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Anti-Allergic Phlorotannins from the Edible Brown Alga, *Eisenia Arborea*

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Eisenia arborea, **an edible brown alga, is occasionally used as a folk medicine due to its anti-allergic effect. In the present study to identify the anti-allergic constituents in the alga, the extract of the alga was purified by partition between solvents and by reversed phase chromatography. Separation of the extract was guided by the inhibitory activity upon β-hexosaminidase release from the rat basophilic leukemia-2H3 cells. HPLC purification afforded six active compounds. Spectral analyses clarified their structures as eckol, 6,6′-bieckol, 6,8′-bieckol, 8,8′-bieckol, phlorofucofuroeckol-A, and phlorofucofuroeckol-B. Most of the phlorotannins exhibited activities similar to or greater than the typical inhibitor, epigallocatechin gallate. Phlorofucofuroeckol-B showed the greatest activity among the tested phlorotannins at 2.8 times greater than epigallocatechin gallate.**

Keywords: *Eisenia arborea*, β-hexosaminidase, phlorotannin, RBL-2H3 cell

Abbreviations: DSCG: disodium cromoglycate, EGCg: epigallocatechin gallate, β-Hex: β-hexosaminidase, HR-FAB-MS: high resolution FAB-Ms, PFF: phlorofucofuroeckol, RBL: rat basophilic leukemia

Introduction

Brown algae are a popular health food in Japan. These algae are known to contain many phlorotannins (Fukuyama *et al.,* 1985, 1989, 1990; Glombitza and Gerstberger, 1985a; Glombitza and Vogels, 1985b). They contain various biological activities, such as antioxidative activity (Nakamura *et al.,* 1996), anti-tumor activity (Kobayashi *et al.,* 2001), anti-HIV activity (Ahn *et al.,* 2004), bactericidal activity (Nagayama *et al.,* 2002), anti-inflammation activity (Shibata *et al.,* 2002, 2003), and so on. However, no reports have been found related to the inhibiting activities of the phlorotannins regarding histamine release from cultured cells.

Our preliminary investigation of the anti-allergic algae found that the dried powder of the brown alga, *E. arborea,* possessed a strong anti-allergic effect on Brown Norway (BN) rats, an allergic model animal. The amount of histamine in the blood of BN rats fed a diet containing the powdered alga (5%) was significantly lower (144.9±63.0 nM, n=5) than that of the animals fed a diet without the alga (311.8±42.0 nM, n=5). The details of these results will be discussed elsewhere in the near future. Although it was postulated that the anti-allergic effect could be due to the algal polysaccharides, the low molecular weight active principle was suggested in the alga, based on the finding that the ethanol extract of the alga showed a similar effect on the BN rats. This finding encouraged us to identify the active constituents of the alga. To monitor the anti-allergic activity of the purified samples, a bioassay system using rat basophilic leukemia (RBL)-2H3 cells was established, according to the procedure reported by Nishibe *et al.* (2001). The RBL cells release histamine which is responsible for the allergy, together with β-hexosaminidase (β-Hex) when they are stimulated by the antigen-antibody reaction or a calcium ionophore (A 23187). The amount of the released histamine is known to be proportional to that of β-Hex (Schwartz *et al.,* 1981). By monitoring the inhibiting activity of the β-Hex release, the M/C (mixture of methanol and chloroform) extract of the dried powder of *E. arborea* was purified and 6 active compounds isolated. Their structures were identified by spectral analyses. They were eckol, 6,6′-bieckol, 6,8′-bieckol, 8,8′-bieckol, phlorofucofuroeckol-A (PFF-A), and phlorofucofuroeckol-B (PFF-B). Most of these phlorotannins showed inhibiting activities similar to or greater than that of the typical inhibitor, epigallocatechin gallate (EGCg), and PFF-B showed a 2.8 times greater activity than EGCg. This result indicated that the anti-allergic effect of *E. arborea* could be due to the histamine release-inhibiting activity of these phlorotannins isolated from the alga.

This paper describes the extraction, purification, and identification of the anti-allergic constituents from *E. ar-*

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borea and their inhibiting activity on histamine release from the stimulated RBL cells, which was estimated by measuring the amount of the released β-Hex.

