Radical Scavenging Activity of Spring Mountain Herbs in the Shikoku Mountain Area and Identification of Antiradical Constituents by Simple HPLC Detection and LC-MS Methods

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The functionality of spring mountain herbs, which were collected in the Kajigamori mountain area of Shikoku area in Japan, was investigated in the course of our studies for utilizing local plant resources. The radical scavenging activity of the extracts from seventeen herbs was measured. Among these herbs, two extracts from Polystichum ovato-paleaceum (Japanese name: Tsuyanashiinode) and Sambucus racemosa subsp. sieboldiana (Japanese name: Niwatoko) showed potent DPPH radical scavenging activity. The material evidence for the potent activity of the extracts was studied by a combination of our developed method for detecting antiradical compounds, LC-MS/MS, and enzymatic hydrolysis.

Key words: mountain herb; radical scavenging activity; Polystichum ovato-paleaceum (Tsuyanashiinode); Sambucus racemosa subsp. sieboldiana (Niwatoko)

Most of the local communities in Japan have travel inconvenience and scarce industry. The result is that these communities have lost their viability due to resident's aging and young people leaving, making it impossible to maintain the living environment. Various approaches, including regional science research, to revitalize such communities are needed to solve this social problem. It should be remembered that, despite the inconvenience and lack of industry, the bio-resources around the local communities, which have supported the traditional way of life, have remained intact. Among these inherent bio-resources, we have focused on edible mountain herbs and investigated useful functions of the herbs for their application to revitalize the local communities. People in the mountain areas that are typical of many local communities used to collect and use as possible industrial products of the mountain areas. We collected 17 species of the mountain herbs in the Kajigamori area of Shikoku according to information about their edibility, and surveyed their radical scavenging activity as indicative of their antioxidant effects and health benefits.

We also identified the potent antiradical constituents in the active herb extracts to obtain material evidence for the radical scavenging activity. We paid particular attention in this study to the required sample scale and adopted suitable methods for small sample amounts. Collecting a large quantity of mountain herbs is difficult in natural mountain areas, because the herbs are sparsely spread and involve collection rights due to recent environmental conservation.

Materials and Methods

Plant materials. Fresh samples of mountain herbs were collected from Kajigamori Park in Otoyoo-cho (Kochi Prefecture, Japan) in March 2011. Their adopted scientific names were selected from three plant dictionaries. 2–4

Instruments. MS and MS² data were obtained by a Xevo QTof MS instrument (ESI mode, Waters Japan, Tokyo, Japan) equipped with an Acuity UPLC system (Waters). MS² is one of the automated MS/MS methods developed by Waters, and MS² data were obtained by the simultaneous acquisition of mass at both high and low collision energy. 5) 1H-NMR data were measured with an ECS-400 spectrometer (JEOL, Tokyo, Japan). Analytical HPLC was performed with an LC-10 gradient system equipped with an SPD-M10AVP Photodiode array (PDA) detector (Shimadzu, Kyoto, Japan). Preparative HPLC was performed with an LC-6A system (Shimadzu).

Preparation of the plant extracts. The collected samples of fresh mountain herbs were cut into pieces and immersed in methanol for 5 d at 23°C. After filtration, each residue was immersed again in methanol for an additional 2 d. The obtained filtrates were combined and evaporated in vacuo to dryness to afford an extract.

DPPH radical scavenging assay. The radical scavenging activity of each extract was assessed by the previously reported method. 6) Briefly, 100 μL of a sample in methanol (2.5 mg/mL) and a 5 mM DPPH (2,2-diphenyl-1-picrylhydrazyl) methanol solution were successively added to 4.8 mL of methanol. The resulting solution was allowed to stand for 30 min at 25°C, before its absorbance at 517 nm was measured. Activity was evaluated by the decrease in absorbance compared to that in a control experiment. This control experiment was similarly conducted without the sample, and blank data were obtained from the sample solution without the addition of DPPH.

Selective detection of the DPPH radical scavenging compound. The method of Masuda et al. 7) was used for this detection. In brief, to a solution (100 μL) of the sample in methanol (2.5 mg/mL) was added 120 μL of a DPPH methanol solution (10 mM). The mixture was stirred and then allowed to stand for 5 min at 25°C. Twenty μL of the solution was injected for analysis by HPLC under the following conditions:

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column, ODS-80Ts (4.6 x 150 mm, Tosoh, Tokyo, Japan); solvent A, 1% acetic acid in H₂O; solvent B, methanol; gradient conditions, solvent B 5%–100% (40 min)–100% (10 min); flow rate, 0.5 mL/min; detection, 280 and 330 nm. The decreased peak intensity was detected by comparing with HPLC data for the non-reacted extract.

