Analyzing the mechanism and experimental validation of *Astilbe chinensis* in the treatment of rheumatoid arthritis through network pharmacology

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Abstract: To unravel the active components and potential mechanisms of *Astilbe chinensis* in treating rheumatoid arthritis (RA), we employed a comprehensive strategy that combined network pharmacology and biological activity verification. Firstly, we identified the relevant compounds in *A. chinensis* from the literature, and RA-related targets were gathered through DisGeNET, GeneCards, and OMIM databases. Subsequently, compound-target and protein-protein interaction networks were constructed to predict the key compounds and promising protein targets of *A. chinensis*. Finally, these predictions were validated through *in vitro* anti-inflammatory activity experiments. A total of 29 potential active compounds and 117 intersecting pharmacological targets were identified. Among them, key active compounds included 3β,6β-dihydroxy olean-12-en-27-oic acid (astilbic acid), 3β-acetoxyolean-12-en-27-oic acid (3-acetyl oleanolic acid), 3β-hydroxyurs-12-en-27-oic acid, 4-*O*-galloylbergenin, and (+)/(-)-catechin. Key targets were identified as AKT1, MMP9, EGFR, CASP3, and HSP90AA1. GO enrichment analysis indicated that signal transduction, proteolysis, and negative regulation of the apoptotic process were closely associated with *A. chinensis* treatment in RA. KEGG pathway analysis indicated that pathways in cancer, osteoclast differentiation, and endocrine resistance might be crucial for *A. chinensis* intervention in RA. This finding suggested that multiple components in *A. chinensis* could regulate various signaling pathways and targets, playing preventive and therapeutic roles in RA. *In vitro* experiments demonstrated that *A. chinensis* extracts inhibited the secretion of inflammatory factors (NO, TNF-α, and IL-6) and significantly suppressed the expression of inducible nitric oxide synthase (iNOS), IL-6, and TNF-α mRNA in LPS-induced RAW 264.7 cells. Collectively, our findings not only established the theoretical basis for the efficacy of *A. chinensis* in treating RA but also provided a direction for subsequent research.

Keywords: *Astilbe chinensis*; Rheumatoid arthritis; Network pharmacology; Anti-inflammatory activity

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1. Introduction

Rheumatoid arthritis (RA) is a chronic inflammatory disease with systemic manifestations characterized by extensive arthrosynovitis and synovial proliferation. This leads to the development of pannus, which invades various structures such as articular cartilage, subchondral bone, ligaments, and tendons, causing damage to articular cartilage, bone, and joint capsule[1]. Without timely intervention, RA can ultimately result in joint deformity and loss of function[2]. Presently, due to an incomplete understanding of the pathogenesis of RA[3], clinical treatment primarily relies on non-steroidal anti-inflammatory drugs, glucocorticoids, immunosuppressive agents, and biological agents. While these treatments exhibit therapeutic effects, prolonged use often comes with side effects such as gastrointestinal reactions, hepatorenal toxicity, urticaria, infections, and other autoimmune diseases[4,5]. Traditional Chinese medicine (TCM), renowned for its long history of treating RA, is increasingly valued for its efficacy and minimal toxic side effects.

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Astilbe chinensis (*Maxim.*) *Franch. et Savat*., also known as "Luoxinfu" in Chinese, belongs to the Saxifragaceae family and is a perennial herb widely distributed in Eurasia and eastern North America. Its rhizome has been utilized in Chinese and Korean traditional medicine as a remedy for cough, antipyretic, analgesic, and arthritis drugs. Both *in vivo* and *in vitro* studies have demonstrated the anti-inflammatory and immunomodulatory effects of *A. chinensis*[6,7]. However, due to the diverse chemical components and the intricate, multitarget synergistic mechanisms of action, the bioactive components of *A. chinensis* and its drug targets for RA therapy remain unclear. Therefore, the application of network pharmacology methods to identify the primary active ingredients of *A. chinensis* and explore the targets of its anti-RA effect holds significant importance in comprehensively elucidating the mechanism of *A. chinensis*'s anti-RA effect from a systems biology perspective.

