Note

Determination of the Antioxidative Activities of Herbs Harvested in Japan by Oxygen Radical Absorbance Capacity Methods

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Oxygen radical absorbance capacity (ORAC) is a method for evaluating antioxidant capacities of both hydrophilic (H-ORAC) and lipophilic compounds (L-ORAC). In this study, we evaluated the antioxidant capacities of eight herbs (Italian parsley, coriander, celery white, watercress, rocket, mustard green, basil, and corn salad) by ORAC. Basil showed the highest antioxidant capacity among the herbs assessed in this study. The harvest season affected both the H- and L-ORAC values of the herbs, except for the H-ORAC value of Italian parsley. The ratios of H- to L-ORAC values of the herbs ranged from 36% to 86%. Total polyphenol contents of herbs were positively correlated with H-ORAC values, and were also affected by harvest season. We found that isoquercitrin and rutin but not ferulic acid contributed to the seasonal change in the H-ORAC value of basil.

Keywords: herb, antioxidant, oxygen radical absorbance capacity (ORAC), quercetin glycosides

Introduction

The reactive oxygen species (ROS), superoxide radical and singlet oxygen, play an important role in health maintenance, such as in the pathogen elimination mechanism (Grant et al., 2012). On the other hand, endogenous enzymes, such as superoxide dismutase and catalase, eliminate superoxide and hydrogen peroxide to protect against oxidative stress in the body (McCord et al., 1985). However, exposure to cigarette smoke and chronic inflammation induce excess ROS production, resulting in damage to biological molecules including proteins, lipids, and nucleic acids. It has been reported that this oxidative damage is related to chronic and degenerative diseases such as cancer (Ames et al., 1995), heart disease (Willcox et al., 2008), and Alzheimer’s disease (Engelhart et al., 2002). Fruits, vegetables, and herbs contain large amounts of antioxidants, which inhibit the decomposition of biological molecules by eliminating ROS. Thus, it is thought that the intake of antioxidant-rich foods helps endogenous antioxidant enzymes to eliminate ROS in the body.

Oxygen radical absorbance capacity (ORAC) is a method that measures the scavenging capacity against peroxyl radicals induced by 2,2'-azobis (2-amidinopropane) dihydrochloride (AAPH) using fluorescein as a fluorescence probe (Ou et al., 2001). Although Ou et al. developed a method to evaluate the activity of hydrophilic antioxidants (H-ORAC), the method showed poor reproducibility (Watanabe et al., 2010). Watanabe et al. improved the original H-ORAC method and evaluated the precision of the method by interlaboratory studies based on the internationally harmonized protocol (Watanabe et al., 2012). The L-ORAC method was developed based on the H-ORAC method, and uses β-cyclodextrin as a solubility enhancer to evaluate the activity of lipophilic antioxidants such as vitamin E and carotenoids (Watanabe et al., 2013).
Table 1. Production areas, cultivation methods, and harvesting times of the eight herbs

<table>
<thead>
<tr>
<th>Area / Cultivation</th>
<th>March</th>
<th>May</th>
<th>September</th>
<th>December</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basil</td>
<td>Ibaraki/water</td>
<td>Ibaraki/water</td>
<td>Gunma/soil</td>
<td>Ibaraki/water</td>
</tr>
<tr>
<td>Corn salad</td>
<td>Ibaraki/water</td>
<td>Ibaraki/water</td>
<td>Ibaraki/water</td>
<td>Ibaraki/water</td>
</tr>
<tr>
<td>Watercress</td>
<td>Ibaraki/water</td>
<td>Ibaraki/water</td>
<td>Ibaraki/water</td>
<td>Ibaraki/water</td>
</tr>
<tr>
<td>Mustard green</td>
<td>Gunma/soil</td>
<td>Gunma/soil</td>
<td>Gunma/soil</td>
<td>Gunma/soil</td>
</tr>
<tr>
<td>Rocket</td>
<td>Ibaraki/water</td>
<td>Ibaraki/water</td>
<td>Ibaraki/water</td>
<td>Chiba/water</td>
</tr>
<tr>
<td>Celery white</td>
<td>Mie/soil</td>
<td>Mie/soil</td>
<td>Mie/soil</td>
<td>Mie/soil</td>
</tr>
<tr>
<td>Coriander</td>
<td>Ibaraki/soil</td>
<td>Ibaraki/soil</td>
<td>Ibaraki/soil</td>
<td>Ibaraki/soil</td>
</tr>
<tr>
<td>Italian parsley</td>
<td>Mie/water</td>
<td>Mie/water</td>
<td>Mie/water</td>
<td>Mie/water</td>
</tr>
</tbody>
</table>

Many traditional herbs have been used as folk medicines for the treatment of health problems (Alok et al., 2014). In Japan, the consumption of several herbs, such as basil and parsley, has been increasing as a result of the westernization of the diet. Herbs contain large amounts of polyphenols that scavenge free radicals. Therefore, in this study, we investigated the effect of harvest season on the ORAC values of herbs. Moreover, we determined the components of basil that contributed to the seasonal change in H-ORAC values.

