



Eriobotrya japonica leaf extract attenuates airway inflammation in ovalbumin-induced mice model of asthma

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ABSTRACT

Ethnopharmacological relevance: *Eriobotrya japonica* leaves has a very long history of medicinal use as an anti-inflammatory and antitussive agent for bronchial inflammation.

Aim of the study: The aim of this study was to evaluate the anti-inflammatory activities of *Eriobotrya japonica* (EJ) leaf water extract in an ovalbumin (OVA)-induced murine asthma model and human tracheal smooth muscle cell (HTSMC).

Materials and methods: Mice were sensitized by intra peritoneal OVA and challenged with nebulized OVA. EJ extract was administered orally at various dose. Bronchoalveolar lavage fluid (BALF) was quantified for nitric oxide (NO), eosinophil peroxidase (EPO), interleukin (IL)-4, IL-13 level and immunoglobulin (Ig) E was quantified in serum. Lung tissue sections were stained with hematoxylin and eosin for assessment of inflammatory cell infiltration whereas mucus production and goblet cell hyperplasia were studied by periodic acid schiff staining. Western blot was done for analysis of pERK1/2 expression and NFκB translocation in HTSMC whereas iNOS and COX-2 expression in RAW264.7 cell.

Results: EJ significantly reduced the levels of BALF's NO, EPO, MMPs, IL-4, IL-13, and serum IgE. It also decreases inflammatory cell infiltration and mucus production. EJ also attenuated the proliferation of HTSMC, inhibits overexpression of ERK 1/2 and translocation of NFκB in HTSMC as well as iNOS and COX-2 expression in RAW 264.7 cell.

Conclusion: Present study suggest that, EJ effectively protects against allergic airway inflammation thus possessing potential therapeutic option for allergic asthma management.

1. Introduction

Asthma is a chronic airway disease featured by bronchial hyperresponsiveness and lung inflammation (Girodet et al., 2011). The most prominent key features include denudation of epithelial lining, goblet cell hyperplasia, sub epithelial thickening, increased airway smooth muscle cell (SMC) proliferation, angiogenesis, and alterations in extracellular matrix (ECM) components by matrix metalloproteinase (MMP) (Mauad et al., 2007). Airway inflammation is regulated by cross-talk of various cytokines. However, the specific pathological role of each cytokine in the pathogenesis of the asthma remains to be fully elucidated. Recently, it has been established that type 2 T-helper cells play a key role in this process. According to *in vivo* animal data,

interleukin (IL)-4 seems to be crucial in the primary allergen sensitization process and IL-13 is more important during secondary exposure to allergen (Chung and Barnes, 1999; Renaud, 2001). More specifically, high expression of IL-13 in the bronchial epithelium of mice causes goblet-cell metaplasia, fibrosis of sub-epithelium, and SMC proliferation in addition to lymphocyte and eosinophil infiltration (Zhu et al., 1999). Furthermore, macrophages are induced by IL-4 and IL-13. In mice, macrophage produce nitric oxide (NO) mediated by the overexpression of inducible nitric oxide synthase (iNOS) (Hodge et al., 2011). Mast cell are activated by IgE, followed by secretion of a variety of mediators, leading to airway tract inflammation and internal remodeling in allergic asthma (Wu, 2011). The eosinophilic inflammatory response in asthma is associated with protein nitration mediated by

