

# Guanmaitong Granule Attenuates Atherosclerosis by Inhibiting Inflammatory Immune Response in ApoE<sup>-/-</sup> Mice Fed High-Fat Diet

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**Background:** Atherosclerosis (AS) is the leading cause of cardiovascular diseases, such as myocardial infarction and stroke. Guanmaitong granule (GMTG) is a TCM (Traditional Chinese medicine) prescribed to treat AS. However, its mechanism remains unclear.

**Methods:** We obtained reliable ingredients and targets of GMTG using the HERB database. AS-related targets were obtained from HERB and GeneCards databases. The target database was constructed by intersecting the ingredients of GMTG with the AS-related targets. STRING and Cytoscape were used to create protein-protein interaction (PPI) network and screen core targets. GO enrichment analysis and KEGG pathway analyses were performed using R. Finally, the ApoE<sup>-/-</sup> mice AS model was induced by a high-fat diet (HFD) for in vivo validation of core pathways and targets.

**Results:** A total of 124 ingredients and 418 potential targets of GMTG for treating AS were obtained. Numerous ingredients and targets were related to *Panax notoginseng*, *Salvia miltiorrhiza*, and *Astragalus*. Most core targets and pathways were involved in the inflammatory immune response. GMTG could decrease serum triglycerides, total cholesterol, low-density lipoprotein-cholesterol, and oxidized low-density lipoprotein level and increase the serum high-density lipoprotein-cholesterol level. Furthermore, GMTG reduced the plaque burden and promoted plaque remodeling by reducing plaque area, lipid deposition, foam cell content, and collagen fiber content in the plaque in the aortic root of ApoE<sup>-/-</sup> mice. GMTG inhibited systemic and plaque inflammatory immune response and increased plaque stability by inhibiting the excessive release of the TLR4/MyD88/NF-κB pathway-induced inflammatory cytokines, tumor necrosis factor, interleukin-6, and interleukin-1 beta.

**Conclusion:** *Radix notoginseng*, *Radix salviae ligulioabae*, and *Radix astragali* are the main ingredients of GMTG for treating AS. Further, GMTG could regulate the level of serum lipids and inhibit inflammatory immune response, which resulted in anti-AS effects such as plaque stabilization, reduction of plaque burden, and plaque remodeling. GMTG is a promising multi-target treatment for AS.

**Keywords:** Guanmaitong granule, atherosclerosis, inflammatory immune response, plaque burden, plaque remodeling

## Introduction

Cardiovascular diseases, especially acute myocardial infarction (AMI), are the leading cause of death, morbidity, and disability worldwide.<sup>1</sup> Atherosclerosis (AS) is a chronic and progressive inflammatory arterial wall disease characterized by intimal lipid accumulation, thickening of the arterial wall, and narrowing of the vascular lumen.<sup>2</sup> Unstable atherosclerotic plaque rupture, platelet aggregation, and thrombosis cause vascular stenosis or occlusion, leading to acute cardiovascular disease,<sup>3,4</sup> a major global health threat that poses a heavy societal burden.<sup>5</sup> Interventional therapy and Western medicine therapies are the main methods of AS treatment.<sup>6</sup> Although revascularization corrects severe stenosis, it does not alter the biological process of AS, and plaque instability is preserved. Statins, lipid-lowering, and anti-inflammatory drugs can be used to treat AS, protect endothelial blood vessels, and even reverse plaques.<sup>7-9</sup> However, long-term, high-dose statin treatment could lead to myopathy/myolysis, diabetes mellitus, liver damage, and other adverse effects.<sup>10-12</sup> The intestinal cholesterol absorption inhibitor ezetimibe only reduces low-density lipoprotein-cholesterol (LDL-C) level and can thus only be used as an adjunct to

statins.<sup>13</sup> The proprotein convertase subtilisin/kexin type 9 (PCSK9) monoclonal antibody significantly reduces serum LDL-C. However, this is a high-cost alternative for the few patients with poor response or intolerance to statins.<sup>14,15</sup> Canakinumab can block the interaction with the interleukin-1 (IL-1) receptor, neutralize the biological activity of human IL-1, and avoid IL-1-induced gene activation and the generation of inflammatory mediators. However, it is expensive, has numerous side effects, and can lead to fatal infections.<sup>16</sup> Therefore, finding safe, effective, and economical anti-AS drugs is imperative.

It is generally believed that AS results from lipid accumulation in the vascular wall. However, the pathogenesis of AS is more complex. Immune cells, inflammation associated with hyperlipidemia, and elevated oxidized low-density lipoprotein (ox-LDL) levels play a crucial role.<sup>17</sup> Extensive experimental and clinical evidence shows that AS is a chronic inflammatory disease,<sup>18</sup> and the inflammatory response plays an essential role in the entire process of atherosclerotic plaque formation. In early AS, vascular endothelial injury, abnormal lipid metabolism, and hemodynamic damage are the leading causes. AS is thought to be accompanied by inflammatory changes in blood flow-mediated endothelial cells (ECs).<sup>19</sup> After the ECs are activated, they expressed monocyte chemoattractant protein 1 (MCP-1), intercellular adhesion molecule 1 (ICAM-1), vascular cell adhesion protein 1 (VCAM-1), and other cytokines, attracting lymphocytes and monocytes, which combine with endothelial biomolecules and infiltrate artery walls, leading to the initiation of inflammation. A large amount of LDL-C is modified to ox-LDL and accumulates in the inner wall of the blood vessels, which promotes the formation of AS plaque.<sup>20</sup> In advanced AS lesions, many inflammatory cells, such as macrophages infiltrate the vascular wall and secrete matrix metalloproteinases to degrade collagen fibers in the extracellular matrix of the plaque, leading to plaque rupture, bleeding, and thrombosis.<sup>21,22</sup> Anti-inflammation therapies provide a new perspective for the developing therapeutic strategies for AS.

Guanmaitong granule (GMTG) is a commonly used prescription of Professor Yitao Xue for treating AS and has had remarkable clinical effects.<sup>23,24</sup> GMTG is composed of the root of Membranous Milkvetch (*Radix Astragali*), root of Ligulilobe sage (*Radix Salviae ligullobae*), *Trichosanthes kirilowii* Maxim (*Trichosanthis Fructus*), rhizome of Chinese Goldthread (*Rhizoma Coptidis*), Sanchi (*Radix Notoginseng*), Figwort Root (*Radix Scrophulariae*), bulb of Thunberg Fritillary (*Bulbus Fritillariae thunbergii*), rhizome of Gaint Knotweed (*Rhizoma Polygoni cuspidati*), Bigflower Cape Jasmine (*Gardenia jasminoides var. grandiflora*), and Oyster (*Crassostrea gigas*). Previous studies have shown that various ingredients of Traditional Chinese medicine (TCM) herbs contained in GMTG have anti-AS effects. Astragaloside IV can prevent ox-LDL-induced ECs injury by reducing oxidative stress and inflammation.<sup>25</sup> The combination of *Astragalus* polysaccharide and hirudin can reduce the apoptosis rate of macrophages induced by ox-LDL, which may be used to treat AS because of the regulation of mitochondrial membrane potential and the expression of related proapoptotic proteins Caspase-3 (CASP3), Bax and anti-apoptotic protein Bcl-2.<sup>26</sup> Tanshinone IIA plays a role by inhibiting LDL oxidation,<sup>27</sup> smooth muscle cell migration and development, arterial intimal monocyte adhesion, pro-inflammatory cytokine expression, platelet aggregation,<sup>28</sup> and macrophage-mediated cholesterol accumulation in AS.<sup>29</sup> Berberine can effectively improve the levels of blood lipids, reduce the area of atherosclerotic plaque in mice, and exert an anti-inflammatory effect by lowering blood CRP and interleukin-6 (IL-6) levels.<sup>30</sup> *Panax notoginseng* saponins can inhibit the formation of foam cells in ApoE<sup>-/-</sup> mice by regulating the TLR4/SYK signaling pathway.<sup>31</sup> However, the specific mechanism of GMTG in treating AS is unclear because of the diverse ingredients of TCM and their complex interaction with the human body. Network pharmacology has become a powerful strategy for studying TCM prescriptions and is a breakthrough method in applying bioinformatics and systems biology in TCM.<sup>32</sup>

The goal of this study was to explore the potential mechanism of action of GMTG in the treatment of AS based on network pharmacology. The core mechanism was validated through ApoE<sup>-/-</sup> mice. The flowchart of the process is shown in Figure 1.

## Materials and Methods

### Database Building and Prediction of Potential Targets

HERB (<http://herb.ac.cn/>), a high-throughput experiment and reference-guided database of TCM, integrates multiple TCM databases containing the most comprehensive list of TCMs and ingredients, providing high-quality and evidence-based links between TCM and modern medicines. Ten TCM herbs in GMTG were screened using the HERB database, with FDR < 0.05 as the screening standard, to obtain the ingredients and targets of GMTG.

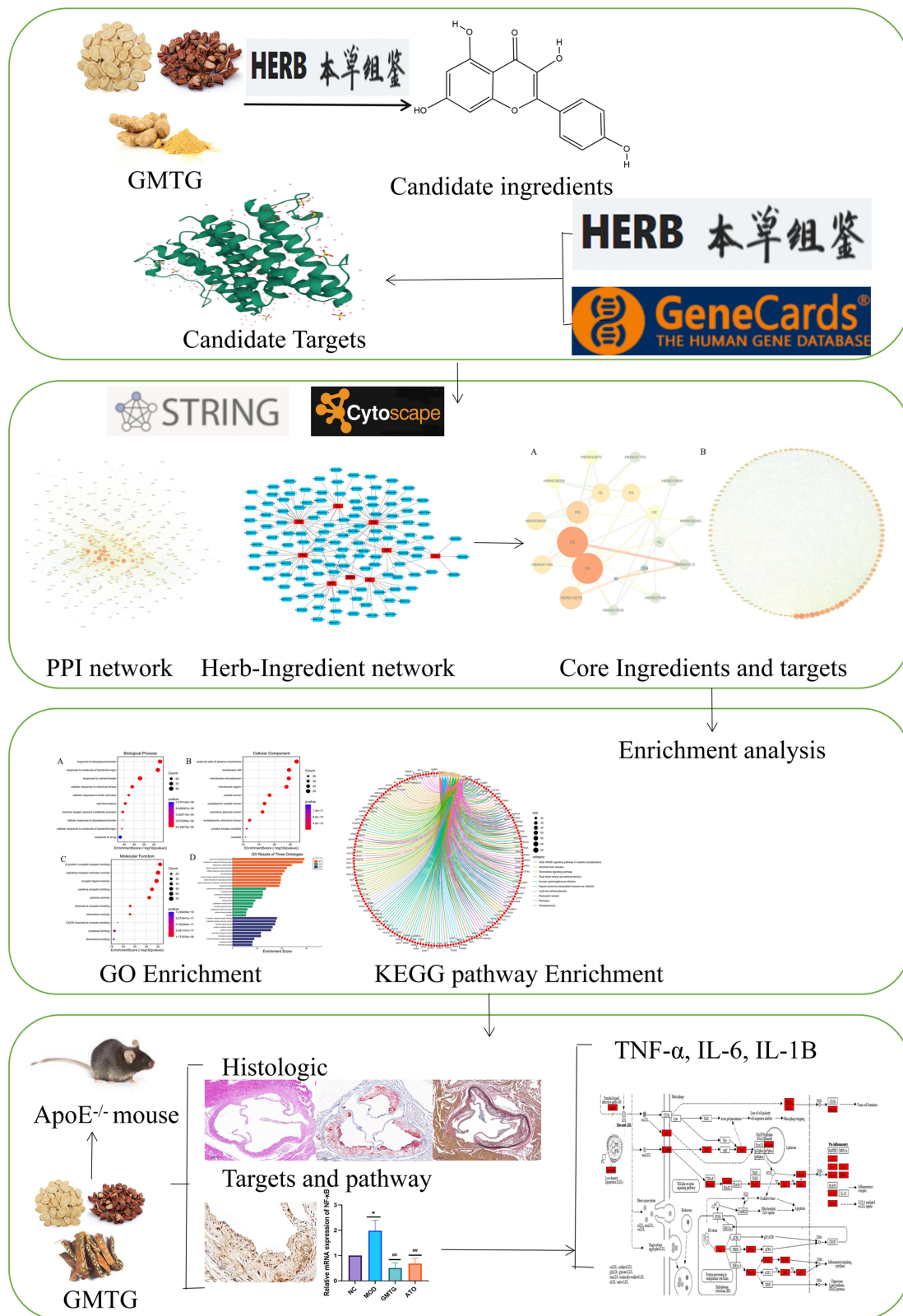


Figure 1 Flow Chart.

