



Comparison of the anti-inflammatory activities of furanocoumarins from the roots of *Angelica dahurica*

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ABSTRACT

Background: The roots of *Angelica dahurica* Bentham et Hooker filius ex Franchet et Savatier (Apiaceae) have traditionally been used for inflammatory skin diseases. *A. dahurica* roots (*Byakushi*) contain furanocoumarins, such as imperatorin and byakangelicin. To elucidate which constituents are responsible for the anti-inflammatory effects, we evaluated the potency of crude *A. dahurica* root extract fractions by monitoring the production of the inflammatory mediator nitric oxide (NO) in hepatocytes.

Methods: The dried roots of *A. dahurica* were collected in South Korea and extracted with methanol. The resulting extract was fractionated into ethyl acetate (EtOAc)-soluble, *n*-butanol-soluble, and water-soluble fractions. Primary cultured rat hepatocytes were treated with interleukin (IL)-1 β and each fraction for 8 h, and then the NO production and lactate dehydrogenase activity in the medium were measured. The expression of inducible nitric oxide synthase (iNOS) was detected by Western blotting, and its mRNA expression level was measured by quantitative reverse transcription-polymerase chain reaction.

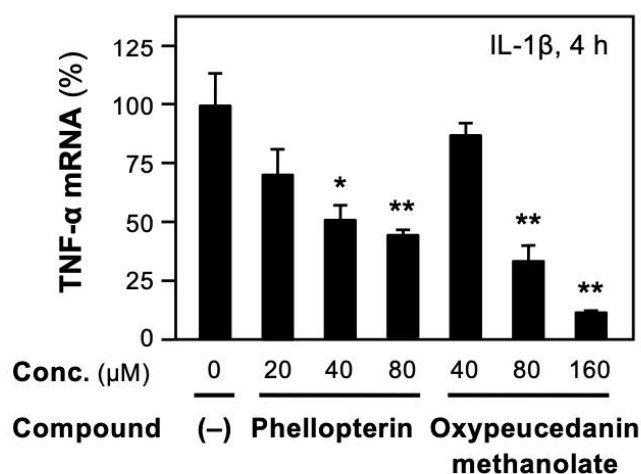
Results: Among the three fractions, the EtOAc-soluble fraction markedly suppressed NO production without showing cytotoxicity and decreased iNOS expression in hepatocytes. From this hydrophobic fraction, we isolated five furanocoumarins: isoimperatorin, imperatorin, phellopterin, oxypeucedanin, and oxypeucedanin methanolate. Phellopterin and oxypeucedanin methanolate significantly suppressed NO production and reduced the mRNA expression of iNOS and tumor necrosis factor α . In contrast, the other three constituents did not affect NO production. Comparison of their chemical structures suggests that a methoxy group at carbon 5 and a side chain at carbon 8 in the furanocoumarin skeleton may be essential for NO production suppression.

Conclusion: These data imply that phellopterin and oxypeucedanin methanolate, which are hydrophobic furanocoumarins, may contribute to the anti-inflammatory effects of *A. dahurica* roots by suppressing iNOS gene expression.

Keywords: Inflammation, nitric oxide, hepatocyte, coumarin, Kampo medicine



Angelica dahurica
(Byakushi)



Furocoumarins in the root of *Angelica dahurica* decrease TNF- α mRNA.

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INTRODUCTION

Angelica dahurica Bentham et Hooker filius ex Franchet et Savatier (Apiaceae) grows in Japan (known as *Yoroigusa*), China, and South Korea (Figure 1). Its roots (called *Byakushi* in Japan) have a bitter taste and are considered to resolve the exterior to emanate pathogens (*Gehyou*) in traditional Japanese medicine [1]. The roots of *A. dahurica* contain

furanocoumarins (also called furocoumarins, e.g., byakangelicin and imperatorin) and monoterpenoids (e.g., α -pinene and camphene). The roots of *A. dahurica* have been included in Kampo formulas, such as *Seijobofuto*, which is used to treat inflammatory skin diseases, including eczema and acne.

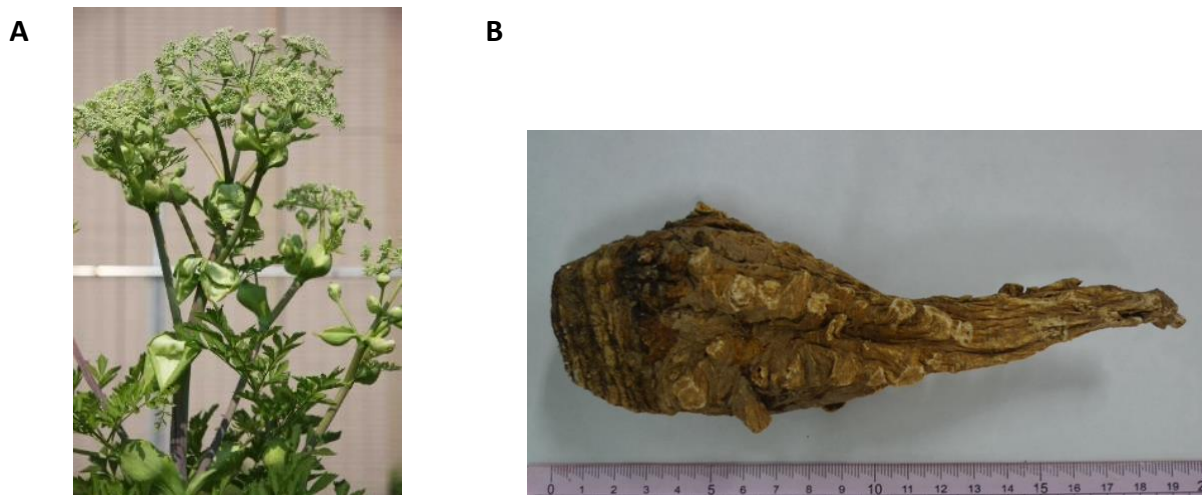


Figure 1. *Angelica dahurica* Bentham et Hooker filius ex Franchet et Savatier (Apiaceae). (A) An aboveground part of *A. dahurica*, which was grown at Biwako-Kusatsu Campus, Ritsumeikan University, Kusatsu, Shiga, Japan. (B) A root of *A. dahurica*, which was collected in South Korea. The same lot of *A. dahurica* root was used in this study. Scale is in cm.

