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Anti-Inflammatory Activity of *Elsholtzia splendens*

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Elsholtzia splendens Nakai has been used in North-East Asia as an ingredient of folk medicines for treating cough, headache and inflammation. The present investigation was carried out to establish its *in vivo* anti-inflammatory activity using several animal models of inflammation and pain. The 75% ethanol extract of the aerial part of *E. splendens* significantly inhibited mouse croton oil-induced, as well as arachidonic acid-induced, ear edema by oral administration (44.6% inhibition of croton oil-induced edema at 400 mg/kg). This plant material also showed significant inhibitory activity against the mouse ear edema induced by multiple treatment of phorbol ester for 3 days, which is an animal model of subchronic inflammation. In addition, *E. splendens* exhibited significant analgesic activity against mouse acetic acid-induced writhing (50% inhibition at 400 mg/kg), while indomethacin (5 mg/kg) demonstrated 95% inhibition. *E. splendens* (5-100 μg/mL) significantly inhibited PGE_2 production by pre-induced cyclooxygenase-2 of lipopolysaccharide-treated RAW 264.7 cells, suggesting that cyclooxygenase-2 inhibition might be one of the cellular mechanisms of anti-inflammation.

Key words: *Elsholtzia splendens* Nakai, Anti-inflammation, Analgesic activity, Cyclooxygenase

INTRODUCTION

Eicosanoids, including prostaglandins (PG) and leukotrienes (LT), are biosynthesized from arachidonic acid by cyclooxygenases (COX) and lipoxygenases (LOX). Since they are deeply associated with inflammatory disorders, acute as well as chronic inflammation, an inhibition of eicosanoid production is one of the important therapeutic strategies for treating various inflammatory diseases. For example, nonsteroidal anti-inflammatory drugs such as indomethacin and diclofenac are general inhibitors of COX-1 (constitutive isoform) and COX-2 (inducible isoform) with varying degrees of potency. Recently developed COX-2 selective inhibitors, including celecoxib, show more promising results in clinical use with fewer side-effects (McMurray and Hardy, 2002). On the other hand, steroidal anti-inflammatory drugs such as dexamethasone impact the inflammatory process, at least partly by suppression of the expression of proinflammatory proteins including COX-2 (Goppelt-Struebe, 1997). Some antiallergic drugs such as zileuton inhibit 5-LOX, thereby reducing concentrations of LTs. In line with these advances, the development of COX/LOX inhibitors or modulators of COX-2 expression may represent a significant avenue in the introduction of new anti-inflammatory agents.

Elsholtzia splendens Nakai (E. s.) has been used in North-East Asia as an ingredient of folk medicines for treating cough, pain and inflammation (Bae, 2000). During our screening program to search for new anti-inflammatory agents, the 75% ethanol extract of E. s. was found to modulate the production of PGE_2 from lipopolysaccharide (LPS)-treated RAW 264.7 cells, a mouse macrophage cell line. This finding prompted us to establish the *in vivo* anti-inflammatory potential of this plant material using several animal models of inflammation, since its anti-inflammatory activity has not been described. In this report, the anti-inflammatory and analgesic activities of E. s. were evaluated and the *in vitro* effect on PG generation was also studied.

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MATERIALS AND METHODS

Chemicals

N-[2-(Cyclohexyl)-4-nitrophenyl]-methane sulfonamide (NS-398) was purchased from Biomol (Plymouth Meeting, PA) and prednisolone from Upjohn Co. (Kalamazoo, MI). Arachidonic acid (AA, 99%), lipopolysaccharide (LPS, *Escherichia coli* 0127:B8), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), croton oil, carboxymethyl cellulose (CMC) and 12-O-tetradecanoylphorbol 13-acetate (TPA) were purchased from Sigma-Aldrich (St. Louis, MO). Dulbecco's modified Eagles medium (DMEM) and other cell culture reagents, including fetal bovine serum (FBS), were obtained from Gibco BRL (Grand Island, NY). Heat-killed *Mycobacterium butyricum* was a product of Difco Lab. (Detroit, MI). EIA kit for PGE_2 and anti-COX-2 antibody (No. 160116) were purchased from Cayman Chem. (Ann Arbor, MI).

