Identification and Antioxidant Activity of Novel Chlorogenic Acid Derivatives from Bamboo (*Phyllostachys edulis*)

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One known and two novel antioxidant compounds have been isolated from bamboo (*Phyllostachys edulis*). The butanol-soluble extract of the bamboo leaves was found to have a significant antioxidant activity, as measured by scavenging the stable 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical and the superoxide anion radical (O_2^-) in the xanthine/xanthine oxidase assay system. Antioxidant activity-directed fractionation of the extract led to the isolation and characterization of three structural isomeric chlorogenic acid derivatives: 3-*O*-(3′-methylcaffeoyl)quinic acid (**1**), 5-*O*-caffeoyl-4-methylquinic acid (**2**), and 3-*O*-caffeoyl-1-methylquinic acid (**3**). Compounds **2** and **3** were isolated and characterized for the first time from the natural products. In the DPPH scavenging assay as well as in the iron-induced rat microsomal lipid peroxidation system, compounds **2** (IC_{50} = 8.8 and 19.2 μM) and **3** (IC_{50} = 6.9 and 14.6 μM) showed ∼2−4 times higher antioxidant activity than did chlorogenic acid (IC_{50} = 12.3 and 28.3 μM) and other related hydroxycinnamates such as caffeic acid (IC_{50} =13.7 and 25.5 μM) and ferulic acid (IC_{50} = 36.5 and 56.9 μM). Among the three compounds, compound **1** yielded the weakest antioxidant activity, and the DPPH scavenging and lipid peroxidation inhibitory activity (IC_{50} = 16.0 and 29.8 μM) was lower than those of chlorogenic and caffeic acids. All three compounds exhibited both superoxide scavenging activities and inhibitory effects on xanthine oxidase. Their superoxide anion (O_2^-) scavenging activities (IC_{50} = **1**, 4.3 μM; **2**, 2.8 μM; and **3**, 1.2 μM) were markedly stronger than those of ascorbic acid (IC_{50} = 56.0 μM), α-tocopherol (IC_{50} > 100 μM), and other test compounds, although their inhibition effects on xanthine oxidase may contribute to the potent scavenging activity. α-Tocopherol exerted a significant inhibitory effect (65.5% of the control) on superoxide generation in 12-*O*-tetradecanoylphorbol-13-acetate-induced human promyelocytic leukemia HL-60 cells, and compound **3** showed moderate activity (36.0%). On the other hand, other compounds including **1**, **2**, chlorogenic acid, and other antioxidants were weakly active (24.8−10.1%) in the suppression of superoxide generation.

Keywords: *Chlorogenic acid derivatives; free radical scavenging; superoxide; xanthine oxidase; Phyllostachys edulis; antioxidant activity*

INTRODUCTION

There are ∼280 species of bamboo within 10 genera, among which *Phyllostachys* and *Sasa albo* are well-known as edible shoots in Asia. The leaves of bamboo have been used in Asian countries as a food wrapping material to prevent food deterioration since ancient times. The leaves have been also utilized clinically in the treatment of hypertension, arteriosclerosis, cardiovascular disease, and certain forms of cancer (*1*). There is now increasing evidence to suggest that many age-related human diseases such as heart disease, cancer, inflammation, arthritis, immune system impairment, and brain dysfunction are the result of cellular damage by free radicals, and antioxidants could play an important role in preventing such diseases (*2*−*4*). Several cancer chemopreventive agents exhibit antioxidant activity through their ability to scavenge oxygen radicals, including singlet oxygen, peroxyl radicals, superoxide, and hydroxyl radicals (*5*, *6*). Studies on the free radical scavenging properties of flavonoids have been permitted characterization of the major phenolic components of naturally occurring phytochemicals as antioxidant. Furthermore, development of plants as sources of antioxidants that can be used to enhance the properties of foods and cosmetics, for both preservation and functional purposes, is currently of major interest.

The traditionally known medicinal effects of bamboo leaves in the treatment of certain diseases are most likely mediated by their antioxidant capacity. In addition, the prolongation effect of food shelf life may be due to the potential antioxidant and antimicrobial components present in bamboo leaves. The presence of antimicrobial benzoic acid and 2,6-dimethoxybenzoquinone has been reported in the bamboo leaves of *Sasa albo-marginata* and *Phyllostachys heterocycla* (*7*, *8*). The polysaccharides isolated from the leaves of bamboo *Sasa albo-marginata* have also been studied as medicinal materials for anti-inflammatory and antitumor activities (*9*). However, there have been no detailed phytochemical studies carried out to date for the antioxidants in bamboo leaves.

For the past decade, we have made efforts to isolate and identify natural flavonoids and polysaccharides with an anticomplementary and antioxidant activity from the medicinal plants of Korea (*10*−*12*). Recently, we observed that the 70% ethanol extracts from the leaves of the bamboo *Phyllostachys edulis*, which grows mainly in southern coastal areas of Korea, showed potent antioxidant capacity based on scavenging activity

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of the stable 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical and superoxide anion radical generated by the xanthine/xanthine oxidase reaction system. We report here the structural characterization and antioxidant potentials of three chlorogenic acid derivatives from bamboo leaves.

Chlorogenic acid is an ester of caffeic acid with quinic acid, which is found naturally in various plants such as coffee beans, apples, and blueberries. Chlorogenic acid and caffeic acid have been shown to inhibit mutagenicity of bay region diol epoxides of polycyclic aromatic hydrocarbons, retinoic acid 5,6-epoxidation, hydroxyl radical formation, and lipid peroxidation (*13*−*15*). However, little is known about the antioxidant effect of the chlorogenic acid derivatives **1**−**3**.

MATERIALS AND METHODS

Plant Material. The leaves of *P. edulis* Gramineae were collected on Goje Island, Korea, in August 1998 under the auspices of the National Agricultural Cooperative Federation. The harvested plant material was lyophilized and milled just prior to the present investigation.

Extraction and Isolation. The lyophilized and milled leaves (500 g) were refluxed in 70% ethanol at 80 ± 2 °C and, then, concentrated and partitioned sequentially to give *n*-hexane (3.4 g), chloroform (9.0 g), ethyl acetate (1.6 g), *n*-butanol (9.3 g), and aqueous ethanolic extracts. Among the solvent fractions, the *n*-butanol extract, which was the highest in yield and quite water-soluble, exhibited the most significant activity in the DPPH and superoxide savenging assays (IC_{50} = 75.8 and 56.0 μg/mL, respectively) as well as for the inhibition of lipid peroxidation. The *n*-butanol-soluble extracts (9.0 g), dissolved in 50 mL of water (pH 10), were adsorbed onto a Diaion HP-20 open column (Pharmacia Fine Chemicals, Piscataway, NJ; 28 × 4 cm i.d.). The unbound fraction from the column was adjusted to pH 3 and separated on an additional Diaion HP-20 column (25 × 2.5 cm i.d.) chromatograph using a stepwise gradient consisting of methanol and water. The active antioxidant material (2.2 g) eluted by 30% methanol was applied to a silica gel 60 G column (12 × 5 cm i.d.) and separated by open column chromatography using a gradient consisting of 1−25% methanol in chloroform. The active fractions were concentrated, dissolved in 1 mL of methanol, and further chromatograped on a Sephadex LH 20 column (Sigma, St. Louis, MO; 95 × 2 cm i.d.) with 95% methanol as the eluent (4 mL fractions, 0.2 mL/min). The fractions (48−60) with antioxidant activity were pooled and concentrated. Rechromatography on a reverse ODS column (8.5 × 1.5 cm i.d.) of the Sephadex LH-20 active material (257 mg) using a 30−70% methanol in water gradient (2 mL fractions, 0.1 mL/min) afforded nine subfractions (fractions 10−18), which were active in the DPPH and superoxide scavenging assays. From the subfractions four antioxidant fractions (A−D) were collected and monitored by preparative silica and ODS TLC using butanol/methanol/water (4:1:2, v/v) and 30% methanol in water as developing solvents, respectively. Fraction A (46 mg) was purified by preparative reversed phase HPLC (C_{18}, 30 × 7.8 mm i.d.) using CH_3CN/water containing 1% trifluoroacetic acid (TFA) (10:90) as the mobile phase at a flow rate of 1.5 mL/min to yield active compound **1** (7.0 mg, t_R =16 min). Fraction B (38 mg) was purified by the same HPLC using CH_3CN/water containing 1% TFA (15:85) as the mobile phase at a flow rate of 1.5 mL/min to yield active compound **2** (5.2 mg, t_R = 20 min). Compound **3** (8.5 mg, t_R = 28 min) was obtained from the fractions C (28 mg) and D (33 mg) on the same preparative HPLC using CH_3CN/water containing 1% TFA (17:83) as the mobile phase. These three purified compounds were subjected to structural analysis.

