

Stigmasterol Inhibits the Osteoclast Differentiation in RA

We initially detected the mRNA expression of NCF4, NFKB1, CYBA, and NCF1 using RT-qPCR analysis. The mRNA expression of NFKB1 ($P < 0.001$), NCF4 ($P < 0.001$), and NCF1 ($P < 0.001$) was increased in the MOD group relative to the NOR group. Compared with MOD group, there was no significant difference in NFKB1, NCF4 and NCF1 mRNA between STG-L group and STG-M group, but decreased in STG-H group, with no significant change observed in the CYBA ($P = 0.099$) mRNA expression among the groups (**Figure 8A**). Then, ELISA was further employed to determine the

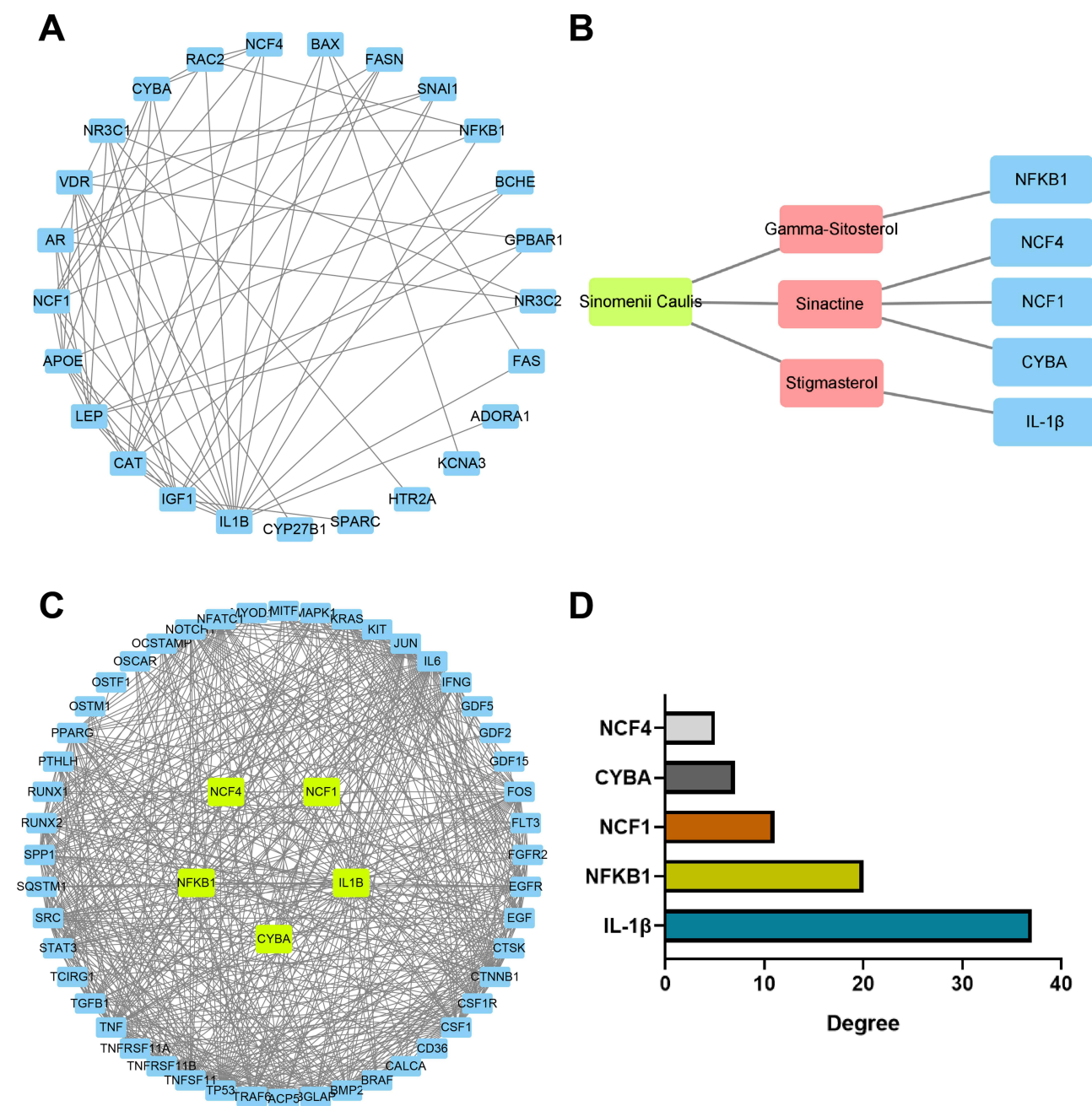


Figure 6 Screening of DEGs regulating osteoclast differentiation in RA. **(A)** PPI network of 33 putative target genes; **(B)** Network of *Sinomenium acutum*-active compounds-target genes. **(C)** PPI network of core genes (NCF4, NFKB1, CYBA, IL-1β, and NCF1) with RA osteoclast differentiation-related genes; **(D)** Degree value of the core genes (NCF4, NFKB1, CYBA, IL-1β, and NCF1).