Materials and Methods

Material, Extraction and Partial Purification (Fig. 1) The brown alga, *E. arborea,* was collected from the Mugizaki coast in Mie prefecture, Japan. The alga was washed, dried in air, and powdered using an ultracentrifugal mill (ZM200, Retsch Co., Ltd., Haan, Germany). The dried powder (500 g) was immersed in 2.5 L of hexane for 24 h, and the hexane extract removed by filtration. The remaining powder was re-extracted with 3.0 L of M/C (methanol: chloroform = 1 : 2), after extraction with 500 mL portions of a mixture of hexane and ethyl acetate (4 : 1 and 1 : 1). The M/C extract was partitioned with 750 mL of water, and then the aqueous methanol layer was extracted with 750 mL of diethyl ether. The ether layer was evaporated to dryness. The residue was re-dissolved in 10 mL of methanol and the solution diluted with 100 mL of water. The water solution was subjected to the reversed phase HPLC analysis and purification.

Reversed Phase HPLC A high performance liquid chromatography (HPLC) system (6A, Shimadzu Co., Kyoto, Japan) was used in this study. An analytical Develosil ODS-5 column (4.6 mm ID × 250 mm L, Nomura Chemical Co., Aichi, Japan, flow rate: 0.5 mL/min) and a preparative Develosil ODS-5 column (10 mm ID × 250 mm L, flow rate: 1.0 mL/min) were used. For the analytical chromatography, a 30-min gradient program between 0.1% TFA (A) and acetonitrile containing 0.1% TFA (B) was employed. For the preparative chromatography, the following three elution conditions were applied. Condition 1: gradient elution between A and B (0–30% B for 120 min, maintain 30% B for 30 min and 30–100% B for 30 min); Condition 2: isocratic elution with 18% B; and Condition 3: isocratic elution with 47% methanol containing 0.1% TFA. The eluate was monitored by UV absorbance at 280 nm.

Structure Analysis The ^{1}H-NMR, ^{13}C-NMR, HSQC, HMBC and ROESY spectra were measured in DMSO-d_6 using a JEOL NM-A-500 spectrometer (JEOL, Tokyo, Japan). The mass analyses were performed using a Kompact Discovery MALDI-TOF mass system (Shimadzu Co. Kyoto, Japan) with 2,5-dihydroxybenzoic acid as the matrix. High resolution FAB-MS was measured by a MS 700 HR-FAB-MS system (JEOL Co.) using glycerin as the matrix. The spectral data are presented in Tables 1 and 2.

Cell Culture RBL-2H3 cells, (Bursumian *et al.,* 1981) obtained from the Japanese Cancer Research Resource Bank, (JCRB, Ibaraki, Osaka, Japan) were cultured in Eagle's Modified Essential Medium (EMEM, MP Biomedicals, Inc., Illkirch, France) supplemented with 20% fetal bovine serum (FBS, Lot. No.: A06072-500, Trace Scientific, Ltd., Melbourne, Australia), 24 mM sodium bicarbonate (Wako Pure Chemical Co., Ltd., Tokyo, Japan), 2 mM L-glutamine (Invitrogen Co., Carlsbad, CA, USA), 75 k unit/L penicillin (Wako), 0.15 mM streptomycin (Wako), 0.1 mM nonessential amino acid (Invitrogen), and 1 mM sodium pyruvate (Cambrex Bio Science, Inc., Walkersville MD, USA) at 37℃ in a humidified atmosphere containing 5% CO_2. The cells were passaged every 4 or 5 days by trypsinization with one medium change between the passage until they attained confluence.

Cell Stimulation and Determination of Released β-Hexosaminidase (β-Hex) The cells were stimulated and the released β-Hex (equivalent to histamine) was determined according to a previously reported procedure (Nishibe *et al.,* 2001) with some modifications.

The RBL cells were precultured for 3 days in EMEM supplemented with 20% FBS and sensitized with anti-dinitrophenyl (DNP) IgE (final 2.5 μg/mL, Sigma-Aldrich Co., St. Louis MO, USA) for 18 hr. The medium was then changed to Tyrode buffer (pH 7.2) (Matsuo *et al.,* 1997), and the test sample solution was added. After incubation for 10 min, the cells were treated with the antigen, DNP-BSA (final 0.5 μg/mL, LSL Co., Ltd., Tokyo, Japan), for 35 min. To stimulate the RBL cells, the calcium ionophore A23187 (Nacalai Tesque, Inc., Kyoto, Japan), was added to the culture medium to make the final concentration of 1 μM, following which, the cells were incubated for 35 min without sensitization by anti-DNP IgE and stimulation by DNP-BSA. The stimulation reaction was stopped by cooling the reaction vessel on ice for 15 min. The culture medium was divided into two wells of a 96-well plate, and the substrate solution (1 mM p-nitrophenyl-N-acetyl-β-D-glucosaminide, Wako) was added to each well. After incubation for 60 min at 37℃, 100 mM sodium bicarbonate was added to each solution to alkalize them and develop a yellow color. The color was measured at the optical density of 405 nm to determine the amount of p-nitrophenol released from the substrate by the action of the released enzyme. The inhibition ratio of the β-Hex was calculated using the following equation:

