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Phenolic Acids and Antioxidant Activities of Wild Ginseng (*Panax ginseng* C. A. Meyer) Leaves

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Abstract The compositions and antioxidant activities of free and hydrolyzed phenolic acids, which are aglycones of esterified phenolic acids, in wild ginseng leaves were investigated. The contents of free and hydrolyzed phenolic acids in the wild ginseng leaves were 422.4 ± 3.5 and 319.6 ± 5.7 mg/100 g, respectively, as gallic acid equivalents. Free phenolic acids were composed of 55.3% benzoic acid derivatives and 44.6% phenylpropanoids. The major constituents of free phenolic acids in the ginseng leaves were syringic (139.4 mg/100 g) and sinapic (131.2 mg/100 g) acids. On the other hand, hydrolyzed phenolic acids in the ginseng leaves were mainly composed of caffeic (59.4 mg/100 g), ferulic (49.5 mg/100 g), and *p*-coumaric (33.8 mg/100 g) acids. Phenylpropanoid content was higher (82.7%) than benzoic acid derivatives (17.3%). IC_{50} values of DPPH radical scavenging activity were 10.2 μg/mL for free phenolic acids and 8.0 mg/mL for hydrolyzed phenolic acids, as gallic acid equivalents. Hydrolyzed phenolic acids also exhibited higher hydroxyl and superoxide radical scavenging activities than free phenolic acids did. These results indicated that the antioxidant activities of the wild ginseng leaves were correlated more closely with phenylpropanoid contents than with total amount of phenolics.

Key words: phenolic acids, antioxidant activities, ginseng leaves, phenylpropanoid

Introduction

Panax ginseng C. A. Meyer (the *Araliaceae* family) is a valuable herb in East Asia that has also gained popularity in western countries because of its pharmacological properties (1-3). *Panax ginseng* is categorized as either cultivated or wild, according to different nurturing methods. Cultivated ginseng is systematically farmed on open land and harvested after 5-6 years when the growth rate and concentration of the active chemical constituents have peaked (4). On the contrary, wild ginseng is planted as seedlings in secluded mountain areas at an altitude between 800 to 1,500 m. Wild ginseng has a slower growth rate and is more sensitive to environmental changes than cultivated ginseng, showing a preference for areas with fluctuating daily temperatures and less exposure to direct sunlight. These differences may produce variations in the active compounds between cultivated and wild ginseng.

It is widely accepted in Korea and China that wild ginseng possesses more active pharmaceutical properties than the cultivated variety. However, only a few studies have been conducted to compare the pharmacological activities of the two types (5). Since most studies have focused on ginseng roots, scientists are less attracted by ginseng leaves. Traditionally, ginseng leaves have been utilized as a tea (6). The biochemical and pharmacological studies of ginseng leaves have mainly concentrated on ginsenosides as effective components. However, non-ginsenoside components have recently received a great deal of attention for their antioxidant activities (7, 8).

Phenolic acids are reportedly present as three different forms of phenolic acids in plant: free, esterified and insoluble-bound (9, 10). For the effective utilization of ginseng leaves as a functional food material, quantitative information on the phenolic acids in ginseng leaves is essential. The purpose of this study was to determine the HPLC profiles of the phenolic acids, including free phenolic acids and aglycones of esterified phenolic acids, in wild ginseng leaves, and also to investigate their antioxidant activities.

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Materials and Methods

Chemicals 2,2-Diphenyl-1-picrylhydrazyl radical (DPPH), epicatechin, L-ascorbic acid, Folin-Ciocalteu reagent, 2-deoxy-D-ribose, xanthine, xanthine oxidase, and nitroblue tetrazolium (NBT) were purchased from Sigma Chemical Co. (St. Louis, MO, USA), as were the following acids: gallic, protocatechuic, gentisic, vanillic, hydrocaffeic, syringic, *p*-hydroxybenzoic, *p*-coumaric, *o*-coumaric, ferulic, sinapic and caffeic. Methanol, acetonitrile and water used for HPLC analysis were obtained from Fisher Scientific (Pittsburgh, PA, USA). All other reagents used in the study were of analytical grade.

Materials Wild *Panax ginseng* C. A. Meyer leaves were collected in late August 2002 from ginseng plants that had been grown for more than 12 years at a mountain in Inje-gun, Gangwon-do, Korea. The collected leaves were freeze-dried, ground to a fine powder through a 60-mesh sieve and kept frozen at -18°C in polyethylene bags during the study.

