

## RESEARCH ARTICLE

# Systematic characterization of the effective constituents and molecular mechanisms of *Ardisiae Japonicae Herba* using UPLC-Orbitrap Fusion MS and network pharmacology

Suxiang Feng<sup>1,2</sup>\*, Jie Yuan<sup>2,3</sup>, Di Zhao<sup>1,2</sup>, Rongrong Li<sup>2,3</sup>, Xuefang Liu<sup>1,2</sup>, Yange Tian<sup>1,2</sup>, Jiansheng Li<sup>2,4</sup>\*

**1** Academy of Chinese Medicine Sciences, Henan University of Chinese Medicine, Zhengzhou, Henan, China, **2** Collaborative Innovation Center for Chinese Medicine and Respiratory Diseases co-constructed by Henan province & Education Ministry of P. R. China, Zhengzhou, Henan, China, **3** College of Pharmacy, Henan University of Chinese Medicine, Zhengzhou, Henan, China, **4** The First Affiliated Hospital, Henan University of Chinese Medicine, Zhengzhou, Henan, China

\* These authors contributed equally to this work.

\* [fengsx221@163.com](mailto:fengsx221@163.com) (SF); [li\\_js8@163.com](mailto:li_js8@163.com) (JL)



## OPEN ACCESS

**Citation:** Feng S, Yuan J, Zhao D, Li R, Liu X, Tian Y, et al. (2022) Systematic characterization of the effective constituents and molecular mechanisms of *Ardisiae Japonicae Herba* using UPLC-Orbitrap Fusion MS and network pharmacology. *PLoS ONE* 17(6): e0269087. <https://doi.org/10.1371/journal.pone.0269087>

**Editor:** Chun-Hua Wang, Foshan University, CHINA

**Received:** March 9, 2022

**Accepted:** May 14, 2022

**Published:** June 15, 2022

**Copyright:** © 2022 Feng et al. This is an open access article distributed under the terms of the [Creative Commons Attribution License](https://creativecommons.org/licenses/by/4.0/), which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

**Data Availability Statement:** All relevant data are within the paper and its [Supporting Information](#) files.

**Funding:** This work was supported by Henan Province science and technology key projects Fund (222102310375), Henan University of Chinese Medicine In-school Support projects Fund (MP2021-14) and Henan scientific research of Chinese Medicine special projects Fund (20-21ZY2153).

## Abstract

### Objective

*Ardisiae Japonicae Herba* (AJH), the dried whole herb of *Ardisia japonica* (Thunb.) Blume [Primulaceae], has been used in treating chronic obstructive pulmonary disease (COPD) in China. However, the material basis and molecular mechanisms of AJH against COPD remain unclear. Therefore, in this study, we attempt to establish a systematic approach to elucidate the material basis and molecular mechanisms through compound identification, network analysis, molecular docking, and experimental validation.

### Methods

Ultra-high performance liquid chromatography-Orbitrap Fusion mass spectrometry (UPLC-Orbitrap Fusion MS) was used to characterize the chemical compounds of AJH. The SwissTargetPrediction, String and Metascape databases were selected for network pharmacology analysis, including target prediction, protein-protein interaction (PPI) network analysis, GO and KEGG pathway enrichment analysis. Cytoscape 3.7.2 software was used to construct a component-target-pathway network to screen out the main active compounds. Auto-dock Vina software was used to verify the affinity between the key compounds and targets. TNF- $\alpha$ -stimulated A549 cell inflammation model was built to further verify the anti-inflammatory effects of active compounds.

### Results

Altogether, 236 compounds were identified in AJH, including 33 flavonoids, 21 Phenylpropanoids, 46 terpenes, 7 quinones, 27 steroids, 71 carboxylic acids and 31 other compounds. Among them, 41 compounds were selected as the key active constituents, which might

**Competing interests:** The authors have declared that no competing interests exist.

**Abbreviations:** AJH, *Ardisiae Japonicae Herba*; COPD, chronic obstructive pulmonary disease; UPLC, Orbitrap Fusion MS, Ultra-high performance liquid chromatography-Orbitrap Fusion mass spectrometry; MTT, Thiazolyl Blue Tetrazolium Bromide; FBS, Fetal bovine serum; ELISA, Enzyme-linked immunosorbent assay; PPI, protein-protein interaction; GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; Bc, betweenness centrality; Cc, closeness centrality; BP, biological process; MF, molecular function; CC, cellular component; TICs, Total ion chromatograms;  $t_R$ , retention time; AKT1, AKT Serine/Threonine Kinase 1, IL6, Interleukin-6; TNF, Tumor necrosis factor; TP53, Tumor Protein P53; MMP9, Matrix Metalloproteinase 9.

exhibit therapeutic effects against COPD by modulating 65 corresponding targets primarily involved in inflammation/metabolism/immune-related pathways. The results of molecular docking showed that the key compounds could spontaneously bind to the receptor proteins with a strong binding ability. Finally, the anti-inflammatory effects of the three active compounds were validated with the decreased levels of Interleukin-6 (IL-6) and Matrix Metalloproteinase 9 (MMP9) in TNF- $\alpha$ -induced A549 cells model.

## Conclusion

This study clarified that AJH may exert therapeutic actions for COPD via regulating inflammation/immune/metabolism-related pathways using UPLC-Orbitrap Fusion MS technology combined with network pharmacology for the first time. This study had a deeper exploration of the chemical components and pharmacological activities in AJH, which provided a reference for the further study and clinical application of AJH in the treatment of COPD.

## Introduction

Chronic obstructive pulmonary disease (COPD) is a serious chronic inflammatory disease mainly characterized by persistent respiratory symptoms and irreversible airflow restriction caused by significant exposure to harmful gases or particles [1]. COPD is considered as the third most life-threatening disease worldwide [2]. Tobacco smoking, biomass fuel exposure, air pollution and host factors are risk factors for COPD [3–5]. Chronic inflammation, which leads to narrow small airways, structural changes and destruction of lung parenchyma, is the primary cause of COPD [6,7]. At present, the commonly used drugs of COPD are bronchodilators, beta 2-agonists, anti-inflammatory agents (such as glucocorticoids, inhaled corticosteroids, antibiotics etc.) and anti-muscarinic drugs in clinical treatment. However, these drugs have limited aftereffect and many side effect [8–10]. In recent years, Chinese medicine (CM) has been widely used in the treatment of COPD because of its remarkable curative effect of reducing the symptoms of cough, phlegm, and delaying the decline of lung function [11]. Pharmacological studies revealed that Chinese medicine (CM) could be applied to treat COPD by regulating various signaling pathways such as JAK2/STAT3, PI3K/Akt/NF-kappaB and EGFR-PI3K-AKT signal pathways, et. al. [12–18].

*Ardisiae Japonicae Herba* (AJH) is the whole herb of *Ardisia japonica* (Thunb.) Blume [Primulaceae], which has anti-inflammatory [19], anti-cancer [20,21], anti-bacterial [22], and anti-viral effects [23,24], has been widely used in clinical treatment of respiratory tract with reliable curative effect, such as chronic bronchitis, pulmonary tuberculosis, upper respiratory tract inflammation and COPD [25–30]. Pharmacodynamic studies clarified that AJH may directly down-regulate the expression of Smad3 and TGF- $\beta$ 1, or inhibit the TGF- $\beta$ 1/Smad3 signaling pathway by increasing the expression of DCN to achieve airway remodeling intervention in COPD rats [31,32]. Moreover, the clinical application of AJH has a history of hundred years and has been recorded in a variety of classical literature [33]. In *Bencao Shiyi* and *Fenlei Caoyao Xing*, AJH was thought to have the effects of reducing swelling, resolving phlegm and relieving cough, and commonly used for cough, wheeze, blood in sputum, chronic bronchitis and damp heat jaundice. Many researches have investigated the capacity of AJH for preventing respiratory diseases, and indicated the efficacy of AJH in the prevention of COPD [34–36]. The main chemical components isolated from AJH are flavonoids, coumarins,

triterpenes, phenols, polysaccharides and essential oils [37–40]. Of these, flavonoids and phenylpropanoids have the activities of anti-inflammation and anti-oxidation by inhibiting the expression of TNF- $\alpha$ , IL-6 [41–43]. Chang et al. isolated and characterized twenty-one triterpene saponins from AJH [21]. Huang et al. isolated quercetin, Ardisinol I and Ardisinol II from AJH [44]. In addition, fifteen compounds from AJH were investigated by K.Y. Yu through HPLC-QTOF-MS [45]. However, there are limited research to investigate the systematical material basis and the molecular mechanisms of AJH, more studies are urgently needed to clarify the bioactive components in AJH, and further clarify its medicinal and clinical application values.

In this study, we developed a comprehensive method to clarify the material basis and molecular mechanisms of AJH for treating COPD by combining UPLC-Orbitrap Fusion MS analysis and network pharmacology. UPLC-Orbitrap Fusion MS technology was performed to characterize the chemical profile of AJH. Network pharmacology and molecular docking were used to conduct data mining on its chemical components to screen the bioactive components of AJH and predict the underlying mechanisms relevant to COPD treatment, and the effects of potentially bioactive components were further validated in TNF- $\alpha$ -induced A549 cells in vitro. The protocol is shown in Fig 1.

## Materials and methods

### Drugs and reagents

Methanol and acetonitrile (HPLC-grade) were obtained from TEDIA (Fairfield, OH, USA), formic acid (MS-grade) was obtained from Thermo Fisher (Waltham, USA). All other chemicals and reagents were analytical grade. Ultra-pure water was obtained through a Milli-QPOD water purification system (Millipore, Merck, USA). *Ardisiae Japonicae* Herba pieces were obtained from Jiangxi Zhangshu Tianqitang Traditional Chinese Medicine Co., Ltd (Jiangxi, China, lot no.: 20190804, 20190728, 20190801), and authenticated by Professor Sui-Qing Chen, Henan University of Chinese Medicine, Zhengzhou, China. The voucher specimens were stored at Scientific Research Center, Henan University of Chinese Medicine, Zhengzhou, China.

