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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

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possibly enhancing cholesterol degradation. (257 Words)

Keywords: puerarin glycosides; low-density lipoprotein receptor; HMG-CoA reductase; antioxidant

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1 **1. Introduction**

2 Isoflavones are plant polyphenolic antioxidants that occur abundantly in
3 soybean and Radix Puerariae (the root of the plant *Pueraria lobata*) (Guerra et al.,
4 2000; Xiong et al., 2006; Xiong FL. et al., 2006). Puerarin (daidzein 8-C-glucoside),
5 the main isoflavone glycoside found in the root of *P. lobata*, has been used for various
6 medicinal purposes in traditional oriental medicine for thousands of years and it is
7 well-known that puerarin protects cells against oxidative stress (Xiong et al., 2006).
8 The antioxidant activity of puerarin is achieved through multiple mechanisms, for
9 example, by reducing H_2O_2-induced elevation of caspase-3 activation (Jiang et al.,
10 2003), scavenging free radicals (Wenli and Yaping, 2005), increasing superoxide
11 dismutase (SOD) activity (Xiong et al., 2006; Xu, 2003), and decreasing
12 malonaldehyde (Xu, 2003). Research has also shown that puerarin reduces serum
13 cholesterol levels (Hsu et al., 2003; Song et al., 1988). These antioxidant activities
14 and cholesterol-lowering effects could beneficially affect the pathophysiology of
15 cardiovascular diseases and atherosclerosis (Song et al., 1988).

16 However, the insolubility of puerarin and other isoflavonoids often limits their
17 bioavailability and may be one reason why many clinical studies have failed to find a
18 positive association between isoflavone intake and the prevention of chronic diseases
19 such as coronary heart disease (Lucas et al., 2001; Tham et al., 1998). The insolubility
20 of isoflavones also hinders industrial applications such as the production of functional
21 foods, food additives, and cosmetic products in which a natural bioactive compound
22 such as puerarin as an active ingredient would be favored. Thus, we developed a
23 novel enzymatic method to synthesize water-soluble puerarin glycosides using the
24 special transglycosylation enzyme *Bacillus stearothermophilus* maltogenic amylase
25 (Li et al., 2004). In the presence of puerarin and maltotriose or soluble starch, the

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26 enzyme transfers a glucosyl unit from soluble starch to puerarin and produces a series
27 of transglycosylated puerarin glycosides. This simple procedure has dramatically
28 increased the water solubility of puerarin up to 168-fold (Li et al., 2004). However, it
29 was not known whether puerarin glycosides retain their isoflavone functions.

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

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

46

47 **2. Materials and methods**

48 ***2.1. Chemicals.***

49 DMEM, fetal bovine serum (FBS), liquid gentamicin reagent solution,
50 penicillin and streptomycin, and trypsin-EDTA were purchased from Join Bio-

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51 Innovation (Seoul, Korea). Enhanced chemiluminescence (ECL) Western blotting
52 detection reagents and Hyperfilm™ ECL were obtained from Amersham Pharmacia
53 Korea (Seoul, Korea). Anti-rabbit IgG and H&L chain-specific peroxidase conjugate
54 were purchased from Calbiochem (Darmstadt, Germany). Anti-HMG-CoA reductase
55 (rabbit polyclonal IgG) was obtained from Upstate (Lake Placid, NY, USA) and
56 monoclonal anti-alpha tubulin clone DM 1a purified mouse immunoglobulin and goat
57 anti-mouse IgG-HRP were purchased Sigma Chemical (St. Louis, MO, USA) and
58 Santa Cruz Biotechnology (Santa Cruz, CA, USA), respectively. PowerScript reverse
59 transcriptase was obtained from Clontech (Palo Alto, CA, USA). The oligo(dT)$_{15}$
60 primer and random hexamers were obtained from Promega (Madison, WI, USA).
61 IQ™ SYBY® Green Supermix was obtained from Bio-Rad (Bio-Rad, Hercules, CA,
62 USA). All other reagents used were purchased from Sigma Chemical (St. Louis, MO,
63 USA). The isoflavone mixture was donated by Pulmuone (Seoul, Korea). HepG2 cells
64 were obtained from the Korean Cell Line Bank (Seoul, Korea).
65
66 ***2.2. Preparation and purification of puerarin and puerarin glycosides.***
67 Puerarin and its glycosides were prepared and assayed using the method of Li
68 et al. (Li et al., 2004). Briefly, *Bacillus stearothermophilus* maltogenic amylase was
69 used for the transglycosylation reaction and maltotriose was used as a donor molecule
70 in the transglycosylation reaction. The Sep-Pak Plus C_{18} cartridge was preconditioned
71 with ethyl acetate, methanol, and water. The transglycosylation solution was loaded
72 on the Sep-Pak Plus C_{18} cartridge. The retained puerarin glycosides were eluted with
73 methanol. The puerarin glycosides solution was purified using a polymeric gel-
74 filtration column (W-251) with recycling preparative HPLC to obtain the final
75 transglycosylation products. Mobile phase is methanol/water (55:45; v/v), flow rate is

76 2.0 ml/min.

77

78 ***2.3. Animals, diets and experimented protocol***

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

92

93 ***2.4. Cell culture, treatments, and viability test***

94 Human hepatoma HepG2 cells were seeded in six-well Falcon plates at 1×10^6
95 cells/ml in DMEM supplemented with 10% FBS, 1% liquid gentamicin reagent
96 solution, and % penicillin and streptomycin. The cells were cultured at 37 °C in a
97 humid atmosphere containing 5% CO_2 until 60–80% confluent and were then used in
98 the real time PCR assays. For the cell viability assay, HepG2 cells were seeded in 24-
99 well Falcon plates at 1×10^4 cells/ml and grown for 48 h. The culture medium was
100 replaced on alternate days, and the cells were kept in medium free of serum and

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101 antibiotics during treatment. In experiments examining the effects of puerarin and its
102 glycosides on cell viability using MTT [3-(4,5-dimethylthiazole-2-yl)-2,5-
103 diphenyltetrazolium bromide] assay, HepG2 cells were incubated in fresh DMEM
104 with or without experimental additives. Cells were exposed to puerarin (0, 100, and
105 200 μM) alone, to puerarin glycosides (0, 100, and 200 μM) alone for 24 h, or to
106 DMEM containing puerarin (0, 100, and 200 μM) and sodium nitroprusside (SNP, 0
107 and 2 mM) together or puerarin glycosides (0, 100, and 200 μM) and SNP (0 and 2
108 mM) together for 24 h. In the cultures used in the real-time PCR assays after reverse
109 transcription, cells were exposed to puerarin (0, 50, 100, and 200 μM) or puerarin
110 glycosides (0, 50, 100, and 200 μM) for 24 h. A stock solution of puerarin was
111 prepared in dimethyl sulfoxide. Control cultures received dimethyl sulfoxide alone.
112 Puerarin glycosides were prepared directly in DMEM. HepG2 cells were treated with
113 lovastatin at concentration of 0, 1, 2.5, 5, 10, and 100 μM, 24h before harvesting the
114 cells. Control cells were treated with 0.1% ethanol. The viability of cultured HepG2
115 cells was quantified by measuring the reduction of MTT by mitochondrial
116 dehydrogenases to produce a dark blue formazan product. Viability was examined
117 using the method of Chung et al. (Chung et al., 2005).
118

119 ***2.5. Antioxidant activity tests***

120 ***2.4.1. Determination of SOD activity***

121 As an important scavenger of intracellular free radicals, the disproportionation
122 activities of the superoxide anion were examined using the method of Marklund and
123 Marklund (Marklund and Marklund, 1974) with minor modification. Briefly, 0.2 ml
124 of puerarin and puerarin glycosides (0, 0.05, 0.1 and 0.5 mM) were placed in the tube
125 and 3 ml of tris-HCl buffer (pH 8.5) and 0.2 ml of pyrogallol (7.2 mM) were added

