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Characterization of β-Hydroxy-β-methylglutaryl Coenzyme A Reductase Inhibitor from *Pueraria thunbergiana*

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This study describes the extraction and characterization of an inhibitor for β-hydroxy-β-methylglutaryl (HMG) coenzyme A (CoA) reductase from *Pueraria thunbergiana*. The maximum HMG-CoA reductase inhibitory activity (IC_{50} = 79 μg) was obtained when *P. thunbergiana* was extracted with 70% ethanol at 30 °C for 12 h. After purification of the HMG-CoA reductase inhibitor by means of systematic solvent extraction, silica gel column chromatography, and HPLC, an active fraction with an IC_{50} of 0.9 μg (4.25 μM) and a yield of 1.3% was obtained. The purified HMG-CoA reductase inhibitor was identified as daidzein ($C_{15}H_{10}O_4$; molecular mass, 254 Da).

KEYWORDS: ***Pueraria thunbergiana*; β-hydroxy-β-methylglutaryl coenzyme A reductase inhibitor**

INTRODUCTION

β-Hydroxy-β-methylglutaryl coenzyme A (HMG-CoA) reductase (EC 1.1.1.34), a rate-limiting enzyme in endogenous cholesterol synthesis, is a 97 kDa glycoprotein and catalyzes the reductive deacylation of HMG-CoA to mevalonate in a two-step reaction (*1*, *2*). Hyperlipemia or coronary heart diseases are caused by increased blood cholesterol levels. Therefore, lowering total cholesterol through the action of a HMG-CoA reductase inhibitor is very important for the remedy or prevention of hyperlipemia.

Many cholesterol-lowering agents, such as cholestyramine, clofibrate, neomycin, plant sterol, triparanol (MER-29), D-throzine, and estrogenic hormones, have been studied and introduced into clinical use (*3*). Nicotinic acid has also been found to reduce both cholesterol and triglyceride in humans. Nicotinic acid decreases lipoprotein synthesis, resulting in a fall in low-density lipoprotein (LDL) cholesterol (*4*). Use of nicotinic acid, however, can cause adverse side effects such as cutaneous vasodilation, rashes, gastrointestinal irritation, hyperuricemia, hyperglycemia, and hepatic dysfunction (*3*). Endo et al. (*5*) reported the isolation of mevastatin (formerly called ML236M or compactin) as a potent inhibitor of HMG-CoA reductase, the rate-limiting enzyme in endogenous cholesterol synthesis. Thus, isolation of mevastatin was a key step toward attaining an effective means of lowering plasma cholesterol in humans. They elucidated the biochemical mechanism that causes the action of mevastatin and showed that mevastatin dramatically lowers LDL and total cholesterol in patients with hypercholesterolemia. Subsequently, three drugs have been marketed in many countries: lovastatin (called mevinolin) or monacolin K, simvastatin, and pravastatin (*6*). In addition to these, many other mevastatin analogues have been synthesized, some of which are now under clinical development (*4*). Mevastatin, a metabolite of *Penicillium citrinum*, was first isolated in 1973 and later isolated from *Penicillium brevicompactum* as an antibiotic. Later, lovastatin was isolated from both *Monascus ruber* and *Aspergillus terreus*. Lovastatin is slightly more potent than mevastatin in inhibiting HMG-CoA reductase. These compounds can be easily converted to the respective open-chain dihydroxy acids. In addition to mevastatin, dihydrocompactin, ML-263A, and ML-236C have been isolated from *P. citrinum*. Furthermore, monacolin J, monacolin L, and dihydromonacolin L acid are all minor metabolites of *M. ruber*, and dihydromevinolin is a product of *Aspergillus terreus*. Extracts of medicinal plants such as *Typha augustifolia*, *Polygonum cuspidatum*, *Crataegus pinnatifida*, and *Polygonum multiflorum* (*7*); orengedokuto (OT), which consists of *Scutellariae radix*, *Coptidis rhizoma*, *Phellodendri cortex*, and *Gardeniae fructus*; and daio-orengedokuto (DOT), which is OT with purgative drugs such as *Rhei rhizoma* added (*8*), showed high HMG-Co A reductase inhibitory activity. Recently, isoflavone compounds found in Korean soybean paste acted as HMG-Co A reductase inhibitors (*9*).

