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Phytochemistry 65 (2004) 3033–3039
PHYTOCHEMISTRY
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Antioxidant activity of phenylpropanoid esters isolated and identified from *Platycodon grandiflorum* A. DC

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Received in revised form 22 June 2004
Available online 6 October 2004

Abstract

Platycodon grandiflorum A. DC (Campanulaceae) is used as a traditional oriental medicine and also as a food in Korea. Here we investigated its antioxidant activity, and isolated and identified its active compounds. Petroleum ether extracts from the whole root of *P. grandiflorum* were fractionated by silica gel column chromatography using a solvent gradient (petroleum ether:diethyl ether, v/v; 9:1–5:5). The 8:2 fraction showed a higher radical scavenging activity than the other fractions, and active compounds were purified from this fraction by reversed-phased HPLC. Two active compounds were identified as coniferyl alcohol esters of palmitic and oleic acids by FAB-MS, UV, IR and NMR spectroscopy. The antioxidant activities of these two compounds, which were evaluated by 1,1-diphenyl-2-picrylhydrazyl (DPPH), superoxide and nitric oxide radical scavenging capacity, were found to be as high as those of BHT or BHA.

Keywords: *Platycodon grandiflorum*; Campanulaceae; Phenylpropanoid; Coniferyl alcohol; Antioxidant; Radical scavenging

1. Introduction

Antioxidants are compounds that delay the oxidation of other molecules by inhibiting the initiation or propagation of oxidizing chain reactions. In the food industry, they have long been used as additives to protect food products from oxidation-related quality deterioration such as rancidity. Recently, more attention was paid to the antioxidants because of increased concerns about oxidative stress in the human body (Halliwell et al., 1995). Oxidative stress, induced by reactive oxygen species (e.g., O_2^-) and nitrogen (e.g., NO^-) species formed during normal metabolic processes, is believed to cause irreversible damage to cellular components such as lipids, proteins and DNA, and can lead to various degenerative diseases (Lopaczynski and Zeisel, 2001; Fang et al., 2002; Ohshima, 2003). It has been reported that the natural antioxidants present in many plants reduce such damage and help prevent mutagenesis, carcinogenesis and aging due to their radical scavenging activities (Cook and Samman, 1996; Beyer et al., 1998). These natural antioxidants have been isolated from various fruits (Luo et al., 2002), vegetables (Boveris et al., 2001) and medicinal plants (Lu and Foo, 2001; Badami et al., 2003), and the identified compounds cover a wide range of phytochemicals including phenolics (e.g., cinnamic acid derivatives and flavonoids) and carotenoids (e.g., lycopene).

The root of *Platycodon grandiflorum* A. DC has been used in Asia as a traditional folk remedy for diseases such as bronchitis, asthma, pulmonary tuberculosis, hyperlipidemia, diabetes and inflammatory disease. Recently, several studies on the extracts of the *P. grandiflorum*

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doi:10.1016/j.phytochem.2004.08.030

root revealed that it contains a wide variety of compounds with immunopharmacological effects (Kim et al., 2000) and preventive effects against hypercholesterolemia and hyperlipidemia (Kim et al., 1995). However, little research has been performed on the antioxidant activity of *P. grandiflorum*. Although Lee and Jeoug (2002) reported that hot water extracts of *P. grandiflorum*, which was grown for an unusually long period of time (23 years), reduced the CCl_4-induced hepatotoxicity possibly due to its $CCl_3^{\bullet}$ radicals scavenging capacity, little was examined in relation to antioxidant activity.

In a related study, it was found that the petroleum ether extracts of the *P. grandiflorum* root have a greater antioxidant capacity than aqueous extracts, and that this ability is related to its phenolic content (Lee et al., 2004). In the present study, the antioxidant phenolic compounds in *P. grandiflorum* were isolated and identified, and their antioxidative activities were compared to some commercial antioxidants, based on their radical scavenging activities.