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126 and then the mixture was shaken and incubated at 25 ℃ for 10 min. 1 ml of stop
127 solution (1 N HCl) was added to each tube. The absorbance of the resulting solution
128 was then measured spectrophotometrically at 420 nm.
129
130 ***2.4.2. DR assay of non-site-specific •OH radical scavenging activity***
131 The non-site-specific •OH radical scavenging activity of puerarin and puerarin
132 glycosides was determined as described by Halliwell et al. (Halliwell et al., 1987)
133 with minor modification. Briefly, 100 mM sodium phosphate buffer (250 μl), 1 mM
134 EDTA (100 μl), 36 mM deoxyribose (100 μl), 1 mM $FeCl_3 \cdot 6H_2O$ (100 μl), 1 mM L-
135 ascorbic acid (100 μl), 10 mM H_2O_2 (100 μl), distilled water (100 μl) and 100 μl of
136 puerarin and puerarin glycosides (0, 0.05, 0.1 and 0.5 mM) were added in the tube and
137 then the tube was mixed by vortexing. In this system, a mixture of Fe^{3+}-EDTA,
138 hydrogen peroxide, and ascorbic acid were used to generate hydroxyl radicals (•OH).
139 The mixture was incubated at 38 ℃ for 1 hour and then 1 ml of 1.0% TBA in 0.05M
140 NaOH and 1 ml of 10% TCA was added to each tube. After mixing, the tube was
141 heated at 100 ℃ for 10 min and then immediately was cooled on ice. The absorbance
142 of the resulting solution was measured at 532 nm. The response degrades the sugar
143 deoxyribose into fragments which under heating with thiobarbituric acid at a low pH,
144 are detected because they generate a pink chromogen. The percent inhibition of the
145 hydroxyl radical was calculated as follows: % Inhib = $[(A_{control(532nm)} -$
146 $A_{sample(532nm)}/A_{control(532nm)}] \times 100$.
147
148 ***2.4.3. LDL oxidation***
149 Fresh human blood from one healthy woman adult volunteer was collected at
150 the Korea University Anam Hospital (Seoul, Korea). LDL was isolated from the

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151 serum according to the method described by Lee et al. (Lee et al., 2003). The protein
152 content of isolated LDL was determined using a Bio-Rad protein kit with BSA
153 (Sigma, St Louis, MO) as the standard. The stock LDL fraction was dialyzed against
154 the degassed PBS (pH 7.4) in the dark for 24 h. The dialysis solution was changed at
155 least four times. Then, the dialyzed LDL was diluted to 250 mg protein/l with 0.01 M
156 sodium phosphate buffer (pH 7.4). For the controls, 40 µl LDL (250 mg/l) was mixed
157 with 5 µl of 50 µM $CuSO_4$ solution and 5 µl of 0.01 M sodium phosphate buffer (pH
158 7.4) and then incubated at 37 ºC for up to 18 h. For the treatment group, 40 µl LDL
159 (250 mg/l) was pre-incubated in the presence or absence of puerarin and puerarin
160 glycosides (100 mM) for 5 min. Then, 5 µl of 50 µM $CuSO_4$ solution were added to
161 initiate the oxidation, followed by incubation at 37 ºC for up to 18 h. The oxidation
162 was then stopped by the addition of 2.5 µl of 27 mM EDTA and cooled at 4 ºC. The
163 degree of LDL oxidation was monitored by measuring the production of thiobarbituric
164 acid-reactive substances. In brief, the LDL-incubation tubes were immediately treated
165 with 100 µl ice-cold 10% trichloroacetic acid to precipitate protein and were
166 incubated for 15 min on ice. The incubation mixture was then centrifuged at 2200 x g
167 for 15 min at 4 ºC. Then, 100 µl supernatant was placed into newly labeled 1.5-ml
168 tubes with screw tops and an equal volume of 0.67% (w/v) thiobarbituric acid was
169 added. The mixture was then heated at 95 ºC for 25 min, followed by cooling on ice.
170 Thiobarbituric acid-reactive substances were then determined by measuring
171 absorbance at 532 nm. Calibration was conducted using a malonaldehyde standard
172 solution prepared from 1,1,3,3-tetramethoxypropane. The thiobarbituric acid-reactive
173 substances value was expressed as µmol malonaldehyde /mg LDL protein.
174
175 ***2.4.4. DPPH radicals scavenging activity***

176 The effect of puerarin and its glycosides on the 2,2-diphenyl-1-picrylhydrazyl
177 (DPPH) radical was estimated according to the method of Singh and Rajini (Singh
178 and Rajini, 2004) with minor modification. 900 μl of various concentration samples
179 (0, 0.00625, 0.0125, 0.025, 0.05, 0.1 and 0.25 mM) were mixed with 300 μl of DPPH
180 solution (1.5 x 10^{-4} M) and then the tube was mixed by vortexing. The mixture was
181 incubated at 37 ℃ for 30 min and the decrease in absorbance at 532 nm was
182 measured. The antioxidant was able to reduce the stable radical DPPH to the yellow-
183 coloured diphenylpicrylhydrazine. The percentage inhibition of DPPH was calculated
184 using the following equation: Radical scavenging activity (%) =
185 $[(A_{sample(517nm)}/A_{control(517nm)})] \times 100$, where $A_{sample(517nm)}$ is the absorbance of the
186 sample and $A_{control(517nm)}$ is the absorbance of the control at 517 nm.
187
188 ***2.4.5. Reducing power***
189 The reducing power of puerarin and its glycosides was determined according
190 to the method of Lee and Yen (Lee and Yen, 2006) with minor modification. Puerarin
191 and its glycosides of various concentrations (1.0 ml), 200 mM phosphate buffer (1.0
192 ml, pH 6.6), and 1% potassium ferricyanide (1.0 ml) were mixed and incubated at 37
193 °C for 20 min. Next, 10% trichloroacetic acid (1.0 ml) was added to the mixture,
194 which was centrifuged at 3000 rpm for 10 min. The supernatant (1.0 ml) was mixed
195 with distilled water (1.0 ml) and 0.1% ferric chloride (0.1 ml), and then the
196 absorbance was measured at 700 nm after standing for 10 min. In the reducing power
197 assay, the presence of reductants (antioxidants) in the sample would result in the
198 reduction of the Fe^{3+}/ferricyanide complex to its ferrous form. Amount of Fe^{2+}
199 complex can then be monitored by measuring the formation of Perl's Prussian blue at
200 700 nm.

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201

202 ***2.5. Transfection and luciferase assays***

203 The cloning of human LDL receptor promoters was performed as described
204 previously (Francova et al., 2004). Briefly, the DNA fragment spanning normal LDL
205 receptor promoter region was inserted into the *Kpn*I and *Sac*I site of pGL3-basic
206 vector (Promega, Madison, WI). HepG2 cells (5×10^5 cells) were plated in 6-well
207 culture plate dishes and then were incubated without antibiotics for 24 h before
208 transfection. The transfection was performed using Metafectene™ (Biontex
209 Laboratories GmbH, München, Germany) according to the manufacturer's protocol.
210 Twenty-four hours after the transfection, the transfected cells were cultured in a
211 DMEM containing puerarin-G (0. 0.1 and 0.2 mM) for an additional 24 h. Luciferase
212 assays were performed using the luciferase assay system (Promega, Madison, WI)
213 according to the manufacturer's protocol.

