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LITHOSPERMUM ERYTHRORHIZON SIEB. ET ZUCC. SUPPRESSES 3-HYDROXY-3-METHYL-GLUTARYL-COA REDUCTASE AND INDUCES LDL RECEPTOR EXPRESSION IN HEPG2 CELLS

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ABSTRACT

We examined the effects of Lithospermum erythrorhizon *Sieb. et Zucc. (LE) on cholesterol metabolism* in vitro. *The ethanolic LE extract (ELE) had total polyphenolic and flavonoid contents of 353 ± 7 and 285 ± 51 mg/dL, respectively. The ELE inhibited Cu^{2+}-mediated LDL oxidation at <400 μg/mL. In HepG2 cells treated with 400 μg/mL ELE, the expression of 3-hydroxy-3-methyl-glutaryl-CoA reductase decreased markedly (45%;* $P < 0.05$*), whereas that of the LDL receptor increased (230%;* $P < 0.05$*). The protein levels of both were altered similarly. The ELE also increased membrane-bound and cell-associated LDL particles, possibly via upregulation of the LDL receptor. In hepatocytes, 400 μg/mL ELE affected surrogate markers of HDL and LDL*

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synthesis: significant expression of apolipoprotein A-I was induced (20%; $P < 0.05$*), whereas that of apoB was suppressed (30%;* $P < 0.05$*). In conclusion, ELE may improve cellular cholesterol metabolism by inhibiting cholesterol biosynthesis and apoB production, accelerating plasma LDL uptake and reducing LDL oxidation.*

PRACTICAL APPLICATIONS

Hypercholesterolemia is a major risk factor for coronary heart disease (CHD), which is the leading cause of mortality in patients with metabolic syndrome. Consequently, therapeutic interventions that prevent or treat metabolic disorders would markedly reduce metabolic syndrome-related death. Lipid-lowering drugs are effective at inhibiting hyperlipidemia, but have undesirable side effects and tolerance issues with long-term intake. Therefore, it is necessary for patients to undergo dietary modification using functional foods, including phytochemicals that have both nutritional and medicinal benefits, to prevent and attenuate hyperlipidemia. This study investigated the antiatherogenic effects of an ethanolic *Lithospermum erythrorhizon* extract (ELE) *in vitro*. The results showed that the ELE inhibited low-density lipoprotein (LDL) oxidation, apoB production and cholesterol synthesis and induced LDL uptake. These results suggest that the ELE protects against atherogenesis and may help to prevent CHD.

INTRODUCTION

CHD is a major health concern worldwide. The development of CHD is closely related to hypercholesterolemia, which is characterized by elevated levels of plasma LDL cholesterol and triglycerides and reduced concentration of HDL cholesterol. Moreover, as CHD is also associated with cellular oxidative stress, oxidized LDL, a substrate for macrophage scavenger receptors, is also a well-known risk factor for CHD. Lipid-lowering drugs are effective for treating and preventing CHD, but they are also costly and often produce undesirable side effects. Furthermore, tolerance issues with long-term intake have been reported (Evans and Rees 2002; Stolk *et al*. 2006).

Dietary supplements or functional foods usually have modest effects and can be tolerated long term, with relatively minor side effects. Consequently, the use of functional foods may be a reasonable prevention method for both healthy and high-risk individuals. The protective effects of phytochemicals against CHD have been widely investigated based on the reported effects of fruits and vegetables (Visioli *et al*. 2000; Joshipura *et al*. 2001; Tucker 2004;

Jensen *et al*. 2008). Such studies have identified numerous phytochemicals with protective effects against CHD, including antioxidant flavonoids and polyphenols.

LE, a traditional medicinal herb in Asia, contains various phytochemicals, such as flavonoids and shikonin compounds (Jian *et al*. 1991; Luo and Li 1992; Nigorikawa *et al*. 2006), and is suggested to have anti-inflammatory (Yoshimi *et al*. 1992; Chen *et al*. 2001; Lee *et al*. 2009), antioxidative (Yingming *et al*. 2004; Han *et al*. 2008), antigonadotropic (Findley and Jacobs 1980; Findley 1981; Winterhoff *et al*. 1988), wound-healing (Hayashi 1977) and antitumor (Staniforth *et al*. 2004) effects. The antioxidative effects of LE include the reduction of LDL oxidation, which in turn could prevent atherosclerosis, although the effects of ethanolic LE extracts on cholesterol metabolism are largely unknown. Therefore, we investigated whether an ELE has CHD-preventive effects *in vitro* and found that it inhibited LDL oxidation. At the RNA and protein levels, the ELE reduced 3-hydroxy-3-methyl-glutaryl-CoA (HMG-CoA) reductase expression, but induced hepatic LDL receptor expression, both of which are advantageous for controlling cholesterol levels. Our results suggest that ELE has potential as a functional food that prevents the development of CHD by favorably altering cholesterol metabolism.

MATERIALS AND METHODS

Preparation of ELE

Lithospermum erythrorhizon was purchased from the Oriental medicinal drug store in Gyeong-doung (Seoul, Korea). Dried Korean LE (12 kg) was extracted overnight in 3.6 L of ethanol. Then, the extract was heated at 45C to evaporate the solvent and lyophilized to obtain 30.6 g of powder. This powder was dissolved in dimethyl sulfoxide (DMSO) for use in the experiments. The final DMSO concentration did not exceed 0.1% throughout the study.

