Evaluation of the Antioxidant Activity of Environmental Plants: Activity of the Leaf Extracts from Seashore Plants

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The antioxidant activity of the methanolic extracts of the leaves of 39 plant species was examined. These leaves were collected from the plants growing on subtropical seashores. The activity was evaluated by three kinds of assay methods, which included the DPPH radical scavenging assay, linoleic acid oxidation assay, and oxidative cell death assay. Two extracts from *Excoecaria agallocha* and *Terminalia catappa* showed remarkably potent activity in all assay systems. The HPLC analysis of the extracts indicated the presence of the same antioxidant and isolation work for the compound identified ellagic acid. The isolated ellagic acid showed strong antioxidant activity in the assay systems used.

Keywords: *Antioxidant activity; seashore plant; DPPH; linoleic acid; oxidative cell death; ellagic acid*

INTRODUCTION

Antioxidants are important inhibitory materials against the oxidative deterioration of food. These antioxidants have received more attention because of their inhibitory activity during the initiation stage of some oxidation-related diseases. Many researchers have sought efficient antioxidants from natural sources and reported their isolation from plants used for crops, vegetables, spices, and herbs (Shahidi, 1997). Screening studies for the antioxidant-related activity on such plants have also been reported (Ganthavorn and Hughes, 1997; Wang et al., 1996; Kim et al., 1994a,b; Tsushida et al., 1994; Banias et al., 1992; Su et al., 1986; Toda et al., 1984). It should be noted that a plant is a large biological entity and a part of it has been used for the above-mentioned applications. Other plants in the environment, which have not been well utilized for humans, should receive attention as an easily accessible source for antioxidants. It is well-known that a plant produces active oxygen species during photosynthesis, in which the plant oxidizes H_2O to O_2 to receive energy for the fixation of carbon dioxide. Normally, the active oxygens produced in plant cells are under control of the plant's own scavenging system. When a plant is exposed to strong sunlight, it is forced to produce a much larger amount of harmful active oxygens in their photosynthetic system, which may cause severe damage to the plant body (Asada and Takahashi, 1987). However, some kinds of plants can grow under these severe conditions, which indicates that they have a special protective system against such harmful species. We expected that these plants have an efficient antioxidant in their chemical constituents for the protective system and planned to evaluate the antioxidant activity of their constituents. For the selection of the kinds of plants, we focused on the seashore in tropical areas. The plants growing there get a large amount of sunlight. They should have an efficient antioxidant system in order to live and may have efficient antioxidants. This paper deals with the antioxidant activity evaluation of such plants and the investigation into the antioxidant constituents of the plants.

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MATERIALS AND METHODS

Plant-Collection Field. The natural seashores of Iriomote Island were chosen for this research purpose because the island belongs to the most southern National Park of Japan and has a subtropical climate. The average accumulated sunray in July is recorded to be 22.5 MJ/m^2 at the nearest Ishigaki meteological station (13.9 MJ/m^2 at the Tokyo meteological station) (Chronological Scientific Tables, 1998).

Materials. Plants were collected in July 1997 and identified by one of the authors (SY) and scientific names for the plants were adopted from Walker (1976) or Hatsusima and Amano (1977). The collected leaves were air-dried for 1 week at room temperature and stored until extraction. Linoleic acid (extra pure grade) was purchased from Nacalai Tesque (Kyoto, Japan) and used without further purification. 2,2′-Azobis(2,4-dimethylvaleronitrile) (AMVN), 1,1-diphenyl-2-picrylhydrazyl (DPPH), and sodium dodecyl sulfate (SDS) were obtained from Wako Chemical Industries (Osaka, Japan). Ethidium bromide was obtained from Katayama Chemical (Osaka, Japan). Other reagents and all solvents were purchased as extra pure grade from Nacalai Tesque (Kyoto, Japan). The HPLC column for analysis was purchased from Tosoh (Tokyo, Japan), and the column for the preparation was purchased from Nomura Chemicals (Seto, Japan). Rats (4-week-old Wistar) were obtained from Nisshin (Tokushima, Japan).

Instruments. Analytical HPLC was carried out using a Shimadzu 6A HPLC system equipped with a diode array detector (SPD-M10AVP, Shimadzu, Kyoto). Preparative HPLC was carried out using a Gulliver HPLC system (JASCO, Tokyo). NMR spectra were recorded by a EX-400 spectrometer (JEOL, Tokyo). MS were recorded by an AX-100 spectrometer (JEOL, Tokyo). Flow cytometric analysis was carried out with a flow cytometer, CytoACE-150 (JASCO, Tokyo).

