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Original Research Article

Regulation of Immunological Effect of *Rubia cordifolia* Extract and Associated MAPKs Pathway in RBL-2H3 Cell-line

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Abstract - *Rubia cordifolia* has been used to treat diseases for many years in China and India. Although the biological properties and major compounds of *R. cordifolia* have been extensively studied, the underlying mechanisms of its biological effects remain elusive. In terms of immunological effects, anti-inflammation effect of macrophage (Raw 264.7) simply has been reported. In this study, *R. cordifolia* was extracted in 70% ethanol and the extract did not affect to macrophage (Raw 264.7) pro-inflammation and T cell (Molt-4). However, in mast cell (RBL-2H3), it showed inhibition of degranulation. The inducing inhibitory effect on degranulation was related to concentration dependent variation in phosphorylation of ERK-1/2 and upregulating the JNK phosphorylation in RBL-2H3 cells. Based on these data, we concluded that *R. cordifolia* newly have anti-allergenic effects in RBL-2H3 and might be used as a therapeutic agent to treat or prevent allergic diseases such as asthma and atopic dermatitis.

Key words - Anti allergy, Immune cells, MAPKs, Mast cells

Introduction

Immune system is essential for our health. If immune system abnormally works, we cannot endure a light infection and are led to death. Immune system consist of cells, which are derived from hematopoietic stem cells, with different functions (Aggarwal and Pittenger, 2005). In this study, among the immune cells, we targeted Macrophage, T cell and Mast cell.

Macrophage is differentiated from monocyte and has various phenotype as tissue environment. Its typical role is phagocytosis of invading microbes (Mosser and Edwards, 2008). LPS (Lipopolysaccharide) as a ligand of TLR4 (Toll-like Receptor 4) of Macrophage and IFN-γ released from T lymphocyte induce M1 Macrophage. It activates transcriptional factor NFκB (Nuclear factor κB) and expresses inflammatory cytokine such as TNF-α, IL-1, IL-6 and IL-12. At the early stage of infection, these responses are essential for innate immunity. On the other hand, IL-4 and IL-13 are recognized to mannose receptor result in M2 Macrophage polarization (Italiani and Boraschi, 2014; Lawrence and Natoli, 2011; Martinez and Gordon, 2014).

T cells are divided to cytotoxic T cell and helper T cell depending on function. Cytotoxic T cell kills bacteria or virus infected cells and involved in adaptive immunity. Helper T cells help development of immune cells by cytokine secretion (Broere *et al.*, 2011; Janeway *et al.*, 2001). Helper T cell has many subtypes such as Th-1, Th-2, Th-17 and regulatory T cell by various transcriptional factors and cytokines. Differentiated helper T cells have individual functions and cytokines (Zhu *et al.*, 2010). Th-1 cells are induced by IFN-γ and T-bet and activating Macrophage by releasing IL-2, IFN-r. Th-2 cells are made by IL-4 and GATA3 and function as B cell activator by IL-4, IL-5 secretion (Abbas *et al.*, 1996; Mosmann and Coffman, 1989).

Mast cells are granulocytes that play a key role in allergy and inflammation. They primarily serve to protect from infections; however, acute or chronic inflammation induces allergic diseases such as asthma, hay fever, and atopic eczema (Kim *et al.*, 2015). Mast cell expresses FcεRI, which has high affinity for IgE; the aggregation of FcεRI induces mast cell activation (Lee *et al.*, 2015). Activated mast cells

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degranulate and release various inflammatory mediators including histamine, β-hexosaminidase, and numerous cytokines (Huang *et al.*, 2008). Intracellular Ca^{2+} levels are increased, which ultimately leads to activation of the mitogen-activated protein kinases (MAPKs) (Namkoong *et al.*, 2012; Nishida *et al.*, 2005).

