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Inhibitory Activity of *Citrus Madurensis* Ripe Fruits Extract on Antigen-induced Degranulation in RBL-2H3 Cells

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Abstract

The purpose of this study was to search edible ripe *Citrus* fruits which are applicable for functional food materials as juice, tea and/or jam with sweet taste and rich aroma. A fifty percent ethanolic extract (CMR-ext) obtained from the edible ripe fruit of *Citrus madurensis* exhibited an inhibitory activity of antigen-induced degranulation in anti-dinitrophenyl (DNP) IgE antibody sensitized rat basophilic leukemia (RBL) -2H3 cells. The inhibitory effect of the CMR-ext on degranulation in RBL-2H3 cells was attributable to 3',5'-di-C-*β*-glucopyranosylphloretin (**1**) which is a constituent of *C. madurensis*. The effect of **1** on Akt and mitogen-activated protein kinases (MAPK) phosphorylation was examined in RBL-2H3 cells. Western blot analysis revealed that **1** (50 μM) inhibited the degranulation by suppression of Akt and p38 phosphorylation.

Keywords: *Citrus madurensis*, 3',5'-di-C-*β*-glucopyranosylphloretin, degranulation inhibition

1. Introduction

Our previous studies (Kubo et al., 1989, Matsuda et al., 1991, Itoh et al., 2009) on *Citrus* fruit have indicated that the extracts of unripe fruits of *C. unshiu* MARKOVICH and *C. hassaku* HORT ex. T. TANAKA showed potent anti-allergic and melanogenesis inhibitory activities, whereas ripe *Citrus* fruit extracts of them had poor activities. It was found that the active constituents of these unripe *Citrus* fruit extracts were several flavonoids, such as hesperidin and narirutin from *C. unshiu* fruit and naringin and neohesperidin from *C. hassaku* fruit. These findings suggested that these unripe *Citrus* fruit may be useful ingredients for anti-allergic agents and/or skin-whitening cosmetics. Recently, on the basis of our investigations, (Fujita et al., 2008, Itoh et al., 2009, Murata et al., 2013, Futamura et al., 2016) several products originated from some unripe *Citrus* fruits are launched in the functional food market with expectation for anti-allergic and tyrosinase inhibitory activities. However, unripe *Citrus* fruits have bitter taste and aren't edible for functional food with sweet taste. On the other hand, there is another market for edible functional food as juice, tea and/or jam with sweet taste and/or rich aroma. Therefore, the purpose of this study was to search edible ripe *Citrus* fruit, which are applicable for functional food materials with sweet taste. Our previous chemotaxonomic report on several *Citrus* fruits indicated that fruit of *C. madurensis* LOUREIRO scarcely contain above mentioned flavanone glycosides (Kubo et al., 2004). Ogawa et al. (2001) reported peels, juice sacs and leaves of *C. madurensis* contain 3',5'-di-C-*β*-glucopyranosylphloretin (**1**). Since *C. madurensis* is a perpetual *Citrus* breed which gives ripe and unripe fruit together, the plant has the advantage of being collectable ripe fruit throughout the year. Thus, we focused on ripe *C. madurensis* fruits.

RBL-2H3 cells originated from rat basophilic leukemia (RBL) have been frequently used to evaluate inhibitory activity of different compounds on type I allergic reaction, and to study IgE-Fcε receptor interactions in relation to intracellular signaling pathways in the process of degranulation (Ortega et al., 1988, Ikawati et al., 2001, Funaba et al., 2003, Choi et al., 2012, Murata et al., 2013). When granules in mast cells or basophils degranulate, an enzyme, *β*-hexosaminidase, is released along with histamine. Therefore, the enzyme is commonly used as the marker of mast cell degranulation or histamine release (Cheong et al., 1998). In fact, several constituents with