214

215 ***2.6. Isolation of total RNA and real time PCR or RT-PCR***

216 Total RNA was extracted from cells using a Sigma TRI reagent kit according
217 to the manufacturer's protocol and suspended in diethylpyrocarbonate (DEPC)-treated
218 water. For cDNA synthesis, 2 μg of total RNA was reverse transcribed using
219 PowerScript reverse transcripase (Clontech) according to Clontech Laboratories
220 protocol, using a combination of oilgo$(dT)_{15}$ primer and random hexamers. Real-time
221 PCR was performed after reverse transcription. *In vitro* PCR primers were designed
222 using published nucleotide sequences for the LDL receptor, HMG-CoA reductase
223 from Skarits et al (Skarits et al., 2003), and the sequences for 18S rRNA from Hasumi
224 et al. (Hasumi et al., 2003).

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225 Real-time PCR was performed by using 12.5 µl iQ SYBR Green Supermix
226 (Bio-Rad), 0.5 µl of each primer (15 µM), 1 µl of cDNA, and 10.5 µl sterile water.
227 The final volume of the reaction mixture was 25 µl. Real-time PCR using the LDL
228 receptor and HMG-CoA reductase templates was performed in one cycle of 3 min at
229 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
230 extension was carried out for 5 min at 72 ºC. Real-time PCR using the template 18S
231 rRNA was performed similarly, except different annealing treatment (18S, 30 s at 60
232 ºC) was used. Following amplification, a melting curve of amplified DNA was
233 analyzed at temperatures between 54 and 95 ºC with a heating rate of 0.2 ºC/s. All
234 real-time PCRs were performed in iCycler iQ (Bio-Rad). During the primer extension
235 step, the increase in fluorescence from the amplified DNA was recorded using the
236 SYBR green optic channel set at a wavelength of 490 nm. Data were collected and
237 viewed using the iCycler iQ optical system software, version 3.1 (Bio-Rad).
238 The following primers were used: for the LDL receptor, F,
239 5'-CAATGTCTCACCAAGCTCTG and R, 5'-TCTGTCTCGAGGGGTAGCTG; for
240 HMG-CoA reductase: F, 5'-TACCATGTCAGGGGTACGTC and R,
241 5'-CAAGCCTAGAGACATAATCATC; and for CYP7A1, F,
242 5'-GCATCATAGCTCTTTACCCAC and R,
243 5'-GGTGTTCTGCAGCAGTCCTGTAAT. The 18S rRNA (F,
244 5'-CGGCTACCACATCCAAGGAA and R, 5'-GCTGGAATTACCGCGGCTGC)
245 transcripts were used as internal controls.
246 *In vivo* PCR primers were designed using published nucleotide sequences for
247 the mouse LDL receptor from Hanaka et al. (Hanaka et al., 2000) and the sequences
248 for mouse HMG-CoA reductase and CYP7A1 from Han et al. (Han et al., 2006) and
249 β-actin from Wood et al. (Wood et al., 2003).

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250 The following *in vivo* primers were used: for the LDL receptor, F,
251 5'-ACTCAGGCAGCAGGAACGAG and R, 5'-GTCATTTTCACAGTCTACCT; for
252 HMG-CoA reductase: F, 5'-GTTCTTTCCGTGCTGTGTTCTGGA and R,
253 5'-CTGATATCTTTAGTGCAGAGTGTGGCAC; and for CYP7A1, F,
254 5'-CCTTGGACGTTTTCTCGCT and R, 5'-GCGCTCTTTGATTTAGGAAG. The β-
255 actin (F, 5'-TGCTGTCCCTGTATGCCTCT and R,
256 5'-AGGTCTTTACGGATGTCAACG) transcripts were used as internal controls.
257 PCR using the LDL receptor primer was performed with an initial cycle of 4 min at
258 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
259 final extension for 5 min at 72 ºC. PCR using the HMG-CoA reductase, CYP7A1, and
260 β-actin primers was performed similarly, with the exception of the annealing
261 temperature (HMG-CoA reductase, 57 ºC; CYP7A1, 52 ºC; β-actin, 50 ºC) and
262 number of cycles (HMG-CoA reductase, 29 cycles; CYP7A1, 30 cycles; β-actin, 23
263 cycles). The β-actin transcripts were used as internal controls.
264

265 ***2.7. Western blotting***

266 The cells and liver were lysed in a buffer containing 10 mM Tris–HCl (pH
267 7.4), 0.1 M EDTA, 10 mM NaCl, 0.5% Triton X-100, and one protease inhibitor
268 cocktail tablet at 4 ºC. The lysate was clarified by centrifugation at 14,000 rpm for 10
269 min at 4 ºC. The protein concentration was determined using a Bio-Rad protein kit
270 with bovine serum albumin (Sigma) as a standard. Equal amounts of protein were
271 boiled in sample buffer (5% ß-mercaptoethanol) for 5 min. Samples were separated
272 using SDS–PAGE and blotted onto a nitrocellulose membrane (0.45 μM Protran
273 Nitrocellulose Transfer Membrane; Schleicher & Schuell BioScience, Dassel,
274 Germany). Nonspecific protein binding sites were blocked by incubation in PBS (pH

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275 7.4), 0.1% Tween 20, and 5% skimmed milk. To examine LDL-receptor, HMG-CoA
276 reductase and monoclonal anti-alpha tubulin expression, the samples were incubated
277 with an anti-LDL receptor (The LDL antibody was a gift from Dr. Allen Cooper),
278 anti-HMG-CoA reductase (rabbit polyclonal IgG) and monoclonal anti-alpha tubulin
279 (mouse immunoglobulin) antibody, respectively (1/2000). After washing several
280 times with PBS 0.1% Tween 20, the membrane was incubated with 1/5000 anti-rabbit
281 IgG or anti-mouse IgG with H&L chain-specific (goat) peroxidase conjugate
282 secondary antibody (Calbiochem, San Diego, CA and St. Louis, MO, USA).
283 Immunoreactive bands were detected by ECL Western blotting detection reagents
284 (Amersham Pharmacia Korea, Seoul, Korea) and exposed to high-performance
285 chemiluminescence film for 10 s. Protein immunoblots were scanned by a 690 Bio-
286 Rad densitometor using the Multi-Analyst program (Bio-Rad, Hercules, CA). The
287 density of each band was quantified using SigmaGel software (Jandel Scientific, San
288 Rafael, CA, USA).
289

290 ***2.8. Statistical analyses***

291 Data from three independent experiments were expressed as means ± S.D.
292 One-way analysis of variance (ANOVA) followed by Tukey's test was used to
293 compare the results from different treatments. Student's t-test was used for
294 comparisons between groups. Data were considered to have statistical differences at P
295 < 0.05.

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296 **3. Results**

297 ***3.1. Transglycosylation reaction and the purification of puerarin glycosides***

298 The purified puerarin was transglycosylated using *Bacillus stearothermophilus*
299 maltogenic amylase to obtain puerarin glycosides (Fig. 1A). The transglycosylated
300 products were separated using preparative liquid chromatography, and puerarin
301 glycoside 2 (puerarin-G2) [maltosyl-α-(1,6)-puerarin], puerarin-G1 [glucosyl-α-(1,6)-
302 puerarin], and puerarin were fractionated. Each puerarin glycoside peak showed a
303 single spot on thin-layer chromatography (data not shown) and a single peak on the
304 HPLC chromatogram (Fig. 1B). Because the enzymatic reaction produced limited
305 amounts of each puerarin glycoside, we mixed equal moles of puerarin-G1 and
306 puerarin-G2. Each puerarin glycoside was added according to its molecular weight so
307 that the mixture contained the same moles of puerarin-G1 and puerarin-G2. We
308 calculated the concentration of the puerarin glycoside average molecular weight as
309 follows: G1 molecular weight, 578 g/mol; G2 molecular weight, 740 g/mol. The
310 average molecular weight of puerarin glycosides was (578 + 740)/2 = 659 g/mol. In
311 the present study, the mixture of puerarin glycosides was used to examine their
312 antioxidant activity and measure the key genes in cholesterol metabolism.
313

314 ***3.2. Antioxidant activities of puerarin and its glycosides***

315 Oxidized LDL is prone to accelerate foam cell formation by macrophage
316 uptake and could cause atherosclerosis (Chu and Liu, 2005; Lau, 2006). We examined
317 the protective effect of puerarin and puerarin glycosides against copper-mediated
318 LDL oxidation. Thiobarbituric acid-reactive substances values were used as an index
319 of LDL oxidation. When LDL was incubated with $CuSO_4$ for up to 8 h, thiobarbituric
320 acid-reactive substances increased in a dose-dependent manner. The puerarin and its

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321 glycosides significantly inhibited thiobarbituric acid-reactive substances formation for
322 up to 12 h (Fig 2A).

