Arch Pharm Res Vol 25, No 3, 329-335, 2002

Archives of Pharmacal Research
http://apr.psk.or.kr

Effects of Sophoraflavanone G, a Prenylated Flavonoid from *Sophora Flavescens*, on Cyclooxygenase-2 and *In Vivo* Inflammatory Response

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(Received March 30, 2002)

Previously, several prenylated flavonoids having a C-8 lavandulyl moiety were found to inhibit cyclooxygenase-1 (COX-1) as well as 5-lipoxygenase (5-LOX), and sophoraflavanone G was the most potent inhibitor against these eicosanoid generating enzymes among 19 prenylated flavonoids tested. In this investigation, effects of sophoraflavanone G on COX-2 induction from RAW 264.7 cells and *in vivo* inflammatory response were studied. Sophoraflavanone G inhibited prostaglandin E_2 (PGE_2) production from lipopolysaccharide (LPS)-treated RAW cells by COX-2 down-regulation at 1-50 uM. Other prenylated flavonoids including kuraridin and sanggenon D also down-regulated COX-2 induction at 10-25 uM, while kurarinone and echinoisoflavanone did not. In addition, sophoraflavanone G showed *in vivo* anti-inflammatory activity against mouse croton oil-induced ear edema and rat carrageenan paw edema via oral (2-250 mg/kg) or topical administration (10-250 ug/ear). Although the potencies of inhibition were far less than that of a reference drug, prednisolone, this compound showed higher anti-inflammatory activity when applied topically, suggesting a potential use for several eicosanoid-related skin inflammation such as atopic dermatitis.

Key words: Flavonoid, Sophoraflavanone, *Sophora flavescens*, Cyclooxygenase, Lipoxygenase, RAW 264.7, Inflammation

INTRODUCTION

Plant flavonoids, known as natural tender drugs, showed anti-inflammatory activity (Harborne and Williams, 2000; Kim *et al.*, 2000). Among these plant constituents, prenylated flavonoids are a group of chemical entities having isoprenyl (3,3-dimethylallyl), geranyl (E-3,7-dimethyl-2,6-octadienyl), 1,1-dimethylallyl and/or lavandulyl (5-methyl-2-isopropyl-hex-4-enyl) moiety to their flavonoid backbone structures. Several previous studies demonstrated the inhibitory activity of some prenylated flavonoids against arachidonic acid (AA) metabolizing enzymes, cyclooxygenases (COX) and lipoxygenases (LOX), which generate proinflammatory mediators; thromboxanes (TX), prostaglandins (PG), hydroxyeicosatetraenoic acids (HETE) and leukotrienes (LT). For example, certain prenylated flavonoids including morusin and kuwanones were shown to inhibit both COX-1 and 12-LOX (Kimura *et al.*, 1986a and b). It was also demonstrated that morusin and artonin E potently inhibited the purified 5-, 12- and 15-LOXs at micromolar concentrations, being most potent on 5-LOX (Reddy *et al.*, 1991). Recently, we have investigated the inhibitory effects of 19 naturally-occurring prenylated flavonoids on COX-1, COX-2, 5-LOX and 12-LOX *in vitro* (Chi *et al.*, 2001a). The prenylated flavonoids tested were sophoraflavanones, sophoraisoflavones, kenusanones and echinoflavanones from several medicinal plants. They showed the different profiles of inhibition depending on the flavonoid backbone structures and prenylated groups attached. Among them, the prenylated flavonoids having a C-8 lavandulyl moiety such as kuraridin, kurarinone and

