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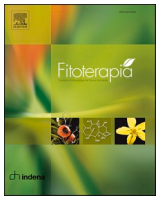

# Structurally diverse limonoids and bio-active evaluation from *Trichilia connaroides*

Dong-Hua Cao [a,b], Jian-Neng Yao [c], Peng Sun [b], Kai-Long Ji [b], Xiao-Nian Li [c], Qiang Cai [d], Chun-Fen Xiao [b], Hua-Bin Hu [b], Zhi-Yong Yu [d,*], You-Kai Xu [b,*]

[a] *The Affiliated Changsha Central Hospital, Hengyang Medical School, University of South China, Changsha 410004, PR China*
[b] *Key Laboratory of Tropical Plant Resources and Sustainable Use, Xishuangbanna Tropical Botanical Garden, Chinese Academy of Sciences, Menglun 666303, PR China*
[c] *State Key Laboratory of Phytochemistry and Plant Resources in West China, Kunming Institute of Botany, Chinese Academy of Sciences, Kunming 650201, PR China*
[d] *Department of Hepatobiliary Surgery, The Second People's Hospital of Yunnan Province, Kunming 650021, PR China*

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ABSTRACT

Four new limonoids, named as trichiconlide G (**1**), 2-hydroxyltrijugin F (**2**), 23-oxo-21-hydroxyltrijugin F (**3**), 21-oxo-23-hydroxyltrijugin F (**4**), along with sixteen known analogues (**5**–**20**) were isolated from the leaves and twigs of *Trichilia connaroides*. Their structures and absolute configurations were determined by spectroscopic analyses, X-ray diffraction analysis, and TD-DFT-ECD calculations. Trichiconlide G (**1**) is one rare naturally occurring 1,2-seco phragmalin-type limonoid bearing a C-7/28 $\delta$-lactone ring. Additionally, 2-hydroxyltrijugin F (**2**), 23-oxo-21-hydroxyltrijugin F (**3**), and 21-oxo-23-hydroxyltrijugin F (**4**) are three naturally occurring limonoids with a rare C-16/8 $\delta$-lactone ring. All isolates were evaluated for their cytotoxic and anti-inflammatory activities. None of compounds exhibited cytotoxicity against five human cancer cell lines A-549, HepG2, 5-8F, Siha, and SCC-4 at the concentration of 40 μM. Compounds **16** and **17** showed moderate anti-inflammatory activity with $IC_{50}$ values of 28.45 ± 2.51 and 22.66 ± 2.01 μM, respectively.

## 1. Introduction

Limonoids, possessing 17$\beta$-furan ring tetranortriterpenoids structurally, are the characteristic secondary metabolites of Meliaceae family in the plant kingdom [1]. This compound class has attracted considerable interest from the research communities of natural products, pharmacology, and synthetic chemistry due to their intriguing structures with important bioactivities [1–4]. The genus *Trichilia* (Meliaceae) comprising 419 species is mainly distributed in tropical regions of America, Africa, and Asia, of which two species and one variant grown in China, namely *T. connaroides*, *T. sinensis*, and *T. connaroides* var. *Microcarpa* [5]. Previously, a number of structurally diverse and highly oxygenated limonoids with a wide spectrum of biological activities, such as cytotoxic [6,7], antifeedant [8,9], anti-HIV [10], and anti-inflammatory properties [11–13], have been isolated from the genus *Trichilia*. *T. connaroides* (Wight et Arn.) Bentv, a tall tree that mainly distributed in the southeast of China, has been applied by local residents as an anti-inflammatory Chinese folk medicine to treat arthritis, pharyngitis, and tonsillitis [14]. In our continuing search for structurally interesting and biologically important limonoids from *Trichilia* species [15–17], four new limonoids (**1**–**4**) and sixteen known analogues (**5**–**20**) were isolated from the leaves and twigs of *T. connaroides* (Fig. 1). Trichiconlide G (**1**) is one rare naturally occurring 1,2-seco phragmalin-type limonoid bearing a C-7/28 $\delta$-lactone ring. Additionally, 2-hydroxyltrijugin F (**2**), 23-oxo-21-hydroxyltrijugin F (**3**), and 21-oxo-23-hydroxyltrijugin F (**4**) are three naturally occurring limonoids with a rare C-16/8 $\delta$-lactone ring. Structural elucidation of these compounds was conducted by comprehensive analyses of the NMR, single crystal X-ray crystallography, and electronic circular dichroism (ECD) data. All compounds were evaluated for their anti-inflammatory effects in the LPS-induced RAW 264.7 cell line and cytotoxicity against five human cancer lines A-549, HepG2, 5-8F, Siha, and SCC-4. Herein, we report the isolation, structural elucidation, and activity evaluation of all the isolates.

* Corresponding author.
*E-mail addresses:* rsyby@139.com (Z.-Y. Yu), xyk@xtbg.ac.cn (Y.-K. Xu).

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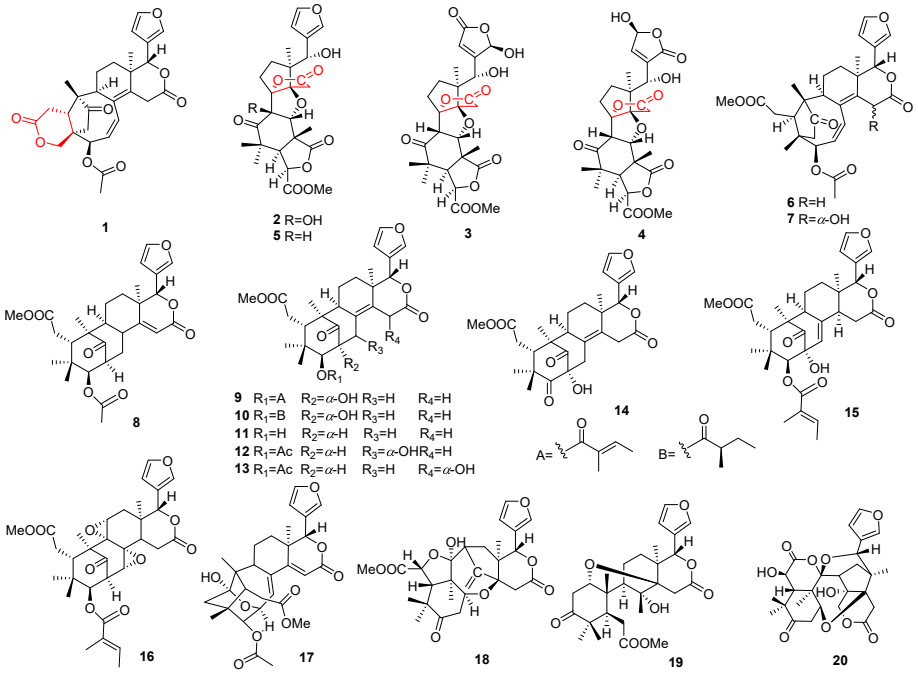



