Arch Pharm Res Vol 32, No 9, 1237-1243, 2009
DOI 10.1007/s12272-009-1909-3

Archives of Pharmaceutical Research
www.springer.com/12272

Anti-inflammatory Activity of the Constituents of the Roots of *Aralia continentalis*

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(Received April 29, 2009/Revised July 17, 2009/Accepted August 5, 2009)

To assess the anti-inflammatory activity of the constituents of the roots of *Aralia continentalis*, *ent*-pimara-8(14),15-diene-19-oic acid (continentalic acid, pimaradienoic acid, compound I), 7β-hydroxy-*ent*-pimara-8(14),15-diene-19-oic acid (compound II), 7-oxo-*ent*-pimara-8(14),15-diene-19-oic acid (compound III), 15α,16α-epoxy-17-hydroxy-*ent*-kauran-19-oic acid (compound IV) and *ent*-kaura-16-en-19-oic acid (kaurenoic acid, compound V), their inhibitory effects against cyclooxygenase-2 (COX-2)-catalyzed PGE_2 and inducible nitric oxide synthase (iNOS)-catalyzed NO production by lipopolysaccharide-treated RAW 264.7 cells were examined. Among the compounds tested, compound III and V moderately inhibited NO production. In addition, compound III weakly inhibited PGE_2 production, while treatment with compounds II and IV at concentrations of up to 100 μM had no significant effects. Conversely, compound I only weakly inhibited PGE_2 and NO production. To elucidate the mechanism by which these changes occurred, the iNOS down-regulating capacity of compound III was investigated. Western blot analysis and an electrophoretic mobility shift assay demonstrated that compound III weakly inhibited COX-2 and iNOS expression at 50-100 μM, and inhibited NF-κB activation. When *in vivo* anti-inflammatory activities of compounds I, III and V were examined, intraperitoneal injection of 4-100 mg/kg of compound I and V significantly inhibited carrageenan-induced paw edema in mice, whereas compound III did not. Taken together, the results of this study suggest that some constituents of *A. continentalis*, especially compounds I, III and V, exert significant anti-inflammatory activity, which suggests that these constituents contribute, at least in part, to the anti-inflammatory action of the roots of *A. continentalis*.

Key words: *Aralia continentalis*, Continentalic acid, Kaurenoic acid, 7-oxo-*ent*-pimara-8(14),15-diene-19-oic acid, Cyclooxygenase, Nitric oxide synthase, Anti-inflammation

INTRODUCTION

Prostaglandins (PG) and nitric oxide (NO) are the principal chemical mediators of inflammation (Gallin and Snyderman, 1999). Cyclooxygenase-2 (COX-2), an inducible isoform of COX, is primarily responsible for the synthesis of large amounts of PGs in inflammatory lesions. In addition, the inducible isoform of nitric oxide synthase (iNOS) produces high concentrations of NO in several inflammatory disorders. Therefore, inhibition and/or down-regulation of these enzymes may lead to anti-inflammatory effects.

The roots of *Aralia continentalis* Kitagawa (Araliaceae) have long been used in Chinese medicine to treat inflammatory disorders, including rheumatism, lumbago, and lameness (Perry, 1990). The methanol extract and dichloromethane fractions of these plant materials were previously reported to inhibit IL-8 production by lipopolysaccharide (LPS)-treated peritoneal macrophages (Lee et al., 1995). The methanol extract from *A. continentalis* has also been found to exert antinociceptive activity against Freund adjuvant-induced pain in rats (Park et al., 2005). Importantly, *A. cordata* extract protected cartilage degradation and inhibited apoptosis, suggesting the potential to inhibit

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osteoarthritis (Bae et al., 2006). Among its constituents, pimaradienoic acid and kaurenoic acid have been shown to exert vasorelaxant action (Tirapelli et al., 2005). When the inhibitory activities against COX-1 and COX-2 were measured, several compounds, including pimaradienoic acid and kaurenoic acid, moderately inhibited COX-1, while only kaurenoic acid inhibited COX-2 weakly (Dang et al., 2005). Pimaradienoic acid has also been found to inhibit PGE_2 and NO production by RAW 264.7 cells via inhibition of NF-κB activation (Kang et al., 2008). Furthermore, the oral administration kaurenoic acid and pimaradienoic acid has been shown to exert analgesic activity (Okuyama et al., 1991) and kaurenoic acid has been found to inhibit acetic acid-induced colitis in rats (Paiva et al., 2002). However, evaluation of the anti-inflammatory activity of the constituents of *A. continentalis* is far from complete. Therefore, the present study was conducted to clearly establish the anti-inflammatory activity of the constituents *in vitro* and *in vivo*.