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sophoraflavanone G possess potent inhibitory activity against COX-1 and 5-LOX at micromolar ranges. Of these three compounds, sophoraflavanone G was the most potent inhibitor of COX-1 from bovine platelets (IC_{50}: 0.1-0.6 uM) and 5-LOX from bovine polymorphonuclear leukocytes (IC_{50}: 0.09-0.25 uM). Its potencies of inhibition against COX-1 and 5-LOX were similar to those of the reference compounds, indomethacin (IC_{50} for COX-1: 0.4-1.3 uM) or nordihydroguaiaretic acid (IC_{50} for 5-LOX: 0.6-0.9 uM), whereas the same compound showed very weak inhibitory activity (less than 20% inhibition at 100 uM) against COX-2. However, it is not understood yet whether sophoraflavanone G inhibits COX-2 induction or not. Since COX-2 is an inducible enzyme producing large amounts of PGs and is a pivotal enzyme to provoke inflammatory response (Needleman and Isakson, 1997), it is meaningful to find out COX-2 modulatory action by sophoraflavanone G. Furthermore, there has been no report to describe *in vivo* anti-inflammatory activity of prenylated flavonoids. To answer these questions, effect of sophoraflavanone G on COX-2 induction from LPS-treated RAW 264.7 cells was investigated and its anti-inflammatory potential was also studied using animal models of inflammation. In addition, effects of several other prenylated flavonoids on COX-2 induction were also demonstrated. As far as our best knowledge, this is the first report showing inhibitory activities of COX-2 expression and *in vivo* inflammatory response by prenylated flavonoids.

MATERIALS AND METHODS

Chemicals

AA (99%), 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT), lipopolysaccharide (LPS, *Escherichia coli* 0127:B8), croton oil and λ-carrageenan were purchased from Sigma Chem. (St. Louis, MO). Prednisolone was from Upjohn (Kalamazoo, MI). NS-398 was obtained from Biomol (Plymouth Meeting, PA). DMEM and other cell culture reagents including FBS were products of Gibco BRL (Grand Island, NY). Protein assay kit was purchased from Bio-Rad (Hercules, CA). Enzyme-linked immunoabsorbant assay (EIA) kit for PGE_2 was obtained from Cayman Chem. (Ann Arbor, MI).

Animals

Male ICR mice (18-22 g) and Spraque-Dawley rats (180-220 g) were obtained from Korea Experimental Animal Center (Seoul). The animals were acclimatized in specific pathogen-free animal facility under the conditions of 20-22°C, 40-60% relative humidity and 12 hr/12 hr (L/D) cycle at least for 7 days prior to experiments.

Isolation of sophoraflavanone G and other prenylated flavonoids (Fig. 1)

Morusin was isolated from the root barks of *Morus alba* L., and sanggenon B and D were obtained from the root barks of commercial crude drug of *Morus mongolica* Schneider. The other prenylated flavonoids used in this study were isolated and structurally identified according to the previously described (Kim *et al.*, 1989; Iinuma *et al.*, 1991; Iinuma *et al.* 1992; Kang *et al.*, 2000).

RAW 264.7 cell culture and measurement of PGE_2 concentrations

RAW 264.7 cells obtained from American Type Culture Collection were cultured in DMEM supplemented with 10% FBS and 1% penicillin-streptomycin under 5% CO_2 at 37°C according to the previously described method (Chi *et al.*, 2001b). Briefly, cells were plated in 96-well plates (2×10^5 cells/well). LPS (1 ug/ml) and the test compounds were added simultaneously and incubated for 24 h, unless otherwise specified. PGE_2 concentration in the medium was measured using EIA kit for PGE_2 according to the manufacturer's recommendation. In order to examine COX-2 inhibitory activity, RAW cells were washed three times with serum-free DMEM after 24 h incubation with LPS. Then, arachidonic acid (100 uM) and test compounds were added. Thirty minute later, PGE_2 concentration was checked using EIA kit. Cytotoxicity of the test compounds against RAW cells was checked using MTT (Mossman, 1983). All compounds used in this study did not show cytotoxicity at the tested concentrations.

