2,2-Diphenyl-1-picrylhydrazyl Radical-Scavenging Active Components from *Polygonum multiflorum* Thunb.

Yong Chen, Mingfu Wang, Robert T. Rosen, and Chi-Tang Ho*

Department of Food Science, Cook College, Rutgers, The State University of New Jersey, 65 Dudley Road, New Brunswick, New Jersey 08901-8520

An activity-directed fractionation and purification process was used to identify the antioxidative components of *Polygonum multiflorum* Thunb. (PM). Dried root of PM was extracted with 95% ethanol and then separated into water, ethyl acetate, and hexane fractions. Among these only the ethyl acetate phase showed strong antioxidant activity by the 2,2-diphenyl-1-picrylhydrazyl (DPPH) test when compared with water and hexane phases. The ethyl acetate fraction was then subjected to separation and purification using silica gel column chromatography and Sephadex LH-20 chromatography. Three compounds showing strong antioxidant activity were identified by spectral methods (^{1}H NMR, ^{13}C NMR, and MS) and by comparison with authentic samples to be gallic acid, catechin, and 2,3,5,4′-tetrahydroxystilbene 2-*O*-*β*-D-glucopyranoside.

Keywords: *Polygonum multiflorum Thunb.; free radical; antioxidant activity; 2,3,5,4′-tetrahydroxystilbene 2-O-β-D-glucopyranoside*

INTRODUCTION

The root of *Polygonum multiflorum* Thunb. (PM) has been used in the Orient as a tonic and antiaging agent since ancient times. Clinically it has been utilized in the treatment of hyperlipidemia, coronary heart disease, neurosis, and other diseases commonly associated with aging (Xiao et al., 1993). The medicinal effects of PM in the treatment of these age-related diseases are possibly mediated by the antioxidant capacity of this plant (Ip et al., 1997) because free-radical-involved oxidative stress has been implicated in the aging process. PM extract has been found both in vitro and in vivo to possess antioxidant activity (Ip et al., 1997; Chen and Zhang, 1987). Research indicates that PM enhances the cellular antioxidant activity (Xiao et al., 1993), increases the function of superoxide dismutase (SOD), significantly inhibits the formation of oxidized lipids (Xiao et al., 1993), and represses lipid peroxidation in rat heart mitochondria (Hong et al., 1994). A more recent study by Ip et al. (1997) revealed that the antioxidant components were contained in the ethyl acetate fraction of PM extract. However, the specific compounds responsible for the antioxidant activity of PM remain unknown. This study was designed to identify the antioxidant constituents in *P. multiflorum* Thunb., to elucidate their chemical structures, and to compare their antioxidant capacities.

MATERIALS AND METHODS

General Procedures. ^{1}H NMR and ^{13}C NMR spectra were obtained on a VXR-200 instrument, and MS analysis was performed on a Micromass Platform II system (Micromass Co., MA) equipped with a Digital DECPc XL560 computer for data analysis. Positive-ion mass spectra were obtained using the heated nebulizer atmospheric pressure chemical ionization (APCI) interface. The ion source temperature was set at 150 °C, and the sample cone voltage was 10 V. Thin-layer chromatography was performed on Sigma-Aldrich silica gel TLC plates (250 μm thickness, 2−25 μm particle size), with compounds visualized by spraying with 5% (v/v) H_2SO_4 in an ethanol solution. Silica gel (130−270 mesh) and Sephadex LH-20 (Sigma Chemical Co., St. Louis, MO) were used for column chromatography. Radical 2,2-diphenyl-1-picrylhydrazyl (DPPH) and silica gel (130−270 mesh) were purchased from Aldrich Chemical Co. (Milwaukee, WI). All solvents used for chromatographic isolation were of analytical grade and purchased from Fisher Scientific (Springfield, NJ).

Plant Material. Dried processed root of PM was purchased from a local Oriental store; it had been imported from China.