MATERIALS AND METHODS

Chemicals

N-[2-Cyclohexyloxy-4-nitrophenyl]methane sulfonamide (NS-398) was obtained from Biomol (Plymouth Meeting, PA). 2-Amino-5,6-dihydro-6-methyl-4H-1,3-thiazine hydrochloride (AMT) was purchased from Tocris Cookson Ltd. (UK). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), prednisolone and lipopolysaccharide (LPS, *Escherichia coli* 0127: B8) were purchased from Sigma (St. Louis, MO). DMEM and other cell culture reagents, including FBS, were products of Gibco BRL (Grand Island, NY). The protein assay kit was purchased from Bio-Rad Lab. (Hercules, CA).

Plant material

The root of *A. continentalis* was purchased from Omni Herb (Seoul), and authenticated by Prof. J. H. Lee (Dong Guk University). A voucher specimen (no. 20080320) was deposited in the laboratory of one of the authors, Prof. J. S. Choi.

Isolation of compound I, II, III, IV and V

The roots of *A. continentalis* (12 kg) were refluxed with methanol (MeOH) for 3 h (3 × 20 L). The total filtrate was then concentrated to dryness *in vacuo* at 40°C in order to render the crude MeOH extract. This extract was suspended in distilled water (H_2O) and successively partitioned with *n*-hexane, methylene chloride (CH_2Cl_2), ethyl acetate (EtOAc) and *n*-butanol (*n*-BuOH), to yield *n*-hexane (360 g), CH_2Cl_2 (40.9 g), EtOAc (104.5 g), and *n*-BuOH (81.3 g) fractions, respectively, as well as a H_2O residue (207.1 g). Repeated chromatography of the *n*-hexane (360 g) over a silica gel column with *n*-hexane:EtOAc solvent system (10:1 to 0:1, gradient), followed by EtOAc:MeOH solvent system (10:1 to 10:3, gradient) afforded 15 subfractions (HF01 ~ HF15). A portion of HF03 (64.0 g) were recrystallized with 100% MeOH, yielding compound I (10 g). A portion of HF03 (62.8 g) successively was separated over a RP-18 column with 100% MeOH and 100% CH_3CN to obtain compound V (2.5 g). Column chromatography of HF09 (17.4 g) were carried out over a silica gel column with *n*-hexane:acetone solvent system (30:1 to 20:1, gradient) to afford 14 subfractions (HF09-1 ~ HF09-14). MPLC chromatography of combined fractions of HF09-7 and HF09-8 (2 g) with H_2O:CH_3CN solvent system (60% CH_3CN to 100% CH_3CN, gradient) were accomplished to obtain compound III (60 mg). MPLC chromatography of combined fractions of HF09-12 and HF09-13 (1.5 g) with H_2O:MeOH solvent system (40% MeOH to 100% MeOH, gradient) were accomplished to obtain compound IV (8 mg). Column chromatography of HF10 (6.1 g) were carried out over a silica gel column with CH_2Cl_2:EtOAc solvent system (50:1 to 0:1, gradient) to afford 6 subfractions (HF10-1 ~ HF10-6). Combined fractions of HF10-3 and HF10-4 (1.8 g) were carried out over the MPLC chromatography with H_2O:MeOH solvent system (80% MeOH to 100% MeOH, gradient) to obtain compound II (10 mg). All isolated compounds I~V were characterized and identified by spectroscopic methods, including ^1H- (400 MHz) and ^{13}C-NMR (100 MHz), as well as by comparison with published data (Shibata et al., 1967; Yahara et al., 1974; Mihashi et al., 1969; Herz et al., 1983; Dang et al., 2005). The structures of compounds I~V [compound I (*ent*-pimara-8(14),15-diene-19-oic acid, continentalic acid, pimaradienoic acid), compound II (7β-hydroxy-*ent*-pimara-8(14),15-diene-19-oic acid), compound III (7-oxo-*ent*-pimara-8(14),15-diene-19-oic acid), compound IV (15α,16α-epoxy-17-hydroxy-*ent*-kauran-19-oic acid), compound V (*ent*-kaura-16-en-19-oic acid, kaurenoic acid)] are shown in Fig. 1.

