

Observations of *Forsythia koreana* methanol extract on mast cell-mediated allergic reactions in experimental models

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Abstract To explore effects of *Forsythia koreana* methanol extract (FKME) on mast cell-mediated allergic and inflammatory properties, the effect of FKME was evaluated on compound 48/80-induced systemic anaphylaxis, ear swelling, and anti-dinitrophenyl (DNP) immunoglobulin E (IgE)-induced passive cutaneous anaphylaxis (PCA). In addition, the effect of FKME was investigated on the histamine release from rat peritoneal mast cells (RPMCs) stimulated by compound 48/80, which promotes histamine release. The human mast cell line HMC-1 was stimulated by phorbol 12-myristate 13-acetate plus calcium ionophore A23187. Acti-

vated HMC-1 can produce several proinflammatory and chemotactic cytokines such as tumor necrosis factor- α (TNF- α), interleukin (IL)-6, and IL-8. Cytokine levels in the culture supernatant were measured by an enzyme-linked immunosorbent assay. Cytotoxicity by FKME was determined by a 3-(4,5-dimethylthiazol-2-yl)-diphenyl-tetrazolium bromide (MTT) assay. FKME inhibited compound 48/80-induced systemic anaphylactic shock and ear swelling in mice. When 1 g/kg FKME was pretreated or posttreated with mice, compound 48/80-induced mice mortality was 50 and 66.7%, respectively. One gram per kilogram of FKME pretreatment inhibited ear-swelling responses derived from compound 48/80 by 29.75%. A PCA reaction was inhibited by 17.9%. In an in vitro model, FKME (1 mg/ml) inhibited histamine release from the RPMCs by 13.8% and TNF- α , IL-6, and IL-8 production from HMC-1 cells by 71.16% ($P<0.001$), 86.72% ($P<0.001$), and 44.6%, respectively. However, FKME had no cytotoxic effects on cell viability. In conclusion, FKME inhibited not only systemic anaphylaxis and ear swelling induced by compound 48/80 but also inhibited a PCA reaction induced by anti-DNP IgE in vivo. Treatment with FKME showed significant inhibitory effects on histamine, TNF- α , IL-6, and IL-8 release from mast cells.

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Introduction

Forsythia koreana can be found in all the areas in Korea. *Forsythiae fructus* is prepared from the dried mature fruit of *F. koreana*, *F. suspensa* or *F. viridissima*, both of which

belong to the family Oleaceae. *F. fructus* has antiviral and anti-inflammation effects (Zhu 1998). Recently, it was reported that *F. fructus* has strong anti-allergy effects and an inhibitory effect in NO production (Kim et al. 2000, 2003); however, there has been no previous demonstration of branches and leaves of *F. koreana*. Thus, the branches and leaves of *F. koreana* were tested for pharmacological activity.

Mast cells are strongly associated with immediate hypersensitivity, an immune reaction resulting from the release of chemical mediators and cytokines after immunoglobulin E (IgE)/Fc ϵ RI-mediated activation (Pearce 1989; Metcalfe et al. 1999; Murrant and Bihari 2000). The significant contribution of mast cells has been implicated in the pathogenesis of diverse inflammatory diseases such as asthma, arthritis, fibrosis, and malignancy (Bradding and Holgate 1999; Krishnaswamy et al. 2001). Compound 48/80 was used to activate mast cells. Compound 48/80 is known as a potent inducer of degranulation and of the release of histamine and other chemical mediators responsible for anaphylactic symptoms (Bronner et al. 1987). Studies on compound 48/80-induced mast cell degranulation and ear-swelling response are regularly performed on a theoretical basis by Kim et al. (1999) (Na et al. 2002). The secretory response of mast cells can be induced by aggregation of their cell surface-specific receptors for IgE by the corresponding antigen (Tkaczyk et al. 2004). It has been established that the anti-IgE antibody induces passive cutaneous anaphylaxis (PCA) as a typical model for mast cell-dependent immediate type allergic reactions (Jeong et al. 2001).

