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REGULAR PAPER

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Prenylated acetophenones from the roots of *Calendula officinalis* and their anti-inflammatory activity

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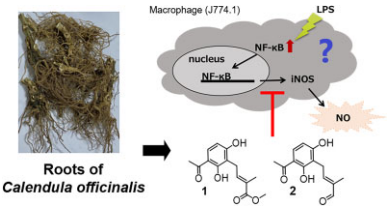
ABSTRACT

Calendula officinalis is a medicinal plant in the Asteraceae family, and it has a broad range of biological activities. In this study, we focused on the roots of *C. officinalis*, which have remarkable anti-inflammatory properties. By using a bioassay-guided fractionation approach, prenylated acetophenones **1** and **2**—of which **1** was previously unknown—were isolated, and their structures were determined by spectroscopic analysis. Both compounds decreased lipopolysaccharide-stimulated NO production in J774.1 cells. This study could lead to the use of the *Calendula* roots as a natural source of inflammatory mediators.

Graphical Abstract



Prenylated acetophenones with anti-inflammatory activity were isolated and determined their structures from the roots of *Calendula officinalis*.

Keywords: *Calendula officinalis*, prenylated acetophenone, anti-inflammatory activity, NO production

Calendula officinalis is a medicinal plant in the Asteraceae family, and it is distributed mainly over large areas of the Mediterranean (Muley, Khadabadi and Banarase 2009; Arora, Rani and Sharma 2013). *C. officinalis* exhibits a broad range of biological activities. In particular, the petals of *C. officinalis* show remarkable anti-inflammatory activity, and oleanane-type triterpene glycosides have been reported as their active compounds (Ukiya *et al.* 2006). In addition, triterpene alcohols (Śliwowski *et al.* 1973; Wilkomirski and Kasprzyk 1979), flavonoid glycosides (Ukiya *et al.* 2006), and carotenoids (Bakó, Deli and Tóth 2002) have been found in the petals of *C. officinalis* and have valuable biological activities. The petals of *C. officinalis* have been used in

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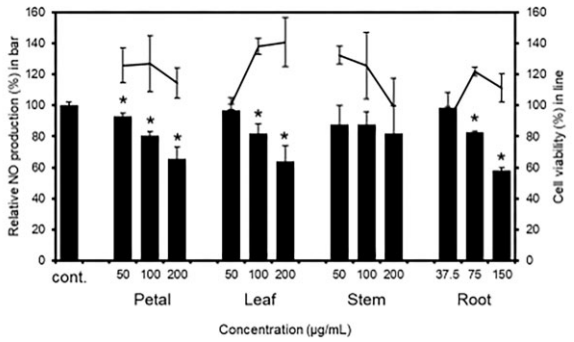

Figure 1. The effect of EtOH extracts of *C. officinalis* on LPS-stimulated NO production and cell viability in J774.1 cells. Cont., control. Data are means±SD ($n = 3$); *mean values are significantly different from those of the control at $P < .05$.

Europe since the 13th century for treating wounds, and a variety of cosmetics have been developed from the plant (Akihisa *et al.* 1996; Andersen *et al.* 2010; Carvalho *et al.* 2018).

In this study, we focused on the anti-inflammatory activity of the roots of *C. officinalis*. In lipopolysaccharide (LPS)-stimulated J774.1 cells, extracts from its roots exhibited the highest anti-inflammatory activity of various parts of *C. officinalis* (petal, leaf, stem, and root) (Figure 1). To the best of our knowledge, there are no detailed studies on the anti-inflammatory activity of the roots of *C. officinalis*. Although oleanane-type triterpene glycosides with anti-inflammatory activity have been isolated from the roots of *C. officinalis* (Wojciechowski *et al.* 1971; Nizyński *et al.* 2015), there may be other active compounds. Therefore, we investigated other anti-inflammatory active compounds from *C. officinalis* roots by a bioassay-guided fractionation approach. A new prenylated acetophenone (**1**) and a known derivative (**2**) were isolated from the roots of *C. officinalis* as active compounds.

