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ORIGINAL ARTICLE

Effect of 1,8-Dihydroxyanthraquinone on the Imbalance of Lipid Metabolism via Regulation of Expression of CYP7A1 and 3-Hydroxy-3-Methylglutaryl Coenzyme A Reductase mRNA in Hyperlipidemic Mice

Jiao Ma, Xu Liu[1], Jiahui Yu[1], Jinghui Sun[1]

Department of Cardiovascular Medicine, Affiliated Hospital of Beihua University, [1]Department of Pharmacology, College of Pharmacy, Beihua University, Jilin, China

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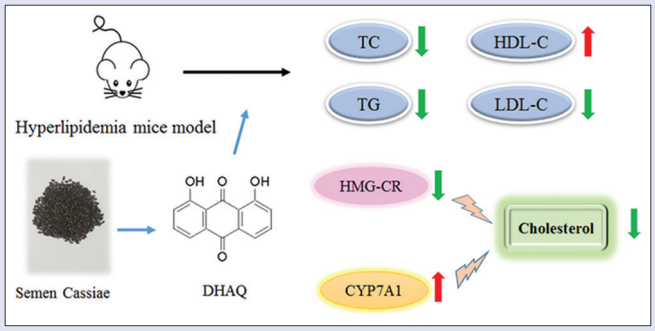
ABSTRACT

Background: *Cassia obtusifolia* is a traditional Chinese medicine used in lowering blood lipids, but the specific compounds and mechanisms responsible for this action are still unknown. Anthraquinones are the primary active components of *C. obtusifolia,* among which 1,8-dihydroxyanthraquinone (DHAQ) exhibits strong biological activities. **Aim:** The effects of DHAQ on blood lipids and the underlying mechanisms were investigated in this study to provide a reliable experimental basis for the development and application of *C. obtusifolia*. **Materials and Methods:** Mice were fed with a high-fat diet for the establishment of a hyperlipidemia mouse model. After the establishment of the model, the mice were intragastrically administered with 5 mg/kg DHAQ once daily, continuously for 6 weeks. Then, the mice were weighed; their lipid/body ratios were calculated; their serum levels of triglyceride (TG), total cholesterol (TC), low-density lipoprotein cholesterol (LDL-C), and high-density lipoprotein cholesterol (HDL-C) were measured. TG and TC contents in the liver tissue samples were measured by the enzyme-linked immunosorbent assay. Reverse transcription–polymerase chain reaction was performed to detect the levels of 7α-hydroxylase (CYP7A1) and 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGCR) mRNA, and Western blot analysis was performed to detect the expression levels of HMGCR and CYP7A1 proteins in the liver tissue of mice. **Results:** Body weights and lipid/body ratios of the hyperlipidemic mice were significantly reduced, and the levels of TG, TC, and LDL-C were significantly reduced in the serum samples. However, the HDL-C content in the serum was significantly increased, and TG and TC contents in the liver tissue were significantly reduced in the DHAQ-treated hyperlipidemic mice. In addition, the expression of CYP7A1 and HMGCR proteins was respectively increased and decreased significantly in the liver tissue of the hyperlipidemic mice. **Conclusion:** DHAQ revealed a hypolipidemic effect in hyperlipidemia mice fed on a high-fat diet, which may be related to the regulation of cholesterol metabolism in the liver.

Key words: 1, 8-dihydroxyanthraquinone, 3-hydroxy-3-methylglutaryl coenzyme A reductase, *Cassia obtusifolia,* CYP7A1, hyperlipidemia

SUMMARY

- 1,8-dihydroxyanthraquinone (DHAQ) has a significant hypolipidemic effect in mice with the high-fat diet-induced hyperlipidemia. DHAQ may regulate the metabolism balance of cholesterol by promoting the conversion of cholesterol into bile acid and inhibiting the synthesis of cholesterol in the liver of mice.



Abbreviations used: DHAQ: 1, 8-dihydroxyanthraquinone; TG: triglyceride; TC: total cholesterol; LDL-C: low-density lipoprotein cholesterol; HDL-C: high-density lipoprotein cholesterol; HMGCR: 3-hydroxy-3-methylglutaryl-coenzyme A reductase; CYP7A1: cholesterol 7α-hydroxylase; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; AI: atherosclerosis index; PMSF: phenylmethylsulfonyl fluoride.

