Antioxidant-Enriched Leaf Water Extracts of *Cinnamomum osmophloeum* from Eleven Provenances and their Bioactive Flavonoid Glycosides

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The study was designed to investigate the antioxidant activities of water extracts of *C. osmophloeum* leaves from eleven provenances (CO1-CO11) and their bioactive phytochemicals. Results revealed that leaf water extracts contained antioxidant phytochemicals and showed efficacy as antioxidants. Among varied leaf water extracts, water extract of CO4 showed the highest total phenolic content (160.9 mg/g) and superior free radical scavenging ability with the IC_{50} values of 10.3 and 16.9 μg/mL for DPPH and superoxide radical scavenging assays, respectively. It was also found to exhibit the best metal chelating ability and reducing power. According to the multiple spectral analyses, bioactive phytochemicals of leaf water extracts were flavonoid glycosides, including kaempferol-7-*O*-rhamnoside and kaempferol-3,7-*O*-dirhamnoside. Leaf water extracts of *C. osmophloeum* with high performance of antioxidant efficacy have great potential as a natural daily supplement.

Keywords: Cinnamomum osmophloeum; *Antioxidant efficacy; Flavonoid glycoside; Kaempferol-7-O-rhamnoside; Phytochemicals*

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INTRODUCTION

Cinnamomum species (cinnamon), belonging to the Lauraceae family, are widely distributed in South and Southeast Asia, and they are commonly used as spices and traditional medicines. Most cinnamons used for flavoring spices are collected from the dried inner bark of *C. cassia*, *C. tamala*, and *C. zeylanicum*. Bioactivities of *Cinnamomum* species, *e.g.* antimicrobial, insecticidal, antioxidant, gastroprotective, anti-inflammatory properties, and anti-hyperuricemia effects, have also drawn great attention from many researchers (Kim *et al.* 2004; Fang *et al.* 2005; Mathew and Abraham 2006; Singh *et al.* 2007; Prasad *et al.* 2009; Eswaran *et al.* 2010). *C. osmophloeum* Kanehira is a species endemic to Taiwan that grows in natural and plantation hardwood forests at elevations below *ca*. 1500 m. *C. osmophloeum* has long been used as a medicinal plant. *C. osmophloeum* parts have been of interest to researchers because the chemical constituents of its leaf essential oil are similar to those of *C. cassia* inner bark oil (Chang *et al.* 2001a). Our previous studies have demonstrated that leaf essential oils from cinnamaldehyde chemotype of *C. osmophloeum* have excellent antitermite, antibacterial,

mosquito larvicidal, antifungal, anti-inflammatory, antidyslipidemic, and anti-hyperuricemia activities (Chang and Cheng 2002; Chang *et al.* 2001a; Cheng *et al.* 2004; Lee *et al.* 2005; Chao *et al.* 2005; Wang *et al.* 2008; Lin *et al.* 2011).

Reactive oxygen species (ROS), such as superoxide radicals, hydroxyl radicals, and hydrogen peroxide, have been found to be important in the initiation and/or progression of diseases such as atherosclerosis, inflammatory injury, cancer, and cardiovascular diseases. More and more people drink functional drinks such as herb teas to reduce the risk of diseases caused by the damage from the over production of free radicals. Functional drinks have been a global trend; many herb infusions, containing single or multiple ingredients, have been proved to be beneficial to our health (Atoui *et al.* 2005; Wojdylo *et al.* 2007; Komes *et al.* 2011). Most antioxidant effects of plant natural products are mainly attributed to phenolic compounds, such as flavonoids, phenolic acids, tannins, *etc*. (Pietta 2000; Komes *et al.* 2011).

Most of the cited reports deal with the antioxidant activity of methanolic/ethanolic extract from *Cinnamomum* species. Bioactivities and compositions of extracts extracted with different solvent would be varied. Water/aqueous extract is generally considered to be safer than organic solvent extract and is easy to obtain by usual brewing. To the best of our knowledge, there has been no research literature concerning the phenolics content, antioxidant activity, and antioxidant phytochemicals of water extract from *C. osmophloeum* leaf. The objectives of this study were to evaluate the antioxidant capacity of leaf water extracts of *C. osmophloeum* from 11 provenances and to investigate the bioactive phytochemicals in the leaf water extract.