The HERB database was searched with the keywords of “atherosclerosis” to obtain AS-related ingredients. Taking the intersection of GMTG and AS ingredients as potential ingredients. The GeneCards database was searched with the keywords of “atherosclerosis” to obtain AS-related targets. The AS-related targets and targets of GMTG were intersected to identify the potential targets.

## Construction of the TCM Herb-Potential Target Network

The TCM herb-potential target network was constructed using Cytoscape software,<sup>33</sup> and the topology of the network was analyzed. The core ingredients of GMTG used to treat AS were obtained by screening the degree values ( $\geq 2.62$  (mean degree value)).

## Protein-Protein Interaction (PPI) Network

The PPI network of potential targets was constructed using STRING<sup>34</sup> and Cytoscape, and the network topology was analyzed. The degree value was screened to identify the core targets of GMTG in the treatment of AS.

## GO and KEGG Enrichment Analyses

Enrichment analysis was performed using Cluster Profiler and Pathview packages in R.  $P < 0.05$  indicated that the enrichment was statistically significant. Pathways with low P-values were selected for experimental verification.

## Drug Preparation

GMTG was composed of 10 single TCM granules: *Radix Astragali* (HUANG QI), *Radix Salviae liguliobae* (DAN SHEN), *Trichosanthis Fructus* (GUA LOU), *Rhizoma Coptidis* (HAUNG LIAN), *Radix Notoginseng* (SAN QI), *Radix Scrophulariae* (XUAN SHEN), *Bulbus Fritillariae thunbergii* (ZHE BEI MU), *Rhizoma Polygoni cuspidati* (HU ZHANG), *Gardenia jasminoides var. grandiflora* (SHUI ZHI), *Crassostrea gigas* (MU LI), which was manufactured by Jiangyin Tianjiang Pharmaceutical Co., Ltd. (Wuxi, Jiangsu, China) and Beijing Kangrentang Pharmaceutical Co., Ltd (Beijing, China). All the above granules were authenticated by the outpatient granule pharmacy of Affiliated Hospital of Shandong University of Traditional Chinese Medicine (Jinan, Shandong, China). The details of GMTG are listed in Tables 1 and 2.

## Establishment of as Model

Fifteen SPF male C57BL/6J mice (8 weeks,  $20 \pm 2$ g) and 48 SPF male ApoE<sup>-/-</sup> All mice were purchased (Vital River Laboratory Animal Technology Co., Ltd., Beijing, China) and raised in a barrier environment. All experimental procedures were in accordance with the standards specified in the Animal Experimental Guidelines of the Chinese Medical Ethics Committee and approved by the Animal Experimental Ethics Committee of Shandong University of Traditional Chinese Medicine (2021–35). We strictly abided by relevant provisions on animal handling.

After 1 week of adaptive feeding with common feed, feeding of common feed was continued in C57BL/6J mice, while ApoE<sup>-/-</sup> mice were fed an HFD (0.15% cholesterol, 21% lard, and 78.85% standard feed; Keao Co., Ltd., Beijing, China) to establish an AS model. The drug dose (based on crude drug content) for mice was 9.1 times that for humans. In this study, the equivalent dose in mice was obtained from the clinical dose in humans (clinical dose of GMTG: 198 g/d, adult human weight 70 kg, mouse dose of GMTG:  $198\text{g}/70\text{kg} \times 9.1 \approx 25.74\text{g}/\text{kg}$ ).<sup>35</sup> After 16 weeks of continuous feeding, three ApoE<sup>-/-</sup> mice were randomly selected for oil red and hematoxylin and eosin (HE) staining of the aorta to verify the model. After successful molding, ApoE<sup>-/-</sup> mice were randomly divided into three groups (n=15 per group): the AS model group (MOD), conventional dose GMTG-treatment group (GMTG), and atorvastatin-treatment group (ATO). C57BL/6J mice were regarded as a control group (NC). NC and MOD groups were administered saline 0.2 mL/d via gavage, the GMTG group was administered GMTG 25.74g/kg/d (0.2mL/20g), and the ATO group was administered atorvastatin calcium (Pfizer Pharmaceuticals, Co., Ltd., New York, USA) 5mg/kg/d (0.2mL) via gavage. All mice were given an intraperitoneal injection of pentobarbital for euthanasia after 8-weeks of intervention.

**Table 1** Composition of GMTG

Academic Name	English Name	Chinese Name	Dose of Crude Drug (g)	Dose of Granule (g)	Ratio	Company	Lot Number
Radix Astragali	Root of Membranous Milkvetch	HUANG QI	30	6	2:10	Beijing Kangrentang Pharmaceutical Co., Ltd	21021381
Radix Salviae ligulobae	Root of Ligulilobe sage	DAN SHEN	30	3	1:10	Beijing Kangrentang Pharmaceutical Co., Ltd	21029101
Trichosanthis Fructus	Trichosanthes Kirilowii Maxim	GUA LOU	15	3	2:10	Jiangyin Tianjiang Pharmaceutical Co., Ltd	21062561
Rhizoma Coptidis	Rhizome of Chinese Goldthread	HUANG LIAN	9	1.5	0.5:3	Jiangyin Tianjiang Pharmaceutical Co., Ltd	21061571
Radix Notoginseng	Sanchi	SAN QI	3	3	3:3	Jiangyin Tianjiang Pharmaceutical Co., Ltd	21082071
Radix Scrophulariae	Figwort Root	XUAN SHEN	15	4.5	3:10	Jiangyin Tianjiang Pharmaceutical Co., Ltd	21041421
Bulbus Fritillariae thunbergii	Bulb of Thunberg Fritillary	ZHE BEI MU	30	3	1:10	Jiangyin Tianjiang Pharmaceutical Co., Ltd	21051551
Rhizoma Polygoni cuspidati	Rhizome of Gaint Knotweed	HU ZHANG	30	2	1:15	Jiangyin Tianjiang Pharmaceutical Co., Ltd	21060251
Gardenia jasminoides var. grandiflora	Bigflower Cape Jasmine	SHUI ZHI	6	1	0.5:3	Jiangyin Tianjiang Pharmaceutical Co., Ltd	20121311
Crassostrea gigas	Oyster	MU LI	30	0.5	0.5:30	Jiangyin Tianjiang Pharmaceutical Co., Ltd	21101671

**Abbreviations:** GMTG, Guanmaitong granule.

**Table 2** Properties and Meridians of GMTG

Chinese Name	Properties	Meridians
HUANG QI	Warm; Sweet	Lung; Spleen
DAN SHEN	Minor cold; Bitter	Liver; Heart
GUA LOU	Sweet, Slightly Bitter, Cold	Lung, Stomach, Large Intestine
HUANG LIAN	Cold; Bitter	Large Intestine; Stomach; Small Intestine; Liver; Heart
SAN QI	Warm; Pungent; Slightly Bitter	Stomach; Liver
XUAN SHEN	Cold; Sweet; Bitter; Salty	Lung; Stomach; Kidney
ZHE BEI MU	Cold; Bitter	Lung; Heart
HU ZHANG	Cold; Bitter	Lung; Liver; Gallbladder
SHUI ZHI	Mild; Bitter; Salty	Liver
MU LI	Minor cold; Salty; Pungent	Liver; Kidney

**Abbreviations:** GMTG, Guanmaitong Granule.

## Sample Collection

After 8 weeks of treatment, blood was collected from the eyeball after anesthesia, allowed to stand at room temperature for 2 h, and then centrifuged at 3500 rpm for 30 min at 4°C. The supernatant was collected for an enzyme-linked immunosorbent assay (ELISA). After exposing the thoracic and abdominal cavity, 0.9% saline was perfused into the apex of the heart until the effluent was clear, the heart and aorta were quickly removed, and the surrounding adipose tissue was discarded. Part of the adipose tissue was fixed with 4% paraformaldehyde for slicing and staining, and the other part was immediately put in liquid nitrogen and transferred to -80 °C for RT-qPCR.

## Measurement of Level of Serum Lipids

The levels of serum total cholesterol (TC) (A111-1-1), serum triglyceride (TG) (A110-1-1), LDL-C (A113-1-1), and serum high-density lipoprotein-cholesterol (HDL-C) (A112-1-1) were measured using kits purchased from Nanjing JianCheng Bioengineering Institute (Jiangsu, China), according to the manufacturer's instructions, and the absorbance was measured by enzyme calibration. The level of serum ox-LDL was detected using an ELISA kit (JL10819, Jianglaibio Co., Ltd., Shanghai, China), and the absorbance at A562 was measured using an enzyme-labeled instrument.

## Enzyme-Linked Immunosorbent Assay (ELISA)

The levels of serum tumor necrosis factor (TNF- $\alpha$ , E-EL-M0049c, Elabscience Biotech, Wuhan, China), IL-6 (JL20268, Jianglaibio Co., Ltd., Shanghai, China), and IL-1 $\beta$  (MM-0040M1, Mmbio, Jiangsu, China) were measured according to the manufacturer's instructions, and the absorbance at A562 nm was measured by enzyme calibration.

## Histological Analysis

Samples were fixed with 4% paraformaldehyde, dehydrated using an alcohol concentration gradient, removed in xylene, embedded in paraffin, and cut into 4  $\mu$ m slices. The sections were stained with HE, Oil red, and Movat, and imaged using a Panoramic MIDI digital scanner (3DHISTECH Ltd., Hungary). Image-Pro Plus 6.0 (Media Cybernetics, Inc., Rockville, MD, USA) was used to analyze the atherosclerotic plaque area, lipid deposition, foam cell content, and collagen fiber content. The formulae are as follows: lipid deposition=red area of oil red staining/plaque area, foam cell content =foam cell area of Movat staining/plaque area, and collagen fiber content=collagen area of Movat staining/plaque area.

