

The anti-allergic effect of the ethyl acetate fraction from an *Ecklonia kurome* extract

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To determine the anti-allergic effects of *Ecklonia kurome*, the ethyl acetate fraction from an *E. kurome* (EEK) extract was compared to the ethyl acetate fraction from an *Eisenia arborea* (EEA) extract and epigallocatechin gallate (EGCG) for inhibitory effects on inflammation in experimental animals, degranulation in inflammatory lymphocytes and enzymatic activities involved in allergic reactions. EEK was found to inhibit mouse ear oedema by inflammatory inducers (arachidonic acid, 12-O-tetradecanoylphorbol-13-acetate and oxazolone) in a dose-dependent manner, through both topical and oral administrations. EEK also inhibited degranulation in rat basophilic leukaemia cells and enzymatic activities (cyclooxygenase-2, soybean lipoxygenase and phospholipase A₂) in a dose-dependent manner. The effects of EEK were generally comparable of those of EEA and EGCG. Therefore, *E. kurome* and the components in EEK have the benefit of being used not only in common foods, but also more widely as a functional food resource in anti-allergic foods.

Keywords: anti-allergic effect; anti-inflammatory effect; *Ecklonia kurome*; phlorotannin; brown algae

Introduction

Ecklonia kurome, a brown alga, grows along the coasts of the Japan Sea, Seto Inland Sea and Kyushu Island (Yoshida, 1998), and is well known as an ingredient in common indigenous food products such as 'Kizami Kurome', 'Maki Kurome', Kurome powder and Kurome essence in Kyushu, Japan. *E. kurome* belongs to the Laminariales, and related species that have food applications and similar geographical distribution are *Ecklonia cava*, *Ecklonia stolonifera*, *Eisenia arborea* and *Eisenia bicyclis*. The brown algae contain abundant amounts of functional food components such as alginate, fucoidan and phlorotannins (Asakawa, Ohkawa, & Tanaka, 1988; Heo, Lee, Song, & Jeon, 2003; Kuda, Kunii, Goto, Suzuki, & Yano, 2007; Ragan & Glombitza, 1986). Among the brown algae, *E. kurome* contains approximately 3% (w/w) phlorotannins, 3% (w/w) fucoidan and 40% (w/w) alginate (Kuda et al., 2007; Shibata et al., 2004). Phlorotannins of this alga possess bactericidal activity on pathogenic bacteria such as methicillin-

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resistant *Staphylococcus aureus* (MRSA; Nagayama, Iwamura, Shibata, Hirayama, & Nakamura, 2002), anti-plasmin activity (Fukuyama et al., 1990) and anti-obesity effects (Kong et al., 2010). Additionally, the phlorotannins have an algicidal effect on red tide microalgae that cause serious damage to aquaculture industries (Nagayama, Shibata, Fujimoto, Honjo, & Nakamura, 2003). Sulfated fucans from this alga exhibit anticoagulant activity (Nishino, Aizu, & Nagumo, 1991).

Although many healthful properties of *E. kurome* have been demonstrated, there might be additional benefits that are as yet unexplored. Allergies plague one-third of the Japanese population (Mikawa, 1997), and have become a social problem in recent years, so the importance of studying the anti-allergic properties of food resources has increased. Although there are some reports of the anti-allergic effects of *E. cava*, *E. stolonifera*, *E. arborea* and *E. bicyclis* extracts (Le, Li, Qian, Kim, & Kim, 2009; Shibata, Nagayama, Tanaka, Yamaguchi, & Nakamura, 2003; Sugiura, Torii, Tanaka, & Matsushita, 2012; Sugiura et al., 2009), the anti-allergic effects of *E. kurome* extracts are unknown, except for a report that crude phlorotannins from this species inhibit a hyaluronidase activity (Shibata, Fujimoto, Nagayama, Yamaguchi, & Nakamura, 2002). To that end, the anti-allergic activity of the phlorotannin-enriched ethyl acetate fraction from an *E. kurome* (EEK) extract was investigated to develop a novel and useful bioactivity of this alga.

