

See discussions, stats, and author profiles for this publication at: <https://www.researchgate.net/publication/312563384>

Anti-HIV diphyllin glycosides from *Justicia gendarussa*

Article in *Phytochemistry* · January 2017

DOI: 10.1016/j.phytochem.2017.01.005

CITATIONS

0

READS

77

11 authors, including:



[Hong-Jie Zhang](#)

Chinese Academy of Sciences

162 PUBLICATIONS 5,028 CITATIONS

[SEE PROFILE](#)



[Dongying Wang](#)

Henan University of Technology

12 PUBLICATIONS 155 CITATIONS

[SEE PROFILE](#)



[Manh Nguyen](#)

Nha Trang University

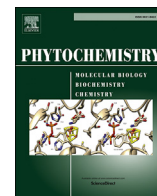
95 PUBLICATIONS 2,093 CITATIONS

[SEE PROFILE](#)

Some of the authors of this publication are also working on these related projects:



N/A, N/A, N/A [View project](#)



Anti-HIV diphyllin glycosides from *Justicia gendarussa*

Hong-Jie Zhang^{a,*}, Emily Rumschlag-Booms^{b,1,2}, Yi-Fu Guan^a, Kang-Lun Liu^a,
Dong-Ying Wang^a, Wan-Fei Li^a, Van Hung Nguyen^c, Nguyen Manh Cuong^d,
Djaja Doel Soejarto^e, Harry H.S. Fong^e, Lijun Rong^{b,**}

^a School of Chinese Medicine, Hong Kong Baptist University, 7 Baptist University Road, Kowloon Tong, Hong Kong Special Administrative Region

^b Department of Microbiology and Immunology, College of Medicine, University of Illinois at Chicago, 835 South Wolcott Avenue, Chicago, IL 60612, USA

^c Institute of Marine Biochemistry of the Vietnam Academy of Science and Technology (VAST), 18 Hoang Quoc Viet road, Cau Giay, Hanoi, Viet Nam

^d Cuc Phuong National Park, Nho Quan, Ninh Binh, Viet Nam

^e Department of Medicinal Chemistry and Pharmacognosy, College of Pharmacy, University of Illinois at Chicago, 833 South Wood Street, Chicago, IL 60612, USA

ARTICLE INFO

Article history:

Received 15 October 2016

Received in revised form

8 January 2017

Accepted 12 January 2017

Available online 19 January 2017

Keywords:

Justicia gendarussa

Acanthaceae

Plant natural products

Arylnaphthalide lignans glycosides

Justiprocumins

Antiviral activity

Anti-HIV

NRTI- and NNRTI-resistant HIV-1 variants

ABSTRACT

In a search for new anti-HIV active leads from over several thousands of plant extracts, we have identified a potent plant lead. The active plant is determined as *Justicia gendarussa* (Acanthaceae), a medicinal plant that has been used for the treatment of injury, arthritis and rheumatism in Asia including China. Our bioassay-guided fractionation of the methanol extract of the stems and barks of the plant led to the isolation of two anti-HIV compounds, justiprocumins A and B. The compounds are identified as new aryl-naphthalide lignans (ANL) glycosides. We further determined that the ANL glycosides are the chemical constituents that contribute to the anti-HIV activity of this plant. Justiprocumin B displayed potent activity against a broad spectrum of HIV strains with IC₅₀ values in the range of 15–21 nM (AZT, IC₅₀ 77–95 nM). The compound also displayed potent inhibitory activity against the NRTI (nucleoside reverse transcriptase inhibitor)-resistant isolate (HIV-1₁₆₁₇₋₁) of the analogue (AZT) as well as the NNRTI (non-nucleoside reverse transcriptase inhibitor)-resistant isolate (HIV-1_{N119}) of the analogue (nevirapine).

© 2017 Elsevier Ltd. All rights reserved.

