

Research Paper

The effects and the mechanisms of naringenin from *Artemisia ordosica* Krasch on allergic rhinitis based on mast cell degranulation model and network pharmacology

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Abstract

Objectives The ethyl acetate extraction of *Artemisia ordosica* Krasch (AOK) root showed anti-allergic rhinitis (AR) effect, while the active compounds and pharmacological targets were unknown.

Methods The P815 degranulation was established by cell counting kit 8 assay, β -hexosaminidase releasing assay and toluidine blue staining. The flavonoids were screened *in vitro*. Then toluidine blue staining and ELISA were carried out to investigate the anti-inflammatory effects of the active compound. Network pharmacology was implemented to explain the mechanisms of the active compound. iGEMDOCK was used to investigate the binding between active compound and hub targets.

Key findings C48/80 was the optimum reagent in triggering P815 degranulation. Naringenin could significantly decrease P815 degranulation. Meanwhile, naringenin could remarkably increase the IL-4 and decrease the tumour necrosis factor- α . The effect of naringenin on AR was achieved by regulating multiple targets (e.g. AKT1, MAPK3, VEGFA) and pathways (e.g. pathways in cancer, VEGF signalling pathway). Nine hub proteins were obtained by topological analysis. Multiple hydrogen bonds and van der Waals forces were formed between the naringenin and the residues of hub proteins.

Conclusions Naringenin might be one of the effective ingredients of AOK against AR. And its effects could achieve through regulating multiple targets and pathways.

Keywords: degranulation; naringenin; network pharmacology; molecular docking; anti-allergy

Introduction

Artemisia ordosica Krasch (AOK) is also called Hara-Shabaga in Mongolian. It mainly distributes in arid-desert areas of the north-west in China to fix sand.^[1] In traditional medicine, AOK is mainly

used for treating mumps, rheumatic arthritis and pharyngalgia.^[2, 3] Nevertheless, the active monomer and anti-inflammatory mechanisms of AOK are still unclear. Thus many studies were focused on investigating the chemical composition and pharmacological effect.

According to the recent research, flavonoids, dicoffeo-quininic acid, polysaccharide, sterols and coumarins were found in AOK.^[2, 4] Flavonoids are the most abundant compounds separated from the AOK.^[2, 5] Moreover, these flavonoids have been proved to have anti-inflammation, insecticide, anti-microbial and antioxidant stress properties.^[6–10]

Our group has previously revealed that the effect of ethyl acetate extract of AOK ameliorated allergic rhinitis (AR) symptoms. Besides, the extract reduced the inflammatory factors as well as increased the anti-inflammatory factors.^[11] Furthermore, acacetin, hyperoside, luteolin and naringenin were separated from the extract (Supplementary results 1–4). However, the activity and mechanism of these compounds for AR have not been well studied. Thus, our study aimed to screen these flavonoids (Figure 1) on AR *in vitro* and elucidate the mechanisms of the active compound against AR by network pharmacology and molecular docking. This study provided a preliminarily theoretical basis for the AOK treatment of AR.

Materials and Methods

Materials

P815 cells were bought from Zhong Qiao Xin Zhou Biotechnology. RPMI-1640 medium and fetal bovine serum (FBS) were obtained from Gibco. 96-well plates and 6-well plates were obtained from Costar. Compound 48/80 (C48/80) and dimethyl sulfoxide (DMSO) were bought from Sigma-Aldrich. Acacetin (CAS 480-44-4), hyperoside (CAS 482-36-0), luteolin (CAS 491-70-3) and naringenin (CAS 480-41-1) were obtained from Chengdu Biopurify Phytochemicals. Calimycin (A23187), ionomycin, dexamethasone (DXM), toluidine blue and *p*-nitrophenyl-*N*-acetyl- β -D-glucosaminide were purchased from Shanghai Yuan-Ye Bio-Technology Company. Cell counting kit 8 (CCK-8) and Triton X-100 were acquired from Beyotime Biotechnology.

Cell culture and buffer

P815 cells were cultured in RPMI-1640 medium with 10% FBS in 5% CO₂ incubator at 37°C.^[12] Cells used for experiment until reaching 80% confluence. The Tyrode's solution (TS) was composed of 116.4 mM NaCl, 5.4 mM KCl, 1.2 mM NaH₂PO₄, 1.6 mM MgSO₄, 18.9 mM NaHCO₃, 1.7 mM CaCl₂, 0.1% glucose and 0.1% bovine serum albumin (BSA) (pH 7.4).

Measurement of cell growth

Cell growth was measured by the CCK-8 kit.^[13] P815 cells were seeded in 96-well plates at different densities (i.e. 1×10^4 , 2×10^4 , 3×10^4 , 4×10^4 per well) and cultured for a period (i.e. 15 h, 24 h, 48 h, 72 h, 96 h) at 37°C. Then 10 μ l of CCK-8 was added and incubated for 2 h. The absorbance was detected at 450 nm by the microplate reader (Biotech, USA).

Mast cells degranulation model *in vitro*

Measurement of stimulants' toxicity

Cell viability was evaluated by the CCK-8 kit. P815 cells (2×10^4 /well) were laid on 96-well plates for 24 h. The culture medium was discarded by centrifugation at 1140 $\times$ g for 10 min. Then TS contained with stimulant was added to the P815 separately and incubated for a different time at 37°C. Lastly, 10 μ l of CCK-8 was added and detected as the steps mentioned in the section 'Measurement of cell growth'.

Measurement of β -hexosaminidase releasing

The release rate of β -hexosaminidase was detected as described.^[14] Briefly, P815 cells were plated in 24-well plates (1.25×10^5 per well) and cultured for 24 h. The culture medium was replaced with TS containing the stimulants by centrifugation at 1140 $\times$ g for 10 min and incubated for different times. After the incubation, 50 μ l of the supernatant was collected via centrifugation at 1520 $\times$ g for 10 min and transferred to a new plate. Cells were then lysed with

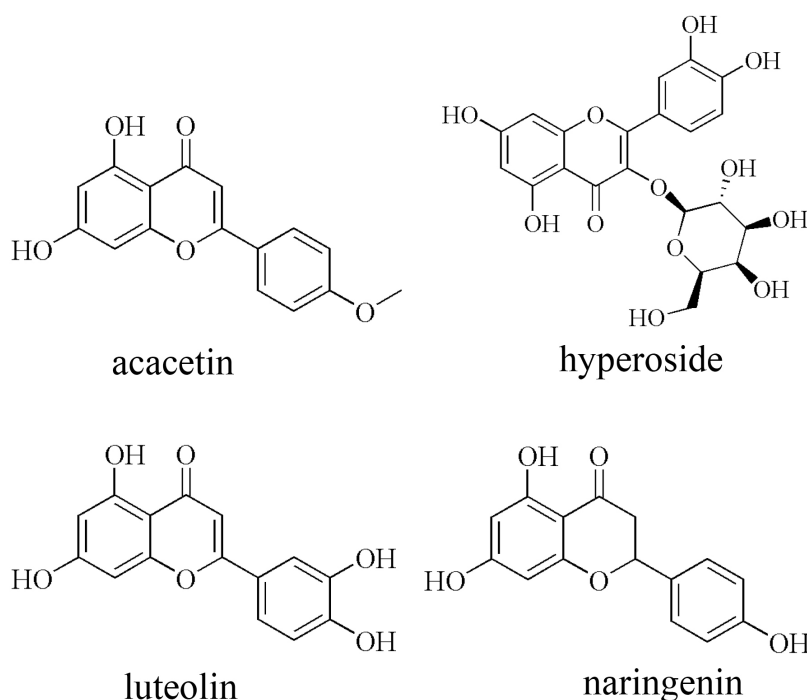


Figure 1 Structure of four flavonoids in AOK.

