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Antioxidant activities of cultivated and wild Korean ginseng leaves

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Abstract

Cultivated and wild ginseng leaves were examined for their various antioxidant activities. Both ginseng leaves were extracted with methanol and sequentially partitioned with solvents in an order of increasing polarity. Among various solvent extracts in cultivated and wild ginseng leaves, EtOAC extracts in both ginseng leaves showed the most powerful scavenging activities against DPPH radicals. Data on other antioxidant activities, measured by inhibition rates against lipid peroxidation and linoleate oxidation, revealed similar results, showing the highest activities in EtOAC extracts, followed by butanol, water, chloroform and hexane extracts, in both cultivated and wild ginseng leaves. EtOAC extracts of wild ginseng leaves contained more phenolics (9.71 g:4.87 g/100 g, dry basis) and flavonoids (3.03 g:2.34 g/100 g, dry basis) than cultivated ginseng leaves. When EtOAC extracts were acid-hydrolyzed, two aglycones of flavonoids, quercetin (0.43 and 0.66 g/100 g, dry basis) and kaempferol (1.23 and 1.50 g/100 g, dry basis) were detected in cultivated and wild ginseng leaves. These differences in concentrations of key antioxidants between two different ginseng leaves seemed to be responsible for their differences in antioxidant activities.

Keywords: Ginseng leaves; Antioxidant activities; DPPH radicals; Quercetin; Kaempferol

1. Introduction

Reactive oxygen species (ROS), including free radicals, such as superoxide anion radicals (O_2^-), hydroxyl radical species (OH·), singlet oxygen ($^1O_2$) and hydrogen peroxide (H_2O_2), are active oxygen species that are often generated by biological oxidation reactions of exogenous factors (Cruitti, 1991; Halliwell & Gutteridge, 1990). These ROS are known to cause aging, cancer and many other ill-effects to the human body (Aruma, 1994; Kehrer, 1993). There are many antioxidants that are introduced to minimize actions of ROS. For example, phenolic compounds can trap the free radicals directly or scavenge them through a series of coupled reactions with antioxidant enzymes (Lewis, 1993; Rao, Paliyath, & Ormrod, 1996).

Ginsengs have gained world-wide reputation for their various nutraceutical activities. Many studies have been conducted to elucidate "magical" healing activities of ginsengs. Although most studies have been focussed on ginseng roots, attention is now increasingly paid to ginseng leaves. Pharmaceutical activities of ginseng leaves mainly come from their abundant polysaccharides, phenolics, flavonoids and ginsenosides (Park, Choi, Boo, Kim, & Lee, 1990; Park, Kwak, Moon, Kim, & Chen, 2004; Xie et al., 2004).

There are two different types of ginseng that are widely consumed by Koreans, cultivated and wild ginseng. Cultivated ginseng is systematically cultivated on an open land with proper control of sunlight by a shield that helps to reduce 1/8–1/13 of total sunlight. Generally, they are harvested after a 5–6-year cultivation period. On the other hand, wild ginseng is cultivated by

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seeding in a deep mountain with an altitude of 800–1500 m. Wild ginseng is slower in growth but more sensitive to environmental changes than cultivated ginseng. At least 10 years are needed for them to grow 4–5 pieces of long leaves. They prefer to grow in an area that has fluctuating daily temperature with less exposure to direct sunlight. Those differences in cultivated periods, as well as cultivated environments may result in differences in composition of active compounds and antioxidant activities between cultivated and wild ginseng leaves.

The objectives of this study were to compare the antioxidant properties and contents, as well as compositions, of potential antioxidant between cultivated and wild ginseng leaves.

2. Materials and methods

2.1. Chemicals

2,2-Diphenyl-1-picryhydrazyl radical (DPPH·), BHT, epicatechin, quercetin, kaempferol, Folin & Ciocalteu's phenol reagent, gallic acid, thiobarbituric acid, sodium dodecyl sulfate and linoleic acid were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All other chemicals used were of analytical grade.

