Antioxidant Properties of Flavone *C*-Glycosides from *Atractylodes japonica* Leaves in Human Low-density Lipoprotein Oxidation

YOUNG-CHAN KIM, MIRA JUN, WOO-SIK JEONG, SHIN-KYO CHUNG

ABSTRACT: Three antioxidant flavone *C*-glucosides, isoorientin, vitexin, and isovitexin, were identified for the 1st time from *Atractylodes japonica* leaves by an activity-guided fractionation with various analytical techniques including column chromatography, high-performance liquid chromatography (HPLC), fast atom bombardment mass spectrometry (FAB-MS), and nuclear magnetic resonance (NMR). Hydroxyl and superoxide anion radicals scavenging activities of these compounds were examined using electron spin resonance (ESR). Inhibitory activities of these compounds on human low-density lipoprotein (LDL) oxidation were evaluated by malondialdehyde (MDA) and thiobarbituric acid-reactive substances (TBARS), both representing intermediates of lipid peroxidation. These flavone *C*-glucosides displayed about 50% of scavenging activity against hydroxyl radicals at the concentrations below 10 μM. The superoxide dismutase (SOD)-equivalent activities of isoorientin, vitexin, and isovitexin at 1 mM were 31.37, 2.71, and 2.63 unit/mL, respectively. Isoorientin at 1 μM exhibited over 60% inhibition of MDA formed during copper-mediated human LDL oxidation. Amounts of free MDA in LDL treated with isoorientin, vitexin, isovitexin, and control were 20.06, 40.73, 34.08, and 48.03 nM/mg protein, respectively. These compounds also prolonged the lag phase time of the conjugated diene formation. There was a positive correlation between the free radical scavenging activities and the inhibitory effects on the LDL oxidation of these compounds. These results suggest that the flavone *C*-glucosides isolated from the leaves of *A. japonica* possess beneficial antioxidant properties against free radicals as well as LDL oxidation.

Keywords: *Atractylodes japonica*, flavone *C*-glycosides, antioxidant, low-density lipoprotein (LDL), electron spin resonance (ESR)

Introduction

Reactive oxygen species (ROS) including superoxide, hydroxyl radicals, singlet oxygen, and hydrogen peroxide are byproducts produced via biological reactions (Wang and Jiao 2000). Increased levels of these ROS generate oxidative stress, which is considered to be associated with degenerative diseases such as diabetes mellitus, atherosclerosis, arthritis, and various cancers (Ames and others 1993; de Zwart and others 1999; Gackowski and others 2001). Many studies have shown that various plants extracts and a number of plant products including polyphenolic substances possess antioxidant properties and act against ROS (Wedworth and Lynch 1995; Lu and Yeap Foo 2001).

Oxidized low-density lipoprotein (LDL) is associated with the development of coronary artery disease, peripheral vascular diseases, and stroke (Stein and others 1999). Oxidized LDL taken by macrophage increases cholesterol level and accumulated cholesterol in the vessel leads to early atherosclerosis. Therefore, protection of LDL oxidation against ROS is considered to be important in the prevention or retardation of the early stages of atherosclerosis (Stein and others 1999). Dietary supplements of antioxidant-rich vegetables and fruits can prevent oxidized LDL mediated vascular injury (Chang and others 2000; Chalas and others 2001). Natural antioxidants such as polyphenols, flavonoids, anthocyanins, vitamin C, and tocopherol suppress the oxidative modification of human LDL and reduce the relative risk of coronary heart disease (Myara and others 1993).

Flavonoids are a secondary metabolite of plants and the most abundant polyphenols in the human diet. They are categorized into several classes according to their chemical structures such as flavonols, flavones, catechins, flavanones, anthocyanidins, and isoflavonoids. Recently, much attention has been paid to their antioxidant properties and their scavenging effect of reactive oxygen species (Burda and Oleszek 2001).

Atractylodes japonica is a valuable medicinal plant, and the roots have been widely used in folk remedy in Asian countries for its pharmacological effects such as pain alleviation and arthritis treatment (Jang and others 2004). From the roots of *A. japonica,* several essential oils such as atractylone, 3-β-hydroxyatratylon, selina-4, hinesol, and β-eudesmol were identified (Kohjyouma and others 1997). Sesquiterpenoid glycosides have been recently found from the methanol extract of rhizome of this plant (Kitajima and others 2003). Biological activities of *A. japonica* such as anti-inflammation, lipoxygenase inhibition, HIV-1 activity suppression, and hypoglycemia have been reported (Konno and others 1985; Resch and others 1998; Min and others 2001). Aqueous extract of *A. japonica* suppressed nitric oxide (NO) formation and PGE2 synthesis by inhibiting nitric oxide synthase and cyclooxygenase-2 mRNA expression in RAW 264.7 macrophages (Jang and others 2004). However, little information is available on the biological properties of *A.*

