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Original Article

Uncovering the Mechanism of Fuzi and Baishao in Treating Rheumatoid Arthritis Using Systems Pharmacology and Molecular Docking

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Abstract

Background and Objectives: Traditional Chinese Medicine has been implemented in clinical practice for thousands of years to treat rheumatoid arthritis (RA). *Aconitum carmichaelii* Debx (Fuzi) and *Paeonia lactiflora* Pall (Baishao) are a common herb-pair that is used in many herbal prescriptions to treat RA. However, the mechanism of Fuzi and Baishao for treating RA remains unclear. Here, we used a systems pharmacology and molecular docking approach to investigate the mechanism of Fuzi and Baishao in the treatment of RA.

Methods: We obtained active compounds and targets through a database search and manual supplementation, followed by network construction and protein-protein interaction construction, which were then verified using molecular docking, Gene Ontology, and Kyoto Encyclopedia of Genes and Genomes analyses.

Results: We obtained 56 active compounds (including a duplicate compound), 102 targets, and 54 pathways using our systems approach. The results indicate that both herbs are involved in IL-17 and tumor necrosis factor signaling pathways through albumin, interleukin-6, glyceraldehyde-3-phosphate dehydrogenase, epidermal growth factor receptor, prostaglandin-endoperoxide synthase 2, and other targets in the treatment of RA. After the combination, the number of targets, pathways, and specific targets on pathways increased.

Conclusion: This research provides new insight into this particular herb pair and novel research directions for the treatment of RA with Fuzi and Baishao.

Keywords: *Aconitum carmichaelii* Debx; *Paeonia lactiflora* Pall; Rheumatoid arthritis; Systems pharmacology.

Abbreviations: ALB, albumin; Baishao, Paeoniae Radix Alba; BP, biological processes; CC, cellular components; CTD, Comparative Toxicogenomics Database; C-T-P, compound-target-pathway; DL, drug-likeness; EGFR, epidermal growth factor receptor; Fuzi, Aconiti Lateralis Radix Praeparata; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GO, gene ontology; KEGG, kyoto encyclopedia of genes and genomes; IFN, interferon; IL, interleukin; MAPK, mitogen-activated protein kinase; MF, molecular functions; OB, oral bioavailability; PPI, protein-protein interaction; PTGS2, prostaglandin-endoperoxide synthase 2; RA, rheumatoid arthritis; TCM, Traditional Chinese Medicine; TCMSP, Traditional Chinese Medicine Systems Pharmacology Database and Analysis Platform; TNF, tumor necrosis factor.

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Introduction

Rheumatoid arthritis (RA) is a widespread chronic joint disease. The Global Burden of Disease data in 2017 showed that the global prevalence of RA was about 0.27%.[1,2] In China, the prevalence of RA is 0.88%, ranking as one of five major musculoskeletal disor-

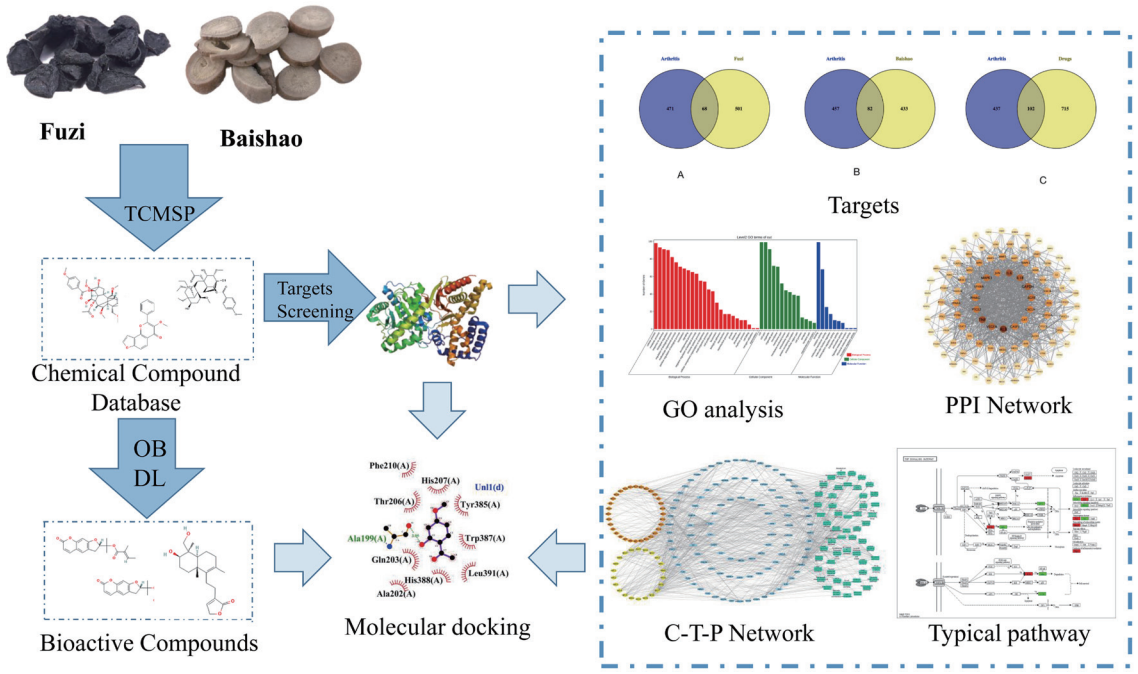

Fig. 1. Flowchart explaining the mechanism of Fuzi and Baishao against RA. C-T-P, compound-target-pathway; DL, drug-likeness; GO, gene ontology; OB, oral bioavailability; PPI, protein-protein interaction; RA, rheumatoid arthritis; TCMSP, Traditional Chinese Medicine Systems Pharmacology Database and Analysis Platform.

ders.[3] If RA is not well controlled, the joints are easily deformed, thus affecting an individual's normal work and life. Despite great efforts by researchers to treat RA, the clinical management of RA remains challenging due to a lack of good treatment options. The current treatments for RA are divided into four groups: non-steroidal anti-inflammatory drugs, immunosuppressive glucocorticoids, disease-modifying anti-rheumatic drugs, and supplemental non-pharmacological treatments.[4] The chronic use of non-steroidal anti-inflammatory drugs can lead to side effects such as gastric ulcers and renal toxicity.[5] Glucocorticoids are also widely used in the treatment of RA, but osteoporosis and hyperglycemia/diabetes are serious side effects that cannot be ignored.[6,7] Disease-modifying anti-rheumatic drugs are targeted for RA inflammation but can cause side effects including increased frequency of infections, elevated cholesterol levels, cytopenia, and gastrointestinal issues.[8] Because of the shortcomings of these treatments, there is an urgent need to find more suitable medicines for RA treatment.