Extraction and Isolation Procedures. The dried root of PM (3380 g) was cut into small pieces and extracted with 95% ethanol (10 L), continuously, at room temperature for 4 weeks. The plant material was filtered off, and the EtOH extracts were combined and concentrated under reduced pressure by a rotary evaporator (Rotavapor R-114, Buchi, Switzerland). The obtained dry extract (165 g) was dissolved in 200 mL of water and then partitioned with hexane and then ethyl acetate. After concentration under reduced pressure, 26.24 g of a dark brown oily substance from the ethyl acetate fraction was obtained. The dry ethyl acetate extract was then subjected to column chromatography (CC) on silica gel, eluted with a solvent mixture of chloroform/methanol with increasing methanol content (30:1, 20:1, 10:1, 8:1, 5:1, 1:1; each 2500 mL), and collected into 41 fractions (A).

DPPH-active fractions A28−A33 were combined to yield 2.5 g of dry extract and then subjected to silica gel eluted with ethyl acetate/methanol/water at a ratio of 15:1:1. A total of 48 fractions (B) were collected, and among these 48 fractions, DPPH-active fractions B24−B29 were combined to obtain 1.43 g of dry extract. This dry extract was then eluted with ethyl acetate/methanol/water/acetic acid at a ratio of 25:1:1:0.1 on the silica gel column to obtain compound **1** (0.98 g). Fractions B32−B35 together were subjected to elution with ethyl acetate/hexane/acetic acid at a ratio of 10:7:1 by silica gel, and compound **2** (48 mg) was obtained.

Fractions A35−A39 were combined and first subjected to a Sephadex LH-20 column (eluted with methanol) and then rechromatographed on a silica gel column using ethyl acetate/

* Corresponding author [fax (732) 932-8004; e-mail ho@aesop.rutgers.edu].

10.1021/jf990092f CCC: $18.00
Published on Web 05/11/1999

methanol/water/acetic acid (30:1:1:0.1), and compound **3** (1.96 g) was obtained.

Structure Determination of Isolated Compounds. *Gallic acid (**1**)*: amorphous powder; APCI, *m/z* 169 $[M - 1]^+$; ^{1}H NMR (200 MHz, CD_3OD) δ 7.07 (2H, s); ^{13}C NMR (50 MHz, CD_3OD) δ 170.7 (s, C-7), 146.7 (s, C-3, 5), 139.9 (s, C-4), 122.2 (s, C-1), 110.6 (d, C-2, 6) [identical with the literature (Gottlieb et al. 1991)].

*Catechin (**2**):* APCI, *m/z* 289 $[M - 1]^+$; ^{1}H NMR (200 MHz, CD_3OD) δ 2.51 (dd, J = 16.1, 8.2 Hz, H-4), 2.85 (1H, dd, J = 16.1, 5.5 Hz, H-4), 4.00 (m, H-3), 5.87 (1H, d, J = 2.2 Hz, H-6), 5.94 (1H, d, J = 2.2 Hz, H-8), 6.74 (2H, m, H-5′,6′), 6.85 (1H, br, s, H-2′); ^{13}C NMR (50 MHz, CD_3OD) δ 28.8 (t, C-4), 69.1 (d, C-3), 83.1 (d, C-2), 95.8 (s, C-8), 96.6 (d, C-6), 115.6 (d, C-2′), 116.4 (d, C-5′), 120.4 (d, C-6′), 132.5 (s, C-1′), 146.5 (s, C-3′, 4′), 157.2, 157.9, 158.1 (s, C-5, C-7, C-8a) [identical with the literature (Foo et al., 1996)].

*2,3,5,4′-Tetrahydroxystilbene 2-O-β-*D*-glucoside (**3**):* brown color powder; APCI, *m/z* 405 $[M - 1]^-$; ^{1}H NMR (200 MHz, CD_3OD) δ 3.25−3.81 (6H, m, sugar H), 4.52 (1H, d, J = 7.6 Hz, H-1″), 6.27 (1H, d, J = 2.7 Hz, H-4), 6.63 (1H, d, J = 2.7 Hz, H-6), 6.77 (2H, d, J = 8.8 Hz, H-2′,6′), 6.92 (1H, d, J = 16.6 Hz, olefinic H), 7.45 (1H, d, J = 8.8 Hz, H-3′,5′), 7.69 (1H, d, J = 16.6 Hz, olefinic H); ^{13}C NMR (50 MHz, CD_3OD) δ 158.6 (s, C-5), 152.3 (s, C-3), 138.2 (s, C-2), 134.0 (d, C-α or C-β), 131.1 (d, C-α or C-β), 130.4 (s, C-1′), 129.5 (d, C-2′, 6′), 122.0 (s, C-1), 116.8 (d, C-3′, 5′), 108.5 (d, C-6), 103.9 (d, C-4), 103.0 (d, C-1), 78.5 (d, C-3″), 78.2 (d, C-5″), 75.8 (d, C-2″), 71.0 (d, C-4″), 62.4 (t, C-6″) [identical with the literature (Nonaka et al., 1982)].