Author Kim is with Korea Food Research Institute, Ginseng Research Group, San 46-1, Baekhyun-dong, Bundang-gu, Sungnam, Gyeonggi 463-746, South Korea. Author Jun is with Department of Food Science, 65 Dudley Rd, Rutgers University, New Brunswick, NJ, 08901. Author Jeong is with Food Science Institute, School of Food & Life Science, Inje University, 607 Obang-dong, Gimhae, Gyeongnam 621-749, South Korea. Author Chung Department of Food Science and Technology, Kyungpook National University, Daegu 702-701, South Korea. Direct inquiries to Author Jeong (Email: jeongws@inje.ac.kr) and Author Chung (Email: kchung@knu.ac.kr).

japonica leaves. In our previous study, leaves of *A. japonica* displayed a marked ROS scavenging activity among 50 kinds of Korean medicinal plants (Kim and Chung 2002).

The aim of the present study was to isolate and identify antioxidants from the leaves of *A. japonica* through an activity-guided fractionation. Three flavone *C*-glucosides were isolated and examined for their antioxidant activities against superoxide anion and hydroxyl radicals using the electron spin resonance (ESR) technique. In addition, the inhibitory effects of these compounds on human LDL oxidation were investigated in vitro.

Materials and Methods

Materials

Leaves of *A. japonica* were obtained from the Pongwha Alpine Medicinal Plant Experiment Station (Pongwha, South Korea). The leaves were dried at room temperature and powdered. Sephadex LH-20 gel, CD_3OD, 1,1-diphenyl-2-picrylhydrazyl (DPPH), 3-*tert*-butyl-4-hydroxyanisole (BHA), α-tocopherol, 5,5,-dimethyl-1-pyrroline-*N*-oxide (DMPO), xanthine/xanthine oxidase (EC1.1.3.22, from milk) were purchased from Sigma Chemicals Co. (St Louis, Mo., U.S.A.). Superoxide dismutase (SOD, EC1.15.1.1, from bovine erythrocyte) was purchased from Wako Pure Chemical Industry, Ltd. (Osaka, Japan). All other chemicals and solvents used in this study were of reagent or HPLC grade.

Activity-guided isolation of active compounds

Dried and powdered leaves of *A. japonica* (250 g) were extracted with methanol (5 × 2 L) and filtered. Total crude plant extract (56.87 g) was collected by a rotary evaporator under 40 °C. The concentrate was then extracted with diethyl ether, ethyl acetate, and *n*-butanol, in successive order. Each extract was tested for its DPPH radical scavenging activity (Blois 1958). Sample with a final concentration of 1 mg/mL was mixed to a DPPH solution (4×10^{-4} *M*) and stored in the dark for 10 min. Then, the absorbance of the mixture was measured at 517 nm. The most active ethyl acetate extract (4.5 g) in DPPH radical scavenging assay was further fractionated using Sephadex LH-20 column (20 × 400 mm) chromatography with 60% methanol as an eluting solvent. The elution was monitored for the absorbance at 280 nm, 5 fractions were obtained based on the absorbance and tested for their DPPH radical scavenging activity. The most active fraction was analyzed and separated by a preparative HPLC system (LC-10A, Shimadzu Co., Tokyo, Japan) attached with a reversed phase Develosil ODS-5 column (20 × 250 mm, Nomura Chemical Co. Ltd., Aichi, Japan) at 254 nm. The mobile phase was 20% MeOH containing 0.1 % trifluoroacetic acid at a flow rate of 4.0 mL/min.

Spectrometric analysis for structure identification

UV/Visible absorption spectra of isolated compounds were determined by a spectrophotometer (UV 1601PC, Schimadzu Co., Tokyo, Japan). ^{1}H-NMR (500 MHz) and ^{13}C-NMR (100 MHz) spectra were obtained on a Varian NMR spectrometer (Varian Unity Plus 500, Palo Alto, Calif., U.S.A.) with CD_3OD as a solvent and TMS as an internal standard. Fast atom bombardment mass spectra (FAB-MS) spectra were obtained using a mass spectrometer (JEOL JMS-DX-705L, JEOL Co., Tokyo, Japan), with 1 *N* a hydrochloric acid aq. HCl-glycerol as the mounting matrix.

