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***Inula japonica* extract inhibits mast cell-mediated allergic reaction and mast cell activation**

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Keywords:

Inula japonica; bone marrow-derived mast cells; eicosanoid; mitogen-activated protein kinases; intracellular Ca^{2+} influx

Abbreviations: IFE, Inulae Flos Extract; BMMCs, Bone marrow-derived mast cells; IL-3, interleukin-3; LTC₄, Leukotriene C₄; SCF, stem cell factor; cPLA₂, cytosolic phospholipase A₂; MAPKs, mitogen-activated protein kinases; PLC γ , phospholipase C γ ; AA, arachidonic acid; ERK1/2, extracellular signal-regulated kinase 1/2; JNK, c-jun N-terminal kinases

1. Introduction

Mast cells are major effector cells of allergic inflammation and increasingly recognized for their roles in innate and adaptive immune responses. When mast cells are activated through IgE-dependent or IgE-independent ways, they release preformed mediators from their granules and produce newly synthesized eicosanoids, chemokines and cytokines (Boyce, 2003; Kalesnikoff and Galli, 2008).

Several lines of evidence indicate that various receptors are expressed on the surface of mast cells. Among them, a high-affinity receptor for IgE (FcεRI) is the most well-studied in IgE/antigen-induced responses, whereas the stem cell factor (SCF; also known as Kit ligand) is a cytokine that binds to the c-Kit receptor (CD117), which also induces mast cells development and activation (Rooski, 2005). Binding of SCF to c-Kit results in dimerization of the receptor followed by activation of its intrinsic tyrosine kinase activity and phosphorylation of key tyrosine residues within the receptor, which leading to a multitude of signaling pathways (Ronnstrand, 2004).

KL and c-Kit binding induces two major signaling pathways by regulating the levels of phosphatidylinositol-4,5-bisphosphate (PIP₂) (OKKenhaug et al., 2007, Rivera and Olivera, 2007). One pathway is the phospholipase Cγ (PLCγ)-mediated hydrolysis of PIP₂ to diacylglycerol (DAG) and inositol-1,4,5-trisphosphate (IP₃). DAG is a protein kinase C (PKC) activator, while IP₃ binds to specific receptors expressed on the endoplasmic reticulum, triggering the release of Ca²⁺ from internal stores. Ca²⁺ is an important intracellular messenger in mast cells because it plays essential role both mast cell degranulation and arachidonic acid (AA) release and metabolism, which requires the translocation of cytosolic phospholipase A₂ (cPLA₂) to intracellular membranes (Evans et al., 2001). The other pathway involves phosphoinositide 3-kinase (PI3K) that phosphorylates PIP₂ to produce PIP₃, which enhances extracellular calcium entry and protein kinase (PKC) activation (Vanhaesebroeck et al., 2001; Wymann and Marone, 2005). Other downstream signal by c-Kit is the Ras/mitogen-activated protein kinases (MAPKs) which have been identified as an important signaling pathway for mitogenic responses and the survival of mast cells (Duronio et al., 1992). The SCF and c-Kit interaction also leads to the activation of many signal transduction pathways. For instance, PI3K regulates the Protein kinase B (Akt), which is a protein-

serine/threonine kinase and has been recognized to modulate a wide range of cellular activities (marone et al., 2008). cPLA₂ is a key enzyme that mediates AA metabolism, which is activated by an increase in the intracellular Ca²⁺ concentration and phosphorylation by MAPKs (Clark et al., 1991; Dennis, 1997). The release of AA from cellular membrane phospholipid by cPLA₂ interacts with 5-lipoxygenase activating protein (FLAP) that presents AA to 5-lipoxygenase (5-LO) for generating leukotrienes (LTs) (Murphy and Gijon, 2007).

The flowers of *Inula japonica* Thunb has long been used in traditional Chinese medicine for the treatment of bronchitis, digestive disorders, and inflammation (Liu et al., 2004). However, the underlying mechanisms for its anti-allergic activity have not been elucidated sufficiently. In addition, the effects of IFE on degranulation and the production of eicosanoid that are mediators of inflammatory and allergic reactions also await clarification. In this study, we investigated the anti-allergic activity of IFE on the production of eicosanoids and degranulation in SCF-induced BMMCs, and examined its major phytochemical compositions.

2. Materials and methods

2.1. Plant material

Dried flowers of *Inula japonica* (Inulae Flos) collected in the Anhui province of China were purchased from Ominherb (Youngehun, Korea), and a voucher specimen has been deposited at the Korea Promotion Institute for Traditional Medicine Industry (DGOM-SB09). The Inulae Flos was extracted with ethanol at a ratio of 1:10 (w/v) and then refluxed for 24 hr at 60 °C. The extracted solution was filtered, and the solvents were evaporated under vacuum at 40 °C (Eyela, Tokyo, Japan), after which they were freeze-dried to obtain the concentrated extract (yield 8 %, w/w). The IFE was dissolved in dimethyl sulfoxide (DMSO) and diluted in the medium so that the final concentration of DMSO was less than 0.01 % v/v and this concentration of DMSO did not induce mast cell activation. A control of DMSO alone was included in all experiments.

2.2. Reagents

The recombinant mouse SCF was purchased from STEMCELL Technologies Inc (Vancouver, BC, Canada). The primary antibodies used in the experiments were as follows: rabbit polyclonal antibodies specific for phospho-ERK1/2, ERK1/2, phospho-p38, p38, phospho-

JNK, JNK, phospho-PLC γ 1, and β -actin were from Cell Signaling Technology, Inc. (Danvers, MA, USA); rabbit polyclonal antibodies for phospho-cPLA $_2$ and PLC γ 1 were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The horseradish peroxidase-conjugated goat anti-rabbit secondary antibody was also from Cell Signaling Technology, Inc. The LTC $_4$ and PGD $_2$ enzyme linked immunoassay (EIA) kit were purchased from Cayman Chemical (Ann Arbor, MI, USA). The enhanced chemiluminescence (ECL) Western blot detection reagent was purchased from Amersham Biosciences, Inc. (Piscataway, NJ, USA).

