

Baolier Capsule's Secret Weapon: Piperine Boosts Cholesterol Excretion to Combat Atherosclerosis

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Purpose: The Baolier capsule (BLEC) is a proprietary Mongolian medicine administered for treating hypercholesterolemia and atherosclerosis (AS). However, the therapeutic effects, primary bioactive ingredients, and potential mechanisms underlying hypercholesterolemia and AS remain unclear. This study aimed to investigate the pharmacological effects, principal active ingredients, and mechanisms of BLEC against hypercholesterolemia and AS.

Methods: Adeno-associated virus tail vein injection was utilized to construct liver-specific LXR α knockout ApoE^{-/-} mice. A high-fat diet was utilized to feed ApoE^{-/-} mice to build hyperlipidemia and AS mouse models. The aorta or liver stained with Oil Red O was used to assess the effect of the drugs on AS or fatty liver formation after the oral administration of BLEC, piperine, statins, or ezetimibe to the mice following the experimental protocol. Biochemical assays were utilized to evaluate the effect of the drugs on serum lipid levels and cholesterol efflux indicators. Transcriptomics was employed to investigate the effect of BLEC on liver gene expression levels. HPLC-MS/MS was used to determine BLEC and its major components in the liver. Western blotting or quantitative reverse transcription polymerase chain reaction was conducted to detect LXR α , ABCA1, ABCG5, ABCG8, and CYP7A1 expression.

Results: Here, we revealed that BLEC decreases lipid levels in the serum and liver, as well as decelerates AS by promoting cholesterol excretion. BLEC and piperine, which are the main components exposed in the target liver tissue, activate LXR α to upregulate ABCA1, ABCG5, ABCG8, and CYP7A1, which promotes cholesterol transport to high-density lipoprotein and excretion to bile and feces. Notably, piperines demonstrated synergistic beneficial effects with atorvastatin or ezetimibe, which are two widely used hypocholesterolemic and anti-atherosclerotic drugs.

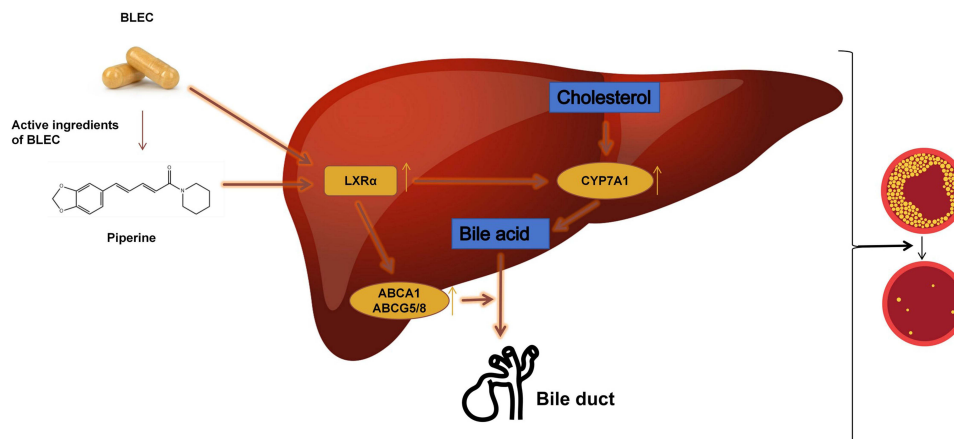
Conclusion: BLEC and its main active ingredient, piperine, promote cholesterol excretion, reduce serum cholesterol levels, inhibit AS, and exhibit good clinical application value and prospects.

Keywords: Baolier Capsule, atherosclerosis, hypercholesterolemia, cholesterol excretion, piperine, LC-MS/MS, transcriptomics

Introduction

Atherosclerotic cardiovascular disease (ASCVD), which is a significant public health issue, is the leading cause of global mortality and imposes a heavy medical and economic burden.¹ Research has revealed that hypercholesterolemia causes ASCVD and reducing low-density lipoprotein cholesterol (LDL-C) levels decreases the risk of major adverse cardiovascular events.²⁻⁴ Clinically, cholesterol reduction is primarily achieved by inhibiting intestinal cholesterol absorption (ezetimibe), increasing the liver uptake of lipoproteins (Proprotein Convertase Subtilisin/Kexin Type 9 [PCSK9] inhibitors), and inhibiting de novo cholesterol synthesis (Statins).⁵⁻⁷ These drugs have been widely prescribed, but a large portion of patients still experience recurrent CVD, and their LDL-C levels fail to reach the target levels

Graphical Abstract



recommended in the guidelines.⁸ Most importantly, none of these existing cholesterol-lowering drugs decrease cholesterol by directly promoting cholesterol catabolism or excretion.

The ATP-binding cassette subfamily G member 5/8 (ABCG5/8) heterodimer helps excrete cholesterol into the bile and intestinal lumen.⁹ Pharmacological LXR α activation increases cholesterol efflux by upregulating ABCG5/G8.¹⁰ However, LXR α increases Sterol Regulatory Element-Binding Protein 1 (SREBP1) that drives fatty acid biosynthetic gene expression, causing detrimental hepatic steatosis and hypertriglyceridemia.^{11,12} Thus, direct LXR agonists are not clinically useful to treat hypercholesterolemia.

Traditional Chinese medicine (TCM), which is one of the intriguing features of traditional Chinese culture, has a history of more than 2000 years, with both unique theories and rich experience.¹³ TCM may be utilized as a complementary and alternative approach to primary and secondary ASCVD prevention.¹⁴ Mongolian medicine, which is part of Mongolian cultural heritage, has evolved from the experience of fighting diseases and absorbed elements from TCM and Tibetan medicine.¹⁵ The Baolier capsule (BLEC), which is a type III Mongolian medicine for lowering blood lipids, has a national invention patent and treats hypercholesterolemia and coronary heart disease.^{16–19} We previously confirmed that BLEC reduces total cholesterol (TC), triglycerides (TG), and LDL-C levels and increase high-density lipoprotein cholesterol (HDL-C) levels through in vivo experiments and network pharmacology.¹⁹ However, the mechanism and main active ingredients by which BLEC reduces cholesterol and thus inhibits AS formation remain unclear.

Piperine is the main active constituent obtained from black pepper (*P. nigrum* L).²⁰ Piperine has exhibited a wide range of biological properties, including antioxidant activity, anti-inflammatory effects, antibacterial, anticancer, neuroprotective, antihypertensive, antidiabetic effects, and hepatoprotective properties.²⁰ The ability to improve the bioavailability of drug molecules by reducing their dosing frequency and dosage.²¹ Piperine significantly reduces TC, triglycerides TG, and LDL-C levels in rats fed a high-lipid diet.²² However, the mechanism by which piperine lowers blood lipids remains unclear.

This study used a comprehensive approach combining in vivo experiments, transcriptomics, and liquid chromatography (LC)-mass spectrometry (MS)/MS to determine the mechanism of BLEC treatment for AS and its major active components (Figure S1). First, we constructed an atherosclerosis (AS) mouse model and observed the therapeutic effects of BLEC on AS by assessing the histopathology of the aortic tissue. Subsequently, we detailed and characterized the expression of the gene profiles at the liver tissue level by conducting transcriptomic analysis. We used the LC-MS/MS method to analyze the liver compounds of BLEC in the mouse model. Finally, we utilized in vivo therapeutic experiments in mice to confirm the effects and pathways of the main active component piperine on hypercholesterolemia

and AS. This study provides an experimental basis for further research on BLEC and its main active component piperine in treating AS and provides new information for improving AS treatment.

Materials and Methods

Materials

The specifications and sources of key reagents as well as the model numbers and manufacturers of the main instruments used in this study are detailed in Table 1.

Table 1 Key Resources Table

Reagent or Resource	Source	Identifier
Antibodies		
ABCA1 (Rabbit mAb)	Proteintech	26564-I-AP
ABCG8 (Rabbit mAb)	Proteintech	24453-I-AP
ABCG5 (Rabbit mAb)	Proteintech	27722-I-AP
CYP7A1 (Rabbit mAb)	Proteintech	18054-I-AP
LXR- α (Rabbit mAb)	Proteintech	14351-I-AP
β -actin (Mouse mAb)	Proteintech	66009-I-Ig
Drug and Chemicals		
BLEC	Inner Mongolia Mongolian Medicine Joint Stock Co., Ltd.	Z20030129
Piperine	MedChemExpress	HY-N0144
Atorvastatin	SUPELCO	PHR1422-IG
Ezetimibe	SUPELCO	PHR1866-IG
Experimental models: Organisms/strains		
ApoE ^{-/-} mice	Gempharmatech	T001458-3
Oligonucleotides		
Control shRNA (sequence: GATCCCCTTCTCCGAACGTGTCACGTTTCAA GAGAACGTGACACGTTCCGGAGAATTTTAA)	Sigma-Aldrich	This paper
LXR α shRNA (sequence: GTCATCTTAGAGCCAGAGGAT)	Sigma-Aldrich	This paper
AAV2/8	Obio Technology Co., Ltd	This paper
Critical commercial assays		
Oil Red O staining solution	Yuan Ye	S19039
Fluorescence Inversion Microscope System	Olympus CellSens Dimension	
Invitrogen TRIzol	Thermo Fisher Scientific	15596026
5×Evo M-MLVRT Master Mix	Rui Zhen	AG11706
2×SYBR Green Pro Taq HS Premix	Rui Zhen	AG11701
Blood glucose monitoring system	OneTouch Ultra Easy	

(Continued)

Table 1 (Continued).

