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Anti-HIV lignans from *Justicia procumbens*

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[ABSTRACT] Twenty-one lignans including three new ones (**1**, **2** and **13**) were isolated from *Justicia procumbens*. The chemical structures of the new lignans were determined by spectroscopic means including 1D and 2D NMR analysis. These compounds were evaluated for their cytotoxic and anti-HIV activities. The new secoisolariciresinol dimethyl ether acetate (**13**) exhibited anti-HIV-1 activity with an IC_{50} value of 5.27 μmol·L^{-1} and a selective index (SI) value of 2.2. The known arylnaphthalene lignan procumbenoside A (**3**) and diphyllin (**8**) demonstrated inhibitory activity against HIV-1 with IC_{50} values of 4.95 (SI > 6.2) and 0.38 μmol·L^{-1} (SI = 5.3), respectively.

[KEY WORDS] *Justicia procumbens*; Antiviral plant; Lignans; Anti-HIV-1 activity

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Introduction

HIV is a virus that causes human acquired immunodeficiency syndrome disease (AIDS), which makes people vulnerable to infection and diseases. About 75 million people have acquired HIV infection and nearly half of them have died since the beginning of the epidemic [1]. There is still no vaccine to prevent HIV infection and the current drugs have no cure for HIV infected diseases. The combination antiretroviral therapy (ART) is the most popular treatment, which would sustain the lives of those infected with HIV as long as the patients take the prescribed drugs [2].

Natural products are known as one of the most important sources for discovery and development of new drugs. Many plant derived compounds have been reported with antiviral effects, and some of them have been investigated for their antiviral efficacy in animal studies and human clinical trials [3]. About 70% of approved antiviral small molecule drugs are derived originally from natural products during 1981 to 2014 [4]. In our previous studies, we have identified several potent anti-HIV-1 compounds from *Justicia* cf. *patentiflora* [5-6]. We further evaluated the anti-HIV activity of the extracts from several other plant species in *Justicia* genus. Among them, the methanol extract of the aerial parts of the plant showed potent inhibitory activity against HIV-1 with an IC_{50} value of 0.12 μg·mL^{-1} and low cytotoxicity with a CC_{50} value more than 20 μg·mL^{-1} using "One-Stone-Two-Birds" antiviral evaluation assay. *J. procumbens* is an annual plant that is widely distributed in southern regions of China, Japan, Vietnam, India and Australia. Its young leaves were reported as a famine food in Malaysia and India, and have been used as herbal tea ingredient in Taiwan [7]. The aerial parts of *J. procumbens* are also used as a popular traditional medicine in China for treatment of fever and inflammation [8]. During the previous phytochemical exploration of this plant, a number of

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Dedicated to Professor SUN Han-Dong on the Occasion of His 80th Birthday

arylnaphthalene lignans such as justicidins A and B have been obtained [9]. These lignans showed antiviral activity against vesicular stomatitis virus (VSV) with MIC values ranging from 0.06−0.25 μg·mL^{-1} and they showed low cytotoxicity against cultured RL-33 cells with MTC values of larger than 31 μg·mL^{-1} [9]. As our continuous efforts to discover antiviral agents from plants, 21 lignans including three new ones (**1**, **2** and **13**) were isolated from *J. procumbens* (Fig. 1). Their antiviral activity against HIV virus was determined by a safe and efficient "One-Stone-Two-Birds" assay protocol, which runs a parallel assay using H5N1 HA constructed HIV virions and VSV-G pseudotyped HIV virions. Herein we report the isolation, identification and biological evaluation of the anti-HIV lignan compounds from *J. procumbens*.