**Fig. 1.** Structures of compounds **1**–**20**.

## 2. Experimental

### 2.1. General experimental procedures

Optical rotations were obtained with a Rudolph Autopol VI polarimeter. UV spectra were measured with a Shimadzu UV-2401A instrument. IR spectra were determined on a Bruker Tensor-27 infrared spectrometer. ECD spectra were measured with a Chirascan circular dichroism spectrometer (Applied Photophysics). The NMR spectra were obtained on a Bruker Avance spectrometer operating at 500 MHz for $^{1}H$ NMR and 125 MHz for $^{13}C$ NMR, using tetramethylsilane as an internal standard. HRESIMS were carried out on a Shimadzu UPLC-IT-TOF mass spectrometer. 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). TLC was performed on plates precoated with silica gel GF254 (10–40 μm), while column chromatography (CC) was run over 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 D101 macroporous resin (Xianlan-xiao New Material Company, China). Spots were detected firstly on TLC (Qingdao Marine Chemical Factory) under 254 nm UV light and then by spraying with 10% $H_2SO_4$ in ethanol for plate heating. All solvents used were of analytical grade (Shanghai Chemical Reagents Co. Ltd), and all solvents used for HPLC were of chromatographic grade (J & K Scientific Ltd.).

### 2.2. Plant material

The twigs and leaves of *Trichilia connaroides* were collected from Menglun town (101°25′ E, 21°41′ N), Mengla Country, Yunnan Province, People's Republic of China at altitude of 570 m in September 2017, and they were identified by one of the authors (Chun-Fen Xiao). A voucher specimen (No. HITBC-0022155) is deposited in the herbarium at Xishuangbanna Tropical Botanical Garden, Chinese Academy of Sciences.

### 2.3. Extraction and isolation

The air-dried powder of the twigs and leaves of *T. connaroides* (6 Kg) was extracted with 95% EtOH at room temperature (40 L × 3), to afford a crude extract (200 g). The extract was then dissolved in 1 L of water and partitioned with EtOAc (1 L × 3), *n*-BuOH (1 L × 3), successively. The EtOAc extract (120 g) was subjected to CC over D101-macroporous absorption resin (8 cm × 120 cm), eluted with MeOH/$H_2O$ (20:80, 40:60, 60:40, 80:20, 100:0, v/v, each 8 L), to afford five major fractions (A–E). Fraction D (80%, 75 g) was separated by silica gel CC (8 cm × 100 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) as the eluent to give six major fractions, Frs. D1–D6 (13.0, 8.2, 18.0, 12.7, 10.4, and 5.2 g, respectively). Fr. D3 (18.0 g) was then subjected to RP-18 silica gel CC (6 cm × 80 cm, MeOH/$H_2O$, 30–90%) to give four subfractions (D3a ~ D3d), according to their TLC profiles. Subfraction D3b (2.5 g) was further purified by semipreparative HPLC (10 mm × 300 mm, $CH_3CN$/$H_2O$, 64:36, v/v, 3 mL/min) to afford compounds **6** (6 mg) and **7** (6 mg). Subsequently, subfraction D3c (1.2 g) was purified by semipreparative HPLC (10 mm × 300 mm, $CH_3CN/H_2O$, 60:40, v/v, 3 mL/min) to afford compounds **2** (6 mg), **3** (4 mg), **4** (8 mg), **5** (4 mg), and **8** (12 mg). Subfraction D3d (3.1 g) was fractionated over a column of Sephadex LH-20 gel (4 cm × 150 cm) eluted with MeOH, and then purified by semipreparative HPLC (10 mm × 300 mm, $CH_3CN/H_2O$, 62:38, v/v, 3 mL/min) to afford compounds **9** (9 mg), **10** (5 mg), and **11** (5 mg). Fraction D4 (12.7 g) was chromatographed on a Sephadex LH-20 gel column (4 cm × 150 cm, MeOH/$H_2O$, 80:20, v/v, each 1 L), affording three subfractions, Fraction D4a~D4c. Subfraction D4a (2.8 g) afforded compounds **15** (4 mg), **16** (6 mg), and **20** (9 mg) by semipreparative HPLC (10 mm × 300 mm, $CH_3CN/H_2O$, 60:40, v/v, 3 mL/min). Subfraction D4c (1.4 g) was purified by semipreparative HPLC (10 mm × 300 mm, $CH_3CN/H_2O$, 57:43, v/v, 3 mL/min) to afford compounds **12** (6 mg), **13** (5 mg), and **14** (4 mg). Fraction D5 (10.4 g) was chromatographed on a RP-18 silica gel column (MeOH/$H_2O$, 30–80%) to give subfraction D5a. Subfraction D5a (2.0 g) was purified by semipreparative HPLC (10 mm × 300 mm, $CH_3CN/H_2O$, 48:52, v/v, 3 mL/min) to give compound **1** (16 mg). Fraction D6 (5.2 g) was separated by CC over Sephadex LH-20 gel (2 cm × 100 cm) eluted with MeOH to give two subfractions (D6a~D6b). After purification by HPLC (10 mm × 300 mm, $CH_3CN$/