2. Results and discussion

2.1. Antioxidant activity and phenolic content

The DPPH radical scavenging activities of fractions eluted during silica gel column chromatography were measured to screen for antioxidant compounds. As shown in Fig. 1(a), the antioxidant activity of fraction II (8:2) was slightly higher than that of the other fractions at a concentration of 50 μg/ml, and decreased in the order I (9:1) > crude, III (7:3) > V (5:5) > IV (6:4). Most fractions exhibited a concentration-dependent increase in their DPPH radical scavenging activities, and especially fraction II showed the highest activity (45.3%) at a concentration of 100 μg/ml. Fraction II was re-loaded on to a silica gel column with a narrower range of solvent gradient, from which six sub-fractions of II-1 (10:0, v/v), II-2 (9:1, v/v), II-3 (8.8:1.2, v/v), II-4 (8.5:1.5, v/v), II-5 (8:2, v/v) and II-6 (MeOH) were obtained. The antioxidant activities of these sub-fractions were in order II-2 > II-4 > II-1, II-3 > II-5, II-6 (MeOH). The highest DPPH radical scavenging activity of fraction II-2 (44.4%) at a concentration of 50 μg/ml was comparable to the 57.9% of BHA (Fig. 1(b)).

It has been reported that antioxidant activities in many plant materials primarily originate from their phenolics (Rice-Evans et al., 1997), indicating a significant positive relationship between total phenolic content and antioxidant activity (Velioglu et al., 1998). Lee et al. (2000) also reported that the antioxidant activity of the fractions separated from Aloe extracts correlated well with their total phenolic content. Thus, we measured total phenolic contents in each fraction and found that the

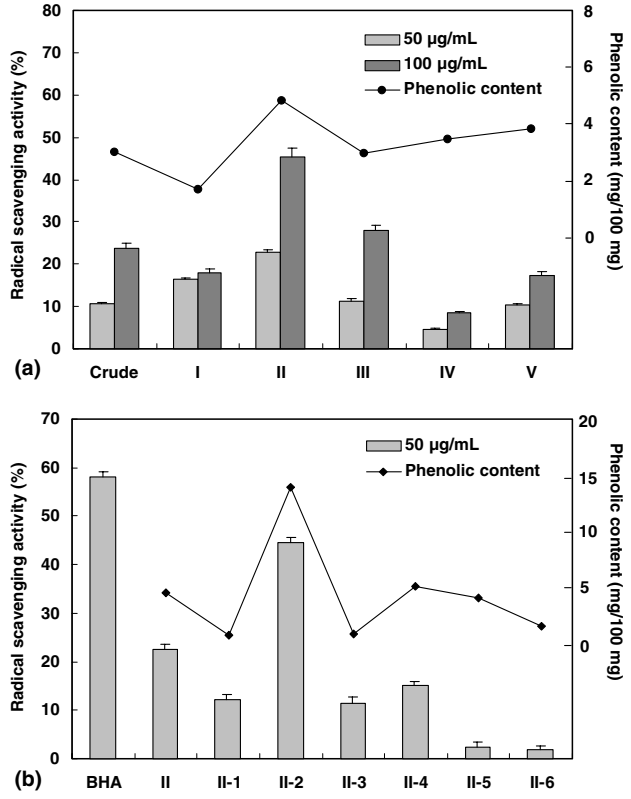
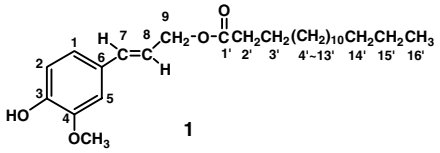


Fig. 1. DPPH radical scavenging activities and total phenolic contents (expressed as ferulic acid equivalents) of the fractions (I–V) (a) and subfractions (II-1–6) from fraction II (b), separated from crude extracts of *P. grandiflorum* by silica gel column chromatography.

antioxidant activities of the fractions from *P. grandiflorum* were also significantly correlated with their phenolic contents, as shown in Fig. 1(a) and (b). In particular, fraction II-2, the most active sub-fraction, had the highest total phenolic content (15 mg of phenolics/100 mg of sample), suggesting that the antioxidant compounds in fraction II-2 had a phenolic structure.

