RESEARCH ARTICLE

Anti-osteoclastogenic diacetylenic components of *Dendropanax morbifera*

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Abstract Methanol (MeOH) extract of the aerial parts of *Dendropanax morbifera* (Araliaceae) has demonstrated a significant dose-dependent inhibitory effect on the RANKL-induced differentiation of bone marrow-derived macrophages to osteoclasts. Bioassay-guided fractionation of the extract resulted in the isolation of a novel diacetylene carboxylic acid (**1**), together with a known diacetylenic compounds (**2**) as phytochemicals to strongly inhibit the osteoclast differentiation. The chemical structure of **1** was determined by spectroscopic analyses as (9Z,16S)-16-O-acetyl-9,17-octadecadiene-12,14-diynoic acid, that is acetyl derivative of **2**. Two diacetylenic components of *D. morbifera*, **1** and **2** exhibited a dose-dependent inhibitory effect on the RANKL-induced formation of tartrate-resistant acid phosphatase-positive multinucleated cells with IC_{50} values of 2.4 and 3.1 μM, respectively. Seven other known components (**3–9**) were also isolated from the extract: dendropanoxide (**3**), friedelin (**4**), epifriedelanol (**5**), α-amyrin (**6**), β-amyrin (**7**), β-sitosterol (**8**), and stigmasterol (**9**). The significant anti-osteoclastogenic activities of **3**, **4**, **5**, and **7** were first reported in this study.

Keywords *Dendropanax morbifera* · Araliaceae · Diacetylene · Osteoclast differentiation

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Introduction

Dendropanax morbifera (Araliaceae) is a subtropical broad-leaved evergreen tree endemic to Korea. The tree is widely distributed in the southern part of the Korean peninsula and Jeju island (Han et al. 1998) and produces a resinous sap that can be used as a varnish. In addition, the leaves and stems of this plant have been used in folk medicine for the treatment of headache, skin diseases, and infection (Park et al. 2004). Recently, biologists and pharmacologists have paid much attention to this plant because it has been reported to have many beneficial effects on human health, such as anti-cancer, anti-oxidant, anti-complement, and anti-diabetic activities (Chung et al. 2011; Moon 2011; Hyun et al. 2013; Kim et al. 2015). In particular, some diacetylenic components such as (3S,8S)-falcarindiol, (3S)-diyene, and (9Z,16S)-16-hydroxy-9,17-octadecadiene-12,14-diynoic acid of *Dendropanax* genus have been reported to be active components responsible for the anti-complement and anti-inflammatory activities (Park et al. 2004; Chung et al. 2011; Chien et al. 2014).

An osteoclast is a type of bone cell that breaks down bone tissue. It is a large multinucleated cell generated originally from the monocytes/macrophages of hematopoietic stem cells. Two essential cytokines, macrophage colony-stimulating factor (M-CSF) and receptor activator of nuclear factor-κB ligand (RANKL), are known

to be involved in osteoclastogenesis, which induces the differentiation of bone marrow-derived macrophages (BMMs) into tartrate-resistant acid phosphatase-positive multinucleated cells (TRAP$^+$-MNCs) (Suda et al. 1999; Boyle et al. 2003). Therefore, a pharmaceutical trial to discover potent substances capable of inhibiting osteoclast differentiation, particularly from natural resources, could be a promising therapeutic strategy for preventing and/or treating bone disorders and related bone fractures (Del Fattore et al. 2008).

During the investigation to identify traditional Korean herbal medicines with anti-osteoclastogenic activity, the methanol extract of aerial parts of *D. morbifera* was found to potently inhibit the RANKL-induced differentiation of BMMs into TRAP$^+$-MNCs (Fig. 1). Therefore, we conducted extensive phytochemical studies of the extract of *D. morbifera*, which finally resulted in the isolation of two diacetylene carboxylic acids, (9Z,16S)-16-O-acetyl-9,17-octadecadiene-12,14-diynoic acid (**1**) and (9Z,16S)-16-

hydroxy-9,17-octadecadiene-12,14-diynoic acid (**2**), as active components responsible for the inhibitory effect on the RANKL-induced differentiation of BMMs into TRAP$^+$-MNCs (Fig. 2).

