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Effects of Mulberry (*Morus alba* L.) Extracts on Lipid Homeostasis in Vitro and in Vivo

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The objective of this study was to investigate the lipid-lowering effects of mulberry water extracts (MWEs). To evaluate the hypolipidemic effect of MWEs, hamsters were fed with either high fat/cholesterol diets (HFCD) or HFCD supplemented with 1 and 2% MWEs for 12 weeks. Plasma total cholesterol (TC) and triglyceride (TG) levels of hamsters fed HFCD with MWEs were significantly reduced by about 30−37% and 16−35%, respectively, as compared to those without MWEs. Similar results were also measured in hepatic TC and TG of hamsters fed HFCD with MWEs. Low-density lipoprotein receptor (LDLR) gene expression and the uptake ability of low-density lipoprotein (LDL) in HepG2 cells were also upregulated by additions of MWEs. MWEs also decreased the gene expressions of enzymes involved in the TG and TC biosyntheses. Results suggest that hypolipidemic effects of MWEs are via an enhancement of LDLR gene expression and the clearance ability of LDL and a decrease in the lipid biosynthesis. Therefore, MWEs could be used as a natural agent against hyperlipidemia.

KEYWORDS: Mulberry extracts; hyperlipidemia; LDL receptor; HMG-CoA reductase; SREBP-1c; SREBP-2

INTRODUCTION

Mulberry, the fruit of *Morus alba* L., is traditionally used in Chinese medicines. It shows some pharmacological effects, such as a fever reduction, liver protection, eyesight improvement, joint strength, urine excretion, blood pressure reduction, and prevention of cardiovascular diseases. Mulberry contains high amounts of water-soluble anthocyanins, which are the most important antioxidative compounds in the plant kingdom (*1*). Recent studies demonstrated that anthocyanins possessed potential biological and pharmacological benefits, such as antioxidant activities and antitumor properties (*2*, *3*). Although mulberry is rich in anthocyanins, the hypolipidmemic effects of mulberry are still not well-documented. Thus, we believe that the hypolipidemic effects of the water extracts from mulberry (MWEs) are worthy of investigation.

A high-fat/cholesterol diet (HFCD) causes an unbalanced lipoprotein metabolism and leads to hyperlipidmemia, characterized as high levels of serum triglyceride (TG) and total cholesterol (TC), as well as a low ratio of HDL/TC (*4*). Higher serum low-density lipoprotein (LDL) results in an increased risk of atherosclerosis and cardiovascular diseases (*5*). Many epidemiological, clinical, and experimental studies indicated that reducing high serum LDL is an effective way to prevent arthrosclerosis and cardiovascular diseases (*6*). Increased hepatic low-density lipoprotein receptor (LDLR) expression results in improved clearance of plasma LDL, which benefits cardiovascular health in humans.

Hepatic LDLR expression regulates LDL cholesterol homeostasis through receptor-mediated endocytosis (*7*, *8*). LDLR gene expression is predominantly regulated in the transcriptional level through a negative feedback mechanism by the intracellular cholesterol pool. This regulation is controlled through the two-step proteolysis of sterol regulatory element-binding proteins (SREBPs) (*9*). In normal levels of intracellular cholesterol, SREBPs reside in the endoplasmic reticulum (ER) and associate with another transmembrane protein, SREBP-cleavage activating protein (SCAP), which provides conditional chaperone activity to the SREBPs. SCAP contains a cholesterol-sensing domain, which responds to the depletion of sterol, thus activating the SCAP-SREBP transporting activity (*10*). The active forms of the SREBPs translocate to the nucleus, bind to its cognate sterol-regulatory element (SRE-1) site of the LDLR promoter, and activate transcription of the LDLR gene (*11*). Inhibition of cholesterol biosynthesis leads to a depletion of intracellular cholesterol and an activation of SCAP-SREBPs transporting activity, thereby resulting in an enhancement of the LDLR expression and subsequently the clearance of LDL from blood (*12*). 3-Hydroxy-3-methylglutaryl coenzyme A reductase (HMG-CoA reductase) is the rate-limiting enzyme in cholesterol biosynthesis. Hence, reducing HMG-CoA reductase expression could attenuate hyperlipidemia via an increase of LDLR expression (*13*).

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SREBPs regulate lipid metabolism (*14*). SREBP-1c controls the transcription of genes involved in fatty acid and TG syntheses (*15*), whereas SREBP-2 regulates the transcription of genes involved in cholesterol biosynthesis (*16*). SREBP-1c and SREBP-2 are structurally related proteins that control lipid homeostasis. SREBP-1c increases the transcription of gene-involved enzymes of hepatic fatty acid synthesis, including fatty acid synthase (FAS) (*17*) and glycerol-3-phosphate acyltransferase (GPAT), a gene involved in the production of TGs (*18*). SREBP-2 targets the enzymes of cholesterol biosynthesis and metabolism, that is, LDLR, HMG-CoA reductase, and SREBP-2 itself (*19*, *20*).

