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Jatrogricaine A: a new diterpenoid with a 5/6/6/4 carbon ring system from the stems of *Jatropha podagrica*

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[ABSTRACT] Jatrogricaine A (**1**), a new diterpenoid possessing a 5/6/6/4 carbon ring system, together with eight known diterpenoids (**2**–**9**) were isolated from the stems of *Jatropha podagrica*. Their structures were elucidated by extensive spectroscopic methods and the absolute configuration of **1** was determined by single crystal X-ray diffraction analysis. All compounds were evaluated for their anti-inflammatory activities *in vitro*, and compound **3** showed significant inhibitory effects against nitric oxide (NO) production in lipopolysaccharide (LPS)-induced RAW264.7 macrophage cells with an IC_{50} of 13.44 ± 0.28 μmol·L^{-1}, being comparable to the positive control, quercetin (IC_{50} 17.00 ± 2.10 μmol·L^{-1}).

[KEY WORDS] *Jatropha podagrica*; Euphorbiaceae; Diterpenoids; Anti-inflammatory activity

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Introduction

The genus *Jatropha* (Euphorbiaceae) comprises approximately 175 species mainly growing in the tropic and subtropical areas of Africa, Asia, and Latin America [1]. Previously phytochemical investigations of this genus revealed that it was a rich resource of structurally intriguing macrocycle diterpenoids, such as tiglianes, daphnanes, lathyreanes, and rhamnofolanes [2–5]. These diverse secondary metabolites exhibited various biological activities, such as cytotoxicity, antibacterial, and anti-inflammatory [5-6].

Jatropha podagrica Hook is a shrub widely cultivated as an ornamental plant in tropical countries. The seed oil of *J. podagrica* is used as a folk medicine by Nigerian for the treatment of rheumatic condition, itch, parasitic skin diseases, and fever [7-8]. Previous chemical investigation of this plant led to the isolation of several diterpenoids [6, 9] and alkaloids [7-8], some of which showed significant antibacterial, neuromuscular-blocking and hypotensive activities. As a part of our effort to discover structurally interesting and biologically active secondary metabolites from genus *Jatropha* [10–12], a new polycyclic diterpenoid with a rare 5/6/6/4 carbon ring system, along with eight known diterpenoids, were isolated from the stems of *J. podagrica*. All the isolates were tested for their inhibitory activities against NO production in LPS-induced RAW264.7 macrophage cells, and compound **3** exhibited significant activity. Herein, we report the isolation, structural elucidation, and bioactivity evaluation of compounds **1**–**9**.

Results and Discussion

The air-dried powder of the stems of *J. podagrica* was extracted with 95% EtOH at room temperature. The concentrated extract was then suspended in H_2O and successively partitioned with petroleum ether and EtOAc. Compounds **1**–**9** were isolated from the EtOAc fraction by various column chromatographic methods (Fig. 1).

Compound **1** was obtained as colorless crystals. The molecular formula $C_{20}H_{28}O_4$ was established by its HR-ESIMS data at *m*/*z* 355.1880 $[M + Na]^+$ (Calcd. 355.1880), indicating seven degrees of unsaturation (DOUs). The IR spectrum displayed absorption bands for hydroxyls (3553 and 3408 cm^{-1}) and carbonyl group (1702 cm^{-1}). The ^1H NMR spectrum showed four singlet methyls [δ_H 1.17, 1.12, 1.08, and 0.99 (each 3H)], a doublet methyl [δ_H 1.26 (3H, d, J = 7.5 Hz)], two oxygenated methines [δ_H 4.73 (1H, t, J = 3.0 Hz) and 3.50 (1H, d, J =

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These authors have no conflict of interest to declare.