2.3. Induction of IgE-mediated passive cutaneous anaphylaxis (PCA) reaction in mice

The ICR mice (Hyochang Science, Daegu, Korea) were kept at a temperature of $22 \pm 1^\circ\text{C}$ and at a relative humidity of $55 \pm 10\%$ and a 12 h/12 h (light/dark) cycle for at least 7 d prior to the experiments throughout the study. IFE was dissolved in DMSO and diluted in 0.5% carboxymethyl-cellulose (CMC). Control mice were administered orally with 0.5% CMC (contain same volume of DMSO) and CMC did not show any cytotoxicity in mice. For PCA, 80 ng of mouse anti-dinitrophenyl (DNP) IgE (Sigma-Aldrich, St. Louis, MO, USA) were intradermally injected into one ear of 7-wk-old male mice, followed 24 h later by oral administration of 100-400 mg/kg IFE or 50 mg/kg fexofenadine-HCl, a histamine H1 receptor antagonist (Korea Pharma, Seoul). One hour later, the mice were intravenously challenged with 60 μg of Ag (DNP-human serum albumin (HSA); Sigma-Aldrich, St. Louis, MO, USA) in 200 μl of PBS containing 1% (w/v) Evans blue. The mice were euthanized 1 h after antigen treatment, and their ears were removed and dissolved with 400 μl formamide at 63°C overnight. The amount of dye extravasation was determined colorimetrically at 630 nm. Experiments using mice were approved by the Institutional Animal Care and Use Committee of Yeungnam University.

2.4. Preparation and activation of BMMCs

Bone marrow cells from male Balb/cJ mice (Sam Taco, INC, Seoul, Korea) were cultured for up to 10 weeks in 50% enriched medium (RPMI 1640 containing 2 mM L-glutamine, 0.1 mM nonessential amino acids, antibiotics and 10% fetal calf serum) and 20 % pokeweed mitogen-stimulated spleen condition medium (PWM-SCM) as a source of interleukin-3 (IL-3). After 3 weeks, > 98% of the cells was found to be BMMCs checked by the previously described procedure (Murakami et al., 1994).

2.5. Determination of LTC_4

BMMCs suspended at a cell density of 1×10^6 cells/ml were seeded in a 96-well plate, pre-incubated with various concentrations of IFE for 1 h, and then stimulated with SCF (30 ng/ml) for 15 min. All reactions were stopped by centrifugation at 120 g at 4 °C for 5 min, and the supernatants were immediately utilized for LTC_4 determination. The level of LTC_4 was determined using enzyme immunoassay (EIA) kit (Cayman Chemical, Ann Arbor, MI, USA) according to the manufacturer's instruction. In these assay conditions, 5-LO dependent LTC_4 generation reached approximately 12 ng/ 10^6 cells. All data were the arithmetic mean of triplicate determinations.

2.6. Determination of PGD_2

To assess COX-2-dependent PGD_2 generation, BMMCs were preincubated with aspirin (1 μ g/ml) for 2 h to irreversibly inactivate the pre-existing COX-1. After washing, BMMCs were activated with SCF (30 ng/ml), IL-10 (100 U/ml) and LPS (200 ng/ml) at 37°C for 7 h with IFE. Concentrations of PGD_2 in the supernatants were measured using PGD_2 EIA kits according to the manufacturer's instruction, and cells were used for western blotting analysis. In these assay conditions, COX-2-dependent phases of PGD_2 generation reached approximately 1.2 ng/ 10^6 cells. All data were the arithmetic mean of triplicate determinations.

2.7. Assay of β -Hexosaminidase (β -Hex) release

β -Hex, a marker of mast cell degranulation, was quantified by spectrophotometric analysis of the hydrolysis of substrate (*p*-nitrophenyl-2-acetamido-2-deoxy- β -D-glucopyranoside, Sigma-Aldrich, USA). Briefly, 25 μ l of cell-free supernatants were mixed with 50 μ l of substrate in 0.1 M citrate, pH 4.5. After incubation for 1 h at 37°C, 175 μ l of stop solution (0.2 M NaOH-glycine) was added to stop the reaction, and absorbance was measured at 405 nm. Values were expressed as percentage release relative to the total β -Hex in the cells. All data was the arithmetic mean of triplicate determinations.

2.8. Measurement of intracellular Ca^{2+} level

Intracellular Ca^{2+} levels were determined with FluoForte™ Calcium Assay Kit (Enzo Life

Sciences, Ann Arbor, MI, USA). BMMCs were preincubated with FluoForte™ Dye-Loading Solution for 1 h at room temperature. After washing the dye from cell surface with PBS, the cells (5×10^4) were seeded into 96-well microplates. Then the cells were pretreated with IFE for 1 h before adding SCF. The fluorescent was measured using a fluometric imaging plated reader at an excitation of 485 nm and an emission of 520 nm (BMG Labtechnologies FLUOStar OPITIMA platereader, Offenburg, Germany). All the assay experiments were independently repeated at least three times.

2.9. SDS-PAGE/Immunoblot analysis

BMMCs were seeded at a cell density of 1×10^6 cells in 1.5 ml tubes and pre-treated with various concentrations of IFE or inhibitors for 1 h prior to the treatment with SCF for 10 min. The cells were then washed with cold PBS and centrifuged at $500 \times g$ at 4 °C. The cell pellets were resuspended in lysis buffer (50 mM Tris-HCl, pH 8.0, 5 mM EDTA, 150 mM NaCl, 1 mM PMSF, 1 % NP40, 0.5 % protease inhibitor cocktail (Merck, Darmstadt, Germany), 1 mM DTT, 1 mM NaF, 1 mM Na_3VO_4) and centrifuged to yield whole-cell lysates. The proteins were separated via 10% SDS-PAGE, transferred to nitrocellulose membranes in 20 % methanol, 25 mM Tris, and 192 mM glycine (Schleicher and Schull, Dassel, Germany), and then blocked via incubation in TTBS (25 mM Tris-HCl, 150 mM NaCl, and 0.2 % Tween 20) containing 5 % non-fat milk. The membranes were subsequently incubated first with a variety of antibodies overnight, washed, and finally incubated for 1 h with a secondary antibody conjugated to horseradish peroxidase. The protein bands were then visualized with an ECL system (Pierce Biotechnology, Rockford, IL, USA).