## Reverse Transcription-Quantitative Polymerase Chain Reaction (RT-qPCR)

Total RNA was extracted from aortic samples using a modified tissue/cell RNA rapid extraction kit (AC0202, Sparkjade, Shandong, China), followed by RNA concentration and purity measurement. Total RNA was then reverse transcribed into cDNA on PCR Thermal Cycler DiceTM (Takara Bio Inc., Kusatsu, Japan) and a SPARK script II-RT Plus Kit (R223-01, Vazyme, Nanjing, China). The cDNA was amplified using 2 $\times$ SYBR Green qPCR Mix (AH0104-B, Sparkjade, Shandong,

**Table 3** Primer Sequences for RT-qPCR Amplification

Name	Sequence 5'-3'
TLR4	F:AATCCCTGCATAGAGGTAGTTCC R:ATCCAGCCACTGAAGTTCTGA
MyD88	F:CATACCCTTGGTGCGCTTA R:CCAGGCATCCAACAACTGC
NF-κB p65	F:ATCGCCACCGATTGAAGAG R:CGGGGTTCAATTGGTCCATT
Actin	F:GGCTGTATCCCCTCCATCG R:CCAGTTGGTAACATGCCATGT

China), and the threshold cycle was recorded using the Light Cycler<sup>®</sup> 96 system (Roche Applied Sciences, Penzberg, Germany) using the following reaction conditions: 95.0 °C, 5 min 1 cycle; 95.0 °C for 10s, 56°C for 30s, 72°C for 30s for 40 cycles, 95°C for 15s, 65°C for 60s, 95°C continuous 1 cycles, and 40°C for 30s. The fold-change in mRNA expression was determined using the  $2^{-\Delta\Delta CT}$  method. Primers for TLR4, MyD88, NF-κB p65, and actin were designed and synthesized by Biosune Biotech (Shanghai, China). (Table 3)

## Immunohistochemistry (IHC)

Immunohistochemical analysis was performed using anti-TLR4 (1:1000, GB11519, Servicebio), anti-MyD88 (1:200, GB11269, Servicebio), and anti-NF-κB p65 (1:400, bs-0465R, Bioss) antibodies. Fixed tissue gradient alcohol dehydration, paraffin embedding, and slicing to a thickness of 4 μm were performed. After deparaffinization and rehydration, the tissue sections were antigen-repaired with citric acid antigen-repair buffer (pH 6.0) and heated for the specified time. After natural cooling, the slides were placed in PBS (pH 7.4) and washed by shaking on a decolorization shaker three times for 5 min each. After treatment with 3% H<sub>2</sub>O<sub>2</sub> and incubation at room temperature in the dark for 25 min, the slides were placed in PBS (pH 7.4) and washed by shaking on a decolorization shaker 3 times for 5 min each. Subsequently, 3% BSA was added to the circle to cover the tissue evenly, and the tissues were sealed for 30 min at room temperature. After gently removing the sealing solution, the sections were placed flat in a wet box with primary antibodies and incubated overnight at 4°C, followed by incubation with secondary antibodies for 30 min. The sections were then stained with diaminobenzidine (DAB) (G1211, Servicebio) and hematoxylin and mounted with neutral balsam. Brownish-yellow granules indicated positive signals. The slides were scanned and evaluated using Image-Pro Plus software.

## Statistical Analysis

We used QQ plots and Shapiro–Wilk test to assess data distribution. Graphpad Prism 9.0.0 software (San Diego, California, USA) was used to analyze and visualize the data. The measurement data are expressed as the mean ± SD. Analysis of variance (ANOVA) and Dunnett's T3 tests were carried out to determine statistical significance for multiple comparisons. The least significant difference (LSD) test was performed under the assumption of equal variances. The indigenous  $\alpha$  level was set to 0.05 (bilateral), and  $P < 0.05$  indicated a statistically significant difference.

## Results

### Screening results of Ingredients and Targets

A total of 124 ingredients and 418 targets of GMTG for AS treatment were screened from the HERB database. *Radix Astragali* contained 24, *Radix Salviae liguliobae* contained 40, *Trichosanthis Fructus* contained 14, *Rhizoma Coptidis* contained 16, *Radix Notoginseng* contained 32, *Radix Scrophulariae* contained 17, *Bulbus Fritillariae thunbergii* contained 12, *Rhizoma Polygoni cuspidati* contained 20, *Gardenia jasminoides* var. *grandiflora* contained 6 ingredients. Eleven ingredients were contained in more than two TCM herbs, eight of which were in *Radix Notoginseng* (Table 4).

A total of 4710 AS-related targets were identified from the GeneCards database. The intersection of GMTG-related and AS-related targets yielded 418 targets for GMTG treatment of AS.

**Table 4** Core Ingredients of GMTG

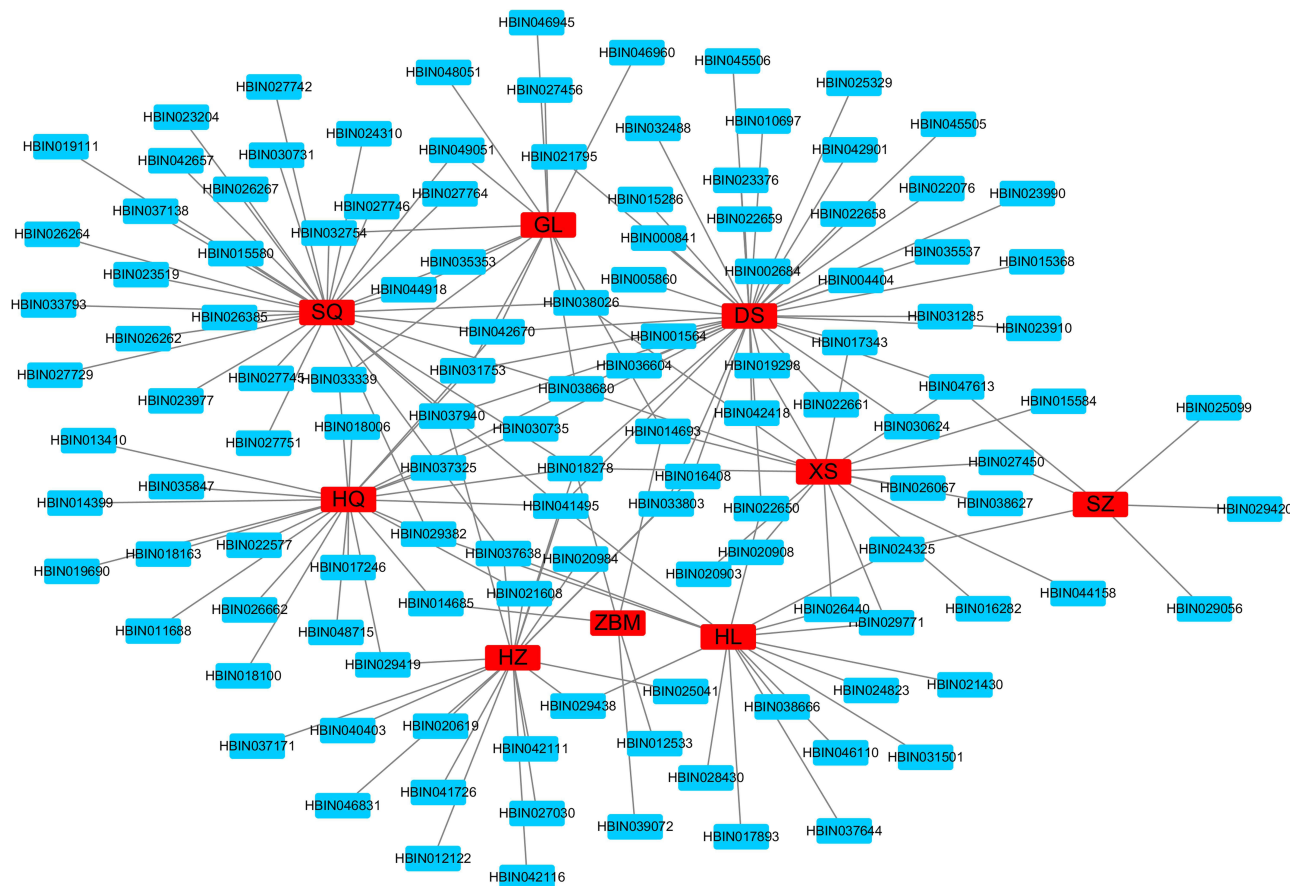
Ingredient	TCM Herbs
β-sitosterol	<i>Radix Astragali, Radix Salviae liguliobae, Radix Notoginseng, Radix Scrophulariae, Bulbus Fritillariae thunbergii, Rhizoma Polygoni cuspidati</i>
Palmitic acid	<i>Radix Astragali, Radix Salviae liguliobae, Trichosanthis Fructus, Radix Notoginseng, Radix Scrophulariae</i>
Quercetin	<i>Radix Astragali, Radix Salviae liguliobae, Rhizoma Coptidis, Radix Notoginseng, Rhizoma Polygoni cuspidati</i>
Oleic acid	<i>Radix Salviae liguliobae, Trichosanthis Fructus, Radix Notoginseng, Radix Scrophulariae</i>
Rutin	<i>Radix Astragali, Radix Salviae liguliobae, Trichosanthis Fructus, Radix Notoginseng</i>
Adenosine	<i>Trichosanthis Fructus, Radix Scrophulariae, Bulbus Fritillariae thunbergii</i>
Higenamine	<i>Radix Astragali, Rhizoma Coptidis, Radix Notoginseng</i>
Kaempferol	<i>Radix Astragali, Radix Salviae liguliobae, Trichosanthis Fructus</i>
Obaculactone	<i>Rhizoma Coptidis, Radix Notoginseng, Rhizoma Polygoni cuspidati</i>
Oleanolic acid	<i>Radix Salviae liguliobae, Radix Notoginseng, Rhizoma Polygoni cuspidati</i>
Ursolic acid	<i>Radix Salviae liguliobae, Radix Scrophulariae, Gardenia jasminoides var. grandiflora</i>