The present study was focused on two aspects of the hypolipidemic effects of MWEs. First, the attenuation of hyperlipidemia in hamsters by supplementing MWEs into HFCD was revealed. Second, human hepatocarcinoma, HepG2 cells, were applied to observe that MWEs increased the LDLR gene expression and its clearance ability of LDL; meanwhile, mediated intracellular lipids biosynthesis was mediated through SREBPs gene expressions.

MATERIALS AND METHODS

Materials. Tris-base, sodium dodecyl sulfate (SDS), NaCl, bovine serum albumin (BSA), Nonidet P-40, gallic acid, and sodium deoxycholat were purchased from Sigma-Aldrich (St. Louis, MO). Phosphate buffer solution (PBS), trypsin-EDTA, fetal bovine serum, and powdered Dulbecco's modified Eagle's medium (DMEM) were purchased from Gibco/BRL (Gaithersburg, MD). Antibody against FAS proteins was purchased from BD Transduction Laboratories (San Diego, CA). Antibody against HMG-CoA reductase proteins was purchased from Millipore Co. (Billerica, MA).

Preparation and Analysis of Nutrient Compositions of MWEs. MWEs were prepared from the fruit of *M. alba* L. (mulberry), which were obtained from the Taichung District Agricultural Research Station in Tai-Pin, Taichung, Taiwan. Briefly, the fruit (100 g) was macerated and stirred with water (200 mL), and the juice was filtered, centrifuged (3000 rpm, 15 min), and concentrated under reduced pressure at 30 °C. Then, the aqueous extract was lyophilized (−80 °C, 12 h) to obtain MWEs and stored at −20 °C before use. The concentration of total polyphenol was analyzed according to a Folin−Ciocalteu assay. Briefly, MWEs (0.1 mg) were dissolved in a test tube with 1 mL of distilled water, and Folin−Ciocalteu reagent (2 N, 0.5 mL) was then added and mixed thoroughly. After an interval of 3 min, 3 mL of 2% Na_2CO_3 solution was added, and the mixture was allowed to stand for 15 min with intermittent mixing. The absorbance of the mixture at 750 nm was measured on a Hitachi U-3210 spectrophotometer (Hitachi, Tokyo, Japan) with gallic acid as the standard. Crude protein was determined by Kjeldahl assay (*21*). The total fat content was determined on a gravimetric basis according to the Soxhlet extraction method (*21*). The total carbohydrate was determined by the phenol−sulfuric acid method (*21*). All of the results were reported as the mean value of at least three replicates.

High-Performance Liquid Chromatography/Electrospray Ionization/Tandem Mass Spectrometry (HPLC/ESI/MS/MS) Analysis of MWEs. Separation of anthocyanins was conducted on a Luna C18(2) column (2.00 mm × 150 mm, 3.0 μm, Phenomenex, Inc., Torrance, CA) using an HPLC system consisting of a Finnigan Surveyor module separation system and a photodiode array (PDA) detector (Thermo Electron Co., MA). A linear gradient from 98% A to 30% B in 45 min was used for the HPLC analysis of anthocyanins. Solvent A was water containing 1% formic acid, and solvent B was acetonitrile containing 0.1% formic acid. The flow rate was 0.2 mL/min. Absorption spectra of anthocyanins were recorded from 240 to 600 nm with the in-line PDA detector. The chromatogram was monitored at 518 nm (*22*). The system was coupled to a Finnigan LCQ Advantage MAX ion trap mass spectrometer and was operated in ESI with positive ionization mode. Samples of 20 μL of extracts were directly injected into the column using a Rheodyne (model 7725i) injection valve. The ion trap instrument was operated at the following settings: capillary voltage; nitrogen was used as the nebulizing and drying gas. The typical operating parameters were as follows: spray needle voltage, 4 kV; ion transfer capillary temperature, 280 °C; nitrogen sheath gas, 45; and auxiliary gas, 0 (arbitrary units). Mass spectra were acquired in a m/z range of 150−1000, with five microscans and a maximum ion injection time of 200 ms. The SIM analysis was a narrow scan event that monitored the m/z value of the selected ion, in a range of 1.0 Th centered on the peak for the molecular ion; this function was used in the analysis of molecular ions of the anthocyanidins. For MS/MS analysis, helium collision gas was introduced in accordance with the manufacturer's recommendations. The MS/MS fragment spectra were produced using normalized collision energies with an increment of 30% and also with wideband activation "off" (*23*).