Extraction of Plant Materials. The dried leaves were cut into small pieces and soaked twice in methanol (leaf weight,

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Table 1. Names of Collected Seashore Plants and Yield of Methanolic Extracts

no.	scientific plant name[a]	common English name[c]	family name[a]	yield, %
1	*Allophylus timorensis* Blume		Sapindaceae	23.1
2	*Asparagus cochinchinesis* Merr.		Liliaceae	25.2
3	*Bidens pilosa* L. var. *radiata* Schultz.-Bip.		Compositae	16.1
4	*Calophyllum inophyllum* L.	Alexandrian laurel	Guttiferae	14.7
5	*Carex pumila* Thunb.		Cyperaceae	7.1
6	*Cassytha filiformis* L.		Lauraceae	11.9
7	*Cerbera manghas* L.		Apocynaceae	7.2
8	*Clerodendrum inerme* Gaertn.	glosy bower	Verbenaceae	18.6
9	*Crinum asiaticum* var. *japonicum* Baker		Amaryllidaceae	18.6
10	*Crossostephium chinense* Makino		Compositae	18.1
11	*Excoecaria agallocha* L.	milky mangrove	Euphorbiaceae	20.3
12	*Flagellaria indica* L.		Flagellariaceae	12.1
13	*Garcinia subelliptica* Merr.		Guttiferae	21.8
14	*Hernandia nymphaeaefolia* Kubitzki	sea horse	Hernandiaceae	5.2
15	*Hibiscus tiliaceus* L.	sea hibiscus	Malvaceae	9.5
16	*Ipomoea pes-caprae* R. Br. subsp. *brasiliensis* Ooststr.		Convolvulaceae	17.0
17	*Ischaemum muticum* L.		Gramineae	11.3
18	*Ixeris lanceolata* Steff. f. *pinnatiloba* Hats.[b]		Compositae	22.9
19	*Lactuca formosana* Maxim.		Compositae	18.6
20	*Limonium wrightii* var. *arbusculum* Hara		Plumbraginaceae	13.4
21	*Liriope spicata* Lour.		Liliaceae	7.1
22	*Lysimachia mauritiana* Lamarck		Primulaceae	13.4
23	*Maytenus diversifolia* Ding Hou		Celastraceae	18.2
24	*Morus australis* var. *glabra* Koidz.		Moraceae	13.3
25	*Pandanus tectorius* Soland. var. *tectorius*	thatch screwpine	Pandanaceae	7.0
26	*Pemphis acidula* J. R. & G.Forst.		Lythraceae	17.4
27	*Peucedanum japanicum* Thunb.		Umbelliferae	16.6
28	*Pongamia pinnata* Merr.		Leguminosae	14.9
29	*Scaevola taccada* Roxb.		Goodeniaceae	6.2
30	*Sesuvium portulacastrum* L.	purslane sesuvium	Aizoaceae	19.7
31	*Sophora tomentosa* L.		Leguminosae	12.5
32	*Spinifex littoreus* Merr.		Gramineae	12.6
33	*Stenotaphorum secundatum* Kuntze[b]		Gramineae	3.3
34	*Terminalia catappa* L.	Indian almond	Combretaceae	14.4
35	*Thespesia populnea* Soland.	potia tree	Malvaceae	12.3
36	*Tournefortia argentea* L. f.		Boraginaceae	6.4
37	*Vitex trifolia* L. var. *trifolia*		Verbenaceae	21.2
38	*Vigna marina* Merr.		Leguminosae	19.5
39	*Wedelia biflora* DC		Compositae	11.0

[a] Cited scientific name and family name for each plant was adopted from Walker (1976). [b] These names were adopted from Hatsusima and Amano (1977), because the above-mentioned dictionary does not cite these plants. [c] Blank indicates name is not cited in Walker (1976).

ca. 7−60 g/methanol, 150−500 mL) for 6−9 days at room temperature. After filtration of the leaves, the solvent of the combined solutions was evaporated off under reduced pressure, giving a methanol extract. The extracts was stored at −30 °C until the assay and analysis.

Measurement for Antiradical Activity using DPPH. To the appropriate amount of sample in methanol solution (4.9 mL) was added 100 μL of 5 mM DPPH in methanol solution. After the solution stood at 37 °C for 0.5 h, the absorbance at 517 nm was measured. For ellagic acid, its methanolic solution was prepared by adding 10 μL of an appropriate amount of ellagic acid in dimethyl sulfoxide to 4.9 mL of methanol. This amount of dimethyl sulfoxide has no effect on the control absorbance. The antiradical activity was evaluated from the decrease value of 517-nm absorption, which was calculated by the following equation: decrease of absorbance = (absorbance of DPPH solution) − (absorbance of DPPH + sample solution) + (absorbance of sample solution).

