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Article

Exploring the Mechanism of Topical Application of Clematis Florida in the Treatment of Rheumatoid Arthritis through Network Pharmacology and Experimental Validation

Ting Lei [1,2], Chang Jiang [1], Li Zhao [1], Jizhou Zhang [1], Qing Xiao [1], Yanhong Chen [1,2], Jie Zhang, Chunquan Zhou [1,*], Gong Wang [1,*] and Jing Han [1,*]

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Abstract: Clematis Florida (CF) is a folk medicinal herb in the southeast of China, which is traditionally used for treating osteoarticular diseases. However, the mechanism of action remains unclear. The present study used network pharmacology and experimental validation to explore the mechanism of CF in the treatment of rheumatoid arthritis (RA). Liquid chromatography-mass spectrometry (LC-MS/MS) identified 50 main compounds of CF, then the targets of them were obtained from TCMSP, ETCM, ITCM and Swiss TargetPrediction databases. RA disease related targets were obtained from DisGenet, OMIM and GeneCards databases. 99 overlapped targets were obtained using Venn diagram. The protein-protein interaction network (PPI)、the compound-target network (CT) and the compound-potential target genes-signaling pathways network (CPS) were built and analyzed. The results showed that the core compounds were screened as oleanolic acid, oleic acid, ferulic acid, caffeic acid, and syringic acid. And the core therapeutic targets were identified as PTGS2 (COX-2), MAPK1, NF-κB1, TNF, RELA by network pharmacology analysis, which belong to the MAPK signaling pathway and NF-κB signaling pathway. The animal experiments indicated that topical application of CF showed significant anti-inflammatory activity in mice model of xylene-induced ear edema and strong analgesic effect on acetic acid-induced withing. Furthermore, in the rat model of adjuvant arthritis (AA), topical administration of CF could alleviate toe swelling and ameliorate joint damage. The elevation of serum content of IL-6, COX-2, TNF-α, IL-1β, and RF caused by adjuvant arthritis were reduced by CF treatment. Western blotting tests showed that CF may exert regulation on the ERK and NF-κB pathway. The results provided a new perspective for the topical application of CF on RA.

Keywords: Clematis Florida; rheumatoid arthritis; network pharmacology; NF-κB; MAPK

1. Introduction

RA is a chronic inflammatory disease primarily affecting the joints [1]. It is the most common form of inflammatory arthritis characterized by chronic synovitis, synovial proliferation, and cellular infiltration. Further, it leads to bone erosion, destruction of articular cartilage, intense joint pain, swelling, and a high rate of disability [2]. Current statistics indicate that RA affects approximately 0.5% to 1% of the global population and the disease affects women more than men [3,4]. The cause of RA is unclear and may be related to genetic, environmental, and immunological factors [5]. Conventional medicine primarily treats RA through non-steroidal anti-inflammatory drugs (NSAIDs), glucocorticoids, and disease-modifying antirheumatic drugs (DMARDs), including newer biologic agents [6]. These treatments aim to manage symptoms and slow disease progression but are not curative. Many anti-RA drugs have limitations such as poor bioavailability or gastrointestinal side effects, which decreased patient compliance. Topical route of drug administration, which bypass

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the first past effect experienced with conventional oral administration, has emerged as a novel option, garnering increasing attention [7]. Topical drug administration provides rapid relief of localized swelling and pain, resulting in superior patience compliance [8].

Clematis Florida (CF) is a folk medicine in southeast China. It was recorded in the Chinese traditional medicine book “Zhong Hua Ben Cao”, which describes its therapeutic effects of energizing the meridians, dispelling wind, activating blood circulation, and relieving pain [9]. Traditionally, CF is prepared by soaking in camellia oil and applied topically to treat trauma, joint inflammation, rheumatic tendon pain, and other inflammatory conditions. Despite its centuries-long usage in folk practices, comprehensive scientific validation to support its efficacy remains scarce. CF belongs to the genus clematis in the ranunculaceae family. Many plants of the genus clematis have a wide range of pharmacological effects including relieving rheumatism pain, treating cervical spondylopathy and scapulohumeral periarthritis, treating hepatic carcinoma and gastrointestinal, etc [10]. Notably, saponin-like compounds and triterpene saponins that extracted from CF have demonstrated in vivo antitumor activity and the capacity to inhibit inflammatory mediators [11,12]. However, the specific compounds and the potential targets of CF for RA treatment still remain unclear. Moreover, the traditional usage of CF is for topical treatment of osteoarthritis-related diseases, but the pharmacological efficacy of topical CF application has not yet been reported. Such exploration not only bridges traditional knowledge with modern medical practice but also potentially expands the therapeutic arsenal against RA, offering hope for more effective and diverse treatment strategies.

The many compounds of Chinese medicines make it complicated to explain the major and minor compounds as well as the main therapeutic targets for the treatment of diseases. The method of network pharmacology makes it possible to elucidate this mechanism [13]. Network pharmacology combines pharmacological and systems biology approaches, which can be used to explain the mechanisms by which Chinese medicines treat diseases. The method emphasizes that the coordination of multiple signaling pathways can improve the therapeutic effect, from the traditional Chinese medicine (TCM) research model of "single drug, single target" to "multi-compound, multi-target, multi-path", revealing the "drug-gene-target" relationship. This may be a good way to explain the mechanism of TCM in treating diseases [14]. In recent years, many studies have used network pharmacology to predict the interactions between compounds in TCM and target genes for specific diseases [15].

In this research, we used LC-MS/MS to detect and identify the chemical constituents of CF. Then network pharmacology method was used to predict potential targets and molecular signal pathways of CF in the treatment of RA, which was verified by animal experiments followed. These experiments aimed at providing essential experimental data for the potential therapeutic application of CF in RA treatment. The process flow of the study, detailing each step from chemical analysis to experimental verification, is clearly depicted in Figure 1.

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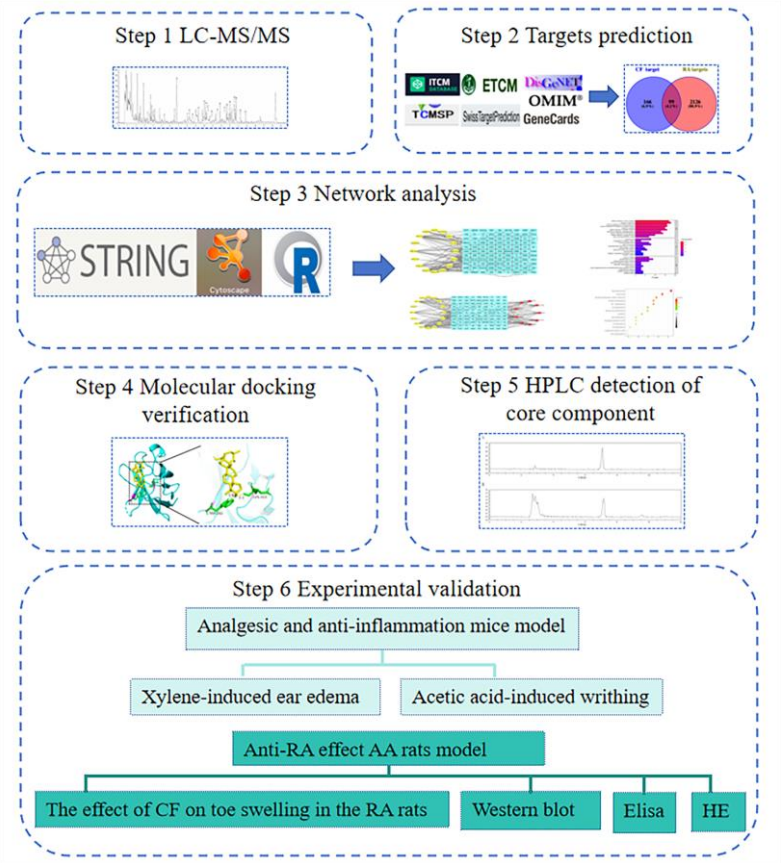

Figure 1. Flowchart of the methodology for studying the mechanism of action of topical application of CF in the treatment of RA.