2.2. Sample preparation

The cultivated ginseng leaves were collected from 6-year-old ginseng plants in the KT&G Center Research Institute, in Gyunggi-do, Korea in late September 2002. The average temperature of the area throughout a year was 13.9 °C. The wild ginseng leaves were collected from over 12-year-old ginseng plants, in late August 2002, which had been cultivated on a mountain with an average temperature of 8.9 °C in Gangwon-do, Korea. The roasting process is necessary for inactivating enzymes and improving flavours of all processed ginseng leaf products. Therefore, roasting of ginseng leaves (moisture, 83–84%) at 160 °C for 5 min was performed before solvent extraction. Then the roasted leaves were cooled at room temperature for a short period of time and rolled. These procedures were repeated three times. The leaves were ground in a mill and passed through 60 mesh sieve. The powder was stored in a freezer for further experiments.

2.3. Sample extraction

One hundred grammes of leaves (60 mesh particle size) were twice extracted with methanol (200 ml) under reflux in water bath at 80 °C for 2 h and then filtered. The filtrate was combined and evaporated to dryness. The dry materials were re-dissolved with 100 ml of distilled water. The solution was consecutively partitioned in a separatory funnel with the equivalent amount of *n*-hexane, chloroform, EtOAC, and *n*-butanol. Each fraction was concentrated in a vacuum evaporator and re-dissolved in methanol to a concentration of 10 mg ml^{-1}.

2.4. Scavenging activity on DPPH radical

The free radical scavenging activity of methanol extracts and their solvents were measured by the 2,2-diphenyl-1-picryl-hydrazyl (DPPH) method proposed by Brand-Williams, Cuvelier, and Berset (1995). 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 samples in different concentrations. After 20 min, the absorbance was measured at 525 nm. The DPPH radical-scavenging activity was calculated according to the following equation.

$$\text{DPPH}^{\bullet}\text{ scavenging activity } (\%) = [(A_0 - A_1)/A_0] \times 100,$$

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

2.5. Antioxidant activity against oxidation of linoleate

The antioxidant activity was assayed using a linoleic acid model system (Osawa & Namiki, 1981). Four milligrammes of samples dissolved in 4 ml of 99.5% (w/v) ethanol were mixed with linoleic acid (2.5% v/v) in 99.5% (w/v) ethanol (4.0 ml), 0.05 M phosphate buffer (pH 7.0, 8.0 ml) and distilled water (4.0 ml) and the mixture was kept in a screw-cap container in the dark at 40 °C for 78 h. The amounts of produced thiobarbituric acid (TBA) were determined according to Sidwell, Salwin, Benca, and Mitchell (1954). One millilitre of this solution was added to 20% trichloroacetic acid (TCA) and 0.75% TBA solution (1.0 ml). This mixture was then placed in a boiling water bath at 100 °C for 30 min. After the mixture was cooled, 2.0 ml of *n*-butanol were added and the mixture was shaken vigorously. After centrifugation at 3000 rpm for 5 min, the organic layer was taken, and its absorbance at 532 nm was measured.

2.6. Antioxidant activity against formation of lipid peroxide

The reaction mixture was composed of 0.2 ml of 25% (w/v) rat liver homogenate in 40 mM Tris–HCl buffer (pH 7.0), 30 mM KCl, 0.5 mM ferrous iron, 0.06 mM ascorbic acid, and various concentrations of the samples in a final volume of 1.0 ml (Bishayee & Balasubramanian, 1971). The mixture was then incubated at 37 °C for 1 h. The lipid peroxide formation was measured by the method of Ohkawa, Oshishi, and Yagi (1979). For this, 0.4 ml of the reaction mixture was treated with