Measurement for Antioxidant Activity against AMVN-Initiated Linoleic Acid Oxidation. To a micelle solution (4 mL) of 2.5 mM linoleic acid in 0.1 M SDS (sodium dodecyl sulfate)−0.05 M phosphate buffer (pH = 7.4) was added 10 μL of sample solution (2.0 g/L of plant extracts or α-tocopherol in methanol, or 5 mM ellagic acid or α-tocopherol in dimethyl sulfoxide), and the solution was sonicated for 30 s. Three milliliters of the solution was placed in a cuvette, and the cuvette was set in the thermocontrolled sample compartment (50.0 ± 0.2 °C) of a spectrophotometer. After the solution was allowed to stand for 15 min, 10 μL of 700 mM AMVN in acetonitrile was added, and the solution was vigorously stirred (absorbance at 234 nm at this stage should be below 1.2). Any increase in the absorbance at 234 nm was recorded for 1 h. Each control experiment was carried out by adding the same vehicle only to the lipid solution.

Measurement for Inhibition Activity against Oxidative Cell Death. The technique for the dissociation of rat thymocyte was similar to that previously described (Nagano et al., 1997). Briefly, thymus glands dissociated from the rats were sliced to a thickness of 0.4−0.5 mm. Thereafter, the slices were gently triturated in chilled Tyrode's solution to dissociate a single thymocyte. Tyrode's solution containing the dissociated thymocyte was passed through a mesh (diameter of 0.05 mm) to remove residues and diluted to the appropriate concentration for this assay. To the cell suspension (ca. 3000 cells) was added the test sample (final concentration: 30−1000 nM for ellagic acid and 10 μg/mL for plant extracts) in dimethyl sulfoxide (final concentration is below 1%), and the suspension was incubated for 30 min at 36 °C. To the suspension was added 30% H_2O_2 (final concentration: 3 mM), and the suspension was incubated at 36 °C. Three hours later, ethidium bromide in DMSO was added to the suspension (final concentration: 10 μM). Fluorescence from the stained cells was measured 2−3 min after the dye addition by a flow cytometer. The excitation wavelength for ethidium was 488 nm, and the emission was detected at a wavelength of 580−620 nm. The fluorescence histogram was analyzed using a software program (JASCO Version 3XX, JASCO, Tokyo). The percent value of the stained cell number to the total cell number was calculated using the same software. The inhibition activity of each sample was evaluated from the inhibition percent, which was obtained by the following equation: inhibition percent = [(stained cell percent of H_2O_2-applied experiment) − (stained cell percent

