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ARTICLE

# The Hypolipidemic Effect of *Hibiscus sabdariffa* Polyphenols via Inhibiting Lipogenesis and Promoting Hepatic Lipid Clearance

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*Hibiscus sabdariffa* extract (HSE) was shown to lower the plasma lipid level and reduce the liver damage. In the present study, we investigated if *Hibiscus sabdariffa* polyphenols (HPE) exerted a hypolipidemic effect and its putative mechanism on liver. HPE exhibited more potency to decrease plasma cholesterol and LDL cholesterol than the crude extract HSE, and increased HDL cholesterol dose-dependently. It decreased the lipid content of hepatocyte through the activation of AMPK and reduction of SREBP-1, thus inhibiting the expression of fatty acid synthase and HMG-CoA reductase. LDLR and LDL binding of HepG2 cells were enhanced when treated with HPE. In conclusion, HPE is worthy of being further investigated and could be developed as an adjunctive for hepatic lipid control and hypolipidemic therapy.

**KEYWORDS: *Hibiscus sabdariffa* polyphenols; LDL; LDL receptor; AMP-activated protein kinase; sterol regulatory element binding protein**

## INTRODUCTION

Non-alcoholic fatty liver (NAFLD) is generally considered to be the liver component of metabolic syndrome, which is frequently accompanied with obesity, dislipidemia, and insulin resistance (*1*). It was estimated that over 20% of the adult population in developed countries have NAFLD, whose occurrence increased even among children. Liver plays an essential role of regulating plasma lipid level through low density lipoprotein (LDL) clearance and high density lipoprotein (HDL) recruitment. However, the lipid uptake must affect the hepatic fat composition and burden the liver function. The degree of fat infiltration of liver is related to the subsequent development of necrosis, inflammation, cirrhosis, and the propensity to progress to hepatocellular carcinoma (*2*). It revealed that obesity induced both steatosis and lymphocyte chemotaxis in the liver. The fatty liver index independently associated with the inflammatory cytokines IL-6 and TNF-α. This evidence suggests that the regulation of hepatic lipid metabolism should be emphasized for preventing dislipidemia and the accompanying illness (*3*).

Sterol regulatory element binding protein (SREBP) is a membrane-bound transcription factor which regulates lipid metabolism and consists of three isoforms as SREBP-1a, -1c and -2 (*4*). In sterol-deficient mice, proteolytic cleavage of SREBPs occurred, thereby releasing the N-terminal mature and active forms and enabling them to enter the nucleus, and binding to the promoters of HMG-CoA reductase, fatty acid synthase and squalene synthase in mouse liver (*5*). Although SREBP-2 is suggested to be selectively involved in cholesterol synthesis, polyunsaturated fatty acid was shown to inhibit the maturation of SREBP-1, thus decreasing the downstream transcription of fatty acid synthase and HMG-CoA reductase (*6*, *4*). In the macrophage and neuroblastoma cells, the expression of HMG-CoA reductase was regulated via SREBP-1 and SREBP-2 respectively (*7*, *8*) .

Many previous studies suggested SREBP was regulated by the upstream AMP-activated protein kinase (AMPK). Alpha-lipoic acid was shown to increase AMPK phosphorylation in the liver cells, thus preventing the insulin-stimulated SREBP-1c expression and the development of NAFLD (*9*). Metformin, a type 2 DM therapautic, decreased hepatic expression of SREBP-1 via regulating AMPK (*10*). The activation of AMPK increased liver fatty acid oxidation and the exocytosis of lipoprotein (*11*). In transgenic mouse models, short-term activation of AMPK led to the increased SREBP-2 expression, whereas chronic activation decreased SREBP-1c and its target genes, resulting in reduced fat storage (*12*).

*Hibiscus sabdariffa* is a common tropical plant which is made as a daily drink in Taiwan. In addition to its use in anti-inflammation, anti-hypertension or anti-liver disorder in traditional medicine, previously we have demonstrated the multifunctional effects of its

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derivatives. *Hibiscus sabdariffa* extract (HSE) was shown to inhibit LDL oxidation, reduce foam cell formation, and decrease vascular smooth muscle cell migration (*13*, *14*). In clinical testing, the extract reduced serum cholesterol in men and women (*15*). There were various ingredients in *Hibiscus sabdariffa*, including organic acids, pectins, polyphenols, e.g., protocatechuic acid, anthocyanin, and flavonoid (*16*). *Hibiscus* anthocyanins induced apoptotic cell death in human leukemia cells and vascular smooth muscle cells (*17*, *18*). Recently, *Hibiscus sabdariffa* polyphenols (HPE) were reported to induce apoptosis in human gastric carcinoma cells via p53 phosphorylation and p38 MAPK/Fas L cascade pathway (*19*). In addition to its antitumor effect, HPE was also demonstrated to prevent the proliferation and migration of vascular smooth muscle cells stimulated by high glucose (*20*).

In the present study, we are going to investigate if HPE exerts the hypolipidemic effect, and the possible effect and putative mechanism of HPE on liver fat metabolism.

## MATERIALS AND METHOD

**Preparation of HSE and HPE.** HSE was prepared from *H. sabdariffa* (Malvaceae) and analyzed as our previous report (*13*). Briefly, *H. sabdariffa L.* (150 g) was macerated with hot water (95 °C, 6000 mL) for 2 h, and the aqueous extract was evaporated under vacuum at −85 °C. The extracted solution was filtered and then lyophilized to obtain 75 g of HSE and stored at 4 °C before use. The final extract of HSE was shown to be composed of polyphenolic acid, anthocyanins, and flavonoids. The concentration of total phenols was analyzed according to the Folin−Ciocalteau method. Briefly, HSE (0.1 mg) was dissolved in a test tube with 1 mL of distilled water; Folin−Ciocalteu reagent (2 N, 0.5 mL) was added and mixed in 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 spectrophotometer (U-3210) with rutin as the standard. The polyphenol content of HSE was estimated about 2%. While preparing the HPE, 100 g of *H. sabdariffa L.* was extracted three times with 300 mL of methanol at 50 °C for 3 h. The extracts were filtered and the solvent was removed with a vacuum rotary evaporator. The residue was dissolved in 500 mL of water (50 °C) and extracted with 200 mL of hexane to remove some of the pigments. The aqueous phase was extracted three times with 180 mL of ethyl acetate, which was then evaporated under reduced pressure. The residue was redissolved in 250 mL of water and was lyophilized to yield approximately 2 g of HPE and stored at −20 °C before use. The presence and proportion of the main constituents of HPE have been identified in our previous publication (*20*) as protocatechuic acid (24.24%), catechin (2.67%), gallocatechins (2.44%), caffeic acid (19.85%), and gallocatechin gallates (27.98%) (**Figure 1**). The polyphenol content of HPE was estimated about 74%.

