Note

Protective Effect of Red-Stemmed Type of *Ipomoea aquatica* Forsk against CCl₄-Induced Oxidative Damage in Mice

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**Summary** Water spinach (*Ipomoea aquatica* Forsk; *I. aquatica*) of the green-stemmed type (green type) is widely consumed, but there also exists a red-stemmed variety (red type). In the present study, the antioxidant capacity of the red type was compared to that of the green type in carbon tetrachloride (CCl₄)-treated mice. CCl₄-induced thiobarbituric acid reactive substrate (TBARS) formation in the liver was significantly suppressed in mice fed 5% red-type *I. aquatica*, while the green type showed no effect. Hydrophobic oxygen radical absorbance capacity (H-ORACₜₙₙ) in the red type showed a lower level than that in the green type; however, lipophilic ORAC (L-ORACₜₙₙ) and total-ORACₜₙₙ levels were significantly higher in the red type than in the green type. α-Tocopherol, anthocyanidin/proanthocyanidin, and β-carotene contents were all significantly higher in the red type than in the green type. These results suggest that the wild red-type *I. aquatica* contains certain lipophilic components that exert antioxidant capacities not only in vitro but also in vivo. Such effective components in the red type would be beneficial phytochemicals for suppressing several diseases related to oxidative stress.

**Key Words** Red-stemmed type of *Ipomoea aquatica* Forsk, antioxidant, oxygen radical absorbance capacity (ORAC), thiobarbituric acid reactive substrates (TBARS), tocopherol

Oxidative stress has been associated with the development of many chronic diseases, including cancer, diabetes, cardiovascular diseases, and hepatitis (1, 2). The excessive generation of reactive oxygen species causes extensive damage to DNA, proteins, and lipids. Hence, current interest is focused on the search for natural antioxidants, especially those of plant origin. Several researchers have demonstrated that various vegetables and fruits possess beneficial effects on the scavenging of free radicals (3, 4).

*Ipomoea aquatica* Forsk (*I. aquatica*), commonly called water spinach, belongs to the family Convolvulaceae, and is a very popular and commonly eaten leafy vegetable in southern China or tropical and subtropical countries. The commercial variety of *I. aquatica* has greenish stems and leaves (green type), whereas the wild varieties have red stems and dark green leaves (red type). Extracts from the green type have traditionally been used for the treatment of nosebleed and high blood pressure (5, 6), and other biological functions such as an antidiabetic (7, 8) and antibiotic (9) have also been reported. Moreover, Prasad et al. (10) have isolated a free radical-scavenging antioxidant from the green type. However, the biological functions of the wild red type have not been clarified.

Carbon tetrachloride (CCl₄) has been used in models for studying free radical-induced liver injury and screening hepato-protective drugs (11). The trichloromethyl free radical, a bioactive metabolite of CCl₄, initiates the lipid peroxidation process in cellular membranes that eventually leads to the progression of liver damage or necrosis (2, 11). Therefore, antioxidants may inhibit all of the oxidative stress induced by CCl₄. In fact, naturally derived antioxidants are reported to counteract both the toxicity and lipid peroxidation induced by many hepatotoxins (12).

In the present study, we performed a comparative investigation of the in vivo antioxidant activities of green- and red-type *I. aquatica* using CCl₄-treated mice, as well as a determination of the in vitro antioxidant capacity and levels of certain antioxidant components.

**Materials and Methods**

**Materials.** One commercial variety (Ton Pai Bai Yao, Serm Sri Seed Co., Thailand) and one wild variety (WC016) of water convolvulus (*I. aquatica*, cv. Thailand)
were used as plant materials. Ton Pai Bai Yao has the same greenish stem and leaves as other commercial varieties and WC016 has the same red stem and dark green leaves as other wild varieties. The seeds of WC016 were obtained from the Asian Vegetable Research and Development Center (AVRDC), Thailand and the Faculty of Horticulture, Chiba University, Japan. Seedlings were cultivated under natural conditions in a greenhouse of the Faculty of Bioresources, Nihon University, Fujisawa, Japan. Daily maximum and minimum air temperatures during cultivation were 40±5°C and 20±5°C, respectively. Stems were sampled every 2–4 wk from August to October 2008.

