

ORIGINAL RESEARCH

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Network pharmacology combining molecular docking to unveil the anti-inflammatory mechanism of *Aconitum Heterophyllum*

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Abstract

Objective The objective of this study was to examine the impact and underlying mechanisms of *Aconitum Heterophyllum* in mitigating cellular inflammation.

Methods Initially, the principal components of *A.heterophyllum* were obtained from relevant literature sources, and the corresponding protein targets of *A.heterophyllum* were searched on Pubchem and Swiss Target Prediction database. Subsequently, the genes linked with inflammation were gathered from PHARMGKB, OMIM, and GeneCards databases. The core targets were screened, and a protein–protein interaction (PPI) network was constructed using Cytoscape 3.9.1. For general targets, the gene ontology (GO) analysis and Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis were conducted. Molecular docking was further applied for the core targets and compounds. Finally, the RAW264.7 cellular inflammation model was constructed, and MTT assay, Griess assay and qRT-PCR assay were used to initially validate the anti-inflammatory effects of *A. heterophyllum* and its underlying mechanisms.

Results In total, 35 compounds and 1322 targets were associated with *A.heterophyllum*, along with 3534 genes implicated in inflammation. The intersection analysis yielded 244 common targets, contributing to the enrichment of 146 pathways. Notably, results of molecular docking revealed strong binding between the active compounds and core targets. Furthermore, in vitro experiments indicated the anti-inflammatory potential of *A.heterophyllum*'s active ingredient through the modulation of the EGFR/JAK2/STAT3 pathway and EGFR-AKT pathway, as well as inhibiting cellular NO release to reduce the inflammatory response.

Conclusion *A.heterophyllum* exerts anti-inflammatory activity by inhibiting EGFR-AKT1 activity and inhibiting EGFR/JAK2/STAT3 signal transduction.

Keywords *Aconitum Heterophyllum*, Anti-inflammatory, Mechanism, Molecular docking, Network pharmacology

Introduction

Inflammation is a biological function. When external stimuli stimulate the body or cause tissue damages, the body will protect itself from infringement through the inflammatory responses [1]. Inflammation can be divided into acute inflammation and chronic inflammation. Acute inflammation progresses rapidly, usually occurs immediately after infection or injury, and lasts for a short time. Chronic inflammation lasts for months

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or years. There are many factors that cause chronic inflammation, such as long-term stimulation of foreign substances or external factors, and persistent acute inflammation [2]. Chronic inflammation is a common feature of diabetes, cancer, obesity, cardiovascular disease, autoimmune diseases and other chronic diseases [3]. Chronic inflammatory diseases affect more than 41 million people worldwide each year, and more than 17 million people die before the age of 70 [4]. The number of deaths due to non-infectious diseases in low-and middle-income countries accounts for nearly 80% of the total, with chronic disease deaths accounting for a large proportion [5]. At present, non-steroidal anti-inflammatory drugs (NSAIDs) are widely used due to their established efficacy, but sometimes they can cause serious adverse reactions and even life-threatening. This highlights a major public health challenge that requires the development of effective and low-toxic anti-chronic inflammation drugs to reduce the impact of chronic inflammatory diseases.

Aconitum heterophyllum is native to the Indus River Basin and the northern Himalayas at an altitude of 2000 to 5000 m [6]. *Aconitum heterophyllum* has a wide range of biological activities such as anti-inflammatory [7], antibacterial [8], anti-renal injury [9], anti-hyperlipidemia [10], and regulation of the central nervous system [11]. The rich secondary metabolites of *Aconitum heterophyllum*, such as atisine [12], heteratisine, bezoylheteratisine [13] and atidine [14], have contributed to its wide range of biological activities. In recent times, the annual consumption of *A. heterophyllum* has exceeded 200 metric tons [15], underscoring its commercial value. *Aconitum heterophyllum*, as a non-toxic and widely used plant medicine, its mechanism of producing various biological activities in the body is not very clear. Therefore, on the basis of the anti-inflammatory activity of *Aconitum heterophyllum*, the anti-inflammatory mechanism was explored in this research from the perspective of anti-inflammatory activity. Network pharmacology provides a promising strategy for unraveling the biological basis of a multi-compound, multi-target system [16], thereby guiding the discovery of mechanisms underlying herbal formulas such as Huangqi Sijunzi Decoction [17], Bushen Hugu Decoction [18], and Wuzang Wenyang Huayu Decoction [19]. As the core methods of network pharmacology, computational and system tools are applied to construct the protein–protein interactions, enabling us to extend the effects of an ingredient on a signal target to its systematic effect on a biological network [20]. Molecular docking is applied to simulate compound ligand–target binding. Both of these approaches were employed to investigate and explain the mechanisms through which *A. heterophyllum* exerts its anti-inflammatory effects,

the results were validated in follow-up in vitro cell experiments.

Materials

RAW 264.7 cells, the mouse monocyte-macrophage, were obtained from China Center for Type Culture Collection. Lipopolysaccharide (LPS) was obtained from Sigma-Aldrich (St. Louis, MO, USA). Thiazolyl Blue (MTT) was obtained from Beijing Labgic Technology Co., Ltd. (Beijing, China). Fetal bovine serum (FBS) was obtained from Zhejiang TianHang Biotechnology Co. Ltd (Hangzhou, China). Dulbecco's Modified Eagle's Medium (DMEM) was obtained from ThermoFisher Biochemical Products Co., Ltd (Beijing, China). Penicillin–streptomycin solution (100×) was obtained from Beijing Aoning Biotechnology Co., Ltd (Beijing, China). Griess Reagent was obtained from Beyotime Biotechnology Co., Ltd (Shanghai, China). TRNzol Universal Reagent was obtained from Tiangen Biotech (Beijing) Co., Ltd (Beijing, China). ABScript II RT Master Mix for qPCR with gDNA remover and TB Green™ Premix Ex Taq™ was obtained from Wuhan ABclonal Biotechnology Co., Ltd (Wuhan, China).

Methods

Network pharmacology analysis of the targets

The compounds of *A. heterophyllum* were obtained from published literature. All the potential pharmacological targets of *A. heterophyllum* were accessed from the Swiss Target Prediction (<http://swisstargetprediction.ch/>). The data retrieval filters were set to “Probability > 0” to ensure the selection of reliable targets.

Furthermore, the related disease targets were searched and analyzed by selecting the species as “Homo sapiens” and inputting the keywords “anti-inflammatory” or “inflammation” into GeneCards Database (<http://www.genecards.org/>), PHARMGKB (<https://www.pharmgkb.org/>) and OMIM (<https://www.omim.org/>). Then, duplicate targets were subsequently removed and the remaining targets were integrated into a comprehensive list, representing the collection of targets related to inflammation for further investigation.

Construction of PPI network

The protein–protein interaction network was constructed by identifying the intersection targets between the *A. heterophyllum*-related genes and the disease targets. This network was developed using the STRING database (<http://string-db.org>) with a confidence score set at “Highest confidence (>0.9)” and the species restricted to “Homo sapiens”. Cytoscape 3.9.1 was employed to build the compound–target–disease network, with each component or target represented by a

node. Further, Cytoscape3.9.1 calculated the parameters of each node in the network diagram. Subsequently, the target nodes with degree values were selected and screened, focusing on those with degree values greater than or equal to twice the median degree value as the core targets, thus identifying the core targets, and constructing the network of “compound-target-disease”.

GO and KEGG enrichment analyses

The list of core targets was uploaded to the DAVID (<https://david.ncifcrf.gov/>) database with $P < 0.05$ as cut-off values and selected the species as “Homo sapiens”. Gene Ontology (GO) analysis was performed to identify enriched biological processes (BP), cellular components (CC), and molecular functions (MF). Additionally, Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis was conducted to gain insights into the pathways related to the biological processes. The enrichment results were then visualized using an online tool (<http://www.bioinformatics.com.cn/>).

