Synergetic Effect and Structure–Activity Relationship of 3-Hydroxy-3-methylglutaryl Coenzyme A Reductase Inhibitors from *Crataegus pinnatifida* Bge.

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The 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR) inhibitors from hawthorn fruit (*Crataegus pinnatifida* Bge.) were isolated and evaluated for their antihyperlipidemic effect induced by high-fat diet in mice. After being further purified with silica and polyamide column chromatography from the fractions (fractions A, F, H, and G) with a high inhibitory rate (IR) to HMGR, 24 chromatographic fractions were obtained, including 8 active fractions with a high IR to HMGR. However, the total inhibitory activity of 24 fractions was decreased by about 70%. From eight active fractions, four compounds were obtained by recrystallization and identified as quercetin (**a**), hyperoside (**b**), rutin (**c**), and chlorogenic acid (**d**), the contents of which in hawthorn EtOH extract were 0.16, 0.32, 1.45, and 0.95%, respectively. The IR values of compounds **a**–**d** to HMGR were 6.28, 9.64, 23.53, and 10.56% at the corresponding concentrations of 0.16, 0.32, 1.45, and 0.95 mg/mL, respectively. It was discovered that the IR of a mixture (2.85 mg/mL) matching the original percentage of compounds **a**–**d** in hawthorn EtOH extract was up to 79.5%, much higher than that of the single compound and the total IR of these four compounds (50.01%). The in vivo results also revealed that the mixture had a more significant lipid-lowering efficacy than the monomers. Structure–activity relationship revealed the inhibitory activity and lowering-lipid ability of compounds **a**–**c** decreased with increasing glycoside numbers. It was concluded that there were synergetic effects on inhibiting HMGR and lowering lipid among compounds **a**–**d**, and the weak hydrophilic ability benefits the inhibition to HMGR and lowering-lipid efficacy.

KEYWORDS: Hawthorn fruit; 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR); antihyperlipidemic effect; synergetic effect; structure–activity relationship (SAR)

1. INTRODUCTION

Hyperlipidemia is a dangerous factor of cardio-cerebrovascular diseases, inducing essential hypertension, coronary heart disease, and atherosclerosis (*1*). It is widely acknowledged that lowering the level of total cholesterol (TC), low-density lipoprotein cholesterol (LDL-C), and triglycerides (TG) can interfere with the progression of atherosclerosis and reduce cardiovascular events in patients with atherosclerosis and cardio-cerebrovascular diseases (*2, 3*). The most widely used lipid-lowering drugs are a class of 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMGR) inhibitors. HMGR is an endoplasmic reticulum (ER) bound enzyme that catalyzes the conversion of hydroxymethylglutaryl-CoA to mevalonate (*4*), an early rate-limiting step in cholesterol biosynthesis, leading to cholesterol-lowering effects. Statins, a competitive inhibitor of HMGR most widely used in clinics, reduce TC, LDL-C, apolipoprotein B, and TG levels (*5*), but they are associated with undesirable side effects such as severe myopathy and statin-associated memory loss (*6*). Myopathy is a serious side reaction, with the potential for rhabdomyolysis (the pathological breakdown of skeletal muscle), leading to acute renal failure (*7*).

Recently, we conducted a rationalized screening to search for HMGR inhibitors from traditional Chinese medicine (TCM). The EtOH extract of hawthorn fruit (*Crataegus pimmatifida* Bge.) showed a very significant inhibition to HMGR by HPLC (*8*) among 73 TCMs. *C. pimmatifida* Bge. (Rosaceae), named Shanzha in Chinese, is widely used as a food and traditional medicine in treating chronic heart failure, high blood pressure, various digestive ailments(*9*), and arrhythmia(*10*), as well as geriatric and antiarteriosclerosis remedies (*11*) by reducing serum cholesterol. It has been documented that hawthorn fruit decreased the serum total cholesterol, LDL-C, and triglycerides in hyperlipidemic humans (*12*).

The major components include flavonoids, proanthocyanidin, triterpenes, organic acids, tannin, flavane and its polymers, and so

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on(*13*). Among them, flavonoids and triterpenes were reported as the main active hypolipidemic constituents (*14*, *15*). However, there had been no reports on the active components of HMGR inhibitors in hawthorn fruit and their lipid-lowering efficacy.

In the present study, we separated and purified the EtOH extract of hawthorn fruit by systematic chromatographic methods using HMGR as the target to research the inhibition to HMGR and the lipid-lowering efficacy of active components and further investigate their synergetic effect and structure−activity relationship.

2. MATERIALS AND METHODS

2.1. Chemicals. Standard quercitrin, hyperoside, rutin, and chlorogenic acid were purchased from the National Institute for the Control of Pharmaceutical Biological Products (Beijing, China). Nicotinamide adenine dinucleotide phosphate (NADPH), 1,4-dithiothreitol (DTT), ethylenediaminetetraacetic acid (EDTA), and 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) were obtained from Sigma Chemical Co. (St. Louis, MO). Other chemicals were purchased from Pharmaceutical and Chemical Reagent Inc. (Chongqing, China). Silica gel (200−300 mesh), polyamide (100−200 mesh), and D-101 macroporuos resin were purchased from Qingdao Marine Chemical Co., Ltd. (Qingdao, China).

2.2. Plant Materials. Hawthorn fruit (*C. pimmatifida* Bge.) was commercially available in Chongqing, China. It was dried at 75 °C in a vibrating blast drier (model VFB-A, Beijing, China) and ground to pass through a 60 mesh screen; the powder was stored in a desiccator at the Chemistry Institute of Pharmacological Resource, Southwest University, China.

2.3. Preparation and Assay of HMGR from Mouse Liver (*8*). Briefly, liver was homogenized in potassium phosphate buffer A (PPB, pH 7.2), containing 50 mmol/L KCl, 40 mmol/L K_2PO_4, 30 mmol/L EDTA, and 10 mmol/L sucrose. The homogenate was centrifuged at 16000*g* for 15 min, and the supernatant was centrifuged at 100000*g* for 60 min. The resulting microsomal fraction was suspended in 3 mL of potassium phosphate buffer A (pH 7.2), containing 250 mmol/L sucrose, 20 mmol/L EDTA, and 50 μmol/L leupeptin, immediately frozen in liquid nitrogen, and stored at −80 °C until analysis.

