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Antinociceptive and Antiinflammatory Effect of a Diterpene Isolated from the Aerial Part of *Siegesbeckia pubescens*

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Abstract - The aerial part of *Siegesbeckia pubescens* (Compositae) has been used to treat rheumatoid arthritis and hypertension in the Oriental medicine. This crude drug has been used without process (SP-0) or with three times-process of steaming and drying (SP-3) or the nine times of that process (SP-9). To search for the antinociceptive anti-inflammatory components from this crude drug, activity-directed fractionation was performed on this crude drug. Since the $CHCl_3$ extract was shown to have a more potent effect than other extracts, it was subjected to silica gel & ODS column chromatography to yield two diterpene compounds (**1**). Compound **1** was structurally identified as ent-16 (H,17-hydroxykauran-19-oic acid, which were tentatively named siegeskaurolic acid A. A main diterpene, siegeskaurolic acid A was tested for the antiiflammatory antinociceptive effects using both hot plate- and writhing antinociceptive assays and carrageenan-induced anti-inflammatory assays in mice and rats. Pretreatment with siegeskaurolic acid A (20 and 30mg/kg) significantly reduced the stretching episodes, action time of mice and carrageenan-induced edema. These results support that siegeskaurolic acid is a main diterpene responsible for antinociceptive and antiiflammatory action of *S. pubescens*. In addition, the assays on SP-0, SP-3 and SP-9 produced the experimental results that SP-9 had more significant effects than other two crude drugs. These results suggest that the processing on the original plant may lead to the higher pharmacological effect

Key words - *Siegesbeckia pubescens*, Compositae, Antinociceptive, Anti-inflammatory, Ent-kaurane-type diterpene

Introduction

Siegesbeckia pubescens (Compositae) is an annual herb which is naturally growing in the mountain of Korea (Kim, 1996). The aerial part has been traditionally used as an antirheumatoid arthritis agent and a tonic in Oriental medicine (Chi *et al.*, 1998). Jiang *et al.* also reported several ent-kaurane-type diterpenoids from *Siegesbeckia pubescens* (Jiang *et al.*, 1992). However, no antinociceptive anti-inflammatory active principle has been revealed from *S. pubescens*. Siegesbeckiae Herba has been used in the Oriental medicine after the repeated processing of steaming and drying. In the traditional medicinal society of Korea, it is evaluated that the crude drug passed through nine times- repetition of the process has the best quality, though the crude drug processed just three times is also used.

Three repetitions of steaming and drying was named SP-3 in this article, which process is called samjung-sampo in the traditional medicinal society of Korea. Likewise, 9 repetitions of that process was named SP-9, which process is called also gujung-gupo. Unprocessed but just dried plant material was named as SP-0. Of the three crude drugs, SP-9 costs highest in the herbal market.

In this research, the antinociceptive and antiiflammatory activities of the MeOH extract of SP-0, SP-3, and SP-9 were compared in animal experiment. Further, in an attempt to isolate the active principle by activity-guided fractionation, phytochemical and pharmacological studies were performed on the herb of SP-9 to reveal the bioactive component of Siegesbeckiae Herba.

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Materials and Methods

Plant material

Siegesbeckia pubescens was collected in Mt. Odae, Pyongchang province, Korea during July in 2004. This plant was identified by Prof. Sang-Cheol Lim (Ph. D., Department of Botanical Resources, Sangji University), a voucher specimen (#natchem-26) was deposited at the same University. To prepare the crude drugs used in the traditional medicines, the plant material was steamed in a pot for 1 h and thereafter dried enough by sunshine. The crude drug passed three times through this processing was labeled as SP-3. Likewise, the crude drug passed through 9 repetition of the process was labeled as SP-9. The crude drug with no steaming but only with drying under sunshine was labeled as SP-0.

Extraction and Isolation

The plant materials, SP-0 (500g), SP-3 (500g) and SP-9 (5kg),

were extracted for 5h three times with MeOH under reflux respectively. Each extracted solution was filtered and dried using a rotatory evaporator under reduced pressure and freeze-dried to give the MeOH extracts of SP-0 (32g), SP-3 (30g), and SP-9 (360g).

