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Antioxidant Activity of 2,3,6-Tribromo-4,5-dihydroxy benzyl methyl ether from *Symphyocladia latiuscula*

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Antioxidant activity of a methanol extract of *Symphyocladia latiuscula* was evaluated by the thiocyanate method in the linoleic acid system. The methanol extract inhibited the peroxidation of linoleic acid in a dose-dependent manner. The MeOH extract was then sequentially partitioned with *n*-hexane, CH_2Cl_2, EtOAc, *n*-BuOH and H_2O. The antioxidant activity of the fractions increased in order of CH_2Cl_2, *n*-hexane, EtOAc, and *n*-BuOH. There was no activity found in H_2O partitoned fraction by the thiocyanate method. Especially, the activities of the fractions of *n*-hexane and CH_2Cl_2 were comparable to that of 2,6-di-*tert*-butylhydroxytoluene (BHT). Column chromatography of the CH_2Cl_2 fraction over silica gel yielded cholesterol (1) and 2,3,6-tribromo 4,5-dihydroxybenzyl methyl ether (2) which were identified by instrumental analysis of MS and ^{1}H- and ^{13}C-NMR. The latter (2) demonstrated significant antioxidant activity.

Key words: *Symphyocladia latiuscula*, antioxidant activity, algae, 2,3,6-tribromo 4,5-dihydroxybenzyl methyl ether, NMR, MS

Introduction

Recently, much attention has focused on the development of safe and effective antioxidants because toxic free radicals play a role in the etiology of many diseases.

We have previously reported a screening result on the MeOH extract of different kinds of seaweed as to their antioxidant activities by assessing the radical scavenging effect on the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical, and the MeOH extract of a red algae, *Symphyocladia latiuscula* was shown to have a strong antioxidant activity (Choi *et al.*, 1993). The MeOH extract and its CH_2Cl_2 soluble fraction also showed strong inhibitory effect on the lipid peroxides produced when a mouse liver homogenate was exposed to the air at 37℃, using 2-thiobarbituric acid (TBA), and prominent inhibitory activity against free radical generation by ACF_2 (Hepatocyte) in dichlorofluoresein (DCF) method (Park *et al.*, 1998). Since the MeOH extract and its CH_2Cl_2 soluble fraction of *S. latiuscula* inhibited lipid peroxidation, we further examined the antioxidant activity by the thiocyanate method in the linoleic acid system. This paper also reports the isolation and structural elucidation of active principle, and its antioxidative activity in model systems.

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Materials and Methods

Algae material

Leafty thalli of *S. latiuscula* were collected at Chungsapo, Pusan in January, 1998 and authenticated by an algologist Prof. K. W. Nam of the Department of Marine Biology, Pukyong National University. A voucher specimen (No. 9801 28) has been deposited in the author's laboratory (J. S. Choi).

Chemicals

Ammonium thiocyanate, ferrous chloride, linoleic acid, L-ascorbic acid, DPPH and 2,6-di-*tert*-butylhydroxytoluene (BHT) were purchased from Sigma Chemical Co. Ltd. (St. Louis, MO). Silica gel (70~230 mesh) and TLC plate (precoated

Kieselgel $60F_{254}$ plate, 0.25 mm) were obtained from E. Merck Co. (Darmstadt, Germany). All other cheimcals used were reagent grade.

Extraction, fractionation and isolation

Shade dried seaweed tissues (580 g) of *S. latiuscula* were extracted with MeOH under reflux. The extracts were concentrated to dryness *in vacuo* at 40℃ to render the MeOH extract (148 g, yield 25.5 %), and then partitioned with *n*-hexane (14.2 g), CH_2Cl_2 (23 g), EtOAc (11.2 g), *n*-BuOH (36.8 g), and H_2O (53.6 g) in sequence to make the corresponding dried extracts. Silica-gel column chromatography of the CH_2Cl_2 fraction (21g) was performed using CH_2Cl_2-MeOH (gradient) as an eluent. This fractionation resulted in 22 subfractions. Of these fractions, further separation of fraction No. 5~9 (5.76 g) was carried out with silica-gel column chromatography using CH_2Cl_2-MeOH (10 : 1) to yield compound 1 (trace) and compound 2 (2.82 g) in the order of increasing polarity, respectively.