**Abbreviations:** GMTG, Guanmaitong Granule.

### Network Construction

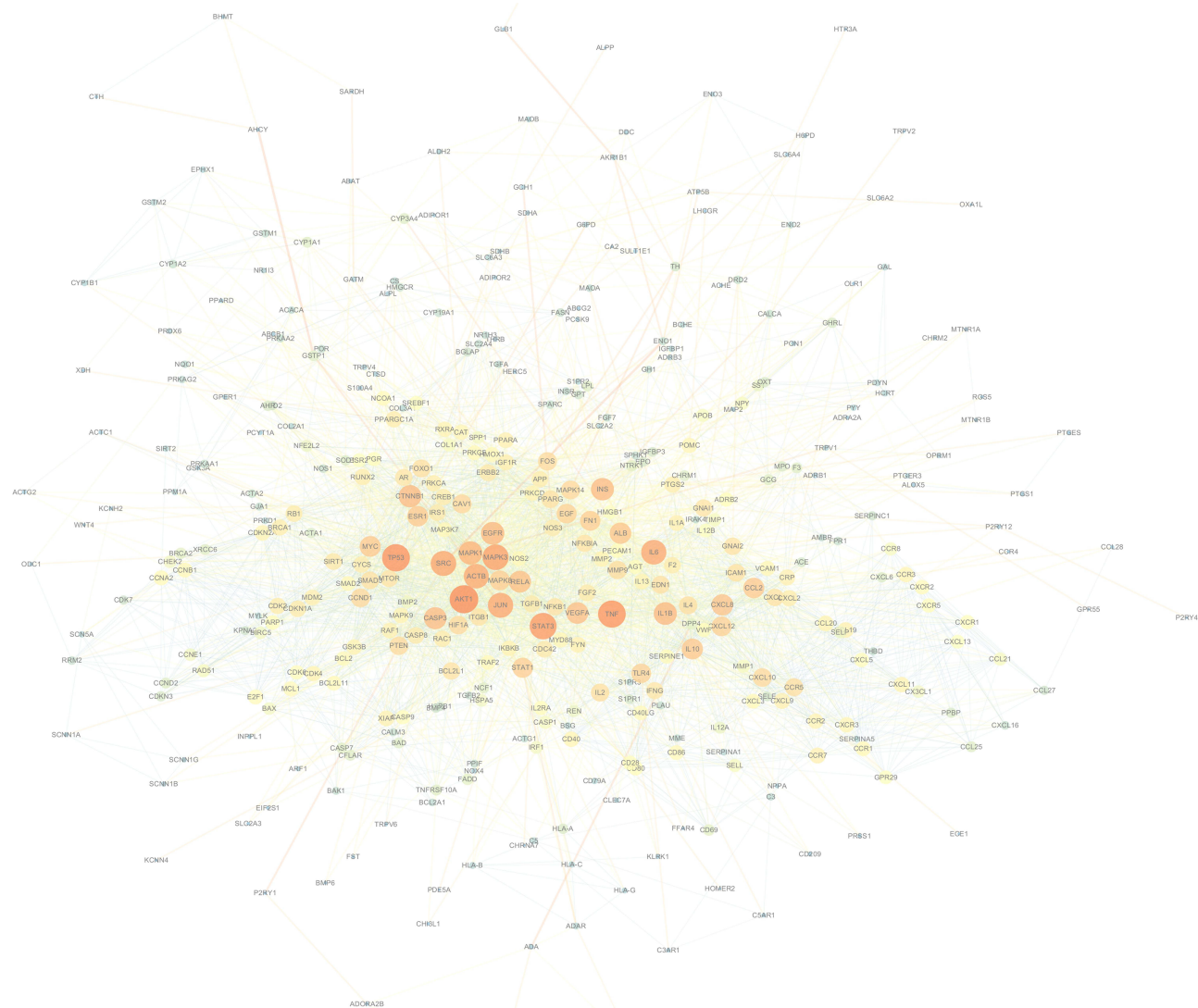
The TCM herb-ingredient network of GMTG used to treat AS (Figure 2) was constructed using Cytoscape software 3.7.0. It contained 133 nodes, including nine TCM herbs and 124 related ingredients. (Appendix 1) The PPI network of potential targets was constructed using STRING and Cytoscape with a confidence of 0.700, and 418 targets were screened and analyzed (Figure 3).

The top 11 ingredients in the network are listed in Table 5. Figure 4A showed the relationship between the TCM herbs and 11 core ingredients. *Radix Notoginseng, Radix Salviae liguliobae,* and *Radix Astragali* contained most of the



**Figure 2** TCM Herb-Ingredient network of GMTG for AS treatment. The red node represents the main TCM herbs of GMTG. The blue node represents the unique ingredient of each TCM herb. The gray edge represents the interaction between TCM herbs and ingredients.





**Figure 3** The PPI network of targets in GMTG for AS treatment. The node represents the potential target of AS treatment. The darker node has a higher degree value. The edge represents the interaction between target.

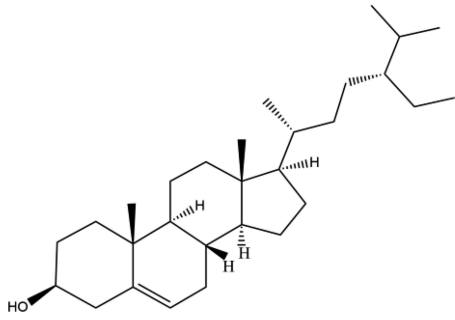
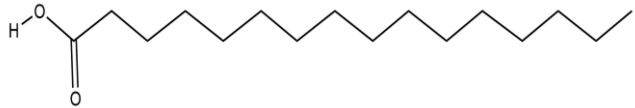
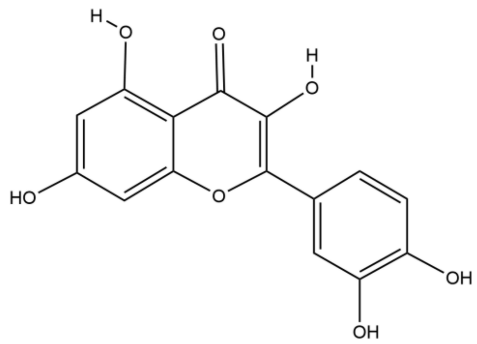
ingredients and were the core TCM herbs in the network. The top 100 targets in the PPI network were selected as core targets according to the degree (Figure 4B). The top 15 core targets based on the degree in the PPI network are listed in Table 6. TNF, IL-6, and IL 1 $\beta$  were the core inflammatory cytokine genes. The targets encoded by AKT1, TP53, EGFR, STAT3, and CASP3 were closely associated with the inflammatory response. MAPK1 and MAPK3 were the MAPK family proteins involved in inflammation.

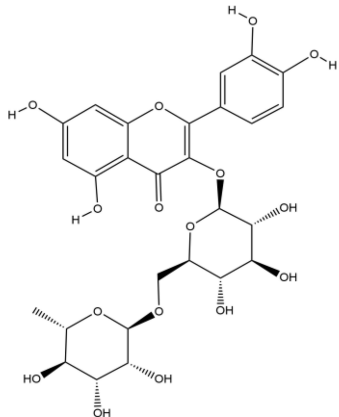
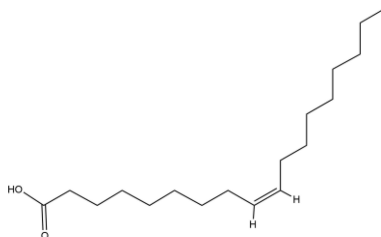
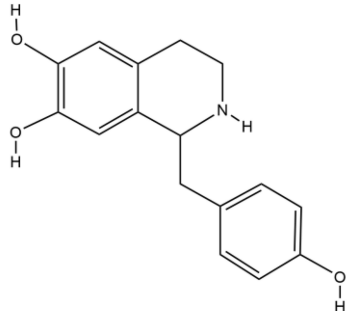
## GO and KEGG Pathway Analysis

Biological process enrichment analysis indicated that the potential targets were mainly enriched in response to lipopolysaccharides, molecules of bacterial origin, nutrient levels, and cellular response to chemical stress and biotic stimulus. (Figure 5A) The enriched cell components were the external side of the plasma membrane and a membrane raft (Figure 5B), and the enriched molecular function terms were primarily G protein-coupled receptor binding, signaling receptor activator activity, and receptor-ligand activity. (Figure 5C) GO enrichment results for the first 10 items are shown in Figure 5D.

KEGG pathway enrichment analysis identified 191 pathways with significant enrichment ( $P < 0.05$ ). The AGE-RAGE signaling pathway in diabetic complications, Lipid and atherosclerosis, TNF signaling pathway, IL-17 signaling pathway,

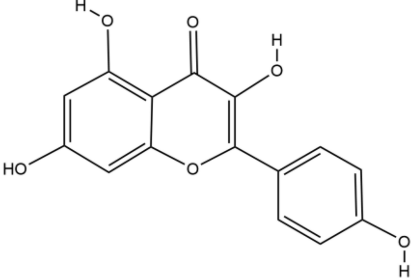
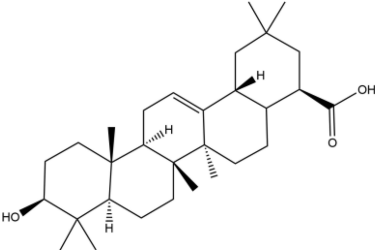
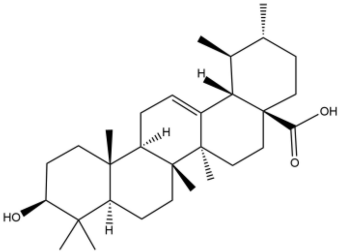
**Table 5** Information of the Top 11 Ingredients in TCM Herb-Ingredient Network

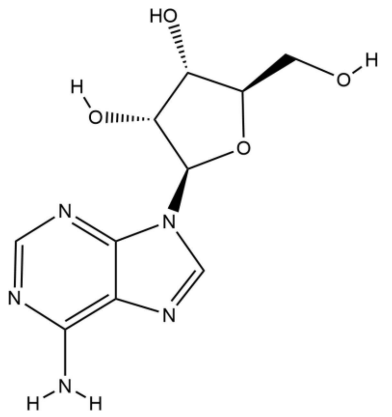
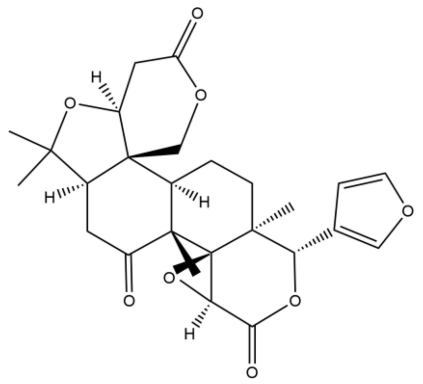
Rank	HERB ID	Name	Degree	OB score	DL	PubChem ID	Molecular Formula	Chemical Structure
1	HBIN018278	Beta-sitosterol	6	36.91390583	0.75	222,284	C <sub>29</sub> H <sub>50</sub> O	
2	HBIN038680	Palmitic acid	5	19.2965647	0.10	985	C <sub>16</sub> H <sub>32</sub> O <sub>2</sub>	
3	HBIN041495	Quercetin	5	46.43334812	0.28	5,280,343	C <sub>15</sub> H <sub>10</sub> O <sub>7</sub>	

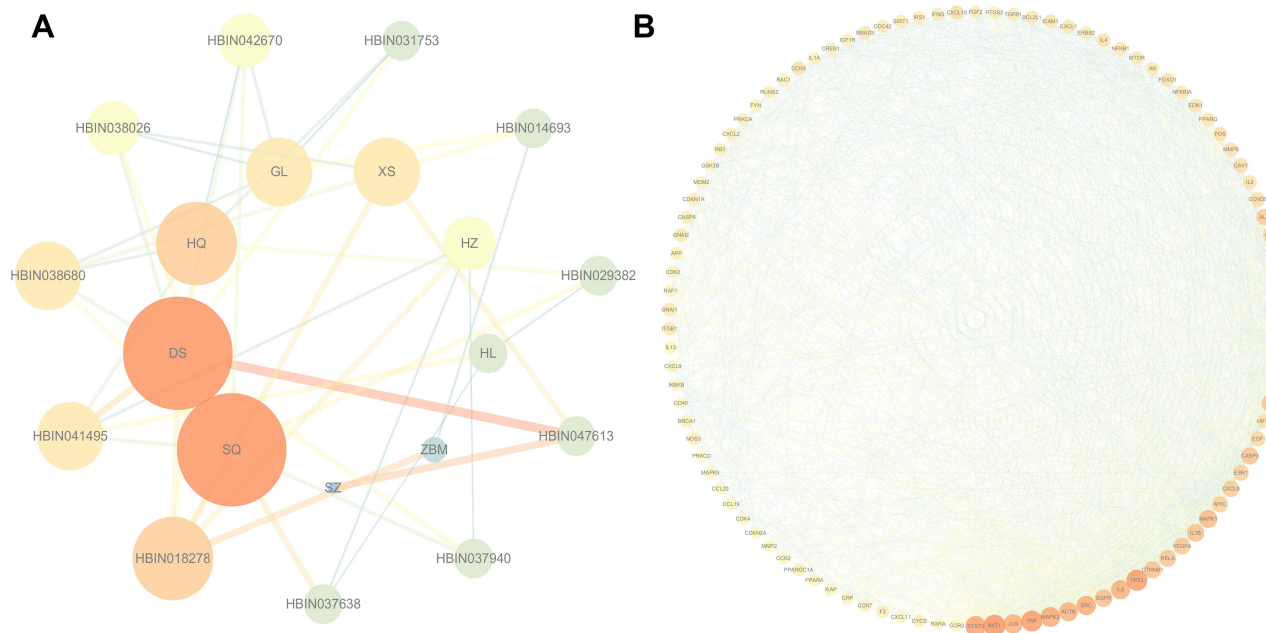
4	HBIN042670	Rutin	4	3.201533128	0.68	5,280,805	C <sub>27</sub> H <sub>30</sub> O <sub>16</sub>	
5	HBIN038026	Oleic acid	4	33.12836481	0.14	445,639	C <sub>18</sub> H <sub>34</sub> O <sub>2</sub>	
6	HBIN029382	Higenamine	3	-	-	114,840	C <sub>16</sub> H <sub>17</sub> NO <sub>3</sub>	

(Continued)

Table 5 (Continued).

