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A new triterpenoid saponin from *Clinopodium chinense* (Benth.) O. Kuntze

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ABSTRACT

A new triterpene saponin, 3β , 16β , 23α , 28β , 30β -pentahydroxyl-olean-11, 13(18)-dien- 3β -yl-[β -D-glucopyranosyl-($1 \rightarrow 2$)]-[β -D-glucopyranosyl-($1 \rightarrow 3$)]- β -D-fucopyranoside, was named Clinoposaponin D (**1**), together with six known triterpene saponins, buddlejasaponin IVb (**2**), buddlejasaponin IVa (**3**), buddlejasaponin IV (**4**), clinopodisides D (**5**), 11α , 16β , 23, 28-Tetrahydroxyolean-12-en- 3β -yl-[β -D-glucopyranosyl-($1 \rightarrow 2$)]-[β -Dglucopyranosyl-($1 \rightarrow 3$)]- β -D-fucopyranoside (**6**) and prosaikogenin A (**7**), and two known triterpenes, saikogenin A (**8**) and saikogenin F (**9**) were isolated from *Clinopodium chinense* (Benth.) O. Kuntze. Their structures were elucidated on the basis of 1D, 2D NMR and MS analysis. Meanwhile, the effects of all compounds on rabbit platelet aggregation and thrombin time (TT) were investigated *in vitro*. Compounds **4** and **7** had significant promoting effects on platelet aggregation with EC₅₀ value at 53.4 and 12.2 μ M, respectively. In addition, the highest concentration (200 μ M) of compounds **2** and **9** shortened TT by 20.6 and 25.1%, respectively.

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1. Introduction

The genus *Clinopodium* comprises nearly 20 species and is mainly distributed in Asia and Europe. *Clinopodium chinense* (Benth.) O. Kuntze (Lamiaceae), a traditional Chinese Medicine (TCM) is popularly used for the treatment of haematuria, allergic dermatitis, dysentery, influenza, heliosis and trauma, etc. (Wang et al. 2013; Zhong et al. 2014). Furthermore, *C. chinense* is one of two medicinal herbs in 'Duan Xueliu', which has long been used as a TCM with an astrictive remedy property, and is officially listed in the Chinese Pharmacopoeia (Pharmacopeia Committee of P. R. China 2010). In recent years, its aqueous or ethanol extract has been found to exhibit haemostatic activities (Zhong et al. 2012). In the course of our research for bioactive constituents from *C. chinense*, one new triterpene saponin named Clinoposaponin D (1), together with six known triterpene saponins (2-7) and two triterpenes (8-9) were obtained. In the present study, we reported the isolation and structural identification of compounds 1-9 and their effects on platelet aggregation and thrombin time (TT) *in vitro*.

2. Results and discussion

Compound **1** was isolated as a white amorphous powder. Its molecular formula was established as $C_{48}H_{78}O_{19}$ on the basis of HR-ESI-MS (m/z 981.5050 [M + Na]⁺, Calcd. for 981.5035; m/z 957.5072 [M – H]⁻, Calcd. for 957.5059). A positive reaction with the Liebermann–Burchard and Molisch reagents indicated that it might be a saponin (Tsuyoshi et al. 2004; Liu et al. 2013).

