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Research Article

Investigation of the Role and Mechanism of *Euonymus alatus* (Thunb.) Siebold in the Treatment of Rheumatoid Arthritis Through Comprehensive Bioinformatic and Experimental Analyses

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Abstract

Background: This study aimed to investigate the main active ingredients and potential mechanism of action of *Euonymus alatus* (Thunb.) Siebold (EA), a traditional Chinese medicine that is used to alleviate symptoms of rheumatoid arthritis (RA). **Methods**: Potential targets of EA and related pathways in the treatment of RA were identified using the Traditional Chinese Medicine Systems Pharmacology (TCMSP) database and analysis tools, SymMap, GeneCards, Swiss Target Prediction database, Metascape, and molecular docking. The anti-inflammatory effect of the predicted core active ingredient in EA was validated using lipopolysaccharide (LPS)-induced RAW 264.7 macrophages *in vitro*. **Results**: The main active ingredients in EA that are influential in the treatment of RA are likely to be flavonoids and polyphenols, such as kaempferol, quercetin, baicalein, wogonin, and oroxylin A. These active ingredients may target AKT1, BCL2, IGF1R, SRC, PTGS2, and EGFR to affect the NF-κB, mTOR, and related signaling pathways, as determined by Kyoto Encyclopedia of Genes and Genomes (KEGG) and Gene Ontology (GO) analyses. Meanwhile, the molecular docking results suggested that these active ingredients most likely target AKT1, BCL-2, and PTGS-2. Experimental studies have shown that the EA active ingredient, baicalein, can suppress macrophage proliferation, TNF-α expression, and reactive oxygen species (ROS) production. **Conclusion**: The active ingredients in EA may target AKT, BCL-2, and PTGS-2 to mediate the regulation of the PI3K/AKT signaling pathway in RA treatment.

Keywords: *Euonymus alatus*; Chinese medicine; rheumatoid arthritis; network pharmacology; RAW264.7; molecular docking

1. Introduction

Rheumatoid arthritis (RA) is a cumulative, chronic autoimmune disorder that can lead to a range of systemic complications. These are primarily defined by proliferative synovitis, pannus development, and erosion of articular cartilage [1]. RA commonly develops in the small joints of the hands and feet, and then further evolves in large joints such as the knees and elbows [2]. Abnormal autoimmune characteristics of RA include the accumulation of lymphocytes, macrophages and other immune cells in joint tissue, causing synovial tissue lesions. RA is associated with an increased number and activation of macrophage-like synovial cells (MLS) and fibroblastic-like synovial cells (FLS), the production of a large number of inflammatory mediators, and the activation of osteoclasts [3]. It can cause the destruction of articular cartilage and bone tissue, and lead to symmetrical joint inflammation, joint deformity, disability and even death [2,4]. RA is a systemic disease that can also induce other systemic diseases, as well as providing favorable development condition for its further progression [4]. If it is not treated in a timely fashion, RA can cause irreversible damage to joints and multiple other organs [5].

Currently, the principal pharmacological agents for RA treatment are non-steroidal anti-inflammatory drugs (NSAIDs), glucocorticoids, and disease-modifying anti-rheumatic drugs (DMARDs) [6]. NSAIDs are effective against endogenous septic fever from any cause, but carry an increased risk of concurrent gastrointestinal bleeding [7]. Although low-dose glucocorticoids do not damage bones and joints, high doses over extended periods can lead to a range of adverse effects, including skin atrophy, striae, rosacea, perioral dermatitis, acne and purpura, as well as systemic reactions such as hyperglycemia, osteoporosis, glaucoma and adrenocortical insufficiency [8,9]. As the main treatment against RA, DMARDs slow the progress of RA and modify its clinical symptoms [10]. Methotrex-

ate, a commonly used DMARD, is associated with various adverse effects, including hepatotoxicity, nephrotoxicity, gastrointestinal disturbances, and bone marrow suppression [11,12]. Biologic DMARDs for RA treatment include TNF-α inhibitors, T-cell regulators, anti-B lymphocyte monoclonal antibodies, and interleukin-6 receptor antibodies. Although these drugs are partially effective against RA, they have non-negligible adverse reactions [13].

In contrast to the adverse reactions observed for chemical and biologic drugs, Traditional Chinese Medicine (TCM) has a superior safety record for RA treatment and is often preferred [14]. Treatment with herbal remedies is an alternative therapy for RA that can offer safer and better outcomes [15]. The TCM *Tripterygium wilfordii* Hook.f. (TWHF) has a long history in the treatment of RA in China [16]. A meta-analysis has shown that TWHF is more effective and less toxic for RA treatment compared to chemical drugs [17]. *Euonymus alatus* (Thunb.) Siebold (EA) belongs to the Celastraceae family which is also part of the same TWHF family. EA has been used in RA treatment for more than 2000 years in Chinese medicine [18]. It is also known as "Guijianyu", or "Weimao" in Chinese. Its medicinal functions are recorded in the National Compilation of Chinese Herbal Medicine as "the promotion of blood circulation through menstruation, the dispersion of stasis, and pain relief". A Chinese medicine book named Zhejiang Folk Herbs in Common Use has described EA as follows: "Water decoction of stem (60~90 g) of Weimao can be used as the 'Monarch drug' in the treatment of rheumatic pain". Some reports have also shown that EA has anti-inflammatory, antioxidant, anti-obesity, and anticancer effects [19]. Moreover, EA was reported to have positive effects on RA treatment, either alone or in combination with other herbs [20].

Network pharmacology is often used to study the composition, targets, and mechanisms of TCM [21]. Some herbal medicines used in RA treatment have been extensively analyzed for their active ingredients and composition through network pharmacology. This approach has identified Celastrol as the main ingredient in TWHF for RA treatment [22]. By assessing the geometric and energetic complementarity between receptors and drug molecules, molecular docking provides a theoretical basis for identifying optimal ligands and conformations in TCM research. In the present study, we investigated the active ingredients and mechanistic basis of EA in the treatment of RA by utilizing network pharmacology, molecular docking, and experimental verification. The results should further our understanding of the clinical effect and mechanism of EA in TCM.

2. Materials and Methods

2.1 Reagents and Materials

RAW 264.7 cells were purchased from the National Collection of Authenticated Cell Cultures (Shanghai, China). Baicalein was obtained from Shanghai Yuanye Bio-Technology Co., Ltd. (Shanghai, China).

2.2 Network Pharmacology and Molecular Docking

2.2.1 Screening of Active Ingredient in EA

The Traditional Chinese Medicine Systems Pharmacology Database and Analysis Tools (TCMSP, https://tcmsp-e.com/) [23] is a dedicated platform for TCM systems pharmacology analysis, while SymMap (http://www.symmap.org/) [24] is a database that integrates TCM with modern medicine. Both systems were used to screen for the active ingredient in EA. These provided comprehensive information on the interactions between ingredients, targets, and diseases in TCM, allowing systematic pharmacological research. After entering these platforms, a Chinese name (Guijianyu), Herb Id (SMHB00153), or Euonymi Alati Ramulus was inputted and retrieved. Bioactive ingredients in EA were selected and screened after setting the following parameters: oral bioavailability (OB $>$30%) [25], drug-likeness (DL $>$0.18) [26], and water partition coefficient (LogP $<$5) [27]. Potential target active ingredients were predicted using Swiss Target Prediction [28] (https://swisstargetprediction.ch/) and PubChem [29] (https://pubchem.ncbi.nlm.nih.gov/). SMILES files of active ingredients from PubChem were downloaded and further retrieved from the Swiss Target Prediction database to obtain target information. Targets with a parameter likelihood $>$0 were selected for further analysis.

2.2.2 RA Target Collection

Targets for RA were extracted from the GeneCards [30] (https://www.genecards.org/) and OMIM [31] (https://www.omim.org/) databases after entering "rheumatoid arthritis" as the keyword. The targets were filtered out by setting the parameter to "Homo sapiens" type, and further processed for deduplication.

