Regular Article

Comparative Analysis of Anti-inflammatory Activity of the Constituents of the Rhizome of *Cnidium officinale* Using Rat Hepatocytes

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The rhizome of *Cnidium officinale* (Umbelliferae) (known as *Senkyu* in Japan; COR) has been used as a crude drug in Japanese Kampo formulas, such as *Jumihaidokuto* (to treat eczema and urticaria) and *Kakkontokasenkyushin'i* (to treat rhinitis). COR contains phthalides, which are thought to be potent principal constituents. Few studies have been reported about the comparison of anti-inflammatory activity of COR constituents. We aimed to identify the constituents in COR and compare their anti-inflammatory activity. COR was extracted with methanol and fractionated into ethyl acetate (EtOAc)-soluble, *n*-butanol-soluble, and water-soluble fractions. Primary cultured rat hepatocytes were used to assess anti-inflammatory activity by monitoring the interleukin (IL)-1*β*-induced production of nitric oxide (NO), an inflammatory mediator. The EtOAc-soluble fraction significantly suppressed NO production without showing cytotoxicity in IL-1*β*-treated hepatocytes, whereas the *n*-butanol-soluble fraction showed less potency, and the water-soluble fraction did not significantly affect the NO levels. Four constituents were isolated from the EtOAc-soluble fraction and identified as senkyunolide A, (3*S*)-butylphthalide, neocnidilide, and cnidilide. Among these phthalides and (*Z*)-ligustilide, senkyunolide A and (*Z*)-ligustilide efficiently suppressed NO production in hepatocytes, whereas the others showed less potency in the suppression of NO production. Furthermore, senkyunolide A decreased the levels of the inducible nitric oxide synthase (iNOS) protein and mRNA, as well as the levels of mRNAs encoding proinflammatory cytokines (*e.g.*, tumor necrosis factor *α*) and chemokine C–C motif ligand 20. These results suggest that senkyunolide A may cause the anti-inflammatory and hepatoprotective effects of COR by suppressing the genes involved in inflammation.

Key words *Cnidium officinale* rhizome; phthalide; senkyunolide A; inflammation; Kampo medicine

INTRODUCTION

Cnidium officinale Makino (Umbelliferae) grows in East Asia, including Japan and China. Cnidium Rhizome (known as *Senkyu* in Japan) is defined by the *Japanese Pharmacopoeia* as the rhizome of *Cnidium officinale*, which is usually passed through hot water.[1)] The crude drug Senkyu has been used to treat pain, heart disease, menstrual disturbances, and fungal infection and has thus been included in several Kampo formulas, such as *Jumihaidokuto* to treat eczema, acne, folliculitis, and urticaria and *Kakkontokasenkyushin'i* to treat sinusitis and allergic rhinitis associated with viscous nasal discharge.[2)]

Many phthalides are isolated from the plants in the family Umbelliferae (=Apiaceae).[3)] In the essential oils of the rhizome of *Cnidium officinale*, 67 volatile compounds were detected, which included five phthalides as major constituents (64.8% of the total weight).[4)] The phthalides included in the rhizome of *Cnidium officinale* were classified into groups by chemical structures: phthalides, such as (3*S*)-butylphthalide, butylidenephthalide, and senkyunolide B, C, and E; dihydrophthalides, such as (*Z*)-ligustilide and senkyunolide A, D, F, and G; and tetrahydrophthalides, such as cnidilide, neocnidilide, ligustilidiol, and senkyunolide H–J.[3)] 3-Butylidenephthalide and (*Z*)-ligustilide are the two major constituents from the Apiaceae plants.[3)]

Phthalides exhibited diverse pharmacological activities, such as vasodilation and anti-inflammatory and antidepressant effects.[3,5)] Senkyunolide A and (*Z*)-ligustilide, which were also included in the rhizome of *Ligusticum striatum* (=*L. chuanxiong*), inhibited the production of proinflammatory mediators, such as nitric oxide (NO) and tumor necrosis factor *α* (TNF-*α*), in lipopolysaccharide (LPS)-treated BV2 cells, a murine microglial cell line.[6)] Senkyunolide H and ligustilidiol, which were isolated from *Cnidium officinale*, inhibited the production of proinflammatory mediators (*e.g.*, prostaglandin E_2 and NO) in LPS-treated RAW264.7 cells, a mouse macrophage line.[7)] Cnidilide also suppressed the production of proinflammatory mediators in RAW264.7 cells.[8)] However, reports comparing the anti-inflammatory activity of constituents in the rhizome of *Cnidium officinale* are very few.

Inflammatory responses are provoked by foreign pathogens derived from bacteria and viruses. For example, LPS is released from the outer membrane of Gram-negative bacteria and becomes active. NO is synthesized by inducible nitric oxide synthase (iNOS) and plays a crucial role under physiological and pathophysiological conditions.[9)] In the liver, LPS stimulates Kupffer cells (resident liver macrophages) to secrete

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interleukin (IL)-1β, which then stimulates hepatocytes to induce the expression of the genes encoding iNOS, proinflammatory cytokines, and chemokines.[10] Primary cultured rat hepatocytes treated with IL-1β are used as an *ex vivo* system to mimic liver injury, and the suppression of NO production is correlated with the anti-inflammatory effect of a constituent. NO is a sensitive marker used to estimate the anti-inflammatory activity of the constituents of crude drugs, such Saposhnikoviae Radix (root and rhizome of *Saposhnikovia divaricata*),[11] Phellodendri Cortex (bark of *Phellodendron amurense* or *Phellodendron chinense*),[12] and Pruni Cortex (bark of *Prunus jamasakura* or *Prunus verecunda*).[13]

In this study, we focused on the anti-inflammatory effects of the rhizome of *Cnidium officinale*. Primary cultured rat hepatocytes were used to monitor NO production in the presence of IL-1β. The constituents were isolated from the rhizome of *Cnidium officinale*. Then, we investigated the potencies of these constituents in the suppression of NO production in the hepatocytes and examined the effects on the expression of inflammatory genes.

MATERIALS AND METHODS

General Experimental Procedures NMR spectra were recorded using a JNM-ECS400 NMR spectrometer (JEOL Ltd., Akishima, Tokyo, Japan) operated at 400 MHz (^{1}H) and 100 MHz (^{13}C) with tetramethylsilane as an internal standard. HPLC analyses were performed using an LC-20AD pump equipped with an SPD-20A UV/VIS detector (Shimadzu Corporation, Kyoto, Japan). Electron ionization (EI)-MS spectra were obtained with a JMS-700 MStation mass spectrometer (JEOL Ltd.). GC-MS analysis was carried out using a Shimadzu 2010 GC system (Shimadzu Corporation) equipped with an AOC-20i autosampler and a DB-5MS capillary column (0.25 mm $\times$ 30 m, 0.25 μm film thickness, Agilent Technologies, Santa Clara, CA, U.S.A.). Column chromatography was run on Silica Gel 60 (Nacalai Tesque Inc., Kyoto, Japan) or Wakogel C-300 HG (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan). Precoated TLC was performed on Silica gel 60 F_{254} plates (FUJIFILM Wako Pure Chemical Corporation). The optical rotations were measured on a DIP-1000 polarimeter (JASCO Corporation, Hachioji, Tokyo, Japan).

Plant Materials and Reagents The rhizome of *Cnidium officinale* Makino collected from Hokkaido, Japan was purchased from Tochimoto Tenkaido Co., Ltd. (Osaka, Japan) and authenticated as Senkyu by Dr. Yutaka Yamamoto (Tochimoto Tenkaido Co., Ltd.). The voucher samples were deposited in the Ritsumeikan Herbarium of Pharmacognosy, Ritsumeikan University, under the code number RIN-CO-24.