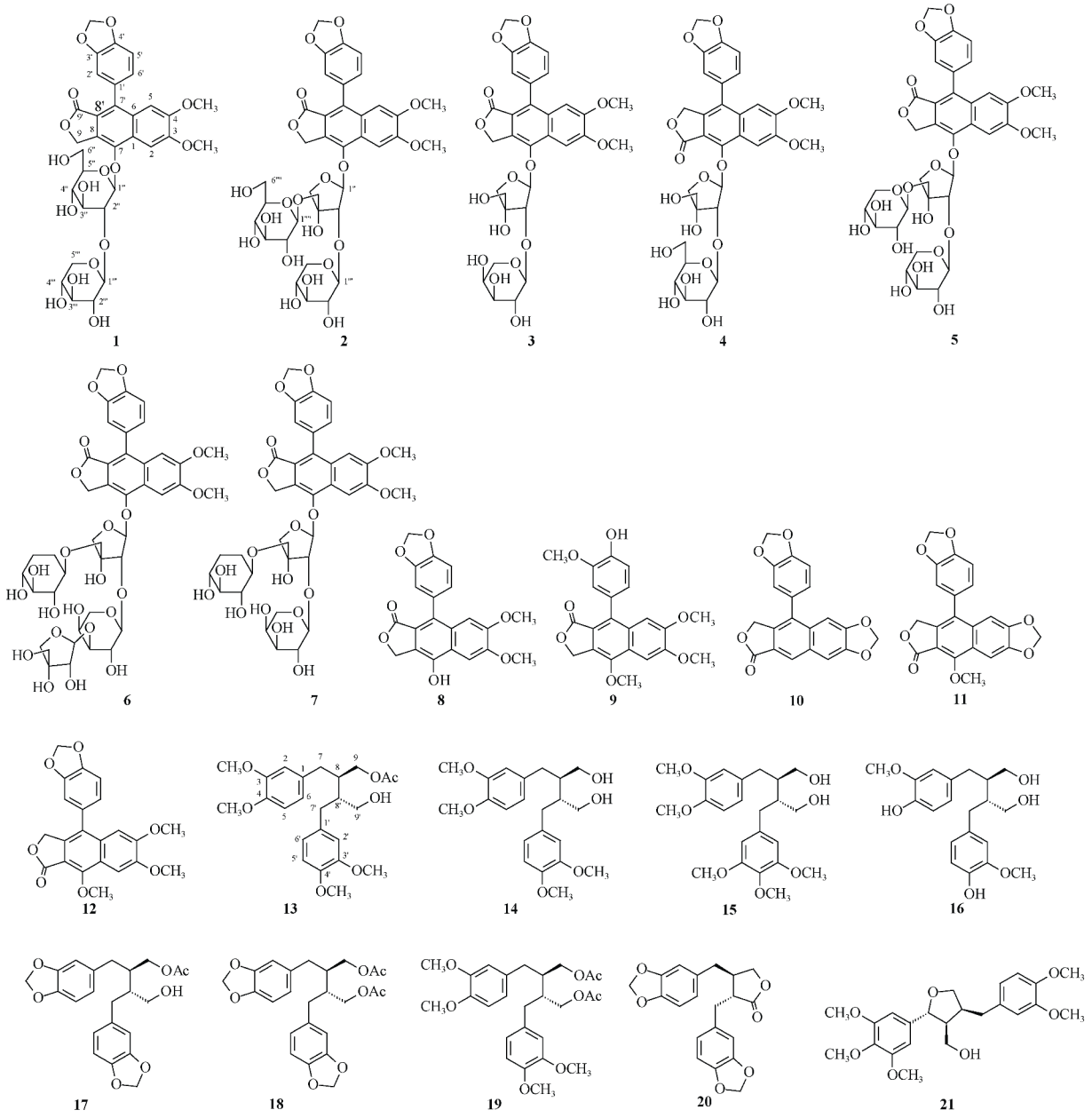

Fig. 1 Chemical structures of compounds 1–21

Results and Discussion

Compound **1** was obtained as a white amorphous powder. Its molecular formula $C_{32}H_{34}O_{16}$ was deduced from the positive HRESI-MS (*m/z* 675.1985 $[M + H]^+$), indicating 16 degrees of unsaturation. Its IR spectrum disclosed the present of hydroxyl (3503 cm^{-1}), lactone ester (1744 cm^{-1}) and aromatic ring (1618 and 1595 cm^{-1}) groups. The NMR spectra of **1** showed two anomeric proton signals at δ_H 5.00 (1H, d, J = 7.8 Hz, H-1″), 4.79 (1H, d, J = 7.6 Hz, H-1‴), and two anomeric carbon signals at δ_C 104.2 (CH, C-1″) and 107.5 (CH, C-1‴) (Table 1), indicating the presence of two sugar units, which

Table 1 ^{1}H (400 MHz) and ^{13}C (100 MHz) NMR data for compounds 1 and 2 (in CD_3OD, δ in ppm, *J* in Hz)

Position	1		2	
	δ_H (*J* in Hz)	δ_C, type	δ_H (*J* in Hz)	δ_C, type
1		131.9, C		130.1, C
2	8.04, s	103.0, CH	7.72, s	102.1, CH
3		153.4, C		153.4, C
4		151.7, C		151.8, C
5	7.04, s	107.0, CH	7.09, s	107.1, CH
6		128.6, C		128.0, C
7		146.0, C		146.2, C
8		132.2, C		131.8, C
9	5.49, d (14.8) 5.76, d (14.8)	69.5, CH_2	5.51, d (14.8) 5.62, d (14.8)	68.9, CH_2
1′		130.0, C		129.4, C
2′	6.78, d (1.2)	111.7, CH	6.82, d (1.6)	111.8, CH
3′		149.0, C		149.0, C
4′		149.0, C		149.0, C
5′	6.94, d (7.6)	109.0, CH	6.98, d (7.6)	109.0, C
6′	6.74, dd (1.2, 7.6)	124.7, CH	6.79, dd (1.6, 7.6)	124.8, CH
7′		137.5, C		136.7, C
8′		120.1, C		120.2, C
9′		172.3, C		172.2, C
1″	5.00, d (7.8)	104.2, CH	5.80, d (2.8)	111.0, CH
2″	3.84, m	84.9, CH	4.92, d (2.9)	86.6, CH
3″	3.70, m	78.2, CH		80.2, C
4″	3.48, m	70.9, CH	3.93, d (10.0) 4.29, d (10.0)	75.8, CH_2
5″	3.37, m	78.1, CH	3.79, d (10.5) 4.10, d (10.5)	71.8, CH_2
6″	3.86, m 3.71, m	62.4, CH_2		
1‴	4.79, d (7.6)	107.5, CH	4.74, d (7.2)	104.9, CH
2‴	3.33, m	76.1, CH	3.31, m	75.0, CH
3‴	3.28, m	78.3, CH	3.31, m	75.3, CH
4‴	3.48, m	71.2, CH	3.54, m	71.2, CH
5‴	3.86, m 3.22, m	67.5, CH_2	3.92, dd (5.6, 11.2) 3.27, m	67.1, CH_2
6‴				-
1⁗		-	4.37, d (7.6)	105.7, CH
2⁗		-	3.28, dd (7.6, 10.4)	78.1, CH
3⁗		-	3.37, dd (4.8, 10.4)	77.8, CH
4⁗		-	3.35, m	71.2, CH
5⁗		-	3.38, m	77.8, CH
6⁗		-	3.56, m 3.52, m	62.1, CH_2
3-OCH_3	4.05, s	57.2, CH_3	4.07, s	57.0, CH_3
4-OCH_3	3.71, s	56.0, CH_3	3.75, s	56.0, CH_3
3′-OCH_2O-4′	6.04, s 6.06, s	102.6, CH_2	6.06, s 6.08, s	102.6, CH_2

