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Characterization of Antioxidants Present in Bitter Tea (*Ligustrum pedunculare*)

ZHEN-YU CHEN,*,† IVAN YUEN FAN WONG,† MAGNOLIA WING SHAN LEUNG,† ZHEN-DAN HE,‡ AND YU HUANG§

Food and Nutritional Science Programme, Department of Biochemistry, and Department of Physiology, The Chinese University of Hong Kong, Shatin, New Territories, Hong Kong, People's Republic of China, and School of Pharmacy, Yunnan University, Kunming, Yunnan, People's Republic of China

The present study examined the antioxidants present in bitter tea (*Ligustrum pedunculare)*. It was found that the crude glycoside fraction strongly protected human low-density lipoprotein (LDL) from oxidation. Further column chromatography led to purification of eight phenylethanoid or monoterpene glycosides: lipedoside A-I, lipedoside A-II, lipedoside B-I, lipedoside B-III, lipedoside B-V, lipedoside B-VI, osmanthuside B, and anatolioside. It was found that lipedoside A-I, lipedoside A-II, lipedoside B-V, and lipedoside B-VI were protective, whereas the other four compounds did not protect human LDL from Cu^{2+}-medicated oxidation. Lipedoside A-I, lipedoside A-II, lipedoside B-V, and lipedoside B-VI also had a scavenging effect on 2,2-diphenyl-1-picrylhydrazyl free radical (DPPH), comparable to that of α-tocopherol. The inhibitory effect of these four phenylethanoid or monoterpene glycosides on oxidation of human LDL and α-tocopherol was dose-dependent at concentrations of 5−40 μM. The present results demonstrate that bitter tea as a beverage contains effective antioxidants that may have benefits similar to those of green tea in terms of antioxidant activity.

KEYWORDS: Antioxidant; bitter tea; green tea; phenylethanoid gycosides; monoterpene glycosides

INTRODUCTION

Tea and bitter tea are popular beverages in China. Tea beverage refers to the brew prepared from the leaves of *Camellia sinensis*. Tea can be classified into three types: green, oolong, and black tea. The main polyphenols in these teas are catechins and theaflavins, which have been shown to have a wide range of biological and pharmaceutical benefits, including anticarcinogenic, antioxidative, and hypolipidemic activities (*1*, *2*). In contrast, bitter tea is brewed from the leaves of 10 species in 5 different families that have been consumed traditionally for a long time in southern China (*3*). We have previously characterized the antioxidants present in one species, *Ligustrum purpuracens*, which is mainly confined to Yunnan Province. It was found that two phenylethanoid glycosides, acteoside and ligupurpuroside A, isolated from this species were effective antioxidants, comparable to catechins purified from green tea (*4*).

The present study was carried out to further characterize the antioxidants present in the species *Ligustrum pedunculare* (LP), which is mainly used to brew bitter tea in Sichuan Province of China. Eight phenylethanoid or monoterpene glycosides were isolated and identified by He et al. (*5*). This study is the first report to demonstrate that the bitter tea brewed from LP contains antioxidants which have potency comparable to that of α-tocopherol.

MATERIALS AND METHODS

Extraction, Isolation, and Identification of Phenylethanoid Glycosides. Dry leaves of LP were purchased from a local store at Chengdu City, Sichuan Province, China. The isolation and purification of phenylethanoid and monoterpene glycosides were carried out according to the method previously described (*5*). As shown in **Figure 1**, the dried leaves (2.15 kg) were extracted with 20 L of hot MeOH four times under reflux. The extract was concentrated in a rotary evaporator to produce a residue of 675 g, which was then dissolved in 1 L of distilled water. The resultant aqueous extract was successively partitioned with hexane, ethyl acetate, and distilled water. The hexane, ethyl acetate, and water layers were evaporated in a rotary evaporator to give residues of 18.9, 184.3, and 174.8 g, respectively. The crude glycoside (2.98 g) was obtained by fractionating H_2O extract in a Diaion HP-20 column, eluted using a mixture of H_2O−MeOH (6:4, v/v). The crude glycoside was then loaded onto a silica column, eluted using varying ratios of $CHCl_3$−MeOH solvent, producing fractions A1, A2, and A3. Fraction A1 was subjected to a Si60 column (Lobar, 40−63 μm) with $CHCl_3$−MeOH (9:1, v/v) as an eluent, leading to purification of

* Corresponding author [telephone (852) 2609-6382; fax (852) 2603-5123; E-mail zhenyuchen@cuhk.edu.hk].
† Food and Nutritional Science Programme, Department of Biochemistry, The Chinese University of Hong Kong.
§ Department of Physiology, The Chinese University of Hong Kong.
‡ School of Pharmacy, Yunnan University.

