Original Research

Ethanol extract of Zhongtian hawthorn lowers serum cholesterol in mice by inhibiting transcription of 3-hydroxy-3-methylglutaryl-CoA reductase via nuclear factor-kappa B signal pathway

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Abstract

Hawthorn is a berry-like fruit from the species of *Crataegus*. In China, it has another more famous name, Shan-Zha, which has been used to improve digestion as a traditional Chinese medicine or food for thousands of years. Moreover, during the last decades, hawthorn has received more attention because of its potential to treat cardiovascular diseases. However, currently, only fruits of *C. pinnatifida* and *C. pinnatifida* var. major are included as Shan-Zha in the Chinese Pharmacopoeia. In this study, our results showed that the ethanol extract of Zhongtian hawthorn, a novel grafted cultivar of *C. cuneata* (wild Shan-Zha), could markedly reduce body weight and levels of serum total cholesterol, triglyceride, low-density lipoprotein cholesterol, and liver cholesterol of hyperlipidemia mice. It could suppress the stimulation effect of high-fat diet on the transcription of 3-hydroxy-3-methylglutaryl-CoA reductase (HMGCR) and p65, and counteract the downregulation of CYP7A1 and LDLR. In addition, the results of luciferase reporter assay and Western blot showed that the transcriptional activity of HMGCR promoter was inhibited by Zhongtian hawthorn ethanol extract in a dose-dependent manner, while overexpression of p65 could reverse this transcriptional repression effect. These results suggested that Zhongtian hawthorn could provide health benefits by counteracting the high-fat diet-induced hypercholesteolemic and hyperlipidemic effects *in vivo*, and the mechanism underlying this event was mainly dependent on the suppressive effect of Zhongtian hawthorn ethanol extract on the transcription of HMGCR via nuclear factor-kappa B (NF-κB) signal pathway. Therefore, this novel cultivar of hawthorn cultivar which has much bigger fruits, early bearing, high yield, cold resistance, and drought resistance, might be considered as a good alternative to Shan-Zha and has great value in the food and medicine industry. In addition, to our best knowledge, this is also the first report that the extract of *Crataegus* could suppress the transcription of HMGCR via NF-κB signal pathway.

Keywords: Zhongtian hawthorn, hypolipidemic, hypocholesteolemic, 3-hydroxy-3-methylglutaryl-CoA reductase, transcriptional regulation, nuclear factor-kappa B

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Introduction

The incidence of hyperlipidemia and hypercholesterolemia is increasing rapidly with the improvement of people's living standard and alteration of lifestyle. Elevated serum lipids, lipoproteins, and cholesterol levels are generally considered to be the most important risk factor for the development of cardiovascular diseases (CVDs). It has been reported that even a 1% decrease in serum cholesterol levels could lower the risk of coronary heart disease by up to 3%.[1] Therefore, lowering serum cholesterol levels is one of the effective means for the prevention of CVDs. Up to now, many cholesterol lowering drugs (such as statins) have been widely used in clinical practice.[2] However, because of the high prices and side effects of these traditional drugs, it is still attractive to develop more effective and safer alternative therapies to lower serum cholesterol.

Hawthorn is a berry-like fruit from the species of *Crataegus*, which is widely distributed in the temperate regions in Asia, Europe, and North America.[3] In China, it has another more famous name, Shan-Zha, which has been documented as a food and traditional Chinese medicine (TCM) for thousands of years. Bing Tang Hu Lu, a very popular traditional Chinese folk snack, is just made by

dipping a string of hawthorns into melted malt sugar. Besides, Shan-Zha is also a well-known TCM which is usually used to improve digestion.[4,5] Furthermore, during the last decades, hawthorn has received more attention because of its activity in the treatment of CVDs, including reduction of plasma cholesterol and triacylglycerol concentrations, improvement of heart function, dilate blood vessels, and treatment of heart arrhythmia.[3,6–10]

There are different varieties of *Crataegus*. The European pharmacopoeia states that *C. monogyna* Jacq. (Lindm.) and *C. laevigata* (Poiret) D.C. (*C. oxyacanthoides* Thuill.) may be used for the treatment of CVDs. The American Herbal Pharmacopoeia and the United States Pharmacopoeia allow for the use of *C. monogyna* Jacq. (Lindm.) and *C. laevigata* (Poiret) D.C. (*C. oxyacanthoides* Thuill.) in natural health products.[11] In the Chinese Pharmacopoeia, the Shan-Zha used in TCM remedy is predominantly *C. pinnatifida* and *C. pinnatifida* var. major.[11–13] However, because the distribution of *C. pinnatifida* and *C. pinnatifida* var. major is limited in the North China, and their fruits is small and low yield, the development and application of Shan-Zha in the modern food and medicine industry is restricted. Besides these two representatives, there are another two varieties of Shan-Zha, *C. cuneata* (wild Shan-Zha) and *C. scabrifolia* (Yunnan Shan-Zha), distributed in the Guangxi province, Yunnan province and Guizhou province of China. Although the experimental evidence of the exact health effects of either *C. cuneata* or *C. scabrifolia* still remained elusive up to now, they has also been used in the folk medicine and food in the South China for a long time.[14] Zhongtian hawthorn is a novel grafted cultivar of *C. cuneata* bred by Pro. Pan of Hezhou University.[15,16] However, so far, whether this novel hawthorn has hypolipidemic and/or hypocholesteolemic effects is still unclear. To resolve this problem, in this study, the fruits of Zhongtian hawthorn were extracted with ethanol, and then its effect on total cholesterol (TC), triglyceride (TG), high-density lipoprotein cholesterol (HDL-C), and low-density lipoprotein cholesterol (LDL-C) in serum of hyperlipidemic mice and the underlying mechanisms were investigated.

Materials and methods

Extraction of Zhongtian hawthorn fruits

Dried fruits of Zhongtian hawthorn were milled to a powder using a grinder. The same batch was used in all the experiments. For the *in vitro* experiments, 20 g of dry hawthorn fruit powder was extracted with 100 mL ethanol using soxhlet apparatus at room temperature for 1.5 h.[17] The ethanol in the obtained extract was removed using the method of vacuum distillation. Finally, about 7 g ethanol extract was obtained from 20 g of dry fruit powder.