However, commercial anti-hyperlipemia drugs, including mevastatin, have some disadvantages, such as high cost for low yield, ineffectiveness in vivo, and some side effects. Therefore, the purpose of this study was to extract, purify, and characterize the HMG-CoA reductase inhibitor from *Pueraria thunbergiana* to produce a new anti-hyperlipemia agent.

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MATERIALS AND METHODS

Materials and Chemicals. Eight hundred and sixty-five kinds of medicinal plants were obtained from the Chodang Nongsan Co. Chungnam, Korea. *Escherichia coli* BL21(DE3) was used as a host for a recombinant plasmid pKFT7-21 containing the HMG-CoA reductase gene, which was obtained from Professor Victor W. Rodwell of Purdue University. All of the chemicals used in this study were of special pure grade and HPLC grade.

Extraction of HMG-CoA Reductase Inhibitor. Powders of the 865 kinds of medicinal plants were each added to water and 70% ethanol (1:40, v/v) and were then shaken for 12 h at 30 °C. Each of the extracts was filtered and lyophilized.

Preparation of Syrian Hamster HMG-CoA Reductase. The catalytic domain of Syrian hamster HMG-CoA reductase with the C-terminal extension of Glu-Glu-Phe (RcatEEF) was overexpressed in *E. coli* BL21(DE3) under the control of the T7 promoter. The construction of pKFT7-21 was described by Frimpong (*2*) *E. coli* BL21-(DE3) freshly transformed with pKFT7-21 was inoculated in 100 mL of LB_{amp} broth. The culture was shaken at 37 °C. When the cell density (A_{600}) reached 0.5−0.7, isopropyl-β-D-thiogalactoside was added to a final concentration of 0.5 mM, and growth was continued further for exactly 5 h. Cells were harvested by centrifugation (10000*g*, 15 min, 4 °C), suspended in 15 mL of buffer A (pH 7.3, 20 mM Na_2PO_4, 50 mM NaCl, 10% glycerol, 100 mM sucrose, and 10 mM dithiothreitol), and used directly for the preparation of cell extract.

Cells suspended in buffer A were broken at duty cycle 40% and output control 4 for 20 min by a sonicator (Branson 450 sonifier) in an ice bath. The cell lysate was ultracentrifuged (100000*g*, 60 min, 4 °C). The pelleted cell debris was discarded, and the volume of the supernatant was frozen in a −70 °C deep freezer before use for an assay of HMG-CoA reductase inhibitory activity. Its protein content was determined according to the method of Bradford using bovine serum albumin as a standard (*10*).

Assay of HMG-CoA Reductase Inhibitory Activity. The HMG-CoA reductase inhibitory activity was assayed spectrophotometrically, following the method of Kleinsek et al. (*11*), whereby the rate of decrease in absorbance at 340 nm due to the oxidation of NADPH was measured. The spectrophotometer was equipped with a cell holder and maintained at 37 °C. The standard assay mixture contained 300 μM HMG-CoA, 500 μM NADPH, 100 mM NaCl, 1.0 mM EDTA, 2 mM dithiothreitol, and 0.5 mM potassium phosphate buffer (pH 7.0) at a final volume of 150 μL. The reaction mixture containing the enzyme (100 μg/150 μL) and all components except HMG-CoA were first monitored to detect any HMG-Co A-independent oxidation of NADHP. To check the inhibitory activities of the fractions from each fraction, evaporated by a SpeedVac (Eyela, Tokyo, Japan), the fractions were dissolved in DMSO. The reaction was then initiated by adding HMG-CoA. One unit of HMG-CoA reductase is defined as the amount of enzyme that catalyzes the oxidation of 1 μmol of NADPH per minute.

The concentration of HMG-CoA reductase inhibitor required to inhibit 50% of the HMG-CoA reductase under the above assay conditions was defined as IC_{50}. A statistical analysis was performed by a Sigma plot of SPSS Inc. using mean values of triplicate measurements ($p \leq 0.05$).

