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Determination of the Antiallergic Activity of Lipophilic Components Isolated from the Red Alga *Pyropia tenuipedalis*

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Abstract

The functional properties of edible lavers are well-known; however, there is limited information on the marine red alga *Pyropia tenuipedalis*, an indigenous species found in the Yamaguchi Prefecture area. In the present study, samples of *P. tenuipedalis* were collected from the Koto River Estuary and cultivated using land-based cultivation techniques. A methanol/chloroform (M/C) extract obtained from naturally grown and land-based cultivated *P. tenuipedalis* showed antiallergic properties, including suppression of ear swelling in the Institute of Cancer Research mouse model, inhibition of inflammation-associated enzymes (i.e., phospholipase A_2, lipoxygenase, cyclooxygenase-2, and hyaluronidase), and anti-degranulation in rat basophilic leukemia-2H3 cells. Chemical composition was determined using thin-layer chromatography. Three glyceroglycolipids, namely digalactosyldiacylglycerol, monogalactosyldiacylglycerol, and sulfoquinovosyldiacylglycerol, were detected in the M/C extracts of natural and cultivated algae and were selected as bioactive candidates. The results demonstrate that glyceroglycolipids in natural and cultivated *P. tenuipedalis* edible algae have promising potential for the development of antiallergic food.

Discipline: Food
Additional key words: antiallergy, anti-inflammation, glyceroglycolipid

Introduction

Indigenous Japanese algae, such as *Monostroma nitidum* (green algae), *Neopyropia tenera* (red algae), and *Neopyropia yezoensis* (red algae), are the most frequently consumed edible products; however, other regional edible species are also being explored. One of these is the red alga *Pyropia tenuipedalis*, an indigenous marine species inhabiting the Yamaguchi Prefecture area that grows by adhering to suboceanic seashells (Abe et al. 2015, Zuccarello et al. 2022). Although *P. tenuipedalis* is a rare species and its relative contribution to the seaweed biomass is limited, it is found in a broad region, including Tokyo Bay, Osaka Bay, Ise Bay, and the Seto Inland Sea (Notoya & Kikuchi 1993, Abe et al. 2015). Furthermore, the amount of free amino acids in *P. tenuipedalis* is comparable to those found in *N. yezoensis*, while the alanine content in *P. tenuipedalis* is higher than in *N. yezoensis* (Miyago & Yasunari 2004), leading to a unique favorite palatability of the alga. Moreover, an oligopeptide and a polysaccharide (porphyran) obtained from *N. yezoensis* have been shown to exhibit antihypertensive and antitumor properties, respectively (Suetsuna 1998, He et al. 2019). Therefore, although *P. tenuipedalis* and *N. yezoensis* are useful as functional food resources, a deeper investigation into the potential of their bioactive algal compounds is required. We used land-based cultivation techniques as described previously (Murase et al. 2018, Abe et al. 2022) to study the antiallergic potential of this local species in developing healthy food products, because the relative biomass of *P. tenuipedalis* in natural ecosystems is very small. Since allergies are a serious public health concern, this study aims to investigate the antiallergic properties of an extract made from a cultivated alga.

Materials and methods

1. Samples and component extraction

(1) Samples

Samples of *P. tenuipedalis* were collected in March

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2011 from the Koto River Estuary in the Yamaguchi Prefecture area and stored at −20°C after air-drying. Land-based cultivation was carried out at the Yamaguchi Prefectural Fisheries Research Center, and algae were collected in February 2016. Subsequently, the samples were washed with tap water to remove all debris, air-dried in an air oven at 35°C for 24 h, and then pulverized with an NR-04 pulverizer (Sansho Industry Co., Ltd., Osaka, Japan).

The antiallergic agent, tea catechin epigallocatechin gallate (EGCG) (Sigma–Aldrich Co., St. Louis, MO, USA), was used as a comparative control for its natural inhibitory properties, as previously described by Tachibana (2011).

