



Cinnamaldehyde derivatives inhibit degranulation and inflammatory mediator production in rat basophilic leukemia cells

Sejin Ahn^a, Eunhee Kim^a, Kooyeon Lee^b, Deug-Chan Lee^{a,c,*}

^a Department of Biomedical Technology, Kangwon National University, Chuncheon, Republic of Korea

^b Department of Bio-health Technology, Kangwon National University, Chuncheon, Republic of Korea

^c Institute of Bioscience and Biotechnology, Kangwon National University, Chuncheon, Republic of Korea

ARTICLE INFO

Article history:

Received 3 March 2016

Received in revised form 7 June 2016

Accepted 18 June 2016

Available online 23 June 2016

Keywords:

4-Chloro-cinnamaldehyde

4-Trifluoro-cinnamaldehyde

Degranulation

Mitogen-activated protein kinases

ABSTRACT

Mast cells play a critical role in allergic diseases. Therefore, development of new therapeutic agents that suppresses the activation of mast cells may help prevent or treat allergic diseases. Here, we investigated the anti-allergic effects of 4-chloro-cinnamaldehyde and 4-trifluoro-cinnamaldehyde in RBL-2H3 cells. β -Hexosaminidase assays revealed that degranulation of RBL-2H3 cells was decreased following treatment with 60 μ M 4-chloro-cinnamaldehyde or 4-trifluoro-cinnamaldehyde. Moreover, quantitative real-time reverse transcription polymerase chain reaction showed that the relative expression levels of tumor necrosis factor- α , interleukin-4, and cyclooxygenase-2 mRNAs were decreased in RBL-2H3 cells treated with 4-chloro-cinnamaldehyde and 4-trifluoro-cinnamaldehyde in a concentration-dependent manner. Additionally, 4-chloro-cinnamaldehyde blocked the phosphorylation of MKKs and MAPKs. These data clearly suggested that 4-chloro-cinnamaldehyde and 4-trifluoro-cinnamaldehyde had inhibitory effects on the inflammatory responses of mast cells and may have potential as novel therapeutic agents for the prevention or treatment of allergic diseases.

© 2016 Elsevier B.V. All rights reserved.

1. Introduction

The prevalence of allergic diseases, such as atopic dermatitis and asthma, has become an important health problem worldwide, causing poor quality of life in children and adults [1–4]. Therefore, it is important to identify new therapeutic agents with application in the prevention and treatment of allergic diseases.

Mast cells play a critical role in allergic and other inflammatory responses. Immunoglobulin E (IgE) is an activator of mast cells [5]. The aggregation of IgE receptors (Fc ϵ RI) on mast cells triggers complicated biological reactions, such as enhancement of calcium influx, rearrangement of the cytoskeleton, degranulation of prestored granules, and activation of enzymes such as protein kinase C (PKC), cyclooxygenase-2 (COX-2), 5-lipoxygenase (5-LO), and mitogen-activated protein kinases (MAPKs) [6–9]. After the activation of intracellular signaling pathways, mast cells release various inflammatory mediators, such as prestored histamine and proteases, de novo-synthesized cytokines, chemokines, and eicosanoids, such as prostaglandins and leukotrienes [10,11].

Rat basophilic leukemia (RBL)-2H3 cells are the tumor analog of rodent mucosal mast cells that secrete various allergic inflammatory mediators. Therefore, RBL-2H3 cells are suitable for the study of allergic responses [12–18]. Phorbol myristate acetate (PMA), a PKC activator, and A23187, a calcium ionophore that functions to increase Ca^{2+} influx, induce the activation of mast cells [7,19,20]. Additionally, the activation of PKC and increased Ca^{2+} influx, stimulated by PMA and A23187, respectively, are critical factors for allergic responses of mast cells [7,19,21,22]. Thus, PMA and A23187 stimulate mast cells to release inflammatory mediators related to allergic responses [12,13,23].

Cinnamon extract has been reported to have anti-oxidative, antineoplastic, antidiabetic, anti-inflammatory, and anti-allergic effects both in vitro and in vivo [16,24–26]. Cinnamon extracts contain various compounds, including cinnamaldehyde, cinnamic acid, and cinnamate [24,27]. Cinnamaldehyde has been reported to inhibit antigen-induced extracellular calcium influx and degranulation in mouse bone marrow-derived mast cells (mBMMCs), human intestinal mast cells (hiMCs) and RBL-2H3 cells through suppression of phospholipase $\text{C}\gamma 1$ [17] and extracellular signal-regulated kinase (ERK) phosphorylation [18], whereas cinnamic acid does not have any inhibitory effects on the degranulation of mast cells [17]. Furthermore, while cinnamaldehyde inhibits allergic responses in mast cells, the inhibitory effects of cinnamaldehyde derivatives on production of allergic inflammatory mediators of mast cells have not been investigated.

* Corresponding author at: Department of Biomedical Technology, Kangwon National University, Chuncheon, Republic of Korea.

E-mail addresses: ajin0317@gmail.com (S. Ahn), hammer345@naver.com (E. Kim), lky@kangwon.ac.kr (K. Lee), dclee@kangwon.ac.kr (D.-C. Lee).

In this study, we report the possible anti-allergic activity of 4-chloro-cinnamaldehyde and 4-trifluoro-cinnamaldehyde by analyzing the mechanisms through which these derivatives affect the release of inflammatory mediators in RBL-2H3 cells stimulated with PMA and A23187.

2. Materials and methods

2.1. Materials

Cinnamaldehyde derivatives were synthesized and purified as described in [28–30]. Cinnamaldehyde and cinnamaldehyde derivatives were dissolved in dimethyl sulfoxide (DMSO) and diluted in Siraganian buffer (25 mM PIPES, 119 mM NaCl, 5 mM KCl, 1 mM CaCl₂, 0.4 mM MgCl₂, 0.1% bovine serum albumin (BSA), 5.6 mM glucose, pH 7.2). The final concentration of DMSO was 0.5%. Phorbol 12-myristate 13-acetate (PMA), A23187 (a calcium ionophore) and U0126 were purchased from Sigma Aldrich (St. Louis, MO, USA). Primary antibodies for total and phosphorylated *c-Jun*, *N*-terminal kinase (JNK), p38, extracellular signal-regulated kinase (ERK), MKK4, MKK3, and MEK1/2 were purchased from Cell Signaling Technology (Beverly, MA, USA). Anti- β -actin antibodies were purchased from Abcam (Cambridge, UK).

2.2. Cell cultures

RBL-2H3 cells were cultured in Dulbecco's modified Eagle's medium (Hyclone, Logan, UT, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 1% penicillin, and 1% streptomycin (Hyclone) in a humidified atmosphere of 5% CO₂ at 37 °C.