As reference standards, twenty-nine pure compounds were used. Twenty-six reference standards, including Methyl salicylate (CHB190925), Palmitic acid (CHB181119), Kaempferol (CHB190127), Embelin (CHB190829), Hydroxygenkwanin (CHB180611), Rapanone (CHB190723), Epigallocatechin gallate (CHB180307), Myricitrin (CHB180611), Isoeugenol (CHB190830), Gallic acid (CHB180114), Ethyl gallate (CHB180116), Caryophyllene oxide (CHB180624), Myristic acid (CHB190102), Taxifolin (CHB190105), Myricetin (CHB180614), Astilbin (CHB190107), Scopoletin (CHB180210), Fraxetin (CHB180112), Cardamomin (CHB180312), Eriodictyol (CHB190123), Cianidanol (CHB180809), Galocatechin (CHB200915), Arctigenin (CHB180131), Epicatechin gallate (CHB180305), Rutin (CHB190110), Nictoflorin (CHB190102) were obtained from Chengdu Cloma Biological Technology Co., LTD (Chengdu, China); Quercetin (MUST-20101104) and Luteolin (MUST-14050411) were obtained from Chengdu Must Biological Technology Co., Ltd. (Chengdu, China); Bergenin (111532–201604) was obtained from China Food and Drug Testing Institute (Beijing, China), and the purity of each compound was  $\geq 98\%$  by HPLC analysis.

Roswell Park Memorial Institute (RPMI) 1640 medium, Radio Immunoprecipitation Assay (RIPA) Lysis Buffer, Thiazolyl Blue Tetrazolium Bromide (MTT) and trypsin-EDTA were obtained from Beijing solarbio science & technology co., ltd (Beijing, China), Fetal bovine serum (FBS) was purchased from Zhejiang Tianhang Biotechnology Co., Ltd. (Zhejiang, China), the ELISA kits for IL-6 and MMP9 (the sensitivity was  $< 0.3$  pg/mL and 5 pg/mL, respectively) were obtained from BOSTER Biological Technology co., ltd (Wuhan, China).

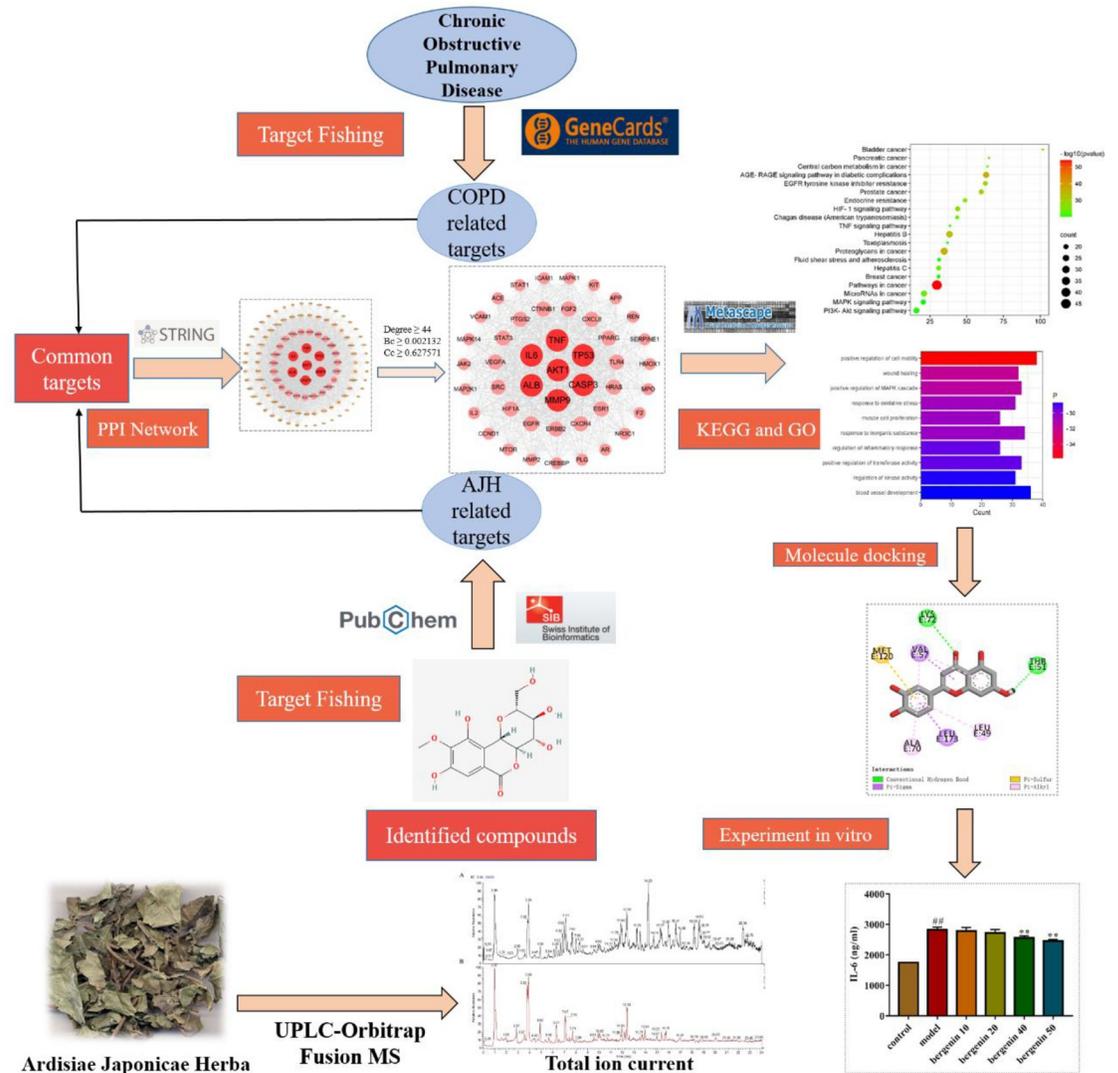


Fig 1. The whole framework of the study.

<https://doi.org/10.1371/journal.pone.0269087.g001>

### Qualitative analysis of AJH

**Sample preparation.** AJH powder (1.0 g) was accurately weighed and added in a 150 mL round-bottom flask with 100 mL methanol, mixed well, reflux-heated for 1h twice and filtered. Obtained filtrates were combined and dried by centrifugation. The residue was redissolved with methanol (HPLC-grade) and diluted to 10 mL volumetric flask. The solution was filtered using 0.22 μm microporous membrane and stored at 4 °C before analysis.

**Standard solution preparation.** Accurately weigh an appropriate amount of each reference standard into a 10 mL volumetric flask, add methanol to dissolve and dilute to the mark, single-component reference stock solutions were obtained and stored at 4 °C before use. Then, proper amounts of reserve solutions were added to a 25 mL volumetric flask, methanol (HPLC-grade) was added to reach scale, the mixed reference solution was obtained. The solutions were filtered through 0.22 μm microporous membranes and stored at 4 °C before use.

**Chromatographic and mass spectrometric conditions.** Chemical identification was conducted on an ultra-performance liquid chromatography-orbitrap fusion mass

(UPLC-Orbitrap Fusion MS) (Thermo Fisher, Waltham, USA) equipped with an electrospray ionization source (ESI). Thermo Scientific Accucore<sup>TM</sup>C<sub>18</sub> column (2.1 mm × 100 mm, 2.6 μm) was applied at a constant flow rate of 0.2 mL/min at 30°C. The mobile phase was made up of methanol (A) and 0.1% formic acid aqueous solution (B), and the gradient elution was as follows: 0~2 min (93~75% B), 2~10 min (75~20% B), 10~15 min (16~12% B), 15~20 min (12~10% B), 22~24 min (10~0% B). The injection volume was 5 μL.

The acquisition parameters of orbitrap fusion were set as follows: Vaporizer temperature: 275°C; Ion Transfer Tube temperature: 300°C; Carrier gas (N<sub>2</sub>); Sheath gas and Aux gas flow were set as 35 arb and 5 arb, respectively; spray voltage were set at 3.5 kV, 2.8 kV (positive and negative ion mode, respectively); collision energy was 35–55 eV. The sample was analyzed in both positive and negative ions Full MS/dd-MS<sub>2</sub> modes with the first-level full scan (resolution: 120,000) and the second-level scan (resolution: 60,000), and the mass range was recorded from m/z 100–1200.

**Data processing.** The chemical compounds of AJH were collected from existing databases, including the Traditional Chinese Medicine Systems Pharmacology Database and the Analysis Platform (TCMSP, <http://lsp.nwu.edu.cn/tcmsp.php>), Traditional Chinese medicine integrative database (TCMID, <http://47.100.169.139:8000/tcmid/>) and SciFinder (<https://scifinder.cas.org/>) database. Then, a database of AJH ingredients was established. AJH was identified by the optimized UPLC-Orbitrap Fusion MS method. The possible chemical composition (with an error of less than 5 ppm) was determined using Xcalibur based on map data, precise molecular weight and resulting fragment ions. The data was analyzed using Compound Discoverer software to integrate ion peak information, attribution information, ChemSpider, mzCloud and other databases of characteristic fragments integrated with existing chemical composition information reports. The structure of compounds was inferred using the cracking prediction of MassFrontier and its cracking rule.

## Network pharmacology

**Target prediction.** The molecular structures of components identified in AJH were downloaded from PubChem database (<https://pubchem.ncbi.nlm.nih.gov/>) and saved in SDF format. These SDF documents were imported to SwissTargetPrediction database (<http://www.swisstargetprediction.ch/>) for target prediction. For more accurate prediction, relevant parameters were set (probability ≥ 0.1). In addition, the COPD-related targets were selected from GeneCards database (<https://www.genecards.org/>) by using “Chronic Obstructive Pulmonary Disease” as the keywords. Then, the overlapping of AJH prediction targets and COPD-related targets was selected as potential targets.

**PPI network construction.** The PPI network construction and analysis were performed on Cytoscape 3.7.2 software. Potential targets screened above were added to STRING database. The screening condition used was “Homo sapiens,” and the other parameters were set by default, a protein-protein interaction (PPI) network was built. Cytoscape 3.7.2 was used for topological analysis of PPI network to realize its visualization. Using three topological parameters, betweenness centrality (Bc), closeness centrality (Cc) and degree, a topology analysis of the PPI network was performed to determine hub genes for further analysis.

**Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis.** Potential targets identified above were uploaded into Metascape database (<https://metascape.org/gp/index.html>) for GO and KEGG analysis to obtain the information of pathways. Bioinformatics Data analysis and Visualization online platform (<http://www.bioinformatics.com.cn/>) was used to conduct GO and KEGG pathway analysis and visualization. In the process, the organism was set as “Homo sapiens”, and significance level was p Ho0.01.

**Construction of component-target-pathway network.** To further clarify the relationship among components, targets and pathways, Cytoscape 3.7.2 software was used to build a component-target-pathway network. The core compound nodes were obtained based on the three parameters: degree, Bc and Cc.