Materials and methods

Plant material

Dendropanax morbifera was cultivated in a greenhouse at Hadong Kyungnam (Korea) and the aerial parts collected in July 2015 and identified by Dr. Jo Chang Soo. A voucher specimen (KR1507) was deposited at the herbarium of the Korea Research Institute of Chemical Technology.

General instrumental procedures

NMR spectra were obtained using a Bruker AM 500 spectrometer using TMS as an internal standard for ^{1}H NMR, ^{13}C NMR, DEPT, HMQC, and HMBC. High-resolution electrospray ionization (HRESIMS) and electron impact (EI) mass spectra were obtained using a Q-Tof micro LC–MS/MS instrument (Waters, Milford, MA, USA) and Varian CP3800-1200L mass spectrometry (Agilent, Lexington, MA, USA), respectively. Preparative-HPLC was performed on a Futecs P-4000 system with a Shim-pack prep-ODS(H) kit column (5 μm, 20 mm × 25 cm). Isolation and purification were carried out using a medium-pressure liquid chromatography (MPLC) system (BUCHI pump Module C-601, silica gel 60, 230–400 mesh, Merck Millipore Sigma, Burlington, MA, USA).

Extraction and isolation

The dried aerial parts (5 kg) of *D. morbifera* were soaked twice in 50 L of methanol (MeOH) at room temperature for 7 days. The concentrated MeOH extract (950 g) was suspended in 10 L of distilled water and then extracted sequentially with equal volumes of ethylacetate (EtOAc) and *n*-butanol (*n*-BuOH), which resulted in 258 g of the EtOAc soluble fraction and 159 g of the *n*-BuOH soluble fraction. The EtOAc fraction (220 g) was subjected to silica gel column (Ø = 15.0 × 60 cm) chromatography with 2 L of MeOH in dichloromethane solution (0, 2, 10, and 50%) in a stepwise gradient manner to obtain four fractions: E1–E4.

E3 (34 g) was subjected to silica gel column chromatography and eluted with EtOAc in *n*-hexane solution (10–50%) in a stepwise gradient manner to obtain five sub-fractions: E31–E35. E34 was further purified by prep-HPLC (Shim-pack, 70% MeOH) to yield 201.0 mg of **1** and 112.0 mg of **2** (Fig. 2). E32 was purified by silica gel
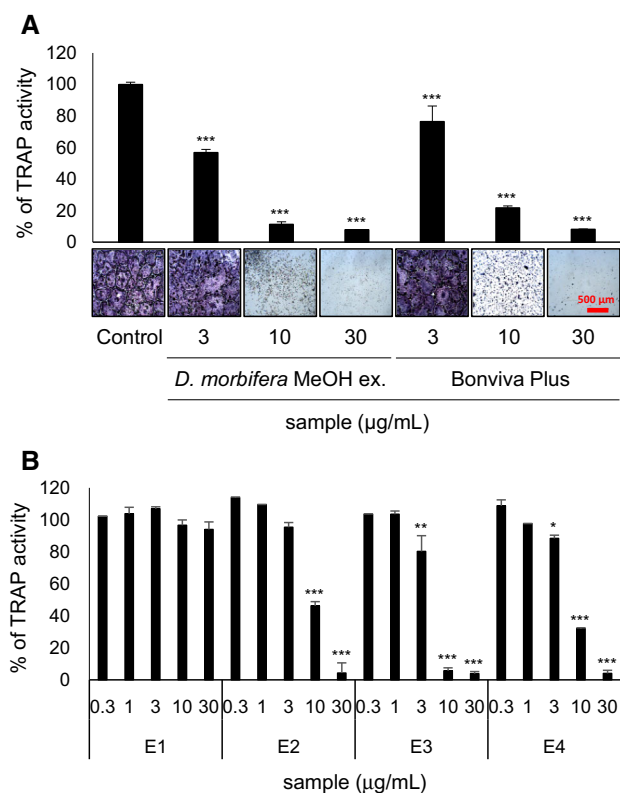

