Food Sci. Biotechnol. Vol. 16, No. 3, pp. 349 ~ 354 (2007)

Food Science and Biotechnology

Identification of Phenolic Compounds and Quantification of Their Antioxidant Activities in Roasted Wild Ginseng (*Panax ginseng* C.A. Meyer) Leaves

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Abstract The objectives of this study were to systemically identify phenolic compounds in roasted wild ginseng (*Panax ginseng* C.A. Meyer) leaves and investigate their radical scavenging activities. Seven phenolic compounds were identified by NMR (H, C, COSY, HMQC, HMBC) and mass (EI-MS, FAB-MS) analyses: 5-caffeoylquinic acid, kaempferol, quercetin, 3,4-dihydroxy-benzoic acid, 4-hydroxy-benzoic acid, 3-(3,4-dihydroxyphenyl)-2-propenoic acid, and 3-(4-hydroxy-3-methoxyphenyl)-2-propenoic acid. Their concentrations ranged from 0.4 (3,4-dihydroxy-benzoic acid) to 7.5 mg (kaempferol) per 100 g of roasted leaves. Among these compounds, 5-caffeoylquinic acid, kaempferol, and quercetin were found exclusively in the leaf portions of the ginseng plants. When their antioxidant activities were measured by DPPH and superoxide anion radical scavenging activity, quercetin, and kaempferol were most effective.

Keywords: roasted wild ginseng leaf, kaempferol, quercetin, antioxidant activity

Introduction

Panax ginseng C.A. Meyer is categorized as either cultivated or wild, according to different nurturing methods. Cultivated ginseng is systematically grown in open land with proper control of sunlight by a shield. Generally, it is harvested after 5-6 years of cultivation when conditions such as growth rate and active chemical constituent concentrations become optimal (1). In contrast, wild ginseng is planted by seeding it in secluded mountain areas at altitudes between 800 and 1,500 m. Wild ginseng is slower in growth, but more sensitive to environmental changes than cultivated ginseng. Such differences account for the different spectrums of active compounds between cultivated and wild ginsengs. It is widely accepted in both Korea and China that wild ginseng has more potent pharmaceutical activity than the cultivated variety. Our previous study revealed that wild ginseng leaves exhibited stronger antioxidant activities than cultivated leaves (2).

Most studies have focused on the antioxidant activities of fresh ginseng leaves. Traditionally, however, *P. ginseng* leaves have been consumed through a conventional tea making process (3). The roasting step in the tea making process is necessary to improve the quality of the leaf tea by inhibiting the activities of deteriorating enzymes such as polyphenol oxidase (PPO). We previously reported that an adequate roasting process did not cause any particular changes in the phenolic compounds (4).

In the present study, we isolated and identified the phenolic compounds in roasted wild ginseng leaves, as well as investigated their antioxidant activities.

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Received September 1, 2006; accepted March 7, 2007

Materials and Methods

Chemicals 2,2-Diphenyl-1-picrylhydrazyl radical (DPPH), xanthine, xanthine oxidase, bovine serum albumin, nitroblue tetrazolium (NBT), epicatechin, and ascorbic acid were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Methanol and water (HPLC grade) were purchased from Honeywell International Inc. (Muskegon, MI, USA). All other chemicals used were of analytical reagent grade.

Materials The fresh wild ginseng leaves were collected in late August, 2002 from ginseng plants that were grown for more than 12 years at a mountain in Inje-gun, Gangwon-do, Korea. As soon as the leaves were collected they were subjected to a tea making process. The fresh leaves were roasted in a stainless steel kettle for 5 min at 160°C. Then, they were immediately cooled at room temperature and rolled into a typical tea shape. These procedures were repeated 3 times. The leaves were then ground in a mill, passed through a 60-mesh sieve, and stored in a freezer until further analysis.

Sequential partition by organic solvents Three hundred g of the ground leaves were extracted twice with boiling water for 10 min, followed by filtration through Whatman No. 2 paper. The water extract was consecutively partitioned in a separatory funnel with equivalent amounts of *n*-hexane, chloroform, ethyl acetate (EtOAC), and *n*-butanol. All fractions were concentrated in a vacuum evaporator and redissolved in methanol. All the extracts were then tested for DPPH radical scavenging activity.

