SHORT REPORT

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A New Diterpenoid with Antitumor Cytotoxicity from Millipede

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Abstract: A new diterpenoid, namely millipedine A (**10**), along with nine known compounds (**1**–**9**), were isolated from the methanol extract of Millipede. Their structures were established on the basis of spectroscopic analysis including one and two-dimensional NMR spectroscopy and comparison with previously reported data. New compound showed moderate cytotoxicity against A549, HCT-116, and SW1990 cell lines in MTT assay.

Keywords: Millipede; diterpenoid; antitumor activities.

1. Insect Source

Millipede is a traditional Chinese medicine, derived from dried worm of the *Myriopoda*, distributed mainly among Gansu, Guangxi, and Sichuan provinces of China [1]. The experimental sample was purchased from Bozhou City, Anhui province, People's Republic of China, in November 2018, and identified as Millipede by Prof. Xiao-Fei Li, Zunyi Medical University. A voucher specimen (ZMCNo. 20181021) of the worm was deposited at the herbarium of the School of Pharmacy, Zunyi Medical University.

2. Previous Studies

The antitumor pharmacological activities of diplopoda family have been reported [2-4]. As far as we know, no systematic chemical component investigations have been reported so far for Millipede.

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3. Present Study

Dried and powdered whole parts of Millipede (5.8 kg) was extracted with 98% methanol under reflux three times (each 3 h) to give an extract (176.0 g), which was suspended in H_2O (2 L) and extracted with cyclohexane (3×2 L), ethyl acetate (3×2 L) and *n*-butanol (3×2 L) successively. After removing the solvent to obtain the cyclohexane extract (70.0 g), the ethyl acetate extract (25.02 g) and the *n*-butanol extract (23.84 g), respectively. The cyclohexane extract and ethyl acetate extract was combined and separated by silica gel medium pressure column chromatography (CC) (70×460 mm, petroleum ether-ethyl acetate 20:1→0:1) to give eleven fractions (Fr.1–11). Fr.5 was purified by recrystallization to give cholesterol (11.8 mg) [7]. Fr.4 was separated by silica gel medium pressure CC (26×460 mm, petroleum ether-ethyl acetate 10:1→0:1) to give eight sections (Fr.4.1–4.8). Fr.4.3 was separated by silica gel medium pressure CC (26×460 mm, petroleum ether-ethyl acetate 20:1→5:1) to give five subfractions (Fr.4.3.1–4.3.5). Fr.4.3.2 was purified by semi-preparative HPLC eluted with 96% methanol (1.0 mL/min) to obtain cholest-4-en-3-one (7.3 mg, t_R 23.0 min) [8]. Fr.4.4 was further separated by semi-preparative HPLC eluted with 95% methanol (1.2 mL/min) to obtain ergosta-4,6,8(14),22-tetraen-3-one (6.9 mg, t_R 12.2 min) [9]. Fr.9 was purified by Sephadex LH-20 CC to afford five subfractions (Fr.9.1–9.5). Fr.9.4 was further separated by semi-preparative HPLC eluted with 83% methanol (6.0 mL/min) to afford three subfractions (Fr.9.4.1–9.4.3). Fr.9.4.3 was purified by normal phase HPLC eluted with 83% *n*-hexane and 17% isopropanol (3.0 mL/min) to obtain cholest-5-en-3β-ol-7-one (19.0 mg, t_R 9.2 min) [7]. Fr.9.4.1 was further separated by semi-preparative HPLC eluted with 82% methanol (6.0 mL/min) to yield aurantiamide acetate (9.6 mg, t_R 5.9 min) [10]. Fr.9.5 was separated by Sephadex LH-20 CC to afford seven subfractions (Fr.9.5.1–9.5.7). Fr.9.5.3 was further separated by semi-preparative HPLC eluted with 96% methanol (25.0 mL/min) to obtain six subfractions (Fr.9.5.3.1–9.5.3.6). Fr.9.5.3.6 was purified by semi-preparative HPLC eluted with 91% methanol (4.0 mL/min) to give four subfractions (Fr.9.5.3.6.1–9.5.3.6.4). Fr.9.5.3.6.4 was further separated by semi-preparative HPLC eluted with 90% methanol (0.8 mL/min) to yield 3-[(*Z*)-octadec-9-enoxy]propane-1,2-diol (25.3 mg, t_R 12.6 min) [11].