EXPERIMENTAL

Plant Materials

Mature leaves of *C. osmophloeum* (Fig. 1) from 11 provenances (CO1 - CO11) were collected from the Tai Power Forest located in Taipei County (CO1), from the Haw-Lin Experimental Forest located in Taipei County (CO2 - CO5), from the Da-Pin-Ting of Taiwan Sugar Farm located in Nantou County in central Taiwan (CO6), and from the Lien Hua-Chin Research Center (CO7 - CO11). The species were identified by Mr. Yen-Ray Hsui of the Taiwan Forestry Research Institute, and voucher specimens were deposited in the laboratory of wood chemistry at the School of Forestry and Resource Conservation, National Taiwan University.

Fig. 1. Photograph of *Cinnamomum osmophloeum* leaves

Chemicals

1,1-Diphenyl-2-picrylhydrazyl (DPPH), hypoxanthine, xanthine oxidase, nitroblue tetrazolium chloride (NBT), trichloroacetic acid (TCA), Folin-Ciocalteu reagent, catechin, gallic acid, and kaempferol were purchased from Sigma Chemical Co. (St. Louis, MO). All other chemicals and solvents used in this experiment were of analytical grade.

Leaf Water Extract

Fresh *C. osmophloeum* leaves (150 g) were subjected to extraction by water for 6 h at 100°C, and the specimen to water ratio was 1:10. The solutions of water extracts were filtered, concentrated by the rotary vacuum evaporator, and lyophilized to remove the water. Yields of water extract from varied sources of leaves were measured based on the dry weight of the leaf, in triplicate.

Chromatography and Spectral Analysis

Water extract (CO4) was fractionated by successive liquid-liquid partition with *n*-hexane, ethyl acetate, and *n*-butanol to obtain four different polar fractions including an *n*-hexane-soluble fraction (11.5%), an ethyl acetate-soluble fraction (10.6%), an *n*-butanol-soluble fraction (35.6%), and a remaining water-soluble fraction (42.3%). Each fraction was tested by DPPH assays in order to determine the most active fraction. After assays, the ethyl acetate soluble fraction was divided into 5 subfractions (EA1-EA5) by column chromatography with Lichroprep RP-18 gels (Merck, Germany).

Based on the activity-guided fractionation procedure of the extract, subfraction EA2 was found to be the most antioxidant-rich fraction. Compounds **1** and **2** were isolated and purified from subfraction EA2 by the HPLC system (HP1100, Agilent, CA) fitted with a RP-18 column (Luna RP-18, 25 cm × 21.2 mm, 5 μm, Phenomenex). The gradient mobile phase consisted of acetone nitrite (A) and ultrapure water (B) at the flow rate of 4 mL/min. The elution program involved a linear gradient from 10 to 55% A to B for 0 to 10 min; 55 to 70% A to B (linear gradient) for 10 to 25 min; and 70 to 100% A to B (linear gradient) for 25 to 30 min. The eluted compounds were detected at 370 nm; Electrospray ionization mass spectrometry (ESI-MS) (negative ion mode); data were collected using a Finnigan MAT-95S mass spectrometer. NMR data such as ^{1}H, ^{13}C, Heteronuclear Single Quantum Coherence (HSQC), and Heteronuclear Multiple Bond Correlation (HMBC) spectra were recorded using a 500 MHz spectrometer after dissolving compounds **1** and **2** in CD_3OD (Bruker, Germany).

Determination of Total Phenolics Content

The amount of total phenolics was measured by means of the Folin-Ciocalteu method using gallic acid as standard (Chang *et al.* 2001b; Yen *et al.* 2012). A 0.5 mL aliquot of diluted extract, 0.5 mL of 1 N Folin-Ciocalteu reagent, and 1 mL of Na_2CO_3 (20%, w/v) were mixed. After 10 min, the absorbance was measured at 730 nm by using a Jasco V-550 UV-Visible spectrophotometer, and against a blank prepared similarly but containing distilled water instead of extract. The concentration of phenolics thus obtained was multiplied by the dilution factor, and the results were expressed as the equivalent to milligrams of gallic acid per gram of extract (mg/g).

DPPH Free Radical Scavenging Activity

The DPPH assay was carried out as reported previously (Chang *et al.* 2001b; Yen *et al.* 2012). Fifty microliters of sample solution were added to 450 µL of 50 mM Tris-HCl buffer (pH 7.4) and 1 mL of 0.1 mM DPPH ethanol solution. After 30 min incubation at ambient temperature, the absorbance was read against a blank at 517 nm in a Jasco V-550 UV-visible spectrophotometer (Tokyo, Japan). The assay was carried out in triplicate. Catechin was used as a positive reference.