Fig. 1 Anti-osteoclastogenic activity of the *D. morbifera* MeOH extract (**a**) and EtOAC fractions, E1–E4 (**b**). BMMs (1 × 10^4 cells/well) were cultured with M-CSF (30 ng/mL) and RANKL (10 ng/mL) in the presence of 0.1% DMSO (vehicle control), *D. morbifera* MeOH extract, Bonviva Plus or EtOAC fractions for 4 days. Multinucleated cells were fixed with 4% formalin, permeabilized with 0.1% triton X-100 in PBS, and stained with TRAP solutions. The stained cells were photographed under a light microscope and the TRAP activity measured at 405 nm. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$
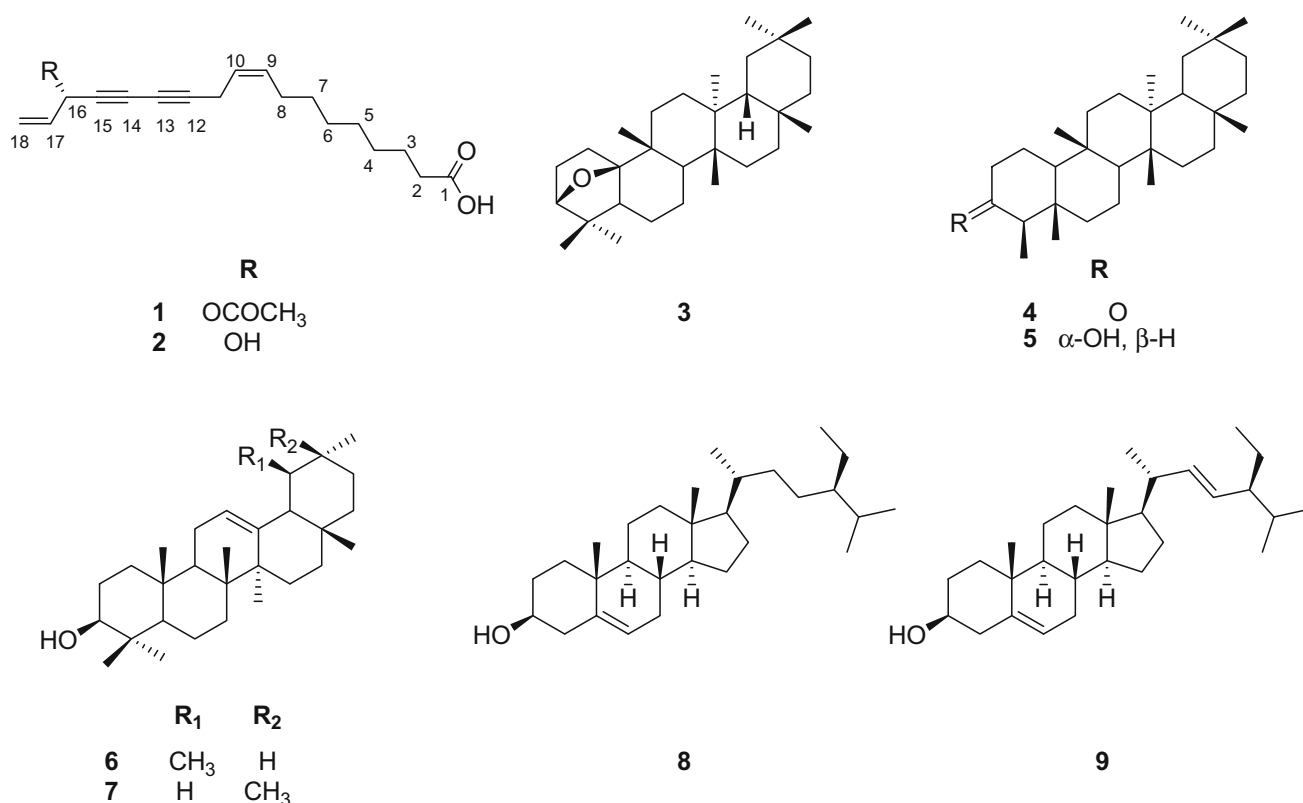
Fig. 2 Phytochemicals (**1**–**9**) isolated from the *D. morbifera* MeOH extract