Materials and methods

General experimental procedures

UV spectra were acquired on a spectrophotometer (V-730 BIO; Jasco, Tokyo, Japan). 1D and 2D NMR spectra were acquired on a 400-MHz spectrometer (Biospin AVANCE III; Bruker, Birellica, MA, USA), with chemical shifts expressed in ppm. The NMR spectra were referenced to residual solvent peaks (CD_3OD: ^1H NMR, 3.30 ppm; ^{13}C NMR, 49.0 ppm). HR-ESI-MS spectra were acquired on an HR-ESI Orbitrap mass spectrometer (Q-Exactive; Thermo Fisher Scientific, Waltham, MA, USA). RP-HPLC separations were performed with a recycling system comprising a prep pump (PU-2086 Plus Intelligent; Jasco), UV detector (UV-2075; Jasco), and Capcell Pak UG 120 C18 column (5 μm, 20 × 250 mm; Osaka Soda, Osaka, Japan), with HPLC-grade solvents. For analytical HPLC, an HPLC pump (PU-4180; Jasco), photodiode array detector (MD-4017; Jasco), and HPLC autosampler (AS-4050; Jasco) were used. Data were analyzed using ChromNAV software (ver. 2; Jasco).

Plant material

The sample was collected in Shiraoi, Hokkaido, Japan in August 2020. The voucher number of the sample is 202008.

Extraction and isolation

The dried sample of *C. officinalis* roots (135 g) was extracted with 50% EtOH (1 L) under stirring at room temperature for 24 h, and the supernatant was collected. The remaining residue was re-extracted with 50% EtOH (1 L). The collected supernatant was filtered, and the filtrates were concentrated at reduced pressure to give the EtOH extract (24 g). This extract was suspended in H_2O (500 mL) and partitioned successively with *n*-hexane (500 mL), ethyl acetate (4 × 500 mL), and 1-butanol (2 × 500 mL) to give *n*-hexane- (40 mg), ethyl acetate- (820 mg), 1-butanol- (3.6 g), and H_2O-soluble extracts (15.4 g), respectively. The ethyl acetate-soluble fraction (820 mg) was subjected to silica gel column chromatography (50 × 210 mm) with gradient elution using *n*-hexane/ethyl acetate mixtures (9:1, 300 mL; 4:1, 400 mL; 7:3, 500 mL; 3:2, 500 mL; 1:1, 500 mL; and 0:1, 600 mL) to yield eight fractions (fr. 1, 28 mg; fr. 2, 18 mg; fr. 3, 27 mg; fr. 4, 43 mg; fr. 5, 28 mg; fr. 6, 21 mg; fr. 7, 39 mg; and fr. 8, 15 mg). Fraction 5 was subjected to preparative RP-HPLC with H_2O–acetonitrile (65:35, 0.1% trifluoroacetic acid) as the eluent, a flow rate of 9.6 mL/min, and a detection wavelength of 210 nm to give **1** (2.3 mg) and **2** (0.2 mg).

Table 1. ^1H NMR (400 MHz) and ^{13}C NMR (100 MHz) data for **1** and **2** in CD_3OD (δ in ppm, J in Hz)

	1		2	
	δ_C	δ_H, (J in Hz)	δ_C	δ_H, (J in Hz)
1	113.4		112.8	
2, 6	163.9		164.0	
3	114.2		114.3	
4	132.3	7.63 (1H, d, 8.8 Hz)	132.5	7.66 (1H, d, 8.8 Hz)
5	108.2	6.42 (1H, d, 8.8 Hz)	108.2	6.43 (1H, d, 8.8 Hz)
7	204.5		204.5	
8	26.2	2.53 (3H, s)	26.2	2.53 (3H, s)
9	23.1	3.50 (2H, d, 7.5 Hz)	23.5	3.50 (2H, d, 7.4 Hz)
10	142.0	6.77 (1H, tq, 1.4, 7.5 Hz)	154.8	6.63 (1H, tq, 1.3, 7.4 Hz)
11	128.2		140.2	
12	12.5	1.97 (3H, d, 1.4 Hz)	9.1	1.97 (3H, d, 1.3 Hz)
13	170.6		197.5	9.32 (1H, s)
14	52.2	3.69 (3H, s)		

Methyl (*E*)-4-(3-acetyl-2,6-dihydroxyphenyl)-2-methylbut-2-enoate (1)

White amorphous solid; HR-ESI-MS *m/z* 265.1069 (calcd for *m/z* 265.1071); UV (MeOH) λ_{max} (log ε) 280 (3.75); ^1H and ^{13}C NMR (CD_3OD 400 MHz) (Table 1).

