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Anti-allergic Effects of Phlorotannins on Histamine Release via Binding Inhibition between IgE and FcεRI

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Two bioactive phloroglucinol derivatives, fucodiphloroethol G (**1**) and phlorofucofuroeckol A (**3**) were first isolated from *Eckolonia cava* (EC) and characterized by nuclear magnetic resonance (NMR) spectroscopic methods, along with eckol (**2**). In this study, anti-allergic activities of phloroglucinol derivatives were assessed on human basophilic leukemia (KU812) and rat basophilic leukemia (RBL-2H3) cell lines using a histamine release assay. Both compounds **1** and **3** exhibited a significant inhibitory activity against histamine release. Meanwhile, the potential inhibitory mechanism was also suggested as the suppression of binding activity between IgE and FcεRI by the cytometric analysis. These results suggested that compounds **1** and **3** could be the key effectors in the crude methanol extract of EC against allergy disease and used as novel candidates for development in the cosmetic and drug industries potentially.

KEYWORDS: Anti-allergic; *Ecklonia cava*; phlorotannins; histamine release; IgE; FcεRI

INTRODUCTION

Allergies are caused by an inappropriate reaction against innocuous environmental proteins (*1, 2*). The prevalence and severity of allergic diseases has increased dramatically during the past decade around the world, especially, affecting an estimated 20% of the population in the developed countries. The current therapies for allergic diseases focus primarily on control of symptoms and suppression of inflammation, without affecting the underlying cause. However, the knowledge about the pathophysiology of allergic diseases has substantially increased, offering new opportunities for therapeutic intervention (*3, 4*). Allergen-specific T helper (Th) cells play a pivotal role in the pathogenesis of allergic hypersensitivity reactions. These Th cells activate a complex immune reaction that triggers the release of potent mediators, such as histamine (*5*), as well as a wide variety of other inflammatory mediators and enhance the recruitment of inflammatory cells, which in turn elicit an inflammatory response that leads to the clinical symptoms of allergic disease. Among the inflammatory substances released from the effector cells, histamine remains the best characterized and most potent vasoactive mediator implicated in the acute phase of immediate hypersensitivity (*6, 7*), and was considered as a marker of degranulation. Inhibition of degranulation in these effector cells is one of a critical step in the prevention of allergic disorders. Although we are all continuously being exposed to harmless antigens, only a certain percentage of the individuals experience adverse immunological reactions to these antigens (*8*). This is due to the fact that the normal immune response to allergens is associated with the induction of tolerance. Abrogation of tolerance or failure to induce tolerance may lead to the induction and perpetuation of active immune responses. Current insights in the pathophysiology of allergy and asthma and in the molecular mechanism of activation of key target cells provide opportunities in the therapy of these diseases. Treatment with anti-IgE has been found beneficial in the treatment of asthma patients, and anti-IgE may also be effective in other allergic disorders, e.g., food allergy and rhinitis (*9*). Compounds inhibiting mast cell activation at the receptor or intracellular level are currently in clinical development (*10*), while strategies to neutralize pro-inflammatory mediators, such as TNF-α, have shown to be promising in the treatment of severe asthma patients (*11*). Chemical mediators cause various pathophysiologic events in acute allergic reactions, such as an increase in vascular permeability, induction of bronchial smooth-muscle contraction or mucus production, and neutrophil chemotaxis (*7*). Thus, it is necessary to reduce the mediator release to prevent and/or alleviate allergic symptoms.

To find novel bioactive natural products with unique structures from marine sources (*12−14*), marine algae were selected as our targets to investigate continuously. In the recent years,

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more and more natural products from marine algae were revealed in widely spectral bioactivities, including anti-cancer, anti-oxidation, anti-allergy, etc. Especially, marine red and brown algae have been shown great interest because of the potential ability to produce the variously bioactive derivatives. Among these derivatives, phlorotannins, which are a class of polymer of phloroglucinol unit usually found from brown algae, have been greatly noticed because of their broad therapeutic perspectives, exhibiting anti-plasmin inhibition (*15*) anti-diabetes (*16*), anti-oxidation (*17*), photo-chemoprevention (*18*), radiation protection (*19*), anti-cancer (*20*), anti-HIV (*21*), and anti-allergic activities (*22, 23*).

As an edible marine brown alga, *Ecklonia cava* is widely distributed at the southern coasts of Korea and Japan only. It is produced plentifully in the Jeju island of Korea (30 000 tons per year) for commercial purposes. *E. cava* is used to produce food ingredients, animal feed, fertilizers, and folk medicine in gynecopathy. In our previous report (*19*), anti-allergic activity of *E. cava* (EC) extract was evaluated by a murine asthma model. To identify the components responsible for the above activity, an ethyl acetate fraction of EC extract, which showed the highest activity in comparison to the other fractions (58% inhibition against histamine release), was purified successfully to afford 10 phloroglucinol derivatives.

In this report, the chemical structures of three bioactive phlorotannins (**1**, **2**, and **3**) were completely assigned by the nuclear magnetic resonance (NMR) techniques. Compound **1** was obtained for the first time from this genus, together with the other two known compounds (**2** and **3**), and the bioactive reports of compound **1** can be found nowhere up to now. Herein, for the first time, the anti-allergic activities of these derivatives on human and rat basophilic leukemia (KU812F and RBL-2H3) cell lines were assessed and the potential mechanism was also suggested by the binding activity between IgE and FcεRI receptor.

