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The anti-inflammatory mechanism of the medicinal fungus puffball analysis based on network pharmacology

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ABSTRACT

Background: Puffball is a common Chinese medicine of edible and medicinal fungus. It promotes hemostasis and muscle regeneration. Recent studies have shown that puffball has a limiting effect on pain and inflammation, but the specific mechanism behind this action remains unclear. This study aimed to reveal the pharmacological mechanism of the anti-inflammatory effect of puffball using the network pharmacology method.

Methods: TCMSP and SwissTargetPrediction were used to analyze and predict the active ingredients and corresponding targets in puffball. The DisGeNET and GeneCards databases were searched to obtain inflammation-related targets, and the PPI network of the cross-genes of puffball ingredients and diseases was constructed through STRING and Cytoscape. The Metascape database was used for GO function enrichment analysis of co-presence genes and KEGG signal pathway enrichment analysis to construct an "ingredient-target-pathway" network and analyze its mechanism of action. Meanwhile, molecular docking was used to verify the ability of active ingredients to bind to key targets.

Results: A total of 6 inflammation-related active ingredients were screened from the 32 candidate compounds of puffball, and 236 targets that correspond to ingredients and 328 inflammation-related targets were collected. In summary, the β-sitosterol and melanin components in puffball can act on signal transducers and activators of transcription (STAT3) and epidermal growth factor receptor (EGFR) targets and are mainly differentiated through Th17 cells signal pathway and cancer-related pathway to play an anti-inflammatory effect.

Conclusion: This study has successfully predicted the active ingredients and potential targets of Puffball's anti-inflammatory effects, which helped to systematically elucidate its mechanism of action. The network pharmacology method can provide new insights for the research and development of edible and medicinal fungi.

1. Introduction

Puffball (*Lasiosphaera* Calvatia), is the dried fruit body of *Lasiosphaera fenzii* Reich, *Calvatiae gigantea* (Batsch ex Pers.) Lloyd, *Calvatia lilacina* (Mont. et Berk.) Lloyd's, which is a commonly used fungus Chinese medicine, "Compendium of Materia Medica" is a masterpiece of pharmacy written by Li Shizhen, a pharmacist in the Ming Dynasty, recorded puffball being used for treating cough and aphonia. Modern pharmacological studies have shown that puffball can significantly reduce the degree of swelling of egg white-induced rat foot swelling and reduce the number of writhing caused by acetic acid in mice [1]. *Calvatia gigantea* fruiting body and liquid fermentation metabolites have obvious anti-inflammatory, analgesic and bacteriostatic effects [2]. However, the specific mechanism behind the puffball's anti-inflammatory action is still unclear, and this represents the main bottleneck restricting its standardized application.

Network pharmacology represents a new drug research model. Based on the "disease-pathway-target-drug" interaction network, this method systematically and comprehensively observes the intervention and impact of drugs on the disease mechanism network [3–5]. Hence, in this paper, we use this effective method to explore the action mechanism of puffball on the inflammation pathway. Fig. 1 illustrates the workflow of this work. First, we analyzed the database to sort out the inflammation-related targets and chemical molecules in puffball. Then, we analyzed the possible interaction between the targets and constructed the network of puffball's inflammation-related targets. Finally,

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we analyzed the related action pathways to summarize the related action mechanism of puffball against inflammation.

2. Materials and methods

2.1. Compound database building

The Chinese Medicine System Pharmacology (TCMSP) database and analysis platform is a distinctive platform that combines pharmacodynamics, pharmacokinetics, network, omics and system analysis to form a unified and complete Chinese medicine System model for pharmacology and pharmacodynamics prediction and validation research. This platform can be used to obtain information about the relationships between drugs, targets and diseases [6]. We retrieved the active ingredients related to puffball in TCMSP, and the parameters to select active ingredients were set as follows: Oral bioavailability (OB) $\geq$ 20% and Drug-likeness (DL) $\geq$ 0.18; then, the target corresponding to the active compound was obtained [7,8]. It is worth mentioning that the OB value reported in some studies is not used as a harsh condition for data screening; thus, to obtain a certain active ingredient base, the OB value was set to 20% [9]. The chemical structure of each active compound with the SMILES format was extracted from PubChem (https://pubchem.ncbi.nlm.nih.gov). Compounds with potentially similar biological activity can be inferred from the principle of structural similarity [10], we obtained the predicted targets using the SMILES string in the SwissTargetPrediction small molecular targets prediction analysis database [11] (www.swisstargetprediction.ch). Since it is allowed to use a low probability threshold to obtain a sufficient number of targets [12], we used a probability $\geq$ 0.1 as the screening condition of the target protein to predict the components (Retrieval date: April 26, 2020). Afterward, the obtained targets were standardized according to the gene names by searching the UniProt Database (http://www.uniprot.org) with the "Homo sapiens" species [13]. Finally, the target library of the puffball ingredients was constructed.