Since the anti-allergic effects of the brown algal ethyl acetate fraction on experimental animals have not been reported, in the present study, anti-allergic tests using a mouse (ICR strain) ear oedema model were conducted. In the ear oedema experiments, three inflammation sensitisers [arachidonic acid (AA), 12-O-tetradecanoylphorbol-13-acetate (TPA) and oxazolone (OXA)] were used, because AA induces inflammatory reactions by prostaglandins (PGs), TPA induces inflammatory reactions by PGs and rapid cyclooxygenase-2 (COX-2) expression and OXA causes a delayed-type (type IV) allergic reaction (Kujubu, Fletcher, Varnum, Lim, & Herschman, 1991; Nakano, 1977; Rao, Currie, Shaffer, & Isakson, 1993). The effects of the brown algal ethyl acetate fraction against the three different types of inflammation were investigated.

In allergic reactions, inflammatory lymphocytes such as mast cells degranulate chemical mediators such as histamine, leukotrienes (LTs) and PGs. The released chemical mediators cause regional inflammatory reactions. LTs and PGs are synthesised in the arachidonic acid cascade, mediated by COX-2, lipoxygenase (LOX) and phospholipase A₂ (PLA₂; Funk, 2001). Therefore, inhibitory effects of EEK on the key enzymes that are involved in the arachidonic acid cascade and acral inflammatory reactions were examined, namely COX-2, LOX and PLA₂. Inhibitory effects of EEK on degranulation in inflammatory lymphocytes were examined using rat basophilic leukaemia (RBL)-2H3 cells, a mast model cell line (Bursimian, Isersky, Petorino, & Siraganian, 1981).

In this paper, we detail the anti-allergic and anti-inflammatory effects of the ethyl acetate fraction from an *E. kurome* extract, and compare it to the anti-allergic effects of the ethyl acetate fraction from an *E. arborea* extract (EEA).

Materials and methods

Materials and sample preparation

E. kurome was collected from an undersea forest of algae near Tsuji Island, Kumamoto, Japan. *E. arborea* (Phaeophyta) was collected at the coast of Mugizaki in Mie, Japan. After washing with tap water to remove all debris, the alga was air-dried and pulverised. Fifty grams of the algal powder was extracted with 150 mL of methanol at 5°C for 48 h,

with occasional shaking. After the extract was concentrated to a small volume with a rotary evaporator, 15 mL of methanol, 30 mL of chloroform and 11.25 mL deionised water were added. The upper layer, corresponding to the non-lipid fraction (Folch, Lees, & Stanley, 1957), was collected and extracted twice with 18.75 mL of ethyl acetate to obtain crude phlorotannins (Ragan & Glombitza, 1986). The ethyl acetate fraction was evaporated to dryness and retained as EEK and EEA (primarily crude phlorotannins) at -20°C until used. The yields of fractions from 50 g of each algal powder were 0.93 g (EEK) and 0.53 g (EEA).

In this study, epigallocatechin gallate (EGCG; Sigma-Aldrich Co., St. Louis MO, USA), a tea catechin, was used as a typical natural inhibitor, since EGCG is well known to possess anti-allergic effect via various inhibition mechanisms (Tachibana, 2011; Yamada, Watanabe, Kaku, Hassan, & Sugano, 1997; Yoshino, Kawaguchi, Yamazaki, & Sano, 2010).

Measurement of the amount of phenolic compounds

The purity of the phenolic compounds in EEK and EEA was measured by the Folin-Denis method (Folin & Denis, 1915) and calculated as the concentration of phloroglucinol that is a standard compound of the phlorotannins.

Anti-inflammatory examinations by mouse ear oedema

Animals

As an ear oedema model, ICR mice were used. The mice (male, 4 weeks old) were purchased from Kyudo Co., Ltd. (Tosu, Saga, Japan) and housed in individual cages kept at $23-26^{\circ}\text{C}$ under a 12 h light/dark cycle until being tested. Food (MF, KBT Oriental Co. Ltd., Tosu, Saga, Japan) and tap water were freely available. All of the animal experiments were performed with permission of the 'Committee for Use and Care of Laboratory Animals' of the National Fisheries University and in compliance with the 'Guideline for Animal Experiments in Research Institutes under the jurisdiction of the Ministry of Agriculture, Forestry and Fisheries'.