Reagent or Resource	Source	Identifier
TC assay kit	Elabscience	E-BC-K109-M
LDL-C assay kit	Elabscience	E-BC-K205-M
HDL-C assay kit	Elabscience	E-BC-K221-M
TG assay kit	Elabscience	E-BC-K261-M
AST assay kit	Nanjing Jiancheng Bioengineering Institute	C010-2-I
ALT assay kit	Nanjing Jiancheng Bioengineering Institute	C009-2-I
TBA assay kit	Elabscience	E-BC-K181-M
Phospholipid assay kit	Wako	296-63801
ACQUITY UPLC I-Class plus	Waters Corporation	
Q-Exactive mass spectrometer	Thermo Fisher Scientific	

Preparation of the BLEC

A solution of 5-g sodium carboxymethylcellulose (CMC–Na) was prepared in a 1000-mL ddH₂O and agitated for 12 h at ambient temperature. Subsequently, the appropriate amount of BLEC powder was measured and combined with 0.5% CMC–Na, and the mixture was then stored at 4°C.

Animals

The Animal Ethics Committee of Southern Medical University (Guangzhou, China) approved this experiment, strictly following the international rules for the care and use of laboratory animals. The research ethical code for the study is 2020-BSGZ-07-01. All experiments were conducted under the “Guidelines for the Care and Use of Laboratory Animals” set by the National Institute for Health of China. The room temperature was regulated at 20°C ± 2°C with 50% ± 20% humidity and equipped with a 12-h light/dark cycle. Male ApoE^{−/−} mice were allowed to eat pure water and food freely. All animals were adapted to the conditions for 1 week. The mice were injected with adeno-associated virus (AAV)2/8-shLXRα (10¹¹ pfu) through their tail veins to develop ApoE^{−/−} mice with hepatocyte-specific knockout of LXRα.

Constructing the as Model and Experimental Groups

The mice were fed a high-fat diet (HFD, 15% fat and 1.2% cholesterol) for 8 weeks. Subsequently, The experimental mice were randomly categorized into groups according to different treatments as described in the figure legends.

Collection of Blood Samples, Liver Tissue, and Aorta

The mice were anesthetized using a mixture of 2–5% inhaled isoflurane (2-chloro-2-difluoromethoxy-1-1-1-trifluoroethane) with oxygen and air. Blood samples were collected after reaching an appropriate anesthesia level. The abdominal and chest cavities were opened in sequence, the heart was exposed, and physiological saline was used to monitor the entire circulatory system from the left ventricle. Tissues (eg, liver, heart) were collected after perfusion. Tissues were quickly frozen in liquid nitrogen and stored in an 80°C freezer. All the mice were weighed before the above processing.

Oil Red O Staining and Imaging

The thoracic and abdominal cavities of the mice were opened after the mice were sacrificed to detect the burden of AS in the mice, and the section from the aortic root to the iliac artery was then extracted and opened longitudinally. Photos were taken with a digital camera against a blue background after staining with the ready-made Oil Red O staining solution. Oil red O staining was performed on aortic root cross-sections and liver tissue, and photographs were taken with a Fluorescence Inversion Microscope System. The AS burden was calculated as the aortic lesion area/total aorta area (%) and the aortic root lesion area/aortic root cross-sectional area (%).

Transcriptomics Analysis

Total RNA from the liver tissue was extracted. RNA isolation and sequencing were conducted exactly as previously described.²³ A computational pipeline was employed to process the raw data from RNA-seq. The sequencing data (in fastq format) were filtered to remove reads with unknown nucleotides. The clean reads were mapped to the mouse reference genome (mm9) by using TopHat v1.4.0. No more than two mismatches were allowed. The mapped reads were assembled into genes and transcripts with Cufflinks v1.3.0. Gene models were downloaded from the University of California, Santa Cruz (UCSC) RefSeq annotations. The gene expression levels were calculated using the fragments per kilobase of transcript per million mapped fragments (FPKM) values in Cufflinks. In analyzing the transcriptomic data, differential expression analysis was conducted using the DESeq (with replicates) functions estimate SizeFactors and nbinomTest or edgeR (without replicates) library. The differentially expressed transcripts with *p-values* of <0.05 and fold change of >2 were statistically significant. Eventually, the R package based on the hypergeometric distribution was used to test the statistical enrichment of differentially expressed transcripts in the Gene Ontology (GO) analysis and the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway, respectively, of which *p-values* of <0.05 indicated significant enrichment.

RNA Isolation, Reverse Transcription, and Quantitative Real-Time Polymerase Chain Reaction (PCR)

Total RNA was extracted from the mouse liver tissue using Invitrogen TRIzol. The cDNA synthesis was conducted in a 10- μ L system using 5 \times Evo M-MLVRT Master Mix. Real-time quantitative PCR was performed using 2 \times SYBR Green Pro Taq HS Premix. The $\Delta\Delta$ Ct method was utilized to analyze its relative expression with ACTIN as a control primer. [Table S1](#) lists the primers used in this study.

Biochemical Assays

Blood glucose levels were accurately measured via the tail vein with the OneTouch UltraEasy blood glucose monitoring system. Whole blood samples were meticulously obtained from the retro-orbital plexus of each mouse, adhering to strict protocols. The blood was allowed to clot naturally at room temperature for 30 min after collection, thereby facilitating serum separation via centrifugation at 1500 g for 10 min. Serum concentrations of total TC, LDL-C, HDL-C, and TG were subsequently identified using high-quality assay kits. Lipids were first extracted from the liver tissue using a meticulous methanol–chloroform (2:1, v/v) protocol for liver lipid profile assessment. The extracted lipids were then analyzed for hepatic TC and TG content utilizing commercial assay kits. Additionally, circulating aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels, which are liver function markers, were assessed using dedicated commercial kits, ensuring a comprehensive assessment of hepatic status. Gallbladder bile, harvested from mice after a 4-h fast, was carefully measured with a precision p10 pipette tip to identify bile volume. Bile concentrations were analyzed by diluting bile of 5 μ L with Milli Q water of 45 μ L, followed by extraction with a chloroform-methanol mixture (2:1) of 200 μ L. The resulting organic and aqueous phases were separated, dried, and reconstituted in ethanol and Milli-Q water, respectively. The organic phase was then evaluated for TC and total bile acids (BAs) using specific assay kits, whereas biliary phospholipid levels were identified with commercial kits from WAKO LabAssay, providing a comprehensive analysis of bile composition. Furthermore, fecal samples were collected over 3 days from individually housed mice, lyophilized, and weighed for consistency. Total fecal BAs were extracted using methanol, whereas fecal

lipids were isolated with a methanol–chloroform (2:1, v/v) mixture. All data obtained from these assays were accurately read and analyzed with the Microplate Manager version 6 software (SoftMax Pro v.7.1).

Extraction of BLEC Components from the Liver Samples

The liver samples stored at -80°C were removed and slowly thawed on ice. The samples were moved from 100 mg to 1.5 mL of Eppendorf tubes, and protein precipitator methanol-acetonitrile of 600 μL (V:V = 4:1, containing L-2-chlorophenylalanine of 4 $\mu\text{g}/\text{mL}$) was added to the samples. The above-mentioned mixed extract was obtained by swirling and oscillating for 1 min, ultrasonic extraction for 10 min in ice water, and static at -40°C overnight. The mixed extract was centrifuged for 10 min (12000 rpm, 4°C) after extraction, and centrifuged at -40°C for 2 h. A supernatant of 500 μL was put into a vial prepared for drying. The dried extract was re-dissolved with methanol-acetonitrile-water (V:V:V = 2:1:1) of 300 μL , and then ultrasonic extraction was performed after 30s of vortexing for 3 min to fully dissolve the extracted substance into the solvent, and the dissolved re-dissolved solution was left to rest overnight at -40°C . The re-dissolved solution that remained overnight was centrifuged for 10 min (12000 rpm, 4°C), and a supernatant of 150 μL was prepared for analysis.

Extraction of the BLEC Components

The Chinese medicine samples were accurately weighed to 100 mg into EP tubes ranging to 1.5 mL, and methanol-water (V:V = 1:1, containing L-2-chlorophenylalanine of 4 $\mu\text{g}/\text{mL}$) of 1 mL was added for dissolution (approximately 80°C), and the drug dissolution was accelerated by vortex oscillation for 2 min. The extracted solution was centrifuged at 12000 rpm and 4°C for 10 min. The supernatant was taken through an organic phase filter membrane of 0.22 μm after centrifugation, combined with methanol-water (V:V = 1:1, containing L-2-chlorophenylalanine of 4 $\mu\text{g}/\text{mL}$) diluted five times, and the extract of 200 μL was taken into an injection vial with a wide liner to be measured. The quality control sample (QC) was prepared by mixing and centrifuging the supernatant of the drug administration and prototype groups in equal volume (the concentration in the QC sample was consistent between the prototype and prototype groups on the machine).

LC-MS/MS Analysis

The metabolomic data analysis was conducted by Shanghai Luming Biological Technology Co., LTD (Shanghai, China). An ACQUITY UPLC I-Class plus fitted with a Q-Exactive mass spectrometer equipped with a heated electrospray ionization (ESI) source was utilized to analyze the metabolic profiling in both ESI-positive and ESI-negative ion modes. An ACQUITY UPLC HSS T3 column (1.8 μm , 2.1×100 mm) was employed in both the positive and negative modes. The binary gradient elution system consisted of (A) water (containing 0.1% formic acid, v/v) and (B) acetonitrile (containing 0.1% formic acid, v/v), and separation was achieved with the following gradient: 0.01 min: 5% B; 2 min: 5% B; 4 min: 30% B; 8 min: 50% B; 10 min: 80% B; 14 min: 100% B; 15 min: 100% B; 15.1 min: 5% B; and 16 min: 5% B. The flow rate was 0.35 mL/min and the column temperature was 45°C . All the samples were maintained at 4°C during the analysis. The injection volume was 5 μL .

The mass ranges from m/z 100 to 1200. Resolutions were set at 70,000 and 17500 for full MS and HCD MS/MS scans, respectively. The Collision energy was set at 10, 20, and 40 eV. The mass spectrometer operated with a spray voltage of 3800 V (+) and 3000 V (–); sheath gas flow rate of 35 arbitrary units; auxiliary gas flow rate of eight arbitrary units; capillary temperature of 320°C ; auxiliary gas heater temperature of 350°C ; S-lens RF level of 50.

