Antioxidative Activities of Phenylethanoid Glycosides from *Ligustrum purpurascens*

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Tea and kudingcha (bitter tea) are the two most popular beverages consumed in China. Tea derived from the leaves of *Camellia sinensis* has been well studied for its various health benefits, but there are very limited data on the biological activities of bitter tea derived from the leaves of *Ligustrum purpurascens* (LP). The present study was carried out to characterize the antioxidants present in the bitter tea brewed from the leaves of LP. It was found that the crude glycoside fraction possessed strong protection against oxidation of human low-density lipoprotein (LDL). The column chromatographic separation led to the isolatation of five phenylethanoid glycosides, namely, acteoside, ligupurpuroside A, *cis*-ligupurpuroside B, *trans*-ligupurpuroside B, and osmanthuside B. When acteoside was heated in the boiling water, it was isomerized to form isoacteoside. Acteoside, isoacteoside, and ligupurpuroside A purified from LP were protective, whereas *cis*-ligupurpuroside B, *trans*-ligupurpuroside B, and osmanthuside B exhibited no protection to human LDL from Cu^{2+}-medicated oxidation. Acteoside, isoacteoside, and ligupurpuroside A were also effective in preventing the peroxyl free radical-induced oxidation of α-tocopherol in human LDL. The antioxidant activities of acteoside, isoacteoside, and ligupurpuroside A were comparable to that observed for a green tea antioxidant, (−)-epicatechin gallate. The inhibitory effect of these three phenylethanoid glycosides on oxidation of human LDL and α-tocopherol was dose-dependent at concentrations of 5−40 μM. The present results suggest that the bitter tea beverage derived from LP contains effective antioxidants that may have an equal benefit as a green tea beverage.

Keywords: *Acteoside; bitter tea; isoacteoside; kudingcha; ligupurpuroside A; Ligustrum purpurascens; phenylethanoid glycoside*

INTRODUCTION

There has been increasing interest in natural antioxidants present in fruits, vegetables, herbs, and beverages. Several studies have suggested that intake of food rich in antioxidants is associated with a lower risk of some chronic diseases (*1*, *2*). Tea is the most popular beverage consumed worldwide. It is brewed from the leaves of *Camellia sinensis*. It is known that tea catechins possess potent antioxidant and free radical scavenging activities. Bitter tea refers to the brew prepared from the leaves of 10 species in 5 different families that are collectively called kudingcha (*3*). Like green tea, bitter tea has also been a popular beverage for a long time in southern China including the provinces of Guangxi, Guizhou, Zhejiang, Yunnan, and Sichuan. Unlike green tea, no kudingcha beverages contain any caffeine. There has been no report to date of the study of the antioxidants possibly present in kudingcha.

In the province of Yunnan, the species *Ligustrum purpurascens* (LP) is used to brew bitter tea. It has been claimed to act as a stimulant to the central nervous system, a diuretic, a treatment for sore throat, an aid to weight loss, and a relief of hypertension (*4*, *5*). However, there were no solid scientific data to confirm these activities. The chemical composition of LP has been previously studied (*6*). Five phenylethanoid glycosides from LP were isolated and identified (Figure 1). They include acteoside, ligupurpuroside A, *cis*-ligupurpuroside B, *trans*-ligupurpuroside B, and osmanthuside B. Isoacetoside is an isomer of acteoside and formed when the leaves of LP were heated in the boiling water. The present study was designed to quantify these phenylethanoid glycosides present in LP and examine their antioxidant activities against the oxidation of human low-density lipoprotein (LDL) and α-tocopherol.

MATERIALS AND METHODS

Extraction, Isolation, and Identification of Phenylethanoid Glycosides. The dried leaves of LP were purchased from a local store in Zhaotong, Yunnan province, China. The isolation and purification of phenylethanoid glycosides were carried out according to the method previously described (*6*). In brief, the dried leaves (520 g) were extracted with 6 L of ethanol four times under reflux. The extract was concentrated in a rotary evaporator and then dissolved in distilled water (Figure 2). The extract was fractionated in a column packed with Diaion HP-20 using 10 L of water first and then 4 L of ethanol as eluting solvents; two factions, A1 and A2, were subsequently obtained. Fraction A1 mainly contained sugar and organic acid components, and fraction A2 contained crude glycosides. Fraction A2 (42 g) was further chromatographed in a silica gel column (2 kg) and eluted using chloroform/methanol ($CHCl_3$/MeOH). Four fractions (B1−B4) were subsequently obtained; each fraction was then subjected to a silica gel column chromatography using various ratios of $CHCl_3$/MeOH as an eluting solvent. Afterward, fraction B1 yielded