The *n*-butanol extract (23.84 g) was separated by silica column (100×800 mm, petroleum ether-ethyl acetate 5:1→0:1) to give three fractions (Fr.A–C). Fr.2 was purified by Sephadex LH-20 CC to afford three subfractions (Fr.B.1–B.3). Fr.2.2 was separated by semi-preparative HPLC eluted with 70%→80%→90%→100% methanol (20.0 mL/min) to give eight sections (Fr.B.2.1–B.2.8). Fr.B.2.8 was purified by recrystallization to yield palmitic acid (14.0 mg) [12]. Fr.C was fractionated by semi-preparative HPLC eluted with 40%→80%→100% methanol (20.0 mL/min) to afford six subfractions (Fr.C.1–C.6). 1*H*-indole-3-carbaldehyde (9.9 mg) [13] was obtained from Fr.C.5 by repeated crystallization. Fr.C.4 was further separated by semi-preparative HPLC eluted with 20% methanol (4.0 mL/min) to yield *p*-hydroxybenzaldehyde (3.9 mg, t_R 11.0 min) [14]. Fr.C.6 was separated by Sephadex LH-20 CC to afford four subfractions (Fr.C.6.1–C.6.4). Fr.C.6.4 was purified by recrystallization to yield compound **10** (7.3 mg).

Millipedine A ***(10)***: white powder; $[\alpha]_D^{20}$ = + 7.49 (*c* = 0.08, MeOH); IR ν_{max} (KBr): = 3400, 2925, 1600 cm^{-1}; ^1H NMR (600 MHz, $CDCl_3$): δ (ppm) = 0.94 (3H, s, 19-CH_3), 0.99 (3H, s, 18-CH_3), 1.18 (3H, s, 20-CH_3), 1.27 (1H, m, H-5), 1.28 (1H, m, H-1), 1.28 (1H, m, H-3), 1.32 (3H, d, *J* = 7.1 Hz, 17-CH_3), 1.33 (3H, d, *J* = 7.1 Hz, 16-CH_3), 1.64 (1H, m, H-6), 1.83 (1H, ddd, *J* = 12.3, 4.1, 2.2 Hz, H-3), 1.91 (1H, m, H-6), 2.55 (1H, m, H-1), 2.75 (1H, m, H-7), 2.95 (1H, m, H-7), 3.26 (1H, m, H-15), 4.01 (1H, tt, *J* = 11.5, 4.1 Hz, H-2), 6.50 (1H, d, *J* = 8.5 Hz, H-11), 6.98 (1H, d, *J* = 8.5 Hz, H-12); ^{13}C NMR (150 MHz, $CDCl_3$): δ (ppm) = 19.2 (CH_2, C-6), 20.5 (CH_3, C-16 and C-17), 22.7 (CH_3, C-19), 26.3 (CH_3, C-20), 27.5 (CH, C-15), 28.7 (CH_2, C-7), 33.5 (CH_3, C-18), 34.9 (C, C-4), 39.6 (C, C-10), 48.9 (CH_2, C-1), 49.1 (CH, C-5), 51.0 (CH_2, C-3), 66.1 (CH, C-2), 114.6 (CH, C-11), 122.9 (CH, C-12), 131.4 (C, C-13), 133.7 (C, C-8), 142.3 (C, C-9), 152.4 (C, C-14); HR-APCI-MS: *m/z* 301.2163 $[M–H]^-$ (calcd. 301.2168 for $C_{20}H_{29}O_2^-$).

