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Two new bioactive lignans from leaves and twigs of *Cleistanthus concinnus* Croizat

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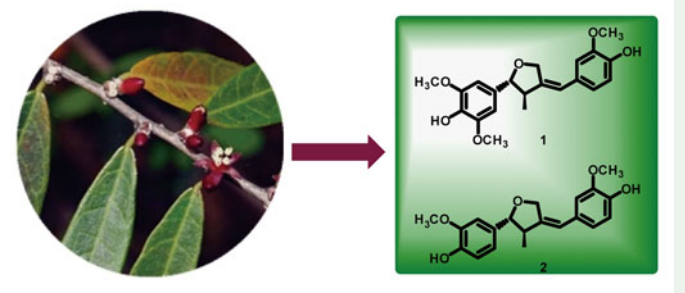
ABSTRACT

Two new lignans (**1**–**2**), along with five known compounds (**3**-**7**) with different structures were isolated from leaves and twigs of *Cleistanthus concinnus* Croizat. The new lignans were elucidated as (7′R,8′S)-3,3′,5′-trimethoxy-4,4′-dihydroxy-7-en-7′,9- epoxy-8,8′-lignan (**1**) and (7′R,8′S)-3,3′-dimethoxy-4,4′-dihydroxy-7-en-7′,9-epoxy-8, 8′-lignan (**2**) by comprehensive spectroscopic analysis including 1D and 2D NMR as well as HREIMS and comparing their NMR data with known compounds in the literature. Among these isolated compounds, compound **1**, **2**, **3**, and **6** were tested for anti- inflammatory effects by inhibiting NO production in lipopolysaccharide (LPS)-stimulated RAW 264.7 cells. Compound **1**, **2**, and **6** exhibit NO inhibitory activity.



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Cleistanthus concinnus Croizat; Euphorbiaceae; lignans; NO inhibitory activity

1. Introduction

The genus *Cleistanthus* belongs to the *Euphorbiaceae* family and consists of approximately 140 species that are mainly distributed Southeast Asian (Li Bingtao 2008).Previously, chemical examination on the genus *Cleistanthus* have led to the

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isolation of lignans and terpenoids (Govindachari et al. 1969; Liu et al. 2015; McGarry et al. 1969; Pegel et al. 1970; Ramesh et al. 2003; Thanh et al. 2011; Trinh et al. 2014). It is most interested in this genus for its bioactive lignans. Among these lignans, cleistanthin A and cleistanthin B show a series of pharmacological activities, for example, antitumor activity, diuresis action and cardiovascular effects (Prabhakaran et al. 1996; Kumar et al. 1998; Pradheepkumar and Shanmugam 1999; Meenakshi and Shanmugam 2000; Parasuraman et al. 2012; Vijayakumar et al. 2014). However, only six plants of the genus *Cleistanthus* have been investigated chemically so far. In order to further broaden the research of chemical constituents and biological activity of this genus, we study on *Cleistanthus concinnus* Croizat from Hainan province and report the isolation and structural elucidation of the two new lignans, **1** and **2**. In addition, new compounds and some known compounds were evaluated for their anti-inflammatory in this paper.

2. Result and Discussion

The 75% EtOH extract from leaves and twigs (26 kg) of *Cleistanthus concinnus* was repeatedly subjected to silica gel, MCI gel and Sephadex LH-20 column chromatography, which led to two new lignans (**1**, **2**) and five known compounds (**3**-**7**). Contrasted with ESIMS and NMR data, the five known compounds were identified as 4′-*O*-demethylepiyangambin (**3**) (Morais et al. 1996), dihydrocubebin (**4**) (Cabanillas et al. 2010), friedelin (**5**) (Leong and Harrison 1999), betulin (**6**) (Salimuzzaman Siddiqui 1988), betulinic acid (**7**) (Salimuzzaman Siddiqui 1988). (see Figure 1)

