Isolation and Identification of Myricitrin, an Antioxidant Flavonoid, from Daebong Persimmon Peel

In-Wook Hwang¹ and Shin-Kyo Chung²

¹Department of Food Science and Nutrition, Dong-A University, Busan 49315, Korea
²School of Food Science and Biotechnology, Kyungpook National University, Daegu 41566, Korea

ABSTRACT: In this study, the antioxidant flavonoid, myricitrin, was isolated and identified from Daebong persimmon peel. The persimmon peel extract was successively fractionated with n-hexane, ethyl acetate, and n-butanol. The ethyl acetate fraction had the strongest antioxidant activities among the solvent fractions and was further fractionated by silica gel and octadecylsilane column chromatography, and preparative high performance liquid chromatography. Three antioxidant compounds were finally isolated, and compound 2 was identified as myricitrin by liquid chromatography/mass spectrometry and ¹H and ¹³C nuclear magnetic resonance. Myricitrin had the strongest antioxidant activities by ferric ion reducing antioxidant power and α,α-diphenyl-2-picrylhydrazyl radical scavenging assays. These results suggested that myricitrin was found as a major antioxidant flavonoid responsible for the strong antioxidant activities of Daebong persimmon peels.

Keywords: antioxidant, myricitrin, persimmon peel, LC/MS, NMR

INTRODUCTION

Free radicals and oxidative stress have been associated with many diseases including atherosclerosis, diabetes mellitus, neurodegenerative diseases, and virus infections (1). Flavonoids are capable of preventing free radical and oxidative stress, thus stopping oxidative damage (2,3). Consequently, flavonoids have become of interest for contemporary health care.

In this respect, persimmon (Diospyros kaki), having various biological effects including antioxidant, anti-diabetic, and anti-cancer (4,5), is one of the useful resources to obtain the natural antioxidant flavonoids. It contains abundant phenolic acids, vitamins, and flavonoids including catechin, epicatechin, and gallocatechin (6). Among the persimmon varieties, Daebong persimmon (Diospyros kaki L. cv. Hachiya) is one of the astringent-type varieties consumed as dried product after removing peels. Consequently, a large amount of persimmon peels are discarded at the production areas. Some researchers had reported persimmon health-benefits include hypocholesterolemic (7), anti-tumor (8), and anti-diabetic effects (9). We also reported the physicochemical properties and pretreatment of Daebong persimmon peel for functional food materials (10), and studied the enzymatic hydrolysis process of Daebong persimmon peels for vinegar fermentation (11).

In this study, we isolated the antioxidant compounds from Daebong persimmon peel by antioxidant activity-guided fractionation and identified the chemical structure by instrumental analyses, including high performance liquid chromatography (HPLC), liquid chromatography/mass spectrometry (LC/MS), ¹H and ¹³C nuclear magnetic resonance (NMR).

MATERIALS AND METHODS

Preparation of Daebong persimmon peel powder and chemicals
Daebong persimmon peels were collected at the production area (Gwangyang, Korea). Persimmon peels were dried in the shade and ground in a roller mill (C.W. Bra-bender Instruments, Inc., South Hackensack, NJ, USA) at 1,000 rpm with a 0.5-mm screen. α,α-Diphenyl-2-picrylhydrazyl (DPPH), 2,4,6-tris(2-pyridyl)-1,3,5-triazine (TPTZ), gallic acid, and Trolox were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). All organic solvents were of analytical grade (Merck KGaA, Darmstadt, Germany), except for HPLC, LC/MS (J.T. Baker Chemical
Co., Phillipsburg, NJ, USA), and NMR (Cambridge Isotope Laboratories, Andover, MA, USA).

**Extraction and isolation of antioxidant compounds**

The isolation scheme of antioxidant from Daebong persimmon peel is shown in Fig. 1. Briefly, persimmon peel powders were extracted with 80% ethanol under reflux for 12 h. This ethanol extract was filtered and concentrated to remove ethanol using rotary evaporation. The resultant extract was successively fractionated with n-hexane, ethyl acetate, and n-butanol. The n-hexane, ethyl acetate, and n-butanol extracts were separately evaporated, whereas the aqueous layer was lyophilized to dryness. Among them, greater antioxidant activity was observed in the ethyl acetate fraction compared with other fractions. The ethyl acetate fraction was subjected to silica gel column chromatography, and was eluted with solvents of increasing polarities. Fraction 2 was further isolated using octadecylsilane (ODS) column chromatography. Finally, fraction 2 was purified by preparative HPLC, and compounds 1, 2, and 3 were obtained. Isolated compounds were analyzed using a NMR spectrometer (400 MHz for $^1$H NMR and 100 MHz for $^{13}$C NMR; Bruker Avance Digital 400, Bruker, Karlsruhe, Germany). Chemical shift ($\delta$) for $^1$H and $^{13}$C NMR are recorded in parts per million (ppm) relative to solvent signals (methanol-d$_4$: $\delta$$_H$ 3.30 and $\delta$$_C$ 49.0) as internal standards.

