Study on the Anti-Inflammatory Effect and Mechanism of Yuxuebi Tablet Based on Network Pharmacology

Xiangka Hu, Ping Shao, Xiaojuan Liu, Ling Han, Liuming Gui, Zengxiaorui Cai, Mushuang Qi, and Chunmei Dai*

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ABSTRACT: Yuxuebi tablet (YXB) is a Chinese patent medicine with the effect of activating blood circulation and dissipating blood stasis and has been used to treat “Bi” syndrome in China. The aim of this study was to reveal its anti-inflammatory efficacy and mechanism. A carrageenan-induced inflammation mouse model was established to demonstrate the anti-inflammatory efficacy of YXB by detecting the paw swelling degree and inflammatory cell infiltration in paws. The active chemical ingredients and anti-inflammatory targets of YXB were obtained through network pharmacology analysis. Finally, the core anti-inflammatory targets of YXB were determined by the ELISA method and western blot. YXB significantly reduced the paw swelling degree and inflammatory cell infiltration in paws. A total of 120 key active components included in YXB interacted with 56 core inflammatory targets (such as TNF, IL1B, IL6, PTGS2, RELA, MAPK1, MAPK8, and MAPK14), mainly involving in the TNF signaling pathway, Toll-like receptor signaling pathway, NF-kappaB signaling pathway, and NOD-like receptor signaling pathway. Further studies in vivo found that YXB reduced the levels of TNF-α, IL-1β, and IL-6 in serum and inhibited the expression of COX-2 and the phosphorylation levels of NF-κB p65, JNK, and p38 protein in paws. Taken together, YXB had a good anti-inflammatory effect, which might be related to inhibiting the phosphorylation of NF-κB, JUN, and p38 and the decrease of COX-2 expression and the levels of inflammatory factors.

1. INTRODUCTION

Inflammation is the body’s adaptive response to infection and tissue injury,¹ with the occurrence and development of a variety of chronic diseases,² such as the “Bi” syndrome characterized by pain and numbness in the limb joints and muscles or swelling and difficulty in flexion-extension in the joints. During the inflammation, different types of cellular mediators are released and activated, including chemokines, cytokines, vasoactive amines, and adhesion molecules.³ The most commonly used anti-inflammatory agents are non-steroidal anti-inflammatory drugs (NSAIDs) such as aspirin and celecoxib, which mainly inhibit cyclooxygenase-2 (COX-2) activity and anticytokine agents such as antitumor necrosis factor (TNF) α.⁴ However, NSAIDs can cause severe gastrointestinal, cardiovascular, hepatic, kidney, and cerebral injury,⁵,⁶ and anticytokine agents can lead to infection from more common bacterial pathogens.⁷ In recent years, more and more traditional Chinese medicines (TCMs) have been used in the treatment of inflammatory diseases because of their good anti-inflammatory effect and low adverse reaction.