The MeOH extract of SP-9 (300g) was suspended in H_2O and partitioned with n-hexane. The n-hexane-soluble portion was concentrated *in vacuo* and freeze-dried to yield the n-hexane extract (6g). The aqueous layer was successively partitioned with $CHCl_3$ and BuOH, respectively, and these layers were also evaporated *in vacuo* and freeze-dried to give masses of $CHCl_3$ (275g) extract and BuOH extract (20g).

The $CHCl_3$ extract (40g) shown as an active fraction by activity-directed fractionation was chromatographed on a silica gel column (600g, 7.0×60.0cm, Merck, Art 7734, Germany) using n-hexane-EtOAc (2:1) as eluent and gave six fractions (Fr. 1-Fr. 6). The concentrated Fr.3 (8.0g) was purified by Sephadex LH-20 column chromatography using MeOH solvent to give compound **1** (3.2g). Compound **1** was also shown in the TLC chromatogram on the MeOH extracts of SP-0, SP-3 and SP-9.

Compound **1**: Colorless needles, mp 208-210°, IR ν_{max} (KBr) cm^{-1}: 3440, 1642, 1442, 1364, 1234, 1023; ^{1}H-NMR ($CDCl_3$, 500 MHz) δ: 0.97 (3H, s, H-20), 0.89 (3H, s, H_b-15), 0.99 (1H, m, H_a-14), 1.00 (1H, m, H-9), 1.03 (1H, m, H-5), 1.17 (3H, s, H-19), 1.84 (1H, m, H_b-14), 1.88 (1H, m, H-16), 2.01 (1H, m, H-13), 3.29-3.31 (2H, m, H-17); ^{13}C-NMR ($CDCl_3$, 125.5 MHz) δ: 42.2 (C-1), 20.4 (C-2), 39.3 (C-3), 45.9 (C-4), 58.3 (C-5), 23.7 (C-6), 44.4 (C-7), 45.2 (C-8), 56.9 (C-9), 41.0 (C-10), 19.9 (C-11), 32.6 (C-12), 39.6 (C-13), 38.0 (C-14), 46.4 (C-15), 44.4 (C-16), 67.7 (C-17), 181.9 (C-18), 29.5 (C-19), 16.3 (C-20).

Animals and Sample Preparation

ICR male mice weighing 20-25g and Sprague-Dawley male rats weighing 100-120g were purchased from the Korean Experimental Animal Co. and maintained under constant conditions (temperature: 20±2℃, dampness: 40-60%, light/dark cycle: 12hr) for two weeks or more. Twenty-four hours before the experiment, animals were given only water. Due to diurnal enzyme activity, animals were sacrificed at fixed time (10:00 A.M.-12:00 A.M.).

The test samples (i.e., three MeOH extracts, compounds **1**) before being orally administered were first dissolved in 10% Tween 80 and diluted with 0.9% saline. The same volume of solvent was administered to the control rats and mice. Extracts were orally administered at 100 and 200mg/kg once a day for a week and compounds **1** was also orally administered at 20 and 30mg/kg as the same way of the extracts. Thirty minutes after the administration the animal experiments were performed. Aspirin and morphine were used for the positive controls.

Writhing assay

The acetic acid abdominal constriction test was performed using mice as described by Whittle (1949). Vehicle, aspirin (100mg/kg) and test solutions (100 and 200mg/kg) were orally administered 30 min before the experiment, and 0.1ml/10g of 0.7% acetic acid-saline was then injected *i.p.* 10 min later, the frequency of writhing in mice was counted for the succeeding 20 min.

Hot plate test

The assay was performed according to the modified of classical hot-plate technique. They were put on a hot plate (Ugo Basile, Italy) heated at 55℃ and the time necessary for the response to the painful stimulus (elevation of the paws, licking, etc) was recorded. The cut-off time was 30s.