Molecular docking

Twenty-two components associated with the core target were selected and docked with ten proteins from the core targets. The 3D structure files of the compounds and proteins were downloaded from the PubChem database (<https://pubchem.ncbi.nlm.nih.gov/>) and PDB database (<https://www.rcsb.org/>) respectively. Then, the compounds were prepared by hydrogenation and designated as ligands in Autodock Vina 4. Similarly, the protein structures were prepared by removing water, adding hydrogens, and being designated as receptors in Autodock Vina 4. After that, the docking box parameters, including center coordinates (center x/y/z) and box size (size x/y/z), were adjusted in Pymol to ensure that the target protein was fully encompassed within the docking box. Autodock Vina 4 accomplished molecular docking and evaluated free binding energies. Finally, the results were imported to Pymol and Ligplot for visualization.

Preparation of the total extract of *A.heterophyllum*

For sample preparation, roots of *A.heterophyllum* were collected from Pakistan by Dr. Sarfaraz Iqbal. The plant material was authenticated by Jun Li in the College of Pharmacy at South-Central Minzu University, Wuhan, Hubei, P.R. China. Approximately 15 kg of dried *A. heterophyllum* roots were finely powdered without any visible lumps and passed through a 30-mesh sieve. The powder was then soaked in ethanol with a volume fraction of 97%, which was five times the volume of the *A.heterophyllum* powder for 24 h at room temperature. This maceration process was repeated six times. Afterward, the extract was filtered, and the solvent was

recovered using a rotary evaporator, resulting in the total extract of *A.heterophyllum*. All the compounds were dissolved in DMSO and then diluted in Dulbecco's Modified Eagle's medium (DMEM, Hyclone, Logan, UT, United States) to achieve the desired concentrations. The final concentration of DMSO in the experimental samples was 0.1%.

Cell cultures and treatments

The cells were cultured in DMEM supplemented with 10% FBS and 1% penicillin–streptomycin solution (100×). The incubator temperature was 37 °C and filled with 5% CO₂. To prepare the cells for experimentation, the following treatments were performed: (1) The cell suspension (5×10^3 cells/well) was plated into 96 well-plates and adhered for 12 h; (2) Transient serum starvation was performed for 12 h to synchronize the cell cycle.

Effect of *A.heterophyllum* on cell viability of RAW264.7

RAW 264.7 cells in logarithmic growth phase were collected and cells were inoculated in 96-well plates at 1×10^3 cells/well. Cells were incubated for 12 h and were subjected to 12 h of starvation after treatment. After this, cells were treated with different concentrations of *A.heterophyllum* (0–200 $\mu\text{g}\cdot\text{mL}^{-1}$) for 24 h. After *A.heterophyllum* treatment, MTT at a final concentration of 0.5 $\text{mg}\cdot\text{mL}^{-1}$ was added and the cells were incubated for 4 h. At the end of the incubation, the methylated filth was dissolved in DMSO and the absorbance was measured at 570 nm. The cell viability was expressed as a percentage of the control culture value.

Effects of *A.heterophyllum* on cell viability of damaged RAW 264.7

Cell viability was assessed using the MTT reduction assay. Briefly, RAW 264.7 cells (5×10^3 cells/well) were treated with *A.heterophyllum* at concentrations ranging from 0.01 $\mu\text{g}\cdot\text{mL}^{-1}$ to 5 $\mu\text{g}\cdot\text{mL}^{-1}$ 24 h at 37 °C. After treatment of the cells with *A.heterophyllum*, cells were incubated with LPS for an additional 24 h. Finally, after a 4 h incubation with MTT (0.5 $\text{mg}\cdot\text{mL}^{-1}$), the methylated filth was dissolved in DMSO and the absorbance was measured at 570 nm. The cell viability was expressed as a percentage of the control culture value.

Nitric oxide measurement

The concentration of nitric oxide (NO) in the culture medium was determined by the Griess reaction. Specifically, the cells were pretreated with *A.heterophyllum* at concentrations ranging from 0.01 $\mu\text{g}\cdot\text{mL}^{-1}$ to 5 $\mu\text{g}\cdot\text{mL}^{-1}$ for 24 h, followed by stimulation with 1 $\mu\text{g}\cdot\text{mL}^{-1}$ of lipopolysaccharide (LPS) for an additional 24 h at 37 °C. After that, 100 μL of supernatant from each well was

mixed with 100 µL of Griess reagent in a new 96-well plate. After incubating for 15 min at room temperature, the optical density (OD) was measured at 540 nm using a microplate reader, and the rate of NO inhibition was calculated.

Inhibition rate (%)=[(OD of model group—OD of drug administration group)/(OD of model group—OD of blank group)] × 100%

RNA Extraction and Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

Quantitative Real-time PCR was employed to determine the gene expression. Total RNA was extracted from RAW 264.7 cells using the Trizol reagent. For mRNA quantification, cDNA was synthesized from 1 µg of total RNA using the PrimeScript RT reagent kit, following the manufacturer’s instructions. Reactions were performed in a 20 µL volume according to the thermal cycler manufacturer’s protocol (Rox), using an ABI 7500 Real-Time PCR system under the condition: 5 min at 95 °C, followed by 40 cycles of 10 s at 95 °C, and 34 s at 58 °C. The primer sequences used for the amplification of target genes are listed in Table 1.

Statistical analysis

All statistical analyses were conducted using Graph-Pad Prism 8 software and Microsoft Excel software. Data were presented as mean ± standard deviation (SD) and were analyzed with one-way analysis of variance (ANOVA).

Results

Active compounds and potential targets of *A. heterophyllum*

A total of 35 chemical compounds of *A.heterophyllum* were identified through a comprehensive literature review (Table 2). By retrieving the Swiss Target Prediction database, 1322 potential targets of *A.heterophyllum* active ingredients were obtained. To focus on targets

Table 2 Information of 35 active compounds

No	Name	CAS	Formula
1	atisine	466–43-3	C ₂₂ H ₃₃ NO ₂
2	hetisine	10,089–23-3	C ₂₀ H ₂₇ NO ₃
3	heteratisine	3328–84-5	C ₂₂ H ₃₃ NO ₅
4	hetidine	28,154–35-0	C ₂₁ H ₂₇ NO ₄
5	atidine	467–91-4	C ₂₂ H ₃₃ NO ₃
6	hetisinone	4829–55-4	C ₂₀ H ₂₅ NO ₃
7	heterophyllisine	15,266–41-8	C ₂₂ H ₃₃ NO ₄
8	heterophyllidine	-	C ₂₁ H ₃₁ NO ₅
9	8-methyllycaconitine	21,019–30-7	C ₃₉ H ₅₅ NO ₁₀
10	14-demethyllycaconitine	-	C ₃₈ H ₅₃ NO ₁₀
11	N-deethyllycaconitine-N-aldeuyde	-	C ₃₈ H ₅₁ NO ₁₁
12	swatinine	-	C ₂₅ H ₄₁ NO ₉
13	heterophyllinine-A	-	C ₂₂ H ₃₃ NO ₂
14	heterophyllinine-B	-	C ₂₅ H ₃₉ NO ₄
15	dihydroatisine	17,934–91-7	C ₂₂ H ₃₅ NO ₂
16	lycoctonine	26,000–17-9	C ₂₅ H ₄₁ NO ₇
17	6β-methoxy-9β-dihydroxylheteratisine	-	C ₂₄ H ₃₇ NO ₆
18	aconitine	302–27-2	C ₃₄ H ₄₇ NO ₁₁
19	dehydroacetylsepaconitine	52,341–40-9	C ₃₅ H ₅₂ N ₂ O ₁₀
20	1α,11,13β-trihydroxylhetisine	-	C ₂₁ H ₂₉ NO ₃
21	6,15β-dihydroxylhetisine	-	C ₂₁ H ₂₉ NO ₂
22	iso-atisine	510–38-3	C ₂₂ H ₃₃ NO ₂
23	steviol	471–80-7	C ₂₁ H ₃₄ O ₃
24	atisenol	84,743–53-3	C ₂₀ H ₃₀ O ₂
25	lappaconitine	-	C ₃₂ H ₄₄ N ₂ O ₈
26	lycaconitine	-	C ₃₆ H ₄₈ N ₂ O ₁₀
27	anthorine	466–43-3	C ₂₂ H ₃₃ NO ₂
28	2-O-cinnamoyl hetisine	-	C ₂₉ H ₃₄ NO ₄
29	atisinium	50,510–23-1	C ₂₃ H ₃₈ NO ₂
30	aconitic acid	585–84-2	C ₆ H ₆ O ₆
31	ferulic acid	537–98-4	C ₁₀ H ₁₀ O ₄
32	cinnamic acid	140–10-3	C ₉ H ₈ O ₂
33	delphatine	25,488–62-4	C ₂₆ H ₄₃ NO ₇
34	13-hydroxylappaconitine	-	C ₃₃ H ₄₈ N ₂ O ₉
35	6-dehydroacetylsepvaconitine	-	C ₃₃ H ₄₆ N ₂ O ₁₀

