

Original Article / 원저

Inhibitory Effects of KM1701 on Airway Cell Infiltration in OVA-Induced Mouse Model

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OVA-유도 쥐 모델에서 기도 세포 침윤에 대한 KM1701의 억제효과

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Abstract

Objectives : The objective of present study is to evaluate anti-inflammatory and anti-allergic effects of Perilla(Perilla frutescens; Labiatae, PF), the roots of Peucedanum praeruptorum(PP) and the root of Scutellaria baicalensis(SB) *in vitro* and anti-asthmatic effects of mixture of PF, PP and SB(PS) on ovalbumin (OVA)-induced asthma in BALB/c mice.

Methods : Anti-inflammatory and anti-allergic effects were observed on the lipopolysaccharide(LPS) treated RAW 264.7 cells through Nitric Oxide(NO) production and RBL-2H3 cells through β -hexosaminidase. Anti-asthmatic effects were observed on the number of inflammatory cells in bronchoalveolar lavage fluid(BALF) and the level of IgE in serum on OVA-induced BALB/c mice.

Results : The treatment of PF, PP and SB(12.5, 25, 50, 100 μ g/ml) resulted in a significant inhibition of NO production in RAW 264.7 cells and mast cell degranulation in RBL-2H3 cells. Oral administration of PS(400mg/kg/day) resulted in a significant reduction in the numbers of eosinophils in BALF and level of IgE in serum.

Conclusion : The oral administration of PS is effective in ameliorating the eosinophilic infiltration *in vivo* and thus can be a good therapeutic candidate for allergic asthma.

Key words : KM1701; Airway Cell Infiltration; OVA-induced Mouse Model

I. Introduction

Allergic diseases, also known as immune disorders, commonly referred to as immediate or type I hypersensitivity reactions with IgE. Crosslinking of the high affinity IgE receptor(Fc ϵ RI) is an essential role in the IgE-mediated allergic reaction¹. Molecular interaction between IgE and allergen on mast cells or basophils leads to allergic reactions inducing the release of inflammatory cytokines resulting in the inflammation leading to clinical symptoms in the target organ².

Asthma is a chronic inflammatory disease of the airways characterized by reduced respiratory function and increased infiltration of leukocytes, especially eosinophils, and it leads to increased hyperresponsiveness, bronchoconstriction and mucus production³. Both eosinophils and T helper(Th) 2 lymphocytes play pathogenic roles associated with increased infiltration of inflammatory cells and mucus secretion in asthma⁴. Eosinophils commonly linked with allergic inflammation act as effector cells by secretion of cytotoxic granule proteins in the pathogenesis of asthma⁵. Airway eosinophils with inflammatory cytokines such as interleukin-13(IL-13) may finally result in airway hyperresponsiveness(AHR) in asthma⁶.

Perilla leaves(*Perilla frutescens*; Labiatae, PF), the roots of *Peucedanum praeruptorum* (PP) and the root of *Scutellaria baicalensis*(SB) are plants used for traditional medicines in East Asia.

Considerable attention has recently been devoted to the anti-allergic, anti-inflammatory, and antitumor-promoting substances in perilla plants⁷. PP is used in alimentary and bronchial disorders and chest pains⁸. SB is used as an anti-inflammatory drug and smooth muscle relaxant⁹. Recently, it is focused that herbal medicine is effective for asthma medications for the treatment of adult patients with moderate and severe allergic asthma¹⁰. Therefore, complementary medicine for asthma treatment has been newly developed. Also, combinations of herbal extracts are more effective because many herbal mixtures have synergistic effects and can neutralize toxic or adverse effects of their individual constituent herbs^{11,12}.

In the present study, we first investigated whether PF, PP and SB exerts anti-inflammatory effects in RAW 264.7 cells and inhibits mast cell degranulation in RBL-2H3 cells. And then, we evaluated the effects of five different ratios of mixture of PF, PP and SB(PS1-5) on OVA-induced asthma in BALB/c mice. These experimental results indicated that a mixture was most effective when the rate of PF, PP and PP was 2:1:1. An optimized combination of PS(KM1701) was thus chosen in this study. We next investigated the effects of KM1701 on mast cell degranulation *in vitro* and eosinophils infiltration *in vivo*.

