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Anti-allergenic activity of polymethoxyflavones from *Kaempferia parviflora*

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ABSTRACT

The inhibitory effects of 13 polymethoxyflavones (PMFs) obtained from *Kaempferia parviflora* extracts were evaluated on RBL-2H3 cell degranulation. These flavones suppressed antigen-induced degranulation in RBL-2H3 cells. 5-Hydroxy-3,7,4',-trimethoxyflavone (KP02) and 5,3',-dihydroxy-3,7,4',-trimethoxyflavone (KP10) showed potent inhibitory activities. KP10 significantly suppressed calcium ionophores, such as A23187 and 2,5-ditert-butylhydroquinone, which promoted calcium influx from the outside of cells and from the endoplasmic reticulum, respectively. However, these activities did not fully explain the PMFs' effects. Immunoblot analysis of KP02 and KP10 showed that the inhibitory effects were attributable to the downregulation of PLCγ1 kinase phosphorylation in the signaling pathways involved in degranulation. The phosphorylation levels of Syk were also suppressed by the addition of KP10. KP02 and KP10 treatment to the cells did not affect the high affinity IgE receptor (FcεRI) levels inside RBL-2H3 cells, but they suppressed the FcεRI levels in the plasma membrane. Therefore, *K. parviflora* PMFs have multiple degranulation mechanisms to suppress RBL-2H3 cells and would be beneficial for alleviating type I allergy symptoms.

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Abbreviations: anti-DNP IgE, anti-dinitrophenyl IgE; BSA, bovine serum albumin; DG, diacylglycerol; DTBHQ, 2,5-ditert-butylhydroquinone; ER, endoplasmic reticulum; FcεRI, high affinity IgE receptors; ITAM, immunoreceptor tyrosine-based activation motif; IP₃, inositol trisphosphate; LAT, linker for activation of T cells; MT, modified Tyrode's; PIP₂, phosphatidylinositol 4,5-bisphosphate; PMA, phorbol 12-myristate 13-acetate; PKC, protein kinase; PLC, phospholipase C; PMF, polymethoxyflavone

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1. Introduction

The incidence of allergic disorders has increased dramatically in developed countries (Yamada, Saito, & Fujieda, 2014). In particular, allergic rhinitis represents a global health care problem that greatly affects daily activity, work productivity, learning, sleep, and quality of life in persons of all ages (Yamada et al., 2014). Allergic reactions represent a dysregulated immune response to a multitude of allergens normally present either in the environment, such as dust and pollen, or in food, such as milk, eggs, and wheat (Campbell, Hill, & Kemp, 1998). A chronic use of drugs for allergic therapy is sometimes associated with adverse effects (Coureau, Bussi eres, & Tremblay, 2008). Ideally, an anti-allergic therapy should be beneficial against different allergic disorders i.e. atopic dermatitis, food allergies, and ultimately be safe on long-term administration. Recent scientific evidence from pre-clinical studies and human clinical trials has highlighted that daily polyphenol intake can improve allergic symptoms (Singh et al., 2011).

Allergy is mainly classified into four types. Among them, the type I allergy is the most common allergic reaction associated with asthma, hives, hay fever, and allergic dermatitis. During the initiation of type I allergic reaction, the allergen binds to IgE molecules, which then bind to high affinity IgE receptors (Fc RI) on the surfaces of mast cells and basophils. The cross-linking of the cell-bound IgE–Fc RI complex with a specific antigen causes the aggregation of Fc RI, which induces a variety of cellular responses, including the release of chemical mediators such as histamine, arachidonic acid metabolites, and cytokines (Kalesnikoff & Galli, 2008). The prevention of allergic diseases using food ingredients has been intensively studied and there is evidence of the preventive effect of dietary polyphenols against allergic diseases (Singh et al., 2011).

Kaempferia parviflora Wall ex. Baker belongs to the family Zingiberaceae. In Thailand, the rhizomes of *K. parviflora* have been used to treat allergies, gastrointestinal disorders, and peptic ulcers (Yenjai, Prasanphen, Daodee, Wongpanich, & Kittakoop, 2004). Tewtrakul, Subhadhirasakul, and Kummee (2008) reported the antiallergic effects of the 7 species of methoxyflavones from *K. parviflora* using RBL-2H3 cells, although detailed mechanism of the action remains unknown. Recently, we isolated and elucidated the structures of 14 polymethoxyflavones (PMFs) and 3 new phenolic glycosides from various *K. parviflora* extracts (Azuma, Kayano, Matsumura, Konishi, Tanaka, & Kikuzaki, 2011; Azuma, Tanaka, & Kikuzaki, 2008).

