

# Tilianin attenuates HDM-induced allergic asthma by suppressing Th2-immune responses via downregulation of IRF4 in dendritic cells

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## ABSTRACT

**Background:** Acacetin 7-O-β-D-glucoside (tilianin) is a major constituent of *Agastache rugosa*, a traditional medicine that has long been used for the treatment of gastrointestinal disorders. Tilianin has a wide variety of pharmacological properties such as cardioprotective, neuroprotective, and anti-atherogenic activities. We recently discovered that tilianin has the ability to suppress MUC5AC expression *in vitro*. In addition, we have established an *in vivo* model of allergic asthma using house dust mite (HDM) that can be applied to tilianin.

**Purpose:** We investigated the effects of tilianin on airway inflammation in a HDM-induced asthma mouse model and associated mechanisms.

**Methods:** Tilianin was treated in splenocytes cultured in Th0 condition and HDM-stimulated bone marrow-derived dendritic cells (BMDCs), and their mRNA expression and cytokines production were determined by quantitative real-time PCR and ELISA. To evaluate the effects of tilianin in an allergic asthma model, mice were sensitized and challenged with HDM. Tilianin was administered prior to challenge by oral gavage and airway hyper-reactivity (AHR) to methacholine, inflammatory cell infiltration, cytokine levels, and airway remodeling were assessed.

**Results:** Tilianin inhibited the production of Th2-related cytokines in splenocytes, which play pivotal roles in allergic airway inflammation. When treated in HDM-stimulated BMDCs, tilianin decreased Th2-skewing cytokine IL-33 and transcription factor IRF4. On the contrary, tilianin increased Th1-skewing regulators, IL-12 and IRF1. In an HDM-induced asthmatic mouse model, tilianin attenuated AHR and airway inflammation. Tilianin suppressed the expression of Th2-related cytokines, IL-13 and IL-33 in lung tissues. As seen in HDM-stimulated BMDCs, tilianin also downregulated the expression of the transcription factor IRF4 but not IRF1.

**Conclusion:** Taken together, these results suggest that tilianin attenuates HDM-induced allergic airway inflammation by inhibiting Th2-mediated inflammation through the selective inhibition of the IRF4-IL-33 axis in dendritic cells.

## Introduction

Allergic asthma is an inflammatory lung disorder characterized by airflow obstruction, airway hyper-responsiveness (AHR), and airway

inflammation (Kim et al., 2010). The pathogenesis of asthma is associated with the initial sensitization and subsequent repeated exposure to environmental antigens. The house dust mite (HDM) is the most prevalent cause of allergic sensitization (Thomas et al., 2010) and a risk

**Abbreviations:** AHR, airway hyper-responsiveness; BALF, bronchoalveolar lavage fluid; BMDC, bone marrow-derived dendritic cell; CD, cluster differentiation; COPD, chronic obstructive pulmonary disease; Crs, compliance; DC, dendritic cells; ELISA, enzyme-linked immunosorbent assay; Ers, elastance; HDM, house dust mite; H&E, hematoxylin, eosin; HPLC, high-performance liquid chromatography; HRESIMS, high-resolution electrospray ionization mass spectrometry; IFN, interferon; IL, interleukin; IRF1, interferon regulatory factor 1; IRF4, interferon regulatory factor 4; NMR, nuclear magnetic resonance; MS, mass spectrometry; OVA, ovalbumin; PAS, periodic acid Schiff; PMA, Phorbol 12-myristate 13-acetate; Rrs, resistance; TCR, T cell receptor; Th2, T helper type 2; UPLC-PDA, ultra performance liquid-photodiode array.

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factor for persistent asthma in human subjects (Johnson et al., 2004).

Allergic asthma is associated with Th2-biased immune responses, which leads to high levels of IgE, airway eosinophilia, and mucus production (Elias et al., 2003). Th2 cytokines, mainly IL-4, IL-5, and IL-13, are the key regulators of the asthmatic phenotype. Therefore, proper control of these cytokines is an important strategy for the treatment of asthma. Naïve CD4<sup>+</sup> T cells can differentiate into various subtypes of effector T cells, including Th1 and Th2 cells. Each T cell subtype is characterized by the expression of hallmark cytokines and master transcription factors. Th1 cells secrete IFN- $\gamma$  and express transcription factor T-bet, whereas Th2 cells are defined by the secretion of IL-4, IL-5, and IL-13 and GATA3 expression (Amsen et al., 2009).

Dendritic cells (DCs) are the primary antigen-presenting cells in the lung that induce allergic sensitization and Th2 immune responses to HDM (Hammad et al., 2010). An increasing number of studies indicate that Th2 development depends on interferon regulatory factor 4 (IRF4) expression in DCs (Ahyi et al., 2009, 2019; Nam and Lim, 2016). IRF4-activated IL-10 and IL-33 production by DCs promotes Th2 responses (Williams et al., 2013). On the other hand, IRF1, another member of the IRF family, induces IL-12 expression in DCs and promotes naïve CD4<sup>+</sup> T cells to differentiate into Th1 cells (Kano et al., 2008).

Leaves of *Agastache rugosa* (Fisch. & C.A.Mey.) Kuntze (Lamiaceae) are traditionally used in East Asia to relieve congestion, diarrhea, headaches, and vomiting (Cao et al., 2017; Desta et al., 2016; Li et al., 2013). We previously reported that tilianin (acacetin 7-O- $\beta$ -D-glucoside) and its derivatives (6'-O-malonyl tilianin, 2'-O-acetyl tilianin, 2'-O-acetyl-6'-O-malonyl tilianin) constitute more than 95% of the methanol extract of *A. rugosa*, and the derivatives are easily converted to tilianin during extraction/concentration process (Lee et al., 2017; An et al., 2018). Tilianin has been also identified in different Asian medicinal plants and exhibits anti-inflammatory, antioxidant, anti-diabetic, and anti-hyperlipidemic effects (Akanda et al., 2019; Zielinska and Matkowski, 2014). Recently, we found that tilianin suppressed the *in vitro* expression of MUC5AC in airway epithelial cells and proposed a potential role of tilianin on asthma (Song et al., 2017). However, the *in vivo* effect of tilianin on asthma and relevant mechanisms have not been explored. In this study, we investigated the effect of tilianin on Th2-immune responses and HDM-induced allergic asthma in mice.

## Materials and methods

### Reagents

*A. rugosa* leaves were collected at Gonyang in Sacheon, South Korea. The plant was authenticated by Dr. Jin-Hyub Paik (Korea Research Institute of Bioscience and Biotechnology), and a voucher specimen (KRIB0059129) was deposited at the International Biological Material Research Center of the Korea Research Institute of Bioscience and Biotechnology (KRIBB), in Daejeon, Korea. The air-dried leaves were chopped and extracted with methanol at room temperature for three days. The combined filtrates was concentrated *in vacuo* at 40 °C to produce a dried extract. The chromatographic separation of the total extract was performed by prep-HPLC using an Atlantis T3 column. Purified compounds were identified by comparing <sup>1</sup>H and <sup>13</sup>C NMR, MS, MS/MS, and HRESIMS data with literature values (Lee et al., 2017; An et al., 2018) (Fig. S1A - S1D). Based on UPLC-PDA analysis, the purity of tilianin was more than 97.0% (Fig. S1E and S1F).

### Mice

Female BALB/c mice were purchased from DBL (Eumseing, South Korea), and DO11.10 TCR transgenic mice were obtained from Taconic (Rensselaer, NY, USA). All experimental procedures were approved by the Institutional Animal Care and Use Committee of Korea Research Institute of Bioscience and Biotechnology (KRIBB-AEC-20008).

### Splenocyte cultures and tilianin treatment

Splenocytes were isolated from DO11.10 mice and cultured in RPMI-1640 media with 10% FBS,  $\beta$ -mercaptoethanol (50  $\mu$ M, Sigma-Aldrich Inc., St Louis, MO, USA), OVA<sub>323-339</sub> peptide (500 nM, Peptron, Daejeon, South Korea), and mIL-2 (2 ng/ml, Biolegend, San Diego, CA, USA) which was designated as the Th0 condition. Tilianin was treated into splenocytes cultured under Th0 conditions for 3 days. Differentiated T cells were prepared by culturing splenocytes in Th0 conditions for 7 days, followed by stimulation with in Phorbol 12-myristate 13-acetate (PMA, 50 ng/ml, Sigma-Aldrich Inc.)/ionomycin (500 ng/ml, Sigma-Aldrich Inc.) or anti-CD3 (1  $\mu$ g/ml, BD Biosciences, San José, CA, USA)/anti-CD28 (1  $\mu$ g/ml, BD biosciences) for 16 h in the presence or absence of tilianin. Data were obtained from three repeated experiments.

### BMDC cultures and tilianin treatment

BMDCs were isolated from BALB/c mice. Isolated cells were cultured in 6-well plates at a density of  $1 \times 10^6$  cells/well with GM-CSF (10 ng/ml, Biolegend, San Diego, CA, USA) on day 0. On day 2 and 4, the media was changed using fresh media with GM-CSF. Non-adherent and loosely adherent cells were used for the assay on day 6. Isolated BMDCs were seeded in 24-well plates at a density of  $1.5 \times 10^6$  cells/well with culture media without GM-CSF. Tilianin treatment was done 1 h before HDM (100  $\mu$ g/ml) treatment. Twenty four h after stimulation with HDM, cell supernatants were used for ELISA, and cell pellets were harvested for total RNA preparation and real-time PCR as described below. All experiments were repeated three times.

### HDM-induced allergic asthma model and treatment

BALB/c mice were randomly divided into four groups: the normal control group (NC), the house dust mite (HDM) sensitization/challenge group (HDM), the tilianin (7.5 mg/kg) treated HDM sensitization/challenged group (Til 7.5), and the tilianin (15 mg/kg) treated HDM sensitization/challenged group (Til 15). Airway inflammation was induced as previously described with minor modification (Choi et al., 2016). Briefly, BALB/c mice were sensitized by i.p. injection of 25  $\mu$ g of purified HDM extract (Greer Laboratories, Lenoir, NC, USA) and 2 mg of aluminum hydroxide (Imject® Alum, Thermo Fisher Scientific, Waltham, MA, USA) on day 0 and day 7. Then, 10  $\mu$ g of HDM was added intranasally for three consecutive days from day 10 to day 12, and the mice were sacrificed 48 h after the last challenge (day 14), as shown in Fig. 4A. Tilianin (7.5 or 15 mg/kg) was dissolved in the vehicle, 20% DMAC, 20% TWEEN80 (Sigma-Aldrich Inc.) and 60% HPBCD (Tokyo Chemical Industry, Tokyo, Japan), and administered orally 1 h before HDM challenges for 3 days. The NC and HDM groups received vehicle only. Two replicated experiments were performed and total number of mice in each group was six.