II. Materials and methods

1. Preparation of the PF, PP and SB

The dried leaves of PF, root of PP and SB

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were purchased from the Kwangmyungdang Medicinal Herbs Market(Ulsan, Republic of Korea). Raw materials were stored in a low temperature warehouse($4\pm 2^{\circ}\text{C}$) until use. The extraction was performed as follow: 100g of raw materials(PS1 PF:PP:SB=1:1:1, PS2 PF:PP:SB=2:1:1, PS3 PF:PP:SB=3:1:1, PS4 PF:PP:SB=2:1:2, PS5 PF:PP:SB=2:2:1) was extracted by refluxing 80°C , 50%(v/v) ethanol(Duksan Pharmaceutical Co. Ltd., Republic of Korea) for 3h two times. Alcoholic extract was filtered through a $0.22\mu\text{m}$ filter and evaporated at 40°C and then freeze-dried. The powder was kept at 4°C before use.

2. Cell culture and cytotoxicity measurement

RAW 264.7 mouse macrophage cells and RBL-2H3 rat basophilic leukemia cells were purchased from the American Type Culture Collection(ATCC; Manassas, VA, USA) and cultured in DMEM supplemented with 10% FBS, 100 U/ml penicillin and $100\mu\text{g}/\text{ml}$ streptomycin at 37°C in a humidified 5% CO_2 atmosphere. The cytotoxicity on RAW 264.7 cells was measured with an 3-(4,5-Dimethylthiazol-yl)-diphenyl tetrazolium bromide(MTT, Sigma, St. Louis, MO, USA)-based colorimetric assay. All experiments were performed 24h after RAW 264.7 cells seeding in 96-well plates. In brief, cells were treated with indicated concentrations of extracts for 24h. MTT solution was added at the end of the treatment to the cell culture media at $0.5\text{mg}/\text{ml}$ final concentration and incubated for 2h at 37°C in the dark. The absorbance at 595nm was determined with a

microplate reader(Infinite F200 PRO, Tecan Trading AG, Switzerland).

3. Measurement of Nitric Oxide(NO)

After RAW 264.7 cells(1.0×10^4 cells/well) were seeded in a 96-well plate, cells were pretreated with extract(12.5 , 25 , 50 , $100\mu\text{g}/\text{ml}$) for 1h prior to stimulation with LPS($1\mu\text{g}/\text{ml}$) for 23h. The NO concentrations were assessed using Griess reagent assay kit(G2930, Promega Korea, Ltd., Korea).

4. Measurement of degranulation

The β -hexosaminidase release inhibition assay using RBL-2H3 cells was performed as previously described¹³). For the β -hexosaminidase inhibition assay, RBL-2H3 cells were seeded onto 24-well plates at 2.0×10^4 cells/well in $400\mu\text{l}$ of medium. The cells were incubated for 24h at 37°C and sensitized with $0.5\mu\text{g}/\text{ml}$ anti-dinitrophenyl(DNP)-IgE, then washed twice with PIPES buffer(25mM PIPES, 119nM NaCl, 5mM KCl, 5.6mM glucose, 0.4mM MgCl_2 , 1mM CaCl_2 , 40mM NaOH, 0.1% BSA, pH7.2) to eliminate free IgE. After washing, the cells were preincubated with either $400\mu\text{l}$ PIPES buffer containing extract(final concentration $100\mu\text{g}/\text{ml}$, respectively) at 37°C for 10 min. RBL-2H3 cells were exposed to $100\mu\text{g}/\text{ml}$ DNP-Human Serum Albumin(HAS) in PBS, followed by incubation at 37°C for 1h. $25\mu\text{l}$ cell culture supernatant were incubated with an equal volume of 5mM substrate solution(5mM p-nitrophenyl-N-acetyl- β -D-glucosaminide dissolved in 0.2M sodium citrate buffer, pH 4.5)

at 37°C for 1.5h. Then, the enzyme reaction was terminated by adding 200 μ l of stop solution(0.1M Na₂CO₃/NaHCO₃, pH 10.0) and the absorbance was measured at 405nm with a microplate reader.

