JPP Journal of Pharmacy and Pharmacology

JPP 2001, 53: 1287–1294

Received October 5, 2000
Accepted June 7, 2001
ISSN 0022-3573

# In-vitro and in-vivo immunomodulatory effects of syringin

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**Abstract**

Syringin was found to possess immunomodulatory activity by which it inhibited the in-vitro immunohaemolysis of antibody-coated sheep erythrocytes by guinea-pig serum through suppression of C3-convertase of the classical complement. In this study, we examined its in-vitro and in-vivo activity on tumour necrosis factor (TNF)-$\alpha$ and nitric oxide (NO) production, CD4+ T cell and CD8+ cytotoxic T cell (CTLL-2) proliferation, and croton oil-, arachidonic acid- and fluorescein-isothiocynate (FITC)-induced mouse ear oedema model. Syringin significantly inhibited both TNF-$\alpha$ production from lipopolysaccharide (LPS)-stimulated RAW264.7 cells and CD8+ T cell (CTLL-2) proliferation in a dose-dependent manner, whereas neither NO production nor CD4+ T cell proliferation were blocked even by high concentrations of syringin. In the in-vivo experiments, syringin also significantly suppressed FITC-induced ear oedema in mice but not the ear oedema induced by croton or arachidonic acid. These results suggest that syringin may be implicated as an immunomodulator having an anti-allergic effect rather than an anti-inflammatory effect. The anti-allergic effect of syringin seems to be due, in part, to inhibition of TNF-$\alpha$ production and cytotoxic T cell proliferation.

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## Introduction

Syringin is a representative phenolic glycoside identified from a variety of medicinal plants such as *Magnolia sieboldii* (Park et al 1996) and *Tinospora cordifolia* (Kapil & Sharma 1997), although there are only a few reports demonstrating its biological activity. This compound, from Kampo formulae (Li et al 1998) and *Acanthopanax senticosus* (Nishibe et al 1990), is known to have an inhibitory effect on bone reabsorption and to possess an anti-stress effect in a chronic swimming stress model.

It has been reported that syringin, an active principle of *Tinospora cordifolia* (a traditional Indian medicinal plant), modulates in-vitro immunohaemolysis of antibody-coated sheep erythrocytes by guinea-pig serum via the inhibition of C3-convertase of the classical complement (Kapil & Sharma 1997). In addition, syringin and related phenolic compounds were identified as major principles in water extracts of some medicinal plants (e.g. Kampo formulae and *Eucommia ulmoides*), that are drunk in Korea and Japan in the form of traditional tea, against allergic symptoms (Li et al 1998; Cho et al, unpublished results). This led us to further examine the general immunomodulating effects of syringin on inflammatory reactions in terms of therapeutic efficacy, since the compound is generally

distributed in the plant kingdom and some phenolic glycosides are reported to have immunomodulating effect (Molnar et al 1989; Ficarra et al 1995). In this study, therefore, we have tried to examine the immunoregulatory effects of syringin. For these, six kinds of in-vitro and in-vivo experimental models were chosen, on the basis that they represented a range of in-vitro and in-vivo immunological phenomena such as allergic and inflammatory symptoms. The data generated with these assays demonstrate that syringin, depending on the assay, significantly inhibits in-vitro tumour necrosis factor (TNF)-$\alpha$ and cytotoxic T cell proliferation and in-vivo FITC-induced ear oedema formation.

## Materials and Methods

### Mice

Male BALB/c and ICR mice (6 weeks old) were purchased from B & K (Fremont, CA). Mice were allowed free access to pelleted diets (Samyang, Daejeon, Korea) and water, and lighting was maintained on a 12-h cycle.