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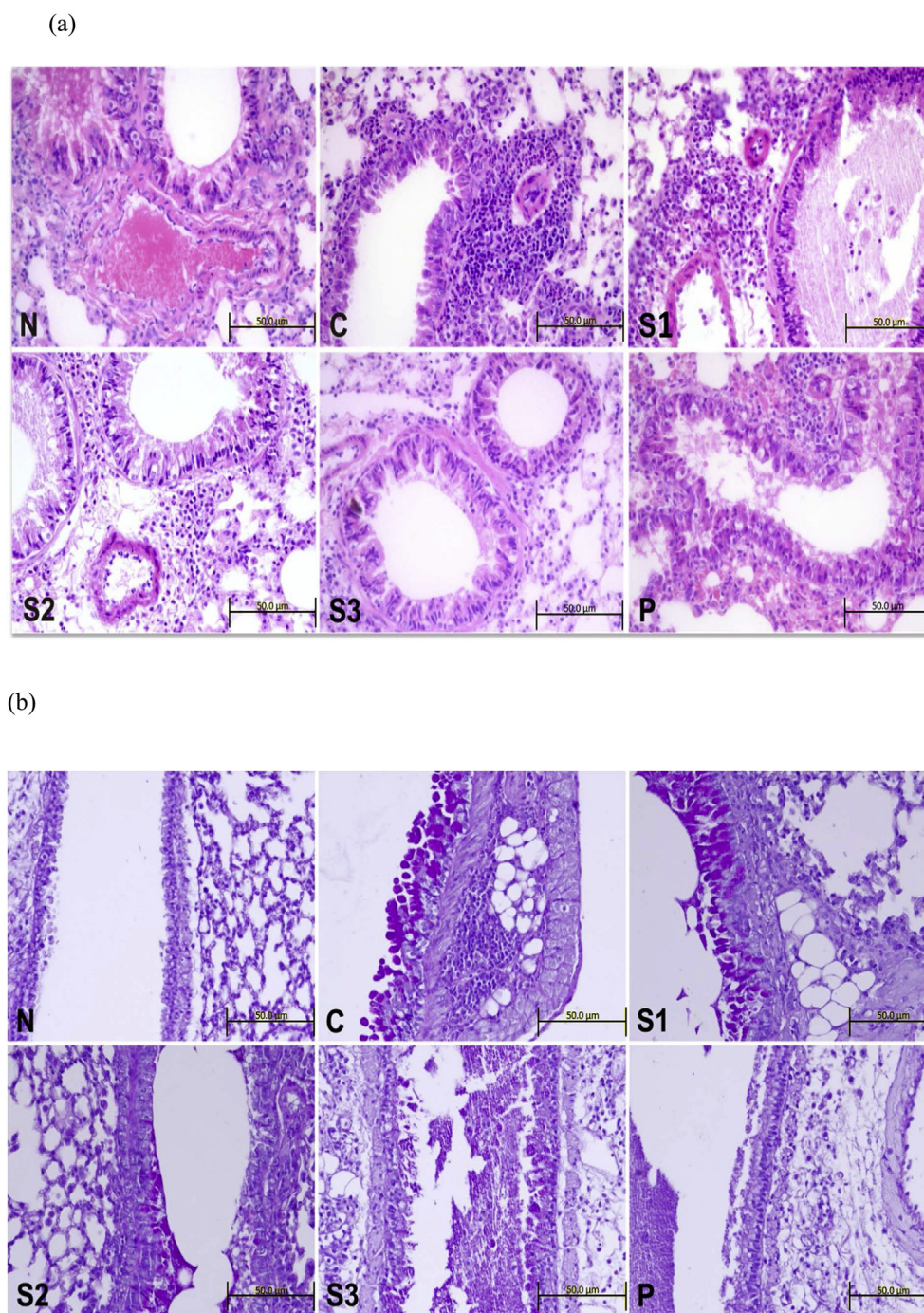


Fig. 1. (a) H/E staining of lungs section. Histological examination of lung section with hematoxylin and eosin stain (magnification x200). (b) PAS staining of lungs section. Histological examination of mucus secretion in lung tissue with periodic acid Schiff (PAS) (magnification x200). N, Normal mice treated PBS only; C, Control; OVA-sensitized/induced mice; P, positive drug control, montelukast (30 mg/kg) + OVA-sensitized/induced mice, S1; (EJ-50 mg/kg) + OVA-sensitized/induced mice, S2; (EJ-100 mg/kg) + OVA-sensitized/induced mice, S3; (EJ-200 mg/kg) + OVA-sensitized/induced mice.

eosinophil peroxidase (EPO), strongly correlating with upregulation of the iNOS (Duguet et al., 2001). Likewise, cyclooxygenase (COX)-2 is constitutively expressed in epithelial cells and the macrophage, promoting the inflammatory prostaglandins synthesis during asthma (Singh et al., 2003). It has been reported that, during airway inflammation, tracheal smooth muscle expresses various adhesion molecules mediated by mitogen-activated protein kinases (MAPKs) signaling and nuclear factor-kappa B (NF- κ B) pathways and up-regulation of adhesion molecules on the surface of the airway epithelium may play a crucial role in mobilization and infiltration of polymorphonuclear neutrophil at sites of inflammation (Lee et al., 2006). Inhibition of NF-

κ B expression attenuates the allergen-induced airway inflammation in a mouse allergic asthma model (Li et al., 2009). Down-regulation of these inflammatory mediators can be potential target in the treatment of various inflammatory diseases including asthma. MMPs are a family of neutral proteolytic enzyme able to degrade ECM components. Among MMPs, 92 kDa gelatinase (MMP-9) is secreted by inflammatory cells involved in asthma, cleaving ECM component. MMP-9 is the major proteinase that induces bronchial remodeling causing the migration of eosinophils and neutrophils across basement membranes (Mautino et al., 1997). Recently, MMPs have been reported to play a crucial role in the infiltration of inflammation inducing cells and mediating airway