Rank	HERB ID	Name	Degree	OB score	DL	PubChem ID	Molecular Formula	Chemical Structure
7	HBIN031753	Kaempferol	3	41.88224954	0.24	5,280,863	C <sub>15</sub> H <sub>10</sub> O <sub>6</sub>	
8	HBIN037940	Oleanolic acid	3	29.02084142	0.76	49,867,939	C <sub>30</sub> H <sub>48</sub> O <sub>3</sub>	
9	HBIN047613	Ursolic acid	3	16.77490232	0.75	64,945	C <sub>30</sub> H <sub>48</sub> O <sub>3</sub>	

10	HBIN014693	Adenosine	3	19,853,17634	0.16	60,961	C <sub>10</sub> H <sub>13</sub> N <sub>5</sub> O <sub>4</sub>	 <p>The chemical structure of Adenosine consists of an adenine base (a fused pyrimidine and imidazole ring system with an amino group at the 6-position) attached to a ribose sugar. The ribose is in its furanose form, with hydroxyl groups at the 2' and 3' positions and a hydroxymethyl group at the 4' position. The adenine base is attached to the 1' carbon of the ribose via a glycosidic bond.</p>
11	HBIN037638	Obaculactone	3	-	-	179,651	C <sub>26</sub> H <sub>30</sub> O <sub>8</sub>	 <p>The chemical structure of Obaculactone is a complex polycyclic molecule. It features a central bicyclic core with multiple fused rings, including a six-membered ring with a ketone group and a five-membered ring with an oxygen atom. There are several stereocenters indicated with wedged and dashed bonds. A furan ring is attached to the structure via a methylene bridge. The overall structure is highly complex and contains multiple oxygen atoms and carbonyl groups.</p>



**Figure 4** Network of core ingredients and core targets. (A) The node represents the TCM herb and target, the darker node has a higher degree. The edge represents the interaction between target, the darker edge has a closer betweenness. (B) The node represents target, the darker node has a higher degree.

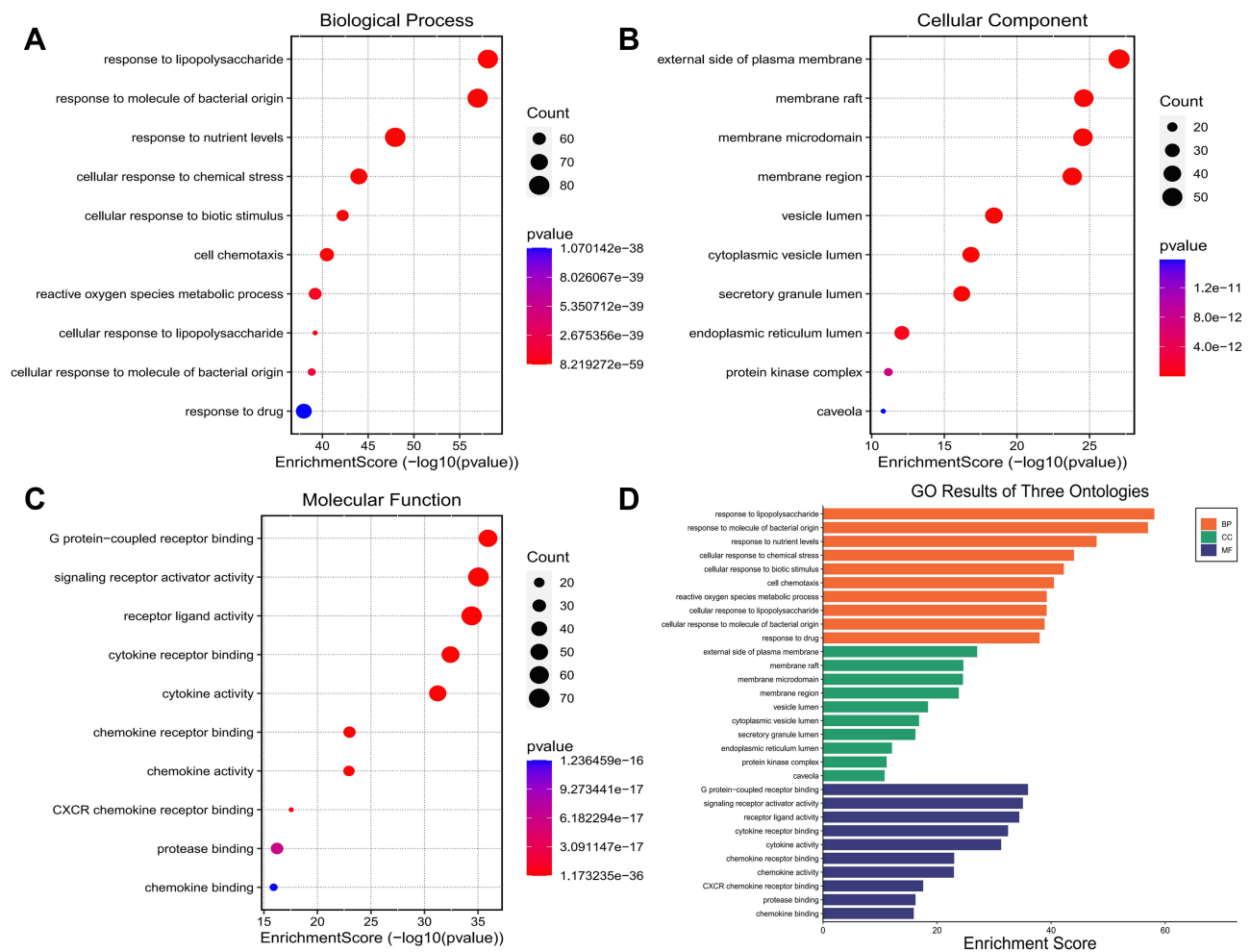
Apoptosis, PI3K-Akt signaling pathway, Toll-like receptor signaling pathway, NF-κB signaling pathway, and many other pathways related to the inflammatory response were among the top 50 pathways ranked by P-value. (Appendix 2) The results of the top 10 KEGG pathways were shown in Figure 6. In addition, the Lipid and atherosclerosis pathway was the most significant and was closely related to core targets TNF, IL-6, and IL-1β.

### Model Verification

Oil red staining from the aortic arch to the thoracic aorta showed atherosclerotic plaque formation in the MOD group, while no apparent plaque occurred in the NC group, which was consistent with the HE staining results. These results indicated the successful construction of the AS model (Figure 7).

**Table 6** Top 15 Targets in PPI Network

Rank	Target	Degree
1	AKT1	108
2	TP53	103
3	TNF	102
4	STAT3	98
5	MAPK3	93
6	SRC	89
7	JUN	88
8	IL6	87
9	ACTB	86
10	MAPK1	78
11	EGFR	75
12	IL1B	74
13	INS	73
14	CTNNB1	72
15	CASP3	71



**Figure 5** GO Enrichment Analysis. (A) Biological Process, (B) Cellular Component, (C) Molecular Function, (D) Results of three Ontologies.

## GMTG Reduced the Serum Lipid Level in ApoE<sup>-/-</sup> Mice

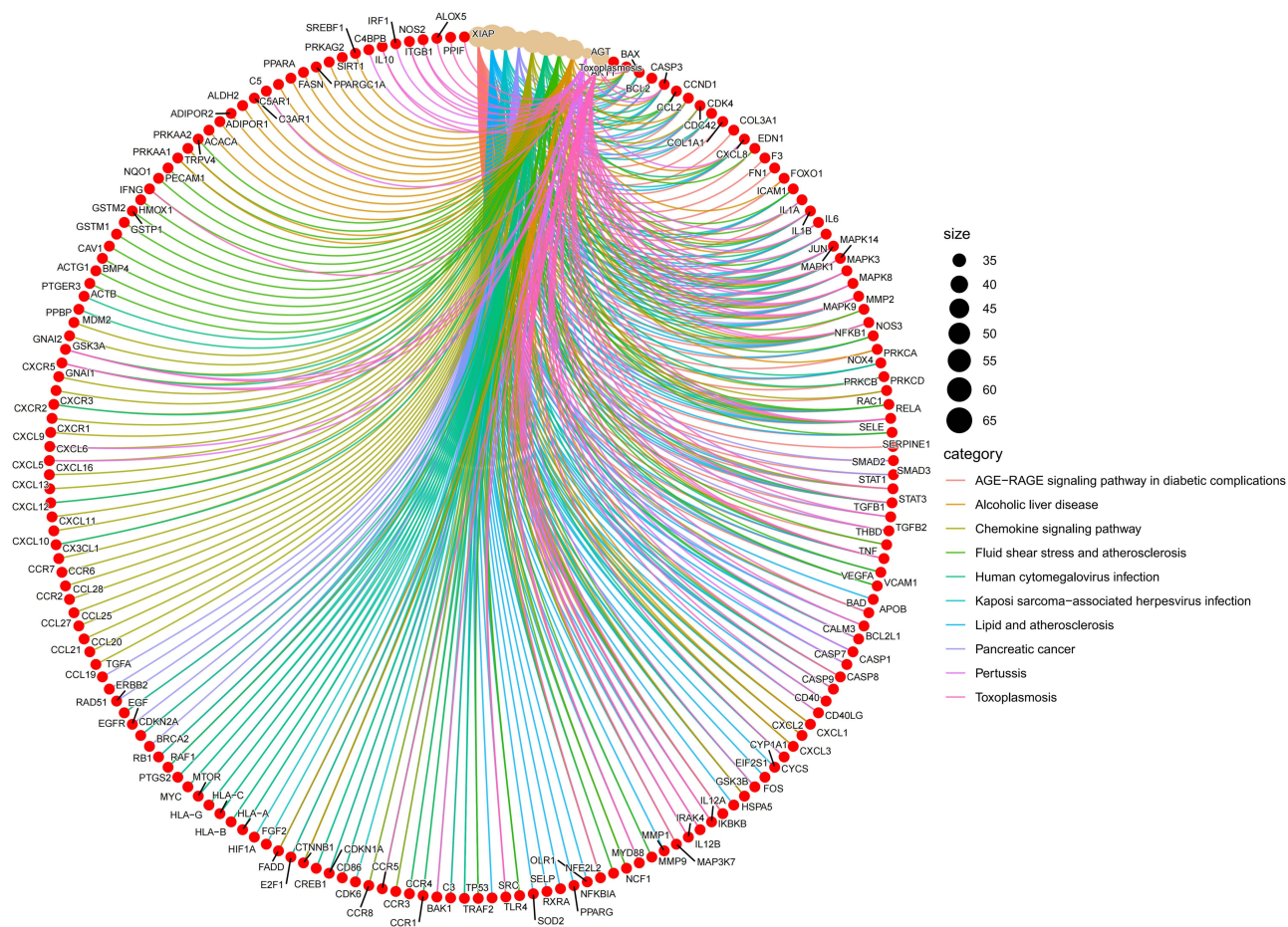
Results showed that GMTG has a lipid-lowering effect. Compared with those in the NC group, the serum TG, TC, LDL-C, and ox-LDL levels in the MOD group were significantly higher, and the HDL-C level was lower, conforming to hyperlipidemia characteristics in the AS model of ApoE<sup>-/-</sup> mice. Compared with the MOD group, the serum TG, TC, LDL-C, and ox-LDL levels in the GMTG and ATO groups were lower. (Figure 8)