Table 1 Primer sequences used for the amplification of target genes

Primer Name	Forward primer (5′–3′)	Reverse primer (5′–3′)
AKT1	CTTTATTGGCTACAAGGAACGG	CAGTCTGAATGGCGGTGGT
EGFR	GCCATCTGGGCCAAAGATACC	GTCTTCGCATGAATAGGCCAAT
JAK2	TTGTGGTATTACGCTGTGTATC	ATGCCTGGTTGACTCGTCTAT
STAT3	AATCTCAACTTCAGACCCGCC AAC	GCTCCACGATCCTCTCTCCAG
iNOS	AGTGAAGCAAAGCCCAACAA	TGGACGGGTGCATGTCA
GADPH	ACCCAGAAGACTGTGGATGG	TCAGCTCAGGGATGACCTTG

relevant to inflammation and anti-inflammatory processes, target genes in GeneCards, PHARMGKB, and OMIM. After removing duplicate entries, 3534 disease-related targets were identified, which were taken at the intersection with the compound targets and obtained 244 common targets.

Network construction

The protein–protein interaction networks of intersection targets were constructed and visualized via the STRING platform and Cytoscape 3.9.1, as depicted in Fig. 1A. In this network, the size of each node corresponds to its

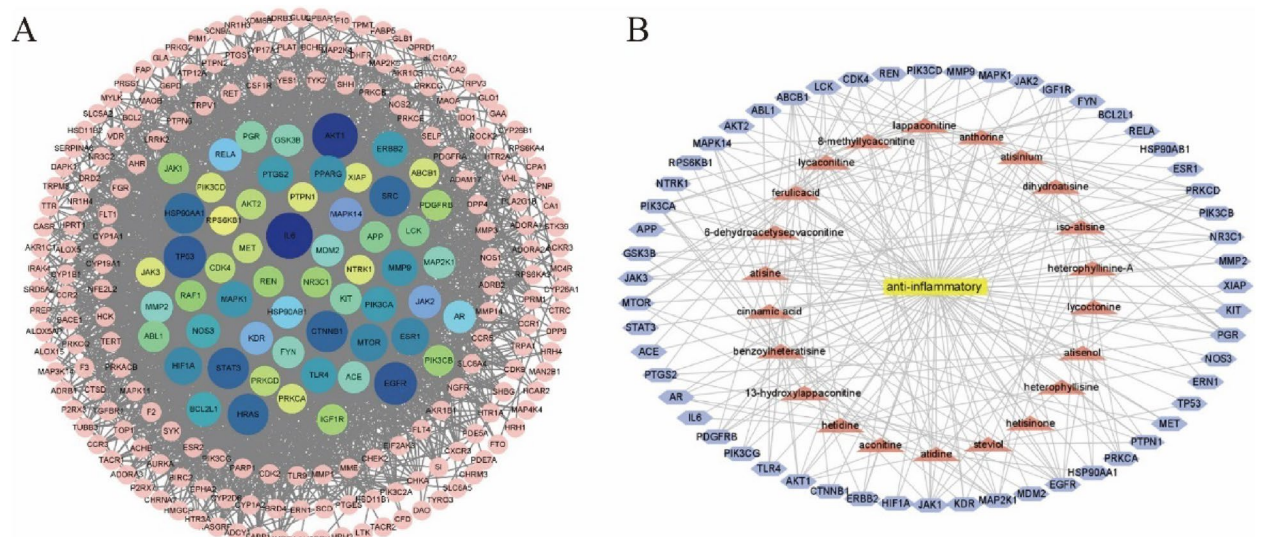


Fig. 1 The results of network construction. **A** The PPI network map of the target protein. **B** The network of “compounds-targets-disease”; Yellow represents the disease. Orange represents the active components. Purple represents the common target of the active components and the disease

degree value, which indicates the number of connections. The targets were arranged in descending order according to their parameter matching score, and the top 55 targets prominently displayed at the center of the graph, were screened as the core targets for further study. As shown in Fig. 1A, IL6, AKT1, EGFR, STAT3, TP53, SRC, HRAS, CTNNB1, and JAK2 exhibited higher weights compared to other targets, implying their important roles in *A.heterophyllum*-regulating anti-inflammatory system. Furthermore, the compounds and core targets were imported to Cytoscape3.9.1 to build a compound-target-disease network that provides insight into the complex effects of *A.heterophyllum* ingredients on inflammation. As illustrated in Fig. 1B, the orange triangles represent the compounds of *A.heterophyllum*, surrounded by corresponding targets.

GO and KEGG enrichment analysis

GO enrichment was performed to understand the mechanisms of the multi-component treatment system. In the STRING database, the 244 common targets were found to be involved in 66 cell components (CC), 111 molecular functions (MF), and 528 biological processes (BP). The top 20 terms of CC, MF and BP were presented as column charts. The main molecular functions identified were ATP binding, and protein kinase activity (Fig. 2A). The most highly enriched CC terms included plasma membrane, cytosol, and cytoplasm (Fig. 2B). From the biological process enrichment analysis, it can be claimed that the mechanism may be related to signal transduction, positive regulation of gene expression and

protein phosphorylation (Fig. 2C). In order to explore the signaling pathways involved in the anti-inflammation of *A.heterophyllum*, KEGG enrichment analysis was conducted (Fig. 2D), revealing a vast array of pathways starting from pathways in cancer, PI3K-Akt signaling pathway, and Proteoglycans in cancer. Notably, high enrichment of EGFR, STAT3, and JAK2 together with AKT1 for the EGFR tyrosine kinase inhibitor resistance and PI3K-Akt signaling pathway suggests that *A.heterophyllum* may activate these two pathways in the treatment of inflammation.

Molecular docking

Twenty-two components corresponding to the core target and top 10 core targets were selected to perform the molecular docking. The vina scores are shown in Table 3.

