







## 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. © 2021 ACG Publications. All rights reserved.

### 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<sub>2</sub>O (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<sub>R</sub>* 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<sub>R</sub>* 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<sub>R</sub>* 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<sub>R</sub>* 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<sub>R</sub>* 12.6 min) [11].

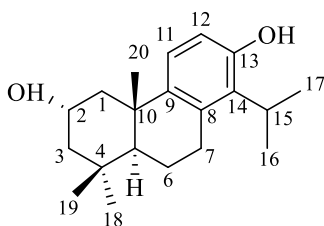
The *n*-butanol extract (23.84 g) was separated by silica column (100×800 mm, petroleum ether-ethyl acetate 5:1→0:1) to give three fractions (Fr.A–C). Fr.2 was purified by Sephadex LH-20 CC to afford three subfractions (Fr.B.1–B.3). Fr.2.2 was separated by semi-preparative HPLC eluted with 70%→80%→90%→100% 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<sub>R</sub>* 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).

*Millipedin A (10)*: white powder;  $[\alpha]_D^{20} = +7.49$  ( $c = 0.08$ , MeOH); IR  $\nu_{\max}$  (KBr): = 3400, 2925, 1600  $\text{cm}^{-1}$ ; <sup>1</sup>H NMR (600 MHz, CDCl<sub>3</sub>):  $\delta$  (ppm) = 0.94 (3H, s, 19-CH<sub>3</sub>), 0.99 (3H, s, 18-CH<sub>3</sub>), 1.18 (3H, s, 20-CH<sub>3</sub>), 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<sub>3</sub>), 1.33 (3H, d,  $J = 7.1$  Hz, 16-CH<sub>3</sub>), 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); <sup>13</sup>C NMR (150 MHz, CDCl<sub>3</sub>):  $\delta$  (ppm) = 19.2 (CH<sub>2</sub>, C-6), 20.5 (CH<sub>3</sub>, C-16 and C-17), 22.7 (CH<sub>3</sub>, C-19), 26.3 (CH<sub>3</sub>, C-20), 27.5 (CH, C-15), 28.7 (CH<sub>2</sub>, C-7), 33.5 (CH<sub>3</sub>, C-18), 34.9 (C, C-4), 39.6 (C, C-10), 48.9 (CH<sub>2</sub>, C-1), 49.1 (CH, C-5), 51.0 (CH<sub>2</sub>, 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]<sup>–</sup> (calcd. 301.2168 for C<sub>20</sub>H<sub>29</sub>O<sub>2</sub><sup>–</sup>).

*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<sub>2</sub>. 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<sub>50</sub>.

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

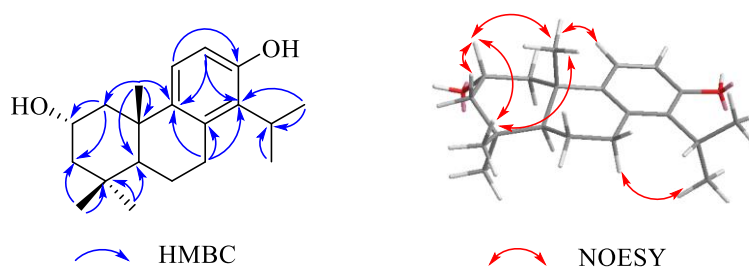


**Figure 1.** The chemical structure of millipedine A (**10**)

Compound **10**, millipedine A, was isolated as white powder, its molecular formula was determined to be C<sub>20</sub>H<sub>30</sub>O<sub>2</sub> on the basis of HR-APCI-MS at *m/z* 301.2163 [M-H]<sup>-</sup>, (calcd for C<sub>20</sub>H<sub>29</sub>O<sub>2</sub><sup>-</sup>, 301.2168), indicating 6 degrees of unsaturation. The IR spectrum of **10** showed absorption bands for hydroxyl group (3400 cm<sup>-1</sup>) [15].