### Materials

Syringin (Figure 1) was purified from *Magnolia sieboldii* (Park et al 1996) and its purity (> 97.5%) was determined by HPLC analysis. Pentoxifylline (oxpentifylline), theophylline, fluorescein-isothiocynate (FITC), indometacin, dexamethasone, nitro-L-arginine methyl ester (L-NAME), sodium carboxylmethylcellulose (Na CMC), MTT (3-(4,5-dimethylthyazol-2-yl)-2,5-diphenyltetrazdium bromide), concanavalin A (Con A), interleukin (IL)-2 and lipopolysaccharide (LPS, from *Escherichia coli* 0111:B4) were purchased from Sigma Chemical Co. (St Louis, MO). Fetal bovine serum (FBS), penicillin, streptomycin and RPMI 1640 were obtained from GIBCO (Grand Island, NY). RAW264.7 (a murine macrophage cell line) and CTLL-2 (an IL-2-dependent murine cytotoxic T lymphocyte cell line) were purchased from ATCC (Rockville, MD). All other chemicals were from Sigma.

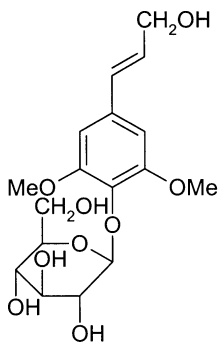


**Figure 1** The chemical structure of syringin.

### In-vitro experiments

*Cell culture*

RAW264.7 cells were maintained in RPMI 1640 supplemented with 100 IU $mL^{-1}$ of penicillin, 100 $\mu$g $mL^{-1}$ of streptomycin and 10% (v/v) FBS. For CTLL-2 cell maintenance, cells were cultured with the same medium containing 25 IU $mL^{-1}$ of IL-2. Cells were grown at 37°C with 5% $CO_2$ in humidified air.

*TNF-$\alpha$ production in-vitro*

The inhibitory effect of syringin on TNF-$\alpha$ production was determined as previously described (Cho et al 1998). Syringin (10 mM) dissolved in hot water was diluted with RPMI 1640; other compounds (10 mM each; dexamethasone, indometacin and A77,1727) were solubilized with solvents (89.1% (v/v) propylene glycol, 10% (v/v) ethanol and 0.9% (v/v) dimethyl sulfoxide). RAW264.7 cells ($1 \times 10^6$ cells/mL) were incubated in the presence of LPS (1 $\mu$g $mL^{-1}$) and various concentrations of syringin and other compounds for 6 h. Supernatants were then collected and assayed for TNF-$\alpha$ content using a mouse TNF-$\alpha$ ELISA kit (Amersham, Little Chalfont, Buckinghamshire, UK).

*Determination of nitric oxide (NO) production*

RAW264.7 cells were seeded in 96-well plates at a density of $1 \times 10^6$ cells $mL^{-1}$. After pre-incubation for 18 h, the various concentrations of syringin or positive control drug (L-NAME) with LPS (1 $\mu$g $mL^{-1}$) were incubated for 24 h under the same conditions (Ding et al 1988; Cho et al 1999). Nitrite in culture supernatants was measured by adding 100 $\mu$L of Griess reagent (1% (w/v) sulfanilamide and 0.1% (w/v) naphthylethylenediamide in 5% (v/v) phosphoric acid) to 100 $\mu$L samples of medium, respectively, for 10 min at room temperature. The nitrite concentration ($\mu$M) was calculated from a standard curve constructed from known concentrations of sodium nitrite.

*CD4+ T lymphocyte proliferation assay*

As T lymphocyte sources, splenocytes were prepared from the spleens of mice killed by cervical dislocation under sterile conditions as described previously (Cho et al 1999). Splenocytes ($5 \times 10^6$ cells $mL^{-1}$) were cultured in flat bottom 96 well microtiter plates in the presence and absence of T lymphocyte mitogen (Con A, 1 $\mu$g

$mL^{-1}$), and in the presence of syringin or A77,1726 in a total volume of 200 $\mu$L/well under the same conditions for 48 h (Cho et al 1999). The CD4+ T lymphocyte proliferation from splenocytes was measured by MTT assay.

*CD8+ IL-2-dependent cytotoxic T cell proliferation assay*

CTLL-2 cells were harvested from IL-2-containing growth medium and washed twice with RPMI 1640 in the absence of both FBS and IL-2. They were re-suspended in growth medium without IL-2 to $5 \times 10^5$ cells $mL^{-1}$. Fifty microliter of cell suspension was placed into each well of a 96-well tissue culture plate and incubated in the presence of syringin and control drugs (dexamethasone, indometacin and A77,1726) and 25 IU $mL^{-1}$ of IL-2 for 48 h (Wlodek et al 1995).

