



Biflavonoid-rich fraction from *Daphne pseudomezereum* var. *koreana* Hamaya exerts anti-inflammatory effect in an experimental animal model of allergic asthma

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ABSTRACT

Ethnopharmacological relevance: *Daphne pseudomezereum* var. *koreana* Hamaya is distributed in the Gangwon-do of South Korea and is traditionally used to treat chronic inflammatory diseases, including rheumatoid arthritis.

Aim of the study: We investigated the anti-inflammatory effect of biflavonoid-rich fraction (BF) obtained from an extract of *D. pseudomezereum* leaves on lipopolysaccharide (LPS)-stimulated RAW264.7 macrophages and mouse model of ovalbumin (OVA)-induced allergic asthma.

Materials and methods: Neochamaejasmin B (NB) and chamaejasmin D (CD) were spectroscopically characterized as major components of BF obtained from the leaves of *D. pseudomezereum*. RAW264.7 cells pretreated with NB, CD and BF and activated by LPS (500 ng/ml) were used to assess the anti-inflammatory effects of these materials *in vitro*. To evaluate the protective effect of BF on allergic asthma, female BALB/c mice were sensitized to OVA by intraperitoneal (i.p.) injection and treated with BF by oral administration (15 or 30 mg/kg).

Results: Pretreatment with BF inhibited LPS-stimulated nitric oxide (NO), TNF- α and IL-6, and led to upregulation of heme oxygenase-1 (HO-1) in RAW264.7 macrophages. Orally administered BF significantly inhibited the recruitment of eosinophils and the production of IL-5, IL-6, IL-13 and MCP-1 as judged by the analysis of BALF from OVA-induced asthma animal model. BF also decreased the levels of IgE in the serum of asthmatic mice. BF suppressed the influx of inflammatory cells into nearby airways and the hypersecretion of mucus by the airway epithelium of asthmatic mice. In addition, the increase in Penh in asthmatic mice was reduced by BF administration. Furthermore, BF led to Nrf2 activation and HO-1 induction in the lungs of mice.

Conclusions: These data have shown the anti-asthmatic effects of BF, and therefore we expect that BF may be a potential candidate as a natural drug/nutraceutical for the prevention and treatment of allergic asthma.

1. Introduction

Allergic asthma is one of the most common chronic respiratory diseases in the world and occurs in both adults and children (Morel et al., 2016). The major pathophysiological characteristics of this disease are

airway inflammation and mucus hypersecretion (Liang et al., 2016; Zhou et al., 2016). Macrophage-derived tumor necrosis factor- α (TNF- α) leads to the recruitment of eosinophils in the lung, and eosinophilic airway inflammation is recognized as a typical feature of allergic asthma (Kim et al., 2012; Thomas, 2001). Th2 cell-derived interleukin-5 (IL-5)

Abbreviations: OVA, ovalbumin; NO, nitric oxide; TNF- α , tumor necrosis factor- α ; IL-5, interleukin-5; IL-6, interleukin-6; IL-13, interleukin-13; IgE, immunoglobulin E; BALF, bronchoalveolar lavage fluid; MCP-1, monocyte chemoattractant protein-1; HO-1, heme oxygenase-1; Nrf2, nuclear factor E2-related factor 2.

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and IL-13 have been shown to play a central role in the pathogenesis of allergic asthma (Corren, 2011; Gao et al., 2016). IL-6 is produced by primary lung epithelial cells and macrophages in response to various stimuli, including allergens (Rincon and Irvin, 2012). Immunoglobulin E (IgE) triggers inflammation (Huang et al., 2014), and mucus overproduction is a major cause of airway obstruction in allergic asthma (Tiwari et al., 2014). Antioxidant protein heme oxygenase-1 (HO-1) can ameliorate the symptoms of allergic asthma (Qian et al., 2017; Ye et al., 2017). Thus, the regulation of inflammatory molecules and mucus production is important in allergic asthma, and HO-1 induction could have beneficial effects in allergic asthma.

Anti-asthmatic synthetic drugs are used to treat or prevent allergic asthma, but these drugs have adverse systemic side effects (Arora et al., 2017). Therefore, it is necessary to circumvent these problems by reducing the side effects and increasing the therapeutic effectiveness of these drugs. Natural compounds have been reported to have multiple biological activities, including antioxidant and anti-inflammatory properties (Rosa et al., 2017; Ueda et al., 2015). *Daphne pseudomezereum* var. *koreana* Hamaya (DP), a shrub of the family Thymelaeaceae, has been collected for the conservation and utilization of plant resources at the National Institute of Biological Resources (NIBR) in Korea [https://species.nibr.go.kr/index.do, accessed 06.01.17]. This plant has been used as a traditional remedy in China and Japan to treat chronic inflammatory disorders such as chronic skin diseases, rheumatoid arthritis, gonorrhea, cutaneous afflictions, aches, rheumatism, high blood pressure, and malaria (Konishi et al., 1993; Xu et al., 2011). Thus, its safety is well-established (Konishi et al., 1993); however, the biological effects of the aerial portion of the plants, which contains bioflavonoids, have not yet been determined. Recently, the development of raw material from *D. pseudomezereum* for use as a health supplement in Korea has increased because of its anti-chronic inflammation properties (Oh et al., 2016). Therefore, we investigated the effects of a biflavonoid-rich fraction (BF) obtained from extracts of *D. pseudomezereum* on lipopolysaccharide (LPS)-stimulated RAW264.7 macrophages. We confirmed that BF inhibits the LPS-induced production of nitric oxide (NO), tumor necrosis factor- α (TNF- α), and interleukin-6 (IL-6). BF also significantly upregulated the expression of HO-1 in RAW264.7 cells in a concentration-dependent manner. Based on previous results (Kim et al., 2012; Thomas, 2001) and on our *in vitro* results that demonstrate the anti-inflammatory and antioxidant properties of BF, we evaluated the protective effects of BF against OVA-induced airway inflammation and mucus secretion in mice.

2. Material and methods

2.1. Chemicals and materials

Lipopolysaccharide (*Salmonella typhimurium*) and dexamethasone were purchased from Sigma-Aldrich (St. Louis, MO, USA). RAW264.7 cells, a mouse macrophage cell line, were purchased from the American Type Culture Collection (TIB-71; ATCC, Manassas, VA, USA) and cultured in Dulbecco's modified Eagle's medium (DMEM; Welgene, Daegu, South Korea) containing 10% heat-inactivated fetal bovine serum (FBS), 100 units/ml penicillin and 100 mg/ml streptomycin (Gibco BRL, Grand Island, NY, USA) at 37 °C in a humidified atmosphere containing 5% CO₂.

2.2. Plant materials and sample preparation

Mass cultivated aerial parts of *Daphne pseudomezereum* var. *koreana* Hamaya (Thymelaeaceae) were collected at Bongyang-ri (Jeongseon landscape architecture farm) in Jeongseon-eup, Republic of Korea, in June 2016 and positively identified by Dr. Jin Seok Kim of the National Institute of Biological Resources (NIBR). After harvesting, the leaves were immediately freeze-dried and stored at -40 °C until analysis. A standard specimen (NIBRVP0000563267, NIBRVP0000600759) was

deposited at the collections preservation facilities of the NIBR in Incheon, Korea.