Extraction of phenolic acids Phenolic acids in the ginseng leaves were extracted according to the method described by Krygier et al (11). Briefly, 10 g of sample powder was extracted 3 times by homogenization (Polytron,

IKA, Germany, 13,000 rpm/20 min) in 30 mL of 80% cold acetone. After centrifugation, the combined supernatants were concentrated to 30 mL under vacuum at 40°C. The aqueous phase was adjusted to pH 2.0 (6 N HCl) and centrifuged to separate a cloudy precipitate. The clear supernatant was extracted 5 times with hexane at a hexane to aqueous phase ratio of 1:1 to remove free fatty acids and other lipid contaminants. The free phenolic acids were then extracted 3 times with diethyl ether-ethyl acetate (1:1, v/v) at a solvent to aqueous phase ratio of 1:1. The ether-ethyl acetate extracts were evaporated to dryness under vacuum at 30°C and then stored at -40°C prior to use. The esterified phenolic acids remaining in the aqueous phase were hydrolyzed with 4 N NaOH for 4 hr under nitrogen at room temperature. Hydrolysates were acidified to pH 2, and the liberated phenolic acids were extracted with diethyl ether-ethyl acetate (1:1, v/v) for analysis.

Determination of total phenolic content Total phenolic contents in the wild ginseng leaves were determined using Folin-Ciocalteu reagent according to the method of Singleton and Lamuela-Raventos (12), with some modifications. Briefly, 1mL of diluted extracts and 1.0 mL of 2-fold diluted Folin-Ciocalteu reagent were mixed. After 3-min reaction, 1.0 mL of 10% sodium carbonate was added. After 1 hr of reaction, the concentration of total phenolics was measured by reading the absorbance at 760 nm, and the reading was compensated to standard gallic acid.

Quantification of phenolic compounds High-performance liquid chromatography (HPLC) was used to determine individual phenolic acids in the wild ginseng leaves using a reversed-phase μ-Bondapak C_{18} column (3.9 mm × 300 mm, Waters, USA), PU-1580 pump, LG-1580-04 gradient, DG-1580-54 degasser and UV-2075 plus detector (JASCO, Tokyo, Japan). The sample volume was 20 μL, and the mobile phase consisted of 0.05% trifluoroacetic acid (A) and acetonitrile (B) with a flow rate of 0.7 mL /min. The following gradient elution was applied: 0-5 min, 2% (B); 5-10 min, 6% (B); 10-50 min, 40% (B); 50-55 min 40% (B); and 55-60 min, 0% (B). The phenolics were detected at 280 nm, and identified by comparing retention times with standard spikes.

DPPH radical scavenging activity Free radical scavenging activity of the wild ginseng leaves was measured by the DPPH method proposed by Brand-Williams et al. (13). Briefly, 100 mM solution of DPPH in ethanol was prepared and 1.0 mL of this solution was added to 0.5 mL of each extract solution at different concentrations. After 20 min, the absorbance was measured at 525 nm. Epicatechin and L-ascorbic acid were used as controls. The DPPH radical scavenging activity was calculated according to the equation below. IC_{50} values were obtained through extrapolation from linear regression analysis and they signified the concentration of sample necessary to scavenge 50% of the DPPH free radicals.

DPPH radical scavenging activity (%) = $[(A_0-A_1)/A_0] \times 100$,

where A_0 is the absorbance of the control, and A_1 is the absorbance in the presence of the test compound.

Superoxide anion radical scavenging activity Superoxide anion radical generated by xanthine/xanthine oxidase system was determined spectrophotometrically by monitoring the NBT product (14). The reaction mixture was prepared by 1.0 mL of 0.05 M phosphate buffer (pH 7.4), 0.04 mL of 3 mM xanthine, 0.04 mL of 3 mM EDTA, 0.04 mL of 0.15% bovine serum albumin, 0.04 mL of 15.0 mM NBT and 0.04 mL of sample solution. After incubation at 25°C for 10 min, the reaction was started by adding 0.04 mL of 1.5 U/mL xanthine oxidase and carried out at 25°C for 20 min. After 20 min, the absorbance of the reaction mixture was measured at 560 nm. Decreased absorbance of the reaction mixture indicates increased superoxide anion scavenging activity.

Superoxide anion radical scavenging activity (%) = $[(A_0-A_1)/A_0] \times 100$,

where A_0 is the absorbance of the control, and A_1 is the absorbance in the presence of the test compound.