2.2.3 Construction of the EA Active Ingredient-Target-RA Disease Network

To identify potential therapeutic targets of EA in the treatment of RA, overlapping targets between EA active ingredient-related targets and RA-related targets were screened using Venny 2.1.0 (by Juan Carlos Oliveros BioinfoGP, CNB-CSIC, Madrid, Spain) software (https://bioinfogp.cnb.csic.es/tools/venny/). Relationships between the active ingredients of EA, common targets, and RA disease were then established and illustrated as a network using Cytoscape v3.9.1 software (Cytoscape Consortium, San Diego, CA, USA) [32]. The degree value of the network was used to predict correlations between the active ingredients of EA and RA targets.

2.2.4 Analysis of Protein-Protein Interaction (PPI)

To study interactions between the active ingredients of EA and common RA targets, we used the STRING database [33] (https://www.string-db.org/) to perform PPI analysis [34]. Overlapping targets were first inputted into the STRING database, and the "Homo sapiens" type was then set for PPI analysis. If the parameter of protein-protein interaction in the PPI network has a confidence level $\geq$0.7, the corresponding targets are screened out [26]. Otherwise, these targets are hidden in the PPI network. The results of PPI analysis were downloaded and set as csv format for further analysis. The PPI network was presented using Cytoscape software.

2.2.5 Gene Ontology (GO) Enrichment Analysis

To better understand the role of common core targets of EA in the treatment of RA, GO analysis [35] was performed using Metascape [36] (http://metascape.org/). Common core targets were classified by GO enrichment analysis into three categories according to their functional similarity: biological processes (BP), cellular components (CC) and molecular functions (MF).

2.2.6 Kyoto Encyclopedia of Genes and Genomes (KEGG) Pathway Enrichment Analysis

To pinpoint key targets for EA-mediated RA therapy, KEGG pathway enrichment [37] analysis of common core targets was carried out using Metascape (http://metascape.org/). Targets identified by PPI analysis were further submitted to Metascape for KEGG pathway enrichment analysis by setting parameters to "Homo sapiens", and the significance threshold to $p < 0.01$. The KEGG enrichment heatmap was generated using an online platform (https://www.bioinformatics.com.cn) for visualization.

2.2.7 Molecular Docking

Molecular docking was performed to verify relationships between the predicted targets and active ingredients identified from the active ingredient-target-disease network, PPI, GO and KEGG analyses. Eight key targets were selected and downloaded from the PDB database by setting parameters of pH 7$\sim$7.5, refinement resolution 1.0$\sim$2.5 Å, and standard gene names [38]. Based on the drug-component-common target-disease network of EA in the treatment of RA (section 3.3), the active ingredients MOL002933 (5,7,4′-trihydroxy-8-methoxyflavone), MOL000552 (5,2′-dihydroxy-6,7,8-trimethoxyflavone), MOL002927 (skullcapflavone II, SFII) and MOL002714 (baicalein) were found to have relatively high degree values in the network and were thus selected for molecular docking analysis. The four active ingredients were prepared for analysis by PubChem. AutoDock Tools (Version 1.5.6, The Scripps Research Institute, La Jolla, CA, USA) were used for receptor (target) preparation, including the removal of water and small molecules, and addition of hydrogen atoms. Molecular docking of the key target (receptor) and active ingredient was further performed by AutoDock Tools [39]. The docking position between ingredient and receptor was displayed by PyMoL software (Version 3.1, Schrödinger, LLC, New York, NA, USA).

2.3 Experimental Validation

2.3.1 Cell Culture and Viability Assay

RAW 264.7 cells (validated by STR profiling and tested negative for mycoplasma) were grown in DMEM at 37 °C in a 5% CO_2 humidified incubator. Upon reaching the logarithmic phase, the cells were seeded into 96-well plates. After 24 h of culture (70–80% confluence), the cells were exposed to a concentration gradient of baicalein (0, 10, 25, 50, 75, 100 µM). The real-time growth status, cellular changes and trends were recorded using Incucyte ZOOM (Sartorius, Göttingen, Germany). Phase object confluence was automatically quantified by IncuCyte ZOOM 2015A software (Sartorius, Göttingen, Germany) according to the built-in phase contrast analysis algorithm.

2.3.2 Measurement of Pro-Inflammatory Cytokines

After 24 h co-incubation with LPS (200 ng/mL) and baicalein, RAW 264.7 cells were lysed using RIPA lysis buffer. The cellular extract was then centrifuged at 12,000 g for 20 min at 4 °C, and the supernatant was collected and heated at 100 °C for 10 min. This was followed by protein separation with SDS-PAGE. Proteins were then transferred onto a PVDF membrane, placed in 5% skimmed milk and incubated at room temperature for 2 h to block non-specific binding. The PVDF membrane was then incubated with primary antibodies (TNF-α, 60291-1-Ig, 1:2000; GAPDH, 60004-1-Ig, 1:2000) obtained from Proteintech (Wuhan, China). After extensive washing with TBST, the membrane was incubated with an HRP-conjugated Affinipure Goat Anti-Mouse IgG(H+L) secondary antibody (SA00001-1, 1:2000; Proteintech, Wuhan, China). Finally, the proteins were visualized with the ECL method.

2.3.3 Determination of ROS

Reactive oxygen species (ROS) were monitored using the 2′,7′-dichlorofluorescein (DCF) assay. RAW 264.7 cells were co-incubated for 24 h with LPS (200 ng/mL) and baicalein (0–100 µM). The cell culture medium was then removed and the cells were incubated with 100 µL of DCFH-DA (10 mM) for 30 min. ROS was assessed based on the DCF fluorescence intensity, as measured by a multifunctional microplate reader (Molecular Devices, San Jose, CA, USA) with 488 nm excitation and 525 nm emission. Incucyte ZOOM 2015A software (Sartorius, Göttingen, Germany) was also used to photograph cell fluorescence.

Table 1. Active ingredients of EA.

Mol ID	Molecule name	OB (%)	DL	LogP
MOL000098	quercetin	46.4333	0.28	1.50
MOL000173	wogonin	30.6846	0.23	2.59
MOL000422	kaempferol	41.8822	0.24	1.77
MOL000525	norwogonin	39.4040	0.21	2.70
MOL000552	5,2′-dihydroxy-6,7,8-trimethoxyflavone	31.7125	0.35	2.55
MOL001790	linarin	39.8437	0.71	–0.18
MOL002714	baicalein	33.5189	0.21	2.33
MOL002776	baicalin	40.1236	0.75	2.07
MOL002908	5,8,2′-trihydroxy-7-methoxyflavone	37.0084	0.27	2.32
MOL002917	5,2′,6′-trihydroxy-7,8-dimethoxyflavone	45.0474	0.80	2.30
MOL002925	5,7,2′,6′-tetrahydroxyflavone	37.0135	0.24	2.07
MOL002926	dihydrooroxylin A	38.7151	0.23	2.55
MOL002927	skullcapflavone II	69.5104	0.44	2.54
MOL002928	oroxylin A	41.3676	0.23	2.59
MOL002933	5,7,4′-trihydroxy-8-methoxyflavone	36.5620	0.27	2.32
MOL004328	naringenin	59.2939	0.21	2.30
MOL005100	5,7-dihydroxy-2-(3-hydroxy-4-methoxyphenyl)chroman-4-one	47.7364	0.27	2.28
MOL005190	eriodictyol	71.7927	0.24	2.03
MOL012245	5,7,4′-trihydroxy-6-methoxyflavanone	36.6269	0.27	2.28
MOL012246	5,7,4′-trihydroxy-8-methoxyflavanone	74.2352	0.26	2.28
MOL013253	5,2′,5′-trihydroxy-6,7,8-trimethoxyflavone	37.4947	0.71	2.60

EA, *Euonymus alatus* (Thunb.) Siebold; OB, Oral bioavailability; DL, drug-likeness; LogP, water partition coefficient.