2.3. Extraction and isolation

Leaves of *D. pseudomezereum* were washed with clean sterile H₂O, cut into small pieces, and mixed with 3 vol of methanol. The mixture was shaken three times in a sonicator for a total of 1 h, and the solvent was evaporated under reduced pressure to yield the crude extract (16.0 g, 10.5%). This total extract (2.0 g) was subjected to medium pressure liquid chromatography (MPLC, Sepbox 2D-5000, Sepiatec, Berlin, Germany) using a reversed-phase silica gel column (Cosmosil 75C18-PERP, 220 g) and a stepwise MeOH-H₂O gradient (10–100% methanol, 30 mL/min, 150 min), yielding 4 fractions (DP Fr.1–4) (Supplementary data, Fig. S1). DP Fr.4 (218.6 mg), a biflavonoid-rich fraction mainly composed of compounds 3 and 4, was chromatographed on an INNO C18 column (20.0 × 250 mm) using a gradient of H₂O-CH₃OH (50%→60%, 50 min) to yield compounds 3 (54.8 mg) and 4 (65.8 mg).

2.3.1. Neochamaejasmin B (3)

Brown amorphous powder; $[\alpha]_D^{20}$ +232.7 (c 0.1, CH₃OH); ECD (MeOH, c 1.8×10^{-3} M) $\lambda_{\max}$ ($\Delta\epsilon$) 307 (+16.40), 281 (−8.15), 249 (+1.55), 233 (−3.19), 221(+2.93) nm; ¹H NMR (CD₃OD) δ 3.14 (1 H, m, H-3), 3.28 (1 H, dd, *J* = 8.9, 3.5 Hz, H-3''), 5.14 (1 H, d, *J* = 8.9 Hz, H-2''), 5.54 (1 H, d, *J* = 4.7 Hz, H-2), 5.75 (1 H, br s, H-5), 5.78 (1 H, br s, H-6''), 5.87 (1 H, br s, H-8), 5.98 (1 H, br s, H-8''), 6.65 (2 H, d, *J* = 8.3 Hz, H-3', 5'), 6.79 (2 H, d, *J* = 8.3 Hz, H-3'', 5''), 6.92 (2 H, d, *J* = 8.3 Hz, H-2', 6'), 7.14 (2H, d, *J* = 8.3 Hz, H-2'', 6''), ¹³C NMR (CD₃OD) δ 49.6 (C-3), 50.7 (C-3''), 81.4 (C-2), 83.2 (C-2''), 96.0 (C-8), 96.4 (C-8''), 97.0 (C-6), 97.3 (C-6''), 103.8 (C-10), 105.0 (C-10''), 116.1 (C-3', 5'), 116.4 (C-3'', 5''), 128.5 (C-2', 6'), 128.7 (C-1'), 129.0 (C-1''), 130.2 (C-2'', 6''), 158.5 (C-4'), 158.9 (C-4''), 163.3 (C-9), 165.1 (C-5, 9''), 165.4 (C-5''), 168.2 (C-7), 168.4 (C-7''), 196.1 (C-4), 198.5 (C-4''); HRESIMS (negative) *m/z* 541.1157 [M-H][−], calcd for C₃₀H₂₁O₁₀, 541.1135.

2.3.2. Chamaejasmin D (4)

Brown amorphous powder; $[\alpha]_D^{20}$ −14.7 (c 0.1, CH₃OH); ECD (MeOH, c 1.8×10^{-3} M) $\lambda_{\max}$ ($\Delta\epsilon$) 311 (−0.51), 295 (+0.19), 249 (+1.55), 257 (+0.13), 223(−0.98) nm; ¹H NMR (CD₃OD) δ 3.31 (1 H, m, H-3), 5.75 (4 H, d, *J* = 8.4 Hz, H-6, 6'', 8, 8''), 5.79 (1H, d, *J* = 12.0 Hz, H-2, 2''), 6.80 (4H, d, *J* = 8.4 Hz, H-3', 5', 3'', 5''), 7.03 (4H, *J* = 8.4 Hz, H-2', 6', 2'', 6''), ¹³C NMR (CD₃OD) δ 49.0 (C-3, 3''), 82.4 (C-2, 2''), 96.2 (C-6, 6'', 8, 8''), 102.7 (C-10, 10''), 116.5 (C-3', 5', 3'', 5''), 129.0 (C-1', 1''), 130.7 (C-2', 6', 2'', 6''), 159.6 (C-4', 4''), 164.3 (C-9, 9''), 165.4 (C-5, 5''), 168.4 (C-7, 7''), 196.8 (C-4, 4''); HRESIMS (negative) *m/z* 541.1144 [M-H][−], calcd for C₃₀H₂₁O₁₀, 541.1135.

2.4. Cell viability assay

RAW264.7 macrophages were seeded in 96-well plates at a density of 1×10^4 cells/well and incubated with BF at various concentrations (2.5, 5, 10 and 20 μ g/ml) for 24 h at 37 °C and 5% CO₂. Cell viability was determined by an MTT assay as described previously (Lee et al., 2018). The absorbance was determined at a wavelength of 570 nm using a Spark™ 10 M multimode microplate reader (Tecan System Inc., CA, USA).

2.5. Nitric oxide assay and cytokine assay

The level of nitrite in the culture medium was examined using Griess reagent. Briefly, Raw264.7 cells were pretreated with neochamaejasmin B (NB), chamaejasmin D (CD) or BF (each, 10 and 20 μ g/ml) for 1 h prior to incubation with LPS (0.5 μ g/ml) for 20 h. After the incubation, 100 μ l of cell culture medium was mixed with an equal volume of Griess reagent [1% sulfanilamide in 5% phosphoric acid and 0.1% N-(1-naphthyl)-ethylenediamine dihydrochloride], and the mixture was

incubated at room temperature (RT) for 10 min. The concentration of nitrite in the mixture was then determined by measuring its absorbance at 540 nm in a microwell plate reader with sodium nitrite as the standard. The levels of IL-6 and TNF- α in the culture medium were measured using an ELISA kit (mouse IL-6 or mouse TNF- α ELISA kit, BD Biosciences, CA, USA) according to the manufacturer's protocol.