LC-MS/MS Data Preprocessing

The original LC-MS/MS data were processed utilizing the software Progenesis QI version 3.0 (Nonlinear, Dynamics, Newcastle, UK) for baseline filtering, peak identification, integral, retention time correction, peak alignment, and normalization. The main parameters were 5-ppm precursor tolerance and 10-ppm product tolerance. Compound identification depended on the precise mass-to-charge ratio (M/z), secondary fragments, and isotopic distribution using The LuMet-TCM, Animal_DB, and Herb databases.

Substances with a total score of >50 points or a total score of >40 points and a secondary matching score of >50 points were retained as the original ingredient for the qualitative substances determined in the QI search database. Substances with an FC value of >10 in the treatment group (administration serum) and control group (blank serum) were utilized as the incoming ingredients. The total content of the relative peak area of the metabolites was set at 100% to obtain a qualitative and quantitative result data matrix after merging and weighing the substances in the positive and negative ion modes. The matrix contains all the information collected from the original data used for analysis, which was the basis for subsequent analysis. The EIC diagram and the MS2 mirror diagram with secondary fragment structure annotations for each determined TCM ingredient and blood component were visualized as well as a pie chart of the quantity and content of all identified TCM components under each chemical classification category.

We extracted the EIC and secondary mass spectra of each component in the data matrix table and annotated the fragment information and fragment structure to further ensure the accuracy of the identification results.

Identification is based on three reference dimensions:

1. The error between the retention time and the retention time of the standard in the database is within ± 0.2 min;
2. The first-order molecular weight error is within 5 ppm;
3. Comparison between the measured MS2 and the standard MS2.

Identification results are confirmed by referring to the retention time of the standard comparison and the dimension of the primary molecular weight error in the absence of secondary fragment peak information.

Statistical Analysis

In the current study, the determination of the sample size did not rely solely on preestablished statistical methods but was informed by previous research results and necessary statistical considerations. The “n” values, representing individual mice, were allocated for *in vivo* experiments, ensuring an appropriate study population representation. Data were consistently expressed as mean values \pm standard deviations, thereby providing a comprehensive and statistically sound depiction of our results. Statistical assessments were conducted with the advanced GraphPad Prism software version 8, ensuring accuracy and precision in our analyses. We used either a one-way analysis of variance (ANOVA) coupled with Tukey’s post hoc test for multiple comparisons, an unpaired, two-tailed Student’s *t*-test for direct comparisons between two groups, or a two-way ANOVA followed by Šidák’s multiple comparisons test, as specified in the respective figure legends, based on the experimental design and data distribution. A statistically significant threshold was set at a *P*-value of <0.05, which is a criterion meticulously adhered to in all analyses, thereby confirming the reliability and significance of our observed effects.

Results

BLEC Attenuates the Development of atherosclerosis in *in vivo*

Similar to our previous studies [10], we administered ApoE^{−/−} mice fed a standard rodent chow or HFD for 12 weeks with BLEC (75 mg/kg/day) and ddH₂O (as the control group) via gavage for 4 weeks to investigate the effects of BLEC on the development of AS in ApoE^{−/−} mice. Atherosclerotic lesions in the aortic root sections and en-face samples were assessed at the end of the experiment. The en-face and aortic root section analyses revealed that the ApoE^{−/−} mice treated with BLEC demonstrated lower average lesion areas than the controls under an HFD, despite no marked differences in the lesions between the two groups fed with the standard rodent chow (Figure 1A-D). Oil Red O-stained lesion quantification in the en-face preparations of the aortas revealed that the treatment with BLEC significantly reduced (23.3%) the lesion area compared with the controls. The Oil Red O-stained aortic root sections were quantified to confirm the positive effects of BLEC on AS, and a significant reduction (25.1%) in the lesion area was observed in the ApoE^{−/−} mice treated with BLEC compared with that observed in the controls. These data indicate that BLEC inhibits AS development in ApoE^{−/−} mice fed with an HFD.

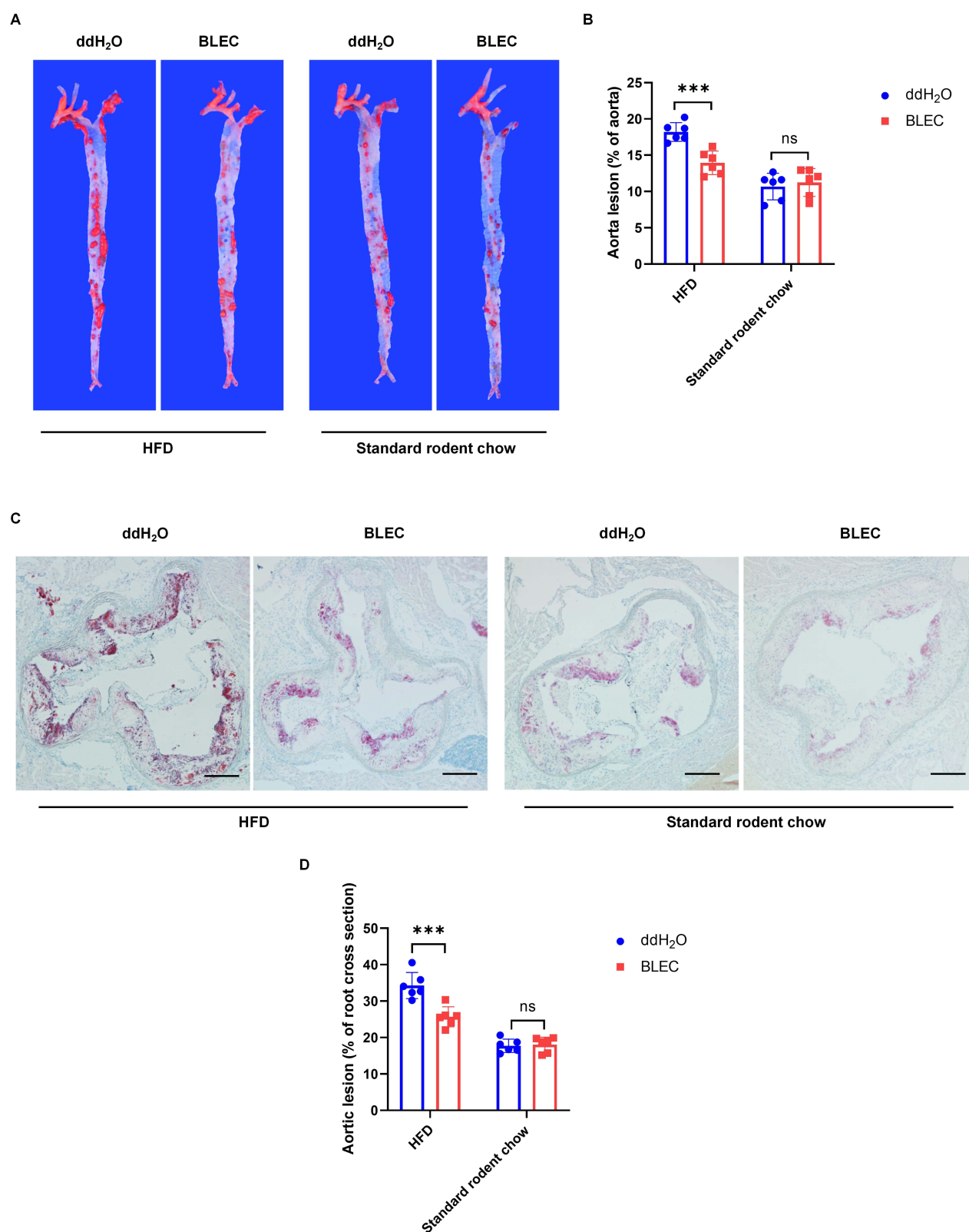


Figure 1 BLEC attenuates the development of AS in vivo. **(A)** Oil red O staining of aorta from ApoE^{-/-} mice fed a standard rodent chow or high-fat diet (HFD) for 8 weeks and administrated with BLEC (75mg/kg/day) and ddH₂O through gavage for 4 weeks; **(B)** Quantification of aorta lesion of aorta from mice treated as in **(A)**. **(C)** Oil red O staining of cross-sections of aorta from ApoE^{-/-} mice treated as in **(A)**. Scale bars, 200 μ m. **(D)** Quantification of aortic lesion of root cross section from mice treated as in **(C)**. Data are mean \pm SD from N=6 mice per group. ***P < 0.001 by unpaired two-tailed t test.

BLEC Modulates Serum Lipid Levels in vivo

The above-mentioned experiments confirmed that ApoE^{-/-} mice treated with BLEC demonstrated smaller atherosclerotic plaques than the control mice. Cholesterol metabolic disorder is a crucial causative factor in AS; thus, we further investigated the effect of BLEC on serum lipid levels in ApoE^{-/-} mice fed with an HFD. Both the BLEC-treated and control mice appeared grossly normal (Figure 2A-D). Mice treated with BLEC demonstrated reduced serum TC levels by 27.4%, as well as lower LDL-C and TG levels by 39.9% and 26.2%, respectively, compared with control mice (Figure 2E-G). Additionally, the HDL-C levels in BLEC-treated mice were increased by 38.7% (Figure 2H). Moreover, the hepatic TC and TG levels in BLEC-treated mice were lower than those in control mice (Figure 2I and J). Consistently, BLEC substantially reduced lipid droplet accumulation in the liver without any liver damage (Figure 2K-M). These results indicate that BLEC inhibits AS development by treating hypercholesterolemia.

