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ORIGINAL RESEARCH

Effects and Potential Mechanism of Zhuyu Pill Against Atherosclerosis: Network Pharmacology and Experimental Validation

Yingying Pan[1,*], Xianrong Feng[2,*], Wei Song[1,*], Xin Zhou[1], Zhen Zhou[3], Gaoyang Chen[1], Tao Shen[1], Xiaobo Zhang[1]

[1]School of Basic Medicine, Chengdu University of Traditional Chinese Medicine, Chengdu, People's Republic of China; [2]Hospital of Chengdu University of Traditional Chinese Medicine, Chengdu, People's Republic of China; [3]Menzies Institute for Medical Research, University of Tasmania, Hobart, Tasmania, Australia

*These authors contributed equally to this work

Correspondence: Xiaobo Zhang; Tao Shen, School of Basic Medicine, Chengdu University of Traditional Chinese Medicine, Chengdu, People's Republic of China, Email zhangxiaobo@stu.cdutcm.edu.cn; shentaotcm@aliyun.com

Background: Atherosclerosis (AS) is an immunoinflammatory disease associated with dyslipidemia. Zhuyu Pill (ZYP) is a classic Chinese herbal compound that has been shown to exhibit anti-inflammatory and lipid-lowering effects on AS in our previous studies. However, the underlying mechanisms by which ZYP ameliorates atherosclerosis have not yet been fully investigated. In this study, network pharmacology and in vivo experiments were conducted to explore the underlying pharmacological mechanisms of ZYP on ameliorating AS.

Methods: The active ingredients of ZYP were acquired from our previous study. The putative targets of ZYP relevant to AS were obtained from TCMSP, SwissTargetPrediction, STITCH, DisGeNET, and GeneCards databases. Protein-protein interactions (PPI) network, Gene Ontology (GO), and Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis were conducted using the Cytoscape software. Furthermore, in vivo experiments were carried out for target validation in apolipoprotein E (ApoE) -/- mice.

Results: Animal experiments revealed that ZYP ameliorated AS mainly through lowering blood lipids, alleviating vascular inflammation, and decreasing the levels of vascular cell adhesion molecule-1 (VCAM1), intercellular adhesion molecule-1 (ICAM1), monocyte chemotactic protein-1 (MCP-1), interleukin 6 (IL-6), and tumor necrosis factor-α (TNF-α). Additionally, the results of Real-Time quantitative PCR revealed that ZYP inhibited the gene expressions of mitogen-activated protein kinase (MAPK) p38, extracellular regulated protein kinases (ERK), c-Jun N-terminal kinase (JNK), and nuclear factor kappa-B (NF-κB) p65. The Immunohistochemistry and Western blot assays showed the inhibitory effect of ZYP on the proteins level of p38, p-p38, p65, and p-p65.

Conclusion: This study has provided valuable evidence on the pharmacological mechanisms of action of ZYP in ameliorating AS that will be useful for forming the rationale of future research studying the cardio-protection and anti-inflammation effects of ZYP.

Keywords: atherosclerosis, Zhuyu Pill, network pharmacology, MAPK, NF-κB

Introduction

Atherosclerosis (AS) is an immunoinflammatory disease and a leading cause of atherosclerotic cardiovascular disease.[1] AS is characterized by the plaque buildup (fatty deposits) in the inner lining of the arteries that could narrow and obstruct arteries, leading to a heart attack or stroke.[1,2] Statins, a class of lipid-lowering drugs, are the mainstay therapy used for preventing AS and cardiovascular disease. However, the adherence to the statin treatment is commonly low. The most common reason for statin discontinuation is statin-associated adverse events, mainly muscle problems, occurring in approximately 7–29% statin users.[3] Some patients are reluctant to take statins at the first place due to concerns of drug side effects.[4]

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Chinese herbal medicine (CHM) is generally considered to be a safe, effective form of complementary medicine that may be used as an adjunct to statins or an alternative treatment for AS and has a favorable safety profile.[5] Zhuyu Pill (ZYP) is a traditional prescription of two CHM mixtures, including *Coptis chinensis* Franch. (Huanglian) and *Tetradium ruticarpum* (A. Jussieu) T. G. Hartley (Wuzhuyu). The formula was firstly documented in the *Taiping Shenghui Fang*, a well-known ancient medical text used for treating gastrointestinal disorders.[6] Many previous studies have shown that ZYP can lower blood lipids, reduce inflammation, and improve cholesterol metabolism.[6–8] Chemical investigations suggested that ZYP mainly contained alkaloids components. According to our previous study, the main bioactive compounds of ZYP include berberine, coptisine, evodiamine, and rutaecarpine, which remained in the blood after the gastrointestinal absorption. Berberine, a quaternary ammonium alkaloid isolated from Huanglian, has been demonstrated to regulate lipid metabolism.[9] Some studies have shown a beneficial effect of berberine for AS by lowering serum lipid levels, improving endothelial dysfunction, inhibiting macrophage inflammation and foam cell formation, activating macrophage autophagy, and regulating the proliferation and migration of vascular smooth muscle cells (VSMCs).[10] Wu et al found that berberine improved serum lipids and systemic inflammation in apolipoprotein E (ApoE) -/- mice with AS induced by high-fat diet.[11] Oxidized low density lipoprotein (ox-LDL) within plaques is regarded as playing a key role in AS pathogenesis. In vitro, it has been proved that berberine inhibited ox-LDL-induced HUVECs proliferation by decreasing the expression of proliferating cell nuclear antigen (PCNA), nuclear factor κB (NF-κB) and oxidized low density lipoprotein receptor 1 (LOX-1), suggesting an anti-atherogenic effect of berberine in the clinic.[12] Another major herb in ZYP, Wuzhuyu, has also been demonstrated that has anti-inflammatory and vascular protective effects.[13] Evodiamine, which is the main active component of Wuzhuyu, has been demonstrated to attenuate VSMC migration through PPAR activation, showing a promising anti-atherogenic effect.[14] The evidence altogether suggests that ZYP has a potential for ameliorating AS by virtue of its anti-inflammatory and lipid-lowering properties.[15] However, the pharmacological mechanisms of action of ZYP for AS has not yet been clear need further investigation.

Because Chinese herbal formula (CHF) like ZYP comprises multiple components, it is difficult to determine how compounds act on different targets to produce synergistic therapeutic effects. Over the recent years, network pharmacology has been increasingly used to investigate the intricate mechanisms of CHM and CHF, which is aligned with the holistic notion of traditional Chinese Medicine (TCM). Several studies have successfully used this approach to investigate the complex mechanism of CHF.[16] In the present study, with the use of multiple databases and computational tools, we sought to investigate the potential molecular mechanisms and pathways of ZYP on AS by constructing a pharmacological network. Further in vivo experiments were used to verify the identified molecular mechanisms and pathways.

Materials and Methods

Collection of Chemical Components and Targets of ZYP

The bioactive components of ZYP were acquired from our previous study. They are berberine, coptisine, evodiamine, and rutaecarpine.[17] Targets of these four chemical components were obtained from the TCMSP database (https://old.tcmsp-e.com/tcmsp.php), the SwissTargetPrediction database (http://www.swisstargetprediction.ch/), and the STITCH database (http://stitch.embl.de/). Since the targets' names collected from the TCMSP database were not standardized, they were transformed using the UniProt database (https://www.uniprot.org). Targets collected from different databases were merged and the duplicates were removed.

Collection of Disease-Associated Targets

The AS-related genes were collected from the following databases: DisGeNET (https://www.disgenet.org/search) and GeneCards (www.genecards.org), using the keyword "atherosclerosis" and the targets enrolled in this study were human genes.

Protein-Protein Interaction (PPI) Data

PPI network was constructed using the overlapped genes between the targets of ZYP active ingredients and AS.[18] We uploaded the genes to the STRING database (https://string-db.org/), with the species limited to "Homo sapiens", and

correlation degree greater than 0.400, as the cut-off confidence score. The common gene targets are shown in a Venn diagram (http://bioinformatics.psb.ugent.be/webtools/Venn/). Next, the network mapping software Cytoscape 3.9.1 (http://www.cytoscape.org) was used to establish a "compounds-targets" network.

GO and KEGG Enrichment Analyses

To further understand the functions of the overlapped genes, the online database Metascape (https://metascape.org) was used to perform GO and KEGG enrichment analyses. The data obtained from the Metascape were then plotted using a bioinformatics online tool (http://www.bioinformatics.com.cn/).

Reagents

Huanglian and Wuzhuyu tablets were bought from the affiliated Hospital of Chengdu University of TCM. The triglycerides (TG) (Cat: A110-1-1), total cholesterol (TC) (Cat: A111-1-1), high-density-lipoprotein cholesterol (HDL-C) (Cat: A112-1-1), and low-density-lipoprotein cholesterol (LDL-C) (Cat: A113-1-1) kits were purchased from Nanjing Jiancheng Bioengineering Inc. (Nanjing, China). The tumor necrosis factor-α (TNF-α) (Cat: MM-0132M1), interleukin 6 (IL-6) (Cat: MM-46428M2), vascular cell adhesion molecule-1 (VCAM-1) (Cat: MM-0129M1), intercellular adhesion molecule-1 (ICAM-1) (Cat: MM-0183M1), and monocyte chemotactic protein-1 (Cat: MCP-1) (MM-0082M1) kits were purchased from Meimian Biotechnology (Nanjing, China). Anti-beta actin (Cat: ab8226) was taken as the loading control and purchased from Abcam (Shanghai, China). Mitogen-activated protein kinase (MAPK) p38 antibody (Cat: BF8015) and phospho-MAPK (Thr180/Tyr182) p38 antibody (Cat: AF4001) were purchased from Affinity (Liyang, China). NF-κBp65 (Cat: A2547) and phospho-NF-κB p65 (Cat: AP0124) were purchased from Abclonal (Wuhan, China). Anti-rabbit and mouse secondary antibodies (Cat: E-AB-1003, E-AB-1001) were purchased from Elabscience (Wuhan, China).