¹H-NMR (500 MHz, pyridine- d_{s}) spectrum showed the presence of five methyl signals [δ_{H} 0.87 (3H, s), 0.96 (3H, s), 1.08 (3H, s), 1.09 (3H, s), 1.12 (3H, s)], three anomeric proton signals $[\delta_{H} 4.95 (1H, d, J = 7.8 Hz), 5.34 (1H, d, J = 7.8 Hz)$ and 5.62 (1H, d, J = 7.8 Hz)] and two olefinic protons mutually coupled [δ_{μ} 6.60 (1H, dd, J = 11.0, 2.9 Hz) and 5.68 (1H, d, J = 11.1 Hz)]. ¹³C-NMR (125 MHz, pyridine- d_s) spectrum showed 48 carbons including five methyl carbons [δ_c 13.2, 19.1, 17.4, 22.4 and 21.2], three anomeric carbons signals [δ_{c} 104.4, 104.5 and 105.5], four olefinic carbons [δ_c 127.4, 126.2, 137.1 and 133.6], which were characteristic olefinic carbons of $\Delta^{11,13(18)}$ -Olean diene. The data above suggested that **1** was a highly oxygenated oleanolic triterpenoid saponin derivative with three sugar moieties (Yamamoto, Miyase, et al. 1993; Liu et al. 1995; Bi et al. 2012; Zhang et al. 2012). The assignment of ¹H-NMR and ¹³C-NMR spectroscopic data of **1** (Table S1) was based on ¹H–¹H COSY (Figure S7), HSQC (Figure S8) and HMBC spectrum (Figure S9). The ¹H-NMR and ¹³C-NMR features of 1 were similar to those of the known compound buddlejasaponin IVb (2) (Table S1), except for the proton signal (H-30) of methylene group that connected to hydroxy group in compound 1. The correlation between $\delta_{\rm H}$ 6.13 (1H, brs, 30-OH) and $\delta_{\rm H}$ 3.67 (2H, s, H-30) in the ¹H–¹H COSY spectrum confirmed their connection. In the HMBC spectrum, the correlations between 30-OH and C-30, H-29 and C-20 proved the linkage of C-30/C-20/C-29. Specific HMBC correlations were given in Figure S10.

Further correlations between 30-OH/H-28 and H-29/H-22 α in the ROESY spectrum (Figure S11) proved that the C-30 hydroxy group was β -oriented and C-29 methyl group was α -oriented. Specific ROESY correlations are given in Figure S12.

The sugar moiety was located at C-3 on the basis of the correlation between the anomeric proton signal at δ_{H} 4.95 (d, J = 7.8 Hz) and carbon signal at δ_{C} 83.0 (C-3) in the HMBC spectrum. Furthermore, the correlations between anomeric proton signals at $\delta_{\rm H}$ 5.62 (d, J = 7.8 Hz, Glu-H-1") and carbon signal at $\delta_{\rm C}$ 77.6 (Fucose-C-2'), and correlation from $\delta_{\rm H}$ 5.34 (d, J = 7.8 Hz, Glu-H-1"') to $\delta_{\rm C}$ 85.2 (Fucose-C-3') proved that sugar–sugar linkages were the same as buddlejasaponin IVb. The type and absolute configuration of the sugars were identified as β -D-fucose and β -D-glucose on the basis of the TLC method comparison with authentic monosaccharides (CH₂Cl₂:MeOH:H₂O = 3:2:0.2, visualisation with ethanol–5% H₂SO₄ spraying) and both coupling constants of anomeric protons were 7.8 Hz. Therefore, the structure of **1** was elucidated as 3β , 16β , 23α , 28β , 30β -pentahydroxyl-olean-11,13(18)-dien- 3β -yl-[β -D-glucopyranosyl-(1 \rightarrow 2)]-[β -D-glucopyranosyl-(1 \rightarrow 3)]- β -D-fucopyranoside and named Clinoposaponin D.

By comparing physical and spectroscopic data (MS, ¹H NMR, ¹³C NMR) with published literatures, the eight known compounds (**2**–**9**) were identified as buddlejasaponin IVb (**2**) (Yamamoto, Miyase, et al. 1993), buddlejasaponin IVa (**3**) (Yamamoto, Suzuki, et al. 1993), buddlejasaponin IV (**4**) (Yamamoto, Suzuki, et al. 1993), clinopodisides D (**5**) (Liu et al. 1995), 11 α , 16 β , 23, 28-tetrahydroxyolean-12-en-3 β -yl-[β -D-glucopyranosyl(1 \rightarrow 2)]-[β -D-glucopyranosyl (1 \rightarrow 3)]- β -D-fucopyranoside (**6**) (Lee et al. 2012), prosaikogenin A (**7**) (Shimizu et al. 1985), saikogenin A (**8**) (Shimizu et al. 1985) and saikogenin F (**9**) (Shimizu et al. 1985), respectively (Figure 1).