2.6. Mouse model with OVA-induced airway inflammation and mucus overproduction

Specific pathogen-free (SPF) female BALB/c mice were purchased from the Koatech Laboratory Animal Center (Pyeongtaek-shi, Gyeonggi-do, Korea). The induction of airway inflammation and the doses and duration of treatment with BF were performed by modifying the previous conditions [Lee et al., 2019; Park et al., 2019; Shin et al., 2019]. After 1 week of acclimatization, the mice were randomly divided into five groups ($n = 6$ in each group). The treatment groups included an NC group (normal control group, phosphate-buffered saline sensitization/challenge), an OVA group (ovalbumin group, OVA sensitization/challenge), a DEX group (OVA sensitization/challenge + oral gavage of 1 mg/kg dexamethasone), a BF 15 group (OVA sensitization/challenge + oral gavage of 15 mg/kg BF), and a BF 30 group (OVA sensitization/challenge + oral gavage of 30 mg/kg BF). On days 0 and 14, the mice were sensitized by intraperitoneal injection of 30 μ g ovalbumin (OVA; Sigma-Aldrich, A5503) and 3 mg aluminum hydroxide (Thermo Scientific, Rockford, IL, USA, #77161) in phosphate-buffered saline (PBS) (200 μ l). Between days 18 and 23, the mice were treated with DEX or BF by oral gavage. On days 21, 22 and 23, the mice were challenged by exposure to an OVA aerosol (1%, w/v, in PBS) for 1 h using an ultrasonic nebulizer (NE-U12; OMRON Corp., Tokyo, Japan). On day 24, enhanced pause (Penh) was assessed using whole body plethysmography (OCP3000, Allmedicus, Korea). DEX was used as a positive control. The animal experiments performed in this study were approved by the Institutional Animal Care and Use Committee of the Korea Research Institute of Bioscience and Biotechnology and performed in compliance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals and Korean national laws regarding animal welfare.

2.7. BALF collection and inflammatory cell counting

To evaluate the numbers of inflammatory cells and the levels of inflammatory cytokines in the animals, collection of bronchoalveolar lavage fluid (BALF) was performed as previously described (Lee et al., 2018). Briefly, tracheal cannulation was performed twice with ice-cold PBS (700 μ l) (total volume, 1.4 ml per mouse). The collected BALF (100 μ l) was centrifuged onto slides for 5 min at 1000 rpm using a Cytospin (Hanil Science Industrial, Seoul, Korea). The slides were then dried at room temperature, and the cells were fixed and stained with Diff-Quik® staining reagent (B4132-1A; IMEB Inc., Deerfield, IL, USA) according to the manufacturer's instructions.

2.8. Determination of cytokine and immunoglobulin E (IgE) levels

The levels of IL-5, IL-6 and IL-13 in BALF were determined using ELISA kits (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. The absorbance was measured at 450 nm using a microplate reader. The blood samples were centrifuged at 3000 rpm for 10 min at 4 °C, and the levels of IgE in the serum were tested using an ELISA MAX™ Deluxe Set (Biolegend, cat no. 432404, San Diego, CA, USA) according to the manufacturer's guidelines.

2.9. Western blot analysis

Mouse lungs obtained 24 h after challenge with BF were homogenized and lysed in lysis buffer using a tissue homogenizer. The proteins in the lung tissue lysates were denatured, resolved on 10–12% SDS

polyacrylamide gels, and transferred to PVDF membranes (Millipore, Billerica, MA, USA). The membranes were blocked with 5% skim milk in TBST for 1 h at room temperature and incubated with specific antibodies against phospho (p)-Nrf2 (Abcam, ab76026, 1:1000), Nrf2 (Santa Cruz, sc-722, 1:1000), HO-1 (Thermo Scientific, PA5-27338, 1:1000), and β -actin (Cell Signaling Technology, 4967S, 1:2500) in 5% skim milk at 4 °C overnight. The membranes were then washed in TBST and incubated with Peroxidase-AffiniPure goat anti-mouse IgG or goat anti-rabbit IgG (1:2000; Jackson ImmunoResearch Laboratories) for 1 h at room temperature. The blots were washed 4 times with TBST and developed using an enhanced chemiluminescence (ECL) kit (Bio-Rad Laboratories Inc., #170-5061). Protein bands were visualized using a LAS-4000 luminescent image analyzer (Fujifilm, Tokyo, Japan).

2.10. Histopathological analysis

Forty-eight hours after the final oral administration of BF and DEX, the asthmatic mice were sacrificed, and their lung tissues were removed. For histological analysis, the lung tissues were fixed in 10% (v/v) neutral-buffered formalin, embedded in paraffin and sliced into 4- μ m sections. A solution of hematoxylin (BBC, Cat No. 3540) and Eosin Y (Thermo Scientific, Cat No. 6766007) (H&E) was used to evaluate the influx of inflammatory cells into the lung, and PAS staining was used to measure mucus production in the lung using a PAS staining kit (IMEB Inc., K7308). The stained lung tissues were visualized using a light microscope to evaluate the degree of inflammatory cell influx (magnification, x100; scale bar 50 μ m) and mucus secretion (magnification, x200; scale bar 100 μ m). The degrees of inflammatory cell influx and mucus secretion in each group were evaluated by two independent observers using a semi-quantitative scope. Inflammatory cell recruitment was evaluated based on H&E staining and was scored according to the following scale: 0, no inflammatory cell recruitment; 0.5, minor recruitment; 1, moderate recruitment; 2, extensive recruitment. Mucus production was evaluated based on PAS staining and was scored according to the following scale: 0, no mucus secretion; 0.5, mild mucus secretion; 1, moderate mucus secretion; 2, distinct mucus secretion; 3, severe mucus secretion.

2.11. Statistical analysis

All quantitative measures are expressed as the mean $\pm$ standard deviation of the values obtained in at least three independent experiments. Statistical significance was determined by a two-tailed Student's t-test for comparisons between two groups. One-way analysis of variance (ANOVA) followed by Dunnett's multiple post hoc test was performed for multiple comparisons. The data were analyzed using SPSS 20.0 (IBM Corp.). $P < 0.05$ was considered to indicate statistical significance.

3. Results

3.1. Identification of phenolic compounds

D. pseudomezereum samples were characterized using UPLC-PDA-QToF-MS with a linear gradient of acetonitrile/water. As shown in Fig. 1, complete chromatographic separation of the phytochemicals was achieved within 14 min using a photodiode array (PDA) detector and total ion current (TIC). The mass spectra of these metabolites, including the experimental m/z values, HRESIMS, molecular formulae, and MS/MS data, were carefully interpreted in the context of the previous literature on the family Thymelaeaceae (Supplementary material, Figs. S1–5) (Ko et al., 2013; Konishi et al., 1993; Wang et al., 2014, 2016). The major metabolites (1–4) are shown as their deprotonated ions in negative ion mode ($[M-H]^-$). According to the target molecular ion of BF (DP Fr.4), the major ions of compounds 3 ($t_R = 6.67$ min) and 4 ($t_R = 7.00$ min) included m/z 541.1157 (calcd. for $C_{30}H_{22}O_{10}$, 541.1135)

