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RESEARCH ARTICLE

Anti-inflammatory activity of compounds from the rhizome of *Cnidium officinale*

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Abstract Five new compounds, 9,3′-dimethoxyhierochin A (**1**), 6-oxo-*trans*-neocnidilide (**2**), (±)-(3*E*)-*trans*-6-hydroxy-7-methoxydihydroligustilide (**3**), (±)-cnidiumin (**4**), and 6-(1-oxopentyl)-salicylic acid methyl ester (**5**), together with twenty known compounds (**6**–**25**), were isolated from the rhizome of *Cnidium officinale*. The chemical structures of new compounds were established by NMR spectroscopic techniques, mass spectrometry, Mosher's method, and CD spectrum. Their anti-inflammatory activities were evaluated against lipopolysaccharide (LPS)-induced nitric oxide (NO) production in macrophage RAW 264.7 cells. Compounds **7**, **13**, and **14** showed inhibitory effects with IC_{50} values of 5.1, 24.5, and 27.8 μM, respectively. In addition, compounds **7**, **13**, and **14** reduced LPS-induced inducible nitric oxide synthase (iNOS) expression and cyclooxygenase-2 (COX-2) protein in a concentration-dependent manner.

Keywords *Cnidium officinale* · Umbelliferae · Anti-inflammatory · NO inhibition

Introduction

Inflammation is a critical host immune response to infection or irritation, and contributes to the initiation and progress of many diseases, including multiple sclerosis, Parkinson's, and Alzheimer's disease (Mosmann 1983). Macrophages are a major cell type that participates in the inflammatory process by producing cytokines, chemokines, and various inflammatory mediators such as nitric oxide (NO), prostaglandin E_2 (PGE_2), interleukin (IL)-6, and tumor necrosis factor (TNF)-α (Heiss et al. 2001). NO is produced by inducible nitric oxide synthase (iNOS) in macrophages, hepatocytes, and renal cells, when stimulated, for example, with lipopolysaccharide (LPS), TNF-α, or IL-1 (Kundu and Surh 2008). The overproduction of NO by iNOS has been implicated in the pathology of some inflammatory disorders, including sepsis, inflammation-mediated tissue damage, and rheumatoid arthritis (Stuehr and Marletta 1985). Therefore, NO production should correlate with the inflammatory process, so that a change in NO levels (e.g., by inhibiting iNOS activity or iNOS protein induction) would represent a means of assessing the effects of compounds on the inflammatory process (Farrell et al. 1992). Cyclooxygenase (COX) is a rate-limiting enzyme that catalyzes the synthesis of prostanoids that mediate inflammation. Prostanoids include arachidonic acid metabolites such as prostaglandins, thromboxanes, and prostacyclins that cause vasodilation, edema, and pain associated with tissue injury. Two isoforms of COX are known, COX-1 and COX-2. Increased COX-2 gene

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expression occurs in response to growth factors, mitogens, and inflammatory mediators (Farrell et al. 1992).

Cnidium officinale (Umbelliferae) is widely cultivated in Chinese, Japan, and Korea and has long been used in traditional medicine as herbal drug to treat pain, inflammation, menstrual disturbance, blood pressure depressant, and deficiency disease of anti-vitamin (Jeong et al. 2009, 2005). Volatile non-polar alkylphthalide derivatives from *C. officinale* have been shown antifungal and smooth muscle relaxing activities. In contract, the more polar components have been less well these activities (Kobayashi et al. 1987). In addition, some authors reported that this plant had pharmacological properties to tumor metastasis and angiogenesis (Onishi et al. 1998). However, the chemical composition and biological anti-inflammatory activities of this plant extracts were not extensively examined. Fractions of the $CHCl_3$ extract from this plant were found to potently inhibit NO production, with an IC_{50} value of 20.8 μg/mL. From these studies, we isolated and investigated five new and twenty known compounds. In this report, the purification and structural elucidation of these compounds are discussed, along with their inhibitory of NO production.

Materials and methods

General experimental procedures

Optical rotations were measured using a JASCO DIP 1000 digital polarimeter. UV spectrum was recorded using a Thermo spectrometer. IR spectrum was recorded using a JASCO FT/IR-4100 spectrometer. The 1D- and 2D- NMR spectra were obtained using Varian Unity Inova 400 MHz and a Bruker Ascend^{TM} 500 MHz spectrometer with tetramethylsilane (TMS) as an internal standard and the chemical shifts were recorded in δ values (ppm). Mass spectrum was recorded using a JEOL JMS-AX 300L and SYNAPT G2 spectrometer. The CD spectrum was measured using a Jasco J-180 spectropolarimeter. Silica gel (Merck, 63–200 μm particle size) and RP-18 (Merck, 75 μm particle size) were used for column chromatography. TLC was performed using Merck silica gel 60 F_{254} and RP-18 F_{254} plates. HPLC was performed using a Waters 2487 Controller system with a UV detector and a YMC Pak ODS-A column (20 × 250 mm, 5 μm particle size, YMC Co., Ltd., Japan), and HPLC solvents were from Burdick & Jackson, U.S.A.

