Anti-inflammatory Activity of Constituents Isolated from Aerial Part of Angelica acutiloba Kitagawa

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Recently, the resources of medicinal plants have been exhausting. The root of Angelica acutiloba is one of the most important ingredients in Japanese Kampo medicine for the treatment of gynecological diseases. In our search for alternative medicinal plant resources of the root of A. acutiloba, we found that its aerial part has the anti-inflammatory potency as well as the root. Phytochemical investigation of the aerial part resulted in the isolation of four compounds including a new dimeric phthalide, namely tokiaerialide (2), along with Z-ligustilide (1), falcarnindiol (3), and bergaptol (4). Next, we investigated the in vitro anti-inflammatory activity of 1-4 in lipopolysaccharide-stimulated RAW264 macrophages. Among the isolated compounds, 1 exhibited the most potent inhibition against lipopolysaccharide-induced production of prostaglandin E2, nitric oxide, and pro-inflammatory cytokines (interleukin-6 and tumor necrosis factor-α). Compounds 3 and 4 also inhibited all inflammatory mediators, but their inhibitory abilities were weaker than those of 1. Furthermore, 1, 3, and 4 strongly also induced heme oxygenase-1. These results suggest that 1, 3, and 4 potentially exert anti-inflammatory activity, and the aerial part of A. acutiloba may be considered to be a useful medicinal resource for inflammatory diseases. Copyright © 2015 John Wiley & Sons, Ltd.

Keywords: Angelica acutiloba; aerial part; Z-ligustilide; tokaerialide; anti-inflammation; macrophages.

INTRODUCTION

Traditional herbal medicines have recently attracted attention, owing to the rising interest in their health benefits. The consumption of medicinal plants is markedly increasing with economic development, and the depletion of medicinal plant resources is becoming recognized as a serious problem. Recently, there has been interest in the effective utilization of non-supplemented medicinal parts of medicinal plants as an approach to solve this problem. The root of Bupleurum falcatum L. (Umbelliferae) is one of the important ingredients in Japanese Kampo medicine (JKM) and traditional Chinese medicine, while its aerial part is not utilized as medicinal material. Our recent study demonstrated that phytochemical analysis of the aerial part of B. falcatum resulted in the isolation of major phenolic components together with low concentrations of saikosaponins (Tung et al., 2015). Furthermore, some isolated compounds showed anti-proliferative activity against HL-60 and HepG2 cancer cell lines. These findings support utilization of the aerial part of B. falcatum as medicinal material, and other medicinal plants also have possibilities to contain active components in non-supplemented medicinal parts.

In Japan, the root of Angelica acutiloba Kitagawa (Umbelliferae) is known as Toki and listed in Japanese Pharmacopeia. A. acutiloba root has been used in JKM for the treatment of gynecological diseases like female reproductive diseases (Tsuchida et al., 1987). In China, the root of Angelica sinensis (Oliv.) Diels (Danggui in Chinese) has been used in traditional Chinese medicine for the same purpose as A. acutiloba (Chao and Lin, 2011). A. acutiloba and Angelica gigas Nakai are commonly used as the substitutes of A. sinensis in the market of Southeast Asia because of the shortage of A. sinensis (Lao et al., 2004).

Phytochemical analyses of the roots of A. acutiloba and A. sinensis have shown that volatile alkylphthalide derivatives are principal components and Z-ligustilide is the main component among the phthalides along with several dimers (Tsuchida et al., 1987; Chao and Lin, 2011; Deng et al., 2006). Because the root of A. acutiloba is the most important herbal medicine in JKM, several biological activities have been elucidated. A. acutiloba root extract relieves insulin resistance induced by high sugar diet in rats (Liu et al., 2011a) and improves advanced glycation end-product-mediated renal injury in a diabetic model rat (Liu et al., 2012). The polysaccharide fraction from the root of A. acutiloba showed a potent anti-tumor activity (Yamada et al., 1990). In the field of inflammation, it has been reported that the water extract of A. acutiloba root inhibited adjuvant arthritis (Cho et al., 1982) and mast cell-derived allergic reactions (Lee et al., 2012). Moreover, Z-ligustilide has

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an anti-asthma activity (Tao et al., 1984). However, because the aerial part of A. acutiloba is not used as an ingredient in KJM, phytochemical investigation of its aerial part and its anti-inflammatory activity has not been studied previously.