Purification of HMG-CoA Reductase Inhibitor. Seventy percent ethanol extracts of *P. thunbergiana* were fractionated stepwise with *n*-hexane, chloroform, ethyl acetate, butanol, and water according to the procedure illustrated in **Figure 1**. The ethyl actate fraction showed high HMG-CoA reductase inhibitory activity (85%). Therefore, the ethyl acetate fraction was subjected to silica gel column chromatography. The active ethyl acetate extracts were dissolved with distilled water and fractionated by means of silica gel column chromatography with ethyl acetate/methanol (80:20,v/v), followed by chloroform/methanol/acetic acid (85:10:5). The active fractions obtained were then applied to a preparative reverse-phase high-performance liquid chromatography (μBonda Pak C_{18} column), equilibrated with deionized water. Isocratic elution was carried out with deionized water/acetonitrile (65:35). The active fraction was collected and lyophilized immediately. The active fraction obtained was subjected to analytical reverse-phase high-performance liquid chromatography (μBonda Pak C_{18}, 10 μm, and 125 Å column), equilibrated with deionized water. An isocratic elution was carried out with 30% acetonitrile.

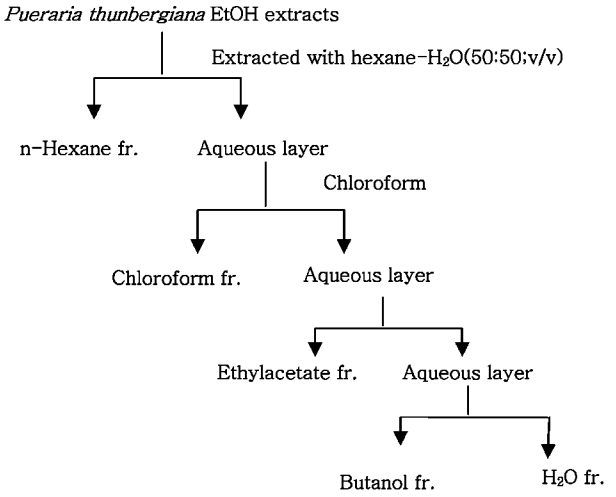

Figure 1. Scheme for extraction and fractionation of HMG-CoA reductase inhibitor from *P. thunbergiana*.

Molecular Weight Determination and Structure Analysis. The molecular weight of the purified HMG-CoA reductase inhibitor from *P. thunbergiana* was determined by LC-MS spectrometry with Mariner [Perseptive Biosystem, ESI-MS(+), C_{18} column, 0.32 × 200 mm], with a gradient of 100% deionized water for 10 min followed 50% acetonitrile for 40 min.

Identification and structure analysis were also performed by ^1H and ^{13}C NMR spectrometry with a Bruker DRX 300 spectrometer and Fourier transform infrared (FT-IR) spectrometry with a Shimadzu FT-IR 8700 spectrometer (Shimadzu Inc.), respectively.

Antihyperlipemia Action of the Purified HMG-CoA Reductase Inhibitor in Vero Cells. Vero cells (ATCC CCL-81), taken from the kidney of an African green monkey, were used as test cells. The cells were routinely grown in a humidified incubator (95% air/5% CO_2) at 37 °C for 24 h. The Vero cells were seeded in each well of a 24 well microplate at (1.0−3.0) × 10^4 cells. Confluent growth was observed after 24 h of incubation for those cells seeded at (3.0−4.0) × 10^4 cells/well. Thus, a concentration of 3.0 × 10^4 cells/well was adopted for this experiment. The concentrations of daidzein showing inhibition of Vero cell growth were selected.

RESULTS

Inhibitory Activities of Various Extracts from Medicinal Plants on HMG-CoA Reductase. The HMG-CoA reductase inhibitory activities of water and ethanol extracts from medicinal plants were investigated. Among 865 kinds of medicinal plants, the water and ethanol extracts of only 25 kinds of medicinal plants showed HMG-CoA reductase inhibitory activity. As shown in **Table 1**, the ethanol extract of *P. thunbergiana* showed the highest HMG-CoA reductase inhibitory activity, at 77% inhibition. Generally, the ethanol extracts showed higher HMG-CoA reductase inhibitory activities than those of water extracts. Ultimately, *P. thunbergiana* was selected for the production of an HMG-CoA reductase inhibitor.