0.1% Triton X-100 for 45 min. The supernatant was obtained and transferred by centrifuging at 2660× g for 10 min. The quantity of β -hexosaminidase was examined by hydrolysis of *p*-nitrophenyl-*N*-acetyl- β -D-glucosaminide (2 mM, 0.1 M citric acid/sodium citrate buffer) for 90 min at 37°C. Then the stop buffer (0.1 M sodium carbonate/sodium bicarbonate, pH 11) was added to stop the reaction. The absorbance was detected at 405 nm. The release rate of β -hexosaminidase was calculated as absorbance of the supernatant at 405 nm/absorbance of the total cell at 405 nm $\times$ 100%.

Measurement of toluidine blue staining

P815 cells were plated in 6-well plates at 6×10^5 per well and incubated with different stimulants. The supernatant was discarded by centrifugation at 1140× g for 10 min. Cells were then incubated with 1 ml of toluidine blue solution for 1 min and washed with phosphate-buffered saline (PBS) twice. The number of degranulation cells was counted in randomly selected three fields under the microscope.

Effect of flavonoids on mast cell degranulation *in vitro*

Measurement of flavonoids' toxicity

P815 cells (2×10^4 /well) were inoculated into 96-well plates and cultured for 24 h. Then cells were pretreated with acetaminophen, hyperoside, luteolin and naringenin separately from 12.5 μ M to 200 μ M for 10 h and 24 h. CCK-8 was added and detected as the steps mentioned in the section 'Measurement of cell growth'.

Measurement of flavonoids' effect on β -hexosaminidase releasing ratio

P815 cells were seeded in 24-well plates (1.25×10^5 per well). After cultivating for 24 h, cells were pretreated with flavonoids for 4 h. Then the culture medium was discarded by centrifugation. TS with 5 μ g/ml C48/80 was added and incubated for 30 min. Then the plates cooled for 10 min to stop the reaction. The release of β -hexosaminidase was detected as the steps mentioned in the section 'Measurement of β -hexosaminidase releasing'. Cells pretreated with 1 μ M DXM for 24 h were regarded as positive control.

Toluidine blue staining

P815 cells were plated in 6-well plates (6×10^5 per well) and incubated with naringenin and C48/80 separately. The protocol of staining is the same as given in the section 'Measurement of toluidine blue staining'. The number of degranulation cells was counted in randomly selected three fields under the microscope.

TNF- α and IL-4 expression assay

The levels of TNF- α and IL-4 were detected by commercialized ELISA kits (Boster Biological Technology, Wuhan, China). Cell supernatant was collected after pretreated with naringenin and C48/80. The specific detection steps were following the manufacturers' instructions.

Statistical analysis

Each experiment was repeated at least three times. Data from each experiment were analysed by SPSS (SPSS 21.0, IBM, USA) software. Significance was evaluated by one-way analysis of variance (ANOVA) followed by Turkey's test. Results were presented as $\bar{X} \pm \text{S.E.M.}$.

Network pharmacological process

Collection of allergic rhinitis and mast cells degranulation target genes

Target genes of AR and mast cell degranulation were collected from GeneCards and Online Mendelian Inheritance in Man (OMIM).^[15, 16] 'Allergic rhinitis', 'anaphylactic rhinitis' and 'mast cells degranulation' were used as keywords.

Collection of naringenin-related target genes

The SMILES format of naringenin was obtained from PubChem. The naringenin-related targets were obtained from the following databases: traditional Chinese medicine integrated database (TCMID), traditional Chinese medicine database and analysis platform (TCMSP), Swiss Target Prediction and STITCH database.^[17–19] The searching species set as 'Homo sapiens'. UniProt Knowledgebase (UniProtKB) was used to correct protein names and extract the gene names.

Analysis of the shared genes

The shared targets of naringenin and AR were acquired by Venny 2.1.0 software and drew the Venn chart.

Protein–protein interaction (PPI) analysis

String database was applied to clarify the interaction of shared proteins.^[20] The searching species set as 'Homo sapiens'. Then the results were visualized by Cytoscape 3.7.2 software.^[21] Degree value analysis and three topological analyses.^[22] Maximal clique centrality (MCC), maximum neighbourhood component (MNC) and edge percolated component (EPC) were carried out, and the top 10 results of each analysis were taken the intersection to identify the hub targets.

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

GO and KEGG pathway enrichment analysis of shared targets were conducted by DAVID bioinformatics resources 6.8.^[23] All the results were performed by R project 4.0.3.

Molecular docking of hub proteins and naringenin

The 3D structure of naringenin was downloaded from PubChem database and converted as MOL2 format by Open Babel 2.4.1. The positive controls of the hub proteins were selected from the DrugBank database. The crystal structures of the hub proteins were obtained from RCSB protein database (PDB). Water molecules and ions were disposed and the binding box parameters were acquired by PyMOL 2.4.1 software. Molecular docking was conducted by iGEMDOCK v2.1.^[24] The docking protocol was set to 70 generation per ligand. The ligand–protein interactions were analysed and visualized by PyMol v2.4.1 and LigPlot v2.2.

Results

Mast cell degranulation model *in vitro*

Cell growth curve

The cell growth curve was measured to determine the optimum density and best experiment time. As shown in [Supplementary Figure 5A](#), 24–48 h was the logarithmic growth phase, and 48–72 h was the stagnate growth phase. Cells started to die from 72 h. The logarithmic growth phase is the optimum study period because of no growth restrictions. Thus, P815 needed to be cultured for 24 h before the experiment. When the density was over 2×10^4 /well, cells grew closer together. However, cells were insufficient when the

density was 1×10^4 /well (Supplementary Figure 5B). Therefore 2×10^4 /well were used for the follow-up experiments.

Cytotoxicity of three stimulants

C48/80, A23187 and ionomycin are common stimulants in triggering degranulation.^[25, 26] The toxicity and the degranulation rate were measured to sift the best stimulant for the following experiments. As it was shown in Supplementary Figure 6A, cells pretreated with 5 μ g/ml C48/80 for 15 min or 30 min did not exhibit significant toxicity (Supplementary Figure 6A). Thus 5 μ g/ml of C48/80 was the applicable concentration. P815 treated with 3 μ M A23187 for 60 min started to display significant toxicity (Supplementary Figure 6B). Both 1 μ M and 3 μ M of A23187 were the best option. Cells presented apparent toxicity when triggered by ionomycin from 1 μ M for 120 min (Supplementary Figure 6C). Consequently, the optimum concentration of ionomycin was 1 μ M and 0.3 μ M.

The release rate of β -hexosaminidase triggered by three stimulants

To investigate the degranulation induced by three stimulates, the release of β -hexosaminidase was measured. As the results shown in Supplementary Figure 7A, three concentrations of C48/80 could significantly increase the release rate of β -hexosaminidase. Both the concentration and the incubating time of the stimulants had a positive correlation with the release rate of β -hexosaminidase. Most pointedly, the release rate of β -hexosaminidase elevated more in the group treated for 30 min. Thus, based on cell viability data, 5 μ g/ml C48/80 pretreated cells for 30 min was the preferred option. A23187 also remarkably increased the release of β -hexosaminidase from 3 μ M to 10 μ M (Supplementary Figure 7B). Combined with the toxicity results

of A23187, 3 μ M A23187 pretreated for 30 min was the practicable condition in triggering degranulation. Similarly, the concentration and the incubating time of ionomycin had positive correlations with β -hexosaminidase release (Supplementary Figure 7C). The release rate of β -hexosaminidase in the ionomycin group elevated remarkably from 0.3 μ M. The increasing amplitude could achieve 40–80%. Thus, combined with the cytotoxicity results, 1 μ M ionomycin pretreated cells for 60 min was an applicable triggering condition.

