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Original Article

Exploration of the molecular mechanism guiding Xinfeng capsule regulatory mechanism for rheumatoid arthritis inflammation

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Abstract: Objectives: Rheumatoid arthritis (RA) is an autoimmune disease characterized by chronic inflammation of the joint synovium. The traditional Chinese medicine Xinfeng capsule (XFC) has a remarkable alleviating effect on inflammatory symptoms, such as joint pain and swelling, in patients with RA. However, the underlying mechanism of action remains to be elucidated. This study intended to conduct network pharmacology, animal experiments, data mining, and molecular docking to explore the molecular mechanism through which XFC can improve the inflammatory symptoms of RA. Methods: The Apriori association rules and a random walk model were employed to evaluate the effect of XFC on the clinical inflammatory indexes of RA. The active ingredients and the potential target genes of XFC were obtained from public databases. Based on the search tool for recurring instances of neighboring genes (STRING) database, the Database for Annotation, Visualization and Integrated Discovery (DAVID) database, Cytoscape software, and molecular docking method, the molecular mechanism by which XFC acts on RA was also analyzed. Finally, an adjuvant arthritis rat model was established to verify the effects of XFC on inflammation-related signaling pathways and inflammatory factors. Results: XFC significantly reduced the level of C-reactive protein (CRP), vascular endothelial growth factor (VEGF), and the erythrocyte sedimentation rate (ESR). The docking space structures of the active ingredients in XFC, namely triptolide and quercetin, and the key targets were stable. Inflammation-related biological processes were identified as the key factors involved in the development of RA, and the regulation of the toll-like receptor (TLR) signaling pathway may be the key link for XFC toward improving the inflammatory state of RA. The expression levels of toll-like receptor 4 (TLR4), myeloid differentiation primary response protein MyD88 (MyD88), interleukin-1 receptor-associated kinase 1 (IRAK1), TNF receptor-associated factor 6 (TRAF6), TGF-beta-activated kinase 1 (TAK1), phospho-Inhibitor of NF-κB kinaseβ (p-IKKβ), phospho-Nuclear factor-k-gene binding (p-NF-κB), and interleukin-1β (IL-1β) can all be decreased by XFC. XFC improves joint inflammation symptoms by lowering pro-inflammatory factors tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), and interferon-γ (INF-γ) levels. Conclusions: XFC could effectively improve the clinical inflammatory indexes of RA. The active ingredients of XFC improved the inflammatory state of RA by regulating the TLR-signaling pathway.

Keywords: Rheumatoid arthritis, toll-like receptor, inflammatory reaction, data mining

Introduction

Rheumatoid arthritis (RA) is a chronic multisystem disease characterized by morning stiffness and symmetrical facet joint pain. The main features of RA are recurrent synovitis and joint bone damage [1]. Current research suggests that the occurrence of RA can be attributed to a combination of genetic and environmental factors, albeit its specific pathogenesis remains unknown. However, the persistent inflammatory imbalance of the joint synovium forms the basic reason for the development of RA [2, 3]. Persistent inflammatory responses are involved in multiple links during the course of RA. In addition to causing synovial inflammation and promoting pannus formation, the inflammatory response stimulates osteoclast differentiation and leads to bone erosion and cartilage destruction in the joints [4-6]. The extent of RA synovial inflammation is related to the expression levels of various inflammatory factors. The signaling

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pathway is located upstream of a variety of inflammatory factors. The Janus-activated kinase Signal transducers and activators of transcription (JAK-STAT), TLR, Ras, and other signaling pathways are located upstream of a variety of inflammatory factors. These pathways play the dual role of cytokine signaling and transcriptional activation of target genes and participate in several processes such as the regulation of the autoimmune inflammatory responses in the body [7-9].

The effective intervention of the RA inflammatory response and the regulation of the inflammatory imbalance can alleviate RA inflammation and repair the synovial joint tissues. Patients with RA require continuous medication to stop the progression of inflammation. However, the long-term use of most drugs induces different degrees of damage to the gas patients' gastrointestinal tract, liver, and kidney functions [10, 11]. Hence, safer and effective alternatives are highly warranted. In this context, the traditional Chinese medicine Xinfeng capsule (XFC) composed of *Tripterygium wilfordii*, *Astragalus* root, and coix seed is beneficial. It has a significant curative effect and is safe and reliable for the clinical treatment of RA. The determination of Astragaloside-II, Astragaloside-IV, Triptol, Celastrol, Wilforgine, and Wilforine in the plasma of rats with adjuvant arthritis was done in our previous study using ultra-high performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS). The integration and classification of the established pharmacokinetic study models of Terpenes and Alkaloids were in line with the features of the classical pharmacokinetic model [12]. Past studies on XFC have focused on its clinical efficacy, including the effects on B-cell activating factor and its receptor (2015), cardiac functions (2015), lipoprotein metabolism (2015), lung functions (2016), the quality of life and safety (2016), and the key proteins of immune inflammation (2022) in RA patients [13-17]. In our past study on the action mechanism of XFC in RA, we evaluated the Actl/NF-κB signaling pathway (2016), lncRNA MAPKAPK5-AS1 (2021), LINC00638, and activating Nrf2/HO-1 pathway (2022) [18-20]. The results of these studies suggested that XFC could significantly improve the inflammatory response of RA. However, systematic network pharmacological studies on the treatment of RA by XFC as well as the combination of animal experiments for verification are still lacking. Therefore, in this study, we evaluated the active components and targets of XFC through network pharmacology combined with molecular docking, analyzed the pathway of target enrichment, and further verified the effects of the XFC on pathway-related proteins and cytokines through animal experiments.

This study has the following novel aspects: First, we found a new mechanism by which XFC improves the inflammatory response of RA through toll-like receptor-signaling pathways, which provides certain references for the clinical treatment of RA. Second, we verified the role of toll-like receptors in the pathogenesis of RA. In addition, we applied a random walk and other models to evaluate the effect of XFC on improving the inflammatory markers of RA. When compared with the traditional data-processing methods, these models offer evident advantages in achieving high-quality data information and exploring deeper rules.

Materials and methods

General data

Data mining case data were obtained from patients with RA from September 2019 to June 2021 at the Department of Rheumatology and Immunology, The First Affiliated Hospital of Anhui University of Chinese Medicine. The control group was treated with methotrexate tablets only (12 mg/week), while the XFC group was treated with XFC (3 capsules, thrice a day) with methotrexate tablets (12 mg/week). The XFC group included 12 male and 110 female patients, with an average age of 55.20 ± 9.58 years and an average disease duration of 10.94 ± 3.20 years. The control group included 10 men and 109 women with an average age of 55.84 ± 9.06 years and an average disease course of 11.42 ± 3.55 years. No significant difference existed in the baseline data (i.e., sex, age, and disease course) between the two groups ($P > 0.05$). The data on serum amyloid A (SAA), vascular endothelial growth factor (VEGF), C-reactive protein (CRP), and the erythrocyte sedimentation rate (ESR) data were collected. This study was conducted after the approval of the ethics committee (No.: 2019AH-12).

