

## Inhibitory Effect of *Alpiniae officinarum* Rhizoma Extract on Degranulation in RBL-2H3 Cells

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**Abstract** - *Alpiniae officinarum* Rhizoma (the rhizome of *Alpinia officinarum* Hance, known as lesser galangal), a family of *Zingiberaceae*, has been used to reduce pain of infection and inflammatory diseases in Asian countries. The present study was focused to evaluate the inhibitory degranulation effect of *Alpiniae officinarum* Rhizoma extract in RBL-2H3 rat basophilic leukemia cells. Cell viability was measured by MTT assay. RBL-2H3 cells were stimulated by phorbol 12-myristate 13-acetate and calcium ionophore A23187. Mast cell degranulation was analyzed by measuring release of  $\beta$ -hexosaminidase in RBL-2H3 cell. Gene expression was measured by qRT-PCR and signaling molecules were detected by immunoblotting. The *Alpiniae officinarum* Rhizoma extract suppressed  $\beta$ -hexosaminidase release in dose-dependent manner and inhibited cyclooxygenase-2 and tumor necrosis factor- $\alpha$  gene expression. Furthermore, it was found that *Alpiniae officinarum* Rhizoma extract reduced mitogen-activated protein kinases, especially phosphorylated p38, at 0.75 mg/ml of *Alpiniae officinarum* Rhizoma extract concentrations. These data show that *Alpiniae officinarum* Rhizoma extract has immunosuppressive effect in mast cell induced allergic inflammation.

**Key words** - Allergic inflammation, COX-2,  $\beta$ -hexosaminidase, Mast cells, Mitogen-activated protein kinases

### Introduction

The rhizome of *Alpiniae officinarum* Hance (*Alpinia officinarum* Rhizoma, AOR), known as lesser galangal, is a family of *Zingiberaceae*. It has been used as a traditional herbal medicine for analgesics and antiphlogistic drugs in Asian countries. Recent studies have demonstrated various properties of AOR such as anti-fungal and anti-bacterial activity (Liu *et al.*, 2012; Zhang *et al.*, 2013), anti-inflammatory activity (Lee *et al.*, 2009) and anti-tumor activity (Ghil, 2013; Zhu *et al.*, 2014). But its immunosuppressive effect on mast cell induced allergic inflammation was not proved by scientific methods.

Allergic reaction occurs because of the excessive response of immune system. It causes symptoms including allergic rhinitis, asthma, anaphylaxis, eczema and urticaria (Kay, 2000). Mast cells play a key role in allergic inflammatory responses. Mast cells are member of immune cells and involved in wound healing, fibrosis and fight against infection. But

excessive or wrong stimulation induces mast cells to cause autoimmunity and allergic reaction (Brown *et al.*, 2008). Basophils have much in common with mast cells. Basophils have many granules and secrete mediators and irritate the body. Furthermore, it is more easily to obtain basophils than tissue-derived mast cells (Siracusa *et al.*, 2013). Therefore, basophils have been used for substitution of mast cells in studies of allergic inflammation. When it is stimulated by IgE, high affinity IgE receptor (Fc $\epsilon$  RI) triggers calcium influx and activates protein kinase C (PKC) and mitogen-activated protein kinases (MAPKs) (Choi *et al.*, 2012). This signaling process induces mast cells to secrete preformed mediators, such as histamine and heparin, by degranulation and produce lipid mediators and cytokines including Interleukin (IL)-4, 10, 13 and tumor necrosis factor (TNF)- $\alpha$  (Moon *et al.*, 2014). These mast cell-derived mediators trigger allergic inflammatory responses. Therefore, regulation of mast cell is important to treat allergic inflammatory diseases.

The objectives of this study were to examine: 1) whether AOR extract inhibits allergic inflammation; 2) if there was an

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inhibitory effect, whether a pro-inflammatory cytokine expression level was down-regulated; and 3) whether AOR extract's inhibitory effect was related with MAPKs pathway.