Animal study

Sixty male mice (approximately 20 g, Experimental Animal Center of Military Medical College) were randomly divided into normal food group (NFD), high-fat group (HFD), simvastatin group (SIM+HFD, 10.0 mg/kg/d), low-dose Zhongtian hawthorn group (ZTL + HFD, 50 mg/kg/d), middle-dose Zhongtian hawthorn group (ZTM + HFD, 90 mg/kg/d), and high-dose Zhongtian hawthorn group (ZTH + HFD, 130 mg/kg/d). The doses used here were calculated and set referring to previous studies on the extract of other *Crataegus* species and the difference of body surface area between mice and human or other animals.[3,8,11,18] After being acclimatized and given free access to water and feed for one week, mice in the NFD groups were kept fed with the basal diet, while the other four groups were fed with the high-fat diet, which consisted of 78.8% basic diet, 10% lard, 10% egg yolk powder, 1% cholesterol, and 0.2% deoxycholate. During the experiment, mice were intragastrically administrated with normal saline (NFD and HFD group), simvastatin (SIM+HFD group), or the ethanol extract of Zhongtian hawthorn (ZTL, ZTM, and ZTH groups), respectively. Body weights of mice were recorded weekly, and food intake was monitored twice a week. Five weeks later, mice were fasted for 12 h, and then blood was collected from angular vein. Serum was separated with centrifugation at 1500 r/min for 30 min at 4°C. The TC, TG, HDL-C, and LDL-C were measured using assay kits (Biosino Bio-technology and Science incorporation, Beijing) according to the manufacturer's instructions. Furthermore, the atherogenic index (AI) and the antiatherogenic index (AAI) were calculated as follows: AI = TC/HDL-C - 1; AAI = HDL-C/TC.

In addition, liver tissues of mice were also collected and immediately frozen in liquid nitrogen for further analysis. All the procedures performed in the animal experiments were in accordance with the ethical standard.

RT-PCR analysis

Total RNA from livers was extracted using TRIzol and then reverse transcribed into cDNA. The mRNA level of 3-hydroxy-3-methylglutaryl-CoA reductase (HMGCR), cholesterol 7α-hydroxylase (CYP7A1), LDL receptor (LDLR), nuclear factor-kappa B (NF-κB) subunit p65, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were quantified by RT-PCR analysis using specific primers (Table 1). The conditions of PCR were as follows: 2 min at 95°C followed by 30 cycles of incubation at 94°C for 15 s, then 54–58°C for 1 min, and 72°C for 30 s. PCR products were electrophoretically separated in 2% agarose gels and the densities of the bands were analyzed with Quantity One software. To further confirm the results, real-time RT-PCR was also performed in an Applied Biosystems StepOne™ real-time PCR system. Data were shown as relative level after being normalized by GAPDH.

Western blot analysis

The proteins of livers were harvested and lysed in Radio-Immunoprecipitation Assay (RIPA) buffer with protease inhibitors. Proteins were separated on 10% sodium dodecyl sulfate (SDS)-polyacrylamide gel and transferred to Polyvinylidene Fluoride (PVDF) membranes. After being immunoblotted with specific rabbit antibodies (Abcam NO. ab174830) overnight at 4°C, the membrane was incubated with IR Dye™-800 conjugated anti-rabbit secondary antibodies for 30 min at room temperature. Specific proteins

Table 1 Primers used in the present study

Gene	Product size (bp)	Primer pair	Primer sequence (5′–3′)*	Annealing temperature (°C)
LDLR	160	Forward	GATTCAGTCCCAGGCAGCGTAT	54
		Reverse	CTTTCTTGATCTTGGCGGGTGTT	
HMGCR	206	Forward	TTATGTCTTTAGGCTTGGTC	56
		Reverse	ACTCAGGGTAATCACTTGC	
CYP7A1	199	Forward	CAGAAGCATAGACCCAAGTGAT	58
		Reverse	TCGGTAGCAGAAGGCATACATC	
HMGCR-promoter	1459	Forward	CGTCGCTAGCGGTCTTACACTGAAGCAGT	58
		Reverse	CGTCAAGCTTGAGCCTTCGACCAATAAGA	
P65	153	Forward	CCAGACCAACAACAACC	58
		Reverse	TCCCGTGAAATACACCT	
GAPDH	201	Forward	ATTCAACGGCACAGTCAAGG	54
		Reverse	GCAGAAGGGGCGGAGATGA	

CYP7A1: cholesterol 7α-hydroxylase; GAPDH: glyceral-dehyde-3-phosphate dehydrogenase; HMGCR: 3-hydroxy-3-methylglutaryl-CoA reductase; LDLR: LDL receptor.
*Restriction enzyme sites of *Nhe*I and *Hind*III were underlined.

were visualized by Odyssey™ Infrared Imaging System (Gene Company, Li-Cor, USA). The expression of β-actin (Santa Cruz NO. sc-47778) was detected as an internal control to show equal loading of the protein samples.

Cloning of HMGCR promoter and luciferase reporter assay

HepG2 human hepatocellular carcinomas cells (ATCC NO. HB-8065) were grown in Dulbecco's modified Eagle's medium (GIBCO Gibco Ltd, Paisley, UK) supplemented with 10% fetal bovine serum (Sijiqing Biological Technology Co., Ltd, Hangzhou, China), penicillin (100 U/mL), and streptomycin (100 μg/mL) at 37°C in a 5% CO_2 incubator. The promoter (−1439∼+20) of HMGCR gene was PCR amplified from the genomic DNA of HepG2 cells using specific primers (Table 1). PCR products were inserted into pGL3 vector and then transformed into *E. coli DH5a*. The recombinant construction was analyzed by restriction enzyme digestion and sequencing to confirm sequence fidelity, and the positive recombinant plasmid was named HMGCR-luc.

To investigate the effect of the Zhongtian hawthorn ethanol extract on the transcriptional activity of HMGCR promoter, the plasmid HMGCR-luc was transfected into HepG2 cells with lipofectamine 2000 (Invitrogen, Shanghai, China). After the transfection, cells were treated with the ethanol extract of Zhongtian hawthorn in different concentrations at 6 h. Twenty-four hours later, the luciferase activity was measured on a Synergy™ 4 plate reader (Bioteck, Instruments, Winooski, VT, USA). Transfection efficiencies were normalized by total protein concentrations of each luciferase assay preparation. In addition, the effect of transfection of the p65-overexpression plasmid (a generous gift from Dr Marty Mayo of the University of North Carolina) on HMGCR promoter activity was also determined using the reporter assay. All experiments were performed at least three times with different preparations of plasmids and cells.