Table 2. Antiradical Activity of Seashore Plant Extracts

no.	plant name for extracts	decrease value of 0.1 mM DPPH abs (517 nm)[a–c] for sample concn 50 mg/L	10 mg/L	5 mg/L
1	*A. timorensis*	0.166 ± 0.011	0.083 ± 0.006	
2	*A. cochinchinesis*	0.279 ± 0.001	0.059 ± 0.002	
3	*B. pilosa* var. *radiata*	0.612 ± 0.011	0.107 ± 0.005	
4	*C. inophyllum*	0.971 ± 0.008	0.207 ± 0.013	
5	*C. pumila*	0.808 ± 0.006	0.158 ± 0.005	
6	*C. filiformis*	0.492 ± 0.008	0.117 ± 0.009	
7	*C. manghas*	0.110 ± 0.011	0.073 ± 0.003	
8	*C. inerme*	0.743 ± 0.002	0.169 ± 0.009	
9	*C. asiaticum* var. *japonicum*	0.327 ± 0.008	0.075 ± 0.007	
10	*C. chinense*	0.408 ± 0.007	0.040 ± 0.001	
11	*E. agallocha*		0.722 ± 0.005	0.573 ± 0.003
12	*F. indica*	0.145 ± 0.001	0.033 ± 0.009	
13	*G. subelliptica*	0.805 ± 0.001	0.174 ± 0.003	
14	*H. nymphaeaefolia*	0.250 ± 0.001	0.070 ± 0.007	
15	*H. tiliaceus*		0.320 ± 0.008	0.181 ± 0.007
16	*I. pes-caprae* subsp. *brasiliensis*	0.399 ± 0.006	0.088 ± 0.010	
17	*I. muticum*	0.448 ± 0.004	0.091 ± 0.005	
18	*I. lanceolata f. pinnatiloba*	0.360 ± 0.009	0.110 ± 0.007	
19	*L. formosana*	0.285 ± 0.006	0.064 ± 0.008	
20	*L. wrightii* var. *arbusculum*	0.495 ± 0.001	0.090 ± 0.010	
21	*L. spicata*	0.219 ± 0.002	0.050 ± 0.009	
22	*L. mauritiana*	0.373 ± 0.006	0.079 ± 0.007	
23	*M. diversifolia*	0.342 ± 0.002	0.084 ± 0.001	
24	*M. australis* var. *glabra*	0.182 ± 0.004	0.062 ± 0.005	
25	*P. tectorius* var. *tectorius*	0.251 ± 0.006	0.054 ± 0.011	
26	*P. acidula*	1.053 ± 0.006	0.205 ± 0.012	
27	*P. japanicum*	0.130 ± 0.004	0.025 ± 0.008	
28	*P. pinnata*	0.417 ± 0.008	0.104 ± 0.008	
29	*S. taccada*	0.414 ± 0.004	0.118 ± 0.013	
30	*S. portulacastrum*	0.347 ± 0.009	0.075 ± 0.010	
31	*S. tomentosa*	0.146 ± 0.011	0.050 ± 0.001	
32	*S. littoreus*	0.421 ± 0.006	0.094 ± 0.006	
33	*S. secundatum*	0.471 ± 0.007	0.113 ± 0.001	
34	*T. catappa*		0.787 ± 0.013	0.570 ± 0.005
35	*T. populnea*	0.598 ± 0.004	0.151 ± 0.000	
36	*T. argentea*	0.379 ± 0.002	0.064 ± 0.009	
37	*V. trifolia* var. *trifolia*	0.324 ± 0.006	0.111 ± 0.001	
38	*V. marina*	0.285 ± 0.004	0.061 ± 0.001	
39	*W. biflora*	0.306 ± 0.008	0.076 ± 0.008	

[a] Mean ± SD ($n = 2$). [b] Absorbance of 0.1 mM DPPH is around 1.0 at 517 nm after incubation. [c] Decrease absorbance value of α-tocopherol (10 mg/L) was 0.524 ± 0.003.

of sample and H_2O_2-applied experiment)]/[(stained cell percent of H_2O_2-applied experiment) − (stained cell percent of nothing-applied experiment)] × 100. Statistical analysis was carried out by using Student's *t* test.

HPLC Analysis of Plant Extracts. Twenty-five milligrams of the extract was dissolved in methanol (2 mL) and passed through a C18 Sep-Pak cartridge (Waters, Milford, CT). The cartridge was successively washed with methanol (1 mL) and acetonitrile (1 mL) to make the sample solution (25 mg/4 mL). Ten microliters of the solution was injected into an HPLC system under the following conditions: column, TSK-ODS-80Ts (4.6 × 150 mm) (Tosoh, Tokyo); solvent A, 0.1% H_3PO_3 in H_2O, solvent B, HPLC-grade acetonitrile; flow rate, 1 mL/min; gradient conditions, A = 90% and B = 10% at 0 min, A = 60% and B = 40% at 40 min, and A = 0% and B = 100% at 50 min in a linear mode. Estimation for the amount of ellagic acid in *E. agallocha* and *T. catappa* was carried out by calculation from corresponding peak area with a standard curve of pure ellagic acid.

Antioxidant Isolation Procedure. Ninety milligrams of the extract was dissolved in methanol and passed through the Sep-Pak C18 cartridge. The solution (0.5 mL/1 operation) was injected into a preparative HPLC under the following conditions: column, Develosil ODS 10/20 (20 × 250 mm); solvent, 50% methanol containing 1% acetic acid; flow rate, 9 mL/min; detection, 254 nm. A peak eluted at 14 min was collected. The collected solution was evaporated in vacuo, giving an amorphous solid (5 mg). ^{1}H NMR (DMSO-d_6, 400 MHz) δ 7.42 (2H, s). EI-MS *m*/*z* 302 (M^+). The solid was used for the structure analysis. One milligram of the solid was acetylated with acetic anhydride (0.5 mL) and pyridine (0.5 mL) to give the tetra-acetate (1 mg) after evaporation of the solvent. ^{1}H NMR ($CDCl_3$, 400 MHz) δ 2.40 (6H, s), 2.48 (6H, s), 8.06 (2H, s). EI-MS *m*/*z* 470 (M^+), 428, 386, 344, 302.