Superoxide Radical Scavenging Capacity

The superoxide radical scavenging assay was carried out as reported previously (Chang *et al.* 2001b). The reaction mixture contained 20 µL of 15 mM Na_2EDTA, 30 µL of 3 mM hypoxanthine, 50 µL of 0.6 mM NBT, 145 µL of 50 mM KH_2PO_4/KOH buffer (pH 7.4), and 5 µL of various concentrations of extracts. The reaction was initiated by the addition 50 µL of xanthine oxidase solution (1 unit in 10 mL of buffer) at 25°C, and the absorbance at 570 nm was recorded every 25 s for 5 min using an ELISA reader (BIO-TEK, PowerWave 340).

Metal Chelating Ability

The chelating effect of ferrous ions from extracts was estimated by the method of Dinis *et al.* with slight modifications (1994). Briefly, 200 µL of different concentrations of the extracts and 740 µL methanol were added into 20 µL of 2 mM $FeCl_2$. The reaction was initiated by the addition of 40 µL of 5 mM ferrozine into the mixture, which was then shaken vigorously and left to stand at ambient temperature for 10 min. Absorbance of the reaction mixture was measured at 562 nm with a Jasco V-550 UV-visible spectrophotometer. The Metal chelating ability was calculated as follows:

$$\text{Metal chelating ability (\%)} = (OD_c - (OD_s - OD_{sb})) / OD_c \times 100 \quad (1)$$

where OD_s is the absorbance of the sample, OD_{sb} is the absorbance of the blank of each sample, and OD_c is the absorbance of the control.

Reducing Power

The reducing power was determined by the method of Chang *et al.* (2001b). Various amounts (final concentration of 12.5, 25, 50, and 100 µg/mL) of extracts were mixed with 0.5 mL of 0.2 M phosphate buffer (pH 6.6) and 0.5 mL of 1% potassium ferricyanide, and the mixture was incubated at 50 °C for 20 min. After adding 0.5 mL of 10% (w/v) trichloroacetic acid, the mixture was centrifuged at 976 × *g* for 10 min in a Hettich Micro22R centrifuge (Tuttlingen, Germany). The supernatant (0.5 mL) was mixed with 0.5 mL of distilled water and 0.1 mL of 0.1% ferric chloride, and the absorbance was read at 700 nm in a Jasco V-500 UV-visible spectrophotometer.

Statistical Analysis

For all the extracts, three samples were prepared for assays of every antioxidant attribute. The data were presented as mean ± standard deviation of three determinations. The significance of difference was analyzed using SAS Scheffe's statistics software and a probability value of $p < 0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

Yields of leaf water extracts from 11 provenances fell within the range of 11.5 to 18.8% based on dry weight of the leaf (Table 1). The amount of total phenolics in leaf water extracts ranged from 52.8 to 166.5 mg/g (Table 1). The water extracts from CO2 and CO4 showed higher total phenolics content (166.5 and 160.9 mg/g).

Table 1. Yields, Total Phenolics Contents, and Antioxidant Activities of Water Extracts from Varied Sources of *C. osmophloeum* Leaf