With its high safety and rich clinical outcomes, Traditional Chinese Medicine (TCM) is widely utilized to treat various diseases.[9] Fuzi is a processed product of the roots of *Aconitum carmichaelii* Debx and is widely used to treat various diseases such as syncope, emetic vomiting, diarrhea, RA, and heart failure.[10,11] Baishao is the dried root of *Paeonia lactiflora* Pall and is widely used in clinical practice. Traditionally, it is mainly used to invigorate blood circulation, regulate menstruation and analgesia, nourish the liver, and alleviate various painful inflammatory conditions.[10] In the clinic, Fuzi and Baishao are usually used as an herb-pair to treat RA. Our previous study demonstrated that the combination of lipid-soluble alkaloids from Fuzi and total glycosides of peony can improve the efficacy of Fuzi in attenuating RA and also reduce its toxicity, as evidenced by reduced concentrations of plasma interleukin (IL)-1β, tumor necrosis factor (TNF)-α, and interferon (IFN)-γ, as well as liver injury-associated markers.[12] However, the constituents of Fuzi and Baishao are very complex, and the specific compounds and mechanisms of Fuzi and Baishao in treating RA are still not fully understood.

Currently, systems pharmacology is widely used as an efficient tool to screen the active compounds and study the mechanisms of complex TCM. Through the compound-target-pathway (C-T-P) model, systems pharmacology predicts the pharmacological effects and potential mechanisms of the bioactive compounds in TCMs and herbal formulations.[13] In recent years, researchers have used the systems pharmacology approach to elucidate the mechanism of TCM and have yielded rich achievements.[13] For example, systems pharmacology has identified potential targets for the treatment of COVID-19 and elucidated the mechanism of action of Xianglian Pill for the treatment of ulcerative colitis and Shenzhi Jiannao formula against vascular dementia.[14–16] In this study, we first applied systems pharmacology to screen the active compounds of Fuzi and Baishao. Then, we elucidated the mechanism of Fuzi and Baishao in the treatment of RA using enrichment analysis and network construction (Fig. 1). This study contributes to the understanding of the mechanism of Fuzi and Baishao in the treatment of RA.

Methods

Chemical compounds obtained and active compounds screening

All the compounds of Fuzi and Baishao were obtained from the Traditional Chinese Medicine Systems Pharmacology Database and Analysis Platform (TCMSP, http://ibts.hkbu.edu.hk/LSP/tcmsp.php), a professional and unique systems pharmacology tool that is designed for TCM.[17] Parameters including oral bioavailability (OB) and drug-likeness (DL) reflecting the drug absorption, distribution, metabolism, and excretion were utilized to screen the effective active ingredients. Models based on P-glycoprotein (P-

gp) and cytochrome P450s were used to calculate OB values for all herbs.[18] Tanimoto coefficient was employed in database DL evaluation.[19] The standards for screening bioactive compounds were OB ≥ 30% and DL ≥ 0.18.[20] Compounds that did not reach the thresholds but reportedly showed strong pharmacological effects were also manually supplemented.

Target screening

To identify targets of the active ingredients of Fuzi and Baishao, the screened active compounds were subjected to various web servers and databases, including Similarity Ensemble Approach (http://sea.bkslab.org/), TCMSP, Swiss Target Prediction (http://www.swisstargetprediction.ch/), SuperPred (https://prediction.charite.de/index.php), and PharmMapper (http://lilab-ecust.cn/pharmmapper/index.html).[17,21–24] It is important to note that targets belonging to Homo sapiens were selected for future analysis. A *P*-value <0.05 was used to filter targets in online services and databases. RA-related targets were obtained from the Comparative Toxicogenomics Database (CTD, http://ctdbase.org/) and DrugBank (https://go.drugbank.com/).[25,26]

Protein-protein interaction (PPI) network construction

The role of a gene or protein *in vivo* is usually regulated by multiple genes, and there are complex relationships between proteins. Complex mechanisms of action between proteins can be mediated by PPI reactions.[27] The STRING (https://string-db.org/, version 11.5) database was used for PPI network construction. The PPI network diagram generated by STRING was imported into Cytoscape (https://cytoscape.org/, version 3.8.2), a professional and open platform used to map and visualize complex networks,[28] for further analysis and refinement; no system nodes were removed in the final network.

Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analyses

We performed GO analysis to screen the roles of identified genes. The biological, molecular, and cellular functions of target genes obtained using GO analysis included biological processes (BP), cellular components (CC), and molecular functions (MF).[29] An online web tool OmicShare (https://www.omicshare.com/tools/) was used to carry out the GO analysis. To understand in which pathways the genes were enriched, we performed KEGG analysis in OmicShare. Pathways with a $P < 0.01$ were selected for final analysis.

Network construction

To better understand and analyze the complex relationship between the active ingredients of Fuzi and Baishao, C-T-P networks were built. The source of the pathway information was extracted from KEGG, and pathways with a $P < 0.01$ were preserved.[30] Because the abundance of disease pathways can impact the correct judgment,[31] the signaling pathways downloaded from KEGG were corrected and screened further, and only non-disease pathways were used in C-T-P network construction. All networks were mapped using Cytoscape.

Molecular docking

The three-dimensional structure formats of albumin (ALB, 7qfe), IL-6 (4o9h), glyceraldehyde-3-phosphate dehydrogenase (GAPDH, 6m61), epidermal growth factor receptor (EGFR, 1m17), and prostaglandin-endoperoxide synthase 2 (PTGS2, 5f19) whose species was restricted to "Homo sapiens" were obtained from RCSB PDB (https://www.rcsb.org/) after the smaller resolution value was chosen. PubChem database (https://pubchem.ncbi.nlm.nih.gov/) was applied for the acquisition of three-dimensional structures of representative compounds and the compounds were saved in "sdf" format. Dehydration, ligand removal, hydrogenation, and molecular docking were performed with the use of AutoDockTools-1.5.6 (https://autodock.scripps.edu/) and PyMOL2.4 (https://pymol.org/2/). The docking results were visualized using Ligplot (https://www.ebi.ac.uk/thornton-srv/software/LigPlus/).

Results

Active compounds of Fuzi and Baishao

After retrieving bioactive chemical compounds by OB and DL from TCMSP, 47 compounds were obtained. The compounds that did not pass OB and DL screening but showed significant pharmacological effects were also manually supplemented. As a result, a total of 56 active compounds were finally obtained (Table 1), among which sitosterol (M35) was the common component of Fuzi and Baishao. In Fuzi, only 34 active compounds passed the strict filtering criteria, and the main compounds in Fuzi were found to be alkaloids, such as deoxyaconitine (M7), hypaconitine (M20), and aconitine (M30). Although Fuzi has promising pharmacological effects and has widespread clinical application, cases of Fuzi poisoning have been reported in China, India, Japan, Korea, and other countries.[32–34] Among them, aconitine (M30) is the main toxic compound, but it is also the main active compound that produces anti-inflammatory, analgesic, and cardiotonic pharmacological effects.[35] Therefore, the most toxic compounds of Fuzi were preserved for the study, including aconitine (M30, OB = 7.87%, DL = 0.23), mesaconitine (M22, OB = 8.7%, DL = 0.25), and hypaconitine (M20, OB = 31.39, DL = 0.26). Less toxic monoester alkaloids in Fuzi, such as benzoylecgonine (M26, OB = 12.83%, DL = 0.25), have also been reported as possible new treatments for mitochondria-related diseases.[36] Benzoylhypaconine (M27, OB = 8.7%, DL = 0.29), also a monoester alkaloid, is a potential anti-inflammatory compound,[37] and coryneine (M28, OB = 3.55%, DL = 0.05) has been shown to act on motor nerve terminals and thus promote the release of acetylcholine.[38] Therefore, we preserved these compounds in our study.