Activated mast cells can produce histamine and a wide variety of other inflammatory mediators and several proinflammatory and chemotactic cytokines such as tumor necrosis factor- α (TNF- α), interleukin (IL)-1, IL-4, IL-6, IL-8, and IL-13 (Artuc et al. 1999; Royer et al. 2001a, b; Stassen et al. 2001). Histamine remains the best-characterized and most potent vasoactive mediator implicated in the acute phase of immediate hypersensitivity (Petersen et al. 1996; Moon et al. 2003). Modulation of the histamine and these cytokines' secretion from mast cells can provide a useful therapeutic strategy for allergic inflammatory diseases.

Cyclosporin A (CsA), an immunosuppressant, was used as a positive control. In a previous report, CsA was found to inhibit cytokine gene expressions such as TNF- α , IL-5, IL-6, and IL-8 (Williams and Coleman 1995; Warbrick et al. 1997).

In this study, the effect of *Forsythia koreana* methanol extract (FKME) was evaluated on both compound 48/80-induced and anti-dinitrophenyl (DNP) IgE-induced allergic reactions. FKME was also investigated regarding phorbol 12-myristate 13-acetate (PMA) and A23187-induced cytokine releases from human mast cells (HMC-1).

Materials and Methods

Materials. Fetal bovine serum (FBS), the α -minimal essential medium and Iscove's Modified Dulbecco's Medium (IMDM) were purchased from Gibco BRL (Grand Island, NY). Compound 48/80, anti-DNP IgE, DNP-human serum albumin (HSA), metrizamide, PMA, A23187 and o-phthalaldehyde (OPA), avidin-peroxidase, 2,2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid), 3-(4,5-dimethylthiazol-2-yl)-diphenyl-tetrazolium bromide (MTT), and other reagents were obtained from Sigma (St. Louis, MO). Antihuman TNF- α /IL-6/IL-8 antibody (Ab), biotinylated antihuman TNF- α /IL-6/IL-8 Ab, and recombinant human (rh) TNF- α /IL-6/IL-8 were purchased from R&D Systems (Minneapolis, MN).

Preparation of FKME. *F. koreana* was collected in Kongju City, South Korea. The *F. koreana* was extracted with 100% methanol (MeOH) for 48 h. The extract was filtered and evaporated on a rotary evaporator under reduced pressure. The concentrated extract was dried using a freeze dryer to result in a solid MeOH extract.

Animals. The original stock of ICR mice (male, 6 wk, 25–30 g) and Wistar rats (male, 8 wk, 180–220 g) were purchased from the Dae-Han Experimental Animal Center (Eumsung, South Korea), and the animals were maintained in the College of Pharmacy, Wonkwang University. The rats were housed five to ten per cage in a laminar airflow room maintained at a temperature of 22 $\pm$ 1 $^{\circ}$ C and a relative humidity of 55 $\pm$ 10% throughout the study. No animal was used more than once. The research was conducted in accordance with the internationally accepted principles for laboratory animal use and care as found in the US guidelines (NIH publication #85-23, revised in 1985).

Compound 48/80-induced systemic anaphylactic reaction. Mice were given an intraperitoneal injection of the mast cell degranulator compound 48/80 (8 mg/kg). FKME dissolved in saline was administered orally 1 h before the injection of compound 48/80. Mortality was monitored for 23 min after induction of an anaphylactic reaction. CsA was intraperitoneally given to the mice 30 min before the injection of compound 48/80.