MAPKs are serine/threonine protein kinases that are highly conserved during evolution. MAPK cascades are initiated by membrane receptors recognizing a wide range of extracellular stimuli. MAPKs play a role in cellular differentiation, proliferation, stress responses, apoptosis, and immune responses. Signal transmission occurs by the sequential phosphorylation of MAPK kinase kinase (MAPKKK), MAPKK, and ultimately MAPK; they are inactivated by MAPK phosphatase. As a result, phosphorylated MAPKs translocate to the nucleus and regulate the phosphorylation of transcription factors for gene expression (Gilfillan and Tkaczyk, 2006; Liu *et al.*, 2007; Seger and Krebs, 1995). In many study, association between MAPKs and allergy is well demonstrated (Lee *et al.*, 2014; Rosenstein *et al.*, 2014). Therefore, materials which can inhibit mast cell degranulation can be used as anti-allergenic or anti-inflammatory medicine.

Rubia cordifolia has been used as a medicinal herb for a long time in India and China (Priya and Siril, 2014). Many studies have revealed that extracts of *R. cordifolia* have a variety of biological effects (Priya and Siril, 2014). Specifically, the rhizome of *R. cordifolia* contains many different classes of phytochemicals which are recognized as biologically active substances including quinines, iridoids, oleanane triterpenoid, bicyclic hexapeptides, and anthraquinones (Patil *et al.*, 2009). The methanolic extracts of *R. cordifolia* have demonstrated potent inhibitory effect in models of acute inflammation (Patel *et al.*, 2010). Patel *et al*. (2011) suggested that *R. cordifolia* has anticancer effects because the methanolic fraction of *R. cordifolia* showed considerable cytotoxicity to both a human cervical cancer cell line and a human larynx carcinoma cell line. In addition, the ethanolic extract of *R. cordifolia* had a wound healing effect in mice (Karodi *et al.*, 2009). Gutpa *et al*. (1993) revealed that extracts of the *R. cordifolia* rhizome inhibited passive cutaneous anaphylaxis (PCA) in mice and rats. In this study, they reported that the chloroform layer, and mollugin isolated from hexane layer, had the most potent inhibitory effects on passive cutaneous anaphylaxis (PCA) and mast cell degranulation. However, it has not been yet elucidated how the extract of *R. cordifolia* rhizome can inhibit the degranulation of mast cells.

In present study, we investigated the immunological effects of the rhizome extract of *R. cordifolia* on typical immune cells which were Mast cell, Macrophage and T cells and also defined the effect related mechanism.

Materials and Methods

Plant material and Extraction

The dried rhizome of *R. cordifolia* was reduced to powder and extracted in 70% EtOH solvent (100 ㎎/㎖, 4°C, 24hr). The supernatant of the extracted solution was isolated by centrifugation (5000 rpm, 5min, 4°C) and stored at -80°C.

Cell culture

In DMEM (Hyclone, Logan, USA), RBL-2H3 cells and Raw 264.7 cells and in RPMI-1640 medium (Hyclone, Logan, USA), MOLT-4 cells were cultured with 10% fetal bovine serum (FBS), 100 U/㎖ penicillin, 100 ㎍/㎖ streptomycin (Hyclone, Logan, USA) and incubated at 37°C at 5% CO_2.

MTT assay for cell viability

The MTT assay was used to determine the cytotoxicity induced by the extract of *R. cordifolia* rhizome. RBL-2H3 cells (1 x 10^5 cells/well) and Raw 264.7 cells (5 x 10^4 cells/well) were cultured in a 24-well plate for 24 hr and treated with various concentrations (from 0 to 1 ㎎/㎖) of the ethanol extract of *R. cordifolia* rhizome, for 24 hr (37°C, 5% CO_2). After removing the supernatant, 5 ㎎/㎖ MTT solution was added to each well and incubated for 4hr. Finally, media were removed, and the formed formazan was dissolved with 300 ㎕ DMSO. Molt-4 cells (2 x 10^5 cells /well) were cultured in a 24-well plate for 24 hr and treated with the ethanol extract of *R. cordifolia* rhizome, for 24 hr (37°C, 5% CO_2). After 5 ㎎/㎖ MTT solution was add to supernatant in each well and incubated for 4hr. Finally, media were removed, and the formed formazan was dissolved with 400 ㎕ DMSO. The absorbance of each sample was detected at 560 ㎚ with microplate reader, Epoch™ (BioTek, USA).

