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Celastrus orbiculatus Thunb. ameliorates high-fat diet-induced non-alcoholic fatty liver disease in guinea pigs

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Celastrus orbiculatus Thunb. (COT) is a traditional Chinese herb. In this study, an experiment was designed to investigate the potential protective effect of COT on the development of non-alcoholic fatty liver disease (NAFLD) induced by high fat diet and to explore the underlying mechanisms. We established a guinea pig model of NAFLD and treated the animals with three doses of COT or 20 mg/kg/d simvastatin (a positive control drug) for 8 weeks. H&E staining of liver tissue sections indicated that COT remarkably improved histopathological change of liver induced by high fat diet. Serum biochemical assays revealed that COT significantly decreased ALT and AST activities in serum. Besides, COT also reduced body weight and liver weight of guinea pigs under high fat diet. Hepatic lipid analysis showed that COT remarkably decreased the contents of total cholesterol (TC), free cholesterol (FC), cholesterol ester (CE) and triglyceride (TG) in liver of guinea pigs fed high fat diet in a dose-dependent manner. The analysis of hepatic genes involved in cholesterol metabolism by quantitative real-time PCR revealed that COT upregulated the mRNA abundance of cholesterol 7α-hydroxylase A1 (CYP7A1) and 3-hydroxy-3-methyl-glutaryl-CoA reductase (HMGCR). Measurement of biochemical parameters in liver indicated that COT attenuated oxidative stress and lowered NO and iNOS levels in guinea pigs under high fat diet. These results reveal that administration of COT effectively ameliorates high-fat diet-induced NAFLD in guinea pigs through decreasing hepatic lipid levels, suppressing oxidative stress and lowering NO and iNOS levels in liver.

1. Introduction

Non-alcoholic fatty liver disease (NAFLD) is the most important cause of chronic liver disease (Bosetti et al. 2007). Although a relatively benign condition in some cases, in others the disease can progress to cirrhosis or even hepatocellular carcinoma (HCC) (Lam and Younossi 2009). Moreover, a large number of studies have suggested that NAFLD significantly increases the risk of cardiovascular diseases (Ioannou et al. 2006). NAFLD is a metabolic disease and, in most developed and developing countries, is highly coincident with dyslipidemia, obesity, metabolic syndrome, and type 2 diabetes (Fan and Farrell 2009). Its pathogenesis involves numerous metabolic and inflammatory pathways and remains incompletely understood. However, the central role of hepatic lipid accumulation has been confirmed in clinical correlation studies and animal models (Petta et al. 2009). Thus, decreasing serum and hepatic lipid levels is crucial to the prevention of NAFLD. Up to now, no particular treatment has been discovered to be safe and highly effective. The only accepted form of management is to increase physical activity, accompanied with weight reduction through lifestyle modification and dietary change. Therefore, there is a requirement for effective pharmacotherapy in NAFLD treatment (Gossard and Lindor 2011).

Traditional Chinese medicine (TCM) has been used to treat liver disease in China since ancient times. Since NAFLD was described in the 1980 s, lots of clinical trials about TCMs for treating NAFLD have been performed in China. Results from these trials suggested that some TCMs indeed are effective for the treatment of NAFLD (Shi et al. 2012). *Celastrus orbiculatus* Thunb. (COT) is a member of the celastraceae family. Its stem, root, leaf, fruit and seed can all be used as Chinese herbs. This plant has been proved to elicit a variety of biological effects through its anti-oxidative and anti-inflammatory properties in the treatment of autoimmune diseases, chronic inflammation and neurodegenerative diseases (Jin et al. 2002). Moreover, our team has found that COT decreases athero-susceptibility by lowering plasma lipids in a previous study. However, the effect of COT on NAFLD, was unknown so far.

In the present study we selected guinea pigs as animal model to test the therapeutic effects of COT on high-fat diet-induced NAFLD. Guinea pigs are useful to investigate lipid metabolism

Table 1: Effect of COT and simvastatin on body weight, liver weight and liver index of guinea pigs after treatment for 8 weeks

Group	Body weight (g)	Liver weight (g)	Liver index (%)
CD	360.25±20.17	5.27±1.39	1.46±0.15
HFD	478.79±30.99**	10.12±2.68**	2.11±0.20*
HFD-L	419.33±27.75$^{\#}$	8.98±2.14$^{\#}$	2.14±0.16
HFD-M	387.1±44.24$^{\#\#}$	6.65±1.65$^{\#}$	1.72±0.18$^{\#}$
HFD-H	402.35±28.77$^{\#\#}$	8.03±2.11$^{\#}$	2.00±0.21
HFD-S	374.43±57.14$^{\#\#}$	6.64±1.89$^{\#}$	1.77±0.23$^{\#}$

Data are presented as mean ± SD (n = 8). $^{*}P < 0.05$, $^{**}P < 0.01$ versus CD group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ versus HFD group. Liver index is liver weight/body weight.

and NAFLD. Like humans, guinea pigs are one of the few species that carry the majority of cholesterol in LDL. This study may provide new insight into treatment options for NAFLD.

