Ethanol extract of *Rubia yunnanensis* inhibits carotid atherosclerosis via the PI3K/AKT signaling pathway

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Received August 5, 2023; Accepted November 13, 2023

DOI: 10.3892/br.2023.1707

Abstract. Atherosclerosis is a multifactorial vascular disease caused by endothelial dysfunction. Because of adverse reactions to drugs used to treat atherosclerosis. For example, statins, which significantly reduce the burden of atherosclerotic disease, have been associated with muscle toxicity. There is a need to identify novel drugs for the prevention and treatment of atherosclerosis *Rubia yunnanensis* is a herbs commonly used in Asian countries for its protective effects against cardiovascular diseases. However, the mechanism of action of *R. yunnanensis* extract in carotid artery atherosclerosis has not been found. The carotid artery is usually used as a site for clinical evaluation of atherosclerosis. The present study aimed to determine the mechanism of action of *R. yunnanensis* extract in the inhibition of carotid atherosclerosis in apolipoprotein E gene knockout ($ApoE^{-/-}$) mice. The mechanism of atherosclerosis inhibition was elucidated by detecting the blood lipid level, carotid artery pathology, and the protein expression of PI3K and AKT. The present study demonstrated that ethanol extract of *R. yunnanensis* reduced lipid levels, intima damage and carotid lipid accumulation and increased p-PI3K/PI3K and p-AKT/AKT protein levels in $ApoE^{-/-}$ mice fed high-fat diet for 12 weeks. It was hypothesized that the effects of *R. yunnanensis* extract may be achieved by regulation of the phosphatidylinositol-3-kinase/protein kinase B signaling pathway. Ethanol extract of *R. yunnanensis* decreased carotid atherosclerosis in $ApoE^{-/-}$ mice fed a high-fat diet via the phosphatidylinositol-3-kinase/protein kinase B signaling pathway. Therefore, *R. yunnanensis* may be a promising option for treating atherosclerosis in the future.

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Key words: Rubia yunnanensis, carotid atherosclerosis, signaling pathway, vascular endothelial damage, PI3K/AKT

Introduction

Atherosclerosis (AS) is a chronic and progressive vascular wall disease caused primarily by plaques formed by lipid deposition under intima artery linings and fibers, mediated by local inflammatory responses of the vessels. The plaques accumulate, resulting in arterial stenosis and decreased elasticity (1). Diet containing high-fat foods, which is one of the main causes of abnormalities in lipid metabolism, as well as changes in poor lifestyle habits such as smoking and reduced exercise, all of which have led to increasing the morbidity and mortality of AS-based cardiovascular disease, with ~16 million people dying from cardiovascular diseases every year worldwide, making AS one of the leading contributing diseases to the global mortality rate (2). AS is a dynamic process, and disorders of lipid metabolism trigger AS, leading to damage of endothelial cells and eventual formation of atherosclerotic plaques (3).

It is well-known that abnormal lipid metabolism and endothelial cell injury are associated with occurrence and progression of AS (4,5). Abnormal lipid metabolism is primarily caused by increased levels of lipids and certain lipoproteins in plasma, leadings to damage of the vascular endothelium and accumulation of adhesion factors on endothelial cells (6). The function of endothelial cells is weakened, causing inflammation and formation of AS (7). Vascular homeostasis depends on the integrity and normal function of endothelial cells (8). Therefore, regulation of blood-lipid disorder and protection of endothelial cells are key for anti-atherosclerosis therapy. Endothelial cell function and atherosclerosis can be regulated by the phosphatidylinositol-3-kinase/protein kinase B (PI3K/AKT) signaling pathway (9).

Rubia yunnanensis is the dried root and rhizome of *R. yunnanensis Diels*, a *Rubiaceae* plant and a unique indigenous medicine in Yunnan, China. *R. yunnanensis* has pharmacological actions, such as anti-myocardial ischemia, anti-oxidation and anti-platelet activity (10). The application of *R. yunnanensis* has been recorded in the 'Southern Yunnan Materia Medica' for treatment of cardiovascular and gastrointestinal disease, menstrual disorder and trauma for hundreds of years (11).

The present study aimed to determine the impact of *R. yunnanensis* alcohol extract in a mouse model of AS, analyze the main components of *R. yunnanensis* alcohol

extract and its effects on carotid AS and blood lipid levels, as well as PI3K and AKT protein expression. The present study aimed to determine whether *R. yunnanensis* regulates the PI3K/AKT pathway to inhibit carotid AS in *ApoE*$^{-/-}$ mice on a high-fat diet (HFD).

Materials and methods

Animals. A total of six C57BL/6J and 18 *ApoE*$^{-/-}$ mice with C57BL/6J genetic background were used to detect blood lipids and pathological indexes. In addition, three C57BL/6J mice and nine *ApoE*$^{-/-}$ mice with C57BL/6J genetic background were used for protein detection. All mice (male; age, 8 weeks; weight, 18-22 g) were purchased from SPF (Beijing) Biotechnology Co., Ltd. [experimental animal certificate no. SCXK (Jing) 2019-0010]. Mice were kept in a cage with stainless steel top and plastic bottom with free access to water and food, and 12/12-h light/dark cycle, temperature of 20-24˚C and humidity of ~50%. All animals received appropriate care in compliance with the 'Guide for the Care and Use of Laboratory Animals' (12). All experiments were approved by the Animal Ethics Committee of Yunnan University of Chinese Medicine and meet the standards of Yunnan University of Chinese Medicine (Kunming, China; approval no. R-062022053).

Mouse model. The mice were randomly divided into four groups (n=6/group): Normal control (C57BL/6J mice), model (*ApoE*$^{-/-}$ mice), ethanol extract of high-dose *R. yunnanensis* (HRY; 2.5 g/kg, *ApoE*$^{-/-}$ mice) and ethanol extract of low-dose *R. yunnanensis* (LRY; 1.25 g/kg, *ApoE*$^{-/-}$ mice). The experimental mice used for WB assay were grouped into 4 groups (n=3/group):. Normal control (C57BL/6J mice), model (*ApoE*$^{-/-}$ mice), ethanol extract of high-dose *R. yunnanensis* (HRY; 2.5 g/kg, *ApoE*$^{-/-}$ mice) and HRY + LY group (HRY; 2.5 g/kg, LY294002; 10 mg/kg, *ApoE*$^{-/-}$ mice). The control group received normal diet; all other groups received HFD diet (40% fat and 1.25% cholesterol). HRY, LRY and HRY + LY group were orally given ethanol extract of *R. yunnanensis* every day for 12 weeks; the other groups were orally given equal volume of normal saline. To investigate whether the PI3K/AKT signaling pathway can be activated by *R. yunnanensis*, we administered ethanol extract of high-dose *R. yunnanensis* along with the PI3K/AKT inhibitor LY294002. HRY + LY group was intraperitoneally injected with LY294002 (Med Chem Express, 10 mg/kg, twice/week for 2 weeks) beginning from the tenth week. The experiment lasted for 12 weeks and no premature death was observed.

Preparation of alcohol extract of R. yunnanensis and analysis of main components by high-performance liquid chromatography (HPLC). R. yunnanensis was acquired from Yunnan Huide Pharmaceutical Co., Ltd., China and identified by Professor Yin Zili of Yunnan University of Chinese Medicine (Yunnan). *R. yunnanensis* extract was obtained after soaking 500 g *R. yunnanensis* powder in 95% ethanol for 24 h, with the extraction heated at 60˚C by reflux. Soaking and heating reflux was repeated four times to obtain the extract. Then, 95% ethanol extract of *R. yunnanensis* was freeze-dried into powder, 0.5 g was taken, methanol was added for ultrasonic dissolution and sample was filtered (0.22 μm microporous membrane filter) and processed by HPLC (Agilent 1260) gradient elution as follows: Mobile phases A and B were acetonitrile and gradient elution, respectively, with 0.1% formic acid solution (10 min, 5-24% A; 10 min, 24% A; 10 min, 24-34% A and 15 min, 34% A and 0~10 min, 95%B→76%B; 10~20 min, 76%B; 20~30 min, 76%B→66%B; 30~45 min, 66%B). The detection wavelength was 280 nm. *R. yunnanensis* and standard products were dissolved in methanol at a flow rate of 1 ml/min and the sample size is 10 μl and column (Agilent TC-C18 (4.6x250 mm, 5 μm)) temperature of 30˚C.