(2) Separation of the methanol/chloroform (M/C) extract

A methanol/chloroform extract was obtained from algae according to a previously described protocol in the literature with minor modifications (Sugiura et al. 2016). Briefly, 10 g of *P. tenuipedalis* powder was extracted using 40 mL of methanol for 3 h on a magnetic hot-plate stirrer. Sunsequently, 80 mL of chloroform was added and continuous extraction was conducted for 1 h. The supernatant was filtered with No. 2 filter paper (Toyo Roshi Kaisya, Ltd., Tokyo, Japan) and partitioned with 30 mL of distilled water. The lipophilic fraction (lower) was recovered and condensed using a rotary evaporator (N-1100, Tokyo Rikakikai Co., Ltd., Tokyo, Japan). The dried residue was dissolved in chloroform, and the solution was retained as the test sample (i.e., the M/C extract) and stored at −20°C.

2. Suppressive effects of the M/C extract on ear swelling

The Institute of Cancer Research (ICR) mouse model was used to study the effects of the M/C extract on ear swelling. ICR male mice aged 4 weeks were purchased from KBT Oriental Co., Ltd. (Tosu, Saga, Japan). To acclimatize to the laboratory conditions, mice were housed in individual cages at 23°C - 26°C under a 12-h light/dark cycle for 7 days until ear swelling tests were performed with a single or twice-administered sample. All animal experiments were approved by the Committee for Use and Care of Laboratory Animals of the National Fisheries University and followed the Guidelines for Animal Experiments in Research Institutes under the Jurisdiction of the Ministry of Agriculture, Forestry and Fisheries (Japan), Approval Numbers: 16–10 (March 31, 2016), 17–8 (March 31, 2017), and 23–4 (March 23, 2023). The mice were fed a solid AIN-93G diet (KBT Oriental) and tap water. The mice maintained good appetite during the experimental period, and diarrhea or abnormal symptoms were not observed in the experimental groups. There were no significant differences in food intake or body weight gain among the tested mice during the experiment (data not shown).

We investigated the suppressive effects of percutaneously- or orally-administered extracts on ear swelling in the ICR mice induced by sensitizers, namely, arachidonic acid (AA), 12-O-tetradecanoylphorbol-13-acetate (TPA), and oxazolone (OXA). Acute chronic inflammation induced by cyclooxygenase-2 (COX-2) expression and delayed-type allergic inflammation were assessed after induction with AA, TPA, and OXA sensitizers (Young et al. 1984, Meurer et al. 1988, Kujubu et al. 1991). Ear swelling experiments were performed after percutaneous and oral administration according to the protocols described by Sugiura et al. (2021).

3. Inhibitory effects of M/C extract on enzymatic activity

Inflammation-related enzymes involved in allergic reactions include phospholipase A_2 (PLA_2), lipoxygenase (LOX), cyclooxygenase-2 (COX-2), and hyaluronidase (HA) (Sakamoto et al. 1980, Funk 2001). Inhibitory effects on the activity of these enzymes were examined as previously described by Sugiura et al. (2021).

4. Anti-degranulation activity

The suppressive effects of M/C extract on antigen-induced degranulation in rat basophilic leukemia (RBL)-2H3 cells (JCRB0023, Health Science Research Resources Bank, Tokyo, Japan) were examined. RBL-2H3 cells are well-known mast cell models (Barsumian et al. 1981). The culture of RBL cells and antigen-mediated degranulation assay were performed as previously described by Sugiura et al. (2021).

5. Identification of active compounds present in the M/C extract

(1) Samples

Two concentrations of the M/C extract (50 and 100 mg/mL) and 1 mg/mL of standard compounds, i.e., digalactosyldiacylglycerol (DGDG; Sigma–Aldrich), monogalactosyldiacylglycerol (MGDG; Sigma–Aldrich), and sulfoquinovosyldiacylglycerol (SQDG; Avanti Polar Lipids, Inc., Birmingham AL, USA), were diluted with chloroform. Because glyceroglycolipids have bioactive potential (Noguchi et al. 2003), three lipophilic bioactive components were chosen as bioactive candidates in our study.

