Antioxidant activities of ethanolic extracts from the twigs of *Cinnamomum osmophloeum*

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Abstract

Cinnamomum osmophloeum Kaneh. is an indigenous tree species in Taiwan. In this study, phytochemical characteristics and antioxidant activities of ethanolic extracts from the twigs of *C. osmophloeum* were investigated for the first time. The extracts were obtained successively using liquid–liquid partition to yield *n*-hexane (Hex), ethyl acetate (EtOAc), *n*-butanol (BuOH) and water fractions. The antioxidant activities of *C. osmophloeum* twig extracts were examined using various antioxidant assays, such as DPPH, NBT, reducing power, lipid peroxidation using mouse brain homogenates, metal chelating ability, and photochemiluminescence (PCL). In addition, total phenolic contents were also determined. Results revealed that the BuOH fraction exhibited the best performance in DPPH assay, NBT assay, reducing power assay and lipid peroxidation using mouse brain homogenates assay. Furthermore, the BuOH fraction has the highest total phenolic contents (496.7 mg of GAE/g). Consequently, kaempferol-7-*O*-rhamnoside was also isolated from the antioxidative BuOH fraction and its activity was also confirmed. These results demonstrated that ethanolic extracts of *C. osmophloeum* twigs have excellent antioxidant activities and thus it has great potential as a source for natural health products.

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Keywords: *Cinnamomum osmophloeum*; Twigs; Extracts; Antioxidant activity; Photochemiluminescence assay

1. Introduction

Plants are potential sources of natural antioxidants. Among the various medicinal and culinary plants, some endemic species are of particular interest because they may be used for producing raw materials or preparations containing phytochemicals with significant antioxidant capacities and health benefits (Exarchou et al., 2002). Reactive oxygen species (ROS) have been found to play an important role in the initiation and/or progression of various diseases such as atherosclerosis, inflammatory injury, cancer, and cardiovascular disease (Halliwell, 1997). Thus, recent studies have investigated the potential of plant products as antioxidants against various diseases induced by free radicals (Hou et al., 2003). Additionally, it has been

determined that the antioxidant effect of plant products is mainly attributed to phenolic compounds, such as flavonoids, phenolic acids, tannins, and phenolic diterpenes (Pietta, 2000).

Cinnamomum osmophloeum Kaneh. (Lauraceae) is an endemic tree that grows in natural hardwood forest of Taiwan at an elevation between 400 and 1500 m. This tree species is of interest to researchers because the chemical constituents of its leaf essential oil are similar to those of *Cinnamomum cassia* bark oil, known as cinnamon oil, commonly used in the food and beverages with great commercial value. Recent phytochemical analyses and biological screenings of *C. osmophloeum* have focused on the leaf essential oil constituents, which have shown excellent anti-bacteria, anti-termite, anti-mite, anti-mildew, anti-mosquito larvae, anti-fungi, and anti-inflammation effects (Chang et al., 2001a; Chang and Cheng, 2002; Chen et al., 2002; Chen and Chang, 2002; Cheng et al., 2004, 2006; Chao et al., 2005). However, to the best of our

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knowledge there is no prior report on the antioxidant activities of *C. osmophloeum* twigs. In this study, we investigated the antioxidant activities of ethanolic extracts of *C. osmophloeum* twigs for the first time and identified their active compounds, following a bioactivity-guided fractionation procedure.

2. Methods

2.1. Chemicals

Analytical grade solvents for extraction and chromatography were purchased from Echo Chemical Co. (Taiwan). 1,1'-Diphenyl-2-picrylhydrazyl radical (DPPH[•]), hypoxanthine, xanthine oxidase, nitroblue tetrazolium chloride (NBT), Folin–Ciocalteu reagent, potassium dihydrogen phosphate (KH₂PO₄), 2-thiobarbituric acid (TBA), ascorbic acid, kaempferol, and (+)-catechin were purchased from Sigma Chemical Co. (St. Louis, MO). Quercetin and gallic acid were purchased from Acros (Belgium). Trichloroacetic acid (TCA) and methanol-*d*₄ were purchased from Merck (Germany). Tris was purchased from Bio-Rad (USA). All other unlabelled chemicals and reagents were purchased from Sigma Chemical Co. (St. Louis, MO).