Plant material

The rhizome of *C. officinale* was purchased from a folk medicine market 'Yakryoung-si' in Daegu, Kerea, in January 2016. Botanical identification was performed by Professor Byung Sun Min and the voucher specimen CUD-2330-1 was deposited at the Herbarium of the College of Pharmacy, Daegu Catholic University, Korea.

Extraction and isolation

The dried rhizome of *C. officinale* (9.8 kg) was extracted three times (3 h × 20 L) with methanol. After solvent was removed under reduced pressure, the residue was suspended in H_2O and then partitioned with *n*-hexane, $CHCl_3$, EtOAc, and *n*-BuOH, successively. By the guided-fractionation-activity, the $CHCl_3$-soluble fraction (107.0 g) was chromatographic (80 × 12 cm, 63–200 μM particle size, Merck) on a silica gel column using a stepwise gradient of *n*-hexan-EtOAc (8:1–0:1, each 10 L) to yield eight fractions (Fr.1–Fr.8) according to their TLC profiles. Fraction 3 (4.5 g) was subsequently subjected to silica gel column chromatography (60 × 3.5 cm) eluting with *n*-hexane–EtOAc (4:1) to yield 4 sub-fractions (Fr. 3–1 to 3–4). Fraction 3.3 (2.5 g) was chromatographed on a silica gel column (60 × 3.5 cm) using a gradient solvent system of *n*-hexane–acetone (6:1–0:1) to give **1** (2.5 mg), **4** (6.0 mg), **6** (100.0 mg), **7** (17.7 mg), **9** (46.0 mg), **11** (12.4 mg), **14** (10.0 mg), **15** (6.1 mg), **18** (57.0 mg), **20** (100.0 mg), **21** (12.0 mg), and **23** (79.0 mg). Fraction 4 (32.0 g) was subjected to silica gel column chromatography (60 × 6.5 cm) using with *n*-hexane–EtOAc (8:1) to yield 6 sub-fractions (Fr. 4–1 to 4–6). Fraction 4.4 was further purified over YMC RP-18 column with MeOH-H_2O (4:1–6:1) and semi-preparative Waters HPLC systems with an isocratic solvent system of 40% MeOH in H_2O + 0.1% trifluoroacetic acid (flow rate 5 mL/min) over 90 min as an eluent, UV detection at 254 and 210 nm to yield **3** (10.0 mg), **5** (2.5 mg), **8** (22.0 mg), **10** (89.0 mg), **12** (79 mg), **13** (6.6 mg), **16** (17.2 mg), **17** (3.4 mg), and **19** (15.9 mg). Fraction 5 (25.0 g) was repeated on a silica gel column (60 × 6.5 cm) using a gradient solvent system of *n*-hexane–acetone (5:1–0:1) resulted in the isolation of **2** (2.5 mg), **22** (4.5 mg), **25** (1.7 mg), and **24** (7.4 mg).

*9,3′-Dimethoxyhierochin A (**1**)*

Yellowish oil; $[\alpha]_D^{24}$ − 3.72 (*c* 0.1, MeOH); UV (MeOH) λ_{max} (logε) nm: 220.5 (4.25); CD (MeOH) λ_{max} nm ($\Delta\varepsilon$) 202.0 (+ 0.75), 228.0 (− 0.23), and 248.5 (+ 0.14); IR (ATR) ν_{max} cm^{-1}: 3432, 1670, 1509, 1034; ^1H and ^{13}C NMR (CD_3OD) data, see Tables 1 and 2; HREIMS *m/z* 386.1731 $[M]^+$ (Calcd for $C_{22}H_{26}O_6$, 386.1729).

Table 1 ^{1}H-NMR data (δ, ppm) for five new compounds

Position	**1**[a]	**2**[c]	**3**[b]	**4**[d]	**5**[a]
2	6.92, d (1.5)				
3		4.30, td (6.4, 4.4)		4.28, d (4.4)	7.06, d (8.0)
4a		2.42, m	2.43–2.61, m	3.80, ddd (7.2, 4.4, 2.8)	7.44, t (8.0)
4b		1.82, ovl[e]			
5a	6.76, d (8.0)	2.53, m	1.94–2.04, m	1.89, m	7.13, d (7.6)
5b				1.74, m	
6	6.80, dd (8.0, 1.5)		4.16, ddd (9.0, 6.0, 3.0)	2.41, m	
7	5.47, d (5.0)	6.34, d (2.8)	3.96, d (3.0)		
8	3.61, ovl[e]				2.88, t (7.2)
9a	3.68, m	3.13, m			1.66, quint (7.6)
9b	3.61, ovl[e]				
10		1.82, ovl[e]	5.28, t (8.0)	1.54, m	1.40, m
11		1.34–1.61, ovl[e]	2.36, dd (14.5, 7.5)	1.35, m	0.96, t (7.6)
12		1.34–1.61, ovl[e]	1.49, dd (14.5, 7.5)	0.91, t (7.2)	
13		0.94, t (6.0)	0.95, t (7.0)		
2′	6.95, ovl[e]				
6′	6.95, ovl[e]				
7′	6.56, d (16.0)				
8′	6.15, d (16.0, 6.0)				
9′	4.06, dd (6.0, 1.0)				
3-OCH_3	3.80, s				
9-OCH_3	3.38, s				
3′-OCH_3	3.86, s				
9′-OCH_3	3.35, s				
7-OCH_3			3.58, s		