In general, when the binding energy of a ligand to a receptor is less than -5 kcal/mol, the two can be considered to have good and appreciable binding affinity. Lower binding energy indicates higher affinity, docking activity, and binding potential, resulting in a more stable conformation. The results revealed that almost all receptor-ligand interactions had binding energies below -5 kcal/mol. Among them, JAK2, AKT1, EGFR, and STAT3 exhibited relatively lower scores in all fractions. Thus, the docking results of the four targets between two key active ingredients with low binding energy are shown in Fig. 3. These findings strongly support the validity of the docking results, based on which we predicted that these

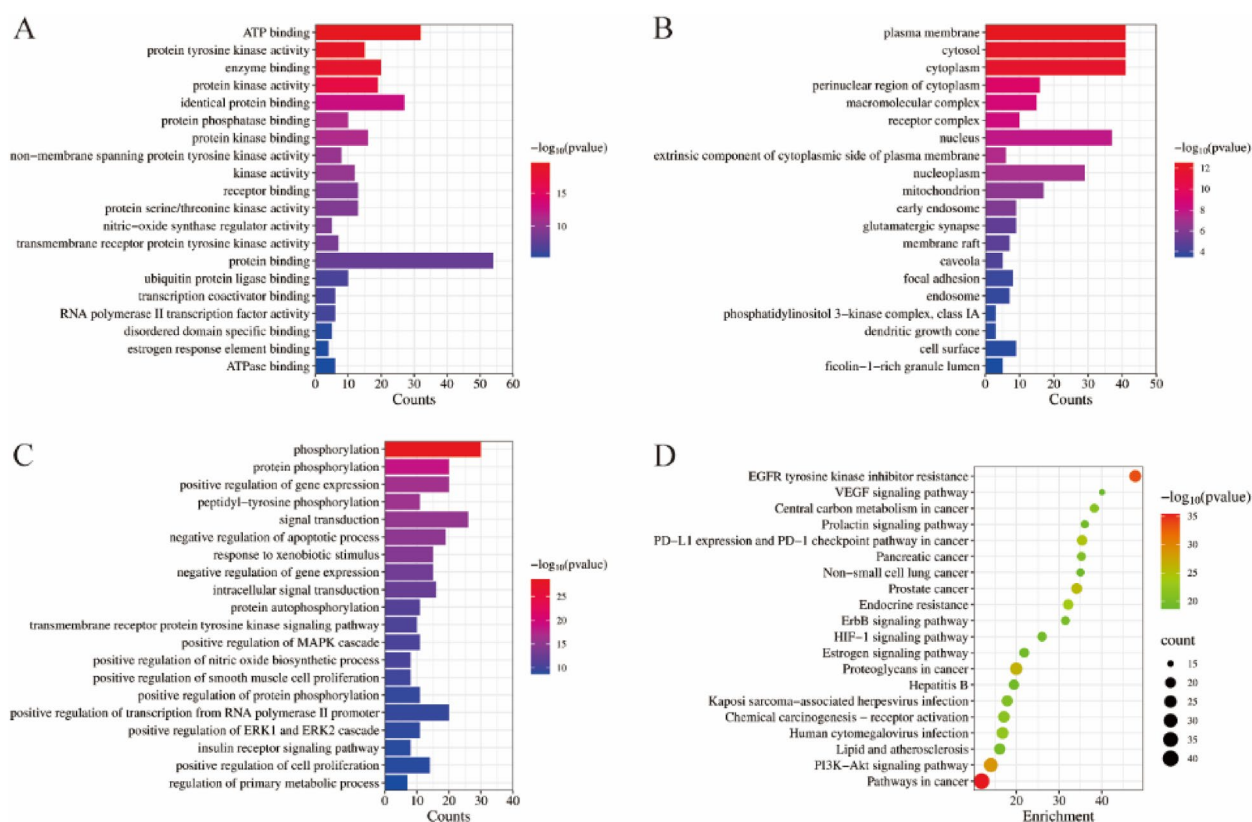


Fig. 2 GO function enrichment and KEGG pathway enrichment analysis (A): MF; (B): CC; (C): BP; (D): KEGG

compounds may play a significant role in the biological processes of anti-inflammatory.

Effect of *A.heterophyllum* on Cell Viability of RAW264.7

Subsequently, RAW264.7 cells were treated with varying concentrations of *A.heterophyllum* solutions to identify an optimal dose for investigating its anti-inflammatory role. Results obtained from MTT illustrated that cell viability was partly inhibited by *A.heterophyllum* ($12.5\text{--}200\ \mu\text{g}\cdot\text{mL}^{-1}$) indicating that *A.heterophyllum* caused prominent cell injury, compared with the normal control group. The RAW264.7 cells treated with *A.heterophyllum* at 3.126 and $6.25\ \mu\text{g}\cdot\text{mL}^{-1}$ showed a trend of slight reduction of cell viability, but there were no notable differences between these two concentrations (Fig. 4A). Given this, concentrations of *A. heterophyllum* at $0.01\text{--}5\ \mu\text{g}\cdot\text{mL}^{-1}$ were applied for the subsequent experiments.

Effects of *A.heterophyllum* on Cell Viability of Damaged RAW 264.7

As shown in Fig. 4B, cells without pharmacological intervention showed a decrease in cell viability (compared

with the normal group, $P < 0.01$) after stimulation with LPS. Pre-incubating the cells with different concentrations of *A. heterophyllum* for 24 h before stimulating the cells with LPS, the four concentrations of *A. heterophyllum* were able to prevent the cells from being damaged to varying degrees (compared with the LPS group, $P < 0.01$).

A.heterophyllum inhibited the release of NO and decreased the expression of iNOS mRNA in LPS-induced RAW264.7

Furthermore, to elucidate the anti-inflammatory role of *A. heterophyllum*, its potential effect on NO levels in RAW264.7 cells was investigated, given the established involvement of NO in inflammatory processes. As Fig. 5A demonstrated, LPS stimulation-induced NO levels for the model group were remarkably higher than that of the control group. However, upon the treatment with 4 different concentrations ($0.01, 0.1, 0.5$, and $5\ \mu\text{g}\cdot\text{mL}^{-1}$) of *A. heterophyllum*, an evident downward trend in NO levels was observed.

The inhibitory effect appeared to slightly weaken with increasing concentrations. For each concentration of *A.heterophyllum*, $0.1\ \mu\text{g}\cdot\text{mL}^{-1}$ of *A.heterophyllum* demonstrated the highest efficacy in reducing NO expression levels. In addition, $0.01\ \mu\text{g}\cdot\text{mL}^{-1}$ of *A. heterophyllum*

Table 3 Scores for molecular docking

Compound/Affinity (kal/mol)	JAK-2	AKT	EGFR	STAT3	IL6	TP53	CTNNB1	HRAS	SRC	HSP90AA1
6-dehydroacetylsepaconitine	−9.1	−9.5	−10.1	−8.3	−7.1	−7.9	−8.8	−6.3	−8.1	−7.2
8-methyllycaconitine	−10.8	−11.2	−8.9	−8.8	−7	−8.1	−9.5	−6.7	−8.7	−9.9
atidine	−9	−9.6	−8	−7	−6.6	−6.8	−6.9	−6.4	−7.6	−7.6
benzoylheteratisine	−10.1	−10	−9	−8.1	−6.9	−7.7	−7.8	−8.5	−8.1	−8.8
dihydroatisine	−9	−9.5	−7.9	−7.6	−6.5	−6.7	−7.3	−6.5	−7.7	−7.5
heterophyllisine	−9	−9.9	−8.1	−7.5	−7	−6.6	−7.4	−6.6	−7.3	−7.8
iso-atisine	−9.5	−9.7	−8.2	−8.6	−7.2	−7.1	−6.6	−7.1	−7.8	−7.8
lappaconitine	−10.2	−10.2	−9	−7.9	−7.5	−8	−8.6	−6.9	−7.7	−8.8
anthorine	−8.2	−6.9	−8.9	−7.8	−9.1	−6.4	−6.7	−8.2	−8.6	−7
atisinium	−8.6	−6.5	−8.3	−7.8	−8.5	−6.2	−6.4	−7	−8.2	−6.3
heterophyllinine-A	-	-	-	-	-	-	-	-	-	-
lycoctonine	−7.2	−5.7	−7.2	−6.7	−7.6	−5.2	−5.8	−6.3	−7.7	−5.7
atisenol	−9.3	−7.1	−8.1	−7.4	−8.9	−7.3	−6.3	−7.1	−9.2	−7
hetisinone	−8.6	−6.8	−8	−6.7	−7.7	−6.7	−7.2	−7.2	−7.9	−6.2
steviol	−9	−6.7	−8.5	−7.5	−8.3	−6.5	−6.2	−7.6	−9	−6.4
aconitine	−7.2	−7	−7.6	−7.8	−8.1	−6.1	−6.1	−6.4	−7.6	−5.5
hetidine	−9.4	−7	−8.5	−8.2	−9.2	−6.8	−6.8	−7.5	−8.7	−7.4
13-hydroxylappaconitine	−10	−7.1	−8.8	−8.7	−9.7	−7.1	−6.7	−7.4	−9.3	−6.3
cinnamic acid	−6.2	−5.5	−6.8	−5.1	−7	−5.4	−4.7	−5.9	−6.3	−6
atisine	−8.8	−6.9	−8.9	−7.7	−9.1	−6.4	−6.7	−8.2	−8.6	−6.8
ferulicacid	−6.6	−6	−7.2	−5.7	−7.3	−5.4	−5.1	−7	−6.7	−6
lycaconitine	−9.6	−7.6	−8.4	−7.6	−8.6	−7.6	−6.9	−6.3	−8.2	−6.2