Acute toxicity experiment and effective dose selection of alcohol extract from R. yunnanensis. To study the adverse effects of ethanol extract of *R. yunnanensis* on mice, acute toxicity test was performed (Data S1).

Blood lipid level determination. The mice were anesthetized by intraperitoneal injection of pentobarbital sodium (40 mg/kg). When mice had no response to tail pinching, 1.5ml blood was collected and serum was obtained by centrifugation at 4˚C and 680 x g for 15 min. Subsequently, total cholesterol (TC; cat. no. A111-1-1), triglyceride (TG; cat. no. A110-1-1), low-density lipoprotein cholesterol (LDL-C; cat. no. A113-1-1) and high-density lipoprotein cholesterol (HDL-C; cat. no. A112-1-1) were measured according to total cholesterol test kit instructions,. triglyceride test kit instructions, low density lipoprotein cholesterol test kit instructions and high-density lipoprotein cholesterol test kit instructions. All kits were bought from Nanjing Jiancheng Bioengineering Institute.

Histological examination of carotid AS. Mice were sacrificed by guillotine decapitation. Death was verified by absence of corneal and eyelash reflex (response to touch) and breathing. The carotid artery was removed under operating microscopes, adipose tissue was removed and carotid artery tissue was fixed at room temperature with 4% paraformaldehyde for 24 h. Samples were stored in -80˚C in liquid nitrogen for protein detection.

For hematoxylin and eosin staining, fixed carotid artery was dehydrated with ascending ethanol gradient, then washed with xylene, paraffin-embedded and cut into translateral sections ~4 μm thick, then stained with hematoxylin and eosin for 5 min each at room temperature. Finally, observations were made using light microscopy (magnification, x40).

For Masson's staining, fixed carotid artery was dehydrated with ascending sucrose gradient. The optimal cutting temperature-embedded carotid artery was transversally cut into sections ~8 μm thick, then slices were soaked in Masson's A solution overnight, rinsed with tap water and soaked for 1 min in Masson's B and C mixed dye. The sections were immersed in Masson's D solution for 6 min, rinsed in tap water, soaked in Masson's E solution for 1 min without rinsing, then soaked in Masson F solution for 30 sec. All steps are performed at room temperature. Finally, stained collagen in carotid artery tissue was examined by light microscopy (magnification, x40). Analysis was performed using Image J software, and the collagen area ratio was calculated as collagen area divided by total area.

For Oil red O staining, fixed carotid artery was dehydrated with ascending sucrose gradient. The OCT-embedded carotid artery was transversally cut into slices ~8 μm thick, immersed in oil red O dye solution for 10 min (away from light) and then the slices were differentiated twice (2 and 5 sec) in 60% isopropyl alcohol. Sections were soaked in distilled water twice (10 sec each time), then soaked with hematoxylin dye for 5 min, washed with distilled water three times (5, 10 and 30 sec), differentiated for 8 sec, washed in distilled water twice (10 sec each), dipped in blue solution for 1 sec, then immersed in tap water twice (5 and 10 sec). All steps are performed at room temperature. Finally, the carotid artery was examined by light microscopy (magnification, x40). Analysis was performed using Image J software (version 1.45S, National Institutes of Health), and lipid area ratio was calculated as lipid area divided by total area.

For Verhoeff's Van Gieson (EVG) staining, fixed carotid artery was dehydrated with increasing sucrose gradient. The OCT-embedded carotid artery was transversally cut into sections ~8 μm thick, then sections were placed in EVG dye solution (EVG dye solution A:B: EVG dye solution C=5:2:2) for 5 min. Sections were stained with EVG dye solution B, then washed with tap water after differentiation until elastic fibers were a purplish black color, and then VG dye solution(EVG dye solution E:D=9:1) was applied the sections were immersed for 3min. All steps are performed at room temperature. Finally, elastic and collagen fibers of carotid artery tissue was examined by light microscopy (magnification, x40).

Western blot analysis. Western blotting was used to measure total and phosphorylated (p-) levels of AKT and PI3K. Samples were prepared used RIPA cracking buffer (cat. no. P0013C; Beyotime Institute of Biotechnology) and lysate was centrifuged at 4˚C, 7,992 x g for 5 min and the supernatant was collected into a pre-cooled Eppendorf tube. BCA protein assay kit (cat. no. P0010; Beyotime Institute of Biotechnology) was used for protein determination and protein (50 μg/lane) were separated via 10% SDS-PAGE. The proteins were then transferred to polyvinylidene difluoride membranes, which were then incubated in 5% buttermilk for 2 h at room temperature. Membranes were rinsed with TBST buffer with 0.1% Tween. Then, the membrane was incubated overnight at 4˚C with primary antibodies (all 1:1,000; all Cell Signaling Technology, Inc.) as follows: Anti-PI3K (cat. no. 4257), anti-p-PI3K (cat. no. 4228), anti-AKT (cat. no. 9272), anti-p-AKT (cat. no. 9271) and anti-β-actin (cat. no. 4967). After the first antibody incubation, the membrane was washed with TBST three times, then incubated with horseradish peroxidase-conjugated polyclonal goat anti-rabbit IgG (1:5,000; cat. no. ab6721; Abcam) at ambient temperature for 1 h, then washed three times in TBST. Finally, chemiluminescence reagent (cat. no. A38555; Cell Signaling Technology, Inc.) was used to observe bands with a Bio-Rad ChemiDoc™ XRS Gel Imaging System (Bio-Rad Laboratories, Inc.). Image Lab™ V4.0 software (Bio-Rad Laboratories, Inc.) was used to detect optical signals and quantify protein levels. All experimental groups were divided into three groups and the experiment was repeated three times.

Statistical analysis. Data were analyzed by GraphPad Prism 8.0 software (GraphPad Software, Inc.; Dotmatics). The measurement data are expressed as mean ± standard deviation. All experiments were repeated three times. If data conformed to normal distribution and the variance was homogeneous, one-way ANOVA was used followed by Tukey's post hoc correction. If the data were normally distributed but with uneven variance, Welch's ANOVA test was used, followed by Tamhane's T2. $P<0.05$ was considered to indicate a statistically significant difference.

Results

HPLC analysis of components of alcohol extract of R. yunnanensis. A total of two primary compounds, namely, *R. yunnanensis* naphthoquinones A and *R. yunnanensis* quinone B (Fig. 1A and B), was detected in the alcohol extract of *R. yunnanensis*. Both compounds have a benzene ring in their structure (Fig. 1C).

Acute toxicity experiment and effective dose selection of alcohol extract from R. yunnanensis. To study the adverse effects of ethanol extract of *R. yunnanensis* on mice, acute toxicity test was performed. Ethanol extract of *R. yunnanensis* had no adverse effects on mice when used in conventional doses (Tables SI and SII). Alcoholic extract of *R. yunnanensis* (2.500, 1.250 and 0.625 g/kg) showed that 0.625 g/kg had no effect on the pathological indices of carotid artery in mice (Fig. S1). Therefore, 2.50 and 1.25 g/kg were selected for this study.

Effect of alcohol extract of R. yunnanensis on blood lipid levels. Compared with the normal control group, serum levels of TC, TG and LDL-C in the model group were significantly increased, while levels of HDL-C (all $P<0.0001$; Fig. 2A-D) were significantly decreased. Compared with the model group, levels of serum TC ($P<0.001$), TG ($P<0.01$) and LDL-C ($P<0.0001$) were decreased in the HRY group, while levels of HDL-C were significantly increased ($P<0.01$). The levels of serum TC, TG and LDL-C were decreased and HDL-C (all $P<0.05$) levels were increased in the LRY group.

Alcohol extract of R. yunnanensis improves the intima of blood vessels. Hematoxylin and eosin staining results showed that in the control group, the vascular structure was complete, smooth and the hierarchy was clear, with no obvious damage or stenosis of the lumen (Fig. 3A). In the model group, vascular endothelium was damaged, the structure and morphology were disorganized, plaque area and the number of lipid nuclei in the plaque was large and the lumen was severely narrowed (Fig. 3B). Compared with the model group, carotid vascular injury were notably reduced in the HRY and LRY groups (Fig. 3C and D).