Microsomal suspensions of 10 μL (80 mg/mL protein) and 10 μL inhibitors were preincubated for 5 min at 37 °C with 80 μL of PPB (pH 6.8), containing 300 mmol/L KCl, 240 mmol/L K_2HPO_4, 6 mmol/L EDTA, and 15 mmol/L DTT. The assay was initiated by adding 10 μL of 50 μmol/L NADPH and 10 μL of HMG-CoA. The incubation was performed at 37 °C for 30 min and terminated by the addition of 500 μL of 0.5 mol/L of NaOH. Then the mixture was centrifuged at 10000*g* for 10 min, and 20 μL of supernatant was taken to measure the content of NADPH by HPLC (*8*). In this reaction, the NADPH would be oxidized to $NADP^+$, causing the decrease in absorbance of NADPH at 340 nm. After adding inhibitors, the reaction velocity was decreased and the depletion of NADPH was correspondingly declined. The changes in the content of NADPH were analyzed, and the inhibitory rate (IR) was calculated according to the following equation:

$$IR = \frac{S_i - S_c}{S_b - S_c} \times 100\% \qquad (1)$$

Here, IR indicates the inhibitory rate and S_i, S_b, and S_c indicate the peak area (mAU*s) of NADPH in inhibitor, blank, and control group, respectively.

HPLC conditions of analyzing NADPH were as follows: chromatographic column, Hypersil C_{18}-ODS (4.6 mm × 200 mm, 5 μm); mobile phase, phosphate buffer/MeOH (78:22, pH 7.2); flow rate, 1 mL/min; injection volume, 20 μL; wavelength of detection, 340 nm; temperature of column, 25 °C.

2.4. Preparation of Hawthorn Fruit Extracts. The dried powder of hawthorn fruit (1000 g) was extracted three times with 15 L of 95% ethanol under ultrasonic vibration. The supernatant was evaporated at 60 °C in a rotary evaporator under reduced pressure to produce 680 g of crude extracts. Then the extract was dissolved in distilled water to obtain supernatant and precipitate (35.2 g, fraction A) by centrifugation. The supernatant was loaded onto the D-101 macroporous adsorption resins

Table 1. Inhibition Rate of Constituents to HMG-CoA Reductase and of Counts

	weight (g)	weight ratio (%)	IR[a] (%)	total inhibitory activity[b]
crude extract	680	100	5.1	34680
precipitation (fraction A)	35.2	5.18	18.4	6476.80
water fraction (fraction B)	448.06	65.89	0.01	44.80
10% EtOH (fraction C)	24.92	3.66	0.2	49.84
20% EtOH (fraction D)	11.92	1.75	0.6	71.52
30% EtOH (fraction E)	16.63	2.45	5.6	931.28
40% EtOH (fraction F)	43.6	6.41	28.3	12338.8
50% EtOH (fraction G)	31.5	4.63	30.3	9544.5
60% EtOH (fraction H)	18.5	2.72	26.3	4865.5
70% EtOH (fraction I)	5.23	0.77	1.5	78.45
80% EtOH (fraction J)	2.09	0.31	2.5	52.25
90% EtOH (fraction K)	4.5	0.66	1.3	58.5
100% EtOH (fraction L)	5.86	0.86	2.2	128.92
total	648.01	95		34641.16

[a] IR indicates the inhibitory rate to HMGR at the concentration of 1 mg/mL. [b] Total inhibitory activity = weight (mg) × IR (%).

column (8 cm × 100 cm), then eluted with different concentrations of EtOH (0, 10, 20, 30, 40, 50, 60, 70, 80, 90, and 100%) to collect various fractions, and concentrated under reduced pressure to obtain condensed fractions designated fractions B, C, D, E, F, H, I, J, K, and L, respectively. Then the IR of fractions A−L to the activity of HMGR was measured by HPLC (**Table 1**).

2.5. Purification of Active Fractions with High Inhibition to HMGR. According to results of section 2.4, four fractions (A, F, G, and H) had a high inhibitory activity to HMGR (**Table 1**) and were further purified.

One gram of fraction A was suspended in MeOH and loaded onto 10 g of silicagel. After MeOH had been evaporated, silica gel with fraction A was put into column (1.5 cm × 70 cm) containing 90 g of silica gel, and then eluted with different ratios of $CHCl_3$/MeOH to produce six fractions (fractions 1−6) (shown in **Table 2**).

Fraction F (1 g) was loaded onto column chromatography on 100 g of polyamide (1.5 cm × 70 cm) and eluted with a gradient of H_2O/MeOH eluent to yield six fractions (fractions 7−12).

Fraction G (1 g) was loaded onto 100 g of polyamide chromatography (1.5 cm × 70 cm) and eluted with a gradient of H_2O/EtOH to yield five fractions (fractions 13−17).

Fraction H (1 g) was loaded onto 100 g of silica gel chromatography (1.5 cm × 70 cm) and eluted with a gradient of $CHCl_3$/MeOH to yield seven fractions (fractions 18−24).

The IR of fractions 1−24 to HMGR was measured by HPLC.

Those fractions with a high inhibition to HMGR were purified with thin layer chromatography (TLC) and recrystallized to obtain eight compounds, **a−h**, respectively. The separation process is shown in **Scheme 1**.

2.6. Structural Identification of Active Compounds. Melting points, UV, IR, ¹H NMR, and TLC were used to identify structures of new compounds. Melting points were determined on an RD-2C electro-thermal melting point apparatus and are uncorrected. The UV spectra were recorded on a Hitachi U-1800 spectrophotometer. The IR spectra were carried out on a Perkin-Elmer IR spectrophotometer. The ¹H and ¹³C NMR spectra were recorded on a Bruker model Avance DMX300 spectrometer (300 MHz for ¹H and 75 MHz for ¹³C) using TMS as an internal standard and DMSO-d_6 as solvent. Macroporous resin was used for crude separations. Analytical TLC was performed on precoated silica gel G plates (Qingdao Marine Chemical Co. Ltd.) and polyamide thin film (Yuanda Chemical Co. Ltd.), visualized with UV light, and then sprayed with 1% vanillin/H_2SO_4 and heated or with 1%AlCl₃/EtOH.