performed worse than that of $0.5 \mu\text{g}\cdot\text{mL}^{-1}$ in this respect, indicating that $0.1 \mu\text{g}\cdot\text{mL}^{-1}$ and $0.5 \mu\text{g}\cdot\text{mL}^{-1}$ may be favorable for follow-up experiments. These findings showed that *A.heterophyllum* could attenuate the inflammatory response preliminarily.

Quantitative Real-time PCR assays were performed to assess the mRNA expression of pro-inflammatory marker iNOS. As expected, the model group exhibited significantly higher iNOS mRNA expression compared to the control group (Fig. 5B), which was partly reversed by *A.heterophyllum* (0.1 and $0.5 \mu\text{g}\cdot\text{mL}^{-1}$). In accordance with the results of the NO concentration measurement, *A.heterophyllum* at a concentration of $0.1 \mu\text{g}\cdot\text{mL}^{-1}$ performed better in inhibiting the expression of iNOS mRNA compared to the concentration of $0.5 \mu\text{g}\cdot\text{mL}^{-1}$.

A.heterophyllum bridle the expression levels of EGFR, STAT3, JAK2, and AKT1 mRNA in LPS-induced RAW264.7

Next, the mRNA expression levels of EGFR, STAT3, JAK2, and AKT1—genes implicated in inflammatory pathology—were measured. The qRT-PCR results demonstrated a similar trend in the expressions of these targets. LPS induced a remarkable activation in the expression of the four genes in RAW264.7 cells in comparison to the control group. Treatment with $0.1 \mu\text{g}\cdot\text{mL}^{-1}$ of *A.heterophyllum* significantly abolished

the LPS-induced effects, while $0.5 \mu\text{g}\cdot\text{mL}^{-1}$ of *A.heterophyllum*, not dramatically though, soothed the high expression as well. EGFR (Fig. 6A), JAK2 (Fig. 6B) and STAT3 (Fig. 6C) expressions experienced a relatively slight increment following LPS treatment and obviously dropped to levels under that of the control group upon $0.1 \mu\text{g}\cdot\text{mL}^{-1}$ *A.heterophyllum* treatment. Regarding AKT1 (Fig. 6D), the treatment effects related to the two concentrations were not that different, both lower than those of the LPS group but slightly higher than the control group. Taken together, these experiments revealed that the LPS-induced levels of mRNA expressions of iNOS, EGFR, STAT3, JAK2, and AKT1 were attenuated by *A.heterophyllum*, suggesting that their involvement in the development of inflammation, and the potential of *A. heterophyllum* as a treatment strategy to inhibit these targets.

Discussion

Inflammation, which is involved in the innate and adaptive immune systems, is a universal defense response to various damaging factors. The persistence of inflammation is a pathophysiological factor in various diseases. According to its duration time, inflammation is divided into acute and chronic inflammation. Acute inflammatory demyelinating polyradiculoneuropathy and septic