levels of IL-6 and IL-1β, suggesting that high concentration of *Stigmasterol* treatment reduced the levels of IL-6 ($P<0.001$) and IL-1β ($P<0.001$) in CIA rats (Figure 8B). Next, the protein levels of osteoclast differentiation-related factors RANKL, ACP5, and Cathepsin K in tissues were evaluated by Western blot analysis. Relative to the NOR group, the tissues from the MOD group presented with higher protein levels of RANKL ($P<0.001$), ACP5 ($P<0.001$) and Cathepsin K ($P<0.001$), while high concentration of *Stigmasterol* treatment decreased these protein levels (Figure 8C and D). It highlights that the high concentration of *Stigmasterol* treatment can inhibit the inflammatory reaction and osteoclast differentiation in RA rats.

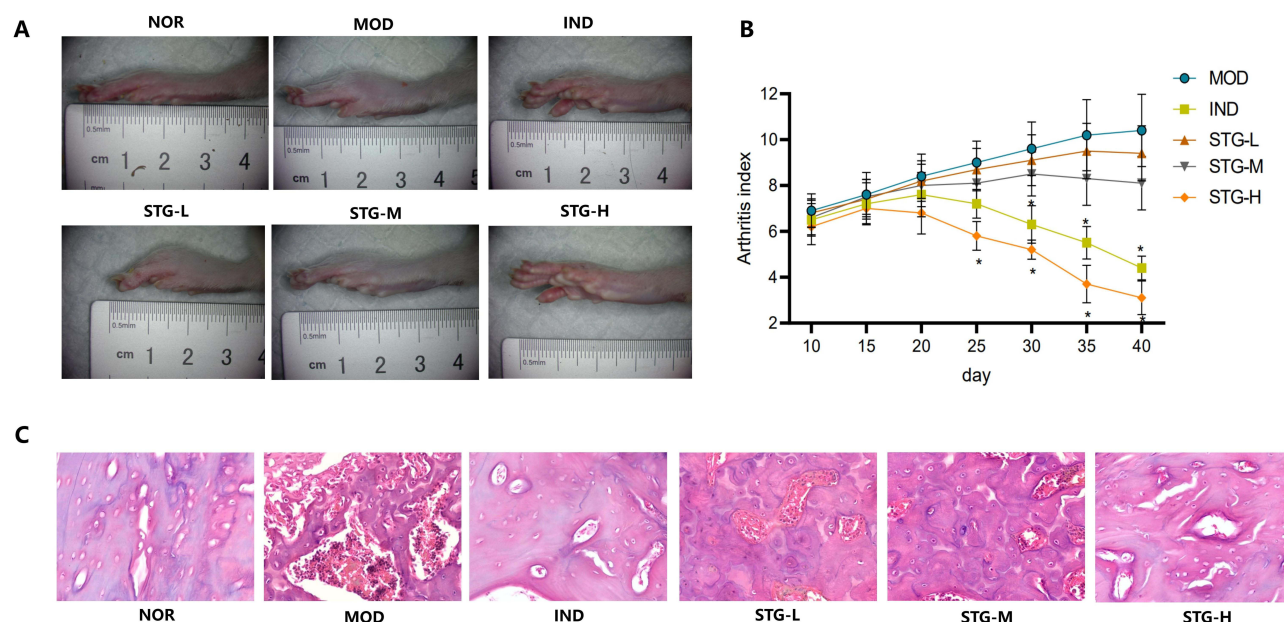


Figure 7 Effects of *Sinomenium acutum* active component on the pathological changes of RA rats. **(A)** Visual observation of the ankle joints of rats. **(B)** AI assessment of joints of rats ($n = 10$); * $p < 0.05$ as compared with the MOD group. **(C)** Pathological changes of ankle joints observed by H&E staining ($n = 10$) (scale bar = 25 μ m).