2.2. Preparation of plant extracts

The twigs of a 13-year-old *C. osmophloeum* Kaneh. were collected at the end of July 2004 from the Taiwan Sugar Company Research Center located in Nantou County in central Taiwan. Diameter of the twigs selected was below 1.5 cm. The species was confirmed by Dr. Yen-Ray Hsui of the Taiwan Forestry Research Institute and voucher specimens were deposited at the laboratory of wood chemistry (School of Forestry and Resource Conservation, National Taiwan University). The dried samples (2.02 kg) were cut into small pieces and soaked in 70% ethanol at ambient temperature for seven days. The extracts were decanted, filtered under vacuum, and then concentrated by a rotary evaporator to obtain dry extracts. The crude extracts were then extracted successively with *n*-hexane (Hex), ethyl acetate (EtOAc), *n*-butanol (BuOH), and water to yield the Hex (4.9%), EtOAc (12.0%), BuOH (50.0%), and water (18.0%) fractions. Each fraction was tested by the various assays in order to determine the most active fraction. After assays, the BuOH fraction was divided into 17 subfractions (BU1–BU17) by column chromatography with Lichroprep RP-18 gels (Merck, Darmstadt, Germany) which was eluted with MeOH/H₂O (gradient elution was performed by changing from 20/80 to 100/0). Compound **1** (retention time = 12.1 min) was isolated and purified from subfraction BU8 by high performance liquid chromatograph (HPLC) on a model PU-980 instrument (Jasco, Japan) with a 250 mm × 21.2 mm, 5-μm Phenomenex Luna RP-18 semipreparative column (Phenomenex, USA). For the mobile phase, solvent A was acetone nitrite while solvent B was H₂O. The elution

conditions were 0–10 min of 10–55% A to B (linear gradient); 10–25 min of 55–70% A to B (linear gradient); 25–30 min of 70–100% A to B (linear gradient). The flow rate was 4 min/ml and the detector employed was Jasco MD-910 photodiode array at 370 nm wavelength. Electrospray ionization mass spectrometry (ESIMS) (negative-ion mode) data were collected using a Finnigan MAT-958 mass spectrometer and NMR data such as ¹H, ¹³C, HSQC (heteronuclear single quantum coherence), and HMBC (heteronuclear multiple bond correlation) spectra of compound **1** dissolved in CD₃OD were recorded using a 500 MHz spectrometer (Bruker, Germany).

2.3. DPPH (1,1-diphenyl-2-picrylhydrazyl) free radical scavenging assay

The scavenging activity of DPPH radical by *C. osmophloeum* twig extracts, their derived fractions, and subfractions were determined using the method reported by Chang et al. (2001b). Initially, 50 μl of the test extracts in DMSO, yielding a series of extracts with different concentrations (1, 5, 10, and 50 μg/ml) in each reaction, were mixed with 1000 μl of 0.1 mM DPPH–ethanol solution and 450 μl of 50 mM Tris–HCl buffer (pH 7.4). DMSO (50 μl) was used as a control for this experiment. After 30 min of incubation at ambient temperature, the reduction of DPPH radical was measured by reading the absorbance at 517 nm. Quercetin, a well-known antioxidant, was used as a positive control. Three replicates were made for each test sample. The inhibition ratio (%) was calculated as follows: % inhibition = [(absorbance of control – absorbance of test sample)/absorbance of control] × 100.

2.4. Superoxide radical scavenging assay (NBT assay)

Measurement of superoxide radical scavenging activity was carried out according to the method described by Kirby and Schmidt (1997) with minor modifications. Reagents in this study were prepared in 50 mM KH₂PO₄–KOH buffer (pH 7.4). The reaction mixture contained 20 μl of 15 mM Na₂EDTA (pH 7.4), 50 μl of 0.6 mM NBT in buffer, 50 μl of xanthine oxidase solution (1 unit in 10 ml of buffer), 30 μl of 3 mM hypoxanthine in 50 mM KOH, 145 μl of buffer, and 5 μl of various concentrations (1, 5, 10, and 50 μg/ml) of extracts or 5 μl of DMSO (as control). The reaction mixture was incubated at ambient temperature, and the absorbance at 570 nm was determined every 30 s up to 8 min using the ELISA reader (BIO-TEK, PowerWave 340). Quercetin was also used as a positive control. Three replicates were made for each test sample. The inhibition ratio (%) was calculated as follows: % inhibition = [(rate of control – rate of test sample)/rate of control] × 100.