Carrageenan-induced paw edema

Initial hind paw volume was determined volumetrically. A 1% solution of carrageenan in saline (0.1ml/rat) was injected subcutaneously into the right hind paw 1 h after the test substances had been administered orally. Test samples, which included extracts and the isolated component, were first dissolved in 10% tween 80 and then diluted by saline. The same volume of tween 80 in saline was administered to the normal control group. Test solutions (each fraction: 100 and 200mg/kg, compound 1: 20 and 30mg/kg) were orally administered for 7 days prior to injecting carrageenan. The control group received vehicle only. Paw volumes were measured up to 5h at intervals of 60 min, and the volume of the edema was measured with a plethysmometer. (Choi *et al.*, 2003) Ibuprofen, an anti-inflammatory drug, was used as a positive control.

Results and Discussion

To compare the antinociceptive and anti-inflammatory effect of the three Siebesbeckiae Herba, three extracts of SP-0, SP-3, and SP-9 were subjected to antinociceptive activity tests using hot plate- and writhing assays in mice and to anti-inflammatory test using carrageenan-induced edema model in rats. As shown in Table 1, oral administration of SP-0 and SP-3 to the mice reduced the stretching episodes exerted by acetic acid at 100 and 200mg/kg dose but the activity was weak. Treatment of mice with SP-9 reduced those stretching epi-

sodes by 20.8% and 32.3%, respectively. Action time was prolonged by treatment with the three MeOH extracts, though SP-9 had the most potent effect. These results suggest that the processing of steaming and drying on *S. pubescens* increases the antinociceptive effect. Nine repetition of the process significantly increased the antinociceptive effect compared 3 repetition or no steaming.

As shown in Table 2, treatment with SP-9 exhibited a most significant antiinflammatory effect in the carrageenan-induced edema model at 100 and 200mg/kg (*p.o.*) of the three MeOH extracts, though every MeOH extract decreased the edema. These results also support that the traditional processing is highly effective for the activity increase of *S. pubescens*. Further, it was also suggested that 9 repetitions of steaming and drying is reasonable for clinical application in the Oriental medicine.

Therefore, the MeOH extract of SP-9 was fractionated into n-hexane-, $CHCl_3$- and BuOH-soluble parts. Since the $CHCl_3$ extract showed more significant antinociceptive and anti-inflammatory effects than other two extracts (data not shown), it was chromatographed on a silica gel column for the isolation. An active subfraction obtained by column chromatography was purified using Sephadex LH-20 chormatography to yield compound **1** (siegeskaurolic acid). On the basis of NMR spectroscopic data, this compound was identified as ent-16αH,17-hydroxykauran-19-oic acid. Our data was in accordance with the literature data. (Jiang *et al.*, 1992)

To demonstrate the antinociceitive and anti-inflammatory effect of the purified compound, siegeskaurolic acid was subjected to the antinociceptive assay using writhing- and hot plate assays in mice and the anti-inflammatory assay in rats. As shown in Table 1, treatment with siegeskurolic acid decreased acetic acid-induced stretching episodes by 28.7% and 43.6% at 20 and 30mg/kg dose, respectively, in the

Table 1. Antinociceptive effect of the MeOH extracts (SP-0, SP-3, and SP-9) of *Siegesbeckia pubescens* herbs by acetic acid-induced writhing syndrome and hot-plate test in mice

Group	Dose (mg/kg, p.o.)	Stretching episodes[1)] (count/20 min)	Inhibition (%)	Action time[2)] (sec)	Increase (%)
Untreated	0	60.6 ± 2.07^{a}	0	8.9 ± 1.33^{e}	0
SP-0	100	57.4 ± 2.41^{b}	5.3	11.6 ± 2.17^{d}	30.3
	200	54.2 ± 2.28^{c}	10.6	$12.8 \pm 1.20^{c,d}$	43.8
SP-3	100	$57.8 \pm 1.93^{a,b}$	4.62	$12.0 \pm 1.16^{c,d}$	34.8
	200	51.0 ± 1.58^{d}	15.8	$13.1 \pm 1.24^{c,d}$	47.2
SP-9	100	48.0 ± 1.59^{e}	20.8	$13.0 \pm 1.09^{c,d}$	46.1
	200	41.0 ± 3.16^{f}	32.3	$13.8 \pm 1.48^{c,d}$	55.1
Aspirin	100	18.0 ± 1.58^{h}	70.3	NT	-
Morphine	10	NT	-	24.5 ± 2.33^{a}	175