were assigned as *β*-glucopyranose and *β*-xylopyranose, respectively [10-12]. Apart from the anomeric carbon signals in each sugar unit, the other oxygenated carbons for *β*-glucopyranose were observed at δ_C 84.9 (CH, C-2"), 78.2 (CH, C-3"), 70.9 (CH, C-4"), 78.1 (CH, C-5") and 62.4 (CH_2, C-6"), and the other oxygenated carbons for *β*-xylopyranose were observed at δ_C 76.1 (CH, H-2‴), 78.3 (CH, C-3‴), 71.2 (CH, C-4‴) and 67.5 (CH_2, C-5‴). The remaining 21 carbon signals in **1** were assigned to an aglycone, which was determined to contain two methoxy groups (δ_C 57.2 and 56.0), one methylenedioxy group (δ_C 102.6), one oxygenated methylene (δ_C 69.5), one carboxyl carbon (δ_C 172.3) and 16 aromatic carbons (δ_C 103.0−153.4) by the analysis of the ^{13}C, DEPT and HSQC NMR spectral data of the compound. On the other hand, the ^{1}H NMR spectra of **1** displayed the proton signals of one 1, 3, 4-trisubstituted benzene ring [δ_H 6.74 (1H, dd, *J* = 7.6 and 1.2 Hz), 6.94 (1H, d, *J* = 7.6 Hz), and 6.78 (1H, d, *J* = 1.2 Hz)], two singlet aromatic protons [δ_H 8.04 and 7.04 (each 1H, s)] and one methylenedioxy group [δ_H 6.04 (1H, s), 6.06 (1H, s)]. The NMR chemical shift signals of the 21 carbons and their bearing protons were found to be very similar to those of diphyllin (**8**), which indicated that the aglycone of the lignan glycoside of **1** belongs to diphyllin. The observation of the distinguished downfield shift for the C-2" carbon signal of the *β*-glucopyranose (δ_C 84.9) and the presence of the HMBC correlations from H-1‴ to C-2" (Fig. 2) revealed that the *β*-xylopyranosyl residue in **1** was connected to C-2" of the *β*-glucopyranose. The *β*-glucopyranosyl residue was further assigned at C-7 of the diphyllin aglycone of **1** due to the presence of the HMBC correlations from H-1" to C-1. The identification of the two sugar units of **1** was confirmed by an acidic hydrolysis of the compound. Compound **1** (1 mg) was treated with 1N HCl for 2 h at 105 °C. The reaction mixture was neutralized with a diluted NaOH solution and partitioned with EtOAc. The aqueous portion was evaporated to obtain the mixture of the D-glucopyranose and D-xylopyranose, which were found to be identical to the respective authentic sugars by a thin layer chromatography (TLC) analysis. Thus, compound **1** was determined as 7-*O*-[*β*-D-xylopyranosyl-(1‴→2″)-*β*-D-glucopyranosyl]-diphyllin, and it has been given the trivial name procumbenoside N.

Compound **2**, isolated as a white amorphous powder, was determined to have a molecular formula of $C_{37}H_{42}O_{20}$ by the positive HRESI-MS (*m/z* 807.2413 $[M + H]^+$) and the analysis of the NMR data. Compound **2** showed similar NMR spectral data (Table 1) to **1** with the only difference on the sugar units. Compound **2** contained three sugar units, which were identified as *β*-apiofuranose, *β*-xylopyranose and *β*-gluopyranose by the presence of the three characteristic anomeric proton [δ_H 5.80 (1H, d, *J* = 2.8 Hz, H-1″), 4.74 (1H, d, *J* = 7.2 Hz, H-1‴) and 4.37 (1H, d, *J* = 7.6 Hz, H-1⁗), respectively] and the three anomeric carbon [δ_C 111.0 (C-1″), 104.9 (C-1‴) and 105.7 (C-1⁗), respectively] NMR signals [13-14]. The apiofuranosyl moiety was assigned to C-7 of the diphyllin aglycone by the presence

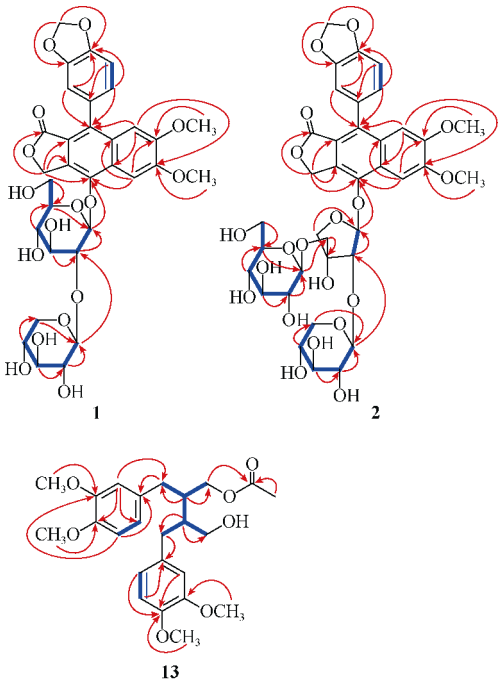

Fig. 2 Key COSY (— in blue bold bond) and HMBC (→ in red) correlations of compounds 1, 2 and 13