Animal Experiment. To establish hyperlipidemia of hamsters, male Gold Syrian hamsters (90−100 g) were housed in colony cages (eight animals per cage) in a room at controlled temperature (25 ± 2 °C) and a 12 h light/12 h dark cycle. The chow (Laboratory Rodent Diet 5001, PMI Nutrition International/Purina Mills LLC, United States) was added with 1% cholesterol and 10% corn oil to obtain the HFCD. Hamsters were divided into four groups: (1) standard chow (control group), (2) HFCD (HFCD group), (3) HFCD supplemented with 1% (w/w) MWEs (1% MWEs group), and (4) HFCD supplemented with 2% (w/w) MWEs (2% MWEs group). All hamsters were fed assigned diets and water ad libitum. Food and water were replenished everyday. After 12 weeks, animals were sacrificed by decapitation. Blood samples and liver tissue were collected, and serum was prepared and stored at −80 °C until analysis. Plasma and liver TC and TG were measured by enzymatic colorimetric methods using commercial kits (Boeheringer Mannheim, Germany). All animals used were handled according to the guidelines of the Instituted Animal Care and Use Committee of Chung Shan Medical University for the care and use of laboratory animals.

Cell Culture and Treatment. HepG2, a kind of human hepatocarcinoma cells was obtained from BCRC (Food Industry Research and Development Institute in Hsin-Chu, Taiwan). Cells were cultured in 90% DMEM medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, and 100 U/mL penicillin. Cells were maintained at 37 °C in a humidified atmosphere of 5% CO_2. To avoid serum lipoproteins interfering with intracellular gene expression, cells were incubated in serum-free DMEM medium supplemented with or without MWEs at experiments. In this study, to avoid cytotoxicity in cell viability, we treated cells with moderate concentrations of MWEs less than 6 mg/mL. Under these conditions, the viability of HepG2 cells was not affected as determined by the MTT assay (data not show).

Reverse Transcription-Polymerase Chain Reaction (RT-PCR). HepG2 cells were plated in culture dishes at a density of 4×10^5 cells/mL. To avoid the interference of lipoprotein from serum, cells were cultured in serum-free DMEM medium and treated with the indicated concentrations of MWEs for 24 h. Total RNA was isolated from the HepG2 cells by using the protocol described by RNeasy Mini Kits (Qiagen, Valencia, CA). For reverse transcription, 4 mg of total cellular RNA was used as templates in a 20 μL reaction containing 4 μL of dNTPs (2.5 mM), 2.5 mL of Oligo dT 20 (10 pmol/mL), and RTase (200 U/mL), and the reaction was performed at 42 °C for 1 h. Afterward, 5 μL of cDNA product was used as the template in PCR amplifications together with the appropriate primers [for LDL receptor, 5′-CAATG TCTCA CCAAG CTCTG-3′ (forward) and 5′-TCTGT CTCGA GGGGT AGCTG-3′ (reverse); HMG-CoA reductase, 5′-AGGTT CCAAT GGCAA CAACA GAAG-3′ (forward) and 5′-ATGCT CCTTG AACAC CTAGC ATCT-3′ (reverse); FAS, 5′-TACAT CGACT GCATC AGGCA-3′ (forward) and 5′-GATAC TTTCC CGTCG CATAC-3′ (reverse); SREBP-1c, 5′-GGATT GCACT TTCGA AGACA TG-3′ (reverse) and 5′-AGGAT GCTCA GTGGC ACTG-3′ (forward); SREBP-2, 5′-TGGCT TCTCT CCCTA CTCCA-3′ (forward) and 5′-GCAGC TGCAA AATCT CCTCT-3′ (reverse); GPAT, 5′-GCCGC TTCTG TTTCT ACCAG-3′ (reverse) and 5′-ACACC GGTTT CTGAC TTTGG-3′; GAPDH, 5′-CGGAGTCAACGGATTTGGTCGTAT-3′ (forward) and 5′-AGCCTTCTCCATGGTTGGTGAAGAC-3′]. The final products were analyzed on 1% agarose gels and detected by ethidium bromide staining. The values of SREBP-2, HMG-CoA reductase, LDLR, SREBP-1c, FAS, and GPAT mRNA were normalized to the value of GAPDH, and values for the HepG2 cells with 1.0, 3.0, 6.0 mg/mL MWEs treatments were expressed relatively to the average values for HepG2 cell without MWEs treatment, which was set to 1.0.

Western Blot Analysis. To analyze the expression of proteins, HepG2 cells (4×10^5 cells/mL) cultured in serum-free DMEM medium were