Measurement of hydroxyl radical and superoxide anion scavenging activity by electron spin resonance (ESR)

The hydroxyl radical was generated by Fenton reaction in the presence of $FeSO_4 \cdot 7H_2O$ and DMPO was used as a spin trapping agent (Calliste and others 2001). The generated hydroxyl radicals were measured by a JES-TE 300 ESR spectrometer (JEOL Ltd., Tokyo, Japan) under the following conditions: modulation frequency, 100 kHz; microwave frequency, 9.78 GHz; microwave power, 4 mW; modulation amplitude, 0.987 G; time constant, 10.24 ms. Four μL of buffer or sample (1 and 10 μL) was mixed with H_2O_2 (10 m*M*, 25 μL), $FeSO_4$ (2 m*M*, 25 μL), and DMPO (48 m*M*, 50 μL) in a quartz capillary. After 3 min, the ESR spectra were recorded at room temperature.

The superoxide anion was generated using xanthine/xanthine oxidase system. The instrumental conditions of ESR spectrometer were as follows: modulation frequency, 100 kHz; microwave frequency, 9.78 GHz; microwave power, 10 mW; modulation amplitude, 0.495 G; time constant, 0.16 ms. Four microliters of buffer or sample (1 and 10 μL) was mixed with xanthine (5 m*M*, 50 μL), xanthine oxidase (0.4 U/mL, 50 μL), and DMPO (900 m*M*, 50 μL) in a quartz capillary. After 3 min, the ESR spectra were recorded at room temperature. The superoxide anion scavenging activity of each sample was compared with the activity of known concentrations of SOD standard.

Isolation of human LDL

Human LDL (density range between 1.006 and 1.063 g/mL) was isolated from healthy female serum by sequential density ultracentrifugation with an ultracentrifuger (Optima TLX, Beckman Co., Palo Alto, Calif., U.S.A.) at 44000 rpm for 16 h. The LDL isolate was dialyzed with a phosphate buffered saline (PBS, pH 7.4) containing 0.01% ethylenediaminetetraacetic acid (EDTA) for 48 h. The EDTA was removed by extensive dialysis with PBS without EDTA for 48 h at 4 °C. The protein concentrations were determined by Bradford assay using bovine serum albumin as a standard.

Oxidation of human LDL

The oxidation of LDL was carried out according to the method of Hussein and others (Hussein and others 2001) with a slight modification. Briefly, samples (1 and 10 μ*M*) were added to a tube containing LDL (0.1 mg protein/mL) and 10 μ*M* $CuSO_4$. The mixture was incubated at 37 °C for 2 h, and the degree of oxidation was terminated by addition of 1 m*M* EDTA and 10 μ*M* butylated hydroxytoluene (BHT). The oxidized LDL solution was then used for further analyses.

Determination of 2-thiobarbituric acid-reactive substances (TBARS)

To determine the effect of isolated compounds on the LDL peroxidation, TBARS value was measured (Esterbauer and Cheeseman 1990). TBARS assay is based on the reaction of malondialdehyde, an end product of lipid peroxidation, with thiobarbituric acid (TBA). After 2 h of incubation (37 °C) of LDL as mentioned previously, 1 mL of 20% tricholoroacetic acid (TCA) was added to the oxidized LDL solution and the mixture was centrifuged at 10000 rpm for 10 min. Then 1 mL of supernatant was mixed with TBA in a screw-capped vial and boiled in water bath at 100 °C for 10 min. After cooling, the amount of TBARS was measured at 532 nm using an UV/VIS spectrophotometer.

Measurement of free malondialdehydes by HPLC

The inhibitory effect of test compounds on free malondialdehyde (MDA) formation in the LDL oxidation process was evaluated (Esterbauer and Cheeseman 1990). An aliquot of the oxidized LDL solution was mixed with an equal volume of acetonitrile and centrifuged at 3000 rpm for 10 min. The supernatant (20 μL) was analyzed by an HPLC equipped with an NH_2 column (4.6 × 250 mm,

Alltech Assoc., Deerfield, Ill., U.S.A.) and a UV detector (267 nm). The mobile phase was CH_3CN:30 m*M* Tris buffer (pH 7.4) (1:9, v/v) at a flow rate of 0.8 mL/min.

Measurement of conjugated dienes

Conjugated dienes were extracted with chloroform and methanol (2:1) from the oxidized LDL solution and centrifuged at 10000 rpm for 10 min. The lower layer was dissolved in cyclohexane, and the absorbance was measured at 234 nm for the conjugated diene quantification (Puhl and others 1994).