MATERIALS AND METHODS

Materials and Chemicals. ^{1}H NMR (400 MHz) and ^{13}C NMR (100 MHz) spectra were recorded on a JEOL JNM-ECP 400 NMR spectrometer (JEOL, Japan), using DMSO-d_6 solvent peak (2.50 ppm in ^{1}H and 39.5 ppm in ^{13}C NMR) as an internal reference standard. For some signals, the chemical shifts approximated the third decimal place. This is to distinguish between signals of very close value but which could nevertheless be clearly differentiated by visual inspection of the spectra. MS spectra were obtained on a JEOL JMS-700 spectrometer (JEOL, Japan). Extraction of EC was performed using Extraction Unit (Dongwon Scientific Co., Korea). Column chromatography was carried out by silica gel 60 (230−400 mesh, Merck, Germany), Sephadex LH-20 (Sigma, St. Louis, MO). Thin-layer chromatography (TLC) was run on precoated Merck Kieselgel 60 F_{254} plates (0.25 mm), and the spots on the TLC plate were detected under a UV lamp (254 and 365 nm) using $CHCl_3$/MeOH/H_2O/acetic acid (65:25:4:3, v/v/v/v) as a development solvent system (*24*). Vanillin−H_2SO_4 was employed as the detecting agent for phenolic compounds (*25*). All of the solvent for column chromatography was of a reagent grade from commercial sources. Dulbecco's modified Eagle's medium (DMEM), RPMI-1640 medium, trypsin−ethylenediaminetetraacetic acid (EDTA), penicillin/streptomycin/amphotericin (10 000 units/mL, 10 000 μg/mL, and 2500 μg/mL, respectively), and fetal bovine serum (FBS) were obtained from Gibco BRL, Life Technologies (Rockville, MD). Human myeloma IgE was purchased from Calbiochem (Gibbstown, NJ). Goat anti-human IgE was purchased from Gentex (San Antonio, TX). Rat anti-DNP monoclonal antibody, unconjugated IgE was purchased from Invitrogen (Carlsbad, CA). Fluorescence isothiocynate (FITC)-conjugated anti-human IgE antibody was purchased from Biosources (Burlingame, CA). All other reagents, such as calcium inonophore (A23187), hydroxyethyl piperazinylethanesulfonic acid (HEPES), L-glutamine, histamine, dimethyl sulfoxide (DMSO), and *ortho*-phthalaldehyde (OPA) were purchased from Sigma Chemicals (St. Louis, MO). The other chemicals and solvents were of analytical grade.

Culture of Cell Lines. The human leukemia cell line, KU812, and rat basophilic leukemia cell line, RBL-2H3, were obtained from the American Type Culture Collection (ATCC) (Manassas, VA). KU812 and RBL-2H3 are maintained in RPMI-1640 medium and DMEM, respectively, supplemented with 10% heat-inactivated FBS, 2 mM L-glutamine, 10 mM HEPES buffer, 100 units/mL penicillin G, and 100 mg/mL streptomycin, and cultured at 37 °C in a humidified atmosphere with 5% CO_2. Cells were subcultured every 3−4 days.

Extraction, Isolation, and Purification of Phlorotannins. The marine edible brown seaweed, EC, was collected from the Jeju island coast of Korea during the period from October 2004 to March 2005. Fresh EC was washed 3 times with water to remove salt. The lyophilized EC was ground into powder before extraction. The dried EC powder (10 kg) was extracted by stirring an extraction unit with MeOH (3 × 5 L) for 10 days. The extract (273 g) was suspended in water and partitioned with *n*-hexane (35.92 g), CH_2Cl_2 (20.49 g), EtOAc (24.87 g), and *n*-BuOH (106 g) in sequence. The EtOAc fraction (24.87 g), which exhibited a most potent anti-allergic activity on human cells, was subjected to a silica gel flash chromatography eluted with hexane/EtOAc/MeOH (gradient) to yield 10 subfractions (F1−F10). The F5 (378.39 mg) with the highest activity on anti-allergy was further purified by Sephadex LH-20 with MeOH only to afford the phlorotannins: compound **1** (102.85 mg), compound **2** (58.30 mg), and compound **3** (47.62 mg), respectively.

3-(4,5-Dimethyl-2-yl)-2,5-diphenyltetrazolium Bromide (MTT) Assay. Cytotoxic efeects of phlorotannins on KU812 and RBL-2H3 cells were measured using the MTT method. The cells were grown in 48-well plates at a density of 1×10^5 cells/well. After 24 h, cells were washed with fresh medium and were treated with different concentrations of phlorotannins. After 48 h of incubation, cells were rewashed and 40 μL of MTT (5 mg/mL) was added and incubated for 4 h. Finally, DMSO (250 μL) was added to solubilize the formazan salt formed, and amount of formazan salt was determined by measuring the OD at 540 nm using an GENios microplate reader (Tecan Austria GmbH, Austria). Relative cell viability was determined by the amount of MTT converted into formazan salt. Viability of cells was quantified as a percentage compared to the control [(OD of treated cells − OD of blank)/(OD of control − OD of blank) × 100], and dose−response curves were developed. The data were expressed as a mean from at least three independent experiments, and $p < 0.05$ was considered significant.

Histamine Release Assay. KU812 cells (5×10^6 cells/mL) were pre-incubated with human IgE (10 μg/mL) for 1 h after 30 min of incubating with different concentrations of samples. The mixture was then incubated with goat anti-human IgE in 30 min. Supernatant was collected for histamine measurement. For A23187 stimulation, KU812 and RBL-2H3 cells are incubated with 2 μM of the stimulant for 30 min after treatment with different concentrations of testing samples and the supernatant was then collected for histamine measurement.

Histamine Measurement. The histamine content was measured by a fluorometric assay, with some modifications (*26*). KU812 cells (5×10^6 cells/mL) were treated with different concentrations of phlorotannins for 30 min. The treated cells were resuspended and stimulated with antibodies or A23187, and supernatants were collected. After centrifugation, 1 N NaOH (50 μL) and 0.2% OPA (50 μL) were added into the supernatant solution (50 μL) and incubated for 5 min. The reaction was terminated by the addition of 3 N HCl (50 μL). The fluorescence intensity was measured through an excitation wavelength at 365 nm and an emission wavelength at 465 nm. The percentage histamine release was calculated as follows: histamine release (%) = (test − negative control)/(positive control − negative control) × 100. The supernatant from the nonstimulated cells was taken as the negative control, and the supernatant from the stimulated cells with anti-FcεRI antibody was the positive control.