Morphological change of P815 triggered by three stimulants

Toluidine blue staining was used to observe degranulation. Compared with the control group, degranulation cells presented lighter blue expanded cell bodies and irregular cell margins. Also, vacuolated cytoplasm was commonly observed. Notably, some cells were entirely ruptured because of serious degranulation (Supplementary Figure 8A–D). Ionomycin induced more degranulation than any other group. But it also led to more cells rupturing than other groups. In contrast, C48/80 could trigger more degranulation than A23187 (Supplementary Figure 8E). Thus, 5 μ g/ml C48/80 pretreated for 30 min was the best reagent for inducing P815 degranulation.

The effect of flavonoids from AOK on mast cell degranulation

The cytotoxicity of flavonoids from AOK

Cells pretreated with hyperoside or naringenin did not show cytotoxicity (Figure 2B and D). Acacetin could significantly reduce cell viability when pretreated with 200 μ M for 24 h (Figure 2A). Also, cells cultivated with 100 μ M luteolin for 24 h and 200 μ M luteolin for 10 h or 24 h showed a remarkable reduction in cell viability (Figure 2C).

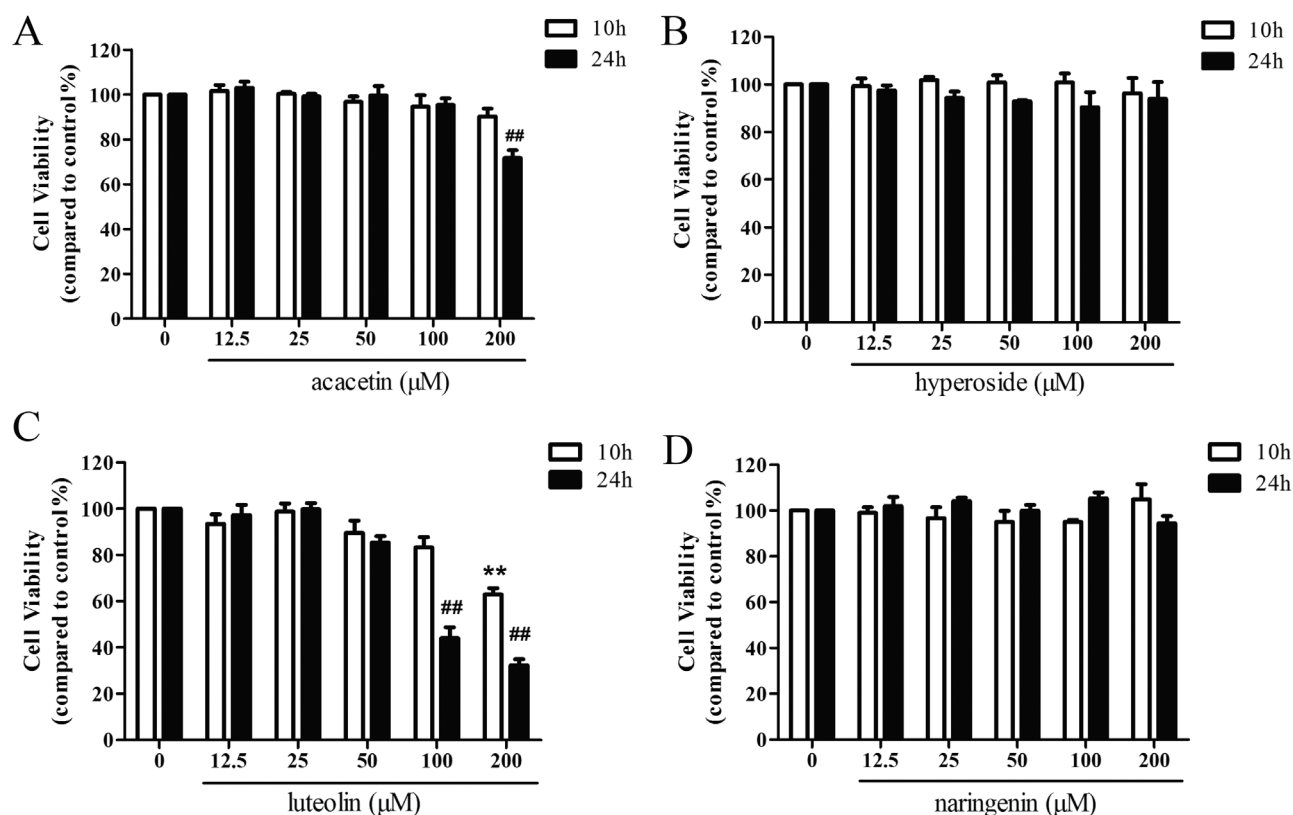


Figure 2 Cytotoxicity of four flavonoids in P815. (A) acacetin; (B) hyperoside; (C) luteolin; (D) naringenin. ** $P < 0.01$; ## $P < 0.01$ versus control group.

Effects of flavonoids on the β -hexosaminidase releasing in P815 cells

Acacetin and hyperoside did not affect the release rate of β -hexosaminidase (Figure 3A and B). Luteolin and naringenin strikingly decreased the release rate of β -hexosaminidase at both concentrations (Figure 3C and D). The release rate in the luteolin group (5 μ M) was $\sim$ 80% of that in C48/80 group, while it was $\sim$ 75% in the naringenin group (5 μ M). DXM (positive control) could reduce the β -hexosaminidase releasing $\sim$ 30% of the C48/80 group (Figure

3E). Naringenin reduced the degranulation of P815 to the same extent as DXM.

Effects of naringenin on P815 cells degranulation

Toluidine blue staining was employed to evaluate the degranulation of naringenin. As the results shown in Figure 4, C48/80 induced degranulation was twice as the control group. Both DXM and naringenin could abate the degranulation remarkably. And there was no significant difference between DXM and naringenin.

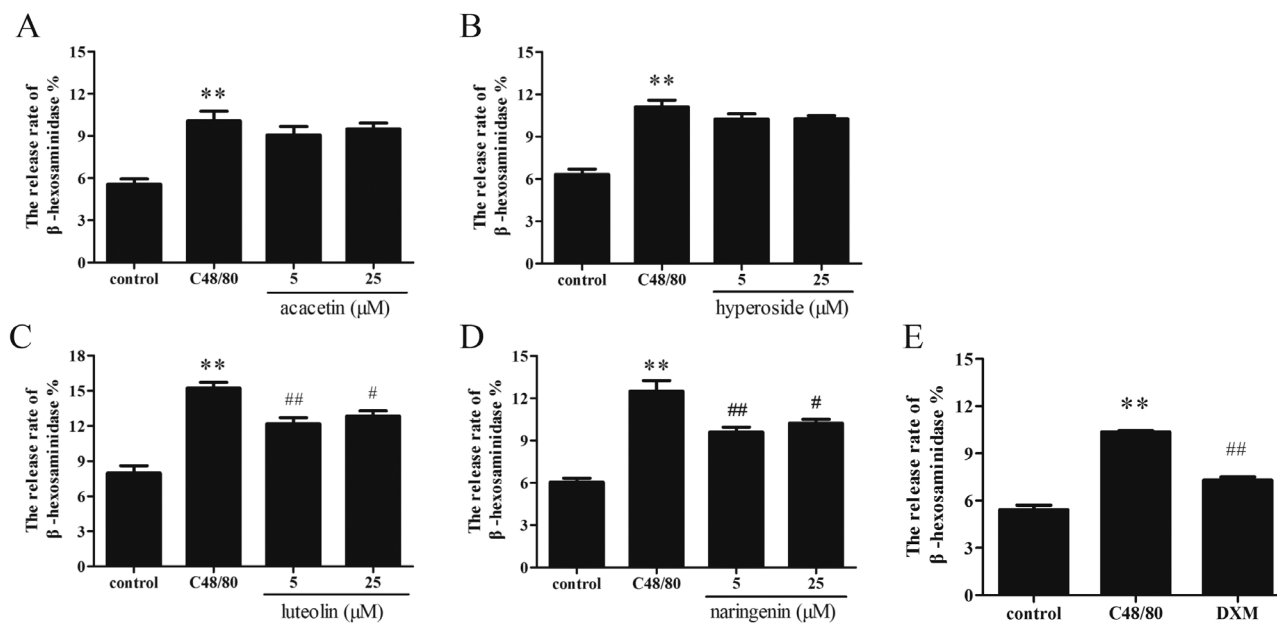


Figure 3 Effects of flavonoids on the β -hexosaminidase releasing in P815 cells. P815 cells pretreated with 5 μ M and 25 μ M flavonoids for 4 h. (A) acacetin; (B) hyperoside; (C) luteolin; (D) naringenin; (E) dexamethasone. P815 cultured with 1 μ M DXM for 24 h was regarded as positive control. ** P < 0.01 versus control group, * P < 0.05 and ## P < 0.01 versus C48/80 group.