ZYP Preparation

ZYP decoction was prepared with Huanglian (*Coptis chinensis* Franch., 5g, Cat: 21080108) and Wuzhuyu (*Tetradium ruticarpum* (A. Jussieu) T. G. Hartley, 5g, Cat: 21110103). The herb mixtures were boiled two times in water. For the first time, the Huanglian and Wuzhuyu tablets were mixed to 40 g at a ratio of 1:1 and heated with 500 mL water at 100 °C for 30 min, and then filtrated to gather the filtrate. Herb broth was collected at the second time by adding 250 mL water to the filtered crude herbs, boiling for another 30 minutes and filtering. After that, a freeze-drying step followed the concentration of both herb broth. According to our previous high performance liquid chromatography (HPLC) results, ZYP contained 36.8 mg/g of berberine, 14.9 mg/g of coptisine, 0.78 mg/g of evodiamine, and 0.33 mg/g of rutecarpine.[17]

Animals and Treatment

Forty 6-8-week-old male ApoE-/- mice and ten 6-8-week-old male C57 mice weighing 20±2g were purchased from Gempharmatech Co., Ltd, Nanjing, China [license number: SCXK (Sichuan) 2020-034]. The mice were adaptively fed for 1 week and had free access to water and food ad libitum. Subsequently, six of C57 mice were randomly selected as the control group (CON, n=6). The ApoE-/- mice were randomised into the following groups: AS model group (MOD, n=6), Atorvastatin group (ARA, 1.5 mg/kg, n=6), low-dose ZYP group (ZYP-L, 1.5 g/kg, n=6), and high-dose ZYP group (ZYP-H, 3g/kg, n=6). The dose of ZYP and ARA were selected as suggested by our previous study results.[19] The dose of raw herbs in ZYP available to normal humans each day was converted to the dose for mice, 1.5g/kg of ZYP was treated as a low dose and 3g/kg as a high dose. The control group was fed with a purified feed (D10012M), and the remaining 4 groups were fed with high-fat feed (D12108C, with regular casein and 1.25% added cholesterol). All the mice were weighed twice a week, and blood was sampled from the mice's tail vein to test lipid levels once a week. After 8 weeks intervention, all the mice fasted for 12h after the final food intake and then were executed for sampling via sodium pentobarbital anesthesia. All procedures in animal experiments were conducted in accordance with the Regulations on Animal Experiments established by the Animal Ethical Committee of Chengdu University of Traditional Chinese Medicine (Ethical No. 2022-23). All the procedures were made gently and quickly in order to minimize the suffering of animal models.

Histological Analysis of Aorta

The aortic arches were fixed in 4% paraformaldehyde for 48h. The fixed tissues were dewatered by a fully automatic dewatering machine. The aortic arches were cut into sections after paraffin embedding. The sections were stained with hematoxylin (Cat: G1004-500ML, Servicebio, Wuhan, China) and eosin (Cat: G1001-500ML, Servicebio, Wuhan, China) (H&E), Oil Red O (Cat: YO7512, Hefei Bomei Technology Co., Ltd., Hefei, China) solution and Masson trichrome stain (Cat: BMB1625, Hefei Bomei Technology Co., Ltd., Hefei, China). Under the microscope, lipid-rich plaque was observed and analyzed with ImageJ.

Cytokine Expression Assessment

The concentrations of TNF-α, IL-6, VCAM-1, ICAM-1, and MCP-1 in the serum were measured using ELISA kits in accordance with the kits' instructions. The final concentrations were calculated based on corresponding standard curves.

Real-Time Quantitative PCR (RT-qPCR) Assay

Total RNA was extracted from the mice aorta tissue using Animal Total RNA Isolation Kit (Cat: RE-03014, FOREGENE, Chengdu, China) and reverse-transcribed into cDNA using PrimeScript RT Reagent Kit (Cat: RR037A, TAKARA BIO INC, Beijing, China) in accordance with the standard protocol. The Real-Time Quantitative PCR (RT-qPCR) assay was conducted using TB Green PremixEX Taq (Cat: RR820A, TAKARA BIO INC, Beijing, China) with the Applied CFX96 Real-Time PCR Detection System. Primer pair sequences are shown in Table 1. In order to quantify the relative levels of targeted gene mRNA transcripts to the control glyceraldehyde-3-phosphate dehydrogenase (GAPDH), the $2^{-\Delta\Delta Ct}$ method was used.

Immunohistochemical Staining

The expression of MAPKp38 and NF-κBp65 in aortic arches tissues were visualized by immunohistochemistry. 4% paraformaldehyde was used to fix the aortic arch tissues, which were then embedded in the paraffin to make slices. Tissues were dewaxed, rehydrated, and blocked with 3% bovine serum albumin (BSA), the sections were incubated with MAPKp38 and NF-Bp65 antibodies (ABclonal Technology Co., Ltd., Wuhan, China, 1:100) at 4 degrees overnight. Next, the sections were incubated with secondary antibodies (Rabbit Anti-Goat IgG, Servicebio, Wuhan, China, 1:100) connected with HRP. After staining with DAB and re-staining with hematoxylin, images were observed under a microscope and the protein expression were analyzed using ImageJ.

Western Blot Assays

A small amount of arterial blood vessel tissues was thoroughly homogenized at low temperature with protein extraction reagent, ice bath for 30min to ensure that the homogenate was fully lysed, then centrifuged at 4°C at 12000rpm for 5min and the supernatant was collected as the total protein solution. The protein concentration was measured using a Bicinchoninic Acid Assay (BCA) kit. A certain amount of denatured proteins were separated by sodium dodecyl sulfate-polyacrylamide (SDS-PAGE) and transferred to a PVDF membrane. The membrane was incubated overnight at 4°C with primary specific antibodies, anti-p38MAPK (1:5000), phospho-p38MAPK (1:1000), anti-NF-κBp65 (1:1000), phospho-NF-κBp65 (1:2000), and β-actin (1:10,000). After washing three times, followed by incubation with secondary

Table 1 The Primer Sequences

Gene Sample	Forward Primer	Reverse Primer
GAPDH	GGTTGTCTCCTGCGACTTCA	TGGTCCAGGGTTTCTTACTCC
MAPKp38	AGGAATTCAATGACGTGTACCT	AGGTCCCTGTGAATTATGTCAG
ERK	CAGCTCAACCACATTCTAGGTA	TCAAGAGCTTTGGAGTCAGATT
JNK	ACCAGAGGTCATTCTCGGCA	TGGGAACAAAACACCACCTTTG
NF-κBp65	AGACCCAGGAGTGTTCACAGACC	GTCACCAGGCGAGTTATAGCTTCAG
IκB-α	CTGGTTTCGCTCTTGTTGAAAT	GGGTAGCATCTGGAGATTTTCC

antibodies (1:3000) at room temperature for 60 min. Enhanced chemiluminescence reagents were used for detection, the results were analyzed by the Image J software.

Statistical Analysis

The results of all experiments are expressed as the mean ± standard deviation (SD). The statistical analyses were performed using GraphPad Prism 8.3.0 software. The differences between groups were evaluated using a one-way analysis of variance (ANOVA). $P<0.05$ was considered to be statistically significant.

Results

Compounds Acquisition and Target Fishing

The four main active ingredients of ZYP (berberine, coptisine, evodiamine, and rutaecarpine) are shown in Table 2. TCMSP, Swiss Target Prediction database, and STITCH database were used to obtain the targets of four compounds. Genes that did not have official gene symbols were excluded. Among 382 active targets being identified, 113 duplicates were removed, leaving 269 included in our analysis.

AS Targets Screening

A search of the Genecards and DisGeNET databases for "atherosclerosis" yielded 6754 active targets. After removing 1568 duplicates, 5186 valid targets were included in the analysis. A total of 198 overlapping genes between the above compounds and AS targets were identified (Figure 1A and B).

PPI Network Analysis

The compound target-AS target PPI network consisting of 198 nodes and 2065 edges with medium connectivity (interaction score ≥0.400) was derived from the data and were then entered into the STRING database. PPI network analysis by Cytoscape and its plug-in MCODE showed that three nodes of the MAPK pathway, MAPK14 (MAPKp38), MAPK1 (ERK) and MAPK8 (JNK) were the key targets (Figure 2A–C).

GO and KEGG Enrichment Analysis

GO enrichment analysis showed that the 198 common targets were primarily enriched in protein phosphorylation, blood circulation, regulation of ion transport, cellular response to lipid, positive regulation of MAPK cascade, inflammatory response, regulation of inflammatory response, and so forth. Significant pathways involved in the lipid metabolism and AS development were identified through the KEGG enrichment analysis which included cAMP signaling pathway, calcium signaling pathway, and NF-κB signalling pathway, the Wnt signaling pathway also ranked high in terms of impact (Figure 3A–B).