ß-hexosaminidase release assay

To invest mast cell degranulation inhibition by the extract of *R. cordifolia* rhizome, we measured the release of β-hexosaminidase as a marker of degranulation. RBL-2H3 cells were cultured in a 24-well plate (2 x 10^5 cells/well) for 24hr. Each well was washed 2 times with Siraganian buffer (119 nM NaCl, 5 nM KCl, 5.6 mM Glucose, 0.4 mM $MgCl_2$, 25 mM PIPES, 1 mM $CaCl_2$, 0.1% BSA, pH 7.2) and treated with the extract of *R. cordifolia* rhizome in Siraganian buffer as vehicle for 1 hr (37°C, 5% CO_2). After incubation, each well was washed 2 times with Siraganian buffer and treated with 1 μM A23187 / 50 nM PMA (Sigma, USA) in Siraganian buffer vehicle for 30 min. at 37°C. An aliquot of each supernatant (20 μl) was transferred to a 96-well plate and incubated with 80 μl of substrate buffer (4-ρ-Nitrophenyl-N-acetyl-β-D-glucosaminide 2 mM, sodium citrate 0.05 M, pH 4.5) for 30 min at 37°C. The reaction was stopped by addition of 200 μl of stop solution (0.1 M $NaHCO_3$, pH 10), and the absorbance of each sample was measured at 405 ㎚ with a microplate reader, Epoch™ (BioTek, USA).

NO assay for pro-inflammatory effect

To evaluate the Pro-inflammation effect in Raw 264.7 cells, Raw 264.7 cells were cultured in 24-well plate (5 x 10^4 cells/well) for 24 hr and treated with ethanol extract of *R. cordifolia* rhizome (1 ㎎/㎖) for 24 hr (37°C, 5% CO_2). 100 μl of supernatants were added to 96-well plate and 100 μl Griess reagent which was mixed with griess reagent A (1% sulfanilic acid in 30% acetic acid) and griess reagent B (1% α-naphtylamine in 30% acetic acid) (1:1). 96-well plate was incubated at 25°C for 15 min and absorbance was measured at 540 ㎚ with a microplate reader, Epoch™ (BioTek, USA).

NO assay for Anti-inflammatory effect

Anti-inflammatory effect determination assay is very similar with Pro-inflammatory assay. Among the above mentioned, the different is that after extract treatment for 1hr, LPS (1 ㎎/㎖) is treated for 24 hr in 37°C, 5% CO_2. N-iminoethyl-$_L$-lysine, L-NIL (10 μM) was used as positive control. After that process, detection method is exactly same with pro-inflammation assay.

RNA isolation

Molt-4 cells (5 x 10^5 cells/well) are cultured in 24-well plate for 24 hr and treated with ethanol extract of *R. cordifolia* rhizome (1 ㎎/㎖) for 6 hr (37°C, 5% CO_2). Cells are harvested for pellet by centrifugation (7500 rcf, 5 min 3 sec, 4°C). To isolate total RNA, AccuZol RNA Extraction Solution (Bioneer) was added to pellet and mixed by pipetting. Then to separate aqueous phase from phenolic phase, 200 μl chloroform was add and centrifuged (12000 rcf, 15 min 30 sec, 4°C). 450 μl of supernatant was moved to new tube and added same amount of isopropanol. After centrifuge, RNA was precipitated to pellet and supernatant was removed. Finally, RNA pellet was washed using 70% EtOH and treated with DNase using DNA-free™ kit from Ambion. The RNA concentration was measured by Epoch™.