Measuring the Total Polyphenol Content

The total polyphenol content of the ELE was measured using a modified Folin–Ciocalteu colorimetric method (Nurmi *et al*. 1996). Briefly, 1 mL of 1 N Folin–Ciocalteu reagent was added to 1 mL of ELE, and the mixture was allowed to stand for 2–5 min. Then, the mixture was incubated with 2 mL of 20% Na_2CO_3 for 60 min at room temperature. The absorbance at 730 nm was determined spectrophotometrically using gallic acid as a standard.

Measuring the Total Flavonoid Content

The total flavonoid content was determined using the aluminum chloride colorimetric assay (Kim *et al*. 2003). Distilled water (4 mL) and 1 mL of ELE were mixed, and then 0.3 mL of $NaNO_2$ was added, followed by 0.3 mL of 10% $AlCl_3$. After incubating this for 5 min at room temperature, 2 mL of 1 M NaOH was added, and the total volume was made up to 10 mL with distilled water. The mixture was vortexed thoroughly, and the absorbance at 510 nm was determined. Catechin was used as a standard.

Cell Cultures

HepG2 cells were purchased from the Korean Cell Line Bank (Seoul, Korea) and grown in Dulbecco's minimum essential medium (WelGENE, Seoul, Korea) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS; WelGENE), 1% (v/v) penicillin–streptomycin (WelGENE) and 0.5% (v/v) gentamicin (WelGENE). Confluent monolayers of cells at 70–80% of confluence were incubated with 0, 200 or 400 μg/mL ELE for 24 h in FBS- and antibiotic-free medium in six-well plates.

Quantification of LDL Oxidation

Normal human plasma was purchased from 3H Biomedical (Uppsala, Sweden). Human LDL particles ($1.025 < d < 1.050$) were isolated from fresh plasma, as described previously (Lee *et al*. 2003). Briefly, plasma was obtained by centrifugation for 30 min at 15C and 4,000 rpm. Then, the LDL was isolated by ultracentrifugation at 111,000 rpm for 1 h 51 min at 15C with added potassium bromide solution. The isolated LDL fraction was dialyzed at 4C in the dark using Spectra/Por® 2 dialysis membranes (Spectrum Laboratories, Rancho Dominguez, CA). During dialysis, 2 L of degassed phosphate-buffered saline (PBS; pH 7.4) was changed four times after dialysis for 12 h each time. The protein content was determined using a modified Bradford assay (Bradford 1976) with a commercial kit (Bio-Rad, Hercules, CA) and bovine serum albumin as a standard (Sigma, St. Louis, MO). Then, the dialyzed LDL was diluted to 250 μg protein/mL with 0.01 M sodium phosphate buffer (pH 7.4). For the control group, 40 μL of 250 μg/mL LDL, 5 μL of 50 μM $CuSO_4$ and 5 μL of 0.01 M sodium phosphate buffer were mixed and incubated at 37C for 10 h. For the experimental groups, 25, 50, 100, 200, 400, 800 or 1,000 μg/mL ELE was added instead of 0.01 M sodium phosphate buffer. Oxidation was stopped by adding 1 μL of 27 mM ethylenediaminetetraacetic acid (EDTA). LDL oxidation was quantified using a TBARS assay performed according to the protocol of the Animal Models of Diabetic Complications Consortium (AMDCC; http://www.amdcc.org/shared/Protocols.aspx). Briefly, the LDL-

incubated tubes were treated with 100 μL of 10% trichloroacetic acid on ice for 15 min. Then, the mixture was centrifuged at 2,200 *g* for 15 min at 4C. Next, 100 μL of the supernatant and an equal volume of 0.67% (w/v) thiobarbituric acid (TBA) were mixed and heated to 95C for 20 min. The TBARS values (expressed as μmol malondialdehyde [MDA]/mg LDL protein) were determined by measuring the absorbance at 532 nm. The assay was calibrated using an MDA standard solution prepared from 1,1,3,3-tetramethoxypropane. The calibration curve was prepared using 0 to 800 μM solutions of 1,1,3,3-tetramethoxypropane.

RT-PCR Analysis

Total RNA extracted from HepG2 cells using TRIzol® reagent (Invitrogen, Carlsbad, CA) was quantified at 260/280 nm. The RNA was stored at −80C until analysis. Single-stranded cDNA was synthesized from 1 μg of the RNA using a cDNA cycle kit (Invitrogen) and oligo dT (Bioneer, Seoul, Korea). The reaction was performed in a final volume of 20 μL for 90 min at 42C and subsequently for 15 min at 70C. The resulting cDNA templates were subjected to PCR amplification (MyCycler™ thermal cycler; Bio-Rad). Each reaction was performed in a final volume of 20 μL containing cDNA, 10× PCR buffer, 2.5 mM dNTPs, 10 mM forward and reverse primers (Bioneer), DNA polymerase (CoreBioSystem, Seoul, Korea) and sterile water. The primers were designed using the published nucleotide sequences for the genes encoding the LDL receptor, HMG-CoA reductase, SREBPs, nuclear receptors, apolipoproteins (apoAI, apoB and apoE) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Table 1). The program consisted of 20–32 cycles of 30 s at 94C, 30 s at 60–62C and 30 s at 72C. The products (10 μL) were electrophoresed at 95 V on 1.2% agarose gels containing ethidium bromide. The relative band densities were determined using Sigma Gel software (SPSS, Chicago, IL).

