Natural Product Sciences
16(3) : 192-197 (2010)

Anti-inflammatory and Anti-allergic Properties of Water Extract from the Seed of *Phaseolus calcaratus* Roxburgh

Minghao Fang[1], Hyoung-Kwon Cho[2], Yun-Pyo Ahn[1], Sang-Jeong Ro[1], Young-Mi Jeon[1], and Jeong-Chae Lee[1,3,*]

[1]*Department of Orthodontics and Institute of Oral Biosciences, BK21 program and School of Dentistry, Chonbuk National University, Jeonju 561-756, Republic of Korea*
[2]*Center for Health Care Technology development, HanPoong Pharmaceutical Co. Ltd., Jeonju 561-201, Republic of Korea*
[3]*Research Center of Bioactive Materials, Chonbuk National University, Jeonju 561-756, Republic of Korea*

Abstract – The seeds of *Raphanus sativus* L. (RSL) and *Phaseolus calcaratus* Roxburgh (PHCR), the root of *Scutellaria baicalensis* (SB), and the flower of *Lonicera japonica* (LJ) have been traditionally used as herbal medicines for anti-inflammation. Unlike the SB and LJ, little information is available for the scientific bases that show the anti-inflammatory mechanisms of RSL and PHCR. In this study, we prepared boiled water extracts from the medicines and determined their potentials in inhibiting nitric oxide (NO) production, cyclooxygenase-2 (COX-2) expression, and tumor necrosis factor (TNF)-α and interleukin (IL)-6 secretion in lipopolysaccharide (LPS)-stimulated RAW 264.7 cells. The effects of the medicines on serum IgE levels in ovalbumin (OVA)-administrated mice were also studied. The medicines inhibited production of TNF-α and IL-6, and COX-2 expression in LPS-stimulated macrophages. Especially, PHCR water extract showed more potent inhibition on TNF-α production than SB and LJ extracts, but RSL extract did not exert these effects. Similar to the cases of SB and LJ, PHCR extract prevented the phosphorylation of IκBα and c-Jun, and the activation of NF-κB-DNA binding. Further, oral supplementation of PHCR extract attenuated significantly serum levels of total and OVA-specific IgE in OVA-treated animals. These results suggest a possibility that PHCR water extract can be used for the treatment of inflammatory and allergic diseases.
Keywords – Traditional medicines, nitric oxide, pro-inflammatory cytokines, IgE lipopolysaccharide, ovalbumin

Introduction

Many investigators have been focused their efforts on developing anti-inflammatory materials from natural resources. The seeds of *Raphanus sativus* L. (RSL) and *Phaseolus calcaratus* Roxburgh (PHCR), the root of *Scutellaria baicalensis* (SB), and the flower of *Lonicera japonica* (LJ) have been traditionally used as anti-inflammatory medicines (Lin and Shieh, 1996; Tae *et al*., 2003; Xu *et al*., 2007; Zhao *et al*., 2007; Barillari *et al*., 2008; Shih *et al*., 2009). Especially, many studies have shown the molecular mechanisms by which SB and LJ exert their anti-inflammatory properties (Kwak *et al*., 2003; Kim *et al*., 2009; Li-Weber, 2009; Shih *et al*., 2009). Phenolic compounds, such as loniceroside C and luteolin, were isolated from LJ and found to be the major active materials contained in the medicine (Kwak *et al*., 2003; Kang *et al*., 2010).

To date, however, little information is available for the scientific bases that show the anti-inflammatory properties of RSL and PHCR seeds, and indicate the mechanisms by which they exert beneficial effects. In addition, there were no reports that demonstrate anti-allergic properties of these medicines. Therefore, the aims of this study were to investigate the anti-inflammatory effects of RSL and PHCR and to compare their effects to that of SB and LJ. To this end, we prepared water extracts from RSL and PHCR seeds, SB root, and LJ flower after boiling, and determined their potentials in inhibiting nitric oxide (NO) production, cyclooxygenase-2 (COX-2) expression, and tumor necrosis factor (TNF)-α and interleukin (IL)-6 secretion in lipopolysaccharide (LPS)-stimulated RAW 264.7 cells. We also examined the mechanism involved in anti-inflammatory action of the extracts. In addition, the inhibitory effects of the water extracts on ovalbumin (OVA)-stimulated IgE production were studied.