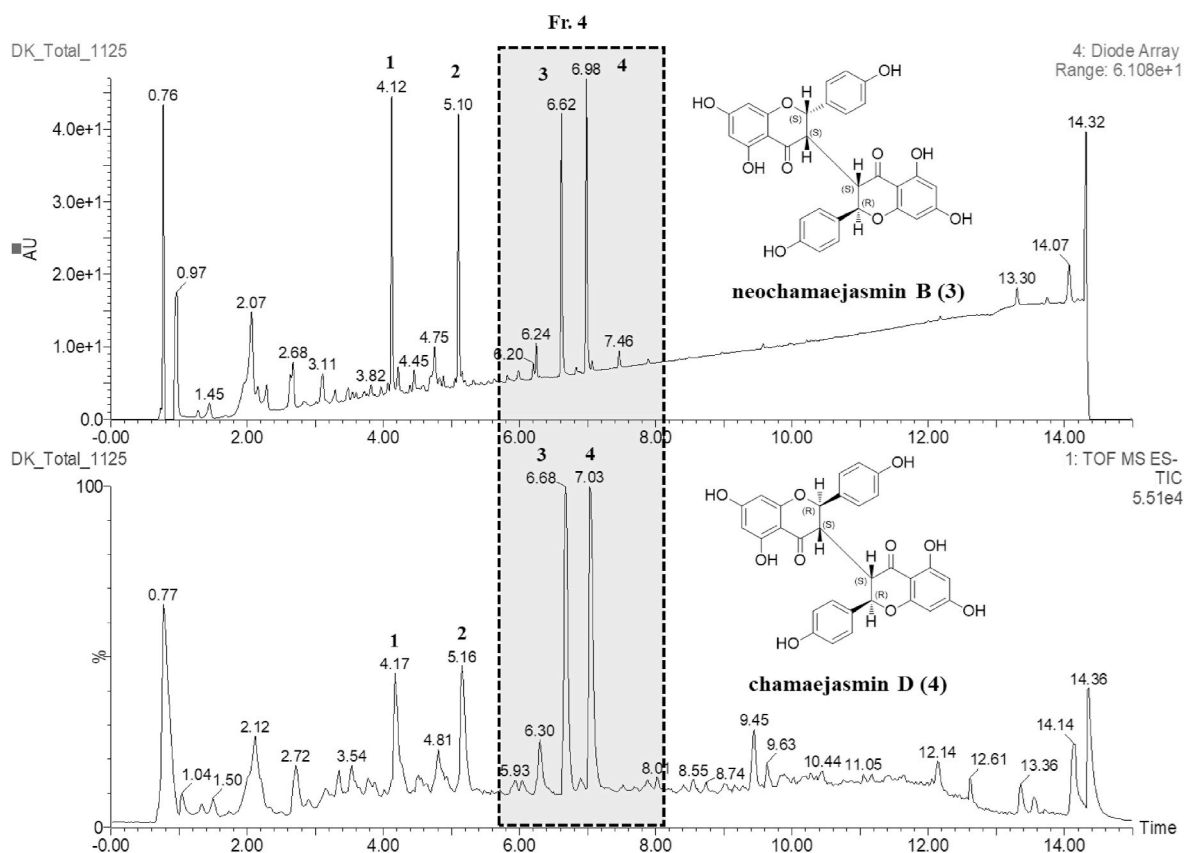


Fig. 1. UPLC-PDA-QToF-MS chromatograms with two active peaks (3 and 4) from the extract of aerial parts of *D. pseudomezereum* A. Gray.

and 541.1144 (calcd. for $C_{30}H_{22}O_{10}$, 514.1135), respectively, and analysis of their high-resolution data compared to the literature led to the assignments of these bioflavonoids. Biflavonoids 3 and 4 had a molecular ion $[M-H]^-$ at m/z 541 and produced MS/MS fragments at 497, 435, 415, 389, 371, and 309. Additionally, the deprotonated molecular ion of flavonoid-like compounds usually produces product ions through a retro-Diels-Alder (RDA) reduction in MS/MS (Ko et al., 2013; Konishi et al., 1993; Wang et al., 2016). Biflavonoids 3 and 4 easily yielded the MS/MS fragment ion m/z 497 $[M-H-CO_2]^-$ after loss of a neutral fragment of 44 Da, corresponding to the loss of a CO_2 moiety. The fragment ion m/z 435 $[^{0,4}B]^-$ was produced by loss of the M-B' ring- C_2 (106 Da) from m/z 541, and m/z 415 $[^{1,3}B]^-$ was produced by the loss of one phloroglucinol ($C_6H_6O_3$, 126 Da); m/z 389 $[^{1,3}B]^-$ was an ion with a B-ring from the first RDA fragmentation. The ^1H and ^{13}C NMR data, in combination with the molecular formulae of the two biflavonoids (3 and 4), indicated symmetry of a tricyclic skeleton with two aromatic rings (Supplementary material, Figs. S6–8). The stereochemistry at the C-2/C-3 and C-2''/C-3'' positions in 3 was determined as *cis-trans* by comparison of the J values ($J_{H-2} = 4.8$ Hz and $J_{H-2''} = 9.0$ Hz) with those of the known 3,3'-biflavanones. The relative configuration of 3 was assigned as identical to that of neochamaejasmin B (3) due to the similarity of their chemical shifts and of the coupling constants of all spectra (Feng et al., 2003). The presence of two carbonyls (δ_C 196.9) and C-2, C-2'' (δ_C 82.5) and C-3, C-3'' (δ_C 48.9) in the ^{13}C -NMR spectrum indicated that the structure of 4 was similar to that of the known C-3/C-3'' biflavanone chamaejasmin D (Wang et al., 2014), and the two compounds had the same planar structures. The similarity of their chemical shifts and coupling constants was confirmed, and their position was identified by COSY and HMBC correlations. Their relative *cis-trans* geometric configurations were determined based on ^1H -NMR coupling constants, and the absolute configurations of 3 (2S, 3S, 2''S, 3''R) and 4 (2R, 3S, 2''R, 3''S) were deduced from their CD spectra (Ferrari et al.,

2003; Wang et al., 2014). Finally, the spectra of compounds 3 and 4 contained ions at m/z 541, indicating that they correspond to neochamaejasmin B (3) and chamaejasmin D (4) based on comparison with the literature on Thymelaeaceae species (Ko et al., 2013; Konishi et al., 1993; Wang et al., 2014, 2016).

3.2. Effect of the biflavonoid fraction (BF) obtained from the extract of *D. pseudomezereum* leaves and its major compounds on LPS-stimulated RAW264.7 macrophages

To assess the anti-inflammatory effects of neochamaejasmin B (NB) and chamaejasmin D (CD), the major components of the biflavonoid fraction (BF) obtained from the leaves of *D. pseudomezereum*, NO assays were performed using cell culture supernatants. As shown in Fig. 2A, an obvious upregulation of nitrite levels was observed in RAW264.7 macrophages after treatment of the cells with 500 ng/ml LPS; this effect was significantly reduced by pretreatment of the cells with NB or CD. The inhibitory effect of BF was more potent than that of either individual compound (NB or CD) at 20 $\mu g/ml$ ($p < 0.05$). The inhibitory effects of BF, NB and CD on TNF- α and IL-6 generation by LPS-stimulated RAW264.7 cells were measured using ELISA assays. Treatment of the cells with NB or CD decreased the levels of TNF- α and IL-6, and BF suppressed the production of these cytokines to a greater degree than NB or CD alone (Fig. 2B & C). There was no significant reduction in cell viability at the concentrations used (data not shown). The results indicate that BF exhibited more potent inhibition than NB or CD alone, suggesting that BF containing NB and CD as major constituents can exert significant effects on the suppression of inflammatory molecules. In addition, the western blotting results indicated that BF upregulated the expression of HO-1 dose-dependently in RAW264.7 cells (Fig. 2D). Collectively, these results indicate that BF is a promising material with anti-asthmatic effects in a murine model.

