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## Original Research Article

# Investigation of the Antiasthmatic Properties of Ethanol Extract of *Callophyllis japonica* in Mice

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## Abstract

***Purpose:*** *To determine whether an ethanol extract from Callophyllis japonica (C. japonica) could attenuate indices of airway inflammation in a murine model of ovalbumin (OVA)-induced asthma.*
***Methods****: The free radical scavenging activity of the C. japonica ethanol extracts (CJE) were investigated using an electron spin resonance (ESR) system. To make develop animal model of asthma, mice were sensitized and challenged with OVA.*
***Results:*** *CJE exhibited considerable scavenging activity of 71.08 ± 0.73, 79.11 ± 6.04%, 75.95 ± 7.01%, and 48.56 ± 5.96% of DPPH, alkyl, superoxide, and hydroxyl radicals, respectively. The successive intraperitoneal administration of CJE reduced the number of eosinophils in bronchoalveolar lavage (BAL) fluid, development of airway hyperresponsiveness (AHR), an increase in pulmonary Th2 cytokines, and allergen-specific immunoglobulin E (IgE).*
***Conclusion:*** *Administration of CJE markedly alleviates all indices of airway inflammation. This study provides evidence that CJE plays a critical role in the amelioration of the pathogenetic process of allergic asthma in mice.*

***Keywords:*** *Asthma, Phenolic contents, Free radical scavenging, Airway hyper-responsiveness, Cytokines, Immunoglobulin E*

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## INTRODUCTION

Asthma is a chronic respiratory disease characterized by airway hyperresponsiveness (AHR), intermittent pulmonary obstruction, and pulmonary and airway inflammation [1]. The inflammatory response in the asthmatic is associated with the infiltration of mast cells, lymphocytes, and eosinophils into the bronchial lumen and lung tissue. There is accompanying increase in the expression of several inflammatory proteins including cytokines, enzymes, and adhesion molecules [2-4]. T-helper 2 (Th2) cells are the predominant lymphocyte population that infiltrates the airways in animal models of asthma [5]. The Th2 cell derived cytokines, including interleukins (IL)-4 and IL-5 are produced by activated $CD4^+$ T cells and play central roles in the pathophysiological

features of allergic inflammation in asthma. They control the key processes of immunoglobulin E (IgE) production, and the differentiation and activation of mast cells and eosinophils [6,7].

Marine algae have been traditionally used for medicinal and dietary purposes in Asia. These algae are rich in vitamins, minerals, dietary fibers, essential fatty acids, enzymes, polysaccharides, and various functional polyphenols. Almost all the algae species had good ability to scavenge free radicals [8].

The red seaweed *Callophyllis japonica* (*C. japonica)* has been traditionally eaten in oriental regions for as dietary component. Previous studies have shown that *C. japonica* extracts have antioxidant activities and radioprotective effects [9,10]. However, their role in asthma and other airway diseases has not been reported. Therefore, we investigated whether *C. japonica* ethanol extracts (CJE) have anti-inflammatory properties against allergic airway reactions in a murine model of ovalbumin (OVA)-induced asthma.

## EXPERIMENTAL

### Preparation of *C. japonica* ethanol extract (CJE)

The red seaweed *C. japonica* was collected along the Coast of Jeju Island in Korea between October 2005 and February 2006. It was authenticated by Dr. Jongchul Lee (Jeju Technopark, Jeju, Korea), and a voucher specimen (no: JBRI 20365) was preserved in the herbarium of Jeju Technopark, Jeju, Republic of Korea. Fresh *C. japonica* was washed three times with tap water and was stored at −20°C. Before extraction, the frozen samples were lyophilized, and homogenized using a grinder. The dried *C. japonica* powder (1 kg) was extracted with 95% ethanol (EtOH) (1:10 w/v) and was evaporated *in vacuo*. The concentrated CJE was freshly dissolved in 10% EtOH (10 mg/ml) before use. Prior to extraction, lyophilized C. japonica was ground into powder by homogenized using a grinder. The dried C. japonica powder (1 kg) was extracted with 95% ethanol (EtOH) (1:10 w/v). After 8 h of extractions at room temperature, the supernatant and the sediment were separated by vacuum filtration. The residue was re-extracted as the first extraction the obtained extraction solutions were combined and concentrated to dryness by vacuum-evaporator at 40°C. The dried extract was weighed and the yield was calculated. The dry extract was kept in dark at 4°C until further analyses.