In recent studies, many PMFs have been shown to have a broad spectrum of biological activities, such as anti-oxidant, anti-inflammatory, and anti-carcinogenic properties (Chinapongtitiwat, Jongaroontaprangsee, Chiewchan, & Devahastin, 2013; Lee, Hsiao, Wright, Chong, Leow, & Ho, et al., 2013; Li, Pan, Lo, Tan, Wang, & Shahidi, et al., 2009). PMFs exclusively exist in the citrus genus which has been used as Chinese herbal medicine for thousands of years. Because nobiletin and tangeretin are the most abundant PMFs in citrus fruits, they have been the focus of primary research owing to their bioactivity (Lee et al., 2013; Li, Wang, Guo, Zhao, & Ho, 2014). Although PMFs have poor aqueous solubility, they have

high intestinal permeability and bioavailability, thereby gaining importance in clinical studies for the benefit of humans in disease prevention and treatment (Lee et al., 2013).

In the present study, we investigated the detailed mechanism of action using isolated PMFs in the degranulation of RBL-2H3 cells.

2. Materials and methods

2.1. Chemicals and reagents

Murine anti-dinitrophenyl IgE (anti-DNP IgE) and DNP-labeled human serum albumin were purchased from Sigma (St. Louis, MO, USA). Fc RI, Na⁺/K⁺-ATPase, PLC 1, p-PLC 1, PLC 2, p-PLC 2 Syk, and p-Syk antibodies were obtained from Cell Signaling Technology (MA, USA). All other chemicals were of reagent grade.

2.2. Cell culture

RBL-2H3 cells (Cell Bank, RIKEN BioResource Center, Tsukuba, Japan) were cultured in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum, 100 U/mL penicillin, and 100  g/mL streptomycin at 37  C in a humidified atmosphere containing 5% CO₂.

2.3. Standard method of antigen-induced  -hexosaminidase inhibitory assay

The release of  -hexosaminidase from activated RBL-2H3 cells was measured as previously described (Kobayashi & Tanabe, 2006). In brief, 2.5   10⁵ cells/well were cultured in a 24-well cell culture plate overnight at 37  C in a humidified 5% CO₂ atmosphere, before incubating for 2 h in growth medium that contained 50 ng/mL of anti-DNP IgE. After washing with modified Tyrode's (MT) buffer, the test sample or a positive control of wortmannin (25  M) was added. After incubating for 10 min, DNP-labeled human serum albumin, final concentration of 50 ng/mL, was added and the culture was incubated for another 30 min. After collecting each supernatant following centrifugation, the cells were extracted in 500  L MT buffer containing 0.1% Triton X-100 for 5 min on ice. The  -hexosaminidase activities of the supernatant and cell lysate were measured as previously reported (Kobayashi et al., 2006). The results were represented as the percentage release of  -hexosaminidase activity as follows:

$$100 \times (A_{\text{supernatant}}) / (A_{\text{supernatant}} + A_{\text{cell lysate}})$$

where A is the absorbance of each well. GraphPad Prism 6 (GraphPad Software, Inc., San Diego, CA, USA) was used to calculate IC₅₀.

2.4. Modified antigen-induced  -hexosaminidase inhibitory assay

After incubation overnight in a 24-well culture plate, RBL-2H3 cells were treated with flavonoids for 24 h. Incubation with anti-DNP IgE and stimulation with DNP-labeled human serum

albumin were performed as described in 2.3. standard method, except the flavonoids were not used. The β -hexosaminidase release rate was measured as in 2.3. standard method, described above.

2.5. Measurement of degradation with calcium (Ca^{2+}) ionophores

RBL-2H3 cells were seeded and treated with various concentrations of flavonoids, as described above, except that they were sensitized using anti-DNP IgE. Either 10 μL of 250 $\mu\text{g/mL}$ A23187, or 10 μL of 500 μM 2,5-ditert-butylhydroquinone (DTBHQ) mixed with 10 μL 0.5 mg/mL phorbol 12-myristate 13-acetate (PMA), was added to each well and the cells were incubated at 37 °C for 30 min. The rate of β -hexosaminidase release was measured as in 2.3. standard method.

2.6. Western blot analysis

RBL-2H3 cells (1.5×10^6 cells) were seeded into 3.5-cm culture dishes. The protein concentration was determined using the Bradford method with bovine serum albumin (BSA) as the standard (Bradford, 1976). A homogenate protein sample (30–40 μg) and plasma membrane protein sample (9 μg) were loaded onto a SDS-PAGE gel (5–7.5%), electrophoresed for 1.5 h, and transferred onto a polyvinylidene fluoride membrane (Millipore, Bedford, MA, USA). The membrane was blocked with 5% BSA and incubated for 1 h at ambient temperature with each primary antibody and for another 1 h with the secondary antibody conjugated to horseradish peroxidase. The proteins were visualized using an enhanced chemiluminescence detection method with the Clarity Western ECL Substrate (Bio-Rad, Hercules, CA, USA). The intensity of each band was determined using the ImageJ software (National Institutes of Health, Bethesda, MD, USA). Each density of the target protein was normalized against that of actin, except for plasma membrane proteins which were normalized against Na^+/K^+ -ATPase. Plasma membrane fractions of RBL-2H3 cells were purified using a Plasma Membrane Protein Extraction Kit from BioVision (Mountain View, CA, USA).

