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Chinese Journal of Natural Medicines 2019, **17**(1): 0027−0032
Chinese Journal of Natural Medicines

•Special topic•

Two new ursane-type nortriterpenes from *Lonicera macranthoides* and their iNOS-inhibitory activities

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Available online 20 Jan., 2019

[ABSTRACT] The flower buds of *Lonicera macranthoides* (Shan Yin-Hua), represent an important traditional Chinese medicine and food ingredient. A phytochemical investigation of the 70% EtOH extract of the flower buds of *L. macranthoides* resulted in the isolation of 12 triterpenoids (**1**−**12**), including two new ursane-type nortriterpenes, 2*α*, 24-dihydroxy-23-nor-ursolic acid (**1**) and 2*α*, 4*α*-dihydroxy-23-nor-ursolic acid (**2**). Their structures were established by multiple spectroscopic methods and comparison with literature data. All isolated compounds were evaluated for their anti-inflammatory effects in LPS-activated RAW264.7 cells. Compounds **1** and **2** exhibited inhibitory effects on iNOS at the concentration of 30 μmol·L^{-1}.

[KEY WORDS] *Lonicerae Flos*; *Lonicera macranthoides*; Ursane-type nortriterpenes; Anti-inflammation; iNOS

[CLC Number] R284.1 **[Document code]** A **[Article ID]** 2095-6975(2019)01-0027-06

Introduction

Lonicerae Flos (also Shan Yin-Hua in Chinese), derived from dried flower buds of *Lonicera macranthoides* Hand. -Mazz., *L. hypoglauca* Miq., *L. confusa* DC. or *L. fulvotomentosa* Hsu et S. C. Cheng, is a commonly used Chinese herbal medicine recorded in *Chinese Pharmacopeia* (2015). It is prescribed commonly in Chinese formulae for its similar heat-clearing and detoxicating effects as those of *Lonicerae japonicae Flos* (Jin Yin−Hua). Modern pharmacological investigation on *Lonicerae Flos* has demonstrated a wide array of bioactive effects, such as antioxidation [1], anti-tumor [2-4], hepatoprotection [5], and anti-inflammation [6]. These bioactive functions are also closely related to the main chemical constituents, including organic acids [7], iridoids [8], triterpenoid saponins [9-15] and flavonoids [1].

As known, triterpenoid saponins were considered as characteristic chemicals specific to *Lonicerae Flos* and often chosen as the chemical/quality control markers, including the series of macranthoidin saponins (macranthoidin B, dipsacoside B, and macranthoidin A) [16]. We therefore focus on the study of triterpenoids from *Lonicerae Flos* to discover potential anti-inflammatory agents. Two new ursane-type nortriterpenes (**1** and **2**), along with ten known triterpenoid saponins (**3**−**12**) were isolated and identified. Anti-inflammatory activities of **1** and **2** were evaluated, and both can obviously inhibited expression of pro-inflammatory proteins inducible nitric oxide synthase (iNOS) and nitric oxide (NO) release in LPS-activated RAW264.7 cells.

Results and Discussion

Compound **1** was obtained as a white amorphous powder with $[\alpha]_D^{25}$ +15.24 (*c* 0.5, CH_3OH). The HR-ESI-MS exhibited a peak at *m/z* 473.3259 $[M - H]^-$ (Calc. 473.3267), corre-

[Received on] 10-Sep.-2018
[Research funding] This work was supported by the National Natural Science Foundation of China (Nos. 81602984 and 81630097) and Science and Technology Planning Project of Guangdong Province (No. 2015B030301005)
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These authors have no conflict of interest to declare.