Instrumental analysis

(a) Electron-Impact Mass spectrometry (EI-MS). EI-MS spectra of isolated compounds were recorded on a SX-102A mass spectrometer operated at a 70-eV accelerating potential.

(b) ^1H- and ^{13}C-NMR. ^1H (600 MHz) and ^{13}C NMR (125 MHz) spectra of the isolated compounds were recorded on a Brucker AM 600 spectrometer with CD_3OD. TMS was used as an internal standard with CD_3OD, and peaks of δ3.40 in ^1H-NMR and 49.3 in ^{13}C-NMR were used as reference peaks to determine the chemical shifts given in the δ value (ppm). Heteronuclear multiple-bond connectivity (HMBC) was measured to confirm the assignments of the NMR spectrum of compound 2.

(c) IR spectroscopy. IR spectral data were obtained by using an infrared spectrometer (FT-IR Spectrometer Spectrum 2000, Perkin Elmer Co.), and the samples were analyzed in a KBr pellet.

Thiocyanate method in a linoleic acid system

Autoxidation of linoleic acid was carried out by using the method of Mitsuda *et al.* (1966). Different amounts of samples dissolved in 0.1 mℓ EtOH (33~520 μg/mℓ) were added to a reaction mixture in a screw cap vial. Each reaction mixture consisted of 2 mℓ of 2.53% linoleic acid in EtOH and 4.0 mℓ of 0.05 M phosphate buffer (pH 7.0). The vial was incubated in an oven at 40℃. At different intervals during incubation, a 0.1 mℓ aliquot of the mixture was diluted with 4.7 mℓ of 75% EtOH, which was followed by adding 0.1 mℓ of 30% ammonium thiocyanate. The absorbance at 500 nm was measured after precisely 3 min from the addition of 0.1 mℓ of 20 mM ferrous chloride in 3.5% hydrochloric acid to the reaction mixture.

DPPH radical scavenging effect

The DPPH radical scavenging effect was evaluated according to the method of Blois (Blois, 1958). Four milliliters of MeOH solution of varing sample concentrations (1.5~45 μM) were added to 1.0 mℓ-DPPH methanol solution (1.5×10^{-1}M). After mixing gently and leaving for 30 min at room temperature, the optical density was measured at 520 nm using a spectrophotometer. The antioxidant activity of each fraction was expressed in terms of IC_{50} (microgram per mℓ or micro molar concentration required to inhibit DPPH radical formation by 50%) and calculated from a log-dose inhibition curve.

Results and Discussion

Antioxidant activities of the MeOH extract and their fractions of *S. latiuscula*

Lipids or unsaturated fatty acids in biological membranes are most susceptible to autoxidation in the presence of oxygen. Especially, linoleic acid is the target of lipid peroxidation (Howard and Ingold, 1967). The antioxidant activities of MeOH extract from *S. latiuscula*, BHT, and L-ascorbic acid as typically synthetic and natural antioxidants, respectively, as measured by the thiocyanate method in the linoleic acid system were perodically investigated over 7 days and the results are shown in Figure 1. The antioxidant effects of different solvent soluble fractions obtained from the MeOH extract, ranging in concentrations from 33 to 520 μg/mℓ, were also investigated, and the results are shown in Figure 2. The antioxidant activities of MeOH extract and all solvent soluble fractions except for H_2O soluble fraction of MeOH extract were observed; the effects of these were dependent on their conentrations. The antioxidant effects of the MeOH extract, various fractions obtained from the MeOH extract, and two kinds of positive controls after incubation with linoleic acid for different intervals are summarized in Tables 1 and 2. On bases of these results, it is apparent that the results show that the antioxidant activities of the MeOH extract and their fractions increased in order of CH_2Cl_2, *n*-hexane, EtOAc, and *n*-BuOH. There was no activity found in H_2O partitioned

