Antibacterial Lignans and Triterpenoids from *Rostellularia procumbens*

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Abstract

One new lignan, rostellulin A (**1**), four known lignans, justin B (**2**), justicidin C (**3**), cilinaphthalide A (**4**), and justicidin A (**5**), and four known triterpenoids, ursolic acid (**6**), euscaphic acid (**7**), 2α-hydroxyursolic acid (**8**), and tormentic acid (**9**), have been isolated from the whole plants of *Rostellularia procumbens*. Their structures were established on the basis of spectral data, including extensive NMR experiments. To our knowledge, compounds **6–9** are known compounds but not previously isolated from *R. procumbens*, **4** was previously reported from other *Rostellularia* species. Antibacterial activities of **1–9** were evaluated against eight bacterial strains with the agar dilution method, and they were found to possess antimicrobial activity with MIC values in the range of 1.56–100 μg/mL. None of the lignans exhibited cytotoxic activity against HCT-8 and Bel-7402 cells at concentrations up to 5 μg/mL.

The widespread use of antibiotics in human diseases and agriculture leads to a serious problem of bacterial resistance [1]. Currently, there is a growing interest to search for antimicrobial substances from natural sources [2]. Traditional Chinese medicine, having been used for the treatment of infectious disorders for centuries, may serve as a rich source for new classes of antibiotics. *Rostellularia procumbens* (*Justicia procumbens*), belonging to the family of Acanthaceae, is a commonly used folk medicine for the treatment of lumbago, fever, cough, and especially for the treatment of infections of the urinary systems in China [3]. The abundance of lignans in the genus *Rostellularia* (*Justicia*) and their broad biological effects has made it a research topic of considerable interest in recent years. To date, a broad spectrum of bioactivities including cytotoxicity, antiplatelet activity, antiviral property, TNF-α inhibitory effect, and the apoptosis inducible effect of justicidin A have been well studied [4], [5], [6], [7], [8], [9], [10], [11], [12], [13]. However, so far there have been no attempts to study the antibacterial activities of chemical constituents in *R. procumbens*, although this herb is most impressive for its effectiveness in the treatment of infectious diseases [3]. In this paper, we describe the isolation, structural characterization of compound **1**, and antimicrobial activities of compounds **1–9** (Fig. **1**) from *R. procumbens*.

Rostellulin A (**1**) was obtained as a white amorphous powder, its molecular formula was assigned as $C_{25}H_{28}O_9$ by HR-ESI-MS (*m/z* = 495.1629 $[M+Na]^+$ calcd. for $C_{25}H_{28}O_9Na$: 495.1631) and NMR spectra. The ^{13}C-NMR and DEPT spectra showed 22 signals for 25 carbons, which were attributable to two methyl, two methoxy groups, four sp^3 methylenes, including a dioxymethylene (δ = 100.6) and two oxygen-bearing substituents (δ = 66.5, 63.7), three sp^3 methines, four sp^2 methines, eight quaternary olefinic carbons, and two carbonyl groups. These data suggested that **1** was an arylnaphthene-type lignan with one symmetrical unit in the structure. The symmetrical moiety was readily identified as a 4-hydroxy-3,5-dimethoxybenzene ring by the interpretation of the ^{1}H-NMR spectrum. ^{13}C-NMR data comparison between **1** and **2** revealed that a methylene (δ = 35.5) and a methine signal (δ = 122.3) in **2** were replaced by an sp^3 methine signal (δ = 48.2) and a quaternary carbon (δ = 128.6) in **1**. This difference implied that a ring was formed via a C-6 to C-7′ bond in **1**, which was evident from HMBC correlations of H-5 to C-7′, and H-7′ to C-6. The relative stereochemistry at C-7′, C-8′, and C-8 in **1** was determined by means of ROESY and selective 1D NOE irradiation experiments. ROESY interactions of H-7′ and H-8 indicated that these two protons are spatially vicinal. Upon irradiation of H-8′, no Overhauser effects were detected between the signals of H-7′ and H-8′, and H-8′ and H-8. In combination with ROESY interactions, this established the relative configurations for H-7′, H-8′, and H-8 to be R^*, S^*, and R^*, respectively. Hence, the structure of rostellulin A was deduced as shown (Fig. **1**).

By comparison of their spectral data with those reported in the literature, compounds **2–9** (Fig. **1**) were identified to be justin B (**2**) [10], justicidin C (**3**) [14], cilinaphthalide A (**4**) [15], justicidin A (**5**) [15], ursolic acid (**6**) [16], euscaphic acid (**7**) [17], 2α-hydroxyursolic acid (**8**) [17], and tormentic acid (**9**) [16]. To the best of our knowledge, compound **4** has been previously reported from other *Rostellularia* species, and compounds **6–9** were isolated from *R. procumbens* for the first time.