1. Introduction

The pandemic AIDS (acquired immunodeficiency syndrome) disease has killed more than 36 million people, and the number of the HIV infected patients exceeds 39 million (UNAIDS, 2016a; 2016b; WHO, 2016a) since its first report in 1981 in the United States (CDC, 1981; Barré-Sinoussi et al., 1983). AIDS is presently the leading cause of death in Africa and ranked as the six leading cause of death worldwide (WHO, 2016b). The efficacy of the drugs available to treat HIV/AIDS patients on the market is diminishing due to drug resistance and side effects. Further, there is no effective vaccine currently available against HIV. Development of new anti-

HIV therapeutics is thus urgently needed.

Higher plants have been used for the treatment of human diseases for thousands of years, and over 130 plant-derived chemicals are important drugs currently in clinical use around the World (Tsang et al., 2007; Taylor, 2005). Plant natural products have been an important source for discovery of anti-HIV agents. For example, calanolide A, a non-nucleoside reverse transcriptase inhibitor (NNRTI), was first isolated from a Malaysian plant *Callophylum lanigerum* (Kashman et al., 1992). It has been investigated continuously in clinical trials (ClinicalTrials.gov, 2016a). Betulinic acid is a triterpene, which can be found in many plant species. One of its derivatives (PA457) had been in Phase II clinical study for the treatment of HIV (ClinicalTrials.gov, 2016b). Ingenol is a diterpenoid isolated from the dried roots of *Euphorbia desmondi* (Evans and Kinghorn, 1974). Its triacetate showed potent anti-HIV activity (Fujiwara et al., 1996a; 1996b). Conocurvone, a trimeric naphthoquinone isolated from an Australian plant *Conospermum incurvum* Lindley (Decosterd et al., 1993), is under drug development by

* Corresponding author.

** Corresponding author.

E-mail addresses: zhanghj@hkbu.edu.hk (H.-J. Zhang), lijun@uic.edu (L. Rong).

¹ These authors contributed equally to this work.

² Present address: Northeastern Illinois University, 5500 N St Louis Ave, Chicago, Illinois 60625, USA.

an Australian company. Its anti-HIV mechanism of action occurs in the late phase of the viral replication cycle.

In the past 15 years, our medicinal plant research program has evaluated antiviral activity of more than 4500 tropical plants, and discovered more than 50 active plant leads against HIV, HCV and avian flu virus (H5N1) (Guan et al., 2016; Hoang et al., 2002; Li et al., 2015; Pan et al., 2014; Rumschlag-Booms et al., 2011; Soejarto et al., 2006; Song et al., 2016; Zhang et al., 2001, 2003a, 2003b, 2003c, 2004, 2005, 2009). Among the active plant leads, a CH₂Cl₂ extract (SV5614) made from the stems and roots of *Justicia gendarussa* (Acanthaceae) showed complete inhibition against HIV replication at a concentration 20 µg/mL. The anti-HIV activity of this plant was further confirmed by evaluation of the MeOH extract (SVA5614) made from the subsequently recollected stem and bark materials of the plant species in the same area, which showed that SVA5614 was able to inhibit HIV replication at a low concentration (IC₅₀ 0.04 µg/mL) without cytotoxicity when tested at much higher concentration of 20 µg/mL.

J. gendarussa is a medicinal plant that has been used for the treatment of various diseases in Asian countries. The plant is a bush of up to about 1.5 m in height, and is found in Southern Asia including China, Vietnam, Thailand, India, Sri Lanka, Pakistan and Indonesia. The whole plant has been used as a native medicinal plant for the treatment of injury from falls and rheumatism in the provinces of China including Guangdong, Guangxi and Taiwan (Jiangsu, 1986). The plant is also widely distributed in the other Southern Asia countries, and is being used in these countries for the treatment of fever, hemiplegia, arthritis, headache, respiratory disorders, muscle pain and digestive trouble (Kavitha et al., 2014; Paval et al., 2009). Bioassay-guided fractionation of the plant extract SVA5614 led to the isolation of two anti-HIV compounds. The compounds, designated as justiprocumins A (**1**) and B (**2**) respectively, belong to aryl-naphthalide lignan (ANL) glycosides, which are determined to be novel molecules (Fig. 1). In addition, we have analyzed the active and non-active fractions of the plant extract using HPLC techniques, and discovered that only the active fractions contained ANL glycosides. The inactive fractions are devoid of these molecules.

