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Antioxidative and hypocholesterolemic activities of water-soluble puerarin glycosides in HepG2 cells and in C57 BL/6J mice

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Abstract

Puerarin is an isoflavone derived from Kudzu roots and has antioxidant and hypocholesterolemic effects; however, its insolubility often limits its biological availability in vivo. Using a novel transglycosylation process, the solubility of puerarin glycosides was increased >100-fold, but it was not known whether these modified puerarin glycosides maintained biological activities. We found that water soluble puerarin glycosides fully maintained antioxidant activities compared with puerarin assessed by radical scavenging activity, reducing power assay, superoxide dismutase activity, and non-site-specific hydroxyl radical scavenging activity. Both puerarin and its glycosides also significantly reduced low-density lipoprotein (LDL) oxidation. Mice fed puerarin glycosides (0.1% w/w) showed significantly reduced plasma total cholesterol levels, thus, we further investigated their hypocholesterolemic mechanisms by assessing several key gene expressions both in vitro and in vivo.

Puerarin and its glycosides induced multiple changes in hepatic cholesterol metabolism. The LDL receptor promoter activity was increased dose-dependently in puerarin glycosides-treated HepG2 cells. Accordingly, the expression of LDL receptor mRNA and protein were also significantly increased in HepG2 cells and mouse livers. The transcription and translation of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase were downregulated both in vitro and in vivo. The cholesterol 7α-hydroxylase (CYP7A1) mRNA levels were not affected in vitro but significantly upregulated in the mouse livers. Collectively, our results show that puerarin and its glycosides are biologically fully active isoflavone and have antioxidant and hypocholesterolemic effects in HepG2 cells and in C57BL/6J mice. In the livers, hypocholesterolemic effects of puerarin glycoside may be achieved by multiple mechanisms including increasing LDL uptake, reducing cholesterol biosynthesis, and
possibly enhancing cholesterol degradation. (257 Words)

*Keywords*: puerarin glycosides; low-density lipoprotein receptor; HMG-CoA reductase; antioxidant
1. Introduction

Isoflavones are plant polyphenolic antioxidants that occur abundantly in soybean and Radix Puerariae (the root of the plant *Pueraria lobata*) (Guerra et al., 2000; Xiong et al., 2006; Xiong FL. et al., 2006). Puerarin (daidzein 8-C-glucoside), the main isoflavone glycoside found in the root of *P. lobata*, has been used for various medicinal purposes in traditional oriental medicine for thousands of years and it is well-known that puerarin protects cells against oxidative stress (Xiong et al., 2006). The antioxidant activity of puerarin is achieved through multiple mechanisms, for example, by reducing H$_2$O$_2$-induced elevation of caspase-3 activation (Jiang et al., 2003), scavenging free radicals (Wenli and Yaping, 2005), increasing superoxide dismutase (SOD) activity (Xiong et al., 2006; Xu, 2003), and decreasing malonaldehyde (Xu, 2003). Research has also shown that puerarin reduces serum cholesterol levels (Hsu et al., 2003; Song et al., 1988). These antioxidant activities and cholesterol-lowering effects could beneficially affect the pathophysiology of cardiovascular diseases and atherosclerosis (Song et al., 1988).

However, the insolubility of puerarin and other isoflavonoids often limits their bioavailability and may be one reason why many clinical studies have failed to find a positive association between isoflavone intake and the prevention of chronic diseases such as coronary heart disease (Lucas et al., 2001; Tham et al., 1998). The insolubility of isoflavones also hinders industrial applications such as the production of functional foods, food additives, and cosmetic products in which a natural bioactive compound such as puerarin as an active ingredient would be favored. Thus, we developed a novel enzymatic method to synthesize water-soluble puerarin glycosides using the special transglycosylation enzyme *Bacillus stearothermophilus* maltogenic amylase (Li et al., 2004). In the presence of puerarin and maltotriose or soluble starch, the
enzyme transfers a glucosyl unit from soluble starch to puerarin and produces a series
of transglycosylated puerarin glycosides. This simple procedure has dramatically
increased the water solubility of puerarin up to 168-fold (Li et al., 2004). However, it
was not known whether puerarin glycosides retain their isoflavone functions.

We recently reported that genistin and its glycosides isoflavones up-regulate
antioxidant genes and scavenge free radicals (Chung et al., 2006). Oxidative stress
plays an important role in the etiology of atherosclerosis thus their antioxidative
properties is believed to improve hepatic lipid metabolism partly by reducing
oxysterol formation. Thus, the possibility exist that puerarin and its glycosides also
have antioxidative properties and the effects on the expression of key genes in
cholesterol metabolism such as the low-density lipoprotein (LDL) receptor, 3-
hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase and cholesterol 7α-
hydroxylase (CYP7A1). Accordingly, we tested the effects of puerarin and its
glycosides on the expression of the key genes, using an approach similar to that used
in previous studies by our group on isoflavones (Chung et al., 2006).

The objectives of this research were to determine if puerarin and its glycosides
have antioxidant effects and whether puerarin and its glycosides inhibit the oxidation
of LDL by their antioxidant effects, in addition to assessing altered cholesterol
metabolism genes such as LDL receptors and HMG-CoA reductase for their
protective activity against atherosclerosis.

2. Materials and methods

2.1. Chemicals.

DMEM, fetal bovine serum (FBS), liquid gentamicin reagent solution,
penicillin and streptomycin, and trypsin-EDTA were purchased from Join Bio-
Innovation (Seoul, Korea). Enhanced chemiluminescence (ECL) Western blotting detection reagents and Hyperfilm™ ECL were obtained from Amersham Pharmacia Korea (Seoul, Korea). Anti-rabbit IgG and H&L chain-specific peroxidase conjugate were purchased from Calbiochem (Darmstadt, Germany). Anti-HMG-CoA reductase (rabbit polyclonal IgG) was obtained from Upstate (Lake Placid, NY, USA) and monoclonal anti-alpha tubulin clone DM 1a purified mouse immunoglobulin and goat anti-mouse IgG-HRP were purchased Sigma Chemical (St. Louis, MO, USA) and Santa Cruz Biotechnology (Santa Cruz, CA, USA), respectively. PowerScript reverse transcriptase was obtained from Clontech (Palo Alto, CA, USA). The oligo(dT)15 primer and random hexamers were obtained from Promega (Madison, WI, USA). IQ™ SYBR® Green Supermix was obtained from Bio-Rad (Bio-Rad, Hercules, CA, USA). All other reagents used were purchased from Sigma Chemical (St. Louis, MO, USA). The isoflavone mixture was donated by Pulmuone (Seoul, Korea). HepG2 cells were obtained from the Korean Cell Line Bank (Seoul, Korea).