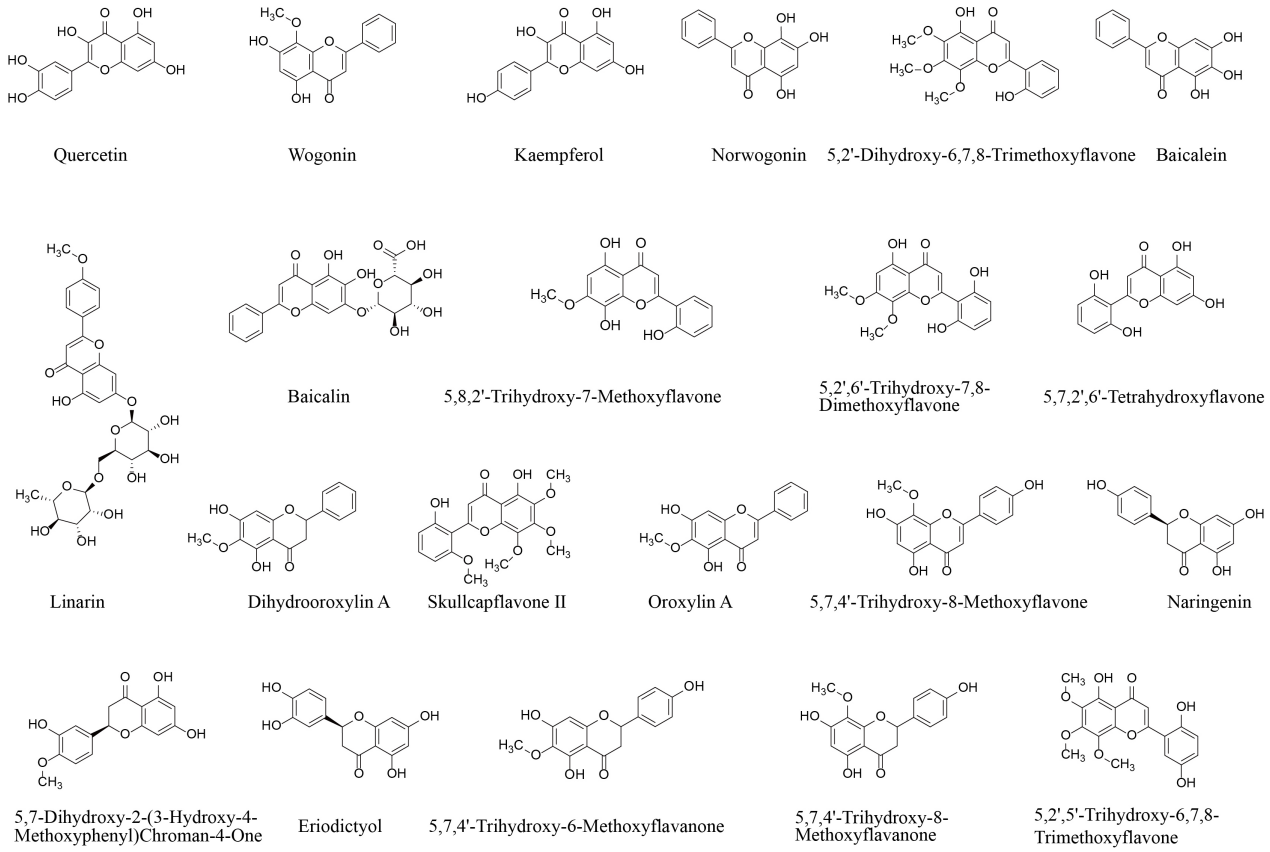

Fig. 1. Chemical structure of the 21 EA active ingredients shown in Table 1. EA, *Euonymus alatus* (Thunb.) Siebold.

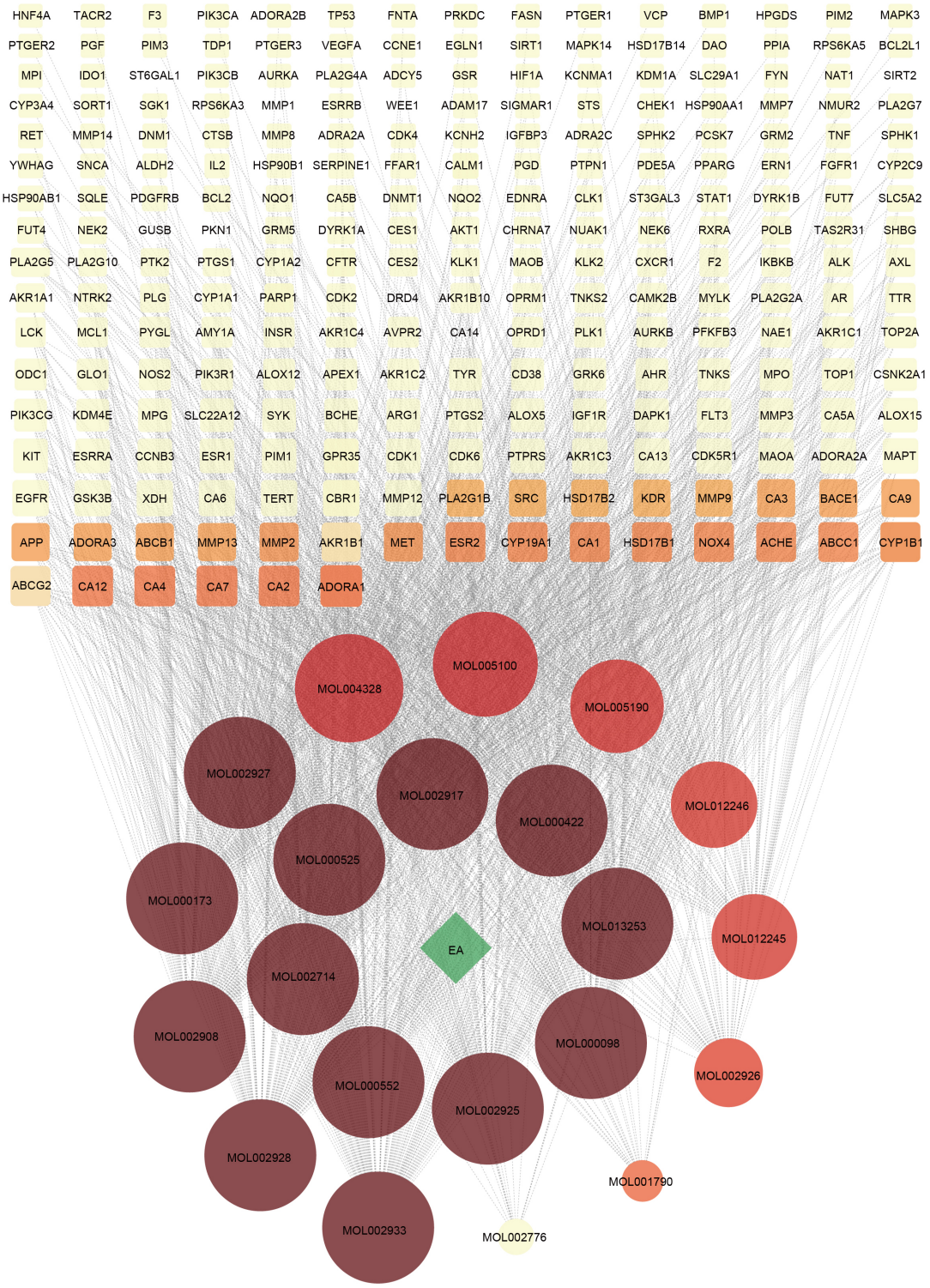

Fig. 2. Drug-ingredient-target network of EA-mediated RA treatment. The network consists of 248 targets and 21 active ingredients. Yellow, orange and red represent different degree values, from low to high respectively. The square represents the target, the circle represents the active ingredient, and the diamond represents the herb drug EA. RA, rheumatoid arthritis.

2.3.4 Statistical Analysis

Data (mean ± SD) was statistically analyzed with GraphPad Prism 10 (GraphPad Software, Inc., San Diego, CA, USA). Group comparisons were made with the Student's *t*-test (for two groups), or one-way ANOVA followed by Bonferroni correction (for multiple groups). A *p*-value of <0.05 was defined as statistical significance.