5. Animal

Six-week-old female BALB/c mice were obtained from Daehan Biolink Co. Ltd.(Seoul, Korea) and kept in an air-conditioned room at room temperature(about 22±1°C) and a humidity of about 55±10%. Animal treatment and maintenance were carried out in accordance with the Principle of Laboratory Animal Care(NIH publication number 85-23, revised 1985) and the Animal Care and Use Guidelines of Kolmar Korea, Sejong, Korea(Approval No. 17-NP-AST-002-P/ Korea Kolmar Animal Test Ethics Committee).

6. Sensitization and airway challenge

The allergic lung inflammation of the mice was induced by ovalbumin(OVA, Sigma Chemical Co., St. Louis, MO, USA) using the established protocol in a previous study 14). The normal group was sensitized and challenged with PBS without OVA and drug treatment. Female BALB/c mice were sensitized by intraperitoneal injections of 20 μ g OVA and 2mg imject® Alum(77161, Termo scientific Korea Ltd., Korea) suspended in 0.1ml saline on days 0, 7 and 14. On days 24-27, animals were challenged with 1% OVA aerosol for 50min. Extract(400mg/kg/day, PO), montelukast(Sigma Chemical Co., St. Louis, MO, USA)(50mg/kg/day, PO) or vehicle(0.5%

Carboxymethyl cellulose, PO) was given 2h before each OVA aerosol challenge. Animal experiments were performed according to the Institutional Guidelines for Animal Care and Use of Kolmar Korea Co., Ltd.

7. Inflammatory cell counts in bronchoalveolar lavage fluid(BALF)

All mice were sacrificed on day 28, and tracheotomies were performed. After ice-cold PBS(1ml) was instilled into the lungs, bronchoalveolar lavage fluid(BALF) was obtained via tracheal cannulation¹⁵⁾. Total inflammatory cell numbers were assessed by counting cells in at least five squares of a hemocytometer, after exclusion of dead cells staining with trypan blue. 100 μ l BALF was loaded onto a slide and centrifuged(200 Xg, 4°C, 10min) to fix the cells onto the slide using a cytopsin machine(Hanil Science Industrial, Korea). Cell pellets was suspended in 1ml of PBS, and 100 μ l of each solution was spun onto a slide. After slides were dried, cells were fixed and stained using Diff-Quick Stain reagents(38721, Sysmex Corporation, Japan) according to the manufacturer's instructions.

8. Measurement of anti-OVA IgE antibodies in serum

The collected blood should be centrifuged immediately to separate the blood cells from the plasma fraction. After centrifugation, the fibrin clot forms in the upper layer; this clot should be squeezed gently using a pair of sterile forceps and subsequently centrifuged to

completely separate the blood cells from the serum. Serum was collected and stored at -7°C . Total IgE and OVA-specific IgE were measured by anti-ovalbumin IgE mouse ELISA kit(500840, Cayman Chemical, MI, USA) according to the manufacturer's instructions.

9. Statistical analysis

All statistical parameters were calculated using Graphpad Prism 5.0 software(GraphPad Software, Inc., La Jolla, CA, USA). Value were expressed as the mean \pm standard error of the mean(SEM). NO, MTT and β -hexosaminidase assays were evaluated by one-way analysis of variance(ANOVA) analysis followed by the Tukey's post hoc test. Animal studies were evaluated by the Student's t-test. Differences with p-value less than 0.05 were considered statistically significant.

III. Results

1. Effect of PF, PP and SB on LPS-induced NO production and β -hexosaminidase from RBL-2H3 cells

The cytotoxic effects of PF, PP and SB in RAW 264.7 cells were measured by the MTT assay. PF, PP and SB did not significantly affect cell viabilities at concentrations of 12.5, 25, 50 and $100\mu\text{g}/\text{mL}$ (Fig. 1A). To examine whether PF, PP and SB inhibit the expression of the NO, we performed Griess reagent assay in RAW 264.7 cells. PF, PP and SB significantly inhibited NO production at concentrations of 12.5, 25, 50 and $100\mu\text{g}/\text{mL}$ (Fig. 1B). And then,

we examined whether degranulation of mast cells was decreased(Fig. 1B, 1C). PF, PP and SB significantly decreased the release of beta-hexosaminidase.