### Determination of phenolic and carbohydrate contents

The phenolic contents of *C. japonica* were determined by the method described by Shetty et al. [11]. Each 1 ml of the algal extract was mixed with 1 ml of 95% EtOH, 5 ml of distilled water, and 0.5 ml of 50% Folin–Ciocalteu reagent (Sigma Chemical, St. Louis, MO). The mixtures were allowed to react for 5 min before 1 ml of 5 % $Na_2CO_3$ was added. Thereafter the mixtures were placed in the dark for 1 h. Absorbance was measured at 725 nm, and a gallic acid standard curve was obtained for the calibration of phenolic content. The total carbohydrate content was measured by the phenol–sulfuric acid method described by Dubois et al. [12], using a mixture of galactose and fucose (1:1 weight ratio) as a standard. The sulfate content in the total carbohydrate of *C. japonica* was measured by the $BaCl_2$/gelation method [13].

### Measurement of free radical scavenging activity by electron spin resonance spectroscopy

Diverse radicals were generated according to the procedures described below and spin adducts were recorded using a JES-FA electron spin resonance (ESR) spectrometer (JEOL Ltd.,Tokyo, Japan). Radical scavenging activity (RSA) was calculated as in Eq 1, in which H and $H_0$ are the relative peak heights of the radical signals with and without sample, respectively.
RSA (%) = $\{(1 H)/(H_o)\} \times 100$ ………………… (1)

#### *2,2-diphenyl-1-picrylhydrazyl radical scavenging activity*

2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity was measured using the method described by Nanjo *et al.* [14].

#### *Alkyl radical scavenging assay*

Alkyl radicals were generated according to the method described by Hiramoto *et al* [15].

#### *Superoxide anion radical scavenging activity*

Superoxide anion radicals were generated by a UV-irradiated riboflavin/EDTA system [16].

#### *Hydroxyl radical scavenging activity*

Hydroxyl radicals were generated by the iron-catalyzed Fenton Haber-Weiss reaction, and the generated hydroxyl radicals were rapidly reacted with nitrone spin traps (5,5-dimethyl-1-pyrroline-N-oxide, DMPO) [17].

## Animals

Female BALB/c mice, 25–30 g, were purchased from Orient Biotech Inc. (Seoul, Korea) and were allowed to acclimatize to our animal facility for at least 1 week. All experimental animals used in this study were maintained under a protocol approved by the Institutional Animal Care and Use Committee of Inje University Medical School, Busan, Republic of Korea (approval ref no. 2009-017). The procedures involving animals and their care conformed to the standard guideline for the use of laboratory animals [18]. National Institutes of Health, USA. Public health service policy on human care and use of laboratory animals. 2002.

## Sensitization and challenge

Mice were sensitized by intraperitoneal (i.p.) injection of 20 µg of OVA and 1.0 mg of aluminum hydroxide adjuvant on days 1 and 15. Mice were injected i.p. with 20 mg/kg CJE dissolved in 200 µl saline of on consecutive days from days 16 to 20. The animals were challenged via the airway with OVA (50 mg/ml of saline) each day from days 22 to 24 [19]. The control mice were exposed to aerosolized saline. Aerosolization was performed for 20 min by placing the mice in a chamber (15 × 25 × 15 cm) connected to an ultrasonic nebulizer (NE-U12, Omron, Tokyo, Japan).

## Bronchoalveolar lavage (BAL) and cell counting

Mice were anesthetized, and the trachea was cannulated while gently massaging the thorax. The lungs were lavaged with 0.7 ml of phosphate buffered saline (PBS). The bronchoalveolar lavage (BAL) fluid samples were collected and the number of cells in a 50 µl aliquot was determined using a hemocytometer. The pellet was resuspended in PBS, and cytospin preparations of BAL cells were stained with Diff-Quik (International Reagents Corp., Kobe, Japan). Approximately 400 cells were counted in each of 4 different random locations [20].

## Histological studies

Two days after the last ovalbumin challenge, the mice were sacrificed, and their lungs were removed. Lung tissues were fixed with 10% (v/v) paraformaldehyde. The specimens were dehydrated and embedded in paraffin. Sections (4 µm) of fixed embedded tissues were cut on a Leica model 2165 rotary microtome (Leica, Nussloch, Germany), placed on glass slides, deparaffinized, and sequentially stained with hematoxylin-2 and eosin-Y for histological examination.

## Assay of Interleukins

The levels of cytokines in BAL fluid were determined by an enzyme-linked immunosorbent assay (ELISA). Levels of IL-4 and IL-5 were measured by ELISA kits (BioLegend, San Diego, CA) [20].