2.2. Acquisition of potential anti-inflammatory targets

The database of DisGeNET (www.disgenet.org) contains information on genes and mutation sites associated with human diseases [14]. Analogously, GeneCards (www.genecards.org) provides concise genomic, proteomic, transcriptional, genetic and functional information on all known and predicted human genes [15]. These two databases were used to construct inflammation-related target libraries by performing automated search using keywords including "inflammation" and "inflammatory". The search results were then passed through other filters, with DisGeNET score gda $\geq$0.2 and GeneCards score $\geq$20 [16]. The Venn Diagram Tool v2.1.0 (https://bioinfogp.cnb.csic.es/tools/venny/index.html) was used to identify the shared target between the composition and the target.

2.3. Constructing a Protein–Protein interaction (PPI) network

STRING (https://string-db.org/) is an online search database for known and predicted protein interactions [17]. It gives a certain weight to the comprehensive results of protein interactions using a scoring mechanism, and finally outputs a comprehensive evaluation score, such that a higher score indicates a closer relationship. We mapped the composition targets into the inflammation targets and a PPI network was then constructed based on the information obtained from the STRING v11.0 database. The background organism was set to "Homo sapiens", a medium confidence score of 0.4 was used as the minimum required interaction score, the results were exported as a tsv file and imported in the Cytoscape v3.7.2 software, and the topological parameters of the networks were analyzed using the Network Analyzer tool [18].

2.4. GO annotation and KEGG enrichment analysis

Using the Metascape (metascape.org) web-based portal for comprehensive gene annotation and analysis resources [19]., we performed the Gene Ontology (GO) annotation and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis, while setting the pre-analysis species to "Homo sapiens". The GO analysis includes three categories: biological process (BP), cellular component (CC) and molecular function (MF). Finally, a bubble chart was plotted using the Bioinformatics (www.bioinformatics.com.cn) free online platform for bioinformatics-related data analysis.

2.5. Ingredient - target - pathway network construction

The ingredient-target-pathway network is constructed by introducing intersection-targets, pathways and selected 6 active ingredients into the Cytoscape software. The nodes with different colors represented different types of clusters, while the edges represented the relationships between the nodes.

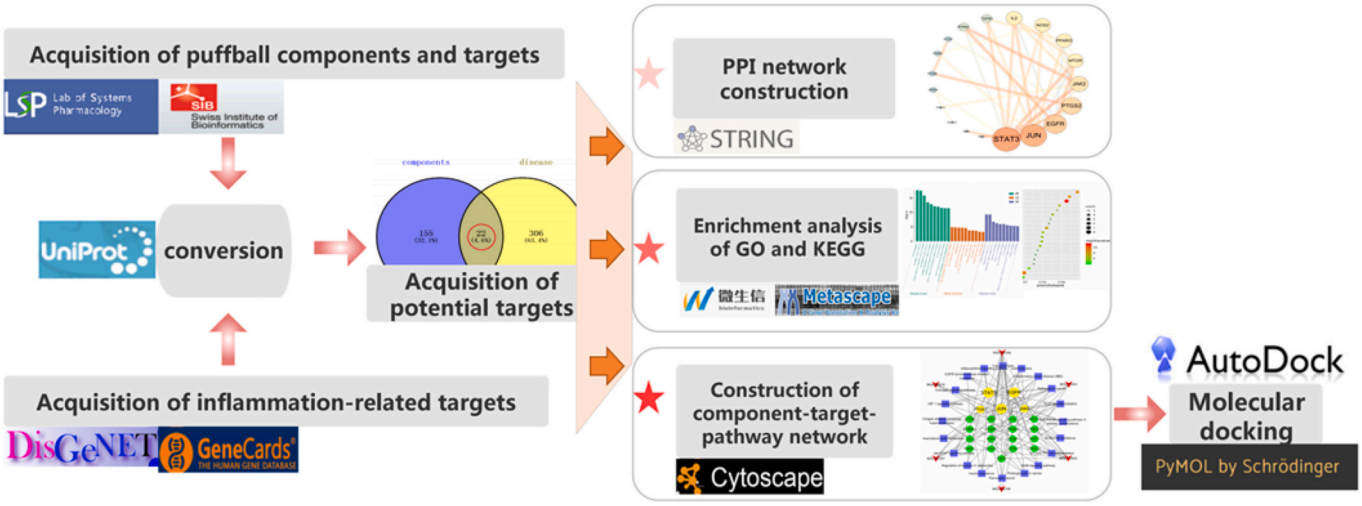

Fig. 1. The experimental process of this work. PPI, protein–protein interaction; GO, gene ontology.