Immunoblot Analysis

HepG2 cells were lysed in ice-cold lysis buffer (10 mM Tris-HCl [pH 7.4], 0.1 M EDTA, 10 mM NaCl and 0.5% Triton X-100) supplemented with a protease inhibitor cocktail (Sigma). The lysate was centrifuged at 14,000 rpm for 10 min at 4C. The protein concentration in each extract was determined with Bradford method (Bradford 1976). The samples were boiled in sample buffer (5% β-mercaptoethanol) for 5 min and separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis. Then, the electrophoresed proteins were transferred onto nitrocellulose membranes (Schleicher & Schuell BioScience, Dassel, Germany) and blocked with 5% nonfat dry milk in PBS (pH 7.4) containing 0.1% Tween 20 for 1 h. The blots were incubated overnight with

TABLE 1.
PCR PRIMER SEQUENCES

Gene	Primer	Sequence*
HMG-CoA reductase	Forward	TACCATGTCAGGGGTACGTC
	Reverse	CAAGCCTAGAGACATAATCATC
SR-BI	Forward	TCTACCCACCCAACGAAGGCT
	Reverse	CCTGAATGGCCTCCTTATCCT
CYP7A1	Forward	GCATCATAGCTCTTTACCCAC
	Reverse	GGTGTTCTGCAGCAGTCCTGTAAT
LDL receptor	Forward	CAATGTCTCACCAAGCTCTG
	Reverse	TCTGTCTCGAGGGGTAGCTG
SREBP-1a	Forward	CTGCTGACCGACATCGAAGAC
	Reverse	GATGCTCAGTGGCACTGACTGTTC
SREBP-1c	Forward	CGGAGCCATGGATTGCACTTTC
	Reverse	GATGCTCAGTGGCACTGACTGTTC
SREBP-2	Forward	CCCTTCAGTGCAACGTGAGTTCAC
	Reverse	TGCCATTGGCCGTTTGTGTC
ApoB	Forward	CGGGAATCTGATGAGGAA
	Reverse	GTGCAAAGTTCCTCCCTA
ApoE	Forward	TAAGCTTGGCACGGCTGTCCAAGGA
	Reverse	ACAGAATTCGCCCCGGCCTGGTACAC
ApoAI	Forward	TGGGATCGAGTGAAGGACCT
	Reverse	CTCCTCCTGCCACTTCTTCTG
GAPDH	Forward	ATGGATGATGATATCGCCGCC
	Reverse	CTCCATGTCGTCCCAGTTGGT

* Primers are shown $5' \rightarrow 3'$.
GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

rabbit anti-LDL receptor (a gift from Dr Allen D. Cooper, Stanford University) or anti-HMG-CoA reductase antibodies (Upstate, Lake Placid, NY) at 4C. After rinsing with PBS plus 0.1% Tween 20, the blots were incubated with peroxidase-conjugated goat anti-rabbit IgG (H&L chain-specific) antibodies (Calbiochem, Darmstadt, Germany). The immunoreactive bands were detected using ECL® Western blotting detection reagents (Amersham-Pharmacia Korea, Seoul, Korea) and exposed to high-performance chemiluminescence film. The immunoreactive bands were imaged with a ChemiDoc XRS system (Bio-Rad) using ECL® Western blotting detection reagents (Amersham-Pharmacia Korea). Each protein band was quantified by densitometric analysis with Gel-Pro Analyzer 4.0 software (Media Cybernetics, Bethesda, MD) and normalized to α-tubulin expression (Santa Cruz Biotechnology, Santa Cruz, CA).

Receptor-Mediated LDL Uptake

Fluorescence-labeled LDL was prepared from 1 mg of isolated human LDL and 300 μg of 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI, 30 mg/mL DMSO; Molecular Probes, Eugene, OR). All unlabeled DiI was removed using a PD-10 column (Amersham-Pharmacia, Uppsala, Sweden). To determine the amounts of membrane-bound and cell-associated LDL, DiI-LDL (30 μg protein/mL) was incubated with 400 μg/mL ELE-treated HepG2 cells for 2 h at 4C and 37C, respectively. Fluorescence was determined using a Perkin Elmer Model LS-5 spectrofluorometer (Perkin Elmer, Waltham, MA) with the excitation and emission wavelengths set at 520 and 578 nm, respectively.

Statistical Analysis

All data are expressed as the mean ± standard error. Two groups were compared using Student's *t*-test. $P < 0.05$ was considered statistically significant.

RESULTS

Measurement of Shikonin and Its Derivatives, Total Flavonoids and Polyphenols in the ELE

The shikonin and acetylshikonin contents in the ELE were analyzed by HPLC (Waters, Milford, MA) using a modified version of the method of Staniforth *et al*. (Staniforth *et al*. 2004). The shikonin and acetylshikonin contents were 2.3 and 2.7%, respectively. The total polyphenolic and flavonoid contents in the ELE were 353 ± 7 and 285 ± 51 mg/dL ($n = 3$), respectively, as determined by the Folin–Ciocalteu and aluminum chloride colorimetric methods.