**Table 1**
$^1$H NMR data for compounds **1**–**4** in $CDCl_3$ at 500 MHz ($\delta$ in ppm and $J$ in Hz).

| Position | 1 | 2 | 3 | 4 |
|---|---|---|---|---|
| 1 | | 4.44, s | 4.48, d (7.8) | 4.65, d (7.9) |
| 2 | 5.78, dd (12.3, 8.3) | | 3.32, d (7.8) | 3.36, d (7.9) |
| 3 | 5.54, d (8.3) | | | |
| 5 | 3.54, overlapped | 3.20, d (8.9) | 3.11, d (8.9) | 3.12, d (8.9) |
| 6$\alpha$ | 2.59, d (6.7) | 5.07, d (8.9) | 5.02, d (8.9) | 5.04, d (8.9) |
| 6$\beta$ | 2.57, d (10.8) | | | |
| 9 | 2.75, m | | | |
| 11$\alpha$ | 1.90, m | 1.44, m | 1.91, td (13.8, 6.3) | 1.83, td (13.6, 5.9) |
| 11$\beta$ | 1.72, m | 2.00, m | 2.42, dd (13.8, 6.3) | 2.39, dd (13.6, 5.9) |
| 12$\alpha$ | 1.23, m | 1.66, m | 1.55, m | 1.31, d (5.4) |
| 12$\beta$ | 1.09, dd (14.4, 2.5) | 2.52, m | 1.77, td (13.8, 5.5) | 1.95, td (13.6, 5.4) |
| 15$\alpha$ | 3.52, overlapped | 3.15, d (19.8) | 3.00, d (19.8) | 3.02, d (19.8) |
| 15$\beta$ | 3.21, dt (21.8, 2.4) | 2.80, d (19.8) | 2.72, d (19.8) | 2.76, d (19.8) |
| 17 | 4.96, s | 4.94, s | 4.56, s | 4.63, s |
| 18 | 1.16, s | 1.04, s | 0.96, s | 0.98, s |
| 19 | 1.04, s | 1.49, s | 1.36, s | 1.42, s |
| 21 | 7.61, br s | 7.40, br s | 5.92, br s | |
| 22 | 6.47, d (1.8) | 6.35, br s | 6.14, br s | 7.18, s |
| 23 | 7.45, t (1.6) | 7.38, t (1.7) | | 6.14, br s |
| 28 | 4.50, d (12.2) 4.17, d (12.2) | 1.43, s | 1.24, s | 1.28, s |
| 29 | 2.47, d (17.8) 2.18, d (17.8) | 1.23, s | 1.06, s | 1.10, s |
| 30 | 6.01, d (12.3) | | | |
| OMe-7 | | 3.84, s | 3.75, s | 3.78, s |
| OAc-2′ | 2.01, s | | | |

**Table 2**
$^{13}$C NMR spectral data for compounds **1**–**4** in $CDCl_3$ at 125 MHz ($\delta$ in ppm).

| Position | 1 | 2 | 3 | 4 |
|---|---|---|---|---|
| 1 | 218.6 | 90.1 | 84.2 | 84.2 |
| 2 | 127.1 | 85.9 | 61.2 | 61.3 |
| 3 | 72.8 | 214.5 | 208.5 | 208.7 |
| 4 | 44.9 | 47.0 | 46.9 | 46.8 |
| 5 | 39.2 | 46.9 | 46.6 | 46.9 |
| 6 | 31.1 | 74.6 | 74.9 | 75.0 |
| 7 | 172.6 | 170.0 | 170.0 | 170.1 |
| 8 | 131.8 | 104.4 | 101.0 | 101.8 |
| 9 | 44.6 | 175.4 | 177.1 | 177.1 |
| 10 | 52.8 | 44.7 | 44.6 | 44.8 |
| 11 | 19.6 | 37.2 | 37.3 | 36.9 |
| 12 | 29.0 | 31.5 | 35.0 | 34.7 |
| 13 | 38.2 | 51.0 | 50.4 | 50.4 |
| 14 | 132.0 | 101.3 | 100.2 | 99.9 |
| 15 | 33.7 | 40.8 | 39.9 | 40.1 |
| 16 | 169.5 | 174.3 | 174.6 | 174.9 |
| 17 | 81.5 | 70.6 | 70.0 | 67.7 |
| 18 | 16.9 | 19.9 | 19.2 | 18.5 |
| 19 | 20.7 | 18.3 | 19.4 | 19.4 |
| 20 | 120.0 | 124.9 | 170.5 | 138.9 |
| 21 | 141.8 | 140.2 | 98.6 | 171.0 |
| 22 | 109.8 | 109.6 | 120.8 | 149.0 |
| 23 | 143.6 | 143.0 | 170.9 | 98.2 |
| 28 | 75.1 | 27.0 | 27.0 | 27.0 |
| 29 | 42.7 | 23.4 | 22.0 | 22.1 |
| 30 | 137.4 | | | |
| OMe-7 | | 53.1 | 53.0 | 53.0 |
| OAc-1′ | 169.9 | | | |
| OAc-2′ | 20.7 | | | |

$H_2O$, 45:55, v/v, 3 mL/min), subfraction D6a (60 mg) yielded compound **19** (8 mg), and subfraction D6b (1.0 g) gave compounds **17** (7 mg) and **18** (6 mg). All of the compounds met the criteria of ≥95% purity, as determined by NMR analysis.

### 2.4. Spectroscopic data

#### 2.4.1. Trichiconlide G (1)

Colorless crystals; $[\alpha]_D^{22.0}$ −110.3 (*c* 0.10, MeOH); UV (MeOH) $\lambda_{max}$ (log $\varepsilon$): 196 (4.2) nm; IR (KBr) $\nu_{max}$: 3436, 2932, 1740, 1631, 1460, 1375, 1230, 1025, 964, 875, 602 cm$^{-1}$; HRESIMS *m/z* 517.1831 $[M + Na]^+$ (calcd for $C_{28}H_{30}O_8Na$, 517.1833); $^1$H NMR ($CDCl_3$), see Table 1 and $^{13}$C NMR ($CDCl_3$), see Table 2.