Coupling constant (J) in Hz are given parentheses

^{1}H- NMR (500 MHz) measured in CD_3OD[a], $CDCl_3$[b] and (400 MHz) in CD_3COCD_3[c], CD_3OD[d]

[e]Overlapped with other signals

*6-Oxo-trans-neocnidilide (**2**)*

Colorless oil; $[\alpha]_D^{24}$ − 23.16 (*c* 0.1, acetone); UV (acetone) λ_{max} (logε) nm: 233 (3.94), 237 (3.60); IR (ATR) ν_{max} cm^{-1}: 1760, 1249, 1025; ^{1}H and ^{13}C NMR (CD_3COCD_3) data, see Tables 1 and 2; HREIMS *m/z* 208.1097 $[M]^+$ (Calcd for $C_{12}H_{16}O_3$, 208.1099).

*(±)-(3E)-trans-6-Hydroxy-7-methoxydihydroligustilide (**3**)*

Colorless oil; $[\alpha]_D^{24}$ − 3.16 (*c* 0.1, $CHCl_3$); UV ($CHCl_3$) λ_{max} (logε) nm: 202.5 (0.92); IR (ATR) ν_{max} cm^{-1}: 3411, 1689, 1360, 1220; ^{1}H and ^{13}C NMR ($CDCl_3$) data, see Tables 1 and 2; HREIMS *m/z* 238.1203 $[M]^+$ (Calcd for $C_{13}H_{18}O_4$, 238.1205).

*(±) Cnidiumin (**4**)*

Colorless oil; $[\alpha]_D^{24}$ − 8.64 (*c* 0.1, MeOH); UV (MeOH) λ_{max} (logε) nm: 219.0 (3.65); IR (ATR) ν_{max} cm^{-1}: 3360, 1710, 1550, 1024; ^{1}H and ^{13}C NMR (CD_3OD) data, see Tables 1 and 2; HREIMS *m/z* 240.1001 $[M]^+$ (Calcd for $C_{12}H_{16}O_5$, 240.0998).

*6-(1-Oxopentyl)-salicylic acid methyl ester (**5**)*

Colorless oil; $[\alpha]_D^{24}$ − 8.72 (*c* 0.1, MeOH); UV (MeOH) λ_{max} (logε) nm: 228.5 (4.35), 309.0 (1.65); IR (ATR) ν_{max} cm^{-1}: 3319, 1712, 1452, 1034; ^{1}H and ^{13}C NMR (CD_3OD) data, see Tables 1 and 2; HREIMS *m/z* 236.1047 $[M]^+$ (Calcd for $C_{13}H_{16}O_4$, 236.1049).

Table 2 ^{13}C-NMR data (δ) for five new compounds

Position	**1**[a]	**2**[c]	**3**[b]	**4**[d]	**4a**[d]	**4b**[d]	**5**[a]
1	134.3	169.0	169.5	172.0	170.5	170.1	123.9
2	110.6			134.8	135.0	134.7	159.1
3	149.1	85.8	148.7	70.3	70.4	69.9	120.5
4a	147.6	28.0	24.6	70.9	70.9	70.6	133.7
5a	116.2	37.5	17.5	25.9	25.9	25.5	119.7
6	119.8	198.8	68.1	24.8	24.8	24.6	142.1
7	89.7	127.1	74.0	139.5	139.6	139.5	205.3
8	52.6	152.2	124.4	170.5	170.5	170.1	47.9
9a	75.7	44.3	154.2	105.9	106.0	106.0	27.4
10		34.9	113.7	35.8	35.9	35.9	23.3
11		28.3	28.2	18.9	18.8	18.8	14.3
12		23.3	22.5	14.3	14.2	14.2	
13		14.3	14.0				
1′	132.3						
2′	116.6 or 112.2						
3′	149.3						
4′	145.5						
5′	130.3						
6′	116.6 or 112.2						
7′	134.4						
8′	124.2						
9′	74.3						
3-OCH_3	56.4						
9-OCH_3	59.3						
3′-OCH_3	56.8						
9′-OCH_3	58.0						
7-OCH_3			58.9				
CO							170.8
OCH_3							53.0

^{13}C- NMR (125 MHz) measured in CD_3OD[a], $CDCl_3$[b] and (100 MHz) in CD_3COCD_3[c], CD_3OD[d]

Preparation of (*S*)- and (*R*)-MTPA ester derivatives

(*R*)-MTPA (α-methoxy-α-(trifluoromethyl)phenyl-acetyl) ester of **3** (**3a**) was prepared using **3** (1.0 mg) and 4-(dimethylamino)-pyridine (0.2 mg) were transferred into an NMR tube. Afterthat, deuterated pyridine (0.5 mL) and (*S*)-(+)-α-methoxy-α-(trifluoromethyl)phenyl-acetyl chloride (6.0 μL) were added into the NMR tube and shaken to mix the sample and MTPA chloride evenly. The reaction NMR tube was permitted to stand at room temperature every 2 h and measured by ^{1}H NMR. In the manner described for (*R*)-MTPA ester of **3**, (*S*)-MTPA ester of **3** (**3b**) was hold as the same way.