Effects of ZYP on Body Weight and Blood Lipid Levels of Mice

The CON group of C57 mice had the greater body weights than the other groups of ApoE-/- mice. The weight of all groups increased with time. The mouse weight of ARA and ZYP groups grew slower. ZYP-H shows a clear effect in slowing weight gain (Figure 4A). The serum levels of the mice, including TC, TG, LDL-C, and HDL-C were detected to

Table 2 The Main Compounds of ZYP

Mol ID	Compounds	OB (%)	DL
MOL001454	Berberine	36.86	0.78
MOL001458	Coptisine	30.67	0.86
MOL003958	Evodiamine	86.02	0.64
MOL002662	Rutaecarpine	40.30	0.60

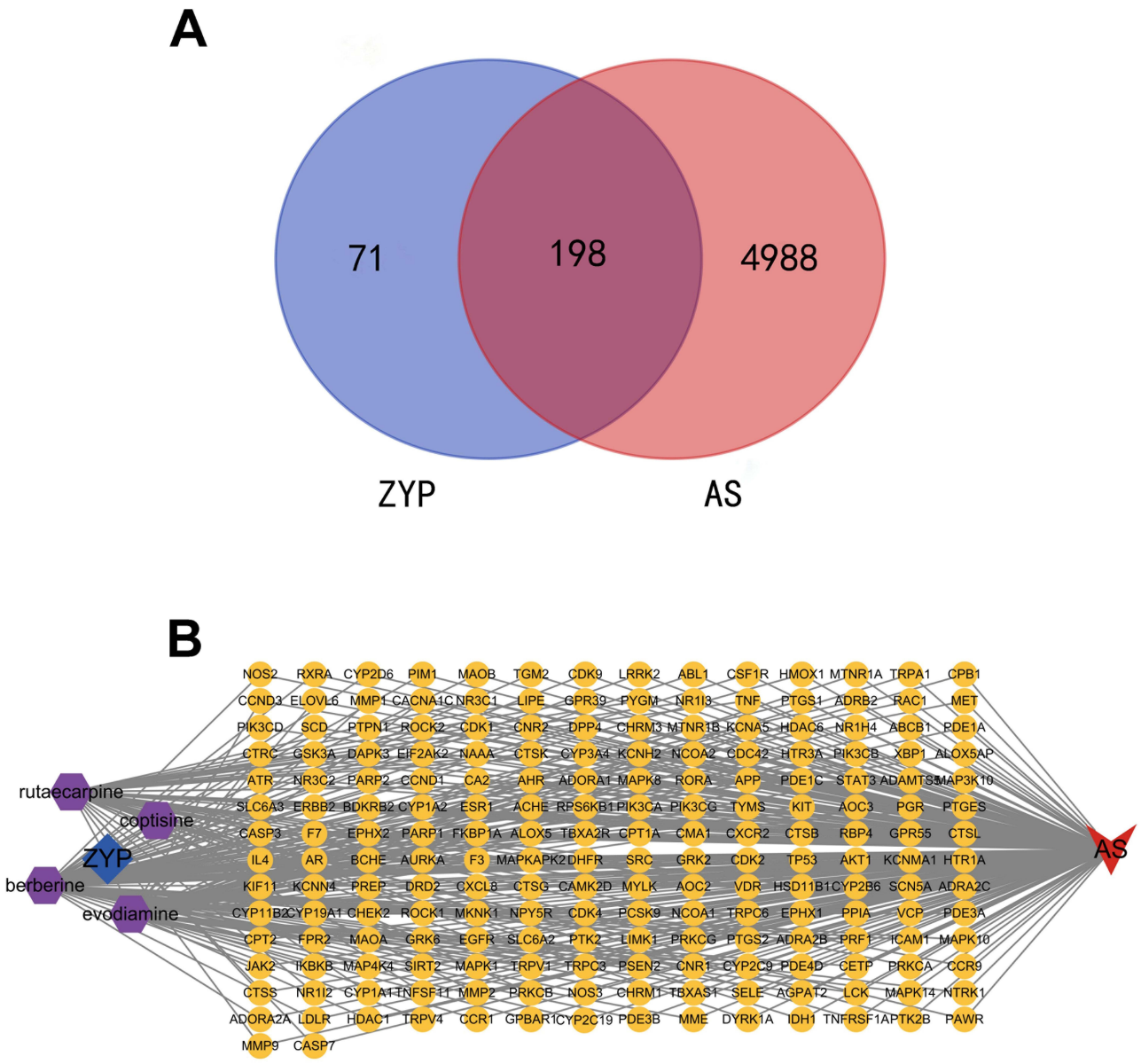

Figure 1 Targets prediction and analysis. (**A**) The Venn diagram of overlapped targets between ZYP and AS. (**B**) Ingredient–target network of AS.

assess the effect of ZYP on blood lipids. Figure 4B–D show that serum TC, TG, and LDL-C levels were lower in CON group. Compared with the CON group, the serum TC, TG and LDL-C levels were significantly higher in the MOD group (P<0.01). The groups treated with 8-week ZYP and ARA had a significantly lower TC, TG, and LDL-C level compared with the MOD group. (P<0.01). In contrast, MOD group had lower HDL-C levels than the CON group, but the values elevated after ZYP intervention (Figure 4E). These results revealed a beneficial effect of ZYP on the lipid metabolism of mice with AS (Figure 4B–E).

ZYP Reduced Lipid Deposition in the Aorta of Mice

The H&E and the Oil Red O-staining revealed the presence of aortic plaque buildups. As shown in Figures, the MOD group displayed greater lipid accumulation in aorta arches than the CON group (P< 0.01). Compared to the MOD group, the atherosclerotic lesions were reduced in the ZYP treated and ARA-treated groups (P<0.01). In addition, the atherosclerotic lesion in the ZYP-H-treated group was smaller than it in the ZYP-L-treated group. High fat diet induced-

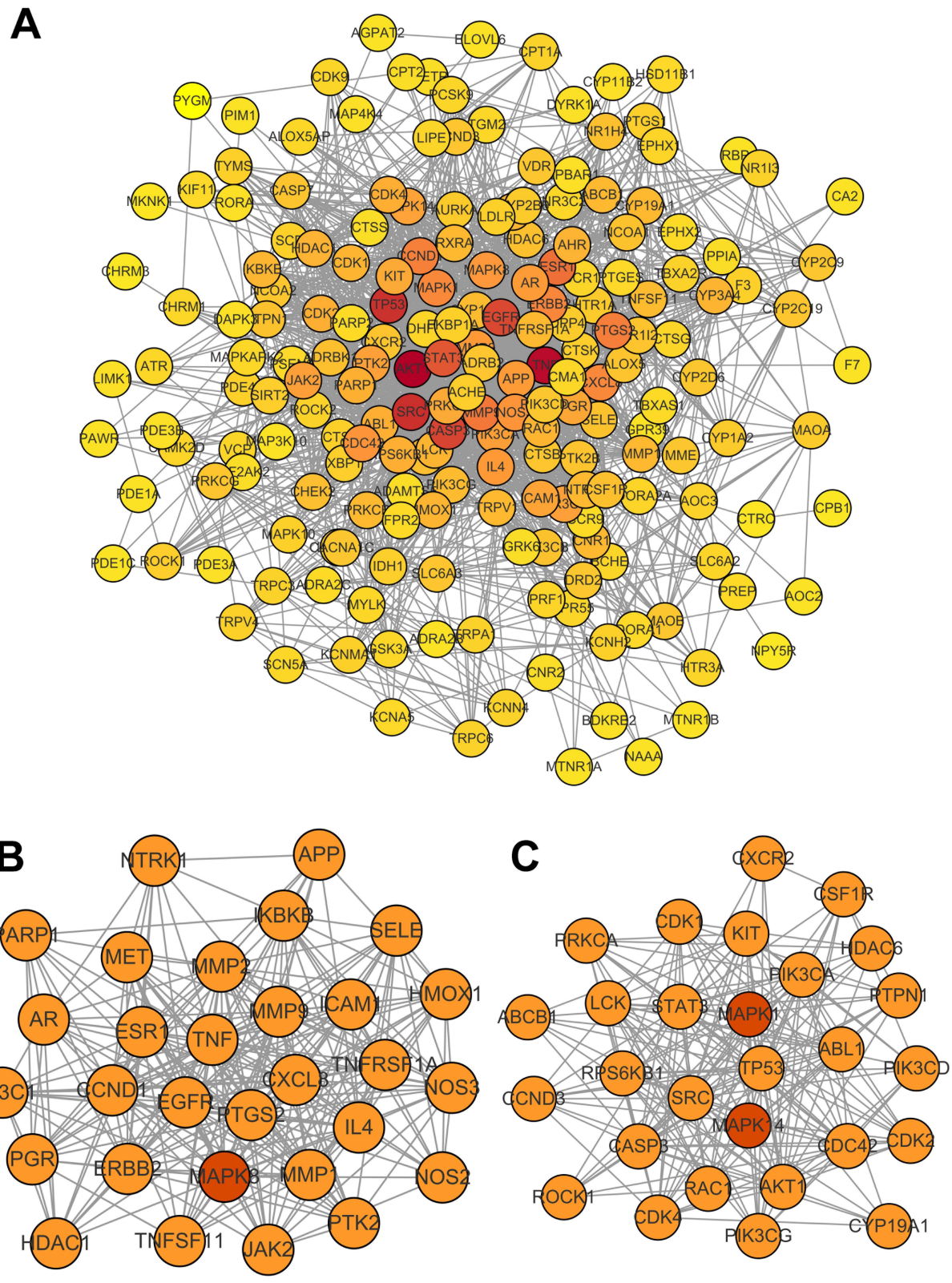

Figure 2 (**A**) PPI network of common targets. (**B** and **C**) The cluster module in the PPI network was obtained by MCODE plug in Cytoscape.

