

***Spirodela polyrhiza* and its Chemical Constituent Vitexin Exert Anti-Allergic Effect *via* ORAI1 Channel Inhibition**

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Published 28 August 2018

Abstract: Intracellular calcium signaling cascades are integral to early and late allergic responses involving mast cell degranulation and type 2 helper T cell activation, respectively. Both the responses are accompanied by the movement of calcium through the calcium release-activated calcium (CRAC) channel, encoded by the *ORAI1* gene. *Spirodela polyrhiza* (L.) Schleid (SP) has anti-inflammatory and anti-allergic effects, but its effect on calcium signaling has not been reported. This study investigated whether a 30% ethanolic SP extract (SP_{EIOH}) and its constituents can reduce CRAC currents (I_{CRAC}), and thus inhibit mast cell degranulation and T cell activation. In Jurkat T lymphocytes, we found that 3 mg/mL SP_{EIOH} inhibited the I_{CRAC} by $81.0 \pm 11.1\%$, whereas one of its constituents vitexin (100 μM) inhibited the I_{CRAC} by $48.9 \pm 8.71\%$. Furthermore, in the RBL-2H3 mast cell, the I_{CRAC} was inhibited by 3 mg/mL SP_{EIOH} ($86.7 \pm 5.83\%$) and 100 μM vitexin ($47.5 \pm 5.67\%$).

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Investigation of human primary T cell proliferation induced by co-stimulation with antibodies to cluster of differentiation 3 and 28, and of RBL-2H3 mast cell degranulation following IgE-antigen complex stimulation revealed that 100 μ M vitexin inhibited both T-cell proliferation (by $34.8 \pm 6.08\%$) and mast cell degranulation (by $36.7 \pm 0.07\%$). These effects were concentration-dependent, and no cytotoxicity was observed. Our findings suggest that vitexin is a promising candidate compound for the development of therapeutic agents to prevent and treat allergic diseases.

Keywords: *Spirodela polyrhiza*; Vitexin; Apigenin 7-O-Glucoside; Calcium Release-Activated Calcium Channel; ORAI1; Anti-Allergic Effect.

Introduction

Allergies are caused by non-microbial environmental antigens that can induce the release of cytokines, such as interleukin (IL)-4, IL-5, and IL-13, from cluster of differentiation (CD) 4+ type 2 helper T cells (Th2 T cells), and also can activate immunoglobulin E (IgE)-mediated degranulation of mast cells (Pillai, 2015). Most allergens are common environmental proteins, animal products (such as dust mite feces, pollen, and pet dander), and chemicals (Lambrecht and Hammad, 2014). Allergic responses can be divided into two phases: the early phase response and late-phase reaction; these are commonly described with the combined term, immediate hypersensitivity. The early phase response develops within a few seconds of exposure to allergen. This is triggered by the release of pre-formed mediators, such as histamine, leukotrienes, and prostaglandins from mast cells, increasing vascular permeability, vasodilation, itchiness, and smooth muscle contraction (Holgate and Polosa, 2008). Subsequently, the late-phase reaction develops slowly, and this inflammatory process is characterized by the accumulation of neutrophils, eosinophils, and Th2 T cells. These are attracted by the chemokines released by mast cells and other cell types (Holgate and Polosa, 2008; Lambrecht and Hammad, 2014).

Intracellular calcium signaling is involved in the signaling cascades that are activated in immunocytes during the early and late phases of allergic responses. Calcium is a well-established second messenger in immunocytes, where it regulates the secretion of pre-formed mediators or cytokines. It also modulates cell proliferation, gene expression, and other functions (Feske, 2007). Normally, the intracellular calcium concentration of resting immunocytes is below 100 nM. However, when an allergen binds to an immunoreceptor, such as the T-cell or B-cell receptor, or an antigen–antibody complex binds to the low-affinity Fc receptor for IgE on mast cells, it generates transient, followed by sustained, intracellular calcium signaling via the Gq protein-coupled receptor (Feske *et al.*, 2012, 2015). Activation of this receptor stimulates phospholipase γ , which hydrolyzes phosphatidylinositol 4,5-bisphosphate to diacyl glycerol and inositol trisphosphate (IP₃). IP₃ binds to the IP₃ receptor on the endoplasmic reticulum (ER), releasing calcium from the ER stores into the cytoplasm, thus generating transient calcium signaling (Feske *et al.*, 2012, 2015). The associated depletion of ER calcium activates store-operated calcium entry

(SOCE), which is responsible for sustained signaling via the calcium release-activated calcium (CRAC) channel encoded by the *ORAI1* gene (Feske, 2010). This calcium signaling modulates the subsequent gene transcription via the calcium-dependent nuclear factor of activated T-cells transcription factor family, which is required for the activation of most immune cells (Feske, 2007).

ORAI1 is ubiquitously expressed in immune cells and is essential for the function of T cells, mast cells, and other immune cells. The genetic deletion of ORAI1 in mice impairs the functions of T and mast cells, and attenuates inflammation in various animal models, including the models of autoimmunity and passive cutaneous anaphylaxis (Baba *et al.*, 2008; Gwack *et al.*, 2008; McCarl *et al.*, 2010; Vig *et al.*, 2008). Moreover, human patients with genetic defects affecting *ORAI1* exhibit severe combined immune deficiency syndrome, and deficits in SOCE and CRAC channel functions (Feske *et al.*, 2006; McCarl *et al.*, 2009). Therefore, the inhibitors of the ORAI1 channel represent an attractive therapeutic target to alleviate inflammatory diseases.

Spirodela polyrhiza (L.) Schleid (SP) is widely distributed in many types of freshwater worldwide and has been used as an ingredient of traditional oriental medicines. The extract of SP has been reported to exhibit anti-inflammatory and anti-allergic effects, such as inhibition of pro-inflammatory cytokine secretion and mast cell degranulation (Ahn *et al.*, 2004; Kim and Ko, 2004; Shyni *et al.*, 2015; Lee *et al.*, 2016). Furthermore, topical application of ethanolic extract of SP alleviates 2,4-dinitrochlorobenzene-induced symptoms of atopic dermatitis in mice (Lee *et al.*, 2016). Recently, we demonstrated that the methanolic extract of SP inhibits the activation of ORAI1 channel and degranulation of mast cells (Nam *et al.*, 2017). However, to the best of our knowledge, no study has determined the effects of each chemical constituent of this extract on T cell activity and mast cell degranulation.

The compounds present in the crude ethanolic extract of SP (SP_{EIOH}) have been identified as luteolin 8-C-glucoside (orientin), apigenin 8-C-glucoside (vitexin), luteolin 7-O-glucoside, apigenin 7-O-glucoside, luteolin and apigenin (Kim *et al.*, 2010; Qiao *et al.*, 2011). Therefore, the present study assessed the effects of SP_{EIOH} and its constituents on the ORAI1 channel by whole-cell patch-clamp technique. We selected the chemical constituent that most effectively inhibited the CRAC and evaluated its effects on mast cell degranulation and T cell proliferation, which require SOCE.