incubated with MWEs for the indicated concentration for 24 h. Cells were washed with ice-cold PBS and lysated in buffer (50 mM Tris-base, pH 8.0, 150 mM NaCl, 0.5% sodium deoxycholate, 1% Nonidet P-40, and 0.1% SDS) plus protease inhibitor cocktail. Cell lysates (50 μg of protein) were mixed with a sample buffer and boiled for 10 min. Then, an equal protein content of total cell lysate from control and MWE-treated samples was resolved on 8% SDS-polyacrylamide gel electrophoresis (PAGE) gels. Proteins were then transferred onto nitrocellulose membranes (Millipore, Bedford, MA) by electroblotting using an electroblotting apparatus (Bio-Rad). Nonspecific binding of the membranes was blocked with Tris-buffered saline (TBS) containing 5% (w/v) nonfat dry milk and 0.1% (v/v) Tween-20 (TBST) for more than 2 h. Membranes were washed with TBST three times for 10 min and incubated with an appropriate dilution of specific primary antibodies in TBST overnight at 4 °C. Subsequently, the membranes were washed with TBST and incubated with an appropriate secondary antibody (horseradish peroxidase-conjugated goat antimouse or antirabbit IgG) for 1 h. After the membrane was washed three times for 10 min in TBST, band detection was revealed by enhanced chemiluminescence using ECL Western blotting detection reagents and exposed ECL hyperfilm in FUJIFILM Las-3000 (Tokyo, Japan). Then, proteins were quantitatively determined by densitometry using FUJFILM-Multi Gauge V2.2 software. The values of HMG-CoA reductase and FAS protein were normalized to the value of β-actin, and values for the HepG2 cells with 1.0, 3.0, and 6.0 mg/mL MWEs treatments were expressed relatively to the average values for HepG2 cell without MWEs treatment, which was set to 1.0.

1,1′-Dioctadecyl-3,3,3′,3′-tetramethyl-indocarbocyanine Perchlorate-LDL (Dil-LDL) Staining and Flowcytometery. Dil-LDL (Molcular Probes), which the LDL labeled with the fluorescent probe, was used to visualize LDL binding sites in cultured cells or to screen for cell mutants defective in the expression of LDL receptors. HepG2 cells (2×10^4 cells/mL) treated with MWEs of the indicated concentrations for 24 h were then treated with Dil-LDL for 4 h at 37 °C to allow Dil-LDL uptake. After treatment, cells were washed and analyzed by flow cytometry (FACScalibur, BD Biosciences) using CellQuestTM Pro software (*24*). The percentage of LDL uptake abilities of HepG2 cell with 1.0, 3.0, and 6.0 mg/mL MWEs treatments was normalized to the value of HepG2 cell without MWEs treatment, which was set to 100%.

Statistical Analysis. The experiment was conducted using a completely random design (CRD). Data were analyzed using analysis of variance (ANOVA). A significant difference was used at the 0.05 probability level and differences between treatments were tested using the least significant difference (LSD) test. All statistical analyses of data were performed using SAS.

Table 1. Nutrient Compositions of MWEs

MWEs	%
protein	19.3
fat	—[a]
carbohydrate	54.4
total polyphenol	10.3

[a] Not detectible.

RESULTS AND DISCUSSION

Nutrient Compounds of MWEs. The nutrient compositions of MWEs are demonstrated in **Table 1**. The protein, fat, carbohydrate, and total polyphenol of MWEs are 19.3, 0, 54.4, and 10.3%, respectively. As we know, protein and carbohydrate are not bioactive compounds for lipid-lowering effects. Thus, we assumed that the polyphenols are major bioactive compounds for lipid-lowering effects in MWEs. Many studies showed that mulberry is rich in anthocyanins, belonging to polyphenols. Therefore, we assayed the anthocyanic compositions of MWEs via a determination of HPLC/ESI/MS/MS. **Figure 1** illustrates the UV chromatograms of the HPLC data of MWEs. Further analysis of the numbered peaks via LC-ESI/MS/MS identified the anthocyanic compounds in MWEs as cyanidine-3-glucoside, cyanidine-3-rutinoside, pelargonidin-3-glucoside, and pelargonidine-3-rutinoside

Table 2. Retention Time, UV−vis, and Mass Spectral Characteristics for the Anthocyanic Compositions of MWEs

peak[a]	compound	R_t[b] (min)	λ (nm)	$[M + H]^+$[c]	MS^2[d]
1	cyanidine-3-glucoside	24.6	512, 279, 244, 327	448	287
2	cyanidine-3-rutinoside	25.5	515, 282, 245, 326	595	448, 287
3	pelargonidin-3-glucoside	26.8	496, 270, 432 sh	432	271
4	pelargonidine-3-rutinoside	27.6	502, 279, 433 sh	578	271, 432

[a] Peak numbering is as in **Figure 1**. [b] R_t, retention time. [c] $[M + H]^+$, positively charged molecular ion. [d] Product ion analysis of molecular ion.