(*R*)-MTPA ester of **3** (**3a**): ^{1}H NMR data (400 MHz, pyridine-d_5) δ_H 2.22 or 2.24 (1H, m, H-5), 5.25 or 5.27 (1H, m, H-6), 4.43 (1H, d, J = 3.2 Hz, H-7) or 4.50 (1H, d, J = 3.0 Hz, H-7).

(*S*)-MTPA ester of **3** (**3b**): ^{1}H NMR data (400 MHz, pyridine-d_5) δ_H 2.24 or 2.22 (1H, m, H-5), 5.27 or 5.25 (1H, m, H-6), 4.50 (1H, d, J = 3.0 Hz, H-7) or 4.43 (1H, d, J = 3.2 Hz, H-7).

Inhibition of nitric oxide production assay

Cell culture

The RAW264.7 cells were maintained in Dulbecco's Modified Essential. These cells were grown at 37 °C in DMEM supplemented with 10% heat-inactivated FBS, penicillin (100 units/mL), and streptomycin sulfate (100 μg/mL) in a humidified atmosphere of 5% CO_2.

MTT assay for cell viability

The RAW264.7 cell viability after 24 h of continuous exposure to the compounds was measured with a colorimetric assay based on the ability of mitochondria in viable cells to reduce MTT (Choi et al. 2018). Briefly, 1×10^4 cells/well treated for 24 h with vehicle or compounds were examined for cell viability. Viability of the macrophages treated with vehicle (0.5% DMSO) only was defined as 100% viable. Survival of macrophage cells after treatment with compounds was calculated using the following formula: viable cell number (%) = OD_{570} (treated cell culture)/OD_{570} (vehicle control) × 100.

Determination of NO production

The level of NO production was determined by measuring the amount of nitric from the cell culture supernatants as described previously. Briefly, the RAW264.7 cells (1×10^5 cells/well) were stimulated with or without 1 μg/mL of LPS for 24 h in the presence or absence of the test compounds (0.5–25 μM). The cell culture supernatant (100 μL) was then reacted with 100 μL of Griess reagent (Mosmann 1983). The remaining cells after the Griess assay were used to test their viability using a MTT based colorimetric assay as previously described.

Western blot analysis

Proteins were extracted from cells in ice-cold lysis buffer (50 mM Tris–HCl, pH 7.5, 1% Nonidet P-40, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 μg/mL leupeptin, 1 mM sodium vanadate, 150 mM NaCl). Fifty microgram of protein per lane was separated by (SDS)-(PAGE) and followed by transferring to a polyvinylidene difluoride membrane. The membrane was blocked with 5% skim milk, and then incubated with the corresponding antibody. After binding of an appropriate secondary antibody coupled to horseradish peroxidase, proteins were visualized by enhanced chemiluminescence according to the instructions of the manufacturer (Jung et al. 2017).

Results and discussion

The $CHCl_3$-soluble fraction from the rhizome of *C. officinale* was subjected repeating the column chromatography, which resulted in the isolation of five new (**1**–**5**) and 20 known compounds (**6**–**25**). By comparison of their physical and spectroscopic data with those reported in the literature, the chemical structures of the known compounds were identified as falcarindiol (**6**) (Ikeda et al. 1998), (3*R*, 8*S*)-heptadeca-1-en-4,6-diyne-3,8-diol (**7**) (Ikeda et al. 1998), conifenyl ferulate (**8**) (Kobayashi et al. 1984), ferulic acid (**9**) (Tominaga et al. 2005), isoferulic acid (**10**) (Tominaga et al. 2005), 3-butilydene-7-hydroxyphthalide (**11**) (Pushan et al. 1984), 2-methoxy-2(4′-hydroxyphenyl)ethanol (**12**) (Ferraboschi et al. 1990), sunkyunolide F (**13**) (Kobayashi et al. 1987), ferulic aldehyde (**14**) (Tominaga et al. 2005), *p*-methoxybenzadehyde (**15**) (Tominaga et al. 2005), 3-buthyl-4-hydroxyphthalide (**16**) (Ohzeki and Mori 2003), senkyunolide C (**17**) (Kobayashi et al. 1987), (*E*)-3-methoxy-1-(3-methoxyphenyl)propane (**18**) (Fleming et al. 2006), coniferol (**19**) (Li and Seeram 2010), oleic acid (**20**) (Li et al. 2012), prenenolone (**21**) (Szendi et al. 1995), falcarrindiol-8-acetate (**22**) (Schmiech et al. 2009), sunkyunolide I (**23**) (Naito et al. 1996), *p*-aldehydephenol (**24**) (Tominaga et al. 2005), and *p*-hydroxyphenol (**25**) (Tominaga et al. 2005) (Fig. 1).