Compound **1** was obtained as colorless oil. Its molecular formula was established as $C_{21}H_{24}O_6$ by positive HRESIMS (*m/z* 395.1467 $[M+Na]^+$, calcd for $C_{21}H_{24}O_6Na$, 395.1465). The IR spectrum indicated a hydroxy group at 3427cm^{-1}. The ^{1}H NMR data of **1** showed the signals for one methyl [δ_H 1.18 (d, $J = 6.6$ Hz, H-9′)], three methoxy

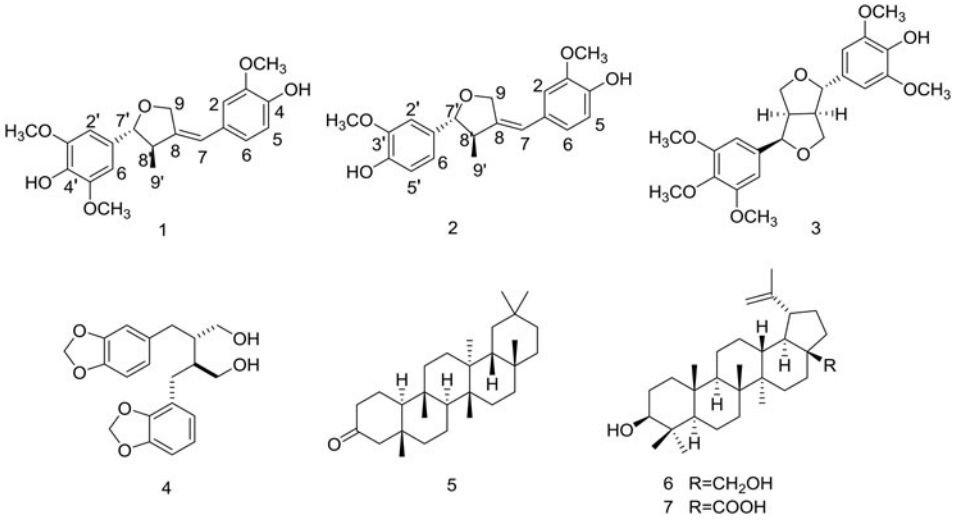

Figure 1. The structures of compound **1–7**.

(δ_H 3.91, 3.90, and 3.90), a double bond [δ_H6.19 (1H, m, H-7)] and an ABX system [δ_H 6.70 (d, $J = 1.8$Hz, H-2), δ_H 6.92 (d, $J = 8.8$ Hz, H-5), 6.70 (overlapped, H-6)]. ^{13}C NMR and DEPT spectra displayed that twelve aromatic carbon signals, one of which was overlapped formed two aromatic rings in the down filed, and one aromatic ring had a symmetric structure. Analysis of the degree of unsaturation and 1D NMR data(see Table S1), there were three rings in the structure. Meanwhile, the correlation sequence of H-7′/H-8′/H-9′ indicated a tetrahydrofuran ring in the ^{1}H-^{1}H COSY spectrum (see Figure S19).

Comparison with (7′R, 8′S)-3, 3′, 4, 5′-tetramethoxy-7-en-7′, 9-epoxy-8, 8′-lignan (Wang et al. 2012) implied that compound **1** had a similar skeleton, only with different substituents in aromatic rings. In the 2D NMR experiments, especially HMBC (Figure S19), the above view was further confirmed. The tetrahydrofuran ring must be in the structure again by the HMBC correlations of H-9 with C-8, C-8′ and H-9′ with C-7′, C-8, and C-8′. The HMBC correlations of H-7 with C-2, C-6, C-8′, and C-9 revealed that the aromatic ring connected with tetrahydrofuran ring by the double bond. On basis of HMBC correlations from H-7′ to C-2′, C-6′, and C-9′ and correlations from H-2′ and H-6′ to C-7′, another aromatic ring located at C-7′. The HBMC correlations of MeO with C-3, C-3′, and C-5′ indicated methoxy group at C-3, C-3′ and C-5′. Moreover, the correlations between protons of the hydroxy group and C-5, C-3′, C-4′ and C-5′ showed the connecting positions of the hydroxy group at C-4 and C-4′.