The antimicrobial properties of the isolated compounds were tested against eight disease-associated microorganisms. The results (Table **1**) showed that compound **8** was found to be most effective against *Staphylococcus epidermidis* with an MIC value of 1.56 μg/mL, and **7** inhibited the growth of *Salmonella typhimurium* with an MIC of 3.125 μg/mL, these data are comparable with those of cefradine. All tested bacteria were not sensitive to **2**, except for *Staphylococcus aureus* with an MIC of 12.50 μg/mL. In addition, the same MIC values of 12.50 μg/mL were observed for **3** against *Salmonella paratyphi* B, **4** against *Escherichia coli*, **8** against *Shigella flexneri* and *Salmonella typhimurium*. In addition, to observe the selectivity of the isolated compounds, the cytotoxic assay was performed with 5-FU (fluorouracil) as positive control (IC_{50} = 0.43 μg/mL and 0.37 μg/

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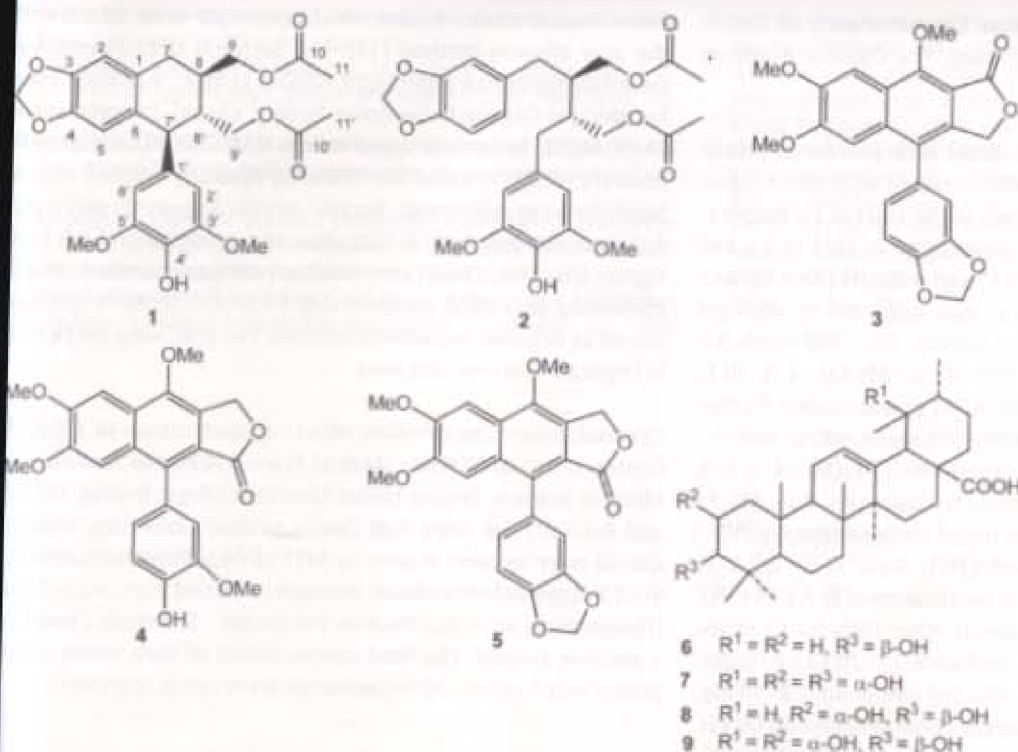

Fig. 1 The structures of compounds **1–9**.

Table 1 Antimicrobial activities of compounds **1–9** (MIC values, µg/mL)

Pathogen	*1*	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*Standards*[a]	
Reference strains											
S. aureus CMCC26001	> 100	12.5	> 100	25	50	50	50	100	25	15	7.5
E. coli CMCC44103	> 100	> 100	> 100	12.5	> 50	50	50	25	> 100	7.5	7.5
S. typhimurium CMCC80087	25	> 100	25	50	50	> 100	3.125	12.5	25	7.5	7.5
S. flexneri CMCC51335	100	> 100	25	50	100	100	> 100	12.5	> 100	3.25	3.25
Clinically isolated strains											
S. epidermidis	50	> 100	> 100	> 100	> 50	50	> 100	1.56	> 100	3.25	7.5
B. subtilis	> 100	> 100	50	> 50	25	> 100	> 100	>100	> 100	3.25	3.25
S. paratyphi A	> 100	> 100	25	> 50	> 50	> 100	> 100	> 100	25	3.25	3.25
S. paratyphi B	50	> 100	12.5	50	> 50	100	> 100	> 100	> 100	3.25	3.25

