Sophora Flavescens Suppresses Degranulation and Pro-inflammatory Cytokines Production through the Inhibition of NF-κB (p65) Activation in the RBL-2H3 cells

Ji Hyo Lyu[1], Sang Eun Park[2], Su Hyun Hong[2], Dong Kyu Kim[2], Woo Shin Ko[1,2], Sang Hoon Hong[1,2*]

1:Clinical Research Center of Oriental Medicine, 2:Department of Oriental Medicine, College of Oriental Medicine, Dongeui University

Sophora flavescens, as a traditional herbal medicine, has been used to treat with a variety of disesases. In previous reports, *S. flavescens* and sophoraflavanone G (a prenylated flavonoid from *S. flavescens*) inhibited cytokines productions in LPS-induced Raw 264.7 macrophages cells and BV2 microglial cells. We examined on the anti-allergic effect of *S. flavescens* on the PMA plus A23187-induced rat leukemia (RBL-2H3) cells. *S. flavescens* inhibited the release of β-hexosaminidase and productions and expressions of tumor necrosis factor (TNF)-α, interleukin (IL)-4 and cyclooxygenase (COX)-2 in a dose-dependent manner on stimulated RBL-2H3 cells, however, *S. flavescens* not affect cell viability. The protein expression level of nuclear factor (NF)-κB (p65) was decreased in the nucleus and suppressed the degradation of inhibitory protein IκB-α protein, the activation of extracellular signal-regulated kinases (ERK) mitogen-activated protein kinase (MAPK) by *S. flavescens*. These results suggest that *S. flavescens* could be involved anti-allergic effect by control of NF-κB (p65) translocation into the nucleus through inhibition of IκB-α degradation and suppression of pro-inflammatory cytokines expression.

Key words : *Sophora flavescens*, pro-inflammatory cytokines, nuclear factor kappa B, Inhibitor kappa B, ERK Mitogen-Activated protein kinase

Introduction

The primary cells of the immune system that are involved in the allergic response are mast cells, basophils, and eisonophils. Mast cells are normally distributed throughout the connective tissue and are involved in the immediate phase of immunoglobulin E (IgE)-mediated allergic reactions[1-3]. The binding of antigens to receptor-bound IgE and the subsquent cross-linking of the high-affinity IgE receptor (FcεR I) is the classical trigger of mast cell activation. Activated mast cells exert their biological effects by releasing preformed and de novo-synthesized mediators, such as histamine, β -hexosaminidase, leukotrienes, prostaglandins, and several pro-inflammatory cytokines[4-7].

Interleukin (IL)-3, IL-4, and tumor necrosis factor-alpha (TNF-α) may facilitate IgE production by B cells[8,9]. TNF-α, one of the pleotropic cytokines, may have an important amplifying effect in asthmatic inflammation and potently stimulates airway epithelial cells to produce cytokines[10,11]. IL-4, a representative Th2 cytokine, plays a pathologic role in the onset of various allergic diseases[12]. Cyclooxygenase (COX) is involved in the inflammatory process and catalyzed the rate-limiting step in the synthesis of prostaglandins from arachidonic acid. COX-1 is expressed constitutively in most tissues and appears to be responsible for maintaining normal physiological functions. Whereas, COX-2 is detected in only certain types of tissues and is induced transiently be growth factors, pro-inflammatory cytokines, tumor promotors, and bacterial toxin[13-16].

Transcription factors belonging to the nuclear factor (NF)-κB family regulate a range of genes that mediate inflammation and cell survival[17]. NF-κB normally resides in the cytoplasm, where it is retained by association with the endogenous inhibitor protein IκB. However, when activated, NF-κB translocates to the nucleus, binds the DNA, and activates genes. The activation involves the phosphorylation, ubiquitination, and degradation of IκB, leading to the nuclear migration of NF-κB[18-21]. Mitogen-activated protein kinases (MAPKs) and NF-κB have important activities as mediators of cellular responses to

* To whom correspondence should be addressed at : Sang Hoon Hong, Clinical Research Center of Oriental Medicine, Dongeui University, Busan, 614-054, Korea

· E-mail : shhong@deu.ac.kr, · Tel : 051-850-7444

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extracellular signals. Some of the MAPKs important to mammalian cells include p38, c-jun N-terminal kinase (JNK), and extracellular signal-regulated kinase (ERK)[22,23].

