

Dieckol isolated from a brown alga, *Eisenia nipponica*, suppresses ear swelling from allergic inflammation in mouse

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Abstract

We previously found a lipophilic fraction of the methanol/chloroform extract of a brown alga, *Eisenia nipponica*, that had an antiallergic effect in a murine ear swelling test. In this study, we purified the active component from the lipophilic fraction using high performance liquid chromatography and analyzed the mass and nuclear magnetic resonance spectra. This uncovered the phlorotannin dieckol, which exhibited antiallergic effects in an ear swelling test using mice sensitized by arachidonic acid, 12-O-tetradecanoylphorbol-13-acetate, and oxazolone. Mechanistic investigations indicated that dieckol suppressed degranulation, chemical mediator release, and the expression of mRNA such as cyclooxygenase-2, interleukin-6, and tumor necrosis factor- α in rat basophilic leukemia-2H3 cells. In summary, we isolated dieckol from *E. nipponica* and demonstrated its antiallergic mechanisms.

Practical applications

As the incidence of allergies increases worldwide, so too does the demand for food components with antiallergic and anti-inflammatory properties. Given this trend, we focused on a brown alga that displays a variety of bioactivities. Here, we have isolated dieckol from the antiallergic lipophilic fraction of *E. nipponica* and found that it possesses diverse physiological activities that may prevent lifestyle-related diseases. Consequently, dieckol or the alga containing this phlorotannin could be used as a health food ingredient to combat not only allergies, but also variety of disorders including the undesirable effects of aging.

KEYWORDS

antiallergy, anti-inflammation, brown alga, cytokine, mouse ear swelling, phlorotannin

1 | INTRODUCTION

In developed countries, the incidence of allergies such as food allergy, asthma, atopic dermatitis, and hay fever has increase, becoming an acute problem (Platts-Mills, 2015). According to the Japanese Ministry of Health, Labor, and Welfare, half of the Japanese people may suffer from allergies, making them a societal problem (Taniguchi, 2012). Natural remedies and foods

possessing antiallergic and anti-inflammatory effects are highly sought-after and recently researchers have endeavored to identify natural compounds and products with antiallergic properties. Prebiotics and probiotics, such as dietary fibers (e.g., oligosaccharides) and lactic acid bacteria, are known to exert antiallergic effects through gut-based immunomodulation (Hardy et al., 2013). Phenolic compounds (polyphenols) are another well-known group of natural antiallergic compounds derived from food products. For

example, tea catechins, flavonoids, and tannins are polyphenols in terrestrial plants with well-established antiallergenic properties (Tachibana, 2011).

There are several reports on the antiallergic or the anti-inflammatory effects of marine products including components of brown seaweeds. For example, marine carotenoids (such as fucoxanthin) and polysaccharides (such as fucoidan) exhibit antiallergic effects by suppressing the bioactivity of inflammatory lymphocytes and by improving the immunological Th1/Th2 balance, respectively (Maruyama et al., 2005; Sakai et al., 2009). The major components of polyphenols in seaweeds are phlorotannins and a few well-known examples such as eckol, 6,6'-bieckol, 8,8'-bieckol dieckol, and phlorofucofuroeckol (PFF)-A, are found in brown algae (Ragan & Glombitza, 1986). These compounds are the secondary photosynthetic metabolites that protect algae from ultraviolet (UV) radiation and consumption by herbivores. In our previous study, six phlorotannins (eckol, 6,6'-bieckol, 6,8'-bieckol, 8,8'-bieckol, PFF-A, and PFF-B) were isolated from the aqueous methanol fraction of the methanol/chloroform (M/C) extract of the brown alga *Eisenia nipponica* (Sugiura et al., 2006, 2007). We also previously reported the antiallergic and anti-inflammatory effects of the isolated phlorotannins (Sugiura et al., 2018).

Previously in vivo experiments demonstrated the antiallergic and anti-inflammatory effects of the lipophilic fraction of extracts from *E. nipponica* (Sugiura et al., 2017). In the present study, we isolated the antiallergic compound in the lipophilic fraction using chromatographic methods, analyzing the mass and nuclear magnetic resonance spectra, and testing its ability to inhibit the activity of lipoxygenase (LOX) involved in allergic reactions (Funk, 2001). Furthermore, to verify the antiallergic effect of the purified compound in vivo, the purified compound was administered to mice to examine its ability to inhibit ear swelling induced by arachidonic acid (AA), 12-O-tetradecanoylphorbol-13-acetate (TPA), and oxazolone (OXA). The mechanism of this suppressive effect was investigated by examining a number of reactions, cyclooxygenase (COX)-2 mRNA expression, cytokine mRNA expression such as interleukin (IL)-6 and tumor necrosis factor (TNF)- α , degranulation, and release of chemical mediators such as histamine, leukotrienes, and prostaglandins (PGs) (Kujubu et al., 1991; Meurer et al., 1988; Rao et al., 1993; Zachariassen et al., 2017). The strength of the antiallergic effect was compared to that of epigallocatechin gallate (EGCG), a typical natural inhibitor.

