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Antioxidative capacity of rhizome extract and rhizome knot extract of edible lotus (*Nelumbo nuficera*)

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Abstract

Extracts from rhizomes (Lotus roots) knot (LRK) and whole rhizomes (LR) were investigated for their antioxidative capacity in comparison with commonly used antioxidants from plant material. Radical scavenging activity was measured spectrophotometrically using the stable radicals 1-diphenyl-2-picrylhydrazyl and 2,2′-azino(3-ethylbenzothioazolino-6-sulfonate), and measured by electron spin resonance (ESR) trapping of the transient carbon-centered 1-hydroxyethyl radical (generated in a Fenton-type reaction). Efficiencies as chain-breaking antioxidants were evaluated by electrochemical measurement of oxygen consumption rate in a peroxidating methyl linoleate emulsion. LRK exhibited high antioxidative capacity, as measured by each of four different methods. LR, however, only showed a significantly high scavenging activity for small carbon-centered radicals, as measured by the ESR method. Total phenol content in the plant extract correlated with the antioxidant capacity, except for the scavenging of carbon-centered radical. Lotus rhizomes knot, as a waste from food production, will be a potential material for extracting antioxidants.

Keywords: Rhizomes (Lotus roots); Antioxidant; Phenolics; Free radicals; ESR; Oxygen consumption; DPPH; ABTS

1. Introduction

Lotus (Nelumbo) is used, not only as an ornamental plant, but also for a dietary staple in Eastern Asia, particularly in China. Rhizomes (Lotus roots), of the "edible lotus" variant of *Nelumbo nucifera* (Slocum & Robinson, 1996), have been widely used and processed as delicious and nutritional foods, and are particularly popular in southern China. To the best of our knowledge, no investigation has been conducted on the antioxidative capacity of Lotus rhizomes. However, observations of a rapid browning of the lotus rhizomes, appearing after removal of the peels, indicate the presence of a significant concentration of polyphenolic compounds in the rhizomes. The fact that rhizome knots become dark and harder after exposure to air proves that polymerization reactions of polyphenols are occurring. Consequently, we were stimulated to investigate the location, composition, structure, and bioactivity of the phenolic compounds in Lotus rhizomes.

Phenolic compounds from vegetables (Scalfi, Fogliano, Pentangelo, Graziani, Giordano, & Ritieni, 2000), fruits (Chang, Tan, Frankel, & Barrett, 2000; Kanazawa & Sakakibara, 2000; Paganga, Miller, & Rice-Evans, 1999; Souquet, Labarbe, Guernevé, Cheynier, & Moutounet, 2000; Wang & Lin, 2000), grains (Zielinski & Kozlowska, 2000), beverages (Daglia, Papetti, Gregotti, Bertè, & Gazzani, 2000; Pellegrini, Simonetti, Gardana, Brenna, Brighenti, & Pietta, 2000; Simonetti, Pietta, & Testolin, 1997), spices (Madsen, Nielsen, Bertelsen, & Skibsted, 1996) and medicinal plants (Hu & Kitts, 2000; Kim, Jung, Kim, & Kim, 2000; Yamaguchi, Saito, Ariga, Yoshimura, & Nakazawa, 2000) have attracted a great deal of attention because of their significant antioxidative activities. Various methods for evaluating antioxidant capacity are available. They include: (1) visible absorption spectroscopy methods, e.g. measuring the 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging activity (Espin, Soler-Rivas, Wichers, & Garcia-Vigera, 2000;