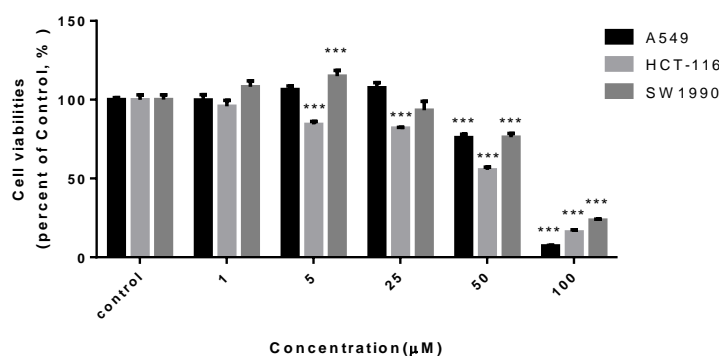
The <sup>1</sup>H NMR spectrum showed the presence of an AB system aromatic ring signals at δ<sub>H</sub> 6.98 (1H, d, *J* = 8.5 Hz) and 6.50 (1H, d, *J* = 8.5 Hz), a isopropyl signals attached to the benzene ring at δ<sub>H</sub> 1.33 (3H, d, *J* = 7.1 Hz), 1.32 (3H, d, *J* = 7.1 Hz), and 3.26 (1H, m), three methyl signals at δ<sub>H</sub> 0.99, 0.94, and 1.18 (each 3H, s), four methylene signals at δ<sub>H</sub> 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<sub>H</sub> 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 <sup>13</sup>C NMR and DEPT spectra showed 20 carbon signals, including five primary carbon signals at δ<sub>C</sub> 20.5, 20.5, 22.7, 26.3, and 33.5, four secondary carbon signals at δ<sub>C</sub> 19.2, 28.7, 48.9, and 51.0, five tertiary carbon signals at δ<sub>C</sub> 27.5, 49.1, 66.1, 114.6, and 122.9, six quaternary carbon signals at δ<sub>C</sub> 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<sub>3</sub> (δ<sub>H</sub> 0.99) to C-4 (δ<sub>C</sub> 34.9), to C-5 (δ<sub>C</sub> 49.1) and 19-CH<sub>3</sub> (δ<sub>H</sub> 0.94) to C-4 (δ<sub>C</sub> 34.9), to C-3 (δ<sub>C</sub> 51.0), indicated that 18-CH<sub>3</sub> and 19-CH<sub>3</sub> were located at C-4. The HMBC correlations of 20-CH<sub>3</sub> (δ<sub>H</sub> 1.18) to C-10 (δ<sub>C</sub> 39.6), to C-1 (δ<sub>C</sub> 48.9), C-9 (δ<sub>C</sub> 142.3), C-5 (δ<sub>C</sub> 49.1), indicated that 20-CH<sub>3</sub> was located at C-10. The HMBC correlations of H-16 (δ<sub>H</sub> 1.33) to C-15 (δ<sub>C</sub> 27.5), to C-17 (δ<sub>C</sub> 20.5), to C-14 (δ<sub>C</sub> 152.4), H-17 (δ<sub>H</sub> 1.32) to C-15 (δ<sub>C</sub> 27.5), C-16 (δ<sub>C</sub> 20.5), C-14 (δ<sub>C</sub> 152.4), positioned the isopropyl group at C-14. The HMBC correlations of H-7 (δ<sub>H</sub> 2.95, 2.75) to C-8 (δ<sub>C</sub> 133.7), to C-9 (δ<sub>C</sub> 142.3), to C-14 (δ<sub>C</sub> 152.4), showed the aromatic hydroxyl located at C-14. The HMBC correlations of H-1 (δ<sub>H</sub> 2.55, 1.28) to C-2 (δ<sub>C</sub> 66.1), and 19-CH<sub>3</sub> (δ<sub>H</sub> 0.94) to C-3 (δ<sub>C</sub> 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<sub>H</sub> 4.01)/19-CH<sub>3</sub> (δ<sub>H</sub> 0.94), H-2 (δ<sub>H</sub> 4.01)/20-CH<sub>3</sub> (δ<sub>H</sub> 1.18), and 19-CH<sub>3</sub> (δ<sub>H</sub> 0.94)/20-CH<sub>3</sub> (δ<sub>H</sub> 1.18) further confirmed the relative stereochemical structures of compound **10**. Accordingly, the structure of compound **10** was established and named millipedine A.

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**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<sub>50</sub> values were 61.81, 46.83, and 70.69  $\mu$ M, respectively (Figure 3).



**Figure 3.** Cytotoxicity of compound **10**

(Results are expressed as mean  $\pm$  SEM. Statistical significance compared to the control, \*\*\*  $p < 0.001$ .)

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## Supporting Information

Supporting Information accompanies this paper on <http://www.acgpubs.org/journal/records-of-natural-products>

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