*MTT assay (colorimetric assay) for measurement of cell proliferation*

Cell proliferation was measured by the conventional MTT assay. At 4 h before culture termination, 10 $\mu$L of MTT solution (10 mg $mL^{-1}$ in phosphate buffered-saline) was added to each well until termination by addition of 15% (w/v) sodium dodecyl sulfate. The optical density (OD) values at 570 nm ($OD_{570-630}$) were measured by a microplate Spectramax 250 microplate reader.

### In-vivo experiments

*Croton-oil-induced mouse ear oedema*

Pretreatment of ICR mice was with drug vehicle (0.5% (w/v) Na CMC) or syringin (0.7, 7, 70 and 700 mg $kg^{-1}$) given orally at 4 h and 1 h, respectively, before croton oil treatment. After the second treatment, croton oil solution (2.5% (v/v)) was then applied to the mouse ear (25 $\mu$L/ear), as described previously (Kim et al 1994). The ear thickness was measured by a dial thickness gauge after 5 h. A control experiment was performed with indometacin (7 and 25 mg $kg^{-1}$) and dexamethasone (1 and 10 mg $mL^{-1}$) under the same conditions. To evaluate curative efficacy, the inhibitory effect of ear oedema by drugs was calculated as follows:

$$\% \text{ of control} = [(D_{ET} - N_{ET})/(C_{ET} - N_{ET})] \times 100 \quad (1)$$

where $D_{ET}$ = ear thickness with syringin or control drugs, $N_{ET}$ = ear thickness of normal untreated group and $C_{ET}$ = ear thickness of control group.

*Arachidonic-acid-induced mouse ear oedema*

ICR mice were pretreated orally with drug vehicle or syringin (0.7, 7, 70 and 700 mg $kg^{-1}$) under the same conditions as in the croton-oil-induced ear oedema experiment. Mice were then treated with 2% (w/v) arachidonic acid solution (25 $\mu$L/ear) as described previously (Kim et al 1994). The ear thickness was measured with a dial thickness gauge after 1 h.

*FITC-induced mouse ear oedema*

The FITC-induced mouse ear oedema experiment was conducted as described previously (Gaspari & Katz 1991; Matsuda et al 1995). Briefly, FITC solution (1% (w/v)) prepared with acetone–dihydrophthalate (1:1) was applied to the abdomen and 5 days later the same concentration of FITC solution was applied to the right ear of BALB/c mice. The ear thickness was measured after 24 h and 48 h. Pretreatment with syringin (3.5, 7, 35 and 70 mg $mL^{-1}$), indometacin (7 and 25 mg $kg^{-1}$) and dexamethasone (1 and 10 mg $mL^{-1}$)) was administered orally once a day and 12 h and 1 h before the second treatment of FITC solution. After evaluating the percentage ear oedema according to equation 2, the drug effect was expressed as a % of control calculated from % of ear oedema of the FITC-treated group:

$$\% \text{ of ear oedema} = [(ET_{AFTER} - ET_{BEFORE})/(ET_{BEFORE}) \times 100] \quad (2)$$

where $ET_{AFTER}$ = ear thickness after FITC treatment and $ET_{BEFORE}$ = ear thickness before FITC treatment.

### Statistical analysis

Unless otherwise stated, all measurements were made from four observations and at least two separate experiments. Data are expressed as mean $\pm$ s.d. Initial analyses of variance were performed, followed by pair-wise comparison of relevant groups using Student's *t*-test. Results with *P* values of 0.05 or less were considered as statistically significant.