Arachidonate

AA-induced ear swelling was caused by the method of Young et al. (1984) with some modifications. Ten microliters of AA (12.5 mg/mL in acetone, stored at -20°C until used; Wako Pure Chemical Industries, Ltd., Osaka, Japan) and 5 μL of test sample dissolved in methanol were mixed and spread on a mouse ear. One hour later, ear swelling was determined with a thickness gauge (547 series, Mitutoyo Corporation, Kawasaki, Kanagawa, Japan).

Eighteen hours after oral administration of a test sample dissolved in 400 μL of 10% Tween 60 solution using a stainless feeding needle, 10 μL of AA was spread on the mouse ear. One hour later, ear swelling was determined with a thickness gauge. The inhibition ratio of the ear swelling was calculated according to the following formula:

$$\text{Inhibition ratio (\%)} = [1 - (T - T_0)/(C - C_0)] \times 100$$

where C_0 is ear thickness without test sample before spreading AA; C is the ear thickness without test sample after spreading AA; T_0 is the ear thickness with test sample before spreading AA and T is the ear thickness with test sample after spreading AA.

12-O-tetradecanoylphorbol-13-acetate

TPA-induced ear swelling was caused by the method of Young et al. (1984) with some modifications. Immediately before use, a stock solution of TPA (Wako; 800 µg/mL in acetone, stored at -20°C until used) was diluted to 80 µg/mL with acetone. Ten microliters of TPA and 5 µL of test sample were mixed and spread on a mouse ear. Four hours later, ear swelling was determined with a thickness gauge.

In the case of oral administration, 400 µL of test sample was administered 3 and 21 h prior to spreading TPA (10 µL) on a mouse ear. Four hours after spreading the TPA, ear swelling was determined with a thickness gauge. The inhibition ratio of the ear swelling was calculated as described above.

Oxazolone

OXA-induced ear swelling was caused by the method of Yoshino et al. (2010) with some modifications. The OXA solution (Sigma-Aldrich) was stored at -20°C until used. Fifty microliters of 1% OXA dissolved in ethanol was spread on the abdominal region of a mouse whose abdominal hair was carefully shaved with an animal shaver (Natsume Seisakusho Co, Ltd., Tokyo, Japan) under anaesthesia with diethyl ether. Five days later, 10 µL of 0.5% OXA dissolved in acetone and 5 µL of test sample were mixed and spread on a mouse ear. Twenty-four hours later, ear swelling was determined with a thickness gauge.

When mice were orally administered test samples, the examination was carried out according to the procedure of Suzuki, Yoshino, Maeda-Yamamoto, Miyase, and Sano (2000) with some modifications. Four-hundred microliters of test sample was orally administered at 1 h prior to the spreading of 1% OXA (50 µL) on the abdominal region of mouse as described above. Five days later, 400 µL of the test sample was orally administered once again at 1 h prior to the spreading of 0.5% OXA (10 µL) on mouse ear. Twenty-four hours later from spreading OXA, ear swelling was determined with a thickness gauge. The inhibition ratio of the ear swelling was calculated as described above.

Anti-degranulation activity

RBL-2H3 cells (JCRB0023, the Health Science Research Resources Bank, Tokyo, Japan) were cultured according to a previous report (Sugiura et al., 2012). Since the amount of degranulation from the mast cells is known to be proportional to β -hexosaminidase (β -Hex; equivalent to histamine) activity (Schwartz, Lewis, Seldin, & Austen, 1981), the degranulation assay by stimulated RBL cells was conducted by measurement of the released β -Hex activity according to the procedure described in a previous report (Sugiura et al., 2012). The experimental conditions of cell stimulation and exposure to tested samples were as follows. Cells (2×10^5 cells/well) were precultured and sensitised overnight with 0.2 µg of anti-dinitrophenyl (DNP) IgE (Sigma-Aldrich) in a 24-well plate. After removal of the anti-DNP IgE, the tested samples were added and the cells were incubated for 10 minutes. Subsequently, the cells were stimulated with 4 µg of DNP-bovine serum albumin (BSA) (LSL Co., Ltd., Tokyo, Japan) for 30 minutes. No inhibition of the β -Hex activity by the tested samples was observed.