**Molecular docking.** The affinity between the core compounds and targets was verified by Autodock Vina software. The 3D structures of core targets in the first five degrees of AJH in COPD treatment were obtained from the RCSB PDB database (<https://www.rcsb.org/>). Pymol 1.8 software was used to remove water molecules and separate the primary ligand. After saving, the structure was imported into Autodock Tools 1.5.6 and saved in “pdbqt” format. Chem 3D software was used to download the mol2 files of the top 10 core compounds, and they were imported into Autodock Tools 1.5.6 and saved in pdbqt format. Finally, docking was carried out through Autodock vina 1.1.2. Discovery Studio 4.5 Client was used to visualize docking results and establish a docking interaction model diagram.

## Experimental validation in vitro

**Cell culture.** A549 cells were from Cell Resource Center of Shanghai Academy of Biological Sciences, Chinese Academy of Sciences (Shanghai, China). Cells were cultured in RPMI 1640 complete medium (containing 10% FBS) and kept in an incubator at 37°C and 5% CO<sub>2</sub>. When the cells were overgrown, they were digested with 0.25% trypsin-EDTA, collected, sub-cultured or tested.

**Cell viability assay.** A549 cells ( $1 \times 10^4$  cells/well) were planted into 96-well plates ( $n = 6$ ), and cultured at 37°C and 5% CO<sub>2</sub> for 24 hours. Then the cells were treated with PBS or different dosages of bergenin (10, 20, 40, 50 µg/mL), luteolin (0.1, 0.2, 0.4, 0.8 µg/mL) and kaempferol (2.5, 5, 10, 20 µg/mL). After incubation for 24 h, added 10 µl MTT (0.5 mg/mL) to each well, and cultured for 4h. Using DMSO (100 µL/well) to replace cell culture. The absorbance was measured at 570 nm using a microplate reader.

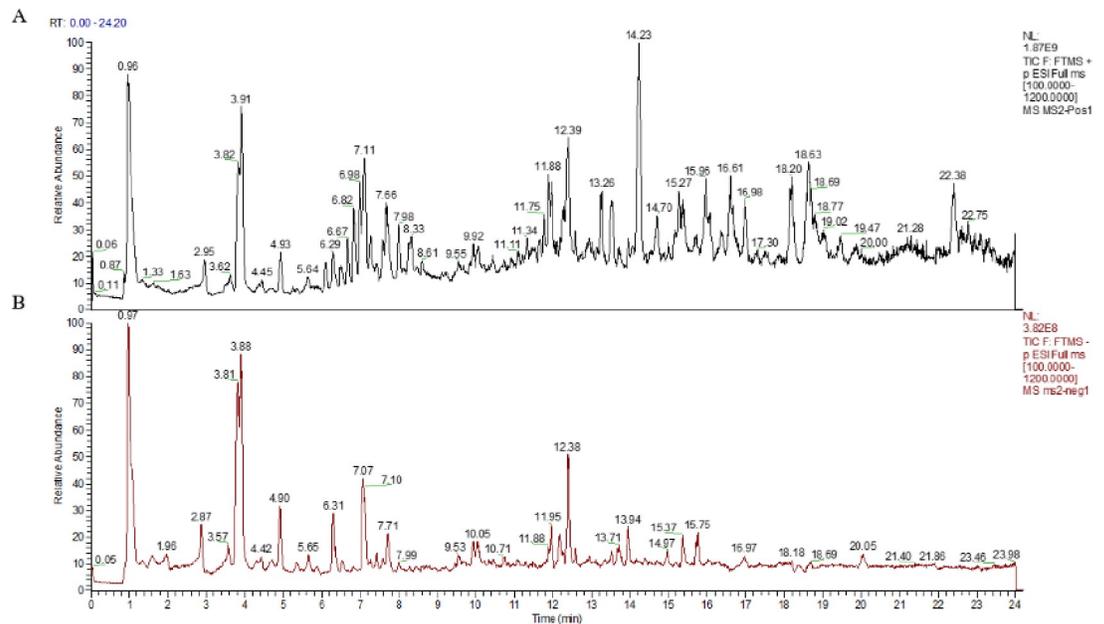
**IL-6, MMP9 expression.** A549 cells ( $2 \times 10^6$  cells/plate) were incubated in 6-well plates ( $n = 6$ ) for 24 h, incubated with TNF- $\alpha$  (10ng/mL) for 24 h. Then treated with bergenin (10, 20, 40, 50 µg/mL), luteolin (0.1, 0.2, 0.4, 0.8 µg/mL), and kaempferol (2.5, 5, 10, 20 µg/mL), and incubated at 37°C with 5% CO<sub>2</sub> in a humidified atmosphere for 48 h. Supernatant was collected, centrifuged and stored at -80°C for testing. RIPA Lysis Buffer 300 µL/well was added to lysate cells. Ten minutes later, the lysate was collected, the supernatant was collected by centrifugation, and levels of IL-6, MMP9 in the supernatant were determined according to the instructions of ELISA kits.

**Statistical analysis.** IBM SPASS Statistic 26 software was applied for statistical analysis, and data was expressed as mean  $\pm$  standard deviation (SD). Analysis of variance (ANOVA) was used to analyze the differences between multiple groups. P-values < 0.05 was considered to be statistically significant.

## Results

### Characterization and identification of components in AJH

Total ion chromatograms (TICs) of AJH are presented in Fig 2 (TICs of the three batches of AJH are as shown in S1 Fig), 236 chemical compounds (162 in ESI<sup>+</sup> and 74 in ESI<sup>-</sup>) were identified in AJH, including 33 flavonoids (F), 21 Phenylpropanoids (P), 46 terpenes (T), 7 quinones (Q), 27 steroids (S), 71 carboxylic acids (C) and 31 others (O) based on the reference standards, ingredients database, mass fragment mode and mass spectrum library with UPLC-Orbitrap Fusion MS system. Among them, 29 compounds (F4, F6, F8, F10, F13-14, F16, F19, F22-23, F25-28, F31, P4, P7, P13, P20, T15, T26, Q4, Q6, C5, C16, C20, O19-20 and



**Fig 2.** The total ion chromatograms (TICs) of AJH (A. positive ion mode. B. negative ion mode).

<https://doi.org/10.1371/journal.pone.0269087.g002>

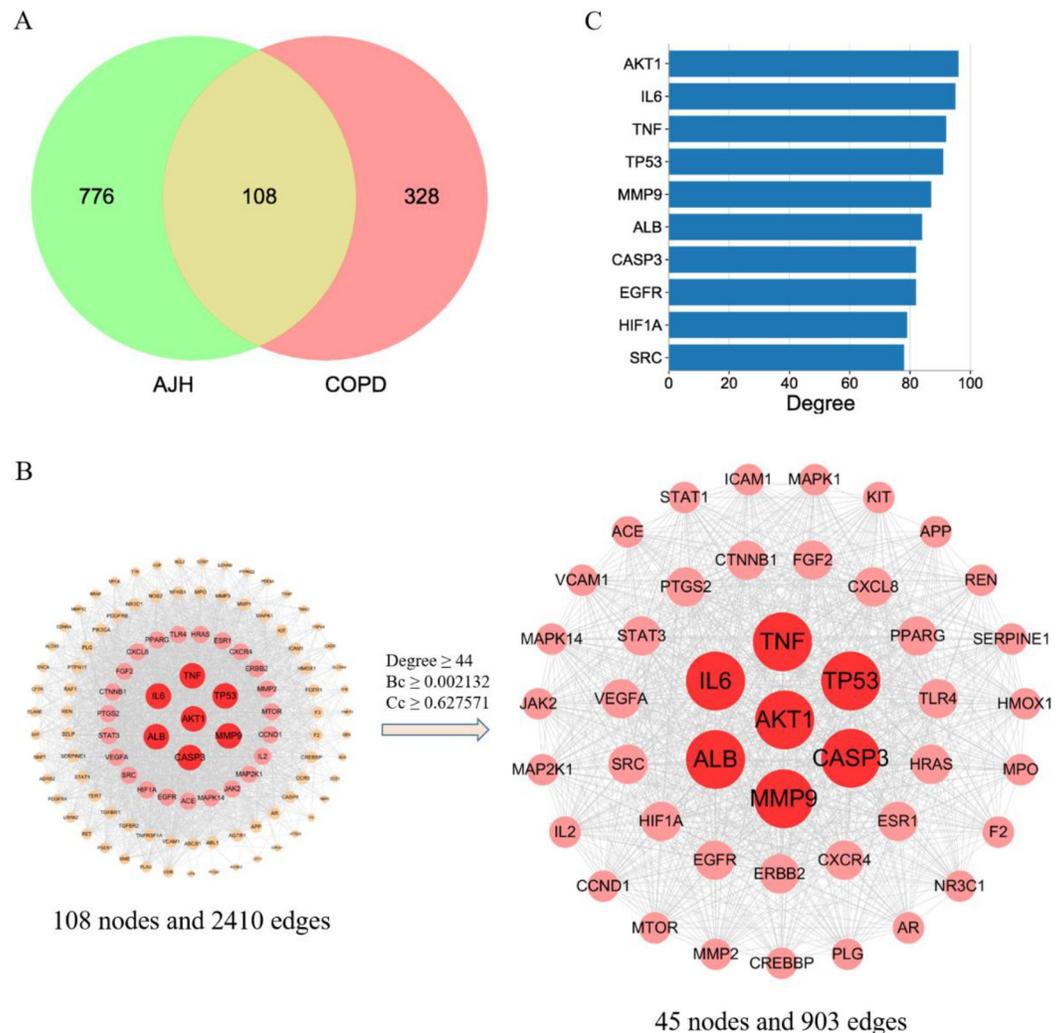
O22) were identified by comparing with the reference standards. The details of 236 components, including compounds name, formula, retention time (tR), and fragments ions are presented in [S1 Table](#).

