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Inhibitory Effect of the Saponins Derived from Roots of *Platycodon grandiflorum* on Carrageenan-Induced Inflammation

Ji Young KIM,[1] Yong Pil HWANG,[1] Dong Hee KIM,[2] Eun Hee HAN,[1] Young Chul CHUNG,[3] Seong Hwan ROH,[4] and Hye Gwang JEONG[1,†]

[1]*Department of Pharmacy, College of Pharmacy, Research Center for Proteineous Materials, Chosun University, Kwangju, South Korea*
[2]*Department of Pathology, College of Oriental Medicine, Daejeon University, Daejeon, South Korea*
[3]*Division of Food Science, Chinju International University, Chinju, South Korea*
[4]*Jangsaeng Doraji Research Institute of Biotechnology, Jangsaeng Doraji Co., Ltd., Chinju, South Korea*

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Previous studies have reported that the saponins isolated from the roots of *Platycodon grandiflorum* A. DC (Campanulaceae), Changkil saponins (CKS), inhibited cyclooxygenase-2 (COX-2) expression in cultured lipopolysaccharide-activated macrophages. The aim of this presented study was to confirm the anti-inflammatory effects of CKS by examining their effect on the inflammatory response induced by carrageenan in a rat by using an acute air pouch inflammation model. CKS significantly reduced the levels of the inflammatory process markers in the air pouch, such as the volume of exudates, the amount of protein and the number of leukocytes and neutrophils. The levels of TNF-α and prostaglandin E_2 (PGE_2) were also markedly lower in the air pouch of the CKS-treated animals than in the controls. An immunoblot analysis showed that CKS reduced the COX-2 expression level in the exudate cells. In addition, CKS significantly reduced the paw edema induced by carrageenan and also markedly reduced the level of PGE_2 production in the inflamed paw. These results suggest that CKS had significant anti-inflammatory effects *in vivo*.

Key words: saponins; *Platycodon grandiflorum*; anti-inflammation; air pouch; carrageenan

Prostaglandins and nitric oxide (NO) are ubiquitous mediators with numerous vascular and inflammatory effects.[1,2] The production of prostaglandins by the constitutive isoenzyme, cyclooxygenase-1, has been implicated in the physiological regulation of the vascular tone and homeostatic functions. In contrast, cyclooxygenase-2 (COX-2) is not generally expressed in resting cells, but is induced in many cell types including macrophages by stimulation with such appropriate pro-inflammatory agents as cytokines and lipopolysaccharide (LPS).[3,4] The activity of COX-2 causes the over-production of prostaglandins, which play a key role in the pathophysiology of arthritis as well as other inflammatory conditions.[1,2,5] Neutrophils are essential for the host defense, and their contribution to the propagation and maintenance of acute and chronic inflammation comprises of several mechanisms. Activated neutrophils produce leukotrienes which participate in the inflammatory response by stimulating the leukocyte functions and regulating vascular permeability.[6]

Some plants contain non-nutritional constituents that may have beneficial biological activities.[7] Platycodi Radix is the root of *Platycodon grandiflorum* A. DC (Campanulaceae; four years old) and has been used as a food and in traditional oriental medicine to treat such adult diseases as bronchitis, asthma and pulmonary tuberculosis, and inflammatory diseases, as well as being taken as a sedative. In addition, its biological significance has previously been reviewed.[8] Previous studies have found that Changkil (CK), which is an aqueous extract of the root from 20-year-old *P. grandiflorum* plants (Lee, S. H., 1991, Patent on the method of cultivating the perennial balloonflower, Patent No. 045971, Korea), prevented hypercholesterolemia and hyperlipidemia.[9] It was recently been shown that CK had protective effects against acetaminophen- and carbon tetrachloride-induced hepatotoxicity and inhibited the progress of hepatic fibrosis in rats.[10–12] It has also been reported that CK and the saponin fraction (CKS) derived from CK had potent antioxidative effects such as superoxide radical scavenging activity *via* the xanthine and xanthine oxidase system, and the inhibition of ROS production by *tert*-butyl hydroperoxide in hepatocytes and the liver.[11,13]