Isolation of Constituents from the Rhizome of *Cnidium officinale* The rhizome of *Cnidium officinale* (951.6 g) was pulverized, extracted with methanol under reflux, and fractionated, as previously described.[13,14] Briefly, the resultant extract (160.4 g) was suspended in water and successively extracted with ethyl acetate (EtOAc) and *n*-butanol (Fig. 1). These layers were concentrated to prepare an EtOAc-soluble

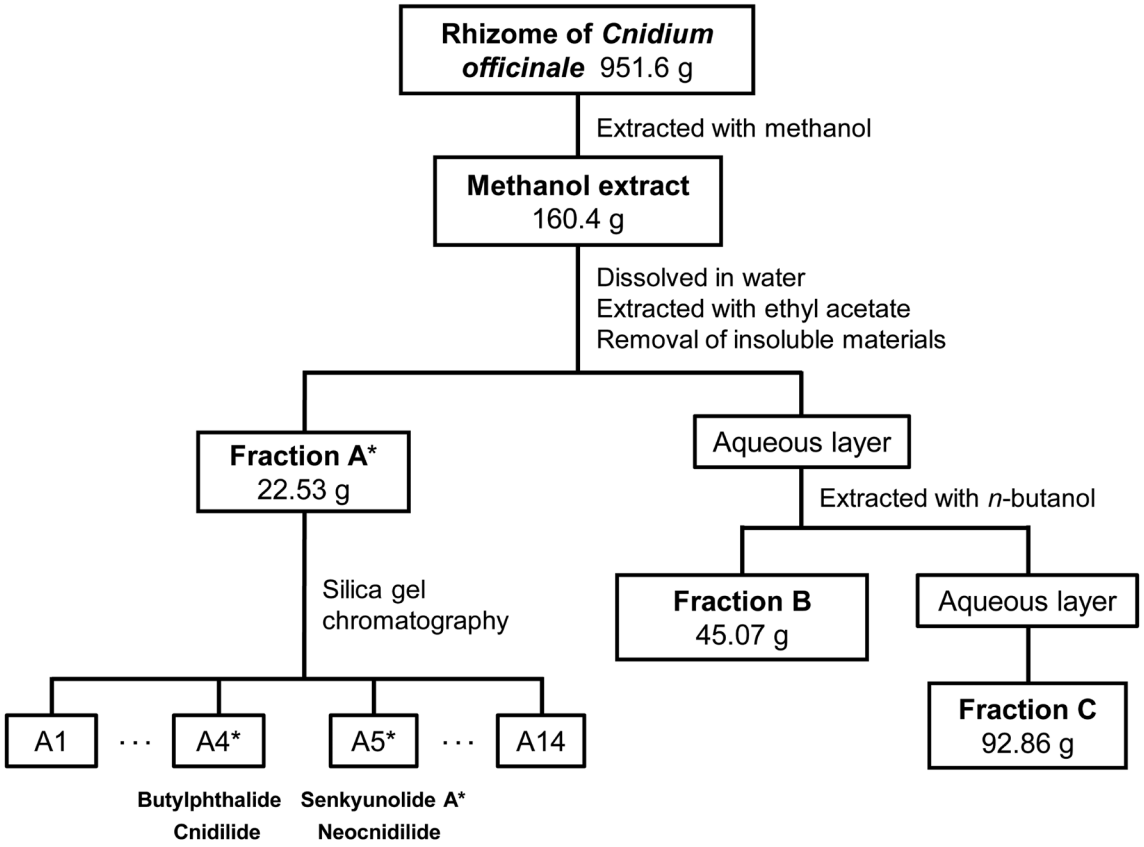

Fig. 1. Purification of Constituents from the Rhizome of *Cnidium officinale*

A flowchart of the procedures used to fractionate constituents from the rhizome of *Cnidium officinale* (COR). The plant material was extracted with methanol, and the dried extract was dissolved in water and sequentially fractionated by hydrophobicity with ethyl acetate (EtOAc), *n*-butanol, and water to obtain fractions A, B, and C, respectively. Constituents are indicated under the relevant subfraction. * A fraction, subfraction, or constituent that significantly inhibited NO induction in this study.

fraction (fraction A, 22.53 g), an *n*-butanol-soluble fraction (fraction B), and a water-soluble fraction (fraction C). Fraction A (10.00 g), which showed NO-suppressing activity, was further purified by silica gel column chromatography (4.0 cm internal diameter (i.d.) × 22.5 cm; Silica Gel 60, 70–230 mesh; Nacalai Tesque, Inc.) by elution with *n*-hexane : ethyl acetate (100 : 0→0 : 100) to yield 14 subfractions (A1 to A14). Subfractions A4 and A5 were further purified.

Subfraction A5 (1.360 g) was fractionated using silica gel column chromatography (Silica Gel 60; 3.0 cm i.d. × 25 cm; *n*-hexane : EtOAc = 70 : 30), resulting in three subfractions, A5.1–A5.3. The subfraction A5.2 was subjected to preparative HPLC (column, Cosmosil 5C18 MS-II (10 mm i.d × 250 mm, Nacalai Tesque Inc.); mobile phase, methanol : water (65 : 35); flow rate = 2.0 mL/min), which resulted in two peaks: compound **1** (8 mg; t_R = 22.4 min) as a colorless oil and compound **3** (2 mg; t_R = 37.1 min) as a white powder.

Compound **1**: $[\alpha]_D^{25}$ −114 (*c* 0.513, $CHCl_3$). EI-MS *m/z* (%): 192 (M^+, 30), 163 (3.0), 135 (5.9), 133 (7.0), 107 (100); high resolution (HR)-EI-MS *m/z* 192.1148 $[M]^+$ (calcd for $C_{12}H_{16}O_2$: 192.1150); ^{1}H-NMR (400 MHz, $CDCl_3$) *δ*: 6.21 (1H, dt, *J* = 9.6, 1.6 Hz, H-7), 5.92 (1H, dt, *J* = 9.6, 4.6 Hz, H-6), 4.93 (1H, dd, *J* = 7.6, 3.6 Hz, H-3), 2.45 (4H, m, H-4 and H-5), 1.88 and 1.54 (each 1H, m, H-8), 1.39 (4H, m, H-9 and H-10), 0.91 (3H, t, *J* = 7.2 Hz, H-11); ^{13}C-NMR (100 MHz, $CDCl_3$) *δ*: 171.3 (C-1), 161.4 (C-3a), 128.3 (C-6), 124.5 (C-7a), 116.9 (C-7), 82.5 (C-3), 31.9 (C-8), 26.7 (C-9), 22.5 (C-5), 22.3 (C-10), 20.8 (C-4), 13.9 (C-11). The assignments of these signals were based on the H–H-correlation spectroscopy (COSY), heteronuclear multiple quantum coherence (HMQC) and heteronuclear multiple bond connectivity (HMBC) spectra. The ^{1}H-NMR spectrum of **1** was identical to the previously reported ^{1}H-NMR spectrum of senkyunolide A.[15] The ^{13}C-NMR spectrum of **1** was identical to the previously published ^{13}C-NMR spectrum of senkyunolide A.[16]