Statistical analysis

All experimental data were presented as the mean ± standard deviations. Data were analyzed using SPSS 17.0 statistical software (SPSS Inc., Chicago, IL, USA). Comparisons among groups were performed with one-way analysis of variance, followed by Tukey's test. Values of $P < 0.05$ were considered statistically significant.

Results

Effects of the ethanol extract of Zhongtian hawthorn on body weight of mice fed with high-fat diet

Obesity is often accompanied by hyperlipidemia. In our present study, after being fed with high-fat diet for three weeks, the body weight of mice in the HFD group became higher than that in the normal-fed group. However, administration of the ethanol extract of Zhongtian hawthorn could dose dependently inhibit the obesity induced by high-fat diet ($R^2 = 39.903$, $F = 2.896$, $P < 0.05$) (Table 2).

Effects of the ethanol extract of Zhongtian hawthorn on serum lipids, lipoproteins, and cholesterol levels of hyperlipidemic mice

Except body weight, the serum lipid indexes of mice were also detected. As shown in Figure 1(a), compared with mice in the NFD group, mice in the HFD group exhibited significant elevations in the level of serum TC, TG, LDL-C, and HDL-C. However, treatment of simvastatin (10.0 mg/kg/d) and the ethanol extract of Zhongtian hawthorn (50, 90, 130 mg/kg/d) could markedly suppress the increased serum TC ($R^2 = 4.021$, $F = 26.951$, $P < 0.01$), TG ($R^2 = 0.432$, $F = 11.362$, $P < 0.01$), and LDL-C ($R^2 = 1.998$, $F = 19.510$, $P < 0.01$) induced by high-fat diet. Besides, although the level of serum HDL-C, the well-known good cholesterol, was also increased by high-fat diet, the ratio of TG ($R^2 = 0.033$, $F = 5.241$, $P < 0.05$), TC ($R^2 = 0.172$, $F = 5.306$, $P < 0.05$), and LDL-C ($R^2 = 0.101$, $F = 7.966$, $P < 0.01$) to HDL-C were all raised in mice of HFD group, whereas all these indexes in mice treated with either simvastatin or the ethanol extract of Zhongtian hawthorn were

Table 2 Effect of the ethanol extract of Zhongtian hawthorn on body weight of mice fed with high-fat diet

	0 day (g)	7 day (g)	14 day (g)	21day (g)	28 day (g)	35day (g)
NFD	25.17 ± 2.08	32.34 ± 2.65	36.41 ± 3.14	37.72 ± 4.32	41.06 ± 4.73	42.20 ± 5.46
HFD	26.34 ± 1.21	35.33 ± 2.81	38.22 ± 3.30	43.22 ± 3.56	46.17 ± 4.03	47.18 ± 3.85
SIM+HFD	27.65 ± 1.35	36.23 ± 1.67	38.57 ± 1.87	42.23 ± 3.57	44.41 ± 2.87	44.35 ± 3.22
ZTL+HFD	26.76 ± 1.63	35.55 ± 1.57	39.07 ± 3.24	42.92 ± 2.57	43.81 ± 2.25	44.67 ± 2.67
ZTM+HFD	26.72 ± 0.89	35.33 ± 1.72	37.86 ± 2.35	40.92 ± 2.74	43.52 ± 3.07	43.05 ± 2.72[b]
ZTH+HFD	26.62 ± 1.23	33.54 ± 2.84	36.03 ± 2.31	40.35 ± 2.35[a]	42.82 ± 2.42[a]	41.57 ± 2.60

Notes: [a]$P<0.05$ versus HFD group; [b]$P<0.05$ versus SIM+HFD group.