CK and the saponin fraction (CKS) derived from CK have recently been reported to have anti-inflammatory activity and reduced the COX-2 expression levels by inhibiting the transcription factor, nuclear factor-κB

† To whom correspondence should be addressed. Tel/Fax: +82-62-230-6639; E-mail: hgjeong@chosun.ac.kr

(NF-κB), as well as prostaglandin E_2 (PGE_2) production *in vitro* in lipopolysaccharide (LPS)-stimulated mouse macrophages.[14] This suggests that CKS exerted its anti-inflammatory effects by inhibiting the production of inflammatory mediators at the inflammatory site. However, little is known regarding its anti-inflammatory effect *in vivo*, even though the anti-inflammatory effects of CKS have been shown in *in vitro* cell culture studies. Therefore, the rat air pouch model of acute inflammation was used in this present study to investigate the anti-inflammatory effects of CKS *in vivo*. The rat air pouch model was developed to evaluate the anti-inflammatory activity of various chemicals.[15–17] The degree of non-immunologically-mediated, carrageenan-induced acute inflammation was characterized by analyzing the contents of the air pouch along with the capacity of the exudates and air pouch lining tissues to produce various inflammatory mediators. It was found that CKS suppressed the carrageenan-induced acute inflammation. This is the first report showing the inhibitory activity of CKS on acute inflammation *in vivo*.

Materials and Methods

Chemicals and materials. The chemicals and cell culture materials were obtained from the following sources: kahweol acetate and carrageenan (Type IV) from Sigma Co.; enzyme immunoassay kits for the PGE_2, antibodies to COX-2 and β-actin from Cayman Chemical Co; Bio-Rad Protein Assay kit from Bio-Rad Laboratories; western blotting detection reagents (ECL) from Amersham Pharmacia Biotech. All other chemicals were of the highest commercial grade available and were used without further purification.

Preparation of CKS. CK refers to the aqueous extract obtained from the twenty-two-year-old roots of *Platycodon grandiflorum* A. DC (Campanulaceae)- which was supplied by Jang Saeng Doraji Co. (Jinju, South Korea). The composition of the *P. grandiflorum* root is reported elsewhere.[9] CK and CKS were prepared by using the method described elsewhere.[10,11,13] CK was subjected to column chromatography over Amberlite XAD-2, Diaion MCl gel HP20 or Kogel BG4600. After removing the saccharides and amino acids with water, the column was eluted with methanol to obtain the saponin fraction of CK and CKS as previously described.[18]

Animals. Male Wistar rats, weighing 150–200 g, were purchased from Dae Han Laboratory Animal Research and Co. (Daejeon, Korea). The animals were provided with Purina Rodent Chow and tap water *ad libitum*, and were maintained in a controlled environment at $21 \pm 2\,^{\circ}C$ and a $50 \pm 5\%$ relatively humidity with a 12 h dark/light cycle. The animals were acclimatized for at least 1 week prior to use. All the animal experiments were performed according to the rules and regulations of the Animal Ethics Committee at Chosun University.

Air pouch model of inflammation. The air pouches were produced by a subcutaneous injection of 20 ml of sterile air into the intra-scapular area of the back. Every 2 days, 10 ml of air was injected into the cavity in order to maintain the space. Seven days after the first air injection, 2 ml of a 1% carrageenan solution dissolved in saline was injected into the pouch to produce an inflammatory response. Either CKS or indomethacin was dissolved in Tween 80/saline (1:99, v/v). The animals were treated by daily gavage for 3 days with the only vehicle, CKS (0.5–5 mg/kg) or indomethacin (5 mg/kg). Twenty-four hours after the final treatment, the animals were injected with the carrageenan solution. The animals were sacrificed by diethyl ether inhalation 24 h after administering the carrageenan. A small incision was made in the pouch wall, and the contents of the air pouch were carefully removed with a sterile Pasteur pipette after injecting 5 ml of ice-cold PBS containing 0.1% EDTA. The total volume of the contents was measured, and the exudate volume was obtained by subtracting 5 ml from this total volume. The protein concentration of the exudate was measured by the Bradford method as reported with a Bio-Rad Protein Assay kit. The exuding cells were separated by centrifugation, and the total number of cells (leukocytes) was counted. Differential counts were microscopically performed after staining air-dried smears with a Wright stain. The TNF-α and PGE_2 levels were determined in the supernatant. The isolated cells were used to determine the COX-2 expression level by using immunoblotting.