Foreign pathogenic bacteria and viruses provoke inflammatory responses. Lipopolysaccharide (LPS) is released from the outer membrane of dead gram-negative bacteria and induces the production of nitric oxide (NO), which is an important proinflammatory mediator synthesized by inducible nitric oxide synthase (iNOS) [2]. In the liver, LPS stimulates Kupffer cells (resident macrophages) to secrete interleukin (IL)-1 β , which then stimulates hepatocytes to induce the iNOS protein [3]. Primary cultured rat hepatocytes treated with IL-1 β are used as an *ex vivo* system to mimic liver injury. Because the suppression of NO production is correlated with an anti-inflammatory effect, NO is a sensitive marker to estimate the anti-inflammatory activity of constituents in crude drugs and functional foods [4].

It was reported that an ethyl acetate extract from the root of *A. dahurica* inhibited the production of NO and prostaglandin E₂ (PGE₂) in two macrophage lines (human THP-1 and mouse RAW264.7) in the presence of LPS [5]. However, there are few reports comparing the anti-inflammatory activities of the constituents of *A. dahurica* roots.

Here, the anti-inflammatory constituents of *A. dahurica* roots were examined, and their effect on NO production in rat hepatocytes was evaluated in the

presence of IL-1 β . The constituents were isolated from *A. dahurica* roots, and we investigated their potency to suppress NO production and the expression of inflammatory genes to clarify the anti-inflammatory effects of *A. dahurica* roots.

MATERIALS AND METHODS

General experimental procedures: Nuclear magnetic resonance (NMR) spectra were recorded on a JNM-ECS400 NMR spectrometer (JEOL Ltd., Akishima, Tokyo, Japan) operated at 400 MHz (¹H) and 100 MHz (¹³C) with tetramethylsilane (TMS) as an internal standard. TMS and deuterated chloroform (CDCl₃) were purchased from Euriso-Top (Saint-Aubin, France). High-performance liquid chromatography (HPLC) analyses were performed using a Prominence HPLC system with an LC 20AT SPD A UV/VIS detector for preparative HPLC (Shimadzu Corporation, Kyoto, Japan). Semipreparative HPLC was performed using a Shimadzu Prominence HPLC system with an SPD-20A Prominence UV/Vis detector (Shimadzu Corporation) and a Cosmosil Cholesterol Packed Column [10 mm internal diameter (i.d.) \times 250 mm, 5 μ m; Nacalai Tesque, Inc., Kyoto, Japan] at a detection wavelength of 254 nm. Column chromatography was run on silica gel 60 (Nacalai Tesque Inc., Kyoto, Japan). Precoated thin-layer chromatography

(TLC) was performed on silica gel 60 F₂₅₄ plates (FUJIFILM Wako Pure Chemical Corporation).

Plant material: The roots of *A. dahurica* Bentham et Hooker filius ex Franchet et Savatier (Apiaceae) collected in South Korea were obtained from Tochimoto Tenkaido Co., Ltd. (Osaka, Japan) and authenticated by Dr. Yutaka Yamamoto (Tochimoto Tenkaido Co., Ltd.). A voucher sample was deposited in the Ritsumeikan Herbarium of Pharmacognosy, Ritsumeikan University, under code number RIN-AD-31.

Extraction of the crude drug and fractionation: Dried *A. dahurica* roots (819.3 g) were crushed, pulverized, and extracted with methanol (MeOH) under reflux. The resultant extract was evaporated *in vacuo* to obtain 282.2 g (34.4% yield from the dried roots). Briefly, the extract was fractionated based on hydrophobicity, according to a previously established method [6]. The resultant extract was suspended in water and successively extracted with ethyl acetate (EtOAc) and *n*-butanol (Figure 2). Insoluble precipitates were removed from the final aqueous layer. Each layer was collected and concentrated by solvent evaporation to prepare an EtOAc-soluble fraction (fraction A), an *n*-butanol-soluble fraction (fraction B), and a water-soluble fraction (fraction C).

Isolation of the constituents from the MeOH extract: Fraction A (27.6 g), which showed high activity to suppress NO production in hepatocytes, was further purified by silica gel 60 (70–230 mesh; Nacalai Tesque, Inc.) column chromatography [4.0 cm i.d. × 22.5 cm] by elution with *n*-hexane:EtOAc (100:0 → 0:100) to yield 12 subfractions (A1 to A12).

Subfractions A2, A5, A7, and A9 were further purified. Subfraction A2 was recrystallized from MeOH to give white needles (152 mg), designated as compound **1**. Subfraction A7 was recrystallized from MeOH to give white dendrite crystals (72 mg), designated as compound **2**. Subfraction A5 was subjected to preparative HPLC using a

Cholesther Packed Column (10 mm i.d. × 250 mm, 5 μm; Nacalai Tesque Inc.; mobile phase, acetonitrile:water (60:40); flow rate, 5.0 mL/min), which resulted in four subfractions, two of which gave compound **3** as white needle crystals [retention time (t_R) = 4.9 min; 20 mg] and compound **4** as yellow dendrite crystals (t_R = 6.1 min; 15 mg). Subfraction A9 was subjected to silica gel 60 column chromatography by elution with *n*-hexane:EtOAc:chloroform (9:9:2) and then with *n*-hexane:EtOAc:chloroform (3:6:1). Next, preparative TLC was performed with *n*-hexane:EtOAc:chloroform (1:8:1) to obtain compound **5** as yellow dendrite crystals (20 mg). The ¹H-NMR and ¹³C-NMR spectra were analyzed to identify the compounds by determining their chemical structures.