After screening, 23 active compounds with promising pharmacological activities were ultimately selected from Baishao. The main compounds of Baishao include the total glucosides of peony. Many of these compounds show excellent pharmacological effects. For example, albiflorin (M47, OB = 12.09%, DL = 0.77) can protect against osteoblast-like MC3T3-E1 cytotoxicity mediated by oxidative stress.[39] Paeonol (M54, OB = 28.79%, DL = 0.04) has been proven to have anti-inflammatory, neuroprotective and anti-tumor effects.[40] Paeoniflorin (M40, OB = 53.87%, DL = 0.79) has anti-inflammatory and immune system modulating effects.[41] Atractylenolide I (M48, OB = 37.37%, DL = 0.15) has been regarded as a candidate drug for the treatment of inflammatory diseases with TNF-α and nitric oxide overproduction. Atractylenolide III (M50, OB = 31.15%, DL = 0.17) shows a similar pharmacological effect but is weaker than atractylenolide I.[42] The pharmacological effects of these compounds partially confirm the suitability of our screening method, and these compounds are taken as representative compounds.

Target fishing of Fuzi and Baishao

Fishing the targets of herbal compounds using wet methods is

Table 1. Bioactive compounds and key parameters of Fuzi and Baishao.

No.	Chemical name	OB (%)	DL	Herb
M1	Carnosifloside I_qt	38.16	0.8	Fuzi
M2	(3R,8S,9R,10R,13R,14S,17R)-3-hydroxy-4,4,9,13,14-pentamethyl-17-[(E,2R)-6-methyl-7-[(2R,3R,4S,5S,6R)-3,4,5-trihydroxy-6-[[(2R,3R,4S,5S,6R)-3,4,5-trihydroxy-6-(hydroxymethyl) oxan-2-yl]oxymethyl]oxan-2-yl]oxyhept-5-en-2-yl]-1,2,3,7,8,10,12,15,16,17-decahydr	41.52	0.22	Fuzi
M3	Jesaconitine	33.41	0.19	Fuzi
M4	Isotalatizidine	50.82	0.73	Fuzi
M5	Ignavine	84.08	0.25	Fuzi
M6	(R)-Norcoclaurine	82.54	0.21	Fuzi
M7	Deoxyaconitine	30.96	0.24	Fuzi
M8	6-Demethyldesoline	51.87	0.66	Fuzi
M9	Benzoylnapelline	34.06	0.53	Fuzi
M10	2,7-Dideacetyl-2,7-dibenzoyl-taxayunnanine F	39.43	0.38	Fuzi
M11	Neokadsuranic acid b	43.1	0.85	Fuzi
M12	Karanjin	69.56	0.34	Fuzi
M13	karakoline	51.73	0.73	Fuzi
M14	Deoxyandrographolide	56.3	0.31	Fuzi
M15	Demethyldelavaine B	34.52	0.18	Fuzi
M16	Demethyldelavaine A	34.52	0.18	Fuzi
M17	Deltoin	46.69	0.37	Fuzi
M18	Delphin_qt	57.76	0.28	Fuzi
M19	11,14-Eicosadienoic acid	39.99	0.2	Fuzi
M20	Hypaconitine	31.39	0.26	Fuzi
M21*	Myristic acid	21.18	0.07	Fuzi
M22*	Mesaconitine	8.7	0.25	Fuzi
M23*	Benzoylmesaconine	8.55	0.27	Fuzi
M24*	Delsoline	17.23	0.63	Fuzi
M25*	Neojiangyouaconitine	9.83	0.26	Fuzi
M26*	Benzoylaconine	12.83	0.25	Fuzi
M27*	Benzoylhypaconine	8.7	0.29	Fuzi
M28*	Coryneine	3.55	0.05	Fuzi
M29*	Denudatine	16.55	0.67	Fuzi
M30*	Aconitine	7.87	0.23	Fuzi
M31*	Neoline	13.32	0.69	Fuzi
M32*	Salsolinol	36.83	0.06	Fuzi
M33*	Songorine	24.46	0.72	Fuzi
M34	Benzoyl paeoniflorin	31.27	0.75	Baishao
M35	Sitosterol	36.91	0.75	Fuzi; Baishao
M36	Beta-sitosterol	36.91	0.75	Baishao
M37	Kaempferol	41.88	0.24	Baishao
M38	(3S,5R,8R,9R,10S,14S)-3,17-Dihydroxy-4,4,8,10,14-pentamethyl-2,3,5,6,7,9-hexahydro-1H-cyclopenta[*a*]phenanthrene-15,16-dione	43.56	0.53	Baishao

(continued)

Table 1. *(continued)*

No.	Chemical name	OB (%)	DL	Herb
M39	Lactiflorin	49.12	0.8	Baishao
M40	Paeoniflorin	53.87	0.79	Baishao
M41	(+)-Catechin	54.83	0.24	Baishao
M42	Mairin	55.38	0.78	Baishao
M43	11alpha,12alpha-epoxy-3beta-23-dihydroxy-30-norolean-20-en-28,12beta-olide	64.77	0.38	Baishao
M44	Albiflorin_qt	66.64	0.33	Baishao
M45	Paeoniflorin_qt	68.18	0.40	Baishao
M46	Paeoniflorgenone	87.59	0.37	Baishao
M47*	Albiflorin	12.09	0.77	Baishao
M48*	Atractylenolide I	37.37	0.15	Baishao
M49*	Atractylenolide II	47.50	0.15	Baishao
M50*	Atractylenolide III	31.15	0.17	Baishao
M51*	Atractylone	41.10	0.13	Baishao
M52*	γ-elemene	23.79	0.06	Baishao
M53	8β-ethoxy atractylenolide III	35.95	0.21	Baishao
M54*	Paeonol	28.79	0.04	Baishao
M55*	Oxypaeoniflorin	21.88	0.78	Baishao
M56*	Gallotannin	7.36	0.03	Baishao

*Compounds with OB < 30% and/or DL < 0.18, yet pharmaceutically validated. OB, oral bioavailability; DL, drug-likeness.

time-consuming, costly, and labor-intensive. In our study, we predicted the targets of active compounds in Fuzi and Baishao using online databases such as Similarity Ensemble Approach, TCMSP, Swiss Target Prediction, and CTD. Using this approach, we identified a total of 1,084 targets including 569 targets in Fuzi and 515 targets in Baishao. We then further screened the targets of Fuzi and Baishao in the drug library and CTD. For Fuzi alone there were 68 targets (Fig. 2a) and for Baishao there were 82 targets (Fig. 2b), and for the combination of the two, there were 102 targets (Fig. 2c) (Table S1). Based on the target fishing methods we used, Fuzi and Baishao may enhance efficacy by increasing the number of targets.