2.2. Isolation and identification of active compounds

As shown in Fig. 2(a), fraction II-2 was further separated by HPLC using a semi- preparative C_{18} reversed phase column with gradient mixtures of MeOH and H_2O, and eluted fractions were collected (fraction/min). The antioxidant activities of collected fractions were easily visualized by using a dot-blot test (not shown), and the fractions, which corresponded to peaks with retention time of 26–28 min, exhibited the highest antioxidant activity. The peaks overlapped at 26–28 min could be better separated into compound **1** (R_t = 27.9 min) and **2** (R_t = 28.3 min) by using an analytical C_{18} reversed phase column and a narrower range of solvent gradient (Fig. 2(b)). The UV absorbance spectra of these two compounds were identical, as shown in Fig. 2(c), indicating that they had similar structures.



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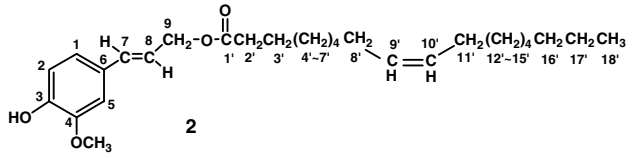

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The FT-IR spectra of compounds **1** and **2** showed the presence of hydroxyl (3450 cm^{-1}) and carbonyl (1740 cm^{-1}) groups, and the ^{1}H and ^{13}C NMR spectra suggested they have similar structures. The ^{1}H NMR spectrum of both compounds exhibited signals typical for a 1,3,4-trisubstituted benzene ring at δ 6.95, 6.93 and 6.89, one *trans* conjugated ethylene at δ 6.60 and 6.17, and methoxyl protons at δ 3.93. This spectrum is consistent with that of coniferyl alcohol except for the methylene (H-9) signal at δ 4.27. The signals of compounds **1** and **2** were slightly shifted to downfield of δ 4.74 and 4.73, respectively, suggesting the presence of an electron withdrawing group, possibly a carboxylic acid connected to the methylene hydroxyl group by an ester bond. The methylene carbon (C-9) resonance at δ 65.34, compared to that of δ 63.82 for coniferyl alcohol, and the carbonyl group in the IR spectra, also supported the presence of a carboxylic acid ester at the end of the side-chain in coniferyl alcohol. Other signals in the ^{1}H and ^{13}C NMR spectra of compounds **1** and **2** exactly corresponded to those of palmitic and oleic acids, respectively, except for the carboxylic carbon (C-1′). Compared to δ 180.5 for palmitic and oleic acids, the signals of both compounds were shifted to upfield of δ 173.9, again suggesting that the carboxylic acid moiety is in its ester form. The suggested structures of compounds **1** and **2** as shown are supported by their molecular weights of 418 and 444, respectively, obtained using FAB-MS and HRFAB-MS with high resolution. The structures explain that the difference in fatty acid chain length resulted in only small differences in retention times and their identical UV absorbance spectra (Fig. 2(b) and (c)). In addition, their UV spectra were similar to that of coniferyl alcohol, as shown in Fig. 2(c), even though the fatty acid moiety made them quite hydrophobic and retarded their elution from the HPLC column (28 min).

Phenolic compounds that occur in lipid-soluble plant extracts have been less well studied than those in water-soluble extracts. In more recent years, some esters between cinnamic acid derivatives and long chain fatty acids or fatty alcohols have been identified from stem barks or specifically suberins of various plants (Houghton, 1989; Balde et al., 1991; Schmutz et al., 1994) and cutins or epicuticular waxes of fruits and leaves (Griffiths et al., 1999; Whitaker et al., 2001; Jetter et al., 2002). Those esters include a wide variety of phenylpropanoid moieties, such as ferulic, caffeic and coumaric acids, and cinnamyl, sinapyl and coumaryl alcohols. The fatty acid or fatty alcohol moieties of the esters also vary in their chain length from C16 to C30.

2.3. Evaluation of antioxidant activity

DPPH radical scavenging has been generally used as a means of quickly evaluating the antioxidant activities of specific compounds or extracts (Wang et al., 1998), whereas recently, more interest has been focused on the scavenging of superoxide and nitric oxide, because they are highly reactive and are practically related with cellular oxidative stress (Taylor, 2001). Table 1 shows the scavenging activities of the isolated compounds **1** and **2** against the DPPH radical, superoxide, and nitric oxide, compared to those for commercial antioxidative compounds, i.e., ascorbic acid, ferulic acid, BHA, BHT and coniferyl alcohol. Among the compounds tested, ferulic acid and ascorbic acid exhibited the strongest activity in all the evaluation tests, and compound **2** showed stronger ($P < 0.01$) activity than compound **1**. In the superoxide scavenging test, compounds **1** and **2** showed much higher ($P < 0.01$) activities than BHA or BHT, whereas their DPPH and nitric oxide scavenging activities were comparable to those of BHA and BHT.