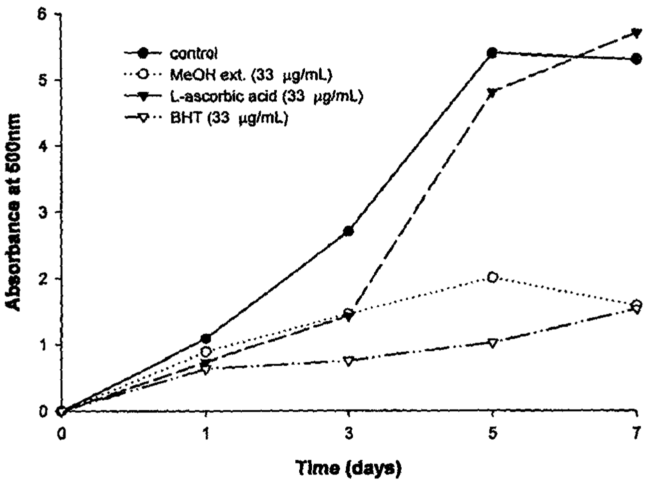

Fig. 1. The comparative antioxidative action of MeOH extract obtained from *S. latiuscula*, typically synthetic and natural antioxidants, BHT and L-ascorbic acid, respectively, for the autoxidation of linoleic acid. The mixture of 2.53% linoleic acid and 0.05 M phosphate buffer of pH 7.0 was incubated at 40℃ for indicated time. Antioxidant activity was measured by the thiocyanate method.

fraction by the thiocyanate method. Especially, the activities of the fractions of *n*-hexane and CH_2Cl_2 soluble fractions were comparable to that of BHT. L-Ascorbic acid did not reduce the oxidation of linoleic acid, which possessed a prooxidant effect, as much as MeOH extract and BHT as seen in Table 1. These orders of the activities of fractions as measured by the thiocyanate method were similar to the antioxidant activities in TBA method and radical scavenging activity reported by us (Park *et al.*, 1998). Recently, the potent antioxidant activities of bromophenols isolated from red algae, *Polysiphonia ulceolate* were reported by Fujimoto *et al.* (1985). They reported that the activities of bromophenol analogs were stronger than that of BHA (butyl hydroxyanisole) by chemiluminescence method. The activities of the fractions indicated that the antioxidants in the fractions were bromophenols because they were known to exist widely in red algae (Kurata and Amiya, 1975) and had the soluble polarity in chloroform. To clarify the contribution of the bromophenols to the antioxidant activity of the fraction, we tried to isolate and identify bromophenols in a strongly active CH_2Cl_2 fraction of MeOH extract from *S. latiuscula*.

Isolation & identification of active compound

Column chromatography on silica gel of the CH_2Cl_2 soluble fraction of the MeOH extract yielded compounds 1 and 2 in the order of increasing polarity, respectively. Compound 1 was readily elucidated as cholesterol by comparison of reported spectroscopic data and finally confirmed by comparison with an authentic sample (Charles and Jacqlynn, 1993). Compound 2, hydroscopic powder,