Inflammation involves multiple events regulated by activation of inflammatory or immune cells. In the inflammatory process, activated macrophages play a key role in inflammatory diseases by producing inflammatory mediators, such as prostaglandin E2 (PGE2), nitric oxide (NO), and pro-inflammatory cytokines (interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α)). (Fujiwara and Kobayashi, 2005; Berenbaum, 2000). Overproduction of inflammatory mediators in macrophages has been implicated in several diseases, including rheumatoid arthritis (Davignon et al., 2013), atherosclerosis (Lind, 2003), asthma (Naik and Wala, 2013), chronic hepatitis (Tilg et al., 1992), and pulmonary fibrosis (Coker and Laurent, 1998). Therefore, inhibition of the production of these inflammatory mediators may thus prevent or suppress a variety of inflammatory diseases.

Heme oxygenases (HO) catalyze the rate-limiting step in heme degradation, resulting in the formation of carbon monoxide, biliverdin, and ferrous iron (Maines, 1988). Several lines of evidence have shown that HO-1, an inducible isoform of HO, acts as a cytoprotective factor against inflammatory responses and oxidative insults through the antioxidant activity of biliverdin and its metabolite, bilirubin, as well as the anti-inflammatory action of carbon monoxide (Otterbein and Choi, 2000). Therefore, the induction of HO-1 is beneficial in response to stressful situations like inflammation.

Our pilot study on A. acutiloba showed that the aerial part extract suppressed PGE2 and NO production with similar potency to the root extract in lipopolysaccharide (LPS)-stimulated murine macrophage-like RAW264 cells, and the phytochemical profile by TLC and HPLC fingerprinting revealed the occurrence of the major compound, Z-ligustilide, at a relatively high concentration in the aerial part rather than the root. In this study, we showed the phytochemical analyses of the aerial part of A. acutiloba. Moreover, we investigated the effects of the isolated compounds on inflammatory mediators and induction of HO-1. Our findings may open the possibility of the aerial part of A. acutiloba as a resource instead of the root.

### MATERIALS AND METHODS

**General procedures.** Optical rotations were measured with a DIP-360 digital polarimeter (JASCO, Easton, USA). NMR spectra were recorded on a JEOL ECX 400 FT-NMR spectrometer (JEOL, Tokyo, Japan) at 20 °C using Jeol 400 FT-NMR spectrometer (JEOL, Tokyo, Japan). (St. Louis, MO, USA). An enzyme-linked immunosorbent assay (ELISA) kit for PGE2 was purchased from Cayman (Ann Arbor, MI, USA). ELISA kits for IL-6 and TNF-α were purchased from Dianclone (Besançon, France). Antibodies against HO-1 and β-actin were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). All other chemicals were obtained from Wako Pure Chemical Industries (Osaka, Japan).

**Preparation of the MeOH extracts of aerial and root parts for the assay of anti-inflammatory activity.** The dried aerial or root parts (100 mg) of A. acutiloba were extracted with MeOH (1.2 mL) under sonication three times at 40 °C individually and then filtered and evaporated with N gas at 60 °C.