2.3. Degranulation and cell viability assay

To confirm the effects of cinnamaldehyde and cinnamaldehyde derivatives on degranulation, β -hexosaminidase activity was measured. Cell viability of RBL-2H3 cells treated with cinnamaldehyde and cinnamaldehyde derivatives was determined using MTT assays. The cells were grown in 24-well plates (2×10^5 cells/mL) for 24 h. After treatment with the indicated chemicals for 1 h, the cells were stimulated with PMA (50 nM) and A23187 (1 μ M) for 30 min. After stimulation, supernatants were used for β -hexosaminidase assays, and cells were used for MTT assays. Supernatants were incubated with substrate buffer (2 mM 4-*p*-nitrophenyl-*N*-acetyl- β -D-glucosaminide in 0.05 M sodium citrate buffer, pH 4.5) for 30 min at 37 °C. The enzyme reaction was terminated by stop buffer (0.1 M NaHCO₃, pH 10), and the absorbance at 405 nm was measured using an Epoch spectrometer (Biotek, Santa Barbara, CA, USA). After stimulation, the cell medium was replaced with 500 μ L MTT solution (5 mg/mL) and incubated for 4 h. The MTT solution was then discarded, and the insoluble formazan products were dissolved in 200 μ L DMSO. Absorbance was measured at 550 nm using an Epoch spectrometer (Biotek).

2.4. Quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR)

Total RNA was isolated using X-zol RNA reagent (PhileKorea, Seoul, Republic of Korea), and the RNA concentration was determined with an Epoch spectrometer (Biotek). To determine the relative gene expression levels of tumor necrosis factor- α (*TNF- α*), interleukin-4 (*IL-4*), and cyclooxygenase-2 (*COX-2*) mRNAs, 500 ng of total RNA was reverse-transcribed to cDNA using ReverTra Ace qPCR RT Master Mix (TOYOBO, Osaka, Japan). The relative gene expression was determined with THUNDERBIRD SYBR qPCR Mix (TOYOBO), and the house-keeping gene large ribosomal protein (*RPLPO*) was used for normalization. The primer pairs were synthesized by Macrogen (Seoul, Republic of Korea) as follows: *TNF- α* (sense, 5'-TGAACCTCGGGGTGATCG-3' and antisense,

5'-GGGCTTGCTACTCGAGTTT-3'), *IL-4* (sense, 5'-TACGGCAACAAGGAA CAC-3' and antisense, 5'-TCTTCAAGCACGGAGGTA-3'), *COX-2* (sense, 5'-TGGTGCCGGGTCGTGATGATG-3' and antisense, 5'-GCAATGCGGTTCTGATACTG-3'), and *RPLPO* (sense, 5'-GTGTTTGACAATGGCAGCAT-3' and antisense, 5'-ACAGACGCTGGCCACATT-3'). qRT-PCR was performed using a StepOne Plus Real-Time PCR system (Applied Biosystems, Foster City, CA, USA).

2.5. Immunoblotting

RBL-2H3 cells were lysed in RIPA buffer (Sigma-Aldrich) containing protease inhibitors (quartett Immunodiagnostika + Biotechnologie Vertriebs GmbH, Berlin, Germany). Equal amounts of protein (30 μ g) in each sample were electrophoresed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) on 10% gels and transferred to polyvinylidene difluoride membranes (Amersham Hybond-P; GE Healthcare, Buckinghamshire, UK). Blotted membranes were blocked with 1% skim milk in TBS containing 0.05% Tween 20 for 1 h. After three washes in TBS containing 0.05% Tween 20, the membranes were incubated with primary antibodies at 4 °C for 16 h. After three washes in TBS containing 0.05% Tween 20, the membranes were incubated with horseradish peroxidase-linked secondary antibodies at room temperature for 1 h. After three washes in TBS containing 0.05% Tween 20, the chemiluminescent signals were detected using WesternBright Peroxide chemiluminescent detection reagent (Advanta, Menlo Park, CA, USA). For re-probing the membranes, the antibodies were stripped off by stripping buffer (10% SDS, 0.5 M Tris-Cl and 0.8% β -mercaptoethanol).

2.6. Statistical analysis

Each experiment was performed independently at least three times. Data were expressed as the mean $\pm$ standard deviation (SD). Control groups and sample groups were compared by one-way analysis of variance (ANOVA) followed by Dunnett's test using SPSS (v.21, for Windows). Differences with *P* values of <0.05 were considered statistically significant.

3. Results

3.1. 4-Chloro-cinnamaldehyde and 4-trifluoro-cinnamaldehyde inhibited the degranulation of RBL-2H3 cells

To evaluate the effect of cinnamaldehyde derivatives on degranulation of RBL-2H3 cells, β -hexosaminidase assay was performed. RBL-2H3 cells were stimulated with A23187 and PMA. RBL-2H3 cells were treated with cinnamaldehyde and seven cinnamaldehyde derivatives at a concentration of 60 μ M for 1 h and stimulated with PMA and A23187 (Fig. 2A). From the seven cinnamaldehyde derivatives, 4-chloro-cinnamaldehyde and 4-trifluoro-cinnamaldehyde significantly reduced the release of β -hexosaminidase in RBL-2H3 cells (Fig. 2A). In addition, the viability of RBL-2H3 cells was not influenced by cinnamaldehyde derivatives when used at a concentration of 60 μ M (Fig. 2B). These results suggested that 4-chloro-cinnamaldehyde and 4-trifluoro-cinnamaldehyde inhibited degranulation and had no cytotoxic effects on RBL-2H3 cells. We next examined the concentration-dependent effects of 4-chloro-cinnamaldehyde and 4-trifluoro-cinnamaldehyde on degranulation of RBL-2H3 cells (Fig. 3). At a concentration of 60 μ M, 4-chloro-cinnamaldehyde and 4-trifluoro-cinnamaldehyde dramatically inhibited degranulation of RBL-2H3 cells compared to the stimulated control group. These results suggested that 4-chloro-cinnamaldehyde and 4-trifluoro-cinnamaldehyde inhibited degranulation of RBL-2H3 cells in a concentration-dependent manner.

3.2. 4-Chloro-cinnamaldehyde and 4-trifluoro-cinnamaldehyde inhibited the gene expression of pro-inflammatory mediators in RBL-2H3 cells

Pro-inflammatory mediators such as TNF- α , IL-4, and COX-2 are crucial for allergic and inflammatory responses [4,16–23]. We measured the effects of 4-chloro-cinnamaldehyde and 4-trifluoro-cinnamaldehyde on the relative gene expression of pro-inflammatory mediators in RBL-2H3 cells (Fig. 4). To assess the effects of 4-chloro-cinnamaldehyde and 4-trifluoro-cinnamaldehyde on the expression of pro-inflammatory mediators, qRT-PCR was performed (Fig. 4). In RBL-2H3 cells stimulated by PMA and A23187, the expression levels of TNF- α , IL-4, and COX-2 mRNAs were increased. However, the induced expression of these mRNAs was blocked in stimulated RBL-2H3 cells treated with 4-chloro-cinnamaldehyde or 4-trifluoro-cinnamaldehyde in a concentration-dependent manner (Fig. 4A–C). These results suggested that 4-chloro-cinnamaldehyde and 4-trifluoro-cinnamaldehyde suppressed the expression of pro-inflammatory mediator genes, such as TNF- α , IL-4, and COX-2 in RBL-2H3 cells stimulated with PMA and A23187.