of the correlations between H-1″ (δ_H 5.80, 1H, d, J = 2.8 Hz, H-1″) and C-7 (δ_C 146.2) in the HMBC spectrum (Fig. 2). The xylopyranosyl moiety was linked to C-2″ of the apiofuranosyl by the presence of the HMBC correlation between H-1‴ (δ_H 4.74, 1H, d, J = 7.2 Hz, H-1‴) and C-2″ (δ_C 86.6), and the glucopyranosyl moiety was linked to C-5″ of apiofuranosyl by the presence of the HMBC correlation between H-1⁗ (δ_H 4.37, 1H, d, J = 7.6 Hz, H-1⁗) and C-5″ (δ_C 71.8). The identification of the three sugar units was confirmed by an acidic hydrolysis reaction of **1** to yield a mixture of *β*-apiofuranose, *β*-xylopyranose and *β*-gluopyranose, which were verified by comparison with the authentic sugars by TLC analysis. Consequently, compound **2** was identified as 7-*O*-[*β*-D-glucopyranosyl-(1⁗→5″)-*β*-D-xylopyranosyl-(1‴→2″)-*β*-D-apiofuranosyl]-diphyllin, and it is given the trivial name procumbenoside O.

Compound **13** was obtained as an amorphous powder and its molecular formula was revealed as $C_{24}H_{32}O_7$ by analysis of the HRESI-MS and NMR data. The NMR spectral data of the compound were highly similar to the known compound secoisolariciresinol dimethyl ether (**14**). Compound **13** was different from **14** only by the presence of an extra acetyl group. The presence of the HMBC correlation of H-9 to C-10 indicated the acetyl group linked to the C-9 as an ester (Fig. 2). Compound **13** was thus determined as secoisolariciresinol dimethyl ether acetate.

In addition to the new lignans (**1**, **2** and **13**), 18 known compounds (**3**−**12** and **14**−**21**) were determined by the direct comparison of their spectroscopic data with those reported in the literatures. These compounds were identified as procumbenoside A (**3**) [13], procumbenoside B (**4**) [14], procumbenoside E (**5**) [12], ciliatoside B (**6**) [15], ciliatoside A (**7**) [15], diphyllin (**8**) [16], cilinaphthalide A (**9**) [17], justicidin E (**10**) [16], justicidin D (**11**) [18], justicidin C (**12**) [18], secoisolariciresinol dimethyl ether (**14**) [19], 5-methoxy-4, 4′-di-*O*-methylsecolariciresinol (**15**) [19], secoisolariciresinol (**16**) [20], hemiariensin (**17**) [21], ariensin (**18**) [22], secoisolariciresinol dimethyl ether diacetate (**19**) [19], hinokinin (**20**) [17] and 5′-methoxy-4′-*O*-methyllariciresinol (**21**) [23], respectively.

All of the lignan isolates and the methanol extract of *J. procumbens* were evaluated for their antiviral activity using "One-Stone-Two-Birds" assay protocol with azidothymidine (AZT) as the positive control compound. Their cytotoxicities were also determined by MTS assay. The new compound secoisolariciresinol dimethyl ether acetate (**13**) and the known compound procumbenoside A (**3**) and diphyllin (**8**) exhibited anti-HIV-1 activity with IC_{50} values of 5.27 (SI 2.2), 4.95 (SI > 6.2) and 0.38 μmol·L^{-1} (SI 5.3), and cytotoxicity against A549 cells with CC_{50} values of 11.6, > 31.0 and 2.02 μmol·L^{-1}, respectively. Compounds **5** and **7** showed modest inhibitory activity against HIV-1 with IC_{50} values of 22.2 (SI > 1.1) and 23.6 μmol·L^{-1} (SI > 1.0), respectively. Compounds **10**, **19** and **20** displayed cytotoxicity with CC_{50} values of 9.2, > 28.2 and > 21.1 μmol·L^{-1} against A549 cells, respectively (Table 2). All the other isolated lignans showed neither anti-HIV-1 activity nor cytotoxicity at a concentration of 10 μg·mL^{-1} tested in A549 cell line.

Diphyllin (**8**), which was also reported as a potential antiviral agent against vesicular stomatitis virus (VSV) [9] and influenza virus [24], displayed the most inhibitory effect against HIV-1 virus. Among the 12 arylnaphthalene lignan isolates, although substitution of a sugar unit at C-7 is required for enhancement of the anti-HIV activity, which was reported by our previous work [5-6], all the presently isolated disaccharide, trisaccharide and tetrasaccharide glycosides displayed less anti-HIV-1 activity or cytotoxicity than diphyllin. The reduced anti-HIV activity of the diphyllin glycosides could be due to the larger sizes of the sugar moieties. Synthesis of a compound library of arylnaphthalene lignans, especially for those containing different types of sugar units, is thus necessary to reveal the structure-activity relationship (SAR) of this type of lignans. It will facilitate elucidation of the antiviral mechanism of action and identification of the antiviral molecular targets of arylnaphthalene lignans. In addition, since the isolated diphyllin in the present study showed weaker antiviral potency than the methanol extract (IC_{50} 0.12 μg·mL^{-1}, SI > 160), it is presumed highly potent antiviral compounds remain to be discovered in this plant.