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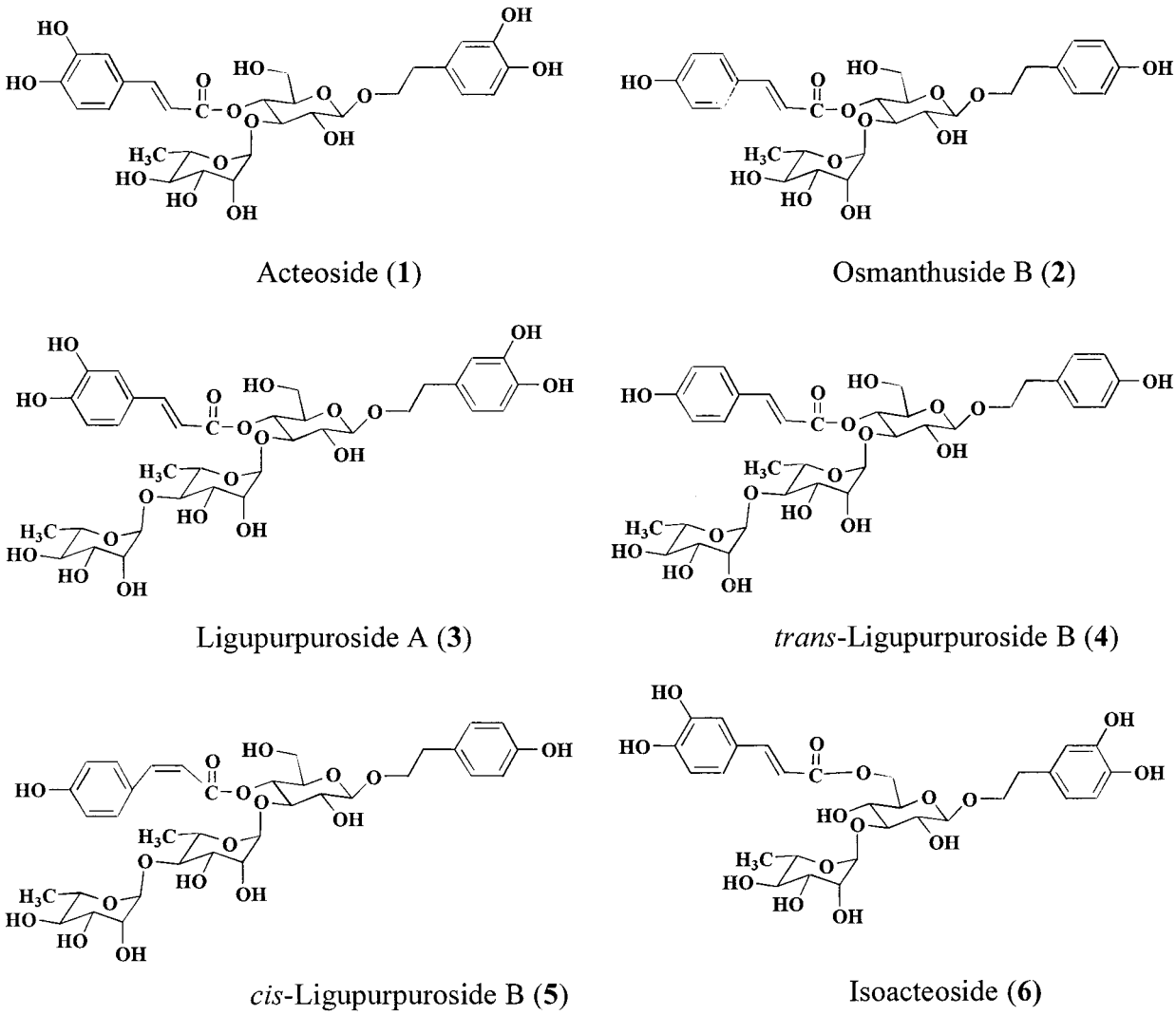

Figure 1. Chemical structures of phenylethanoid glycosides present in a bitter tea beverage derived from LP.

132 mg of osmanthuside B, whereas fractions B1 and B2 gave 65 mg of *cis*-ligupurpuroside B and *trans*-ligupurpuroside B. Fractions B3 nd B4 were subjected to a silica column eluted with $CHCl_3$/MeOH (7:3, v/v) and then purified in a commercial column packed with Si 60 (Lobar, 40−63 μm, 250 × 25 mm, Merck, Darmstadt, Germany) followed by further purification in a glass column packed with Sephadex LH-20. Two glycosides were obtained, namely, acteoside and ligupurpuroside A.

Isoacteoside was obtained after the incubation of acteoside at 100 °C. In brief, 16.1 mg of acteoside was dissolved in 100 mL of distilled water and heated at 100 °C for 8 h. Approximately 80% of the acteoside was converted to isoacteoside. A Sephadex LH-20 column (100 μm, Pharmacia Fine Chemicals Co., Ltd.) was then used to separate isoacteoside from acteoside, and the purity was tested using HPLC equipped with a C-18 column (Hypersil ODS, 250 × 4.6 mm, 5 μm, Alltech, Deerfield, IL).