RBL-2H3 Cell Stimulation and Assay for β-Hexosaminidase Release. The release of mast cell mediators by exocytosis was monitored using the β-hexosaminidase assay as described previously,

with some modifications (*3*). RBL-2H3 cells were plated at 5×10^5 cells/400 μL/well in 24-well culture plates and were cultured overnight with anti-DNP IgE (0.5 μg/mL, final concentration). The supernatants were removed, and the cells were washed 3 times with releasing buffer (25 mM Pipes at pH 7.2, 125 mM NaCl, 2.7 mM KC1, 5.6 mM glucose, 1 mM $CaCl_2$, and 0.1% BSA). Then, the cells were pre-incubated at 37 °C for 15 min in the presence of various concentrations of test compounds. Next, they were stimulated by the addition of 10 ng/mL DNP−BSA for 20 min. Aliquots (10 μL) of the medium and cell lysate, which was obtained by the addition of 200 μL of 0.1% Triton X-100, were incubated with 10 μL of 1 M *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide in 0.1 M sodium citrate (pH 4.5) at 37 °C for 1 h. At the end of the incubation, 250 μL of carbonate buffer containing 0.1 M Na_2CO_3 and 0.1 M $NaHCO_3$ (pH 10) was added, and then absorbance because of the formation of *p*-nitrophenol was measured at 405 nm. The net percent release of β-hexosaminidase was calculated using the following equation:

$$\text{net percent release} = [(A - C)/(B - C)] \times 100$$

where A is the amount of β-hexosaminidase in the extracellular fluid, B is the total content of β-hexosaminidase in the cells, and C is the amount of β-hexosaminidase in the extracellular fluid from the nonstimulated cells.

$$\text{inhibition (\%)} = [(1 - \text{net percent release involving test compounds})/\text{net percent release of the stimulated cells}] \times 100$$

Flow Cytometry Analysis of Binding Activity between Human IgE and FcεRI Receptor on KU812F Cell. The binding activity between human IgE and FcεRI receptor in KU812F cell was analyzed by an indirect immunofluorescence flow cytometric method. Briefly, KU812F cells (1×10^6 cells/mL) were incubated with human IgE antibody (10 μg/mL). The cell was then washed twice with ice-cold phosphate-buffered saline (PBS) and incubated with FITC-conjugated anti-human IgE antibody (2.5 μg/mL). After washing with ice-cold PBS, the cells were suspended in 1 mL of PBS and subjected to flow cytometer (Beckman Coulter Epics XL, Ramsey, MN), with a wavelength range between 475 and 525 nm. The percentage of positive cells was calculated by the arbitrary 1% cutoff channel position determined by the negative control.

Statistics. Results are presented as mean ± standard error of the mean. Student's *t* test was used to determine the level of significance.

RESULTS AND DISCUSSION

Compound **1** was isolated as an off-white powder. The molecular formula was established as $C_{24}H_{18}O_{12}$ by LREIMS, which showed a $[M + H]^+$ peak at *m/z* 499.08, in addition to ^{1}H and ^{13}C NMR and DEPT spectroscopic analyses. This molecular formula revealed 16 unsaturation degrees. The ^{1}H NMR spectrum in **1** showed 8 aromatic protons and 10 phenolic hydroxyl signals at δ 9.11 (3H, s, OH-2, 2′, 6′), 8.99 (1H, s, OH-4), 8.93 (2H, s, OH-4, 6‴), 8.95 (2H, s, OH-3″, 5″), 8.57 (1H, s, OH-2‴), and 8.47 (1H, s, OH-4‴), which were clearly supported by the ^{13}C and DEPT NMR spectra as well (see **Table 1**). All data mentioned above were found to be consistent with those reported previously. Compound **1** was completely elucidated as fucodiphloroethol G (*27*).

Compound **1** (Fucodiphloroethol G): off-white powder. ^{1}H NMR (DMSO-d_6, 400 MHz) δ: 5.83 (1H, d, J = 2.8 Hz, H-3), 5.52 (1H, d, J = 2.8 Hz, H-5), 5.84 (2H, br s, H-3′, 5′), 5.98 (1H, d, J = 2.3 Hz, H-4″), 5.85 (1H, d, J = 2.3 Hz, H-6″), 5.90 (2H, br s, H-3‴, 5‴), 9.11 (3H, s, OH-2, 2′, 6′), 8.99 (1H, s, OH-4), 8.93 (2H, s, OH-4, 6‴), 8.95 (2H, s, OH-3″, 5″), 8.57 (1H, s, OH-2‴), 8.47 (1H, s, OH-4‴). ^{13}C NMR (DMSO-d_6, 100 MHz): see **Table 1**. LREIMS *m/z*: 499.08, $[M + H]^+$ ($C_{24}H_{18}O_{12}$) (**Figure 1**).