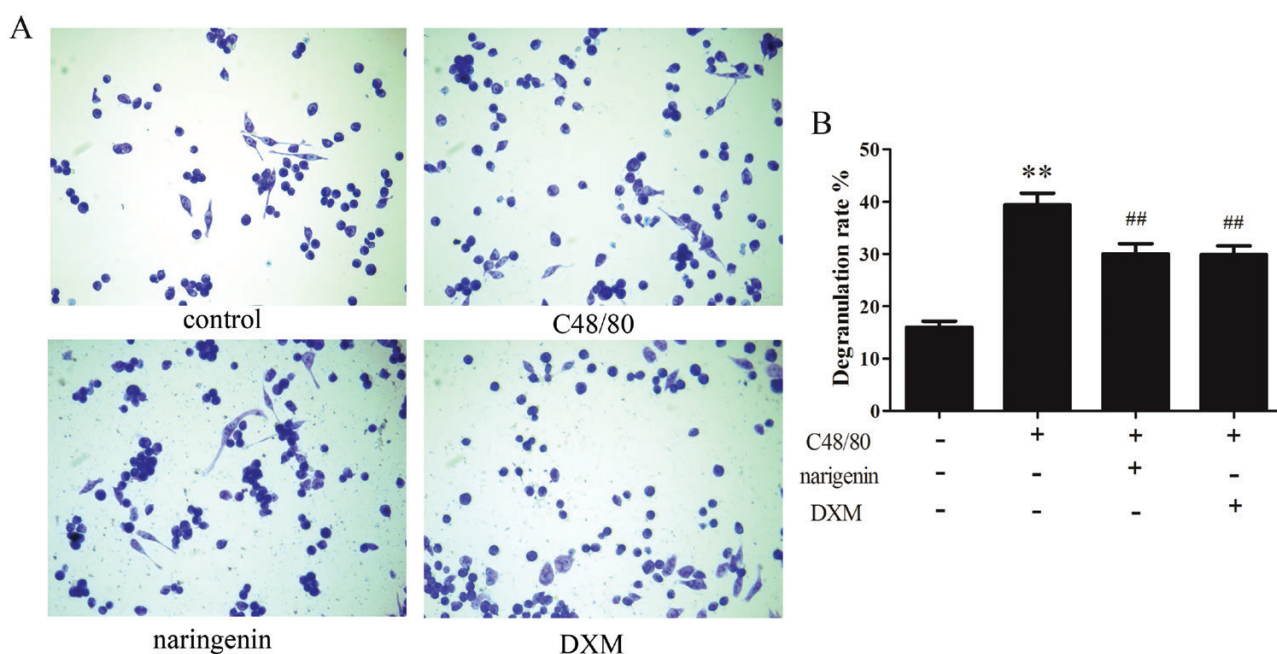


Figure 4 Effects of naringenin on the degranulation in P815 cells. (A) toluidine blue staining; (B) statistical results. P815 cells were pretreated with 5 μ M and naringenin for 4 h. P815 cells cultured with 1 μ M dexamethasone for 24 h was regarded as positive control. ** P < 0.01 versus control group, ## P < 0.01 versus C48/80 group.

Effects of naringenin on TNF- α and IL-4

TNF- α and IL-4 were assessed to investigate the effects of naringenin on inflammation-related factors. The results in Figure 5A revealed that naringenin significantly reduced the level of TNF- α , which was ~74% of the C48/80 group, while TNF- α in DXM was ~60% of the C48/80 group. P815 cells triggered by C48/80 could increase the release of IL-4. In contrast, in cells pretreated with naringenin or DXM, the IL-4 was released much more than that in C48/80 group (Figure 5B).

Network pharmacological strategy for predicting therapeutic targets of naringenin on allergic rhinitis

The shared targets of naringenin, allergic rhinitis and mast cell degranulation

A total of 2075 genes related to AR were acquired by eliminating 134 duplicate targets. Excluding the 23 duplicated genes, a total of 1443 genes associated with mast cell degranulation were obtained.

And 135 naringenin-related genes were collected from TCMSP, TCMID, STITCH and Swiss Target Prediction. The shared targets of disease and naringenin were 33 (Figure 6). The specific shared genes were shown in Table 1.

Establishment and analysis of the PPI network of shared targets

PPI network figured out the interaction of shared genes. The results of the PPI network revealed that an intricate relationship existed among the shared targets. As shown in Figure 7, there were 33 nodes and 209 edges. The shade and the size of the nodes changed with the degree value. The top 10 genes in terms of degree value were AKT1, MAPK3, VEGFA, PTGS2, SRC, CASP3, MAPK1, CAT, MMP2 and BDNF (Figure 8A). The results of three topological analyses were shown in Figure 8. Nine hub genes of naringenin were obtained, namely SRC, PTGS2, MAPK1, CASP3, CAT, MAPK3, MMP2, VEGFA and AKT1.

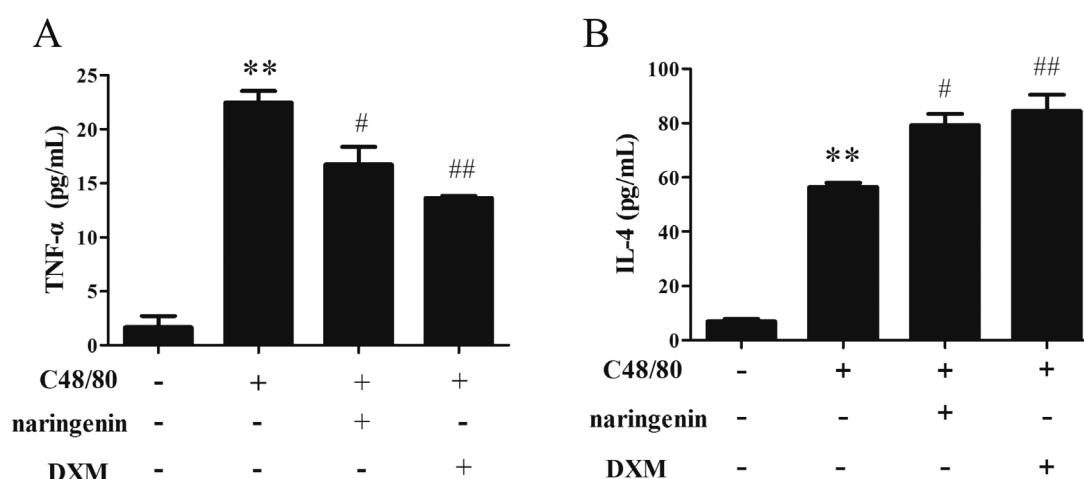


Figure 5 Effects of naringenin on TNF- α and IL-4 level in P815 cells. P815 cells pretreated with 5 μ M naringenin for 4 h. (A) TNF- α ; (B) IL-4. P815 cultured with 1 μ M dexamethasone for 24 h was regarded as positive control. ** P < 0.01 versus control group, # P < 0.05 and ## P < 0.01 versus C48/80 group.