Murine cell culture

J774.1 macrophage cells were obtained from the National BioResource Project of Ministry of Education, Culture, Sports, Science and Technology, Japan. The cells were cultured in RPMI 1640 medium (Nacalai Tesque, Tokyo, Japan) containing 10% foetal bovine serum (Life Technologies, Carlsbad, CA, USA) and 1% penicillin/streptomycin (Life Technologies) under an atmosphere of 5% CO_2 at 37 °C.

Measurement of NO

NO production in J774.1 cells was measured by following a previously reported method with a slight modification (Kurata *et al.* 2019; Inui *et al.* 2021). The cells were placed in 96-well plates at a density of 1×10^5 cells/well. After preincubation overnight, the cells were incubated in a medium containing 0.1% LPS (Sigma-Aldrich, St. Louis, MO, USA) with or without the test

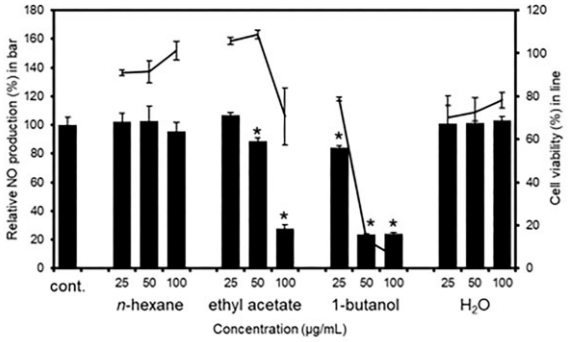

Figure 2. The effect of each soluble fractions of *C. officinalis* on LPS-stimulated NO production and cell viability in J774.1 cells. Cont., control. Data are means±SD ($n = 3$); *mean values are significantly different from those of the control at $P < .05$.

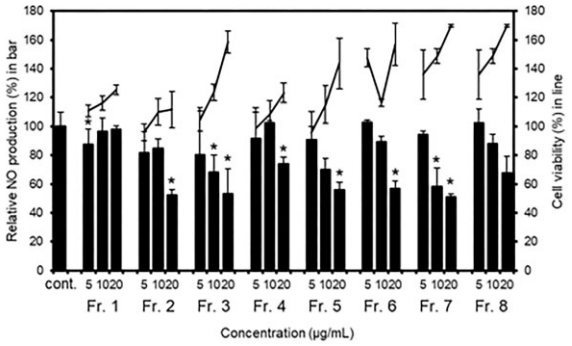

Figure 3. The effect of each fraction of ethyl acetate extracts from the roots of *C. officinalis* on LPS-stimulated NO production and cell viability in J774.1 cells. Cont., control. Data are means±SD ($n = 3$); *mean values are significantly different from those of the control at $P < .05$.

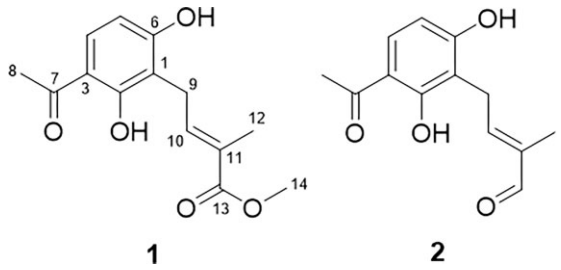

Figure 4. Chemical structures of **1** and **2**.