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

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

337 The non-site-specific hydroxyl radical scavenging activity of puerarin and
338 puerarin glycosides was quantified using a deoxyribose assay (Table 2). Puerarin also
339 increased non-site-specific hydroxyl radical scavenging activity. Both puerarin and its
340 glycoside were effective at quenching hydroxyl radicals. 0.5 mM puerarin, puerarin
341 glycoside, and vitamin E (reference compound) showed non-site-specific hydroxyl
342 radical scavenging activity with 43.2 ± 0.4%, 45.3 ± 2.0% and 69.8 ± 2.5%,
343 respectively. Puerarin glycoside was slightly more effective than puerarin, but the
344 effects were lower than those of vitamin E treatment (Table 2).

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345 The effects of puerarin and its glycosides on radical scavenging activity and
346 reducing power were further tested (Fig. 2B, C). DPPH reactivity is commonly used
347 to screen for the free-radical-scavenging ability of antioxidative phytochemicals. In
348 Fig. 2B, the radical scavenger activities of puerarin and its glycosides at various
349 concentrations are compared with ascorbic acid. Radical scavengers were evaluated
350 by their reactivity to a stable free radical, DPPH$^{\bullet}$. This method is based on the
351 reduction of methanolic DPPH solution in the presence of a hydrogen-donating
352 antioxidant, due to the formation of the non-radical form DPPH-H by the reaction.
353 The puerarin and its glycosides significantly increased radical scavenging activity
354 between 6.25 and 100 μM in a dose-dependent manner. When 0.1 mM of puerarin,
355 puerarin glycosides and ascorbic acid was tested, they increased radical scavenging
356 activity by 70.3 ± 5.5, 71.7 ± 2.7 and 79.6 ± 1.7%, respectively. A similar effect was
357 found with ascorbic acid at high concentrations (100 and 250 μM).
358 Figure 2C shows the reducing power of puerarin and its glycosides at various
359 concentrations compared to the reducing power of ascorbic acid. The reducing power
360 of puerarin and its glycosides significantly increased in a dose-dependent manner, but
361 both had lower reducing power than ascorbic acid (Fig. 2C).
362 In these results, puerarin and its glycosides have strong antioxidant activities
363 that could protect LDL from oxidation.
364
365 ***3.3. Transcriptional activation of LDL receptor expression by puerarin glycosides***
366 Initially, we investigated the effects of puerarin glycosides on the expression
367 of the LDL receptor gene. The vector containing –AAGGACTGGAGTGG to –
368 GACACAGCAGGTCGTGATCCG region (LDL-R sequence map was showed at
369 www.ucl.ac.uk/fh) of human LDL receptor promoter was transfected to HepG2 cells

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370 then puerarin glycosides were treatment for 24 h. We found that the promoter activity
371 was significantly increased in a dose-dependent manner (Fig. 3A). The puerarin
372 glycosides activated LDL receptor promoter activity by 42 ± 5.0-fold compared with
373 controls at 0.2 mM concentration.
374

375 ***3.4. Effect of puerarin and its glycosides on the key genes in cholesterol metabolism***
376 ***and synthesis***

377 To determine the effect of concentration of puerarin and its glycosides on the
378 cholesterol gene expression, we first performed dose-response studies in which
379 HepG2 cells were cultured with various concentrations (0, 50, 100, and 200 μM) of
380 puerarin and its glycosides for 24 h. We performed SYBR Green real-time PCR.
381 Melting curve analysis revealed the presence of single pick only (Supplementary Fig.
382 2A-D) and forth cycles of amplification were examined, the fluorescence profile of
383 LDL receptor at 25th cycles, HMG-CoA reductase at 21th cycles, CYP7A1 at 21th
384 cycles, 18S samples at 10th cycles showed increase of fluorescent signals
385 (Supplementary Fig. 1A-D). We showed that LDL receptor mRNA levels increased
386 dose-dependently in puerarin and its glycoside-treated cells (Fig. 3B). We also
387 investigated their effect on LDL-receptor protein levels using α-tubulin as a reference.
388 Western blot results were analyzed using SigmaGel software (Jandel Scientific) and
389 LDL-receptor levels in puerarin and its glycoside-treated cells were all upregulated
390 compared to controls (Fig. 3C).

391 In contrast, exposure to puerarin glycoside (50, 100, and 200 μM) resulted in
392 HMG-CoA reductase mRNA and protein levels that were slightly lower than in the
393 unexposed control; HMG-CoA reductase mRNA and protein levels in 50,100 or 200
394 μM puerarin-treated cells also decreased (Fig. 4A, B). Lovastatin was used as a

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395 positive control and treatment of lovastatin significantly reduced HMG-CoA
396 reductase mRNA. Lovastatin was more effective than puerarin and puerarin
397 glycosides however, both puerarin and its glycosides also significantly reduced HMG
398 CoA reductase transcription (Fig. 4A). HMG-CoA reductase protein levels were also
399 decreased at 10 and 100 μM of lovastatin-treated HepG2 cells. Puerarin and its
400 glycosides (100 μM) were slightly more effective than lovastatin (Fig. 4B). The
401 expression of CYP7A1, which encodes cholesterol 7-α hydroxylase, was not changed
402 (Fig. 4C).
403
404 ***3.5. Plasma lipid levels in C57BL/6J mice***
405 The *in vivo* hypocholesterolemic activity of puerarin glycosides (0.05% and
406 0.1%-puerarin glycosides) was studied with C57BL/6J mice. After puerarin glycodies
407 consumption for 1, 2 and 3 weeks respectively, plasma levels of total cholesterol in all
408 experiment groups (0.05% and 0.1% puerarin glycosides-groups) were all lower than
409 that in the control group (Fig. 5A). Plasma levels of total cholesterol in the 0.05%-
410 and 0.1%-puerarin glycosides consumption for 3 weeks were reduced to 20.0% ($P <$
411 0.05) and 28.3% ($P < 0.05$) of that in the control-group, respectively (Fig. 5A). The
412 diet supplemented with 0.05 and 0.1%-puerarin glycosides for 3 weeks reduced
413 plasma levels of triglyceride compared with this in control group (Fig. 5B). There was
414 slight decrease of plasma glucose levels in the 0.1% puerarin glycoside-group after
415 consumption for 2 and 3 weeks respectively (Fig. 5C).
416
417 ***3.6. Liver expression of LDL receptor, HMG-CoA reductase and CYP7A1***
418 When the animals received the puerarin glycosides, CYP7A1 and LDL
419 receptors mRNA increased significantly compared with this in control group (Fig.