Table 1 Protein names and gene names of the shared targets

No.	Protein	Gene	No.	Protein	Gene
1	Hepatocyte growth factor receptor	MET	18	Plasminogen activator inhibitor 1	SERPINE1
2	Mast/stem cell growth factor receptor Kit	KIT	19	Phospholipase A2, membrane-associated	PLA2G2A
3	RAC-alpha serine/threonine-protein kinase	AKT1	20	Prostaglandin E2 receptor EP4 subtype	PTGER4
4	Tyrosine-protein kinase Lck	LCK	21	Adiponectin	ADIPOQ
5	Catalase	CAT	22	Adenosine receptor A3	ADORA3
6	Superoxide dismutase [Cu-Zn]	SOD1	23	Cytochrome P450 3A4	CYP3A4
7	Peroxisome proliferator-activated receptor gamma	PPARG	24	Multidrug resistance-associated protein 1	ABCC1
8	Tyrosine-protein kinase SYK	SYK	25	Prostaglandin G/H synthase 1	PTGS1
9	Vascular endothelial growth factor receptor 2	KDR	26	Prostaglandin E2 receptor EP2 subtype	PTGER2
10	Apoptosis regulator Bcl-2	BCL2	27	Aldo-keto reductase family 1 member B1	AKR1B1
11	Glutathione S-transferase P	GSTP1	28	Heme oxygenase 1	HMOX1
12	Prostaglandin G/H synthase 2	PTGS2	29	Mitogen-activated protein kinase 1	MAPK1
13	Caspase-3	CASP3	30	Cytochrome P450 2C9	CYP2C9
14	72 kDa type IV collagenase	MMP2	31	Mitogen-activated protein kinase 3	MAPK3
15	ATP-dependent translocase ABCB1	ABCB1	32	Vascular endothelial growth factor A	VEGFA
16	Proto-oncogene tyrosine-protein kinase Src	SRC	33	Phosphatidylinositol 4,5-bisphosphate 3-kinase catalytic subunit gamma isoform	PIK3CG
17	Brain-derived neurotrophic factor	BDNF			

GO and KEGG pathway enrichment analysis

The shared genes were conducted GO and KEGG enrichment analysis to explore the mechanisms of naringenin on AR. GO enrichment analysis contained three parts: biology process (BP), molecular function (MF) and cellular component (CC). There were 272 outcomes in GO enrichment analysis, of which BP accounted for 76% (Figure 9). We selected the top 10 results of each part according to

the *P*-value to visualize the results (Figure 10). The results demonstrated that the shared genes are mainly distributed in mitochondrion, extracellular region, endoplasmic reticulum membrane, endoplasmic reticulum, extracellular region and other CC. They regulated MF such as protein serine/threonine kinase activity and protein kinase activity and affected protein phosphorylation, activation of MAPK activity, phosphatidylinositol-mediated signalling, oxidative stress and other biological processes (BP). There were 87 results in KEGG pathway enrichment analysis altogether. The bubble diagram presented the pathways with *P* < 0.01 (Figure 11). KEGG analysis showed that various signalling pathways were involved in

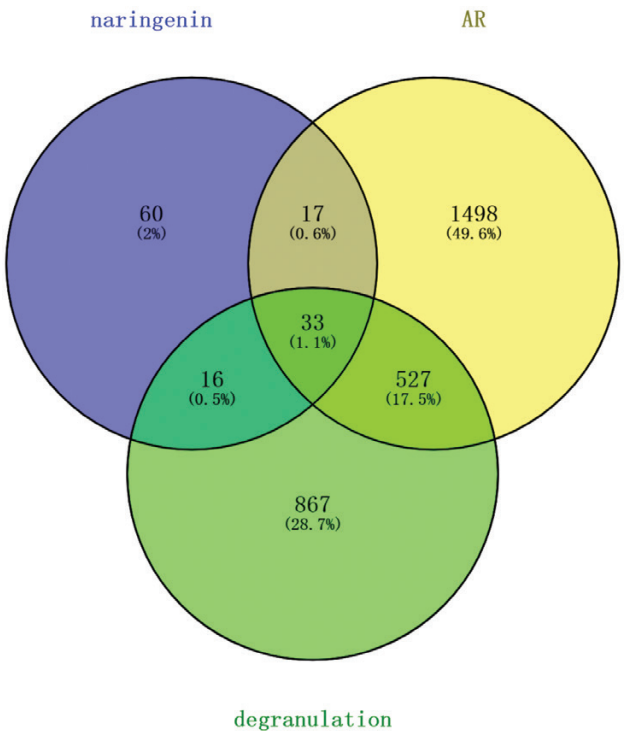


Figure 6 The Wayne chart of the shared targets.

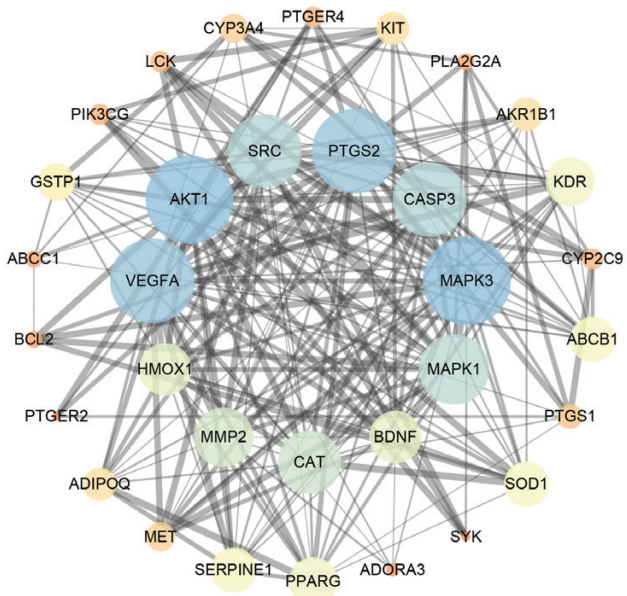


Figure 7 The protein-protein interaction (PPI) network of 33 shared targets.

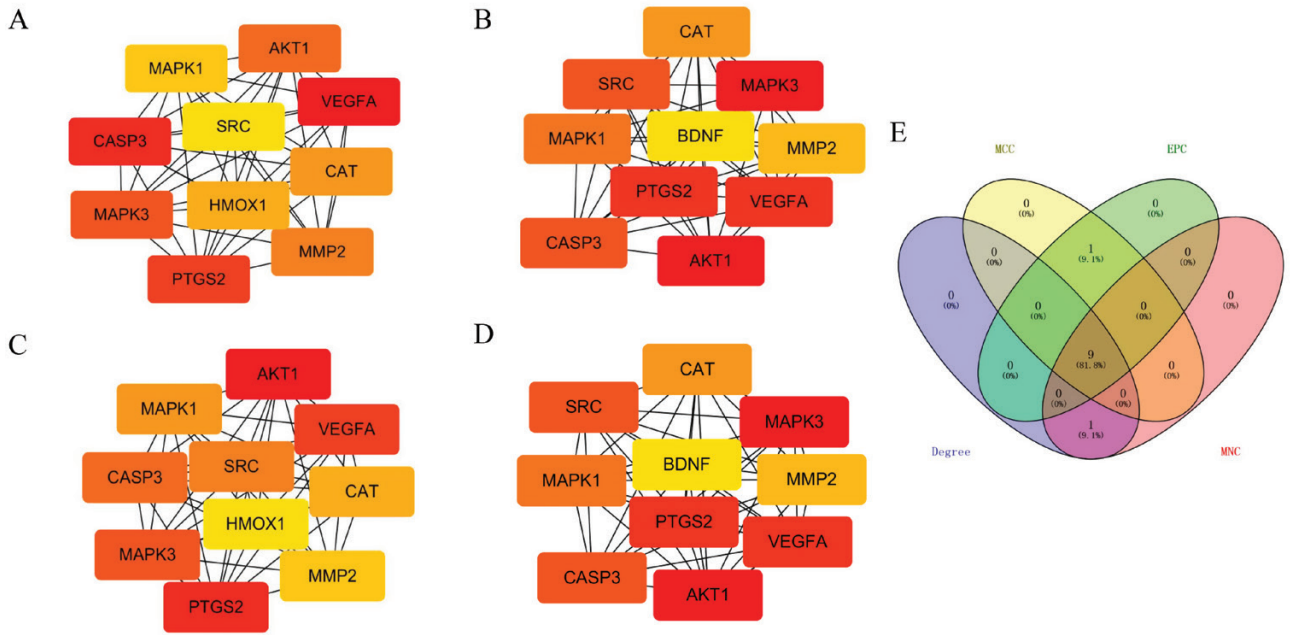


Figure 8 Topological analysis of the shared targets and Wayne diagram. (A) degree analysis; (B) MCC analysis; (C) MNC analysis; (D) EPC analysis; (E) the Wayne diagram of four analyses.