Blood platelets play an essential role in haemostasis by involving in the formation of thrombi to prevent blood loss and maintain vascular integrity (Huang et al. 2014). The previous study showed that total saponins of *C. chinense* significantly increased the rat platelet aggregation (Liu et al. 1985). Therefore, the platelet aggregation effect of isolated compounds was evaluated. The results showed that compounds **1**, **2**, **4**, **6**, **7** and **9** exhibited varying degrees to promote rabbit platelet aggregation at concentrations up to 100 μ M. Additionally, compounds **4** and **7** exhibited potent platelet aggregation activity and their EC₅₀ value were 53.4 and 12.2 μ M, respectively. Interestingly, their effects on promoting platelet aggregation activity were stronger than polyphyllins II at the concentration of 100 μ M (Table S2). To determine the effect of the isolated compounds **2** and **9** remarkably shortened TT by 20.6 and 25.1%, respectively. Similarly, thrombin (0.006 U) remarkably shortened TT by 22.0%.

3. Experimental

3.1. General experimental procedures

NMR spectra were measured on Bruker AV-500 NMR (Bruker Corporation, Switzerland) with TMS as an internal standard. Mass spectra were obtained on a Mariner API-TOF mass spectrometer (Applied Biosystems, USA).UV spectra (Shelton, Connecticut) were obtained on a Shimadzu UV-2401 UV/vis spectrometer (Shimadzu, Japan). All solvents were analytical grade (Tianjin Chemical Plant). Column chromatography was performed on silica gel (100–200 mesh, 200–300 mesh, Qingdao Marine Chemical Co., Ltd., China), macroporous resin D101 (Mitsubishi Chemical Holdings Corp. Japan), Sephadex LH-20 (40–75 µm, Pharmacia, Sweden) and MCI GEL CHP 20P (75–150 µm, Mitsubishi Chemical Co., Japan). Thin-layer chromatography was performed on silica gel GF254 (Qingdao Marine Chemical Co., Ltd., China).



Figure 1. Structures of compounds 1-9.

3.2. Plant material

The herbs of *C. chinense* (Benth.) O. Kuntze were collected from Putian city, Fujian province, China, and identified by Prof. Sheban Pu from Department of Pharmacognosy, School of Traditional Chinese Pharmacy, China Pharmaceutical University, Nanjing, China. The voucher specimen (CPU-CC20100820) was deposited in the Herbarium of China Pharmaceutical University.

3.3. Isolation and identification

Dried aerial parts of C. chinense (6 kg) were extracted with 80% EtOH (3×60 L) under reflux. After removal of solvent under vacuum, the extract was first suspended in H₂O and then partitioned with petroleum ether, EtOAc and n-BuOH saturated with water, successively. The n-BuOH soluble (226 g) was subjected to a column chromatography of macroporous resin D101 (2000 g, 10×100 cm column), washing with H₂O, ethanol-H₂O mixtures with increasing amount of ethanol to give four fractions (Fr. I–IV). Fr. III (50 g) was subjected to YMC RP-18 column chromatography, eluting with CH₂OH-H₂O (30:70-100:0) to yield four fractions (Fr. A–D). Fr. B (12 g) was firstly subjected to YMC RP-18 CC using a step gradient of CH₂OH-H₂O (50:50-70:30), to obtain four fractions (Fr. B1-Fr. B4). Fr. B2 (1.4 g) was further purified by YMC RP-18 CC with CH₃OH-H₃O (50:50), and Sephadex LH-20 (methanol) to yield compound 1 (64 mg). Fr. C (26 g) was fractionated by silica gel column chromatography with CH₂Cl₂-CH₃OH (20:1-1:1) to afford 6 fractions (Fr. C1-C6). Fr. C1 (230 mg) was subjected to silica gel CC using a step gradient of petroleum ether-acetic ether (2:1-1:1) to yield compound 9 (32 mg). Fr. C2 (160 mg) was subjected to silica gel CC using a step gradient of CH₂Cl₂-CH₂OH (20:1–10:1) and Sephadex LH-20 (methanol) to yield compound **7** (5 mg). Fr. C3 (15 g) was subjected to YMC RP-18 CC using a step gradient of CH₂OH-H₂O (40:60-100:0) to obtain compounds 3 (3200 mg), 4 (3600 mg), 5 (32 mg), 6 (220 mg) and 2 (3500 mg). Fr. D (7 g) was firstly subjected to silica gel CC using a step gradient of CH₂Cl₂-CH₃OH (20:1-10:1) and further purified by preparative TLC with CH₂Cl₂-EtOAc (1:2) to yield compound 8 (16 mg). All the pure compounds were structurally elucidated through 1D NMR, 2D NMR, HR-ESI-MS, ESI-MS, UV spectra and literature data.