[a] Left column is for cefradine and right is for gentamycin.

mL for HCT-8 and Bel-7402 cells, respectively), all the lignans exhibited no cytotoxic effects against HCT-8 and Bel-7402 cells at concentrations up to 5 µg/mL. The isolation of antibacterial compounds from *R. procumbens* lends supports to the traditional use of this herb in infectious diseases. The identification of the antibacterial principles provides a rational basis for quality control of herbal remedies derived from *R. procumbens*.

Materials and Methods

General: Melting point was determined on an XRC-1 micromelting apparatus. Optical rotation was determined on a JASCO-20C digital polarimeter. UV spectrum was recorded on a Shimadzu UV-2401PC spectrophotometer. IR spectrum was obtained with a Bruker Tensor 27 FT-IR spectrophotometer with KBr pellets. ^{1}H-NMR (400 MHz) and ^{13}C-NMR (100 MHz) spectra were recorded at 300 K on a Bruker AM-400 spectrometer with TMS as an internal reference. ^{1}H-^{1}H COSY, HMQC, HMBC, and ROESY spectra were measured with a DRX-500 spectrometer. EI-MS (70 eV) were recorded on a VG Auto Spec-3000 spectrometer. HR-ESI-MS was carried our with an API QSTAR Pulsar 1 spectrometer. Silica gel (200–300 mesh and 10–40 µm) for column chromatography and GF_{254} for TLC were obtained from Qingdao Marine Chemical Factory (Qingdao, P. R. China). Sephadex LH-20 was obtained from Amersham Pharmacia Biotech (Upsala, Sweden). Fractions were monitored by TLC and spots were visualized after spraying with 10% H_2SO_4 in ethanol followed by heating.

Plant material: The whole plants of *R. procumbens* were collected in Malong, Yunnan Province, in July 2005. The plant material was identified by Prof. Heng Li, and a voucher specimen (No.

CHYX0354) is deposited at the State Key Laboratory of Phytochemistry, Kunming Institute of Botany, The Chinese Academy of Sciences.

Extraction and isolation: The air-dried and powdered whole plants of *R. procumbens* (17 kg) were extracted with 80% ethanol under reflux (2×25 L). After removal of the solvent by evaporation, the residues (1700 g) were suspended in H_2O (3 L), and then partitioned with EtOAc (3×3 L) and *n*-BuOH (3×3 L), successively. The EtOAc extract (160 g) was subjected to silica gel column chromatography (CC) (8.5×120 cm, 200–300 mesh, 3.0 kg) eluted with petroleum ether (60–90 °C)-Me_2CO (4:1, 20 L) to give four fractions (Frs. A - D). Fr. A (14 g) was further fractionated by silica gel CC (5×70 cm, 200–300 mesh, 600 g) with increasing polarity of petroleum ether (60–90 °C)-EtOAc (4:1, 3:1, 2:1, 1:1, 0:1, each 1.5 L) to yield three fractions (Frs. A1 - A3). Fr. A3 (5 g) was subjected to vacuum liquid chromatography (VLC) (3.5×10 cm, 10–40 μm) eluted with $CHCl_3$-Me_2CO (20:1, 2 L) to give four fractions A3.1 - A3.4. Final purification of fr. A3.1 by VLC (2×7 cm, 10–40 μm) with petroleum ether (60–90 °C)-EtOAc (5:1, 400 mL) as solvents, and Sephadex LH-20 (1.5×70 cm) with $CHCl_3$-MeOH (6:4, 200 mL) afforded compounds **2** (19 mg) and **3** (27 mg). Fr. A 3.3 was repeatedly chromatographed by VLC (2×5 cm, 10–40 μm) eluted with petroleum ether (60–90 °C)-Me_2CO (5:1, 200 mL) and Sephadex LH-20 (1.5×70 cm) with MeOH to yield compound **6** (10 mg). Fr. B (75 g) was subjected to silica gel CC (7.5×70 cm, 200–300 mesh, 1.5 kg) eluted with $CHCl_3$-MeOH-H_2O (80:18:2, 15 L) to yield six fractions (Frs. B1 - B6). Silica gel CC (5×70 cm, 200–300 mesh, 900 g) of fr. B1 (31 g) with gradient $CHCl_3$-Me_2CO as eluent (15:1 → 0:1, each 800 mL) to afford eight fractions B1.1 - B1.8. Compounds **1** (8 mg) and **4** (7 mg) were obtained from fr. B1.1 (500 mg) by VLC (2×5 cm, 10–40 μm) with $CHCl_3$-Me_2CO (15:1, 300 mL) and Sephadex LH-20 gel filtration with $CHCl_3$. Repeated chromatography of fr. B1.2 (700 mg) by VLC (2×7 cm, 10–40 μm) with $CHCl_3$-Me_2CO (10:1, 400 mL) and Sephadex LH-20 with $CHCl_3$-MeOH (6:4, 200 mL) afforded **5** (12 mg) and **7** (120 mg). From fr. B1.4 (3 g), compounds **8** (60 mg) and **9** (70 mg) were purified by VLC (3.5×10 cm, 10–40 μm) using increasing polarity of $CHCl_3$-Me_2CO (10:1 → 5:1, each 100 mL) followed by gel filtration on Sephadex LH-20 with MeOH as eluent.