type I allergic inhibitory activity have been isolated from several natural resources, such as *Mentha* × *piperita* LINNE var. *citrata* BRIQ leaves (Sato & Tamura, 2015), *Coix lachryma-jobi* LINNE var. *ma-yuen* STAPF bran (Chen et al., 2012), *Arachis hypogaea* LINNE skins (Tomochika et al., 2011) and *Caesalpinia sappan* LINNE root and heartwood (Yodsaoue et al., 2009) by using degranulation inhibitory activity assay in RBL-2H3 cells. In the present work, we evaluate the degranulation inhibitory activity of 50% ethanolic extract of edible ripe fruit of *C. madurensis* (CMR-ext) and **1** isolated from CMR-ext by use of RBL-2H3 cells. In several reports (Mastuda et al., 2002, Murata et al., 2013) concerning with anti-allergic constituent of plant resources, it was noticed that a flavonoid, baicalein, exhibited anti-degranulation activity. Thus we used baicalein as a positive control agent.

2. Materials and Methods

2.1 Reagents

Monoclonal mouse IgE anti-dinitrophenyl (anti-DNP IgE) was purchased from Yamasa Corporation (Tokyo, Japan). DNP-labeled human serum albumin (DNP-HSA) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Fetal bovine serum (FBS) was purchased from Nichirei Bioscience (Tokyo, Japan). All primary antibodies were obtained from Cell Signaling Technology (Danvers, MA, USA), including Extracellular signal Regulated Kinase (ERK) (#4695), P-ERK (#4376), p38 (#9212), P-p38 (#9215), Akt (#4691), P-Akt (#2965) and *β*-actin (#4967). A horseradish peroxidase- labeled secondary antibody (anti-rabbit IgG HRP linked whole antibody, NA934-1ML) and chemiluminescent (ECL) kit were obtained from GE Healthcare (Tokyo, Japan). Baicalein was obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Other chemical and biochemical reagents were of reagent grade and were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan) and/or Nacalai Tesque, Inc. (Kyoto, Japan) unless otherwise noted.

2.2 Plant Materials and Extraction

Fruits of *C. madurensis* (cv. Shikikitsu in Japanese) were collected in the Experimental Farm, Kindai University (34° 2′ N, 135° 11′ E, 17 m ASL), located in Wakayama Prefecture, Japan in April, 2012. The *C. madurensis* trees are grown in the ground for the purpose of the genetic resources preservation. Ripe and unripe fruits were collected from two trees which were propagated by grafting (the height of trees, 2.5 m; canopy width, 3.6 m; the age of trees, 40 years old; the life span of trees, 50-70 years). The data of cultivation environment are as follows: annual mean temperature, 17.6 ℃; maximum temperature, 34.5 ℃ and 28.8 ℃ (soil); minimum temperature, -0.1 ℃ and 8.3 ℃ (soil); annual rainfall, 2,283 mm/year. The collected fruits were visually classified by the color of fruit; i.e., fruits with whole yellow and whole green appearance were defined as ripe and unripe fruits, respectively (Figure 1). Physical data of ripe and unripe fruits (n = 20) was as follows: diameters of fruits; 34.6 ± 4.3 mm (ripe fruits), 24.6 ± 3.6 mm (unripe fruits), fresh weight of fruits; 14.9 ± 5.5 g (ripe fruits), 7.2 ± 2.4 g (unripe fruits). The samples were identified by the Experimental Farm, Kindai University, air-dried at 50 ℃ for 72 h in an automatic air-drying apparatus (Vianove Inc., Tokyo, Japan), and powdered. Voucher specimens of ripe and unripe fruits (Shikikitsu Ripe Fruits: CMR201204 and Shikikitsu Unripe Fruits: CMU201204) are deposited in the Experimental Farm, Kindai University. The each fruits powder (10 g) was extracted with 50% ethanol (EtOH) (100 ml) for 2 h under reflux. The extract was evaporated under reduced pressure and then lyophilized to give the 50% EtOH extract of ripe fruits (CMR-ext) in 46.7% yield. The yield of 50% EtOH extract of unripe fruits was 30.8%.