Data mining model

In the Apriori association rule model analyses, the variables were assigned and XFC was used as the explanatory variable. The XFC group was assigned a value of 1 with XFC, and the control group without XFC was assigned a value of 0. An unchanged and increased inflammatory index was assigned a value of 0, while a decreased index was assigned a value of 1. The Apriori association rules are in the form of left-hand-side (LHS) and right-hand-side (RHS) item sets. When the LHS set occurs, the RHS set may occur. In the Apriori association rule mining, two parameters (support and confidence) are essential. The support has a form revealing the probability of A and B occurring at the same time. Confidence in this form reveals the conditional probability of B under the A premise. The minimum confidence and minimum support were designed to be 50% and 90%, respectively. The SPSS Modeler 15.0 software was used to analyze the correlation between XFC and the clinical inflammatory indexes. The calculation method is shown in formula (1). A random walk model was established using the ORACLE (10 g; Oracle Corp., Redwood Shores, CA, USA) to observe for improvement by XFC on the clinical inflammatory indexes. For the random walk model, a walker moves either up (u(i) = +1) or down (u(i) = -1) one unit length (u) for each step i of walk. A random walk naturally motivates quantification of this correlation by calculating the 'net displacement' (y) of the walker after I steps, which is the sum of the unit steps u(i) for each step i. The calculation method is shown in formula (2).

$$\text{Support } (X \rightarrow Y) = \sigma \frac{(X \cup Y)}{N}$$

$$\text{Confidence } (X \rightarrow Y) = \sigma \frac{(X \cup Y)}{\sigma(X)} \quad (1)$$

$$\text{life } (X \rightarrow Y) = \text{confidence } \frac{(X \rightarrow Y)}{\sigma(Y)}$$

$$y(I) = \sum_{i=1}^{I} u(i) \quad (2)$$

Screening of the XFC active ingredients and potential targets

The RA-related differentially expressed genes were obtained by searching the Gene Expression Omnibus (GEO) database (https://www.ncbi.nlm.nih.gov/geo/). The active ingredients of *T. wilfordii*, *Astragalus,* and Coix seeds were screened in the Traditional Chinese Medicine Systems Pharmacology Database and Analysis Platform (TCMSP) database (https://old.tcmsp-e.com/index.php). The screening conditions were oral bioavailability (OB) ≥ 30% and drug sample (DL) ≥ 0.18. DrugBank database (https://go.drugbank.com/) was used to obtain the active ingredient-binding target genes. The active ingredient-binding target genes and RA differentially expressed genes were intersected to obtain the potential targets of XFC acting on RA. A visual network diagram of "traditional Chinese medicine-active ingredients-potential targets" was generated by the Cytoscape 3.8.0 software. We obtained the protein interaction network between the potential targets through the STRING database (https://cn.string-db.org/) [21]. The topological parameters of all points in the protein interaction network were further analyzed by the Maximal Clique Centrality (MCC) algorithm in Cytoscape, while the centrality score of each target in the network was calculated, from which the top 15 were shortlisted.

Molecular docking of the XFC active components and potential genes

The protein 3D structures of the 15 potential targets were downloaded from the Protein Data Bank (PDB) database (https://www.rcsb.org/). The 3D structures of the XFC active ingredients were downloaded through the Pub Chem database (https://pubchem.ncbi.nlm.nih.gov/). Molecular docking was performed using the Auto Dock 4.2.6 software. Pymol 2.2.0 software was used to visualize the best results for the docking-binding activity. The key targets were determined based on the size of the binding energy. The key targets were entered into the Database for Annotation, Visualization and Integrated Discovery (DAVID) database (https://david.ncifcrf.gov/) for Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analyses.

Experimental animals

The experimental animals were purchased from the Anhui Experimental Animal Center (license number: SYXK 2005-02). A total of 15 clean-grade male Wistar rats (average body weight: 206.14 ± 22.75 g) were randomly

assigned to 5 groups. The normal control (NC) group, the model control (MC) group, the Low-dose XFC group, the Medium-dose XFC group, and the High-dose XFC group contained three rats each. The rats in the XFC and MC groups were injected with 0.1 mL of Freud's complete adjuvant from the left hind footpad toward the outer ankle joint to establish the arthritis model. Based on the XFC clinical human once-daily dose, the XFC low, medium, and high dosing groups (0.3 g/kg, 0.6 g/kg, and 1.2 g/kg) were converted to rat dosing and multiplied by 2.5, 5, and 10 times to generate the XFC low, medium, and high dosing groups. Following eighteen days, XFC was given once daily to the normal control and model control rats in a volume of 10 mL/kg, whereas saline was given in accordance with the same protocol. Each group was administered this for 14 consecutive days, once a day. This animal experiment was approved by the Animal Ethics Committee (No.: AHUCM-rats-2021022).

ELISA

The levels of IL-6 (JYM0646Ra), IFN-γ (JYM-0654Ra) and TNF-α (JYM0635Ra) (Genemei Biotechnology Corp., Wuhan, China) were diluted in accordance with the kit instructions. The absorbance value was measured with a microplate reader (Leidu Corp., Shenzhen, China). The standard substance concentration and the corresponding absorbance value were employed to draw a standard curve, which was then used to calculate the final serum sample concentrations of TNF-α, IFN-γ and IL-6 after considering the dilution factor.

Western blotting

The collected synovial tissues were added to the RIPA lysis buffer (Beyotime Corp., Shanghai, China), centrifuged at 12500×g for 20 minutes, and the total protein was extracted. The proteins were separated by electrophoresis on polyacrylamide gels (Solarbio Corp., Beijing, China) and electrotransferred onto the PVDF membranes (Millipore Corp., Bedford, MA, USA). TLR4 (1:1500, bs-20594R, Bioss, China), MyD88 (1:1000, sc-74532, Santa Cru, USA), IRAK1 (1:1000, bs-28003R, Bioss, China), TRAF6 (1:1000, bs-2830R, Bioss, China), IL-1β (1:1000, ab315084, abcam, UK), TAK1 (1:1000, bs-3585R, Bioss, China), IKKβ (1:1000, bsm-51550M, Bioss, China), p-IKKβ (1:1000, ab194519, abcam, UK), NF-KB (1:2000, 66535-1-Ig, Proteintech, China), p-NF-kB (1:1000, 3033S, CST, UK), GAPDH (1:2000, TA-08, Zsbio, China) and the PVDF membranes (IPVH00010, Millipore) were incubated for 2 h. The secondary antibodies used were anti-rat (1:20000, ZB-2305, Zsbio, China) or anti-rabbit (1:20000, ZB-2301, Zsbio, China). A chemiluminescence imaging system was used for imaging, and the gray value of the band was calculated using GAPDH (Zsbio Corp., Beijing, China) as the internal reference.

Statistical analyses

Statistical analysis of the clinical and experimental research data was performed using SPSS 25.0 software. The measurement data were represented by $\bar{x} \pm s$. The comparison between the two groups was performed by t-test. Comparisons between multiple groups were made using one-way ANOVA. The difference was considered to be statistically significant at $P < 0.05$.