HPLC analysis of the constituents: The extract and isolated compounds were dissolved in MeOH at a concentration of 1.0 mg/ml. The solutions were filtered through a 0.45 μm Millipore filter unit (Advantec, Tokyo, Japan), and the filtrates (10 μL) were injected onto the Cosmosil 5C₁₈ MS-II HPLC column (4.6 mm i.d. × 150 mm, 5 μm; Nacalai Tesque, Inc.). The UV detection wavelength was 270 nm. The mobile phase was a binary eluent of (A) water and (B) acetonitrile under the following gradient conditions: 0–10 min, linear gradient from 30% to 45% B; 10–25 min, linear gradient from 45% to 60% B; 25–35 min, linear gradient from 60% to 65% B; 35–45 min, linear gradient from 65% to 100% B; 45–60 min, isocratic at 100% B. The flow rate was 1.0 mL/min, and the column temperature was 40 °C.

Each compound confirmed by NMR spectral analysis was accurately weighed and dissolved in MeOH to make 1.00 mg/5 mL stock solutions, and 2.5, 5.0 and 10.0 μL of each standard solution were analyzed in triplicate. The calibration curve for each standard compound was constructed by plotting the peak areas (y) against the amount injected (x), and the regression equation was calculated in the form $y = Ax + B$: compound **1** (isoimperatorin), $y = 31067674.29x + 13333.50$ ($R^2 = 1.0000$); **2** (oxypeucedanin), $y = 24903707.48x - 533.62$ ($R^2 =$

= 0.9999); **3** (imperatorin), $y = 23508476.19x + 8,781.67$ ($R^2 = 1.0000$); **4** (phellopterin), $y = 41433464.76x + 8451.17$ ($R^2 = 1.0000$); and **5** (oxypeucedanin methanolate), $y = 15407659.86x - 521.59$ ($R^2 = 0.9999$). Fraction A was accurately weighed and dissolved in MeOH to prepare a 1.0 mg/mL solution and 10.0 μ L was analyzed in triplicate. The peak areas of each compound in the sample solution were compared to the calibration curves, and the amount of each compound in 10 μ L of sample solution was calculated. The amount of compound **1** in 10.0 μ L of the sample solution (10.0 μ g of fraction A) was calculated to be 0.332 μ g, and its content in fraction A was 3.32%. The amounts and contents of the other compounds were similarly calculated.

Preparation of primary cultured rat hepatocytes: Specific pathogen-free male Wistar rats were obtained from Charles River Laboratories Japan Inc. (Yokohama, Japan), housed at 21–23 °C on a 12-h light-dark cycle, and fed a CRF-1 diet (Charles River Laboratories Japan) with water available *ad libitum*. The animals were acclimated for at least a week. Hepatocytes were isolated from the livers of the Wistar rats by collagenase perfusion [7]. The isolated cells resuspended in Williams' E medium were centrifuged and seeded at 1.2×10^6 cells per 35-mm diameter dish. The hepatocytes were incubated at 37 °C for 2 h. After the medium was changed, the hepatocytes were further incubated at 37 °C overnight for assays the following day. All animal care and experimental procedures were performed in accordance with the laws and guidelines of the Japanese government and were approved by the Animal Care Committee of Ritsumeikan University, Biwako-Kusatsu Campus (No. BKC2020-045).

Measurement of NO levels and lactate dehydrogenase (LDH) activity: Each fraction or constituent was added to the hepatocyte medium with 1 nM rat IL-1 β , and the dishes were incubated at 37 °C for 8 h. The nitrite level (a stable metabolite of NO) in the medium was measured using the Griess method [8–9]. LDH activity in the medium

was estimated using an LDH Cytotoxicity Detection kit (Takara Bio Inc., Otsu, Shiga, Japan) to monitor cytotoxicity. The half-maximal inhibitory concentration (IC₅₀) value of each fraction or constituent was calculated to determine its ability to suppress NO production unless the fraction or constituent was cytotoxic [9].

Direct NO quenching activity: Each fraction or constituent (dissolved in dimethyl sulfoxide) was added to medium containing 25 μ M NaNO₂ and incubated at 37 °C for 1.5 h as previously described [10]. The final concentration of each constituent was 50 μ M (phellopterin) and 120 μ M (oxypeucedanin methanolate). The medium was then mixed with Griess reagent [9] and incubated at 23 °C for 5 min. The absorbance at 540 nm was measured to determine the reduction of nitrite.

Western blot analysis: Hepatocytes were treated with IL-1 β and each fraction or constituent at 37 °C, and whole-cell lysates were prepared [11–12]. The lysates were resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and blotted onto Sequi-Blot membranes (Bio-Rad, Hercules, CA, USA). Immunostaining was performed using primary antibodies that were raised against rat iNOS (Thermo Fisher Scientific, Waltham, MA, USA) and rat β -tubulin (Cell Signaling Technology Inc., Danvers, MA, USA) and then a horseradish peroxidase-conjugated anti-immunoglobulin Fc antibody (Cell Signaling Technology Inc.). Protein was visualized using enhanced chemiluminescence blotting detection reagents (GE Healthcare Biosciences Corp., Piscataway, NJ, USA) and detected using an Amersham Imager 600 (GE Healthcare). To measure intensity of the bands, densitometry was performed using ImageJ software (<http://imagej.nih.gov/ij/>), and the band intensity of the iNOS was normalized by that of β -tubulin (internal control). The intensity of the iNOS band in the presence of IL-1 β alone was set at 1.

Quantitative reverse transcription-polymerase chain reaction (RT-PCR): Hepatocytes were treated with IL-1 β and/or each constituent at 37 °C for 4 h, and total RNA was prepared [13]. The cDNA was reverse-transcribed with an oligo(dT) primer, and then quantitative PCR was performed with the primers for iNOS, elongation factor 1 α (EF) [13], and tumor necrosis factor α (TNF- α) mRNAs [14]. Relative mRNA levels were estimated in triplicate by quantitative PCR with a Thermal Cycler Dice Real Time system (Takara Bio Inc.), and the obtained values were normalized to EF mRNA, which was used as the internal control [13–14].