2.5. Reducing power assay

The reducing power of *C. osmophloeum* twig extracts was determined according to the method of Oyaizu (1986) with

minor modifications. Briefly, 0.5 ml of sample with different concentrations (6.25–50 µg/ml) was mixed with 0.5 ml of a 0.2 M phosphate buffer (pH 6.6) and 0.5 ml of a 1% potassium ferricyanide solution. The mixture was incubated in a water bath at 50 °C for 20 min. Subsequently, 0.5 ml of a 10% (w/v) trichloroacetic acid solution was added, and the mixture was then centrifuged at 3000 rpm for 10 min. Finally, 0.5 ml of the supernatant layer solution was mixed with 0.5 ml of distilled water and 0.1 ml of 0.1% ferric chloride, and the absorbance of the reaction mixture was measured at 700 nm. Three replicates were made for each test sample. Increased absorbance of the reaction mixture indicated increased reducing power of the sample.

2.6. Lipid peroxidation assay using mouse brain homogenates

This assay was carried out according to the method described by Chang et al. (2001b). The brain of young adult male Balb/c mice were dissected and homogenized in ice-cold Tris–HCl buffer (20 mM, pH 7.4) to produce a 1/10 homogenate. The homogenate was centrifuged at 12,000g for 15 min at 4 °C. 1 ml aliquots of the supernatant were incubated with the test samples in the presence of 10 µM FeSO₄ and 0.1 mM ascorbic acid at 37 °C for 1 h. The reaction was terminated by addition of 1.0 ml trichloroacetic acid (28%, w/v) and 1.5 ml thiobarbituric acid (1%, w/v) in succession, and then the solution was heated at 100 °C. After 15 min, the color of the malondialdehyde (MDA)–TBA complex was measured at 532 nm. (+)-Catechin, a well-known antioxidant, was used as a positive control. Three replicates were made for each test sample. The inhibition ratio (%) was calculated as follows: % inhibition = [(absorbance of control – absorbance of test sample)/absorbance of control] × 100.

2.7. Metal chelating assay

The chelating effect of ferrous ions from *C. osmophloeum* twig extracts was estimated by the method of Dinis et al. (1994) with slight modifications and then compared with quercetin. Briefly, 200 µl of different concentrations of the extracts and 740 µl methanol were added into 20 µl of 2 mM FeCl₂. The reaction was initiated by the addition of 40 µl of 5 mM ferrozine into the mixture, which was then shaken vigorously and left standing at ambient temperature for 10 min. The absorbance of the reaction mixture was measured at 562 nm. Three replicates were made for each test sample. The ratio of inhibition of ferrozine–Fe²⁺ complex formation was calculated as follows: % inhibition = [(absorbance of control – absorbance of test sample)/absorbance of control] × 100.

2.8. Photochemiluminescence (PCL) assay

The luminol-photochemiluminescence assay was carried out with the procedure described by Popov and Lewin

(1999) and with the standard protocol adapted. The extracts of *C. osmophloeum* twigs were measured in the Photochem (Analytik Jena AG, Jena, Germany) system with the kits of antioxidant capacity of lipid-soluble substances (ACL) and antioxidant capacity of water-soluble substances (ACW), where the luminol plays a double role as the photosensitizer and the radical detecting agent. The hydrophilic antioxidants were measured using the ACW kit. Briefly, 1.5 ml reagent 1 (buffer solution pH 10.5), 1 ml reagent 2 (water), 25 µl reagent 3 (photosensitizer), and 10 µl of the sample solution were mixed and measured. The lipophilic antioxidants were measured using the ACL kit. In short, 2.3 ml reagent 1 (methanol), 200 µl reagent 2 (buffer solution), 25 µl reagent 3 (photosensitizer), and 10 µl of the sample solution were mixed and measured. In the ACW kit, the antioxidant potential was assayed by means of the lag phase at different concentrations and expressed as µmol of ascorbic acid/g used as a standard to obtain a calibration curve. While in the ACL kit, the antioxidant potential was then determined by means of the area under the curve at different concentrations and expressed as µmol of trolox/g used as a standard for obtaining a calibration curve. Three replicates were made for each test sample.