TNF-α and PGE_2 assay. The levels of TNF-α and PGE_2 in the supernatant were determined by an enzyme-linked immunosorbant assay (ELISA) with commercially available kits according to the manufacturer's instructions.

Immunoblot analysis. Cell lysates were prepared by treating the cells with a lysis buffer (150 mM NaCl, 100 mmol Tris at pH 8.0, 1% Tween 20, 50 mmol diethyldithiocarbamate, 1 mmol EDTA, 1 mmol phenylmethylsulfonyl fluoride, 10 µg/ml of aprotinin, 10 µg/ml of trypsin inhibitor, and 10 µg/ml of leupeptin). SDS–PAGE was performed on 10% polyacrylamide gel under reducing conditions. The resolved proteins were transferred to a polyvinylidene difluoride membrane. After blocking, the membrane was incubated with the anti-COX-2 polyclonal antiserum or monoclonal anti-β-actin. The secondary antibody to IgG conjugated to horseradish peroxidase was used. The blots were probed with the ECL western blot detection system according to the manufacturer's instructions.

Histology. The excised pouch tissues were fixed in 10% formalin in a 0.01 M phosphate buffer (pH 7.4) and embedded into paraffin wax blocks. The sections were stained with haematoxylin and eosin.

Paw edema model of inflammation. The animals were treated by daily gavage for 3 days with the only vehicle, CKS (0.5–5 mg/kg) or indomethacin (5 mg/kg). Twenty-four hours after the final treatment, the animals were injected with the carrageenan (0.05 ml; 3% w/v in saline) into the subplantar area of the right hind paw. The volumes of the injected and contralateral paws were measured 3 and 5 h after inducing an edema by using a plethysmometer. The edema volume is expressed for each animal as the difference between the carrageenan-injected and contralateral paws. After determining the extent of the paw edema (5 h), the animals were sacrificed by cervical dislocation, and the right hind paws were homogenized in 2 ml of saline. Aliquots of the supernatant were used to determine the PGE_2 level as already described.

HPLC analysis of CKS. The analysis of CKS was performed by an HPLC system (Shimadzu, MD) consisting of a UV detector (SPD-10A), a pump (LC-10AD), and an automatic injector (SIL-10A). The wavelength of the UV detector was 210 nm, and a reversed phase column (Gemini C18 5Φ μmol, 4.6 × 150 mm, Phenomenex, CA, USA) was used. The column temperature was maintained at 40 °C by a column temperature controller (TS130, Phenomenex, CA). The flow rate was 0.7 ml/min. The mobile phase consisted of methanol and 50 mmol phosphate buffer (pH 3.0), a stepwise gradient being used from 27% methanol to 60% methanol.

Statistical analyses. Each result is expressed as the mean ± SD, and the data was analyzed by one-way ANOVA followed by Dunnett's test or Student's t test for significant difference. A P value < 0.05 is considered significant.

Results

Effect of CKS on the volume, protein amount and cell count in the exudate from an air pouch

The air pouches obtained from the control or the CKS-treated rats were assessed 24 h after the carrageenan challenge. Initially, the inflammatory process in an the air pouch was evaluated by measuring the volume and protein content of the exudate as well as the differential cell count of the infiltrated exudate. The volume and total protein content of the air pouch exudate were significantly lower in the CKS-treated animals than in the controls (Fig. 1A). The leukocyte count in the exudate was also significantly lower in the CKS-treated animals than in the controls (Fig. 1B). A differential analysis showed that more than 82% of the exudate cells (leukocytes) obtained from the control animals were neutrophils and that the neutrophil count in the exudate was also significantly higher in the CKS-treated animals (Fig. 1B). Indomethacin, which was used as a reference compound, significantly reduced the measured parameters.