2.7. Quantitative Analysis of Active Components in Hawthorn Fruit Extract. For quantification of active components in hawthorn fruit extract, a Waters HPLC system equipped with a 510 pump, a UV detector, and a Symmetry Hypersil C_{18}-BDS (4.6 × 250 mm, 5 μm) was used. Quercetin, hyperoside, rutin, and chlorogenic acid in methanol were injected into the column as external standard compounds. After injection, the column was eluted with a linear gradient of acetonitrile in water from

Table 2. Contents and IR of Chromatographic Fractions to HMGR

fraction	weight (mg)	weight ratio (%)	IR[a] (%)	total activity[b]
A	1000	100.0	18.4	184.0
1	99	9.9	2.5	2.5
2	78	7.8	39.6	30.9
3	255	25.5	2.4	6.1
4	149	14.9	4.6	6.9
5	50	5.0	0.2	0.1
6	154	15.4	10.3	15.9
sum	785	78.5		62.3
F	1000	100.0	28.3	283.0
7	61	6.1	5.9	3.6
8	80	8.0	30.4	24.3
9	283	28.3	16.5	46.7
10	92	9.2	1.9	1.7
11	80	8.0	11.1	8.9
12	170	17.0	7.9	13.4
sum	766	76.6		98.7
G	1000	100.0	30.3	303.0
13	72	7.2	3.4	2.4
14	308	30.8	30.4	93.6
15	164	16.4	16.5	27.1
16	80	8.0	0.3	0.2
17	153	15.3	4.8	7.3
sum	777	77.7		130.7
H	1000	100.0	26.3	263.0
18	120	12.0	6.9	8.3
19	15	0.8	39.6	5.9
20	65	6.5	2.4	1.6
21	128	12.8	30.4	38.9
22	73	7.3	1.9	1.4
23	30	3.0	2.6	0.8
24	312	31.2	11.9	37.1
sum	713	74.3		93.9

[a] IR indicates the inhibitory rate to HMGR at the concentration of 1 mg/mL. [b] Total inhibitory activity = weight (mg) × IR (%).

10 to 50% (containing 0.3% KH_2PO_4) over 30 min before returning to initial conditions for 10 min. The flow rate was 1 mL/min, and effluent was monitored at 360 nm.

Before being analyzed, the EtOH extract of hawthorn fruit was dissolved in methanol, and after a brief centrifugation (1000*g*, 10 min), a 5 mL aliquot of the supernatant was passed through a 2 mL cleanup column and eluted with methanol. Twenty microliters of collected eluant was used to analyze the content of active compounds by HPLC.

2.8. Animal Experiments. Kunming mice (20 ± 2 g) of both genders were purchased from Animal Breeding Center of the Third Military Medical University (Chongqing, China). Animals were cared for according to the institutional guidelines of Chongqing City Laboratory Animal Administration Committee of China. Mice were housed in an air-conditioned room (24 ± 2 °C, 55 ± 10% relative humidity, 15 air changes in 1 h, and 12 h light cycle) with 5 mice per cage. The regular and the high-fat and high cholesterol (HFHC) diets contained 2% cholesterol, 10% lard, 10% yolk powder, and 0.5% sodium deoxycholate, purchased from Animal Breeding Center of the Third Military Medical University (Chongqing, China).

After 1 week of accommodation period, the mice were divided into normal control (HFHC free), model (HFHC diet), compound **a** (2.85 mg/kg/day), compound **b** (2.85 mg/kg/day), compound **c** (2.85 mg/kg/day), compound **d** (2.85 mg/kg/day), and mixture of compounds **a**−**d** (2.85 mg/kg/day), and 10 mice were included in each group. The mixture of compounds **a**−**d** was matched according to their ratio in hawthorn fruit extract (0.16:0.32:1.42:0.95). Mice in the normal control were fed regular mouse diet. Other animals were fed HFHC diet for 2 months to get the hyperlipidemic mice. Then hyperlipidemic mice were untreated or treated with above extracts using a stomach tube for 6 weeks, respectively.

Six hours after the last treatment, the mice were sacrificed, their blood was collected, and the serum for the measurement of cholesterol levels was obtained by centrifugation. Then total cholesterol (TC), triglyceride (TG), high-density lipoprotein-cholesterol (HDL-C), and low-density lipoprotein-cholesterol (LDL-C) were assayed using commercially available kits.

2.9. Statistics. The data were analyzed by SPSS11.5 software and expressed as mean ± standard deviation (SD), and one-way analysis of variance (ANOVA) was used for statistical evaluation. Differences were accepted as statistically significant at *P* values of < 0.05.

3. RESULTS AND CONCLUSION

3.1. IR of Fractions A−L to the Activity of HMGR. Table 1 showed the IR of hawthorn fruit extract to the activity of HMGR. From 1000 g of dried powder of hawthorn fruit, 680 g of crude EtOH extract was obtained. Its IR to HMGR was only 5.1%, and the total inhibitory activity was about 34680.

Then the crude extract was dissolved in water to get the precipitate and supernatant. The IR of precipitate (35.2 g, fraction A) was up to 18.4%, and the total inhibitory activity was about 6476.8. From the supernatant, 11 fractions (fractions B−L) were obtained. Among them, fractions F, G, and H had high inhibitory activities to HMGR, their IRs being up to 28.3, 30.3, and 26.3%, respectively. According to the data in **Table 1**, the total activity of fractions A−L was 34641.16. This indicated that there had been no loss of total inhibitory activity. The inhibitory activities of fractions A, F, G, and H accounted for 95.9%, but their weights were only 18.9% of total weight.

3.2. IR of Fractions 1−24 to the Activity of HMGR. Fractions A, F, G, and H were further purified with different column chromatographies to obtain 24 fractions (fractions 1−24) (**Table 2**).

Six fractions were obtained from precipitate (fraction A). Among them, fraction 2 showed the highest inhibitory activity to HMGR, up to 39.6%, 2-fold higher than that of the precipitate. However, after purification by column chromatography, the loss of weight was only 21.5%, and total activity was up to 66.2%, compared with that of fraction A (**Table 2**). Fraction 2 was further purified with TLC and recrystallized in MeOH, and a pure compound (**a**) with yellow needle-like crystals was obtained and identified as quercetin.