Correspondence:

Prof. Jinghui Sun,
College of Pharmacy, Beihua University, 3999 Binjiang East Road, Jilin 132013, China.
E-mail: sunjinghui2008@126.com

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INTRODUCTION

Hyperlipidemia is an important risk factor for various chronic diseases, including atherosclerosis, cardiocerebrovascular disorders, and non-alcoholic fatty liver, seriously endangering human health.[1] It is believed that one of the primary risk factors for coronary heart diseases is hypercholesterolemia;[2] therefore, regulating the blood lipid level is an important factor in the prevention and treatment of the aforementioned disorders. However, the clinical symptoms of hyperlipidemia are not very clear, and the treatment compliance of most patients is poor, which results in a satisfactory control effect on hyperlipidemia. Currently, statins are the primary lipid-modulating drugs used in the clinical setting. However, the long-term use of statins will induce some adverse reactions, such as rhabdomyolysis and hepatic injury.[3,4] Therefore, it is of great significance to develop

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a safe and green product for the prevention and treatment of hyerlipidemia.

Traditional Chinese medicines and their extracts have a unique therapeutic effect on hyperlipidemia.[5,6] They are more popular because of their less side effects, mild and lasting effects, and multi-target characteristic.[7] *Cassia obtusifolia* is the mature and dry seed of *C. obtusifolia* L.[8] It is a traditional Chinese medicine and has been used in East Asia for thousands of years. It is used as the monarch medicine in the prescriptions of some famous Chinese patent medicines for regulating blood lipid levels, such as Jueming Jiangzhi Tablets, Xuezhining Pills, and Zhikang Granules.[9-11] It has been reported that both *C. obtusifolia* extract and its active parts have a hypolipidemic effect, but the specific active components and their mechanism of action are still unclear.[12-14] Anthraquinones are the primary active components of *C. obtusifolia*, among which 1,8-dihydroxyanthraquinone (DHAQ) has strong biological activities.[15] Therefore, in this study, we investigated that the lipid-lowering effects of DHAQ and its mechanism were investigated in mice with hyperlipidemia induced by high-fat diet, so as to provide a reliable experimental basis for its further development and application.

MATERIALS AND METHODS

Reagents

The following reagents were obtained: DHAQ (DHAQ, with a purity more than 98%, Shanghai Macklin Biochemical Co., Ltd., Shanghai, China); enzyme-linked immunosorbent assay (ELISA) kits for the detection of triglyceride (TG), total cholesterol (TC), low-density lipoprotein cholesterol (LDL-C), and high-density lipoprotein cholesterol (HDL-C) (BioSino, Beijing, China); and cholesterol 7α-hydroxylase (CYP7A1) polyclonal antibodies (ABclonal, Wuhan, China); Cholesterol CYP7A1 polymerase chain reaction (PCR) primers and 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMGCR) were designed by Dingguo Changsheng Biotechnology Co., Ltd (Beijing, China).

Experimental animals and procedures

A total of 30 mice were randomly divided into two groups: normal control (CON) group (n = 15, an equal volume of the solvent) and control + DHAQ (CON + DHAQ) group (n = 15, 5 mg/kg DHAQ) (the mice in these two groups were on the normal diet). Next, another 30 mice were divided into hyperlipidemia model (MOD) group (n = 15, an equal volume of normal solvent) and model + DHAQ (MOD + DHAQ) group (n = 15, 5 mg/kg DHAQ) (the mice in these two groups were on a high-fat diet for the establishment of a hyperlipidemia mouse model). At the same time when the mice were maintained on a high-fat diet, the mice in CON + DHAQ and MOD + DHAQ groups were administered with DHAQ (5 mg/kg) via gavage, whereas those in CON and MOD groups were administered with an equal volume of the solvent, once a day for 6 weeks, and all the mice were weighed once a week during the administration. After the administration, the mice were fasted for 12 h and then anesthetized with ether. Their body lengths (the distance from the tip of the nose to the outer edge of the anus) were measured for the calculation of Lee's index with the following formula:

Lee's index = (Body weight [g] $\times 10^3$/body length [CM]) $^{1/3}$.