Shentong-Zhuyu decoction (STZY) was first recorded in the “Yilin Gaicuo” written by Wang Qingren during the Qing Dynasty and had been documented for the treatment of shoulder pain, arm pain, low back pain, leg pain, or body aches. STZY has been recognized by the Chinese National Administration of Traditional Chinese Medicine as a classic prescription of Chinese medicine. Yuxuebi tablet (YXB) is a Chinese patent medicine approved by the National Medical Products Administration and derived from the addition and reduction of Shentong-Zhuyu decoction. It is produced by China Resources Liaoning Benxi Third Pharmaceutical Co., Ltd. and composed of Boswellia carterii Birdw (named “Ru xiang” in Chinese; RX), Clematis chinensis Osbeck (named “Wei ling xian” in Chinese; WLX), Carthamus tinctorius L. (named “Hong hua” in Chinese; HH), Salvia miltiorrhiza Bunge (named “Dan shen” in Chinese; DS), Commiphora myrrha (Nees) Engl. (named “Mo yao” in Chinese; MY), Cyathula
officinalis Kuan (named “Chuan niu xi” in Chinese; CNX), Conioselinum anthriscoides ‘Chuanxiong’ (named “Chuan xiong” in Chinese; CX), Angelica sinensis (Oliv.) Diels (named “Dang gui” in Chinese, DG), Curcuma longa L. (named “Jiang huang” in Chinese; JH), Cyperus rotundus L. (named “Xiang fu” in Chinese; XF), and Astragalus mongholicus Bunge (named “Huang qi” in Chinese; HQ). YXB has the effect of activating blood circulation, dissipating blood stasis, and relieving pain without obvious adverse reactions and can be used to treat “Bi” syndrome caused by the invasion of evil “Qi” in the theory of Chinese medicine, such as rheumatoid arthritis and knee osteoarthritis.\(^8\) In the YXB, the chemical composition of RX, such as boswellic acid type and non-terpenoid type, could affect the levels of cytokines and oxygen species and inhibit the synthesis of leukotriene, COX-1/2, and 5-lipoxygenase (5-LOX).\(^9\) The extracts from WLX inhibited the levels of nitric oxide (NO), prostaglandin E2 (PGE2), TNF-\(\alpha\), interleukin-1beta (IL-1\(\beta\)), and interleukin-6 (IL-6) in RAW 264.7 cells induced by lipopolysaccharide and alleviated paw edema and inflammatory cell infiltration of carrageenan-induced mice.\(^11\)\(^,\)\(^12\) The aqueous extract from HH could improve neutrophilic lung inflammation in mice by activating nuclear factor E2-related factor 2 (Nrf2).\(^13\) DS could downregulate TNF-\(\alpha\), COX-2, inducible nitric oxide synthase (iNOS), and ILs by activating Nrf2, thus inhibiting the inflammatory response.\(^14\) Other TCMs included in YXB also have good anti-inflammatory effects.\(^15\)\(^\sim\)\(^21\) However, as a complex Chinese traditional patent medicine, the anti-inflammatory mechanisms of YXB are still poorly understood.

Network pharmacology has been a popular research method in recent years and has been widely used in the study of the mechanism of action of TCMs.\(^22\) It is to build a network of drug, disease, and therapeutic targets by linking published drug data with the disease, gene expression information, and protein–protein interaction data, better revealing the characteristics of multicomponent drug synergy, in line with the complex characteristics of the human body and providing a prerequisite for the discovery of effective natural drugs.\(^23\)\(^,\)\(^24\) The aim of this paper was to study the anti-inflammatory effect and mechanism of YXB based on network pharmacology and experimental verification of carrageenan-induced inflammation mice.

2. RESULTS

2.1. YXB Inhibited the Paw Edema Induced by Carrageenan. Changes in the thickness of the paw after inflammation were used to evaluate the swelling degree of the paw.\(^25\) Compared with the control group, the swelling degree of the right hind paw in the model group increased significantly (\(P < 0.01\)), suggesting that carrageenan induced inflammation successfully. YXB 3.0 g/kg and Cele 0.08 g/kg markedly suppressed the swelling degree of carrageenan-induced mice paw (\(P < 0.05\)) (Figure 1). These results indicated the potent anti-inflammatory effect of YXB.

2.2. YXB Reduced Inflammatory Cell Infiltration in the Paw. To further observe the anti-inflammatory effect of YXB on carrageenan-induced inflammation mice, the morphological changes of the paw were observed by the hematoxylin and eosin (H&E) staining method. Only a few inflammatory cells were found in the control group, but subcutaneous injection of carrageenan resulted in massive inflammatory cell infiltration in the model group. Cele and YXB could alleviate the inflammatory cell infiltration, especially YXB 1.50 g/kg and 3.00 g/kg and Cele 0.08 g/kg (Figure 2). These results suggested that YXB could reduce the infiltration of inflammatory cells in local tissues.

**Figure 1.** Effect of YXB on paw swelling in mice. Data were represented as mean ± SD, \(n = 6\). *\(P < 0.05\), **\(P < 0.01\) vs control. *\(P < 0.05\), **\(P < 0.01\) vs model.