naringenin against AR (e.g. VEGF signalling pathway, pathways in cancer, NF- κ B signalling pathway, PI3K-Akt signalling pathway, HIF signalling pathway, B-cell receptor signalling pathway). Both the GO and KEGG enrichment analysis indicated that the mechanisms of naringenin against AR might be through regulating MF, BP and multiple signalling pathways.

Molecular docking of hub proteins and naringenin

Molecular docking was carried out to evaluate the interaction between hub proteins and naringenin. iGEMDOCK is a common software in predicting the interaction of ligand and protein. Binding energy illustrates the binding affinity between compound and protein. The lower the energy is, the more stable the binding conformation is. As the docking results shown in Table 2, the binding

energy of naringenin with hub proteins was close to the positive controls. The binding energy of PTGS2, CAT, MMP2 and AKT1 was much lower than the positive control, implying the binding of these hub proteins to naringenin was more stable than that of positive control. Binding conformations of naringenin with PTGS2, CAT, MMP2 and AKT1 were presented in Figure 12. Naringenin was embedded into the binding pocket of CAT and formed three H-bonds with Arg 112 (2.76 Å, 2.81 Å) and Asn 148 (2.59 Å) (Figure 12A and E). In addition, van der Waals forces were formed in naringenin with CAT at Ala 76, Lys 77, Val 74, Phe 153 and Val 116. Although naringenin formed five H-bonds with AKT1 at Gly159 (3.19 Å), Lys179 (3.24 Å), Gly294 (2.84 Å) and Thr195 (2.77 Å, 3.10 Å). However, most of these interactions are weak hydrogen bonds (Figure 12C and G). The H-bond with His 120 (2.95 Å) was the only hydrogen bond of naringenin formed with MMP 2. Leu116, Leu137, Thr 143, Tyr 42, Leu 83 and Ile 141 were also responsible for naringenin binding to MMP 2 (Figure 12B and F). Ser 530 (3.01 Å) was the only amino residues formed H-bond upon binding of PTGS2 to naringenin. Val 349, Tyr 355, Ala527, Leu 352, Phe 518, Met 522, Trp 387, Val 523 and Tyr 385 all contributed to lowering the binding energy of PTGS2 to naringenin.

Discussion and Conclusion

Mast cell degranulation is involved in AR, asthma and other allergic diseases.^[27,28] Prevention of mast cell degranulation is a vital strategy in drug development against AR. C48/80, A23187 and ionomycin are common stimulants in triggering degranulation.^[25,26] However, the types and doses of agents inducing mast cell degranulation vary from the studies. Our results showed that these three stimulants could significantly cause the degranulation of P815 without affecting cell viability. P815 treated with 5 μ g/ml C48/80 for 30 min was the best option. Cells treated with 3 μ M A23187 for 30 min or 1 μ M

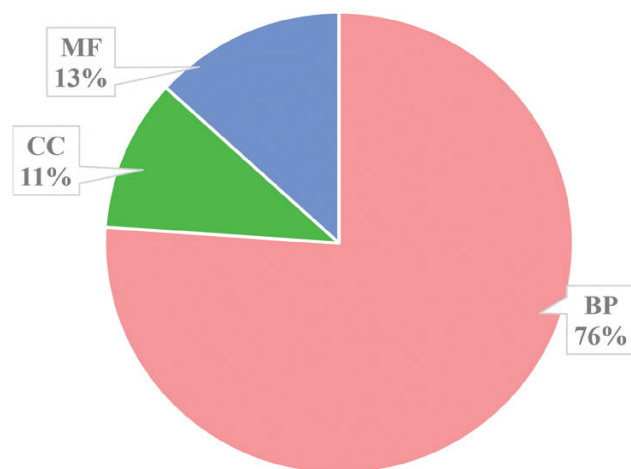


Figure 9 Pie chart of GO functional enrichment analysis.

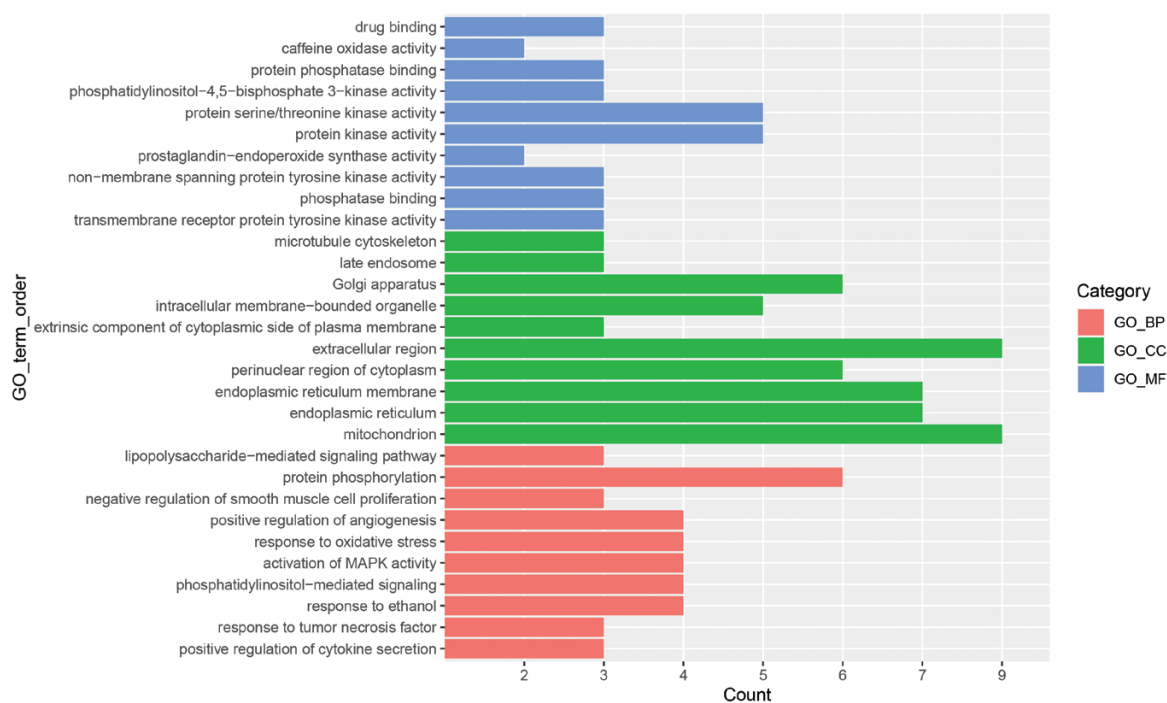


Figure 10 Bar charts of GO functional enrichment analysis.