The blood samples (0.8–1.0 mL) were collected through the inner canthus vein of each mouse via a capillary method, and the serum was separated and cryopreserved for further analysis. The adipose tissue around the liver of mice was carefully separated, and the liver was washed with normal saline precooled at 4°C, and the water on the surface of the liver was absorbed with a piece of filter paper and then the weight of the organ was weighed for the calculation of the mice organ indexes. The formula for the calculation of organ index is as follows.

Organ index = organ mass (g)/body weight (g) ×100%

The left liver lobe was fixed in 10% neutral formalin, and the remaining liver tissue was frozen at − 80°C in a deep freezer until further analysis [Figure 1]. The experimental procedures were approved by Beihua University Laboratory Animal Ethics Committee (No. 20190018).

Measurement of serum biochemical indicators

TG, TC, LDL-C, and HDL-C levels in the serum samples were measured by ELISA technique. The tests were performed based on the manufacturer's instructions, and the atherosclerosis index (AI) of mice was calculated using the following formula:

$$AI = \frac{TC - HDL - C}{HDL - C}$$

Measurement of hepatic lipid contents

The samples of liver tissue (0.1 g) were added into 0.9 mL of normal saline (1: 9 w/v) on ice, and the liver homogenate was prepared with a mass fraction of 0.1 by an electric homogenizer (15,000 rpm, 30 s). The concentration of protein in the homogenate was measured by Coomassie brilliant blue method. TC and TG contents in the liver tissue were detected as per the manufacturer's instructions (Applygen Technologies Inc., Beijing, China).

Reverse transcription–polymerase chain reaction

A high-purity total RNA rapid extraction kit (centrifugal-column type) was used to extract the total RNA from the samples of liver tissue. We performed a one-step reverse transcription PCR (RT-PCR) using the transcription kit to synthesize and amplify the cDNA by PCR amplification reaction. The sequence numbers of all genes were sought out from GenBank. Primer 6.0 software (PREMIER Biosoft, San Francisco, CA, USA) was used to design the primers, and the primers were synthesized by Beijing Dingguo Changsheng Bioengineering Company, in which glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the internal reference gene [Table 1].

The amplification conditions of the samples were as follows: predenatured at 94°C for 3 min, denatured at 94°C for 30 s, annealed for 30 s (annealing temperature: 55°C for CYP7A1 and GAPDH, 58°C for HMGCR), extended at 72°C for 30 s, with 30 cycles, and then extended at 72°C for 5 min. Then, the samples were kept at 4°C for use. The results of the gel electrophoresis on 10 μL PCR products were recorded, and the images were photographed by a gel imaging system (Tanon 1600 gel Image system, Shanghai Tanon Science and Technology Co., Ltd., Shanghai, China), and the optical density (OD) of the photographs was scanned and analyzed by a gel imaging system, in which OD values of HMGCR and CYP7A1 were used to represent the relative amount of HMGCR and CYP7A1 mRNA.

Western blot analysis

The liver tissue was cut into small pieces to which 10 times the volume of lysis buffer was added (containing phenylmethylsulfonyl fluoride) and homogenized at 15,000 rpm on ice for 1 h. Then, the homogenate was centrifuged at 4°C and 12000×g for 10 min, and the supernatant was collected for the determination of protein content. The protein content was determined by bicinchoninic acid method. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis was used to separate the proteins in the samples, and the proteins were transferred onto the PVDF membrane at a constant current (200 MA). Skim milk (5%) was used to block the membranes for 1 h, and then the membranes were first incubated with the primary antibodies (HMGCR, CYP7A1, and GAPDH polyclonal antibodies) and then with the second antibodies