## GMTG Inhibited the Level of Serum Inflammatory Cytokines in ApoE<sup>-/-</sup> Mice

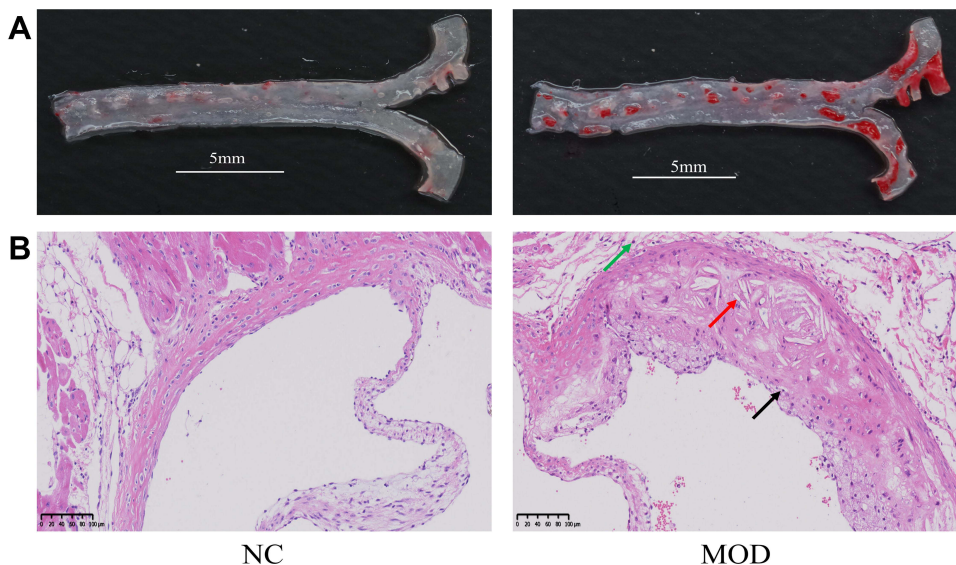
Based on the results of PPI and Cytoscape analyses, we selected inflammatory cytokines, including TNF- $\alpha$ , IL-6, and IL-1 $\beta$ , as the core targets for experimental verification. Compared with those in the NC group, the levels of serum TNF- $\alpha$ , IL-6, and IL-1 $\beta$  in the MOD group were increased. Compared with those in the MOD group, the levels of the above inflammatory cytokines in the GMTG and ATO groups were significantly decreased. (Figure 9)

## GMTG Ameliorated Atherosclerotic Plaque Lesions and Pathological Damage in ApoE<sup>-/-</sup> Mice

Oil red staining showed that atherosclerotic plaques in the thoracic aorta of the MOD group were significantly formed, and after GMTG and ATO treatment, the formation of aortic plaque was reduced. (Figure 10A) HE staining showed obvious atherosclerotic plaques in the aortic root of the MOD group, and a lipid core and cholesterol crystals were also formed, with inflammatory cell infiltration. (Figure 10B) The results were consistent with the oil red staining results. Oil red staining



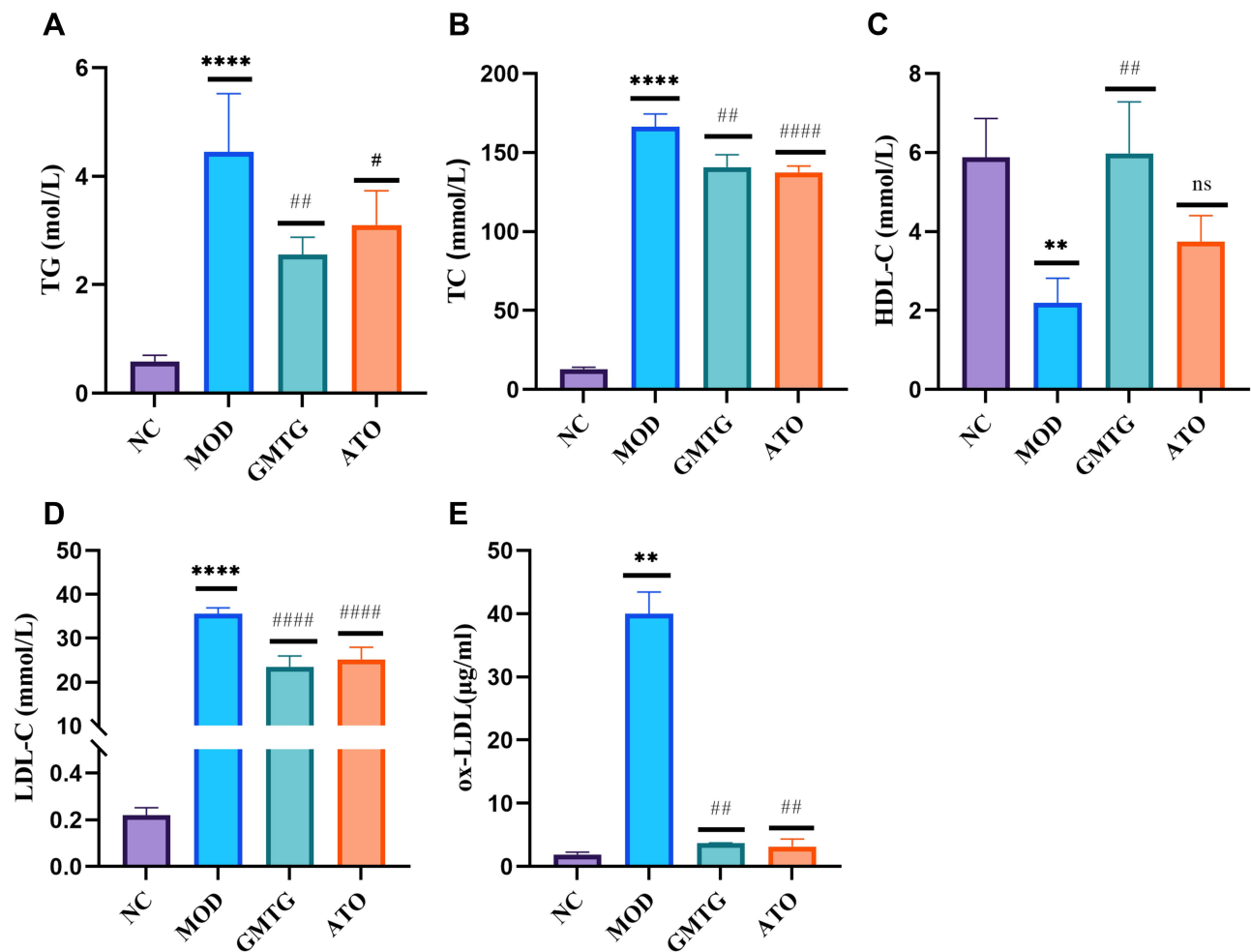
**Figure 6** KEGG pathway Enrichment Analysis. The red node represents target, the yellow node represents pathway, the line represents the relationship between pathway and target.



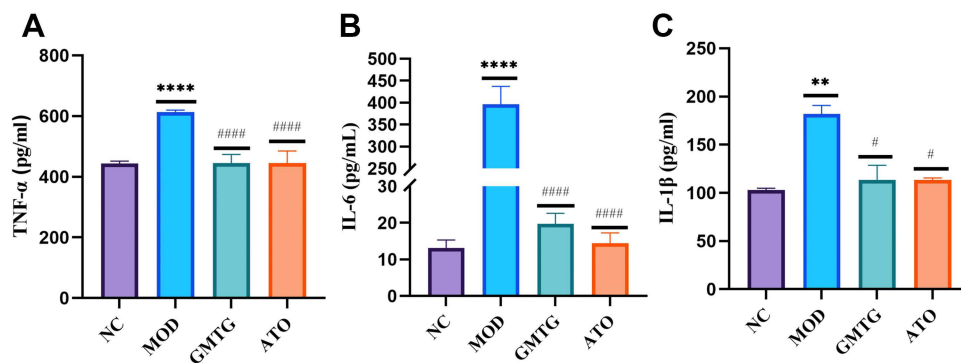
**Figure 7 (A)** Oil red staining of thoracic aorta, **(B)** HE staining. The black arrow represents plaque, the red arrow represents cholesterol crystals, the green arrow represents lymphocyte infiltration.

analysis of aortic root showed that GMTG and ATO reduced the atherosclerotic plaque area and lipid deposition in aortic root compared to that in the MOD group. (Figure 10C, D and E) In addition, the results of Movat staining showed that the content of foam cells in the GMTG group was lower than that in the MOD group, and the content of collagen fibers was higher than