2. Results

2.1. Main Compounds in CF Extract

To determine the composition of CF, the CF samples were analyzed by LC-MS/MS in both positive and negative ionization modes (Figure 2). In the base peak chromatogram (BPC) of CF sample, a total of 50 compounds were identified (Supplementary file 1). 166 CF targets were identified by searching these compounds in the TCMSP, ETCM, ITCM databases (Supplementary file 2). 2126 RA related targets were identified from DisGenet, OMIM and GeneCards databases (Supplementary file 3). Finally, 99 overlapped targets were identified by Venn diagram (Figure 3a). A total of 16 compounds that reactive to 99 overlapped targets were considered main compounds in CF for the treatment of RA.

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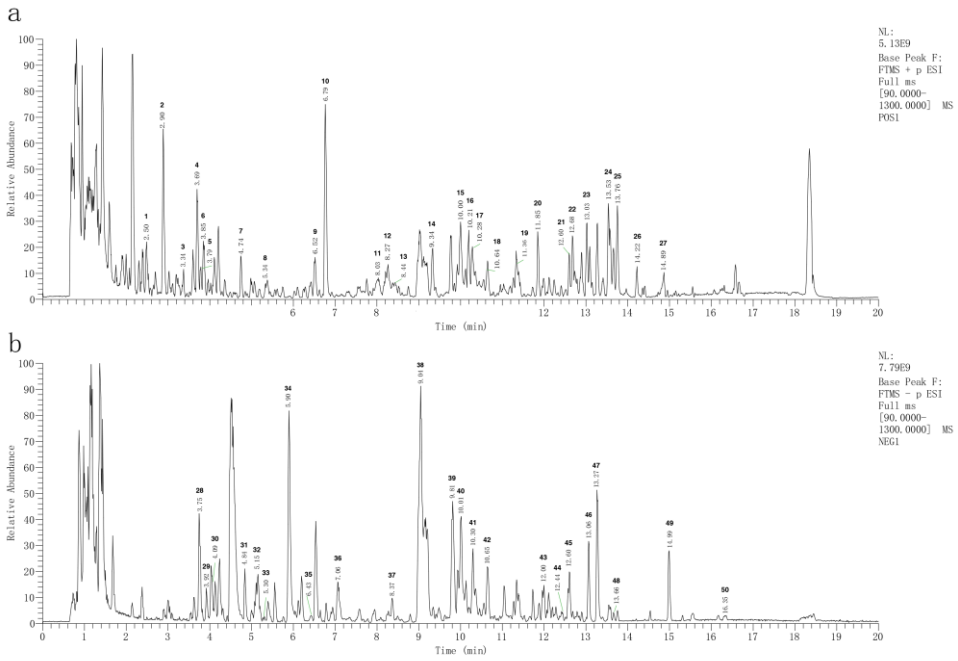

Figure 2. Total ion current diagram of CF extraction solution; (a) The positive ion detection mode. (b) The negative ion detection mode.

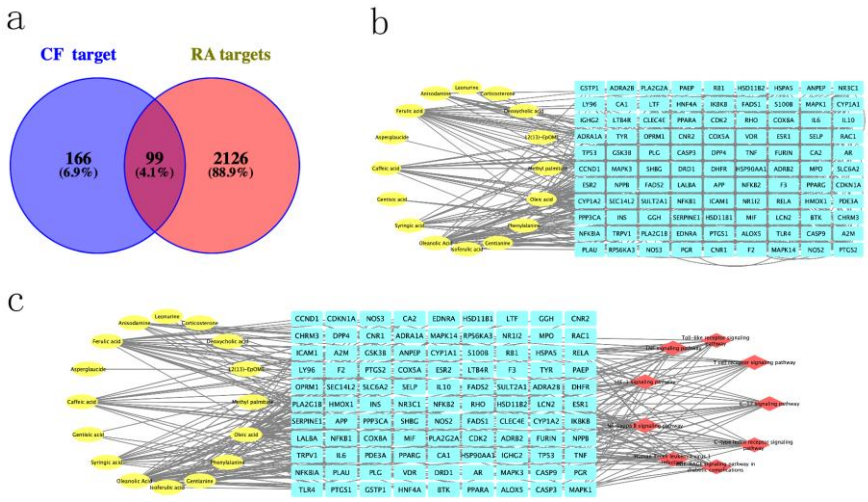

Figure 3. Network pharmacology analysis of CF for treating RA; (a) Venn diagrams of potential targets of CF and RA; (b) Plot of association between CF compounds and RA. 16 yellow nodes on the left represent the main compounds of CF. 99 CF targets for the treatment of RA were labeled blue on the right side; (c) CTS network of CF in treatment of RA. Yellow nodes indicate the main compounds of CF, red nodes denote the 9 signaling pathways identified from the KEGG analysis of the PPI network, and blue nodes symbolize the overlapped target genes linked to both CF and RA.

2.2. Construction of CT Network and Screening Core Compounds in CF

A Compound-Target (CT) network was constructed to visualize the interaction of CF compounds and candidate RA targets using Cytoscape software (Figure 3b). 99 targets were regulated by 16 compounds in the CT network. Based on the CytoNCA analysis, the average degree value of all compounds was found to be 11.75. Compounds with degree values greater than average were considered core compounds (Supplementary file 4). 5 core compounds were screened, namely oleic acid, oleanolic acid, ferulic acid, caffeic acid, and syringic acid.

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2.3. Screening of Main Targets

String database was used to analyze the KEGG pathway of overlapped targets. All pathways were sorted according to false discovery rate (FDR) values. The top 30 pathways with lowest FDR values were listed (Supplementary file 5). 9 pathways of the top 30 pathways that show the highest correlation with inflammation were identified (Table 1). The corresponding targets in these pathways have been identified as the main targets of CF in the treatment of RA.

Table 1. Targets corresponding to inflammatory pathways.

Pathway	Target
IL-17 signaling pathway	LCN2, GSK3B, IL6, NF-κB1, TNF, PTGS2, CASP3, HSP90AA1, MAKP1, IKBKB, MAPK3, MAPK14, NF-κBIA, RELA
C-type lectin receptor signaling pathway	NF-κB1, IKBKB, NF-κBIA, RELA, PTGS2, PPP3CA, MAPK14, MAPK3, MAPK1, TNF, IL6, IL10, NF-κB2, CLEC4E
AGE-RAGE signaling pathway in diabetic complications	ICAM1, NOS3, F3, TNF, IL6, NF-κB1, SERPINE1, MAPK3, CASP3, RELA, MAPK1, CCND1, MAPK14
Human T-cell leukemia virus 1 infection	CDK2, RB1, CCND1, CDKN1A, MAPK3, PPP3CA, TP53, NFKBIA, NF-κB1, TNF, MAPK1, RELA, IL6, IKBKB, NF-κB2, ICAM1
NF-kappa B signaling pathway	PLAU, ICAM1, PTGS2, RELA, TNF, NF-κB1, NF-κB2, IKBKB, TLR4, NF-κBIA, LY96, BTK
HIF-1 signaling pathway	NOS2, NOS3, NF-κB1, INS, CDKN1A, SERPINE1, HMOX1, TLR4 MAPK3, MAPK1, RELA, IL6
TNF signaling pathway	TNF, IL6, RELA, NF-κB1, CASP3, IKBKB, PTGS2, MAPK1, MAPK3 , ICAM1, MAPK14, NF-κBIA
Toll-like receptor signaling pathway	TNF, NF-κB1, LY96, TLR4, RELA, MAPK1, MAPK3, MAPK14, IL6, IKBKB, NF-κBIA
T cell receptor signaling pathway	IL10, IKBKB, TNF, NF-κB1, NF-κBIA, GSK3B, MAPK14, RELA, MAPK1, MAPK3, PPP3CA

2.4. GO and KEGG Pathway Enrichment Analysis

According to the GO enrichment analysis, the top 10 with the smallest p value of each part were selected as prominent biological processes, including stress-activated MAPK cascade and I-kappaB/NF-kappaB complex (Figure 4a). The results of KEGG pathway analysis showed that CF treatment of RA involved AGE-RAGE signaling pathway in diabetic complications, NF-kappa B signaling pathway, HIF-1 signaling pathway, VEGF signaling pathway (Figure 4b).