*Inhibitory effects on enzymatic activities**Phospholipase A₂*

Assays for the inhibitory effects of test samples on PLA₂ activity were conducted according to the method of Sugiura et al. (2009). Diheptanoyl Thio-PC (1.66 mM;

Cayman Chemical Co., Ann Arbor, MI, USA) dissolved in dimethyl sulfoxide (DMSO) was diluted with 25 mM Tris-HCl buffer (pH 7.5). One-hundred microliters of Diheptanoyl Thio-PC solution and 5 μ L of sample or DMSO (solvent control) were mixed, and 5 μ L of 5,5'-dithio-bis-(2-nitrobenzoic acid) (50 mM; Sigma-Aldrich) dissolved in Tris-HCl buffer was added. After incubating the reaction mixture at room temperature for 5 minutes, background absorbance at 415 nm (OD_{415}) was measured using a spectrophotometer (MTP-120, Corona Electric Co. Ltd., Hitachinaka, Ibaraki, Japan). Immediately, to start the enzymatic reaction, 5 μ L of porcine pancreatic PLA₂ (25 units; Sigma-Aldrich) diluted with Tris-HCl buffer was added. Seventy-five minutes after the addition of PLA₂, the increase in OD_{415} by 5-thio-2-nitrobenzoic acid produced from the reaction was measured. The inhibition ratio of the PLA₂ activity was calculated according to the following formula:

$$\text{Inhibition ratio (\%)} = [1 - (T_{75} - T_0) / (C_{75} - C_0)] \times 100$$

where C_0 is the OD_{415} without test sample before PLA₂ was added; C_{75} is the OD_{415} without test sample 75 minutes after PLA₂ was added; T_0 is the OD_{415} with test sample before PLA₂ was added and T_{75} is the OD_{415} with test sample 75 minutes after PLA₂ was added.

Cyclooxygenase-2

COX-2 enzyme assays were conducted using the method of Kawakami et al. (2009) with some modifications. One thousand seven hundred twenty microliters of 0.1 M Tris-HCl buffer (pH 8.0), 10 μ L of 3 μ M hematin (Sigma-Aldrich), 10 μ L of 330 μ M tryptophan (Wako) and test sample or control solvent (0.1 M Tris-HCl buffer including 10% DMSO) were mixed. The mixture and 10 μ L of ovine COX-2 (6.6 units; Cayman) were mixed continuously and incubated at 25°C for 5 minutes. After adding 50 μ L of 30 μ M linoleic acid diluted with ethanol as substrate to the mixture to start the enzymatic reaction, optical density at 235 nm (OD_{235}) was measured using a spectrophotometer (U-1800, Hitachi High Technologies Corp., Tokyo, Japan). After incubating the mixture at 25°C for 5 minutes, the increase in OD_{235} due to conjugated diene production (9- or 13-hydroxyoctadecadienoic acid) by COX-2 activity on linoleic acid was determined. The inhibition ratio of the COX-2 activity was calculated according to the following formula:

$$\text{Inhibition ratio (\%)} = [1 - (T_5 - T_0) / (C_5 - C_0)] \times 100$$

where C_0 is the OD_{235} without test sample when linoleic acid was added; C_5 is the OD_{235} without test sample 5 minutes after addition of linoleic acid; T_0 is the OD_{235} with test sample when linoleic acid was added and T_5 is the OD_{235} with test sample 5 minutes after addition of linoleic acid.

Lipoxygenase

Soybean lipoxygenase (SBL, Type I-B, Sigma-Aldrich) was used as an alternatives to human 5-lipoxygenase (Komoda, Morimitsu, Hirota, & Hirota, 1995). The inhibitory effects of test samples on SBL activity were conducted according to the method of Komoda et al. (1995). After 2 mL of the SBL (100 units) in 0.2 M borate buffer (pH 9.0)

and 20 μ L of test samples or methanol (solvent control) was mixed, the mixture was incubated at room temperature for 5 minutes. After 50 μ L of 0.4 mM linoleic acid diluted with ethanol as the substrate was added, optical density at 234 nm (OD_{234}) was measured. After 20 minutes, the increase in OD_{234} due to conjugated diene production by SBL activity from linoleic acid was determined. The inhibition ratio of the SBL activity was calculated according to the following formula:

$$\text{Inhibition ratio (\%)} = [1 - (T_{20} - T_0) / (C_{20} - C_0)] \times 100$$

where C_0 is the OD_{234} without test sample when linoleic acid was added; C_{20} is the OD_{234} without test sample 20 minutes after addition of linoleic acid; T_0 is the OD_{234} with test sample when linoleic acid was added and T_{20} is the OD_{234} with test sample 20 minutes after addition of linoleic acid.