2 | MATERIALS AND METHODS

2.1 | Materials

E. nipponica samples were collected from the coast of Mugizaki in Mie Prefecture, Japan. Recently, the specific name was established from *E. arborea* growing off the coast of Japan (Kawai et al., 2020). Harvested algae were dried and ground into powder as previously outlined by Sugiura et al. (2008). EGCG (Sigma-Aldrich, St.

Louis, MO, USA) was used as a typical natural inhibitor, since it exhibits well-known antiallergic and anti-inflammatory properties (Tachibana, 2011).

2.2 | Extraction and purification

2.2.1 | Methanol/chloroform (M/C) extraction

M/C extraction and preparation of lipophilic fraction were performed according as previously reported (Sugiura et al., 2016) with slight modifications (Figure 1). First, *E. nipponica* powder was defatted using the method in Sugiura et al. (2007) to avoid contamination of lipophilic components such as pigments, then, 200 g of it was extracted using 800 ml of methanol for 3 hr on a magnetic hot plate stirrer at 50°C. Next, 1,600 ml of chloroform was added, and continuous extraction was conducted for 1 hr. After filtering the supernatant (No. 2 filter paper, Toyo Roshi Kaisha, Ltd., Tokyo, Japan), it was partitioned with 600 ml of water. The lipophilic (lower) fraction was recovered and condensed using a rotary evaporator, and the dried residue was dissolved in chloroform. The solution was retained and stored at 4°C for further analysis.

2.2.2 | Sample preparation procedures

After removing the chloroform from the lipophilic fraction, the residue was dissolved in hexane. This solution was then applied onto a CIG column (25 $\times$ 400 mm; Kusano Scientific Co., Tokyo, Japan) filled with silica gel (Wakogel C-300, Wako Pure Chemical Co., Osaka, Japan). The column was sequentially eluted with hexane, chloroform, chloroform: acetone (9:1, v/v), and finally pure acetone at a flow rate of 12 ml/min using a medium pressure pump (SSC-3471; Senshu Scientific Co., Ltd., Tokyo, Japan).

The LOX inhibiting activity was used as a screening method of antiallergic component (Chung et al., 2009; Sugiura et al., 2009), with the acetone fraction showing an obvious effect. This fraction was, therefore, purified by reversed-phase chromatography. Chromatorex octadecylsilyl (ODS) silica gel (100–200 mesh; Fuji Silysia Chemical Ltd., Aichi, Japan) was suspended in methanol and the suspension poured into a glass column (20 $\times$ 450 mm). After the column was washed with water, the acetone fraction dissolved in a small amount of chloroform: methanol (1:1, v/v) was added to the column. The column was sequentially eluted with acetonitrile: water = 1:4 (v/v), 2:3 (v/v), 3:2 (v/v), 100% acetonitrile, and then, methanol. The 2:3 (v/v) fraction showed LOX-inhibiting activity, therefore it was afforded further HPLC analysis and purification.

2.2.3 | Analytical and preparative HPLC

An HPLC system (pump, PU-980, JASCO Corporation, Tokyo, Japan; UV detector, LaChrom L-7400, Hitachi High-Tech Science