## Materials and Methods

### Preparation of AOR extract

In order to extract water and lipid soluble biological active ingredients, 100 mg of dried rhizome of *Alpinia officinarum* Hance was extracted by being placed on shaker (Nanoentek, Korea. Programmed with mode F7 and 20 rpm) in 1 ml of 70% ethanol at 4°C for 24 hr. After centrifugation at 9,000 g for 5 min by 1730MR (Labogene, Korea), the supernatant was obtained.

### Cell culture

RBL-2H3 rat basophilic cell line was used to evaluate suppressive effect of extract on mast cell. The cells were cultured in DMEM medium (Hyclone, USA) with 10% fetal bovine serum, 100 U/ml penicillin and 0.1 mg/ml of streptomycin at 37°C under 5% CO<sub>2</sub>. The cells were cultured for 24 hr and treated with extract.

### MTT assay

The cytotoxicity of extract was measured by MTT colorimetric assay.  $1 \times 10^5$  cells were cultured in 24 well plate for 24 hr and AOR extract was treated. After 1 hr, 50 nM of phorbol 12-myristate 13-acetate (PMA) and 1 µM of calcium ionophore A23187 was added for 30 min. After replacing the media, 50 µl of Thiazolyl Blue Tetrazolium Bromide (MTT) solution (Sigma, USA) was added and incubated for 4 hr. Subsequently, the media was removed and 300 µl of DMSO was added to dissolve formazan crystal. The solvent was moved to 96 well plate. The absorbance of each sample was detected by spectrophotometer, Epoch<sup>TM</sup> (BioTek, USA), at 550 nm.

### β-hexosaminidase release assay

Release of β-hexosaminidase was measured to evaluate inhibitory effect of extract on mast cell degranulation.  $1 \times 10^5$  cells were cultured in 24 well plate for 24 hr and AOR extract was treated for 1 hr. Then the cells stimulated by 50 nM of

PMA and 1 µM of A23187 for 30min. After stimulation, 20 µl of supernatant was moved to 96 well plate. 80 µl of substrate buffer was treated each well and incubated at 37°C for 30 min. 200 µl of 0.1 M carbonate buffer (pH 10) was used to stop reaction. The absorbance was measured by spectrophotometer, Epoch<sup>TM</sup>, at 405 nm. The release of β-hexosaminidase was calculated by percentage compared to PMA/A23187 stimulated control.

### RNA isolation and qRT-PCR

The cell pellets were lysed with 1 ml of X-zol reagent (Phile Korea Technology, Korea). Then 200 µl of chloroform was added and centrifuged to separate aqueous phase from phenolic phase (12000 rcf, 15 min, 4°C). To precipitate the RNA from aqueous phase, 500 µl of isopropanol was added. After centrifuging, the supernatant was removed and RNA pellet was remained. The RNA pellet was washed with 70% EtOH. DNase treatment was done using DNA-free<sup>TM</sup> kit (Ambion, USA). The RNA concentration was measured by Epoch<sup>TM</sup>.

Reverse transcription was conducted with ReverTraAce® qPCR RT Master Mix (TOYOBO, Japan). Five hundred µl of total RNA in 10 µl reaction volume containing 2 µl of 5x RT Master Mix was reverse transcribed into cDNA. The process was carried out by thermal program of 65°C for 5 min, 37°C for 15 min, followed by 50°C for 5 min, 98°C for 5 min.