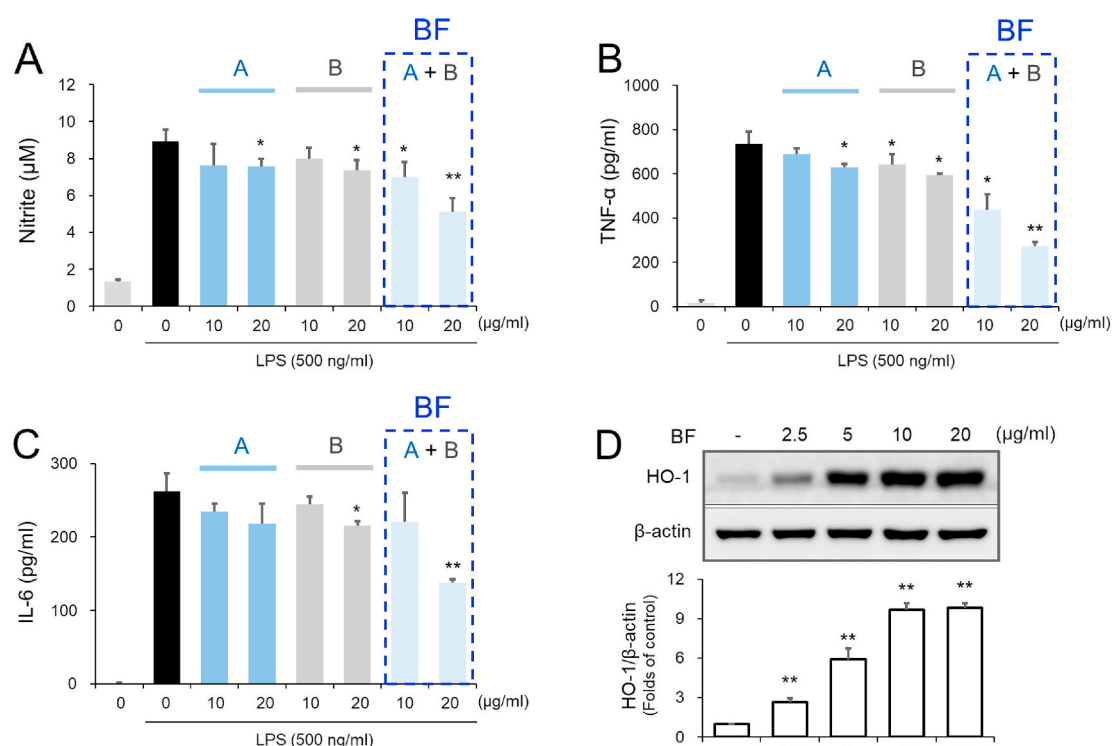


Fig. 2. A biflavonoid-rich fraction (BF) obtained from *D. pseudomezereum* A. Gray suppresses LPS-induced inflammation and upregulates HO-1 expression *in vitro*. (A–C) The production of NO, TNF-α and IL-6 in RAW264.7 macrophages was examined by NO and ELISA assays. (D) The protein level of HO-1 in cell lysates was detected by western blotting. Densitometry of the indicated immunoblot bands was performed using ImageJ software. The data are expressed as the mean ± standard deviation (S.D). * $p < 0.01$ vs. control group. * $p < 0.05$, ** $p < 0.01$ vs. LPS treatment alone. A: neochamaejasmin B, B: chamaejasmin D, BF: biflavonoid-rich fraction.

3.3. Effect of BF on eosinophil recruitment in OVA-induced asthmatic mice

To induce allergic asthma, intraperitoneal administration of ovalbumin (OVA) and OVA inhalation was conducted in BALB/c mice as shown in Fig. 3. The regulatory effects of BF on the recruitment of inflammatory cells were confirmed using Diff-Quick® staining. As shown in Fig. 4, OVA sensitization/inhalation markedly increased the numbers of eosinophils, macrophages and neutrophils in the BALF of mice compared with the NC group ($p < 0.01$) (Fig. 4A–C). However, treatment with BF dose-dependently decreased the numbers of these cells compared with the OVA group ($p < 0.05$). In the treated animals, the number of eosinophils was decreased by 54.12% (DEX), 39.22% (BF 15), and 50.20% (BF 30). The number of macrophages was decreased by 42.71% (DEX), 26.10% (BF 15), and 40.34% (BF 30), and the number of neutrophils was decreased by 47.79% (DEX), 29.41% (BF 15), and 49.26% (BF 30). In total, the inhibitory effects of 30 mg/kg of BF were

similar to those of 1 mg/kg DEX, which was used as a positive control.

3.4. Effect of BF on inflammatory cytokines and IgE in OVA-induced asthmatic mice

Next, the inhibitory effect of BF on IL-5, IL-6 and IL-13 was confirmed using ELISA assays. As shown in Fig. 5, elevated levels of IL-5, IL-6 and IL-13 were observed in the BALF of asthmatic mice, whereas decreased levels of these molecules were detected in the BF-treated group compared with the OVA group ($p < 0.05$) (Fig. 5A–C). Treatment of the animals with 30 mg/kg BF decreased the IL-5, IL-6 and IL-13 levels to 56.60%, 77.23%, and 78.50%, respectively, of the control levels. To evaluate the effect of BF on IgE, blood was collected from mice on day 25, and serum was isolated. As shown in Fig. 6, OVA-sensitization/inhalation led to marked upregulation of IgE generation. However, administration of 15 or 30 mg/kg BF greatly inhibited OVA-induced IgE production; treatment with BF at 30 mg/kg BF inhibited

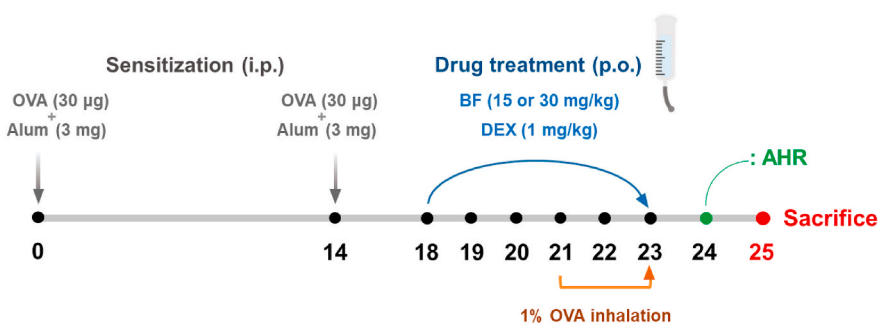


Fig. 3. Experimental procedure. Female BALB/c mice were divided into five groups ($n = 6$ in each group), sensitized by ovalbumin (OVA) and alum injection (i.p.) on days 0 and 14 and exposed to a 1% OVA aerosol on days 21–23. Oral administration of BF (15 or 30 mg/kg) or DEX (1 mg/kg) was performed on days 18–23. The level of AHR was measured on day 24. On day 25, the mice were sacrificed, and BALF, serum and lung tissues were collected.