### Bronchoalveolar lavage fluid (BALF) and cell analysis

BALF was obtained by injecting cold PBS using a tracheal cannula with a 20 G blunt needle. The total number of inflammatory cells were counted by using a hemacytometer after trypan blue staining. To identify differential cell counts, 100  $\mu$ l of BALF was centrifuged onto slides using Cytospin (Hanil Science Industrial, Seoul, Korea). Then, slides were dried, fixed, and stained using the Diff-Quick staining reagent (B4132-1A; IMEB Inc., Deerfield, IL, USA).

### Assessment of airway hyperresponsiveness (AHR)

Forty-eight h after the last HDM challenge, the AHR was assessed using a *flexiVent* system (SCIREQ, Inc., Montreal, Canada) according to the manufacturer's protocol. Briefly, mice were anesthetized using a

**Table 1**

Primer sequences for quantitative real-time PCR

| Genes                      | Sense (5' to 3')      | Antisense (5' to 3') | Length (bp) |
|----------------------------|-----------------------|----------------------|-------------|
| <i>Gapdh</i>               | CCTGCACCACCAACTGCTTA  | GTCTTCTGGGTGGCAGTGAT | 109         |
| <i>Irfng</i>               | ATCTGGAGGAACTGGCAAAA  | GCTGATGGCTGATTGTCTT  | 108         |
| <i>Il5</i>                 | ATCAAACTGTCCTGGGGGT   | TCTCCTCGCCACACTTCTCT | 99          |
| <i>Il13</i>                | CCCTGGATTCCCTGACCAAC  | CCAGGGATGGTCTCTCCTCA | 211         |
| <i>Il10</i>                | GGCAGCCTTGACAGAAAAGAG | GCTTGGCAACCAAGTAACC  | 274         |
| <i>Il33</i>                | AGAGATCCTTGCTTGGCAGT  | AGCACCTGGTCTTGCTCTTG | 199         |
| <i>Il12a</i><br>(IL-12p35) | CCCTTGCCCTCTAAACCAC   | TAGTAGCCAGGCAACTCTCG | 226         |
| <i>Il12b</i><br>(IL-12p40) | CGAGACTCTGAGCCACTCAC  | AGACAGAGACGCCATTCCAC | 237         |
| <i>Irf1</i>                | TCTCGGGCATCTTTGCGTTC  | CGGAACAGACAGGCATCCTT | 270         |
| <i>Irf4</i>                | TCTCTGCCAGCCAGCAGGT   | CCTGGGACTCAGGTGGGGCA | 211         |
| <i>Gata3</i>               | AGGCAACCACTGCCGTCCT   | TTTGGCCCATCCAGCCAGG  | 135         |
| <i>Muc5ac</i>              | CCAGCAATCCCCTTTCCGAT  | CCCTGCGGACAGTTGATCTT | 292         |

mixture of ketamine and xylazine (Rompun, Bayer Korea, Seoul, Korea) and the anesthetized mice were tracheostomized using an 18 G metal cannula. Mice received methacholine using a nebulizer (Aeroneb) and were sequentially exposed to increasing doses of methacholine in PBS; 0, 12.5, 25, and 50 mg/ml (Sigma-Aldrich Inc.).

#### Histopathological analysis

For histopathological analysis, lung sections were stained with hematoxylin, eosin (H&E, Sigma-Aldrich Inc.), and periodic acid Schiff (PAS, Abcam, Cambridge, UK) according to the manufacturers' protocols. Based on the severity of the inflammatory lesion in lung tissue, inflammatory scores ranged from 0 to 3 (0, no inflammatory lesion was observed; 1, focal infiltration of inflammatory cells around airway; 2, multiple infiltration of inflammatory cells around airway; 3, diffused lesion with massive infiltration of inflammatory cells around airway) were determined. To quantify the mucin production, the number of PAS-positive cells in the airway was counted and the length of the basement membrane in each airway was measured using ImageJ version 1.52a (National Institutes of Health, Bethesda, MD, USA). The results were given as mean number of PAS-positive cells per micrometer of basement membrane.

#### Quantitative real-time reverse transcription (RT)-PCR

Total RNA was extracted from cell pellets or homogenized lung tissues. For RNA isolation, TRIzol reagent (Ambion, Carlsbad, CA, USA) was used according to the manufacturer's protocol. cDNA was synthesized from 1 µg of total RNA using the ReverTra Ace First Strand cDNA Synthesis Kit (Toyobo, Osaka, Japan). For real-time PCR, the iQ SYBR Green supermix (Bio-Rad, Hercules, CA, USA) and an S1000™ Thermal Cycler (Bio-Rad) were used. Information on primers and their sequences is provided in Table 1. Relative gene expression levels were evaluated by their ratio to Gapdh mRNA.

#### Western blot analysis

Cell lysates and tissue lysates were harvested with RIPA buffer (Biosesang, Seongnam, South Korea) containing a protease inhibitor cocktail and a phosphatase inhibitor. Proteins were fractionated on 10% SDS-polyacrylamide gels. Gels were transferred to polyvinylidene fluoride membranes, and the membranes were incubated for 1 h in 5% skim milk in TBS-T buffer to block non-specific binding. Membranes were incubated with primary antibodies, recognizing IRF4 (Cell Signaling Technology, Danvers, MA, USA), GATA3, or GAPDH (Santa Cruz Biotechnology, Dallas, TX, USA), followed by incubation with an HRP-conjugated secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA, USA). Signals were developed using an enhanced

chemiluminescence system (Thermo Fisher Scientific).

#### Measurement of cytokine secretion levels by ELISA

Levels of cytokines in cell supernatants or BALF were analyzed with a commercially available specific OptEIA sandwich ELISA kit (BD Biosciences) following the manufacturer's instructions.

#### Statistical analysis

Data were analyzed and graphed with GraphPad Prism software (ver. 6.07) and are presented as means ± SEMs. Statistical analysis was calculated using analysis of variance (ANOVA) followed by a multiple comparison test with Dunnett's adjustment. *P* values ≤ 0.05 were considered statistically significant.

## Results

#### Tilianein differentially regulates cytokine production in splenocytes

We first assessed the effects of tilianein on Th1 and Th2 cytokine production since an imbalance between the Th1 and Th2 response is a critical factor in the development of asthma. Splenocytes from DO 11.10 TCR mice were treated with tilianein and cultured in Th0 conditions for three days. Tilianein suppressed IL-13 production by up to 37% (*p* < 0.0001), but increased IFNγ production by up to 20% (*p* < 0.0001) (Fig. 1B). Quantitative real-time PCR analysis revealed that the Th2-related cytokine IL-4 and IL-13 expression were significantly inhibited, while the Th1-related cytokine IFNγ expression was increased by tilianein in a dose-dependent manner (Fig. 1C). Other Th1 and Th2 cytokines, TNF-α and IL-10, were reduced at the highest dose of tilianein (20 µM). A well-known immunosuppressive drug, dexamethasone inhibited all cytokines we measured regardless of subtype specificity (Fig. 1B and C). To determine the effect of tilianein on differentiated effector T cells, splenocytes from DO 11.10 mice were cultured in Th0 conditions for seven days, and then stimulated with anti-CD3/anti-CD28 or PMA/ionomycin in the presence or absence of tilianein. Interestingly, tilianein did not significantly suppress IL-13 production in differentiated effector T cells (Fig. 1D). These data suggested that the inhibitory effect of tilianein on IL-13 production in splenocytes might require antigen-presenting cells. Thus, we designed further experiments to study the direct effect of tilianein on antigen-presenting cells.

#### Tilianein regulates cytokine expression in BMDCs

Dendritic cells (DCs) are professional antigen-presenting cells in the spleen and contribute to the differentiation of T cells by producing cytokines (Kumar et al., 2019). Given the opposite effects of tilianein on Th2