Sophora flavescens, as a traditional herbal medicine, has been used to treat with a variety of disesases. Previously, *S. flavescens* inhibited cytokines productions in LPS-induced Raw 264.7 cells and BV2 cells[24,25]. However, study on anti-allergic effect of *S. flavescens* in mast cells has not been identified. We used rat leukemia (RBL-2H3) cells to investgate the effect of the *S. flavescens* on the degranulation, pro-inflammatory cytokines secretion and expression, NF-κB activation, IκB-α degradation, and MAPKs activation.

Materials and Methods

1. Preparation of *S. flavescens*

The S. flavescens was identified and authenticated by Professor W.S. Ko, College of Oriental Medicine, Dongeui University (Busan, Korea). *S. flavescens* a one day dose for human adults were boiled with distilled water at 100 ℃, and the whole mixture is decocted until the volume is reduced by half. The extract water (400 ㎖) was filtered through 0.22 ㎛ filter and the filtrate was freeze-dried (yield, 5.53 g) and kept at 4℃. The dried filtrate was dissolved in phosphate buffered saline (PBS) and filtered through 0.22 ㎛ filter before use.

2. Reagents

Phorbol 12-myristate 13-acetate (PMA), calcium ionophore A23187, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and p-nitro-phenyl-N-β-D-glucosaminide were purchased from Sigma Chemical Co. (St. Louis, MO). Dulbecco's Modified Eagle's Medium (DMEM) containing L-glutamine (200 mg/L) and FBS were purchased from Hyclone (Logan, UT). TNF ELISA kit (BD OptEIATM Rat TNF ELISA Set), IL-4 ELISA kit (BD OptEIATM Rat IL-4 ELISA Set) were purchased from BD Biosciences (Franklin Lakes, NJ). Anti-NF-κB (p65), IκB-α, β-actin, p38, ERK, JNK, and phosphorylated-p38, -ERK, -JNK polyclonal antibodies were purchased from Cell Signaling Technology (Beverly, MA). Phosphatase labeled affinity purified antibody to rabbit IgG and BCIP/NBT phosphatase substrate were purchased from KPL (Gaithersburg, MD).

3. Cells culture

RBL-2H3 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) with 10 % (v/v) heat-inactivated fetal bovine serum (FBS), 100 U/㎖ Penicillin and 100 ㎍/㎖ Streptomycin in a humidified incubator with 5 % CO_2. In all experiments, RBL-2H3 cells were treated for 1 h with the presence of the indicated concentrations of *S. flavescens* prior to stimulation with 50 nM PMA plus 1.0 μM A23187 in serum-free DMEM.

4. MTT assay

The cell viability of *S. flavescens* was assessed using the MTT assay[26] in the remaining cells after Griess reaction. The MTT solution (0.5 ㎎/㎖) was added to each well. After incubation for 3 h at 37 ℃ and 5 % CO_2, the supernatant were removed and formed formazan crystals in viable cells were measured at 540 ㎚ with a microplate reader. The percentage of cell viability was calculated against untreated cells. All experiments were performed in triplicate well.

5. β-hexosaminidase assay

β-hexosaminidase was measured in both supernatant and pellet fractions using a previously reported method[7]. Briefly, RBL-2H3 cells (3×10^5 cells) were treated for 1 h with the presence of the indicated concentrations of *S. flavescens* prior to stimulation with 50 nM PMA plus 1 μM A23187 and incubated at 37 ℃ for 60 min. After stimulation, 50 ㎕ of each sample was incubated with 50 ㎕ of 1 mM p-nitro-phenyl-N-β-D-glucosaminide dissolved in 0.1 M citrate buffer, pH 5, in 96 well microtiter plate at 37 ℃ for 1 h. The reaction was terminated with 200 ㎕/well of 0.1 M carbonate buffer, pH 10.5. The plate was read at 405 ㎚ in an ELISA reader. The inhibition percentage of β-hexosaminidase release was calculated using the following equation :

$$\beta\text{-hexosaminidase release}(\%) = \frac{A_{405} \text{ of sup.}}{A_{405} \text{ of sup.} + A_{405} \text{ of pellet}} \times 100$$

where is A405 is absorption of measured at 405 ㎚ and sup. is supernatant.