Clinoposaponin D (1): White amorphous powder: $[\alpha]_D^{20} = -22.74(c = 0.1, \text{MeOH}), \text{ m.p.}$ 268–270 °C; UV (MeOH, λ_{max}, nm): 240, 251, 260 nm; HR-ESI-MS: *m/z* 981.5050 (Calcd. for C₄₈H₇₈O₁₉ Na [M + Na]⁺, 981.5035), 957.5072 (Calcd. for C₄₈H₇₇O₁₉ [M – H]⁻, 957.5059). ¹H-NMR (500 MHz, pyridine-d₅) δ: 1.84 (1H, m, H-1), 1.08 (1H, m, H-1), 2.28 (1H, m, H-2), 2.03 (1H, m, H-2), 4.19 (1H, dd, J = 12.1, 4.6 Hz, H-3), 1.66 (1H, d, J = 9.8 Hz, H-5), 1.84 (1H, m, H-6), 1.45 (1H, m, H-6), 1.45 (1H, m, H-7), 1.35 (1H, d, J = 8.7 Hz, H-7), 2.09 (1H, s, H-9), 6.60 (1H, dd, J = 10.7, 2.1 Hz, H-11), 5.68 (1H, d, J = 10.7 Hz, H-12), 1.59 (1H, dd, J = 3.7, 12.7 Hz, H-15), 2.23 (1H, d, J = 12.7 Hz, H-15), 4.31 (1H, m, H-16), 2.75 (1H, d, J = 14.5 Hz, H-19), 2.27 (1H, d, J = 14.1 Hz, H-19), 2.16 (1H, td, J = 13.3, 3.8 Hz, H-21), 1.50 (1H, m, H-21), 3.07 (1H, dt, J = 14.7, 3.8 Hz, H-22), 1.76 (1H, td, J = 13.8, 3.6 Hz, H-22), 3.73 (1H, d, J = 10 Hz, H-23), 4.42 (1H, dd, J = 10.5, 4.75 Hz, H-23), 1.08 (3H, s, H-24), 0.96 (3H, s, H-25), 0.87 (3H, s, H-26), 1.12 (3H, s, H-27), 4.28 (2H, m, H-28), 1.09 (3H, s, H-29), 3.67 (2H, s, H-30), δ4.95 (1H, d, J = 7.8 Hz, H-1'), 4.70 (1H, dd, J = 7.8 Hz, H-1'), 4.70 (1H, J = 9.7, 7.8 Hz, H-2'), 4.12 (1H, dd, J = 9.7, 3.1 Hz, H-3'), 4.22 (1H, m, H-4'), 3.63 (1H, m, H-5'), 1.44 (3H, d, J = 6.3 Hz, H-6'), δ5.62 (1H, d, J = 7.8 Hz, H-1"), 4.12 (1H, m, H-2"), 4.21 (1H, m, H-3"), 4.21 (1H, m, H-4"), 3.65 (1H, m, H-5"), 4.35 (1H, m, H-6"), 4.31 (1H, m, H-6"), δ 5.34 (1H, d, J = 7.8 Hz, H-1"'), 4.03 (1H, t, J = 8.3 Hz, H-2"'), 4.28 (1H, m, H-3"'), 4.27 (1H, m, H-4"'), 3.98 (1H, m, H-5"'), 4.50 (1H, dd, J = 11.8, 2.5 Hz, H-6"'), 4.35 (1H, m, H-6"'); ¹³C-NMR (125 MHz, pyridine-d_c), δ: 38.7 (C-1), 26.4 (C-2), 83.0 (C-3), 44.2 (C-4), 48.2 (C-5), 18.7 (C-6), 32.8 (C-7), 40.8 (C-8), 54.9 (C-9), 36.9 (C-10), 127.4 (C-11), 126.2 (C-12), 137.1 (C-13), 44.7 (C-14), 35.4 (C-15), 76.8 (C-16), 45.3 (C-17), 133.6 (C-18), 33.8 (C-19), 38.7 (C-20), 30.3 (C-21), 29.8 (C-22), 65.0 (C-23), 13.2 (C-24), 19.1 (C-25), 17.4 (C-26), 22.4 (C-27), 64.6 (C-28), 21.2 (C-29), 73.5 (C-30), 104.4 (C-1'), 77.6 (C-2'), 85.2 (C-3'), 72.0 (C-4'), 70.9 (C-5'), 17.6 (C-6'), 104.5 (C-1"), 76.6 (C-2"), 79.2 (C-3"), 72.6 (C-4"), 77.8 (C-5"), 63.6 (C-6"), 105.5 (C-1"'), 75.8 (C-2"'), 78.8 (C-3"'), 72.4 (C-4"'), 78.9 (C-5"'), 63.0 (C-6"').