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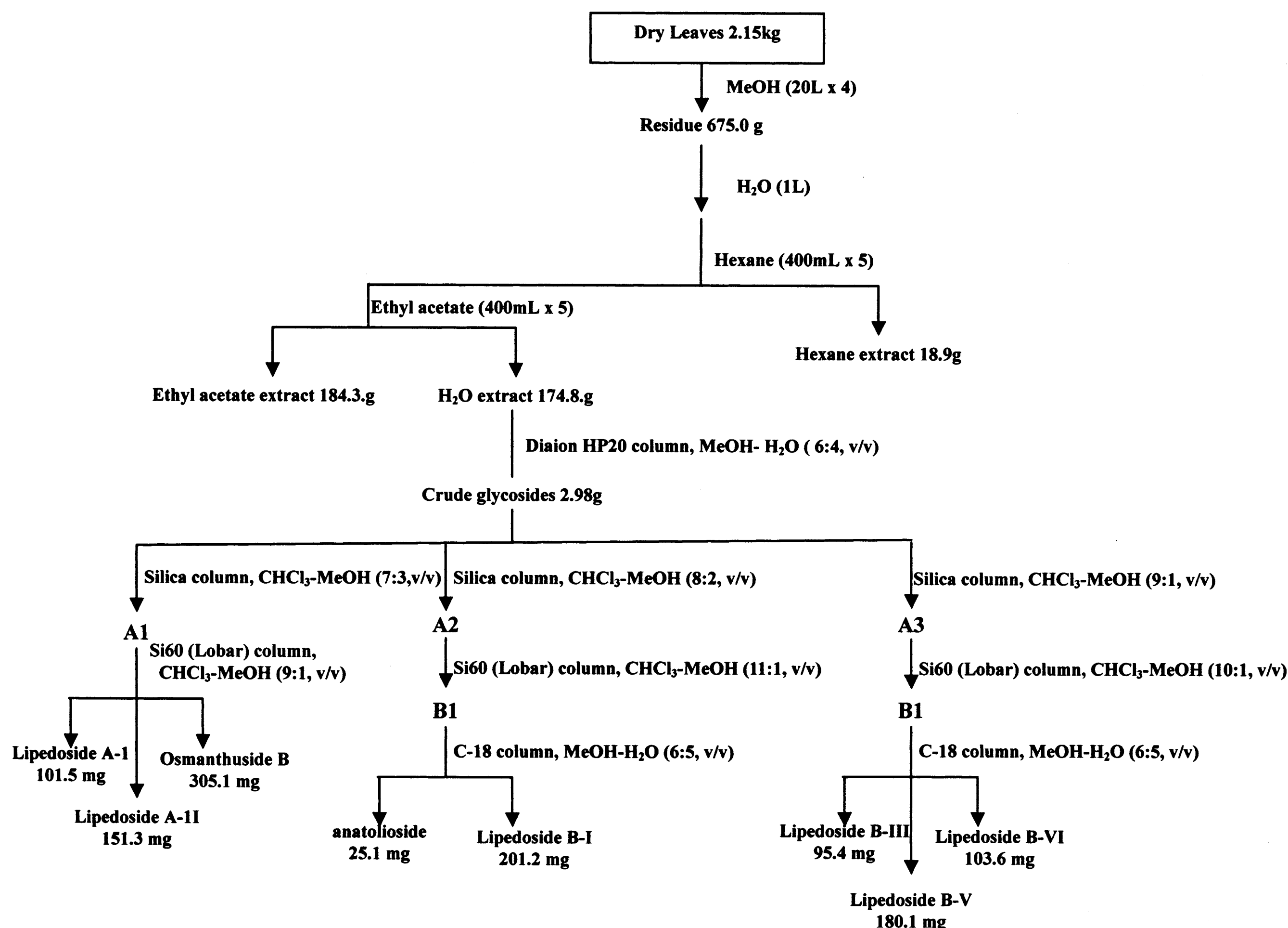