2.2. Preparation and purification of puerarin and puerarin glycosides.

Puerarin and its glycosides were prepared and assayed using the method of Li et al. (Li et al., 2004). Briefly, Bacillus stearothermophilus maltogenic amylase was used for the transglycosylation reaction and maltotriose was used as a donor molecule in the transglycosylation reaction. The Sep-Pak Plus C18 cartridge was preconditioned with ethyl acetate, methanol, and water. The transglycosylation solution was loaded on the Sep-Pak Plus C18 cartridge. The retained puerarin glycosides were eluted with methanol. The puerarin glycosides solution was purified using a polymeric gel-filtration column (W-251) with recycling preparative HPLC to obtain the final transglycosylation products. Mobile phase is methanol/water (55:45; v/v), flow rate is
2.3. Animals, diets and experimented protocol

The mice were obtained from Orient Bio (Gyeonggi-Do, Korea). Female and male C57BL/6J mice were housed in a specific-pathogen-free system at 21-25 °C and humidity-controlled room with a 12 h on/12 h off light and then were divided into three groups of 10-17 mice. Animal care and handing was performed under protocols approved by the Committee on Animal Experimentation of the Hae-Eun Biotech Research Committee. In the feeding study, mice aged 7 weeks were fed normal chow or chow containing puerarin glycosides (0.05% and 0.1%) for 3 week. After feeding, the mice were fasted overnight (16-19 h), and blood samples were collected in purple-topped EDTA tube once a week. Plasma sample were obtained from the blood by centrifugation at 3,000 rpm for 15 min. The total cholesterol level was determined by enzymatic methods (Asanpharm, South Korea). The mice were killed after 3 weeks and several organs were quick-frozen in liquid nitrogen and stored at -80 °C for total RNA and protein extraction.

2.4. Cell culture, treatments, and viability test

Human hepatoma HepG2 cells were seeded in six-well Falcon plates at $1 \times 10^6$ cells/ml in DMEM supplemented with 10% FBS, 1% liquid gentamicin reagent solution, and % penicillin and streptomycin. The cells were cultured at 37 °C in a humid atmosphere containing 5% CO$_2$ until 60–80% confluent and were then used in the real time PCR assays. For the cell viability assay, HepG2 cells were seeded in 24-well Falcon plates at $1 \times 10^4$ cells/ml and grown for 48 h. The culture medium was replaced on alternate days, and the cells were kept in medium free of serum and
antibiotics during treatment. In experiments examining the effects of puerarin and its
glycosides on cell viability using MTT [3-(4,5-dimethylthiazole-2-yl)-2,5-
diphenyltetrazolium bromide] assay, HepG2 cells were incubated in fresh DMEM
with or without experimental additives. Cells were exposed to puerarin (0, 100, and
200 µM) alone, to puerarin glycosides (0, 100, and 200 µM) alone for 24 h, or to
DMEM containing puerarin (0, 100, and 200 µM) and sodium nitroprusside (SNP, 0
and 2 mM) together or puerarin glycosides (0, 100, and 200 µM) and SNP (0 and 2
mM) together for 24 h. In the cultures used in the real-time PCR assays after reverse
transcription, cells were exposed to puerarin (0, 50, 100, and 200 µM) or puerarin
glycosides (0, 50, 100, and 200 µM) for 24 h. A stock solution of puerarin was
prepared in dimethyl sulfoxide. Control cultures received dimethyl sulfoxide alone.
Puerarin glycosides were prepared directly in DMEM. HepG2 cells were treated with
lovastatin at concentration of 0, 1, 2.5, 5, 10, and 100 µM, 24h before harvesting the
cells. Control cells were treated with 0.1% ethanol. The viability of cultured HepG2
cells was quantified by measuring the reduction of MTT by mitochondrial
dehydrogenases to produce a dark blue formazan product. Viability was examined
using the method of Chung et al. (Chung et al., 2005).

2.5. Antioxidant activity tests

2.4.1. Determination of SOD activity

As an important scavenger of intracellular free radicals, the disproportionation
activities of the superoxide anion were examined using the method of Marklund and
Marklund (Marklund and Marklund, 1974) with minor modification. Briefly, 0.2 ml
of puerarin and puerarin glycosides (0, 0.05, 0.1 and 0.5 mM) were placed in the tube
and 3 ml of tris-HCl buffer (pH 8.5) and 0.2 ml of pyrogallol (7.2 mM) were added
and then the mixture was shaken and incubated at 25 °C for 10 min. 1 ml of stop solution (1 N HCl) was added to each tube. The absorbance of the resulting solution was then measured spectrophotometrically at 420 nm.

2.4.2. DR assay of non-site-specific \( \cdot OH \) radical scavenging activity

The non-site-specific \( \cdot OH \) radical scavenging activity of puerarin and puerarin glycosides was determined as described by Halliwell et al. (Halliwell et al., 1987) with minor modification. Briefly, 100 mM sodium phosphate buffer (250 µl), 1 mM EDTA (100 µl), 36 mM deoxyribose (100 µl), 1 mM FeCl\(_3\)·6H\(_2\)O (100 µl), 1 mM L-ascorbic acid (100 µl), 10 mM H\(_2\)O\(_2\) (100 µl), distilled water (100 µl) and 100 µl of puerarin and puerarin glycosides (0, 0.05, 0.1 and 0.5 mM) were added in the tube and then the tube was mixed by vortexing. In this system, a mixture of Fe\(^{3+}\)-EDTA, hydrogen peroxide, and ascorbic acid were used to generate hydroxyl radicals (\( \cdot OH \)). The mixture was incubated at 38 °C for 1 hour and then 1 ml of 1.0% TBA in 0.05M NaOH and 1 ml of 10% TCA was added to each tube. After mixing, the tube was heated at 100 °C for 10 min and then immediately was cooled on ice. The absorbance of the resulting solution was measured at 532 nm. The response degrades the sugar deoxyribose into fragments which under heating with thiobarbituric acid at a low pH, are detected because they generate a pink chromogen. The percent inhibition of the hydroxyl radical was calculated as follows: % Inhib = \[
\frac{(A_{\text{control}(532\text{nm})} - A_{\text{sample}(532\text{nm})})}{A_{\text{control}(532\text{nm})}} \times 100.
\]