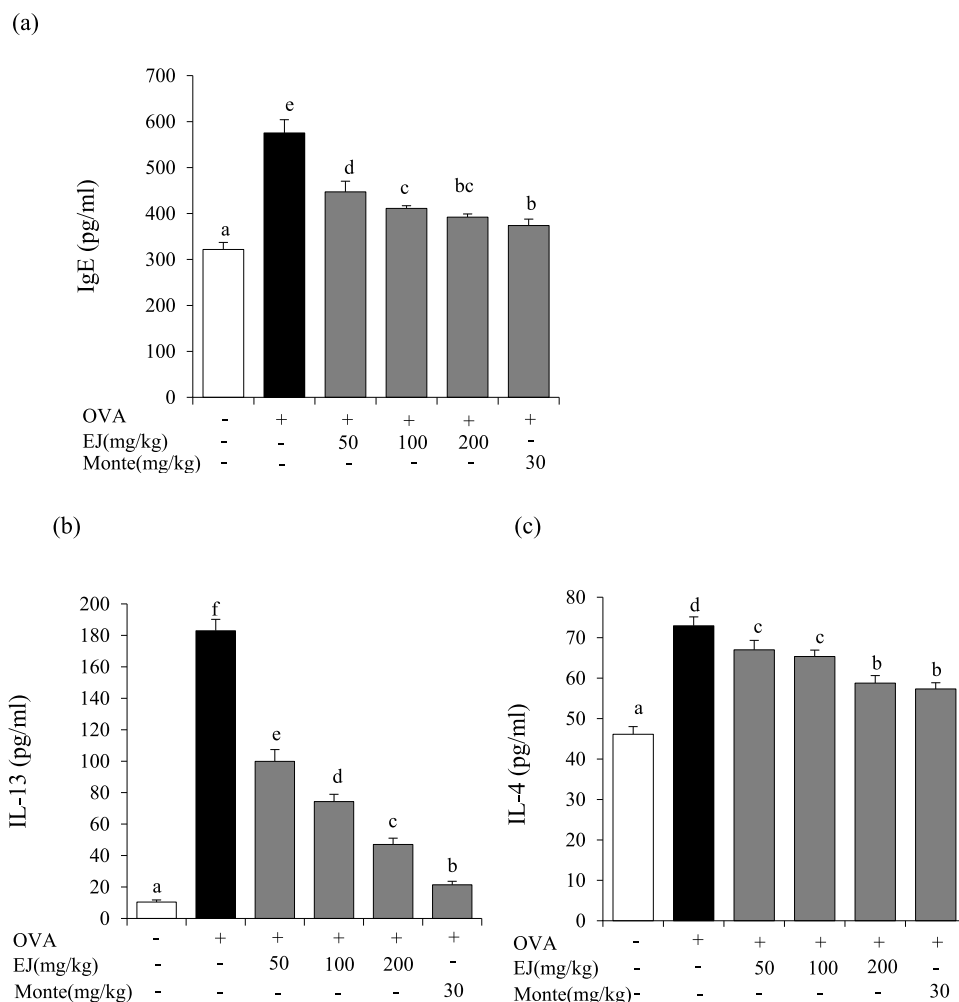


Fig. 2. (a) IgE level in serum and the levels of IL-13 (b) and IL-4 (c) in BALF were measured using ELISA kits. Each value is the mean $\pm$ SD of three independent experiments. Bars with the same letter are not significantly different by Duncan's post-hoc test of multiple comparisons ($p < 0.05$). Monte = Montelukast.

hyper-responsiveness in ovalbumin-induced murine model of asthma (Kumagai et al., 1999).

Several herbal plants serve as a source of medicine to prevent and cure variety of disease like cancer (Panth et al., 2017), atherosclerosis (Lee et al., 2016). The leaves of loquat, *Eriobotrya japonica* (Thunb.) Lindl. (EJ), family Rosaceae is widely used in Chinese herbal treatment as an anti-inflammatory and antitussive agent for bronchial inflammation (Liu et al., 2016). Similarly, in Korea, the leaves *E. japonica* have been widely used as a traditional medicine with beneficial effects for chronic inflammatory diseases including, asthma, phlegm and chronic bronchitis (Cha et al., 2011). It is also use in Japanese folk medicine as a diuretic, digestive, and antipyretic agent. Various potent phytochemicals like flavonoids, tannins, triterpenes, sesquiterpenes, and glycosides have been found in the leaves of this plant exhibiting antioxidant, antiviral, cytotoxic, and hypoglycemic properties (Shih et al., 2010). In present study, we aim to explore the activity of EJ leaves extract on OVA induced mice model of asthma.

2. Materials and methods

2.1. Plant material

E. japonica leaves were collected from Mokpo, Jeollanam-do, Republic of Korea on September 2014 and taxonomically identified by Professor Hui Kim (plant taxonomist), College of Natural Sciences, Mokpo National University, Jeollanam-do, Republic of Korea. A

voucher specimen was deposited in the scientific herbarium at the Department of Oriental Medicine Resources, College of Natural Sciences, Mokpo National University, under number H. Kim 10052.

2.2. Preparation of EJ water extract

200 g of EJ dried leaves was placed on round bottom flask and heated with distilled water at 60 °C continuously for a period of 48 h. Then, extract was filtered through vacuum filter and the filtrate was concentrated by evaporating the water in rotary evaporator. The final extract was stored at -70 °C overnight and then subjected for freeze drying for 5 days. After 5 days, the fine powder of extract was collected and weighed. Percentage yield was 65.6 g (32.8%).

2.3. In vivo experiment

Healthy female BALB/c mice (4 weeks old) were brought from the Orient Co. Ltd (Seoul, Republic of Korea) and acclimatize for 1 week before start of experiment. The mice were divided into 6 group containing 6 mice in each group; Normal (only PBS), Control (only OVA), S1 (OVA + EJ 50 mg/kg/day), S2 (OVA + EJ100 mg/kg/day), S3 (OVA + EJ 200 mg/kg/day), and Positive Drug Control (OVA + Montelukast-30 mg/kg/day). Mice were supplied with standard rodent chow and drinking water in free access and kept in 12/12 h' light/dark cycle. All experimental procedures were carried under the guideline of Mokpo National University Lab Animal Research