Materials and Methods

Chemicals  Fluorescein and Trolox were purchased from Sigma-Aldrich (Milwaukee, WI, USA). 2,2'-azobis-2-methylpropanimidamide dihydrochloride (AAPH) was obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Randomly methylated β-cyclodextrin (RMCD) was obtained from Junsei Chemical (Tokyo, Japan). All other chemicals were of reagent grade.

Herbs  Herbs were harvested in September and December, 2012 and March and May, 2013 and were gifted from S & B Foods, Inc. (Tokyo, Japan). The herbs used in this study were as follows: Ocimum basilicum (basil); Valerianella locusta (corn salad); Nasturtium officinale (watercress); Brassica juncea (mustard greens); Eruca sativa (rocket); Apium graveolens (celery white); Coriandrum sativum (coriander); and Petroserum crispum var. neapolitanum (Italian parsley). These herbs were chosen as commonly consumed herbs in Japan. The production areas, cultivation methods (in water or soil), and harvesting times of these herbs are described in Table 1. Watercress and the other herbs were cultivated for 30 and 50 days, respectively, after sowing, and were harvested each season when they reached a commercially standard grade.

Preparation of herb extracts  Edible parts of the herbs (about 50–200 g each) were cut into small pieces, snap-frozen in liquid nitrogen, and lyophilized; samples were then pulverized using a grinder mill (GM-200; Retsch, Haan, Germany). Freeze-dried samples from each season were obtained in triplicate for each month. These samples (1 g each) were extracted using an accelerated solvent extractor (ASE-350; Dionex, San Jose, CA, USA), as described previously (Watanabe et al., 2012, 2013). Briefly, each sample was extracted with n-hexane and dichloromethane (1:1), followed by extraction with MWA (methanol:water:acetic acid = 90:9.5:0.5). For L-ORAC measurement, n-hexane-dichloromethane extracts of herbs were dried under nitrogen gas and re-dissolved in dimethylsulfoxide (DMSO, 5 mL). For H-ORAC measurement, MWA extracts were made up to a volume of 50 mL with MWA.

H-ORAC measurement  H-ORAC values of herb extracts were measured according to the methods described by Watanabe et al. (Watanabe et al., 2012). Briefly, MWA extracts of herbs were 10-fold diluted with assay buffer solution (75 mmol/L phosphate buffer, pH 7.4), and the 10-fold diluted sample was further diluted 5, 25, and 125-fold with 10% (v/v) MWA in the assay buffer solution to obtain the approximate H-ORAC value of each sample. Trolox calibration solutions (50, 25, 12.5, and 6.25 μmol/L in assay buffer) were made to obtain a standard curve. Diluted samples, Trolox calibration solution, and assay buffer solution (35 μL) were added to the wells of a 96-well plate, and 115 μL of fluorescein (110.7 nmol/L), and 50 μL of AAPH (31.7 mmol/L) were added to each well, and then incubated at 37°C. The fluorescence intensity was monitored every 2 min for 90 min by a micro plate reader (POWERSCAN HT; DS Pharma Biomedical Co., Ltd., Osaka, Japan). The approximate H-ORAC of each sample was calculated on the basis of the standard curve for Trolox. Next, the 10-fold diluted sample was further diluted with 10% (v/v) MWA in the assay buffer solution in reference to the approximate H-ORAC value of each sample. The fluorescence intensity of each well of the 96-well micro plate was monitored after the addition of fluorescein and AAPH solutions. The H-ORAC value of samples was finally determined based on the converged dilution ratio.