RAW 264.7 cell culture and measurement of NO and PGE_2 concentrations

RAW 264.7 cells obtained from American Type Culture Collection (ATCC) were cultured with DMEM supplemented with 10% FBS and 1% antibiotics (100 U/mL penicillin and 100 μg/mL streptomycin) in a 5% CO_2 atmosphere at 37°C. The cells were activated with LPS as previously described (Chi et al., 2001). Briefly, cells were plated in 96-well plates (2×10^5 cells/well).

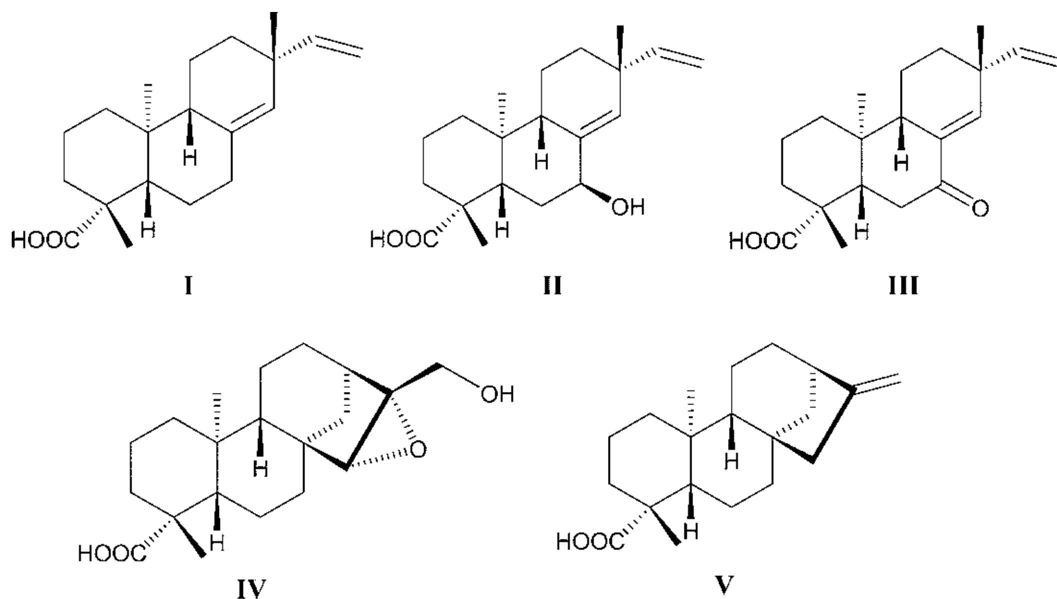

Fig. 1. Chemical structures of the constituents isolated from the roots of *A. continentalis*

After pre-incubation for 2 h, test compounds and LPS (1 mg/mL) were added and incubated for 24 h unless otherwise specified. Test compounds dissolved in DMSO were diluted to appropriate concentrations with serum-free DMEM. The final concentration of DMSO was adjusted to 0.1% (v/v). Cell viability was assessed using MTT assay as described previously (Mossman, 1983). PGE_2 concentration in the medium was measured using an ELISA kit for PGE_2 (Cayman Chem. Co.) according to the manufacturer's reco-mmendations. To assess NO production, the stable conversion product of NO, nitrite (NO_2^-), was measured using Griess reagent and the optical density was determined at 550 nm.

