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Chemical constituents from the twigs and leaves of *Trichilia sinensis* and their biological activities

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ABSTRACT

Phytochemical investigation on the twigs and leaves of *Trichilia sinensis* led to the isolation of two previously undescribed limonoids (*i.e.*, trichiliasinenoids D and E, **1** and **2**), two previously undescribed phenolic acids (**3** and **4**), and one previously undescribed natural phenolic acid dimer (**5**), together with 11 known compounds (**6-16**). Their structures were elucidated by extensive spectroscopic analysis (IR, UV, HRESIMS, 1D and 2D NMR) and chemical techniques. The potential anti-inflammatory activities of all the compounds were evaluated in lipopolysaccharide-stimulated RAW 264.7 cells. Among these isolates, compounds **1**, **2**, **6**, and **11-13** expressed weak NO inhibition. The antibacterial activities of all the compounds against bacteria were also tested *in vitro*. Compound **16** exhibited moderate antibacterial activities against *Escherichia Coli*.

1. Introduction

Trichilia (Meliaceae) is a genus of perennial herbs that were mainly distributed throughout the tropical America and Africa, India, Indochina, and the Malay Peninsula. There are approximately 86 species in this genus, of which two species and one variant grow in China (Chen et al., 1997). The genus *Trichilia* is well known for producing different types of limonoids with a wide range of biological activities (*e.g.* antifeedant, antibacterial, anticancer, and anti-inflammatory activities) (Tan and Luo, 2011; Zhang and Xu, 2017). *Trichilia sinensis* Bentv, a shrub native to southern China and Vietnam, has been used in folk medicine to treat diseases including abdominal pain caused by *Ascaris lumbricoides*, chronic osteomyelitis, scabies, and eczema (Editorial Committee of Chinese Materia Medica and The Administration Bureau of Traditional Chinese Medicine, 2000). Previous phytochemical investigations on *T. sinensis* indicated that the plant was a rich source of mexicanolide-type and phragmalin-type limonoids. Limonoids from this plant were reported to possess anti-inflammatory, cytotoxic, and acetylcholinesterase (AChE) inhibitory activities (Cao et al., 2017; Liu et al., 2017, 2016; Xu et al., 2013). In our previous study, three novel limonoids with an unprecedented C-29-C-7 connecting carbon skeleton were isolated from the twigs and leaves of *T. sinensis* collected from Xishuangbanna, China (Cao et al., 2017). As a continuing phytochemical investigation on this plant, two previously undescribed limonoids (*i.e.*, trichiliasinenoids D and E, **1** and **2**), two previously undescribed phenolic acids (**3** and **4**), and one previously undescribed natural phenolic acid dimer (**5**), together with 11 known compounds (**6–16**) were isolated (Fig. 1). These compounds were evaluated for their anti-inflammatory activities *via* the inhibition of lipopolysaccharide (LPS)-induced NO production in RAW264.7 cells and cytotoxicities toward RAW264.7 cells. Furthermore, the antibacterial activities of these compounds were tested against Gram-positive bacteria (*Staphylococcus aureus* and *Candida albicans*) and Gram-negative bacteria (*Escherichia coli* and *Pseudomonas aeruginosa*). Herein, we

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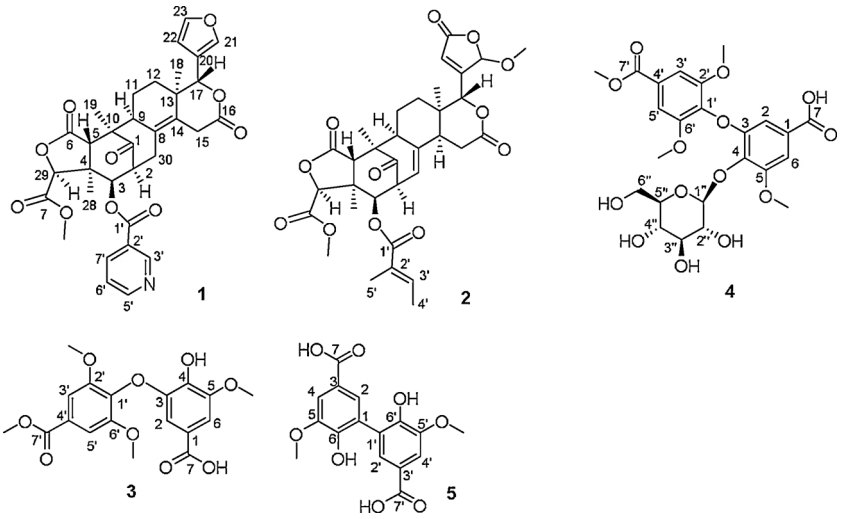

Fig. 1. The chemical structures of compounds **1**–**5**.

report the isolation, structural elucidation, and anti-inflammatory and antibacterial activities of the sixteen compounds (**1–16**) from the twigs and leaves of *T. sinensis*.