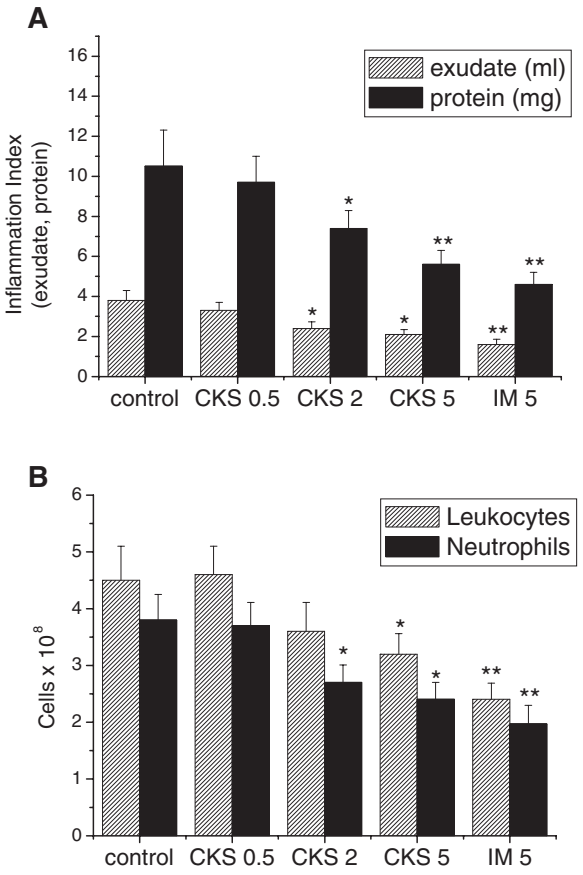

Fig. 1. Effects of CKS on the Volume, Protein Content and Cell Count in Exudates from the Air Pouches.

The animals were pretreated with CKS (mg/kg) or indomethacin (IM, mg/kg) before the carrageenan injection. The animals were sacrificed 24 h later, and the exudate from eash air pouch was collected. The volume of the exudate and protein amount (A) and the numbers of leukocytes and neutrophils in the exudate were assessed (B). Each value is expressed as the mean ± SD of two individual experiments, performed on 6 rats in each group. $^{*}P < 0.05$ and $^{**}P < 0.01$ compared with the control.

Effect of CKS on the release of inflammatory mediators

At the inflammatory sites, many cells including macrophages/monocytes, neutrophils, fibroblasts and T lymphocytes generated a variety of inflammatory mediators. The content of the inflammatory mediators in the exudate from each animal was measured. As shown in Fig. 2, the concentrations of such inflammatory mediators as TNF-α and PGE_2 were markedly lower in the exudates from the CKS-treated animals. Indomethacin (5 mg/kg) significantly reduced the PGE_2 level without affecting the concentration of the TNF-α.

Effect of CKS on COX-2 expression

The expression of COX-2 within the exudate cells was analyzed by immunoblotting in order to determine if the decreased PGE_2 level in the air pouch from the CKS-treated animals had been caused by the inhibition of COX-2 up-regulation. The results showed that the