Western blot analysis

To measure the protein levels of iNOS, Western blotting technique was used (Chi et al., 2001). RAW 264.7 cells were cultured in 6-well plates (5×10^6 cells/well) in the presence or absence of LPS (1 mg/mL) with/without test compounds for 16-20 h. After cell homogenates were prepared, the supernatant was obtained by centrifugation at 15,000 g for 30 min. Using Tris-glycine gels (8%), electrophoresis was carried out and bands were blotted to PVDF membranes. The membranes were incubated with iNOS antibody (N32030, Transduction Lab.) and the bands were visualized by chemiluminescent reagent (Amersham).

Electrophoretic mobility shift assay (EMSA)

RAW cells were treated with LPS and various concentrations of test compounds for 3 h. To prepare nuclear fractions, the cells were washed with PBS, harvested and resuspended in 400 μL of buffer A (10 mM HEPES, 10 mM KCl, 0.1 mM EDTA, 1mM DTT, 0.5 mM PMSF, pH 7.9) for 15 min on ice. After 10% NP-40 (25 μL) was added, the tubes were vortexed vigorously for 10 sec. The nuclei were collected by centrifugation at 5,000 rpm for 3 min and the supernatant was saved as the cytosolic fraction. The nuclei were lysed in buffer B (20 mM HEPES, 0.4 M NaCl, 1 mM EDTA, 1 mM DTT, 1 mM PMSF, pH 7.9). Nuclear transcription factor-κB (NF-κB) consensus oligonucleotide (Promega) was phosphorylated by T4 polynucleotide kinase (10 units) with 10 μCi of [γ-^{32}P] ATP (3,000 Ci/mmol) at 37°C for 10 min. Unincorporated oligonucleotides were removed by microspin G-25 column (Amersham, UK). Nuclear extract containing 5 μg protein was incubated with ^{32}P-labeled NF-κB consensus oligonucleotide in gel shift binding buffer at room temperature for 20 min. The incubation mixture was subjected to electrophoresis on a 4% polyacrylamide gel in TBE buffer (0.5X) at 350 V. The gel was dried and exposed to X-ray film overnight at -70°C.

λ-Carrageenan (CGN)-induced paw edema in mice

To examine the *in vivo* anti-inflammatory activity, the mouse CGN-induced paw edema assay was uaed according to the previously described procedures (Winter et al., 1962) with slight modification. Specific pathogen-free male ICR mice were purchased from Orient Bio and acclimatized in the animal facility for at least 7 days prior to the experiment with lab chow

and water *ad libitum*. All animal experiments were carried out according to the strict guideline of animal care by Korea Food and Drug Administration and Kangwon National University. Test compounds or reference compound dissolved in DMSO (0.05 mL/mouse) were administered intraperitoneally to mice. One h later, 1% CGN (w/v) dissolved in pyrogen-free sterile saline solution (0.05 mL/paw) was injected into the right hind paw and paw volume was measured using plethysmometer (Ugo Basil, Italy) 5 h later. A paw volume increase from the initial non-treated paw volume was regarded as edema.

$$\% \text{ inhibition of paw edema} = \frac{(\text{CGN-treated control group} - \text{test group})}{(\text{CGN-treated control group} - \text{control group without CGN})} \times 100$$

Statistical analysis

Experimental values were presented as arithmetic mean ± S.D. The unpaired student's *t*-test and ANOVA with Turkey test were used to determine the statistical significance.