Specimen	Yield (%)	Total phenolics content (mg/g)	IC_{50} (μg /mL)		
			DPPH	Superoxide radical	Metal chelating ability
CO1	17.2 ± 0.2 ^{a,b}	134.5 ± 0.9 ^{d}	36.7 ± 0.4 ^{e}	35.9 ± 4.0 ^{a}	385.2 ± 19.5 ^{c}
CO2	18.8 ± 0.1 ^{a}	166.5 ± 2.4 ^{a}	18.1 ± 0.2 ^{f}	12.1 ± 1.1 ^{c}	528.2 ± 43.1 ^{a,b}
CO3	18.7 ± 0.8 ^{a}	102.1 ± 1.5 ^{e}	37.5 ± 0.4 ^{e}	37.5 ± 4.3 ^{a}	620.7 ± 84.2 ^{a}
CO4	11.6 ± 1.1 ^{d}	160.9 ± 1.4 ^{b}	10.3 ± 0.1 ^{g}	16.9 ± 0.4 ^{b,c}	153.2 ± 9.6 ^{f}
CO5	15.0 ± 0.5 ^{b,c}	147.2 ± 0.3 ^{c}	19.2 ± 0.0 ^{f}	20.0 ± 1.1 ^{b,c}	205.6 ± 16.9 ^{d,e}
CO6	11.5 ± 1.5 ^{d}	64.1 ± 0.1 ^{g,h}	57.6 ± 0.6 ^{b}	44.3 ± 4.9 ^{a}	308.1 ± 33.6 ^{c,d}
CO7	14.0 ± 0.7 ^{c,d}	73.6 ± 0.7 ^{f}	47.7 ± 0.8 ^{d}	14.6 ± 0.5 ^{b,c}	380.3 ± 6.5 ^{c}
CO8	13.4 ± 0.1 ^{c,d}	62.4 ± 0.6 ^{h}	54.4 ± 0.4 ^{c}	23.2 ± 1.8 ^{b}	335.6 ± 14.5 ^{c}
CO9	15.0 ± 0.4 ^{b,c}	52.8 ± 0.1 ^{i}	84.7 ± 1.2 ^{a}	38.9 ± 4.3 ^{a}	420.4 ± 14.0 ^{b,c}
CO10	16.1 ± 0.4 ^{a,b,c}	69.5 ± 0.8 ^{g,f}	46.5 ± 1.6 ^{d}	19.7 ± 0.4 ^{b,c}	404.2 ± 27.8 ^{c}
CO11	14.8 ± 0.4 ^{b,c}	72.2 ± 0.2 ^{f}	57.0 ± 1.2 ^{b,c}	21.6 ± 1.5 ^{b,c}	570.7 ± 59.9 ^{a}
Catechin	-	-	2.9 ± 0.3 ^{h}	3.8 ± 0.1 ^{d}	ND

Note: ND: Not determined. Numbers followed by different letters (a-i) are statistically different at the probability level of $p < 0.05$ according to the Scheffe's analysis.

The scavenging effect of leaf water extracts on DPPH radical increased linearly with increasing concentrations (Fig. 2a). The leaf water extracts from varied provenances of *C. osmophloeum* leaf exhibited significant inhibitory activity. As listed in Table 1, the IC_{50} values for scavenging DPPH radicals of 11 water extracts of *C. osmophloeum* were below 60 μg/mL, except that of water extract of CO9. Catechin is the positive control with an IC_{50} value of 2.9 μg/mL. Among these water extracts, CO4 exhibited the best free radical scavenging activity, the IC_{50} value of water extract of CO4 was found to be 10.3 μg/mL. Water extracts from CO2 and CO5 also showed superior antiradical property. These results indicated that *C. osmophloeum* leaf water extracts could be used as a natural alternative to replace the synthetic antioxidant, *e.g.* butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT), in food and/or pharmaceutical industries. Diverse antioxidant products were derived from forest bioresources. Such studies are beneficial to sustainable utilization of forest biomass (Ebringerova *et al.* 2008; Kang *et al.* 2011; Luis *et al.* 2012).

The water extracts also exhibited a significant superoxide radical scavenging activity. IC_{50} values of the superoxide radical scavenging capacity of water extracts were all below 45 μg/mL (Table 1). The IC_{50} value of superoxide radical scavenging assay for catechin was 3.8 μg/mL. Among water extracts, CO2, CO4, CO5, CO7, CO8, CO10, and

CO11 showed better superoxide radical scavenging activity, with the IC_{50} values ranged from 12.1 to 23.2 μg/mL.

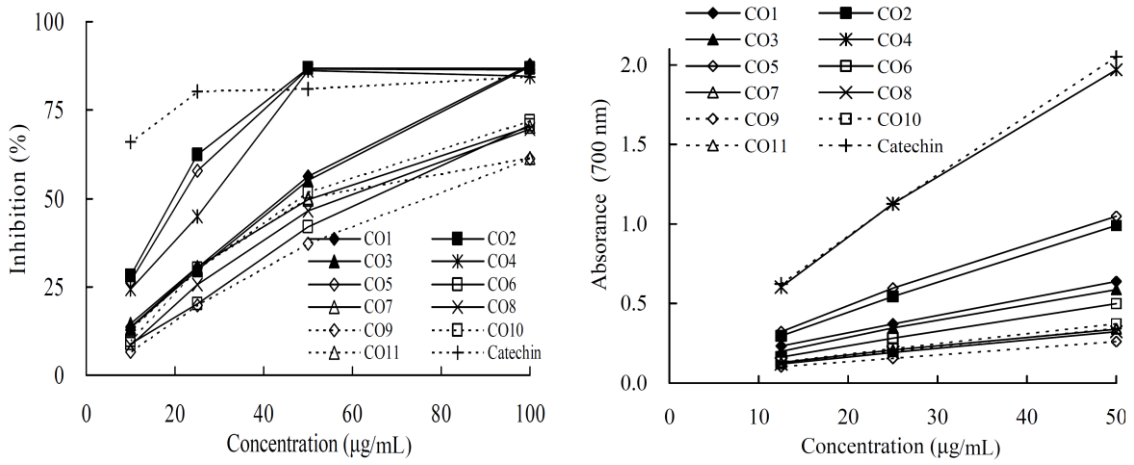


Fig. 2. Antioxidant activities of leaf water extracts from *C. osmophloeum* leaves.
(a) DPPH free radical scavenging activity; (b) Reducing power