Compound **1** was yellowish oil with a molecular formula of $C_{22}H_{26}O_6$ determined by high-resolution electron impact mass spectrometry (HREIMS) with *m/z* of 386.1731 (Calcd for $C_{22}H_{26}O_6$, 386.1729). The ^{1}H NMR spectrum of **1** indicated the existence of a pair of *meta*-coupled aromatic protons at δ_H 6.95 (2H, overlapped, H-2′ and H-6′); dihydrofuran moiety at δ_H 5.47 (1H, d, J = 5.0 Hz, H-7) and 3.61 (1H, m, H-8), one set of ABX system at δ_H 6.92 (1H, d, J = 1.5 Hz, H-2), 6.80 (1H, dd, J = 8.0, 1.5 Hz, H-6) and 6.76 (1H, d, J = 8.0 Hz, H-5), two methylene groups with an oxygen function at δ_H 3.68 (1H, m, H-9a), 3.61 (1H, m, H-9b), 4.06 (2H, dd, J = 6.0, 1.0 Hz, H-9′), and *trans*-olefinic protons at δ_H 6.56 (1H, d, J = 16.0 Hz, H-7′) and 6.15 (1H, dd, J = 16.0, 6.0 Hz, H-8′) (Table 1). The ^{13}C NMR spectrum of **1** showed 22 signals assignable to two benzene rings, an olefinic group, three oxygenated groups, four methoxy groups, and an aliphatic group (Table 2), indicating the presence of neolignan derivative, comparable to hierochin A isolated from *Anastatica hierochuntica* (Yoshikawa et al. 2003). Both had closely related structures, except for replacement of hydroxy groups into methoxy groups being located at C-9 and C-3′ in **1**. This was also confirmed by a heteronuclear multiple bond correlation (HMBC) experiment, showing significant correlation from the signals at δ_H 3.38 (OCH_3-9) and 3.86 (OCH_3-3′) to the signals at δ_C 75.7 (C-9) and 149.3 (C-3′), respectively (Fig. 2). In NOESY spectrum, the correlation between the H-2/H-6 and H-8; H-7 and H-9 indicated the 7, 8-*trans* relative configuration. Furthermore, the CD spectrum of **1** showed negative Cotton effect at 228.0 (Δε − 0.23) and two positive Cotton effects at 202.0 (+ 0.75) and 248.5 (+ 0.14), which indicated that it had a 7*S*, 8*R* configuration, comparable to (2*S*,3*R*)-(+)-DCA (Hirai et al. 1994). Therefore, it was concluded that **1** had a structure as shown in Fig. 1, and was named 9,3′-dimethoxyhierochin A. Compound **1** was the first lignan,