RESULTS

In RAW 264.7 cells, LPS treatment highly induces COX-2 and iNOS for 24 h (Chi et al., 2001), which produce large amount of PGE_2 and NO, respectively. In the present study, PGE_2 and NO concentrations increased to 61.0 ± 2.3 nM and 52.0 ± 3.7 μM from the control level of 1.0 ± 0.3 nM and 0.2 ± 0.0 μM, respectively (n = 3). When the inhibitory activity of the compounds were examined under this condition, compound III weakly inhibited COX-2-catalyzed PGE_2 production, while other compound I, II, IV and V did not significantly inhibit PGE_2 production at concentrations up to 100 μM (Fig. 2A). Compound I, II and IV rather slightly increased PGE_2 production at the higher concentrations. Against NO production, compound I, III and V showed inhibition. Particularly, compound III moderately inhibited iNOS-catalyzed NO production (Fig. 2B). As expected, NS-398 (COX-2 inhibitor) and AMT (iNOS inhibitor) potently inhibited PGE_2 and NO production with 96.8% and 96.7% inhibition at 0.1 and 1.0 μM, respectively. MTT bioassay demonstrated that only compound V (kaurenoic acid) showed 78.6% and 5.1% cytotoxicity at 100 and 50 μM, respectively; therefore, the concentration of compound V evaluated was limited to 50 μM. No other compounds tested showed a cytotoxic effect on RAW cells at concentrations up to 100 μM (data not shown).

To elucidate the cellular inhibitory mechanism of most PGE_2- and NO-inhibitory molecule, compound III, Western blot analysis was employed. As shown in Fig. 3A, compound III weakly down-regulated COX-2 and iNOS induction at 50-100 μM. Moreover, compound III did inhibit NF-κB activation as revealed by EMSA (Fig. 3B). These results suggest that compound III may inhibit COX-2-catalyzed PGE_2 and iNOS-catalyzed NO production at least in part by iNOS down-regulation through an inhibition of NF-κB activation.

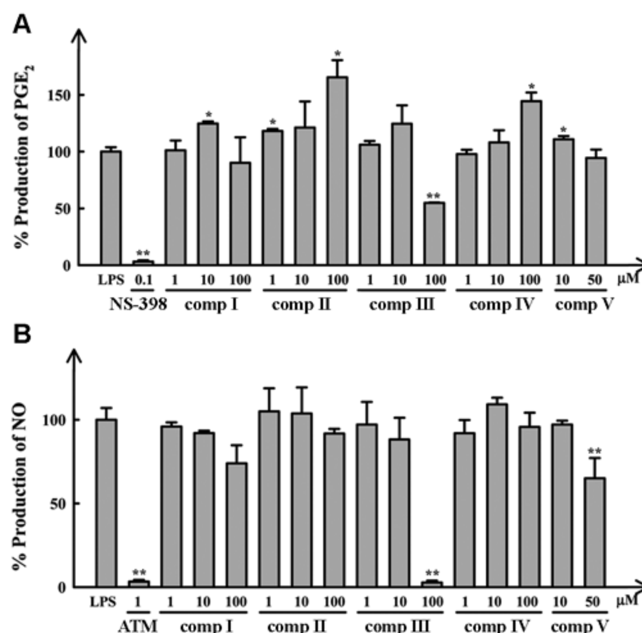

Fig. 2. Effects on PGE_2 and NO production from LPS-treated RAW 264.7 cells. **A** Inhibition of COX-2-catalyzed PGE_2 production from LPS-treated RAW 264.7 cells, **B** Inhibition of iNOS-catalyzed NO production from LPS-treated RAW 264.7 cells, LPS and the test compounds were simultaneously added and the cells were incubated for 24 h (n = 3), $^*p < 0.05$, $^{**}p < 0.001$. Significantly different from the LPS-treated control group.

In addition, *in vivo* anti-inflammatory activity of the compounds showing anti-inflammatory activity *in vitro* was examined. By intraperitoneal injection, compound I and V dose-dependently inhibited CGN-induced paw edema in mice (Table I). In particular, compound V potently inhibited paw edema by more than 70% at 100 mg/kg, i.p. Comparing with the inhibitory activity of the reference compound, prednisolone, compound V shows promise for development as a new anti-inflammatory agent. However, compound III did not significantly inhibit edema when administered at 4-100 mg/kg, even though it showed meaningful anti-inflammatory activity *in vitro*.