**Animals and Experimental Design.** Male Syrian golden hamsters were purchased from the National Laboratory Animal Breeding and Research Center (Taipei, Taiwan). Hamsters were housed and acclimated in laboratory conditions (18−23 °C, humidity 55−60%, 12 h light/dark cycle) for at least 1 week before each study. All hamsters were fed with appropriate diet and free access to water and were weighed every week. After one week of acclimation, the hamsters were randomly divided into six groups ($n$ = 8 per group). The hamsters were fed with different diets: a standard Purina Chow (Purina Mills, Inc.) for the control group, a calorie-rich-fat diet containing 0.2% cholesterol and 10% coconut oil with standard Purina Chow for the high-fat diet (HFD) group, HFD with HSE 1% or 2% (w/w) for the HSE group, and HFD with HPE 0.1% or 0.2% (w/w) for the HPE group. These experiments were terminated after 10 weeks. All the animals were sacrificed, and the serum and liver samples were collected for further investigations. All animal experimental protocol used in this study was approved by the Institutional Animal Care and Use Committee of the Chung Shan Medical University (IACUC, CSMU), Taichung, Taiwan.

**Serum Biochemical Assays.** The serum sample was collected using EDTA tubes and centrifuged at 3,000 rpm for 10 min at 4 °C. Concentration of triglycerides, total cholesterol, LDL cholesterol (LDL-C), and HDL cholesterol (HDL-C) were measured by enzymatic colorimetric methods using commercial kits (HUMAN, Germany).

**Determination of Total Cholesterol and Triglycerides in Liver.** After removal from the animals, part of the samples of fresh liver were collected for analyzing the lipid contained. Liver lipids were extracted as described previously (*21*). Briefly, liver (1.25 g) was homogenized with chloroform/methanol (v/v, 1:2), and then added and thoroughly mixed with chloroform (1.25 mL) and distilled water (1.25 mL). After centrifugation (1500*g* for 10 min), the lower clear organic phase solution was transferred into a new glass tube and then lyophilized. The lyophilized powder was dissolved in chloroform/methanol (v/v, 1:2) as the liver lipid extract and stored at −20 °C for less than 3 days. The liver cholesterol and triglycerides in the lipid extracts were measured by enzymatic colorimetric methods using commercial kits (HUMAN, Germany).

**Cell Culture.** Human hepatocyte cell line, HepG2, was obtained from American Type Culture Collection and grown in minimum essential medium (MEM) supplemented with 10% fetal bovine serum, 100 unit/mL penicillin, 100 μg/mL streptomycin, and 2 mM L-glutamine (HyClone, Thermo Scientific). The cells were cultured at 37 °C in a humidified atmosphere of 95% air−5% $CO_2$. Once the monolayers had become approximately 80% confluent, the media were replaced with MEM containing 5% LPDS for 24 h prior to experiments.

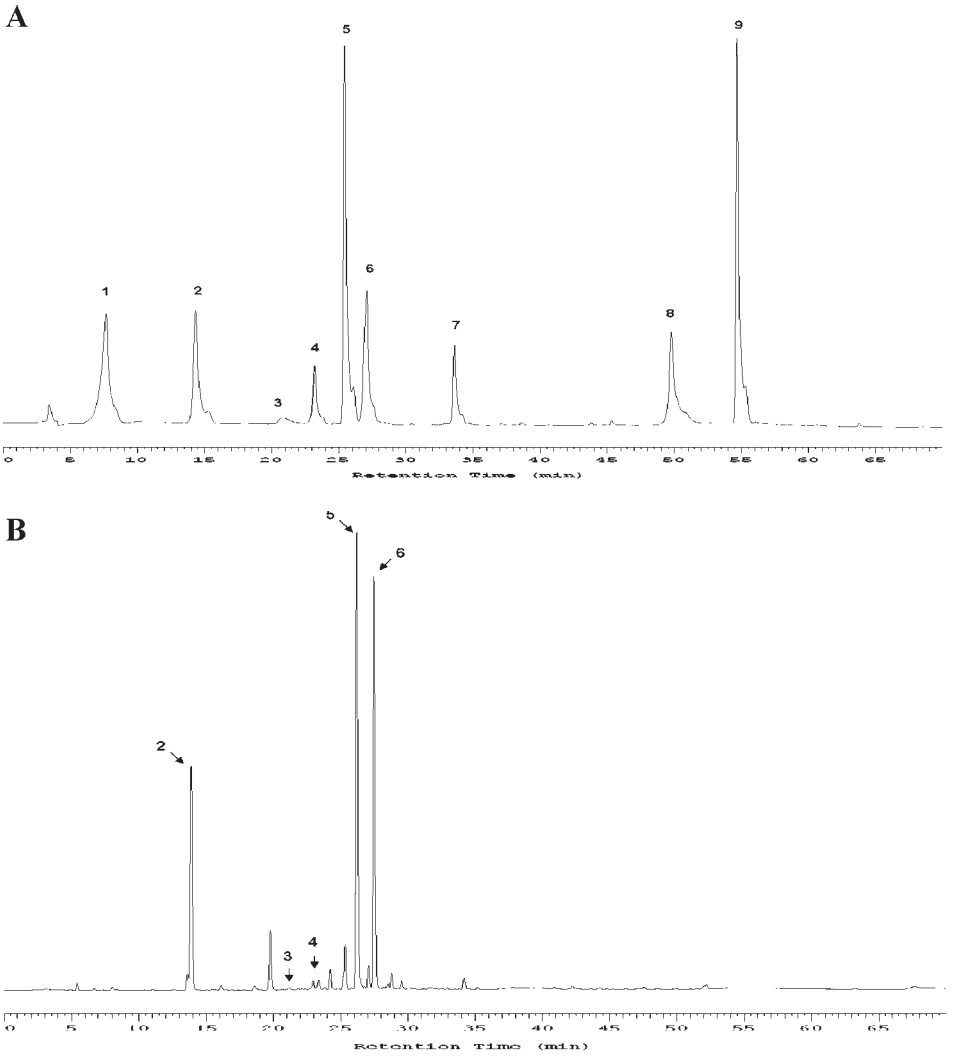
**Cytotoxicity Assay.** HepG2 cells were seeded at a density of $1 \times 10^6$ cells/mL in 24-well plate and incubated with compounds at various concentrations for 24 h. Thereafter, the medium was changed and cells were incubated with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, 0.5 mg/mL) for 4 h. The viable cell was directly proportional to the production of formazan. Following dissolution in isopropanol, the result was read at 563 nm with a spectrophotometer (Hitachi, U-3210).

**Determination of Cellular Total Cholesterol and Triglyceride Content.** The determinations of cellular total cholesterol and triglyceride content were essentially described previously (*22*). Cells were washed twice in PBS, cellular lipids were extracted in situ using two 30 min incubations with 1.0 mL of hexanes−isopropanol 3:2 (v/v), and the residual cell protein was determined after digestion in 0.1 N NaOH. The lipid extracts were centrifuged (1500*g* for 10 min), and the suspension was transferred into a new glass tube and then lyophilized. The lyophilized powder was dissolved in chloroform/methanol (v/v, 1:2), then stored and analyzed as the liver lipid extract.