column chromatography and eluted with 10% EtOAc in *n*-hexane solution to yield **8** and **9**.

E2 (20 g) was rechromatographed on silica gel and eluted with EtOAc in *n*-hexane solution (2–50%) in a stepwise gradient manner to obtain five sub-fractions: E21–E25. E21 was further purified through silica gel column chromatography and eluted with 3% MeOH in MC solution to yield 10.0 mg of **3** and 14.5 mg of **4**. E22 was dissolved in EtOAc and allowed to crystallize at room temperature to yield 120.0 mg of **5**. The remaining filtrate of E22 was fractionated into five sub-fractions (E221–E225) using silica gel column chromatography and eluted with EtOAc in *n*-hexane in a stepwise gradient manner. (3–50%). E224 was crystallized in EtOAc at room temperature to yield a mixture (490.7 mg) of **6** and **7**.

(9Z,16S)-16-O-acetyl-9,17-octadecadiene-12,14-diynoic acid (1): Viscous liquid; $[\alpha]_D^{20}$-43.6 (*c* 0.50, CH$_3$OH); ^{1}H NMR (CDCl$_3$, 500 MHz); ^{13}C NMR (CDCl$_3$, 125 MHz) (Table 1); HRESIMS *m/z* 330.1825 (calculated for C$_{20}$H$_{26}$O$_4$, 330.1831).

Cell culture and osteoclast differentiation

All cell culture materials were purchased from HyClone (Logan, UT, USA). Bone marrow-derived cells (BMCs) were obtained from 5-week-old male ICR mice (Damool Science, Daejeon, Korea) by flushing the femurs and tibias with α-MEM supplemented with antibiotics (100 units/mL penicillin and 100 μg/mL streptomycin). BMCs were cultured in α-MEM supplemented with 10% fetal bovine serum (FBS) with 30 ng/mL of mouse recombinant M-CSF (R&D Systems, Minneapolis, MN, USA) for 1 day. After 1 day, non-adherent BMCs were plated on a Petri dish and cultured for 3 days in the presence of M-CSF (30 ng/mL). After non-adherent cells were washed out, adherent cells were used as BMMs. For the induction of osteoclastogenesis, BMMs were cultured in the presence of 10 ng/mL of mouse recombinant RANKL (R&D Systems) and 30 ng/mL of M-CSF for 4 days to differentiate them into mature TRAP$^+$-MNCs. All experimental procedures for BMC cultures and osteoclast differentiation were performed in strict accordance with the recommendations in the Standard Protocol for Animal Study of Korea Research Institute of Chemical Technology (KRICT; No. 2012-7D-02-01). The protocol (ID No. 7D-M1) was approved by the Institutional Animal Care and Use Committee of KRICT.

TRAP staining and activity assay

For the induction of osteoclastogenesis, isolated BMMs (1 × 10^4 cells/well) were cultured with M-CSF (30 ng/