Hydroxyl radical scavenging activity Competition between deoxyribose and the sample against hydroxyl radical generated from the Fe^{3+}/ascorbate/EDTA/H_2O_2 system was measured to determine the hydroxyl radical scavenging activity (15). The reaction mixture consisted of 0.30 mL of 0.02 M sodium phosphate buffer (pH 7.0), 0.15 mL of 10 mM 2-deoxyribose, 0.15 mL of 10 mM $FeSO_4$, 0.15 mL of 10 mM EDTA, 0.15 mL of 10 mM H_2O_2, 0.525 mL of H_2O, and 0.075 mL of sample solution. The reaction was started by the addition of hydrogen peroxide. After incubation at 37°C for 2 hr, the reaction was stopped by adding 0.75 mL of 2.8% trichloroacetic acid and 0.75 mL of 1.0% thiobarbituric acid. After boiling the mixture for 20 min followed by ice-cooling, the absorbance was measured at 520 nm. Hydroxyl radical scavenging ability was evaluated as the inhibition rate of 2-deoxyribose oxidation by hydroxyl radical.

Hydroxyl radical scavenging activity (%) = $[(A_0-A_1)/A_0] \times 100$,

where A_0 is the absorbance of the control, and A_1 is the absorbance in the presence of the test compound.

Results and Discussion

Phenolic acid contents in the wild ginseng leaves This study was designed to investigate the profiles of free and hydrolyzed phenolic acids from the wild ginseng leaves extracted by solvent extraction and alkali hydrolysis. Total phenolic acid contents of free and hydrolyzed phenolic acids from the wild ginseng leaves were determined (data not shown). The concentrations of free and hydrolyzed phenolic acids were 422.4 and 319.6 mg gallic acid equivalent/100 g dry weight, respectively. The contents of free phenolic acids were slightly higher than those of

hydrolyzed phenolic acids in the ginseng leaves. The major free phenolic acids identified in the wild ginseng leaves were protocatechuic, *p*-hydroxybenzoic, vanillic, *p*-coumaric, syringic, hydrocaffeic and sinapic acids (Fig. 1 (A)). Syringic and sinapic acids were the major constituents in free phenolic acids of the wild ginseng leaves and their contents were 139.4 and 131.2 mg/100 g, respectively (Table 1). The total percentage distribution of benzoic acid derivatives and phenylpropanoids in the wild ginseng leaves were 55.3 and 44.6%, respectively.

Figure 1 (B) is an HPLC chromatogram of hydrolyzed phenolic acids from esterified phenolic acids in the ginseng leaves. Six phenolic acids were present in the ginseng leaves. Caffeic (59.4 mg/100 g), *p*-coumaric (33.8 mg/100 g) and ferulic (49.5 mg/100 g) acids were the principal phenolic aglycones liberated from their esterified phenolic acids in the ginseng leaves (Table 1). Most of the phenolic acids in hydrolyzed phenolic acids existed as phenylpropanoids. The percentage distributions of benzoic acid derivatives and phenylpropanoids of hydrolyzed phenolic acids in the wild ginseng leaves were 17.3 and 82.7%, respectively. These results were consistent with a previous report indicating that phenylpropanoids are either bound to cell wall polymers or occur in simple esters (16).

Table 1. Individual phenolic acid contents in wild ginseng leaves

Name	tR (min)[1)]	Free phenolic acids (mg/100 g of leaves)	Hydrolyzed phenolic acids[2)] (mg/100 g of leaves)
Benzoic acids			
Protocatechuic	8.8	1.63	t[3)]
p-Hydroxybenzoic	11.8	8.16	t
Vanillic	15.9	23.45	2.53
Syringic	18.8	139.40	29.47
Cinnamic acids			
Hydrocaffeic	13.2	3.12	t
Caffeic	16.5	-	59.47
p-Coumaric	19.6	4.98	33.88
Ferulic	22.3	-	49.56
Sinapic	24.0	131.23	10.21
Total		311.97	185.12

[1)]tR: retention time.
[2)]Determined as a hydrolysate from esterified phenolic acids.
[3)]t: trace amount (less than 1 mg/100 g dry ginseng leaves).
All values are dry basis. Values are means of three determinations.

Antioxidant activities of phenolic acids As shown in Table 2, free and hydrolyzed phenolic acids from the wild ginseng leaves exhibited good antioxidant activity. Hydrolyzed phenolic acids from the ginseng leaves had an IC_{50} value of 8.0 μg/mL for DPPH radical scavenging activity, as gallic acid equivalent. The IC_{50} of the free and hydrolyzed phenolic acids were 21.9 and 17.0 μg/mL for hydroxyl radicals, respectively. Both types of phenolic acids were also able to scavenge superoxide anion radicals generated from the xanthine/xanthine oxidase system. Hydrolyzed phenolic acids from the ginseng leaves were more efficient than free phenolic acids in terms of superoxide radical scavenging activities.