**Extraction and isolation of compounds 1-4.** The pulverized air-dried aerial part (79.0 g) was extracted with MeOH (1.0 L × 3 times) at 40 °C with sonication. The combined extracts were concentrated to a dark brown residue (15.3 g). The obtained crude extract was partitioned with a solvent mixture of hexane-MeOH–H2O (200 mL:200 mL:20 mL, v/v/v) to afford a hexane layer (2.60 g after removing the solvent) and MeOH–H2O layer, which was then evaporated and further partitioned between EtOAc and water (200 mL:200 mL) to obtain an EtOAc residue (1.40 g). The hexane extract (2.60 g) was loaded onto a silica gel column chromatography on a silica gel column (30×300 mm) and eluted with a gradient of hexane–EtOAc (8:1→2:1, v/v) to afford additional compound 1 (120 mg). The EtOAc extract was chromatographed on a silica gel column (30×300 mm) and eluted with hexane–EtOAc (8:1) to yield compound 2 (80 mg). Finally, fraction 5 (145 mg) was chromatographed on a silica gel column (20×400 mm) with hexane–EtOAc (5:2, v/v) to afford compounds 3 (150 mg) and 4 (80 mg). Compound 1, 3, and 4 were identified as Z-ligustilide (Mitsuhashi et al., 1963; Tsuchida et al., 1987), falcarindiol (Tanaka et al., 1977), and bergaptol (Caceres et al., 2001; Girennavar et al., 2007) by the comparisons of spectral data, respectively.

**Compound 2:** pale-yellow oil; [α]D 25 +21° (c 0.15, CHC13); ESI-MS m/z (% base peak): 381 (39), 191 (100); HR-ESI-MS m/z: 381.2079 [M+H]+ (caled for C24H32O4, 381.2065); 1H-NMR (CDCl3, 400 MHz) δ:
Anti-inflammatory evaluation

Cell culture. The murine macrophage-like cell line, RAW264 (RCB No. 0535), was obtained from the RIKEN BioResource Center Cell Bank and cultured at 37 °C in a 5% CO₂ atmosphere in Dulbecco's modified Eagle's medium (DMEM) containing 10% FBS. To stimulate the cells, the medium was replaced with fresh DMEM, and then LPS was added in the presence or absence of compounds to the indicated concentrations. Jolkinolide B (Uto et al., 2012) was used as a positive control to assess the test validity of anti-inflammatory activity of isolated compounds.

Measurement of PGE₂ and cytokines. RAW264 cells were plated at 4 × 10⁵ cells/well in 24-well plates and then treated with or without the compounds before exposure to 50 ng/mL LPS. After 12 h of incubation, the level of nitrite (an indicator of NO synthesis) was measured using the Griess reaction. Briefly, the cell culture medium was mixed with an equal volume of Griess reagent (1% sulfanilamide in 0.1% naphthylethlenediamine dihydrochloride and 5% phosphoric acid in water). The absorbance was measured with a microplate reader at 540 nm.

Measurement of NO. RAW264 cells were plated at 4 × 10⁵ cells/well in 24-well plates and treated with or without the compounds before exposure to 50 ng/mL LPS. After 12 h of incubation, the level of nitrite (an indicator of NO synthesis) was measured using the Griess reaction. Briefly, the cell culture medium was mixed with an equal volume of Griess reagent (1% sulfanilamide in 0.1% naphthylethlenediamine dihydrochloride and 5% phosphoric acid in water). The absorbance was measured with a microplate reader at 540 nm.

Western blotting analysis. RAW264 cells were plated at 1 × 10⁶ cells/well in 6-cm dish and treated with or without the compounds before exposure to 50 ng/mL LPS. After 12 h of incubation, the harvested cells were lysed, and the supernatants were boiled for 5 min. Equal amounts of lysate protein were run on 10% SDS-PAGE and electrophoretically transferred to PVDF membrane. After blotting, the membrane was incubated with specific primary antibody overnight at 4 °C and further incubated for 1 h with HRP-conjugated secondary antibody. Bound antibodies were detected by ECL Plus Western Blotting Detection System with a LAS-4000 mini (GE Healthcare, Princeton, NJ, USA).

RNA isolation and RT-PCR analysis. RAW264 cells were plated at 1 × 10⁶ cells/well in 6-cm dish and treated with or without the compounds before exposure to 50 ng/mL LPS. After 6 h of incubation, total RNA was extracted using Trizol Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. From each sample, 1 μg of total RNA was used to synthesize cDNA using PrimeScript first-strand cDNA Synthesis Kit (Takara Bio, Shiga, Japan). Then, PCR analyses were performed on aliquots of cDNA preparations to detect HO-1 and GAPDH using TaKaRa Ex Taq (Takara Bio). After initial denaturation for 2 min at 94 °C, the PCR primers used in this study are as follows: HO-1, sense 5′-CTGTGTAACCTCTGGCTTCC-3′ and antisense 5′-CCACACTACCTGAGTCTACC-3′; GAPDH, sense 5′-GACCCCTTCTAGGACCTCAAC-3′ and antisense 5′-CATACCGAGAAATGAGCTTG-3′. PCR products were separated on 2% agarose gels and photographed under UV excitation after ethidium bromide staining.