Transcriptomics Analysis Reveals the Signaling Pathways for BLEC Treatment of Hypercholesterolemia

A comprehensive transcriptomics analysis was conducted to determine differentially changed transcripts within the study groups. This attempt aimed to decipher in detail the potential mechanisms underlying BLEC treatment for hypercholesterolemia. The liver is the most important organ for regulating cholesterol metabolism; therefore, the transcriptomics

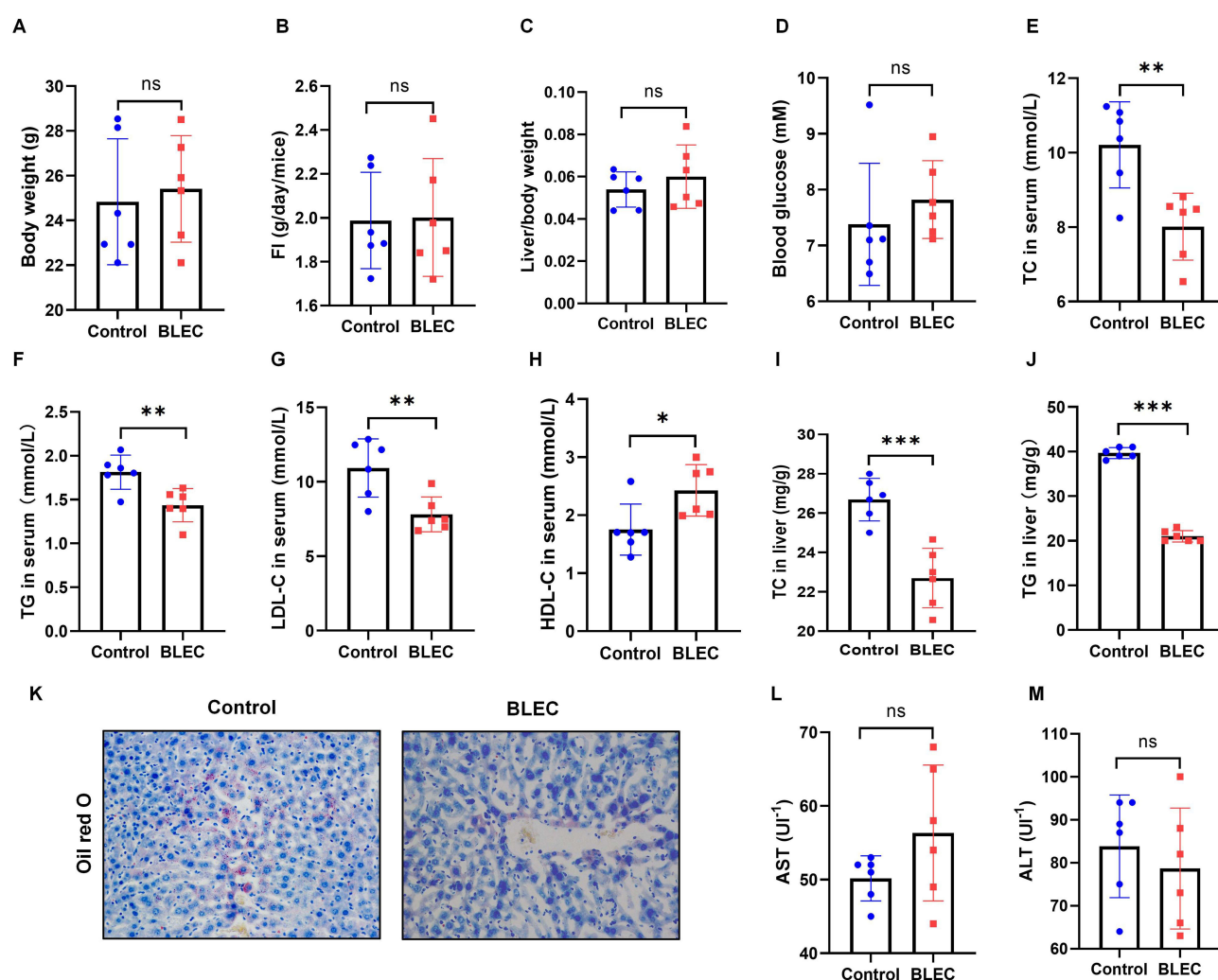


Figure 2 BLEC modulates serum lipid levels in vivo. ApoE^{-/-} mice fed a high-fat diet (HFD) for 8 weeks and administrated with BLEC (75mg/kg/day) and ddH₂O through gavage for 4 weeks. (A) Body weight. (B) Food intake (FI). (C) Liver-to-body weight ratio. (D) Blood glucose. (E) Serum TC. (F) Serum TG. (G) Serum LDL-C. (H) Serum HDL-C. (I) Liver TC. (J) Liver TG. (K) Oil Red O stains of mouse liver sections. Scale bars, 100 μ m. (L) Serum levels of AST. (M) Serum levels of ALT. Data are mean \pm SD from N=6 mice per group. *P < 0.05, **P < 0.01 and ***P < 0.001 by unpaired two-tailed *t* test.

analysis revealed that a total of 1349 transcripts were identified to be differentially expressed in liver tissues between BLEC-treated and control mice (Table S2) using two criteria: an expression level change of ≥ 2.0 -fold and a *P*-value of ≤ 0.05 (from Student's *t*-test). Figure S2A shows the related transcript abundances and frequencies in the BLEC-treated and control mice livers. Among the 1349 transcripts, 660 were upregulated, whereas 689 were downregulated, as shown in the volcano plot (Figure S2B). Differentially changed transcript enrichment analysis was conducted using the GO functional analyses and KEGG enrichment pathway to gain information into the functional attribution of BLEC regulation. The differentially changed transcripts were mostly enriched for biological process (BP) terms associated with fatty acid and lipid metabolic process according to the functional annotations in the GO database for cellular component (CC) terms related to mitochondrion and for molecular function (MF) terms correlated with oxidoreductase activity and ABC-type transporter activity (Table S3). Figure 3A illustrates the BP, CC, and MF GO enrichment terms for the differentially changed transcripts. The 45 potential KEGG pathways were enriched within the groups. The top 20 most significant KEGG pathways were selected for the scatter plot (Table S4 and Figure 3B). We then conducted chord diagram analysis on the differentially changed transcripts by focusing on three relevant pathways of fatty acid and lipid metabolic process, ABC-type transporter activity, and bile secretion. We revealed that the ATP-binding cassette transporter A1 (ABCA1), ABCG5, ABCG8, and cholesterol 7 α -hydroxylase (CYP7A1) were associated with the three relevant pathways (Figure 3C and D). Among them, ABCA1, ABCG5, ABCG8, and CYP7A1 expressions were significantly upregulated. Together, these data indicate that BLEC may treat hypercholesterolemia and AS by upregulating genes associated with cholesterol efflux, thereby promoting cholesterol excretion.

BLEC Increases Cholesterol Excretion

We investigated whether or not BLEC promotes hepatic cholesterol excretion. Strikingly, the BLEC-treated mice exhibited increased bile volume and concentrations of biliary cholesterol and BAs in the gallbladder (Figure 4A-C). The total biliary cholesterol was increased by 73.9% in BLEC-treated mice compared with that in control mice, and the increase of total biliary BAs increased by 153.4% (Figure 4D and E). Additionally, BLEC decreased the cholic acid-to-muricholic acid ratio, and the biliary phospholipid concentration was slightly increased (Figure 4F-H). The composition changes in the BAs of BLEC-treated mice may contribute to their lower cholesterol levels, as cholic acid better promotes cholesterol absorption in the intestine than muricholic acid. Consistently, the bile from the control mice was clear, whereas those from the BLEC-treated mice were opaque (Figure 4I). Consistently, ABCA1, ABCG5, ABCG8, CYP7A1, and liver X receptor α (LXR α) expressions were elevated (Figure 4J and K), indicating higher cholesterol and BA levels in bile and lower lipid levels in serum and liver. These results reveal that BLEC treats hypercholesterolemia and AS because it promotes cholesterol excretion in the liver.

LC-MS/MS Analysis of the Chemical Constituents of BLEC for Promoting Cholesterol Excretion and Treating as

The BLEC test solution was first analyzed with LC-MS/MS to further confirm the effective components of BLEC in promoting cholesterol excretion and treating AS. Figures S3 and S4 present the typical total ion chromatograms of BLEC in both the positive and negative modes. All compound structures were characterized or tentatively characterized according to their MS data, referring to previous literature and spectrum libraries (MassBank <http://www.massbank.jp/>, NIST <https://www.nist.gov/> et al) data. The BLEC test solution exhibited 1611 significant constituents. Table S5 summarizes their accurate MS and MS/MS data. Flavonoids, terpenes, and phenylpropanoids are the top three classes of compounds with the highest variety in the ranking of the types and contents of BLEC components, with contents of 17.76%, 17.56%, and 14.33% respectively (Figure 5A); whereas terpenes, alkaloids, and steroids are the top three classes of compounds with the highest content at 26.57%, 15.62%, and 14.85% respectively (Figure 5B), but piperine, as one of the top 10 compounds in BLEC content, has the highest proportion, reaching 27.45% of the top ten compounds (Figure 5C).