Statistical analysis

Data are expressed as means $\pm$ SD. Statistical analyses were performed using Student's *t*-test. $P < 0.05$ was considered statistically significant.

Results

The degree of purity of phenolic compounds in EEK and EEA

When the phenolic compounds (phlorotannins) of EEK and EEA were measured by Folin-Denis method, the purity values were 74.1% (*E. kurome*) and 72.0% (*E. arborea*). From these purity values, the main components of the fractions were considered to be algal phenolic compounds, i.e., phlorotannins.

The anti-inflammatory effect on mouse ear oedema

Percutaneous administration

EEK, EEA and EGCG exhibited anti-allergic effects on mouse ear oedema induced by each sensitizer. The effects of EEK were generally comparable to that of EEA and EGCG (Table 1). At a dose of 1 mg/ear, the effects of EEK on all inflammatory reactions tended to be stronger than those of EEA. Since inhibition ratios of EEK and EGCG in ear oedema induced by TPA and OXA were over 50% at a dose of 0.1 mg/ear,

Table 1. Inhibitory effects of percutaneous administrations of EEK, EEA and EGCG on each sensitizer-induced ear edema in male ICR mice.

Sensitizer		AA		TPA		OXA	
Sample dose (mg/ear)		0.1	1	0.1	1	0.1	1
Inhibition ratio (%)	EEK	40.5 $\pm$ 13.5	87.1 $\pm$ 9.7	56.5 $\pm$ 12.3	81.8 $\pm$ 17.1	73.4 $\pm$ 8.9	93.0 $\pm$ 11.5
	EEA	46.3 $\pm$ 12.7	66.8 $\pm$ 18.0	34.7 $\pm$ 7.6	79.7 $\pm$ 18.3	30.6 $\pm$ 10.7	82.9 $\pm$ 13.3
	EGCG	49.5 $\pm$ 11.3	75.8 $\pm$ 24.3	70.1 $\pm$ 12.2	87.9 $\pm$ 9.1	78.9 $\pm$ 6.5	103.9 $\pm$ 2.8

Notes: EEK, ethyl acetate fraction from *Ecklonia kurome* extract; EEA, ethyl acetate fraction from *Eisenia arborea* extract; EGCG, epigallocatechin gallate; AA, arachidonic acid; TPA, 12-O-tetradecanoylphorbol-13-acetate; OXA, oxazolone. Data are expressed as means $\pm$ SD ($n = 5$).

Table 2. Inhibitory effects of oral administrations of EEK, EEA and EGCG on each sensitizer-induced ear edema in male ICR mice.

Sensitizer		AA		TPA		OXA	
Sample dose (mg/body)		0.1	1	0.1	1	0.1	1
Inhibition ratio (%)	EEK	32.2 ± 11.2	66.9 ± 16.0	45.7* ± 8.7	65.7 ± 24.4	49.3 ± 4.9	82.8* ± 7.1
	EEA	39.2 ± 11.1	64.9 ± 7.0	31.3 ± 4.8	68.8 ± 4.8	15.2 ± 9.6	75.8 ± 11.3
	EGCG	20.7 ± 17.9	67.8 ± 12.5	22.9 ± 14.8	49.5 ± 15.3	48.8 ± 7.1	68.9 ± 9.0

Notes: *indicates $P < 0.05$ against EGCG. EEK, ethyl acetate fraction from *Ecklonia kurome* extract; EEA, ethyl acetate fraction from *Eisenia arborea* extract; EGCG, epigallocatechin gallate; AA, arachidonic acid; TPA, 12-O-tetradecanoylphorbol-13-acetate; OXA, oxazolone. Data are expressed as means ± SD ($n = 5$). Asterisks indicate that the inhibition ratio of EEK is significantly higher than that of EGCG.

0.01 mg/ear of the samples were examined. In the ear oedema by TPA, the inhibition ratios of EEK and EGCG were 29.0% and 42.1%, respectively. In the ear oedema induced by OXA, inhibition ratios of EEK and EGCG were 46.2% and 42.2%, respectively.