2.10. Optimization of HPLC Conditions

The separation of analytes was carried out using a Shimadzu HPLC system consisted of a SIL-20A auto sampler and LC-20 pump coupled with a SPD-M20A diode array detector (DAD). Each sample was analyzed on a SunFire™ C18 column (250 mm x 4.6 mm, 5 μm , Waters, Milford, USA). The mobile phase consisted of water (A) and acetonitrile (B), which were applied in the gradient elution as follows: 20-40% B at 0-35 min, and 40-55% B at 35-45min. The flow rate of the mobile phase was 1.0 ml/min, and the detection wavelength was set at 210 nm.

2.11. Statistical Analysis

All experiments described were performed three or more times. Average values were expressed as means $\pm$ s.e.m. Student's *t*-test was used for the comparison of two independent groups. For all tests, a *P*-value < 0.05 was considered statistically significant.

3. Results

3.1. IFE suppresses the mast cell-mediated passive cutaneous anaphylaxis reaction in mice

We first examined the anti-allergic activity of IFE by using the passive cutaneous anaphylaxis (PCA) mouse model. A local extravasation was induced by a local injection of IgE followed by an antigenic challenge (Lu et al., 2011). An oral administration of IFE (100-400 mg/kg) 1 h before injection of antigen significantly suppressed the mast cell-mediated PCA reaction in a dose-dependent manner (27.5% inhibition at 100 mg/kg, 48.7% inhibition at 200 mg/kg and 60.2% inhibition at 400 mg/kg). The suppressive effect of IFE at the highest dose was comparable to that of 50 mg/kg fexofenadine-HCl (Ciprandi et al., 2003), histamine H1 receptor antagonist used as a positive control (Fig. 1).

3.2. Effect of IFE on SCF-induced degranulation

The *in vivo* result led us to investigate the mechanism of the anti-allergic activity of IFE on mast cells activation. First, we examined the cytotoxic effect of IFE on BMDCs using the MTT assay and found that it did not affect cell viability at 50 μ g/ml (data not shown). Thus, we decided to use IFE at a dose less than 50 μ g/ml for subsequent experiments. Degranulation was monitored by determining the release of β -Hex because histamine release by activated mast cells parallels the release of β -Hex. In order to determine whether IFE inhibits β -Hex release, BMDCs were pre-treated with various doses of IFE for 1 h at 37°C. After treatment with SCF for 15 min, the supernatants were collected for determination of β -Hex. As shown in Fig. 2A, SCF treatment significantly increased β -Hex release. Pre-treatment with IFE resulted in a dose-dependent suppression of β -Hex release. Calcium influx of mast cells is critical to degranulation. To investigate the mechanism of IFE on the inhibition of degranulation, we evaluated the level of intracellular Ca^{2+} . SCF induced the

1 increase of intracellular Ca^{2+} , and IFE pre-treatment decreased the intracellular Ca^{2+} level in
2 SCF-stimulated BMMC (Fig. 2B).

3 The early activation of $\text{PLC}\gamma$ results in the generation of IP_3 and DAG (El-Sibai and
4 Backer, 2007). These two second messengers lead to an increase in Ca^{2+} and activation of
5 PKC, which is a regulator of calcium influx during mast cell degranulation. The increase of
6 $\text{PLC}\gamma 1$ on BMMCs stimulated with SCF reached its maximum within 5 min (data not shown).
7 IFE pre-treatment on BMMCs suppressed SCF-induced $\text{PLC}\gamma 1$ phosphorylation in a dose-
8 dependent manner (Fig. 2C), indicating that the inhibitory effect of IFE on degranulation was
9 mediated through the inhibition of intracellular Ca^{2+} influx, with a concomitant reduction in
10 $\text{PLC}\gamma 1$ phosphorylation.

11 12 3.3. Inhibitory effect of IFE on 5-LO dependent LTC_4 and COX-2 dependent PGD_2 13 generation

14 Lipid mediators like ILTs can initiate, amplify inflammatory responses, and influence the
15 magnitude of subsequent immune responses (Harizi et al., 2008). In order to determine
16 whether IFE inhibits 5-LO dependent LTC_4 generation, cells were pre-treated with various
17 doses of IFE for 1 hr and then stimulated with SCF for 15 min. As shown in Fig. 3A, IFE
18 consistently inhibited LTC_4 generation in a dose-dependent manner. The inhibitory activity of
19 the highest concentration of IFE was weaker than that of licofelone (COX-2/5-LOX dual
20 inhibitor) which was used as a positive control (Vidal et al., 2007).

21 It is well-known that PGD_2 is one of the major prostanoids secreted from the activated
22 mast cell and has long been implicated in the etiology and manifestation of inflammation and
23 allergic diseases. Therefore, we investigated the effect of IFE on COX-2 dependent PGD_2
24 generation. COX-2 expression was induced with the triad SCF/IL-10/LPS, which is the most
25 efficient induction condition for COX-2 in BMMCs (Moon et al., 1998). To assess COX-2
26 dependent PGD_2 generation, BMMCs were pre-treated with aspirin to abolish any pre-
27 existing COX-1 activity, followed by a brief wash, and then stimulated with the SCF/IL-
28 10/LPS for 7 h with or without IFE. The PGD_2 generation was dose-dependently inhibited by
29 IFE (Fig. 3B, upper), with a concomitant reduction of COX-2 protein (Fig. 3B, lower). The
30 inhibitory effect of the highest concentration of IFE was comparable to that of licofelone.
31 This result clearly showed that inhibition of PGD_2 generation was accompanied by a
32 reduction in COX-2 protein expression.

3.4. IFE inhibits phosphorylation of cPLA₂ and phosphorylation of MAPKs

cPLA₂ is a key enzyme that mediates AA metabolism. The post-translational activity of cPLA₂ is mediated by two signals: calcium-induced translocation from the cytosol to the membranes including the nuclear envelope, and phosphorylation of the enzyme by MAPKs such as ERK, JNK and p38 (Kurosawa et al., 2009). Previously, we reported that MAPKs phosphorylation was induced in SCF- or SCF/IL-10/LPS-stimulated BMMCs (Jin et al., 2011; Lu et al., 2012). To examine whether the inhibition of SCF-induced phosphorylation of cPLA₂ by IFE was controlled through the blockage of MAPKs phosphorylation, BMMCs were pre-treated with IFE or MAPKs inhibitors for 1h before stimulating with SCF for 10 min. The phosphorylation of cPLA₂ was suppressed in a dose-dependent manner by IFE (Fig. 4A). Moreover, MAPKs inhibitors (SP600125, SB203580, and U0126) also almost completely blocked cPLA₂ phosphorylation like our previous report (Jin et al., 2011), suggesting that IFE-mediated inhibition of cPLA₂ phosphorylation occurred through MAPK-dependent pathways.