2.6. Molecular docking

Three-dimensional structure of core target proteins used for molecular docking in the PPI network was obtained from the Protein Data Bank database (www.rcsb.org) [20]. The solvent and organic were removed through the PyMOL v2.4 open source molecular graphics tool [21]. The AutoDockTools v1.5.6 interface was used to add hydrogen and partial charges to the proteins and ligands [22]. The inflammation-related components were retrieved from the PubChem database in sdf format, and the energy was minimized using the MM2 force field in the Chem3D software. Grid maps were produced using the AutoGrid system. Docking parameters were set to 10 automated runs for a population size of 150, with a maximum number of energy evaluations of 2,500,000 for each docking experiment using the Lamarckian genetic algorithm as the search method and a maximum number of generations of 27,000. We used a gene mutation rate of 0.02 and a crossover rate of 0.8. The results of the docking computations were ranked by the binding affinity. Finally, we used the PyMOL molecular graphic system, Discovery Studio Visualizer to visualize the conformations and interactions between the ligands and the target proteins.

3. Results

3.1. Acquisition results of puffball active ingredients and targets

From the results of the TCMSP search and network construction, a total of 6 core anti-inflammatory ingredients were screened out of the 32 candidate ingredients obtained from the initial search, as shown in Table 1. Overall, a total of 236 ingredient-related targets were identified from the TCMSP and SwissTargetPrediction databases.

3.2. Acquisition of potential anti-inflammatory targets

A total of 328 inflammation-related targets were identified from DisGeNET and GeneCards. Fig. 2 shows the Venn diagram of the ingredient and inflammation targets, such that the common part of the two was regarded as to include potential anti-inflammatory targets.

3.3. Protein–protein interaction (PPI) network analysis results

The intersection targets were imported into the STRING data platform to obtain the PPI function network. Among the 22 intersection targets, 19 targets showed medium interactions (with a confidence score $\geq$ 0.4) in the PPI map generated by the STRING database. The targets of TRPV1, PARP1 and PDE5A that were not connected to the network were removed, and the Cytoscape software was used to perform the topological analysis to screen important core targets in the network. As shown in Fig. 3, the size and color settings of the node reflect the size of the degree, while the thickness and color settings of the edge reflect the combined score. The closer the color to red, the higher the corresponding value. The results of the topological analysis show that the three targets with a higher degree value among the anti-inflammatory targets of puffball are signal transducers and activators of transcription (STAT3, degree = 11), transcription factor AP-1 (JUN, degree = 10) and epidermal growth factor receptor (EGFR, degree = 8), which play a major role in the network.

Table 1
Bioactive ingredients of puffball.

Mol ID	Molecule Name	OB (%)	DL	PubChem ID	Number of targets
MOL000357	Sitogluside	20.63	0.62	5742590	15
MOL000358	beta-sitosterol	36.91	0.75	222284	70
MOL000798	ergosta-7,22-diene-3β-ol	43.51	0.72	102597281	23
MOL000816	ergosta-7,22-dien-3-one	44.88	0.72	101449383	12
MOL013051	ergosta-7,22-dien-3-one	44.88	0.72	129360209	13
MOL013053	melanin	26.4	0.67	6325610	75

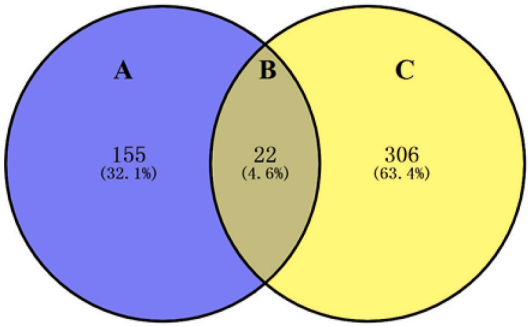

Fig. 2. The intersection of active ingredient targets(A+B) and inflammation targets(B+C) represented in 22 targets(B). This Venn diagram was built using the Venn Diagram Tool v2.1.0 platform.