Oral administration

As shown in Table 2, EEK, EEA and EGCG administered orally exhibited anti-allergic effects on mouse ear oedema induced by each sensitizer. The effects of EEK were generally comparable to that of EEA and EGCG. At a dose of 0.1 mg/ear, the effects of EEK on the inflammatory reactions induced by TPA and OXA tended to be stronger than those of EEA. The effects of EEK tended to be stronger than those of EGCG. In particular, 0.1 and 1 mg of EEK exhibited more significant inhibitory effects on ear oedema induced by TPA and OXA, respectively, than EGCG.

Anti-degranulation activity

As shown in Figure 1, EEK, EEA and EGCG suppressed the degranulation in RBL cells in a dose-dependent manner. The inhibition ratios of EEK at 5, 10, 25 and 50 µg/mL were 5.3%, 45.1%, 82.6% and 100%, respectively. Also, the inhibition ratios of EEA at 5, 10, 25 and 50 µg/mL were 19.3%, 40.6%, 75.4% and 100%, respectively. Thus, the effects of EEK were approximately comparable to EEA. At 10 µg/mL, the effect of EEK was significantly ($P < 0.05$) stronger than that of EGCG, and at 25 and 50 µg/mL, it was comparable to EGCG. The 50% inhibition concentration (IC_{50}) values of EEK and EEA were 11.5 µg/mL (EEK) and 15.3 µg/mL (EEA). Since induction of cell death by components of the extracts was suspected due to the lack of degranulation in RBL cells at 50 µg/mL, the induction of cell death was checked by exclusion of Trypan blue. The survival ratio was 96.3% (EEK) and 96.1% (EEA), and cell death by the extract was not observed (data not shown).

Inhibitory effects on enzymatic activities

As shown in Table 3, EEK, EEA and EGCG exhibited inhibitory effects on all enzymatic activities tested (PLA_2 , COX-2 and SBL). The inhibitory effects of EEK on PLA_2 and COX-2 activity tended to be weaker than that of EEA, and effects on SBL tended to be stronger than those of EEA. The inhibitory effect of EEK on SBL activity was significantly ($P < 0.01$) stronger than that of EGCG. The inhibitory effects of EEK on

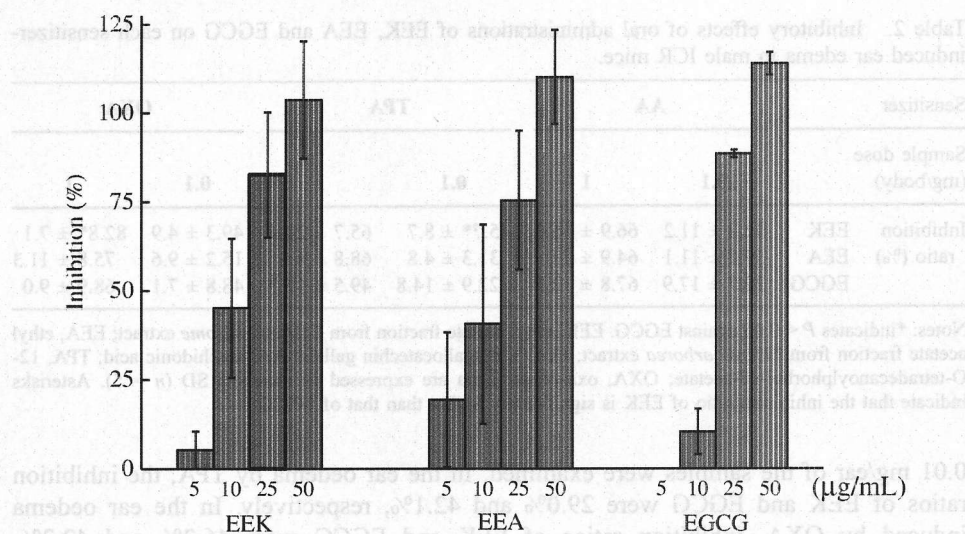


Figure 1. The dose-dependent inhibitory effects of EEK, EEA and EGCG on degranulation in RBL-2H3 cells stimulated by the antigen-antibody reaction.