2. Materials and methods

2.1. Screening active compounds of *A. chinensis* and RA-related targets

The primary components of *A. chinensis* were extracted from literature sources[8,9], and their SDF structural formulas were obtained from the PubChem database (https://pubchem.ncbi.nlm.nih.gov/)[10]. Subsequently, each SDF file was uploaded individually to the Pharmmapper platform (http://lilab-ecust.cn/pharmmapper/submitfile.html) for target prediction[11]. Targets with a z-score of ≥ 0.6 were selected from the predicted results. These predicted targets were then converted into standardized gene symbols using the UniProt database (https://www.uniprot.org/)[12].

To compile potential targets for *A. chinensis*, duplicate targets were removed after merging the obtained targets. The next step involved identifying RA-related targets by searching databases with the keywords "Rheumatoid arthritis" and "Homo sapiens". The integration of three databases was performed, including DisGeNET (https://www.disgenet.org/)[13], GeneCards (https://www.genecards.org/)[14], and OMIM (https://omim.org/search/advanced/)[15]. Specifically, in the GeneCards database, targets with a relevance score greater than or equal to the quartile were extracted, and these targets were then merged with those from other databases to obtain potential RA targets after removing duplicates. Finally, the intersection set of potential *A. chinensis* targets and RA-related targets was determined.

2.2. Constructing the "herb-component-target" network

Venny 2.1 (http://www.bioinformatics.com.cn/static/others/jvenn/index.html) was utilized to construct the intersection target of the drug target and disease target, serving as the candidate targets for *A. chinensis* against RA. Subsequently, Cytoscape 3.8.2 software was employed to construct the network of "herb-component-target". This network, combined with the screened main active ingredients, core targets, and focused main signal pathways, was employed to systematically analyze the potential mechanism of *A. chinensis* in treating RA. Notably, targets with "betweenness centrality", "closeness centrality", and "degree centrality" greater than the median were selected as the core nodes of the network. "betweenness centrality" indicates the number of shortest paths between nodes, "closeness centrality" describes the inverse of the reciprocal distances, and the "degree centrality" parameter represents the number of edges linked to the node.

2.3. Constructing the target protein-protein interaction (PPI) network

The intersection targets were input into the STRING database (https://string-db.org/)[16]. After filtering out

scattered targets, PPI network was constructed with the minimum interaction threshold set to "highest confidence" (≥ 0.4), while the rest of the parameters were maintained at their default values. The data were then imported into Cytoscape 3.8.2 for visualization and to identify core targets. In this network, nodes represent different targets, and edges symbolize the relationships between these targets.

2.4. GO/KEGG enrichment analyses and KEGG pathway diagram drawing

Based on the provided data, GO and KEGG enrichment analyses were conducted on the DAVID platform (https://david.ncifcrf.gov/) with a significance threshold set at "$P < 0.01$". The results were considered statistically significant at this level. The visualization of the histogram and bubble plot for the core targets in the core network was accomplished using the Hiplot online tool (https://hiplot.com.cn/). In the bar chart, a smaller *P*-value indicates higher enrichment, and the length of the bar corresponds to the number of enriched genes. To explore the interactions between key genes and pathways of *A. chinensis* and RA, the disease-drug intersection target was uploaded to the KEGG database. The most relevant pathway to the topic was selected, and the mapper tool was utilized to construct the KEGG pathway map.

2.5. *In vitro* anti-inflammatory activity evaluation

Macrophages and their expression and secretion products play a crucial role in the pathogenesis of RA[17]. Therefore, a classic lipopolysaccharide (LPS)-induced macrophage inflammation model was chosen to assess the impact of *A. chinensis* extracts on the release of inflammatory mediators, including nitric oxide (NO), tumor necrosis factor (TNF-α), and interleukin-6 (IL-6). RAW264.7 cells (provided by the Chinese Academy of Traditional Chinese Medicine) were treated with varying concentrations (15, 30, and 60 mg/mL) of *A. chinensis* extracts for 4 h. Subsequently, they were stimulated with LPS (0.1 μg/mL) for 16 h (for TNF-α and IL-6) or 24 h (for NO). The culture medium was then analyzed for the production of nitrite, TNF-α, and IL-6 using ELISA kits (obtained from Jiangsu Boshen Biotechnology Co., Ltd.) following the provided instructions. For the gene expression studies, cells were pre-treated with different concentrations of *A. chinensis* extracts for 4 h and then stimulated with LPS (0.1 μg/mL) for 12 h. The relative expression of nitric oxide synthase (iNOS), TNF-α, and IL-6 mRNA was evaluated using real-time reverse transcription-polymerase chain reaction (RT-PCR), with the results expressed as the ratio of optimal density relative to glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