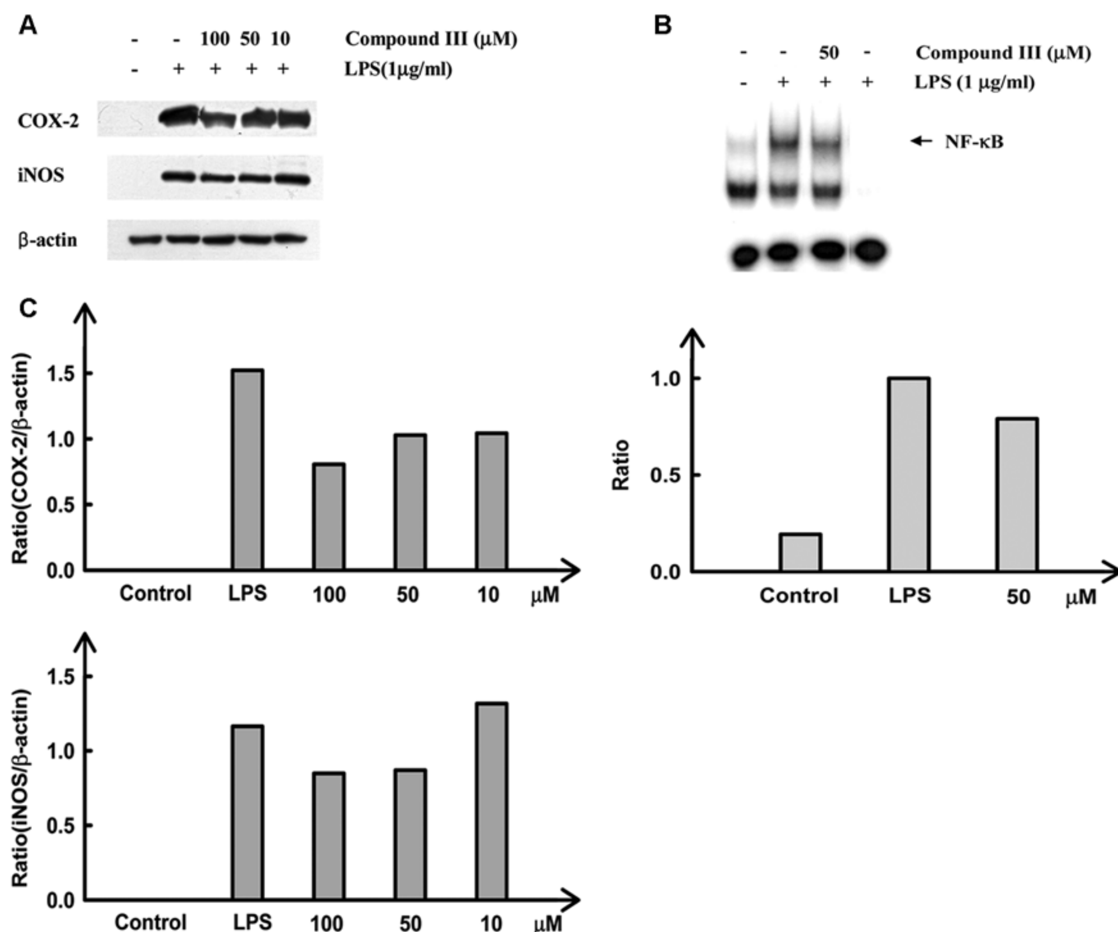

Fig. 3. Effects of compound III on COX-2 and iNOS expression. (**A**) Western blotting analysis, (**B**) EMSA, competitor, (**C**) Densitometric analysis was carried out using Image J 1.37v program (Wayne Rasband, NIH, USA).

Table I. Inhibition of λ-carrageenan (CGN)-induced paw edema in mice

Compounds	Dose (mg/kg)	Increased paw volume (mL)	% inhibition
CGN	-	0.176 ± 0.009[a]	
Prednisolone	20	0.078 ± 0.035*[,b,c]	55.7
ent-Pimara-8(14), 15-diene-19-oic acid (compound I)	4	0.202 ± 0.054[a]	-
	20	0.158 ± 0.026[a]	10.2
	100	0.136 ± 0.026*[,a,b]	22.7
7-Oxo-*ent*-pimara-8(14), 15-diene-19-oic acid (compound III)	4	0.170 ± 0.012[a]	3.4
	20	0.184 ± 0.065[a]	-
	100	0.165 ± 0.062[a]	6.2
ent-Kaura-16-en-19-oic acid (compound V)	4	0.160 ± 0.016[a]	9.1
	20	0.134 ± 0.034*[,a,b]	23.9
	100	0.050 ± 0.022*[,c]	71.6