sponding to a molecular formula $C_{29}H_{46}O_5$ with seven degrees of unsaturation. IR absorption bands at 3412 and 1691 cm^{-1} indicated the presence of hydroxyl and ketone groups. The ^1H NMR (600 MHz, in C_5D_5N) of **1** displayed an olefinic proton at δ_H 5.45 (1H, t, J = 3.5 Hz), five methyls [δ_H 1.18 (3H, s), 0.99 (3H, s), 0.98 (3H, d, J = 6.7 Hz), 0.97 (3H, d, J = 6.2 Hz), and 0.82 (3H, s)], an oxygenated methylene [δ_H 4.34 (1H, dd, J = 10.5, 10.5 Hz), 3.89 (1H, dd, J = 10.5, 2.0 Hz)], and two oxygenated methines [δ_H 4.26 (1H, ddd, J = 11.0, 9.3, 6.0 Hz), 4.06 (1H, dd, J = 9.3, 6.0 Hz)]. In the ^{13}C NMR (DEPT 135) spectra, 29 carbon signals could be revealed as five methyls, nine methylenes (an oxygenated at δ_C 62.1), nine methines (two oxygenated at δ_C 80.3 and 69.2), and six quaternary carbons (a carbonyl at δ_C 180.2, Table 1). Such evidence mentioned above suggested that 1 might be a highly oxygenated nortriterpenoid. An analysis of key COSY and HMBC correlations were used to establish the planar structure of compound **1**, as shown in Fig. 2. The spin system of H_2-1/H-2/H-3/H-4 (H_2-24)/H-5/H_2-6/H_2-7 deduced from ^1H-^1H COSY experiment, together with the correlations of H_3-25 to C-1, C-5, C-9, and C-10, and of H_3-26 to C-7, C-8, C-9, and C-14, confirmed the linkage and assignment of rings A and B. The substructure of ring C was confirmed by the COSY correlations of H-9/H_2-11/H-12, and HMBC correlations of H_3-27 to C-8, C-13, C-14, and C-15, and of H-12 to C-13, and C-14. Similarly, rings D and E were established by the COSY correlations of H_2-15/ H_2-16, and H-18/H-19(H_3-29)/H-20(H_3-30)/H_2-21/H_2-22, together with the HMBC correlation peaks of H-18/C-13, H_2-16/C-17, 18, 22, 28, and H_2-22/C-16, 17, 18, 28.

Table 1 ^1H (600 MHz) and ^{13}C NMR (150 MHz) data of compounds 1 and 2 (in C_5D_5N, J in Hz)

No.	1			2		
	δ_C		δ_H (J in Hz)	δ_C		δ_H (J in Hz)
1	48.6	CH_2	2.20 (dd, 12.6, 4.6) 1.21	48.3	CH_2	2.28 1.42
2	69.2	CH	4.26 (ddd, 11.0, 9.3, 4.6)	69.7	CH	4.08 (ddd, 11.1, 9.6, 4.4)
3	80.3	CH	4.06 (dd, 9.3, 6.0)	86.0	CH	3.93 (d, 9.6)
4	50.5	CH	2.62	75.6	C	
5	48.9	CH	1.38	56.7	CH	1.62
6	24.1	CH_2	1.33 1.01	18.3	CH_2	2.28 1.51
7	33.3	CH_2	1.55 1.27	33.4	CH_2	1.64 1.40
8	40.4	C		40.6	C	
9	47.3	CH	1.69 (dd, 11.3, 6.2)	48.5	CH	1.81 (dd, 10.8, 6.9)
10	38.4	C		37.5	C	
11	24.4	CH_2	1.92	24.2	CH_2	2.03
12	125.8	CH	5.45 (t, 3.5)	125.9	CH	5.48 (t, 3.5)
13	139.7	C		139.7	C	
14	42.9	C		42.9	C	
15	29.0	CH_2	2.28 (td, 12.6, 4.6)	29.0	CH_2	2.30
16	25.2	CH_2	2.11 (dd, 12.6, 4.6)	25.2	CH_2	2.09
17	48.4	C		48.4	C	
18	53.9	CH	2.61 (d, 10.8)	53.9	CH	2.62 (d, 11.3)
19	39.8	CH	1.46	39.8	CH	1.45
20	39.7	CH	1.02	39.8	CH	1.03
21	31.4	CH_2	1.43	31.4	CH_2	1.39
22	37.8	CH_2	1.95	37.8	CH_2	1.97
24	62.1	CH_2	4.34 (dd, 10.5, 10.5) 3.89 (dd, 10.5, 2.0)	19.6	CH_3	1.51 (s)
25	17.0	CH_3	0.82 (s)	17.0	CH_3	0.99 (s)
26	17.9	CH_3	0.99 (s)	17.9	CH_3	1.07 (s)
27	24.3	CH_3	1.18 (s)	24.2	CH_3	1.14 (s)
28	180.2	C		180.2	C	
29	17.7	CH_3	0.98 (d, 6.7)	17.8	CH_3	0.98 (d, 6.3)
30	21.8	CH_3	0.97 (d, 6.2)	21.8	CH_3	0.96 (d, 6.3)

