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In-vitro and in-vivo anti-inflammatory action of the ethanol extract of *Trachelospermi caulis*

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Abstract

In this study, we aimed to investigate the anti-inflammatory activity, antinociceptive activity and the action mechanism of *Trachelospermi caulis* extract. The anti-inflammatory effects were investigated using arachidonic acid, 12-*O*-tetradecanoylphorbol 13-acetate or carrageenan-induced oedema assays. Antinociceptive activity, using the acetic acid-induced writhing model, was also tested in mice. The extract exhibited dose-dependent and significant ($P < 0.05$ at 100–400 mg kg^{-1}) anti-inflammatory and antinociceptive activity in the animals. To further understand the mechanism of activity, we investigated whether the extract inhibited the expression of inducible nitric oxide synthase (iNOS), the production of nitric oxide (NO) and the expression of TNF-α from murine macrophage RAW 264.7 cells. Similar to the in-vivo activity, the iNOS expression, NO production and TNF-α expression were found to be dose dependent and significantly suppressed by the extract through the inhibition of the p38 MAP kinase/NF-κB pathway. Taken together, the results presented here suggest that *T. caulis* extract may be useful for the treatment of various inflammatory diseases.

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Introduction

Trachelospermi caulis, known as Nak-Suk-Deung in Korea, is the dried leafy stem of *Trachelospermi asiaticum* var. *intermedium* Naki (Apocyanaceae). This herb extract contains arctiin and several glucosides, including tracheloside, nortracheloside and matairesinoside (Inagaki et al 1973; Nishibe et al 1973; Sakushima et al 1973). The extract is known to have antipyretic and analgesic activity and has traditionally been used as a folk remedy in Korea for the treatment of various inflammatory diseases, including rheumatoid arthritis and abscesses. Although some constituents of *T. caulis* extract have been identified, to our knowledge there have been no studies on the anti-inflammatory effects of *T. caulis* extract aimed at proving its pharmacological activity.

In this study, we investigated the anti-inflammatory effect of *T. caulis* extract using arachidonic acid, 12-*O*-tetradecanoylphorbol 13-acetate (TPA) or carrageenan-induced oedema in animals. Antinociceptive activity, using an acetic acid-induced writhing model, was tested in mice. At the same time, the effects of the extract on the expression of iNOS, NO production and TNF-α expression were investigated in lipopolysaccharide (LPS)-stimulated RAW 264.7 macrophages. As a result, *T. caulis* extract was identified as a potential medicinal herb for treating inflammatory diseases, including rheumatoid arthritis.

Materials and Methods

Reagents

To measure the in-vivo anti-inflammatory effects, arachidonic acid, 12-*O*-tetradecanoylphorbol 13-acetate (TPA), carrageenan and acetic acid were purchased from Sigma-Aldrich (St Louis, MO). Cell culture reagents were obtained from GIBCO/Invitrogen (Carlsbad, CA). The Tris-glycine polyacrylamide gels were purchased from Novex (San Diego, CA).

Anti-iNOS, anti-phospho-p38, and anti-actin antibodies were from Santa Cruz (Santa Cruz, CA). Phospho-NF-κB p65 (Ser536) was purchased from Cell Signaling Technology Inc. (Beverly, MD). LPS and Griess reagents were from Sigma-Aldrich (St Louis, MO). As positive control, Joins, a combination herbal drug containing extracts of *Trichosanthes kirilowii, Prunella vulgaris* and *Clematis mansshurica*, commonly used for the treatment of rheumatoid arthritis in Korea, was obtained from Sun Kyung Pharmaceutical Co. Ltd (Seoul, Korea).

Preparation of the ethanol extract of *T. caulis*

The extracts were obtained from Hunan Guohua Pharmaceutical Co., Ltd in China, where the *T. caulis* extract was prepared by first chopping the dried medicinal plants with a domestic mixer and then adding 10 parts of 30% ethanol. After allowing the mixture to stand at 60–70°C for 2 h, the ethanol extracts were filtered and concentrated in 60–70°C bath water under reduced pressure. The extract's yield was approximately 2.5%. The voucher specimen (Sinil-1007) was deposited at the Life Science R&D Center, Sinil Pharmaceutical Co. Ltd, Korea. The extracts were dissolved to 300 mg mL^{-1} in 100% dimethyl sulfoxide (DMSO), diluted to 30–300 μg mL^{-1} with the media (0.1% DMSO) for in-vitro study with RAW 264.7 macrophages, and suspended in 5% Arabic gum for the in-vivo studies. As for the control studies, 0.1% DMSO and 5% Arabic gum were utilized for the in-vitro and in-vivo studies, respectively.

