

Monoester-Type C₁₉-Diterpenoid Alkaloids from *Aconitum carmichaelii* and Their Cardiotoxicity

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Abstract: A new (1) and six known (2-7) monoester-type C₁₉-diterpenoid alkaloids were isolated from the lateral roots of *Aconitum carmichaelii* Debx. Their structures were determined by spectroscopic techniques and calculations of NMR chemical shifts. Compound **1** (1-*epi*-hokbusine A) was an aconitine-type diterpenoid alkaloid possessing an unusual 1β-methoxy group. According to the main toxicity of the lateral roots of *A. carmichaelii*, the cardiotoxic effects of the isolates were evaluated using H9c2 rat myocardial cells and zebrafish embryos. The results showed that all the monoester-type C₁₉-diterpenoid alkaloids exhibited cardiotoxicity, and compound **6** was found to be the most toxic compound.

Keywords: *Aconitum carmichaelii*; monoester-type C₁₉-diterpenoid alkaloids; H9c2 cells; zebrafish embryos; cardiotoxicity. © 2021 ACG Publications. All rights reserved.

1. Introduction

The lateral roots of *Aconitum carmichaelii* Debx. (Ranunculaceae), named Fuzi in Chinese, were first documented in *Shennong's Materia Medica* (*Shennong Bencao Jing* in Chinese) approximately 2000 years ago and have been widely used in China [1–2]. In modern clinical applications, Fuzi is extensively used to treat heart failure, but it has great cardiotoxicity, including tachycardia, atrioventricular block, cardiogenic shock, and cardiac arrest [3–6]. Thus, the remarkable cardiotoxicity of Fuzi has attracted more and more attentions. C₁₉-diterpenoid alkaloids are confirmed as the main effective and toxic constituents of Fuzi and can be divided into three categories (diester-type, monoester-type, and aminoalcohol-type) based on the number of ester bonds. Diester-type C₁₉-diterpenoid alkaloids have a stronger cardiotoxicity than monoester-type C₁₉-diterpenoid alkaloids,

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2.3. Extraction and Isolation

For extraction and preliminary fractionation of Fuzi, refer to reference [10]. Separation of portion E (12 g) was performed via RP-MPLC eluted by a gradient solvent system (10-100% MeOH in H₂O) to get 14 fractions (E₁-E₁₄). Fraction E₁ was purified by preparative TLC (CH₂Cl₂/MeOH/NH₃·H₂O, 15:1:0.1) and HPLC (MeOH/H₂O, 38:62) to afford compound **6** (3.1 mg). E₂₋₁-E₂₋₆ were afforded by chromatographing E₂ over Sephadex LH-20 column chromatography (petroleum ether/CH₂Cl₂/MeOH, 2:2:1). Successive purification of subfraction E₂₋₆ by TLC (CH₂Cl₂/MeOH/NH₃·H₂O, 10:1:0.1) and RP HPLC (35% MeOH in H₂O, containing 0.1% acetic acid) yielded compound **5** (2.5 mg). Fraction E₃ was separated by silica gel column chromatography (petroleum ether/Me₂CO/diethylamine, 10:1:0.1 to 3:1:0.1), followed by preparative TLC (CH₂Cl₂/MeOH/NH₃·H₂O, 10:1:0.1) to yield compound **2** (4.2 mg). Fraction E₄ was further divided into E₄₋₁-E₄₋₃ by silica gel column chromatography (petroleum ether/Me₂CO/diethylamine, 10:1:0.1 to 3:1:0.1). Purification of subfraction E₄₋₂ by RP semipreparative HPLC (44% MeCN in H₂O, containing 0.1% TFA) afforded compound **1** (4.2 mg). Subfraction E₄₋₃ was purified by preparative TLC (petroleum ether/Me₂CO/diethylamine, 3:1:0.1) to yield compound **3** (3.6 mg). Fraction E₇ was chromatographed via a Sephadex LH-20 column (CH₂Cl₂/MeOH, 1:1) to obtain E₇₋₁-E₇₋₄. Subfraction E₇₋₃ was further purified by RP-HPLC (20% MeCN in H₂O) to furnish compounds **4** (6.3 mg) and **7** (4.5 mg).