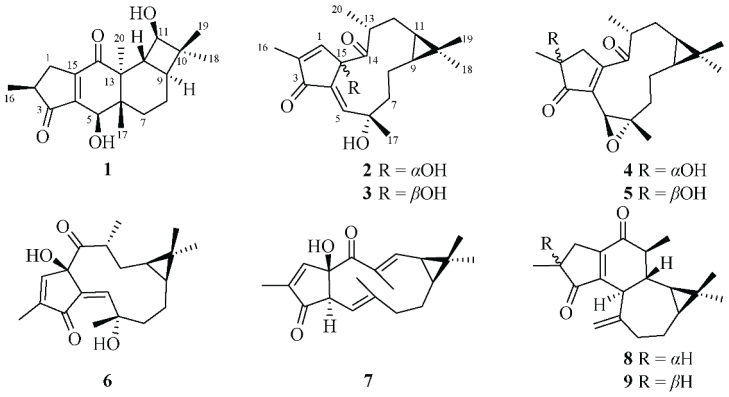

Fig. 1 The structures of compounds 1–9

8.7 Hz)], and a series of aliphatic multiplets. The ^{13}C NMR combined with DEPT experiment resolved 20 carbon signals consistent with five methyls, three methylenes, five methines (two oxygenated), a tetrasubstituted double bond (δ_C 157.6 and 147.6), two carbonyl groups (δ_C 214.8 and 204.9), and three sp^3 quaternary carbons. As three of the seven DOUs were attributed to one double bond and two carbonyl groups, the remaining four DOUs required that **1** was tetracyclic. Above information was very similar to those of euphoractine T [13], a known diterpene with a 5/6/6/4 carbon ring system, except for the replacement of an oxymethine in euphoractine T by a methylene in **1**. Combined with the upfield-shifted chemical shift of C-2 in **1** with respect to that in euphoractine T (δ_C 41.5 in **1**; δ_C 51.1 in euphoractine T), **1** was assigned as 1-dehydroxylated derivative of euphoractine T.

The planar structure of **1** was further supported by the detailed interpretation of 2D NMR spectra. Two spin systems as depicted in Fig. 2 were assigned by the ^1H-^1H COSY of H-1α/H-2/H_3-16 and H_2-7/H_2-8/H-9/H-12/H-11. These fragments, quaternary carbons, double bonds, and carbonyls were further linked by HMBC correlations from H_3-16 to C-1/C-2/C-3, H_3-17 to C-5/C-6/C-7/C-13, H_3-18/19 to C-9/C-10/C-11, and H_3-20 to C-6/C-12/C-13/C-14, which generated a 5/6/6/4 carbon ring system.

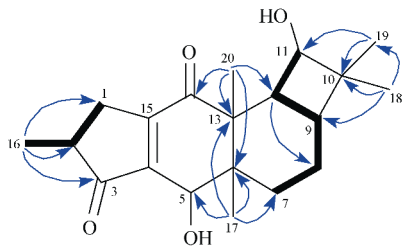

Fig. 2 ^1H–^1H COSY (▬) and key HMBC (→) correlations of 1

The relative configuration of **1** was established by the NOESY experiment. The NOE correlations (Fig. 3) of H-12/H_3-17 and H_3-19 indicated these protons were on the same side and were arbitrarily assigned as β-orientation. Thus, the NOE correlations of H-11/H-9, H_3-20, and H_3-18 as well as H_3-20/H-5 assigned that those protons are α-oriented. The relative configuration of C-2 could not be assigned due to the long distance between C-2 and other chiral centers. Fortunately, the crystals of **1** were obtained from a mixed solution of CH_2Cl_2–MeOH (1 : 5) and single-crystal X-ray crystallographic analysis using anomalous scattering of Cu Kα radiation (Fig. 4) was performed. The final refinement with a Flack parameter of –0.14 (13) permitted the establishment of the absolute configuration of **1** as 2*S*, 5*R*, 6*S*, 9*S*, 11*R*, 12*R*, 13*S*. Compound **1** was given a trivial name jatrogricaine A.