Results

Apriori association rule analysis of XFC and RA inflammatory indexes

In the Apriori association rule model, the minimum confidence and minimum support were designed to be 50% and 90%, respectively. When the support degree was > 90%, the improvement degrees of XFC on VEGF and ESR increased significantly, which were 1.046 and 1.014, respectively. XFC resulted in a low improvement in CRP, which was 0.992, but met the minimum support and confidence. The improvement in SAA caused by XFC was 0.968, which was higher than that in CRP; however, the support and confidence were the lowest. The results of the Apriori association rule analysis indicated that XFC was closely related to the improvement in VEGF, ESR, and CRP (**Figure 1A**). Supplementary Table 1 shows the details of the Apriori association rule analysis for XFC and RA inflammatory indexes.

Comparison of the clinical inflammatory indexes before and after XFC treatment

A comparison of the differences in CRP, ESR, SAA, and VEGF before and after the treatment revealed that the XFC group was better than

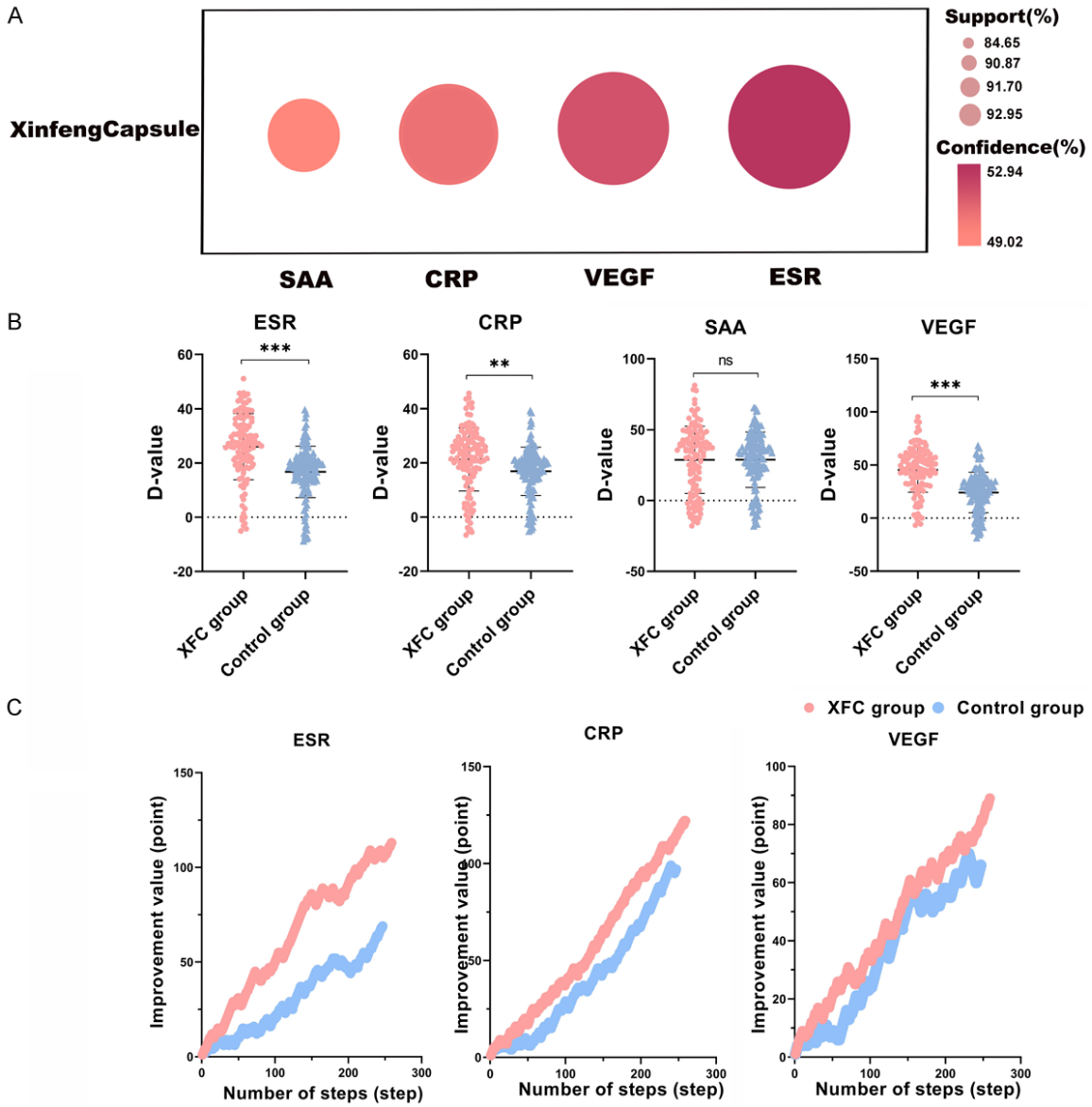

Figure 1. Improvement in the inflammatory indexes of RA patients by XFC treatment. A. The Apriori association rule model analysis of the association among XFC and ESR, CRP, SAA, and VEGF. B. Comparison of the differences in ESR, CRP, SAA, and VEGF between the XFC and control groups before and after treatment. C. The depiction of the random walk model to evaluate the improvement in the effect of XFC on CRP, ESR, and VEGF.

the control group in terms of reduced CRP, ESR, and VEGF ($P < 0.01$ or $P < 0.001$) (**Figure 1B**). The random walk model was further used to evaluate the effect of XFC on CRP, ESR, and VEGF in patients with RA. The improvement or the improvement coefficients of inflammatory indexes in the XFC and control groups were 0.642 and 0.566 for ESR, 0.623 and 0.605 for CRP, and 0.642 and 0.612 for VEGF, respectively. The higher the improvement or improvement coefficients, the more obvious is the improvement index of the corresponding group and better is the treatment effect (**Figure 1C**). These results suggested that XFC has a good effect on improving clinical inflammatory indexes.

Screening of XFC active ingredients and the potential targets

Based on the gene chip data of the GEO gene database GSE55235, the differentially expressed genes in RA were identified. All component data of T. wilfordii, Astragalus, and coix