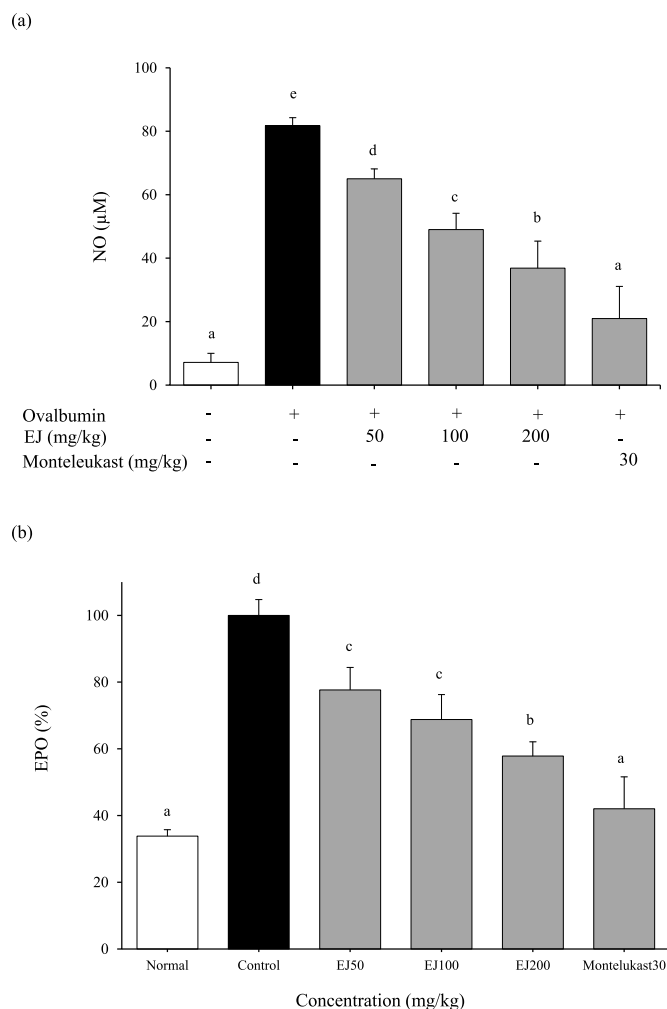
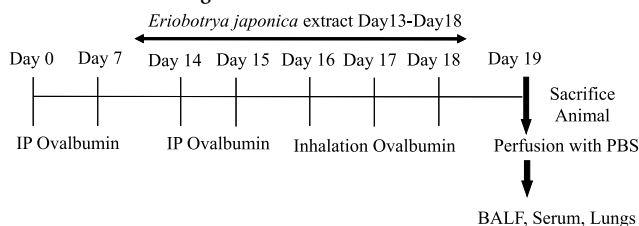


Fig. 3. Quantification of NO (a) and EPO (b) in BALF. Each value is the mean $\pm$ SD of three independent experiments. Bars with the same letter are not significantly different by Duncan's post-hoc test of multiple comparisons ($p < 0.05$).

Committee.

2.3.1. OVA-induced allergic asthma



Intraperitoneal (IP) injection of 20 μ g OVA emulsified in 2 mg aluminum hydroxide in 200 μ L phosphate buffer saline (PBS; pH 7.4) were injected in mice for sensitization on days 0, 7 and 14. On day 16, 17 and 18 after initial sensitization, mice received an airway challenge with nebulized OVA (1% w/v in PBS) continuously for 30 min. EJ was dissolved in PBS and prepared fresh daily before oral treatment to mice as gastric gavages at doses of 50 mg/kg, 100 mg/kg or 200 mg/kg once daily from day 13–18. Normal and positive drug control mice were orally administered with PBS and montelukast (30 mg/kg) respectively. Montelukast is widely used anti-asthmatic drug developed as a cysteinyl leukotriene receptor antagonist. On final day, mice were paralyzed by cervical dislocation 48 h after the last challenge, and a surgery was

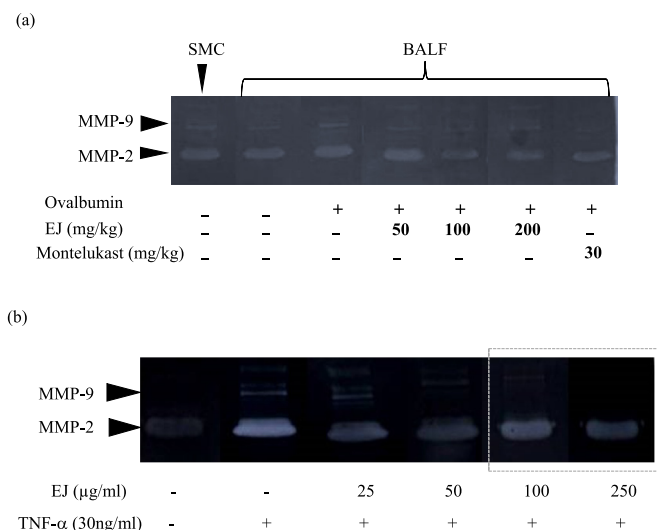


Fig. 4. Effect of EJ on MMP-9 enzymatic action in BALF and HTSMC suspension. (a) Quantification of MMP-9 in BALF of different groups were done by gelatin gel electrophoresis. TNF- α treated HTSMC suspension was used as a control marker of 92 kDa MMP-9 and 72 kDa MMP-2 in first lane (SMC). (b) HTSMC pretreated with EJ for 1 h were stimulated by 30 ng/ml of TNF- α for 24 h. The cell supernatant medium was subjected to gelatin gel electrophoresis for quantification of MMPs.