Note: The values on the x-axis are expressed as the concentration of the fractions or EGCG. The inhibition ratio was calculated from the results of triplicate experiments. Data are expressed as means $\pm$ SD. The reproducibility was confirmed by repeated runs. EEK, ethyl acetate fraction from *E. kurome* extract; EEA, ethyl acetate fraction from *E. arborea* extract; EGCG, epigallocatechin gallate.

Table 3. The inhibitory effects of EEK, EEA and EGCG on enzymatic activities involved in allergic reactions.

Samples	IC ₅₀ (µg/mL)		
	PLA ₂	COX-2	SBL
EEK	1618.9 $\pm$ 106.2	434.6 $\pm$ 164.0	35.9** $\pm$ 3.2
EEA	1023.3 $\pm$ 527.6	350.0 $\pm$ 229.1	321.0 $\pm$ 209.7
EGCG	1091.4 $\pm$ 22.8	154.4 $\pm$ 108.9	488.7 $\pm$ 29.6

Note: The inhibition ratio was calculated from the results of triplicate experiments. Data are expressed as means $\pm$ SD. The reproducibility was confirmed by repeated runs. **indicates that IC₅₀ values of EEK are significantly ($P < 0.01$) lower than those of EGCG.

EEK, ethyl acetate fraction from *E. kurome* extract; EEA, ethyl acetate fraction from *E. arborea* extract; EGCG, epigallocatechin gallate; PLA₂, phospholipase A₂; COX-2, cyclooxygenase-2; SBL, soybean lipoxygenase.

PLA₂ and COX-2 activities were weaker than those of EGCG, and inhibition of PLA₂ activity was the weakest of the tested enzymatic activities.

Discussion

In this study, phlorotannin-enriched EEK and EEA extracts inhibited mouse ear oedema induced by sensitizers (AA, TPA and OXA; Tables 1 and 2), degranulation in RBL cells (Figure 1) and activities of enzymes (PLA₂, COX-2 and SBL) involved in allergic reactions (Table 3), to a similar degree as inhibition by EGCG.

As shown in Table 3, the inhibitory effects of EEK were generally comparable to those of EEA. However, the inhibitory effect of EEK on SBL activity tended to be

stronger than that of EEA, and the inhibitory effects of EEK on PLA₂ and COX-2 tended to be weaker than those of EEA. Such differences of the effects between *E. kurome* and *E. arborea* may be due to differences in phlorotannin composition between the two brown algal species. Phlorotannins are abundant in these algae and exhibit anti-allergic and anti-inflammatory effects as described above (Nguyen, Choi, Kim, & Jung, 2012; Ragan & Glombitza, 1986). For *E. kurome*, eckol, dieckol, 6,6'-bieckol, 8,8'-bieckol and phlorofucofuroeckol (PFF)-A have been identified (Fukuyama et al., 1990; Nagayama et al., 2003). In addition to the phlorotannins identified in *E. kurome*, 6,8'-bieckol and PFF-B have also been identified in *E. arborea* (Sugiura et al., 2007). Therefore, the differences of the effects between the two algae may be attributed to the chemical composition of the phlorotannins. In fact, when the phlorotannin composition of EEK used in this study was analysed by high-performance liquid chromatography (HPLC) using phlorotannin standards obtained from *E. arborea* (Sugiura et al., 2009; Tsukui et al., 2006), eckol, 6,6'-bieckol, 8,8'-bieckol, dieckol and PFF-A were detected (preliminary data not shown). These phlorotannins possess inhibitory effects on the enzymatic activities and degranulation in RBL cells (Shibata et al., 2003; Sugiura et al., 2007, 2009), and the preliminary data are consistent with previous reports (Fukuyama et al., 1990; Nagayama et al., 2003). As described in the Results, since the purity values of phenolic compounds (phlorotannins) in EEK and EEA were 74.1% and 72.0%, respectively, it is postulated that the active components in the ethyl acetate fraction from the *E. kurome* extract are phlorotannins.