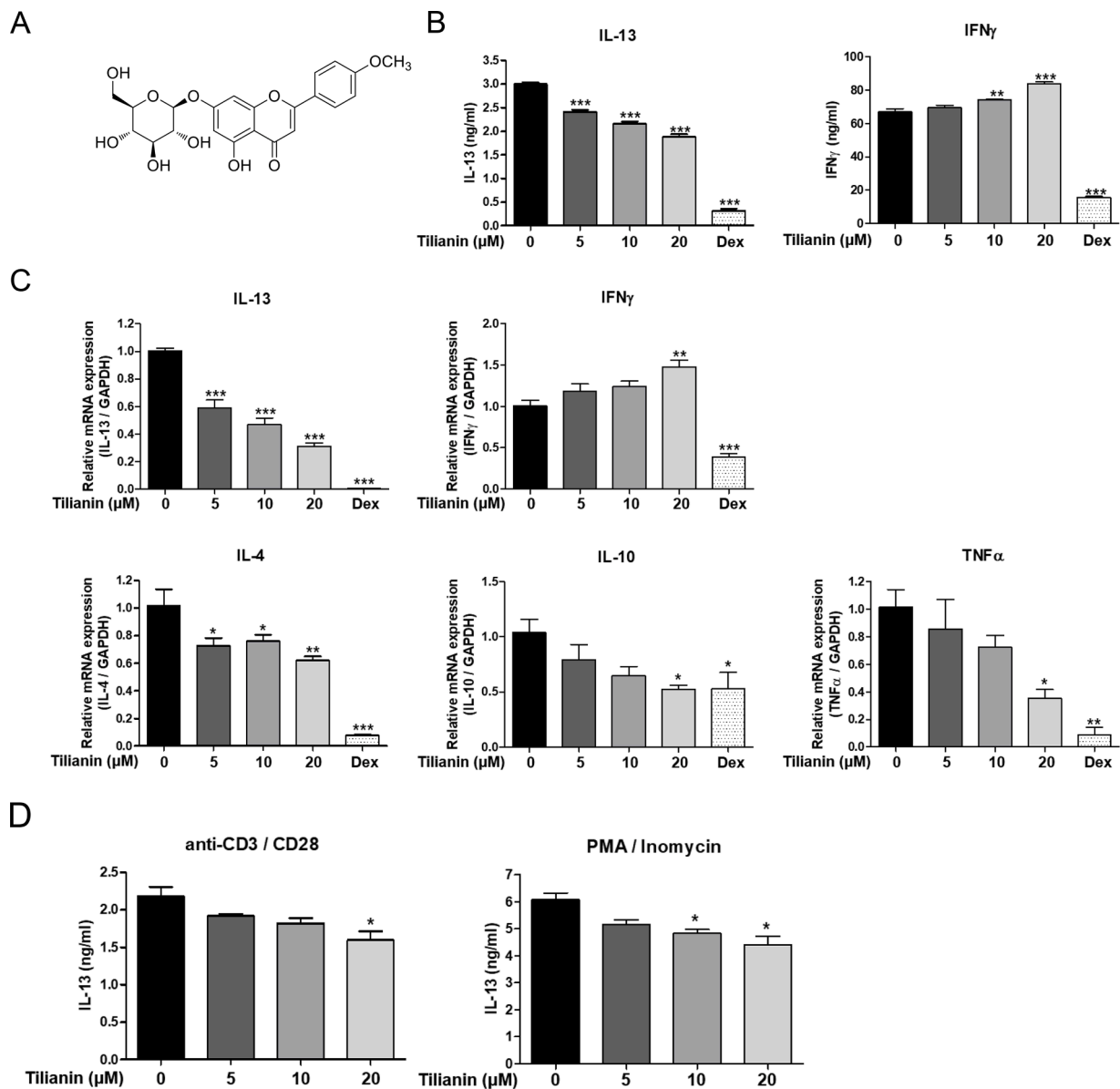


**Fig. 1.** Effect of tilianin on cytokine production in splenocytes. (A) Chemical structure of tilianin. (B) The levels of secreted IL-13 and IFN $\gamma$  in the supernatant of splenocytes cultured in Th0 condition with tilianin or dexamethasone. (C) mRNA levels of *IL-4*, *IL-13*, *IL-10*, *IFN $\gamma$* , and *TNF- $\alpha$*  in splenocytes cultured in Th0 conditions with tilianin or dexamethasone were determined by real-time PCR. The data were normalized to *Gapdh* mRNA expression. (D) Secreted IL-13 levels from effector T cells stimulated with anti-CD3/28 or PMA/ionomycin in the presence or absence of tilianin were analyzed by ELISA. All experiments were repeated 3 times and representative results are presented. Statistics represented as mean  $\pm$  SEM of each group; \*  $p < 0.05$ , \*\*  $p < 0.01$ , and \*\*\*  $p < 0.001$ , compared with the untreated control.

and Th1 cytokine production in splenocytes (Fig. 1A), we speculated that tilianin might differentially regulate the Th1-skewing cytokine IL-12 and the Th2-skewing cytokines IL-33 and IL-10 in DCs. To test this hypothesis, we generated BMDCs and stimulated them with HDM, the major cause of Th2-allergic response, in the presence or absence of tilianin. Tilianin inhibited the expression of IL-33 at every time point but upregulated the expression of IL-12p40 at later time points compared with the HDM only groups (Fig. 2A). To evaluate the dose-dependency of tilianin, BMDCs were treated with HDM along with three different doses of tilianin (5, 10, 20  $\mu$ M) and were analyzed at the time points previously shown to have the most significant difference between the HDM only group and the HDM plus tilianin treated groups (6 h for IL-33 and 24 h for IL-12p40). Tilianin decreased the HDM-induced expression of IL-13 (6.4-fold) up to 1.0-fold ( $p < 0.0001$ ) and IL-10 (6.2-fold) to 2.7-fold ( $p = 0.0035$ ) (Fig. 2B). On the other hand, the levels of IFN $\gamma$ , IL-12p40, and IL-12p35 transcripts were significantly increased by

tilianin (20  $\mu$ M) up to 5.2-, 4.4-, and 4.9-folds, respectively (Fig. 2B). Consistently, HDM-induced IL-12p40 secretion in supernatants were also increased by tilianin up to two-fold (Fig. 2C). Taken together, we concluded that tilianin differentially regulates Th1- and Th2-related cytokine production in DCs.

#### Tilianin differentially regulates IRF4 and IRF1 in BMDCs

Next, we investigated transcription factors that are involved in the differential regulation of cytokine expression by tilianin. It has been shown that IRF1 and IRF4 in DCs play a critical role in the development of Th1 and Th2 cells (Lohoff and Mak, 2005). IRF4 promotes Th2 cell development by upregulating IL-10 and IL-33 expression in DCs (Williams et al., 2013). Whereas IRF1 promotes the expression of IL-12 in DCs and inhibits cytokine production from Th2 cells (Buccione et al., 2018). Real-time PCR analysis revealed that tilianin suppressed the

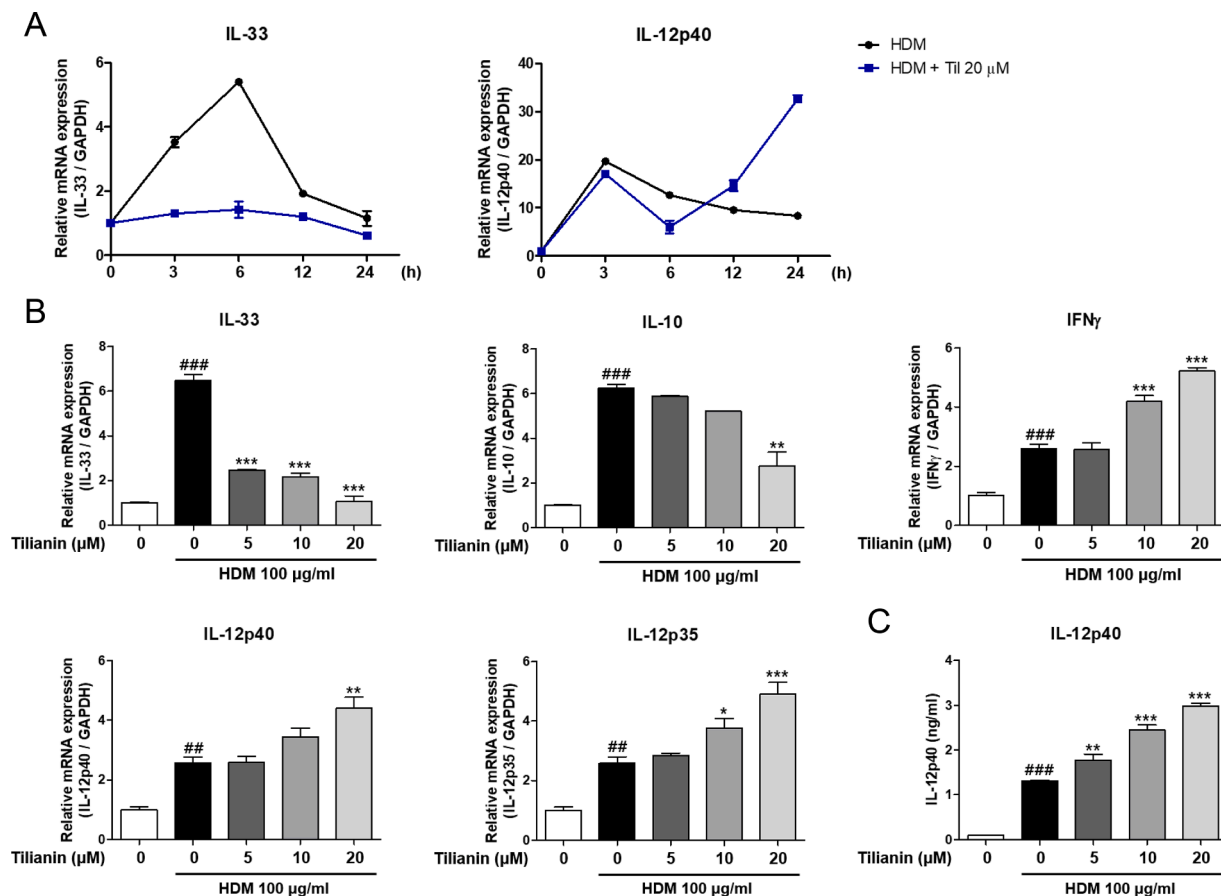


**Fig. 2.** Effect of tilianin on transcriptional expression of cytokine in BMDCs. (A) Kinetics of mRNA expression levels of *IL-33* and *IL-12p40* in BMDCs stimulated by HDM (100 μg/ml) with or without tilianin (20 μM) for the indicated times. The mRNA levels were measured by real-time PCR and normalized to *Gapdh* mRNA expression. (B) mRNA levels of *IL-33*, *IL-10*, *IFNγ*, *IL-12p40*, and *IL-12p35* in BMDC stimulated with HDM (100 μg/ml) with or without tilianin. (C) The protein level of *IL-12p40* in BMDC stimulated with HDM (100 μg/ml) with or without tilianin. All experiments were repeated 3 times and representative results are presented. Statistics represented as mean ± SEM of each group; ###  $p < 0.001$ , compared with untreated control, \*  $p < 0.05$ , \*\*  $p < 0.01$ , and \*\*\*  $p < 0.001$ , compared with HDM-treated group.

expression of *IRF4* at 6 h and increased the expression of *IRF1* at 24 h in HDM-treated BMDCs (Fig. 3A). The expression patterns of *IRF4* and *IRF1* correlated with the expression of *IL-33* and *IL-12p40*. The effects of tilianin on *IRF4* or *IRF1* in BMDCs occurred in a dose-dependent manner (Fig. 3B). The expression of *IRF4* and *IRF1* in HDM-treated BMDCs were increased to 3.9- and 4.8-folds, respectively. Tilianin reduced the expression of *IRF4* up to 1.5-fold, while increased *IRF1* expression up to 8.63-fold. We confirmed that tilianin dose-dependently inhibited *IRF4* protein, but not *IRF1* protein (Fig. 3C). These results suggest that tilianin differentially regulates the expression of Th1- and Th2-related cytokines by controlling the balance of *IRF1* and *IRF4* in BMDCs.

#### Tilianin attenuates HDM-induced airway inflammation

Since *IRF4* expression in DCs is critical for Th2-mediated immune responses (Williams et al., 2013), we evaluated the effect of tilianin on Th2-mediated airway inflammation *in vivo* using an HDM-induced asthma model (Fig. 4A). The number of total and differential inflammatory cells in BALF, including macrophages, neutrophils, eosinophils, and lymphocytes, was significantly increased in the HDM-treated group (HDM) compared to the normal control group (NC) (Fig. 4B and C). Oral administration of tilianin (15 mg/kg) in HDM-treated mice reduced the total number of inflammatory cells in BALF by 30% ( $p = 0.0181$ ), particularly the eosinophils and neutrophils were reduced by 36% ( $p = 0.0257$ ) and by 58% ( $p = 0.0118$ ), respectively, when compared to the HDM-treated mice (Fig. 4C). To determine the effect of tilianin on

airway function, mice were exposed to methacholine aerosols. Compared with the normal control group, HDM-treated mice had markedly higher total respiratory system resistance (Rrs) and elastance (Ers) values and lower compliance (Crs) values. This upregulation of Rrs and Ers and downregulation of Crs were reversed by the administration of tilianin (Fig. 4D).