Plant materials

E. s. was collected in October, 2000 at Mt. Kyuryong, Taejon, Korea. Its identification was confirmed by one of the authors, Dr. K. Bae and a voucher specimen has been deposited in the herbarium of the College of Pharmacy, Chungnam National University, under the registration number CNU 1814. The dried and chipped aerial part of E. s. (200 g) was extracted with 1,500 mL of 75% ethanol at room temperature for 1 week (repeated twice). The combined solution was evaporated to dryness under vacuo (20 g) and used throughout this study.

Animals

Male ICR mice (16-20 g) and Sprague-Dawley (SD) rats (150-180 g) were obtained from SLC (Japan). Animals were fed with laboratory chow (Purina Korea) and water *ad libitum*. They were acclimatized in a specific, pathogen-free, animal facility at 20-22°C, 40-60% relative humidity and 12 h/12 h (light/dark) cycle for at least 7 days.

In vivo anti-inflammatory activity

In order to evaluate the inhibitory activity of E. s. against animal models of acute inflammation, mouse croton oil-induced and arachidonic acid-induced ear edema were employed according to the previously described procedures (Kim *et al.*, 1993). Briefly, 2.5% croton oil in acetone was topically applied to ears of mice (20 μL/ear). Five hours later, the ear thickness was measured using a dial thickness gauge (Lux Scientific Instrument, USA). In the case of arachidonic acid-induced edema, 2% inflammagen was smeared and the ear thickness measured one hour later. E. s. and reference drugs dispersed in 0.5% CMC were administered orally 1 h prior to the treatment of each inflammagen. To measure the anti-inflammatory activity in an animal model of subchronic inflammation, TPA-induced ear edema assay (multiple treatment of TPA) was carried out using a slightly modified procedure of Stanley *et al.* (1991). Briefly, TPA dissolved in acetone (3 μg/10 μL) was topically applied to ears of mice once a day for three days. E. s. and prednisolone dispersed in 0.5% CMC were orally administered once a day for 3 consecutive days, 2 h after the TPA treatment. At 12 h intervals, the ear thickness was measured using a dial thickness gauge. For evaluating the inhibitory activity against an animal model of chronic inflammation, rat adjuvant-induced arthritis (AIA) was used according to the previously reported procedure (Kim *et al.*, 1999). Briefly, an arthritic inflammation was provoked by injection of *Mycobacterium butyricum* (0.6 mg/rat) dissolved in mineral oil to the rat right hind paw. E. s. and prednisolone were orally administered daily. The swelling of the treated and untreated paws was measured using plethysmometer (Ugo basile, Italy) for 20 days.

Analgesic activity

For measuring analgesic activity, the standard acetic acid-induced writhing test was employed according to the previously described procedure of Bentley *et al.* (1983). Briefly, E. s. and indomethacin were orally administered to mice. One hour later, 100 μL of acetic acid (0.7%) was administered intraperitoneally and the incidence of writhing was counted for 10 min starting 10 min after the administration of acetic acid solution.

RAW 264.7 cell culture, measurement of PGE_2 concentration and Western blotting of COX-2

RAW 264.7 cells obtained from American Type Culture Collection were cultured in DMEM supplemented with 10% FBS and 1% antibiotics under 5% CO_2 at 37°C, and activated with LPS. All procedures, including Western blotting technique, were based on previously described methods (Chi *et al.*, 2001). Briefly, cells in 96-well plates (2×10^5 cells/well) were treated with LPS (1 μg/mL) and various concentrations of E. s. for 24 h, unless otherwise specified. E. s. was dissolved in DMSO and diluted with serum-free DMEM into appropriate concentrations. Cell viability was accessed by MTT bioassay (Mossman, 1983). PGE_2 concentration in the media was measured using an EIA kit following the manufacturer's recommendation. To determine the direct inhibitory activity of COX-2 by E. s., cells were incubated with LPS (1 μg/mL) for 24 h in order to allow for COX-2 induction, and washed thoroughly three times with serum-free DMEM. E. s. and AA (10 μM) were added and incubated for another 30 min. PGE_2 concentration in the media was measured using an EIA kit as described above. For Western blotting of COX-2, cells were cultured in 6-well plates (5×10^6 cells/well) in the presence or absence of LPS (1 μg/mL), either with or

without E. s. for 20 h. After preparing the cell homogenate, proteins were separated on 4-15% Tris-glycine gel (Novex Lab.) by electrophoresis and bands were blotted to PVDF membrane. The COX-2 band was visualized using anti-COX-2 antibody and horseradish peroxide-conjugated secondary antibody.