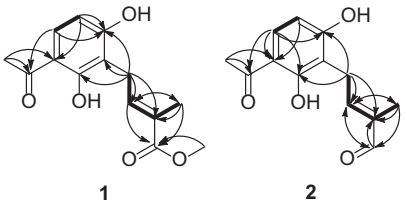

Figure 5. Connectivities (bold line) determined by ^{1}H–^{1}H COSY spectra and significant HMBC correlations (solid arrows) observed for **1** and **2**.

sample at various concentrations for 24 h. All tested samples were dissolved in dimethyl sulfoxide (DMSO) and diluted with 0.2% LPS in RPMI 1640 medium with a final DMSO concentration of <0.2% (v/v). N^G-monomethyl-L-arginine (L-NMMA; Dojindo Laboratories, Kumamoto, Japan) was used as a positive control, and NO production was determined by a Griess assay. The supernatant medium (100 μL) was aliquoted and mixed with 100 μL Griess reagent (1:1 v/v 1% sulfanilamide and 0.1% *N*-(1-naphthyl) ethylenediamine dihydrochloride in 5% H_3PO_4) in 96-well plates. Absorbance at 540 nm was measured on a microplate reader (SpectraMax 190; Molecular Devices, Sunnyvale, CA, USA).

MTT assay

Cell viability was evaluated by the MTT assay. MTT reagent (5 μL) was added to each well of the residual medium mentioned above, and the plate was incubated for 3 h under an atmosphere of 5% CO_2 at 37 °C. The supernatant of each well was removed and mixed with DMSO (200 μL). Absorbance was measured at 535 nm using a microplate reader.

Statistical analysis

All data were expressed as the means±standard deviations. Differences between means were compared by Student's *t*-test. For all statistical tests, differences with *P* values of <.05 were considered significant.

Results and discussion

Isolation and structural elucidation of anti-inflammatory compounds

To screen anti-inflammatory compounds from the roots of *C. officinalis*, we evaluated LPS-stimulated NO production in J774.1 cells treated with each soluble fraction sample (*n*-hexane, ethyl acetate, 1-butanol, and H_2O extracts). The ethyl acetate and 1-butanol fractions exhibited high activity (Figure 2). Although the 1-butanol fraction showed high activity, the cell viability decreased substantially. Thus, the ethyl acetate fraction was further fractionated. In the fractions separated by silica gel column chromatography, all fractions except frs. 1 and 4 showed high activity (Figure 3). We isolated **1** and **2** as the active compounds from fr. 5.

Compound **1** was obtained as a white amorphous solid using reverse-phase (RP)-HPLC (Figure 4). Its molecular formula was determined to be $C_{14}H_{16}O_5$ by HR-ESI-MS, with $[M+H]^+$ at *m/z* 265.1069 (calcd for *m/z* 265.1071). The ^{1}H NMR spectrum of **1** revealed the signals assigned to 2 *ortho*-coupled aromatic protons at δ_H 6.42 (d, $J = 8.8$ Hz, H-5) and 7.63 (d, $J = 8.8$ Hz, H-4) (Table 1). The methyl proton (δ_H 2.53, H-8) and carbonyl carbon (δ_C 204.5, C-7) suggested the presence of an acyl group. The HMBC correlations from H-8 to C-3 (δ_C 114.2) and from H-4 to C-7 (δ_C 204.5) established the presence of an acetophenone moiety (Figure 5). The olefin proton derived from a prenyl group was observed at δ_H 6.77 (tq, $J = 1.4, 7.5$, H-10). The signals for the other protons in the prenyl group were assigned by the ^{1}H–^{1}H COSY and HMBC correlations. In addition, the signals at δ_H 3.69 (s, H-14) and δ_C 170.6 (C-13) suggested the presence of a methyl ester group. The HMBC correlation of H-12 (δ_H 1.97) to C-13 revealed that a prenyl group was esterified. The HMBC correlations of H-9 (δ_H 3.50) to C-1 (δ_C 113.4) and C-2,6 (δ_C 163.9) showed that the methyl esterified prenyl group was attached to C-1. Further, NOE between H-9 and H-12 was observed in the NOESY spectrum of **1**, suggesting that the double bond of C-10 and C-11 is *E* configuration. Based on these spectroscopic analyses, **1** was determined to be a new prenylated acetophenone, methyl (*E*)-4-(3-acetyl-2,6-dihydroxyphenyl)-2-methylbut-2-enoate.