The current paper reports the isolation, identification and biological evaluation of the anti-HIV ANL glycoside from the plant *J. gendarussa*.

2. Results and discussion

The dried, milled plant material (4.0 kg) was extracted with MeOH to afford a MeOH extract (SVA5614, 155 g). Chromatographic fractionation of SVA5614 over a silica gel column afforded the active fraction F26, which was subjected to preparative HPLC separation to yield 8 fractions (F41–F48). The active fractions F45 and F48 were found to consist of pure compounds, which were given the names of justiprocumins A (**1**) and B (**2**), respectively. The two new compounds were elucidated to belong to aryl-naphthalide lignan (ANL) glycosides with each containing two methoxy and a methylenedioxy groups by comparison of their ¹H and ¹³C NMR data to those of known ANL compounds (Table 1) (Yang et al., 2006; Liu et al., 2008). The γ-lactone carbonyl carbon was assigned at C-9' rather than at C-9 due to the presence of the HMBC long-range correlations of H₂-9 to C-7. Analysis of 1D (¹H, ¹³C and DEPT) and 2D (¹H-¹H COSY, HMQC and HMBC) NMR data determined the two methoxy groups at C-4 and C-5, and the methylenedioxy group at C-3' and C-4' to form a piperonyl (1, 3-benzodioxole) group (Figs. S1–S14, Supplementary data).

Justiprocumin A (**1**), [α]_D²⁰ –26.2° (c 0.55 in MeOH), was obtained as a white powder with a molecular formula of C₃₅H₃₈O₁₇ determined by positive HRESIMS ([M+H]⁺ m/z 731.2217, calcd 731.2187) and NMR studies (Table 1 and Figs. S1–S3, Supplementary data). The ¹H and ¹³C NMR spectra of **1** exhibited the sugar signals with the anomeric proton at δ_H 4.852 (1H, d, J = 7.9 Hz) and carbon at δ_C 106.35 (d), and the sugar signals with the anomeric proton at δ_H 4.319 (1H, d, J = 7.7 Hz) and carbon at δ_C 104.72 (d).

Compound **1** was hydrolysed by acid to determine the two sugar units. Justiprocumin A (**1**, 1.5 mg) was heated with 1N HCl for 4 h at 105 °C. The reaction mixture was neutralized and partitioned with AcOEt. The aqueous solution was evaporated to afford the mixture of D-glucose and D-quinovose, which were identified in comparison with the authentic samples of the corresponding sugars by TLC and HPLC analysis. The glucopyranosyloxy group was linked to C-4'' of quinovopyranosyl group to form a disaccharide group due to the presence of the HMBC correlation of H-1''' to C-4'' (Fig. 2 and Fig. S6, Supplementary data). The disaccharide unit was further assigned at C-7 of the aryl-naphthalide lignan due to the presence of the HMBC correlation of H-1'' to C-7. In the NMR spectra of **1**, we observed