Quantification of cytokine gene expression by qRT-PCR

0.5 ㎍ of isolated RNA was used for cDNA synthesis (Rever Tra Ace^R QPCR RT kit (TOYOBO, Japan)). It is conducted by the thermal program at 37°C for 15min, 50°C for 5 min and 98°C for 5 min. quantitative RT-PCR was performed with 1.5 μl of cDNA, 5 μl Master mix (SYBRs Green qPCR Master Mix (TOYOBO, Japan)) and 0.5 μl of each sense and antisense primer (10 ㎜ol/μl). Total reaction volume, 20 μl was stuffed with nuclease free water. Primer IFN-γ (S:CCA ACC TAA GCA AGA TCC CA, A:GGGTC ACCTGACACATTCAA), IL-4 (S:CAC CTT ACA GGA GAT CAT CAA AAC T, A:TCC TTC TCA GTT GTG TTC TTG G) was used for analysis cytokine gene expression levels and HPRT (S:CCT GGC GTC GTG ATT AGT G, A:TGA GGA ATA AAC ACC CTT TCC A) was used for normalization. The reaction was performed in a real- time detection system, StepOnePlus™ REALTIMEPCRSYSTEMS (Applied Biosystems, USA). The results were analyzed using the ΔΔCt method.

Immunoblotting

To investigate the MAPK signaling pathway, RBL-3H2 cells were cultured in 60 ㎜ dish (6 x 10^5 cell/㎖), and treated as described above in the β-hexosaminidase release assay. The cell pellet is collected, washed with PBS, and then lysed with RIPA buffer (Sigma, USA) containing protease and

phosphatase inhibitor. Protein concentration of lysed sample was determined using the BCA assay. Cellular protein (35 ㎍) was loaded in 10% SDS-PAGE gel and transferred to poly vinylidene difluoride (PVDF) membrane (GE healthcare, USA). The membrane was blocked with 1% skim milk in TBS-T for 1hr. The membrane was incubated with primary antibodies against p38, ERK 1/2, JNK, phosphorylated p38, phosphorylated ERK 1/2, phosphorylated JNK (Cell signal, USA) and β-actin (Santacruz, USA) (1:1000) in 1% skim milk and shaken at 4°C for 16hr. The membrane was washed 3 times with PBS-T and incubated with horseradish peroxidase (HRP)-conjugated secondary antibody (1:5000, Santacruz, USA) for 1 hr and washed. Protein signals were visualized using ECL solution (Advasta, USA).

Statistical analysis

Each experiment was repeated at least 3 times. The results were represented as mean ± SD. Statistical significance was determined through one-way analysis of variance (ANOVA) with Bonferroni correction using SPSS (v.21, for Windows). P value < 0.05 was considered as statistically significant.

Results and Discussions

Traditionally, medicinal herbs have been used to treat many immune disorders including allergy and inflammation. Medicines originating from natural products have fewer side effects and continuous effects compared to chemically synthesized medicines (Mohamed *et al.*, 2012). In this study, we investigated the pharmacological effects of the rhizomal extract of *R. cordifolia*.

RBL-2H3 cells belong to rodent mast cell line commonly used in the fields of allergy and inflammation research. Mast cells are activated by aggregation of FcεRI with IgE, and release numerous inflammatory mediators including histamine and the enzyme β-hexosaminidase. Since β-hexosaminidase correlates with histamine, it is used as a marker of mast cell degranulation. In this context, we measured β-hexosaminidase levels in the supernatants of RBL-2H3 cells activated by PMA and A23187 and treated with extract of *R. cordifolia* rhizome. The extract of the *R. cordifolia* rhizome decreased β-hexosaminidase release in a concentration−dependent manner, starting at a concentration of 0.5 ㎎/㎖ which inhibited release by more than 40% (Fig. 1A). This suggests that the extract of *R. cordifolia* rhizome has an inhibitory effect on mast cell degranulation, and is similar to previously published studies (Gupta *et al.*, 1993). Also, the cytotoxicity of *R. cordifolia* extract was evaluated using MTT assay. RBL-2H3 cells were activated with PMA and A23187 and treated with various concentrations of *R. cordifolia* extract. *R cordifolia* extract did not significantly affect the viability of RBL-2H3 cells at concentrations up to 1 ㎎/㎖.