Studies on the pharmacology of target organs have revealed that after the oral TCM prescription administration, their components migrate to the target organs, serving as the direct substances that act directly on the body. We administered BLEC (75 mg/kg/day) to mice via gavage for seven consecutive days, with water available but no food for 12 h before

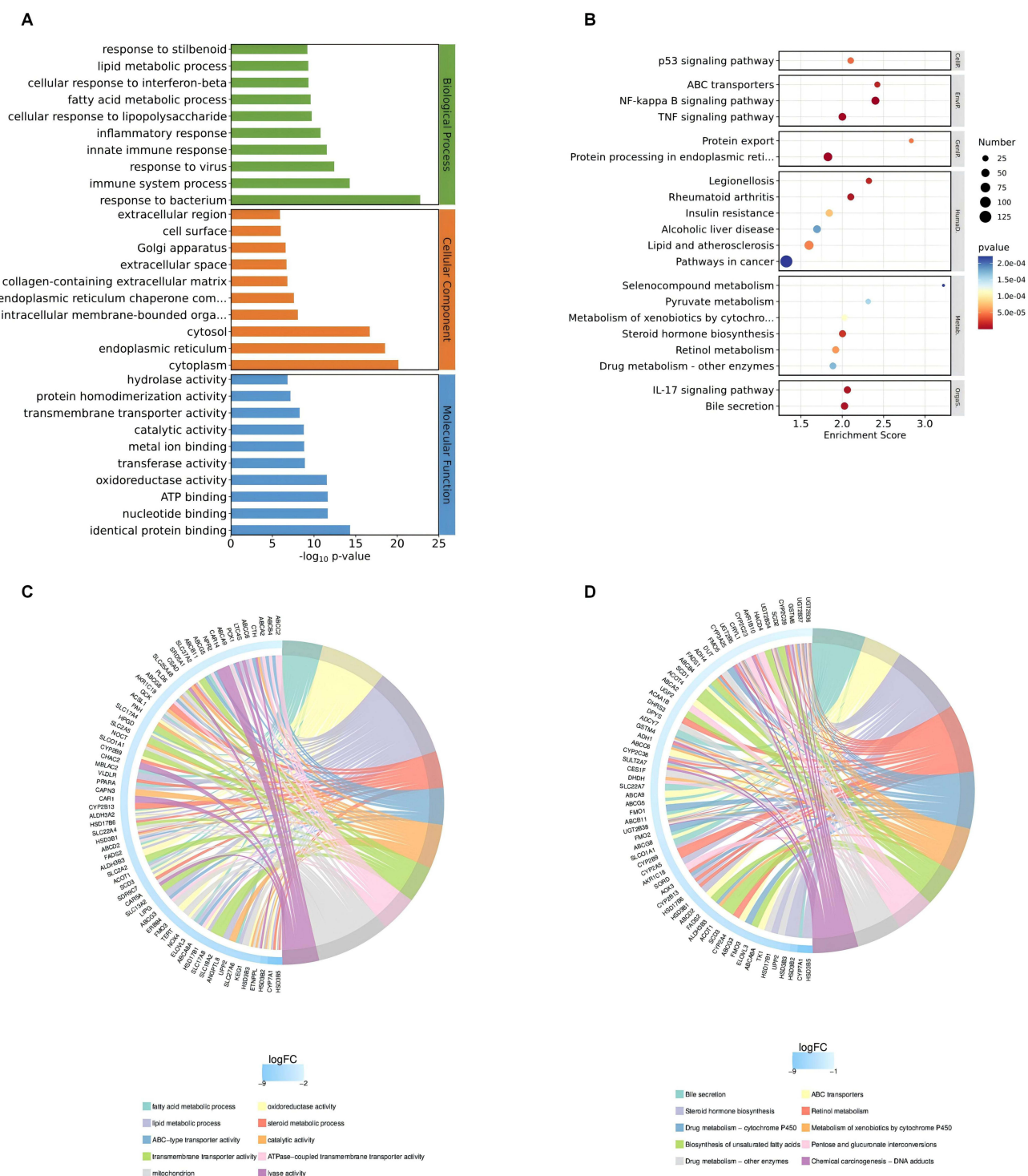


Figure 3 Transcriptomics analysis reveals signaling pathways for BLEC treatment of hypercholesterolemia. **(A)** GO term enrichment analysis. **(B)** KEGG enrichment analysis. **(C)** Chordogram analysis of GO enrichment of differentially expressed transcripts. **(D)** Chordogram analysis of KEGG enrichment of differentially expressed transcripts.

the last dose, to identify the substances that directly regulate liver cholesterol metabolism through BLEC. We extracted the mice's livers 4 hours after the last administration. Candidate ions with high response from the liver samples of the BLEC-treated group and low or no response in the liver samples of the control group were selected to avoid interference from the biological matrix. These candidate ions were inferred as prototype constituents or metabolites based on the

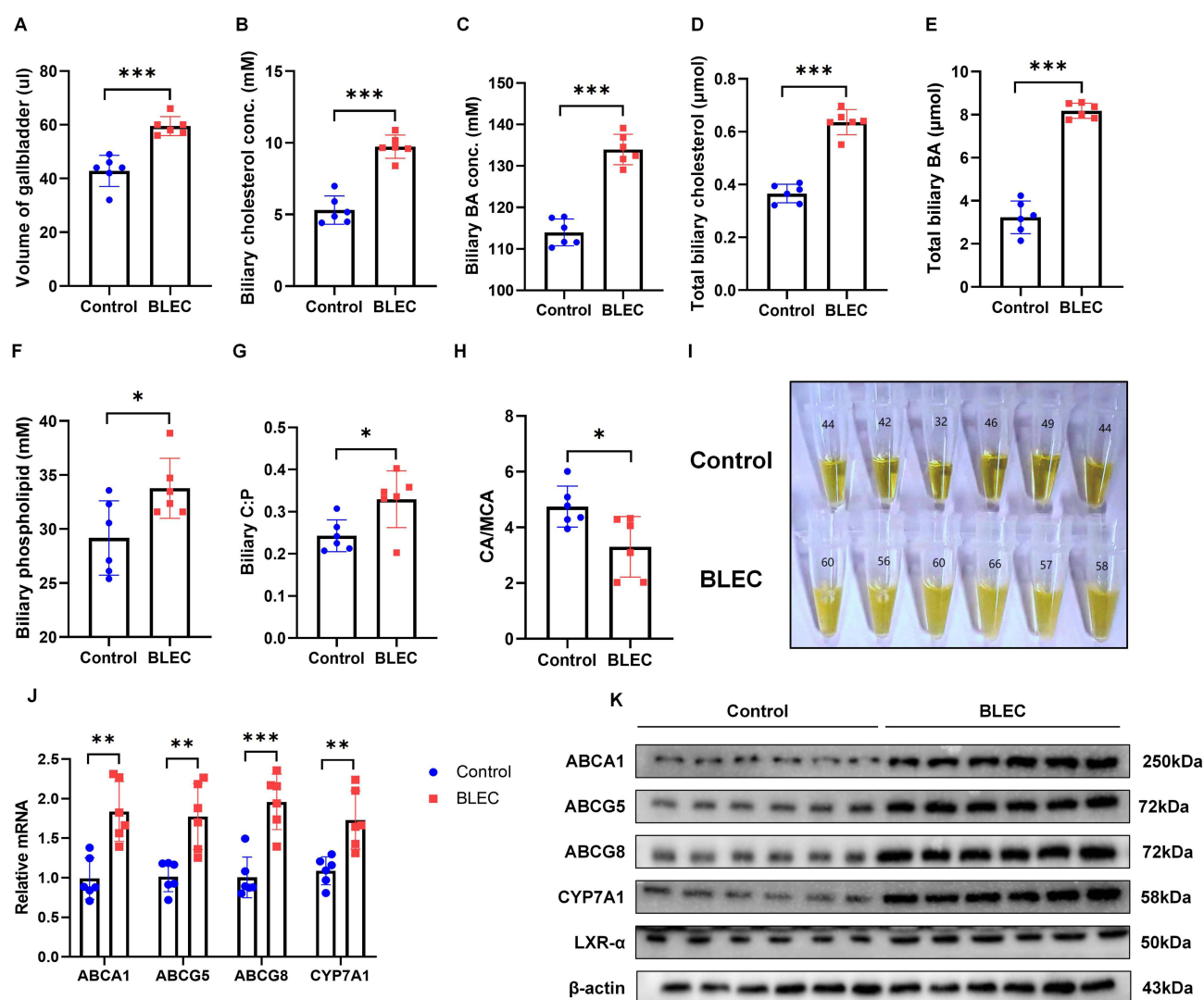


Figure 4 BLEC increases cholesterol excretion. ApoE^{-/-} mice fed a high-fat diet (HFD) for 8 weeks and administrated with BLEC (75mg/kg/day) and ddH₂O through gavage for 4 weeks. (A) Volume of bile in gallbladder. (B) Biliary cholesterol concentration. (C) Biliary BAs concentration. (D) Total biliary cholesterol. (E) Total biliary BAs. (F) Biliary phospholipid. (G) Biliary cholesterol: phospholipid ratio (C: (P)). (H) The ratio of cholic acid (CA) to muricholic acid (MCA). (I) Representative images of the bile. (J) Expression of the genes in mouse liver measured by RT-qPCR. (K) Expression of the protein in mouse liver measured by Western blot. Data are mean±SD from N=6 mice per group. *P < 0.05, **P < 0.01 and ***P < 0.001 by unpaired two-tailed t test.

primary ions and tandem MS/MS fragmentations, as well as compared with the relevant reference literature on the BLEC formula and its components in vitro and in vivo. A total of 38 constituents were determined as prototype constituents in mouse liver samples of the BLEC-treated group (Table S6). Of these, piperine was the highest content prototype component (Figure 5D). Additionally, we identified the predominant components in the liver samples of BLEC identified by LC-MS/MS and the active components of BLEC selected from the TCMSP database in our previous studies¹⁹ as piperine, tanshinone IIa, neocryptotanshinone, and piperlonguminine (Figure 5E). These research results indicate that piperine is the main active ingredient in BLEC.

We then investigated the effect of piperine on lipid levels in hyperlipidemic mice. These ApoE^{-/-} mice were fed HFD for 12 weeks and then treated with control solvent or different piperine doses (1, 3, 10, 30, and 100 mg/kg) through a gastric tube daily for 4 weeks (Figure 5F). The results revealed a significant, dose-dependent reduction in blood lipid levels among the mice treated with piperine (Figure 5G-J). The effect of piperine at 30 mg/kg was optimal for improving lipid levels. These results reveal that piperine is the main active component of BLEC in treating hyperlipidemia and AS.