Materials and Methods

Reagents and Chemicals

All chemicals were purchased from Sigma-Aldrich (Merck, St. Louis, MO, USA), except N-[4-[3,5-bis(trifluoromethyl)-1H-pyrazol-1-yl]phenyl]-4-methyl-1,2,3-thiadiazole-5-carboxamide (BTP2) and IP₃, which were purchased from Tocris Bioscience (Bristol, UK) and Merck Millipore (Billerica, MA, USA), respectively. A stock solution of 20 mM IP₃ was prepared in distilled water (H₂O). All other stock solutions were prepared in dimethyl sulfoxide and stored at −20°C until further analysis.

Cell Culture

The cell lines HEK293T (catalog no. CRL-3216) and RBL-2H3 (catalog no. CRL-2256) were procured from the American Type Culture Collection (ATCC; Manassas, VA, USA) and grown in Dulbecco's modified Eagle's medium (Life Technologies, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (Welgene Inc., Gyeongsan, Korea) and 1% penicillin/streptomycin (P/S; Life Technologies). Jurkat T lymphocytes (catalog no. TIB-152) were also purchased from the ATCC and grown in Roswell Park Memorial Institute (RPMI)-1640 medium (Life Technologies) supplemented with 10% fetal bovine serum and 1% P/S. All cells were maintained in a humidified incubator with 10% carbon dioxide (CO₂) at 37°C, except the Jurkat T lymphocytes (5% CO₂).

Cell Transfection

The presence of human stromal interaction molecule 1 (STIM1) protein in the ER membrane is necessary to activate the CRAC currents (I_{CRAC}). We therefore co-transfected HEK293T cells with human (h) STIM1 and ORAI1 expression vectors (Prakriya, 2013). Detailed procedure for the co-transfection of hORAI and hSTIM1 has been described previously (Nam and Lee, 2016). Briefly, the HEK293T cells were triple-transfected with a mammalian expression vector carrying the hORAI1, hSTIM1, and enhanced green fluorescent protein (pEGFP-N1; Life Technologies) at a ratio of 4.5:4.5:1 using the Lipofectamine Plus reagent (Life Technologies), according to the instruction of the manufacturer. The hORAI1 and hSTIM1 clones were gifted by Dr. Sung Joon Kim, Seoul National University, Korea. The experiments were performed 24–36 h after transfection.

SP_{EtOH} Preparation

Dried whole aquatic parts of SP were obtained from the Kwangmyungdang Medicinal Herbs Co., Ltd. (Ulsan, Republic of Korea). Two-hundred grams of SP were pulverized and extracted with 30% ethanol for 3 h prior to filtering (Whatman no. 1). The resultant SP_{EtOH} was concentrated using a rotary vacuum evaporator, with a yield of 9.3%. The product obtained was pulverized and stored at –20°C.

SP_{EtOH} Analysis

The SP_{EtOH} and the relevant standard compounds were analyzed using the high-performance liquid chromatography (HPLC) 1290 system (Agilent, Santa Clara, CA, USA) at the Korea Basic Science Institute (Seoul, Korea). Ten microliters of 10 mg/mL SP_{EtOH} was separated using the Poroshell 120 SB-C18 column (3.0 mm × 100 mm, 2.7 μm; Agilent) and detected at a wavelength of 340 nm. A mobile phase gradient (A, 0.05% formic acid; B, 0.05% formic acid in acetonitrile) was used with 5–10% B for 3 min, 10–30% B for 2 min, 30–40% B for 5 min, 40–90% B for 2.5 min, followed by equilibration with 5% B. The flow rate was 0.5 mL/min.

Electrophysiology

The conventional whole-cell patch-clamp current recordings were performed using the Axopatch 200B amplifier (Molecular Devices, Sunnyvale, CA, USA) and Digidata 1440A interface (Molecular Devices), as described previously (Nam and Lee, 2016). Briefly, the cells were transferred to a perfusion chamber with a glass bottom and allowed to settle for 5–10 min. The bath solution was then perfused at 10 mL/min at room temperature. The patch pipettes were pulled from the borosilicate glass capillaries (World Precision Instruments, Inc. Sarasota, FL, USA) using a flaming/brown micropipette puller (Model P-97, Shutter Instrument, Novato, CA, USA), and then fire-polished to a resistance of 2–3 MOhm (Narishige, East Meadow, NY, USA). The I_{CRAC} was negligible, and only the cells with tight seals (> 16 GOhm) were therefore selected for analysis. Immediately after establishing the whole-cell configuration, ramp-like pulses from -130 to $+50$ mV over 100 ms were applied every 20 s at a holding potential of $+10$ mV, to obtain the current–voltage relationship (I – V) curves. The bath solution contained 135 mM NaCl, 3.6 mM KCl, 1 mM $MgCl_2$, 10 mM $CaCl_2$, 5 mM D-glucose, and 10 mM HEPES, and the pH was adjusted to 7.4. The pipette solution contained 130 mM Cs-glutamate, 20 mM BAPTA, 1 mM $MgCl_2$, 3 mM MgATP, 0.002 mM sodium pyruvate, and 20 mM HEPES, and the pH was adjusted to 7.2. When the I_{CRAC} reached a steady-state, we serially added the indicated concentrations of SP_{EtOH} or the other test compounds to the bath solution. At the end of each experiment, we also confirmed the basal current using BTP2, which is a potent inhibitor of ORAI1 channel activation.

β -Hexosaminidase Assay

The activity of β -hexosaminidase was quantified by spectrophotometric analysis of p-nitrophenyl N-acetyl-d-glucosamine hydrolysis, as described previously (Nam *et al.*, 2017). Briefly, RBL-2H3 mast cells (ATCC) were seeded in 48-well culture plates (3×10^5 cells/mL) and incubated overnight. The cells were sensitized with 1.0 mg/mL anti-dinitrophenyl (DNP)-IgE for 3 h, and then washed four times with phosphate-buffered saline (PBS). The cells were then exposed to different concentrations of SP_{EtOH} (0.03, 0.1, 0.3, and 1 mg/mL) or vitexin (10, 30, and 100 μ M) dissolved in piperazine-N, N'-bis(2-ethanesulfonic acid) (PIPES) buffer (119 mM NaCl, 5 mM KCl, 25 mM PIPES, 5.6 mM glucose, 1 mM $CaCl_2$, 0.4 mM $MgCl_2$, and 0.1% bovine serum albumin; pH 7.2) and stimulated with 800 ng/mL DNP-horse serum albumin (HSA) for 1.5 h. The medium was then harvested and incubated with 1 mM p-nitrophenyl N-acetyl-D-glucosamine in citrate buffer (pH 4.5) for 1.5 h at 37°C. The reaction was stopped by adding sodium carbonate buffer (pH 10.0), and the absorbance was read at 405 nm. The activity of β -hexosaminidase was expressed as a percent of the absorbance at 405 nm.