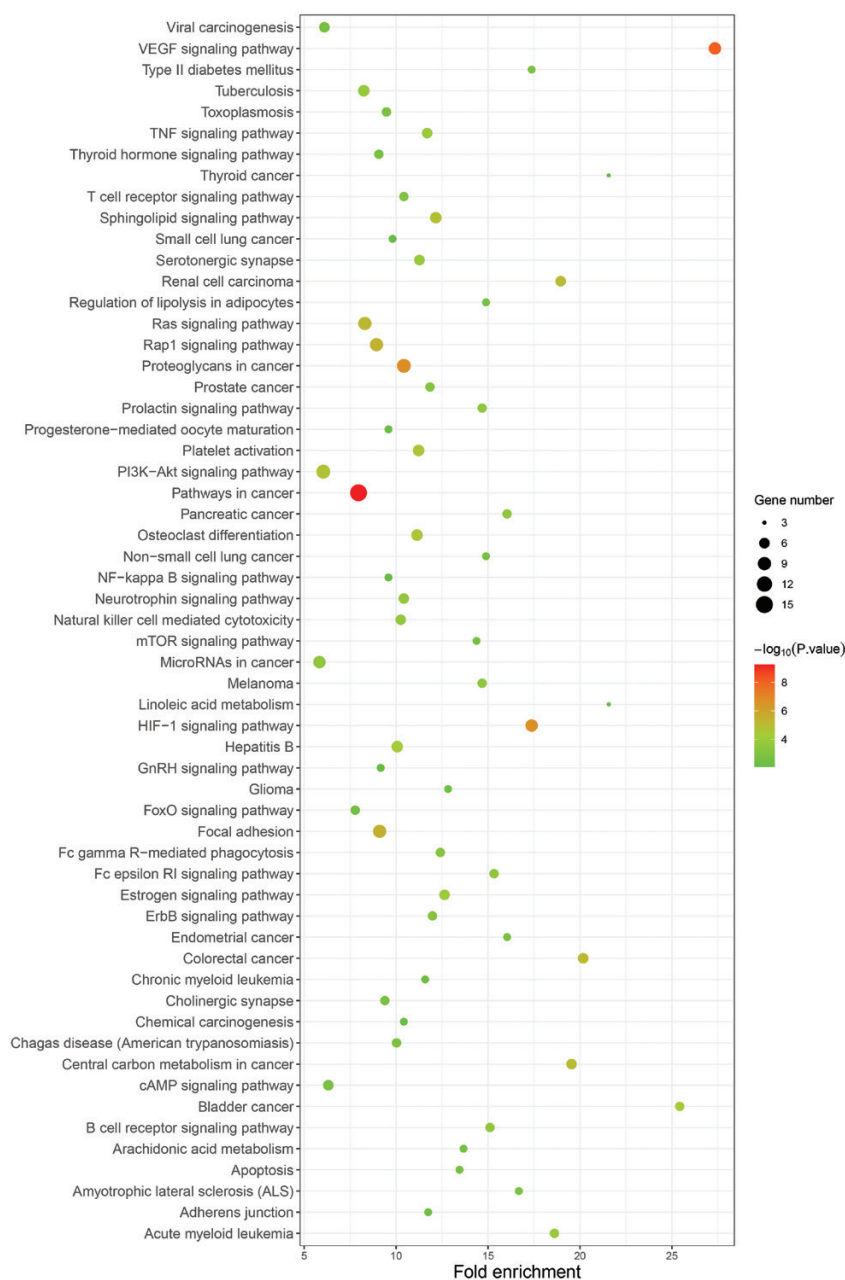


Figure 11 Bubble diagram of KEGG enrichment analysis.

Table 2 The docking results of naringenin and the hub proteins

Target	UniProt ID	PDB ID	Engery (kcal/mol)	Positive control	
SRC	P12931	6e6e	−95.72	−108.95	Tirbanibulin
PTGS2	P35354	2h8h	−108.86	−101.48	Nabumetone
MAPK1	P28482	5ikr	−101.86	−111.53	Ulixertinib
CASP3	P42574	2ojj	−109.14	−114.73	Minocycline
CAT	P04040	3kjf	−113.47	−57.11	Fomepizole
MAPK3	P27361	1dgh	−88.63	−108.71	Seliciclib
MMP2	P08253	2waj	−107.02	−79.42	Captopril
VEGFA	P15692	1hov	−84.43	−95.93	Vandetanib
AKT1	P31749	1vpf	−100.38	−96.58	Genistein

ionomycin for 1 h were both the preferred agents. Finally, C48/80 was chosen as the trigger reagent because of the higher release rate of β -hexosaminidase and fewer cell damage.

As we all know, flavonoids are the most abundant components in fruits, vegetables and food supplements.^[8] A broad range of pharmacological activities have been confirmed, such as antibacterial,

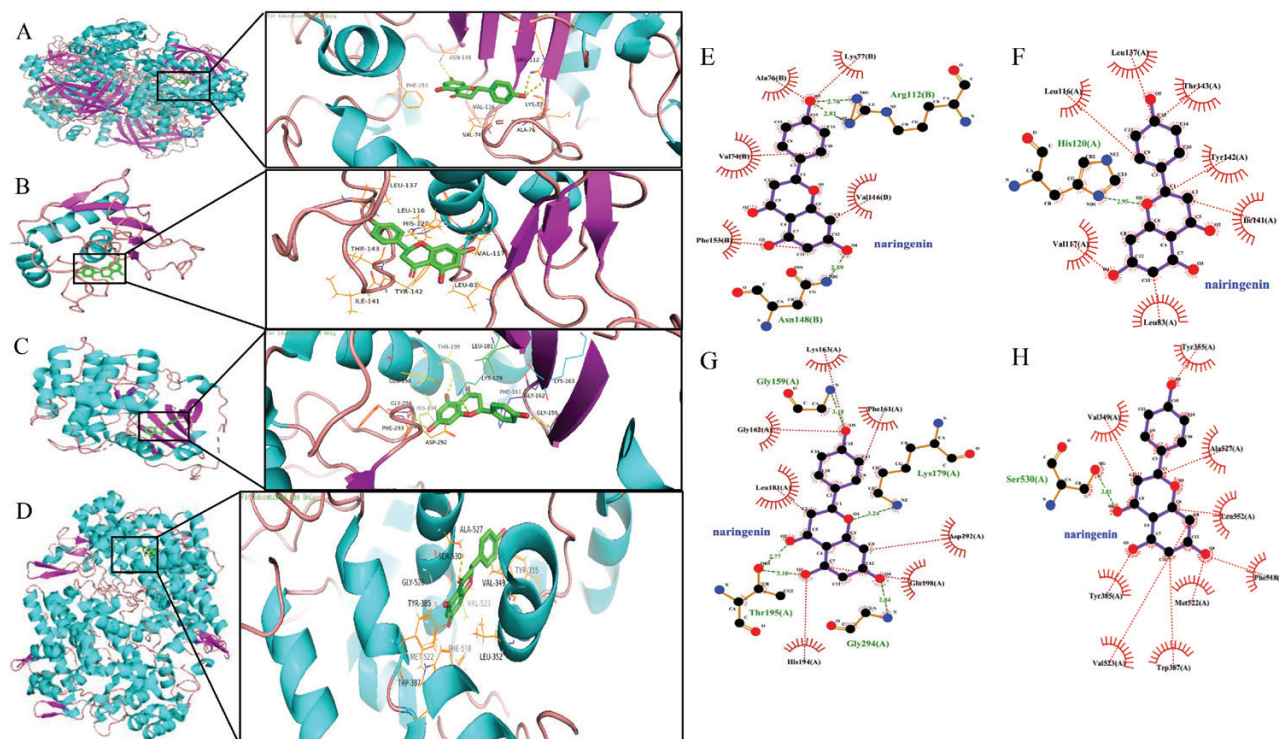


Figure 12 Main interaction of naringenin with CAT (A) and (E), MMP2 (B) and (F), AKT1 (C) and (G), PTGS2 (D) and (H).