Six purified phenylethanoid glycosides were subjected to verification of their chemical structures on the basis of their spectral data of UV, LC-MS, FAB-MS, ^{1}H NMR, and ^{13}C NMR and comparison with those of published data (*6*) and reference compounds (Figure 1).

HPLC Analysis of Phenylethanoid Glycosides. Acteoside, ligupurpuroside A, *cis*-ligupurpuroside B, *trans*-ligupurpruposide B, and osmanthuside B were analyzed using a Shimadzu LC-10AD HPLC (Tokyo, Japan) equipped with a ternary pump delivery system. In brief, 5 μL of samples (1 mg/mL in methanol) was injected onto a C-18 column (Hypersil ODS, 250 × 4.6 mm, 5 μm, Alltech) maintained in a cold water bath of 4 °C, via a rheodyne valve (20 μL capacity, Shimadzu, Tokyo, Japan). An initial eluting mixture of H_2O and acetonitrile (80:20, v/v) was used at a flow rate of 1 mL/min. From 15 to 43 min, the percentage of acetonitrile was increased to 100%. Separated glycosides were monitored using a UV detector at 227 nm (Shimadzu SPD-10AV). A typical HPLC chromatogram of the ethanol extract derived from LP is shown in Figure 3.

LDL Isolation. Fresh blood was collected from healthy subjects at the Prince of Wales Hospital, The Chinese University of Hong Kong, Shatin, Hong Kong. To prevent lipoprotein modification, EDTA (0.1%) and NaN_3 (0.05%) were added. LDL was isolated from plasma according to the method described previously (*7*). To minimize the oxidation of LDL, the centrifuge tubes containing plasma were flushed with nitrogen gas. First, the plasma was centrifuged at 1500*g* for 15 min to remove cells and cell debris. A NaCl/KBr solution (153 g of NaCl, 354 g of KBr, and 100 μg of EDTA dissolved in 1 L of water, 1.33 g/mL) was then added to increase the plasma density to 1.019. The plasma was recentrifuged at 160000*g* at 4 °C for 20 h. After the top layer containing chylomicron and very low-density lipoprotein (VLDL) was removed, the density of remaining plasma fractions was increased to 1.064 and they were recentrifuged at 160000*g* for an additional 24 h. The top LDL fraction was collected and then flushed with nitrogen and stored at −70 °C. The protein content of isolated LDL was determined using Lowry's method (*8*).

LDL Oxidation. The stock LDL fraction (5 mg of protein/mL) was dialyzed against 100 volumes of the degassed dialysis solution (pH 7.4) containing 0.01 M sodium phosphate, 0.9% NaCl, 10 μM EDTA, and 0.05% NaN_3 in the dark for 24 h.