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Joyeux, Lobstein, & Mortier, 1995; Tadolini, Juliano, Piu, Franconi, & Cabrini, 2000), determining the ability to scavenge the radical cation of 2,2′-azinobis (3-ethylbenzothiazoline-6-sulfonate) (ABTS) in aqueous and/or lipophilic systems (Arnao, Cano, & Acosta, 1999; Degenhardt, Knapp, & Winterhalter, 2000; Miller & Rice-Evans, 1997; Yu & Ong, 1999), and assaying 2-thiobarbituric acid reactive substances of oxidized lipids (Lee, Weintraub, & Yu, 2000); (2) fluorescence methods, e.g. assaying oxygen radical absorbance capacity (Naguib, 2000; Wang & Lin, 2000); (3) chemiluminescence methods (Whitehead, Thorpe, & Maxwell, 1992); (4) electron spin resonance (ESR; Madsen et al., 1996; Noda, Kohno, Mori, & Packer, 1999; Yamaguchi, Ariga, Yoshimura & Nakazawa, 2000); and (5) electrochemical methods, measuring oxygen consumption of peroxidating lipids electrochemically (Baron, Skibsted, & Andersen, 1997; Mikkelsen, Sosniecki, & Skibsted, 1992; Møller, Madsen, Aaltonen, & Skibsted, 1999) or determining the redox potential of antioxidants (Jørgensen & Skibsted, 1993; Jørgensen, Madsen, Thomsen, Dragsted, & Skibsted, 1999). These assays measure the antioxidative activity at different stages of lipid oxidation, and a better strategy is to combine methods yielding information on any antioxidant interference at different stages of oxidative damage.

In the assay-guided investigation the total phenol contents of plant extracts were determined; the antioxidative capacities of rhizome knot extract and whole rhizome extract were evaluated using four different methods, and their antioxidative activities were further compared with other commonly used antioxidants, including plant extracts with known polyphenolic contents.

2. Materials and methods

2.1. Chemicals

Horse heart metmyoglobin (MMb, type III), methyl linoleate and Tween 20 were obtained from Sigma (St. Louis, MO, USA). 6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), quercetin dihydrate, 2,2-diphenyl-1-picrylhydrazyl hydrate (DPPH) and gallic acid monohydrate were purchased from Aldrich Chemical Co. (Steinheim, Germany). 4-Pyridyl-1-oxide-*N*-*tert*-butylnitrone (POBN), analytical grade hydrogen peroxide (30%) and ascorbic acid were supplied by Fluka Chemika (Switzerland). Folin-Ciocalteu-reagent, and analytical grade iron(II) sulphate heptahydrate, ethanol and potassium persulfate were obtained from Merck (Darmstadt, Germany) and the diammonium salt of 2,2′-azino-(3-ethylbenzthiazoline-6-sulfonic) acid (ABTS) was from Applichem (Darmstadt, Germany). Water was purified through a Millipore Q-plus purification train (Millipore, Bedford, MA, USA).

2.2. Plant extracts

Rhizome (Lotus roots) knot extract (LRK), whole rhizomes extract (LR), green tea extract (GTP), white and red grape seed extracts (WGE and RGE), Gingko biloba leave extract (GBE) and tea pigments (TP) were donated by the Laboratory of Natural Product Chemistry at the Department of Food Science and Technology at Huazhong Agricultural University, P.R. China. The extracts of Lotus root knot and whole Lotus roots were obtained by the following procedure: 10 g of dry and ground sample were extracted with 70 ml of 50% aqueous ethanol on a water bath for 30 min at 40 °C. The extraction was repeated twice, and the three extracts pooled, centrifuged and filtered, followed by evaporation of the solvent by rotary evaporation at 35 °C. The residues were then chromatographed on Sephadex LH-20, eluted by water and 50% aqueous ethanol. The 50% aqueous ethanol eluant was taken to dryness by rotary evaporation, and the residues were refrigerated and freeze-dried.

2.3. Analysis of total phenolic compound

Total phenols of LRK, LR, GTP, WGE and RGE were analyzed according to the method of Zoecklin, Fugelsang, Gump, and Nury, (1995) using gallic acid as standard, and the results were expressed as milligrams of gallic acid equivalents (GAE) per 100 mg of dry extracts and μM total phenol.

2.4. Determination of total flavone glycosides

Total flavone glycosides of GBE were determined according to the method of Hasler and Sticher (1992).