Compound **3**: $[\alpha]_D^{24}$ −64.1 (*c* 0.239, $CHCl_3$). EI-MS *m/z* (%): 194 (M^+, 6.0), 149 (2.1), 137 (4.8), 109 (12), 108 (100); HR-EI-MS *m/z* 194.1300 $[M]^+$ (calcd for $C_{12}H_{18}O_2$: 194.1307); ^{1}H-NMR (400 MHz, $CDCl_3$) *δ*: 6.78 (1H, dd, *J* = 6.4, 3.2 Hz, H-7), 3.98 (1H, ddd, *J* = 8.8, 7.2, 5.2 Hz, H-3), 2.46–2.55 (1H, m, H-3a), 2.36–2.40 and 2.31–2.35 (each 1H, m, H-6), 2.14–2.26 and 1.91–1.98 (each 1H, m, H-5), 2.02–2.10 and 1.12–1.22 (each 1H, m, H-4), 1.70–1.84 (2H, m, H-8), 1.48–1.60 (2H, m, H-9), 1.32–1.46 (2H, m, H-10), 0.93 (3H, t, *J* = 6.8 Hz, H-11). ^{13}C-NMR (100 MHz, $CDCl_3$) *δ*: 170.3 (C-1), 135.3 (C-7), 131.1 (C-7a), 85.4 (C-3), 43.1 (C-3a), 34.3 (C-8), 27.5 (C-9), 25.4 (C-4), 25.0 (C-6), 22.6 (C-10), 20.8 (C-5), 13.9 (C-11). The assignments of these signals were based on the H–H-COSY, HMQC and HMBC spectra. The ^{1}H- and ^{13}C-NMR spectra of **3** were identical to the previously published ^{1}H- and ^{13}C-NMR spectra of neocnidilide.[17]

Subfraction A4 was fractionated by silica gel column chromatography (Wakogel C-300 HG; 1.5 cm i.d. × 30 cm). Subfraction A4.2 was subjected to preparative HPLC (column, Cosmosil 5C18 MS-II (10 mm i.d × 250 mm); mobile phase, acetonitrile:water (65 : 35); flow rate = 2.0 mL/min), yielding compound **2** (6 mg; t_R = 6.8 min) as a colorless oil and compound **4** (16 mg; t_R = 7.9 min) as a colorless oil.

Compound **2**: $[\alpha]_D^{25}$ −54.1 (*c* 0.465, $CHCl_3$). EI-MS *m/z* (%): 190 (M^+, 7.3), 148 (2.3), 144 (2.1), 133 (100); HR-EI-MS *m/z* 190.0984 $[M]^+$ (calcd for $C_{12}H_{14}O_2$: 190.0994); ^{1}H-NMR (400 MHz, $CDCl_3$) *δ*: 7.90 (1H, d, *J* = 8.0 Hz, H-7), 7.67 (1H, t, *J* = 7.2 Hz, H-5), 7.53 (1H, td, *J* = 7.6, 0.8 Hz, H-6), 7.44 (1H, d, *J* = 7.6 Hz, H-4), 5.48 (1H, dd, *J* = 7.8, 4.4 Hz, H-3), 2.01–2.08 and 1.72–1.81 (each 1H, m, H-8), 1.33–1.52 (4H, m, H-9 and 10), 0.91 (3H, t, *J* = 6.4 Hz, H-11). ^{13}C-NMR (100 MHz, $CDCl_3$) *δ*: 170.8 (C-1), 150.2 (C-3a), 134.0 (C-5), 129.1 (C-6), 126.2 (C-7a), 125.8 (C-7), 121.8 (C-4), 81.5 (C-3), 34.5 (C-8), 26.9 (C-9), 22.5 (C-10), 13.9 (C-11). The assignments of these signals were based on the H–H-COSY, HMQC and HMBC spectra. The ^{1}H- and ^{13}C-NMR spectra of **2** were identical to the previously published ^{1}H- and ^{13}C-NMR spectra of (3*S*)-butylphthalide.[17]

Compound **4**: $[\alpha]_D^{24}$ −149 (*c* 0.500, $CHCl_3$). EI-MS *m/z* (%): 194 (M^+, 0.45), 150 (54), 107 (15), 93 (59), 79 (100); HR-EI-MS *m/z* 194.1291 $[M]^+$ (calcd for $C_{12}H_{18}O_2$: 194.1307); ^{1}H-NMR (400 MHz, $CDCl_3$) *δ*: 5.95 (1H, ddt, *J* = 10.0, 5.6, 2.0 Hz, H-6), 5.87 (1H, dt, *J* = 10.0, 3.6 Hz, H-7), 4.44 (1H, dt, *J* = 8.4, 5.6 Hz, H-3), 3.17 (1H, m, H-7a), 2.49 (1H, m, H-3a), 2.11–2.17 and 1.94–2.04 (each 1H, m, H-5), 1.72–1.80 and 1.54–1.63 (each 1H, m, H-8), 1.72–1.80 and 1.27–1.35 (each 1H, m, H-4), 1.44–1.53 and 1.35–1.44 (each 1H, m, H-9), 1.35–1.44 (2H, m, H-10), 0.93 (3H, t, *J* = 6.8 Hz, H-11). ^{13}C-NMR (100 MHz, $CDCl_3$) *δ*: 176.7 (C-1), 130.4 (C-6), 120.9 (C-7), 81.7 (C-3), 42.4 (C-7a), 37.4 (C-3a), 29.1 (C-8), 28.0 (C-9), 23.3 (C-5), 22.5 (C-10), 19.2 (C-4), 13.9 (C-11). The assignments of these signals were based on the H–H-COSY, HMQC and HMBC spectra. The ^{1}H- and ^{13}C-NMR spectra of **4** were identical to the previously published ^{1}H- and ^{13}C-NMR spectra of cnidilide.[17]

Compound **5**: (*Z*)-Ligustilide (Standard solution for crude drugs determination; FUJIFILM Wako Pure Chemical Corporation) was used as a standard for the analyses.

Analyses of Phthalides by GC-MS To detect (3*S*)-butylphthalide (**2**), cnidilide (**4**), senkyunolide A (**1**), neocnidilide (**3**), and (*Z*)-ligustilide (**5**) and measure the content of each constituent, GC-MS analyses were performed. The analytical conditions were as follows: electron ionization mode, injector and transfer line temperature, 200 and 270 °C, respectively; oven temperature programmed from 50 to 300 °C at 10 °C/min; carrier gas, helium (1 mL/min); splitless mode; ionization energy, 70 eV; ionization current, 60 μA. (3*S*)-Butylphthalide, cnidilide, senkyunolide A, neocnidilide, and (*Z*)-ligustilide were accurately weighed and dissolved in EtOAc to make stock solutions of 1.00 mg/mL. Then, (3*S*)-butylphthalide standard solutions (2.00, 4.00, and 8.00 μg/mL) were prepared by diluting the stock solutions to make calibration curves. Series of cnidilide, senkyunolide A, neocnidilide, and (*Z*)-ligustilide standard solutions (5.00, 10.0, and 20.0 μg/mL) were similarly prepared. A calibration curve of each standard compound was calculated by plotting peak areas of base ion peak (*y*) against a series of injection amounts (*x*, μg). The base peak ion [(3*S*)-butylphthalide, *m/z* 133; cnidilide, *m/z* 79; senkyunolide A, *m/z* 107; neocnidilide, *m/z* 108; (*Z*)-ligustilide, *m/z* 148] was selected. The calibration equation and correlation coefficient of the five standard compounds were as follows; (3*S*)-butylphthalide, $y = 45827.29x - 4127.00$ ($R^2 = 1.0000$); cnidilide, $y = 88159.47x - 46181.50$ ($R^2 = 1.0000$); senkyunolide A, $y = 84567.90x - 136752.50$ ($R^2 = 1.0000$); neocnidilide, $y = 59692.27x - 43696.50$ ($R^2 = 0.9996$); (*Z*)-ligustilide, $y = 25522.99x - 21664.50$ ($R^2 = 1.0000$). Fraction A was accurately weighed and dissolved in EtOAc to make a sample solution of 0.100 mg/mL. The peak areas of base