420 6A). LDL receptor protein levels also were significantly higher in 0.1% puerarin
421 glycosides consumption (Fig. 6B). HMG-CoA reductase mRNA levels were
422 suppressed by 14.5% after 0.1% puerarin glycosides consumption (Fig. 6B).
423

424 **4. Discussion**

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

432 It, however, was not determined whether modifying the chemical structure
433 would affect the functional properties of puerarin. Thus, we initially compared
434 antioxidant activity of puerarin with its glycosides. First, the results showed that
435 puerarin and its glycosides suppressed LDL oxidation *in vitro* and this demonstrated
436 that puerarin glycosides as well as puerarin possess antioxidant potency against Cu^{2+}-
437 mediated LDL oxidation. Our results are in line with previously reported data that
438 soybean genistein showed the inhibitory effects on LDL oxidation (Kapiotis et al.,
439 1997; Lai and Yen, 2002; Ruiz-Larrea et al., 1997; Vedavanam et al., 1999). This
440 suggests that inhibition of LDL oxidation may be one of the mechanisms that account
441 for the anti-atherogenic properties of puerarin and its glycosides. The antioxidant
442 activity of puerarin and its glycosides was further analyzed using four independent
443 methods: SOD activity, DPPH radical-scavenging activity, reducing power assay, and
444 DR assay of non-site-specific $^{\bullet}OH$ radical scavenging activity. Surprisingly, all results

445 consistently supported similar levels of antioxidative effects for both puerarin and its
446 glycosides.

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

460 Both in vitro and in vivo results were similar with minor variations. In cells,
461 puerarin and its glycosides induced the LDL receptor promoter activity, mRNA and
462 protein levels. Both chemicals reduced HMG-CoA reductase mRNA and proteins as
463 well. The effects of puerarin glycosides were comparable in cultured cells, thus
464 puerarin glycosides were tested in animals. Puerarin glycosides efficiently lowered
465 plasma cholesterol levels and the mRNA but not the protein levels of LDL receptor
466 were induced. HMG CoA reductase was clearly lowered both in mRNA and protein
467 levels. Interestingly, CYP7A1 transcript was increased in mouse liver as well. The
468 observed significant increase in the LDL receptor, both in mRNA and protein levels,
469 should result in a higher net removal of LDL particles from the circulation. In

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470 addition, HMG-CoA reductase expression was slightly reduced both in mRNA and
471 protein levels and this may cause a reduced hepatic cholesterol biosynthesis. Puerarin
472 glycosides consumption induced hepatic CYP7A1 mRNA levels in mouse liver but
473 not in HepG2 cells. Thus, our results suggest that puerarin and its glycosides may
474 generate hypocholesterolemic effect by multiple mechanisms.
475 The hypocholesterolemic effects of puerarin and its glycosides were similar to
476 hypocholesterolemic effects of soy isoflavones. There are many studies supporting
477 hypocholesterolemic effects of soy isoflavones. For example, fa/fa Zucker rat fed 38
478 and 578mg isoflavones/kg of soy isoflavones resulted in 21 and 29% of total
479 cholesterol after 70 days of feeding (Peluso et al., 2000) and rats fed soy isoflavones
480 (10 mg) 20 weeks showed 50% of VLDL+LDL cholesterol after 20 week (Wagner et
481 al., 2003). We observed significant reductions in total cholesterol within 3 weeks and
482 this may suggest that puerarin could generate a quicker response than soy isoflavones
483 in lowering plasma cholesterol. This will be investigated in the future. Puerarin and its
484 glycosides showed hypocholesterolemic effects, however, these isoflavones were
485 much more ineffective than lovastatin. At 0.1 mM concentration, lovastatin down-
486 regulated HMG-CoA reductase approximately 90%, however, puerarin and its
487 glycosides exerted approximately 40% reductions in HepG2 cells. Thus, it is
488 reasonable that these isoflavones may be useful as a dietary supplements however, we
489 cannot completely rule out the possibility as a therapeutic applications, if isoflavones
490 are safe and tolerant in humans.
491 We also found that puerarin glycoside significantly lowered plasma glucose
492 and triglyceride levels. This is interesting because agents having these functions could
493 be applied to treat metabolic syndrome and Type II diabetes. Yan et al. showed that
494 CYP7A1 mRNA expression was increased in rats fed high cholesterol-diet containing

495 puerarin (Yan et al., 2006) and our findings are in line with their results. In addition, it
496 was previous shown that Kudzu water extract significantly suppressed intracellular
497 cholesterol production in HepG2 cells. They speculated that the extract may contain
498 HMG-CoA reductase inhibitors (Lee et al., 2002), however, our results show that
499 puerarin isoflavones directly regulate HMG-CoA reductase gene expression.
500 We also found that puerarin glycosides lowered plasma glucose and
501 triglyceride levels. This is interesting since agents with these properties could be
502 useful to treat metabolic syndrome and Type II diabetes. One potential mechanism
503 behind this finding is an activation of sterol regulatory element binding proteins
504 (SREBPs). SREBPs are cellular sterol sensors and three isoforms regulate cellular
505 fatty acid and cholesterol levels, respectively. One of the isoform, SREBP-1c is
506 regulated by insulin as well as cellular cholesterol levels and the activation of SREBP-
507 1c could up-regulated glucose transporters in adipocyte (Im et al., 2006). The insulin-
508 sensitive glucose transporter 4 (GLUT 4), primarily expressed in muscle and
509 adipocytes, has a major role in glucose uptake and metabolism (Huang and Czech,
510 2007; Watson et al., 2004) and its mRNA and protein levels were increased by
511 administration of puerarin (Hsu et al., 2003). After 3 weeks, we found that the plasma
512 glucose levels in mice fed puerarin glycosides (0.1% w/w) were significantly lowered
513 than control group. This may be due to the activation of SREBP dependent GLUT 4
514 upregulation. We plan to investigate the mechanism of puerarin on glucose and
515 triglyceride metabolism in the future.
516 In conclusion, our results show that puerarin and its glycosides are
517 biologically fully active isoflavones and have antioxidant and hypocholesterolemic
518 effects. In the livers, hypocholesterolemic effects of puerarin glycoside may be
519 achieved by multiple mechanisms including increasing LDL uptake, reducing

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520 cholesterol biosynthesis, and possibly enhancing cholesterol degradation. Puerarin
521 glycosides may be effective as versatile biomaterials considering their enhanced water
522 solubility and bioactivity and could be used to develop functional foods and cosmetic
523 products enriched with active isoflavonoids.
524

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

Addition	Viability (%)
Control	100.0 ± 7.3
2 mM SNP	64.6 ± 1.5[a]
0.1 mM Puerarin	106.5 ± 5.0
0.2 mM Puerarin	109.3 ± 7.2
0.1 mM Puerarin-G	97.0 ± 5.3
0.2 mM Puerarin-G	101.0 ± 6.9
0.1 mM Puerarin + 2 Mm SNP	85.2 ± 0.2[ab]
0.2 mM Puerarin + 2 mM SNP	94.0 ± 1.6[ab]
0.1 mM Puerarin-G + 2 mM SNP	111.6 ± 0.3[ab]
0.2 mM Puerarin-G + 2 mM SNP	121.0 ± 5.7[ab]

Data are means ± S.D. (n = 4), [a]$P < 0.05$ compared to control, [b]$P < 0.05$ compared to 2 mM SNP. Puerarin-G indicates puerarin glycosides.

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Table 2. Effects of puerarin and puerarin glycosides on superoxide dismutase (SOD) activity and non-site-specific hydroxyl radical scavenging activity

Concentration of antioxidants (Mm)	% of SOD activity			% Inhibition of non-site-specific hydroxyl radical scavenging activity		
	Ascorbic acid	Puerarin	Puerarin-G	Vitamin E	Puerarin	Puerarin-G
0	0.0	0.0	0.0	0.0	0.0	0.0
0.05	58.1 ± 1.6[a]	35.2 ± 1.8[b]	33.8 ± 0.6[c]	53.1 ± 0.7[d]	32.4 ± 3.8[b]	37.7 ± 0.1[c]
0.1	62.3 ± 1.6[a]	37.2 ± 1.0[b]	42.1 ± 1.7[c]	54.1 ± 0.8[d]	37.5 ± 1.4[b]	39.4 ± 0.5[c]
0.5	71.3 ± 2.1[a]	57.4 ± 1.7[b]	55.9 ± 1.2[c]	69.8 ± 2.5[d]	43.2 ± 0.4[b]	45.3 ± 2.0[c]

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 for 10 min. The resulting solution was then determined by measuring absorbance at 420 nm. Reference compound is ascorbic acid. % of SOD activity = 100 − ($A_{sample(420nm)}$/ $A_{control(420nm)}$ × 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, $FeCl_3{\cdot}6H_2O$, L-ascorbic acid, H_2O_2 and the mixture were incubated at 38 . The absorbance of the resulting solution was measured at 532 nm. Reference compound is vitamin E and % activity is 100 − ($A_{sample(420nm)}$/$A_{control(420nm)}$ × 100). Puerarin-G indicates puerarin glycosides and reference control is ascorbic acid or vitamin E.