2.4.3. LDL oxidation

Fresh human blood from one healthy woman adult volunteer was collected at the Korea University Anam Hospital (Seoul, Korea). LDL was isolated from the
serum according to the method described by Lee et al. (Lee et al., 2003). The protein content of isolated LDL was determined using a Bio-Rad protein kit with BSA (Sigma, St Louis, MO) as the standard. The stock LDL fraction was dialyzed against the degassed PBS (pH 7.4) in the dark for 24 h. The dialysis solution was changed at least four times. Then, the dialyzed LDL was diluted to 250 mg protein/l with 0.01 M sodium phosphate buffer (pH 7.4). For the controls, 40 μl LDL (250 mg/l) was mixed with 5 μl of 50 μM CuSO₄ solution and 5 μl of 0.01 M sodium phosphate buffer (pH 7.4) and then incubated at 37 ºC for up to 18 h. For the treatment group, 40 μl LDL (250 mg/l) was pre-incubated in the presence or absence of puerarin and puerarin glycosides (100 mM) for 5 min. Then, 5 μl of 50 μM CuSO₄ solution were added to initiate the oxidation, followed by incubation at 37 ºC for up to 18 h. The oxidation was then stopped by the addition of 2.5 μl of 27 mM EDTA and cooled at 4 ºC. The degree of LDL oxidation was monitored by measuring the production of thiobarbituric acid-reactive substances. In brief, the LDL-incubation tubes were immediately treated with 100 μl ice-cold 10% trichloroacetic acid to precipitate protein and were incubated for 15 min on ice. The incubation mixture was then centrifuged at 2200 x g for 15 min at 4 ºC. Then, 100 μl supernatant was placed into newly labeled 1.5-ml tubes with screw tops and an equal volume of 0.67% (w/v) thiobarbituric acid was added. The mixture was then heated at 95 ºC for 25 min, followed by cooling on ice. Thiobarbituric acid-reactive substances were then determined by measuring absorbance at 532 nm. Calibration was conducted using a malonaldehyde standard solution prepared from 1,1,3,3-tetramethoxypropane. The thiobarbituric acid-reactive substances value was expressed as μmol malonaldehyde /mg LDL protein.

2.4.4. DPPH radicals scavenging activity
The effect of puerarin and its glycosides on the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical was estimated according to the method of Singh and Rajini (Singh and Rajini, 2004) with minor modification. 900 μl of various concentration samples (0, 0.00625, 0.0125, 0.025, 0.05, 0.1 and 0.25 mM) were mixed with 300 μl of DPPH solution (1.5 x 10⁻⁴ M) and then the tube was mixed by vortexing. The mixture was incubated at 37 °C for 30 min and the decrease in absorbance at 532 nm was measured. The antioxidant was able to reduce the stable radical DPPH to the yellow-coloured diphenylpicrylhydrazine. The percentage inhibition of DPPH was calculated using the following equation: Radical scavenging activity (%) = 

\[
\left( \frac{A_{\text{sample}(517\text{nm})}}{A_{\text{control}(517\text{nm})}} \right) \times 100,
\]

where \(A_{\text{sample}(517\text{nm})}\) is the absorbance of the sample and \(A_{\text{control}(517\text{nm})}\) is the absorbance of the control at 517 nm.

2.4.5. Reducing power

The reducing power of puerarin and its glycosides was determined according to the method of Lee and Yen (Lee and Yen, 2006) with minor modification. Puerarin and its glycosides of various concentrations (1.0 ml), 200 mM phosphate buffer (1.0 ml, pH 6.6), and 1% potassium ferricyanide (1.0 ml) were mixed and incubated at 37 °C for 20 min. Next, 10% trichloroacetic acid (1.0 ml) was added to the mixture, which was centrifuged at 3000 rpm for 10 min. The supernatant (1.0 ml) was mixed with distilled water (1.0 ml) and 0.1% ferric chloride (0.1 ml), and then the absorbance was measured at 700 nm after standing for 10 min. In the reducing power assay, the presence of reductants (antioxidants) in the sample would result in the reduction of the Fe³⁺/ferricyanide complex to its ferrous form. Amount of Fe²⁺ complex can then be monitored by measuring the formation of Perl’s Prussian blue at 700 nm.
2.5. Transfection and luciferase assays

The cloning of human LDL receptor promoters was performed as described previously (Francova et al., 2004). Briefly, the DNA fragment spanning normal LDL receptor promoter region was inserted into the *Kpn*I and *Sac*I site of pGL3-basic vector (Promega, Madison, WI). HepG2 cells (5 x 10⁵ cells) were plated in 6-well culture plate dishes and then were incubated without antibiotics for 24 h before transfection. The transfection was performed using Metafectene™ (Biontex Laboratories GmbH, München, Germany) according to the manufacturer’s protocol. Twenty-four hours after the transfection, the transfected cells were cultured in a DMEM containing puerarin-G (0, 0.1 and 0.2 mM) for an additional 24 h. Luciferase assays were performed using the luciferase assay system (Promega, Madison, WI) according to the manufacturer’s protocol.

2.6. Isolation of total RNA and real time PCR or RT-PCR

Total RNA was extracted from cells using a Sigma TRI reagent kit according to the manufacturer’s protocol and suspended in diethylpyrocarbonate (DEPC)-treated water. For cDNA synthesis, 2 μg of total RNA was reverse transcribed using PowerScript reverse transcriptase (Clontech) according to Clontech Laboratories protocol, using a combination of oligo(dT)₁₅ primer and random hexamers. Real-time PCR was performed after reverse transcription. *In vitro* PCR primers were designed using published nucleotide sequences for the LDL receptor, HMG-CoA reductase from Skarits et al. (Skarits et al., 2003), and the sequences for 18S rRNA from Hasumi et al. (Hasumi et al., 2003).
Real-time PCR was performed by using 12.5 µl iQ SYBR Green Supermix (Bio-Rad), 0.5 µl of each primer (15 µM), 1 µl of cDNA, and 10.5 µl sterile water. The final volume of the reaction mixture was 25 µl. Real-time PCR using the LDL receptor and HMG-CoA reductase templates was performed in one cycle of 3 min at 95 ºC followed by 40 cycles of 30 s at 95 ºC, 30 s at 62 ºC, and 30 s at 72 ºC. A final extension was carried out for 5 min at 72 ºC. Real-time PCR using the template 18S rRNA was performed similarly, except different annealing treatment (18S, 30 s at 60 ºC) was used. Following amplification, a melting curve of amplified DNA was analyzed at temperatures between 54 and 95 ºC with a heating rate of 0.2 ºC/s. All real-time PCRs were performed in iCycler iQ (Bio-Rad). During the primer extension step, the increase in fluorescence from the amplified DNA was recorded using the SYBR green optic channel set at a wavelength of 490 nm. Data were collected and viewed using the iCycler iQ optical system software, version 3.1 (Bio-Rad).