3. Results

3.1. Targets collection and component target network construction

A total of 48 chemical compounds from *A. chinensis* were identified from the literature, and 29 SDF structural formulas were obtained from the PubChem database. Potential targets were predicted using the Pharmmapper database. After merging and eliminating duplicates, a set of 269 potential drug targets with a z-score of ≥ 0.6 was considered as core active components. Additionally, 652 targets were obtained from the DisGeNET database, 1518 RA-related targets were acquired from the GeneCards database, and 183 targets were obtained from the OMIM database. After removing redundancies, a total of 1869 RA-related targets were obtained for subsequent analysis. After further removal of duplicates, 117 common targets of *A. chinensis* and RA were identified. The compound-target Venn diagram is presented in Figure 1A.

The 117 intersection targets were recognized as key proteins involved in the treatment of RA with *A. chinensis*. The relationship between compounds and key targets is illustrated in Figure 1B, where circles represent chemical compositions and diamonds represent component targets. The size and color depth are positively correlated with the degree, while the degree is related to the extent of the component's role in the disease. A larger value indicates a greater likelihood of the ingredient treating the disease. According to the network topology analysis results, the top 10 ingredients with moderate values in the core nodes of the network are as follows: 3β,6β-dihydroxy olean-12-en-27-oic acid (astilbic acid), 3β-acetoxyolean-12-en-27-oic acid (3-acetyl oleanolic acid), 3β-hydroxyurs-12-en-27-oic acid, 4-*O*-galloylbergenin, (+)-catechin, (-)-catechin, β-sitosterol palmitate, β-sitosterol, daucosterol, and 4-hydroxybenzoic acid.

3.2. PPI network construction

A total of 115 targets were imported into the STRING platform to construct a PPI network (PPI enrichment *P*-value < $1.0e^{-16}$). As depicted in Figure 2, the network comprised 115 nodes, and 1194 edges were also obtained using this platform. The average node degree and local clustering coefficient were 37.0 and 0.618, respectively. The node color represents the degree of the target, with a darker shade indicating a higher degree. The inner circle highlights the core targets.

Based on the topological parameters, 20 key targets were identified: AKT1, MMP9, EGFR, CASP2, HSP90AA1, IL2, SRC, ESR1, PPARG, MMP2, HSP90AB1, KDR, STAT1, CCL5, RHOA, ANXA5, BCL2L1, MDM2, MAPK8, and HRAS.

3.3. GO and KEGG enrichment analyses

The GO functional enrichment and KEGG pathway annotation visual analyses of the 115 core targets were conducted using the DAVID platform. A total of 583 GO enrichment items were identified, comprising 431 biological processes (BP), 49 cellular components (CC), and 103 molecular functions (MF). In Figure 3A, the top 10 significantly enriched terms ($P < 0.01$) for *A. chinensis* in the treatment of RA are presented, with