To investigate another immunological effect of *R. cordifolia* rhizome extract, we tested Macrophage and T helper cell activation effect in, respectively, Raw 264.7 cell line and Molt-4 cell line. Molt-4 cells were treated with *R. cordifolia* rhizome extract (1 ㎎/㎖) and cell toxicity was not shown. About T helper cell activation, we analyzed gene expression level of typical Th1 cytokine (IFN-γ) and Th2 cytokine (IL-4). However, the result was not significant (Fig. 1B). In Raw 264.7 cell, 1 ㎎/㎖ of extraction did not affect to cell viability but also no effect in terms of pro-inflammation. There was no difference between treated sample and control (Fig. 1C). However, anti-inflammatory effect was significant (Fig. 1D) and this result accorded closely with reported researches (Ghosh *et al.*, 2010; Kasture *et al.*, 2001; Patel *et al.*, 2010). Thus, these data demonstrate that *R. cordifolia* extract has inhibition effect of degranulation in mast cell excepting already known effect.

Therefore, to determine which signaling pathways are regulated by the extract of *R. cordifolia* rhizome in mast cell, we evaluated the phosphorylation of JNK, p38, and ERK-1/2 MAPK pathways via western blot. These MAPKs respond to many different stimuli (Liu *et al.*, 2007), and their signaling cascade plays a critical role in immune cell signal transmission (Johnson and Lapadat, 2002). The phosphorylation level of p38 (upper band) was not significantly changed by any concentration of *R. cordifolia* rhizome extract, and phosphorylation of JNK increased proportionally with the concentration of the extract. However, phosphorylation of ERK-1/2 has concentration dependent variation (Fig. 2).

Taken together, extract of *R. cordifolia* rhizome inhibits mast cell degranulation and this effect was shown by alteration of the phosphorylation of ERK-1/2 and JNK (Fig.