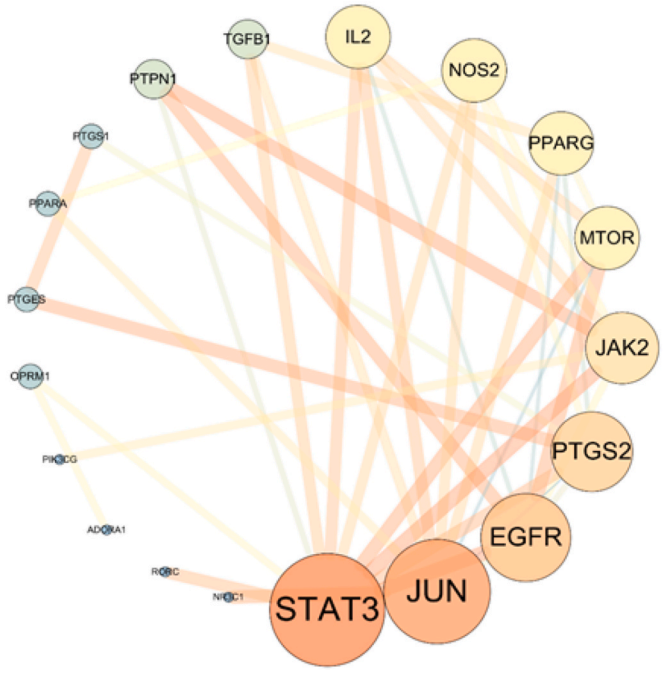

Fig. 3. PPI of the intersection targets. The size of the node represents the node degree of the target protein in the network. STAT3, Signal transducer and activator of transcription; JUN, Transcription factor AP-1; EGFR, epidermal growth factor receptor; PTGS2, prostaglandin endoperoxide synthase 2; JAK2, Tyrosine-protein kinase; MTOR, Serine/threonine-protein kinase mTOR; PPARG, Peroxisome proliferator-activated receptor gamma; NOS2, Nitric oxide synthase; IL-2, Interleukin 2; TGF-β1, Transforming growth factor beta-1; PTPN1, Tyrosine-protein phosphatase non-receptor type 1; PTGS1, Prostaglandin-endoperoxide synthase 1; PPARA, Peroxisome proliferator-activated receptor alpha; OPRM1, Mu opiate receptor; NR3C1, Nuclear receptor subfamily 3 group C member 1; RORC, RAR-related orphan receptor C; PIK3CG, PI3-kinase subunit gamma; ADORA1, Adenosine receptor A1. PPI network diagram was constructed via the cytoscape platform.

3.4. Molecular mechanism of the puffball anti-inflammatory effect

We performed a Metascape enrichment analysis of the 22 overlapping genes for GO and KEGG and obtained 650 biological processes (BP), 26 cellular ingredients (CC) and 38 molecular functions (MF)

under the GO entry. The p-value determines the results of the correlation test [23], the top ten of each category were sorted according to the LogP value from small to large, and a histogram was drawn (Fig. 4). The top rank belonged to the blood circulation, circulatory system process and cellular response to organic cyclic compound enrichment in BP's, RNA polymerase II transcription factor complex, transcription factor complex and post synapse enrichment in CC's and nuclear receptor activity, transcription factor activity, direct ligand regulated sequence-specific DNA binding and steroid hormone receptor activity in MF's.

The KEGG enrichment analysis on potential anti-inflammatory targets resulted in 34 signal pathways, which were classified according to the LogP value from small to large, of which 16 pathways were related to inflammation (Fig. 5). Among the inflammation-related pathways, Th17 cell differentiation signaling pathway has a high enrichment degree, and can be regarded as the main anti-inflammatory pathway. The meaning of concentration and p-value represents a highly correlated way in which active ingredients exert their anti-inflammatory effects.

3.5. Construction of the ingredient-target-pathway network

Using the Cytoscape software, a network graph with 48 nodes and 115 edges was constructed (Fig. 6). Specifically, the nodes included 6 active ingredients of puffball, 22 potential anti-inflammatory targets and 16 signal pathways. It can be seen from Fig. 6 that each active ingredient can correspond to multiple targets within multiple pathways, which fully reflects the multi-ingredient, multi-target and multi-pathway mechanism of the puffball anti-inflammatory effect. The ingredient attribute nodes in the network graph, MOL000358 (β-sitosterol, degree value 11) and MOL013053 (melanin, degree value 9), have the highest degree value; these can be considered as the core nodes in the network with a major anti-inflammatory effect. The core target STAT3 (degree value 11) is connected with multiple pathways, such as Th17 cell differentiation, pathways in cancer or inflammatory bowel disease (IBD), adipocytokine signaling pathway, etc. The active ingredient MOL000357 (Sitogluside) has a direct effect on STAT3.

3.6. Molecular docking results

We analyzed the stability of the docking conformation according to the lowest binding affinity. The lower the binding affinity, the more stable the binding of the compound to the ligand and the greater the interaction possibility. As shown in Table 2, the molecular docking binding affinity between the active ingredients of puffball and the key node STAT3 is less than −5.00 kJ/mol, while the most stable compound for docking binding is MOL000816 (ergosta-7,22-dien-3-one). We obtained 2D maps of the interactions and 3D forms of the hydrophobic pockets of the complex through Discovery Studio Visualizer (Fig. 7). Sitogluside has a docking binding affinity of −5.93 kJ/mol, with the carbonyl group at position 1 of the independent six-membered ring and the hydroxyl groups at positions 3,5 forming hydrogen bonding interactions with residues MET660 and LYS658, respectively, with the remainder bound in hydrophobic pockets surrounded by hydrophobic residues GLU638, VAL637, TYR640, THR641 and the polar residue TYR657. The beta-sitosterol has a docking binding affinity of −6.84 kJ/mol, ILE659, TYR640, TYR657, VAL637 and GLU638 forming a hydrophobic pocket encapsulating the cyclopenta[a]phenanthren portion, in addition to the hydrophobic interaction of TYR640 with the branched alkanes on cyclopentane. ergosta-7,22-diene-3β-ol has a docking binding affinity of −7.15 kJ/mol, TYR640, VAL637, GLN644, GLU638 etc. form a more pronounced hydrophobic pocket and MET648 also has a hydrophobic effect with the hydroxyl group on the ring. As two compounds with the same name but different structures, ergosta-7,22-dien-3-one showed the best docking performance and the form of action was more different, GLU638, GLN644 etc. formed hydrophobic pockets, in addition, MOL000816 with a docking binding affinity of −7.43 kJ/mol produced pi alkyl interactions with TYR640 and pi-sigma interactions with TYR640, while MOL013051 with a docking binding affinity of −7.62 kJ/mol produced pi-alkyl interactions with TRP623,TYR657 and TYR640. For the docking fraction of −6.16 melanin formed conventional hydrogen bond with LYS658, TYR640, GLN644,GLU638, alkyl interaction with ILE659 and van der Waals forces with MET660,