2.5. Oxygen consumption assay

Two-hundred and fifty microlitres of 28.2 mM methyl linoleate (dissolved in methanol) were mixed with 62.5 μl of 0.04g/ml Tween-20 in methanol, and methanol was removed with a stream of nitrogen, followed by adding 2.50 ml of 5.0 mM thermostatted (25 °C) air-saturated phosphate buffer (pH = 6.8) and 10 μl of sample extract or pure compound at different concentrations. Then 25 μl of 0.20 mM MMb aqueous solution were added in order to initiate oxidation. Immediately after MMb addition, measurements of the oxygen consumption were started by injection of the sample into a thermostatted (25.0 ± 0.1 °C) 70 μl measuring cell (Chemiware, Viby J., Denmark) with no headspace. The relative oxygen consumption was measured with a Clark electrode connected to a multichannel analyser ReadOx-4H (Sable Systems, Henderson, NEV, USA) and recorded at time intervals of 5 s for 20 min. The electrode was calibrated by a two-point calibration with anoxic and air-saturated buffers thermostatted at 25 °C.

The initial oxygen consumption rate $V(O_2)$ in μmol l^{-1} s^{-1} was calculated from:

$$V(O_2) = -\text{slope}\ [O_2]_{\text{initial}} 10^6/100 \quad (1)$$

The slope (per cent O_2 per second) was calculated from the oxygen consumption in the 80 to 40% interval relative to the initial 100% oxygen concentration corresponding to water saturated with air, $[O_2]_{\text{initial}} = 2.6\times10^{-4}$ mol l^{-1} at 25 °C. The influence of each of the extracts on the initial rate of oxygen consumption was expressed as an antioxidative index relative to the rate in the absence of extract:

$$I_{\text{oxygen}} = \frac{V(O_2) \text{ with extract present}}{V(O_2) \text{ without extract present}} \quad (2)$$

2.6. ESR assay

Four millilitres of 0.0032 mol l^{-1} POBN (dissolved in 1.0 mol l^{-1} aqueous ethanol) were mixed with 20 μl of 0.0022 mol l^{-1} $FeSO_4$, followed by addition of 50 μl of 5.0 mg ml^{-1} extract sample or 50 μl of water as a reference. Finally, 80 μl of 0.034 mol l^{-1} H_2O_2 were added and the mixture was transmitted to the quartz cell. The concentration of the mixture was as follows: 0.9639 mol l^{-1} ethanol, 3.05 mmol l^{-1} POBN, 9.91μmol l^{-1} $FeSO_4$ and 0.65 mmol l^{-1} H_2O_2. Exactly 2 min after the addition of the H_2O_2 solution, measurements were made on the JEOL FR 30 ESR spectrometer (JEOL Ltd., Tokyo, Japan). The internal manganese standard (the marker) was fixed at 700. The measurements were carried out at room temperature with the following settings: Microwave power 4 mW; centre field 334.954 mT; Sweep width 7. 5 mT; Sweep time 4 min; modulation width 0.1 mT; amplitude 630; conversion time C 0.3 s.

The degree of inhibition (I_{ESR}) was calculated from the peak height of the ESR signal for the trapped radical by:

$$I_{\text{ESR}} = \left(1 - \frac{\text{Peak height}_{\text{sample}}}{\text{Peak height}_{\text{reference}}}\right) \times 100\% \quad (3)$$

2.7. Free radical scavenging activity

The method of Joyeux et al. (1995) based on scavenging of the stable radical DPPH, as modified by Tadolini et al. (2000), was used to determine the free radical-scavenging activity of the extracts. Methanolic solution (1.5 ml) of DPPH (20 mg l^{-1}) were added to 0.75 ml of methanolic solution of extract or pure compound, and 0.75 ml of methanol was used as blank. Determination of DPPH concentration was carried out spectrophotometrically (absorbance at 517 nm) after 5 min incubation at room temperature, using a Cintra 40 UV-Visible Spectrometer (GBC Scientific Equipment, Australia). Most extract solutions were coloured and corrections for background absorbances of each sample were applied. The relative inhibition of the antioxidants against DPPH· was calculated according to the following equation:

$$\%\ \text{Inhibition} = \left(1 - \frac{\text{Absorbance}_{\text{sample}} - \text{Absorbance}_{\text{background}}}{\text{Absorbance}_{\text{blank}}}\right) \times 100\% \quad (4)$$