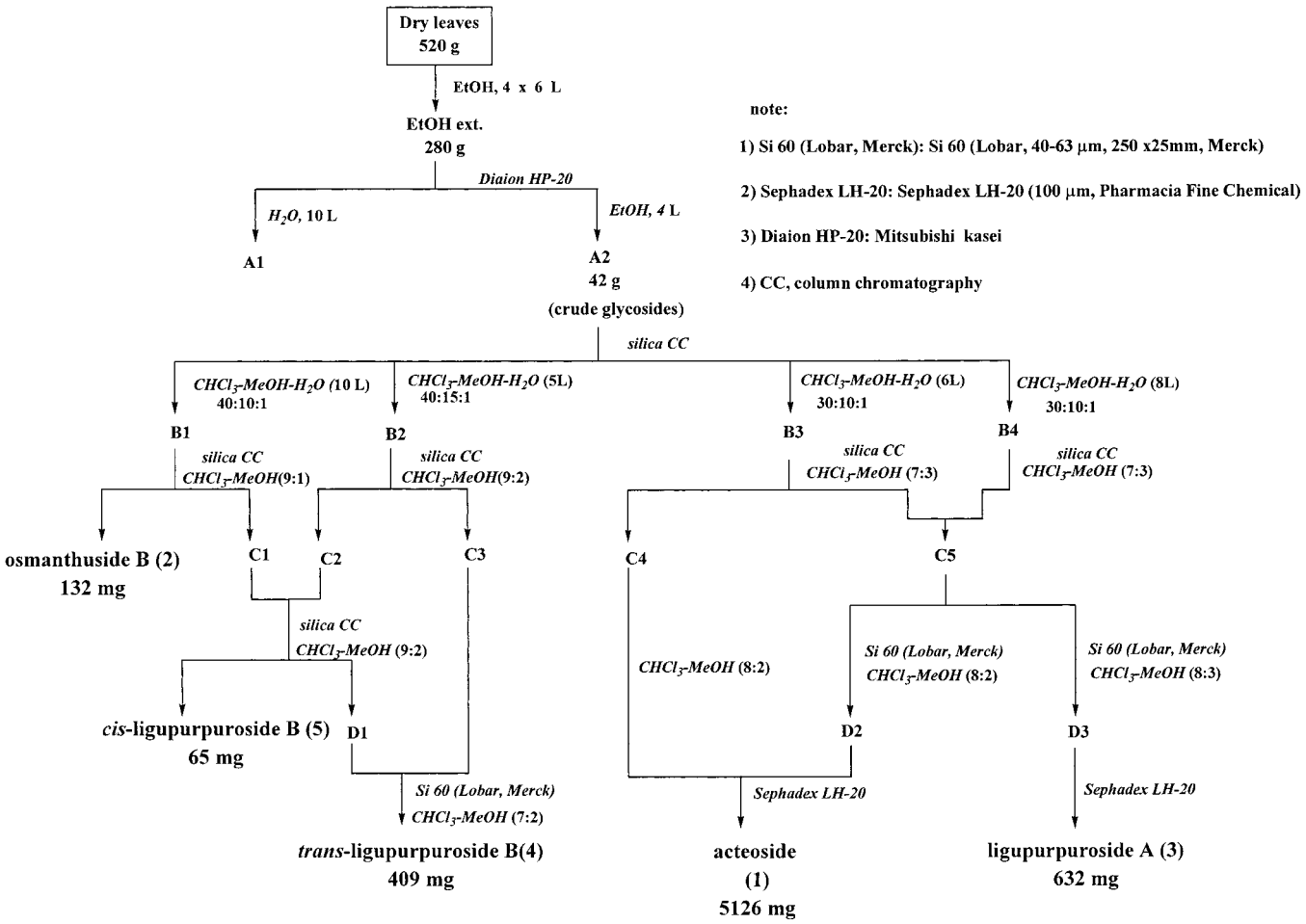

Figure 2. Separation and purification of phenylethanoid glycosides from LP.

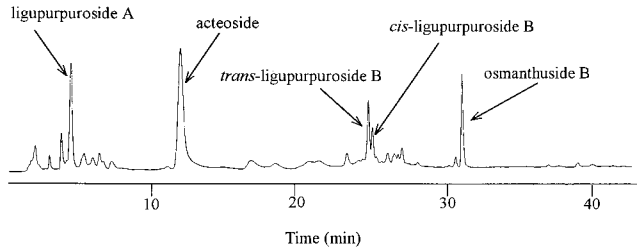

Figure 3. HPLC profile of phenylethanoid glycosides present in an ethanol extract of LP. See text for the conditions and Table 1 for percent composition.

The dialysis solution was changed four times. Oxidation of LDL was conducted as previously described by Puhl et al. (*9*). LDL protein (150 μg) was incubated in a mixture containing 5 μM $CuSO_4$ at 37 °C for up to 32 h. The oxidation was then stopped by the addition of 25 μL of 1.0% EDTA and cooled at 4 °C. The degree of LDL oxidation was monitored by measuring the production of thiobarbituric acid-reactive substances (TBARS) as previously described (*10*). After the reaction was stopped by the addition of EDTA at 4 °C, 2 mL of 0.67% thiobarbituric acid and 15% trichloroacetic acid in 0.1 N HCl solution were added to the LDL-incubated tube. The incubation mixture was then heated at 95 °C for 1 h, cooled on ice, and centrifuged at 1000*g* for 20 min. TBARS was determined by measuring the absorbance at 532 nm. Calibration was done with a malondialdehyde (MDA) standard solution prepared from tetramethoxypropane, although thiobarbituric acid reacts with a number of lipid oxidation products including other aldehydes and peroxyl radicals. The value was expressed as nanomoles of MDA per milligram of LDL protein.

Interactions of Phenylethanoid Glycosides with Cu^{2+}. The chelation reaction between each tested phenylethanoid and Cu^{2+} was examined according to the method previously described by Brown et al. (*11*). In brief, stock solutions of each tested phenylethanoid glycoside (1 mM) were prepared in water. Then the solution was diluted to 250 μM in a cuvette, and the absorption spectra were recorded at wavelengths between 200 and 350 nm. To detect any chelation or interaction between phenylethanoid glycosides and Cu^{2+}, scans with the addition of 250 and 500 μM $CuSO_4$ were taken and compared with the spectrum for each tested phenylethanoid glycoside alone. If a chelation reaction occurs, changes in the spectra of phenylethanoid glycosides are expected.