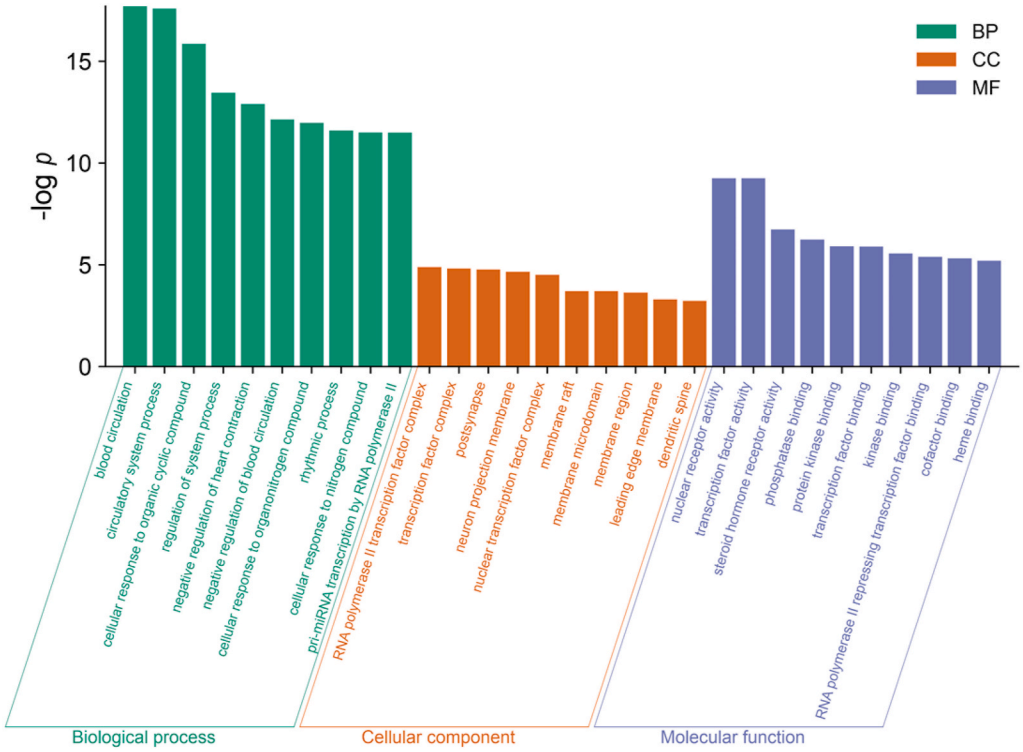

Fig. 4. GO function analysis histogram. BP is marked by dark cyan, CC is marked by sienna and MF is marked by steel blue. The bar chart was constructed through the bioinformatics platform. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