It is worth noting that each compound corresponds to multiple targets. For example, paeonol (M54) can act on PTSG1, PTGS2, BCL2, MAPK1, TNF, NFKBIA, AKR1C2, ABCG2, MAPT, CA2, CA3, MIF, CYP1A2, STS, FOS, ALOX5, CYP2C19, JUN, STAT1, and AKR1C1. These targets correspond to 56 active compounds in Fuzi and Baishao. Some of these compounds and their corresponding targets have been validated by other studies. For instance, phenol (M54) from Baishao could potentially act on 20 targets including TNF and mitogen-activated protein kinase (MAPK)-1. Interestingly, phenol can suppress the production of pro-inflammatory cytokines such as TNF-α.[40] Similarly, paeoniflorin (M40) may act on 28 targets including TNF, IL-6, and MAPK1. Interestingly, it has been shown that paeoniflorin could decrease the activity of TNF-α, the expression of IL-6, improve the pain threshold and reduce arthritis symptoms in RA rats.[43] These studies partially validate the results of our target fishing.

PPI network construction of Fuzi and Baishao

To better understand the properties and relationships of the 102 targets, three PPI networks were established (Fig. 3, Figs. S1 and S2). The complicated PPI network shows that there is an intricate relationship among these targets in the treatment of RA. In the PPI: nodes represent genes, proteins, small molecules, or other entities;

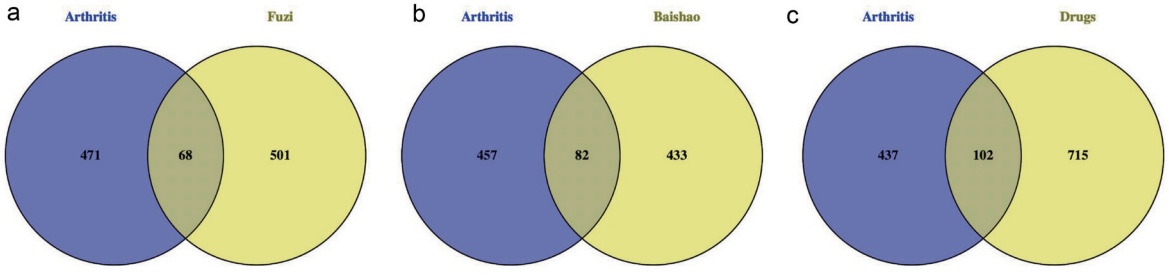

Fig. 2. Venn diagram showing the intersection of drugs and disease. (A) The intersection of Fuzi and arthritis. (B) The intersection of Baishao and arthritis. (C) The intersection of Fuzi-Baishao and RA-related targets. RA, rheumatoid arthritis.

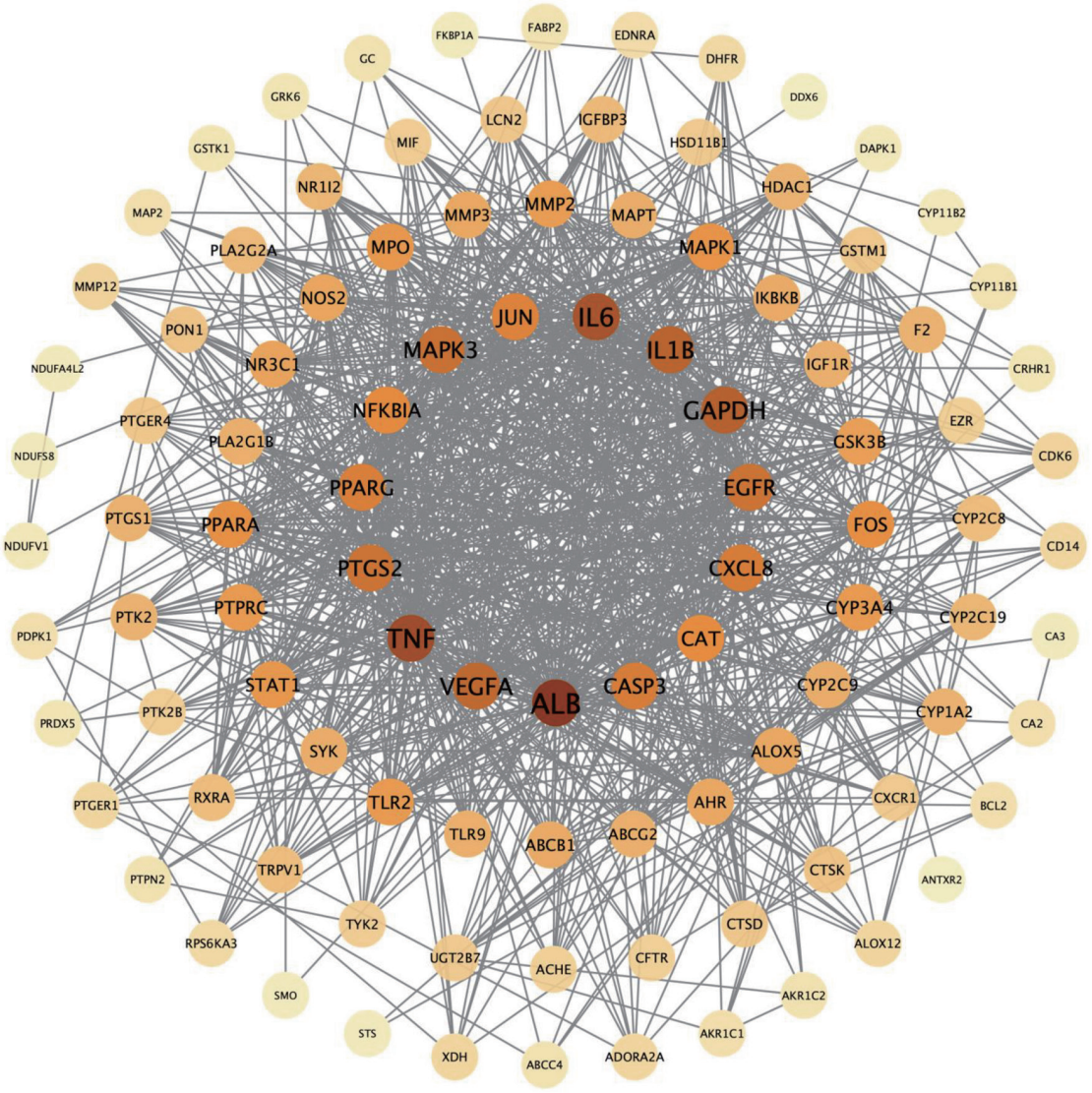

Fig. 3. PPI network showing the relationship between the targets of Fuzi and Baishao. Shades of color indicate high or low degree values. Nodes that are not linked to other nodes are excluded. PPI, protein-protein interaction.