## Results

### In-vitro experiments

*Effect on TNF-$\alpha$ production*

Syringin significantly inhibited LPS-induced TNF-$\alpha$ production in murine RAW264.7 macrophages at concentrations of 125 $\mu$M and above in a dose-dependent manner (Figure 2). The compound was not cytotoxic at the tested concentration for 6 h incubation (data not shown), suggesting that a specific pharmacological action may be involved in the inhibitory activity of syringin. Similarly, pentoxifylline (oxpentifylline), a non-

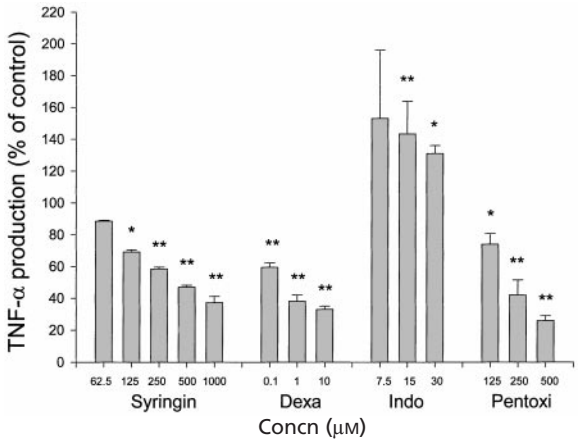


**Figure 2** Effects of syringin, dexamethasone (Dexa), indometacin (Indo) and pentoxifylline (oxpentifylline; Pentoxi) on TNF-$\alpha$ production from RAW264.7 cells stimulated by LPS for 6 h. TNF-$\alpha$ production was assayed from culture supernatants of RAW264.7 cells ($1 \times 10^6$ cells/mL) stimulated by LPS (1 $\mu$g mL$^{-1}$) in the presence of various concentrations of testing compounds. Data represent mean $\pm$ s.d. of 4 observations; $^{**}P < 0.01$ and $^{*}P < 0.05$ compared with controls. Similar results were obtained by separate experiments.

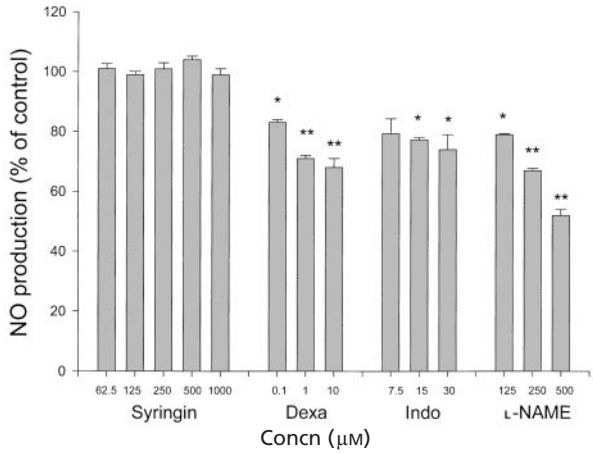


**Figure 3** Effect of syringin, dexamethasone (Dexa), indometacin (Indo) and L-NAME on NO production from RAW264.7 cells stimulated by LPS. RAW264.7 cells ($1 \times 10^6$ cells/mL) were incubated with various concentrations of testing compounds in the presence or absence of 1 $\mu$g mL$^{-1}$ of LPS for 24 h. Supernatants were collected and assayed for nitrite. Data represent mean $\pm$ s.d. of 4 observations; $^{**}P < 0.01$ and $^{*}P < 0.05$ compared with control. Similar results were obtained by separate experiments.

selective phosphodiesterase (PDE) inhibitor used in this experiment as a positive control, also significantly suppressed TNF-$\alpha$ production in a dose-dependent manner. The other non-selective PDE inhibitor, theophylline, displayed a lesser effect than syringin (data not shown). In addition, dexamethasone showed a significant inhibitory effect, whereas indometacin enhanced TNF-$\alpha$ production, as reported previously (Lang et al 1995).

*Effect on NO production*

Syringin was added to murine RAW264.7 macrophages simultaneously with LPS and accumulation of NO was measured after 24 h incubation. Syringin did not attenuate the production of NO (Figure 3) and cell viability (data not shown) even at high concentrations, as was reported previously (Park et al 1996). In contrast, L-NAME inhibited NO production in LPS-stimulated RAW264.7 cells in a dose-dependent manner. Unlike syringin, however, dexamethasone and indometacin showed a significant suppressive effect.