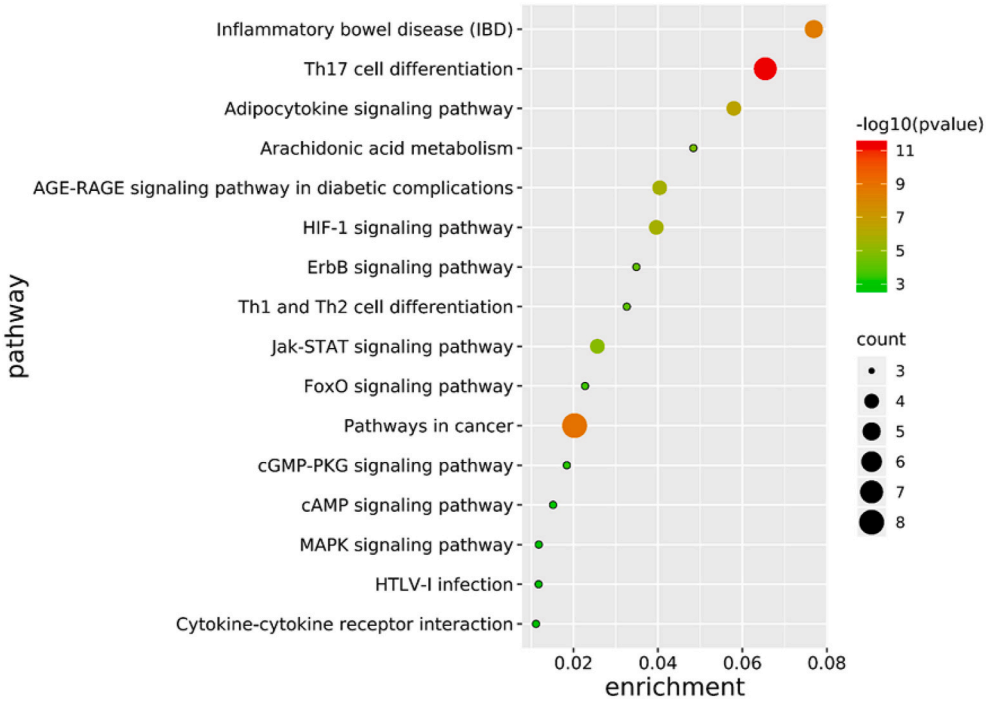

Fig. 5. Dot plot of the KEGG pathway enrichment analysis. The horizontal axis represents the enrichment rate of the input genes in the pathway, while the vertical axis represents the pathway name. The color scale indicates different thresholds of the p-value, and the size of the dot indicates the number of genes corresponding to each term. The bubble map was constructed through the bioinformatics platform. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

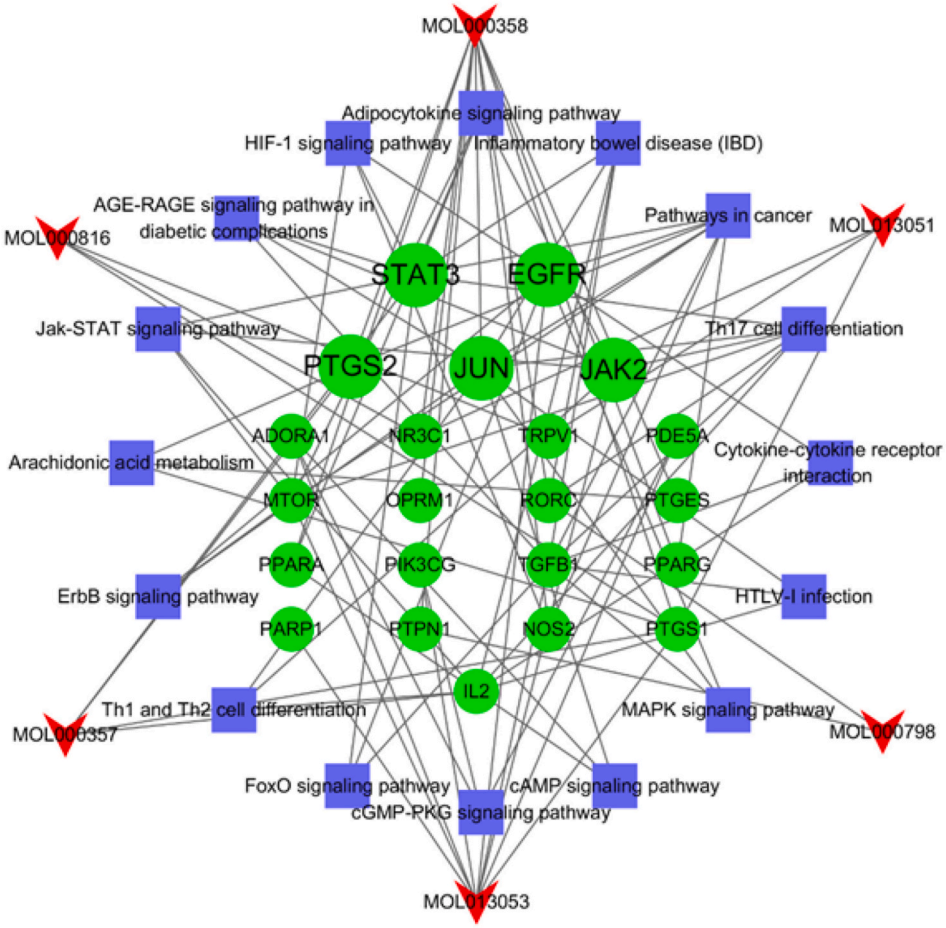

Fig. 6. Ingredient-target-pathway network diagram. The active ingredient is displayed as a red V shape, the intersection target is displayed as a green circle, and the signal path is displayed as a blue rounded rectangle. This network diagram was constructed via the cytoscape platform. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Table 2
Molecular docking results of active ingredients and STAT3.*