0.2 ml of 8.1% sodium dodecyl sulfate (SDS), 1.5 ml of 0.8% TBA, and 1.5 ml of 20% acetic acid solution adjusted to pH 3.5 with NaOH. The total volume was made up to 4 ml with distilled water and the mixture was kept in a water bath at 95 °C for 1 h. After the mixture was cooled, 1 ml of distilled water and 5 ml of *n*-butanol were added, followed by vigorous shaking. After centrifugation at 4000 rpm for 10 min, the organic layer was taken and its absorbance at 532 nm was measured. The inhibition rates against formation of lipid peroxide of rat liver were determined by comparing the absorbance of the sample-added groups with those of the controls in which no sample was added.

$$\text{Inhibition rates against formation of lipid peroxide (\%)} = [(A_0 - A_1)/A_0] \times 100,$$

where A_0 was the absorbance of the control groups and A_1 was the absorbance in the sample added groups.

2.7. Determination of total phenolic contents

Total phenolic contents of fractions were determined according to the method of Singleton and Lamuela-Raventos (1999) with minor modification. One millilitre of each fraction and 1.0 ml of diluted Folin–Ciocalteu reagent were mixed. After a 3-min incubation, 1.0 ml of 10% sodium carbonate was added and the mixture was incubated for 1 h. The absorbance at 760 nm was measured and converted to phenolic contents according to the calibration curve of gallic acid.

2.8. Determination of total flavonoid content

Total flavonoid content was determined by the colorimetric method described previously (Woisky & Salatino, 1998; Kumazawa, Hamasaka, & Nakayama, 2004). To 0.5 ml of ginseng leaf extracts, 0.5 ml of 2% $AlCl_3$ ethanol solution was added. After 1 h at room temperature, the absorbance was measured at 420 nm. Total flavonoid contents were calculated as kaempferol from a calibration curve.

2.9. Extraction and quantification of flavonoids

Flavonoid aglycone was extracted by the method of Hertog, Hollman, and Katan (1999). The EtOAC fractions (0.1 g) were weighed and dispersed in 40 ml of 62.5% aqueous methanol containing 2 g/l of 2 (3)-*tert*-butyl-4-hydroxyanisole (BHA). The mixture was then ultrasonicated for 5 min. To this extract, was added 10 ml of 6 M HCl with careful mixing. The sample was bubbled with nitrogen for 40–60 s, after which the flask was tightly sealed. Hydrolysis was carried out in a shaking water bath at 90 °C for 2 h. After hydrolysis, the extract was allowed to cool and filtered, and it was made up to 100 ml with methanol, followed by sonication for 5 min. The extract was filtered through a 0.2-μm membrane filter prior to injection into the HPLC. Chromatographic equipment consisted of a Jasco liquid chromatograph model equipped with PU-1580 pump, LG-1580-04 gradient, DG-1580-54 degasser and UV-2075 plus detector (JASCO, Tokyo, Japan). Samples were injected at ambient temperature into a reversed-phase μ-Bondapack column (3.9 mm × 300 mm, Waters). The mobile phases were: (A) 0.05% trifluoroacetic acid in water and (B) 0.05% trifluoroacetic acid in acetonitrile with a flow rate of 0.7 ml/min. Gradient elution was applied as follows: 0–16 min, 5% (B); 16–22 min, 14% (B); 22–50 min, 35% (B); 50–55 min 50% (B); and 55–60 min, 0% (B). The flavonoids were detected at 370 nm.

2.10. Statistical analysis

All experimental data were analyzed using analysis of variance (ANOVA) and significant differences among means from triplicate analysis at ($P < 0.05$) were determined by Duncan's multiple range test using the statistical analysis system (SAS).