*Effect on CD4+ T lymphocyte proliferation*

One method of controlling the progression of rheumatoid arthritis is to attenuate the proliferation or activation of T cells, since they play a central role in the pathophysiological state of the diseases (Panayi et al 1992). To evaluate the inhibitory effect of syringin on CD4+ T cell proliferation, we employed a splenocyte

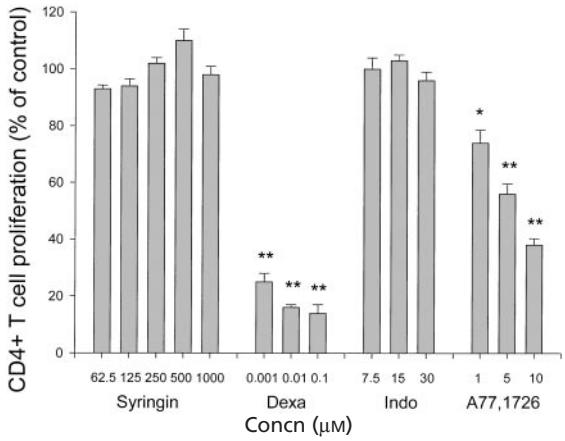


**Figure 4** Effect of syringin, dexamethasone (Dexa), indometacin (Indo) and A77,1726 on CD4+ T lymphocyte proliferation stimulated by Con A (1 $\mu$g mL$^{-1}$). Splenocytes ($5 \times 10^6$ cells/mL) were incubated with various concentrations of testing compounds in the presence of Con A for 48 h. Cell proliferation was assayed by a conventional MTT method. Data represent mean $\pm$ s.d. of 4 observations; $^{**}P < 0.01$ and $^{*}P < 0.05$ compared with control. Similar results were also obtained by separate experiments.

proliferation assay in which a common T cell mitogen (Con A) selectively induced CD4+ T cell proliferation from splenocytes. As shown in Figure 4, syringin caused no reduction in Con A-induced lymphocyte prolifer-

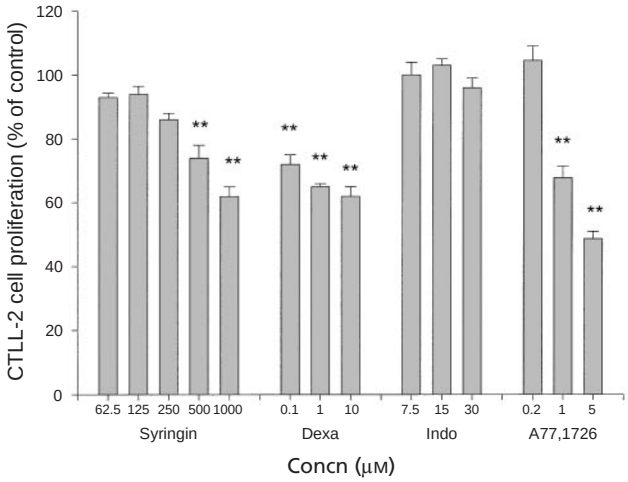


**Figure 5** Effect of syringin, dexamethasone (Dexa), indometacin (Indo) and A77,1726 on CTLL-2 proliferation stimulated by 25 IU $mL^{-1}$ of IL-2. CTLL-2 cells ($5 \times 10^5$ cells/mL) were incubated with various concentrations of testing compounds in the presence of 25 IU $mL^{-1}$ of IL-2 for 48 h. Cell proliferation was assayed by a conventional MTT method. Data represent mean ± s.d. of 4 observations; $^{**}P < 0.01$ compared with control.. Similar results were also obtained by separate experiments. Basal and stimulated OD values of CTLL-2 cells were 0.15 ~ 0.20 and 1.00 ~ 1.10, respectively.