degree represents the number of nodes that it interacts with; and edges represent interactions between nodes. The number of edges represents the number of protein interactions, and the average node degree is the average of the degrees of connectivity of all components and targets.[44] The number of edges in the PPI network of Baishao was 696 and the average node degree was 17.0 (Fig. S1). In contrast, the number of edges in the network in Fuzi was 416 and the average node degree was 12.2 (Fig. S2). Both the numbers of edges and the average nodes of the individual Fuzi and Baishao PPI networks were smaller than the combinatorial PPI network of Fuzi and Baishao. In the combinatorial PPI network, the top five targets by degree value were ALB, IL-6, GAPDH, EGFR, and PTGS2.

GO analysis of Fuzi and Baishao

To elucidate the properties of target proteins, GO analysis containing BP, CC, and MF was analyzed (Fig. 4). The top five biological processes were cellular process, metabolic process, response to stimulus, biological regulation, and regulation of the biological process. Cell, cell part, organelle, organelle part, and membrane were the top five cellular components. The top five molecular functions included binding, catalytic activity, molecular transducer activity, molecular function regulator, and transcription regulator activity. Fuzi and Baishao were also separately subjected to GO analysis to better elucidate the pharmacological effects of their combination (Figs. S3 and S4). In Fuzi, the top five biological processes were the cellular process, metabolic process, biological regulation, response to stimulus, and multicellular organismal process. The cell, cell parts, organelle, organelle parts, and membrane were the top five cell components. The top five molecular functions included binding, catalytic activity, molecular transducer activity, molecular function regulator, and transcription regulator activity. In Baishao, the top five biological processes were the cellular process, metabolic process, biological regulation, response to

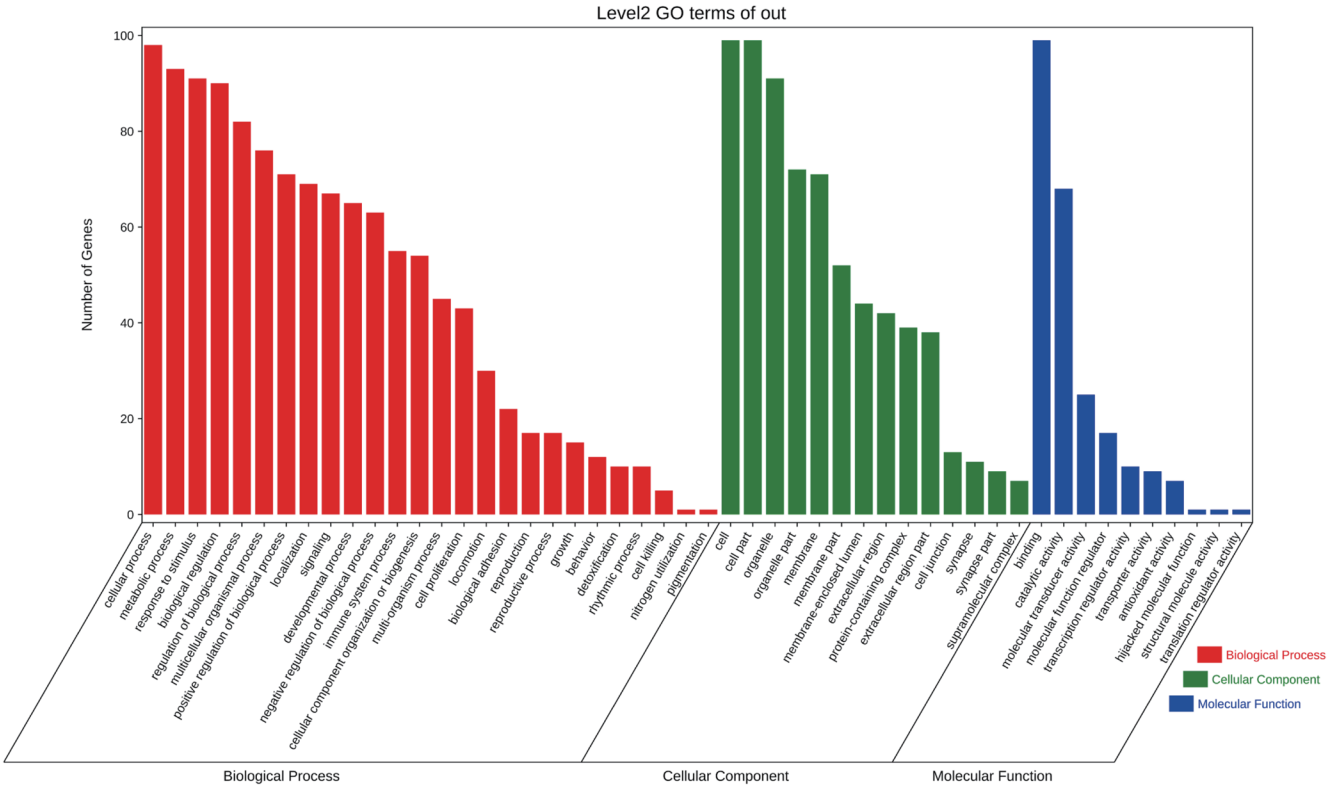

Fig. 4. GO enrichment analysis showing the number of targets participating in BP, CC, and MF. BP, biological processes; CC, cellular components; GO, gene ontology; MF, molecular functions.

stimulus, and regulation of the biological process. The top five fits in cellular components and molecular functions were identical to that of Fuzi. GO enrichment showed that the main difference was in the biological process. The response to a stimulus was in the fourth position for Fuzi and Baishao alone, but it rose to the third position when the two were combined, while the biological regulation dropped to the fourth position. It is noteworthy that for Fuzi, the regulation of the biological process appeared in the top five after binding, while the multicellular organismal process dropped out of the top five. It is also noteworthy that among these targets, we re-ran the GO enrichment of the specific targets to Fuzi and Baishao and found that the targets of Fuzi acted mainly on inflammation (Fig. S5), while the targets of Baishao acted on another regulated metabolism (Fig. S6).