The 1D NMR spectrum of **2** resembled that of **1**. The typical signal of an aldehyde group was observed at δ_H 9.32

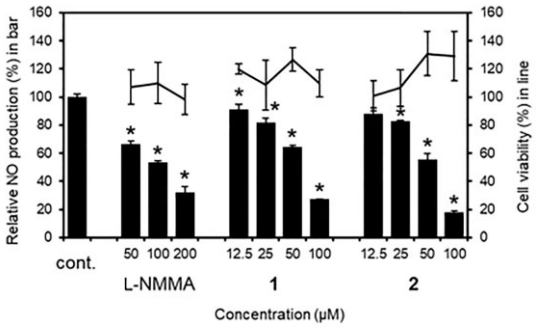

Figure 6. The effect of **1** and **2** on LPS-stimulated NO production and cell viability in J774.1 cells. N^G-monomethyl-L-arginine (L-NMMA) was used as a positive control. Cont., control. Data are means±SD ($n = 3$); *mean values are significantly different from those of the control at $P < .05$.

(H-13) in the ^{1}H NMR spectrum of **2**. The HMBC correlation of H-13 to C-11 (δ_C 140.2) established the presence of an aldehyde prenyl group. Therefore, the structure of **2** was assigned as the analog of **1** shown in Figure 4. Although **2** has been isolated from *Doronicum pardalianches* (Bohlmann and Abraham 1979) and *Osteospermum* species (Jakupovic *et al.* 1988), this is the first time it has been isolated from a species in the *Calendula* genus.

Anti-inflammatory activity

The *in vitro* anti-inflammatory activities of **1** and **2** were evaluated. Both compounds exhibited a concentration-dependent decrease in NO production rates (Figure 6). The IC_{50} values of **1** and **2** were 69.2 and 57.4 μM, respectively. Compared with the positive control of L-NMMA (IC_{50} 138.2 μM), **1** and **2** showed 2-fold higher activity. Moreover, the cell viability rates of **1** and **2** were higher than 100% at a concentration of 100 μM.

The expression of inducible NO synthase (iNOS) in LPS-stimulated macrophage cells produces NO, which causes cancer and other diseases (Lee *et al.* 2013). In a previous study, paeonol, which has an acetophenone skeleton same as **1** and **2**, has been reported as an iNOS expression inhibitor (Himaya *et al.* 2012). Compounds **1** and **2** did not show NO scavenging activity (data not shown). This result suggested that **1** and **2** may decrease LPS-stimulated NO production via inhibiting iNOS expression in macrophage cells. A geranylated acetophenone analog has been reported as a microsomal prostaglandin E_2 synthetase-1 (mPGES-1) inhibitor (Svouraki *et al.* 2017). Because prostaglandin E_2 also induces iNOS expression (Lee *et al.* 2013), it is possible that **1** and **2** suppressed iNOS expression by inhibiting mPGES-1. Thus, this study might lead to the use of the *Calendula* roots as a natural source of inflammatory mediators.

Conclusion

In this study, 2 prenylated acetophenones (**1** and **2**), including the new compound **1**, were isolated from the roots of *C. officinalis* by bioassay-guided fractionation. The compounds exhibited remarkable anti-inflammatory activity without cytotoxicity. This study might lead to the use of the *Calendula* roots as a source of naturally occurring inflammatory mediators.

Supplementary material

Supplementary material is available at *Bioscience, Biotechnology, and Biochemistry* online.

Data availability

The data underlying this article are available in the article and in its online supplementary material.

Author contribution

W.K., R.M., and S.M. performed the structural elucidation of the isolated compounds. W.K. evaluated the anti-inflammatory activity of all samples. M.F. and Y.S. collected the samples. The manuscript was prepared by R.M. S.K. supervised all the processes in the experiments and the manuscript preparation. All authors have read and approved the final manuscript. W.K. and R.M. contributed equally to this work.

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Disclosure statement

No potential conflict of interest was reported by the authors.