Preparation of EtOAC fractions The EtOAC fraction, which showed the highest DPPH scavenging activity of the sequentially partitioned organic solvents, was further fractionated to a EtOAC-soluble neutral fraction and an

aqueous fraction by adding 0.2 M phosphate buffer solution (pH 8.0). Then, the aqueous fraction was adjusted to pH 3.0 with 1.0 N HCl and further extracted with EtOAC to obtain the EtOAC-soluble acidic fraction. Both the EtOAC-neutral and acidic fractions were passed through a Sepabeads SP-850 column (ion exchange resin; Mitsubishi, Tokyo, Japan) and washed with distilled water. After washing, the adsorbates were eluted with 75% ethanol and concentrated to dryness.

Fractionation of EtOAC-soluble neutral fraction The EtOAC-soluble neutral fraction was subjected to further separation through a silica gel column (0.063-0.20 mm, 3× 30 cm, Merck, Darmstadt, Germany) by $CHCl_3$/MeOH with a stepwise gradient (Fig. 1). The fractions with the highest DPPH radical scavenging activity were further fractionated on Sephadex LH-20 (MeOH, 0.56 mL/min, Pharmacia, Piscataway, NJ, USA) followed by final purification through preparative HPLC on a reverse phase μ-Bondapak C_{18} column (7.8×300 mm, Waters, Milford, MA, USA). Preparative HPLC spectra were performed on a Jasco PU-1580 pump, with a LG-1580-04 gradient, DG-1580-54 degasser, and UV-2075 plus detector (Jasco, Tokyo, Japan). The mobile phases were (A) 0.05% phosphoric acid and (B) methanol, with a flow rate of 3.0 mL/min. Gradient elution was applied as follows: 0-25 min, 30-64.4% B; 25-30 min, 64.4-90% B; 35-40 min, 90-30% B. The active compounds were detected at 320 nm.

Fractionation of EtOAC-soluble acidic fraction The EtOAC-soluble acidic fraction was subjected to a silica gel column (0.063-0.20 mm, 3×30 cm, Merck) and eluted with a stepwise gradient of $CHCl_3$/MeOH (Fig. 1). Each fraction was tested for DPPH scavenging activity, and the