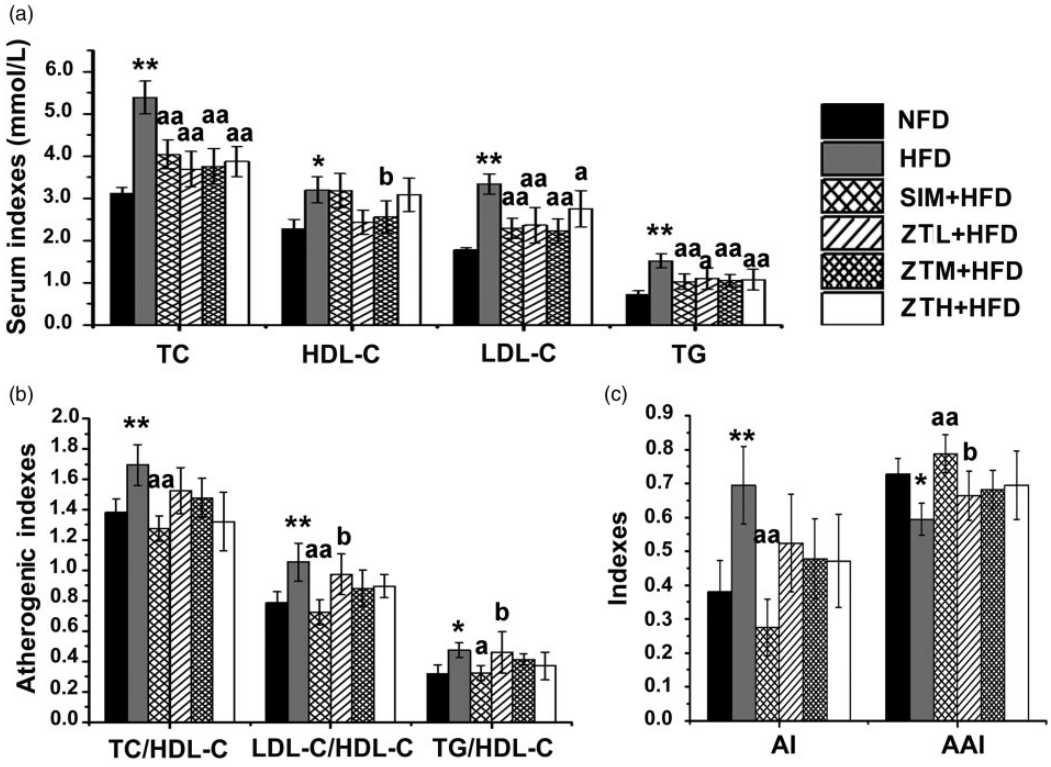

Figure 1 Effects of the ethanol extract of Zhongtian hawthorn on serum indexes in mice fed with high-fat diet. (a) The level of serum total cholesterol (TC), total triglyceride (TG), high-density lipoprotein cholesterol (HDL-C), and low-density lipoprotein cholesterol (LDL-C); (b) the ratio of serum TG, TC, and LDL-C to HDL-C; (c) atherogenic index (AI) and antiatherogenic index (AAI). The data were shown as the mean ± standard deviation. Notes: ** $P < 0.01$ versus normal food (NFD) group;* $P < 0.05$ versus NFD group; [aa]$P < 0.01$ versus high-fat (HFD) group; [a]$P < 0.05$ versus HFD group; [bb]$P < 0.01$ versus simvastatin-treated HFD (SIM+HFD) group; [b]$P < 0.05$ versus SIM+HFD group

reduced, and the effects of Zhongtian hawthorn ethanol extract exhibited a dose-dependent manner (Figure 1(b)). Furthermore, the ethanol extract of Zhongtian hawthorn could also decrease the AI ($R^2 = 0.140$, $F = 5.789$, $P < 0.01$) and increase the AAI ($R^2 = 0.030$, $F = 5.665$, $P < 0.01$), implying that it might block the progression of atherosclerosis (Figure 1(c)). Furthermore, treatment of simvastatin (10.0 mg/kg/d) and the ethanol extract of Zhongtian hawthorn could also reduce the content of liver cholesterol ($R^2 = 20.107$, $F = 60.111$, $P < 0.01$) (Figure 3(c)).

Effects of the ethanol extract of Zhongtian hawthorn on transcription and expression of metabolic enzymes of cholesterol

To investigate the molecular mechanisms underlying the cholesterol-reducing effects of the Zhongtian hawthorn ethanol extract, the expression of cholesterol metabolic enzymes in liver of mice was detected with RT-PCR and western blot. As shown in Figure 2, similar to simvastatin, the ethanol extract of Zhongtian hawthorn could suppress the stimulation effect of high-fat diet on the transcription of HMGCR and p65, the major subunit of NF-κB transcription complex, and counteract the downregulation of CYP7A1 and LDLR. Moreover, as cholesterol and HMGCR were the most sensitive indexes affected by Zhongtian hawthorn ethanol extract in Figures 1 to 3(c), the effect of Zhongtian hawthorn ethanol extract on the expression of HMGCR, the key enzyme in cholesterol biosynthesis was further confirmed using real-time RT-PCR and western blot. Consistent with the semi-quantitative RT-PCR analysis, the results of real-time RT-PCR and western blot also showed that both mRNA and protein level of HMGCR were upregulated by high-fat diet, whereas the ethanol

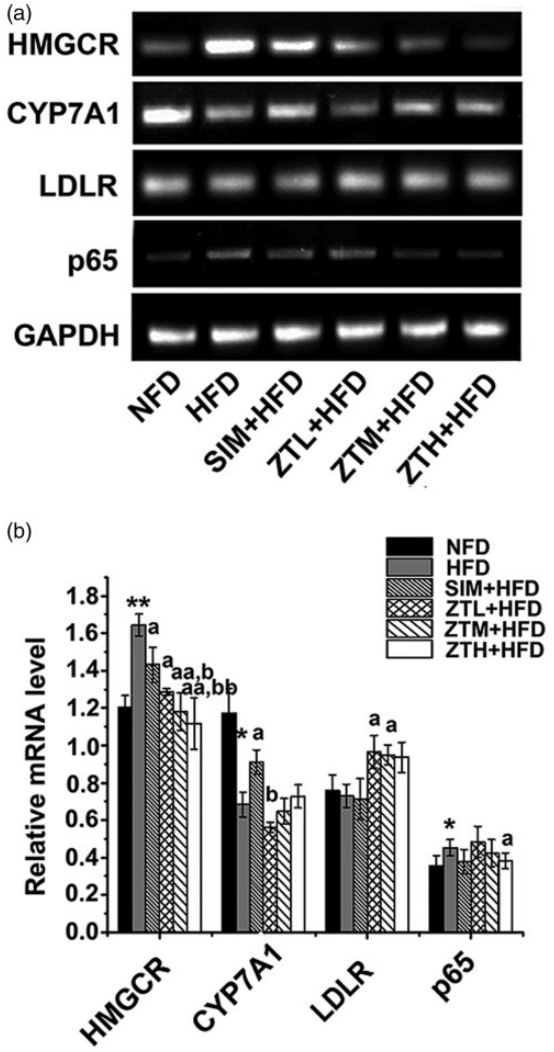

Figure 2 Effects of the ethanol extract of Zhongtian hawthorn on mRNA levels of metabolic enzymes of cholesterol in mice fed with high-fat diet. (a) The mRNA level of 3-hydroxy-3-methylglutaryl-CoA reductase (HMGCR), nuclear factor-kappa B (NF-κB) subunit p65, cholesterol 7α-hydroxylase (CYP7A1), and LDL-receptor (LDLR) in liver was detected by RT-PCR. (b) The relative quantity was determined with the ratio of band density of genes to that of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) using the Quantity One software. ** $P < 0.01$ versus NFD group; * $P < 0.05$ versus NFD group; $^{aa}P < 0.01$ versus HFD group; $^{a}P < 0.05$ versus HFD group; $^{bb}P < 0.01$ versus SIM+HFD group; $^{b}P < 0.05$ versus SIM+HFD group

extract of Zhongtian hawthorn could dose dependently suppress this effect (Figure 3).