Ethanol extract of R. yunnanensis reduces the content of collagen fiber. After Masson staining, collagen fibers were blue and smooth muscle cells were red. Levels of aortic blue staining in the model group was significantly higher than that in the control group and the degree of fibrosis was significantly increased ($P<0.0001$; Fig. 4A, B and E). Compared with the model, the

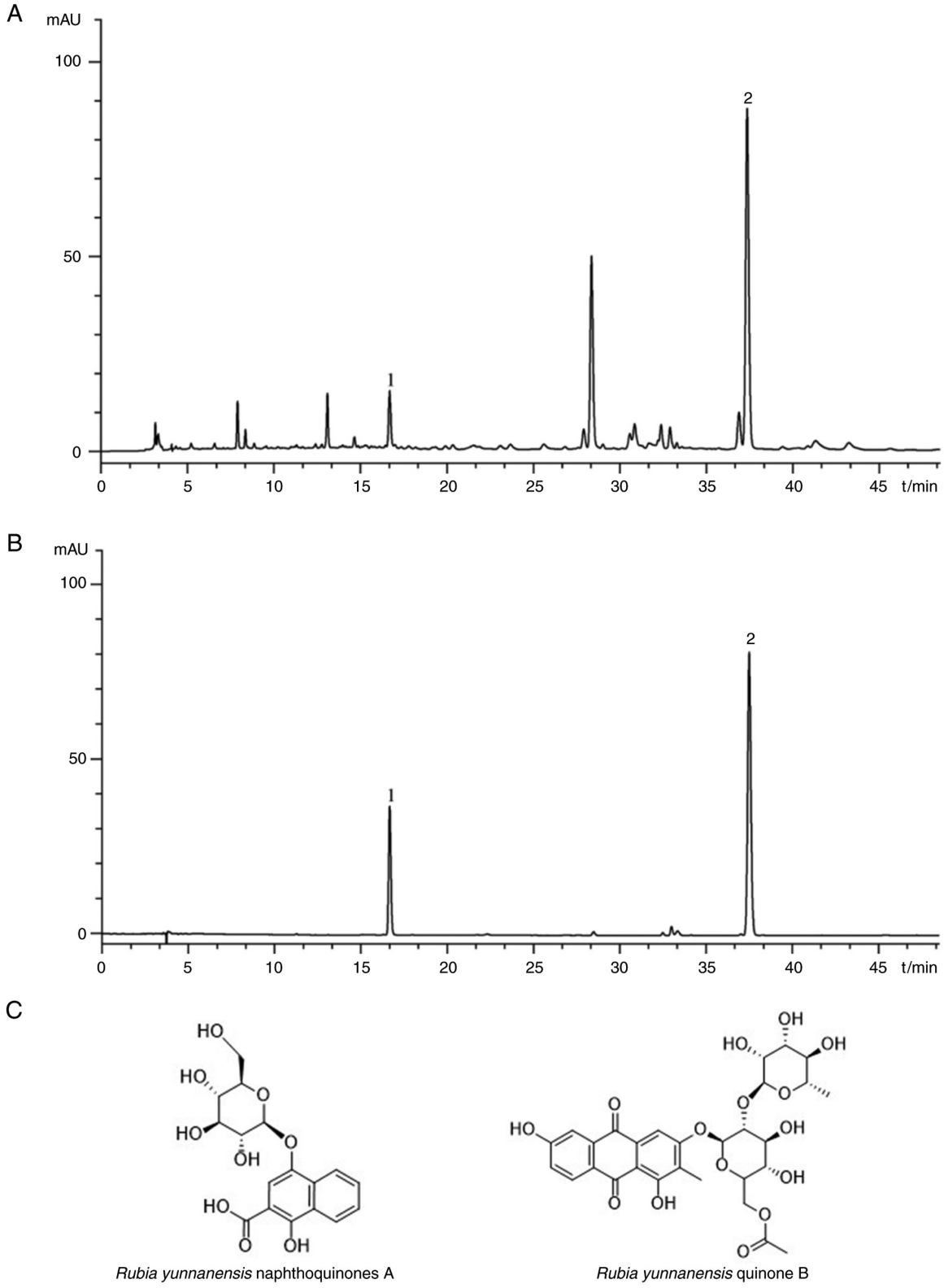

Figure 1. Analysis of chemical constituents of ethanol extracts from *Rubia yunnanensis*. Chromatogram of (A) ethanol extract of *Rubia yunnanensis* and (B) standards. (C) Structure of two main chemical components in the ethanol extract of *Rubia yunnanensis*.

collagen fiber content in HRY was significantly decreased (P<0.0001; Fig. 4C and E). The collagen fiber content of LRY was also significantly decreased (P<0.0001; Fig. 4D and E).

Ethanol extract of R. yunnanensis inhibits lipid accumulation in carotid artery. Oil red O staining showed no lipid deposition in the carotid artery of the control group (Fig. 5A and E). Compared with the control group, there was a large amount of lipid deposition in the carotid artery of the model group (P<0.0001; Fig. 5B and E). Compared with the model, the area of carotid artery lipid content in HRY was significantly reduced (P<0.0001; Fig. 5C and E). The area of carotid artery

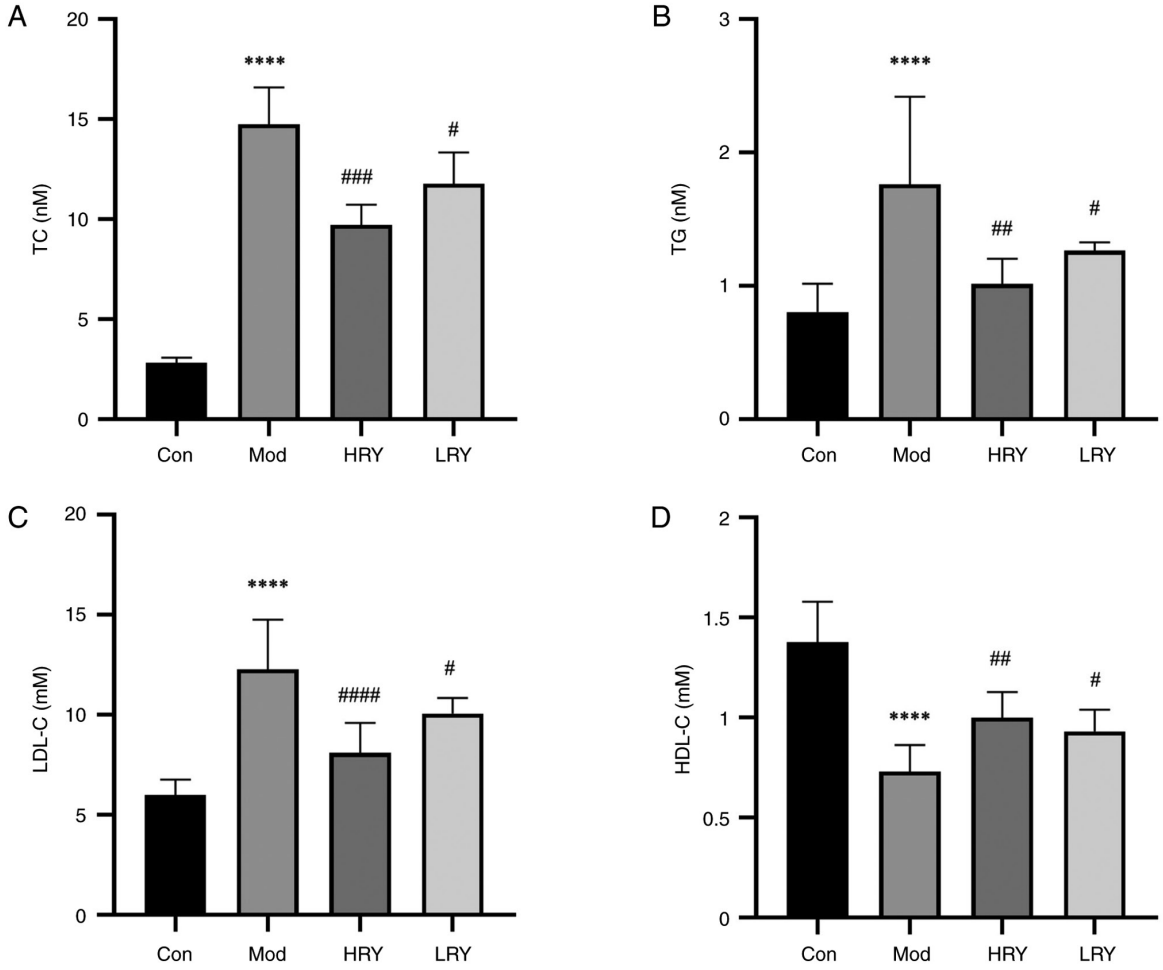

Figure 2. Ethanol extract of RY decreases the levels of blood lipids in a carotid atherosclerosis model. (A) TC, (B) TG, (C) LDL-C and (D) HDL-C levels. n=6. ****P<0.0001 vs. Con. $^{\#}$P<0.05, $^{\#\#}$P<0.01, $^{\#\#\#}$P<0.001 and $^{\#\#\#\#}$P<0.0001 vs. Mod. RY, *Rubia yunnanensis*; H, high; L, low; TC, total cholesterol; TG, triglyceride; DL-C, density lipoprotein cholesterol; Con, control; Mod, model.

lipid content also decreased significantly in the LRY group (P<0.0001; Fig. 5D and E).