The Effect of ELE on the Cu^{2+}-induced LDL Oxidation *In Vitro*

Isolated human LDL was incubated with $CuSO_4$ and various concentrations of ELE for 10 h, and the amount of oxidation was measured using a TBARS assay. The ELE had biphasic effects on LDL oxidation. At 25, 50, 100, 200 and 400 μg/mL, ELE significantly inhibited oxidation, whereas at high concentrations (800 and 1,000 μg/mL), the ELE induced LDL oxidation as compared with the controls (Fig. 1). At >500 μg/mL, however, the ELE showed significant cytotoxicity in a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (data not shown). These data suggest that ELE protects against LDL oxidation without cytotoxicity at concentrations of up to 500 μg/mL.

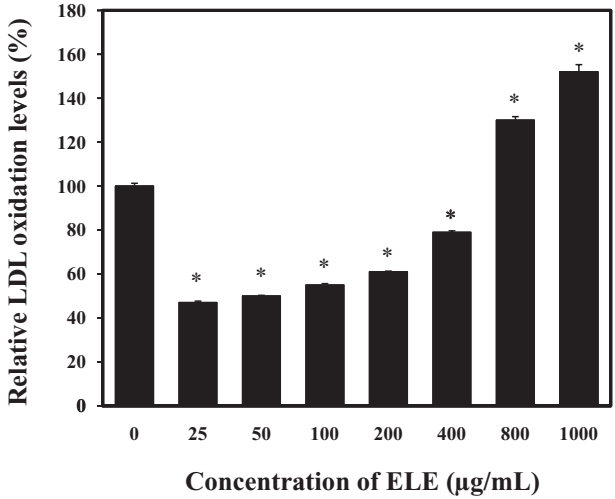

FIG. 1. EFFECT OF THE ETHANOLIC EXTRACT OF ELE ON CU^{2+}-INDUCED LDL OXIDATION
The isolated human LDL was incubated with ELE or sodium phosphate buffer (control) at 37C for 10 h. LDL oxidation was quantified using a TBARS assay.
The data are expressed as the mean ± standard error of three independent experiments.
*Significantly different from the control ($P < 0.05$).

Effects of ELE on the Expression of the LDL Receptor, HMG-CoA Reductase, *CYP7A1*, ApoB and ApoAI in HepG2 Cells

To investigate the effect of ELE on the mRNA expression of genes involved in cholesterol metabolism, we treated HepG2 cells with 200 or 400 μg/mL ELE for 24 h and then performed RT-PCR analysis. We assessed the expression of HMG-CoA reductase, LDL receptor, *CYP7A1* and *SR-BI*. At 200 and 400 μg/mL ELE, the expression of HMG-CoA reductase was significantly suppressed (42 and 45%, respectively; $P < 0.05$; Fig. 2), whereas the expression of the LDL receptor increased significantly by 230% at 400 μg/mL ELE ($P < 0.05$). By contrast, no effect on the expression of *CYP7A1* or *SR-BI* was observed. As HMG-CoA reductase and the LDL receptor are regulated by SREBPs, we examined the effect of ELE on *SREBP* mRNA expression. The expression levels of three *SREBP* genes, *SREBP-1a*, *SREBP-1c* and *SREBP-2*, were significantly reduced by treatment with 200 and 400 μg/mL ELE, except for the expression of *SREBP-1c* at 200 μg/mL ELE (Fig. 3). The expression of apolipoproteins, the structural components of lipoproteins that play an important role in lipid transport and metabolism, was also analyzed in HepG2 cells incubated with ELE. At

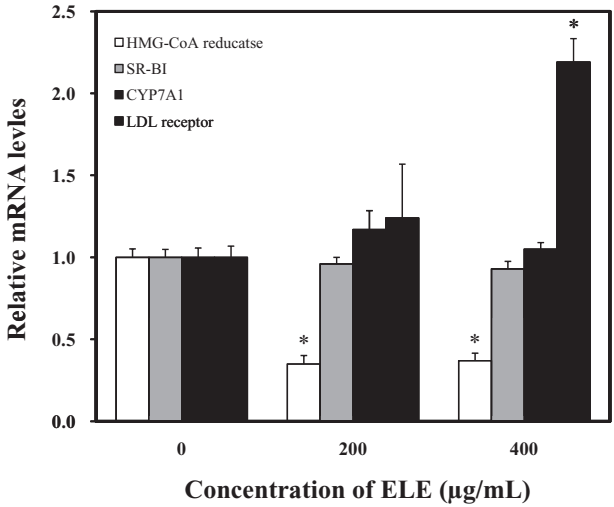

FIG. 2. EFFECT OF THE ELE ON THE mRNA EXPRESSION OF HMG-COA REDUCTASE, *SR-BI*, *CYP7A1* AND LDL RECEPTOR IN HEPG2 CELLS

HepG2 cells were treated with 0, 200 or 400 μg/mL ELE for 24 h. Total RNA was extracted from HepG2 cells. HMG-CoA reductase, *SR-BI*, *CYP7A1* and LDL receptor mRNA levels were then measured by RT-PCR. The relative band densities were determined using Sigma Gel software (SPSS) with *GAPDH* as a reference.

The data are expressed as the mean ± standard error of three independent experiments.

*Significantly different from the control ($P < 0.05$).

400 μg/mL, the expression of apoAI increased significantly, whereas the expression of apoB decreased and no effect on apoE was observed (Fig. 4).