Data are means ± S.D. (n = 3), $^{a}P < 0.001$ compared to control (ascorbic acid 0 mM); $^{b}P < 0.001$ compared to control (puerarin 0 mM); $^{c}P < 0.001$ compared to control

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(puerarin-G 0 mM); [d]$P < 0.05$ compared to control (vitamin E 0 mM).

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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) Cu^{2+}-induced LDL oxidation; the reaction mixture containing human LDL, $CuSO_4$, sodium phosphate buffer with puerarin (or puerarin glycosides) or without puerarin (or puerarin glycosides) was incubated. The degree of LDL oxidation by $CuSO_4$ 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 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

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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

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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 α-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 α-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 ℃,

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respectively.

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Figure 1A Chung *et al.*

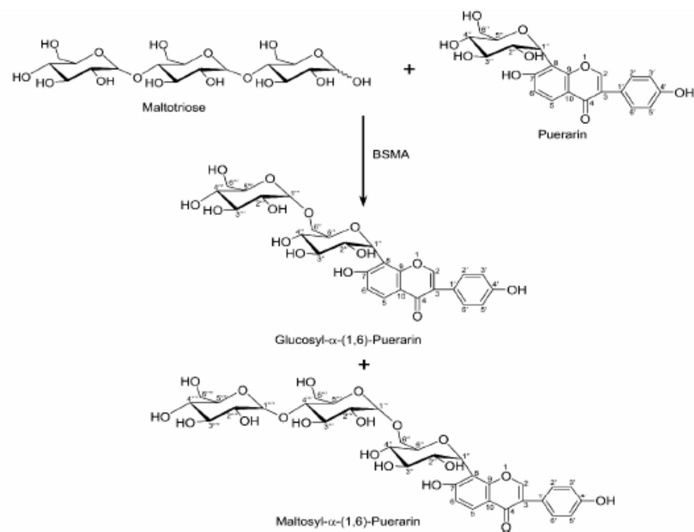

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Figure 1B Chung *et al.*

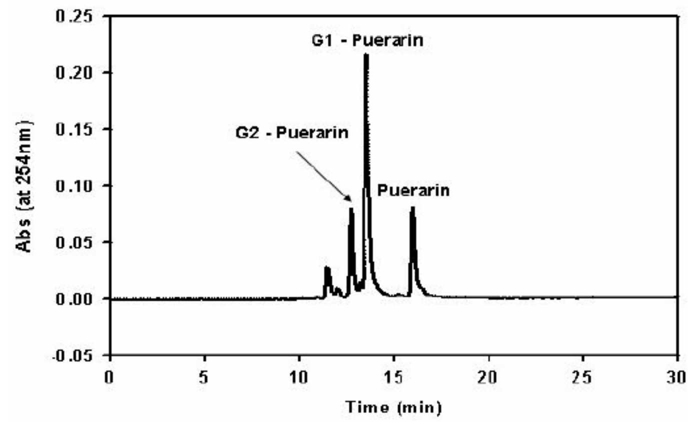

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Figure 2A Chung *et al.*

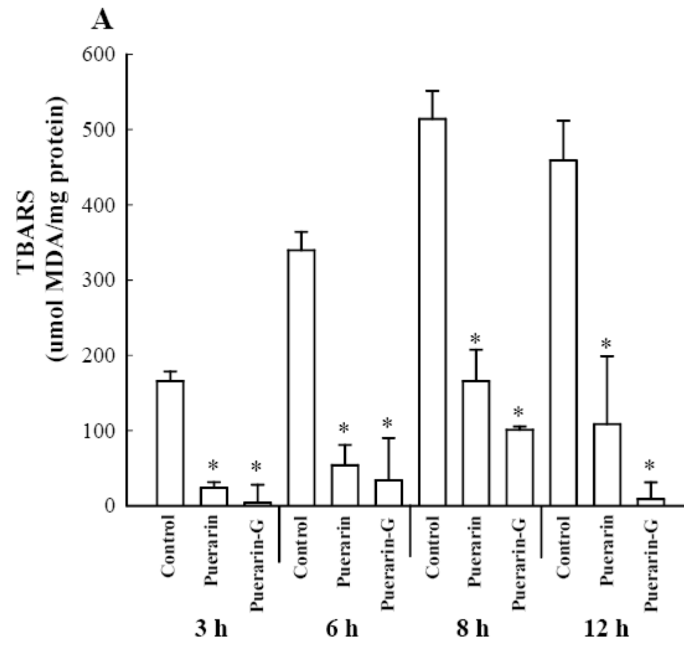

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Figure 2B Chung *et al.*

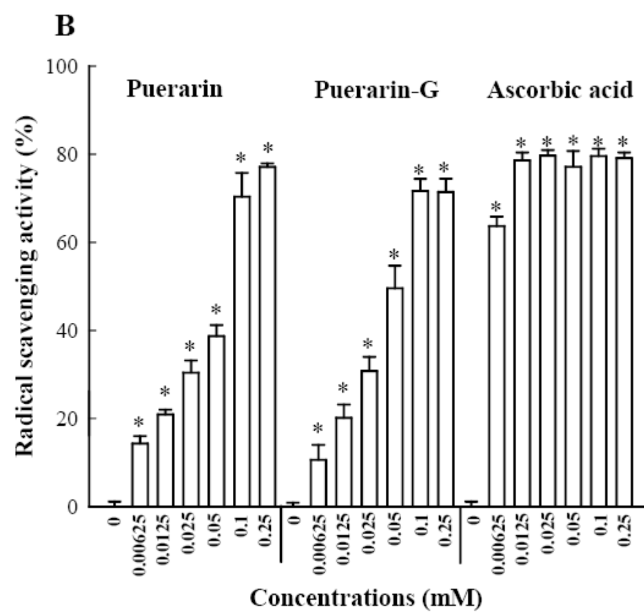

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Figure 2C Chung *et al.*

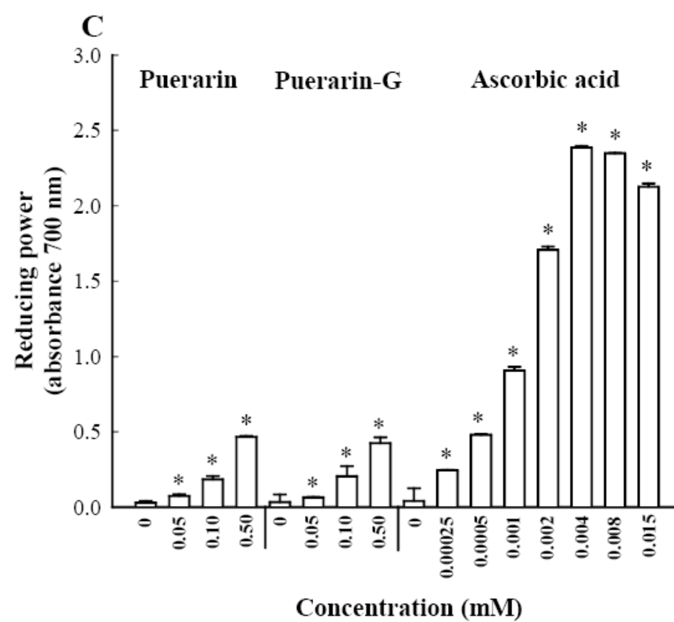

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Figure 3A Chung *et al.*

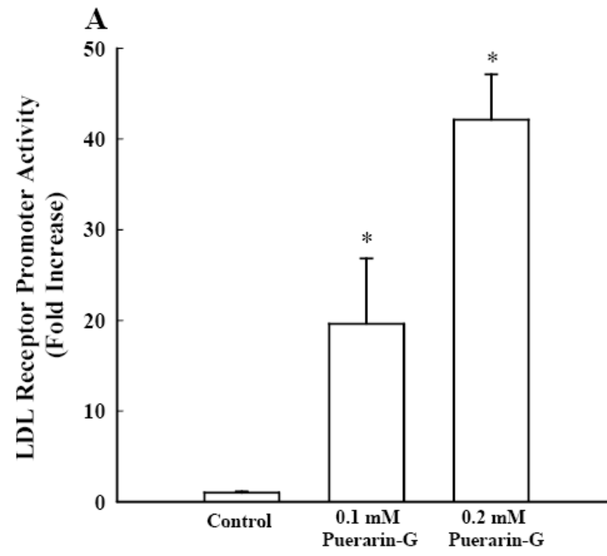

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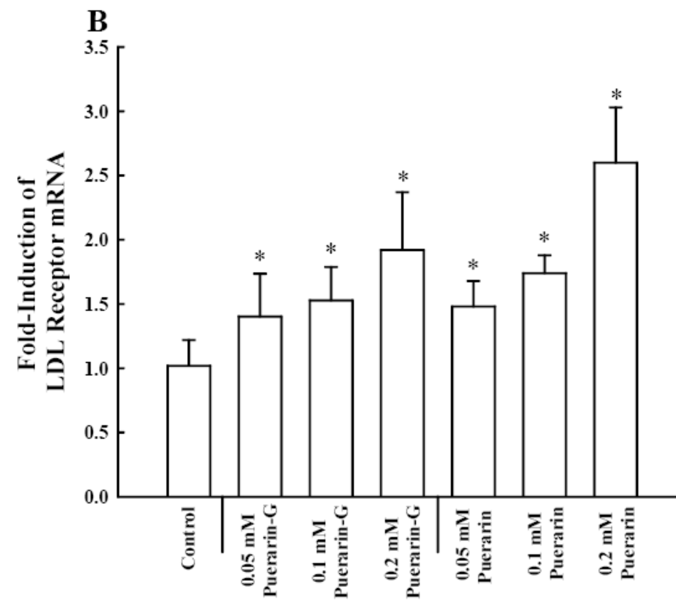
Figure 3B Chung *et al.*



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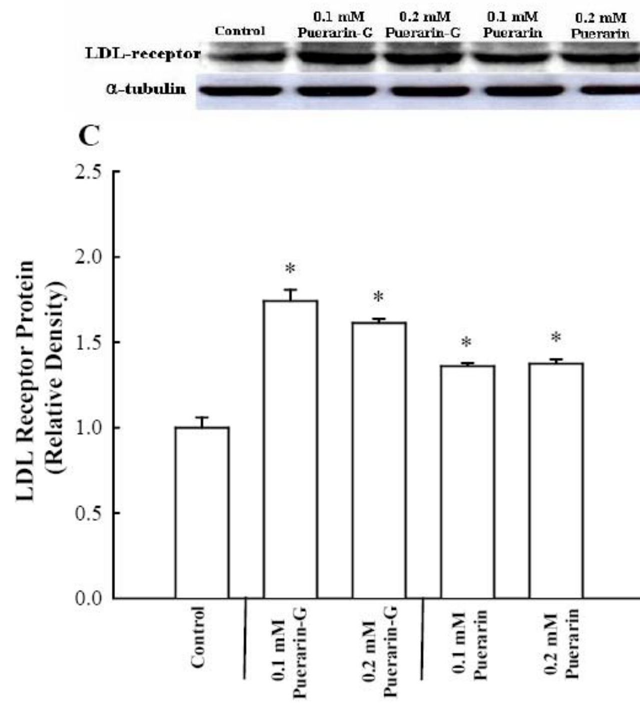