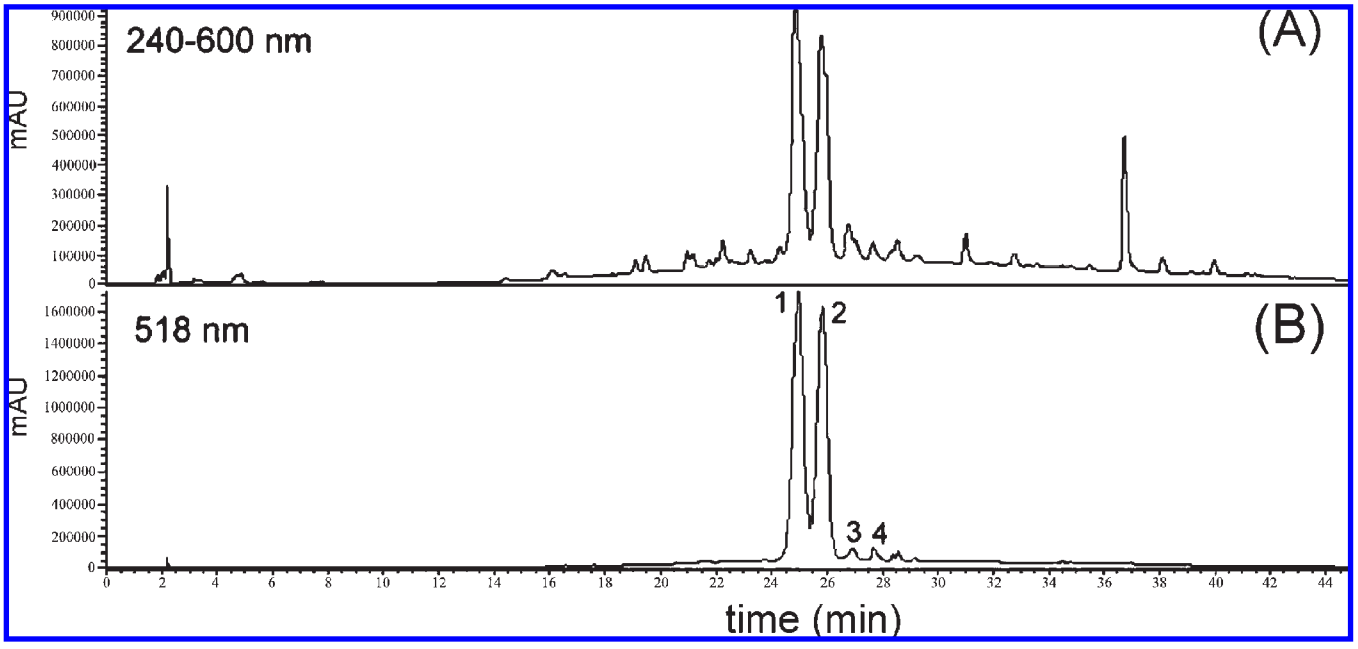

Figure 1. HPLC/UV chromatogram of MWEs. Chromatograms were monitored at scan ranges of 240−600 (**A**) and 518 nm (**B**), which correspond to the identified anthocyanic compositions. Anthocyanic compositions corresponding to peaks 1−4 in panels **A** and **B** are marked. Peaks: 1, cyanidine-3-glucoside; 2, cyanidine-3-rutinoside; 3, pelargonidin-3-glucoside; and 4, pelargonidine-3-rutinoside.