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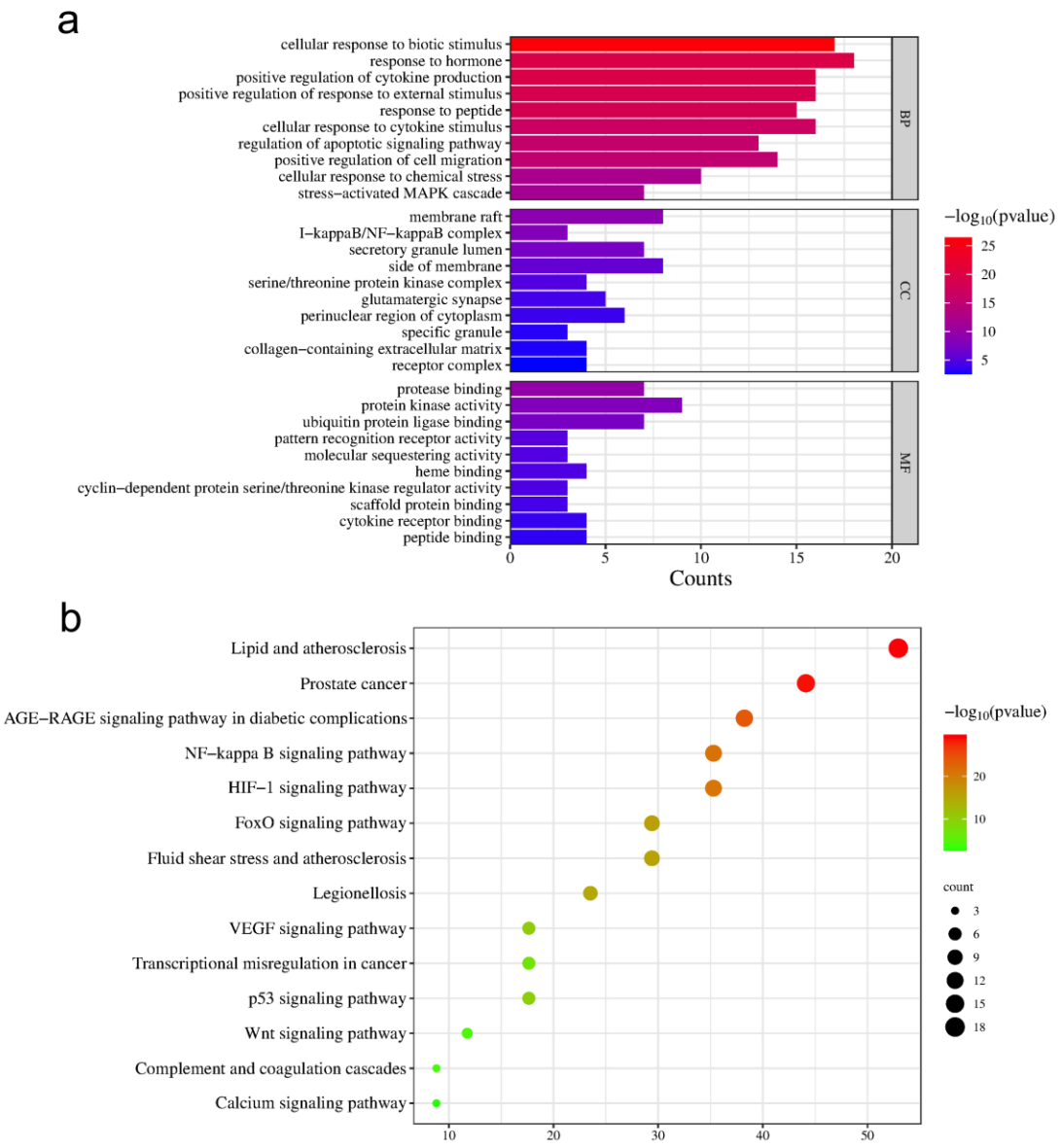

Figure 4. GO and KEGG functional analysis. (a) GO analysis for CF treatment of RA; (b) KEGG analysis for CF treatment of RA.

2.5. Construction of CPS Network and Screening of Core Targets

Cytoscape was used to construct a compound-potential target genes-signaling pathways (CPS) network (Figure 3c). Utilizing the Cyto-NCA tool within Cytoscape, we identified the core targets of CF. Targets were ranked based on their degree value from the lowest to the highest (Table 2). Furthermore, targets with a degree value 3 times higher than the average were identified as the core targets. These core targets are PTGS2, MAPK1, NFκB1, TNF, and RELA.

Table 2. Ranking of targets according to degree value from high to low.

Target	Full name of target	Target alias	Degree
PTGS2	2Prostaglandin G/H Synthase 2	COX-2	15
MAPK1	Mitogen-Activated Protein Kinase 1	ERK2	11
NFκB1	Nuclear factor kappa-B	P50	11
TNF	Tumor necrosis factor	TNF-alpha	11

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RELA	V-rel reticuloendotheliosis viral oncogene homolog A	NFκB P65	11
MAPK3	Mitogen-activated protein kinase 3	ERK1	9
PTGS1	Prostaglandin-endoperoxide synthase 1	COX-1	9
IL6	Interleukin 6	HGF	9
NFκBIA	NF-Kappa-B Inhibitor Alpha Antibody	IKBA	8
IKBKB	Inhibitor of nuclear factor kappa-B kinase subunit beta	IKK2	8
MAPK14	Recombinant Human Mitogen-Activated Protein Kinase 14	CSPS	8
ADRB2	beta-2 adrenergic receptor	ADRB2R	8

2.6. Molecular Docking Effectiveness

Molecular docking was used to validate the interaction between core compounds of CF (oleanolic acid, syringic acid, oleic acid, caffeic acid and ferulic acid) and the core targets identified by CPS network (MAKP1, NFκB1, TNF-α and PTGS2) (Figure 5). The free binding energies between the compounds and their corresponding targets were relatively high (Table 3). That means these core compounds of CF may play a significant role in RA treatment by targeting these core targets.