The effect of the ethanol extract of Zhongtian hawthorn on the transcriptional activity of HMGCR promoter

Since the mRNA level of HMGCR was markedly decreased by Zhongtian hawthorn ethanol extract, we then wondered whether the transcriptional activity of HMGCR promoter was affected by Zhongtian hawthorn extract. To confirm this issue, we constructed the HMGCR-promoter-driven luciferase reporter plasmid, and then the reporter assay was performed. As shown in Figure 4, the transcriptional activity of HMGCR promoter was inhibited by the ethanol extract of Zhongtian hawthorn in a dose-dependent manner ($R^2 = 6.908 \times 10^7$, $F = 40.313$, $P < 0.05$), whereas overexpression of p65 could reverse this transcriptional repression. Furthermore, the results of Western blot also showed that the ethanol extract of Zhongtian hawthorn could suppress the expression of HMGCR and p65, while overexpression of p65 could antagonize the inhibitory effect of Zhongtian hawthorn ethanol extract on the expression of HMGCR (Figure 5). Taken together, these results indicated that suppression of NF-κB signal pathway was essential in the inhibitory effect of Zhongtian hawthorn ethanol extract on HMGCR transcription.

Discussion

Clinical and experimental studies have proven that consumption of high-fat diet is one of the major contributors of CVDs due to the development of abnormal lipid metabolism as well as atherosclerosis.[19,20] Natural products possessing hypocholesteolemic and/or hypolipidemic properties are commonly used as functional foods or medicines to prevent the development of CVDs.[20–23] In the present study, it was demonstrated that the ethanol extract of Zhongtian hawthorn, a novel grafted cultivar of *C. cuneata* (wild Shan-Zha in the South China), could provide health benefits by counteracting the high-fat diet-induced hypercholesteolemic and hyperlipidemic effects *in vivo*, and the transcriptional regulation of HMGCR via NF-κB signal pathway might be implicated in the hypocholesteolemic effect of Zhongtian hawthorn ethanol extract.

The metabolism of cholesterol, including biosynthesis, catabolism, and reverse cholesterol transport, is a key element of the development of CVDs. In the present study, our results showed that the ethanol extract of Zhongtian hawthorn could markedly downregulate HMGCR, the rate-limiting enzyme in cholesterol biosynthesis, while upregulate CYP7A1, the cholesterol 7α-hydroxylase which is responsible for catalyzing the metabolic conversion of cholesterol into bile acid.[24] In addition, the level of LDLR, an important receptor in the transport of cholesterol-carrying lipoprotein into cells,[25] was also slightly elevated by the ethanol extract of Zhongtian hawthorn. These results suggested that the major function of Zhongtian hawthorn ethanol extract in the improvement of hypercholesterolemia might be carried out by inhibiting the production of cholesterol and promoting the elimination of cholesterol, and it might also have little stimulating effect on the transport of cholesterol.

HMGCR is a highly conserved, membrane-bound enzyme present in archaea, prokaryotes, and eukaryotes. It is the rate-controlling enzyme of the mevalonate pathway, the metabolic pathway in sterol and isoprenoid biosynthesis. The paradoxical upregulation in hepatic HMGCR is observed in patients with hypercholesteolemic, non-alcoholic fatty liver disease and obesity, while HMGCR inhibitors, such as statins, are considered as one of the most effective classes of drugs for reducing serum cholesterol.[26] The cholesterol from diet could feedback regulate the transcription, translation, or activity of HMGCR, and

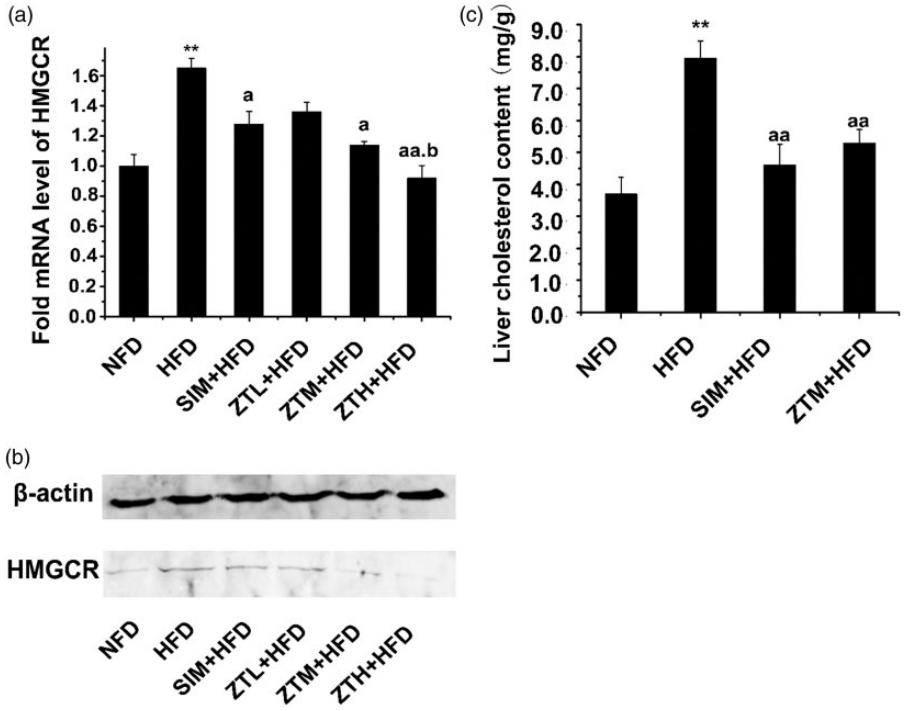

Figure 3 Effects of the ethanol extract of Zhongtian hawthorn on mRNA and protein levels of HMGCR, and the cholesterol content in the liver of mice. (a) The mRNA level detected by real-time RT-PCR. (b) The protein level detected by western blot. (c) The cholesterol content in the liver. ** $P < 0.01$ versus NFD group; * $P < 0.01$ versus NFD group; $^{aa}P < 0.01$ versus HFD group; $^{a}P < 0.05$ versus HFD group; $^{b}P < 0.05$ versus SIM+HFD group

this feedback regulation might play an important role in resistance/susceptibility to dietary cholesterol. In general, patients or animal strains with higher basal levels of liver HMGCR are more resistant to dietary cholesterol and exhibit a greater cholesterol-lowering effect in response to statins.[27] However, the sensitivity between different animal species and different people is various and even conflicting. For instance, rabbits and hamsters exhibit lower levels of hepatic HMGCR and more sensitive to dietary cholesterol than rats. Even within the same species, Sprague-Dawley, Wistar-Furth, Spontaneously Hypertensive, Lewis, and Wistar-Kyoto rats exhibited much higher hepatic HMGCR activities and more resistant to dietary cholesterol than Buffalo, Brown Norway, and Copen-hagen 2331 rats.[27] Besides, although adequate evidence suggested that dietary cholesterol challenges could suppress the expression of HMGCR, many reports also showed that high-fat diet feeding could stimulate the expression of HMGCR and cholesterol accumulation in the liver.[28–32] Here, our results were quite consistent with the latter literatures. Further investigation focused on the detailed mechanisms underlying the different response to dietary cholesterol was still needed to be performed.