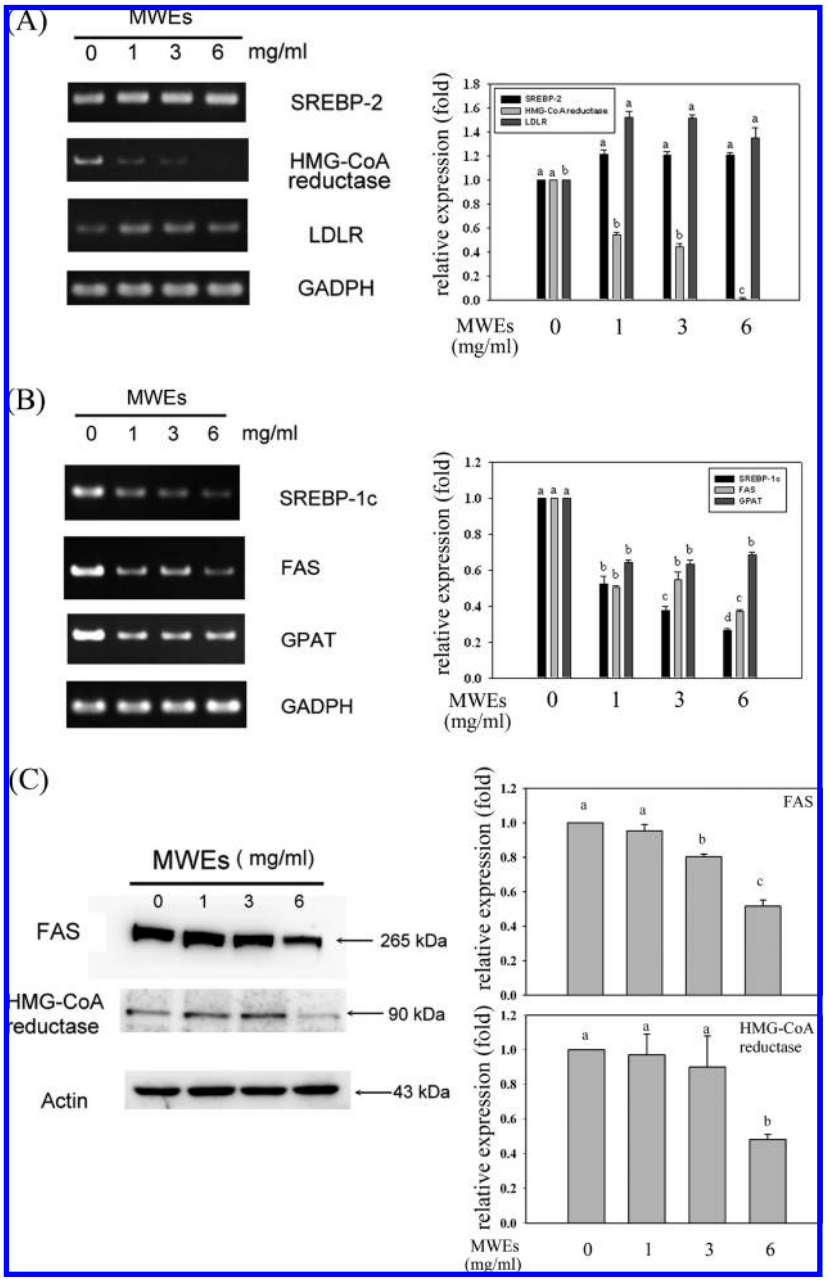

Figure 2. SREBP-2, HMG-CoA reductase, and LDLR mRNA expressions (**A**); SREBP-1c, FAS, and GPAT mRNA expressions (**B**); and FAS and HMG-CoA reductase protein expressions (C) of HepG2 cells as affected by MWEs. The data are given as means ± SDs ($n = 3$). Mean values in each target gene or protein expressions with different letters indicate a significant difference ($p < 0.05$).

Table 3. Effect of MWEs on Plasma and Hepatic TG, Cholesterol Levels, and Risk Ratio in HFCD-Fed Hamsters[a]

	plasma TG	plasma TC	plasma HDL	plasma LDL	LDL/HDL	hepatic TC	hepatic TG
control	87.14 ± 8.59 c	110.57 ± 12.80 d	92.86 ± 8.09 c	14.71 ± 1.80 d	0.16 ± 0.02 d	26.86 ± 2.65 d	317.46 ± 64.58 c
HFCD	250.00 ± 17.32 a	358.57 ± 30.24 a	143.57 ± 26.41 b	139.29 ± 16.94 a	1.00 ± 0.20 a	112.74 ± 7.50 a	657.14 ± 107.52 a
1% MEWs	175.00 ± 16.58 b	300.00 ± 19.80 b	183.86 ± 19.73 a	102.14 ± 23.25 b	0.56 ± 0.11 b	87.00 ± 12.95 b	569.00 ± 48.07 b
2% MEWs	159.29 ± 22.63 b	233.57 ± 30.92 c	166.43 ± 18.87 a	60.71 ± 16.67 c	0.37 ± 0.15 c	72.57 ± 10.55 c	526.86 ± 58.61 b

[a] Data are given as means ± SD ($n = 8$). Mean values with different letters in each testing parameter were significantly different ($p < 0.05$). HDL, high-density lipoprotein.

with retention times of 24.6, 25.5, 26.8, and 27.6 min, respectively, as summarized in **Table 2**.

Although major compositions of MWEs were carbohydrate and protein (Table**1**); however, polyphenols are mentioned as the major bioactive component in berry fruit extract, which owns lipid-lowering effects in the literature (*25*, *26*). Several literatures showed that fruit contains polyphenols, which demonstrated very good health characteristics (*27*, *28*). Haruenkit et al. (*27*) analyzed nutritional values of three tropic fruits, durian, mangosteen, and snake fruit, and durian contains the highest total polyphenols. Meanwhile, durian showed the best cholesterol-lowering and antioxidant effect in high cholesterol-fed rats than the other two fruits. In a comparison of total polyphenols in our MWEs and durian, much higher total polyphenols were detected in our