Protection of Phenylethanoid Glycoside to α-Tocopherol in LDL. The protective role of each tested phenylethanoid to α-tocopherol in human LDL was studied as we previously described (*12*). Human LDL was isolated as previously described above. The stock LDL fraction (5 mg of protein/mL) was dialyzed against 100 volumes of the degassed dialysis solution (pH 7.4) containing 0.01 M sodium phosphate, 0.9% NaCl, 10 μM EDTA, and 0.05% NaN_3 in the dark for 24 h. Oxidation of α-tocopherol in LDL was induced by 2,2′-azobis-(2-amidinopropane) dihydrocholoride (AAPH). The LDL (150 μg of protein/mL) was incubated in sodium phosphate buffer (pH 7.4, 10 mM) at 37 °C with constant stirring. The various amounts of each phenylethanoid glycoside were added before the addition of 1.0 mM AAPH. An aliquot of the incubation solution (1 mL) was recovered and chilled in ice. One milliliter of ethanol containing 0.5 mg of butylated hydroxytoluene as an antioxidant and 1.0 μg of tocopherol acetate as an internal standard was added and immediately extracted with 2 mL of hexane. The hexane was evaporated under a gentle stream of nitrogen, and the resulting extract was redissolved in 100 μL of ethanol and subjected to HPLC analysis for determination of α-tocopherol in LDL.

Table 1. Content of Some Phenylethanoid Glycosides Present in *L. purpurascens*[a]

	content (g/100 g of dry leaves)
acteoside	2.1
ligupurpuroside A	0.7
cis-ligupurpuroside B	0.1
trans-ligupurpuroside B	0.2
osmanthuside B	0.5

[a] Average of three separate extractions.

HPLC Analysis of α-Tocopherol in LDL. α-Tocopherol in LDL was determined using a Shimadzu LC-10AD HPLC equipped with a ternary pump delivery system (*12*). In brief, 10 μL of the extract dissolved in ethanol was injected onto a C-18 column (Microsorb MV, 250 × 4.6 mm, i.d., 5 μm, Rainin, Woburn, MA) via a rheodyne valve (20 μL capacity; Cotati, CA). Methanol was used as an eluting solvent at a flow rate of 2 mL/min. α-Tocopherol was monitored by using a diode array detector at either 200 or 280 nm and quantified according to the amount of α-tocopherol acetate standard added.

Statistics. Data are expressed as means ± SD. The analysis of variance (ANOVA) when applicable was used for statistical evaluation of significant differences in protection to LDL oxidation by each phenylethanoid glycoside using Sigmastat (Jandel Scientific Software, San Rafael, CA). Differences were considered significant when $p < 0.05$.

RESULTS

The antioxidant activity of the water fraction (A1) and the total glycoside fraction (A2) derived from the leaves of LP (Figure 3) was first tested in LDL oxidation under the same conditions. It was found that fraction A2 possessed strong protection against oxidation to human LDL. It was therefore targeted for isolation and purification of possible antioxidants. According to He et al. (*6*), the column chromatographic separations led to the purification of five phenylethanoid glycosides (Figure 1). As shown in Table 1, HPLC analysis found that acteoside was most abundant (2.1 g/100 g of dry leaves) followed by ligupurpuroside A, osmanthuside B, and *trans*-ligupurpuroside B.

When the leaves of LP or pure acteoside was heated in boiling water, HPLC analysis demonstrated that a new peak following that of acteoside gradually became bigger with heating. This peak was later identified as isoacteoside, which is an isomer of acteoside. The structure of isoacteoside was confirmed using ^{1}H NMR and ^{13}C NMR. The ^{1}H NMR spectrum of isoacteoside was similar to that of acteoside, except for differences in the chemical shifts of H-4 (acteoside, δ 4.81; isoacteoside, δ 3.41) and 2H-6 (acteoside, δ 3.63 and 3.84; isoacteoside, δ 4.34 and 4.50) in their glucosyl moiety. The ^{13}C NMR chemical shifts of isoacteoside were close to those of acteoside, but slight differences were observed in the shifts at C-3, C-4, and C-6 in its glucosyl moiety (acteoside, δ 81.66, 70.69, 62.49; isoacteoside, δ 84.45, 70.94, 65.20).

Six purified phenylethanoid glycosides demonstrated various antioxidant activities (Figure 4). *cis*-Ligupurpuroside B, *trans*-ligupurpuroside B, and osmanthuside B showed no or little antioxidant activity, whereas acteoside, isoacteoside, and ligupurpuroside A were protective to human LDL, comparable to the activity of (−)-epicatechin gallate (ECG). These three phenylethanoid glycosides demonstrated a dose-dependent antioxidant activity in Cu^{2+}-mediated LDL oxidation (Figure 5). In most cases, LDL was oxidized significantly within 4 h in the absence of any antioxidant. Addition of 10−20 μM acteoside, isoacteoside, and ligupurpuroside A extended the lag time to >10 h. Acteoside showed a stronger antioxidant activity than isoacteoside (Figure 5).