2. Investigations and results

2.1. COT significantly reduced body weight and liver weight of guinea pig under high fat diet

At the beginning of the study, all guinea pigs were divided into 6 groups randomly and the initial body weight showed no significant difference among these groups. After a high fat diet for 8 weeks, the final body weight, liver weight and liver index were, however, significantly different among these groups. As shown in Table 1, the three parameters of HFD group were all remarkably increased compared with that of the CD group ($P < 0.01$). Compared with the HFD group, COT reduced body weight by 12.4%, 19.2% and 16.0% in HFD-L, HFD-M and HFD-H group respectively and simvastatin reduced body weight by 21.8%. Compared with the HFD group, COT also reduced liver weight by 11.3%, 34.3% and 20.7% in the HFD-L, HFD-M and HFD-H groups respectively and simvastatin reduced liver weight by 34.4%. In addition, middle dosage (5 g crude drug/kg/d) of COT and simvastatin (20 mg/kg/d) decreased the liver index compared with the HFD group.

2.2. COT ameliorated the pathological change of liver and reduced liver injury in guinea pigs under high fat diet

To assess histological changes, H&E staining of liver tissue sections from each group was examined. The CD group showed a normal lobular architecture with central veins and radiating hepatic cords (Fig. 1A). After high fat diet for 8 weeks, a large amount of lipid droplets appeared in hepatocytes and denaturation even necrosis presents in parts of hepatocytes in HFD group. The pathological change was significantly attenuated by COT in a dose-dependent manner. The effect of high dosage of COT was similar to that of simvastatin.

To evaluate the degree of liver injury, we carried out an analysis of serum ALT and AST activities. Our study indicated that a high fat diet increased serum ALT and AST by 3.4 and 2 fold respectively compared with CD group, whereas COT and simvastatin reversed the increase. Compared with the HFD group, COT decreased ALT activity by 40%, 53% and 58% in HFD-L, M and H groups, respectively and simvastatin decreased it by 62%. Compared with the HFD group, COT also decreased AST activity by 4%, 26% and 31% in the HFD-L, and H groups, respectively and simvastatin decreased it by 51%. This result was consistent with morphologic changes observed in the liver.

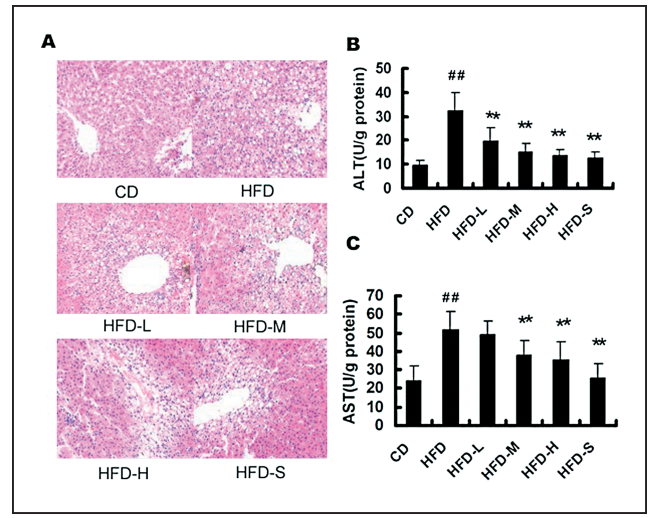

Fig. 1: COT and simvastatin ameliorated the pathological change of liver and lessened liver injury in guinea pigs fed high fat diet. The histological change of liver was displayed by HE staining and the status of liver injury was reflected by the activities of ALT and AST in serum. (A) shows the representative HE staining sections of hepatic tissue of various groups. (B) and (C) show the activities of ALT and AST in serum respectively. Data are presented as mean ± SD (n = 8). $^{\#\#}P < 0.01$ versus CD group; $^{**}P < 0.01$ vs HFD group.