To assess the anti-inflammatory effects of tilianin, histological analysis of lung tissues was performed using H&E and PAS staining. Massive infiltration of inflammatory cells around the airway and hyperplasia of goblet cells (PAS-positive cells) in the bronchus were observed in HDM-treated mice compared with control mice (Fig. 5). Both doses of tilianin (7.5 and 15 mg/kg) decreased lung inflammatory score significantly to 1.5 ( $p = 0.0033$ ) and 1.3 ( $p = 0.0003$ ), respectively (Fig. 5A). Number of PAS positive cells per 1 μm of basement membrane in the airway was also decreased in Til 15 group compared to HDM group (from 0.2 to 0.13,  $p = 0.474$ ) (Fig. 5B).

We further investigated transcript levels of cytokines in lung tissues of the normal control group, the HDM treated group, and the tilianin treated groups (7.5 mg/kg or 15 mg/kg) using real-time RT-PCR. Tilianin (15 mg/kg) suppressed the mRNA expression of *IL-13* (1.2-fold,  $p = 0.0004$ ) and *IL-33* (2.2-fold,  $p = 0.0004$ ). The expression of the mucin production-associated gene *MUC5AC* was also decreased in Til 7.5 (from 8.6-fold to 6.4-fold,  $p = 0.0039$ ) and Til 15 (from 8.6-fold to 3.7-fold,  $p = 0.0011$ ). However, transcript levels of *IL-5* and *IFNγ* were not affected by tilianin (Fig. 6A). Next, we examined the levels of asthma-associated Th2 cytokines in BALF using an ELISA. Both doses of

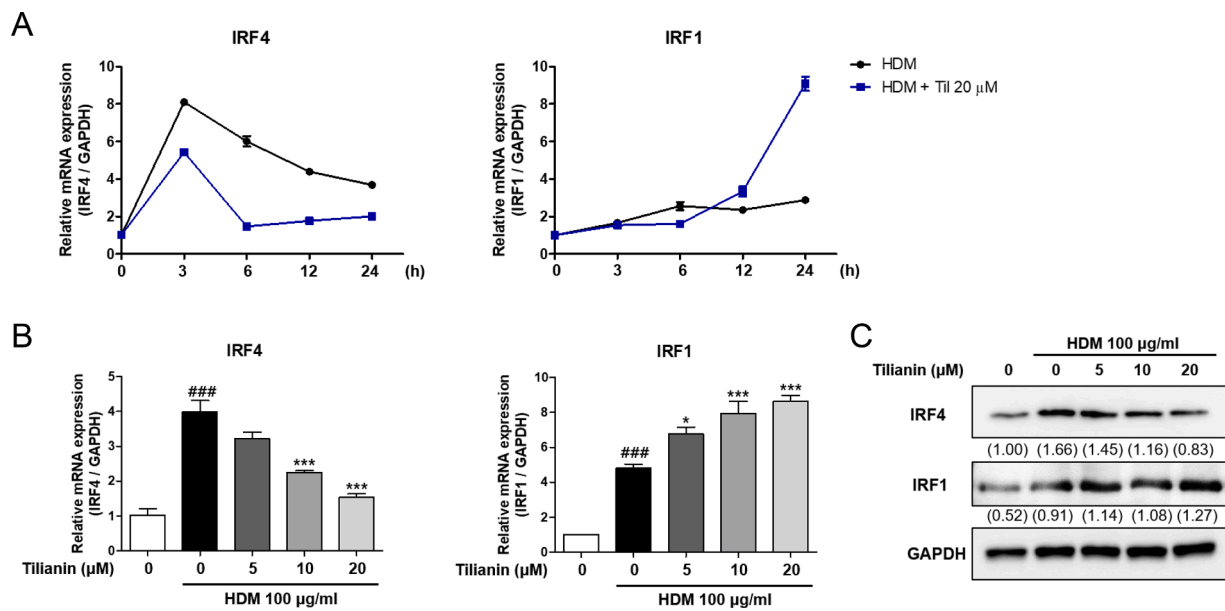


**Fig. 3.** Effect of tilianin on protein levels of IRF1 and IRF4 in BMDC. (A) Kinetics of mRNA levels of *IRF4* and *IRF1* in BMDCs stimulated by HDM (100  $\mu$ g/ml) with or without tilianin for the indicated time. The mRNA levels of *IRF4* and *IRF1* in cells were measured by real-time PCR. (B) Transcript levels of *IRF4* and *IRF1* in BMDCs stimulated by HDM (100  $\mu$ g/ml) with or without tilianin (5, 10, 20  $\mu$ M) for 6 or 24 h, respectively. The mRNA expression was normalized to *Gapdh* mRNA expression. (C) Protein levels of IRF4 and IRF1 were detected by western blot. The numbers are noted below the blotting image indicate the relative band density. All experiments were repeated 3 times and representative results are presented. The quantitative western blot data were normalized with GAPDH. Statistics are represented as mean  $\pm$  SEM of each group;  $^{###}p < 0.001$ , compared with untreated control,  $^{*}p < 0.05$ ,  $^{**}p < 0.01$ , and  $^{***}p < 0.001$ , compared with BMDCs treated with HDM alone.

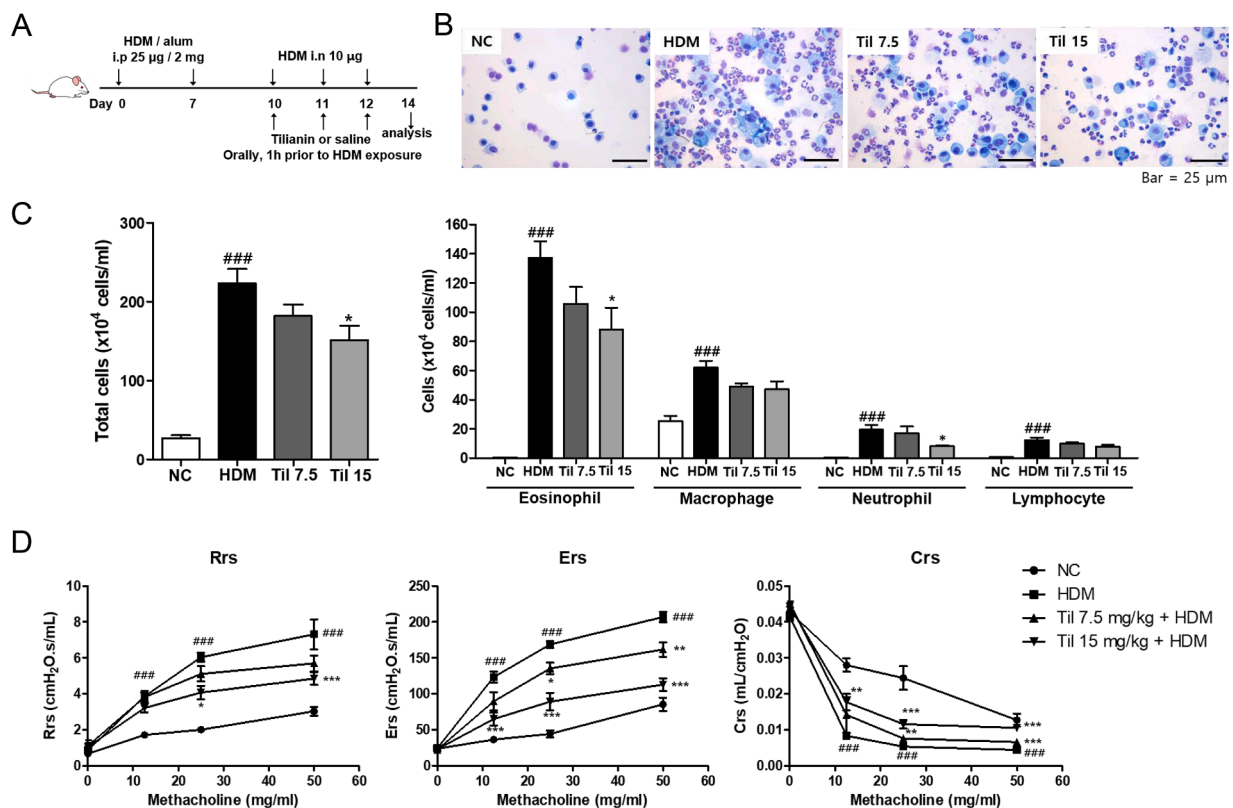


**Fig. 4.** Effect of tilianin on airway hyperresponsiveness in HDM-induced asthma model. (A) A schematic illustration of HDM sensitization, challenge, and drug treatment in this study. (B and C) Total and differential cells in the BALF of mice. (D) The values of Respiratory system resistance (Rrs), Elastance (Ers), and Compliance (CrS) were determined two days after the last HDM challenge using methacholine challenge tests. NC; normal control mice treated with saline only, HDM; HDM-sensitized/challenged mice, Til 7.5 and Til 15; Tilianin (7.5 and 15 mg/kg) + HDM-sensitized/challenged mice. Representative results from three replicated experiments are shown. All data are represented as the mean  $\pm$  SEM ( $n = 6$ ).  $^{#}p < 0.05$ ,  $^{###}p < 0.001$ , compared with normal control (NC);  $^{*}p < 0.05$ ,  $^{**}p < 0.01$ , and  $^{***}p < 0.001$ , compared with HDM group.