Statistical analysis: The results in the figures and tables are representative of at least three independent experiments that yielded similar results. The values are presented as the means \pm standard deviation (SD). Differences were analyzed using Student’s *t*-test followed by Bonferroni’s correction. Statistical significance was set at $P < 0.05$ and $P < 0.01$.

RESULTS

Inhibition of NO production by the crude fractions from the A. root extract: The roots of *A. dahurica* were extracted with MeOH and successively fractionated based on hydrophobicity with EtOAc (fraction A), *n*-butanol (fraction B), and water (fraction C), as shown in Figure 2. Then, the effects of the *A. dahurica* root extract and its crude fractions on NO production in hepatocytes in the presence of IL-1 β were examined.

Among the crude fractions, the addition of fraction A to the medium significantly reduced IL-1 β -induced NO production in a dose-dependent manner. In contrast, fraction B was less effective, and fraction C did not cause a significant change in NO production (Figure 3A). Medium including each fraction showed much lower LDH activity than that of the whole cell extract (data not shown), suggesting that all fractions were not cytotoxic at the concentrations tested. Furthermore, Western blot analysis indicated that fraction A, as well as fraction B, suppressed the iNOS protein expression that was induced by IL-1 β in hepatocytes (Figure 3B).

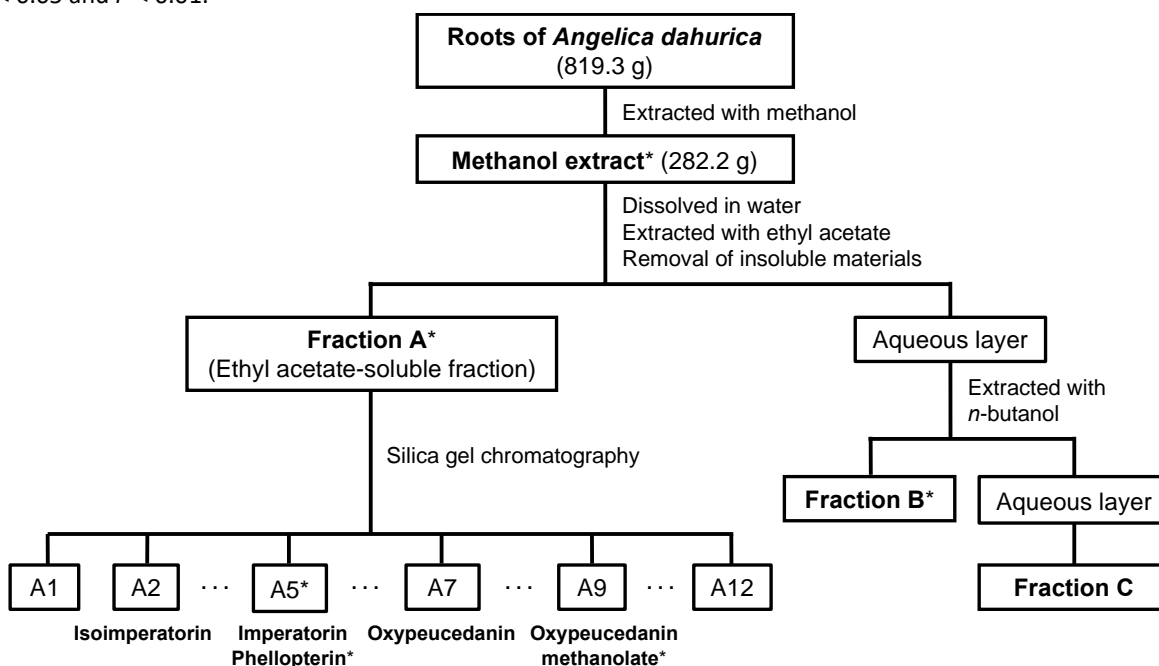


Figure 2. Isolation of the constituents from the root of *A. dahurica*. A flowchart of the procedure to isolate the constituents is shown schematically. The dried plant material was extracted and partitioned into three fractions based on hydrophobicity. The recovery of the extract from the dried roots was 34.4%. Constituents isolated from fraction A are depicted under the relevant subfraction. * Extract, fraction, or constituent that suppressed NO induction in hepatocytes.