In addition to its role in regulating cholesterol homeostasis, HMGCR has also been regarded as a novel target of anticancer therapy in recent years.[33] Therefore, research on the regulation of HMGCR has been remaining the subject of great interest. The sterol regulatory element-binding protein (SREBP) has been considered as the key factor in regulating the transcription of HMGCR. SREBP forms a complex in the ER membrane with SREBP cleavage-activating protein (SCAP). SCAP could escort SREBP from ER to Golgi, where SREBP is activated by two sequential proteolytic events, allowing it to enter the nucleus and activate the transcription of HMGCR. Besides, accumulating studies suggested that the nuclear factor kappa B (NF-κB) pathway also played important roles in the transcriptional regulation of HMGCR. The active form of NF-κB is usually a heterodimer of p65 and p50. The p65 protein has a transactivation domain in their C-termini and is considered as a main subunit of NF-κB, while p50 has no intrinsic ability to activate transcription. Previous studies have demonstrated that increased NF-κB signal is correlated with obesity and numerous metabolic disorders, and plays important roles in the high-fat diet-induced hypercholesterolemia and atherosclerosis, whereas suppression of NF-κB signal pathway is involved in the antiatherosclerosis and cholesterol-lowering effects of statins and other pharmacologic agents.[34–40] Furthermore, it has been demonstrated that there is a cross-talk between MyD88-NF-κB and SCAP-SREBP2 pathways in the lipopolysaccharides-induced inflammatory response, upregulation of HMGCR, and cholesterol disorders,[41] and the promoter region of both SREBP-2 and HMGCR contains putative NF-κB response elements and they are all direct target genes of NF-κB.[42,43] Similarly, here, our results showed that the repression of NF-κB pathway was also implicated in the transcriptional inhibition of HMGCR by Zhongtian hawthorn ethanol extract.

Currently, except *C. pinnatifida* and *C. pinnatifida* var. major, the representative Shan-Zha listed in the Chinese Pharmacopoeia, *C. cuneata* (wild Shan-Zha) and

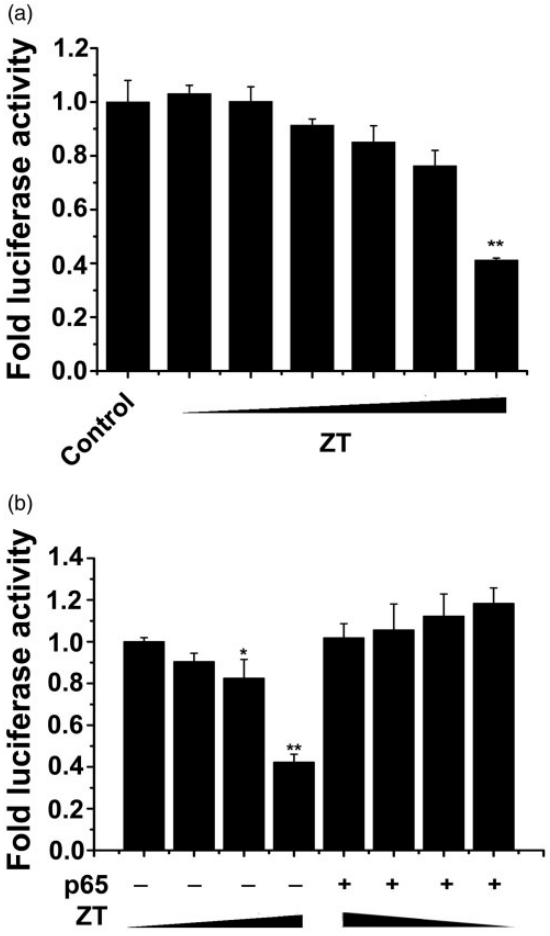

Figure 4 Effects of the ethanol extract of Zhongtian hawthorn on the transcriptional activity of HMGCR promoter. (a) HepG2 cells were transfected with 0.2 μg of HMGCR-luc plasmids and treated with 0.0, 0.2, 0.4, 0.6, 0.8, and 1.0 μg of the ethanol extract of Zhongtian hawthorn (ZT), and then the luciferase reporter assay was performed to detect the transcriptional activity of HMGCR promoter. (b) HepG2 cells were transfected with 0.2 μg of HMGCR-luc plasmids and treated with 0.0, 0.3, 0.6, and 1.0 μg of the ethanol extract of ZT combined with transfection of p65 expression plasmid or the vehicle, and then the luciferase reporter assay was performed to detect the transcriptional activity of HMGCR promoter. Values were normalized as the relative luciferase activity (fold). ** $P < 0.01$ versus control, n = 3

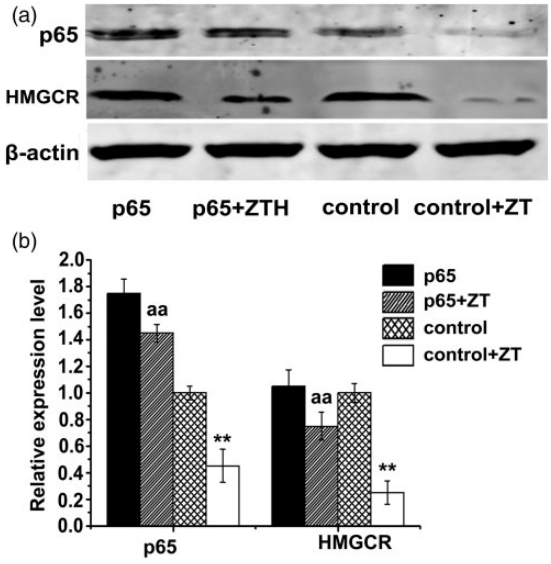

Figure 5 Antagonistic effects between p65 overexpression and the ethanol extract of Zhongtian hawthorn on the expression of HMGCR in HepG2 cells. (a) HepG2 cells were transfected with 0.2 μg of p65 expression plasmid and treated with 1.0 μg of the ethanol extract of Zhongtian hawthorn (ZT), and then the protein levels of p65 and HMGCR were detected by Western blot. (b) The relative quantity was determined with the ratio of band density of genes to that of β-actin using the Quantity One software. ** $P < 0.01$ versus control; $^{aa}P < 0.01$ versus p65, n=3