**MS and MS² measurement of the active peak compounds.** The same HPLC conditions were employed as those used in the previous experiment except for an injection volume of 10 μL. The MS and MS² data for the active peaks were obtained under the following conditions: capillary voltage, 3.0 kV (ESI positive) or 2.6 kV (ESI negative); cone voltage, 3.0 kV; capillary temperature, 250°C; desolvation temperature, 400°C; cone gas flow rate, 50 L/h; desolvation gas flow rate, 800 L/h; MS² low-collision energy, 6 V; MS² high-collision energy, from 20 to 150 V. The elemental composition of each peak compound was calculated by Mass Lynx software (V. 4.1, Waters) from the high-resolution MS data of the corresponding protonated, deprotonated or ion-adducted molecular ion.

**Photo isomerization of chlorogenic acid.** Chlorogenic acid (60 mg, Nacalai Tesque, Kyoto, Japan) was dissolved in a 0.2 M phosphate buffer (pH 6.8, 12 mL). The solution was poured into three glass Petri dishes (10 cm diameter), and the dishes were placed on ice under sunlight for 1.5 h. The solution was separated by preparative HPLC to give small sample for measurement. This antiradical (radical scavenging) activity is well-known to be closely related to the antioxidant activity that provides useful functions from edible plant resources.

**Results and Discussion**

Edible mountain herbs were collected from Kajigamori Park in the Kajigamori area of Otoyō Town, which was well-known religious holy area, but now is a typically depopulated rural part of Shikoku. The identified plant names (both scientific and Japanese), collected part of plants and weights, and extract weights are listed in Table 1. Large-scale collection of the mountain herbs was difficult, so we chose evaluation by the radical scavenging activity which needed only a small sample for measurement. This antiradical (radical scavenging) activity is well-known to be closely related to the antioxidant activity that provides useful functions from edible plant resources.

Figure 1 shows the activity of the methanol extracts from 17 mountain herbs which was obtained by the DPPH radical scavenging assay. Expressed by the percentage scavenging activity, itadori (P. cuspidatum), iwabako (C. ramonoides), uwabamisou (E. umbellatum var. majus), tsuyanashinio (P. ovato-paleaceum), tebakomomijigasa (C. tebakoensis), nagabamomiijichigo (R. palmatis), niwato (S. racemosa subsp. sieboldiana), fuki (P. japonicus), momijigasa (C. delphiniifolia), yamaajisai (H. serratum), and ryoubu (C. barbinervis) each showed potent activity at a concentration of 50 μg/mL (Fig. 1A). Re-measurement of these plant extracts at a lower concentration (10 μg/mL) revealed tsuyanashinio (P. ovato-paleaceum) and niwato (S. racemosa subsp. sieboldiana) to both have very potent activity (Fig. 1B). There have been limited reports on the radical scavenging activity and related antioxidant activity of edible mountain herbs in scientific literature so far published. The radical scavenging activity of various fern plants has been reported by Ding et al. and although tsuyanashinio (P. ovato-paleaceum) belongs to the fern family, no activity of tsuyanashinio itself has yet been cited. The antioxidative properties of western niwato (S. racemosa subsp. sieboldiana) has been investigated by Dawidowicz and coworkers, however, no report on the antioxidative activity of Japanese niwato (S. racemosa subsp.
The potent antiradical activity of these two herbs, which was apparent at a concentration of 10 mg/L, was comparable to that of Okinawan medicinal herbs. The efficiency of this activity was estimated to be more potent than that of most aqueous extracts from various edible plants, and of organic solvent extracts from some medicinal and aromatic plants, which have recently been investigated, suggesting that these two herbs have possible health-promoting functions as antioxidant-related agents.