Figure 1. Separation and purification of phenylethanoid and monoterpene glycosides in bitter tea.

lipedoside A-I (101.5 mg), lipedoside A-II (151.3 mg), and osmanthuside B (25.1 mg). Fraction A2 was loaded onto a Si60 column eluted with $CHCl_3$−MeOH (11:1, v/v), followed by purification on a C-18 column washed with a solvent of MeOH−H_2O (6:5, v/v), yielding 25 mg of anatolioside and 201 mg of lipedoside B-I. Similarly, fraction A3 was loaded onto a Si60 column eluted with $CHCl_3$−MeOH (10:1, v/v), followed by purification on a C-18 column washed with a mixture of MeOH−H_2O (6:5, v/v), leading to 95.4 mg of lipedoside B-III, 103.6 mg of lipedoside B-IV, and 180.1 mg of lipedoside B-V. Each purified phenylethanoid or monoterpene glycoside was subjected to verification of their chemical structures (**Figure 2**) on the basis of their UV, LC-MS, FAB-MS, ^{1}H NMR, and ^{13}C NMR spectral data, according to He et al. (*5*).

HPLC Analysis of Phenylethanoid Glycosides. Lipedoside A-I, lipedoside A-II, lipedoside B-I, lipedoside B-III, lipedoside B-V, lipedoside B-VI, osmanthuside B, and anatolioside were analyzed using a Shimadzu LC-10AD HPLC (Tokyo, Japan), equipped with a ternary pump delivery system. In brief, 10 μL of total glycosides extracts (1 mg/mL in methanol) was injected onto a C-18 column (Hypersil ODS, 250 × 4.6 mm, 5 μm, Alltech, Deerfield, IL), maintained in a cold water bath at 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%. The separated glycosides were monitored using a UV detector at 227 nm (Shimadzu SPD-10AV, Tokyo, Japan). A typical HPLC chromatogram for a mixture of phenylethanoid and monoterpene glycosides derived from LP is shown in **Figure 3**.

Preparation of Low-Density Lipoprotein (LDL). Human LDL was isolated as described previously (*6*). In brief, 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. 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. 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 was removed, the density of the remaining plasma fractions was increased to 1.064, and the plasma was recentrifuged at 160000*g* for an additional 24 h. The top LDL fraction was collected, flushed with nitrogen, and stored at −70 °C. The protein content of isolated LDL was determined according to Lowry et al. (*7*).

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. The dialysis solution was changed four times. Oxidation of LDL was conducted as previously described by Puhl et al. (*8*). 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 addition of 25 μL of 1.0% EDTA, and the mixture was cooled at 4 °C. The degree of LDL oxidation was monitored by measuring the production of thiobarbituric acid-reactive substances (TBARS) as previously described (*9*). After the reaction was stopped by 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