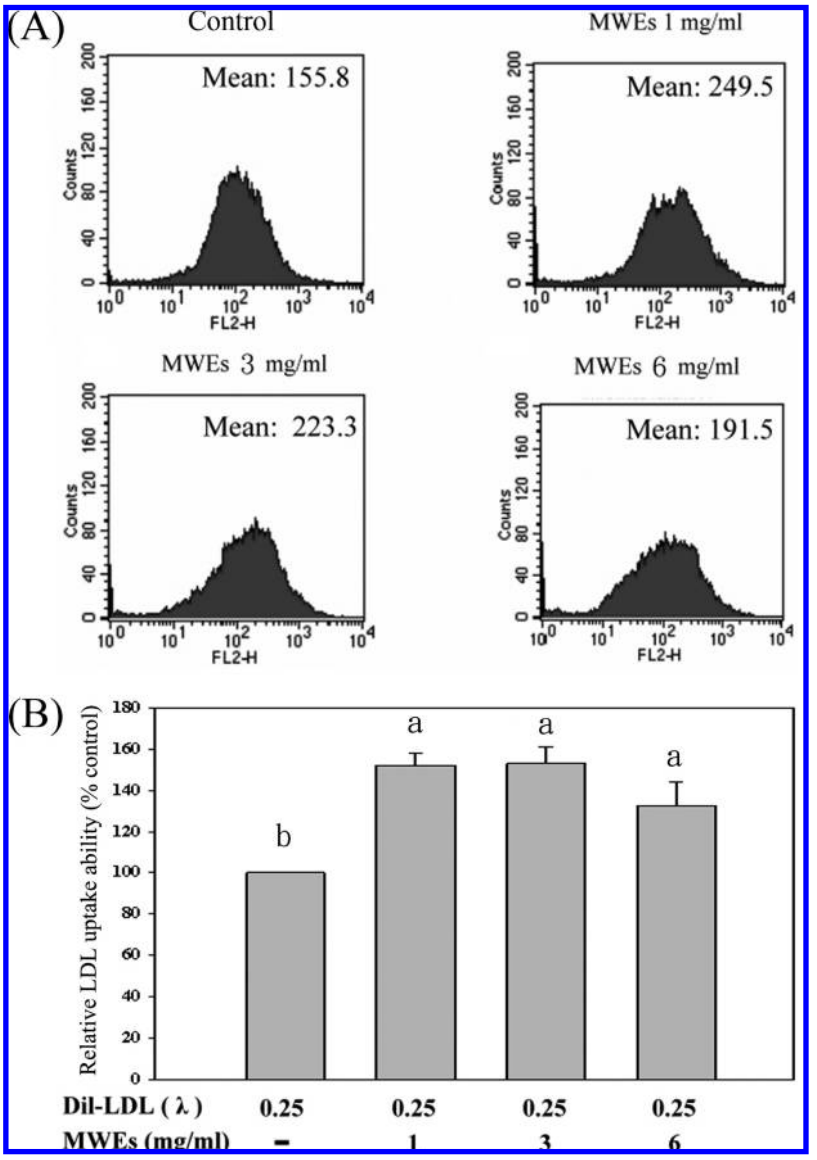

Figure 3. LDL uptake ability in HepG2 cells treated with MWEs. The data are given as means ± SDs ($n = 3$). Mean values in bars with different letters indicate a significant difference ($p < 0.05$).

MWEs [10300 mg gallic acid equivalent (GAE)/100 g dried MWEs vs 309.7 mg GAE/100 g durian]. Besides, anthocyanin belongs to polyphenols and is a natural red food colorant. **Table 2** and **Figure 1** also show that mulberry contains high amounts of anthocyanins where cyanidine-3-glucoside and cyanidine-3-rutinoside are the major components. Some metabolites of cyaniding-3-glucoside are produced by the intestinal microflora in rat, such as protocatechuic acid (*29*). Thus, we believe that the lipid-lowering effects of polyphenol-rich MWEs in a high-fat/cholesterol habit warrant a further investigation.

Hypolipidemic Effects of MWEs in HFCD-Fed Hamsters. To verify the hypolipidemic effects of MWEs in vivo, an animal study was conducted in hamsters fed with HFCD. After 12 weeks, the plasma TG level was increased ($p < 0.05$) in the HFCD-fed hamsters (HFCD, 1% MEW, and 2% MEW groups) rather than the control group but was decreased ($p < 0.05$) by about 30 and 37% in the HFCD-fed hamsters supplemented with 1 and 2% MWEs, respectively (**Table 3**). Similarly, increased ($p < 0.05$) plasma TC and LDL were observed in the HFCD-fed hamsters when compared to the control group at the end of experiment. Meanwhile, plasma TC and LDL levels in the HFCD-fed groups with MWEs were also lower ($p < 0.05$) than those without MWEs. In addition, MWEs increased ($p < 0.05$) HDL levels in HFCD-fed groups, which resulted in lower LDL/HDL ratios. HFCD resulted in increased ($p < 0.05$) hepatic TC and TG levels as compared to the normal diet (control group) (**Table 3**). However, hepatic TC and TG in HFCD-fed groups were reduced ($p < 0.05$) by supplementing MWEs.

Phenol-rich green, oolong, and black tea extracts show a hypolipidemic effect, where it could be explained by lowest fat absorption in rats drinking green tea extracts (*30*). Hypolipidemic and antiatherogenic effects of apple phenols are mainly via a promotion of cholesterol catabolism and inhibition of intestinal absorption of cholesterol (*31*), while apple phenols exert an improvement of lipid profiles (HDL and non-HDL/HDL) by supplementation (*32*). Similar results were demonstrated in our study. Plasma lipids and cholesterol profiles in HFCD-fed hamsters were improved by supplementing MWEs (**Table 2**). The plasma TC was decreased when HFCD-fed hamsters supplemented with 1 and 2% MWEs by 16.33 and 34.86%, respectively, were compared to the HFCD group. The same effects of lowering plasma TG were observed in HFCD hamster supplemented MWEs (plasma TG reduction: 1% MWEs, 30.00%; 2% MWEs, 36.28%). Yang, Wang, and Chen (*30*) also reported that

phenol-rich green, oolong, and black tea extracts decreased hepatic TG contents, which resulted from the less fat absorption. Although HFCD significantly increased hepatic TC and TG contents in HFCD-fed hamsters, MWEs supplementations indeed reduced ($p < 0.05$) hepatic TC and TG contents (**Table 3**).