It is known that the antioxidant activity of a phenylpropanoid compound is closely associated with its structure, such as substitutions on the aromatic ring and side chain structure (Natella et al., 1999; Nenadis et al., 2003; Shahidi and Wanasundara, 1992). However, little is known about the antioxidant activity of the fatty acid ester of phenylpropanoids. As shown in Table 1, two fatty acid esters of coniferyl alcohol also exhibited antioxidant activities, even though they showed significantly lower ($P < 0.01$) activities than that of coniferyl alcohol in all three radical scavenging tests. Since the fatty acid moieties are attached to the side-chain hydroxyl group functionality of coniferyl alcohol, it is unlikely that they affect the aromatic hydroxyl group that is responsible for antioxidant activity. Instead, the fatty acids change the polarity of the compounds becoming more hydrophobic, and thus might give negative influences on their antioxidant activity that were assayed in aqueous conditions. Similarly, the small differences in radical scavenging activities between compounds **1** and **2** also appeared to be due to their different solubility in aqueous solution. However more studies are required to confirm this.

The phenolic esters found in plants are related to the main functions of suberin and cuticle as a hydrophobic transport barrier against water loss over the plant

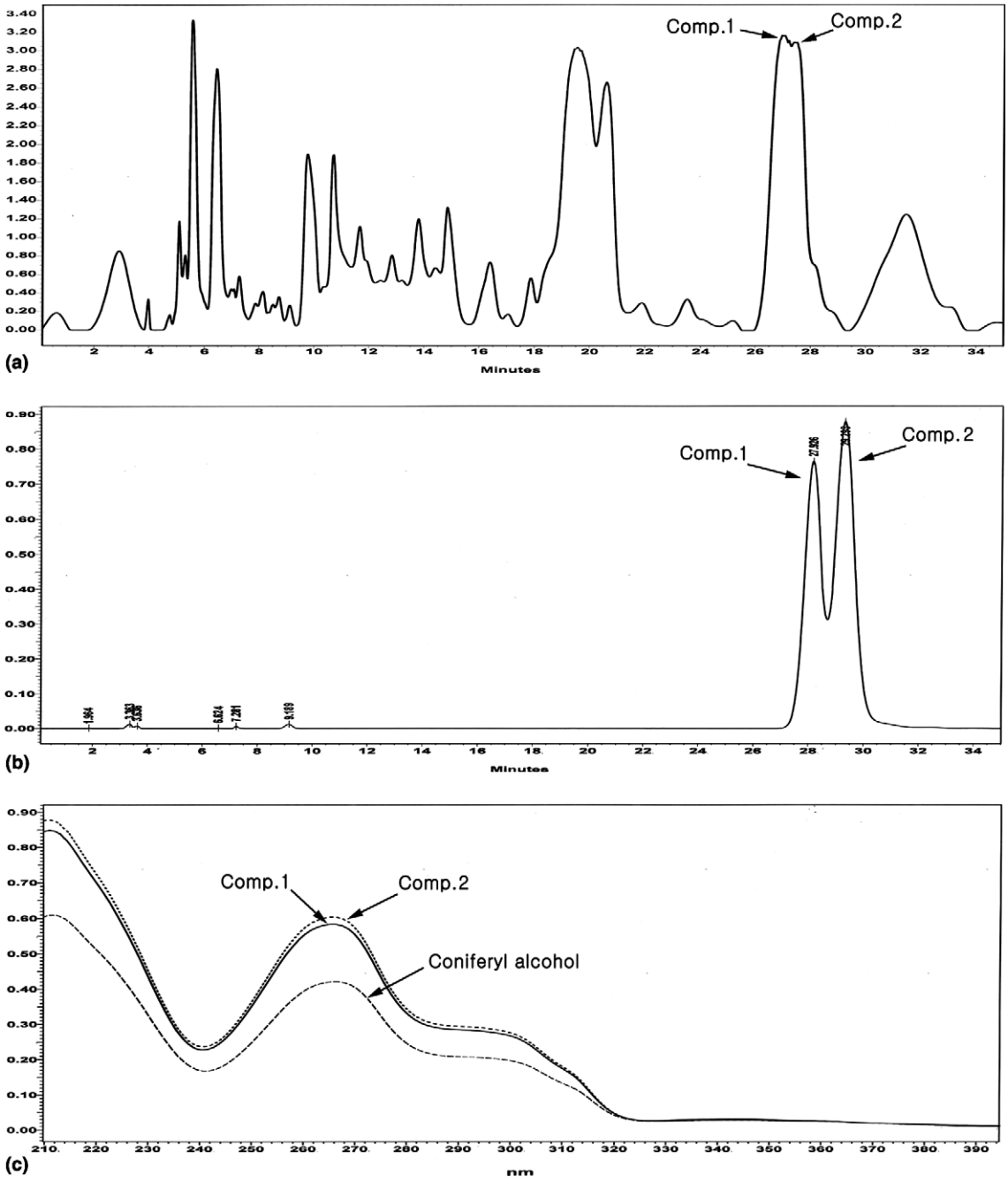

Fig. 2. Reversed phase HPLC chromatograms of active compounds (**1** and **2**) of *P. grandiflorum* detected at 254 nm (a, b). and UV spectra of the compounds (c).

surface (Riederer and Schreiber, 1995) and as a protectant from pathogens or environmental harm. For example, the accumulation of ferulic acid esters has been reported during the suberization of wound healing potato tubers (Bernards and Lewis, 1992) and of tree bark (Adamovics et al., 1977). In addition to their role as a physical barrier, these lipophilic plant surface layers are also important as a protectant from oxidation caused by environmental oxygen. Some researchers reported that the antioxidant activity of the apple cuticle is negatively related