Anti-asthmatic Activity of an Ethanol Extract from *Saururus chinensis*

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As an attempt to find bioactive medicinal herbs exerting anti-asthmatic activity, the effects of an ethanol extract from the parts of *Saururus chinensis* were evaluated in both *in vitro* and *in vivo*. The ethanol extract of *S. chinensis* (ESC) inhibited generation of the cyclooxygenase-2 (COX-2) dependent phases of prostaglandin D2 in bone marrow-derived mast cells in a concentration-dependent manner with an IC50 value of 14.3 μg/ml. ESC also inhibited leukotriene C4 production with an IC50 value of 0.3 μg/ml. This demonstrates that ESC has COX-2/5-lipoxygenase dual inhibitory activity. In addition, this compound inhibited degranulation reaction in a dose dependent manner, with an IC50 value of 1.3 μg/ml. An ovalbumin induced mouse asthmatic animal model was used to determine its *in vivo* anti-asthmatic activity. The oral administration (50–200 mg/kg) of ESC reduced the number of infiltrated eosinophils in a bronchoalveolar lavage fluid. Furthermore, ESC (100 mg/kg) inhibited the eotaxin and IL-4 mRNA expression levels. These results suggest that the anti-asthmatic activity of *S. chinensis* might in part occur via the inhibition of eicosanoid generation, degranulation as well as the down regulation of IL-4 and eotaxin mRNA expression.

Key words *Saururus chinensis*; cyclooxygenase-2; 5-lipoxygenase; mast cell; asthma; eosinophil

*Saururus chinensis* (Saururaceae) is a perennial herb distributed in China and southern Korea and the aerial part of *S. chinensis* has been used for folk medicine for the treatment of edema, jaundice, gonorrhea, and several inflammatory diseases in Korea. Previous phytochemical studies of the genus *Saururus* have demonstrated the presence of more than 20 lignans, as well as flavonoids, anthraquinones, and furanoditerpenes. Neolignan derivatives isolated from this plant, which are tetrahydrofuran type, are known to have a variety of biological activities such as inhibition of cell adhesion, anti-inflammatory, murine neuroleptic, hepatoprotective and hypercholesterolemia activities.

Asthma is an inflammatory disease of the airways, and the current focus in managing asthma is the control of inflammation. In bronchial asthma, various factors result in infiltration of mast cells, eosinophils and Th2 lymphocytes into the lesions with downstream mediators, leading to the formation of asthmatic phenotypes such as mucous hyperproduction, airway hyper-responsiveness (AHR), and submucosal thickness. A bronchoalveolar lavage (BAL) from allergic asthma consistently showed a higher number of eosinophils, mast cells, and mast cell-derived mediators than the BAL from normal, nonallergic subjects. When activated, many of the effector cells, including the mast cells in asthma, produce a variety of cytokines and newly synthesized eicosanoids. Eicosanoids, which include prostaglandins (PGs) and leukotrienes (LTs) are inflammatory mediators that are biosynthesized in many cell types by cyclooxygenases (COX) and lipoxygenases (LOX) and are strongly associated with inflammatory disorders, acute as well as chronic inflammation. The inhibition of eicosanoid production is one of the important therapeutic strategies in various inflammatory diseases. Among the eicosanoid generating enzymes, an inducible isoform of COX-2 was found to be essential for the production of prostanoids in inflammatory sites. Arachidonic acid can also be converted to LTs via the action of 5-LOX. LTs have biological properties that would be expected for molecules that participate in the pathogenesis of bronchial asthma, and there is evidence for their formation in the airways of asthmatic individuals. Therefore, the dual inhibition of COX-2/5-LOX is believed to be the ideal treatment for allergic diseases and asthma.

Although many biological activities of *S. chinensis* have been reported, the anti-asthmatic activity of *S. chinensis* has not been reported to date. As part of an ongoing investigation of new biologically active compounds from medicinal herb, the present study was designed to determine the mechanism underlying the anti-asthmatic effect of *S. chinensis*.

**MATERIALS AND METHODS**

**Materials** The aerial parts of *S. chinensis* were collected at College of Pharmacy Yeungnam University (Herbal Garden) and identified by professor Ki-Hwan Bae (College of Pharmacy, Chungnam National University, Daejeon, Korea). A voucher specimen has been deposited at the herbarium of the College of Pharmacy, Yeungnam University (YNU 6024). The dried aerial parts of *S. chinensis* (1 kg) were cut into small pieces and extracted repeatedly with 70% ethanol (EtOH, 2 l). The solution was filtered through filter paper and to give a powered extract (100.3 g).