Studies were extended to determine the effect of IFE or MAPK inhibitors on SCF-induced phosphorylation of ERK1/2, JNK and p38 using Western blot analysis. BMMCs were treated with the indicated concentrations of IFE or MAPKs inhibitors for 1 h prior to SCF treatment. SCF-stimulated BMMCs led to MAPKs phosphorylation, whereas treatment with IFE or MAPK inhibitors significantly inhibited this effect (Fig. 4B).

3.5. HPLC Analysis of IFE

HPLC analysis of sesquiterpene lactones from IFE was carried out on a Shimadzu HPLC system, which depends on the established LC condition. Three sesquiterpene lactones 1-*O*-acetylbritannilactone (1), britanin (2) and tomentosin (3) were isolated by authors, and the chemical structures were determined by the comparison of their NMR spectral data with the previously published data (Kazuo et al., 1981; Zhou et al., 1993; Kim et al., 2004). As shown in Fig. 5, the three peaks were assigned by comparing their UV spectra and retention times with those of each reference compound and by spiking sample with reference compounds. Compounds 1, 2 and 3 could be detected at concentrations of 10.8 %, 3.0 % and 2.9 %, based on the peak areas, respectively.

4. Discussion

Inula japonica, a well-known traditional medicinal herb, possesses diverse biological activities and pharmacological functions such as hypoglycemic and hypolipidemic activities (Shan et al., 2006). Although the anti-inflammatory activities of *Inula* species (Han et al., 2004; Hernandez et al., 2005; Hernandez et al., 2007; Qin et al., 2009; Qin et al., 2010; Whan Han et al., 2001) have been demonstrated mostly in mouse macrophage cell line Raw 264.7 cells, those of *Inula japonica* in mast cells and its mechanism have not been reported. In this study, we show that IFE reduced inflammatory allergic responses in mast cells by using the PCA mouse model. We also show that IFE inhibited the synthesis of LTC₄ and PGD₂ and degranulation by suppressing cPLA₂, MAPKs, PLCγ1 activation and intracellular Ca²⁺ release in BMMCs.

PCA is one of the most important *in vivo* models of anaphylaxis in local allergic reaction (Lu et al., 2011). IFE effectively suppressed the local allergic reaction in this mouse model. When administered with 100 mg/kg IFE, substantial inhibition was observed; but when administered orally of IFE at 400 mg/kg, the PCA reaction was strongly inhibited comparable of that achieved with fexofenadine-HCl. This result led us to evaluate IFE's mode of action on BMMCs. Mast cells represent a major source of histamine, proteases, and other potent chemical mediators implicated in a wide variety of inflammatory and immunologic processes (Boyce, 2003). Activation of BMMCs with FcεRI cross-linking or SCF or SCF/IL-10/LPS triggers biphasic responses: the immediate responses which release the preformed granule contents such as histamine and newly generated lipid mediators such as LTC₄ and PGD₂, and the delayed responses which induces COX-2 expression and leads to the delayed generation of PGD₂ (Moon et al., 1998). Upon stimulation with SCF, mast cells release β-Hex, a marker of degranulation. Therefore, the inhibitory effect of IFE on SCF-induced β-Hex release from BMMCs was examined. As shown in Fig. 2A, IFE dose-dependently inhibited β-Hex release in BMMCs. Thus we investigated the implication of PLCγ1 phosphorylation in the modulation of degranulation by IFE. PLCγ1-mediated Ca²⁺ signal is essential for mast cell degranulation (Metcalf et al., 2009). The activated PLCγ1 leads to the hydrolysis of PIP₂-4,5 and production of DAG and IP₃, which induces the activation of PKC and intracellular Ca²⁺ release, respectively (Gilfillan and Rivera, 2009). We showed that SCF-induced phosphorylation of PLCγ1 and Ca²⁺ release was inhibited by IFE (Fig. 2B), suggesting that the suppression of β-Hex release may be mediated by PLCγ1 phosphorylation in BMMCs.

LTC₄ plays major roles in the several inflammatory diseases including asthma, psoriasis, rheumatoid arthritis, and inflammatory bowel disease. LTC₄ biosynthesis is initiated by the action of 5-LO translocation to the nuclear membrane where binds with FLAP, and use AA as substrate released (Murphy and Gijon, 2007). COX also converts AA, which is released from the plasma membrane via the action of cPLA₂, into PGH₂ and then into PGD₂ (Rajakariar et al., 2006). In this study, when BMMCs were stimulated with SCF or a combination of SCF/IL-10/LPS with or without IFE or licofelon, both LTC₄ and PGD₂ generation were significantly inhibited. These results suggested that IFE has a COX-2/5-LOX dual inhibitory activity. The 85 kDa cPLA₂ is specific to glycerophospholipids containing AA in the *sn*-2 position and plays a role in mediating AA release for eicosanoid production (Luo et al., 2008). Activation of cPLA₂ is related to several mechanisms, including activation of MAPKs and PKC (Nito et al., 2008). In this study, SCF-induced cPLA₂ phosphorylation was suppressed by IFE or MAPKs inhibitors such as U0126, SP600125 and SB203580, demonstrating that cPLA₂ was regulated by IFE as shown in Fig. 4. We next demonstrated that SCF-induced phosphorylation of ERK, JNK and p38 was abolished by IFE in a dose-dependent manner, suggested that IFE inhibited LTC₄ and PGD₂ generation through MAPK-mediated cPLA₂ pathway.

HPLC analysis of the marker compounds from IFE was a multi-step gradient elution, accomplished on a reversed-phase C18 column. Three sesquiterpenes were identified as major components in IFE extracts. Our recent here results showed that britanin and tomentosin inhibited the generation of eicosanoids and degranulation in SCF-induced BMMCs (data not shown). Therefore, anti-allergic activity of IFE could be caused in part by britanin and tomentosin. Further experiments to clarify its mechanism are under investigation in our lab.