**Preparation of Protein Extract of HepG2 Cells.** The proteins of cells were harvested in a cold RIPA buffer (1% NP-40, 50 mM Tris−base, 0.1% SDS, 0.5% deoxycholic acid, 150 mM NaCl, pH 7.5) containing leupeptin (17 μg/mL) and sodium orthovanadate (10 μg/mL). The cell suspension was vortexed at 4 °C for 1 h. All mixtures were then centrifuged at 12,000 rpm at 4 °C for 10 min, and the protein concentration of the supernatants was determined with the Coomassie blue total protein reagent (Kenlor Industries, Inc., USA) using bovine serum albumin as standard.

**Western Blot Analysis.** Equal amounts of protein samples (50 μg) were subjected to SDS−polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes (Millipore, Bedford, MA). Membranes were blocked with 5% nonfat milk powder with 0.1% Tween-20 in TBS and then incubated with the primary antibody at 4 °C overnight. Afterward, membranes were washed three times with 0.1% Tween-20 in TBS and incubated with the secondary antibody conjugated to horseradish peroxidase (GE Healthcare, Little Chalfont, Buckinghamshire, U.K.). Antibodies of SREBP-1, PPARα, and β-actin were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies of HMG CoA reductase and fatty acid synthase were from Upstate Biotechnology (Lake Placid, NY) and Transduction Laboratory (Lexington, KY) respectively. The anti-pAMPK and anti-AMPK were purchased from Cell Signaling Technology (Beverly, MA). Band detection was thereafter revealed by enhanced chemiluminescence using ECL Western blotting detection reagents and exposed in FUJFILM Las-3000 (Tokyo, Japan). Protein quantitative was determined by densitometry using FUJFILM-Multi Gauge V2.2 software.

**Reverse Transcription-Polymerase Chain Reaction (RT-PCR) Assay.** To determine the transcription level expression of HMG-CoA reductase (HMGCoARed), SREBP-1c, LDL receptor (LDLR), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in the HepG2 cells, total RNA was extracted using TriSolution Reagent Plus kits (GENEMARK Technology Co., Ltd.). cDNA synthesis and PCR amplification were performed according to the following procedures. For reverse transcription,




| Peak No. | Compound | Retention time (min) | Proportion (%) |
|---|---|---|---|
| 2 | PCA | 13.80 | 24.24 |
| 3 | Catechin | 21.00 | 2.67 |
| 4 | GC | 23.30 | 2.44 |
| 5 | Caffeic acid | 25.60 | 19.85 |
| 6 | GCG | 27.02 | 27.98 |
| | unknown | | 22.82 |

**Figure 1.** The HPLC chromatogram of HPE. (**A**) HPLC chromatogram of nine kinds of standard polyphenols (1 mg/mL; 10 $\mu$L). Peaks: 1, gallic acid; 2, protocatechuic acid (PCA); 3, catechin; 4, gallocatechin (GC); 5, caffeic acid; 6, gallocatechin gallate (GCG); 7, rutin; 8, quercetin; 9, naringenin. (**B**) HPLC chromatogram of free polyphenols from HPE (10 mg/mL, 10 $\mu$L).

4 $\mu$g of total cellular RNA were used as templates in a 20 $\mu$L reaction containing 4 $\mu$L of dNTP (2.5 mM), 2.5 $\mu$L of Oligo dT (10 pmol/$\mu$L), and RTase (200 unit/$\mu$L); the reaction was performed at 42 °C for 1 h. Afterward, 5 $\mu$L of cDNA was used as a template for PCR amplification with the appropriate primers. The HMGCR primers were forward, 5′-AGGTTCCAATGGCAACAACAGAAG-3′, and reverse, 5′-ATGCTCCTTGAACACCTAGCATCT-3′, which amplified a 828 bp fragment, run for 31 cycles at 95 °C for 1 min, 63 °C for 1 min and 72 °C for 1 min. The SREBP-1c primers were forward, 5′-AGGATGCTCAGTGGCACTG-3′, and reverse, 5′-GGATTGCACTTTCGAAGACGTG-3′, which amplified a 110 bp fragment, run for 31 cycles at 95 °C for 1 min, 58 °C for 1 min and 72 °C for 1 min. The LDLR primers were forward, 5′-CAATGTCTCACCAAGCTCTG-3′, and reverse, 5′-TCTGTCTCGAGGGGTAGCTG-3′, which amplified a 600 bp fragment, run for 31 cycles at 95 °C for 1 min, 63 °C for 1 min and 72 °C for 1 min. The GAPDH mRNA served as an internal control. The GAPDH primers were forward, 5′-TCCCTCAAGATTGTCAGCAA-3′, and reverse, 5′-AGATCCACAACGGATACATT-3′, which amplified a 309 bp fragment, run for 30 cycles at 95 °C for 1 min, 55 °C for 1 min and 72 °C for 2 min. PCR products were visualized on 2% agarose gels stained with ethidium bromide.