Ethanol extract of R. yunnanensis decreases content of elastic fiber. EVG staining showed that elastic fibers of the inner wall of blood vessels in the normal control group were intact and without interruption (Fig. 6A). In the model group, a large number of elastic fibers were clustered in the intima of blood vessels (Fig. 6B). The aggregation of elastic fibers in the HRY and L groups was less than that in the model (Fig. 6C and D).

Protein expression of PI3K, AKT and p-PI3K and p-AKT is upregulated in carotid artery. The phosphorylation of PI3K and AKT was measured by western blotting. Mice were treated with LY294002 dissolved in ethanol extract of *R. yunnanensis*. Western blotting showed that compared with the normal control group, the protein ratios of p-PI3K/PI3K and p-AKT/AKT in the carotid tissues of the model group were significantly decreased and p-PI3K and p-AKT (all P<0.0001; Fig. 7B-E) protein expression also decreased significantly. Compared with the model group, the protein ratios of p-PI3K/PI3K (P<0.001; Fig. 7B) and p-AKT/AKT (P<0.0001; Fig. 7C) in carotid tissue in HRY were significantly increased and p-PI3K (P<0.0001; Fig. 7D) and p-AKT (P<0.001; Fig. 7E) protein expression was significantly increased. LY294002 inhibitor eliminated this effect, and compared with HRY group, the protein ratios of p-PI3K/PI3K (P<0.05; Fig. 7B), and p-AKT/AKT (P<0.01, Fig. 7C) in carotid tissue in HRY + LY were significantly decreased and p-PI3K (P<0.001; Fig. 7D) and P-AKT (P<0.01; Fig. 7E) protein expression was significantly decreased. HRY activated the PI3K/AKT signaling pathway after carotid endothelial cell injury and LY294002 inhibited the PI3K/AKT signaling pathway.

Discussion

Carotid AS is widely recognized as a complex pathophysiological disease associated with abnormal lipid metabolism and endothelial dysfunction (13,14). The AS mouse model of ApoE$^{-/-}$ induced mice on HFD is frequently used in animal experiments to assess drug efficacy and mechanisms in atherosclerosis; this model resembles the disease course of atherosclerosis (15,16). The aim of the present study was to use an established model of carotid AS in *ApoE*$^{-/-}$ mice induced by HFD and to explore the mechanism of inhibition of carotid AS by an ethanol extract of *R. yunnanensis*.

Chinese herbal medicine has been used to prevent and treat disease for >100 years. *R. yunnanensis* has been used by

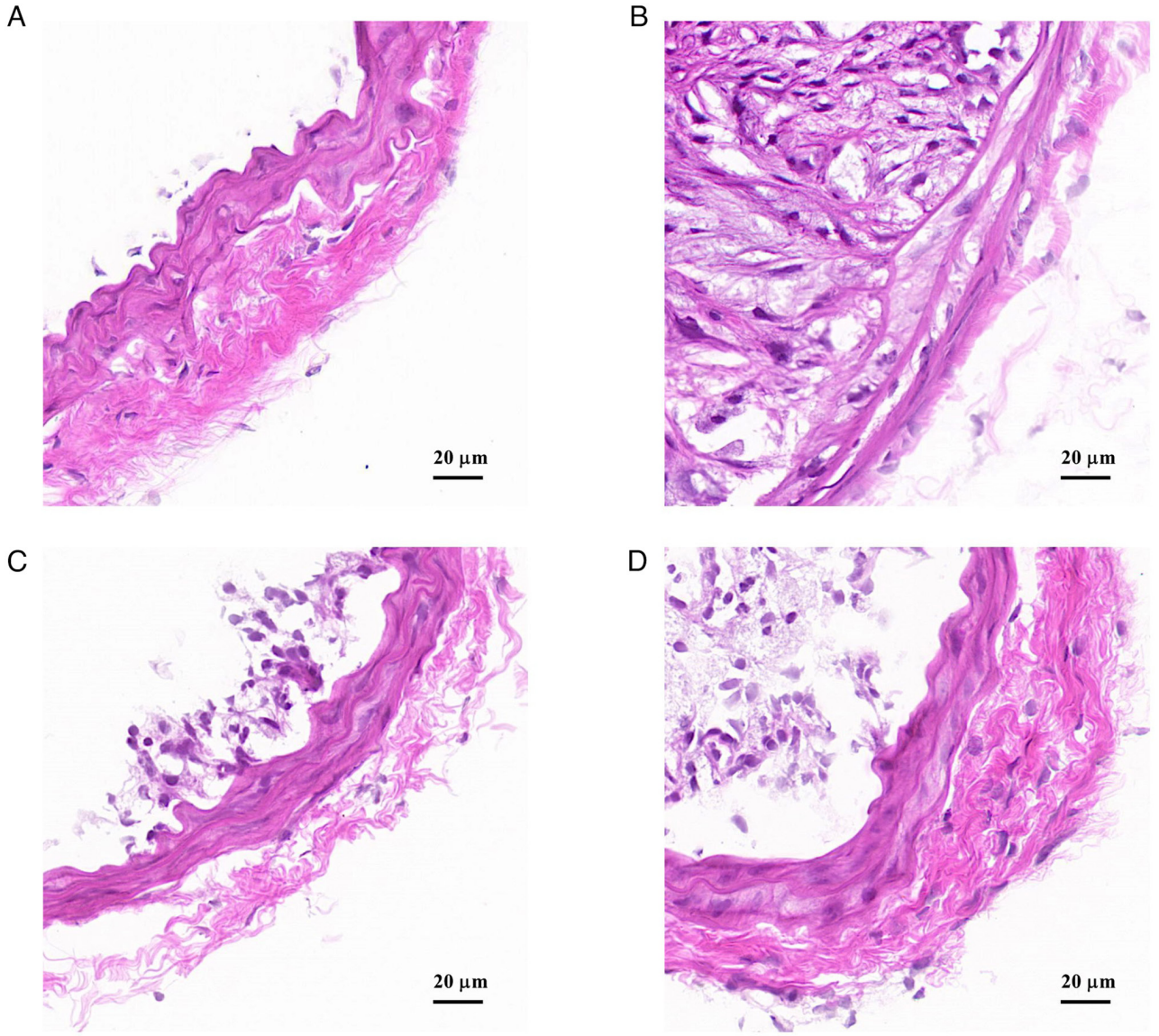

Figure 3. Effect of ethanol extract of RY on carotid artery vascular structure. Hematoxylin and eosin staining (magnification, x40) of (A) control, (B) model, (C) HRY and (D) LRY. RY, *Rubia yunnanensis*; H, high; L, low.

Yunnan peoples to treat cardiovascular disease (17). Previous studies have shown that *R. yunnanensis* extract has significant pharmacological effects such as lowering blood lipids and antiplatelet aggregation (17,18). Ren *et al* (19) conducted HPLC on *R. yunnanensis* samples and identified two primary components, *R. yunnanensis* naphthoquinones A and *R. yunnanensis* quinone side B (19). Therefore, in the present study, the chemical composition of ethanol extract of *R. yunnanensis* was analyzed; *R. yunnanensis* naphthoquinones A and *R. yunnanensis* quinone B were the main compounds.