5. Conclusion

Our previous study demonstrated that IFE showed anti-asthmatic activity through the down-regulating the production of Th2 cytokines and IgE in the OVA-induced airway inflammation model (Park et al., 2011). Here, we showed that IFE suppressed mast cell-mediated allergic reactions, eicosanoid generation, and degranulation through the suppression of SCF-mediated several signaling pathways. The present results together with our previous report on IFE provide a rationale for the use of IFE in the treatment of allergic inflammatory

diseases.

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Figure legends

Fig. 1. IFE inhibits IgE/Ag-induced PCA reaction. IFE was oral administered one hour prior to the challenge with antigen. Each amount of dye was extracted and measured as described in Materials and methods. Each bar represents the mean $\pm$ s.e.m. (Data were from three independent experiments, in each experiment, n = 8 animals). * $P < 0.05$ and ** $P < 0.01$ were compared to IgE/Ag sensitized mice.

Fig. 2. Effect of IFE on SCF-induced β -Hex release, intracellular Ca^{2+} level, and PLC γ 1 phosphorylation in BMMCs. BMMCs were pre-treated with IFE (1, 5, 10 $\mu\text{g/ml}$) for 1 h and incubated with SCF (100 ng/ml) for 10 min. (a) β -Hex released into the supernatant and (b) intracellular Ca^{2+} level were determined. (c) The cells were harvested for measurements of total or phosphorylated PLC γ 1 by Western blot analysis. Data were from three independent experiments. Values are shown as the mean $\pm$ s.e.m. ** $P < 0.05$ and *** $P < 0.01$ were compared to SCF-stimulated BMMCs.

Fig. 3. Effect of IFE on LTC $_4$ generation and PGD $_2$ generation. (a) BMMCs were pre-incubated with the indicated concentrations of IFE for 1 h. All the cells were stimulated with SCF (30 ng/ml) for 15 min. LTC $_4$ released into the supernatant was determined. (b) BMMCs

were pre-incubated with 1 µg/ml of aspirin for 2 h to abolish pre-existing COX-1 activity, followed by a brief washing and then stimulated with SCF/IL-10/LPS for 7 h. PGD₂ released into the supernatant was quantified by a PGD₂ EIA Kit (upper), and the cells were used for immunoblotting for COX-2 (lower). Data were from three separate experiments. Values are shown as the mean ± s.e.m. * $P < 0.05$ and ** $P < 0.01$ were compared to SCF-stimulated BMMCs.

Fig. 4. Effect of IFE on cPLA₂ phosphorylation and MAP kinases phosphorylation in BMMCs.

(a) BMMCs were pre-incubated with the indicated concentrations of IFE for 1 h and stimulated with SCF (30 ng/ml) for 15 min. The phosphorylation of cPLA₂ was evaluated via Western blot analysis. p-cPLA₂/β-actin protein levels were determined by measuring immunoblot band intensities by scanning densitometry. The results from three separate experiments as relative ratios (%) are represented. Data were from three independent experiments. Values are shown as the mean ± s.e.m. * $P < 0.05$ and ** $P < 0.01$ were compared to SCF-stimulated BMMCs. (b) BMMCs were pre-treated with IFE (1, 5, 10 µg/ml), U0126 (25 µM), SP600125 (25 µM) and SB203580 (30 µM) for 1 h and then stimulated with SCF (30 ng/ml) for 10 min. The levels of phosphorylation of MAPKs were evaluated via Western blot analysis. Similar results were observed in three independent experiments.

Fig. 5. HPLC chromatograms of IFE, and marker compounds 1-O-acetylbritannilactone (1), britanin (2) and tomentosin (3)

Abstract

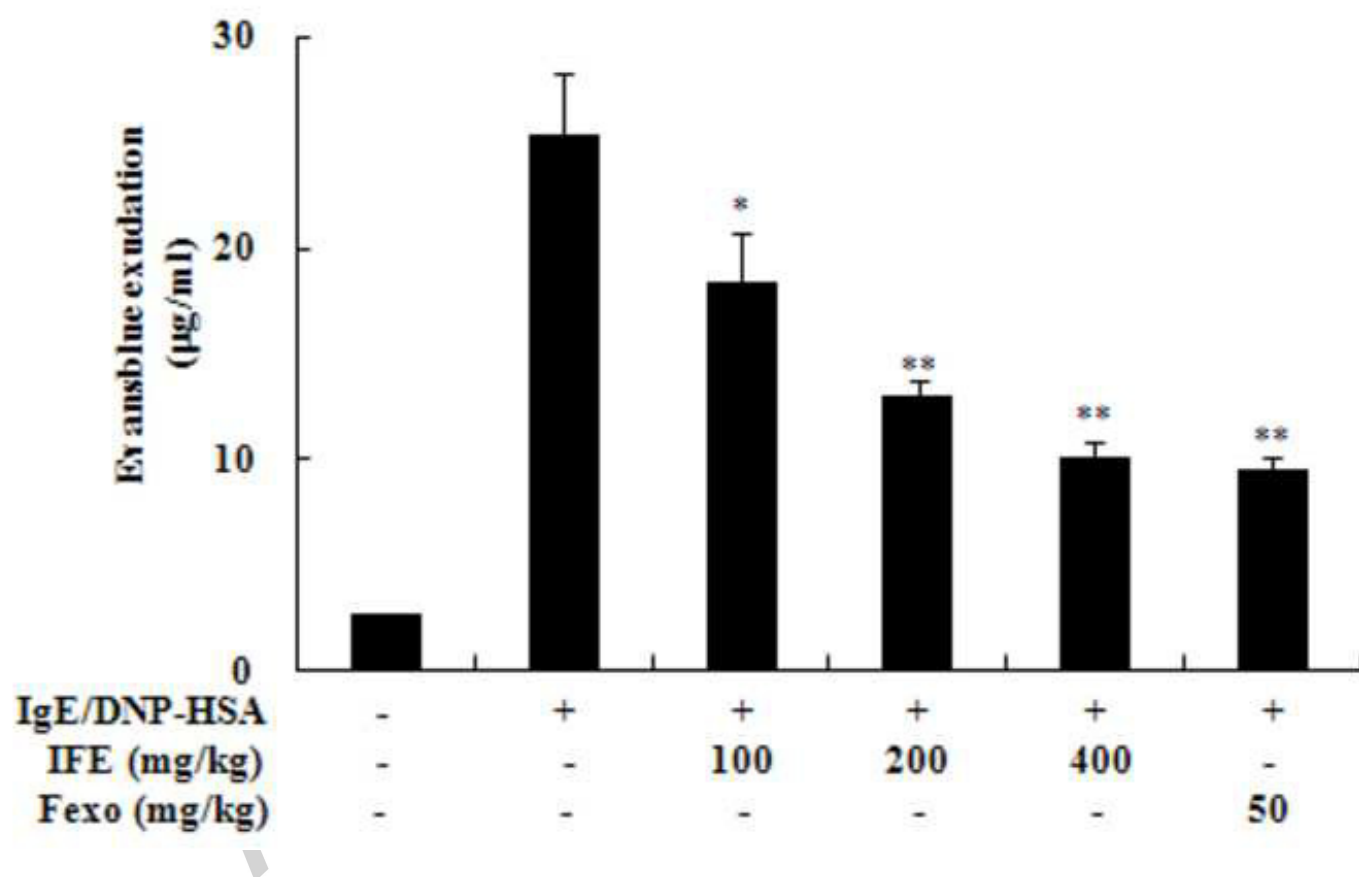
Ethnopharmacological relevance: The flowers of *Inula japonica* (*Inulae Flos*) has long been used in traditional medicine for the treatment of bronchitis, digestive disorders, and inflammation. However, the mechanisms underlying its anti-inflammatory effects remain yet to be elucidated. The objectives of this study were 1) to assess the anti-allergic activity of the ethanol extract of flowers of *Inula japonica* extract (IFE) *in vivo*, 2) to investigate the mechanism of its action on mast cells *in vitro*, and 3) to identify its major phytochemical compositions.