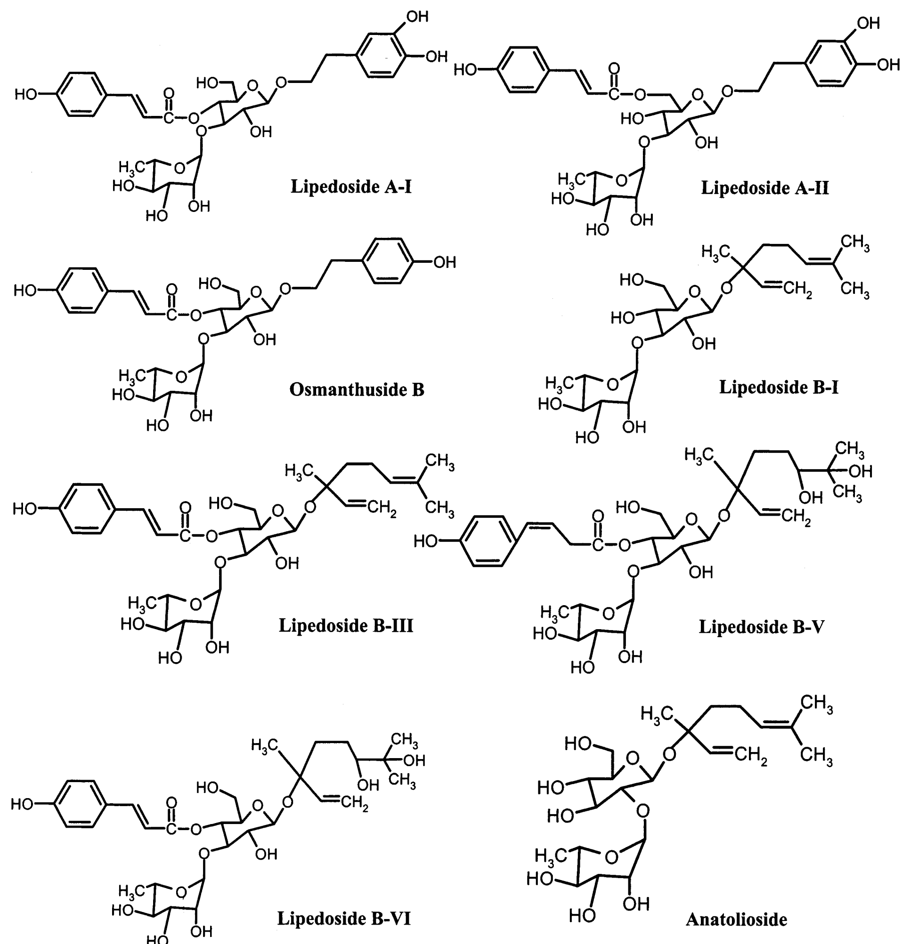

Figure 2. Chemical structures of phenylethanoid and monoterpene glycosides present in a bitter tea beverage derived from the plant *L. pedunculare.*

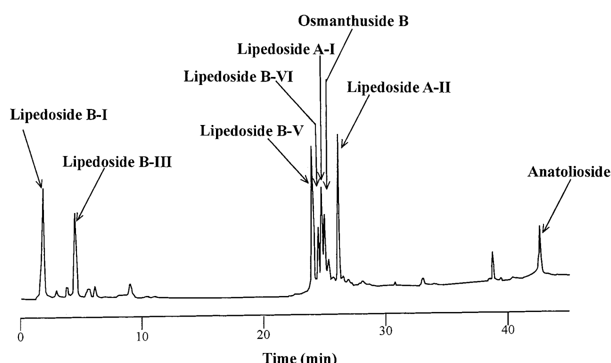

Figure 3. HPLC profile of phenyethanoid and monoterpene glycosides isolated from *L. pedunculare.* See text for the conditions and percent composition in the leaves of *L. pedunculare.*

radicals. The value was expressed as nanomoles of MDA per milligram of LDL protein.

Free Radical Scavenging Assay. Anti-free radical activities of phenylethanoid and monoterpene glycosides from LP were also examined as previously described by Blois (*10*). α-Tocopherol was used as a reference antioxidant. In brief, 0.5 mL of methanol containing 60 μM of each tested glycoside was mixed in a test tube with 2.5 mL of methanol containing 75 μM 2,2-diphenyl-1-picrylhydrazyl (DPPH), which is a stable free radical and has a typical absorbance at 517 nm. The reaction mixture was maintained in the dark at room temperature for 90 min, and the absorbance at 517 nm was then recorded. The free radical scavenging activity was calculated by using the following equation:

$$\text{scavenging activity (\%)} = [A_a - (A_b - A_c)]/A_a \times 100$$

where A_a is the absorbance of the incubation DPPH solution without addition of the tested glycosides, A_b is the absorbance of the incubation mixture containing both the tested glycoside and DPPH, and A_c is the absorbance of the blank solution without DPPH.

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

RESULTS

The antioxidant activity of the water extract, which contained most of the phenylethanoid and monoterpene glycosides, was first tested in LDL oxidation. It was found that this fraction strongly protected human LDL from oxidation. The HPLC analysis found that lipedoside B-I (6.1 g in 100 g of dry leaves) was most abundant, followed by lipedoside A-I (1.9 g), lipedoside B-III (1.4 g), osmanthuside B (1.3 g), lipedoside B-V (0.8 g), lipedoside B-VI (1.0 g), anatolioside (0.2 g), and lipedoside A-II (0.1 g).