Effect of ELE on LDL Receptor and HMG-CoA Reductase Protein Expression in HepG2 Cells

Because the mRNA expression of HMG-CoA reductase increased significantly, and that of the LDL receptor decreased following ELE exposure, we examined the expression of HMG-CoA reductase and the LDL receptor at the protein level. HepG2 cells were treated with 200 and 400 μg/mL ELE for 24 h. LDL receptor expression increased by 34 and 50% at 200 and 400 μg/mL, respectively, compared with the controls (Fig. 5A), whereas HMG-CoA reductase expression decreased by 57 and 44% at 200 and 400 μg/mL, respectively, similar to the results obtained at the RNA level (Fig. 5B).

Effect of ELE on Receptor-Mediated LDL Uptake in HepG2 Cells

As the ELE induced both RNA and protein expression of the LDL receptor, which plays a crucial role in clearing circulating LDL cholesterol

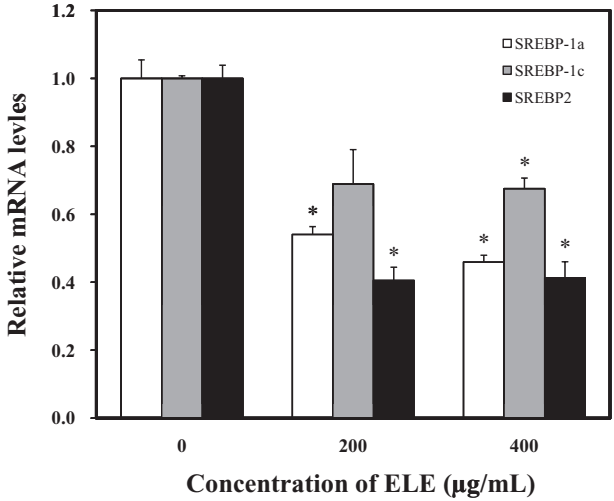

FIG. 3. EFFECT OF ELE ON THE MRNA EXPRESSION OF *SREBP-1A*, *SREBP-1C* AND *SREBP-2* IN HEPG2 CELLS

HepG2 cells were treated with 0, 200 or 400 μg/mL ELE for 24 h. Total RNA was extracted from HepG2 cells and *SREBP-1a*, *SREBP-1c* and *SREBP-2* mRNA levels were measured by RT-PCR. The relative band densities were determined using Sigma Gel software (SPSS) with *GAPDH* as a reference.

The data are expressed as the mean ± standard error of three independent experiments.

*Significantly different from the control ($P < 0.05$).

from the blood, we investigated the LDL receptor-mediated uptake of LDL cholesterol following ELE exposure. To measure the uptake of LDL, we incubated HepG2 cells treated with 400 μg/mL ELE for 24 h with DiI-labeled LDL cholesterol and determined the amounts of membrane-bound and cell-associated LDL. As shown in Fig. 6, the amounts of both membrane-bound and cell-associated LDL increased significantly, and the levels were similar to each other.

DISCUSSION

We investigated the hypocholesterolemic effects of ELE *in vitro* by quantifying its role in LDL oxidation and the expression of genes involved in cholesterol metabolism. In HepG2 cells, ELE increased the expression of the LDL receptor and decreased the expression of HMG-CoA reductase at both the RNA and protein levels. The ELE enhanced receptor-mediated LDL uptake, possibly caused by the upregulation of the LDL receptor. The ELE also

inhibited LDL oxidation via its antioxidant activities. The ELE treatment strongly protected against LDL oxidation initiated by Cu^{2+} ions. Flavonoids and polyphenolic antioxidants in the ELE may inhibit LDL oxidation and change receptor affinity because of their lipophilic nature (Kelley *et al*. 1976; Yamamoto *et al*. 2000; Chen *et al*. 2005; Kurosawa *et al*. 2005; Han *et al*. 2008). Oxidized LDL particles have a low affinity for the LDL receptor, but a markedly higher affinity for macrophage scavenger receptors such as SR-A and CD36, which can accelerate foam-cell formation in the major blood vessels. Consequently, the prevention of LDL oxidation by antioxidative ELE compounds may increase the affinity of LDL for the LDL receptor, as shown by our LDL uptake results.

Plasma cholesterol homeostasis is maintained largely by hepatic synthesis and removal from the circulation. The former is largely controlled by HMG-CoA reductase, whereas the latter is regulated mainly by hepatic uptake of LDL particles and the subsequent metabolism of hepatic cholesterol into bile acids. The LDL receptor and CYP7A1 are involved in the latter process. In this study, ELE downregulated HMG-CoA reductase and