peak ion of (3*S*)-butylphthalide, cnidilide, senkyunolide A, neocnidilide, and (*Z*)-ligustilide in the sample solution were fit to the calibration curves, and the amounts of each compound in 10.0 μL of the sample solution calculated. The amounts of (3*S*)-butylphthalide, cnidilide, senkyunolide A, neocnidilide, and (*Z*)-ligustilide in 10.0 μL of the sample solution (1.00 μg of fraction A) were calculated to be 56.43, 79.09, 126.8, 96.81, and 94.36 ng, respectively; therefore, the contents of these compound in fraction A were 5.643, 7.909, 12.68, 9.681, and 9.436%, respectively.

Animal Experiments and Primary Cultured Rat Hepatocytes 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. Male Wistar rats (Charles River Laboratories Japan, Inc., Yokohama, Japan) were housed at 21–23 °C under a 12 h light–dark cycle and fed with a CRF-1 diet (Charles River Laboratories Japan) with water available *ad libitum*. The animals were acclimated to their housing for a week. Hepatocytes were isolated from the livers of Wistar rats according to a previously published method.[12,18] Briefly, the liver was perfused with collagenase, and the dispersed cells were centrifuged, resuspended, and seeded at 1.2×10^6 cells per 35 mm diameter dish. The cells were incubated at 37 °C for 2 h. After the replacement of the medium, the cells were further incubated at 37 °C overnight.

Estimation of NO Production and Lactate Dehydrogenase (LDH) Activity Each fraction or constituent was added to the medium on day 1, and the hepatocytes were incubated for 8 h. Nitrite (a stable metabolite of NO) in the medium was measured using the Griess method to measure NO levels.[19,20] The NO levels in the presence and absence of IL-1β in the medium were set at 100 and 0%, respectively. The IC_{50} against nitrite was determined in triplicate for at least three different concentrations of a fraction or a constituent.[21] Loxoprofen sodium (Kolon Life Science, Inchon, South Korea) was used as a positive control.[21] The LDH activity in the medium was measured using an LDH Cytotoxicity Detection Kit (TaKaRa Bio Inc., Kusatsu, Japan) to estimate cytotoxicity. An IC_{50} value of a fraction or a constituent was calculated to determine its ability to inhibit NO production when the compound did not show cytotoxicity.

Direct NO-Quenching Activity Each constituent was added to a medium containing 25 μM $NaNO_2$ and incubated at 37 °C for 1.5 h, according to the previously described method.[20] This medium was then mixed with Griess reagent[19] and incubated at room temperature for 5 min. The absorbance at 540 nm was measured to determine the reduction in nitrite by the constituent.

Western Blot Analysis Western blotting was performed as previously described.[22] Briefly, hepatocytes were treated with 1 nM IL-1β and a fraction or constituent for 8 h and lysed in the presence of a protease inhibitor cocktail (Nacalai Tesque, Inc.). The resultant lysates were run on a 10% sodium dodecyl sulfate-polyacrylamide gel and blotted onto a Sequi-Blot membrane (Bio-Rad, Hercules, CA, U.S.A.). After blocking with 5% Difco skim milk (BD Biosciences, San Jose, CA, U.S.A.), immunostaining was performed using primary antibodies against iNOS (Clone 54; BD Biosciences) and β-tubulin (Cell Signaling Technology Inc., Danvers, MA, U.S.A.) and then horseradish peroxidase-conjugated anti-immunoglobulin Fc antibody. The protein was visualized with Enhanced Chemiluminescence Blotting Detection Reagents (GE Healthcare Biosciences Corp., Piscataway, NJ, U.S.A.) and detected using an Amersham Imager 600 (GE Healthcare).

Quantitative RT-PCR Total RNA was prepared from rat hepatocytes using Sepasol I Super G solution (Nacalai Tesque, Inc.) and purified with the TURBO DNA-free Kit (Applied Biosystems).[12,13] The cDNA was reverse-transcribed from total RNA and amplified by PCR with the primers that were previously described.[23] The mRNA levels were quantitatively measured in triplicate by real-time PCR using SYBR Green I and the Thermal Cycler Dice Real Time System (TaKaRa Bio Inc.). When a single peak of melting temperature was observed on the dissociation curve of the amplified product from each mRNA, the threshold cycle (Ct) value was used for the subsequent calculation. The relative mRNA levels were calculated from the obtained Ct values according to the $\Delta\Delta$Ct method. The Ct values were normalized to elongation factor 1α (EF) mRNA,[24] which is encoded by a house-keeping gene and used as an internal control. The normalized mRNA lev-

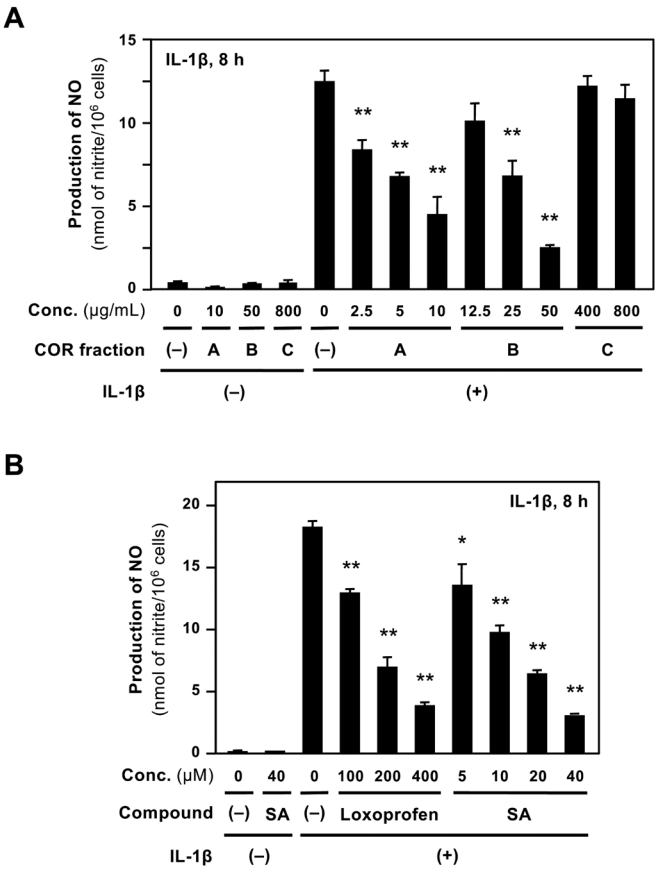

Fig. 2. Fraction A of COR Extract and Senkyunolide A Inhibit NO Production in Hepatocytes

(A) The effect of COR fractions on NO production and the expression of inducible nitric oxide synthase (iNOS). Hepatocytes were treated with IL-1β (1 nM) in the presence or absence of each COR fraction (A to C) for 8 h. The effects of COR fractions on NO production. The levels of nitrite (a major metabolite of NO) in the medium were measured in triplicate. The values are the mean± standard deviation (S.D.) ($n = 3$). *$p < 0.05$ and **$p < 0.01$ *versus* IL-1β alone. (B) The effect of senkyunolide A on NO production. Hepatocytes were treated with IL-1β in the presence or absence of senkyunolide A for 8 h. Senkyunolide A alone was added to the medium as a negative control, *i.e.*, senkyunolide A-treated and IL-1β-untreated control. SA, senkyunolide A. As a positive control, loxoprofen was added to the medium. The levels of nitrite were measured and shown similarly to (A).

els in the total RNA from the hepatocytes treated with IL-1β alone were set at 100%.