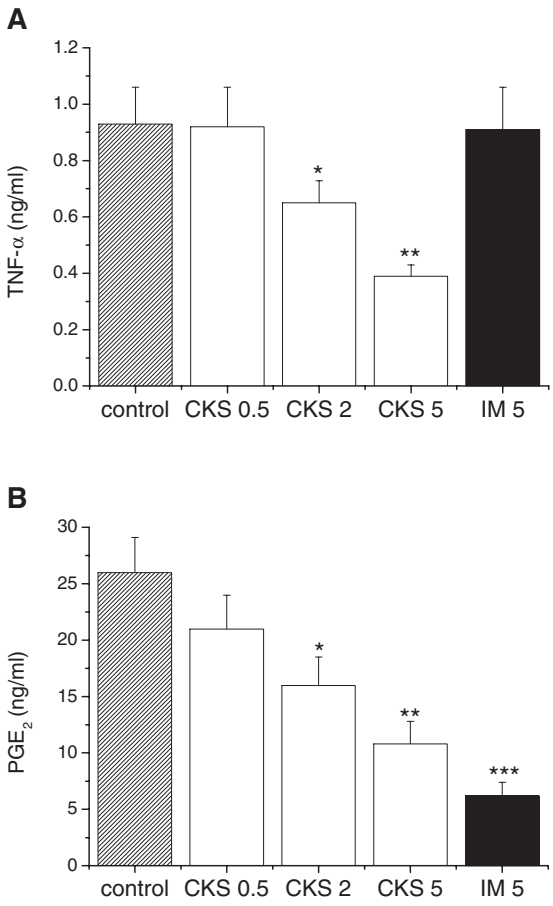

Fig. 2. Effects of CKS on the Release of Inflammatory Mediators.

The animals were pretreated with CKS (mg/kg) or indomethacin (IM, mg/kg) before the carrageenan injection. The animals were sacrificed 24 h later, and the exudate from each air pouch was collected. The levels of TNF-α (A) and PGE_2 (B) in the exudate were measured. Each values is expressed as the mean $\pm$ SD of two individual experiments, performed on 6 rats in each group. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.005$ compared with the control.

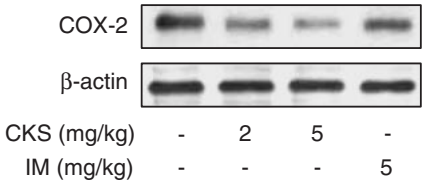

Fig. 3. Effects of CKS on the Expression of COX-2 in the Exudate Cells.

The animals were pretreated with CKS (mg/kg) or indomethacin (IM, mg/kg) before the carrageenan injection. The animals were sacrificed 24 h later, and the exudate in each air pouch was collected. The isolated exudate cells were pooled from 6 rats in each treatment group. The cell lysate (30 μg of protein) was separated by SDS–PAGE, transferred to a nitrocellulose membrane and blotted with an anti-COX-2 or β-actin antibody, as described in the Materials and Methods section. One of two representative experiments is shown.

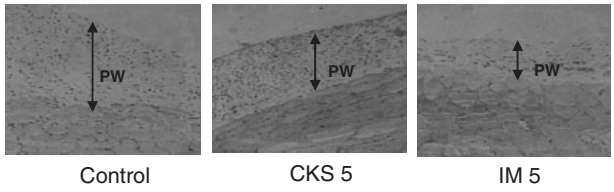

Fig. 4. Histological Change in the Pouch Tissues of the CKS-Treated Rats.

The animals were pretreated with CKS (mg/kg) or indomethacin (IM, mg/kg) before the carrageenan injection. The animals were sacrificed 24 h later and the air pouch was removed from each rat, fixed and embedded in paraffin. Sections were stained with hematoxylin-eosin. PW, inflamed pouch wall. 100×.

COX-2 expression level in the exudate cells from the CKS-treated animals was significantly lower than that from the control animals (Fig. 3). This suggests that CKS might have inhibited the up-regulation of COX-2 expression in the exudate cells at the inflammatory site. As expected, indomethacin did not affect the expression of COX-2.

Histological analysis of the inflammatory air pouch

The inflammatory response in the pouch tissues was also examined histologically. The infiltration of many inflammatory cells such as neutrophils, macrophages and lymphocytes was observed in the pouch wall, and the tissue edema contributed to the enlargement of the wall in the vehicle-treated animal (Fig. 4). In contrast, the pouch wall of a CKS-treated animal consisted of thin connective tissue and showed little evidence of an acute inflammatory response. These findings suggest that the CKS treatment suppressed the acute inflammatory response in the pouch wall.