$$\text{Inhibition ratio}(\%) = [1 - (T - NC)/(PC - NC)] \times 100$$

where NC is the OD_{405} of the blank cell medium without the test sample and the stimulations; PC is the OD_{405} of the cell medium without the test sample, but with stimulation; and T is the OD_{405} of the cell medium with both the test sample and stimulation.

Results and Discussion

Extraction and Isolation of Active Constituents The flow diagram for the extraction and partial purification of the active constituents from the alga is shown in Figure 1 (Folch *et al.,* 1957; Shibata *et al.,* 2002). The M/C extract (yield: 430 mg) obtained by the extraction was re-dissolved in water containing a small amount of methanol, and the solution was subjected to further purification.

The solution was analyzed by RP-HPLC, and the chromatogram (Fig. 2) indicated that it contained 6 major components together with several minor substances. To obtain sufficient amounts of the pure substances for the bio-activity tests and for the structure analyses, the remaining solution was separated by preparative RP-HPLC (elution condition 1 in the experimental section).

This chromatographic separation afforded two active fractions which included active compounds 1–4 (Fr. 1) and active compounds 5 and 6 (Fr. 2), and they showed 60.6±0.8% and 78.3±15.6% inhibitions on β-Hex release from RBL cells at a concentration of 0.1 mg/mL, respectively. The other fractions were inactive. These active fractions were subjected to the second RP-HPLC purification procedure. To purify the active Fr. 1, elution Condition 2 was employed, and four active compounds, 1: eckol, 2: 6,6′-bieckol, 3: 6,8′-bieckol, and 4: 8,8′-bieckol were obtained. To purify the active Fr. 2, elution Condition 3 was applied, and two active compounds, 5: phlorofucofuroeckol-B (PFF-B) and 6: phlorofucofuroeckol-A (PFF-A) were produced. The yields of compounds 1–6 were 1: 11.3 mg, 2: 4.1 mg, 3: 3.3 mg, 4: 22.3 mg, 5: 3.3 mg and 6: 5.3 mg, respectively, from 500 g of the dried alga.

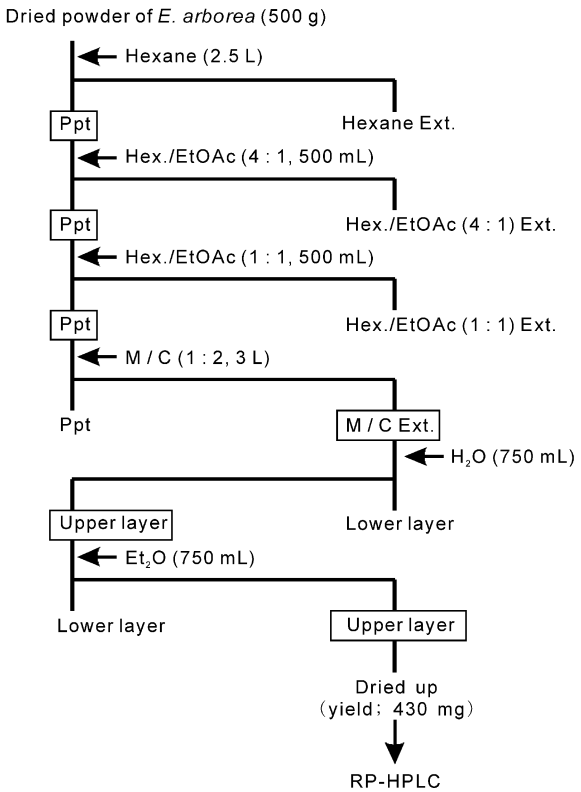

Fig. 1. Flow diagram for extraction and partial purification of the phlorotannins from *E. arborea*.