2.4. Spectral Data

1-epi-Hokbusine A (1): white powder; $[\alpha]_D^{20}$ -11.7 (*c* 0.05, MeOH); ¹H (acetone-*d*₆, 600 MHz) and ¹³C NMR (acetone-*d*₆, 150 MHz) data, see Tables 1 and 2. IR (ATR) ν_{\max} 3365, 2923, 1676, 1278, 1099, 718 cm⁻¹; (+)-HR-ESI-MS *m/z* 604.3121 [M + H]⁺ (calcd. for C₃₂H₄₆NO₁₀, 604.3122).

2.5. Cell culture

H9c2 cells were cultured in accordance with literature methods [10].

2.6. Determination of H9c2 Cell Viability

The H9c2 cell viability was assessed by the activity of mitochondrial dehydrogenases using a colorimetric MTT method. Exponentially growing cells were plated into 96-well plates (7 × 10³ cells per well) and incubated for 24 h. Then the cells were treated with the isolated compounds at concentrations of 1.56, 3.125, 6.25, 12.5, 25, and 50 μM for 24 h. Then, 0.5 mg/mL MTT was added into each well. After a 4-hour incubation at 37°C, the purple formazan crystals were dissolved in 0.18 mL of DMSO. The optical density ratios were measured on an automated MK3 microplate spectrophotometer (Thermo Fisher Scientific, Waltham, MA, US) at 490 nm.

2.7. Zebrafish Husbandry

Zebrafish of wild-type AB strain obtained from China Zebrafish Resource Center were maintained and raised according to the routine procedures [15]. All zebrafish were kept at 28.5°C under a 14-h light/10-h dark cycle. The embryos were generated by natural spawning and maintained in the embryonic medium (15 μM NaCl, 500 μM KCl, 1 mM CaCl₂, 500 μM NaHCO₃, 74 μM Na₂HPO₄, 1 mM MgSO₄, and 120 μM KH₂PO₄ in distilled H₂O). The zebrafish experiments were performed under the approval of the Institutional Animal Care and Use Committee of Chengdu University of Traditional Chinese Medicine.

2.8. Assessment of Zebrafish Malformation Rates and Heart Rates

Zebrafish embryos were distributed into 24-well microplates after 48 h post fertilization (hpf), and at least 15 fish were placed in each well. One well of embryos were treated with 0.1% (v/v) DMSO embryo medium (the control group), and other wells were treated with various concentrations of the isolated alkaloids (6.25, 12.5, 25, and 50 μ M). Plates were cultivated at 28.5°C for 24 h. The acute toxicity of zebrafish exposed to the isolated alkaloids was determined by observing the changes in zebrafish morphology and examining the malformation rates under a Leica M165Fic fluorescence microscope (Leica Microsystems). At the same time, 60-second video images were collected for measurable assessment of the heart rates.

2.9. Data Analysis

Data analyses were prepared using SPSS 19.0 software (IBM Corp.). Data are shown as the mean \pm standard deviation (SD). Statistical significance was assessed by one-way analysis of variance (ANOVA), followed by a least-significant-difference (LSD) test. Figures were manufactured using GraphPad Prism software Version 5.0 (GraphPad Software, Inc.). In all comparisons, $P < 0.05$ indicated a statistically significant difference.