The eight purified phenylethanoid or monoterpene glycosides demonstrated varying antioxidant activity (**Figure 4**). Lipedoside B-I, lipedoside B-III, anatolioside, and osmanthuside B showed no or little antioxidant activity, whereas lipedoside A-I, lipedoside A-II, lipedoside B-V, and lipedoside B−VI protected human LDL at a level comparable to that of acetoside, which was the major antioxidant in another bitter tea species, called *L. purpurascens* (**Figure 4**). Lipedoside A-I, lipedoside A-II,

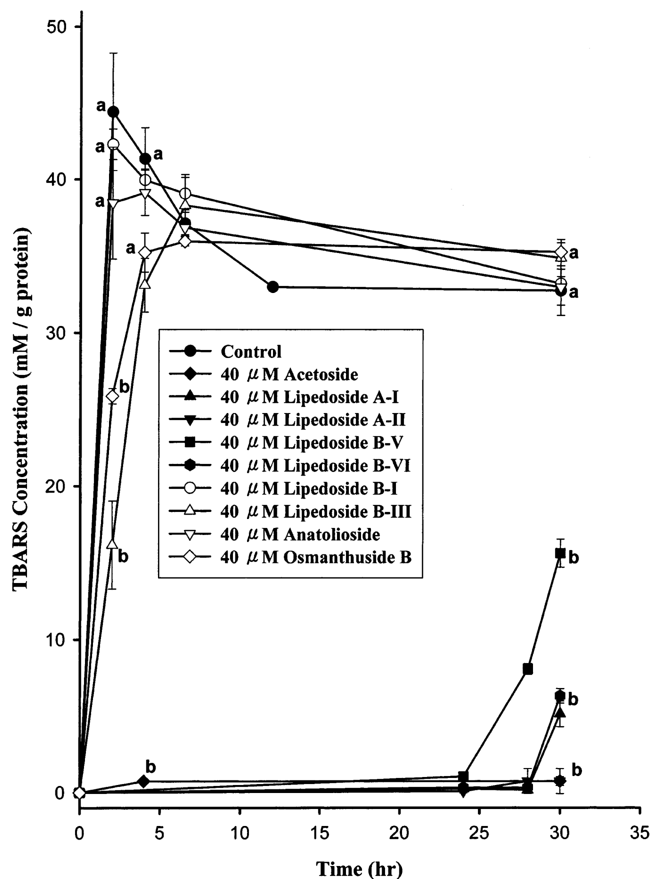

Figure 4. Inhibitory effect of eight phenylethanoid or monoterpene glycosides (40 μM) isolated from *L. pedunculare* on production of thiobarbituric acid-reactive substances (TBARS) in Cu^{2+}-mediated oxidation of human low-density lipoprotein (LDL). The LDL (150 μg of protein/mL) was incubated in sodium 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. Acetoside isolated from the species *L. purpurascens* was used as a reference antioxidant. Means at the same time point with different letters (a, b, c) differ significantly at $p < 0.05$.

lipedoside B-V, and lipedoside B-VI demonstrated a dose-dependent antioxidant activity in Cu^{2+}-mediated LDL oxidation (**Figure 5**). In most cases, LDL was oxidized significantly within 2 h in the absence of any antioxidant. Addition of 10−20 μM lipedoside A-I, lipedoside A-II, lipedoside B-V, or lipedoside B-VI extended the lag time to more than 8 h. These phenylethanoid and monoterpene glycosides, at a concentration of 40 μM, completely protected LDL from oxidation for 24 h.

The free radical scavenging effect of these phenylethanoid or monoterpene glycosides was also examined (**Figure 6**). The result was consistent with that of LDL oxidation. Lipedoside B-I, lipedoside B-III, anatolioside, and osmanthuside B showed no or little DPPH scavenging activity, but lipedoside A-I, lipedoside A-II, lipedoside B-V, and lipedoside B-VI demonstrated free radical scavenging potency comparable to that of α-tocopherol under the same experimental conditions (**Figure 6**).