ApoE-/- mice receiving ZYP showed a significantly smaller AS plaques, and the ZYP intervention effects were dose-dependent (Figure 5A–D). According to the results of Masson staining, ZYP or ARA increased collagen content. However, the difference across groups was not statistically significant (Figure 5E–F).

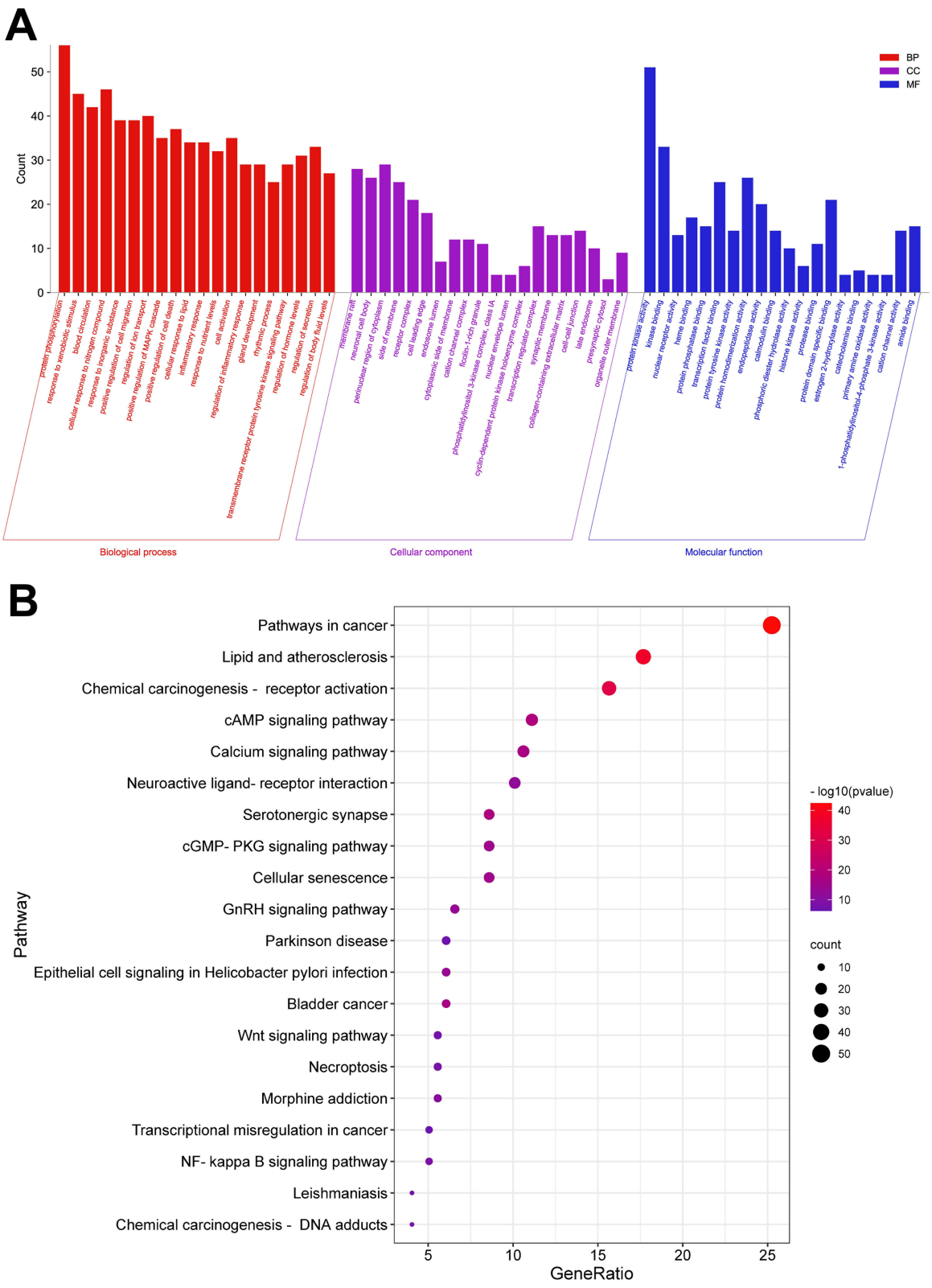

Figure 3 The GO (**A**) and KEGG pathway enrichment (**B**) analysis of 198 therapeutic genes of ZYP on AS.

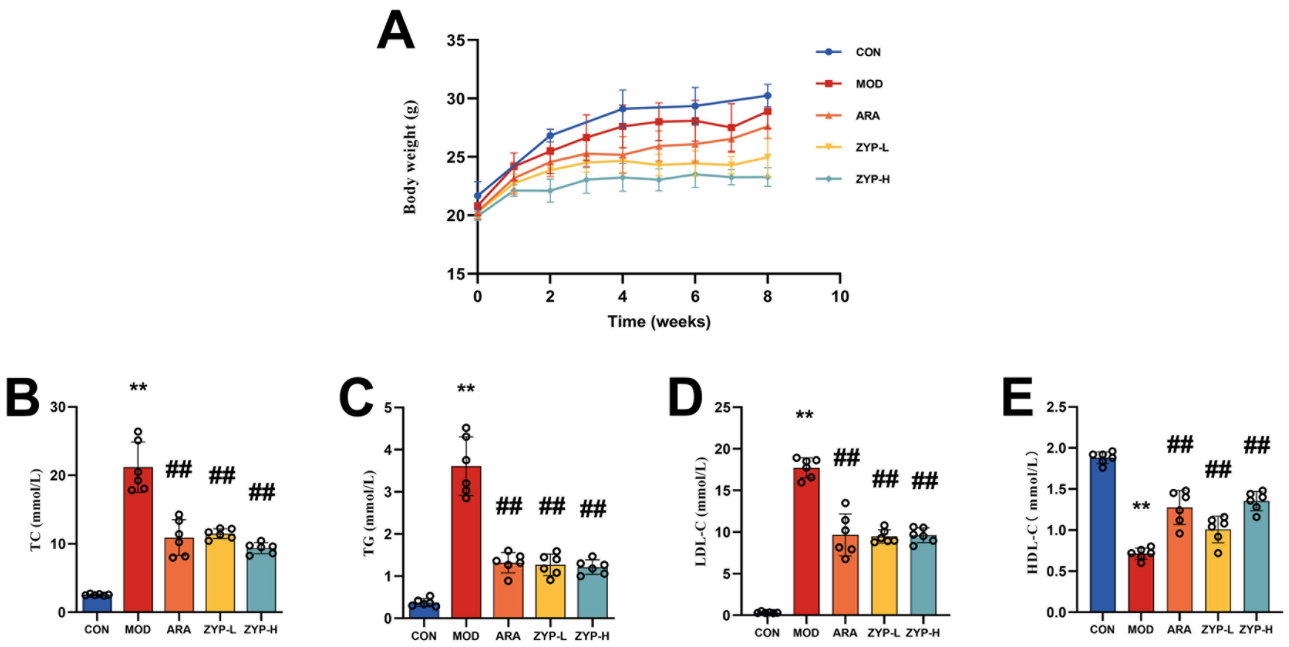

Figure 4 ZYP reduced lipid levels in ApoE-/- mice: (**A**) Body weights, (**B**) serum total cholesterol (TC) level, (**C**) serum triglyceride (TG) level, (**D**) serum low-density lipoprotein cholesterol (LDL-c) level, and (**E**) serum high-density lipoprotein cholesterol (HDL-c) level. Data are represented as means ± SD for n=6 per group. (**P<0.01 vs CON group, ##P<0.01 vs MOD group).
Abbreviations: CON, control group; MOD, model group; ARA, atorvastatin group; ZYP-L, Zhuyu Pill low dose; ZYP-H, Zhuyu Pill high dose.

Effect of ZYP on Expression of Inflammatory Cytokines in Mice

The serum levels of inflammatory cytokines TNF-α and IL-6 were assessed by ELISA kits. The levels of TNF-α and IL-6 were higher in the MOD group than the CON group (P<0.01). Eight weeks' intervention with ZYP or ARA significantly decreased the expression levels of TNF-α and IL-6, with a greater reduction seen in the ZYP-H group than the ZYP-L group and ARA group (Figure 6A–B). In addition, the MOD group showed an increased expression of ICAM-1, VCAM-1 and MCP-1, of which the levels were significantly lower in the ZYP and ARA groups (P< 0.01) (Figure 6C–E).