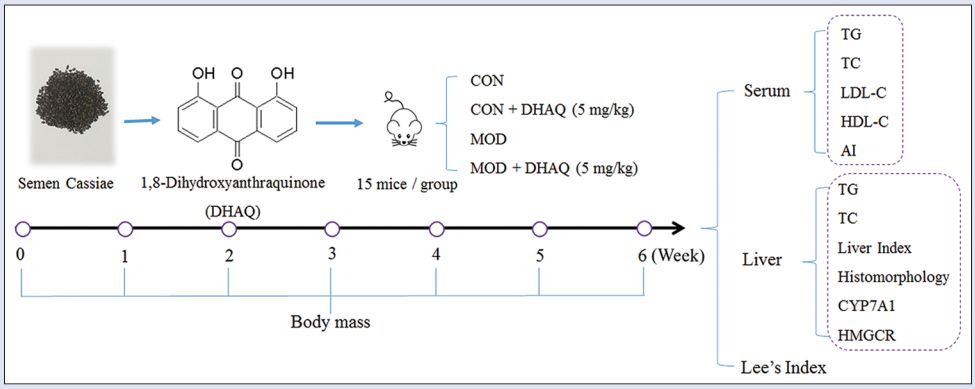

Figure 1: The experimental protocol

Table 1: Primers used for quantitative real-time polymerase chain reaction

Genes	Primer sequences
CYP7A1	Forward 5' TTTGGGGAATTGCCGTGTTG 3' Reverse 5' ACGGAATCAACCCGTTCTCC 3'
HMGCR	Forward 5' GCGGCAGCTTGAGATCAT 3' Reverse 5' AGCTCAGCCATTTTGCCAG 3'
GAPDH	Forward 5' GGCAAGTTCAACGGCACAG 3' Reverse 5' GCCAGTAGACTCCACGACAT 3'

GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; HMGCR: 3-hydroxy-3-methylglutaryl coenzyme A reductase; CYP7A1: Cholesterol 7α-hydroxylase

for 1 h at 4°C overnight. Then, they were washed thrice with TBST buffer. Electrochemiluminescence working fluid was dripped onto the membranes for 1–2 min for the chemiluminescence, the residual liquid was removed, and the images were analyzed by a gel imaging system, in which the optical density of target bands was measured for calculating the expression levels of the proteins.

Statistical analysis

The data were analyzed using SPSS 20.0 software (IBM SPSS Inc., Chicago, IL, USA) and represented as mean ± standard deviation. The data among multiple groups were compared using one-way analysis of variance and those between two groups were compared using the least significant difference *t*-test, in which a value of $P < 0.05$ was considered a statistically significant difference.

RESULTS

Effects of 1,8-dihydroxyanthraquinone on blood lipid contents

According to the results, the serum TC, TG, and LDL-C levels and AI of mice were significantly lower than those of MOD group ($P < 0.05$ or $P < 0.01$), whereas the serum HDL-C levels were significantly higher ($P < 0.05$) in MOD + DHAQ group than that of MOD group. The serum TC, TG, and LDL-C levels and AI of mice in CON + DHAQ group were not significantly different from those in CON group ($P > 0.05$), indicating that DHAQ had no significant effect on blood lipids of healthy mice [Figure 2].

Effect of 1,8-dihydroxyanthraquinone on the body weight of mice

Our results [Figure 3] showed that the initial body weight of mice was not significantly different among the groups ($P > 0.05$); with the extension of feeding days, the body weight of mice in MOD group and MOD + DHAQ group in which the mice were fed with the high-fat diet increased faster than that in CON group; on days 21 and 42 after the feeding, compared with that in CON group, the body weight of mice was significantly increased in MOD group ($P < 0.05$), whereas compared with that in MOD group, the body weight of mice was significantly decreased in MOD + DHAQ group ($P < 0.05$).

Effect of 1,8-dihydroxyanthraquinone on fat/body ratio in mice

The results in Figure 4 showed that the fat mass and Lee's index of hyperlipidemic mice were significantly decreased in MOD + DHAQ group ($P < 0.05$) compared with those in MOD group.

Effects of 1,8-dihydroxyanthraquinone on liver lipid contents and liver pathological changes in mice

Figure 5 shows that compared with those in the CON group, TC and TG levels in the liver tissue of mice were significantly increased in the MOD group ($P < 0.01$). On the 6th week after the administration of DHAQ, TC and TG levels in the liver tissue of mice in MOD + DHAQ group were significantly decreased ($P < 0.01$). The hematoxylin and eosin (H and E) staining of liver tissue showed that in CON group, the structure of liver lobules was complete; the shape of liver cells was round; the nucleus was located at the center; the cytoplasm was stained evenly; and the structure of portal area around the lobules was normal. However, in MOD group, the structure of hepatic lobules was disordered; the central vein of lobules was dilated; the volume of liver cells was increased; and fat vacuoles were visible in the cytoplasm. In contrast to the MOD group, the structure of hepatic lobules was significantly improved, almost recovered to normal and the steatosis of intralobular hepatocytes was significantly alleviated in MOD + DHAQ group, suggesting that DHAQ mitigates the lipid accumulation in the liver tissue of hyperlipidemic mice.