3. Results and Discussion

3.1. Structure Elucidation

Compound **1** was separated as a white powder possessing a molecular formula of $C_{32}H_{45}NO_{10}$, based on a quasi-molecular ion at m/z 604.3121 $[M + H]^+$ (calcd. for $C_{32}H_{46}NO_{10}$, 604.3122) in its HRESIMS. The IR spectrum of **1** showed characteristic absorptions for the carbonyl (1676 cm^{-1}) and OH (3365 cm^{-1}) functionalities. The 1H NMR spectrum of compound **1** (Table 1) showed signals attributed to five methoxy groups (δ_H 3.18, 3.32, 3.37, 3.41, and 3.70), six oxymethine groups [δ_H 3.42 (1H, d, $J = 6.0$ Hz, H-16), 3.71 (1H, brs, H-1), 4.26 (1H, dd, $J = 6.6, 1.8$ Hz, H-6), 4.29 (1H, brd, $J = 4.2$ Hz, H-3), 4.70 (1H, d, $J = 6.0$ Hz, H-15), and 4.87 (1H, d, $J = 5.4$ Hz, H-14)], an isolated oxymethylene group [δ_H 3.43 (1H, overlapped, H-18a) and 3.59 (1H, d, $J = 7.8$ Hz, H-18b)], a *N*-methyl group [δ_H 3.15 (3H, s, H₃-20)], an isolated *N*-methylene group [δ_H 3.45 (1H, d, $J = 12.0$ Hz, H-19a) and 3.65 (1H, d, $J = 12.0$ Hz, H-19b)], a characteristic *N*-methine group [δ_H 3.47 (1H, s, H-17)], and a monosubstituted aromatic ring [δ_H 7.53 (2H, t, $J = 7.8$ Hz, H-3' and H-5'), 7.65 (1H, m), and 8.06 (2H, dd, $J = 7.8, 1.2$ Hz, H-2' and H-6')]. The ^{13}C NMR (Table 2) and DEPT data exhibited 32 carbons signals, including six methyls (five oxygenated, δ_C 50.3, 55.5, 59.1, 59.3, and 62.5), four methylenes (one oxygenated, δ_C 77.4), 16 methines (six oxygenated, δ_C 70.2, 76.8, 80.0, 80.8, 82.8, and 94.5), and six quaternary carbons (two oxygenated, δ_C 75.4 and 83.5; one aromatic, δ_C 131.1; and one carbonylic, δ_C 166.3). These spectroscopic data revealed that compound **1** was a typical benzoyleconitine-type diterpenoid alkaloid with five methoxy and three hydroxy groups. The planar structure of compound **1** was further confirmed to be the same as hokbusine A [16] by the 1H - 1H COSY and HMBC correlations (Figure 2). However, in comparison with the ^{13}C NMR data of hokbusine A, C-1, C-2, and C-3 in **1** were shielded by $\Delta\delta_C$ -1.9, -3.7, and -1.5 ppm, respectively, whereas C-17 and C-19 were deshielded by $\Delta\delta_C$ +5.3 and +2.1 ppm. These differences suggested that compound **1** and hokbusine A might be C-1 epimers [16]. In the ROESY spectrum of **1**, the correlations of H-10 with H-6 and H-14, of H-6 with H-9 and OMe-16, of H-15 with OMe-8 and H-18a, of H-3 with H-18a, of H-12b with H-14; and of H-12a with H-1, H-16, and H-17, demonstrated that OH-3, OH-15, and OMe-6 were α -oriented, while OMe-1, H-14, and OMe-16 groups were in the β -orientation. Meanwhile, in the 1D-NOE experiment of **1**, irradiation of H-7 enhanced H-15, and irradiation of H-6 enhanced H-9, while irradiation of H-12a enhanced H-1, H-16, and H-17.