Quantitative RT-PCR was performed using SYBR® Green qPCR Master Mix (TOYOBO, Japan). The mixture contained 1.5 µl of cDNA samples, 5 µl of qPCR Master Mix, and 0.5 µl of each forward and reverse primer (10 pmol/µl), then RNase free water was added to make up for 20 µl of total reaction volume. The primers for COX-2 (F : TGG TGC CGG GTC TGA TGA TG, R : GCA ATG CGG TTC TGA TAC TG), IL-4 (F : TAC GGC AAC AAG GAA CAC, R : TCT TCA AGC ACG GAG GTA ), and TNF-α (F : TGA ACT TCG GGG TGA TCG, R : GGG CTT GTC ACT CGA GTT TT) were used to analyzed gene expression level and RPLP0 (F : GTG TTT GAC AAT GGC AGC AT, R : ACA GAC GCT GGC CAC ATT) was used as an internal control. The reaction was conducted in a real-time detection system, StepOnePlus<sup>TM</sup> REALTIME PCR SYSTEMS (Applied Biosystems, USA). The PCR profiles contained an initial

denaturation step at 95°C for 1 min, followed by 40 cycles of amplification step including denaturation at 95°C for 15s, annealing at 58°C for 30s, and elongation at 95°C for 15s with a melting curve analysis at 95°C for 15s, 60°C for 30s, and 95°C for 15s. The result was analyzed by  $\Delta\Delta C_t$  method.

### Immunoblotting

To determine the signaling mechanism, the cells were treated with AOR extract for 1 hr and stimulated by 50 nM of PMA and 1  $\mu$ M of A23187 for 10 min. The cell pellets were lysed in RIPA buffer (Sigma, USA) containing protease & phosphatase inhibitor (Thermo scientific, USA). The concentration of lysed protein samples was measured by BCA methods. Thirty  $\mu$ g of protein were loaded in 10% SDS-PAGE gel and transferred to PVDF membrane (GE healthcare, USA). Blotted membrane was blocked with 5% skim milk in TBS-T for 1 hr and incubated with primary antibody against p38, ERK, JNK, phosphorylated p38, phosphorylated ERK, phosphorylated JNK and  $\beta$ -actin (Cell signal, USA) in 1% skim milk for 16 hr at 4°C. Then the membrane was washed with TBS-T for 3 times. After treatment of horseradish peroxidase-linked secondary antibody (Santacruz, USA) for

1 hr and washing, protein signals were detected using Western Bright Peroxide chemi luminescent detection reagent (Advasta, USA). Densitometry of blots was performed using UltraQuant software (version 2013.5.10.1, Aplegen, USA).

## Results

### Effect of AOR extract on cell viability in RBL-2H3 cells

The cytotoxic effect of AOR extract was measured by MTT assay (Fig. 1). AOR extract was treated to cells for 1 hr and PMA/A23187 was added to stimulate the cells for 30 min. As a result, AOR extract did not significantly affect the cell viability ranging from 0 to 0.75 mg/ml of AOR concentrations. However, the cell viability was decreased by  $83.59 \pm 1.29$  % in 1 mg/ml of AOR extract treated cells. Therefore, following experiments were conducted from 0 to 0.75 mg/ml.

### Effect of AOR extract on degranulation of PMA/A23187 induced RBL-2H3 cells

Degranulation was measured by  $\beta$ -hexosaminidase release assay and it is a simple method to investigate degranulation of mast cells. The effect of AOR extract on  $\beta$ -hexosaminidase

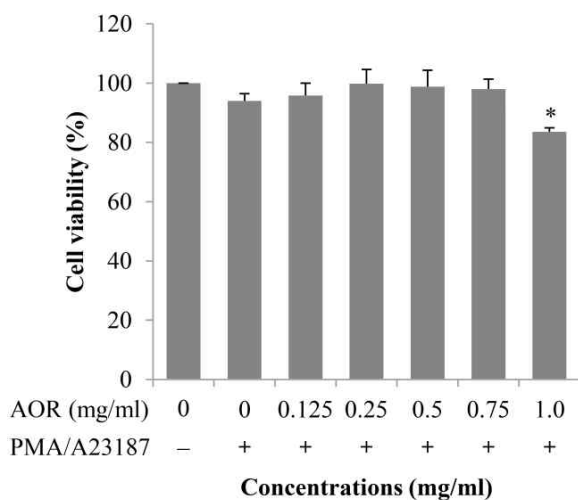


Fig. 1. Effect of AOR extract on cell viability in RBL-2H3 cells. Cell viability was measured by MTT assay. The cells were treated with AOR extract for 1 hr and stimulated by PMA/A23187 for 30 min.