T Lymphocyte Isolation

The experiments with human blood were approved by the Institutional Review Board (IRB), Dongguk University College of Medicine (2017-07-003 IRB). The peripheral

blood samples were obtained from whole blood samples collected from healthy donors. The peripheral blood mononuclear cells were obtained from the blood samples using the Ficoll-Paque Plus medium (GE Healthcare, Chicago, IL, USA). The T cells were isolated using the Pan-T Cell Isolation Kit (Miltenyi Biotec, Bergisch Gladbach, Germany), with a slightly modified protocol. Briefly, the cells were counted and 10 μ L of Pan T Cell Biotin–Antibody Cocktail was added per 10^7 cells. After incubation at 4°C for 5 min, 30 μ L of buffer and 20 μ L of Pan T Cell MicroBead Cocktail were added, and the mixture was incubated at 4°C for 10 min prior to separation on a LS column. (Miltenyi Biotec)

T Cell Proliferation Assay

The purified T cells were washed twice with PBS and resuspended in PBS to a concentration of 10^5 and 10^6 cells/mL. Carboxyfluorescein succinimidyl ester (CFSE; Thermo Fisher Scientific, Waltham, MA, USA) was added and incubated at room temperature for 10 min. To stop the reaction, cold complete media was added, and the cells were incubated on ice for 5 min. The CFSE-labeled cells were seeded in 96-well plates at 2×10^5 cells/well, and co-stimulated with 5 μ L/mL antihuman CD3 (Thermo Fisher Scientific) and 2 μ L/mL antihuman CD28 (Thermo Fisher Scientific). After incubation with RPMI-1640 for 3 d, the proliferation of CD4+ T cells was analyzed by fluorescence-activated cell sorting. The allophycocyanin-conjugated antihuman CD4 (BD Pharmingen) was used to identify CD4+ T cells, according to the protocol of the manufacturer, with slight modifications.

Cytotoxicity Assay

The Jurkat T cells were plated in 96-well plates at a density of 1×10^4 cells per well, treated with different concentrations of SP_{EIOH} (0.001–1 mg/mL), vitexin (0.1–100 μ M), apigenin 7-glucoside (0.1–100 μ M), and BTP2 (0.01–10 μ M), and then cultured for different periods — 24, 48, and 72 h. The control group was the vehicle (DMSO). Cell toxicity was determined using the Cell Counting Kit-8 (CCK-8; Dojindo, Rockville, MD, USA). Ten microliters of CCK-8 solution was added to each well and incubated at 37°C for 4 h. The absorbance was measured at 450 nm using a microplate reader (BioTek Instruments Inc, Winooski, USA).

Statistical Analysis

All data are expressed as mean $\pm$ standard error of the mean. The N values refer to the number of individual groups. For multiple comparisons, we used the one-way analysis of variance with Newman Keuls *post hoc* correction. The *P* values < 0.05 were considered statistically significant, as compared to that of the respective control. The statistical analyses were performed using Prism 6.0 (GraphPad Software; La Jolla, CA, USA) and Origin 8.0 (MicroCal, LLC, Northampton, MA, USA).

Results

Inhibition of I_{CRAC} by SP_{EtOH} in hORAI1 and hSTIM Co-Transfected HEK293T Cells

We performed whole-cell patch clamp on the HEK293T cells that co-expressed hORAI1 and hSTIM (HEK_{ORAI1}). With 20 mM BAPTA and 20 μ M IP₃ in the patch pipette solution, the I_{CRAC} increased gradually as the ER calcium store was depleted by the activation of IP₃ receptor. Figure 1A shows a typical I_{CRAC} recording; serial application of SP_{EtOH} concentration-dependently inhibited the I_{CRAC} at -120 mV (Fig. 1B) by $11.8 \pm 1.8\%$ ($n = 8$), $35.1 \pm 11.1\%$ ($n = 4$), and $69.7 \pm 6.1\%$ ($n = 5$) at concentrations of 0.1, 0.3, and 1 mg/mL, respectively (Fig. 1C).

SP_{EtOH} -Mediated Inhibition of I_{CRAC} in T Cells and Mast Cells

The I_{CRAC} is important to activate immune cells, such as CD4+ T cells and mast cells, involved in allergic reactions. Therefore, we investigated whether the SP_{EtOH} also modulated endogenously expressed ORAI1 channels, which can generate the I_{CRAC} in T lymphocytes and mast cells. As shown in Figs. 2A and 2B, the patch-clamp recording of the

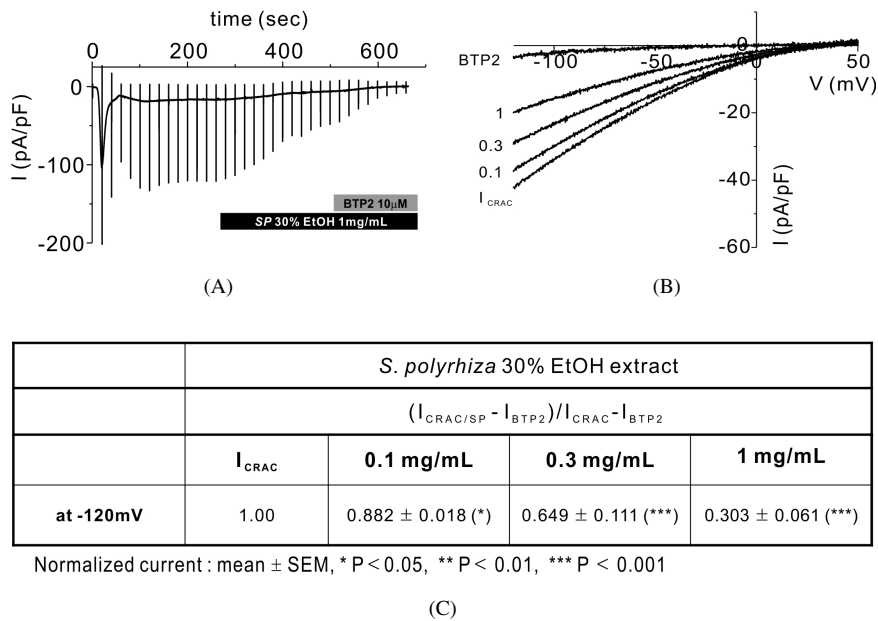


Figure 1. Inhibitory effect of 30% ethanolic extract of *Spirodela polyrhiza* (SP_{EtOH}) on the calcium release-activated calcium channel current (I_{CRAC}) in the HEK293T cells co-transfected with human *ORAI1* and stromal interaction molecule 1 (*STIM1*; HEK_{ORAI1}). (A) A typical recording of the I_{CRAC} and its inhibition by SP_{EtOH} (1 mg/mL) and BTP2 (10 μ M). (B) Representative I_{CRAC} current (I)-voltage (V) relationship curves. (C) A summary of the normalized (%) I_{CRAC} values at -120 mV, which were calculated by subtracting the BTP2-resistant current (I_{BTP2}) from the current observed in the presence of SP_{EtOH} at the indicated concentrations. The P values < 0.05 were considered significant (* $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$).