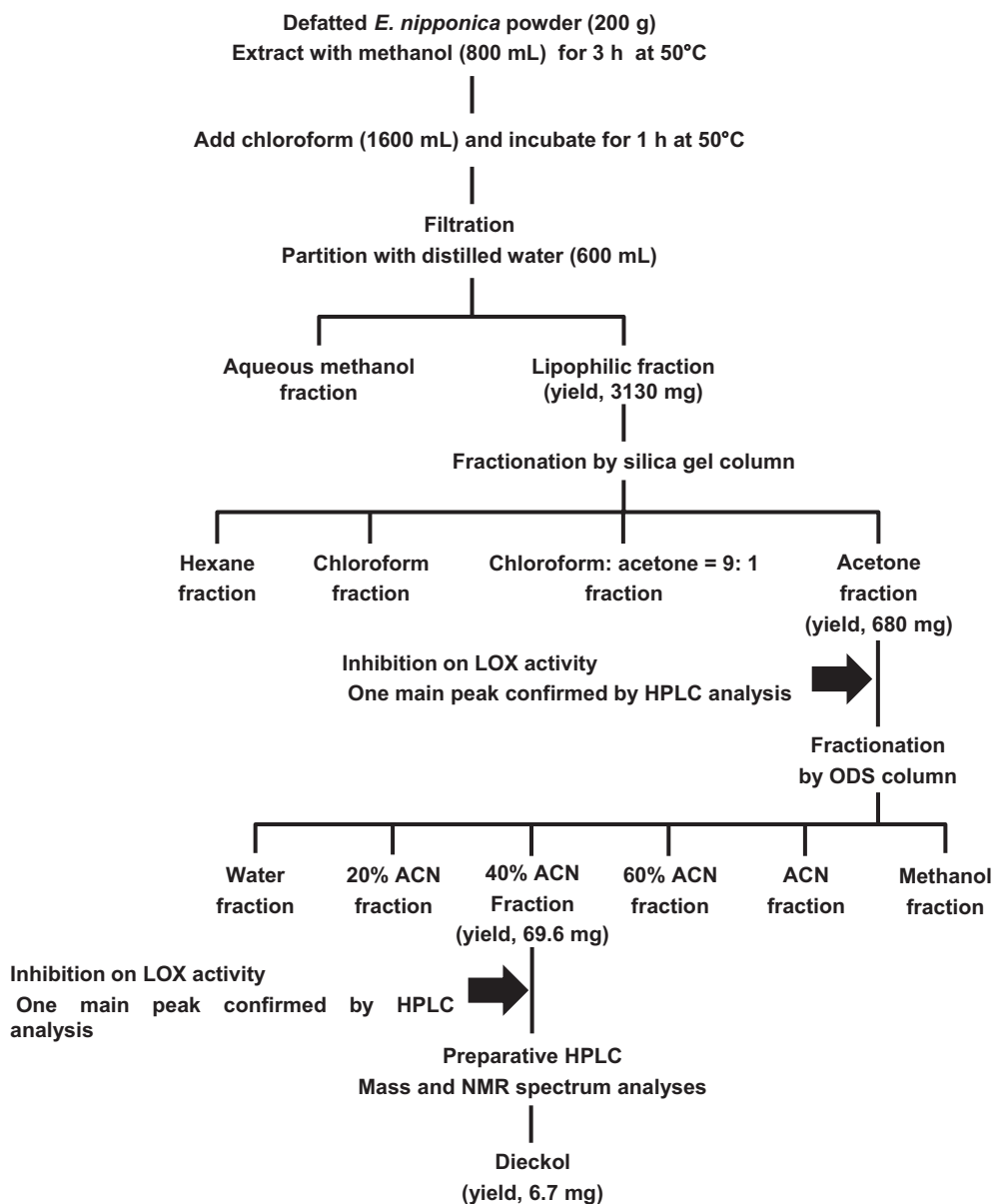


FIGURE 1 Isolation and identification of dieckol from *E. nipponica* ACN, acetonitrile

Corporation, Tokyo, Japan; data processor, D-2500 Chromato-Integrator, Hitachi High-Technologies Corporation, Tokyo, 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, 1.0 ml/min) and a preparative Develosil ODS-5 column (10 mm ID × 250 mm L, Nomura Chemical Co.; flow rate, 2.0 ml/min) were used. For analytical chromatography, a 60-min gradient program between 0.1% trifluoroacetic acid (TFA) and acetonitrile containing 0.1% TFA followed by isocratic elution with acetonitrile containing 0.1% TFA for 15 min was employed. The eluate was monitored through UV absorbance at 230 nm. For preparative chromatography, an isocratic elution with 20% acetonitrile containing 0.1% TFA was used. The eluate was monitored through UV absorbance at 280 nm, which was applied to distinguish each peak derived from the high amount of component by lowering the sensitivity.

2.2.4 | Structure analysis

^1H -NMR, ^{13}C -NMR, HSQC, and HMBC spectra were measured in CD_3OD using a JEOL NM-A-500 spectrometer (JEOL, Tokyo, Japan). Mass spectrometry (MS; electrospray ionization (ESI) mass) analyses were performed using an LC-MS system (LCMS-2010EV; Shimadzu Co., Kyoto, Japan). The ESI mass conditions were as follows: nebulizer gas, 1.5 ml/min; curved desolvation line temperature, 250°C; heat block temperature, 200°C; detector, 1.5 kV; drying gas, 0.1 MPa. The LC system was composed of the following: pump, LC-20AD; column oven, CPO-20A (temperature, 40°C); detector, SPD-20A (PDA). A separative TSKgel ODS-100V HPLC column (3 μM , 2 mm ID × 75 mm L, Tosoh Corporation, Tokyo, Japan; flow rate, 0.2 ml/min) was used. H_2O (A) and methanol (B) were employed as the mobile phase. The following elution conditions were applied: isocratic elution with 10% B for 10 min (condition 1) and gradient elution between A and B (condition

2, 10%–100% B for 3 min, maintain 100% B for 2 min). After applying of these conditions, the mobile phase was restored to condition 1.

2.3 | Anti-inflammatory effects on mouse ear swelling

2.3.1 | Animals

Institute of Cancer Research (ICR) mice were used in the ear swelling model. Four-week-old male mice were purchased from KBT Oriental Co., Ltd. (Tosu, Saga, Japan) and housed in individual cages kept at 23°C–26°C under a 12-hr light/dark cycle until experimentation. A solid AIN-93G diet (KBT Oriental) and tap water were freely available. All animal experiments were conducted with permission from 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 (approval number: 17–8; March 31, 2017).

2.3.2 | Mouse ear swelling test

Evaluations of samples' ability to suppress mouse ear swelling were performed as previously described (Sugiura et al., 2013, 2018). AA, TPA, and OXA were used as sensitizers for the induction of acute, subacute, and delayed-type allergic inflammation, respectively (Yoshino et al., 2010; Young et al., 1984). Samples were dissolved in solution and percutaneously or orally administered to the mice.