(2) Thin-layer chromatography (TLC) analysis

TLC was used to identify chemical compounds in the M/C extract, as previously described with minor modifications (Araki et al. 1986). Briefly, a solvent

comprising chloroform, methanol, and distilled water (70:21:3, v/v) was added to the developing chamber, which was equilibrated for approximately 1 h. Subsequently, the samples were spotted onto a TLC plate (Silica gel 60, 20 cm × 20 cm, Merck KGaA, Darmstadt, Germany). The plate was dehydrated using an air dryer and placed in a chamber in a fume hood for 75 min. The plate was then removed, air-dried for 5 min, and sprayed with anthrone reagent (0.05% anthrone [v/v] and 1% thiourea [v/v] dissolved in 66% sulfate [v/v]). Finally, the plate was dried at 110°C for 10 min, and violet-colored spots were captured using an electronic camera. Rf values of the spots were calculated using the following formula:

$$Rf = A / B,$$

where A is the distance between the spot and the original line and B is the distance between the solvent and the original line.

(3) Suppressive effects of glyceroglycolipid standards on mouse ear swelling

The method described above was used to examine the suppressive effects of percutaneously administered standards on AA-induced ear swelling to determine whether glyceroglycolipids are the active components in the M/C extract.

6. Statistical analysis

The data are expressed as mean ± standard deviation. Statistical comparisons were performed by Tukey's test using Excel Statistics software, version 2018 (Social Survey Research Information Co., Ltd., Tokyo, Japan). A value of $P < 0.05$ was considered as statistically significant.

Results

1. Suppressive effects of the M/C extract on ear swelling

The M/C extracts of natural and cultivated algae suppressed ear swelling induced by sensitizers in mice (Table 1). The suppressive effect of the extract obtained from naturally grown algae was similar to that observed with the extract obtained from cultivated algae after percutaneous administration. Furthermore, the natural algae M/C extract had a suppressive effect on AA and TPA-induced ear swelling compared with that of the EGCG treatment. Moreover, no significant differences were observed in oral administration among the tested groups, except for the suppressive effects of the M/C extract obtained from naturally grown algae on TPA-induced ear swelling. In the oral administration experiments, the M/C extract obtained from land-based cultivated algae had greater suppressive ratios than those obtained after percutaneous administration.

2. Inhibition of inflammation-related enzymes and anti-degranulation activity

The inhibitory effects of land-based cultivated algae on the enzymatic activities of COX-2 and HA were significantly greater than those observed with the M/C extract obtained from naturally grown algae and EGCG treatment. In contrast, the inhibitory effects of land-based cultivated algae on the enzymatic activity of PLA_2 and LOX were significantly smaller than those observed with naturally grown algae M/C extract and EGCG treatment. Furthermore, the M/C extract inhibition ratio obtained from land-based cultivated algae at 300 μg/mL concentration was 20.8% ± 3.9% with regard to the enzymatic activity of PLA_2. Moreover, the inhibitory effects of the extract obtained from naturally grown algae on the activities of PLA_2 and HA were significantly

Table 1. Suppressive effects of the M/C extract on mouse ear swelling induced by AA, TPA, and OXA sensitizers

Samples			Natural growth	Land-based cultivation	EGCG
Suppression ratio (%)	Precutaneous	AA	59.6 ± 22.9 ab	30.4 ± 18.8 a	81.6 ± 12.7 b
		TPA	65.6 ± 19.3 ab	36.2 ± 15.0 a	83.3 ± 13.9 b
		OXA	40.1 ± 9.7 a	25.4 ± 14.6 a	79.2 ± 27.2 b
	Oral	AA	49.7 ± 8.2	43.6 ± 12.6	48.1 ± 18.1
		TPA	29.4 ± 4.8 a	57.7 ± 11.4 b	52.1 ± 9.7 b
		OXA	36.7 ± 3.3	37.0 ± 9.1	44.1 ± 12.7

Sample dose: 0.1 mg/mouse. Values are presented as mean ± standard deviation of four experiments (n = 4). Differences between samples with different letters are statistically significant ($P < 0.05$).
Abbreviations: AA, arachidonic acid; TPA, 12-O-tetradecanoylphorbol-13-acetate; OXA, oxazolone; EGCG, epigallocatechin gallate

greater than those observed after EGCG treatment (Table 2).

Figure 1 shows that the M/C extract obtained from naturally grown and land-based cultivated algae had anti-degranulation activity in RBL cells. The IC_{50} values of the M/C extract obtained from naturally grown and cultivated algae were 55.6 (± 7.9) and 73.9 (± 17.0) μg/mL, respectively. These values were significantly larger than those observed after EGCG treatment (9.3 ± 1.0 μg/mL).