Western blotting of COX-1 and COX-2

For measuring protein level of COX-1/COX-2 expression, RAW cells were cultured in 100 mm plates in the presence or absence of LPS (1 ug/ml) with/without the test compounds for 20 h following the previously described procedure (Chi *et al.*, 2001b). After preparing the cell homogenate, the supernatant was obtained by centrif-ugation. Using 8% Tris-glycine gel, electrophoresis was carried out and bands were blotted to PVDF membranes. Antibody against COX-1 (No-160109, Cayman Chem.) or COX-2 (No-160116, Cayman Chem.) was incubated and bands were visualized by the treatment of secondary antibody and DAB reagent (Vector Lab.).

In vivo anti-inflammatory activity

In order to evaluate the inhibitory activity of the test compounds in animal models of inflammation, mouse croton oil-induced ear edema assay and rat λ-carrageenan

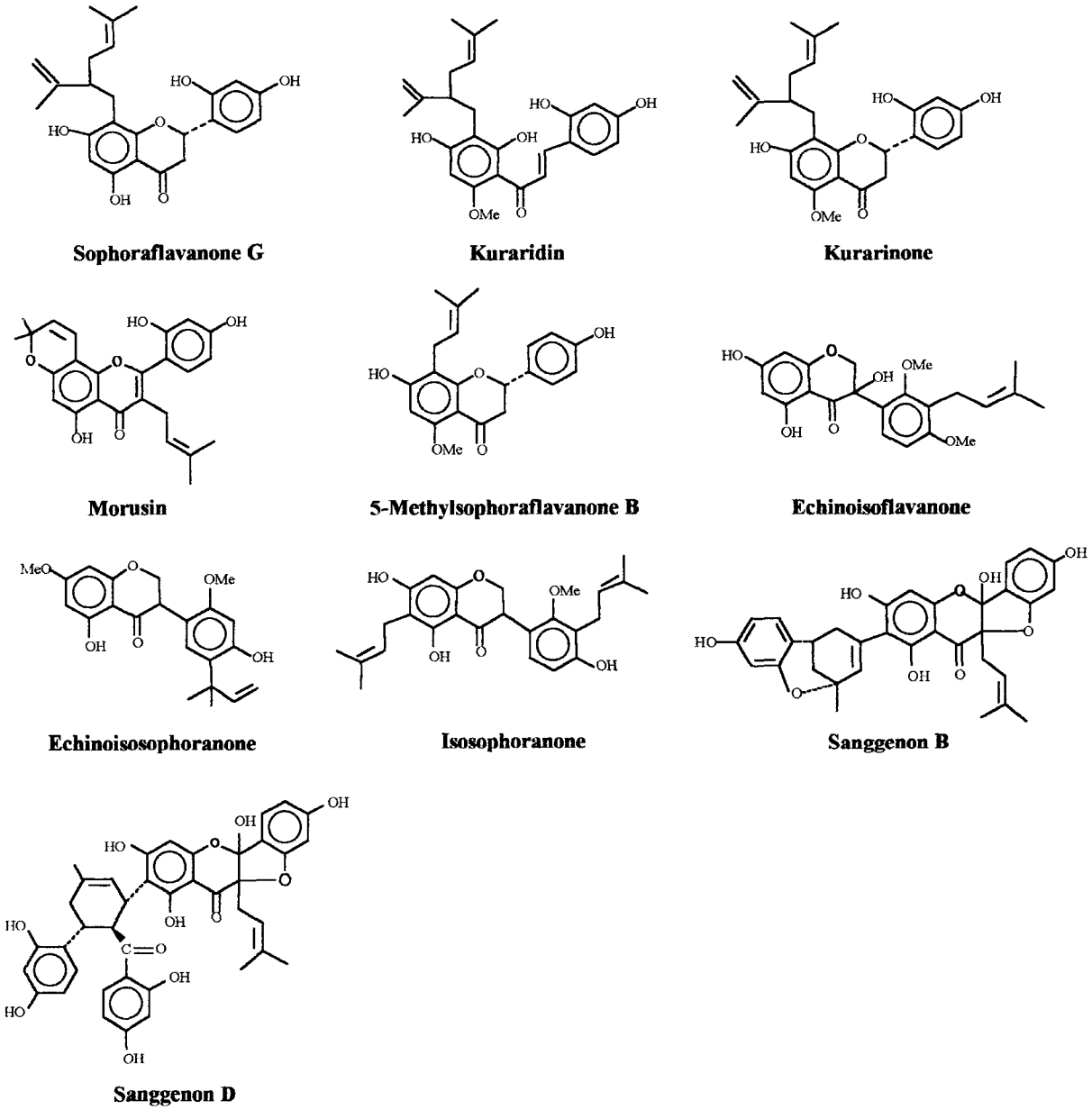

Fig. 1. Chemical structures of the prenylated flavonoids used in this study

paw edema assay were carried out following the established procedure (Kim *et al.*, 1993; Winter *et al.*, 1983). In brief, the test compounds including sophoraflavanone G dissolved in 1% carboxymethyl cellulose (CMC) were orally administered to mice. One hour later, 2.5% croton oil dissolved in acetone (20 ul/ear) was topically applied to the inner and the outer surfaces of ear. The ear thickness was measured 5 h after croton oil treatment using dial thickness gauge (Lux Scientific Instrument, USA). Percent inhibition of ear edema was calculated compared to the control group treated with vehicle and croton oil only. In order to determine topical activity, the test compounds dissolved in acetone were applied topically to ears of mice. After 30 min, croton oil was applied to induce ear edema. Measuring ear thickness increased and calculation of inhibition were carried out as same as in the oral treatment. In rat paw edema assay, 1% carrageenan solution was subplantally injected to the right hind paw. Test compounds dispersed in 1% CMC were orally administered one hour before carrageenan injection. Five hours later after carrageenan injection, paw volume was measured using plethysmometer (Ugo Basile, Italy).

Statistical analysis

All values were presented as arithmetic mean ±S.D.