Similarly, six fractions (fractions 7−12) were obtained from fraction F. The loss of total weight and total inhibitory activity were 23.4 and 65.2%, respectively. Among six fractions, fractions 8, 9, and 11 had strong inhibitory activities, their IR values being 30.4, 16.5, and 11.1%, respectively. After purification by TLC and recsytallization in MeOH, compounds **b**, **c**, and **d** were obtained from fractions 8, 9, and 11, respectively, and identified as hyperoside (**b**), rutin (**c**), and chlorogenic acid (**d**).

After purification with polyamide, five fractions were obtained from fraction G. The loss of total weight and total inhibitory activity were 23.3 and 56.9%, respectively. Two fractions (14 and 15) with high IR to HMGR values were obtained from fraction G. After purification by TLC and recrystallization in MeOH, compounds **e** and **f** were produced from fractions 14 and 15, respectively, and identified as hyperoside (**b**) and rutin (**c**).

After purification on a silica gel column, seven fractions were produced from fraction H. According to the data in **Table 2**, the loss of total weight and inhibitory activity of these fractions were 25.7 and 64.3%, respectively. From these fractions, two fractions (19 and 21) with high IR to HMGR values were obtained. After purification by TLC and recrystallization in MeOH, compounds **g** and **h** were produced from fractions 19 and 21, respectively, and identified as quercetin (**a**) and hyperoside (**b**).

During the period of separating active compounds with the different column chromatographies, we observed that the total

Scheme 1. Scheme of Separating Active Compounds with High Inhibition to HMGR from *Crataegus pinnatifida* Bge.
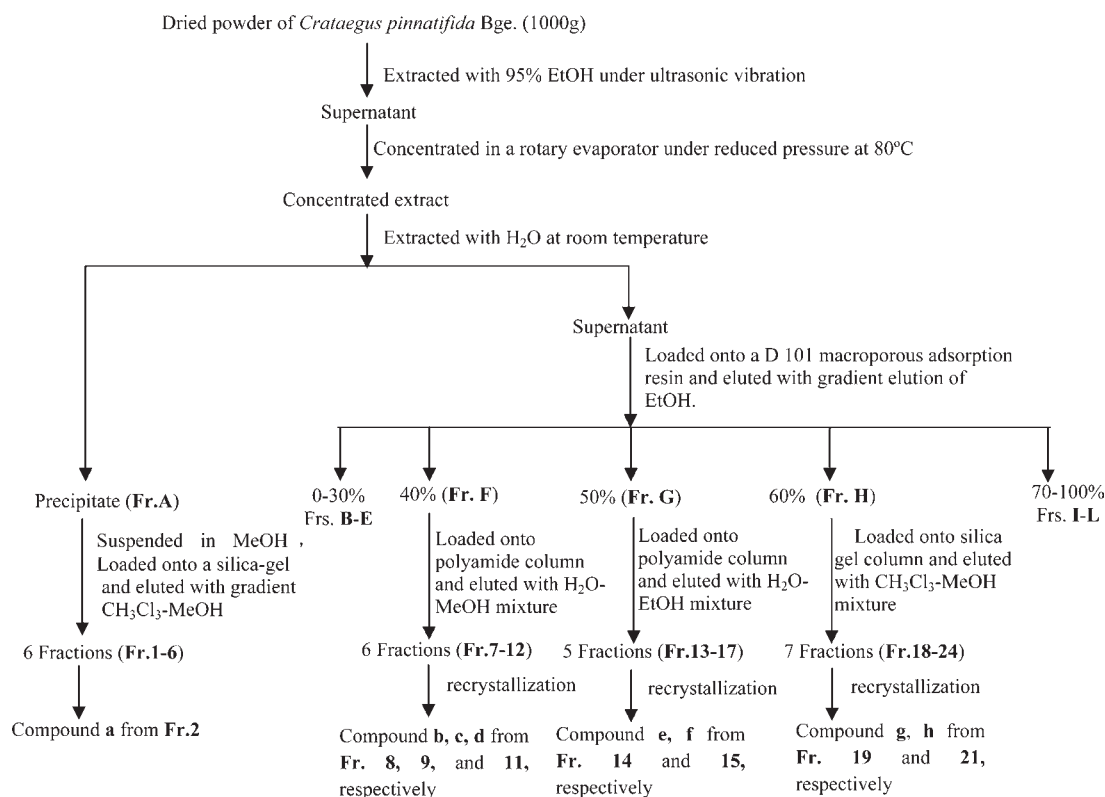
weight of those fractions (A, F, G, and H) with a high IR was reduced by 20−30%, and the total activity (fractions 1−24) was reduced by 50−70%. This indicated that further purification to crude extract weakened the inhibitory activity of active components to HMGR, and it was speculated that there was a synergetic effect among the active components.

3.3.   Structural Identification of Active Compounds from Hawthorn Fruit. Compounds **a** and **g** were negative in Molish's reaction and positive in HCl−Mg reaction. Both of them had the same structural properties: yellow needle-like crystal (MeOH), mp 310−312 °C; EI-MS (*m/z*) 302 [M + 1]; Anal. Calcd $C_{15}H_{10}O_7$; UV λ_{max} (MeOH) 359 nm; IR v (cm^{-1}) 3416.82 (OH stretching vibration), 1662.1 (C=O stretching vibration), 1612.2, 1560.4, 1522.6, 1450.7 (aromatic ring skelton vibration), 1383.8, 1320.8, 1320.1, 1264.2, 1200.7, 1170.0, 1132.3, 1093.1, 1015.2, 942.1, 864.4, 842.4, 824.9; ^{1}H NMR (300 MHz, DMSO-d^6) δ 12.49 (s, 1H, 5-Ar−OH), 10.78 (s, 1H, 7-Ar−OH), 9.60 (s, 1H, 4′-Ar−OH), 9.37 (s, 1H, 3-Ar−OH), 9.31 (s, 1H, 3′-Ar−OH), 7.67 (s, 1H, 2′-Ar−H), 7.54 (d, 1H, *J* = 8.4 Hz, 6′-Ar−H), 6.88 (d, 1H, *J* = 8.4 Hz, 5′-Ar−H), 6.41 (s, 1H, 8-Ar−H), 6.19 (s, 1H, 6-Ar−H); ^{13}C NMR (75 MHz, DMSO-d^6) δ 175.87 (C-4), 163.91 (C-7), 160.75 (C-5), 156.16 (C-9), 147.73 (C-4′), 146.82 (C-2), 145.09 (C-3′), 135.77 (C-3), 121.98 (C-1′), 120.00 (C-6′), 115.63 (C-2′), 115.09 (C-5′), 103.04 (C-10), 98.21 (C-6), 93.38 (C-8). According to the results of structural identification and color reaction, compounds **a** and **g** were confirmed as quercetin, 4*H*-1-benzopyran-4-one,   2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxy, assigned as compound **a**.