6. Enzyme-linked immunosorbent assay for pro-inflammatory cytokines (TNF-α, IL-4)

Each cytokines concentration in RBL-2H3 cells were measured with commercially available Rat TNF, IL-4 ELISA kit (BD Biosciences), according to the manufacture's protocol. Color development was measured at 450 ㎚ using an automated microplate ELISA reader.

7. Isolation of total RNA from cells and Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

Total RNA was isolated as per the manufacture's instructions. Briefly, cells were lysed additional Trizol reagent

(Invitrogen, Carlsbad, CA) and the cell lysate was passed through the pipette several times. 0.2 ㎖ of chloroform was added per 1 ㎖ of Trizol reagent. The tubes were shaken vigorously and incubated at room temperature for 2-3 min. The samples were centrifuged at 14,000 g for 20 min. The aqueous phase was transferred to a fresh tube and RNA was precipitated by the addition of 0.5 ㎖ isopropanol. The RNA pellet was air-dried and resuspended in nuclease-free water. The concentration of RNA was estimated spectrophotometrically. Three microgram RNAs were reverse-transcribed using M-MLV reverse transcriptase (Promega, Madison, WI). Single stranded cDNA was amplified by PCR with primers(Table 1).

PCR amplifications were done in a 20 ㎕ PCR PreMix (Bioneer Co., Korea) containing 10 mM Tris-HCl, 40 mM KCl, 1.5 mM $MgCl_2$, 250 μM dNTP, 1 unit of Taq polymerase. Amplifications were carried out in a PCR machine (ASTEC PC802) using an initial denaturation at 95 ℃ for 5 min followed by 30 cycles (TNF-α, COX-1 : 35 cycles) of denaturation for 60 sec at 95 ℃, annealing for 60 sec at 52 ℃ (COX-1, COX-2 : 55 ℃) and extension for 60 sec at 72 ℃. This was concluded with a final extension for 7 min at 72 ℃. Amplicons were separated in 1 % agarose gels in 0.5× TBE buffer at 100 V for 30 min, stained with ethidium bromide and visualised under UV light. GAPDH (Glyceraldehyde-3-phosphate dehydrogenase) was used as an internal control to evaluate relative expressions of TNF-α, IL-4 and COX-2.

Table 1. Oligonucleotide primers used for PCR in this study.

Target gene	Oligonucleotide sequences (5' to 3' direction)	Expected size	Accession number
TNF-α	CGTCTACTCCTCAGAGCCCC TCCACTCAGGCATCGACATT	226 bp	NM012675
IL-4	AACACTTTGAACCAGGTCAC AGTGCAGGACTGCAAGTATT	330 bp	X16058
COX-1	ACTGGTCTGCCTCAACACCA CAAGGGTGAGACCCCAAGTT	223 bp	S67721
COX-2	TGACCAGAGCAGAGAGATGA CATAAGGCCTTTCAAGGAGA	250 bp	S67722
GAPDH	GGCCAAAAGGGTCATCATCT GTGATGGCATGGACTGTGGT	201 bp	NM017008

8. Western blot analysis

Cell extracts were prepared by detergent lysis procedure. Cells (5×10^6 cells) were scraped, washed once with PBS and resuspended in lysis buffer. Equal amounts of protein were separated electrophoretically using 10 % SDS-PAGE, and then the gel was transferred to nitrocellulose membranes. Blots were blocked for at least 2 h with 5 % non-fat dry milk. The blot was incubated with NF-κB (p65), IκB-α, β-actin, p38, ERK, JNK, and phosphorylated-p38, -ERK, -JNK polyclonal antibodies at 4℃ and secondary antibodies at room temperature were detected by the AP (BCIP/NBT phosphatase substrate) system according to the recommended procedure.

9. Preparation of nuclear extract

The treated cells were washed and centrifuged and then resuspended in hypotonic buffer and incubated on ice for 30 min. After centrifugation at 14,000 rpm, 4 ℃ for 20 min, the supernatant cytoplasmic protein was collected. The remnants were resuspended in extraction buffer, incubated on ice for 30 min, and then centrifuged (14,000 rpm, 4 ℃, 20 min), after which the supernatant nuclear extract was collected.

10. Statistical analysis

Data is presented as the mean ± SE (standard error) of at least three separate experiments. Comparisons between two groups were analyzed using Student's t-test. P values less than 0.05 considered be statistically significant.