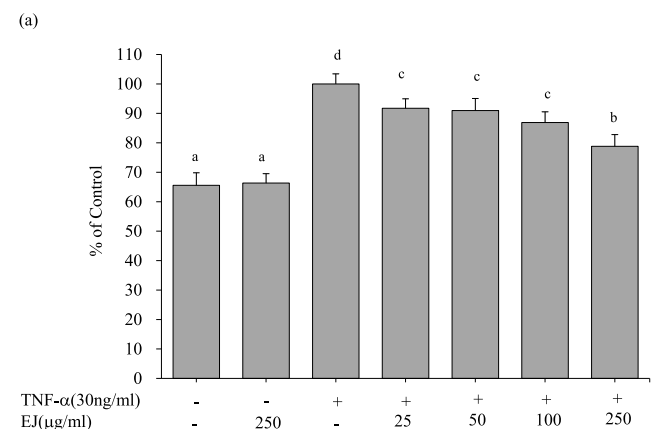


Fig. 5. Effect of EJ on HTSMC proliferation. Each value is the mean $\pm$ SD of three independent experiments. Bars with the same letter are not significantly different by Duncan's post-hoc test of multiple comparisons ($p < 0.05$).

performed. To get BALF, after tracheostomy, PBS (0.5 mL) was infused into the lung and withdrawn via tracheal cannulation multiple times (total volume of 1.5 mL was collected in an eppendorf tube). BALF was centrifuged and the supernatant obtained from BALF was stored at -70°C for biochemical analysis. Lungs tissue were obtained and stored at -70°C until used for staining.

2.3.2. Measurement of the levels of cytokines (IL-4, IL-13) and IgE

The levels of IL-4, IL-13 in BALF and IgE in serum were measured using enzyme-linked immunosorbent assay (ELISA) kits (Abcam, UK) according to the manufacturer's protocols. Microtiter plates coated with anti-IgE antibody and cytokines antibodies were incubated with serum and BALF respectively of each group mice. The plates were then washed several times as indicated and finally incubated for 10 min in the dark and the absorbance was then measured at 450 nm.

2.3.3. Histology

After the dissection of lungs, section of tissue was fixed in 10% neutral buffered formalin for 4 h. Then, tissue was subjected to

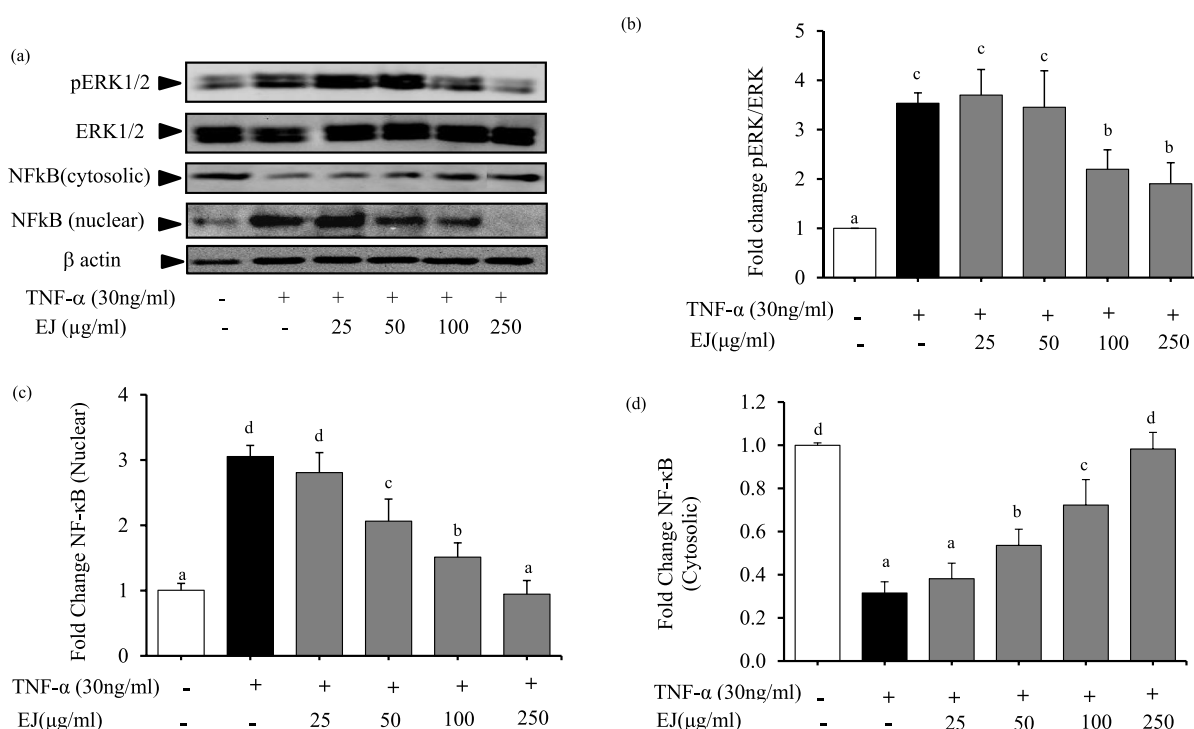


Fig. 6. Effect of EJ on cell signaling molecules of HTSMC. (a) Western blot was carried out for measurement of phosphorylated ERK1/2 expression and NFκB translocation in HTSMC. β-actin was used for normalization. (b) Normalized quantitative data for pERK1/2 protein expression. (c) Normalized quantitative data for NFκB nuclear protein expression. (d) Normalized quantitative data for NFκB cytosolic protein expression. Values are expressed as mean $\pm$ SD, $n = 3$ independent experiments. Bars with the same letter are not significantly different at $p < 0.05$.