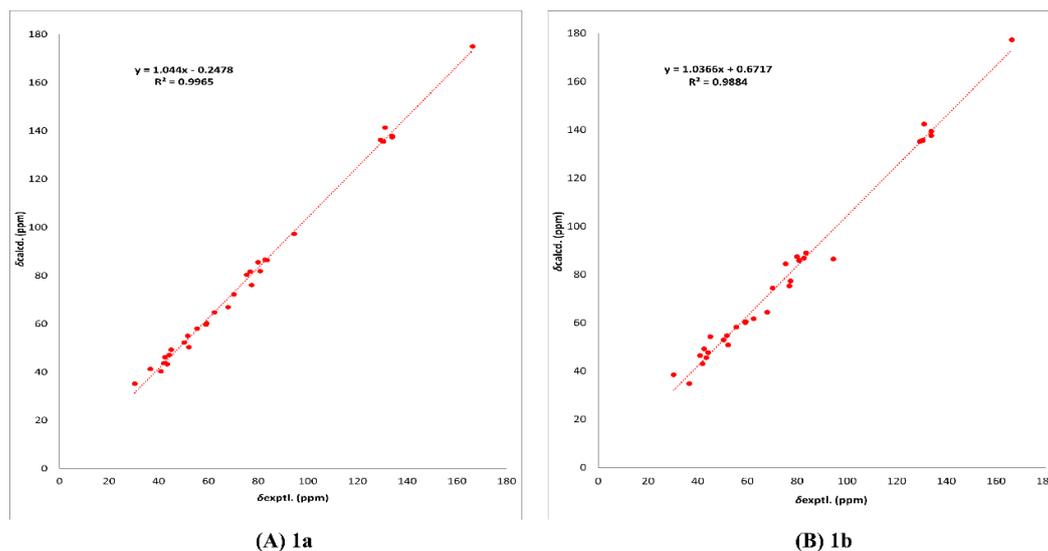


Figure 4. Correlation plots of experimental ^{13}C NMR chemical shifts versus corresponding calculated ^{13}C NMR chemical shifts for **1a** (A) and **1b** (B).

By comparing the experimental and literature NMR data, six known compounds were identified as 14-benzoylmesaconine (**2**) [19], 14-benzoylaconine (**3**) [20], 14-benzoylneoline (**4**) [21], carmichaenine C (**5**) [22], (-)-(A-b)-14 α -benzyloxy-*N*-ethyl-1 α ,8 β ,15 α -trihydroxy-6 α ,16 β ,18-trimethoxyaconitane (**6**) [23], and 14-*O*-cinnamoylneoline (**7**) [24].

Table 1. ^1H NMR data for compound **1** (600 MHz) and hokbusine A [16]

No.	hokbusine A	1	No.	hokbusine A	1
1	3.22 brt (6.4)	3.71 brs	16	3.25 m	3.42 d (6.0)
2a	2.02 m	1.64 dt (16.2, 4.2)	17	2.87 brs	3.47 s
2b	2.34 m	2.38 d (16.2)	18a	3.55 d (8.7)	3.43 (overlapped)
3	3.75 dd (8.2, 5)	4.29 brd (4.2)	18b	3.61 d (8.7)	3.59 d (7.8)
4	-	-	19a	2.50 m	3.45 d (12.0)
5	2.10 m	2.60 d (6.6)	19b	2.73 m	3.65 d (12.0)
6	4.03 d (7.0)	4.26 dd (6.6, 1.8)	20	2.38 s	3.15 s
7	2.85 brs	3.12 d (1.8)	1'	-	-
8	-	-	2'/6'	8.01 d (7.4)	8.06 dd (7.8, 1.2)
9	2.58 t (6.0)	2.63 dd (7.2, 5.4)	3'/5'	7.43 t (7.4)	7.53 t (7.8)
10	2.07 m	2.56 m	4'	7.54 t (7.5)	7.65 m
11	-	-	7'	-	-
12a	2.07 m	1.93 m	OCH ₃ -1	3.28 s	3.41 s
12b	2.70 m	2.33 m	OCH ₃ -6	3.28 s	3.37 s
13	-	-	OCH ₃ -8	3.12 s	3.18 s
14	4.82 d (5.1)	4.87 d (5.4)	OCH ₃ -16	3.71 s	3.70 s
15	4.52 d (6.0)	4.70 d (6.0)	OCH ₃ -18	3.30 s	3.32 s