Compounds **b**, **e**, and **h** were all positive in Molish's reaction and HCl−Mg reaction, indicating they were a kind of flavonoid glycoside. Those in absolute methanol were hydrolyzed by 2% H_2SO_4 at 60 °C and then neutralized with NaOH to remove H_2SO_4. After being concentrated under reduced pressure, the residue was recrystallized in ethyl acetate to get the yellow crystal. Its mp was 310−312 °C. The R_f value was the same as for

quercetin, indicating the aglycone is quercetin. Another result of the TLC chromatogram confirmed they contained galactose. Their structural properties were as follows: yellow amorphous powder (MeOH), mp 234−236 °C; ESI-MS (*m/z*) 465 [M + 1]; Anal. Cacld $C_{21}H_{20}O_{12}$; UV λ_{max} (MeOH) 359 nm; IR v (cm^{-1}) 3436.6 (OH stretching vibration), 2923.2 (saturated C−H stretching vibration), 1654.5 (C=O stretching vibration), 1607.1, 1505.1, 1452.4 (aromatic ring skelton vibration), 1384.4, 1363.9, 1305.6, 1261.7, 1205.6, 1172.7, 116.4, 1134.8, 996.2, 819.7, 796.7; ^{1}H NMR (300 MHz, DMSO-d^6) δ 12.73 (s, 1H, 5-Ar−OH), 10.96 (s, 1H, 7-Ar−OH), 9.83 (s, 1H, 4′-Ar−OH), 9.25 (s, 1H, 3′-Ar−OH), 7.78−7.75 (dd, 1H, *J* = 5.9 Hz, 6′-Ar−H), 7.62 (s, 1H, 2′-Ar−H), 6.91 (d, 1H, *J* = 8.5 Hz, 5′-Ar−H), 6.49 (d, 1H, *J* = 1.4, 8-Ar−H), 6.29 (s, 1H, 6-Ar−H), 5.47 (d, 1H, *J* = 7.6, H−Ar-1″), 5.23 (br s, 1H, galactosyl OH), 4.95 (br s, 1H, galactosyl OH), 4.53 (br s, 2H, galactosyl OH), 3.74 (s, 1H, galactosyl H), 3.66−3.26 (m, 6H, H-2″−6″); ^{13}C NMR (75 MHz, DMSO-d^6) δ 177.48 (*C*-4), 164.10 (C-7), 161.22 (C-5), 156.28 (C-2, C-9), 148.44 (C-4′), 144.81 (C-3′), 133.49 (C-3), 121.98 (C-6′), 121.10 (C-1′), 115.95 (C-5′), 115.17 (C-2′), 103.92 (C-10), 101.81 (C-1″), 98.65 (C-6), 93.48 (C-8), 75.83 (C-5″), 73.19 (C-3″), 71.20 (C-2″), 67.92 (C-4″), 60.13 (C-6″). According to the results of structural identification and color reaction, it was confirmed that compounds **b**, **e**, and **h** are hyperoside, 4*H*-1-benzopyran-4-one, 2-(3,4-dihydroxyphenyl)-3-(β-D-galactopyranosyloxy)-5,7-dihydroxy, assigned as compound **b**.

Compounds **c** and **f** were all positive in HCl−Mg reaction, aluminum chloride (AlCl$_3$) reaction, and Molish's reaction. Both of them had the same structural properties: yellow needle-like crystal (MeOH), mp 195−198 °C; ESI-MS (*m/z*) 610 [M + 1]; Anal. Calcd $C_{27}H_{30}O_{16}$; UV λ_{max} (MeOH) 357 nm; IR v (cm^{-1}) 3429.3 (OH stretching vibration), 2983.5, 2937.7, 2901.1 (saturated C−H stretching vibration), 1654.1 (C=O stretching vibration), 1599.8, 1505.9, 1454.5 (aromatic ring skelton vibration), 1362.4, 1295.8, 1233.6, 1203.3, 1168.8, 1124.2, 1091.9, 1061.4, 1042.1,

1014.0, 969.7, 944.0, 911.9, 879.7, 827.7, 807.9; ^{1}H NMR (300 MHz, DMSO-d^6) δ 12.69 (s, 1H, 5-Ar$-$OH), 10.93 (s, 1H, 7-Ar$-$OH), 9.77 (s, 1H, 4'-Ar$-$OH), 9.28 (s, 1H, 3'-Ar$-$OH), 7.63 (d, 2H, J = 7.4, 6'-Ar$-$H), 6.92 (d, 1H, J = 8.6 Hz, 5'-Ar$-$H), 6.47 (s, 1H, 8-Ar$-$H), 6.28 (d, 1H, 6-Ar$-$H), 5.43 (d, 1H, J = 6.3 Hz, 1''-H), 4.47 (d, 1H, J = 9.3 Hz, 1'''-H), 1.07 (d, 3H, J = 5.9 Hz, 6'''-H); ^{13}C NMR (75 MHz, DMSO-d^6) δ 177.37 (C-4), 164.05 (C-7), 161.22 (C-5),156.42 (C-2), 156.60 (C-9), 148.40 (C-4'), 144.74 (C-3'), 133.31 (C-3), 121.58 (C-1'), 121.18 (C-6'), 116.27 (C-5'), 115.22 (C-2'), 103.97 (C-10), 101.19 (C-1''), 100.73 (C-1'''), 98.66 (C-6), 93.57 (C-8), 76.46 (C-3''), 75.91(C-5''), 74.07 (C-2''), 71.85 (C-4''), 70.56(C-4'''), 70.37(C-2'''), 70.01(C-3'''), 68.23 (C-5'''), 66.99 (C-6''), 17.71 (C-6'''). It was confirmed that compounds **c** and **f** were rutin, 4H-1-benzopyran-4-one, 3-[(6-O-(6-deoxy-α-L-mannopyranosyl)-β-D-glucopyranosyl]-oxy]-2-(3,4-dihydroxyphenyl)-5,7-dihydroxy, assigned as compound **c**.