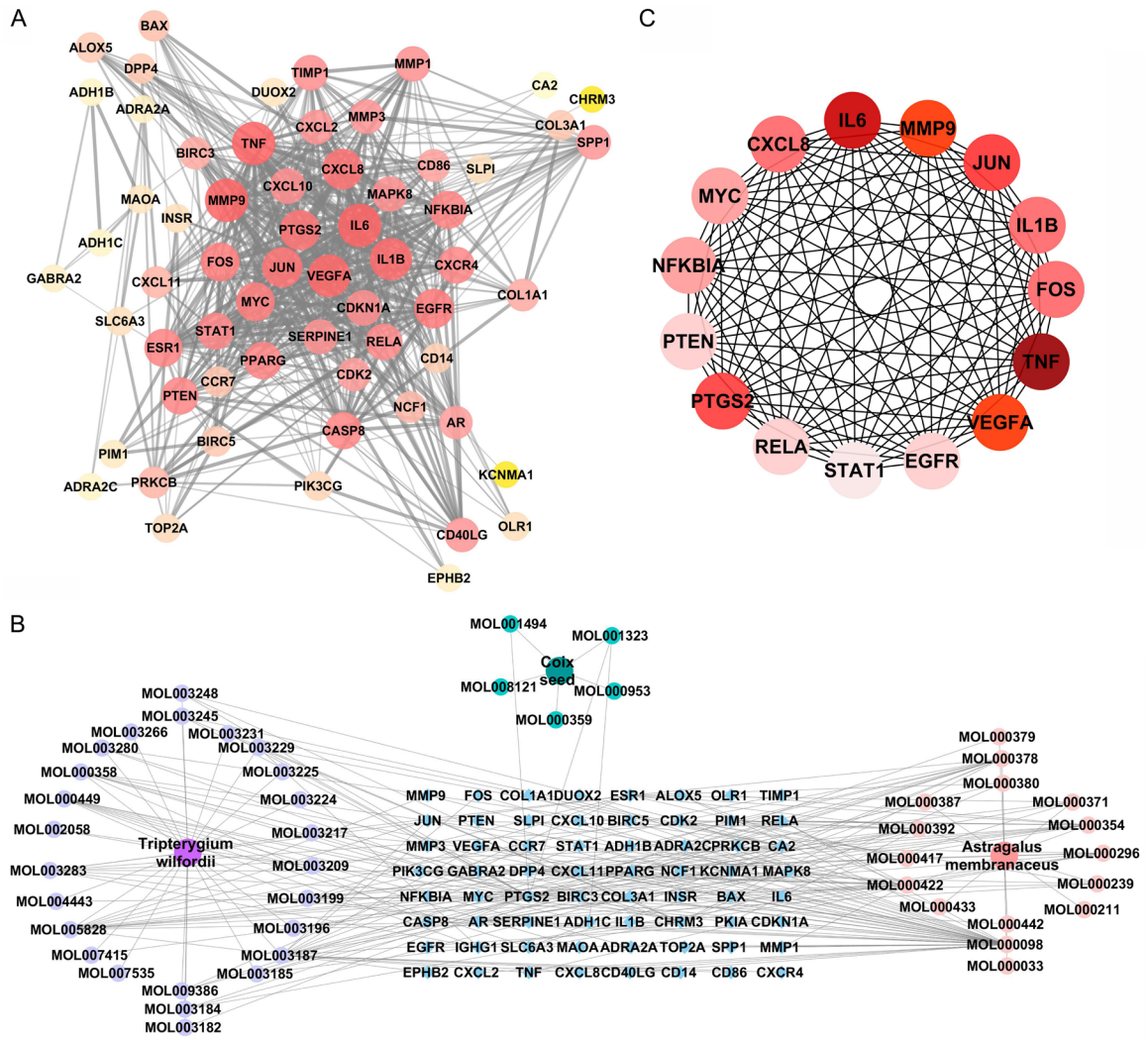

Figure 2. Screening of the active ingredients and the targets of XFC. A. A PPI network diagram depicting the 64 potential targets; B. A diagram depicting the association among Traditional Chinese medicine, active ingredients, and the potential target network; C. A diagram depicting the top 15 potential network targets.

seed were retrieved from TCMSP. The obtained active ingredients were entered into the DrugBank database to obtain active ingredient-binding target genes. A total of 64 potential targets of XFC used for the treatment of RA were obtained by taking the intersection of the active ingredient-binding target genes and the differentially expressed genes in RA (**Figure 2A**; Supplementary Table 2). The potential targets were combined with 16 active ingredients of T. wilfordii, 25 active ingredients of Astragalus, and 5 active ingredients of coix seed (Supplementary Table 3). A network diagram of the traditional Chinese medicine XFC-active components-potential targets was constructed (**Figure 2B**). The significance of 64 potential targets was analyzed using the topological MCC algorithm, and the top 15 of them were tumor necrosis factor (TNF), Interleukin 6 (IL6), recombinant matrix metalloproteinase 9 (MMP9), VEGFA, prostaglandin-endoperoxide synthase 2 (PTGS2), jun proto-oncogene (JUN), c-x-c motif chemokine ligand 8 (CXCL8), fos proto-oncogene (FOS), MYC proto-oncogene (MYC), epidermal growth factor receptor (EGFR), interleukin 1β (IL1β), NFKB inhibitor alpha (NFKBIA), phosphatase and tensin homolog (PTEN), RELA proto-oncogene (RELA), and signal transducer and activator of transcription 1 (STAT1) (**Figure 2C**).