Results

1. Effect of S. flavescens on cell viability and degranulation from RBL-2H3 cells

We examined whether *S. flavescens* could affect the degranulation on RBL-2H3 cells. Degranulation of PMA plus A23187-induced mast cells were measured by β -hexosamindase assay as described in Materials and Methods. The release of β-hexosaminiase decreased significantly with all concentrations of *S. flavescens* (Fig. 1A, $P<0.005$). The inhibition rates at the doses of 1.0 ㎎/㎖ and 2.0 ㎎/㎖ were 24.75 % and 38.06 %, respectively. The cell viability effect of *S. flavescens* on RBL-2H3 cells was evaluated by MTT assay. *S. flavescens* concentrations from 1.0 ㎎/㎖ to 2.0 ㎎/㎖ had no effect on cell survival(Fig. 1B).

2. Effect of *S. flavescens* on secretion and mRNA expression of TNF-α

We determined whether *S. flavescens* could regulate secretion and mRNA expression of TNF-α in RBL-2H3 cells. Cells were pre-treated with various concentration of S. flavescens and then A23187 challenge for 8 h. Treatment with *S. flavescens* dose-dependently suppressed TNF-α secretion induced by A23187 in RBL-2H3 cells(Fig. 2A). The inhibition rates at the doses of 1.0 ㎎/㎖ and 2.0 ㎎/㎖ were 10.21 % and 47.57 %, respectively. Also TNF-α mRNA expression decreased significantly by *S. flavescens* in dose-dependent manner (Fig. 2B). In contrast to TNF-α, the level of GAPDH mRNA expression remained the same under these conditions.

3. Effect of *S. flavescens* on secretion and mRNA expression of IL-4

We determined whether *S. flavescens* could regulate secretion and mRNA expression of IL-4 in RBL-2H3 cells. Cells were pre-treated with various concentration of *S. flavescens* and then A23187 challenge for 8 h. Treatment with *S. flavescens* dose-dependently suppressed IL-4 secretion induced by A23187 in RBL-2H3 cells(Fig. 3A). The inhibition rates at the doses of 1.0 ㎎/㎖ and 2.0 ㎎/㎖ were 13.99 % and 25.51 %, respectively. Also IL-4 mRNA expression decreased significantly by *S. flavescens* in dose-dependent manner (Fig. 3B). In contrast to IL-4, the level of GAPDH mRNA expression remained the same under these conditions.

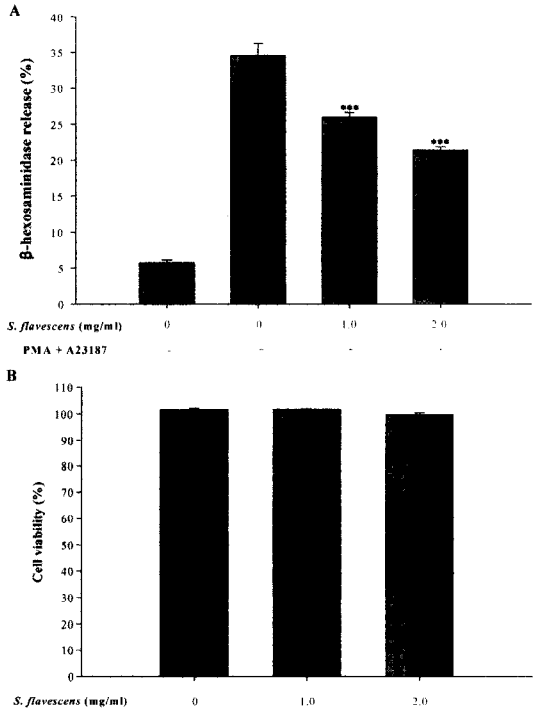
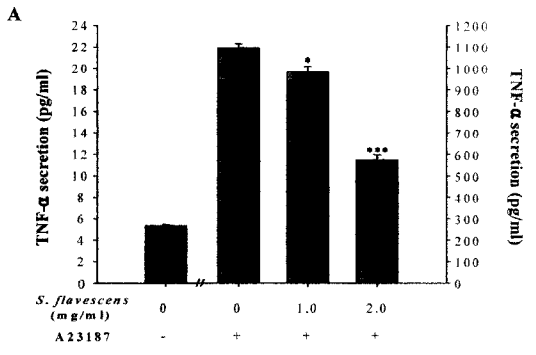


Fig. 1. Effects of *S. flavescens* on the degranulation and cell viability in RBL-2H3 cells. Cells were treated with the indicated concentration of *S. flavescens*. Degranulation was assessed by β-hexosaminidase release into the supernatant. β-hexosaminidase released into the medium is presented as mean ± SEM (n=4). *** P < 0.005; significantly different from the stimulated group (A). Cell viability was evaluated by MTT assay. Data represent the mean ± SE of three independent experiments (B).