2.3. COT modulated cholesterol metabolism and attenuated significantly lipid deposition in the liver of guinea pigs under high fat diet

As shown in Fig. 2, after high fat diet for 8 weeks, the levels of TG, TC, FC and CE in the liver were significantly increased in the HFD group compared to the CD group ($P < 0.01$). Compared with the HFD group, COT lowered TC, FC and CE levels in livers in a dose-dependent manner and the effect of high dose COT was

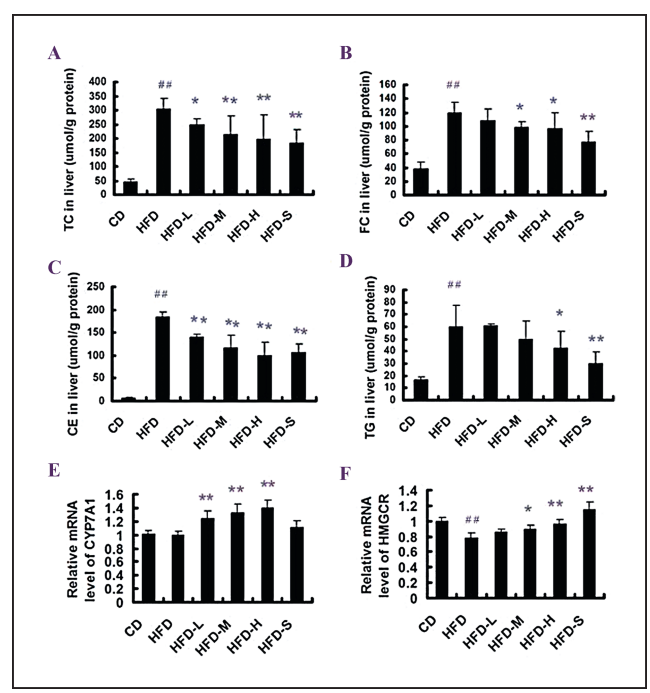

Fig. 2: COT and simvastatin modulated cholesterol metabolism and attenuated significantly lipid deposition in liver of guinea pig fed high fat diet. The levels of TC (A), FC (B), CE (C) and TG (D) in liver of guinea pigs were determined by enzyme method after 8 weeks' treatment. The mRNA abundance of CYP7A1 (E) and HMGCR (F) in liver, which were analyzed by quantitative real-time PCR, were calculated after adjusting for β-actin using the $2 - \Delta\Delta Ct$ method. Data are presented as mean ± SD (n = 8). $^{\#\#}P < 0.01$ versus CD group; $^{*}P < 0.05$, $^{**}P < 0.01$ versus HFD group.

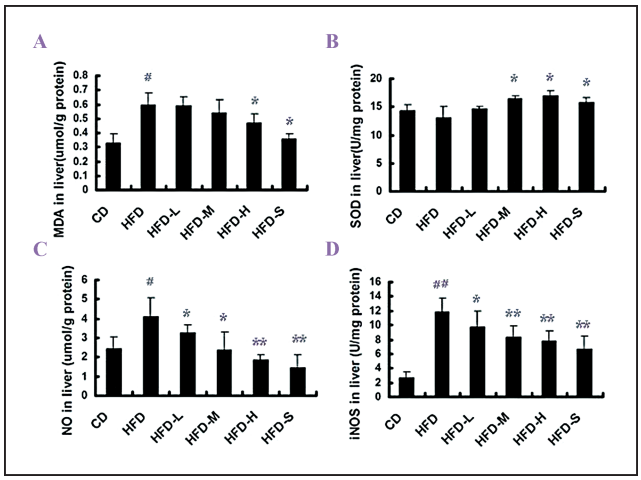

Fig. 3: Effect of COT and simvastatin on the levels of MDA, SOD, NO and iNOS in liver of guinea pigs fed high fat diet. (A) shows the content of MDA in liver. (B) shows the activity of SOD in liver. (C) shows the content of NO in liver. (B) shows the activity of iNOS in liver. Data are presented as mean ± SD (n = 8). $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ versus CD group; $^{*}P < 0.05$, $^{**}P < 0.01$ versus HFD group.

similar to that of simvastatin (Fig. 2A, B and C). Besides, high doses of COT and simvastatin also decreased TG levels by 28% and 47% respectively compared with HFD group (Fig. 2D).
Cholesterol synthesis in the liver includes 30 steps of enzymatic reactions, in which HMGCR is a rate-limiting enzyme. Compared with that of the CD group, the mRNA level of HMGCR was significantly decreased in the HFD group ($P < 0.01$). Compared with the HFD group, COT up-regulated the HMGCR mRNA levels in a dose–dependent manner and simvastatin further increased the level by 46% and its effect was more significant than that of COT($P < 0.01$) (Fig. 2F). Another important step in cholesterol metabolism is the conversion of cholesterol into bile acid through cytochrome P450-meidiated oxidation in liver. The rate-limiting enzyme for the dominant pathway of bile acid synthesis is cytochrome P450 7A1 (CYP7A1). As shown in Fig. 2E, COT significantly up-regulated the CYP7A1 mRNA level by 25%, 34% and 41% in the HFD-L, M, H groups, respectively, while simvastatin had no significant influence on its level.