KEGG analysis of Fuzi and Baishao

After GO analysis, we further subjected the screened targets to KEGG analysis, and only the non-disease-associated pathways were preserved. In the end, 54 pathways including the IL-17 signaling pathway, linoleic acid metabolism, and some others were enriched (Table. S2). Most of these pathways have been confirmed to be relevant in the treatment of RA. For example, TNF and IL-6 are defined as the therapeutic targets of RA, and regulation of linoleic acid metabolism and arachidonic acid metabolism are widely applied in the treatment of RA.[45–48] To better understand the pharmacological effects after the combination, we subjected Fuzi and Baishao to separate KEGG enrichment analyses (Table S3). There were 45 pathways when Fuzi was used alone, and 43 pathways when Baishao was used alone. When they were combined, the number of signaling pathways increased to 54. It is noteworthy that there were multiple targets in one pathway. The TNF signaling pathway plays an influential role in the treatment of RA, and TNF is considered an important target for RA treatment.[45,49] Our analysis indicated that Fuzi and Baishao work on different targets in the TNF pathway (Fig. 5). Fuzi acted on 6 targets including FOS, IL6, IKBKB, CASP3, MMP3, and PTGS2. In addition to the above 6 targets, Baishao acted on IL-1β, NF-κB-IA, and JUN. Finally, the combination of Fuzi and Baishao acted on 9 targets after removing the duplication term. The above evidence indicates that the combination of Fuzi and Baishao increases efficacy by increasing the number of targets, the number of signaling pathways, and the number of targets on specific signaling pathways.

C-T-P network construction of Fuzi and Baishao

To elucidate the specific mechanism of the potentiation effect of Fuzi and Baishao, a C-T-P network (Fig. 6) containing 56 compounds, 102 targets, and 54 pathways was created using the Cytoscape platform. To compare the results of enhanced pharmacological effects after the combination of the two herbs, separate C-T-P networks of Fuzi and Baishao were also plotted (Figs. S7 and S8). In the C-T-P network, one active ingredient can act on multiple targets, and at the same time, one pathway has multiple targets. When Fuzi and Baishao were used separately to treat RA, there were 34 active components of Fuzi and 23 ingredients of Baishao. However, when they were combined, a total of 56 ingredients were screened. When Fuzi and Baishao acted alone on RA, the number of targets was 68 and 82, and the number of pathways was 45 and 43, respectively. The number of targets increased to 102 and the

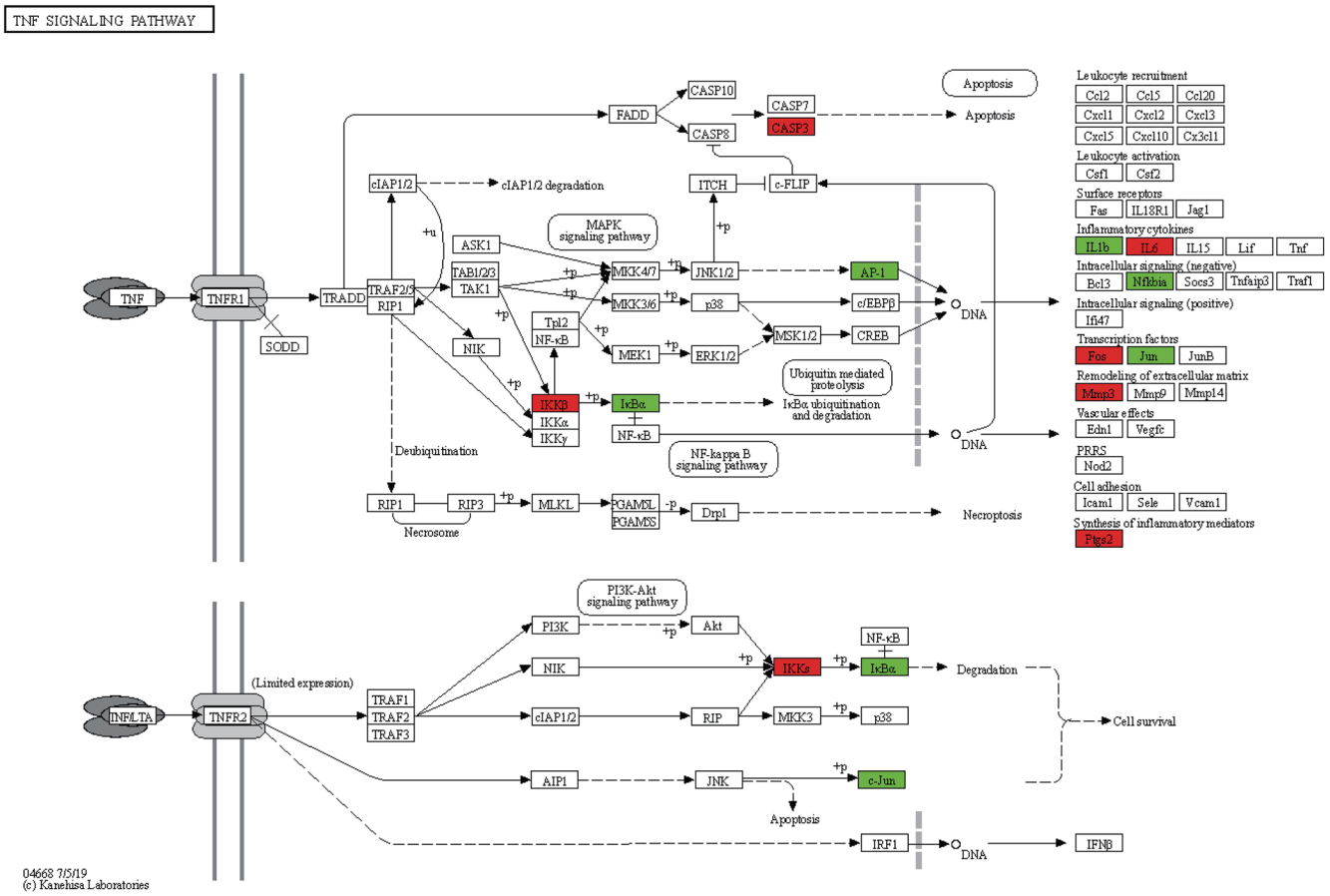

Fig. 5. Distribution of targets of Fuzi and Baishao on the TNF signaling pathway. Red represents the target shared by Fuzi and Baishao, and green represents the targets of Baishao. The compression pathway is derived from KEGG. KEGG, kyoto encyclopedia of genes and genomes; TNF, tumor necrosis factor.

number of pathways increased to 54 when they were combined. It is evident that both targets and pathways were increased after the combination of the two herbs. These results suggest that Fuzi and Baishao used together can treat RA by influencing more targets and pathways, compared to using them alone. The results reflect the properties of herbal medicine to treat disease holistically.

