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Two new flavonoid–triterpene saponin meroterpenoids from *Clinopodium chinense* and their protective effects against anoxia/reoxygenation-induced apoptosis in H9c2 cells

Yin-Di Zhu[a,b,1], Rong-Chang Chen[b,1], Hong Wang[a], Hai Jiang[c], Xue-Li Huang[a], Mei-Ling Zhang[a], Ling-Yu Li[b], Zhen Hu[d], Xu-Dong Xu[b,*], Chao-Jie Wang[a,*], Xiao-Xia Ye[a,*], Jun-Shan Yang[b]

[a] School of Pharmacy, Wenzhou Medical University, Wenzhou 325035, China
[b] Key Laboratory of Bioactive Substances and Resources Utilization of Chinese Herbal Medicine, Ministry of Education, Institute of Medicinal Plant Development, Peking Union Medical College and Chinese Academy of Medical Sciences, Beijing 100193, China
[c] Key Laboratory of Chinese Materia Medica, Ministry of Education, Heilongjiang University of Chinese Medicine, Harbin 150040, China
[d] The Second Affiliated Hospital & Yuying Children's Hospital of Wenzhou Medical University, Wenzhou Medical University, Wenzhou 325027, China

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ABSTRACT

Two new flavonoid–triterpene saponin meroterpenoids, clinoposides G (**1**) and H (**2**) were isolated from the aerial parts of *Clinopodium chinense* (Benth.) O. Kuntze. Their structures were elucidated through spectroscopic and electronic circular dichroism (ECD) analyses. Compounds **1** and **2** were evaluated for their protective effects against anoxia/reoxygenation(A/R)-induced injury in H9c2 cells. A/R treatment severely injured the H9c2 cells, which was accompanied by apoptosis. Both **1** and **2** pretreatment significantly inhibited cell injury and apoptosis, improved mitochondrial membrane potential, increased activities of antioxidant enzymes, and reduced the levels of the inflammatory cytokines. In addition, the presence of **1** and **2** significantly decreased the protein level of p65 and increased the level of Nrf2 in cell nucleus. Unique chemical structure and good biological activity of **1** and **2** elucidated the potential of meroterpenoids as a promising reagent for treating heart disease.

1. Introduction

Clinopodium is a genus in the family Lamiaceae, which contains approximately 22 species occurring in tropical and subtropical regions of Southeast Asia [1]. The aerial parts of *C. chinense*, couple with *Clinopodium polycephalum*, which are known as “duanxueliu” in China, have been used as a traditional folk medicine for the treatment of hematuria, skin trauma, influenza and allergic dermatitis [2]. Numerous chemical constituents have been reported from the genus *Clinopodium* including flavonoids [3], triterpenoid saponins [4], phenylpropanoids [5], diterpenes [6] and quinoids [7], as well as volatile and fatty oils, and these components exhibit diverse biological activities, including helostatic [8], anti-hyperglycemic [9], anti-tumour [10] and cardioprotective activity [11].

Our previous studies proved that total flavonoids from *Clinopodium chinense* (Benth.) O. Kuntze showed significantly protective effect against doxorubicin-induced cardiotoxicity and ischemic heart disease [12]. Further chemical studies found six novel favonoid-triterpene saponin meroterpenoids with unprecedented structures in the aerial parts of *Clinopodium chinense* [13]. In the present study, we examined the protective effects of two new flavonoid–triterpene saponin meroterpenoids, clinoposides G (**1**) and H (**2**) (Fig. 1), against anoxia/reoxygenation(A/R)-induced H9c2 cells injury in vitro. This function relies on the activation of Nrf2 and inhibition of NF-κB.

2. Experimental

2.1. General experimental procedures

Optical rotation values were determined on a Perkin-Elmer 341 digital polarimeter equipped with a sodium lamp (589 nm) and a 1-dm microcell in MeOH. UV spectra were recorded on a Shimadzu UV2550 spectrometer in MeOH. ECD spectra were measured in MeOH on a JASCO J-815 spectropolarimeter. NMR spectra were acquired on Bruker AV III 600 MHz (the chemical shift values are reported as δ values with TMS as an internal standard). HR-ESI-MS spectra were measured on a LTQ Orbitrap XL mass spectrometer (Thermo Scientific, Waltham, MA, USA). Column chromatography was performed over

* Corresponding authors.
E-mail addresses: xdxu@implad.ac.cn (X.-D. Xu), chjwang@wmu.edu.cn (C.-J. Wang), yxx@wmu.edu.cn (X.-X. Ye).
[1] These authors contribute equally to this work.