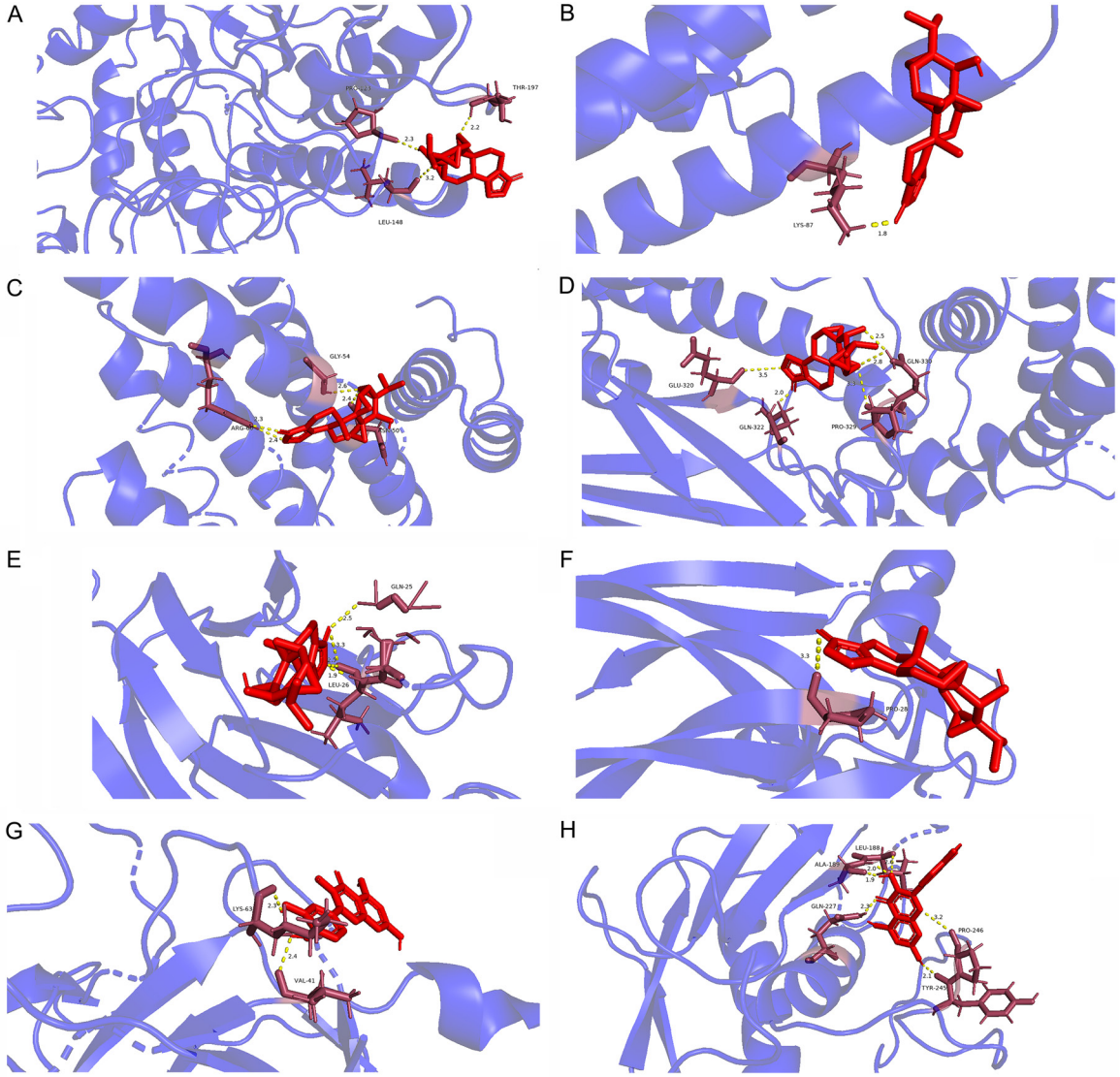

Figure 3. Molecular docking of XFC active components and the key target genes. A. FOS and triptolide; B. JUN and triptolide; C. RELA and triptolide; D. STAT1 and triptolide; E. TNF and triptolide; F. VEGFA and triptolide; G. IL1B and quercetin; H. MMP9 and quercetin.

Molecular docking of XFC active ingredients and the potential targets

The active components, such as triptolide, quercetin, and formononetin, were docked with the top 15 potential targets, that is TNF, MMP9, VEGFA, PTGS2, JUN, FOS, EGFR, IL1β, PTEN, RELA, and STAT1. The binding energy was < 0, which implied that the potential gene and the active ingredient could bind spontaneously; the lower the binding energy, the more stable the binding conformation. The binding energy was < -4.25 kcal/mol, which indicated a certain binding activity. The binding energy of triptolide to FOS, JUN, RELA, TNF, VEGFA, and STAT1, while that of quercetin to MMP9 and IL1B was < -4.25 kcal/mol. This result indicated that the docking structure was stable (**Figure 3A-H**). Triptolide and quercetin were identified as the active ingredients of XFC. FOS, JUN, RELA, TNF, VEGFA, STAT1, MMP9, and IL1β were identified as the key targets of XFC in the treatment of RA.

KEGG and GO enrichment analysis

GO and KEGG enrichment analysis of the key targets was performed using the DAVID data-

base. The screening condition was $P < 0.05$, and the top 15 entries were selected for display. A total of 63 signaling pathways were obtained from KEGG analysis, which included the TLR signaling pathways (**Figure 4A**). A total of 101 entries were obtained from GO biological process (BP) analysis, including responses to cytokines and inflammatory responses (**Figure 4B**). A total of 8 entries were obtained from GO cellular component (CC) analysis, including the extracellular region and secretory granules (**Figure 4C**). A total of 17 entries were obtained from GO molecular function (MF) analysis, including the tumor necrosis factor-receptor binding and cytokine activity (**Figure 4D**). This result suggests that influencing the biological processes related to inflammation and the TLR signaling pathway may be an important mechanism through which XFC treats RA.

Effects of XFC on joint symptoms, inflammatory factors and TLR signaling pathway in rats with adjuvant arthritis

We looked into the function of the TLR signaling pathway, the inflammatory response, and the impact of XFC on joint inflammation using animal research. The findings showed that the MC group's rats' toe swelling degree, number of swollen joints, and arthritis index were all significantly higher than those of the NC group, while the inflammatory factors TNF-α, IL-6, and INF-γ had significantly higher levels ($P < 0.01$). This suggests that an imbalance of inflammatory factors and successful modeling causes joint swelling and other symptoms. Following XFC delivery, we observed that TNF-α and IL-6 levels were considerably lower ($P < 0.05$ or $P < 0.01$), as well as toe swelling, the number of swollen joints, and the arthritis index in the XFC high, medium, and low dose groups compared with the MC group. Compared to the XFC low and medium dose groups, the XFC high-dose group showed significantly reduced toe swelling, the number of swollen joints, the arthritis index, and TNF-α, IL-6, and INF-γ levels (**Figure 5A**, **5B**).

To learn more about XFC's role in the TLR signaling pathway and its mechanism in arthritis. Using WB experiments, we identified the target proteins upstream and downstream of the TLR4 signaling pathway and the degrees of protein phosphorylation. The findings showed that in the synovial tissues of the rats in the MC group, there were significantly higher levels of upstream TLR4, MyD88, IRAK1, TRAF6, and TAK1 protein expression than in the NC group and downstream levels of IKKβ, NF-κB phosphorylation, and IL-1β ($P < 0.01$). This implies that the generation of inflammatory factors is induced, and the TLR4 signaling pathway is activated. Following XFC administration, it was discovered that the protein expression levels of TLR4, MyD88, IRAK1, TRAF6, TAK1, p-IKKβ, p-NF-κB, and IL-1β were considerably reduced in the high, medium, and low dose groups compared with the MC group ($P < 0.05$ or $P < 0.01$). In comparison to the XFC medium- and low-dose groups, the TLR4, MyD88, IRAK1, TRAF6, TAK1, p-IKKβ, p-NF-κB, and IL-1β protein expression levels were further decreased in the high-dose group (**Figure 5C-F**). This shows that by blocking the TLR4 signaling pathway's activation, XFC may further lower the degree of inflammation.

Discussion

The pathogenesis of RA involves the overactivation of inflammation-related signaling pathways and the imbalance of downstream cytokines [22, 23]. XFC, the traditional Chinese medicine compound preparation, can effectively alleviate the inflammatory manifestations of RA, such as joint pain and swelling. In this study, Apriori association rules and random walk model were employed to observe the improvement in the clinical inflammatory indexes by XFC. These results indicated that XFC could significantly reduce CRP, ESR, and VEGF in patients with RA. The random walk model results demonstrated that XFC was better than a single immunosuppressant in improving ESR, CRP, and VEGF. In past studies, the Xinfang capsule was found to effectively reduce ESR, CRP, disease activity score in 28 joints (DAS28), and other inflammatory indexes in RA patients [14]. Sun et al. suggested that XFC could improve the morning stiffness of the joints as well as the disease activity index in RA [24].

