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New Anti-Inflammatory Formulation Containing *Synurus deltoides* Extract

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Synurus deltoides was previously found to possess significant anti-inflammatory activity especially against chronic inflammation, and strong analgesic activity *in vivo*. In this study, new anti-inflammatory formulation containing *S. deltoides* extract as a major ingredient was prepared and *in vivo* activity was evaluated. The plausible action mechanism was also investigated. The new formulation (SAG) contains 1 part of *S. deltoides* extract, 0.9 part of *Angelica gigas* extract and 0.9 part of glucosamine sulfate (w/w). SAG inhibited dose-dependently edematic response of arachidonic acid (AA)- and 12-*O*-tetradecanoyl 13-acetate (TPA)-induced ear edema in mice, which is an animal model of acute inflammation. SAG showed 44.1% inhibition of AA-induced ear edema at an oral dose of 50 mg/kg. In an animal model of chronic inflammation, SAG clearly reduced the edematic response of 7-day model of multiple treatment of TPA (38.1% inhibition at 200 mg/kg/day). Furthermore, SAG (50-800 mg/kg/day) as well as *S. deltoides* extract (285 mg/kg/day) significantly inhibited prostaglandin E_2 production from the skin lesion of the animals of 7-day model. These results were well correlated with *in vitro* finding that SAG as well as *S. deltoides* extract reduced cyclooxygenase (COX)-1- and COX-2-induced prostanoid production, measured in mouse bone marrow-derived mast cells. Therefore, these results suggest that SAG possesses anti-inflammatory activity *in vivo* against acute as well as chronic inflammatory animal models at least in part by inhibition of prostaglandin production through COX-1/COX-2 inhibition. And COX inhibition of SAG is possibly contributed by *S. deltoides* extract among the ingredients. Although the anti-inflammatory potencies of SAG were less than those of currently used anti-inflammatory drugs, this formulation may have beneficial effect on inflammatory disorders as a neutraceutical.

Key words: *Synurus deltoides* Aiton Nakai, *Angelica gigas* Nakai, Glucosamine sulfate, Anti-inflammatory activity, Cyclooxygenase, Prostaglandin

INTRODUCTION

The conventional anti-inflammatory drugs such as steroidal anti-inflammatory drugs (SAID) and nonsteroidal anti-inflammatory drugs (NSAID) are used for treatment of most of acute inflammatory disorders. These drugs are also used to treat chronic inflammatory disorders including rheumatoid arthritis (RA) without altering the disease process. On long-term use, however, they may produce frequent adverse effects. Thus, it is necessary to develop new anti-inflammatory agents for prolonged and safe use. In this respect, plant-derived anti-inflammatory nutraceuticals may be suitable for several chronic inflammatory disorders including RA.

Synurus deltoides Aiton, Nakai (Compositae) is an edible plant and largely distributed at North-East Asia including Korea (Lee, 2002). It has long been used as a folk medicine for treating inflammatory edema, bleeding, vomiting, and urinary inflammation, etc. (Choi, 1991). A few chemical and pharmacological studies were reported previously concerning *S. deltoides* and related species. Yoshitama *et al.* (1980) detected anthocyanins in this plant. Zarembo *et al.* (2001) measured 20-hydroxyecdysone content. Ham *et al.* (1997) have shown that the same plant possessed antimutagenicity *in vitro*. Recently, we have proven that *S. deltoides* extract possessed signifi-

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cant anti-inflammatory activity especially against an animal model of chronic inflammation, and strong analgesic activity (Park *et al.*, 2004). From this plant material, ursolic acid and scopoletin were successfully isolated as active principles. These previous findings indicate that *S. deltoides* extract may be a new candidate for anti-inflammatory/antirheumatic agent. Therefore, in this study, new anti-inflammatory formulation (SAG) containing *S. deltoides* extract as a major ingredient has been developed and its anti-inflammatory potential including cellular action mechanism was established.

MATERIALS AND METHODS

Materials

Arachidonic acid (AA, 99%), 12-*O*-tetradecanoylphorbol 13-acetate (TPA), aspirin, lipopolysaccharide (LPS, Escherichia coli 0127:B8), recombinant mouse c-Kit ligand (KL), IL-10 and carboxymethylcellulose (CMC) were purchased from Sigma Chem. (St. Louis, MO). Prednisolone was a product of Upjohn Co. (Kalamazoo, MI). Cell culture reagents including RPMI 1640 and fetal calf serum were obtained from Gibco BRL. (Grand Island, NY). Glucosamine hydrochloride was purchased from Exquim S. A. (Spain).