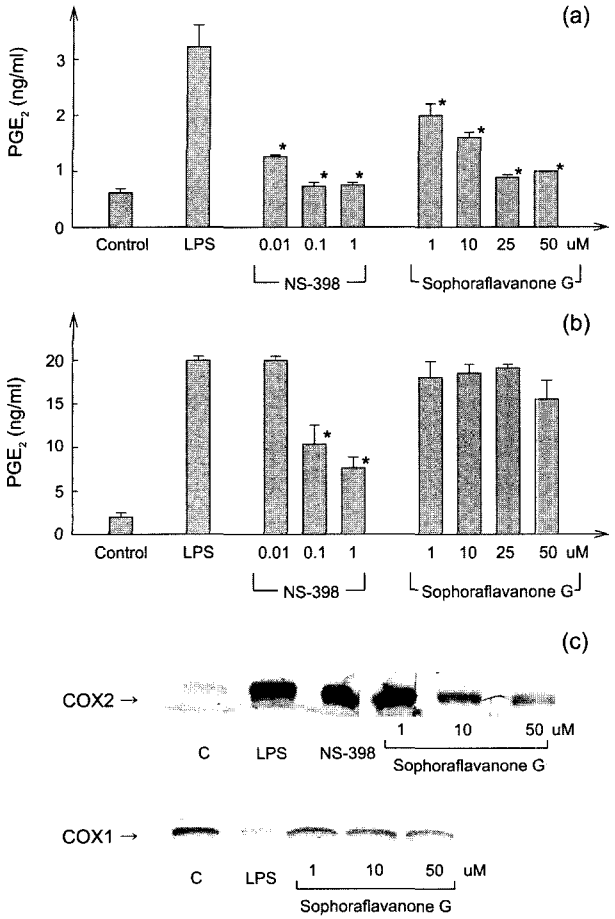

Fig. 2. Effect of sophoraflavanone G on PGE_2 production from LPS-treated RAW 264.7 cells (a) Inhibition of PGE_2 production from LPS-treated RAW 264.7. Test compounds including sophoraflavanone G were added simultaneously with LPS treatment. After 24 h incubation, PGE_2 concentrations of the medium were measured by EIA as described in Materials and Methods., (b) Inhibition of PGE_2 production from COX-2 pre-induced RAW 264.7. RAW cells were incubated with LPS for 24 h. After washing thoroughly with serum-free DMEM, arachidonic acid (100 uM) and test compounds were added and incubated for another 30 min in order to check COX-2 inhibition. PGE_2 concentrations of the medium were measured by EIA. *: $P < 0.01$, Significantly different from LPS-treated control group (n=3)., (c) Effects on COX-2 expression from LPS-treated RAW 264.7 cells (Western blot). Same amount of protein (10 ug) was loaded in each lane. Induction of COX-2 was markedly reduced by sophoraflavanone G, while the level of COX-1 was not effectively reduced.

One-way ANOVA was used to determine the statistical significance. IC_{25} and IC_{50} values were calculated using linear regression analysis. All experiments were performed at least twice and they gave the similar results.

RESULTS

Under the standard condition, RAW 264.7 cells treated with LPS produced 3.23 ± 0.39 ng PGE_2/ml medium compared to basal level (without LPS) of 0.62 ± 0.08 ng PGE_2/ml during 24 h incubation. When the test compounds were simultaneously added with LPS, sophoraflavanone G inhibited PGE_2 production concentration-dependently (1-50 uM) as