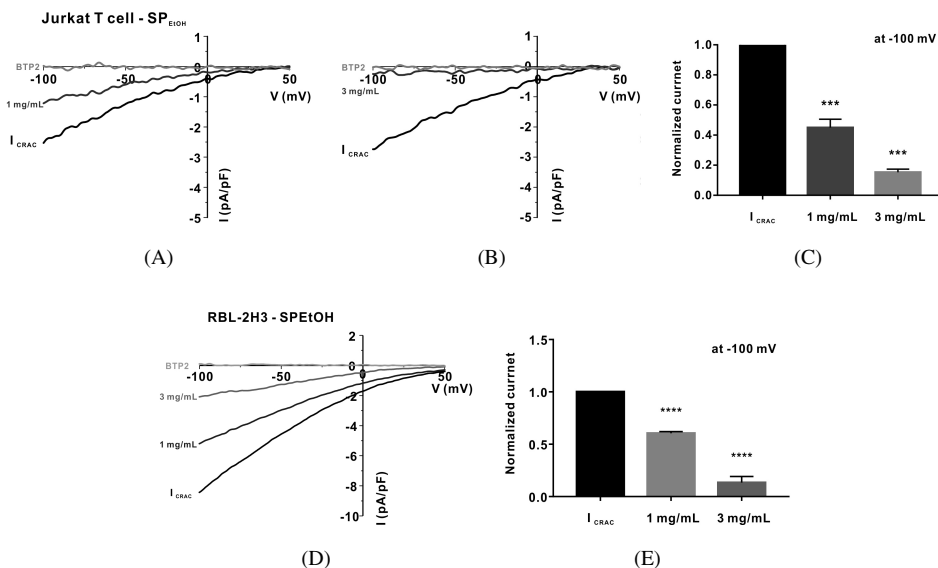
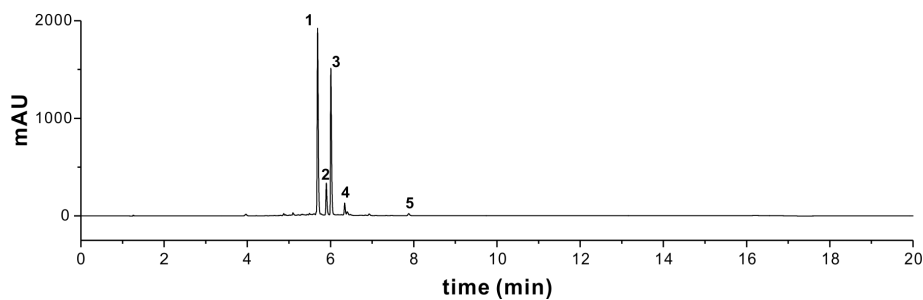


Figure 2. Inhibition of the calcium release-activated calcium channel current (I_{CRAC}) by 30% ethanolic extract of *Spirodela polyrhiza* (SP_{EtOH}) in the Jurkat CD4+ T lymphocytes and RBL-2H3 mast cells. (A, B) Representative I_{CRAC} current (I)-voltage (V) relationship curves in control cells and in those treated with 1 mg/mL (A) and 3 mg/mL (B) SP_{EtOH} . The basal current was confirmed using 10 μ M BTP2. (C) The normalized histogram of I_{CRAC} inhibition by SP_{EtOH} at -120 mV. The P values < 0.05 were considered significant (*** $P < 0.001$). (D) A typical I_{CRAC} in the RBL-2H3 cells and its inhibition by 1 and 3 mg/mL SP_{EtOH} . The basal current was also confirmed using 10 μ M BTP2. (E) A summary of the inhibition rate of I_{CRAC} by SP_{EtOH} in the RBL-2H3 cells at -120 mV. The P values < 0.05 were considered significant (**** $P < 0.0001$).

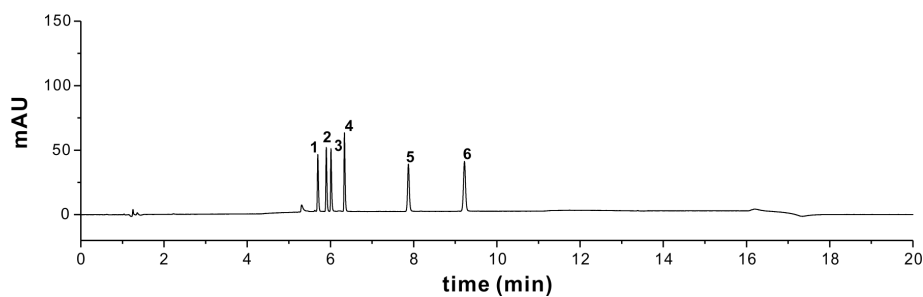
I_{CRAC} in the Jurkat T cells (immortalized human CD4+ T lymphocytes) showed that the bath application of 1 and 3 mg/mL SP_{EtOH} concentration-dependently inhibited the I_{CRAC} by $55.1 \pm 5.5\%$ ($n = 15$) and $84.7 \pm 2.1\%$ ($n = 17$), respectively (Fig. 2C). The experiment was repeated in RBL-2H3 cells (rat basophilic leukemia cells). The results indicated that 1 and 3 mg/mL SP_{EtOH} can effectively inhibit the I_{CRAC} by $39.55 \pm 1.67\%$ ($n = 4$) and $86.7 \pm 5.83\%$ ($n = 3$), respectively (Figs. 2D and 2E).

Analysis of SP_{EtOH} Constituents

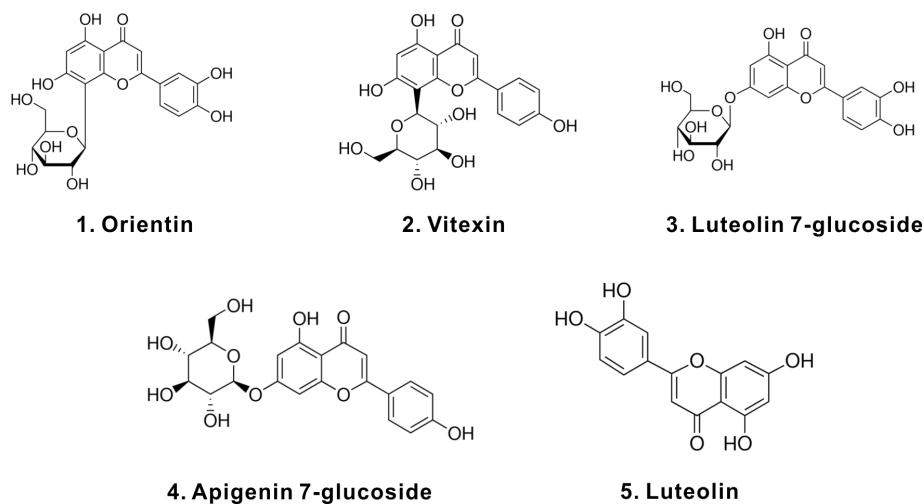
To elucidate the bioactive components that can inhibit the activation of ORAI1 channel, we performed the HPLC analysis. The HPLC analysis of the SP_{EtOH} revealed five peaks eluting at 5.689 min (1), 5.899 min (2), 6.008 min (3), 6.339 min (4), and 7.880 min (5) (Fig. 3A). Under the same HPLC conditions, we also analyzed six standard compounds based on a previous report (Qiao *et al.*, 2011): orientin (5.695 min), vitexin (5.901 min), luteolin 7-O-glucoside (6.014 min), apigenin 7-O-glucoside (6.339 min), luteolin (7.874 min), and apigenin (9.222 min) (Fig. 3B). We thus identified five of these flavonoids in SP_{EtOH} ; apigenin was not detected (Fig. 3C). The concentration of these constituents in SP_{EtOH} was determined by comparison of the peak areas with those of a known amount



(A)



(B)



(C)

Figure 3. Analysis of the constituents of the 30% ethanolic extract of *Spirodela polyrhiza* (SP_{EtOH}). High-performance liquid chromatograms of six standard compounds (A) and of SP_{EtOH} (B). The standard compounds were: 1, orientin; 2, vitexin; 3, luteolin 7-O-glucoside; 4, apigenin 7-O-glucoside; 5, luteolin; 6, apigenin. (C) The structures of the compounds identified within SP_{EtOH}.