2. Results and discussion

Compound **1** was isolated as a white, amorphous powder. Its molecular formula $C_{33}H_{33}NO_{10}$ was established by HRESIMS (*m/z* 626.2004 $[M + Na]^+$, calcd as 626.1997), corresponding to 18 degrees of unsaturation. The IR spectrum indicated the presence of ester/ketone (1722 cm^{-1}) and olefinic (1625 cm^{-1}) groups. The ^{13}C NMR (Table 1) as well as DEPT and HSQC spectra of **1** displayed 33 carbon resonances as four methyls (including one O-methyl), four methylenes, 13 methines (including seven sp^2 and three oxygenated sp^3), and 12 quaternary carbons (including five carbonyl ones). A *β*-substituted furan ring (δ_H 6.46, br s; 7.44, br s, and 7.55, br s; δ_C 110.1, 120.3, 141.6, and 143.3), a nicotinoyl (δ_H 7.52, dd, J = 8.2, 4.8 Hz; 8.23, dt, J = 8.2, 1.7 Hz; 8.89, dd, J = 4.8, 1.7 Hz; 9.27, d, J = 1.7 Hz; δ_C 123.8, 124.0, 137.1, 151.7, and 155.5), a keto carbonyl (δ_C 214.9), and four ester/lactone carbonyls (δ_C 163.9, 168.8, 169.7, and 172.2) were evident from the NMR data (Table 1). The above observations suggested that **1** was a mexicanolide-type limonoid (Xu et al., 2013). Its NMR data showed great similarity to those of trichiliasinenoid A (Cao et al., 2017), suggesting their close structures. Detailed analysis of the 1D (^1H and ^{13}C NMR) and 2D NMR data (^1H–^1H COSY, HSQC, and HMBC) (Fig. 2a) indicated that both compounds shared the same skeleton, and the major differences were the presence of a C-8-C-14 double bond instead of a C-8-C-30 one in **1**. The conclusion was supported by the significant downfield shift for C-8 and C-30 ($\Delta\delta_C$ -15.2 and -88.8, respectively) and upfield shift for C-14 ($\Delta\delta_C$ 89.4) as determined by the HMBC correlations from H-30 (δ_H 2.64) to C-8 and C-14.

The relative configuration of **1** was determined by the ROESY data (Fig. 2b). The ROESY correlations of H-5/H-17, H-17/H-11*β*, H-17/H-15*β*, H-15*β*/H-30*β* indicated that these protons were all *β*-oriented. The ROESY correlations of H-2/H-3, H-3/H-28, H-2/H-30*α*, H-30*α*/H-15*α*, H-15*α*/H_3-18, H_3-28/H-9, H-9/H_3-19, H-9/H-11*α* revealed the *α*-orientation of the corresponding protons. Therefore, the structure of compound **1** was finally established as depicted and was named trichiliasinenoid D.

Compound **2**, obtained as a white amorphous powder, possessed a molecular formula of $C_{33}H_{38}O_{12}$ as deduced from the HRESIMS peak at *m/z* 625.2275 ($[M-H]^+$, calcd for $C_{33}H_{37}O_{12}$, 625.2291). The IR spectrum implied the presence of γ-lactone and ester/ketone carbonyl functionalities on the basis of the absorption bands at 1793 and 1748 cm^{-1}, respectively. Analysis of the ^1H and ^{13}C NMR data for **2** suggested a mexicanolide-type limonoid structure with a tigloyloxy group (δ_H 1.82, s; 1.82, d, J = 4.5 Hz; 6.68, q, J = 4.5 Hz; δ_C 12.2, 14.9, 127.0, 141.9) for **2**. Comparison of its NMR data with those of

Table 1
^1H and ^{13}C NMR data of compounds **1** and **2**.

position	**1**		**2**	
	δ_H (multi, J in Hz)	δ_C	δ_H (multi, J in Hz)	δ_C
1		214.9		213.6
2	3.35, dd (9.1, 6.0)	47.9	3.54, dd (8.6, 6.0)	49.3
3	5.54, d (9.1)	73.3	5.27, d (8.6)	70.6
4		47.4		47.4
5	4.38, s	45.3	4.11, s	44.6
6		172.2		172.6
7		169.7		168.2
8		126.8		140.3
9	2.21, dd (12.2, 5.1)	51.7	2.35, dd (11.6, 5.0)	55.9
10		51.3		48.8
11α	2.11, m	17.1	2.11, m	21.5
11β	1.86, m		2.02, m	
12α	1.52, m	29.6	1.85, m	34.7
12β	1.25, m		1.73, m	
13		38.6		37.6
14		134.8	2.27, d (6.7)	45.8
15α	3.13, d (18.4)	33.2	2.81, dd (18.9, 6.7)	29.3
15β	3.13, d (18.4)		2.71, d (18.9)	
16		168.8		167.0
17	5.42, s	81.2	5.21, s	76.6
18	1.00, s	17.1	1.07, s	21.7
19	1.47, s	15.4	1.41, s	15.2
20		120.3		162.2
21	7.55, br s	141.6	5.73, s	103.7
22	6.46, br s	110.1	6.28, s	122.7
23	7.44, br s	143.3		169.1
28	1.32, s	23.3	1.28, s	22.7
29	4.53, s	81.3	4.52, s	81.9
30α	2.64, d (15.5)	32.2	5.26, d (6.0)	122.6
30β	2.26, dd (15.5, 6.0)			
OMe-7	3.30, s	53.3	3.59, s	52.9
OMe-21			3.70, s	58.5
1′		163.9		166.2
2′		123.8		127.0
3′	9.27, d (1.7)	151.7	6.68, q (4.5)	141.9
4′			1.82, d (4.5)	14.9
5′	8.89, dd (4.8, 1.7)	155.5	1.82, s	12.2
6′	7.52, dd (8.2, 4.8)	124.0		
7′	8.23, dt (8.2, 1.7)	137.1		