Animals

Male BALB/cJ and ICR mice were purchased from Orient Co. (Seoul, Korea). Animals were maintained in a specific pathogen-free environment under the experimental conditions of 22 ± 1°C, 40-60% relative humidity and 12 h/12 h (light/dark) cycle feeding with pellet chow and water *ad libitum*. Animals were used after acclimatization for at least 7 days.

Preparation of anti-inflammatory formulation (SAG)

The aerial parts of *S. deltoides* and roots of *Angelica gigas* (*A. gigas*) were collected from North-Eastern area of China in 2003 and were dried. These plant materials were identified by one of the authors, Dr. K. Bae (College of Pharmacy, Chungnam National University, Korea). The chopped aerial parts of *S. deltoides* (3.0 kg) were refluxed in 70% ethanol (30 L). After filtration, the filtrate was dried *in vacuo* (625 g). The same procedure using *A. gigas* (3 kg) yielded 1,000 g extract. The water content of the final dried extracts of *S. deltoides* and *A. gigas* were 30.6 and 29.2%, respectively. The anti-inflammatory formulation (SAG) was a mixture of 1 part of *S. deltoides* as a major constituent, 0.9 part of *A. gigas* and 0.9 part of glucosamine, based on the dry weight. Throughout the investigation, SAG was used without any further treatment.

Preparation of mouse bone marrow-derived mast cells (BMMC) and measurement of prostaglandin D_2 (PGD_2)

Bone marrow cells from the male BALB/cJ mice were cultured for up to 10 weeks in 50% enriched medium (RPMI 1640 containing 2 mM L-glutamine, 0.1 mM nonessential amino acids, antibiotics and 10% fetal calf serum) and 50% WEHI-3 cell conditioned medium as a source of IL-3. After 3 weeks, >98% of the cells were found to be BMMC as determined by a procedure described previously (Bingham *et al.*, 1996; Moon *et al.*, 1998). For measuring the effect of SAG on COX-2, cells were suspended at a density of 5×10^5 cells/mL in enriched medium and were pre-incubated with aspirin (10 μg/mL) for 2 h in order to irreversibly inactivate preexisting COX-1. After washing, BMMC were activated with KL (100 ng/mL), IL-10 (100 U/mL) and LPS (100 ng/mL) at 37°C for 8 h in the presence or absence of SAG and *S. deltoides* extract previously dissolved in dimethylsulfoxide (DMSO). For measuring COX-1 activity, cells without aspirin pretreatment were incubated at 37°C for 2 h in the presence of activators. All the reactions were stopped by centrifugation at 120 g at 4°C for 5 min. From the supernatant, the concentration of PGD_2 was determined by using an ELISA kit (Cayman Chem., Ann Arbor, MI) according to the manufacturer's instruction.

AA-induced and TPA-induced ear edema (acute inflammation)

For an animal model of acute inflammation, mouse ear edema assay was carried out according to the slightly modified method (Kim *et al.*, 1993) of the original procedure of Tonneli *et al.* (1965). SAG dissolved in 0.5% CMC or prednisolone in the same vehicle was administered orally to ICR mice (18-22 g) at the indicated doses 1 h prior to inflammagen treatment. As an inflammagen, AA (2%) or TPA (3 μg/ear) dissolved in acetone (20 μL) was topically applied to both the ears of mice. One hour later in case of AA treatment or three hours later for TPA treatment (3-h model), ear thickness of the mice was measured by using an engineering gauge (Mitutoyo Co., Japan). The increase in thickness on comparison with that of the vehicle-treated control group was regarded as edema.

Multiple TPA treatment-induced ear edema (chronic inflammation)

For provoking chronic inflammation, TPA was applied to both the ears of ICR mice for 7 days (7-day model). TPA (1 μg/20 μL acetone) was applied daily to the inner and outer surface of mouse ear as described previously (Park *et al.*, 2001). SAG and *S. deltoides* extract dissolved in 0.5% CMC were orally administered 1 h prior to TPA

treatment. Control group received only TPA and vehicle. On the day of sacrifice (7th day), SAG and *S. deltoides* extract were administered, and 1 h later, TPA was applied. After three hours of the final TPA treatment, the thickness of both the ears of mice was measured using an engineering gauge. Immediately after, mice were sacrificed for measuring the concentration of prostanoid. Ear samples from three randomly selected mice per group were removed and biopsies (5×5 mm) were subjected to homogenization as described below. In this experiment, the vehicle-treated control group received only acetone and vehicle. Ear thickness of the vehicle-treated control group was measured, but no significant change was observed for 7 days.