#### 2.4.2. 2-hydroxyltrijugin F (2)

White, amorphous powder; $[\alpha]_D^{22.1}$ −108.3 (*c* 0.15, MeOH); UV (MeOH) $\lambda_{max}$ (log $\varepsilon$): 195 (3.9) nm; IR (KBr) $\nu_{max}$: 3436, 2956, 1789, 1759, 1713, 1631, 1467, 1388, 1228, 1068, 984, 602 cm$^{-1}$; HRESIMS *m/z* 541.1680 $[M + Na]^+$ (calcd for $C_{26}H_{30}O_{11}Na$, 541.1680); $^1$H NMR ($CDCl_3$), see Table 1 and $^{13}$C NMR ($CDCl_3$), see Table 2.

#### 2.4.3. 23-oxo-21-hydroxyltrijugin F (3)

White, amorphous powder; $[\alpha]_D^{22.1}$ −43.2 (*c* 0.17, MeOH); UV (MeOH) $\lambda_{max}$ (log $\varepsilon$): 195 (3.8) nm, 193 (3.8) nm; IR (KBr) $\nu_{max}$: 3432, 2925, 1790, 1772, 1751, 1701, 1632, 1467, 1384, 1226, 1073, 967, 554 cm$^{-1}$; HRESIMS *m/z* 557.1627 $[M + Na]^+$ (calcd for $C_{26}H_{30}O_{12}Na$, 557.1629); $^1$H NMR ($CDCl_3$), see Table 1 and $^{13}$C NMR ($CDCl_3$), see Table 2.

#### 2.4.4. 21-oxo-23-hydroxyltrijugin F (4)

White, amorphous powder; $[\alpha]_D^{22.2}$ −34.9 (*c* 0.25, MeOH); UV (MeOH) $\lambda_{max}$ (log $\varepsilon$): 196 (3.7) nm; IR (KBr) $\nu_{max}$: 3441, 2925, 1783, 1758, 1709, 1632, 1442, 1344, 1260, 1030, 984, 544 cm$^{-1}$; HRESIMS *m/z* 533.1666 $[M - H]^-$ (calcd for $C_{26}H_{29}O_{12}$, 533.1665); $^1$H NMR ($CDCl_3$), see Table 1 and $^{13}$C NMR ($CDCl_3$), see Table 2.

### 2.5. X-ray crystallographic analysis of compound 1

Colorless crystals of trichiconlide G (**1**) were obtained by recrystallization from MeOH. The X-ray crystallographic data were obtained on a Bruker APEX DUO CCD diffractometer equipped with graphite monochromatic Cu K$\alpha$ radiation ($\lambda$ = 1.54178 Å) at 100 (2) K. The structure was solved by direct method with SHELXS-97 (Sheldrick 2008) and refined with full-matrix least-squares calculations on $F^2$ by using SHELXS-97 (Sheldrick 2008). All non-hydrogen atoms were refined anisotropically. The hydrogen atom position were geometrically idealized and allowed to ride on their parent atoms. Crystal data of **1**, Orthorhombic, $C_{28}H_{30}O_8$, space group $P2_12_12_1$ with a = 10.3978 (2) Å, b = 12.8609 (3) Å, c = 17.5647 (4) Å, and V = 2348.84 (9) Å$^3$; Z = 4; Dc = 1.398 Mg/m$^3$; and F (000) = 1048. Crystal size: 0.500 × 0.400 × 0.180 mm$^3$. Independent reflections 4410 [$R_{(int)}$ = 0.0480]. The final indices were $R_1$ = 0.0514 and $wR_2$ = 0.1247 ($I > 2\sigma(I)$). The final indices were $R_1$ = 0.0515 and $wR_2$ = 0.1248 (all data). The goodness of fit on $F^2$ was 1.068. Crystallographic data for **1** have been deposited at the Cambridge Crystallographic Data Center with the deposition number of CCDC 2012583. A copy of the data can be obtained free of charge on application to CCDC, 12 Union Road, Cambridge CB2 1EZ, UK [tel: (+44) 1223–336-408; fax: (+44) 1223–336-033; e-mail: deposit0@ccdc.cam.ac.uk].

### 2.6. 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 DMEM/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 RAW 264.7 macrophages were seeded in 96-well plates with 1 × 10$^4$