## Measurement of OVA-specific serum levels of IgE

The OVA-specific serum IgE levels were determined by ELISA using samples collected 12 h after the last OVA challenge. In brief, a 96-well microtitre plate was coated with OVA (10 mg/ml) and was then treated with mouse sera, followed by biotin-conjugated rat anti-mouse IgE (Pharmingen, San Diego, CA). Then avidin-horseradish peroxidase (HRP) solution was added to each well. The optical density of these units was measured at 405 nm [21].

## Determination of airway hyper-responsiveness

Airway hyper-responsiveness (AHR) was measured 3 days after the last OVA challenge in mice in an unrestrained, conscious state, according to the method of Hamelmann *et al* [22]. Mice were placed in a barometric plethysmographic chamber (All Medicus Co., Seoul, Korea), and baseline readings were taken and averaged for 3 min. Aerosolized methacholine (Mch) was then nebulized in increasing concentrations (from 2.5 to 50 mg/ml) through an inlet of the main chamber for 3 min. Readings were taken and were averaged for 3 min after each nebulization. Bronchopulmonary resistance is expressed as an enhanced pause (Penh) and were calculated as follows: Penh = (Te/RT-1) x (PEF/PIF), where Te is expiratory time, RT is relaxation time, PEF is peak expiratory flow, and PIF is peak inspiratory flow, according to the manufacturer's protocol. The results are expressed as the percentage increase of Penh following challenge with each concentration of Mch, where the baseline Penh (after saline challenge) is expressed as 100%.

## Statistical analysis

Data values are presented as mean ± SEM. Significant differences among the groups were determined using an unpaired Student's *t*-test. A value of $p < 0.05$ was considered statistically significant. Statistical analysis was performed using Prism 5 for Windows software (GraphPad Software, Inc., La Jolla, CA, USA).

## RESULTS

### Phenolic and Carbohydrate Contents of *C. japonica* extract

The yield of *C. japonica* EtOH extract was 9.3 % (% dry wt. of alga). In the chemical analysis, the total phenolic content of *C. japonica* EtOH extract was determined to be 39.8 ± 0.7 mg/g ($n$ = 3). However, low amounts of carbohydrate (2.1 ± 0.3 mg/g, $n$ = 3) were detected in *C. japonica*. The sulfate content of *C. japonica* was determined to be 0.11 mg/g (sulfate/total sugar).

### Antioxidant activity of ethanol extract of *C. japonica*

Figure 1 shows the free radical scavenging activities of the extract on DPPH, alkyl, superoxide, and hydroxyl radicals. At 200 □g/ml, exhibited considerable scavenging activity of 71.08 ± 0.73, 79.11 ± 6.04, 75.95 ± 7.01, and 48.56 ± 5.96 % of DPPH, alkyl, superoxide, and hydroxyl radicals, respectively. The values were compared with those of vitamin C (Vit C, 200 □g/ml) used as the standard.

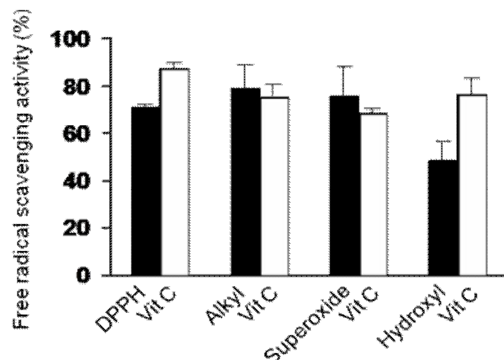


**Fig 1:** Free radical scavenging activity of CJE using an ESR spectrometer. Free radical scavenging activities: DPPH radical scavenging activity, alkyl radical scavenging activity, superoxide radical scavenging activity, hydroxyl radical scavenging activity. Each value is expressed as mean ± SEM (n = 3).

### CJE decrease the number of inflammatory cells in BAL fluids of OVA-sensitized and -challenged mice

Compared with those in the control group, the total cell numbers in the BAL fluids were significantly increased ($p < 0.05$) by approximately 10-fold 2 days after the last OVA challenge (Fig 2). The numbers of eosinophils in the BAL fluid increased significantly ($p < 0.05$). In the CJE-treated group, the numbers were reduced significantly in comparison with the OVA-exposed group.