L-ORAC measurement  L-ORAC values of herb extracts were measured according to the methods described by Watanabe et al. (Watanabe et al., 2013). Briefly, DMSO solutions of the lipophilic fractions of herbs were diluted with diluent [7% (w/v) RMCD dissolved in 50% (v/v) acetone], and the 10-fold diluted sample
was 5, 25, 125-fold further diluted with 10% (v/v) DMSO in the diluent. Trolox™ calibration solutions (50, 25, 12.5, and 6.25 μmol/L in 10% DMSO in the diluent) were made to obtain a standard curve. Diluted samples, Trolox™ calibration solution, and assay buffer solution (35 μL) were added to the wells of a 96-well plate, and fluorescein (115 μL, 110.7 nmol/L in assay buffer solution) and AAPH (50 μL, 31.7 mmol/L in assay buffer solution) were added to each well, and then incubated at 37°C. Fluorescence intensity was monitored every 2 min for 120 min using a micro plate reader. The approximate L-ORAC of each sample was calculated based on the standard curve for Trolox™. Next, the 10-fold diluted sample was further diluted with 10% DMSO in the diluent in reference to the approximate L-ORAC value of each sample. The fluorescence intensity of each well of the 96-well micro plate was monitored after the addition of fluorescein and AAPH solutions. The L-ORAC value of samples was finally determined based on the converged dilution ratio.

Measurement of total polyphenols in herb extracts Total polyphenols of herb extracts were measured by the Folin-Ciocalteu method. The MWA extracts of herbs were diluted 2, 4, 8-fold with 50% MWA solution. Diluted samples (80 μL) were mixed with Folin-Ciocalteu reagent [56 μL, 37.5% (v/v)] in a 96-well plate, and incubated for 5 min at room temperature. Sodium bicarbonate solution [2% (w/v), 120 μL] was added to each well and incubated for 15 min at room temperature, and absorbance at 750 nm was then measured using a micro plate reader. Gallic acid was used as a standard and total polyphenol contents were expressed as gallic acid equivalent (GAE).

Liquid chromatography (LC)-MS analysis An HPLC system by Waters (Milford, MA, USA), consisting of an autosampler (cooled to 10°C; injection volume: 10 μL), a column oven set at 30°C, and a triple quadrupole mass spectrometer (Xevo® TQD; Waters) with an electrospray interface operating in the negative mode, was used. A YMC-Ultra HT Pro C18 column (75 mm × 2.0 mm, 2.0 μm; YMC Co., Ltd., Kyoto, Japan) was used for separation with water (A) and methanol (B) as eluents, both acidified with 0.1% (v/v) acetic acid at a flow rate of 0.2 mL/min with gradient elution (0 min: 95% A, 5 min: 95% A, 20 min: 30% A, 25 min: 30% A, 30 min: 5% A, 32 min: 5% A, and 35 min: 95% A). Cone and desolvation gas flows were set to 50 and 1,000 L/h, respectively. The capillary voltage was set at 3.0 kV, the source temperature at 150°C, and the desolvation temperature at 650°C. The cone voltage was optimized for each standard compound using the Waters IntelliStart MS Console (Waters). Multiple Reaction Monitoring (MRM) was used for the identification of polyphenols in the basil extracts by comparing the retention times and MRM mass transitions with standards (ferulic acid, quercetin, isoquercitrin, rutin, and luteolin). The precursor, product ions, and retention time (min) were determined after the optimization of MS/MS as follows: m/z = 193.0 and 133.9 at 15.9 min for ferulic acid, m/z = 301.0 and 150.9 at 20.3 min for quercetin, m/z = 462.9 and 300.1 at 17.0 min for isoquercitrin, m/z = 609.0 and 301.0 at 17.7 min for rutin, m/z = 285.0 and 132.9 at 20.7 min for luteolin. Data acquisition and analysis were performed using MassLynx 4.1 (TargetLynx) software (Waters).

Statistical analysis Statistical analyses were performed using factorial ANOVA followed by the Tukey-Kramer multiple-comparison test. Data are expressed as means ± SD. The level of significance was defined as p < 0.05.