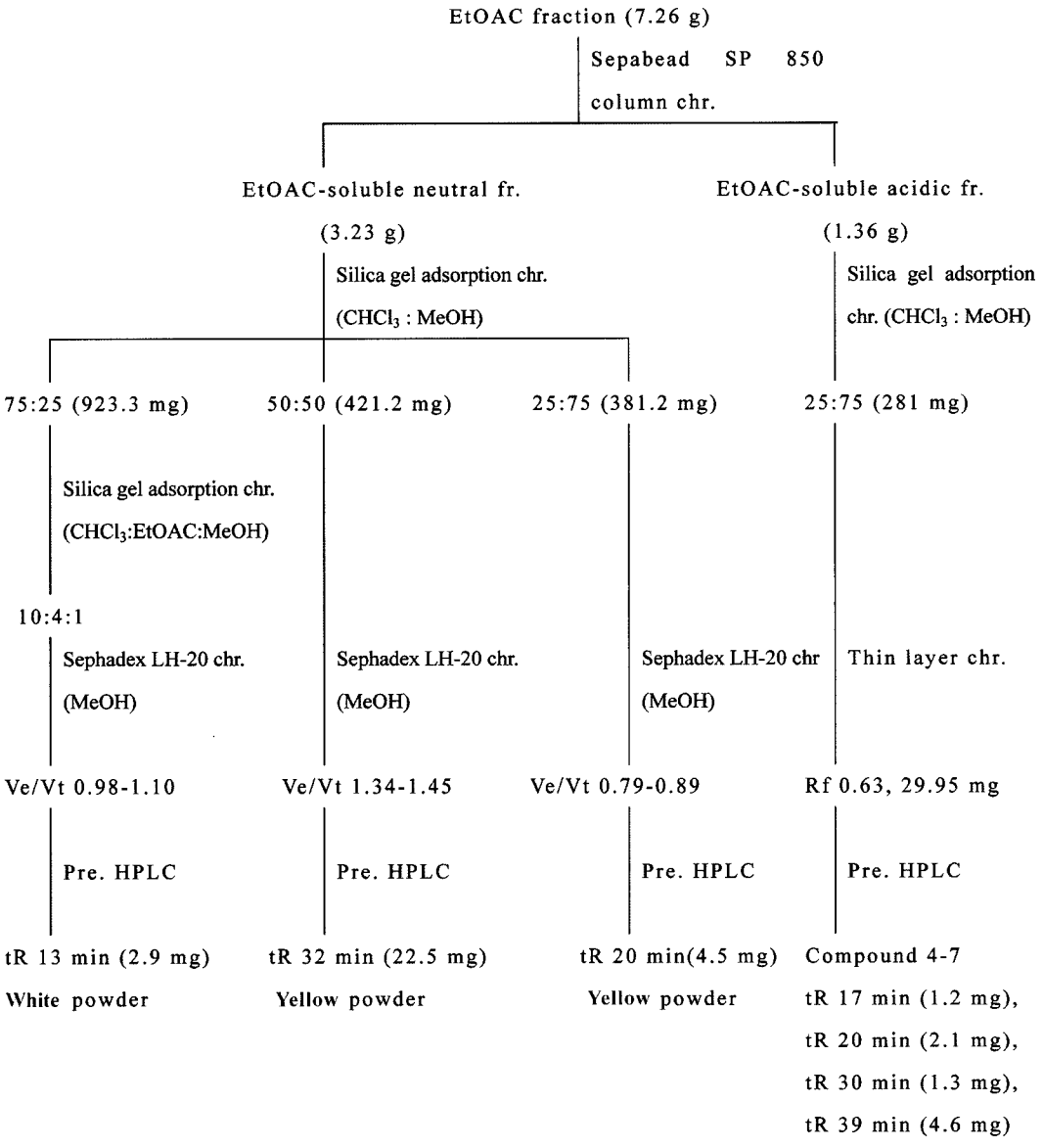

Fig. 1. Purification of phenolic compounds in the EtOAC-soluble neutral and acidic fractions of roasted wild ginseng leaves. Ve/Vt, elution volume/total volume; tR, retention time.

fraction with the highest antioxidant activity was further separated on TLC (silica gel 60 F_{254}, Merck) that was developed by toluene/EtOAC/formic acid (5/4/1, v/v/v) and visualized at 280 nm. Each spot was removed, redissolved in methanol, and tested for antioxidant activities. The most active spot (R_f 0.63) was further purified by preparative HPLC on a reverse phase μ-Bondapak C_{18} column (7.8×300 mm, Waters). The mobile phases were (A) 0.05% phosphoric acid and (B) methanol, with a flow rate of 3.0 mL/min. The gradient started from 98 to 50% of solvent A for 50 min. The sample volume was 0.1 mL and the eluate was observed at 280 nm.

EI-MS, FAB-MS, and NMR analyses Mass data were obtained with a JMS-AX 505WA mass spectrophotometer (Jeol Ltd., Tokyo, Japan). ^1H and ^{13}C NMR, ^1H-^1H COSY, HMQC, and HMBC data were obtained with a Bruker AMX-500 spectrometer (Bruker Analytik GmbH, Rheinstetten, Germany) operating at 500 MHz. The samples were dissolved in deuterated methanol (CD_3OD).

DPPH radical scavenging activity The free radical scavenging activity of the ginseng leaves was measured using the DPPH method proposed by Brand-Williams *et al.* (5). Briefly, a 0.1 mM solution of DPPH in ethanol was prepared, and 1.0 mL of this solution was added to 0.5 mL of each of the extracts of different concentrations. After 10 min of reaction time the absorbance was measured at 525 nm, and the reading was converted to DPPH radical scavenging activity (%) according to the follow equation;

$$\text{DPPH radical scavenging activity (\%)} = [1-(A_{\text{sample (525 nm)}}/A_{\text{control (525 nm)}})] \times 100$$

The ED_{50} value signified the concentration of sample necessary to scavenge 50% of the DPPH free radicals. ED_{50} values were obtained through extrapolation from linear regression analysis.