3. Results and discussion

Fig. 1 shows the DPPH radical-scavenging activity of various solvent extracts from cultivated and wild ginseng leaves with various concentrations. As a positive control, epicatechin extraction was also examined for DPPH radical scavenging activities. EtOAC extracts of both cultivated and wild ginseng leaves showed the best results through all concentrations. Among the other sol-

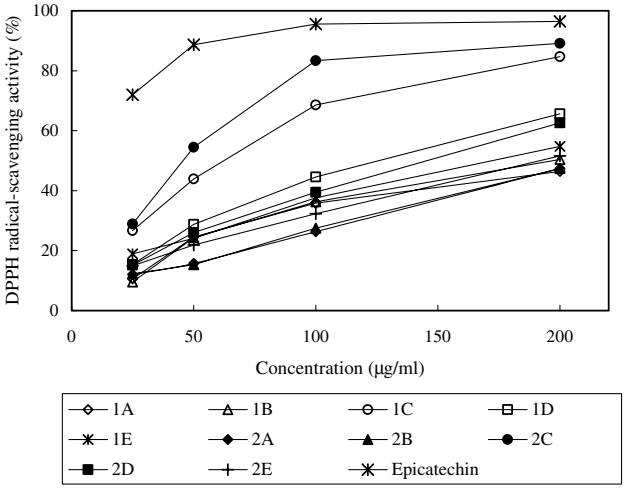

Fig. 1. Changes in DPPH radical scavenging activities according to different concentration of solvent extracts from cultivated (1) and wild ginseng leaves (2). A, hexane fraction; B, chloroform fraction; C, EtOAC fraction; D, butanol fraction; E, water fraction.

vent extracts, butanol extracts seemed relatively effective in scavenging activity, followed by water, chloroform and hexane extracts, in both cultivated and wild ginseng leaves. Within the same solvent extract groups, extracts of wild ginseng leaves exhibited better (not statistically significant) radical-scavenging activities than those of cultivated ginseng leaves. At the concentration of 25 μg/ml, significant differences in scavenging activities were observed between epicatechin (71.9%) and EtOAC extracts of both cultivated (26.6%) and wild ginseng leaves (28.9%). However, as concentration of EtOAC extracts in the assay system increased, the differences in scavenging activities between epicatechin and EtOAC extracts became less significant. A close to linear correlation between DPPH radical-scavenging activity and concentrations of polyphenolic compounds in various vegetable and fruits has been reported (Pyo, Lee, Logendra, & Rosen, 2004; Robards, Prenzeler, Tucker, Swatsitang, & Glover, 1999). These reports indicated that the radical-scavenging capacity of extracts might be mostly affected by the presence and position of the phenolic hydroxyl group. The anti-radical activity of phenolic compounds depends on their molecular structure, that is, on the availability of phenolic hydrogens and on the possibility of stabilization of the resulting phenoxyl radicals formed by hydrogen donation (Catherine, Rice-Evans, Nicholas, & George, 1996; Ramarathnam, Ochi, & Takeuchi, 1997).

When linoleic acid was readily oxidized by incubating it at 40 °C for up to 78 h, concentrations of TBARS that were measured at 532 nm were abruptly increased after 20 h (Fig. 2). However, addition of EtOAC extracts of both cultivated and wild ginseng leaves effectively suppressed production of TBARS. Their suppressing activities on TBARS were even better than those of epicatechin. Eberhardt, Lee, and Liu (2000) reported that a hydrophilic antioxidant was less effective in an oil-in-water emulsion system, whereas the opposite was true for the hydrophobic antioxidants, such as phenolic acids, flavonols and flavanones.

Formation of lipid peroxide in livers of SD rats was induced by mincing liver tissues and adding a mixture of $FeCl_2$ and H_2O_2. The amounts of oxidative products after incubation with solvent extracts, BHT and epicatechin were measured and compared (Fig. 3). As previously observed, EtOAC extracts were most efficient in preventing formation of oxidative products in all solvent extract groups. But their inhibiting activities were not as efficient as epicatechin. At less than 150 μg/ml, EtOAC extracts of wild ginseng leaves were better than BHT in suppressing formation of oxidative products from linoleic acid. Generally, as observed through all experiments, wild ginseng leaves exhibited more powerful antioxidant activities than cultivated ginseng leaves.