Animals

Male ICR mice (aged 6 weeks) and male Wistar rats (aged 6 weeks) were purchased from the Dae Han Experimental Animal Center (Eumseong, Korea). They were housed in the animal facilities of the Life Science R&D Center, Sinil Pharmaceutical Co. Ltd. The 10 mice and 6 rats were placed in a cage in a laminar airflow cabinet at a temperature of 22 ± 1°C, and relative humidity of 55 ± 10% was maintained throughout the study. The animal study was conducted in accordance with guidelines issued by the US National Institutes of Health (NIH publication # 85-23, 1985) and the protocol was pre-approved by the Institutional Ethics Committee of Sinil Pharmaceutical Co. Ltd.

Arachidonic acid-induced ear oedema assay

Plant extracts were orally administered 1 h before the topical application of 2% arachidonic acid dissolved in acetone (20 μL/ear) to the right ears of the mice. For evaluation of the activity, two different processes were followed: the thickness of each ear was measured 4 h after induction of inflammation using gauge calipers (Ozaki Co., Tokyo, Japan); and the animals were extinguished by deep ether anesthesia 1 h after the topical treatment. Six-millimeter diameter disks were removed from the ears of the mice and weighed in a balance.

12-*O*-tetradecanoylphorbol 13-acetate (TPA)-induced ear oedema assay

Plant extracts were orally administered 1 h before each mouse received 2.5 μg of TPA (12-*O*-tetradecanoylphorbol 13-acetate) dissolved in 20 μL of acetone (Young & De Young et al 1989). This was applied by automatic pipette in 20-μL volumes to both the anterior and posterior surfaces of the right ear. The left ear (control) received the same volume of acetone, simultaneously with TPA. Joins (400 mg kg^{-1}) was used as the reference drug. Two different processes were used for evaluation of the activity as described in the previous section for the arachidonic acid-induced ear oedema assay.

Carrageenan-induced paw oedema assay

The carrageenan-induced oedema assay was conducted on male rats following the procedure of Winter et al (1962). The *T. caulis* extract (20–400 mg kg^{-1}), Joins (400 mg kg^{-1}) or the vehicle were given orally 60 min before the injection of carrageenan (1% w/v in saline solution) into the subplantar region of the right hind paw. The contralateral paw was injected with an equal volume of saline. The paw volumes were determined hourly by plethysmometer (Ugo Basile, Italy) for 4 h.

Acetic-acid-induced writhing test

The acetic-acid-induced writhing method was used with adaptations as described by Koster et al (1959). One hour after orally administering *T. caulis* extract (20–400 mg kg^{-1}), Joins (400 mg kg^{-1}) or the vehicle to a group of eight mice, each mouse was given 0.7% aqueous solution of acetic acid (10 mL kg^{-1} body weight) intraperitoneally. Each mouse was placed in a transparent observation cage 10 min after the acetic acid injection and the number of writhes per mouse was counted for 10 min. Writhing was defined as a contraction of the abdominal muscles together with a stretching of the hind limbs (Hernández-Pérez & Rabanal 2002).

Cell culture and stimulation

Murine macrophage RAW 264.7 cells were cultured at 37°C in Dulbecco's Modified Eagle Medium (DMEM), containing 10% fetal bovine serum (FBS), penicillin (100 U mL^{-1}), and streptomycin sulfate (100 μg mL^{-1}), in a humidified atmosphere of 5% CO_2. The cells were treated with the extract 30 min before their treatment with 1-μg mL^{-1} LPS for 4 h, or as indicated in the legends, washed with 1× PBS, and then used for immunoblot analysis.

Western blot analysis

The LPS-stimulated cells were treated with *T. caulis* extract and then washed with 1× PBS and lysed on ice for 30 min using a lysis buffer (20 mM HEPES, pH 7.5, 150 mM NaCl, 1% Nonidet p-40, 10% glycerol, 60 mM octyl β-glucoside, 10 mM NaF, 1-mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride, 2.5 mM nitrophenylphosphate, 0.7 μg mL^{-1} pepstatin, and a protease inhibitor tablet). Lysates were centrifuged at 12 000 rev min^{-1} at 4°C for 15 min and the supernatants were dissolved in a 2×Laemmli buffer. Proteins were separated by SDS-PAGE and transferred onto a nitrocellulose membrane (Schleicher & Schuell, Dachen, Germany). Rabbit anti-iNOS, anti-phospho-p38, anti-phospho-NF-κB or other antibodies

were used as primary antibodies. Horseradish peroxidase-conjugated antibodies were used as secondary antibodies. Band detection was conducted using an enhanced chemiluminescence (ECL) detection system (Amersham Biosciences, Sweden).