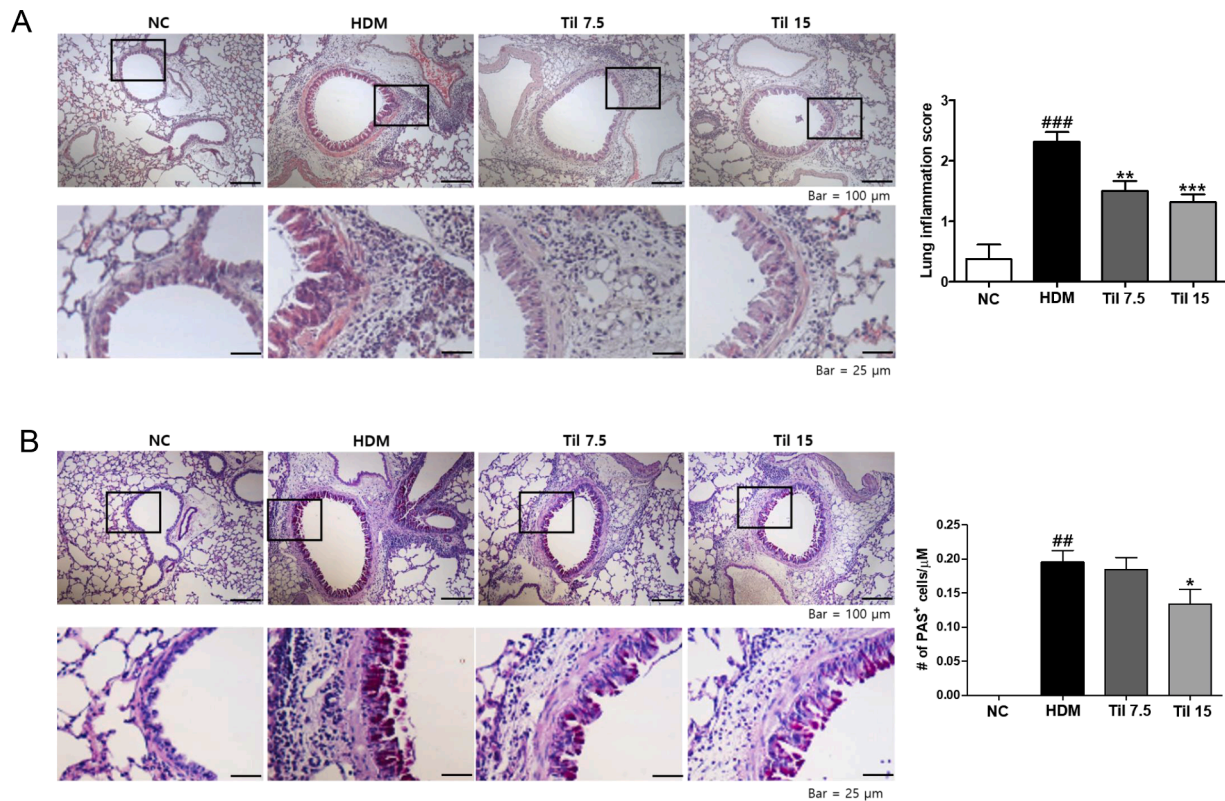


**Fig. 5.** Effects of tilianin on airway inflammation and mucus secretion in HDM-induced asthma model. (A) Lung tissue sections were stained with H&E and severity of inflammation in lung section was scored ranged from 0 to 3. (B) Lung tissue sections were stained with PAS and number of PAS-positive cells per 1  $\mu\text{m}$  of basement membrane in the airway were counted. NC; normal control mice treated with saline only, HDM; HDM-sensitized/challenged mice, Til 7.5 and Til 15; Tilianin (7.5 and 15 mg/kg) + HDM-sensitized/challenged mice.

tilianin inhibited HDM-induced IL-13 secretion in BALF by up to 66% (Fig. 6B). HDM-induced IL-5 secretion in BALF were also suppressed by tilianin up to 46% ( $p = 0.0006$ ) (Fig. 6B). Compared to dexamethasone, tilianin reduced the main features of asthma to a similar extent (Fig. S2). Taken together, these results suggest that tilianin attenuates HDM-induced allergic inflammation by inhibiting asthma-related inflammatory cytokines.

#### *Tilianin inhibits the IRF4 expression level in HDM-induced airway inflammation model*

To confirm whether tilianin has similar effects on the regulation of IRF4 and IRF1, as seen in previous *in vitro* BMDC experiments, we evaluated the effect of tilianin on the mRNA expression of IRF4 and IRF1 in lung tissues of HDM-induced mice. As expected, HDM treatment increased the mRNA level of IRF4 (1.3-fold,  $p = 0.0104$ ) and reduced IRF1 (0.4-fold,  $p < 0.0001$ ) compared with NC. These alterations of IRF4 and IRF1 expression were reversed by tilianin (15 mg/kg) to 0.71- ( $p < 0.0001$ ) and 0.75-folds ( $p = 0.0316$ ), respectively (Fig. 7A). Western blot analysis revealed that HDM-induced IRF4 protein levels in lung tissues were almost completely suppressed up to 37% ( $p = 0.0002$ ) in tilianin-administrated mice (Fig. 7B). However, IRF1 protein was barely detectable in this condition (Fig. S3). Protein levels of GATA3, the master regulator of Th2 differentiation, were also decreased up to 58% ( $p < 0.0001$ ) in tilianin-administrated mice (Fig. 7B). Taken together, these data suggest that tilianin inhibits Th2-mediated airway inflammation by suppressing IRF4 in the HDM-induced asthma mouse model.

#### **Discussion**

Allergic asthma is a heterogeneous disorder with different

phenotypes caused by various factors. Steroids have been used as the primary treatment for asthma. However, long-term treatment of these drugs can cause multiple side effects, including steroid tolerance, mood changes, high blood pressure, and muscle weakness (Brown et al., 2002; Hopkins and Leinung, 2005; Lehmann and Ott, 2008). As a consequence, novel targets as well as natural plant compounds are being actively investigated for integrative asthma therapies, with the aim of developing new drugs to replace steroids (Lin et al., 2019).

In this study, we showed the reciprocal regulatory role of tilianin from *A. rugosa*, a traditional medicine, on Th1 and Th2 cytokine expression. Th2-related cytokines, IL-4, IL-5, and IL-13, play essential roles in allergic inflammation by mediating airway eosinophilia, mucus hypersecretion, and inflammatory cytokine overexpression. Thus, the overexpression of Th2 cytokines is a critical factor in asthma development. For this reason, the inhibition of excessive Th2 cytokines is an important treatment strategy for allergic asthma (Woodruff et al., 2009). Importantly, tilianin inhibited Th2 cytokine IL-13 but not Th1 cytokine IFN $\gamma$ . Thus, unlike steroids that have broad immune suppressive effects, tilianin might have an ability to restore Th1/Th2 balance to suppress allergic responses by inhibiting Th2 responses specifically.

DCs play a pivotal role in the Th2-immune response to specific antigen. When HDM is exposed to the body, DCs act as a bridge connecting innate immunity to adaptive immunity via expressing MHC molecules, CD80 and CD86, on cell membranes and secreting cytokines such as IL-33 and IL-10 in response to allergens like Der p1 to promote the differentiation of Th2 cells (Lambrecht and Hammad, 2009). IRFs in DCs play critical roles in Th2 development (Kumar et al., 2019). The upregulation of IRF4 in DCs can promote naïve CD4<sup>+</sup> T cells to differentiate into Th2 cells while inhibiting differentiation to Th1 cells (Akbari et al., 2014; Gao et al., 2013; Huber and Lohoff, 2014). Conversely, IRF1, another member of the IRF family, promotes Th1 development while

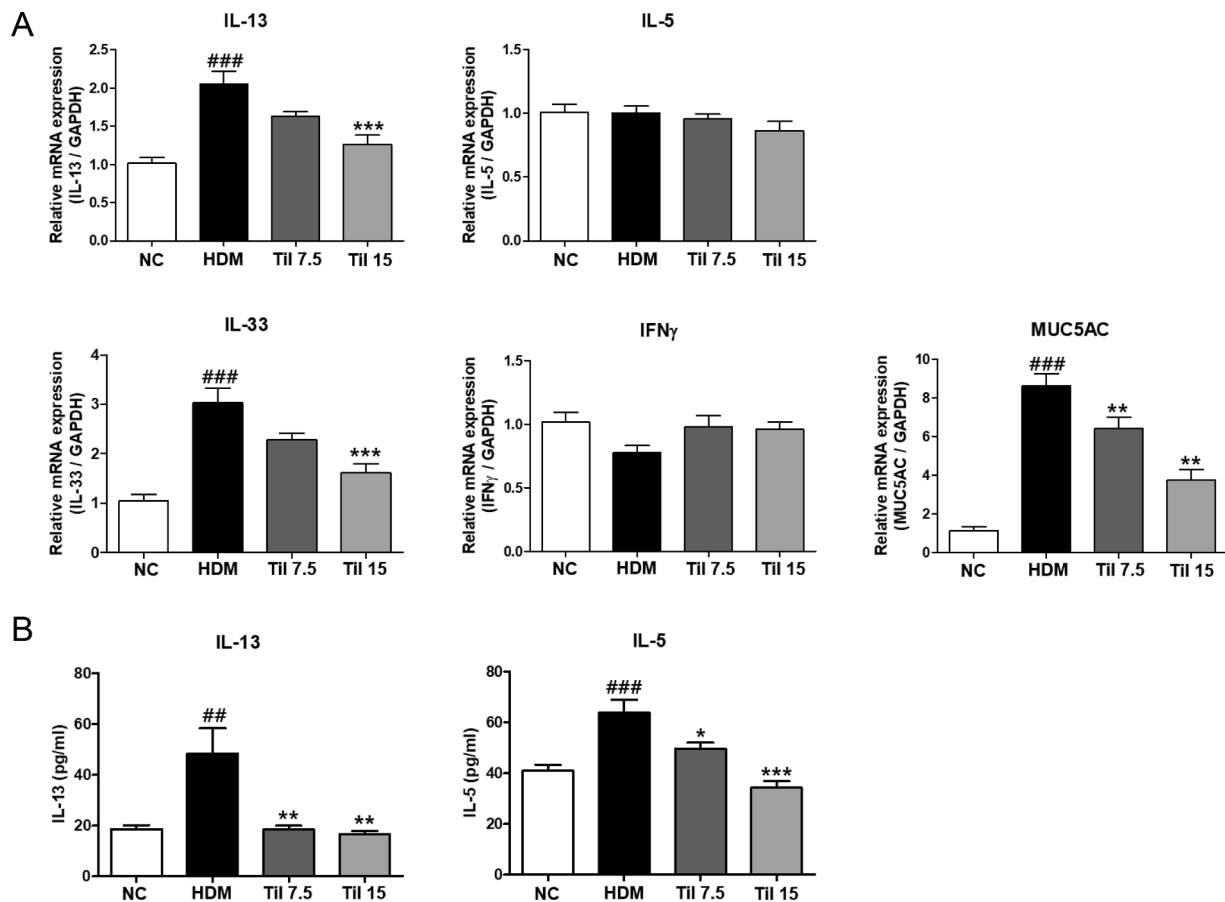


**Fig. 6.** Effects of tilianin on mRNA expression and secretion of inflammatory cytokines in BALF and lung tissues. (A) The mRNA levels of asthma-related cytokines and *MUC5AC* were determined by real-time RT-PCR. The mRNA expression data were normalized to *Gapdh* mRNA expression. (B) The levels of Th2-related cytokines were measured using an ELISA. NC; normal control mice treated with saline only, HDM; HDM-sensitized/challenged mice, Til 7.5 and Til 15; Tilianin (7.5 and 15 mg/kg) + HDM-sensitized/challenged mice. Representative results from three replicated experiments are shown. All data are represented as the mean  $\pm$  SEM ( $n = 6$ ). #  $p < 0.05$ , ##  $p < 0.01$ , ###  $p < 0.001$ , compared with normal control (NC); \*  $p < 0.05$ , \*\*  $p < 0.01$ , and \*\*\*  $p < 0.001$ , compared with HDM group.

inhibiting cytokine production from Th2 cells (Lohoff and Mak, 2005). IRF1 induces IL-12 expression in DCs, which promotes naïve CD4<sup>+</sup> T cells to differentiate into Th1 cells (Kano et al., 2008; Qiao et al., 2013). It has been shown that HDM activates IRF4 in DCs, which promotes Th2 responses via the upregulation of IL-10 and IL-33 (Williams et al., 2013). We showed that tilianin inhibited IRF4 expression while it enhanced IRF1 in HDM-treated BMDCs. The kinetics of tilianin effects on IRF1 and IRF4 expression was well correlated with its effects on IL-12 and IL-33 expression. Thus, it appears that the inhibitory effect of tilianin on the Th2 immune response is mediated by its differential effects on IRF4 and IRF1 expression in DCs. At present, we do not understand the mechanism by which tilianin suppresses IRF4. Further studies will be necessary to elucidate the molecular mechanism of the reciprocal regulation of IRF1 and IRF4 by tilianin. Based on the inhibitory effect of tilianin on Th2 responses, we decided to examine the inhibitory effect of tilianin on asthma. Using an HDM-induced animal model, we demonstrated that tilianin ameliorates allergic asthma. Tilianin reduced the hallmarks of asthma, including AHR, inflammatory cell infiltration in BALF, mucus production, and Th2 cytokine levels. Interestingly, the reciprocal effect of tilianin on IRF1 and IRF4 seen in BMDC was recapitulated *in vivo*.