Vitamin E Reference Standard (HPLC grade) was purchased from Eisai Food & Chemical (Tokyo, Japan). This kit contains \( \alpha \)-tocopherol, \( \delta \)-tocopherol, \( \beta \)-tocopherol, and \( \delta \)-tocopherol in each vial. Cyanidin chloride (P grade), \( \alpha \)- and \( \beta \)-carotene standard, and lutein (commercial name: Xanthophyll) (HPLC grade) were obtained from Chromadex (CA, USA), Wako Pure Chemical Industries, Ltd. (Osaka, Japan), and Sigma (MO, USA), respectively.

**Animals and treatment.** Male ICR mice, 8 wk of age (25–30 g), obtained from Japan SLC, Inc. (Shizuoka, Japan), were randomly divided into six groups of six mice each (A–F). Mice in groups A and D were fed an AIN-93G-based regular diet for 2 wk, mice in groups B and E were fed the regular diet supplemented with 5% freeze-dried commercial variety of \( I. \) aquatica, and E were fed the regular diet supplemented with the lipophilic ORAC \(_{FL} \) (L-ORAC \(_{FL} \)) assay. Hydrophilic extraction was performed with 2×10 mL of AWA, which was used in the hydrophilic ORAC \(_{FL} \) (H-ORAC \(_{FL} \)) assay. Both H-ORAC \(_{FL} \) and L-ORAC \(_{FL} \) assays were performed on a MTP-32 microplate reader (Corona, Ibaraki, Japan) with fluorescence filters for an excitation wavelength of 485 nm and an emission wavelength of 520 nm at 37°C. Trolox calibration solutions were used for the standard assay. The final ORAC values were calculated according to the method of Wu et al. (15). Data are expressed as micromoles of Trolox equivalents (TE) per gram of dried weight (\( \mu \)mol of TE/g). Total antioxidant capacity (Total-ORAC \(_{FL} \)) was calculated combining both H-ORAC \(_{FL} \) and L-ORAC \(_{FL} \).

**Quantification of tocopherols and carotenoids.** The contents of tocopherols and carotenoids in the extracts used in the L-ORAC \(_{FL} \) assay were analyzed by using HPLC (LaChrom Elite, Hitachi High-Technologies Corporation, Tokyo, Japan). The sample was mixed with each standard material. Retention time, peak shape and increase of peak area were confirmed by HPLC. Then, sample identification was confirmed by comparing retention times and absorption spectra to those of standard materials. Quantification was accomplished using calibration of the standards.

Separation of \( \alpha \), \( \beta \), \( \gamma \), and \( \delta \)-tocopherol was carried out on a Waters Spherisorb NH \(_{2} \) Column, 3.5 \( \mu \)m, 4.6×250 mm (Waters, MA, USA), and elution was performed with an isocratic solvent mixture of hexane/ethyl acetate (80 : 20 v/v) at a flow rate of 1.0 mL/min. The samples were filtered through a 0.45-\( \mu \)m Millipore-FH filter (Millipore, MA, USA). The injection volume was 20 \( \mu \)L, and the detection wavelength was 295 nm.

Separation of \( \alpha \)- and \( \beta \)-carotene and lutein was achieved on a Waters XBridge C18 Column, 5 \( \mu \)m, 4.6×250 mm (Waters), and elution was performed with an isocratic solvent mixture of acetone/ethanol (84 : 16 v/v) at a flow rate of 1.0 mL/min. Twenty microliters of the filtered samples were injected into the chromatograph, which was recorded at 470 nm.

**Quantification of anthocyanidin and proanthocyanidin.** Anthocyanidin and proanthocyanidin concentrations in the L-ORAC extracts were determined by the butanol-HCl method (16, 17). Briefly, 200 \( \mu \)L of L-ORAC extraction or cyanidin chloride standards (25, 50, 100, 500, 1,000 nm) was added to 800 \( \mu \)L of butanol-HCl (95 : 5, v/v) and absorbance was determined at 550 nm for anthocyanidin concentration. For the determination of proanthocyanidin concentration, the sample/butanol-HCl mixture was heated for 30 min at 95°C, and absorbance of the anthocyanidin products was measured at 550 nm. Anthocyanidin and proanthocyanidin contents in L-ORAC extracts were expressed as milligram per gram of dried sample.