*Author for correspondence
Tel: +82-63-270-4049; E-mail: leejc88@jbnu.ac.kr

Experimental

Chemicals and laboratory wares – Unless otherwise specified, chemicals and laboratory wares were purchased from Sigma Chemical Co. (St. Louis, MO, USA) and Falcon Labware (Becton-Dickinson, Franklin Lakes, NJ, USA), respectively.

Preparation of water extracts – The dried traditional medicines were obtained from traditional herbal market, located in Jeonju (Korea) and identified by Dr. H.K. Cho, director of Center for Health Care and Technology Development, HanPoong Pharmaceutical Co. Ltd. (Korea). The voucher specimens (HP-RSLS, HP-PHCRS, HP-SBR and HP-LJF) were deposited at the Center. Briefly, the dried seeds of RSL (200 g) and PHCR (200 g), SB root (200 g) and LJ flower (200 g) were boiled in 2 L of distilled water for 3 h, respectively. The extracts were centrifuged at 3,200 rpm for 30 min and the supernatants were lyophilized to give 2.529 g, 2.098 g, 6.448 g and 4.227 g of crude powers for RSL, PHCR, SB and LJ, individually. The extracts were stored at –20 °C until use.

Cell culture and treatment – RAW 264.7 macrophage cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS; HyClone, Logan, UT, USA), 2 mM L-glutamine and antibiotics. When the cells had reached 70 - 80% confluence in culture plates, they were treated with each of the water extracts (0 - 100 μg/mL) just before the addition of 1 μg/mL LPS.

Measurement of NO production – Nitrite concentration in culture medium was determined as an indicator of NO produced by LPS-stimulated macrophages. In brief, conditioned media (100 μL) were prepared from the cultures incubated with each of extracts in the presence and absence of 1 μg/mL LPS for 48 h and then mixed with the same volume of Griess reagent. Absorbance of the mixtures was measured at 540 nm using ELISA reader (Packard Instrument Co., Downers Grove, IL, USA).

Measurement of cytokines – The amount of cytokines was determined by ELISA at the Bank for Cytokine Research (Chonbuk National University). Cells cultured in 24-well culture plates were pretreated with different concentrations of water extracts 30 min before exposure to 1 μg/mL LPS. After 48 h incubation, culture supernatants were assessed by ELISA using TNF-α- or IL-6-specific OptEIA™ kit. The amount of cytokines produced was calculated from standard curves generated using known concentrations of recombinant cytokine proteins.

Western blot analysis – Equal protein amounts for each sample were separated by 12% SDS-PAGE and blotted onto PVDF membranes. The blots were probed with primary and secondary antibodies and then developed with enhanced chemiluminescence (Amersham Pharmacia Biotech, Buckinghamshire, UK) prior to exposure to X-ray film (Eastman-Kodak, Rochester, NY, USA). All antibodies (COX-2, β-actin, p-IκBα, p-c-Jun) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Electrophoretic mobility shift assay (EMSA) – The DNA-protein binding reactions were performed for 30 min at room temperature with 10 - 15 μg protein in the 20-μl buffer containing 1 μg/mL BSA, 0.5 μg/mL poly (dI-dC), 5% glycerol, 1 mM DTT, 1 mM PMSF, 10 mM Tris-Cl (pH 7.5), 50 mM NaCl, 30,000 cpm of [α-^{32}P] dCTP-labeled oligonucleotides, and the Klenow fragment of DNA polymerase. The samples were separated on 6% polyacrylamide gels and the dried gels were exposed to X-ray films (Eastman Kodak Co.) for 12 - 24 h at –70 °C.