2.8. Total antioxidant activity

The ABTS radical cation decolorization method as described by Re, Pellegrini, Proteggente, Pannala, Yang, and Rice-Evans (1999) was adopted to compare the antioxidant activity of LRK and LR with other antioxidants. The decrease in concentration of preformed radical cation ($ABTS^{\cdot+}$ generated by oxidation of ABTS with potassium persulfate) on exposure to the extract solution was spectrophotometrically measured in comparison with standards of Trolox. After 3 min incubation of 1.0 ml of $ABTS^{\cdot+}$ solution and 10 μl of solutions of extracts or pure compound or standard solutions of Trolox, measurements were carried out at 734 nm at room temperature. The effect of each extract and pure compound on scavenging $ABTS^{\cdot+}$ was expressed as Trolox equivalent antioxidant capacity (TEAC), and defined as the concentration (mM) of Trolox with equivalent antioxidant activity to a 0.5 mg m^{-1} concentration of each extract or pure compound.

3. Results and discussion

Phenolic compounds are considered as the most important antioxidative components of herbs and other plant materials, and good correlation between the concentration of plant phenolics and the total antioxidant capacity has been reported (Madsen et al., 1996; Pellegrini et al., 2000). In the present study of the antioxidant capacity of Lotus root, the total phenol contents of the two type of Lotus root extract were determined for comparison with the contents of other plant materials known to have antioxidative activity. As may be seen from the analytical results presented in Table 1, the root knot extract (LRK) has a very high content of phenolic compounds which is even higher than the content of green tea polyphenols (GTP). Thus, the preparation has been receiving particular attention due to its high content of polyphenols.

Table 1
Content of total phenolics in extracts of lotus root in comparison with selected plant extracts[a]

	LRK	LR	GTP	WGE	RGE	GBE
Total phenols (g/100 g dry extract)	77.0±0.5	31.9±0.9	72.6±0.8	58.9±0.7	56.7±1.0	
Total flavone glycoside (g/100 g dry extract)						19.2±0.8

[a] Values are the means of three determinations, expressed as means±S.D. LRK, lotus root knot extract; LR, lotus root extract; GTP, green tea polyphenols; WGE, white grape seed extract; RGE, red grape seed extract; GBE, Ginkgo biloba leaf extract.

Table 2
Antioxidative index, I_{oxygen} based on oxygen depletion rate in peroxidating methyl linolate emulsion for extracts of Lotus root in comparison with water-souble antioxidants and green tea polyphenols[a]

Ascorbic acid		Trolox		LRK		LR		GTP[b]	
C (μM)	I_{oxygen}	C (μM)	I_{oxygen}	C[b] (μM)	I_{oxygen}	C (μM)	I_{oxygen}	C(μM)	I_{oxygen}
20	0.26±0.06	0.89	0.52±0.05	1.81	0.35±0.08	3.75	0.65±0.11	0.85	0.59±0.05
60	0.21±0.05	1.76	0.45±0.07	3.62	0.18±0.07	7.51	0.52±0.06	1.71	0.39±0.09
100	0.20±0.06	2.64	0.34±0.04	5.44	0.14±0.05	15.01	0.26±0.06	3.41	0.24±0.05
140	0.20±0.04	3.48	0.26±0.10	7.25	0.12±0.05	22.52	0.19±0.03	5.12	0.20±0.07
180	0.19±0.03	4.40	0.21±0.07	9.06	0.09±0.03	30.02	0.16±0.05	6.82	0.16±0.04
		6.67	0.25±0.02	10.87	0.13±0.04	37.53	0.23±0.04	8.53	0.24±0.10

[a] Red grape extract was found to have $I_{oxygen} = 0.26$ at a concentration of 4.76 μM total phenols. RK, lotus root knot ectract; LR, lotus root extract; GTP, green tea polyphenols.
[b] Concentrations of extracts (LRK,LR and GTP) are expressed as μM total phenols. Each value of the antioxidative index is mean of two determinations±S.D.