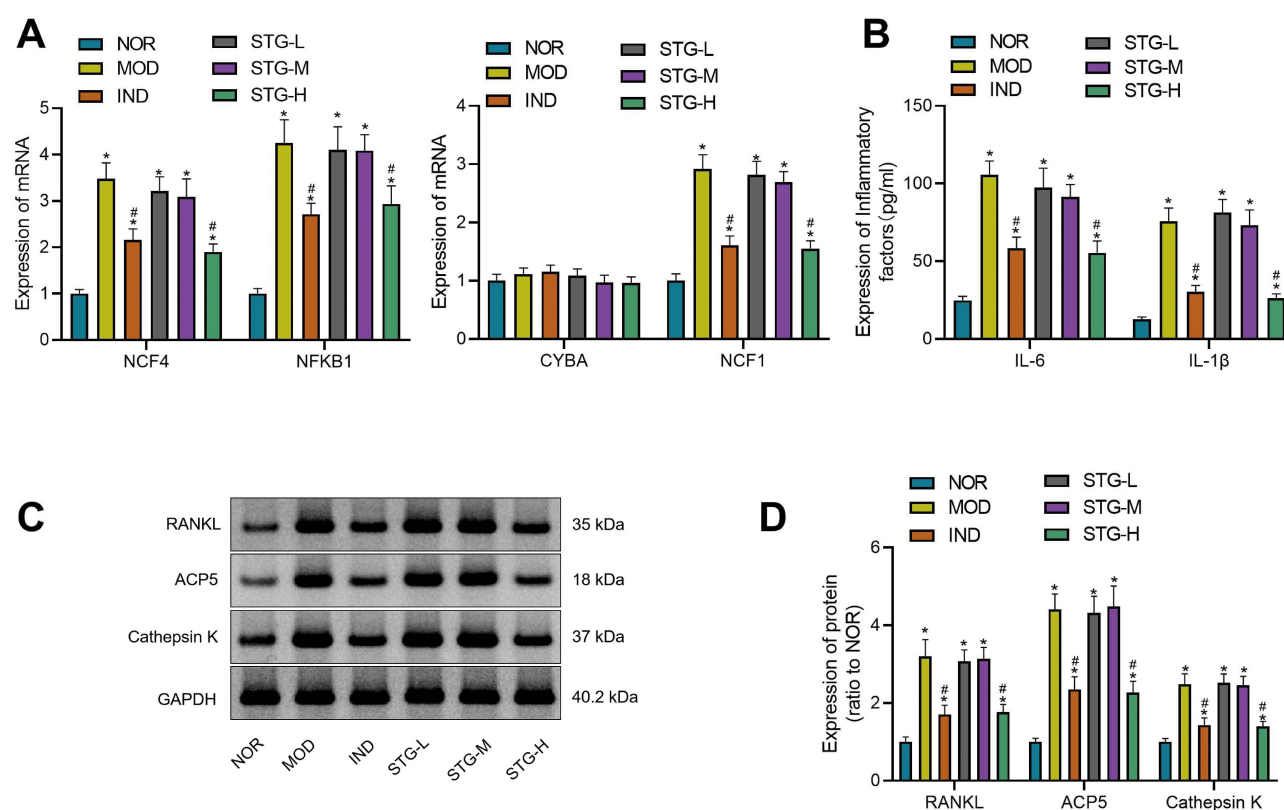


Figure 8 Effects of *Sinomenium acutum* active component on osteoclast differentiation. **(A)** mRNA expression of NCF4, NFKB1, CYBA, and NCF1 by RT-qPCR. **(B)** Levels of IL-6 and IL-1 β by ELISA; * $p < 0.05$ as compared with the NOR group; # $p < 0.05$ as compared with the MOD group. **(C&D)** Protein level of osteoclast differentiation-related factors RANKL, ACP5, and Cathepsin K by Western blot; * $p < 0.05$ as compared with the NOR group; # $p < 0.05$ as compared with the MOD group. Single factor analysis of variance was used for all experimental results. The experiments were repeated three times.