These two edible mountain herbs could be selected for useful plant resources possessing potent radical scavenging ability. Both researchers and food consumers nowadays require scientific evidence for the useful functions of food stuffs, so we next identified the main antiradical substances in the two extracts as the evidence for their potent activity. It is well-known that plants have various secondary metabolites including antiradical compounds. An efficient and highly selective detection method for the potent antiradical compound in a raw extract is therefore required. We have developed a selectable method for detecting DPPH radical scavenging compounds in crude plant extracts. This method is notable for requiring a small sample size and only a conventional HPLC instrument, and also can rank the radical scavenging efficiency of various antiradical constituents in an extract. Figures 2 and 3 respectively show the detection data for tsuyanashiinode (P. ovato-paleaceum) and niwatoko (S. racemosa subsp. sieboldiana). In Fig. 2, the three peaks at retention times of 16.2, 19.3, and 20.3 min were substantially decreased by DPPH addition, indicating these peaks (1 at 16.2 min, 2 at 19.3 min, and 3 at 20.3 min) should have the strongest radical scavenging activity in the extract of tsuyanashiinode (P. ovato-paleaceum). In Fig. 3, the three peaks at retention times of 4.4 min (peak 4), 19.4 min (peak 5) and 23.8 min (peak 6) were also decreased by DPPH addition, indicating these to be the peaks for the potent antiradical constituents of niwatoko (S. racemosa subsp. sieboldiana).

The detected peak compounds (Fig. 4) were mainly identified by the LC-MS method under the same HPLC analytical conditions. Peak compound 1 showed a deprotonated molecular ion at m/z 341.0876 in the ESI-negative mode, revealing 1 to have the molecular formula of C_{15}H_{18}O_{9}. In addition to this ion peak, two fragment peaks at m/z 179.0351 (C_{9}H_{7}O_{4}) and m/z 161.0224 (C_{9}H_{5}O_{3}), and its UV spectrum [\lambda_{\text{max}} 332, 310 (sh) nm], which was obtained by a photo diode array (PDA) detector with the HPLC instrument, suggested the presence of a caffeoyl group in 1. The elemental composition of the remaining structure was calculated to...
be C₆H₁₁O₆. This element count and relatively faster retention time of I in the HPLC data strongly indicated the remaining structure to be a typical water-soluble biomolecule such as hexose. To confirm the presence of hexose, we applied an enzymatic hydrolysis reaction to the crude extract to detect the hydrolyzed aglycon (caffeic acid). Figure 5 shows the analytical HPLC data for the tsuyanashiinode (P. ovato-paleaceum) extract enzymatically hydrolyzed by β-D-glucosidase. Peak 1 disappeared during the reaction and a new peak at a retention time of 21.5 min appeared. This 21.5-min peak was identified to be caffeic acid by comparison with an authentic sample. These data confirmed I to be a β-D-glucoside of caffeic acid. The glucosidic linkage was determined by clear observation of the C₉H₅O₃ fragment ion in the MS² mode (an automated MS/MS measurement method), which was derived by cleavage of the ester bond (CO–O) of the caffeoyl moiety. Peak
compound 1 was thus identified as depicted by structure 1 (Fig. 4). This compound has also been identified as a potent antiradical compound in a parasitic plant of South Asia.14)

The molecular formula of peak compound 2 was determined to C_{16}H_{18}O_{9} from the deprotonated molecular ion (m/z 353.0867), which was obtained by the LC-MS analysis. The typical UV spectrum of 2 [\lambda_{\text{max}} 337, 300 (sh) nm] suggested the presence of a caffeoyl moiety, and the fragment ion at m/z 191.0538 (C_{7}H_{11}O_{6}) also suggested the presence of a quinic acid. There have been many isomeric caffeoyl quinic acids found in nature, although the most widely distributed one is chlorogenic acid (3-caffeoylquinic acid). We therefore compared authentic chlorogenic acid with peak 2 by PDA-HPLC and determined that 2 was chlorogenic acid from the same retention time and UV spectrum as those of the authentic sample. Chlorogenic acid is a widely distributed antioxidant in fruits and vegetables.