2.7. Statistical analysis

Data were represented as the mean $\pm$ SD. Statistical analyses were performed using one-way analysis of variance and Dunnett's or Fisher's test to identify significant differences between groups.

3. Results and discussion

3.1. Effects of *K. parviflora* extracts on the antigen-mediated degranulation of RBL-2H3 cells

We examined the effects of *K. parviflora* extracts on the antigen-mediated degranulation of RBL-2H3 cells. Table 1 shows that the CH_2Cl_2 extract and EtOAc soluble fraction had potent inhibitory effects.

3.2. Effects of *K. parviflora* flavones on the antigen-mediated degranulation of RBL-2H3 cells

Next, we studied the effects of the flavones isolated from the CH_2Cl_2 extract and EtOAc soluble fraction. Previously, we reported the identification of PMFs isolated from a CH_2Cl_2 extract of *K. parviflora* and an EtOAc-soluble fraction (Azuma et al., 2011). The chemical structures are shown in Fig. 1. All the flavones have 7-methoxyflavone as their basic structure and they have inhibitory effects at 100 μM (Table 2A). We hypothesized that these flavones may inhibit the activity of β -hexosaminidase. Then, we measured the inhibitory activities of 5-hydroxy-3,7,4'-trimethoxyflavone (KP02) and 5,3'-dihydroxy-3,7,4'-trimethoxyflavone (KP10) using the cell lysate from RBL-2H3. As a result, KP02 and KP10 both did not inhibit β -hexosaminidase at 100 μM (data not shown).

We could not calculate the IC_{50} values for KP04, KP11, KP12, and KP13. The flavones were classified into three groups based on their activity levels. The highest activity group, where $\text{IC}_{50} < 15 \mu\text{M}$, comprised KP01, KP02, KP07, KP08, KP09, and KP10. In these flavones, a hydroxyl group was substituted at C5. The group with the second highest activity levels, where $\text{IC}_{50} < 100 \mu\text{M}$, comprised KP05 and KP06. In these flavones, a methoxyl group was substituted at C5 but C3 was not substituted simultaneously. The structure characteristics of KP12 and KP13 placed them in second group, but their activities were low. The group with the lowest activity levels, where $\text{IC}_{50} > 100 \mu\text{M}$, comprised KP03, KP04, and KP11. In these flavones, methoxyl groups were substituted at both C3 and C5.

Matsuda et al. screened over 50 types of flavonoids based on their anti-degranulation activity using RBL-2H3 cells (Mastuda, Morikawa, Ueda, Managi, & Yoshikawa, 2002). They also reported the activities of the 7-methoxyflavone derivatives. When the hydroxyl group was substituted at C5, substitutions with methoxyl groups at both C3' and C4' resulted in higher activity levels than substitutions with methoxyl groups at C4'. This structure–activity relationship remained the

Table 1 – Effects of *K. parviflora* extracts on the degranulation of RBL-2H3 cells.

	Secretion (% of control)			
	0 $\mu\text{g/mL}$	100 $\mu\text{g/mL}$	500 $\mu\text{g/mL}$	1000 $\mu\text{g/mL}$
CH_2Cl_2 extract	100.0 $\pm$ 0.6	32.9 $\pm$ 1.8**	7.2 $\pm$ 0.1**	14.0 $\pm$ 1.6**
EtOAc soluble fraction	100.0 $\pm$ 1.3	32.8 $\pm$ 1.3**	11.2 $\pm$ 0.2**	16.0 $\pm$ 1.7**
H_2O soluble fraction	100.0 $\pm$ 2.5	106.7 $\pm$ 5.2	101.4 $\pm$ 2.9	100.3 $\pm$ 2.0
Antigen-mediated degranulation was induced using lower than 1000 $\mu\text{g/mL}$ of the CH_2Cl_2 extract and EtOAc- and H_2O -soluble fractions. Released β -hexosaminidase was used as a marker of degranulation. The values represent the mean $\pm$ standard error (n = 3). Statistical analyses were performed using Dunnett's multiple comparisons test (**P < 0.01).				