Multiplets and or overlapped signals are reported without designating multiplicity.

The planar structure of **1** is similar to asiatic acid (**12**), implying that they might possess the same relative configurations. NOE correlations of H-5/H-9/H_3-27/H-19/H_3-30, H_3-25/H_3-26, and H-18/H_3-29 indicated that A/B, and B/C rings were *trans*-fused, D/E rings were *cis*-fused, and CH_3-27 was *α*-oriented in 1 (Fig. 2). NOE correlations of H_3-25/H-2/H_2-24, and H-3/H-5 and the coupling constants of H-2, H-3 and H-4 ($J_{2,3}$ = 9.3 Hz, $J_{3,4}$ = 6.0 Hz) indicated that the configurations of 2, 3-OH and 4-CH_2OH were 2*α*, 3*β*, 4*β*. Hence, the structure of **1** was elucidated as a new ursane-type nortriterpene, named 2*α*, 24-dihydroxy-23-nor-ursolic acid (Fig. 1).

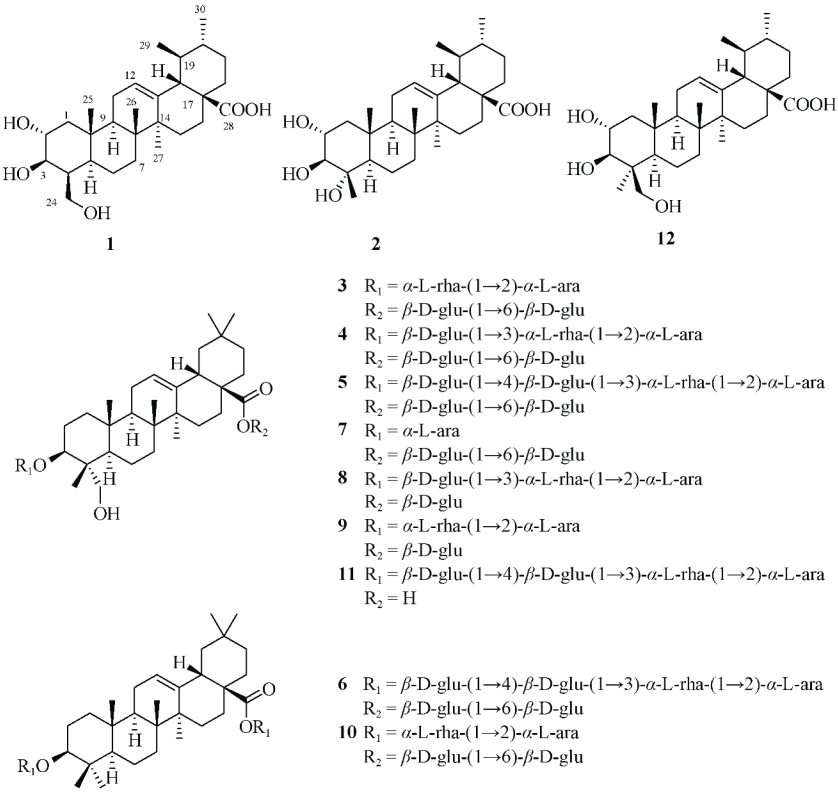

Fig. 1 The structures of compounds 1−12 isolated from *Lonicerae Flos*

Compound **2** was obtained as a white amorphous powder with $[\alpha]_D^{25}$ +6.36 (*c* 0.5, CH_3OH). Based on the HR-ESI-MS data, its molecular formula was established as $C_{29}H_{46}O_5$ (seven degrees of unsaturation). IR absorption spectrum revealed the presence of hydroxyl (3427 cm^{-1}) and ketone (1691 cm^{-1}) groups. All the proton and carbon signals of **2** were assigned unambiguously by ^{1}H-^{1}H COSY, HSQC, and HMBC experiments (Table 1). The ^{13}C NMR data of 2 were similar to 1 except that a hydroxymethyl group (C-24, δ_C 62.1) in 1 was replaced by a methyl (C-24, δ_C 19.6) in **2**, and C-4 was an oxygenated quaternary carbon (δ_C 75.6) in **2** instead of tertiary carbon in 1 as indicated by DEPT experiment. Thus, **2** was possibly a 4-OH isomer of **1**. The differences were verified by HMBC correlations of H_3-24/C-3, 4, 5 and H-3/C-2, 4, 24. Additionally, compound **2** had same relative configurations with 1 as indicated by the NOE data and coupling constants, and it was consequently identified as 2*α*, 4*α*-dihydroxy-23-nor-ursolic acid (Fig. 1).