Instrumentation. ^1H NMR and ^{13}C NMR spectra were measured on a Bruker model AMX-500 (Silberstreifen, Germany) instrument at 500 and 125 MHz, respectively. Compounds were analyzed in CD_3OD with tetramethylsilane (TMS) as an internal standard. $^1H-^{13}C$ HMQC and $^1H-^{13}C$ HMBC experiments were performed with the same NMR instrument at 499.9 MHz. Electrospray ionization (ESI) mass spectra were obtained using a Micromass Quattro II (Altrincham, U.K.) mass spectrometer. An electrospray energy of −3.5 kV and sample infusion solvent of 50% aqueous acetonitrile were employed. Low- and high-resolution FABMS (HR-FABMS) were measured on a JMS-700 Mstation mass spectrometer (JEOL Ltd.). The CI reagent gas used was methane, and glycerol was used as the FAB matrix. IR spectra were taken on a Jasco FTIR-430 (Easton, MD) spectrometer with KBr disks, and UV spectra were measured on a Shimadzu model UV-2401 spectrometer. $[\alpha]_D$ was measured on an AUTOPOL III polarimeter (Rudolph Research), and thermal analysis for melting points was performed on a DSC 2010 (TA Instruments, Inc., Leatherhead, U.K.). HPLC was performed with a Waters model 2690-allience series (Milford, MA) equipped with a Waters 996 photodiode array (PDA) detector and a Waters SymmertryPrep C_{18} (7 μm, 300 × 7.8 mm i.d.) or a Waters Symmertry C_{18} (5 μm, 150 × 2.1 mm i.d.) column.

ESR Measurements. Electron spin resonance (ESR) spectra were obtained using a Bruker model ESP-300s ESR (Silberstreifen, Germany) spectrometer at room temperature (25 °C). Operation conditions of the ESR spectrometer were as follows: microwave power, 10 mW; sweep time, 41.9 s; time constant, 5.12 s; modulation amplitude, 1.04 G; and microwave frequency, 9.78 GHz. The control ESR spectrum was obtained using 200 μM DPPH in ethanol solution. Each reaction mixture contained in a total volume of 5 mL, 200 μM DPPH plus 0.1 mM α-tocopherol, plus 0.1 mM compound **1**, plus 0.1 mM compound **2**, plus 0.1 mM compound **3**, respectively. The reactions were performed at 25 °C under air for 10 min, and then the intensity of the ESR signal of the reaction mixtures (0.5 mL) was measured in a glass tube centered in the microwave cavity.

General Conditions. Thin-layer chromatography (TLC) was performed on Kieselgel 60 F 254 plates (20 × 20 cm 0.25 mm; Merck, Darmstadt, Germany) or reversed phase C_{18} silica gel UV 254 plates (20 × 10 cm, 0.25 mm; Merck) with compounds visualized under UV light. Silica gel 60 G (200 mesh, Merck), Sephadex LH-20 (25−100 μm, Pharmacia Fine Chemicals), Diaion HP-20 (Supelco, Bellefonte, PA), C_{18} Sep-Pak cartridge (Waters, Milford, MA), and ODS-silica gel (Pharmacia Fine Chemicals) were used for column chromatography.

DPPH Scavenging Assay. Assay for DPPH free radical scavenging potential is based on the scavenging activity of stable DPPH free radicals (*16*). Reaction mixtures containing test samples dissolved in methanol and 200 μM DPPH (Sigma) in ethanolic solution in a 96-well microtiter plate were incubated at 37 °C for 30 min. After the reaction, absorbance was then measured at 520 nm, and percent inhibition was calculated. IC_{50} values denote the concentration of sample required to scavenge 50% of the DPPH free radicals.

Lipid Peroxidation Inhibition Assay. Five 8-month-old rats (Sprague Dawley, Korea) were killed by decapitation, and the livers were excised, rapidly washed, and homogenized in 8 volumes (v/w) of 5 mM Tris-HCl buffer (pH 7.4) containing 0.25 M sucrose and 0.1 mM EDTA. Subcelluar fractionation was carried out using differential centrifugation, and the pelleted microsomes were diluted with the same buffer, frozen in liquid nitrogen, and stored at −70 °C until use. Reaction mixtures contained 10 μL of the rat microsomes, 500 μL of 0.1 mM Tris-HCl buffer (pH 7.4) containing 12.5 μM $FeSO_4 \cdot 7H_2O$, and 40 μL of 2 mM ascorbic acid. Test samples (20 μL) in 5% dimethyl sulfoxide (DMSO) or 1.5% Tween 80 were added to the reaction mixture and incubated at 37 °C for 30 min followed by centrifugation. After the addition of 0.3 mL of thiobarbituric acid (Sigma) to the supernatant, the tubes were placed in a boiling water bath for 5 min. Absorbance was then measured at 530 nm, and the percent inhibition of lipid peroxidation by sample was calculated. IC_{50} values denote the concentration of sample required to inhibit 50% of microsomal lipid peroxidation.

Superoxide Anion Radical Scavenging Activity. The superoxide anion radical scavenging activity was performed using the method of Okamura et al. (*17*) with some modification. This assay is based on the removal rate of xanthine/xanthine oxidase-generated superoxide by measuring the reduction of nitro blue tetrazolium (NBT). The sample solution (0.1 mg/mL) in 5% DMSO or 1.5% Tween 80 was added to 1 mL of a mixture of 0.1 mM xanthine and 0.2 mM NBT (Sigma) in 50 mM potassium phosphate buffer (pH 7.5) containing 0.05 mM EDTA. Xanthine oxidase (0.1 mL) (Sigma; 0.8 unit/mL) diluted in 50 mM phosphate buffer (pH 7.5) was added, and the resulting mixture was incubated at 37 °C for 20 min. Addition of 2 mL of 2.5 N HCl to the mixtures terminated the reaction, followed by increase of coloration of NBT, which was measured at 540 mM. The percent of removal rate by sample was calculated relative to the control. IC_{50} values were calculated from the concentration of sample required to reduce 50% of the NBT.

Xanthine Oxidase Inhibition Assay. The xanthine oxidase activity with xanthine as the substrate was measured according to the method of Noro et al. (*33*) with some modification. The assay mixture consisted of 0.1 mL of test sample solution, 0.3 mL of 50 mM phosphate buffer (pH 7.5), and 0.1 mL of xanthine oxidase (Sigma; 0.5 unit/mL) solution. After preincubation of the mixture at 25 °C for 15 min, the reaction was initiated by adding 0.2 mL of 0.15 mM xanthine solution and then incubated at 25 °C for 30 min. Adding 0.3 mL of 1 N HCl stopped the reaction, and the absorbance of the assay mixture at 290 nm was measured spectrophotometrically. A blank was prepared in the same way, but the enzyme solution was added to the assay mixture after the addition of 1 N HCl. One unit of xanthine oxidase was defined as the amount of enzyme producing 1 μM uric acid per minute at 25 °C. Xanthine oxidase inhibitory activity was expressed as the percentage inhibition of XO in the above assay system, calculated as $(1 - B/A) \times 100$, where A is the activity of the enzyme without test material and B is the activity of the enzyme with test material. The IC_{50} value, 50% inhibitory concentration of test compound, was calculated by linear regression analysis.

Assay for Inhibition of TPA-Induced Superoxide Generation in HL-60 Cells. The test for inhibition of TPA-induced superoxide generation was carried out according to the method of Miller et al. (*18*). Human promyelocytic leukemia HL-60 cells (5×10^5 cells/mL) were inoculated in RPMI 1640 (Gibco RBL, Grand Island, NY) supplemented with 10% FBS (Gibco RBL). The cells were preincubated with 1.25% DMSO at 37 °C in 5% CO_2 incubation for 4 days to differentiate into granulocyte-like cells. The cells were then washed with phosphate-buffered saline (PBS, pH 7.4), and suspended at a density of 1×10^6 cells/mL. The test compound dissolved in 5 μL of DMSO was added to the cell suspension, and the mixture was washed twice with PBS to remove extracellular test compounds. Free radical formation was induced by an addition of 8 μM TPA (Nakarai Tesque) followed by the addition of 100 μM of the test agents with cytochrome *c* (160 μM). After incubation for 1 h at 37 °C, cytochrome *c* reduction was measured at 515 nm using an ELISA reader (Bio-Rad model 3550). The percent inhibition of radical formation with TPA by the test compounds was calculated by subtracting the value obtained by TPA alone. Blank reaction mixtures were prepared in which cell suspensions were omitted, and these values were subtracted from the treatment groups.