**Statistical analysis.** The data of in vitro experiments are presented as means±SE. Data were assessed by...
Results
To evaluate the in vivo antioxidant activity of *I. aquatica*, CCl₄-induced lipid peroxidation was measured in the liver of mice fed each type of *I. aquatica* for 2 wk. As shown in Fig. 1, the TBARS level in the mice treated with saline alone was not affected by either type of *I. aquatica*. On the other hand, CCl₄-induced TBARS formation was significantly suppressed by the red type, while the green type did not show an effect (Fig. 1).

In general, vegetables that are dark green in color, such as spinach, have strong antioxidant capacities (15). To evaluate the in vitro antioxidant capacity of *I. aquatica*, we next performed an ORAC assay using their hydrophilic (H-ORAC) and lipophilic (L-ORAC) extracts. As shown in Table 1, H-ORAC in the red type showed a lower level than that in the green type; however, the L-ORAC level was significantly higher in the red type than in the green type. Total-ORAC in the red type was significantly higher than in the green type (Table 1).

Next, we analyzed certain major lipophilic antioxidants, such as α-tocopherol, β-tocopherol, γ-tocopherol, δ-Tocopherol, anthocyanidins/proanthocyanidins, and carotenoids in L-ORAC extracts of both types of *I. aquatica*.

Student’s t-tests. Differences were considered significant at p<0.05.

<table>
<thead>
<tr>
<th><em>I. aquatica</em></th>
<th>H-ORAC₃₁₀</th>
<th>L-ORAC₃₁₀</th>
<th>Total-ORAC₃₁₀*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Green type</td>
<td>199 ± 11</td>
<td>166 ± 11</td>
<td>365 ± 12</td>
</tr>
<tr>
<td>Red type</td>
<td>59.4 ± 4.0*</td>
<td>443 ± 35</td>
<td>503 ± 32*</td>
</tr>
</tbody>
</table>

ORAC data was expressed as micromoles of Trolox equivalents per gram of dried samples (μmol of TE/g). Values are expressed as means±SE (n=4). *Total ORAC = H-ORAC + L-ORAC. Each value was significantly different between the green type and the red type of *I. aquatica*.

Fig. 1. Inhibitory effect of the red type of *Ipomoea aquatica* Forsk on CCl₄-induced lipid peroxidation in mouse liver. Values are expressed as means±SE (n=6). Values with different letters are significantly different at p<0.05. Data were assessed by one-way ANOVA and Tukey’s multiple comparison test.

Fig. 2. Vitamin E content in L-ORAC extract of *Ipomoea aquatica* Forsk. Data is expressed as microgram per gram of dried weight (DW). Values are expressed as means±SE (n=4). UD: Under the detection limit. *p<0.05 represents a significant difference compared to the green type of *Ipomoea aquatica* Forsk.

Fig. 3. Total anthocyanidin and proanthocyanidin contents in L-ORAC extracts of *Ipomoea aquatica* Forsk. Data is expressed as microgram of cyanidin-chloride equivalents per gram of dried sample. Values are expressed as means±SE (n=4). *p<0.05 represents a significant difference compared to the green type of *Ipomoea aquatica* Forsk.

Fig. 4. Carotenoid contents in L-ORAC extracts of *Ipomoea aquatica* Forsk. Data is expressed as microgram of cyanidin-chloride equivalents per gram of dried sample. Values are expressed as means±SE (n=4). *p<0.05 represents a significant difference compared to the green type of *Ipomoea aquatica* Forsk.
tocopherol in both types (Fig. 2). The content of γ-tocopherol was nearly identical in both types. Δ-Tocopherol was under the detection limit in both types (Fig. 2).

Levels of anthocyanin and its precursor proanthocyanidin were significantly higher in the red type compared to the green type; the proanthocyanidin level in the red type was twice as high as in the green type (Fig. 3).

β-Carotene content was significantly higher in the red type than in the green type (Fig. 4), while α-carotene was only detected in the red type, and lutein levels did not differ between the two types (Fig. 4).