2.3 Anti-degranulation Activity

Anti-degranulation activity was examined according to the method of Murata et al. (2013). RBL-2H3 cells (Japan Health Sciences Foundation, Osaka, Japan) were cultured in Enhanced Eagle's Minimum Essential Medium (EMEM) supplemented with 10% FBS and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin) in a CO_2 incubator (37 ℃, 5% CO_2). Cells were inoculated in a 24-well plate at 2×10^5 cells/ well with 400 μl of culture medium. After 24 h incubation in a CO_2 incubator, 100 μl of anti-DNP IgE dissolved with EMEM (final concentration 0.45 μg/ml) was added to each well to start cell sensitization, followed by incubation in a CO_2 incubator for 24 h. Cells were washed twice with 500 μl of siraganian buffer {NaCl 119 mM, KCl 5 mM, $MgCl_2$ 0.4 mM, piperazine-1,4-bis(2-ethanesulfonic acid) (PIPES) 25 mM, NaOH 40 mM; pH 7.2} and 180 μl of siraganian (+) buffer (siraganian buffer supplemented with 5.6 mM glucose, 0.1% BSA and 1 mM calcium chloride) was added.

The test sample was dissolved with dimethyl sulfoxide (DMSO) and diluted with siraganian (+) buffer to a final DMSO concentration of 0.1% v/v. In the control group, DMSO solution diluted with siraganian (+) buffer to 0.1% of final concentration was used instead of the sample solution. An aliquot of 20 μl of test solution were added to each well, and then incubated for 0.5 h in a CO_2 incubator. Degranulation was induced by adding 50 μl of DNP-HSA dissolved with siraganian (+) buffer (final concentration 0.01 μg/ml), followed by incubation in a

CO_2 incubator for 0.5 h. A portion of the supernatant (50 μl) was transferred to a 96-well plate and 50 μl of *β*-hexosaminidase substrate, *p*-nitrophenyl-*N*-acetyl-*β*-D-glucosamide dissolved with 0.1 M citric acid aqueous solution (final concentration 0.5 mM), was added. After incubation in a CO_2 incubator for 1 h, 100 μl of alkaline buffer (0.05 M $NaHCO_3$ and 0.05 M Na_2CO_3, pH 10) was added to terminate the reaction, and absorbance at 405 nm was measured using a microplate reader (Sunrise Rainbow Thermo, Tecan Japan Co., Ltd., Kanagawa, Japan). Baicalein was used as a positive control agent.

To evaluate the cytotoxic effect of test sample against RBL-2H3 cells, the cell viability was determined by a 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2*H*-tetrazolium (WST-8) assay using a commercial kit (Cell count reagent SF). RBL-2H3 cells (2.2×10^3 cells/well) were exposed to control or the sample solutions in 96-well plates for 48 h. DMSO solution diluted with siraganian (+) buffer (0.1%, v/v) served as the solvent control. After control or the sample solutions were exposed, 10 μl of Tetra Color ONE solution was added to each well, and the 96-well plate was continuously incubated at 37 ℃ for 4 h, then the OD values for each well were measured at wavelength 450 nm using a microplate reader.

The inhibition (%) of the release of *β*-hexosaminidase by the test samples was calculated by the following equation,

$$\text{Inhibition (\%)} = [1-(T-B-N)/(C-N)] \times 100$$

Control (C): DNP-BSA (+), test sample (-); Test (T): DNP-BSA (+), test sample (+); Blank (B): DNP-BSA (-), test sample (+); Normal (N): DNP-BSA (-), test sample (-).