RESULTS

Isolation and Identification of Active Compounds. A preliminary free radical scavenging assay revealed that the scavenging percentages of DPPH radical with hot water, 70% ethanol, and methanol extracts of bamboo leaves were 45.5, 75.6, and 68.2%, respectively (data not shown). The lyophilized and milled leaves of *P. edulis* were prepared by extraction with 70% ethanol in water at 80 ± 2 °C and partitioned between hexane and water. The more polar layer was then partitioned with chloroform, ethyl acetate, and *n*-butanol. The dried subfractions were subjected to comparison of antioxidant activities including free radical scavenging and suppression of lipid peroxidation. As shown in Figure 1, DPPH radical scavenging activity of the fractions tested, at a concentration of 100 μg/mL, was in the order *n*-butanol subfraction (79.1%) > 70% ethanol extract (70.0%) > ethyl acetate subfraction (56.4%) > final aqueous layer (41.0%) > hexane subfraction (37.3%) > chloroform subfraction (30.4%). The percent of superoxide scavenging of hexane, chloroform, ethyl acetate, *n*-butanol, and final aqueous subfractions was 27.8, 41.5, 50.0, 62.3, and 30.6%, respectively. Similarly, the *n*-butanol subfraction was the most effective in inhibiting rat microsomal lipid peroxidation, suppressing it by 77.3% of control. The most effective *n*-butanol extract was subjected to chromatographic separation to obtain compounds **1**−**3**, based on antioxidant activities. Structural analysis of the three compounds was performed using spectroscopic methods.

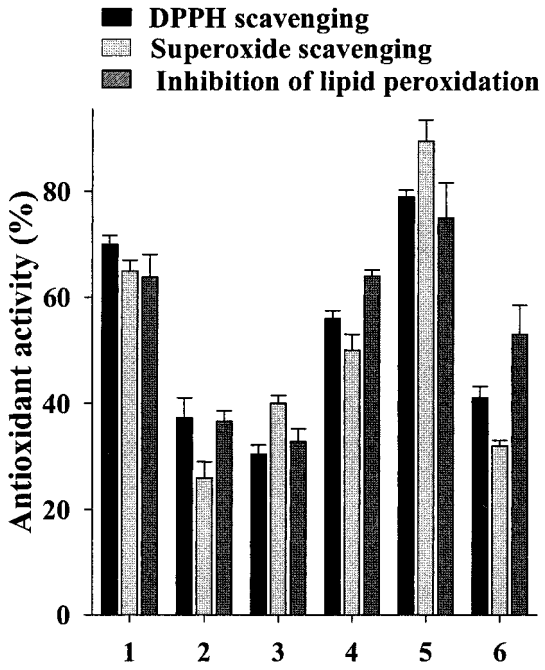

Figure 1. Antioxidant activity of organic solvent-partitioned subfractions of 70% ethanolic extracts from *P. edulis*. Experiments were carried out at 100 μg/mL concentrations of samples as described under Materials and Methods. Error bars represent standard deviations of the mean (n = 3). Columns 1 represent antioxidant activity of the 70% ethanolic extract; 2, of the hexane subfraction in the organic solvent partition; 3, of the chloroform subfraction; 4, of the ethyl acetate subfraction; 5, of the *n*-butanol subfraction; 6, of the final aqueous layer. The *n*-butanol subfraction (columns 5) comprises compounds **1**−**3**.

Compound ***1****:* pale yellow powder (7.0 mg); $[\alpha]^{25}_D$ −39.2 (ethanol, *c* 0.3); mp 195−197 °C; UV λ_{max} in MeOH (nm) 244sh, 299, 328; +NaOH 266sh, 310, 374; +$AlCl_3$ 246sh, 300, 329; +$AlCl_3$ + HCl 245sh, 299, 328; +NaOAc 369; +NaOAc + H_3BO_3 249sh, 305, 349; IR (KBr) ν_{max} 3420, 3250, 1730, 1679, 1523, 1442 cm^{-1}; TLC positive reaction with $FeCl_3$ and bromocresol green; ESI-MS, *m/z* 369 $[M + H]^+$; FABMS *m/z* 369 $[M + H]^+$, *m/z* 391 $[M + Na]^+$; HR-FABMS (glycerol matrix), *m/z* 369.1185 $[C_{17}H_{20}O_9 + H]^+$; ^1H NMR (CD_3OD, 500 MHz) δ 7.57 (1H, 15.9 Hz, H-7′), 7.04 (1H, d, 2.0 Hz, H-2′), 6.93 (1H, dd, 8.2 Hz, 2.0 Hz, H-6′), 6.76 (1H, d, 8.4 Hz,

H-5′), 6.28 (1H, d, 15.9 Hz, H-8′) 5.34−5.36 (1H, m, H-3), 4.09−4.13 (1H, m, H-5), 3.73 (OCH_3), 3.66 (1H, dd, 7.8 Hz, 3.3 Hz, H-4), 1.98−2.22 (4H, m, H-2, H-6); ^{13}C NMR (CD_3OD, 125 MHz) δ 176.1 (COO^-), 168.1 (C-9′), 149.2 (C-3′), 146.4 (C-7′), 146.2 (C-4′), 127.1 (C-6′), 123.7 (C-1′), 116.8 (C-5′), 115.1 (C-8′), 114.7 (C-2′), 75.8 (C-4), 73.8 (C-1), 72.6 (C-3), 67.3 (C-5), 53.6 (OCH_3), 39.8 (C-2), 36.1 (C-17).

Compound ***2****:* white powder (5.2 mg); $[\alpha]^{25}_D$ −47.2 (ethanol, *c* 0.2); mp 200−201 °C; UV λ_{max} in MeOH (nm) 244sh, 296, 328; +NaOH 261sh, 308, 372; +$AlCl_3$ 263sh, 306, 351; +$AlCl_3$ + HCl 244sh, 296, 328; +NaOAc 274, 334, 375; +NaOAc + H_3BO_3 252sh, 303, 348; IR (KBr) ν_{max} 3433, 2950, 1735, 1680, 1631, 1519, 1445 cm^{-1}; TLC positive reaction with $FeCl_3$ and bromocresol green; ESI-MS, *m/z* 369 $[M + H]^+$; FABMS *m/z* 369 $[M + H]^+$; *m/z* 391 $[M + Na]^+$; HR-FABMS (glycerol matrix), *m/z* 369.1185 $[C_{17}H_{20}O_9 + H]^+$; ^1H NMR (CD_3OD, 500 MHz) δ 7.61 (1H, 15.9 Hz, H-7′), 7.06 (1H, d, 2.0 Hz, H-2′), 6.95 (1H, dd, 8.2 Hz, 2.0 Hz, H-6′), 6.78 (1H, d, 8.2 Hz, H-5′), 6.35 (1H, d, 15.9 Hz, H-8′) 4.23−4.30 (3H, m, H-3, 4, 5), 3.75 (OCH_3), 1.99−2.21 (4H, m, H-2, H-6); ^{13}C NMR (CD_3OD, 125 MHz) δ 176.5 (COO^-), 168.9 (C-9′), 149.5 (C-3′), 146.9 (C-4′), 146.8 (C-7′), 127.9 (C-6′), 123.0 (C-1′), 116.5 (C-5′), 115.8 (C-8′), 115.1 (C-2′), 74.3 (C-4), 73.9 (C-1), 71.3 (C-3), 70.6 (C-5), 52.9 (OCH_3), 40.8 (C-2), 36.4 (C-17).

Compound ***3****:* white powder (8.5 mg); $[\alpha]^{25}_D$ −16.9 (water, *c* 0.35); mp 202−203 °C; UV λ_{max} in MeOH (nm) 245sh, 300, 329; +NaOH 266sh, 310, 374; +$AlCl_3$ 355; +$AlCl_3$ + HCl 245sh, 299, 328; +NaOAc 338, 377sh; +NaOAc + H_3BO_3 254, 306, 351; IR (KBr) ν_{max} 3423, 2957, 1734, 1686, 1631, 1522, 1443 cm^{-1}; TLC positive reaction with $FeCl_3$ and bromocresol green; ESI-MS, *m/z* 369 $[M + H]^+$; FABMS *m/z* 369 $[M + H]^+$; *m/z* 391 $[M + Na]^+$; HR-FABMS (glycerol matrix), *m/z* 369.1185 $[C_{17}H_{20}O_9 + H]^+$; ^1H NMR (CD_3OD, 500 MHz) δ 7.51 (1H, 15.9 Hz, H-7′), 7.04 (1H, d, 2.0 Hz, H-2′), 6.94 (1H, dd, 8.2 Hz, 2.0 Hz, H-6′), 6.77 (1H, d, 8.4 Hz, H-5′), 6.20 (1H, d, 15.9 Hz, H-8′) 5.26−5.30 (1H, m, H-3), 4.12−4.15 (1H, m, H-5), 3.71 (1H, dd, 7.5 Hz, 3.1 Hz, H-4), 3.70 (OCH_3), 1.93−2.23 (4H, m, H-2, H-6); ^{13}C NMR (CD_3OD, 125 MHz) δ 175.4 (COO^-), 168.3 (C-9′), 149.7 (C-3′), 147.2 (C-4′), 146.8 (C-7′), 127.7 (C-6′), 123.0 (C-1′), 116.5 (C-5′), 115.1 (C-2′, C-8′), 75.8 (C-4), 72.6 (C-1), 72.1 (C-3), 70.3 (C-5), 53.0 (OCH_3), 38.1 (C-6), 37.8 (C-2).