Table 1 ^{1}H, ^{13}C NMR spectroscopic data of **1** and **2** in CDCl$_3$

Position	1 (δ_H)	2 (δ_H)	1 (δ_C)	2 (δ_C)
1			179.3	179.5
2	2.37, t (J = 7.5 Hz)	2.36, t (J = 7.5, 7.4 Hz)	33.9	34.1
3	1.66, m	1.65, m	24.6	24.7
4	1.36, m	1.37, m	28.9	28.9
5	1.36, m	1.37, m	29.1	29.1
6	1.36, m	1.37, m	29.1	29.1
7	1.36, m	1.37, m	29.1	29.1
8	2.04, dt (J = 7.2, 6.9 Hz)	2.05, dt (J = 7.2, 6.7 Hz)	27.1	27.1
9	5.52, m	5.52, m	132.3	132.9
10	5.40, m	5.40, m	121.9	122.0
11	3.05, d (J = 6.9 Hz)	3.04, d (J = 7.0 Hz)	17.7	17.7
12			80.4	80.1
13			70.8	71.2
14			64.6	64.1
15			71.9	74.3
16	4.92, d (J = 5.8 Hz)	4.93, d (J = 5.4 Hz)	64.0	63.4
17	5.89, ddd (J = 17.5, 10.3, 5.8 Hz)	5.95, ddd (J = 17.0, 10.2, 5.4 Hz)	133.0	136.1
18	5.56, d (J = 17.5 HZ)	5.48, d (J = 17.0 HZ)	119.5	117.0
	5.35, d (J = 10.3 HZ)	5.25, d (J = 10.2 HZ)		
19 (C=O)			169.6	
20 (OCOCH$_3$)	2.12, s		20.9	

mL) and RANKL (10 ng/mL) in the presence of 0.1% DMSO (vehicle control) or the test sample for 4 days. Extracts and chemicals were reconstructed to 30 mg/mL and 30 mM DMSO stock solution, and diluted to 1:1000 with the culture medium. Then, cells were fixed with 3.7% formalin for 5 min and permeabilized with 0.1% Triton X-100 for 10 min before staining with TRAP solution (Sigma-Aldrich, St. Louis, MO, USA) for 10 min to visualize TRAP$^+$-MNCs. To measure TRAP activity, cells were fixed with 3.7% formalin for 5 min, permeabilized with 0.1% Triton X-100 for 10 min, and treated with TRAP buffer (100 mM sodium citrate pH 5.0, 50 mM sodium tartrate) containing 3 mM *p*-nitrophenyl phosphate (Sigma-Aldrich) at 37 °C for 5 min. Reaction mixtures were transferred into new plates containing an equal volume of 0.1 N NaOH and optical density (OD) measured at 405 nm. TRAP activity was performed in triplicate, and commercially available Bonviva Plus® (ibandronate sodium) was used as a reference drug.

Cytotoxicity assay

BMMs were plated in a 96-well plate in triplicate at a density of 1×10^4 cells/well for 24 h. The cells were then treated with isolated constituents **1** and **2** for 3 days. Compounds **1** and **2** were reconstructed to 30 mM DMSO stock solution, and diluted to 1:1,000 with the culture medium. Therefore, 0.1% DMSO was used as the vehicle

control. The supernatants of cultured media were transferred to a new 96-well plate. Lactate dehydrogenase (LDH) activity was measured in triplicate using the Cyto-Tox96 Non-Radioactive Cytotoxicity Assay (Promega, Madison, WI, USA) according to the manufacturer's protocol. Absorbance was measured using the Hidex sense beta plus microplate reader (Hidex, Turku, Finland) at 490 nm.

Statistical analysis

All quantitative values are presented as the mean ± SD. Statistical differences were analyzed using Student's *t* test. $P < 0.05$ was considered significant.

Results

Anti-osteoclastogenic activity of the MeOH extract of *D. morbifera*

The MeOH extract obtained from the aerial parts of *D. morbifera* dose-dependently inhibited the formation of TRAP$^+$-MNCs, with the concentration required for a 50% inhibitory effect (IC$_{50}$) on TRAP activity being 3.49 μg/mL (Fig. 1a). The reference drug, Bonviva Plus®, also dose-dependently inhibited the formation of TRAP$^+$-MNCs, with an IC$_{50}$ of 6.37 μg/mL.