Effect of CKS on the paw edema

An intraplantar injection of carrageenan to the rats caused an inflammatory reaction. A pretreatment with CKS caused a dose-dependent reduction in the hind paw edema (Fig. 5A). Indomethacin (5 mg/kg) was used as the reference compound, showing a significant reduction in swelling after administering the carrageenan. The level of the edema was determined by examining the PGE_2 level (Fig. 5B) in the homogenate of an inflamed paw. The PGE_2 level was significantly reduced by CKS in a dose-dependent manner. Indomethacin also significantly reduced the PGE_2 level.

HPLC analysis of CKS

The HPLC spectrum of CKS resulted in peaks six different platydodins (saponins) with the following retention times (Rt, min) (Fig. 6): platycoside E (1, Rt 85.1 min), platycodin D_3 (2, Rt 93.0 min), polygalacin D_2 (3, Rt 101.9 min), platycodin D_2 (4, Rt 107.3 min), deapioplatycodin D (5, Rt 109.9 min), and platycodin D (6, Rt 111.3 min). No other peaks could be correctly identified as platydodins.

Discussion

P. grandiflorum is commonly used in traditional oriental herbal medicines and has beneficial effects on

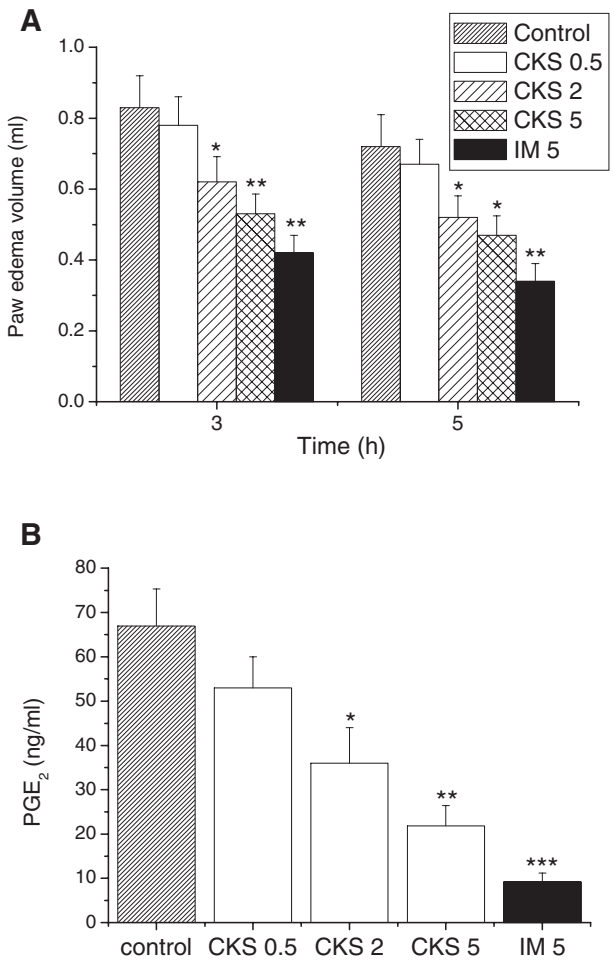

Fig. 5. Effects of CKS on Carrageenan-Induced Paw Edema.