The three pure compounds exhibited characteristic phenolic color reactions, that is, purplish brown with $FeCl_3$ and dark brown with bromocresol green (*19*). IR absorptions of the compounds **1**−**3** showed the same pattern, suggesting the presence of hydroxyl (3420−3433 cm^{-1}) and ester carbonyl (1730−1735 cm^{-1}) groups. In addition, their UV absorption patterns [λ_{max} in MeOH (nm) 244−245sh, 296−300, 328−329] were nearly identical to each other and similar to those of caffeic, chlorogenic, and ferulic acids, indicating the presence of a $-CH{=}CH-CO_2H$ group linked to the phenyl ring (*20*). The FABMS and ESI-MS of the three phenolic acids showed the same molecular ions $[M + H]^+$ at *m/z* 369 and $[M + Na]^+$ ions at *m/z* 391, which were in accordance with a chlorogenic acid containing a methoxyl or hydroxyl group. Their isomeric structures, as shown in Figure 2, were determined by NMR and HR-FABMS in combination with NMR spectral data of an authentic chlorogenic acid and compared with the data reported for other phenolic acids.

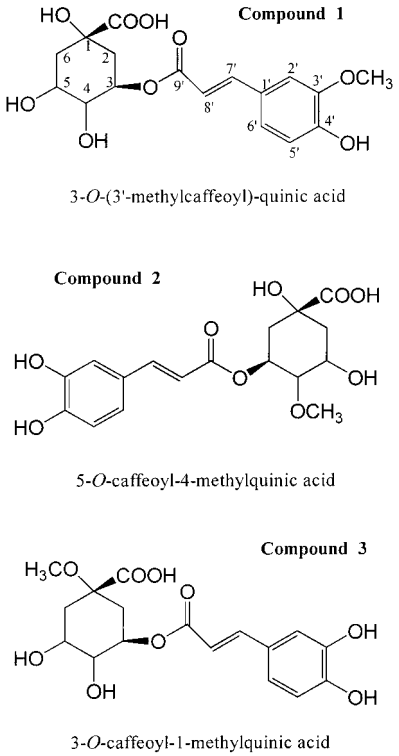

Figure 2. Chemical structures of antioxidative chlorogenic derivatives **1**−**3** isolated from *P. edulis*.

Compound **1** was obtained as a pale yellow amorphous powder. In the HR-FABMS, a peak corresponding to $[M + H]^+$ was observed at *m/z* 369.1185, showing the molecular formula of **1** to be $C_{17}H_{20}O_9$. The ^1H NMR spectrum showed 11 signals including one AMX spin system (δ_H 7.04, 6.76, and 6.93) in the aromatic region originating from a 1,3,4-trisubstitued benzene ring (*21*), one trans conjugated olefinic methine at δ_H 7.57 and 6.28 (J = 15.9 Hz), and a methoxyl proton signal at δ_H 3.73 (3H, s). The ^{13}C NMR spectrum exhibited 17 signals comprising carboxyl acid carbonyl (δ_C 176.1) and ester carbonyl (δ_C 168.1) carbons, one methoxyl (δ_C 53.6) carbon, and two hydroxylated aromatic carbons (δ_C 149.2 and 146.2), which were characterized by matching with an HMQC spectrum. The ^1H and ^{13}C NMR data were similar to those of the authentic sample of chlorogenic acid, except the presence of methoxyl signals, indicating that **1** was a chlorogenic methyl ether, which was in good agreement with the result of MS analysis. To establish connectivity of carbons through two or three bonded long-range couplings, HMBC experiments were carried out. The HMBC correlations clearly revealed the methoxyl group attached to the C-3′ carbon in the caffeoyl moiety of chlorogenic acid, thus confirming compound **1** as chlorogenic acid 3′-methyl ether; 3-*O*-(3′-methylcaffeoyl)quinic acid (see Figure 2). This is a known compound isolated from unroasted coffee bean (*40*). The identification of chlorogenic acid 3′-methyl ether by us agreed in part with the results of Corse et al. (*40*).

The molecular formula of compound **2**, $C_{17}H_{20}O_9$, was determined to be identical to that of compound **1** on the basis of HRMS. The ^1H and ^{13}C NMR spectra were similar to those of compound **1** and also showed a methoxyl proton and carbon signals at δ_H 3.75 (3H, s) and δ_H 52.9, respectively. The only difference between compounds **1** and **2** in ^1H NMR was that the multiple signals at δ_H 4.23−4.30 (3H), assigned to protons of H-3,

Table 1. Antioxidant Activity of Chlorogenic Acid Derivatives 1−3 from *P. edulis* and Related Phenolics and Antioxidants

compound	antioxidant activity (IC_{50}, μM)	
	DPPH[a]	MTBA[b]
1	16.0 ± 0.34	29.8 ± 0.07
2	8.8 ± 0.06	19.2 ± 0.22
3	6.9 ± 0.07	14.6 ± 0.37
chlorogenic acid	12.3 ±0.12	28.3 ± 0.64
caffeic acid	13.7 ± 0.10	25.5 ± 0.53
ferulic acid	36.5 ± 0.23	56.9 ± 1.48
ascorbic acid	49.5 ± 0.35	−[c]
α-tocopherol	40.6 ± 0.29	50.1 ± 0.26
(−)-epigallocatechin	3.7 ± 0.03	36.6 ± 0.45

[a] DPPH free radical scavenging activity. [b] Inhibition of iron-induced rat liver microsomal lipid peroxidation. [c] Not determined; each value is the mean ± SD (n = 3).

H-4, and H-5, were observed, and this indicated that the esterification between quinic and caffeic acids existed at the 4- or 5-position of compound **2**. Analysis of the HMQC and HMBC spectra allowed the complete assignments for the ^{1}H and ^{13}C NMR of compound **2**. In the HMBC experiments, key correlation peaks were observed between the H-5 proton (δ_H 4.09−4.13, m) in the quinic moiety and the C-9′ carbon (δ_C 168.9) of the caffeoyl moiety and between the methoxyl proton (δ_H 3.75) and the C-4 carbon of the quinic moiety. The HMBC data confirmed the location of the methoxyl group at C-4 and the esterification site of chlorogenic acid at C-5. Thus, the structure of compound **2** was identified as 5-*O*-caffeoyl-4-methylquinic acid (Figure 2).

Similarly, compound **3** obtained as a white amorphous powder and showed ^{1}H and ^{13}C NMR resonances comprising typical methoxyl signals (δ_H 3.70 and δ_C 53.0) almost identical to those of compounds **1** and **2**. The positions of methoxyl and ester carbonyl substitution were determined by HMBC. The methoxyl proton signal at δ_H 3.70 showed a strong cross-peak with the carbon of the carboxyl carbonyl group (δ_C 175.4) and weak cross-peaks with carbons at δ_C 168.3, 72.8, and 39.8, from which its presence at the 1-position was deduced. Another key correlation peak was observed between the H-3 multiplet signals and the C-9′ carbon at δ_C 168.3. In addition, compound **3** also showed a bathochromic shift upon the addition of $AlCl_3$, indicating 3′,4′-dihydroxyl substitution (*21*). Consequently, the methoxyl group of compound **3** was at the C-1, and the esterification is located between C-3 and C-9′. Therefore, we assigned compound **3** as 3-*O*-caffeoyl-1-methylquinic acid.

Antioxidant Activities of Chlorogenic Acid Derivatives 1—3. To evaluate the three chlorogenic acid derivatives **1**−**3** for antioxidant potential, their DPPH free radical scavenging and lipid peroxidation inhibitory activities were compared with those of chlorogenic acid as well as other structurally related hydroxycinnamates such as caffeic and ferulic acids and other selected standard antioxidants including epigallocatechin, ascorbic acid, and α-tocopherol (Table 1). In terms of DPPH scavenging activity, epigallocatechin was found to be the most potent antioxidant (IC_{50} = 3.7 μM), whereas ascorbic acid showed the lowest activity (IC_{50} = 49.5 μM). Compounds **2** and **3** were also highly active in the DPPH scavenging and were stronger than chlorogenic acid, caffeic acid, ferulic acid, ascorbic acid, and α-tocopherol. In the DPPH scavenging assay as well as in the iron-induced rat microsomal lipid peroxidation system, compounds **2** (IC_{50} = 8.8 and 19.2 μM) and **3** (IC_{50} = 6.9 and 14.6 μM) showed ∼2−4 times higher antioxidant activity than did chlorogenic acid (IC_{50} = 12.3 and 28.3 μM), caffeic acid (IC_{50} = 13.7 and 25.5 μM), ferulic acid (IC_{50} = 36.5 and 56.9 μM), and α-tocopherol (IC_{50} = 40.6 and 50.1 μM). Among the three compounds, compound **1** was the weakest in antioxidant activity, and the DPPH scavenging and lipid peroxidation inhibitory activities (IC_{50} = 16.0 and 29.8 μM) were a little lower than those of chlorogenic and caffeic acids (Table 1). In this experiment, epigallocatechin present in tea plants and known as a potent free radical scavenger (*22*) showed the highest DPPH quenching, but the inhibition effect on lipid peroxidation was lower than those of compounds **2** and **3**. Ferulic acid exhibited the lowest activity in the suppression of lipid peroxidation. In the preliminary assay of the iron-induced lipid peroxidation, ascorbic acid showed a prooxidant activity (data not shown), and the presence and involvement of metal ions and oxygen in the prooxidant activity of ascorbic acid has been reported (*26*, *39*). To determine whether compounds **1**−**3** directly react with and scavenge free radical signals, ESR measurements of DPPH treated by **1**−**3** were performed in which the same concentration of α-tocopherol was compared (Figure 3). With the addition of compounds **1**−**3** and α-tocopherol, ESR signals of DPPH were very rapidly and clearly scavenged in the presence of **1**−**3**, whereas α-tocopherol did not cause complete radical scavenging. These findings emphasize that the three bamboo isolates could be better antioxidants than the commercially available α-tocopherol in the radical scavenging process.