Superoxide anion radical scavenging activity The superoxide anion radical was generated by the xanthine/xanthine oxidase system and determined spectrophotometrically by monitoring the concentration of the NBT end product (6). The reaction mixture consisted of 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 indicated increased superoxide radical scavenging activity. The scavenging effect was calculated using the equation described for DPPH.

Results and Discussion

Identification of phenolic compounds in the EtOAC-soluble neutral fraction Compound **1** was obtained as a white powder, and the positive FAB-MS showed an $[M+H]^+$ ion peak at *m/z* 354. Final identification of this

Table 1. ^{13}C NMR and ^1H NMR data of compounds 1-3[1)]

Position	Compound 1		Compound 2		Compound 3	
	δC	δH	δC	δH	δC	δH
1	121.92					
2	115.37	7.06 (1H, d, 1.90)	148.21		148.21	
3	147.23		137.30		137.30	
4	149.71		177.54		177.54	
5	116.60	6.79 (1H, d, 8.17)	158.43		158.43	
6	123.13	6.97 (1H, dd, 1.90, 8.17)	99.44	6.18 (s)	99.44	6.18 (s)
7	146.93	7.57 (1H, d, 15.90)	165.82		165.82	
8	115.32	6.27 (1H, d, 15.90)	94.62	6.40 (s)	94.62	6.40 (s)
9	168.79		162.69		162.69	
10			104.82		104.82	
1'	76.25		123.89		123.89	
2'	38.88	217-2.25 (2H, m)	130.84	8.10 (d, 8.82)	116.11	7.57 (d, 9.00)
3'	71.39	4.19-4.17 (1H, br s)	116.46	6.92 (d, 8.83)	144.46	
4'	72.10	3.74 (1H, br d, 11.59)	160.73		148.73	
5'	73.57	5.34 (1H, br d, 4.28)	115.37	6.90 (d, 8.83)	115.37	6.85 (d, 9.10)
6'	38.33	2.08-2.10 (2H, br d)	129.72	8.08 (d, 8.82)	129.72	7.57 (d, 9.00)
7'	177.15					

[1)]Assignments confirmed by 2D COSY, HMQC, and HMBC experiments.

compound was obtained by NMR spectrometry using the 1D ^{1}H spectrum, and the heteronuclear 2D HMQC and HMBC correlation spectra (Table 1 and Fig. 2). The ^{1}H-NMR spectrum of compound **1** displayed proton signals located at *d* 7.57 (1H, d, *J*=15.90 Hz, H-7 caffeoyl), 7.06 (1H, d, *J*=1.90 Hz, H-2 caffeoyl), 6.97 (1H, dd, *J*=1.90 and 8.17 Hz, H-6 caffeoyl), 6.79 (1H, d, *J*=8.17 Hz, H-5 caffeoyl), 6.27 (1H, d, *J*=15.90 Hz, H-8 caffeoyl), 5.34 (1H, br d, *J*=4.28 Hz, H-5 quinic), 4.19-4.17 (1H, br s, H-3 quinic), 3.74 (1H, br d, *J*=11.59 Hz, H-4 quinic), 2.17-2.25 (2H, m, H-2 quinic), and 2.08-2.10 (2H, br d, H-6 quinic). From the comparison of these data with those reported by Azuma *et al.* (7), the compound was identified as 5-caffeoylquinic acid and its concentration was 0.93 mg/100 g of dried leaf tea.