2.4 | Suppression of degranulation, chemical mediator release, and COX-2, IL-6, and TNF- α gene expression

RBL-2H3 cells were purchased from the Health Science Research Resources Bank (JCRB0023, Tokyo, Japan). Cell culture, cell stimulation, and anti-degranulation assays were conducted according to previously described methods (Sugiura et al., 2018). RBL cells were stimulated using either an antigen–antibody reaction or the calcium ionophore A23187. Since the degranulation from mast cells is proportional to β -hexosaminidase (β -Hex; equivalent to histamine) activity (Schwartz et al., 1981), the anti-degranulation assay measured the β -Hex activity. ELISA kits for leukotriene B₄ (LTB₄) and prostaglandin E₂ (PGE₂) (Cayman Chemical Co., Ann Arbor, MI, USA) or for histamine (SPI-BIO, Montigny-le-Bretonneux, France) were used to determine the released chemical mediators from RBL cells stimulated by the antigen–antibody reaction according to the manufacturer's instructions. The suppressive effects of the test samples on COX-2, IL-6, and TNF- α gene expression in RBL cells stimulated with A23187 were investigated according to the method of Matsui et al. (2013). Although it is suspected that the samples included cell

death, this was not detected using a Trypan blue exclusion assay. In the anti-degranulation assay, no inhibition of β -Hex activity by the samples was observed.

2.5 | Statistical analysis

Data are expressed as mean $\pm$ SD. Comparisons between dieckol and EGCG groups were performed using Student's *t* test. In the study on mRNA expression, multiple comparisons between groups were performed using Dunnett's multiple range test with the Excel Statistics 2016 software (Social Survey Research Information Co., Ltd., Tokyo, Japan). *p* < .05 was considered statistically significant.

3 | RESULTS

3.1 | Extraction and isolation of active component

The flow diagram for the method of extraction and purification of the active components from the alga is shown in Figure 1. The yield of the lipophilic fraction from dried *E. nipponica* powder was 3,130 mg. An acetone fraction (yield, 680 mg) obtained from the lipophilic fraction using a silica gel (Wakogel C-300) column inhibited LOX activity (inhibition ratio 76.9% at a concentration of 1.0 mg/ml) and was a main component in HPLC analysis, so it was applied to an ODS (Chromatorex) column for further fractionation. The 40% eluate from the column (yield, 69.6 mg) showed strong LOX-inhibiting activity (inhibition ratio 86.0% at a concentration of 1.0 mg/ml) and the preparative HPLC separation of the eluate yielded 6.7 mg of a pure active compound. The IC₅₀ value of the purified compound for the LOX-inhibition assay was 427.9 μ g/ml.

3.2 | Structure analysis

The structure of the isolated compound was analyzed by mass spectra, ¹H-NMR, ¹³C-NMR, HSQC, and HMBC spectra. The NMR spectral data are summarized in Table 1 and the elucidated structures are shown in Figure 2. The compound showed its [M + H]⁺ ion at M/Z = 743 in the LC-MS analysis, indicating a molecular weight of 742. Thus, the isolated compound was identified as dieckol by comparing their spectra with those reported elsewhere (Chowdhury et al., 2014).

3.3 | Antiallergic and anti-inflammatory effects of dieckol

3.3.1 | Suppression of mouse ear swelling

Percutaneous administration of the isolated compound led to suppression of ear swelling induced by any of the three sensitizers, and

TABLE 1 ^1H - and ^{13}C -NMR spectra of dieckol in CD_3OD

Position	^1H	Position	^{13}C
2', 4', 6'	5.88	1'	161.9
		2', 6	95.4
		3', 5'	160.2
		4'	97.7
		6'	95.8
3	6.09	1	124.6
6	6.05	2	147.1
8	6.02	3	99.5
		4, 5a	144.2
		4a, 4a''	154.5
		7	157.8
		8	99.8
		9, 9''	146.9
		9a	125.6
		10a	138.7
2'', 6''	6.01	1''	126.2
		2'', 6''	96.2
		3'', 5''	152.4
		4''	143.3
3''	6.11	1'', 2''	147.3
6''	5.94	3''	99.4
8''	5.91	4''	143.4
		5a''	144.3
		6''	95.9
		7''	156.0
		8''	99.9
		9a''	147.4
		10a''	138.5

this was significantly greater than that of EGCG, a natural typical inhibitor; dieckol strongly suppressed the AA-induced ear swelling by 87.0%, whereas the suppression ratio of EGCG was approximately 50%–60%. When administered orally, the suppressive effects of dieckol on ear swelling induced by AA and TPA were comparable with those of EGCG, with a ratio of approximately 15%–40%. The suppressive effect of dieckol on OXA-induced ear swelling was greater than that of EGCG. In vivo experiments showed that dieckol was more efficient than EGCG (Table 2).