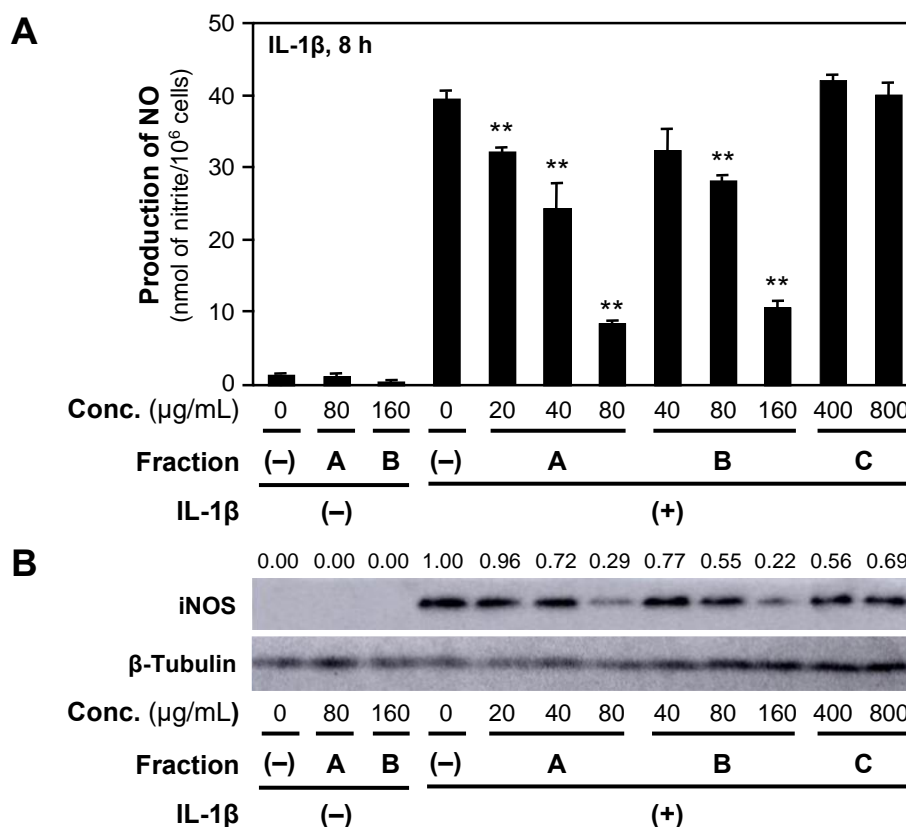


Figure 3. Fraction A of the *A. dahurica* root extract suppressed NO and iNOS induction. **(A)** The effects of crude fractions A to C from an *A. dahurica* root extract on NO production. Hepatocytes were treated with 1 nM IL-1 β in the presence or absence of each fraction for 8 h. NO levels were measured by determining the amount of nitrite in the medium. The results represent the mean \pm SD ($n = 3$). When compared with the total LDH activity of the whole-cell extract (100%), the LDH activity in each medium containing a fraction was very low (data not shown). * $P < 0.05$ and ** $P < 0.01$ versus IL-1 β alone. **(B)** The effects of the fractions on iNOS expression. Hepatocytes were treated similarly to (A). The cell extracts (20 μ g per lane) were subjected to Western blot analysis to detect iNOS (130 kDa) and β -tubulin (55 kDa; internal control). After densitometry, the band intensity of iNOS was normalized by that of β -tubulin and indicated by each lane.

Then, the IC₅₀ values of the *A. dahurica* root extract and fractions A and B were calculated and are shown in Table 1. Fractions A and B showed lower IC₅₀ values for NO production than the extract. Because fraction A effectively suppressed NO production, it was expected that this fraction (the EtOAc-soluble fraction) may contain hydrophobic constituents that suppress IL-1 β -induced NO production.

Isolation of the constituents of fraction A from the *A. dahurica* root extract: When fraction A from the *A. dahurica* root extract was analyzed by HPLC, many peaks were observed (Figure 4A). Therefore, fraction A was

fractionated into 12 subfractions by silica gel chromatography, which were further purified by silica gel chromatography and other methods, as described in the *Materials and Methods*. To determine the chemical structures of the purified constituents, we analyzed their ¹H- and ¹³C-NMR spectra (Supplementary Data) and compared them with the previously reported spectra of the relevant compounds [15–17]. Finally, we isolated five furanocoumarins and identified them as isoimperatorin (compound 1), imperatorin (3), phellopterin (4), oxypeucedanin (2), and oxypeucedanin methanolate (5) (Figure 4B).

Table 1. Fractionation of an *A. dahurica* root extract and their effects on NO production.

Fraction	Amount [g]	Yield [%] ^a	IC ₅₀ [μg/mL] ^b
Methanol extract	282.2	—	254 ± 38.4
A (ethyl acetate-soluble) fraction	27.6	10.7	25.7 ± 4.0
B (<i>n</i> -butanol-soluble) fraction	21.1	8.2	60.6 ± 17.1
C (water-soluble) fraction	208.3	81.0	NA

^aThe percentage is calculated as the weight of each fraction divided by the sum of the three fractions (100%). ^bThe half-maximal inhibitory concentration of NO production in IL-1β-treated hepatocytes (mean ± SD). At least three experiments were performed to determine these values. NA, not applied due to low activity.