The effects of extraction temperature and time on the HMG-CoA reductase inhibitory activity of the ethanol extracts were then determined. The HMG-CoA reductase inhibitor was maximally extracted by shaking at 30 °C for 12 h (data not shown).

Purification of HMG-CoA Reductase Inhibitor. Table 2 illustrates the purification steps and the inhibitory activity of the HMG-CoA reductase inhibitor isolated from *P. thunbergiana*. Among several organic solvent extracts, ethyl acetate extracts showed the highest HMG-CoA reductase inhibitory

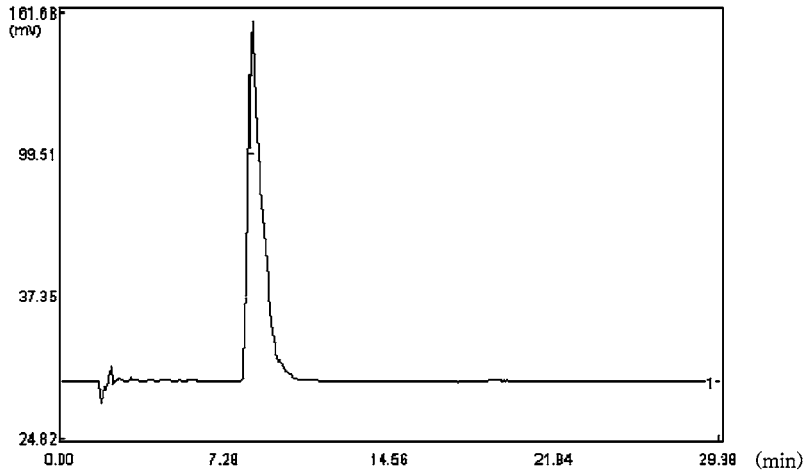

Figure 2. Analytical reverse phase HPLC profile of active fraction obtained from preparative reverse phase HPLC.

Table 1. HMG-CoA Reductase Inhibitory Activity of the Various Extracts from Second-Selected Medicinal Plants (Percent Inhibition)

scientific name	part used	water extract	EtOH extract
Pueraria thunbergiana	radix	nd[a]	77[b]
Glicyrrhizae radix	radix	72	27
Angelica koreana Maximowicz	radix	15	72
Curcuma longa Linne	radix	51	43
Rehmannia glutinosa Liboschitz var. *purpurea* Makino	radix	57	nd
Agastache rugosa	herba, stem	71	1
Platycodon grandiflorum A. De Candolle	radix	46	72
Prunus persica Batsch	seed	64	26
Hordeum vulgare	seed	71	nd
Akebia quinata	radix	43	68
Angelica dahurica Bentham et Hooker	radix	57	69
Rubus coreanus Miquel	fruit	7	71
Acorus graminueus Solander	radix, stem	32	51
Lindera strichnifolia Villars	radix	65	54
Coix lachrymajobi	fruit	70	11
Leonurus sibircus Linne	whole	57	15
Artemisia princeps	whole	nd	50
Cnidium officinale Makino	radix, stem	68	68
Paeonia lactiflora var. *hortensis*	radix	58	42
Polyporus umbellatus Fries	radix	68	72
Rhizoma atractylodes lancea D.C	fruit	60	63
Achyranthes japonica	radix	71	57
Angelicagigas sp.	fruit	nd	50
Crataegus pinnatifida	fruit	50	nd

[a] Not determined. [b] Mean value of triplicate measurements.

Table 2. Purification of HMG-CoA Reductase Inhibitor from *P. thunbergiana*

purification step	IC_{50} (μg)	solid yield (%)
ethanol extracts	79	100
ethyl acetate extracts	67	66.8
first silica gel column chromatography	57	53.7
second silica gel column chromatography	27	24.6
preparative HPLC	2.5	3.1
analytical HPLC	0.9	1.3

activity. The active fraction from the silica gel column chromatography of the ethyl acetate extracts showed 27 μg of IC_{50} and 24.6% of activity yield. After preparative HPLC, the active fraction showed 2.5 μg of IC_{50} and 3.1% activity yield. The active fraction from the above HPLC was collected and subjected to an analytical HPLC. One peak containing HMG-CoA reductase inhibitory activity was observed (**Figure 2**). After the purification steps, HMG-CoA reductase inhibitor with an IC_{50} of 0.9 μg was obtained, and the activity yield was 1.3%.