Statistical analyses. The experimental results are presented as mean ± SE. Each experiment was repeated at least three times. Data were compared using Student’s t test, and P values less than 0.05 were considered to be significant.

RESULTS

Effects of extracts of aerial and root parts of A. acutiloba on LPS-induced PGE₂ and NO production

To assess the anti-inflammatory potency of the aerial part of A. acutiloba, we compared the effect of MeOH extracts of its aerial and root parts on LPS-induced PGE₂ and NO production in RAW264 cells. As shown in Fig. 1A, the aerial part extract significantly attenuated LPS-induced PGE₂ with similar potency to the root extract. Moreover, the inhibitory potency of NO production by the aerial part extract was stronger than that by root extract (Fig. 1B). These results indicate that the aerial part of A. acutiloba might contain the anti-inflammatory compounds as well as the root. The cytotoxicity of the extracts of aerial and root parts to cells was assessed using MTT assay, and the cell viability was not affected in the concentration range tested (Supporting Information Fig. S1). Thus, the inhibitory effects were not attributable to cytotoxic effects.

Isolation and structural elucidation of the compounds

The MeOH extract of the aerial part of A. acutiloba was partitioned with hexane–MeOH–water (10:10:1, v/v/v) and then between ethyl acetate and water, followed by column chromatography to yield a new phthalide dimer (2) along with Z-ligustilide (1) (Chen et al., 2007), falaricndiol (3) having anti-nociceptive (Tanaka et al., 1977) and anti-tumor activities (Jin et al., 2012), and bergapten (4) having radical scavenging activity (Girennavar et al., 2007; Fig. 2).

Z-Ligustilide (1) was known to be one of the major compounds of the A. acutiloba root (Tsuchida et al., 1987; Miyazawa et al., 2004; Fukuda et al., 2009). We
obtained 1 from both the hexane and EtOAc extracts of the aerial part, and its content was 0.228 dry weight (dw) 
%, although the other alkyl phthalides, including butylidene phthalide, butylphthalide, senkyunolide A, 
and tokinolide A were isolated from the A. acutiloba root (Tsuchida et al., 1987; Kim et al., 2006). HPLC fin-
gerprint of the aerial and root parts showed that 1 is 
one of the major compounds of the aerial part as well 
as root (Supporting Information Fig. S2). In addition, 
the minor compound falcariol (3) was also found in 
the root part. Meanwhile, the coumarin component 
bergaptol (4) with low yield was isolated for the first 
time from A. acutiloba.