It is interesting to note that several recent reports have also suggested that tilianin may be beneficial for pulmonary diseases. In toxic particle-induced chronic obstructive pulmonary disease (COPD), tilianin inhibits

the neutrophil infiltration via suppressing CXCL2, IL-17/STAT3 signaling pathway (Kim et al., 2020). In a human airway epithelial cell line, tilianin inhibits the expression of MUC5AC induced by EGF (Song et al., 2017). In addition, the aglycone of tilianin, acacetin, also improve asthma symptoms in OVA-sensitized mice (Huang and Liou, 2012). Collectively, these results suggest that tilianin may have therapeutic potential for the treatment of allergic asthma.

## Conclusion

We demonstrated that tilianin has selective inhibitory effects on Th2-responses by suppressing IRF4 and also attenuates allergic inflammation in an HDM-induced asthma mouse model. Therefore, these findings will be useful for the development of tilianin as a novel anti-asthmatic drug.

## CRediT authorship contribution statement

**Soo-Jin Park:** Formal analysis, Investigation, Writing - original draft. **Kiram Lee:** Methodology, Formal analysis. **Min-Ah Kang:** Investigation. **Tae-hyoun Kim:** Data curation. **Hyun-Jae Jang:** Resources. **Hyung Won Ryu:** Resources. **Sei-Ryang Oh:** Project administration, Funding acquisition. **Hyun-Jun Lee:** Methodology, Writing - review & editing, Supervision, Project administration, Funding

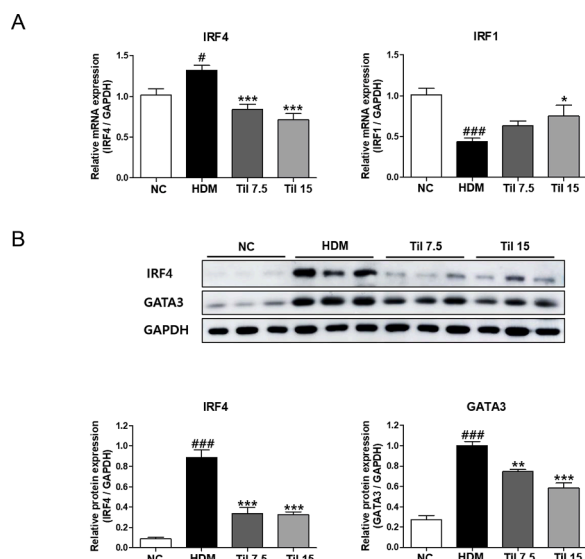


**Fig. 7.** Effects of tilianin on the expression of IRF4 and GATA3 in lung tissues. (A) The transcript levels of *IRF4* and *IRF1* in lung tissues were determined by real-time RT-PCR. Data were normalized to *Gapdh* gene expression. (B) The protein levels of IRF4 and GATA3 in lung tissues were analyzed by western blot. The western blot was quantitated using ImageJ. The levels of IRF4 and GATA3 were calculated over GAPDH. NC; normal control mice treated with saline only; HDM; HDM-sensitized/challenged mice, Til 7.5 and Til 15; Tilianin (7.5 and 15 mg/kg) + HDM-sensitized/challenged mice. Representative results from three replicated experiments are shown. All data are represented as the mean  $\pm$  SEM ( $n = 6$ ). <sup>#</sup>  $p < 0.05$ , <sup>###</sup>  $p < 0.001$ , compared with normal control (NC); \*  $p < 0.05$ , \*\*  $p < 0.01$ , and \*\*\*  $p < 0.001$ , compared with HDM group.

acquisition.

## Declaration of Competing Interest

The authors declare no competing financial interest.

## Acknowledgments

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## Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.phymed.2020.153392.

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Figure S1

A

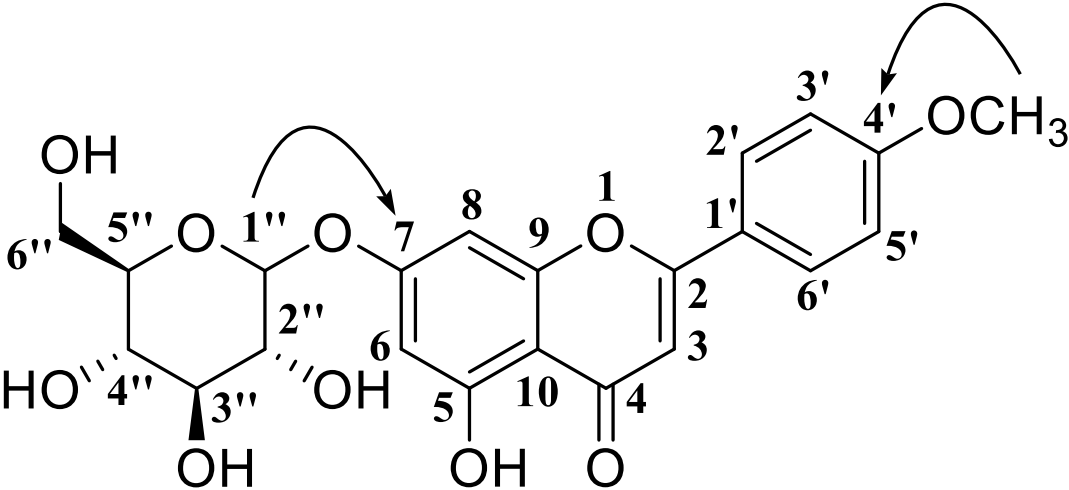


Figure S1

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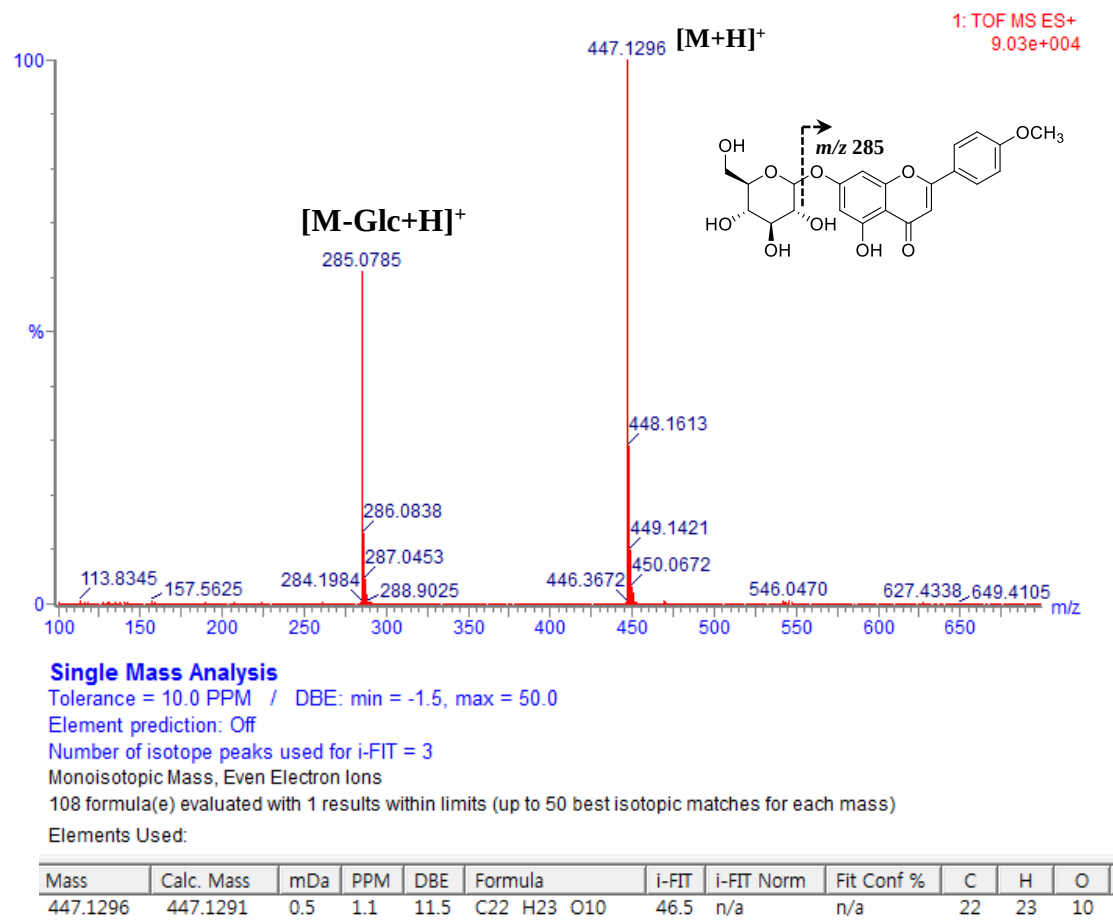