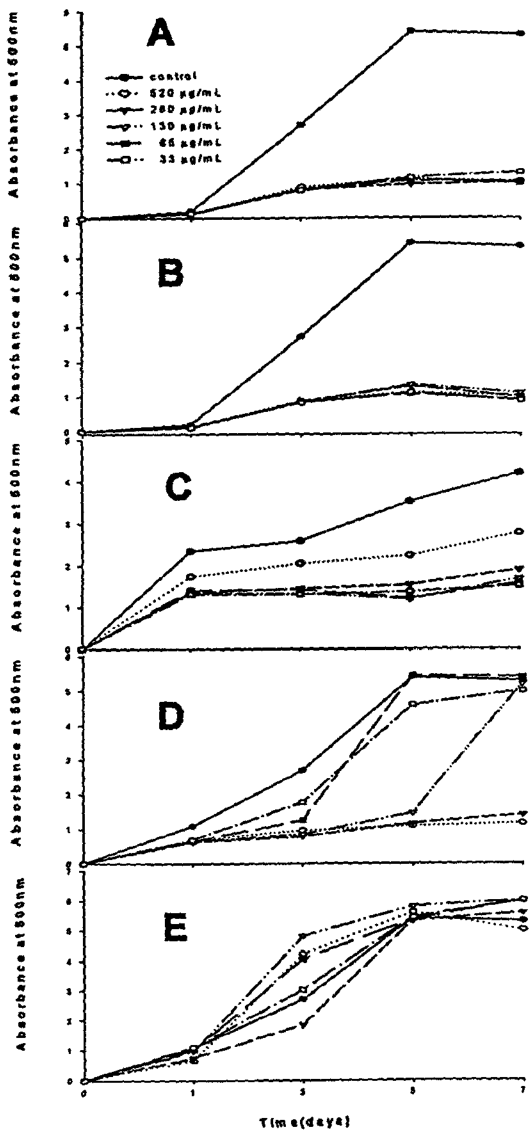

Fig. 2. Antioxidative action of different solvent soluble fractions obtained from MeOH extract of *S. latiuscula* for the autoxidation of linoleic acid. The mixture of 2.53% linoleic acid and 0.05 M phosphate buffer of pH 7.0 was incubated at 40℃ for indicated time. Antioxidant activity was measured by the thiocyanate method.
A: *n*-Hexane fraction B: CH_2Cl_2 fraction
C: EtOAc fraction D: *n*-BuOH fraction
E: H_2O fraction

Table 1. The comparative antioxidative action of MeOH extract obtained from *S. latiuscula*, typically synthetic and natural antioxidants, BHT and L-ascorbic acid, respectively

Sample	Time (days)	Absorbance at 500 nm	Inhibition (%)[a]
	0	0[c]	–
Control[b]	1	1.09	–
	3	2.70	–
	5	5.40	–
	7	5.30	–
MeOH ext. (33 μg/mℓ)	1	0.89	18.9
	3	1.46	46.7
	5	2.00	63.0
	7	1.58	75.0
L-ascorbic acid (33 μg/mℓ)	1	0.74	32.0
	3	1.43	47.0
	5	4.81	11.0
	7	5.70	–
BHT (33 μg/mℓ)	1	0.64	41.7
	3	0.76	71.8
	5	1.02	71.7
	7	1.53	80.8

Measurement was done by the thiocyanate method after incubation of every other day for 7 days
[a]Inhibition % (Capacity to inhibit the peroxide formation in linoleic acid)={1−(absorbance of sample at 500 nm/absorbance of control at 500 nm)}×100
[b]Control was incubated only with linoleic acid but without the samples
[c]Results are presented as means of triplicate experiments

showed a broad hydroxyl and CH stretching absorptions at 3,420 and 2,924 cm^{-1}, respectively. Strong absorption band observed at 501 cm^{-1} indicated the halogenated nature. The parent molecular ion peak at *m/z* 380 (7.5%) with the isotope peaks, *m/z* 390 (20.2%), 392 (20.9%) and 394 (9.1%) in the EI-MS, were consistent with the molecular formular of $C_8H_7O_3Br_3$. The presence of strong intense peaks corresponding M^+-OCH_3 at *m/z* 357 (34.2%), 359 (95.8%), 361 (100%) and 363 (35.7%) suggested that a methoxyl group was attached. A successive lose of bromine unit from the M^+-OCH_3, which resulted in debrominated ion at *m/z* 117, showed the presence of tribromine unit. The ^{1}H-NMR spectrum of compound 2 in CD_3OD exhibited the presence of a methoxyl (δ3.39) and a methylene (δ4.79) protons (Table 3).