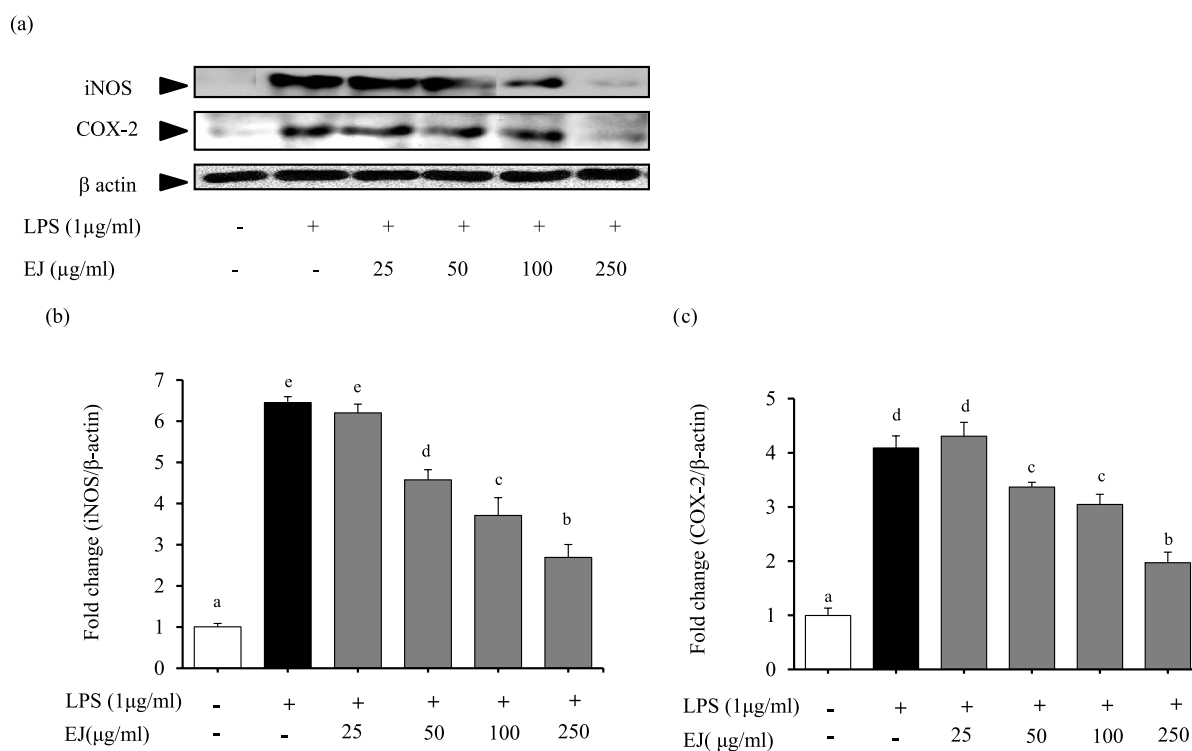


Fig. 7. Effect of EJ on expression of iNOS and COX-2 in RAW264.7 cell. (a) Western blot was carried out for measurement of iNOS and COX-2 expression in RAW 264.7 cell. β-Actin was used for normalization. (b) Normalized quantitative data for iNOS protein expression. (c) Normalized quantitative data for COX-2 protein expression. Values are expressed as mean $\pm$ SD, $n = 3$ independent experiments. Bars with the same letter are not significantly different at $p < 0.05$.

dehydration gradually from 50% ethanol to 100% ethanol and finally on xylene for different time interval applying vacuum. Tissues were then embedded in paraffin, microtome 5 μ m thickness, stained with H&E solution (hematoxylin; Sigma HHS-16, and eosin; Sigma HT110316) and periodic acid–schiff (PAS; Sigma SLBC9804) to estimate inflammatory cell infiltration and mucus production, respectively.

2.3.4. Colorimetric measurement of NO production and EPO assay in BALF

Quantification of NO in BALF was done by Griess reagent. Briefly, 100 μ l of BALF was mixed with 100 μ l of Griess reagent. The absorbance of chromophore formed during diazotization of nitrite with sulfanilamide and subsequent coupling with naphthyl-ethylene-diamine dihydrochloride was read at 540 nm. Quantification in terms of μ M of NO was done according to standard curve obtained from NaNO₃ (Lee et al., 2015).