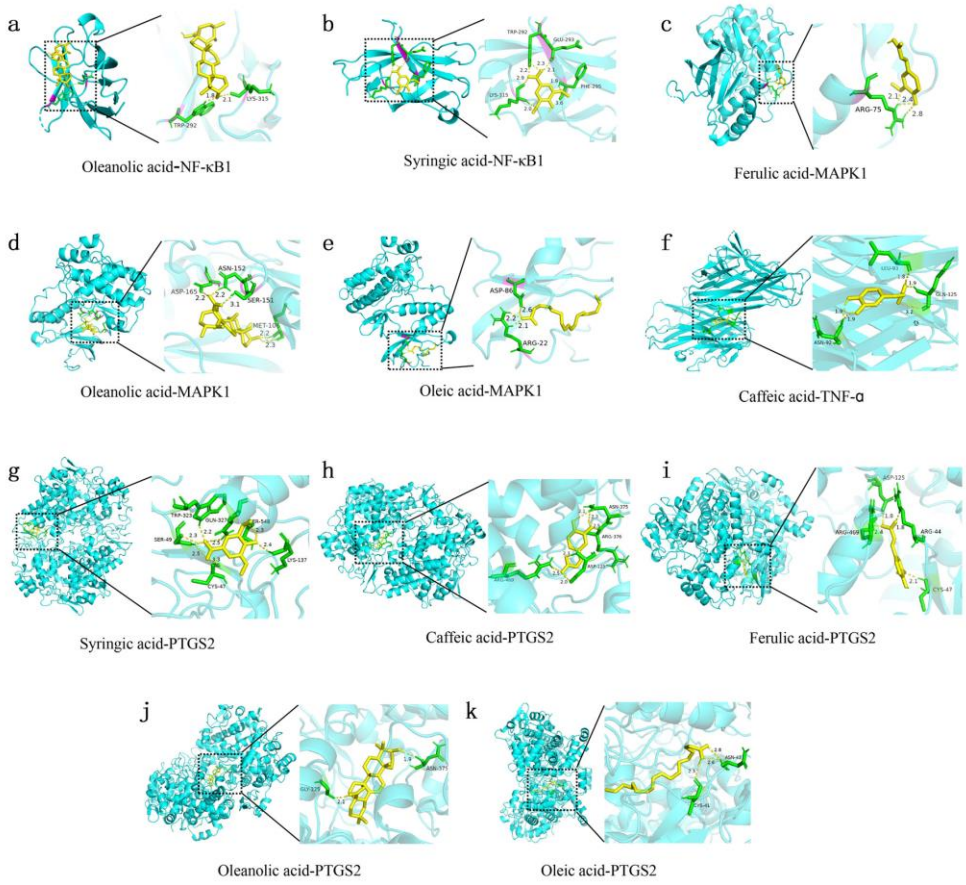

Figure 5. Pattern diagram of molecular docking. (a) Molecular docking of oleanolic and NF κ B1; (b) Molecular docking of syringic acid and NF κ B1; (c) Molecular docking of ferulic acid and MAPK1; (d) Molecular docking of oleanolic acid and MAPK1; (e) Molecular docking of oleic acid and MAPK1; (f) Molecular docking of caffeic acid and TNF-α; (g) Molecular docking of syringic acid and PTGTS2; (h) Molecular docking of caffeic acid and PTGTS2; (i) Molecular docking of ferulic acid and PTGTS2; (j) Molecular docking of oleanolic acid and PTGTS2; (k) Molecular docking of oleic acid and PTGTS2.

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Table 3. Molecular docking scores of the five core compounds and four target.

Target	PDB ID	Compound	Affinity/(kcal.mol-1)
NF-κB1	1U36	Oleanolic acid	-6.6
NF-κB1	1U36	Syringic acid	-3.87
MAPK1	4S31	Ferulic acid	-4.57
MAPK1	4S31	Oleanolic acid	-8.14
MAPK1	4S31	Oleic acid	-3.15
TNF-α	2AZ5	Caffeic acid	-5.37
PTGTS2	5F19	Syringic acid	-5.6
PTGTS2	5F19	Caffeic acid	-5.83
PTGTS2	5F19	Ferulic acid	-5.2
PTGTS2	5F19	Oleanolic Acid	-10.31
PTGTS2	5F19	Oleic acid	-5.27

2.7. HPLC Measurement of Quality Control Substance Oleanolic Acid Content

The HPLC results indicate that the oleanolic acid content in CF is 1.34% (Figure 6). This quantification is crucial because oleanolic acid is one of the key bioactive compounds contributing to the therapeutic properties of CF.

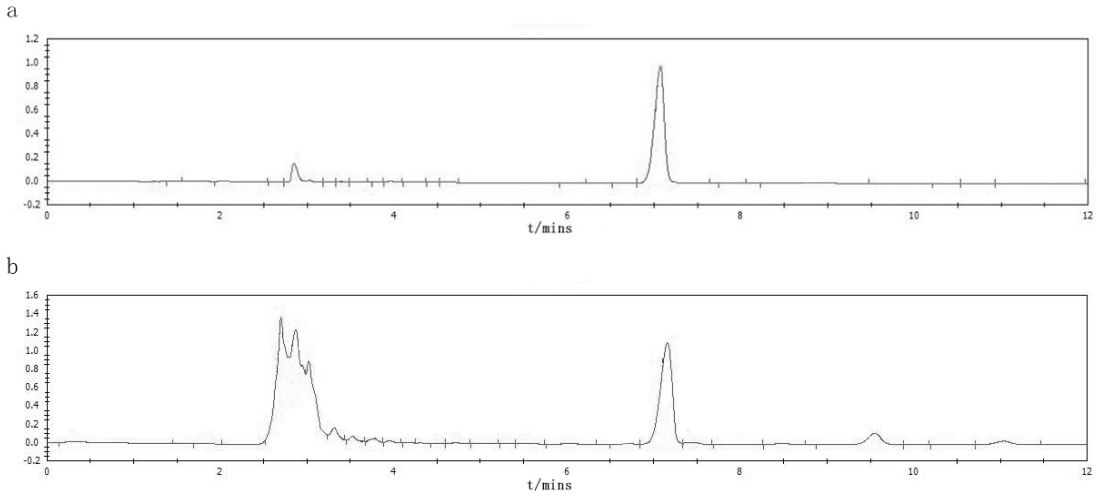

Figure 6. Results of HPLC. (a) Chromatogram of control; (b) Chromatogram of the CF sample.

2.8. CF Alleviated Xylene-Induced Mouse Ear Edema

The anti-inflammatory effect of CF was assessed using xylene-induced mouse ear edema. As shown in Figure 7a, xylene application induced significant ear edema. After the intervention of CF-H administration, the degree of ear edema significantly decreased and the effects showed a dose-dependent manner ($P < 0.001$).

2.9. CF Showed Analgesic Activity in Acetic Acid-Induced Writhing Model

In the treatment of RA, it is important for drugs to exert analgesic effects to alleviate pain and stiffness. Acetic acid-induced writhing tests were performed to examine the analgesic activity of CF (Figure 7b). In the acetic acid-induced writhing test, CF-H treatment significantly decreased the number of writhes ($P < 0.001$). The effects also showed a dose-dependent manner. The data indicated that CF was effective in ameliorating the pain induced by chemical stimulation.

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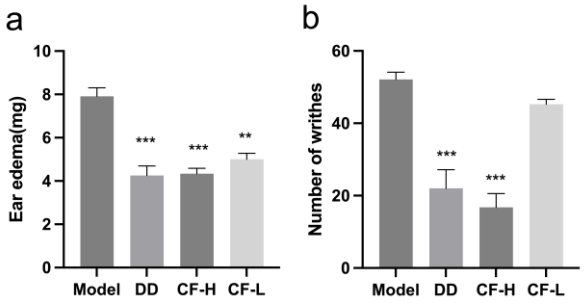

Figure 7. Effects of CF on inflammation and pain in mice (Mean±SEM, n=8). (a) Anti-inflammatory effect of CF on xylene-induced ear edema in mice; (b) Analgesic effect of CF in the acetic acid-induced writhing model. $^{**}P$ <0.01, $^{***}P$ <0.001 vs. Model group.

2.10. CF Alleviated the Severity of Arthritis in Rats

Figure 8a-b showed the effect of CF on toe swelling in the adjuvant arthritis (AA) model rats. Animals in model group showed significant toe swelling after injection of Freund's adjuvant (P < 0.001). The degree of toe swelling in CF-H group was significantly reduced compared to the model group when the treatment continued for 14 days（P < 0.01）. There was no significant difference in the effect of reducing toe swelling between the CH-H and DD groups. The CF-L group showed no efficacy in reducing toe swelling, indicating a dose-dependent response of the treatment. The results indicate the efficacy of the CF-H treatment in alleviating RA symptoms. During the experiment, no significant difference in the body weight of the animals in different groups were observed (Figure 8c), indicating that the test did not affect general condition of the rats. This result may be due to the fact that the administration route was topical, which has little influence on the general condition of rats.