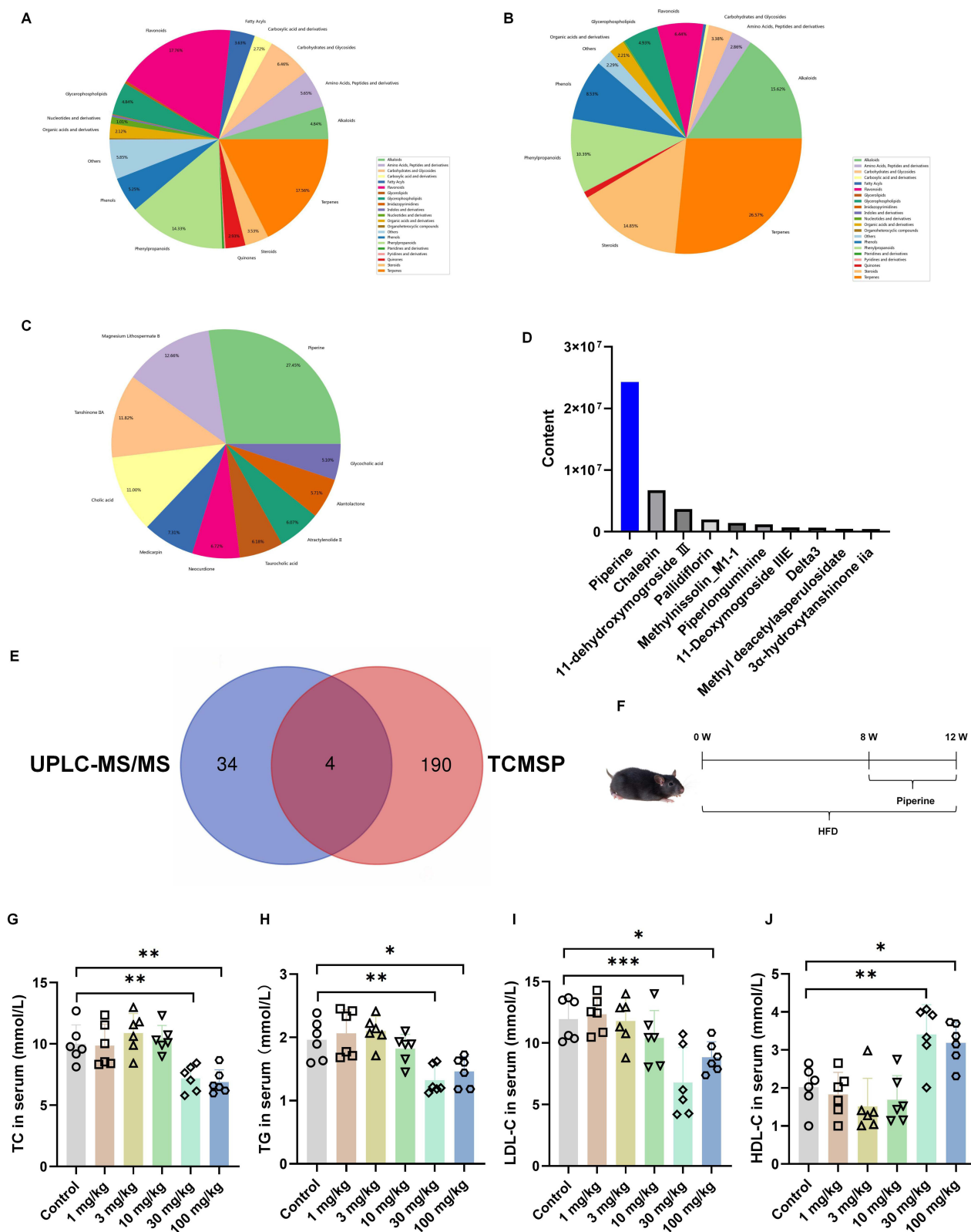


Figure 5 UPLC-MS/MS analysis of chemical constituents of BLEC for promoting cholesterol excretion and treating AS. **(A-C)** Distribution chart of source categories **(A)**, distribution chart of content **(B)** and distribution chart of the top ten compounds **(C)** by content of BLEC chemical constituents by UPLC-MS/MS analysis. **(D)** The top 10 prototype constituents in mice liver samples of the BLEC-treated group. **(E)** Identification of main active ingredients by taking an intersection of components in the liver samples of BLEC determined by UPLC-MS/MS and the active components of BLEC selected from TCMSP database. **(F)** Schematic overview of the experimental design in the mice model. **(G-J)** ApoE^{-/-} mice fed a high-fat diet (HFD) for 8 weeks and administrated with Piperine (0, 1, 3, 10, 30, 100 mg/kg/day) and ddH₂O through gavage for 4 weeks. Serum TC **(G)**. Serum TG **(H)**. Serum LDL-C **(I)**. Serum HDL-C **(J)**. Data are mean±SD from N=6 mice per group. *P < 0.05, **P < 0.01 and ***P < 0.001 by one-way ANOVA.

Piperine Increases Cholesterol Excretion and Requires LXR α

ABCA1, ABCG5, ABCG8, and CYP7A1 expressions are mainly regulated by liver X receptor α (LXR α) at the transcriptional level. Therefore, we subsequently tested whether piperine could promote cholesterol excretion in the liver and whether its metabolic benefits were based on LXR α . First, we developed ApoE $^{-/-}$ mice with liver LXR α -specific knockout (TKO) by tail vein injection of AAV. We subsequently administered TKO and ApoE $^{-/-}$ mice (WT) fed with HFD for 8 weeks with piperine (30 mg/kg/day) and ddH $_2$ O (as the control group) via gavage for 4 weeks. All mice demonstrated similar body weight, food intake, and blood glucose levels, but knockout of LXR α caused drastic lipid droplet accumulation in the liver, ballooning hepatocytes, and increased AST and ALT (Figure 6A and Figure S5A-D). Piperine substantially reduced the hepatic lipid content as well as TC and TG in the serum, and LXR α ablation completely abrogated these effects (Figure 6B-E). The LDL-C was markedly decreased, whereas HDL-C was increased in piperine-treated mice compared with control mice (Figure 6F and G). The total biliary cholesterol was increased by approximately two-fold in the piperine-treated mice compared with the control mice but reduced to the basal level in the TKO treated with piperine (Figure 6H-M). Additionally, the fecal cholesterol was increased by 118.0% in piperine-treated mice, which was blunted by LXR α deficiency (Figure 6N). These results are consistent with the expressional alterations of ABCA1, ABCG5, ABCG8, and CYP7A1. ABCG5/G8 excretes cholesterol to the bile and the cholesterol is then removed from the body through feces, whereas ABCA1 transports cholesterol to HDL (Figure 6O-R). These results reveal that piperine is the main active component of BLEC in treating hypercholesterolemia and AS, and its metabolic benefits require LXR α .

Piperine Synergizes with Statin/Ezetimibe

We investigated whether piperine exhibits synergistic lipid-lowering and anti-AS effects with the existing drugs: atorvastatin and ezetimibe (EZ). Piperine or atorvastatin treatment alone similarly decreased TC and TG in ApoE $^{-/-}$ mice. Notably, piperine and atorvastatin coadministration profoundly decreased the serum and hepatic lipid levels in ApoE $^{-/-}$ mice (Figure 7A-D). The body weight and other metabolic parameters were comparable (Figure S6A-D). Piperine upregulated ABCA1, ABCG5, ABCG8, and CYP7A1 expressions in mice regardless of atorvastatin treatment (Figure 7E-H). Consistent with the results in Figure 7E-H, piperine notably increased the total biliary cholesterol, total biliary BAs, and fecal cholesterol in ApoE $^{-/-}$ mice regardless of atorvastatin treatment. Atorvastatin alone did not affect the biliary cholesterol, BAs, or fecal cholesterol levels (Figure 7I-K). However, all mice demonstrated similar AST and ALT (Figure 7L and M). Finally, we treated the mice with piperine and EZ. Piperine functioned similarly in the absence or presence of EZ in ApoE $^{-/-}$ mice (Figure 5N-Z and Figure S4E-H). Piperine and EZ synergistically decreased TC and TG in the serum and liver of WT mice, although individual treatment improved the lipid profiles (Figure 7N-Q). Piperine continuously increased the total biliary cholesterol, total biliary BAs, and fecal cholesterol, not causing liver damage in ApoE $^{-/-}$ mice even with EZ (Figure 5R-V). However, piperine cannot upregulate ABCA1, ABCG5, ABCG8, and CYP7A1 under EZ treatment (Figure 7W-Z). More importantly, piperine can prevent AS whether used alone or in combination with statins or EZ, and even with both statins and EZ, but the triple combination demonstrated the most significant inhibitory effect on AS (Figure 8). These studies indicate that piperine exhibits therapeutic potential for treating hypercholesterolemia and AS.

Discussion

This study revealed that BLEC significantly reduced serum cholesterol levels and inhibited AS. Subsequent transcriptomic analysis revealed that BLEC mainly affects the hepatic cholesterol excretion pathway, and UPLC-MS/MS analysis indicated that piperine is the main active component responsible for the effects of BLEC. Further in vivo validation demonstrated that piperine upregulated cholesterol efflux genes (ABCA1/ABCG5/8/CYP7A1) by activating LXR α , thereby promoting hepatic cholesterol excretion. Most importantly, piperine exhibited a good clinical application value.