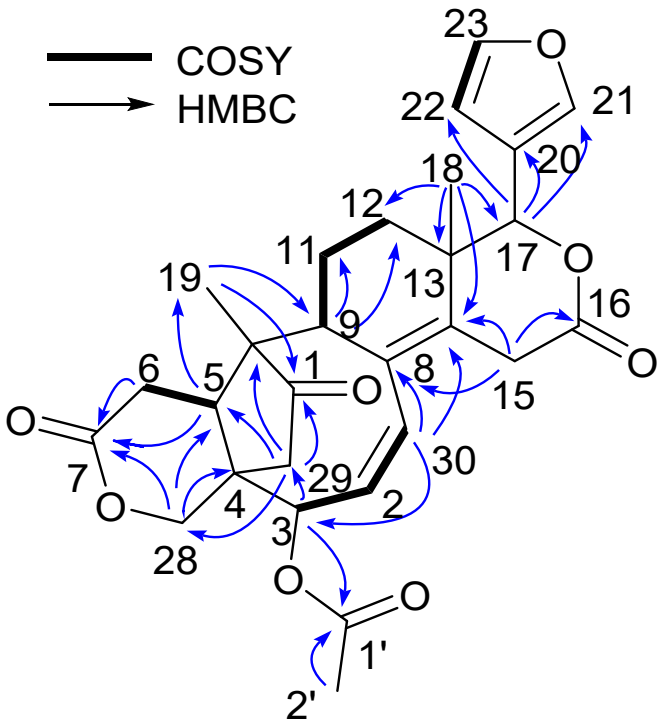



**Fig. 2.** $^{1}H—^{1}H$ COSY, selected HMBC correlations of compound **1**.

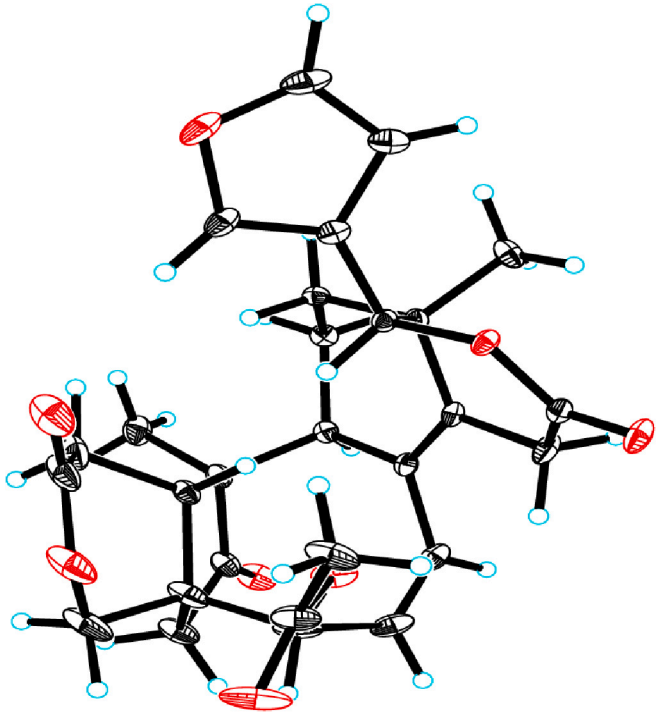


**Fig. 3.** Single-crystal X-ray structure of Trichiconlide G (**1**).

cells/well and allowed to adhere for 12 h at 37 °C in a humidified atmosphere containing 5% $CO_2$. After that the RAW 264.7 macrophages were pretreated with compounds for 24 h, followed by 1 mg/L LPS for 24 h. L-NMMA (NG-monomethyl L-arginine, Sigma) was used as a positive control. 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 at OD 550 as previously reported [18]. All experiments were performed in three independent replicates. Statistical analysis was calculated using SPSS 21.0 software.

### 2.7. Cytotoxicity assay

The obtained compounds were tested for their cytotoxic activity against the A-549, HepG2, 5-8F, Siha, and SCC-4 cancer cell lines using the MTS method as previously reported [19]. Briefly, all cells were cultured in DMEM medium containing 10% fetal bovine serum and 100 U/mL penicillin/streptomycin in a humidified incubator in a 5% $CO_2$ atmosphere at 37 °C. Then, 100 μL adherent cells was seeded into each well ($1 \times 10^4$ cells/well) of 96-well cell culture plates and allowed to adhere for 18 h before test drug addiction. Each tumor cell line was exposed to a test compound at concentrations of 0, 0.064, 0.32, 1.65, 8, and 40 μM in DMSO in triplicate for 24 h, with cisplatin as the positive control. After 24 h incubation, 20 μL MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxy-phenyl)-2-(4-sulfophenyl)-2H-tetrazolium] solution was added to each well, which were incubated for another 4 h to give a formazan product. The OD value of each well was measured at 490 nm using a Biorad 680 instrument. The $IC_{50}$ value of each compound was calculated by the Reed and Muench method [20].

## 3. Results and discussion

Compound **1** was obtained as colorless crystals. It possessed a molecular formula of $C_{28}H_{30}O_8$ based on a sodium adduct (+)-HRESIMS ion at *m/z* 517.1831 $[M + Na]^+$ (calcd. 517.1833), indicating 14 indices of hydrogen deficiency. The IR spectrum showed absorption bands at 1740 and 1631 $cm^{-1}$, indicative of the presence of carbonyl and olefinic groups, respectively. Apart from the easily distinguishable signals for a typical *β*-substituted furan ring ($\delta_H$ 7.61, br s; 7.45, t, $J = 1.6$ Hz; 6.47, d, $J = 1.8$ Hz), an acetyl group ($\delta_H$ 2.01, s), the $^{1}H$ NMR spectrum of **1** (Table 1**)** also presented two singlet methyl protons at $\delta_H$ 1.04 and 1.16. Additionally, the $^{13}C$ NMR spectrum (Table 2) of **1** showed one keto-carbonyl ($\delta_C$ 218.6), three ester carbonyls ($\delta_C$ 172.6, 169.9, and 169.5), and eight olefinic carbons ($\delta_C$ 143.6, 141.8, 137.4, 132.0, 131.8, 127.1, 120.0, and 109.8). These functionalities accounted for 8 indices of hydrogen deficiency, thus requiring six additional rings in the structure of **1**. The aforementioned data suggested that **1** was a hexacyclic limonoid [1]. The $^{1}H$ and $^{13}C$ NMR data of **1** showed high similarity to those of trichiconlide F [21], with the only difference being the replacement of the tigloyloxyl group at C-3 by an acetyl group, which was evidenced by the HMBC correlations from H-3/$H_3$-2′ to C-1′ (Fig. 2) and the molecular weights of **1**, showing 40 mass units less than that of trichiconlide F.

The above deduction and the relative configuration of **1** was verified by a single-crystal X-ray diffraction study by using Cu Kα radiation [Flack parameter of 0.25 (9)] (Fig. 3). To further clarify its absolute configuration, the X-ray geometry of **1** was directly used for ECD calculation. The theoretical calculated ECD data was in good agreement with the experimental spectra in the 190–400 nm region (Fig. 5). Thus, the absolute configuration of **1** was finally determined as 3*R*, 4*R*, 5*S*, 9*S*, 10*S*, 13*R*, 17*R* as shown in Fig. 1, and named as trichiconlide G.