Statistical Analysis The results are representative of at least three independent experiments that yielded similar findings. The values are presented as the mean± standard deviation (S.D.). The differences were analyzed using Student's *t*-test followed by Bonferroni correction. The significance was set at $p < 0.05$ and $p < 0.01$.

RESULTS

Suppression of NO Production by Crude Fractions from COR Extract The rhizome of *Cnidium officinale* (COR) was extracted with methanol according to a previously published method.[14,22] The resultant COR extract was successively partitioned into three fractions by hydrophobicity using EtOAc (fraction A), *n*-butanol (fraction B), and water (fraction C).

Because NO production is induced by IL-1β in rat hepatocytes,[10] the effects of COR fractions A, B, and C on NO production were examined using hepatocytes. The addition of fraction A or B into the medium decreased IL-1β-induced NO production in a dose-dependent manner, whereas fraction C did not cause a significant change (Fig. 2A). The LDH activity in the medium including each fraction showed much lower LDH activity than that of whole cell extract (data not shown), suggesting that all fractions showed no cytotoxicity at the concentrations applied. The IC_{50} values of COR extract and fractions A and B were calculated according to the criteria described in Materials and Methods and are summarized in Table 1. Fractions A and B showed lower IC_{50} values than

Table 1. Fractionation of a *Cnidium officinale* Rhizome Extract and Its Effects on Nitric Oxide Production

Fraction	Yield [%][a)]	IC_{50} [μg/mL][b)]	Constituent (Compound)	Content [%][c)]	IC_{50} [μM][b)]
Methanol extract	100	42.6±11.0	—	—	
A (ethyl acetate-soluble)	14.0	4.58±1.46	—	100	
			Senkyunolide A (**1**)	12.68	17.2±5.51
			Neocnidilide (**3**)	9.681	NA
			(*Z*)-Ligustilide (**5**)	9.436	12.3±3.15
			Cnidilide (**4**)	7.909	NA
			(3*S*)-Butylphthalide (**2**)	5.643	NA
B (*n*-butanol-soluble)	28.1	19.9±0.49	—	—	
C (water-soluble)	57.9	NA	—	—	

a) The percentage is calculated as the weight of each fraction divided by the sum of three fractions. *b*) The half-maximal inhibitory concentration of nitric oxide (NO) production in IL-1β-treated hepatocytes (mean ± standard deviation). At least three experiments were performed to determine these values. *c*) The content of each constituent was measured by GC-MS and shown as a percentage of the dry weight of fraction A. NA, not applied due to low activity.

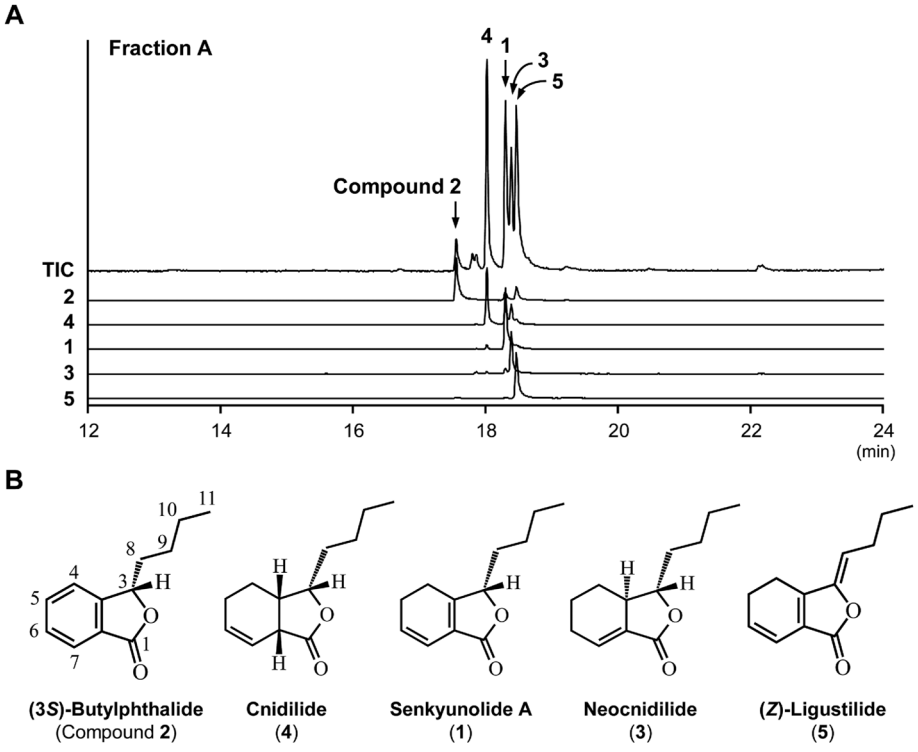

Fig. 3. Constituents Present in the Rhizome of *Cnidium officinale*

(A) GC-MS profiles of fraction A of COR extract. GC-MS analyses were performed under Materials and Methods. Total ion chromatogram (TIC) and mass chromatograms of constituents are shown. Under the chromatogram of TIC, five chromatograms are aligned, which correspond to the peaks of the constituents: (3*S*)-butylphthalide (compound **2**), *m*/*z* 133; cnidilide (**4**), *m*/*z* 79; senkyunolide A (**1**), *m*/*z* 107; neocnidilide (**3**), *m*/*z* 108; (*Z*)-ligustilide (**5**), *m*/*z* 148. Peaks of compounds **2** and **5** are 3-fold magnified along the *y*-axis. (B) Chemical structures of the constituents present in fraction A of the rhizome of *Cnidium officinale*. Constituents detected by the GC-MS analysis are depicted. The order of the constituents corresponds to those of the peaks in (A).