Nitric oxide assay

The nitrite concentration in the culture medium was measured as an indicator of NO production based on the Griess reaction. One-hundred microlitres of each supernatant was mixed with the same volume of Griess reagent (1% sulfanilamide in 5% phosphoric acid and 0.1% naphthylethylenediamine dihydrochloride in water), and the resulting mixture was then incubated at room temperature for 10 min. Its absorbance at 540 nm was then measured in a microplate reader. A freshly cultured medium was used as a blank in all experiments.

Quantitation of arctiin in the extract of *T. caulis*

To identify arctiin as the active component of *T. caulis*, the ethanol extract was dissolved in 50% methanol. Before injection onto HPLC, all samples were filtrated through a 0.2-μm microspin PVDF filter. Arctiin content was determined by an HPLC system (Jasco Inc., Easton, MD) equipped with an AS2051 auto-sampler and a UV detector 2075. The constituents of the extract were separated by a C_{18} Cosmosil column (10 μm, 4.6 × 250 mm) using a mobile phase containing 50% methanol at a flow rate of 0.8 mL min^{-1}. Arctiin was detected by measuring absorbance at 280 nm and quantified using purified arctiin as a standard.

Statistical analysis

Results are expressed as mean ± s.e.m. calculated from the data. Statistical analysis was performed by one-way analysis of variance followed by Bartlett's test for comparing control and treatment groups. If Bartlett's test confirmed the homogeneity of variance, Dunnett's multiple comparison tests were then applied to compare each experiment group with the control group. *$P < 0.05$ or **$P < 0.01$ was considered statistically significant.

Results

12-*O*-tetradecanoylphorbol 13-acetate (TPA)-induced mouse ear oedema analysis

The thickness and weight of oedema were measured after the topical administration of TPA. The effects of *T. caulis* extract and Joins on TPA-induced mouse ear oedema are summarized in Table 1. The administration of Joins (400 mg kg^{-1}) significantly decreased the TPA-induced mouse oedema as compared with the control group ($P < 0.01$). The effect of the extract of *T. caulis* was dose dependent and showed significant effects in the test. The extract of *T. caulis* showed maximal inhibition at 400 mg kg^{-1} with an inhibition ratio of 54.3% for oedema weight and 65.7% for oedema thickness, which was a similar potency to Joins (45.7% for weight and 53.7% for thickness at 400 mg kg^{-1}).

Arachidonic-acid-induced mouse ear oedema analysis

The thickness and weight of oedema after the topical administration of arachidonic acid were measured as described in the methods section. The effects of *T. caulis* extract and Joins on arachidonic-acid-induced mouse ear oedema are summarized in Table 1. The administration of Joins (400 mg kg^{-1}) significantly decreased the arachidonic-acid-induced mouse oedema compared with the control group ($P < 0.01$). The *T. caulis* extract also showed dose-dependent and significant effects in the test; it caused maximal inhibition at 400 mg kg^{-1} with an inhibition ratio of 56.1% for oedema weight and 61.8% for oedema thickness, which was a similarly potency to Joins (38.2% for weight and 52.7% for thickness at 400 mg kg^{-1}).

Carrageenan-induced oedema analysis

The extract exhibited a significant suppression of carrageenan-induced inflammation in a dose-dependent manner in rats compared with the control groups (Table 2). Joins significantly inhibited inflammation induced by carrageenan compared with the control group ($P < 0.05$ at 3 h and 4 h).

Table 1 Effects of *Trachelospermi caulis* extract (TC) on 12-*O*-tetradecanoylphorbol 13-acetate (TPA)- or arachidonic acid (ARA)-induced ear oedema in mice

Group	Dose (mg kg^{-1})	TPA-induced oedema		ARA-induced oedema	
		Weight (mg)	Thickness (mm)	Weight (mg)	Thickness (mm)
TC	0	15.9 ± 0.27	0.67 ± 0.01	15.2 ± 0.27	0.55 ± 0.004
	20	14.5 ± 0.61	0.58 ± 0.02**	14.3 ± 0.35	0.55 ± 0.005
	100	8.4 ± 0.51**	0.27 ± 0.01**	8.6 ± 0.44**	0.26 ± 0.006**
	400	7.3 ± 0.31**	0.23 ± 0.01**	6.6 ± 0.30**	0.21 ± 0.010**
Joins	400	8.6 ± 0.57**	0.31 ± 0.02**	9.3 ± 0.47**	0.26 ± 0.007**

TC or Joins were administered orally 1 h before the TPA or arachidonic acid treatment. Values are expressed as mean ± s.e.m., n = 8. *$P < 0.05$, **$P < 0.01$, compared with the vehicle-treatment group (0).