2.9. Determination of total phenolics

Total phenolic contents were determined according to the Folin–Ciocalteu method (Chang et al., 2001b). The concentrations of total phenols in extracts were measured by UV spectrophotometer (Jasco UV-550, Japan), according to a colorimetric oxidation/reduction reaction. Briefly, 0.5 ml of extract solution was mixed with 0.5 ml of 1 N Folin–Ciocalteu reagent. The mixture was kept within 2–5 min, followed by the addition of 1.0 ml of 20% Na₂CO₃. After 10 min of incubation at ambient temperature, the mixture was centrifuged for 8 min (12,000g). The absorbance of the supernatant was measured at 730 nm. The results were expressed as gallic acid equivalents (GAE) in milligrams per gram of extracts. Each test was repeated three times, and the results were averaged.

2.10. Quantification of kaempferol-7-O-rhamnoside

Kaempferol-7-O-rhamnoside was isolated from subfraction BU8. The method described by Wang et al. (2003), with a slight modification, was employed to quantify the kaempferol-7-O-rhamnoside. The individual peak area corresponding to kaempferol-7-O-rhamnoside, serving as the index compound, in the HPLC profile of BuOH fraction was determined at the observed maximal absorbance of OD₃₇₀. A standard calibration curve of kaempferol-7-O-rhamnoside was obtained with a series of standard compound concentrations. Quantification of the index compound in the BuOH fraction was then performed by HPLC analysis. The peak area of the candidate compound in the chromatogram of the BuOH fraction was then

defined, and its content in the extracts was obtained on the basis of the quantity calculated from the standard calibration curve.

2.11. Statistical analysis

All results are expressed as mean $\pm$ SD ($n = 3$). The significance of difference was calculated by Scheffé's test, and results with $P < 0.05$ were considered to be statistically significant.

3. Results and discussion

3.1. DPPH radical scavenging activity of *C. osmophloeum* twig extracts

DPPH assay has been extensively used for screening antioxidant activity because it can accommodate many samples in a short period and is sensitive enough to detect active ingredients at low concentrations (Sánchez-Moreno, 2002). DPPH radical scavenging activity of crude extracts and their derived fractions of *C. osmophloeum* twigs were investigated. As shown in Fig. 1, the highest activity was observed in the BuOH fraction, while crude extract and EtOAc fraction also showed good inhibitory effects. In the presence of the 10 $\mu\text{g/ml}$ test sample, the DPPH radical inhibition of twig extracts and their derived fractions decreased in the following order: BuOH (70.3%) > crude (69.0%) > EtOAc (54.4%) > water (21.1%) > Hex (16.8%). Quercetin (a positive control), a well-known antioxidant, shows 88.7% inhibition on DPPH radical at a concentration of 10 $\mu\text{g/ml}$. As shown in Table 1, the EC_{50} value of quercetin, crude, Hex, EtOAc, BuOH and water fractions were 2.2, 7.1, 33.5, 9.8, 6.9 and 31.7 $\mu\text{g/ml}$, respectively. Among the fractions tested, the highest antioxidant activity was observed for the BuOH fraction. According to the results reported by Wang et al. (2003), hot-water extract from *Luctuca indica* was found to possess significant DPPH

Table 1

Total phenolic contents and EC_{50} values (DPPH free radical scavenging assay) of crude extracts and their derived fractions of *C. osmophloeum* twigs

Specimens	EC_{50} ($\mu\text{g/ml}$)	Total phenolics (mg of GAE/g)
Crude extract	7.1 ± 0.1^d	313.4 ± 7.8^c
Hex fraction	33.5 ± 0.4^a	–
EtOAc fraction	9.8 ± 0.7^c	404.7 ± 23.6^b
BuOH fraction	6.9 ± 0.1^d	496.7 ± 17.6^a
Water fraction	31.7 ± 0.1^b	149.9 ± 0.3^d
Quercetin	2.2 ± 0.1^e	–

Results are mean $\pm$ SD ($n = 3$). Numbers followed by different letters (a–e) are significantly different at the level of $P < 0.05$ according to the Scheffé's test.

radical scavenging activity, with an EC_{50} value of 12.2 $\mu\text{g/ml}$. These results indicate that *C. osmophloeum* twig extracts and the BuOH fraction have better performance against DPPH radical. Furthermore, comparison with the results obtained by Chen (2003) shows that *C. osmophloeum* twig extracts are much better than the *C. osmophloeum* leaf essential oil ($\text{EC}_{50} = 88.4 \mu\text{g/ml}$), indicating that *C. osmophloeum* twig extracts have better potential source as antioxidants than its leaf essential oil.