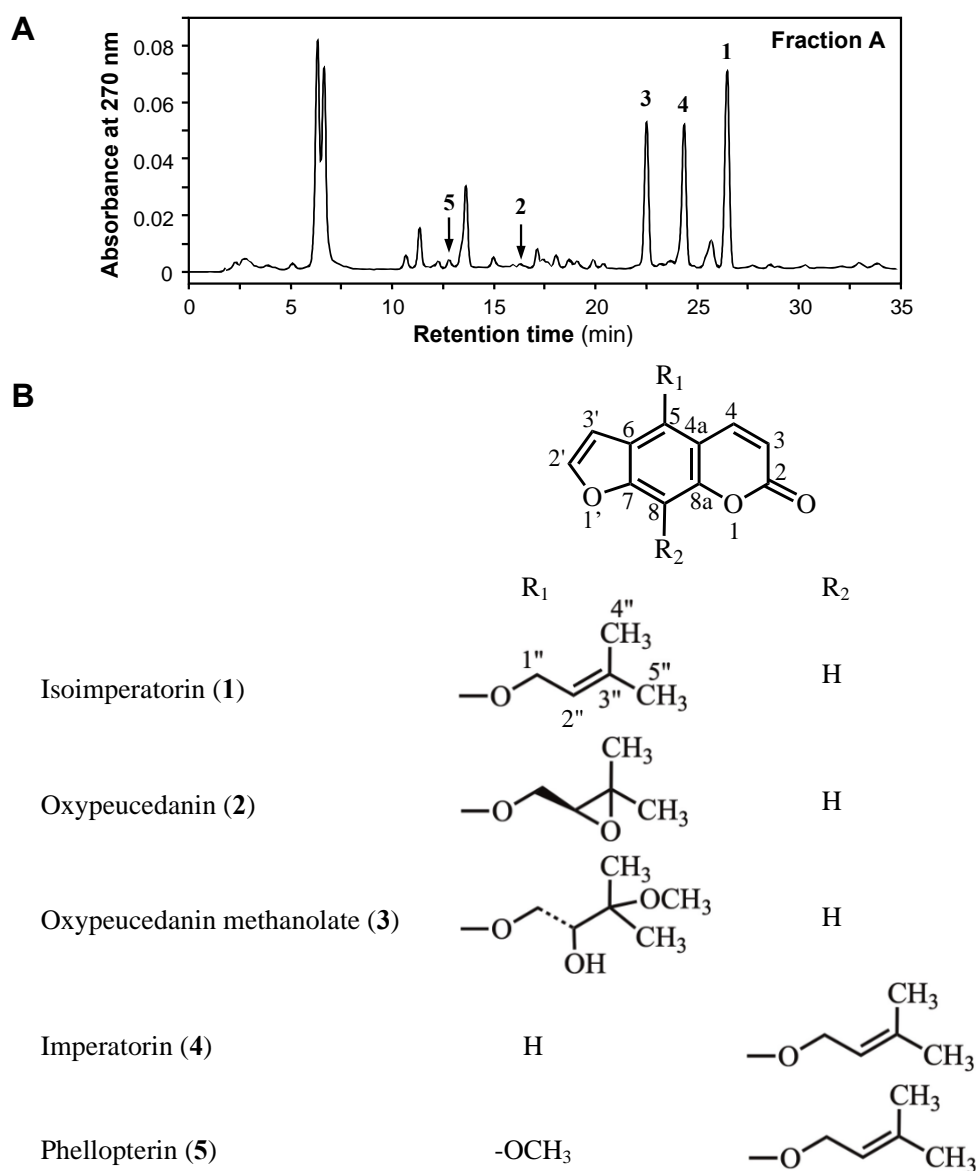


Figure 4. The furanocoumarins in fraction A from an *A. dahurica* root extract. (A) HPLC chromatogram of fraction A from the *A. dahurica* root extract. HPLC was performed using a Cosmosil 5C18 MS-II column. The UV detection wavelength was 270 nm. The mobile phase was a binary eluent of water and acetonitrile, as described in the *Materials and Methods*. Each peak indicated by a number corresponds to the relevant compound number in (B). The retention times (t_R) of the peaks were $t_R = 26.5$ min (compound 1), $t_R = 16.3$ min (2), $t_R = 22.5$ min (3), $t_R = 24.4$ min (4), and $t_R = 12.8$ min (5). (B) Chemical structures of the furanocoumarins in the *A. dahurica* root.

Table 2. Constituents isolated from the *A. dahurica* root and their effects on NO production.

Constituent (Compound)	Content [% of fraction A] ^a	Content [% of extract] ^b	IC ₅₀ [μM] ^c
Isoimperatorin (1)	3.32	0.325	NA
Imperatorin (3)	3.23	0.316	NA
Phellopterin (4)	2.00	0.196	50.6 ± 15.9
Oxypeucedanin methanolate (5)	0.302	0.030	122 ± 21.4
Oxypeucedanin (2)	0.0988	0.010	NA

^aThe content of each constituent was measured by HPLC and is presented as a percentage of the dry weight of fraction A. ^bThe content of each constituent is shown as a percentage of the dry weight of the MeOH extract when 9.8% of fraction A was recovered from the MeOH extract. ^cThe half-maximal inhibitory concentration of NO production in IL-1β-treated hepatocytes (mean ± SD). At least three experiments were performed to determine these values. NA, not applied due to low activity.

Next, HPLC analysis was performed to estimate the content of each constituent in fraction A. The contents of isoimperatorin (3.32%) and imperatorin (3.23%) were higher in fraction A than the other constituents (Table 2).

Inhibition of NO production by the constituents of fraction A: We examined whether the furanocoumarins in fraction A from an *A. dahurica* root extract could suppress NO induction in IL-1β-treated hepatocytes. When isoimperatorin (1), imperatorin (3), and oxypeucedanin (2) were examined, these compounds did not affect NO production at final concentrations up to 340 μM (data not shown). As shown in Figure 5A, phellopterin (4) and oxypeucedanin methanolate (5) significantly suppressed NO production in the presence of IL-1β, whereas they did not induce NO production in the absence of IL-1β. Neither furanocoumarin showed an increase in LDH activity in the medium (data not shown), which suggests that they are nontoxic. When the IC₅₀ values were calculated, the value for phellopterin was the lowest, suggesting that phellopterin exhibited the highest potency to suppress NO among the five furanocoumarins tested (Table 2).

When the direct NO-quenching activities of phellopterin (4) and oxypeucedanin methanolate (5) were measured, no significant changes in NO levels were observed when compared with the NO levels in the medium containing NaNO₂ alone (data not shown). These

data imply that phellopterin and oxypeucedanin methanolate did not directly quench NO.

Inhibition of iNOS gene expression by phellopterin and oxypeucedanin methanolate: The effects of phellopterin (4) and oxypeucedanin methanolate (5) on *iNOS* gene expression were further investigated. When each furanocoumarin was added to the hepatocyte medium, the increase in *iNOS* protein expression that was induced by IL-1β was suppressed (Figure 5B).

Next, quantitative RT-PCR analysis of *iNOS* mRNA was performed using total RNA from hepatocytes, as described in the *Materials and Methods*. As shown in Figure 6A, the expression of *iNOS* mRNA increased in the presence of IL-1β alone (a positive control), whereas the *iNOS* mRNA level in the absence of IL-1β was much lower than that of the positive control (data not shown). When phellopterin or oxypeucedanin methanolate was added to the medium with IL-1β, *iNOS* mRNA levels decreased in a dose-dependent manner. No amplification was observed when cells were individually treated with phellopterin or oxypeucedanin methanolate (data not shown). These results suggest that phellopterin and oxypeucedanin methanolate suppressed *iNOS* gene expression by reducing *iNOS* mRNA levels.