Results are presented in the mean  $\pm$  SD of three independent experiments. \*Value significantly different from control cells (\* $p < 0.05$ ).

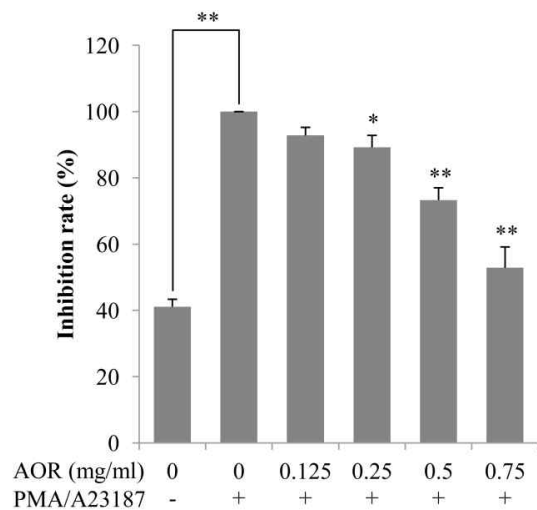


Fig. 2. Effect of AOR extract on degranulation in PMA/A23187 stimulated RBL-2H3 cells. The degranulation was investigated by measuring  $\beta$ -hexosaminidase release. AOR extract was treated for 1 hr and then PMA/A23187 was added to stimulate the cells. Results are presented in the mean  $\pm$  SD of three independent experiments. \*Value significantly different from control cells (\* $p < 0.05$ , \*\* $p < 0.001$ ).

release from RBL-2H3 cells is shown in Fig. 2. According to the result,  $\beta$ -hexosaminidase release was reduced on the concentration-dependent manner.  $\beta$ -hexosaminidase release was decreased by 52.85% in 0.75 mg/ml of AOR extract treated cells. This result shows that AOR extract inhibits degranulation in stimulated RBL-2H3 cells.

#### Effect of AOR extract on COX-2, IL-4, and TNF- $\alpha$ gene expression level in activated RBL-2H3 cells

Activated mast cells trigger allergic inflammation through production of lipid mediators, chemokines, and pro-inflammatory cytokines. To analyze gene expression level, qRT-PCR was performed (Fig. 3). As a result, gene expression level of COX-2 was significantly reduced compared to PMA/A23187 stimulated cells. COX-2 gene expression reached  $0.43 \pm 0.11$

fold in 0.75 mg/ml of AOR extract treated cells (Fig. 3A). TNF- $\alpha$  gene expression was suppressed by AOR extract. The gene expression level was reduced by  $0.55 \pm 0.05$  fold in 0.25 mg/ml of AOR extract treated cells (Fig. 3B). IL-4 gene expression level was also reduced but there was no significant change. It was reduced only  $0.71 \pm 0.08$  fold in 0.125 mg/ml of AOR extract treated cells (Fig. 3C).

#### Effect of AOR extract on MAPKs signaling

MAPK signal pathway is important for cellular activities. To analyze the inhibitory mechanism of AOR extract on RBL-2H3 cells, the phosphorylated MAPKs was evaluated by immunoblotting. The cells were treated with AOR extract for 1 hr and stimulated by PMA/A23187 for 30 min. The protein was extracted from the cell pellet. As shown in Fig. 4,