The following primers were used: for the LDL receptor, F, 5'-CAATGTCTCACCAAGCTCTG and R, 5'-TCTGTCTCGAGGGGTAGCTG; for HMG-CoA reductase: F, 5'-TACCATGTCAGGGGTACGTC and R, 5'-CAAGCCTAGAGACATAATCATC; and for CYP7A1, F, 5'-GCATCATAGCTCTTTACCCAC and R, 5'-GGTGTTCTGCAGCAGTCCTGTAAT. The 18S rRNA (F, 5'-CGGCTACCACATCCAAGGA and R, 5'-GCTGGAATTACCGCGGCTGC) transcripts were used as internal controls.

*In vivo* PCR primers were designed using published nucleotide sequences for the mouse LDL receptor from Hanaka et al. (Hanaka et al., 2000) and the sequences for mouse HMG-CoA reductase and CYP7A1 from Han et al. (Han et al., 2006) and β-actin from Wood et al. (Wood et al., 2003).
The following *in vivo* primers were used: for the LDL receptor, F, 5'-ACTCAGGCAGCAGGAACGAG and R, 5'-GTCATTTTCACAGTCTACCT; for HMG-CoA reductase: F, 5'-GTTCTTTCCGTGCTGTGTTCTGGA and R, 5'-CTGATATCTTTAGTGCAAGTGTGGC; and for CYP7A1, F, 5'-CCTTGGAACGTTTTTCTCGCT and R, 5'-GCGCTCTTTGATTTAGGAAG. The β-actin (F, 5'-TGCTGTCCCTGTATGCCTCT and R, 5'-AGGTCTTTACGGATGTCAACG) transcripts were used as internal controls.

PCR using the LDL receptor primer was performed with an initial cycle of 4 min at 94 ºC; followed by 30 cycles of 30 s at 94 ºC, 30 s at 42 ºC, and 30 s at 72 ºC; and a final extension for 5 min at 72 ºC. PCR using the HMG-CoA reductase, CYP7A1, and β-actin primers was performed similarly, with the exception of the annealing temperature (HMG-CoA reductase, 57 ºC; CYP7A1, 52 ºC; β-actin, 50 ºC) and number of cycles (HMG-CoA reductase, 29 cycles; CYP7A1, 30 cycles; β-actin, 23 cycles). The β-actin transcripts were used as internal controls.

### 2.7. Western blotting

The cells and liver were lysed in a buffer containing 10 mM Tris–HCl (pH 7.4), 0.1 M EDTA, 10 mM NaCl, 0.5% Triton X-100, and one protease inhibitor cocktail tablet at 4 ºC. The lysate was clarified by centrifugation at 14,000 rpm for 10 min at 4 ºC. The protein concentration was determined using a Bio-Rad protein kit with bovine serum albumin (Sigma) as a standard. Equal amounts of protein were boiled in sample buffer (5% β-mercaptoethanol) for 5 min. Samples were separated using SDS–PAGE and blotted onto a nitrocellulose membrane (0.45 µM Protran Nitrocellulose Transfer Membrane; Schleicher & Schuell BioScience, Dassel, Germany). Nonspecific protein binding sites were blocked by incubation in PBS (pH
7.4), 0.1% Tween 20, and 5% skimmed milk. To examine LDL-receptor, HMG-CoA reductase and monoclonal anti-alpha tubulin expression, the samples were incubated with an anti-LDL receptor (The LDL antibody was a gift from Dr. Allen Cooper), anti-HMG-CoA reductase (rabbit polyclonal IgG) and monoclonal anti-alpha tubulin (mouse immunoglobulin) antibody, respectively (1/2000). After washing several times with PBS 0.1% Tween 20, the membrane was incubated with 1/5000 anti-rabbit IgG or anti-mouse IgG with H&L chain-specific (goat) peroxidase conjugate secondary antibody (Calbiochem, San Diego, CA and St. Louis, MO, USA). Immunoreactive bands were detected by ECL Western blotting detection reagents (Amersham Pharmacia Korea, Seoul, Korea) and exposed to high-performance chemiluminescence film for 10 s. Protein immunoblots were scanned by a 690 Bio-Rad densitometor using the Multi- Analyst program (Bio-Rad, Hercules, CA). The density of each band was quantified using SigmaGel software (Jandel Scientific, San Rafael, CA, USA).

2.8. Statistical analyses

Data from three independent experiments were expressed as means ± S.D. One-way analysis of variance (ANOVA) followed by Tukey’s test was used to compare the results from different treatments. Student’s t-test was used for comparisons between groups. Data were considered to have statistical differences at $P < 0.05$. 
3. Results

3.1. Transglycosylation reaction and the purification of puerarin glycosides

The purified puerarin was transglycosylated using *Bacillus stearothermophilus* maltogenic amylase to obtain puerarin glycosides (Fig. 1A). The transglycosylated products were separated using preparative liquid chromatography, and puerarin glycoside 2 (puerarin-G2) [maltosyl-\(\alpha\)-(1,6)-puerarin], puerarin-G1 [glucosyl-\(\alpha\)-(1,6)-puerarin], and puerarin were fractionated. Each puerarin glycoside peak showed a single spot on thin-layer chromatography (data not shown) and a single peak on the HPLC chromatogram (Fig. 1B). Because the enzymatic reaction produced limited amounts of each puerarin glycoside, we mixed equal moles of puerarin-G1 and puerarin-G2. Each puerarin glycoside was added according to its molecular weight so that the mixture contained the same moles of puerarin-G1 and puerarin-G2. We calculated the concentration of the puerarin glycoside average molecular weight as follows: G1 molecular weight, 578 g/mol; G2 molecular weight, 740 g/mol. The average molecular weight of puerarin glycosides was \((578 + 740)/2 = 659 \text{ g/mol}\). In the present study, the mixture of puerarin glycosides was used to examine their antioxidant activity and measure the key genes in cholesterol metabolism.