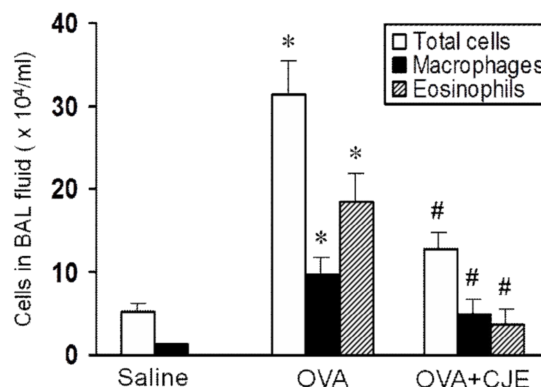


**Fig 2:** Effects of CJE on the recruitment of inflammatory cells in OVA-induced allergic asthmatic mice. Groups of mice were treated with saline, OVA-inhaled mice administered saline (OVA), and OVA-inhaled mice administered 20 mg/kg of CJE, respectively. The BAL cells were collected 2 days after the last OVA-challenge. Each value indicates the mean ± SEM from five separate experiments (n = 6 per group). *p < 0.05 vs. saline; #p < 0.05 vs. OVA.

### Effects of CJE on lung tissue in OVA-induced asthma

Marked influxes of inflammatory cells into the airway and around the blood vessels were detected in the OVA-sensitized/challenged mice (Fig 3B, OVA) but not in the saline-treated control mice (Fig 3, Saline). Mice treated with the CJE evidenced marked reductions in the infiltration of inflammatory cells within the peribronchiolar and perivascular regions (Fig 3, OVA+CJE). Mucus hypersecretion and airway occlusion were the prominent histopathological features of the murine asthmatic lung. Mucus cell hypertrophy and airway luminal narrowing caused by the secreted mucus were observed in the OVA-sensitized/challenged mice (Fig 3, OVA). The administration of CJE induced a marked improvement in luminal narrowing in the airway (Fig 3, OVA+CJE).

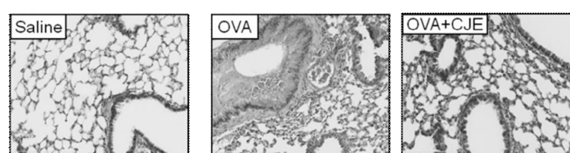


**Fig 3:** CJE inhibit pathological changes in lung tissue of OVA-sensitized and -challenged mice. Lungs were removed 2 days after the last airway challenge. Sections were stained by hematoxylin and eosin staining (×200). Six animals were assigned to each group.

### Effect of CJE on the regulation of levels of bronchoalveolar lavage fluid cytokines in OVA-sensitized and OVA-challenged mice

Four hours after the last airway challenge, BAL fluids were obtained. The levels of IL-4 and IL-5 in the BAL fluids were significantly increased ($p < 0.05$) by airway challenge with OVA, when compared with that of the control. The administration of CJE reduced the concentrations of IL-4 and IL-5 ($p < 0.05$) (Fig 4A).

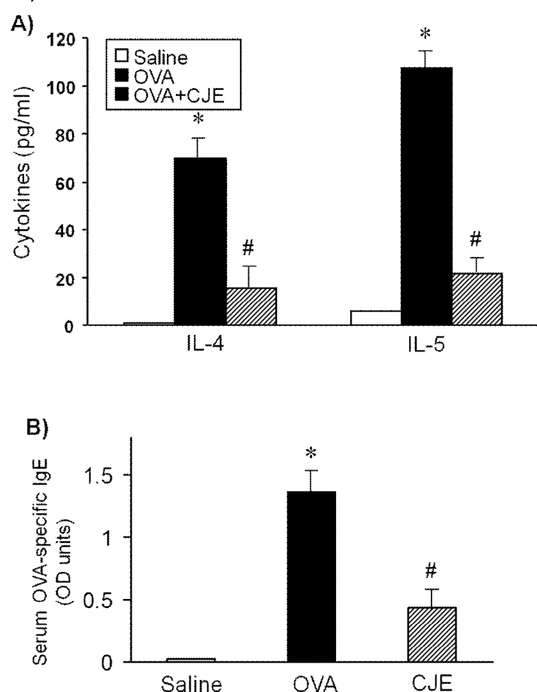


**Fig 4:** The effect of CJE (20 mg/kg) on IL-4 and IL-5 cytokine levels. The IL-4 and IL-5 cytokine levels in the BAL fluids were measured by an ELISA kit. B) Effects of CJE on serum OVA-specific IgE antibody levels. Each value indicates the mean ± SEM from five separate experiments (n = 6 per group). **p* < 0.05 vs. saline; #*p* < 0.05 vs. OVA.

### Effect of CJE on OVA-specific serum IgE levels

Serum levels of OVA-specific immunoglobulins were measured 12 h after the final airway challenge. We found that sensitization and challenge with OVA resulted in increased ($p < 0.05$) serum levels of OVA-specific IgE when compared with saline-treated mice. Treatment of sensitized mice with CJE resulted in a 72% reduction ($p < 0.05$) in OVA-specific IgE (Fig 4B).