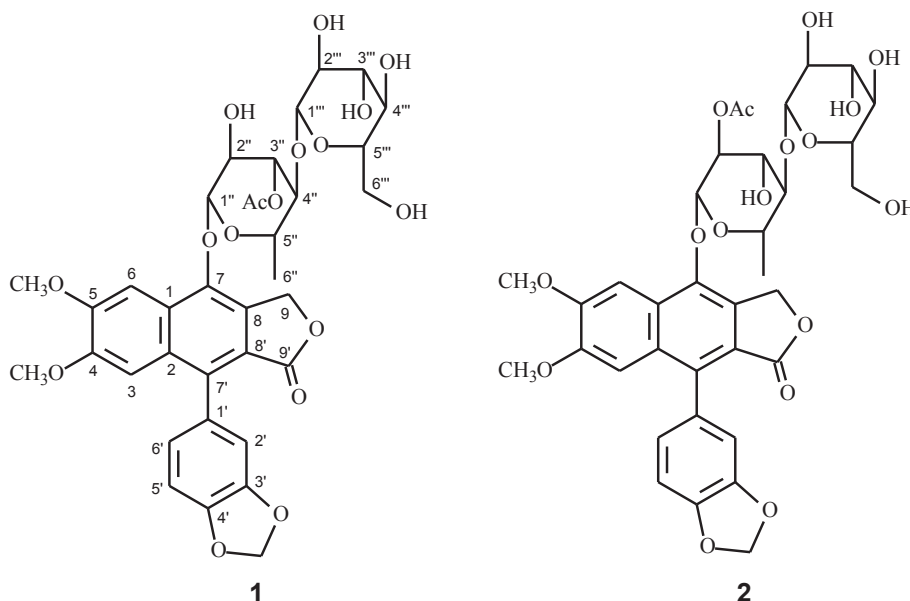


Fig. 1. Structures of compounds **1** and **2**.

Table 1
 ^1H (500 MHz) and ^{13}C (100 MHz) NMR data of compounds **1** and **2** in methanol- d_4 .

No	1		2	
	δ_{H} (J in Hz)	δ_{C}	δ_{H} (J in Hz)	δ_{C}
1		128.87		127.36
2		131.88		131.60
3 ^a	7.011 s	106.93	6.962 s	106.91
	6.992 s		6.955 s	
4		151.71		151.77
5		153.37		153.25
6 ^a	8.052 s	102.62	7.496 s	101.86
	8.044 s		7.493 s	
7		146.24		145.95
8 ^a		132.18		127.57
				127.50
9 ^a	5.550 d (15.2)	69.05	5.471 brs	68.63
	5.546 d (15.2)			
	5.418 d (15.1)		5.471 brs	
	5.415 d (15.2)			
1' ^a		129.91		129.85
		129.89		
2' ^a	6.766 d (1.8)	111.75	6.712 d (1.6)	111.75
	6.725 overlap		6.657 d (1.6)	
3'		148.99		148.93
4'		148.99		148.93
5' ^a	6.929 d (7.8)	108.97	6.908 d (7.9)	108.94
	6.919 d (7.7)			
6' ^a	6.723 dd (7.9, 1.9)	124.73	6.685 dd (7.9, 1.7)	124.74
	6.715 dd (7.7, 1.8)		6.665 dd (7.8, 1.9)	
7'		137.75		136.75
8' ^a		119.96		120.14
		119.92		120.11
9'		172.08		171.76
1'' ^a	4.852 d (7.9)	106.35	5.184 d (8.1)	101.86
	4.842 d (7.9)		5.181 d (8.0)	
2'' ^a	3.862 dd (9.2, 7.7)	73.73	5.266 dd (9.7, 7.9)	75.17
	3.858 dd (9.4, 8.0)		5.248 dd (9.8, 7.9)	
3''	5.035 t (9.0)	76.79	3.756 brt (9.0)	74.12
4''	3.603 t (9.4)	81.93	3.453 t (9.1)	85.77
5''	3.522 dq (9.3, 5.9)	73.03	3.668 m	72.91
6'' ^a	1.425 d (6.0)	18.13	1.465 d (6.2)	18.07
	1.422 d (6.1)		1.464 d (6.1)	
1'''	4.319 d (7.7)	104.72	4.431 d (7.8)	105.01
2'''	3.100 dd (9.2, 8.0)	75.31	3.229 dd (9.3, 8.0)	75.07
3'''	3.325 t (8.8)	77.93	3.377 t (9.0)	77.93
4'''	3.220 dd (9.5, 8.7)	71.89	3.272 brt (9.4)	71.51
5'''	3.285 ddd (8.6, 6.2, 2.4)	78.04	3.371 m	78.22
6'''	3.918 dd (11.6, 2.1)	63.04	3.914 dd (11.9, 2.1)	62.59
	3.667 dd (11.6, 6.2)		3.645 dd (11.7, 6.1)	
3'-OCH ₂ O-4' ^a	6.045 m	102.62	6.036 m	102.58
	6.029 m		6.018 m	
4-OCH ₃ ^a	3.683 s	55.99	3.679 s	56.01
	3.675 s		3.676 s	
5-OCH ₃ ^a	3.961 s	56.69	3.975 s	56.68
	3.959 s		3.972 s	
2''-OCOCH ₃ ^a			2.113 s	21.30
			2.111 s	
2''-OCOCH ₃ ^a				171.76
3''-OCOCH ₃	2.186 s	21.46		
3''-OCOCH ₃		173.11		