Cumulatively, these results indicate that XFC could improve the clinical inflammatory indexes of RA. However, how XFC regulates the clinical inflammatory response in RA needs to be ascertained. In this study, the active ingredients of XFC and the key targets acting on RA

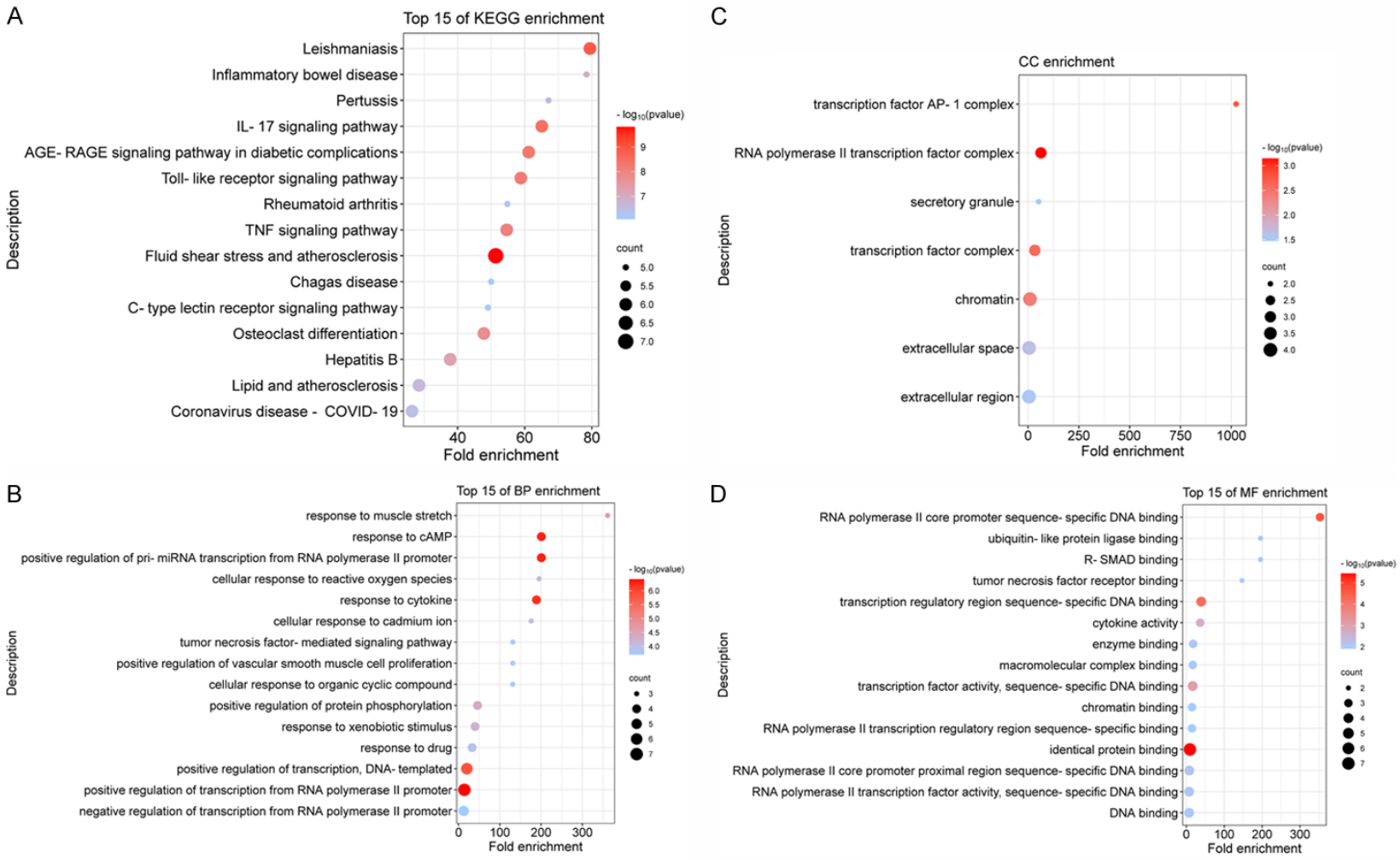

Figure 4. KEGG and GO enrichment analyses. A. Top 15 entries in the KEGG enrichment analysis; B. Top 15 entries in the GO BP enrichment analysis; C. Entries in the GO CC enrichment analysis; D. Top 15 entries in the GO MF enrichment analysis.

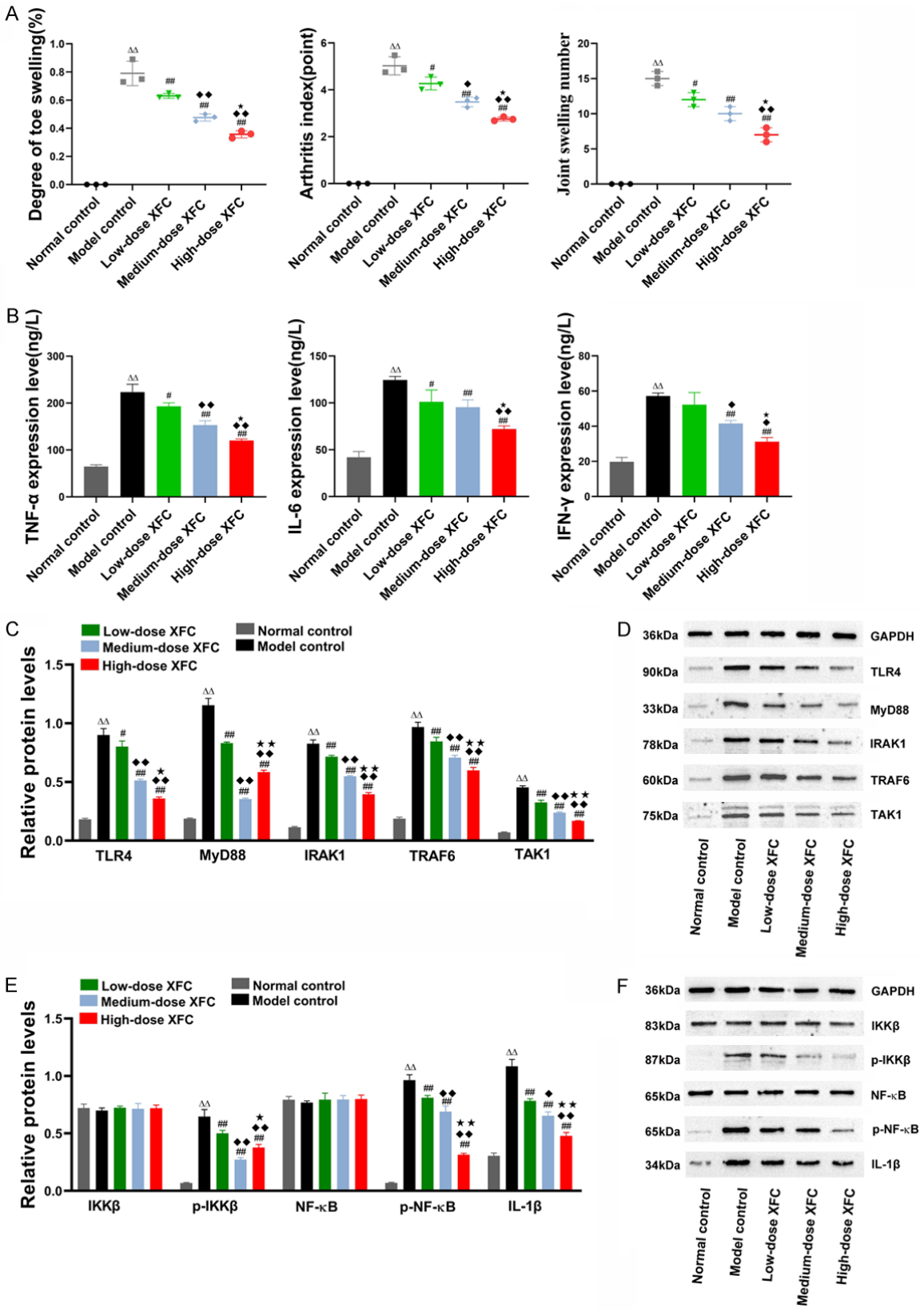

Figure 5. Effects of XFC on joint symptoms, inflammatory factors and TLR signaling pathway in rats with adjuvant arthritis. A. Effect of XFC on joint inflammatory symptoms; B. Effect of XFC on inflammatory factors; C, D. Effect of XFC on TLR4 signaling pathway proteins; E, F. Effect of XFC on TLR4 signaling pathway protein phosphorylation and downstream inflammatory factors. Note: Compared with the Normal control group, $^{\Delta\Delta}P < 0.01$; compared with the Model control group, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$; compared with the Low-dose XFC group, $^{\blacklozenge}P < 0.05$, $^{\blacklozenge\blacklozenge}P < 0.01$; compared with Medium-dose XFC group, $^{\star}P < 0.05$, $^{\star\star}P < 0.01$.