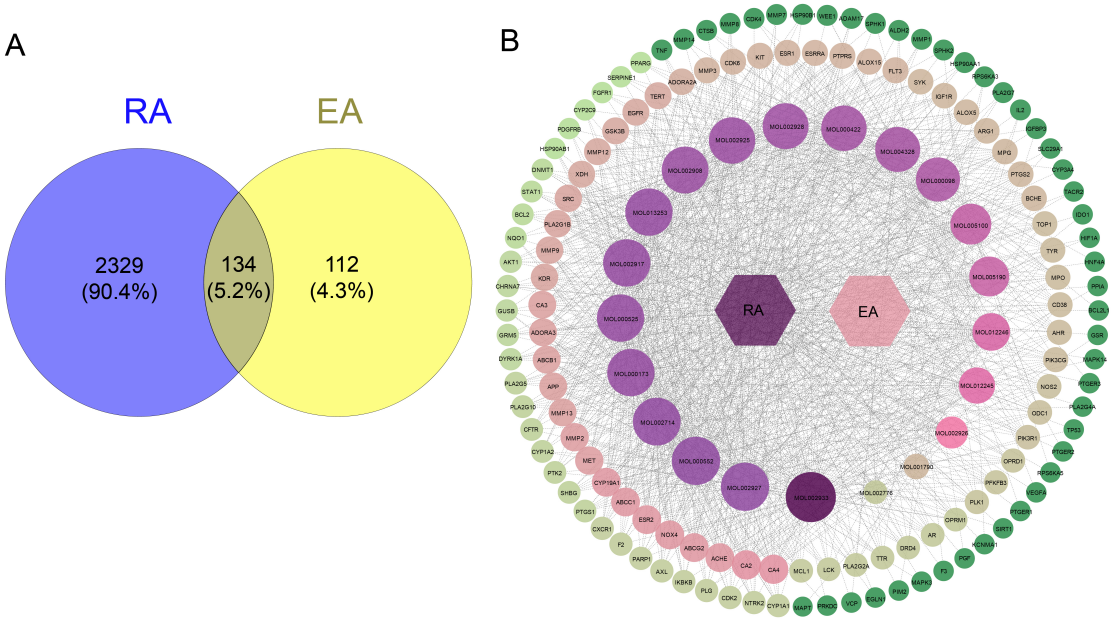

Fig. 3. Venn diagram and drug-ingredient-target-disease network diagram for EA-mediated RA treatment. (A) Overlap of RA- and EA-related targets. (B) Network visualization of EA active compounds and the corresponding potential targets in RA treatment. Nodes are colored by degree value (light green to purple, low to high). Compounds are in the inner ring, and targets are in the surrounding rings.

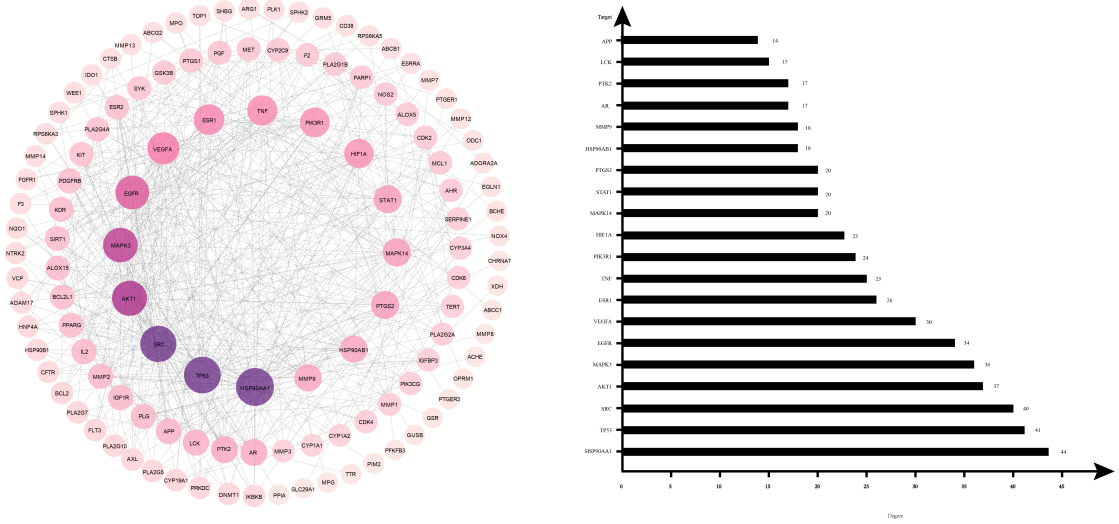

Fig. 4. PPI network diagram and statistical analysis of high degree targets in the PPI network. The darker the cycle, the higher the degree of target. The nodes for HSP90AA1, TP53, SRC, AKT1, MAPK3, EGFR, VEGFA, ESR1, TNF, PIK3R1, HIF1A, MAPK14, STAT1, PTGS2, HSP90AB1, MMP9, AR, PTK2, LCK and APP have a relatively high degree value in the PPI network. PPI, protein-protein interaction.

3. Result

3.1 EA Active Ingredients and Potential Targets in the Treatment of RA

By searching the TCSMP, SymMap, Swiss Target Prediction and PubChem databases, 21 active ingredients (Table 1) and chemical structures (Fig. 1) were identified in EA, as well as 248 potential targets for EA-mediated RA treatment. The topology of the drug-ingredient-target network for EA in the treatment of RA revealed 268 nodes (total number of ingredients, targets, and drugs) and 1814 edges (connections among nodes) (Fig. 2). Topological analysis also revealed high degree ingredients, such as quercetin (MOL000098), kaempferol (MOL000422), baicalein (MOL002714), wogonin (MOL000173), and