The relative configuration of **1** was determined by the ROESY spectrum (Figure S2) and (7′R, 8′S)-3, 3′, 4, 5′-tetramethoxy-7-en-7′, 9-epoxy-8, 8′-lignan (Wang et al. 2012).The ROESY correlations of H-7′/Me-9′ suggested the β-orientation of H-7′ and the methyl group. Therefore, the structure of compound **1** was defined as(7′R, 8′S)-3, 3′, 5′-trimethoxy-4, 4′-dihydroxy-7-en-7′, 9-epoxy-8, 8′-lignan.

Compound **2** was isolated as a colorless oil, and was assigned the molecular formula $C_{20}H_{22}O_5$ from the HRESIMS (*m/z* 365.1365 $[M + Na]^+$ (calcd for $C_{20}H_{22}O_5Na$, 365.1359). The ^{13}C NMR data and DEPT of 1 show 20 carbon signals corresponding to the presence of three tertiary methyl (two methoxy at δc 55.9), an oxygenated methylene (δc 70.2), two methine (one oxygenated methine at δc 87.3), seven olefinic methines, eight quaternary carbon (exocyclic double bond at δc 144) . In the HMBC spectrum (Figure S19), correlation signals of compound **2** and those of compound **1** were comparable, including a same tetrahydrofuran ring and two aromatic rings. By analysis of ^{1}H NMR and HMBC data, confirmed that compound **2** had two ABX systems, and the hydroxy group located at C-4 and C-4′ as well as two methoxy groups at C-3 and C-3′.

The correlations of H-7′/Me-9′ suggested they located in the same side by the ROESY spectrum. In contrast with compound **2**, the structure of compound **1** was decided as (7′R,8′S)-3,3′-dimethoxy-4,4′-dihydroxy-7-en-7′,9-epoxy-8,8′-lignan.

Compound **1**,**2**,**3**, and **6** were tested for anti- inflammatory effects by inhibiting NO generation with lipopolysaccharide (LPS)-induced RAW264.7 cells. As a result, compound **1**, **2**, and **6** exhibited NO inhibitory activity at the concentration of 25 μM, but no more than 50% (see Table S2). However, compound **3** had no inhibitory activity at the same concentration. Furthermore, the IC_{50} of compounds **1**, **2**, and **6** was also evaluated (Table S3).

3. Experimental

3.1. General experimental procedures

UV spectra were recorded on a SHIMADZU UV-2401 spectrophotometer. Optical rotations were measured on a JASCO P-1020. IR spectra were obtained with a TENOR 27 spectrophotometer using KBr pellets. NMR spectroscopic data were recorded on BRUKER spectrometer with TMS a**s** an internal standard. HRESIMS were acquired with Agilent 6500 Q-TOF mass spectrometer. Semi-preparative HPLC was carried out on an Agilent 1100 series LC with a Waters X-Bridge Prep Shield RP18 (10×150 mm) column. Silica gel G_{254} (100-200 mesh, $300-400$ mesh, Qingdao Marine Chemical, Inc., Qingdao, PR China) and Sephadex LH-20 ($40-70\mu$m, Amerisham Pharmacia Biotech AB, Uppsala, Sweden) were used for column chromatography (CC).

3.2. Plant material

The leaves and twigs of *Cleistanthus concinnus* Croizat were collected and identified by Dr. Yan-Hui Fu(Hainan Normal University) from Hainan Province, People's Republic of China, in September 2015. A voucher specimen (No. 20150920) was deposited at the State Key Laboratory of Phytochemistry and Plant Resource in West China, Kunming Institute of Botany, Chinese Academy of Science (CAS).