Compound **2** was obtained as a yellow powder, and the positive FAB-MS showed an $[M+H]^+$ ion peak at *m/z* 287. From the EI-MS, the molecular formula $C_{15}H_{10}O_6$ was deduced for compound **2**. The complete structure of compound **2** was elucidated by 1D and 2D NMR experiments at 500 Hz. The ^{1}H-NMR spectrum of compound **2** displayed proton signals located at δ 6.18 (1H, s) and 6.40 (1H, s) ascribable to H-6 and H-8, the protons of ring A, and proton signals at δ 8.10 (1H, d, *J*=8.82 Hz, H-2'), 6.92 (1H, d, *J*=8.83 Hz, H-3'), 6.90 (1H, d, *J*=8.83 Hz, H-5'), and 8.08 (1H, d, *J*=8.82 Hz, H-6') indicative of a 4'-disubstitution of ring B. A low-field signal at 177.54 ppm in the ^{13}C NMR spectrum for C=O (C-4) along with the signals at 99.44 (C-6) and 94.62 (C-8) ppm are typical of a flavonol structure with a C-3 hydroxyl. The HMQC spectra established all of the correlations between the protons and carbons of compound **2**, whereas the HMBC spectrum showed relationships for H-2'/C-4', C-2 and C-6', and H-3'/C-5' and C-1', and H-6/C-10 and C-8 (Fig. 2). Thus, compound **2** was identified as kaempferol by comparisons of the MS and NMR spectra with the literature data (8, 9), and its concentration was 7.5 mg/100 g of dried leaf tea.

Compound **3** showed a UV spectrum typical of flavonols, with absorption bands at 257 and 370 nm (data not shown); the molecular weight of 302 was determined by FAB-MS. This fragment corresponded to the molecular ion of quercetin $[(M+H)^+$ at *m/z* 302]. From the EI-MS, the molecular formula $C_{15}H_{10}O_7$ was deduced for compound **3.** It was identified as quercetin by comparisons of the MS and NMR spectra with the literature data (8, 9). Its concentration was 1.5 mg/100 g of dried leaf tea.

Identification of phenolic compounds in the EtOAC-soluble acidic fraction The identification of 4 phenolic compounds from the acidic EtOAC fraction was performed by EI-MS techniques (Fig. 3). According to fragment signals of the NIST/EPA/MSDC library data, compound **4** was identified as 3,4-dihydroxy-benzoic acid with a 92% match. The molecular weight of compound **5** was estimated as *m/z* 138, and the other fragment ions were *m/z* 121 ($M-OH^+$) and 93 ($M-COOH^+$). According to the NIST/EPA/MSDC library data, compound **5** was suggested as 4-hydroxy-benzoic acid (95%). Compound **6** and **7** were identified as 3-(3,4-dihydroxyphenyl)-2-propenoic acid (94%) and 3-(4-hydroxy-3-methoxyphenyl)-2-propenoic acid (91%), because their fragment ions were identical to those of the NIST/EPA/MSDC library data. Our previous study using HPLC has proved the presence of these phenolic acids in wild ginseng leaves (10).