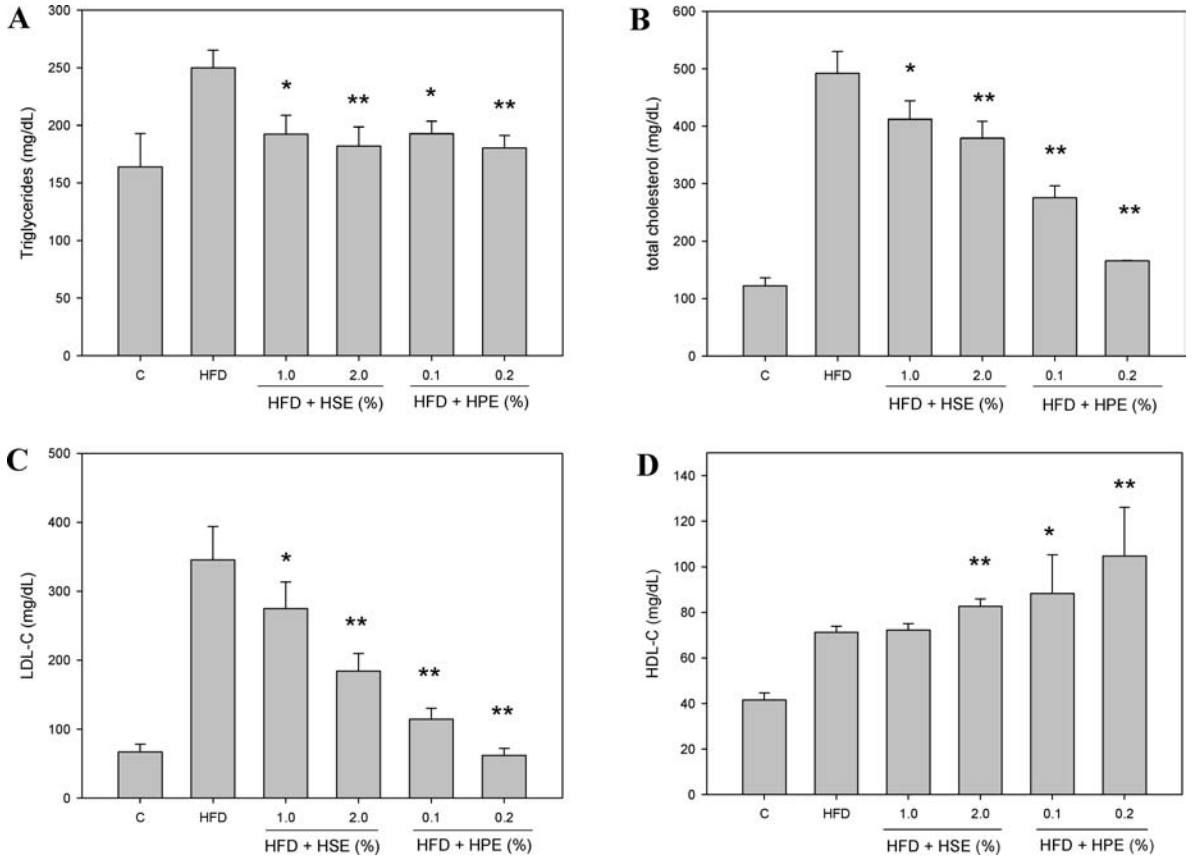



**Figure 2.** Effect of HSE and HPE on plasma lipid levels. The hamsters were fed with HFD for 10 weeks with or without different doses of HSE or HPE. HFD contained 0.2% cholesterol and 10% coconut oil. (**A**) Triglyceride. (**B**) Total cholesterol. (**C**) LDL cholesterol. (**D**) HDL cholesterol. Data are presented as means ± SD ($n$ = 8 for each group). *$p < 0.05$, **$p < 0.001$, as compared with the HFD-fed group.

**LDL Uptake Assay.** HepG2 cells were seeded in 6-well culture plates at a density of $1 \times 10^6$ cells/well, and cultured in medium containing 10% FBS overnight. Cells were washed with warm PBS, incubated with medium containing lipoprotein-deficient serum (LPDS), and treated with HSE or HPE for 18 h. After the treatment, cells were incubated with the fluorescent 1,1′-dioctadecyl-3,3,3′,3′- tetramethylindocarbocyanin perchlorate (DiI)-LDL (Molecular Probes, Inc.) at a concentration of 6 $\mu$g/mL for 4 h. Cells were then detached from the plates by incubation with trypsin−EDTA at 37 °C for 10 min. Intracellular fluorescence dye was detected by Nikon epifluorescent microscope (Nikon TE300) using a rhodamine filter connected to CoolSNAP-Pro *CF* DIGITAL KIT (Media Cybernetics, Inc.). Afterward, cells were pelleted, washed once, and resuspended in PBS on ice for FACScan (Becton Dickinson).

**Statistical Analysis.** Data were analyzed using an unpaired $t$ test and represented as means ± SD. A value of $p < 0.05$ was considered statistically significant. In the animal experiments, all data collected were analyzed using an unpaired $t$ test after one-way ANOVA testing showed a significant difference among all the groups ($P < 0.001$).

## RESULTS AND DISCUSSION

**HPE Decreases the Plasma Lipid Level.** The hamsters were fed with HFD for 10 weeks with or without HSE or HPE. The serum triglyceride and total cholesterol levels were thereby enhanced by HFD about 1.6- and 4.5-fold respectively. To be compared with the HFD-fed only group, HSE and HPE showed to decrease serum triglyceride and total cholesterol, although HPE exhibited more potency to inhibit total cholesterol (**Figure 2**). LDL cholesterol level was reduced 20% and 45% when treated with 1% and 2% HSE respectively, whereas 0.1% and 0.2% demonstrated superior ability in lowering serum LDL cholesterol. It was shown that 0.1% HPE decreased more than 60% of LDL cholesterol. The level of HDL cholesterol was only significantly altered by 2% HSE, whereas it was enhanced by HPE dose-dependently.

**HPE Decreases the Liver Lipid Levels and the Lipid Content of Hepatocytes.** The change of hepatic lipid is associated with liver disorder, metabolic disregulation, and the variety of lipoprotein phases. **Figure 3** showed that, in the HFD-fed group, the liver cholesterol increased 5-fold and the triglyceride increased about 2-fold compared with the controls. Treatment of HSE and HPE significantly decreased the elevation of cholesterol and triglyceride induced by HFD. Similarly, HPE exhibited more potent in lowering hepatic lipid. Body weight of the experimental subjects showed no significant change throughout the course (**Figure 4**).

For investigating the related modulating pathway transduced by HPE, first we tested the dose range which would be applied on HepG2 cells in the following experiments. It showed that the cells were tolerant for various doses of HSE treatment, while treated with HPE the cytotoxicity generated over 1.0 mg/mL (**Figure 5**). Therefore, it was considered as the maximum treatment dose or HSE and HPE in our experiments. **Figure 6** showed that both HSE and HPE decreased dose-dependently the cellular contents of cholesterol and triglyceride. Among all the doses used, HPE was shown to be slightly more effective than HSE. Especially at 1.0 mg/mL, HPE showed its cholesterol-lowering ability far beyond HSE.

**HPE Regulates Lipid Synthesis and the Phosphorylation of AMPK.** HSE and HPE dose-dependently reduced the expression of fatty acid synthase in liver cells (**Figure 7**). At 0.5 mg/mL, HPE decreased 75% and 69% of fatty acid synthase and HMG-CoA reductase, respectively, which showed its superior effect than HSE especially at lower dose.

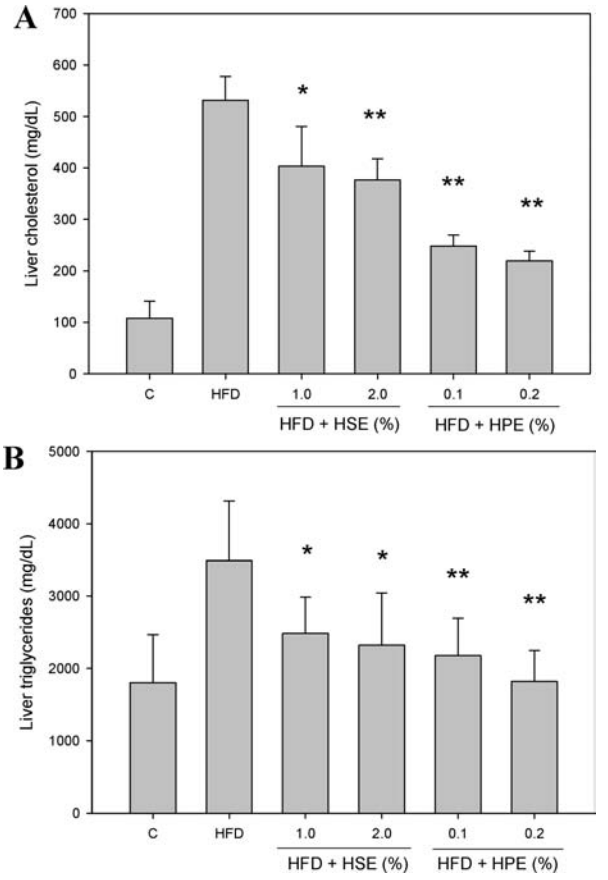



**Figure 3.** Effect of HSE and HPE on liver lipid levels. The hamsters were fed with HFD for 10 weeks with or without different doses of HSE or HPE. HFD contained 0.2% cholesterol and 10% coconut oil. (**A**) Triglyceride. (**B**) Total cholesterol. Data are presented as means ± SD ($n$ = 8 for each group). *$p$ < 0.05, **$p$ < 0.001, as compared with the HFD-fed group.