Table 3. Physicochemical Properties of the Purified HMG-CoA Reductase Inhibitor

appearance	pale yellow
soluble	ethanol methanol DMSO
insoluble	water hexane chloroform
UV (λ_{max}) (MeOH)	221, 238 nm
molecular formula	$C_{15}H_{10}O_4$
molecular weight	254
melting point	323 °C
ingredient name	daidzein (4′,7-dihydroxyisoflavone)

Molecular Mass and Structural Analysis of the HMG-CoA Reductase Inhibitor. As shown in **Figure 3**, the molecular mass of the purified HMG-CoA reductase inhibitor was found to be roughly 254.13 Da by means of LC-MS analysis (**Figure 3**).

The purified HMG-CoA reductase inhibitor was identified as daidzein ($C_{15}H_{10}O_4$) by means of an instrumental analysis of ^{1}H NMR (**Figure 4**),^{13}C NMR (**Figure 5**), and FT-IR (**Figure 6**). Its structure is shown in **Figure 6**. Daidzein is known as one of the isoflavone compounds that are founds in natural plants, and Sung et al. (*9*) reported that three kinds of isoflavone compounds, genistein, daidzein, and glycitein, from Korean soybean paste showed high HMG-CoA reductase inhibitory activity.

Properties of the HMG-CoA Reductase Inhibitor. The physicochemical properties of the purified HMG-CoA reductase inhibitor were investigated (**Table 3**). The purified HMG-CoA reductase inhibitor was pale yellow in color and was soluble in ethanol, methanol, and DMSO. The maximum absorption spectra of the purified HMG-CoA reductase inhibitor dissolved in methanol were 221 and 238 nm.

The HMG-CoA reductase inhibition pattern of the purified HMG-CoA reductase inhibitor from the ethanol extract of *P. thumbergiana* was investigated by Lineweaver−Burk plot (**Figure 7**). It was predicted to be a competitive inhibition pattern on HMG-CoA reductase, suggesting that HMG-CoA reductase

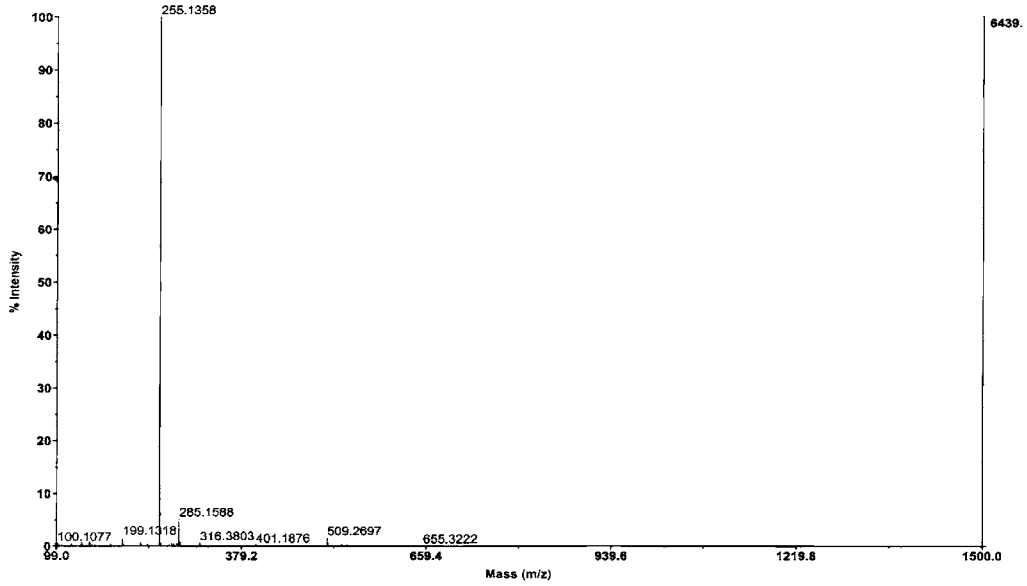

Figure 3. LC–mass spectrum of the purified HMG-CoA reductase inhibitor.