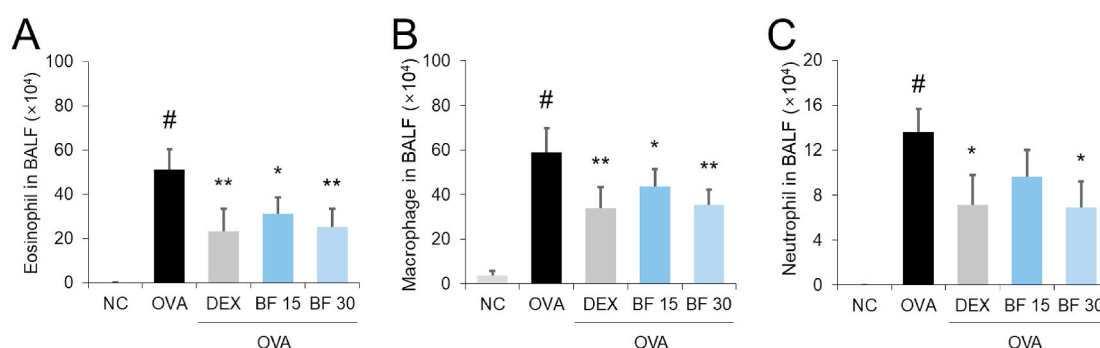


Fig. 4. BF reduces the recruitment of eosinophils in the BALF of OVA-induced asthmatic mice. (A–C) The numbers of eosinophils, macrophages and neutrophils were measured using Diff Quik® staining. The data are expressed as the mean $\pm$ standard deviation (S.D) of the values obtained for six mice in each group. [#] $p < 0.01$ vs. normal control group. ^{*} $p < 0.05$, ^{**} $p < 0.01$ vs. OVA treatment alone. NC: normal control, OVA: ovalbumin, DEX: dexamethasone.

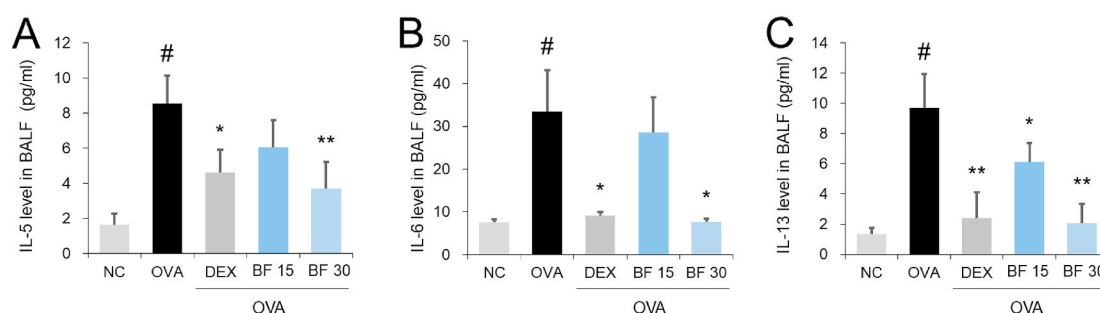


Fig. 5. BF decreases the levels of inflammatory cytokines in the BALF of OVA-induced asthmatic mice. (A–C) The levels of IL-5, IL-6, IL-13 were measured using ELISA assays. The data are expressed as the mean $\pm$ standard deviation (S.D) of the values measured in six mice in each group. [#] $p < 0.01$ vs. normal control group. ^{*} $p < 0.05$, ^{**} $p < 0.01$ vs. OVA treatment alone.

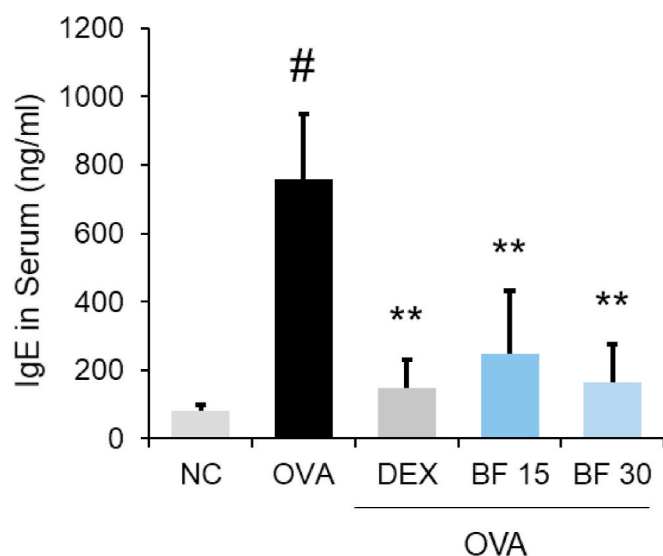


Fig. 6. BF reduces the levels of immunoglobulin E in the serum of OVA-induced asthmatic mice. The levels of IgE and histamine were measured using ELISA assays. The data are expressed as the mean $\pm$ standard deviation (S.D) of the values measured in six mice in each group. [#] $p < 0.01$ vs. normal control group. ^{*} $p < 0.05$, ^{**} $p < 0.01$ vs. OVA treatment alone.

IgE production by 79.0%.

3.5. Effect of BF on inflammatory cell influx, mucus secretion and AHR in OVA-induced asthmatic mice

In OVA-induced allergic asthma mouse models, influx of

inflammatory cells into the lung is a consistent pathological feature (Gurusamy et al., 2016; Kim et al., 2019). Therefore, we evaluated the inhibitory effect of BF on inflammatory cell recruitment. Our results showed that inhalation of OVA led to an increased influx of inflammatory cells into peribronchial lesions in the lung tissue compared with the NC group (Fig. 7A and C). However, treatment with BF effectively decreased this influx compared with the OVA group. BF also significantly reduced the OVA-induced increase in MCP-1 levels in BALF (Fig. 7D). To test whether BF affects OVA-induced mucus secretion, we assessed the amount of mucus in the airway using periodic acid Schiff (PAS) staining. The results showed that OVA inhalation markedly increased the production of mucus in the bronchial airways of the OVA group, and this level was reduced after BF administration (Fig. 7B and E). In particular, the inhibitory effects of 30 mg/kg BF were similar to those of 1 mg/kg DEX. To assess the inhibitory effect of BF on AHR, enhanced pause (Penh) was evaluated on day 24. As shown in Fig. 7F, aerosol inhalation of methacholine (MCH, 12.5 or 25 mg/ml) led to upregulation of Penh values, whereas administration of BF dose-dependently reduced them.