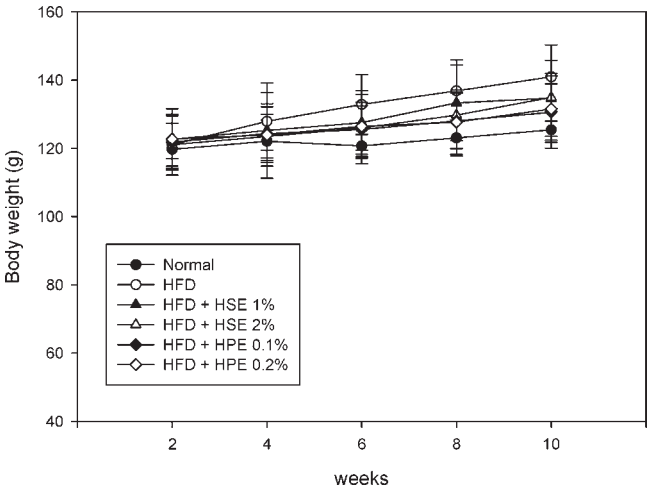



**Figure 4.** Effect of HSE or HPE on the body weight change of HFD-fed animals. The hamsters were grouped and fed with different diets: normal diet (control), HFD, HFD containing 1% HSE (HFD + 1% HSE), HFD containing 2% HSE (HFD + 2% HSE), HFD containing 0.1% HPE (HFD + 0.1% HPE), and HFD containing 0.2% HPE (HFD + 0.2% HPE).

As shown in **Figure 7**, HSE and HPE did not alter the protein level, but the phosphorylation of AMPK. This result suggested that AMPK might be involved in HPE-induced regulation of lipid synthesis. The previous reports have demonstrated the importance of AMPK in regulating lipid metabolism. Activation of

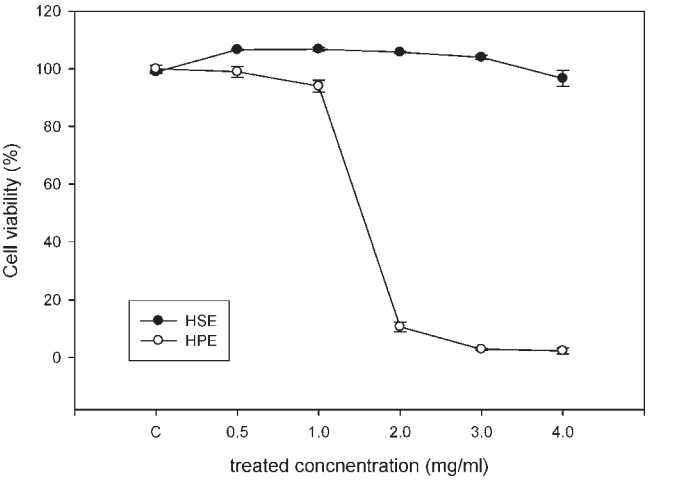



**Figure 5.** Dose dependent cytotoxicity of HSE or HPE on HepG2. HepG2 cells were incubated with various concentrations of HSE or HPE for 24 h. Data are presented as means ± SD from three independent experiments ($n$ = 3 for each independent experiment).

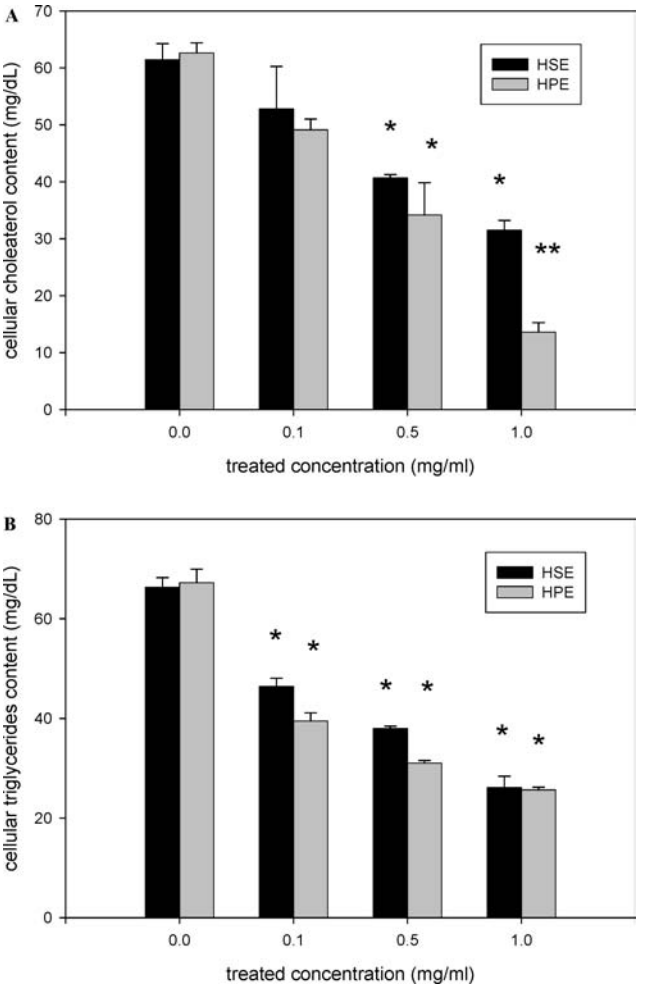



**Figure 6.** Effect of HSE and HPE on reducing the cellular lipid content. HepG2 cells were incubated for 6 h with or without different concentrations of HSE (**A**) or HPE (**B**). Intracellular lipids were extracted and analyzed for quantifying the contents of cholesterol and triglyceride. Data are presented as means ± SD from three independent experiments ($n$ = 3 for each independent experiment).

AMPK simultaneously inhibited the fatty acid and cholesterol synthesis in rat hepatocyte via inactivating the acetyl CoA