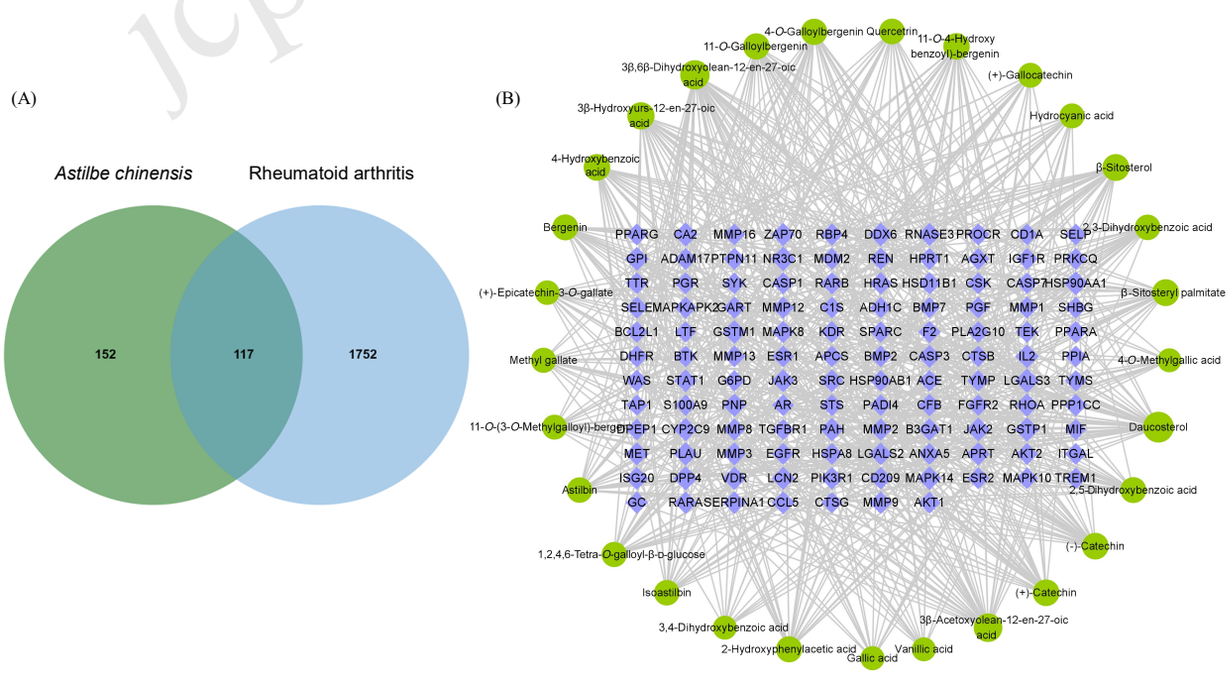

Figure 1. (A) Wayne diagram of the active ingredients of *A. chinensis* and RA; (B) Active ingredient-RA target network.

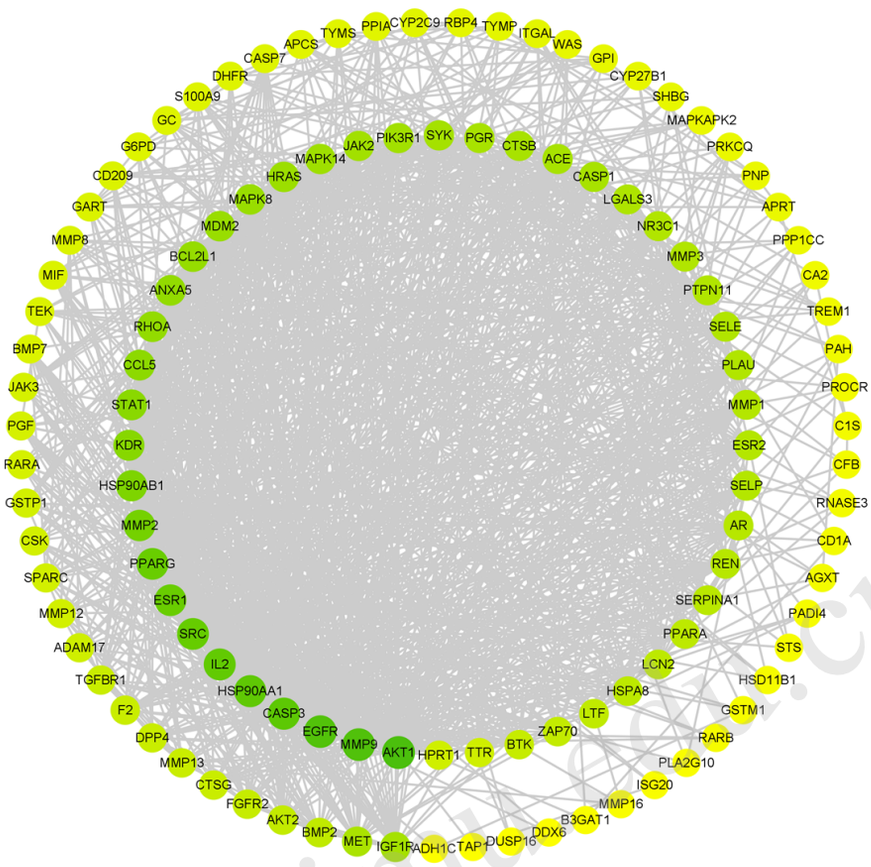

Figure 2. PPI network.

the column length in the histogram reflecting the number of target genes in the respective functional pathway.