shown in Fig. 2a. The reference compound, NS-398 (COX-2 inhibitor) potently inhibited PGE_2 production at 0.01-1 uM as expected. IC_{50} values for sophoraflavanone G and NS-398 were 2.7 and < 0.01 uM, respectively. When added after COX-2 was fully induced (24 h after LPS treatment), sophoraflavanone G did not effectively reduce PGE_2 production (Fig. 2b), whereas NS-398 potently inhibited (IC_{50}=0.01 uM). In all above experiments, sophoraflavanone G was not cytotoxic against RAW cells at the tested concentrations when judged by MTT bioassay. Next, COX-2 expression level was checked with western blotting. Fig. 2c clearly demonstrated that sophoraflavanone G down-regulated COX-2 expression from LPS-treated RAW cells at 10 and 50 uM, while the same compound did not affect the concentration of COX-1 at 1-50 uM. Effects of other prenylated flavonoids on COX-2 expression were also examined using the same experimental protocol at non-cytotoxic concentrations (10 and 25 uM). As shown in Fig. 3, sanggenon D down-regulated COX-2 induction at 10-25 uM. Kuraridin and sanggenon B inhibited COX-2 expression only at high concentration (25 uM). In contrast, other prenylated flavonoids including kurarinone and isosophoranone did not effectively reduced COX-2 expression up to 25 uM

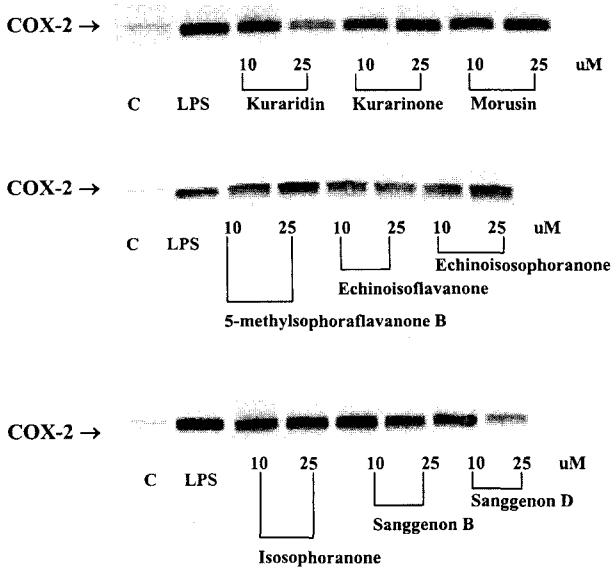

Fig. 3. Effects of prenylated flavonoids on COX-2 expression (Western blot) RAW cells were treated with LPS and each test compound. Same amount of protein (10 ug) was loaded in each lane of electrophoresis. Kuraridin (25 uM), sanggenon B (25 uM) and sanggenon D (10 and 25 uM) markedly suppressed COX-2 induction.