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No.	hokbusine A	1	No.	hokbusine A	1
1	82.7	80.8	16	93.4	94.5
2	34.0	30.3	17	62.6	67.9
3	71.7	70.2	18	76.7	77.4
4	43.7	44.2	19	50.1	52.2
5	45.8	42.5	20	42.8	42.0
6	83.3	82.8	1'	56.5	131.1
7	41.6	43.4	2'/6'	130.0	130.4
8	82.5	83.5	3'/5'	128.7	129.3
9	45.4	45.0	4'	133.3	133.9
10	41.9	40.9	7'	166.4	166.3
11	50.7	51.7	OCH ₃ -1	56.5	55.5
12	36.4	36.6	OCH ₃ -6	58.8	59.1
13	75.1	75.4	OCH ₃ -8	50.0	50.3
14	79.7	80.0	OCH ₃ -16	62.9	62.5
15	77.8	76.8	OCH ₃ -18	59.4	59.3

3.2. Cardiotoxicity On H9c2 Cells

The cardiotoxicity of seven isolated monoester-type C₁₉-diterpenoid alkaloids (**1-7**) were evaluated using H9c2 cells and zebrafish embryos. As shown in Figure 5, all the alkaloids exhibited significant toxicity in H9c2 cells at concentrations of 1.56, 3.125, 6.25, 12.5, 25, and 50 μM. Among them, compounds **2** and **6** showed the strongest toxicity in a dose-dependent manner. Particularly, the survival rates of compounds **2** and **6** at 50 μM decreased to 46.73 ± 3.81% and 48.80 ± 4.54%, respectively. Comparison of compounds **2** and **3** suggested that the monoester-type C₁₉-diterpenoid alkaloids with a N-CH₃ group had stronger cytotoxicity than those with a N-CH₂CH₃ group. When compared with compound **4**, introduction of an α-OH group at C-15 in compound **6** improved the cytotoxicity. In addition, replacement of a benzoyl group (**4**) by a cinnamyl group (**7**) resulted in loss of the cytotoxicity. Therefore, it can be inferred that the alkyl group at N, the acyloxy group at C-14, and the hydroxy group at C-15 have significant effects on the cytotoxicity of monoester-type C₁₉-diterpenoid alkaloids in H9c2 cells.

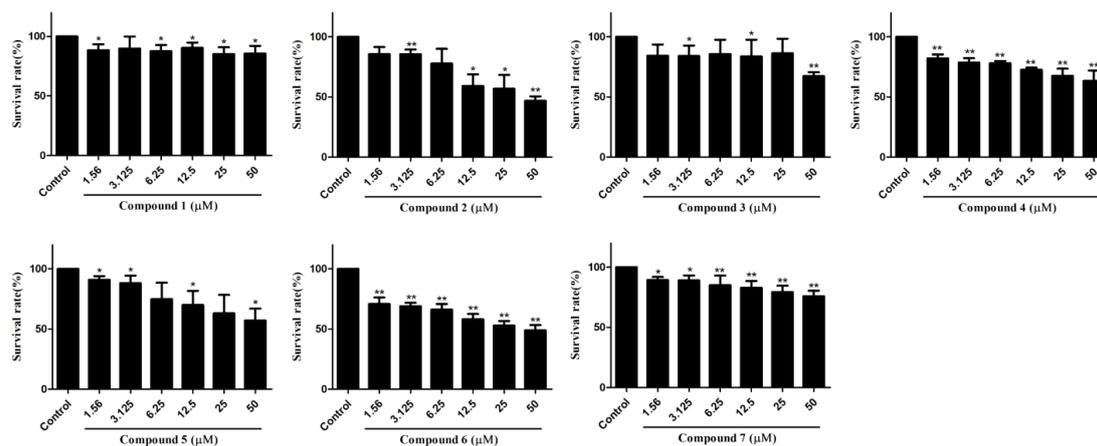


Figure 5. Cytotoxic effects of compounds **1-7** in H9c2 cells. Results are showed as the mean \pm SD for three individual experiments. * $P < 0.05$, ** $P < 0.01$ vs the control group.