Furthermore, 136 KEGG pathway items were obtained, and 15 highly probable signaling pathways were selected based on the False Discovery Rate (FDR) value to generate the bubble map. As illustrated in Figure 3B, the therapeutic targets were predominantly associated with pathways in cancer, osteoclast differentiation, endocrine resistance, proteoglycans in cancer, and the C-type lectin receptor signaling pathway.

For BP, the top five terms included signal transduction, proteolysis, negative regulation of apoptotic process, positive regulation of cell proliferation, and positive regulation of cell migration. In CC, the top five terms were extracellular cytoplasm, plasma membrane, extracellular region, extracellular space, and extracellular exosome. In MF, the prominent terms were identical protein binding, zinc ion binding, protein serine/threonine/tyrosine kinase activity, enzyme binding, and serine-type endopeptidase activity.

3.4. Endocrine resistance signaling pathway construction

As an illustrative example, the potential targets and mechanisms of *A. chinensis* in the treatment of RA within the endocrine resistance signaling pathway are depicted in Figure 4. According to the KEGG enrichment analysis results, 15 intersection targets between disease and drug were identified in the endocrine resistance signaling pathway. These targets included SRC, MMP2, PIK3R1, MAPK14, ESR1, MMP9, EGFR, ESR2, IGF1R, MAPK10, MAPK8, AKT2, MDM2, AKT1, and HRAS (the red targets represent the intersection targets between disease and drug).

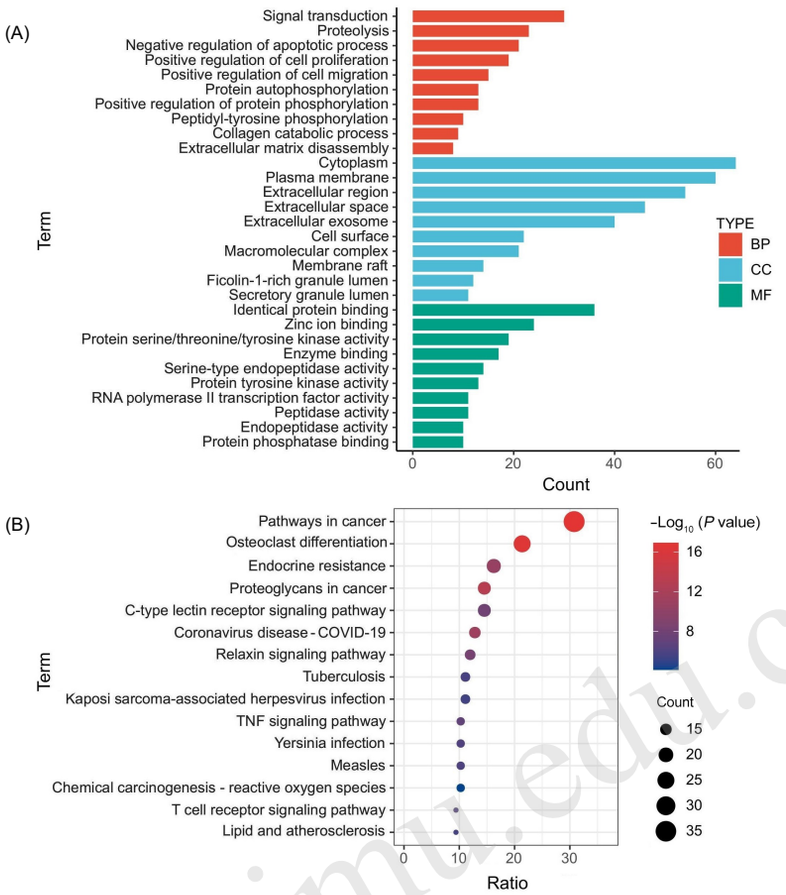

Figure 3. Enrichment analysis. (A) GO function analysis diagram; (B) KEGG pathway enrichment bubble diagram.