Ten known compounds (Fig. 1) were obtained and identified as dipsacoside B (**3**) [14], macranthoidin A (**4**) [14], macranthoidin B (**5**) [17], lonimacranthoide II (**6**) [13], akebia saponin D (**7**) [18], 3*β*-*O*-(*β*-D-glucopyranosyl-(1→3)-*α*-L-rhamnopyranosyl-(1→2)-*α*-L-arabinopyranosyl)-hederagenin-28-*O*-(*β*-D-glucopyranosyl) ester (**8**) [12], thalictoside VI (**9**) [19], 3*β*-*O*-(*α*-L-rhamnopyranosyl-(1→2)-*α*-L-arabinopyranosyl)-28-*O*-(*β*-D-glucopyranosyl-(1→6)-*β*-D-glucopyranosyl) oleanolic acid (**10**) [20], macranthoside B (**11**) [21], and asiatic acid (**12**) [22] by comparing their spectroscopic data to previously published data. In addition, compounds **9** and **12** were firstly reported from *Lonicera* species, and compound **10** was isolated from this plant for the first time.

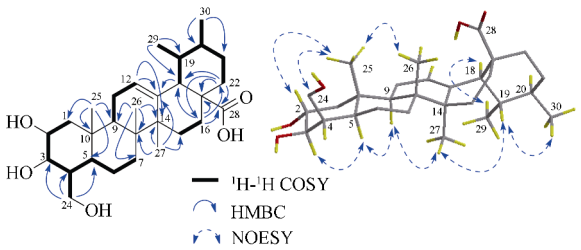

Fig. 2 Key ^{1}H-^{1}H COSY, HMBC, and NOESY correlations of compound 1

Nitric oxide (NO) is pro-inflammatory mediator produced by macrophages during inflammatory response. Occurrence of inflammation is always accompanied by overproduction of NO, and is promoted by inducible nitric oxide synthases (iNOS)[23-24]. Accordingly, inhibiting iNOS is a common anti-inflammatory mechanism. Herein, the anti-inflammatory activities of compounds **1** and **2** were assayed by detecting the expression levels of release of NO and iNOS proteins in LPS-activated RAW264.7 cells. As shown in Fig. 3, two new compounds (**1** and **2**) significantly inhibited expression of iNOS in LPS-induced RAW264.7 cells, at the same time NO release was decreased when cells were treated by the compounds. The results indicated that compounds **1** and **2** were likely to have anti-inflammatory properties and could suppress NO release in inflammatory responses to a certain extent.