Materials and Methods: The anti-allergic activity of IFE was evaluated using mouse bone marrow-derived mast cells (BMMCs) *in vitro* and a passive cutaneous anaphylaxis (PCA) animal model *in vivo*. The effects of IFE on mast cell activation were evaluated in terms of degranulation, eicosanoid generation, Ca^{2+} influx, and immunoblotting of various signaling molecules.

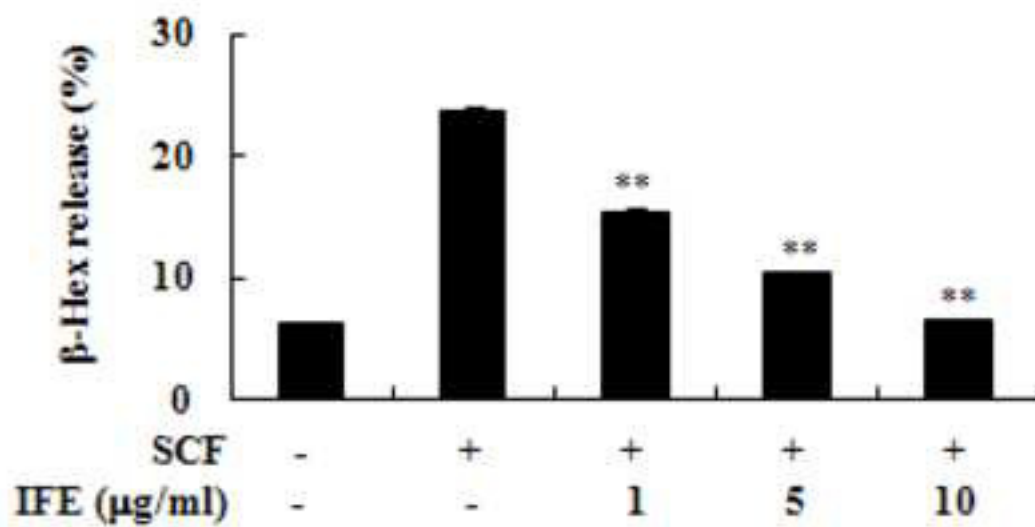
Results: IFE inhibited degranulation and the generation of eicosanoids (PGD_2 and LTC_4) in stem cell factor (SCF)-stimulated BMMCs. Biochemical analysis of the SCF-mediated signaling pathways demonstrated that IFE inhibited the activation of multiple downstream signaling processes including mobilization of intracellular Ca^{2+} and phosphorylation of the mitogen-activated protein kinases (MAPKs), $\text{PLC}\gamma 1$, and cPLA_2 pathways. When administered orally, IFE attenuated the mast cell-mediated PCA reaction in IgE-sensitized mice. Its major phytochemical composition included three sesquiterpenes, 1-*O*-acetylbritannin lactone, britanin and tomentosin.

Conclusions: This study suggests that IFE modulates eicosanoids generation and degranulation through the suppression of SCF-mediated signaling pathways that would be beneficial for the prevention of allergic inflammatory diseases. Anti-allergic activity of IFE may be in part attributed particularly to the presence of britanin and tomentosin as major components evidenced by a HPLC analysis.