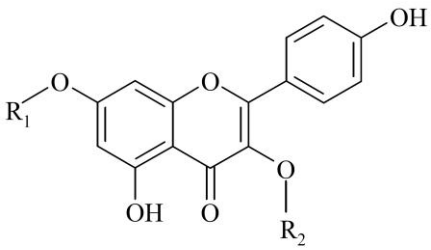
Metal chelating ability is vital since it can reduce the concentration of the catalyzing transition metal in lipid peroxidation (Duh *et al.* 1999). The IC_{50} values of the metal chelating ability of water extracts from *C. osmophloeum* were shown in Table 1. The absorbance of ferrozine–Fe^{2+} complex decreased linearly with a dose dependent effect. The highest activity was observed in the water extract of CO4, with an IC_{50} value of 153.2 μg/mL. The reducing power of various water extracts were shown in Fig. 2b. The reducing power of CO4 extract was the highest among all the extracts. Due to the high reducing power, it could be considered that constituents in the water extracts were good electron donors, and could terminate oxidation chain reactions by reducing the oxidized intermediates into the stable form.

Many studies have revealed that the phenolic contents in plants are related to their antioxidant activities, and the antioxidant activities of phenolic compounds are probably due to their redox properties, which allow them to act as reducing agents, hydrogen donors, and singlet oxygen quenchers. Further data analysis showed that there was a positive correlation between the total phenolic content of water extract and DPPH (R^2 = 0.83), confirming the antioxidant capacity of water extract correlates closely with its total phenolics content.

Two bioactive phytochemicals were isolated and identified from ethyl acetate soluble fraction of water extract from CO4 by using HPLC, MS, and 1D and 2D NMR spectrometry. The structures of identified flavonoid glycosides, kaempferol-7-*O*-rhamnoside (**1**) and kaempferol-3,7-*O*-dirhamnoside (**2**), are shown in Fig. 3.

Compound **1** was isolated and identified from active subfraction of CO4 water extract, and its characteristics are shown as follows: yellow amorphous solid; ESI-MS *m*/*z* 431; ^{1}H-NMR (CD_3OD, 500 MHz) δ 1.26 (3H, d, *J* = 6.1 Hz, H-6’’), 3.47 (1H, t, *J* = 9.5 Hz, H-4’’), 3.62 (1H, dd, *J* = 6.1, 9.5 Hz, H-5’’), 3.82 (1H, dd, *J* = 3.2, 9.5 Hz, H-3’’), 4.01 (1H, brs, H-2’’), 5.54 (1H, brs, H-1’’), δ 6.38 (1H, s, H-6), 6.69 (1H, s, H-8), 6.88 (2H, d, *J* = 8.2 Hz, H-3’, H-5’), 8.06 (2H, d, *J* = 8.2 Hz, H-2’, H-6’); ^{13}C-NMR (CD_3OD, 125 MHz) δ 18.1 (C-6’’), 71.2 (C-5’’), 71.7 (C-2’’), 72.1 (C-3’’), 73.6 (C-4’’),

95.3 (C-8), 99.8 (C-6), 99.9 (C-1''), 106.1 (C-10), 116.3 (C-3'), 116.3 (C-5'), 123.5 (C-1'), 130.8 (C-2'), 130.8 (C-6'), 137.5 (C-3), 148.7 (C-2), 157.7 (C-9), 160.7 (C-4'), 162.2 (C-5), 163.2 (C-7), 177.4 (C-4). Based on the spectral data, compound **1** was confirmed as kaempferol-7-*O*-rhamnoside.



1 R_1 = Rha, R_2 = H
2 R_1 = Rha, R_2 = Rha

Fig. 3. Structures of flavonoid glycosides compounds.
Compound **1**: Kaempferol-7-*O*-rhamnoside; compound **2**: kaempferol-3,7-*O*-dirhamnoside.