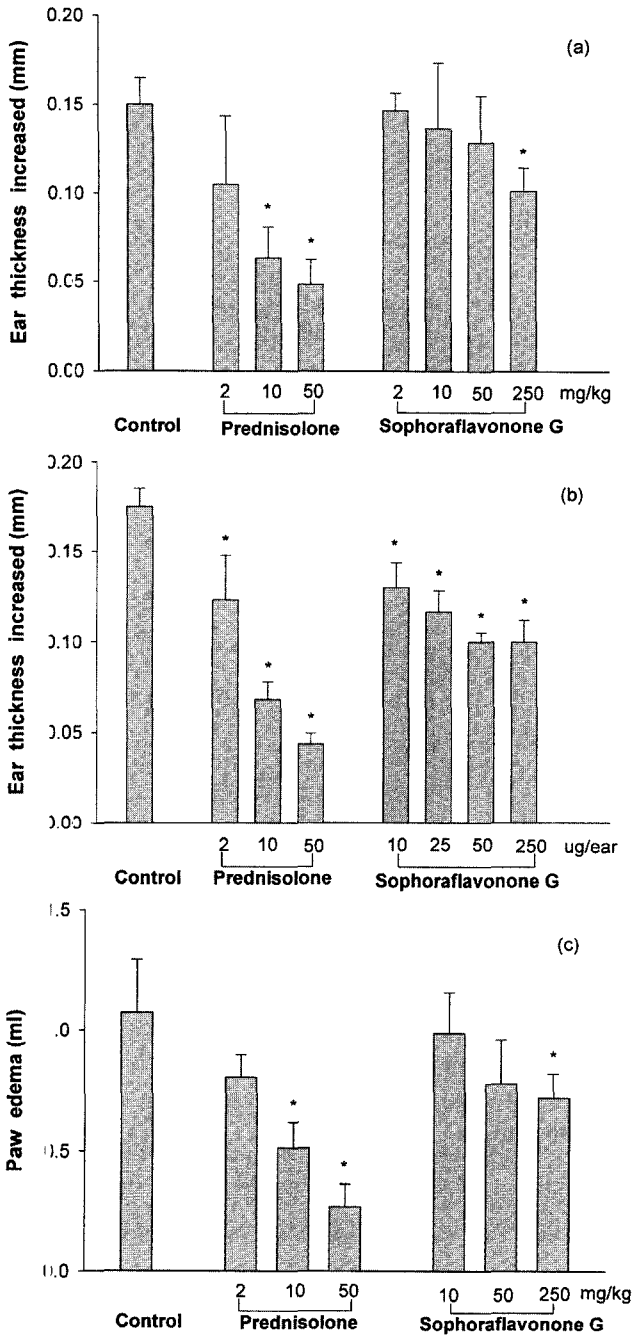

Fig. 4. *In vivo* anti-inflammatory activity of sophoraflavanone G
(a) Inhibition of sophoraflavanone G against croton oil-induced mouse ear edema by oral administration. (b) Inhibition of sophoraflavanone G against croton oil-induced mouse ear edema by topical application, (c) Inhibition of sophoraflavanone G against rat carrageenan paw edema by oral administration, *:P < 0.05, Significantly different from inflammagen-treated control group (n = 5).

from LPS-treated RAW 264.7 cells.