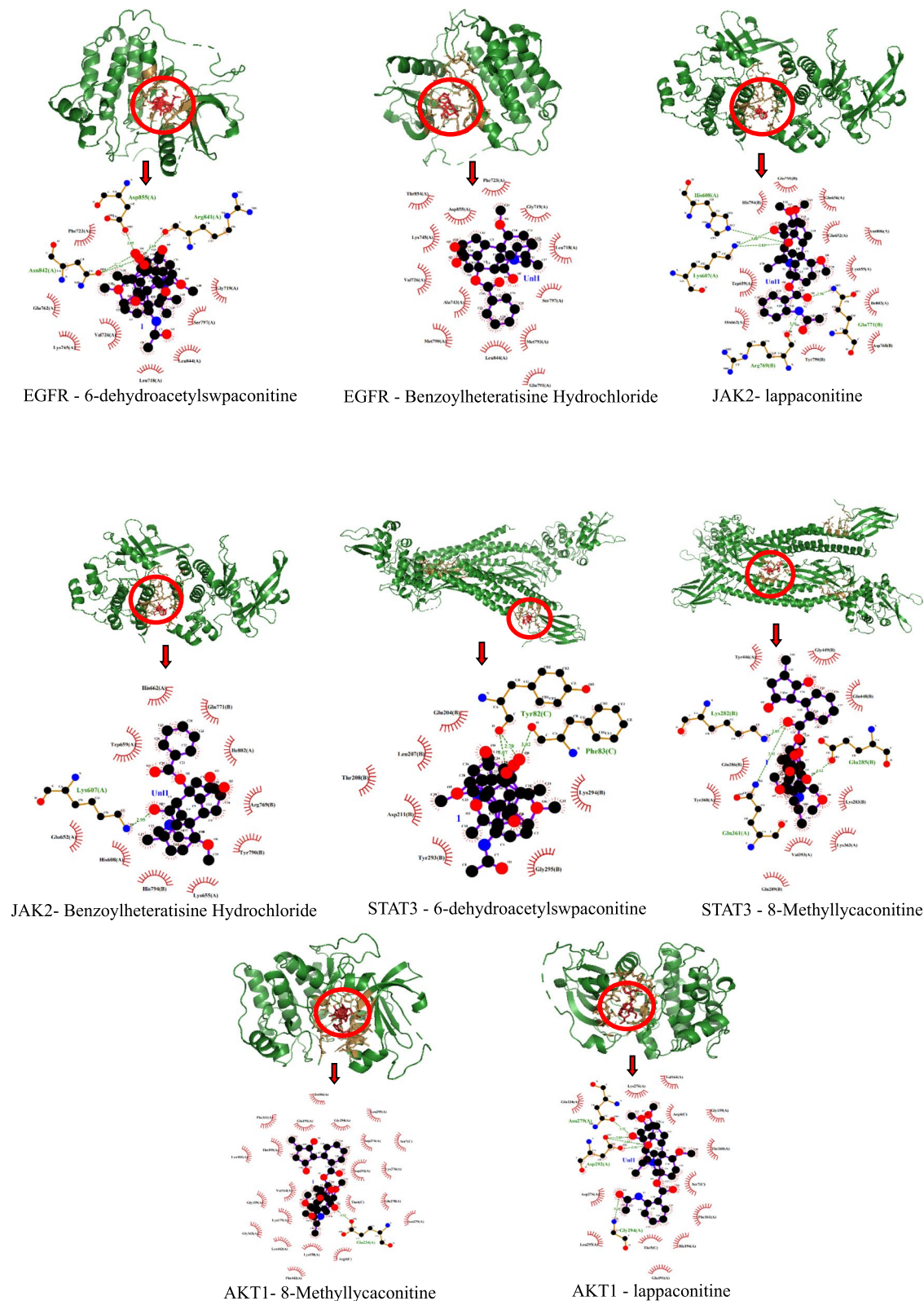


Fig. 3 Molecular docking results of AKT1, JAK2, EGFR and STAT3 with compounds

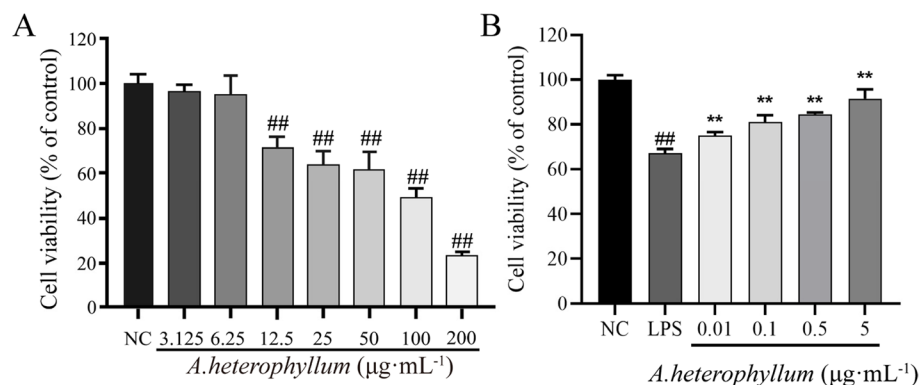


Fig. 4 Results of cell viability assay. **A** Effect of *A.heterophyllum* on cell viability of RAW264.7. **B** Effects of *A.heterophyllum* on cell viability of damaged RAW 264.7. $N=6$, mean $\pm$ SD. ^{##} $P < 0.01$, compared with normal control; ^{**} $P < 0.01$, compared with the LPS group

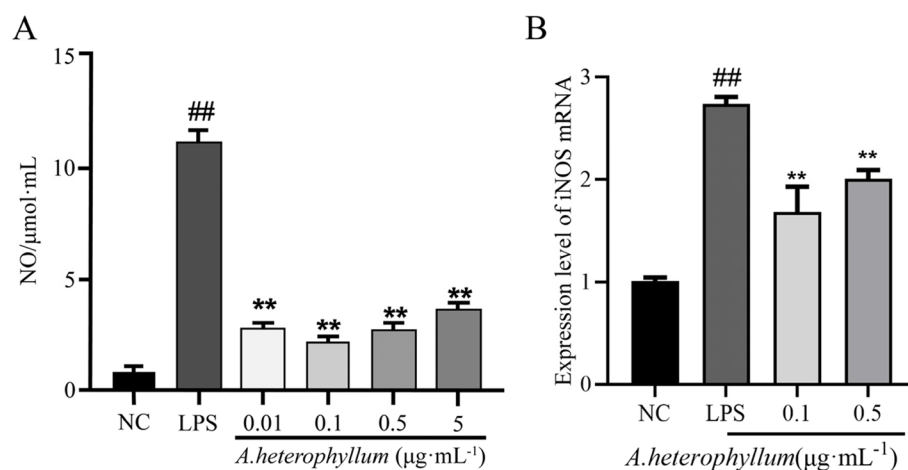


Fig. 5 Effects of *A.heterophyllum* on NO release and iNOS mRNA expression level of LPS-induced RAW264.7. **A** NO release level; **B**: iNOS mRNA expression level. $N=6$, mean $\pm$ SD. ^{##} $P < 0.01$, compared with normal control; ^{**} $P < 0.01$, compared with the LPS group

shock are typical acute inflammatory diseases, while chronic inflammation significantly affects the progression of atherosclerosis, cancer, obesity, and other diseases [21]. Recent studies have already provided the long-term coexistence of acute and chronic inflammation in rheumatoid arthritis, chronic obstructive pulmonary disease, asthma, and ulcerative colitis [22]. Due to social pressures and increased economic competitiveness, many people in modern society have varying degrees of stress, which may also lead to sleep disorders when people feel too much pressure. Most people are likely to de-stress through poor lifestyle and dietary habits. Common risk factors for chronic inflammation include smoking, stress, sleep problems, obesity, unhealthy diet, etc. [23]. Therefore, chronic inflammation is likely to lurk in most people's bodies and pose a threat to life and health. Modern studies have shown that many traditional Chinese medicines have

good anti-inflammatory effects while having small side effects and are easier to obtain, and have great development value.

A.heterophyllum contains abundant secondary metabolites, which, as clarified in modern studies, have explicit potential in the treatment of inflammatory conditions. For instance, atidine, as well as iso-atidine, could reduce inflammatory tissue injury driven by reactive oxygen species metabolism through radical scavenging effects [8]. Heteratisine competitively inhibits acetylcholinesterase and butyrylcholinesterase, which may explain its use in the treatment of neuropathic pain due to neuroinflammation¹¹. Moreover, lappaconitine can suppress the release of the common inflammatory mediator, NO, thereby modulating inflammation [24]. Considering that the involved mechanisms are poorly reported, this research aims to probe into the mechanism by which *A.heterophyllum* attenuates inflammation.

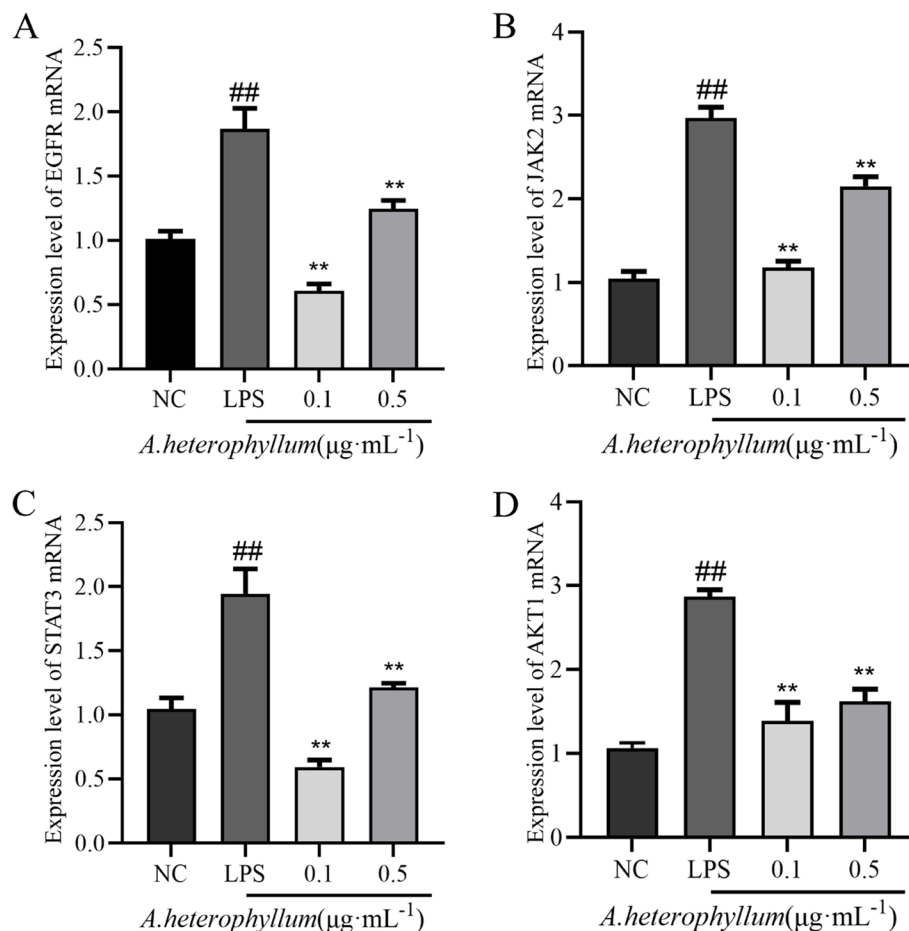


Fig. 6 Effects of *A.heterophyllum* on LPS-induced inflammation on related genes. **A** The effect of the key components in *A.heterophyllum* on EGFR; **(B)**: The effect of the key components in *A.heterophyllum* on JAK2; **(C)**: The effect of the key components in *A.heterophyllum* on STAT3; **(D)**: The effect of the key components in *A.heterophyllum* on AKT1. $N=3$, mean $\pm$ SD. ^{##} $P<0.01$, compared with normal control; ^{**} $P<0.01$, compared with the LPS group

Through constructing the PPI network, IL6, TP53, CTNNB1, especially JAK2, AKT1, EGFR, and STAT3 were methodically obtained as the core targets. Pro-inflammatory cytokines such as CCL-2 and CCL-5 would experience an intense upstream due to the upstream transactivation of EGFR [25]. The knocking out of JAK2 would downregulate the expression of TNF- α and IL-6 while increasing the expression of IL-10 [26]. Synergistic interactions between NF- κ B and STAT3 induce the hyper-activation of NF- κ B, followed by the production of IL-6. This triggers a positive feedback loop of NF- κ B activation by the IL-6–STAT3 axis and plays a role in chronic inflammation [27]. Molecular docking results indicated that the targets were capable of binding to chemical compounds from *A.heterophyllum*, as the binding energy was generally less than -6 kcal/mol. These findings suggest that the anti-inflammatory effects of *A. heterophyllum* depend on the modulation of EGFR, JAK2, and STAT3.