3.2. Antioxidant activities of puerarin and its glycosides

Oxidized LDL is prone to accelerate foam cell formation by macrophage uptake and could cause atherosclerosis (Chu and Liu, 2005; Lau, 2006). We examined the protective effect of puerarin and puerarin glycosides against copper-mediated LDL oxidation. Thiobarbituric acid-reactive substances values were used as an index of LDL oxidation. When LDL was incubated with CuSO\(_4\) for up to 8 h, thiobarbituric acid-reactive substances increased in a dose-dependent manner. The puerarin and its...
glycosides significantly inhibited thiobarbituric acid-reactive substances formation for up to 12 h (Fig 2A).

These results suggest that the inhibition of LDL oxidation by puerarin and its glycosides may be one of the mechanisms that prevent atherosclerosis.

At 2 mM, SNP, a widely used nitric oxide donor, reduced cell viability. However, cell viability, reduced by SNP, was enhanced when the HepG2 cell was treated with puerarin and its glycosides in a dose-dependent manner (Table 1). Among antioxidant systems, SOD is a major scavenger of ROS. The percent of SOD activity was significantly increased by treatment of puerarin and its glycosides (Table 2). SOD activity was positively associated with puerarin and its glycosides concentrations, but under the same experimental conditions, puerarin and its glycosides showed weaker SOD activity than ascorbic acid (Table 2). Ascorbic acid, puerarin, and puerarin glycosides were used in the concentration range of 0-0.5 mM for SOD activity test. SOD activity of puerarin and puerarin glycoside (0.5 mM) and ascorbic acid (0.5 mM), reference compound, were 71.3 ± 2.1%, 57.4 ± 1.7% and 55.9 ± 1.2% of the initial concentration, respectively.

The non-site-specific hydroxyl radical scavenging activity of puerarin and puerarin glycosides was quantified using a deoxyribose assay (Table 2). Puerarin also increased non-site-specific hydroxyl radical scavenging activity. Both puerarin and its glycoside were effective at quenching hydroxyl radicals. 0.5 mM puerarin, puerarin glycoside, and vitamin E (reference compound) showed non-site-specific hydroxyl radical scavenging activity with 43.2 ± 0.4%, 45.3 ± 2.0% and 69.8 ± 2.5%, respectively. Puerarin glycoside was slightly more effective than puerarin, but the effects were lower than those of vitamin E treatment (Table 2).
The effects of puerarin and its glycosides on radical scavenging activity and reducing power were further tested (Fig. 2B, C). DPPH reactivity is commonly used to screen for the free-radical-scavenging ability of antioxidative phytochemicals. In Fig. 2B, the radical scavenger activities of puerarin and its glycosides at various concentrations are compared with ascorbic acid. Radical scavengers were evaluated by their reactivity to a stable free radical, DPPH•. This method is based on the reduction of methanolic DPPH solution in the presence of a hydrogen-donating antioxidant, due to the formation of the non-radical form DPPH-H by the reaction. The puerarin and its glycosides significantly increased radical scavenging activity between 6.25 and 100 µM in a dose-dependent manner. When 0.1 mM of puerarin, puerarin glycosides and ascorbic acid was tested, they increased radical scavenging activity by 70.3 ± 5.5, 71.7 ± 2.7 and 79.6 ± 1.7%, respectively. A similar effect was found with ascorbic acid at high concentrations (100 and 250 µM).

Figure 2C shows the reducing power of puerarin and its glycosides at various concentrations compared to the reducing power of ascorbic acid. The reducing power of puerarin and its glycosides significantly increased in a dose-dependent manner, but both had lower reducing power than ascorbic acid (Fig. 2C).

In these results, puerarin and its glycosides have strong antioxidant activities that could protect LDL from oxidation.

3.3. Transcriptional activation of LDL receptor expression by puerarin glycosides

Initially, we investigated the effects of puerarin glycosides on the expression of the LDL receptor gene. The vector containing –AAGGACTGGAGTGG to –GACACAGGCTGTGATCCG region (LDL-R sequence map was showed at www.ucl.ac.uk/fh) of human LDL receptor promoter was transfected to HepG2 cells
then puerarin glycosides were treatment for 24 h. We found that the promoter activity was significantly increased in a dose-dependent manner (Fig. 3A). The puerarin glycosides activated LDL receptor promoter activity by $42 \pm 5.0$-fold compared with controls at 0.2 mM concentration.

3.4. Effect of puerarin and its glycosides on the key genes in cholesterol metabolism and synthesis

To determine the effect of concentration of puerarin and its glycosides on the cholesterol gene expression, we first performed dose-response studies in which HepG2 cells were cultured with various concentrations (0, 50, 100, and 200 μM) of puerarin and its glycosides for 24 h. We performed SYBR Green real-time PCR. Melting curve analysis revealed the presence of single pick only (Supplementary Fig. 2A-D) and forth cycles of amplification were examined, the fluorescence profile of LDL receptor at 25th cycles, HMG-CoA reductase at 21th cycles, CYP7A1 at 21th cycles, 18S samples at 10th cycles showed increase of fluorescent signals (Supplementary Fig. 1A-D). We showed that LDL receptor mRNA levels increased dose-dependently in puerarin and its glycoside-treated cells (Fig. 3B). We also investigated their effect on LDL-receptor protein levels using $\alpha$-tubulin as a reference. Western blot results were analyzed using SigmaGel software (Jandel Scientific) and LDL-receptor levels in puerarin and its glycoside-treated cells were all upregulated compared to controls (Fig. 3C).

In contrast, exposure to puerarin glycoside (50, 100, and 200 μM) resulted in HMG-CoA reductase mRNA and protein levels that were slightly lower than in the unexposed control; HMG-CoA reductase mRNA and protein levels in 50,100 or 200 μM puerarin-treated cells also decreased (Fig. 4A, B). Lovastatin was used as a
positive control and treatment of lovastatin significantly reduced HMG-CoA reductase mRNA. Lovastatin was more effective than puerarin and puerarin glycosides however, both puerarin and its glycosides also significantly reduced HMG-CoA reductase transcription (Fig. 4A). HMG-CoA reductase protein levels were also decreased at 10 and 100 µM of lovastatin-treated HepG2 cells. Puerarin and its glycosides (100 µM) were slightly more effective than lovastatin (Fig. 4B). The expression of CYP7A1, which encodes cholesterol 7-α hydroxylase, was not changed (Fig. 4C).