The animals were pretreated with CKS (mg/kg) or indomethacin (IM, mg/kg) before the carrageenan injection. After inducing inflammation with carrageenan, the volume of the paw after 3 h and 5 h (A) and the PGE_2 level in inflamed paw after 5 h (B) were measured as described in the Materials and Methods section. Each value is expressed as the mean ± SD of two individual experiments, performed on 6 rats in each group. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.005$ compared with the control.

inflammatory diseases.[8] Previous studies have demonstrated that CKS isolated from *P. grandiflorum* had antioxidative and anti-inflammatory effects.[11,13,19] Furthermore, CKS has been reported to inhibit COX-2 expression as well as the subsequent production of PGE_2 by LPS-activated macrophages *in vitro*.[14] These studies suggest that CKS might function as an anti-inflammatory agent, possibly by suppressing the production of a number of inflammatory mediators. This present study documents for the first time that CKS inhibited the carrageenan-induced acute inflammation in a rat air pouch model. The degree of non-immunologically-mediated, carrageenan-induced acute inflammation was determined by analyzing the contents of the air pouch. This was manifested by the amount of fluid exuded, protein content, number of exudate cells (particularly neutrophils), and the content of the inflammatory mediators. This model has been used to estimate the anti-inflammatory effects of various drugs.[15–17]

A CKS pretreatment reduced the level of fluid exudation, protein content, the number of exudate cells (especially neutrophils), and the content of the inflammatory mediators, including TNF-α and PGE_2, in the air pouch exudate (Figs. 1 and 2). A histological analysis of the pouch tissues also demonstrated the anti-inflammatory effect of CKS (Fig. 4). This suggests that CKS inhibited the function of inflammatory cells that produced these pro-inflammatory mediators, highlighting the *in vivo* anti-inflammatory effect of CKS. It is probable that neutrophils and macrophages were the targets of the anti-inflammatory effects of CKS, because they contributed toward the majority of exudate cells and are known to produce the inflammatory mediators that were assessed.[15] It should be noted that there was a lower number of neutrophils in the exudates, which is indicative of the anti-inflammatory activity of CKS (Fig. 1). Whether the decrease in number of polymorphonuclear leukocytes in the exudate was a primary effect of CKS on the neutrophil function or an effect secondary to the inhibition of the local production of chemotactic or chemokinetic molecules remains to be determined. Monocytes/macrophages are the main sources of chemokines, as well as of TNF-α and PGE_2.[20] Therefore, the suppressive effect on monocytes/macrophages that produced large amounts of the inflammatory mediators is likely to be a major part of the anti-inflammatory action of CKS. However, a direct effect of CKS on the function of each of the cellular components in the inflammatory response to carrageenan would be needed to determine the cellular basis for the anti-inflammatory effect of CKS.

The induction of COX-2 greatly increases the level of PGE_2 synthesis, which contributes to the pathophysiology of various inflammatory processes. PGE_2 overproduction as a result of COX-2 expression *in vivo* has been reported for chronic inflammatory conditions such as rheumatoid arthritis,[2] as well as in experimental models of inflammation such as the air pouch and paw edema.[5,15–17,21] These results suggest that CKS is effective in treating experimental inflammation, and is accompanied by a decrease in the TNF-α and PGE_2 levels (Fig. 2). TNF-α can stimulate the release of other cytokines, including IL-1β and chemokines, as well as the expression of adhesion molecules and inducible enzymes.[22] COX-2 is primarily responsible for PGE_2 produced in inflammation. It is interesting that the anti-inflammatory action of CKS might inhibit the up-regulation of COX-2 at the inflammatory sites and, as a result, exert its anti-inflammatory effect. Hence, COX-2 is up-regulated in the air pouch and catalyzes the production of a large amount of PGE_2. Moreover, inflammation in the air pouch model is suppressed by non-steroidal anti-inflammatory drugs (NSAID), which inhibit COX-2, indicating a key role for inducible COX-2 in this model of acute inflammation.[15,16] In this study, the immunoblot analysis showed that CKS significantly