Table 1. Quantification of the Five Major Chemical Constituents of Ethanolic Extract of *Spirodela polyrhiza* (SP_{EIOH}) (1 mg/mL)

	Retention Time (min)	Average Area	Average Amount (μg/mL)	*Calculated Amount (μg/mg)	Dilution Factor
Orientin	5.695	338.6	8.8373	88.3725	100
Vitexin	5.901	56.8	1.3211	13.2108	100
Luteolin 7-Glucoside	6.014	252.6	6.9729	69.7295	100
Apigenin 7-Glucoside	6.339	217.4	3.9347	3.9347	10
Luteolin	7.874	60.2	1.1274	1.1274	10
Apigenin	9.222	30.6	0.4760	0.0476	1

Note: *Calculated amount = (average amount × dilution factor)/10 (mg).

Table 2. Effects of the Major Five Chemical Constituents of Ethanolic Extract of *Spirodela polyrhiza* (SP_{EIOH}) on the *I*_{CRAC}

	Apigenin 7-Glucoside	Vitexin	Luteolin	Luteolin 7-Glucoside	Orientin	Component Mixture (Contained in 1 mg/mL SP _{EIOH})
Contained in 1 mg/mL SP _{EIOH}	10 μM	30 μM	4 μM	150 μM	200 μM	
Inhibition Rate	10.23 ± 1.3 (%) (n = 5)	23.24 ± 4.07 (%)*** (n = 4)	6.14 ± 1.42 (%) (n = 5)	4.55 ± 2.64 (%) (n = 4)	0.15 ± 6.3 (%) (n = 4)	3.06 ± 2.89 (%) (n = 4)

Notes: The results shown are percentile inhibition rate as compared with that of the controls, which were normalized to 100%, and are presented as mean ± standard error of the mean. The values within parentheses indicate the total number (*n*) of experiments at each concentration. The *P* values < 0.05 were considered significant (***) (*P* < 0.001).

of analytical standard compound (Table 1). The levels determined were 88.37 μg/mg orientin, 13.21 μg/mg vitexin, 69.73 μg/mg luteolin 7-O-glucoside, 3.93 μg/mg apigenin 7-O-glucoside, and 1.12 μg/mg luteolin.

Effects of SP_{EIOH} Constituents on the I_{CRAC}

The quantification data were used to calculate the concentration of each component in 1 mg/mL SP_{EIOH}, and whole-cell patch-clamp of HEK_{ORAI1} cells was performed. As shown in Table 2, the *I*_{CRAC} was inhibited by 23.24 ± 4.07% and 10.23 ± 1.3% in the presence of 30 μM vitexin and 10 μM apigenin 7-O-glucoside, respectively (*n* = 5). The other constituents and mixture of these compounds inhibited the *I*_{CRAC} by < 10%.

The effects of serial bath application of 10, 30, and 100 μM vitexin and apigenin 7-O-glucoside on the *I*_{CRAC} in the HEK_{ORAI1} cells were then determined (Fig. 4). The *I*_{CRAC} was significantly inhibited by 19.7 ± 4.71%, 30.9 ± 4.95%, and 40.6 ± 5.86% in the

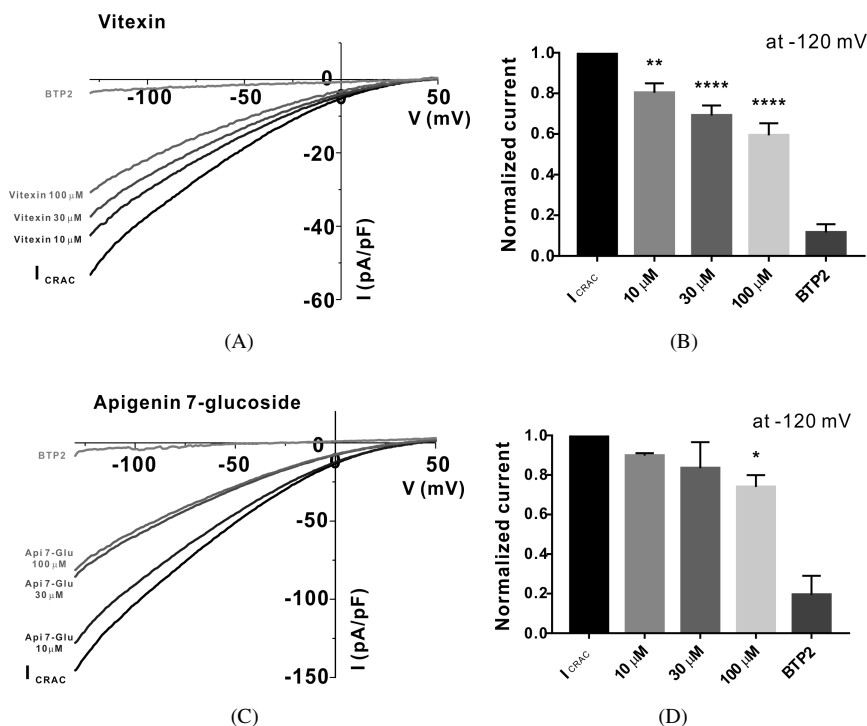


Figure 4. Vitexin and apigenin 7-O-glucoside inhibited the calcium release-activated calcium channel current (I_{CRAC}) in a concentration-dependent manner in HEK_{Orai1}. (A, C) Representative I_{CRAC} current (I)-voltage (V) relationship curves for control cells and those treated with the indicated concentrations of vitexin or apigenin 7-O-glucoside. (B, D) The normalized histograms of I_{CRAC} inhibition by the indicated treatments at -120 mV. The P values < 0.05 were considered significant (** $P < 0.01$ and **** $P < 0.0001$).

presence of 10, 30, and 100 μ M vitexin, respectively ($n = 12$). However, apigenin 7-O-glucoside only produced statistically significant inhibition at 100 μ M ($26.1 \pm 6.06\%$; Fig. 4D; $n = 5$).

In human Jurkat CD4 $^{+}$ T lymphocytes, the inhibition rate of IP₃-induced I_{CRAC} by vitexin was higher than that by the SP_{EIOH} (Fig. 5A). The inhibition rate of I_{CRAC} by vitexin was $29.7 \pm 6.79\%$, $40.3 \pm 8.52\%$, and $48.9 \pm 8.71\%$ at a concentration of 10, 30, and 100 μ M, respectively (Fig. 5B). The results of the same experiment in the Jurkat CD4 $^{+}$ T lymphocytes revealed that the I_{CRAC} produced in RBL-2H3 can be effectively inhibited by vitexin by $10.3 \pm 2.07\%$, $24.2 \pm 4.88\%$, and $47.5 \pm 5.67\%$ at a concentration of 10, 30, and 100 μ M, respectively (Figs. 5C and 5D).