EEK and EEA exhibited anti-allergic effects on mouse ear oedema in both percutaneous and oral administrations of the extract (Tables 1 and 2) in a manner comparable to EGCG (Yoshino et al., 2010). Thus, the ethyl acetate fractions inhibited not only inflammatory reactions induced by AA and TPA, but also the type IV allergic reaction induced by OXA. PGs produced by COX-2 activity are involved in AA-induced inflammation, while rapid COX-2 expression and LTs produced by LOX activity are involved in TPA-induced inflammation (Kujubu et al., 1991; Rao et al., 1993). LTs, PGs and histamine that cause various inflammatory reactions are released by degranulation in mast cells. In a previous study, a phlorotannin-enriched methanol/chloroform extract of *E. arborea* strongly suppressed LT B₄ and prostaglandin D₂ release from RBL cells (Sugiura et al., 2009). This suggests that the inhibitory effects of EEK on the mouse ear oedema may be attributed to mechanisms that inhibit enzymatic activities involved in allergic reactions and degranulation in inflammatory cells as mentioned above, and the active components that suppressed the mouse ear oedema may be phlorotannins as detected in the preliminary analysis. To better understand the effect of purified phlorotannins from *E. kurome*, further studies are needed.

In this study, the ethyl acetate fractions administered orally suppressed mouse ear oedema to a similar degree as EGCG. These results for EGCG are consistent with previous reports that when administered orally, TPA- or OXA-induced mouse ear oedema was suppressed (Huang et al., 2006; Suzuki et al., 2000). Since EGCG is absorbed into human plasma (about 2.0%; Nakagawa, Okuda, & Miyazawa, 1997) and the fractions exhibited an inhibitory effect on mouse ear oedema by oral administration as mentioned above, the active components might be absorbed into gut along with EGCG and might exert the inhibitory effect. For the phlorotannins regarded as the candidate active component of the fractions, further studies for the inhibitory effect on mouse ear oedema and absorption using cultured cells and/or animal models are needed.

According to a report by Nagayama et al. (2002), when ICR mice were orally administered crude *E. kurome* phlorotannins, no abnormal symptoms for weight gain and

diet consumption were observed. For anti-allergic and anti-inflammatory drugs, the inhibitory ratios of hydrocortisone (an anti-inflammatory drug) on mouse ear oedema were 77% at 0.2 mg/ear (AA), 50% at 0.03 mg/ear (TPA) and 73% at 0.05 mg/ear (OXA; Banno et al., 2005; Suzuki et al., 2000; Valderramas et al., 2008). The IC_{50} value of tranilast (an anti-degranulation drug) on degranulation in RBL cells was 15.3 $\mu\text{g/mL}$ (Matsubara, Masaki, Ohmori, Karasawa, & Hasegawa, 2004). The IC_{50} values of Me-Indoxam (sPLA₂ inhibitor), zileuton (a 5-LOX inhibitor) and celecoxib (a COX-2 inhibitor) were 0.22 $\mu\text{g/mL}$, 0.54 $\mu\text{g/mL}$ and 0.02 $\mu\text{g/mL}$, respectively (McMillan, Spruce, Crawley, Walker, & Foster, 1992; Penning et al., 1997; Singer et al., 2002). In the present study, the ethyl acetate fractions possessed comparable (anti-degranulation) and/or more moderate anti-allergic and anti-inflammatory effects than above mentioned drugs. Moreover, the fractions administered orally also suppressed the mouse ear oedema as mentioned above, and dried the alga from which the fraction was made contains no more than about 3% (w/w) of phlorotannins (Shibata et al., 2004). Therefore, the suppression by EEK seems to be not as drastic as drugs but moderate as a functional food. Thus, an ethyl acetate fraction from brown algal methanol/chloroform extract is rather suitable for functional foods.

Taken together with the case of *E. kurome* products as described in the Introduction, the alga may be safe and very suitable for functional food resources. However, to be effectively used as a functional food resource, further studies are needed as follows: the relation between efficacy and safety of the alga, and *in vivo* studies for a detailed understanding of anti-allergic mechanisms such as the effect on cytokine balance using animal models.

Conclusions

The ethyl acetate fraction from an *E. kurome* methanol/chloroform extract suppressed mouse ear oedema induced by inflammation sensitisers (AA, TPA and OXA) and demonstrated inhibitory effects on degranulation in RBL cells and enzymatic activities (PLA₂, COX-2 and SBL) involved in allergic reactions, to a similar degree as the ethyl acetate fraction from an *E. arborea* methanol/chloroform extract that is known to possess anti-allergic effects. Thus, *E. kurome* and its components possess anti-allergic and anti-inflammatory effects, and have the capability to serve as a functional food resource for allergies.

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