3.3. Cardiotoxicity On Zebrafish Embryos

After treatment with the isolated monoester-type C_{19} -diterpenoid alkaloids at 6.25, 12.5, 25, and 50 μM , the zebrafish embryos developed abnormally with symptoms of cardiotoxicity. As shown in Figure 6, the representative toxic compounds (**2** and **6**) had notable cardiotoxic effects on the heart morphology of zebrafish, mainly including the serious pericardial edema and yolk sac edema. Furthermore, Figure 7 showed that the malformation rates of zebrafish treated with the monoester-type C_{19} -diterpenoid alkaloids increased significantly, especially the malformation rates of zebrafish treated with compounds **2** and **6** at 25 and 50 μM . In addition to the malformation rates, the heart rates of zebrafish were also affected by the above alkaloids (Figure 8). Particularly, the average heart rate of the control zebrafish embryos was 164.5 ± 3.42 beats/min, while the average heart rates increased to 179.5 ± 1.92 (compound **1**), 192.5 ± 7.72 (compound **2**), 173.5 ± 4.44 (compound **3**), 172.5 ± 3.42 (compound **5**), and 188 ± 8.64 beats/min (compound **6**) at 50 μM . These results indicated that the isolated monoester-type C_{19} -diterpenoid alkaloids had cardiotoxic effects on H9c2 cells and zebrafish embryos.

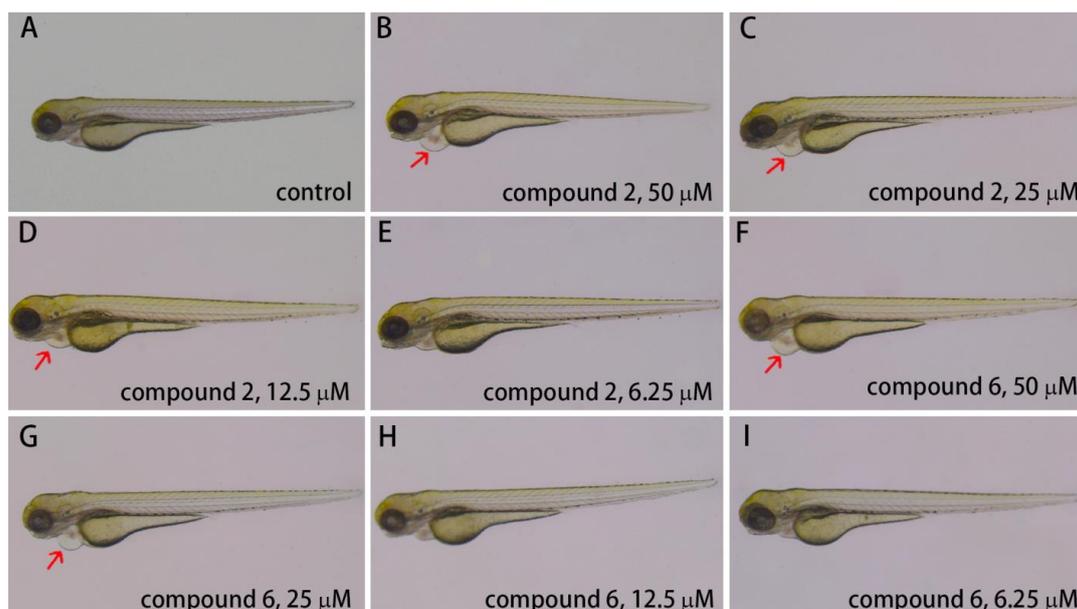


Figure 6. Effects of compounds **2** and **6** on the heart morphology of zebrafish.

