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Original article

Three minor new compounds from the aerial parts of *Leonurus japonicus*

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ABSTRACT

Phytochemical investigation of the aerial parts of *Leonurus japonicus* led to the isolation of one new labdane diterpenoid, leojaponin D (**1**) and two new ionone derivatives, leojapononones A and B (**2** and **3**), together with seven known diterpenoids (**4**–**10**). Their structures were elucidated by extensive 1D and 2D NMR spectroscopic data and by comparison with data reported in the literature. Selected isolates were evaluated their effects on Jurkat IL2 secretion.

1. Introduction

Leonurus japonicus (Labiatae), commonly called Chinese motherwort, is a herbaceous flowering plant native to several regions in Asia, including China, Korea, Japan and Cambodia. For thousands of years in China, the aerial part of *Leonurus japonicus* has been primarily used to treat menoxenia, dysmenorrhea, amenorrhea, lochia, oliguresis, ulcerations and other diseases in women, and thus is named "Yi Mu Cao" [1,2]. Phytochemical investigation on this species has led to discover various natural compounds with different structural patterns, including alkaloids, flavonoids, glycosides, diterpenoids and triterpenoids, among which diterpenoids are the major constituents [3]. Our previous research on the chemical constituents of *L. japonicus* has led to the isolation of three diterpenoids [4], especially leojaponin A, which is the first example of clerodane-type diterpenoid obtained from *L. japonicus*. As part of our ongoing program to discover structurally interesting and potential bioactive chemical constituents, we reinvestigated this plant, and obtained three additional new compounds, including one labdane-type diterpenoid, leojaponin D (**1**), and two ionone derivatives, leojapononones A and B (**2** and **3**), together with seven known diterpenoids, leojaponins A–C (**4–6**) [4], leoheteronin D (**7**) [5], villenol (**8**) [6], leoheterin (**9**) [7] and 3α-acetoxy-7β-hydroxy-15-O-methylleopersin C(**10**) [8] (Fig. 1). Herein, we report the isolation and structure elucidation of the new compounds, as well as the effect on Jurkat IL2 secretion of selected isolates.

2. Experimental

2.1. Plant material

The aerial parts of *L. japonicus* were collected in Xichang county, Sichuan Province, China, and identified by Prof. Xi-Wen Li, Kunming Institute of Botany. A voucher specimen (KIB 20120601) was deposited at the State Key Laboratory of Phytochemistry and Plant Resources in West China, Kunming Institute of Botany, Chinese Academy of Sciences.

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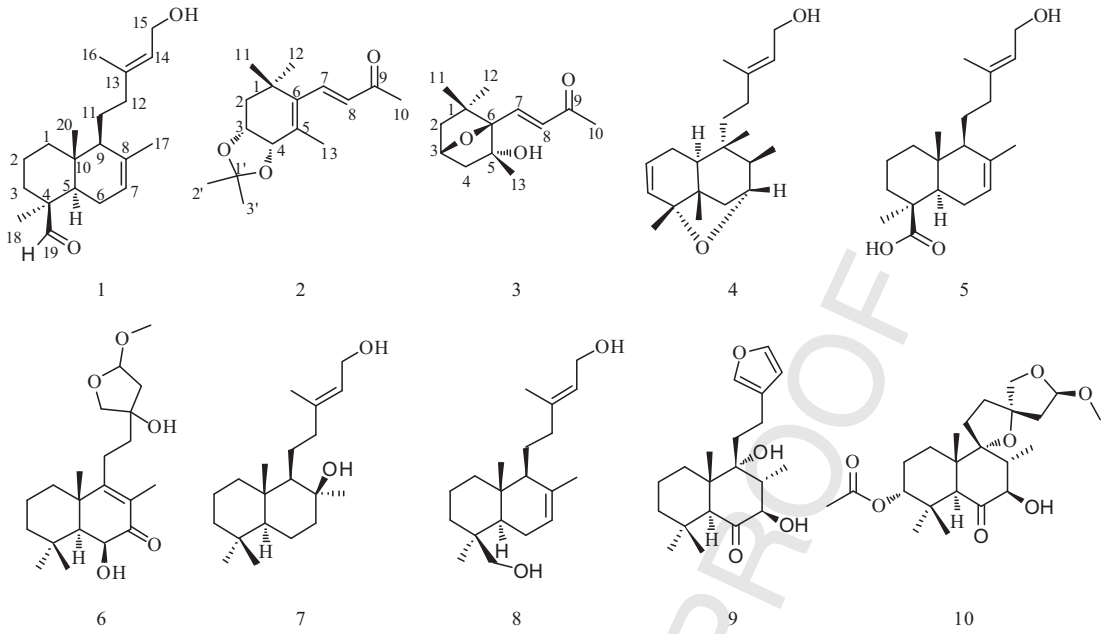

Fig. 1. Structures of compounds **1–10**.