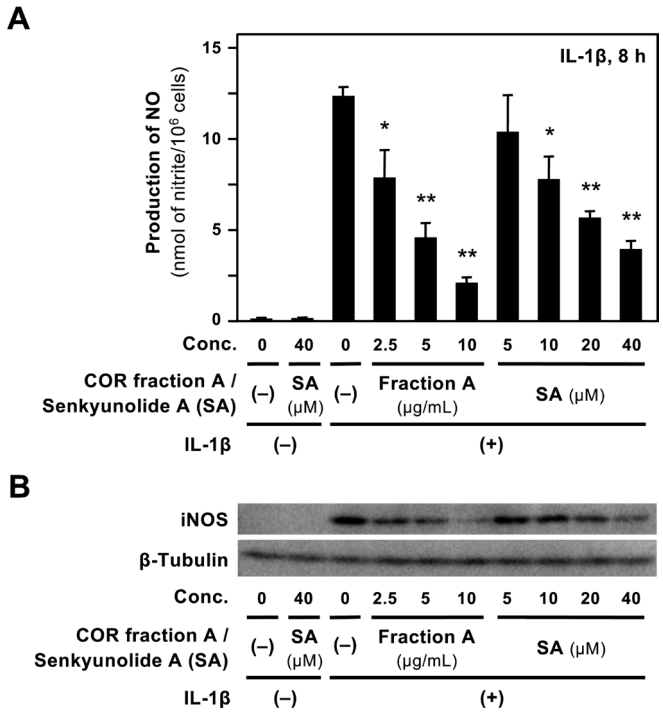

Fig. 4. Senkyunolide A Suppresses the *iNOS* Gene Expression

(A) The effects of senkyunolide A on NO production and iNOS expression in hepatocytes. Hepatocytes were treated with IL-1β (1 nM) in the presence or absence of senkyunolide A (SA) for 8 h. Senkyunolide A alone was added to the medium as a negative control, *i.e.*, senkyunolide A-treated and IL-1β-untreated (SA, IL-1β(−)) control. As a positive control, COR fraction A was added to the medium. The effects of senkyunolide A on NO production. The levels of nitrite (a major metabolite of NO) in the medium were measured in triplicate. The values are the mean± standard deviation (S.D.) ($n=3$). $*p<0.05$ and $**p<0.01$ *versus* IL-1β alone. (**B**) The effects of senkyunolide A on iNOS expression. From the hepatocytes treated in (A), the cell extracts (20 μg per lane) were prepared and analyzed by Western blotting to detect iNOS (130 kDa) and β-tubulin (55 kDa; internal control). SA, senkyunolide A.

the methanol extract in NO production in the hepatocytes. Because fraction A markedly suppressed NO production, it was expected that this EtOAc-soluble fraction may include hydrophobic constituents that suppress IL-1β-induced NO production.

Isolation of Constituents from Fraction A of COR Extract The constituents of COR fraction A, which markedly suppressed NO production, were purified by silica gel chromatography and preparative HPLC, as described in Materials and Methods. Two compounds showed two distinct peaks in the HPLC chromatogram. Therefore, we isolated them from subfraction A5.2 and identified them as senkyunolide A (**1**) and neocnidilide (**3**). Two other compounds were isolated from subfraction A4.2 and identified as (3*S*)-butylphthalide (**2**) and cnidilide (**4**).

GC-MS analysis was performed to estimate the content of each constituent isolated from COR fraction A (Fig. 3). As shown in Table 1, the content of senkyunolide A (12.68%) was the highest in fraction A among the constituents.

Suppression of NO Production by the Constituents of COR Fraction A in Hepatocytes Next, we examined whether these phthalides in COR fraction A could suppress NO induction in IL-1β-treated hepatocytes. Because senkyunolide A (**1**) was most abundant in COR fraction A, this constituent was first examined. Loxoprofen, *i.e.*, a non-steroidal anti-inflammatory drug (NSAID), was used as a positive control to compare activity to suppress NO production. Previously, we reported that loxoprofen suppressed NO production in IL-1β-treated hepatocytes.[21] As shown in Fig. 2B, senkyunolide A (**1**) much more efficiently suppressed NO production than loxoprofen (a positive control). In contrast, senkyunolide A alone [SA, IL-1β(−)] did not induce NO production in the hepatocytes.

Senkyunolide A and (*Z*)-ligustilide (**5**) significantly suppressed NO production in hepatocytes, whereas (3*S*)-butylphthalide (**2**), neocnidilide (**3**), and cnidilide (**4**) did not (Table 1). Senkyunolide A and (*Z*)-ligustilide exhibited the highest potency in NO suppression, but did not show cytotoxicity (data not shown). When the NO-quenching activity of senkyunolide A was measured, no significant changes in NO levels were observed compared with the NO levels in the medium containing $NaNO_2$ alone (data not shown). These results suggest that senkyunolide A did not directly quench NO. Therefore, we used senkyunolide A for the subsequent experiments as a constituent characteristic of COR extract.

Inhibition of *iNOS* Gene Expression by Senkyunolide A Next, the effects of senkyunolide A in COR fraction A on *iNOS* gene expression were further investigated. When senkyunolide A was added with IL-1β to the medium for hepatocytes, it inhibited the induction of NO production and the iNOS protein (Fig. 4). Senkyunolide A alone [SA, IL-1β(−)] did not induce NO production or iNOS protein.

Quantitative RT-PCR analysis of the total RNA from the hepatocytes was performed using the EF mRNA as an internal control, and the Ct values were normalized by those of EF mRNA, as described in Materials and Methods. As shown in Fig. 5A, the expression of iNOS mRNA increased in the presence of IL-1β alone (a positive control). When senkyunolide A and IL-1β were added to the medium with hepatocytes, it decreased iNOS mRNA levels in a dose-dependent manner. In the absence of IL-1β, the iNOS mRNA levels were 0.019% of those of the positive control (IL-1β alone). This value in the absence of IL-1β was not shown in Fig. 5A. Senkyunolide A also reduced the levels of the iNOS antisense transcript, which interacts with and stabilizes the iNOS mRNA[24] (data not shown). These results suggest that senkyunolide A in COR fraction A suppressed *iNOS* gene expression by reducing iNOS mRNA levels.

Downregulation of Proinflammatory Genes by Senkyunolide A It was expected that senkyunolide A might suppress the genes that are involved in inflammation other than the *iNOS* gene. Therefore, we examined whether senkyunolide A inhibits the expression of the genes encoding proinflammatory cytokines, such as IL-6 and TNF-α, and chemokines, such as chemokine C–C motif ligand 20 (CCL20).

Quantitative RT-PCR analysis revealed that these mRNA levels were induced by IL-1β alone, as shown in Figs. 5B–D. When senkyunolide A and IL-1β were added to the medium, it decreased the expression of IL-6, TNF-α, and CCL20 mRNAs in the hepatocytes. In the absence of IL-1β, the levels of IL-6 and TNF-α mRNAs were 0.12 and 0.75%, respectively, of those of the positive control (IL-1β alone). Although the CCL20 mRNA levels were also measured in the absence of IL-1β, their percentage could not be calculated due to very low expression (data not shown). Therefore, these mRNA levels in the absence of IL-1β were not shown in Fig. 5.