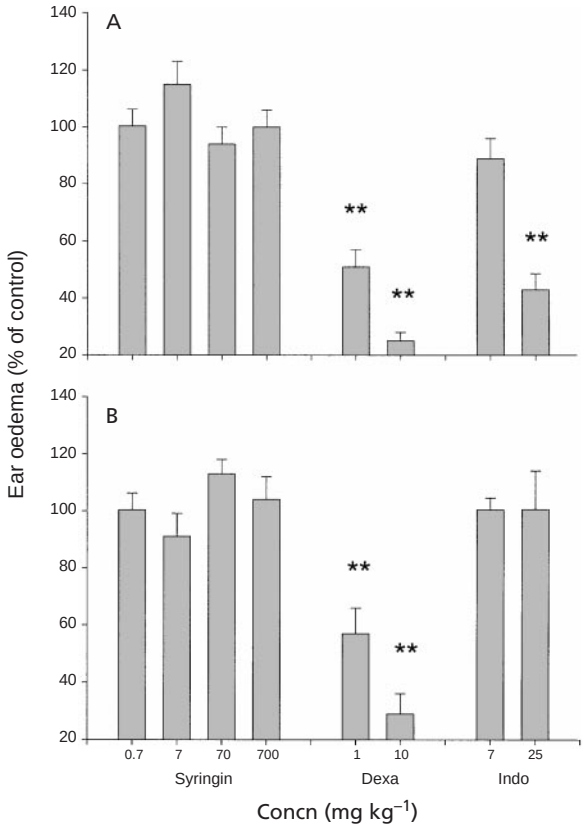


**Figure 6** Effect of syringin, dexamethasone (Dexa) and indometacin (Indo) on arachidonic-acid- (A) or croton-oil-induced (B) mouse-ear oedema. Arachidonic acid (2%) and croton oil (2.5%) solution were topically applied (25 $\mu$L/ear) to the left ear of ICR mice (n = 7). The thickness of the oedema was measured with a constant-pressure thickness gauge 1 or 4 h after treatment and the data (mean ± s.d.) were expressed as the change in thickness between treated and untreated ears. $^{**}P < 0.01$ compared with control. Similar results were also obtained by separate experiments.

ation at any of the tested concentrations, whereas a standard control drug, A77,1726 (an active metabolite of leflunomide), strongly suppressed T cell proliferation. In addition, dexamethasone, but not indometacin, potently inhibited the proliferation.

*Effect on CD8+ cytotoxic T lymphocyte (CTLL-2) cell proliferation*

To examine the inhibitory effect of syringin on CD8+ cell proliferation, CTLL-2 cells were used as a model system. The standard cell proliferation induced by IL-2 showed a linearity ($r^2 = 0.938$) at final concentrations in the range 0.3–40 IU $mL^{-1}$ (data not shown). Syringin significantly suppressed CTLL-2 cell proliferation in a dose-dependent manner. As positive control drugs, A77,1726 and dexamethasone dose-dependently inhibited the proliferation, whereas indometacin showed no effect (Figure 5).

## In-vivo experiments

*Croton-oil-induced ear oedema*

It is known that croton oil stimulates arachidonic acid production via activation of phospholipase $A_2$ ($PLA_2$), resulting in oedema formation (Kim et al 1994, 1998, 1999a; Seeds & Bass 1999). Under our conditions, croton oil also strongly induced ear swelling up to 54%, and the anti-inflammatory effect of syringin was evaluated under these conditions. Figure 6A shows that syringin had no effect on ear oedema formation at doses of up to 700 mg $kg^{-1}$. Conversely, control drugs, dexamethasone and indometacin, significantly abolished the inflammation, as reported previously (Ohmori et al 1988).

*Arachidonic-acid-induced ear oedema*

Arachidonic acid induces prostaglandin $E_2$-mediated oedema formation via the activation of cyclooxygenase (COX) (Simon 1999). We also employed this in-vivo model to test anti-inflammatory effect of syringin. As expected, arachidonic acid treatment apparently swelled

mouse ears up to 67%. Under our study conditions, the standard compound dexamethasone strongly inhibited arachidonic-acid-induced ear swelling, whereas syringin and indometacin did not (Figure 6B).