RESULTS AND DISCUSSION

Antioxidant Activity of Plant Extracts. Thirty-nine kinds of plant leaves were collected from plants growing on the seashores because leaves are the most illuminated part of the plant and may have an efficient antioxidant system (Asada and Takahashi, 1987). However, no antioxidant investigation on the collected plant leaves has been reported yet. After air-drying, the leaves were soaked in methanol to extract the organic constituents. The yields of the extracts, as summarized in Table 1, varied between 3% and 25% based on the weight of the dry leaves. To evaluate the antioxidant activity of the extracts, a radical scavenging assay using DPPH was first employed, because radical trapping is one of the most important properties of chain-breaking types of antioxidants (Niki, 1987) and the DPPH method is one of the most effective procedures to estimate the amount of radical trapping materials in the extracts. DPPH is a stable radical species and shows absorbance at 517-nm, while reduced DPPH does not have a 517-nm absorbance (Blois, 1958). The activity was evaluated by the decreasing value of the 517 nm absorbance, which

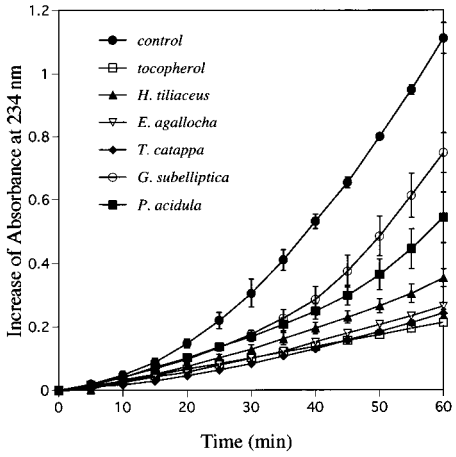

Figure 1. Inhibitory activity of five plant extracts and α-tocopherol against AMVN-induced linoleic acid oxidation in 0.1 M SDS micelle system (concentration for extracts and α-tocopherol, 5 mg/L; AMVN, 2.3 mM; linoleic acid, 2.5 mM). Control experiment was carried out by addition of the same amount of vehicle (methanol). Each bar shows SD.

is summarized in Table 2. All extracts were measured at the concentration of 10 mg/L against 0.1 mM DPPH. The extracts that showed decreasing values below 0.3 were examined again at the concentration of 50 mg/L, while three extracts that had values above 0.3 were examined under diluted conditions (5 mg/L). From the comparison of the values, the extracts of *E. agallocha* and *T. catappa* showed potent radical trapping activity and the activity was stronger than that of the pure natural antioxidant, α-tocopherol (10 mg/L, absorbance decrease 0.524 ± 0.003). The extracts of *H. tiliaceus, G. subelliptica,* and *P. acidula* showed moderate activity.

A possible and important target of radical oxidation, not only in the food but also in the living cells is the unsaturated lipid. The antioxidant activities of two strong extracts and three moderately active extracts were examined using linoleic acid as the oxidation substrate. These results are shown in Figure 1. All examined extracts showed inhibitory activity in this system. The extracts of *E. agallocha* (5 mg/L) and *T. catappa* (5 mg/L) showed strong activity which is comparable to that of α-tocopherol (5 mg/L). The activity of the other extracts was weaker than that of α-tocopherol. The activity order obtained in this system is in good agreement with the data in the DPPH system.

Some antioxidants protect cells from death due to oxidative stress induced by external active oxygens such as H_2O_2 (Oyama et al., 1994). In the cells, H_2O_2 produces a hydroxyl radical, and the antioxidant can trap the radical after its permeation into the cells (Oyama et al. 1998). The cell protective activity of the extracts against H_2O_2-induced oxidative cell damage was examined by a reported protocol using a flow cytometer (Nagano et al., 1997). The activity was evaluated using a percent inhibition value obtained from the relative dead cell number; the data are summarized in Table 3. Table 3 shows that the inhibition activity varied and some extracts promoted cell death. Among them, the extracts of *E. agallocha* and *T. catappa* showed a strong inhibitory activity, which is similar to the results from previous assay systems. The *P. acidula* extract, a moderately active extract in the previous two systems, showed a 33.6% inhibition, while *G. subelliptica,* another moderately active extract, showed, interestingly, a very promotive activity (−42.6%). The activities of the other extracts do not correlate well with that in the previous systems. Such promotive extracts may contain a cytotoxic material for used cells or may generate active oxygen species, as do some polyphenols (Stadler et al., 1996).