2.2. Extraction and isolation

The air dried aerial parts of *L. japonicus* (15.0 kg) were extracted with 95% EtOH (3 L × 40 L) at room temperature, and the combined solvents were evaporated *in vacuo* to yield a residue (1.5 kg). The residue was subjected to a silica gel column (10 kg, 100–200 mesh) eluting with $CHCl_3$–Me_2CO (1:0, 9:1, 8:2, 2:1, 1:1, 0:1) to afford fractions A–F. Fraction A (120 g) was decolorized using MCI gel (90% MeOH–H_2O), and the concentrated elute was chromatographed *via* a silica gel CC (1.2 kg, 200–300 mesh) eluting with petroleum ether–Me_2CO gradient (100:1–0:1) to afford subfractions A1–A6. Fraction A2 (11.0 g) was subjected to a RP-18 CC (500 g, MeOH–H_2O gradient, 40–100%) to afford subfractions A2.1–A2.5. Compound **1** (1.1 mg) was isolated from fraction A2.2 followed by repeated column chromatography over silica gel (17 g, $CHCl_3$–Me_2CO gradient, 100:1–1:1). Fraction A3 (23.0 g) was separated by medium-pressure column chromatography on RP-18 (600 g, MeOH–H_2O gradient, 40–100%) to give subfractions A3.1–A3.3. Fraction A3.1.2 (4.2 g) was separated by Sephadex LH-20 CC ($CHCl_3$–MeOH), and then by repeated column chromatography over silica gel (3 g, petroleum ether–Me_2CO gradient, 30:1–1:1) to give subfractions A3.1.2.1–A3.1.2.8. Compound **2** (1.2 mg) and **3** (1.1 mg) were finally purified by semi-preparative HPLC (60% MeCN–H_2O) from fraction A3.1.2.4 (306.2 mg). Fraction B (70.0 g) was subjected to MCI gel (90% MeOH–H_2O) and chromatographed on silica gel (petroleum ether–Me_2CO, 30:1–0:1) to afford subfractions B1–B7. Fraction B3 (11.3 g) was chromatographed *via* a RP-18 column (30–100% gradient MeOH–H_2O) to furnish B3.1–B3.7. Fraction B3.2 (0.06 g) was purified by semi-preparative HPLC (62% MeCN–H_2O) to give compounds **4** (12.0 mg) and **7** (3.0 mg). Fraction B3.3 (0.25 g) was purified by Sephadex LH-20, and finally by semi-preparative HPLC (50% MeCN–H_2O) to give compounds **5** (14.0 mg), **8** (8.1 mg), and **9** (6.3 mg). Fraction B3.4 (0.08 g) was purified by LH-20 and semi-preparative HPLC (42% MeCN–H_2O) to yield compounds **6** (6.0 mg) and **10** (11.4 mg).

2.3. The immune activity assay

Proliferation assay: Jurkat T cells (5000 cells/well) were seeded into 96 well plate. Compounds **4–10** were dissolved in DMSO and added into cells with final concentration of 20 μmol/L. The cell viability was measured with AlamarBlue (Invitrogen Inc.) after 72 h.

ELISA assay to detect the secretion of Interleukin 2 (IL-2): Jurkat T cells (105 cells/well) were seeded into 96 well plate and incubated with compounds as indicated in the presence of PMA (40 nmol/L) and Ionomycin (1 μmol/L) for 12 h. The amount of IL-2 in medium was measured with the kit from BD biosciences. Briefly, the capture antibody in coating buffer (0.1 mol/L Sodium carbonate, pH 9.5) (1:500) was coated onto 96 well plate for overnight at 4 °C. After washing, medium from cultured cells was added into plate and incubated for 2 h at room temperature. Detector antibodies were used to incubate with captured IL-2 and developed with TMB substrate after stopping the reaction with stopping solution. Plates were read immediately at 450 nm with Envision from PE company.