with scald development during storage (Anet, 1974; Meir and Bramlage, 1988), but these workers only identified a few lipid-soluble antioxidant compounds, such as tocopherols. Other researchers also detected the presence of lipophilic phenolics that have antioxidant activities in the cuticle or epicuticular wax of apples, but the active compounds were not identified (Ju and Bramlage, 1999). From this point of view, the

Table 1
DPPH, superoxide and nitric oxide radical scavenging activities of the compounds **1** and **2** in comparison with other antioxidants

Samples	IC_{50} (μM)[a]		
	DPPH	O_2^-	NO^-
Compound **1**	48.23 ± 1.33	85.75 ± 1.81	53.82 ± 4.12
Compound **2**	34.94 ± 0.61	75.00 ± 2.24	36.45 ± 3.87
Coniferyl alcohol	19.91 ± 0.93	69.43 ± 2.56	20.33 ± 0.16
Ferulic acid	4.14 ± 0.38	33.06 ± 0.45	4.56 ± 0.12
Ascorbic acid	7.70 ± 0.39	55.90 ± 4.94	8.25 ± 0.59
BHA	21.33 ± 1.39	200.96 ± 3.40	56.47 ± 2.41
BHT	39.91 ± 2.32	307.10 ± 7.72	95.11 ± 2.25

[a] Mean ± standard deviation of three replicates.

antioxidant activities of phenylpropanoid esters, which were firstly observed in the present study, would help understand the biological significance of suberin and cuticle in plants, as a surface antioxidant against the oxidative stress. Moreover, two phenylpropanoid esters isolated from *P. grandiflorum* root are supposed to originate particularly from the suberin of the root, but more studies are required.

3. Experimental

3.1. Materials

Chopped and dried roots of *P. grandiflorum* A. DC (Campanulaceae family) cultivated in Youngju, Korea, were purchased from the oriental herbal market and stored in a refrigerator (4 °C). Silica gel 60 (70–230 mesh ASTM; 63–200 μm) was purchased from Merck Ltd. (Darmstadt, Germany) for open CC. 1,1-Diphenyl-2-picrylhydrazyl (DPPH), xanthine (99%), xanthine oxidase (25 U), butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), sodium nitroprusside (SNP), nitro blue tetrazolium chloride (NBT) and sodium phosphate were purchased from Sigma–Aldrich Chemical Co. (St. Louis, MO, USA). Griess reagent was obtained from Promega Co. (Madison, USA). All solvents and chemicals used were of analytical grade.