EPO assay is a colorimetric assay based on the oxidation of o-phenylenediamine (OPD, P7288, Sigma) by EPO in the presence of hydrogen peroxide (Sigma, H1009). Briefly a volume of 50 μ l of BALF supernatant was added into a 96-well plate in duplicate. Then substrate solution of 0.1 mM OPD, 0.05M Tris pH 8.0, Triton X-100 (Sigma), and 1 mM H₂O₂ was added to each well. After 60 min incubation at room temperature, the reaction was stopped by adding 50 μ l of 4M sulfuric acid. Colorimetric absorbance was measured at 492 nm with a micro plate reader.

2.3.5. Gelatin zymography

Gelatin zymography of BALF for MMP-9 expression was done as describe previously (Jun et al., 2016).

2.4. In vitro experiment

2.4.1. Proliferation assay

HTSMC proliferation was assessed by using MTT assay as described previously (Paudel et al., 2016). TNF- α was used as a HTSMC proliferation inducer for 24 h. The conversion of MTT to formazan by alive HTSMC was dissolved in dimethyl sulfoxide (DMSO) and absorbance was read at 540 nm.

2.4.2. Gelatin zymography

Gelatin zymography of HTSMC supernatant was done as describe previously (Jun et al., 2016).

2.4.3. Western blot analysis

Western blot for phosphorylated and non-phosphorylated form of ERK1/2, and NF κ B (for HTSMC lysate) whereas iNOS and COX-2 (for RAW 264.7 cell lysate) was done as described previously (Paudel et al., 2016).

2.5. Statistical analysis

SPSS was used for statistical analysis of data. Each data was expressed as mean $\pm$ standard deviation. Groups were compared by using one- way analysis of variance (ANOVA) followed by Duncan's post-hoc test of multiple comparisons. Bars with the same letter are not significantly different by Duncan's multiple range tests ($p < 0.05$).

3. Results

3.1. In vivo

3.1.1. Histological examination

Fig. 1a shows the H/E staining of lungs section. Inflammatory cell infiltration was markedly reduced by EJ treatment as compared to control group (only OVA treated group). Fig. 1B shows PAS staining of lungs section. PAS staining is indicated by dark blue stain of mucous producing goblet cell. EJ treatment attenuates goblet cell infiltration as

compared to control group (only OVA treated group).

3.1.2. Effect of EJ on IgE level in serum and IL-4, IL-13 in BALF

Fig. 2(A) shows effect of EJ on level of IgE in serum. Treatment of EJ can reduced the IgE level in mice serum in a dose dependent manner. Similarly (Fig. 2b and c), shows the level of IL-13 and IL-4 respectively in BALF. EJ can significantly attenuated the level of IL-4 and IL-13 in BALF as compared to control OVA treated group.

3.1.3. Quantification of NO and EPO in BALF

Fig. 3a Shows the NO scavenging activity of EJ on BALF. There was a dose dependent scavenging of NO level in BALF as compared only OVA treated group. Eosinophil cell infiltration on lungs was evaluated by EPO assay. Fig. 3b Shows the dose dependent inhibition of EPO in BALF by EJ treatment as compared to only OVA treated group.

3.1.4. Effect of EJ on expression of MMP-9 in BALF

The MMP-9 expression in BALF was carried out by gelatin zymography. MMP-9 expression was significantly increased by OVA, however, treatment of EJ inhibit the MMP-9 expression in BALF as shown in Fig. 4a.

3.2. In vitro

3.2.1. Effect of EJ on the enzymatic action of MMP-9

Fig. 4b Depicts the effect of EJ on the enzymatic action of MMP-9 in HTSMC. To examine the inhibitory effect on MMP-9 enzymatic action, EJ pretreated HTSMC was stimulated with TNF- α for 24 h. Then, the condition medium was subjected to gelatin zymography. EJ showed dose dependent inhibition on the enzymatic action of MMP-9.

3.2.2. Effect of EJ on proliferation of HTSMC

Fig. 5 Shows the effect of various concentrations of EJ on the proliferation of HTSMC induced by 30 ng/ml of TNF- α . There was a dose dependent inhibition of the proliferation of HTSMC by EJ within safe dose concentration.

3.2.3. Effect of EJ on phosphorylation of ERK1/2, NF κ B in HTSMC

The effect of EJ on phosphorylation of ERK 1/2, NF κ B was carried out by Western blot analysis. Phosphorylation of ERK was up regulated with TNF- α (30 ng/ml) while EJ pretreatment inhibited the ERK expression (Fig. 6a). TNF- α treatment increase the pERK1/2 expression by 3.5-fold while EJ treatment lower the expression up to 1.9-fold (Fig. 6b). Similarly, EJ also inhibit the translocation of NF κ B from cytosol to nucleus (Fig. 6a.). TNF- α treatment increase the NF κ B nuclear expression by 3-fold while EJ treatment lower the expression up to 1-fold (Fig. 6c.). Correspondingly, NF κ B cytosolic expression was decreased by TNF- α treatment and increased by EJ (Fig. 6d.).

3.2.4. Effect of EJ on iNOS and COX-2 expression in RAW 264.7

In RAW264.7 cell, iNOS and COX-2 expression was increased with LPS treatment while suppressed by EJ in a dose dependent manner as shown in Fig. 7a. LPS treatment increase the iNOS expression by 6-fold (Fig. 7b.) and COX-2 by 4-fold (Fig. 7c) while EJ treatment lower the expression of both iNOS and COX-2.

