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Two new isopimarane diterpenes from the feces of *Trogopterus xanthipes*

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ABSTRACT

Chemical investigation of Trogopterus Feces has led to the isolation of two new isopimarane diterpenes, wulingzhic acid A (1) and wulingzhic acid B (2). Their structures were elucidated by chemical and extensive spectral analysis. Compounds 1 and 2 exhibited weak activity of antithrombin and moderate activity of antiplatelet aggregation *in vitro*.

1. Introduction

Trogopterus Feces, called "Wulingzhi", are the dry stool of *Trogopterus xanthipes* Milne-Edwards (Petauristidae). Trogopterus Feces have the function of invigorating blood and relieving pain, and are often used in treatment of amenorrhea, menses pain and postpartum abdominal pain in traditional Chinese medicine. Modern studies have indicated that Trogopterus Feces mainly consisted of the chemical constituents of triterpenoids, diterpenoids and phenolic acids, and possess the pharmacological action of antiplatelet aggregation and anti-inflammatory [1–3]. In our present study different solvent extracts of Trogopterus Feces were screened, which showed that the ethyl acetate extract was the important active part. So the ethyl acetate extract was chemically investigated and two new isopimarane diterpenes were isolated. Wulingzhic acid was an isopimarane diterpene from Trogopterus Feces, which was reported to have pharmacological functions of antiplatelet aggregation and antibiosis [1]. In this study, we deal with the isolation and structural elucidation of two new isopimarane diterpenes, wulingzhic acid A (**1**) and wulingzhic acid B (**2**) (Fig. 1), and report some of their anticoagulative activities. Their structures were elucidated by means of chemical and extensive spectroscopic analysis.

2. Experimental

2.1. General experimental procedures

Optical rotations were measured on a JASCO P-1020 polarimeter. IR spectra were taken on a Nicolet IR-100 FT-IR spectrometer in KBr discs. NMR spectra were measured on a Bruker AV-500 MHz (500 MHz for ^{1}H NMR and 125 MHz for ^{13}C NMR) using TMS as internal standard and chemical shifts were recorded as δ values. ESI-MS and HR-ESI-MS spectra were obtained on a Micromass Q/TOF Mass Spectrometer. Anticoagulative assay was performed on coagulation analysis instrument LG-PABER-I. The blood sample was treated on Anke TDL-40B centrifugal machine. Silica gel for column chromatography (CC) (200–300 mesh) and TLC plates (10–40 μm) were the products of Qingdao Marine Chemical Co., Ltd (Qingdao, China). Thrombin was purchased from Xisen Sanhe Co., Ltd (Leling, China). Adenosine diphosphate (ADP) was purchased from Beijing Zhongqin Scientific Instrument Co. Ltd (Beijing, China). Rabbit (3.8 kg) was supplied by Shanghai Sikelai Experimental Animal Co., Ltd (Shanghai, China).

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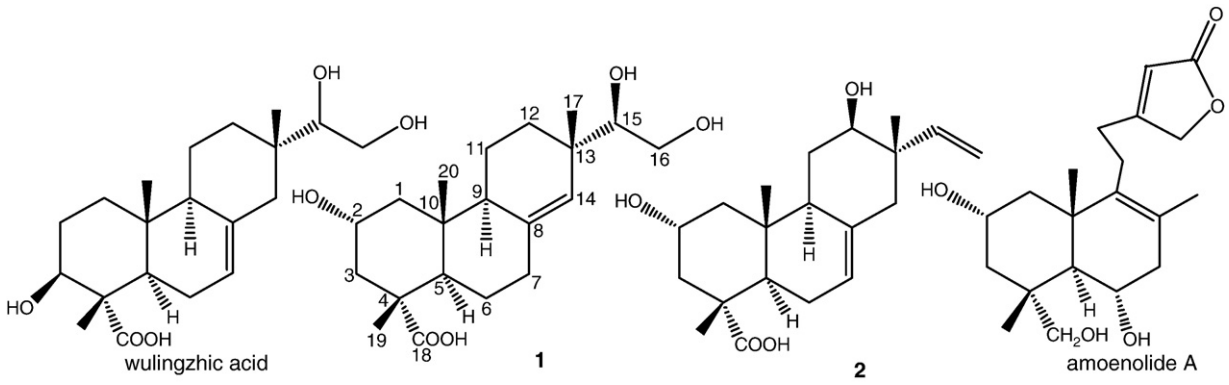

Fig. 1. Structures of wulingzhic acid and compounds **1**–**2**.

2.2. Materials

Trogopterus Feces were collected in June of 2008 from Hebei province of China, and identified as the dry stool of *T. xanthipes* by associate professor Nianyun Yang of Nanjing University of Traditional Chinese Medicine. A voucher specimen (GS-20080610) was kept in the Herbarium of Nanjing University of Traditional Chinese Medicine.