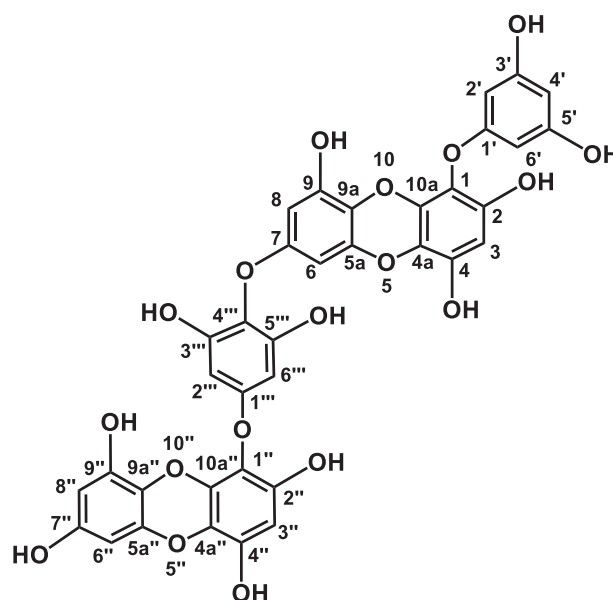
3.3.2 | Suppressive effects on reactions in RBL-2H3 cells

To investigate the suppressive mechanisms of dieckol on mouse ear swelling, we performed in vivo experiments using RBL cells. Specifically, we examined its effects on degranulation, chemical mediator release, and expression of mRNAs such as COX-2 and cytokines, which are all involved in mouse ear swelling induced by

the three sensitizers (Kujubu et al., 1991; Meurer et al., 1988; Rao et al., 1993; Zachariassen et al., 2017).

As shown in Table 3, dieckol exhibited stronger suppression of degranulation in RBL cells by antigen–antibody reaction than EGCG. Its IC_{50} value was $7.7 \pm 4.2 \mu\text{M}$, and the efficacy was approximately four times than that of EGCG. Although a suppressive effect of dieckol on degranulation in RBL cells stimulated by A23187 was observed, it was weaker than that of EGCG. Dieckol also suppressed chemical mediator release (Table 4). The suppressive effect of dieckol on LTB_4 and PGE_2 release from RBL cells stimulated by antigen–antibody reaction was comparable to EGCG. On another note, the suppressive effects of dieckol on histamine release from RBL cells were significantly greater than that of EGCG.

Using RBL cells, suppressive effects on COX-2 mRNA expression by dieckol were compared with those of EGCG. The result was that

**FIGURE 2** Chemical structure of dieckol**TABLE 2** Suppressive effect of dieckol on mouse ear swelling induced by the three sensitizers

Administration	Sensitizer	Suppression ratio (%)	
		Dieckol	EGCG
Percutaneous	AA	87.0** $\pm$ 14.6	51.2 $\pm$ 7.5
	TPA	85.8* $\pm$ 17.6	58.7 $\pm$ 10.1
	OXA	77.7** $\pm$ 11.2	54.6 $\pm$ 6.0
Oral	AA	36.6 $\pm$ 14.8	22.6 $\pm$ 1.5
	TPA	16.3 $\pm$ 4.7	26.8 $\pm$ 7.3
	OXA	40.7* $\pm$ 4.7	27.4 $\pm$ 1.9

Note: Sample dose was 150 nmol/mouse. Data were calculated from the results of quadruplicate experiments ($n = 4$). Values are presented as means $\pm$ standard deviations. Asterisks indicate more significant efficacy compared with that of the EGCG group (*, $p < .05$; **, $p < .01$). Abbreviations: AA, arachidonic acid; EGCG, epigallocatechin gallate; OXA, oxazolone; TPA, 12-O-tetradecanoylphorbol-13-acetate.

TABLE 3 Suppressive effect of dieckol on degranulation in RBL-2H3 cells

Stimulation	IC ₅₀ value (μM)	
	Dieckol	EGCG
Antigen-antibody reaction	7.7** ± 4.2	30.5 ± 2.6
A23187	90.6 ± 3.1	27.7 ± 0.3

Note: Data were calculated from the results of triplicate experiments ($n = 3$). Values are presented as means ± standard deviations. Reproducibility was confirmed through repeated runs. Asterisks indicate more significant efficacy compared with that of the EGCG group (**, $p < .01$). A23187, calcium ionophore; EGCG, epigallocatechin gallate

the suppressive activities of both substances at a concentration of 100 μM were essentially equivalent in that they both completely suppressed COX-2 mRNA expression. A difference was observed at 50 μM, wherein EGCG did not show any suppression and dieckol completely suppressed COX-2 mRNA. No suppression was observed for dieckol and EGCG at 10 μM (Figure 3). Moreover, the suppressing effects of dieckol on cytokine mRNA expression (IL-6 and TNF-α) were also compared with that of EGCG. In the case of IL-6, any concentration of both compounds tested significantly suppressed IL-6 mRNA expression and completely suppressed mRNA expression at both 50 and 100 μM. Regarding TNF-α, although only 100 μM EGCG completely suppressed mRNA expression, any tested concentration of dieckol significantly suppressed mRNA expression (Figure 4). Accordingly, the suppressive effects of dieckol on COX-2 and TNF-α mRNA expression were stronger than those of EGCG. Thus, a narrow threshold in efficacy of the samples was observed.