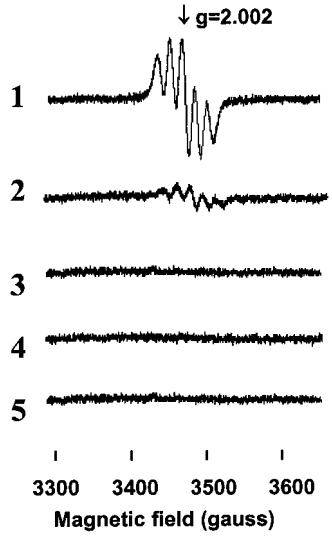

Figure 3. ESR spectra of DPPH radical and the reaction of DPPH with bamboo antioxidants **1**−**3** and α-tocopherol. Spectra: 1, 0.5 mL of 200 μM DPPH in ethanol solution; 2, plus 0.1 mM tocopherol; 3, plus 0.1 mM compound **1**; 4, plus 0.1 mM compound **2**; 5, plus 0.1 mM compound **3**.

Superoxide Scavenging and Xanthine Oxidase (XO) Inhibitory Activities of 1—3. XO is a key enzyme that catalyzes the oxidation of hypoxanthine or xanthine to uric acid. During the reoxidation of XO, molecular oxygen acts as an electron acceptor, producing superoxide radical and hydrogen peroxide (*33*). Consequently, XO is considered to be the important biological source of superoxide radicals, and inhibition of XO is an effective therapeutic approach for hyperuricemia causing goat and kidney stones (*41*, *42*). It has been reported that some flavonoids and the structurally

Table 2. Superoxide Scavenging Activity and Xanthine Oxidase Inhibition of Chlorogenic Acid Derivatives 1−3 and the Related Phenolics and Antioxidants

compound	O_2^- (IC_{50}, μM)[a]	XO (IC_{50}, μM)[b]
1	4.3 ± 0.02	6.7 ± 0.06
2	2.8 ± 0.03	28.3 ± 0.32
3	1.2 ± 0.01	26.2 ± 0.14
chlorogenic acid	6.9 ±0.12	20.1 ± 0.51
caffeic acid	5.1 ± 0.19	25.0 ± 0.53
ferulic acid	32.4 ± 0.47	>50
ascorbic acid	56.0 ± 1.01	>100
α-tocopherol	>100	>100
epigallocatechin	5.3 ± 0.33	>100

[a] Superoxide anion scavenging activity in the X/XO system; [b] Inhibitory activity of XO; the XO measurement of compounds **1**−**3** and chlorogenic, caffeic, and ferulic acids was feasible only for concentrations <50 μM due to overlapping absorbance of these compounds and uric acid at 295−300 nm. Other compounds were analyzed up to the highest concentration tested (100 μM); each value is the mean ± SD (n = 3).

related antioxidants inhibit XO and have superoxide-scavenging activities (*45*). With this viewpoint, we evaluated both the superoxide scavenging activity using an X/XO system and the inhibitory effects on XO. Dual IC_{50} values of the chlorogenic acid (CGA) derivatives **1**−**3** and other test antioxidants are listed in Table 2. CGA derivatives **1**−**3**, epigallocatechin, caffeic acid, and CGA exerted potent O_2^- scavenging activity (IC_{50} < 10 μM). The above most potent superoxide scavengers also showed inhibition effects on XO (IC_{50} = 6.7−28.3 μM), whereas only epigallocatechin (IC_{50} > 100 μM) was ineffective in inhibiting the enzyme. Among the XO inhibitory compounds, compound **1** exhibited the strongest inhibition effect (IC_{50} = 6.7 μM) on XO. Ascorbic and ferulic acids produced relatively weak O_2^- scavenging (IC_{50} = 56.0 and 32.4 μM) and no XO inhibitory activity up to the highest concentration tested (IC_{50} > 100 and > 50 μM). α-Tocopherol did not scavenge superoxide or inhibit XO activity up to the highest concentration tested (IC_{50} > 100 μM).

Effects of 1—3 against Superoxide Generation. Reactive oxygen intermediates originated from superoxide anion (O_2^-) have been shown to cause an oxidant-mediated DNA damage and certain form of cancer. Although the role of the different enzymatic and metal catalysts in specific pathological processes is still being clarified, it has been clearly demonstrated that increased O_2^- generation is implicated in many human diseases (*28*). Several cancer chemopreventive agents exhibit antioxidant activity through their ability to scavenge oxygen radicals and suppress generation of O_2^- in cells (*46*, *47*). In particular, TPA-type tumor promoters are reported to trigger O_2^- generation in epithelial cells and leukocytes, through the X/XO and NADPH oxidase systems, respectively (*32*, *46*). To find out whether the XO inhibitory compounds **1**−**3** and other antioxidants also affect O_2^- generation through the NADPH oxidase system, an inhibitory activity on the superoxide generation was measured using TPA-induced HL-60 cells. Interestingly, as shown in Figure 4, α-tocopherol was the most active (65.5%) in suppressing TPA-induced superoxide generation with cultured HL-60 cells. Compounds **1** and **2** as well as other test compounds responded poorly in this inhibition assay of the superoxide generation, whereas compound **3** was only moderately active (36.0%), although not as potent as that of tocopherol. No cytotoxicity by MTT assay was detected at test concentrations of all compounds.

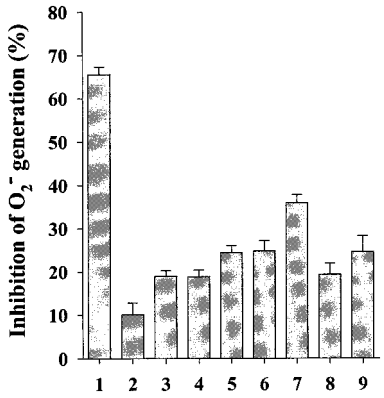

Figure 4. Comparison of inhibition effect of compounds **1**−**3** and selected natural antioxidants on O_2^- generation as measured in TPA-induced HL-60 cells. HL-60 cells (1×10^6 cells/well) grown in RPMI 1640 and differentiated with 1.3% DMSO for 4 days were treated by 8 mM TPA and followed by the addition of 0.5 mg/mL of test compound together with 160 mM cytochrome *c*. After an incubation of 1 h at 37 °C, cytochrome *c* reduction was measured at 515 nm. Error bars represent standard deviations of the mean (n = 3). Columns: 1, α-tocopherol; 2, ascorbic acid; 3, caffeic acid; 4, chlorogenic acid; 5, compound **1**; 6, compound **2**; 7, compound **3**; 8, epigallocatechin; 9, ferulic acid.

DISCUSSION

The aim of this research was to isolate and characterize antioxidant compounds from the leaves of bamboo (*P. edulis*), which is a well-known edible shoot in Korea, as well as to evaluate their antioxidant activity. Three structural isomeric CGA derivatives, **(1)** 3-*O*-(3′-methylcaffeoyl)quinic acid, **(2)** 5-*O*-caffeoyl-4-methylquinic acid, and **(3)** 3-*O*-caffeoyl-1-methylquinic acid, were isolated by using antioxidant activity-directed purification including DPPH and O_2^- scavenging and MTBA methods. Compounds **2** and **3**, reported here for the first time, were methyl substituted at the quinic moiety (C-4 and C-1) of CGA, whereas compound **1** was methyl substituted at the caffeoyl group (C-3′) of CGA.