Statistical analysis

All values were represented as arithmetic mean±S.D. One-way ANOVA was used to determine the statistical significance.

RESULTS

Against animal models of acute inflammation, E. s. showed a dose-dependent anti-inflammatory activity by oral administration (Table I). At oral doses of 100-400 mg/kg, E. s. significantly inhibited mouse croton oil-induced ear edema (44.6% inhibition at 400 mg/kg), while prednisolone (10 mg/kg) demonstrated 64.6% inhibition. Against arachidonic acid-induced ear edema, E. s. showed a slightly weaker anti-inflammatory activity (22.9% inhibition at 400 mg/kg), whereas indomethacin showed potent inhibition (52.1% inhibition at 10 mg/kg). In a subchronic model of skin inflammation provoked by multiple treatment of TPA, E. s. (50-200 mg/kg/day) inhibited edema for up to 60 h dose-dependently (Table II). Prednisolone (2 mg/kg/day) showed similar or slightly less inhibitory activity against the same animal model compared to the E. s.-treated group (200 mg/kg/day). However, E. s. did not show significant inhibitory activity in rat AIA, an animal model of chronic inflammation, at doses of 5-200 mg/kg/day for 20 days, while prednisolone (5-10 mg/kg/day) did show potent inhibition (data not shown). On the other hand, E. s. showed significant analgesic activity on acetic acid-induced writhing at doses of 100-400 mg/kg with an IC_{50} value of approximately 400 mg/kg (Fig. 1). Indomethacin showed potent analgesic activity (91.2% inhibition at 5 mg/kg).

Table I. Inhibition of mouse ear edema by *E. splendens*

Group[a]	Dose (mg/kg)	Ear thickness increased (mm)	
		Croton oil[b]	Arachidonic acid[c]
Control	–	0.11 ± 0.01 (–)[d]	0.08 ± 0.01 (-)
Prednisolone	10	0.04 ± 0.02* (64.6)	NT[e]
Indomethacin	10	NT	0.04 ± 0.01* (52.1)
E. splendens	100	0.10 ± 0.02 (9.3)	0.09 ± 0.01 (-)
	200	0.08 ± 0.01 (23.1)	0.08 ± 0.01 (6.2)
	400	0.06 ± 0.02* (44.6)	0.06 ± 0.01 (22.9)

[a]All compound were dissolved in 0.5% CMC and orally administered to mice 1 h prior to the treatment of inflammagen. [b]Croton oil-induced ear edema, [c]Arachidonic acid-induced ear edema, [d]Values in parenthesis represent % inhibition of ear edema compared to the control group. [e]NT: Not tested. *: P<0.01, Significantly different from the control group (n = 6, arithmetic mean ± S.D.).

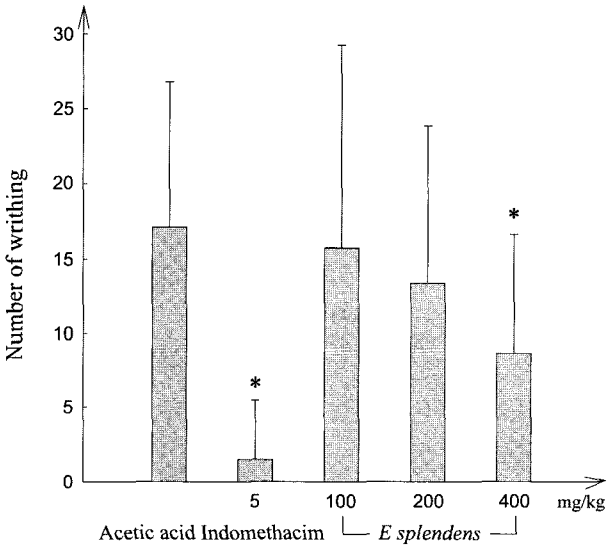

Fig. 1. Analgesic activity of *E. splendens* in mice. The standard acetic-acid-induced writhing in mice was employed. The acetic acid-treated control group showed 17.1 ± 9.7 incidences of writhing in 10 min. n = 8, *: P<0.05, Significantly different from the acetic acid-treated control group.