## Network pharmacology analysis

**Potential targets prediction.** After removing repeated targets, 884 targets were collected based on 236 primarily identified compounds using the SwissTargetPrediction database ([S2 Table](#)). Only 207 compounds had relevant targets when the probability  $\geq 0.1$ . In addition, 6412 COPD-related genes were collected from the GeneCards database. To ensure the precision of target collection, based on the parameter of “Relevance score,” the index above the third median value was selected as the key index, and 436 COPD-related targets were obtained ([S2 Table](#)). After taking the intersection of 884 predicted targets and 436 COPD-related targets, 108 common targets were obtained which means that they could be the main potential targets of AJH in COPD treatment (Fig 3A)

**Construction and analysis of the PPI network.** After inputting these common targets into STRING database, we obtained a PPI network comprising 108 nodes and 2410 edges (Fig 3B). Using the three parameters degree, Bc and Cc, the index above the median value was selected as the key index, the threshold value of screening was degree  $\geq 44$ , Bc and Cc, the index above the median value was selected as the [Fig 3](#). In addition, genes in the top ten degree were selected ([Fig 3C](#)), the results showed that AKT Serine/Threonine Kinase 1 (AKT1), Interleukin-6 (IL6), Tumor necrosis factor (TNF), Tumor Protein P53 (TP53) and Matrix Metalloproteinase 9 (MMP9) were the most vital targets of PPI, which may be the main targets of AJH in COPD treatment.

**GO and KEGG pathway enrichment analysis.** GO and KEGG enrichment analysis on the 108 potential targets was conducted through Metascape database to gain insight into the mechanisms of AJH in treating COPD. Accordingly, 2, 297 GO terms and 167 KEGG



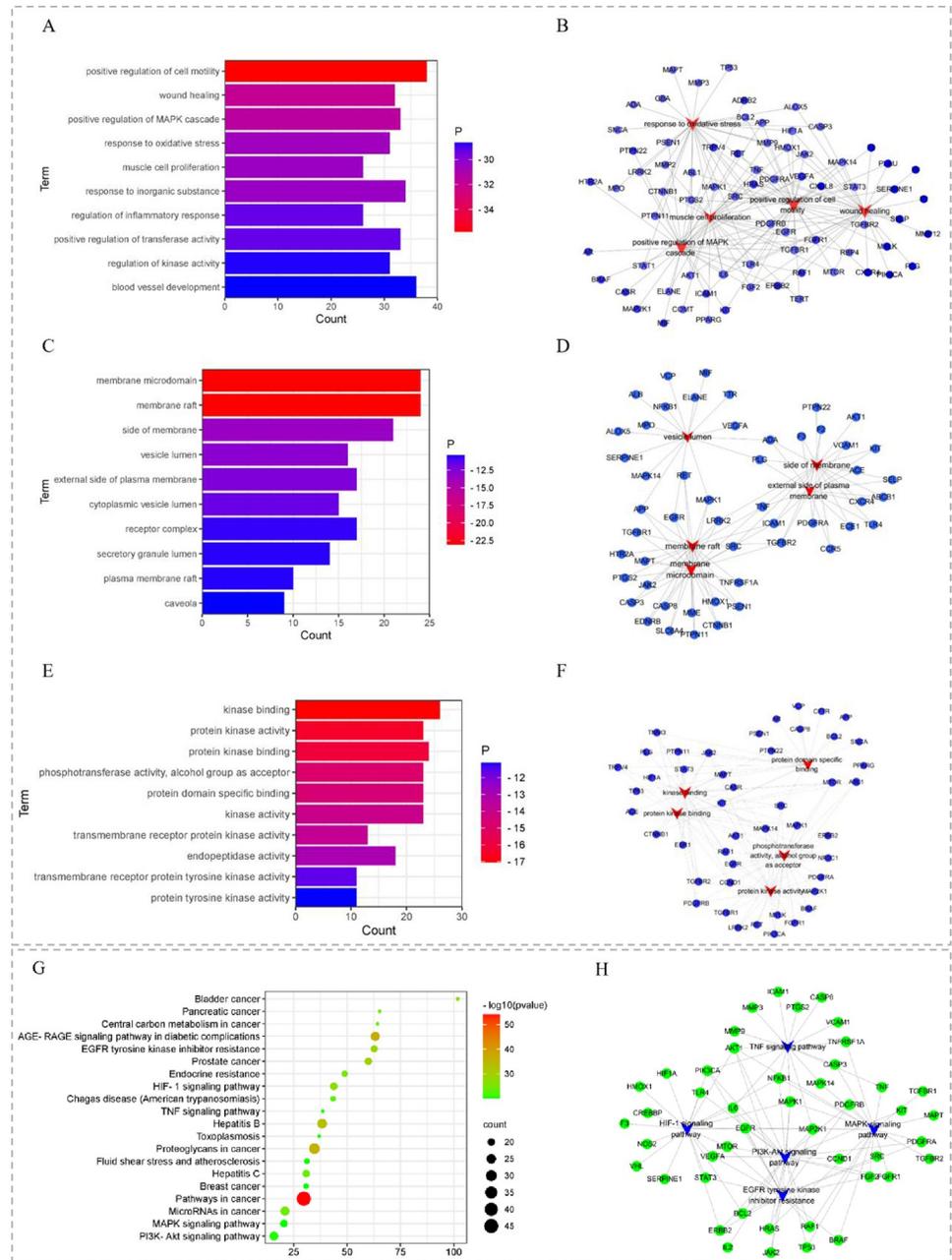
**Fig 3. Network of common targets.** (A) Intersection of Venn diagram. (B) PPI network of common targets. (C) Targets in the top ten degree.

<https://doi.org/10.1371/journal.pone.0269087.g003>

pathways with P-value valuerdingly, 2, 297 (S2 Table). Moreover, the term/pathway-target networks were constructed (as shown in Fig 4).

Gene ontology enrichment analysis consisted of three parts: biological process (BP), cellular component (CC) and molecular function (MF). The top 10 important terms in BP, CC and MF are shown in Fig 4A–4F. The enrichment results showed that 2, 063 enrichment terms are related to BP, which mainly cover positive regulation of cell motility, positive regulation of MAPK cascade, response to oxidative stress and regulation of inflammatory response. In addition, 134 enrichment results were related to MF, including kinase binding, protein kinase activity and phosphotransferase activity. 100 CC items were also collected, of which membrane raft, membrane microdomain and side of membrane were the most enriched. The top 20 KEGG pathways enriched were shown in Fig 4G and 4H. Among them, the pathways significantly related to COPD treatment include EGFR tyrosine kinase inhibitor resistance, HIF-1, TNF, PI3K-Akt and MAPK signaling pathway, etc.

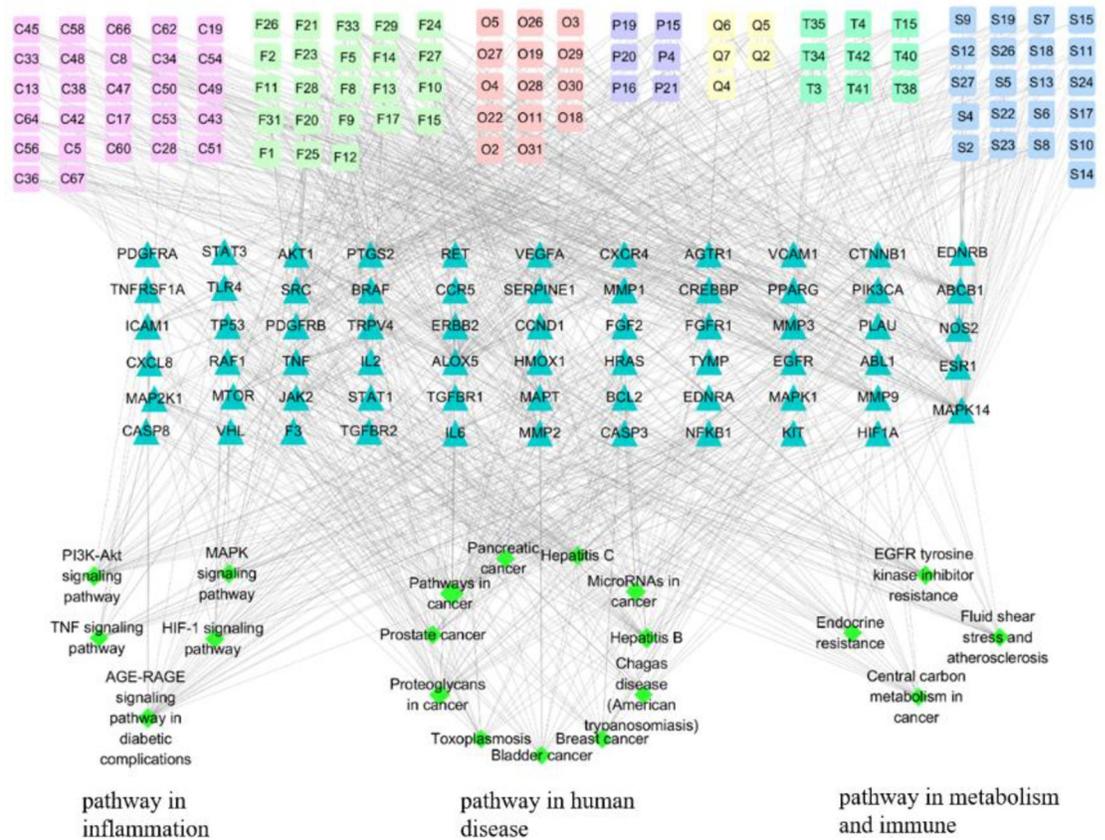
**Component-target-pathway network.** The component-target-pathway network (Fig 5) was constructed to clarify the contact among components, key targets and pathway, which



**Fig 4. GO and KEGG enrichment of potential targets.** (A, C, E: Top 10 significantly enriched terms in BP, CC and MF, respectively. B, D, F: The top five terms and corresponding targets in BP, CC and MF, respectively). (G) Top 20 pathways enriched. (H) Pathway-target network (the five COPD-related pathway and corresponding targets).

<https://doi.org/10.1371/journal.pone.0269087.g004>

comprised 190 nodes and 1042 edges. The rectangle nodes represent 105 components identified in AJH, the triangle nodes represent 65 COPD-related targets, and the diamond nodes represent the top 20 enriched pathways. The edges represent interactions between compounds, targets and pathways. According to the topological parameters, 41 hub components were obtained, including 12 flavonoids, 11 steroids, 7 carboxylic acids, 6 terpenes, 2 phenylpropanoids, 1 quinone, 1 ketone and 1 phenol. The detailed information of components with degree values in the top twenty are shown in [Table 1](#). Among them, Hydroxygenkwanin, luteolin,



**Fig 5. Component-target-pathway network comprised 190 nodes and 1042 edges.** The rectangle nodes represent 105 components identified in AJH, the triangle nodes represent 65 COPD-related targets, and the diamond nodes represent the top 20 enriched pathways. The edges represent interactions between compounds, targets and pathways.