2. Inhibitory effects of PS1-5 on the infiltration of total leukocytes, eosinophils into the airways and the productio of IgE in a mouse model of asthma

We induced asthma in BALB/c mice by ovalbumin sensitization and challenge. And then, mice were treated with PS1-5($400\text{mg}/\text{kg}/\text{day}$) and examined the alternation of total cells in the BAL fluid and the level of anti-DNP IgE in serum in order to estimate the effects of PS1-5 on asthma. All treatment groups significantly reduced the numbers of total inflammatory cells, and PS2 is most reduced the number of increased eosinophils(Fig. 2A, 2B). Using hematology analyzer, PS2 is most reduced the number of WBC. And this result is similar to precedents(Fig. 2C). We investigated the effects of PS1-5 to inhibit the production of anaphylactic IgE. Treatments of PS1-5 significantly reduced the production of IgE(Fig. 2D).

3. Inhibitory effects of KM1701 on β -hexosaminidase from RBL-2H3 cells and the infiltration of total leukocytes in a mouse model of asthma

We optimized combination rate 2:1:1 mixture of PF, PP and SB(KM1701), because the present results indicated that KM1701 was most

effective. To optimize dose of KM1701, We evaluated inhibitory effects of KM1701 on mast cell degranulation and infiltration of immune cells related asthma in BALB/c mice by ovalbumin sensitization and challenge. KM1701 significantly decreased the release of beta-hexosaminidase(Fig. 3A) in dose-dependent manner and the number of immune cells related asthma cells in BALF(Fig. 3B).

IV. Discussion

In the present study, PF, PP and SB effectively reduced NO production in LPS-induced RAW 264.7 cells and the release of beta-hexosaminidase in RBL-2H3 cells. Therefore, these medicinal plants had potent inhibitory effects on mast cell degranulation and anti-inflammatory properties. The suppressive effects of PS on airway inflammation were noted by a marked decrease in recruitment of inflammatory cells into BALF and in levels of IgE in serum. These results demonstrated the potent anti-inflammatory effect of PS in OVA-challenged mice.

Macrophage has essential roles in the initiation and maintenance of inflammation. LPS-stimulated macrophage results in the production of NO, playing a key role in the pathogenesis of inflammation. We investigated whether PF, PP and SB show potent inflammatory effects in LPS-induced RAW 264.7 cells. The results showed that all extracts inhibit the NO production. RBL-2H3 cells, rat basophilic leukemia cells, have properties of mucosal-type mast cells and express several Fcε

RI on the membrane surface. RBL-2H3 cells have been widely used for mast cell degranulation through the antigen-induced aggregation of FcεRI¹⁶. The β-hexosaminidase is usually released with histamine when mast cells are immunologically activated, during the cross-linking of FcεRIs¹⁷. Therefore, to evaluate anti-allergic activities of either compounds or herbal extracts, β-hexosaminidase is used as a marker of mast cell degranulation^{16,18,19}. We investigated whether PF, PP and SB could inhibit mast cell degranulation in RBL-2H3 cells. The results showed that all extracts inhibit mast cell degranulation.

Asthma, an inflammatory disease of the lungs and airways, is characterized by increased infiltration of leukocytes, especially eosinophils, and reduced airway function. The inflammation causes airway hyperresponsiveness, bronchoconstriction, and mucus production¹⁵. Eosinophils are the major cell types that can be recruited to sites of immunological or inflammatory responses²⁰. The increased numbers of eosinophils in BALF is a hallmark of asthma. In the present study, PS-treated mice had significantly reduced lung eosinophilia in BALF compared with PBS-treated mice. IgE has been closely related with Th2-type responses. Treatment of PS to OVA sensitized/challenged mice significantly decreased serum IgE levels. Therefore, these results indicate that oral administration of PS inhibited IgE production and eosinophilic infiltration.

V. Conclusion

In the present study, PF, PP and SB significantly inhibited the production of NO and the release of beta-hexosaminidase in LPS-activated RAW 264.7 cells and IgE-sensitized RBL-2H3 cells, respectively. We also demonstrated for the first time that PS attenuates the production of IgE and eosinophils infiltration in OVA-sensitized/challenged allergic

asthma model mice. These results suggest that PS can abrogate the symptoms of asthma via anti-inflammatory activities and inhibitory effects on mast cell degranulation *in vitro* and *in vivo*. Our findings suggest that PS may be promising candidates for the development of a potent therapeutic agent for allergic asthma.