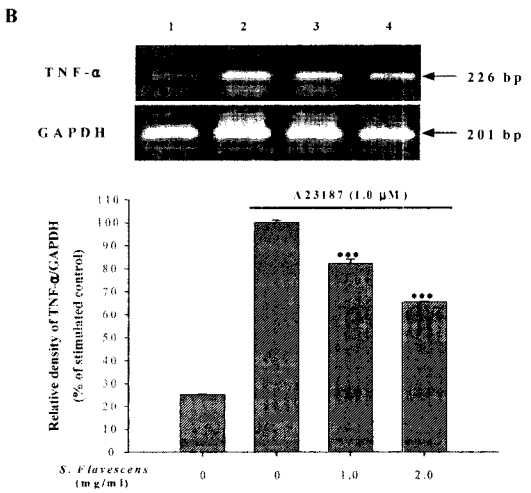


Fig. 2. *S. flavescens* reduces the TNF-α secretion and mRNA expression in RBL-2H3 cells. TNF-α concentration was measured from cell supernatants using ELISA method. Vertical bars represent as the mean ± SE from 4 wells. * P < 0.05, *** P < 0.005; significantly different from the stimulated group (A). Total RNA was isolated, TNF-α mRNA expression was detected by RT-PCR analysis (B). Lane 1, negative control group; lane 2, positive control group (only treated stimulus); lane 3, *S. flavescens* 1.0 ㎎/㎖ + stimulus; lane 4, *S. flavescens* 2.0 ㎎/㎖ + stimulus.

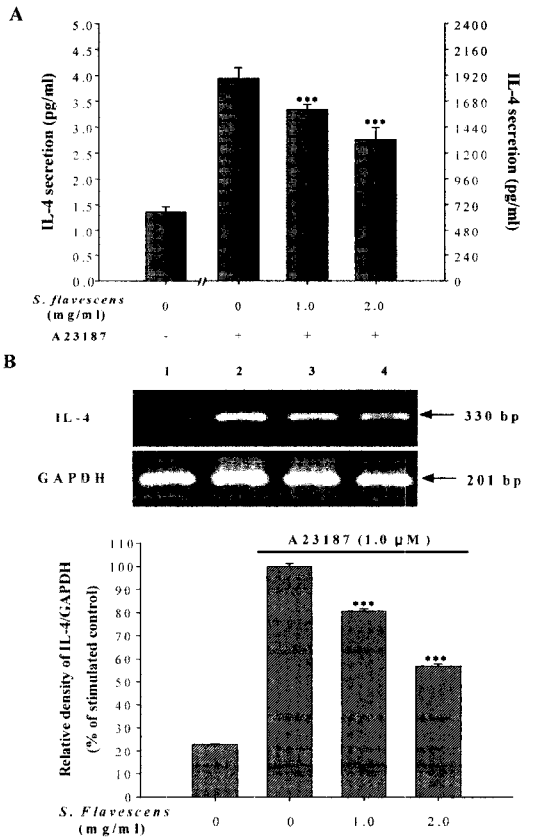


Fig. 3. *S. flavescens* reduces the IL-4 secretion and mRNA expression in RBL-2H3 cells. IL-4 concentration was measured from cell supernatants using ELISA method. Vertical bars represent as the mean ± SE from 4 wells. *** P < 0.005; significantly different from the stimulated group (A). Total RNA was isolated, IL-4 mRNA expression was detected by RT-PCR analysis (B). Lane 1, negative control group; lane 2, positive control group (only treated stimulus); lane 3, *S. flavescens* 1.0 ㎎/㎖ + stimulus; lane 4, *S. flavescens* 2.0 ㎎/㎖ + stimulus.

4. Effect of *S. flavescens* on mRNA expression of COX-2

We examined the effect of *S. flavescens* on A23187-induced COX-1 and COX-2 mRNA expressions, the cells were treated with *S. flavescens* for 1 h. And the cells were treated with A23187 for 4 h and then performed the RT-PCR analysis. COX-2 mRNA expression was increased by A23187 but increased COX-2 mRNA expression was significantly down-regulated by treated with *S. flavescens*, whereas COX-1 mRNA levels showed no change after such treatment(Fig. 4).