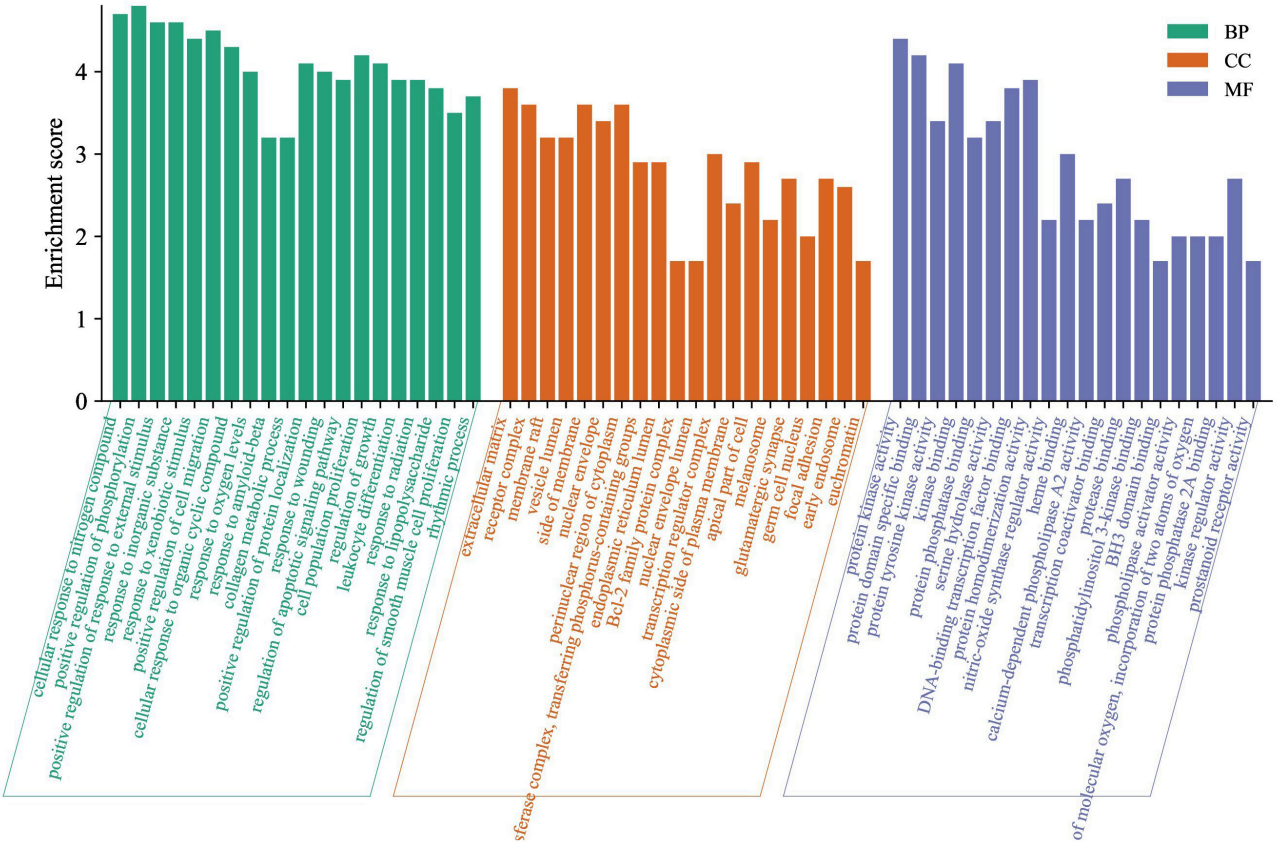

Fig. 5. GO analysis showing convergence of the common targets with the PI3K/AKT signaling pathway. BP, biological processes; CC, cellular components; MF, molecular functions; GO, Gene ontology.

oroxylin A (MOL002928), as well as high degree targets such as ADORA1 (21), CA2 (20), CA7 (20), CA12 (20), and CA4 (20).

3.2 Common EA-Related Targets for RA Treatment

To identify the common targets of EA ingredients for RA treatment, 2463 RA-related targets were first obtained by searching the GeneCards and OMIM database. An intersection analysis of targets related to both EA and RA was then performed using a Venn diagram (Fig. 3A). This revealed 134 overlapping targets, accounting for 5.2% of the total number of EA- and RA-related targets.

3.3 Drug-Ingredient-Common Target-Disease Network of EA in the Treatment of RA

To better understand the relationship between EA ingredients and RA-related targets, we constructed a drug-ingredient-common target-disease network using Cytoscape. This network (Fig. 3B), which integrates 134 overlapping targets from the Venn diagram (Fig. 3A) and 21 bioactive compounds, comprised 158 nodes and 1177 edges. Topological assessment revealed high degree values for specific ingredients such as MOL002933 (5,7,4′-trihydroxy-8-methoxyflavone), MOL002927 (skullcapflavone II, SFII), MOL002714 (baicalein) and MOL000552 (5,2′-dihydroxy-6,7,8-trimethoxyflavone). Hence, these ingredients are more likely to be the active ingredients in EA-mediated RA treatment.

3.4 PPI Analysis of the Common Targets for EA-Mediated RA Treatment

To elucidate interactions among common targets, a protein-protein interaction (PPI) network was generated from the above 134 overlapping targets using the STRING database (Fig. 4). Among these, 120 showed interactions. The PPI network revealed 134 nodes and 547 edges. The average node degree was 8.16, and the average local clustering coefficient in the interaction network was 0.506. The targets with high node degree values included HSP90AA1 (Heat Shock Protein 90α family class A member 1), TP53 (tumor protein P53), and SRC (proto-oncogene tyrosine-protein kinase Src). SRC is a signal molecule in the vascular epidermal growth factor receptor signaling pathway, which mediates the SRC/ERK/CREB pathway. The main pathological cells in RA are FLS. Overexpression of SRC promotes the migration and invasion of FLS, as well as the negative regulation of MMP2 and MMP9 [40]. TP53 is a suppressor of inflammation, with dysfunction of TP53 related to autoimmune diseases [41]. TP53 could regulate the inflammatory response in FLS and promote RA

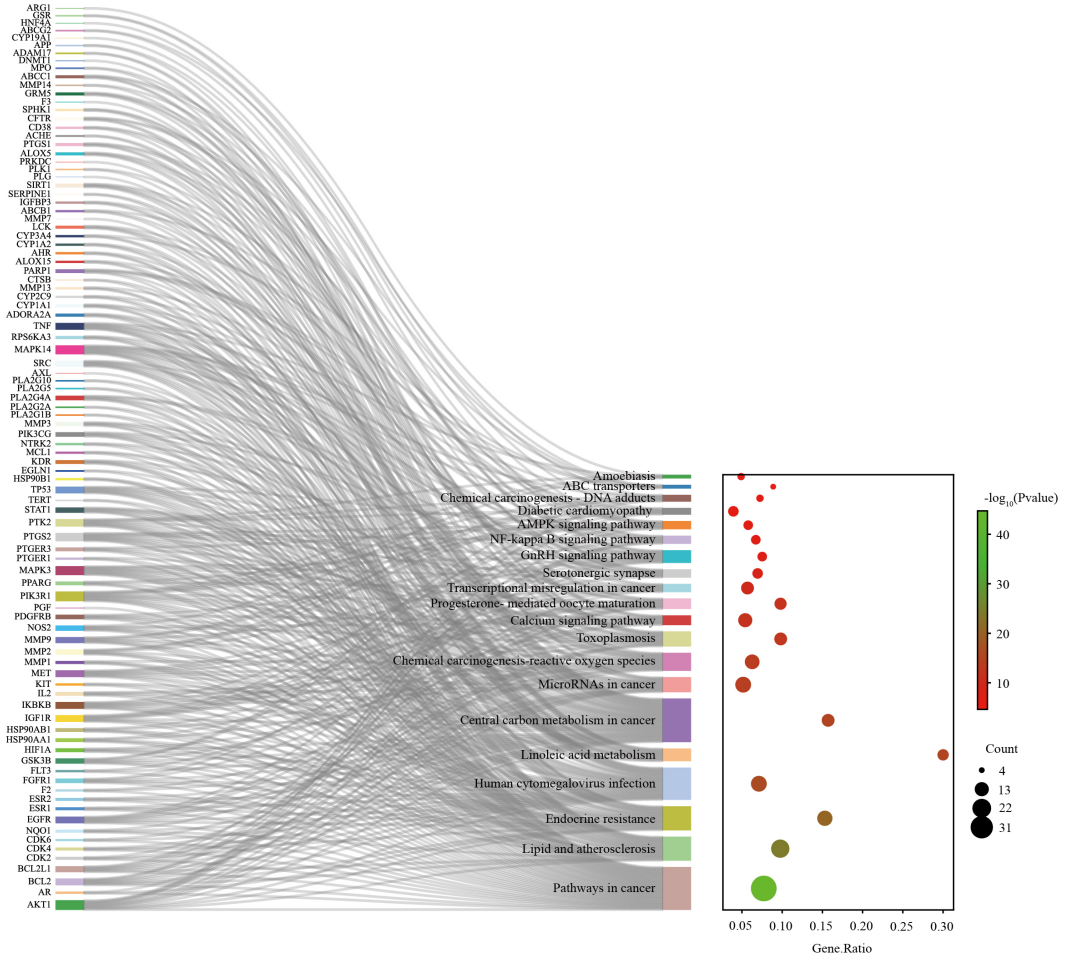

Fig. 6. Sankey-Bubble diagram of KEGG pathway enrichment analysis. The relationship between common targets and 20 signaling pathways is shown. The size of each circle corresponds to the number of enriched targets. The larger area indicates the greater degree of enrichment. Different colors of circle represent different *p* values, with the values gradually increasing from green to red. KEGG, Kyoto Encyclopedia of Genes and Genomes.

[42]. HSP90AA1 is an essential heat shock protein and a well-characterized member of the Hsp90 subfamily [43]. Down-regulation of HSP90AA1 can influence the immune response and inflammation to alleviate RA [44].