Identification of phytochemicals in *D. morbifera*

Next, extensive phytochemical investigation of *D. morbifera* extract was conducted to identify the active ingredients responsible for its anti-osteoclastogenic property. E3 (IC_{50} on TRAP activity; 5.84 μg/mL) exhibited the most potent anti-osteoclastogenic activity, and E4 (7.76 μg/mL) and E2 (9.46 μg/mL) also demonstrated anti-osteoclastogenic activities (Fig. 1b). In this study, E3 and E2 were further subjected to repeated column chromatography, which finally led to the isolation of two active components, **1** and **2**, as well as other constituents (**3–9**) (Fig. 2).

Compound **1** was obtained as a viscous liquid. The molecular formula was determined to be $C_{20}H_{26}O_4$ at *m/z* 330.1825 (calc. 330.1831) $[M+Na]^+$ by HRESIMS. The ^1H NMR spectra of **1** exhibited a similar pattern to that of **2**. The ^1H NMR spectrum of **2** showed signals for five olefinic protons at δ_H 5.95 (ddd, *J* = 17.0, 10.2, 5.4 Hz), δ_H 5.52 (m), δ_H 5.48 (d, *J* = 17.0 Hz), δ_H 5.40 (m) and δ_H 5.25 (d, *J* = 10.2 Hz), a methine signals at δ_H 4.93 (d, *J* = 5.4 Hz), and fourteen aliphatic protons at δ_H 3.04 (d, *J* = 7.0 Hz, 2H), δ_H 2.36 (t, *J* = 7.5, 7.4 Hz, 2H), δ_H 2.05 (dd, *J* = 7.2, 6.7 Hz, 2H), δ_H 1.65 (m, 2H) and δ_H 1.37 (m, 8H). When the ^1H NMR spectrum of **1** was compared with that of **2**, an acetoxy signal newly observed at δ_H 2.12 (s, 3H) in **1**. By comparing the ^{13}C spectrum of **1** with that of **2**, additional acetoxy signals at δ_C 169.6 and δ_C 20.9 were observed for **1**. In the HMBC spectrum, the typical correlation between H-16 (δ_H 5.92) and C-19 (δ_C 169.6) indicated that the acetoxy group was attached at C-16, and that the hydroxyl group at the C-16 position of **2** was replaced with an acetoxy group to become **1**. The absolute stereochemistry at C-16 of **2** was reported to be an *S*-configuration, a compound from *D. morbifera* (Park et al. 2004). In this study, **1** and **2** had a negative optical rotation value, $[\alpha]_D^{20}$-43.6 (*c* 0.50, CH₃OH) and $[\alpha]_D^{20}$-15.9 (*c* 0.25, CH₃OH), suggesting the absolute stereochemistry at C-16 of **1** to be *S*-configuration. To the best of our knowledge, **1** had never been reported in this species, or even from other natural plant sources. However, **1** appears to be an artefact derived from **2** rather than a genuine novel constituent of the species, as **1** is easily obtained by acetylation of **2** with pyridine and acetic anhydride at room temperature. The chemical structure and stereochemistry of **2** was determined to be (9Z,16S)-16-hydroxy-9,17-octadecadiene-12,14-diynoic acid in a direct comparison to spectroscopic data in the literature (Park et al. 2004). Thus, the chemical structure of **1** was established to be (9Z,16S)-16-O-acetyl-9,17-octadecadiene-12,14-diynoic acid (Fig. 2). Both compounds **1** and **2** were somewhat unstable, even with refrigeration, and there was some decomposition into

insoluble matter in MeOH at room temperature within a month.

The other isolated components (**3–9**) were identified as dendropanoxide (**3**) (Motoo et al. 2005), friedelin (**4**) (Trinh et al. 2007), epifriedelanol (**5**) (Trinh et al. 2007), α-amyrin (**6**) (Liliana et al. 2012), β-amyrin (**7**) (Liliana et al. 2012), β-sitosterol (**8**) (Virgilio et al. 2015), and stigmasterol (**9**) (Virgilio et al. 2015) by comparing their spectroscopic data to the literature (Fig. 2).