Compound **2**, colorless powder, had a molecular formula of $C_{26}H_{30}O_{11}$ as deduced from the sodium adduct (+)-HRESIMS ion at *m/z* 541.1680 $[M + Na]^+$ (calcd. 541.1680). Its IR absorptions showed the presence of hydroxyl (3436 $cm^{-1}$), *γ*-lactone (1760 $cm^{-1}$), and carbonyl (1713 and 1631 $cm^{-1}$). The $^{13}C$ NMR data (Table 2) displayed 26 carbon resonances, which were further classified by the DEPT experiment as five methyls, three methylenes, seven methines, and eleven quaternary carbons, including one ketone ($\delta_C$ 214.5), three ester carbonyls ($\delta_C$ 175.4, 174.3, and 170.0), a *β*-substituted furan ring ($\delta_C$ 143.0, 140.2, 124.9, and 109.6), one methoxyl group ($\delta_C$ 53.1), three oxymethines ($\delta_C$ 90.1, 74.6, and 70.6), and two oxyquaternary carbons ($\delta_C$ 104.4 and 101.3). The NMR data of **2** showed similarities to those of trijugin F (**5**) [22], except for the presence of an additional hydroxy group at C-2 ($\delta_C$ 85.9) of **2**, which was confirmed by the HMBC correlations from H-1 to