Discussion

Network pharmacology is a new research approach that integrates systems biology, bioinformatics, network science and other disciplines. It analyzes the direct molecular relationship between medicines and treatment objects and reveals the systematic pharmacological mechanism of medicines from the perspective of systemic level and biological network, thereby guiding R&D and clinical diagnosis and treatment of new drugs.³⁸ In our study, the possible molecular mechanism of *Sinomenium acutum* in the treatment of RA was explored in combination of bioinformatics analysis on relevant active compounds and targets of *Sinomenium acutum* and animal experimental verification.

With the development of science and technology, the extraction technology of effective compounds of TCMs is gradually mature. More and more TCMs are widely used in clinical treatment of RA, such as *Sinomenium acutum*, *Total Glucosides of Paeony* and *Tripterygium Wilfordii*.³⁹ Among them, *Sinomenium acutum* is first recorded in Compendium of Materia Medica, with bitter and pungent in taste, neutral in nature, and attributive to liver and spleen channels. It has been reported to have good therapeutic effects on anti-inflammatory, sedation, analgesia, anti-hypertension, anti-arrhythmia, and immunosuppression.^{28,40} *Sinomenium acutum* has been also implicated to inhibit angiogenesis, synovial hyperplasia, and bone destruction, thereby alleviating the disease process of RA.⁴¹ In our investigation, 15 active compounds of *Sinomenium acutum* and 231 corresponding target genes were initially screened by network pharmacological analysis. *Sinomenine* is regarded as main active chemical component of *Sinomenium acutum* in most current researches, including immunosuppression, arthritis improvement, anti-inflammatory, and other pharmacological characteristics. However, in recent years, more and more in-depth researches on *Sinomenium acutum* and the action mechanism reveal that, in addition to *Sinomenine*, *Sinomenium acutum* also contains other active compounds playing anti-inflammatory and anti-bone destruction roles; for example, *Stigmasterol*, *Magnoflorine*, and *Michelalbaine* are the active compounds contribute to the regulation of immune and inflammatory responses.⁴² The exploration for other active compounds of *Sinomenium acutum* involving in RA treatment is critical for the investigation of the treatment mechanism of RA.

On this basis, GeneCards database was conducted to screen the genes related to RA development, and 96 RA-related target genes of the active compounds were obtained. However, these data screened by network pharmacology can only reflect the correlation but cannot clarify the biological activities such as up-regulation or down-regulation in the process of disease treatment. Therefore, we analyzed the potential targets and mechanisms of *Sinomenium acutum* on RA from its active compounds. Our study subsequently analyzed the DEGs in GEO-datasets related to RA, including GSE10500, GSE29746, GSE56649, GSE77289 and GSE97779. Then, the intersection between them and the 96 RA-related target genes of the active compounds was analyzed, and 33 genes were finally screened. Moreover, GO functional analysis and KEGG pathway enrichment analysis were employed in this study, which demonstrated that the target genes regulated by *Sinomenium acutum* may participate in several important signal pathways such as MAPK and p53 signal pathways, which can regulate inflammatory response and osteoclast differentiation. We screened five core genes (NCF4, NFKB1, CYBA, IL-1 β and NCF1), which were identified to be closely related to osteoclast differentiation in RA according to PPI network results. Among these core genes, IL-1 β ranked first in the Degree value. Furthermore, as indicated by network pharmacology analysis, for the active compounds of *Sinomenium acutum*, *Stigmasterol* targets IL-1 β , *Sinactine* targets NCF4, NCF1, and CYBA, and *Gamma-Sitosterol* targets NFKB1. Accordingly, the network pharmacology network of *Sinomenium acutum* regulating RA development was constructed, which has important guiding significance for subsequent research.