Discussion

Lipid peroxidation of biomembranes is one of the principle causes of CCl₄ toxicity (18). This is evidenced by the elevation of TBARS and the decrease in the activity of free radical scavenging enzymes in CCl₄-treated animals. On the other hand, potent antioxidants are known to decrease the level of TBARS through anti-peroxidation (19, 20).

In the present study, although the H-ORAC₅₅ level was significantly higher in the green-type I. aquatica than in the red type, L-ORAC₅₅ and total-ORAC₅₅ levels were significantly higher in the red type, and only the red type could decrease TBARS level in the liver of CCl₄-treated mice. These results demonstrate that antioxidant components in the green-type H-ORAC₅₅ extract were insufficient to decrease TBARS level, but what was predominant in the red-type L-ORAC₅₅ extract effectively suppressed CCl₄-induced liver peroxidation.

To determine which lipophilic constituents contributed to the higher L-ORAC₅₅ level in the red type, we next analyzed some of the major lipophilic antioxidants such as vitamin E (tocophers) and anthocyanidin/proanthocyanidin.

Among the various forms of vitamin E, α-tocopherol has the highest antioxidant activity (21). In the present study, the α-tocopherol level in the red type was significantly higher than that in the green type. Many researchers have demonstrated that α-tocopherol suppresses CCl₄-induced lipid peroxidation in the liver of rats (22–24). Therefore, α-tocopherol in the red type is considered to contribute to the significant suppression of the TBARS level in CCl₄-treated mice.

In the process of hydrophilic extraction by AWA (70% acetone) in this study, both types of I. aquatica were separated into a green aqueous layer; and a yellowish-red acetone layer; the former aqueous layer was measured as H-ORAC₅₅, and latter acetone layer, which was much more abundant in the red type than in the green type, was combined with the lipophilic extracts and measured as L-ORAC₅₅. Therefore, it was considered that this yellowish-red acetone layer contributed to the higher L-ORAC₅₅ level in the red type. Purplish and reddish fruits and vegetables generally contain much more anthocyanin, which is one of the major antioxidants in plants. Anthocyanin is a water-soluble pigment; however, its aglycon anthocyanidin and precursor proanthocyanidin are better extracted in 70% acetone (25, 26). In the present study, both anthocyanidin and proanthocyanidin levels were significantly higher in L-ORAC extracts of the red type than of the green type; specifically, the proanthocyanidin level in the red type was almost twice that of the green type. Anthocyanins and their aglycons are potent antioxidants (27, 28) that prevent CCl₄-induced liver damage in rats (29). Moreover, proanthocyanidins are reported to exert higher radical-scavenging activities than anthocyanins (26) or activities that are fifty times higher than vitamin E (30). Therefore, anthocyanin and proanthocyanidin are considered to contribute to the higher value of L-ORAC₅₅ in the red type and significant suppression of the TBARS level in mice fed the red type of I. aquatica.

On the other hand, the green type of I. aquatica is known to be a good source of carotenoids, especially β-carotene and lutein (31); however, the ORAC₅₅ assay does not measure the antioxidant activity of carotenoids because, chemically, it is not a chain-breaking antioxidant (32). In the present study, the β-carotene content in L-ORAC extracts was significantly higher in the red type than in the green type. β-carotene is reported to protect against lipid peroxidation induced by CCl₄ (33). Therefore, it is also considered that the antioxidant capacity of β-carotene, which was not reflected in the L-ORAC₅₅ level in the red type, could effectively contribute to the significant suppression of CCl₄-induced TBARS level. We have also detected some polyunsaturated fatty acids, whose antioxidant activities are also not measured by the ORAC₅₅ assay (32), in the red type (unpublished data). Furthermore, involvement of other lipophilic polyphenols in the red type on the suppression of lipid peroxidation in mice could not be clarified in the present study. Further studies are needed to elucidate the most effective components which contributed to the suppression of lipid peroxidation in the liver of mice fed the red type of I. aquatica.

In summary, the results presented here showed that the wild red type of I. aquatica contains certain effective components that exert antioxidant capacities in vivo. Such effective components would be beneficial phytochemicals for suppressing several diseases related to oxidative stress.

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References