C. scabrifolia (Yunnan Shan-Zha) were also used in the folk medicine and food in the South China.[14] However, the experimental evidence of the exact effects of either *C. cuneata* or *C. scabrifolia* on hyperlipidemia and/or hypercholesterolemia still remained elusive. Here, our data showed that a novel grafted cultivar of *C. cuneata*, Zhongtian hawthorn, could inhibit the high-fat diet-induced hyperlipidemia and hypercholesterolemia *in vivo*. Furthermore, compared with the other varieties of hawthorn, Zhongtian hawthorn has much bigger fruits and is early bearing, high productive, cold, and drought resistant.[15,16] Therefore, Zhongtian hawthorn might be considered as a good alternative to Shan-Zha and has great value in the food and medicine industry. In addition, to our best knowledge, this is also the first report that the extract of *Crataegus* could suppress the transcription of HMGCR via NF-κB signal pathway.

Authors' contribution: H-JH and X-GL contributed equally to this work. X-GL and L-WP designed the experiments. H-JH, Q-QD, AM, G-LS, Q-TW, and X-YC performed the experiments. H-JH and X-GL analyzed data. HZ, T-CZ, and L-WP contributed reagents, materials, and tools. H-JH and X-GL wrote the paper.

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DECLARATION OF CONFLICTING INTERESTS

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

REFERENCES

1. Manson JE, Tosteson H, Ridker PM, Satterfield S, Hebert P, O'Connor GT, Buring JE, Hennekens CH. The primary prevention of myocardial infarction. *N Engl J Med* 1992;**326**:1406–16
2. Taylor F, Huffman MD, Macedo AF, Moore TH, Burke M, Davey Smith G, Ward K, Ebrahim S. Statins for the primary prevention of cardiovascular disease. *Cochrane Database Syst Rev* 2013;**1**:CD004816
3. Dahmer S, Scott E. Health effects of hawthorn. *Am Fam Physician* 2010;**81**:465–8