Hypothesizing that EGFR, JAK2, STAT3, and AKT1 could be blocked by *A.heterophyllum*, and then alleviate inflammation, qRT-PCR was introduced to test the expression levels of the targets' mRNA. The results demonstrated that *A.heterophyllum* manifestly reduced the LPS-induced iNOS secretion in RAW264.7 cells in accordance with the data on NO release, and the increase in the expression of the four targets was diminished by *A.heterophyllum* remarkably. The autophosphorylation of active EGFR tyrosine residue dimer can recruit AKT1 to the cell membrane and phosphorylate later. Then the PI3K-AKT signaling pathway is activated [25], leading to the significant production of the inflammatory factors IL-1 β [28] and the subsequent induction of iNOS expression [29]. Thus, by inhibiting EGFR and AKT1, *A.heterophyllum* could decrease the activation of iNOS and partially contributes to the inflammatory response.

JAK2 has been shown to mediate through EGFR as well [30]. The JAK2/STAT3 signaling pathway is known to be

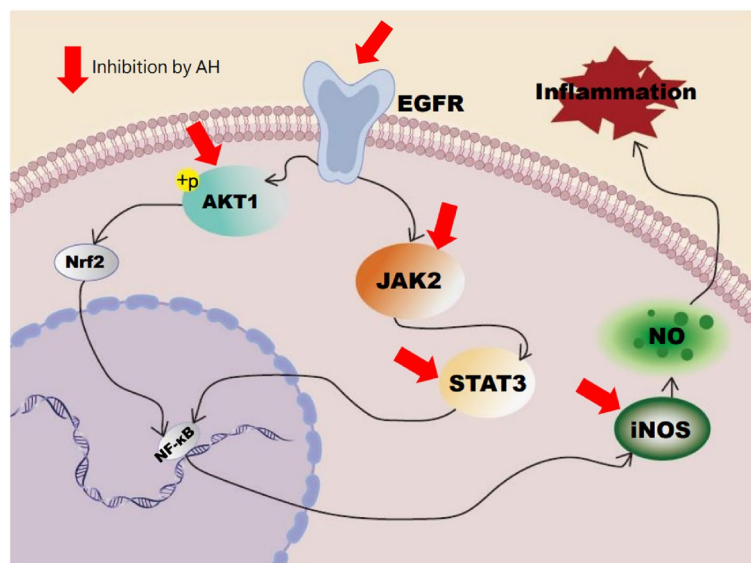


Fig. 7 Possible mechanism of actions of *A.heterophyllum* as an anti-inflammatory agent

over-activated in cells and animal models of inflammation, and plays a crucial role in regulating inflammation development [31]. According to the reports, phosphorylation of JAK2 and STAT3 can induce iNOS expression [32]. Excessive production of NO resulting from the excessive metabolism of iNOS can lead to sepsis, posing a risk of further progression into a systemic inflammatory response syndrome [33]. Furthermore, activation of the JAK2/STAT3/NF- κ B pathway strongly induced the expression of TNF- α [34], which in turn activates the NF- κ B/iNOS pathway and promotes neuroinflammatory development [35]. The qRT-PCR results confirmed that EGFR, JAK2, STAT3, and iNOS were activated in inflammatory RAW264.7 cells, consistent with the aforementioned reports. Conversely, by inhibiting JAK2/STAT3 pathway, *A.heterophyllum* can reduce the expression of iNOS and mitigate inflammatory response, aligning with the speculated mechanism.

Although further study is needed to clarify this mechanism in detail, these results suggested the protective effects of *A.heterophyllum* on LPS-induced inflammatory response through regulating EGFR/JAK2/STAT3 pathway and EGFR/AKT1 pathway and inhibiting the expression of iNOS associated with the release of NO. This forms a complete negative regulation circuit that further alleviates the progression of inflammation.

Conclusion

The results of this study suggest that *A.heterophyllum* exerts anti-inflammatory activity by inhibiting EGFR-AKT1 activity and inhibiting EGFR/JAK2/STAT3 signal transduction (Fig. 7).

Acknowledgements

We appreciate Dr. Sarfaraz Iqbal, who donated the roots of *A. heterophyllum*. We also want to thank Professor Jun Li for identifying *A. heterophyllum*.

Authors' contributions

Xiang Meng: Methodology, writing—original draft, software, data curation. Xinyi Xu: Methodology, validation, software, data curation. Leiling Shi: review. Yupeng Liu: Data curation. Jun Deng: Methodology. Xianju Huang: Conceptualization, Methodology, writing—review and editing, funding acquisition.

Funding

This work was supported by Hubei Science and Technology Innovation Base (platform) (2024CFC003). Hubei International Science and Technology Cooperation Base (SH2311) and the modern transmission and innovation research team of Traditional Chinese Medicine, South Central Minzu University.

Data availability

The datasets used and/or analyzed among the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