*2.4 Isolation and Identification Procedure for **1***

The following isolation procedure for **1** was carried out according to the methods of Ogawa et al. and Sato et al. (2001, 2006). A suspension of CMR-ext (100 g) in water (500 ml) was extracted successively with hexane (500 ml × 2), ethyl acetate (AcOEt, 500 ml × 5), and butanol (BuOH, 500 ml × 5). Evaporation of the solvent gave a hexane-soluble fraction (0.07 g; yield from CMR-ext: 0.07%), an AcOEt-soluble fraction (2.6 g; 2.6%), and a BuOH-soluble fraction (26.4 g; 26.4%). The aqueous layer was evaporated under reduced pressure and then lyophilized to give a water-soluble fraction (70.8 g; 70.8%). A part of the BuOH-soluble fraction (25.3 g) was loaded on a Diaion HP-20 (6.5 × 27 cm, Mitsubishi Chemical) column. Elution with MeOH and water in increasing proportions monitored with TLC [Merck No. 1.15685 silica gel 60 RP-18 $F_{254}S$, water: MeOH (1:1, v/v), detection; UV and 10% H_2SO_4 followed with heating] gave 22 chromatographic fractions of 500 ml each. TLC analysis of the collected fractions allowed us to assemble them into three fractions (Fr. 1 to 3). Fr. 1 [fr.1 to 6, elution solvent; water / MeOH (1:1), yield; 18.0 g], Fr. 2 [fr. 7 to 17, water to water / MeOH (1:1), 4.6 g], and Fr. 3 [fr. 18 to 22, water/MeOH (1:1) to MeOH， 1.1 g]. Fr. 2 (4.5 g) was submitted to column chromatography over 225 g of silica gel (Merck No. 1.09385 silica gel 60, 4.6 × 27 cm). Elution with chloroform ($CHCl_3$) and MeOH in increasing proportions monitored with TLC [Merck No. 1.05715 silica gel 60 F_{254}, $CHCl_3$/MeOH/H_2O (6:4:1, v/v), detection; UV and 10% H_2SO_4 followed with heating] gave 21 chromatographic fractions of 450 ml of each. TLC analysis of the collected fractions allowed us to assemble them into five fractions (Fr. 2-1 to 2-5). Fr. 2-1 [fr. 1-8, elution solvent; $CHCl_3$/MeOH (10:1) to (4:1), 0.8 g], Fr. 2-2 [fr. 9, $CHCl_3$/MeOH (7:3), 0.28 g], Fr. 2-3 [fr. 10-12, $CHCl_3$/MeOH (7:3), 1.14 g], Fr. 2-4 [fr. 13-16, $CHCl_3$/MeOH (7:3), 0.63 g], and Fr. 2-5 [fr. 17-21, $CHCl_3$/MeOH (7:3) to MeOH, 1.29 g]. Fr. 2-4 showed a single spot (*Rf* -value: 0.30) on TLC [$CHCl_3$/MeOH/H_2O (6:4:1, v/v). Purification of Fr. 2-4 was carried out by preparative HPLC [SunFire Prep C18 OBD, 19 i.d. × 250 mm, mobile phase: 18% acetonitrile (CN_3CN) containing 0.1% trifluoroacetic acid, 15 ml/min, detection UV 280 nm]. Identification of the compound was carried out by the comparison of the physicochemical data of the purified compound with those of the reported data (Ogawa et al., 2001, Sato et al., 2006) for the known **1** (Figure 2).

*2.5 HPLC Determination of **1** in CMR-ext*

Content of **1** in CMR-ext was determined by HPLC analysis described in previous report (Itoh et al., 2009) with minor modification. An accurately weighed CMR-ext (50 mg) was added in a volumetric 100 ml flask. After addition of MeOH up to 100 ml, the sample of CMR-ext was extracted by ultrasonic radiation for 30 min at room temperature. After filtration with a membrane filter (0.45 μm, GL Sciences Inc. Tokyo, Japan), an aliquot of 10 μl of the sample solution was injected into the HPLC system. The HPLC system consisted of a Shimadzu SCL-10Avp (Shimadzu, Kyoto) with a Shimadzu pump unit LC-20AT, Shimadzu UV-Vis detector SPD-10Avp and Chromato-PRO (Run Time Corporation, Kanagawa, Japan). The TSK gel ODS-120T (4 μm, 250 × 4.6 mm i.d.) column (Tosoh Co., Tokyo) was used at 37 ℃. The mobile phase was a gradient system of a solution A [0.1% H_3PO_4 in distilled water: CH_3CN (9:1 v/v)] and solution B [0.1% H_3PO_4 in distilled water: CH_3CN (2:8 v/v)] in the following ratio 0 min, solution A: solution B 9:1; for 30 min, 3:7 v/v. The flow rate was 0.8 ml/min; detection

was at UV 280 nm; and the t_R for **1** was 15.3 min. The peak area ratios *versus* concentrations of **1** (r = 0.9999) yielded straight-line relationships in the range of 0.625-40 μg/ml with the above correlation coefficients. Under this condition, narirutin, naringin, hesperidin and neohesperidin were eluted at the t_R of 16.8, 17.4, 17.8, and 18.4 min, respectively.