Measurement of PGE_2 concentration

As an index of cyclooxygenase (COX-1/COX-2) activity, PGE_2 concentration from the skin biopsies was measured essentially by following the previously described procedure (Park *et al.*, 2001). In brief, a biopsy sample was homogenized in 100 mM phosphate buffer (pH 7.4) containing 1 mM EDTA and 10 μM indomethacin. After centrifugation at 1,500 g for 10 min, 50 mM citrate buffer (pH 3.5) was added to the supernatant. The mixture was centrifuged again at 2,500 g for 10 min. The resulting supernatant was applied to a 6 mL Sep-Pak C_{18} cartridge (Waters Associate, U.S.A.) and eluted with 5 mL of ethyl acetate containing 1% methanol. The eluent was dried under N_2 stream and PGE_2 concentration was measured with an ELISA kit (Cayman Chem., Ann Arbor, MI) according to the manufacturer's instruction.

Analgesia

Acetic acid-induced writhing test was used to measure analgesic activity according to the previously described procedure of Bentley *et al.* (1983). SAG or aspirin dissolved in 0.5% CMC was administered orally to ICR mice at the indicated doses, 1 h prior to acetic acid injection. Acetic acid (0.7%, 100 μL) was administered intraperitoneally and numbers of writhing were counted for 10 min immediately after 10 min of the administration of acetic acid solution.

Statistical analysis

Experimental results were represented as arithmetic mean±S.D. One-way ANOVA test was used for evaluation of statistical significance.

RESULTS AND DISCUSSION

The anti-inflammatory activity of SAG was studied by employing several experimental animal models. The effect of SAG on COX was also investigated to explore the plausible mechanism of action. For this purpose, mouse mast cell culture was used. SAG, at concentrations of 100-500 μg/mL clearly inhibited PGD_2 production by COX-1 (40.5-79.9% inhibition) upon incubation with the mast cells. The IC_{50} value was found to be 300.0 μg/mL (Fig. 1A). To examine the effect of SAG on COX-2, mast cells were initially treated with aspirin to inactivate preexisting COX-1, and then the activators were added to induce COX-2 activity. After 8 h of COX-2 activation, PGD_2 concentration was measured in the medium. Upon simultaneous treatment with SAG, the formulation was also found to inhibit PGD_2 production by COX-2 at concentrations of 300-500 μg/mL with an IC_{50} value of