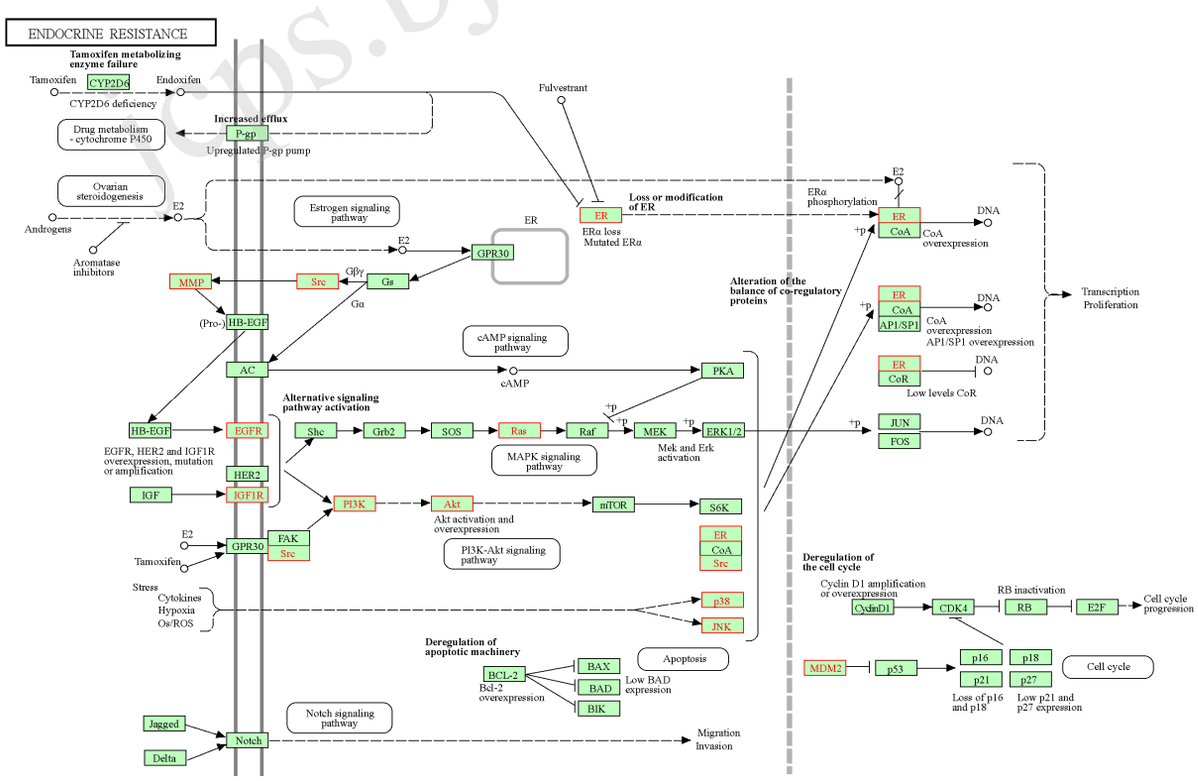

Figure 4. Potential targets and mechanism of *A. chinensis* in endocrine resistance signaling pathway.

3.5. The *in vitro* anti-inflammatory activity of *A. chinensis* extracts

Cytokines produced by macrophages play a crucial role in the pathogenesis of inflammatory development in RA. As illustrated in Figure 5, LPS stimulation significantly increased the concentration of inflammatory cytokines (NO, TNF-α, and IL-6) compared to the control group. Importantly, the *A. chinensis* extract demonstrated a clear suppression of LPS-induced NO production in a dose-dependent manner (Fig. 5A). Consistent with the inhibitory effect on NO production, a dose-dependent reduction in TNF-α levels was also observed with *A. chinensis* extract (Fig. 5B). However, in terms of IL-6 production, the *A. chinensis* extracts exhibited a significant inhibitory effect at high concentrations (60 μg/mL), while no obvious effect was observed at lower concentrations (Fig. 5C).

Cytokines produced by macrophages undergo catalysis by related upstream proteins and are primarily regulated at the transcriptional level. Therefore, the expression level of iNOS was assessed using RT-PCR. The results indicated a significant up-regulation in the expression of the iNOS gene in response to LPS, and *A. chinensis* extract exhibited a notable inhibition of this up-regulated iNOS expression in a dose-dependent manner (Fig. 6A). Furthermore, the effects of *A. chinensis* extract on LPS-induced TNF-α and IL-6 mRNA expression were also examined. The results demonstrated a potent inhibitory effect of *A. chinensis* extract on LPS-induced TNF-α mRNA expressions in a concentration-dependent manner (Fig. 6B). Similarly, concerning IL-6 mRNA expression, there was an enhanced down-regulatory effect in a dose-dependent manner (Fig. 6C). These findings suggested that *A. chinensis* extract might target the transcriptional regulation of iNOS, TNF-α, and IL-6 genes.