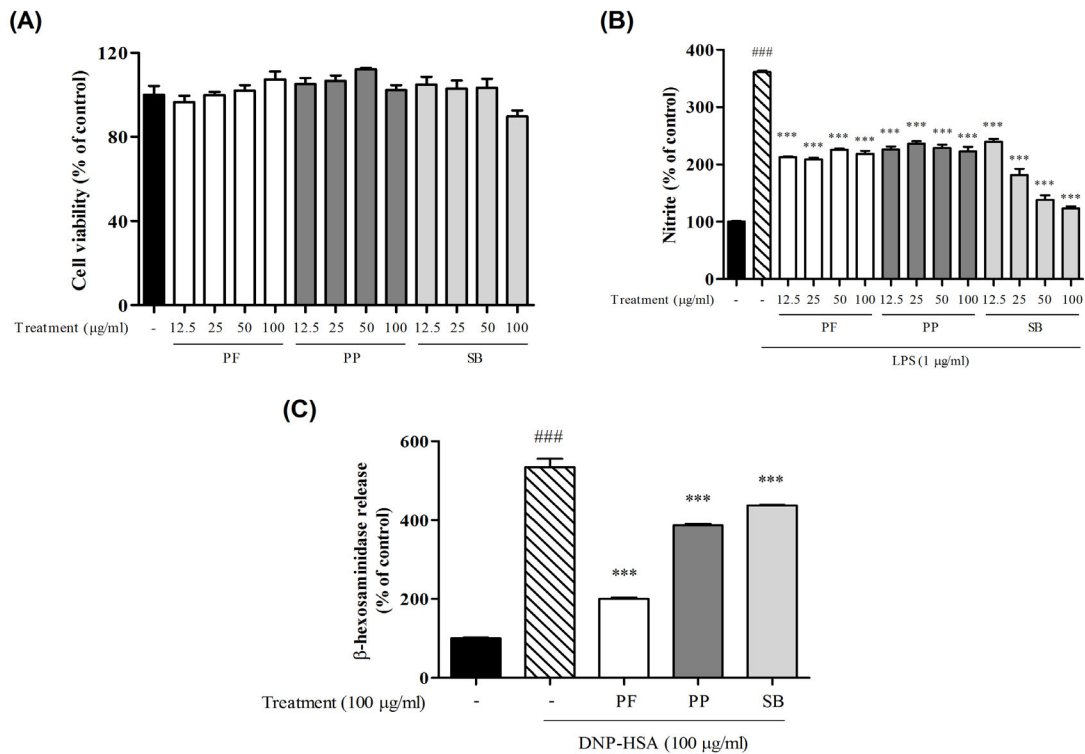


Fig. 1. Inhibitory Effects of PF, PP and SB on Release of NO Production from LPS-Induced RAW 264.7 Cells and β -hexosaminidase from IgE-Sensitized RBL-2H3 Cells

(A) RAW 264.7 cells were treated with the indicated concentrations of PF, PP and SB for 24h and cell viability was determined by an MTT assay. (B) Cells were pretreated with extract(12.5, 25, 50, 100 μ g/ml) for 1h prior to stimulation with LPS(1 μ g/ml) for 23h. Production of Nitric Oxide were measured by Griess reaction assay. (C) RBL-2H3 cells were treated with DNP-HSA(100 μ g/ml) for 1h. Values are represented the mean \pm SEM(n=3); ###p<0.001, significantly different from control, ***p<0.001, significantly different from negative control.