Table 2 Effects of *Trachelospermi caulis* (TC) on carrageenan-induced paw oedema in rats

Group	Dose (mg kg^{-1})	Percent increase of paw oedema			
		1 h	2 h	3 h	4 h
Control	0	22.6 ± 2.18	50.0 ± 2.30	88.6 ± 1.66	83.3 ± 2.72
TC	100	16.6 ± 0.40*	48.8 ± 0.48	58.8 ± 1.01**	56.6 ± 1.80**
	200	11.7 ± 1.65**	41.2 ± 2.78	49.0 ± 2.91**	41.2 ± 3.11**
	400	13.5 ± 2.25*	39.5 ± 4.66	45.2 ± 4.30**	42.7 ± 5.45**
Joins	400	24.5 ± 1.32	40.2 ± 3.42	43.2 ± 3.90**	47.5 ± 3.37**

TC or Joins were administered orally 1 h before the carrageenan treatment. Values were expressed as mean ± s.e.m., n = 8. *$P < 0.05$, **$P < 0.01$ compared with the control group.

Compared with Joins, *T. caulis* extract showed a similar anti-inflammatory effect in the majority of points studied.

Acetic-acid-induced writhing test

Antinociceptive activity was evaluated by the acetic-acid-induced writhing test. The effect of *T. caulis* extract in mice is shown in Figure 1. It was found that *T. caulis* extract displayed a significant and dose-dependent inhibition of the writhing responses induced by acetic acid when compared with the control group, with a maximal inhibitory value of 45.8% at a dosage of 400 mg kg^{-1} ($P < 0.01$). It exhibited similar activity to Joins (49.4% inhibition at 400 mg kg^{-1}) in this model.

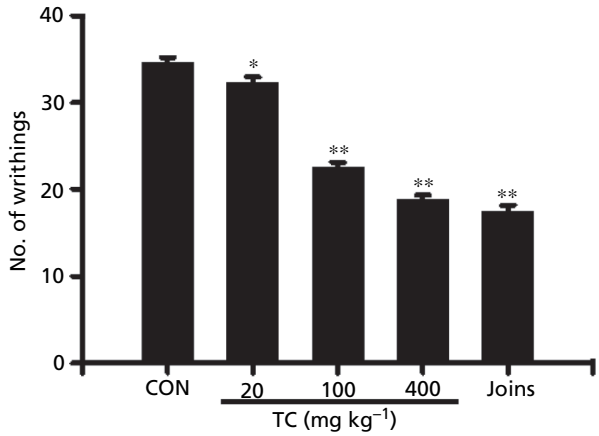

Figure 1 Antinociceptive activity of *Trachelospermi caulis* on acetic-acid-induced writhing mouse model. One hour after receiving oral injection of *T. caulis* (TC, 20–400 mg kg^{-1}), Joins (400 mg kg^{-1}) or vehicle, each mouse was given intraperitoneally 0.7% aqueous solution of acetic acid (10 mL kg^{-1} body weight). Each mouse was placed in a transparent observation cage 10 min after the acetic acid injection and the number of writhes per mouse was counted for 10 min. The writhing activity consisted of a contraction of the abdominal muscles together with a stretching of the hind limbs. Data are represented as mean ± s.e.m. of 8 mice per group. *$P < 0.05$, **$P < 0.01$ compared with vehicle-treated controls (CON).

Effect of *T. caulis* extract on the expression of iNOS, NO production and TNF-α expression

To identify the anti-inflammatory mechanism, the effect of *T. caulis* extract on the LPS-induced expression of iNOS protein, NO production and TNF-α expression in RAW 264.7 macrophages was measured. Western blot experiments showed the induction of iNOS and TNF-α in the cells 4 h after LPS (1 μg mL^{-1}) treatment. The expression of iNOS and TNF-α was significantly suppressed by *T. caulis* extract in a dose-dependent manner (Figure 2A and 2C). RAW 264.7 macrophages were stimulated with LPS (1 μg mL^{-1}) for 24 h to induce NO production. The production of NO was estimated from the accumulation of nitrite, a stable product of NO metabolism, in the medium using the Griess reagent as described in the methods section. LPS-stimulated RAW 264.7 cells produced 14.1 ± 1.1 μM nitrite over 24 h. When the cells were incubated with *T. caulis* extract (30–300 μg mL^{-1}), the production of nitrite was significantly inhibited in a dose-dependent manner (Figure 2B) and the viability of the cells was not affected (data not shown).