[1), 2)] Methods indicate writhing-, and hot plate tests, respectively. NT (not tested). Values represent means±S.D. (n=10).
[a – h] Values sharing the same superscript letter are not significantly different each other ($p<0.05$) by Duncan's multiple range test.

Table 2. Inhibitory effect of the MeOH extracts (SP-0, SP-3, and SP-9) of *Siegesbeckia pubescens* herbs on carrageenan-induced edema of the hind paw in rats

Group	mg/kg (p.o)	1 h	2 h	3 h	4 h	5 h
		ml				
Untreated	0	1.29 ± 0.08^{t}	2.42 ± 0.06^{de}	2.99 ± 0.07^{a}	2.41 ± 0.05^{de}	1.95 ± 0.08^{lm}
SP-0	100	1.28 ± 0.07^{t}	2.21 ± 0.05^{ijk}	2.76 ± 0.08^{b}	2.38 ± 0.03^{def}	1.90 ± 0.05^{lmno}
	200	1.30 ± 0.09^{t}	2.17 ± 0.03^{k}	2.65 ± 0.04^{c}	2.31 ± 0.07^{fgh}	1.87 ± 0.07^{mnop}
SP-3	100	1.25 ± 0.03^{t}	2.30 ± 0.07^{fghi}	2.70 ± 0.05^{bc}	2.36 ± 0.06^{efg}	1.92 ± 0.061^{mn}
	200	1.27 ± 0.07^{t}	2.24 ± 0.04^{hijk}	2.61 ± 0.03^{c}	2.27 ± 0.05^{ghij}	1.85 ± 0.07^{mnop}
SP-9	100	1.28 ± 0.04^{t}	2.20 ± 0.06^{jk}	2.65 ± 0.07^{c}	2.31 ± 0.08^{fgh}	1.83 ± 0.04^{nopqr}
	200	1.26 ± 0.02^{t}	1.98 ± 0.04^{l}	2.47 ± 0.05^{d}	2.25 ± 0.04^{hijk}	1.80 ± 0.07^{pqr}
Ibuprofen	100	0.82 ± 0.03^{v}	1.22 ± 0.05^{t}	1.49 ± 0.04^{s}	1.23 ± 0.08^{t}	0.97 ± 0.05^{u}

Values represent means ± S.D. (n=10). Values sharing the same superscript letter are not significantly different each other ($p<0.05$) by Duncan's multiple range test.

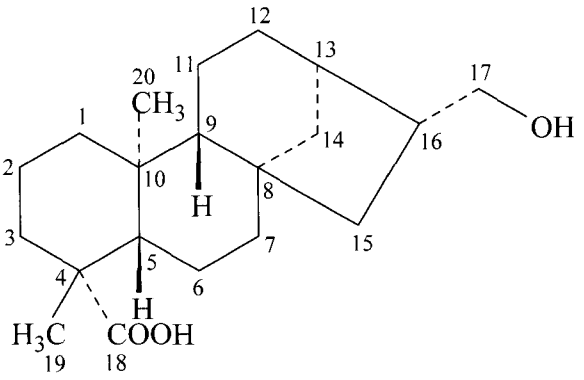

Fig. 1. Structure of compound 1 isolated from *S. pubescens*

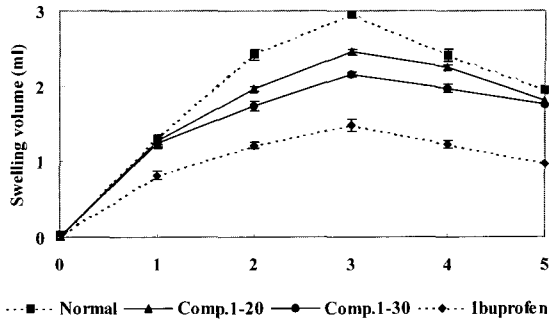

Fig. 3. Effects of siegeskaurolic acid (comp. **1**) isolated from the roots of *S. pubescens* on carrageenan-induced paw edema in rats. Values represent means±S.D. (n=10).