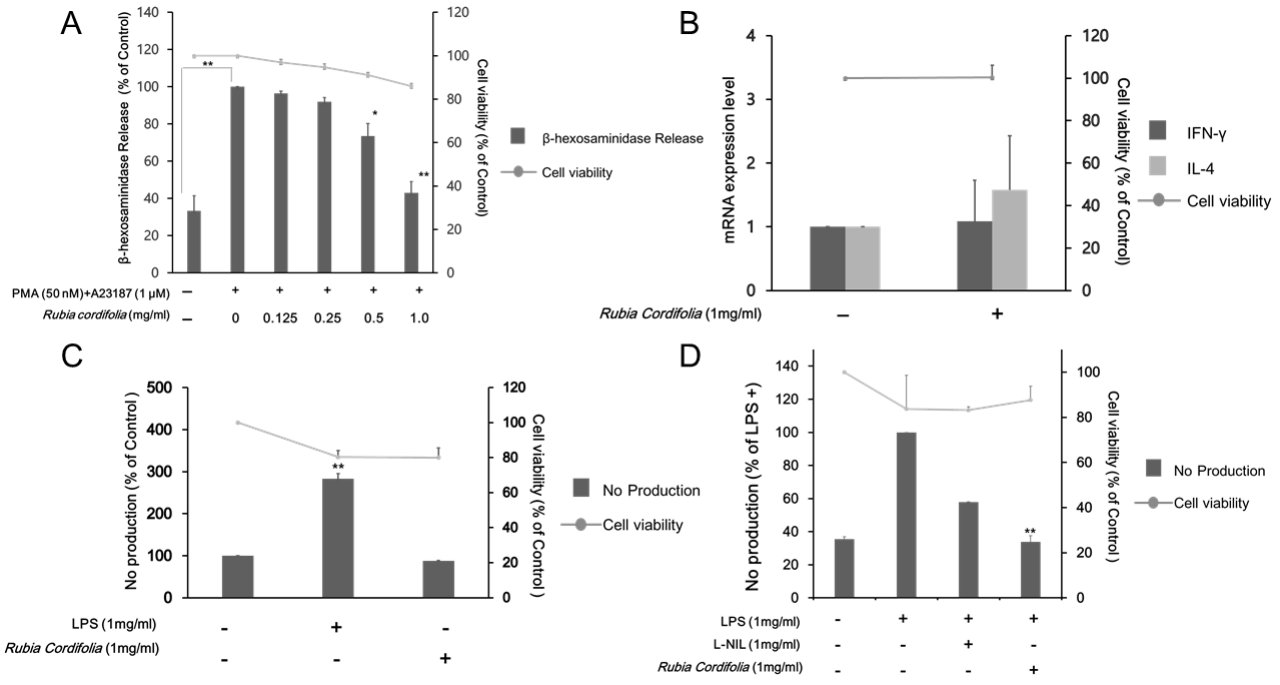

Fig. 1. Immunological effects of *R.cordifolia* rhizome extract. (A) Extract of *R.cordifolia* rhizome inhibits β-hexosaminidase release. The cells were treated with various concentration of extraction of *R.cordifolia* rhizome for 1hr and treated with 1 μM A23187/50 nM PMA for 30min. (B) T cells were not activated by 1 ㎎/㎖ of *R.cordifolia* extract. Expression level of IFN-γ and IL-4 measured by qRT-PCR were not significant. In Raw 264.7 cell, (C) pro-inflammatory effect was not affected by *R.cordifolia* extract (1 ㎎/㎖) and (D) Anti-inflammatory effect by *R.cordifolia* extract (1 ㎎/㎖) was considerable. NO productivity is detected by absorbance at 540 ㎚. Cell toxicity by extract of *R.cordifolia* rhizome was not shown in all cell line. The results are manifested as the mean ± SD of three independent experiments. *P < 0.05, **P < 0.01 : significantly different from control group.

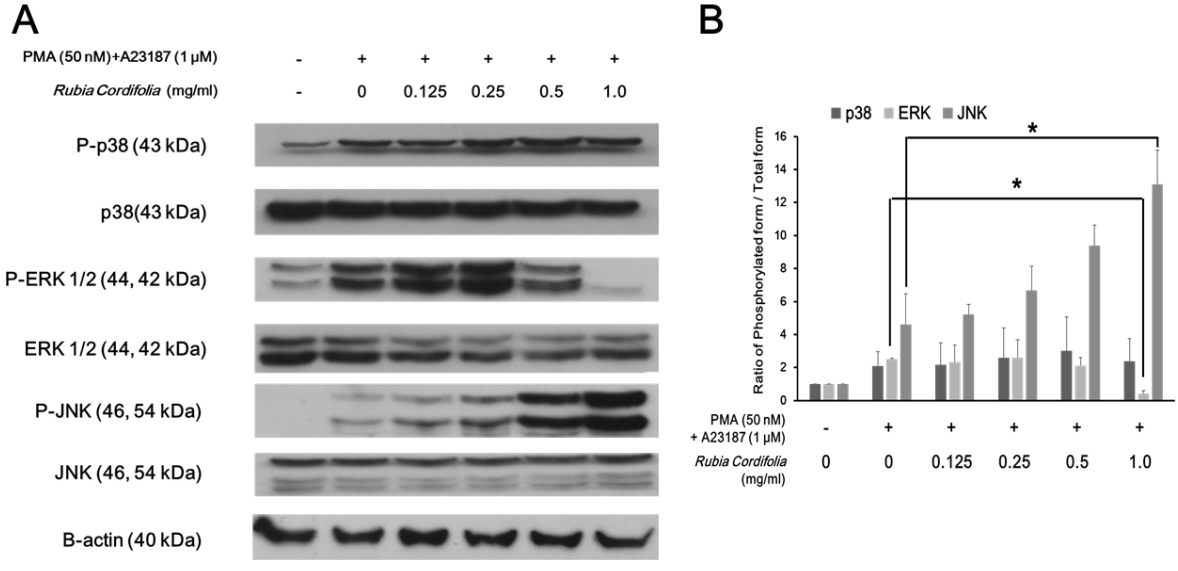

Fig. 2. Effect of extract of *R.cordifolia* rhizome on the phosphorylation of MAPKs. (A) Phosphorylation of MAPKs was determined by Immunoblotting assay. (B) Densitometric analysis of MAPKs phosphorylation. The results show p38 was not significant but ERK 1/2 had concentration dependent variation and JNK was increased by *R.cordifolia* concentration.