Data were measured in $CDCl_3$ at 600 MHz (^1H NMR, J in H) and 150 MHz (^{13}C NMR).

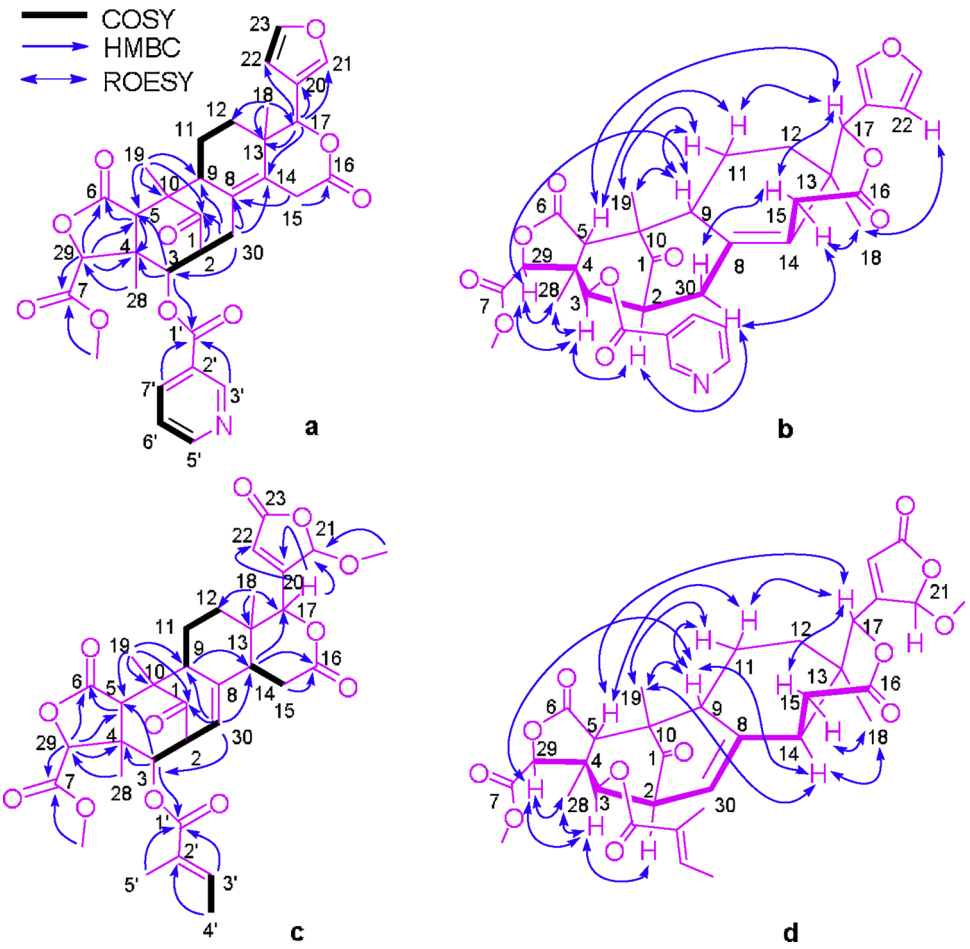

Fig. 2. Selected ^{1}H-^{1}H COSY, HMBC, and ROESY correlations of compounds **1** and **2**.

trichiliasinenoid B (Cao et al., 2017) suggested that, both compounds shared similar structural features, the only difference being the replacement of the furan ring by a 21-methoxybutenolide moiety at C-17 in **2**. This was verified by the HMBC correlations of H-17 (δ_H 5.21) with C-20 (δ_C 162.2), C-21 (δ_C 103.7), and C-22 (δ_C 122.7), and of OMe-21 (δ_H 3.70) with C-21 (δ_C 103.7). The relative configuration of **2** was established by a ROESY experiment (Fig.2d). The C-21 configuration in **2** was not assigned by the available data. Thus, the structure of **2** was established as drawn and was named trichiliasinenoid E.