Stigmasterol is a phytosterol with a chemical structure close to cholesterol, participating in the immune regulation process for the treatment of corresponding diseases.³² In osteoarthritis-related studies, the inhibitory effects of *Stigmasterol* on the inflammatory response in chondrocytes have been implicated, with properties of anti-inflammatory and anti-catabolism.⁴³ A CIA model was constructed in this study for in vivo verification. The modeled animals were administrated by gavage with *Stigmasterol* solution. Surprisingly, it was found that *Stigmasterol* significantly reduced the release of inflammatory factors IL-6 and IL-1 β in CIA rats, thereby improving the joint pathological injury after RA. According to the research results, *Stigmasterol* was identified as the effective component participating in the treatment mechanism of *Sinomenium acutum* in RA, but the specific mechanisms warrant further deepened investigation.

Yokota et al demonstrated that the degree of osteoclast differentiation was negatively correlated with bone density of RA patients, which accompanied by inflammatory reactions.⁴⁴ In our study, the suppression of *Stigmasterol* on the levels of osteoclast differentiation-related factors (RANKL, ACP5 and Cathepsin K) was also observed. In the process of osteoclast differentiation, RANKL and its receptor has been discovered to activate osteoclasts and differentiate the osteoclasts into mature osteoclasts after the collection of osteoclast precursor cell membrane. The dynamic changes of RANKL and its receptor may have an impact on the differentiation and maturation of osteoclasts and the expression related to bone resorption activity.⁴⁵ Moreover, ACP5 and Cathepsin K proteins are regarded as markers of osteoclast differentiation, the expressions of which to some extent reflect the level of osteoclast differentiation.⁴⁶ We also demonstrated that *Stigmasterol* treatment could downregulate the expression of NFKB1, CYBA and NCF1. It is well known that NF- κ B activation can regulate various pathological reactions, including inflammatory response, immune response, oxidative stress, cell proliferation and apoptosis, and free radical damage, with its mediation of a variety of signal transduction pathways, which function importantly in the occurrence and development of RA. Otherwise, CYBA and NCF1 are also widely reported to be involved in the regulation of inflammatory response.⁴⁷ Our investigation confirmed the inhibition of *Stigmasterol* on osteoclast differentiation, inflammatory response, and histopathological damage of RA. In addition, it is also found that high concentration of *Stigmasterol* treatment can downregulate the levels of NFKB1, NCF4 and NCF1.

However, the current research still has limitations, and the mechanism of *Stigmasterol* regulating osteoclast differentiation in RA needs to be further explored. It is still unclear whether the screened *Sinactine* and *Gamma-Sitosterol* are also involved in the regulation of these target genes. In our future study, in order to further seek the new research ideas and potential therapeutic targets for the treatment of RA, we will further experimentally investigate the specific molecular mechanism of other active compounds of *Sinomenium acutum* in the treatment of RA based on this research.

Conclusion

The current study combined network pharmacology and bioinformatics analysis to identify the involvement of *Sinomenium acutum* in regulation of osteoclast differentiation in RA, and determine the involved core genes (NCF4, NFKB1, CYBA, IL-1 β , and NCF1) and the effective active component *Stigmasterol*. Then, animal experimental validations provide the evidence for the suppression of *Stigmasterol* in AI score and histopathological injury of joints of CIA rats, accompanied with downregulated levels of inflammatory factors (IL-6 and IL-1 β), osteoclast differentiation-related factors (RANKL, ACP5, and Cathepsin K). Besides, *Sinomenium acutum* can reduce the mRNA expression of NFKB1, NCF4, and NCF1. It concludes that *Stigmasterol* as an active component of *Sinomenium acutum* could inhibit the osteoclast differentiation, inflammatory response, and histopathological damage in RA, and downregulate the expression of NFKB1, NCF4, and NCF1. In short, our study uncovers that the active component of *Sinomenium acutum*, *Stigmasterol* is beneficial for ameliorating joint injury in RA, providing a theoretical basis for the drug development of *Stigmasterol* in the treatment of RA.

Data Sharing Statement

The data that supports the findings of this study are available on request from the corresponding author upon reasonable request.

Ethics Approval and Consent to Participate

The current study was approved by the Experimental Animal Welfare and Ethics Committee of The First Hospital of Hunan University of Traditional Chinese Medicine and conducted according to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health. Extensive efforts were made to minimize the suffering of the included animals.

Consent for Publication

Consent for publication was obtained from the participants.

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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 declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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