The LC-MS analysis of peak compound 3 gave a deprotonated molecular ion at m/z 353.0866, indicating the same molecular formula, C_{16}H_{18}O_{9}, as that of 2. A fragment ion due to the quinic acid moiety was also observed at m/z 191.0535. These data indicated that 3 might be one of the isomers of chlorogenic acid. Possible regioisomers concerning the position of the caffeoyl moiety of chlorogenic acid were synthesized according to the method of Brandl et al.,16) although none of these synthesized isomers coincided with 3. An intensive examination of the UV spectrum of 3 revealed the absence of a shoulder peak at 310 nm and \lambda_{\text{max}} shifted to 320 nm in comparison with the spectrum of chlorogenic acid. These data indicated 3 to be a cis-caffeoyl isomer of chlorogenic acid. We therefore identified 3 by comparing with cis-chlorogenic acid which had been synthesized by a photo isomerization reaction of chlorogenic acid. LC-MS studies on constituents of edible plants have recently revealed the presence of cis-hydroxycinnamoyl quinic acids as minor constituents along with major chlorogenic acid,17) however, the production mechanism for these cis-hydroxycinnamic acids in plant bodies is still unclear. We were able to selectively detecting cis-chlorogenic acid as the main antiradical compound in the extract of tsuyanashinode (P. ovato-paleaceum) in this investigation.

Peak 4 presented in Fig. 3, which shows the HPLC data for niwatoko (S. racemosa subsp. sieboldiana),
indicates antiradical response and a typical phenolic UV absorption maximum at 279 nm. However, no reliable MS data for the peak could be obtained, because the peak contained many impurities due to its too rapid elution just after solvent shock. Hence, separation and the LC-MS analysis for the peak were reexamined.

A C30 reversed-phase column (Nomura Chemical, Kasugai, Japan) showed satisfactory retention of the peak compound in the column (data not shown) and the LC-MS analysis of the peak afforded a clear protonated molecular ion at $m/z = 152.0677$, revealing the molecular formula of peak compound 4 to be $C_8H_{10}NO_2$. These data, including its UV spectrum, indicate that compound 4 is possibly dopamine. Co-injection of authentic dopamine into the HPLC instrument using the same C30 column clarified that 4 was identical to dopamine. Dopamine is well known to be a catecholamine neurotransmitter, and its potent antioxidant activity in a lipid oxidation system has also been reported. 18)

Peak 5 in the HPLC data was thought to contain several compounds from its unsymmetrical shape. The LC-MS analysis of the peak afforded a clear protonated molecular ion at $m/z = 152.0677$, revealing the molecular formula of peak compound 4 to be $C_8H_{10}NO_2$. These data, including its UV spectrum, indicate that compound 4 is possibly dopamine. Co-injection of authentic dopamine into the HPLC instrument using the same C30 column clarified that 4 was identical to dopamine. Dopamine is well known to be a catecholamine neurotransmitter, and its potent antioxidant activity in a lipid oxidation system has also been reported. 18)

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6 to be determined as quercetin digalactoside, and at least one of the galactoses was attached at the 3-position of the quercetin structure. A time-course analysis of the enzymatic reaction was carried out to observe an intermediate peak at a retention time of 27.3 min. The UV spectrum of this intermediate peak indicated that it was 3-O-glycosidated quercetin. Non-sugar substituted ions of the A and B rings of quercetin moiety (m/z 153.0153 and 137.0240, respectively) were also observed in the MS data for 6,21 indicating that 6 was a quercetin 3-O-β-D-galactosyl-β-D-galactoside, although the linkage between the two sugars is still unclear from these obtained data. Quercetin is one of the most potent antioxidants of plant constituents. Although glycosidation at the 3-position of quercetin decreases its activity,22 various 3-glycosides of quercetin, such as rutin, are still recognized as potent food antioxidants by their strong antiradical ability. It is therefore likely that, 6 is one of the main antioxidant in niwatoko (S. racemosa subsp. sieboldiana) together with dopamine and chlorogenic acid.

In conclusion, we screened the radical scavenging activity of spring mountain herbs from Shikoku mountains as one of the antioxidation-related function of these bio-resources. Of the herbs examined in this investigation, tsuyanashiinode (P. ovato-paleaceum) and niwatoko (S. racemosa subsp. sieboldiana) were recognized to have potent antiradical activity. Material evidence for the potent radical scavenging activity of the two herbs was demonstrated by a caffeoyl β-D-glucoside, chlorogenic acid, and cis-chlorogenic acid as the main active constituents in tsuyanashiinode (P. ovato-paleaceum), and by dopamine, chlorogenic acid, and a quercetin digalactoside in niwatoko (S. racemosa subsp. sieboldiana). We hope that these potent antiradical mountain herbs can help to revitalize the local communities as useful bio-resources.

Acknowledgments

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