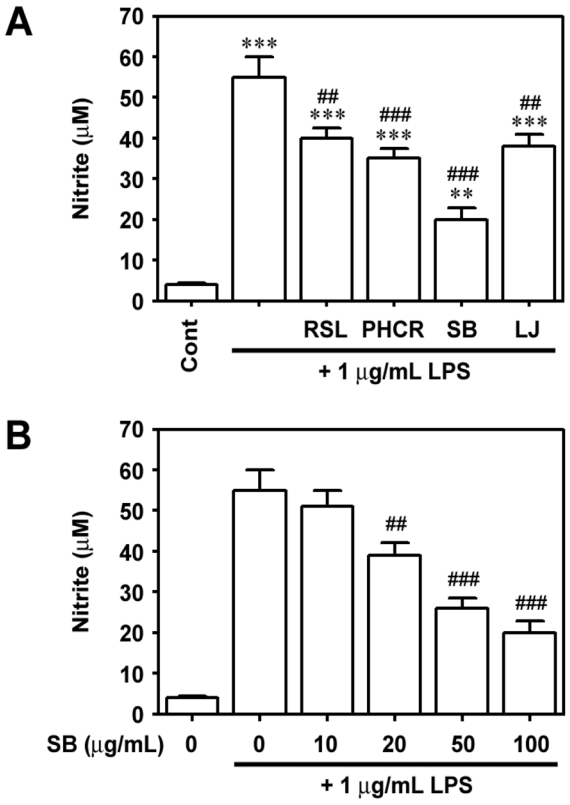

Fig. 1. Effects of water extracts from traditional medicines on NO production in LPS-stimulated RAW 264.7 cells.

(A) Cells were incubated with 100 μg/mL of each extract in the presence and absence of 1 μg/mL LPS for 48 h and the level of NO in the conditioned media was measured. (B) Cells were cultured with the increasing doses of SB water extract in the presence of 1 μg/mL LPS for 48 h, and the level of NO produced was then determined. $^{**}P < 0.01$ and $^{***}P < 0.001$ vs. the non-treated control cells. $^{\#\#}P < 0.01$ and $^{\#\#\#}P < 0.001$ vs. the LPS treatment alone.

The oligonucleotide primer sequences specific for NF-κB were: 5'-AAG GCC TGT GCT CCG GGA CTT TCC CTG GCC TGG A-3' and 3'-GGA CAC GAG GCC CTG AAA GGG ACC GGA CCT GGA A-5'.

Allergen sensitization and challenge – In brief, unsensitized BALB/c animals received IP injections of aluminium hydroxide only in PBS (group I). The mice in group II were sensitized on days 1 and 7 by IP injection of 50 mg OVA adsorbed with 2 mg aluminium hydroxide in PBS and challenged on days 17 and 19 by IP injection with the same dose of OVA. Mice in groups III, IV, V and VI were orally administrated with 2.5 mg/mouse (100 mg/kg body weight) of RSL, PHCR, SB and LJ extracts, respectively, 24 h prior to each of the allergen challenge.

Evaluation of OVA-specific IgE level – After 24 h of the last challenge, mice were anesthetized by inhalation of diethyl ether and whole blood was collected by cardiac puncture using a syringe. Blood samples were then centrifuged at 10,000 × *g* for 10 min at 4 °C and the supernatants were used as serum samples to measure serum IgE levels. The levels of total and OVA-specific IgE in the mice sera were determined by ELISA at the Bank for Cytokine Research (Chonbuk National University, Chonju, Korea). Total IgE concentrations in the sera were calculated from a standard curve of mouse IgE. Sample levels of OVA-specific IgE were expressed as arbitrary units (AU).

Statistical analysis – All data are expressed as mean ± standard deviation (SD). One-way analysis of variance (ANOVA; SPSS version 18.0 software) followed by Scheffe's test was applied to determine differences between the groups. A value of $P < 0.05$ was considered significant.