Antioxidants interrupt lipid (and protein) oxidation, either in the propagation phase (chain-braking mechanism) or by protecting the oxidation substrates against the first formed radicals in the initiation phase. Accordingly, evaluation of plant material for antioxidative activity should not depend only on a single method, but it should include measurement of reactions characteristic of both the initiation and the propagation phase (Schwarz et al., 2001).

Oxygen consumption is characteristic of the propagation phase and the effect of the antioxidants on the progress of lipid oxidation, during which peroxyl radicals are chain-carrying, is directly measured in the assay of the rate of depletion of oxygen in a peroxidating lipid emulsion. The plant extracts explored in the present study showed different effects on the oxygen depletion in the assay, based on metmyoglobin-initiated oxidation of methyl linoleate. As may be seen from Table 2, the antioxidative indices of LRK, LR and other selected antioxidants showed different concentration dependencies. Notably, LRK is most effective in halting the oxygen consumption by the peroxidating lipid emulsion and comparable to the effective chain-braking antioxidant Trolox. LR is the second least effective and only more effective than ascorbic acid, which is not normally considered effective in such O/W-emulsions, although the concentration effects of the antioxidants are different. The green tea polyphenols (GTP) are comparable, although not quite as efficient as LRK. The overall ranking for the five plant extracts and the two water-soluble antioxidants is accordingly, when correction for concentration effects is made: LRK > GTP > Trolox > RGT > LR > Ascorbic acid. The effect as a chain-breaking antioxidant is clearly correlated to the polyphenol contents of the extract, with LKR being superior even to GTP. Ascorbic acid is often considered as a prooxidant in such aqueous food models and, for LR prooxidative components, may also be present. It should also be noted that LRK is still effective at a rather low concentration.

Scavenging of non-lipid radicals is of importance for protection against early events in oxidative damage, i.e. formation of first radicals by metal catalysis or light exposure (Skibsted, 2000). The antioxidative capacity of the plant extracts was accordingly investigated, in terms of their ability to scavenge small carbon-centered radicals. Assays based on scavenging of the hydroxyl radical (Madsen et al., 1996) are found to overestimate the antioxidant efficiencies (Schwarz et al., 2001) and the Fenton chemistry-based assay may be improved by adding alcohol. The mechanism of forming the small carbon-centered radical (Rosen, Britigan, Halpern, & Pou, 1999) is

$$Fe^{2+} + H_2O_2 \rightarrow \cdot OH + OH^- + Fe^{3+} \quad (5)$$

$$CH_3CH_2OH + \cdot OH \rightarrow CH_3 \cdot CHOH + H_2O \quad (6)$$

followed by the spin-trapping reaction:

$$CH_3 \cdot CHOH + POBN \rightarrow POBN\text{-}CH(CH_3)OH \quad (7)$$

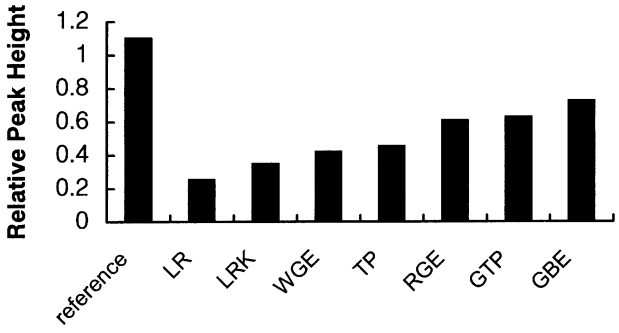

Fig. 1. Relative peak height of ESR spectra in the $FeSO_4/H_2O_2$/EtOH/POBN assay for plant extracts. LR, Lotus root extract; LRK, Lotus rood knot extract; GTP, green tea polyphenol; WGE, with grape seed extract; RGE, red grape seed extract; GBE, Gingko biloba leave extract; TP, tea pigment. Values are the means of three determinations.