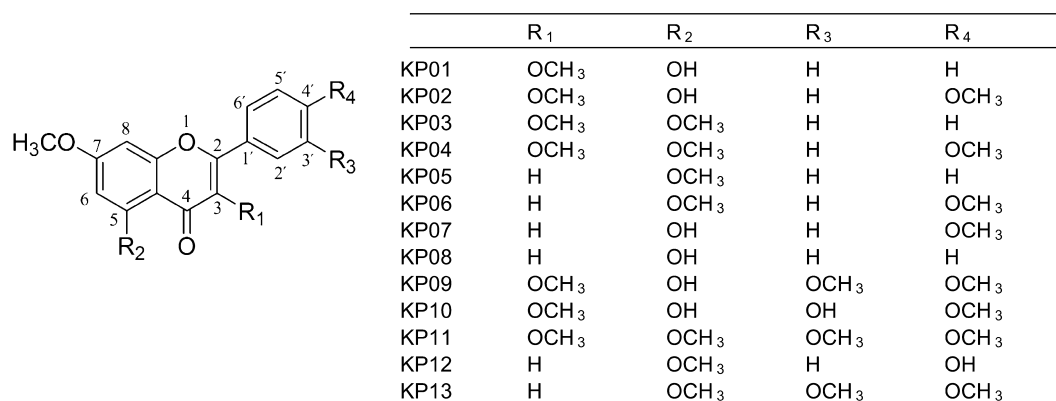


Fig. 1 – The chemical structures of flavanones in the CH₂Cl₂ extract and EtOAc-soluble fraction obtained from *K. parviflora*.

same if the C5 hydroxyl group was changed into a methoxyl group. Our data agreed with the structure–activity relationships reported by Mastuda et al. (2002).

3.3. Effects of *K. parviflora* flavones on the antigen-mediated degranulation of RBL-2H3 cells in modified method

When RBL-2H3 cells were stimulated by the antigen with flavones, it was possible that the flavones simply inhibited the binding of the antigen and antibody. Then, we modified the degranulation assay method, where the antigen was added to the RBL-2H3 cells after preincubation overnight before washing away the flavones. To the best of our knowledge, we were the first to show this modified method. In this modified degradation test, most of the flavones also significantly inhibited degradation at a concentration of 30 μ M or 100 μ M, except for KP11 and KP13 (Table 2B). It was then possible to calculate the IC₅₀ values for KP02, KP07, KP09, and KP10. These data suggest that the *K. parviflora* flavones affected the RBL-2H3 cells. These flavones were polymethoxylated, and therefore were hydrophobic compounds. LogP is a hydrophobic index, where values >2 are considered to indicate high hydrophobicity (Shichijo, Yamamoto, Tsujishita, Kimata, Nagai, & Kokubo, 2003). We thus calculated the LogP values using the Marvin Sketch program (<http://www.chemaxon.com/marvin/>). All the flavones from *K. parviflora* had LogP values that exceeded 2. This suggests that these flavones may be readily absorbed into cells and may function inside the cells.

3.4. Comparison of the effects of *K. parviflora* flavones and other flavonoids

We compared the activities of *K. parviflora* flavones with those of other flavonoids, such as quercetin, hesperetin, (–)-epigallocatechin-3-O-gallate (EGCG), and genistein, with which we determined the anti-degradation activity using the RBL-2H3 cell assay. Quercetin, hesperetin, EGCG, and genistein significantly inhibited degranulation at 100 μ M (Table 2A). We were able to calculate IC₅₀ for quercetin only. However, KP01, KP02, KP07, KP08, KP09, and KP10 had lower IC₅₀ values than quercetin. Furthermore, it was not possible to calculate the IC₅₀ values for the flavonoids tested using the modified degranulation assay (Table 2B). Our data suggested that the *K. parviflora*

flavones had higher activity levels than the other common flavonoids reported to have antidegradation activities (Kaneko, Kanesaka, Yoneyama, Tominaga, Jirillo, & Kumazawa, 2010; Maeda-Yamamoto, Inagaki, Kitaura, Chikumoto, Kawahara, & Kawakami et al., 2004; Tedeschi, Lorini, Galbiati, Gibelli, & Miadonna, 2000). Quercetin and genistein are reported to inhibit the PKT phosphorylation signaling pathway (Kaneko et al., 2010; Tedeschi et al., 2000). Therefore, the *K. parviflora* PMFs may also inhibit this cell signaling pathway.

3.5. Effects of KP02 and KP10 on Ca²⁺ ionophore-induced degranulation of RBL-2H3 cells

To clarify the mechanisms that underlie the inhibitory effects of the *K. parviflora* flavones on antigen-stimulated degranulation, we determined the degranulation-mediated Ca²⁺ influx downstream of degranulation using the highest inhibitory activity, where KP10 and KP02 had the highest inhibitory activity at standard method and modified method, respectively (Table 2). We then selected KP02 and KP10 to elucidate the inhibitory mechanisms and measured the anti-degranulation effect stimulated by A23187, which is a Ca²⁺ ionophore that promotes the influx of Ca²⁺ from outside the cell (Table 3A). KP02 did not have an inhibitory effect, whereas KP10 significantly inhibited degranulation. Next, we measured the anti-degranulation effect stimulated by DTBHQ, which is a Ca²⁺ ionophore that promotes Ca²⁺ influx from the endoplasmic reticulum (ER). DTBHQ stimulation alone could not induce degranulation; therefore, both DTBHQ and PMA, an activator, were added to RBL-2H3 cells. The DTBHQ and PMA stimulation assay was also performed using KP02 and KP10 (Table 3B). We found that KP10 had a higher inhibitory activity than KP02 in this assay.