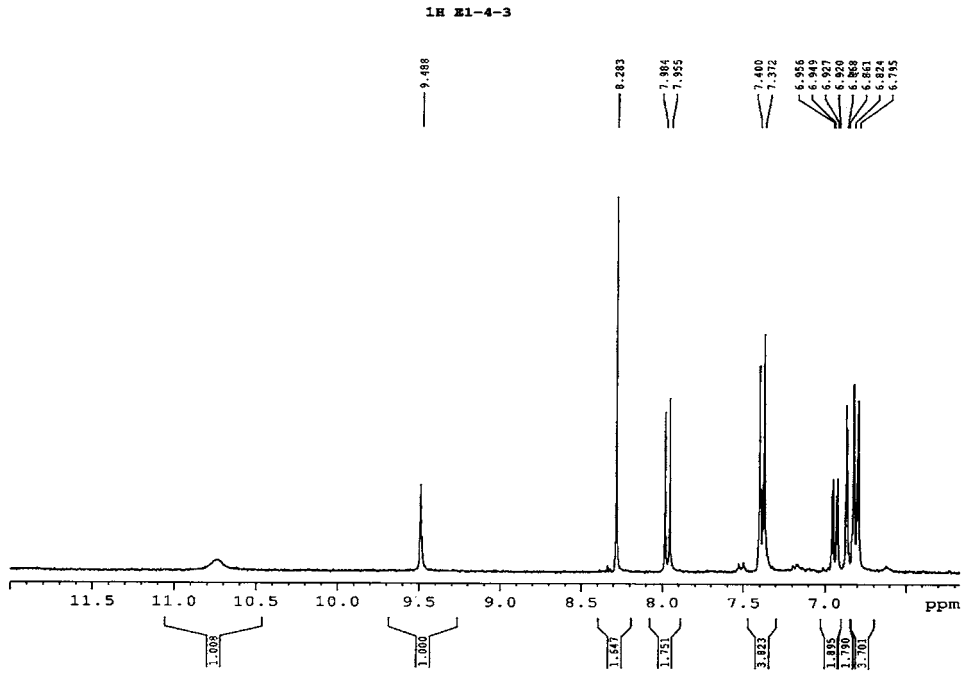

Figure 4. ^{1}H NMR spectrum of the purified HMG-CoA reductase inhibitor in DMSO-d_6 at 300 MHz.

from *P. thumbergiana* binds competitively with the substrate, at the active site of HMG-CoA reductase.

Anti-hyperlipemia Action of the Purified HMG-CoA Reductase Inhibitor in Vero Cells. Vero cell is a green monkey kidney cell. Its cholesterol formation by HMG-CoA reductase is very important in cell formation and growth. That is, inhibition of its HMG-CoA reductase would lead to growth inhibition. Therefore, the anti-hyperlipemia action of the purified inhibitor was investigated by adding various concentrations of the purified HMG-CoA reductase inhibitor into a culture broth of Vero cells. As shown in **Figure 8**, the Vero cells clearly changed into a circular shape at 5.0 μM daidzein. These results indicated that growth of the Vero cells was significantly inhibited at 5.0 μM of the purified inhibitor. Furthermore, the purified inhibitor was very effective in preventing cholesterol biosynthesis.

DISCUSSION

To obtain a potent inhibitor of HMG-CoA reductase that will thereby function as a cholesterol-lowering agent, a screening process was performed on water extracts and 70% ethanol extracts of medicinal plants. The ethanol extracts from *P. thunbergiana* showed the highest HMG-CoA reductase inhibitory activity, whereas it had not been determined in water extracts, suggesting that the inhibitor may be a hydrophobic substance.

It was known that feedback suppression of cholesterol is mediated through changes in the activity of HMG-CoA reductase (*12*, *13*). Changes in the reductase activity are closely related to changes in the overall rate of cholesterol synthesis (*5*), and this suggests that the inhibition of HMG-CoA reductase would be an effective means of lowering plasma cholesterol in humans.