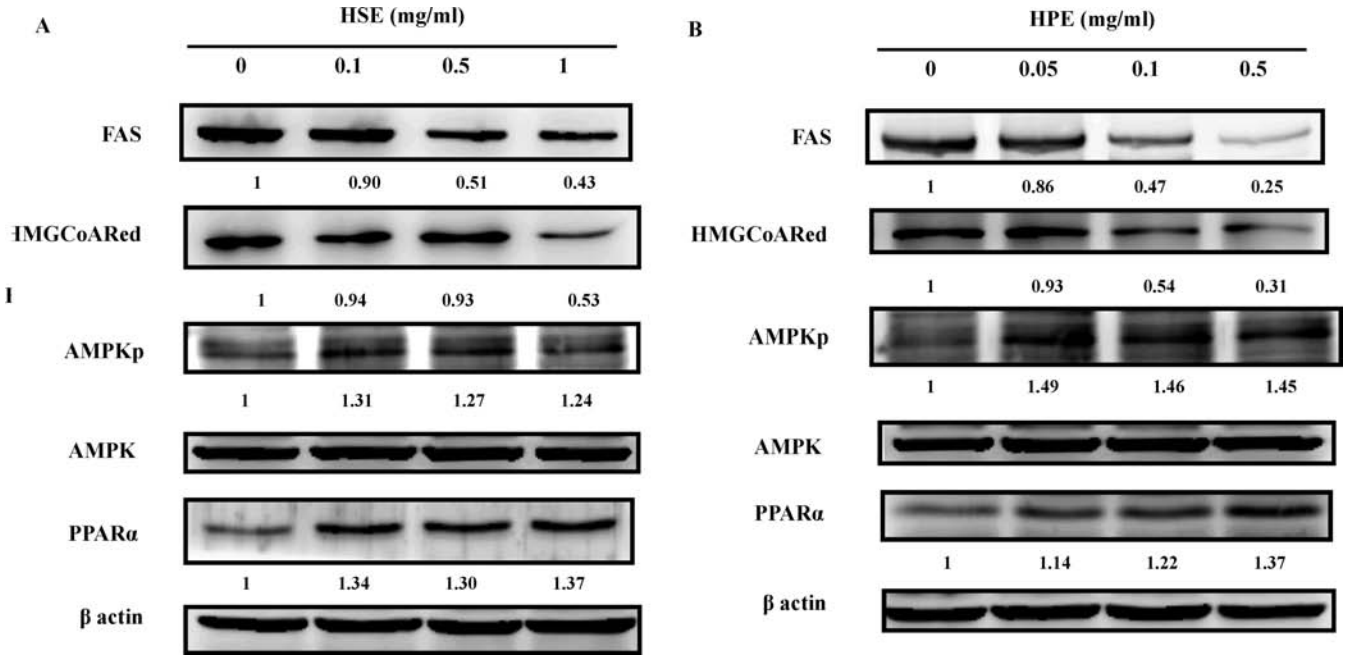



**Figure 7.** Effect of HSE and HPE on lipogenic-regulating enzymes. HepG2 cells were incubated for 6 h with or without different concentrations of HSE (**A**) or HPE (**B**). Cell lysates were resolved by 10% SDS−PAGE. The phosphorylation of AMPK was assessed by immunoblots with phospho-Thr-172 AMPK (pAMPK) antibodies. The number in the bottom indicates the fold compared with that of the control. Three independent experiments were conducted which showed similar pattern of changes.

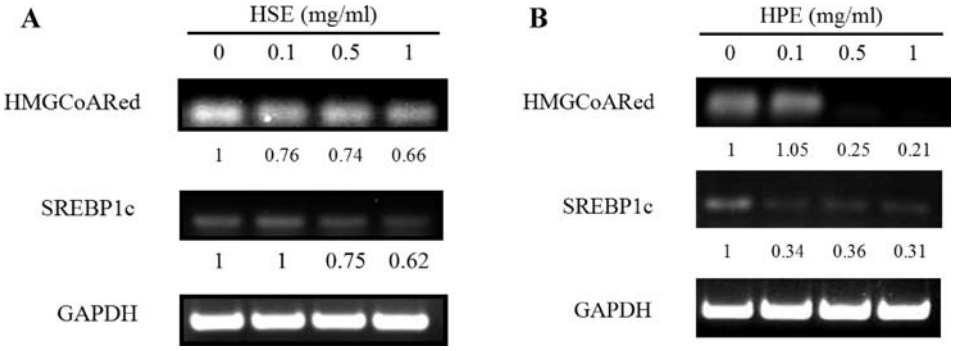



**Figure 8.** Effect of HSE and HPE on the transcription of lipogenic-regulating molecules. HepG2 cells were incubated for 6 h with or without different concentrations of HSE (**A**) or HPE (**B**). RT-PCR was used for the mRNA determination. The number in the bottom indicates the fold compared with that of the control. Three independent experiments were conducted which showed similar pattern of changes.

carboxylase (ACC) and HMG CoA reductase respectively (*23*). As malonyl CoA produced by ACC-2 is exclusively involved in regulation of fatty acid oxidation, that produced by ACC-1 is utilized in fatty acid synthesis. It was shown that activation of AMPK regulated the switch of fatty acid synthesis or fatty acid oxidation through the modulation of these two isoforms in response to different cellular stresses (*24*).

**HPE Inhibits the Expression of SREBP-1 and Its Target Transcription.** The ratio of SREBP-1 isoforms was shown to vary in different tissues over a 50−100-fold range. In the liver and adipose, mRNA of SREBP-1c is 9- and 3-fold more abundant than SREBP-1a respectively, whereas in spleen SREBP-1a is 10 times more abundant than SREBP-1c (*6*).

In order to understand the mechanism transduced by HSE and HPE, we further observed the mRNA expression of recognized markers involved in lipid metabolism (**Figure 8**). Similarly, HPE was shown to be more potent to regulate lipid metabolism. At 0.5 mg/mL, HPE decreased 75% of the expression of HMG-CoA and two-thirds of the expression of SREBP-1, which was reported as the transcription regulator of fatty acid synthase and HMG-CoA reductase. Compared with the result shown in **Figure 7**, the HPE-induced inhibition of SREBP-1 occurred consistently with the reduction of lipid synthetic enzyme.

**AMPK Activation Mediates the HPE-Induced Regulation of Lipid Synthesis.** In comparison of the results shown in **Figure 7** and **Figure 8**, it was demonstrated that HPE-induced regulation of lipid synthesis was almost abolished by Compound C, the selective AMPK inhibitor (**Figure 9**). HPE-induced reduction of SREBP-1, fatty acid synthase and HMG-CoA reductase was inhibited by AMPK inhibition. These results suggested that AMPK phosphorylation plays the pivotal role in HPE-transduced regulation of lipid synthesis. Cellular lipids were extracted and analyzed to confirm whether AMPK mediated the lipogenesis change induced by HPE. It was shown that AMPK inhibition blocked the HPE-induced reduction of cholesterol and triglyceride.

Actually, in addition to the lipid synthesis, many previous reports have demonstrated the pivotal role of AMPK to the downstream regulation of lipid oxidation. In the liver of SCD-1 knockout mice, in parallel with the activation of AMPK, the phosphorylation of acetyl-CoA carboxylase at Ser-79 was increased and its enzymatic