were analyzed through network pharmacology. The results suggested that FOS, JUN, RELA, TNF, VEGFA, STAT1, MMP9, and IL1β were involved in the XFC treatment of the RA disease process. Past studies have established that activated synovial cells secrete inflammatory factors and recruit T cells, B cells, and macrophages to the joint cavity so as to "respond" to each other. Among them, inflammatory factors, such as TNF-α and IL-1β, are the key substances for the mutual "response" between the cells [25, 26]. VEGFA is involved in the migration of monocytes/macrophages and the formation of pannus in RA synovial inflammation [27]. MMP9 can control several aspects of inflammation, such as the regulation of various cytokines and chemokines as well as the survival of inflammatory cells [28]. XFC is prepared from natural medicines such as *T. wilfordii*, and the research and development of natural medicinal plants have been a research hotspot in this direction [29]. Molecular docking is commonly employed to simulate the binding pattern and affinity between small molecular compounds contained in natural drugs and targets [30]. The results suggested that the structure of triptolide and quercetin was stable with the key target, indicating that triptolide and quercetin may be the key active components of XFC. Triptolide is an epoxidized diterpene lactone compound with anti-inflammatory, immunomodulatory, and antitumor effects. WEN et al. reported that triptolide could reduce IL-1β and TNF-α in the treatment of RA as well as significantly reduce the degree of bone erosion [31]. It has been demonstrated that the inhibition of triptolide in the expression of agonist-induced inflammatory chemokines is mediated by targeting TLR early signaling, especially TLR4 signaling in RAW 264.7 cells [32]. Quercetin is a flavonoid compound with several biological activities, such as the inhibition of the expression and secretion of inflammatory mediators, antioxidation, immune regulation, and the inhibition of the production of matrix metalloproteinases [33]. Past studies have reported that quercetin inhibits the mitogen-activated protein kinase (MAPK)/TLR4 pathway to decrease cytokine production and T helper cell 17 (Th17) polarization, thereby exerting an anti-inflammatory effect [34]. Furthermore, quercetin significantly alleviates joint inflammation by reducing the levels of cytokines IL-1β, IL-6, and IL-17 and matrix metalloproteinases MMP3 and MMP9 and has the potential to prevent joint inflammation [35]. These findings suggest that triptolide and quercetin may be the most important components of XFC that alleviate the inflammatory response in RA and that their action mechanism is related to TLR signaling and inflammatory factors.

Enrichment analysis revealed that inflammation-related biological processes are the key factors in the occurrence of RA and that the regulation of the TLR signaling pathway may be the key link for XFC in improving the inflammatory state of RA. The regulation of the TLR signaling pathway may be the key link in the treatment of RA with XFC. Past studies have demonstrated that the TLR signaling pathway is the key to regulating RA inflammation and autoimmunity [36]. TLRs are a class of ancient type I transmembrane protein receptors that are widely expressed in immune cells. TLRs not only play an important role in activating immune responses but also participate in the pathogenesis of inflammatory diseases [37]. Their aberrant activation may be the key to autoimmune inflammatory diseases. Dysfunctional TLR-mediated responses are characteristic of patients with RA and are involved in the establishment of a chronic inflammatory state [38]. The TLR4 signaling pathway has been shown to have a critical role in developing RA illness. The most common cells, fibroblast-like synoviocytes (FLS) and macrophage-like synoviocytes (MLS) in the synovial tissues of RA patients, express TLR4. TLR4 expression increases with the severity of the patient's disease [39, 40]. We examined the activation of upstream and downstream target proteins of the TLR4 signaling pathway and their phosphorylation using an adjuvant arthritis rat model to study the possible function of TLR4 in RA. The MC group exhibited considerably more significant levels of TLR4, MyD88, IRAK1, TRAF6, and TAK1 protein expression when compared to the NC group. Additionally, there was a substantial increase in the downstream levels of IKKβ, NF-κB phosphorylation, and IL1β. This is in line with earlier study findings. TLR4 protein expression was increased in the synovial tissues of mice used as RA model subjects by Li et al. [41]. According to earlier research, MyD88's carboxyl-terminus binds to the intracellular TIR region of TLR4, while MyD88's amino-terminus binds to the amino-terminus of IL-1 IRAK to activate IRAK.

Activated IRAK reactivates TRAF6, and TRAF6 activates the inhibitor of IKKs complex [42]. The IKKs complex phosphorylates the inhibitor of NF-κB, activating NF-κB. NF-κB is subsequently transported into the nucleus to trigger the production of particular genes and activate cytokines like IL-1 and IL-6 [43]. Rats in the MC group had significantly higher levels of the inflammatory markers TNF-α, IL-6, and INF-γ, as well as degree of toe swelling, the number of swollen joints, and the arthritis index compared to the NC group. This suggests that an imbalance of inflammatory chemicals causes symptoms like joint swelling. Relevant studies have exhibited that the serum concentrations of IL-6 and TNF-α in RA are significantly enhanced [44, 45]. TNF-α and IL-6 are abundant in patients with RA. TNF-α is chiefly produced by macrophages and is one of the key actors causing inflammation [46]. IL-6 can induce the accumulation of neutrophils at the site of inflammatory injury and stimulate the differentiation of osteoclasts, which ultimately leads to inflammatory reactions, such as joint swelling and cartilage erosion [47].

After XFC intervention, the degree of toe swelling, number of swollen joints, and arthritis index were all significantly decreased; at the same time, the levels of TNF-α and IL-6 were significantly reduced. Compared to the MC group, the protein expression levels of TLR4, MyD88, IRAK1, TRAF6, TAK1, p-IKKβ, p-NF-κB, and IL-1β were significantly reduced in the XFC high, medium, and low dose groups. Cytokine networks and signaling pathways may interact in the mechanism by which XFC controls inflammatory factor imbalance. This suggests that XFC's active ingredients, quercetin and aphidicolin, may inhibit the phosphorylation of IKKβ and NF-κB, as well as the overexpression of the TLR4 signaling pathway. This, in turn, reduces the production of downstream pro-inflammatory cytokines, like IL-6, IL-1β, and TNF-α, thus ameliorating RA's inflammatory state.