Licochalcone D and kaempferol have been reported to inhibit the degranulation caused by Ca²⁺ ionophores (Itoh, Ninomiya, Yasuda, Koshikawa, Deyashiki, & Nozawa, et al., 2009; Tanifuji, Aizu-Yokota, Funakoshi-Tago, Sonoda, Inoue, & Kasahara, 2010). In the present study, KP10 exhibited significant inhibitory effects after stimulation with both A23187 and DTBHQ, but the inhibitory effect was lower compared with that on antigen-stimulated degranulation. Furthermore, KP02 did not inhibit the stimulatory effect of A23185. These data suggest that KP02 and KP10 mainly inhibit in the different mechanisms from degranulation-mediated Ca²⁺ influx.

Table 2 – Effects of *K. parviflora* flavones, quercetin, hesperetin, (–)-Epigallocatechin-3-O-gallate (EGCG), and genistein on the antigen-stimulated degranulation of RBL-2H3 cells: (A) using the standard method; (B) using the modified method.

(A) Standard method	Secretion (% of control)				IC ₅₀ (μM)
	0 μM	10 μM	30 μM	100 μM	
KP01	100.0±3.9	36.3±6.4**	30.3±1.3**	38.4±3.4**	4.1
KP02	100.0±1.4	40.5±3.0**	21.7±1.2**	21.0±0.5**	7.2
KP03	100.0±1.4	96.2±5.8	90.2±5.9	71.7±1.6**	>100 μM
KP04	100.0±10.9	104.9±8.7	97.2±1.4	54.5±4.5**	–
KP05	100.0±6.9	97.2±6.0	73.7±3.3**	37.8±4.6**	69.2
KP06	100.0±10.9	87.5±6.3	58.2±14.4**	40.5±2.6**	46.7
KP07	100.0±3.9	52.2±2.8**	36.4±2.2**	39.1±2.1**	11.0
KP08	100.0±3.9	41.4±1.6**	31.3±1.0**	32.7±1.7**	6.7
KP09	100.0±2.2	40.5±5.4**	38.2±2.0**	32.5±5.9**	5.9
KP10	100.0±2.2	15.4±1.2**	7.4±0.4**	0.1±0.2**	3.5
KP11	100.0±2.2	84.8±5.4	86.3±3.5	74.0±2.9**	–
KP12	100.0±0.7	77.2±10.1	76.6±8.9	67.4±4.5**	–
KP13	100.0±0.7	76.4±4.6	59.1±1.5	49.6±4.2**	–
Quercetin	100.0±7.1	61.3±10.2**	38.2±0.8**	55.6±10.2**	23.3
Hesperetin	100.0±6.2	101.1±4.5	90.5±4.5*	46.6±1.0**	–
EGCG	100.0±2.9	96.2±3.4	101.1±5.8	92.1±2.6*	–
Genistein	100.0±2.9	71.6±0.7**	57.7±2.1**	62.1±2.2**	–
(B) Modified method	Secretion (% of control)				IC ₅₀ (μM)
	0 μM		30 μM	100 μM	
KP01	100.0±8.5		68.7±5.3**	56.7±3.2**	–
KP02	100.0±6.9		45.2±1.1**	21.6±0.9**	25.5
KP03	100.0±8.6		82.8±7.2*	89.2±10.0	–
KP04	100.0±8.5		56.4±1.5**	63.5±9.8**	–
KP05	100.0±8.5		77.2±12.8*	102.1±2.9	–
KP06	100.0±8.5		64.8±4.8**	52.9±8.7**	–
KP07	100.0±8.6		56.0±1.6**	42.7±4.4**	42.0
KP08	100.0±7.2		89.9±4.5*	23.9±1.9**	–
KP09	100.0±7.2		58.4±2.1**	27.9±1.7**	40.3
KP10	100.0±7.2		49.5±6.5**	24.9±2.2**	29.5
KP11	100.0±8.6		95.6±9.2	107.3±11.1	–
KP12	100.0±8.6		84.8±8.1	75.7±9.4*	–
KP13	100.0±7.2		100.9±10.6	91.9±5.1	–
Quercetin	100.0±7.1		96.2±13.2	78.0±4.6*	–
Hesperetin	100.0±7.1		110.9±6.0	90.1±5.3	–
EGCG	100.0±7.8		93.0±4.6	103.9±7.4	–
Genistein	100.0±7.8		71.9±4.6**	62.9±5.0**	–

Values represent the mean ± standard error (n = 3). Statistical analyses were performed using Dunnett's multiple comparisons test (*P < 0.05; **P < 0.01).