3. Results and discussion

Compound **1** was obtained as a colorless oil, $[\alpha]^{24.4}_{D}$ – 92.2 (*c* 0.11, MeOH); UV (MeOH) λ_{max} (log ε) 203 (4.06) nm. The molecular formula of **1** was assigned as $C_{20}H_{32}O_2$ from its HR-EIMS (*m/z* 304.2402 $[M]^+$, calcd. 327.2295 $[M+Na]^+$) with five degrees of unsaturation. The IR spectrum revealed the presence of a hydroxyl group (3427 cm^{-1}) and a carboxyl group (1724 cm^{-1}). The ^1H NMR spectrum (Table 1) displayed signals of four tertiary methyl groups and one olefinic proton signal (δ_H 5.17). The ^{13}C NMR and DEPT spectra (Table 1) exhibited 20 carbon resonances attributed to four methyls, seven methylenes, five methines (one carbonyl and two olefinics) and four quaternary carbons (two olefinics). On the base of the HSQC spectrum, all protons were assigned unambiguously to their corresponding carbons. The ^1H–^1H COSY and HSQC spectra established the spin systems for the molecular structure fragments of C-1–C-2–C-3, C-5–C-6–C-7, C-9–C-11–C-12 and C-14–C-15, and the connectivity was confirmed by the HMBC experiment (Fig. 2). The HMBC correlations from Me-18 to C-3, C-4 and C-5, from H-19 to C-4, and from H-5 to C-4 and C-18 indicated that the quaternary carbon C-4 was connected with C-18 and C-19 and C-3 was linked to C-5 through C-4. The HMBC correlations from H-1 and H-5 to C-10 and C-20 indicated that the quaternary C-10 was connected

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Table 1
NMR data of compounds **1–3** ($CDCl_3$, TMS, δ in ppm, J in Hz).[a]

No.	**1**		**2**		**3**	
	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H
1α	35.2 (t)	1.29 (overlap, 1 H)	35.5 (s)		44.1 (s)	
1β		1.48 (overlap, 1H)				
2α	17.1 (t)	1.63 (overlap, 1H)	41.2 (t)	1.71 (m, 2H)	48.3 (t)	1.63 (d, 1H, 11.4)
2β		1.63 (overlap, 1H)				1.84 (dd, 1H, 6.0, 11.4)
3α	33.3 (t)	1.42 (overlap, 1H)	71.8 (d)	4.39 (overlap, 1H)	75.5 (d)	4.41 (d, 1H, 6.0)
3β		1.32 (overlap, 1H)				
4α	48.7 (s)		75.9 (d)	4.39 (overlap, 1H)	47.7 (t)	1.69 (d, 1H, 12.1)
4β						2.05 (dd, 1H, 6.2, 12.1)
5	34.9 (d)	1.80 (overlap, 1H)	129.2 (s)		82.0 (s)	
6α	25.2 (t)	1.52 (overlap, 1H)	140.2 (s)		91.1 (s)	
6β		1.80 (overlap, 1H)				
7	118 .8 (d)	5.17 (m, 1H)	141.9 (d)	7.19 (d, 1H, 16.4)	130.3 (d)	6.88 (d, 1H, 16.1)
8	136.7 (s)		133.1 (d)	7.19 (d, 1H, 16.4)	141.4 (d)	6.37 (d, 1H, 16.1)
9	53.5 (d)	1.25 (t-like, 1H)	198.2 (s)		197.7 (s)	
10	35.5 (s)		25.9 (q)	2.32 (s, 3H)	28.1 (q)	2.29 (s, 3H)
11α	29.7 (t)	1.61 (m, 1H)	28.1 (q)	1.48 (s, 3H)	25.5 (q)	1.46 (s, 3H)
11β		1.36 (m, 1H)				
12	41.3 (t)	2.05 (t-like, 2H)	27.9 (q)	1.41 (s, 3H)	31.7 (q)	0.87 (s, 3H)
13	139.9 (s)		18.6 (t)	1.89 (s, 3H)	31.2 (q)	1.20 (s, 3H)
14	123.2 (d)	5.41 (t, 1H, 6.8)				
15	59.3 (t)	4.16 (d, 2H, 6.8)				
16	16.4 (q)	1.68 (s, 3H)				
17	23.3 (q)	1.64 (s, 3H)				
18	14.5 (q)	1.10 (s, 3H)				
19	206.3 (d)	9.24 (s, 1H)				
20	22.1 (q)	0.94 (s, 3H)				
1′			108.4 (s)			
2′			27.4 (q)	1.06 (s, 3H)		
3′			29.0 (q)	1.14 (s, 3H)		