Figure S1

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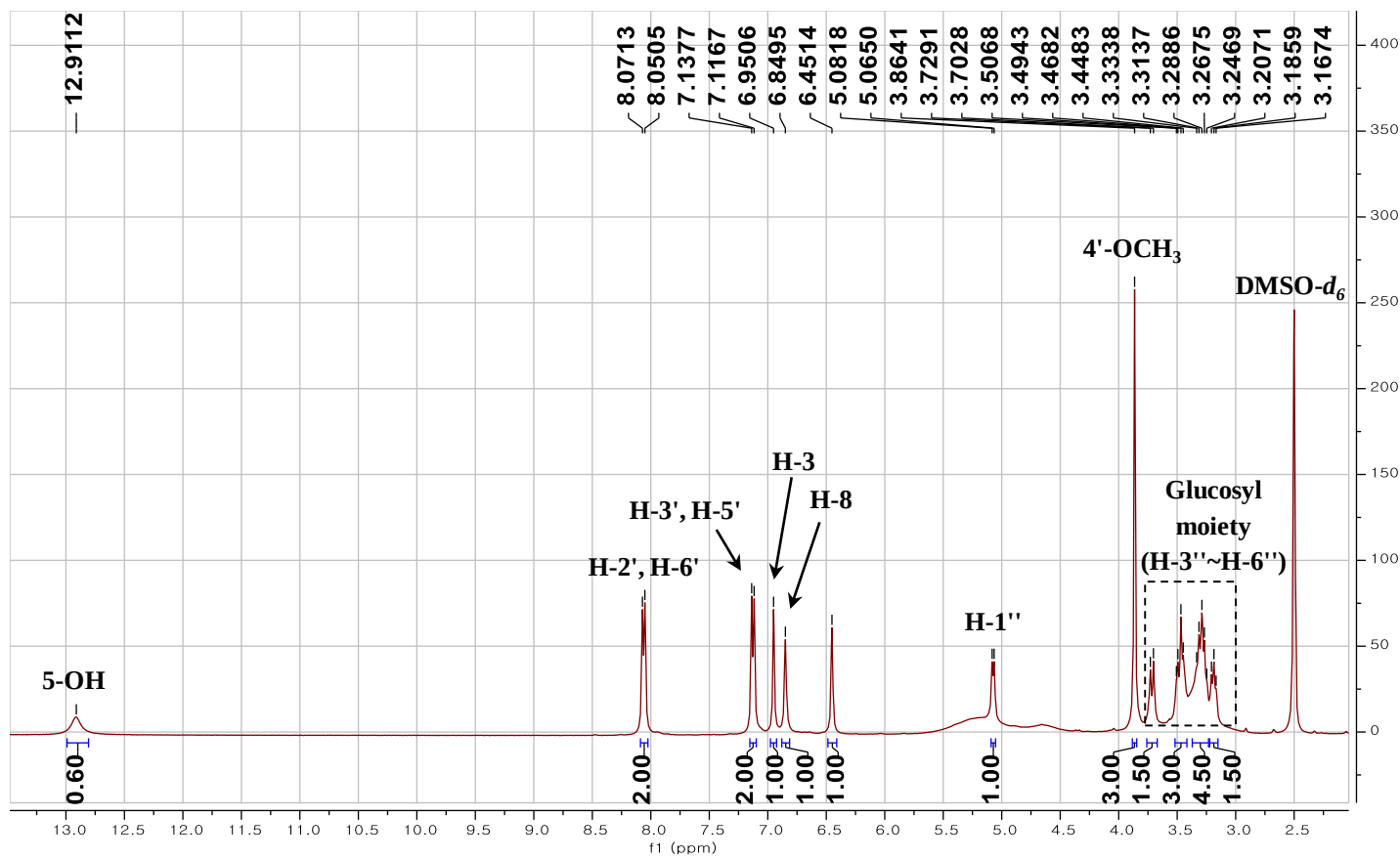


Figure S1

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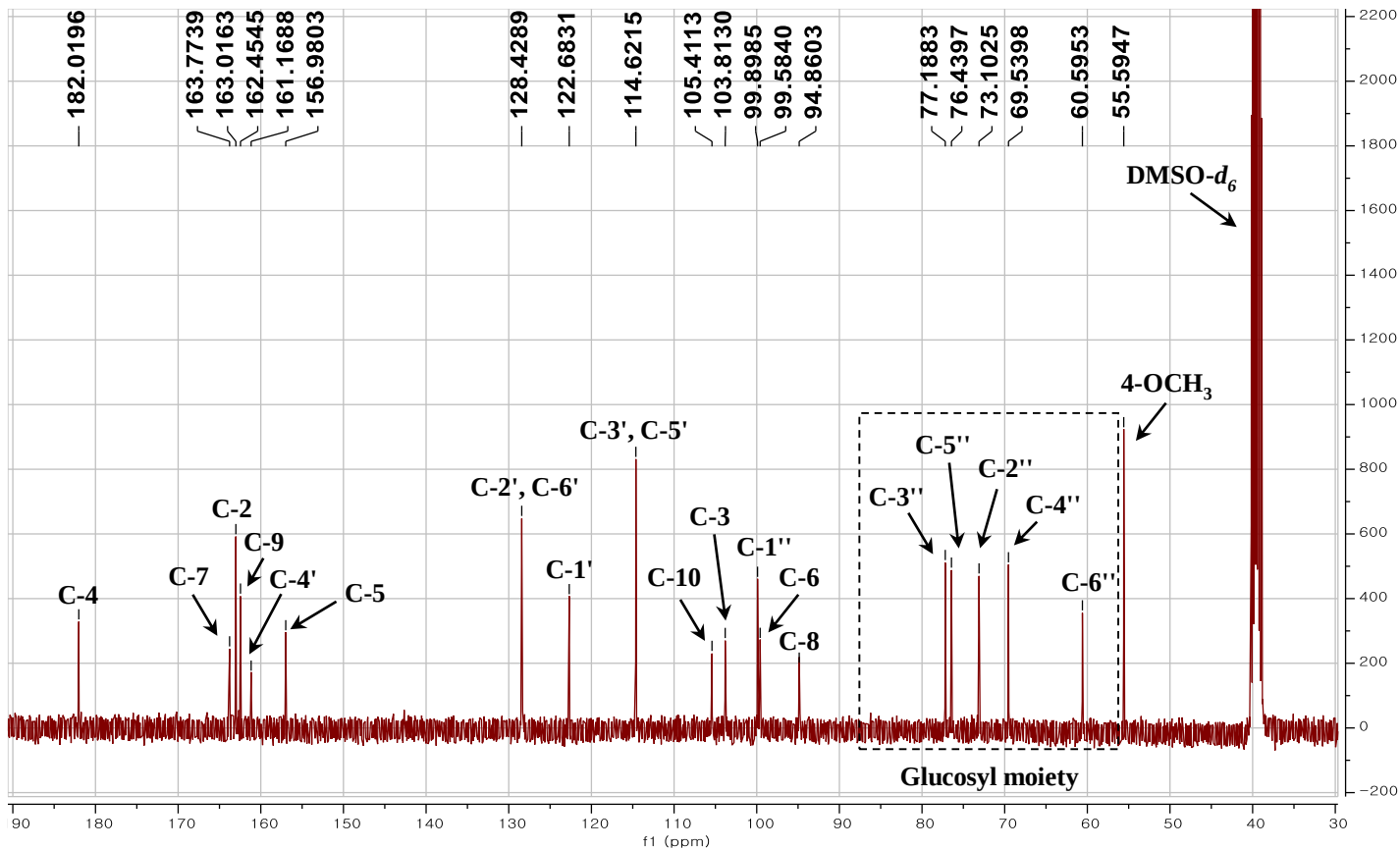


Figure S1

E

① Instrument

| Instrument |                                               |
|------------|-----------------------------------------------|
| LC-MS      | [Waters] AQUITY™ Ultra Performance LC-QTOF-MS |

② Solvents

| Solvent   |                      |
|-----------|----------------------|
| Solvent A | 0.1% Formic acid/DW  |
| Solvent B | 0.1% Formic acid/CAN |

④ Column

ACQUITY UPLC® BEH C18 1.7µm 2.1X100mm Column

⑤ Ion Mode

ESI+

⑥ Absorbance

UV : Max Plot

⑦ Injection Volume

2 µl

③ Method - Gradient

| Time(min) | Flow (mL/min) | %A | %B |
|-----------|---------------|----|----|
| (Initial) | 0.400         | 80 | 20 |
| 1.00      | 0.400         | 80 | 20 |
| 7.00      | 0.400         | 50 | 50 |
| 8.50      | 0.400         | 2  | 98 |
| 9.00      | 0.400         | 2  | 98 |
| 9.20      | 0.400         | 80 | 20 |
| 11.00     | 0.400         | 80 | 20 |