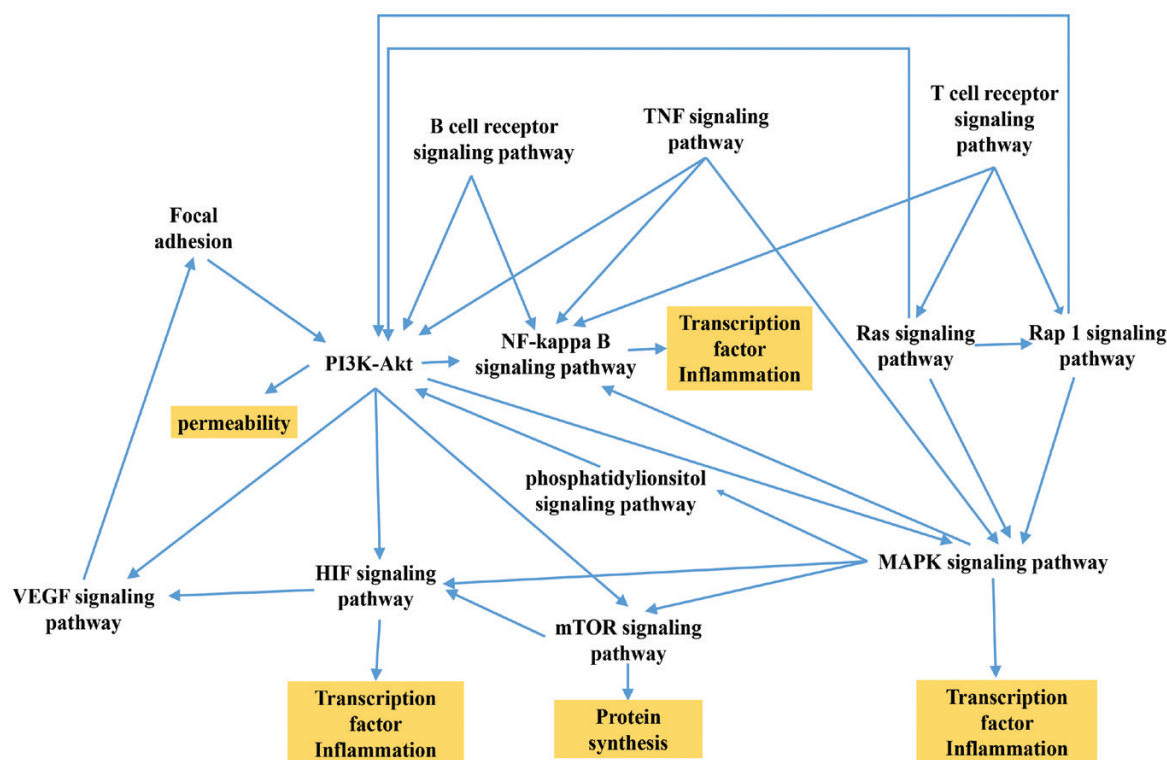


Figure 13 The interaction of each pathway.

anti-inflammatory, anti-allergic, anti-cancer.^[29] AOK is a traditional Mongolian herbal medicine. Various flavonoids such as isosakuranetin, cirsimaritin, rhamnetin, eupatolitin, genkainin and others were isolated from AOK.^[5] Although studies have found that

flavonoids from AOK have antioxidant and antibacterial effects, the anti-allergy property has not yet been investigated. Our group previously found that ethyl acetate extract of AOK ameliorated the AR symptoms and regulated the expression of inflammation-related

factors in guinea pigs. Besides, HPLC-MS analysis implied that these four flavonoids were abundant in the ethyl acetate layer.^[11] Thus, our study investigated the effects of four flavonoids that have been isolated from AOK on degranulation in mast cells. Both β -hexosaminidase release and toluidine blue staining revealed that naringenin reduced degranulation of P815 to a similar extent as the DXM. Moreover, naringenin decreased the level of the inflammatory factor TNF- α while increased the anti-inflammatory factor IL-4.

Network pharmacology is an approach to initially elucidate the targets and mechanisms of compounds on disease. In this study, 33 shared genes associated with naringenin and diseases were obtained from six databases. The PPI network of these shared genes was established. Finally, nine hub genes were identified through the analysis, namely SRC, PTGS2, MAPK1, CASP3, CAT, MAPK3, MMP2, VEGFA and AKT1. The GO and KEGG enrichment analysis indicated that the effect of naringenin affected biological processes such as activation of MAPK activity, response to oxidative stress, protein phosphorylation and phosphatidylinositol-mediated signalling through regulating pathways in cancer, VEGF signalling pathways, PI3K-Akt signalling pathway, HIF signalling pathway, Rap 1 signalling pathway, NF- κ B signalling pathway and other pathways. And these pathways are not separated but have cross-talk with each other (Figure 13). These signalling pathways above were related to the occurrence and the development of AR. Chen *et al.*^[30] found that allergic airway inflammation was associated with PI3K/Akt/HIF/VEGF pathways both in human primary cultured nasal epithelial cells and the nasal mucosa of a murine AR model. Studies also proved that PKC δ /PI3K/Akt/mTOR/HIF α /VEGF pathway played an important role in allergic airway inflammation.^[31] Also, the MAPK pathway was involved in AR pathological process.^[32] Besides, numerous studies had confirmed the anti-inflammatory property of naringenin. Park *et al.*'s^[33] research showed that naringenin inhibited the inflammation in BV2 microglia through downregulating NF- κ B and inactivating of Akt/MAPK signalling pathways. Alberca^[34] discovered the immunomodulatory properties of naringenin correlated with NF- κ B, PI3K/AKT and MAPK axis in different cell types. Furthermore, the anti-allergy effect of naringenin had been investigated *in vivo* and *in vitro*. However, none of these discussed molecular mechanisms.^[35, 36]

The molecular docking analysis found naringenin could embed in the binding pocket of hub proteins through hydrogen bonds and van der Waals forces. Notably, the binding energy between naringenin with PTGS2, CAT, MMP2 and AKT1 was much lower than the positive controls. It indicated that naringenin combined more stable with these hub proteins.

Based on the group's previous research on the ethyl acetate extract of AOK, our study further identified naringenin as one of the active components of AOK against AR and preliminarily elucidated its mechanism by network pharmacology and molecular docking. However, there are several limitations of network pharmacology and molecular docking as follows. (1) Network pharmacology is based on the spectral data obtained from experiments. Alternatively, most of the spectral information currently used for network calculations is from different experimental conditions. Therefore the results may occasionally present false negatives. (2) The number of small molecule compounds and their targets that have been evaluated is limited, so it still cannot fully reveal their pharmacological effects. (3) Molecular docking is the result of semi-flexible docking. Although the binding of small molecules and proteins can be highly mimicked, the conformation of small molecules and proteins will change *in vivo* due to their flexible structure. (4) Since the naringenin molecule has

several hydroxyl groups, it is easier to form hydrogen bonds with protein, so the binding energy of molecular docking is much lower. Therefore, the binding of naringenin with the hub protein needs to be further confirmed by experiments. In summary, our study provided a preliminary theoretical basis for AR treatment with AOK extract. There needs further support to explore AOK therapeutic use in clinical medicine due to the limitations of network pharmacology and molecular docking technology.

Supplementary Material

Supplementary data are available at *Journal of Pharmacy and Pharmacology* online.

Author Contributions

YN and ZS performed the experiments. QZ and CS analyzed the data. YN and ZS collected data and interpreted the results. SW provided intellectual support and research assistance. YN, ZS and CS edited the manuscript. BX revised the manuscript.

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Conflict of Interest

All author declared that no conflict of interests exists.

Data Availability

All original data generated in the course of the study or the third-party data analyzed in the article appear in the submitted article.

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