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Figure 4A Chung *et al.*

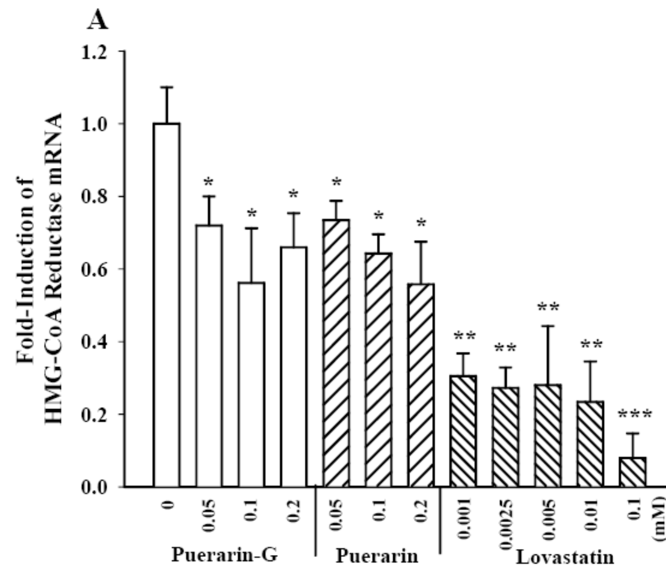

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Figure 4B Chung *et al.*

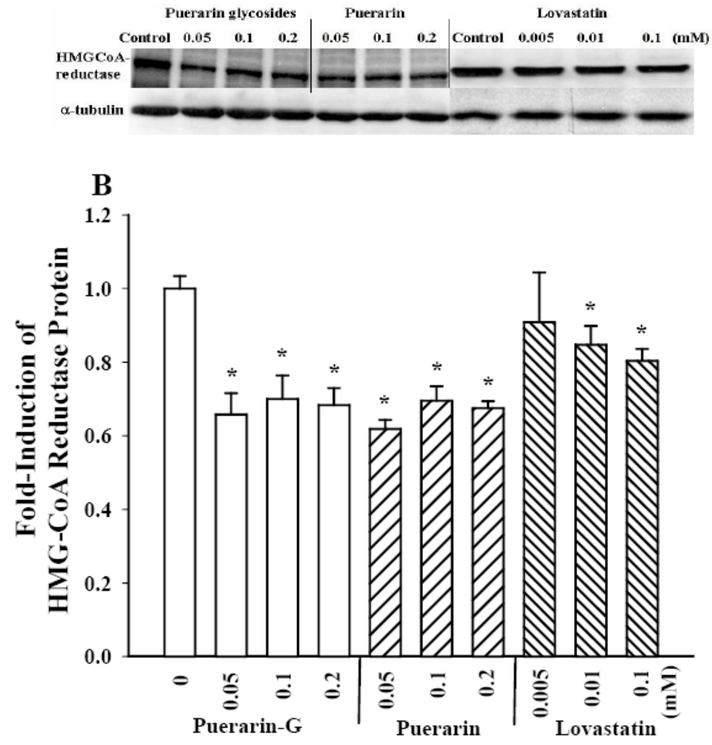

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Figure 4C Chung *et al.*

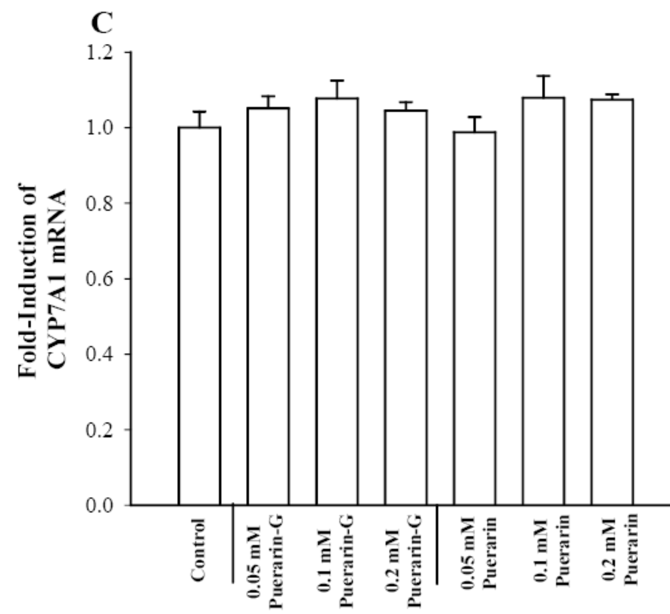

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Figure 5A Chung *et al.*

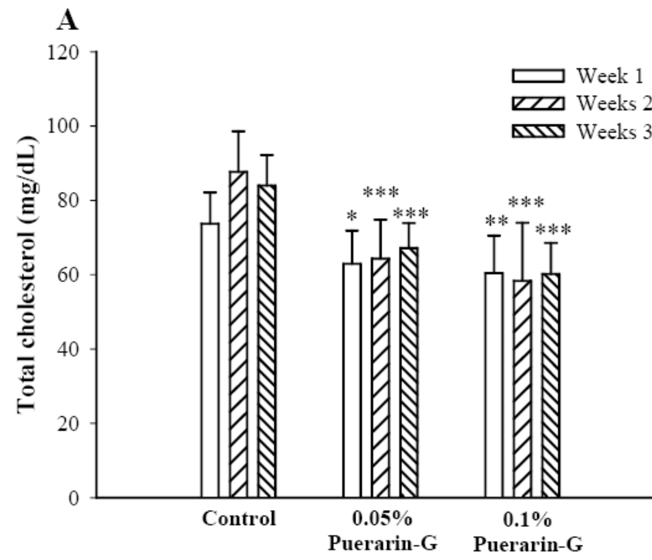