Lipid metabolism disorder affects initiation and progression of carotid AS (20). Lipids include TG and TC. Elevated TG will cause functional damage to vascular endothelial cells, strong lipid metabolism, excessive generation of oxygen free radicals, promotion of adhesion molecule expression, oxidative damage, foam cell formation, inflammation and the development of carotid AS (21,22). Increased TC promotes the adhesion of white blood cells and the aggregation of platelets, causing release of growth factors and monocyte infiltration, facilitates the multiplication of smooth muscle cells, accelerating their entry into the intima, and promotes the production of collagen fibers, making fatty plaques change into fibrous plaques (23,24). Lipid can combine with apolipoprotein to form lipoprotein, which is the primary form of lipid transported in the body (25). Most TC is found in LDL-C. High levels of LDL-C are associated with inflammation and lipid accumulation, which stimulates plaque formation (26). HDL-C reverses the transport of TC to the liver, thereby decreasing TC content in the body. When HDL-C levels are significantly decreased, reverse transport of TC will be inhibited, which increases the TC content in the body and increases the risk of AS (27). Here, the lipid levels and carotid artery pathology were examined in a mouse model of carotid AS. Ethanol extract of *R. yunnanensis* lowered levels of TC, TG and LDL-C, whereas the levels of HDL-C increased and damage to endothelial cells and accumulation of lipids, collagen and elastic fibers decreased. These findings indicated that induced disruption of lipid metabolism in the mouse model could be

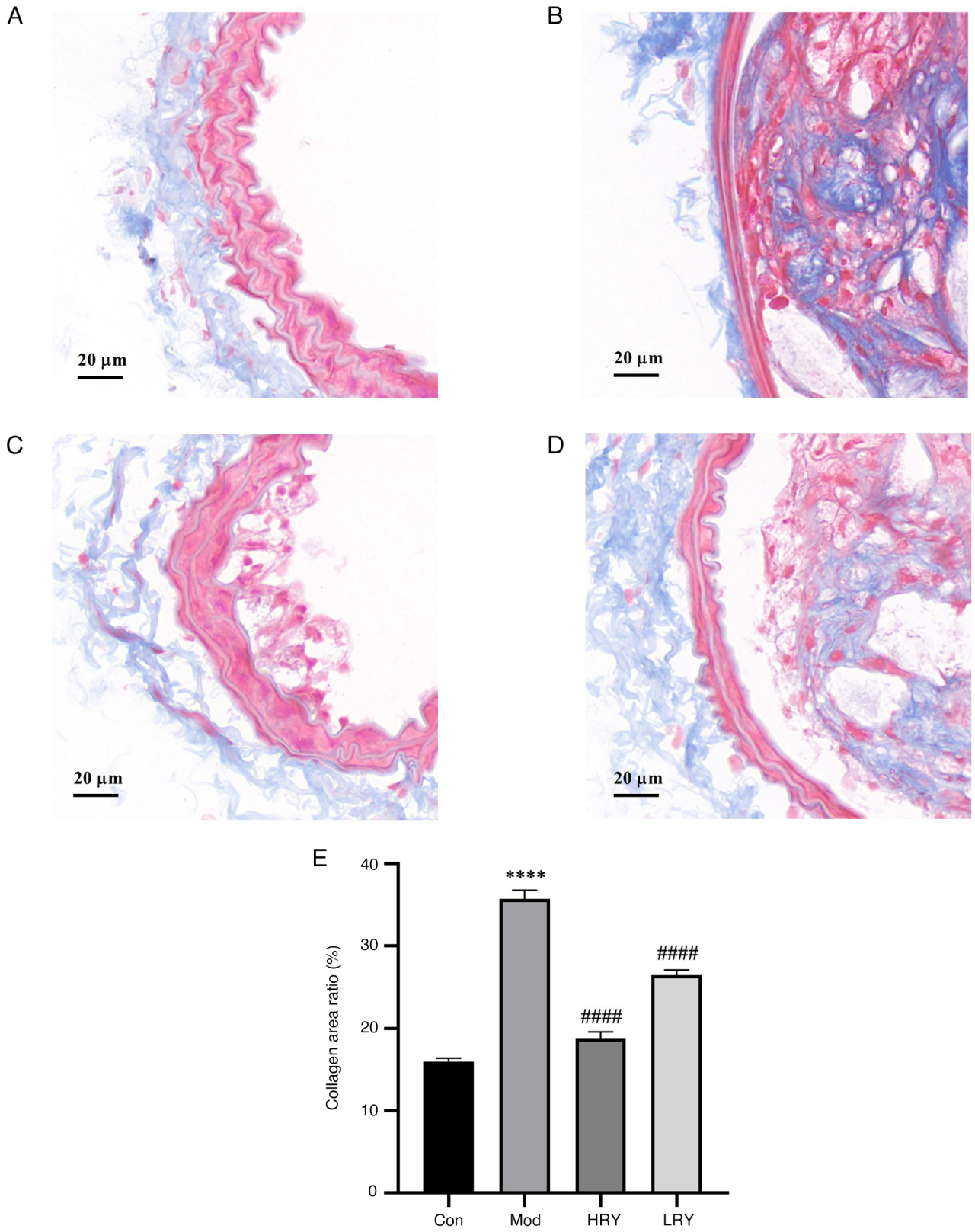

Figure 4. Effects of ethanol extract of RY on carotid vascular collagen fibers. Masson's staining of (A) control, (B) model and (C) HRY and (D) LRY. (E) Carotid collagen area/total visual field area. n=3. ****P<0.0001 vs. Con; $^{####}$P<0.0001 vs. Mod. RY, *Rubia yunnanensis*; H, high; L, low; Con, control; Mod, model.

regulated by ethanol extract of *R. yunnanensis*, which reduced plaques in the carotid artery.

Damage to vascular endothelial cells caused by lipid metabolism disorders is involved in the formation of carotid AS (28). The innermost layer of the blood vessel wall is attached by the vascular endothelium, which has a single layer of endothelial cells acting as a physical wall between blood flow and the blood vessel wall, regulating