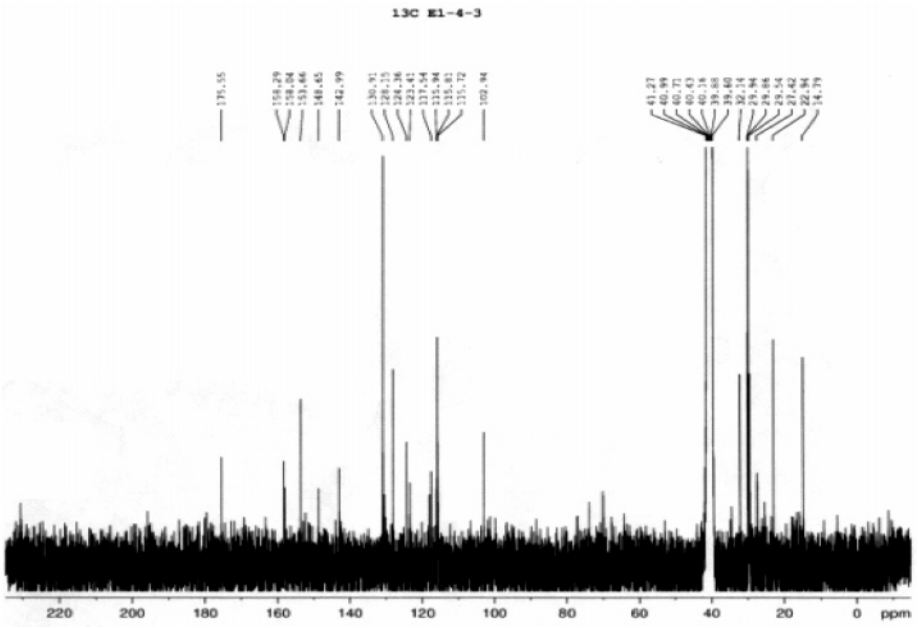

Figure 5. ^{13}C NMR spectrum of the purified HMG-CoA reductase inhibitor in DMSO-d_6 at 300 MHz.

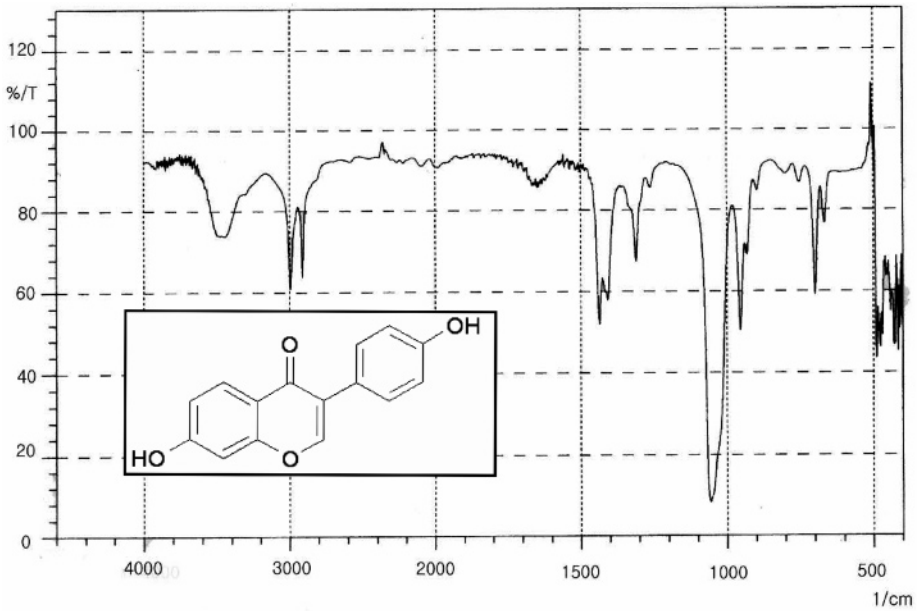

Figure 6. FT-IR spectrum of purified HMG-CoA reductase inhibitor. (Inset) Structure of purified HMG-CoA reductase inhibitor, daidzein.