**Animals** Female ICR mice (16—20 g) and BALB/c mice (16—20 g) were obtained from Hyochang Science (Daegu, Korea) and fed with laboratory chow (Purina, Seoul, Korea).
Korea) and water ad libitum. Animals were acclimatized in a specific pathogen-free animal facility under the conditions of 20–22 °C, 40–60% relative humidity, and 12 h/12 h (light/dark) cycle at least for 7 d.

Preparation and Activation of Bone Marrow-Derived Mast Cells (BMMC) Bone marrow cells from male BALB/c mice were cultured for up to 10 weeks in 50% enriched medium (RPMI 1640 containing 2 mm l-glutamine, 0.1 mm nonessential amino acids, antibiotics and 10% fetal calf serum) and 50% WEHI-3 cell conditioned medium as a source of IL-3. After 3 weeks >98% of the cells were found to be BMMC when checked by the previously described procedure.20

Determination of PGD$_2$. To measure the inhibitory activity on COX-2 by an ethanol extract of $S$. chinensis (ESC), cells suspended at a cell density of 5 x 10$^5$ cells/ml in enriched medium were preincubated with aspirin (10 μg/ml) for 2 h to irreversibly inactivate preexisting COX-1. After washing, BMMC were activated with KL (100 ng/ml), IL-10 (100 U/ml) and LPS (100 ng/ml) at 37 °C for 8 h in the presence or absence of ESC previously dissolved in dimethylsulfoxide (DMSO). All reactions were stopped by centrifugation at 1200 x g for 5 min. The supernatant and cell pellets were immediately frozen in liquid N$_2$ and stored at −80 °C for further analysis. Under the conditions employed, COX-2-dependent phases of PGD$_2$ generation reached 1.6 ng/ml cells without ESC.

Effect of ESC on 5-LOX Activity BMMC suspended in enriched medium at cell density of 1 x 10$^6$ cells/ml were pretreated with ESC for 15 min at 37 °C and stimulated with KL (KL: 100 ng/ml). After 20 min of stimulation, the supernatants were isolated for further analysis by EIA. LTC$_4$ was determined using an enzyme immunoassay kit (Cayman Chemical) according to manufacturer’s instruction. Under the conditions employed, the amount of LTC$_4$ without ESC was reached approximately 500 pg/ml cells.

Assay of β-hexosaminidase (HEX) release β-HEX, a marker of mast cell degranulation, was quantitated by spectrophotometric analysis of the hydrolysis of $p$-nitrophenyl-2-acetamido-2-deoxy-β-D-glucopyranoside (Sigma). Briefly, after harvesting supernatant, cells were lysed in the same volume of medium by freeze and thaw three times. Ten microliters of the BMMC lysate or supernatant samples were mixed with 50 μl of β-HEX substrate solution (1.3 mg/ml $p$-nitrophenyl-2-acetamido-2-deoxy-β-D-glucopyranoside in 100 mm sodium citrate, pH 4.5) in each well of 96-well plates and then incubated at 37 °C for 60 min. The reaction was stopped by adding 140 μl of 0.2 M Glycin–NaOH (pH 10.7). The absorbance at 410 nm was measured in a microplate reader. The percentage of β-HEX released into the supernatant was calculated by the following formula: [S/(S+P)] x 100, where S and P are the β-HEX contents of supernatant and cell pellet, respectively.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR) Total RNA from mouse lung was extracted with TRIzol® reagent (Invitrogen, Carlsbad, CA, U.S.A.) according to manufacturer’s instructions and quantified by measuring optical density at 260 nm and then detected by applying to 1.2% formaldehyde-agarose gels. RT-PCR was carried out using RNA PCR kit (Takara, Shiga, Japan) by thermal cycler (GeneAmp PCR system 2400, Perkin-Elmer, Norwalk, CT, U.S.A.). One microgram of total RNA from each sample was used as template for reverse transcription reaction using 2.5 pmol oligo (dT)$_12$ primers and 5 units of avian myeloblastosis virus (AMV) reverse transcriptase in 10 mm Tris–HCl (pH 8.3), 50 mm KCl, 5 mm MgCl$_2$, 1 unit/μl RNase inhibitor and 1 mm dNTP mixture. The reaction was performed for 10 min at 30 °C, 30 min at 42 °C, heated to 99 °C for 5 min and chilled to 4 °C on ice. Two microliters of RT product was used for PCR. PCR was carried out in a final volume of 20 μl containing 1 mm MgCl$_2$, 1X PCR buffer, 1 mm dNTP mixture, 1 Unit Taq DNA polymerase (Takara), 0.2 μm each primer. Amplifications were carried out using a thermal cycler with the following profile: 5 min at 94 °C before the first cycle, 30 s for denaturation at 94 °C, 30 s for primer annealing, 30 s for extension at 72 °C, and 5 min at 72 °C after the last cycle. Primer sequences and PCR product sizes were as follows: eotaxin, 5′-CCA AGG ACT TG(TTT)TTG CT(T)CT GAT G-3′ and 5′-ATT CTG TGT GCT TGG CAT GGT AGC-3′, 495 bp, 25 cycles; IL-4, 5′-ACG GCA CAG AGC TAT TGA TG-3′ and 5′-ATG GTG CAG TAC TAC GA-3′, 454 bp, 35 cycles. The PCR products were analyzed by 1.2% agarose gel electrophoresis with ethidium bromide staining and UV irradiation. Actin-specific primers (5′-CAC CGG CCA CCA GTT GCA CA-3′ and 5′-CAG GTC CCG GCC AGC CAG GT-3′, 574 bp, 25 cycles) were used as the positive controls.