Ear-swelling response. Compound 48/80 was freshly dissolved in saline and injected intradermally into the dorsal aspect of a mouse ear using a microsyringe with a 28-gauge hypodermic needle. Ear thickness was measured with a digimatic micrometer (Mitutoyo, Kawasaki, Japan) under mild anesthesia. The ear-swelling response represented an increment in the thickness above baseline control values. The ear-swelling response was determined 40 min after the

compound 48/80 or vehicle injection. FKME was administered orally 1 h before the compound 48/80 infection (100 µg/site). The values obtained thus appear to represent the effect of compound 48/80 rather than the effect of the vehicle injection (physical swelling), as the ear-swelling response evoked by physiologic saline returned to nearly baseline thickness levels within 40 min.

PCA reaction. PCA was performed as previously described (Shin et al. 1997). An IgE-dependent cutaneous reaction was generated by sensitizing the skin with an intradermal injection of anti-DNP IgE followed 48 h later with an injection of DNP-HSA 48 h later into the mice tail vein. The DNP-HSA was diluted in phosphate-buffered saline (PBS). The mice were injected intradermally with 100 ng of anti-DNP IgE into each of three dorsal skin sites that had been shaved 48 h earlier. The sites were outlined with a water-insoluble red marker. Forty-eight hours later, each mouse received an injection of 200 µl of the 1:1 mixture of 1 mg/ml DNP-HSA in PBS and 4% Evans blue via the tail vein. One hour before this injection, FKME was administered orally. The mice were killed, and the dorsal skin was removed for measurement of the pigment area. The amount of dye was then determined colorimetrically after extraction with 1 ml of 0.1 N KOH and 9 ml of a mixture of acetone and phosphoric acid (5:13). The absorbent intensity of the extraction was measured at 620 nm by a spectrometer, and the amount of dye was calculated with the Evans blue measuring line.

Preparation of RPMCs. Rat peritoneal mast cells (RPMCs) were isolated as previously described (Jippo-Kanemoto et al. 1993). In brief, rats were anesthetized by ether and injected with 20 ml of Tyrode buffer B (NaCl, glucose, NaHCO₃, KCl, and NaH₂PO₄) containing 0.1% gelatin into the peritoneal cavity; the abdomen was gently massaged for about 90 s. The peritoneal cavity was carefully opened, and the fluid containing peritoneal cells was aspirated by Pasteur pipette. The peritoneal cells were then sedimented at 150×g for 10 min at room temperature and resuspended in Tyrode buffer B. Mast cells were separated from the major components of rat peritoneal cells (i.e., macrophages and small lymphocytes) according to the method described by Yurt et al. (1977). In brief, peritoneal cells suspended in 1 ml of Tyrode buffer B were layered onto 2 ml of 0.225 g/ml metrizamide (density 1.120 g/ml) and centrifuged at room temperature for 15 min at 400×g. The cells remaining at the buffer-metrizamide interface were aspirated and discarded; the cells in the pellet were washed and resuspended in 1 ml of Tyrode buffer A (10 mM HEPES, 130 mM NaCl, 5 mM KCl, 1.4 mM CaCl₂, 1 mM MgCl₂, 5.6 mM glucose, and 0.1% bovine serum albumin) containing calcium.

Histamine assay. The RPMC suspensions (2×10^5 cells/ml) were preincubated for 10 min at 37° C before the addition of compound 48/80 for stabilization. The cells were preincubated with FKME for 20 min and then incubated for 15 min with compound 48/80 (6 µg/ml). The reaction was stopped by cooling the tubes in ice. The cells were separated from the released histamine by centrifugation at 400×g for 5 min at 4° C. Residual histamine in the cells was released by disrupting the cells with perchloric acid and centrifugation at 400×g for 5 min at 4° C. The histamine content was measured by the *o*-phthaldialdehyde spectrofluorometric procedure of Shore et al. (1959). The fluorescent intensity was measured at 440 nm (excitation of 360 nm) using a spectrofluorometer.

The inhibition percentage of histamine release was calculated using the following equation:

$$\% \text{ Inhibition} = (A - B) \times 100 / A$$

In the equation, A is a histamine release without FKME, and B is a histamine release with FKME.