Effect of *T. caulis* extract on activating phosphorylation of p38 MAP kinase/NF-κB pathway

Because p38 MAP kinase is necessary for iNOS expression induced by LPS in RAW 264.7 macrophages (Chen & Wang 1999), the effect of *T. caulis* extract on the activation of p38 in LPS-stimulated RAW 264.7 cells was tested. As shown in Figure 3A, the extract inhibited the activating phosphorylation of p38 in a dose-dependent manner. Next, the phosphorylation of NF-κB, a pleiotropic regulator critical for the expression of iNOS in the cells (Xie et al 1994), was checked. The NF-κB p65 was phosphorylated at Ser276 and Ser536 for full transcriptional activity (Ghosh & Karin 2002). *T. caulis* extract also suppressed the activating phosphorylation of NF-κB (Figure 3B).

Quantitation of arctiin in the extract of *T. caulis*

Tracheloside and arctiin have been reported as active components inhibiting TPA-induced inflammation (Nishibe et al 1997). HPLC analysis was conducted to determine the

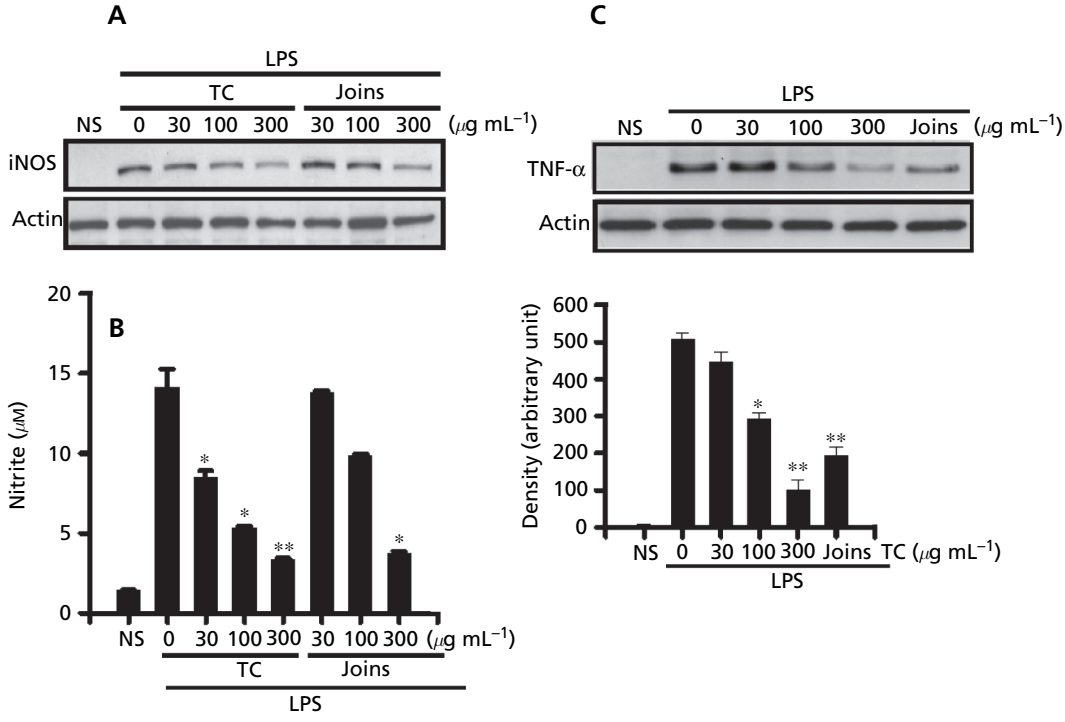

Figure 2 Effect of *Trachelospermi caulis* on LPS-induced iNOS expression, NO production and TNF-α expression in murine macrophage RAW 264.7 cells. A. The cells were treated with *T. caulis* (TC) or Joins in the indicated doses 30 min before incubating them with 1 μg mL^{-1} LPS for 4 h, and the levels of iNOS protein were determined using Western blot analysis. B. The concentration of nitrite in the culture media was monitored through the Griess reaction. One-hundred microlitres of each supernatant was mixed with the same volume of Griess reagent (1% sulfanilamide in 5% phosphoric acid, and 0.1% naphthylethylenediamine dihydrochloride in water) and the resulting mixture was then incubated at room temperature for 10 min. Its absorbance at 540 nm was then measured in a microplate reader. C. The cells were treated with TC or Joins in the indicated doses 30 min before incubating them with 1 μg mL^{-1} LPS for 4 h, and the levels of TNF-α protein were determined using Western blot analysis. The data are presented as the means ± s.e.m. from three independent experiments. *$P < 0.05$, **$P < 0.01$ compared with the control (0).