Table 3 – Effects of KP02 and KP10 on calcium ionophore-induced degranulation in RBL-2H3 cells. (A) 5 μg/mL A23187; (B) 5 μM 2,5-ditert-butylhydroquinone (DTBHQ) and phorbol 12-myristate 13-acetate (PMA).

(A)	Secretion (% of control)				IC ₅₀ (μM)
	0 μM	10 μM	30 μM	100 μM	
A23187					
KP02	100±5.4	104.8±5.1	98.1±7.7	97.5±8.2	–
KP10	100±2.6	72.0±3.3**	46.8±2.2**	30.2±1.8**	25.7
(B)	Secretion (% of control)				IC ₅₀ (μM)
	0 μM	10 μM	30 μM	100 μM	
DTBHQ + PMA					
KP02	100±3.0	101.1±2.6	90.3±3.3	87.0±3.4*	–
KP10	100±3.5	102.8±0.7	73.5±1.7**	38.3±2.1**	68.82

The values represent the mean ± standard error (n = 3). Statistical analyses were performed using Dunnett's multiple comparisons test (*P < 0.05; **P < 0.01).

3.6. Effect of KP02 and KP10 on the signaling pathways involved in antigen-stimulated degranulation

We used immunoblot analyses to examine how KP02 and KP10 influence the signaling pathways involved in antigen-stimulated degranulation. The signaling pathways related to degranulation involve phosphorylation of the Src family nonreceptor tyrosine kinases Lyn after cross-linkage of the antigen to FcεRI via IgE. The activated Lyn kinase then phosphorylates the β- and γ-chains of immunoreceptor tyrosine-based activation motif (ITAM), and the activated γ-ITAMs recruit and phosphorylate the Syk kinase to initiate the signaling cascade (Kalesnikoff & Galli, 2008; Kopeć, Panaszek, & Fal, 2006; Kraft & Kinet, 2007; Metcalfe, Peavy, & Gilfillan, 2009). The activated Syk then phosphorylates the adaptor molecule LAT (linker for activation of T cells), before the activated LAT forms a complex with other adaptor proteins such as phospholipase C (PLC) γ1/2 (Saitoh, Arudchandran, Manetz, Zhang, Sommers, & Love et al., 2000). The phosphorylated PLCγ1/2 in the complex hydrolyzes phosphatidylinositol 4,5-bisphosphate to inositol

1,4,5-triphosphate, which is an inducer of intracellular Ca²⁺ mobilization, and diacylglycerol (DG) then activates protein kinase C (PKC) (Kopeć et al., 2006). First, to determine the optimum stimulation time for detecting phosphorylation, we tested a time course of the phosphorylation of PLCγ1/2 during several stimulation periods (Fig. 2A). PLCγ1 and -2 were phosphorylated most at 1–2 min and 0.5–1 min, respectively. Therefore, we used the detection time of 1 min. As shown in Fig. 2B and C, the phosphorylation levels of PLCγ1 were suppressed by the addition of KP02 and KP10. The phosphorylation levels of Syk were also suppressed by the addition of KP10. These results suggest that KP02 and KP10 suppress antigen-induced degranulation in RBL-2H3 cells by downregulating the signaling pathways involved in degranulation.

3.7. Effects of KP02 and KP10 on the cell surface expression of FcεRI

The affinity receptor for IgE, FcεRI, initiates the signaling events in mast cells. Multivalent allergens can crosslink