Determination of the Scavenging Effect on DPPH Radicals. This method was adapted from that of Chen and Ho (1995). Basically, the tested compounds were added into 1.0×10^{-4} M ethanol solution of DPPH to make the final concentration to be 30 μM. After thorough mixing, the solutions were kept in the dark for 30 min. Thereafter, the absorbency of the samples was measured using a spectrophotometer (Milton Roy, model 301) at 517 nm against ethanol without DPPH as the blank reference. Each sample was duplicated in the test, and the values were averaged. For the determination of IC_{50}, each of the purified compounds was made into eight different concentrations for DPPH tests. IC_{50} was obtained by extrapolation from linear regression analysis.

RESULTS AND DISCUSSION

This DPPH scavenging activity-directed fractionation and isolation experiment confirmed the report by Ip et al. (1997) that antioxidant compounds are present in the ethyl acetate fraction. DPPH is a free radical compound and has been widely used to test the free radical scavenging ability of various chemicals (Dinis et al., 1994; Wang et al., 1998). In this experiment, after determination of the presence of DPPH scavenging compounds in the ethyl acetate fraction, which was subsequently eluted into 41 fractions, a thorough DPPH test was conducted toward these 41 fractions. The result is shown in Figure 1. Fractions A28−A33 were combined, and TLC showed the presence of several compounds. Therefore, the combined fraction was then subjected to another round of fractionation and DPPH testing (Figure 2). A yield of 0.98 g of pure compound **1** from fractions B24−B29 was determined to be gallic acid; 48 mg of compound **2** was purified from fractions B32−B35 and proved to be catechin; and from fractions A35−A40, ∼1.96 g of compound **3** was isolated and determined to be 2,3,5,4′-tetrahydroxystilbene 2-*O*-β-D-glucoside. Their structures, as shown in Figure 3, were determined by NMR and confirmed by the literature. These three purified compounds demonstrated DPPH free radical scavenging activity following the order of gallic acid > catechin > 2,3,5,4′-tetrahydroxystilbene

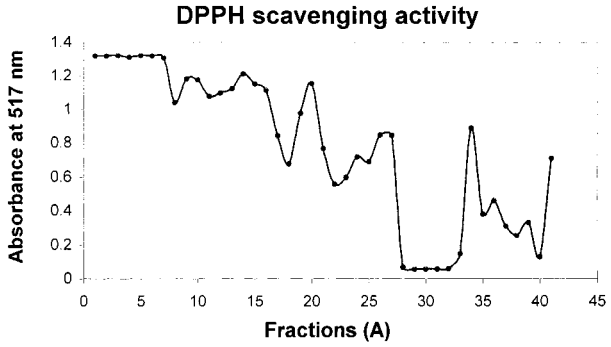

Figure 1. DPPH test for the 41 fractions obtained from the column chromatography of the ethyl acetate extract of *P. multiforum* Thunb.

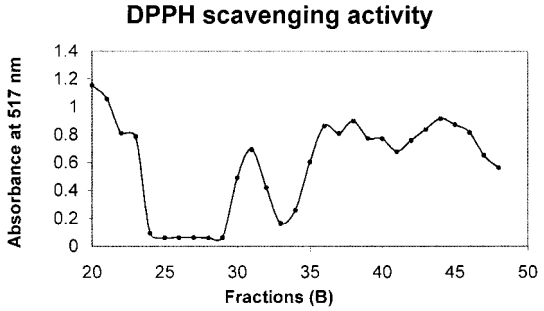

Figure 2. DPPH test for the 48 fractions from the second column chromatography of the combined active fractions of the first column chromatography.