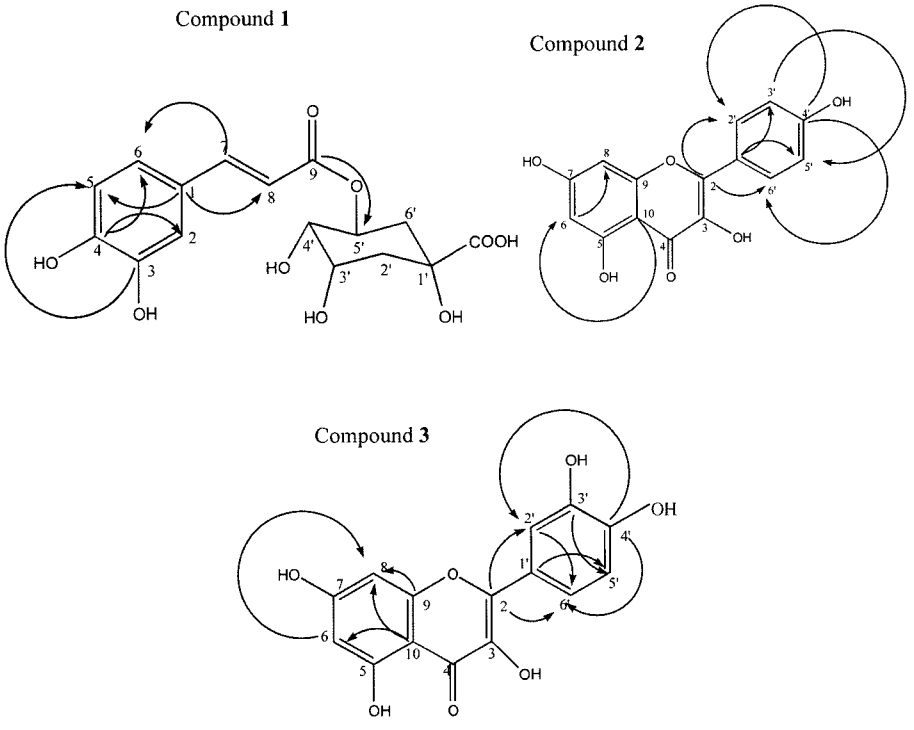

Fig. 2. Structures of the active compounds 1-3. Compound 1. 5-caffeoylquinic acid; 2. kaempferol; 3. quercetin.

Table 2. DPPH and superoxide anion radical scavenging activities of separated compounds from roasted wild ginseng leaves[1)]

Compound	DPPH ED_{50} (μM)	O_2^- ED_{50} (μM)[1)]
5-Caffeoylquinic acid (**1**)	111.1	212.1
Kaempferol (**2**)	49.7	72.8
Quercetin (**3**)	35.0	62.9
3,4-Dihydroxy-benzoic acid (**4**)	279.0	389.8
4-Hydroxy-benzoic acid (**5**)	608.3	1103.4
3-(3,4-Dihydroxyphenyl-2-propenoic acid (**6**)	79.3	167.1
3-(4-Hydroxy-3-methoxyphenyl)-2-propenoic acid (**7**)	329.9	546.6
Epicatechin	34.4	86.1
L-Ascorbic acid	79.5	170.3

[1)]ED 50 values were obtained and signified the concentration of sample necessary to scavenge 50% of DPPH and superoxide anion radicals.

Antioxidant activities of phenolic compounds in the EtOAC-soluble fractions As shown in Table 2, compound **1-7** were all able to reduce free radicals. The DPPH radical scavenging activities of the separated compounds in the EtOAC-soluble fraction, as compared to the positive controls (epicatechin and L-ascorbic acid), decreased in the order of epicatechin ≈ compound **3** > **2** > L-ascorbic acid ≈ compound **6** > **1** > **4** > **7** > **5**; their ED_{50}