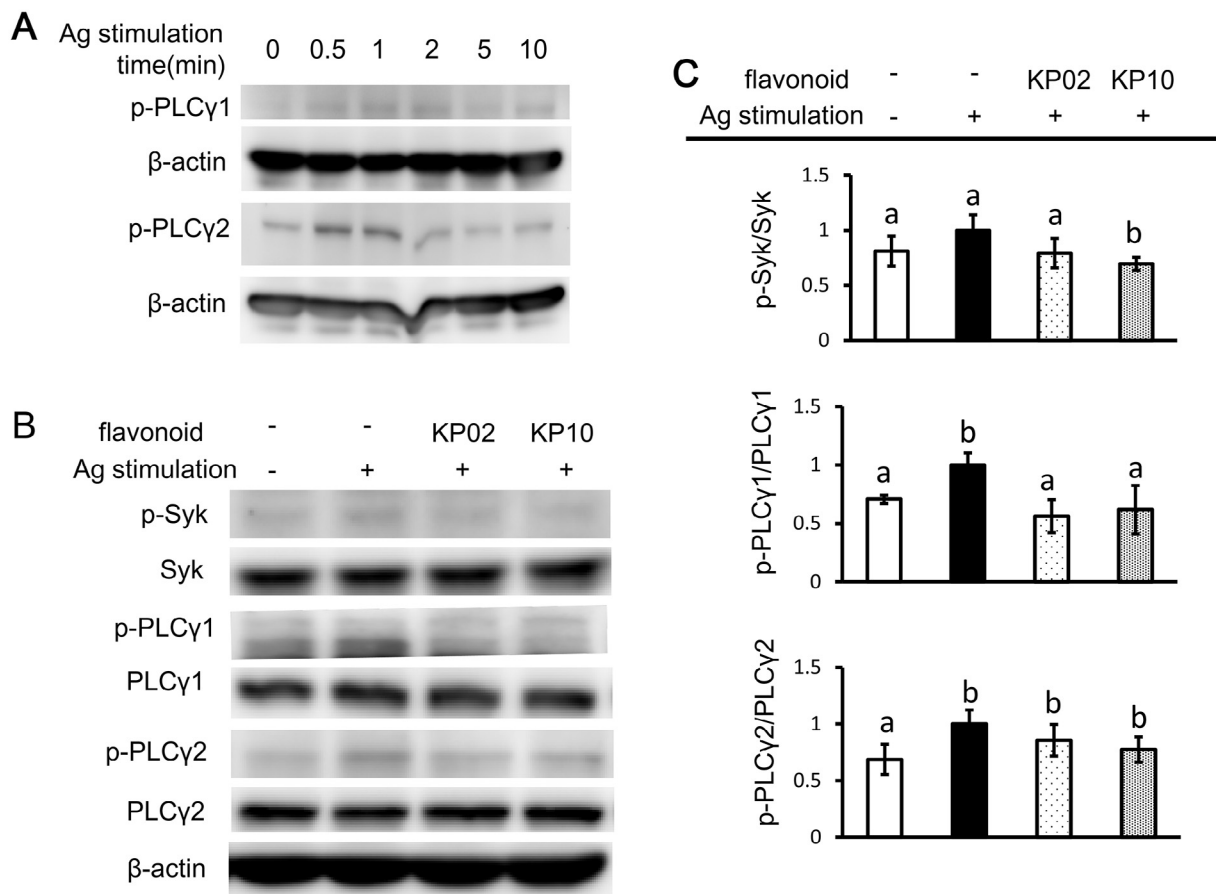


Fig. 2 – Effects of KP02 and KP10 on the signaling pathway involved in the degranulation of RBL-2H3 cells stimulated by antigen. (A) After antigen-induced stimulation, RBL-2H3 cells were stimulated with antigen for the times indicated and lysates were used to prepare blots with antiphospho PLCγ1 and 2. (B) After sensitization and 1 h of incubation with flavonoids, the RBL-2H3 cells were stimulated with the antigen for 1 min. Each cell lysate was subjected to immunoblot analysis. In the figure, p-PLCγ 1, p-PLCγ 2, and p-Syk represent phosphorylated PLCγ 1, PLCγ 2, and Syk, respectively. Three independent experiments were performed, with the representatives shown. (C) The ratio of p-Syk/Syk, p-PLCγ1/PLCγ1, and p-PLCγ2/PLCγ2 in (B). Statistical analyses were performed using Fisher's exact test. Means not labeled with a common letter differ at a time point; $P < 0.05$.