content of arctiin and tracheloside in the extract (Figure 4). Arctiin was detected at 7.59 min retention time and tracheloside was not detected. The fractions containing arctiin were confirmed by mass spectroscopic analysis compared with the authentic arctiin (data not shown). One gram of the extract contained approximately 47 mg of arctiin. To elucidate the role of arctiin in the inhibition of the NO production from RAW 264.7 cells, arctiin was used to treat the cells. As shown in Figure 4C, the production of NO was suppressed by arctiin in a dose-dependent manner.

Discussion

In this study, the pharmacological basis for the traditional usage of *Trachelospermi caulis* extract for the treatment of various inflammatory diseases, including rheumatoid arthritis, was investigated. Several animal models for the study of anti-inflammatory activity were used (arachidonic-acid-induced or 12-*O*-tetradecanoylphorbol 13-acetate-induced mouse ear oedema, or carrageenan-induced rat paw oedema), as was the mouse acetic-acid-induced writhing model for measurement of antinociceptive activity. All the studies with animal models clearly demonstrated that *T. caulis* extract has anti-inflammatory and antinociceptive activity. At the same time, whether *T. caulis* extract inhibits iNOS expression, TNF-α expression or NO production was also investigated. It was found that *T. caulis* extract significantly suppressed the expression of iNOS and NO production. It is clear from our findings that *T. caulis* extract has potent in-vitro and in-vivo anti-inflammatory and antinociceptive activity.

T. caulis, the dried leafy stem of *T. asiaticum* var. *intermedium* Nakai or *T. jasminoides* var. *pubescens* Makino, is commonly used as a folk remedy in Korea to remove the collateral obstruction and local fever from rheumatoid arthritis, and muscular contracture, and to bring down swelling from sore throats and carbuncles. However, to our knowledge there have been no reports on the in-vitro and in-vivo anti-inflammatory activity of *T. caulis* aimed at proving its pharmacological actions. This study established the anti-inflammatory activity of *T. caulis* in several experimental animal models, each of which represented a different type of inflammation. First, the effects of *T. caulis* extract in a topical inflammatory model, the TPA-induced ear oedema, were studied in mice. The results showed that the extracts inhibited TPA-induced ear oedema in mice (Table 1) in a dose-dependent manner, indicating the presence of active substances endowed with anti-inflammatory activity. It has been established that the phorbol ester (TPA) exerts its inflammatory action through protein kinase C activation with subsequent