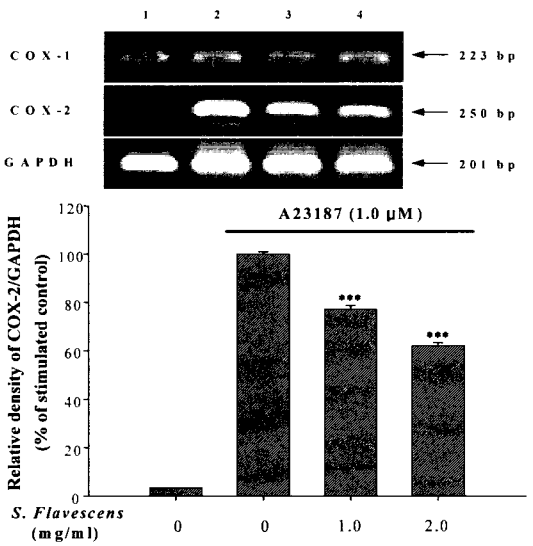


Fig. 4. *S. flavescens* inhibits the COX-2 mRNA expression in RBL-2H3 cells. Total RNA was isolated, COX-1, COX-2 mRNA was analyzed by RT-PCR analysis. Lane 1. negative control group; lane 2. positive control group (only treated stimulus); lane 3. *S. flavescens* 1.0 mg/ml + stimulus; lane 4. *S. flavescens* 2.0 mg/ml + stimulus.

5. Effect of *S. flavescens* on NF-κB (p65) activation and IκB-α degradation

We examined the effects of *S. flavescens* on NF-κB (p65) activation and IκB-α degradation using Western blot analysis. RBL-2H3 cells were pre-treated with *S. flavescens* for 1 h before PMA plus A23187 stimulation. We stimulated the cells with PMA plus A23187 for 30 min (IκB-α) and 2 h (NF-κB). In the stimulated cells, the activation level of NF-κB was increased in the nucleus. *S. flavescens* inhibited the PMA plus A23187-induced the nuclear translocation of NF-κB (p65). Also *S. flavescens* inhibited the PMA plus A23187-induced degradation of IκB-α(Fig. 5).

6. Effect of *S. flavescens* on ERK MAPK activation

We investigated whether MAPKs (p38, JNK, and ERK) signaling pathways are involved in the suppression of pro-inflammatory cytokines expressions by *S. flavescens*, the activation of MAPKs was assessed Western blot analysis. RBL-2H3 cells were treated with *S. flavescens* and PMA plus A23187 for 2 h. The treatment of cells with PMA plus A23187 increased the phosphorylation of ERK whereas *S. flavescens* suppressed phosphorylation of ERK MAPK activation, but did not affect the phosphorylation of p38 and JNK(Fig. 6).

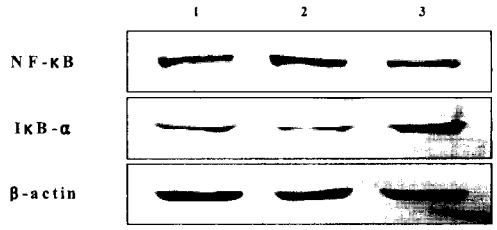


Fig. 5. *S. flavescens* suppresses the expression of NF-κB (p65) and the degradation of IκB-α in RBL-2H3 cells. The cell extract were assayed Western blot analysis for NF-κB (p65) in the nuclear extract and IκB-α in the cytoplamic extract. Lane 1. negative control group; lane 2. positive control group (only treated stimulus); lane 3. *S. flavescens* 2.0 mg/ml + stimulus.

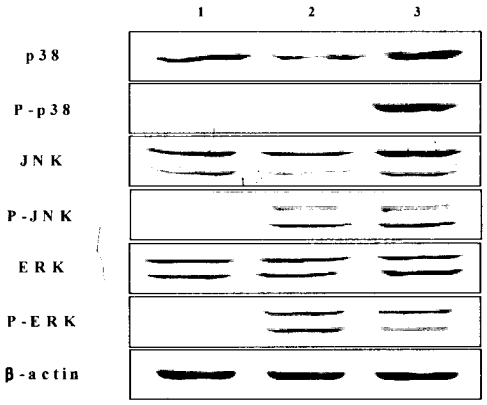


Fig. 6. *S. flavescens* inhibits the phosphorylation of ERK MAPK in RBL-2H3 cells. The cell extract were assayed Western blot analysis for the phosphorylation of p38, ERK, and JNK. Lane 1. negative control group; lane 2. positive control group (only treated stimulus); lane 3. *S. flavescens* 2.0 mg/ml + stimulus.