**Figure 2.** Effect of YXB on inflammatory cell infiltration of mouse paw tissue (H&E).

2.3. Potential Active Ingredients and Targets of YXB for Anti-Inflammation. A total of 352 potential active compounds of YXB were obtained by searching TCMSP and TCMID databases: 17 for RX, 37 for WLX, 38 for HH, 61 for DS, 35 for MY, 4 for CNX, 54 for CX, 61 for DG, 52 for JH, 29 for XF, and 19 for HQ. Some TCMs had the same chemical...
composition, and 1215 targets were obtained from the SwissTargetPrediction.

Five-hundred inflammation targets were collected from the Genecards database. The overlap targets of YXB and inflammation were screened using Venny 2.1, and 137 genes were obtained. The results suggested that these targets might be the anti-inflammatory targets of YXB (Figure 3).

2.4. Protein–Protein Interaction (PPI) Network Analysis. One-hundred and thirty seven YXB-anti-inflammatory targets were imported into STRING 11.5, and the PPI network was visualized using Cytoscape 3.7.2. The network consisted of 114 nodes and 496 edges where each node represented a gene and each edge connected to two genes (Figure 4). According to the topological parameter analysis of the network, the median degree of nodes was 6, and there were 56 targets whose degree values were higher than the median, which indicated that these targets were in the center of the network and closely related to other targets. That was to say they might be the key anti-inflammatory targets of YXB (Table 1).

2.5. Screening of Key Ingredients of YXB. To further screen the key ingredients of YXB, we established the YXB-potential active ingredient-target network (Figure 5). The network consisted of 348 nodes (281 for potential active ingredients, 56 for core targets, and 11 for TCMs) and 1948 edges. The topological parameter analysis of the network showed that the median degree value of 281 active compounds was 6, and the number of compounds whose degree values were higher than the median was 120. These compounds

Figure 3. Overlap targets of YXB and inflammation.

Figure 4. YXB-anti-inflammatory target PPI network. Nodes represent proteins, and lines represent relationships between proteins and proteins. The larger the node, the closer the color was to red, indicating that its degree value was higher and the protein was more important. The thicker the line, the bluer the color was, indicating that the protein binding was stronger.
might play a key role in the anti-inflammatory process of YXB. The top 10 compounds in the degree value are shown in Table 2.

2.6. Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) Enrichment. The 56 core targets were imported into the DAVID database for enrichment analysis to obtain the biological process (BP), molecular function (MF), cellular component (CC), and KEGG. The top 20 terms are shown in Figure 6. According to the results, YXB-anti-inflammatory core targets were involved in the BP, MF, and CC. The BP included positive regulation of transcription from RNA polymerase II promoter, positive regulation of NF-kappaB transcription factor activity, and positive regulation of the nitric oxide biosynthetic process and inflammatory response. In terms of MF, the targets were mainly related to protein kinase activity, transcription factor binding, protein serine/threonine kinase activity, and protein phosphatase binding. As for the CC, there were mainly cytosol, membrane raft, and nucleoplasm. In addition, 99 pathways were revealed from the KEGG pathway enrichment analysis, including the TNF signaling pathway, Toll-like receptor signaling pathway, NF-kappaB signaling pathway, NOD-like receptor signaling pathway, and so on. Four pathways closely related to inflammation were selected to construct the pathway-target network (Figure 6).

Combined with the analysis of “YXB-potential active ingredient-target network” and “Anti-inflammatory pathway-target network of YXB”, PTGS2 (protein name: Cyclooxygenase-2, COX-2), RELA (protein name: Nuclear factor NF-kappaB p65 subunit, NF-xB p65), MAPK1 (protein name: Mitogen-activated protein kinase 1, ERK2), MAPK8 (protein name: Mitogen-activated protein kinase 8, JNK1), MAPK14 (protein name: Mitogen-activated protein kinase 14, p38), TNF (protein name: Tumor necrosis factor, TNF-alpha), IL1B (protein name: Interleukin-1 beta, IL-1beta), and IL6 (protein name: Interleukin-6, IL-6) were selected for further study of the anti-inflammatory mechanism of YXB because of their high degree value and close relation to inflammation (Figure 7).