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

Received: 12 December 2024 Revised: 29 January 2025 Accepted: 12 February 2025

Published online: 04 March 2025

References

- Bai J, Zhang Y, Tang C, et al. Gallic acid: Pharmacological activities and molecular mechanisms involved in inflammation-related diseases. *Biomed Pharmacother*. 2021;133: 110985. <https://doi.org/10.1016/j.biopha.2020.110985>.
- Kishore N, Kumar P, Shanker K, Verma AK. Human disorders associated with inflammation and the evolving role of natural products to overcome. *Eur J Med Chem*. 2019;179:272–309. <https://doi.org/10.1016/j.ejmech.2019.06.034>.
- Chen Z, Ding W, Yang X, Lu T, Liu Y. Isoliquiritigenin, a potential therapeutic agent for treatment of inflammation-associated diseases. *J Ethnopharmacol*. 2024;318: 117059. <https://doi.org/10.1016/j.jep.2023.117059>.
- WHO. Noncommunicable diseases. Accessed December 11, 2024. <https://www.who.int/zh/news-room/fact-sheets/detail/noncommunicable-diseases>.
- Sarup S, Obukhov AG, Raizada S, Atre R, Baig MS. Drug repurposing in the treatment of chronic inflammatory diseases. *Future J Pharm Sci*. 2024;10(1):152. <https://doi.org/10.1186/s43094-024-00730-1>.
- Malik ZA, Bhat JA, Ballabha R, Bussmann RW, Bhatt AB. Ethnomedicinal plants traditionally used in health care practices by inhabitants of Western Himalaya. *J Ethnopharmacol*. 2015;172:133–44. <https://doi.org/10.1016/j.jep.2015.06.002>.
- Rajakrishnan R, Alfathan AH, Al-Ansari AM, et al. Therapeutic efficacy of the root tubers of *Aconitum heterophyllum* and its substitute *Cyperus rotundus* in the amelioration of pylorus ligation induced ulcerogenic and oxidative damage in rats. *Saudi J Biol Sci*. 2020;27(4):1124–9. <https://doi.org/10.1016/j.sjbs.2019.12.025>.
- Ahmad H, Ahmad S, Shah SAA, et al. Antioxidant and anticholinesterase potential of diterpenoid alkaloids from *Aconitum heterophyllum*. *Bioorg Med Chem*. 2017;25(13):3368–76. <https://doi.org/10.1016/j.bmc.2017.04.022>.
- Konda VGR. Antioxidant and nephroprotective activities of *Aconitum heterophyllum* root in glycerol induced acute renal failure in rats. *J Clin Diagn Res*. 2016; 10(3):FF01–FF05. <https://doi.org/10.7860/JCDR/2016/10798.7388>.
- Subash AK, Augustine A. Hypolipidemic effect of methanol fraction of *Aconitum heterophyllum* wall ex Royle and the mechanism of action in diet-induced obese rats. *J Adv Pharm Technol Res*. 2012;3(4):224–8. <https://doi.org/10.4103/2231-4040.104713>.
- Nisar M, Ahmad M, Wadood N, Lodhi MA, Shaheen F, Choudhary MI. New diterpenoid alkaloids from *Aconitum heterophyllum* Wall: Selective butyrylcholinesterase inhibitor. *J Enzyme Inhib Med Chem*. 2008;24(1):47–51. <https://doi.org/10.1080/14756360801906202>.
- Malhotra N, Kumar V, Sood H, Singh TR, Chauhan RS. Multiple genes of mevalonate and non-mevalonate pathways contribute to high aconites content in an endangered medicinal herb. *Aconitum heterophyllum* Wall. *Phytochemistry*. 2014;108:26–34. <https://doi.org/10.1016/j.phytochem.2014.08.025>.
- Jacobs WA, Craig LC. THE ACONITE ALKALOIDS. *J Biol Chem*. 1942;143(3):589–603. [https://doi.org/10.1016/S0021-9258\(18\)72589-1](https://doi.org/10.1016/S0021-9258(18)72589-1).
- Jowett HAD. XCIX.—Contributions to our knowledge of the aconite alkaloids. Part XIII. On atisine, the alkaloid of *Aconitum heterophyllum*. *J Chem Soc Trans*. 1896;69(0):1518–1526. <https://doi.org/10.1039/CT8966901518>.
- Wani TA, Kaloo ZA, Dangroo NA. *Aconitum heterophyllum* Wall. ex Royle: A critically endangered medicinal herb with rich potential for use in medicine. *J Integr Med*. 2022;20(2):104–113. <https://doi.org/10.1016/j.joim.2021.12.004>.
- Li S. Network target: a starting point for traditional Chinese medicine network pharmacology. *China J Chin Materia Med*. 2011;36(15):2017–20.
- Cui Y, Wang H, Wang D, et al. Network pharmacology analysis on the mechanism of Huangqi Sijunzi Decoction in treating cancer-related fatigue. *Khan F, ed. J Health Eng*. 2021;2021:1–10. <https://doi.org/10.1155/2021/9780677>.
- Sang T, Zhang T, Wang J, Zheng Y. Network pharmacology and molecular docking analysis on molecular targets and mechanisms of Bushen Hugu Decoction in the treatment of malignant tumor bone metastases. *Ergün S, ed. BioMed Res Int*. 2022;2022:1–16. <https://doi.org/10.1155/2022/2055900>.
- Sun Y, Wen X, Li Z, Xu L, Xia M. Integrated network pharmacology and proteomics to reveal the cognitive improvement effect of Wuzang Wenyang Huayu decoction on vascular dementia. *Chin Med J (Engl)*. 2022;135(19):2380–2. <https://doi.org/10.1097/CM9.0000000000001958>.
- Wang Y, Yuan Y, Wang W, et al. Mechanisms underlying the therapeutic effects of Qingfei Yin in treating acute lung injury based on GEO datasets, network pharmacology and molecular docking. *Comput Biol Med*. 2022;145: 105454. <https://doi.org/10.1016/j.compbiomed.2022.105454>.
- Whitesell J. Inflammatory Neuropathies. *Semin Neurol*. 2010;30(04):356–64. <https://doi.org/10.1055/s-0030-1267279>.
- Nathan C. Nonresolving inflammation redux. *Immunity*. 2022;55(4):592–605. <https://doi.org/10.1016/j.immuni.2022.03.016>.
- Gupta A, Agarwal V. Inflammation as a shared mechanism of chronic stress related disorders with potential novel therapeutic targets. *Naunyn Schmiedeberg Arch Pharmacol*. 2024;397(11):8383–94. <https://doi.org/10.1007/s00210-024-03205-5>.
- Pang L, Liu CY, Gong GH, Quan ZS. Synthesis, in vitro and in vivo biological evaluation of novel lappaconitine derivatives as potential anti-inflammatory agents. *Acta Pharm Sin B*. 2020;10(4):628–45. <https://doi.org/10.1016/j.apsb.2019.09.002>.
- Singh D, Attri B, Gill R, Bariwal J. Review on EGFR inhibitors: critical updates. *Mini-Rev Med Chem*. 2016;16(999):1–1. <https://doi.org/10.2174/1389557516666160804162457>.
- Wang T, Chen D, Wang P, Xu Z, Li Y. miR-375 prevents nasal mucosa cells from apoptosis and ameliorates allergic rhinitis via inhibiting JAK2/STAT3 pathway. *Biomed Pharmacother*. 2018;103:621–7. <https://doi.org/10.1016/j.biopha.2018.04.050>.
- Hirano T. IL-6 in inflammation, autoimmunity and cancer. *Int Immunol*. 2021;33(3):127–48. <https://doi.org/10.1093/intimm/dxaa078>.
- Gong Y, Qiu J, Jiang T, et al. Maltol ameliorates intervertebral disc degeneration through inhibiting PI3K/AKT/NF- κ B pathway and regulating NLRP3 inflammasome-mediated pyroptosis. *Inflammopharmacology*. 2023;31(1):369–84. <https://doi.org/10.1007/s10787-022-01098-5>.
- Sun K, Luo J, Jing X, et al. Hyperoside ameliorates the progression of osteoarthritis: An in vitro and in vivo study. *Phytomedicine*. 2021;80: 153387. <https://doi.org/10.1016/j.phymed.2020.153387>.
- Wei X, Zhang B, Liang X, et al. Higenamine alleviates allergic rhinitis by activating AKT1 and suppressing the EGFR/JAK2/c-JUN signaling. *Phytomedicine*. 2021;86: 153565. <https://doi.org/10.1016/j.phymed.2021.153565>.
- Betts BC, Abdel-Wahab O, Curran SA, et al. Janus kinase-2 inhibition induces durable tolerance to alloantigen by human dendritic cell-stimulated T cells yet preserves immunity to recall antigen. *Blood*. 2011;118(19):5330–9. <https://doi.org/10.1182/blood-2011-06-363408>.
- Quintás-Cardama A. The role of Janus kinase 2 (JAK2) in myeloproliferative neoplasms: Therapeutic implications. *Leuk Res*. 2013;37(4):465–72. <https://doi.org/10.1016/j.leukres.2012.12.006>.
- Verma N, Tripathi SK, Chaudhury I, Das HR, Das RH. iNOS-targeted 10-23 DNAzyme reduces LPS-induced systemic inflammation and mortality in mice. *Shock*. 2010;33(5):493–9. <https://doi.org/10.1097/SHK.0b013e3181c4ecbb>.
- Li Q, Cheng Y, Zhang S, Sun X, Wu J. TRPV4-induced Müller cell gliosis and TNF- α elevation-mediated retinal ganglion cell apoptosis in glaucomatous rats via JAK2/STAT3/NF- κ B pathway. *J Neuroinflammation*. 2021;18(1):271. <https://doi.org/10.1186/s12974-021-02315-8>.
- Zheng S, Wang C, Lin L, et al. TNF- α impairs pericyte-mediated cerebral microcirculation via the NF- κ B/iNOS axis after experimental traumatic brain injury. *J Neurotrauma*. 2023;40(3–4):349–64. <https://doi.org/10.1089/neu.2022.0016>.

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