Culture of HMC-1 cells. Human mast cell line HMC-1 cells were grown in IMDM medium supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin, 10 µM monothioglycerol, and 10% heat-inactivated FBS at 37° C in 5% CO₂ at 95% humidity. Cells (5×10^5 cells/500 µl) were treated with FKME for 30 min before stimulation with 50 nM PMA plus 1 µM A23187 and incubated at 37° C for 8 h (Kim et al. 2003).

MTT assay. To test the viability of the cells, a MTT colorimetric assay was performed as described previously (Kim et al. 2001). Briefly, HMC-1 cells (1×10^6 cells/ml) were incubated for 8 h after stimulation in the absence or presence of FKME (1 mg/ml). After an addition of MTT solution, the cells were incubated at 37° C for 4 h. The crystallized MTT was dissolved, and the absorbance was measured at 540 nm.

Assay of TNF-α, IL-6, and IL-8 secretion. Secreted TNF-α, IL-6, and IL-8 level in supernatants from HMC-1 cells were measured by a sandwich enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's protocol (R&D Systems, Spokane, WA). Absorption of the avidin-horseradish peroxidase color reaction was measured at 405 nm and compared with serial dilutions of human TNF-α, IL-6, and IL-8 recombinant as a standard.

Statistical analysis. The results are expressed as the mean ± SEM or the mean ± SE for a number of experiments. Statistical significance was compared between each treated group and control using an analysis of variance, with a post

hoc test of the means according to Tukey's method. Mann–Whitney's *U* test was used on histamine assay, as the results of the histamine assay did not have normality. For all tests, a *P* value less than 0.05 was considered significant.

Results

Effect of FKME on compound 48/80-induced systemic anaphylaxis. To assess the contribution of FKME in anaphylactic reactions, the *in vivo* model of systemic anaphylaxis was used initially. As a nonimmunologic stimulator, compound 48/80 (8 mg/kg) was used. After the injection of the compound 48/80, the mice were monitored for 23 min, after which the mortality rate was determined. As shown in Table 1, an oral administration of saline as a control induced a fatal reaction in 100% of each group. When the FKME was orally administered at concentrations of 0.01–1 g/kg 1 h before the compound 48/80 injections, the mortality was reduced (Table 1). When FKME (1 g/kg) was orally administered 5 min after the compound 48/80 injections, mice mortality was observed at 66.7% (Table 2). CsA (5 mg/kg) completely suppressed compound 48/80-induced mortality (Table 1).

Effect of FKME on the ear-swelling response. The fact that an intradermal application of compound 48/80 can induce an ear-swelling response in normal mice has been confirmed in previous studies (Na et al. 2002). One hundred micrograms of compound 48/80 was injected per ear site. As shown in Table 3, when mice were pretreated with 1 g/kg FKME for 1 h, the ear-swelling responses derived from compound 48/80 were inhibited by 29.75% (Table 3).

Table 1. Effect of FKME pretreatment on compound 48/80-induced systemic anaphylactic shock

FKME concentration (g/kg) ^a	Compound 48/80 (8 mg/kg) ^b	Mortality (%) ^c
None (saline)	+	100.0
0.01	+	50.0
0.1	+	50.0
1	+	50.0
1	–	0
CsA (5 mg/kg) ^d	+	0

^a The groups of mice (*n*=6, male, 6 wk, 25–30 g) were orally pretreated with 200 µl of saline, or FKME was given at various doses 1 h before the compound 48/80 injection.

^b The compound 48/80 solution was intraperitoneally given to the groups of mice.

^c Mortality (%) is presented as the 'no. of dead mice×100/total no. of experimental mice.'

^d CsA was used as a positive control and given intraperitoneally to the group of mice.