Effects of 1,8-dihydroxyanthraquinone on the expression of genes responsible for lipid metabolism in the liver

CYP7A1 can convert cholesterol into bile acids in hepatocytes, leading to the decrease in the level of cholesterol in hepatocytes. Therefore, the expression of CYP7A1 was detected at the mRNA and protein levels in the liver tissue of mice. Our results showed that CYP7A1 mRNA and protein expression levels in the liver tissue of hyperlipidemic mice in MOD + DHAQ group were significantly increased compared with those in the MOD group ($P < 0.05$) [Figure 6]. HMGCR is a key

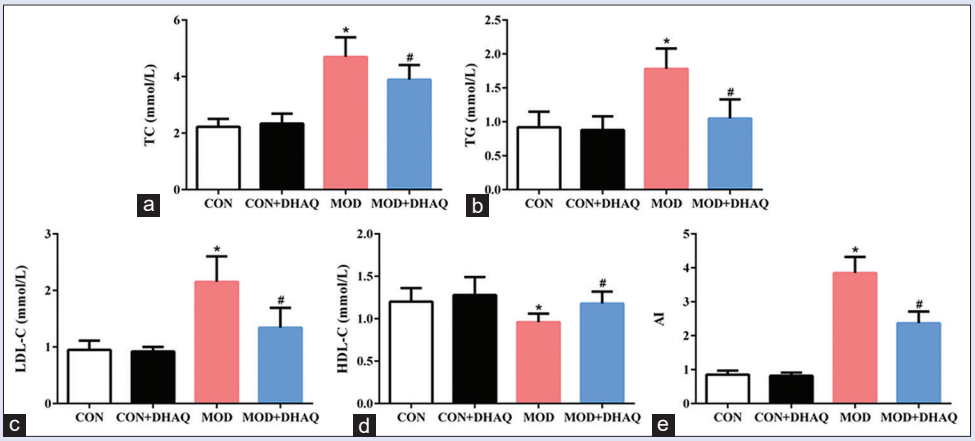

Figure 2: Effects of 1,8-dihydroxyanthraquinone on the serum total cholesterol (a), triglyceride (b), low-density lipoprotein cholesterol (c), high-density lipoprotein cholesterol (d) and atherosclerosis index (e) levels in hyperlipidemic rat. *$P < 0.05$, versus control group; #$P < 0.05$, versus model group

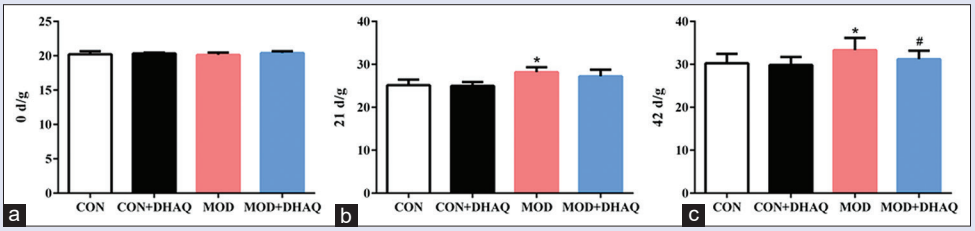

Figure 3: Effect of 1,8-dihydroxyanthraquinone on the body weight of mice on day 0 (a), 21 (b) and 42 (c) after the feeding with the high-fat diet. *$P < 0.05$, versus control group; #$P < 0.05$, versus model group