Molecular docking

Based on the results of the PPI, the top five targets with degree values as core targets were molecularly docked to the representative compounds that have been shown in studies to have significant activity against RA. The stability between the two drugs can be judged by the binding energy; the lower the binding energy, the better the affinity between targets and compounds. The results showed that these binding energies were all less than −5 kcal/mol, indicating that the compound had a high affinity with proteins, especially the binding energy of paeoniflorin and GAPDH (−10.1 kcal/mol). The binding energy of the compound and the protein is shown in Figure 7A, and an image of the optimal docking of receptor and ligand was visualized. The results showed that phenol binds to Ala199 in PTGS2 via a hydrogen bond. Albiforin forms one hydrogen bond with Ser122, Thr184, and Thr153 in GAPDH and two hydrogen bonds with Thr182 and Arg234. The structure of aliform is linked to Arg117, Arg186, and His146 in ALB via one hydrogen bond. Paeoniflorin can interact with Tyr161 and His146 in ALB via one hydrogen bond and interact with Arg117 and Arg186 via two hydrogen bonds (Fig. 7B).

Discussion

Fuzi and Baishao, both of which have excellent clinical efficacy, are extensively used as traditional Chinese herbs. The combination of Fuzi and Baishao has pharmacological effects such as pain suppression and regulation of menstruation and paralysis, among others. Although Fuzi and Baishao have been the focus of scholarly research, the mechanism of their use in combination with the treatment of RA has remained unclear. Here, we evaluated the mechanism of Fuzi and Baishao in RA using a systems pharmacology approach. We screened the active compounds and potential targets of the two herbs and demonstrated that one active compound can link to different targets and different targets can be connected to the same active compound, with a significant increase in the potential targets when the two are used together. We also found that Fuzi and Baishao can treat RA through 56 active compounds, 102 targets, and 54 pathways, which represents a significant increase in the number of compounds, targets, and pathways compared to Fuzi and Baishao used alone.

The progression of RA is associated with the continuous production of inflammatory cytokines and deregulation of anti-inflammatory cytokines.[50–53] Because of the importance of cy-

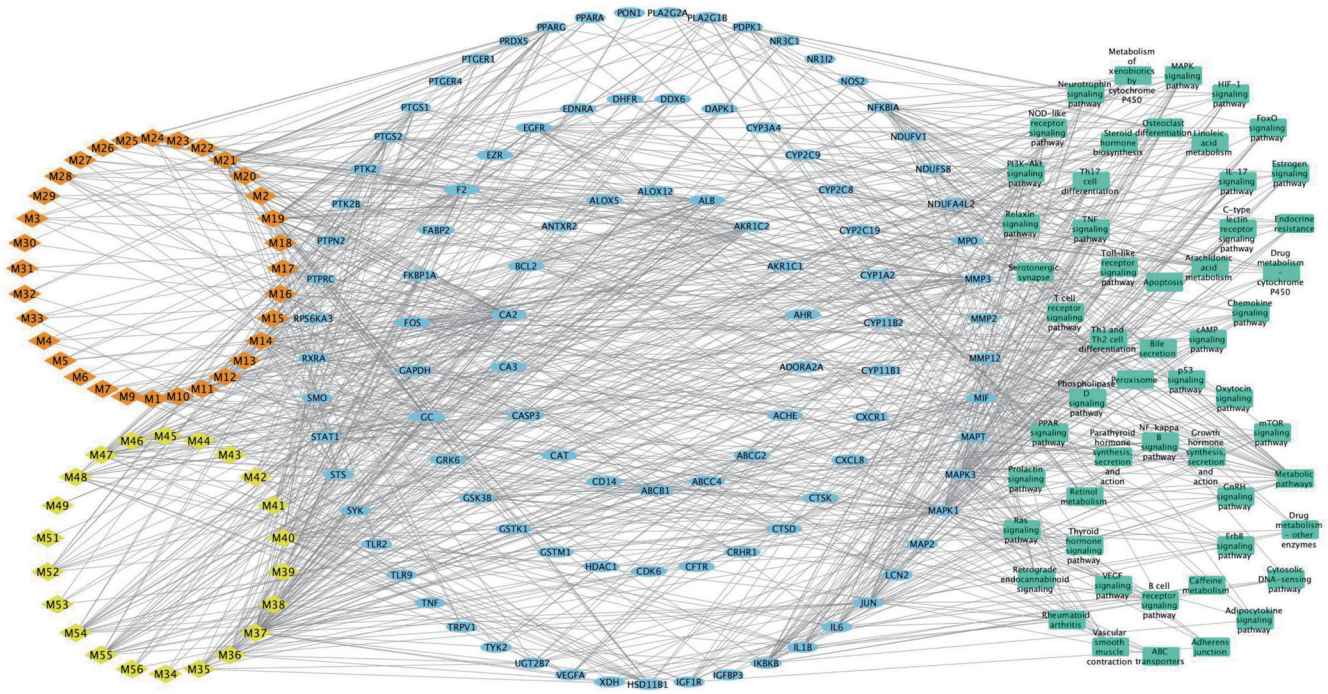

Fig. 6. The C-T-P network showing the combined mechanism of Fuzi and Baishao. C-T-P, compound-target-pathway.

tokines, cytokine-targeted therapies have been developed, such as those targeting the important pro-inflammatory cytokines TNF-α and IL-1.[54] Here, we found out that Fuzi and Baishao can target pro-inflammatory cytokines, including TNF and IL-6, to improve RA. Meanwhile, the results of some studies suggest that the combination of alkaloids with total glucosides of peony significantly reduces the concentration of cytokines and increases the anti-inflammatory effect of alkaloids, resulting in a more effective treatment of RA.[12] In our study, PPI network analysis showed that IL-6 was one of the top five targets with higher activity in protein interactions and good affinity with representative compounds, which confirms the reliability of our study. In addition, Fuzi and Baishao