Experimental

General experimental procedure

Optical rotations were measured with a Horiba SEPA-300 polarimeter (HORIBA Ltd., Kyoto, Japan). A Tenor 27 spectrophotometer was used for scanning IR spectroscopy with

KBr pellets (Bruker, Karlsruhe, Germany). ^{1}H (400 MHz) and ^{13}C NMR (100 MHz) spectra were recorded on a Bruker Ascend^{TM} instrument (Bruker, Karlsruhe, Germany). The HRESI-MS were performed on an Agilent Technologies 6540 time-of-flight mass spectrometer (Agilent Tchnologies, Inc., California, USA). Analytical HPLC was performed on an Agilent Technologies Series 1100 HPLC with DAD detector (Agilent Technologies, Inc., California, USA) equipped with an analytical Thermo-C_{18} (150 mm × 4.6 mm) column. Semi-preparative HPLC was performed on an Agilent Technologies Series 1100 HPLC with DAD detector equipped with a semi-preparative Alltima-C_{18} (5 μm; 250 mm × 10 mm) column. Preparative HPLC was performed on a Waters Preparative HPLC 2545 System (Waters, Massachusetts, USA) with a UV detector equipped with a Phenomenex LUNA-C_{18} column (120 Å, 12 μm; 250 mm × 50 mm) column. Column chromatography was performed with silica gel (100−230 mesh, DAVISIL; 230−400 mesh, Merck, New Jersey, USA). Reversed-phase flash chromatography was accomplished with RP-18 silica gel (40−63 μm, Merck, New Jersey, USA). Fractions were monitored by TLC using silica gel 60 F_{254} TLC sheets (Merck, New Jersey, USA), and spots were visualized by heating Si gel plates sprayed with 5% H_2SO_4 in EtOH. Polyethylenimine (PEI) was purchased from Invitrogen. Fetal bovine serum (FBS) was purchased from Life Technologies. Human embryonic kidney 293T cells and human lung epithelia A549 cancer cells were purchased from American Type Culture Collection (ATCC). Influenza A virus (H5N1) hemagglutinin (HA) plasmid, influenza A virus (H1N1) neuraminidase (NA) plasmid and pHEF-VSV-G (vesicular stomatitis virus glycoprotein [VSV-G]) plasmid were obtained from Prof. RONG Li-Jun, University of Illinois at Chicago, Illinois, USA. ELISA plate reader was provided by Bio-Rad, Hercules, Califonia, USA. The HIV vector pNL4-3.Luc. R-E was obtained through the AIDS Research and Reference Reagent Program (Division of AIDS, NIAID, NIH). Other chemicals and reagents used in the bioassay were commercially available.

Plant material

The aerial parts of *J. procumbens* were collected in Ningde City of Fujian Province, China, in July 2013. The identification was conducted by Prof. CHEN Hu-Biao in School of Chinese Medicine, Hong Kong Baptist University. A voucher specimen (SHA00026) is momentarily available for inspection at the Quality Research Laboratory/Phytochemistry Laboratory, School of Chinese Medicine, Hong Kong Baptist University.

Extraction and isolation

The air-dried aerial parts of the plant materials (18.0 kg) were exhaustively extracted with MeOH for 4 times (4 × 60 L) at room temperature (12 h each time) and filtered to yield a filtrate. Concentration of the filtrate under vacuum gave a brown residues, which were dissolved in H_2O, followed by successive partitions of petroleum ether (4 × 5 L), EtOAc (4 × 5 L) and *n*-BuOH (4 × 5 L) to afford petroleum ether-soluble, EtOAc-soluble, *n*-BuOH-soluble and H_2O-soluble extracts after dryness *in vacuo*. The EtOAc soluble fraction (154.0 g), which demonstrated the most potent anti-HIV-1 activity among the four portions, was chromatographed over a silica gel column (100−230 mesh; 10 cm × 150 cm), eluting with gradient petroleum ether/Me_2CO (8 : 1, 10 L; 4 : 1, 10 L; 3 : 1, 10 L; 1 : 1, 10 L), followed by CH_2Cl_2/MeOH (8 : 2, 10 L; 7 : 3, 10 L; 0 : 10 10 L) solutions to afford 140 fractions (F1−F140). The combined fractions F92−F126 (47.8 g) were chromatographed over a silica gel column (100−230 mesh; 5 cm × 100 cm), eluting with gradient CH_2Cl_2/EtOAc (9 : 1, 5 L; 8 : 2, 5 L; 7 : 3, 5 L), followed by CH_2Cl_2/MeOH (8 : 2, 5 L; 8 : 2, 5 L; 7 : 3, 5L; 0 : 10, 5L) solutions to afford 50 sub-fractions (SFI1−SFI50). The combined sub-fractions SFI 22−45 (40.6 g) were subjected to a RP-18 silica gel column (40−63 μm; 3.5 cm × 50 cm), eluting with MeOH/H_2O (8 : 2) solvent system to yield fraction SFI51 and SFI52. Absorbed on silica gel (230−400 mesh), SFI51 (11.4 g) was subjected to a silica gel column (230−400 mesh; 5 cm × 100 cm) separation, eluting with gradient CH_2Cl_2/MeOH (95 : 5, 2 L; 90 : 10,

Table 2 Inhibitory activity of the extract and compounds against HIV-1

Compound	IC_{50} ± SD (μmol·L^{-1})[a]	CC_{50} ± SD (μmol·L^{-1})[b]	SI[c]
JPM[d]	0.12 ± 0.04	> 20	> 160
3	4.95 ± 0.26	> 31.0	> 6.2
5	22.2 ± 1.5	> 25.8	> 1.1
7	23.6 ± 1.4	> 25.8	> 1.0
8	0.38 ± 0.03	2.02 ± 0.03	5.3
10	cytotoxic[e]	9.2 ± 0.2	-
13	5.27 ± 0.03	11.6 ± 0.2	2.2
19	cytotoxic[e]	> 28.2	-
20	cytotoxic[e]	> 21.1	-
AZT	0.0031 ± 0.0004	> 10	> 3200
paclitaxel	-	0.0042 ± 0.0019	-

[a] Inhibitory activity against HIV-1 pseudovirus (HIV/HA); [b] Cytotoxic activity against A549 cells; [c] Selective Index (= CC_{50}/IC_{50}); [d] MeOH extract of the aerial parts of *J. procumbens*, and the activity data are expressed in μg·mL^{-1}; [e] These compounds displayed cytotoxicity at a concentration of 10 μg·mL^{-1}