3.3. 4-Chloro-cinnamaldehyde inhibited activation of the MKKs-MAPKs pathway in RBL-2H3 cells

As shown in Figs. 1–4, 4-chloro-cinnamaldehyde and 4-trifluoro-cinnamaldehyde exhibited similar structures and inhibitory activities on RBL-2H3 cell activation. Therefore, in the next experiment, we chose only 4-chloro-cinnamaldehyde to examine the mechanisms mediating the expression of pro-inflammatory mediators in response to treatment with 4-chloro-cinnamaldehyde and 4-trifluoro-cinnamaldehyde in RBL-2H3 cells. To identify the signaling pathway mediating the inhibitory effects of 4-chloro-cinnamaldehyde on the gene expression of pro-inflammatory mediators, the phosphorylated forms of MAPKs were determined by immunoblotting analysis (Fig. 5A). ERK, JNK, and p38 were phosphorylated after stimulation by PMA and A23187 in RBL-2H3 cells. However, despite stimulation with PMA and A23187, the phosphorylation of MAPKs were reduced significantly in RBL-2H3 cells treated with 60 μ M 4-chloro-cinnamaldehyde compared with that in the control. While the phosphorylation of p38 was suppressed by only 60 μ M 4-chloro-cinnamaldehyde, the phosphorylation of ERK and JNK were suppressed by both 40 and 60 μ M 4-chloro-cinnamaldehyde. In addition, the inhibitory effect of 4-chloro-cinnamaldehyde on ERK phosphorylation was compared to that of U0126 (Fig. 5B and C). U0126 induces suppression of ERK1/2 phosphorylation through specific inhibition of MEK 1/2 activity, which is ERK1/2 kinases [31]. In this reason, U0126 was widely used for MKK-MAPK

pathway analysis [13,15,32]. For comparative analysis of the efficacy between 4-chlorocinnamaldehyde and U0126, we performed immunoblotting analysis in both concentration-dependent (Fig. 5B) and combinational manners (Fig. 5C). As shown in Fig. 5B, ERK phosphorylation was significantly decreased in 2 μ M and 5 μ M of U0126 treated RBL-2H3 cells. In case of 4-chlorocinnamaldehyde treated RBL-2H3 cells, ERK phosphorylation was strongly suppressed at 30 μ M and 60 μ M concentration. Both U0126 and 4-chlorocinnamaldehyde attenuated the phosphorylation of ERK in concentration dependent manner. We showed the results of combinational manner in Fig. 5C. In case of single treatment of 15 μ M 4-chlorocinnamaldehyde, ERK phosphorylation didn't change significantly. On the other hand, in case of co-treatment of 15 μ M 4-chlorocinnamaldehyde and 2 μ M U0126, the weak attenuation of ERK phosphorylation was observed. Similar to these results, in case of co-treatment of 30 μ M 4-chlorocinnamaldehyde and 2 μ M U0126, ERK phosphorylation was strongly reduced compared to the case of single treatment of 30 μ M 4-chlorocinnamaldehyde. Taken together, these results suggested that 4-chloro-cinnamaldehyde significantly inhibited the phosphorylation of MAPKs in RBL-2H3 cells stimulated with PMA and A23187. To confirm this effect, the phosphorylation of MKKs were determined by immunoblotting analysis of the same PVDF membranes used for immunoblotting analysis of MAPKs (Fig. 5A). When RBL-2H3 cells were stimulated with PMA and A23187, MKKs were phosphorylated. In RBL-2H3 cells treated with from 40 to 60 μ M 4-chloro-cinnamaldehyde, while the phosphorylation of MEK1/2 and MKK4 were inhibited compared with that in the stimulated control group, the phosphorylation of MKK3/6 was inhibited only at the concentration of 60 μ M 4-chloro-cinnamaldehyde, as expected. Therefore, these results indicated that 4-chloro-cinnamaldehyde significantly inhibited the MKKs-MAPKs signaling pathway in RBL-2H3 cells stimulated with PMA and A23187 (Fig. 6).

4. Discussion

In the present study, we investigate the inhibitory effect of cinnamaldehyde derivatives on degranulation and inflammatory mediator production in RBL-2H3 cells. We present the evidence that 4-chloro-cinnamaldehyde and 4-trifluoro-cinnamaldehyde, from the seven cinnamaldehyde derivatives, prevent degranulation in RBL-2H3 cells. We also provide the evidence that 4-chloro-cinnamaldehyde and 4-trifluoro-cinnamaldehyde suppress pro-inflammatory mediator expression via inhibition of MAPK signaling in RBL-2H3 cells. Based on these evidences, 4-chloro-cinnamaldehyde and 4-trifluoro-cinnamaldehyde could be a potential therapeutic agent for mast cell-related diseases.

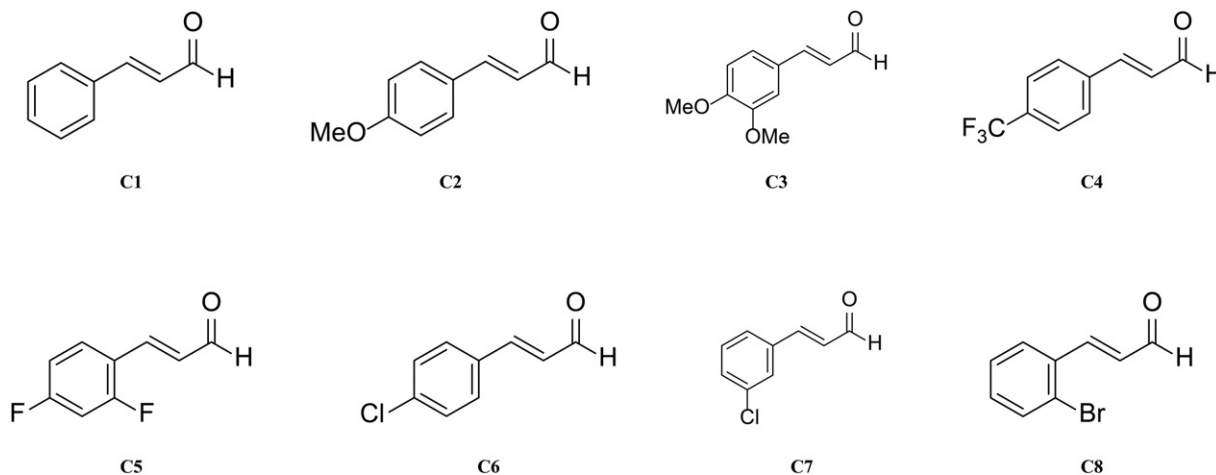


Fig. 1. Structures of cinnamaldehyde and cinnamaldehyde derivatives. **C1:** Cinnamaldehyde. **C2:** 4-Methoxy-cinnamaldehyde. **C3:** 3,4-Dimethoxy-cinnamaldehyde. **C4:** 4-Trifluoromethyl-cinnamaldehyde. **C5:** 2,4-Fluoro-cinnamaldehyde. **C6:** 4-Chloro-cinnamaldehyde. **C7:** 3-Chloro-cinnamaldehyde. **C8:** 2-Bromo-cinnamaldehyde.