3.2. Superoxide radical scavenging activity of *C. osmophloeum* twig extracts

Superoxide radical was generated by the hypoxanthine–xanthine oxidase and NBT systems in this assay. The decrease in absorbance at 570 nm with the presence of antioxidants indicates the consumption of superoxide radical in the reaction mixture. Fig. 2 shows the superoxide radical scavenging activity of ethanolic extracts from the twigs of *C. osmophloeum* compared with quercetin. In the presence of the 10 $\mu\text{g/ml}$ test sample, the superoxide radical inhibition of *C. osmophloeum* twig extracts and their derived fractions decreased in the following order: BuOH (63.6%) > crude (62.1%) > EtOAc (55.3%) > water

Fig. 1. Free-radical scavenging activity of ethanolic extracts from twigs of *C. osmophloeum* measured by DPPH assay. Results are mean $\pm$ SD ($n = 3$).

Fig. 2. Superoxide radical scavenging activity of ethanolic extracts from twigs of *C. osmophloeum* measured using NBT assay. Results are mean $\pm$ SD ($n = 3$).

(32.1%) > Hex (10.0%). The EC_{50} value of quercetin, crude, Hex, EtOAc, BuOH and water fractions were 3.5, 6.3, >100, 7.3, 4.9 and 30.9 $\mu\text{g/ml}$, respectively. These results revealed that the BuOH fraction possessed the highest antioxidant activity, which was similar to the DPPH radical scavenging activity results. Results reported by Tung et al. (2005) demonstrated that the crude extract and its BuOH fraction from *Acacia confusa* twigs also showed excellent inhibitory activity against superoxide radical with EC_{50} values of 25.4 and 6.3 $\mu\text{g/ml}$, respectively. Comparing these results indicates that *C. osmophloeum* twig extracts would be an excellent source as a natural antioxidant and merit further investigation.

3.3. Reducing power of *C. osmophloeum* twig extracts

The reducing power of a compound may serve as a significant indicator of its potential antioxidant activity (Meir et al., 1995). In this assay, the yellow color of the test solution changes to green depending on the reducing power of test specimen. The presence of reductants in the solution causes the reduction of the Fe^{3+} /ferricyanide complex to the ferrous form. Therefore, Fe^{2+} can be monitored by the measurement of the absorbance (OD value) at 700 nm (Zou et al., 2004). As shown in Fig. 3, the reducing power of *C. osmophloeum* twig extracts and their derived fractions at 50 $\mu\text{g/ml}$ are as follows: BuOH fraction (OD value = 1.9) > crude extract (OD value = 1.8) > EtOAc fraction (OD value = 1.6) > water fraction (OD value = 0.7) > Hex fraction (OD value = 0.3). The reducing power of the BuOH fraction was the highest among all the fractions, and it increased linearly with increasing concentration. Shon et al. (2004) reported that red onion, yellow onion, and white onion extracts had absorption values of 0.17, 0.12 and 0.12, respectively at a concentration of 1 mg/ml in the reducing power assay. Comparing the two results shows that the reducing power of *C. osmophloeum* twig extracts and their derived fractions are much better than that of the onion extracts.

Fig. 3. Reducing power of ethanolic extracts from twigs of *C. osmophloeum*. Results are mean $\pm$ SD ($n = 3$).

3.4. Lipid peroxidation assay using mouse brain homogenates of *C. osmophloeum* twig extracts

Lipid peroxidation is a free radical-related process in biologic systems that may occur under enzymatic or nonenzymatic control. There has been an increasing interest in lipid peroxidation because formation of cytotoxic products such as MDA and 4-hydroxynonenal can influence cell function and the course of major human diseases (Sevanian and Ursini, 2000). Therefore, in this assay, pink color of MDA–TBA complex was detected at 532 nm and the inhibitory effects of *C. osmophloeum* twig extracts and their derived fractions on both ferric ion and ascorbic acid-induced lipid peroxidation on mouse brain homogenates were also calculated.