Table 3. Inhibitory Effect of Seashore Plant Extracts against H_2O_2-Induced Cell Death

no.	plant extract (10 mg/L)	% of stained cell[a]	% inhibition
	control	7.2 ± 6.4[b]	
	H_2O_2 (3 mM)	63.0 ± 7.6[b]	
1	*A. timorensis*	76.6 ± 5.0[c]	−24.0
2	*A. cochinchinesis*	70.3 ± 6.5	−12.8
3	*B. pilosa* var. *radiata*	73.3 ± 3.1	−18.2
4	*C. inophyllum*	69.2 ± 24.8	−11.0
5	*C. pumila*	45.5 ± 14.2[c]	30.9
6	*C. filiformis*	61.6 ± 7.1	2.4
7	*C.manghas*	69.9 ± 4.6	−12.2
8	*C. inerme*	50.2 ± 21.6	22.6
9	*C. asiaticum* var. *japonicum*	73.8 ± 6.7	−19.0
10	*C. chinense*	62.7 ± 19.0	0.6
11	*E. agallocha*	21.2 ± 6.5[c]	72.9
12	*F. indica*	57.6 ± 9.5	9.6
13	*G. subelliptica*	87.1 ± 9.3[c]	−42.6
14	*H. nymphaeaefolia*	64.5 ± 6.3	−2.7
15	*H. tiliaceus*	54.8 ± 25.9	14.6
16	*I. pes-caprae* subsp. *brasiliensis*	86.7 ± 8.8[c]	−41.9
17	*I. muticum*	54.2 ± 17.2	15.5
18	*I. lanceolata* f. *pinnatiloba*	57.2 ± 15.6	10.2
19	*L. formosana*	66.7 ± 9.8	−6.4
20	*L. wrightii* var. *arbusculum*	62.4 ± 7.7	1.0
21	*L. spicata*	66.8 ± 13.0	−6.7
22	*L. mauritiana*	72.6 ± 19.5	−16.9
23	*M. diversifolia*	64.0 ± 12.8	−1.8
24	*M. australis* var. *glabra*	76.1 ± 26.5	−23.1
25	*P. tectorius* var. *tectorius*	65.4 ± 6.0	−4.2
26	*P. acidula*	44.0 ± 14.6[c]	33.6
27	*P. japanicum*	55.3 ± 23.5	13.6
28	*P. pinnata*	63.8 ± 11.0	−1.3
29	*S. taccada*	67.8 ± 7.0	−8.4
30	*S. portulacastrum*	52.7 ± 23.1	18.2
31	*S. tomentosa*	68.6 ± 7.7	−9.8
32	*S. littoreus*	48.7 ± 17.0	25.3
33	*S. secundatum*	63.1 ± 7.9	−0.2
34	*T. catappa*	28.8 ± 9.0[c]	60.5
35	*T. populnea*	36.5 ± 28.2	46.8
36	*T. argentea*	67.0 ± 10.4	−7.0
37	*V. trifolia* var. *trifolia*	50.9 ± 21.6	21.5
38	*V. marina*	71.4 ± 6.7	−14.8
39	*W. biflora*	79.8 ± 20.7	−28.8

[a] Mean ± SD ($n = 4-6$). [b] $n = 20$. [c] Significant ($P < 0.005$) from H_2O_2 experiment.

Isolation and Identification of an Antioxidant. In all three assay systems used, two extracts from *E. agallocha* and *T. catappa* always showed strong antioxidant activity. The constituents of the two extracts were analyzed and compared by HPLC with gradient elution. The data are shown in Figure 2. In both data sets, a typical sharp peak was observed at the retention time of 17.6−17.7 min. The UV spectra for the peak in both sets of data were very similar as shown in Figure 3, indicating that the same compound exists in both extracts and may play an important role in the activity of both extracts. To identify this compound, isolation was carried out using the extracts of *T. catappa.* The preparative HPLC separation described in the Materials and Methods section gave compound **1** as a colorless solid. The EI-MS of **1** gave a molecular ion peak at *m*/*z* 302. Acetylation of **1** with acetic anhydride and pyridine gave a tetraacetate (**2**), which was confirmed by the MS molecular and fragment ion peaks (*m*/*z* 470, 428, 386, 344, and 302). The ^{1}H NMR of **1** showed only an aromatic signal at 7.42 ppm; however, the acetylated compound **2** showed two signals due to the phenolic