Compound 2 was obtained as pale yellow oil with 
$\alpha$ $^{20}_{D} -21$° (c 0.15, CHCl$_3$). Its molecular formula was 
determined to be C$_{24}$H$_{28}$O$_{4}$ based on the observed 
molecular peak at m/z 381.2079 [M + H]$^+$ (calcd for 
C$_{24}$H$_{29}$O$_{4}$, 381.2065) in the HR-ESI-TOF-MS spec-
trum. The 1H-NMR (Supporting Information Fig. S3) 
and H–H COSY spectra displayed the presence of a butyl 
side chain [δ 2.78 (t, J = 8.0 Hz, H-8), 1.25 (m, H-9), 1.10– 
1.20 (overlapped, H-10), and 0.89 (t, J = 6.8 Hz, H-11)] 
and butylidene side chain [δ 4.86 (t, J = 7.6 Hz, H-8$'$), 
2.18 (dt, J = 14.0, 6.8 Hz, H-9$'$), 1.10–1.20 (overlapped, 
H-10$'$), and 0.92 (t, J = 7.2 Hz, H-11$'$)]. In addition, four 
olefinic protons accounting for two isolated double bonds 
[δ 5.97 (dt, J = 9.6, 4.4 Hz, H-6), 6.15 (dt, J = 9.6, 2.0 Hz, 
H-7, 6.20 (dd, J = 10.4, 6.4, 2.0 Hz, H-6$'$), and 5.80 (br d, 
J = 10.4 Hz, H-7$'$)] were observed. The 13C-NMR 
spectrum (Supporting Information Fig. S4) associated with 
the DEPT spectrum revealed 24 carbons including two ester carbonyl signals [δ 169.4 (C-1) and 172.8 (C-1$'$)], six 
olefinic carbons, three quaternary carbons, a methine, 
eight methylenes, and two methyls. The 24 carbon signals 
and their resonance pattern suggested that 2 might be a phthalide dimer formed by two ligustilide units (Tsuchida 
et al., 1987; Deng et al., 2006). The upfield shift of the 
olefinic proton signal of the butylidene side chain [δ 4.86 
t (J = 7.6 Hz)] indicated that it was not affected by 
deshielding of the neighboring lactone and indicated 
the butylidene side chain in the ligustilide moiety has 
a Z-geometry form (Tsuchida et al., 1987; Deng et al., 
2006; Chen et al., 2007). The 1H- and 13C-NMR spectra 
data of 2 were found to be similar to those of 
sinaspirolide (Deng et al., 2006) and neodiligustilide 
(Chen et al., 2007), which further suggested the struc-
ture of 2 as a dimeric ligustilide forming a cyclobutane 
ring. This hypothesis was confirmed by the presence 
of the prominent fragment ion of the monomer m/z 
191 in the ESI-MS spectrum (Supporting Information 
Fig. S5). The interlinkage between the two monomers 
was established by the HMBC correlations of H-8/C-3$'$, 
H-8/C-4$'$, and H-7$'$/C-3, respectively, supporting the 
carbon bonding of C-3$'$→C-7$'$ and C-8→C-3$'$ to build 
the fused cyclobutane ring. In addition, the HMBC spectrum of 2 showed correlations of H-6/C-7a, H-7/ 
C-1, H-7/C-5a, H-8/C-3, H-8/C-5a, H-8/C-9, H-6/C-7a, 
H-7/C-1$'$, H-7/C-3$'$a, and H-8/C-3$'$a confirmed the
Effect of the isolated compounds on PGE2, NO, IL-6, and TNF-α production in LPS-stimulated RAW264 macrophages

To examine the anti-inflammatory effect of the isolated compounds, we first measured the cytotoxicity of 1–4 against RAW264 cells using MTT assay (Supporting Information Fig. S6). Cell viability was not altered by 1–4 below 25 μM. Therefore, cells might be treated with 1–4 at concentrations below 25 μM in the following investigations of anti-inflammatory effect.

To investigate the effect of 1–4 on LPS-induced PGE2, NO, IL-6, and TNF-α production, RAW264 cells were treated with 1–4 at concentrations of <25 μM for 30 min before exposure to 50 ng/mL LPS for 12 h. As shown in Fig. 4A and B, the treatment of 1 between 3.125 and 25 μM strongly suppressed both LPS-induced PGE2 (IC50 = 1.05 μM) and NO production (IC50 = 8.45 μM). Compounds 2, 3, and 4 also strongly reduced PGE2 (2, IC50 = 15.61 μM; 3, IC50 = 7.78 μM; 4, IC50 = 7.18 μM) and NO (2, IC50 > 25 μM; 3, IC50 = 11.24 μM; 4, IC50 = 11.85 μM), but their inhibitory potency against PGE2 and NO was weaker than that of 1. As shown in Fig. 4C and D, 1 exhibited suppression of IL-6 and TNF-α. On the other hand, compounds 3 and 4 weakly reduced IL-6 and TNF-α. Compound 2 exhibited the suppression of TNF-α at only 25 μM, but showed no inhibition of IL-6 at any tested concentration. Overall, the rank order of inhibitory potencies against inflammatory mediators was 1 > 3 = 4 > 2. These results suggest that 1 was the most potent inhibitor among the four isolated compounds.