<https://doi.org/10.1371/journal.pone.0269087.g005>

kaempferol, Idebenone, Myricetin 3-O-galactoside, Linoleic acid, Alpinetin, Bergenin and Digitoxigenin showed strong interactions with 10 or more COPD targets, and might be the potential bioactive constituents of AJH against COPD.

**Validation by molecular docking.** The top ten core compounds were docking with the corresponding five key targets, and the results were analyzed. The basic information about ligands and proteins is shown in Table 2. The results showed that the binding free energy ( $\Delta G$  in kcal/mol) of compounds for binding to key targets was negative (as shown in S2 Table), indicating the ligands molecules could bind to receptor proteins spontaneously. In addition, the binding energy was smaller than -5.0 kJ/mol, which further proved the strong binding energy. The heat map revealed the difference of binding energy between different compounds and targets, as shown in Fig 6.

Luteolin has the best binding ability with five core targets. Visualization of the docking results was performed using Discovery Studio 4.5 Client software. The docking of the luteolin, kaempferol and bergenin with target proteins was primarily through thirteen forces, including conventional hydrogen bond, unfavorable donor-donor, unfavorable acceptor-acceptor, unfavorable bump, carbon hydrogen bond, amide-pi stacked, pi-donor hydrogen bond, pi-sigma, pi-pi stacked, pi-sulfur, sulfur-x, pi-alkyl, van der waals (S2 Fig). Luteolin has the strongest binding to MMP9 through 6 forces, mainly including conventional hydrogen bond, which formed hydrogen bonds with MET247 and ARG249 residues. Kaempferol has the strongest

**Table 1. Topological properties of hub components.**

ID	components	Bc	Cc	Degree
F31	Hydroxygenkwanin	0.03934312	0.43661972	15
F25	Luteolin	0.03669989	0.43661972	14
F28	Kaempferol	0.01581494	0.4025974	11
Q5	Idebenone	0.01506443	0.34521158	11
F33	(-) Alpinetin	0.02813808	0.38847118	10
C45	Linoleic acid	0.02027674	0.4200542	10
F12	Myricetin 3-O-galactoside	0.01617418	0.37170264	10
P4	Bergenin	0.01301043	0.39641944	10
S7	Digitoxigenin	0.02640662	0.42234332	10
F24	Laricitrin	0.00713272	0.34521158	9
S8	(3beta,5beta,12beta)-3,12,14-Trihydroxycardanolide	0.02181039	0.4200542	9
C8	Mycophenolic acid	0.021414	0.35632184	9
C66	Bis(2-ethylhexyl) phthalate	0.01380206	0.38271605	9
C56	Farnesyl acetate	0.02283748	0.37897311	9
F29	Naringenin	0.02506296	0.37897311	9
O29	2-Methylcardol monoene	0.00942	0.388471	9
T4	Helenalin	0.024118	0.3875	9
S12	Alfaxalone	0.014573	0.388471	9
S4	Cortisol	0.015075	0.388471	9
T34	18-β-Glycyrrhetic acid	0.014072	0.41779	8

<https://doi.org/10.1371/journal.pone.0269087.t001>

binding to MMP9 through 5 forces, which formed hydrogen bonds with ALA189, GLN227, LEU188 and VAI223 residues. Bergenin has the strongest binding to TNF through 4 forces, which formed hydrogen bonds with SER99, HIS73 and residues. These results clarified that the active ingredients had better binding properties to protein targets.

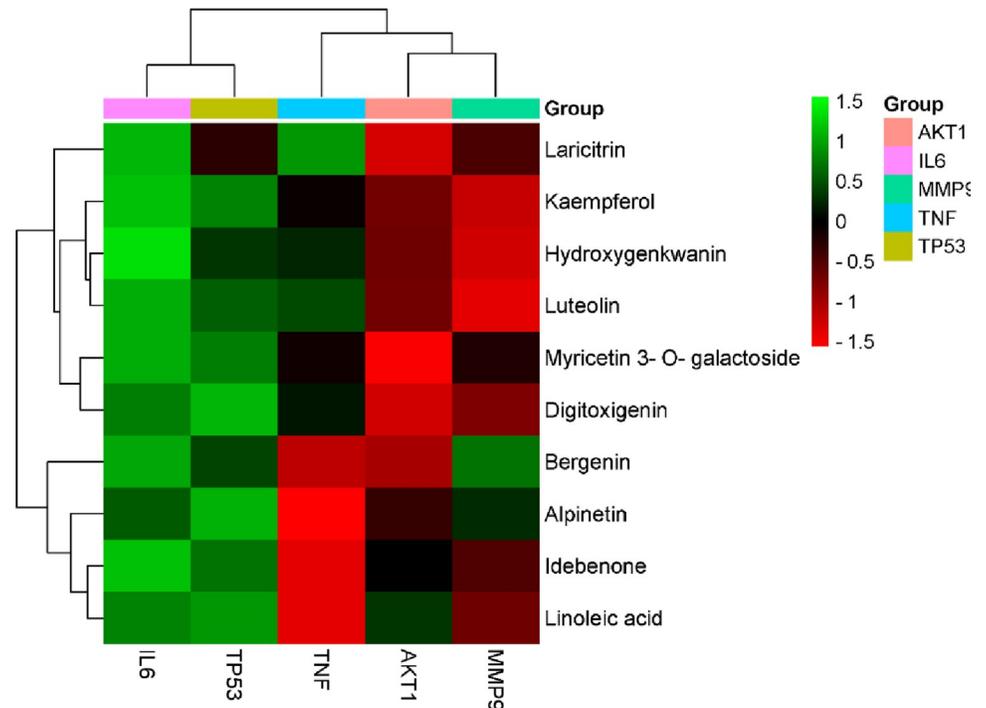
## Experimental validation

According to the network pharmacology, molecular docking analysis and references, three bioactive components including luteolin, kaempferol, bergenin had the better molecular docking results. To further evaluate the results obtained by systematic pharmacologic analysis, these components were selected from AJH to examine the potential effects of anti-inflammatory by using TNF-α-stimulated A549 cells. We employed ELISA assay for IL-6, MMP9 to confirm the predicted anti-inflammatory effects of three bioactive components. Moreover, MTT assay and cytokine expression assay were performed at concentrations that did not affect cell activity by comparing references and conducting preliminary experiments.

**Table 2. The basic information of ligand and protein.**

Targets	PDB ID	Ligand ID	Ligand Name
AKT1	2F7Z	6EA	(1S)-1-(1H-INDOL-3-YLMETHYL)-2-(2-PYRIDIN-4-YL- [1,7] NAPHTHYRIDIN-5-YLOXY)-EHYLAMINE
IL6	5FUC	NAG	2-acetamido-2-deoxy-beta-D-glucopyranose
TNF	6OP0	A7A	(R)- {1- [(2,5- dimethyl phenyl) methyl] -6-(1-methyl-1H-pyrazol-4-yl)-1H-benzimidazol-2-yl} (pyridin-4-yl) methanol
TP53	5O1C	9GZ	5-(4-fluorophenyl)-3-iodanyl-2-oxidanyl-4-pyrrol-1-yl-benzoic acid
MMP9	6ESM	B9Z	(2-{S})-2- [2- [4-(4- methoxyphenyl) phenyl] sulfanylphenyl] pentanedioic acid

<https://doi.org/10.1371/journal.pone.0269087.t002>



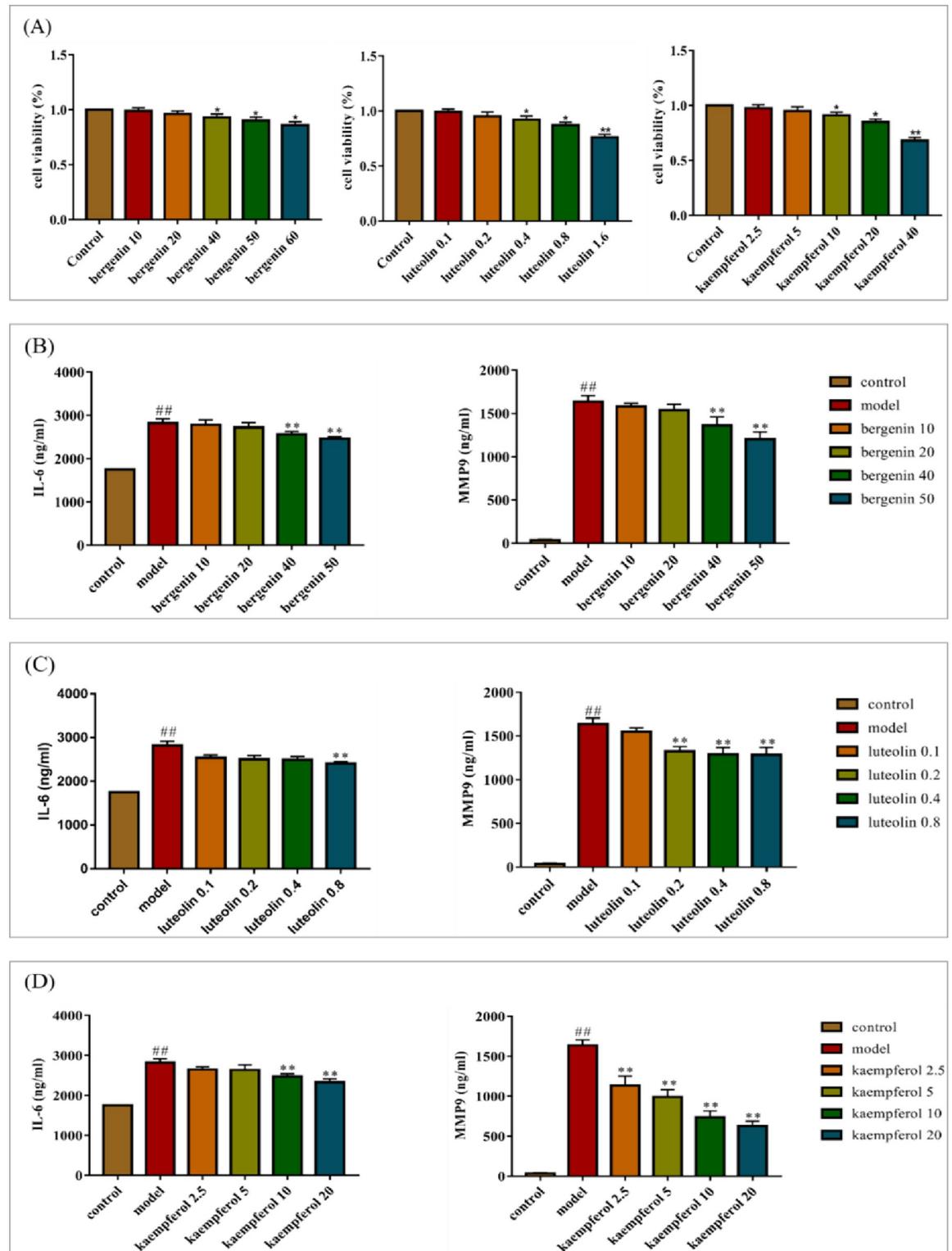
**Fig 6. The binding energy between the core compounds with core targets in AJH.**