Cell Viability Assay: A549, HCT-116 and SW1990 cell lines was obtained from the Cell Bank of the Zunyi Medical University. The cells were maintained in DMEM containing 10% fetal bovine serum

(Taixin Bio Technol Co., Beijing, China) at 37 °C in a humidified incubator containing 5% CO_2. The MTT assay was used to evaluate A549, HCT-116 and SW1990 cells viability. Briefly, A549, HCT-116 and SW1990 cells were plated in 96-well plates (500 cells/well) for 12 h and then incubated with compound **10** in various concentrations for an additional 48 h. Then, the prepared MTT solution (20 μL, 5 mg/mL) was added, and the cells were incubated for another 2 h. After the formazan that formed was fully dissolved in DMSO and the absorbance was read at 490 nm on a microplate reader. Data were analyzed using GraphPad Prism 6 software, and nonlinear regression analysis (dose-response) was used to determine the IC_{50}.

The diterpenoid compound millipedine A (**10**) (Figure 1) was obtained from the methanol extract of Millipede by semi-preparative HPLC purification and recrystallization.

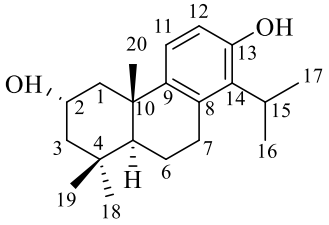

Figure 1. The chemical structure of millipedine A (**10**)

Compound **10,** millipedine A, was isolated as white powder, its molecular formula was determined to be $C_{20}H_{30}O_2$ on the basis of HR-APCI-MS at *m/z* 301.2163 $[M–H]^-$, (calcd for $C_{20}H_{29}O_2^-$, 301.2168), indicating 6 degrees of unsaturation. The IR spectrum of **10** showed absorption bands for hydroxyl group (3400 cm^{-1}) [15].

The ^1H NMR spectrum showed the presence of an AB system aromatic ring signals at δ_H 6.98 (1H, d, J = 8.5 Hz) and 6.50 (1H, d, J = 8.5 Hz), a isopropyl signals attached to the benzene ring at δ_H 1.33 (3H, d, J = 7.1 Hz), 1.32 (3H, d, J = 7.1 Hz), and 3.26 (1H, m), three methyl signals at δ_H 0.99, 0.94, and 1.18 (each 3H, s), four methylene signals at δ_H 1.28 (1H, m, H-1), 2.55 (1H, m, H-1), 1.28 (1H, m, H-3), 1.83 (1H, ddd, J = 12.3, 4.1, 2.2 Hz, H-3), 1.64 (1H, m, H-6), 1.91 (1H, m, H-6), 2.75 (1H, m, H-7), and 2.95 (1H, m, H-7), as well as five methyne signals at δ_H 4.01 (1H, tt, J = 11.5, 4.1 Hz), 1.27 (1H, m), 6.50 (1H, d, J = 8.5 Hz), 6.98 (1H, d, J = 8.5 Hz), and 3.26 (1H, m). The ^{13}C NMR and DEPT spectra showed 20 carbon signals, including five primary carbon signals at δ_C 20.5, 20.5, 22.7, 26.3, and 33.5, four secondary carbon signals at δ_C 19.2, 28.7, 48.9, and 51.0, five tertiary carbon signals at δ_C 27.5, 49.1, 66.1, 114.6, and 122.9, six quaternary carbon signals at δ_C 34.9, 39.6, 131.4, 133.7, 142.3, and 152.4. These NMR data above showed that compound **10** has an abietane diterpene skeleton [16]. The HMBC correlations (Figure 2) of 18-CH_3 (δ_H 0.99) to C-4 (δ_C 34.9), to C-5 (δ_C 49.1) and 19-CH_3 (δ_H 0.94) to C-4 (δ_C 34.9), to C-3 (δ_C 51.0), indicated that 18-CH_3 and 19-CH_3 were located at C-4. The HMBC correlations of 20-CH_3 (δ_H 1.18) to C-10 (δ_C 39.6), to C-1 (δ_C 48.9), C-9 (δ_C 142.3), C-5 (δ_C 49.1), indicated that 20-CH_3 was located at C-10. The HMBC correlations of H-16 (δ_H 1.33) to C-15 (δ_C 27.5), to C-17 (δ_C 20.5), to C-14 (δ_C 152.4), H-17 (δ_H 1.32) to C-15 (δ_C 27.5), C-16 (δ_C 20.5), C-14 (δ_C 152.4), positioned the isopropyl group at C-14. The HMBC correlations of H-7 (δ_H 2.95, 2.75) to C-8 (δ_C 133.7), to C-9 (δ_C 142.3), to C-14 (δ_C 152.4), showed the aromatic hydroxyl located at C-14. The HMBC correlations of H-1 (δ_H 2.55, 1.28) to C-2 (δ_C 66.1), and 19-CH_3 (δ_H 0.94) to C-3 (δ_C 51.0), established the presence of a hydroxyl at C-2. The vicinal coupling constants of H-2 (tt, J = 11.5, 4.1 Hz) showed H-2 at axial orientation, means the hydroxyl at C-2 with α configuration [17]. The NOESY correlations of H-2 (δ_H 4.01)/19-CH_3 (δ_H 0.94), H-2 (δ_H 4.01)/20-CH_3 (δ_H 1.18), and 19-CH_3 (δ_H 0.94)/20-CH_3 (δ_H 1.18) further confirmed the relative stereochemical structures of compound **10**. Accordingly, the structure of compound **10** was established and named millipedine A.