DISCUSSION

There are very limited data on the health benefits of drinking bitter tea. Bitter tea has been claimed to be a stimulant to the central nervous system, a diuretic, a sore throat reliever, and an anti-hypertensive (*11*, *12*). However, there were no solid scientific data to substantiate these biological and pharmaceutical activities. None of these claims specified the species. Unlike the green tea derived from *Camellia sinensis*, bitter tea is a general name referring to 10 species in 5 families, and its chemical composition varies with species (*3*). The present study is the first report of examining the antioxidant activity of bitter tea derived from LP. Previous studies demonstrated that oxidation of human LDL is one of the risk factors in development of atherosclerosis (*12*−*18*) and that dietary antioxidants lower the incidence of coronary heart diseases (*19*−*24*). The present results demonstrate clearly that the bitter tea derived from LP contained at least four antioxidants, lipedoside A-I, lipedoside A-II, lipedoside B-V, and lipedoside B-VI, which were effective in protecting human LDL from oxidation.

The four antioxidants present in LP possessed free radical scavenging activity comparable to that of α-tocopherol (**Figure 6**), which is the primary antioxidant known to protect human LDL from oxidative modification (*19*). The antioxidant activity of lipedoside A-I, lipedoside A-II, lipedoside B-V, and lipedoside B-VI is also speculated to be as potent as that of tea catechins. As shown in **Figure 4**, these four phenylethanoids and monoterpenes showed anti-LDL oxidation activities similar to that of acetoside, which was purified from the species *L. purpurascens*. Our previous study showed that acetoside could protect human LDL from oxidation at a level comparable to that of (−)-epicatechin gallate (ECG), one of the strongest antioxidants in green tea (*4*). In this regard, the bitter tea derived from LP possessed at least the same antioxidant potency as catechins present in the green tea on the basis of the same concentrations.

The biochemical mechanisms by which lipedoside A-I, lipedoside A-II, lipedoside B-V, and lipedoside B-VI act as antioxidants may be related to their function as chelators to inactivate Cu^{2+}, used in initiation of free radicals (*12*), or as primary antioxidants by directly reducing the formation of free radicals mediated by Cu^{2+}. To test the second possibility, a DPPH-induced oxidation assay was conducted, proving that these phenylethanoid and monoterpene glycosides had a direct free radical-scavenging activity. It was also noticed that the antioxidant activity of lipedoside A-I, lipedoside A-II, lipedoside B-V, and lipedoside B-VI was related to their number of hydroxyl groups, i.e., their electron- or hydrogen-donating potency. In fact, lipedoside B-I and lipedoside B-III showed no or little protection to human LDL because they had two separate phenol moieties. In contrast, lipedoside A-I and lipedoside A-II inhibited strongly oxidation of human LDL because they were characterized by having two catechol moieties. The structures of lipedoside B-V [3-(6,7-dihydroxy-3,7-dimethyloct-1-enyl)-(3-α-L-rhamnopyranosyl)-(4-*O-cis-p*-coumaroyl)-β-D-glucopyranoside] and lipedoside B-VI [3-(6,7-dihydroxy-3,7-dimethyloct-1-enyl)-(3-α-L-rhamnopyranosyl)-(4-*O-trans-p*-coumaroyl)-β-D-glucopyranoside] are very similar, having two hydroxy groups at positions 6 and 7. Thus, they are more vulnerable to loss of a proton. The antioxidant activity observed for lipedoside A-I, lipedoside A-II, lipedoside B-V, and lipedoside B-VI is most likely attributable to their proton-donating capacity.

Many studies have addressed the health benefits of drinking green tea because it contains four major catechin antioxidants, (−)-epicatechin (EC), (−)-epigallocatechin (EGC), (−)-epicatechin gallate (ECG), and (−)-epigallocatechin gallate (EGCG). Research on the chemistry and potential health benefits of bitter tea is in its primary stage. The species *L. purpuracens*