2.7. YXB Decreased the Levels of Serum Inflammatory Factors. The levels of TNF-alpha, IL-1beta, and IL-6 in the serum were significantly increased in the model group (P < 0.01), showing that the acute inflammation model was successful, compared with the control group. However, YXB 3.0 g/kg markedly decreased the levels of TNF-alpha and IL-1beta (P < 0.05). Meanwhile, Cele 0.08 g/kg and each dose of YXB markedly inhibited the production of IL-6 (P < 0.05) (Figure 8A-C).

2.8. YXB Inhibited the Expression of Target Proteins. Western blot analysis revealed that the expression of COX-2 and Phospho-NF-kappaB p65 (p-NF-xB p65) in paw tissue of the model group were increased (P < 0.05). However, YXB and Cele decreased the expression of COX-2 and p-NF-xB p65 (P < 0.05) (Figure 8A-C). Meanwhile, compared with the control group, the expression of phospho-38 (p-p38) in the paw tissue of the model group was significantly increased (P < 0.01), and the expression of phospho-JNK (p-JNK) and phospho-ERK1/2 (p-ERK1/2) was also increased (P > 0.05). As expected, YXB and Cele inhibited the expression of p-JNK and p-p38 (P < 0.05). However, there was no significant difference in the expression of p-ERK1/2 (Figure 9D-G).

3. DISCUSSION

Inflammation is the first sign of infection and injury, divided into acute inflammation, which develops quickly and lasts for a short time, and chronic inflammation, which develops slowly and lasts for a long time. Uncontrolled inflammation is a major cause of the development of many chronic diseases. Bi syndrome is the symptom causing inflammation of limb joints and muscles under the stimulation of external factors such as wind, cold, dampness, and heat. YXB is a common Chinese patent medicine used to treat “Bi” syndrome. In this study, the carrageenan-induced inflammation mouse model was used to evaluate the anti-inflammatory effect and mechanism of YXB. Subcutaneous injection of carrageenan in mice is a common animal model for screening anti-inflammatory drugs, which can cause edema and cellular infiltration in paw. In this research, we found that a high dose of YXB could effectively reduce the swelling degree of paws caused by carrageenan and...
the infiltration of inflammatory cells in paws, indicating that it had a good anti-inflammatory effect.

YXB is composed of 11 TCMs, which contains many kinds of compounds and has the characteristics of multiple targets and multiple pathways. Therefore, we analyzed the anti-inflammatory mechanism of YXB through network pharmacology. By establishing the network of "YXB-potential active ingredient-target network", we found that 120 active compounds might have acted on 56 key targets related to inflammation. Among them, the top 10 compounds with anti-inflammatory effects were quercetin, beta-sitosterol, luteolin, kaempferol, stigmasterol, myristicin, isorhamnetin, caffeic acid, hederagenin, and methyl eugenol, which were sorted by the degree value and reported in the literature.

Figure 5. YXB-potential active ingredient-target network. Circles represent protein targets, diamonds and triangles represent active ingredients, and hexagons represent TCM. The larger the node, the closer the color was to red, indicating that the degree value was higher.

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reported that quercetin, a flavonoid widely found in natural plants, could inhibit LPS-induced macrophage inflammation and oxidative stress, and it also reduced the expression of C-C motif chemokine ligand (CCL) 17, CCL22, IL-4, IL-6, interferon (IFN)-γ, and TNF-α in atopic dermatitis by local administration. For beta-sitosterol, it inhibited the NF-κB pathway and activated the heme oxygenase-1 (HO1)/nuclear factor erythroid 2-related factor 2 (Nrf2) pathway to exert an anti-inflammatory effect. Luteolin affected M1/M2 polarization of macrophages and played an anti-inflammatory role by downregulating phosphorylated signal transducer and activator of transcription 3 (p-STAT3) and upregulating p-STAT6. Stigmasterol and kaempferol significantly inhibited the NF-κB and MAPK pathways mediated by Toll-like receptor 4 (TLR4)/myeloid differentiation factor-88 adaptor protein (MyD88) and reduced the release of TNF-