3.3. Extraction and isolation

The air-dried leaves and twigs (26 kg) of *Cleistanthus concinnus* were extracted three times with 75% EtOH at room temperature. After the solvent EtOH was evaporated in vacuum, the residue was required, which was suspended in H_2O and then extracted with EtOAc, and n-BuOH. The EtOAc-soluble fraction (670 g) was subjected to normal phase Silica gel (100-200 mesh) with a gradient elution of petroleum ether-EtOAc (petroleum ether,100:1, 50:1, 20:1 to 1:1) and then $CHCl_3$-MeOH (50:1, 20:1, 10:1, 5:1, 1:1) to give 9 major fractions (Fr.1-Fr.9). The Fr.4 (80 g) was chromatographed over MCI gel eluting with gradient of MeOH-H_2O (v/v, from 30:70 to 100:0) to obtain eight portions (Fr.4a-4h). Fr.4d (15g) was divided to four fractions (Fr.4d-1-Fr.4d-4) by a silica gel column eluted with petroleum ether-Me_2CO (50:1 to 5:1). Fr.4d-2 (2.8g) was separated over Sephadex LH-20 gel column, and further purified by semi-preparative HPLC (Phenomenex Luna C18, 5μm, 250×10mm, MeCN-H_2O, 33:67) to get compound **1** (12 mg). Similarly, Fr.4d-3 (3.8g) was once again subjected to silica gel column chromatography with petroleum ether-Me_2CO (100:1 to 5:1) to yield four subfractions (Fr.4d-3a-Fr.4d-3d). Then Fr.4d-3b (862mg) was separated by preparative HPLC (Waters Xselect CSH prep C18, 5μm, 19×150mm; MeCN-H_2O, v/v, from 20:80 to 90:10, flow rate, 10 mL/min) to get 4 fractions (Fr.4d-3b-1-Fr.4d-3b-4). Fr.4d-3b-3 (164mg) was separated by semi-preparative HPLC (Phenomenex Luna C18, 5μm, 250×10mm, MeOH-H_2O, 60:40) to give compound **2** (4.4 mg).With the same purification procedures, **3**(5.4 mg) and **4** (10.0 mg) was obtained from Fr.4c. **5** (9.5 mg), **6** (10.8 mg), **7** (13.0 mg) was obtained from Fr.5.

3.3.1. (7′R, 8′S)-3, 3′, 5′-trimethoxy-4, 4′-dihydroxy-7-en-7′, 9-epoxy-8, 8′-lignan(1)

Colorless oil; $[\alpha]_D^{21}$ -35.8 (I_2 0.23MeOH); UV (MeOH) λ_{max} (log$\mathcal{E}$) 207 (4.46) nm, 268 (3.92) nm, 294 (3.57) nm; IR (KBr) ν_{max} 3427, 2962, 2938, 2877, 2844, 1745, 1614, 1517, 1463, 1429, 1382, 1273, 992, 576 cm^{-1}; ^{1}H NMR data ($CDCl_3$, 500 MHz) δ_H: 6.92 (1H, d, $J = 8.8$ Hz, H-5), 6.7 (1H, d, $J = 1.8$ Hz, H-2) , 6.7 (1H, m, H-6), 6.65(2H, s, H-2′, H-6′), 6.19 (1H, m, H-7), 5.64 (1H, s, 4-OH), 5.53 (4′-OH), 5.00, 4.7 (2H, m, H-9), 4.19 (1H, d, $J = 9.6$ Hz, H-7′), 3.91 (3H, s, 3-OCH_3), 3.90 (6H, s, 3′, 5′-OCH_3), 2.68 (1H, m, H-8′), 1.18 (3H, d, $J = 6.6$ Hz, H-9′).^{13}C NMR data ($CDCl_3$, 125 MHz) δ_C: 147 (C-3′, C-5′), 146.5 (C-4), 144.5 (C-3), 143.8 (C-8), 134.5 (C-4′), 131.5 (C-1′), 129.9 (C-1), 121.3 (C-6), 119.8 (C-7), 114.6 (C-5), 110.7 (C-2), 103.3 (C-2′, C-6′), 87.6 (C-7′), 70.2 (C-9), 56.3 (3′-OCH_3, 5′-OCH_3), 55.9 (3-OCH_3), 47.7 (C-8′), 14.2 (C-9′).positive ESIMS *m/z* 395 $[M + Na]^+$; HRESIMS *m/z* 395.1467 $[M + Na]^+$ (calcd for $C_{21}H_{24}O_6Na$, 395.1465).