Table 1. NMR Data for Phlorotannins in DMSO-d_6

position	δ_c (mult)[a]		
	1[b]	**2**[c]	**3**[d]
1	123.3 (s)	122.9 (s)	122.5 (s)
2	154.5 (s)	145.6 (s)	147.2 (s)
3	95.7(d)	97.9(d)	98.3 (d)
4	153.0 (s)	141.5 (s)	142.1 (s)
		122.9 (s)	122.7 (s)
5	92.3 (d)		
		142.2 (s)	134.1 (s)
6	150.3 (s)	93.6(d)	103.3 (s)
7		152.6 (s)	103.5 (s)
8		98.3 (d)	146.6 (s)
9			99.2(d)
		122.3(s)	
10			150.5 (s)
		136.8 (s)	
11			120.0 (s)
			149.6 (s)
			151.0 (s)
13			94.8(d)
14			144.9 (s)
			126.4 (s)
			137.0 (s)
1′	121.9 (s)	160.0 (s)	160.2 (s)
2′	151.1 (s)	93.5(d)	93.7(d)
3′	94.7(d)	158.4 (s)	158.8 (s)
4′	154.6 (s)	96.0(d)	96.2(d)
5′	94.7(d)	158.4 (s)	158.8 (s)
6′	151.1 (s)	93.5(d)	93.7(d)
1″	156.5 (s)		159.9 (s)
2″	101.6 (s)		93.4(d)
3″	157.2 (s)		159.0 (s)
4″	96.8(d)		96.7(d)
5″	157.8 (s)		159.0 (s)
6″	93.3 (d)		93.4(d)
1‴	101.0 (s)		
2‴	157.1 (s)		
3‴	94.7(d)		
4‴	157.2 (s)		
5‴	94.7(d)		
6‴	157.1 (s)		

[a] Recorded at 100 MHz. [b] Fucodiphloroeckol G. [c] Eckol. [d] Phlorofucofuroeckol A.

Compound **2** was isolated as a light brown powder. On the basis of the above data and values reported previously, compound **2** was assigned as a known compound, eckol (*23*) (**Figure 1**).

Compound **2** (eckol): light brown powder (lyophilized). ^{1}H NMR (DMSO-d_6, 400 MHz) δ: 9.54 (1H, s, OH-9), 9.45 (1H, s, OH-4), 9.21 (2H, s, OH-2, 7), 9.16 (2H, s, OH-3′, 5′), 6.14 (1H, s, H-3), 5.96 (1H, d, J = 2.8 Hz, H-8), 5.80 (1H, d, J = 1.7 Hz, H-4′), 5.78 (1H, d, J = 2.8 Hz, H-6), 5.72 (2H, J = 1.7 Hz, H-2′, 6′). ^{13}C NMR (DMSO-d_6, 100 MHz): see **Table 1**. LREIMS *m/z*: 373.00 $[M + H]^+$ ($C_{18}H_{12}O_9$).

Compound **3** was isolated as a light brown powder. The molecular formula was established as $C_{30}H_{18}O_{14}$, with 22 unsaturated degrees on the basis of EIMS, ^{13}C, and DEPT NMR analyses. The ^{1}H NMR spectrum in **3** showed two AB_2 systems at δ 5.82 (2H, t, J = 2.2 Hz), 5.75 (2H, d, J = 2.2 Hz), and 5.71 (2H, d, J = 2.2 Hz), three nonsubstituted aromatic resonances at δ 6.71 (1H, s), 6.42 (1H, s), and 6.29 (1H, s), and nine phenolic hydroxyl groups signals at δ 10.17 (1H, s), 9.92 (1H, s), 9.88 (1H, s), 9.48 (1H, s), 9.23 (2H, s), 9.21 (2H, s), and 8.23 (1H, s). The ^{13}C NMR signals of **3** revealed 30 signals, among these resonances, including the presence of 9 aromatic sp^2 carbons, with the remaining 21 signals assigned as O-bearing sp^2 quaternary aromatic carbons. According to the above data, together with the comparison to the those data from

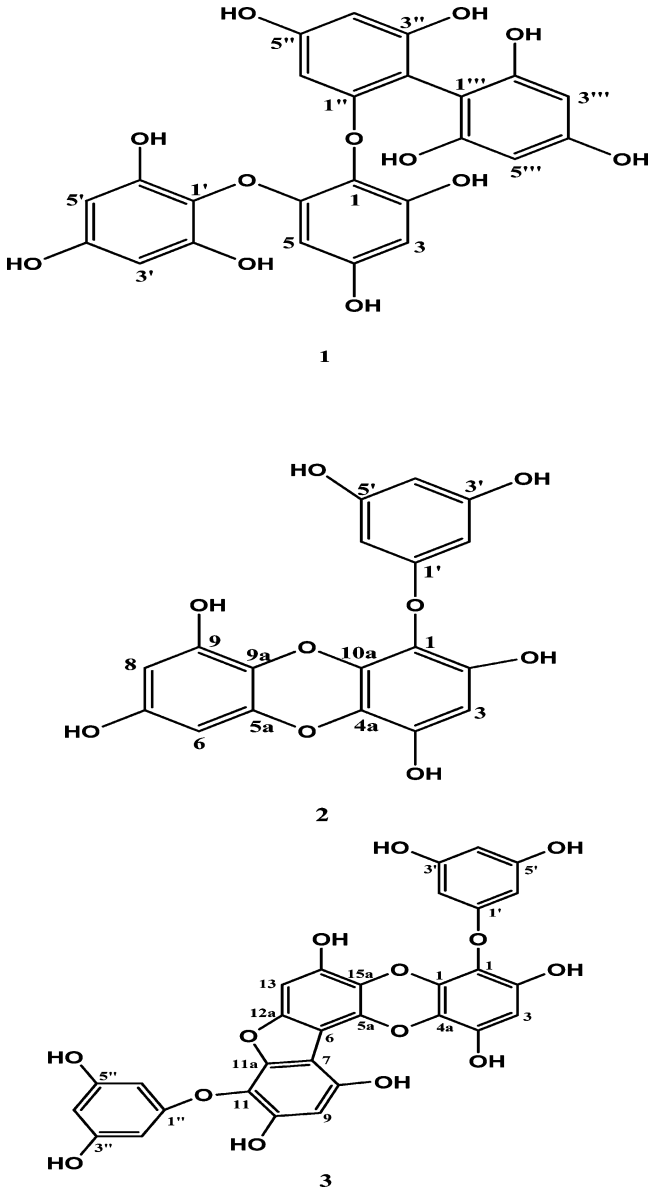

Figure 1. Chemical structures of phlorotannins isolated from *E. cava*.

literature reported, compound **3** was identified as a known phloroglucinal derivative, phlorofucofuroeckol A (*23*).