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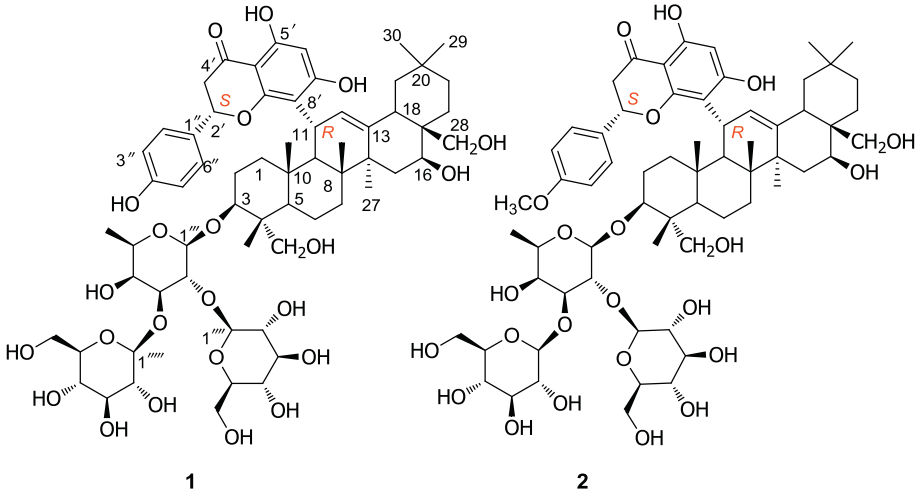

Fig. 1. Structures of compounds **1** and **2**.

D101 macroporous resin (The Chemical Plant of Nankai University, Tianjin, China), silica gel (100–200 mesh, Qingdao Haiyang Chemical Co., Ltd., Qingdao, China), Sephadex LH-20 (Pharmacia, Uppsala, Sweden), and C-18 reversed-phase silica gel (50 μm, YMC CO., LTD., Kyoto, Japan). HPLC was conducted on a CXTH LC-3000 HPLC system with a CXTH LC-3000 UV spectrophotometric detector (UV detection at 210 nm) and a YMC (250 × 10 mm; flow rate 2.0 mL/min) semi-preparative column packed with C18 (5 μm, YMC CO., LTD., Kyoto, Japan). TLC was performed on precoated silica gel GF254 plates (Yantai chemical industry research institute, Yantai, China), and spots were detected with UV lights (254 and 365 nm) and further sprayed with 10% H_2SO_4 reagent followed by heating to 100 °C. GC analysis was carried out on an Agilent 6890 N gas chromatograph equipped with a FID detector (injector temp. 250 °C; detector temp. 250 °C; N_2 as carrier gas). Fucose was purchased from Alfa Aesar, and D-glucopyranose was purchased from Sinopharm Chemical Reagent Co., Ltd. All the solvents used for column chromatography and HPLC were of analytical grade (Beijing Chemical Works, China). Quercetin and ginsenoside Rb 1 (purity > 99%) were obtained from Shanghai Winherb Medical S & T Development (Shanghai, China). Cell culture products were purchased from Gibco BRL (Grand island, NY). TUNEL kit and JC-1 were purchased from Sigma-Aldrich (St. Louis, MI). The kits for determining LDH, SOD, and CAT were obtained from the Jiancheng Bioengineering Institute (Nanjing, China). The ELISA kits for determining TNF-α, IL-6, IFN-γ, and MCP-1 were from R&D Systems (Wiesbaden, Germany). Primary antibodies against p-p65, Nrf2, and *β*-actin were from Santa Cruz Biotechnology (CA). Horseradish peroxidase (HRP)-conjugated secondary antibodies were from CW biotech (Beijing, China).