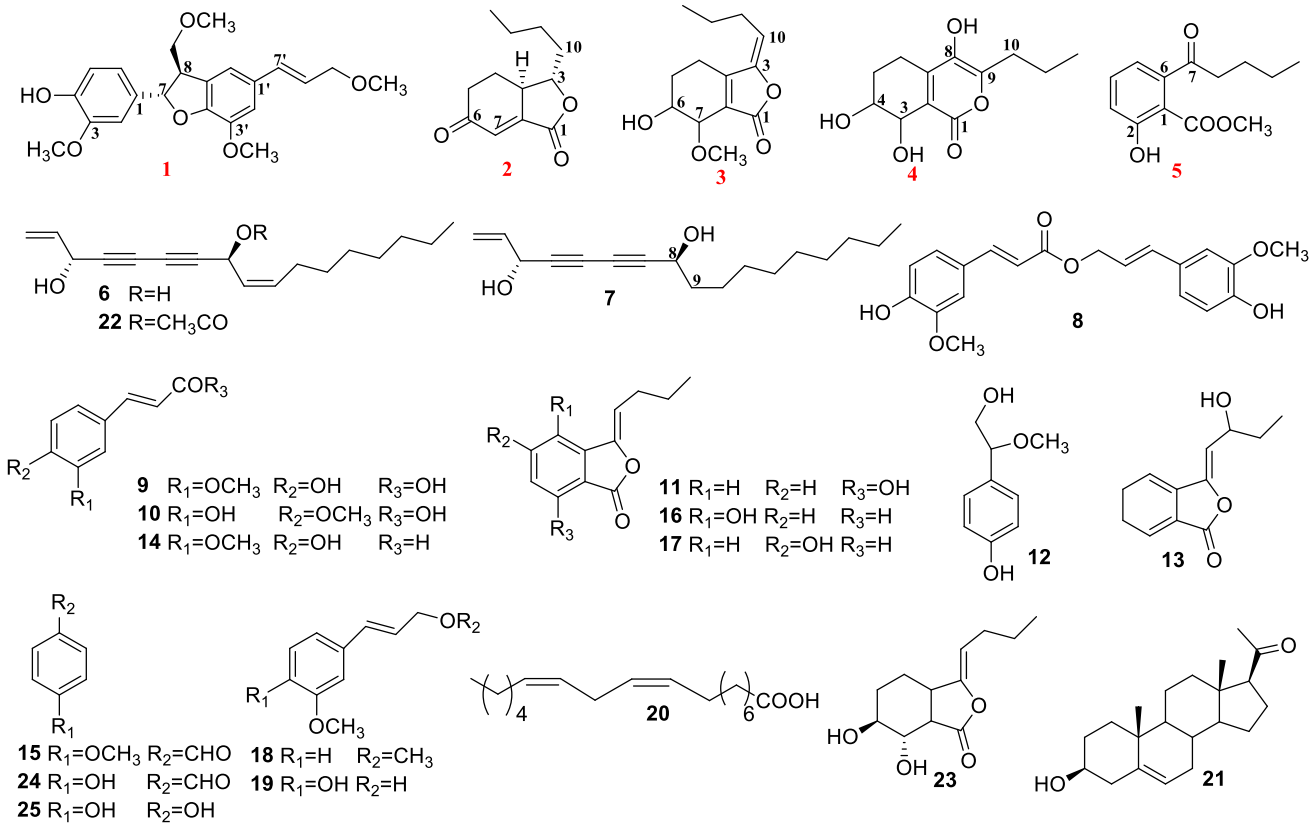

Fig. 1 Structures of compounds isolated from the rhizome of *C. officinale*

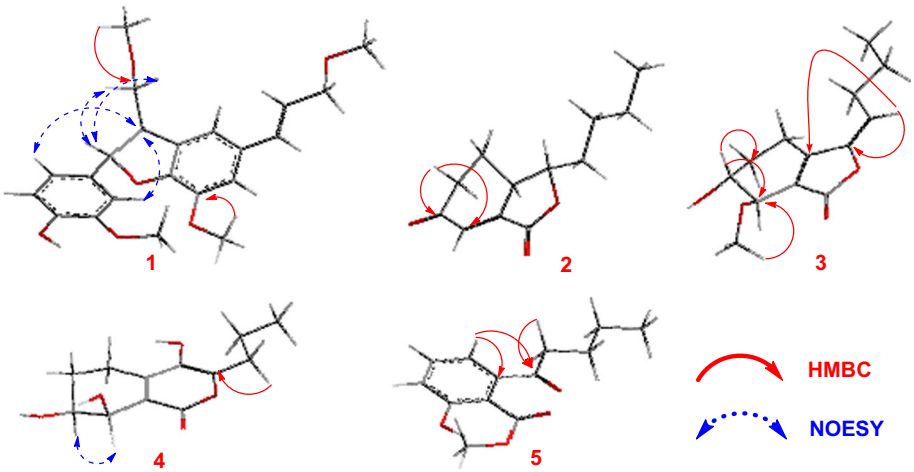

Fig. 2 The selected HMBC and NOESY correlations for new compounds **1**–**5**

which isolated and elucidated structure from *C. officinal* (Li et al. 2015).

Compound **2** was colorless oil. The molecular formula of **2** was determined to be $C_{12}H_{16}O_3$ by HREIMS with *m/z* of 208.1097 (Calcd for $C_{12}H_{16}O_3$, 208.1099). The ^{1}H NMR spectrum of **2** displayed the signals of the isobenzofuran moiety at δ_H 6.34 (1H, d, J = 2.8 Hz, H-7), 4.30 (1H, td, J = 6.4, 4.4 Hz, H-3), 3.13 (1H, m, H-9), 2.53 (2H, m, H-5), 2.42 and 1.82 (each 1H, m, H-4a and H-4b), and the butyl side chain with chemical shifts at δ_H 1.82 (2H, overlapped, H-10), 1.34-1.61 (each 2H, overlapped, H-11, 12), 0.94 (3H, t, J = 6.0 Hz H-13). The ^{13}C NMR spectrum showed 12 carbon signals, comprising an oxygenated, five methylenes, one methyl, two methines, and three quaternary including two carbonyl groups (δ_C 198.8 and 169.0). The above data indicated that **2** was a phthalide derivative being similar to *trans*-neocnidilide (Fischer and Gijbels 1987), excluding for the replacement of methylene group

into carbonyl group at C-6 in **2**. The result was confirmed by the correlation between δ_H 2.53 (H-5) and δ_C 198.8 (C-6)/127.1 (C-7) in the HMBC spectrum (Fig. 2). In the NOESY spectrum, correlation of H-3 (δ_H 4.30) and H-9 (δ_H 3.13) were not observed, together with a coupling constant of H-3 (J = 6.4, 4.4 Hz) indicated that **2** has the 3, 9-*trans* configuration (Oguro and Watanabe 2011; Fischer and Gijbels 1987). Furthermore, the relative configuration of **2** was determined to be the same as stereoisomers of sedanolide by comparison of negative optical rotation with $[\alpha]_D^{24}$ − 71.3 (Oguro and Watanabe 2011), which led to determination the absolute stereostructure of **2** as 3*S*,9*R* configuration. On the basis of the above data, structure of **2** was elucidated as shown and it was named 6-oxo-*trans*-neocnidilide.