Compound **3** was obtained as white amorphous power. Its HRESIMS displayed a pseudomolecular ion peak $[M+Na]^+$ at *m/z* 401.0843 (calcd 401.0843), corresponding to the molecular formula of $C_{18}H_{18}O_9$. The IR absorptions showed the presence of hydroxy (3426 cm^{-1}), carbonyl (1718 cm^{-1}), and aromatic (1502 and 1598 cm^{-1}) groups. The ^{1}H NMR spectrum of **3** (Table 2) showed signals for a symmetric 2′,6′-dimethoxy-1′,4′-disubstituted benzene ring (δ_H 7.40, br s, 2 H; 3.79, s, 6 H) and an asymmetric 1,3,4,5- or 1,3,4,6-tetrasubstituted benzene ring (δ_H 6.74, br s; 7.28, br s), and two additional methoxy groups [δ_H 3.90 (3H), 3.89 (3H)]. The ^{13}C NMR (with DEPT) spectrum (Table 2) with ten aromatic carbon signals (three methines and seven quaternary carbons) and four methoxy signals (δ_C 56.8, 56.8, 56.8, and 52.9) were in agreement with the above deduction. Besides, two carboxyl/ester carbonyls (δ_C 169.7, 167.9) were also observed in the ^{13}C NMR spectrum. Structural details of **3** were further established on the basis of 2D NMR data (Fig. 3). The HMBC correlations from H-3′/H-5′ to C-7′ (δ_C 167.9), C-4′ (δ_C 128.7), and C-1′ (δ_C 137.7), as well as from the two symmetric methoxy groups to C-2′/C-6′ (δ_C 154.5) and from the methoxy at δ_H 3.90 to C-7′ suggested the presence of a methyl 2′,6′-dimethoxy-1′-*O*-substituted benzoate. The carboxyl resonance at δ_C 169.7 was attached to C-1 by the HMBC correlations from H-2/H-6 to C-1 and C-7. HMBC correlations from the remaining methoxyl (δ_H 3.89) to C-5 and from H-6 to C-1 (δ_C 121.5), C-2 (110.2), C-4 (δ_C 141.7), C-5 (δ_C 149.4), C-7 (δ_C 169.7), as well as from H-2 to C-1 (δ_C 121.5), C-3 (δ_C 147.1), C-4 (δ_C 141.7), C-6 (108.5), C-7 (δ_C 169.7) indicated the presence of a 3,4,5-tri-*O*-substituted benzoic acid fragment. Connection of the two benzoyl groups *via* a C_3-O-$C_{1'}$ ether linkage was deduced from their chemical shifts (δ_C 137.7 for C-1′ and δ_C 147.1 for C-3) and HMBC correlations above (Li et al., 2008). Thus, compound **3** was established and named 3-(2′,6′-dimethoxy-4′-(methoxycarbonyl)phenoxy)-4-hydroxy-5-methoxybenzoic acid.

Compound **4** was obtained as white amorphous powder and gave a sodiated molecular ion $[M+Na]^+$ at *m/z* 563.1372 (calcd 563.1371) in the HRESIMS, suggesting a molecular formula of $C_{24}H_{28}O_{14}$ for **4**. The IR spectrum showed the absorption bands of hydroxy (3426 cm^{-1}), carbonyl (1721 cm^{-1}), and aromatic (1599, 1501 cm^{-1}) groups. The ^{1}H and ^{13}C NMR spectra of **4** (Table 2) showed, except for signals for an extra hexosyl moiety (δ_H 3.26–3.78, 6 H and 5.28, 1 H; δ_C 62.6, 71.4, 75.7, 77.8, 78.4, 104.8), distinct signals similar to those for compound **3**. The noticeable differences were only observed for C-1 (Δ + *ca* 6 ppm), C-3 (Δ + *ca* 5 ppm), C-4 (Δ - *ca* 2 ppm), C-5 (Δ + *ca* 5 ppm) in accordance with typical glycosylation shifts of such a phenyl group with glycosylation occurred at C-4 (Miyase et al., 1988). HMBC correlation between H-1″ (δ 5.28, d, J = 7.5 Hz) and C-4 (δ 139.6) was further verified the location of the glucosyl group at C-4. The hexose was determined to be D-glucose by GC analysis of the hexose methyl ether prepared from methylation of its hydolysate, while the β-configuration of the glucose was established by the large coupling constant of the anomeric proton (J = 7.5 Hz). Thus, the structure of **4** was established

Table 2
^{1}H and ^{13}C NMR spectroscopic data for compounds **3–5**.