3.5 GO Analysis Suggests the PI3K/AKT Signaling Pathway Participates in EA-Mediated RA Treatment

The 120 common targets for EA in RA treatment were systematically evaluated using GO enrichment analysis, with a parameter setting of $p < 0.01$. Metascape analysis revealed the top 20 entries for BP, CC, and MF. The interrelationships among these significant pathways were further assessed through topological analysis and correlation mapping (Fig. 5). The top 5 pathways in BP were the cellular response to nitrogen compound (GO:1901699), positive regulation of response to external stimulus (GO:0032103), cell population proliferation (GO:0008283), positive regulation of cell migration (GO:0030335), and response to xenobiotic stimulus (GO:0009410). The high degree pathways were the cellular response to nitrogen compound (54), while the highest degree target was SRC (16) in the GO analysis. The top 5 pathways in CC were the perinuclear region of cytoplasm (GO:0048471), vesicle lumen (GO:0031983), extracellular matrix (GO:0031012), receptor complex (GO:0043235), and side of membrane (GO:0098552). The highest degree pathway was the perinuclear region of cytoplasm (24), while the highest degree target was APP (10). The top 5 pathways in MF were protein kinase activity (GO:0004672), protein domain specific binding (GO:0019904), kinase binding (GO:0019900), DNA-binding transcription factor binding (GO:0140297), and protein homodimerization activity (GO:0042803). The highest degree pathway was protein kinase activity (27), while the highest degree target was TP53 (10). AKT1 and PIK3R1 both had a high degree in the PPI network (Fig. 4), while the SRC, APP and TP53 pathways were linked to activation of the PI3K/AKT signaling pathway [45–47]. Furthermore, the PI3K/AKT signaling path-

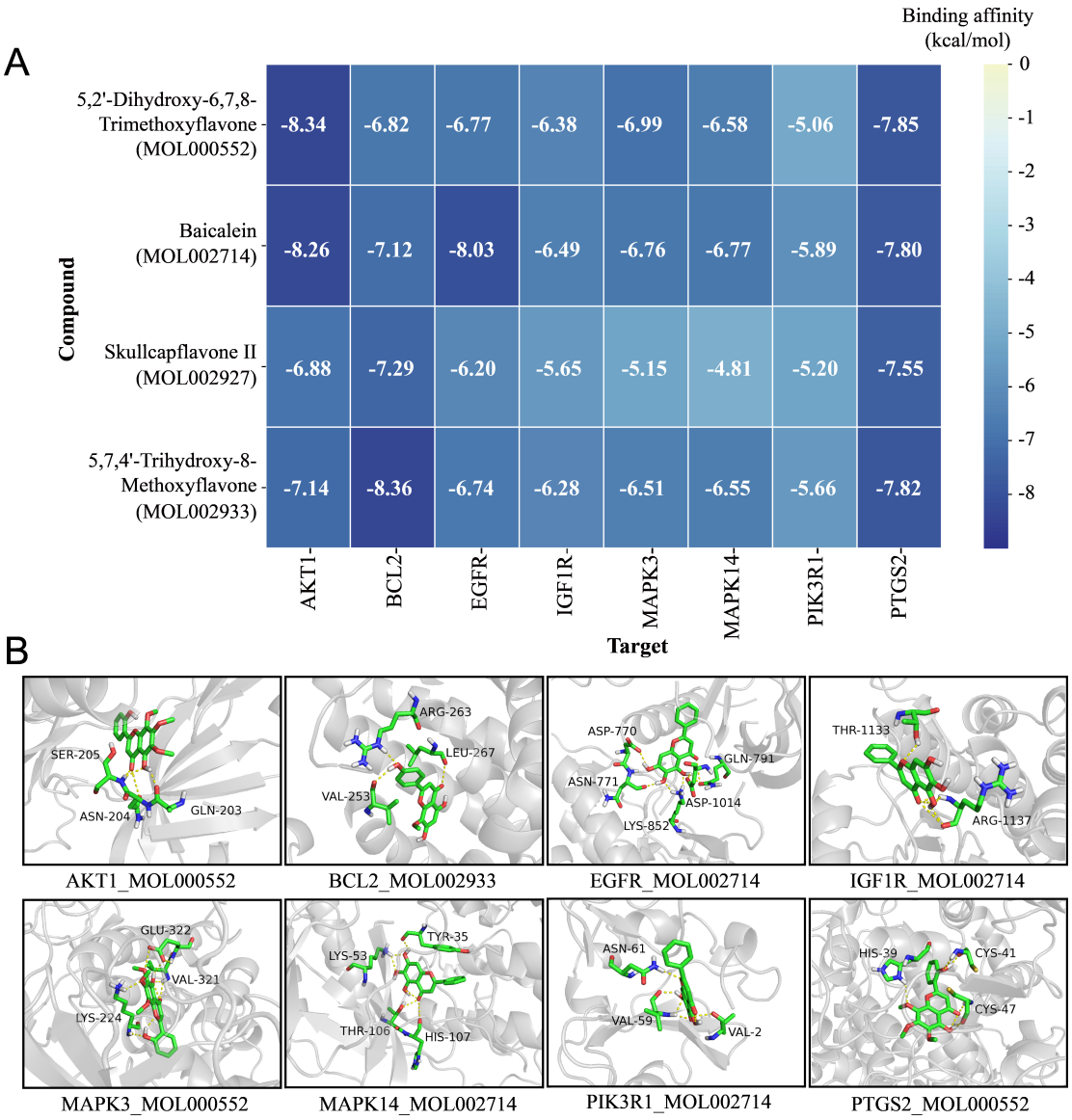

Fig. 7. Molecular docking of EA active ingredients and RA-related targets. (A) Binding affinity heat map between targets and active compounds. A darker blue indicates a stronger molecular interaction. (B) Docking results with the lowest binding energy are displayed in cartoons.

way regulated downstream signals, including RAS mutants, prolactin signaling, inflammasome regulation, and apoptosis of synovial fibroblasts [48,49]. Moreover, these key network targets converged on the PI3K/AKT signaling pathway and included the biological processes and GO enrichment. Thus, we deduced the PI3K/AKT signaling pathway may contribute to EA-mediated RA treatment.

3.6 AKT1, BCL2 and PTGS2 May Be Key Targets in EA-Mediated RA Treatment

To further understand the 120 common targets of EA in RA treatment, we conducted KEGG pathway enrichment analysis with a parameter setting of $p < 0.01$. This identified 20 signaling pathways that were statistically significant. The top 5 included central carbon metabolism in cancer (hsa05230), pathways in cancer (hsa05200), human cytomegalovirus infection (hsa05163), lipid and atherosclerosis (hsa05417), and endocrine resistance (hsa01522). The highest degree pathway was central carbon metabolism in cancer (58), and the highest degree target was AKT1 (12) (Fig. 6).

To better understand the relationship between EA active ingredients and RA-related targets, we first selected 8 targets in the top 5 signal pathways revealed by KEGG enrichment analysis: AKT1 (PDB: 4EJN), BCL2 (PDB: 6QGK), EGFR (PDB: 5XDK), IGF1R (PDB: 1K3A), MAPK3 (PDB: 6GES), MAPK14 (PDB: 2GFS), PIK3R1 (PDB: 1H9O), PTGS2 (PDB: 5F19). These were docked with the predicted EA active ingredients of 5,2′-dihydroxy-6,7,8-trimethoxyflavone, baicalein, SFII, and