4 | DISCUSSION

In our previous study, the lipophilic fraction of an *E. nipponica* extract was found to exhibit antiallergic and anti-inflammatory properties (Sugiura, Usui, & Miyata, 2017). In this follow-up study, we isolated

TABLE 4 Suppressive effect of dieckol on chemical mediator release from RBL-2H3 cells stimulated by antigen-antibody reaction

Chemical mediators	IC ₅₀ value (μM)	
	Dieckol	EGCG
Histamine	31.7** ± 2.3	246.6 ± 70.2
LTB ₄	32.8 ± 1.1	48.3 ± 27.6
PGE ₂	40.4 ± 3.7	28.7 ± 0.4

Note: Data were calculated from the results of the triplicate experiments ($n = 3$). Values are presented as means ± standard deviations. Reproducibility was confirmed through repeated runs. Asterisks indicate more significant efficacy compared with that of the EGCG group (**, $p < .01$).

Abbreviations: EGCG, epigallocatechin gallate; LTB₄, leukotriene B₄; PGE₂, prostaglandin E₂.

the active component from the lipophilic fraction and examined its antiallergic effects. Preparation by silica gel and ODS column chromatography led to an acetone fraction containing the biologically active compound. Using preparative HPLC, mass and NMR spectra analyses revealed the compound to be dieckol, a phlorotannin. Dieckol has been isolated from several brown algae such as *Eisenia bicyclis*, *Ecklonia cava*, *Ecklonia kurome*, and *Ecklonia stolonifera* and is a typical phlorotannin (Fukuyama et al., 1985; Kang et al., 2003; Myung et al., 2005; Nakamura et al., 1996). Although other phlorotannins have been isolated from *E. nipponica* in the ethyl ether extract of the aqueous methanol fraction obtained through M/C extraction (Sugiura et al., 2007), a measurable amount of dieckol was not found. In the present study, dieckol was isolated from the lipophilic fraction, while in *Eisenia* or *Ecklonia* species extracts it is generally found in the water-soluble fraction. Since the defatting step might explain this difference, we compared the relative amounts of dieckol in the aqueous methanol and/or lipophilic fractions from defatted and non-defatted powder as measured by HPLC. This analysis conditions were as follows: an analytical Develosil ODS-5 column (4.6 mm ID × 150 mm L, Nomura Chemical Co.; flow rate, 0.5 ml/min) was used with an isocratic elution with 30% acetonitrile containing 0.1% TFA. The eluate was monitored with UV absorbance at 230 nm. From these analyses, we found that the amount of dieckol in the aqueous methanol fraction from non-defatted powder (829.0 μg/g powder) was significantly higher than that of lipophilic fraction (577.6 μg/g powder) ($p < .05$). However, the amount of dieckol found in the aqueous methanol fraction from defatted powder (136.6 μg/g powder) was significantly lower than that of lipophilic fraction (205.8 μg/g powder) ($p < .05$). This result could be explained by the structure of dieckol exhibiting both lipophilicity and hydrophilicity. Dieckol is more lipophilic than eckol and bieckols and may migrate to more lipophilic than aqueous methanol fractions from defatted powder due to an increase of dieckol lipophilicity from the reduction of other lipophilic compounds after defatting.

The in vivo antiallergic effect of the isolated dieckol was demonstrated in that percutaneous and oral dieckol administration suppressed ear swelling induced by three sensitizers in mice (Table 2), which corroborates other reports. Dieckol was reported to alleviate passive cutaneous anaphylactic reactions and atopic dermatitis in NC/Nga mice (Ahn et al., 2015; Yang et al., 2016), and supporting literature confirms the in vivo effects observed in the present study. Chemoattractant adhesion and migration of inflammatory leukocytes into the topical inflammatory site both lead to participate in ear swelling (Hirasawa et al., 2009). Because dieckol exhibited suppressive effects on chemoattractant reactions and cell adhesion of lymphocytes (Kang et al., 2015; Kim et al., 2012), it supports a suppressive mechanism for this drug in the animal model used here. Furthermore, there are several reports on the anti-inflammatory mechanisms of dieckol, which suppress the aforementioned in vivo allergic and inflammatory reactions. In murine BV2 microglia cells, dieckol exhibited anti-inflammatory effects by blocking the expression of inducible nitric oxide synthase, COX-2, and nuclear factor-κB proteins, nitric oxide and PGE₂ production, and the generation of