References

Akihisa T, Yasukawa K, Oinuma H *et al.* Triterpene alcohols from the flowers of Compositae and their anti-inflammatory effects. *Phytochemistry* 1996;**43**:1255-60.

Andersen FA, Bergfeld WF, Belsito DV *et al.* Final report of the Cosmetic Ingredient Review Expert Panel amended safety assessment of *Calendula officinalis*-derived cosmetic ingredients. *Int J Toxicol* 2010;**29**:221S-43S.

Arora D, Rani A, Sharma A. A review on phytochemistry and ethnopharmacological aspects of genus *Calendula*. *Pharmacogn Rev* 2013;**7**:179-87.

Bakó E, Deli J, Tóth G. HPLC study on the carotenoid composition of *Calendula* products. *J Biochem Biophys Methods* 2002;**53**:241-50.

Bohlmann F, Abraham WR. Naturally occurring terpene derivatives. Part 188. A new sesquiterpene alcohol and other constituents from *Doronicum pardalianches*. *Phytochemistry* 1979;**18**:668-71.

Carvalho AR, Diniz RM, Suarez MAM *et al.* Use of some Asteraceae plants for the treatment of wounds: from ethnopharmacological studies to scientific evidences. *Front Pharmacol* 2018;**9**:1-11.

Himaya SWA, Ryu BM, Qian ZJ *et al.* Paeonol from *Hippocampus kuda* Bleeler suppressed the neuro-inflammatory responses *in vitro* via NF-κb and MAPK signaling pathways. *Toxicol Vitr* 2012;**26**:878-87.

Inui S, Hosoya T, Yoshizumi K *et al.* Phytochemical and anti-inflammatory properties of Senegalese propolis and isolated compounds. *Fitoterapia* 2021;**151**:104861.

Jakupovic J, Zdero C, Paredes L *et al.* Sesquiterpene glycosides and other constituents from *Osteospermum* species. *Phytochemistry* 1988;**27**:2881-6.

Kurata T, Misawa N, Hosoya T *et al.* Isolation and identification of components from Wasabi (*Wasabia japonica* Matsumura) flowers and investigation of their antioxidant and anti-inflammatory activities. *Food Sci Technol Res* 2019;**25**:449-57.

Lee AS, Ellman MB, Yan D *et al.* A current review of molecular mechanisms regarding osteoarthritis and pain. *Gene* 2013;**527**:440-7.

Muley BP, Khadabadi SS, Banarase NB. Phytochemical constituents and pharmacological activities of *Calendula officinalis* Linn (Asteraceae): a review. *Trop J Pharm Res* 2009;**8**:455-65.

Nizyński B, Alsoufi ASM, Pączkowski C *et al*. The content of free and esterified triterpenoids of the native marigold (*Calendula officinalis*) plant and its modifications in *in vitro* cultures. *Phytochem Lett* 2015;**11**:410-7.

Śliwowski J, Dziewanowska K, Kasprzyk Z. Ursadiol: a new triterpene diol from *Calendula officinalis* flowers. *Phytochemistry* 1973;**12**:157-60.

Svouraki A, Garscha U, Kouloura E *et al*. Evaluation of dual 5-lipoxygenase/microsomal prostaglandin E2 synthase-1 inhibitory effect of natural and synthetic acronychia-type isoprenylated acetophenones. *J Nat Prod* 2017;**80**:699-706.

Ukiya M, Akihisa T, Yasukawa K, Tokuda H *et al*. Anti-inflammatory, anti-tumor-promoting, and cytotoxic activities of constituents of marigold (*Calendula officinalis*) flowers. *J Nat Prod* 2006;**69**:1692-6.

Wilkomirski B, Kasprzyk Z. Free and ester-bound triterpene alcohols and sterols in cellular subfractions of *Calendula officinalis* flowers. *Phytochemistry* 1979;**18**:253-5.

Wojciechowski Z, Jelonkiewicz-Konador A, Tomaszewski M *et al*. The structure of glycosides of oleanolic acid isolated from the roots of *Calendula officinalis*. *Phytochemistry* 1971;**10**:1121-4.