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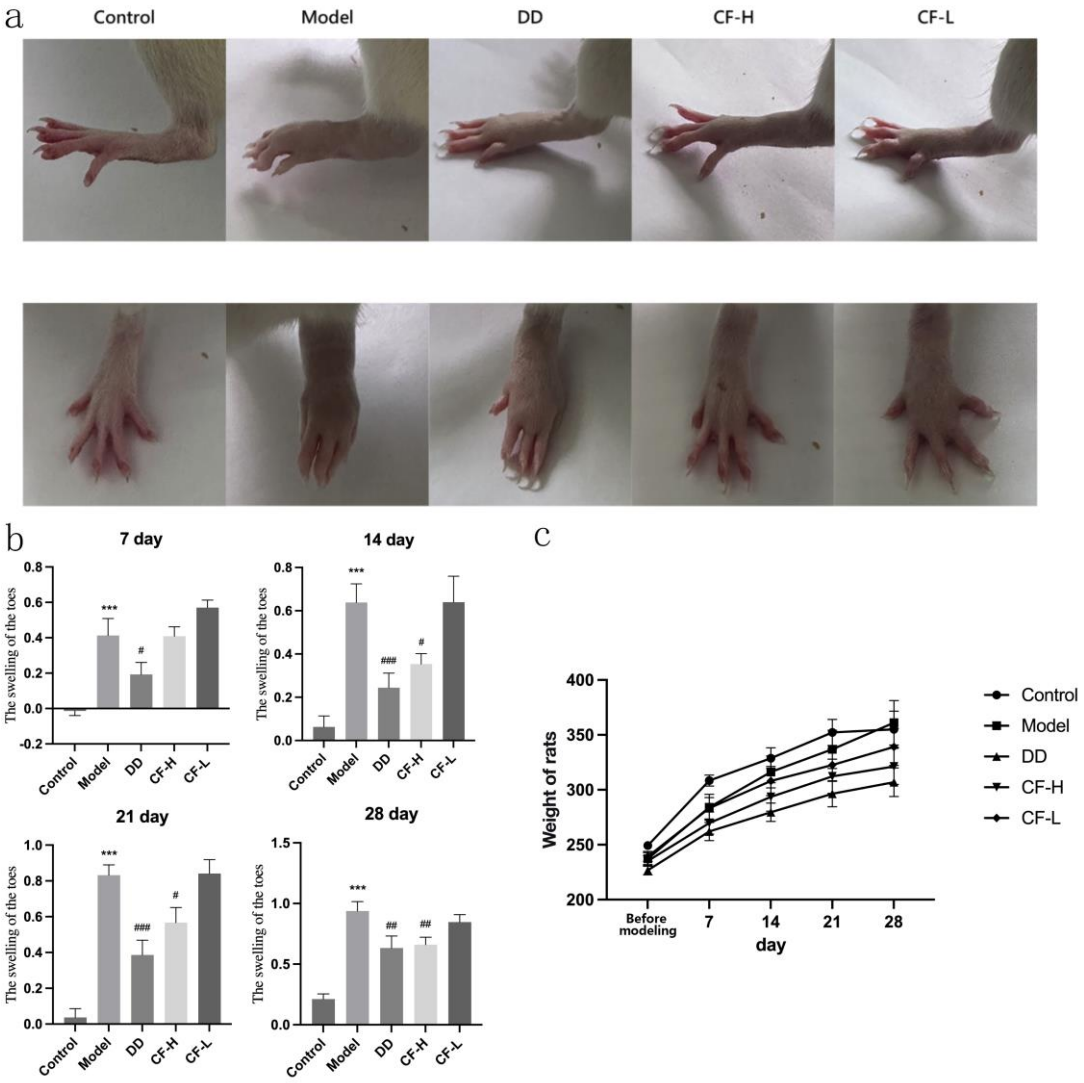

Figure 8. Amelioration effects of CF on the arthritis in AA rats (Mean±SEM, n=8). (a) Macroscopic changes of arthritis of the hind limbs in rats were shown; (b) Comparison of toe swelling in rats of different dosing times in each group; (c) Changes in body weight of rats. $^{***}P$ <0.001 vs. Control group; $^{\#}P$<0.05, $^{\#\#}P$<0.01, $^{\#\#\#}P$<0.001 vs. Model group.

2.11. Effects of CF Extracts on Serum IL-6, COX-2, TNF-α, IL-1β and RF in RA Rats

Figure 9a-e presents the serum levels of inflammatory factors in RA rats. In the RA model group, there was a significant increase in the serum levels of IL-6, COX-2, TNF-α, RF and IL-1β compared to the control group ($P<0.01$). The CF-H groups showed a significant reduction in the serum levels of the inflammatory factors ($P<0.01$). As positive control, DD treatment could significantly reduce the levels of RF, COX-2, TNF-α and IL-1β ($P<0.05$). Compared with DD group, the CF-H group had more significant effect on reducing the serum COX-2 level ($P<0.001$). As for IL-6 and TNF-α, the reduction effects of CF-H group were better than the DD group, although there was no significant difference in the comparison. CF-L groups exhibited notably lower serum levels of COX-2, TNF-α, IL-6 and IL-1β compared to the model group ($P<0.05$, $P<0.01$). The results indicated CF could reduce the level of serum inflammatory factors in a dose-dependent manner, and the effect of CF-H group is even better than that of positive control. This finding verified the targets identified in the network pharmacology analysis of CF for RA treatment.

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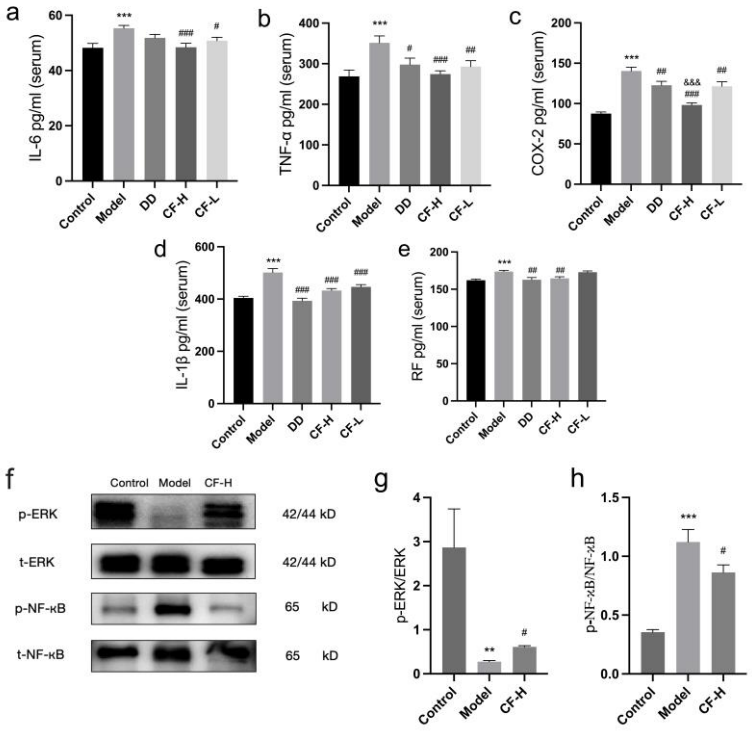

Figure 9. Effects of CF extracts on serum inflammation factors and ptoteins expression in RA rats. (a) serum IL-6 level; (b) serum TNF-ɑ level; (c) serum COX-2 level; (d) serum IL-1β level; (e) serum RF level (Mean±SEM , n=8); (f) Western blot analysis; (g) Effect of CF on p-ERK, ERK proteins in rats; (h) Effect of CF on NF-κB, p-NF-κB proteins in rats.(Mean±SEM , n=5). $^{**}P$<0.01, $^{***}P$<0.001 vs. Control group; $^{\#}P$<0.05， $^{\#\#}P$<0.01,$^{\#\#\#}P$<0.001 vs. Model group; $^{\&\&\&}P$<0.001 vs. DD group.