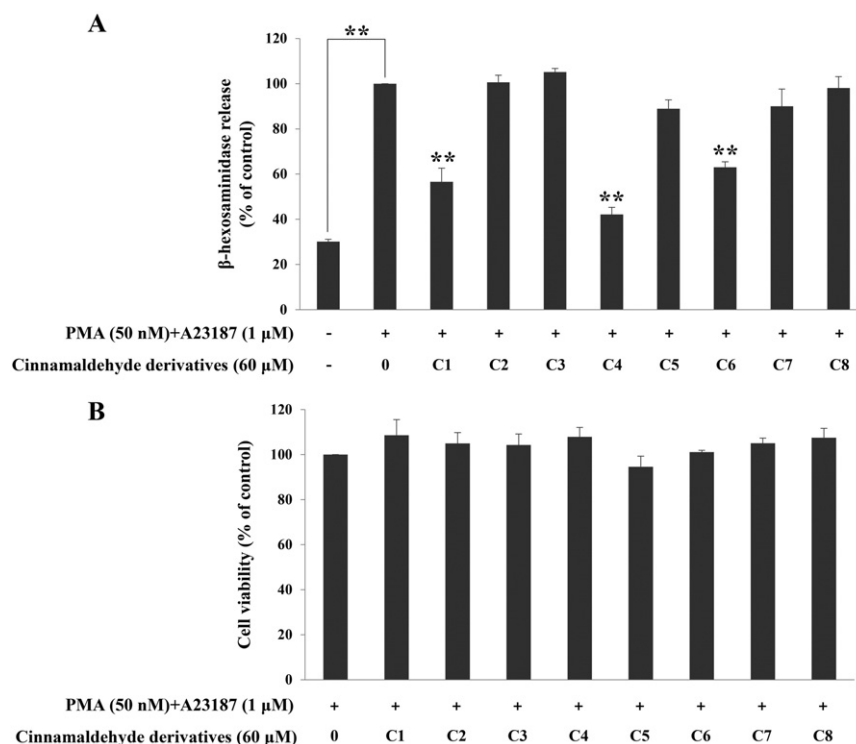


Fig. 2. Effects of cinnamaldehyde and cinnamaldehyde derivatives on β -hexosaminidase release and cell viability in RBL-2H3 cells. **A:** β -Hexosaminidase release in RBL-2H3 cells treated with 60 μ M cinnamaldehyde or cinnamaldehyde derivatives. RBL-2H3 cells were treated with 60 μ M cinnamaldehyde and cinnamaldehyde derivatives for 1 h and then stimulated with PMA and A23187. Data are expressed as a percentage of the β -hexosaminidase activity. **B:** Viability of RBL-2H3 cells treated with cinnamaldehyde and cinnamaldehyde derivatives followed by stimulation. Results are presented as the mean $\pm$ SD of three independent experiments. $^{**}P < 0.01$. **C1:** Cinnamaldehyde. **C2:** 4-Methoxy-cinnamaldehyde. **C3:** 3,4-Dimethoxy-cinnamaldehyde. **C4:** 4-Trifluoromethyl-cinnamaldehyde. **C5:** 2,4-Fluoro-cinnamaldehyde. **C6:** 4-Chloro-cinnamaldehyde. **C7:** 3-Chloro-cinnamaldehyde. **C8:** 2-Bromo-cinnamaldehyde.

Cinnamon extract has anti-oxidative, antineoplastic, antidiabetic, anti-inflammatory, and anti-allergic effects both in vitro and in vivo [16,24–26]. Ethanolic cinnamon extract has been shown to inhibit degranulation and phosphorylation of ERK 1/2 in RBL-2H3 cells and human intestinal mast cells [16]. Inhibitory effects of cinnamon water extract on phosphorylation of ERK1/2 and scavenger activity in macrophage has been revealed [26]. Cinnamaldehyde, the major constituents of *Cinnamomi cortex* [27], possesses versatile biological activities [17,18,33–35]. In macrophage, cinnamaldehyde suppressed the production of pro-inflammatory mediators such as COX-2 and iNOS

through attenuation of activation of ERK1/2, p38 and NF- κ B [33,34]. In addition, cinnamaldehyde inhibited activation of signaling pathway related with allergic inflammation in RBL-2H3 cells and mBMMCs [17,18]. Cinnamaldehyde inhibited phosphorylation of phospholipase C γ 1 (PLC γ 1) [17] and ERK1/2 [18]. Not only cinnamaldehyde, also its derivatives have diverse biological activities [35–40]. In particular, *p*-hydroxycinnamic acid, 4-hydroxy-3-methoxycinnamaldehyde and α -bromo-4-chlorocinnamaldehyde have the immunomodulatory effects in mammalian cells [36,37,39]. In coxsackie virus B3 (CVB3)-treated rat cardiomyocytes, α -bromo-4-chlorocinnamaldehyde inhibited the

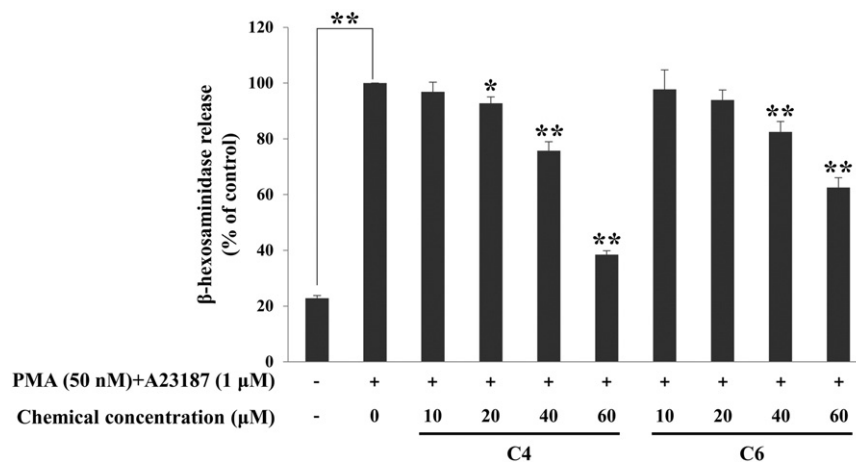


Fig. 3. Concentration-dependent effects of 4-chloro-cinnamaldehyde and 4-trifluoro-cinnamaldehyde on β -hexosaminidase release in RBL-2H3 cells. RBL-2H3 cells were treated with 10, 20, 40 and 60 μ M 4-chloro-cinnamaldehyde (C6) or 4-trifluoro-cinnamaldehyde (C4) for 1 h and stimulated with PMA and A23187. Data are expressed as percentage of the β -hexosaminidase activity relative to the stimulated control. Results are presented as the mean $\pm$ SD of three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$ compared with stimulated RBL-2H3 cells.