3.4. Acid hydrolysis of compound 1

Compound **1** (1.4 mg) was heated in 3 mol L⁻¹ HCl–CH₃OH (5 mL) for 3 h in a sealed tube at 90 °C. The mixture was then extracted with EtOAc. The aqueous layer was evaporated to dryness with ethanol in vacuo at 50 °C until neutral. The residues were determined in comparison with authentic monosaccharides using HPTLC (CHCl₃:CH₃OH:H₂O = 3:2:0.2, visualisation with ethanol–5% H₂SO₄ spraying). For the sugars of compound **1**, the *R*_f values of glucose and fucose by TLC were 0.32 and 0.40, respectively.

3.5. Effect of compounds on platelet aggregation

The blood platelet aggregation test was performed according to the method of Born et al. (1963). Briefly, whole blood samples were collected from the carotid of urethaneanesthetised rabbits and mixed with 3.8% sodium citrate (9:1 v/v), followed by centrifuging at 1000 rpm for 10 min. The supernatants were obtained and used as platelet rich plasma (PRP). While the remaining blood sample was centrifuged at 3000 rpm for10 min and the supernatants were collected as platelet poor plasma (PPP). The effect of individual compounds on the platelet aggregation was measured by the Born's Light Transmission Aggregometry method using Agg-RAM-Aggregometer (Helena Laboratories, Beaumont, Texas). Briefly, the amount of platelet in PRP with PPP was adjusted to 3×10^{11} L⁻¹. The light transmission was set at 0% with PRP and at 100% with PPP. PRP (225 µL) was incubated with 25 µL of vehicle (final concentration 0.3% DMSO) or different concentrations of individual compounds in turbidimetric cup at 37 °C for 5 min. The changes in the light transmittance of the reaction mixture were continuously recorded for 5 min and the maximal aggregation was recorded. Polyphyllins II and ADP were used as positive control.

3.6. TT assay

PPP was prepared referring to the method in Section 3.5. TT was determined by a coagulation-analysis instrument (LG-PABER-I, Steellex Co., China) (Liu et al. 2010). A reagent kit according to the manufacturer's recommendations was used to assay TT. Briefly, 50 μ L of PPP and 10 μ L of individual compounds solution (100, 200 μ M) was incubated for 3 min at 37 °C, followed by addition of 100 μ L of thrombin solution. Shortly after adding thrombin solution, the coagulation-analysis instrument was started up to record TT.

3.7. Statistical analysis

All data are expressed as mean \pm SD. Statistical differences among groups were analysed by the one-way analysis of variance (ANOVA) and two-tailed Student's *t*-test. A value of *p* < 0.05 was considered significant.

4. Conclusions

In this study, seven triterpenoid saponins and two triterpenes including a new triterpenoid saponin were isolated and elucidated from *C. chinense* (Benth.) O. Kuntze. Among them, compounds **4** and **7** markedly promoted rabbit platelet aggregation and compounds **2** and **9** significantly shortened plasma TT, which indicated that compounds **4** and **7** could accelerate the thrombosis through promoting platelet aggregation so as to stop the bleeding and compounds **2** and **9** could affect the function of fibrinogen in plasma. Such results suggest that compounds **2**, **4**, **7** and **9** might be the main haemostatic ingredients of *C. chinense*. Furthermore, the $\Delta^{11,13(18)}$ -Olean diene saponins and 13, 28-epoxy-Olean-11-ene saponins exhibited better haemostatic activity when compared to the Δ^{12} -Oleanene saponins in *C. chinense* (Benth.) O. Kuntze.

Supplementary material

Supplementary material relating to this article is available online, alongside Figures S1–S12, Tables S1 and S3.

Disclosure statement

No potential conflict of interest was reported by the authors.

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