<https://doi.org/10.1371/journal.pone.0269087.g006>

The MTT kits were used to assess the cytotoxicity to select proper concentrations of bergenin, luteolin and kaempferol. As shown in Fig 7A, bergenin, luteolin, kaempferol showed cytotoxicity to A549 cells at 60, 1.6 and 40  $\mu\text{g/mL}$ , respectively. Therefore, bergenin (10, 20, 40, 50  $\mu\text{g/mL}$ ), luteolin (0.1, 0.2, 0.4, 0.8  $\mu\text{g/mL}$ ), kaempferol (2.5, 5, 10, 20  $\mu\text{g/mL}$ ) were selected for subsequent experiments. Compared with controls, the contents of IL6 and MMP9 in A549 cells were significantly increased ( $p < 0.01$ , Fig 7) after TNF- $\alpha$  induction. Compared with the model group, the content of IL6 and MMP9 were decreased in varying degrees after the treatment of three compounds with different concentrations. In A549 cells co-cultured with TNF- $\alpha$  and three compounds, the expression levels of IL-6 and MMP9 reduced significantly ( $p < 0.01$ ) in a dose-reliant pattern. Bergenin with low dose (10  $\mu\text{g/mL}$ ) has no anti-inflammatory effects (Fig 7B–7D).

## Discussion

In this study, the chemical components of AJH were identified through a comprehensive approach, including rapid analysis of chemical components, network pharmacology analysis and experiment in vitro, and the molecular mechanisms of its anti-COPD against COPD was discussed. Firstly, the total ion chromatograms of AJH were obtained by UPLC-Orbitrap Fusion MS. Different mobile phase systems and elution gradients were investigated to optimize reliable and effective chromatography conditions. It was found that the elution effect of methanol was better than that of acetonitrile, and the separation effect of each chromatographic peak was better. Adding 0.1% formic acid can improve the peak shape and the mass spectrum response, so we used methanol-0.1% formic acid water as the mobile phase. In addition, due to the different response modes of various compounds in AJH, positive and negative ion-scanning modes were selected for simultaneous detection line monitoring. By using



**Fig 7. Effect of bergenin, luteolin, kaempferol on A549 cells.** A: The effect of bergenin, luteolin, kaempferol on A549 cell viability were detected by MTT analysis. B-D: The effects of different concentrations of bergenin, luteolin, kaempferol on the contents of IL6, MMP9 in COPD model were determined by ELISA, respectively. Values represented as mean  $\pm$  SD, n = 6. <sup>##</sup>P < 0.01 versus control group. <sup>\*\*</sup>P < 0.01, <sup>\*</sup>P < 0.05, versus model group.

<https://doi.org/10.1371/journal.pone.0269087.g007>

the Compound Discoverer 3.0 software to match the original MS raw data to mzCloud, Chemspider and other built-in databases, a total of 236 chemical constituents (162 in ESI+ and 74 in ESI-) were clarified or preliminarily characterized, mainly including flavonoids, phenylpropanoids and terpenes, which is consistent with the literature [46]. Previous study had shown fifteen compounds from AJH were investigated by K.Y. Yu through HPLC-QTOF-MS [45]. Compared with previous studies on the chemical constituents of AJH, this study identified and compared the chemical constituents of AJH *in vitro* more comprehensively and systematically. It could be seen that the established UPLC-Orbitrap Fusion MS method has more sensitivity to identify the chemical components in AJH, and the results provided more chemical material basis information for further study of AJH.

Network pharmacology can reflect the contact among components/targets and pathways, and the molecular mechanisms of drug therapy can be clarified by constructing a “component-target-pathway” network. In the present study, 111 of the 236 components characterized of AJH were thought to have potentially critical anti-COPD effects through acting on 108 related targets. To further explore the key pharmaceutically bioactive compounds and underlying mechanisms of AJH against COPD, a multidimensional component-target-pathway network was further constructed and visualized using Cytoscape v3.7.2. Based on three topological parameters, forty-one key active components were screened out, including twelve flavonoids, eleven steroids, seven carboxylic acids, six terpenes, two phenylpropanoids, one quinone and two other compounds, which proved that AJH had a multicomponent synergistic effect in the treatment of COPD. Of these, flavonoids have the activities of anti-inflammation and anti-oxidation by inhibiting the expression of TNF- $\alpha$ , IL-6 and COX-2, which may have greater potential effects on COPD [47–49]. It has been reported that luteolin and kaempferol exert therapeutic effects on COPD by inhibiting lung inflammation through reducing the release of inflammatory cytokines such as IL-6, IL-12 and TNF- $\alpha$  [50,51]. Alpinetin play a protective role in COPD by decreasing the activities of TGF- $\beta$ 1, TNF- $\alpha$ , and  $\alpha$ -SMA, inhibiting apoptosis and reducing the release of inflammatory cytokines [52]. Hydroxygenkwanin showed antitumor activity on Non-Small Cell Lung Cancer cells by promoting the degradation of EGFR [53]. Laricitrin is an efficacious immunoadjuvant with great potential to improve the inhibitory effects of lung cancer on differentiation, maturation and function of dendritic cells [54]. In addition, idebenone, linoleic acid, bergenin and digitoxigenin also have relatively high degree values. Among them, bergenin, the main phenylpropanoids in AJH, can significantly improve lung damage and reduce the levels of inflammatory factors such as TNF- $\alpha$ , IL-6, IL-1 $\beta$  and PEG2, and can significantly improve lung function and blood gas indexes in COPD model rats [55,56]. The mechanisms of these active ingredients cover multi-aspects of the pathogenesis of COPD, and the targets/pathways obtained are basically consistent with our predicted results, indicating that the comprehensive pharmacological strategy has certain predictive accuracy.

The results of network pharmacology showed that these active components may effectively improve the pathologic status of COPD patients by modulating sixty-five corresponding targets primarily involved in inflammation and oxidation related pathways. Among the predicted potential targets, AKT1, IL6, TNF, TP53, MMP9, CASP3 and ALB had high frequency, and they were involved in several biological processes, including positive regulation of MAPK cascade, positive regulation of cell motility, response to oxidative stress, regulation of inflammatory response, which were closely related to the pathogenesis of COPD. KEGG enrichment analysis showed that the potential targets were highly related to human disease for cancer, infectious diseases, metabolic diseases, immune system and signal transduction, including the pathways in cancer, AGE-RAGE signaling pathway in diabetic complications, endocrine resistance, EGFR tyrosine kinase inhibitor resistance, HIF-1, TNF, MAPK and PI3K-Akt signaling

pathway, etc. Combined with the COPD pathogenesis and common signaling pathways, it is inferred that AJH exerted therapeutic effects on COPD through the following aspects: anti-inflammatory, antioxidant and immune regulation. TNF- $\alpha$  is one of the key signal molecules of high secretion of airway mucus, and it is also a biological indication of COPD grade and curative efficacy [57]. TNF- $\alpha$  can activate neutrophils and macrophages to release more cytokines such as IL-6 and IL-8, thus speeding up the inflammatory response process [58]. Abnormal activation of TNF signaling pathway plays a key role in goblet cell metaplasia and airway mucus hypersecretion [59]. The binding of advanced glycation end-product receptor (RAGE) to AGEs (glycosylated and oxidized proteins, lipids or nucleic acids) can induce pro-inflammatory response, and the AGE-RAGE pathway has become a significant signal transduction pathway, affecting immune and oxidative stress responses by activating MAPK and NF- $\kappa$ B pathways [60,61]. Thus, we speculated that AJH might play an anti-inflammatory and antioxidant role in the treatment of COPD by regulating TNF, MAPK signaling pathway and AGE-RAGE signaling pathway in diabetic complications. In addition, the enrichment results showed that AJH could indirectly participate in the treatment of COPD through central carbon metabolism and endocrine resistance.

We selected molecular docking to validate the binding of core compounds and targets related to inflammation and oxidative stress. The binding free energy of compounds to the key targets was smaller than -5.0 kJ/mol, which indicated that the ligand molecules could spontaneously bind to receptor proteins with strong binding ability. Studies have shown that alveolar epithelial cells A549 are one of the mediators for COPD detection [62,63]. Inflammation is the most important cause of COPD [6]. Based on our previous research, TNF- $\alpha$  can inhibit the growth of A549 cells, induce the secretion of inflammatory factors such as IL8 and MMP-9, and activate NF- $\kappa$ B and MAPK signaling pathway, which are consistent with the pathological development mechanism of COPD [64]. Therefore, we selected TNF- $\alpha$  induced A549 cells as the *in vitro* inflammatory model. Based on the results of network pharmacology and molecular docking, three bioactive components (luteolin, kaempferol and bergenin) with high response values were selected to perform some verification experiments. Experiments *in vitro* showed that these three bioactive components could inhibit the increase of IL-6 and MMP9, and alleviate the pathological manifestations of COPD.

## Conclusion

In summary, this study clarified the components of AJH systematically, and explored the molecular mechanisms of AJH against COPD using an integrative strategy for the first time. Totally, 236 components were characterized using UPLC-Orbitrap Fusion MS, of which forty-one key bioactive components screened in AJH may effectively improve the pathologic status of COPD patients by modulating sixty-five corresponding targets primarily involved in inflammation/ metabolism/immune-related pathways. This study provided a comprehensive exploration into the chemical characteristics, pharmacological effects of AJH, and provided a reference for the further study and clinical application of AJH in the treatment of COPD. However, more experiments are needed to further demonstrate the deeper mechanisms, which will be the focus of our research in the future.