Anti-osteoclastogenic activity of phytochemicals in *D. morbifera*

Since Bonviva Plus® is a mixture of vitamin D3 (or cholecalciferol; 240 mg) and ibandronate sodium (168 mg), the effects of all isolated components (**1–9**) on the RANKL-induced formation of TRAP⁺-MNCs were evaluated at 30 μM compared to those of vitamin D3 and ibandronate sodium (Fig. 3). Both of vitamin D3 and ibandronate sodium used as the positive controls strongly inhibited the TRAP activity, and also two diacetylenic fatty acids (**1** and **2**) strongly inhibited the TRAP activity. Compounds **3**, **4**, **5**, and **7** also significantly inhibited the TRAP activity, but in the following dose-dependent experiments, the anti-osteoclastogenic activities of **1** and **2** were confirmed by TRAP staining and measuring its activity. Both **1** and **2** dose-dependently inhibited the RANKL-induced formation of TRAP⁺-MNCs (Fig. 4a) and TRAP activity (Fig. 4b). The IC_{50} values of **1** and **2** for
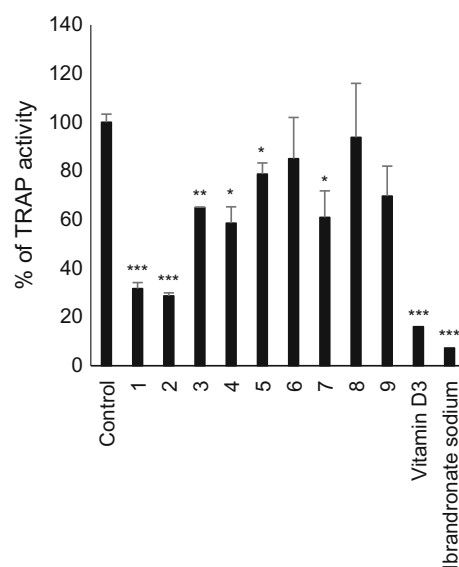

Fig. 3 Effects of phytochemicals isolated from *D. morbifera* MeOH extract on osteoclast differentiation. BMMs (1×10^4 cells/well) were cultured with M-CSF (30 ng/mL) and RANKL (10 ng/mL) in the presence of 0.1% DMSO (vehicle control) or 30 μM of other isolated phytochemicals (**1–9**) for 4 days. The TRAP activity of osteoclasts was measured at 405 nm. *p < 0.05; **p < 0.01; ***p < 0.001
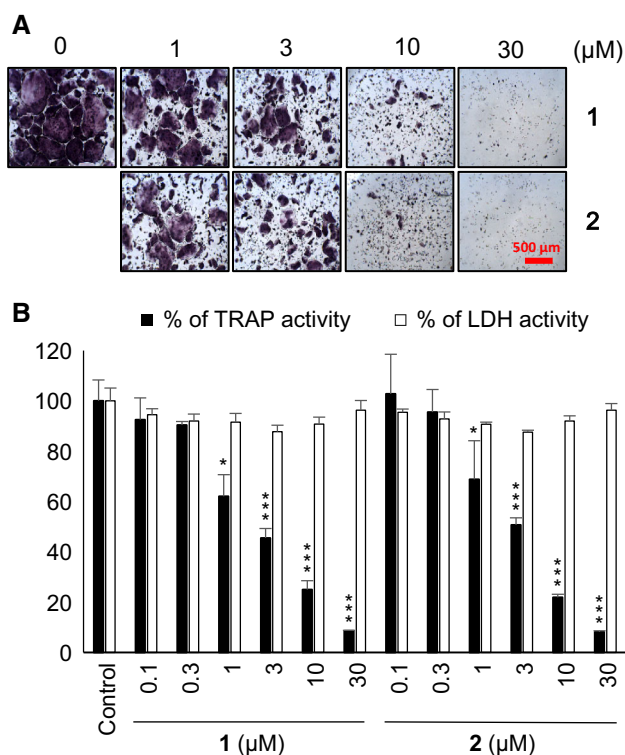
Fig. 4 Anti-osteoclastogenic activity of compounds **1** and **2**. BMMs (1×10^4 cells/well) were cultured with M-CSF (30 ng/mL) and RANKL (10 ng/mL) in the presence of 0.1% DMSO (vehicle control) or the indicated compounds for 4 days. Multinucleated cells were fixed, permeabilized, and stained with TRAP solutions. The stained cells were photographed under a light microscope (**a**) and the TRAP activity of osteoclasts measured at 405 nm (**b**; black bar). The cytotoxicity of the compounds was also evaluated (**b**; white bar). $*p < 0.05$; $***p < 0.001$