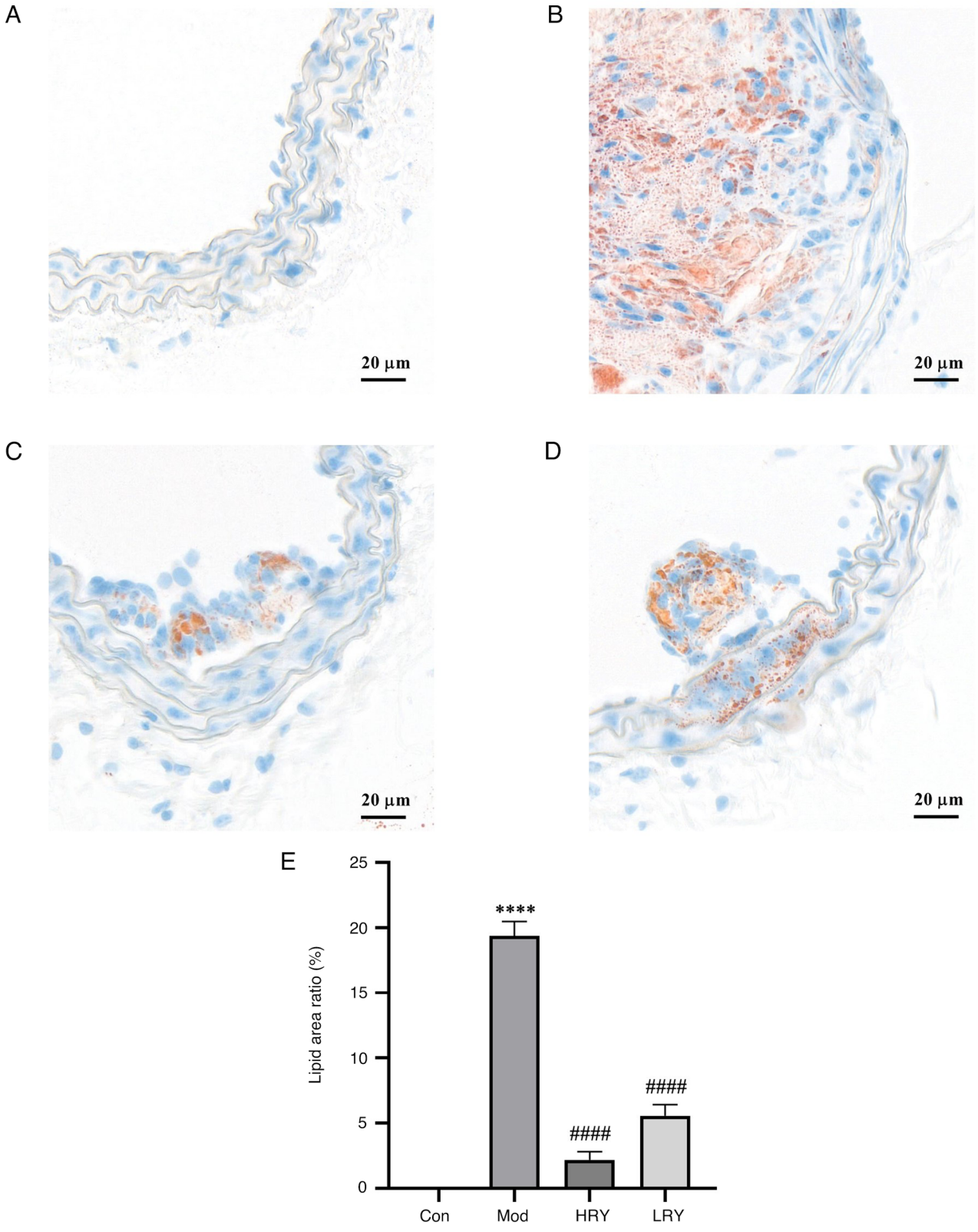

Figure 5. Effect of ethanol extract of RY on lipid accumulation in carotid artery. Oil Red O staining of (A) control, (B) model and (C) HRY and (D) LRY. (E) Carotid lipid-red area/total visual field area. n=3. ****P<0.0001 vs. Con; $^{####}$P<0.0001 vs. Mod. RY, *Rubia yunnanensis*; H, high; L, low; Con, control; Mod, model.

the exchange of molecules (29). Intravascular environmental homeostasis is associated with endothelial cells. Endothelial cells regulate vascular active factors, such as adhesion and chemotactic molecules, to maintain their balance and stability (30). Studies have found that vascular endothelial cells prevent the accumulation of inflammatory factors to prevent AS (31,32). When the vascular endothelium is damaged, LDL enters the subendothelial layer and

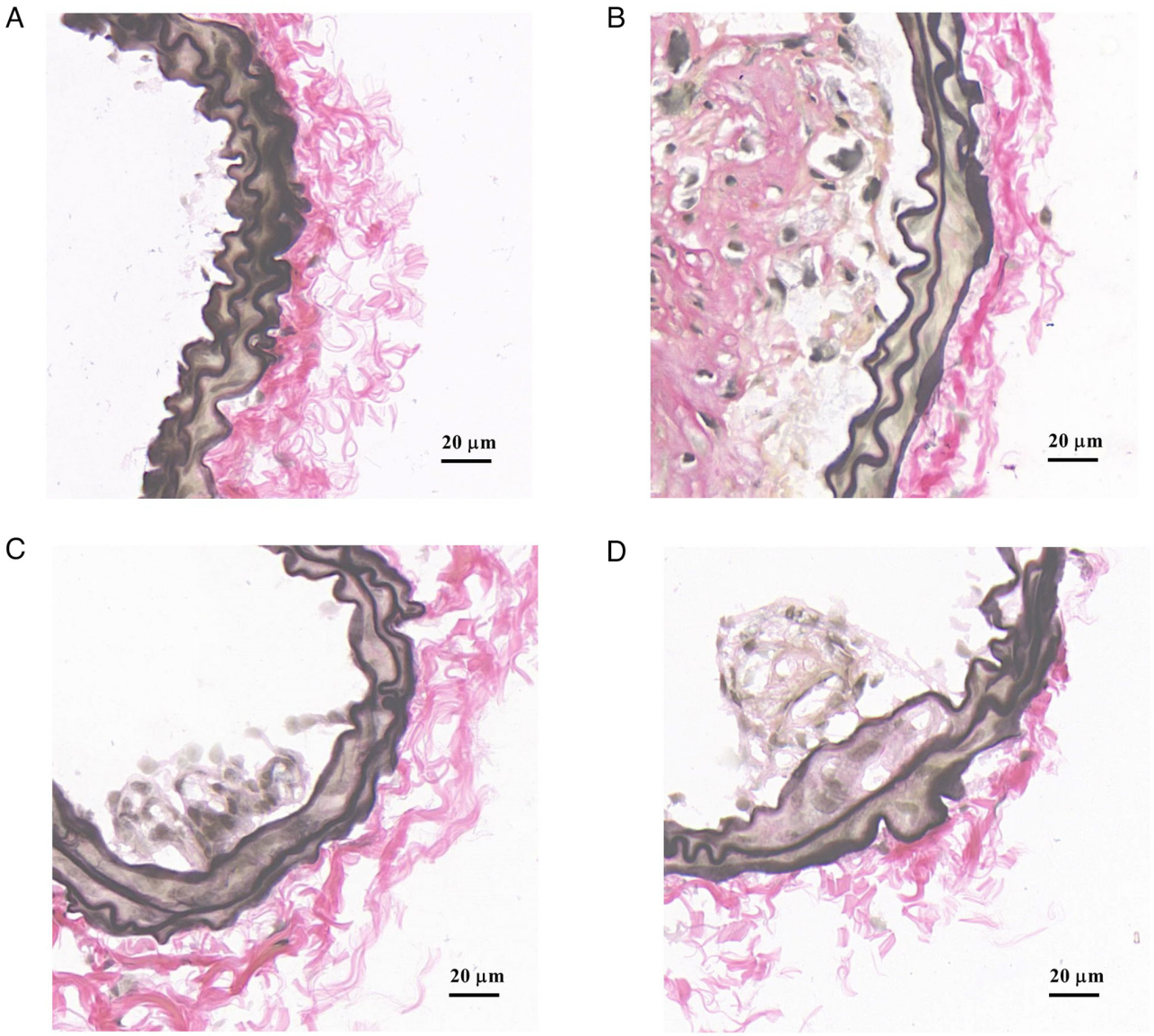

Figure 6. Effect of ethanol extract of RY on carotid vascular elastic fiber. Verhoeff's Van Gieson staining of (A) control, (B) model, (C) HRY and (D) LRY. RY, *Rubia yunnanensis*; H, high; L, low.

is oxidized, resulting in secretion of various cell adhesion molecules and inflammation, prompting monocytes to adhere to the endothelial surface, resulting in formation of foam cells and accelerating the formation of AS (33). The PI3K/AKT pathway is one of the most common signaling pathways in living organisms and it is involved in AS, the proliferation and migration of epithelial cells, endothelial dysfunction, lipid metabolism and other processes required for maintaining normal body function (34-36). PI3K is an important kinase that promotes cell proliferation and inhibits apoptosis by activating many downstream factors. AKT is a key downstream target enzyme in PI3K activation that acts in a number of cellular processes (37,38). AKT activates downstream factors by conducting the signal sent by PI3K, thereby regulating cell proliferation and apoptosis (39). Investigations have shown that upregulation of PI3K/AKT pathway activity promotes vascular regeneration and inhibits vascular endothelial apoptosis, the multiplication of smooth muscle cells and development of AS (40,41).