*FITC-induced ear oedema*

FITC is known to act as a hapten which is able to induce allergic reactions such as type IV hypersensitivity (Gaspari & Katz 1991). Under our study conditions, FITC significantly increased ear swelling up to 46% at 24 h and 34% at 48 h. Unlike croton-oil- and arachidonic-acid-induced ear oedema experiments, syringin significantly attenuated the oedema formation measured at 24 h and 48 h, in a dose-dependent manner, although its effect was less than those of the standard compound, dexamethasone. In contrast, indometacin did not affect the FITC-induced ear swelling (Figure 7).

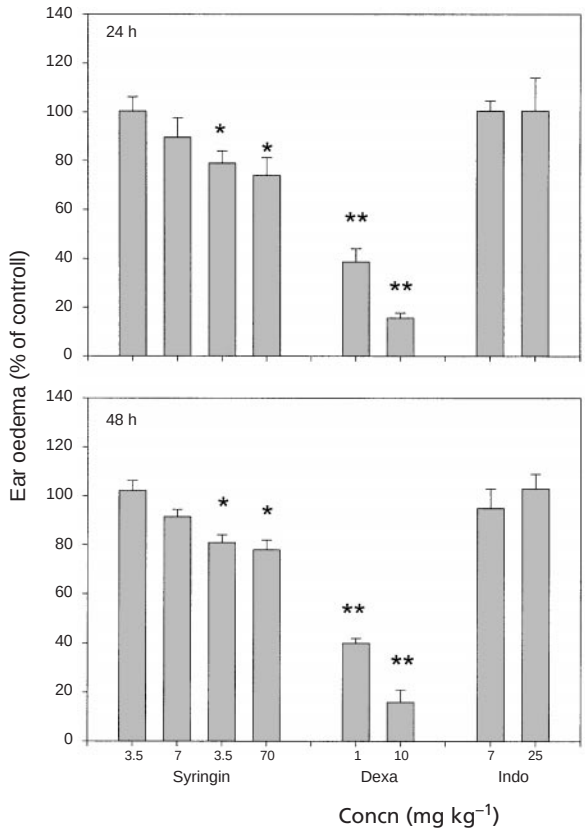


**Figure 7** Effect of syringin, dexamethasone (Dexa) and indometacin (Indo) on FITC-induced mouse-ear oedema. FITC (1%) was topically applied (25 $\mu$L/ear) to the left ear of BALB/c mice (n = 25). The thickness of the oedema was measured with a constant-pressure thickness gauge 24 and 48 h after treatment and the data (mean $\pm$ s.d.) were expressed as the change in thickness between treated and untreated ears. $^{**}P < 0.01$ and $^{*}P < 0.05$ compared with control. Similar results were also obtained by separate experiments.

## Discussion

Although syringin is one of most abundant glycosides in the plant kingdom, there have been only a few reports concerning its biological activity. Reports providing evidence of the immunoregulatory role of syringin are especially sparse (Kapil & Sharma 1997). In this study, we examined both the in-vitro and in-vivo immunomodulatory effects of syringin using activated macrophages and T lymphocytes, and arachidonic-acid-, croton-oil-, and FITC-induced mouse ear oedema models.

Syringin significantly inhibited TNF-$\alpha$ production and CD8+ CTLL-2 cell proliferation, whereas neither NO production nor CD4+ T lymphocyte proliferation was affected by the compound even at high concentrations. These in-vitro effects seem to be not due to non-specific cytotoxicity, since syringin did not suppress normal cell viability at pharmacologically effective doses (data not shown). On the other hand, the aglycone from syringin, an intestinal metabolite (Kim et al 1999b), showed a weak TNF-$\alpha$ inhibitory activity (16.5%) at 100 $\mu$M (data not shown), suggesting that the whole structure may be necessary for biological activity. Syringin also significantly attenuated FITC-induced ear oedema, but not croton-oil- or arachidonic-acid-induced oedema. These inhibitory patterns of syringin were strikingly different from those of dexamethasone and indometacin, implying that syringin may possess a distinguishable pharmacological mechanism from the control drugs.