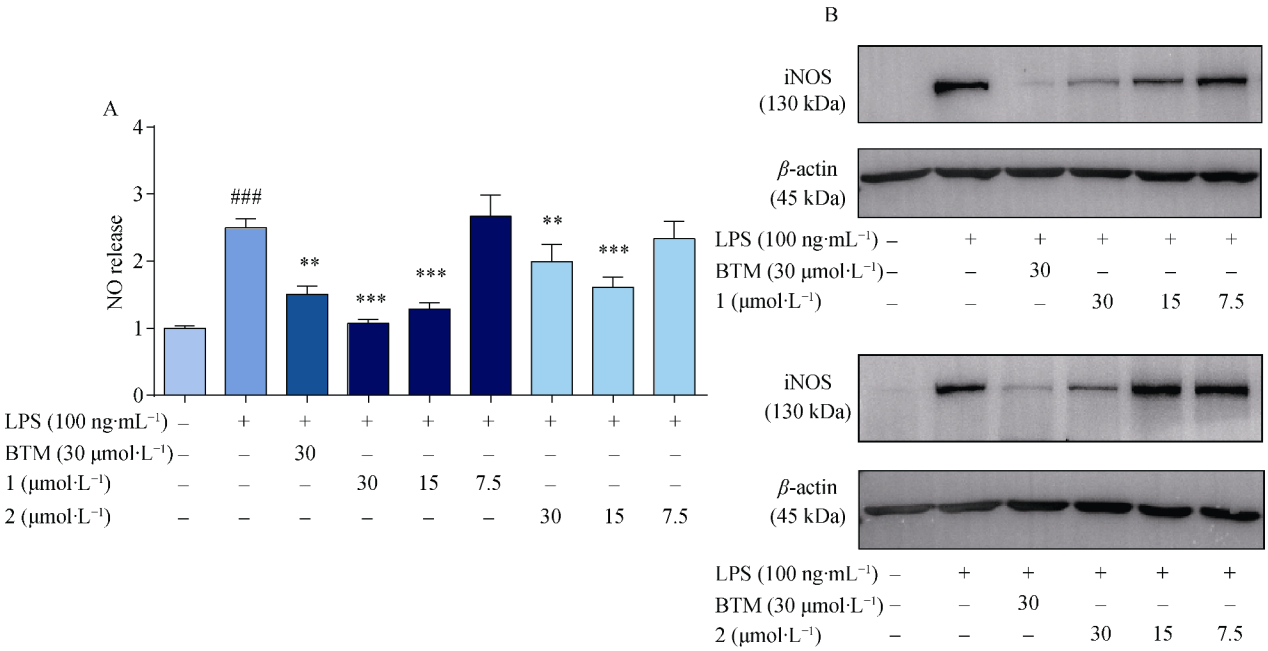

Fig. 3 Effects of compounds 1 and 2 on iNOS and NO release in LPS-activated RAW264.7 cells. A) Both compounds can reverse increase of NO release at 30 and 15 μmol·L^{-1}. NO release level of normal cells was normalized as 1. Results are given in mean ± SD, n = 5. One-way ANOVA was used in statistic analysis, $^{**}P < 0.01$ *vs* LPS group, $^{***}P < 0.001$ *vs* LPS group, $^{\#\#\#}P < 0.001$ *vs* normal group. B) Representative Western blot image showed that expression of iNOS decreased upon treatment by compounds 1 at 30 and 15 μmol·L^{-1}, and 2 at 30 μmol·L^{-1}

As the use of *L. macranethoides* is associated with anti-inflammation, finding new anti-inflammatory compounds provides more clues to knowing the essence of pharmacology of this plant, and active compounds are potentially useful in quality control.

Experimental Part

General experimental procedures

Column chromatography (CC) was carried out on HP-20 macroporous resin (Diaion, Mitsubishi Chemical Corporation, Tokyo, Japan), silica gel (200−300 mesh; Qingdao Marine Chemical Ltd., Qingdao, China), ODS-silica gel (12 nm, S-50 μm; YMC Co., Ltd., Kyoto, Japan), and Sephadex LH-20 (GE Healthcare Biosciences AB, Uppsala, Sweden); TLC was performed on precoated silica gel plates (SGF254, 0.2 mm; Yantai Chemical Industry Research Institute, China). Analytical HPLC was performed on a Waters 2695 separations module equipped with a 2998 photodiode array detector using an RP-18 column (5 μm, 4.6 mm × 250 mm; COSMOSIL). Semi-preparative HPLC was carried out on a Waters 1515 isocratic HPLC pump equipped with a 2489 UV/ visible detector and RP-18 column (5 μm, 10.0 mm × 250 mm, COSMOSIL).