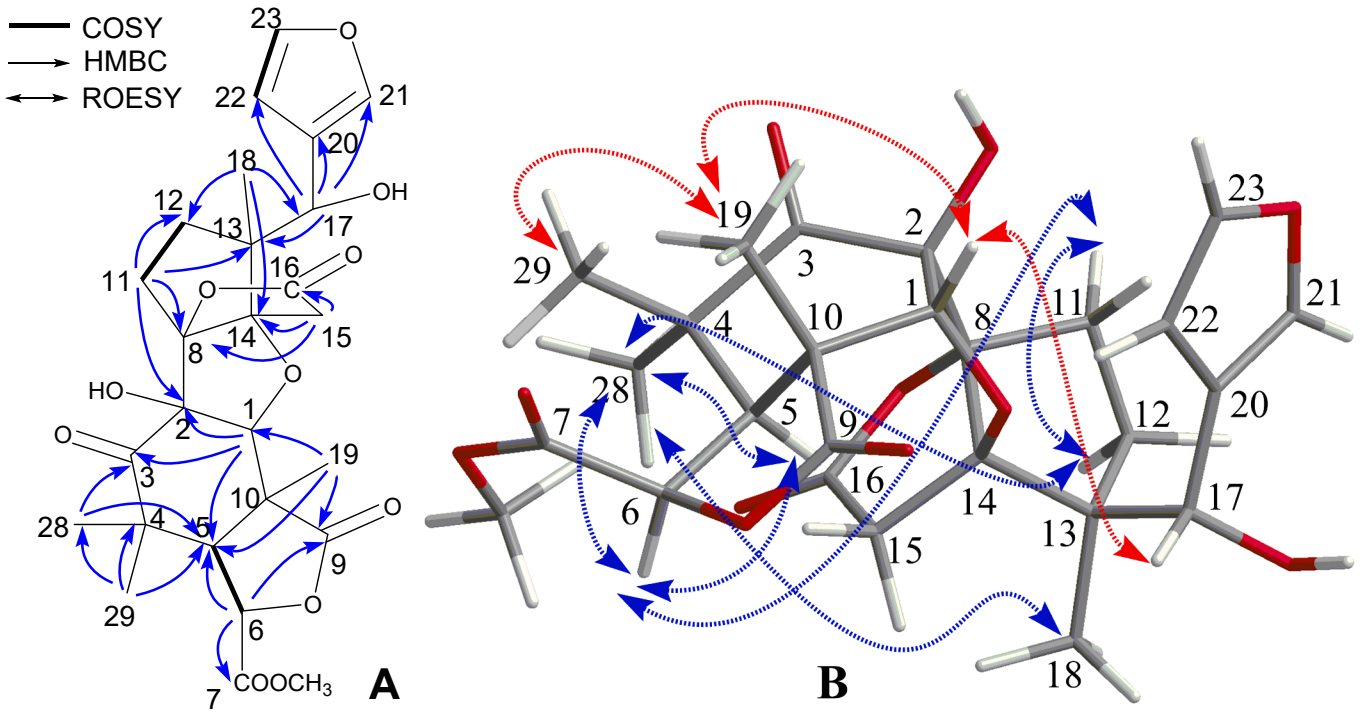



**Fig. 4.** $^{1}H—^{1}H$ COSY, selected HMBC (A) and ROESY (B) correlations of compound **2.**

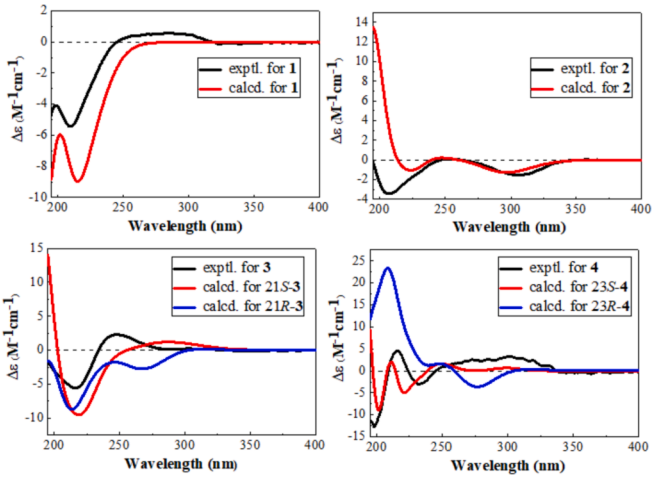



**Fig. 5.** Experimental and calculated ECD spectra of compounds **1**–**4.**

C-2 and from $H_2$-11 to C-2/C-8 (Fig. 4A). As a result, the carbon signals of C-1, C-2, C-3, and C-8 were shifted downfield by $\Delta\delta_C$ 5.2, 23.9, 5.1, and 2.4, respectively, due largely to the inductive effect of the 2-OH as compared with the proton in **2**. The relative configuration of **2** was established by ROESY correlations (Fig. 4B) and by comparing the NMR data with those of trijugin F [22]. The H-5 and H-6 protons were assigned in an $\alpha$-orientation by the ROESY correlation of H-5/H-6 and their coupling constants, which were closely comparable to those of trijugin F. Subsequently, Me-28 and Me-18 were fixed as $\alpha$-oriented by the ROESY correlations of H-5/$H_3$-28, H-6/$H_3$-28, H-6/H-11$\alpha$, H-11$\alpha$/12$\alpha$, H-12$\alpha$/$H_3$-28, and $H_3$-18/$H_3$-28. The ROESY correlations of H-1/H-17, H-1/$H_3$-19, and $H_3$-19/$H_3$-29 indicated H-1, H-17, Me-19, and Me-29 to be the $\beta$-oriented. The absolute configuration of **2** was determined by comparison of the calculated electronic circular dichroism (ECD) data with their experimental ones, in combination with biosynthetic considerations [22]. In the 190–400 nm region (Fig. 5), both the

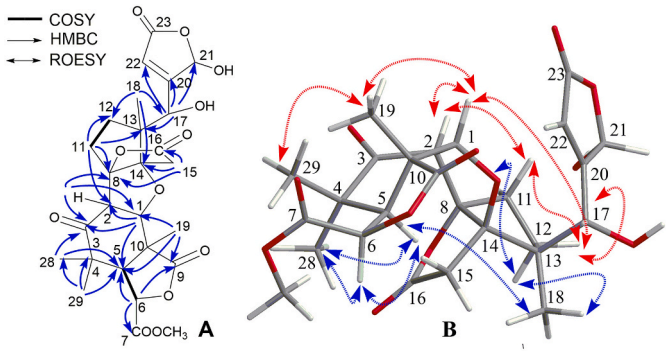



**Fig. 6.** $^{1}H—^{1}H$ COSY, key HMBC (A) and ROESY (B) correlations for compound **3.**

experimental ECD spectrum and the calculated one for **2** showed a negative first Cotton effect around 215 nm and the same trend for other parts. Therefore, qualitative analysis of the calculated and experimental

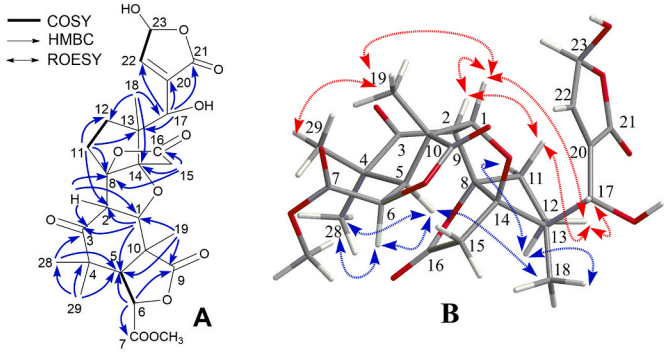
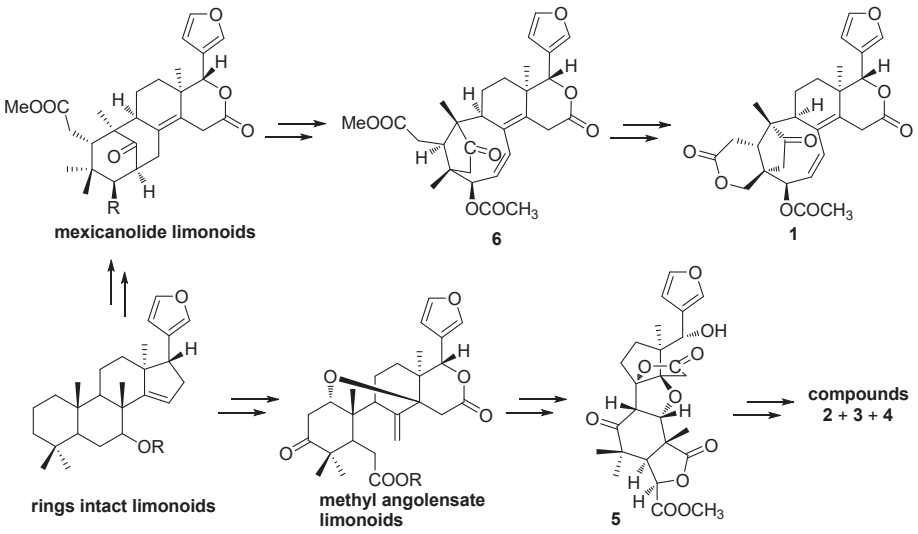



**Fig. 7.** $^{1}H—^{1}H$ COSY, key HMBC (A) and ROESY (B) correlations for compound **4.**




**Scheme 1.** Hypothetical biosynthetic pathway of **1**–**4**.

ECD spectra allowed the assignments of the absolute configuration of **2** as shown in Fig. 1, and compound **2** was named as 2-hydroxyltrijugin F.