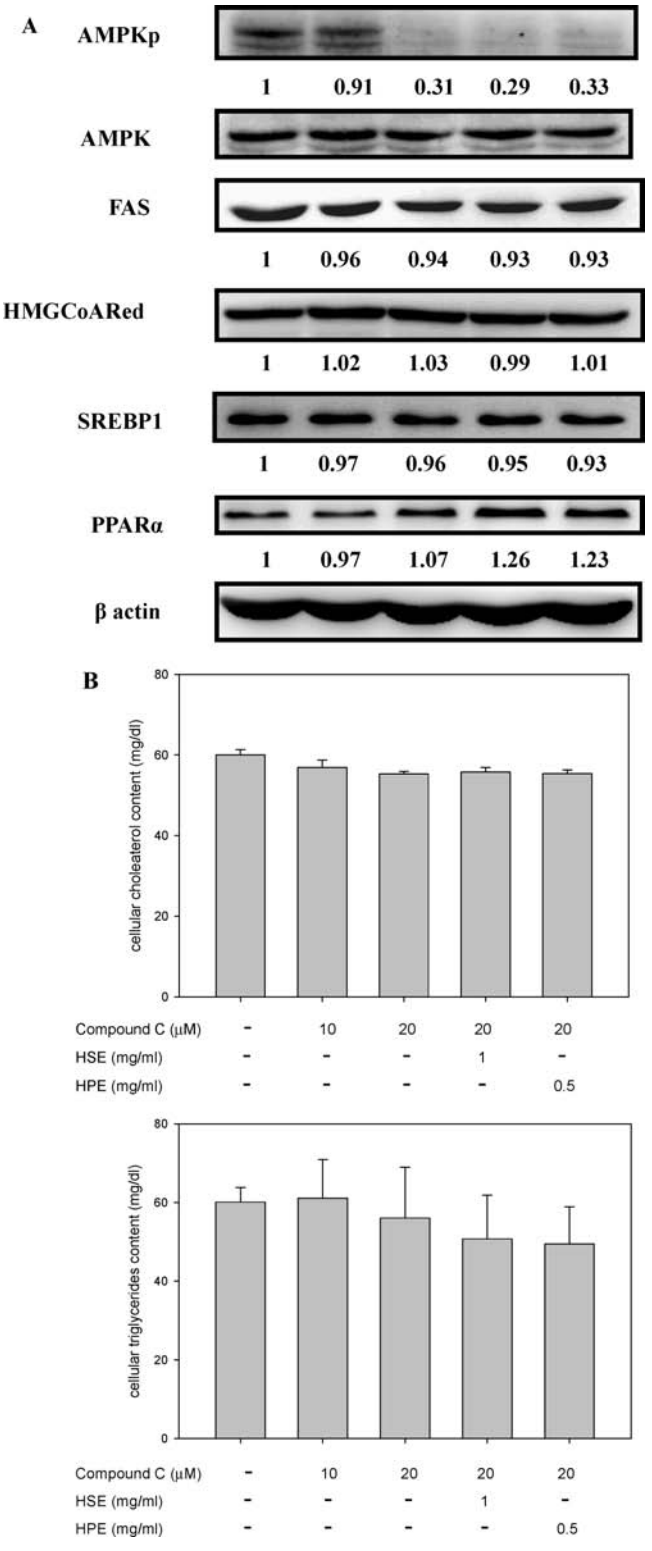



**Figure 9.** The pivotal role of AMPK in HSE and HPE-induced regulation of lipogenesis. Cells were pretreated with the selective AMPK inhibitor Compound C (10 or 20 $\mu$M) for 1 h, thereafter treated with HSE (1 mg/mL) or HPE (0.5 mg/mL) for 6 h. (**A**) Cell lysates were resolved by 10% SDS−PAGE. The number in the bottom indicates the fold compared with that of the control. Three independent experiments were conducted which showed a similar pattern of changes. (**B**) Intracellular lipids were extracted and analyzed for quantifying the contents of cholesterol and triglyceride.

activity was decreased, resulting in decreased level malonyl-CoA, thus derepressing CPT-1 and fatty acid oxidation (*25*).

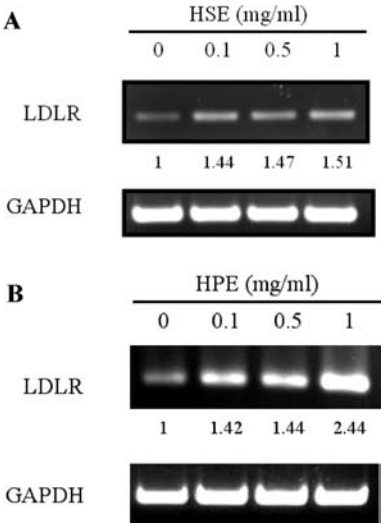



**Figure 10.** Effect of HSE or HPE on LDLR expression. HepG2 cells were incubated for 6 h with or without different concentrations of HSE (**A**) or HPE (**B**). RT-PCR was used for the mRNA determination. The number in the bottom indicates the fold compared with that of the control. Three independent experiments were conducted which showed a similar pattern of changes.

Furthermore, activation of AMPK could exert different effects according to the roles of diverse tissues or cells. In contrast to skeletal muscle in which AMPK stimulates fatty acid oxidation, AMPK activation inhibits lipogenesis and fatty acid oxidation in adipocytes. This discrepancy conserves ATP in adipose tissue and shares fatty acid for exportation to other tissues under the condition of stress or emergency demand (*26*).

Although the present investigation also indicated the essential role of AMPK for lipid metabolism, lipid is not the only metabolite regulated by AMPK. The carbohydrate metabolism is also involved for energy producing. In normal mouse liver, AMPK activation decreased the expression of genes involved in lipogenesis and glycolysis, hence lowering the circulating lipids and glycogen synthesis. The constitutive expression of AMPK abolished hyperglycemia and decreased gluconeogenesis in STZ-induced diabetic mice (*27*). It has been reported that SREBP-increased elevation of fatty acid synthetase, PPARα or triglyceride was reversed by AMPK activation, thus promoting glucose oxidation and insulin secretion (*28*). Therefore, AMPK could be considered as a therapeutic target for the treatment of diabetes or metabolic syndrome.

According to the results shown in **Figure 7B** and **Figure 9A**, PPARα was also induced by HPE in parallel with the AMPK activation. PPARs is one of family of ligand-activated transcription factors, which are classified as α, $\beta/\delta$ and $\gamma$. Among the three isoforms, PPARα is expressed abundantly in liver, kidney and skeletal muscle (*29*). The activation of PPARα mediates lipoprotein metabolism, enhances hepatic uptake, and increases the esterification of free fatty acids. Moreover, it promotes mitochondrial fatty acid uptake and oxidation (*30*). PPARα regulated pathway is enhanced in diabetic heart, which relies primarily on fatty acid oxidation for energy demand (*31*). It was shown that α2 AMPK was required for fatty acid oxidation or PPARα gene transcription (*32*). In the muscle, activation of AMPK/p38 MAPK/PPARα promoted the downstream gene expression of lipid metabolism (*33*). However, in the present investigation, HPE-induced PPARα expression was not affected by AMPK inhibition. The roles of HPE-induced PPARα and the involved signal transduction pathways will be further investigated in the near future.