^a The NMR signals were shown to be doubling due to some degree of hindered rotation of the aryl naphthalene bond.

additional signals at δ_{H} 2.185 (3H, s) and δ_{C} 21.46 (d), 173.11 (s) ascribed to an acetyloxy group, which was assigned to C-3'' due to the presence of the HMBC correlations of the acetyl methyl protons to C-3''. Accordingly, **1** was determined as 9-(1,3-benzodioxol-5-yl)-4-[(3-O-acetyl-6-deoxy-4-O- β -D-glucopyranosyl- β -D-glucopyranosyl)oxy]-6,7-dimethoxy-naphtho[2,3-c]furan-1(3H)-one, and was given the trivial name justiprocumin A.

Justiprocumin B (**2**), $[\alpha]_{\text{D}}^{20} -32.0^\circ$ (c 0.74, MeOH), was obtained as a white powder with the same molecular formula (C₃₅H₃₈O₁₇) to that of **1**, which was determined by positive HRESIMS ($[\text{M}+\text{H}]^+$ m/z 731.2216, calcd 731.2187) and NMR studies (Table 1 and

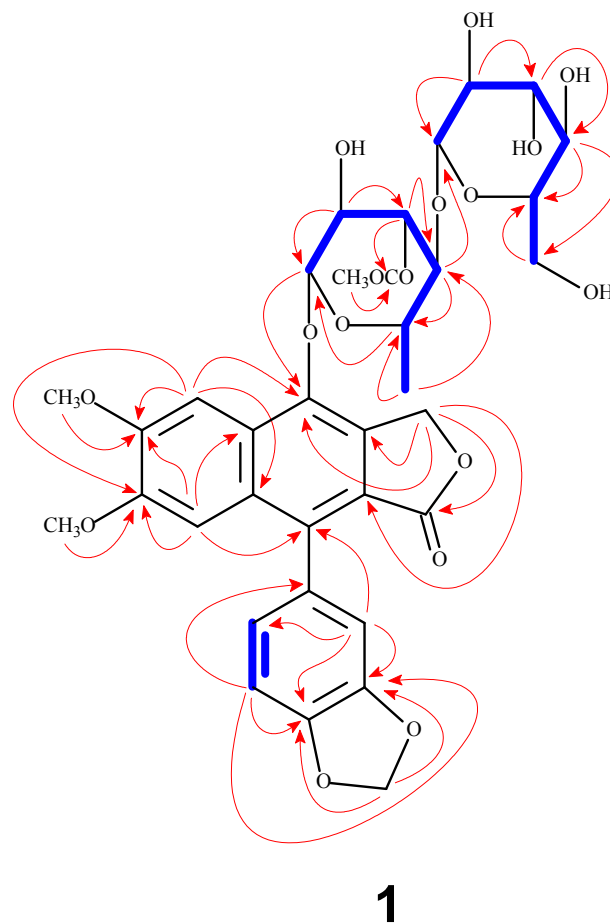


Fig. 2. Key COSY (— in blue bold bond) and HMBC (→ in red) correlations of compound **1**. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Figs. S8–S10, Supplementary data). The NMR data of this compound is very similar to those of **1**, suggesting that **2** possess a structure similar to **1**. Compound **2** differs from **1** only by the position of an acetyloxy group, which was determined at C-2'' by the presence of the HMBC correlations of the acetyl methyl protons to C-2'' (Fig. 3 and Figs. S6 and S13, Supplementary data). In combination of analysis of the 2D NMR data including ^1H - ^1H COSY, HMQC and HMBC spectral data, **2** was determined as 9-(1,3-benzodioxol-5-yl)-4-[(2-O-acetyl-6-deoxy-4-O- β -D-glucopyranosyl- β -D-glucopyranosyl)oxy]-6,7-dimethoxy-naphtho[2,3-c]furan-1(3H)-one, and was given the trivial name justiprocumin B.