2.2. Plant material

The aerial parts of *C. chinense* were purchased from Chinese Medicinal Material Markets (Bozhou, China) in 2013. The plant was identified by Dr. Shichun Yu at the Anhui University of Traditional Chinese Medicine, Anhui, China and a voucher specimen (20130312) was deposited in the Key Laboratory of Bioactive Substances and Resources Utilization of Chinese Herbal Medicine, Ministry of Education, Beijing, China.

2.3. Extraction and isolation

The dried and powdered plant materia (15 kg) were extracted twice (2 h each time) with 100 L of 70% ethanol (v/v) at 80 °C to afford a crude extract (3000 g, 20% yield) after filtration and removal of the solvent using a rotary evaporator. The crude extract was suspended in H_2O and partitioned successively with petroleum ether, ethyl acetate, and *n*-BuOH to obtain three fractions. The *n*-BuOH extract (350 g) was subjected to D101 column (700 mm × 250 mm) eluted with mixtures of EtOH − H_2O (1:4, 1:1, 6:1, and 1:0), the fractions eluted with EtOH − H_2O (1:1 and 6:1) were combined and evaporated at 55 °C under vacuum to afford approximately 180 g of the total saponins. The total saponins (180 g) were subjected to SiO_2 column chromatography (720 g, 100–200 mesh, 8 × 60 cm) using a gradient of $CHCl_3$/MeOH (from 1:0 to 0:1, flow rate 40 mL/min) to obtain fractions A–H. Fraction G (30 g) was purified by flash chromatography on RP-18 eluted with MeOH − H_2O mixtures of decreasing polarity (30:70 to 90:10) to afford fractions G1–G9. The fraction G6 (250 mg) was further purified by Sephadex LH-20 (MeOH) to afford 4 fractions (Frs G6.1–6.4). Fraction G6.4 (40 mg) was separated by ODS HPLC (eluent MeOH/H_2O, 72:28) to isolate **2** (4.3 mg, t_R = 29 min). The fraction G8 (320 mg) was chromatographed Sephadex LH-20 (MeOH) to afford 5 fractions (Frs G8.1–8.5). Fraction G8.5 (80 mg) was separated by ODS HPLC (eluent MeOH/H_2O, 67:33) to isolate **1** (8.2 mg, t_R = 38 min).

Clinoposide G (**1**): Yellow powder; $[\alpha]_D^{20}$ − 7.1(c 0.14, MeOH); UV (MeOH) λ_{max} (log ε) 206 (4.43), 296 (4.13), 345 (3.53) nm; IR (film) ν_{max} 3368, 2942, 2879, 1636, 1613, 1382, 1247, 1070, 837 cm^{-1}; ^1H and ^{13}C NMR (Table 1); HR-ESI-MS: *m/z*1213.5829 $[M-H]^-$ (calcd for $C_{63}H_{89}O_{23}$, 1213.5795).

Clinoposide H (**2**): Yellow powder; $[\alpha]_D^{20}$ − 5.7 (c 0.07, MeOH); UV (MeOH) λ_{max} (log ε) 212 (4.35), 297 (4.07), 344 (3.53) nm; IR (film) ν_{max} 3353, 2938, 1636, 1612, 1379, 1250, 1070, 832; ^1H and ^{13}C NMR (Table 1); HR-ESI-MS: *m/z*1251.5896 $[M + Na]^+$ (calcd for $C_{64}H_{92}O_{23}Na$, 1251.5927).

2.4. ECD calculations

A conformational search for compound **1** (the substituted triheteroglycan was simplified to a methyl group) was performed via Spartan'14 software using the MMFF force field calculation, the conformers generated were further optimized at the gas-phase B3LYP/6-31G(d) level via Gaussian 09 suite of programs. Time-dependent density functional theory (TD-DFT) at the B3LYP/6-311G + (d,p) level was employed to calculate excitation energy and rotatory strength of the conformers and the solvent effect of methanol were taken into consideration using a conductor-like polarizable continuum model (CPCM). The CD curves were simulated by SpecDis 1.64 program using a Gaussian function with a bandwidth σ of 0.24 eV and UV shift of − 12 nm [14].