Effect of ZYP on Gene Expressions of MAPK/NF-κB Signaling Pathways in the Aorta

The relative expression of MAPKp38, ERK, JNK, NF-κBp65 and IκB-α, mRNA is shown in the Figure 7. The MOD group had a greater expression of three genes in the MAPK Signalling pathways, including MAPKp38, ERK, and JNK, than the CON group (P<0.01), and the expression decreased after the treatment of ZYP and ARA. The ZYP-H showed the greatest beneficial effect, followed by ARA and ZYP-L (all P<0.01) (Figure 7A–C). The expression of NF-κBp65 gene in MOD group was higher than in CON group and decreased after interventions. Similarly, the ZYP-H showed the greatest beneficial effect, followed by ARA and ZYP-L (all P<0.01). There was no significant difference in the expression of IκB-α between the MOD and CON groups, nor between the ZYP and MOD groups (Figure 7D–E).

ZYP Improves as Through MAPK/NF-κB Signaling Pathways

The immunohistochemistry and Western blot results showed that expression of p38 and p65 was increased in the MOD group compared to the CON group and decreased to varying degrees after administration, with the most significant effect in the ZYP-H group (Figures 8, 9A and 9C). The expression of p-p38 was significantly higher in the MOD group compared to the CON group (P <0.01), and significantly lower in all dosing groups compared to the MOD group (P <0.05), with ZYP-L group lower than ARA group and ZYP-H group lower than ZYP-L group (Figure 9B). Statistical analysis of p-p65 expression levels yielded a significant increase in the MOD group compared to the CON group (P<0.01) and a significant decrease in the ARA group compared to the model group (P<0.01). The inhibitory effect of ZYP was dose-dependent, with the ZYP-H group (P<0.01) being lower than the ZYP-L group (P < 0.05) (Figure 9D).

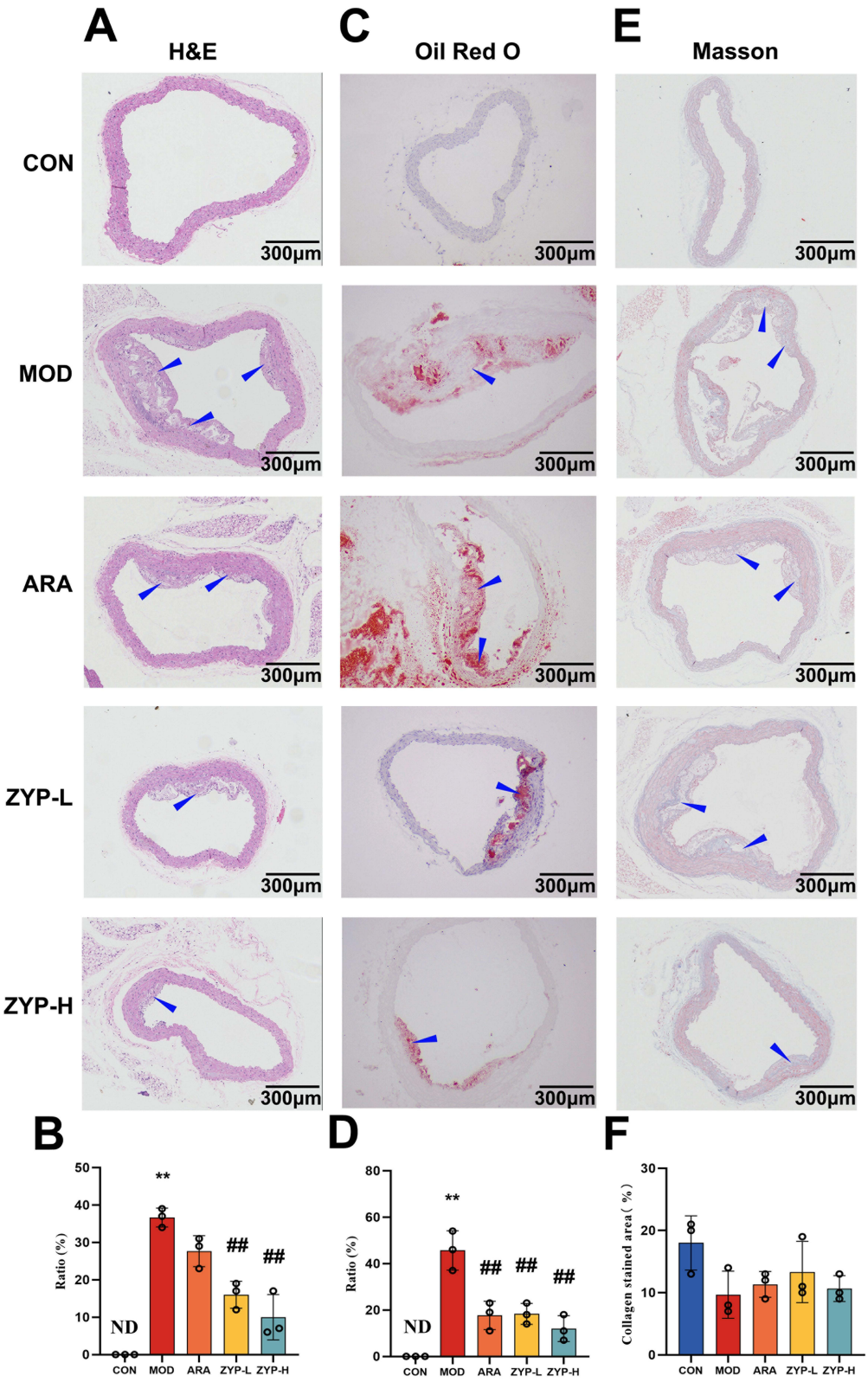

Figure 5 ZYP reduced lipid deposition in the aorta of ApoE-/- mice (original magnification × 80). (**A**) H&E staining of aortic arches, (**C**) Oil red o staining of the aortic arches (**B** and **D**) The areas of plaques were calculated respectively by ImageJ analysis software. (**E**) Masson staining of aortic arches, (**F**) The collagen content was quantified by ImageJ analysis software. All the blue triangles point to areas of plaque. Data are represented as means ± SD; n=3 per group. (**P<0.01 vs CON group, $^{\#\#}P$<0.01 vs MOD group).

Abbreviations: CON, control group; MOD, model group; ARA, atorvastatin group; ZYP-L, Zhuyu Pill low dose; ZYP-H, Zhuyu Pill high dose.

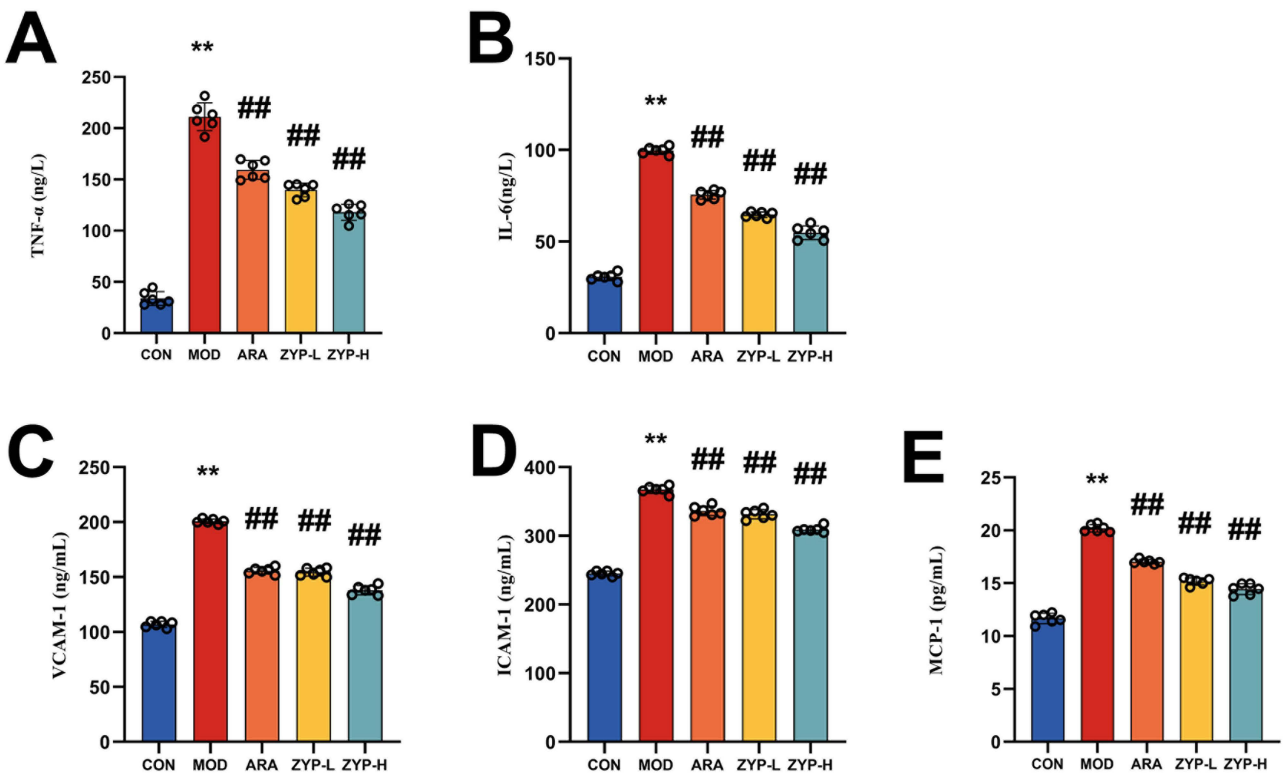

Figure 6 ZYP reduced the expression of serum inflammatory factors in ApoE-/- mice: (**A**) Serum tumor necrosis factor-alpha (TNF-α) level; (**B**) serum interleukin-6 (IL-6) level; (**C**) vascular cell adhesion molecule-1 (VCAM-1), (**D**) intercellular adhesion molecule-1 (ICAM-1). (**E**) Monocyte chemoattractant protein-1 (MCP-1). Data are represented as means ± SD; n = 6 per group in serum level. (**P<0.01 vs CON group, $^{\#\#}P$<0.01 vs MOD group).
Abbreviations: CON, control group; MOD, model group; ARA, atorvastatin group; ZYP-L, Zhuyu Pill low dose; ZYP-H, Zhuyu Pill high dose.