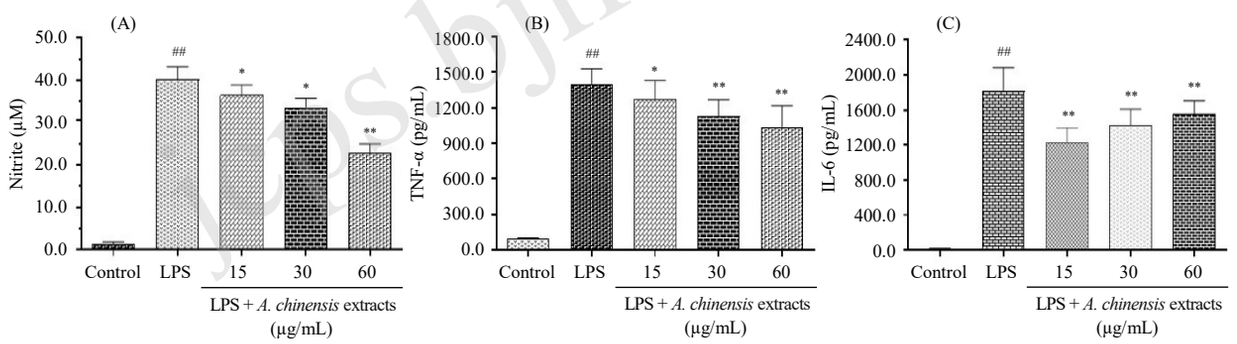

Figure 5. Determination results of nitrite, TNF-α, and IL-6 contents in culture medium in each group. ($\bar{x} \pm s$, $n=3$). $^{\#\#}P < 0.01$ *vs*. the control group, while $^{*}P < 0.05$ and $^{**}P < 0.01$ *vs*. the LPS-treated group.

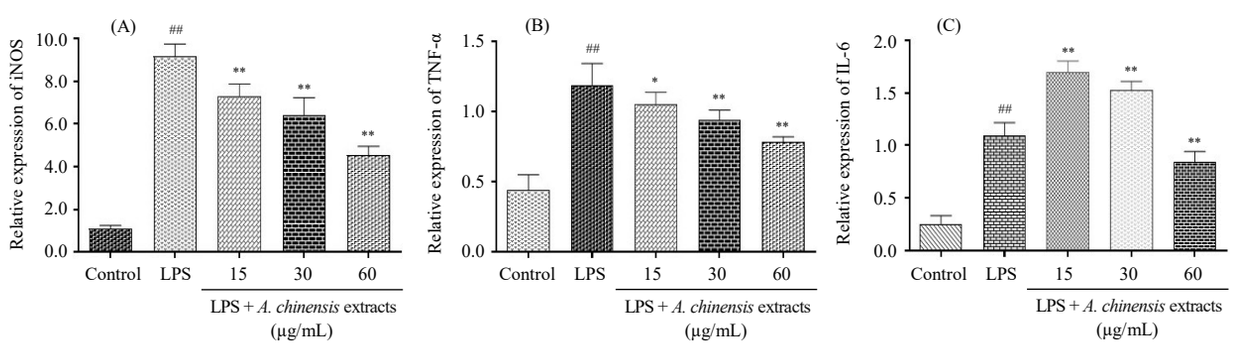

Figure 6. Inhibitory effect of *A. chinensis* extracts on iNOS, TNF-α, and IL-6 mRNA expressions in LPS-induced RAW264.7 cells. ($\bar{x} \pm s$, $n=3$). $^{\#\#}P < 0.01$ *vs*. the control group, while $^{*}P < 0.05$ and $^{**}P < 0.01$ *vs*. the LPS-treated group.

4. Discussion

RA is a chronic, progressive, and invasive autoimmune disease characterized by joint synovitis and extra-articular lesions as its primary clinical manifestations[18]. Current treatment strategies primarily aim to reduce joint swelling and pain, control the progression of arthritis, prevent and minimize joint damage, and promote the repair of damaged joints and bones[19]. TCM possesses the advantage of coordinating biological systems in the treatment of RA, thanks to its multi-component and multitarget characteristics. However, these features also contribute to the challenges in identifying active ingredients and elucidating the mechanisms of drug action.