position	3		4		5	
	δ_H (multi, J in Hz)	δ_C	δ_H (multi, J in Hz)	δ_C	δ_H (multi, J in Hz)	δ_C
1		121.5		127.5		125.6
2	6.74,br s	110.2	6.74, d (2.0)	109.4	7.68, d (2.0)	127.5
3		147.1		152.0		122.0
4		141.7		139.6	7.60, d (2.0)	112.2
5		149.5		154.5		148.7
6	7.28, br s	108.5	7.34, d (2.0)	109.0		150.2
7		169.7		169.0		170.0
1′		137.7		136.6		125.6
2′		154.5		154.4	7.68, d (2.0)	127.5
3′	7.40, br s	107.8	7.43, br s	107.9		122.0
4′		128.7		129.0	7.60, d (2.0)	112.2
5′	7.40, br s	107.8	7.43, br s	107.9		148.7
6′		154.5		154.4		150.2
7′		167.9		167.8		170.0
OMe-2′, 6′	3.79, s	56.8	3.81, s	56.8		
OMe-5	3.89, s	56.8	3.91, s	57.1	3.87, s	56.5
OMe-5′					3.87, s	56.5
OMe-7′	3.90, s	52.9	3.92, s	53.0		
1″			5.28, d (7.5)	104.8		
2″			3.53, m	75.7		
3″			3.42, m	77.8		
4″			3.42, m	71.4		
5″			3.26, m	78.4		
6″			3.68, dd (12.0, 5.0) 3.78, dd (12.0, 2.9)	62.6		

All data were measured in CD_3OD at 500 MHz (^{1}H NMR) and 125 MHz (^{13}C NMR).

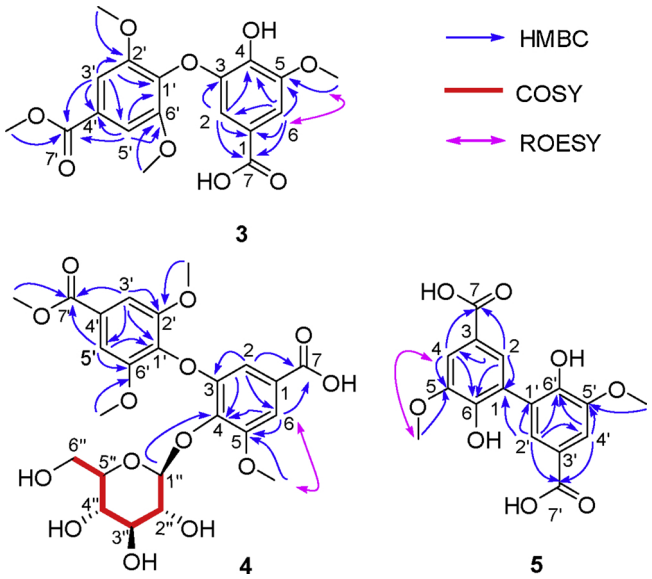

Fig. 3. Selected ^{1}H-^{1}H COSY, HMBC, and ROESY correlations of compounds **3–5**.

and named 3-(2′,6′-dimethoxy-4′-(methoxycarbonyl)phenoxy)-5-methoxy-4-O-β-D-glucopyranosylbenzoic acid.

Compound **5** was obtained as white amorphous power. The HRESIMS displayed a pseudomolecular ion at m/z 357.0572 $[M+Na]^+$ (calced 357.0581), consistent with a molecular formula of $C_{16}H_{14}O_8$. The IR absorption bands indicated the presence of hydroxy (3508 cm^{-1}), conjugated carboxyl (1692 cm^{-1}), and aromatic (1491 and 1599 cm^{-1}) groups. Taking into consideration the molecular formula, two identical methoxy 1,5,6-trisubstituted benzoyloxy groups (δ_H 7.60, d, J = 2 Hz, 2H; 7.68, d, J = 2 Hz, 2 H; 3.87, s, 6H; δ_C 56.5, 125.6, 127.5, 122.0, 112.2, 148.7, 150.2, 170.0) were easily recognized by the ^{1}H and ^{13}C NMR spectra (Table 2). 2D NMR experiments were further conducted to figure out the structural details. The carboxyl resonance at 170.0 was attached to C-3 by the HMBC correlation from H-2 and H-4 to C-7. The HMBC correlations of H-4/C-2, C-5, C-6, C-7 and the ROSEY correlation of H-4/OCH_3-5 indicated the methoxy group was attached to C-5 (Fig.3). The hydroxy group was assigned to C-6 by the HMBC correlation from H-2 to C-1, C-4, C-6, C-7, as well as from H-4 to C-2, C-5, C-6, C-7 (Fig.3). Two 5-methoxy-6-hydroxyl-1-substituted benzoic acid fragments were thus established. Connection of the two subunits *via* a C_1-$C_{1'}$ linkage was deduced from their chemical shifts (δ_C 125.6 for C-1 and C-1′) and HMBC correlations above (Li et al., 2008). Compound **5** was therefore established as depicted and was named 6,6′-dihydroxy-5,5′-dimethoxy-[1,1′-biphenyl]-3,3′-dicarboxylic acid.