Considering the pathological mechanism of stimuli used in in-vitro and in-vivo experiments, syringin is supposed to act as an immunomodulator displaying an anti-allergic effect rather than a conventional anti-inflammatory effect. Thus, syringin attenuated the FITC-induced ear swelling (Figure 7), but not ear swelling induced by activated $PLA_2$ and COX (Seeds & Bass 1999; Simon 1999). Additionally, the effect of syringin was strikingly different from that of indometacin (Figure 6) or aspirin (data not shown). Finally, rat footpad oedema triggered by carrageen was also not decreased by syringin (data not shown), supporting the findings of the mouse experiments.

FITC is an allergenic hapten which is capable of inducing type IV hypersensitivity similar to contact dermatitis (Gaspari & Katz 1991). Although the pathophysiological mechanism is not fully elucidated, it has been reported that histamine release plays a critical role

in FITC-induced allergic symptoms (Matsuda et al 1995). To address the possibility that syringin alters histamine release, a passive cutaneous anaphylaxis model was employed. However, syringin displayed no significant effect (data not shown), suggesting that blockade of histamine release could be ruled out from syringin pharmacology. FITC has been reported to enhance the production of monocyte chemoattractant protein-1, IL-8 and TNF-$\alpha$ from immune or inflammatory cells (Lummus et al 1998). In particular, hapten-induced TNF-$\alpha$ production is known to play an important role in initiating cutaneous immune responses through migrating epidermal Langerhans cells (Wang et al 1997) and stimulating the production of other cytokines such as IL-1 and IL-6 (Sekut & Connolly 1996; Bellanti 1998) in allergen-induced contact dermatitis. In these terms, our results regarding the anti-TNF-$\alpha$ effect of syringin could suggest the other possibility that syringin may suppress the biosynthesis of TNF-$\alpha$ locally secreted in FITC-induced ear oedema, resulting in an anti-allergic effect. To support this hypothesis, histopathological experiments such as in-situ hybridization should be further added. Another explanation for the pathological mechanism would be the suppressive effect of syringin on CD8+ cytotoxic T cell proliferation. Because these cells are also known to participate in the pathophysiology of allergic diseases (Kalish & Askenase 1999; Kehren et al 1999), the prevention of the expansion of the CD8+ T cell population by syringin may be also implicated as an inhibitory mechanism (Figure 3). To fully address the exact inhibitory mechanism, however, several experiments should be followed.

In terms of a biochemical target(s) of syringin, our data suggest that COX-, $PLA_2$- and histamine-mediated pathways could be excluded (Figure 6; data not shown). Moreover, considering that inhibitors of common pathways in the mitogenic response, such as NF-$\kappa$B activation or protein tyrosine phosphorylation, display multi-potential immunomodulatory activity in activated macrophages and lymphocytes (Jackson et al 1998; Manthey et al 1998; Cho et al 2000), these common pathways might not be exerted in pharmacological targets of syringin. However, one possible biochemical target of syringin is speculated to be the sigma ligand, which was originally found in distinct regions of the CNS, but is also expressed on immune cells (Derocq et al 1995). Thus, it is possible that syringin may act as a weak agonist of sigma ligands. The first reason for this is the structural similarity between syringin and the ligand agonist, SR31747, which is reported to inhibit endotoxin-induced pro-inflammatory cytokines (Derocq et al 1995). Another is the ability of syringin to modulate the stress condition which is related to brain function (Nishibe at al 1990). Finally, the in-vivo effect seems to be dependent on sex and experimental conditions (data not shown), suggesting that hormone production may be involved in syringin pharmacology. To exactly explain the hypothesis, however, further studies should be conducted concerning the sigma ligand.

In conclusion, we have shown that syringin dose-dependently inhibited FITC-induced mouse ear oedema, TNF-$\alpha$ production and CTLL-2 cell proliferation. Our results presented here suggest that syringin, as an immunomodulator, may possess anti-allergic activity rather than anti-inflammatory activity. However, more experiments should be carried out to explain the exact inhibitory mechanism and to demonstrate other immunomodulatory effects of syringin.

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