The ^{13}C-NMR spectrum showed eight signals of carbons; two carbons from benzylic methylene at δ 76.36 with methoxyl at δ58.38, three brominated carbons at δ119.28, 114.75 and 129.43, and two oxygen-bearing tetrahedral carbons at δ146.23 and 144.39 (Table 3). Therefore, compound 2 was suggested to be a tribrominated dihydroxy benzyl methyl ether. Detailed analysis of the ^{1}H- and ^{13}C-NMR spectra, aided by HMBC experiments (Bax

Table 2. Antioxidant activities of various solvent soluble fractions obtained from the MeOH extract of *S. latiuscula* as measured by the thiocyanate method after incubation of every other day for 7 days at a concentration of 33 μg/mℓ

Sample	Time (days)	Absorbance at 500 nm	Inhibition (%)[a]
	0	0[c]	–
Control[b]	1	1.09	–
	3	2.70	–
	5	5.40	–
	7	5.30	–
n-Hexane fr.	1	0.66	28.3
	3	0.80	70.3
	5	1.6	78.4
	7	1.32	75.1
CH_2Cl_2 fr.	1	0.60	35.4
	3	0.83	69.4
	5	1.11	79.4
	7	0.89	83.2
EtOAc fr.	1	0.66	35.4
	3	0.94	65.4
	5	3.66	32.2
	7	4.44	17.0
n-BuOH fr.	1	0.70	36.4
	3	1.78	34.1
	5	4.60	14.8
	7	5.00	5.7
H_2O fr.	1	1.10	0.0
	3	3.02	0.0
	5	5.44	0.0
	7	6.00	0.0

[a]Inhibition % (Capacity to inhibit the peroxide formation in linoleic acid)={1−(absorbance of sample at 500 nm/absorbance of control at 500 nm)}×100
[b]Control was incubated only with linoleic acid but without the samples
[c]Results are presented as means of triplicate experiments

Table 3. ^{1}H- and ^{13}C-NMR data of compound 2 in CD_3OD

Position	compound 2*	
	δ_H	δ_C
1	——	129.43
2	——	114.75
3	——	114.19
4	——	146.23
5	——	144.39
6	——	119.28
CH_2O	4.79s	76.36
OCH_3	3.39s	58.38

*Assignments are based on analysis of HMBC data

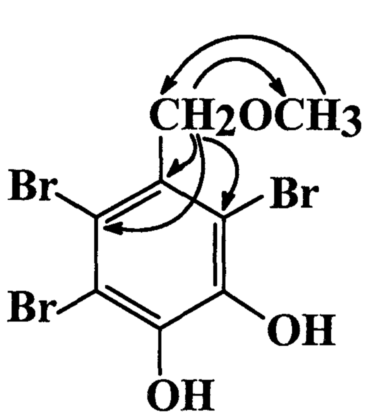

Fig. 3. HMBC correlation of compound 2.

and Summers, 1986), enabled establishment of full assignment of compound 2.

In the HMBC spectrum (Fig. 3), the signals at δ 129.43, 119.28 and 114.75 showed coupling of methylene protons (δ3.39). Accordingly, the signals at δ129.43, 119.28 and 114.75 were assigned to be C-1, C-6 and C-2, respectively. The methoxyl group was found to be attached to methylene according to long-range C-H coupling between OCH_3 (δ3.39) and methylene (δ76.36) in the HMBC spectrum. Two oxygenated carbon at δ144.39 & 146.23 and another brominated carbon at δ114.19 were assigned to be C-4, C-5 and C-3, respectively. Thus, compound 2 must be 2,3,6-tribromo-4,5-dihydroxybenzyl methyl ether which was previously isolated from the red algae (Kurata and Amiya, 1973). Most likely compound 2 seems to be the artifact which was derived by methylation of the corresponding alcohol during extraction procedure (Kurata and Amiya, 19 75). This was the first assignment of 2,3,6-tribromo-4,5-dihydroxy benzyl methyl ether from *Symphyocladia latiuscula* by the analysis of ^{1}H-NMR, ^{13}C-NMR and MS.

The antioxidative activity of 2,3,6-tribromo 4,5-dihydroxy benzyl methyl ether (2)

The antioxidant activity of compound 2 was examined by using the thiocyanate method and radical scavenging activity on DPPH radical. As seen in Figure 4, compound 2 showed a remarkable antioxidative activity measured by the thiocyanate method. Compound 2 inhibited the autoxidation of linoleic acid by 50% at the concentration of 47.28 μM after incubation for 3 days. Its 50% inhibitory concentration is seen in Table 4. BHT, a known synthetic antioxidant, inhibited linoleic acid autoxidation by 50% at 30. 80 μM after incubation for 3 days.