In addition to the five previously undescribed compounds (**1-5**), eleven known compounds named β-hydroxypropiovanillone (**6**) (Karonen et al., 2004), kaempferol-3-O-rutinoside (**7**) (Feng et al., 2007), kaempferol 3-O-β-D-glucopyranoside-7-O-α-L-rhamnopyranoside (**8**) (Szewczyk et al., 2014), Kaempferol 3-O-glucoside (**9**) (Senatore et al., 1999), meliavosin (**10**) (Rogers et al., 1998), stigmast-4-ene-3β,6β-diol (**11**) (Qu et al., 2009), 7-ketositosterol (**12**) (Byung et al., 2007), lyoniside (**13**) (Zheng et al., 2011), 3,4,6-trimethoxy phenyl-O-D-glucoside (**14**) (Kimura et al., 1984), 1-hexadecanoyl propan-2,3-diol (**15**) (Misra and Siddiqi, 2000), and 3,5-dimethoxy-4-hydroxy-benzaldehyde (**16**) (Yuan et al., 2016) were also obtained and structurally determined by comparing their spectroscopic data with those in the literature. All the compounds were isolated from the plant for the first time.

All the isolates were evaluated for their inhibitory effects on nitric oxide (NO) production stimulated by LPS in RAW264.7 cells using L-NMMA (NG-monomethyl L-arginine) as the reference compound with an IC_{50} value of 35.5 μM. The cytotoxicity of the tested samples were assessed by the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay at the concentrations (≤100 μM) showing no cytotoxicity. Among the isolates, compounds **1**, **2**, **6**, and **10-13** exhibited weak NO inhibition and the rest compounds were inactive (IC_{50} ≥100 μM) (Table 3). All the isolated compounds were also evaluated for their *in vitro* antibacterial activities against Gram-positive bacteria (*Staphylococcus aureus* and *Candida albicans*) and Gram-negative bacteria (*Escherichia coli and Pseudomonas aeruginosa*) using a microbroth dilution method for the determination of the minimum inhibitory concentrations (MICs) (Supporting Information, Table S1). Compound **16** showed moderate inhibitory activity against *Escherichia coli*.

Table 3
Effect of compounds **1–16** on LPS-induced NO production in RAW264.7 cells[a,b].

Compound	IC_{50}/μM	CI (95%, n = 3)
1	93.8	[91.4, 95.9]
2	88.3	[87.4, 89.8]
3-5	> 100	–
6	92.5	[91.8, 92.9]
7-8	> 100	–
9	> 100	–
10	73.9	[73.4, 74.3]
11	92.7	[92.3, 93.1]
12	76.5	[76.2, 76.8]
13	93.1	[92.6, 94.1]
14-16	> 100	–
L-NMMA [c]	35.5	[32.7, 38.1]

[a] Results are expressed as IC_{50} values in μM.
[b] Compounds with IC_{50} > 100μM are not shown.
[c] Positive control.

3. Experimental

3.1. General experimental procedures

Optical rotations were obtained with a JASCO P-1020 polarimeter. UV spectra were measured with a Shimadzu UV-2401 A instrument. IR spectra (KBr) were determined on a Bruker Tensor-27 infrared spectrometer. 1D and 2D NMR spectra were recorded on Bruker DRX-500 and Bruker Avance III 600 spectrometers with TMS as an internal standard. ESIMS and HRESIMS were recorded on an AutoSpec Premier P776 instrument. Semi-preparative HPLC was performed on a Waters 600 pump system with a 2996 photodiode array detector by using a YMC-Pack ODS-A column (300 × 10 mm, S^{-5} μm). Silica gel (200 − 300 mesh, Qingdao Marine Chemical Factory, Qingdao, China), Sephadex LH-20 gel (40 − 70 μm, Amersham Pharmacia Biotech AB, Uppsala, Sweden), C18-reversed phase silica gel (250 mesh, Merck) and MCI gel (CHP20/P120, 75 − 150 μm, high-porous polymer, Mitsubishi Chemical Corporation, Tokyo, Japan) were used for column chromatography (CC). Pre-coated silica gel GF254 plates (Qingdao Haiyang Chemical Co. Ltd) were used for analytical TLC. All solvents used for CC were of analytical grade (Shanghai Chemical Reagents Co. Ltd), and all solvents used for HPLC were of spectral grade.

3.2. Plant material

The twigs and leaves of *T. sinensis* were collected from Xishuangbanna Tropical Botanical Garden (XTBG), Chinese Academy of Science (CAS), Mengla Country, Yunnan Province, People's Republic of China in May 2017, and they were identified by one of the authors (Chun-Fen Xiao). A voucher specimen (No. HITBC-028935) is deposited in the herbarium at XTBG.