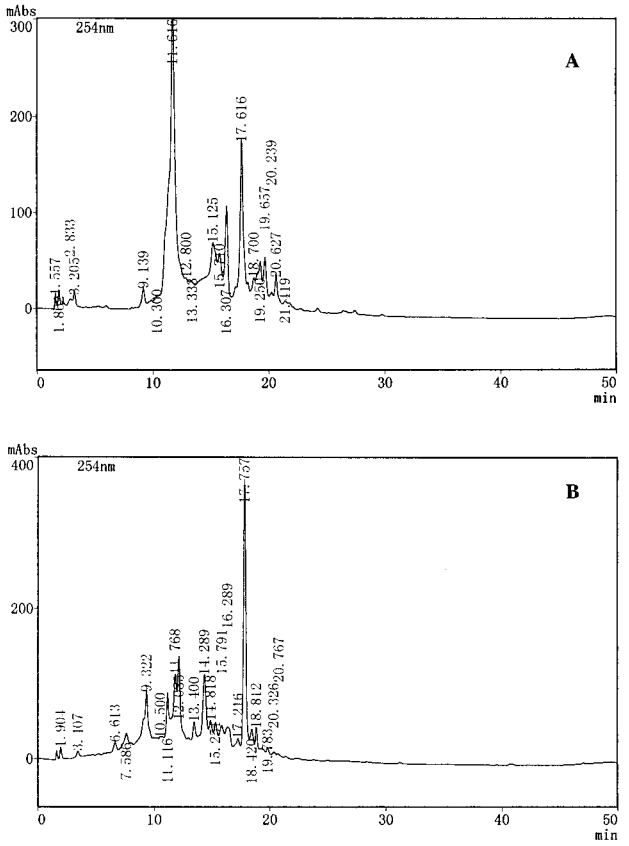

Figure 2. HPLC profiles of the extracts from *E. agallocha* (data A) and *T. catappa* (data B).

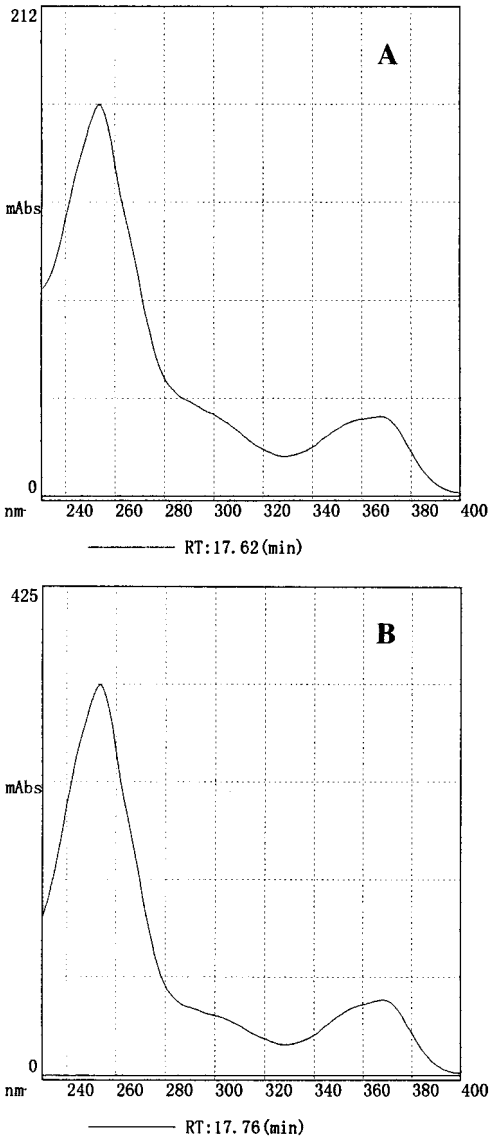

Figure 3. UV spectra of 17.6−17.7-min peaks in HPLC data from *E. agallocha* (data A) and *T. catappa* (data B).

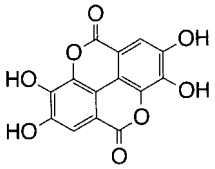

Figure 4. Structure of ellagic acid (**1**).