The cyclopentanoperhydrophenanthrene structure of cholesterol hinders the human body from efficiently degrading cholesterol molecules.^{3,24} Therefore, similar to sodium-glucose cotransporter two inhibitors that increase glucose excretion to lower blood sugar and bring cardiovascular benefits,²⁵ increasing cholesterol excretion is also an ideal lipid-lowering strategy. However, effective targets and drugs to promote cholesterol excretion in clinical practice remain

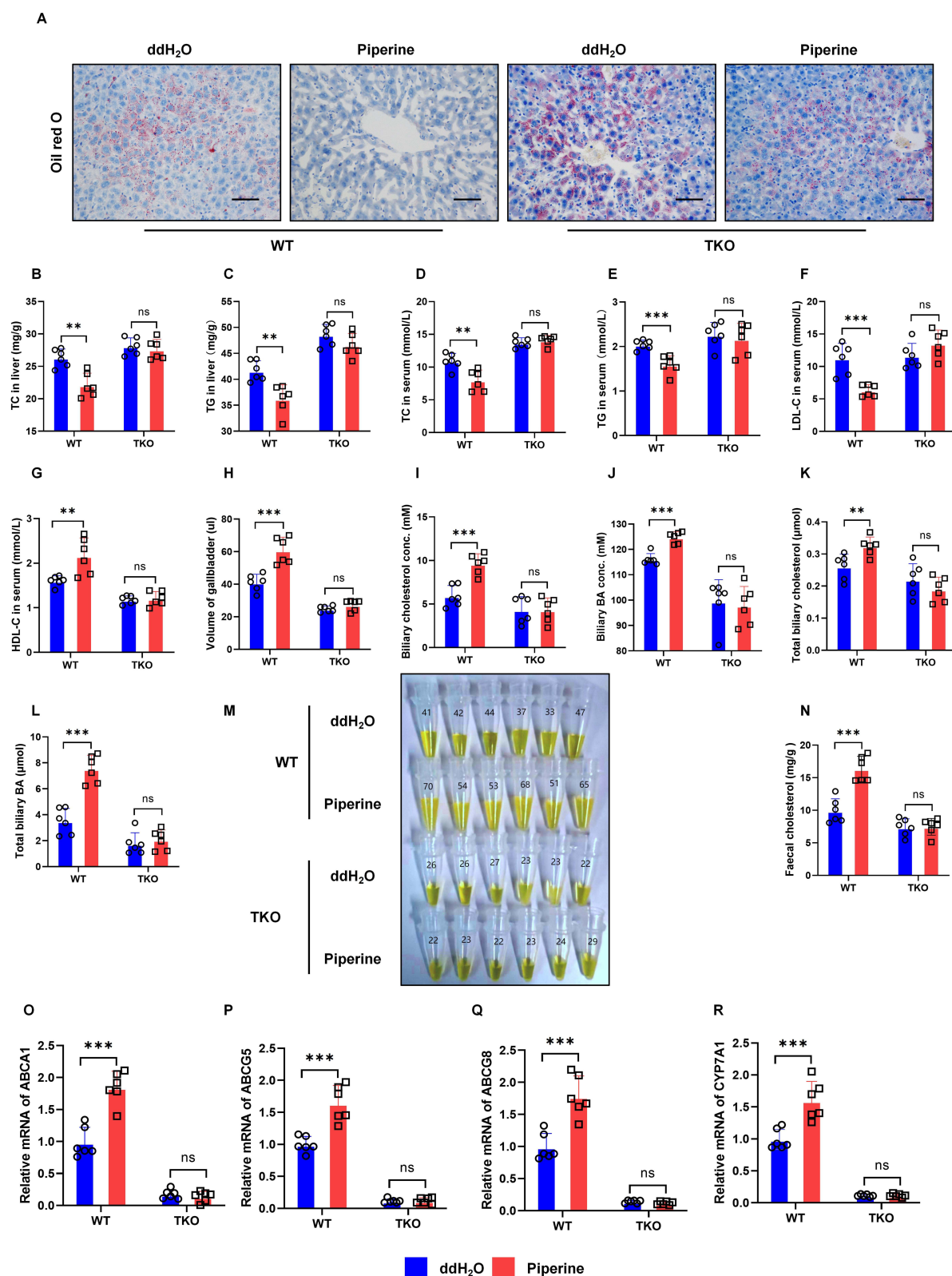


Figure 6 Piperine increases cholesterol excretion require LXR α . ApoE^{-/-} mice with liver LXR α -specific knockout (TKO) and ApoE^{-/-} mice (WT) fed a high-fat diet (HFD) for 8 weeks and administrated with piperine (30mg/kg/day) and ddH₂O through gavage for 4 weeks. **(A)** Oil Red O stains of mouse liver sections. Scale bars, 100 μ m. **(B)** Serum TC. **(C)** Serum LDL-C. **(D)** Serum TG. **(E)** Serum HDL-C. **(F)** Liver TC. **(G)** Liver TG. **(H)** Volume of bile in gallbladder. **(I)** Biliary cholesterol concentration. **(J)** Biliary BAs concentration. **(K)** Total biliary cholesterol. **(L)** Total biliary BAs. **(M)** Representative images of the bile. **(N)** Faecal cholesterol. **(O-R)** Expression of ABCA1 (**O**), ABCG5 (**P**), ABCG8 (**Q**), CYP7A1 (**R**) in mouse liver measured by RT-qPCR. Data are mean \pm SD from N=6 mice per group. **P < 0.01 and ***P < 0.001 by unpaired two-tailed t test.

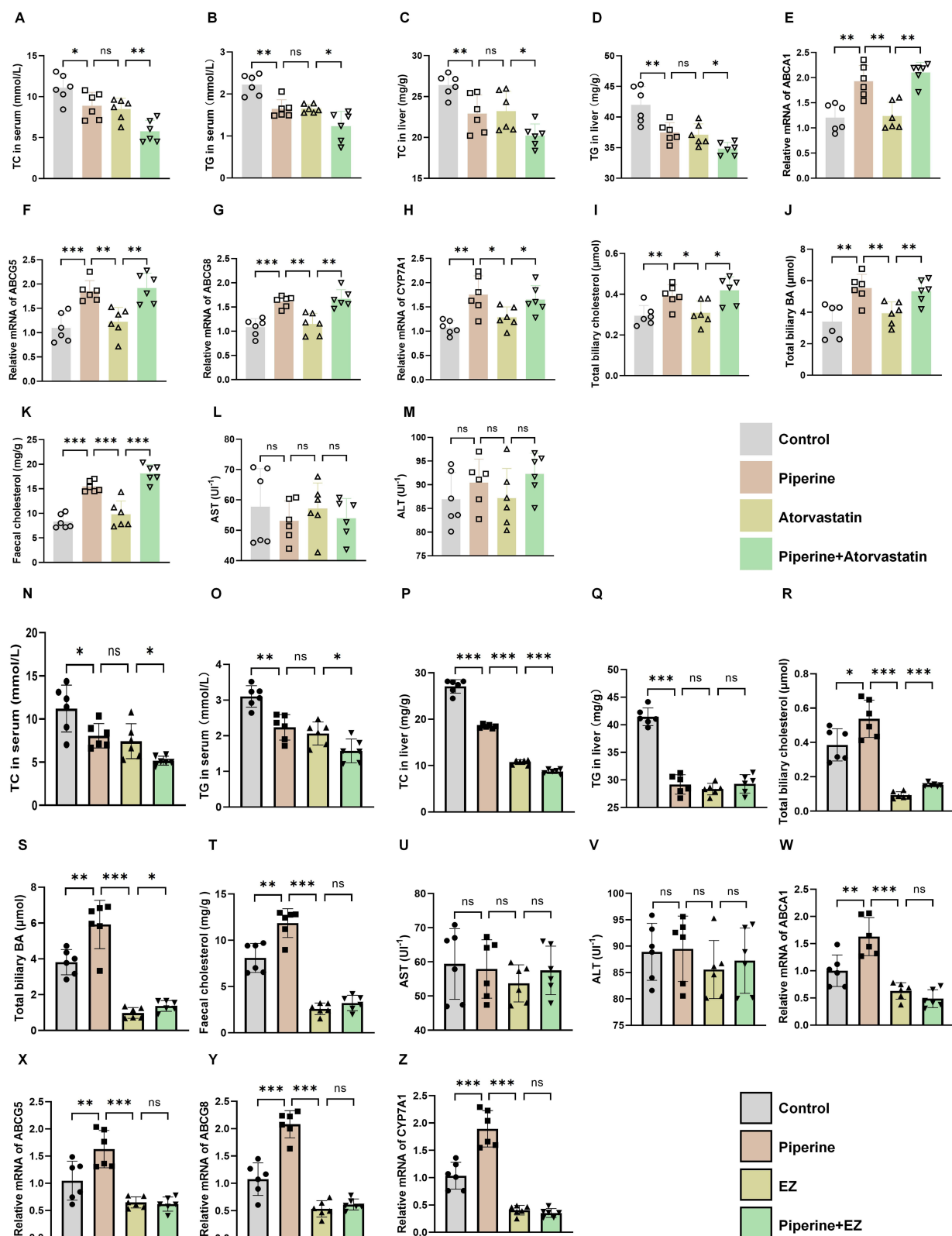


Figure 7 Piperine synergizes with statin/ezetimibe for serum blood lipids. Some 8-week-old male $ApoE^{-/-}$ mice were randomly grouped and fed a high-fat diet (HFD) for 8 weeks. Mice were gavaged with ddH₂O, piperine (30mg/kg/day), atorvastatin (30mg/kg/day) or piperine and atorvastatin for 4 weeks. (A) Serum TC. (B) Serum TG. (C) Liver TC. (D) Liver TG. (E-H) Expression of ABCA1 (E), ABCG5 (F), ABCG8 (G), CYP7A1 (H) in mouse liver measured by RT-qPCR. (I) Total biliary cholesterol. (J) Total biliary BAs. (K) Faecal cholesterol. (L) Serum levels of AST. (M) Serum levels of ALT. Some 8-week-old male $ApoE^{-/-}$ mice were randomly grouped and fed a HFD for 8 weeks. Mice were gavaged with ddH₂O, piperine (30mg/kg/day), EZ (10mg/kg/day) or piperine and EZ for 4 weeks. (N). Serum TC. (O) Serum TG. (P) Liver TC. (Q) Liver TG. (R) Total biliary cholesterol. (S) Total biliary BAs. (T) Faecal cholesterol. (U) Serum levels of AST. (V) Serum levels of ALT. (W-Z) Expression of ABCA1 (W), ABCG5 (X), ABCG8 (Y), CYP7A1 (Z) in mouse liver measured by RT-qPCR. Data are mean±SD from N=6 mice per group. *P < 0.05, **P < 0.01 and ***P < 0.001 by one-way ANOVA.