| PubChem CID/SID | compound name   | structure | degree | betweeness centrality | closeness centrality | TCMs                |
|-----------------|-----------------|-----------|--------|-----------------------|---------------------|---------------------|
| 5280343         | quercetin       |           | 45     | 0.0064621             | 0.37513514          | HH, MY, CNX, XF, HQ |
| 222284          | beta-sitosterol |           | 35     | 0.00861617            | 0.39476878          | WLX, HH, MY, CNX, CX, DG, XF |
| 5280445         | luteolin        |           | 33     | 0.00658523            | 0.40395809          | HH, DS, XF          |
| 5280863         | kaempferol      |           | 30     | 0.00477005            | 0.38684504          | HH, XF, HQ          |
| 5280794         | Stigmasterol    |           | 30     | 0.00648001            | 0.39476678          | WLX, HH, MY, DG, JH, XF |
| 4276            | myristicin      |           | 21     | 0.00573265            | 0.375948            | WLX, HH, DG         |
| 5281654         | isorhamnetin    |           | 20     | 0.00306913            | 0.3603229           | XF, HQ              |
| 689043          | caffeic acid    |           | 20     | 0.00495797            | 0.37351991          | DS, CX              |
| 73299           | hederagenin     |           | 16     | 0.00580735            | 0.38427464          | WLX, HQ             |
| 7127            | methyl eugenol  |           | 15     | 0.00395166            | 0.34596211          | WLX, MY, CX         |
α, IL-1β, IL-6, and IL-18. Myristicin, isorhamnetin, hederagenin, and methyl eugenol could eliminate reactive oxygen species and inhibit the expression of inflammatory factors and had antioxidant and anti-inflammatory effects. Therefore, as a compound Chinese medicine preparation, the key anti-inflammatory compounds of YXB have synergistic effects.

Through network pharmacology analysis, we found that the key anti-inflammatory targets of YXB included TNF-α, IL-1β, IL-6, COX-2, NF-κB p65, ERK1/2, JNK, and p38. TNF-α, IL-1β, and IL-6 were mainly produced by monocytes. TNF-α binding to the receptors TNFR1 and TNFR2 could activate downstream NF-κB and MAPK signaling pathways and participate in the inflammatory response and apoptosis. IL-1β could rapidly stimulate the transcription of hundreds of gene mRNA in macrophages, epithelial cells, and endothelial cells by activating NF-κB and MAPK signaling pathways. TNF-α and IL-1β played a synergistic role in the development of inflammation and were the main activators of IL-6 expression. IL-6 could activate NF-κB and other signaling pathways and further enhance the transcription of mRNA for inflammatory cytokines, such as IL-6, TNF-α, and IL-1β.

In addition, IL-6 could also promote overproduction of VEGF, increasing angiogenesis and vascular permeability, which were the pathological features of inflammatory lesions. COX-2, the key enzyme for prostaglandin synthesis, was highly expressed in inflammatory diseases and the main target of anti-inflammatory agents. Activation of NF-κB could promote the transcription of COX-2. ERK1/2, JNK, and p38 were members of the highly conserved protein kinase family in

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**Figure 6.** GO and KEGG enrichment analysis of anti-inflammatory targets of YXB.

**Figure 7.** Anti-inflammatory pathway-target network of YXB. Circles represent protein targets; the arrows represent pathways.
the MAPK signaling pathway, which could promote the production of TNF-α, IL-1β, and IL-6. In animal experiments, we found that YXB could effectively reduce the levels of TNF-α, IL-1β, and IL-6 in the serum of carrageenan-induced inflammatory mice. Similarly, YXB significantly reduced the expression of COX-2, p-NF-κB p65, p-JNK, and p-p38, while it had little effect on expression of p-ERK.