Compound **3**: light brown powder (lyophilized). ^{1}H NMR (DMSO-d_6, 400 MHz) δ: 10.17 (1H, s, OH-14), 9.92 (1H, s, OH-4), 9.88 (1H, s, OH-10), 9.48 (1H, s, OH-2), 9.23 (2H, s, OH-3″,5″), 9.21 (2H, s, OH-3′, 5′), 8.23 (1H, s, OH-8), 6.71 (1H, s, H-13), 6.42 (1H, s, H-9), 6.29 (1H, s, H-3), 5.82 (2H, t, J = 2.2 Hz, H-4′, 4″), 5.75 (2H, d, J = 2.2 Hz, H-2′, 6′), 5.71 (2H, d, J = 2.2 Hz, H-2″, 6″). ^{13}C NMR (DMSO-d_6, 100 MHz): see **Table 1**. LREIMS *m/z*: 603.12 $[M + H]^+$ ($C_{30}H_{18}O_{14}$) (**Figure 1**).

The cytotoxicity levels of phlorotannins (**1**, **2**, and **3**) were assessed using MTT assays on these two cell lines before testing of phlorotannins for their anti-allergic effects on two cell lines (KU812 and RBL-2H3), As shown in parts **A** and **B** of **Figure 2**, concentrations of the tested compounds were used up to 5 times higher than in consequent testing experiments. Phlorotannins exerted a low toxicity on testing cell lines. At a concentration of 500 μM, the lowest cell viabilities of KU812 and RBL-2H3 were 95.28 and 98.03% for compound **1**, respectively. In the other treatment, the compounds showed no significant cytotoxic effects to the testing cells.

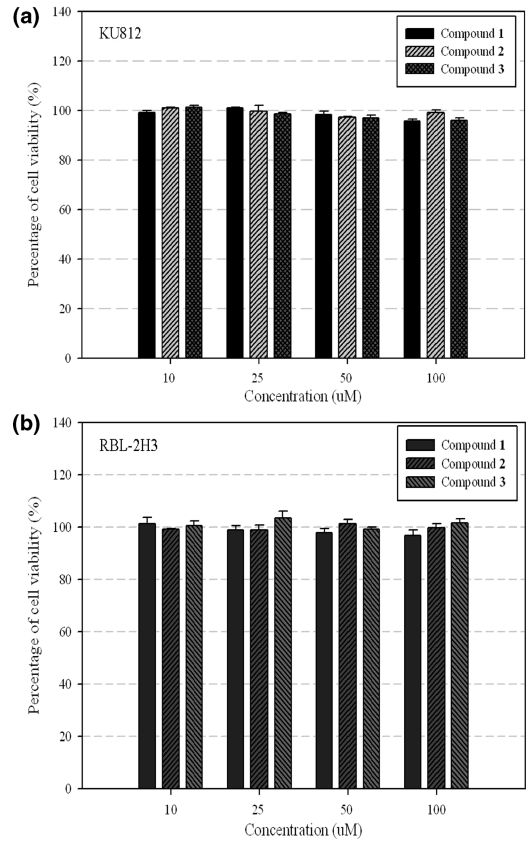

Figure 2. (A) Cytotoxicity levels of phlorotannins with different concentrations on human basophilic leukemia (KU812) assessed by MTT assays. Cells were cultured in RPMI-1640-free FBS medium and incubated for 24 h after treatment. Each value is the average of triplicate cultures, and each bar indicates mean ± standard deviation (SD). (B) Cytotoxicity levels of phlorotannins with different concentrations in rat basophilic leukemia (RBL-2H3) assessed by MTT assays. Cells were cultured in DMEM-free FBS medium and incubated for 24 h after treatment. Each value is the average of triplicate cultures, and each bar indicates mean ± SD.

The effects of phlorotannins (**1**, **2**, and **3**) on histamine release stimulated by antibodies were assessed by the inhibition of the compounds on the degranulation of KU812 cells stimulated by IgE and anti-IgE antibodies. KU812 cells (1×10^6 cells/mL) were pre-incubated with human IgE (10 μg/mL) for 1 h after 30 min of incubating with different concentration of samples. The mixture was then incubated with goat anti-human IgE in 30 min. Histamine content in the supernatant was measured by a fluorometic method. As shown in **Figure 3A**, at the highest concentration (100 μM) of compounds **1**, **2**, and **3**, the relative levels of histamine release in KU812 cell were 23.97, 44.26, and 34.54%, respectively.

The inhibitory effects of phlorotannins (**1**, **2**, and **3**) on the degranulation of KU812 cell mediated by calcium ionophore (A23187) were assessed. KU812 cells were incubated with 2 μM A23187 for 30 min after treatment with different concentrations of samples. Histamine content in the supernatant was measure by a fluorometic method. As shown in **Figure 3B**, at the highest concentration of compounds **1**, **2**, and **3**, the relative levels of histamine release in KU812 cells were 27.40, 61.15, and 37.05%, respectively.