The potent anti-HIV ANL glycosides (**1** and **2**) have been identified from the MeOH extract of *J. gendarussa*. We further analyzed the active and non-active fractions of the plant extract using TLC and HPLC-UV techniques (Fig. S15, Supplementary data). We discovered that only the active fractions contained ANL glycosides, suggesting that the ANL glycosides are the active components responsible for the anti-HIV activity of this plant. An herbal product formulation enriched with ANL glycosides may be amenable to become a therapeutic candidate for the treatment of HIV/AIDS disease.

Justiprocumin B (**2**) was evaluated for its anti-HIV-1 activity against the broadness of the spectrum of HIV strains. Four HIV-1 clinical isolates, BAL and SF162 (both are M-tropic), LAV0.04 (T-tropic), and 89.6 (dual tropic), were tested against compound **2** using a standardized human peripheral blood mononuclear cell culture (PBMC) assay (Japour et al., 1993). Compound **2** displayed

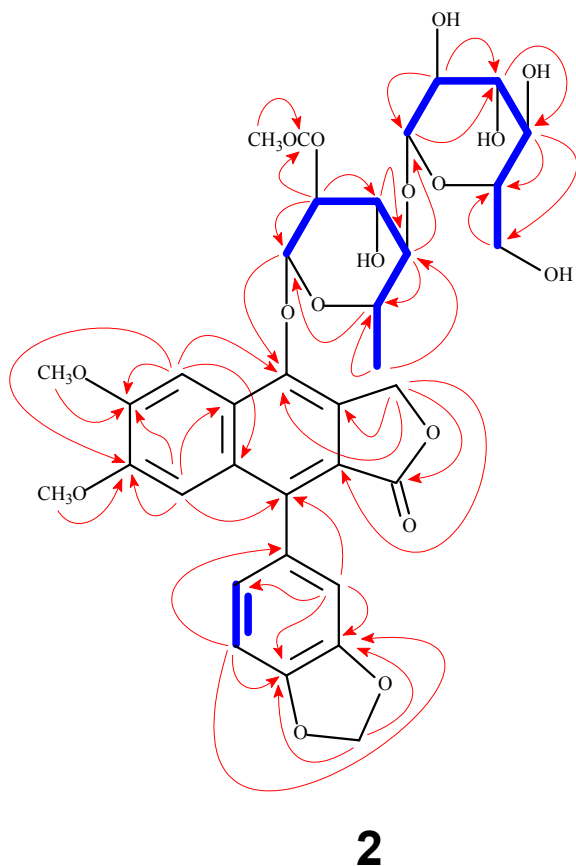


Fig. 3. Key COSY (→ in blue bold bond) and HMBC (→ in red) correlations of compound **2**. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

potent inhibition activity against the particle production of all four HIV-1 isolates (Fig. S16, Supplementary data). It gave the IC_{50} values ranging from 14 to 21 nM as compared to the IC_{50} values of 77–95 nM for the clinically used drug AZT (zidovudine) (Table 2).