Chlorogenic acid (5-*O*-caffeoylquinic acid) is widely recognized to be an antioxidant for human low-density lipoprotein (LDL) (*14*, *44*). It is also known as a scavenger for superoxide anion and nitric oxide and as an inhibitor against formation of conjugated diene from linoleic acid peroxidation (*14*, *29*, *34*). There are also some papers (*15*, *37*) on the detection and determination of chlorogenic acid isomers, neochlorogenic acid (3-*O*-caffeoylquinic acid), and cryptochlorogenic acid (4-*O*-caffeoylquinic acid) from plum and prune. The CGA isomers have been reported to have almost the same antioxidant activities (*37*). Interestingly, Hu et al. (*31*) have recently reported that bamboo *Phyllostachys nigra* leaf extract (BLE) was shown to exhibit free radical scavenging in different model systems. They investigated the antioxidant behavior of BLE in vitro and found the presence of caffeic and chlorogenic acids and luteolin 7-glucoside responsible for the antioxidant activity of BLE. However, the results may not be sufficient to evaluate the bamboo antioxidants, because the antioxidant identification was conducted only by HPLC analysis. The authors suggested the possibility of inevitable errors in the individual antioxidant identification. We observed very similar HPLC patterns and UV absorption properties between CGA and CGA-3′-methyl ether under various analyzing conditions.

In the current study, we attempted to evaluate the antioxidant potency of **1**−**3** with CGA and other anti-

oxidants in vitro. The antioxidant activity of **1**−**3** and other phenolic acids in our assay system (Table 1) was observed to have an order of effectiveness of **3** > **2** > CGA ≅ caffeic acid > **1** > ferulic acid. The action mechanism of phenolics (CGA, caffeic, and ferulic acids) as antioxidants has been proposed from the studies on the autoxidation of linoleic acid micelles through the direct inhibition related to the H-donating ability of phenol (*34*). The antiradical activity of phenolic compounds depends on their molecular structure, that is, on the availability of phenolic hydrogens and on the possibility for stabilization of the resulting phenoxyl radicals formed by hydrogen donation (*30*). Generally, antioxidant activity was shown to increase with an increase in hydroxyl groups and a decrease in glycosylation. For example, the ability of the hydroxycinnamates to enhance the inhibition of LDL to oxidation shows that CGA and caffeic acid have a higher peroxyl radical scavenging ability than monophenolics such as *p*-coumaric acid (*29*, *30*, *35*). Methoxylation of one hydroxyl group of caffeic acid to form ferulic acid decreases the efficiency of the scavenging reaction with peroxyl radicals. Ferulic acid is much more effective than *p*-coumaric acid because the electron-donating methoxyl group allows increased stabilization of the resulting aryloxyl radical through electron delocalization after hydrogen donation by the hydroxyl group (*34*). In our results, it is noteworthy that the introduction of a methoxyl function at the quinic moiety of CGA, which was observed in compounds **2** and **3**, considerably increased antioxidant activity (Table 1). However, the introduction of a methoxyl function at the caffeoyl moiety (compound **1)** was ineffective in enrichment of antioxidant activity. This is very consistent with the above antioxidant properties of general phenolic acids (*30*, *34*).

Previous structure−activity relationship studies on cinnamic acids and derivatives have pointed out the importance of *o*-3′,4′ -dihydroxyl groups to the antiradical efficacy. This possibility has been supported by the recent findings (*24*, *25*) that polyphenols inhibited free radical formation and the propagation of free radical reactions through the chelating of transition metal ions. We also obtained a similar result that compound **1** lacking *o*-3′,4′-dihydroxyl groups was less effective for scavenging the radicals and delaying the iron-induced lipid peroxidation than compound **2** or **3**. Moreover, we observed that **2** and **3** caused UV bathochromic shifts by Al^{3+} ion chelating in the presence of $AlCl_3$ (see UV λ_{max}). The results confirmed with the reported minimal responses toward Cu^{2+} ion chelation of kaempferol lacking the catechol structure (*26*). In addition, it is of interest to note that caffeic acid containing the *o*-3′,4′-dihydroxyl groups, but lacking a methoxyl-substituted quinic acid moiety in CGA, was shown to have a much lower antioxidant activity than compounds **2** and **3** (Table 1). The enhanced antioxidant potential of the bamboo phenolics was probably due to increased hydrophilicity of the compounds by esterification. Particularly, CGA isomers showed quite similar antioxidant activity (*37*); thus, it seems that the position of esterification on the quinic moiety of CGA has no influence on the antioxidant activities. Consequently, we conclude that the correlation between their hydrogen-donating antioxidant properties and their metal chelation and hydrophilicites is the important factor for antioxidant potentials of **1**−**3**.

In our iron-induced lipid peroxidation experiments (Table 1), α-tocopherol and epigallocatechin showed a relatively low activity when compared to those of **1**−**3**, CGA, and caffeic acid. These data were not strange to the authors, and other workers also obtained similar results (*34*, *39*, *48*, *49*). CGA and caffeic acid prevent the chain initiation of lipid peroxidation by scavenging peroxy radical ($LOO^{\bullet}$), and they are known to be more effective radical scavengers than Trolox, α-tocopherol, and ascorbic acid (*48*). However, the two methods, DPPH and MTBA assays, used in our study did not always give the same results (data not shown) due to their different reaction conditions and different substrates of product monitored. Other researchers have also proposed this; all methods did not give always the same results for antioxidant activity (*49*).

In the LDL oxidation model, the partition coefficient between the aqueous and the lipophilic phases may influence the accessibility of antioxidant to free radicals (*25*). Trolox, a water-soluble form of tocopherol, was 2 times more potent antioxidatively than α-tocopherol, indicating that some water solubility was advantageous in the LDL oxidation system (*48*). Additionally, there are some conflicting results concerning the beneficial effect of enhanced lipophilicity. Making vitamin C lipophilic by the synthesis of 2-*O*-alkylascorbic acid enhances its protective effect against LDL oxidation (*52*). Recently, Chalas et al. (*35*) has reported that ethyl esterification increased the lipophilicity of phenolic acids and enhanced the antioxidant properties of caffeic, sinapic, and ferulic acids. On the other hand, ferulic acid *β*-glucuronide, which has the hydrophilic sugar moiety in addition to the hydrophobic acid moiety, is stronger than ferulic acid in the LDL oxidation system. Ferulic acid itself does not associate with the lipid portion of LDL and exerts its antioxidant properties from the aqueous phase (*25*, *34*). Hence, in the lipid peroxidation system, the correlation between antioxidant activity and the degree of lipophilicity of the compound remains unclear and not fully understood.

In view of the crucial role of superoxide anion (O_2^-) in the development of inflammation and age-related disease, and the suggested role of superoxide generation inhibition as a contributing factor in the process (*2*, *27*, *32*), we evaluated the effects of compounds **1**−**3** on superoxide scavenging and generation in vitro and in vivo (Table 2 and Figure 4). In the superoxide scavenging assay using the X/XOD system, all three compounds **1**−**3** exerted dual activity, that is, both superoxide scavenging and XO inhibitory activities. CGA and caffeic acid also showed a similar dual activity (Table 2). Generally, the *o*-dihydroxyl bearing compounds showed potent O_2^- scavenging activity, but no correlation was observed between their XO inhibition and the superoxide scavenging as well as their antioxidant activities. It was interesting that the superoxide scavenging efficacy of compound **1** bearing the *o*-methoxyl group was considerably high, in contrast to the lower antioxidant activity than that of CGA. This reflected the fact that the superoxide scavenging activity of the compound **1** was mainly attributed to the potent inhibition against XO (*41*). According to their effect on XO and superoxide, the test antioxidants could be classified into following groups: (a) potent O_2^- scavenger without XO inhibitory activity (epigallocatechin), (b) XO inhibitors with an additional O_2^- scavenging activity (**2**, **3**, CGA, and caffeic acid), (c) XO inhibitor without additional O_2^-

scavenging activity (**1**), and (d) weak O_2^- scavengers without XO inhibitory activity (ferulic acid, ascorbic acid, and α-tocopherol). On the basis of these results, **2** and **3** can be useful in the search for better compounds against O_2^- involved pathological process such as inflammation, aging, and cancer than XO inhibitors without an additional superoxide scavenging activity.

When compared with the antioxidant activity of the test compounds in this study, no correlation was found between the superoxide radical scavenging activity and their inhibitory effect on XO activity. In our assay system, epigallocatechin (EGC) did not inhibit XO at a concentration of 100 μM (Table 2), although it exhibited a strong O_2^- scavenging. Some researchers have also obtained the same results as ours (*41*), whereas some others reported that EGC inhibited XO activity through a mixed-type mode (*51*). We cannot explain clearly in the present data, why EGC does not show XO inhibition. It was shown that many flavonoids including luteolin, chrysin, kaempferol, quercetin, myricetin, and isorhamnetin inhibited XO activity in either mixed-type mode or competitive mode (*50*). Apparently, the structural difference of flavonoids influences the inhibitory effect on XO and superoxide scavenging. However, the mechanism of inhibition of XO by flavonoids is not clearly understood, and it has been known so far that the extent of XO inhibition by structurally related flavonoids and phenolic is quite different. Recently, Moini et al. (*43*) have reported that a flavonoid extract from French maritime pine bark (PBE) selectively inhibits XO through binding to the enzyme rather than by the redox activity, but the active components of PBE, which are responsible for this effect on enzyme, remain to be elucidated.