2.6 Western Blot Analysis

RBL-2H3 cells were treated using the same method as described above. The harvested cells were lysed with a lysis buffer (Cell Signaling Technology) and centrifuged at 9,200 × g for 10 min. The protein concentration of the supernatant was determined with a Protein Assay (Bio-Rad, Hercules, CA, USA). A solution of the same protein concentration was prepared and subjected to electrophoresis followed by transfer of protein to PVDF membranes at 60 V for 4 h. The resulting membranes were blocked with Blocking One-P solution for 20 min at room temperature for phosphorylated protein and 5% skimmed milk solution (TBST: in mM; NaCl 137, KCl 2.7, Tris 25 and 0.05% Tween, pH 7.4) for 1 h at room temperature for non-phosphorylated proteins (β-actin). Antibodies were diluted with blocking buffers in the ratio antibody: blocking buffer (1:1,000). The membrane was treated with the antibody solution and the membrane was washed with TBST. Anti-rabbit IgG HRP linked whole antibody were diluted in the same manner as the primary antibody and the solution was used to immerse each membrane. After washing the membrane with TBST, the proteins on membranes were visualized using ECL detection system.

2.7 Statistical Analysis

The experimental data were evaluated for statistical significance using Bonferroni/Dunn's multiple-range test with GraphPad Prism for Windows, Ver. 5 (GraphPad Software Inc., 2007).

3. Results and Discussion

Inhibitory activity of CMR-ext on antigen-induced degranulation was determined by measuring inhibitory activity of β-hexosaminidase release in RBL-2H3 cells according to the method of Murata et al. (2013). Dinitrophenyl-labeled human serum albumin (DNP-HSA) was used as an antigen. As shown in Table 1, treatment of CMR-ext (3.1 to 200 μg/ml) significantly inhibited degranulation induced by DNP-HSA from RBL-2H3 cells sensitized with anti-DNP IgE. Cytotoxicity of test samples against RBL-2H3 cells were evaluated by measuring cell proliferation using a commercial kit. CMR-ext didn't show any significant effects on cell proliferation at the concentration of 3.1 to 200 μg/ml. Baicalein inhibited degranulation without any significant effects on cell proliferation at 50 μM. Since *C. madurensis* is a perpetual *Citrus* breed which gives ripe and unripe fruit together, both fruit were collectable at the same time. Therefore, we compared the degranulation inhibitory activity of the ripe fruit extract (CMR-ext) with that of the unripe fruit extract. Unripe fruit extract significantly inhibited degranulation without any significant effects on cell proliferation at 12.5, 50 and 200 μg/ml, however the unripe extract showed less activity than CMR-ext (Table 1). These results suggested that ripe *C. madurensis* fruit may be applicable for functional food materials as juice, tea and/or jam with sweet taste and rich aroma. Thus, we focused on ripe *C. madurensis* fruits.

1-a; Ripe fruits 1-b; Unripe fruits

Figure 1. Photographs of ripe and unripe fruits of *C. madurensis*

Table 1. Inhibitory activities of CMR-ext and unripe *C. madurensis* fruit extract on DNP-HSA induced degranulation in RBL-2H3 cells

Samples	Concentration (μg/ml or μM)	Inhibition (%)	Cell proliferation (%)
Run 1			
Control			100.0±0.1
Ripe *C. madurensis*	3.1 μg/ml	30±5	101.9±2.8
fruit extract (= CMR-ext)	12.5	51±3	100.9±0.9
	50	53±3	103.5±0.7
	200	59±3	99.2±2.7
Baicalein	50 μM	76±1	107.6±2.2
Run 2			
Control			100.0±3.3
Unripe *C. madurensis*	3.1 μg/ml	4±5	96.8±1.8
fruit extract	12.5	24±3	97.9±0.7
	50	28±4	99.3±2.6
	200	34±4	107.5±3.0
Baicalein	50 μM	70±3	103.3±3.0

Each value in inhibition represents the mean ± S.D. of 3 experiments. Baicalein was used as a positive control agent. Each value in cell proliferation represents the mean ± S.D. of 3 experiments.