2.12. Protein Expression of p-ERK, ERK, p-NF- κ B and NF- κ B in Rats

Compared with the control group, the expression of p-ERK/ERK in the joint tissues of rats in the model group was significantly reduced (P<0.01), and the expression of p-NF-κB/NF-κB was significantly increased (*P*<0.001). Compared with the model group, the expression of p-ERK/ERK showed an increased and the expression of p-NF-κB/NF-κB showed a decrease in CF-H group (*P*<0.05). These results indicate that CF can modulate the expression of proteins in the ERK and NF-κB signaling pathways, thereby exerting its anti-RA effects (Figure 9f-h).

2.13. Histological Analysis of Rats

The ankle joint tissues of rats in the control group showed no signs of inflammation or damage. The synovial tissue was structurally intact, with no inflammatory infiltration, indicative of normal joint health. The rats in model group exhibited significant pathological changes. A large number of inflammatory cells infiltrated the joint cavity, and the articular cartilage structure was destructed notably. In the groups treated with CF at different dosages, a significant improvement was observed in joint tissue health. Only a small amount of inflammatory cell infiltration in the ankle joints, and the structure of the articular cartilage remained largely intact. Notably, the CF-H treatment group showed a more pronounced improvement compared to the CF-L group, suggesting a dose-dependent effect of the treatment. In the group treated with DD, the infiltration of inflammatory cells and joint destruction were reduced compared to the model group (Figure 10). The findings suggest that CF has a therapeutic effect on RA.

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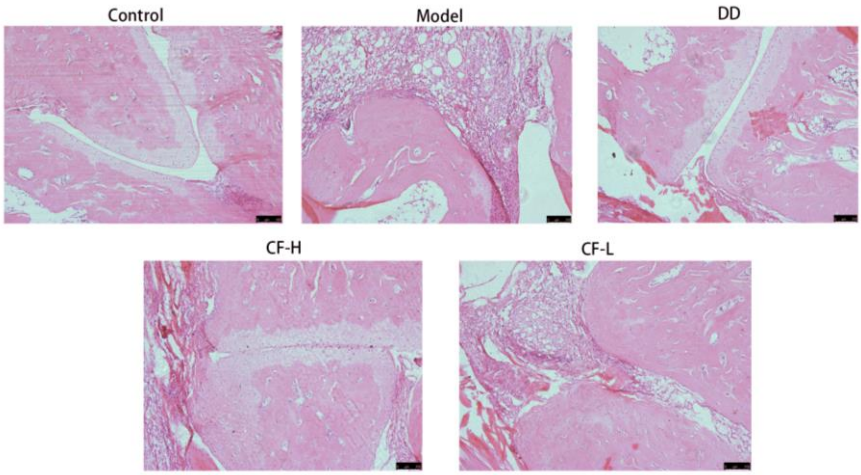

Figure 10. Morphology of synovial tissue of ankle joint of rats in various groups.

3. Discussion

In this study, an integrated strategy combining LC-MS/MS and network pharmacology was used to elucidate the possible effective compounds and core therapeutic targets of CF in anti-RA effects. Then, animal experiments were conducted to observe the therapeutic effects of topical administration of CF on RA and to verify the targets screened by network pharmacological. Our results showed that topical application of CF could mitigated inflammation, showed analgesic activity, improve the symptoms of RA model rats and reduce the levels of serum inflammatory factors IL-6, COX-2, TNF-α, IL-1β and RF. Moreover, CF has significant effects on NF-κB and ERK MAPK pathways in joint tissue. The results validate the findings of network pharmacology analysis.

There have been no reports on the analysis of the complete composition of CF using LC-MS/MS methods before. In the present study, LC-MS/MS revealed that CF contains a rich variety of chemical compounds. Through the analysis of the CT network, the core compounds of CF in its anti-RA action were identified as oleanolic acid, oleic acid, ferulic acid, caffeic acid, and syringic acid. All these compounds have been reported to possess anti-inflammatory bioactivity. Oleanolic acid, a pentacyclic triterpenoid compound, is widely distributed in nature and exhibits various biological activities including anti-tumor, anti-inflammatory, antiviral, and antioxidant properties [16]. Literature indicates that CF is rich in saponins, primarily oleanane-type triterpene saponins [17]. Oleic acid, a monounsaturated fatty acid, is known for its anti-inflammatory and antioxidant effects [18]. Ferulic acid has a variety of biological activities, especially in oxidative stress, inflammation, vascular endothelial injury, fibrosis, apoptosis and platelet aggregation [19]. Caffeic acid is a widely distributed hydroxycinnamic acid salt and phenylpropanoid metabolite in plant tissues, with multiple biological activities such as antioxidant, anti-cancer, antiviral, anti-inflammatory, and anti-diabetic effects [19–22]. Syringic acid can inhibit the release of inflammatory mediators, alleviate inflammation, and possesses additional properties such as antioxidant [23,24]. In this study, it was found that the ethanol extract of CF applied topically had significant anti-inflammatory and analgesic activity and significant pharmacological effect against RA. These effects may be the result of a synergistic action of multiple compounds.

Meanwhile, network pharmacology revealed that 99 potential targets of CF were responsible for the RA treatment. Among them, PTGS2, MAPK1, NF-κB1, TNF and IL6 were the core targets, which has been experimental validated. PTGS2（Prostaglandin G/H Synthase 2）, also known as COX-2, significantly promotes prostaglandin production in synovial tissue of RA patients. It is one of the common targets for the treatment of RA [25]. It, along with IL-6 and TNF-α, are inflammatory factors that play a key role in the pathogenesis of RA. When the expression of COX-2 was inhibited, the feeling of pain can be relieved [26]. IL-6, a member of the pro-inflammatory cytokine family, is an important inflammatory mediator in the RA disease process [27]. Excessive and sustained

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dysregulation of IL-6 synthesis can have pathological effects on chronic immune-mediated diseases. TNF plays crucial roles in various cellular processes, including apoptosis, cell survival, and immune regulation [28]. TNF-α directly affects osteocyte RANKL expression and increases osteoclastogenesis [29].

NF-κB is the core activated protein in a wide range of autoimmune diseases, including RA [30]. Activated NF-κB can participate in the inflammatory response by regulating cytokines, adhesion molecules and chemokines. The release of activated NF-κB into the nucleus also induces the downstream production of pro-inflammatory cytokines such as COX2, TNF-α, IL-6 and IL-1β. They will further exacerbate the inflammatory response [31]. In our study, ELISA results showed that the serum COX-2, TNF-α and IL-1β levels in RA rats were significantly elevated. After the administration of CF intervention, their level decreased significantly. Meanwhile, the activation of NF-κB p65 was inhibited by CF, indicating that the activation of the NF-κB signaling pathway in rat synovial tissue was effectively inhibited. As mentioned above, COX-2, TNF-α, IL-6, IL-1β and RF may be induced by the activation of NF-κB, so NF-κB was verified as one of the key targets for CF to treat RA.

MAPK1 (Mitogen-Activated Protein Kinase 1), also known as ERK1 (Extracellular Signal-Regulated Kinase 1), is a pivotal member of MAPK family. The MAPK signaling pathway plays a positive role in the cellular activity of synoviocytes, chondrocytes, and bone marrow mesenchymal stem cells in knee joints [32]. Studies have shown that activating the MAPK pathway may regulate the secretory metabolism of cartilage, thereby promoting the production of extracellular matrix by chondrocytes and protecting cartilage [33]. The phosphorylation and activation of ERK1/2 and Akt may upregulates the expression of cell cycle proteins in MSCs35 [34]. Activation of ERK MAPK pathway could suppress the autophagy of chondrocytes and promote the proliferation of chondrocytes, since the ERK MAPK pathway is associated with a multiple of growth factor signaling pathways that regulate cell proliferation and tissue homeostasis [35]. In the present study, we found that the expression of p-ERK1/2 protein in CFA-induced arthritis rats was elevated by CF treatment, suggesting that CF may promote the proliferation of chondrocytes by targeting the ERK1/2 signaling pathway, which may provide protection for chondrocytes.