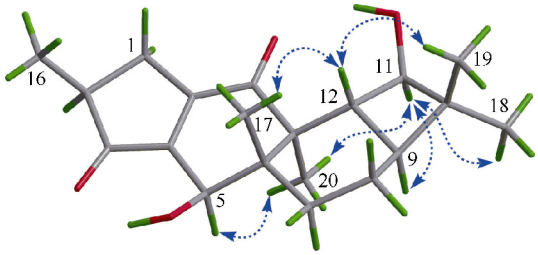

Fig. 3 Key NOE correlations of 1

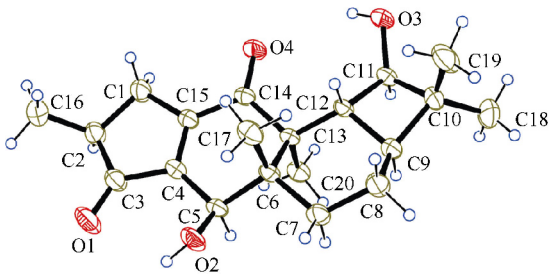

Fig. 4 X-ray crystal structure of 1

The known compounds, 15-*epi*-4*E*-jatrogrossidentadion (**2**) [14], 4*E*-jatrogrossidentadion (**3**) [14], 2-hydroxyisojatrogrossidion (**4**) [14], 2-*epi*-hydroxyisojatrogrossidion (**5**) [14], 4*Z*-jatrogrossidion (**6**) [14], jatrocurcasenone D (**7**) [15], and sikkimenoids A (**8**) and B (**9**) [16], were identified by comparison of their observed NMR data with those reported in

literatures.

All compounds were evaluated for their inhibitory effects on NO production induced by LPS in RAW264.7 macrophage cells. Compound **3** exhibited pronounced inhibition of NO production with an IC_{50} value of 13.44 ± 0.28 μmol·L^{-1}, being comparable to the positive control, quercetin (IC_{50} 17.0 ± 2.10 μmol·L^{-1}). To investigate whether the inhibitory activities of compound **3** was generated from its cytotoxicity, the effects on LPS-induced RAW264.7 macrophage cells viability were measured using the MTT method. The result showed that compound **3** (up to 80 μmol·L^{-1}) did not show any significant cytotoxicity with LPS treatment for 24 h.

In conclusion, a new diterpene with a 5/6/6/4 carbon ring system was isolated from *Jatropha podagrica*. This type of diterpenoid is rare in nature and only 16 derivatives have been reported so far [13, 17–20]. Compound **1** represents the first example of this compound class from genus *Jatropha*. Biosynthetically, the 5/6/6/4 carbon ring system might derive from the intramolecular cyclization of the macrocyclic deterpenes skeleton such as the co-isolated lathyranes **4** and **5** [13]. Moreover, compound **3** showed pronounced anti-inflammatory activities against nitric oxide (NO) production in lipopolysaccharide (LPS)-induced RAW264.7 macrophage cells, which expended the pharmaceutical usage of this plant.

Experimental

General Experimental Procedures

The X-ray data were collected using an Agilent Xcalibur Nove X-ray diffractometer. Melting points were measured on an X-4 melting instrument and were uncorrected. Optical rotations were measured on a Rudolph Autopol I automatic polarimeter, UV spectra on a Shimadzu UV-2450 spectrophotometer, and IR spectra on Bruker Tensor 37 infrared spectrophotometers. 1D and 2D NMR spectra were measured on Bruker AM-400 spectrometers at 25 ℃. ESIMS was measured on a Finnigan LCQ Decainstrument, and HR-ESIMS was performed on a Waters Micromass Q-TOF spectrometer. Column chromatography was performed on silica gel (300–400 mesh, Qingdao Haiyang Chemical Co., Ltd.), reversed-phase C_{18} (RP-C_{18}) (12 nm, S-50 μm, YMC Co., Ltd.), Sephadex LH-20 gel (Amersham Biosciences), and MCI gel (CHP20P, 75–150 μm, Mitsubishi Chemical Industries Ltd.). A Shimadzu LC-20 AT equipped with a SPD-M20A PDA detector was used for HPLC. An YMC-pack ODS-A column (250 mm × 10 mm, S-5 μm, 12 nm) and a Phenomenex Lux cellulose-2 chiral column (10 mm × 250 mm, 5 μm) were used for semi-preparative HPLC separation. All solvents used were of analytical grade (Guangzhou Chemical Reagents Company, Ltd.).