In this experiment, we assessed the inhibitory effects of phlorotannins (**1**, **2**, and **3**) on degranulation in RBL-2H3 cells mediated by calcium ionophore (A23187). RBL-2H3 cells were incubated with 2 μM of A23187 for 30 min after treatment with different concentrations of samples. As shown in **Figure 3C**,

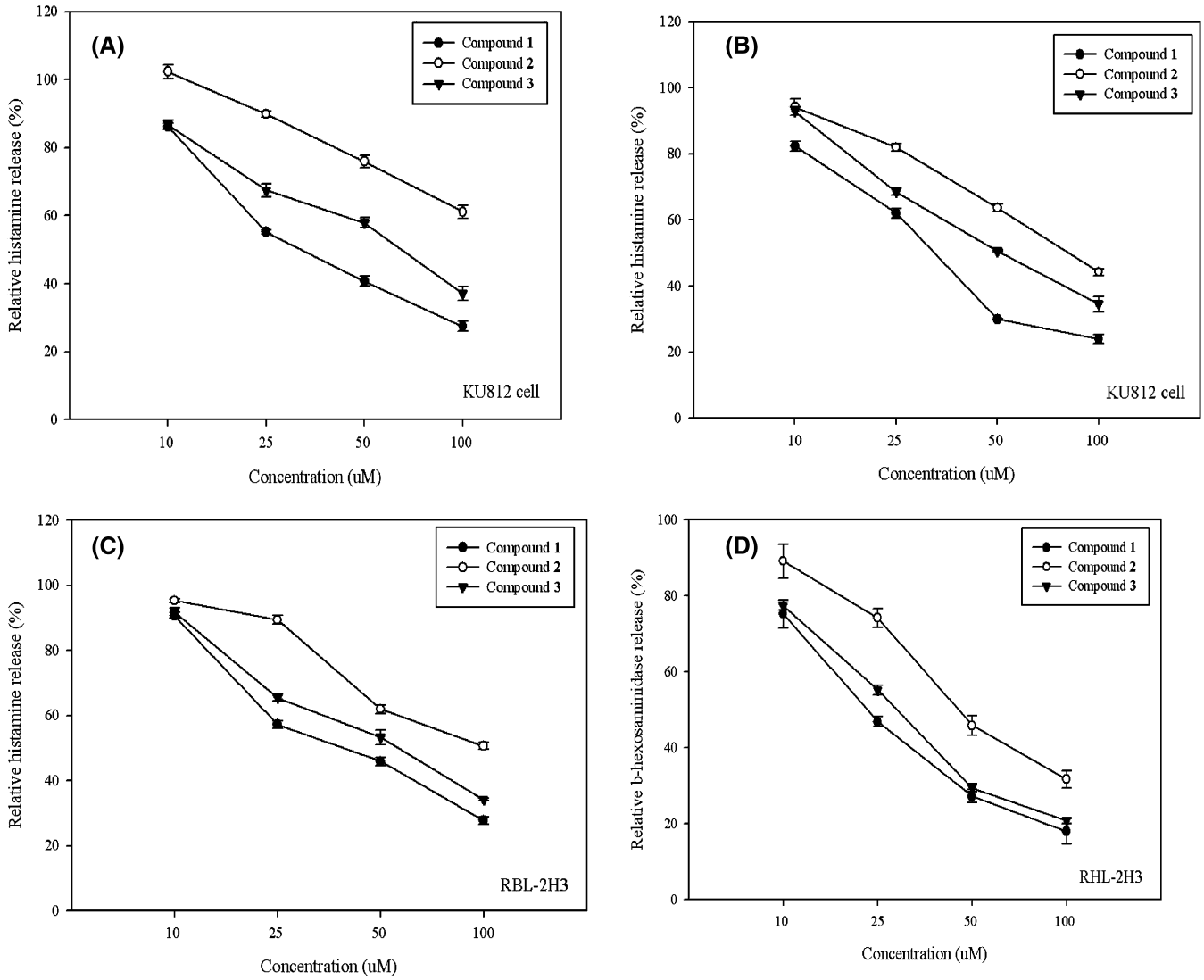

Figure 3. (A) Effects of phlorotannins on FcεRI-sensitized histamine release in KU812 cell. KU812 cells were incubated in the presence of compounds with different concentrations (0, 12.5, 25, 50, and 100 μM) for 30 min. Then, KU812F cells were stimulated by human IgE antibody for 1 h. Histamine release was triggered by incubating with anti-human IgE antibody for 30 min. Histamine contents were determined using a fluorometric method. Each value is the average of triplicate cultures, and each error bar indicates the SEM ($n = 3$). Significant difference is from the control at $p < 0.05$ (Student t test). (B) Effects of phlorotannins on A23187-sensitized histamine release in KU812 cell. KU812 cells were incubated in the presence of testing compounds with different concentrations (0, 12.5, 25, 50, and 100 μM) for 30 min. Then, KU812F cells were stimulated by A23187 (2 μM) for 30 min. Histamine contents were determined using a fluorometric method. Each value is the average of triplicate cultures, and each error bar indicates the SEM ($n = 3$). Significant difference is from the control at $p < 0.05$, respectively (Student t test). (C) Effects of phlorotannins on A23187-sensitized histamine release in RBL-2H3. The cells were incubated in the presence of testing compounds with different concentrations (0, 12.5, 25, 50, and 100 μM) for 30 min. Then, RBL-2H3 cells were stimulated by A23187 (2 μM) in 30 min. Histamine contents were determined using a fluorometric method. Each value is the average of triplicate cultures, and each error bar indicates the SEM ($n = 3$). Significant difference is from the control at $p < 0.05$ (Student t test). (D) Effects of phlorotannins on FcεRI-sensitized histamine release in RBL-2H3. The cells were incubated in the presence of testing compounds with different concentrations (0, 12.5, 25, 50, and 100 μM) for 30 min. Then, RBL-2H3 cells were stimulated by rat IgE antibody in 1 h. Histamine release was triggered by adding 0.5 μM DNP−BSA followed by 30 min of incubation. β-Hexosaminidase contents were also determined using a fluorometric assay. Each value is the average of triplicate cultures, and each error bar indicates the SEM ($n = 3$). Significant difference is from the control at $p < 0.05$ (Student t test).

at the highest concentration of compounds **1**, **2**, and **3**, the relative levels of histamine release in RBL-2H3 cells were 27.73, 50.63, and 34.18%, respectively.