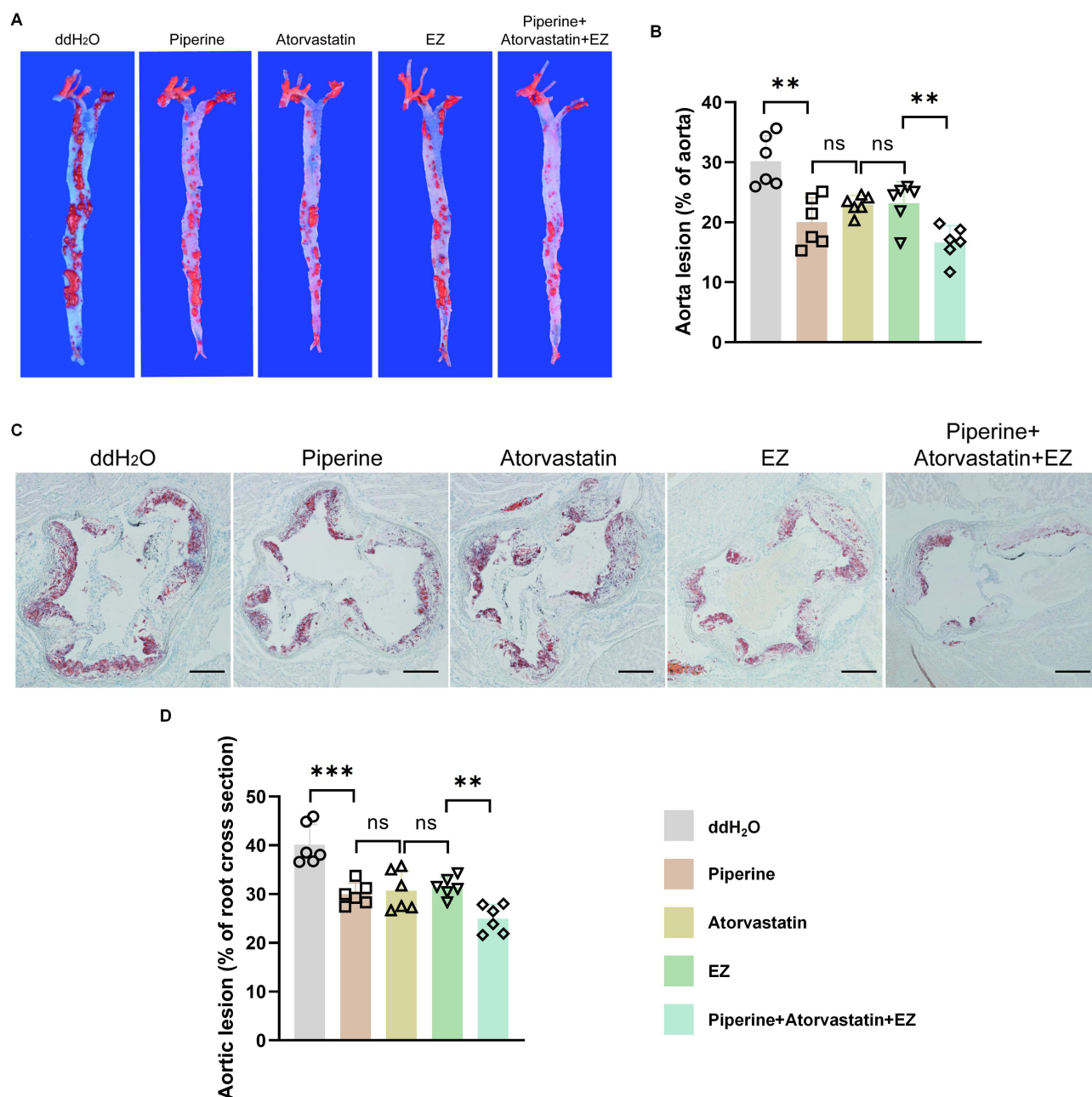


Figure 8 Piperine synergizes with statin and ezetimibe for treating AS. **(A)** Oil red O staining of aorta from ApoE^{-/-} mice fed a high-fat diet (HFD) for 8 weeks and administrated with ddH₂O, piperine (30mg/kg/day), atorvastatin (30mg/kg/day), EZ (10mg/kg/day) or piperine, atorvastatin and EZ through gavage for 4 weeks; **(B)** Quantification of aorta lesion of aorta from mice treated as in **(A)**. **(C)** Oil red O staining of cross-sections of aorta from ApoE^{-/-} mice treated as in **(A)**. Scale bars, 200 μ m. **(D)** Quantification of aortic lesion of root cross section from mice treated as in **(C)**. Data are mean \pm SD from N=6 mice per group. **P < 0.01 and ***P < 0.001 by one-way ANOVA.

unavailable. Mongolian medicine, as a precious TCM treasure, believes that improving the function of “the liver to separate the essence of blood from the turbidity” can treat AS.²⁶ This profound and unique understanding is comparable to the advanced concept of lowering cholesterol levels and preventing AS by increasing cholesterol excretion. Our research confirms that the Mongolian medicine BLEC promoted cholesterol excretion, lowered cholesterol levels, and prevented AS. Clarifying the main active ingredients of TCM compound preparations is crucial for improving the modernization level of Chinese medicine, ensuring the stability and predictability of drug efficacy, and promoting the recognition and application of Chinese medicine in the international pharmaceutical market.²⁷ Concurrently, our research confirms that piperine is the main active ingredient of BLEC in lowering cholesterol levels and preventing AS.

Piperine, as an important active alkaloid extracted from plants, such as *Piperis Fructus* and *Piperis Longi Fructus*, has made significant progress in recent years in lowering blood lipid levels and inhibiting foam cell formation, thereby preventing AS.^{28–30} Studies on hyperlipidemic rat models have revealed that it significantly reduced serum TC and TG levels, which helps improve lipid metabolism disorders.^{22,28,29} Piperine and its derivatives, such as piperlongumine (GBN), have been proven to exhibit various biological activities, including antioxidant, anti-inflammatory, anti-tumor, anti-seizure, and immunomodulatory effects, which are related to AS, in addition to regulating blood lipid levels.^{31–36} However, direct in vivo experimental evidence of the effect of piperine on AS remains unavailable. Piperine's clinical application still faces some issues despite its many advantages in reducing blood lipids and preventing AS. First, the toxicity of piperine is relatively high, which greatly limits its widespread clinical use.³⁷ Second, the pharmacokinetic characteristics of piperine remain unclear, and its absorption, distribution, metabolism, and excretion processes require further in-depth research.³⁸ Additionally, the lipid-lowering mechanism of piperine has not been fully elucidated, and further exploration of its molecular targets and signaling pathways is warranted. Our research confirms that piperine exerts lipid-lowering and anti-atherosclerotic effects by activating LXR α . Moreover, it plays a good role in lipid-lowering and anti-atherosclerotic effects when combined with clinical drug statins and EZ.

Although piperine has dose-dependency, caution should be taken regarding the toxicity of piperine. Future research should focus on how to reduce the toxicity of piperine.

LXR α , as key transcription factors regulating lipid balance, have attracted much attention in recent years for their role in cholesterol metabolism and transport processes, providing new information for treating hypercholesterolemia and AS. LXR α controls the expression of a series of genes in a tissue-dependent manner, thereby regulating cholesterol absorption, transport, efflux, and excretion, and they are important sensors and regulators for maintaining the cholesterol balance in the body.^{11,12} Studies have revealed that activating LXR α significantly improves reverse cholesterol transport, which is the process of transferring excess cholesterol within cells through HDL to the liver for transformation and clearance, thereby effectively inhibiting AS occurrence and development. In this process, LXR α upregulates the expression of cholesterol efflux regulatory proteins, such as ABCA1, ABCG1, ABCG5/G8, and CYP7A1, thereby promoting cholesterol transport from peripheral tissues to the liver and reducing the intestinal absorption of dietary cholesterol.^{10,39} However, its clinical application still faces some challenges despite the significant potential of LXR α in regulating cholesterol metabolism. First, LXR α activation may cause some undesirable side effects, such as increasing the expression of genes involved in fatty acid biosynthesis, resulting in hepatic steatosis and hypertriglyceridemia.^{11,12} This limitation hinders the feasibility of using LXR α agonists directly for treating hypercholesterolemia. Second, the performance of different LXR α agonists in animal experiments indicated differences, such as some agonists may increase TC and TG, whereas others may only elevate HDL-C levels.^{11,12} This indicates that the selectivity and mechanism of action of LXR α agonists still require further research and optimization. Our research confirms that piperine can not only activate LXR α , promote cholesterol excretion, and lower TC, TG, and LDL-C levels but also inhibit hepatic steatosis. Thus, piperine is an effective way to lower cholesterol levels and prevent AS, with good clinical application potential.

However, this study has certain limitations. First, we have confirmed that piperine is the main active ingredient of BLEC through multi-omics integration and molecular biological validation using doses equivalent to the recommended animal experiment doses, but these are significantly higher than the clinical dosages. Thus, further pharmacological and toxicological studies are warranted to guide the determination of the clinical dose range. Second, our study confirmed that piperine promoted liver cholesterol excretion and anti-AS by activating LXR α without promoting liver steatosis, but the detailed mechanism by which piperine activates LXR α remains unclear.

Conclusion

In summary, our research reveals that BLEC and its active component piperine exerted anti-atherosclerotic effects by regulating blood lipid levels. Mechanistically, BLEC and piperine activated LXR α to upregulate the expression of genes related to cholesterol efflux, thereby promoting cholesterol excretion, reducing cholesterol levels, and attenuating AS progression. More importantly, our research confirms that piperine has a synergistic effect with existing lipid-lowering drugs, which enhances its potential clinical application prospects. Although piperine has dose-dependency,

caution should be taken regarding the toxicity of piperine. Future research will focus on elucidating the mechanism by which piperine stimulates LXR α and synthesizing new piperine derivatives with low toxicity and strong ability to promote cholesterol excretion. Additionally, there will be an exploration of the potential applications of BLEC or piperine in other lipid-associated diseases, such as non-alcoholic fatty liver disease. Therefore, our research will provide a novel and promising therapeutic strategy for promoting cholesterol excretion in treating AS and reducing the risk of CVD. It also provides a theoretical foundation and injects new vitality into the field of new drug development.

Data Sharing Statement

Data will be made available on request.

Acknowledgments

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Disclosure

All authors disclosed no conflicts of interest.

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