Effects of Apigenin 7-O-Glucoside and Vitexin on Primary CD4 $^{+}$ T Cell Proliferation and Mast Cell Degranulation

The SP_{EIOH} and its chemical components vitexin and apigenin 7-O-glucoside were tested to determine whether they are effective in the activation of allergic disease-related T cells and

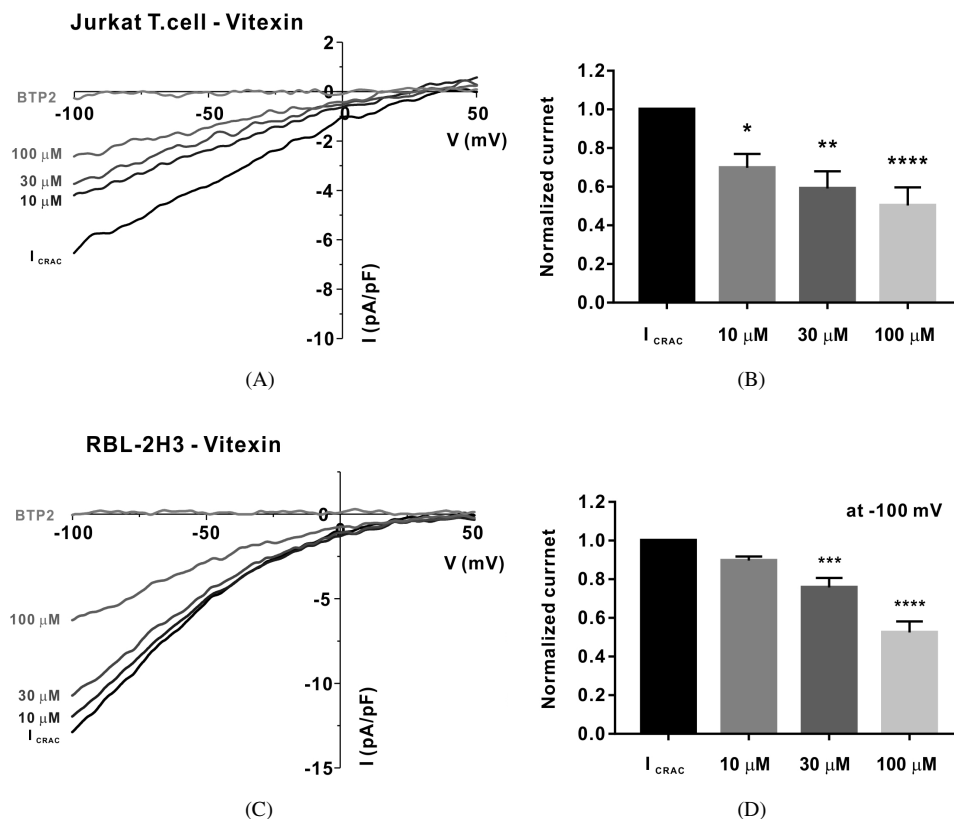
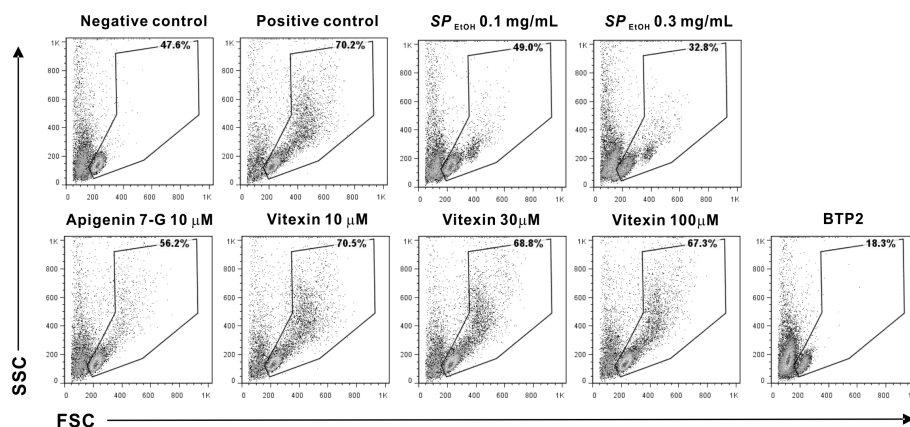


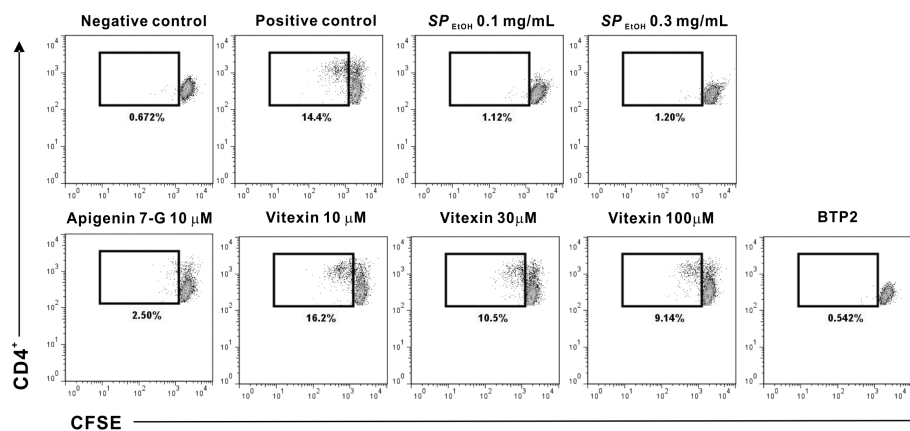
Figure 5. Inhibition of the calcium release-activated calcium channel current (I_{CRAC}) by vitexin in the Jurkat T lymphocytes and RBL-2H3 cells. (A) Typical recordings of I_{CRAC} and its inhibition by 10, 30, and 100 μ M vitexin. (B) The normalized histogram of I_{CRAC} at -100 mV for the control cells and those treated 10, 30, or 100 μ M vitexin, or 10 μ M BTP2. The P values < 0.05 were considered significant (* P < 0.05, ** P < 0.01, and **** P < 0.0001). (C) The representative current (I)-voltage (V) curve of the I_{CRAC} and its inhibition by 10, 30, and 100 μ M vitexin in the RBL-2H3 cells. (D) A summary of the normalized inhibition percent of the I_{CRAC} by 10, 30, and 100 μ M vitexin in the RBL-2H3 cells. The P values < 0.05 were considered significant (*** P < 0.05 and **** P < 0.0001).