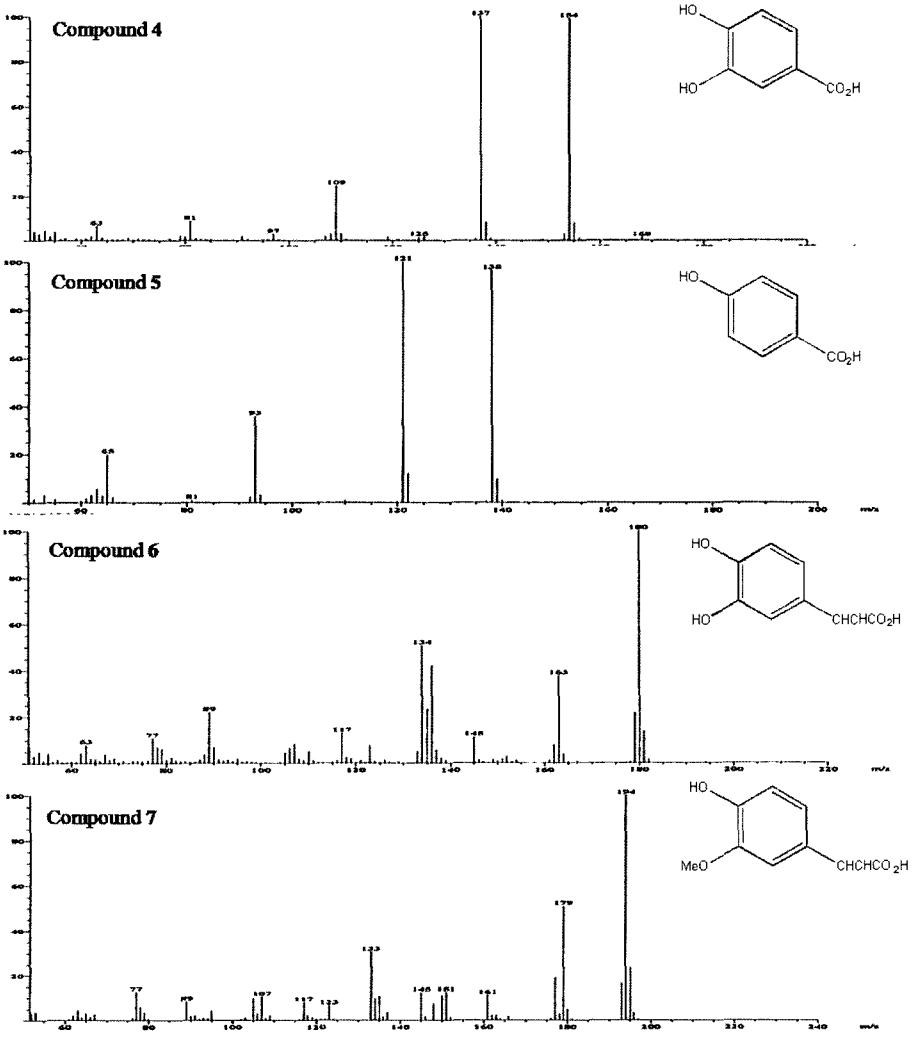

Fig. 3. EI-MS spectrum of the active compounds 4-7. Compound 4. 3,4-dihydroxy-benzoic acid; 5. 4-hydroxy-benzoic acid; 6. 3-(3,4-dihydroxyphenyl)-2-propenoic acid; 7. 3-(4-hydroxy-3-methoxyphenyl)-2-propenoic acid.

values were 34.4, 35.0, 49.7, 79.5, 79.3, 111.1, 279.0, 329.9, and 608.3 μM, respectively. When the superoxide anion radical scavenging activity of each compound was measured by the xanthine-xanthine oxidase system, compound **7** exhibited the highest superoxide anion radical scavenging activity. The ED_{50} value of compound **3** was 62.9 μM, which was almost equivalent to 86.1 μM for epicatechin.

The antioxidant activities of edible plants are primarily due to such phenolic compounds as flavonoids and phenolic acids (11). The differences in antioxidative activities between the phenolic acids and flavonols might be attributed to the number of hydroxyl groups in their molecular structures (12). Among the phenolic acids, 3-(3,4-dihydroxyphenyl)-2-propenoic acid showed a relatively higher radical scavenging activity, followed by 3,4-dihydroxy-benzoic, 5-caffeoylquinic, 3-(4-hydroxy-3-methoxyphenyl)-2-propenoic, and 4-hydroxy-benzoic acids, respectively. It has been found that phenyl-propanoids are more active antioxidants than benzoic acid derivatives, and a hydroxyl group in the ortho- or para-position increases the antioxidant activity, whereas the insertion of a methoxy group in monophenols decreases their antioxidant capacity (13). 5-Caffeoylquinic acid is the esterified form of caffeic acid, with quinic acid on the carboxyl group, and this esterification results in decreased antioxidant activity (14). The flavonoids contain a number of phenolic hydroxyl groups attached to ring structures, conferring their antioxidant activities. The ortho-dihydroxy structure in the B ring confers a higher stability to the radical form and participates in electron delocalization (15). Thus, the dihydroxylations in the 3' and 4' positions of the B ring of quercetin caused higher radical scavenging activity than the single hydroxyl group in the 4 position of kaempferol's B ring.

In conclusion, we isolated and identified phenolic compounds in roasted wild ginseng leaves, and our results represent the first report of systemical identification and quantification of these antioxidants. Finally, we found that two flavonoids, kaempferol, and quercetin, were the major compounds responsible for strong radical scavenging activities in roasted wild ginseng leaves.

Acknowledgments

This work was supported by a grant from Agricultural Research Promotion Center, Ministry of Agriculture and Forest, Korea.

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