Discussion

AS is a chronic disease that mainly occurs in large and medium-sized arteries. Inflammation and lipid accumulation are hallmarks of AS, which are accompanied by smooth muscle cell proliferation, apoptosis, necrosis, and fibrosis.[20] AS can cause coronary artery stiffness and stenosis and further lead to myocardial infarctions and strokes. The rupture of atherosclerotic plaque can also lead to a cardiovascular event.[21] Atorvastatin for lipid-lowering and aspirin for antiplatelet aggregation are commonly used in clinical practice for AS and prevention of cardiovascular disease. However, due to the complex pathogenesis of AS, these treatments may not achieve satisfactory therapeutic effects.[22] ZYP has been demonstrated to exert a promising beneficial effect on AS, which appears to act primarily by decreasing blood lipids, inhibiting inflammation, and preventing the buildup of plaques in arteries. In our previous study, we observed that ZYP exerted anti-atherogenic effects in mice. However, the underlying mechanisms of the action are not clearly defined.

During AS progression, immune and inflammatory cells invade the aortic wall, resulting in fatty deposits building up on the wall of affected arteries.[23] Mitogen-activated protein kinase (MAPK) is a highly conserved serine/threonine-like protein kinases in eukaryotes. MAPKp38 is involved in the inflammatory pathway. Various extracellular and intracellular stimuli, including oxidative stress, cytokines, and growth factors, can activate MAPKp38 and promote inflammation.[24] A body of evidence showed that MAPKp38 is a potential mediator in the inflammatory response to AS.[25] Studies using both patient-derived tissues and animal models have demonstrated that MAPKp38 is activated in macrophages and participates in the formation of atherosclerotic plaque lesions.[26,27] They found that MAPKp38 plays a key role in the positive feedback mechanism that leads to foam cell formation, an important process of AS development. Activated by oxLDL, p38 in turn takes up oxLDL to produce lipid-rich macrophage foam cells.[28] AS involves a persistent inflammatory response in its progression.[29] Cytokines that promote inflammation are produced in response to p38 activation. Based on this, previous clinical studies of the MAPK inhibitors showed a significant efficacy in ameliorating AS. NF-κB is the major transcription factor in inflammatory response and its role in AS has been widely accepted. The NF-κB is

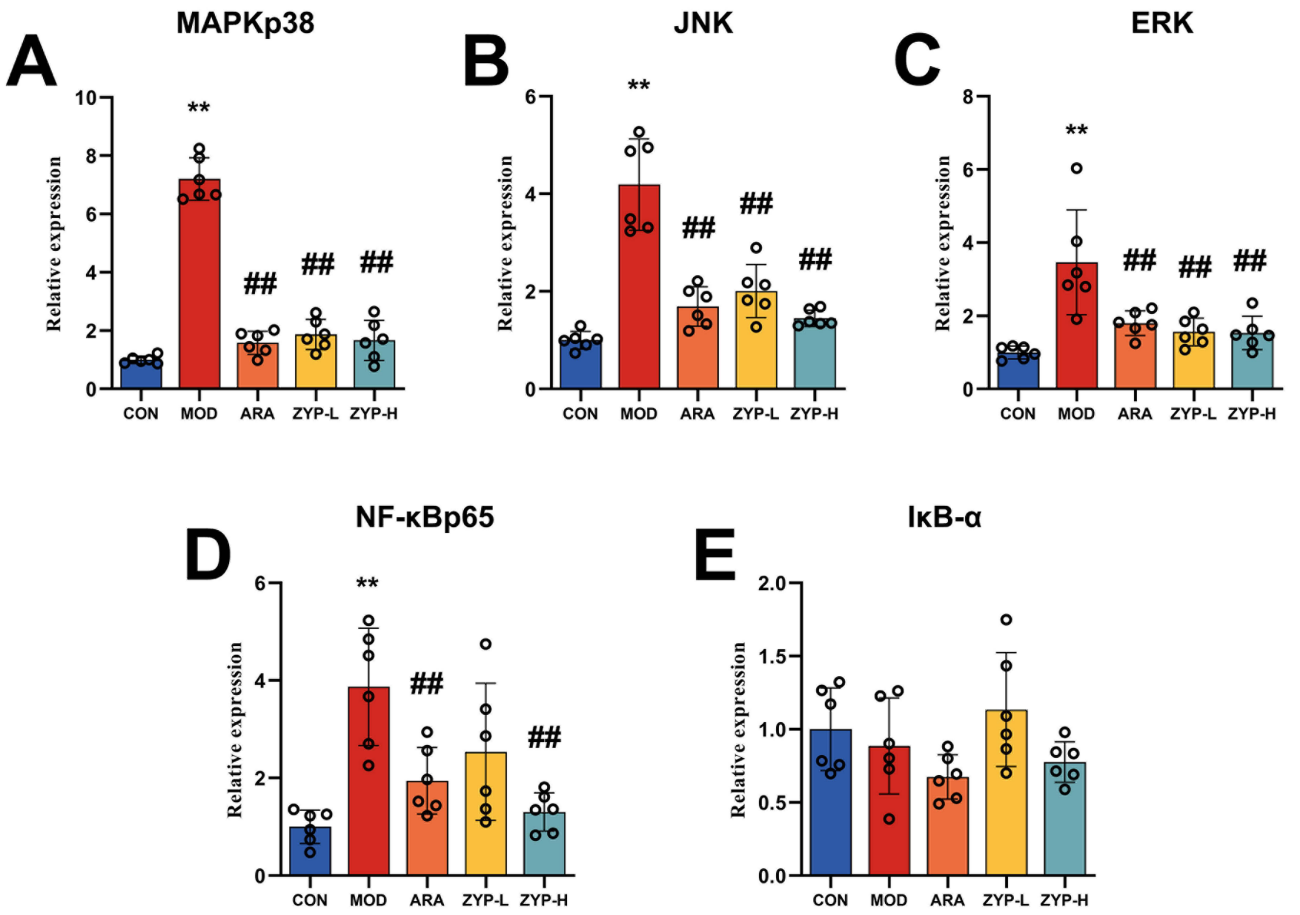

Figure 7 Relative mRNA expression of MAPK and NF-κB signalling pathway. (**A**) MAPKp38 relative mRNA expression; (**B**) JNK relative mRNA expression; (**C**) ERK relative mRNA expression; (**D**) NF-κBp65 relative mRNA expression; (**E**) IκB-α relative mRNA expression. Data are represented as means ± SD; n = 6 per group. (** *P*<0.01 vs CON group, ## *P*<0.01 vs MOD group).

Abbreviations: CON, control group; MOD, model group; ARA, atorvastatin group; ZYP-L, Zhuyu Pill low dose; ZYP-H, Zhuyu Pill high dose.

activated in human atherosclerotic plaques and regulating inflammatory activity of NF-κB could limit disease progression in mice.[30,31] Dyslipidemia is an important contributor to the progression of AS. NF-κB is involved in breaking cholesterol homeostasis by regulating reverse cholesterol transport. It contributes to foam cell formation and atherogenesis by reducing ABCA1-driven cholesterol efflux. Inflammation is present at all stages of AS from happening. NF-κB can not only up-regulate the inflammation levels, such as increasing the expression of TNF-α, IL-1β, IL-6, ICAM-1 and VCAM-1, but also promotes foam cell formation, VSMC proliferation, calcification, plaque development and rupture.[32] Researchers[33] found that the activity of NF-κB was dramatically increased in low-density lipoprotein receptor (LDLR) -/- mice after long-term feeding of high-fat diet to accelerate AS. More evidence revealed that NF-κB phosphorylation was increased after high-fat feeding in ApoE-/- mice.[33]

Due to the complex compositions and multiple targets of CHM, a clear clarification of the curative mechanism of CHM is always challenging.[34] Network pharmacology is a subject integrating pharmacology, computational biology and other disciplines that has been extensively applied to the field of TCM.[35,36] Considering the holistic notion of TCM theory and the clinical value of evidence-based medicine, this approach could be used to discover the targets of CHM, predict the treatment effects, and untangle the mechanism of CHM on treating diseases.[37] Based on the results of network pharmacology, ZYP can regulate 198 targets to treat AS. PPI cluster showed that MAPK1, MAPK8, and MAPK14 were the core targets. GO analysis revealed that the 198 common targets were significantly enriched in protein phosphorylation, positive regulation of MAPK cascade, cellular response to lipid, inflammatory response, regulation of inflammatory response, etc. KEGG enrichment analysis revealed significant pathways involved in lipid metabolism and AS, with the