TCM has shown distinct advantages in treating RA, with ethnobotanical medicinal plants being an important source for developing anti-RA drugs [36]. CF is a plant in the ranunculaceae family that primarily known for its anti-inflammatory and analgesic effects. Medicinal products derived from the Clematis genus are frequently used to treat RA, boost immunity, and as an adjunct therapy for cancer [37,38]. However, current research indicates that Clematis genus drugs possess certain toxicity [10]. Preliminary studies by our research group revealed that the oral median lethal dose (LD50) of CF in mice is 60.08g/kg, iindicating potential toxicity at high oral dosages [39]. Nevertheless, our previous studies have also shown that topical application of CF does not elicit toxic responses [40]. Topical administration of TCM in treating RA offers multiple advantages, such as reducing the side effects associated with oral administration, avoiding the first-pass effect in the gastrointestinal tract, ease of use, and the ability to immediately discontinue treatment if adverse reactions occur [41]. To sum up, CF is a safe and effective topical drug in the treatment of RA.

4. Materials and Methods

4.1. LC-MS/MS Analysis

4.1.1. Sample Preparation

The sample of CF were sourced from the Min Dong She Green Herb Development Association (Ningdei, China). The sample were identified by Zehao Huang from Fujian University of Traditional Chinese Medicine and stored in the Materia Medica, Fujian Academy of Chinese Medical Science. 20 mg sample of CF powder and 300 μL of 40% methanol solution were mixed using the vortex mixing method. Every mixture was centrifuged at 16000 r/min (4℃) for 15 minutes. The resulting supernatant was collected for analysis.

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4.1.2. Instruments and Analytical Conditions

The chemical composition of CF was detected and scanned using a UHPLC-Q-Exactive-Focus mass spectrometer (Thermo Scientific, Bremen, Germany) equipped with an ESI source. The mobile phase consisted of 0.1% methanol in water (phase A) and 0.1% methane in acetonitrile (phase B). The gradient elution scheme was as follows: 0 - 17.0 min, 5%-98% B; 17.0 - 17.2 min, 98%-5%B; 17.2 - 20.0 min, 5%B. The time-of-flight mass range was 90-1300; the spray voltage was 3800 volts for ESI+ and 3500 volts for ESI-; the sheath gas flow rate was 40 L/min; the ion transfer tube temperature was set at 320℃; and the nebulization temperature was maintained at 350℃. MS data were acquired using the mass spectrometer and processed with ProteoWizard software.

4.1.3. Screening of Main Compounds of CF

Compounds exhibiting high abundance values were selected from the mass spectra. Additionally, relevant literature was reviewed to supplement our understanding of the main constituents of CF. This comprehensive approach enabled us to finalize the main constituents of CF.

4.2. Network Pharmacology Analysis

4.2.1. Candidate Therapeutic Targets of CF Screening

The TCMSP database (https://old.tcmsp-e.com/tcmsp.php), the ETCM database (http://www.tcmip.cn/ETCM/index.php), and the ITCM database (http://itcm.biotcm.net) were utilized to predict all targets associated with the main compounds identified by mass spectrometry. Swiss TargetPrediction (http://www.swisstargetprediction.ch/result.php) was employed to predict the compounds that lacking target information and these targets were focused with a confidence level of 0.8 or higher. These targets were then standardized into gene names used the UniProt database (https://www.uniprot.org/). Ultimately, the prediction results from all these databases were compiled and organized.

4.2.2. Construction of RA-Related Target Database

The DisGenet database (https://www.disgenet.org/), the OMIM database (https://www.omim.org/), and the GeneCards database (https://www.genecards.org/) were conducted to screen the targets related to “arthritis”. Subsequently, the data obtained from these sources were merged and converted into standard gene names in Uniprot.

4.2.3. Intersection between Main Compounds and Disease Targets

The online tool Venny 2.1 (https://bioinngp.cnb.csic.es/tools/venny/index.html) was employed to intersect the disease targets with the drug targets to identify the possible targets of CF against RA.

4.2.4. Construction of CT Network

The String database (https://cn.string-db.org) was used to analyze the CF and RA targets that overlapped. The organism was set to Homo sapiens and the minimum required intaraction score was >0.7. Cytoscape 3.7.1 software was used for network topology analysis. Subsequently, the core compounds within this network were analyzed using the CytoNCA 2.1.6 plugin in Cytoscape.

4.2.5. CPS Network Construction and Core Target Acquisition

String was used to perform KEGG pathways enrichment analyse. We prioritized pathways based on their FDR from lowest to highest. Among the top 30 pathways, 9 pathways that were related to inflammation were selected for primary analysis. The targets within these 9 inflammation-related pathways, which are directly involved in treating RA were designated as the primary targets. They were imported into Cytoscape for the construction of the CPS network. Core targets were identified through the CytoNCA plugin for Cytoscape.

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4.2.6. GO and KEGG Enrichment Analysis

The identified targets were analyzed for GO and KEGG pathway enrichment using R software (https://www.r-project.org). ClusterProfiler, GOplot and Pathview are the major visualization packages included in the R package.

4.2.7. Molecular Docking

Molecular docking was conducted to validate the interaction between core compounds and their corresponding target. The SDF format files of the core compounds were downloaded from the PubChem database (https://pubchem.ncbi.nlm.nih.gov) and converted into MOL2 format files. The PDB structure files of the target proteins were acquired from the RCSB PDB database (https://www.rcsb.org). These targets were prepared by removing water molecules and adding hydrogen atoms using AutoDock Tools 1.5.7. Molecular docking simulations were performed with AutoDock Vina 1.2.3, and the results were visualized using PyMOL software.

4.3. Quality Control of CF Extract

According to the report, CF was rich in saponins, primarily oleanane-type triterpene saponins [17]. And oleanolic acid was commonly used as a quality control substance for medical plants of clematis [42]. So oleanolic acid was determined as a quality control substance for CF extract using HPLC method.

4.3.1. Sample and Standard Solution Preparation

A sample of 10g CF powder was dissolved in 150 ml methanol by ultrasonication for 70 minutes, then filtered using 0.45μm membrane filter. A standard solution of oleanolic acid was prepared by accurately weighing 5.0 mg of the oleanolic acid standard. This oleanolic acid standard was dissolved in 5.0 mL of methanol to create the stock solution.

4.3.2. Instruments and Analytical Conditions

HPLC analysis was carried out by Leaps UHPLC system (Chromai, Beijing, China). The chromatography was performed on a Hypersil ODS C18 column (250 mm × 4.6 mm, 5 μm). Water (10%A) and acetonitrile (90%B) were used as mobile phase solvents, utilizing an isocratic elution system. The mobile phase flow rate was kept at 1 mL/min. The column temperature was kept constant at 35℃, and the samples were detected at 205 nm. The final content of the analytes was quantified using the external standard method.

4.4. Experimental Validation

4.4.1. Animals

All animals were purchased from Beijing Huafukang Bio-technology Co (Beijing, China). Animals were kept under conventional laboratory conditions. All animals were fed water and standard maintenance food ad libitum. The acclimation period for animals was one week. Animal experimental procedures and methods were approved by the Animal Ethics Committee of Fujian Academy of Traditional Chinese Medicine. (FJATCM-IAEC2023024).

4.4.2. Preparation of the CF Extract

A sample of 1000g CF powder was crushed and subjected to extraction with 70% ethanol at 90℃, performed twice. The extract was subsequently concentrated under reduced pressure at 70℃ to obtain 298g extract. Dilute the above extract with water to 0.8g/ml as a CF-High dose sample and 0.4g/ml as a CF-Low dose sample. This extract was prepared and reserved for further use.