According to the report by Ogawa et al. (2001), *C. madurensis* are thought to originate from natural hybrids between the genera *Citrus* and *Fortunella*, and contain **1** in their peels, juice sacs and leaves (Figure 2) (Ogawa et al., 2001). Recently, **1**, a component of *C. madurensis* peels, was reported to have tyrosinase inhibitory activity (Lou et al., 2012) and antioxidant activity (Yu et al., 2013). To identify active constituents of CMR-ext, at first, we isolated **1** (pale yellow powder, isolation yield, 0.32% from CMR-ext) according to the method of Ogawa et al. and Sato et al. (2001, 2006), and identified its chemical structure on the basis of several NMR spectral data (^{1}H-NMR, ^{13}C-NMR, and DEPT) analysis, thereafter the degranulation inhibitory activity of **1** was examined. As shown in Table 2, **1** inhibited degranulation at the concentration of 50, 100 and 200 μM without any significant effects on cell proliferation. The content (mg/g of extract) of **1** in CMR-ext was determined by HPLC analysis. As a result, CMR-ext contained 11.0 mg/g of **1**. Thus, a part of the degranulation inhibitory activity of CMR-ext is attributable to **1**. Comparison of the inhibitory activity of CMR-ext with that of **1** gave a hypothesis that other constituents of CMR-ext may also contribute to the activity. The HPLC analysis revealed that the contents of several flavonoids in CMR-ext were as follows: hesperidin, 0.9 mg/g, neohesperidin, 0.9 mg/g, while narirutin and naringin were not detected. These data were in accordance with those of reports of Kawaii et al. (1999). Our previous paper (Murata et al., 2013) reported that hesperidin and neohesperidin showed a weak degranulation inhibitory activity in RBL-2H3 cells. To identify other active ingredients, further studies are required, and now undergoing.

Table 2. Inhibitory activity of 3',5'-di-C-*β*-glucopyranosylphloretin (**1**) on DNP-HSA induced degranulation in RBL-2H3 cells

Samples	Concentration (μM)	Inhibition (%)	Cell proliferation (%)
Control			100.0±0.1
1	50	10±2	98.7±1.2
	100	32±2	103.9±4.8
	200	46±2	106.7±1.9
Baicalein	50	83±0	107.6±2.2

Each value in inhibition represents the mean ± S.D. of 3 experiments. Baicalein was used as a positive control agent. Each value in cell proliferation represents the mean ± S.D. of 3 experiments.

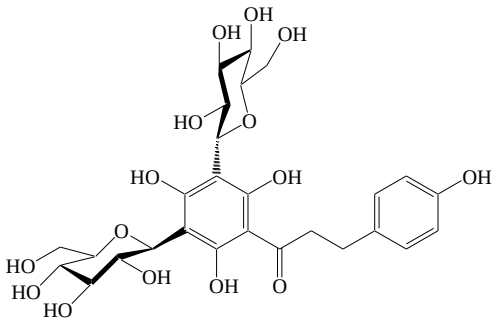

Figure 2. The chemical structure of 3',5'-di-C-*β*-glucopyranosylphloretin (**1**)

Type I allergy is defined as hypersensitive reaction. As illustrated in Figure 3, this type of allergy is known to be evoked by antigen-induced activation of Fcε receptors expressed on the surface of mast cells and basophils (Tomochika et al., 2011). Crosslinking of IgEs is essential, and is the first step triggering the signaling cascades such as Akt and MAPK that lead to degranulation of chemical mediators such as histamine, arachidonic acid metabolites and neutral proteases (Tomochika et al., 2011).