Table II. Inhibition of TPA-induced mouse ear edema (multiple treatment) by *E. splendens*

Group[a]	Dose (mg/kg/day)	Ear thickness increased (mm)				
		12 h[b]	24 h	36 h	48 h	60 h
Control	–	0.18 ± 0.01[c]	0.18 ± 0.02	0.20 ± 0.02	0.20 ± 0.02	0.21 ± 0.02
Prednisolone	2	0.14 ± 0.03 (25.0)[d]	0.16 ± 0.01 (10.5)	0.17 ± 0.02 (16.4)	0.16 ± 0.02 (19.5)	0.16 ± 0.03 (23.6)
E. splendens	50	0.17 ± 0.01 (3.7)	0.16 ± 0.02 (6.7)	0.19 ± 0.03 (9.0)	0.16 ± 0.02 (17.5)	0.18 ± 0.01 (13.0)
	100	0.15 ± 0.01 (16.7)	0.13 ± 0.03 (24.8)	0.17 ± 0.04 (16.4)	0.17 ± 0.02 (12.7)	0.17 ± 0.01 (15.0)
	200	0.12 ± 0.02* (32.4)	0.13 ± 0.01 (25.7)	0.15 ± 0.04 (28.7)	0.15 ± 0.04 (22.9)	0.16 ± 0.03 (23.6)

[a]TPA (3.0 μg/ear/day) was topically applied to ears of mice for three days. All compound were dissolved in 0.5% CMC and orally administered to mice 2 h after TPA treatment once a day for three consecutive days. [b]Time after first application of TPA, [c]Arithmetic mean ± S.D. (n = 6), [d]Values in parenthesis represent % inhibition of ear edema compared to each column of the control group. *: p<0.01, Significantly differ ent from each column of the control group.