2.4. COT suppressed oxidative stress and reduced NO and iNOS levels in hepatic tissue of guinea pigs under high fat diet

MDA, an end product of lipid peroxidation, is one of the most reliable and widely used indices of oxidative stress. The toxic effects of reactive oxygen species (ROS) can be reduced by antioxidants such as superoxide dismutase (SOD) (Seddon et al. 2007). In our study, we determined the levels of MDA and SOD in liver. Compared with that of the CD group, the level of MDA was significantly increased, whereas the activity of SOD did not change in the HFD group. Compared with the HFD group, high doses of COT and simvastatin significantly lowered the MDA levels by 23% and 43% respectively (Fig. 3A). Meanwhile, compared with that of the HFD groups, the activity of SOD was promoted by 27%, 31% and 17% in HFD-M, H and S group respectively (Fig. 3B).
NO levels and iNOS activity in liver were also determined in the study. The increase of these parameters can result in inflammation and liver damage. As shown in Fig. 3C and 3D, NO levels and iNOS activity in the liver were increased in the HFD group compared with CD group ($P < 0.01$), whereas COT and simvastatin reversed the increase.

Table 2: Contents of total flavonoids and its components (rutin, kaempferol and quercetin) from stem of COT

Item	Content in crude drug (mg/g)	Content in concrete (mg/g)
Total flavonoids	7.46 ± 0.22	84.60 ± 0.22
Rutin	1.80 ± 0.32	20.41 ± 0.32
Kaempferol	2.80 ± 0.24	31.75 ± 0.24
Quercetin	0.0072 ± 0.15	0.082 ± 0.15

Data are presented as mean ± SD of at least three independent samples.

3. Discussion

With the growing epidemic of obesity, a great deal of attention has focused on metabolic syndrome and its associated hepatic manifestation, NAFLD. NAFLD is now recognized as an important health burden. Therefore, efficacious pharmacotherapy will rescue patients from slowly progressing disease with possible progression to irreversible changes (Siebler and Galle 2006). In this study, COT clearly protected guinea pigs from HFD-induced NAFLD, as evidenced by significant reductions in body weight and liver weight (Table 2) as well as histopathological improvement (Fig. 1). Specifically, COT decreased serum ALT and AST levels (Fig. 1), which suggested that COT improved the hepatocellular damage induced by NAFLD. Elevated serum ALT and AST levels have been used as surrogate biomarkers for NAFLD and steatohepatitis in clinical settings. When the hepatocyte is injured, cellular membrane is disrupted and the leakage of the enzyme into extracellular fluid occurs. Therefore they can be detected at abnormal levels in serum (Hennes et al. 1990).
The pathophysiology of NAFLD is still subject to intensive research and several players and mechanisms have been suggested. The most recent prevailing concept is the "multiple hit" hypothesis (McCullough 2006). The first hit is a reversible accumulation of fat in hepatocytes (Chitturi et al. 2002). The subsequent hits involve a combination of oxidative stress, lipid peroxidation, cell death and pro-inflammatory cytokines (Anstee and Goldin 2006). A retention of lipids within hepatocytes is a prerequisite for the development of the disease. In this study, we discovered for the first time that COT could significantly lower TC, FC, CE and TG levels in liver of guinea pig under high fat diet.
The chelesterol level in liver is determined by the balance between production and clearance. On one hand, cholesterol can be derived from food intake or hepatic biosynthesis. On the other hand, cholesterol can be converted into bile acid in the liver and further excreted into the intestine for elimination from the body with feces. CYP7A1 is the rate-limiting enzyme of the conversion of cholesterol into bile acid. In this study, our data indicated the mRNA abundance of CYP7A1 in the liver was increased by COT, therefore, we hypothesize that the lipid-lowering effect of COT probably is related to a promotion of cholesterol clearance through the up-regulation of CYP7A1 expression. In addition, compared with the HFD group, COT slightly increased the level of HMGCR mRNA, a rate-limiting enzyme of cholesterol biosynthesis. This was probably caused by a negative feedback mechanism owing to the decrease of cholesterol content in the liver. Simvastatin can inhibit the conversion of HMG-CoA into mevalonate by competitive blocking of HMGCR and the endogenous cholesterol synthesis in the liver is remarkably reduced. Meanwhile, inhibition of cholesterol synthesis should cause an overexpression of HMGCR as a result of a feedback-regulatory mechanism (Hampton et al. 1996). Similar to previous studies, our findings also indicated that simvastatin up-regulated HMGCR mRNA levels in the liver. However, simvastatin has no effect on CYP7A1 mRNA levels.