**Instrumental analyses**

UV/visible (Vis) analysis was conducted to confirm the polyphenol type of fractions using UV/Vis spectrophotometer (UV 1601 PC, Shimadzu Co., Kyoto, Japan) in the wavelength range of 200 $\sim$ 600 nm. LC/MS analysis was conducted to identify the molecular weight of isolated compounds. Determination was performed with a 6410 Triple Quadrupole LC/MS system (Agilent Technologies). Electro spray ionization-MS was performed in the positive ion mode (600°C, 20 psi). Data were collected using a mass scan from 0 to 460 m/z, at 1.5 s per scan. $^1$H and $^{13}$C NMR spectra of isolated compounds were recorded on a NMR spectrometer (400 MHz for $^1$H NMR and 100 MHz for $^{13}$C NMR; Bruker Avance Digital 400, Bruker, Karlsruhe, Germany). Chemical shift ($\delta$) for $^1$H and $^{13}$C NMR are recorded in parts per million (ppm) relative to solvent signals (methanol-d$_4$: $\delta$$_H$ 3.30 and $\delta$$_C$ 49.0) as internal standards.

**Antioxidant activities**

To test the antioxidant activities, DPPH radical scavenging activity (12) and ferric ion reducing antioxidant power (FRAP) assays (13) were conducted. The DPPH radical scavenging activities of the extracts and fractions were determined by measuring the decrease in absorbance of methanolic DPPH solution at 517 nm in the presence of the sample. The initial concentration of DPPH was 0.1 mM and the reading was taken after allowing the solution to stand for 30 min. In cases where the absorbance decreased too much (when the solution turned yellow) before the 30 min period, the sample was appropriately diluted. The results are expressed in $\mu$M gallic acid equivalents ($\mu$M GAE).

The FRAP assay was performed using TPTZ solution. The working solution was prepared by mixing 25 mL of acetate buffer (pH 3.6), 2.5 mL of TPTZ solution (10 mM), and 2.5 mL of FeCl$_3$·6H$_2$O solution (20 mM), and...
warmed at 37°C before every determination. Then, 175 µL of this working solution was mixed with 25 µL of the sample solution. After incubation at 37°C for 30 min in the dark, the absorbance of the sample solution was measured at 590 nm. Results are expressed in µM trolox equivalents (µM TE). All of the tests were performed in triplicate.

**Statistical analysis**
The values were expressed as the mean±standard deviation. The results were evaluated through analysis of variance with Duncan’s multiple range test \((P<0.05)\) using Statistical Analysis System software version 9.3 (SAS Institute Inc., Cary, NC, USA).

**RESULTS AND DISCUSSION**

**Isolation of antioxidant compounds**
Isolation process of antioxidant compounds is shown in Fig. 1. The extraction yield was 17.5 g of extract per 100 g of persimmon peel powder. The ethanol extract was fractionated with \(n\)-hexane, ethyl acetate, and \(n\)-butanol by their polarity. The yields of fractions including \(n\)-hexane fraction, ethyl acetate fraction, \(n\)-butanol fraction, and aqueous fraction were 365 mg, 1.45 g, 3.24 g, and 12.16 g, respectively. Among them, the ethyl acetate fraction showed the strongest antioxidant activity. Thus, antioxidant compounds of ethyl acetate fraction were isolated in further experiments. The ethyl acetate fraction was separated with chloroform/methanol by step-wise elution using a silica gel column chromatography, and three fractions were obtained. Among them, fraction 2 showed the strongest antioxidant activity and the highest amount (975.8 mg). Thus, fraction 2 was separated by ODS column chromatography, and fraction 2-1 (466.2 mg) and 2-2 (505.6 mg) were obtained. Finally, prep-HPLC was conducted to completely separate with Fr. 2-2.

As a result, fraction 2-2 was separated to three compounds (compound 1, 35.2 mg; compound 2, 180.6 mg; compound 3, 274.3 mg). In HPLC chromatogram (Fig. 2), it was supposed that compound 2 was pure.

**Identification of isolated compound**
To identify the chemical structure of compound 2, UV/Vis absorption spectrum, LC/MS, \(^1\)H and \(^{13}\)C NMR
analyses were conducted. The instrumental data are shown in Fig. 3 and described as follows:

Compound 2 (myricitrin, myricetin 3-O-rhamnoside; 180.6 mg). UV, $\lambda_{\text{max}}$ nm 257, 360 (MeOH); LC/MS, m/z 303, 465 (M+H)$^+$; $^1$H NMR (in CD$_3$OD), $\delta$ 6.19 (1H, d, 1.8 Hz, H-6), 6.35 (1H, d, 2.3 Hz, H-8), 6.94 (2H, s, H-2', 6'), 5.30 (1H, d, 1.8 Hz, H-1'), 4.21 (1H, dd, 3.2, 1.8 Hz, H-2'), 3.76~3.78 (1H, dd, 9.4, 3.4 Hz, H-3'), 3.31~3.34 (1H, m, H-4'), 3.48~3.54 (1H, m, H-5'), 0.94~0.96 (3H, m, H-6'); $^{13}$C NMR (in CD$_3$OD), $\delta$ 159.2 (C-2), 136.1 (C-3), 179.5 (C-4), 163.1 (C-5), 99.7 (C-6), 164.0 (C-7), 94.6 (C-8), 158.4 (C-9), 105.6 (C-10), 121.7 (C-1), 109.6 (C-2', 6'), 146.7 (C-3', 5'), 137.7 (C-4'), 103.5 (C-1'), 71.7 (C-2'), 72.0 (C-3'), 73.2 (C-4'), 71.9 (C-5'), 17.5 (C-6').