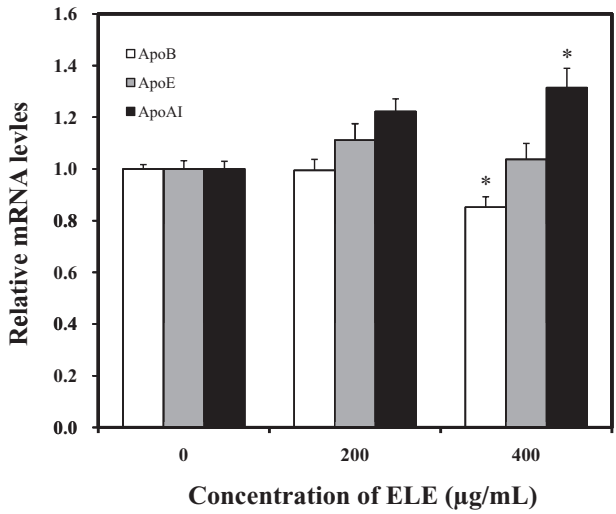

FIG. 4. EFFECT OF ELE ON THE MRNA EXPRESSION OF apoB, apoE AND apoAI IN HEPG2 CELLS

HepG2 cells were treated with 0, 200 or 400 μg/mL ELE for 24 h. Total RNA was extracted from HepG2 cells and apoB, apoE and apoAI mRNA levels were measured by RT-PCR. The relative band densities were determined using Sigma Gel software (SPSS) with *GAPDH* as a reference. The data are expressed as the mean ± standard error of three independent experiments. *Significantly different from the control ($P < 0.05$).

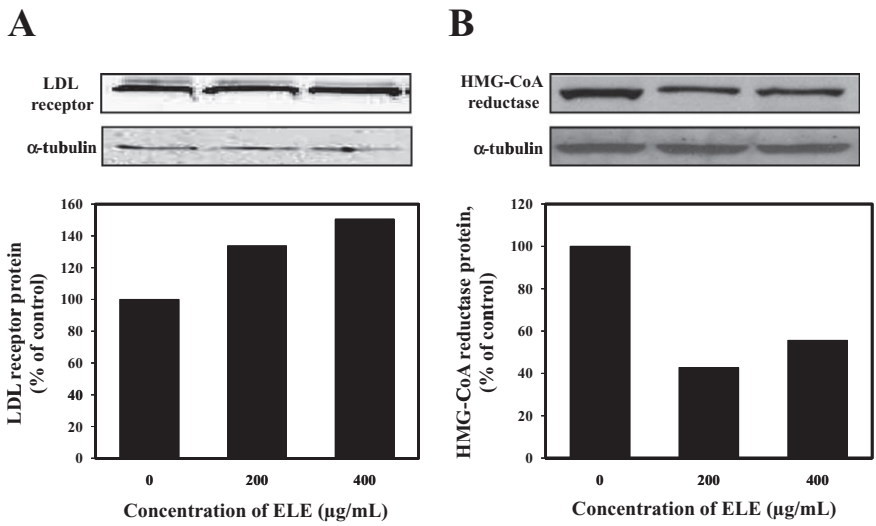

FIG. 5. EFFECT OF ELE ON THE PROTEIN EXPRESSION OF THE LDL RECEPTOR (A) AND HMG-COA REDUCTASE (B) IN HEPG2 CELLS

HepG2 cells were treated with 0, 200 or 400 μg/mL ELE for 24 h. Proteins were isolated from HepG2 cells and separated by electrophoresis. LDL receptor and HMG-CoA reductase protein levels were determined by immunoblot analysis using specific antisera and quantified by image analysis using Gel-Pro Analyzer 4.0 software (Media Cybernetics).

upregulated the LDL receptor, both of which could reduce the plasma cholesterol concentration. However, the expression of *CYP7A1*, which encodes cholesterol 7-α hydroxylase, a rate-limiting enzyme in the classical pathway of bile acid formation, was unaltered. Under normal circumstances, HMG-CoA reductase and the LDL receptor are induced on the depletion of cholesterol in hepatocytes via the activation of SREBPs (Brown and Goldstein 1997; Shimano 2001). These genes were regulated in opposite directions in this study: the LDL receptor was upregulated, whereas HMG-CoA reductase was downregulated. The ELE may reduce HMG-CoA reductase expression in a sterol-independent manner via the downregulation of SREBPs (Lloyd 1995), especially SREBP2. The induction of the LDL receptor may be triggered later by a reduction in cellular cholesterol synthesis and the enhanced uptake of plasma LDL particles. LE has been suggested to inhibit acyl-CoA acetyltransferase, which produces the cellular storage form of cholesterol involved in the formation of foam cells (An *et al*. 2007). By regulating multiple cholesterol-related genes, ELE may lower cellular and plasma cholesterol levels.

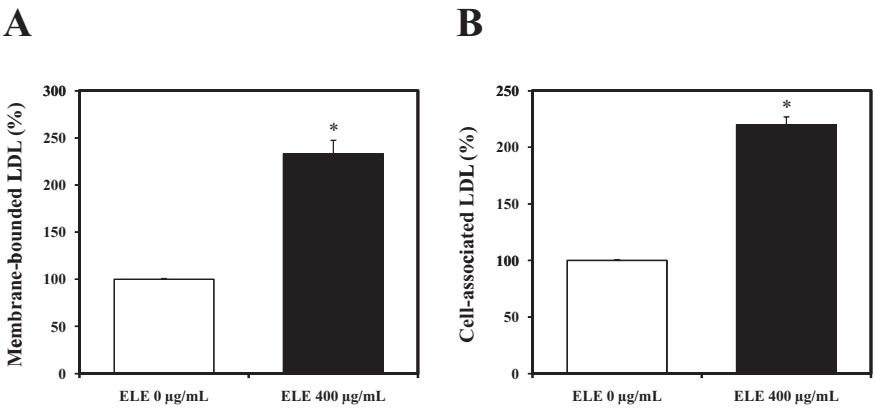

FIG. 6. EFFECT OF ELE ON RECEPTOR-MEDIATED LDL UPTAKE BASED ON THE MEASUREMENT OF MEMBRANE-BOUND (A) AND CELL-ASSOCIATED (B) LDL IN HEPG2 CELLS

Fluorescence (DiI)-labeled LDL was incubated with 400 µg/mL ELE-treated HepG2 cells for 2 h at 4C and 37C to measure membrane-bound and cell-associated LDL, respectively. Receptor-mediated LDL uptake was determined by spectrofluorometer.