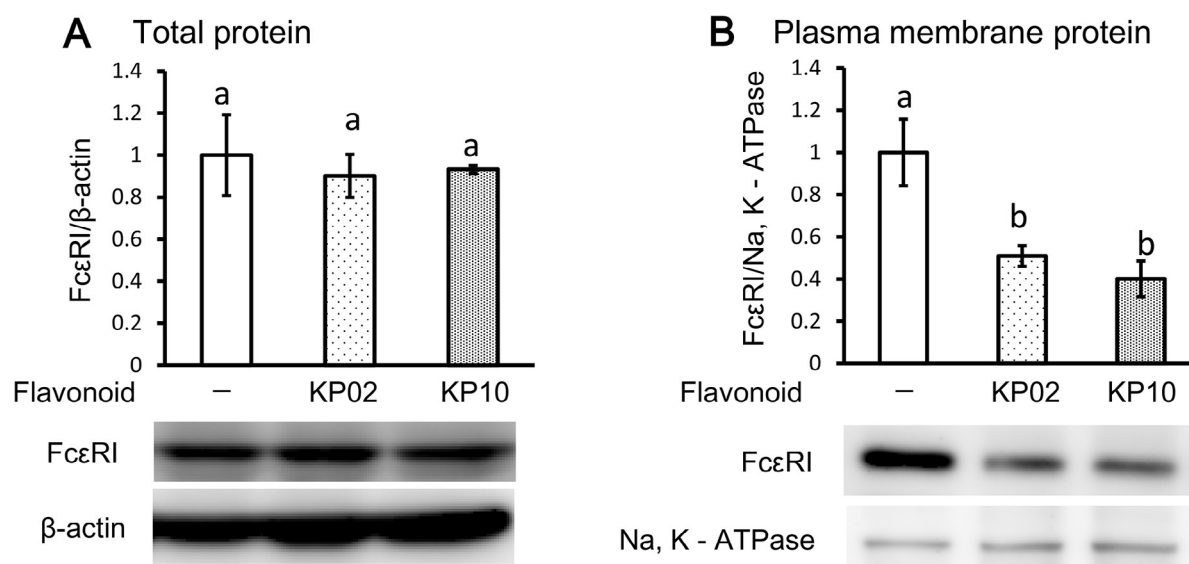


Fig. 3 – Effects of polymethoxyflavone on the cell surface expression of FcεRI. (A) After preincubation with 100 μM KP02 and KP10, the RBL-2H3 cells were lysed. Each cell lysate was submitted to immunoblot analysis. **(B)** After preincubation with 100 μM KP02 and KP10, the plasma membrane was isolated from RBL-2H3 cells. Each plasma membrane fraction was submitted to immunoblot analysis. Three independent experiments were performed, with the representatives shown. Statistical analyses were performed using Fisher's exact test. Means not labeled with a common letter differ at a time point; $P < 0.05$.

allergen-specific IgE bound by FcεRI to initiate signaling events that ultimately lead to mast cell activation, thereby resulting in the secretion of allergic mediators and induction of gene transcription for various inflammatory cytokines (Abramson, Licht, & Pecht, 2006). Signal transduction via FcεRI plays an essential role in IgE-mediated allergic responses; therefore, the expression of FcεRI is critical for the antigen-dependent activation of mast cells. Furthermore, the *K. parviflora* flavones had high anti-degranulation effects after preincubation overnight (Table 2B). In this study, we investigated the effects of KP02 and KP10 on the surface expression of FcεRI in RBL-2H3 cells. The cells were cultured overnight with 100 μM KP02 and KP10 before the total cell FcεRI and cell surface FcεRI levels were analyzed by western blotting. The treatments with KP02 and KP10 did not affect the FcεRI levels in RBL-2H3 cells (Fig. 3A). However, the treatments with KP02 and KP10 suppressed the FcεRI levels in the plasma membrane of RBL-2H3 cells (Fig. 3B). These data suggest that KP02 and KP10 suppress the translocation of FcεRI. The mRNA expression of FcεRIα did not change (data not shown). EGCG and (-)-epigallocatechin-3-O-(3-O-methyl)-gallate (EGCG3'Me) suppress FcεRI expression in human basophilic KU812 cells (Fujimura, Umeda, Yamada, & Tachibana, 2008). Both EGCG and EGCG3'Me suppress the expression of both the mRNA and protein of FcεRI. These data suggest that the *K. parviflora* flavones suppress FcεRI on the cell surface by different mechanisms compared with EGCG and EGCG3'Me.

inhibitory effects of KP02 and KP10 on antigen-stimulated degranulation in RBL-2H3 cells were demonstrated and these effects were related to the suppression of degranulation due to Ca^{2+} influx, phosphorylation of Syk and PLCγ1, and translocation of FcεRI to the cell surface (Fig. 4). *K. parviflora* and PMFs may be beneficial for ameliorating the symptoms of type I allergic responses.

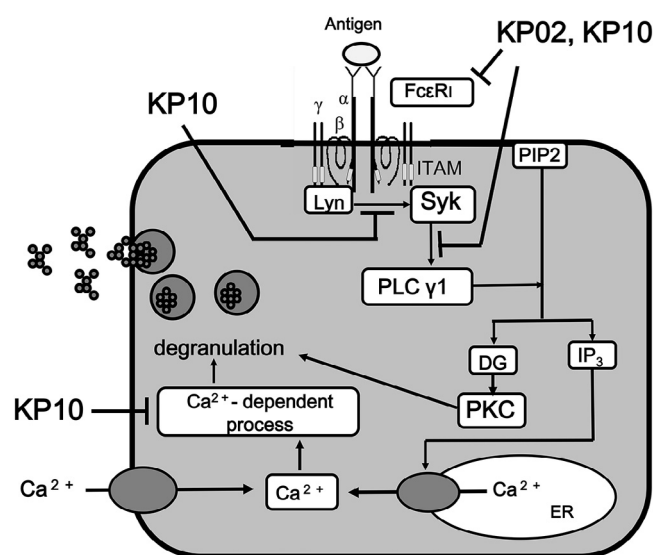


Fig. 4 – Scheme showing the inhibitory effects of KP02 and KP10 on antigen-stimulated degranulation in RBL-2H3 cells. DG, diacylglycerol; ER, endoplasmic reticulum; IP₃, inositol trisphosphate; ITAM, immunoreceptor tyrosine-based activation motif; PIP₂, phosphatidylinositol 4,5-bisphosphate; PKC, protein kinase; PLC, phospholipase C.

4. Conclusions

PMFs isolated from *K. parviflora* were found to be capable of inhibiting antigen-stimulated degranulation in RBL-2H3 cells. The

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