TRAP activity were calculated to be 2.41 and 3.11 μM, respectively (Fig. 4b). They did not exhibit a distinct cytotoxic effect on BMMs below 30 μM, suggesting that their anti-osteoclastogenic activity was not due to their potential to inhibit the survival of BMMs.

Discussion

In this study, we found anti-osteoclastogenic activity of the MeOH extract of aerial parts of *D. morbifera* with an IC_{50} for TRAP activity comparable to that of Bonviva Plus®. Bonviva Plus® is the most widely used medicine in clinics; it contains ibandronate sodium monohydrate and concentrated cholecalciferol at a ratio of 1:1. The anti-osteoclastogenic activity of ibandronate has been well reported in vitro (Zhang et al. 2013).

Further bioassay-guided fractionation of the extract resulted in the isolation of a new diacetylene carboxylic acid (**1**) and related congener (**2**) as active principles responsible for the inhibitory effect on osteoclast differentiation. Compounds **1** and **2** exhibited potent inhibitory effects on the RANKL-induced differentiation of BMMs into TRAP⁺-MNCs in a dose-dependent manner. Compounds **1** and **2** are diacetylene carboxylic acids, generally called plant polyacetylenes, and consist of at least two triple carbon–carbon bonds, which are frequently produced by higher plants of the Apiaceae and Araliaceae families. More than 1100 different acetylenes and biogenetically related substances have been identified in plants. The most common polyacetylenes are falcarinol-type aliphatic C_{17}-polyacetylenes, which are produced from C_{18}-polyacetylene carboxylic acid by decarboxylation (Christensen and Brandt 2006; Dawid et al. 2015) and have been reported to have a variety of health-promoting metabolic effects as well as potential anti-cancer, anti-fungal, and anti-inflammatory properties.

Compounds **3**, **4**, **5**, and **7** also significantly inhibited TRAP activity at 30 μM. Compound **3** exhibits several biological activities, including antiplasmodial activity (Chung et al. 2009), antidiabetic activity (Moon 2011), and autophagy-inducing activity (Lee et al. 2013). Compound **4** also exhibits several biological activities, including antioxidant, anti-inflammatory, analgesic, and antipyretic activities (Antonisamy et al. 2011; Sunil et al. 2013). Several biological activities of compounds **5** and **7,** such as anti-cancer activity and anti-inflammatory activity (Holanda Pinto et al. 2008), have also been reported. However, this is the first report of their anti-osteoclastogenic activity, though their anti-osteoclastic potential was relatively less than that of compounds **1** and **2**.

In summary, *D. morbifera* exhibited anti-osteoclastogenic activity, and its potential could be due to several anti-osteoclastogenic phytochemicals. Compound **1**, a novel phytochemical, showed anti-osteoclastogenic activity, as well as that of known compounds **2**, **3**, **4**, **5**, and **7**, were first reported in this study, suggesting that the anti-osteoclastogenic activity of *D. morbifera* and its phytochemicals could serve as novel natural resources for treating osteoclast-related disorders, such as osteoporosis, rheumatoid arthritis, periodontal disease, and cancer bone metastasis.

Compliance with ethical standards

Conflict of interest The authors have declared no conflicts of interest.

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