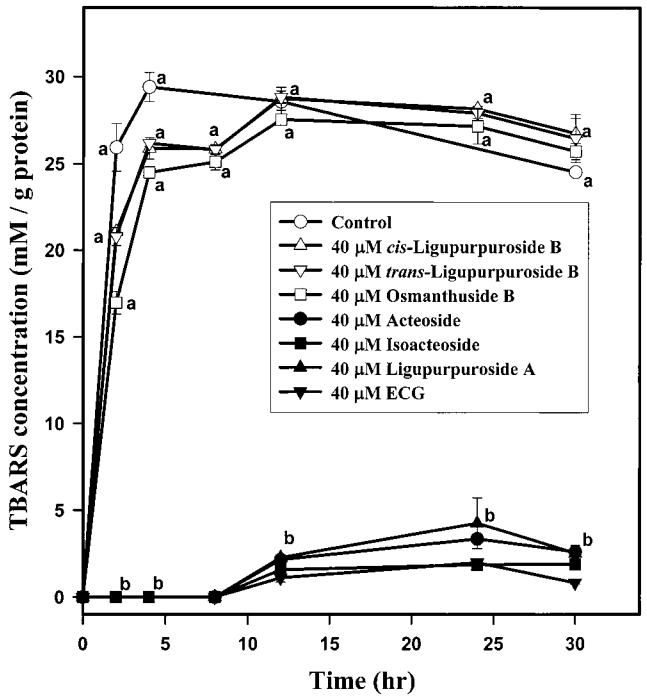

Figure 4. Inhibitory effect of six phenylethanoid glycosides (40 μM) isolated from LP on the production of TBARS in Cu^{2+}-mediated oxidation of human LDL. The LDL (150 μg of protein/mL) was incubated in sodium phosphate buffer (pH 7.4) containing 5 μM $CuSO_4$. The oxidation was conducted at 37 °C. Data are expressed as mean ± SD of $n = 6-8$ samples. ECG from green tea was used as a reference antioxidant. Means at the same time point with different letters differ significantly at $p < 0.05$.

The protective effect of six phenylethanoid glycosides on α-tocopherol in human LDL was also examined (Figure 6). *cis*-Ligupurpuroside B, *trans*-ligupurpuroside B, and osmanthuside B showed no protection to α-tocopherol. This was in agreement with that observed in the TBARS test. In contrast, acteoside, isoacteoside, and ligupurpuroside A were strongly protective to α-tocopherol in human LDL. These three phenylethanoid glycosides also demonstrated a dose-dependent protecting role to α-tocopherol in human LDL (Figure 7).

DISCUSSION

Green, oolong, and black tea beverages from the leaves of the plant *C. sinensis* have been extensively studied for their antioxidant, anticarcinogenic, and hypolipidemic activities. However, there are very limited data on bitter tea, especially the beverage from the species LP cultured in the provine of Yunnan, China. Many studies suggest that oxidation of human LDL is one risk factors in the development of cardiovascular disease (*13*−*19*). The present results demonstrated clearly that the bitter tea derived from LP contained at least two antioxidants, namely, acteoside and ligupurpuroside A, which were effective in protecting human LDL from oxidation.

α-Tocopherol is a primary antioxidant protecting human LDL from oxidative modification (*20*−*22*). Several reports suggest that vitamin E supplementation is associated with a lower risk of coronary heart disease