Compound **3** was colorless oil. Its molecular formula was determined to be $C_{13}H_{18}O_4$ by HREIMS with *m/z* of 238.1203 (Calcd for $C_{13}H_{18}O_4$, 238.1205). The NMR spectroscopic features similar to those of **2**, except that the resonances of 6-oxocyclohexen and butyl group in **2** were replaced by those 6-hydroxy-7-methoxycyclohexen unit with chemical shifts at δ_H 4.16 (1H, ddd, J = 9.0, 6.0, 3.0 Hz, H-6), 3.96 (1H, d, J = 3.0 Hz, H-7), 3.58 (3H, s, OCH_3-7) and butylidene moiety at δ_H 5.28 (1H, t, J = 8.0 Hz, H-10) in **3**. These data showed a structure related to (*Z*)-6-hydroxy-7-methoxydihydroligustilide (Bae et al. 2011), except with the configuration of double bond of butylidene moiety in **3**. The butylidene moiety was found to be connected to the phthalide unit at C-3 by a correlation between δ_H 5.28 (H-10) and δ_C 148.7 (C-3)/154.2 (C-9) in the HMBC spectrum (Fig. 2). In addition, the location of hydroxyl group at C-6 and methoxyl group at C-7 were confirmed by correlation peaks from the signals at δ_H 4.16 (H-6) and 3.58 (OCH_3-7) to the carbon at δ_C 17.5 (C-5) and 74.0 (C-7), respectively (Fig. 2). To determine the absolute configuration of C-6, a modified Mosher's method was used to prepare (*R*)- and (*S*)-α-methoxy-α-trifluoromethylphenylacetic (MTPA) esters (**3a** and **3b**) (Su et al. 2002) (Fig. 3). The ^{1}H-NMR spectra of these two ester derivatives were acquired, and the $\Delta\delta_H$ values

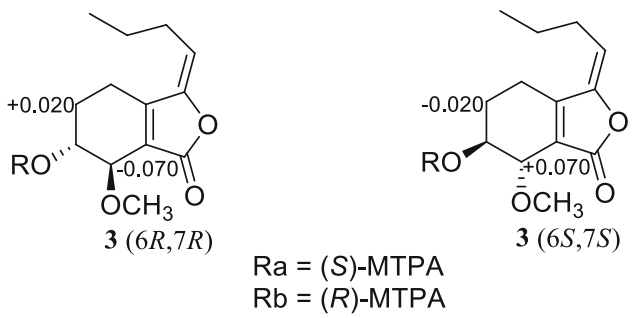

Fig. 3 Results with the modified Mosher's method for **3**

[$\Delta\delta_H = \delta_S - \delta_R$] are shown as Fig. 3. The H-5, H-6 and H-7 proton signals of **3a**/**3b** were shifted downfield and separated two signals at δ_H 2.22 (m)/2.24(m), 5.27 (m)/5.25 (m), and 4.50 (d, J = 3.0 Hz)/4.43 (d, J = 3.2 Hz), respectively, suggested that **3** was a pair of (6*R*)- and (6*S*)-isomers. In the NOESY spectrum, the proton H-7 was not irradiated upon other one, together with coupling constant of H-7 (J = 3.0 Hz) indicated the 6, 7-*trans* relative configuration (Pushan et al. 1984). Therefore, **3** was suspected to be a racemic mixture of the *trans*-isomers, (6*S*,7*S*) and (6*R*,7*R*) (Fig. 1). In addition, the stereochemistry of double bond reported as 5.5 and 5.3 ppm for *E*- and *Z*-butylidenephthalide, suggesting an *E*-form between C-3 and C-10 (Grech et al. 1994). The structure of **3** was conclusively elucidated as the mixture of (±)-(3*E*)-*trans*-6-hydroxy-7-methoxydihydroligustilide.

Compound **4** was colorless oil. The molecular formula of **4** was determined to be $C_{12}H_{16}O_5$ by HREIMS with *m/z* of 240.1001 (Calcd for $C_{12}H_{16}O_5$, 240.0998). The NMR spectrum of **4** exhibited the presence of isochromene signals with chemical shifts at δ_H 4.28 (1H, d, J = 4.4 Hz, H-3), 3.80 (1H, ddd, J = 7.2, 4.4, 2.8 Hz, H-4), 2.41 (2H, m, H-6), 1.89 1.74 (each 1H, m, H-5a, 5b), and the propyl side chain at δ_H 1.54 (2H, m, H-10), 1.35 (2H, m, H-11), 0.91 (3H, t, J = 7.2 Hz H-12). The ^{13}C NMR spectrum showed 12 carbon signals, including two oxygenated, four methylenes, one methyl, and five quaternary carbons including one carbonyl group (δ_C 172.0). The above results indicated that **4** had the same planar structure resembled those of EI-1941-3, isolating from the broths *Farrowia* sp. (Shoji et al. 2005), excluding with **4** less a carbonyl group at position of C-6 and more a hydroxyl and a vinyl group between C-8 and C-9 than EI-1941-3. From the HMBC spectrum, the structure of **4** was further confirmed by the presence of correlations from δ_H 1.54 (H-10) to δ_C 105.9 (C-9) (Fig. 2). In NOESY spectrum, correlation of H-3 and H-4 together with the coupling constant of H-3 (J = 4.4 Hz) demonstrated that 3,4-*cis* relative configuration (Solfrizzo et al. 2004). In storage time, **4** was unstable and separated into two isomers with carbon signals displaying as Table 2 suggested that **4** was suspected to be a racemic mixture of the *cis*-isomers, **4a** (3*S*,4*R*) and **4b** (3*R*,4*S*) (Fig. 1). The structure of **4** was elucidated and it was named (±)-cnidiumin.