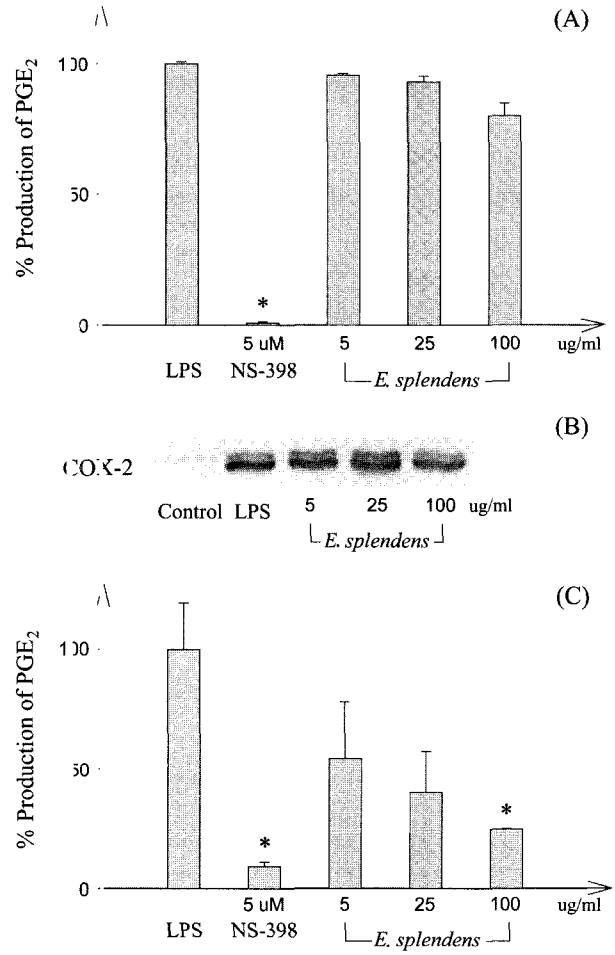

Fig. 2. Inhibition of PGE_2 production from LPS-treated RAW 264.7 cells by *E. splendens*. (A) Effect of pretreatment of E. s. on PGE_2 production. E. s. and LPS were simultaneously added to RAW cells. After 24 h, PGE_2 concentration was measured using an EIA kit as described in Materials and Methods. LPS treatment produced 22.4 ± 0.2 ng/mL of PGE_2 over 24 h from the basal PGE_2 level of 0.5 ± 0.1 ng/mL. One hundred percent PGE_2 production in this figure represented the difference between these two values. (B) Effect of E. s. on COX-2 expression (Western blotting). E. s. and LPS were simultaneously added to RAW cells. After 20 h, cells were harvested and homogenized. The same amount of protein was used for electrophoresis (10 μg/lane). (C) Effect of E. s. on PGE_2 production by pre-induced COX-2. To induce COX-2, LPS was treated on RAW cells for 24 h. After washing thoroughly, E. s. and arachidonic acid were added and incubated for another 30 min. PGE_2 concentration in the media was measured using an EIA kit. One hundred percent production represented 17.3 ± 3.2 ng/mL of PGE_2. n = 3, *: $P<0.005$, significantly different from the LPS-treated control.

In order to determine the cellular mechanism of anti-inflammation, the effects on COX-2 activity and expression were investigated using a mouse macrophage-like cell line, RAW 264.7 cells. When LPS (1 μg/mL) was treated, RAW 264.7 cells induced a high level of COX-2, concomitantly producing a large amount of PGE_2. The peak time for the highest induction of COX-2 was previously determined to be 10-20 h after LPS treatment (Chi *et al.*, 2001). When added simultaneously with LPS, E. s. (5-25 μg/mL) did not significantly inhibit PGE_2 production for 24 h incubation period as shown in Fig. 2A. The highest concentration of E. s. (100 μg/mL) only inhibited PGE_2 production by 19.9%, while as expected, NS-398 (COX-2 inhibitor) potently inhibited PGE_2 production (99.2% inhibition at 5 μM). In accordance with this result, E. s. did not significantly suppress COX-2 induction over the same concentration range, as revealed by Western blotting experiment (Fig. 2B). Instead, E. s., when added after COX-2 was fully induced and incubated for shorter time period (30 min), strongly inhibited PGE_2 production in a concentration-dependent manner (Fig. 2C). E. s. and NS-398 at the tested concentrations did not show any cytotoxicity judged by MTT assay, indicating that the inhibition of PGE_2 production by E. s. was not associated with its cytotoxicity.

In order to evaluate its acute toxicity, E. s. was orally administered to mice up to 3.2 g/kg (2 fold dilution). For 14 days after administration, no apparent toxicity, including death or any change of major organ (lung, heart, liver, spleen and kidney) weight, was observed (data not shown).

DISCUSSION

All results from this study clearly demonstrated that E. s. inhibited both acute and subchronic inflammation dose-dependently by oral administration. E. s. also showed analgesic activity, although at a potency far less than that of indomethacin. Moreover, the absence of any significant acute toxicity of this plant material by oral administration suggests that it is practically nontoxic and may be used safely in human at moderate doses. E. s., however, did not significantly inhibit the chronic inflammation in rat AIA test at the tested doses.

E. s. showed less than 20% inhibition of PGE_2 production even at the highest concentration tested (100 μg/mL) from LPS-treated RAW cells for a 24 h period, when added simultaneously with LPS. This result is in accordance with the lack of significant suppression of COX-2 induction by E. s. In contrast, E. s. strongly reduced PGE_2 production by pre-induced COX-2 in the 30 min incubation experiment, suggesting that E. s. may possess a direct COX-2 inhibitory activity without affecting COX-2 expression. The precise reason for the absence of profound reduction of PGE_2 production for 24 h incubation is not clear at present. Nevertheless, it is strongly speculated that active principle(s) of E. s. may undergo extensive metabolism in RAW cell culture, rapidly changing to inactive metabolite(s) for 24 h incubation, whereas E. s. inhibited PGE_2 production in 30 min incubation by direct inhibition of COX-2. This speculation may also partly explain the experimental finding that E. s. did not affect AIA in rats, a chronic model of

inflammation, where continuous inhibition of inflammatory stimuli over the entire disease process is necessary to express the inhibitory activity. It is well known that nonsteroidal anti-inflammatory drugs (NSAID) inhibit COX-1/COX-2 while steroidal anti-inflammatory drugs (SAID) inhibit proinflammatory gene expression including COX-2. Thus E. s. behaves like NSAID with respect to the cellular action mechanism.