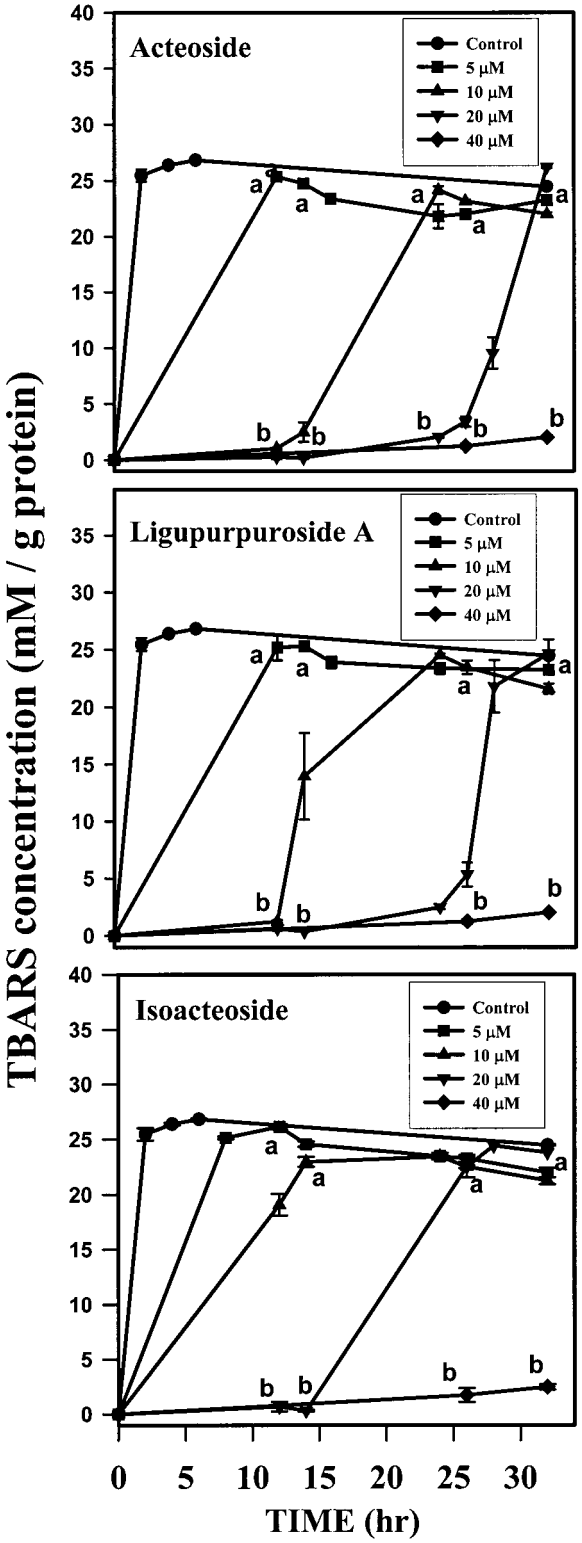

Figure 5. Dose-dependent inhibitory effect of acteoside, isoacteoside, and ligupurpuroside A on the production of TBARS in Cu^{2+}-mediated oxidation of human LDL. The LDL (150 μg of protein/mL) was incubated in sodium phosphate buffer (pH 7.4) containing 5 μM $CuSO_4$. The oxidation was conducted at 37 °C. Data are expressed as mean ± SD of n = 6−8 samples. Means at the same time point with different letters differ significantly at $p < 0.05$.

in both men and women (*23*, *24*). The level of serum α-tocopherol is inversely correlated with the mortality rate of cardiovascular disease (*25*, *26*). The present results clearly show that acteoside and ligupurpuroside A present in the leaves of LP protected not only human

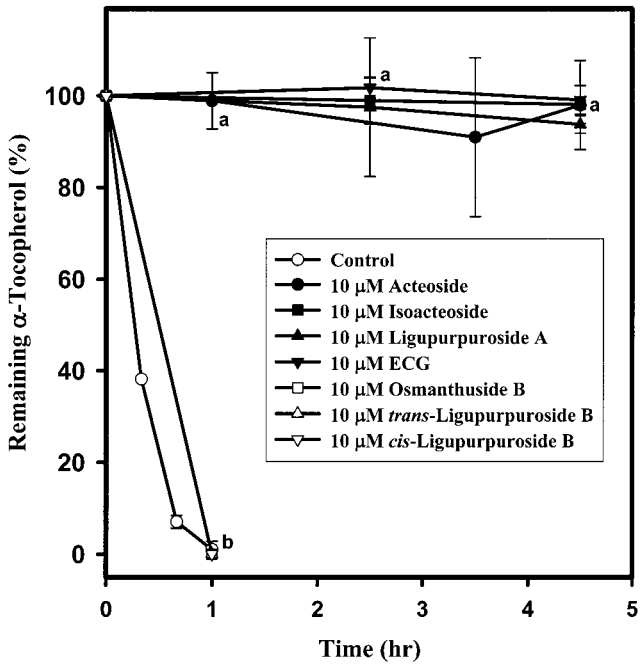

Figure 6. Protective effect of six phenylethanoid glycosides (10 μM) isolated from LP on α-tocopherol in human LDL. The LDL (150 μg of protein/mL) was incubated in sodium phosphate buffer (pH 7.4), and the oxidation of α-tocopherol in LDL was induced by 1 mM AAPH at 40 °C. Data are expressed as mean ± SD of n = 6−8 samples. ECG from green tea was used as a reference antioxidant. Means at the same time point with different letters differ significantly at $p < 0.05$.

LDL but also α-tocopherol from oxidation in vitro. Acteoside and ligupurpuroside A as natural antioxidants may be beneficial if they are absorbed and circulated in the blood.

The biochemical mechanisms by which acteoside, isoacteoside, and ligupurpuroside A act as antioxidants remain unclear at the present time. Probably, they protect human LDL from oxidation by one of the following mechanisms: (i) they function as chelators to inactivate Cu^{2+} used in the initiation of free radicals (*11*); and (ii) they act as a primary antioxidant by directly reducing the formation of free radicals mediated by Cu^{2+}. To test the first possibility, the interaction between Cu^{2+} and each phenylethanoid glycoside was examined. Addition of equimolar Cu^{2+} did not cause any major spectral shift of acteoside, isoacteoside, and ligupurpuroside at wavelengths between 200 and 400 nm, indicating that these three phenylethanoid glycosides cannot chelate Cu^{2+} (data not shown). Instead, it was believed that the antioxidant activity of these phenylethanoid glycosides was related to their number of hydroxyl groups, that is, their electron- or hydrogen-donating potency. In fact, osmanthuside B, *cis*-ligupurpuroside B, and *trans*-ligupurpuroside B showed no or little protection to human LDL because they had only two separate phenol moieties. In contrast, acteoside, isoacteoside, and ligupurpuroside A strongly inhibited the oxidation of human LDL and α-tocopherol because they were characerized by having two catechol moieties. Thus, they are more vulnerable to loss of a proton or an electron due to resonance delocalization. The antioxidant activities observed for acteoside, isoacteoside, and ligupurpuroside A are most likely attributable to their proton-donating capacities.