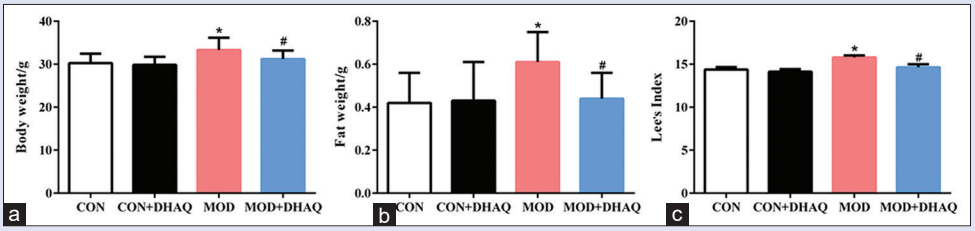

Figure 4: Effect of 1,8-dihydroxyanthraquinone on the body weight (a), fat weight (b) and Lee's index (c) in hyperlipidemic rat. *$P < 0.05$, versus control group; #$P < 0.05$, versus model group

enzyme responsible for the synthesis of cholesterol in the liver. Our results demonstrate that the high-fat diet might induce the expression of HMGCR in the liver tissue of mice, and the expression levels of HMGCR mRNA and protein in the liver tissue of mice were significantly decreased in the MOD + DHAQ group ($P < 0.05$) [Figure 6]. These results suggest that DHAQ plays an important role in regulating the lipid metabolism by promoting the conversion of cholesterol into bile acids and inhibiting the cholesterol synthesis in the liver of hyperlipidemic mice.

DISCUSSION

C. obtusifolia has been used as an edible and medicinal herb for a long time, and it is often consumed in the form of tea, with a significant hypolipidemic effect. Anthraquinones are the primary active components of *C. obtusifolia*. Li *et al.*[16] found that *C. obtusifolia* anthraquinones might significantly reduce the level of TC, TG, and LDL-C and increase the level of HDL-C in the serum of experimental hyperlipidemia rats, and the inhibition of intracellular cholesterol synthesis might be one of its hypolipidemic mechanisms. Mei *et al.*[17] confirmed that cholesterol-lowering probiotics in combination with *C. obtusifolia* anthraquinone might significantly reduce the blood lipid level and the liver lipid deposition in rats with non-alcoholic fatty liver disease (NAFLD). The anthraquinones in *C. obtusifolia* are mainly chrysophanol, emodin, rhein, emodin methyl ether (physcion), obtusifolin, aloe emodin, chrysoobtusin, obtusin, and aurantioobtusin,[18] but there is no report on which component is the active monomer. It has been reported that DHAQ is one of the most active anthraquinones.[15] Our results showed that DHAQ might significantly reduce the body weight, fat weight, Lee's index, and liver index of obese mice induced by high-fat diet; regulate the level of TC, TG, HDL-C, and LDL-C in the serum of mice; and reduce the liver lipid deposition, indicating that DHAQ has a good hypolipidemic effect.

Hypercholesterolemia is one of the most important risk factors for atherosclerosis and related diseases, and lowering blood cholesterol level is very important to prevent cardiovascular diseases.[19] *C. obtusifolia*

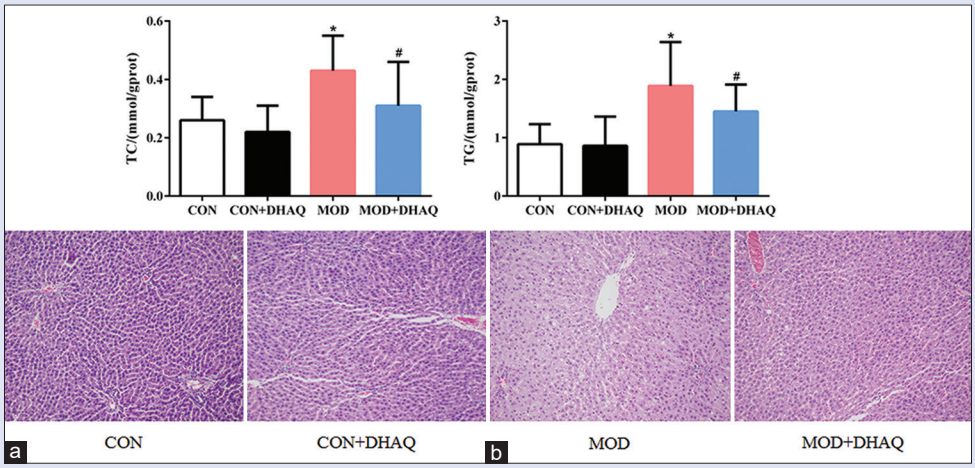

Figure 5: Effect of 1,8-dihydroxyanthraquinone on the total cholesterol (a) and triglyceride (b) levels and histomorphological changes in the liver. *$P < 0.05$, versus control group; #$P < 0.05$, versus model group