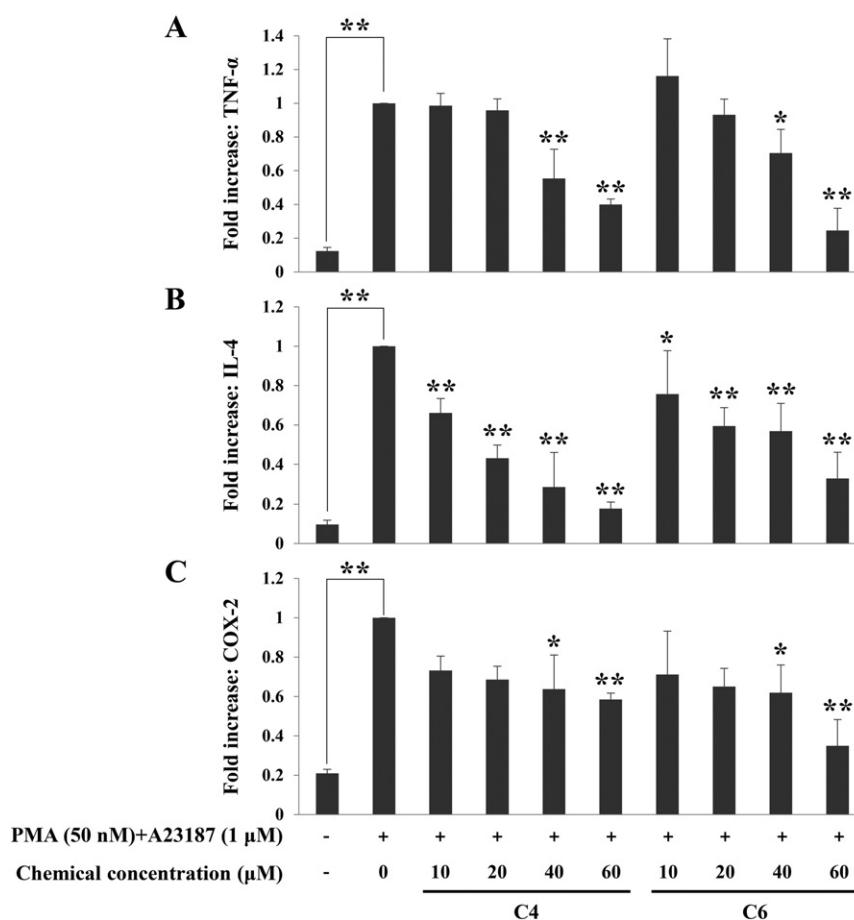


Fig. 4. Effects of 4-chloro-cinnamaldehyde and 4-trifluoro-cinnamaldehyde on *TNF-α*, *IL-4*, and *COX-2* gene expression. RBL-2H3 cells were treated with 10, 20, 40 and 60 μM 4-chloro-cinnamaldehyde (C6) or 4-trifluoro-cinnamaldehyde (C4) for 1 h and stimulated with PMA and A23187. The relative gene expression levels of *TNF-α*, *IL-4*, and *COX-2* were determined by qRT-PCR. The *RPLPO* gene was used as the endogenous control. **A:** Relative expression of *TNF-α* mRNA. **B:** Relative expression of *IL-4* mRNA. **C:** Relative expression of *COX-2* mRNA. Results are presented as the mean ± SD of three independent experiments. **P* < 0.05, ***P* < 0.01 compared with stimulated RBL-2H3 cells.

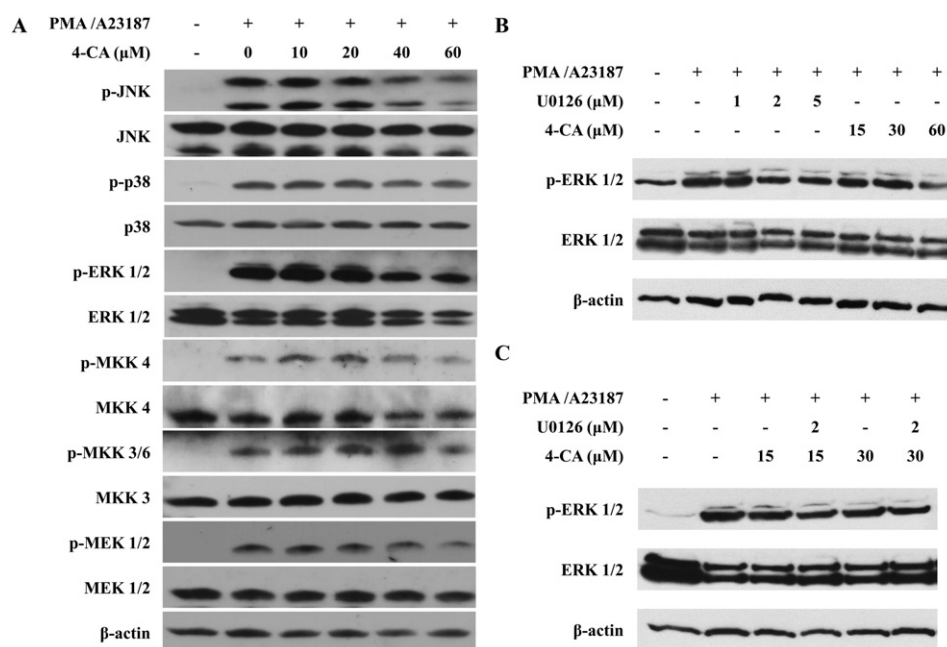


Fig. 5. Effects of 4-chloro-cinnamaldehyde (4-CA) on the phosphorylation of MAPKs and MKKs. **A:** Immunoblotting analysis of MAPKs and MKKs phosphorylation. **B:** Concentration-dependent effect of 4-chloro-cinnamaldehyde on ERK phosphorylation compared to U0126. **C:** Combination effect of 4-chloro-cinnamaldehyde and U0126 on ERK phosphorylation. RBL-2H3 cells were treated with several concentration of 4-chloro-cinnamaldehyde for an hour and stimulated with PMA (50 nM) and A23187 (1 μM). MAPKs and MKKs (A) were measured in the same PVDF membrane.

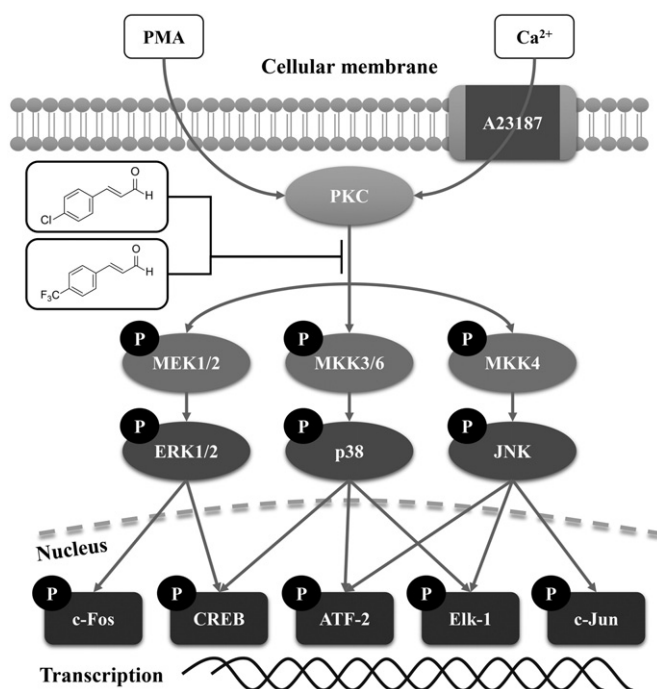


Fig. 6. Schematic diagram of the signaling pathway suppressed by 4-chloro-cinnamaldehyde and 4-trifluoromethyl-cinnamaldehyde in RBL-2H3 cells.