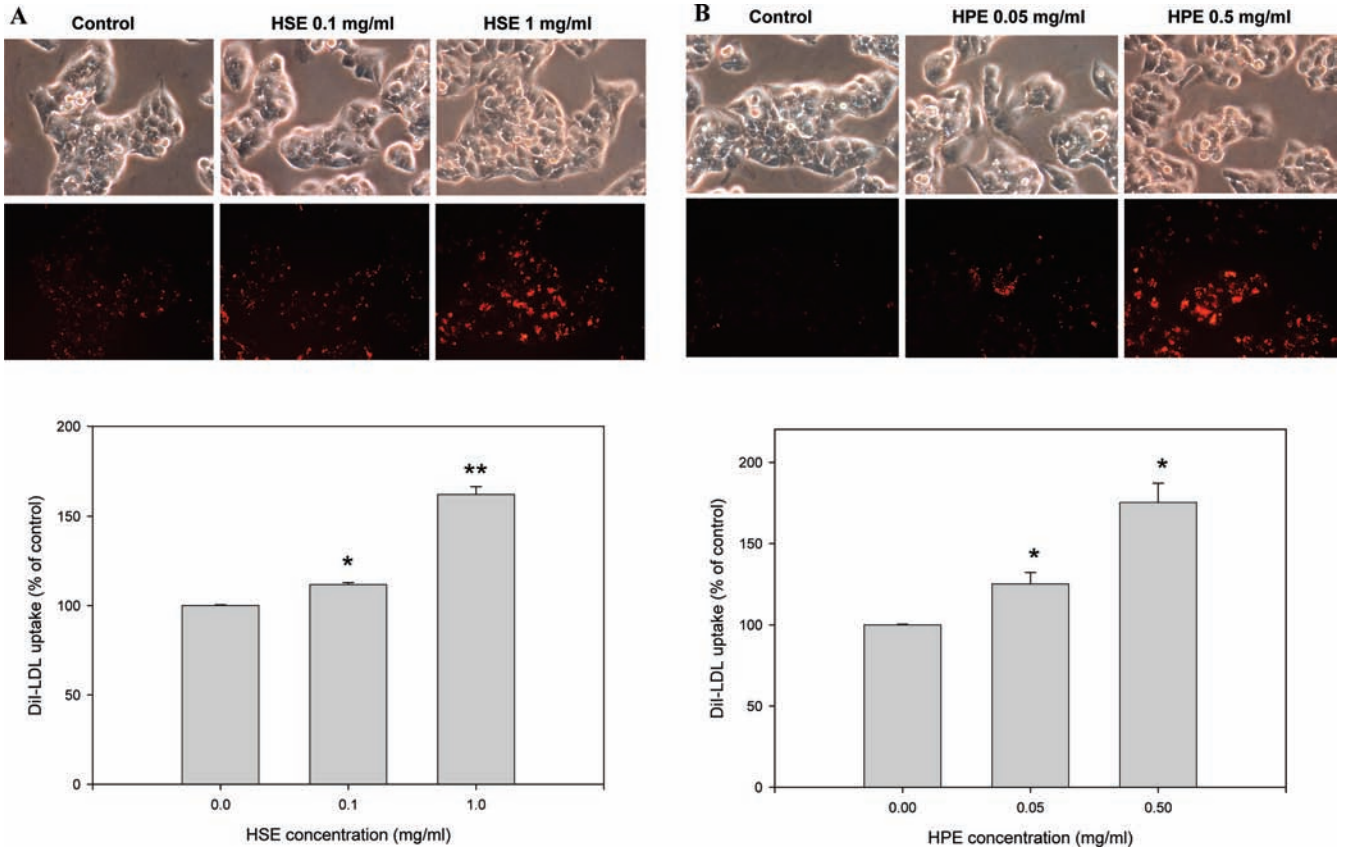



**Figure 11.** Effect of HSE and HPE on increasing LDL uptake. HepG2 cells cultured with medium containing 10% LPDS were treated with the indicated concentrations of HSE (**A**) or HPE (**B**) for 18 h. After treatment, DiI-LDL (6 $\mu$g/mL) was added to the medium, and cells were trypsinized 4 h later (photograph magnification, 400×). The uptake of DiI-LDL was measured by FACScan (Becton Dickinson) with $2 \times 10^4$ cells per sample. The mean fluorescence value of the control is expressed as 100%. Data are presented as means ± SD from three independent experiments ($n = 3$ for each independent experiment). *$p <$ 0.05, **$p < 0.001$, as compared with the control.

**LDLR Expression and LDL Uptake Are Enhanced by HPE.** The expression of LDLR was increased 1.5-fold and 2.5-fold when treated with 0.5 mg/mL and 1.0 mg/mL HPE, respectively (**Figure 10**), implying that HPE could exert its hypolipidemic effect through the elevation of hepatic clearance.

For investigating if the LDL uptake was increased, we observed the LDL binding on cells treated with different conditions. **Figure 11** showed that cells treated with 0.5 mg/mL HPE exhibited similar LDL uptake ability as cells treated with 1 mg/mL HSE, which was demonstrated to increase 70% of the LDL binding.

Among the different risk factors resulting in disregulation of lipid metabolism, high LDL cholesterol level is identified as the most important one which is frequently accompanied with metabolic syndrome or cardiovascular disease. No known pathway exists in mammals by which the carbon atoms of steroid ring can be used as a source of metabolic energy. Therefore, the plasma cholesterol level is regulated by the rate of biosynthesis and uptake through the liver LDLR. We observed that HPE increased the expression of LDLR mRNA. In parallel to the result, there existed more LDLR on the liver cell surface to accomplish more LDL uptake and hepatic clearance, thus reducing the plasma cholesterol concentration.

It has been reported that LDLR expression is predominantly regulated by SREBP2. Fenofibrate, a PPARα ligand, induced LDLR expression by increasing maturation of SREBP2 (*34*). Furthermore, in the rat liver and hepatoma cell line Fao, PPARα was demonstrated to regulate Insig, the ER membrane protein, hence reducing the amount of SREBP-2 and the expression of LDLR (*35*). Although the other lipid metabolic genes could be regulated by SREBPs, the elevation of LDLR seemed to be only transduced by SREBP2.

**The Hepatic Hypolipidemic Effect of HPE.** In the present investigation, we have demonstrated the hypolipidemic and hepatic fat-lowering effect of HPE, which activates AMPK in liver cells, hence decreasing SREBP-1 and the transcription of its target genes, HMG-CoA reductase and fatty acid synthase. Furthermore, HPE enhances the expression of LDLR, thus increasing the LDL uptake and hepatic clearance. These results suggest that although HPE promotes LDL transport from plasma into liver, it inhibits the hepatic neo-lipid synthesis, thereby exerting its protect effect.

The polyphenol content of HSE and HPE was estimated about 2% and 74% respectively. As observed in the present investigation, HPE possesses a better effect for regulating lipid metabolism compared with HSE. The advantage of HPE and its polyphenol composition should contribute indispensably for HSE, which has been demonstrated on the hepatic protective and hypolipidemic effect (*36*, *13*). As shown in **Figure 1**, HPE is mainly composed of PCA, caffeic acid, GCG. It was reported that PCA increased the hepatic LDLR, apoE and LCAT, thus lowering the plasma total cholesterol level (*37*). Caffeic acid attenuated the hyperlipidemic status in alcohol-induced rats (*38*). Recently, GCG-rich catechins demonstrated strong activity in lowering serum triglyceride and cholesterol, and increasing the hepatic LDLR expression (*39*).

HPE is worthy of being further investigated and developed, whereas the purification is not easily approached or necessary for the population. Since *Hibiscus sabdariffa* is one of the

easily-cultivated and inexpensive herbals in the tropical area, it is suggested to popularize the daily drink as a nutraceutical for hepatic lipid control or hypolipidemic adjuvant.

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