Ingredients	Name	3D coordinates of active sites(x, y, z)	Binding affinity (kJ/mol)	Hydrophobic interaction	Hydrogen bond
MOL000357	Sitogluside	2.9742, 2.6772, −1.0782	−5.93	+	+
MOL000358	beta-sitosterol	4.5555, 3.4521, −0.5393	−6.84	+	–
MOL000798	ergosta-7,22-diene-3β-ol	14.6244, −16.1294, 0.7104	−7.15	+	–
MOL000816	ergosta-7,22-dien-3-one	7.4163, 3.978, 0.3019	−7.62	+	–
MOL013051	ergosta-7,22-dien-3-one	14.9214, −16.2275, 0.2738	−7.43	+	–
MOL013053	melanin	13.8995, −15.6341, 0.1721	−6.16	+	+

* "+" indicates the presence of the interaction and "−" indicates its absence.

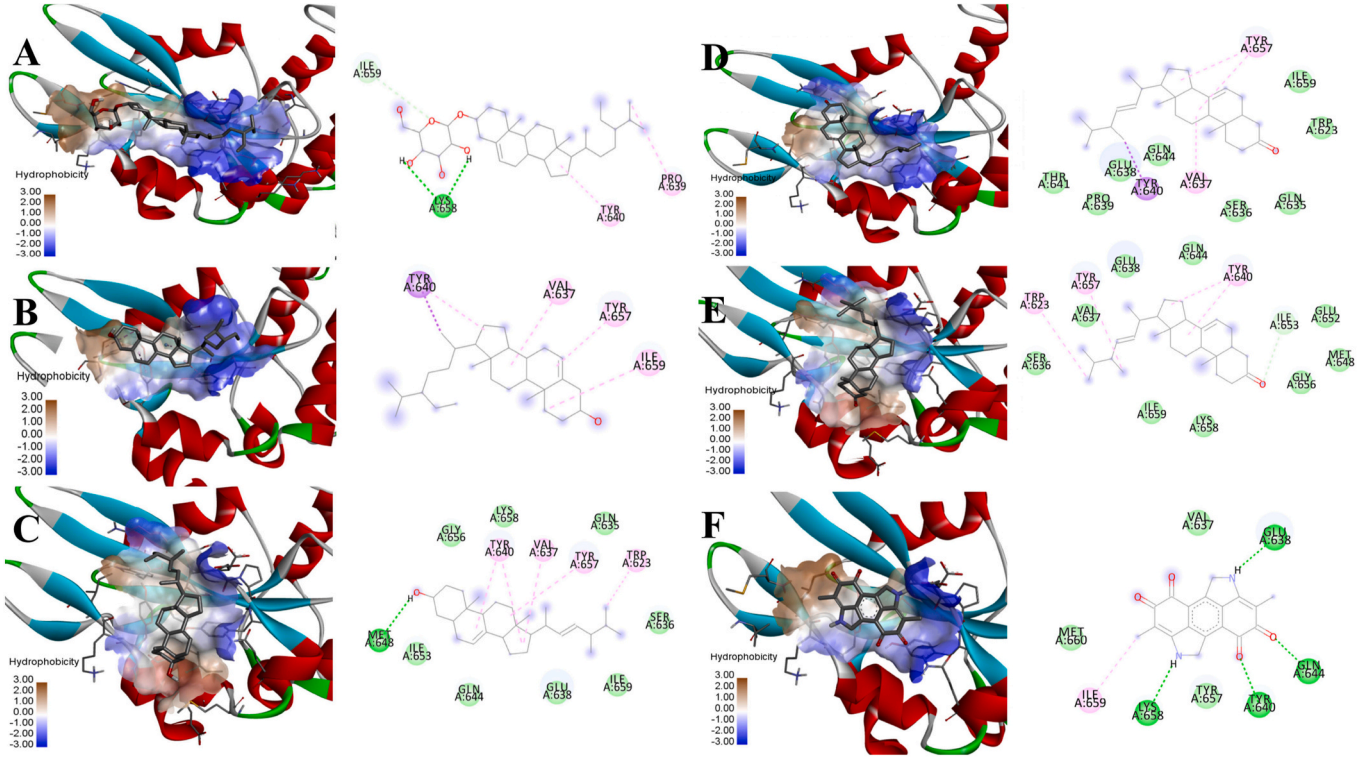

Fig. 7. Hydrophobic binding poses of 6 core compounds to STAT3 and 2D interaction diagrams. (A) Sitogluside, (B) beta-sitosterol, (C) ergosta-7,22-diene-3β-ol, (D) ergosta-7,22-dien-3-one (MOL000816), (E) ergosta-7,22-dien-3-one (MOL013051), (F) melanin. This image was created with Discovery Studio Visualizer.

VAL637. The non-covalent interactions are mainly hydrophobic interactions, and a few hydrogen bonds exist.