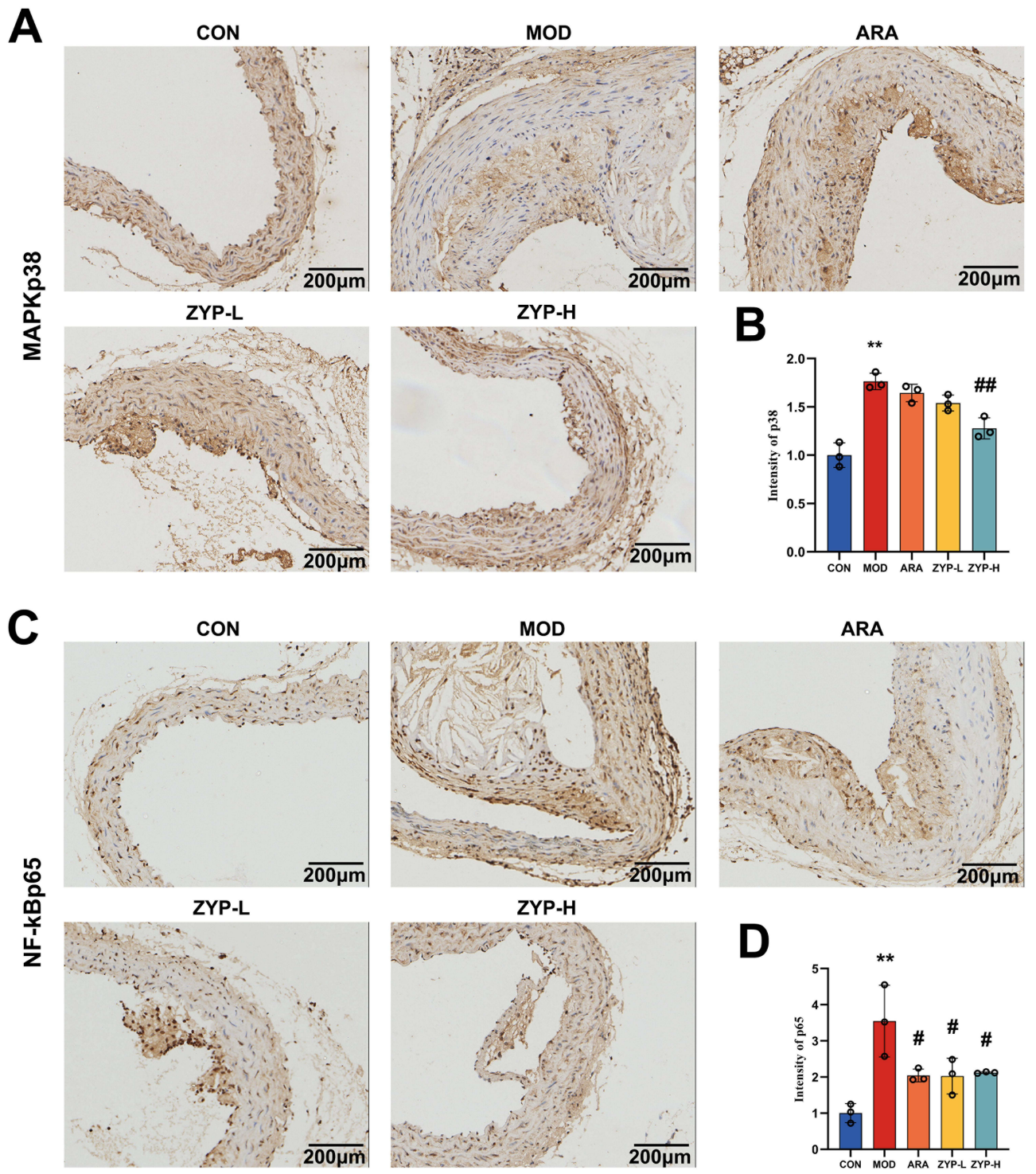

Figure 8 Immunohistochemistry results of MAPKp38 and NF-κBp65 (original magnification × 100). (**A** and **B**): Representative protein expression of MAPKp38; (**C** and **D**): representative protein expression of NF-κBp65. Data are represented as means ± SD; n=3 per group. (**P<0.01 vs CON group, $^{\#}P$<0.05, $^{\#\#}P$<0.01 vs MOD group).
Abbreviations: CON, control group; MOD, model group; ARA, atorvastatin group; ZYP-L, Zhuyu Pill low dose; ZYP-H, Zhuyu Pill high dose.

NF-κB signalling pathway also ranking high in terms of impact. Previous literature suggested that MAPK and NF-κB were two classical pathways involved in the pathogenesis of AS. Therefore, we chose these two pathways for further experimental validation.

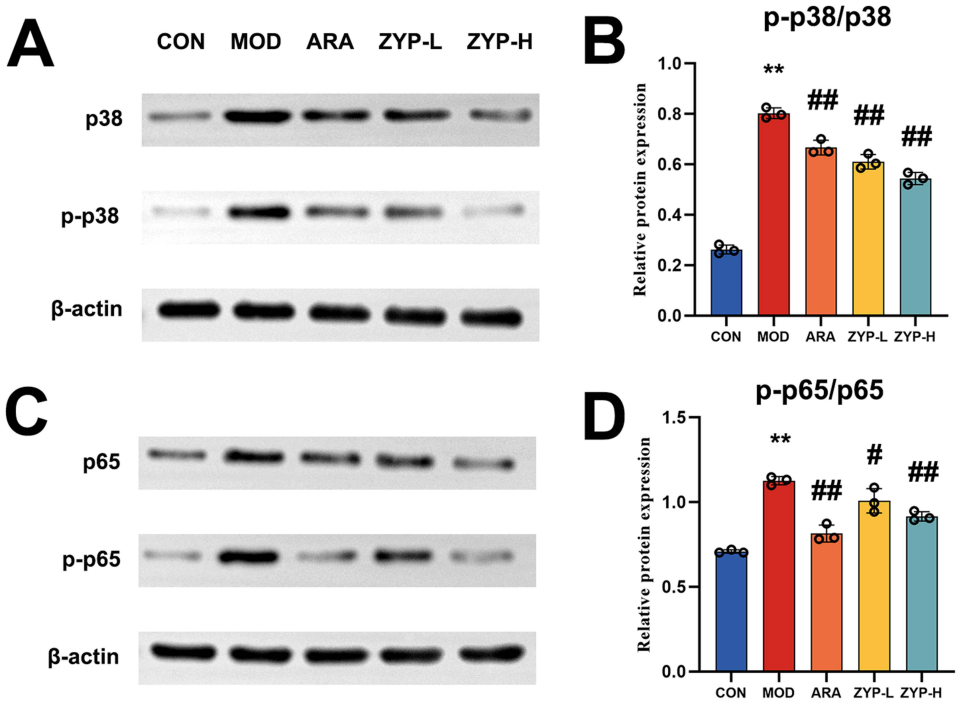

Figure 9 The protein level of MAPK and NF-κB signalling pathway. (**A** and **C**): The proteins expression of p38, p-p38, p65, and p-p65; (**B** and **D**): representative protein expression of p-p38/p38 and p-p65/p65. Data are represented as means ± SD; n=3 per group. (**P<0.01 vs CON group, #P<0.05, ##P<0.01 vs MOD group).
Abbreviations: CON, control group; MOD, model group; ARA, atorvastatin group; ZYP-L, Zhuyu Pill low dose; ZYP-H, Zhuyu Pill high dose.

In vivo, we constructed an AS model by feeding ApoE-/- mice with high fat diet. Compared with the MOD group, ZYP decreased the serum TC, TG and LDL-C levels, reduced the serum inflammatory cytokines VCAM1, ICAM1, MCP-1, IL-6, and TNF-α, and reduced lipid deposition in the aorta, indicating its beneficial effect on AS. Besides, the RT-qPCR assay revealed that ZYP inhibited the expressions of MAPKp38, ERK, and JNK, as well as NF-κBp65. The pivotal proteins on the MAPK and NF-κB pathways were verified through immunohistochemical staining and Western blot assays. High fat diet intake upregulated the expressions of MAPKp38 and NF-κBp65 of the aorta in the MOD group. ZYP reduced the proteins expression, indicating that the inhibition of MAPK and NF-κB pathways may be involved in the therapeutic effect of ZYP on AS.

Conclusion

In summary, this comprehensive study explored the role and relevant mechanisms of ZYP in ameliorating AS by network pharmacology and in vivo experiments. The results suggest that ZYP exerted its potential protective effect on AS mainly through MAPK and NF-κB pathways, which provides more insight into the mechanism of ZYP for prevention of AS. Moreover, network pharmacology was proved to be useful in pharmacological studies on TCM.

Data Sharing Statement

Raw data relevant to the conclusions of this study will be provided by the corresponding authors upon reasonable request.

Ethics Approval and Informed Consent

The animal research involved in this study was approved by the animal ethics review committee of Chengdu University of Traditional Chinese Medicine (Ethical No. 2022-23).

Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically

reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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Disclosure

The authors declare that there is no conflict of interest.