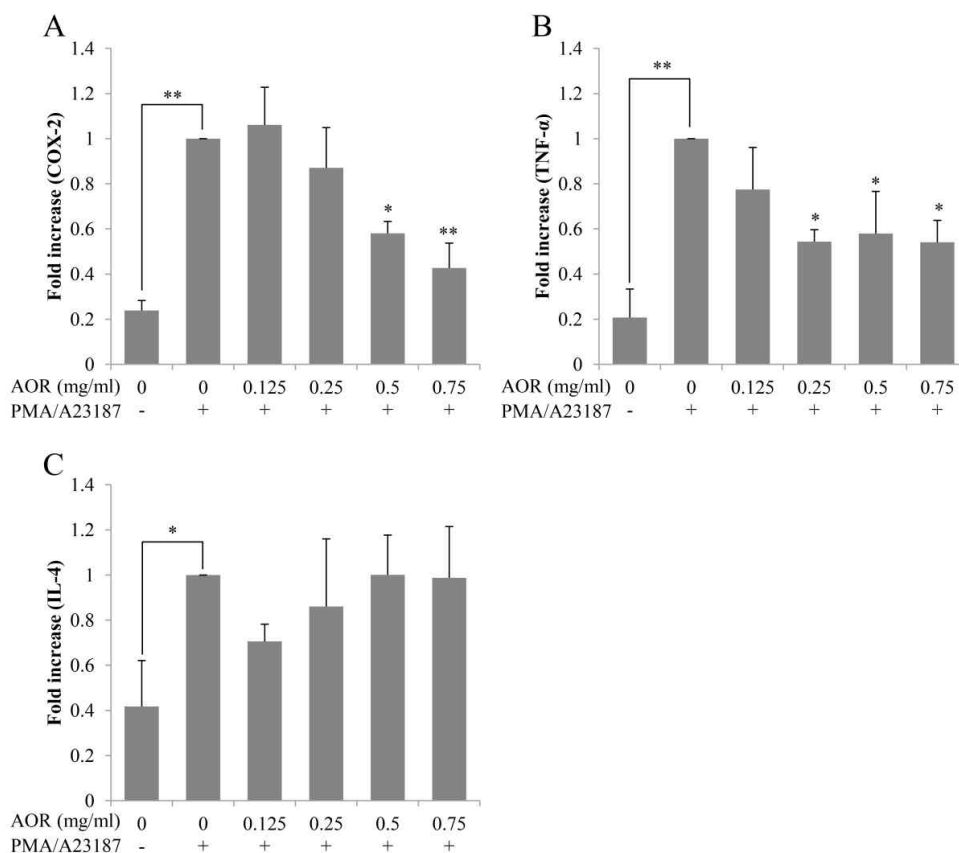


Fig. 3. Effect of AOR extract on gene expression level according to the concentrations. AOR extract was treated for 1 hr and then PMA/A23187 was added to stimulate the cells. The expression of COX-2, TNF- $\alpha$  and IL-4 was detected by qRT-PCR. The results were analyzed with fold change values. (A) COX-2 gene expression; (B) TNF- $\alpha$  gene expression; (C) IL-4 gene expression. Results are presented in the mean  $\pm$  SD of three independent experiments. \*Value significantly different from control cells (\* $p$  < 0.05, \*\* $p$  < 0.001).