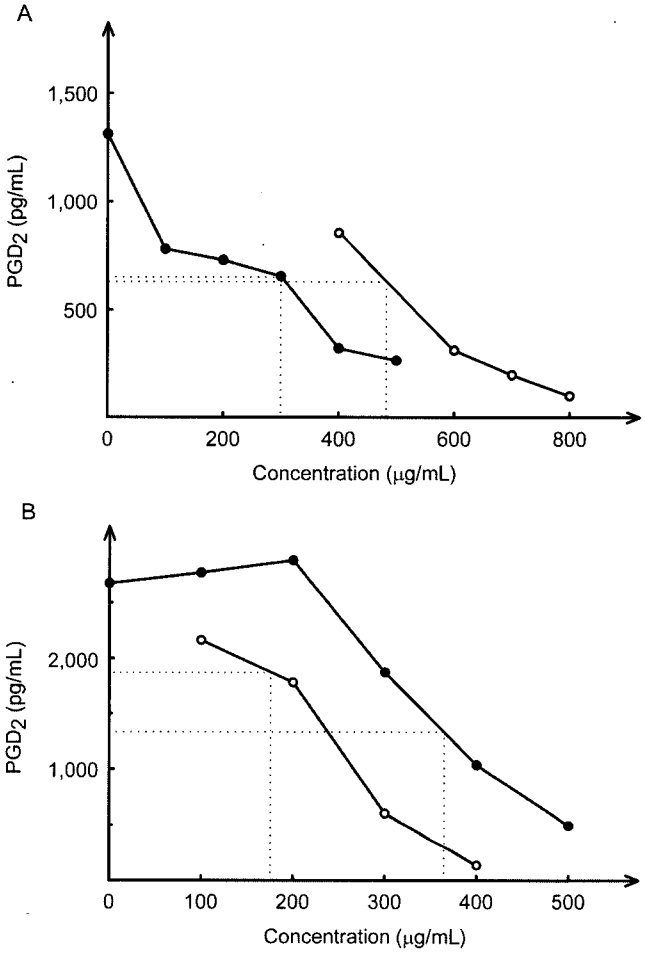

Fig. 1. Inhibition of PGD_2 production in BMMC by SAG. (A) COX-1-derived PGD_2 production. BMMC were treated with activators for 2 h, and PGD_2 concentration was measured in the medium. Control group without SAG or *S. deltoides* extract produced 1,312 pg/mL of PGD_2. (B) COX-2-derived PGD_2 production. BMMC was initially treated with aspirin to inactivate preexisting COX-1, and was further incubated for 8 h with activators to induce COX-2 expression. Test compounds were simultaneously treated with activators for 8 h. Control group produced 2,676 pg/mL of PGD_2. The symbols (○) and (●) indicate *S. deltoides* and SAG, respectively. Values are represented as arithmetic means derived from triplicate determinations.

363.9 μg/mL (Fig. 1B). The parallel experiment with *S. deltoides* extract gave the IC_{50} values of 482.0 and 176.5 μg/mL against COX-1 and COX-2 reactions, respectively. Under the same condition, the IC_{50} value of NS-398 (a selective COX-2 inhibitor) for COX-2-induced PGD_2 production was less than 10 nM (data not shown). Therefore, it was clearly indicated that SAG as well as *S. deltoides* extract reduced prostanoid production by COX-1 and COX-2. However, at present, it is not known whether SAG or *S. deltoides* extract inhibits PGD_2 production by COX-2 inhibition and/or COX-2 down-regulation. A further investigation is required to unveil the detailed inhibitory mechanism of PGD_2 production .

Against an acute inflammation model of mouse ear edema provoked by AA, orally administered SAG (12.5-800 mg/kg) exhibited a dose-dependent inhibitory activity (7.5-48.4%, Fig. 2A). Prednisolone (a reference SAID) was found to potently inhibit ear edema (73.1% inhibition at 10 mg/kg). SAG was also tested on another acute inflammation model of TPA-induced ear edema (3-h model). Upon oral administration, SAG (50-800 mg/kg) showed 4.9-19.7% inhibition in a dose-dependent manner (Fig. 2B), but the results were not statistically significant. Under the same condition, prednisolone potently inhibited ear edema (73.8% inhibition) at a concentration of 10 mg/kg. On the other hand, SAG considerably inhibited the chronic inflammation due to multiple treatment of TPA-induced ear edema (7-day model) at the oral doses of 12.5-800 mg/kg/day in a dose dependent manner, as shown in Fig. 3A. Especially, SAG showed significant

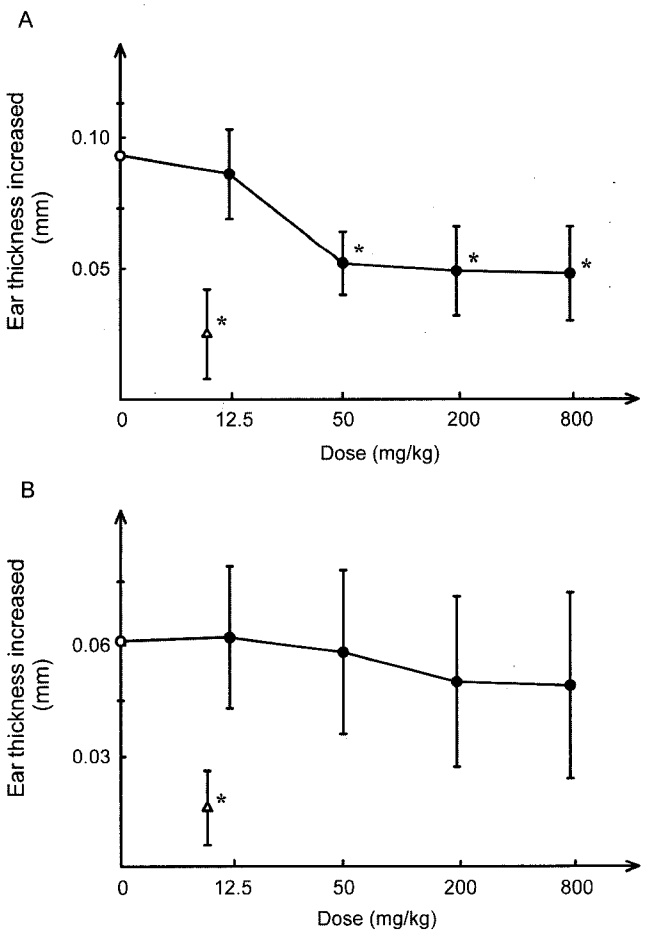

Fig. 2. Inhibition of acute inflammation by SAG. (A) AA-induced ear edema. The increase in ear thickness of the AA-treated group was 0.093 ± 0.020 mm (n = 10). (B) TPA-induced ear edema (3 h-model). The increase in ear thickness of the TPA-treated group was 0.061 ± 0.016 mm (n = 10). The symbolic representations are given as, Prednisolone (△, 10 mg/kg), SAG (●). *: P < 0.01 was considered to be significantly different from the inflammagen-treated group.