Superoxide generation from leukocytes has been reported to depend largely upon the action of multicomponent NADPH oxides (*32*). In the superoxide generation assay (Figure 4), α-tocopherol suppressed strongly TPA-induced superoxide in HL-60 cells. Compound **3** also showed a moderate activity in the inhibition assay of superoxide generation, whereas other compounds, including **1**, **2**, and other test antioxidants, responded poorly. This explains that the most superoxide scavenging and XO inhibitory compounds, such as **1**−**3**, CGA, and caffeic acid, have no influence on O_2^- generation through NADPH oxidase system (*32*). As more hydrophilicity, in general, is known as a decreasing factor of test compounds for cellular incorporation, the cellular uptake rate is one of the crucial factors for inhibition of O_2^- generation (*27*, *46*). Thus, the weak activity of test compounds except for α-tocopherol may be due to more hydrophilicity causing low efficiency for cellular incorporation. α-Tocopherol did not scavenge superoxide at a concentration of 100 μM (Table 2), indicating that it preferentially suppresses the NADPH oxidase system responsible for superoxide generation in TPA-induced HL-60 cells. It is also proposed that the high lipophilicity of tocopherol might be the propensity to interact with the cells and to penetrate into the lipid bilayer. Murakami et al. (*27*) also proposed that the importance of the geranyloxyl group for the inhibition of TPA-induced skin tumor promotion in mice might be related to a high cellular uptake of auraptene and more favorably interact with target sites. However, we cannot explain clearly with the present data (Figure 4) why compound **3** exhibited a higher activity than compounds **1** and **2**, why EGC exhibited weaker activity than other test compounds, and why α-tocopherol suppressed superoxide strongly in HL-60 cells. Further in vivo studies and more information are needed to elucidate the above questions.

In summary, three CGA derivatives from the leaves of bamboo *P. edulis* have been isolated and identified as (**1**) 3-*O*-(3′-methylcaffeoyl)quinic acid, (**2**) 5-*O*-caffeoyl-4-methoxylquinic acid, and (**3**) 3-*O*-caffeoyl-1-methoxylquinic acid. **2** and **3** were new CGA methyl ether compounds, whereas **1** was isolated first by Corse et al. (*40*). They all showed strong free radical and superoxide scavenging activities and inhibition of lipid peroxidation. Compound **3** was the most active, followed by compounds **2** and **1**, and other related antioxidants such as CGA and other cinnamic acid derivatives exhibited weaker antioxidant activity than the bamboo isolates. We conclude that it is likely that the higher antioxidant and superoxide scavenging activity in the CGA methyl ether compounds than in other phenolic acids and antioxidants is mediated through *o*-dihydroxyl and methoxyl properties of the molecules, as well as inhibition of xanthine oxidase. The active sites of CGA derivatives **1**−**3**, which are responsible for effects on XO, remain to be elucidated. Additional assay models investigating antioxidative and inhibition mechanism against XO as well as further in vivo studies upon superoxide generation are needed to address the bioavailability of the bamboo CGA derivatives.

LITERATURE CITED

(1) Shibata, M.; Yamatake, M.; Sakamoto, M.; Kanamori, K.; Takagi, K.; Okabe, S. Pharmacological studies on bamboo grass. *Nippon Yakurigaku Zasshi* **1975**, *71*, 481−485.

(2) Perry, G.; Raina, A. K. L; Nonomura, A.; Wataya, T.; Sayre, L. M.; Smith, M. A. How important is oxidative damage? Lessons from Alzheimer's disease. *Free Radical Biol. Med*. **2000**, *28*, 831−834.

(3) Carr, A.; Frei, B. The role of natural antioxidants in preserving the biological activity of endothelium-derived nitric oxide. *Free Radical Biol. Med*. **2000**, *28*, 1806−1814.

(4) Gao, Z.; Huang, K.; Yang, X.; Xu, H. Free radical scavenging and antioxidant activities of flavonoids extracted from the radix of *Scutellaria baicalensis* Georgi. *Biochim. Biophys. Acta* **1999**, *1472*, 643−650.

(5) Ito, H.; Miyake, M.; Nishitani, E.; Mori, K.; Hatano, T.; Okuda, T.; Takasaki, M.; Nishino, H.; Yoshida, T. Antitumor promoting activity of polyphenols from *Cowania mexicana* and *Coleogyne ramosissima*. *Cancer Lett*. **1999**, *143*, 5−13.

(6) Wei, H. C.; Frenkel, K. Relationship of oxidative events and DNA oxidation in SENCAE mice to in vivo promoting activity of phorbol ester-type tumor promoter. *Carcinogenesis* **1993**, *14*, 1195−1201.

(7) Chuyen, N. V.; Kurata, T.; Kato, H.; Fujimaki, M. Antimicrobial activity of Kumazasa (*Sasa albo-marginata*). *Agric. Biol. Chem*. **1982**, *46*, 971−978.

(8) Nishina, A.; Hasegawa, K.; Uchibori, T.; Seino, H.; Osawa, T. 2,6-Dimethoxy-*p*-benzoquinone as an antibacterial substance in the bark of *Phyllostachys heterocycla* var. *pubescens*, a species of thick-stemmed bamboo. *J. Agric. Food Chem*. **1991**, *39*, 266−269.

(9) Okabe, S.; Takeuchi, K.; Takagi, K.; Shibata, M. Stimulatory effect of the water extract of bamboo grass (Folin solution) on gastric acid secretion in pylorus-ligated rats. *Jpn. J. Pharmacol*. **1975**, *25*, 608−610.

(10) Kweon, M.-H.; Kwak, J.-H.; Ra, K.-S.; Sung, H.-C.; Yang, H.-C. Structural characterization of a flavonoid compound scavenging superoxide anion radical isolated from *Capsella bursa-pastoris*. *J. Biochem. Mol. Chem*. **1996**, *29*, 423−428.

(11) Kweon, M.-H.; Jang, H.; Lim, W.-J.; Jang, H.-I.; Kim, C.-W.; Yang, H.-C.; Hwang, H.-J.; Sung, H.-C. Anticomplementary properties of polysaccharides isolated from fruit bodies of mushroom *Pleurotus ostreatus*. *J. Microbiol. Biotechnol.* **1999**, *9*, 450−456.

(12) Kweon, M.-H.; Lim, W.-J.; Yang, H.-C.; Sung, H.-C. Characterization of two glucans activating an alternative complement pathway from the fruiting bodies of mushroom. *Pleurotus ostreatus*. *J. Microbiol. Biotechnol.* **2000**, *10*, 267−271.

(13) Tanaka, T.; Kojima, T.; Kawamori, T.; Wang, A.; Suzui, M.; Okamoto, K.; Mori, H. Inhibition of 4-nitro-quinoline-1-oxide-induced rat tongue carcinogenesis by the naturally occurring plant phenolics caffeic, ellagic, chlorogenic, and ferulic acids. *Carcinogenesis* **1993**, *14*, 1321−1325.

(14) Ohnish, M.; Morishita, H.; Iwahashi, H.; Toda, S.; Shirataki, Y.; Kimura, M.; Kido, R. Inhibitory effects of chlorogenic acids on linoleic acid peroxidation and hemolytic. *Phytochemistry* **1994**, *36*, 579−583.

(15) Kasai, H.; Fukada, Z.; Yamaizumi, S.; Sugie, S.; Mori, H. Action of chlorogenic acid in vegetables and fruits as an inhibitor of 8-hydroxydeoxyguanosine formation in vitro and in a rat carcinogenesis model. *Food Chem. Toxicol.* **2000**, *38*, 467−471.

(16) Chen, Y.; Wong, M.; Rosen, R. T.; Ho, C.-T. 2,2-Diphenyl-1-picrylhydrazyl radical scavenging active components from *Polygonum multiflorum* Thunb. *J. Agric. Food Chem.* **1999**, *47*, 2226−2228.

(17) Okamura, H.; Mimura, A.; Yakou, Y.; Niwano, M.; Takahara, Y. Antioxidant activity of tannins and flavonoids in *Eucalptus rostrata*. *Phytochemistry* **1993**, *33*, 557−561.

(18) Miller, E. G.; Gonzales-Sanders, A. P.; Couvillon, A. M.; Wight, J. M.; Hasegawa, S.; Lam, L. K. T.; Sunahara, G. I. Inhibition of oral carcinogenesis by green coffee beans and limonoid glucosides. In *Food Phytochemicals for Cancer Prevention*; Huang, M.-T., Osawa, T., Ho, C.-T., Rosen, R. T., Eds.; ACS Symposium Series 546; American Chemical Society: Washington, DC, 1994; pp 220−229.

(19) Hostettmann, K.; Hostettmann, M. Isolation techniques for flavonoids. In *The Flavonoids: Advances in Research*; Harbone, J. B., Mabry, T. J., Eds.; Chapman and Hall Press: New York, 1982; pp 1−16.

(20) Markham, K. R. Techniques of flavonoid identification. In *Biological Techniques Series*; Treherne, J. E., Rubery, P. H., Eds.; Academic Press: London, U.K., 1982; pp 15−49.