3. Analysis of active components in M/C extract

The amounts of the M/C extract of natural and cultivated algae in 10 g of the algal powder were 274.6 and 265.3 mg, respectively. Figure 2 shows the TLC analysis of the M/C extract obtained from naturally grown and land-based cultivated algae, which showed spots attributable to the three standard glyceroglycolipids. The Rf values of the spots agreed with those observed for the standard compounds (Table 3). The percutaneously administered standards suppressed AA-induced ear swelling, and the suppressive ratio at a dose of 0.1 mg/mouse was approximately 50% - 80% (Table 4). This observation suggests that the M/C extract may contain glyceroglycolipids as bioactive compounds. However, notable unknown spots were also observed (Fig. 2).

Discussion

In this study, the lipophilic M/C extract obtained from naturally grown and land-based cultivated *P. tenuipedalis* showed antiallergic activity through

Table 2. Inhibitory effects of the M/C extract on the activity of enzymes involved in allergic reactions

Samples		Natural growth	Land-based cultivation	EGCG
IC_{50} values (μg/mL)	PLA_2	18.2 ± 5.2 a	>300 b	230.2 ± 107.6 c
	LOX	211.7 ± 75.2 a	1,865.6 ± 197.1 b	380.9 ± 21.4 a
	COX-2	6.5 ± 0.1 a	1.0 ± 0.7 b	4.8 ± 0.4 c
	HA	33.3 ± 0.9 a	19.8 ± 1.5 b	91.3 ± 6.7 c

Values are presented as mean ± standard deviation of three experiments (n = 3). Reproducibility was confirmed by repeated runs. Differences between samples with different alphabet letters are statistically significant ($P < 0.05$). Abbreviations: PLA_2, phospholipase A_2; LOX, lipoxygenase; COX-2, cyclooxygenase-2; HA, hyaluronidase; EGCG, epigallocatechin gallate

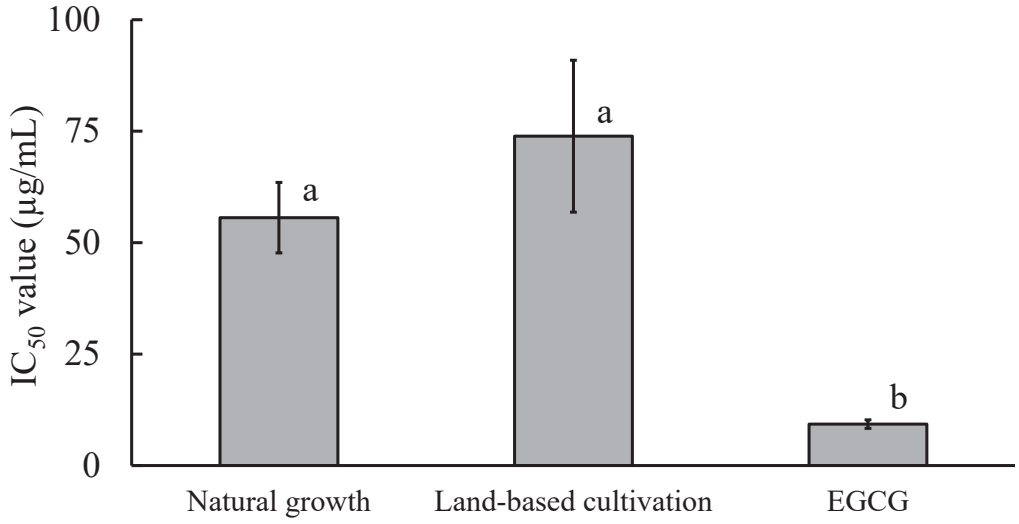

Fig. 1. Suppressive effect of the algal extract on degranulation in RBL-2H3 cells stimulated with antigen
Values are presented as mean ± standard deviation of three experiments (n = 3). Reproducibility was confirmed through repeated runs. Differences between samples with different alphabet letters are statistically significant ($P < 0.05$). Abbreviation: EGCG, epigallocatechin gallate