The data are expressed as the mean ± standard error of three independent experiments.

*Significantly different from the control ($P < 0.05$).

The expression of apoB and apoAI, surrogate markers for plasma LDL and HDL, was also altered, and the expression of SR-BI, the cellular receptor for HDL, slightly decreased. These data suggest that ELE may increase the concentration of HDL while reducing the level of LDL. This possibility should be investigated further in animals. In this study, ELE inhibited LDL oxidation at low concentrations and promoted it at high concentrations. Therefore, its dosage and effects on humans should be investigated *in vivo*. Shikonin and its derivatives are thought to be the major bioactive compounds in ELE (Jian *et al*. 1991; Luo and Li 1992; Zhang *et al*. 2009), and the sum of both shikonin and acetylshikonin in the ELE was approximately 5%, implying that a small quantity of shikonin and its derivatives may be effective. However, the interaction of shikonin-derived compounds with other phytochemicals in the ELE may produce synergistic effects on cholesterol metabolism. This should be verified using single-compound experiments.

In conclusion, the antioxidative phytochemicals in ELE may improve cholesterol metabolism by preventing LDL oxidation and regulating the expression of genes involved in cholesterol metabolism. According to our results, ELE may confer desirable health benefits in the prevention of CHD.

NOMENCLATURE

apoAI	apolipoprotein A-I
apoB	apolipoprotein B
CHD	coronary heart disease
HDL	high-density lipoprotein
LDL	low-density lipoprotein
LE	*Lithospermum erythrorhizon* Sieb. et Zucc
ELE	ethanolic extract of *Lithospermum erythrorhizon* Sieb. et Zucc.
SR-BI	scavenger receptor class-B, type I
SREBP	sterol regulatory element binding protein
TBARS	thiobarbituric acid relative substances

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REFERENCES

AN, S., PARK, Y.D., PAIK, Y.K., JEONG, T.S. and LEE, W.S. 2007. Human ACAT inhibitory effects of shikonin derivatives from *Lithospermum erythrorhizon*. Bioorg. Med. Chem. Lett. *17*, 1112–1116.

BRADFORD, M.M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. *72*, 248–254.

BROWN, M.S. and GOLDSTEIN, J.L. 1997. The SREBP pathway: Regulation of cholesterol metabolism by proteolysis of a membrane-bound transcription factor. Cell *89*, 331–340.

CHEN, C.Y., MILBURY, P.E., LAPSLEY, K. and BLUMBERG, J.B. 2005. Flavonoids from almond skins are bioavailable and act synergistically with vitamins C and E to enhance hamster and human LDL resistance to oxidation. J. Nutr. *135*, 1366–1373.

CHEN, X., OPPENHEIM, J. and HOWARD, O.M. 2001. Shikonin, a component of antiinflammatory Chinese herbal medicine, selectively blocks chemokine binding to CC chemokine receptor-1. Int. Immunopharmacol. *1*, 229–236.

EVANS, M. and REES, A. 2002. The myotoxicity of statins. Curr. Opin. Lipidol. *13*, 415–420.

FINDLEY, W.E. 1981. The antigonadotropic activity of *Lithospermum ruderale*. II. The inhibition of LRF-induced gonadotropin release *in vitro*. Contraception *23*, 157–162.

FINDLEY, W.E. and JACOBS, B.R. 1980. The antigonadotropic activity of *Lithospermum ruderale* I. The lack of steroid-like activity at the receptor level. Contraception *21*, 199–205.

HAN, J., WENG, X. and BI, K. 2008. Antioxidants from a Chinese medicinal herb – *Lithospermum erythrorhizon*. Food Chem. *106*, 2–10.

HAYASHI, M. 1977. Pharmacological studies of Shikon and Tooki. (3) Effect of topical application of the ether extracts and Shiunko on inflammatory reactions. Nippon Yakurigaku Zasshi *73*, 205–214.

JENSEN, G.S., WU, X., PATTERSON, K.M., BARNES, J., CARTER, S.G., SCHERWITZ, L., BEAMAN, R., ENDRES, J.R. and SCHAUSS, A.G. 2008. *In vitro* and *in vivo* antioxidant and anti-inflammatory capacities of an antioxidant-rich fruit and berry juice blend. Results of a pilot and randomized, double-blinded, placebo-controlled, crossover study. J. Agric. Food Chem. *56*, 8326–8333.

JIAN, Y., ZHOU, Y. and ZHANG, H. 1991. Quality of Chinese drug Zicao. Zhongguo Zhong Yao Za Zhi *16*, 524–526, 573–524.

JOSHIPURA, K.J., HU, F.B., MANSON, J.E., STAMPFER, M.J., RIMM, E.B., SPEIZER, F.E., COLDITZ, G., ASCHERIO, A., ROSNER, B., SPIEGELMAN, D. *ET AL*. 2001. The effect of fruit and vegetable intake on risk for coronary heart disease. Ann. Intern. Med. *134*, 1106–1114.