Table 2. Effect of FKME posttreatment on compound 48/80-induced systemic anaphylactic shock

FKME concentration (g/kg) ^a	Compound 48/80 (8 mg/kg) ^b	Mortality (%) ^c
None	+	100.0
1	+	66.7

^a The groups of mice (*n*=6, male, 6 wk, 25–30 g) were orally pretreated with 200 µl of saline or FKME was given at 1 g/kg doses 5 min after the compound 48/80 injection.

^b The compound 48/80 solution was intraperitoneally given to the groups of mice.

^c Mortality (%) is presented as the 'no. of dead mice×100/total no. of experimental mice.'

Effect of FKME on PCA. A local injection of anti-DNP IgE followed by an intravenous antigenic challenge was given. Anti-DNP IgE was injected into dorsal skin sites. After 48 h, all animals were injected intravenously with DNP–HAS containing Evans blue dye. The cutaneous anaphylactic reaction was best visualized by the extravasation of the dye. When FKME was orally administered to a mouse, a PCA reaction was inhibited (Table 4).

Effect of FKME on the histamine release from RPMCs. FKME dose dependently inhibited the compound 48/80-induced histamine release at concentrations of 0.01–1 mg/ml. At a concentration of 1 mg/ml, the inhibition rate reached 13.8% (Fig. 1).

Effect of FKME on HMC-1 viability. To test the cytotoxic effect of FKME, a MTT assay was performed with the HMC-1 cells. Figure 2 shows the viability of cells incubated for 8 h after stimulation in the absence or presence FKME (1 mg/ml). The OD values for the absence or presence FKME were 1.855±0.056 and 1.878±0.033, respectively. FKME did not significantly affect cell viability and had no toxicity on HMC-1 cells (Fig. 2).

Effect of FKME on cytokine secretion from HMC-1 cells. The inhibitory effect of FKME was examined in terms of the PMA plus A23187-induced production of TNF-α, IL-6,

Table 3. Effect of FKME on compound 48/80-induced ear swelling response in mice

FKME addition (g/kg) ^a	Thickness (mm)	Inhibition (%)
None	0.201±0.02	–
1	0.141±0.00	29.75

Twenty microliters of compound 48/80 (100 µg/site) were applied intradermally.

^a The mice (male, 6 wk, 25–30 g) were orally administered with the indicated concentration of FKME for 1 h prior to the compound 48/80 application. The data represent the mean ± SE (*n*=5).

Table 4. Effect of FKME on 48 h passive cutaneous anaphylaxis in mice

FKME addition (g/kg) ^a	Amount of dye (μg/ml) ^b	Inhibition (%)
None	0.75±0.05	—
1	0.62±0.07	17.9

^a FKME dissolved with distilled water was orally administered to mice 1 h before the challenge.

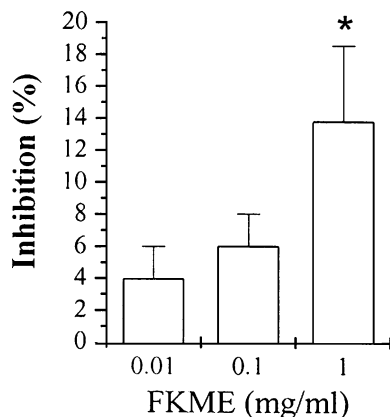
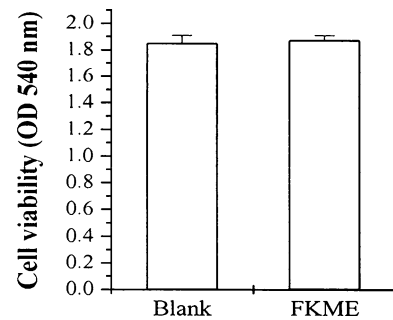
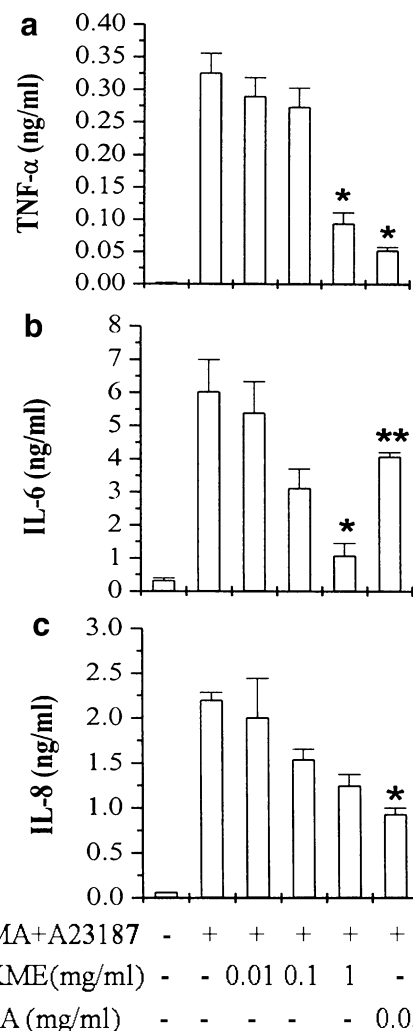
^b Each value indicates means ± SE ($n=3$, male, 6 wk, 25–30 g).