Results shown in Fig. 4 revealed that extracts of *C. osmophloeum* twigs and their derived fractions have an excellent activity in suppressing lipid peroxidation on mouse brain homogenates. BuOH fraction had better inhibitory effect than (+)-catechin at all concentrations, more than 50% of the inhibitory activity of lipid peroxidation was observed at the concentration of 5 $\mu\text{g/ml}$. It is also noteworthy that (+)-catechin, which has an EC_{50} value of ~ 20 $\mu\text{g/ml}$, showed less effectiveness than the BuOH fraction and EtOAc fraction. Chang et al. (2001b) also reported that extracts of *A. confusa* bark and heartwood had excellent inhibitory effects in lipid peroxidation on mouse brain homogenates. These results indicate that twigs of *C. osmophloeum* might also have the same antioxidant potential as *A. confusa* bark and heartwood.

3.5. Metal chelating ability of *C. osmophloeum* twig extracts

The chelating effect of ferrous ions by *C. osmophloeum* twig extracts and their derived fractions was estimated using the method of Dinis et al. (1994). Ferrozine can quantitatively form complexes with Fe^{2+} . In the presence of samples possessing chelating activity, the formation of complexes is decreased. Therefore, measurement of the rate of color reduction helps to estimate the chelating activity of the samples.

As shown in Fig. 5, the water fraction showed a better inhibitory effect with 66.4% of inhibition at a concentration of 2500 $\mu\text{g/ml}$, followed by Hex fraction (43.7%), crude extract (40.3%), BuOH fraction (31.9%), and EtOAc fraction (30.3%), respectively. These results indicate that *C. osmophloeum* twig extracts showed poor metal chelating activity.

3.6. Antioxidant capacity of *C. osmophloeum* twig extracts using PCL assay

The PCL method, which is easy and rapid to perform, presents numerous advantages: it does not require high temperatures to generate radical and is more sensitive, can be measured in a few minutes even at nanomolar range (Sacchetti et al., 2005). The activities of water-soluble sub-

Fig. 4. Effects of *C. osmophloeum* twig extracts and their derived fractions on both ferric ion and ascorbic acid-induced lipid peroxidation on mouse brain homogenates. Results are mean $\pm$ SD ($n = 3$).

Fig. 5. Metal chelating ability of ethanolic extracts from twigs of *C. osmophloeum*. Results are mean $\pm$ SD ($n = 3$).

stances were expressed as ascorbic acid equivalents, while the lipid-soluble substances were expressed as trolox equivalents. The water-soluble antioxidant amounts of *C. osmophloeum* twig extracts and their derived fractions ranged from 91.3 to 3820.0 μmol of ascorbic acid/g, while the amount of lipid-soluble antioxidants ranged from 380.0 to 2278.7 μmol of trolox/g. As shown in Table 2, the antioxidant potential of *C. osmophloeum* twig extracts and their derived fractions in ACW systems decreased in the

following order: EtOAc fraction > BuOH fraction > crude extract > water fraction > Hex fraction. Meanwhile, in ACL systems, they are as follows: EtOAc fraction > BuOH fraction > crude extract > Hex fraction > water fraction. It is obvious that the EtOAc fraction shows the best antioxidant potential with 3820.0 μmol of ascorbic acid/g in ACW systems and 2278.9 μmol of trolox/g in ACL systems. Lee et al. (2004) reported that, using the PCL method, the lipid and water antioxidant activities of soybean extracts ranged from 2.4 to 4.4 μmol of trolox/g soybean extracts and from 174.2 to 430.9 μmol of ascorbic acid/g soybean extracts, respectively. Comparing the aforesaid results obtained by PCL methods shows that the antioxidant potential of *C. osmophloeum* twig extracts and their derived fractions are much better than that of the soybean extracts.