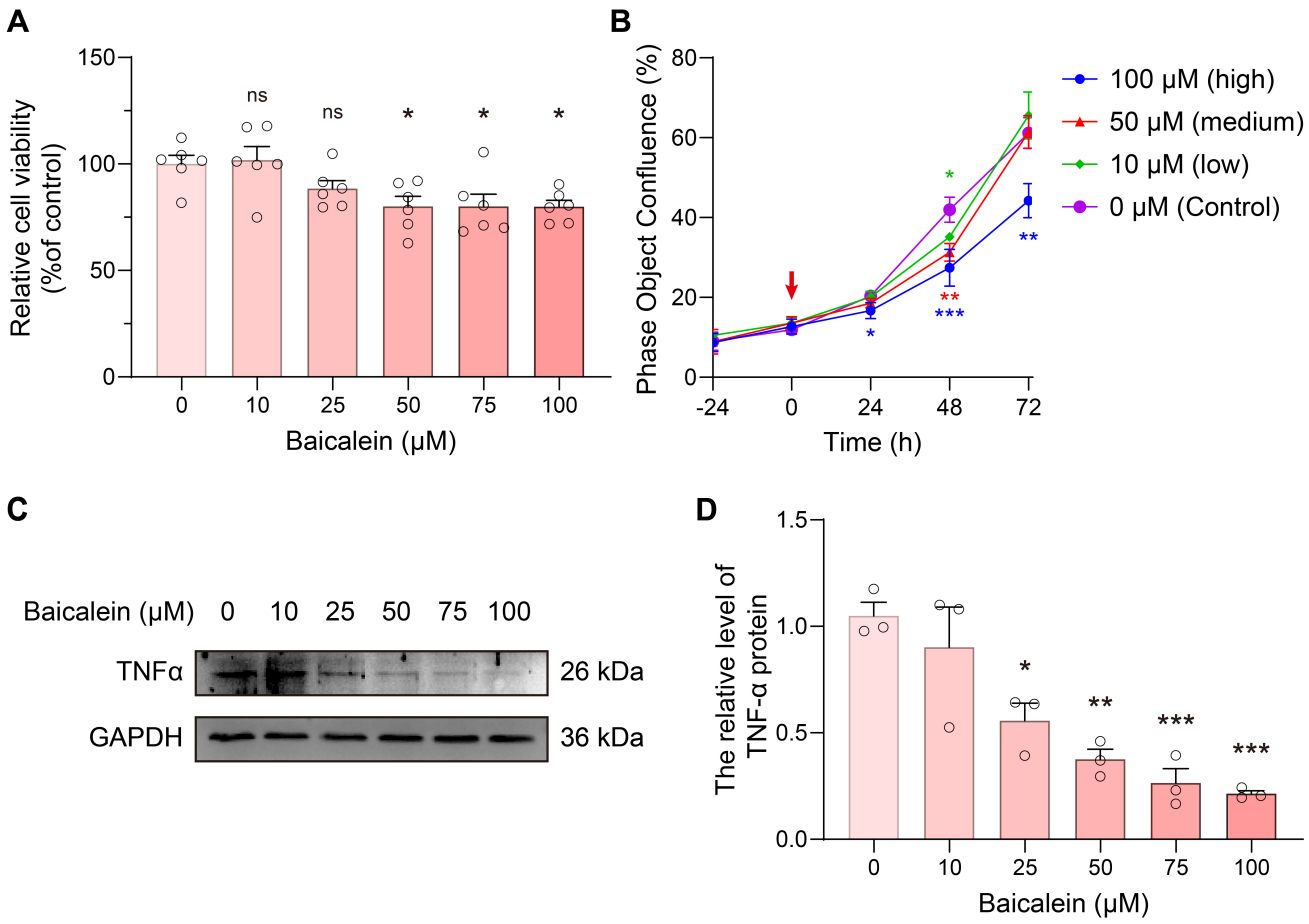

Fig. 8. Baicalein suppressed RAW 264.7 cell proliferation and decreased expression of the inflammatory cytokine TNF-α. (A) CCK-8 assay showing RAW 264.7 cell viability after treatment with different baicalein concentrations. (B) Active cell imaging analysis showing phase object confluence of RAW 264.7 cells after treatment with different baicalein concentrations. Red arrow: different concentrations of baicalein treatment. (C) Representative Western blot analysis showing that baicalein decreased TNF-α expression in a dose-dependent manner in RAW 264.7 cells. (D) Grayscale analysis of Western blot results. n = 3. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ns, not significant.

5,7,4′-trihydroxy-8-methoxyflavone. Molecular docking results showed that AKT1, BCL2, and PTGS2 had the strongest affinities with these active ingredients (Fig. 7).

3.7 Baicalein Decreases LPS-Induced Inflammation in RAW 264.7 Cells

Molecular docking analysis revealed that baicalein had the best binding activity with multiple potential targets. Extensive literature has previously shown that baicalein, a natural flavonoid, has significant anti-inflammatory activity [50]. Baicalein also has a well-documented pharmacological effect in *Scutellaria baicalensis* Georgi [51]. Moreover, baicalein showed relatively high value according to ingredient analysis of EA, the drug-component-common target-disease network analysis, and molecular docking analysis. Baicalein may therefore be suitable for exploring the anti-inflammatory potential of EA. Consequently, we used CCK-8 assay [52] and a real-time live-cell imaging assay to assess the effect of baicalein on RAW 264.7 cell viability. Compared to the control group, baicalein did not affect the viability of RAW 264.7 cells at low concentrations (Fig. 8A,B). However, baicalein suppressed cell proliferation at concentrations of 50–100 μM (Fig. 8A,B). Baicalein was also found to inhibit expression of the inflammatory cytokine TNF-α in a dose-dependent manner in RAW 264.7 cells (Fig. 8C,D).

3.8 Baicalein Decreases LPS-Induced ROS Generation in RAW 264.7 Cells

Since baicalein can suppress the production of key pro-inflammatory cytokines such as TNF-α (Fig. 8C), we next investigated whether it can also mediate anti-inflammation effects by influencing terminal inflammatory mediators (e.g., ROS, PGE2, NO) in macrophage cells. Treatment with 10 μM baicalein significantly decreased LPS-induced ROS levels in RAW 264.7 cells, as observed by a significant increase in DCFH-DA fluorescence intensity in LPS treated group, and a significant decrease in

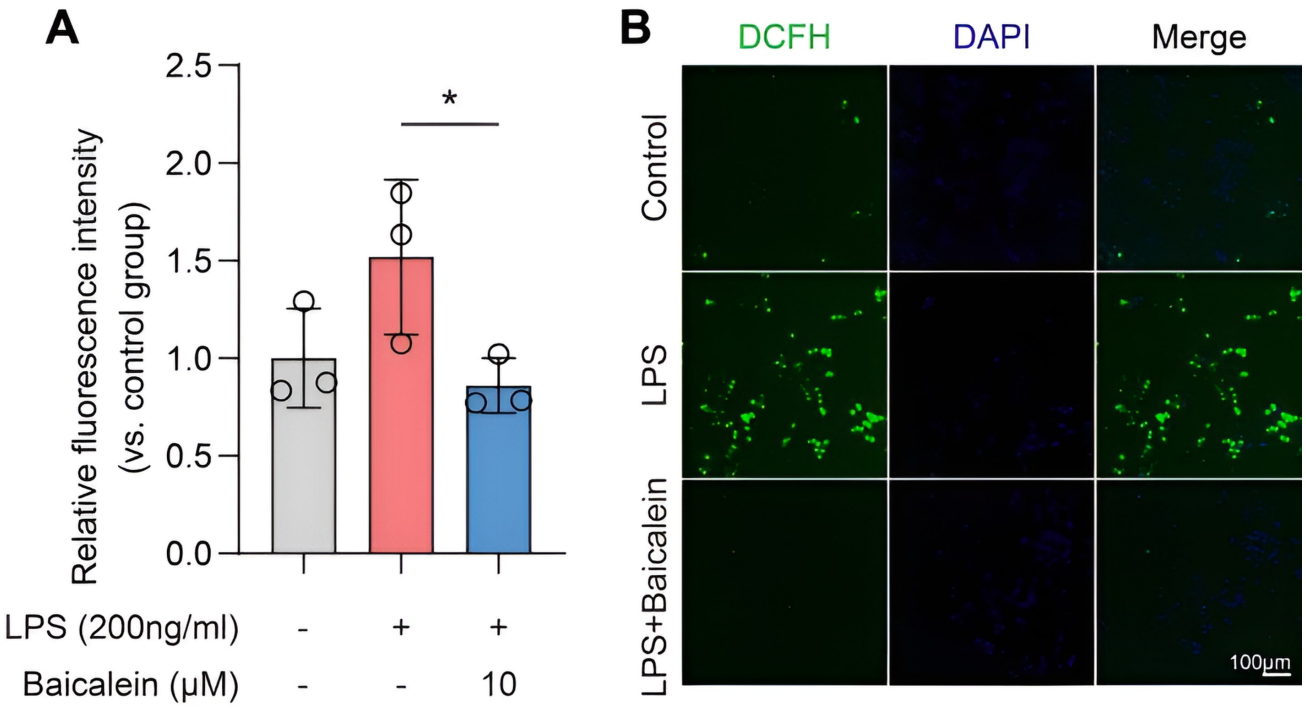

Fig. 9. Baicalein attenuates LPS-induced ROS generation in RAW 264.7 cells. (A) Graph of fluorescence intensity showing that baicalein decreases ROS generation. *$p < 0.05$. (B) Cell fluorescence photographs showing that baicalein decreases ROS generation. Scale bar = 100 μm. ROS, Reactive oxygen species.