production of TNF- α , IL-1 β and IL-6 via suppression of TLR4 expression and NF- κ B activation [39]. *p*-hydroxycinnamic acid [36] and 4-hydroxy-3-methoxycinnamaldehyde [37] attenuated *anti*-CD3/CD28 or PMA/A23187 induced T cell activation via suppression of ERK1/2, p38 phosphorylation and NF- κ B activation in Jurkat cells [36,37]. However, the immunomodulatory effects of other cinnamaldehyde derivatives such as 4-chlorocinnamaldehyde and 4-trifluoro-cinnamaldehyde have not been reported yet. In this context, we investigated the immunosuppressive effects of 7 cinnamaldehyde derivatives on allergic responses in vitro and their potential mechanism of intracellular action.

Mast cells are key effector cells in the allergic inflammatory responses [5,8,11,41,42]. Activation of mast cells induces intracellular signals leading to secretion of histamine, proteases, prostaglandins, leukotrienes and cytokines, causing allergic inflammation followed by allergic diseases [8,11]. Mast cells have many secretory granules for storage of inflammatory mediators, such as histamine and serotonin, as well as other proteins [8,10,43]. When mast cells are stimulated by Fc ϵ RI or A23187, the lysosomal hydrolases such as β -hexosaminidase and cathepsin D are released with inflammatory mediators such as histamine and serotonin [10]. Granule exocytosis, referred to as degranulation, is regulated by membrane-membrane fusion proteins termed SNAREs [10,44]. During degranulation, the secretory granules of mast cells can fuse with plasma membrane using SNAREs, and prestored molecules, such as histamine and β -hexosaminidase, are then released into the extracellular environment [8,43,44]. Thus, the release of β -hexosaminidase is widely used for an indicator of degranulation of mast cells [12,14–17,19,23,45]. In this context, the effect of cinnamaldehyde derivatives on degranulation was assessed by β -hexosaminidase assay. As shown in Figs. 2 and 3, not only cinnamaldehyde, 4-chloro-cinnamaldehyde and 4-trifluoro-cinnamaldehyde also significantly decreased β -hexosaminidase release and had no cytotoxic effect on RBL-2H3 cells.

The effects of IgE and PMA/A23187 on mast cells are known to activate PKC and extracellular Ca²⁺ uptake [6,11,23,42,44,46]. These effects control degranulation, cytoskeleton rearrangement, and gene expression of pro-inflammatory cytokines in mast cells [5–8, 19,46]. TNF- α is involved in recruitment neutrophils, promotion of T-cell activation, migration, and maturation of antigen-presenting

cell [10,13,16,21,45]. IL-4 is a central factor of not only Th2 cell immune responses but also B cell development and isotype switching [9,12,14,42,45]. Increased expression of COX-2 leads to the release of prostaglandins, which affect inflammatory symptoms such as pain, fever, and swelling [47,33].

MAPKs control the expression of pro-inflammatory cytokines in immune cells [48]. The MAPK pathways are stimulated by various extracellular factors, followed by distinct intracellular responses involving a series of phosphorylation events [48–50]. There are three groups of MAPKs: ERK, JNK, and p38. ERK is phosphorylated by MEK1/2, JNK is phosphorylated by MKK4, and p38 is phosphorylated by MKK3/6 [51]. The phosphorylated MAPKs are activated and subsequently affect their substrates, including ATF2, Elk-1, c-Fos, and c-Jun. [48–50]. These substrates function as transcription factors for inflammatory cytokines [48,49]. Thus, MAPK pathways are therapeutic targets for inflammatory diseases, such as asthma [51,52]. In this study, despite stimulation with PMA and A23187, 4-chloro-cinnamaldehyde and 4-trifluoro-cinnamaldehyde inhibited expression of mRNA for TNF- α , IL-4, and COX-2 in RBL-2H3 cells (Fig. 4A–C). Therefore, we deduced that 4-chloro-cinnamaldehyde and 4-trifluoro-cinnamaldehyde act as intracellular modulators and inhibit intracellular signaling pathways. For identify the mechanism of 4-chloro-cinnamaldehyde and 4-trifluoro-cinnamaldehyde in suppressing pro-inflammatory mediator production, we investigated activation of MKKs–MAPKs pathway. For investigation, we chose only 4-chloro-cinnamaldehyde and 4-trifluoro-cinnamaldehyde because two chemicals exhibited similar structures and effects on RBL-2H3 activation. Compared to the control, the phosphorylation of MAPKs and MKKs were significantly reduced in RBL-2H3 cells treated with 4-chloro-cinnamaldehyde (Figs. 5 and 6). As the current data, it is difficult to completely describe the relationship between chemical structure and activity. As a result, it showed a low activity, when the electron donating group (EDG) was introduced to the phenyl ring of cinnamaldehyde (C2, C3). However, when substituted to the electron withdrawing group (EWG) in the *para* position of the phenyl ring was showed a higher activity (C4, C6). The research for the structure activity relationship (SAR) is in progress in our laboratory.

In conclusion, this study showed that 4-chloro-cinnamaldehyde and 4-trifluoro-cinnamaldehyde inhibited the gene expression of pro-inflammatory mediators by blocking MKKs–MAPKs signaling and degranulation in RBL-2H3 cells. Inflammation is the essential part of immune response against pathogens in normal condition. However, excessive inflammation can lead to the onset of inflammatory disease such as atopic dermatitis and asthma [1,3,4,53]. Although further study will be required to investigate the inhibitory effects of 4-chloro-cinnamaldehyde and 4-trifluoro-cinnamaldehyde in vivo, our results of attenuated level of inflammatory mediators clearly indicated that 4-chloro-cinnamaldehyde and 4-trifluoro-cinnamaldehyde may have potential as therapeutic agents against allergic diseases mediated by mast cells based on the mechanism of blocking MKKs–MAPKs signaling pathway.

Acknowledgements

This study was supported by 2013 Research Grant from Kangwon National University (No. C1009950-01-01).

References

- [1] D.Y.M. Leung, M. Boguniewicz, M.D. Howell, I. Nomura, Q.a. Hamid, New insights into atopic dermatitis, *J. Clin. Invest.* 113 (2004) 651–657.
- [2] C.L. Carroll, R. Balkrishnan, S.R. Feldman, A.B. Fleischer, J.C. Manuel, The burden of atopic dermatitis: impact on the patient, family, and society, *Pediatr. Dermatol.* 22 (2005) 192–199.
- [3] M. Caminati, I. Durić-Filipović, S. Arasi, D.G. Peroni, Z. Živković, G. Senna, Respiratory allergies in childhood: recent advances and future challenges, *Pediatr. Allergy Immunol.* 26 (2015) 702–710.
- [4] J.N. Larsen, L. Broge, H. Jacobi, Allergy immunotherapy: the future of allergy treatment, *Drug Discov. Today* 21 (2016) 26–37.