It is well established that lipid peroxidation is one of the reactions set into motion as a consequence of the formation of free radical in cells and tissues (Nishibori and Namiki, 1985).

As seen in Figure 6, compound 2 showed a remarkable antioxidant activity measured by the radical scavenging activity on DPPH radical. Its 50 % inhibitory concentration showed scavenging activity on DPPH radical at a concentration of 7.45 μM. On the other hand, L-ascorbic acid, a natural antioxidant, scavenged DPPH radical by almost 50 % at 15.33 μM.

Antioxidant compounds acting in living systems are classified into preventive antioxidants and

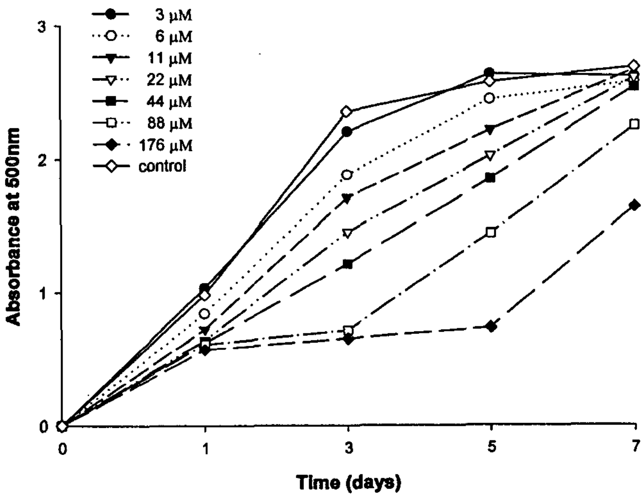

Fig. 4. Antioxidative action of 2,3,6-tribromo-4,5-dihydroxy benzyl methyl ether for the autoxidation of linoleic acid. The mixture of 2.53% linoleic acid and 0.05 M phosphate buffer of pH 7.0 was incubated at 40℃ for indicated time. Antioxidant activity was measured by the thiocyanate method.

Table 4. Antioxidant activities of 2,3,6-tribromo-4,5-dihydroxy benzyl methyl ether obtained from *S. latiuscula*, typically synthetic and natural antioxidants, BHT and L-ascorbic acid, respectively

Sample	Time (days)	IC_{50}[a] (μM)
2,3,6-tribromo-4,5-dihydroxy benzyl methyl ether	1	>176.0
	3	47.1
	5	107.4
	7	>176.0
L-ascorbic acid	1	>388.0
	3	>388.0
	5	>388.0
	7	>388.0
BHT	1	>308.0
	3	30.8
	5	50.8
	7	100.9

Measurement was done by the thiocyanate method after incubation of every other day for 7 days
[a]IC_{50} (Mean of 50% inhibitory concentration, μM)

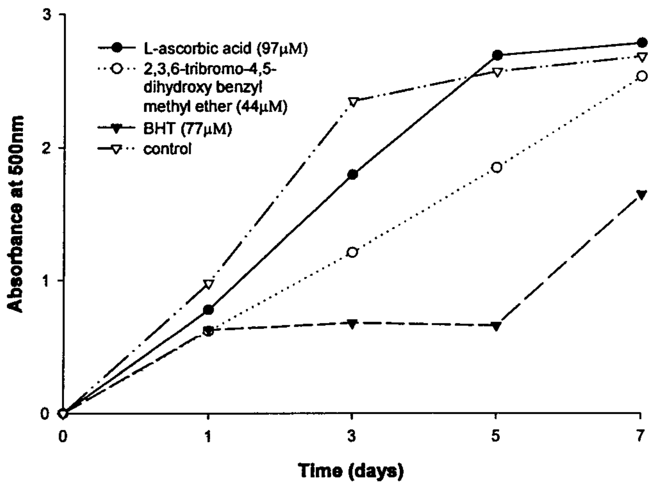

Fig. 5. The comparative antioxidative action of 2,3,6-tribromo-4,5-dihydroxy benzyl methyl ether, typical synthetic and natural antioxidants, BHT and L-ascorbic acid, respectively, for the autoxidation of linoleic acid. The mixture of 2.53% linoleic acid and 0.05 M phosphate buffer of pH 7.0 was incubated at 40℃ for indicated time. Antioxidant activity was measured by the thiocyanate method.