## Supporting information

**S1 Fig. The total ion chromatograms (TICs) of AJH in three batches.**  
(PDF)

**S2 Fig. The docking results of luteolin, kaempferol and bergenin binding to the five targets.**

(PDF)

**S1 Table. The detailed information of components identified in AJH based on UPLC-Orbitrap Fusion MS.**

(XLSX)

**S2 Table. The details of network pharmacology results.**

(XLSX)

**S1 File.**

(DOCX)

## Author Contributions

**Conceptualization:** Suxiang Feng, Jie Yuan.

**Data curation:** Di Zhao, Rongrong Li, Yange Tian.

**Visualization:** Suxiang Feng, Xuefang Liu.

**Writing – original draft:** Suxiang Feng, Jie Yuan.

**Writing – review & editing:** Suxiang Feng, Jiansheng Li.

## References

1. Singh D, Agusti A, Anzueto A, Barnes PJ, Bourbeau J, Celli BR, et al. Global Strategy for the Diagnosis, Management, and Prevention of Chronic Obstructive Lung Disease: the GOLD science committee report 2019. *Eur Respir J*. 2019; 53(5). Epub 2019/03/09. <https://doi.org/10.1183/13993003.00164-2019> PMID: 30846476.
2. Beran D, Zar HJ, Perrin C, Menezes AM, Burney P, Forum of International Respiratory Societies working group c. Burden of asthma and chronic obstructive pulmonary disease and access to essential medicines in low-income and middle-income countries. *Lancet Respir Med*. 2015; 3(2):159–70. Epub 2015/02/15. [https://doi.org/10.1016/S2213-2600\(15\)00004-1](https://doi.org/10.1016/S2213-2600(15)00004-1) PMID: 25680912.
3. Kohansal R, Martinez-Cambor P, Agusti A, Buist AS, Mannino DM, Soriano JB. The natural history of chronic airflow obstruction revisited: an analysis of the Framingham offspring cohort. *Am J Respir Crit Care Med*. 2009; 180(1):3–10. Epub 2009/04/04. <https://doi.org/10.1164/rccm.200901-0047OC> PMID: 19342411.
4. Gan WQ, FitzGerald JM, Carlsten C, Sadatsafavi M, Brauer M. Associations of ambient air pollution with chronic obstructive pulmonary disease hospitalization and mortality. *Am J Respir Crit Care Med*. 2013; 187(7):721–7. Epub 2013/02/09. <https://doi.org/10.1164/rccm.201211-2004OC> PMID: 23392442.
5. Yuan C, Chang, Lu G, Deng X. Genetic polymorphism and chronic obstructive pulmonary disease. *Int J Chron Obstruct Pulmon Dis*. 2017; 12:1385–93. Epub 2017/05/27. <https://doi.org/10.2147/COPD.S134161> PMID: 28546746; PubMed Central PMCID: PMC5436778.
6. van Rijn AL, van Boheemen S, Sidorov I, Carbo EC, Pappas N, Mei H, et al. The respiratory virome and exacerbations in patients with chronic obstructive pulmonary disease. *PLoS One*. 2019; 14(10): e0223952. Epub 2019/10/28. <https://doi.org/10.1371/journal.pone.0223952> PMID: 31647831; PubMed Central PMCID: PMC6812800.
7. Cong W, Lu J, Xiang S, Liu Z. Advances in the role of inflammatory signaling pathways in chronic obstructive pulmonary disease. *Journal of Shanxi Medical University*. 2018; 49(03):305–10. <https://doi.org/10.13753/j.issn.1007-6611.2018.03.021>
8. Vogelmeier CF, Criner GJ, Martinez FJ, Anzueto A, Barnes PJ, Bourbeau J, et al. Global Strategy for the Diagnosis, Management, and Prevention of Chronic Obstructive Lung Disease 2017 Report. GOLD Executive Summary. *Am J Respir Crit Care Med*. 2017; 195(5):557–82. Epub 2017/01/28. <https://doi.org/10.1164/rccm.201701-0218PP> PMID: 28128970.

9. Tashkin DP, Celli B, Senn S, Burkhart D, Kesten S, Menjoge S, et al. A 4-year trial of tiotropium in chronic obstructive pulmonary disease. *N Engl J Med*. 2008; 359(15):1543–54. Epub 2008/10/07. <https://doi.org/10.1056/NEJMoa0805800> PMID: 18836213.
10. Calzetta L, Ritondo BL, Matera MG, Pezzuto G, Cazzola M, Rogliani P. Investigational treatments in phase I and II clinical trials: a systematic review in chronic obstructive pulmonary disease (COPD). *Expert Opin Investig Drugs*. 2020; 29(7):723–38. Epub 2020/05/14. <https://doi.org/10.1080/13543784.2020.1769064> PMID: 32401655.
11. Chao J, Dai Y, Verpoorte R, Lam W, Cheng YC, Pao LH, et al. Major achievements of evidence-based traditional Chinese medicine in treating major diseases. *Biochem Pharmacol*. 2017; 139:94–104. Epub 2017/06/22. <https://doi.org/10.1016/j.bcp.2017.06.123> PMID: 28636884.
12. Xiang FS, Jie ZH, Di Z, Rong LR, Hang DX, Yang WP, et al. Chemical profiling and quantification of Tanreqing injection, a systematic quality control strategy equipped with UPLC-Q-Orbitrap fusion MS. *Journal of Liquid Chromatography & Related Technologies*. 2020;43(7–8).
13. Chen S, Ji S, Shang L, Xie W, Zhang L, Liu T, et al. Mechanism of Modified Erchentang on Expression of Transforming Growth Factor-beta 1 (TGF- $\beta$  1) and Histone Deacetylase 2 (HDAC2) Gene in Rats with Chronic Obstructive Pulmonary Disease. *Chinese Journal of Experimental Traditional Medical Formulae*. 2017; 23(10):147–54.
14. Zhong Y, Chen J, Zhang W, Lv J, Lan Y. Based on JAK2/STAT3 signal pathway to explore the mechanism of Wenfei Fuyang Qutan Recipe on airway inflammation in COPD rats. *Liaoning Journal of Traditional Chinese Medicine*. 1–12.
15. Xu F, Lin J, Cui W, Kong Q, Li Q, Li L, et al. *Scutellaria baicalensis* Attenuates Airway Remodeling via PI3K/Akt/NF-kappaB Pathway in Cigarette Smoke Mediated-COPD Rats Model. *Evid Based Complement Alternat Med*. 2018; 2018:1281420. Epub 2018/06/05. <https://doi.org/10.1155/2018/1281420> PMID: 29861765; PubMed Central PMCID: PMC5971322.
16. Feng F, Du J, Meng Y, Guo F, Feng C. Louqin Zhisou Decoction Inhibits Mucus Hypersecretion for Acute Exacerbation of Chronic Obstructive Pulmonary Disease Rats by Suppressing EGFR-PI3K-AKT Signaling Pathway and Restoring Th17/Treg Balance. *Evid Based Complement Alternat Med*. 2019; 2019:6471815. Epub 2019/02/26. <https://doi.org/10.1155/2019/6471815> PMID: 30800170; PubMed Central PMCID: PMC6360623.
17. Zhang L, Tian Y, Zhu L, Liu S, Ma J, Wu M, et al. Mechanism of Bufeiyishen Formula antioxidant stress in rats with chronic obstructive pulmonary disease via Nrf2 signaling pathway. *China Journal of Traditional Chinese Medicine and Pharmacy*. 2020; 35(5):6.
18. Zhao P, Liu X, Dong H, Tian Y, Feng S, Zhao D, et al. Bufeiyishen Formula Restores Th17/Treg Balance and Attenuates Chronic Obstructive Pulmonary Disease via Activation of the Adenosine 2a Receptor. *Front Pharmacol*. 2020; 11:1212. Epub 2020/08/28. <https://doi.org/10.3389/fphar.2020.01212> PMID: 32848801; PubMed Central PMCID: PMC7427463.
19. Liu W, Yang D, Yu S, Huang L, Chen S, Guo L. Studies on pharmacological action of aidicha. *Lishizhen Medicine and Materia Medica Research*. 2009; 20(12):3002–3.
20. Li Q, Li W, Hui LP, Zhao CY, He L, Koike K. 13,28-Epoxy triterpenoid saponins from *Ardisia japonica* selectively inhibit proliferation of liver cancer cells without affecting normal liver cells. *Bioorg Med Chem Lett*. 2012; 22(19):6120–5. Epub 2012/09/04. <https://doi.org/10.1016/j.bmcl.2012.08.027> PMID: 22940450.
21. Chang X, Li W, Jia Z, Satou T, Fushiya S, Koike K. Biologically active triterpenoid saponins from *Ardisia japonica*. *J Nat Prod*. 2007; 70(2):179–87. Epub 2007/01/25. <https://doi.org/10.1021/np0604681> PMID: 17243725.
22. Amin MN, Banik S, Ibrahim M, Moghal MMR, Anonna SN. A Study on *Ardisia solanacea* for Evaluation of Phytochemical and Pharmacological Properties. *International Journal of Pharmacognosy and Phytochemical Research*. 2015; 7(1).
23. Dat NT, Bae K, Wamiru A, McMahan JB, Le Grice SF, Bona M, et al. A dimeric lactone from *Ardisia japonica* with inhibitory activity for HIV-1 and HIV-2 ribonuclease H. *J Nat Prod*. 2007; 70(5):839–41. Epub 2007/04/03. <https://doi.org/10.1021/np060359m> PMID: 17397219.
24. Piacente S, Pizza C, De Tommasi N, Mahmood N. Constituents of *Ardisia japonica* and their in vitro anti-HIV activity. *J Nat Prod*. 1996; 59(6):565–9. Epub 1996/06/01. <https://doi.org/10.1021/np960074h> PMID: 8786362
25. Kobayashi H, Mejía E. The genus *Ardisia*: a novel source of health-promoting compounds and phytopharmaceuticals. *Journal of Ethnopharmacology*. 2005; 96(3):347–54. <https://doi.org/10.1016/j.jep.2004.09.037> PMID: 15619551
26. Zeng L, Wang Z, He C. Reserach Progress on Chemical Composition and Pharmacological Action of *Ardisia japonica*. *Guangxi Sciences*. 2019; 26(5):6.