[a] ^1H NMR and ^{13}C NMR data were recorded at 400 MHz and 100 MHz, respectively.

with C-1 and C-5. The relationships from H-20 to C-10 and C-9, from H-11 to C-10, and from H-11, H-12 and H-20 to C-9, suggested that C-20 and C-12 were linked to C-9 through C-10 and C-11 respectively. The above evidence implied that **1** was a labdane-type diterpenoid, which was highly similar to the known diterpenoid, villenol [6]. A careful comparison of their NMR data suggested that the main difference resulted from the hydroxymethyl (C-19, δ_C 64.7) in villenol oxidized to an aldehyde group (C-19, δ_C 206.3) in compound **1**, which was further supported by the HMBC correlations of H-3 and H-5 with C-19. The ROESY correlation of H-15 with Me-16 disclosed that the double bond between C-13 and C-14 was E geometry. The relative configurations of **1** were established by analysis of its ROESY data. Considering the structures of labdane-type diterpenoids previously isolated from the species *L. japonicus*, Me-20 was supposed to be β-oriented [5]. The correlations of Me-20 with H-6β, H-11β and H-3β, of H-6α with H-5, of H-5 with H-9 and Me-18 revealed that H-5, H-9 and Me-18 were all α-oriented. Thus, compound **1** is elucidated as 15-hydroxy-labda-7, 13E-diene-4-al, named leojaponin D.

Compound **2** was isolated as a colorless oil, $[\alpha]^{17.0}_D$ + 3.4 (c 0.07, MeOH); UV (MeOH) λ_{max} (log ε) 202 (3.69) nm. Its molecular formula was determined as $C_{16}H_{24}O_3$ by its HR-EIMS (*m/z* 264.1728 [M] $^+$, calcd. 264.1725), implying five degrees of unsaturation. The IR spectrum revealed the existence of a carboxyl group (1724 cm^{-1}). Its ^1H NMR and ^{13}C NMR (Table 1) spectra displayed signals of six methyls, one methylene, four methines (including two oxymethines) and five quaternary carbons (including two olefinic carbons). In addition to two methyl singlets at δ_c 27.4 and 29.0 and one quaternary carbon at δ_c 108.4 (s), indicating an isopropylidene group, the other 13 carbons implied an ionone derivative, in which C-3 and C-4 were oxygenated. Careful comparison of NMR data of 2 with known compound 3α,4α-isopropyliden-β-ionol showed close structural similarities[9].

The main difference resulted from the hydroxymethine (C-9, δ_c 68.1) in 3α,4α-isopropyliden-β-ionol oxidized to a carboxyl (C-9, δ_c 198.2) in **2**, which was confirmed by the HMBC correlations of H-7, H-8 and H-10 with C-9 (Fig. 3). The relative configurations of **2** were established by analysis of its ROESY data. The stereochemistry of H-3 and H-4 were both assigned β-orientated according to

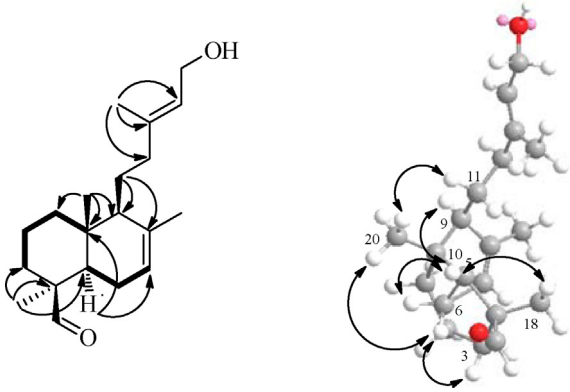

Fig. 2. Key HMBC (H → C), ^1H–^1H COSY (–) and ROESY (H⌒H) correlations of **1**.

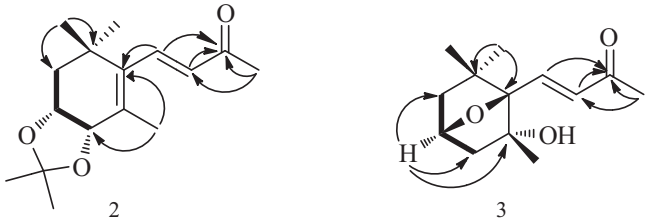

Fig. 3. Selected HMBC (H → C) and ^1H–^1H COSY (–) correlations of **2** and **3**.