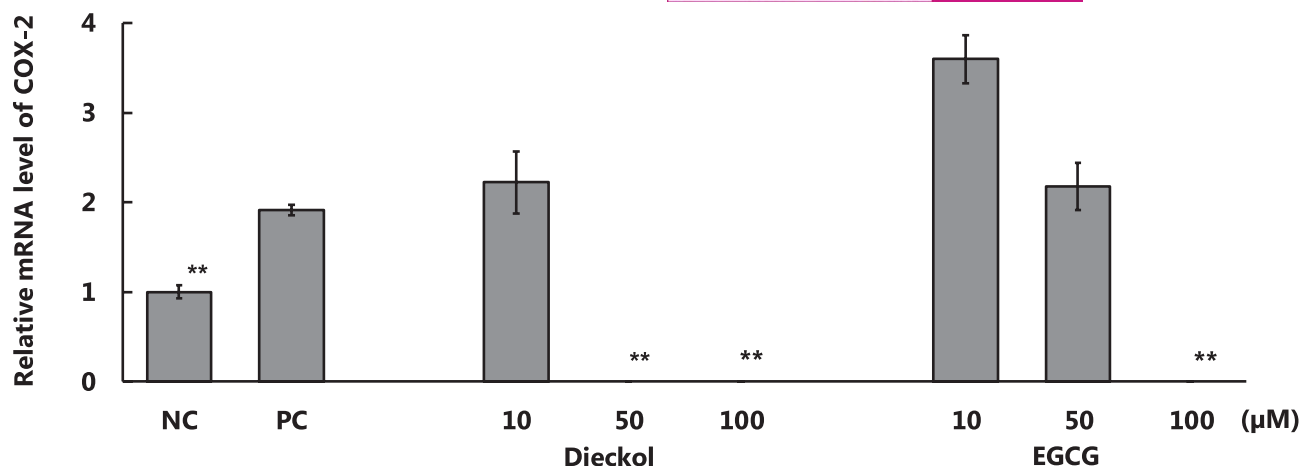


FIGURE 3 Suppressive effects of dieckol on COX-2 mRNA expression in RBL cells stimulated by A23187. Values were calculated from the results of duplicate experiments and are expressed as mean $\pm$ SD. Reproducibility was confirmed through repeated runs. Asterisks indicate that the mRNA level of the test group was significantly lower than that of PC (*, $p < .01$). NC, negative control without A23187; PC, positive control with A23187; EGCG, epigallocatechin gallate

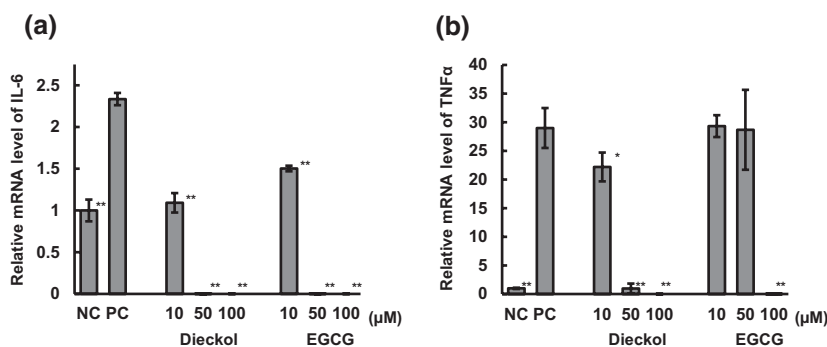


FIGURE 4 Suppressive effects of dieckol on cytokine mRNA expression in RBL cells stimulated by A23187. A, IL-6; B, TNF- α . Values were calculated from the results of duplicate experiments and are expressed as mean $\pm$ SD. Reproducibility was confirmed through repeated runs. Asterisks indicate that the mRNA level of the test group was significantly lower than that of PC (*, $p < .05$, **, $p < .01$). A23187, calcium ionophore; IL-6, interleukin-6; TNF- α , tumor necrosis factor- α ; NC, negative control without A23187; PC, positive control with A23187; EGCG, epigallocatechin gallate

pro-inflammatory cytokines in the mitogen-activated protein kinase (MAPK) cascade (Jung et al., 2009), as well as to the inhibition of enzymatic activities (phospholipase A_2 and LOX) involved in arachidonic acid (AA) cascade (Shibata et al., 2003). Since the AA cascade is related to the MAPK cascade and mRNA expression of COX-2, IL-6, and TNF- α , and all are involved in ear swelling (Hirasawa et al., 1995; Kujubu et al., 1991; Meurer et al., 1988; Rao et al., 1993; Zachariassen et al., 2017), these anti-inflammatory mechanisms would contribute to the suppression of mouse ear swelling observed in the present study (Table 2), confirmed by mRNA expression suppression of COX-2, IL-6, and TNF- α (Figures 3 and 4). Since degranulation and chemical mediator release are also involved in ear swelling (Kujubu et al., 1991; Meurer et al., 1988; Rao et al., 1993), the suppressive effect of dieckol on degranulation and chemical mediator release from RBL cells (Tables 3 and 4) can be considered as one of the suppressive mechanisms of dieckol in ear swelling. These results are confirmed by reports on the compound's suppression of PGE₂

synthesis and degranulation via the suppression of binding activity between IgE and Fc ϵ RI (Jung et al., 2009; Le et al., 2009).

In the present study, dieckol exhibited total suppression effects comparable to and/or stronger than EGCG. Since dieckol more significantly suppressed histamine release and COX-2 and TNF- α mRNA expression than EGCG, this could explain the suppression of mouse ear swelling. From the data presented in Table 3, dieckol suppressed degranulation stimulated by the antigen-antibody reaction with a low IC₅₀ value. As shown in Table 2, the suppressive effect of orally administered dieckol on ear swelling was milder than that of percutaneous administration, which may be due to decreased absorption due to passage through the intestinal tract. Nevertheless, since the suppressive effect of dieckol was comparable to and/or stronger than that of EGCG administered orally, dieckol can be said to be sufficiently efficacious. Corona et al. (2016) reported the absorption and metabolism of crude phlorotannin in a human study, finding that at 6–24 hr