KELLEY, C.J., HARRUFF, R.C. and CARMACK, M. 1976. Polyphenolic acids of *Lithospermum ruderale*. II. Carbon-13 nuclear magnetic resonance of lithospermic and rosmarinic acids. J. Org. Chem. *41*, 449–455.

KIM, D.O., JEONG, S.W. and LEE, C.Y. 2003. Antioxidant capacity of phenolic phytochemicals from various cultivars of plums. Food Chem. *81*, 321–326.

KUROSAWA, T., ITOH, F., NOZAKI, A., NAKANO, Y., KATSUDA, S., OSAKABE, N., TSUBONE, H., KONDO, K. and ITAKURA, H. 2005. Suppressive effects of cacao liquor polyphenols (CLP) on LDL oxidation and the development of atherosclerosis in Kurosawa and Kusanagi-hypercholesterolemic rabbits. Atherosclerosis *179*, 237–246.

LEE, J.H., JUNG, K.M., BAE, I.H., CHO, S., SEO, D.B., LEE, S.J., PARK, Y.H. and LIM, K.M. 2009. Anti-inflammatory and barrier protecting effect of *Lithospermum erythrorhizon* extracts in chronic oxazolone-induced murine atopic dermatitis. J. Dermatol. Sci. *56*, 64–66.

LEE, S.J., MOYE, L.A., CAMPOS, H., WILLIAMS, G.H. and SACKS, F.M. 2003. Hypertriglyceridemia but not diabetes status is associated with VLDL containing apolipoprotein CIII in patients with coronary heart disease. Atherosclerosis *167*, 293–302.

LLOYD, D.B. and THOMPSON, J.F. 1995. Transcriptional modulators affect in vivo protein binding to the low density lipoprotein receptor and 3-hydroxy-3-metnylglutaryl coenzyme A reductase prototers. J. Biol. Chem. *270*, 25812–25818.

LUO, S. and LI, T. 1992. Determination of naphthoquinone in Zicao. Zhongguo Zhong Yao Za Zhi *17*, 552–554.

NIGORIKAWA, K., YOSHIKAWA, K., SASAKI, T., IIDA, E., TSUKAMOTO, M., MURAKAMI, H., MAEHAMA, T., HAZEKI, K. and HAZEKI, O. 2006. A naphthoquinone derivative, shikonin, has insulin-like actions by inhibiting both phosphatase and tensin homolog deleted on chromosome 10 and tyrosine phosphatases. Mol. Pharmacol. *70*, 1143–1149.

NURMI, K., OSSIPOV, V., HAUKIOJA, E. and PIHLAJA, K. 1996. Variation of total phenolic content and individual low-molecular-weight phenolics in foliage of mountain birch trees (*Betula pubescens* ssp *tortuosa*). J. Chem. Ecol. *22*, 2023–2040.

SHIMANO, H. 2001. Sterol regulatory element-binding proteins (SREBPs): Transcriptional regulators of lipid synthetic genes. Prog. Lipid Res. *40*, 439–452.

STANIFORTH, V., WANG, S.Y., SHYUR, L.F. and YANG, N.S. 2004. Shikonins, phytocompounds from *Lithospermum erythrorhizon*, inhibit the transcriptional activation of human tumor necrosis factor alpha promoter *in vivo*. J. Biol. Chem. *279*, 5877–5885.

STOLK, M.F., BECX, M.C., KUYPERS, K.C. and SELDENRIJK, C.A. 2006. Severe hepatic side effects of ezetimibe. Clin. Gastroenterol. Hepatol. *4*, 908–911.

TUCKER, K.L. 2004. Dietary intake and coronary heart disease: A variety of nutrients and phytochemicals are important. Curr. Treat. Options Cardiovasc. Med. *6*, 291–302.

VISIOLI, F., BORSANI, L. and GALLI, C. 2000. Diet and prevention of coronary heart disease: The potential role of phytochemicals. Cardiovasc. Res. *47*, 419–425.

WINTERHOFF, H., GUMBINGER, H.G. and SOURGENS, H. 1988. On the antigonadotropic activity of *Lithospermum* and *Lycopus* species and some of their phenolic constituents. Planta Med. *54*, 101–106.

YAMAMOTO, H., INOUE, K. and YAZAKI, K. 2000. Caffeic acid oligomers in Lithospermum erythrorhizon cell suspension cultures. Phytochemistry *53*, 651–657.

YINGMING, P., YING, L., HENGSHAN, W. and MIN, L. 2004. Antioxidant activities of several Chinese medicine herbs. Food Chem. *88*, 347–350.

YOSHIMI, N., WANG, A., MORISHITA, Y., TANAKA, T., SUGIE, S., KAWAI, K., YAMAHARA, J. and MORI, H. 1992. Modifying effects of

fungal and herb metabolites on azoxymethane-induced intestinal carcinogenesis in rats. Jpn. J. Cancer Res. *83*, 1273–1278.

ZHANG, Y., QIAN, R.Q. and LI, P.P. 2009. Shikonin, an ingredient of *Lithospermum erythrorhizon*, down-regulates the expression of steroid sulfatase genes in breast cancer cells. Cancer Lett. *284*, 47–54.