4. Discussion

During airway remodeling, an asthmatic' demonstrate the inflammatory cell infiltration in airways as revealed by histopathological analyses of bronchial biopsies. Predominantly, cell in these infiltrates are mast cells, basophils, eosinophils, monocytes, and T lymphocytes (Jeffery, 2001). There is a marked loss of airway function occurring in multiple parts of lung compartments, characterized as goblet cell hyperplasia, SMC proliferation and hypertrophy, collagen deposition, loss of integrity of airway epithelial barrier, angiogenesis (Holgate, 2008).

Ovalbumin is an allergen used to induce asthma in BALB/c mice allowing feasible method to study the pathogenic mechanism of asthma as this experimental model strongly co-relates to features of human asthma and facilitate studies of potential therapeutic agents (Nials and Uddin, 2008). In our study, OVA was successful to induce asthma like feature in BALB/c mice. Our results revealed that EJ possess potent anti-inflammatory activity during asthma manifestation, attenuating pro-inflammatory mediators including IL-4, IL-13, NO production and EPO expression in BALF of BALB/c mice, iNOS and COX-2 expression in RAW264.7. EJ also decrease goblet cell hyperplasia and inflammatory cell infiltration as well as down regulate the IgE level. Furthermore, EJ inhibited TNF- α induced HTSMC proliferation via ERK 1/2 and NF- κ B pathway.

Although there is availability of potent synthetic anti-inflammatory steroidal drugs to treat airway remodeling, the possible side effect and high cost limits it as a drug of choice in asthma (Royce and Tang, 2009). Montelukast is popular choice of physician in combination therapy of asthma. However, United States' food and drug administration has added the information on leukotriene inhibitors dated August 28, 2009, presenting the case of neuropsychiatric events including agitation, aggression, anxiousness, dream abnormalities, hallucination, depression, insomnia, irritability, restlessness, suicidal thinking and behavior (Philip et al., 2009). Being an herbal medicine *Eriobotrya japonica* leaf extract provides a potential therapeutics alternative for asthma prevention and treatment.

Inflammation of airways starts with the infiltration of immune cell to the lungs space as well as release of inflammatory mediators like nitric oxide, iNOS and COX-2 by macrophage, IgE by mast cell, IL-4 and IL-13 by Th2 cell (Girodet et al., 2011). In our result, treatment of EJ significantly attenuated IL-4 and IL-13 level in BALF and IgE in serum of BALB/c mice. Also, there was decrease in goblet cell hyperplasia and inflammatory cell infiltration as revealed by result of PAS and H/E staining of lungs tissue respectively. Among alterations in the structure of the asthmatic airways, increased airway smooth muscle mass regulated by the proinflammatory cytokines including IL-1 β and TNF- α may represent a key feature contributing to airflow obstruction (Khan, 2013). COX-2 expressions have also been found to be increased during asthma. Inflammatory cells produce COX-2 via stimulation with toxic materials such as LPS, allergens, and chemicals (Redington et al., 2001). Increased COX-2 expression induces increased expression of prostaglandins, which aggravate inflammatory responses. Sang-Hyun Kim et al. reported the anti-inflammatory effect of leaves of *Eriobotrya japonica* correlating with attenuation of p38 MAPK, ERK, and NF- κ B activation in mast cells (Kim and Shin, 2009). In our result, extract of EJ leaves showed anti-inflammatory activity by attenuation of iNOS and COX-2 in RAW264.7 cell. Similarly, MAPK (JNK and ERK) and NF- κ B pathways are also involved in airway SMC proliferation and migration (Tang et al., 2013). Also, recent study shows that IgE can induces proliferation in human airway smooth muscle cells via MAPK pathways (Redhu et al., 2013). In our study, EJ dose dependently inhibited the proliferation of tracheal SMC induced by TNF- α and also the phosphorylation of ERK and translocation of NF- κ B from cytosol to nucleus. Lemjabbar et al., demonstrated that human bronchial epithelial cells were able to produce major constitutive 92 kDa gelatinase and upregulate its production in response to inflammatory injury, thus playing a role in the remodeling of airways (Lemjabbar et al., 1999). In addition, BALF of patients undergoing mechanical ventilation for severe asthma exacerbation contained increased concentrations of MMP-3 and MMP-9 compared with subjects with mild asthma (Lemjabbar et al., 1999). In consistence with this result, our result showed that, EJ leaf extract inhibits the MMP-9 expression in both HTSMC and BALF. Taken together, EJ possess the property to down regulate the inflammatory response in allergic asthma.

5. Conclusion

This study showed that EJ leaf extract demonstrated the anti-asthmatic action through inhibition of inflammatory mediators that are up regulated by macrophage, mast cell, eosinophil, Th2 lymphocyte, airway smooth muscle cell during asthma progression. These findings strongly suggest that EJ can prevent asthma by attenuating inflammatory response in asthma mediated by allergen and alteration in various cell involved.

Conflicts of interest

The authors declare that there are no conflicts of interest.

Author's contribution

The authors' responsibilities were as follows—DWK: designed the research; TMK and KRP conducted the research; KRP analyzed the data and wrote the manuscript; and all authors read and approved the final manuscript.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jep.2019.112082>.

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