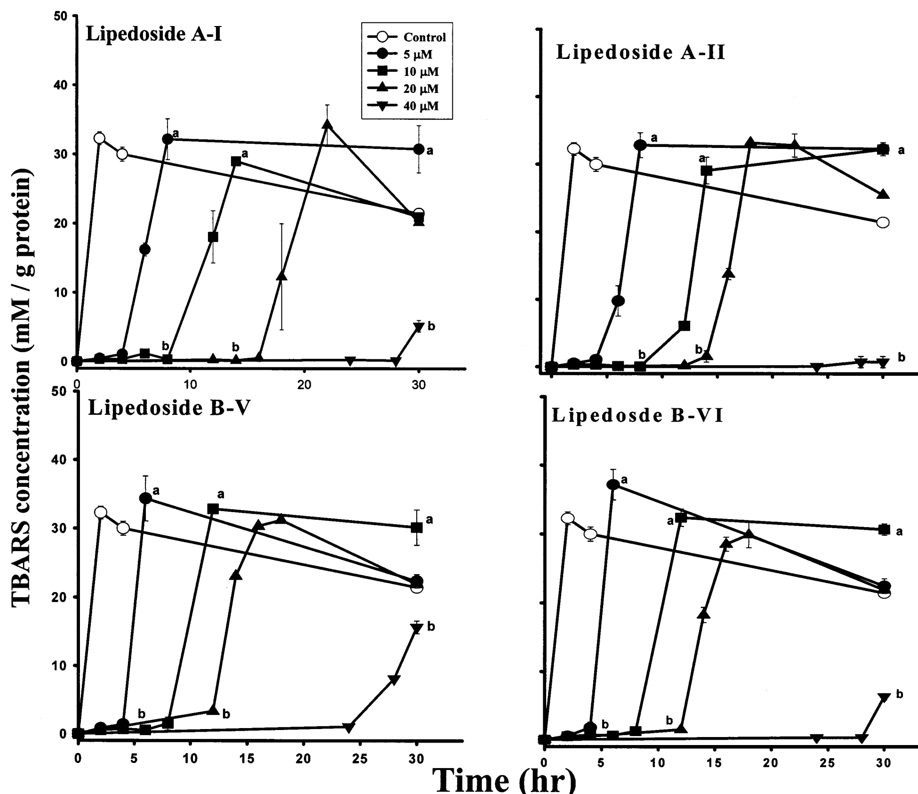

Figure 5. Dose-dependent inhibitory effect of lipedoside A-I, lipedoside A-II, lipedoside B-V, and lipedoside B-VI on production of thiobarbituric acid-reactive substances (TBARS) in Cu^{2+}-mediated oxidation of human low-density lipoprotein (LDL). The LDL (150 μg of protein/mL) was incubated in sodium 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 (a, b) differ significantly at $p < 0.05$.

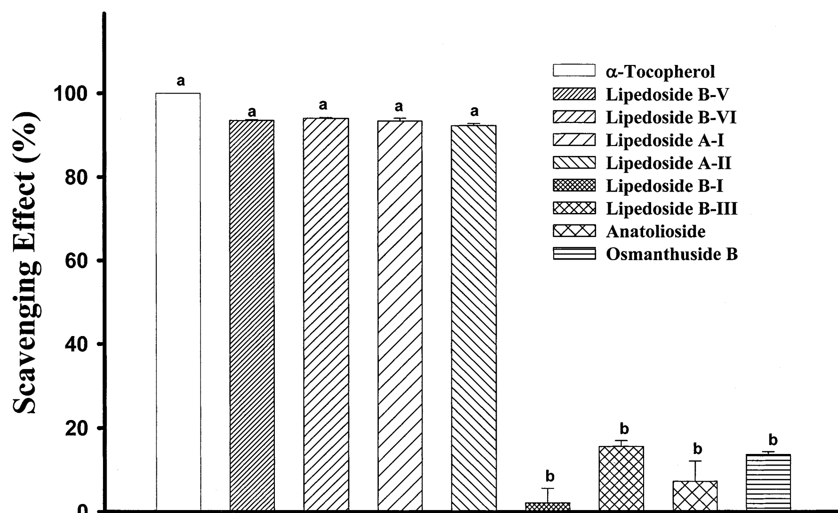

Figure 6. Free radical scavenging effects of eight phenylethanoid or monoterpene glycosides (10 μM) isolated from *L. peduncular.* 2,2-Diphenyl-1-picrylhydrazyl DPPH was used as a stable free radical. α-Tocopherol was used as a reference antioxidant. Means with different letters (a, b) differ significantly at $p < 0.05$.

has been previously shown to contain two antioxidants, acteoside and ligupurpuroside A. The present study is the first report on the antioxidants present in the species *L. pedunculare*. The potency of the bitter tea antioxidants is comparable to that of α-tocopherol and green tea catechins. We are currently investigating the absorption, metabolism, and excretion of these phenyethanoid and monoterpene glycosides and exploring other possible health benefits, including anticarcinogenic, cholesterol-lowering, and cardiovascular relaxing activities, associated with drinking bitter tea.

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