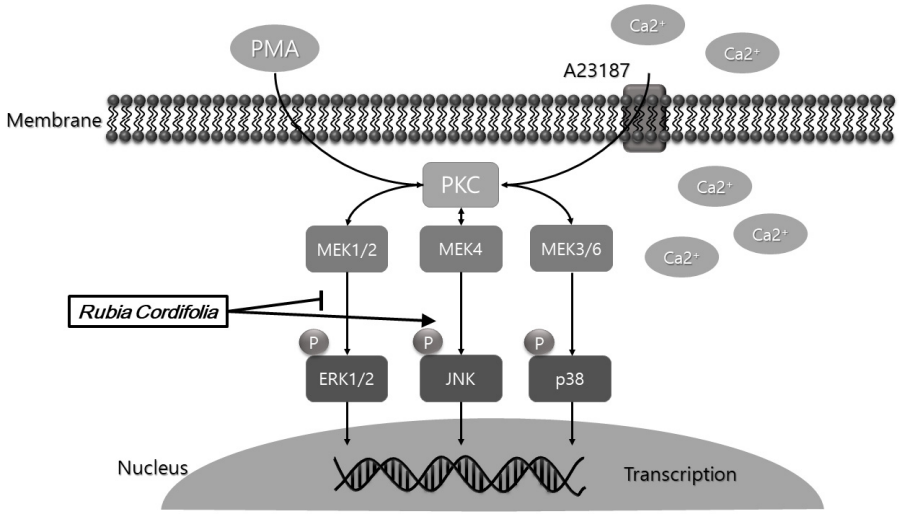

Fig. 3. Schematic diagram. Mast cell activated with PMA and A23187 works on MAPKs. Treatment of *R.cordifolia* in mast cell led to concentration dependent variation of ERK1/2 phosphorylation and increasing of JNK phosphorylation.

3). ERK plays a key role in cellular differentiation and proliferation (Robert, 2012). It is well known that not only in mast cells, but also in other inflammatory cells such as T-cells and B-cells, targeting MAPK can reduce inflammatory effects in allergenic diseases (Simard *et al.*, 2010). In IgE-stimulated mast cells, treatment with an inhibitor of ERK-1/2 attenuated the production of cysteinyl leukotrienes (cysLTs), which are known as primary inflammatory lipid mediators (Cho *et al.*, 2004). In addition, Kim *et al*. (2014) demonstrated that the expression of pro-inflammatory cytokines (IL-1ß and IL-6) is decreased through inhibition of the ERK1/2 pathway. Therefore, a substance that targets the ERK1/2 pathway can be used as an effective treatment for inflammation and allergies. The full name of JNK is a c-Jun N-terminal kinase and the phosphorylation of JNK activates the transcription factor, AP-1 (Liu *et al.*, 2007). The generally known function of JNK and AP-1 is cell death and apoptosis (Karin *et al.*, 1997). In terms of immunological response, JNK activation caused TNF and IL-10 secretion (Liu *et al.*, 2007) and inhibition of bone-marrow-derived mast cell survival (Jeffrey *et al.*, 2006). Accordingly, showing cell toxicity in exceed treatment of *R. cordifolia* (1 ㎎/㎖) is regarded as effect of JNK pathway.

In present study, we confirmed that the already known effect of *R. cordifolia* extract, that is an anti-inflammatory effect in Macrophage and there was no effect on Macrophage pro-inflammation and T cell activation. However, in mast cell, degranulation inhibitory effect was manifested. Also, we demonstrated that this effect is associated with the alteration of phosphorylation in ERK-1/2 and JNK of MAPKs. These results support the use of the extract of *R. cordifolia* rhizome as an anti-allergenic treatment.

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