Discussion

Allergic reaction is caused by the breaking of the Th1/Th2 balance, or by induction of a Th2 lymphocyte-dominant immune response[27-29]. And this reaction is induced by the rapid local and systemic release of inflammatory mediators such as histamine, serotonin, heparin, β-hexosaminidase and various pro-inflammatory cytokines from mast cells that are located throughout the human body[7,30,31]. Mast cells and basophils play important roles on the pathogenesis of allergic diseases. The aggregation of FcεR I by antigens (allergens) results in tyrosine phosphorylation and Ca^{2+} influx via Ca^{2+} release-activated Ca^{2+} channels. The elevation of intracellular free Ca^{2+} levels plays an essential role in the degranulation process[32]. The β-hexosaminidase assay has been widely used to monitor RBL-2H3 mast cell degranulation[33-36]. We observed that anti-allergic effects of *S.*

flavescens on PMA plus (or) A23187-induced on RBL-2H3 cells. As shown in Fig. 1, *S. flavescens* inhibited β-hexosaminidase release induced by PMA plus A23187 in RBL-2H3 cells. However, *S. flavescens* had no effect on cell survival all concentrations.

Mast cells release the cytokines implicated in the allergic reactions (immediate-type hypersensitivity), which include IL-3, -4, -6, -13, and TNF-α[29,37]. TNF-α is a cytokine that mediates a multitude of inflammatory events and is considered to be an important initiator of the inflammatory response[25,38]. IL-4 plays a major role in B cell activation and isotype switching, resulting in the generation of IgE antibodies[29,39]. Jung et al reported that *S. flavescens* inhibited TNF-α secretion and mRNA expression in LPS-stimulated BV2 microglial cells[25]. In recent years, it has been demonstrated that COX-2 plays important roles in various tumor and inflammatory diseases. COX-2, one of the major mediators of the inflammatory reactions, is also strongly induced in activated monocytes and macrophages[40,41]. Previously, sophoraflavanone G, a prenylated flavonoid from *S. flavescens*, down-regulated COX-2 induction from LPS-stimulated Raw 264.7 cells[24]. Similarly, the present study shows that *S. flavescens* significantly suppressed pro-inflammatory cytokines (TNF-α and IL-4) secretion and mRNA expression in activated RBL-2H3 cells(Fig. 2, 3). Also, the expression of COX-2 mRNA was decreased by *S. flavescens*(Fig. 4).

NF-κB ia a key transcription factor required for the expression of many inflammatory involved genes including COX-2, hypoxia-inducible factor (HIF)-1α, and inflammatory cytokines[42]. Activation of NF-κB is dependent on the phosphorylation and degradation of IκB-α, an endogenous inhibitor that binds to NF-κB in the cytoplasm[41]. Once activated, MAPKs can translocate from cytoplasm to nucleus, leading to the phosphorylation of a multitude of transcription factors and altered gene transcription[43-45]. Recent studies in a variety of cultured cells indicate that induction of COX-2 is regulated by the MAPKs[46]. In previous report, *S. flavescens* inhibited the phosphorylation of ERK MAPK in LPS-stimulated BV2 microglial cells[25]. Moreover, we have shown that S. flavescens suppressed activation of NF-κB (p65) in nucleus and degradation of IκB-α in cytoplasm(Fig. 5). And among the MAPKs, *S. flavescens* reduced the activation of ERK MAPK but not of p38 or JNK(Fig. 6).

In conclusion, we have shown that *S. flavescens* can modulate the allergic reaction induced by PMA plus (or) A23187 in RBL-2H3 cells. *S. flavescens* inhibited degranulation of mast cells and expression of pro-inflammatory cytokines genes through regulation of the NF-κB (p65) activation and Iκ B-α degradation. In addition, the phosphorylation of ERK MAPK activation regulated by the *S. flavescens*. Therefore, our results suggest that *S. flavescens* significantly suppressed the release of inflammatory mediators, so this herb could be used as an anti-allergic drug.

Acknowledgements

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