3.5. Plasma lipid levels in C57BL/6J mice

The in vivo hypocholesterolemic activity of puerarin glycosides (0.05% and 0.1%-puerarin glycosides) was studied with C57BL/6J mice. After puerarin glycosides consumption for 1, 2 and 3 weeks respectively, plasma levels of total cholesterol in all experiment groups (0.05% and 0.1% puerarin glycosides-groups) were all lower than that in the control group (Fig. 5A). Plasma levels of total cholesterol in the 0.05%- and 0.1%-puerarin glycosides consumption for 3 weeks were reduced to 20.0% (P < 0.05) and 28.3% (P < 0.05) of that in the control-group, respectively (Fig. 5A). The diet supplemented with 0.05 and 0.1%-puerarin glycosides for 3 weeks reduced plasma levels of triglyceride compared with this in control group (Fig. 5B). There was slight decrease of plasma glucose levels in the 0.1% puerarin glycoside-group after consumption for 2 and 3 weeks respectively (Fig. 5C).

3.6. Liver expression of LDL receptor, HMG-CoA reductase and CYP7A1

When the animals received the puerarin glycosides, CYP7A1 and LDL receptors mRNA increased significantly compared with this in control group (Fig.
LDL receptor protein levels also were significantly higher in 0.1% puerarin glycosides consumption (Fig. 6B). HMG-CoA reductase mRNA levels were suppressed by 14.5% after 0.1% puerarin glycosides consumption (Fig. 6B).

4. Discussion

Puerarin is an isoflavone abundant in kudzu roots (*Pueraria lobata*), a well-known Asian folk medicine, which has been reported to have comprehensive isoflavonoid functions. However, its low water solubility has limited its formulation into food products and hindered effective digestive absorption by dietary intake. Thus, we developed water-soluble puerarin glycosides by enzymatic transglycosylation (Li et al., 2004), which exhibited dramatically higher water solubility compared to puerarin itself.

It, however, was not determined whether modifying the chemical structure would affect the functional properties of puerarin. Thus, we initially compared antioxidant activity of puerarin with its glycosides. First, the results showed that puerarin and its glycosides suppressed LDL oxidation *in vitro* and this demonstrated that puerarin glycosides as well as puerarin possess antioxidant potency against Cu$^{2+}$-mediated LDL oxidation. Our results are in line with previously reported data that soybean genistein showed the inhibitory effects on LDL oxidation (Kapiotis et al., 1997; Lai and Yen, 2002; Ruiz-Larrea et al., 1997; Vedavanam et al., 1999). This suggests that inhibition of LDL oxidation may be one of the mechanisms that account for the anti-atherogenic properties of puerarin and its glycosides. The antioxidant activity of puerarin and its glycosides was further analyzed using four independent methods: SOD activity, DPPH radical-scavenging activity, reducing power assay, and DR assay of non-site-specific •OH radical scavenging activity. Surprisingly, all results
consistently supported similar levels of antioxidative effects for both puerarin and its
glycosides.

Additionally, we evaluated the molecular basis of the hypocholesterolemic
effects of puerarin and its glycosides on HepG2 cells and C57BL/6J mice in the level
of gene expression. The HepG2 cell line has been used as a model system in
numerous studies on cholesterol metabolism. Although the cell line share many
characteristics of hepatocyte physiology, it might show different responsiveness to
exogenous compounds. Therefore, the results from the HepG2 cells were confirmed in
animal experiments *in vivo*. Plasma cholesterol reduction could be achieved by
several mechanisms. First, uptake of dietary cholesterol could be inhibited in the
gastrointestinal track; second, LDL removal by receptor mediated uptake could be
increased in the liver; third, hepatic cholesterol biosynthesis could be reduced; fourth,
the level of CYP7A1, which encodes cholesterol-degrading enzyme, cholesterol 7α-
hydrolase, could be increased in the liver. In this study, we focus on the effects of
puerarin isoflavones on the liver metabolism

Both in vitro and in vivo results were similar with minor variations. In cells,
puerarin and its glycosides induced the LDL receptor promoter activity, mRNA and
protein levels. Both chemicals reduced HMG-CoA reductase mRNA and proteins as
well. The effects of puerarin glycosides were comparable in cultured cells, thus
puerarin glycosides were tested in animals. Puerarin glycosides efficiently lowered
plasma cholesterol levels and the mRNA but not the protein levels of LDL receptor
were induced. HMG CoA reductase was clearly lowered both in mRNA and protein
levels. Interestingly, CYP7A1 transcript was increased in mouse liver as well. The
observed significant increase in the LDL receptor, both in mRNA and protein levels,
should result in a higher net removal of LDL particles from the circulation. In
addition, HMG-CoA reductase expression was slightly reduced both in mRNA and protein levels and this may cause a reduced hepatic cholesterol biosynthesis. Puerarin glycosides consumption induced hepatic CYP7A1 mRNA levels in mouse liver but not in HepG2 cells. Thus, our results suggest that puerarin and its glycosides may generate hypocholesterolemic effect by multiple mechanisms.

The hypocholesterolemic effects of puerarin and its glycosides were similar to hypocholesterolemic effects of soy isoflavones. There are many studies supporting hypocholesterolemic effects of soy isoflavones. For example, fa/fa Zucker rat fed 38 and 578mg isoflavones/kg of soy isoflavones resulted in 21 and 29% of total cholesterol after 70 days of feeding (Peluso et al., 2000) and rats fed soy isoflavones (10 mg) 20 weeks showed 50% of VLDL+LDL cholesterol after 20 week (Wagner et al., 2003). We observed significant reductions in total cholesterol within 3 weeks and this may suggest that puerarin could generate a quicker response than soy isoflavones in lowering plasma cholesterol. This will be investigated in the future. Puerarin and its glycosides showed hypocholesterolemic effects, however, these isoflavones were much more ineffective than lovastatin. At 0.1 mM concentration, lovastatin down-regulated HMG-CoA reductase approximately 90%, however, puerarin and its glycosides exerted approximately 40% reductions in HepG2 cells. Thus, it is reasonable that these isoflavones may be useful as a dietary supplements however, we cannot completely rule out the possibility as a therapeutic applications, if isoflavones are safe and tolerant in humans.