Structure Analysis and Identification of the Isolated Phlorotannins The structures of the isolated compounds were analyzed by mass spectra, ^{1}H-NMR, ^{13}C-NMR, ^{1}H-^{1}H COSY, HSQC, HMBC and ROESY spectra. The spectral data are summarized in Tables 1 and 2, and the elucidated structures are shown in Fig. 3.

Compounds 1, 2, 4, and 6 were identified as eckol, 6,6′-bieckol, 8,8′-bieckol, and phlorofucofuroeckol-A (PFF-A), respectively, by comparing their spectra with those reported in the references (Fukuyama *et al.*, 1985, 1989, 1990).

Compound 3 showed its $[M+H]^{+}$ ion at M/Z=743 in the MALDI-TOF Ms analysis, and this result indicated its molecular weight of 742. The molecular weight was the same as those of the bieckols, and it indicated that compound 3 could be an isomer of them. Compound 3 showed 28 carbon signals, 22 signals containing one carbon, 5 signals containing two carbons, and one signal containing four carbons in its ^{13}C-NMR spectrum (Table 1). These results suggested that compound 3 was an asymmetric molecule. Most of the ^{1}H and ^{13}C signals resembled those of eckol, except for the signals for the 6- and 8′-protons and carbons. In compound 3, the protons corresponding to H-6 and H-8′ of two eckol moieties were lost, and the C-6 and C-8′ signals shifted to 99.5 and 104.5 ppm from 93.8 and 98.5 ppm of the corresponding carbons of the eckols, respectively. These analyses, in addition to the ^{1}H-^{1}H COSY, HSQC, HMBC analyses, clearly suggested that compound 3 was 6,8′-bieckol. Although the existence of 6,8′-bieckol had already been pointed out by Glombitza *et al.* (1985b), no spectral data for the intact phlorotannin were indicated. Only those of its per-

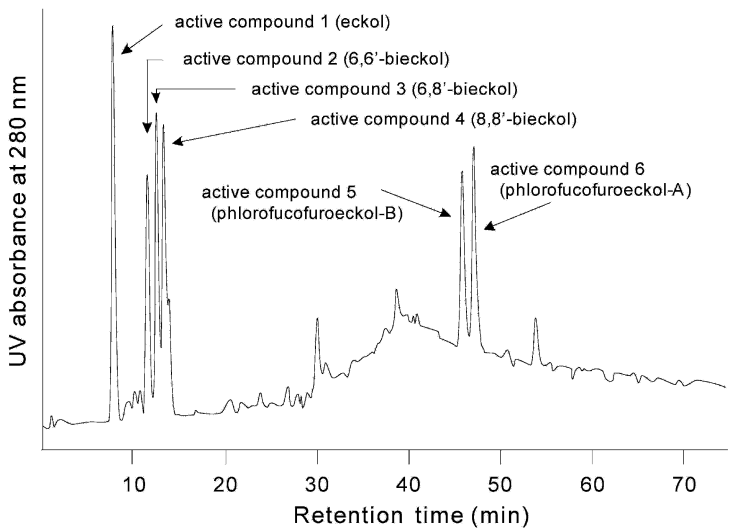

Fig. 2. Analytical RP-HPLC chromatogram of the partially purified phlorotannins from *E. arborea*. The elution conditions are listed in the "Materials and Methods" section.

Table 1. Ms and NMR spectra of compound 1–4.