### Effect of CJE on airway hyper-responsiveness

In OVA-sensitized and -challenged mice, the dose-response curve of percent Penh was shifted to the left compared with that of control mice (Fig 5). In addition, the percent Penh produced by methacholine administration (at concentrations from 2.5 mg/ml to 50 mg/ml) increased significantly ($p < 0.05$) in the OVA-sensitized and -challenged mice compared with controls. Moreover, OVA-sensitized and -challenged mice that were treated with CJE showed a concentration-response curve that shifted ($p < 0.05$) to the right compared with that of untreated mice.

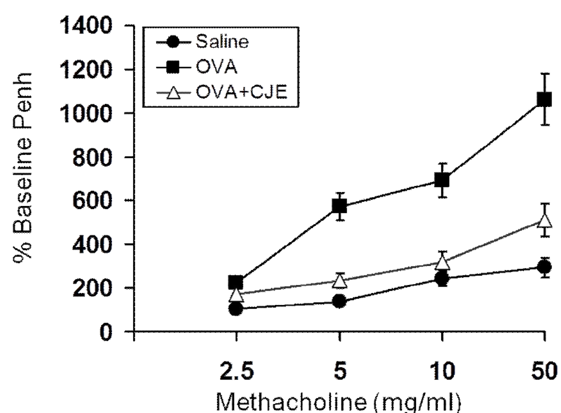


**Fig 5:** Effects of CJE on airway responsiveness in OVA-sensitized and -challenged mice. Each value indicates the mean ± SEM from five separate experiments (n = 6 per group). *p < 0.05 vs. saline; #p < 0.05 vs. OVA.

## DISCUSSION

Airways are unique in both their exposure to high levels of environmental oxidants and their unusually high concentration of extracellular oxidants [23]. Therefore, the role of oxidative stress in asthma is gaining increasing scientific attention. In recent years, many marine algae extracts have been demonstrated to have strong antioxidant properties [24,25]. On the basis of the information, before investigating the effects of a CJE in a murine model of OVA-induced asthma, we assessed the polyphenolic contents of the CJE. Polyphenols from marine algae, called phlorotannins, have received the greatest attention and have been investigated extensively because they have high capacity for scavenging free radicals and have few side effects [26]. Our study has shown that the total phenolic content was high in the EtOH extract of *C. japonica*. This finding of high phenolic and low carbohydrate content in red algae is consistent with previously published results for brown algae [27].

As shown in Figure 1, we found that the ethanol fraction from the CJE exhibited strong free radical scavenging activity. This suggests that CJE should be considered as a drug for the prevention or treatment of ROS-related diseases.

Airway allergen challenge causes a significant tissue infiltration of eosinophils in both humans and mice [28]. In the present study, CJE treatment noticeably suppressed infiltration of eosinophils in BAL fluids of mice (Fig 2). The results of histological examination of the lung section are in agreement with the cell numbers in BAL fluids (Fig 3). CJE remarkably reduced the infiltration of inflammatory cells in the peribronchiolar and perivascular areas of the mice. Administration of the CJE also resulted in a marked improvement of occlusion of airway diameter [29].

Th2 lymphocytes play essential roles in the initiation and development of asthma by releasing Th2 cytokines [30,31]. Of these, IL-4 especially induces isotype switching in B cells, from IgG to IgE production, regulation of the chemokines required for eosinophil migration, and might increase mucus secretion in allergic airways [32-34]. In this study, treatment with CJE attenuated the increase in IgE (Fig. 4B). The CJE-induced reduction in IgE could be attributed to a decrease in IL-4 levels. IL-5 is most specific to eosinophils and is the major hematopoetin responsible for eosinophil growth, differentiation, mobilization, recruitment, activation, and survival, which induces the release of pro-inflammatory mediators [35]. It has been reported that the infiltration of eosinophils into the asthmatic lung leads to degranulation, resulting in airway epithelial damage and the development of AHR [36]. Therefore, our results indicate that the CJE inhibits eosinophil influx, which might prevent oxidative burst.

## CONCLUSION

Our results strongly indicate that administration of CJE markedly alleviates certain asthmatic features, including the Th2 cytokine production that leads to pulmonary eosinophilia, AHR, and increased IgE levels in an experimental mouse model. Our results support the hypothesis that CJE might prove to be a useful therapeutic approach to allergic airway diseases.

## ACKNOWLEDGEMENT

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