Results and Discussion

Activity-guided isolation and identification of antioxidant compounds

Crude extract of *A. japonica* leaves was sequentially fractionated by liquid-liquid partition with diethyl ether, ethyl acetate, *n*-butanol, and water. Each fraction was examined for the hydroxyl and DPPH radical scavenging activity. Ethyl acetate fraction, which showed the strongest antioxidant activity in both assays (data not shown), was further fractionated using Sephadex LH-20 column chromatography eluted with 60% methanol. Individual column subfractions from ethyl acetate fraction were monitored for their absorbance at 280 nm and tested for the antioxidant activity by DPPH radical scavenging assay. As shown in Figure 1, the absorbance pattern of the fractions closely overlaps with the DPPH radical scavenging activity pattern. The fractions were combined into 5 groups based on the patterns of the absorbance and the antioxidant activity; Group I, fr. 7-11; Group II, fr. 12-18; Group III, fr. 19-25; Group IV, 26-37; Group V, 38-56. Group IV exhibited the highest antioxidant activity with 80% DPPH radical scavenging; therefore, it was further purified using semi-preparative HPLC. Three pure compounds were isolated from group IV, and their structures were identified by comparison of their Miss., ^{1}H- and ^{13}C-NMR data with those reported in the previous literature (Adinarayana and others 1980; Krauze-Baranowska and Cisowski 1995; Hamed and others 1997; Maatooq and others 1997; Senatore and others 2000; Latte and others 2002). The isolated flavone *C*-glucosides were identified as isoorientin, vitexin, and isovitexin, and all of these phenolic compounds are isolated from the *A. japonica* for the 1st time (Figure 2).

Compound 1 (67 mg). UV (MeOH), max 270, 349 nm; FAB-MS, m/z 449 $(M+H)^+$; ^{1}H-NMR (in CD_3OD) 7.61(1H, m, H2′), 7.48 (1H, d, H6′), 6.76 (1H, d, H5′), 6.27 (1H, m, H8), 6.08 (1H, m, H6), 5.13 (1H, d, H1″), 3.62 (1H, dd, H6″a), 3.61 (1H, dd, C6″b), 3.38 (1H, t, C2″), 3.34 (1H, t, C3″), 3.20 (1H, t, C4″), 3.13 (1H, ddd, C5″); ^{13}C NMR (in CD_3OD) 183.96 (C4), 166.21 (C2), 164.89 (C7), 162.00 (C5), 158.67 (C9), 151.03 (C4′), 147.01 (C3′), 135.59 (C3), 120.31 (C6′), 123.51 (C1′), 114.13 (C2′), 116.77 (C5′), 105.17 (C10), 75.29 (C1″), 109.13 (C6), 95.20 (C8), 82.61 (C5″), 80.11 (C3″), 72.59 (C2″), 71.79 (C4″), 62.87 (C6″). (isoorientin; luteolin-6-*C*-glucoside)

Compound 2 (45 mg). UV (MeOH), max 270, 329 nm; FAB-MS, m/z 433 $(M+H)^+$; ^{1}H-NMR (in DMSO-d_6) 7.60(1H, m, H2′), 7.57(1H, d, H6′), 6.85(1H, d, H5′), 6.38(1H, m, H8), 6.19(1H, m, H6), 5.17 (1H, d, H1″), 3.77 (1H, dd, H5″a), 3.51 (1H, dd, H2″), 3.50 (1H, ddd, H4″), 3.39 (1H, t, H3″), 3.09 (1H, dd, H5″b); ^{13}C NMR (in CD_3OD) 181.95 (C4), 163.78 (C7), 160.28 (C5), 162.67 (C2), 155.89 (C9), 161.04 (C4′), 115.78 (C3′), 102.35 (C3), 128.78 (C6′), 121.48 (C1′), 128.78 (C2′), 115.78 (C5′), 103.83 (C10), 73.27 (C1″), 98.10 (C6), 104.50 (C8), 78.54 (C3″), 70.24 (C2″), 70.41 (C4″), 81.72 (C5″). (vitexin, apigenin-8-*C*-glucoside)