		compound 1 (eckol)		compound 2 (6,6'-bieckol)		compound 3 (6,8'-bieckol)					compound 4 (8,8'-bieckol)	
	$[M+H]^+$	373		743		743					743	
moiety	position	1H	13C	1H	13C	position	1H	13C	position	1H	13C	1H	13C
PG[a]	1'		160.4		160.4	1''		160.4	1'''		160.4		160.4
	2',6'	5.71	93.7	5.74	93.7	2'',6''	5.71	93.7	2''',6'''	5.74	93.9	5.72	94
	3',5'		158.8		158.8	3'',5''		158.7	3''',5'''		158.7		158.8
	4'	5.84	96.2	5.79	96.2	4''	5.78	96.3	4'''	5.78	96.2	5.78	96.3
major skeleton	1		123.2		123.6	1		123.6	1'		123.4		123.4
	2		145.9		145.4	2		145.8	2'		145.4		145.9
	3	6.13	98.2	6.07	97.8	3	6.02	97.9	3'	6.06	97.9	6.14	98.2
	4		141.9		141.9	4		141.9	4'		141.8		141.9
	4a		122.2		122	4a		122.4	4a'		122		122.5
	5a		142.6		141.4	5a		141.6	5a'		140.9		141.3
	6	5.79	93.8		99.7	6		99.5	6'	5.94	93.6	5.94	93.9
	7		153		151.3	7		151.5	7'		151.5		151.8
	8	5.95	98.5	6.03	97.9	8	6.15	98.2	8'		104.5		104.4
	9		146.1		144.5	9		144.8	9'		144.3		144.6
	9a		122.6		122.7	9a		123	9a'		122.9		123.1
	10a		137.2		137.2	10a		137.3	10a'		137.2		137.3
OH[b]	2	9.19		9.13		2 or 2'	9.11		7 or 7'	8.64		9.17	
	3',5'	9.15		9.15			9.46			8.74		9.14	
	4	9.46		9.07		3'',5'' or	9.12		9 or 9'	7.93		9.44	
	7	9.19		8.62		3''',5'''	9.15			9.27		7.95	
	9	9.52		9.26		4 or 4'	9.06					8.80	
							9.18						

a: PG is an abbreviation of phloroglucinol moiety. b: OH indicates hydroxyl group.

Table 2. Ms and NMR spectra of compound 5 and 6.

	compound 5 (PFF-B)			compound 6 (PFF-A)		
	$[M+H]^+$	603		603		
moiety	position	1H	13C	position	1H	13C
PG[a]	1'		160.3	1'		160.3
	2',6'	5.76	93.7	2',6'	5.75	93.8
	3',5'		158.9	3',5'		158.9
	4'	5.82	96.5	4'	5.82	96.4
	1''		159.9	1''		160
	2'',6''	5.71	93.5	2'',6''	5.71	93.6
	3'',5''		159	3'',5''		159.1
	4''	5.82	96.3	4''	5.82	96.6
major skeleton	1		122.2	1		122.5
	2		146.3	2		147
	3	6.19	98.6	3	6.28	98.4
	4		142	4		142.1
	4a		123	4a		122.7
	5a		141.4	5a		134.0
	6	6.75	91.5	6		103.3
	6a		150.3	7		103.5
	7a		149.2	8		146.5
	8		120.4	9	6.42	99.2
	9		150.4	10		150.4
	10	6.48	98.6	11		120.2
	11		145.5	11a		149.5
	12		104.9	12a		150.9
	13		108.0	13	6.70	94.9
	14		137.2	14		144.8
	14a		125.8	14a		126.4
	15a		136.7	15a		136.9
OH[b]				2-OH	9.47	
				4-OH	9.92	
				8-OH	8.19	
				10-OH	9.88	
				14-OH	10.18	
				3',5'-OH	9.21	
				3'',5''OH	9.23	

a: PG is an abbreviation of phloroglucinol moiety. b: OH indicates hydroxyl group.