mast cells. As a positive control, we isolated and stimulated human primary CD4+ T cells with anti-human CD3 and CD28 antibodies for 72 h. The experimental groups were exposed to these antibodies in the presence of 0.1 or 0.3 mg/mL SP_{EtOH} ; 10 μ M apigenin 7-O-glucoside; or 10, 30, or 100 μ M vitexin. Flow cytometric analysis of these CFSE-labeled cells indicated that the SP_{EtOH} and apigenin 7-O-glucoside exhibited potent inhibitory effects on CD4+ T cell proliferation (Figs. 6B and 6C). However, the SP_{EtOH} and apigenin 7-O-glucoside also exhibited cytotoxic effects when compared with that on the control cells (Fig. 6A). Interestingly, vitexin showed less potent inhibitory effect on CD4+ T-cell proliferation at a concentration of 10, 30, and 100 μ M (17.7%, 30.23%, and 34.8% inhibition, respectively) when compared with that of the other treatments, and less cytotoxicity

(Fig. 6). To confirm the cytotoxicity of vitexin, vitexin, apigenin, and BTP2 (a potent small molecule inhibitor of the I_{CRAC}) were tested using the CCK-8 kit, which can determinate cell viability in cell proliferation and cytotoxicity. As shown in Figs. 7A and 7C, vitexin and BTP2 did not show cytotoxicity even at a high concentration of 100 μ M. However,



(A)



(B)

Figure 6. Vitexin significantly inhibited the proliferation of the CD4⁺ Jurkat T lymphocytes with minimal cytotoxicity. (A) Flow cytometric gating strategy for human lymphocytes. The dead cells were excluded by selecting all live cells from the forward scatter (FSC) and side scatter (SSC). The positive control cells were co-stimulated with anti-human CD3 and CD28 antibodies. The 30% ethanolic extract of *Spirodela polyrhiza* (SP_{EtOH} ; 0.3 mg/mL) exhibited strong cytotoxicity. Apigenin 7-O-glucoside and BTP2 also reduced cell viability, as compared with that of the positive control. (B) Flow cytometric analysis of the carboxyfluorescein succinimidyl ester (CFSE)-labeled CD4⁺ T cells co-stimulated with anti-CD3/CD28 3 d after culture with the indicated concentration of apigenin 7-O-glucoside or vitexin. (C) The percent proliferation of CD4⁺ T cells, as compared with that of the control cells that were not stimulated with any antibodies. The x-axis represents the concentration (μ M). The P values < 0.05 were considered significant (* $P < 0.05$, *** $P < 0.001$, and **** $P < 0.0001$).

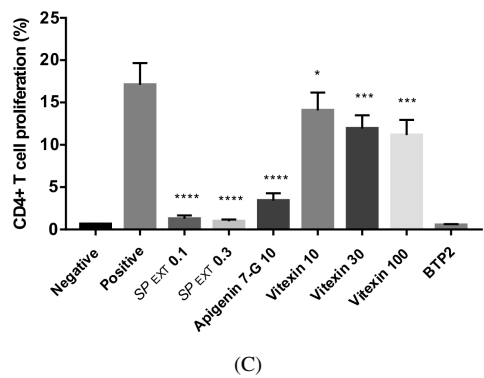


Figure 6. (Continued)

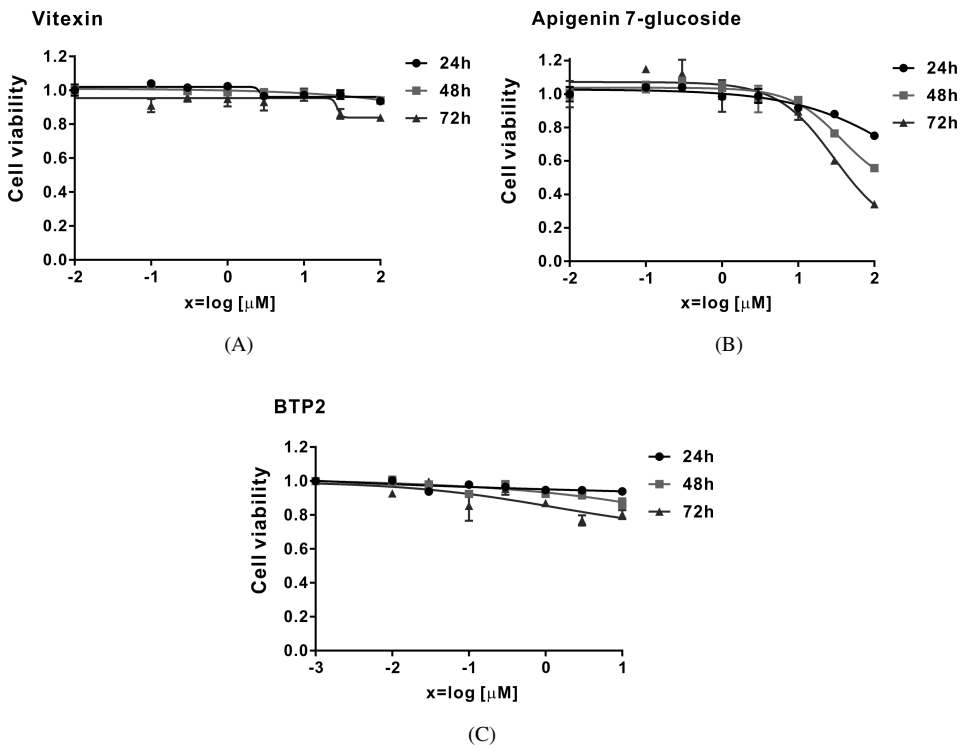


Figure 7. Cytotoxicity of vitexin, apigenin 7-O-glucoside, and BTP2 in the Jurkat T lymphocyte. (A) Cell viability was measured after exposing the cells to different concentrations of vitexin (0.01–100 μM) for 24 (black circle), 48 (gray rectangle), and 72 (black triangle) h. (B) The viability of Jurkat T lymphocytes after exposure to 0.01–100 μM apigenin 7-O-glucoside for 24 (black circle), 48 (gray rectangle), and 72 (black triangle) h. (C) The cytotoxicity of Jurkat T lymphocytes treated with different concentrations of BTP2 (I_{CRAC} inhibitor) (0.01–10 μM) for 24 (black circle), 48 (gray rectangle), and 72 (black triangle) h. The results showed that the viability of Jurkat T cells decreased only marginally even after treatments with 100 μM vitexin and BTP2; however, apigenin 7-O-glucoside exhibited cytotoxicity at a concentration of over 10 μM.

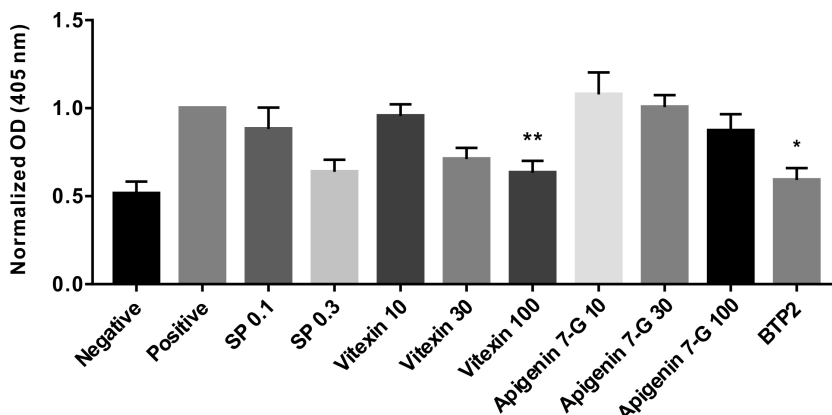


Figure 8. Inhibition of the activity of β -hexosaminidase in the IgE-activated RBL-2H3 mast cells. The activity of β -hexosaminidase activity is shown in the presence of the indicated compounds. The x-axis represents the concentration (μ M). The P values < 0.05 were considered significant (* $P < 0.05$ and ** $P < 0.01$).

apigenin 7-O-glucoside showed cytotoxicity at a concentration of 30μ M; 40% of the cell died 72-h after treatment with 30μ M apigenin 7-O-glucoside treatment (Fig. 7B).