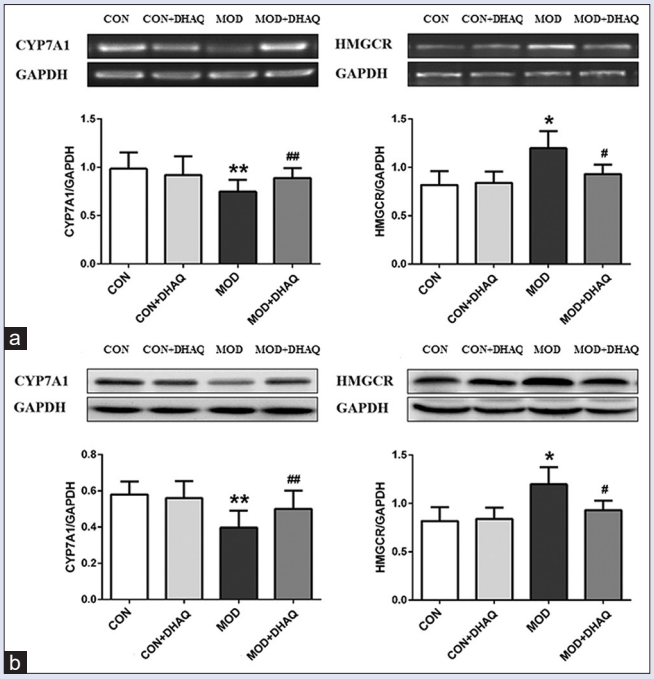

Figure 6: Effects of 1,8-dihydroxyanthraquinone on the expression of hepatic cholesterol-related synthetic factors in hyperlipidemic rat.(a) mRNA expressions of CYP7A1 and 3-hydroxy-3-methylglutaryl coenzyme A reductase. (b) Expressions of CYP7A1 and 3-hydroxy-3-methylglutaryl coenzyme A reductase proteins. *$P < 0.05$, **$P < 0.01$, versus control group; #$P < 0.05$, ##$P < 0.01$, versus model group

anthraquinones can inhibit the synthesis of cholesterol in cells, and combined with cholesterol-lowering probiotics, anthraquinones can upregulate the expression of key factors of liver cholesterol metabolism, such as CYP7A1 and LDL receptor, and downregulate the expression of HMGCR and peroxisome proliferator-activated receptor (PPAR-γ) in NAFLD rats.[16,17] Our results also indicated that DHAQ, the primary active substance of anthraquinones, might significantly decrease the abnormally elevated serum TC and LDL-C levels and improve the disorder of cholesterol metabolism induced by high-fat diet in mice. HMGCR, a key enzyme in the cholesterol synthesis of liver, can transform hydroxymethylglutaryl coenzyme A into mevilonic acid (MVA), which is the rate-limiting step in the synthesis of cholesterol in the liver.[20] The primary mode for the body to clear excess cholesterol is to convert the cholesterol into bile acid in the liver and excrete it out of the body. Cholesterol is converted into 7α-hydroxycholesterol by CYP7A1 and the subsequent enzymatic reactions catalyze the conversion of 7α-hydroxycholesterol into bile acid, in which CYP7A1 is the key enzyme of cholesterol catabolism.[21] Therefore, HMGCR and CYP7A1 play an important role in maintaining the homeostasis of cholesterol. To explore the mechanism of regulating cholesterol metabolism of DHAQ, the expression of HMGCR and CYP7A1 were detected in the liver tissue of mice in this study. Our results showed that after the administration of DHAQ for 6 weeks, the expression of HMGCR and CYP7A1 in the liver tissue of mice decreased significantly, suggesting that DHAQ could not only inhibit the synthesis of cholesterol under *in vivo* conditions but also promote the cholesterol to be converted into bile acid, and then excreted out of the body, thus regulating the lipid metabolism.

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Conflicts of interest

There are no conflicts of interest.

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