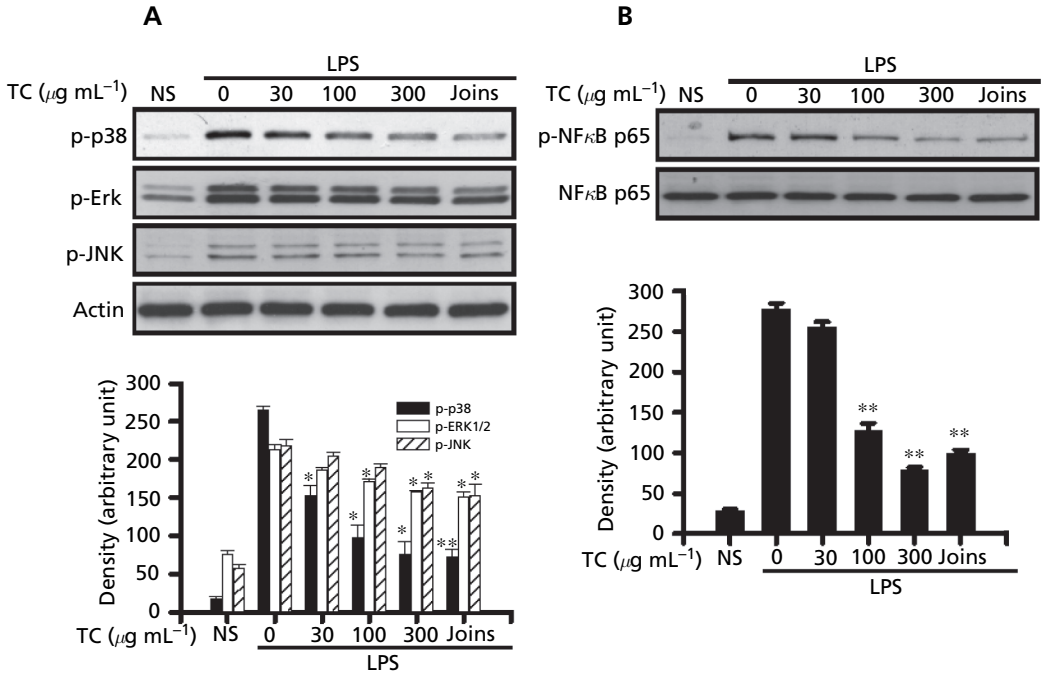

Figure 3 Effect of *Trachelospermi caulis* on the activating phosphorylation of p38 and NF-κB in murine macrophage RAW 264.7 cells. A. The cells were treated with *T. caulis* (TC) or Joins in the indicated doses 30 min before incubating them with 1 μg mL^{-1} LPS for 30 min and the activating phosphorylation of p38, ERK and JNK were measured using specific antibodies by Western blot analysis as described in the methods section. B. The cells were treated with TC or Joins in the indicated doses 30 min before incubating them with 1 μg mL^{-1} LPS for 10 min, and the activating phosphorylation of NF-κB was measured by Western blot analysis as described previously. The data are presented as the means ± s.e.m. from three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$ compared with the control.

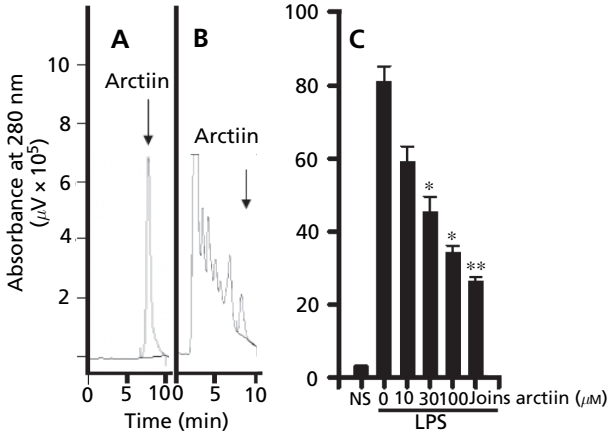

Figure 4 HPLC chromatograms of arctiin present in *Trachelospermi caulis*. This analysis was conducted by an HPLC system (Jasco Inc., Easton, MD) equipped with an AS2051 auto-sampler and a UV detector 2075. The extract was separated by a C_{18} Cosmosil column (10 μm, 4.6 × 250 mm) using a mobile phase containing 50% methanol at a flow rate of 0.8 mL min^{-1}. Arctiin was detected by measuring the absorbance at 280 nm. A. Chromatogram of arctiin. B. Chromatogram of *T. caulis* (TC) extract. The fraction appearing at 7.59 min, as indicated by the arrow, was arctiin. C. The concentration of nitrite in the culture media was monitored through the Griess reaction.

activation of the NF-κB pathway and phospholipase A_2 stimulation to produce several inflammatory mediators, such as nitric oxide (Chen et al 1998) and prostaglandins (Just et al 1998). As shown in Figure 3, the extract inhibited both p38 MAP kinase and NF-κB signals, which are important for iNOS expression and NO production. Therefore, the extract may have an inhibitory effect in TPA-induced inflammation by interfering with the mediator. Arachidonic acid-induced ear oedema is a good in-vivo model useful for evaluating lipoxygenase inhibitors (Young & De Young 1989). The results in Table 1 show that *T. caulis* extract caused significant ($P < 0.01$) inhibition of oedema compared with the control group of mice. The inhibitory potency was similar to that of Joins, which was used as a reference drug. These results led to the measurement of whether or not the extract inhibited the cyclooxygenase pathway. Unexpectedly, it showed minimal effect on the expression of COX-2 and PGE_2 production in LPS-stimulated RAW 264.7 macrophages (data not shown). This result seems to suggest that there may be another mechanism inhibiting arachidonic-acid-induced oedema in the mouse model. Carrageenan-induced rat paw oedema is a suitable experimental model for evaluating the anti-oedematous effect and is believed to be biphasic; the first phase (1 h) involves the release of serotonin and histamines, and the second phase (>1 h) is mediated by prostaglandin, a cylooxygenase product. Continuity between the two phases is provided by the kinins (Vinegar et al 1969), which accompany the leucocytes. *T. caulis* extract inhibited the carrageenan-induced