Results and Discussion
In this study, we investigated the antioxidative activities of eight herbs by ORAC methods. As shown in Fig. 1a-d, basil, a member of the Lamiales family, exhibited the highest H-ORAC values among the herbs assessed in this study for each month except September. The H-ORAC values of basil ranged from 4453.2 ± 55.2 (September) to 14380.1 ± 1082.2 (March) μmol TE/100 g FW. These herbs were harvested from the same production areas except for basil and rocket, which were harvested in September and December, respectively (Table 1). These results show that the H-ORAC values of all herbs were affected by harvest season. It is well known that H-ORAC values are correlated with the total polyphenol content of vegetables and fruits (Takebayashi et al., 2010). Thus, we evaluated the total polyphenol content of the herbs by the Folin-Ciocalteu method, and investigated whether the H-ORAC values were correlated with the total polyphenol content of the herbs. The correlation coefficients between H-ORAC values and the total polyphenol content of herbs were as follows: basil (0.99), corn salad (0.96), watercress (0.59), mustard greens (0.66), rocket (0.86), celery white (0.93), coriander (0.61), and Italian parsley (0.36); thus, significant positive correlations were observed for all of the herbs used in this study. The L-ORAC values of basil ranged from 2931.9 ± 133.8 (December) to 6223.7 ± 929.8 (September) μmol TE/100 g FW and were the highest among the herbs assessed in this study for each month except March (Fig. 1e-h). Interestingly, the L-ORAC values of basil increased in September, which was a contrasting trend to that of the H-ORAC values. The ratio of H- to L-ORAC values of the herbs ranged from 36% to 86%, indicating that both hydrophilic and lipophilic compounds contributed to the antioxidative activities of the herbs.

We next attempted to clarify the components of basil that contributed to its H-ORAC values, because it showed the highest antioxidative activity and the most seasonal change among the herbs used in this study. It was reported that basil contains ferulic acid, quercetin, isoquercitrin, rutin, and luteolin (Vlase et al., 2014). Thus, we quantified these components in the MWA extracts of basil using LC-MS (Fig. 2). The contents of isoquercitrin and rutin tended to increase in March and May, whereas the content of ferulic acid was not affected by harvest season (Fig. 2a-c). Quercetin and luteolin were not detected in this experiment. Next, we investigated whether the contents of these compounds in basil
Fig. 1. The ORAC values of eight herbs harvested in each season. The H-ORAC values of eight herbs harvested in (a) March, (b) May, (c) September, and (d) December were measured. The L-ORAC values of eight herbs harvested in (e) March, (f) May, (g) September, and (h) December were measured. Data are expressed as means ± SD. Bars with differing letters have significantly different ($p < 0.05$) values according to the Tukey-Kramer multiple comparisons test.

Fig. 2. The amounts of polyphenolic compounds in the MWA extracts of basil from each season, and their correlation with H-ORAC values. The amounts of (a) ferulic acid, (b) isoquercitrin, and (c) rutin in the MWAs extracts of basil from each season were measured by LC-MS. Data are expressed as means ± SD. Bars with differing letters have significantly different ($p < 0.05$) values according to the Tukey-Kramer multiple comparisons test. Correlation coefficients between the contents of the compounds and H-ORAC values of basil were calculated from the data in Figs. 1 and 2a-c for (d) ferulic acid, (e) isoquercitrin, and (f) rutin.
were correlated with H-ORAC values (Fig. 2d-f). The correlation coefficients between the contents of the compounds and H-ORAC values of basil were as follows: ferulic acid (0.26), isoquercitrin (0.81), and rutin (0.80); these results suggest that isoquercitrin and rutin might contribute to the seasonal change in H-ORAC values of basil. Next, we assessed the H-ORAC values of these compounds. The H-ORAC values of ferulic acid, isoquercitrin, and rutin were 19417.5, 20182.3, and 14440.4 μmol TE/g, respectively. Their contributions to the H-ORAC values of basil were as follows: ferulic acid (2.0% to 8.6%), isoquercitrin (1.1% to 7.6%), and rutin (2.0% to 18.9%). These results suggest that basil contains hydrophobic antioxidants other than these compounds. Further study is needed to clarify the other compounds that contribute to the H-ORAC values of basil.

This study revealed that the harvest season affects the antioxidative activities of herbs. Furthermore, the capacities of hydrophilic antioxidants were positively correlated with the total polyphenol content. These results indicated that polyphenols are the main contributors to hydrophilic antioxidative activities, and that the total polyphenol content is also affected by the harvest season. However, further study is needed to clarify the effects of harvest season on the antioxidative activities of herbs. We also observed that basil showed the highest antioxidative activities among the herbs assessed in this study. Moreover, the basil used in this study contained quercetin glycosides not but the aglycone, and quercetin glycosides but not ferulic acid might contribute to the seasonal change in H-ORAC values of basil. Our results provide information on the antioxidative activities of herbs harvested in each season, and might be useful in the development of cultivation techniques to produce herbs with strong antioxidative activities.

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References