We also found that puerarin glycoside significantly lowered plasma glucose and triglyceride levels. This is interesting because agents having these functions could be applied to treat metabolic syndrome and Type II diabetes. Yan et al. showed that CYP7A1 mRNA expression was increased in rats fed high cholesterol-diet containing
puerarin (Yan et al., 2006) and our findings are in line with their results. In addition, it was previous shown that Kudzu water extract significantly suppressed intracellular cholesterol production in HepG2 cells. They speculated that the extract may contain HMG-CoA reductase inhibitors (Lee et al., 2002), however, our results show that puerarin isoflavones directly regulate HMG-CoA reductase gene expression.

We also found that puerarin glycosides lowered plasma glucose and triglyceride levels. This is interesting since agents with these properties could be useful to treat metabolic syndrome and Type II diabetes. One potential mechanism behind this finding is an activation of sterol regulatory element binding proteins (SREBPs). SREBPs are cellular sterol sensors and three isoforms regulate cellular fatty acid and cholesterol levels, respectively. One of the isoform, SREBP-1c is regulated by insulin as well as cellular cholesterol levels and the activation of SREBP-1c could up-regulated glucose transporters in adipocyte (Im et al., 2006). The insulin-sensitive glucose transporter 4 (GLUT 4), primarily expressed in muscle and adipocytes, has a major role in glucose uptake and metabolism (Huang and Czech, 2007; Watson et al., 2004) and its mRNA and protein levels were increased by administration of puerarin (Hsu et al., 2003). After 3 weeks, we found that the plasma glucose levels in mice fed puerarin glycosides (0.1% w/w) were significantly lowered than control group. This may be due to the activation of SREBP dependent GLUT 4 upregulation. We plan to investigate the mechanism of puerarin on glucose and triglyceride metabolism in the future.

In conclusion, our results show that puerarin and its glycosides are biologically fully active isoflavones and have antioxidant and hypocholesterolemic effects. In the livers, hypocholesterolemic effects of puerarin glycoside may be achieved by multiple mechanisms including increasing LDL uptake, reducing
cholesterol biosynthesis, and possibly enhancing cholesterol degradation. Puerarin glycosides may be effective as versatile biomaterials considering their enhanced water solubility and bioactivity and could be used to develop functional foods and cosmetic products enriched with active isoflavonoids.

Acknowledgements

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5. References


Wenli, Y., Yaping, Z., 2005. Chemiluminescence evaluation of oxidative damage to biomolecules induced by singlet oxygen and protective effects of antioxidants. Biochim Biophys Acta 1725, 30-34.


Table 1. Effect of puerarin and puerarin glycosides on SNP cytotoxicity

<table>
<thead>
<tr>
<th>Addition</th>
<th>Viability (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>100.0 ± 7.3</td>
</tr>
<tr>
<td>2 mM SNP</td>
<td>64.6 ± 1.5&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>0.1 mM Puerarin</td>
<td>106.5 ± 5.0</td>
</tr>
<tr>
<td>0.2 mM Puerarin</td>
<td>109.3 ± 7.2</td>
</tr>
<tr>
<td>0.1 mM Puerarin-G</td>
<td>97.0 ± 5.3</td>
</tr>
<tr>
<td>0.2 mM Puerarin-G</td>
<td>101.0 ± 6.9</td>
</tr>
<tr>
<td>0.1 mM Puerarin + 2 mM SNP</td>
<td>85.2 ± 0.2&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td>0.2 mM Puerarin + 2 mM SNP</td>
<td>94.0 ± 1.6&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td>0.1 mM Puerarin-G + 2 mM SNP</td>
<td>111.6 ± 0.3&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td>0.2 mM Puerarin-G + 2 mM SNP</td>
<td>121.0 ± 5.7&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Data are means ± S.D. (n = 4), <sup>a</sup><i>P</i> < 0.05 compared to control, <sup>b</sup><i>P</i> < 0.05 compared to 2 mM SNP. Puerarin-G indicates puerarin glycosides.
Table 2. Effects of puerarin and puerarin glycosides on superoxide dismutase (SOD) activity and non-site-specific hydroxyl radical scavenging activity

<table>
<thead>
<tr>
<th>Concentration of antioxidants (Mm)</th>
<th>% of SOD activity</th>
<th>% Inhibition of non-site-specific hydroxyl radical scavenging activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ascorbic acid</td>
<td>Puerarin</td>
<td>Puerarin-G</td>
</tr>
<tr>
<td>0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>0.05</td>
<td>58.1 ± 1.6a</td>
<td>35.2 ± 1.8b</td>
</tr>
<tr>
<td>0.1</td>
<td>62.3 ± 1.6a</td>
<td>37.2 ± 1.0b</td>
</tr>
<tr>
<td>0.5</td>
<td>71.3 ± 2.1a</td>
<td>57.4 ± 1.7b</td>
</tr>
</tbody>
</table>

SOD activity; the reaction mixture containing tris-HCl buffer and pyrogallol with various concentration samples (0, 0.05, 0.1, 0.5 mM) was incubated at 25 °C for 10 min. The resulting solution was then determined by measuring absorbance at 420 nm. Reference compound is ascorbic acid. % of SOD activity = 100 − (Asample(420nm)/Acontrol(420nm) × 100).

Non-site-specific hydroxyl radical scavenging activity; the various concentration samples (0, 0.05, 0.1, 0.5 mM) were mixed with sodium phosphate buffer, EDTA, deoxyribose, FeCl₃·6H₂O, L-ascorbic acid, H₂O₂ and the mixture were incubated at 38 °C. The absorbance of the resulting solution was measured at 532 nm. Reference compound is vitamin E and % activity is 100 − (Asample(420nm)/Acontrol(420nm) × 100).

Puerarin-G indicates puerarin glycosides and reference control is ascorbic acid or vitamin E.

Data are means ± S.D. (n = 3), aP < 0.001 compared to control (ascorbic acid 0 mM); bP < 0.001 compared to control (puerarin 0 mM); cP < 0.001 compared to control.
(puerarin-G 0 mM); $^{d}P < 0.05$ compared to control (vitamin E 0 mM).
Figures legends

**Figure 1.** Production of puerarin glycosides using *Bacillus stearothermophilus* maltogenic amylase (BSMA). (A) Enzymatic reaction scheme. (B) Preparative HPLC chromatogram of puerarin and its glycosides. Puerarin glycosides were analyzed into maltosyl-α-(1,6)-puerarin (puerarin-G2) and glucosyl-α-(1,6)-puerarin (puerarin-G1) (A) and the separated peaks of puerarin-G2 and puerarin-G1 were fractioned by preparative HPLC (B).