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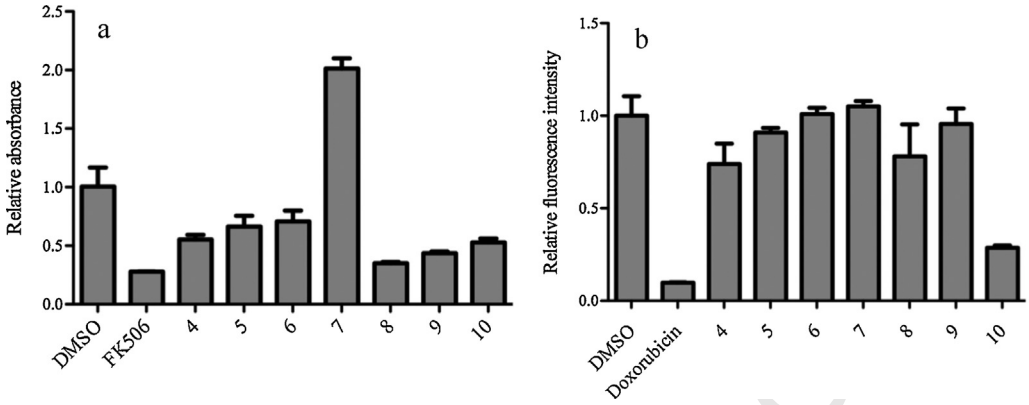

Fig. 4. Effect of compounds on the secretion of IL-2 and proliferation of Jurkat T cells. (a) Jurkat T cells were seeded into 96 well plate, and treated with compounds as indicated in the presence of PMA and ionomycinity was measured by alarmablue after the treatment of the compounds as indicated for 72 h.SA. (b) Jurkat T cells were plated into 96 well plate and the cell viability was measured by alarmablue after the treatment of the compounds as indicated for 72 h.

the correlations between these two protons and Me-11. Thus, compound **2** is determined as 3α,4α-isopropyliden-β-ionone, and given the trivial name as leojaponone A.

Compound **3** was isolated as a colorless oil with $[\alpha]^{14.0}_{D}$ −19.2 (*c* 0.11, MeOH) and UV (MeOH) λ_{max} (log ε) 225 (3.79) nm, which gave the molecular formula $C_{13}H_{20}O_3$ from its HREIMS (*m/z* 224.1409 $[M]^+$, calcd. 224.1412), requiring four degrees of unsaturation. The IR spectrum revealed a hydroxyl group (3433 cm^{-1}) and a carboxyl group (1666 cm^{-1}). The ^1H and ^{13}C NMR data (Table 1) of **3** were highly similar to those reported for crotalionoside C [10], which was also one ionone derivative. A careful comparison of their 1D NMR data, together with HMBC and ^1H–^1H COSY analysis indicated that the difference was due to a hydroxymethine (C-9, δ_c 69.3) in crotalionoside C replaced by a carboxyl group (C-9, δ_c 197.7) in **3**. This was confirmed by HMBC correlations from H-7, H-8 and H-10 to C-9. In the ROESY spectrum, the correlations of Me-11 with H-2β and H-4β indicated that they were all β-oriented. Correlations of H-3 with Me-13 implied that they were all α-oriented. Therefore, compound **3** is determined as megatigma-7-en-3,6-epoxy-5-hydroxy-9-one, and named as leojaponone B.

Considering the immune activity of diterpenoids previously isolated from the plants of the genus *Leonurus* [11], compounds **4**–**10** were tested for their *in vitro* effect on the secretion of IL-2 and the proliferation in Jourkat T cells [12]. Except for compound **7**, all compounds exhibited the inhibition of IL-2 secretion upon the activation of T cell by PMA and ionomycin at the dose of 20 μmol/L. However, compound **10** also interfered the proliferation of Jurkat T cells (Fig. 4). New compounds (**1**–**3**) were not tested their bioactivities currently for the mass limitation.

4. Conclusion

In summary, three new minor compounds, including one labdane-type diterpenoid (**1**) and two ionone derivatives (**2** and **3**), together with seven known diterpenoids, were isolated from the aerial parts of *L. japonicus*. This investigation could shed new light on the further understanding of the chemical constituents of *L. japonicus*.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.cclet.2015.05.004.

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