Compound **d** was blue in ferric chloride (FeCl$_3$) reaction: yellow powder (MeOH/H$_2$O), mp 207$-$209 °C; ESI-MS (m/z) 354 [M + 1]; Anal. Calcd$_{16}$H$_{18}$O$_9$; UV λ_{max} (MeOH) 328.5 nm; IR v (cm^{-1}) 3389.5 (OH stretching vibration), 2956.0, 2928.8, 2857.1 (C$-$H stretching vibration), 2626.4, 1685.6 (C=O stretching vibration), 1638.9, 1614.3, 1528.7, 1517.7, 1442.8, 1384.1 (aromatic ring skelton vibration), 1289.4, 1252.4, 1190.4, 1158.8, 1133.6, 1114.1, 1086.2, 1038.1, 970.2, 818.4; ^{1}H NMR (300 MHz, H$_2$O-d^6) δ 7.47 (d, 1H, J = 15.9 Hz, α-H), 7.03 (s, 1H, 2'-H), 6.96 (d, 1H, J = 8.3 Hz, 6'-H), 6.83 (d, 1H, J = 8.3 Hz, 5'-H), 6.17 (d, 1H, J = 15.9 Hz, β-H), 5.25$-$5.18 (m, 1H, 3-H), 4.19 (d, J = 3.0 Hz, 5-H), 3.80 (dd, 1H, J = 8.5 Hz, 4-H), 2.22$-$2.00 (m, 4H, 2-H, 6-H); ^{13}C NMR (75 MHz, H$_2$O-d^6) δ 1176.57 (C-7'), 168.13 (C-9), 146.60 (C-4), 145.71 (C-3,), 143.72 (C-7), 126.39 (C-1), 122.24 (C-6), 115.65 (C-5), 114.65 (C-2), 113.78 (C-8), 74.40 (C-1'), 70.93 (C-4'), 70.13 (C-3'), 68.70 (C-5'), 36.08 (C-6'), 35.95 (C-2'). It was confirmed that compound **d** was chlorogenic acid, (1S,3R,4R,5R)-3-[[3-(3,4-dihydroxyphenyl)-1-oxo-2-propenyl]oxy]-1,4,5-trihydroxycyclohexanecarboxylic acid.

The structures of compounds **a**$-$**d** are shown in **Figure 1**.

3.4. Quantification of Four Active Components in Hawthorn Fruit Extract. The HPLC chromatogram of the ethanol extract of hawthorn fruit is shown in Figure**2**. There are more than 10 peaks in the extract, which accounted for about 4%. In 100 g of dried extract, chlorogenic acid (**d**) accounted for 0.95 g; rutin (**c**), 1.42 g; hyperoside (**b**), 0.32 g; and quercetin (**a**), 0.16 g. In addition, ursolic acid accounted for 0.32 g; oleanolic, 0.08 g; and maslinic acid, 0.08 g. The total content of compounds **a**$-$**d** was about 2.85 g in 100 g of dried extract of hawthorn fruit.

3.5. IR of Four Monomers and Their Mixture to HMGR. According to the ratio of the four monomers in crude extract of hawthorn fruit, quercetin (**a**), hyperoside (**b**), rutin (**c**), and chlorogenic acid (**d**) were adjusted to concentrations of 0.16, 0.32, 1.42, and 0.95 mg/mL with PPB (pH 6.8), respectively. Then their IR to the activity of HMGR was analyzed by HPLC. The IR values of compounds **a**$-$**d** at 1 mg/mL were 6.28, 9.64, 23.53, and 10.56%, respectively. Their total IR was about 50.01% (Table**3**). According to their ratio in hawthorn fruit extract, the mixture of compounds **a**$-$**d** was matched with the concentration of 2.85 mg/mL. The IR of the matched mixture was up to 79.5%, much higher than that of the sum of four monomers.

3.6. Effects of Four Monomers and Their Mixture on Lipid-Lowering Efficacy in Mice. As mixture of compounds **a**$-$**d** showed an improved IR to HMGR in vitro, the effects of four monomers and their mixture on lipid-lowering efficacy in vivo were investigated.

After a 6 week treatment, all mice showed good health status, and no mortality was recorded during the whole experimental
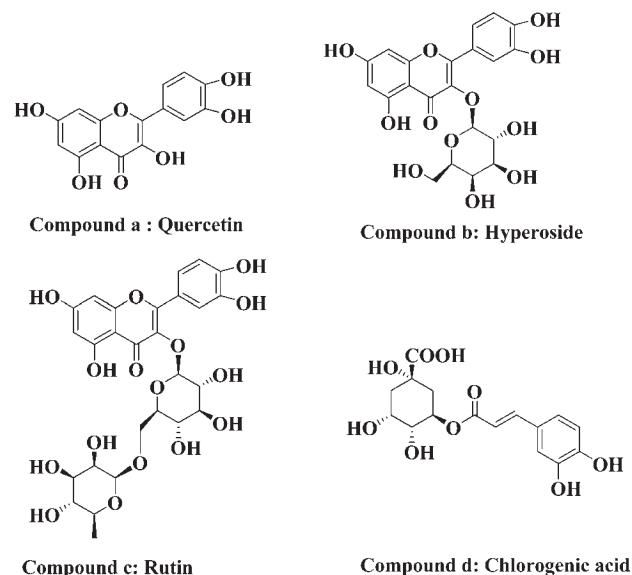

Figure 1. Structures of compounds **a**$-$**d**: (**a**) quercetin; (**b**) hyperoside; (**c**) rutin; (**d**) chlorogenic acid.

period. There were no significant differences in the body weights among different groups during treatment, suggesting the mixture or compounds **a**$-$**d** was safe and well tolerated in the mice.