Compound 3 (25 mg). UV (MeOH), max 271, 333 nm; FAB-MS, m/z 433 $(M+H)^+$; ^{1}H-NMR (in CD_3OD) 8.05 (2H, d, H2′, 6′), 6.88 (2H, d, H3′, 5′), 6.39 (1H, d, H8), 6.20 (1H, H6), 5.24 (1H, d, H1″), 3.69 (1H, dd, H6″a), 3.58 (1H, dd, H6″b), 3.43 (1H, dd, H2″), 3.35 (1H, t, H3″), 3.29 (1H, t, H4″), 3.20 (1H, ddd, H5″); ^{13}C NMR (in CD_3OD) 183.99 (C4), 164.96 (C7), 162.78 (C5), 162.02 (C4′), 158.68 (C9), 166.13 (C2), 103.84 (C3), 129.51 (C2′), 129.31 (6′), 123.07 (C1′), 117.00 (C3′), 117.08 (C5′), 105.16 (C10), 75.29(C1″), 109.18 (C6), 95.25 (C8), 82.61 (C5″), 80.13 (C3″), 72.59 (C2″), 71.79 (C4″), 62.87 (C6″). (isovitexin; apigenin-6-*C*-glucoside)

Flavone *C*-glucosides are widely distributed in many plants. In recent studies, isoorientin, vitexin, and isovitexin have been identified in cucumber leaves (Krauze-Baranowska and Cisowski 1995; McNally and others 2003), barley leaves (Norbaek and others 2003), and *Pelargonium reinforme* (Latte and others 2002). Vitexin and isovitexin were also identified from *Vitex lucens* and bamboo leaves (Zhang and others 2005), and isoorientin from *Polygonum oriental,* lemongrass (Cheel and others 2005), and St. John's Wort (Jurgenliemk and Nahrstedt 2002).

ROS scavenging activities of isolated compounds by ESR

For understanding of antioxidant activities of isolated compounds, their scavenging activity against hydroxyl radicals as well as superoxide anion radicals were determined using ESR along with

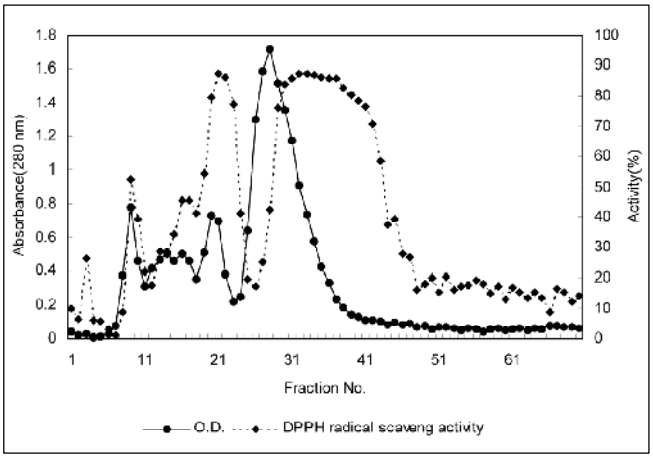

Figure 1—Chromatogram of the ethyl acetate fraction by Sephadex LH-20 column chromatography eluted with 60% methanol

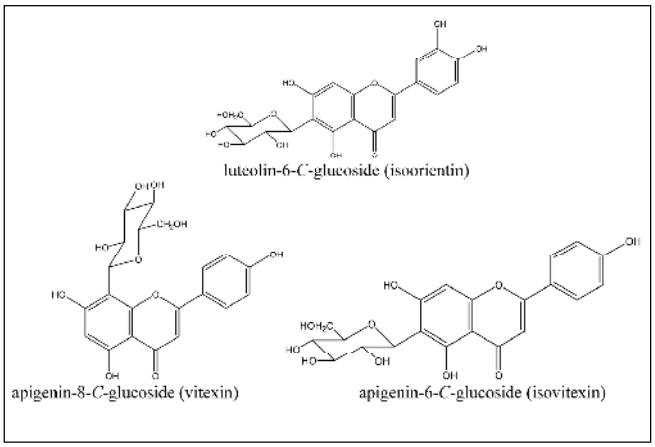

Figure 2—Structures of 3 flavone *C*-glucosides isolated from *Atracylodes japonica*

Table 1—Hydroxyl and superoxide anion radicals scavenging activities of the flavone *C*-glucosides isolated from *Atractylodes japonica* leaves[a]

	Hydroxyl radical		Superoxide anion			
	Inhibition (%)		Inhibition (%)		SOD equivalent activity (unit/mL)	
	1 μ*M*	10 μ*M*	0.1 m*M*	1 m*M*	0.1 m*M*	1 m*M*
Isoorientin	50.06 ± 3.15	49.92 ± 1.56	48.5 ± 1.14	79.4 ± 0.75	6.96 ± 1.01	31.37 ± 3.47
Vitexin	55.93 ± 1.59	42.01 ± 0.94	18.6 ± 2.82	30.1 ± 5.50	1.38 ± 0.13	2.71 ± 0.66
Isovitexin	48.88 ± 1.63	56.12 ± 3.28	21.5 ± 2.78	29.5 ± 0.91	1.44 ± 0.04	2.63 ± 0.15
α-Tocopherol	23.25 ± 3.90	58.32 ± 2.88	NE[b]	NE	NE	NE
BHA	30.48 ± 4.70	57.65 ± 4.00	NE	NE	NE	NE

[a]Values are expressed as means ± S.D. from triplicate measurements. BHA = 3-*tert*-butyl-4-hydroxyanisole (BHA); SOD = superoxide dismutase (SOD).
[b]No effect.