The antioxidant activity of phenolic acids arises from the hydroxyl substitution reactivity on the aromatic ring of the phenol moiety. Previous reports demonstrated that the difference in antioxidant capacity could be ascribed to individual molecular structures. The following are some possible explanations: increasing the number of hydroxyl groups may increase antioxidant activity (17); but insertion of a methoxy group in monophenols may decrease the antioxidant activity (18); the presence of the -CH=CH-COOH groups in phenylpropanoids may increase antioxidant activity more than the carboxylate group in benzoic acids (19, 20). For example, caffeic, sinapic, ferulic, and p-coumaric acids were found to be more active than protocatechuic, syringic, vanillic, and p-hydroxybenzoic acids (19). From this study, hydrolyzed phenolic acids showed higher antioxidant activities and higher

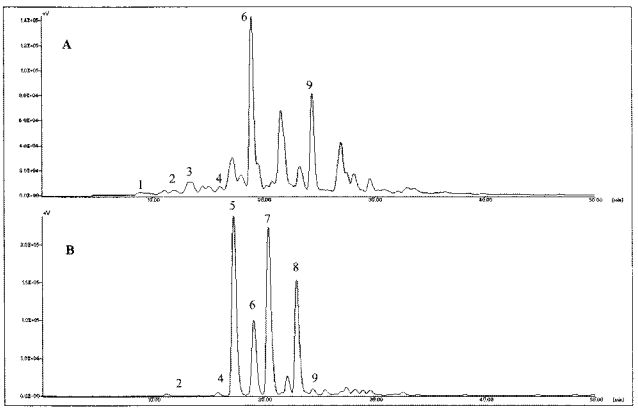

Fig. 1. HPLC chromatograms of free (A) and hydrolyzed phenolic acids (B) from wild ginseng leaves at 280 nm. Peaks: 1. protocatechuic acid; 2. *p*-hydroxybenzoic acid; 3. hydrocaffeic acid; 4. vanillic acid; 5. caffeic acid; 6. syringic acid; 7. *p*-coumaric acid; 8. ferulic acids; 9. sinapic acid.

Table 2. Radical scavenging activities of phenolic acids[1)] from wild ginseng leaves

Phenolic compounds	DPPH (μg gallic acid equivalent/mL)	OH · (μg gallic acid equivalent/mL)	O_2^{-} (μg gallic acid equivalent/mL)
Free phenolic acids	10.23	21.96	119.71
Hydrolyzed phenolic acids[2)]	8.01	17.06	110.21
Epicatechin	4.12	15.02	54.12
L-Ascorbic acid	14.01	19.12	88.13

[1)]All values are dry basis.
[2)]Determined as a hydrolysate from esterified phenolic acids.
Values are means of three determinations. Each amount is represented as an IC_{50} value.

phenylpropanoid content; 44.6% for free phenolic acids vs. 82.7% for hydrolyzed phenolic acids. These results indicated that the antioxidant activities were highly correlated with the ratio of phenylpropanoids to benzoic acid derivatives.

Recently, it has been shown that phenylpropanoids can protect low-density lipoprotein (LDL) from oxidative modifications (21, 22) and thereby reduce atherogenesis (23). They also exhibit inhibitory effects on tumor promotion (24) and can block the formation of mutagenic compounds such as nitrosamine (25). It has been known that the vast proportion of phenylpropanoids present in the diet exists in the ester form, which cannot be absorbed through the gastrointestinal tract wall. However, it has also been demonstrated that the microbial esterase present in mammal intestines (rats and humans) can release free phenolic acids (ferulic, caffeic, and *p*-coumaric acids) into the lumen, and that free phenylpropanoids can be absorbed into the circulatory system (26). Even though most phenylpropanoids are present as the esterified form in wild ginseng leaves, they could be absorbed by enzymatic degradation in the gastrointestinal tract. With this consideration, the consumption of wild ginseng leaves may play a role in the prevention of free radical-involved human diseases.

In conclusion, this study identified and quantified 9 different phenolic acids in free and hydrolyzed phenolic acids from wild ginseng leaves by HPLC. Hydrolyzed phenolic acids with higher phenylpropanoid contents showed higher radical scavenging activities. These results confirmed that the antioxidant activities of phenylpropanoids are more effective than those of benzoic acid derivatives.

Acknowledgments

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