4. CONCLUSIONS

In summary, this study showed that YXB exerted an anti-inflammatory effect, which might be related to inhibiting the phosphorylation of NF-κB p65, JNK, and p38, downregulating expression of COX-2, and reducing the levels of TNF-α, IL-1β, and IL-6. It provides a theoretical basis for YXB to treat "Bi" syndrome and has a better understanding of the anti-inflammatory mechanism of YXB, providing reference for searching safe and effective anti-inflammatory agents.

5. MATERIALS AND METHODS

5.1. Materials. Male KM mice, weighing 16–18 g, were purchased from the Animal Laboratory Centre of Jinhzhou Medical University (Animal license number: SCXK [Liao] 2019-0007). YXB (NO: 20210303) was supplied by China Medical University (Animal license number: SCXK [Liao] 20210301). Celecoxib Capsules (Cele) (NO: DK1011) was purchased from Pfizer. Carrageenan (NO: C804872) was purchased from Shanghai Macklin Biochemical Co., Ltd. Isoflurane (NO: 32791) was purchased from Shanghai Wanleibio Co., Ltd. β-actin Antibody (NO: AC026) and HRP Goat Anti-Rabbit IgG (H + L) (NO: AS014) were purchased from Abclonal. An Ultra-High Sensitivity ECL Kit (NO: BLS23A) was purchased from Biosharp. Mouse TNF-α, IL-1β, and IL-6 ELISA kits were purchased from R&D.

5.2. Screening the Active Ingredients and Targets of YXB. The compound information of YXB was obtained from the TCMSP database (https://old.tcmsp-e.com/tcmsp.php) and TCMID database (http://119.3.41.228:8000/tcmid/). Bioavailability (OB) ≥ 30% and drug-like (DL) ≥ 0.18 were used as parameters to screen active ingredients in the TCMSP database. The compounds obtained from the TCMID database were imported into SwissADME (http://www.swissadme.ch/) in the style of 2D structure files to screen active ingredients. The two conditions must be met at the same time. That was to say GI absorption was high and at least two of the Druglikeness (Lipinski, Ghose, Veber, Egan and Muegge) were “yes”. The targets of active ingredients were predicted by the SwissTargetPrediction database (http://www.swisstargetprediction.ch/).

5.3. Collecting Inflammation Targets. The keyword of “inflammation” was searched in the GeneCards database (https://www.genecards.org/). The targets were ranked by “Relevance score”, and the first 500 targets were selected to improve their reliability. All targets were standardized by Uniprot (http://beta.uniprot.org/).

5.4. PPI Analysis. The intersection targets of action targets of YXB and inflammatory targets, the potential anti-inflammatory targets of YXB, were obtained using Venny 2.1.0 (https://bioinfogp.cnb.csic.es/tools/venny/index.html) and imported into STRING 11.5 (https://www.string-db.org/). The organism was limited to homo sapiens, and the minimum required interaction score was set as highest confidence (0.900) to construct a PPI network model. The result of PPI was visualized using Cytoscape 3.7.2. Topology parameter analysis was carried out, and degree, betweenness centrality, and closeness were the key indicators to measure the importance of nodes. Targets whose degree values were greater than the median, the key anti-inflammatory targets of YXB, were selected for further analysis.

5.5. Go and KEGG Enrichment Analysis. To understand the enrichment of the collective targets in BPs, CCs, MFs, and pathways, DAVID Bioinformatics Resources 6.8 (https://david.ncifcrf.gov/) was used for the analysis of GO and KEGG of core targets of YXB anti-inflammatory effects, and then the results were visualized using R project.

5.6. Animal Experiment. All procedures involved in animal experiments in this study were approved by the Ethics Committee of Jinhzhou Medical University (SYXX [Liao] 2019-0007), and the guidelines for the Guide for the Care and Use of Laboratory Animals issued by the National Institutes of Health of the United States were strictly followed.