2 L; 85 : 15, 2 L; 80 : 20, 2 L; 75 : 25, 2 L; 70 : 30, 2 L; 60 : 40, 2 L; 0 : 100, 2 L) to give fractions SFI53−SFI78. SFI57 (0.7 g), 59 (1.5 g), 60 (1.3 g), 65 (0.1 g) and 66 (1.3 g) were subjected to a RP-18 silica gel column (40−63 μm; 3.5 cm × 50 cm) separation, eluting with gradient MeOH/H_2O (4 : 6, 0.5 L; 5 : 5, 0.5 L; 6 : 4, 0.5 L; 7 : 3, 0.5 L; 1 : 0, 1 L) solutions to afford fractions SFI79−SFI83, SFI84−SFI88, SFI89−SFI93, SFI94−SFI98 and SFI99−SFI103, respectively. SFI81, 86, 87, 92, 97 and 101 were further subjected to preparative HPLC separation on the Phenomenex LUNA-C_{18} column (12 μm; 250 mm × 50 mm), respectively, eluting with an isocratic MeCN/H_2O (3 : 7) at a flow rate of 20 mL·min^{-1} to obtain compounds **1** (3.1 mg), **2** (11.7 mg), **3** (6.5 mg), **4** (1.0 mg), **5** (10.0 mg), **6** (3.5 mg), **7** (2.5 mg), **8** (1.1 mg) and **15** (9.1 mg). The combined fractions F50−F91 (40.8 g) were separated by a silica gel (100−230 mesh; 5 cm × 100 cm) column, eluting with the gradient CH_2Cl_2/EtOAc (9 : 1, 5 L; 9 : 1, 5 L; 8 : 2, 5 L) to afford sub-fractions SFII1−SFII46. The combined sub-fractions SFII43−SFII46 (15.8 g) were separated by a RP-18 silica gel column (40−63 μm; 3.5 cm × 50 cm), eluting with an MeOH/H_2O (8 : 2) solvent system to yield fraction SFII47, which was further subjected to the same RP-18 silica gel column (40−63 μm; 3.5 cm × 50 cm), eluting with gradient MeOH/H_2O (6 : 4, 1 L; 5 : 5, 1 L; 4 : 6, 1 L; 3 : 7, 1 L; 0 : 1, 2 L) solutions to yield fractions SFII48−SFII52. SFII48−SFII51 were further subjected to preparative HPLC separation on the Phenomenex LUNA-C_{18} column (12 μm; 250 mm × 50 mm), respectively, eluting with an isocratic MeCN/H_2O (3 : 7) at a flow rate of 20 mL·min^{-1} to obtain compounds **13** (6.5 mg), **14** (22.9 mg), **16** (8.9 mg) and **21** (3.2 mg). The combined fractions F16−F49 (17.7 g) were subjected to a RP-18 silica gel column (40−63 μm; 3.5 cm × 50 cm), eluting with an MeOH/H_2O (8 : 2) solvent system to yield fraction SFIII1, which was further subjected to the same RP-18 silica gel column (40−63 μm; 3.5 cm × 50 cm), eluting with gradient MeOH/H_2O (6 : 4, 1 L; 5 : 5, 1 L; 4 : 6 1 L; 3 : 7 1 L; 0 : 1, 2 L) solutions to yield fractions SFIII2−SFIII11. SFII5−SFII9 were subjected to preparative HPLC separation on the Phenomenex LUNA-C_{18} column (12 μm; 250 mm × 50 mm), respectively, eluting with an isocratic MeCN/H_2O (5 : 4) at a flow rate of 20 mL·min^{-1} to obtain compounds **9** (6.4 mg), **10** (3.4 mg), **11** (1.7 mg), **12** (11.4 mg), **17** (5.3 mg), **18** (6.9 mg), **19** (7.4 mg) and **20** (7.2 mg).

Procumbenoside N (**1**). White powder; $[\alpha]_D^{20}$ +9.1° (*c* 0.12, MeOH); UV (MeOH) λ_{max} (log ε) 201.4 (3.74), 225.2 (3.65), 261.8 (4.43), 294.7 (3.32), 314. 8 (3.52), 354.6 (3.09) nm; IR (film) ν_{max} 3503, 2891, 1744, 1618, 1508, 1480, 1458, 1437, 1396, 1341, 1262, 1230, 1127, 1062, 1037, 934, 853, 770 cm^{-1}; ^{1}H and ^{13}C NMR data, see Table 1; HRESI-MS *m/z* 675.1985 $[M + H]^+$ (Calcd. for $C_{32}H_{35}O_{16}$ 675.1925).

Procumbenoside O (**2**). White powder; $[\alpha]_D^{20}$ −82.4° (*c* 0.12, MeOH); UV (MeOH) λ_{max} (log ε) 201.6 (3.53), 225.4 (3.32), 261.7 (4.21), 294.1 (3.23), 315.2 (3.26), 356.1 (3.10) nm; IR (film) ν_{max} 3503, 2894, 1744, 1618, 1508, 1480, 1459, 1437, 1397, 1341, 1262, 1231, 1127, 1062, 1039, 934, 853, 770 cm^{-1}; ^{1}H and ^{13}C NMR data, see Table 1; HRESI-MS *m/z* 807.2413 $[M + H]^+$ (Calcd. for $C_{37}H_{43}O_{20}$, 807.2348).