Effect of the isolated compounds on the induction of HO-1 protein and mRNA expressions

Because HO-1 is known to be a novel protective gene with potent anti-inflammatory effect, we examined whether 1–4 enhance HO-1 protein expression in LPS-stimulated RAW264 cells. As shown in Fig. 5, HO-1 protein and RNA levels were increased by the treatment of 1–4. Among these compounds, compound 1 is the strongest inducer of HO-1 expression at protein and mRNA, and 3 and 4 also strongly increased HO-1 expression. However, the induction potency of 2 was weaker than those of 1, 3, and 4. These data suggested that 1, 3, and 4 are potent HO-1 inducer at the level of protein synthesis.

DISCUSSION

In this study, we found that the aerial part of A. acutiloba has anti-inflammatory potency similar to the root part. Phytochemical analysis of the aerial part resulted in the isolation of four compounds including a new dimeric phthalide, tokiaerialide (2). Furthermore, we demonstrated that the aerial part contains a major marker constituent Z-ligustilide (1) together with falcarnidil (3) and bergaptol (4), and the concentrations of Z-ligustilide in the aerial part is remarkably high (0.228 dw%) as compared with that in roots (approximately 0.05 dw%; Tsuchida et al., 1987; Miyazawa et al., 2004; Fukuda et al., 2009). To our knowledge, this is the first report of a chemical profile of the aerial part of A. acutiloba.

To further investigate the mechanism underlying the anti-inflammatory effect of isolated compounds, we examined the effects of 1–4 on inflammatory mediators. Our data showed that 1 exerted the most potent inhibition of LPS-induced production of PGE2, NO, IL-6, and TNF-α. This evidence has a good agreement with previous result (Chung et al., 2012). Compounds 3 and 4 also
suppressed all inflammatory mediators, but their inhibitory abilities were weaker than those of 1. Additionally, 1, 3, and 4 strongly induced HO-1 expression. Based on these findings, 1, 3, and 4 may contribute to the anti-inflammatory activity of the aerial part of *A. acutiloba*.

Previously, almost all researches on herbal medicines have been focusing on the chemical and biological analyses of medicinal part defined by the Japanese Pharmacopeia. However, it becomes important to consider the utilization of non-supplemented medicinal parts for the resource protection of medicinal plants because it is well known that the resource of native medicinal plants like licorice has been exhausted (Marui et al., 2014; Yamamoto and Tani, 2005). This is the reason why we started to investigate the aerial part of medicinal plants. Resource of ginsenosides, major bioactive compounds in the root of *Panax ginseng*, has been supplied from the leaves of American ginseng based on an investigation (Li et al., 1996). In our previous study, we

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**Figure 4.** Effect of 1–4 on LPS-induced production of PGE2 (A), NO (B), IL-6 (C), and TNF-α (D) in RAW264 cells. RAW264 cells were treated with the indicated concentrations of 1–4 for 30 min before incubation with 50 ng/mL LPS for 12 h. The concentrations of PGE2, nitrite, IL-6, and TNF-α were determined as described in the Materials and Methods section. Data are expressed as the means ± SE of three independent experiments. *p < 0.05 vs. LPS alone.

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isolated major phenolic components together with saikosaponins from the aerial part of *B. falcatum*, and some isolated components suppressed the proliferation of cancer cell lines (Tung *et al.*, 2015). As a second trial, we isolated four compounds including a major marker constituent Z-ligustilide (1) and new compound tokaerialide (2) from the aerial part of *A. acutiloba* and clarify that 1, 3, and 4 suppress LPS-induced inflammatory mediators and induce HO-1 in this study. These evidences may approach the development of not only a potential resource in JKM prescription having anti-inflammatory activities instead of *A. acutiloba* root but also new usage of non-supplemented part of medicinal herbs. From these findings, we suggest that other medicinal plants also may have possibilities to contain active components in non-supplemented medicinal parts.

Acknowledgements

The authors are grateful for the financial support from the Genkai town (Saga prefecture, Japan).

Conflict of Interest

The authors have declared that there is no conflict of interest.

REFERENCES


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