Protocol for Allergen Sensitization/Challenge and ESC Treatment Six weeks old female BALB/c were sensitized by intraperitoneal (i.p) administration on days 0, 7, and 14 with 100 μg of OVA in PBS mixed with equal volumes of alum (1 mg) as an adjuvant in a total volume of 200 μl. On days 22 and 24, mice were exposed to aerosolized OVA (1% OVA in PBS) or PBS for 1 h. ESC or dexamethasone (Dexa) was treated for 7 times orally at every 12 h from 1 d before the first challenge to 1 h before the second challenge. The challenge and treatment protocol is shown in Fig. 4A.

BAL Procedure and Determination of Eosinophil Number Eighteen hours after the last aerosol challenge, mice were sacrificed. To obtain BAL fluid (BALF), mice were anesthetized, a tracheal cannula was inserted in each mouse via a midcervical incision, and the airway of each mouse was lavaged three times with 1.5 ml of PBS. BALF was immediately centrifuged (2 min, 4 °C, 1600 g). After removing the supernatant, the cells were resuspended in 0.5 ml of PBS. Ten microliters of the BALF was used for total leukocytes with hemocytometer and the remaining samples were cytospined by staining and UV irradiation. Actin-specific primers (5′-CAC CGG CCA CCA GTT GCA CA-3′ and 5′-CAG GTC CCG GCC AGC CAG GT-3′, 574 bp, 25 cycles) were used as the positive controls.

Statistical Analysis All values were represented as an arithmetic mean ± S.D. One-way ANOVA was used to determine the statistical significance.

RESULTS AND DISCUSSION Murakami et al. reported that BMMC exhibit biphasic PGD$_2$ biosynthetic responses over time, as well as COX-1-dependent immediate and COX-2-dependent delayed responses. The immediate PGD$_2$ generation occurring within
2 h is associated with the coupling of COX-1 with the delayed PGD$_2$ generation, which is observed after several hours of culturing (during 2—10 h). This is associated with the de novo induction and function of COX-2 after stimulation with particular combinations of cytokines and LPS. This cell model also appears to be suitable for examining the effect of 5-LOX inhibitors because the immediate LTC$_4$ generation elicited by the IgE-dependent or cytokine-initiated stimulus occurs in BMMC via 5-LOX. COX-2-dependent PGD$_2$ generation was inhibited in a dose-dependent manner in the presence of ESC when the BMMC were activated with a combination of cytokines and LPS with an IC$_{50}$ value of approximately 14.3 µg/ml (Fig. 1). Under the conditions employed, COX-2-dependent phases of PGD$_2$ generation without the pretreatment of ESC reached approximately 1.6 ng/10$^6$ cells.

Arachidonic acid can also be converted to LTs by the action of 5-LOX in BMMC and the inhibition of 5-LOX is believed to be the ideal treatment for allergic diseases and asthma. Therefore, the inhibitory activity of ESC on the generation of LTC$_4$ in the BMMC was examined. Figure 2 shows that the BMMC stimulated with KL for 15 min produced approximately 500 pg/ml LTC$_4$, and preincubation of the BMMC with ESC resulted in the dose-dependent suppression of LTC$_4$ biosynthesis with an IC$_{50}$ value of 0.3 µg/ml.