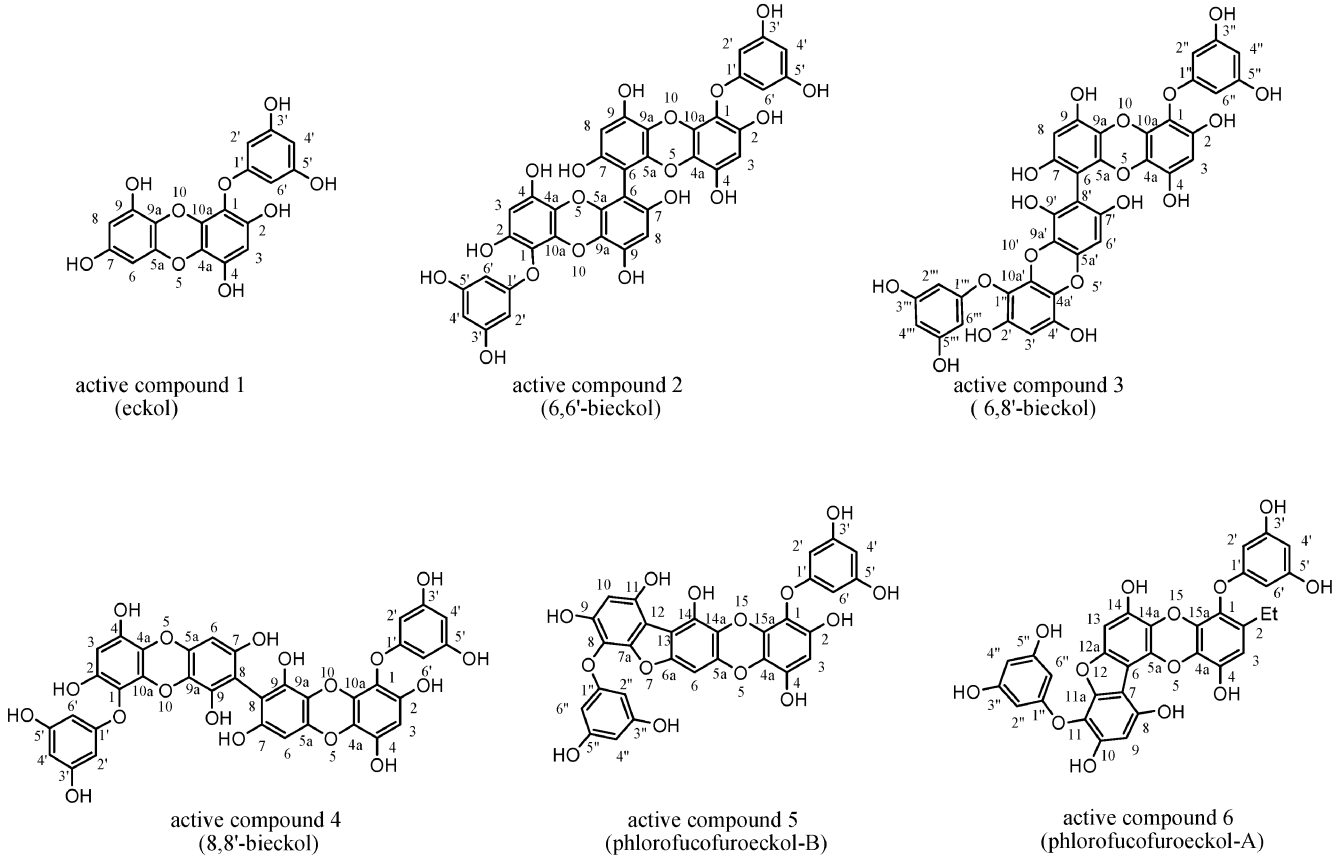

Fig. 3. Structures of the phlorotannins isolated from *E. arborea.*

Table 3. The inhibitory effects of the active compounds on β-Hex release from RBL cells.

Stimulation pattern	Concentration (μM)	Active compounds						
		1[c]	2	3	4	5	6	EGCg[d]
A[a]	5	43.4 ± 14.4	15.5 ± 2.5	6.1 ± 4.1	16.4 ± 4.1	38.3 ± 0.2	27.7 ± 4.1	0
	10	65.2 ± 7.8	40.9 ± 1.8	30.0 ± 0.8	20.7 ± 17.8	59.3 ± 14.9	34.7 ± 12.7	13.6 ± 5.7
	25	74.2 ± 6.2	79.1 ± 2.5	58.8 ± 1.2	46.8 ± 12.7	**90.4 ± 1.5**	45.5 ± 0.6	**59.1 ± 3.9**
B[b]	25	68.9 ± 2.1	47.6 ± 0.6	66.6 ± 0.3	31.6 ± 2.2	84.8 ± 2.5	54.1 ± 13.4	69.8 ± 13.4

The inhibition ratios were calculated from the results of duplicate experiments and were given as means ± SEM, n = 2. The reproducibility was confirmed by repeated runs. The cells were exposed to various concentrations of the test samples for 45 min. Compound 1, eckol; 2, 6,6'-bieckol; 3, 6,8-bieckol; 4, 8,8'-bieckol; 5, phlorofucofuroeckol-B; 6, phlorofucofuroeckol-A.

a, Stimulation by antigen-antibody reaction; b, Stimulation by calcium ionophore, A23187; c, Concentration of compound 1 used in the assay was ten times higher than those of the other samples; d, EGCg was used as the positive control.

acetate were reported. Accordingly, this paper is the first one that shows the exact spectral data for the intact 6,8′-bieckol.