The results obtained in the present study may have some implications for individuals who regularly con-

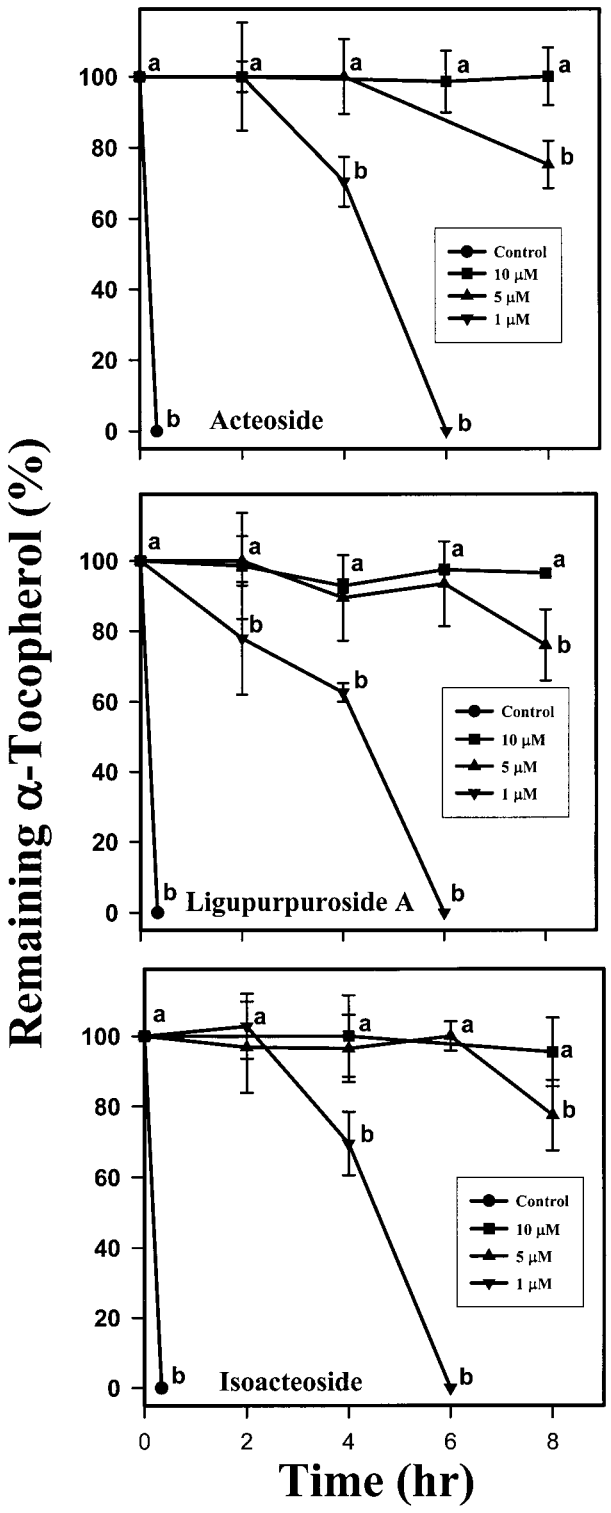

Figure 7. Dose-dependent protective effect of acteoside, acteoside, and ligupurpuroside A on α-tocopherol in human LDL. The LDL (150 μg of protein/mL) was incubated in sodium phosphate buffer (pH 7.4), and the oxidation of α-tocopherol in LDL was induced by 1 mM AAPH at 40 °C. Data are expressed as mean ± SD of $n = 6-8$ samples. Means at the same time point with different letters differ significantly at $p < 0.05$.

sume bitter tea derived from LP. It is known that green tea contains four major catechin antioxidants, namely, (−)-epicatechin (EC), (−)-epigallocatechin (EGC), ECG, and (−)-epigallocatechin gallate (EGCG). To our best knowledge, this is the first study to demonstrate that the bitter tea beverage derived from LP also contains two antioxidants, namely, acteoside and ligupurpuroside A, which are comparable to those in green tea. We are currently investigating the absorption, metabolism, and excretion of acteoside and ligupurpuroside A and exploring other possible health benefits including cholesterol-lowering and cardiovascular relaxing activities associated with drinking the bitter tea derived from LP.

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