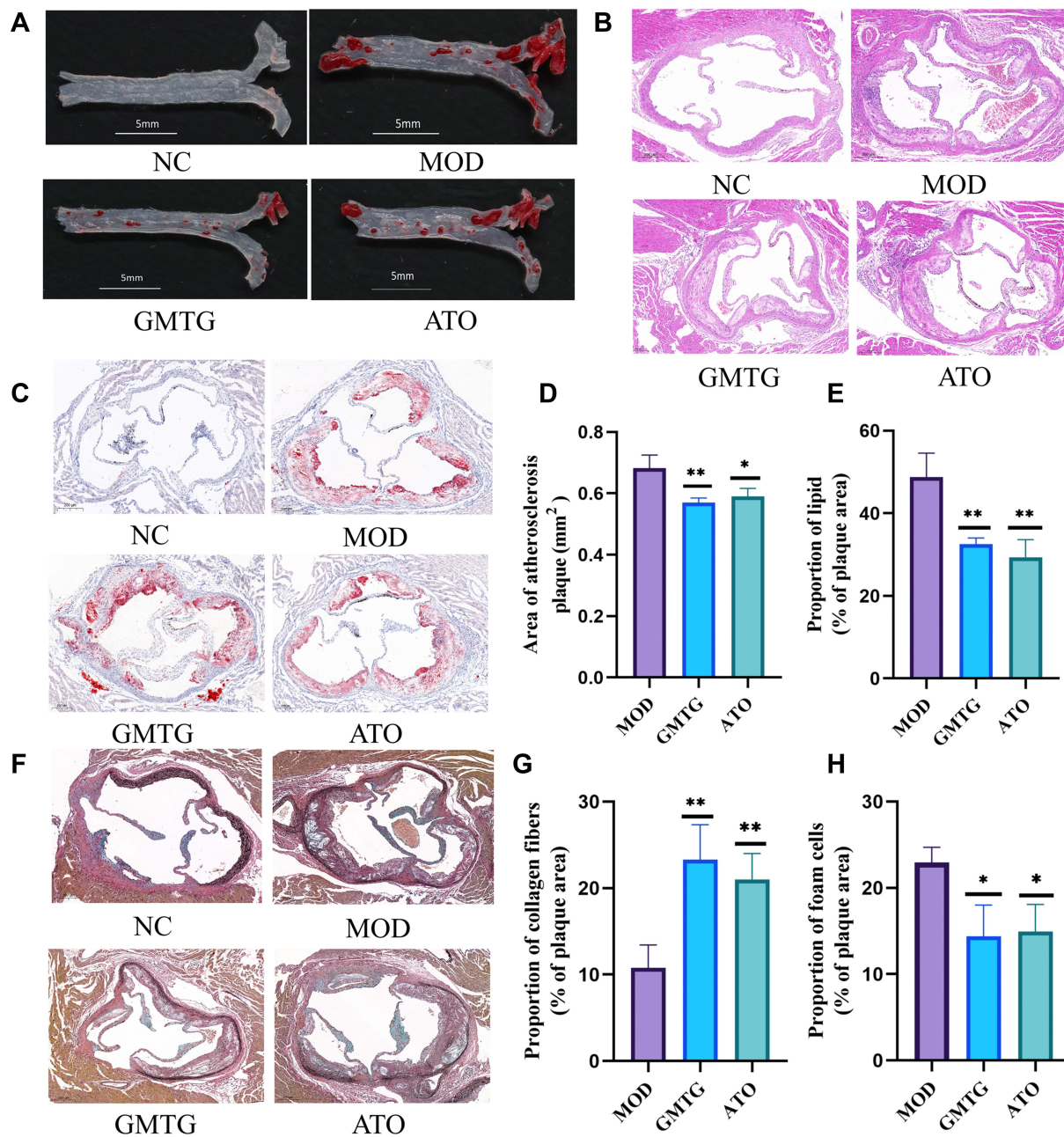


**Figure 8** GMTG treatment reduced levels of serum lipid. (A) TG, (B) TG, (C) HDL-C, (D) LDL-C, (E) ox-LDL. Data are presented as mean  $\pm$  SD (n = 3). \*\*P<0.01, \*\*\*P<0.0001, vs NC group; #P<0.05, ##P<0.01, ####P<0.0001, ns means no statistical significance, vs MOD group.



**Figure 9** GMTG treatment alleviates levels of serum inflammatory cytokines. (A) TNF- $\alpha$ , (B) IL-6, (C) IL-1 $\beta$ . Data are presented as mean  $\pm$  SD (n = 3). \*\*P<0.01, \*\*\*P<0.0001, vs NC group; #P<0.05, ##P<0.01, ####P<0.0001, vs MOD group.

that in the MOD group. (Figure 10F, G and H) In summary, GMTG could reduce the area of aortic atherosclerotic plaque, reduce plaque lipid deposition and plaque foam cell content, and increase plaque collagen fiber content, thereby reducing the burden of plaque and promoting plaque remodeling to play an anti-AS role.

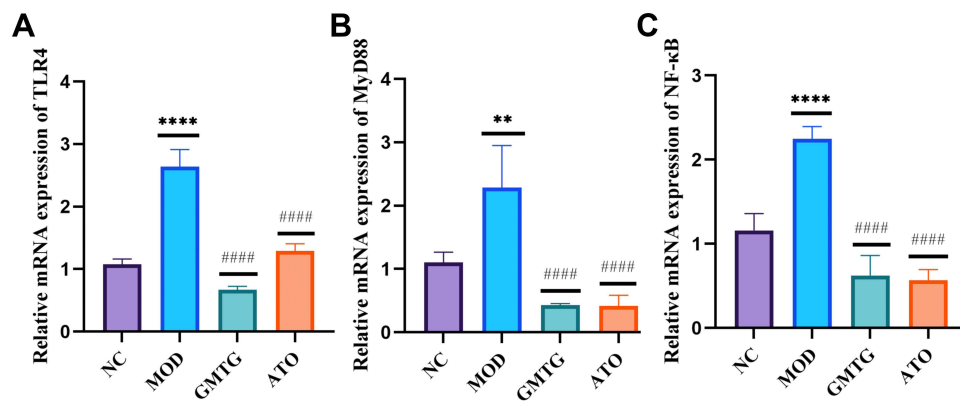


**Figure 10** GMTG treatment alleviates atherosclerotic plaque lesions and pathological damage in ApoE<sup>-/-</sup> Mice. (A) Oil red staining of the thoracic aorta, (B) HE staining, (C) Oil red staining of the aortic root, (D) Area of atherosclerosis plaque, (E) Proportion of lipid, (F) Movat staining, nucleus and elastic fibers were black, proteoglycans were blue, collagen fibers were red, muscle fibers were pink, and foam cells were pale purple. (G) Proportion of collagen fibers, (H) Proportion of foam cells. Data are presented as mean ± SD (n = 3). \*P<0.05, \*\*P<0.01, ns means no statistical significances MOD group.

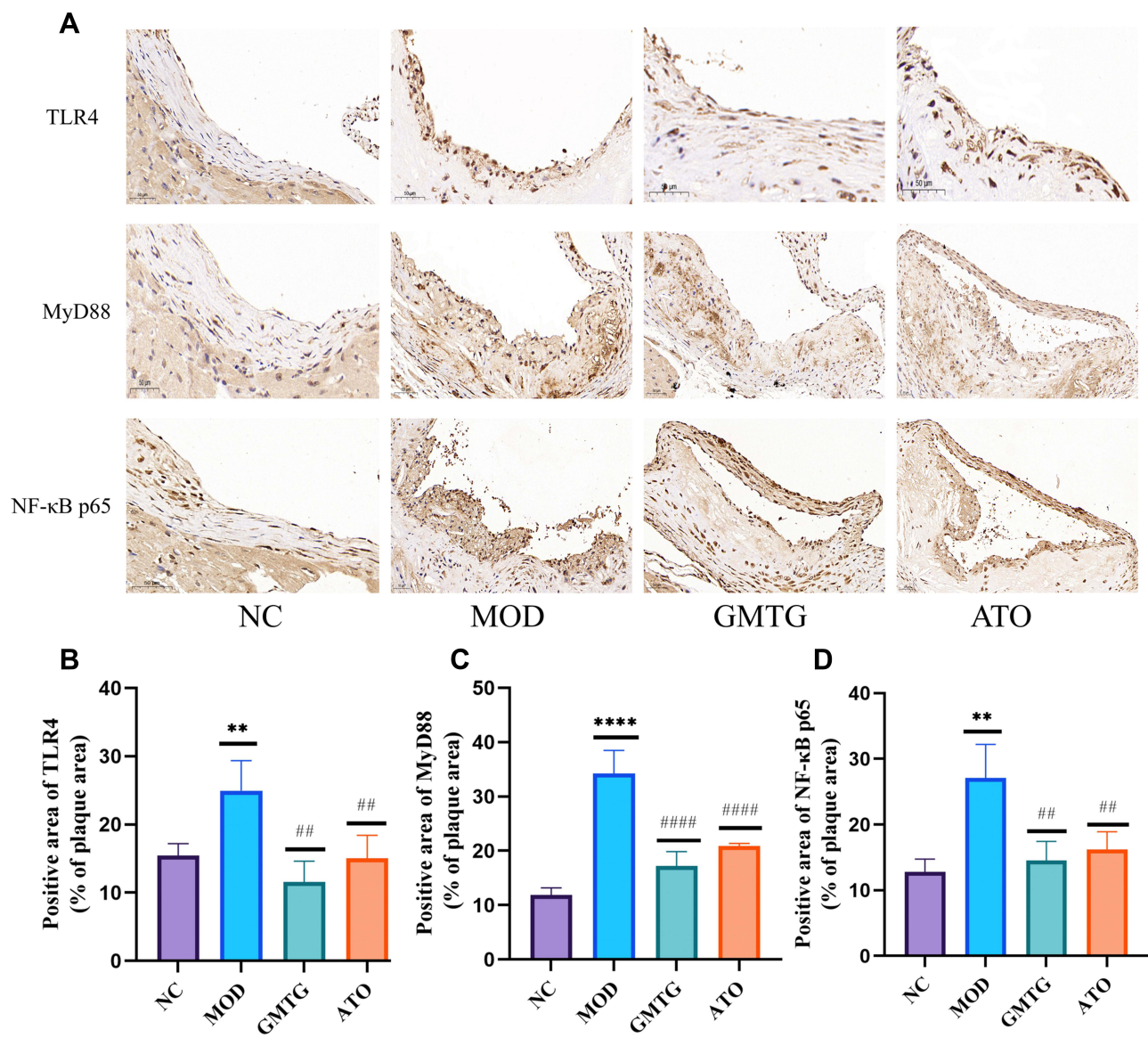
## GMTG Regulated the TLR4/MyD88/NF-κB Signaling Pathways in ApoE<sup>-/-</sup> Mice

Based on the results of KEGG pathway enrichment and PPI analyses, the TLR4/MyD88/NF-κB signaling pathway in AS was selected as the core inflammation-related pathway for experimental verification. The mRNA levels of TLR4, MyD88, and NF-κB in the MOD group were higher than those in the NC group (Figure 11). Compared with those in the MOD group, TLR4, MyD88, and NF-κB mRNA levels were significantly decreased after GMTG or ATO treatment.

The IHC results showed that the positive areas of TLR4, MyD88, and NF-κB in the MOD group were higher than those in the NC group, and the positive areas of TLR4, MyD88, and NF-κB in the GMTG and ATO groups were lower than those in the MOD group (Figure 12). It was consistent with RT-qPCR results. The above results showed that GMTG



**Figure 11** GMTG treatment regulates mRNA expressions of TLR4, MyD88, NF-κB in ApoE<sup>-/-</sup> Mice. (A) The relative mRNA expression of TLR4, (B) MyD88, (C) and NF-κB of the RT-qPCR results. Data are presented as mean ± SD (n = 3). \*\*P<0.01, \*\*\*\*P<0.0001, vs NC group, ####P<0.0001, vs MOD group.



**Figure 12** GMTG treatment regulates protein expressions of TLR4, MyD88, and NF-κB in ApoE<sup>-/-</sup> mice. (A) The positive area of TLR4, (B) MyD88, (C) and NF-κB of IHC results. Data are presented as mean ± SD (n = 3). \*\*P<0.01, \*\*\*\*P<0.0001, vs NC group; ##P<0.01, ####P<0.0001, vs MOD group.

could inhibit the inflammatory immune response of atherosclerotic plaque through the TLR4/MyD88/NF- $\kappa$ B pathway in ApoE<sup>-/-</sup> mice and has the anti-AS effect of stabilizing the plaque.

## Discussion

In this study, the main ingredients and mechanisms of GMTG in treating AS were comprehensively and reliably analyzed using a high-quality database of TCM. The key targets of the core signaling pathway and downstream inflammatory cytokines were verified in vivo by constructing an ApoE<sup>-/-</sup> mice AS model, which provided objective data supporting the anti-AS effect of TCM, that is the inhibition of inflammatory and immune response in AS.