Oxidative stress is considered a key event in the progression of NAFLD (Koek et al. 2011). It is mirrored by the increase of lipid peroxidation products (i.e., MDA) in patients with NAFLD and in animal models (Videla et al. 2004; Serviddio et al. 2008; Gaemers et al. 2011). The toxic effects of ROS can be inhibited by antioxidants such as SOD (Sanyal et al. 2010). Under our experimental conditions, the high oxidative damage induced by a 8-week high fat diet was confirmed by a the increase of MDA. However, COT and simvastatin significantly lowered MDA content and increased SOD activity in the liver. Similar to previous studies, our findings indicated that COT has antioxidative properties.

Besides ROS, reactive nitrogen species (RNS) also contribute to hepatocyte damage and inflammatory cells activation (Lirussi et al. 2007). Among RNS, peroxynitrite, which is generated from NO and the anion superoxide, determines cell injury by protein oxidation and nitrosylation, which causes dysfunction of several enzymes including the components of mitochondrial respiratory chain (Trujillo et al. 2008). NO is produced from L-arginine by any of three NO synthases (NOS), two of them are of constitutive type, including neuronal type (nNOS) and endothelial type (eNOS) and one is inducible (iNOS) (Forstermann et al. 1994). McKim et al. (2003) reported that iNOS was required for the pathogenesis of early alcohol-induced hepatitis by production of nitric oxide-derived peroxynitrite. In our study, COT reduced iNOS activity and suppressed NO synthesis in hepatic tissue of NAFLD. These data indicates that the decrease of ROS and RNS may be an important mechanism for COT to prevent NAFLD.

In summary, our data showed that administration of COT effectively ameliorates high-fat diet-induced NAFLD in guinea pigs, as proved by histopathological improvement, decrease of serum ALT and AST activities and significant reduction in body and liver weight. The preliminary exploration of the underlying mechanisms indicates that COT protects against hepatic injury by targeting several pathways: 1) COT significantly lessens lipid deposition in liver by modulating cholesterol metabolism; 2) COT suppresses oxidative stress in liver; 3) COT lowers NO and iNOS levels in liver. It can be inferred that COT administration may be a useful therapeutic herb for avoiding or treating NAFLD. However, extracting the effective components of COT and testing their pharmacological function need to be explored further so as to determine the relevance of COT in preventing NAFLD.

4. Experimental

4.1. Plant material

The stems of COT were collected from Mountain Tai in China and identified by Professor Li Tongde in School of Pharmacy, Taishan Medical University. The dried stems were minced using a grinder.

4.2. Preparation of COT extract and determination of medical components

The powder (500 g) was soaked with 55% ethanol (7500 ml) overnight. The combined extract was filtered and concentrated into concrete (11.34 g crude drug/g). Then the concrete was diluted to solutions of 2.5, 5.0, 10.0 g crude drug/ml with 5% sodium carboxymethylcellulose and stored at 4 °C. Meanwhile, the medical components of concrete were determined. The content of total flavoids was detected by ultraviolet absorption spectrophotometer (UAS) and its components (kaempferol, rutin and quercetin) were detected by capillary zone electrophoresis (CZE) method. The result was shown in Table 2.