- [5] A.M. Gilfillan, C. Tkaczuk, Integrated signalling pathways for mast-cell activation, *Nat. Rev. Immunol.* 6 (2006) 218–230.
- [6] Y. Yanase, I. Hide, S. Mihara, Y. Shirai, N. Saito, Y. Nakata, M. Hide, N. Sakai, A critical role of conventional protein kinase C in morphological changes of rodent mast cells, *Immunol. Cell Biol.* 89 (2011) 149–159.
- [7] O. Pernas-Sueiras, A. Alfonso, M.R. Vieytes, L.M. Botana, PKC and cAMP positively modulate alkaline-induced exocytosis in the human mast cell line HMC-1, *J. Cell. Biochem.* 99 (2006) 1651–1663.
- [8] T.C. Theoharides, D. Kempuraj, M. Tagen, P. Conti, D. Kalogeromitros, Differential release of mast cell mediators and the pathogenesis of inflammation, *Immunol. Rev.* 217 (2007) 65–78.
- [9] N. Hirasawa, Y. Sato, Y. Fujita, K. Ohuchi, Involvement of a phosphatidylinositol 3-kinase-p38 mitogen activated protein kinase pathway in antigen-induced IL-4 production in mast cells, *Biochim. Biophys. Acta Bioenerg.* 1456 (2000) 45–55.
- [10] N. Puri, P.A. Roche, Mast cells possess distinct secretory granule subsets whose exocytosis is regulated by different SNARE isoforms, *Proc. Natl. Acad. Sci. U. S. A.* 105 (2008) 2580–2585.
- [11] V. Heib, M. Becker, C. Taube, M. Stassen, Advances in the understanding of mast cell function, *Br. J. Haematol.* 142 (2008) 683–694.
- [12] Y. Choi, M.S. Kim, J.K. Hwang, Inhibitory effects of panduratin A on allergy-related mediator production in rat basophilic leukemia mast cells, *Inflammation* 35 (2012) 1904–1915.
- [13] Y. Lu, Y.T. Jeong, X. Li, M.J. Kim, P.H. Park, S.L. Hwang, J.K. Son, H.W. Chang, Emodin isolated from polygoni cuspidati radix inhibits TNF- α and IL-6 release by blocking NF- κ B and MAP kinase pathways in mast cells stimulated with PMA plus A23187, *Biomol. Ther.* 21 (2013) 435–441.
- [14] S.H. Lee, H.J. Shin, D.Y. Kim, D.W. Shim, T.J. Kim, S.K. Ye, H.S. Won, S. Koppula, T.B. Kang, K.H. Lee, Streptochlorin suppresses allergic dermatitis and mast cell activation via regulation of Lyn/Fyn and Syk signaling pathways in cellular and mouse models, *PLoS One* 8 (2013).
- [15] S. Tanifuji, E. Aizu-Yokota, M. Funakoshi-Tago, Y. Sonoda, H. Inoue, T. Kasahara, Licochalcones suppress degranulation by decreasing the intracellular Ca²⁺ level and tyrosine phosphorylation of ERK in RBL-2H3 cells, *Int. Immunopharmacol.* 10 (2010) 769–776.
- [16] Y. Hagenlocher, I. Bergheim, S. Zacheja, M. Schäffer, S.C. Bischoff, A. Lorentz, Cinnamon extract inhibits degranulation and de novo synthesis of inflammatory mediators in mast cells, *Allergy Eur. J. Allergy Clin. Immunol.* 68 (2013) 490–497.
- [17] N. Kageyama-Yahara, X. Wang, T. Katagiri, P. Wang, T. Yamamoto, M. Tominaga, M. Kadowaki, Suppression of phospholipase C γ 1 phosphorylation by cinnamaldehyde inhibits antigen-induced extracellular calcium influx and degranulation in mucosal mast cells, *Biochem. Biophys. Res. Commun.* 416 (2011) 283–288.
- [18] Y. Hagenlocher, K. Kießling, M. Schäffer, S.C. Bischoff, A. Lorentz, Cinnamaldehyde is the main mediator of cinnamon extract in mast cell inhibition, *Eur. J. Nutr.* 54 (2015) 1297–1309.
- [19] M. Funaba, T. Ikeda, M. Abe, Degranulation in RBL-2H3 cells: regulation by calmodulin pathway, *Cell Biol. Int.* 27 (2003) 879–885.
- [20] M. Vig, J.P. Kinet, Calcium Signaling in Immune Cells, Vol. 102009 21–27.
- [21] D. Kempuraj, B. Madhappan, S. Christodoulou, W. Boucher, J. Cao, N. Papadopolou, C.L. Cetrulo, T.C. Theoharides, Flavonols inhibit proinflammatory mediator release, intracellular calcium ion levels and protein kinase C theta phosphorylation in human mast cells, *Br. J. Pharmacol.* 145 (2005) 934–944.
- [22] B.D. Baram, R. Adachi, O. Medalia, M. Tuvim, B.F. Dickey, Y.A. Mekori, R. Sagi-eisenberg, Exocytosis of lysosomes in mast cells, *J. Exp. Med.* 189 (1999) 1649–1657.
- [23] Y.S. Lee, S. Hur, T.Y. Kim, Homoisoflavanone prevents mast cell activation and allergic responses by inhibition of Syk signaling pathway, *Allergy Eur. J. Allergy Clin. Immunol.* 69 (2014) 453–462.
- [24] P.V. Rao, S.H. Gan, Cinnamon: a multifaceted medicinal plant, *Evid. Based Complement. Alternat. Med.* 2014 (2014).
- [25] J. Gruenewald, J. Freder, N. Armbruester, Cinnamon and health, *Crit. Rev. Food Sci. Nutr.* 50 (2010) 822–834.
- [26] H. Kang, S.-H. Park, J.-M. Yun, T.-G. Nam, Y.-E. Kim, D.-O. Kim, Y.J. Kim, Effect of cinnamon water extract on monocyte-to-macrophage differentiation and scavenger receptor activity, *BMC Complement. Altern. Med.* 14 (2014) 90.
- [27] R. Wang, R. Wang, B. Yang, Extraction of essential oils from five cinnamon leaves and identification of their volatile compound compositions, *Innovative Food Sci. Emerg. Technol.* 10 (2009) 289–292.
- [28] G. Battistuzzi, S. Cacchi, G. Fabrizio, An efficient palladium-catalyzed synthesis of cinnamaldehydes from acrolein diethyl acetal and aryl iodides and bromides, *Org. Lett.* 5 (2003) 777–780.
- [29] S. Noël, C. Luo, C. Pinel, L. Djakovitch, Efficient heterogeneously palladium-catalysed heck arylation of acrolein diethyl acetal. Selective synthesis of cinnamaldehydes or 3-arylpropionic esters, *Adv. Synth. Catal.* 349 (2007) 1128–1140.