Compound **2** (kaempferol-3,7-*O*-dirhamnoside) was a yellow amorphous solid; ESI-MS *m/z* 579; ^{1}H-NMR (CD_3OD, 500 MHz) δ 0.92 (3H, d, *J* = 5.6 Hz, H-6''), 1.25 (3H, d, *J* = 6.2 Hz, H-6'''), 3.32-3.33 (1H, m, H-4''), 3.47 (1H, t, *J* = 9.1, 3.4 Hz, H-4'''), 3.48 (1H, dd, *J* = 9.1, 5.6 Hz, H-5''), 3.60 (1H, dd, *J* = 9.1, 6.2 Hz, H-5'''), 3.70 (1H, dd, *J* = 9.1, 3.4 Hz, H-3''), 3.82 (1H, dd, *J* = 9.1, 3.4 Hz, H-3'''), 4.01 (1H, brs, H-2'''), 4.21 (1H, dd, *J* = 3.4, 1.7 Hz, H-2''), 5.39 (1H, s, H-1''), 5.55 (1H, brs, H-1'''), 6.47 (1H, d, *J* = 2.0 Hz, H-6), 6.73 (1H, d, *J* = 2.0 Hz, H-8), 6.93 (2H, d, *J* = 8.7 Hz, H-3', H-5'), 7.79 (2H, d, *J* = 8.7 Hz, H-2', H-6'); ^{13}C-NMR (CD_3OD, 125 MHz) δ 17.7 (C-6''), 18.2 (C-6'''), 71.3 (C-2''), 71.7 (C-2'''), 71.9 (C-5'''), 72.1 (C-5'''), 72.1 (C-3''), 72.1 (C-3'''), 73.2 (C-4''), 73.6 (C-4''), 95.6 (C-8), 98.7 (C-6), 100.6 (C-1'''), 103.5 (C-1''), 107.6 (C-10), 116.6 (C-3'), 116.6 (C-5'), 122.4 (C-1'), 132.0 (C-2'), 132.0 (C-6'), 136.5 (C-3), 158.1 (C-2), 159.9 (C-9), 161.8 (C-4'), 163.1 (C-5), 163.6 (C-7), 179.8 (C-4).

The spectral data of identified flavonoid glycosides were consistent with the published literature values (Fico *et al.* 2003). Some studies also reported that *Cinnamomum* species were rich in flavonoids, especially kaempferol glycosides (Nakano *et al.* 1983; Fang *et al.* 2005; Prasad *et al.* 2009; Lin *et al.* 2011).

IC_{50} values of kaempferol-7-*O*-rhamnoside obtained from DPPH and superoxide radical scavenging assay were 11.7 and 29.5 μg/mL, respectively (Table 2). Kaempferol-3,7-*O*-dirhamnoside showed no antioxidant activity below 100 μg/mL, revealing that replacing the hydroxyl group at C-3 with sugar glycoside significantly decreased the antioxidant activity of the compound. Hydrolysis of kaempferol glycoside to kaempferol would occur during digestion in human stomach with stomach acids, and the antioxidant property of water extract would be more efficacious. Several reports have demonstrated that flavonoids are the bioactive phytochemicals, and present excellent antioxidant and anti-inflammatory activities (Fang *et al.* 2005; Jayaprakasha *et al.* 2006; Pietarinen *et al.* 2006; Lin *et al.* 2011).

Table 2. Antioxidant Activities of Flavonoid Glycosides from *C. osmophloeum* Leaf

Specimens	IC_{50} (μg /mL)	
	DPPH	Superoxide radical
Kaempferol-7-*O*-rhamnoside	11.7 ± 0.6 [a]	29.5 ± 0.0 [a]
Kaempferol-3,7-*O*-dirhamnoside	＞100	＞100
Kaempferol	4.5 ± 0.1 [b]	4.8 ± 0.1 [b]
Catechin	2.9 ± 0.3 [c]	3.8 ± 0.1 [c]

Note: Numbers followed by different letters (a-c) are statistically different at the probability level of $p < 0.05$ according to the Scheffe's analysis.

CONCLUSIONS

This is the first report demonstrating that the water extracts from *C. osmophloeum* leaf possess beneficial antioxidant activity, as evidenced in the DPPH free radical assay, superoxide radical scavenging activity assay, reducing power assay, and metal chelating assay. The bioactive phytochemicals, kaempferol glycosides, were isolated and identified from water extract based on the bioactivity-guided fractionation procedure. Results concluded that water extract of *C. osmophloeum* leaf has remarkable potential for use as a natural dietary supplement.

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