Conclusions

In this study, by establishing the Apriori association rules and a random walk model, it was found that XFC significantly improved the RA inflammatory indexes, such as ESR, CRP, and VEGF. The results of network pharmacology and molecular docking indicated that XFC has the potential to improve the inflammatory state of RA by regulating the TLR signaling pathway via the active ingredients triptolide and quercetin. Animal research demonstrated that XFC reduced the upregulation of the TLR4 signaling pathway and the levels of inflammatory factors IL-6, TNF-α, and IL-1β, thereby attenuating the joint inflammatory state associated with RA. However, these studies had some limitations. Owing to time constraints, this study was only verified through animal experiments and did not include cell experiments. In the future, we plan to perform RA synovial cell culture to further verify these conclusions from a cellular perspective. Second, this study only explored the mechanism by which XFC improved the inflammatory response of RA through the toll-like receptor signaling pathway. However, whether XFC can regulate other signaling pathways to alleviate RA warrants further confirmation.

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Written informed consent was obtained from each patient.

Disclosure of conflict of interest

None.

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Supplementary Table 1. Apriori association rule analysis for XFC and RA inflammatory indexes

Items (LHS ⇒ RHS)	Support	Confidence	Lift
{XFC} ⇒ {ESR}	92.95%	51.34%	1.014
{XFC} ⇒ {VEGF}	91.70%	52.94%	1.046
{XFC} ⇒ {CPR}	90.87%	50.23%	0.992
{XFC} ⇒ {SAA}	84.65%	49.02%	0.968

Supplementary Table 2. 64 potential targets of XFC for RA treatment

Number	Targets
1	ADH1B
2	ADH1C
3	ADRA2A
4	ADRA2C
5	ALOX5
6	AR
7	BAX
8	BIRC3
9	BIRC5
10	CA2
11	CASP8
12	CCR7
13	CD14
14	CD40LG
15	CD86
16	CDK2
17	CDKN1A
18	CHRM3
19	COL1A1
20	COL3A1
21	CXCL10
22	CXCL11
23	CXCL2
24	CXCL8
25	CXCR4
26	DPP4
27	DUOX2
28	EGFR
29	EPHB2
30	ESR1
31	FOS
32	GABRA2
33	IGHG1
34	IL1β
35	IL6
36	INSR
37	JUN
38	KCNMA1
39	MAOA

40	MAPK8
41	MMP1
42	MMP3
43	MMP9
44	MYC
45	NCF1
46	NFKBIA
47	OLR1
48	PIK3CG
49	PIM1
50	PKIA
51	PPARG
52	PRKCB
53	PTEN
54	PTGS2
55	RELA
56	SERPINE1
57	SLC6A3
58	SLPI
59	SPP1
60	STAT1
61	TIMP1
62	TNF
63	TOP2A
64	VEGFA

Supplementary Table 3. Active ingredients of XFC

MOL ID	Active ingredient Name	Drug
MOL000211	Mairin	Astragalus membranaceus
MOL000239	Jaranol	Astragalus membranaceus
MOL000296	Hederagenin	Astragalus membranaceus
MOL000033	(3S,8S,9S,10R,13R,14S,17R)-10,13-dimethyl-17-[(2R,5S)-5-propan-2-yloctan-2-yl]-2,3,4,7,8,9,11,12,14,15,16,17-dodecahydro-1H-cyclopenta[a]phenanthren-3-ol	Astragalus membranaceus
MOL000354	Isorhamnetin	Astragalus membranaceus
MOL000371	3,9-di-O-methylnissolin	Astragalus membranaceus
MOL000378	7-O-methylisomucronulatol	Astragalus membranaceus
MOL000379	9,10-dimethoxypterocarpan-3-O-β-D-glucoside	Astragalus membranaceus
MOL000380	(6aR,11aR)-9,10-dimethoxy-6a,11a-dihydro-6H-benzofurano[3,2-c]chromen-3-ol	Astragalus membranaceus
MOL000387	Bifendate	Astragalus membranaceus
MOL000392	Formononetin	Astragalus membranaceus
MOL000417	Calycosin	Astragalus membranaceus
MOL000422	Kaempferol	Astragalus membranaceus
MOL000433	FA	Astragalus membranaceus
MOL000442	1,7-Dihydroxy-3,9-dimethoxy pterocarpene	Astragalus membranaceus
MOL000098	Quercetin	Astragalus membranaceus
MOL003182	(+)-Medioresinol di-O-beta-D-glucopyranoside_qt	Tripterygium wilfordii
MOL003184	81827-74-9	Tripterygium wilfordii
MOL003185	(1R,4aR,10aS)-5-hydroxy-1-(hydroxymethyl)-7-isopropyl-8-methoxy-1,4a-dimethyl-4,9,10,10a-tetrahydro-3H-phenanthren-2-one	Tripterygium wilfordii
MOL003187	Triptolide	Tripterygium wilfordii
MOL003196	Tryptophenolide	Tripterygium wilfordii
MOL003199	5,8-Dihydroxy-7-(4-hydroxy-5-methyl-coumarin-3)-coumarin	Tripterygium wilfordii
MOL003209	Celallocinnine	Tripterygium wilfordii
MOL003217	Isoxanthohumol	Tripterygium wilfordii
MOL003224	Tripdiotolnide	Tripterygium wilfordii
MOL003225	Hypodiolide A	Tripterygium wilfordii
MOL003229	Triptinin B	Tripterygium wilfordii
MOL003231	Triptoditerpenic acid B	Tripterygium wilfordii
MOL003245	Triptonoditerpenic acid	Tripterygium wilfordii
MOL003248	Triptonoterpene	Tripterygium wilfordii
MOL003266	21-Hydroxy-30-norhopan-22-one	Tripterygium wilfordii
MOL003280	TRIPTONOLIDE	Tripterygium wilfordii
MOL000358	Beta-sitosterol	Tripterygium wilfordii
MOL000449	Stigmasterol	Tripterygium wilfordii
MOL002058	40957-99-1	Tripterygium wilfordii
MOL003283	(2R,3R,4S)-4-(4-hydroxy-3-methoxy-phenyl)-7-methoxy-2,3-dimethylol-tetralin-6-ol	Tripterygium wilfordii
MOL004443	Zhebeiresinol	Tripterygium wilfordii
MOL005828	Nobiletin	Tripterygium wilfordii
MOL007415	[(2S)-2-[[(2S)-2-(benzoylamino)-3-phenylpropanoyl]amino]-3-phenylpropyl] acetate	Tripterygium wilfordii
MOL007535	(5S,8S,9S,10R,13R,14S,17R)-17-[(1R,4R)-4-ethyl-1,5-dimethylhexyl]-10,13-dimethyl-2,4,5,7,8,9,11,12,14,15,16,17-dodecahydro-1H-cyclopenta[a]phenanthrene-3,6-dione	Tripterygium wilfordii
MOL009386	3,3'-bis-(3,4-dihydro-4-hydroxy-6-methoxy)-2H-1-benzopyran	Tripterygium wilfordii
MOL001323	Sitosterol alpha1	Coix Seed
MOL001494	Mandenol	Coix Seed
MOL000359	Sitosterol	Coix Seed
MOL008121	2-Monoolein	Coix Seed
MOL000953	CLR	Coix Seed