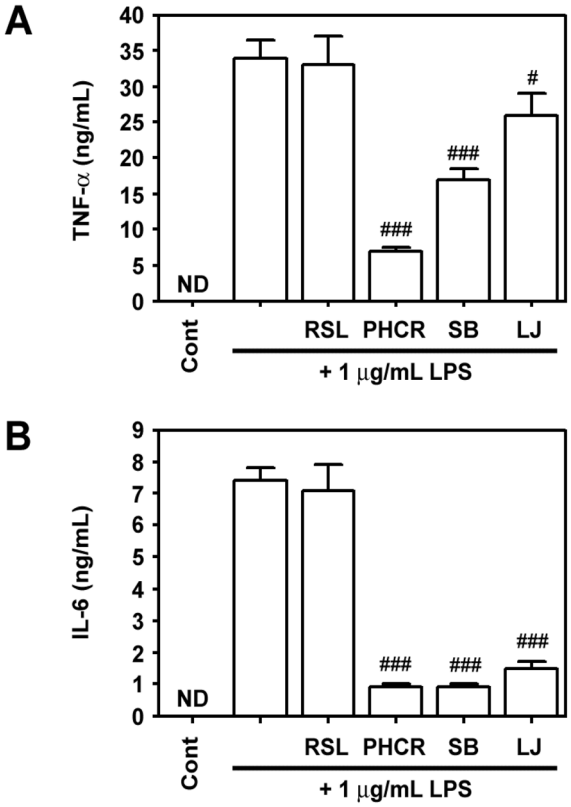

Fig. 2. Effects of water extracts from traditional medicines on the production of pro-inflammatory cytokines in LPS-stimulated RAW 264.7 cells.

Cells were stimulated with 1 μg/mL LPS in the presence of 100 μg/mL of each extract and after 48 h of co-incubation, the levels of TNF-α (A) and IL-6 (B) in the conditioned culture supernatants were measured by ELISA. $^{\#}P < 0.05$ and $^{\#\#\#}P < 0.001$ vs. the LPS treatment alone. ND, not detected.

Results

Effects of water extracts on NO production in LPS-stimulated macrophage cells – When RAW 264.7 cells were exposed to 1 μg/mL LPS for 48 h, the nitrite concentration in a conditioned medium increased to approximately 14-fold (55 ± 4.9 μM), compared to that of the untreated control (4.0 ± 0.5 μM) (Fig. 1A). All the

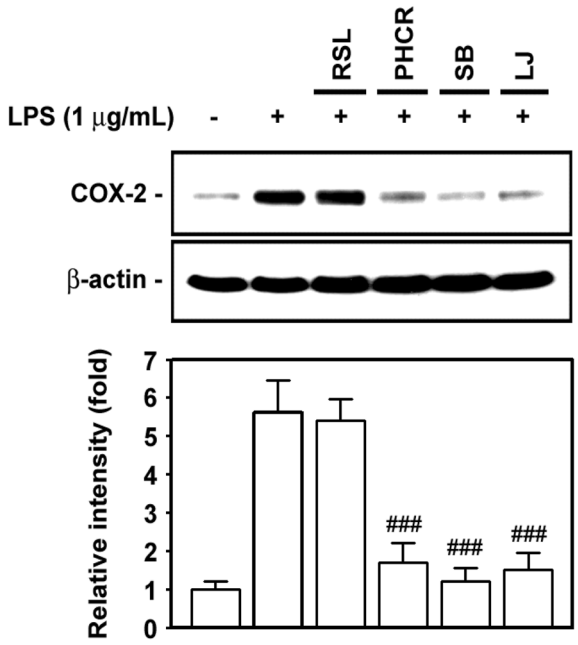

Fig. 3. Suppression by water extracts from traditional medicines on COX-2 expression in LPS-stimulated RAW 264.7 cells.