3.6. Effect of BF on the expression of HO-1 in the lung

In our *in vitro* experiments, BF caused upregulation of HO-1 expression (Fig. 2D). Based on this result and on the importance of HO-1 induction in the anti-asthmatic response, we next investigated whether BF administration affects HO-1 induction in the lungs of mice. The results of Western blot analysis showed that HO-1 expression was markedly increased in the BF group compared with the NC group. Because Nrf2 a transcription factor that controls the expression of genes that encode antioxidant enzymes and its activation leads to HO-1 induction (Ye et al., 2017), it was necessary to determine whether HO-1 induction was caused by Nrf2 activation. The results presented in Fig. 8 show that BF

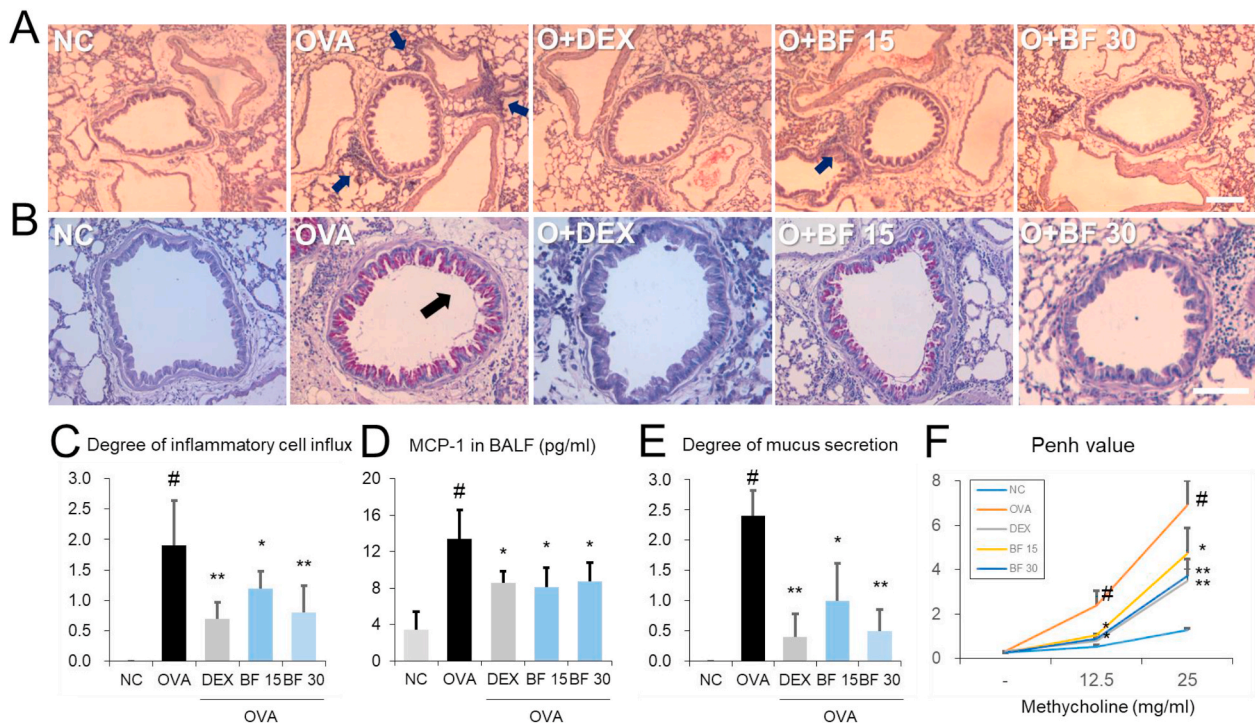


Fig. 7. BF attenuates the influx of inflammatory cells and the overproduction of mucus in the lung of OVA-induced asthmatic mice. (A) The influx of inflammatory cells was evaluated using an H&E staining kit. (B) The production of mucus was examined using a PAS staining kit. (C) Quantitative analysis of inflammatory cell influx. (D) The level of MCP-1 in the BALF was determined using an ELISA assay. (E) Quantitative analysis of mucus secretion. (F) Penh values were determined using whole body plethysmography. The data are expressed as the mean $\pm$ standard deviation (S.D) of the values measured in six mice in each group. [#] $p < 0.01$ vs. normal control group. ^{*} $p < 0.05$, ^{**} $p < 0.01$ vs. OVA treatment alone. Penh: enhanced pause.

upregulated the activation of Nrf2 in a concentration-dependent manner.

4. Discussion

Macrophages link the innate and adaptive immune responses that occur in allergic airway inflammation by expressing inflammatory molecules such as NO, TNF- α and IL-6 (Huang et al., 2017). NO plays an important role in allergic asthma by inducing an imbalance between the oxidant and antioxidant pathways, and increased levels of NO are also observed in asthmatic patients (Gao et al., 2016; Prado et al., 2011). TNF- α and IL-6 are pro-inflammatory cytokines that trigger the activation of NF- κ B and induce macrophage activation, pulmonary inflammation and mucus overproduction (Chun et al., 2018; Fujii et al., 2017; Liu et al., 2015). The concentrations of these molecules are specifically elevated in asthmatic patients compared with control subjects (Jiang and Zhu, 2016; Neveu et al., 2010; Rincon and Irvin, 2012). Accumulating evidence suggests that the antioxidant protein HO-1 is

indispensable for the promotion of anti-inflammatory activity and that it has protective effects in pulmonary inflammatory disease, including allergic asthma (Liu et al., 2015; Qian et al., 2017; Ye et al., 2017). Thus, inhibitory regulation of NO, TNF- α and IL-6 production and upregulation of HO-1 may play a role in the modulation of allergic asthma. Interestingly, in our *in vitro* study, it was found that BF not only exerts anti-inflammatory activity but also has antioxidant effects; thus, a protective effect of BF on airway inflammatory response was expected.

An increase in eosinophil infiltration in lung tissue and BALF is the major hallmark of patients with allergic asthma and animal models of OVA-induced allergic airway inflammation (Walsh et al., 2010). The recruitment of macrophages and neutrophils involves an increase in mucus hypersecretion and results in airway obstruction in the pathological process of allergic asthma (de Almeida et al., 2017; Lai and Rogers, 2010; Monteseirin, 2009; Ono et al., 2012). It is well known that IL-5 promotes eosinophil survival, differentiation and migration (Rincon and Irvin, 2012). Previous studies showed that the levels of IL-6 were increased in the bronchial epithelial cells of patients with asthma and in