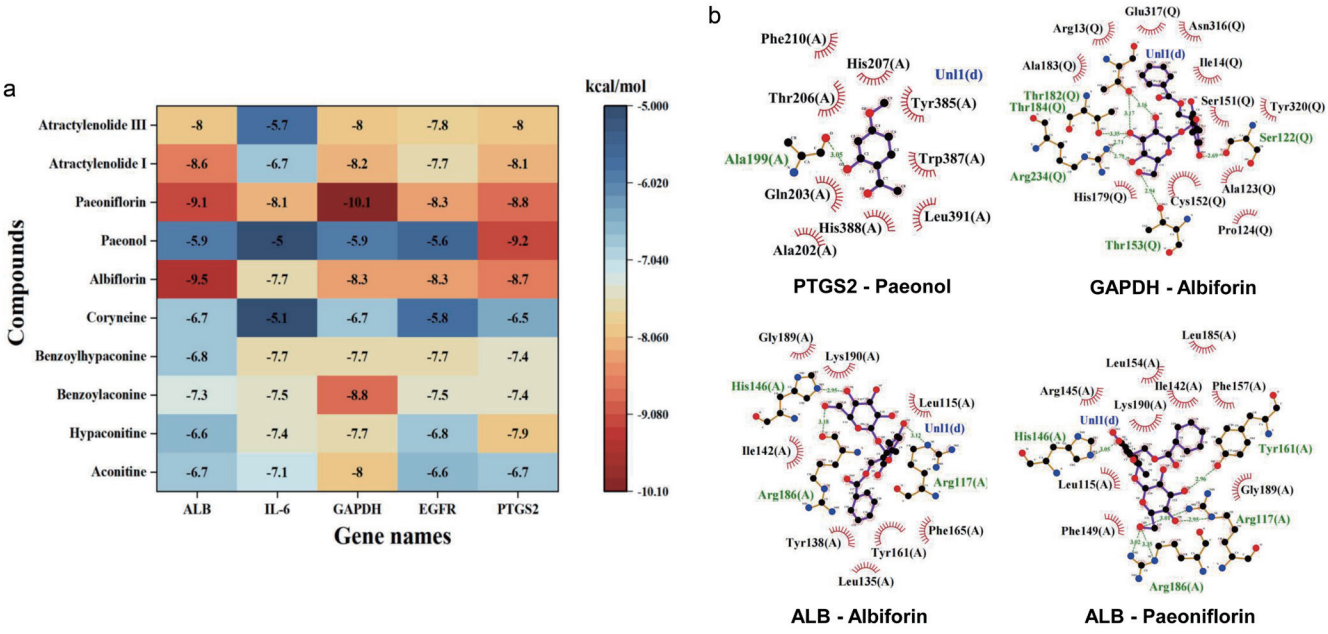

Fig. 7. Molecular docking of representative compounds and core targets. (A) Heat map of estimated binding energy. (B) Image of the optimal docking of compound-target pairs, including PTGS2 and Paeonol; GAPHD and Albiforin; ALB and Albiforin; ALB and Paeoniflorin. ALB, albumin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PTGS2, prostaglandin-endoperoxide synthase 2.

could target cytokine-related pathways such as the TNF signaling pathway. These results suggest that the regulation of inflammatory status is one of the major mechanisms of these two herbs in the treatment of RA.

Our study has several limitations. First, many factors influence the therapeutic effect of herbal medicine, such as decoction time, time of administration, and route of administration. One of the shortcomings of systematic pharmacology is that it cannot give direct dose-phenotype results since it only searches drugs and diseases through databases. It was noted that the ratio of the use of Fuzi and Baishao varies in different formulas. For example, in Zhenwu Tang, the ratio is 1:1 for the treatment of retention of water caused by kidney/spleen yang deficiency.[55] Another classic formula from the same work, the Shaoyao-Gancao-Fuzi Decoction, has a 3:1 ratio of Fuzi to Baishao, which has significant anti-inflammatory and analgesic effects.[56] The optimal ratio of Fuzi to Baishao for the treatment of RA requires further validation. In addition, although some of Fuzi and Baishao's targets have been confirmed through literature searches and molecular docking, many of the predicted targets still need to be validated. Even if all targets are known, due to algorithmic shortcomings, this computerized method may not be able to obtain targets that appear in real *in vivo* conditions. Furthermore, Fuzi is a strongly toxic drug and its toxicity cannot be ignored in the treatment of RA.[57] Our study only demonstrates that Baishao enhances the effects of Fuzi in the treatment of RA but cannot elucidate whether there is any toxicity. Also, only online databases and literature searches were artificially used to determine the targets, but there are thousands of components in one herbal medicine. Thus, our study may not include all compounds in Fuzi and Baishao. Lastly, some metabolites may be biologically active, but not all of them are currently known, so not all targets can be predicted.

Recently, the gut microbiota has become a new direction for identifying the occurrence and development of disease, especially in autoimmune diseases such as RA. Studies have shown that an increase in gut microbiota such as Prevotella and a decrease in bacteria such as Bifidobacterium are involved in the process of RA.[58,59] The gut microbiota can synthesize and release certain anti-inflammatory metabolites, such as short-chain fatty acids, to mitigate the disease process.[60,61] In our study, some compounds were not identified as bioactive compounds because they did not meet the screen standard. However, one such compound, myristic acid, has been demonstrated to modify the composition of the gut microbiota,[62] and has been shown to have activity against bacterial infection and multi-species biofilm formation.[63] Therefore, other compounds excluded in our experiment may also improve RA through modulating the gut microbiota. In addition to small molecule compounds, large molecule compounds such as polysaccharides may regulate the composition of the gut microbiota to control disease progression.[64,65] However, in our study, only small molecule compounds were selected, and big molecule compounds were omitted. Therefore, future studies are needed to study the effects of compounds like myristic acid and polysaccharides on RA.

Conclusions

Fuzi and Baishao, a classical herbal pair in Chinese medicine, are widely used in the clinical treatment of RA. We found out that the effects of Fuzi and Baishao on RA can be attributed to the action of 56 bioactive components that act as a holistic network with 102 targets and 54 pathways. We also found that the regulation of inflammatory status is one of the major mechanisms of Fuzi and Baishao in the treatment of RA. Compared to using Fuzi and Baishao alone, the use of the two in combination can affect more targets and pathways and thus increase efficacy. This study provides new ideas for the development of drug targets in the treatment of RA.

Supporting information

Supplementary material for this article is available at https://doi.org/10.14218/FIM.2023.00044.

Table S1. The detailed target genes.

Table S2. The enriched KEGG pathways in Fuzi and Baishao combination.

Table S3. The enriched KEGG pathways in Fuzi and Baishao alone.

Fig. S1. PPI network showing the relationship between the targets of Fuzi. PPI, protein-protein interaction.

Fig. S2. PPI network showing the relationship between the targets of Baishao. PPI, protein-protein interaction;

Fig. S3. GO enrichment analysis showing the number of targets of Fuzi participating in biological process, cellular component, and molecular function. GO, gene ontology.

Fig. S4. GO enrichment analysis showing the number of targets of Baishao participating in biological process, cellular component, and molecular function. GO, gene ontology.

Fig. S5. GO enrichment analysis showing the number of specific targets of Fuzi participating in biological process, cellular component, and molecular function. GO, gene ontology.

Fig. S6. GO enrichment analysis showing the number of specific targets of Baishao participating in biological process, cellular component, and molecular function. GO, gene ontology.

Fig. S7. The compound-target-pathway network showing the combined mechanism of Fuzi.

Fig. S8. The compound-target-pathway network showing the combined mechanism of Baishao.

Acknowledgments

There is nothing to declare.

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Conflict of interest

SJY has been an editorial board member of *Future Integrative Medicine* since September 2021. These authors declare that there are no conflict of interests regarding this work.

Author contributions

Methodology (JL, SJY, and PC); validation (YXZ and HMZ); for-

mal analysis (DDZ); writing-original draft preparation (YXZ and HMZ); software (DDZ); writing-review and editing (JL and SJY). All authors have read and agreed to the published version of the manuscript.

Data sharing statement

All relevant data are within the paper and its Supporting Information files.

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