4. McGovern PE, Zhang J, Tang J, Zhang Z, Hall GR, Moreau RA, Nunez A, Butrym ED, Richards MP, Wang CS, Cheng G, Zhao Z, Wang C. Fermented beverages of pre- and proto-historic China. *Proc Natl Acad Sci USA* 2004;**101**:17593–8
5. Cheng ME, Peng HS. Development of the origin and application of shanza in ancient and modern. *Zhonghua yi shi za zhi* 2011;**41**:336–8
6. Jurikova T, Sochor J, Rop O, Mlcek J, Balla S, Szekeres L, Adam V, Kizek R. Polyphenolic profile and biological activity of Chinese hawthorn (Crataegus pinnatifida BUNGE) fruits. *Molecules* 2012;**17**:14490–509
7. Wu J, Peng W, Qin R, Zhou H. Crataegus pinnatifida: chemical constituents, pharmacology, and potential applications. *Molecules* 2014;**19**:1685–1712
8. Chang WT, Dao J, Shao ZH. Hawthorn: potential roles in cardiovascular disease. *Am J Chinese Med* 2005;**33**:1–10
9. Wang J, Xiong X, Feng B. Effect of crataegus usage in cardiovascular disease prevention: an evidence-based approach. *Evid Based Complement Alternat Med* 2013;**2013**:149363
10. Kuo DH, Yeh CH, Shieh PC, Cheng KC, Chen FA, Cheng JT. Effect of shanzha, a Chinese herbal product, on obesity and dyslipidemia in hamsters receiving high-fat diet. *J Ethnopharmacol* 2009;**124**:544–50
11. Edwards JE, Brown PN, Talent N, Dickinson TA, Shipley PR. A review of the chemistry of the genus Crataegus. *Phytochemistry* 2012;**79**:5–26
12. Rigelsky JM, Sweet BV. Hawthorn: pharmacology and therapeutic uses. *Am J Health Syst Pharm* 2002;**59**:417–22
13. Chen JD, Wu YZ, Tao ZL, Chen ZM, Liu XP. Hawthorn (shan zha) drink and its lowering effect on blood lipid levels in humans and rats. *World Rev Nutr Diet* 1995;**77**:147–54
14. Fen YX, Gao GY, Chen HB, Si JY, Wang LW. Studies on the utilization of hawthorn resources in China (in Chinese). *Med Res Commun* 1996;**25**:18–19
15. Pan ZT. Zhongtian Dashanzha, a new hawthorn cultivar for fresh market in the South of China (in Chinese). *J Fruit Sci* 2011;**28**:186–7
16. Pan ZT. Characteristics and cultivation techniques of Zhongtian hawthorn (in Chinese). *Southern Hortic* 2010;**21**:17–18
17. Lin Y, Vermeer MA, Trautwein EA. Triterpenic acids present in hawthorn lower plasma cholesterol by inhibiting intestinal ACAT activity in hamsters. *Evid Based Complement Alternat Med* 2011;**2011**:801272
18. Zick SM, Gillespie B, Aaronson KD. The effect of Crataegus oxycantha special extract WS 1442 on clinical progression in patients with mild to moderate symptoms of heart failure. *Eur J Heart Failure* 2008;**10**:587–93
19. Keevil JG, Cullen MW, Gangnon R, McBride PE, Stein JH. Implications of cardiac risk and low-density lipoprotein cholesterol distributions in the United States for the diagnosis and treatment of dyslipidemia: data from National Health and Nutrition Examination Survey 1999 to 2002. *Circulation* 2007;**115**:1363–70
20. Wan CW, Wong CN, Pin WK, Wong MH, Kwok CY, Chan RY, Yu PH, Chan SW. Chlorogenic acid exhibits cholesterol lowering and fatty liver attenuating properties by up-regulating the gene expression of PPAR-alpha in hypercholesterolemic rats induced with a high-cholesterol diet. *Phytother Res* 2013;**27**:545–51
21. Li Q, Wu JH, Guo DJ, Cheng HL, Chen SL, Chan SW. Suppression of diet-induced hypercholesterolemia by scutellarin in rats. *Planta Med* 2009;**75**:1203–8
22. Prasad K, Lee P. Suppression of hypercholesterolemic atherosclerosis by pentoxifylline and its mechanism. *Atherosclerosis* 2007;**192**:313–22
23. Yan LP, Chan SW, Chan AS, Chen SL, Ma XJ, Xu HX. Puerarin decreases serum total cholesterol and enhances thoracic aorta endothelial nitric oxide synthase expression in diet-induced hypercholesterolemic rats. *Life Sci* 2006;**79**:324–30
24. Vlahcevic ZR, Pandak WM, Heuman DM, Hylemon PB. Function and regulation of hydroxylases involved in the bile acid biosynthesis pathways. *Semin Liver Dis* 1992;**12**:403–19
25. Zhang L, Reue K, Fong LG, Young SG, Tontonoz P. Feedback regulation of cholesterol uptake by the LXR-IDOL-LDLR axis. *Arterioscler Thromb Vasc Biol* 2012;**32**:2541–6
26. Caballero F, Fernandez A, De Lacy AM, Fernandez-Checa JC, Caballeria J, Garcia-Ruiz C. Enhanced free cholesterol, SREBP-2 and StAR expression in human NASH. *J Hepatol* 2009;**50**:789–96
27. Ness GC, Gertz KR. Hepatic HMG-CoA reductase expression and resistance to dietary cholesterol. *Exp Biol Med* 2004;**229**:412–6
28. Ness GC, Chambers CM. Feedback and hormonal regulation of hepatic 3-hydroxy-3-methylglutaryl coenzyme A reductase: the concept of cholesterol buffering capacity. *Proc Soc Exp Biol Med* 2000;**224**:8–19
29. Wu N, Sarna LK, Hwang SY, Zhu Q, Wang P, Siow YL, OK. Activation of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase during high fat diet feeding. *Biochim Biophys Acta* 2013;**1832**:1560–8
30. Park Y, Park EM, Kim EH, Chung IM. Hypocholesterolemic metabolism of dietary red pericarp glutinous rice rich in phenolic compounds in mice fed a high cholesterol diet. *Nutr Res Pract* 2014;**8**:632–7
31. Ness GC. Physiological feedback regulation of cholesterol biosynthesis: role of translational control of hepatic HMG-CoA reductase and possible involvement of oxylanosterols. *Biochim Biophys Acta* 2015;**1851**:667–73
32. Lin P, He YR, Lu JM, Li N, Wang WG, Gu W, Yu J, Zhao RH. In vivo lipid regulation mechanism of polygoni multiflori radix in high-fat diet fed rats. *Evid Based Complement Alternat Med* 2014;**2014**:642058
33. Altwairgi AK. Statins are potential anticancerous agents (review). *Oncol Rep* 2015;**33**:1019–39
34. Pamukcu B, Lip GY, Shantsila E. The nuclear factor-kappa B pathway in atherosclerosis: a potential therapeutic target for atherothrombotic vascular disease. *Thromb Res* 2011;**128**:117–23
35. de Winther MP, Kanters E, Kraal G, Hofker MH. Nuclear factor kappaB signaling in atherogenesis. *Arterioscler Thromb Vasc Biol* 2005;**25**:904–14
36. Real JT, Martinez-Hervas S, Garcia-Garcia AB, Civera M, Pallardo FV, Ascaso JF, Vina JR, Chaves FJ, Carmena R. Circulating mononuclear cells nuclear factor-kappa B activity, plasma xanthine oxidase, and low grade inflammatory markers in adult patients with familial hypercholesterolaemia. *Eur J Clin Invest* 2010;**40**:89–94
37. Lenglet S, Quercioli A, Fabre M, Galan K, Pelli G, Nencioni A, Bauer I, Pende A, Python M, Bertolotto M, Spinella G, Pane B, Palombo D, Dallegri F, Mach F, Vuilleumier N, Montecucco F. Statin treatment is associated with reduction in serum levels of receptor activator of NF-kappaB ligand and neutrophil activation in patients with severe carotid stenosis. *Mediat Inflamm* 2014;**2014**:720987
38. Rudofsky G, Reismann P, Groener JB, Djuric Z, Fleming T, Metzner C, Grafe IA, Bierhaus A, Nawroth PP. Identical LDL-cholesterol lowering but non-identical effects on NF-kappaB activity: high dose simvastatin vs combination therapy with ezetimibe. *Atherosclerosis* 2012;**223**:190–6
39. Lin HL, Shen KP, Chang WT, Lin JC, An LM, Chen IJ, Wu BN. Eugenosedin-A prevents high-fat diet increased adhesion molecules through inhibition of MAPK- and p65-mediated NF-kappaB pathway in rat model. *J Pharm Pharmacol* 2013;**65**:300–9
40. Andrade JM, Paraiso AF, de Oliveira MV, Martins AM, Neto JF, Guimaraes AL, de Paula AM, Qureshi M, Santos SH. Resveratrol attenuates hepatic steatosis in high-fat fed mice by decreasing lipogenesis and inflammation. *Nutrition* 2014;**30**:915–9
41. Li LC, Varghese Z, Moorhead JF, Lee CT, Chen JB, Ruan XZ. Cross-talk between TLR4-MyD88-NF-kappaB and SCAP-SREBP2 pathways mediates macrophage foam cell formation. *Am J Physiol Heart Circ Physiol* 2013;**304**:H874–884
42. Zhao GJ, Tang SL, Lv YC, Ouyang XP, He PP, Yao F, Tang YY, Zhang M, Tang YL, Tang DP, Cayabyab FS, Tian GP, Tang CK. NF-kappaB suppresses the expression of ATP-binding cassette transporter A1/G1 by regulating SREBP-2 and miR-33a in mice. *Int J Cardiol* 2014;**171**:e93–95
43. Naito M, Bomsztyk K, Zager RA. Renal ischemia-induced cholesterol loading: transcription factor recruitment and chromatin remodeling along the HMG CoA reductase gene. *Am J Pathol* 2009;**174**:54–62

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