Compounds **3** and **4** were a pair of isomer. They had the same molecular formulas of $C_{26}H_{30}O_{12}$ as determined by their respective (+)-HRESIMS ion at *m/z* 557.1627 $[M + Na]^+$ (calcd. 557.1629) and (−)-HRESIMS ion at *m/z* 533.1666 $[M - H]^-$ (calcd. 533.1665). Analyses of the NMR data (Tables 1 and 2) of **3** and **4** indicated that both compounds possessed very similar structures to that of trijugin F (**5**) [22] except for the C-17 substituent, where the presence of a 21-hydroxybutenolide group [$\delta_H$ 5.92 (H-21) and 6.14 (H-22); $\delta_C$ 170.5 (C-20), 98.6 (C-21), 120.8 (C-22), and 170.9 (C-23)] in **3** and a 23-hydroxybutenolide moiety [$\delta_H$ 7.18 (H-22) and 6.14 (H-23); $\delta_C$ 138.9 (C-20), 171.0 (C-21), 149.0 (C-22), and 98.2 (C-23)] in **4** replaced the *β*-substituted furanyl moiety in trijugin F (**5**), respectively. These assignments were confirmed by HMBC data (Fig. 6A and Fig. 7A). The relative configuration of the limonoid core in compounds **3** and **4** was identical to those of trijugin F (**5**) on the basis of $^1$H NMR data and a ROESY experiment (Fig. 6B and Fig. 7B), but the relative configuration of C-21 in **3** and C-23 in **4** could not be assigned *via* the available ROESY data. In the ROESY spectrum of **3** and **4**, the correlations of H-5/H-6, H-5/$H_3$-18, H-5/$H_3$-28, H-6/$H_3$-28, H-11*α*/H-12*α*, and H-12*α*/$H_3$-18 indicated that H-5, H-6, Me-28, and Me-18 were *α*-oriented. The ROESY correlations of H-1/H-2, H-1/H-12*β*, H-1/$H_3$-19, H-2/H-11*β*, H-11*β*/H-12*β*, H-12*β*/H-17, and $H_3$-19/$H_3$-29 revealed that H-1, H-2, Me-19, and Me-29 were *β*-oriented. To determine the configuration of C-21 in **3** and C-23 in **4**, the TD-DFT theory method at B3LYP/6-31g(d) level was adopted to calculate the theoretical CD spectrum of two tentative C-21 epimers (21*R*)-**3** and (21*S*)-**3** as well as two tentative C-23 epimers (23*R*)-**4** and (23*S*)-**4**. By comparing the experimental CD spectrum with the theoretically calculated CD curves (Fig. 5), the absolute configuration of **3** and **4** were tentatively assigned as 1*S*, 2*S*, 5*S*, 6*R*, 8*S*, 10*R*, 13*S*, 14*R*, 17*R*, 18*S*, 19*R*, 21*R* and 1*S*, 2*S*, 5*S*, 6*R*, 8*S*, 10*R*, 13*S*, 14*R*, 17*R*, 18*S*, 19*R*, 23*S*, respectively, as shown in Fig. 1, and compound **3** and **4** were named as 23-oxo-21-hydroxyltrijugin F and 21-oxo-23-hydroxyltrijugin F, respectively.

In addition to the four previously undescribed compounds (**1**–**4**), sixteen known analogues were identified as trijugin F (**5**) [22], andhraxylocarpin C (**6**) [23], trichiliton A (**7**) [24], 3*β*-acetoxycarapin (**8**) [25], $\Delta^{8,14}$-2-hydroxy-6-deoxyswietenine (**9**) [26], cipadesin N (**10**) [27], proceranolide (**11**) [28], khasenegasin L (**12**) [29], godavarin J (**13**) [30], 2*α*-hydroxymexicanlide (**14**) [31], methyl 3*β*-tigloyloxy-2-hydroxy-1-oxo-meliac-8(30)-enate (**15**) [32], trichanolide C (**16**) [12], andhraxylocarpin B (**17**) [23], 12-deacetoxyltrijugin A (**18**) [15], methyl 8*α*-hydroxy-8,30-dihydroangolensate (**19**) [22], trichilin B (**20**) [33] by comparing their spectroscopic data with those in the literatures.

**Table 3**
Effect of compounds **1**–**20** on LPS-induced NO production in RAW264.7 cells.[a,b]

| Compound | $IC_{50}$/μM | Compound | $IC_{50}$/μM | Compound | $IC_{50}$/μM |
|---|---|---|---|---|---|
| **1** | 48.64 ± 1.28 | **8** | >50 | **16** | 28.45 ± 2.51 |
| **2** | >50 | **9** | >50 | **17** | 22.66 ± 2.01 |
| **3** | 46.52 ± 1.83 | **10** | 41.70 ± 0.58 | **18** | >50 |
| **4** | >50 | **11** | >50 | **19** | >50 |
| **5** | >50 | **12** | N.T. | **20** | N.T. |
| **6** | >50 | **13** | N.T. | | |
| **7** | >50 | **14** | N.T. | | |
| **L-NMMA**[b] | 14.17 ± 1.08 | **15** | >50 | | |

[a] Data are represented as mean ± SEM; N.T.: Not tested.
[b] Positive controls.

The biosynthetic pathways for **1**–**4** are shown in Scheme 1. The precursors for **1** were considered to co-isolated mexicanolide-type limonoids [24], which were transformed to intermediate **6** by a series of oxidations and skeletal rearrangements process. Then, intermediate **6** would undergo hydrolysis, oxidation, and dehydration to afford compound **1**. Compound **5** might be biosynthetically derived from co-isolated methyl angolensate-class limonoids *via* a pinacol rearrangement [34], and then oxidation to afford compounds **2**–**4**.

In this study, all the isolates were evaluated for their cytotoxicity against five human tumor cell lines A-549, HepG2, 5-8F, Siha, and SCC-4 using the MTS method, but none of them were active at the concentration of 40 μM. Additionally, all the isolates, except for compounds **12**, **13**, **14**, and **20** (insufficient quantities), were evaluated for their anti-inflammatory effects in the LPS-induced RAW 264.7 cell line. Compounds **16** and **17** showed moderate anti-inflammatory activity with $IC_{50}$ values of 28.45 ± 2.51 and 22.66 ± 2.01 μM, respectively; Compounds **1**, **3**, and **10** exhibited weak anti-inflammatory activity with $IC_{50}$ values of 48.64 ± 1.28, 46.52 ± 1.83, and 41.70 ± 0.58 μM, respectively (Table 3).

## Declaration of competing interest

The authors declare no conflict of interest.

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*Declaration of Competing Interest*

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fitote.2021.105001.

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