We screened ingredients and targets related to GMTG through high-throughput experiments of TCM and reference database HERB to ensure the data obtained were comprehensive and reliable. The HERB database, also known as BENCAO ZUJIAN, was jointly developed by the Peking University of TCM, Institute of Computing Technology of the Chinese Academy of Sciences, and Institute of Kidney Research of West China Hospital of Sichuan University. It is advantageous because of the ability to cross-reference multiple TCM database, including the most comprehensive list of TCM herbs and ingredients compiled to date, which makes it convenient to use. In addition, it provides high-quality, evidence-based links between TCM herbs and modern drugs by linking targets, diseases, and TCM herbs/ingredients by integrating the results of high-throughput experimental results, literature search, and statistical inference results of TCM herbs/ingredients.<sup>36</sup> Therefore, considering the high-quality of retrieval results from the HERB database, we directly search for ingredients and targets related to TCM herbs to reduce the false positive rate of indirect searching, providing reliable data supporting further experimental research.

ApoE<sup>-/-</sup> mice develop atherosclerotic lesions naturally, however, they mostly form stable plaques and fail to form a vulnerable plaque model.<sup>37</sup> Our goal was to simulate the complex pathological changes in the late stage of AS, especially the mechanism of vulnerable plaque and plaque rupture in the late stage. Based on the existing research,<sup>38</sup> we constructed an HFD-induced atherosclerotic vulnerable plaque model using ApoE<sup>-/-</sup> mice. First, 8-weeks-old ApoE<sup>-/-</sup> mice were fed an HFD for 16 weeks. After 8 weeks of continuous administration, pathological changes in plaque and various indicators were detected. In the MOD group, large lipid cores, foam cells and cholesterol crystals, infiltration of inflammatory cells, and the rupture of some plaques were observed in the typical aortic root plaque.<sup>37</sup>

The inflammatory response is involved in all stages of AS development.<sup>39</sup> CANTOS experiment confirmed the correctness of AS inflammation theory and opened the second era of AS prevention and treatment. It focused on anti-inflammatory benefits.<sup>16</sup> TCM herbs have therapeutic potential in preventing and treating AS inflammation and immunity because of their multi-target effects and overall regulation. Analysis of the composition of GMTG showed that *Radix Notoginseng*, *Radix Salviae ligulioabae*, and *Radix Astragali* play a more important role in the formula, which contains many ingredients and targets for the treatment of AS by GMTG. These three TCM herbs are commonly used for the clinical treatment of AS.<sup>40</sup> Existing studies have shown that various main ingredients of GMTG have anti-AS effects, including  $\beta$ -sitosterol, palmitic acid, quercetin, etc.  $\beta$ -sitosterol can reduce cholesterol synthesis by inhibiting cholesterol synthase expression and regulate inflammation by regulating the hypothalamus-pituitary-adrenal axis.<sup>41</sup> Palmitic acid, a saturated fatty acid, is the leading free fatty acid in plasma lipids. It may lead to an inflammatory reaction,<sup>42-45</sup> cell dysfunction, and even cell death<sup>46,47</sup> by inducing oxidative stress and continuous endoplasmic reticulum stress in cardiomyocytes. Kaempferol is a flavonoid antioxidant, which has anti-inflammatory and anti-atherosclerotic effects.<sup>48,49</sup> It can increase the expression of Bcl-2 by silencing the mitochondrial pathway mediated by regulatory factor 1 and play a protective role in hypoxic myocardial cells. Kaempferol can effectively reduce vascular inflammation and prevent AS development.<sup>50</sup> Rutin is an anti-inflammatory, anti-oxidant, anti-allergic, and antiviral flavonoid molecule, known to have anti-atherosclerotic and autophagy-inducing properties, which could inhibit ox-LDL-mediated macrophage inflammation and foam cell formation by inducing autophagy and modulating PI3K/ATK signaling, showing potential in treating atherosclerosis.<sup>51</sup> Oleic acid is a monounsaturated fatty acid, which can stimulate vascular smooth muscle cells proliferation and migration.<sup>52</sup> It also has an anti-inflammatory effect, reducing LDL oxidation and lowering cholesterol.<sup>53-55</sup> Higenamine has anti-thrombotic, anti-apoptotic, antioxidant, anti-inflammatory, and immunomodulatory effects.<sup>56</sup> Oleanolic acid and ursolic acid belong to pentacyclic triterpenoid acids, and they are isomers, so their pharmacological effects are almost the same. They have been proved to have pharmacological effects such as anti-inflammatory, anti-atherosclerosis, lowering lipid, and inhibiting smooth muscle cell proliferation.<sup>57</sup> Adenosine, an extracellular signal molecule, has the effects of anti-inflammatory and anti-thrombotic, reducing

blood pressure and heart rate, and may delay the occurrence of early atherosclerotic events.<sup>58,59</sup> Obaculatone has anti-inflammatory, analgesic, and antioxidant effects, which can regulate blood lipids and prevent atherosclerosis.<sup>60,61</sup>

Dyslipidemia is one of the most critical risk factors for AS. It is well known that hypercholesterolemia is the primary risk factor for atherosclerosis cardiovascular disease (ASCVD), and treatment for reducing LDL-C has become the cornerstone of primary and secondary prevention of ASCVD.<sup>62</sup> However, there is still a residual cardiovascular risk after effectively lowering LDL-C levels using statins or combined lipid-lowering strategies.<sup>63,64</sup> Recent studies have shown that inflammation and cholesterol are both pathogenic factors of AS. AS develop only when these two factors coexist.<sup>65</sup> CANTOS showed that the cardiovascular benefits of canakinumab were directly related to the reduction in IL-6.<sup>16</sup> Furthermore, evidence shows that hypertriglyceridemia has a causal relationship with an increased risk of AS,<sup>66,67</sup> TG-rich lipoprotein and its residues are also independent risk factors for AS.<sup>65</sup> Ox-LDL is a causal factor of AS and plays an important role in the formation by promoting inflammation and lipid deposition in the arterial wall. LDL-C is oxidized and modified to ox-LDL in the intimal region of the blood vessel, which induces the injury and adhesion of ECs to monocytes and chemotaxis to the subcutaneous tissue, allowing the formation of macrophage-derived foam cells, which leads to the dissolution of fibrous caps and eventually affects plaques stability. It plays an important role in all aspects of AS.<sup>68</sup> Overall, reducing circulating TC, TG, LDL-C, and ox-LDL levels is beneficial to AS, which is consistent with the results of this study. GMTG can regulate lipid levels by reducing the TG, TC, LDL-C, and ox-LDL levels, and increasing HDL-C level. In addition, GMTG reduced the levels of circulating TNF- $\alpha$ , IL-6, and IL-1 $\beta$  in ApoE<sup>-/-</sup> mice, indicating that it has anti-inflammatory and lipid-lowering effects.

We identified a large number of pathways that might be involved in the treatment of AS by GMTG through enrichment analysis. The TLR4 pathway not only exists in the top one pathway set, but its downstream cytokines, including TNF- $\alpha$ , IL-6, and IL-1 $\beta$ , are also the core targets in the PPI network. Therefore, we selected the TLR4 pathway and its downstream inflammatory cytokines for further experimental verification. Studies have shown that the TLR4 pathway might be involved in the inflammatory and immune responses in AS.<sup>69,70</sup> The activation of Toll-like receptors and MyD88 is an important part of the immune and inflammatory mechanism of AS and plaque formation.<sup>71</sup> NF- $\kappa$ B is a downstream transcription factor of the TLR4 pathway. Activated NF- $\kappa$ B translocates into the nucleus and binds to its related DNA motif to induce the transcription of target genes, including pro-inflammatory cytokines, such as TNF, IL-1, and mitogen.<sup>72</sup> The TLR4/MyD88/NF- $\kappa$ B pathway is an important inflammatory signal transduction pathway in the body and is closely related to the development of AS. Upregulation of TLR4 protein expression in vascular ECs can activate NF- $\kappa$ B through MyD88 and induce the production and release of pro-inflammatory cytokines such as TNF- $\alpha$ , IL-6, and IL-1 $\beta$ , to promote the formation of AS plaques.<sup>73–75</sup> TNF- $\alpha$  is a pro-inflammatory cytokine closely related to AS, thrombosis, and plaque rupture. It can promote the endocytosis and transport of LDL-C in vascular ECs,<sup>76</sup> inhibit the formation of nitric oxide synthase, stimulate ECs to express adhesion molecules, and lead to vascular endothelial dysfunction.<sup>77,78</sup> As an important pro-inflammatory cytokine, IL-6 is involved in the inflammatory response and aggravates the development of AS.<sup>79</sup> IL-1 $\beta$ , another important pro-inflammatory cytokine, is involved in various autoimmune inflammatory reactions and cell activities, including cell proliferation, differentiation, and apoptosis.<sup>80–83</sup> IL-1 $\beta$  also synergistically induced VEGF production with TNF and IL-6, and plays a role in angiogenesis.<sup>80</sup> Our study showed that GMTG could inhibit the TLR4/MyD88/NF- $\kappa$ B pathway, reduce the release of downstream inflammatory cytokines TNF- $\alpha$ , IL-6, and IL-1 $\beta$ , and play an anti-AS role by inhibiting plaque inflammation and increasing plaque stability.

Although GMGT seems a little better than statin in lipid regulation and anti-inflammation, most of the results were not statistically significant. Thus, further research is necessary.

## Conclusion

Overall, the effective mechanism of GMTG in treating AS involves the regulation of targets and pathways in various biological processes, especially inflammation and immune response. The results showed that GMTG could exert anti-inflammatory and immune-regulating effects on AS partially via the TLR4/MyD88/NF- $\kappa$ B signaling pathway. GMTG could also regulate the levels of serum lipids, reduce plaque burden, promote plaque remodeling and increase plaque stability. In addition, GMTG can potentially reduce the risk of residual cardiovascular inflammation. Therefore, GMTGs could be a potential therapeutic drug with multiple anti-AS effects, which warrants further study.

## Abbreviations

AMI, Acute myocardial infarction; AS, Atherosclerosis; ASCVD, Atherosclerosis cardiovascular disease; DAB, Diaminobenzidine; ECs, Endothelial cells; ELISA, Enzyme-Linked Immunosorbent Assay; GMTG, Guanmaitong granule; LDL-C, Low-density lipoprotein-cholesterol; HDL-C, High-density lipoprotein-cholesterol; HFD, High-fat diet; ICAM-1, Intercellular adhesion molecule 1; IHC, Immunohistochemistry; IL-1 $\beta$ , Interleukin-1 beta; IL-6, Interleukin-6 LDL-C, Low density lipoprotein-cholesterol; MCP-1, Monocyte chemoattractant protein 1; MyD88, Myeloid differentiation primary response protein MyD88; NF- $\kappa$ B, Transcription factor p65; ox-LDL, oxidized low density lipoprotein; PCSK9, Proprotein convertase subtilisin/kexin type 9; RT-qPCR, Reverse transcription quantitative polymerase chain reaction; SYK, Tyrosine-protein kinase SYK; TC, Total cholesterol; TCM, Traditional chinese medicine; TG, Triglycerids; TLR4, Toll-like receptor 4; TNF- $\alpha$ , Tumor necrosis factor; VCAM-1, Vascular cell adhesion protein 1.

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## Disclosure

The authors declare that there are no conflicts of interest in this work.

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