The mice were fed a high-fat and -cholesterol (HFHC) diet for 2 months. Compared to the normal diet group, the levels of TC, TG, and LDL-C increased (p < 0.01) and that of HDL-C decreased (p < 0.05) in the hyperlipidemic group. Then, hyperlipidemic mice were treated with compounds **a**$-$**d** or matched mixture from hawthorn fruit orally for 6 weeks or left untreated. The levels of TC, TG, and LDL-C in therapy groups decreased in different degrees and that of HDL-C increased in the blood of the hyperlipidemic mice (**Table 4**). Among these monomers, quercetin (**a**) showed the highest lipid-lowering effect (p < 0.05). The IR values of hyperoside, rutin, and chlorogenic acid decreased in order, which was similar to the result of their inhibitory activity to HMGR. In general, the lipid-lowering efficacy of compounds **a**$-$**d** was not perfect; the content of TC, TG, and LDL-C showed no difference from that of the hyperlipidemic control. However, the mixture of compounds **a**$-$**d** could significantly reduce the levels of TC, TG, and LDL-C by 46.5, 49.6, and 58.1%, respectively (p < 0.01). This confirmed that there was a positive synergetic effect on lowering lipid among these four compounds, similar to that of HMGR.

3.7. Structure$-$Activity Relationship of Compounds a$-$c to HMGR and Lipid-Lowering Efficacy. As seen in the structures of compounds **a**$-$**c** in **Figure 1**, they contained a quercetin glycone. Hyperoside (**b**) is a monoglycoside of compound **a**, linked with a β-D-galatose on the 3-OH of quercetin. Rutin (**c**) is a disaccharide glycoside of compound **a**, linked with a disaccharide of 6-O-L-rhamnosyl-D-glucose at the 3-OH of quercetin. In general, the introduction of the glycosyl group increased the hydrophilic ability of a compound. The greater the number of the glycosyl group is, the stronger is the hydrophilic ability. From the data of **Table 5**, the IR values of quercetin, hyperoside, and rutin to HMGR were 39.6, 30.4, and 16.5%, respectively, which gradually decreased with the increase of glycosyl group number. Similarly, the efficacy of quercetin, hyperoside, and rutin in lowering TC, TG, and LDL-C was decreased with the increase of glycosyl group number (**Table 4**). Quercetin (**a**), without a glycosyl group, showed the highest inhibitory activity and lipid-lowering effect. This indicated that the activities of inhibition to HMGR and lowering lipid were related to its hydrophilic ability. The weak

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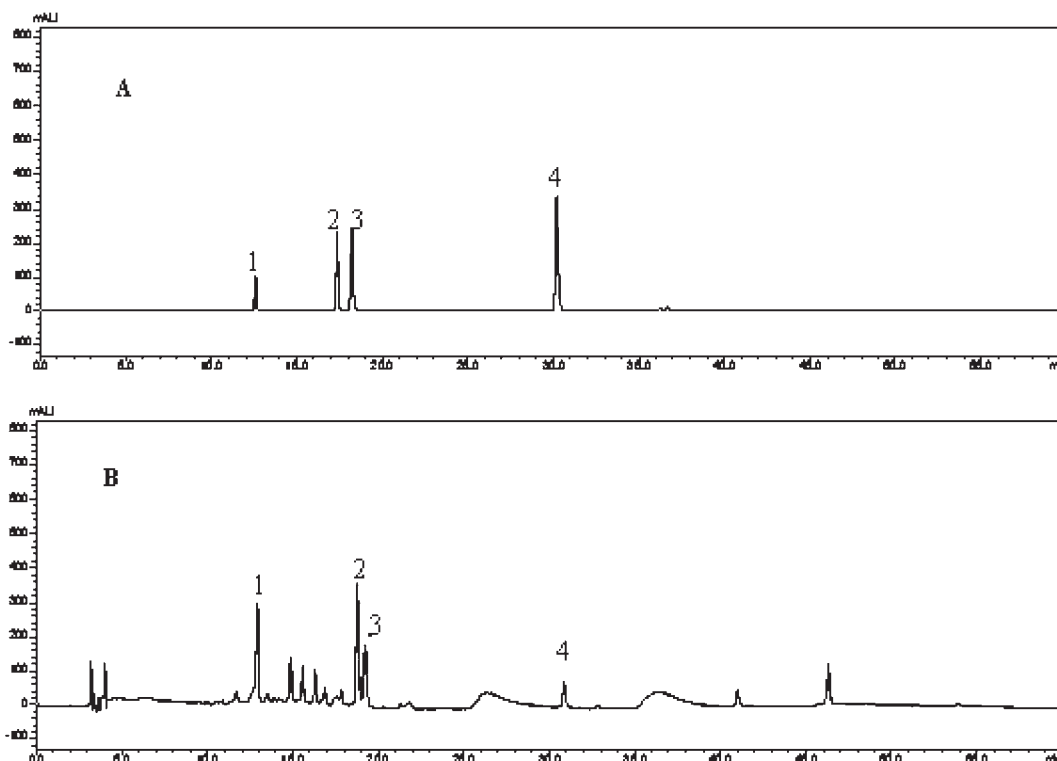
Figure 2. HPLC chromatogram of ethanol extract of hawtorn fruit: (**A**) chromatogram of standard compounds **a**−**d**; (**B**) chromatogram of sample (1, chlorogenic acid; 2, rutin; 3, hyperoside; 4, quercetin). Symmetry Hypersil C_{18}-BDS (4.6 × 250 mm, 5 μm) was used and was eluted with a linear gradient of acetonitrile in water from 10 to 50% (containing 0.3% KH_2PO_4) over 30 min before returning to initial conditions for 10 min. The flow rate was 1 mL/min, and effluent was monitored at 360 nm.

Table 3. Inhibition Rate of Compounds from Hawthorn Fruit to HMG-CoA Reductase

		quercetin	hyperoside	rutin	chlorogenic acid	total[a]	mixture[b]
concentration (mg/mL)		0.16	0.32	1.42	0.95	2.85	2.85
IR (%)		6.28	9.64	23.53	10.56	50.01	79.5

[a] Total indicated the sum of compounds **a**−**d**. [b] Mixture indicated compounds **a**−**d** were matched according to the ratio of compounds **a**−**d** in hawthorn fruit extract, which was 0.16:0.32:1.42:0.95, respectively. IR indicated the inhibitory rate at the corresponding concentration in **Table 3**.

hydrophilic ability benefited the inhibition to HMGR and lipid-lowering efficacy.