2.3. Extraction and isolation

The air-dried and powdered Trogopterus Feces (3 kg) were extracted with 60% C_2H_5OH (2 × 50 L) for 2 h under reflux, and the combined extracts were concentrated under reduced pressure. The resulting extract (370 g) was then suspended in H_2O and extracted successively with ethyl acetate, *n*-butanol to give the respective extracts after solvent removal. The combined ethyl acetate layers were evaporated under reduced pressure to leave the residue (277 g), which was chromatographed on silica gel (2 kg) eluting with $CHCl_3$–CH_3OH, stepwise gradient (100:0 → 5:1) and 5 fractions were collected. Fr. 3 (10 g) was separated by silica gel [CH_2Cl_2–CH_3OH (20:1)] to obtain compounds **1** (150 mg) and **2** (100 mg).

Wulingzhic acid A (**1**): white powder, $[\alpha]_D^{20}$ +49.0 (*c* 0.10, CH_3OH); IR (KBr) λ_{max} 3430, 2911, 1695, 1454, 1389 1258, 1034, 965, 720 cm^{-1}; ^1H NMR (CD_3OD, 500 MHz), see Table 1; ^{13}C NMR (CD_3OD, 125 MHz), see Table 1; ESI-MS: m/z *m/z* 351 $[M-H]^-$, 387 $[M+Cl]^-$, 703 $[2\,M-H]^-$ and 739 $[2\,M+Cl]^-$; HR-ESI-MS: m/z 351.2163 $[M-H]^-$ ($C_{20}H_{31}O_5$, calc. 351.2171).

Wulingzhic acid B (**2**): white powder, $[\alpha]_D^{20}$ +17.7 (*c* 0.11, CH_3OH); IR (KBr) λ_{max} 3430, 2909, 1693, 1461 1270, 1060, 722 cm^{-1}; ^1H NMR (CD_3OD, 500 MHz), see Table 1; ^{13}C NMR (CD_3OD, 125 MHz), see Table 1; ESI-MS: m/z 333 $[M-H]^-$ and 667$[2\,M-H]^-$; HR-ESI-MS: m/z 333.2059 $[M-H]^-$ ($C_{20}H_{29}O_4$, calc. 333.2066).

2.4. Clotting time of rabbit plasma thrombin

Evaluation of anticoagulative activities of the different solvent extracts and compounds **1**–**2** was performed by using thrombin time (TT) method. All samples were dissolved in ethanol respectively. Rabbit common carotid artery was cut off to take a sample of blood, which was mixed with anticoagulant (3.8% sodium citrate) in the proportion of 9 to 1, and the mixture was centrifuged at 2500 rpm for 15 min to collect the plasma. The plasma (50 μL) was put in a plastic cup for 3 min at 37 °C, and 100 μL thrombin solution of 15 U mL^{-1} diluted by 0.1 mol mL^{-1} pH 7.4 Tris–HCl buffer was also put in the plastic cup along with 10 μL sample solution, meanwhile, the coagulation analysis instrument was started up so that the thrombin clotting time was recorded. The same experiment was done for the positive control drug heparin sodium and the blank solvent ethanol. Each analyte was tested several times, and an average value was applied. TT prolongation rate was calculated to assess the anticoagulative activities of the samples.

Table 1
^1H (500 MHz) and ^{13}C (125 MHz) NMR spectral data of compounds **1**–**2** in CD_3OD (δ, ppm; *J*, Hz).

	1		**2**	
	^1H (m, *J* Hz)	^{13}C	^1H (m, *J* Hz)	^{13}C
1α	1.09 (t, 11.9)	48.5	1.08 (t, 11.9)	48.5
1β	2.02 (m)		2.10 (m)	
2	3.80 (dddd, 11.6, 11.6, 4.3, 4.3 Hz)	64.9	3.82 (dddd, 11.7, 11.7, 4.3, 4.3 Hz)	64.8
3α	1.67 (t, 11.6)	46.4	1.68 (t, 11.5)	46.5
3β	1.89 (m)		1.89 (m)	
4		44.7		46.2
5	1.91 (d, 2.8)	50.4	2.05 (m)	45.9
6α	1.27 (m)	25.0	1.76 (m)	25.8
6β	1.34 (m)		1.94 (m)	
7α	2.15 (m)	36.2	5.37 (brd, 5.1)	122.6
7β	2.27 (ddd, 14.5, 5.6, 2.9)			
8		137.3		135.3
9	1.85 (m)	52.8	2.04 (m)	52.4
10		39.7		37.7
11α	1.64 (m)	19.6	1.78 (m)	29.6
11β	1.52 (m)		1.43 (m)	
12α	1.30 (m)	31.5	3.52 (dd, 11.6, 5.6)	75.8
12β	1.47 (m)			
13		38.9		43.1
14	5.40 (brs)	129.9	1.93 (m)	45.9
15	3.24 (dd, 8.7, 2.6)	81.2	5.89 (dd, 17.5, 10.8)	148.3
16a	3.61 (dd, 11.2, 2.6)	64.0	5.02 (dd, 17.5, 1.4)	112.4
16b	3.43 (dd, 11.2, 8.7)		5.00 (dd, 10.8, 1.4)	
17	0.98 (s)	22.7	0.84 (s)	15.1
18		181.4		181.3
19	1.21 (s)	18.5	1.27 (s)	18.8
20	0.87 (s)	16.2	0.98 (s)	16.5