chain-breaking ones. On basis of the fact that bromophenol derivative 2 isolated from *S. latiuscula* inhibited autoxidation of linoleic acid and scavenged the DPPH radical, it apparently acted as the chain-breaking antioxidant.

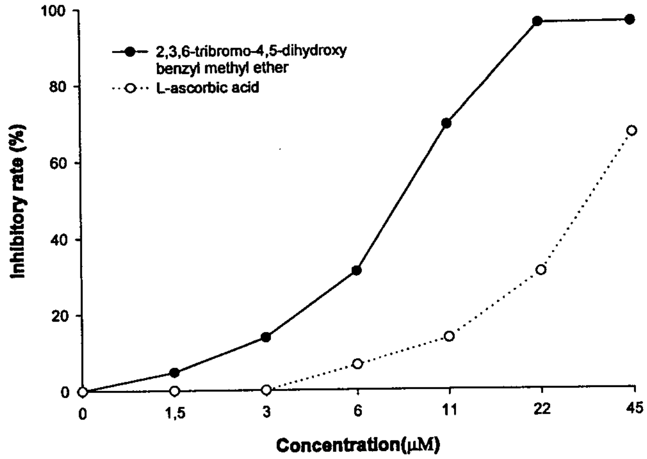

Fig. 6. DPPH radical scavenging effect of 2,3,6-tribromo-4,5-dihydroxy benzyl methyl ether.

A radical scavenging effect of phenolic compounds isolated from natural sources has been widely studied (Yoshida *et al.*, 1989). Phenolic compounds react with the free radical formed during autoxidation, and generate a new radical which is stabilized by the resonance effect of the aromatic nucleus (Cuvelier *et al.*, 1992). The high radical scavenging property of compound 2 with catechol group is probably due to a superior stability of radical derived from catechol compound to that of phenoxyl radical (Ruiz Larrea *et al.*, 19 94). In a sense of successive screening test for antioxidant principles in marine algae, Sakata *et al.* (1994) tested algal extracts for antioxidative activity and found that the lipid fractions of the green algae *Enteromorpha* sp. and the brown algae, *Undaria pinnatifida* showed potent antioxidative activities in the conventional test. They identified pheophytin a, one of the degradative products of chlorophyll a, as an active principle (Antonius *et al.*, 1992). Fujimoto *et al.* (1980) also screened extracts from 21 algal species for antioxidant activity and reported that more than half of them exhibited this effect to some extent. In particular, the chloroform-soluble fraction extracted from several species of brown algae, *Eisenia bicyclis* and *Undaria pinnatifida*, showed excellent antioxidant activities. And they also found that bromophenols which were isolated from a red algae, *Polysiphonia ulceolate*, showed a remarkable antioxidant activities (Fujimoto *et al.*, 1985). Park *et al.* (1991) demonstrated the presence of two effective natural antioxidant compounds in three edible algae *Laminaria sinclairii*, *Undaria pinnatifida* and *Enteromorpha linza*; these were confirmed to be benezene-derivative substances. Furoglucinol related compounds were also identified as antioxidants from brown algae such as *Eisenia bicyclis* (Maruyama *et al.*, 1990). Recently, we reported phloroglucinol as one of antioxidant principles from a brown algae, *Ecklonia stolonifera* (Lee *et al.*, 1996).

The present work would tend to indicate that the methanol extract of *S. latiuscula* and their fractions and its component, 2,3,6-tribromo-4,5-dihydroxy benzyl methyl ether, may be useful for the treatment of oxidative damage. Further investigation of antioxidant principles are now in progress.

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