DMPO in both radical system. The ESR free radical method relates to the effect of antioxidant on the initiation of oxidation (Madsen and others 1996). Superoxide anion radicals were generated by xanthine/xanthine oxidase system. Hydroxyl radicals were generated photochemically by hemolytic cleavage of H_2O_2 by Fenton reaction in the presence of $FeSO_4$ and a spin-trapping agent DMPO. The ESR spectra of hydroxyl radical and superoxide anion spin adduct are shown in Figure 3. Figure 3a and 3b demonstrate typical ESR spectra of DMPO-hydroxyl and DMPO-superoxide spin adducts obtained under controlled conditions. With regard to the consumption of hydroxyl and superoxide anion radical, the antioxidant competes for the hydroxyl radicals with DMPO resulting in decrease of spin adducts, which reflects its scavenging ability (Unno and others 2002). In both radical systems, the intensity of ESR spectra decreased when treated with the isolated compounds to the reaction mixture, indicating the potent radical scavenging properties of tested compounds.

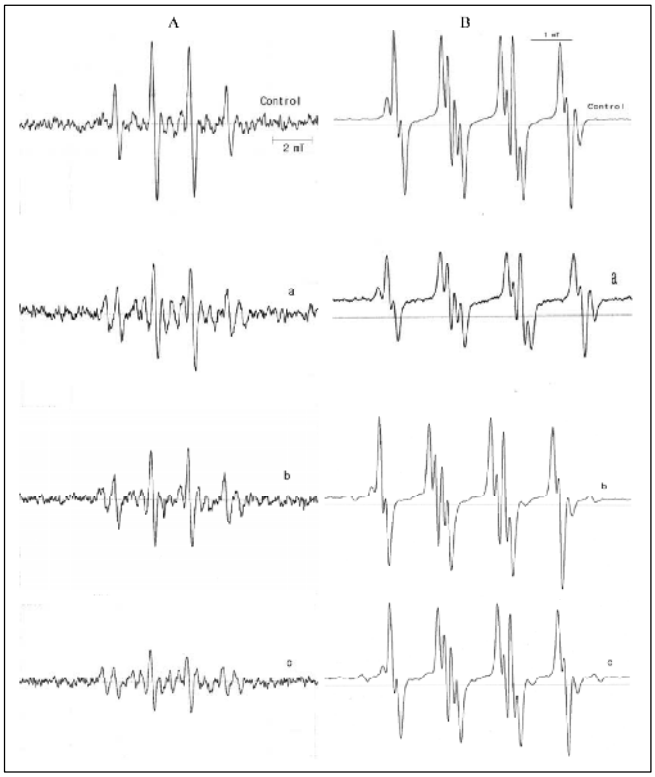

Figure 3—Electron spin resonance (ESR) spectrum of hydroxyl radical and superoxide anion spin adduct of 5,5,-dimethyl-1-pyrroline-*N*-oxide (DMPO) produced xanthine/xanthine oxidase system; (A) hydroxyl radical; (B) superoxide anion, (a) luteolin-6-*C*-glucoside; (b) apigenin-8-*C*-glucoside; (c) apigenin-6-*C*-glucoside.