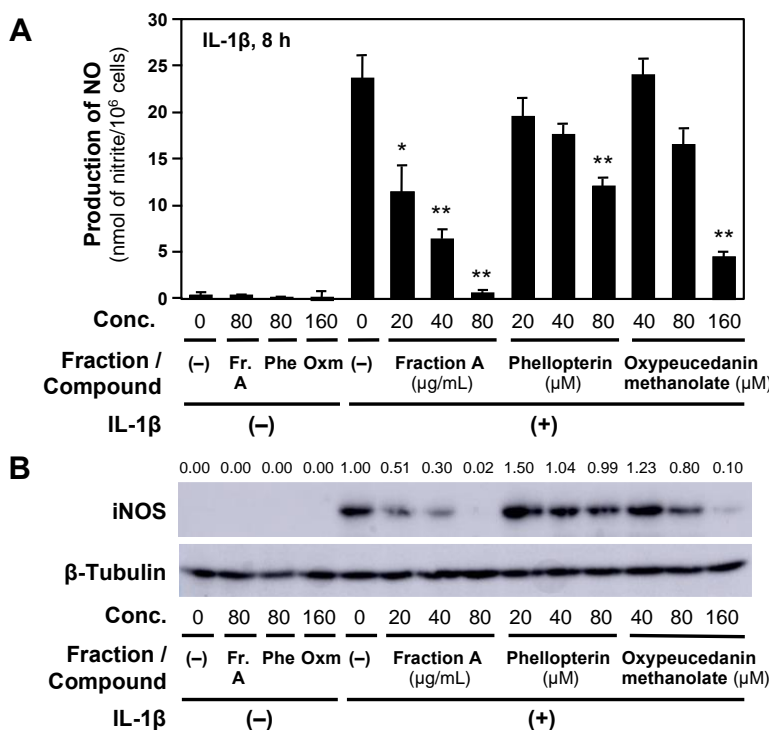


Figure 5. Phellopterin and oxypeucedanin methanolate suppress IL-1β-induced *iNOS* gene expression. (A) The effects of phellopterin and oxypeucedanin methanolate on NO production. Hepatocytes were treated with 1 nM IL-1β in the absence or presence of fraction A from the *A. dahurica* root extract, phellopterin (Phe), or oxypeucedanin methanolate (Oxm) for 8 h. Then, the NO levels in the medium were measured. The data represent the mean ± SD ($n = 3$). * $P < 0.05$ and ** $P < 0.01$ versus IL-1β alone. (B) The effects of phellopterin and oxypeucedanin methanolate on iNOS protein levels. The hepatocytes were treated similarly to (A), and the cell extracts were subjected to Western blot analysis to detect the expression of iNOS and β-tubulin. After densitometry, the band intensity of iNOS was normalized by that of β-tubulin and indicated by each lane.

Furthermore, it was expected that phellopterin and oxypeucedanin methanolate might suppress other genes that are involved in inflammation. Therefore, we measured the expression levels of TNF-α mRNA in IL-1β-treated hepatocytes. Quantitative RT-PCR analysis revealed that IL-1β induced the TNF-α mRNA levels (Figure 6B). When

phellopterin or oxypeucedanin methanolate was added to the medium, both furanocoumarins decreased the expression of the TNF-α mRNA. No amplification was observed when cells were individually treated with phellopterin or oxypeucedanin methanolate (data not shown).

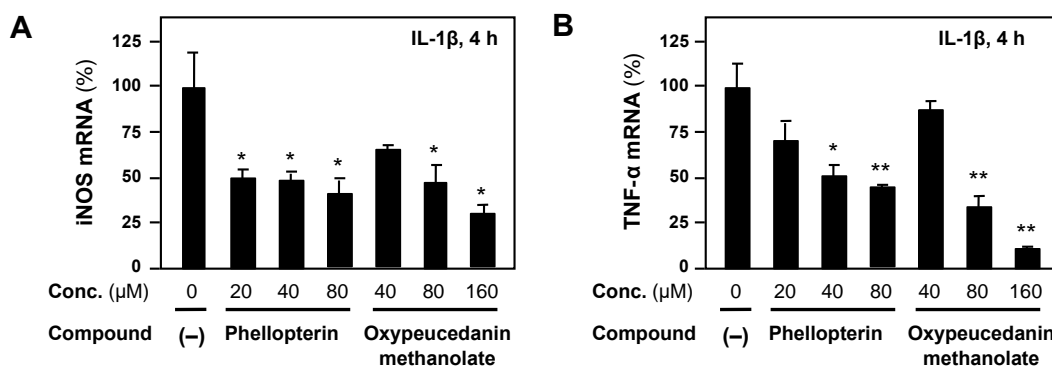


Figure 6. Phellopterin and oxypeucedanin methanolate inhibit the expression of proinflammatory genes. Total RNA was prepared following incubation with 1 nM IL-1β in the presence of fraction A, phellopterin, or oxypeucedanin methanolate for 4 h. Quantitative RT-PCR was carried out to detect *iNOS* mRNA (A) and TNF-α mRNA (B) using EF mRNA as an internal control. The expression level of each mRNA was normalized to that of EF mRNA and is expressed as a percentage (mean ± SD, $n = 3$). * $P < 0.05$ and ** $P < 0.01$ versus IL-1β alone.

DISCUSSION

In the current study, five furanocoumarins were purified and identified from the hydrophobic fraction (A) of an *A. dahurica* root extract. HPLC analyses provided the content of each constituent in the extract. The recovery rates were 34.4% of the MeOH extract from the dried roots (Figure 2) and 9.8% of fraction A from the extract. Therefore, the content of imperatorin was calculated to be 0.109% in dried roots (Table 2). Previously, Pfeifer *et al.* reported that the dried roots of *A. dahurica* contained 0.09–0.28% imperatorin [18], which is comparable to our results. Similarly, the content of isoimperatorin (0.112% in the dried roots) is also comparable to the result from this report (0.04–0.10%) [18].