Therefore, to investigate the inhibition mechanism of degranulation by **1**, phosphorylation of Akt and relevant MAPKs (p38 and ERK) was examined by Western blot analysis. DNP-HSA induction at 0.01 μg/ml in RBL-2H3 cells led to phosphorylation of Akt, p38 and ERK (Figure 4). In our preliminary examination of DNP-HSA induction (0.01 μg/ml) on the phosphorylation of Akt, p38 and ERK, these were phosphorylated 15 min after induction, and phosphorylation peaked at 1 h (data not shown). In RBL-2H3 cells pretreated with **1** at 50 μM, phosphorylation of Akt and p38 was suppressed at 1 h, whereas phosphorylation of ERK was not suppressed (Figure 4). Significant effect was not observed in the expression level of Akt, p38, ERK and *β*-actin with or without DNP-HSA and/or **1**. These results suggest that **1** inhibits DNP-HSA-induced degranulation in RBL-2H3 cells by suppression of Akt and p38 phosphorylation as one of the degranulation inhibition mechanisms.

In conclusion, CMR-ext significantly inhibited DNP-HSA-induced degranulation in anti- DNP IgE antibody sensitized RBL-2H3 cells, without any effects on cell proliferation. It was revealed that a part of the degranulation inhibitory activity of the CMR-ext was attributable to **1**. To the best of our knowledge, this is the first report on degranulation inhibitory activity of **1**. Western blot analysis suggested that **1** inhibited degranulation by suppression of Akt and p38 phosphorylation.

Thus, ripe *C. madurensis* fruit may be applicable for functional food materials as juice, tea and/or jam with sweet taste and rich aroma in expectation of anti-type I allergic effect. There is an advantage that ripe fruits of this plant can be collected throughout the year due to a perpetual breed. However, further investigations are required to examine *in vivo* effects in animals and to reveal other active constituents.

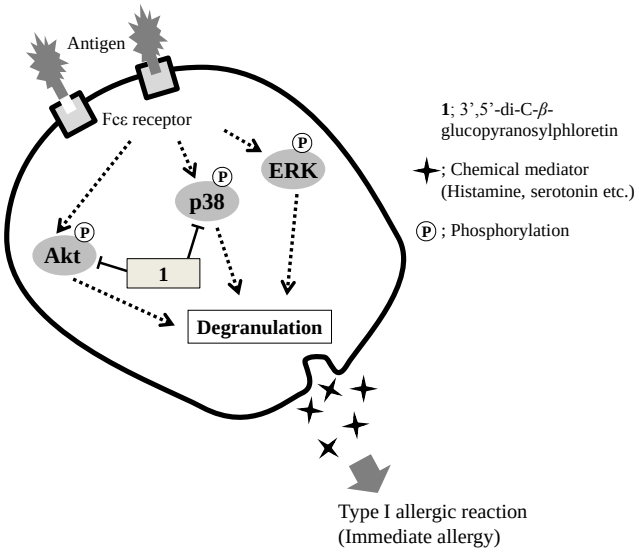

Figure 3. Schematic representation showing the inhibition of **1** on degranulation in RBL-2H3 Cells

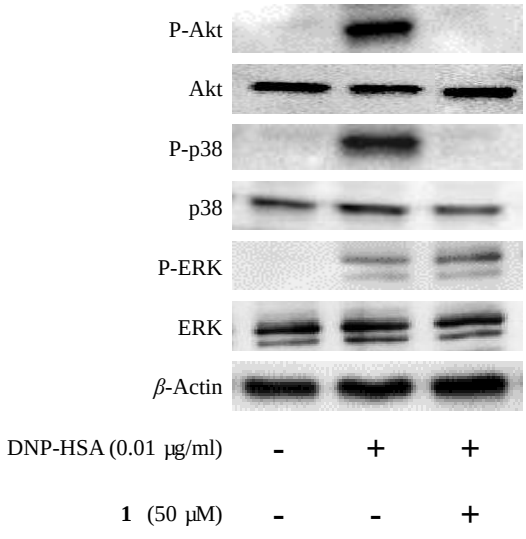

Figure 4. Effect of **1** on Akt, p38 and ERK phosphorylation in RBL-2H3 cells

RBL-2H3 cells were treated with **1** (50 μM) for 1 h, and degranulation was induced with DNP-HSA (0.01 μg/ml) for 0.5 h. Phosphorylation of Akt, p38 and ERK was determined with Western blot analysis.

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