Compound 5 showed its $[M+H]^+$ ion at M/Z=603 in the MALDI- TOF-Ms analysis, and its molecular weight was estimated to be 602. Its molecular formula was determined to be $C_{30}H_{18}O_{14}$ on the basis of its HR-FAB-MS spectrum (positive, in glycerin, m/z=603.0717, Δ 2.0 mmu). The molecular formula was the same as that of PFF-A, and suggested compound 5 could be an isomer of PFF-A. Most of the NMR signals of compound 5 resembled those of PFF-A except for C-5a, C-6, C-6a, C-8, C-10, C-12, C-13, C-14, H-6 and H-10. A comparison of their ^{1}H and ^{13}C NMR spectra and analyses of the ^{1}H-^{1}H COSY, HSQC, HMBC, ROESY spectra of compound 5 revealed its structure as shown in Fig. 3. The details of the structure analyses will be reported elsewhere (Sugiura *et al.,* 2006). Compound 5 was a new structural isomer of PFF-A, and it was called phlorofucofuroeckol-B (PFF-B).

Inhibitory Activities on β-Hex Release from RBL-2H3 Cells by the Isolated Phlorotannins The degree of inhibition of the β-Hex release by the test sample is summarized in Table 3, together with that of a positive control, epigallocathecin gallate (EGCg). All of the isolated phlorotannins showed inhibitory effects to various degrees. Although compound 1, eckol, showed a lesser activity than the positive control by several to ten times, the other isolated phlorotannins showed inhibitory activities similar to or greater than EGCg. The newly identified pholorofucofuroeckol-B (PFF-B) showed the strongest activity

among the tested samples, including EGCg at any dose. The IC_{50} value of PFF-B was 7.8μM. This result indicated that PFF-B had a 2.8–6.0 times greater inhibitory activity than those of ECGg (IC_{50}=22.0μM) or Tranilast (IC_{50}=46.6 μM) (Matsubara *et al.,* 2004), a clinically used anti-allergic drug.

Similar inhibition tendencies by the phlorotannins were observed when the cells were stimulated by the ionophore, A23187, as shown in Table 3. PFF-B again showed the greatest inhibitory activity among the tested phlorotannins or ECGg. These results implied that the phlorotanins could inhibit not only cellular events triggered by the antigen-antibody reaction before calcium influx caused by A23187, but also cellular events later than the calcium influx until degranulation of the RBL cells.

The results showed that although eckol, with a lower molecular weight, exhibited weaker activity, the other phlorotannins with higher molecular weights showed stronger activities comparable to EGCg, indicating that the molecular size or the number of phenol groups could be an important factor for expressing their activities. The importance of the molecular size in exhibiting such an inhibition was reported for apple polyphenols (Kanda *et al.,* 1998). The apple condensed tannin (ACT) that had a larger molecular size showed a greater histamine release inhibitory activity than the monomer of ACT, epicathecin. Although the reason why PFF-B possessed a greater activity than the others is not presently clear , its unique structure might be the reason for its strong activity.

This paper reported the first finding that the phlorotannins, the major polyphenols of algae, possessed an inhibitory activity on β-Hex release from cultured cells. A similar inhibitory effect has been studied for the polyphenols of terrestrial plants, such as tea (Matsuo *et al.,* 1997), perilla (Ueda *et al.,* 2002), persimmon (Kotani *et al.,* 2000), as well as on those of clinically used drugs: disodium cromoglycate (DSCG) and Tranilast (Kakegawa *et al.,* 1992). The inhibition mechanism was well investigated for the tea catechin, EGCg. EGCg inhibited the tyrosine phosphorylation of the protein kinase involved in the RBL degranulation (Yamashita *et al.,* 2000) and expression of the IgE receptor in the cultured human basophile leukemia cells, KU812 (Fujimura *et al.,* 2002). In addition, the tea catechins inhibited hyalulonidase (Kakegawa *et al.,* 1985b), which is closely related to the inflammatory reaction. The same enzyme inhibition was observed for the clinically used drugs, DSCG or Tranilast (Kakegawa *et al.,* 1985a). The phlorotannins were also reported to inhibit the enzyme (Shibata *et al.,* 2002). The similarities in the inhibitory effects between the phlorotannins and tea catechins or the clinical drugs indicated the possibility that similar mechanisms could be involved in the expression of their anti-allergic effects.

Moreover, an improvement in the allergy symptoms of the model rats fed a diet including the alga, *E. arborea,* could be due to the inhibitory effects of the isolated phlorotannins on both the enzyme and histamine release. The results of the present study revealed one of the reasons why *E. arborea* is popular as a folk medicine or as a health food in Japan.

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