Secoisolariciresinol dimethyl ether acetate (**13**). Amorphous powder; ^{1}H NMR (in CD_3OD, 400 MHz): δ 6.81 (1H, d, *J* = 8.6 Hz, H-2′), 6.80 (1H, *d*, *J* = 8.1 Hz, H-2), 6.58−6.65 (4H, m, H-5/6/5/6′), 4.23 (1H, dd, *J* = 6.0, 11.2 Hz, H-9*α*), 3.99 (1H, dd, *J* = 6.6, 11.2 Hz, H-9*β*), 3.79−3.80 (6H, s, 3/3′-OCH_3), 3.70−3.71 (6H, s, 4/4′-OCH_3), 3.68 (1H, dd, *J* = 6.0, 10.9 Hz, H-9′*α*), 3.51 (1H, dd, *J* = 6.5, 10.9 Hz, H-9′*β*), 2.55−2.70 (4H, m, H-7/7′), 2.16 (1H, m, H-8), 2.03 (3H, s, Ac-CH_3), 1.92 (1H, m, H-8′); ^{13}C NMR (in CD_3OD, 100 MHz): δ 172.9 (C, Ac-CO), 150.3 (C, C-4/4′), 148.7/148.6 (C, C-3/3′), 135.1 (C, C-1), 134.6 (C, C-1′), 122.5 (CH, C-6/6′), 113.7/113.6 (CH, C-5/5′), 112.9/112.8 (CH, C-2/2′), 65.9 (CH_2, C-9), 62.6 (CH_2, C-9′), 56.5 (CH_3, 3/3′-OCH_3), 56.2 (CH_3, 4/4′-OCH_3), 44.3 (CH, C-8), 40.5 (CH, C-8′), 35.8 (CH_2, C-7), 35.5 (CH_2, C-7′), 20.9 (CH_3, Ac-CH_3); HRESI-MS *m/z* 455.2072 $[M + Na]^+$ (Calcd. for $C_{24}H_{32}O_7Na$, 455.2046).

"One-Stone-Two-Birds" antiviral evaluation assay

HIV/VSV-G or HIV/HA virions were prepared as described previously [5, 25]. The virions were generated, respectively, by co-transfecting with either 0.5 μg VSV-G envelope expression plasmid or 0.5 μg hemagglutinin (HA) envelope expression plasmid with 0.5 μg neuraminidase (NA) expression plasmid and 2 μg replication-defective HIV vector (pNL4-3-Luc-RE) into human embryonic kidney 293T cells (90% confluent) in six-well plates *via* PEI (Invitrogen). The HIV vector pNL4-3.Luc. R.E. was obtained through the AIDS Research and Reference Reagent Program (Division of AIDS, NIAID, NIH).

The HIV-1 vector pNL4-3.Luc. R.E. was co-transfected with the H5N1 HA and NA constructs to generate HIV virions with bird flu HA on the viral surface (HIV/HA). This pNL4-3 was derived from an infectious molecular clone of SI, T-tropic virus, and is replication deficient since the HIV is Env^- and Vpr^-. Also the luciferase gene (LUC) carried by this recombinant HIV vector serves as the reporter for HIV replication. The evaluation principle is that the level of the luciferase activity in the cells should be proportional to the level of viral entry and replication. If a compound (or fraction) can interfere with HIV replication/or HA-mediated viral entry, the level of the luciferase activity in the infected cells will be reduced. Thus, using this protocol, we were able to identify fractions or compounds capable of inhibiting HIV replication.

Target A549 human lung cells were seeded at 1 × 10^4 cells per well (96-well plate) in complete DMEM and incubated at 37 °C, 5% CO_2, with high humidity. Twenty-four hours later, 10 μL sample with serial concentrations in 10% aqueous DMSO and 190 μL of the pseudovirus with target cells were incubated at the final concentrations of 10, 5, 2.5, 1.25, 0.625 and 0.3125 μmol·L^{-1}. Twenty-four hours

post-infection, all media containing sample and virus were removed from target cells and replaced with fresh and complete DMEM. Forty-eight hours post-infection, 50 μL of neolite luciferase substrate (PerkinElmer, Waltham, Massachusetts, USA) was added to each well and plates were incubated at room temperature for 5 to 10 min. The experiment was repeated three times. Luciferase activity was measured by an EnVision plate reader (PerkinElmer). The infection rate was measured as relative light units (RLUs) in the infected cells. Virus alone with DMSO was used as the negative control; and virus with azidothymidine (AZT, ≥ 98%, HPLC, Sigma-Aldrich Chemical Co., St Louis, Missouri, USA) was used as positive control.

Cytotoxicity assay

Cytotoxicity was determined by MTS assay with a slight modification [5]. Approximately 0.5×10^4/well A549 human lung cells were seeded in a 96-well tissue culture plate in DMEM supplemented with FBS and penicillin/streptomycin (day 0). DMSO (5 μL) alone or compounds in 10% aqueous DMSO were added to the 95 μL cell suspension with cells on the following day with the final concentrations of 10, 5, 2.5, 1.25, 0.625 and 0.3125 μmol·L^{-1} and incubated at 37 °C in 5% CO_2 for 24 h. After 24 h, all media were removed and replaced with 100 μL fresh complete DMEM. After 48 h post initial addition of DMSO or compound, 20 μL of CellTiter 96 Aqueous One Solution was added per well. After gentle mixing, plates were incubated at 37 °C in 5% CO_2 for 4 h. 25 μL of a 10% SDS solution was added per well and plates were stored at room temperature for approximately 12 h. Absorbance was measured at 450 nm using a plate reader. Paclitaxel was used as positive control. The experiment was repeated three times. Percentage growth inhibition was calculated as: [*OD* (cells + samples) – *OD* (day 0 only cells)]/[*OD* (cells + 10% DMSO) – *OD* (day 0 only cells)] = % survival, cytotoxicity = 1 – % survival.