Using primary cultured rat hepatocytes, we estimated the potencies of the constituents to suppress IL-1 β -induced NO production to evaluate their anti-inflammatory effects. Comparative analysis of the five constituents was performed using hepatocytes. The IC₅₀ value of phellopterin (50.6 μ M) was comparable to values previously obtained (39.0 μ M (luteolin from the leaves of *Perilla frutescens* Britton var. *crispa* form *viridis*) [19] and 51.0 μ M (nobiletin from the peel of *Citrus unshiu* [20])), suggesting that phellopterin may possess comparable potency to those compounds. The IC₅₀ value of oxypeucedanin methanolate (122 μ M) was also comparable to the earlier determined values of 109 μ M (γ -schizandrin from the fruit of *Schisandra chinensis*) [11] and 129 μ M [(–)-epicatechin] [21].

Phellopterin and oxypeucedanin methanolate efficiently suppressed NO production in hepatocytes, whereas isoimperatorin, imperatorin, and oxypeucedanin were less potent. Xanthotoxine and bergapten are present in the roots of *Angelica acutiloba* Kitagawa, the roots and rhizomes of *Saposhnikovia divaricata* (Turcz.) Schischk, and the roots and rhizomes of *Glehnia littoralis* Fr. Schmidt ex Miq. [22]. These two furanocoumarins, however, did not affect NO production in hepatocytes [22]. When their chemical structures were compared (Figure 4B), a methoxy group at carbon 5 and a side chain at carbon 8, which are

present in phellopterin, seem to be essential to suppress NO production. Although the content of phellopterin and oxypeucedanin methanolate was low, *A. dahurica* roots contain many other furanocoumarins, such as byakangelicin and byakangelicol, whose activity is not evaluated. It is likely that many furanocoumarins are involved in the anti-inflammatory activity of *A. dahurica* roots. More studies using the furanocoumarins are necessary to elucidate the structure–activity relationship of furanocoumarins in the future.

Macrophages produce various proinflammatory mediators, such as NO and PGE₂. In LPS-treated rat peritoneal macrophages, imperatorin had the most potent inhibitory potency against PGE₂ production among isoimperatorin, imperatorin, phellopterin, oxypeucedanin methanolate, and byakangelicin [23]. In IL-1 β -treated hepatocytes, phellopterin suppressed NO production, whereas imperatorin did not (Table 2). This result may be due to differences in cell types and signal transduction pathways. Indeed, the potency to suppress NO production by anti-inflammatory drugs is not the same as that of PGE₂ production in LPS-treated RAW264.7 cells [9].

As a pharmacokinetic study, we did not perform an experiment to orally administer phellopterin or oxypeucedanin methanolate to animals. In general, when a drug is orally administered, the drug concentration in blood rapidly becomes high and then gradually decreases. Because *A. dahurica* roots contain many potent furanocoumarins, a total concentration of these furanocoumarins in blood might be high enough to exhibit an activity to suppress NO production when an *A. dahurica* root extract is orally administered to animals.

The present study suggests that phellopterin and oxypeucedanin methanolate reduced both the protein and mRNA levels of the *iNOS* gene in IL-1 β -treated hepatocytes, resulting in suppression of *iNOS* gene expression (Figures 5, 6A). These two furanocoumarins downregulated the mRNA expression of TNF- α (Figure 6B). Our previous reports suggest that transcription factors, *i.e.*, nuclear factor κ B (NF- κ B) and CCAAT/enhancer-binding protein β

(C/EBP β), in the IL-1 signaling pathway are involved in the molecular mechanisms of the pharmacological activity of crude drugs in Kampo medicine [11,24]. Indeed, phellopterin and oxypeucedanin methanolate decreased the levels of type 1 IL-1 receptor mRNA in rat hepatocytes (data not shown). More studies are required to clarify the involvement of these transcription factors in this pathway.

There is a possibility that fraction B, *i.e.*, *n*-butanol-soluble fraction, also contains anti-inflammatory constituents. We selected fraction A, because its potency to suppress NO production was 2.36-fold higher than that of fraction B (Table 1). Fraction B contains amphipathic constituents, such as alkaloids and glycosides. Indeed, fraction B of an *A. dahurica* root extract contained alkaloids (*e.g.*, dahurine A), but not glycosides [25–26]. Such alkaloids may possess anti-inflammatory activity.

It is expected that several furanocoumarins from *A. dahurica* roots may exhibit various pharmacological activities other than anti-inflammatory effects. Han *et al.* reported that phellopterin, but not imperatorin or bergapten, promoted the differentiation of 3T3-L1 cells (a mouse fetal fibroblast cell line) to adipocyte-like cells [27]. Furthermore, phellopterin decreased blood glucose levels in type 2 diabetes mellitus model mice that were induced by high-fat diets and streptozotocin [27]. Recently, Tsai *et al.* reported that imperatorin inhibited cAMP-specific phosphodiesterase (PDE) 4 and alleviated psoriasisiform dermatitis [28]. It is unknown whether phellopterin or oxypeucedanin methanolate has PDE activity. If they have PDE activity, they may contribute anti-inflammatory effects. Detailed studies of the furanocoumarins in *A. dahurica* roots and their pharmacological effects, including antidiabetic activity, remain to be investigated in the future.

CONCLUSION

The results of the current study on hydrophobic constituents suggest that two furanocoumarins, phellopterin and oxypeucedanin methanolate, may be responsible for the anti-inflammatory activities of *A.*

dahurica roots by suppressing proinflammatory gene expression.

List of Abbreviations: NO: nitric oxide, IL: interleukin, IC₅₀: half-maximal inhibitory concentration, SD: standard deviation, i.d.: internal diameter, NMR: nuclear magnetic resonance, *t_R*: retention time.

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Competing Interests: HA performed this study as an undergraduate student of the College of Life Sciences, Ritsumeikan University. RO, TI, and TS performed the experiments as graduate students of the Graduate School of Life Sciences, Ritsumeikan University. YN performed the experiments as a graduate student of the Graduate School of Pharmaceutical Sciences, Ritsumeikan University. All authors have no financial interests or conflicts of interest.

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