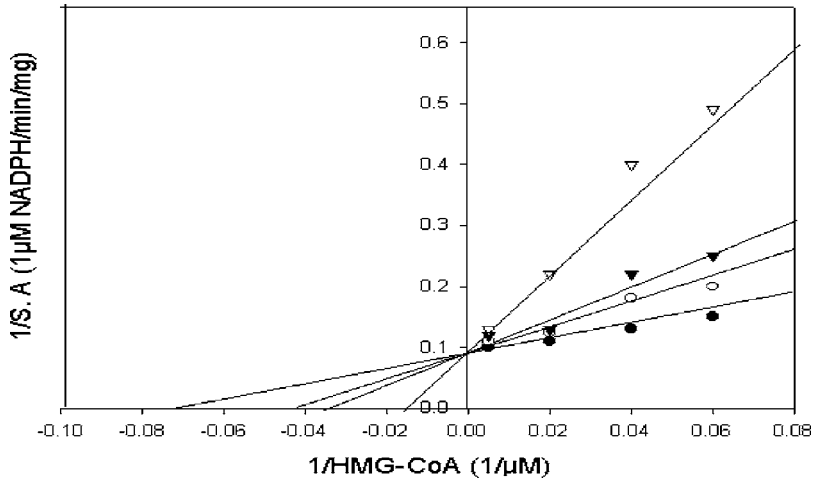

Figure 7. Lineweaver–Burk plot for the inhibition of HMG-CoA reductase by daidzein: ●, without daidzein; ○, 5 μM daidzein; ▲, 10 μM daidzein; ▽, 20 μM daidzein; S.A, specific activity.

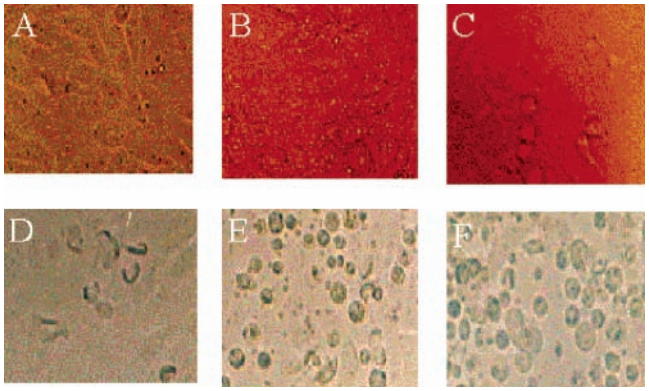

Figure 8. Phase-contrast microscopy of Vero cells. The cells were incubated for 24 h with various concentrations of purified daidzein: (**A**) control, 0.0 μM; (**B**) 0. 5 μM daidzein; (**C**) 2.5 μM daidzein; (**D**) 5.0 μM daidzein; (**E**) 10.0 μM daidzein; (**F**) 12.5 μM daidzein.

Various HMG-CoA reductase inhibitors have been isolated from many microorganisms. Mevastatin, dihydrocompactin, ML-236A, and ML-236C were isolated from *P. citrium* (*14*), and lovastatin was isolated from *M. ruber* and *A. terreus* (*15*, *16*). Meanwhile, several chemically synthesized compounds, fluvastatin, cerivastatin, strovastatin, rosuvastatin, and HR780, have been developed as effective cholesterol-lowering agents through the inhibition of HMG-CoA reductase (*17*−*21*). Similarly, some chemically modified HMG-CoA reductase inhibitors, including simvastatin and pravastatin, have been developed from natural products. However, most commercial HMG-CoA reductase inhibitors have adverse effects, including cutaneous vasodilation, rashes, gastrointestinal discomfort, hyperuricemia, hyperglycemia, and hepatic dysfunction (*4*). We report here a novel HMG-CoA reductase inhibitor extracted from *P. thunbergiana*: daidzein. *P. thunbergiana* has long been used to prevent coughs and cold symptoms and has been used as a tonic medicine. Furthermore, daidzein is known as one of the major isoflavones in soybeans and other plants, and it exhibits many biological activities, including the reduction of breast tumor formation (*22*), antioxidation (*23*), antiproliferation (*24*), antiangiogenic activity (*25*), and the inhibition of cytokinetic action and growth factors (*26*). Therefore, the HMG-CoA reductase inhibitor from *P. thunbergiana* will be very useful in the preparation of antihyperlipemia drugs and functional foods without any side effects.

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