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4.4.3. Xylene-Induced Mouse Ear Edema

32 ICR male mice (18-22g, 7-9 weeks) were randomly divided into 4 groups with 8 mice in each group, which included xylene model group (0.9% nomarl saline); positive control group (Diethylamine Diclofenate, DD), CF-High dose group (CF-H), and CF-Low dose group (CF-L). 20 μL of CF was applied on both sides of the right ear in the CF-H and CF-L group mice, and an equal amount of nomarl saline was applied to the mice in model group. Mice in positive control group were appled with DD for a thickness of 1mm. The administration was performed once every 10 minutes for a total of 6 times. After the last administration for 10 minutes, 40 μL of xylene was applied to the right ear, whereas the left ear was left untreated. After 30 minutes, the mice were sacrificed. The ears were collected using a biopsy punch with a diameter of 6 mm and weighed. Ear edema was defined as the difference in weight between the right ear and the left ear.

4.4.4. Acetic Acid-Induced Abdominal Writhing Response

32 ICR male mice (18-22g, 7-9 weeks) were randomly divided into 4 groups with 8 mice in each group, which included model group (0.9% nomarl saline); positive control group (Diethylamine Diclofenate, DD), CF-High dose group (CF-H), and CF-Low dose group (CF-L). One day before the experiment, the hair covering on the abdomen of mice has been removed with 8% sodium sulfide. On the second day after hair removed, mice in CF-H and CF-L groups received topical application of 0.05ml CF on the abdomen of mice. The mice in the model group was treated with the same dose of nomarl saline, while the positive control group was treated with 0.05g of DD. The administration was performed once daily for 5 days. On the 5th day, the drugs were administrated twice with a 30 minute interval. After the last administration for 1 hour, mice were intraperitoneally injected with 0.2 ml of 0.6% acetic acid per mouse. The writhe was defined as the contraction of the abdomen and pelvic rotation, followed by the extension of the hind limbs. the number of writhe in each group of mice were observed and recorded within 5-20 minutes after injection of acetic acid.

4.4.5. AA Model Preparation

40 SD rats (half male and half female, 160-260g, 6-8 weeks). The rats were securely positioned in a supine posture within an immobilizer. Subsequently, 0.1 ml of Freunds Complete Adjuvant (FCA, Beyotime, Shanghai, China) was injected intradermally into the dorsum of the left hind toe. One minute following the injection, the rats were returned to their cages.

4.4.6. Experimental Grouping and Drug Administration

The rats were divided into 5 groups with 8 rats in each group, which included control group (0.9% nomarl saline); model group (0.9% nomarl saline); positive control group (Diethylamine Diclofenate, DD), CF-High dose group (CF-H), and CF-Low dose group (CF-L). All groups except the normal group received an injection of 0.1 ml of FCA in the left hind toe to make the AA model. From the second day after inflammation onset, the CF treatment groups received topical applications of CF-H (0.8g/ml) and CF-L (0.4g/ml) doses to the affected toes daily for 28 consecutive days. The positive control group received a daily topical application of 0.2g DD (10mg/g, Novartis Pharma, Beijing, China), continuing for 28 days. The model control group and the normal control group were treated with 0.9% nomal saline applied topically.

4.4.7. Detection the Degree of Toe Swelling in Rats

Foot volumes and body weights of rats were measured on the day before the AA model was made, and 7, 14, 21, and 28 days after modeling. A total of 5 measurements were taken. The degree of toe swelling for each group of rats was then calculated. The degree of toe swelling as calculated as (toe volume after modeling-toe volume before modeling)/toe volume before modeling*100%.

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4.4.8. Determination of Serum Inflammatory Factor

After the final toe volume measurement, the rats were euthanized under sodium pentobarbital anesthesia. Subsequently, 5 ml of blood was collected from the abdominal aorta. The blood was then centrifuged at 5000r/min for 10 minutes to separate the serum. The contents of COX-2, TNF-α, IL-6, IL-1β and RF in the serum were determined using the ELISA kits (Elabscience, Wuhan, China, E-EL-R0792c, E-EL-R2856c, E-EL-R2856c, E-EL-M0037; Mlbio, Shanghai, China, ML-EA-A02702).

4.4.9. Hematoxylin and Eosin Staining

Ankle joints tissues were harvested and fixed in 4% paraformaldehyde, followed by decalcification with 10% neutral buffered EDTA. The tissues were obtained, fixed, decalcified, embedded in paraffin, and sectioned at a thickness of 5μm. The joint sections underwent deparaffinization using xylene and dehydrated in a gradient ethanol series. The sections were stained with hematoxylin for 5 minutes, differentiated with 1% hydrochloric acid ethanol for 30 seconds, treated with 0.2% ammonia for bluing, and stained with 0.5% eosin for 10 minutes. Finally, the sections were observed under a light microscope.

4.4.10. Western Blotting

Joint tissue was obtained after the final toe volume measurement. Total protein was extracted using the column tissue protein extraction kit (Yaenzyme, China). For every 10mg of tissue, 100μL of lysis buffer was added. The mixture was thoroughly ground using the Servicebio high-speed cryogenic tissue grinder (Servicebio, China). The lysate was transferred to an ultrafiltration centrifuge tube and centrifuged at 13,000 r/min(4℃) for 2 minutes. The protein concentration was determined using a BCA protein assay kit. 15μg of protein sample was subjected to Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) and then transferred to the 0.45μm PVDF membrane (Yaenzyme, China). The gel blocking was performed with blocking solution for 30 minutes, followed by incubation with primary antibodies overnight at 4℃: NF-κB p65 (1:4000, Cell Signaling Technology, Rabbit mAb, #3033S), NF-κB (1:1000, BIOSS, Mouse mAb, bsm-33117M), ERK p44/42 (1:2000, ABclonal, Rabbit mAb, A4782), ERK (1:1000, Cell Signaling Technology, Rabbit mAb, #4695S). After washing three times with TBST, the membrane was incubated with HRP-conjugated goat anti-rabbit secondary antibody (1:5000, Cell Signaling Technology, #7074S) or HRP-conjugated goat anti-mouse secondary antibody (1:10000, BIOSS, bs-0296G-HRP) at 26℃ for 2 hours. After washing three times with TBST, the proteins were made visible through ECL chemiluminescent substrate (Yaenzyme, China) and the intensities were measured using ImageJ software (n=5).

4.5. Statistical Analysis

Data were expressed as Mean±SEM, and were analyzed by one-way analysis of variance (ANOVA), when the series of each group were normally distributed. The LSD-test was used to compare between groups when the variance was the same; Games-Howell was used to compare between groups when the variance was not the same. The significance level was $P<0.05$. All statistical analyses were performed using SPSS 20.0 software.

5. Conclusion

In this study, LC-MS/MS and network pharmacology were used to screen and predict the core compounds of CF in treating RA as oleanolic acid, oleic acid, ferulic acid, caffeic acid, and syringic acid. The key targets were determined to be PTGS2（COX-2）, MAPK1（ERK1）, IL6, TNF, and RELA, primarily involving the ERK MAPK pathway and NF-κB pathway. The pharmacological effects and mechanisms of the topical application of CF in treating RA were also validated through animal experiments. The results of this study provide theoretical support for the use of CF in the topical treatment of RA and establish a theoretical basis for its use. The present study demonstrates that CF is a new and valuable natural potential drug for the topical treatment of RA.

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Author Contributions: Jing Han, Chunquan Zhou and Gong Wang contributed to the design of this study. Ting Lei, Jizhou Zhang, Yanhong Chen, Jie Zhang, Chunquan Zhou conducted the animal experiments. Ting Lei, Li Zhao and Chang Jiang conducted the LC-MS experiments and network pharmacology analysis. Ting Lei and Jin Han wrote the main manuscript text. All the authors participated in the interpretation of results. All the authors have read and approved the final manuscript.

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Data Availability Statement: The datasets presented in this study are available in online repositories. All data generated or analysed during this study can be obtained upon reasonable request to the corresponding author.

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Conflicts of Interest: We declare that we have no financial and personal relationships with other people organizations that can inappropriately influence our work, there is no professional or other personal interest of any nature or kind in any product, service and/or compary that could beconstrued as influ-encing the position presented in, or the review of, the manuscript entitled.

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