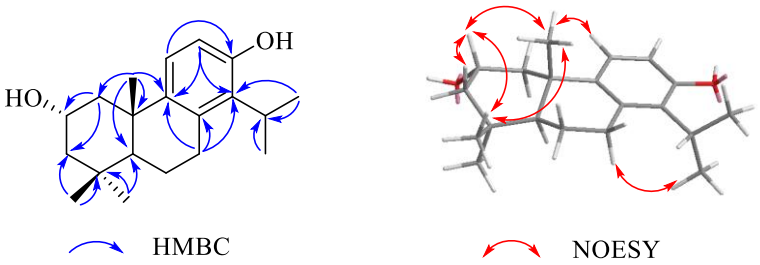

Figure 2. Key HMBC and NOESY correlations of compound **10**

The *in vitro* cytotoxicities of compound **10** against A549, HCT-116 and SW1990 cell lines were tested by the MTT assay [18-20]. Compound **10** exhibited moderate cytotoxicity against A549, HCT-116, and SW1990 cell lines in a dose-dependent manner, and the IC_{50} values were 61.81, 46.83, and 70.69 μM, respectively (Figure 3).

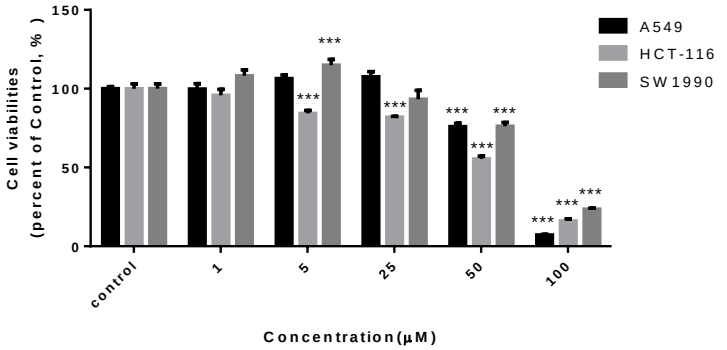

Figure 3. Cytotoxicity of compound **10**
(Results are expressed as mean ± SEM. Statistical significance compared to the control, *** p <0.001.)

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Supporting Information

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