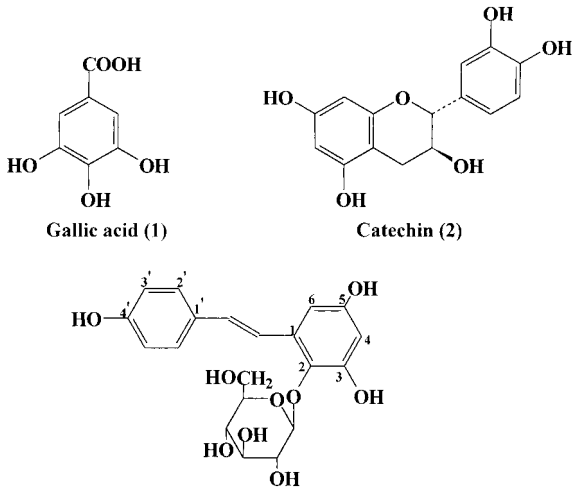

Figure 3. Structures of gallic acid, catechin, and 2,3,5,4′-tetrahydroxystilbene 2-*O*-β-D-glucopyranoside.

2-*O*-β-D-glucoside, conforming to the result obtained in the initial fractionation process. Gallic acid is a naturally occurring polyphenol with antioxidant capacity. It is essential to the antioxidant activity of black tea (Ho et al., 1992; Shiraki et al., 1994). However, there are reports that gallic acid has dual effects either as an antioxidant or as a prooxidant depending on its concentration (Yen and Hsieh, 1998; Aruoma et al., 1993). In this experiment, gallic acid showed the strongest DPPH radical scavenging activity with an IC_{50} of 10.60 μM compared to catechin and 2,3,5,4′-tetrahydroxystilbene 2-*O*-β-D-glucoside (Table 1). Catechin also exists naturally in a number of plants and has antioxidant activity. It exhibited a strong capacity for scavenging $^{\bullet}$OH free radical (Hanasaki et al., 1994) and suppressed

Table 1. IC_{50} in Scavenging DPPH Free Radicals

compound	IC_{50} (μM)
gallic acid	10.60
catechin	18.56
2,3,5,4′ -tetrahydroxystilbene 2-*O*-β-D-glucoside	33.24

cytotoxicity induced by H_2O_2 (Nakayama, 1994) and by iron, revealing its iron-chelating ability (Morel et al., 1993). 2,3,5,4′-Tetrahydroxystilbene 2-*O*-β-D-glucoside, regarded as the major active component for the medicinal effect of PM, also demonstrated a strong capacity for quenching DPPH radicals, although not as potent as that of gallic acid and catechin. It was first isolated from the root of PM by a research group in Japan (Kimura et al., 1983). Studies have shown that it can reduce the elevation of lipid levels (Arichi et al., 1982) and prevent liver injury induced by diets high in peroxidized lipids (Kimura et al., 1983).

In summary, the DPPH-directed fractionation and identification study has resulted in the identification of three compounds from the root of *P. multiflorum* Thunb. They all showed strong free radical scavenging activity. This not only helped further our understanding of the antioxidant activity of PM but also has an implication in the food industry for use as a natural antioxidant.

LITERATURE CITED

Arichi, H.; Kimura, Y.; Ojuda, H.; Baba, K.; Kozawa, M.; Arichi, S. Effects of stilbene components of the roots of *Polygonum cuspidatum* Sieb et Zucc. on lipid metabolism. *Chem. Pharm. Bull.* **1982**, *30*, 1766−1770.

Aruoma, O. I.; Murcia, A.; Butler, J.; Halliwell, B. Evaluation of the antioxidant and prooxidant action of gallic acid and its derivatives. *J. Agric Food Chem.* **1993**, *41*, 1880−1885.

Chen, C. W.; Ho, C.-T. Antioxidant properties of polyphenols extracted from green and black teas. *J. Food Lipids* **1995**, *2*, 35−46.