References

1. Falk E. Pathogenesis of atherosclerosis. *J Am Coll Cardiol*. 2006;47(8 Suppl):C7–C12. doi:10.1016/j.jacc.2005.09.068
2. Zhu Y, Xian X, Wang Z, et al. Research progress on the relationship between atherosclerosis and inflammation. *Biomolecules*. 2018;8(3). doi:10.3390/biom8030080
3. Zhou Z, Albarqouni L, Curtis AJ, Breslin M, Nelson M. The safety and tolerability of statin therapy in primary prevention in older adults: a systematic review and meta-analysis. *Drugs Aging*. 2020;37(3):175–185. doi:10.1007/s40266-019-00736-y
4. Šimić I, Reiner Ž. Adverse effects of statins - myths and reality. *Curr Pharm Des*. 2015;21(9):1220–1226. doi:10.2174/1381612820666141013134447
5. Shaito A, Thuan DTB, Phu HT, et al. Herbal medicine for cardiovascular diseases: efficacy, mechanisms, and safety. *Front Pharmacol*. 2020;11:422. doi:10.3389/fphar.2020.00422
6. Zhang X, Gao R, Zhou Z, et al. Uncovering the mechanism of Huanglian-Wuzhuyu herb pair in treating nonalcoholic steatohepatitis based on network pharmacology and experimental validation. *J Ethnopharmacol*. 2022;296:115405. doi:10.1016/j.jep.2022.115405
7. Zhou X, Ren F, Wei H, et al. Combination of berberine and evodiamine inhibits intestinal cholesterol absorption in high fat diet induced hyperlipidemic rats. *Lipids Health Dis*. 2017;16(1):239. doi:10.1186/s12944-017-0628-x
8. Yu H, Liu C, Zhang F, et al. Efficacy of Zhuyu Pill intervention in a cholestasis rat model: mutual effects on fecal metabolism and microbial diversity. *Front Pharmacol*. 2021;12:695035. doi:10.3389/fphar.2021.695035
9. Zhang L, Wu X, Yang R, et al. Effects of berberine on the gastrointestinal microbiota. *Front Cell Infect Microbiol*. 2020;10:588517. doi:10.3389/fcimb.2020.588517
10. Xing L, Zhou X, Li AH, et al. Atheroprotective effects and molecular mechanism of berberine. *Front Mol Biosci*. 2021;8:762673. doi:10.3389/fmolb.2021.762673
11. Wu M, Yang S, Wang S, et al. Effect of berberine on atherosclerosis and gut microbiota modulation and their correlation in high-fat diet-fed ApoE-/- mice. *Front Pharmacol*. 2020;11:223. doi:10.3389/fphar.2020.00223
12. Xu RX, Sun XC, Ma CY, et al. Impacts of berberine on oxidized LDL-induced proliferation of human umbilical vein endothelial cells. *Am J Transl Res*. 2017;9(10):4375–4389.
13. Liao JF, Chiou WF, Shen YC, Wang GJ, Chen CF. Anti-inflammatory and anti-infectious effects of Evodia rutaecarpa (Wuzhuyu) and its major bioactive components. *Chin Med*. 2011;6(1):6. doi:10.1186/1749-8546-6-6
14. Ge X, Chen S, Liu M, Liang T, Liu C. Evodiamine attenuates PDGF-BB-induced migration of rat vascular smooth muscle cells through activating PPARγ. *Int J Mol Sci*. 2015;16(12):28180–28193. doi:10.3390/ijms161226093
15. Poznyak A, Grechko AV, Poggio P, Myasoedova VA, Alfieri V, Orekhov AN. The diabetes mellitus-atherosclerosis connection: the role of lipid and glucose metabolism and chronic inflammation. *Int J Mol Sci*. 2020;21(5). doi:10.3390/ijms21051835
16. Jiang N, Li H, Sun Y, et al. Network pharmacology and pharmacological evaluation reveals the mechanism of the sanguisorba officinalis in suppressing hepatocellular carcinoma. *Front Pharmacol*. 2021;12:618522. doi:10.3389/fphar.2021.618522
17. Yu H, Liu C, Wang J, et al. miRNA and miRNA target genes in intervention effect of Zhuyu pill on cholestatic rat model. *J Ethnopharmacol*. 2022;283:114709. doi:10.1016/j.jep.2021.114709
18. Szklarczyk D, Gable AL, Lyon D, et al. STRING v11: protein-protein association networks with increased coverage, supporting functional discovery in genome-wide experimental datasets. *Nucleic Acids Res*. 2019;47(D1):D607–d613. doi:10.1093/nar/gky1131
19. Zhang Y, Du M, Wang J, Liu P. Astragaloside IV relieves atherosclerosis and hepatic steatosis via MAPK/NF-κB signaling pathway in LDLR(-/-) mice. *Front Pharmacol*. 2022;13:828161. doi:10.3389/fphar.2022.828161
20. Libby P, Bornfeldt KE, Tall AR. Atherosclerosis: successes, surprises, and future challenges. *Circ Res*. 2016;118(4):531–534. doi:10.1161/CIRCRESAHA.116.308334
21. Ruiz-León AM, Lapuente M, Estruch R, Casas R. Clinical advances in immunonutrition and atherosclerosis: a review. *Front Immunol*. 2019;10:837. doi:10.3389/fimmu.2019.00837
22. van Leuven SI, Kastelein JJ. Atorvastatin. *Expert Opin Pharmacother*. 2005;6(7):1191–1203. doi:10.1517/14656566.6.7.1191
23. Zheng Q, Duan L, Lou Y, et al. Slfn4 deficiency improves MAPK-mediated inflammation, oxidative stress, apoptosis and abates atherosclerosis progression in apolipoprotein E-deficient mice. *Atherosclerosis*. 2021;337:42–52. doi:10.1016/j.atherosclerosis.2021.10.010
24. Coulthard LR, White DE, Jones DL, McDermott MF, Burchill SA. p38(MAPK): stress responses from molecular mechanisms to therapeutics. *Trends Mol Med*. 2009;15(8):369–379. doi:10.1016/j.molmed.2009.06.005

25. Zhang M, Gao J, Zhao X, et al. p38α in macrophages aggravates arterial endothelium injury by releasing IL-6 through phosphorylating megakaryocytic leukemia 1. *Redox Biol*. 2021;38:101775. doi:10.1016/j.redox.2020.101775
26. Cheng F, Twardowski L, Fehr S, et al. Selective p38α MAP kinase/MAPK14 inhibition in enzymatically modified LDL-stimulated human monocytes: implications for atherosclerosis. *FASEB J*. 2017;31(2):674–686. doi:10.1096/fj.201600669R
27. Shafi S, Codrington R, Gidden LM, Ferns GA. Increased expression of phosphorylated forms of heat-shock protein-27 and p38MAPK in macrophage-rich regions of fibro-fatty atherosclerotic lesions in the rabbit. *Int J Exp Pathol*. 2016;97(1):56–65. doi:10.1111/iep.12167
28. Reustle A, Torzewski M. Role of p38 MAPK in atherosclerosis and aortic valve sclerosis. *Int J Mol Sci*. 2018;19(12):3761. doi:10.3390/ijms19123761
29. Wolf D, Ley K. Immunity and Inflammation in Atherosclerosis. *Circ Res*. 2019;124(2):315–327. doi:10.1161/CIRCRESAHA.118.313591
30. Brand K, Page S, Rogler G, et al. Activated transcription factor nuclear factor-kappa B is present in the atherosclerotic lesion. *J Clin Invest*. 1996;97(7):1715–1722. doi:10.1172/JCI118598
31. Mallavia B, Recio C, Oguiza A, et al. Peptide inhibitor of NF-κB translocation ameliorates experimental atherosclerosis. *Am J Pathol*. 2013;182(5):1910–1921. doi:10.1016/j.ajpath.2013.01.022
32. Yu XH, Zheng XL, Tang CK. Nuclear Factor-κB activation as a pathological mechanism of lipid metabolism and atherosclerosis. *Adv Clin Chem*. 2015;70:1–30.
33. Hajra L, Evans AI, Chen M, Hyduk SJ, Collins T, Cybulsky MI. The NF-kappa B signal transduction pathway in aortic endothelial cells is primed for activation in regions predisposed to atherosclerotic lesion formation. *Proc Natl Acad Sci U S A*. 2000;97(16):9052–9057. doi:10.1073/pnas.97.16.9052
34. Zhang X, Gao R, Zhou Z, et al. A network pharmacology based approach for predicting active ingredients and potential mechanism of Lianhuaqingwen capsule in treating COVID-19. *Int J Med Sci*. 2021;18(8):1866–1876. doi:10.7150/ijms.53685
35. Yuan Z, Pan Y, Leng T, et al. Progress and prospects of research ideas and methods in the network pharmacology of traditional Chinese medicine. *J Pharm Pharm Sci*. 2022;25:218–226. doi:10.18433/jpps32911
36. Zhang X, Shen T, Zhou X, et al. Network pharmacology based virtual screening of active constituents of Prunella vulgaris L. and the molecular mechanism against breast cancer. *Sci Rep*. 2020;10(1):15730. doi:10.1038/s41598-020-72797-8
37. Zhang GB, Li QY, Chen QL, Su SB. Network pharmacology: a new approach for Chinese herbal medicine research. *Evid Based Complement Alternat Med*. 2013;2013:621423. doi:10.1155/2013/621423

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