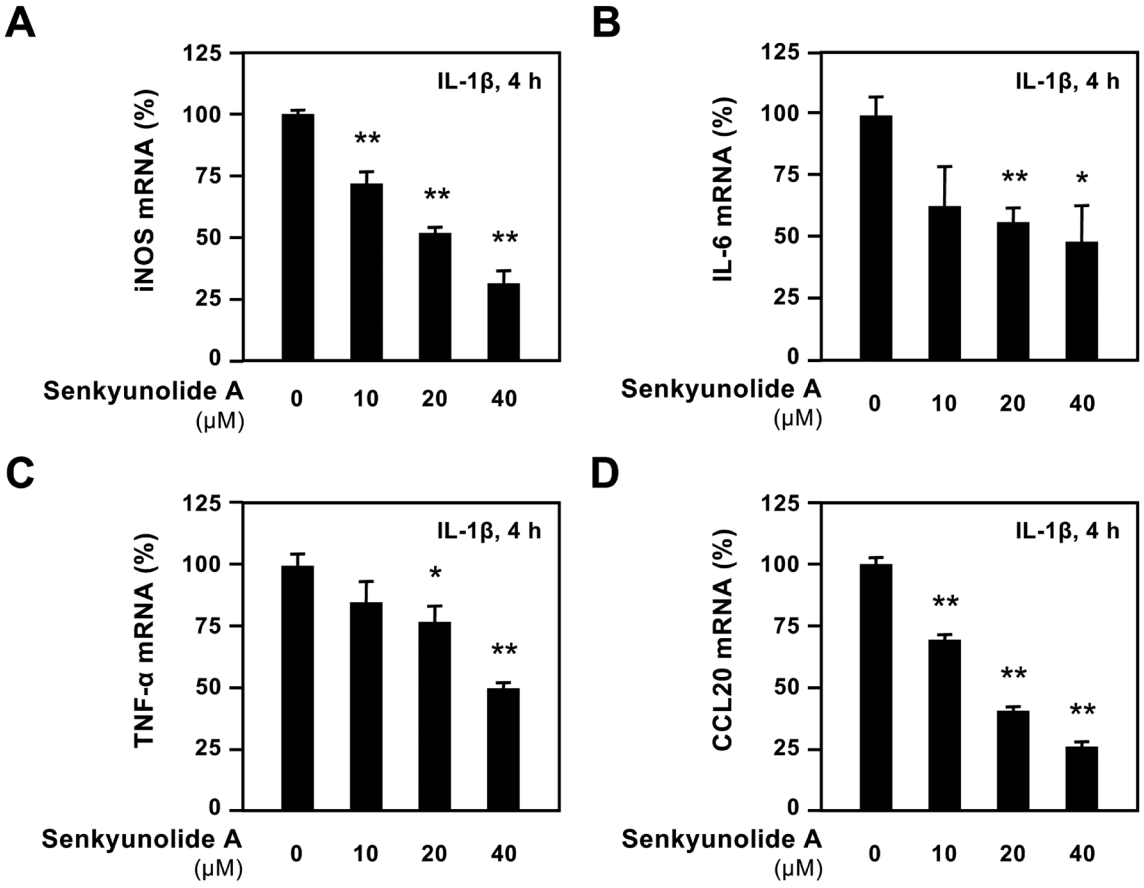

Fig. 5. Senkyunolide A Downregulates the Expression of Proinflammatory Genes

After incubation with IL-1β (1 nM) and senkyunolide A for 4 h, total RNA was prepared from the hepatocytes and analyzed by quantitative RT-PCR. The mRNA levels were measured using elongation factor 1α (EF) mRNA as an internal control: iNOS mRNA (A), IL-6 mRNA (B), tumor necrosis factor α (TNF-α) mRNA (C), and chemokine C–C motif ligand 20 (CCL20) mRNA (D). The mRNA expression levels were normalized to that of EF mRNA. The normalized mRNA levels in the total RNA from the hepatocytes treated with IL-1β alone were set at 100%. The mRNA levels that were measured in the total RNA from IL-1β-untreated hepatocytes are not shown in the figure due to very low expression levels (see details in the text). The relative mRNA levels were represented as the mean± S.D. ($n = 3$) of the resulting percentage. *$p < 0.05$ and **$p < 0.01$ *versus* IL-1β alone.

DISCUSSION

In this study, five phthalides from the rhizome of *Cnidium officinale* were purified, and their chemical structures were identified. GC-MS analyses provided the content of each constituent in a COR extract (Fig. 3, Table 1). Given that the content of senkyunolide A was 12.68% in fraction A and the yield of fraction A was 14.0% (Table 1), the content of senkyunolide A is calculated as 1.78% in COR extract. There is a previous report that a COR extract contained 1.18% senkyunolide A,[25] which is comparable to our results.

Using primary cultured rat hepatocytes, we compared anti-inflammatory potencies of these constituents to inhibit NO production in response to IL-1β. This hepatocyte system is one of the *in vitro* models of liver injury to assess the anti-inflammatory effects of constituents of crude drugs.[11,21] Therefore, we systematically performed comparative analyses of the five COR constituents using the hepatocytes. In contrast, RAW264.7 and BV2 cells are derived from leukemia and microglia, respectively, and show macrophage features. These cell lines are used to evaluate the suppression of NO production in response to lipopolysaccharide (LPS).[21] However, not all the phthalides identified in this study were assessed using each cell line. For example, only (*Z*)-ligustilide and senkyunolide A were evaluated using BV2 cells.[6] The five phthalides identified in this study were analyzed together, and their anti-inflammatory activities were compared by monitoring NO production in the hepatocytes.

Senkyunolide A and (*Z*)-ligustilide (dihydrophthalides) efficiently suppressed NO production, whereas (3*S*)-butylphthalide (phthalide) and cnidilide and neocnidilide (tetrahydrophthalides) showed less potency in hepatocytes. When their chemical structures are compared, three double bonds between carbon-3–8, carbon-3a–7a, and carbon-6–7, which are present in dihydrophthalides, seem to be essential to suppressing NO production. In RAW264.7 cells, there is a report that (*Z*)-ligustilide inhibited NO production.[26] More studies using other phthalides are needed to clarify the structure–function relationship of phthalides in the future.

The IC_{50} values of senkyunolide A (17.2 μM) and (*Z*)-ligustilide (12.3 μM) with the IC_{50} values of other constituents in crude drugs were compared: 25.1 μM (naringenin from the bark of *Prunus jamasakura*),[13] 15.8 μM (limonin from the bark of *Phellodendron amurense*),[12] and 11.7 μM (isoliquiritigenin from the roots and stolons of *Glycyrrhiza uralensis*).[20] These results imply that senkyunolide A and (*Z*)-ligustilide may possess comparable potency to those of limonin and isoliquiritigenin, respectively.

The present study suggests that senkyunolide A in COR fraction A suppressed *iNOS* gene expression by reducing iNOS mRNA levels in IL-1β-treated hepatocytes (Figs. 4, 5A). Furthermore, senkyunolide A inhibited the expression of IL-6 and TNF-α and CCL20 mRNAs (Figs. 5B–D). In LPS-treated BV2 cells, senkyunolide A also inhibited the production of NO and TNF-α.[6] Together, these data indicate that senkyunolide A possesses anti-inflammatory activity in both hepatocytes and BV2 cells and may be one of the principal constituents responsible for the anti-inflammatory effects of COR.

The molecular mechanisms of the pharmacological activity of senkyunolide A may be involved in the signal transduction pathway *via* the transcription factor, nuclear factor κB (NF-κB). Our previous studies suggest the involvement of NF-κB.[12,13,21,22] To support this hypothesis, COR extract decreased iNOS and TNF-α expression by suppressing nuclear translocation of NF-κB in BV2 cells.[27] On the contrary, senkyunolide A did not affect the nuclear translocation of NF-κB in the same cell line.[6] It remains unclear whether NF-κB is involved in the decreased expression of iNOS and TNF-α. It is expected that several COR constituents are involved in various pharmacological activities other than anti-inflammatory effects. Detailed investigation of the phthalides in the rhizome of *Cnidium officinale*, as well as their pharmacological effects, will be necessary in the future.

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Conflict of Interest F.N.N. and Y.N. performed this study as graduate students of the Graduate School of Life Sciences, Ritsumeikan University and the Graduate School of Pharmaceutical Sciences, Ritsumeikan University, respectively. S.T. performed this study as an undergraduate student of the College of Life Sciences, Ritsumeikan University. The other authors declare no conflict of interest.

Supplementary Materials The online version of this article contains supplementary materials.