Data analysis

Data represent the results of three independent experiments. Standard deviation was calculated using Microsoft Excel. The IC_{50} and CC_{50} values of the results were performed using GraphPad Prism version 6 (GraphPad Software, San Diego, CA, USA).

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References

[1] Deeks SG, Lewin SR, Bekker LG. The end of HIV: still a very long way to go, but progress continues [J]. *PLoS Med*, 2017, **14(11)**: e1002466.

[2] Williams BG, Granich R. Ending AIDS: myth or reality [J]. *Lancet*, 2017, **390**(10092): 357.

[3] Martin KM, Ernst E. Antiviral agents from plants and herbs: a systematic review [J]. *Antivir Ther*, 2003, **8**(2): 77-90.

[4] Newman DJ, Cragg GM. Natural products as sources of new drugs from 1981 to 2014 [J]. *J Nat Prod*, 2016, **79**(3): 629-661.

[5] Zhang HJ, Rumschlag-Booms E, Guan YF, *et al*. Potent inhibitor to drug-resistant HIV-1 strains identified from the medicinal plant *Justicia gendarussa* [J]. *J Nat Prod*, 2017, **80**(6): 1798-1807.

[6] Zhang HJ, Rumschlag-Booms E, Guan YF, *et al*. Anti-HIV diphyllin glycosides from *Justicia gendarussa* [J]. *Phytochemistry*, 2017, **136**: 94-100.

[7] Tanaka T. *Tanaka's Cyclopedia of Edible Plants of the World* [M]. Keigaku Publishing Company, Tokyo, 1976: 634.

[8] Yang MH, Wu J, Cheng F, *et al*. Complete assignments of ^{1}H and ^{13}C NMR data for seven arylnaphthalide lignans from *Justicia procumbens* [J]. *Magn Reson Chem*, 2006, **44**(7): 727-730.

[9] Asano J, Chiba K, Tada M, *et al*. Antiviral activity of lignans and their glycosides from *Justicia procumbens* [J]. *Phytochemistry*, 1996, **42**(3): 713-717.

[10] Qin HL, Yu DQ, Cong PZ. *^{13}C NMR Spectra of Natural Organic Products* [M]. Chemical Industry Press, Beijing, 2011.

[11] Cabrita L, Andersen OM. Anthocyanins in blue berries of *Vaccinium padifolium* [J]. *Phytochemistry*, 1999, **52**(8): 1693-1696.

[12] Wu WW, Miao LP, Wang XJ, *et al*. A new lignan glycoside procumbenoside E from *Justicia procumbens* L. [J]. *Chin J Pharm*, 2012, **43**: 669-672.

[13] Day SH, Lin YC, Tsai ML, *et al*. Potent cytotoxic lignans from *Justicia procumbens* and their effects on nitric oxide and tumor necrosis factor-*α* production in mouse macrophages [J]. *J Nat Prod*, 2002, **65**(3): 379-381.

[14] Weng JR, Ko HH, Yeh TL, *et al*. Two new arylnaphthalide lignans and antiplatelet constituents from *Justicia procumbens* [J]. *Arch Pharm (Weinheim)*, 2004, **337**(4): 207-212.

[15] Day SH, Chiu NY, Tsao LT, *et al*. New lignan glycosides with potent antiinflammatory effect, isolated from *Justicia ciliate* [J]. *J Nat Prod*, 2000, **63**(11): 1560-1562.

[16] Fukamiya N, Lee KH, Antitumor agents, 81. Justicidin-A and diphyllin, two cytotoxic principles from *Justicia procumbens* [J]. *J Nat Prod*, 1986, **49**(2): 348-350.

[17] Day SH, Chiu NY, Won SJ, *et al*. Cytotoxic lignans of *Justicia ciliate* [J]. *J Nat Prod*, 1999, **62**(7): 1056-1058.

[18] Ohta K, Munakata K. Justicidin C and D, the 1-methoxy-2, 3-naphthalide lignans, isolated from *Justicia procumbens* [J]. *Tetrahedron Lett*, 1970, **11**: 923-925.

[19] Chen CC, Hsin WC, Huang YL. Six new diarylbutane lignans from *Justicia procumbens* [J]. *J Nat Prod*, 1998, **61**(2): 227-229.

[20] Liu B, Liu MT, Gan ML, *et al*. Chemical constituents from roots of *Machilus yaoshansis* [J]. *China J Chin Mat Med*, 2012, **37**(9): 1227-1231.

[21] Badheda LP, Prabhu BR, Mulchandani NB. Lignans from *Piper cubeba*. Part 3. Lignans of *Piper cubeba* [J]. *Phytochemistry*, 1987, **26**: 2033-2036.

[22] Hernandez JD, Roman LU, Espineira J, *et al*. Ariensin, a new lignan from *Bursera ariensis* [J]. *Planta Med*, 1983, **47**(4): 215-217.

[23] Kato MJ, Yoshida M, Gottlieb OR. Lignoids and arylalkanones

from fruits of *Virola elongate* [J]. *Phytochemistry*, 1990, **29**: 1799-1810.

[24] Chen HW, Cheng JX, Liu MT, e*t al*. Inhibitory and combinatorial effect of diphyllin, a v-ATPase blocker, on influenza viruses [J]. *Antiviral Res*, 2013, **99**(3): 371-382.

[25] Li WF, Wang J, Zhang JJ, *et al*. Henrin A: a new anti-HIV *ent*-kaurane diterpene from *Pteris henryi* [J]. *Int J Mol Sci*, 2015, **16**(11): 27978-27987.

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