Chen, K. J.; Zhang, W. P. Advances on anti-aging herbal medicines in China. *Abstr. Chin. Med.* **1987**, *1*, 309−330.

Dinis, T. C.; Maderia, V. M.; Almeida, L. M. Action of phenolic derivatives (acetaminophen, salicylate, and 5-aminosalicylate) as inhibitors of membrane lipid peroxidation and as peroxyl radical scavengers. *Arch. Biochem. Biophys.* **1994**, *315*, 161−169.

Foo, L. Y.; Newman, R.; Waghorn, G.; McNabb, W. C.; Ulyatt, M. J. Proanthocyanidins from *Lotus corniculatus*. *Phytochemistry* **1996**, *41*, 617−624.

Gottlieb, H. E.; Kumar, S.; Sahai, M.; Ray, A. B. Ethyl brevifolin carboxylate from *Flueggea microcarpa*. *Phytochemistry* **1991**, *30*, 2435−2438.

Hanasaki, Y.; Ogawa, S.; Fukui, S. The correlation between active oxygens scavenging and antioxidative effects of flavonoids. *Free Radical Biol. Med.* **1994**, *16*, 845−850.

Ho, C.-T.; Chen, Q.; Shi, H.; Zhang, K. Q.; Rosen, R. T. Antioxidative effect of polyphenol extract prepared from various Chinese teas. *Prev. Med.* **1992**, *21*, 520−525.

Hong, C. Y.; Lo, Y. C.; Tan, F. C.; Wei, Y. H.; Chen, C. F. Astragalus membranceus and *Polygonum multiflorum* protect rat heart mitochondria against lipid peroxidation. *Am. J. Chin. Med.* **1994**, *22*, 63−70.

Ip, S. P.; Tse, A. S. M.; Poon, M. K. T.; Ko, K. M. Antioxidant activities of *Polygonum multiflorum* Thunb., *in vivo and in vitro*. *Phytother. Res.* **1997**, *11*, 42−44.

Kimura, Y.; Ohminami, H.; Okuda, H.; Baba, K.; Kozawa, K.; Arichi, S. Effects of stilbene components of roots of *Polygonum spp.* on liver injury in peroxidized oil-fed rats. *Planta Med.* **1983**, *49*, 51−54.

Morel, I.; Lescoat, G.; Cogrel, P.; Sergent, O.; Pasdeloup, N.; Brissot, P.; Cillard, P.; Cillard, J. Antioxidant and iron-chelating activities of the flavonoids catechin, quercetin and diosmetin on iron-loaded rat hepatocyte cultures. *Biochem. Pharmacol.* **1993**, *45*, 13−19.

Nakayama, T. Suppression of hydroperoxide-induced cytotoxicity by polyphenols. *Cancer Res.* **1994**, *54*, 1991s−1993s.

Nonaka, G.-I.; Miwa, N.; Nishioka, I. Stilbene glycoside gallates and proanthocyanidins from *Polygonum multiflorum*. *Phytochemistry* **1982**, *21*, 429−432.

Shiraki, M.; Hara, Y.; Osawa, T.; Kumon, H.; Nakayama, T.; Kawakishi, S. Antioxidative and antimutagenic effects of theaflavins from black tea. *Mutat. Res.* **1994**, *323*, 29−34.

Wang, M. F.; Li, J. G.; Rangarajan, M.; Shao, Y.; LaVoie, E. J.; Huang, T.-C.; Ho, C.-T. Antioxidantive phenolic compounds from sage (*Salvia officinalis*). *J. Agric. Food Chem.* **1998**, *46*, 4869−4873.

Xiao, P. G.; Xing, S. T.; Wang, L. W. Immunological aspects of Chinese medicinal plants as antiaging drugs. *J. Ethnopharmacol.* **1993**, *38*, 167−175.

Yen, G. C.; Hsieh, C. L. Antioxidant activity of extracts from Du-zhong (*Eucommia ulmoides*) toward various lipid peroxidation models *in vitro*. *J. Agric. Food Chem.* **1998**, *46*, 3952−3957.

Received for review February 5, 1999. Revised manuscript received March 23, 1999. Accepted March 28, 1999.

JF990092F