- [30] B.P. Joshi, A. Sharma, A.K. Sinha, Efficient one-pot, two-step synthesis of (E)-cinnamaldehydes by dehydrogenation-oxidation of arylpropanes using DDQ under ultrasonic irradiation, *Tetrahedron* 62 (2006) 2590–2593.
- [31] J.V. Duncia, J.B. Santella, C.A. Higley, W.J. Pitts, J. Wityak, W.E. Fietze, F.W. Rankin, J.H. Sun, R.A. Earl, A.C. Tabaka, C.A. Teleha, K.F. Blom, M.F. Favata, E.J. Manos, A.J. Daulerio, D.A. Stradley, K. Horiuchi, R.A. Copeland, P.A. Scherle, J.M. Trzaskos, R.L. Magolda, G.L. Trainor, R.R. Wexler, F.W. Hobbs, R.E. Olson, MEK inhibitors: the chemistry and biological activity of U0126, its analogs, and cyclization products, *Bioorg. Med. Chem. Lett.* 8 (1998) 2839–2844.
- [32] M. Jin, K.H. Son, H.W. Chang, Luteolin-7-O-glucoside suppresses leukotriene C(4) production and degranulation by inhibiting the phosphorylation of mitogen activated protein kinases and phospholipase C γ 1 in activated mouse bone marrow-derived mast cells, *Biol. Pharm. Bull.* 34 (2011) 1032–1036.
- [33] D.H. Kim, C.H. Kim, M.S. Kim, J.Y. Kim, K.J. Jung, J.H. Chung, W.G. An, J.W. Lee, B.P. Yu, H.Y. Chung, Suppression of age-related inflammatory NF- κ B activation by cinnamaldehyde, *Biogerontology* 8 (2007) 545–554.
- [34] B.H. Kim, Y.G. Lee, J. Lee, J.Y. Lee, J.Y. Cho, Regulatory effect of cinnamaldehyde on monocyte/macrophage-mediated inflammatory responses, *Mediat. Inflamm.* 2010 (2010).
- [35] G. Brackman, T. Defoirdt, C. Miyamoto, P. Bossier, S. Van Calenbergh, H. Nelis, T. Coenye, Cinnamaldehyde and cinnamaldehyde derivatives reduce virulence in vibrio spp. by decreasing the DNA-binding activity of the quorum sensing response regulator LuxR, *BMC Microbiol.* 8 (2008) 149.
- [36] H.S. Lee, Y.D. Kim, B.R. Na, H.R. Kim, E.J. Choi, W.C. Han, H.K. Choi, S.H. Lee, C.D. Jun, Phytocomponent p-hydroxycinnamic acid inhibits T-cell activation by modulation of protein kinase C- θ -dependent pathway, *Int. Immunopharmacol.* 12 (2012) 131–138.
- [37] U. Akber, B.R. Na, Y.S. Ko, H.S. Lee, H.R. Kim, M.S. Kwon, Z.Y. Park, E.J. Choi, W.C. Han, S.H. Lee, H.M. Oh, C.D. Jun, Phytocomponent 4-hydroxy-3-methoxycinnamaldehyde ablates T-cell activation by targeting protein kinase C- θ and its downstream pathways, *Int. Immunopharmacol.* 25 (2015) 130–140.
- [38] I.A. Ismail, H.S. Kang, H.J. Lee, H. Chang, J. Yun, C.W. Lee, N.H. Kim, H.S. Kim, J.I. Yook, S.H. Hong, B.M. Kwon, 2-Hydroxycinnamaldehyde inhibits the epithelial-mesenchymal transition in breast cancer cells, *Breast Cancer Res. Treat.* 137 (2013) 697–708.
- [39] Y. Zhang, W. Cao, Y.H. Xie, Q. Yang, X.Q. Li, X.X. Liu, S.W. Wang, The comparison of α -bromo-4-chlorocinnamaldehyde and cinnamaldehyde on coxsackie virus B3-induced myocarditis and their mechanisms, *Int. Immunopharmacol.* 14 (2012) 107–113.
- [40] G. Brackman, S. Celen, U. Hillaert, S. van Calenbergh, P. Cos, L. Maes, H.J. Nelis, T. Coenye, Structure-activity relationship of cinnamaldehyde analogs as inhibitors of AI-2 based quorum sensing and their effect on virulence of vibrio spp, *PLoS One* 6 (2011).
- [41] K.N. Rao, M.A. Brown, Mast cells: multifaceted immune cells with diverse roles in health and disease, *Ann. N. Y. Acad. Sci.* 1143 (2008) 83–104.
- [42] R. Pawankar, M. Okuda, H. Yssel, K. Okumura, C. Ra, Nasal mast cells in perennial allergic rhinitis exhibit increased expression of the Fc ϵ RI, CD40L, IL-4, and IL-13, and can induce IgE synthesis in B cells, *J. Clin. Invest.* 99 (1997) 1492–1499.
- [43] E.J. Blott, G.M. Griffiths, Secretory lysosomes, *Nat. Rev. Mol. Cell Biol.* 3 (2002) 122–131.
- [44] A. Lorentz, A. Baumann, J. Vitte, U. Blank, The SNARE machinery in mast cell secretion, *Front. Immunol.* 3 (2012) 1–17.
- [45] F. Huang, K. Yamaki, X. Tong, L. Fu, R. Zhang, Y. Cai, R. Yanagisawa, K. Inoue, H. Takano, S. Yoshino, Inhibition of the antigen-induced activation of RBL-2H3 cells by sinomenine, *Int. Immunopharmacol.* 8 (2008) 502–507.
- [46] P. Draber, V. Sulimenko, E. Draberova, Cytoskeleton in mast cell signaling, *Front. Immunol.* 3 (2012) 1–18.
- [47] C. Charlier, C. Michaux, Dual inhibition of cyclooxygenase-2 (COX-2) and 5-lipoxygenase (5-LOX) as a new strategy to provide safer non-steroidal anti-inflammatory drugs, *Eur. J. Med. Chem.* 38 (2003) 645–659.
- [48] C. Dong, R.J. Davis, R.A. Flavell, Mapkinases in the immune response, *Annu. Rev. Immunol.* 20 (2002) 55–72.
- [49] M.H. Cobb, MAP kinase pathways, *Prog. Biophys. Mol. Biol.* 71 (1999) 479–500.
- [50] L. Chang, M. Karin, Mammalian MAP kinase signalling cascades, *Nature* 410 (2001) 37–40.
- [51] A. Akinleye, M. Furqan, N. Mukhi, P. Ravella, D. Liu, MEK and the inhibitors: from bench to bedside, *J. Hematol. Oncol.* 6 (2013) 27.
- [52] X. Chu, X. Ci, J. He, M. Wei, X. Yang, Q. Cao, H. Li, S. Guan, Y. Deng, D. Pang, X. Deng, A novel anti-inflammatory role for ginkgolide B in asthma via inhibition of the ERK/MAPK signaling pathway, *Molecules* 16 (2011) 7634–7648.
- [53] D.B. Peden, Development of atopy and asthma: candidate environmental influences and important periods of exposure, *Environ. Health Perspect.* 108 (2000) 475–482.