and IL-8 from HMC-1 cells. The culture supernatant was assayed for each cytokine level using the ELISA method. FKME dose dependently inhibited production of TNF- α , IL-6, and IL-8 in PMA plus A23187-stimulated HMC-1 cells. One milligram per milliliter of FKME treatment blocked TNF- α , IL-6, and IL-8 production by nearly 71.16, 86.72, and 44.6%, respectively, compared with no treatment of FKME ($P<0.001$). CsA (0.05 mg/ml) also inhibited TNF- α , IL-6, and IL-8 production (Fig. 3).

Discussion

This study showed that a FKME pretreatment profoundly affects compound 48/80-induced systemic anaphylaxis, ear-swelling response, histamine release from RPMCs, and anti-DNP IgE-induced PCA reaction. In addition, FKME inhibited PMA plus A23187-induced cytokine secretions from HMC-1. However, a FKME pretreatment did not inhibit mortality.

It is believed that stimulation of mast cells with compound 48/80 initiates the activation of a signal transduction pathway, which leads to a histamine release. There have been several reports that found compound 48/

**Figure 1.** Effect of FKME on a compound 48/80-induced histamine release from RPMCs. RPMCs (2×10^5 cells) were preincubated with various concentrations of FKME at 37° C for 10 min prior to incubation with compound 48/80. Each datum represents the mean ± SEM. * $P<0.05$; significantly different from the control value.**Figure 2.** Effect of FKME on the cell viability in HMC-1 cells. Cell viability was evaluated by a MTT assay 8 h after FKME treatment (1 mg/ml) in HMC-1 cells. Data represent the mean ± SEM of three independent experiments.**Figure 3.** Inhibition of TNF- α (a), IL-6 (b), and IL-8 (c) secretion by FKME or CsA in PMA plus A23187-stimulated HMC-1 cells. Cells were pretreated with FKME or CsA (0.05 mg/ml) for 30 min and then challenged with PMA (50 nM) plus A23187 (1 μ M) for 8 h. TNF- α , IL-6, and IL-8 concentrations were measured from cell supernatants using the ELISA method. Values are the mean ± SEM of duplicate determinations from three separate experiments. * $P<0.001$, ** $P<0.01$; significantly different from the stimulated group.

80 able to activate G proteins (Mousli et al. 1990a, b; Shin and Lee 2003). Recently, Chadi et al. (2000) announced that compound 48/80 activates mast cell phospholipase D (PLD) via heterotrimeric guanosine triphosphate-binding proteins. They found that recombinant $G_{\beta 2 \gamma 2}$ subunit markedly synergized PLD activation by compound 48/80 in permeabilized RBL-2H3 cells. Murine mast cells are a good experimental model for the study of compound 48/80-induced histamine release (Alfonso et al. 2000). A report found that compound 48/80 increased the permeability of the lipid bilayer membrane by causing a perturbation of the membrane indicates that the membrane permeability increase may be an essential trigger for the release of mediators from mast cells (Tasaka et al. 1986). Thus, it is possible to hypothesize that FKME can act on the lipid bilayer membrane affecting the prevention of the perturbation being induced by compound 48/80.