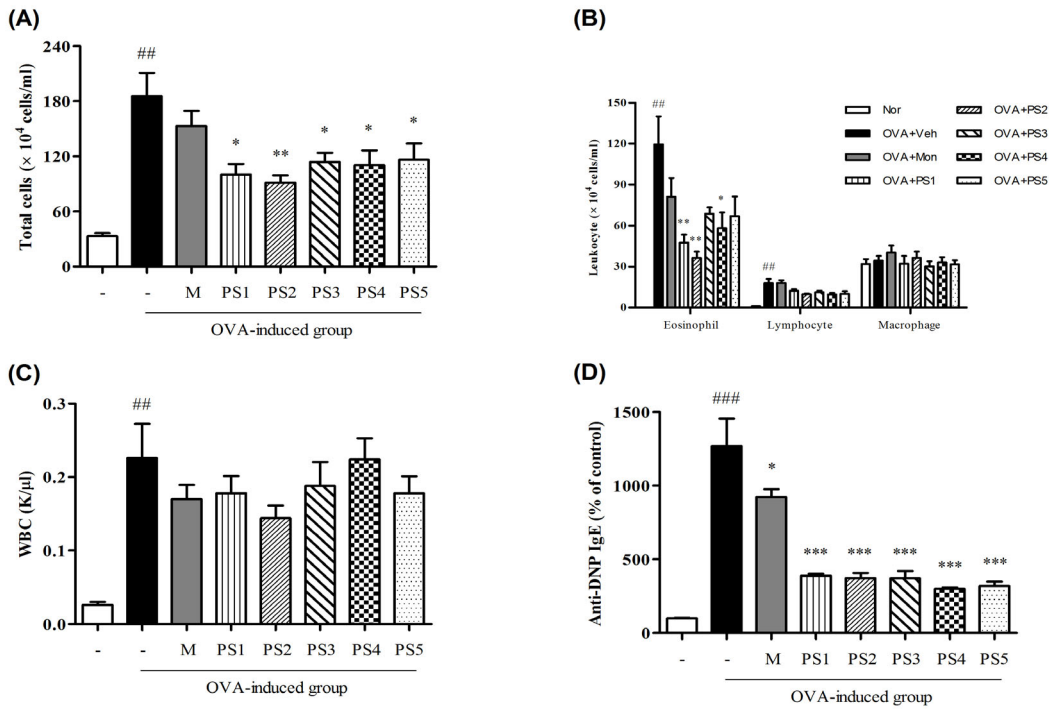


Fig. 2. Effects of PS1-5 on BAL Fluid Cell Infiltration and IgE Levels of OVA-Challenged Mice
BAL fluids and serum were obtained from sensitized mice 24h after the last saline aerosol or 10mg/ml OVA aerosol challenge. Inflammatory cell counts (A), leukocyte counts (B), WBC counts using hematology analyzer (C) in BAL fluids and IgE levels in serum (D) were measured. Differential cell count was performed on a minimum of 500 cells to identify eosinophil, macrophage, and lymphocyte. Values are represented the mean \pm SEM(n=12). ##p<0.01 and ###p<0.001, significantly different from control, *p<0.05, **p<0.01 and ***p<0.001, significantly different from negative control. M and Mon, Montelukast.

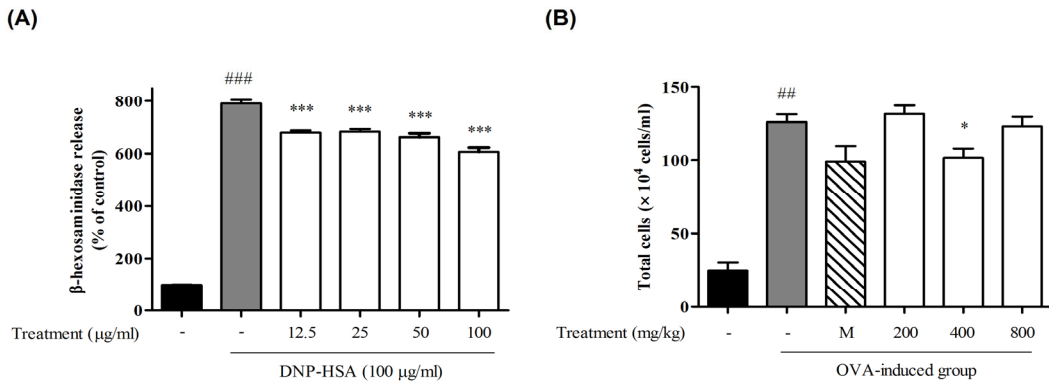


Fig. 3. Effects of KM1701 on Release of β -hexosaminidase from IgE-Sensitized RBL-2H3 Cells and BAL Fluid Cell Infiltration of OVA-Challenged Mice
(A) RBL-2H3 cells were treated with DNP-HSA for 1h. (B) BAL fluids and serum were obtained from sensitized mice 24h after the last saline aerosol or 10mg/ml OVA aerosol challenge. Inflammatory cell counts in BAL fluids were measured. Values are represented the mean \pm SEM. ##p<0.01 and ###p<0.001, significantly different from control, *p<0.05 and ***p<0.001, significantly different from negative control. M, Montelukast.

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