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Figure 5B Chung *et al.*

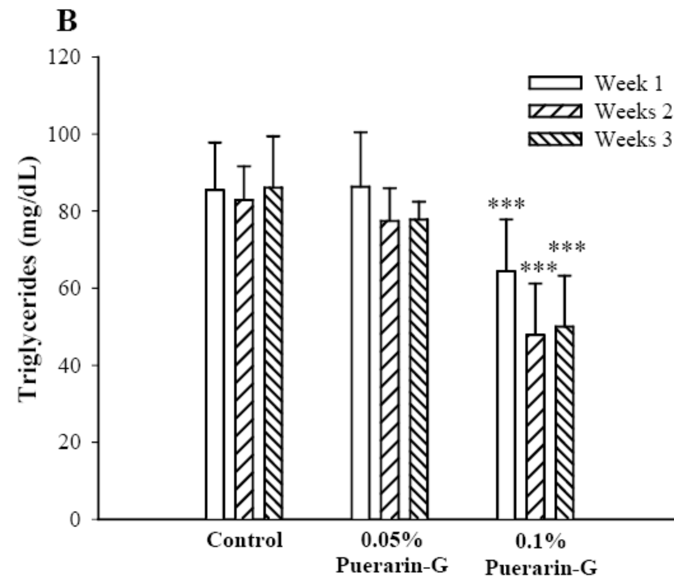

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Figure 5C Chung *et al.*

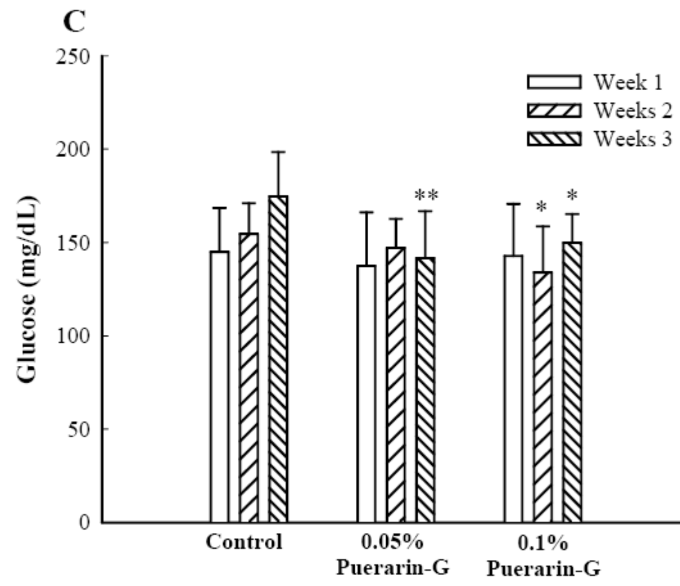

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Figure 6A Chung *et al.*

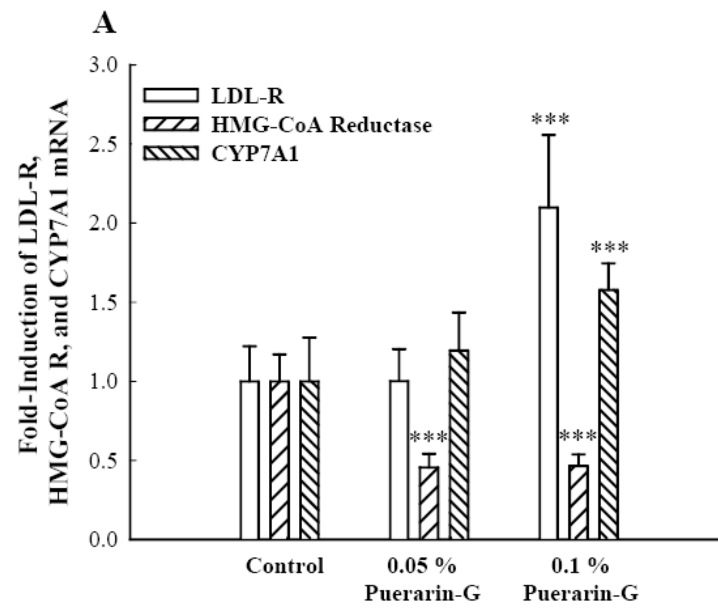

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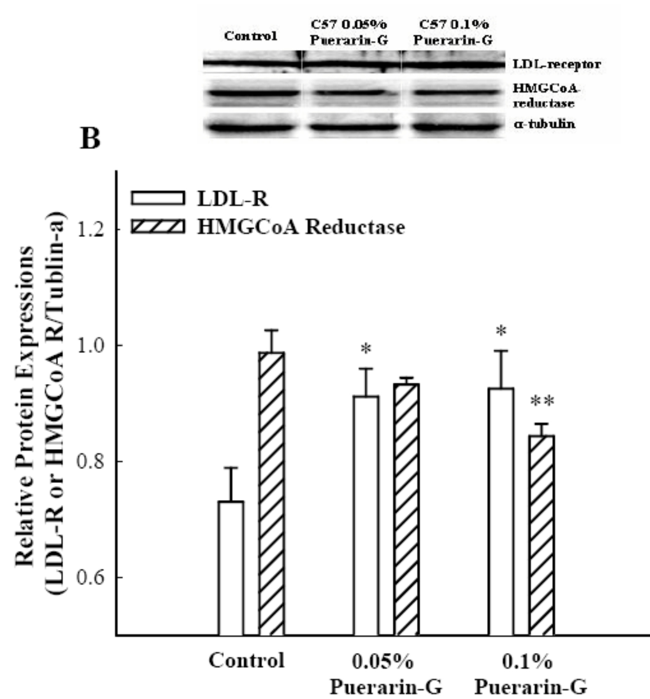
Figure 6B Chung *et al.*



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