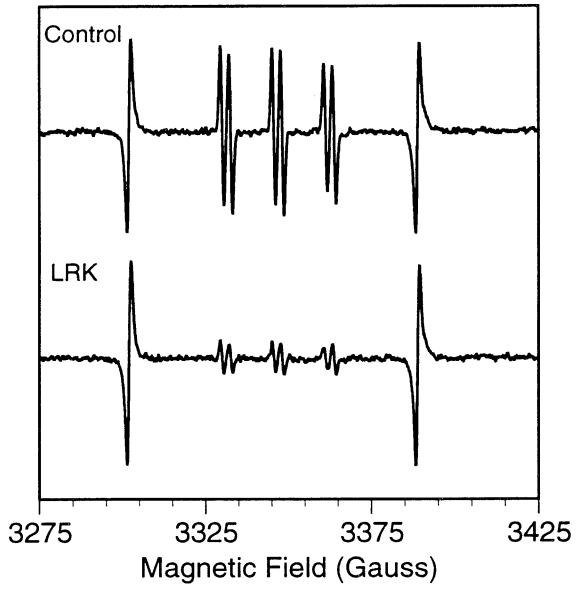

Fig. 2. ESR spectra of the $FeSO_4/H_2O_2$/EtOH/POBN system. Control shows the spectra for the assay without antioxidant. Lotus root knot extract (LRK) shows the spectra after adding LRK.

In this assay the concentration of ethanol in the reaction mixture should be in excess compared to the steady-state concentration of •OH in order to ensure that the reaction of •OH with POBN becomes insignificant.

Fig. 1 illustrates that the plant extracts tested have scavenging activity, LR and LRK being the most efficient scavengers and GBE and GTP being the least efficient. The significant radical scavenging activity of LR may be ascribed to components other than polyphenols in LR. Although GTP showed a remarkably high retarding effect on O_2 consumption, it was less efficient in scavenging small carbon-centered free radicals than LR. This finding is, however, in agreement with what was observed for scavenging of •OH by GTP (Noda et al., 1999). The effect on the ESR signal of the spin trap by LRK can be seen in Fig. 2 and the dose-dependent increase of antioxidative activity of both LRK and LR is illustrated in Fig. 3.

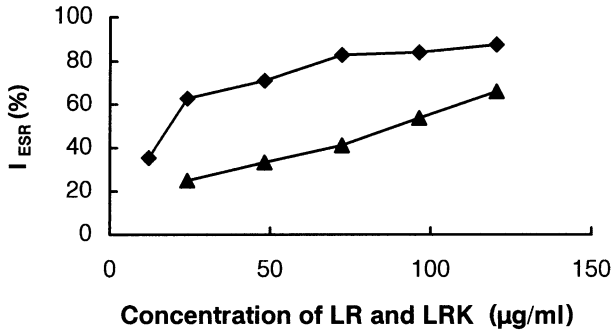

Fig. 3. Effects of concentration of lotus root knot extract (LRK) and lotus root extract (LR) on the degree of inhibition (I_{ESR}) for small carbon-centered free radicals. ▲, LRK; ■, LR.

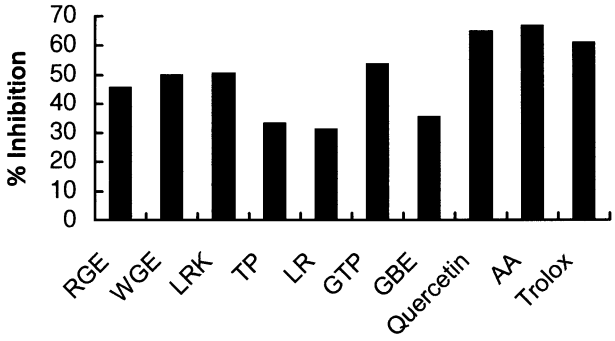

Fig. 4. DPPH free radical-scavenging activity of plant extract and selected antioxidants. See Table 1 and Fig. 1 for abbreviations.