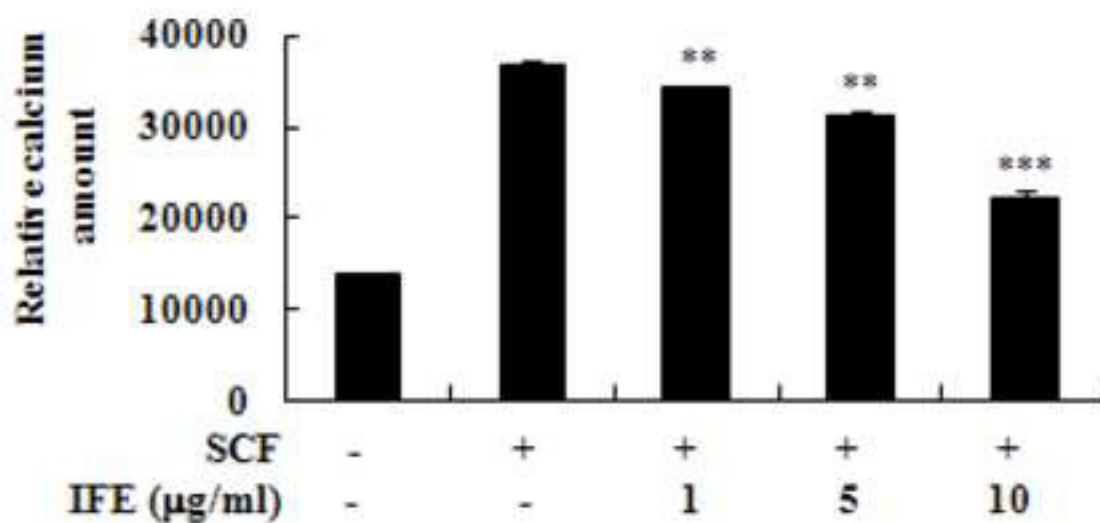
Fig.1



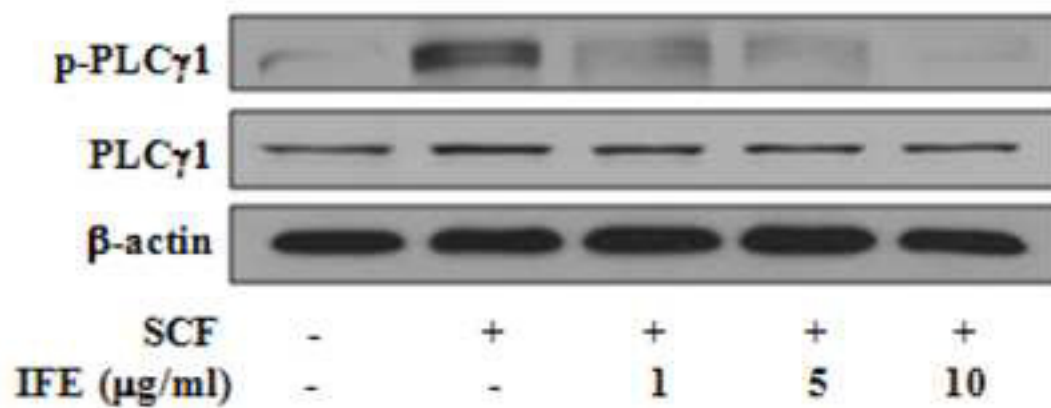
(A)



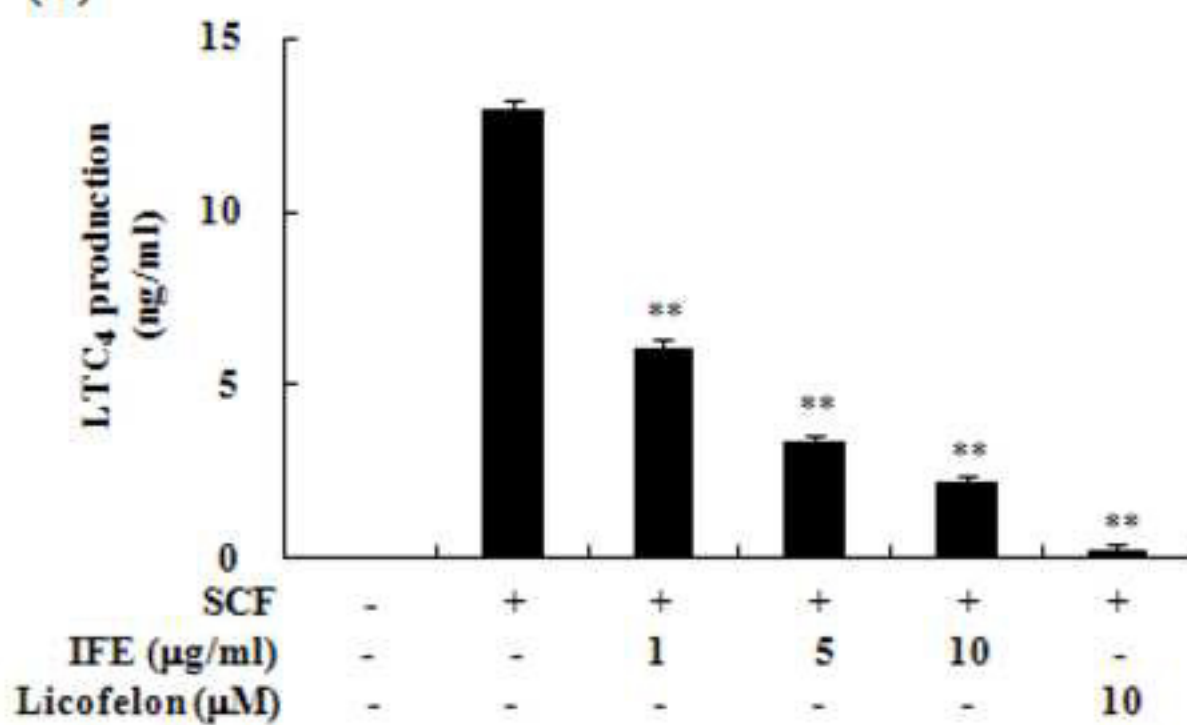
(B)



(C)



(A)



(B)