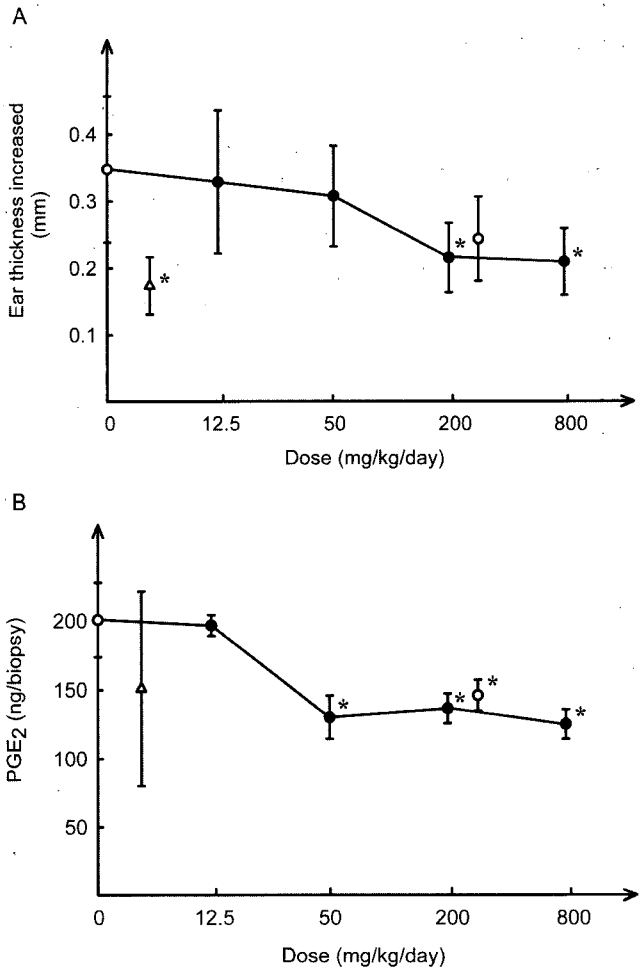

Fig. 3. Inhibition of multiple TPA-treated edema by SAG (7-day model). (A) Inhibition of ear edema. The increase in ear thickness of the multiple TPA-treated group was 0.348 ± 0.109 mm (n = 10). *: P < 0.01 was considered to be significantly different from the TPA-treated group. (B) Inhibition of PGE_2 production. PGE_2 concentration of the TPA-treated group drastically increased to 200.9 ± 27.2 ng/biopsy from the basal level of 4.5 ± 0.0 ng/biopsy (n = 3). *: P < 0.02 was considered to be significantly different from the TPA-treated group. The symbolic representations are given as, Prednisolone (△, 5 mg/kg/day), *S. deltoides* (○, 285 mg/kg/day), SAG (●).

inhibitory activity at 200 and 800 mg/kg/day (38.1 and 39.6% inhibition, respectively), while prednisolone (5 mg/kg/day) showed stronger inhibition (50.0%). The parallel experiment with *S. deltoides* extract showed 30.0% inhibition at 285 mg/kg/day. In order to verify the mechanism of action, PGE_2 concentration was measured from the skin lesion, in which PGE_2 is present as a major prostanoid. Multiple treatment with TPA drastically increased PGE_2 concentration in the skin, while SAG treatment significantly reduced PGE_2 production (Fig. 3B). The reduction in PGE_2 concentration by SAG was 3.2-36.3% at the oral doses of 12.5-800 mg/kg/day, whereas prednisolone (5 mg/kg/day) showed 24.8% inhibition. *S. deltoides* extract also reduced PGE_2 concentration by 26.9% at a dosage of 285 mg/kg/day. The inhibitory activities of PGE_2 production by SAG and *S. deltoides* extract were well correlated with the findings of *in vitro* study by using BMMC, as described above. These results strongly suggest that SAG may behave as an inhibitor of prostanoid production, although it is still not clear how much portion of the edematic response is contributed by PGE_2 produced by COX-1/COX-2 in this animal model. Taken together, it is demonstrated that SAG may inhibit acute as well as chronic inflammation in animal models, at least in part by inhibiting prostanoid production. It is also demonstrated that the inhibition of prostanoid production by SAG is possibly due to the presence of *S. deltoides* extract in the formulation.