The present study investigated the effects of ethanol extract of *R. yunnanensis* on protein expression levels of p-AKT/AKT and p-PI3K/PI3K in carotid arteries of the AS mouse model. The aim was to determine whether the ethanol extract of *R. yunnanensis* upregulates phosphorylation of PI3K and AKT protein in the AS model to alleviate endothelial cell damage. As expected, a HFD induced abnormal blood lipid levels in *ApoE*$^{-/-}$ mice and caused severe pathological carotid AS, with hematoxylin and eosin staining showing endothelial cells were irregularly arranged and a large number of AS plaques had formed, indicating that carotid AS had caused serious endothelial cell damage. Concurrently, p-PI3K and p-AKT protein content and p-PI3K/PI3K and p-AKT/AKT ratios were decreased. These results indicated that phosphorylation of PI3K and AKT was inhibited in carotid AS, suggesting that the activity of the PI3K/AKT signaling pathway was inhibited. However, following treatment with *R. yunnanensis* extract, the expression of p-PI3K and p-AKT and the ratios of p-PI3K/PI3K and p-AKT/AKT increased, indicating that *R. yunnanensis*

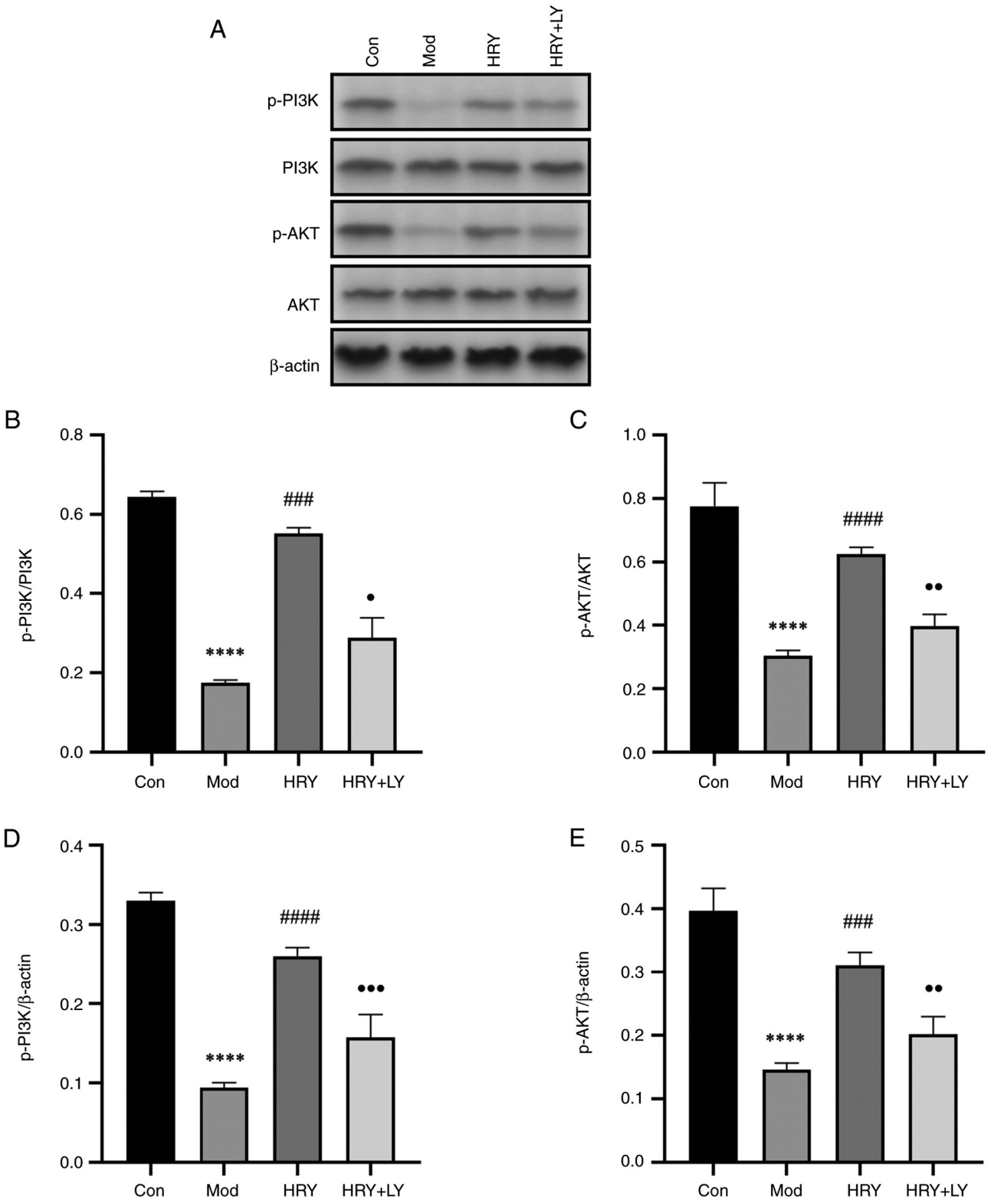

Figure 7. Effect of ethanol extract of RY on expression of p-PI3K and p-AKT in carotid artery tissue. (A) Representative western blotting of total and p-PI3K and AKT. Quantitative analysis of (B) p-PI3K/PI3K, (C) p-AKT/AKT, (D) p-PI3K/β-actin and (E) p-AKT/β-actin ratio. n=3 per group. ****P<0.0001 vs. Con. $^{###}$P<0.001, $^{####}$P<0.0001 vs. Mod. •P<0.05, ••P<0.01, •••P<0.001 vs. HRY. RY, *Rubia yunnanensis*; H, high; p-, phosphorylated.

alcohol extract activated the PI3K/AKT signaling pathway. To evaluate the role of the PI3K/AKT signaling pathway in inhibition of AS by *R. yunnanensis* alcohol extract, PI3K/AKT inhibitor LY294002 (42) was used to treat AS induced by HFD following intervention with RY + H. LY294002 blocked the PI3K/AKT signaling, suggesting that activity of PI3K/AKT signaling pathway could be upregulated by the ethanol extract of *R. yunnanensis*.

In summary, in *ApoE*$^{-/-}$ mice fed HFD for 12 weeks, *R. yunnanensis* extract lowered blood lipid levels, inhibited

carotid artery lipid accumulation and decreased elastic fiber thinning loss and intimal hyperplasia. *R. yunnanensis* extract decreased blood lipid levels and alleviated endothelial damage, inhibiting the occurrence and development of AS via upregulation of the PI3K/AKT pathway. The extract of *R. yunnanensis* mainly contains *R. yunnanensis* naphthoquinones A and *R. yunnanensis* quinone B. Overall, *R. yunnanensis* is a promising treatment for AS. Future research should investigate the mechanism of action of *R. yunnanensis* in the treatment of AS to provide a theoretical basis for its potential drug use.

Acknowledgements

The authors would like to thank Professor Yin Zili (Yunnan University of Chinese Medicine, Yunnan) for assistance with the identification service.

Funding

The present study was supported by the Xingdian Talent Support Program-Special for Young Talent (grant no. XDYC-QNRC-2022-0284) and National Administration of Traditional Chinese Medicine High-level Key Discipline Construction Project 'Dai Medicine'.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

GL, LY and XD designed the experiments and analyzed data. JC and PC analyzed data. XD and GL wrote and revised the manuscript. JC and PC confirm the authenticity of all the raw data. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