The inhibitory effects of phlorotannins (**1**, **2**, and **3**) on degranulation of RBL-2H3 cells mediated by IgE stimulation via a granular enzyme (β-hexosaminidase) were evaluated. RBL-2H3 cells were incubated with 0.5 μg/mL of IgE antibody for 1 h after treatment with different concentrations of samples. Degranulation of the cells was triggered by adding DNP−BSA to the plate. As shown in **Figure 3D**, at the highest concentration of compounds **1**, **2**, and **3**, the relative levels of β-hexosaminidase release in KU812 cell were 18.02, 31.67, and 20.80%, respectively.

In this experiment, binding activity between IgE and FcεRI receptor in KU812 cells was analyzed by an indirect fluorescent flow cytometric method. As shown in parts **A**−**C** of **Figure 4**, at the highest concentration, compound **1** showed the strongest inhibition on binding number at 30.58%, followed by compound **3** of 34.23% and compound **2**of 47.60%, compared to the blank treatment. The percentage of positive cells in the staining pattern

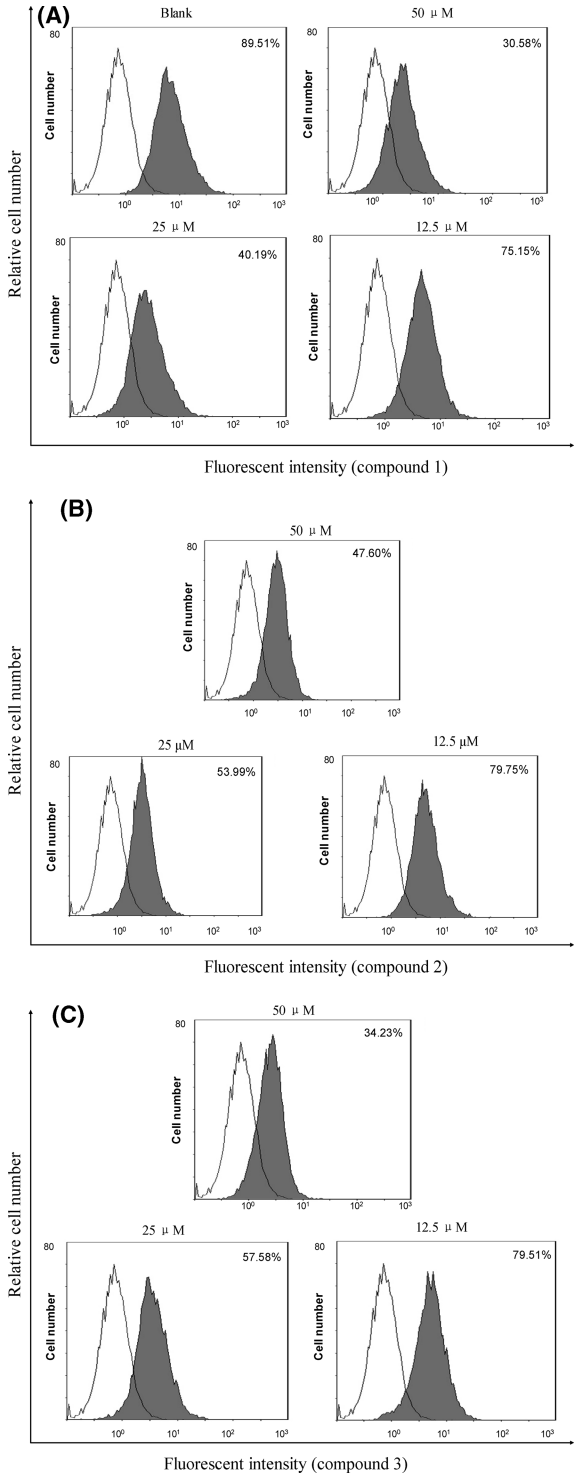

Figure 4. Effects of phlorotannins on binding activity between IgE and FcεRI on the KU812 cell surface. The percentage of binding between IgE and FcεRI by treating with compounds **1**, **2**, and **3** was displayed in A, B, and C, respectively. The cells were incubated with different concentrations of testing samples and human IgE (10 μg/mL), followed by incubation with FITC-conjugated anti-human IgE antibody, and subjected to flow cytometer (Beckman Coulter Epics XL, Ramsey, MN), with a wavelength range between 475 and 525 nm. In the graphs, the white curve regions represent the relative cell number and fluorescent intensity before treating IgE and tested compounds and the gray curve regions represent the relative cell number and fluorescent intensity after treating IgE for blank and treating IgE and tested compounds at different concentrations for sample measurements.

Table 2. Inhibition Activity of Phlorotannins on Degranulation

cell lines and stimulant	compound (IC_{50}, μM)		
	1	**2**	**3**
KU812 + human IgE	44.20	83.84	65.81
KU812 + A23187	49.61	>100	69.44
RBL-2H3 + rat IgE	31.65	62.35	38.87
RBL-2H3 + A23187	55.12	92.85	64.06

was calculated with the arbitrary 1% cutoff channel position determined by the negative control.

There was no significant cytotoxic effect of phlorotannins on testing cell lines as shown in parts **A** and **B** of **Figure 2**. This result supported that the sequent experiments may not be influenced by the toxic effects of the tested phlorotannins (**1**, **2**, and **3**). Therefore, the highest concentrations of these phlorotannins (100 μM) can be safely used to evaluate the inhibitory effects on human basophilic leukemia (KU812) and rat basophilic leukemia (RBL-2H3), which belong to mast cell lines.