4. DISCUSSION

C. pimmatifida Bge. (Rosaceae) is widely used as a food and traditional medicine in the treatment of chronic heart failure, high blood pressure, arrhythmia, and various digestive ailments (*9*), as well as geriatric and antiarteriosclerosis remedies(*11*). It has been documented that it decreased the serum total cholesterol, LDL-C, and triglyceride in hyperlipidemic humans (*12*). In hawthorn fruit, the active ingredients mainly contained flavonols (quercetin, hyperoside, rutin, and vetixin), flavanols (epicatechin), phenylpropanoids (chlorogenic acid), triterpenes (ursolic acid, oleanolic acid, and maslinic acid) and other ingredients. It was recorded that flavonoids and triterpenes were the active ingredients of hypolipidemic (*15*), but it is not clear whether these ingredients can inhibit the HMGR activity and lower the lipid level in blood of mice.

To confirm the hypolipidemic activity and mechanism of action, the inhibitory activity of various fractions from hawthorn fruit to HMGR was traced. We separated and purified the EtOH extract of hawthorn fruit by systematic chromatographic methods using HMGR as the target to obtain and identify the active compounds with high inhibitory activity to HMGR. The lipid-lowering efficacy of these components was verified by animal experiments.

From **Table 1**, the IR and the total inhibitory activity of crude extract with EtOH were about 5.1% and 34680, respectively. After being purified with D-101 macroporous adsorption resin, four main active fractions (fractions A, F, G, and H) were obtained. Their total weight was 128.8 g, and the total inhibitory activity was 33225.6, which was near that of crude extract. After further purification with different column chromatography to these four active fractions, we observed that the loss of weight was about 30%, but the loss of inhibitory activity was up to 70% (**Table 2**). This indicated that further purification to crude extract weakened the inhibitory activity of active components to HMGR, and it was speculated that there was a synergetic effect among the active components. From the chromatographic fractions, four compounds were obtained by recrystallization in MeOH and identified as quercetin (**a**), hyperoside (**b**), rutin (**c**), and chlorogenic acid (**d**), which indicated that flavonoids were the active ingredients with a high inhibitory activity to HMGR in hawthorn fruit.

To further investigate the synergetic effect on the HMGR activity, we compared the IR values of **a**, **b**, **c**, and **d** and their mixture matched according to the ratio in crude extract. It was discovered that the IR of the mixture to HMGR was up to 79.5%, much higher than that of any of the single compounds and the total IR of their sum (50.01%). In addition, ursolic acid, oleanolic acid, and maslinic did not inhibit the activity of HMGR (data not shown). Although triterpenes, especially ursolic acid, were reported as the main active hypolipidemic constituents, the present results indicate triterpenes could lower lipid (*15*) not by the mechanism of inhibiting HMGR activity.

Table 4. Lipid-Lowering Efficacy of Compounds **a**−**d** and Mixture (*n* = 10, Mean ± SD)

group	dose (mg kg^{-1})	TC^a (mmol L^{-1})	TG^a (mmol L^{-1})	LDL-C^a (mmol L^{-1})	HDL-C^a (mmol L^{-1})
normal control		2.40 ± 0.25	0.79 ± 0.11	1.49 ± 0.08	1.56 ± 0.10
hyperlipidemic control		6.58 ± 0.53##	3.04 ± 0.41##	4.59 ± 0.74##	1.34 ± 0.13#
compound **a**	2.85	4.12 ± 0.65*	1.66 ± 0.33*	2.17 ± 0.30	1.47 ± 0.23
compound **b**	2.85	5.13 ± 0.67	1.83 ± 0.57	2.56 ± 0.56	1.38 ± 0.26
compound **c**	2.85	5.88 ± 0.64	2.54 ± 0.48	3.28 ± 0.34	1.40 ± 0.34
compound **d**	2.85	6.15 ± 0.60	2.87 ± 0.60	3.77 ± 0.44	1.39 ± 0.14
mixture^b	2.85	3.52 ± 0.18**	1.53 ± 0.32**	1.92 ± 0.25**	1.51 ± 0.12

^{a} #, $p < 0.05$, ##, $p < 0.01$ versus normal control group; *, $p < 0.05$, **, $p < 0.01$ versus hyperlipidemic control group. ^{b} The ratio of compounds **a**−**d** in the mixture was 0.16:0.32:1.42:0.95.

Table 5. IR of Compounds **a**−**c** to HMG-CoA Reductase

	quercetin	hyperoside	rutin
concentration (mg/mL)	1	1	1
IR^a (%)	39.6	30.4	27.1

^{a} IR indicates the inhibitory rate to HMGR at the concentration of 1 mg/mL.

The result of animal tests in vivo further confirmed that the lipid-lowering efficacy of the matched mixture was better than that of every monomer and showed a significant difference ($p < 0.01$). Therefore, compounds **a**−**d** had not only the inhibitory activity to HMGR in vitro but also the lipid-lowering efficacy in vivo. The better lipid-lowering activity of compounds **a**−**d** was realized by their synergetic inhibition to HMGR.

Structure−activity relationship study revealed that the inhibitory activities to HMGR and lipid-lowering efficacy were related with the glycosyl numbers in compounds **a**−**c**. Quercetin without a glycosyl group showed the highest activity, then hyperoside with a monoglycoside. Therefore, the weak hydrophilic ability benefits the inhibition to HMGR and lipid-lowering efficacy.

ABBREVIATIONS USED

HMGR, hydroxy-3-methylglutaryl coenzyme A reductase; TC, total cholesterol; LDL-C, low-density lipoprotein cholesterol; TG, triglyceride; HDL-C, high-density lipoprotein-cholesterol; HFHC, high fat and high cholesterol; NADPH, nicotinamide adenine dinucleotide phosphate; DTT, 1,4-dithiothreitol; EDTA, ethylenediaminetetraacetic acid; SAR, structure−activity relationship.

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