**Figure 2.** Antioxidant activities of puerarin and its glycosides. (A) Cu²⁺-induced LDL oxidation; the reaction mixture containing human LDL, CuSO₄, sodium phosphate buffer with puerarin (or puerarin glycosides) or without puerarin (or puerarin glycosides) was incubated. The degree of LDL oxidation by CuSO₄ was monitored by measuring the production of thiobarbituric acid-reactive substances (TBARS). TBARS were then determined by measuring absorbance at 532 nm. (B) DPPH radical-scavenging activity; various concentration samples (0, 0.00625, 0.0125, 0.025, 0.05, 0.1 and 0.25 mM) were mixed with DPPH solution, and the mixture were incubated at 37°C and expressed as absorbance change at 517 nm. Reference compound is ascorbic acid. (C) reducing power assay; puerarin and its glycosides of various concentrations (0, 0.05, 0.1 and 0.5 mM), phosphate buffer and potassium ferricyanide were mixed and incubated at 37°C and expressed as absorbance change at 700 nm. Reference compound is ascorbic acid. Data are the mean values ± standard deviation (n = 4). *P < 0.05 vs. control.

**Figure 3.** Effect of puerarin and its glycosides on the expression of the LDL receptor. (A) the LDL receptor promoter activity assay. HepG2 cells were transfected with the
luciferase vector containing the LDL receptor promoter and, 24 h after the transfection, the cells were incubated in the medium containing 0, 0.1 or 0.2 mM puerarin glycoside (Puerarin-G) for an additional 24 h. The activity of the control was set at 1 and the relative luciferase activities were presented as fold induction relative to that of the vehicle control; (B) real time PCR of the LDL receptor gene. The LDL receptor mRNA levels in each sample were normalized to the quantity of 18S rRNA (18S). “Fold-induction” of target gene mRNA in real-time PCR results was calculated as follows: d Threshold cycle (dCt) = (Ct of LDL receptor mRNA) – (Ct of 18S mRNA); ddCt = (dCt of mRNA in treated cells) – (dCt of mRNA in untreated cells); fold-induction = 2^{-ddCt}; (C) Western blot of the LDL receptor. Cellular proteins were separated and identified using SDS–PAGE and western blotting. The density of each band of LDL-R protein was quantified using SigmaGel software (Jandel Scientific, San Rafael, CA). The LDL receptor protein levels in each sample were normalized to the quantity of α-tubulin. All values are means ± SD values of three tests. *P < 0.05 compared with vehicle control.

**Figure 4.** Effect of puerarin and its glycosides on the expression of HMG-CoA reductase. (A) real time PCR of the LDL receptor gene; (B) Western blot of the HMG-CoA reductase; (C) real-time PCR of CYP7A1. Data are obtained as described in figure 3. Lovastatin was used as a positive control. Data are the means ± standard deviation (n = 4). *P < 0.05 vs. control.

**Figure 5.** Effects of puerarin glycosides on plasma total cholesterol (A), triglycerides (B), and glucose (C) concentration. C57BL/6J mice were fed normal chow or chow containing puerarin glycosides (0.05% and 0.1%) for 3 week and blood samples were
collected once a week. Data are the means ± standard deviation (n = 10-17). \( *P < 0.05 \) vs. control.

**Figure 6.** Effects of puerarin glycosides on the expression of LDL receptor, HMG-CoA reductase and CYP7A1 mRNA (A) and protein (B) in C57BL/6J mice given 0.05% and 0.1% puerarin glycoside diet for 3 weeks. A, LDL-receptor and CYP7A1 mRNA level was induced by 0.1% puerarin glycosides (puerarin-G); HMGCoA-reductase mRNA level was decreased by 0.05% and 0.1% puerarin glycosides. The mRNA levels in each sample were normalized to the quantity of \( \alpha \)-tubulin. B, LDL-receptor protein was induced by 0.05% and 0.1% puerarin-G; HMGCoA-reductase protein level was decreased by 0.1% puerarin glycosides. The protein levels in each sample were normalized to the quantity of \( \alpha \)-tubulin. Data are obtained as described in figure 3. Data are the means ± standard deviation (n = 6-12). \( *P < 0.05 \) vs. control; \( **P < 0.01 \) vs. control; \( ***P < 0.001 \) vs. control.

**Supplementary Figure legends**

**Supplementary Figure 1.** Amplification plot of real-time PCR. (A) LDL receptor; (B) HMG-CoA reductase; (C) CYP7A1; (D) 18S rRNA (18S). cDNA was detected with SYBR Green. The x-axis indicates the PCR cycle number and the y-axis indicates the fluorescence intensity. Threshold cycle (Ct) of LDL receptor, HMG-CoA reductase, CYP7A1 and 18s rRNA were 25, 21, 21, and 10 cycles, respectively.

**Supplementary Figure 2.** Malt curve of real-time PCR. (A) LDL receptor; (B) HMG-CoA reductase; (C) CYP7A1; (D) 18S rRNA (18S). The x-axis indicates the temperature (celsius). The melting point (Tm) of LDL receptor, HMG-CoA reductase, CYP7A1, 18S rRNA from melting curves were 87.7, 89.5, 86.5, and 86.6 °C,
respectively.
Figure 1A Chung et al.
Figure 1B Chung et al.
Figure 2A Chung et al.
Figure 2B Chung et al.
Figure 2C Chung et al.
Figure 3A Chung et al.
Figure 3B Chung et al.
Figure 3C Chung et al.
Figure 4A Chung et al.
Figure 4B Chung et al.

The figure shows a bar chart with the title "Fold-Induction of HMG-CoA Reductase Protein." The x-axis represents various concentrations of Puerarin-G, Puerarin, and Lovastatin, while the y-axis represents the fold-induction of HMG-CoA reductase protein. The chart includes error bars indicating variability. The treatment groups are labeled with "Control," "0.05 mM," "0.1 mM," and "0.2 mM," with different concentrations for each group. Asterisks indicate statistical significance. The graph suggests a dose-dependent effect on the fold-induction of the protein.
Figure 4C Chung et al.

[Graph showing Fold Induction of CYP71A1 mRNA with different treatments]
Figure 5A Chung et al.
Figure 5B Chung et al.
Figure 5C Chung et al.
Figure 6A Chung et al.
Figure 6B Chung et al.

![Graph showing relative protein expressions of LDL-R and HMGCoA Reductase with control and Puerarin-G treatments.]