3.3.2. (7′R,8′S)-3,3′-dimethoxy-4,4′-dihydroxy-7-en-7′,9-epoxy-8,8′-lignan (2)

Colorless oil; $[\alpha]_D^{21}$ -38.8 (c 0.2MeOH); UV (MeOH) λ_{max} (log$\mathcal{E}$) 203 (4.60) nm, 228 (4.22) nm, 273 (4.02) nm; IR (KBr) ν_{max} 3421, 3073, 2963, 2937, 2875, 2847, 1756, 1721, 1603, 1517, 1462, 1432, 1382, 1274, 1124, 820, 776 cm^{-1}; ^{1}H NMR data ($CDCl_3$, 500 MHz)δ_H: 6.96 (1H, d, $J = 1.5$ Hz, H-2′), 6.90 (2H, d, $J = 8.4$ Hz, H-5, H-5′), 6.69 (1H, d, $J = 1.8$ Hz, H-2), 6.7 (1H, m, H-6), 6.87 (1H, dd, $J = 8.4$ Hz, $J = 1.5$ Hz, H-6′), 6.19 (1H, m, H-7),5.62 (2H, brs, 4, 4′-OH), 4.99, 4.7 (2H, m, H-9), 4.21 (1H, d, $J = 9.6$, H-7′), 3.91 (6H, s, 3,3′-OCH_3), 2.67 (1H, m, H-8′), 1.17 (3H, d, J = 6.6, H-9′). ^{13}C NMR data ($CDCl_3$, 125 MHz) δ_C: 146.7 (C-3′), 146.4 (C-4), 144.5 (C-3), 144 (C-8), 145.5 (C-4′), 132.3 (C-1′), 129.9 (C-1), 121.3 (C-6), 120 (C-6′), 119.7 (C-7), 114.5 (C-5′),114 (C-5), 110.7 (C-2), 108.7 (C-2′), 87.3 (C-7′), 70.2 (C-9), 55.9 (3, 3′-OCH_3), 47.6 (C-8′), 14.2 (C-9′).positive ESIMS *m/z* 365 $[M + Na]^+$; HRESIMS *m/z* 365.1365 $[M + Na]^+$ (calcd for $C_{20}H_{22}O_5Na$, 365.1359).

3.4. NO production inhibitory assay

Murine macrophages RAW264.7 cells were seeded into 96-well plates. The cells were pretreated with 10μM, 25μM, 50μM, and 100μM concentrations of indicated compounds for 4 h and then stimulated with LPS (1 μg/mL) for 24 h. Without any drugs and L-NMMA-positive drugs were used as the control groups. The NO production was evaluated by determining the nitrite concentration in the cultured RAW264.7 cells with Griess reagent. Then, the absorbance was measured with a microplate reader at 570nm.

4. Conclusion

In this paper, two new lignans and five known compounds were isolated from leaves and twigs of *Cleistanthus concinnus*. Interestingly, the open-loop new lignans were not only first found from this genus, but also displayedsome anti-inflammatory activitiy at the same concentration. However, bicyclic oxygen lignans did not show any anti-inflammatory activity at the concentration of 25 μM. The slight differences in the structure of these compounds lead to large differences in activity, which is worthy of further study.

Supplementary materials

Supplementary material related to this article is available online, alongside Tables S1–S3, and Figures S1–S19.

Disclosure statement

No potential conflict of interest was reported by the authors.

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