When sophoraflavanone G was tested in mouse ear edema model, it inhibited croton oil-induced ear edema in differing potencies depending on the routes of administration. By oral treatment, sophoraflavanone G showed 32% inhibition at the highest dose tested (250 mg/kg), while prednisolone, a reference steroidal anti-inflammatory drug, showed potent inhibition (30-68% inhibition at 2-50 mg/kg). IC_{25} and IC_{50} values were 134 and 8.5 mg/kg for sophoraflavanone G and prednisolone, respectively (Fig. 4a). Interestingly, sophoraflavanone G showed much higher anti-inflammatory activity by topical application in the same model. IC_{25} and IC_{50} values for sophoraflavanone G and prednisolone were 9.8 and 6.9 ug/ear, respectively (Fig. 4b). For rat paw edema, sophoraflavanone G showed weak anti-inflammatory activity (33% inhibition at 250 mg/kg), while prednisolone showed 25-75% inhibition at 2-50 mg/kg (Fig. 4c).

DISCUSSION

The present investigation clearly demonstrated that sophoraflavanone G inhibited PGE_2 production from LPS-treated RAW 264.7 cells. However, the same compound did not effectively blocked PGE_2 production when added after COX-2 was fully induced in RAW cells. These results strongly support the previous finding that sophoraflavanone G did not directly inhibit COX-2 (Chi *et al.*, 2001a). Sophoraflavanone G clearly down-regulated COX-2 induction without significantly affecting COX-1 level, suggesting that sophoraflavanone G inhibits PGE_2 production from LPS-treated RAW cells, at least in part, by inhibition of COX-2 expression. Other prenylated flavonoids such as kuraridin and sanggenon D also inhibited COX-2 induction, while kurarinone and echinoisoflavanone did not. Previously, various types of flavonoids including flavones, flavonols, isoflavones and biflavonoids were proved to inhibit the expression of inflammation-related inducible enzymes such as COX-2 and inducible nitric oxide synthase (NOS type 2) in macrophages or macrophage-like cell lines (Kim *et al.*, 1999; Liang *et al.*, 1999; Cheon *et al.*, 2000). And it was suggested that a down-regulation of the expression of these enzymes might be one of anti-inflammatory action mechanisms of flavonoids. Although no structural-activity relationship in COX-2 down-regulation among the prenylated flavonoids is found, our study has revealed that certain prenylated flavonoids possess the similar properties with common flavonoids, which may contribute to *in vivo* anti-inflammatory activity of this group of plant constituents.

In addition to down-regulating potential of inflammation-related inducible enzymes, certain flavonoids directly inhibit AA metabolizing enzymes depending on their chemical structures. For instance, quercetin and several biflavonoids are inhibitors of phospholipase A_2 (PLA_2) (Lindahl and Tagesson, 1993; Chang *et al.*, 1994). Some

flavones, flavonols and prenylated flavonoids inhibit COX-1, 5-LOX and/or 12-LOX with differing potencies. Recently, several catechins, quercetin and sanggenon D were proved to inhibit COX-2, but not selective over COX-1 (Noreen *et al.*, 1998; Chi *et al.*, 2001a). The present study and our previous investigation (Chi *et al.*, 2001a) demonstrated that sophoraflavanone G, in particular, inhibits the enzyme activities of COX-1, 5-LOX and 12-LOX as well as down-regulates COX-2 induction, leading to the reduction of eicosanoid production.

The present study has also shown that sophoraflavanone G possesses anti-inflammatory activity *in vivo*. Especially, this compound showed much higher anti-inflammatory activity via topical application compared to the activity by oral treatment. A lavandulyl moiety attached to flavonoid backbone structure may increase the skin penetration rate (bioavailability) due to its hydrophobic property. These results suggest that this type of prenylated flavonoids may have a potential for anti-inflammatory agents, especially for several eicosanoid-related skin inflammation such as atopic dermatitis.

In conclusion, sophoraflavanone G inhibited COX-2 induction from LPS-treated RAW 264.7 cells, mainly by COX-2 down-regulation. The same compound showed anti-inflammatory activity *in vivo* against mouse croton oil-induced ear edema and rat carrageenan paw edema.

ACKNOWLEDGEMENTS

This investigation was financially supported by grant No. 98-0403-11-01-3 from the interdisciplinary research program of the KOSEF and greatly acknowledged.

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