Optical rotations: Jasco P-1020 digital polarimeter (Jasco, Tsukuba, Japan). UV spectrum: Jasco V-550 UV/VIS spectrophotometer (Jasco, Tsukuba, Japan). IR spectrum: Jasco FT/IR-480 spectrometers (Jasco, Tsukuba, Japan). HR-ESI-MS: Waters Synapt G2 QTOF mass spectrometer F (Waters Corp, Milford, MA, USA). ESI-MS: Finnigan LCQ Advantage MAX (Thermo Fisher Scientific, Boston, MA, USA). NMR: Bruker AVANCE-600 NMR spectrometer (chemical shift values are presented with TMS as the internal reference; Bruker, Billerica, MA, USA).

Plant material

Dry flower buds of *Lonicera macranthoides* Hand.-Mazz. (*Lonicerae Flos*) were purchased from Bozhou Kunyuan Pharmaceutical Co., Ltd. (Bozhou, China) in November 2014, and was identified as *Lonicera macranthoides* Hand.-Mazz. by Prof. ZHOU Guang-Xiong , Jinan University. A voucher specimen

(No. 28-78-56-3) was deposited in the Institute of Chinese Medicine and Natural Product Research, Jinan University.

Extraction and isolation

The dried flower buds of *L. macranthoides* (35.0 kg) were extracted with 70% EtOH under heat-reflux for two times, 2 h each time. The extraction was concentrated under reduced pressure to give a crude extract (8.5 kg). The crude extract was suspended in H_2O then subjected to HP-20 macroporous resin and eluted gradiently with H_2O−EtOH (100 : 0, 30 : 70, 50 : 50, 95 : 5) to give four fractions (LM1−4). Fr. LM4 (340.0 g) was subject to column chromatography on SiO_2 eluting with $CHCl_3$−CH_3OH in gradient (100 : 0, 98 : 2, 95 : 5, 90 : 10, 80 : 20, 70 : 30, 60 : 40, 0 : 100 *V/V*), to yield 23 fractions (Fr. 1 to 23). Fr. 16, 18, 19, and 21 were purified by Sephadex LH-20 with CH_3OH−H_2O (50 : 50, *V/V*) to give **3** (6.6 g), **4** (7.7 g), **6** (50.0 mg) and **5** (23.5 g), respectively. Fr. 10 (9.9 g, eluted by $CHCl_3$−CH_3OH in 95 : 5, *V/V*) was separated on ODS CC with CH_3OH−H_2O gradiently (50 : 50, 70 : 30, 90 : 10, 100 : 0, *V/V*), yielding 8 fractions (Fr. 10.1−Fr. 10.8) and **1** (Fr. 10.7, 159.0 mg). Fr. 10.6 was prepared using semi-preparative HPLC with CH_3CN−H_2O (45 : 55, *V/V*) to give **2** (4.7 mg, t_R = 15.5 min) and **12** (6.2 mg, t_R = 18.3 min). Fr. 13 (8.2 g) was separated into five subfractions (Fr.13.1−13.5) by ODS CC, using CH_3OH-H_2O (50 : 50, 70 : 30, 100 : 0, *V/V*) as the eluent. Then, Fr. 13.2 was purified on semi-preparative HPLC with CH_3CN-H_2O (35 : 65, *V/V*) to give **7** (60.0 mg, t_R = 15.5 min), **8** (25.5 mg, t_R = 19.4 min) and **9** (12.7 mg, t_R = 21.7 min), while Fr. 13.4 (CH_3CN−H_2O, 50 : 50, *V/V*) to offer **10** (365.0 mg, t_R = 14.5 min) and **11** (175.7 mg, t_R = 20.0 min).