UV/Vis absorption spectra of compound 2 showed specific absorption peaks at 257 and 360 nm (data not shown). This result indicated that compound 2 could be assumed as a flavonol (14,15). Molecular mass of compound 2 could be assumed as a flavonol (14,15). Molecular mass of compound 2 was 465 [M+H]$^+$, consistent with previous studies (16-18). The fragment ions at m/z 303 might be the aglycone ion from myricitrin whose rhamnosyl group was cleaved ([M-(C$_6$H$_{12}$O$_5$)+H]$^+$). Collision induction dissociation of the clusters led to the fragment at m/z 465, [M+H]$^+$, which, in turn, gave rise to the aglycone myricetin after rhamnose loss. Considering this result and previous research (18,20), compound 2 was identified as a myricitrin (Fig. 3). These NMR data were in good agreement with those of myricitrin whose rhamnosyl group was cleaved from myricitrin whose rhamnosyl group was cleaved (22). Myricitrin has three hydroxyl groups at 3', 4', and 5' positions. Rhamnose was attached at C-3 position, and it has three hydroxyl groups. Flavonoid glycosides such as astragalin, isouqueritin, and myricitrin are contained in persimmon leaves (21). In addition, myricitrin was also isolated from Nymphaea lotus (22), Chrysobalanus icaco (23), and Polygonum aviculare (24).

Antioxidant activities of isolated compounds

Antioxidant activities of isolated compounds were determined by DPPH radical scavenging activity and FRAP assay. The values of DPPH and FRAP were ranged from 105.18~165.75 µM GAE and 682.79~1,609.56 µM TE, respectively (Table 1). Compound 1 and 3 had similar activities and showed no significant differences. Compound 2, identified as a myricitrin, showed the strongest antioxidant activities. Thus, myricitrin is a compound that has the greatest impact on the antioxidant activities of Daebong persimmon peel. Myricitrin is rhamnose glycoside of myricetin contained in various plants, and it has been reported that has strong antioxidant activity as radical scavenger and metal-ion chelator (25). In addition, myricitrin has been reported to inhibit the aldose reductase activity (18) and the oxidation of low-density lipoprotein (26), and has anti-malarial effect (27). It also showed lower IC$_{50}$ values for ·OH and ·O$_2^-$ than ascorbic acid in radical scavenging activity by electron spin resonance measurement (20). The reason of these activities is that flavonoids having hydroxyl groups in the B-system.

**Table 1.** Antioxidant activities of isolated compounds from Daebong persimmon peels.

| Samples (1 mg/mL) | DPPH (µM GAE) | FRAP (µM TE) |
|------------------|---------------|---------------|
| Compound 1       | 138.27±3.46$^a$ | 703.09±90.18$^b$ |
| Compound 2       | 165.75±1.57$^a$ | 1,609.56±90.88$^a$ |
| Compound 3       | 105.18±5.03$^c$ | 682.79±40.87$^a$ |

DPPH, α,α-diphenyl-2-picrylhydrazyl radical scavenging activity; FRAP, ferric ion reducing antioxidant power; GAE, gallic acid equivalent; TE, trolox equivalent. Different letters (a-c) in a column are significantly different at $P<0.05$ by Duncan’s multiple range test.
ring can act as strong radical scavengers through formation of a hydrogen bond with the semiquinone radical of the B-ring (28). In a previous study, the pyrroloimidazolones in the B-ring of myricitrin also showed stronger inhibition effects of low-density lipoprotein oxidation induced by copper ion and \( \alpha,\alpha'-\text{azobis-(2-amidinopropane dihydrochloride than the } \text{O-dihydroxy-substituted flavonoid} \) (29). In terms of the importance of the B-ring, other researchers reported that myricetin reacts 6 times more quickly than quercetin to the galvinoxyl radical and concluded that flavonoid reactivity depends highly on the substitution pattern of OH groups in the B-ring (29). Moreover, flavanol glycosides showed stronger antioxidant activity than that of aglycones due to the number and localization of hydroxyl groups as well as hydrogen bonds (30). The role of the hydrogen bonds is to repair the damage in the structure of these flavonols when they donate hydroxyl hydrogens to free radicals. It is of crucial importance for human health because it can prevent the formation of new and perhaps more potent free radicals (31). Thus, myricitrin-rich persimmon peel can be utilized as a natural and strong antioxidant food material.

**AUTHOR DISCLOSURE STATEMENT**

The authors declare no conflict of interest.

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