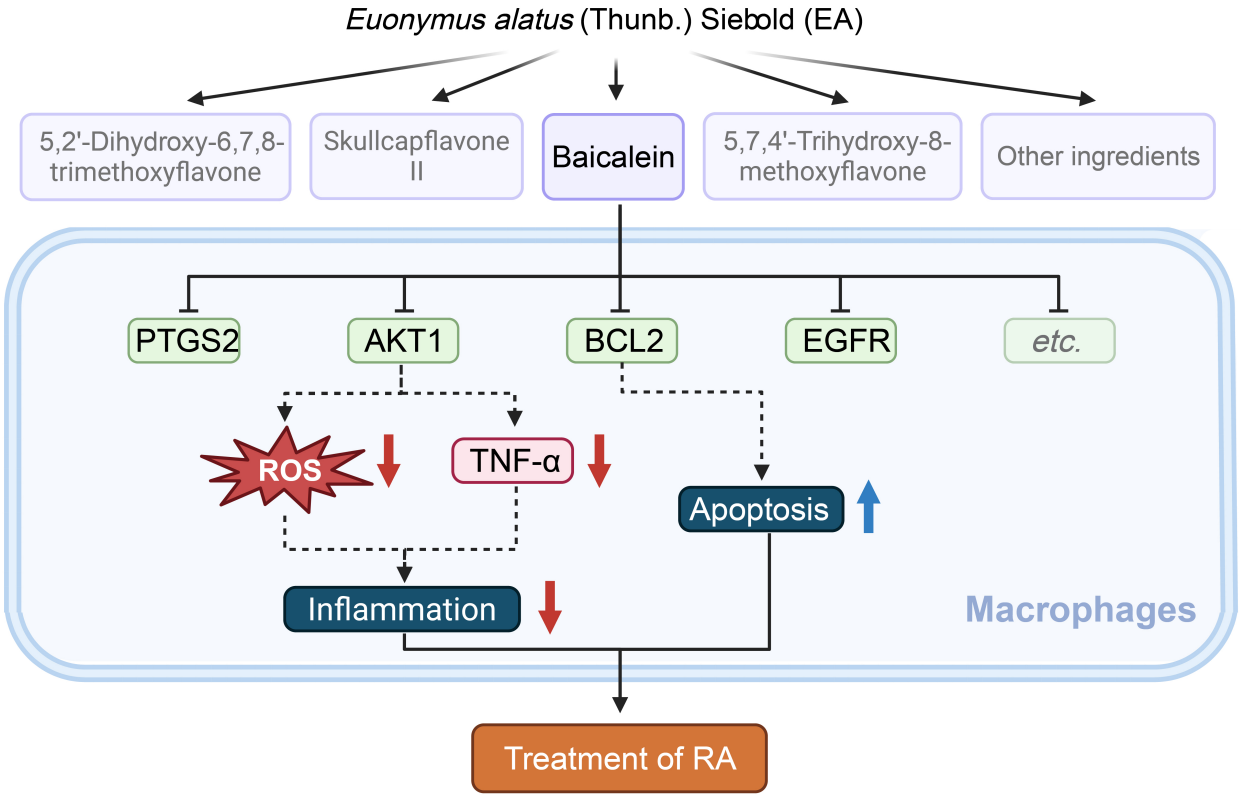

Fig. 10. Proposed mechanism for EA in the treatment of RA. The RA treatment effects of EA may be due to the action of its active ingredients on RA-related targets (e.g., BCL2, AKT, PTGS2), resulting in decreased inflammation and induced cell apoptosis. Created in BioRender. Zeng, J. (2025) https://BioRender.com/2464pqd.

LPS+Baicalein treated group (Fig. 9). Thus, baicalein decreases LPS-induced ROS generation in RAW 264.7 cells.

4. Discussion

This study investigated the potential role of EA in the treatment of RA and its mechanism of action using network pharmacology, molecular docking and experimental verification. This identified 21 active ingredients in EA and 134 overlapping targets implicated in RA therapy. PPI, GO, and KEGG analyses revealed that AKT1, BCL2, MAPK3, EGFR, PIK3R1, MAPK14, and PTGS2 may be important candidate targets in EA-mediated RA treatment. These converged onto the PI3K/AKT signaling pathway, which is known to be extensively and aberrantly activated in RA synovial cells [53]. GO analysis suggested the EA active ingredients can affect RA via the regulation of cellular apoptosis and anti-inflammation through five pathways: regulation of cell migration, response to LPS, regulation of the apoptotic signaling pathway, the BCL2 family protein complex, and prostanoid receptor activity. Moreover, the top five pathways identified by KEGG analysis (cancer, endocrine pathways etc.) are involved in the progression of RA. In addition, molecular docking results demonstrated that four EA active ingredients exhibited favorable binding affinities toward eight potential targets related to RA. Taken together, these findings indicate that EA-mediated RA treatment is likely to involve the regulation of cellular inflammation and apoptosis (Fig. 10).

Study has shown that baicalein can suppress the production of key pro-inflammatory cytokines (e.g., IL-6, TNF-α, IL1-β) and terminal inflammatory mediators (e.g., ROS, PGE2, NO) in macrophages [54]. Another study reported that the ability of baicalein to attenuate inflammatory cell infiltration in RA is mediated through the suppression of PI3K, IKK, NF-κB, and AKT phosphorylation [55]. Furthermore, baicalein was reported to bind to PTGS and change its spatial conformation, thereby altering its biological activity [56]. Baicalein, a key component of EA, was found in the present study to inhibit RAW 264.7 cell proliferation and to decrease TNF-α expression and ROS production by these cells. Our study also suggests that AKT1, BCL2, MAPK3, EGFR, PIK3R1, MAPK14, and PTGS2 may be key candidate targets in EA-mediated RA treatment. AKT is a major player in the PI3K/AKT signaling pathway, serving as an upstream regulator of gene expression in RA synovial fluid neutrophils and participating in the formation of neutrophil extracellular traps (NETs) in these cells [57]. PTGS2 is highly expressed in vascular endothelium, synovial lining cells, chondrocytes, and sub-synovial fibroblast-like cells of the RA synovium. It catalyzes the conversion of arachidonic acid to PGH2, contributing to the production of PGE2 [58]. A previous study showed that phosphorylation of PI3K/AKT can activate IL-1β, induce the expression of pro-inflammatory factors TNF-α and IL-6, and promote the development of joint inflammation in rats [59]. Therefore, we propose that EA-mediated effects in RA treatment may be due to the actions of its active ingredients, such as baicalein, on targets that suppress inflammation in RA and induce cell apoptosis via the PI3K/AKT pathway, and possibly also other signaling pathways (Fig. 10).

5. Conclusions

In conclusion, our results highlight the complex relationship among chemical ingredients, targets and pathways in the EA-mediated treatment effects in RA. Baicalein and other ingredients in EA may target AKT1, BCL2, MAPK3, EGFR, PIK3R1, MAPK14, and PTGS2 to mediate RA treatment effects via the PI3K/AKT signaling pathway, or other apoptosis signaling pathways. Further experimental verification is needed to fully characterize the specific mechanism of EA-mediated RA therapy.

Availability of Data and Materials

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

Author Contributions

SL and QW conceived this study, QW performed data analysis and interpreted results with the assistance from JZ. QW, JZ, YW, HL, GL, XZ, HS, and YD performed the research, analyzed the data, participated in experiments and drafted the article. SL and HS reviewed and edited the manuscript; SL and HS supervised the study. All authors read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

Not applicable.

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Conflict of Interest

The authors declare no conflict of interest.

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