The genus *Elsholtzia* is an ornamental, usually aromatic herb, belonging to the Labiatae family. In Korea, five species are distributed (Lee, 1996), and the aerial part has been used since ancient time as an ingredient of folk remedies for cough, inducing sweat, blood circulation, rheumatism, inflammation, headache, and diuretics (Bae, 2000). Among the species of the genus *Elsholtzia*, the constituents of *E. cristata* (=*E. ciliata*) have been comparatively well studied. Previously, elsholtzia ketone, 3-methyl-2-(3-methylbut-3-enoyl)-furan, dehydro-elsholtzia ketone, and monoterpenes were isolated from *E. cristata* (Kobold *et al.*, 1987). Apigenin, apigenin-7-*O*-glucoside, luteolin-7-*O*-glucoside and linarin were successfully isolated (Lee *et al.*, 1988). In addition, β-sitosterol, ursolic acid, 2α-hydroxyursolic acid, tormentic acid, β-sitosterol glucoside, apigenin and luteolin were also identified (Isobe and Noda, 1992). The essential oil of Elsholtzia species has been reported to possess antibacterial and antifungal activities (Kobold *et al.*, 1987). In contrast, the chemistry and pharmacological activity of E. s have not been described. To the best of our knowledge, this is the first report demonstrating the *in vivo* anti-inflammatory activity of E. s. and providing a scientific basis for the medicinal use of this plant material. The isolation of the active principles from E. s. is currently being investigated.

All results from the present investigation suggest that E. s. may be used as an anti-inflammatory agent with analgesic activity. The inhibition of prostanoid production by direct COX-2 inhibition may be, at least in part, one of the cellular action mechanism(s) of anti-inflammation by E. s.

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REFERENCES

Bae, K. (Ed.), in *The medicinal plants of Korea*. Kyohaksa Co., Seoul, pp.435, (2000).

Bentley, G. A., Newton, S. H., and Star, J., Studies on the anti-nociceptive action of alpha-agonist drugs and their interactions with opioid mechanisms. *Brit. J. Pharmacol.*, 79, 125-134 (1983).

Chi, Y. S., Cheon, B. S., and Kim, H. P., Effect of wogonin, a plant flavone from Scutellaria radix, on the suppression of cyclooxygenase-2 and the induction of inducible nitric oxide synthase in lipopolysaccharide-treated RAW 264.7 cells. *Biochem. Pharmacol.*, 61, 1195-1203 (2001).

Goppelt-Struebe, M., Molecular mechanisms involved in the regulation of prostaglandin biosynthesis by glucocorticoids. *Biochem. Pharmacol.*, 53, 1389-1395 (1997).

Isobe, T., and Noda, Y., Studies on chemical constituents of Elsholtzia herb. *J. Chem. Soc.* (Japan), 4, 423-425 (1992).

Kim, H. K., Namgoong, S. Y., and Kim, H. P., Anti-inflammatory activity of flavonoids: Mice ear edema inhibition. *Arch. Pharm. Res.*, 16, 18-24 (1993).

Kim, H. K., Son, K. H., Chang, H. W., Kang, S. S., and Kim, H.P., Inhibition of rat adjuvant-induced arthritis by gingketin, a biflavone from *Gingko biloba* leaves. *Planta Med.*, 65, 465-467 (1999).

Kobold, U., Vostrowsky, O., Bsetman, H. J., Bisht, J. C., Pant, A. K., Melkani, A. B., and Mathela, C. S., Terpenoids from Elsholtzia species, 2. Constituents of essential oils from a new chemotype of *Elsholtzia cristata*. *Planta Med.*, 55, 268-271 (1987).

Lee, W. C. (Ed.), in *Standard illustration of Korean plants*. Academy, Seoul, pp.302-303 (1996).

Lee, Y., Lee, I., Woo, W., and Park, C., Flavonoids of *Elsholtzia cristata*. *Arch. Pharm. Res.*, 11, 247-249 (1988).

Mossman, T., Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxic assays. *J. Immunol. Methods*, 65, 55-63 (1983).

McMurray, R. W., and Hardy, K. J., COX-2 inhibitors: today and tomorrow. *Am. J. Med. Sci.*, 323, 181-189 (2002).

Stanley, P. L., Steiner, S., Havens, M., and Tramposch, K. M., Mouse skin inflammation induced by multiple topical applications of 12-O-tetradecanoylphorbol-13-acetate. *Skin Pharmacol.*, 4, 262-271 (1991).