At the first look, the more active compounds **1** and **3** showed the most strongly and dose-dependent inhibition of histamine release in both cell lines (KU812 and RBL-2H3) and both kinds of stimulants (IgE and anti-IgE; A23187). In the antibody-stimulated treatment, compound **1**, fucodiphloroethol G, showed the highest inhibitory activity. The IC_{50} values against histamine release were 31.65 μM on RBL-2H3 cells and 44.20 μM on KU812 cells, respectively. Compound **3**, phlorofucofuroeckol A, exerted higher effective inhibition on the degranulation level (IC_{50} = 38.87 μM) of RBL-2H3 cells and on KU812 cells at IC_{50} = 65.81 μM (**Table 2**). In both cases, the active compounds in the antibody-stimulated case showed a stronger degranulation inhibition compared to A23187 treatments, as shown as parts **A** and **C** of **Figure 3**. This evidence, on one hand, suggested that the active compounds may be useful for the prevention of allergen−IgE-induced allergic reactions. On the other hand, the result also proposed multifunctional inhibitory effects of phlorotannins on histamine release.

In all cases, compound **2**, which has the less hydroxyl function groups, showed less inhibitory effect on histamine release on both cell lines. Compound **1**, which has 11 hydroxyl functional groups, exhibited a significant inhibitory effect comparable to the active compound (*3*). Compounds **1** and **3** showed stronger activity, indicating that the molecular construction and numbers of phenol groups could be an important factor for exerting their activities (*23*). The same evidence was also reported with apple polyphenols (*28*).

Calcium ionophore mediated histamine release by selectively increasing intracellular Ca^{2+} (*29*), which in turn caused the degranulation in mast cells and basophils (*30, 31*). This increase may be correlated with the increase of cell-membrane permeability (*32*). In our present study, the isolated phloroglucinol compounds were able to inhibit histamine release stimulated by A23187 in both cell lines. A possible explanation for this phenomenon is that pholorotannins might stabilize the cell membrane, thus alleviated the increase of intracellular Ca^{2+} levels correlated with the decrease of histamine release from the cell. The interaction between cathecins and polyphenolic compounds from green tea was also reported (*33*). It was reported that cathecins from green tea were able to inhibit membrane leakage and make the membrane structure tight (*34*).

The inhibitory effects of these phlorotannins on degranulation by antibody stimulation also indicated other possible mechanisms. It was supported by the inhibition mechanism of tea

cathecins well-investigated previously. Epigallocatechin gallate (EGCg) inhibited tyrosine phosphorylation of the protein kinase involved in RBL-2H3 degranulation (*35*). EGCg also suppressed the expression of FcεRI in KU812F cells (*36*). It was also reported that phlorotannins exibited an inhibitory effect on the hyaluronidase enzyme (*37*). The suggested mechanisms may be responsible for the inhibitory effects of phlorotannins on histamine release.

Moreover, flow cytometric analysis offered strong evidence that compounds **1**, **2**, and **3** were able to suppress the binding between IgE and FcεRI receptor in a dose-dependent manner (**Figure 4**). Cross-linking of FcεRI induced by the complex formation of IgE with allergens is a critical step in the IgE-mediated allergic reaction pathway; therefore, inhibition by binding between IgE and FcεRI receptor has been a target for developing anti-allergic drugs. Previous reports indicated that monoclonal antibodies specific for IgE are an effective adjunct therapy for asthma (*37*). There is also evidence that showed that binding of monomeric IgE to FcεRI receptor in mast cell and basophil not only stimulates the survival and proliferation of nonstimulated cells (*38*) but also increases the expression of FcεRI receptor on the cell surface (*39*). On the one hand, compounds **1** and **3** showed a strong suppression on binding activity between IgE and its receptor (30.58 and 34.23%) at the highest concentration (parts **A** and **C** of **Figure 4**), while compound **2**, eckol, which possesses the less functional groups and molecular weight, showed a lower inhibitory effect (47.60%, **Figure 4B**), indicating that the diverse structural construction with more less hydroxyl functional groups plays an important role in inhibiting the binding of IgE and its receptors. In a previous study, tea polyphenol, (−)-epigallocatechin-3-*O*-gallate, inhibited mast cell RBL-2H3 degranulation via binding to the lipid raft 67RL on the cell surface (*36*). Other research revealed that inhibition of histamine release from RBL-2H3 cells by EGCg was mediated by inhibiting tyrosine phosphorylation of proteins induced by antigen and A23187 (*35*). Inhibition of the intracellular Ca^{2+} level elevated via FcεRI activation is also a possible mechanism.

Conclusively, according to the present report phlorotannins (**1**, **2**, and **3**) isolated from brown algae, *E. cava*, exhibited anti-allergic activities. Compounds **1** (fucodiphloroethol G) and **3** (phlorofucofuroeckol A) showed the strongest inhibitory effects. It could be one of the reasons that *E. cava* is used as folk medicine against allergy disease in Korea and Japan. In addition, on the basis of chemistry studies, it should be much clear to understand the key role of phlorotannins as potential candidates for treating this kind of diseases as well. Furthermore, the structural differences among these phlorotannins lead to different inhibitory effects not only because of the number of phenolic hydroxyl groups but also the linkages of phenol units. This study revealed one possible mechanism of degranulation inhibition: the tested compounds suppress the binding between IgE and FcεRI receptor. Therefore, it could be suggested that compounds **1** and **3** can be the potential candidates for pharmaceutical, food industry, and cosmetic use after more investigation.

ABBREVIATIONS USED

EC, *E. cava*; KU812, human basophilic leukemia cell line; RBL-2H3, rat basophilic leukemia cell line; Th, T helper cell line; HIV, human immunodeficiency virus; TLC, thin-layer chromatography; DMSO, dimethyl sulfoxide; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; FITC, fluorescence isothiocynate; FcεRI, high-affinity immunoglobulin E receptor; HEPES, hydroxyethyl piperazinylethanesulfonic acid; OPA, *ortho*-phthalaldehyde; ATCC, American Type Culture Collection; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; OD, optical density; EGCg, epigallocatechin gallate.

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