3.7. Total phenolic contents in twig extracts of *C. osmophloeum*

The total phenolics in crude extracts and their derived fractions of *C. osmophloeum* twigs were determined according to the Folin–Ciocalteu method and expressed as GAE. As shown in Table 1, the total phenolic contents in BuOH

Table 2

Antioxidant activity of ethanolic extracts from twigs of *C. osmophloeum* measured using PCL methods

Specimens	ACW ^a (μmol of ascorbic acid/g)	ACL ^b (μmol of trolox/g)
Crude extract	1086.7 $\pm$ 6.5	1751.7 $\pm$ 4.5
Hex fraction	91.3 $\pm$ 2.9	728.7 $\pm$ 3.5
EtOAc fraction	3820.0 $\pm$ 12.0	2278.7 $\pm$ 9.5
BuOH fraction	1326.7 $\pm$ 30.5	1889.7 $\pm$ 5.9
Water fraction	182.3 $\pm$ 3.1	380.0 $\pm$ 0.7

^a ACW: Antioxidant activity of water-soluble substances.

^b ACL: Antioxidant activity of lipid-soluble substances. Results are mean $\pm$ SD ($n = 3$).

fraction showed the highest amount (496.7 mg of GAE/g), followed by EtOAc fraction (404.7 mg of GAE/g), crude extract (313.4 mg of GAE/g), and water fraction (149.9 mg of GAE/g). 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 (Chang et al., 2001b). The aforesaid results demonstrated that the free radical scavenging effect of each fraction from the crude extracts of *C. osmophloeum* twigs correlates closely with their phenolic contents.

3.8. Identification and quantification of active compound and its antioxidant activity

Among the BuOH subfractions, BU8 was found to have an excellent DPPH radical scavenging activity with an EC₅₀ value of 6.4 µg/ml. To find out its active component, compound **1** was isolated and identified from BU8 by using HPLC, MS, and 1D- and 2D-NMR spectrometry. It was characterized as kaempferol-7-*O*-rhamnoside and its characteristics analyzed are shown as follows: yellow amorphous solid; ESIMS *m/z* 431; ¹H NMR (CD₃OD, 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'); ¹³C NMR (CD₃OD, 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). The spectral data of kaempferol-7-*O*-rhamnoside were in good agreement with the published values (Fico et al., 2003). From the quantification by HPLC, there was 38.3 µg of kaempferol-7-*O*-rhamnoside in 1 mg of BuOH fraction.

To determine the antioxidant activities of kaempferol-7-*O*-rhamnoside, DPPH and NBT assays were performed. Quercetin and kaempferol were used as the positive control. In Table 3, kaempferol-7-*O*-rhamnoside showed 26.9 µM and 68.1 µM of EC₅₀ values in DPPH assay and NBT assay, respectively. Kaempferol-7-*O*-rhamnoside was a well-known flavanoid glycoside, which has been reported to possess excellent inhibitory effect on rat aldose

Table 3
EC₅₀ values (µM) of kaempferol-7-*O*-rhamnoside in DPPH scavenging activity and superoxide radical scavenging activity

Compounds	DPPH radical	Superoxide radical
Kaempferol-7- <i>O</i> -rhamnoside	26.9	68.1
Kaempferol	14.2	13.1
Quercetin	4.5	4.3

reductase (Shimizu et al., 1984). Several studies indicated that flavonoid glycosides also have excellent antioxidant activities (Ko et al., 2005). These results provide promising baseline information for the potential use of the crude extract of *C. osmophloeum* twigs as well as the isolated compounds as antioxidative supplements. The EC₅₀ of kaempferol-7-*O*-rhamnoside was slightly higher compared with that of the BuOH fraction as shown above, indicating that some active components in the BuOH fraction of *C. osmophloeum* twigs might not have been isolated and it is worth further investigation.

4. Conclusions

C. osmophloeum has been used as a medicinal plant in Taiwan. To our knowledge, this is the first report demonstrating that the ethanolic extract from *C. osmophloeum* twigs has antioxidant activity as seen in the DPPH free radical assay, superoxide radical scavenging activity assay, reducing power assay, lipid peroxidation assay using mouse brain homogenates, and PCL assay. The antioxidant constituent was also isolated and identified from *C. osmophloeum* twigs based on the bioactivity-guided fractionation procedure. Furthermore, our results showed that crude ethanolic extract of *C. osmophloeum* twigs might have good potential as a source for natural health products due to its antioxidant activities.

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