4. Discussion

Puffball is included in the Chinese Pharmacopoeia 2015 edition as one of the few medicinal fungi, which has the effect of reducing inflammation and hemostasis and is widely used in the clinical practice to treat sore throat, cough, aphasia, vomiting and hemoptysis. Based on existing research, we used the method of network pharmacology to explore the potential mechanism of the puffball fungi antiinflammation effect; our analysis included the related targets, components, biological processes and pathways.

The core target in the PPI network was found to be STAT3, which can be activated by EGFR, transforming growth factor beta (TGF-β) [24], interleukin 6 (IL-6) and other cytokines and growth factors through phosphorylation [25]. Tumor necrosis and intrinsic signaling events lead to the recruitment of bone marrow-derived cells and cytokines and cause chemotaxis. The secretion of chemical factors and inflammation are among the prerequisites for the tumor cells to invade and grow far away, and the inflammatory mechanism may further support the implantation and growth of tumor cells. However, STAT3 plays an important role in liver inflammation and maintaining homeostasis [26]. As a regulator of the inflammatory response, STAT3 dimer is destroyed by LOXL3 deacetylation and oxidation of the lysine residues, which inhibits its transcriptional activity, thereby regulating the differentiation of $CD4^+$ T cells into helper T cells (Th17) or regulatory T cells to regulate the inflammation [27,28].

Multi-part network modeling is unequivocally helpful for the study of mechanisms of action in traditional medicine [29]. It can be observed from the interaction network diagram of potential active ingredients, targets and pathways that 22 target proteins correspond to 6 potentially related active ingredients, among which β-sitosterol and melanin are the core active ingredients in this network. β-sitosterol is one of the components of plant sterols, and it is widely present in various plant seeds, such as plant oils and nuts [30]. Studies have shown that β-sitosterol has a wide range of anti-inflammatory effects on peritoneal macrophages and other surrounding tissues. Affecting microglial cell activity by inhibiting p38, ERK, and NF-κB pathway activation reduces lipopolysaccharide-induced tumor necrosis factor-α (TNF-α) expression [9]. Another component with potential anti-inflammatory activity in the network is melanin, which is a high-molecular-weight polymer of tyrosinase, with antioxidants and cellular protection features. Grape melanin has inhibitory effects on adjuvant-induced arthritis in rats [31], as a natural product with anti-inflammatory and immune regulatory effects, it can reduce the level of IL-1 protein in the serum of

non-alcoholic liver disease model rats and set the anti-inflammatory cytokines levels back to normal [32]. Sitogluside in combination with beta-sitosterol regulates the proliferation of human peripheral blood lymphocytes and significantly enhances NK cell activity [33]. The molecular docking results showed that the core target STAT3 docked well with the 6 active compounds in the puffball, with ergosta-7, 22-dien-3-one and its isomers being the most stable compound bound to STAT3 if viewed purely on the basis of affinity. Ergosta-7, 22-dien-3-one intervenes in the inflammatory response and regulates the expression of IL-1 and IL-6 [34]. Similarly, ergosta-7,22-dien-3-ol significantly reduces LPS-induced NO levels and cell viability damage [35].

In order to explore the action mechanism of puffball, we focused on analyzing the biological processes and signaling pathways that are associated with inflammation-related diseases. BP's results demonstrate that the puffball anti-inflammatory effect mainly focuses on blood circulation and circulatory system process, which shows that the above-mentioned processes may be related to inflammation. For organic ring compounds, certain Chinese patent medicines can promote blood circulation and eliminate blood stasis by inhibiting inflammation-related molecules [12]. Th17 is a subset of T cells that can secrete IL-17, TGF-β, IL-6, IL-23 and IL-21, all of which can promote the differentiation of Th17 cells with low levels. In patients with Th17 coronary heart disease, IL-5 inhibits the differentiation of Th17 cells [13]. In addition, Th17 cells are associated with inflammatory bowel disease and central nervous system inflammation [14, 15].

5. Conclusions

In summary, the way puffball exerts its anti-inflammatory effect may depend on active ingredients, such as β-sitosterol and melanin, acting on certain targets, such as STAT3 and EGFR, and inhibiting the differentiation of Th17 cells through biological processes, such as blood circulation and circulatory system process. Due to the limitations of computational methods in systems biology, the actual treatment effect cannot be guaranteed to be consistent with the predicted results. As a follow-up of this study, we intend to conduct *in vivo* and *in vitro* experiments on related target pathways to verify the predicted results of network pharmacology and molecular docking.

CRediT authorship contribution statement

Hongshi Bu: Conceptualization, Investigation, Methodology, Writing-original draft.

Xiaohuan Li: Conceptualization, Visualization.

Liming Hu: Software.

Jia Wang: Writing-original draft.

Yang Li: Formal analysis, Validation.

Tianyi Zhao: Resources, Writing-review&editing.

Huan Wang: Resources, Project administration.

Shumin Wang: Supervision, Funding cquisition, acquisition, Resources, Project administration.

Declaration of competing interest

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.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.imu.2021.100549.

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