All compounds were intraperitoneally administered (n = 5). The paw volume of the untreated control group was 0.121 ± 0.009 mL. *$p < 0.05$, significantly different from the CGN-treated group by unpaired Student's *t*-test. [a,b,c]$p < 0.05$, significantly different from each other by one-way ANOVA followed by Turkey analysis.

DISCUSSION

The present investigation has clearly shown that several constituents from *A. continentalis* possess anti-inflammatory activity *in vitro* and *in vivo*. Particularly, compound III shows significant *in vitro* anti-

inflammatory activity and compound V possesses potent *in vivo* anti-inflammatory activity.

The results of the present study also revealed some discrepancy between the *in vitro* and *in vivo* results. Compound III possessed higher anti-inflammatory activity *in vitro* than compound V; however, the *in vivo* potency of compound V was much higher than that of compound III. Indeed, compound III did not significantly inhibit CGN-induced paw edema in mice when administered by peritoneal injection. The exact reason for these differences is currently unknown. It is possible that the bioavailability of compound III through the peritoneal cavity is very low. Conversely, the *in vivo* anti-inflammatory activity of compound V was potent, even though it did not possess high anti-inflammatory activity *in vitro*. These findings suggest that another cellular mechanism(s) of compound V, such as the inhibition of proinflammatory cytokine release, may exist. This notion should be examined further.

It was previously reported that 10-100 μM of compound I inhibited PGE_2 and NO production by LPS-treated raw cells, primarily via COX-2 and iNOS down-regulation (Kang et al., 2008). However, in the present study, the inhibitory activity of compound I on PGE_2 production was negligible and its inhibition of NO production was very weak. These differences may be explained, at least in part, by the different sensitivities of the *in vitro* systems used in different studies. Specifically, 200 ng/mL LPS was used to stimulate RAW cells in the previously conducted study, whereas 1 μg/mL LPS was used in the present study. Because the inhibitory action against less activated cells is believed to be higher and more sensitive, the NO-inhibitory activity and iNOS-down-regulatory activity of compound I in RAW cells activated by treatment with 200 ng/mL LPS may be more sensitive. It is currently not known if the inhibitory activity of compound I is intrinsic or an experimental artifact. It is also significant to mention that the most NO-inhibitory compound among the derivatives tested, compound III, inhibits COX-2 and iNOS induction via NF-κB inhibition although the potencies were weak.

The roots of *A. continentalis* have been widely used as an anti-inflammatory drug in Chinese medicine. Among their constituents, several compounds were previously examined for the anti-inflammatory activity. Dang et al. (2005) demonstrated that diterpenes including continentalic acid possessed COX-1 inhibitory activity, while their COX-2 inhibitory activities were weak. Kang et al. (2008) claimed that compound I inhibits PG and NO production mainly by COX-2 and iNOS down-regulation. Here we demonstrated that compound III down-regulates COX-2 and iNOS expression, and that compounds I and V exert *in vivo* anti-inflammatory activity. Taken together, these findings indicate that the combined activities of the constituents of the roots of *A. continentalis* may contribute, at least in part, to its anti-inflammatory effects.

In conclusion, the results of the present study demonstrate that several constituents of the roots of *A. continentalis* possess anti-inflammatory activity *in vitro* and *in vivo*. Specifically, compound III showed significant inhibitory activity against PGE_2 and NO production by LPS-treated RAW 264.7 cells. And compound V showed potent inhibition against CGN-induced edema in the paws of mice when administered intraperitoneally at doses of 4-100 mg/kg.

ACKNOWLEDGEMENTS

This study was financially supported by the research fund of Studies on the Identification of the Efficacy of Biologically Active Components from Oriental Herbal Medicines from Korean Food and Drug Administration (2007) and post-BK21 project from Ministry of Education, Korea.

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