Effects of MWEs on Gene and Protein Expressions of Enzymes Related to Lipid Metabolism, as Well as the Uptake Ability of LDLR. To investigate the mechanism of MWEs on lipid homeostasis, the HepG2 cells were applied in the present study. In the presence of 1.0, 3.0, and 6.0 mg/mL of MWEs, a higher ($p < 0.05$) LDLR gene expression was observed as compared to the absence of MWEs (**Figure 2A**), whereas the lower ($p < 0.05$) HMG-CoA reductase gene expressions resulted from additions of MWEs (**Figure 2A**). However, MWEs showed no ($p > 0.05$) effects on SERBP-2 gene expressions. In TG biosynthesis, SREBP-1c, FAS, and GPAT gene expressions were downregulated ($p < 0.05$) by additions of MWEs (**Figure 2B**). Besides, the largest ($p < 0.05$) reductions of SREBP-1c and FAS gene expression were detected in the presence of 6 mg/mL MWEs. We further examined the effects of MWEs on the protein expressions of HMG-CoA reductase and FAS via the HepG2 cell model. Data revealed that the suppressions ($p < 0.05$) in protein expression of HMG-CoA reductase and FAS in HepG2 cells were detected until treatment of 3 and 6 mg/mL of MWEs, respectively, as compared to those without MWEs treatment (**Figure 2C**). Because of increased LDLR gene expression in HepG2 cell treated with MWEs, the LDLR activity was determined by the Dil-LDL uptake ability assay via a flow cytometric analysis. The results showed that MWEs increased ($p < 0.05$) the uptake ability of LDLR, which elevates the receptor-mediated endocytosis of LDL (**Figure 3**). The percentages of LDL uptake ability in HepG2 cells as shown were enhanced by approximately 55, 55, and 36% in the presence of 1.0, 3.0, and 6.0 mg/mL MWEs, respectively.

The HepG2 cell line was first introduced in 1979 (*33*). Dietschy indicated that a regulation of hepatic LDLR and HMG-CoA reductase activity could be observed in HepG2 cells (*34*). Hence, previous literature also applied the HepG2 cell line to study lipid metabolism (*35*−*37*). Next, the hypolipidemic mechanism of MWEs is explored via a HepG2 cell model. As we know, the lipid metabolism in mammals is highly related to expressions of serum cholesterol clearance (i.e., LDLR), TC biosynthesis (i.e., SREBP-2 and HMG-CoA reductase), and TG biosynthesis (i.e., SREBP-1c, FAS, and GPAT). Increased LDLR gene expression and LDL uptake ability in HepG2 cells were analyzed in MWEs treatment groups, which could explain the fact of lower plasma cholesterol and improved cholesterol profile (**Figures 2A** and **3** and **Table 3**). SREBP-1c increases the transcription of gene-involved enzymes of hepatic fatty acid synthesis, including FAS (*17*) and GPAT, a gene involved in the production of TGs (*18*). MWEs also downregulated SREBP-1c gene expression (**Figure 2B**), which may affect the following lipogenic enzymes' expressions. HMG-CoA reductase, FAS, and GPAT are rate-limiting enzymes in cholesterol, fatty acid, and TG biosyntheses, respectively (*13*, *17*, *18*). Meanwhile, decreased HMG-CoA reductase and FAS gene and protein expressions by supplementing MWEs were demonstrated in **Figure 2**, which are associated with the lower hepatic TC and TG contents of HFCD-fed hamsters supplemented with MWEs (**Table 3**).

In conclusion, the hypolipidemic effects of polyphenol-rich MWEs could account for decreased HMG-CoA reductase, FAS, and GPAT expressions; meanwhile, increased LDLR expression and uptake ability. The precious data in this study not only benefit further health research but also suggest that MWEs are valued to develop as an ingredient of hypolipidemic agents in further health markets.

ABBREVIATIONS USED

DiI-LDL, 1,1′-dioctadecyl-3,3,3′,3′-tetramethyl-indocarbocyanine perchlorate-LDL; FAS, fatty acid synthase; GAE, gallic acid equivalent; GPAT, glycerol-3-phosphate acyltransferase; HFCD, high-fat/cholesterol diets; HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; LDLR, low-density lipoprotein receptor; MWEs, mulberry water extracts; SCAP, SREBP-cleavage activating protein; SREBPs, sterol regulatory element-binding proteins; TC, total cholesterol; TG, triglyceride.

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