writhing assay. Treatment with that compound (20 and 30mg/kg dose, *p.o.*) prolonged the action time by 59.6% and 88.8%, respectively.

Aspirin was used for the positive control to compare the peripherally mediated analgesic antinociceptive activity in the writhing assay, and morphine was also used to compare a centrally mediated one. Both aspirin and morphine showed potent activities in writhing assay and hot plate assay, respectively, as shown in Fig. 2. The antinociceptive activity shown by a diterpene tested indicates that this compound might possess centrally and peripherally mediated antinociceptive properties.

It is well known that the carrageenan-induced edema is appeared as two phases (phase 1 and –2 edema). The phase 1 edema, which is caused by histamine and serotonin release, is induced immediately after the carrageenan-injection and reaches maximum edema at 30 min and then disappeared (Crunkhon *et al.*, 1971). However, phase 2 edema, which is caused by kinins, proteases and prostaglandins begins to appear at about 1 h after the injection. (Lewis *et al.*, 1975; Moncada *et al.*, 1975) Siegeskaurolic did not exhibit antinflammatory activity within 1h, but showed the activity from 1h after carrageenan injection. These findings suggest that the anti-inflammatory property of siegeskaurolic acid is associated with the inhibition of phase 2 edema.

In conclusion, it was revealed that siegeskaurolic acid is a main diterpene of Siegesbeckiae Herba responsible for the antinociceptive and antiiflammatory activity. In addition, it was also suggested that repetition of steaming and drying on the plant source contributes to the increase of pharmacological potency.

Acknowledgements

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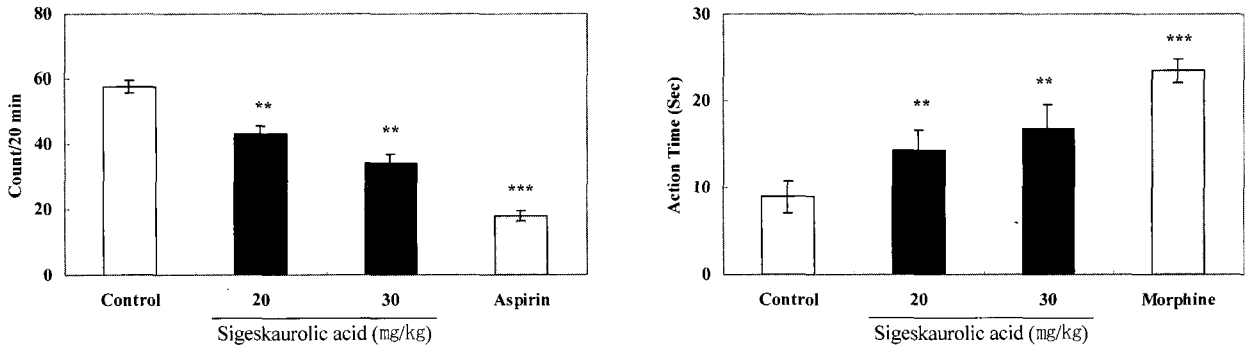

Fig. 2. Antinociceptive effect of siegeskaurolic acid (comp. **1**) isolated from the roots of *S. pubescens* by acetic acid-induced writhing and hot plate method in mice.
Methods indicate writhing- (upper), and hot plate tests (down), respectively. Values represent means(S.D. (n=10). *, **, *** Astericks indicate the means which are significantly different ($p<0.05$, $P<0.01$ and $p<0.001$), respectively, from the control.

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