paw oedema at all time points (1–4 h from the carrageenan challenge) (Table 2). These results suggest that the extract inhibited the previously-mentioned biphasic inflammation induced by carrageenan. However, the mechanism of this action is not at all clear in the model. Next, measurements to determine whether *T. caulis* extract had an antinociceptive activity were taken using the acetic-acid-induced writhing mouse model. The results showed that oral administration of the extract suppressed the abdominal constriction that followed acetic acid, indicating that it has an analgesic effect at the doses assayed (Figure 1). Constriction induced by acetic acid is considered to be a non-selective antinociceptive model since acetic acid acts indirectly by inducing the release of endogenous mediators, which stimulate the nociceptive neurons that are sensitive to non-steroidal anti-inflammatory drugs, narcotics and other centrally active drugs (Collier et al 1968; Bighetti et al 1999). Thus, the results of this writhing test alone did not ascertain whether the antinociceptive effects of *T. caulis* extract are central or peripheral.

To further understand the mechanism of its action, we investigated whether *T. caulis* extract inhibited the expression of iNOS and NO production in LPS-stimulated RAW 264.7 macrophages. NO is an important mediator in the inflammatory process, and is produced at inflamed sites by an inducible enzyme, NOS. High levels of NO have been described in a variety of pathological processes, including various forms of inflammation, circulatory shock and carcinogenesis (Ohshima & Bartsch 1994; Szabo 1995; MacMicking et al 1997). Therefore, an inhibitor of NOS may be effective as a therapeutic agent for inflammatory diseases (Koo et al 2001). The results of this study demonstrated that the extract inhibits LPS-induced NO production in a dose-dependent manner in LPS-stimulated RAW 264.7 macrophages. This suppression was correlated with the down-regulation of the expression of iNOS protein in the cells (Figure 2). These findings may indicate that *T. caulis* extract possesses in-vivo anti-inflammatory effects through the inhibition of the production of NO. The inducible expression of iNOS in macrophages is regulated mainly at the transcriptional level, particularly by NF-κB (Xie et al 1994). Recently, the p38 MAP kinase was reported as the upstream kinase of NF-κB activation in the same RAW 264.7 cells (Kim et al 2006). The results of previous studies, as well as our own, suggest that the anti-inflammatory activity of *T. caulis* extract may be mediated by the inhibition of p38 MAP kinase and NF-κB pathway. However, we could not exclude the possibility that ERK and JNK MAP kinases contributed to NO production because *T. caulis* extract at least partially inhibited both MAP kinases (Figure 3A).

T. caulis contains arctiin, tracheloside, nortracheloside and matairesinol monoglucoside (Inagaki et al 1973; Nishibe et al 1973; Sakushima et al 1973). Less is known, however, about the pharmacological actions of the components at present. Tracheloside and arctiin are transformed into aglycones trachelogenin and arctigenin, respectively, by gastric juices and intestinal bacteria in rats (Nose et al 1992, 1993) and both have been shown to effectively inhibit 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced ear oedema in mice (Nishibe et al 1997). In our phytochemical research, we have identified the presence of arctiin in *T. caulis* extract (Figure 4B). The arctiin inhibited the NO production in RAW 264.7 macrophages (Figure 4C). Although we could not exclude the possibility that other components besides arctiin contributed to the anti-inflammatory activity of the extract, previous and our present results strongly suggest that the effects of *T. caulis* extract observed in this work were mostly, or at least partially, due to the presence of this compound.

Conclusions

These results clearly demonstrate that *Trachelospermi caulis* extract has significant anti-inflammatory effects in-vitro and in-vivo. It is also possible to conclude that the extract displays antinociceptive activity in the acetic-acid-induced writhing animal model. The activity of *T. caulis* extract identified in this study may be related to its inhibitory effects against the production of nitric oxide (NO) through the inhibition of both p38 MAP kinase and NF-κB pathway. Thus, it is concluded that *T. caulis* extract has in-vitro and in-vivo anti-inflammatory effects against the acute and proliferative phases of inflammation, which may provide a scientific basis to explain why *T. caulis* extract has been so commonly used as a Korean folk medicine.

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