Plant material

The stem of *Jatropha podagrica* were collected in the Xishuangbanna city, Yunnan Province, China, in May 2016, and were authenticated by Associate Professor XU You-Kai of Xishuangbanna Tropical Botanical Garden. A voucher specimen (accession number: FDS-201605) has been deposited at the School of Pharmaceutical Sciences, Sun Yat-sen University.

Extraction and isolation

The air-dried powder of the stems of *J. podagrica* (10 kg) was extracted with 95% EtOH (3 × 10 L) at room temperature to give a crude extract (600 g). The extract was suspended in H_2O (2 L) and successively partitioned with petroleum ether (PE, 3 × 2 L), and EtOAc (3 × 2 L) to yield two corresponding portions. The EtOAc extract (100 g) was subjected to MCI gel column eluted with MeOH–H_2O (5 : 5 → 10 : 0) to afford Frs. I−IV. Fr. II was subjected to silica gel column (PE/EtOAc, 10 : 1 → 0 : 1) to afford Frs. IIA−IID. Fr. IID was loaded onto a Sephadex LH-20 column and eluted with MeOH to give Fr. IID1–Fr. IID2, then Fr. IID2 was subjected to silica gel column (PE/CH_2Cl_2, 1 : 1 → 0 : 1) to afford **4** (8 mg) and **5** (10 mg). Separation of the Fr. III (14 g) was subjected to silica gel column (PE–CH_2Cl_2, 10 : 1 → 0 : 1) to get Fr. IIIA and IIIB. Fr. IIIA was subjected to silica gel column (PE–EtOAc, 20 : 1 → 1 : 1) to afford Fr. IIIA1 and IIIA2. Fr. IIIA1 was purified on HPLC with a Phenomenex Lux chiral column (CH_3CN– H_2O, 4.5 : 5.5, 3 mL·min^{-1}) to obtain **1** (2 mg, t_R 11 min). Fr. IIIB was loaded onto a Sephadex LH-20 column and eluted with MeOH to give Fr. IIIB1–Fr. IIIB3. Fr. IIIB1 (200 mg) was subjected to silica gel column (PE–EtOAc, 20 : 1 → 1 : 1) to afford Fr. IIIB1a−Fr. IIIB1c, then Fr. IIIB1a was purified on a HPLC system equipped with a YMC column (MeOH–H_2O, 8 : 2, 3 mL·min^{-1}) to give **2** (28 mg, t_R 13 min) and **3** (35 mg, t_R 17 min). Fr. IIIB1b was purified on HPLC (MeOH–H_2O, 7.5 : 2.5, 3 mL·min^{-1}) to give **6** (18 mg, t_R 9 min) and **7** (16 mg, t_R 12 min). Fr. IV was separated by column chromatography on reversed-phase silica gel C_{18} eluted with a MeOH–H_2O gradient (5 : 5 → 10 : 0) to give Fr. IVA–Fr. IVD, and then Fr. IVB was loaded onto a Sephadex LH-20 column and eluted with MeOH to afford Fr. IVB1 and Fr. IVB2, and then Fr. IVB2 was subjected to further purification by HPLC with a Phenomenex Lux chiral column (MeOH–H_2O, 9.2 : 0.5, 3 mL·min^{-1}) to give **8** (8 mg, t_R 18 min) and **9** (10 mg, t_R 22 min). The purity of compounds **1**–**9** was greater than 95% as determined by ^{1}H NMR spectrum.

Jatrogricaine A (1)

Colorless crystal, mp 205–210 °C; $[\alpha]^{20}_D$ +36 (*c* 0.13, MeOH); UV (MeOH) λ_{max} (log ε) 207 (3.67) nm, IR (KBr) ν_{max} 3553, 3408, 2948, 2924, 2864, 1702, 1671, 1460, 1393, 1373, 1320, 1289, 1245, 1228 cm^{-1}; ^{1}H and ^{13}C NMR data, see Table 1; HR-ESIMS *m/z* 355.1880 $[M + Na]^+$ (Calcd. for $C_{20}H_{28}O_4Na$, 355.1880).