Rostellulin A (**1**): $C_{25}H_{28}O_9$, white amorphous powder, m.p. 175–177 °C; *Rf* 0.58, silica gel GF_{254}, $CHCl_3$-Me_2CO (5:1); $[\alpha]_D^{19}$: +43.94 (*c* 0.11, $CHCl_3$); UV ($CHCl_3$): λ_{max} (log ε) = 241 (3.85), 293 (3.45) nm; IR (KBr): ν_{max} = 3441, 2923, 1729, 1609, 1484, 1262, 1240, 1116, 1038, 936 cm^{-1}; ^{1}H-NMR (400 MHz, $CDCl_3$): δ = 6.60 (1H, s, H-2), 6.22 (1H, s, H-5), 2.79 (2H, m, H-7), 2.24 (1H, m, H-8), 4.19 (1H, dd, *J* = 11.5, 3.5 Hz, H-9a), 4.04 (1H, m, H-9b), 2.0 (each 3H, each s, H-11 and H-11′), 6.31 (each 1H, each s, H-2′ and H-6′), 3.70 (1H, d, *J* = 10.5 Hz, H-7′), 1.97 (1H, m, H-8′), 4.05 (1H, dd, *J* = 11.5, 3.5 Hz, H-9′a), 3.98 (1H, dd, *J* = 11.5, 3.5 Hz, H-9′b), 5.80 (2H, s, OCH_2O), 3.80 (each 3H, each s, 2×OMe); ^{13}C-NMR (100 MHz, $CDCl_3$): δ = 132.4 (C-1), 107.9 (C-2), 146.0 (C-3), 145.8 (C-4), 109.3 (C-5), 128.6 (C-6), 32.8 (C-7), 35.5 (C-8), 66.5 (C-9), 170.8 (C-10 or C-10′), 20.8 (C-11, C-11′), 133.7 (C-1′), 106.0 (C-2′, C-6′), 147.2 (C-3′, C-5′), 134.8 (C-4′), 48.2 (C-7′), 43.5 (C-8′), 63.7 (C-9′), 170.9 (C-10′ or C-10), 100.6 (OCH_2O), 56.4 (2×OMe); HR-ESI-MS: *m/z* = 495.1629 $[M + Na]^+$ (calcd. for $C_{25}H_{28}O_9Na$: 495.1631).

Antimicrobial assay: Antibacterial properties were tested with the agar dilution method [18]. The bacterial strains employed were *Staphylococcus aureus* CMCC26001 (CMCC: National Center for Medical Culture Collections; Beijing, China), *Escherichia coli* CMCC44103, *Salmonella typhimurium* CMCC80087, and *Shigella flexneri* CMCC51335, and the following clinically isolated strains: *Staphylococcus epidermidis*, *Bacillus subtilis*, *Salmonella paratyphi*-A, *Salmonella paratyphi*-B. Cefradine and gentamycin (both from Sigma; Shanghai, China) were used as reference standards, plates containing only MHA medium and 1% DMSO in MHA medium served as negative and solvent controls. The tests were performed in triplicate and repeated once.

Cytotoxic assay: The cytotoxic effects against human HCT-8 (Cell Center, Institute of Basic Medical Science, Chinese Academy of Medical Science, Beijing Union Medical College, Beijing, China) and Bel-7402 cell lines (Cell Center, Wuhan University, Wuhan, China) were assayed *in vitro* by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] method [19], with 5-FU (fluorouracil; Shanghai Hualian Pharm. Inc.; Shanghai, China) as a positive control. The final concentration of each tested compound was 5 μg/mL. All experiments were run in triplicate.

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