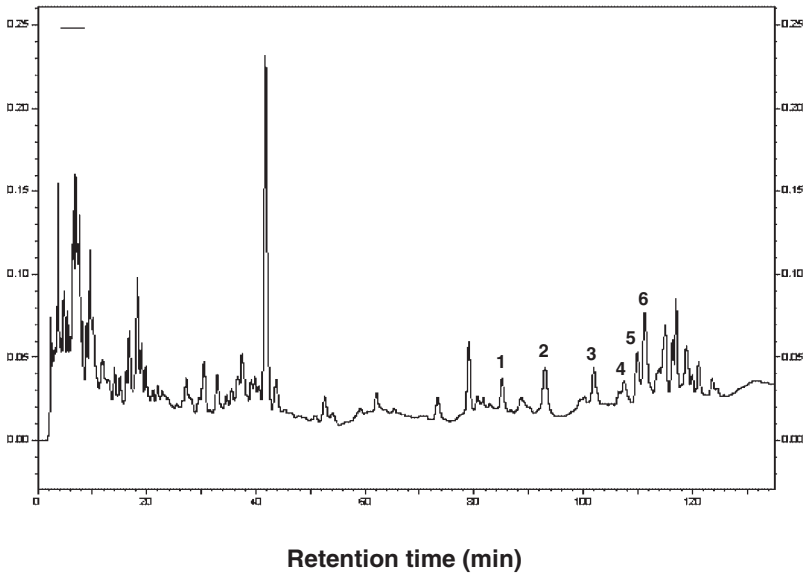

Fig. 6. HPLC Chromatograms of CKS.
The peaks for platycoside E (1, Rt 85.1 min), platycodin D_3 (2, Rt 93.0 min), polygalacin D_2 (3, Rt 101.9 min), platycodin D_2 (4, Rt 107.3 min), deapioplatycodin D (5, Rt 109.9 min), and platycodin D (6, Rt 111.3 min) are labeled on the chromatogram.

inhibited COX-2 expression in the exudate cells (Fig. 3). This suggests that the decrease in PGE_2 level was due to the inhibition of COX-2 expression, and this inhibitory effect on COX-2 expression was correlated with the *in vitro* effect on macrophages.[14,19] In the other inflammation model used in this study, the paw edema induced by carrageenan, CKS also had potent inhibitory effects on the level of paw swelling and the production of PGE_2 in a homogenate of the inflamed paw (Fig. 5). This suggests that the up-regulation of COX-2 with the increased local production of PGE_2 was the key event in carrageenan-induced inflammation, and that the secretion of other mediators was a secondary event that amplified the inflammatory response. Therefore, CKS might partly suppress the up-regulation of COX-2 at the inflammatory site. Alternatively, the decrease in PGE_2 level with the CKS treatment may be caused by the inhibitory effect of CKS on the release of cytokine. It has been reported that the stimulation of macrophages/monocytes, fibroblasts and epithelial cells with such cytokines as IL-1β and TNF-α led to PGE_2 production.[23] It has recently been reported that CKS suppressed the LPS-activated production of PGE_2 and COX-2 expression by inhibiting the activation of NF-κB in cultured macrophages.[14] Therefore, the inhibitory mechanism of CKS for the release of cytokine might depend on the *in vivo* suppression of COX-2 expression. However, since little is known regarding the precise contents and components of saponins in CKS, we could not correctly identify which peak represented which kind of platydodins (Fig. 6). In addition, the precise mechanism by which components of CKS exert anti-inflammatory effects is still largely unknown, even though the anti-inflammatory effects of CKS have been shown in this study. We have just gleaned a little information on the components of saponins in CKS which contained platycoside E, platycodin D_3, polygalacin D_2, platycodin D_2, deapioplatycodin D and platycodin D. Therefore, additional studies will be needed to identify the precise contents and composition of saponins in CKS and provide the answer to which components of CKS exert anti-inflammatory effects and elucidate the mechanisms involved.

In conclusion, CKS inhibited carrageenan-induced acute inflammation and the production of a variety of proinflammatory mediators *in vivo*. The anti-inflammatory action of CKS can partly be explained by its capacity to inhibit the production of cytokines as well as COX-2 expression at the inflammatory sites. Therefore, CKS might be of interest in the search for new anti-inflammatory agents. Furthermore, future studies will be needed to determine its therapeutic efficacy in various inflammatory diseases.

Acknowledgments

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