X-ray crystallographic analysis of 1

$C_{20}H_{28}O_4$, M = 332.44, monoclinic, space group $P2_1$ (no. 4), a = 9.9044 (1) Å, b = 7.4188 (1) Å, c = 12.5208 (2) Å, β = 99.585 (1), V = 907.17 (2) Å^3, Z = 2, T = 294.7 (6) K, μ (Cu Kα) = 0.669 mm^{-1}, D_c = 1.2170 g·cm^{-3}, 16576 reflections

measured (7.16° ≤ 2^{θ} ≤ 144.12°), 3521 unique (R_{int} = 0.0257, R_{sigma} = 0.0166) which were used in all calculations. The final R_1 was 0.0352 (**I** >= 2σ (I)) and wR_2 was 0.0937. Flack parameter = –0.14 (13). Crystallographic data for the structure of **1** have been deposited in the Cambridge Crystallographic Data Centre (deposition number: CDCC 1589314).

Table 1 ^{1}H (500 MHz) and ^{13}C (125 MHz) NMR data of 1 in $CDCl_3$

No.	δ_H, multi. (*J* in Hz)	δ_C, type	No.	δ_H, multi. (*J* in Hz)	δ_C, type
1α	2.87, ddd (19.0, 6.6, 3.7)	31.6, CH_2	11	3.50, d (8.6)	73.7, CH
1β	2.35, dt (19.0, 2.5)				
2	2.57, m	41.5, CH	12	2.06, dd (13.0, 8.6,)	45.3, CH
3		214.8, C	13		54.0, C
4		157.6, C	14		204.9 C
5	4.73, t (3.0)	71.0, CH	15		147.6 C
6		48.0, C	16	1.26, d (7.5)	16.1, CH_3
7α	1.56, m	30.4, CH_2	17	0.99, s	14.3, CH_3
7β	1.87, dt (13.9, 3.6)				
8α	1.43, m	21.2, CH_2	18	1.12, s	28.3, CH_3
8β	1.54, m				
9	1.25, m	37.1, CH	19	1.08, s	15.1, CH_3
10		44.0, C	20	1.17, s	14.0, CH_3

Cell culture

The RAW264.7 mouse macrophage cell line was purchased from the Cell Bank of Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (Shanghai, China), and was cultured in Dulbecco's modified Eagle medium (DMEM, Gibco Invitrogen Corp., Carlsbad, CA, USA) which was supplemented with 10% FBS, 100 Units/mL penicillin and 100 μg·mL^{-1} streptomycin. The cells were placed at 37 °C in a humidified incubator containing 5% CO_2.

MTT assay

The cytotoxicity of the isolated compounds toward RAW264.7 cells was determined by MTT assay. RAW264.7 cells were planted in 96-well plates (5 × 10^3/well) for 24 h. Then they were treated with test samples which dissolved in DMSO and diluted in 100 μL DMEM making the final drug concentration 50 μmol·L^{-1} and 1% DMSO. 1% DMSO served as solvent control. Wells without cells contain only 100 μL DMEM were served as blank control. 24 h later, 20 μL solution MTT was added in each well. After incubation for 4 h, the medium was removed and 100 μL DMSO was added in each well, then the absorbance (A) was detected at 490 nm using a microplate reader. The inhibition of cell growth was calculated according to the following formula: % Inhibition = [1– (A_{sample} – A_{blank}) / ($A_{solvent}$ – A_{blank})] × 100.

Inhibitory activity toward NO

NO release was assessed by a colorimetric assay based on a diazotization reaction using the Griess reagent system. RAW264.7 cells were planted in 96-well plates (5 × 10^3/well) for 24 h and then pre-incubated with different concentrations of compounds for 1 h before stimulation with LPS (1.0 μg·mL^{-1}) for 24 h. The NO concentration in culture medium was determined by Griess reagent kit, then the absorbance (A) was measured at 540 nm using a microplate reader. The inhibition of NO release was calculated according to the following formula: Inhibition (%) = [1 – (A_{sample} – A_{blank}) / (A_{model} – A_{blank})] × 100. The experiments were performed in triplicates, and the data were presented as the mean ± SD. Quercetin was used as a positive control.

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