3.2. Extraction and isolation procedures

Platycodon grandiflorum roots 500 g were ground into powders that were subsequently dispersed in petroleum ether (4 l) for 3 days at room temperature with shaking. The solvent was exchanged everyday, and the extracted solvents were combined, filtered, and concentrated by vacuum-evaporation at 40 °C. The crude extract was then subjected to silica gel CC (25 mm diameter, 300 mm length). The extract (0.4 g) dissolved in a small amount of petroleum ether (4 ml) was loaded onto the column, and eluted stepwise with petroleum ether Et_2O mixtures from 9:1 (v/v; I) to 1:1 (v/v; V), at a flow rate of 4 ml/min. Each fraction (100 ml) collected was tested for antioxidant activity using DPPH as reactant. For subfractionation, the collected fractions were re-subjected again to CC using a narrower range of petroleum ether/Et_2O gradient.

The most active subfractions collected were further separated using a HPLC (Waters 2690-Alliance series, Milford, MA, USA) equipped with a Waters 996 photodiode array (PDA) detector. A semi-preparative reversed phase column (Waters μ-Bondapak C_{18}; 10 μm, 300 × 7.8 mm) was used for separations, with a solvent gradient of MeOH–H_2O from 90:10 to 100:0 at a flow rate of 1.5 ml/min. Eluted fractions were collected based on the UV absorbance profiles (200–400 nm), with antioxidant activities of each fraction quickly determined by dot-blot testing on a TLC plate stained with DPPH (0.4 mM) (Soler-Rivas et al., 2000). Active compounds were further purified by HPLC equipped with a Waters analytical column (μ-Bondapak C_{18}; 10 μm, 300 × 3.9 mm) using a narrower solvent gradient.

3.3. Identification of the active compounds

Low- and high-resolution (HR) Fast Atom Bombardment Mass Spectrometry (FAB-MS) used a JMS-700 Mstation mass spectrometer (JEOL Ltd., Tokyo, Japan) with *m*-nitrobenzyl alcohol (M-NBA) as matrix in the FAB^+ mode (Cs ion emission). Infrared (IR) spectra were obtained on a FT-IR430 (Jasco International Co., Tokyo, Japan) spectrometer with KBr disks. ^1H and ^{13}C NMR spectra were obtained using a Varian® model UI500 (Varian Inc., Melbourne, Australia) at 500 MHz.

3.3.1. Palmitic acid ester of coniferyl alcohol (compound *1*)

UV λ_{max} (MeOH) nm (log ε) 262 (1.3), 300sh (1.1); IR (KBr) ν_{max} 3450, 2930, 1740, 1520, 1470, 1180 cm^{-1}; FAB-MS *m/z* 419.3 $[M + H]^+$; HRFAB-MS *m/z* 419.3141, calculated for $[C_{26}H_{42}O_4 + H]^+$ *m/z* 419.3161; ^1H NMR ($CDCl_3$, 500 MHz), δ 6.95 (1H, *d*, *J* = 2.0 Hz, H-5), 6.93 (1H, *dd*, *J* = 8.0, 2.0 Hz, H-1), 6.89 (1H, *d*, *J* = 8.0 Hz, H-2), 6.60 (1H, *d*, *J* = 16.0 Hz, H-7), 6.17 (1H, *dt*, *J* = 15.5, 6.5 Hz, H-8), 4.74 (2H, *dd*, *J* = 7.0, 1.5 Hz, H-9), 3.94 (3H, OCH_3), 2.37 (2H, *t*, *J* = 8.0 Hz, H-2′), 1.67 (2H, *m*, H-3′), 1.23–1.38 (24H, *m*, H-4′–15′), 0.91 (3H, *t*, *J* = 7.0 Hz, H-16′); ^{13}C NMR ($CDCl_3$, 500 MHz), δ 173.94 (C-1′), 146.87 (C-4), 146.13 (C-3), 134.59 (C-7), 129.09 (C-6), 121.22 (C-8), 120.88 (C-5), 114.68 (C-2), 108.62 (C-1), 65.34 (C-9), 56.15 (OCH_3), 34.58 (C-2′), 32.09 (C-14′), 29.33–29.96 (C-4′–13′), 25.18 (C-3′), 22.86 (C-15′), 14.29 (C-16′).