In this study, when FKME was orally administered at various concentrations (0.01–1 g/kg), the effect did not change in a dose-dependent manner (Table 1). It is thought that the maximum effective concentration of FKME is 0.01 g/kg on compound 48/80-induced mortality. From this, it is suggested that exceeding the treatment of the proper dose of FKME does not necessarily improve the effect of FKME on compound 48/80-induced mortality. Further study, however, is necessary regarding the regulation of compound 48/80-induced mortality with various concentrations of FKME.

A FKME-administered mouse is protected from IgE-mediated local allergic reactions. The mechanism of the protection against anti-DNP IgE, while not clear at present, may be suggested only in a number of particular conditions. It is conceivable that FKME inhibits the initial phase of immediate-type allergic reactions, most likely through interference with the degranulation system.

HMC-1 cells activated by PMA plus A23187 are useful in an in vitro model system for studying of the multifunctional effects of the immune and inflammatory reactions (Hosoda et al. 2002; Shin et al. 2003). It was showed that FKME inhibited the production of inflammatory cytokines such as TNF- α , IL-6, and IL-8 from PMA plus A23187-stimulated HMC-1 cells. TNF- α is an essential cytokine in many pathological conditions, including allergic diseases, rheumatoid arthritis, and pulmonary fibrosis (Camusi et al. 1991). TNF- α induced production and release of eosinophil chemotactic factors such as eotaxin (Sato et al. 2001; Hoeck and Woisetschlager 2001) and regulated on activation, normal T cell expressed and secreted (Hashimoto et al. 2001) from fibroblasts and epithelial cells. IL-6 and IL-8 play a major role in triggering and sustaining the allergic inflammatory response. IL-6 is abundantly expressed in inflammatory regions such as rheumatoid arthritis synovial tissue and deficiencies in IL-6 prevent and/or reduce the severity of experimental arthritis (Feldmann et al. 1996;

Alonzi et al. 1998; Boe et al. 1999). IL-6 is also released in asthma. There is evidence for increased release of IL-6 from alveolar macrophages from asthmatic patients after an allergen challenge, and increased basal release, compared with non-asthmatic subjects (Gosset et al. 1991; Broide et al. 1992). IL-8 is the most extensively studied member of the entire chemokine superfamily, with its major actions being a neutrophil chemoattractant and activator. Free IL-8 has been detected in the sera and bronchial tissue of subjects with severe atopic asthma but not in samples from normal subjects (Shute et al. 1997). In the present study, it was shown that FKME treatment inhibited TNF- α , IL-6, and IL-8 production by approximately 71.16, 86.72, and 44.6%, respectively, compared with no treatment of FKME. CsA, an immunosuppressant, was used as a positive control. CsA significantly suppressed TNF- α , IL-6, and IL-8 production. Interestingly, inhibition of IL-6 production by treatment of 1 mg/ml FKME was more effective than a treatment with CsA. Although 1 mg/ml FKME for cells is thought an excess of quantity, the data in the experiments of this study indicated that FKME may play a role as inhibitor of the aforementioned cytokines.

In conclusion, the data here indicated that FKME inhibited not only systemic anaphylaxis and ear swelling induced by compound 48/80 but also PCA reactions induced by anti-DNP IgE in vivo. In addition, FKME showed significant inhibitory effect on histamine and cytokines release from mast cells. The anti-allergic and anti-inflammatory effect of FKME is thus revealed. However, further studies regarding the biological mechanism of FKME are needed.

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