REFERENCES

1) The Committee on the Japanese Pharmacopoeia. *The Japanese Pharmacopoeia, 17th edn*. The Minister of Health, Labour and Welfare, Japan (2016).
2) Arai S, Hioki C, Kosoto H, Yoshikawa M, Muramatsu S, Katai S, Namiki T, Hanawa T, Togo T eds. *Textbook of Traditional Japanese Medicine: Research on the standardization of traditional Japanese medicine, Part 1: Kampo*. Tokyo, Japan. promoting integrated medicine (subsidized by the Health and Labour Sciences Research Grant, Japan 2010–2011). (2011). http://kampotextbook.sakura.ne.jp/
3) Beck JJ, Chou SC. The structural diversity of phthalides from the Apiaceae. *J. Nat. Prod.*, **70**, 891–900 (2007).
4) Choi HS, Kim MSL, Sawamura M. Constituents of the essential oil of *Cnidium officinale* Makino, a Korean medicinal plant. *Flavour Fragrance J.*, **17**, 49–53 (2002).
5) Chan SS, Cheng TY, Lin G. Relaxation effects of ligustilide and senkyunolide A, two main constituents of *Ligusticum chuanxiong*, in rat isolated aorta. *J. Ethnopharmacol.*, **111**, 677–680 (2007).
6) Or TC, Yang CL, Law AH, Li JC, Lau AS. Isolation and identification of anti-inflammatory constituents from *Ligusticum chuanxiong* and their underlying mechanisms of action on microglia. *Neuropharmacology*, **60**, 823–831 (2011).
7) Bae KE, Choi YW, Kim ST, Kim YK. Components of rhizome extract of *Cnidium officinale* Makino and their *in vitro* biological effects. *Molecules*, **16**, 8833–8847 (2011).
8) Lee YM, Lee YR, Kim CS, Jo K, Sohn E, Kim JS, Kim J. *Cnidium officinale* extract and butylidenephthalide inhibits retinal neovascularization *in vitro* and *in vivo*. *BMC Complement. Altern. Med.*, **16**, 231 (2016).
9) Colasanti M, Suzuki H. The dual personality of NO. *Trends Pharmacol. Sci.*, **21**, 249–252 (2000).
10) Kitade H, Sakitani K, Inoue K, Masu Y, Kawada N, Hiramatsu Y, Kamiyama Y, Okumura T, Ito S. Interleukin 1β markedly stimulates nitric oxide formation in the absence of other cytokines or lipopolysaccharide in primary cultured rat hepatocytes but not in Kupffer cells. *Hepatology*, **23**, 797–802 (1996).
11) Kamino T, Shimokura T, Morita Y, Tezuka Y, Nishizawa M, Tanaka K. Comparative analysis of the constituents in Saposhnikoviae Radix and Glehniae Radix cum Rhizoma by monitoring inhibitory activity of nitric oxide production. *J. Nat. Med.*, **70**, 253–259 (2016).
12) Fujii A, Okuyama T, Wakame K, Okumura T, Ikeya Y, Nishizawa M. Identification of anti-inflammatory constituents in Phellodendri Cortex and Coptidis Rhizoma by monitoring the suppression of nitric oxide production. *J. Nat. Med.*, **71**, 745–756 (2017).
13) Yamauchi Y, Okuyama T, Ishii T, Okumura T, Ikeya Y, Nishizawa M. Sakuranetin downregulates inducible nitric oxide synthase expression by affecting interleukin-1 receptor and CCAAT/enhancer-binding protein *β*. *J. Nat. Med.*, **73**, 353–368 (2019).
14) Ohno N, Yoshigai E, Okuyama T, Yamamoto Y, Okumura T, Sato K, Ikeya Y, Nishizawa M. Chlorogenic acid from the Japanese herbal medicine Kinginka (Flos Lonicerae japonicae) suppresses the expression of inducible nitric oxide synthase in rat hepatocytes. *HOAJ Biol.*, **1**, 2 (2012).
15) Yamagishi T, Kaneshima H. Constituents of *Cnidium officinale* Makino. Structure of senkyunolide and gas chromatography-mass spectrometry of the related phthalides. *Yakugaku Zasshi*, **97**, 237–243 (1977).
16) Zhang D, Teng H, Li G, Liu K, Su Z. Separation and purification of *Z*-ligustilide and senkyunolide A from *Ligusticum chuanxiong* Hort. with supercritical fluid extraction and high-speed countercurrent chromatography. *Sep. Sci. Technol.*, **41**, 3397–3408 (2006).
17) Tsukamoto T, Ishikawa Y, Miyazawa M. Larvicidal and adulticidal activity of alkylphthalide derivatives from rhizome of *Cnidium officinale* against *Drosophila melanogaster*. *J. Agric. Food Chem.*, **53**, 5549–5553 (2005).
18) Kanemaki T, Kitade H, Hiramatsu Y, Kamiyama Y, Okumura T. Stimulation of glycogen degradation by prostaglandin E_2 in primary cultured rat hepatocytes. *Prostaglandins*, **45**, 459–474 (1993).
19) Green LC, Wagner DA, Glogowski J, Skipper PL, Wishnok JS, Tannenbaum SR. Analysis of nitrate, nitrite, and [^{15}N]nitrate in biological fluids. *Anal. Biochem.*, **126**, 131–138 (1982).
20) Tanemoto R, Okuyama T, Matsuo H, Okumura T, Ikeya Y, Nishizawa M. The constituents of licorice (*Glycyrrhiza uralensis*) differentially suppress nitric oxide production in interleukin-1β-treated hepatocytes. *Biochem. Biophys. Rep.*, **2**, 153–159 (2015).
21) Inaba H, Yoshigai E, Okuyama T, Murakoshi M, Sugiyama K, Nishino H, Nishizawa M. Antipyretic analgesic drugs have different mechanisms for regulation of the expression of inducible nitric oxide synthase in hepatocytes and macrophages. *Nitric Oxide*, **44**, 61–70 (2015).
22) Takimoto Y, Qian HY, Yoshigai E, Okumura T, Ikeya Y, Nishizawa M. Gomisin N in the herbal drug gomishi (*Schisandra chinensis*)

suppresses inducible nitric oxide synthase gene *via* C/EBP*β* and NF-*κ*B in rat hepatocytes. *Nitric Oxide*, **28**, 47–56 (2013).
23) Dwijayanti DR, Okuyama T, Okumura T, Ikeya Y, Nishizawa M. The anti-inflammatory effects of Indonesian and Japanese bitter melon (*Momordica charantia* L.) fruit extracts on interleukin-1*β*-treated hepatocytes. *Functional Foods in Health and Disease*, **9**, 16–33 (2019).
24) Matsui K, Nishizawa M, Ozaki T, Kimura T, Hashimoto I, Yamada M, Kaibori M, Kamiyama Y, Ito S, Okumura T. Natural antisense transcript stabilizes inducible nitric oxide synthase messenger RNA in rat hepatocytes. *Hepatology*, **47**, 686–697 (2008).
25) Lim EY, Kim JG, Lee J, Lee C, Shim J, Kim YT. Analgesic effects of *Cnidium officinale* extracts on postoperative, neuropathic, and menopausal pain in rat models. *Evid. Based Complement. Alternat. Med.*, **2019**, 9698727 (2019).
26) Chung JW, Choi RJ, Seo EK, Nam JW, Dong MS, Shin EM, Guo LY, Kim YS. Anti-inflammatory effects of (*Z*)-ligustilide through suppression of mitogen-activated protein kinases and nuclear factor-*κ*B activation pathways. *Arch. Pharm. Res.*, **35**, 723–732 (2012).
27) Lee SH, Lee JH, Oh EY, Kim GY, Choi BT, Kim C, Choi YH. Ethanol extract of *Cnidium officinale* exhibits anti-inflammatory effects in BV2 microglial cells by suppressing NF-*κ*B nuclear translocation and the activation of the PI3K/Akt signaling pathway. *Int. J. Mol. Med.*, **32**, 876–882 (2013).