*3.3.2. Oleic acid ester of coniferyl alcohol (compound **2**)*

UV λ_{max} (MeOH) nm (log ε) 262 (1.3), 300sh (1.1); IR (KBr) ν_{max} 3450, 2930, 1740, 1530, 1470, 1180 cm^{-1}; FAB-MS *m/z* 445.3 $[M + H]^+$; HRFAB-MS, *m/z* 445.3332, calculated for $[C_{28}H_{44}O_4 + H]^+$ *m/z* 445.3318; ^1H NMR ($CDCl_3$, 500 MHz), δ 6.95 (1H, *d*, *J* = 2.0 Hz, H-5), 6.93 (1H, *dd*, *J* = 8.0, 2.0 Hz, H-1), 6.89 (1H, *d*, *J* = 8.0 Hz, H-2), 6.60 (1H, *d*, *J* = 16.0 Hz, H-7), 6.17 (1H, *dt*, *J* = 15.5, 6.5 Hz, H-8), 5.37 (2H, *m*, H-9′, 10′), 4.73 (2H, *dd*, *J* = 6.5, 1.5 Hz, H-9), 3.93 (3H, OCH_3), 2.37 (2H, *t*, *J* = 8.0 Hz, H-2′), 2.03 (4H, *m*, H-8′, 11′), 1.67 (2H, *m*, H-3′), 1.24–1.40 (20H, *m*, H-4′–7′, 12′–17′), 0.91 (3H, *t*, *J* = 7.0 Hz, H-18′); ^{13}C NMR ($CDCl_3$, 500 MHz) δ173.95 (C-1′), 146.87 (C-4), 146.14 (C-3), 134.60 (C-7), 130.26 (C-9′), 129.99 (C-10′), 129.10 (C-6), 121.22 (C-8), 120.88 (C-5), 114.68 (C-2), 108.62 (C-1), 65.34 (C-9), 56.15 (OCH_3), 34.62 (C-2′), 32.15 (C-16′), 29.29–30.01 (C-4′–7′, 12′–15′), 27.47 (C-8′), 27.41 (C-11′), 25.21 (C-3′), 22.92 (C-17′), 14.35 (C-18′).

3.4. Phenolic content

Total phenolic contents were determined using the procedure of Singleton et al. (1999). Analyses were carried out in triplicate, all results expressed as ferulic acid equivalents.

3.5. Evaluation of DPPH radical scavenging activity

DPPH radical scavenging activity was measured as described by Hwang et al. (2001). DPPH radical scavenging activity (%) was calculated using [(absorbance of control − absorbance of sample)/absorbance of control] × 100, and IC_{50} was defined as the concentration of sample showing 50% DPPH radical scavenging activity.

3.6. Evaluation of superoxide radical scavenging activity

The superoxide radical-scavenging activity was determined according to the procedure of Okamura et al. (1993) with some modification. The sample solution in 10% DMSO was added to 0.1 ml of a mixture of 0.2 mM hypoxanthine and 0.1 mM NBT in 50 mM potassium phosphate buffer (pH 7.5) containing 0.05 mM EDTA in a 96-well flat-bottom microplate. Xanthine oxidase (0.1 ml), diluted with 50 mM phosphate buffer (pH 7.5) to 0.08 U/ml, was added to the mixture, and the microplate was incubated at 37 °C for 20 min. The addition of 0.1 ml of 1 N HCl to the mixture terminated the reaction, and the absorbance at 540 nm was measured. The IC_{50} value was defined as the concentration of sample required to reduce the NBT by 50%.

3.7. Evaluation of nitric oxide (NO) radical scavenging activity

The scavenging activity of the nitric oxide radical was measured as described by Yen et al. (2001) with some modification. Samples of different concentrations were added to 50 μl of sodium nitroprussude (5 mM) in phosphate buffer saline (PBS) in a 96-well flat-bottom microplate, and the reaction mixture was incubated at 25 °C for 150 min in the dark. The amount of NO produced by sodium nitroprusside was assayed by measuring nitrite accumulation using the Griess reaction (Green et al., 1982). The IC_{50} value was defined as the concentration of sample required to inhibit the nitric oxide radical by 50%.

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