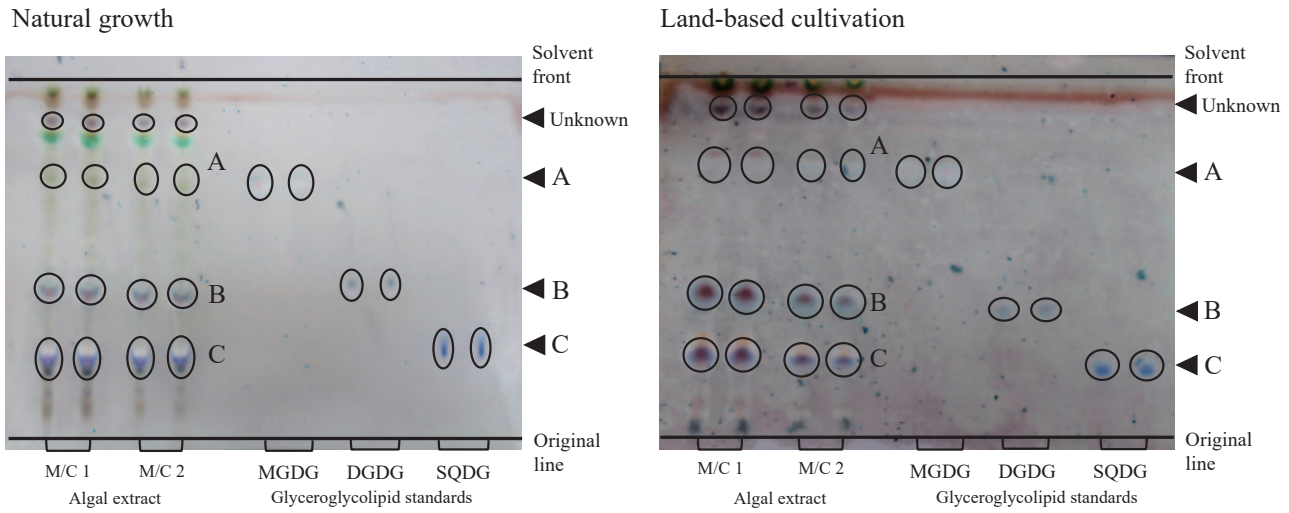

Fig. 2. Component analysis of the algal extract by thin-layer chromatography
Abbreviations: DGDG, digalactosyldiacylglycerol; MGDG, monogalactosyldiacylglycerol; SQDG, sulfoquinovosyldiacylglycerol
M/C 1, M/C 2, and each of glyceroglycolipid standards were applied at 100 mg/mL, 50 mg/ml, and 1 mg/mL, respectively.
A, B, and C indicate MGDG, DGDG, and SQDG mobility, respectively.

Table 3. Average Rf values for spots in thin-layer chromatography

		Natural growth	Land-based cultivation	MGDG	DGDG	SQDG
Spot	Unknown	0.878	0.906			
	A	0.726	0.748	0.724		
	B	0.416	0.390		0.397	
	C	0.228	0.223			0.214

Abbreviations: DGDG: digalactosyldiacylglycerol; MGDG, monogalactosyldiacylglycerol; SQDG, sulfoquinovosyldiacylglycerol.
A, MGDG mobility; B, DGDG mobility; C, SQDG mobility

Table 4. Suppressive effects of the glyceroglycolipid standards on mouse ear swelling induced by AA sensitizer

Samples	MGDG	DGDG	SQDG
Suppression ratio (%)	51.6 ± 10.8	60.4 ± 27.3	78.0 ± 21.8

Sample dose: 0.1 mg/mouse. The samples were percutaneously administered to mice's ear. Values are presented as means ± standard deviation of four experiments (n = 4). No statistically significant differences were observed among the test groups ($P > 0.05$).
Abbreviations: AA, arachidonic acid; MGDG, monogalactosyldiacylglycerol; DGDG, digalactosyldiacylglycerol; SQDG, sulfoquinovosyldiacylglycerol

different inhibitory mechanisms, including the suppression of the allergic inflammation response in a mouse model, inflammation-related enzymes, and degranulation in RBL cells (Fig. 1; Tables 1, 2). In mice, the activity of inflammation-related enzymes and degranulation is involved in allergic reactions, such as ear swelling (Funk 2001, Kerdel et al. 1987, Meurer et al. 1988, Sakamoto et al. 1980, Young et al. 1984). These findings agree with our interpretation that anti-degranulation and inflammation-related enzyme inhibition constitute the suppressive mechanism of the M/C extract on allergic inflammation in mice.