The next step was performed to see the compositional differences in active compounds between cultivated and wild ginseng leaves. Total phenolic and flavonoid contents of various solvent extracts from both ginseng leaves are summarized in Table 1. EtOAC extracts, which exhibited the best antioxidant activities in all sol-

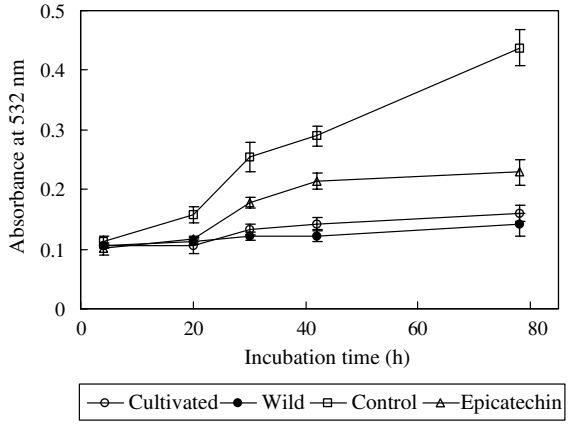

Fig. 2. Changes in autoxidation rate of linoleic acid on EtOAC extracts from cultivated and wild ginseng leaves.

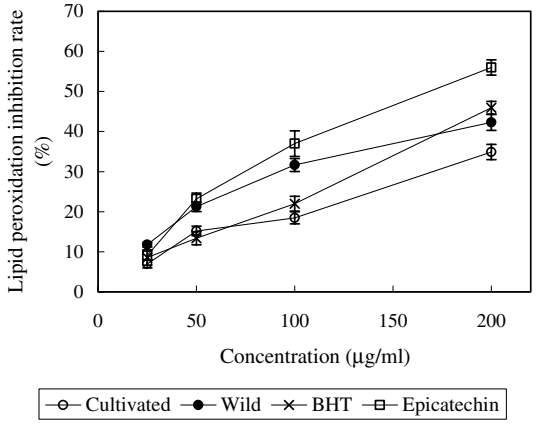

Fig. 3. Changes in lipid peroxiation inhibition activities according to different concentration of EtOAC extracts from cultivated and wild ginseng leaves.

Table 1
Comparison of total flavonoid and phenolic contents between various extracts of cultivated and wild ginseng leaves extracts (g/100 g, dry basis)

Extracts	Cultivated		Wild	
	Flavonoids[a]	Phenolics[b]	Flavonoids[a]	Phenolics[b]
n-Hexane	–	0.33 ± 0.01	–	0.31 ± 0.03
Chloroform	0.07 ± 0.01	1.32 ± 0.11	0.08 ± 0.01	0.69 ± 0.23
EtOAC	2.34 ± 0.06	4.87 ± 0.58	3.03 ± 0.05	9.71 ± 1.01
n-Butanol	1.76 ± 0.10	1.45 ± 0.20	1.58 ± 0.07	1.31 ± 0.51
Water	0.21 ± 0.01	1.03 ± 0.19	0.12 ± 0.02	0.83 ± 0.01

Values are means of three replicates ± SD.
–, not detected.
[a] As equivalent to kaempferol.
[b] As equivalent gallic acid.

vent extracts of cultivated and wild ginseng leaves, possessed the highest concentrations of polyphenols and flavonoids. EtOAC extracts of wild ginseng leaves had higher concentrations of total phenolics and flavonoids (9.71 and 3.03 g/100 g, respectively) than those of cultivated ginseng leaves (4.87 and 2.34 g/100 g, respectively). When EtOAC extracts were acid-hydrolyzed, two flavonoid aglycones, quercetin and kaempferol, were identified (Fig. 4). When concentrations of two flavonoid aglycones were compared between the two ginseng leaves, considerably higher amounts of both quercetin (0.66:0.43 g/100 g) and kaempferol (1.50:1.23 g/100 g) were found in wild ginseng leaves (Table 2). More efficient antioxidant activity in wild ginseng leaves originate from the presence of higher concentrations of such potent antioxidant compounds.