The plant extracts and selected antioxidants were also examined for their radical-scavenging capacities toward the stable free radical DPPH•. The mechanism of scavenging DPPH• by radical scavengers (Espin et al., 2000) can be briefly expressed as:

$$\underset{\text{(purple)}}{\text{DPPH}\cdot} + (\text{AH})_n \rightarrow \underset{\text{(yellow)}}{\text{DPPH-H}} + (\text{A})_n \quad (8)$$

As shown in Fig. 4, LRK showed a better scavenging capacity for DPPH· than LR. Ascorbic acid, quercitin, Trolox and GTP were found to be the most efficient scavengers and it must be concluded that these results are in agreement with the result of the oxygen consumption assay, except for the efficiency of ascorbic acid. The relationship between the concentration of LRK and LR and the scavenging efficiency against DPPH is shown in Fig. 5. The results from the DPPH scavenging assay are in agreement with the oxygen depletion assay and seem to reflect the efficiency of the extracts to halt the propagation of lipid oxidation, while the ESR spin trapping assay reflect rather the capacity to prevent initiation of oxidation.

The ABTS radical cation decolorization assay is in the improved version (Re et al., 1999) applicable for the study of both water-soluble and lipid-soluble antioxidants. The total antioxidant activity of selected plant extracts was assessed by this system, in which potassium

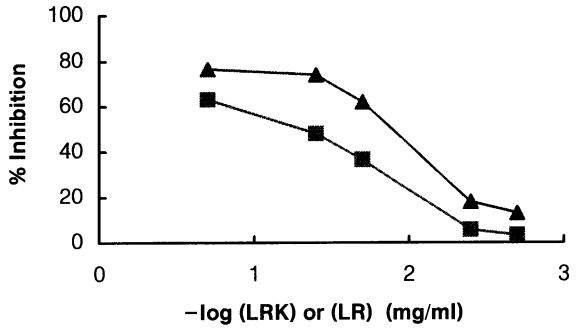

Fig. 5. Effects of concentration of lotus root knot extract (LRK) and lotus root extract (LR) on the scavenging activity for DPPH free radicals. ▲,- LRK; ■,- LR.

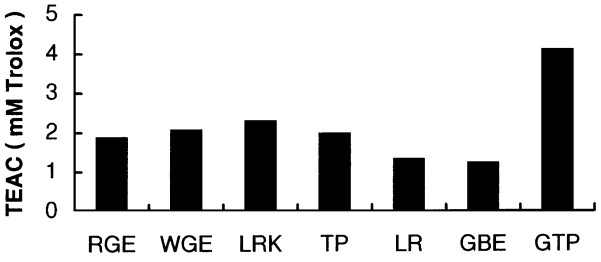

Fig. 6. Total antioxidant activity (expressed as Trolox equivalents in mM in 0.5 mg extract ml^{-1} solutions) of plant extracts. See Table 1 for abbreviations.

persulfate (rather than MMb and H_2O_2) was used for forming the ABTS free radical prior to addition of the potential antioxidant.

The experimental results (Fig. 6) indicate that LRK, with the highest value of total phenol, ranked the highest according to the TEAC value, except for GTP, and LR had a relatively low TEAC value in correspondence with its low total phenolic content. This result is in general agreement with that of Pietta, Simonetti, and Mauri (1998) and similar to those found in the oxygen consumption assay and the DPPH scavenging assay.

In conclusion, LRK exhibited a distinctive antioxidative capacity in comparison with commonly used plant extracts. Based on the high antioxidative activity, Lotus root knots should be considered as an additive to other products for oxidative protection. The knowledge of composition and structure of phenolic compounds in LRK is important for a better understanding of its function and physiochemical properties, such as phase distribution. We have, therefore, embarked on an assay-guided study on composition and structure of the active compounds of LRK, which should further help to add value to this waste product from Asian food production.

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