3.3. Extraction and isolation

The dried and powered twigs and leaves of *T. sinensis* (5.0 kg) were percolated with 95% aqueous EtOH (40 L) three times (for seven days each time) at room temperature. Removal of the solvent from the combined extracts *in vacuo* afforded a crude residue (274 g), which was then suspended in distilled H_2O and successively partitioned with EtOAc and *n*-BuOH. The EtOAc-soluble fraction (145 g) was separated over a MCI gel column (8 cm × 100 cm) chromatography and eluted with MeOH-H_2O (20:80, 40:60, 60:40, 80:20, 100:0, v/v, each 8 L) to give five fractions. The fourth fraction (68 g) was chromatographed on a silica gel column (6 cm × 70 cm, 200–300 mesh) with gradient mixtures of $CHCl_3$-MeOH (100:0, 50: 1, 20:1, 10:1, 5: 1, 2:1, 1: 1, v/v, each 4 L) elution to yield seven fractions, Frs. A–G (6.5, 4.2, 20.4, 12.7, 5.2, 8.6 and 3.2 g, respectively). Fr. B (4.2 g) was subjected to Sephadex LH-20 (2 cm × 100 cm) eluted with $CHCl_3$-MeOH (1: 1, v/v) to give six sub-fractions (B-1 ~ B-6), according to their TLC profiles. Sub-fraction B-4 was recrystallized to give **15** (30 mg). Fr. C (20.4 g) was further separated by a silica gel column (6 cm × 70 cm, 200–300 mesh, petroleum ether/acetone, from 50/1 to 1/1, v/v, each 1 L) to yield eight fractions, Fr. C-1 ~ C-8. Sub-fraction C-3 (5 g) was purified by repeated CC over Sephadex LH-20 (2 cm × 100 cm; MeOH) and semi-preparative HPLC (10 mm × 300 mm, MeCN/H_2O, 52:48, v/v, 3 mL/min) to yield **1** (6 mg), **2** (4 mg), and **16** (5 mg). Sub-fraction C-5 (6.5 g) was fractionated by a reversed silica gel column (RP-18, 5 cm × 40 cm) eluted with MeOH/H_2O (30:70, 40:60, 50:50, 60:40, 70:30, 80:20, 90:10, 100:0, v/v, each 1 L) to give eight sub-fractions, C-5a ~ C-5 h. Sub-fraction C-5e (74 mg) was separated by semi-preparative HPLC (10 mm × 300 mm, MeCN/H_2O, 45: 55, v/v, 3 mL/min) to give **10** (33 mg), **11** (19 mg), and **12** (16 mg). Fr. D (12.7 g) was subjected to CC (RP-18, 5 cm × 40 cm, MeOH/H_2O, 30:70, 50:50, 70:30, 90:10, and 100:0, v/v, each 1 L) to yield five fractions, Fr. D-1 ~ D-5. Fr. D-3 was separated by CC over Sephadex LH-20 (2 cm × 100 cm) eluted with MeOH to give four fractions, further purification of which by normal-phase preparative thin-layer chromatography using $CHCl_3$/acetone (10: 1) afforded **3** (8 mg), **4** (11 mg), **5** (5 mg), and **14** (15 mg), respectively. Fr. E (5.2 g) was chromatographed on an ODS (2 cm × 40 cm) column eluted with a MeOH/H_2O gradient (30:70, 40:60, 50:50, 60:40, 70:30, 80:20, 90:10, v/v, each 1 L) to yield seven sub-fractions, E-1 ~ E-7. Sub-fraction E-5 (134 mg) was subjected to semi-preparative HPLC (10 mm × 300 mm, MeCN/H_2O, 30: 70, v/v, 3 mL/min) to give compounds **7** (15 mg), **8** (7 mg), **9** (41 mg), and **13** (23 mg). Sub-fraction E-6 (80 mg) was purified by normal-phase preparative thin-layer chromatography ($CHCl_3$/MeOH, 10: 1, v/v) to give **6** (5 mg). All of the compounds met the criteria of ≥ 95% purity, as determined by NMR analysis.

3.3.1. Trichiliasinenoid D (1)

White power; $[\alpha]_D^{25.7}$ -24.9 (*c* 0.06, MeOH); UV (MeOH) λ_{max} (log ε): 203 (2.97), 261 (2.20) nm; IR (KBr) ν_{max}: 3428 (H_2O), 2927, 1722, 1625, 1385, 1275, 1029, 743 and 582 cm^{-1}; HRESIMS *m/z* 626.2004 $[M+Na]^+$ (calcd for $C_{33}H_{33}NO_{10}Na$, 626.1997); ^1H and ^{13}C NMR data, see Table 1.

3.3.2. Trichiliasinenoid E (2)

White power; $[\alpha]_D^{25.7}$ -6.0 (*c* 0. 15, MeOH); UV (MeOH) λ_{max} (log ε): 203 (3.17) nm; IR (KBr) ν_{max}: 3429 (H_2O), 2924, 1793, 1748, 1631, 1440, 1384, 1259, 1212, 1118, 1053, 995 and 583 cm^{-1}; HRESIMS *m/z* 625.2275 $[M-H]^+$ (calcd for $C_{33}H_{37}O_{12}$, 625.2291); ^1H and ^{13}C NMR data, see Table 1.