Cells were exposed to 100 μg/mL of each extract prior to exposure to 1 μg/mL LPS and after 24 h of incubation, whole protein lysates from the cells were adjusted for the analysis of COX-2 expression by immunoblotting. Quantification of COX-2 protein was measured by densitometric analysis after normalizing the bands to that of β-actin. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, and $^{\#\#\#}P < 0.001$ vs. the LPS treatment alone.

medicines diminished significantly LPS-stimulated NO production, where SB extract showed the most efficient activity. As expected, SB extracts showed a dose-dependent suppression on NO production; the addition at 20 and 100 μg/mL reduced nitrite concentration to 39 ± 3.1 μM ($P < 0.01$) and 20 ± 2.9 μM ($P < 0.001$), respectively, compared to the LPS treatment alone (Fig. 1B).

Inhibitory effects of the medicines on LPS-induced cytokine secretion and COX-2 expression in RAW 264.7 cells – Secretion of TNF-α and IL-6 was measured using the culture media of cells stimulated with 1 μg/mL LPS alone or in combination with 100 μg/mL of each of the extracts for 48 h. LPS treatment alone increased the level of TNF-α by 34 ± 2.5 ng/mL in the cells, whereas this was reduced to 7 ± 0.5, 17 ± 1.5, or 26 ± 3.0 ng/mL by treating the cells with 100 μg/mL of PHCR, SB, or LJ extract, respectively (Fig. 2A). Similarly, these extracts showed a marked inhibitory activity on LPS-stimulated IL-6 production in the cells (Fig. 2B). LPS-stimulated COX-2 expression in RAW 264.7 cells was also inhibited almost completely by treatment with 100 μg/mL of each of PHCR, SB, or LJ extract (Fig. 3). RSL extract did not show any significant inhibition on cytokine production and COX-2 expression in LPS-stimulated macrophages (Figs. 2 and 3).

Suppression of IκBα and c-Jun phosphorylation, and NF-κB-DNA binding by the medicines – LPS treatment increased the level of phosphorylated IκBα to 3.6-fold ($P < 0.001$), compared to the untreated control value (Fig. 4A). This increase was suppressed by treating the cells with the extracts from PHCR, SB, or LJ. LPS also increased DNA-NF-κB binding in the cells, and this was inhibited apparently by adding PHCR extract at 50 μg/mL (Fig. 4B). These medicines also attenuated