(21) Agrawal, P. K. Methods for signal assignment. In *Carbon-13 NMR of Flavonoids*; Agrawal, P. K., Ed.; Elsevier Press: New York, 1989; pp 5−31.

(22) Nanjo, F.; Goto, K.; Seto, R.; Suzuki, M.; Sakai, M.; Hara, Y. Scavenging effects of tea catechins and their derivatives on 1,1-diphenyl-2-picrylhydrazyl radical. *Free Radical Biol. Med.* **1996**, *21*, 895−902.

(23) Brown, J. E.; Khodr, H.; Hider, R. C.; Rice-Evans, C. A. Structural dependence of flavonoids interactions with Cu^{2+} ions: implications for their antioxidant properties. *Biochem. J.* **1998**, *330*, 1173−1178.

(24) Iwahashi, H. Some polyphenols inhibit the formation of pentyl radical and octanoic acid radical in the reaction mixture of linoleic acid hydroperoxide with ferrous ions. *Biochem. J.* **2000**, *346*, 265−273.

(25) Rice-Evans, C. A.; Miller, N. J.; Paganga, G. Structure-antioxidant activity relationships of flavonoids and phenolic acids. *Free Radical Biol. Med.* **1996**, *20*, 933−956.

(26) Stoyanovsky, D. A.; Wu, D.; Cederbaum, A. I. Interaction of 1-hydroxyethyl radical with glutathione, ascorbic acid and α-tocopherol. *Free Radical Biol. Med.* **1998**, *24*, 132−138.

(27) Murakami, A.; Kuki, W.; Takahashi, Y.; Yonei, H.; Nakamura, Y.; Ohto, Y.; Ohigashi, H.; Koshimizu, K. Auraptene, a citrus coumarin, inhibits 12-*O*-tetradecanoylphorbol-13-acetate-induced tumor promotion in ICR mouse skin, possibly through suppression of superoxide generation in leukocytes. *Jpn. J. Cancer Res.* **1997**, *88*, 443−452.

(28) Lin, J.-K.; Chen, P.-C.; Ho, C.-T.; Lin-Shiau, S.-Y. Inhibition of xanthine oxidase and suppression of intracellular oxygen species in HL-60 cells by theaflavin-3,3′-digallate, (−)-epigallocatechin-3-gallate, and propyl gallate. *J. Agric. Food Chem.* **2000**, *48*, 2736−2743.

(29) Kono, Y.; Kobayashi; Tagawa, S.; Adachi, K.; Ueda, A.; Sawa, Y.; Shibata, H. Antioxidant activity of polyphenolics in diets rate constants of reactions of chlorogenic acid and caffeic acid with reactive species of oxygen and nitrogen. *Biochim. Biophys. Acta* **1997**, *1335*, 335−342.

(30) Silva, F. A. M.; Borges, F.; Guimaräes, C.; Lima, J. L. F. C.; Matos, C.; Reis, S. Phenolic acids and derivatives: studies on the relationship among structure, radical activity, and physicochemical parameters. *J. Agric. Food Chem.* **2000**, *48*, 2122−2126.

(31) Hu, C.; Zhang, Y.; Kitts, D. D. Evaluation of antioxidant and prooxidant activites of bamboo *Phyllostachys nigra* Var. *Henonis* leaf extract in vitro. *J. Agric. Food Chem.* **2000**, *48*, 3170−3176.

(32) Murakami, A.; Makashima, M.; Koshiba, T.; Maoka, T.; Nishino, H.; Yano, M.; Sumida, T.; Kim, O. K.; Koshimizu, K.; Ohigashi, H. Modifying effects of carotenoids on superoxide and nitric oxide generation from stimulated leukocytes. *Cancer Lett.* **2000**, *149*, 115−123.

(33) Noro, T.; Oda, Y.; Toxhio, M.; Ueno, A.; Fukushima, S. Inhibition of xanthine oxidase from the flowers and buds of *Daphne genkwa*. *Chem. Pharm. Bull.* **1983**, *31*, 3984−3987.

(34) Rice-Evans, C. A.; Miller, N. J.; Paganga, G. Antioxidant properties of phenolic acid compounds. *Trends Plant Sci.* **1997**, *2*, 152−158.

(35) Chalas, J.; Claise, C.; Edeas, M.; Messaoudi, C.; Vergnes, L.; Abella, A.; Lindenabum, A. Effect of ethyl esterification of phenolic acids on low-density lipoprotein oxidation. *Biomed. Pharmacother.* **2001**, *55*, 54−60.

(36) Baderschneider, B.; Winterhalter, P. Isolation an characterization on novel benzoates, cinnamates, flavonoids, and lignans from Riesling wine and screening for antioxidant activity. *J. Agric. Food Chem.* **2001**, *49*, 2788−2798.

(37) Nakatani, N.; Kayano, S.; Kikuzaki, H.; Sumino, K.; Katagiri, K.; Mitani, T. Identification, quantitative determination, and antioxidative activities of chlorogenic acid isomers in prune (*Prunus domestica* L.). *J. Agric. Food Chem.* **2000**, *48*, 5512−5516.

(38) Yan, X.; Suzuki, M.; Ohnishi-Kameyama, M.; Sada, Y.; Nakanishi, T.; Nagata, T. Extraction and identification of antioxidants in the rots Yacon (*Smallanthus sonchifolius*). *J. Agric. Food Chem.* **1999**, *47*, 4711−4713.

(39) Fukumoto, L. R.; Mazza, G. Assessing antioxidant and prooxidant activities of phenolic compounds. *J. Agric. Food Chem.* **2000**, *48*, 3597−3604.

(40) Corse, J.; Sondheimer, E.; Lundin, R. 3-Feruloylquinic acid, a 3′ methyl ether of chlorogenic acid. *Tetrahedron* **1962**, *18*, 1207−1210.

(41) Cos, P.; Ying, Li; Calomme, J. P.; Chmanga, K.; Poel, B. V.; Pieters, L.; Vlietinch, A. J.; Berghe, D. V. Structure−activity relationship and classification of flavonoids as inhibitors of xanthine oxidase and superoxide scavengers. *J. Nat. Prod.* **1998**, *61*, 71−76.

(42) Lavelli, V.; Peri, C.; Rizzolo, A. Antioxidant activity of tomato products as studied by model reactions using xanthine oxidase, myeloperoxidase, and copper-induced lipid peroxidation. *J. Agric. Food Chem.* **2000**, *48*, 1442−1448.

(43) Moini, H.; Guo, Q.; Packer, L. Enzyme inhibition and protein-binding action of the procyanidin-rich French maritime pine bark extract, Pycnogenol: effect on xanthine oxidase. *J. Agric. Food Chem.* **2000**, *48*, 5630−5639.

(44) Heinonen, I. M.; Meyer, A. S.; Frankel, E. N. Antioxidant activity of berry phenolics on human low-density lipoprotein and liposome oxidation. *J. Agric. Food Chem*. **1998**, *46*, 4107−4112.
(45) Nagao, A.; Seki, M.; Kobayashi, H. Inhibition of xanthine oxidase by flavonoids. *Biosci., Biotechnol., Biochem*. **1999**, *63*, 1767−1790.
(46) Murakami, A.; Ohura, S.; Nakamura, Y.; Koshimizu, K.; Ohigashi, H. 1′-Acetoxychavicol acetate, a superoxide anion generation inhibitor, potently inhibits tumor promotion in ICR mouse skin. *Oncology* **1996**, *53*, 386-391.
(47) Nakamura, Y.; Ohto, Y.; Murakami, A.; Osawa, T.; Ohigashi, H. Inhibitory effects of curcumin and tetrahydrocurcuminoids on the tumor-induced reactive oxygen species generation in leukocytes in vitro and in vivo. *Jpn. J. Cancer Res*. **1998**, *89*, 361−370.
(48) Vinson, J. A.; Kabbagh, Y. A.; Serry, M. M.; Jang, J. Plant flavonoids, especially tea flavonoids, are powerful antioxidant using an in vitro oxidation model for heart disease. *J. Agric. Food Chem*. **1995**, *43*, 2000−2002,
(49) Warner, K.; Measuring tocopherol efficacy in fat and oils. In *Antioxidant Methodology*; Aruoma, O. I., Cuttett, S. L., Eds.; AOCS Press: Champaign, IL, 1997; pp 223−233.
(50) Nagao, A.; Seki, M.; Kobayashi, H. Inhibition of xanthine oxidase by flavonoids. *Biosci., Biotechnol., Biochem*. **1999**, *63*, 1767−1790.
(51) Aucamp, A.; Gaspar, A.; Hara, Y.; Apostolides, Z. Inhibition of xanthine oxidase by catechin from tea (*Camellia sinensis*). *Anticancer Res*. **1997**, *17*, 4381−4386.
(52) Liu, Z.; Ma, L. P.; Liu, Z. L. Making vitamin C lipophilic enhances its protective effects against free radical induced peroxidation of low-density lipoprotein. *Chem. Phys. Lipids* **1998**, *95*, 49−57.

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