acetoxyl groups at 2.40 and 2.48 ppm along with a low-field-shifted aromatic signal (8.06 ppm). Based on these results, **1** was indicated to be ellagic acid (Figure 4). Its spectroscopic data and HPLC retention time were all identical with the authentic ellagic acid. Ellagic acid belongs to hydrolyzable tannins, and the strong antioxidant activity of tannins was reported by Okuda and co-workers (Yoshida et al., 1989). The antioxidant activity of ellagic acid has already been reported, and the activity is much stronger than that of α-tocopherol (Su et al., 1988). The activity of ellagic acid was measured by our three methods, which is shown in Figures 5 and 6 and Table 4. In the anti-DPPH assay, 10 μM ellagic acid showed an absorbance decrease of 0.92 for 0.5 h at 37 °C, while the same amount of α-tocopherol showed 0.18 (Table 4). These data revealed that ellagic acid was much more effective than α-tocopherol. Figure 5 shows the inhibitory activity of the linoleic acid oxidation of ellagic acid. Ellagic acid (12.5 μM) showed inhibitory activity; however, the activity was weaker than that of the same concentration of α-tocopherol. Figure 6 shows the effect of four doses of ellagic acid on cell protection activity. One hundred nanomolar of ellagic acid demonstrated 30% inhibition, and 1 mM showed 80% inhibition. These effective concentration values were similar to those obtained by antioxidant-active flavonoids (Oyama et al., 1994) and curcuminoids (Masuda et al., 1998). The amount of ellagic acid in the extracts was estimated from the peak area of the ellagic acid peak in the HPLC chromatogram, and the amounts in the extracts of *E. agallocha* and *T. catappa*. leaves were 1.59 ± 0.13 and 1.95 ± 0.02 mg/g dry weight of leaves, respectively. Daniel et al. (1989) have investigated the quantity of ellagic acid in various nuts and fruits and reported 0.3−1.5 mg in 1 g of dry material as the total amount of ellagic acid after acid hydrolysis containing ellagic acid derivatives. The amount in the two seashore plant leaves is higher than these nuts and fruits. The high amount of ellagic acid in the seashore plant leaves contributed to the strong antioxidant activity of the extracts. Although many other compounds in the extracts were observed by HPLC analysis, the antioxidative contribution of these compounds could not be examined. An assay-guided isolation of these antioxidative compounds is necessary for further antioxidant investigation of seashore plants.

CONCLUSIONS

The antioxidant activity of 39 kinds of seashore plant leaves was investigated using 3 assay methods. The assay results indicated that some seashore plants could

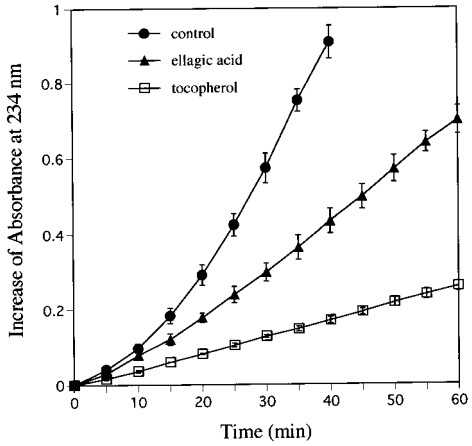

Figure 5. Inhibitory activity of ellagic acid and α-tocopherol against AMVN-induced linoleic acid oxidation in 0.1 M SDS micelle system (concentration for ellagic acid and α-tocopherol, 12.5 μM; AMVN, 2.3 mM; linoleic acid, 2.5 mM). Control experiment was carried out by addition of the same amount of vehicle (dimethyl sulfoxide). Each bar shows SD.

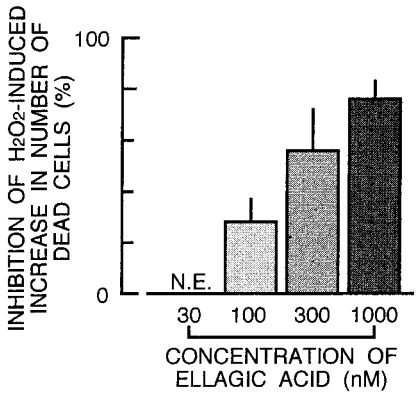

Figure 6. Inhibitory activity of ellagic acid against 3 mM H_2O_2-Induced cell death. Each column and bar show the average and SD of four to six experiments, respectively.

Table 4. Antiradical Activity of Ellagic Acid and α-Tocopherol

	decease value of 0.1 mM DPPH abs (517 nm)[a] for concn		
sample[b]	5 μM	10 μM	20 μM
ellagic acid	0.449 ± 0.009	0.919 ± 0.008	*c*
α-tocopherol	*c*	0.184 ± 0.010	0.441 ± 0.004

[a] Mean ± SD (n = 3). [b] Each sample solution (5 mM) was prepared with dimethyl sulfoxide and added. [c] Not examined.

be good antioxidant sources. The potent activities of two plant extracts, *E. agallocha* and *T. catappa*, were observed in all the assay systems used. Ellagic acid was isolated as the common constituent from the two extracts. The potent antiradical and cell protective activities of ellagic acid were confirmed.

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