3.3.3. 3-(2′,6′-dimethoxy-4′-(methoxycarbonyl)phenoxy)-4-hydroxy-5-methoxybenzoic acid (3)

White power; UV (MeOH) λ_{max} (log ε): 211 (4.05), 257 (3.53), 287 (3.14) nm; IR (KBr) ν_{max}: 3426, 2924, 1718, 1598, 1502, 1384, 1217, 1126 and 766 cm^{-1}; HRESIMS *m/z* 401.0843 $[M+Na]^+$ (calcd for $C_{18}H_{18}O_9Na$, 401.0843); ^1H and ^{13}C NMR data, see Table 2.

3.3.4. 3-(2′,6′-dimethoxy-4′-(methoxycarbonyl)phenoxy)-5-methoxy-4-O-β-D-glucopyranosylbenzoic acid (4)

White power; $[\alpha]_D^{25.7}$ -2.4 (*c* 0. 1, MeOH); UV (MeOH) λ_{max} (log ε): 211 (4.05), 257 (3.53), 287 (3.14) nm; IR (KBr) ν_{max}: 3426, 2942, 1721, 1599, 1501, 1418, 1340, 1217, 1086 and 764 cm^{-1}; HRESIMS *m/z* 563.1372 $[M+Na]^+$ (calcd for $C_{24}H_{28}O_{14}Na$, 563.1371); ^1H and ^{13}C NMR data, see Table 2.

3.3.5. 6,6′-dihydroxy-5,5′-dimethoxy-[1,1′-biphenyl]-3,3′-dicarboxylic acid (5)

White power; UV (MeOH) λ_{max} (log ε): 211 (4.05), 257 (3.53), 287 (3.14) nm; IR (KBr) ν_{max}: 3508, 3422, 2943, 1692, 1599, 1491, 1410, 1269, 1039 and 769 cm^{-1}; HRESIMS *m/z* 357.0572 $[M+Na]^+$ (calcd for $C_{16}H_{14}O_8Na$, 357.0581); ^1H and ^{13}C NMR data, see Table 2.

3.4. Determination of absolute configuration of sugar moieties

Compound **4** (3 mg) was separately dissolved in 1 N HCl and refluxed for 6 h. After removal of HCl by evaporation and extraction with CH_2Cl_2, the H_2O extract was again evaporated and dried *in vacuo* to afford a residue that contained the monosaccharide. The residue was dissolved in pyridine (1 mL), and 2 mg L-cysteine methyl ester hydrochloride was added to the solution. The mixture was kept at 60 °C for 2 h and then the solvent was evaporated under a stream of N_2, to give a residue. The residue was then trimethylsilylated *via* a reaction with N-(trimethylsilyl) imidazole (0.2 mL) for 2 h. The mixture was partitioned between *n*-hexane and H_2O (2 mL each), and the *n*-hexane extract was analyzed by GC under the following conditions: L-chirasil-Val-column (25 m × 0.25 mm, i.d.); detection, FID; detector temperature, 280 °C; injection temperature, 250 °C; column temperature, 270 °C; and carrier, N_2 gas, 250 kPa (Ma et al., 2018). The presence of D-glucose in the acid hydrolysate of compound **4** was verified by comparison of the retention

times of their derivatives to those of corresponding control samples prepared by the same procedure. The retention time of D-glucose was 24.78 min.

3.5. Assay for inhibition ability toward LPS-induced NO production and cytotoxicity testing

The RAW 264.7 macrophages (obtained from Kunming Institute of Zoology, Chinese Academy of Sciences) were maintained in DEMEM/high-glucose medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% (v/v) newborn calf serum and antibiotics (100 U/mL penicillin and 0.1 g/L streptomycin) at 37 °C in the presence of 5% CO_2. The cell viability was determined by MTS assay before the nitric oxide (NO) production assay, and the NO production was measured by the accumulation of nitrite in the culture supernatants using the Griess Reagent System as previously reported (Li et al., 2014). All experiments were performed in three independent replicates, and L-NMMA (NG-monomethyl L-arginine) (Sigma) was used as a positive control. Statistical analysis was calculated using SPSS 21.0 software.

3.6. Antimicrobial assays

The purified compounds were tested against Gram-positive bacteria (*Staphylococcus aureus* ATCC25923 and *Candida albicans* ATCCY0109) and Gram-negative bacteria (*Escherichia coli* ATCC25922 and *Pseudomonas aeruginosa* ATCC27853) using a microbroth dilution method in a 96-well microtiter plate, as reported previously (Wu et al., 2011). All microbial strains were provided by the National Institute for the Control of Pharmaceutical and Biological Products (NICPBP, China). Bacteria were seeded at 1×10^6 cells per well (200 μL) in a 96-well plate containing Mueller-Hinton broth (meat extracts 0.2%, acid digest of casein 1.75%, starch 0.15%) with different concentrations (from 1 to 512 μg/mL) of each test compound. Positive control: amikacin for *Staphylococcus aureus* and *Escherichia Coli*; vancomycin for *Pseudomonas aeruginosa*; fluconazole for *Candida albicans*. The minimum inhibitory concentration (MIC) was defined as the lowest concentration giving no visible growth after incubation at 37 °C for 18–20 h.

Conflict of interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.phytol.2018.11.020.

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