The radical scavenging activities of the flavone *C*-glucosides were compared with those of commercial antioxidants such as α-tocopherol and BHA as described in Table 1. The superoxide anion scavenging activity of these compounds was also expressed as SOD-equivalent activity (unit/mL). For the hydroxyl radicals, all the isolated flavone *C*-glucosides showed about 50% scavenging activity at both 1 and 10 μ*M*. The hydroxyl radical scavenging activities of these compounds were higher than those of α-tocopherol and BHA at 1 μ*M* but lower at 10 μ*M*. Among the flavone *C*-glycosides, there was no significant difference in hydroxyl radical scavenging activity according to the number of hydroxyl groups or position of substituted sugar. Similar results were available in the study by Rice-Evans and others (1996). In superoxide anion scavenging activity, SOD-equivalent activity was calculated from the calibration curve using bovine erythrocyte SOD. α-Tocopherol and BHA in ethanol were used, which implies that the solubility might not influence their activity. At both concentrations (0.1 m*M* and 1 m*M*), all 3 flavone *C*-glycosides showed higher superoxide anion scavenging activity than α-tocopherol and BHA. Isoorientin, which possesses hydroxyl group on C3′ and C4′, exhibited marked hydroxyl radical inhibition around 79.4% and 48.5% at 1 m*M* and 0.1 m*M*, respectively. Vitexin and isovitexin displayed similar inhibitory activity (around 30% and 20% at 1 m*M* and 0.1 m*M*, respectively), demonstrating that position of substituted sugar had no influence on the activity. α-Tocopherol and BHA, however, showed no scavenging properties on superoxide at any given concentration. Antioxidant mechanism of flavones *C*-glucoside, which are much more polar compounds than α-tocopherol and BHA, would be different from that of lipophilic antioxidant. A negative effect of lipophillic antioxidants such as α–tocopherol, BHA, and BHT has been reported in a pyrogallol autoxidation system (Kim and others 1995). In SOD-equivalent activity, isoorientin with the strongest activity showed 6.96 unit/mL and 31.37 unit/mL at the concentration of 0.1 m*M* and 1 m*M*, respectively. Vitexin and isovitexin showed less than 3 unit/mL at both concentrations.

It is a general notion that the number of hydroxyl groups substituted on B-ring contributes to free radical scavenging activity of flavonoids (Husain and others 1987). Ortho dihydroxyl groups at C3′ and C4′ of B-ring in isoorientin give stronger radical scavenging activity than vitexin and isovitexin having only 1 hydroxyl group. The contribution of 3′, 4′-dihydroxy structure of luteolin (aglycone of isoorientin) to higher antioxidant activity in TEAC was reported in earlier study (Rice-Evans and others 1996). The position of substituted sugar did not play an important role in ROS scavenging activity. Several studies were accomplished on antioxidant property of isoorientin. Isoorientin from lemongrass showed inhibitory

activity toward DPPH (IC_{50}, 9-10 μM) and inhibited lipid peroxidation by 70% at 100 μg/mL (Cheel and others 2005). Suppression on xanthine oxidase activity and lipoxygenase activity by isoorientin were determined (Budzianowski and others 1991; Lin and others 2002). Vitexin from *Acer palmatum* was shown to inhibit superoxide radicals by 70% and DPPH radicals by 60% at 100 μg/mL (Kim and others 2005). Other biological properties of *C*-glycosylflavones such as anti-platelet, vasorelaxing, antibacterial, and hypoglycemic effects have also been studied (Andrade-Cetto and Wiedenfeld 2001; Lin and others 2002).

Inhibitory effects of flavone *C*-glucosides on human LDL oxidation

The antioxidative effect of the isolated flavone *C*-glucoside was further investigated by analyzing their ability to inhibit human LDL oxidation induced by $CuSO_4$. The inhibitory activity of compounds on the oxidation of human LDL was determined by the TBARS value, which generally measures the secondary oxidation products, mostly carbonyl compounds such as malondialdehyde (MDA) (Shahidi and Hong 1991). Marked inhibitions on the lipid peroxidation by the isolated flavone *C*-glucosides were observed as shown in Figure 4. They all exhibited better inhibitory properties than BHA and α-tocopherol and showed more than 65% of LDL oxidation inhibition at 100 μM. Vitexin and isovitexin showed relatively weaker activity at 10 μM, but in the case of isoorientin, maintained stronger activity. By addition of isoorietin, vitexin, and isovitexin, the levels of free MDA were decreased to 8.54, 4.47, and 10.83 nM/mg protein, respectively, compared with that of control 48.03 nM/mg at 100 μM. At same concentration, the inhibition of free MDA production was 77% to 90% in HPLC analyses, but lower inhibition (18% to 67%) was detected by spectrophotometry. It was assumed that in the case of the TBA test, all TBARS reacted to the thiobarbituric acid and increased the absorbance because tested samples showed lower inhibition rates than those using the HPLC method.

Inhibition of conjugated diene production by flavone *C*-glucoside is presented in Figure 5. Conjugated diene formation curves show 3 phases of oxidation, including induction, propagation, and the termination phase (Iuliano and others 1999). The induction period of control was about 30 min, and the rate of oxidation increased rapidly. With the addition of the tested compounds, the induction period was prolonged. Isovitexin and vitexin extended the lag time compared with the control, but final absorbance was similar to that of control. Isoorientin not only increased the lag time of the oxidation reaction about twice more than control but also reduced the maximum rate of diene formation. Isoorientin exhibited the strongest inhibition in human LDL peroxidation, as well as in ROS scavenging activity. Similarly, luteolin, the aglycone of isoorientin, exerted better activity than apigenin, aglycones of vitexin, and isovitexin (O'Reilly and others 2000). A positive correlation between ROS scavenging activity and the prevention of LDL oxidation was observed. These results suggest that isolated flavone *C*-glucoside can effectively inhibit both hydroxyl radicals and superoxide anion radicals and can also confer very efficient inhibition against lipid peroxidation.