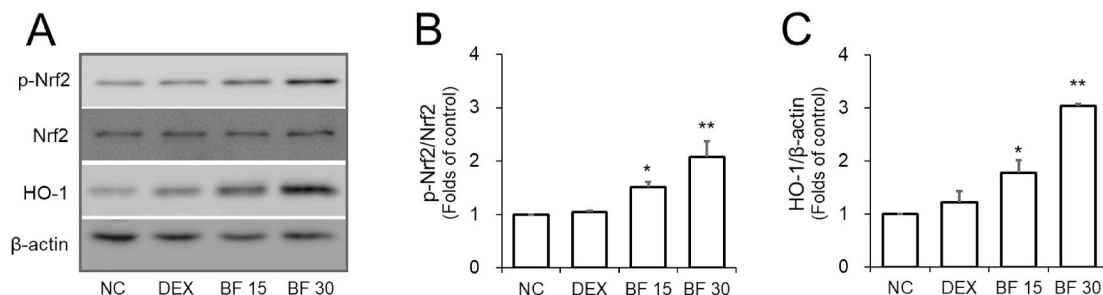


Fig. 8. BF upregulates the expression of HO-1 and induces the activation of Nrf2 in the lungs of mice. The levels of HO-1 expression and Nrf2 phosphorylation were determined using Western blot analysis. The data are expressed as the mean $\pm$ standard deviation (S.D) of the results obtained in six mice in each group. [#] $p < 0.01$ vs. normal control group. ^{*} $p < 0.05$, ^{**} $p < 0.01$ vs. OVA treatment alone. HO-1: heme oxygenase-1; Nrf2, Nuclear factor E2-related factor 2.

an allergic asthma model (Bao et al., 2017; Marini et al., 1992; Stadnyk, 1994). The high IL-6 level correlated with increased IL-13 levels in patients with asthma. (Rincon and Irvin, 2012). IL-13 leads to the production of IgE by B lymphocytes (Deo et al., 2010), and the binding of antigen-specific IgE to receptors on the surface of mast cells induces the production of inflammatory mediators, including histamine (Galli and Tsai, 2012), and contributes to airway hyperresponsiveness (AHR) and mucus production (Corren, 2013; Mayr et al., 2002). Furthermore, serum IgE levels are elevated in animal models of asthma (Kim et al., 2018; Xu et al., 2018). Corren reported that IL-5 and IL-13 antagonist therapy may be efficacious and useful in treating patients with severe asthma by controlling persistent IL-5 and IL-13 activity (Corren, 2011, 2013). Interestingly, improvements in allergic airway inflammation were accompanied by a reduction in the levels of these cytokines in asthmatic animal models (de Almeida et al., 2017). In this study, BF effectively reduced the levels of IL-5, IL-6, IL-13 and IgE, which are important markers and the major therapeutic targets in allergic asthma. These inhibitory effects were similar to those reported in previous studies [Lee et al., 2019; Shin et al., 2019]. Thus, these results suggest that BF has a regulatory action that protects against airway inflammation induced by OVA.

Mucus produced by the airway epithelium acts as a protective barrier against inhaled foreign irritants. However, excessive mucus makes breathing difficult and leads to airway remodeling and AHR (Lai and Rogers, 2010; Lee et al., 2019). Hence, regulation of mucus overproduction may improve breathing problems and lung function. Previous studies reported that IL-13 is closely associated with mucus hypersecretion and AHR (Corren, 2011; Gao et al., 2016), and, in the present study, we confirmed the regulatory effect of BF on IL-13 production; therefore, BF was expected to exert a protective effect against mucus hypersecretion and AHR. In the present study, BF inhibited OVA-induced mucus secretion and AHR. These results suggest that BF effectively controls the overproduction of mucus and decreases AHR, two of the major characteristics of allergic asthma, indicating that BF may have the potential to reduce asthmatic symptoms.

The level of MCP-1 is closely related to the increase in airway inflammatory response because this compound induces the recruitment of inflammatory cells (Lee et al., 2014; Yuk et al., 2017). BF effectively reduced inflammatory cell influx into the nearby airway, and the levels of MCP-1 were clearly reduced by BF administration. These results imply that the inhibitory effect of BF on MCP-1 levels may lead to inhibition of inflammatory cell influx, suggesting that BF may be useful as a therapeutic adjuvant in allergic asthma.

As mentioned earlier, the targeting of eosinophil recruitment and inflammatory cytokines has been studied for its therapeutic potential in treating airway inflammatory responses. Accumulating evidence has already shown that HO-1 has biological anti-inflammatory effects and that its induction can ameliorate airway inflammation by attenuating inflammatory cell influx and the production of inflammatory molecules (Liu et al., 2016; Xu et al., 2015). It is also well known that attenuation of mucus overproduction and NF- κ B activation are accompanied by HO-1 induction (Liu et al., 2015; Ye et al., 2017). Thus, strategies to increase HO-1 induction appear to be very important in the treatment of allergic asthma. We confirmed that BF led to HO-1 induction in RAW264.7 cells. After confirming that BF administration reduced inflammatory cell influx and the levels of inflammatory molecules and mucus overproduction, we focused on the effect of BF in HO-1 induction because of its protective effects against airway inflammation and mucus overproduction. In this study, oral administration of BF led to increased HO-1 expression in the lungs of mice. Thus, we expect that the observed protective effects of BF on airway inflammation are, in part, related to HO-1 induction.

5. Conclusion

From the major constituents (3 and 4) of BF obtained from the leaves

of *D. pseudomezereum*, we isolated one rare compound (4, chamaejasmin D) and showed that it possesses the 2R, 3S, 2''R, 3''S motif, along with its isomer (3, neochamaejasmin B). BF has an inhibitory effect on the production of macrophage-derived inflammatory molecules by RAW264.7 macrophages. BF also significantly attenuates the recruitment of inflammatory cells, including eosinophils, as well as the levels of IL-5, IL-6, IL-13 and IgE and the overproduction of mucus in a murine model. These effects of BF are accompanied by HO-1 induction. Collectively, these abilities of BF suggest that it may be suitable for the prevention and treatment of allergic asthma. To the best of our knowledge, this is the first report showing that BF has anti-inflammatory effects and that it exerts protective effects on OVA-induced airway inflammation. However, additional mechanistic studies are needed to advance the current knowledge regarding the effects of BF.

5.1. Declaration of competing interests

The authors declare that there are no conflicts of interest.

CRediT authorship contribution statement

Jae-Won Lee: Conceptualization, writing-original draft, preparation. **Hyung Won Ryu:** Conceptualization, writing-original draft, preparation. **Doo-young Kim:** performed the experiments, Formal analysis. **Ok-Kyoung Kwon:** performed the experiments, Formal analysis. **Hyun-Jae Jang:** performed the experiments, Formal analysis. **Hyuk Joon Kwon:** performed the experiments, Formal analysis. **Soo-Young Kim:** performed the experiments, Formal analysis. **Su Ui Lee:** performed the experiments, Formal analysis. **Sung-Man Kim:** performed the experiments, Formal analysis. **Eun Sol Oh:** performed the experiments, Formal analysis. **Hye In Ahn:** performed the experiments, Formal analysis. **Kyoung-Seop Ahn:** Conceptualization. **Sei-Ryang Oh:** All authors have read and approved the published version of the manuscript, funding acquisition, Conceptualization, writing-original draft, preparation.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jep.2020.113386>.

Supporting information description

Spectroscopic data for neochamaejasmin B and chamaejasmin D: UV 254 nm chromatogram of BF (Fig. S1), MS and HRESIMS (Figs. S2–5), NMR spectra (Figs. S6–7), and CD spectra (Fig. S8).

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