Mast cell degranulation was investigated using the rat basophilic leukemia RBL-2H3 cell line. As shown in Fig. 8, the IgE-mediated degranulation of these cells increased the activity of β -hexosaminidase by 50% when compared with that of the positive control (assigned a value of 1). Vitexin concentration-dependently inhibited this degranulation by $4.4 \pm 6.6\%$, $29.0 \pm 6.4\%$, and $36.7 \pm 6.8\%$ at 10, 30, and 100μ M, respectively ($n = 5$). However, apigenin 7-O-glucoside only inhibited degranulation by $12.8 \pm 9.4\%$ at a concentration of 100μ M, and this effect was not statistically significant.

Overall, these results suggest that the SP_{ETOH} and vitexin can inhibit the I_{CRAC} , as well as effectively inhibit both T-cell proliferation and mast cell degranulation, which are subsequently activated by the activation of I_{CRAC} . In particular, vitexin has negligible cytotoxic effect. Therefore, it is expected that vitexin will be useful to develop immunosuppressive drugs.

Discussion

Exaggerated immune responses to allergic stimuli can lead to diseases, such as atopic dermatitis (eczema), allergic rhinitis, and allergic asthma; these conditions frequently occur in a single individual (Rhodes *et al.*, 2001; Sicherer and Leung, 2014). Current therapeutic management aims to reduce relapse and inflammation, and to alleviate symptoms, such as itching. Combinations of immunosuppressive agents, such as corticosteroids and antihistamines, are usually employed to inhibit the activation of lymphocytes and mast cells (Griffiths *et al.*, 2017; Okubo *et al.*, 2017). However, these agents can induce severe adverse effects and may not exhibit sufficient efficacy. Therefore, new strategies are needed to develop novel effective therapies for allergic diseases. From this

perspective, intracellular calcium signaling through the ORAI1 ion channel, which plays an important role in both T-cell activation and mast cell degranulation, provides a promising target for drug development in the future.

In the present study, we examined the effects of SP_{EtOH} on I_{CRAC} and cellular activities relevant to allergic disease. The whole-cell patch-clamping (Figs. 1 and 2) found that SP_{EtOH} effectively inhibited the I_{CRAC} in $\text{HEK}_{\text{ORAI1}}$ cells and T lymphocytes, although to a slightly less extent (69.7% vs. 55.1% inhibition at 1 mg/mL). This is probably due to the presence of different ORAI subtypes in the T cells. Recent reports suggest that naive T cells express more ORAI2 than ORAI1, and the intracellular calcium signal is changed by the expression ratio of these subtypes as cells differentiate. This modulates the magnitude of SOCE and regulates T-cell-mediated immune responses (Vaeth *et al.*, 2017).

The HPLC analysis confirmed the presence of five of the six previously reported chemical constituents of SP_{EtOH} : orientin, vitexin, luteolin 7-O-glucoside, apigenin 7-O-glucoside, and luteolin (Fig. 3). Based on the results of the quantitative analysis, we assessed whether these compounds inhibited the I_{CRAC} at the level present in 1 mg/mL SP_{EtOH} . Vitexin and apigenin 7-O-glucoside inhibited the I_{CRAC} . Vitexin is an apigenin flavone glucoside that has been shown to display a great variety of activities, including anti-oxidant, anticancer, anti-inflammatory, and neuroprotective effects (He *et al.*, 2016). Although the biological activities of apigenin 7-O-glucoside have not been studied extensively, it has been reported to show anti-oxidant, antidiabetes, and antiviral activities (Knipping *et al.*, 2012; Babenko and Kharchenko, 2015; Rocchetti *et al.*, 2017). However, to the best of our knowledge, no study has investigated whether these compounds inhibit the activation of ORAI1 ion channels. Vitexin (10–100 μM) exhibited a concentration-dependent inhibition of the Jurkat T cell I_{CRAC} by 29.7–48.9%. In contrast to that of the SP_{EtOH} , vitexin showed more potent I_{CRAC} inhibitory effects in the Jurkat T cells than in $\text{HEK}_{\text{ORAI1}}$ (Figs. 4 and 5). Apigenin 7-O-glucoside (10–100 μM) also exhibited a concentration-dependent inhibition of the I_{CRAC} (by 10.3–26.1%), although to an extent less than vitexin.

We also investigated whether these I_{CRAC} inhibitors within the SP_{EtOH} , vitexin and apigenin 7-O-glucoside, suppressed T cells and inhibited mast cell degranulation. The SP_{EtOH} and apigenin 7-O-glucoside strongly inhibited the proliferation of CD4+ T cells that were induced by the co-stimulation with anti-CD3 and anti-CD28 antibodies; however, some cytotoxicity was observed. Although vitexin was less effective than apigenin 7-O-glucoside, no cytotoxicity was observed (Fig. 6). Extensive studies on vitexin have not reported of cytotoxicity *in vitro* at concentrations of up to 200 $\mu\text{g/mL}$ (He *et al.*, 2016). Furthermore, even repeated treatments with a high dose of vitexin (10 mg/kg, intraperitoneally) over a 7-day period were safe in mice (Borghi *et al.*, 2013). Vitexin also showed strong inhibitory effects on mast cell degranulation, which is responsible for histamine release, whereas apigenin 7-O-glucoside did not exhibit any significant effect on this process (Fig. 8).

In summary, our electrophysiological study quantified the inhibitory effects of SP_{EtOH} and its five chemical constituents on the activation of ORAI1 channel, which is responsible for the I_{CRAC} . Calcium influx through the I_{CRAC} is critical for the initial calcium signaling

in CD4⁺ T-cell activation and mast cell degranulation. We found that vitexin significantly inhibited the I_{CRAC} and also effectively inhibited T-cell proliferation and mast cell degranulation, with no cytotoxic effects. Thus, vitexin is a candidate inhibitor of the ORAI1 channel, a key therapeutic target to treat allergic diseases. The present findings suggest that vitexin represents a potent candidate for the development of therapeutic agents to prevent and treat allergic diseases. Future studies should aim to increase the potency of this effect by synthesizing and analyzing structurally related compounds. The present study is expected to provide a strong basis to develop anti-allergic agents using oriental herbs in the future.

Acknowledgments

This research was supported by the Convergence of Conventional Medicine and Traditional Korean Medicine R&D program funded by the Ministry of Health & Welfare (Korea) through the Korean Health Industry Development Institute (KHIDI) [grant number HI16C0766, to W. K. Kim].

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