In addition, when the analgesic activity was measured by employing acetic acid-induced writhing assay, SAG showed 11.2-31.3% inhibition at the doses of 12.5-800 mg/kg, as shown in Fig. 4. Aspirin exhibited stronger inhibition at doses of 25 and 100 mg/kg (35.7 and 49.0% inhibition, respectively). For an examination of the acute toxicity, SAG was two fold (up to 5 g/kg) orally administered to mice. During 14 days after administration, any significant toxic effect including the death of the animal and change in body and main organ weight was not observed (data not shown).

SAG considerably and significantly inhibited AA-induced edema and multiple TPA treatment-induced edema (7-day model). These results may be partly explained on the basis of the nature of inflammation in animal models. As demonstrated above, SAG inhibited prostanoid synthesis COX-1/COX-2 inhibition. It was previously reported that eicosanoids, such as PGF_{2a} and leukotriene B_4 synthesized by COX-1 and 5-lipoxygenase were pivotal to provoke an edematic response in AA-induced ear edema (Rao *et al.*, 1993). It has also been reported that the newly induced COX-2 may also be partly responsible for the edema produced by multiple treatment with TPA (7-day model), which was similar to 3-day multiple treatment in TPA model (Chi *et al.*, 2003). However, it is not yet clear

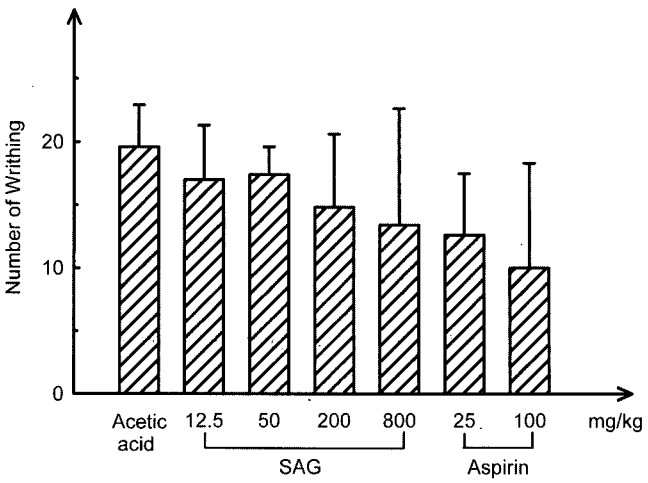

Fig. 4. Analgesic activity of SAG in mice. The standard acetic acid-induced writhing test was employed to measure the analgesic activity of SAG in mice. Acetic acid was intraperitoneally injected and test compounds were orally administered. The number of writhing of the acetic acid-treated control group was 19.6 ± 3.3 (n = 5).

why SAG was less inhibitory on TPA-induced edema (3-h model). Although COX-2 was also induced in this model (Jang and Pezzuto, 1998) and COX/LOX inhibitors reduce the edema, it may be speculated that the portion of the edematous response contributed by COX-1/COX-2 is less when compared with those of other animal models, such as AA-induced edema.

Among the components of SAG formulation, *S. deltoides* extract was previously demonstrated to possess anti-inflammatory and analgesic activity (Park *et al.*, 2004). *Angelica gigas* has been traditionally used as an anti-inflammatory agent and was also reported to possess similar activity (Choi *et al.*, 2003). Moreover, glucosamine is a component of the mammalian joint space and is known to have a favorable protective effect on joint erosion. The combined action of these three components may give beneficial effect on the inflammatory disorders, especially on COX-1/COX-2 involved diseases. In the present study, the combination of these three natural products produces SAG, which possesses inhibitory activity against acute as well as chronic inflammation.

In conclusion, the present investigation clearly demonstrated that SAG exhibits significant and dose-dependent inhibitory activity against acute as well as chronic inflammation in animal models. SAG also showed analgesic activity. It is proposed that these activities might be mediated through reduced production of prostanoid by inhibition of COX-1/COX-2. Although the activity of SAG was not so potent when compared to the conventionally used drugs, SAG appears to have a potential as a safe anti-inflammatory agent at moderate doses.

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