4.3. Animals and treatments

Forty eight male England short-hair guinea pigs (260∼310 g, 5 months old) were purchased from the experimental animal center of Taibang Biological Products Co., Shandong, China. All experiments were approved by the laboratory animals' ethical committee of Taishan Medical University and followed national guidelines for the care and use of animals. All guinea pigs were randomly allocated into 6 groups: regular chow diet group (CD), high fat diet group (HFD, 10% lard+ 10% yolk power+ 0.30% cholesterol + 79.7% grass), HFD with low dosage of COT group (HFD-L, HFD + 2.5 g crude drug/kg/d), HFD with middle dosage of COT group (HFD-M, HFD + 5.0 g crude drug/kg/d), HFD with high dosage of COT (HFD-H, HFD + 10.0 g crude drug/kg/d) and HFD with simvastatin group (HFD-S, HFD + 20 mg/kg/d). Every group is composed of 8 guinea pigs. COT and simvastatin were dissolved in 5% sodium carboxymethylcellulose with the concentrations of 2.5, 5.0, 10.0 g COT crude drug/mL and 20 mg simvastatin/mL respectively. The drugs (COT or simvastatin) and vehicle (5% sodium carboxymethylcellulose) were given by oral gavage once daily for 8 weeks.

At the end of 8 weeks, animals were put on an overnight fast, and anesthetized with ketamine hydrochloride intravenously prior to sacrifice. Serum samples were collected into heparinized tubes (50 U/mL). Liver samples were dissected out and washed immediately with an ice-cold saline to remove as much blood as possible. One part of the liver samples was immediately stored at − 80 °C for future analysis, another part was excised and fixed in 10% formalin solution for histopathologic analysis.

4.4. Activity testing of COT on NAFLD

4.4.1. Alanine transferase (ALT) and aspartate transferase (AST) activities in serum

In order to show the status of liver injury, the activities of ALT and AST in serum were detected by UV-lacate dehydrogenase method and UV-malate dehydrogenase method respectively on an automatic biochemical analysis instrument (7600-020, Hitachi) according to the manufacturer's instructions (Nanjing Jiancheng Bioengineering Institute, China).

4.4.2. Pathological examination of liver (H&E staining)

A portion of liver tissue that was instantly fixed in 10% phosphate buffered formalin, was processed by routine histology procedures and then embedded in paraffin. Tissue sections (5 μm) were stained with haematoxylin and eosin (H&E) for histopathological examination.

4.4.3. Measurement of hepatic lipid

For quantification of hepatic triglyceride and cholesterol levels in the liver, liver tissues were homogenized and homogenates were centrifuged at 1000 × g for 15 min. The supernatants were used for the assay of hepatic lipid. Concentrations of total cholesterol (TC), free cholesterol (FC) and triglyceride (TG) were determined by enzymatic methods according to the manufacturer's instructions (Applygen Technologies Inc., China). cholesterol ester (CE) was calculated as TC minus FC.

4.4.4. Malondialdehyde (MDA), superoxide dismutase (SOD), nitric oxide (NO) and induced NO synthase (iNOS)

Liver tissues were homogenized on ice. Homogenates were centrifuged at 1000 × g for 15 min at 4 °C. The supernatants were used immediately for further assays. The contents of MDA and NO and the activities of SOD and iNOS were determined by using commercially available kits (Nanjing Jiancheng Bioengineering Research Institute, Nanjing, China) according to the manufacturer's instructions.

4.4.5. Quantitative real-time PCR

A RT-PCR assay was used to detect the expression of 3-hydroxy-3-methylglutaryl- CoA reductase (HMGCR) and cholesterol 7α-hydroxylase A1 (CYP7A1) in the liver. These are hepatic key enzymes of cholesterol metabolism. Primer Design 4.1 Software was used to design the followings primers: β-actin: forward primer:5'-TTACTA CTTTGCTGCGTTACACC-3', reverse primer:5'-CATGCCAATCTCATCTCGTTT -3'(length of 78 bp); CYP7A1: forward primer:5'-CAGTATGCTGCTGTTTATG-3', reverse primer:5'-GTTCTCGGTGGTGTTTCC -3'(length of 335 bp); HMGR: forward primer:5'-TGATAGCACCAGCAGATT-3', reverse primer:5'-TATAAAGG TTGCGTCCAG-3'(length of 68 bp). The primers were synthesized by TaKaRa. Total liver RNA was isolated by TRIZOL Reagent (*Invitrogen*). cDNA synthesis was performed using MuLV Reverse Transcriptase (Applied Biosystems). Real-time PCR was performed using a SYBR-green PCR master mix kit (TianGen Biotech). The data was analyzed by using Rotor-gene Q software ver.1.7(Qiagen). Relative mRNA levels were calculated by the $2^{-\Delta\Delta Ct}$ method and normalized against β-actin. Each experiment was repeated three times.

4.5. Statistical analysis

Results are shown as the mean ± SD for at least three independent experiments. Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by Student–Newmann–Keuls multiple comparison tests with the SPSS 13.0 software for Windows. P-values less than 0.05 were considered statistically significant.

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