Compound **5** was colorless oil with a molecular formula of $C_{13}H_{16}O_4$ determined by HREIMS with *m/z* of 236.1047 (Calcd for $C_{13}H_{16}O_4$, 236.1049). The ^{1}H NMR spectrum of **5** displayed one set of AB_2 type aromatic protons at δ_H 7.44 (1H, t, J = 8.0 Hz, H-4), 7.13 (1H, d, J = 7.6 Hz, H-5), and 7.06 (1H, d, J = 8.0 Hz, H-3); an oxopentyl group at δ_H 2.88 (2H, t, J = 7.2 Hz, H-8), 1.66 (2H, quint, J = 7.6 Hz, H-9), 1.40 (2H, m, H-10), and 0.96 (3H, t, J = 7.6 Hz, H-11); a methoxy group at δ_H 3.86 (3H, s). The ^{13}C NMR

spectrum showed 13 carbon signals, consist of one aromatic ring, three methylenes, one terminal methyl, one methoxy, and two carbonyl groups (δ_C 205.3 and 170.8). On the basis of the above spectral analyses, indicated that **5** was a salicylic acid derivative similar to 6-acylsalicylates (Tamura et al. 1996), apart from the oxopentyl group instead of oxobutyl group in 6-acylsalicylates. In the HMBC experiment, the signals of δ_H 2.88 (H-8) and 7.13 (H-5) showed correlation δ_C 205.3 (C-7) and 142.1 (C-6), respectively, which indicated that oxopentyl group was connected to phenyl ring at C-6. Therefore, it was determined that **5** had a structure as shown in Fig. 1, and was named 6-(1-oxopentyl)-salicylic acid methyl ester.

The inhibitory effects of the 25 compounds isolated from *C. officinale* were evaluated on RAW246.7 cells by NO production. As shown in Table 3, **7** showed the strongest inhibitory potency with IC_{50} 5.1 μM, following **13** and **14** with IC_{50} values of 24.5 and 27.8 μM, respectively, but the others were inactive. The presence of a double bond at position 9 and 10 of **6** did not seem to influence the inhibition of NO production in comparison of **7**. In addition, the presence of that aldehyde moiety at position 3 of **14**, strongly showed inhibitor of NO production in comparison with the attached carboxylic unit in **9**. RAW264.7 cells were pretreated for 30 min with **7**, **13**, and **14** followed by stimulation with LPS (1 μg/mL) for 18 h. Total lysates were prepared, and the expression levels of iNOS and COX-2 were determined by western blot. Compounds **7**, **13**, and **14** were shown to suppress LPS-induced iNOS and COX-2 protein expression (Fig. 4). This is a first report for the anti-inflammatory of constituents from *C. officinale*. With result of this research, it may contribute a part of screening test for treatment of diseases which related to the anti-inflammatory response from herb.

Table 3 Inhibition of NO production in macrophage RAW264.7 cells by 25 compounds

Compounds	IC_{50} values (μM)	Compounds	IC_{50} values (μM)
1	> 50	**14**	27.8 ± 4.2
2	> 50	**15**	> 50
3	> 50	**16**	> 50
4	> 50	**17**	> 50
5	> 50	**18**	> 50
6	> 50	**19**	> 50
7	5.1 ± 0.6	**20**	> 50
8	> 50	**21**	> 50
9	> 50	**22**	> 50
10	> 50	**23**	> 50
11	> 50	**24**	> 50
12	> 50	**25**	> 50
13	24.5 ± 3.2	Celastrol[a]	1.0 ± 0.1

[a]Positive control for NO production

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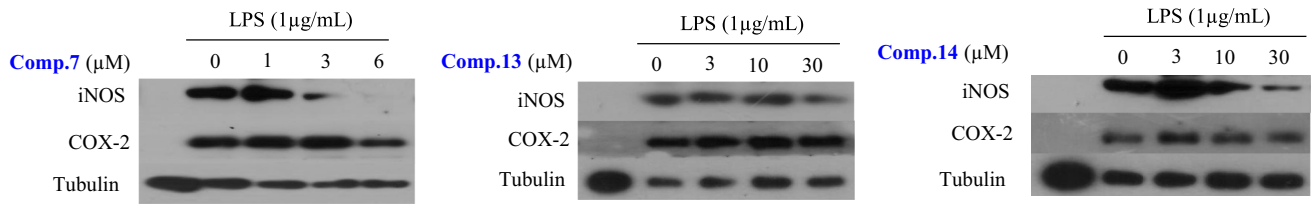

Fig. 4 Inhibition of LPS-induced iNOS and COX-2 expression in RAW264.7 cells by compounds **7**, **13** and **14**

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