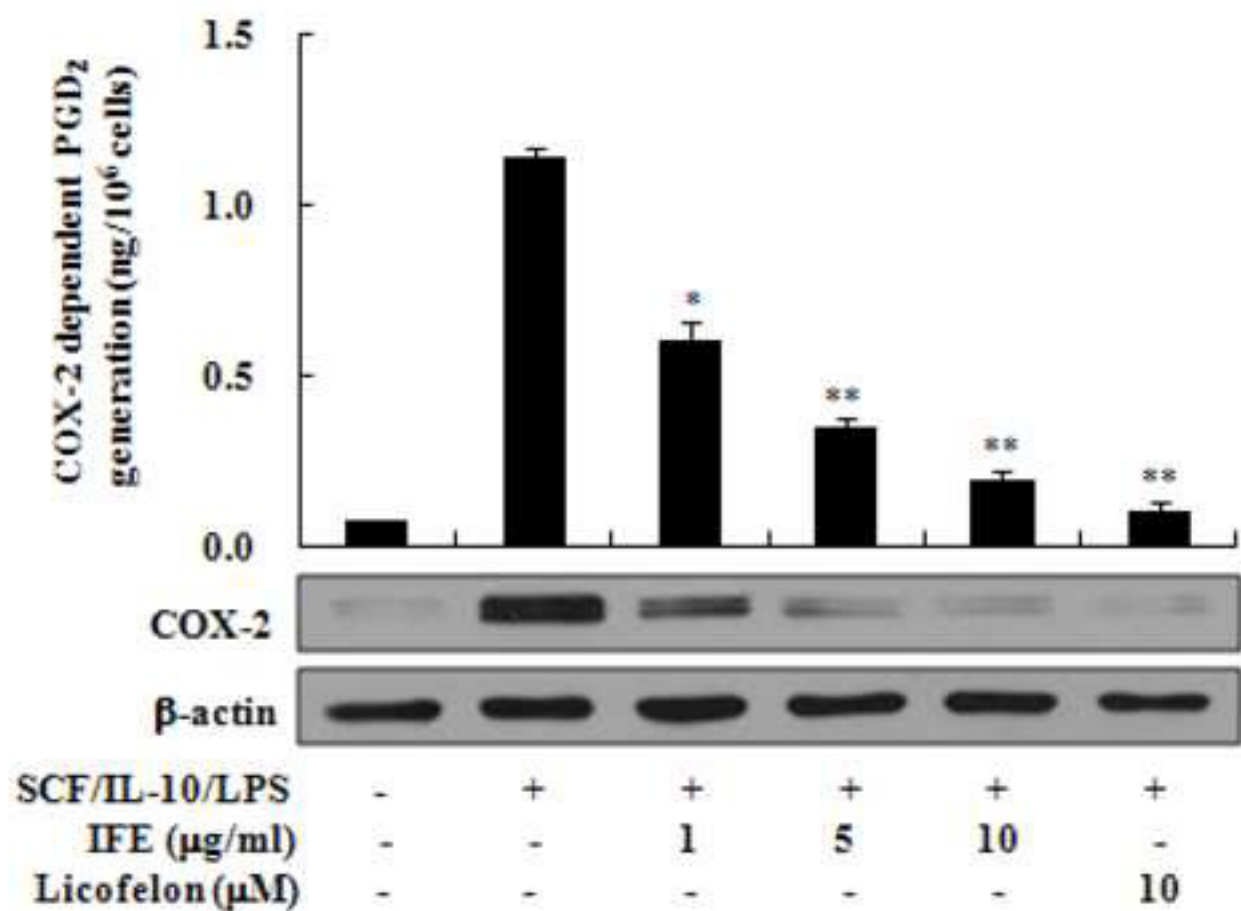


Fig 4

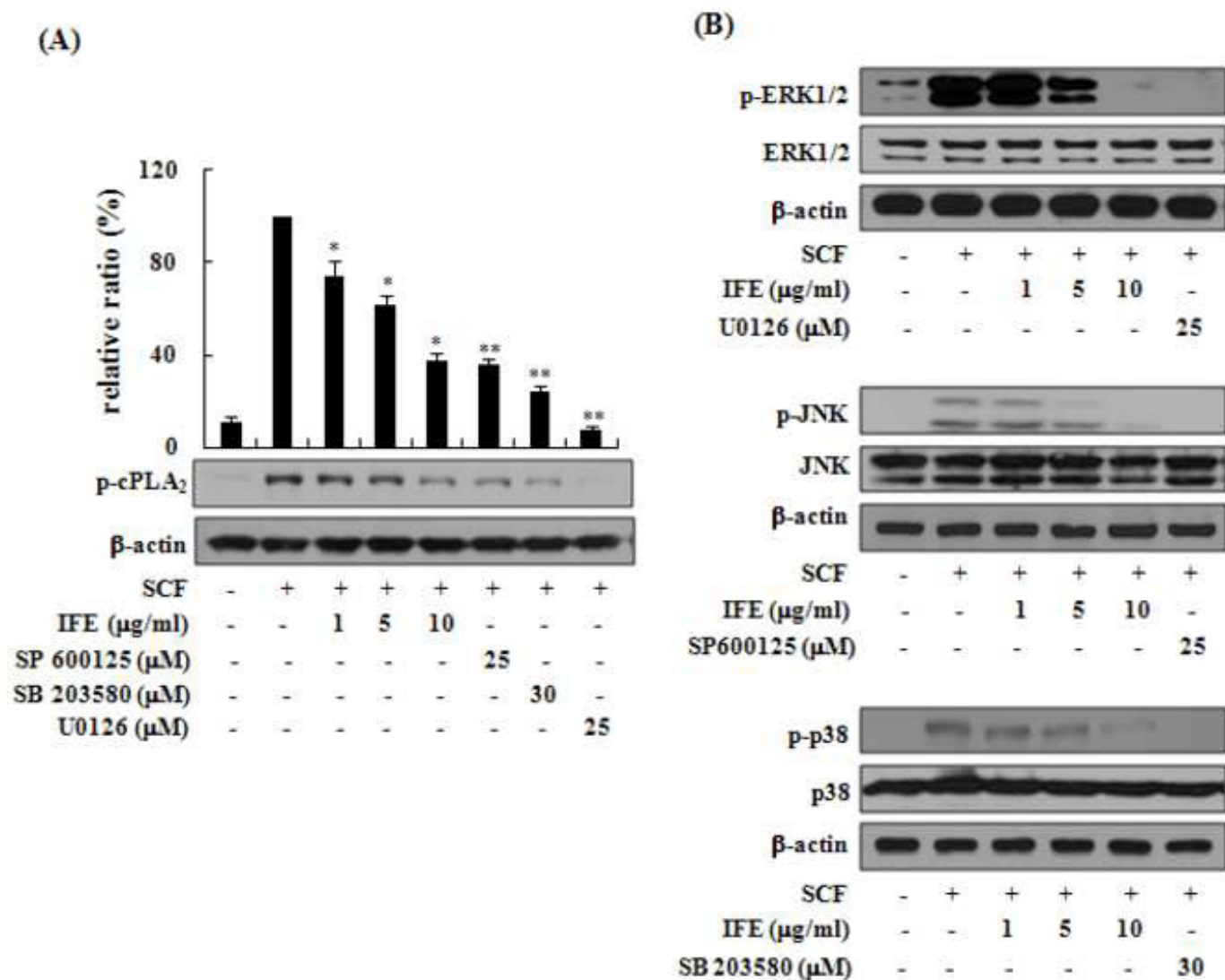


Fig. 5