Flavonoids are one of the most powerful antioxidants found in plants. Typically, they possess one or more of the following structural elements that are considered important to their antioxidant activities: an *o*-diphenol group in ring B; a 2–3-double bond conjugated with the 4-oxo function, and hydroxyl groups at positions 3 and 5 (Rice-Evans, Miller, & Pagana, 1996). The antioxidant activities of ginseng leaf can be contributed by phenolic compounds, such as quercetin and kaempferol aglycones or glycosides. As shown throughout this study, wild ginseng leaves were better in scavenging against free radicals and inhibiting oxidation reactions occurring in lipid than cultivated ginseng leaves. To understand and compare antioxidant properties between the two ginseng leaves, we plan to isolate each phenolic compound from the two ginseng leaves and investigate its characteristic antioxidant properties.

Table 2
Concentrations of quercetin and kaempferol in EtOAC extracts of cultivated and wild ginseng leaves (g/100 g, dry basis)

Components	Cultivated	Wild
Quercetin	0.43 ± 0.02	0.66 ± 0.03
Kaempferol	1.23 ± 0.07	1.50 ± 0.02

Values are means of three replicates ± SD.

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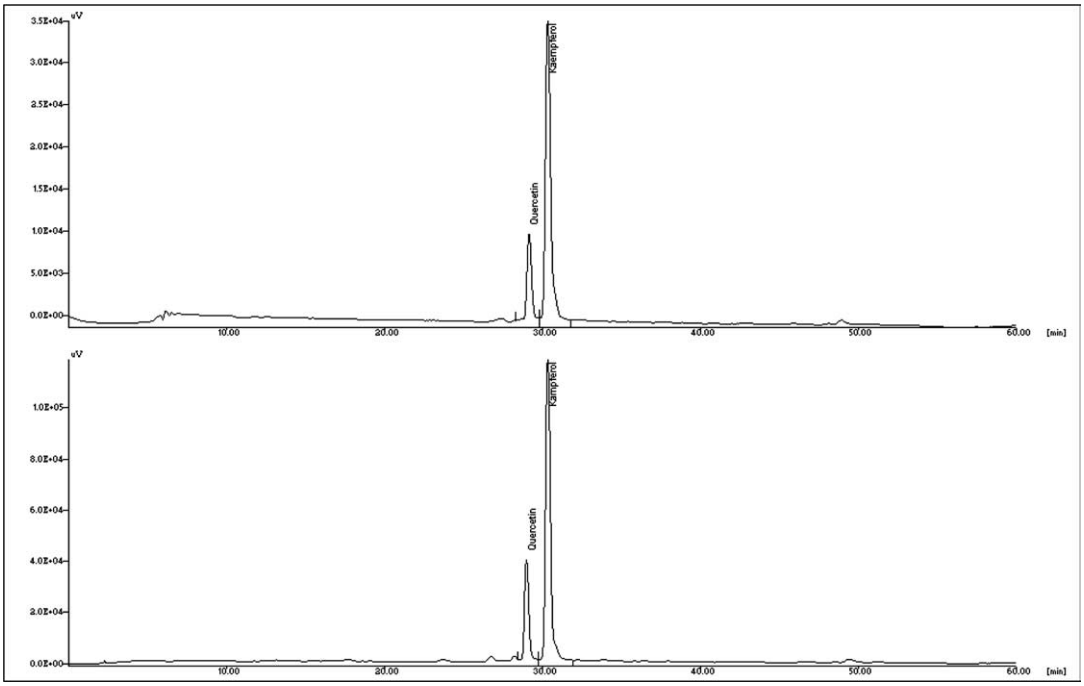

Fig. 4. HPLC chromatograms of aglycons of flavonoids in EtOAC extracts form cultivated and wild ginseng leaves.

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