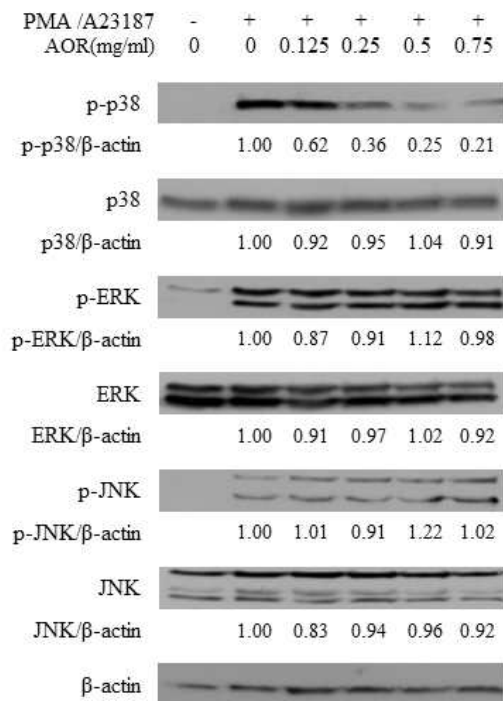


Fig. 4. Effect of AOR extract on MAPKs signaling pathway. AOR extract was treated for 1 hr and then PMA/A23187 was added to stimulate the cells. Phosphorylated p38 was affected by AOR extract. All bands were quantified by densitometric analysis. The intensity of bands was normalized with respect to  $\beta$ -actin.

phosphorylated p38, ERK and JNK was significantly increased by PMA/A23187. Phosphorylated p38 was suppressed by 0.75 mg/ml of AOR extract. However, phosphorylated ERK and JNK were not affected by AOR extract. Therefore, the signal mechanism of AOR extracts could be represented as shown in Fig. 5.

## Discussion

Mast cells induce allergic inflammatory responses, such as rhinitis, asthma, eczema, and asthma, as the cells were sensitized (Kay, 2000; Brown *et al.*, 2008). In this study, RBL-2H3 rat basophilic leukemia cells were used to evaluate the immunosuppressive effect of AOR extract. Basophils are similar with mast cells, so RBL-2H3 cells are used in allergic inflammation model (Park *et al.*, 2010; Siracusa *et al.*, 2013). RBL-2H3 cells were stimulated by PMA and A23187. PMA activates mast cells by activation of PKC pathway and A23187 is involved in calcium influx and degranulation. For this reason, PMA/A23187 is used in studies of allergic reaction instead of IgE-Fc $\epsilon$ RI sensitization (Ludowyke *et al.*, 1996; Ashmole and Bradding, 2013).

Stimulated and activated mast cells release allergic mediators.

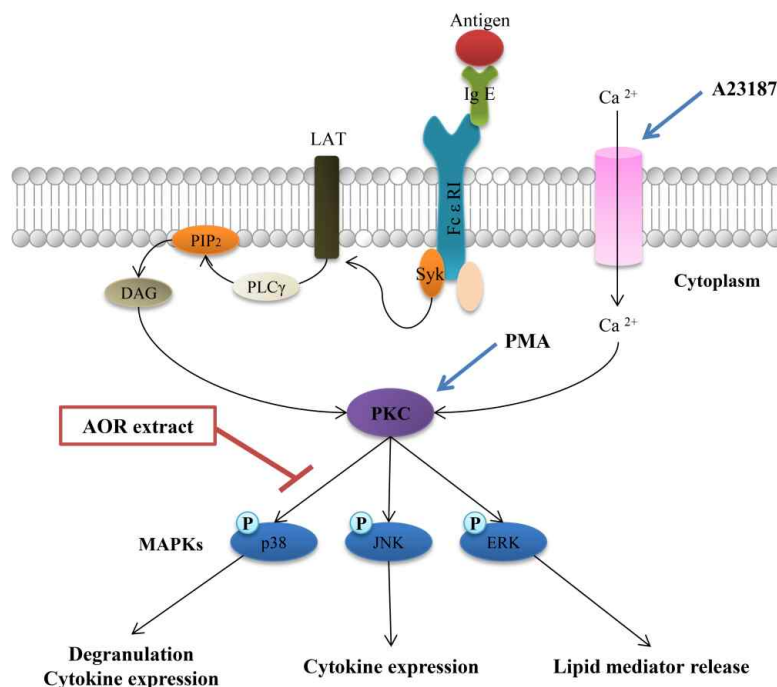


Fig. 5. Signal pathway affected by AOR extract in RBL-2H3 cells.