Cele is a selective inhibitor of COX-2. In this study, low and high doses of Cele in clinical use were selected as the positive control.
Mice were fed with standard laboratory food and water for 1 week under a 12 h light/dark cycle. The humidity was set at 60–70%, and the temperature was 23 ± 2 °C. The mice were randomly divided into seven groups: control, model, Cele 0.04 g/kg, Cele 0.08 g/kg, YXB 0.75 g/kg, YXB 1.50 g/kg, and YXB 3.00 g/kg (n = 6). Cele and YXB were diluted in 0.5% carboxymethylcellulose sodium to form a suspension for gavage. The dose of Cele and YXB was converted according to the dose relationship between the adult and mouse in the third edition of Pharmacological Research Methodology of Traditional Chinese Medicine edited by Chen Qi.

The treatment lasted for 7 days. To evaluate the effect of YXB on paw edema, 100 μL of 1% carrageenan suspension (W/V, containing 0.9% saline) was subcutaneously injected into the right hind paw of mice 30 min after the last administration, except for the control group, which received the same amount of sterile saline. After the experiment, the mice were killed by cervical dislocation, and the right paw was immediately dissected. Part of the paw was prepared for histological analysis, and the other parts were stored at −80 °C for western blotting.

5.7. Paw Edema Evaluation. The thickness was measured before inflammation (0 h) and at 2, 4, and 6 h after the intraplantar injection of carrageenan by vernier calipers. The degree of swelling was calculated as the thickness of the paws after inflammation minus that of the paws before inflammation.

5.8. Hematoxylin and Eosin Staining. The part of right paws of mice were collected and fixed in 10% neutral buffered formalin for 24 h and then decalcified in 10% EDTA for 15 days. Thereafter, the right paws were embedded in paraffin and cut into five-micrometer section. Subsequently, H&E were used for staining, and morphological changes were observed by using a light microscope (OLYMPUS, NO: DP72).

5.9. Evaluation of the Serum Levels of Cytokines. After six hours of inflammation, mice were subjected to light anesthesia induced by isoflurane, and blood samples were collected by retroorbital sinus puncture in the absence of anticoagulant and centrifuged at 35 000 r/min for 10 min at 4 °C. The supernatant was stored at −80 °C and used for measurement of TNF-α, IL-1β, and IL-6 by ELISA.

5.10. Western Blotting Analysis. The soft tissue from paw was harvested and homogenized in lysis buffer containing 1% PMSF and 2% phosphatase inhibitor cocktail. The BCA protein detection kit was used to determine the protein level of the extract. Protein samples were separated by 10% SDS-PAGE and transferred to a polyvinylidene fluoride (PVDF) membrane. After blocking in Tris-buffered saline containing Tween 20 (TBST) and 5% bovine serum albumin at room temperature for 1 h, PVDF membranes were washed three times in TBST and incubated overnight at 4 °C with primary antibodies of COX-2 (1:500), NF-κB p65 (1:500), p-NF-κB p65 (1:500), p-jNK (1:1000), p-ERK1/2 (1:500), p-p38 (1:500), p38 (1:1000), ERK1/2 (1:500), p-ERK1/2 (1:500), and β-actin (1:1000). After washed with TBST three times at room temperature, the PVDF membranes were incubated with HRP Goat Anti-Rabbit IgG (H + L) (1:10000) for 1 h and washed three times with TBST. Proteins were scanned by using an Ultra-High Sensitivity ECL Kit. The protein expression was analyzed using Image J software.

5.11. Statistical Analysis. The data were recorded using the software SPSS 26.0 and reported as the mean ± standard deviation (mean ± SD). One-way analysis of variance analysis was used to evaluate the significance of the differences between groups with normal distribution; otherwise, the independent sample test was used for the nonparametric test. P < 0.05 was considered statistically significant.
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Complete contact information is available at: https://pubs.acs.org/10.1021/acsomega.2c04641

Author Contributions
X.H., P.S., X.L., and L.H. contributed equally to this work.

Notes
The authors declare no competing financial interest.

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