Conclusions

Lipid peroxidation is believed to play a key role in the development of atherosclerosis. In fact, there are indications that the atherogenicity of high plasma LDL levels is caused to a major extent by the accompanying elevated LDL oxidation products. This study demonstrated that flavone *C*-glucosides from *A. japonica* possess strong ROS scavenging and LDL oxidation inhibitory actions, indicating that it is a very effective protector against oxidative stress. Thus, the leaves of *A. japonica* might be beneficial resources with

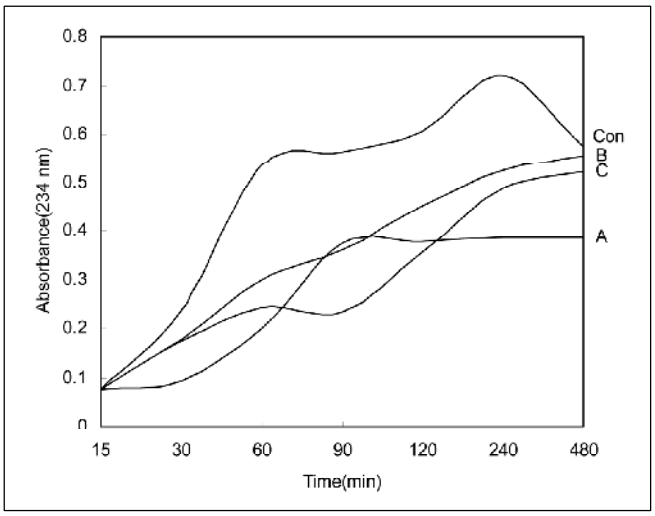

Figure 5—Inhibitory effects of conjugated diene formation on the $CuSO_4$-induced low-density lipoprotein (LDL) peroxidation by flavone *C*-glucosides. (a) luteolin-6-*C*-glucoside; (b) apigenin-8-*C*-glucoside; (c) apigenin-6-*C*-glucoside.

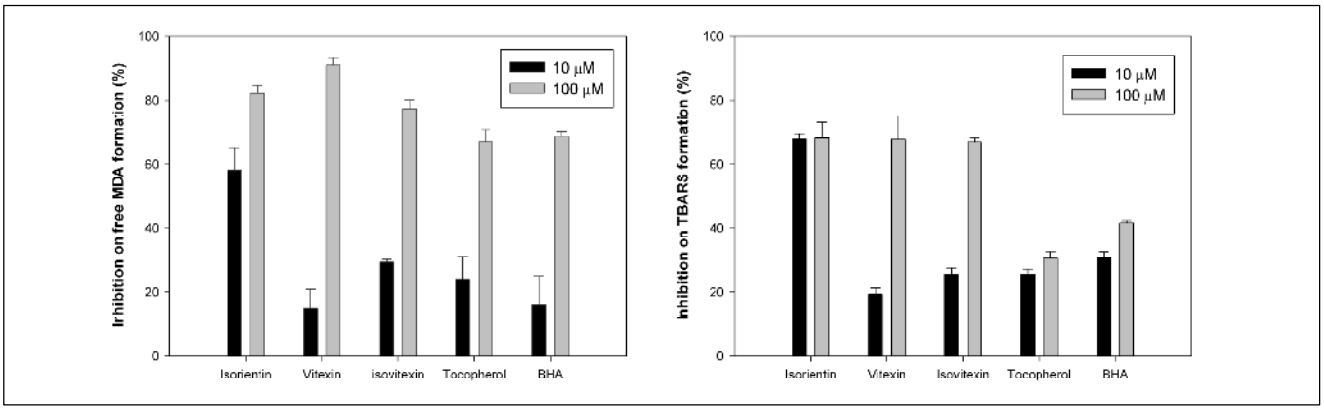

Figure 4—% Inhibition of flavone *C*-glucosides on thiobarbituric acid-reactive substances (TBARS) and malondialdehyde (MDA) formation in human low-density lipoprotein (LDL) peroxidation induced by $CuSO_4$

antioxidant property inhibiting LDL oxidation, which will prevent human diseases in which free radicals are involved.

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