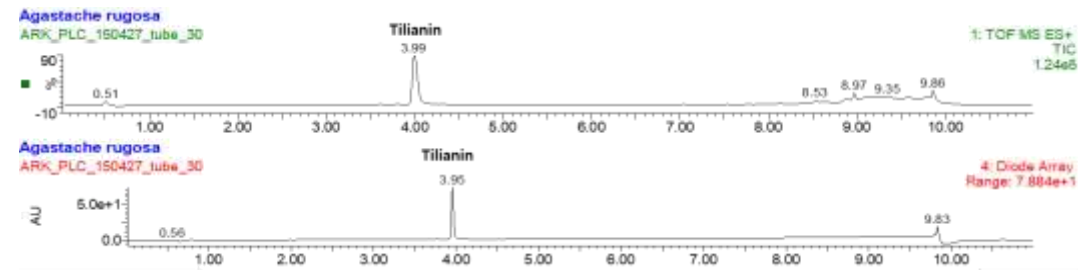


Figure S1

F

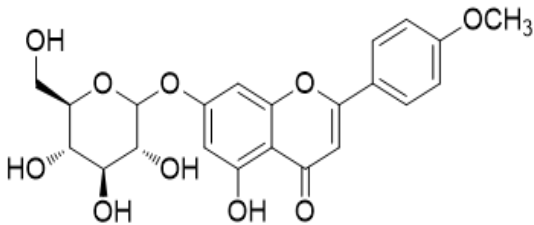
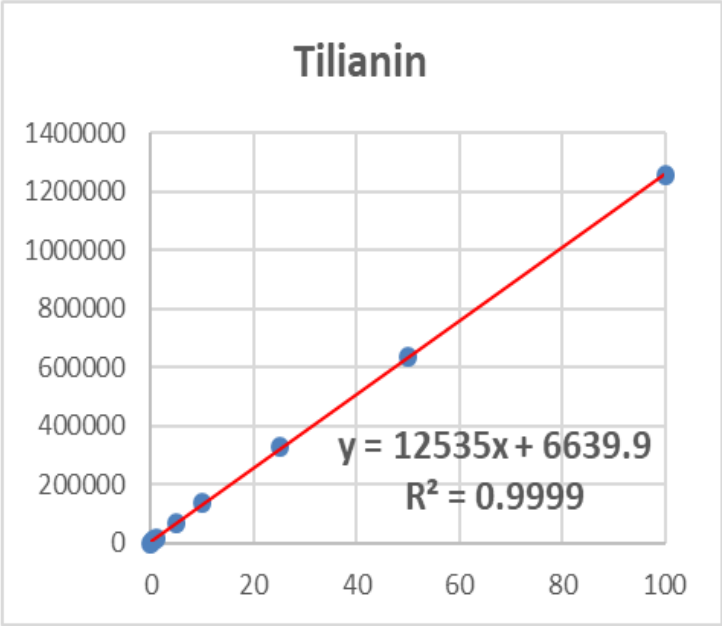
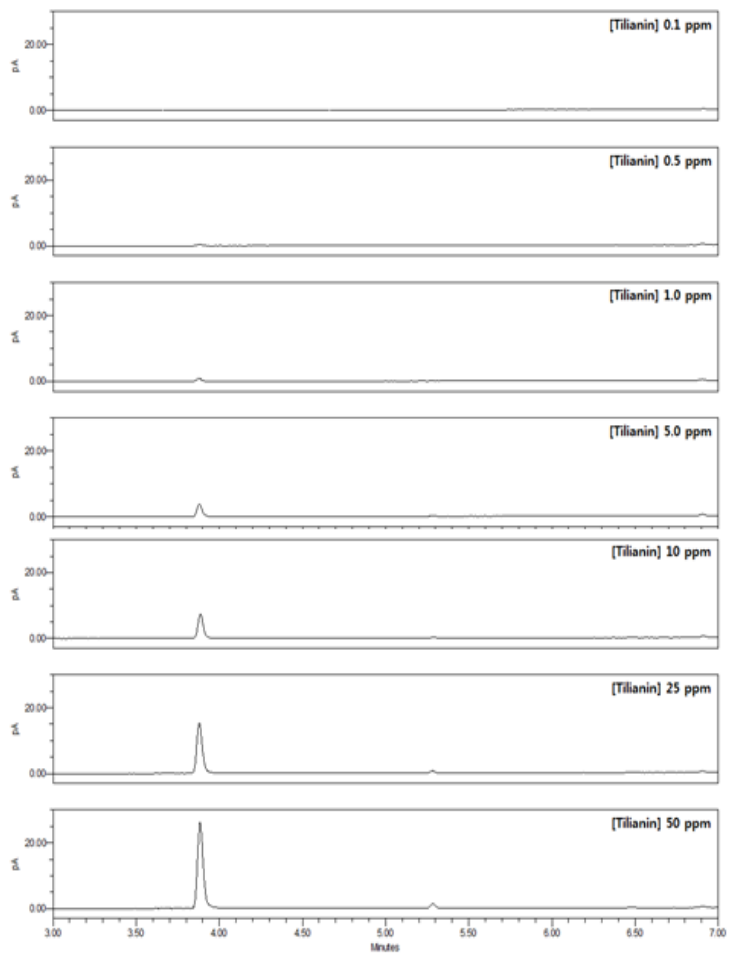
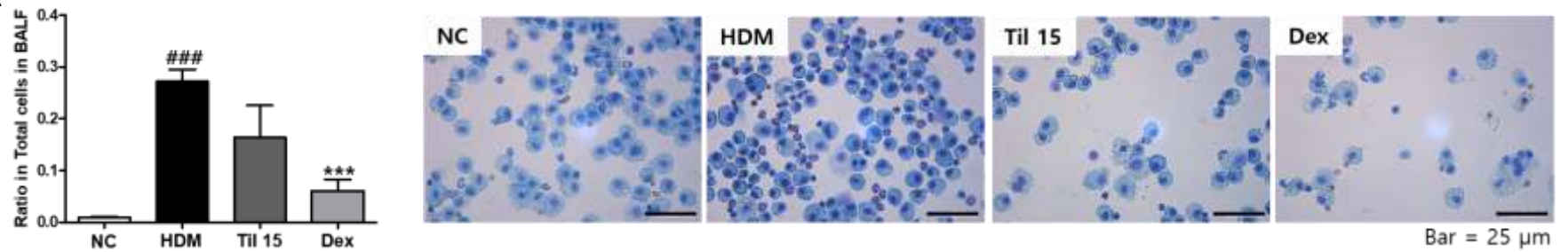
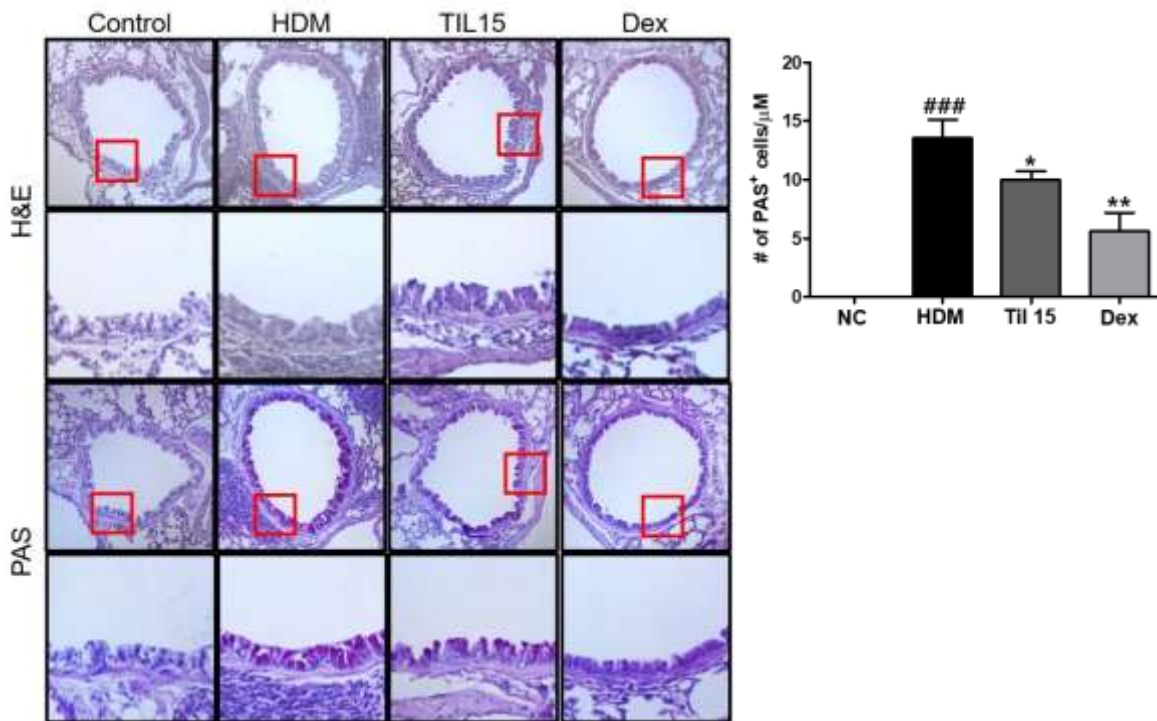


Figure S2

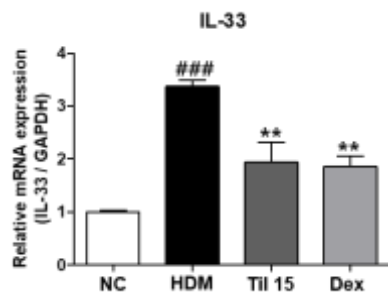
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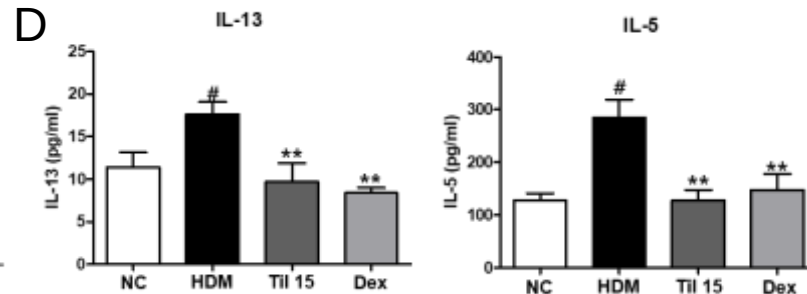
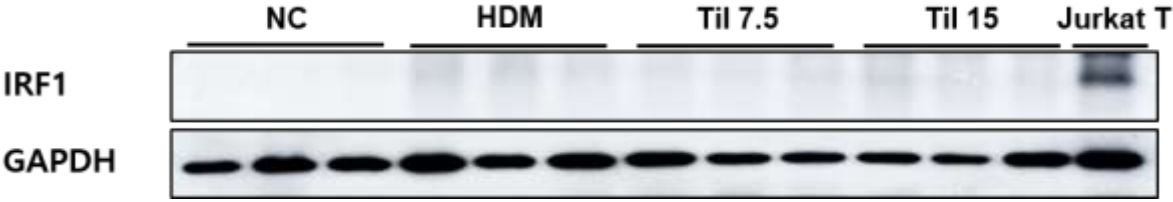


Figure S3



## Supplementary figure captions

### Figure S1. Molecular structure and purity of tilianin extracted from *A. rugosa*.

(A) Structure and HMBC Structure and HMBC ( $\rightarrow$ ) correlation of tilianin. (B) ESIMS and HRESIMS data of tilianin. (C)  $^1\text{H}$ -NMR spectrum of tilianin (400 MHz,  $\text{DMSO}-d_6$ ). (D)  $^{13}\text{C}$ -NMR spectrum of tilianin (100 MHz,  $\text{DMSO}-d_6$ ). (E) UPLC-PDA-QToF-MS chromatogram of isolated tilianin using prep-HPLC from *A. rugosa* extracts. (F) Calibration curves of isolated tilianin from *A. rugosa* based on UPLC-PDA at UV 254 nm.

### Figure S2. Comparison of the effects of tilianin and dexamethasone on HDM-induced allergic asthma.

(A) Ratio of eosinophils and representative pictures showing cell types in BALF of HDM induced allergic asthma treated with either tilianin or dexamethasone. (B) Representative pictures of H&E or PAS stained lung tissue sections in HDM-induced allergic asthma treated with either tilianin or dexamethasone. Lower panel of each stained section shows magnified image highlighted by the red color square of upper image (Left panel). The number of goblet cells in epithelial layer normalized by length of circumference of airway (Right panel). (C) The expression of IL-33 in the lung tissues measure by quantitative RT-PCR. (D) Protein levels of IL-13 and IL-5 in lung tissues analyzed by ELISA.

**Figure S3. Effects of tilianin on the expression of IRF1 in lung tissues.** The protein levels of IRF1 in lung tissues were analyzed by western blot. NC; normal control mice treated with saline only, HDM; HDM-sensitized/challenged mice, Til 7.5 and Til 15; Tilianin (7.5 and 15 mg/kg) + HDM-sensitized/challenged mice. Representative results from three replicated experiments are shown.