2α, 24-dihydroxy-23-nor-ursolic acid (1)

White amorphous powder; $[\alpha]_D^{25}$ +15.24 (*c* 0.5, CH_3OH); IR (KBr) ν_{max}: 3412, 2928, 1691, 1455, 1377, 1279, 1065 cm^{-1}. ^1H NMR (600 MHz, pyridine-d_5) and ^{13}C NMR (150 MHz, pyridine-d_5): see Table 1; HR-ESI-MS: *m/z* 473.3259 $[M - H]^-$ (Calcd. for $C_{29}H_{45}O_5$, 473.3267).

2α, 4α-dihydroxy-23-nor-ursolic acid (2)

White amorphous powder; $[\alpha]_D^{25}$ +6.36 (*c* 0.5, CH_3OH); IR (KBr) ν_{max}: 3427, 2925, 2861, 1691, 1638, 1455, 1382, 1264, 1093, 564 cm^{-1}; ^1H NMR (600 MHz, pyridine-d_5) and ^{13}C NMR (150 MHz, pyridine-d_5): see Table 1; HR-ESI-MS: *m/z* 473.3260 $[M - H]^-$ (Calcd. for $C_{29}H_{45}O_5$, 473.3267).

Assay of anti-inflammatory activity in LPS-stimulated RAW264.7 macrophages

The murine macrophages RAW264.7 cell line was purchased from American Type Culture Collection (ATCC, Manassas, VA, USA) and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Gibco BRL Co., Grand Island, NY, USA), penicillin G (100 $U \cdot mL^{-1}$), streptomycin (100 $mg \cdot mL^{-1}$), and L-glutamine (2 $mmol \cdot L^{-1}$) (Gibco BRL Co., Grand Island, NY, USA). Cells were grown at 37 °C in a humidified atmosphere containing 5% CO_2. All of the tested compounds were dissolved in DMSO in 120 $mmol \cdot L^{-1}$, and work concentration was 30 $\mu mol \cdot L^{-1}$ (DMSO final concentration was less than 0.1 % in the assay).

For NO release level detection, RAW264.7 cells were seeded in 96-well plates at 1×10^5 cells/well. The cells were pretreated with the test compounds or betamethasone (BTM, positive control, 30 $\mu mol \cdot L^{-1}$) for 2 h and then stimulated with LPS (100 $ng \cdot mL^{-1}$) for another 24 h. Then NO concentration in the medium supernatants were tested by NO detection kit (Beyotimes, Shanghai, China).

For iNOS expression detection, cells were plated at a density of 1×10^6 cells/well in 6-well plates. Pretreatment and LPS-stimulation were conducted in same procedure as in NO release experiment. After LPS-stimulation, total cellular proteins were extracted by using RIPA lysis buffer (Cell Signaling Technology, Boston, MA, USA) containing 1× protease inhibitor mix (Roche Applied Science, Germany). Protein concentration was determined using BCA protein assay kit (Beyotimes, Shanghai, China). Thirty micrograms of proteins per lane were separated by 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene fluoride (PVDF) membranes (GE Healthcare Life Sciences, Buckinghamshire, UK). The membranes were blocked in 5% skimmed milk and then incubated with primary antibodies (iNOS and *β*-actin both were rabbit-originated and were from Cell Signaling Technologies, Boston, MA, USA) at 4 °C overnight. Afterward, the membranes were incubated with HRP-linked anti-rabbit secondary antibody (Abcam, MA, US) at room temperature for 1.5 h. Then, the protein bands were visualized by adding HRP substrate chemiluminescence reagent (Millipore Corporation, MA, US), and the images were acquired by Tanon 5200 Western Blot Chemiluminescence Imaging System (Tanon Techonologies, Shanghai, China).

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Cite this article as: MEI Yu-Dan, ZHANG Nan, ZHANG Wei-Yang, TANG Jin-Shan, ZHOU Hua, YU Yang, YAO Xin-Sheng. Two new ursane-type nortriterpenes from Lonicera macranthoides and their iNOS-inhibitory activities [J]. *Chin J Nat Med*, 2018, **17**(1): 27-32.