following oral administration, the main metabolites—composed of phloroglucinol oligomers—were detected in plasma and urine in addition to moderate absorption of low-molecular weight phlorotannin. In the present study in mice, oral administration of dieckol suppressed mouse ear swelling induced by AA or TPA with inflammation peaking within 1 to 4 hr, comparable to EGCG. However, the suppressive effect of dieckol on OXA-induced ear swelling—perhaps due to moderate absorption of phlorotannins—reached peak inflammation 5 days later and was significantly higher than that of EGCG (Table 2). Since dieckol suppressed the ear swelling related to the delayed-type allergic reaction sensitized by OXA, it might have utility in the treatment of chronic allergic diseases such as atopic dermatitis. Our in vitro experiments demonstrated differences in the rank order of IC_{50} values while the suppression of mRNA expression was dramatic, therefore, further in vivo studies for absorption, metabolism, and suppression of chronic allergic reactions are warranted.

In the present study, EGCG was used as a positive example of a natural typical inhibitor since its antiallergic effects are well reported (Tachibana, 2011) and that it is a food-grade phenolic compound comparable to dieckol. Several pharmaceutical reagents show strong antiallergic effects such as dexamethasone and wortmannin which exhibit strong suppression of mouse ear swelling and degranulation or chemical mediator release in RBL cells, respectively (Carlson et al., 1985; Ivetić Tkalečić et al., 2012; Nigorikawa et al., 2014; Yano et al., 1993). Compared to the dose used in present study, the efficacies of these pharmaceutical reagents would be comparable to and/or higher than that of dieckol. However, because our interest is focused on the potential of dieckol as a health food ingredient with antiallergic or anti-inflammatory activity and food safety comparable to EGCG, a more moderate effect than that seen with the aforementioned pharmaceutical agent might be desirable.

In terms of structure-activity correlations, hydroxy groups in the dieckol molecule play an important role in the inflammatory reaction (Kim, Ku, Lee, et al., 2012), since the amount and locations of hydroxy groups in terrestrial polyphenols such as tea catechins and flavonoids are critical (Matsuo et al., 1997; Takano-Ishikawa et al., 2006). Therefore, the amount and locations of hydroxy groups in the dieckol molecule may explain its higher efficacy compared to EGCG. In our previous studies, we investigated the antiallergic effects of eckol and bieckols (bieckols and dieckol are dimers of eckol) that are 6, 6'-, 6, 8'-, and 8,8'-bieckol isolated from *E. nipponica* (Sugiura et al., 2007, 2013, 2017, 2018). We found that the antiallergic effects of bieckols tended to be equivalent if not stronger than those of eckol. When the present data on dieckol were compared with those of the aforementioned reports, dieckol also exhibited slightly stronger antiallergic effects than those observed with eckol. The difference between the effects between eckol and the other dimers may derive from the number of hydroxy groups present in their molecular structure. When the antiallergic efficacy of dieckol is compared to antiallergic effects of PFF-A and PFF-B derived from *E. nipponica* in previous studies (Sugiura et al., 2013,

2018), suppression of mouse ear swelling by dieckol was comparable to and/or lower than that of PFFs. Regarding the suppression of degranulation and chemical mediator release in RBL cells, dieckol was comparable to and/or stronger than PFFs. Thus, there may be another as-yet-unappreciated structure-activity correlation besides difference in hydroxy groups. Although differences are seen across phlorotannins, dieckol is expected to act as an antiallergic compound when included as a health food ingredient.

In conclusion, dieckol, a type of phlorotannin, was isolated from *E. nipponica*. In vivo experiments revealed that dieckol suppressed mouse ear swelling attributable to the suppression of degranulation, chemical mediator release (histamine, LTB_4 and PGE_2), and mRNA expression of COX-2, IL-6, and TNF- α . There are some reports of the physiological activities of dieckol such as antidiabetic effects (Lee et al., 2010), anticancer effects (Yoon et al., 2013), the chemoprevention of vascular disease (Kim et al., 2012), and anti-Alzheimer's disease effects (Kwak et al., 2015). Therefore, *E. nipponica*, including phlorotannins like dieckol, could have utility as a health food material. Although the isolation of dieckol from the alga and the antiallergic effect of the extracted compound are presented here for the first time, further studies on the bioavailability of phlorotannins and alga are needed.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTION

Yoshimasa Sugiura: Conceptualization; Data curation; Formal analysis; Investigation; Methodology; Project administration; Validation; Writing-original draft; Writing-review & editing. **Masakatsu Usui:** Data curation; Formal analysis; Investigation; Methodology; Writing-review & editing. **Hirotaka Katsuzaki:** Data curation; Formal analysis; Investigation; Methodology; Writing-review & editing. **Kunio Imai:** Data curation; Formal analysis; Investigation; Methodology; Writing-review & editing. **Ryusuke Tanaka:** Methodology; Writing-review & editing. **Teruo Matsushita:** Data curation; Methodology; Writing-review & editing. **Masaaki Miyata:** Data curation; Methodology; Supervision; Writing-review & editing.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author, Y. Sugiura, upon reasonable request.

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