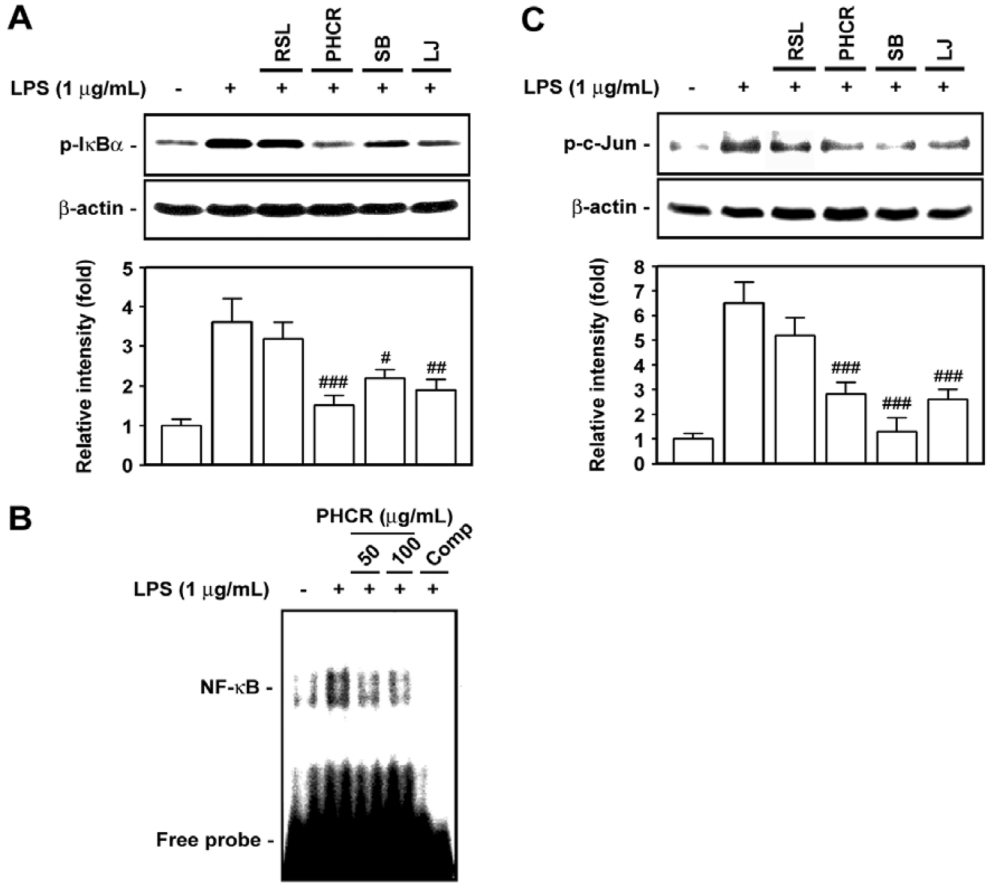

Fig. 4. Effects of water extracts from traditional medicines on IκBα and c-Jun phosphorylation, and NF-κB DNA binding in LPS-stimulated RAW 264.7 cells.

(A) Cells were exposed to 100 μg/mL of each extract in the presence of 1 μg/mL LPS for 1 h, and the levels of p-IκBα in whole protein lysates were detected by Western blotting. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, and $^{\#\#\#}P < 0.001$ vs. the LPS treatment alone. (B) Cells were also exposed to the indicated doses (50 and 100 μg/mL) of PHCR extract in the presence of 1 μg/mL LPS for 1 h, and then nuclear fractions were prepared from the cells to analyze NF-κB binding activity by EMSA. Comp, 50x addition of NF-κB primers. (C) The levels of p-c-Jun in the same protein lysates were detected by Western blotting. $^{\#\#\#}P < 0.001$ vs. the LPS treatment alone.

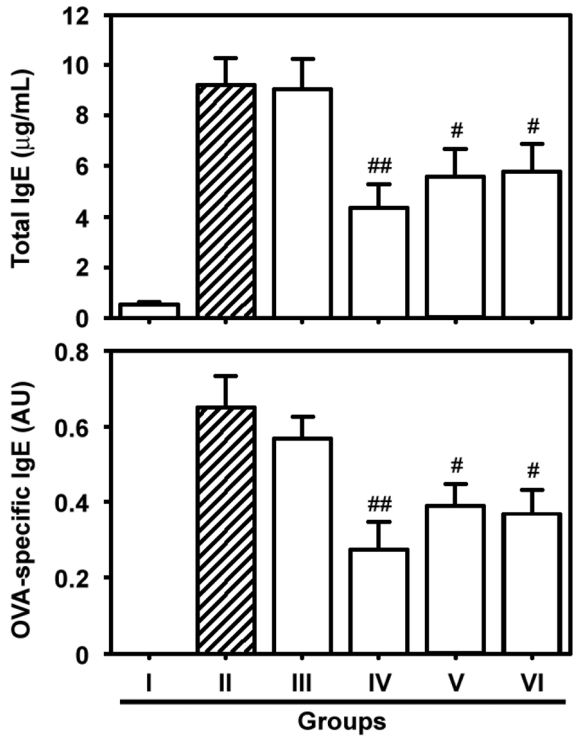

Fig. 5. Evaluation of serum IgE levels in mice subjected to OVA or in combination with the medicinal extracts.

The levels of total and OVA-specific IgE in mice sera were measured by ELISA and total IgE levels were calculated from a standard curve of mouse IgE. The levels of OVA-specific IgE were expressed as arbitrary units (AU).

significantly LPS-induced phosphorylation of c-Jun (Fig. 4C). However, RSL extract did not affect the phosphorylation of IκBα and c-Jun in LPS-stimulated cells.