As mentioned above, the lipophilic M/C extract exhibited antiallergic properties. Moreover, DGDG,

MGDG, and SQDG glyceroglycolipids accounted for approximately 62% of the total lipids present in *N. yezoensis* (Araki et al. 1986) and have been reported to exert anti-inflammatory effects in an inflammation mouse model (Bruno et al. 2005). In the present study, TLC method confirmed the presence of these glyceroglycolipids in the M/C extract (Fig. 2 ; Table 3). Percutaneously administered glyceroglycolipid standards exhibited suppressive effects on ear swelling (Table 4). Hence, they were the key ingredients responsible for the antiallergic activity. In contrast, glyceroglycolipids are usually digested into acylglycerol and fatty acids in the intestine, and only fatty acids are absorbed by the body (Sugawara 2007). Furthermore, approximately 80% - 90% of the fatty acids composing glyceroglycolipids in *N. yezoensis* are palmitic acid and eicosapentaenoic acid (EPA) (Araki et al. 1986). Moreover, EPA possesses antiallergic properties (Calder 2001). These studies suggest similarities in the fatty acid composition of glyceroglycolipids in *P. tenuipedalis* and *N. yezoensis*. Additionally, the suppressive mechanisms of ear swelling by percutaneous administration may differ from those of oral administration. This implied that the percutaneously administered glyceroglycolipids found in the M/C extract suppressed mouse ear swelling by anti-degranulation and inhibited inflammation-related enzymatic activity (PLA_2, LOX, COX-2, and HA). In contrast, oral administration results in digestion into acylglycerol, palmitic acid, and EPA, and the absorbed EPA may exhibit an antiallergic effect. Additionally, an unknown component was detected. Because the Rf value for acyl sterol glucoside is greater than that for MGDG (Hirayama & Matsuda 1973), we suggest that the unidentified glycolipid may be acyl sterol glucoside. Thus, further studies are required to unequivocally identify all components in the M/C extract and to clarify the fatty acid component of glyceroglycolipids in *P. tenuipedalis*.

We found differences in the antiallergic efficacy of M/C extracts obtained from naturally grown and land-based cultivated algae. In the percutaneous administration of the ear swelling test, the suppressive effects of the M/C extract obtained from naturally grown algae were greater than those observed using the M/C extract of cultivated algae. Moreover, the inhibitory effects of the M/C extract obtained from natural algae on the activity of PLA_2 and LOX enzymes were significantly higher than those observed using the extract of cultivated algae (Tables 1, 2). The concentration of typical pigments in lavers (e.g., carotenoids and chlorophylls) depends on the growth environment (Amano & Noda 1978). β-Carotene and lutein carotenoids demonstrated PLA_2 and LOX inhibiting activity (Lomnitski et al. 1993, Song et al. 2010) and suppressed ear swelling in mice (Horváth et al. 2015). Therefore, the differences in the efficacy of M/C extracts may be due to variations in carotenoid concentrations. As the Rf values of pigments are greater than those of glyceroglycolipids (Henry et al. 1983, Davidi et al. 2014), spots higher than MGDG may indicate the presence of pigments, including carotenoids and chlorophylls (Fig. 2). However, the presence of bioactive pigments and other lipophilic components should also be examined in future studies, although DGDG, MGDG, and SQDG glycolipids are likely the active components in the M/C extract.

In conclusion, the present study pioneered a report on the antiallergic properties of an extract obtained from the red alga *P. tenuipedalis*. Our results indicated that DGDG, MGDG, and SQDG glyceroglycolipids are likely active compounds in the *P. tenuipedalis* extract. Furthermore, we also demonstrated the antiallergic potential of the algal extract obtained from land-based cultivation techniques, showing that cultivated algae may be a beneficial edible laver that could attract international interest.

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