All animal experiments were approved by the Animal Ethics Committee of Yunnan University of Traditional Chinese Medicine (approval no. R-062022053).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Chen J, Zhang X, Millican R, Sherwood J, Martin S, Jo H, Yoon YS, Brott BC and Jun HW: Recent advances in nanomaterials for therapy and diagnosis for atherosclerosis. Adv Drug Deliv Rev 170: 142-199, 2021.
2. Legein B, Temmerman L, Biessen EA and Lutgens E: Inflammation and immune system interactions in atherosclerosis. Cell Mol Life Sci 70: 3847-3869, 2013.
3. Geovanini GR and Libby P: Atherosclerosis and inflammation: Overview and updates. Clin Sci (Lond) 132: 1243-1252, 2018.
4. Usai MV, Bosiers MJ, Bisdas T, Torsello G, Beropoulis E, Kasprzak B, Stachmann A and Stavroulakis K: Surgical versus endovascular revascularization of subclavian artery arteriosclerotic disease. J Cardiovasc Surg (Torino) 61: 53-59, 2020.
5. Sun Y, Gao Y, Zhou L, Lu Y, Zong Y, Zhu H, Tang Y, Zheng F, Sun Y and Li Y: A multi-target protective effect of Danggui-Shaoyao-San on the vascular endothelium of atherosclerotic mice. BMC Complement Med Ther 23: 60, 2023.
6. Lusis AJ: Atherosclerosis. Nature 407: 233-241, 2000.
7. Hansson GK: Inflammation, atherosclerosis, and coronary artery disease. N Engl J Med 352: 1685-1695, 2005.
8. Williams IL, Wheatcroft SB, Shah AM and Kearney MT: Obesity, atherosclerosis and the vascular endothelium: Mechanisms of reduced nitric oxide bioavailability in obese humans. Int J Obes Relat Metab Disord 26: 754-764, 2002.
9. Xing SS, Yang XY, Zheng T, Li WJ, Wu D, Chi JY, Bian F, Bai XL, Wu GJ, Zhang YZ, *et al*: Salidroside improves endothelial function and alleviates atherosclerosis by activating a mitochondria-related AMPK/PI3K/Akt/eNOS pathway. Vascul Pharmacol 72: 141-152, 2015.
10. Zhang R, Miao Y, Chen L, Yi S and Tan N: De Novo transcriptome analysis reveals putative genes involved in anthraquinone biosynthesis in Rubia yunnanensis. Genes (Basel) 13: 521, 2022.
11. Yi S, Lin Q, Zhang X, Wang J, Miao Y and Tan N: Selection and validation of appropriate reference genes for quantitative RT-PCR analysis in Rubia yunnanensis diels based on transcriptome data. Biomed Res Int 2020: 5824841, 2020.
12. Larmann J, Jurk K, Janssen H, Müller M, Herzog C, Lorenz A, Schmitz M, Nofer JR and Theilmeier G: Hepatic overexpression of soluble urokinase receptor (uPAR) suppresses diet-induced atherosclerosis in low-density lipoprotein receptor-deficient (LDLR-/-) mice. PLoS One 10: e0131854, 2015.
13. Liu S, Liu Y, Liu Z, Hu Y and Jiang M: A review of the signaling pathways of aerobic and anaerobic exercise on atherosclerosis. J Cell Physiol 238: 866-879, 2023.
14. Zhou ZX, Ren Z, Yan BJ, Qu SL, Tang ZH, Wei DH, Liu LS, Fu MG and Jiang ZS: The role of ubiquitin E3 ligase in atherosclerosis. Curr Med Chem 28: 152-168, 2021.
15. Kolovou G, Anagnostopoulou K, Mikhailidis DP and Cokkinos DV: Apolipoprotein E knockout models. Curr Pharm Des 14: 338-351, 2008.
16. Nakashima Y, Plump AS, Raines EW, Breslow JL and Ross R: ApoE-deficient mice develop lesions of all phases of atherosclerosis throughout the arterial tree. Arterioscler Thromb 14: 133-140, 1994.
17. Gao Y, Su Y, Huo Y, Mi J, Wang X, Wang Z, Liu Y and Zhang H: Identification of antihyperlipidemic constituents from the roots of Rubia yunnanensis Diels. J Ethnopharmacol 55: 1315-1321, 2014.
18. Liou MJ and Wu TS: Triterpenoids from Rubia yunnanensis. J Nat Prod 65: 1283-1287, 2002.
19. Ren H, Wang X, Gao L, Niu L and Li J: Characteristic spectrum and quantitative study of the index components of the Yi medicine Rubia yunnanensis Chin Med Mater 45: 1400-1404, 2022 (In Chinese).
20. Yang R, Powell-Braxton L, Ogaoawara AK, Dybdal N, Bunting S, Ohneda O and Jin H: Hypertension and endothelial dysfunction in apolipoprotein E knockout mice. Arterioscler Thromb Vasc Biol 19: 2762-2768, 1999.
21. Nader MA, el-Agamy DS and Suddek GM: Protective effects of propolis and thymoquinone on development of atherosclerosis in cholesterol-fed rabbits. Arch Pharm Res 33: 637-643, 2010.
22. Calan M, Calan O, Gonen MS, Bilgir F, Kebapcilar L, Kulac E, Cinali T and Bilgir O: Examination of adhesion molecules, homocysteine and hs-CRP in patients with polygenic hypercholesterolemia and isolated hypertriglyceridemia. Intern Med 50: 1529-1535, 2011.
23. Meyer-Lindemann U, Mauersberger C, Schmidt AC, Moggio A, Hinterdobler J, Li X, Khangholi D, Hettwer J, Gräßer C, Dutsch A, *et al*: Colchicine Impacts leukocyte trafficking in atherosclerosis and reduces vascular inflammation. Front Immunol 13: 898690, 2022.
24. Massberg S, Brand K, Gruner S, Page S, Müller E, Müller I, Bergmeier W, Richter T, Lorenz M, Konrad I, *et al*: A critical role of platelet adhesion in the initiation of atherosclerotic lesion formation. J Exp Med 196: 887-896, 2002.
25. Vega GL and Grundy SM: Hypercholesterolemia with cholesterol-enriched LDL and normal levels of LDL-apolipoprotein B. Effects of the step I diet and bile acid sequestrants on the cholesterol content of LDL. Arterioscler Thromb Vasc Biol 16: 517-522, 1996.

26. Badimon L, Vilahur G and Padro T: Lipoproteins, platelets and atherothrombosis. Rev Esp Cardiol 62: 1161-1178, 2009 (In English, Spanish).
27. Calabresi L, Gomaraschi M, Simonelli S, Bernini F and Franceschini G: HDL and atherosclerosis: Insights from inherited HDL disorders. Biochim Biophys Acta 1851: 13-18, 2015.
28. Gimbrone MA Jr and Garcia-Cardena G: Endothelial cell dysfunction and the pathobiology of atherosclerosis. Circ Res 118: 620-636, 2016.
29. Aird WC: Endothelium as an organ system. Crit Care Med 32 (5 Suppl): S271-S279, 2004.
30. Corre I, Paris F and Huot J: The p38 pathway, a major pleiotropic cascade that transduces stress and metastatic signals in endothelial cells. Oncotarget 8: 55684-55714, 2017.
31. Rafieian-Kopaei M, Setorki M, Doudi M, Baradaran A and Nasri H: Atherosclerosis: Process, indicators, risk factors and new hopes. Int J Prev Med 5: 927-946, 2014.
32. Poznyak A, Grechko AV, Poggio P, Myasoedova VA, Alfieri V and Orekhov AN: The diabetes mellitus-atherosclerosis connection: The role of lipid and glucose metabolism and chronic inflammation. Int J Mol Sci 21: 1835, 2020.
33. Marchio P, Guerra-Ojeda S, Vila JM, Aldasoro M, Victor VM and Mauricio MD: targeting early atherosclerosis: A focus on oxidative stress and inflammation. Oxid Med Cell Longev 2019: 8563845, 2019.
34. Li Y, Xu Q, Shi M, Gan P, Huang Q, Wang A, Tan G, Fang Y and Liao H: Low-level laser therapy induces human umbilical vascular endothelial cell proliferation, migration and tube formation through activating the PI3K/Akt signaling pathway. Microvasc Res 129: 103959, 2020.
35. Chen L, Qin L, Liu X and Meng X: CTRP3 Alleviates Ox-LDL-Induced inflammatory response and endothelial dysfunction in mouse aortic endothelial cells by activating the PI3K/Akt/eNOS Pathway. Inflammation 42: 1350-1359, 2019.
36. Zhou YJ, Xu N, Zhang XC, Zhu YY, Liu SW and Chang YN: Chrysin improves glucose and lipid metabolism disorders by regulating the AMPK/PI3K/AKT signaling pathway in insulin-resistant HepG2 cells and HFD/STZ-Induced C57BL/6J mice. J Agric Food Chem 69: 5618-5627, 2021.
37. Manning BD and Toker A: AKT/PKB signaling: Navigating the network. Cell 169: 381-405, 2017.
38. Li Q, Li N, Cui HH, Tian XQ, Jin C, Chen GH and Yang YJ: Tongxinluo exerts protective effects via anti-apoptotic and pro-autophagic mechanisms by activating AMPK pathway in infarcted rat hearts. Exp Physiol 102: 422-435, 2017.
39. Jing R, Zhong QQ, Long TY, Pan W and Qian ZX: Downregulated miRNA-26a-5p induces the apoptosis of endothelial cells in coronary heart disease by inhibiting PI3K/AKT pathway. Eur Rev Med Pharmacol Sci 23: 4940-4947, 2019.
40. Liu J, Xu P, Liu D, Wang R, Cui S, Zhang Q, Li Y, Yang W and Zhang D: TCM Regulates PI3K/Akt signal pathway to intervene atherosclerotic cardiovascular disease. Evid Based Complement Alternat Med 2021: 4854755, 2021.
41. Luo L, Liang H and Liu L: Myristicin regulates proliferation and apoptosis in oxidized low-density lipoprotein-stimulated human vascular smooth muscle cells and human umbilical vein endothelial cells by regulating the PI3K/Akt/NF-κB signalling pathway. Pharm Biol 60: 56-64, 2022.
42. Guo J, Jie W, Shen Z, Li M, Lan Y, Kong Y, Guo S, Li T and Zheng S: SCF increases cardiac stem cell migration through PI3K/AKT and MMP-2/-9 signaling. Int J Mol Med 34: 112-118, 2014.