27. Peng L, Yang Y, Wu X. Application Research of Miao Medicine in the Pulmonary Disease. *Academic Journal of Shanghai University of Traditional Chinese Medicine*. 2016; 30(5):5.
28. Ge Z, Fan X. Effect of Miao Nationality's Herbs for Relieving Cough and Reducing Phlegm Combined with Chinese Medicine on Antagonizing TGF- $\beta$ 1 in COPD Model of Rats. *Journal of Basic Chinese Medicine*. 2006; 012(004):289–91.
29. Yuan X. Regulation of Wnt signaling pathway in airway remodeling in COPD rats and intervention of *Ardisiae Japonicae Herba* and *Lespedezacuneata*. *Guizhou University of Traditional Chinese Medicine*. 2014.
30. Luo X. Study on the mechanism of rHSG regulating airway remodeling in COPD rats and the effect of *Ardisiae Japonicae Herba* and *Lespedezacuneata* on it: *Guizhou University of Traditional Chinese Medicine*; 2014.
31. Liu M, Zhou X, Li G, Wang M. Exploring the regulation mechanism of *Ardisiae Japonicae Herba* and *Lespedezacuneata* on TGF- $\beta$ 1/Smad3 pathway in COPD rats based on DCN. *Journal of Medicine & Pharmacy of Chinese Minorities*. 2019; 25(11):26–9. <https://doi.org/10.16041/j.cnki.cn15-1175.2019.11.018>
32. Ge Z, Luo X, Yuan X, Li B. Effects of Miao Medicine *Ardisiae Japonicae Herba* and *Lespedezacuneata* on the proliferation of airway fibroblasts and the expression of rHSG and PDGF in COPD rats. *Lishizhen Medicine and Materia Medica Research*. 2016; 27(4):3.
33. Zhan Y, editor *Modern research on Miao medicine Ardisia japonica*. 2003 National Symposium on Miao Medicine; 2003; Longli, Guizhou, China.
34. Peng L, Yang Y, Wu X. Application Research of Miao Medicine in the Pulmonary Disease. *Academic Journal of Shanghai University of Traditional Chinese Medicine*. 2016; 30(05):90–4.
35. Ge Z, Fan X. Effect of Miao Nationality's Herbs for Relieving Cough and Reducing Phlegm Combined with Chinese Medicine on Antagonizing TGF- $\beta$ 1 in COPD Model of Rats. *Journal of Basic Chinese Medicine*. 2006;(04):289–91.
36. Zhou X, Ge Z, Bi F. Miao Medicine Lung Frame with Warm Air Fried Western Medicine Treatment Chronic Obstructive Pulmonary Disease at Stable Phrase Effect Analysis. *Chinese Journal of Experimental Traditional Medical Formulae*. 2012; 18(15):305–7.
37. Hu W, Jiang X, Zhang T, Yang H. Compared analysis on the constituents and their contents of the volatile oil from different parts in *Ardisia japonica*. *Guangdong Agricultural Sciences*. 2013; 40(03):66–70.
38. Ma L, Li W, Wang H, Kuang X, Li Q, Wang Y, et al. A simple and rapid method to identify and quantitatively analyze triterpenoid saponins in *Ardisia crenata* using ultrafast liquid chromatography coupled with electrospray ionization quadrupole mass spectrometry. *J Pharm Biomed Anal*. 2015; 102:400–8. Epub 2014/12/03. <https://doi.org/10.1016/j.jpba.2014.10.013> PMID: 25459939.
39. Chen Y, Du K, Li J, Bai Y, An M, Tan Z, et al. A Green and Efficient Method for the Preconcentration and Determination of Gallic Acid, Bergenin, Quercitrin, and Embelin from *Ardisia japonica* Using Nonionic Surfactant Genapol X-080 as the Extraction Solvent. *Int J Anal Chem*. 2018; 2018:1707853. Epub 2018/03/01. <https://doi.org/10.1155/2018/1707853> PMID: 29487621; PubMed Central PMCID: PMC5816874.
40. Xiao Z, Wen X, Pan T, Ouyang B, Huang Y, Tian X. Analysis of Monosaccharide Composition in *Ardisia japonica* Polysaccharide by TLC and HPLC. *China Medical Herald*. 2016; 13(30):130–3.
41. Hu N, Wang C, Dai X, Zhou M, Li Y. Phillygenin inhibits LPS-induced activation and inflammation of LX2 cells by TLR4/MyD88/NF- $\kappa$ B signaling pathway. *Journal of Ethnopharmacology*. 2019; 248:112361. <https://doi.org/10.1016/j.jep.2019.112361> PMID: 31683033
42. Li Z, Cao Q, Wu W, Gao Y, Zhang K. Study on adsorption and separation of flavonoid glycosides in *Radix Astragali* by acrylic/acrylic acid copolymer gel grafted with paos-cyclodextrin. *Journal of Traditional Chinese Veterinary*. 2019; 38(02):17–21. <https://doi.org/10.13823/j.cnki.jtcvm.2019.02.004>
43. Zhou S, Wen H, Han X, Li H. Phillygenin protects against osteoarthritis by repressing inflammation via PI3K/Akt/NF- $\kappa$ B signaling: In vitro and vivo studies. *Journal of Functional Foods*. 2021; 80(9):104456.
44. Huang B, Chen W, Hu Y, Qiu C, Zhang W, Liang B. Study on chemical constituents of anti tuberculosis Chinese herbal medicine *Ardisia japonica*. *Acta Pharmaceutica Sinica*. 1981;(01):29–32.
45. Yu KY, Gao W, Li SZ, Wu W, Li P, Dou LL, et al. Qualitative and quantitative analysis of chemical constituents in *Ardisiae Japonicae Herba*. *J Sep Sci*. 2017; 40(22):4347–56. Epub 2017/09/20. <https://doi.org/10.1002/jssc.201700667> PMID: 28926203.
46. Xu C, Zhang L, Wang Y, Wei S, Qin R, Chi M. Study on HPLC Fingerprint of Miao Medicine *Ardisia japonica*. *Journal of Chinese Medicinal Materials*. 2014; 37(09):1570–3.
47. Chanput W, Krueyos N, Ritthiruangdej P. Anti-oxidative assays as markers for anti-inflammatory activity of flavonoids. *International Immunopharmacology*. 2016;40. <https://doi.org/10.1016/j.intimp.2016.08.038> PMID: 27598863

48. Chen CY, Peng WH, Tsai KD, Hsu SL. Luteolin suppresses inflammation-associated gene expression by blocking NF-kappaB and AP-1 activation pathway in mouse alveolar macrophages. *Life Sciences*. 2007; 81(23–24):1602–14. <https://doi.org/10.1016/j.lfs.2007.09.028> PMID: 17977562
49. Pan H, Duan G, Zhu P, Li C, Wang Z. Study on adsorption and separation of flavonoid glycosides in *Radix Astragali* by acrylic/acrylic acid copolymer gel grafted with paos-cyclodextrin. *Journal of Traditional Chinese Veterinary*. 2019; 38(2):6.
50. Li Y. Effects of luteolin on COPD and its anti-inflammatory mechanism. *Journal of Modern Medicine & Health*. 2016; 32(08):1216–8.
51. Ja CM, Prasad PR, Won CJ, Kyung SJ, Jin CD, Il PY. Inhibitory effects of kaempferol-3-O-rhamnoside on ovalbumin-induced lung inflammation in a mouse model of allergic asthma. *International immunopharmacology*. 2015; 25(2).
52. Su Y, Tao X, Xu J. Protective effect of Alpinetin on rats with chronic obstructive pulmonary disease. *Food Science & Nutrition*. 2020; 8(12). <https://doi.org/10.1002/fsn3.1952> PMID: 33312544
53. Leu YL, Wang TH, Wu CC, Huang KY, Chen CY. Hydroxygenkwanin Suppresses Non-Small Cell Lung Cancer Progression by Enhancing EGFR Degradation. *Molecules*. 2020; 25(4):941. <https://doi.org/10.3390/molecules25040941> PMID: 32093124
54. Wei-An Chang, Jen-Yu Hung, Shu-Fang Jian, et al. Laricitrin ameliorates lung cancer-mediated dendritic cell suppression by inhibiting signal transducer and activator of transcription 3. *Oncotarget*. 2016.
55. Wu H, Wang I, Wu J. Effects of bergenin on lung function and blood gas analysis in the rats of chronic obstructive pulmonary disease. *World Clinical Drugs*. 2017; 38(04):248–51.
56. Tang Q, Wang Q, Sun Z, Kang S, Fan Y, Hao Z. Bergenin Monohydrate Attenuates Inflammatory Response via MAPK and NF-kappaB Pathways Against *Klebsiella pneumoniae* Infection. *Front Pharmacol*. 2021; 12:651664. Epub 2021/05/22. <https://doi.org/10.3389/fphar.2021.651664> PMID: 34017253; PubMed Central PMCID: PMC8129520.
57. G. SJ, Annalicia V, G. DA, E. OHP, Felicia G, V. BR, et al. Biomarkers of progression of chronic obstructive pulmonary disease (COPD). *Journal of Thoracic Disease*. 2014; 6(11).
58. Kany S, Vollrath JT, Relja B. Cytokines in Inflammatory Disease. *International Journal of Molecular Sciences*. 2019; 20(23). <https://doi.org/10.3390/ijms20236008> PMID: 31795299
59. K T, B J, J SJ, R BP, T D-P, F UI, et al. Activation of epidermal growth factor receptors is responsible for mucin synthesis induced by cigarette smoke. *American journal of physiology Lung cellular and molecular physiology*. 2001; 280(1). <https://doi.org/10.1152/ajplung.2001.280.1.L165> PMID: 11133506
60. Logan SM, Storey KB. Pro-inflammatory AGE-RAGE signaling is activated during arousal from hibernation in ground squirrel adipose. *PeerJ*. 2018; 6:e4911. Epub 2018/06/12. <https://doi.org/10.7717/peerj.4911> PMID: 29888131; PubMed Central PMCID: PMC5991297.
61. Sparvero LJ, Asafu-Adjei D, Kang R, Tang D, Amin N, Im J, et al. RAGE (Receptor for Advanced Glycation Endproducts), RAGE Ligands, and their role in Cancer and Inflammation. *BioMed Central*. 2009; 7(1).
62. Hou H, Zhang G, Li H, Chen T, Gao Y, Song L, et al. Establishment of chronic obstructive pulmonary disease cell model in vitro by stimulating A549 and RAW264.7 cells with CSE and LPS *Journal of Guangxi Medical University*. 2020; 37(10):1772–7. <https://doi.org/10.16190/j.cnki.45-1211/r.2020.10.003>
63. Xie K, Liang Y, Bian Q, Li Y, Zhang K, ZHANG Y, et al. Mechanism of Bufeifei Yishen Formula in treating COPD on autophagy of A549 cells by PI3K/AKT/mTOR signaling pathway. *China Journal of Traditional Chinese Medicine*. 2021; 36(04):2294–8.
64. Fang K. The establishment of model of inflammation by A549 alveolar epithelial cells and the effects of the three Tiao-Bu Fei-Shen extracts on inflammatory response via regulating NF-κB signaling pathway [Master]: Henan University of Chinese Medicine; 2015.