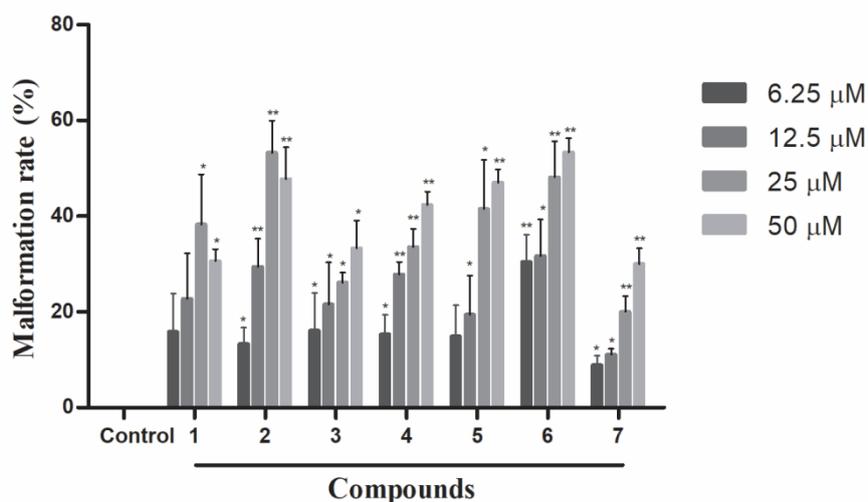
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Figure 7. Effects of compounds 1-7 on malformation rates of zebrafish. Results are presented as the mean \pm SD for three individual experiments. * $P < 0.05$, ** $P < 0.01$ vs the control group.

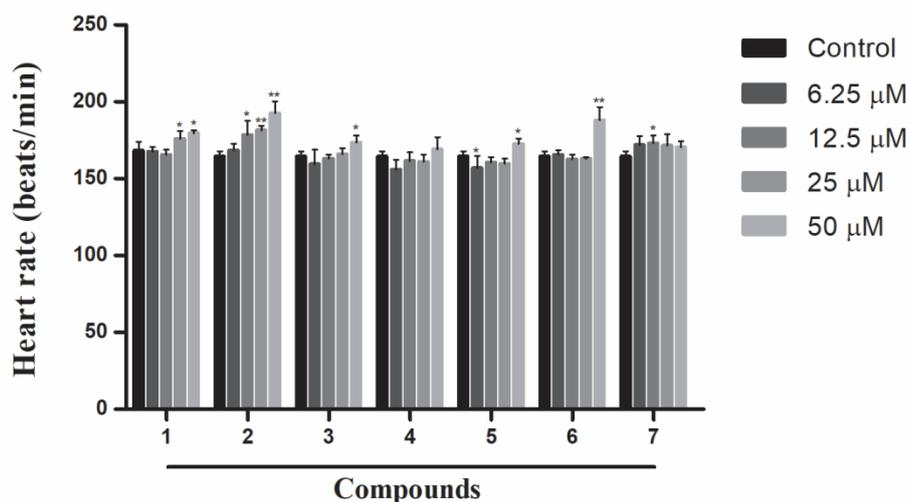


Figure 8. Effects of compounds 1-7 on heart rates of zebrafish. Results are presented as the mean \pm SD for three individual experiments. * $P < 0.05$, ** $P < 0.01$ vs the control group.

4. Conclusion

So far, more than 80 C₁₉-diterpenoid alkaloids have been isolated from Fuzi [4,14,25]. It is worth noting that the substituent group at C-1 is mostly α -oriented [4,26,27]. However, in this study, we obtained a monoester-type C₁₉-diterpenoid alkaloid possessing an unusual 1 β -methoxy group (**1**). In addition, researchers have increasingly focused on the cardiotoxicity of diester-type C₁₉-diterpenoid alkaloids in Fuzi, but few studies have looked at the cardiotoxicity of monoester-type C₁₉-diterpenoid alkaloids. This study demonstrated that all the isolated monoester-type C₁₉-diterpenoid alkaloids had cardiotoxic effects on H9c2 cells and zebrafish embryos. Thus, this study has greatly improved the understanding of the cardiotoxicity of Fuzi.

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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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Author Contribution

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