Mast cells are implicated in the pathology and mortality of anaphylaxis and other allergic disorders by virtue of their ability to be activated through Fc$_	ext{ε}$RI bound antigen-specific IgE. Mast cells may also be activated by various cytokines through each cytokine receptor. Activation through any of these receptors leads to release of a number of biologically active molecules, including histamine, serotonin, proteoglycans and neutral proteases. Among these molecules, histamine is one of the most important chemical mediators in the pathologic allergic reaction. When mast cells are activated by various stimuli, the release of histamine bears a close parallel to that of β-HEX, which is one of degranulation marker. Therefore, the inhibitory activity of ESC on the degranulation reaction in the BMMC was examined. As shown in Fig. 3, ESC caused the dose-dependent inhibition of β-HEX release with an IC$_{50}$ value of 1.3 µg/ml. Under the conditions employed, β-HEX was released about 23—27% without the pretreatment of ESC.

In a murine asthma model, not only eosinophilia in BALF and lung but also the AHR were reduced in genetically mast-cell-deficient mice. However, the eosinophilia and AHR were restored by adoptive transfer of BMMC, proving mast cells as key effector cells for the pathophysiology of bronchial asthma. On allergen challenge of airways, the mast cells release the newly synthesized mediators. Among those mediators, LTs and PGs whose increased production has been demonstrated in asthma have significant effects on the eosinophil activation and chemotaxis. Because ESC inhibited both PGD$_2$ and LTC$_4$ production in BMMC, these results have led us to determine the biological activity of ESC in vivo animal model. When 50—200 mg/kg of ESC was administered orally to the mice 7 times, the number of eosinophils was weakly reduced in a dose-dependent manner (Fig. 4B). The number of total leukocytes in the BALF obtained from the PBS-challenged group was 0.88 ± 0.21 × 10$^5$ cells, but relatively few eosinophils were detected in this group. On the other hand, the total number of leukocytes (6.2 ± 1.5 × 10$^5$) and eosinophils (3.84 ± 0.04 × 10$^3$) in the BALF of the OVA-challenged lungs was significantly higher compared with the PBS-challenged group (data not shown). We could speculate from the result that the reduced eosinophil recruitment into the airway may result from the
decreased LTC₄ and PGD₂ production from mast cells by ESC directly or indirectly. The chemokine termed eotaxin is a potent eosinophil chemotactic factor that plays a central role in eosinophilic airway inflammation in asthma. Clinical and experimental investigations have reported a strong correlation between the presence of CD4⁺ T₉₂ cells and disease severity, suggesting an integral role for these cells in the pathophysiology of asthma. Among the T₉₂ cytokines, high levels of IL-4 and IL-13 were produced at in an asthmatic lung and are believed to be the key regulators of many hallmark features of disease. The effect of ESC on the mRNA expression of the eosinophil % was expressed as a percentage in total leukocytes. Data were represented as arithmetic means ± S.D. (n=6). **p<0.01 was considered significantly different from the OVA-induced mice using an one-way ANOVA test.

Fig. 4. Effect of in Vivo Administration of ESC on Eosinophil Recruitment in BALF after Inhalation of OVA

(A) Immunization protocol. All mice were sensitized by intraperitoneally with OVA (100 µg/ml) on days 0, 7 and 14. The mice were then exposed to aerosolized OVA or PBS for 1h on days 22 and 24. The mice were orally administered with ESC or dexamethasone (Dexa) for 7 times at 12 h intervals from days 21 to 23. (B) Eosinophils from BALF were counted using a hematocytometer and stained with Diff-Quick solution. The eosinophil % was expressed as a percentage in total leukocytes. Data were represented as arithmetic means ± S.D. (n=6). **p<0.01 was considered significantly different from the OVA-induced mice using an one-way ANOVA test.

Fig. 5. Expression of Eotaxin and IL-4 mRNAs in the Lung Tissue

Eighteen hours after the second aerosol challenge, mice were sacrificed and lungs were taken. Total RNA from lungs of mice was subjected to RT-PCR analysis. Orally administrated amount for ESC and dexamethasone (Dexa) was 100 mg/kg and 5 mg/kg, respectively.

the level of T₉₂ cytokines and an eotaxin accompanied by the enhanced accumulation of eosinophils in the lung. Therefore, the reduction of infiltrated eosinophils in the lung by ESC may occur in part via the inhibition of eicosanoid generation, which reduced directly or indirectly the level of eotaxin and T₉₂ cytokines such as IL-4 mRNA expression.

This paper is the first to show that S. chinensis has anti-asthmatic activity in vivo animal model. The results from this study provide an additional rationale for using S. chinensis for treating inflammation related disorders.

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