A. chinensis has a rich history of medicinal use in folk traditions, valued for its efficacy in clearing away heat and toxic materials, reducing swelling and relieving pain, expelling wind-damp, and addressing issues such as colds, fever, arthralgia, inflammation, chronic bronchitis, pain, and headaches. Modern pharmacological research has revealed that *A. chinensis* exhibits antitumor and cancer-preventive properties, anti-RA effects, immune-enhancing capabilities, anti-allergic and antibacterial activities, anti-gout attributes, as well as anti-inflammatory properties. Moreover, it has been found to effectively reduce carbuncle swelling. In this study, the network pharmacology approach was employed to investigate the intricate network relationships of *A. chinensis*, considering its multiple components, targets, and pathways in the treatment of RA. Through this approach, a total of 29 active ingredients in *A. chinensis* were identified, with triterpenoid saponins and flavonoids emerging as the main active components in the treatment of RA. Additionally, 117 key targets were pinpointed, including AKT1, MMP9, EGFR, CASP2, HSP90AA1, IL2, SRC, ESR1, PPARG, MMP2, and others. KEGG enrichment analysis revealed 15 key pathways related to RA, indicating that pathways in cancer, osteoclast differentiation, endocrine resistance, proteoglycans in cancer, and the C-type lectin receptor signaling pathway were closely associated with the anti-RA effect of *A. chinensis*.

To validate the accuracy of the network predictions, *in vitro* anti-inflammatory activity of *A. chinensis* extract was assessed. The results demonstrated that *A. chinensis* extracts effectively inhibited the release of inflammatory factors NO, TNF-α, and IL-6 to varying degrees. Prior studies have supported these findings, showing that *A. chinensis* ethanol extract can suppress inflammation in macrophages through the NF-κB pathway, inhibiting the release of NO and prostaglandin E2, and suppressing the expression of inducible iNOS and COX-2 proteins[20]. This consistency between network pharmacology predictions and *in vitro* anti-inflammatory activity results suggests a high level of accuracy in the network pharmacology predictions.

In summary, in contrast to currently utilized non-steroidal anti-inflammatory drugs, glucocorticoids, and biologics in clinical practice, *A. chinensis* not only reduced joint inflammation and inhibited disease progression and irreversible bone destruction but also regulated immunity. This dual-action approach aligns with the goal of treating both symptoms and underlying causes, highlighting a unique advantage of *A. chinensis* in the treatment of RA.

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基于网络药理学分析落新妇干预类风湿性关节炎的作用机制及实验验证

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摘要: 本研究以网络药理学和体外实验相结合的综合策略为基础, 预测落新妇改善风湿性关节炎的可能活性成分与作用机制。首先从文献中获取落新妇的有效化合物, 从DisGeNET、GeneCards和OMIM数据库分别筛选出与RA相关的靶点, 然后构建化合物-靶标网络和蛋白-蛋白相互作用网络, 以预测落新妇可能的关键成分及药物靶点, 最后通过体外实验进行验证。最终筛选出29个潜在活性成分和117个交叉药理靶标, 其关键活性成分可能是落新妇酸、3β-乙酰基齐墩果酸、3β-羟基齐墩果-12-烯-27-酸、4-*O*-没食子酰岩白菜素和儿茶素, 关键作用靶点可能是AKT1、MMP9、EGFR、CASP3和HSP90AA1; GO富集分析显示信号转导、蛋白水解和细胞凋亡过程的负调控与落新妇治疗RA机制密切相关; KEGG通路分析表明癌症、破骨细胞分化和内分泌抵抗信号通路与落新妇干预RA有关。体外实验表明落新妇提取取物可以抑制巨噬细胞炎症因子NO、TNF-α和IL-6的分泌, 降低诱导型一氧化氮合酶(iNOS)、TNF-α和IL-6在LPS诱导的RAW 264.7细胞中的mRNA表达。本研究增加了中药落新妇及其有效成分抗RA药理作用的新认识, 也为后续研究提供思路与依据。

关键词: 落新妇; 类风湿性关节炎; 网络药理; 抗炎活性