In early phase, preformed mediators, such as histamine and heparin are secreted (Jung *et al.*, 2011; Jeong *et al.*, 2013).  $\beta$ -hexosaminidase is a lysosomal enzyme which is released with preformed mediators by degranulation.  $\beta$ -hexosaminidase release is easy to be detected by colorimetric method compared to histamine release. Therefore,  $\beta$ -hexosaminidase release is measured to show inhibitory effect on degranulation. In this study,  $\beta$ -hexosaminidase release was significantly reduced by AOR extract on dose-dependent manner. Therefore, it was found that AOR extract inhibited degranulation of mast cells.

Activated mast cells secrete lipid mediators such as leukotriene and prostaglandin. They also produce chemokines and cytokines including IL-4, 5, 10, 13, and TNF- $\alpha$  (Kopeck *et al.*, 2006; Chung *et al.*, 2012). COX-2 is an enzyme that converts arachidonic acid to prostaglandin E<sub>2</sub>, a lipid mediator, and causes inflammation, pain and fever. It was reported that COX-2 expression increases in A23187 stimulated rat cells (Giuliano and Warner, 2002). Mast cell-derived TNF- $\alpha$  is one of the cytokines that triggers acute phase reaction and systemic inflammation. TNF- $\alpha$  is preformed and stored in the granules of mast cell or newly produced by mast cell activation (Lu *et al.*, 2013). TNF- $\alpha$  activates neutrophils and other effector cells (Locksley *et al.*, 2001; Metcalfe *et al.*, 2009). TNF- $\alpha$  is established in the therapy of rheumatoid arthritis and other chronic inflammation, so it could be a target for therapy of allergic inflammatory diseases (Harvima *et al.*, 2014). IL-4 is an important cytokine in allergic reaction. IL-4 stimulates T helper type 2 cells and induces IgE production in B cells. IL-4 causes IgE mediated allergic reaction (Hirasawa *et al.*, 2000). In the present study, we analyzed gene expression level of COX-2, TNF- $\alpha$  and IL-4 in AOR extract treated RBL-2H3 cells by qRT-PCR. As a result, COX-2 and TNF- $\alpha$  gene expression was suppressed by AOR extract. IL-4 gene expression level was also reduced but there was not significant change ranging from 0 to 0.75 mg/ml of AOR extract. This result suggests that AOR extract inhibits degranulation and inflammatory response in mast cells but has no direct effect on IgE production.

Mitogen-activated protein kinases (MAPKs) mediate intracellular signaling associated with various cellular activities. There are three types of MAPK pathway in mammals including extracellular signal-regulated kinases (ERK), p38

and c-Jun N-terminal kinases (JNK). Each pathway activated by specific cascades (Chang and Karin, 2001; Kim and Choi, 2010; Namkoong *et al.*, 2012). In this study, we investigated the effect of AOR extract on MAPK signal pathway in RBL-2H3 cells. In the 0.75 mg/ml of AOR extract treated cells, phosphorylated form of p38 was inhibited. p38 is affected by PKC pathway, and involved in degranulation of mast cells (Smolen *et al.*, 2000). ERK induces release of arachidonic acid and involved in lipid mediator production (Paunovic and Harnett, 2013). JNK is related with cytokine and gene expression but it was not affected by AOR extract in this study. According to the other research, it was demonstrated that IL-4 production is regulated by JNK at the transcription step. IL-4 production was controlled by JNK activation and not related with ERK and p38 (Chen *et al.*, 2006). Therefore, this result shows that AOR extract inhibits allergic inflammatory responses through suppression of phosphorylated p38 but not affects IL-4 production.

In conclusion, we demonstrated that AOR extract significantly suppressed degranulation of PMA/A23187 stimulated RBL-2H3 cells. The gene expression level, COX-2 and TNF- $\alpha$  was also reduced by AOR extract. Furthermore, AOR extract inhibited MAPK signaling pathway, especially p38 activation. These results indicate that extract could be used for immunosuppressive agent or dietary supplement.

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