Inhibitory effects of the medicines on IgE production in OVA-exposed mice – Level of total IgE was increased to approximately 18-fold (9.2 ± 1.1 μg/mL) in OVA-sensitized and challenged mice (group II), as compared with unsensitized mice (group I, 0.52 ± 0.09 μg/mL) (Fig. 5). Increased IgE level was reduced significantly by oral administration with the extracts from PHCR, SB and LJ, where the PHCR extract was the most potent inhibitor. Similarly, increased OVA-specific IgE level was diminished by the combined administration with the medicines. These results indicate that PHCR extract exerts anti-allergic activity by inhibiting the production of serum IgE.

Discussion

In this study, we demonstrate that the water extracts from the medicines tested inhibit significantly NO production in LPS-stimulated macrophage cells, and SB extract is the most potential inhibitor (Fig. 1). Similar to this, the medicines showed the inhibitory potentials on pro-inflammatory cytokines and COX-2 expression in LPS-stimulated macrophages (Figs. 2 and 3). PHCR extract showed more potent inhibition on TNF-α production than other extracts, whereas RSL extract did not have the effect. It was previously reported that the boiled but not non-boiled LJ extract inhibited COX-2 protein and mRNA expression in IL-1β-stimulated A549 cells (Xu *et al*., 2007). In parallel with previous studies, the current finding supported the anti-inflammatory potentials of LJ and SB. This study also revealed for the first time that the water extract from PHCR seed has a potential for anti-inflammation, especially in inhibiting pro-inflammatory cytokines, although this needs further investigations.

NF-κB and AP-1 transcription factors are key regulators of intracellular signaling involved in the production of NO and pro-inflammatory cytokines, and in the COX-2 expression in responses to microbial pathogens (Xagorari *et al*., 2002; Gaestel *et al*., 2007). Numerous studies have highlighted a closed relation between the inhibition of transcription factors and the anti-inflammation induced by traditional medicines. Actually, SB and LJ are known to exert anti-inflammatory activities by suppressing NF-κB and MAPK signaling pathways (Kim *et al*., 2009; Kang *et al*., 2010). Consistent with these studies, the present findings show that in addition to SB and LJ, PHCR seed extract blocks efficiently the phosphorylation of IκBα and c-Jun, and NF-κB-DNA binding activity (Fig. 4). However, additional experiments are required for the elucidation of MAPK roles in regulating transcription factors in PHCR extract and/or LPS-exposed macrophages.

Accumulating evidence has implicated the beneficial roles of phenol containing compounds on anti-inflammation. Oroxylin A, a flavonoid isolated from SB root, showed suppressive effects on NO production, and iNOS and COX-2 expression by inhibiting NF-κB activation (Shih *et al*., 2009). SB also contains various flavonoidic compounds including Wogonin, Baicalein, and Beicalin, which are known to have anti-inflammatory and anti-oxidant potentials (Li-Weber, 2009). A bioflavonoid, ochnaflavone, and a new trierpenoid saponin, loniceroside C, were isolated from LJ and found to have anti-inflammatory potential (Kwak *et al*., 2003). The polyphenols, such as catechin, quercetin, myricetin and myricetin-rutinosides, are also identified as the active components of the ethanol extract from *Phaseolus angularis* Wight, a kind of Adzuki beans (Zhao *et al*., 2007). With this regard, it can be considered that the polyphenols are to be the compounds responsible for the anti-inflammation

induced by PHCR water extract. More detailed experiments will be needed in order to clarify the relationship between the compounds and the anti-inflammation.

In summary, this study demonstrates the anti-inflammatory potentials of water extract from PHCR seed and the possible mechanisms involved. We also show that oral administration of PHCR extract decreases serum IgE level in OVA-treated animals (Fig. 5). These anti-inflammatory and anti-allergic properties of PHCR water extract strongly suggest a possibility that can be used for the treatment of inflammatory and allergic diseases.

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Received July 2, 2010
Revised August 16, 2010
Accepted August 20, 2010