The compound was further evaluated for its efficacy against the drug-resistant HIV isolates NRTI (nucleoside reverse transcriptase inhibitor)-resistant isolate (HIV-1₁₆₁₇₋₁) and NNRTI (non-nucleoside reverse transcriptase inhibitor)-resistant isolate (HIV-1_{N119}) using the standardized human peripheral blood mononuclear cell culture (PBMC) assay (Japour et al., 1993). As shown in Table 2, justiprocumin B (**2**) much more effectively inhibited the HIV-1 resistant isolate HIV-1₁₆₁₇₋₁ in comparison with AZT, which as a NRTI, was effective against LAV and HIV-1_{N119}. Compound **2** also displayed potent inhibition activity against the nevirapine resistant isolate HIV-1_{N119} with an IC_{50} value of 495 nM. The results clearly demonstrate that justiprocumin B (**2**) is a potent inhibitor against both NRTI- and NNRTI-resistant HIV-1 isolates.

In the present study we report the isolation and structure identification of two new ANL glycosides (**1** and **2**) from higher

plants. Justiprocumin B (**2**) displayed broad spectrum and potent anti-HIV-1 activity including its anti-HIV activity against NRTI- and NNRTI-resistant HIV-1 variants. The chemical structures of the two compounds were identified as ANL glycosides based on the spectroscopic methods including 1D and 2D NMR spectral data (Fig. 1). Many resonance signals in the 1H NMR spectra of the two compounds were split into two, indicating atropisomerism due to the stereo hindrance of the bond rotation between C-1' and C-7' (Fig. S17, Supplementary data) (Charlton et al., 1996; Ren et al., 2014), which may provide a rationale for chemical modification of the aryl-naphthalene lignans by introducing large functional groups at C-3, C-2' and C-6' in order to increase the structural rigidity. We thus consider that an ANL molecule with less flexibility will have a three-dimensional structure with fixed stereochemistry, which is more likely to be attached to specific viral targets without binding to other proteins or receptors that may cause toxicity, making them more attractive as anti-HIV drug candidates (Eckard et al., 2006).

Natural aryl-naphthalene lignans have been reported to have a variety of biological activities including antibacterial, antitumor and anti-HIV activities (Zhang et al., 2007; Lee et al., 2005; Tuchinda et al., 2008; Chang et al., 1995; Ren et al., 2014). In one study, anti-HIV activity was observed for two natural aryl-naphthalene lignans that showed moderate inhibitory effect on HIV-1 reverse transcriptase activity (IC_{50} 3.5 and 5.5 μ M, respectively), and much less inhibitory activity on human DNA polymerase- α (IC_{50} 289 and 989 μ M, respectively) (Chang et al., 1995). However, to our knowledge, no anti-HIV activity has been reported for aryl-naphthalene lignan glycosides. We have discovered a new and potent anti-HIV ANL compound which is amenable for further study as an anti-HIV lead compound.

3. Conclusion

Two new anti-HIV lead compounds [justiprocumins A (**1**) and B (**2**)] were successfully isolated from the stems and the barks of *J. gendarussa* using our “One-Stone-Two-Birds” bioassay evaluation guided system. The cell-based evaluation protocol was using the viral entry mediated glycoprotein (GP) incorporated onto another virus to produce so-called pseudovirion particles, which retained the viral entry property but with alleviated safety concerns (Li et al., 2015; Rumschlag-Booms et al., 2011). The potency of the anti-HIV activity of the isolated compounds was confirmed by testing justiprocumin B (**2**) against a broad spectrum of HIV strains. Compound **2** showed potent inhibition activity against the HIV-1 clinical isolates including the NRTI- and NNRTI-resistant HIV-1 isolates. The efficacy of **2** against the resistant HIV-1 isolates may warrant further investigation of the ANL compounds as a drug candidate for treatment of the HIV infected diseases in combination with other anti-HIV therapeutic drugs.

4. Experimental

4.1. General experimental procedures

Optical rotations were measured with a Perkin-Elmer model 241

Table 2
Inhibition activity of justiprocumin B (**2**) against HIV clinical strains and resistant strains.

Strains (IC_{50} , nM)							
Compound	Bal: M-Tropic	89.6: Dual-Tropic	SF162: M-Tropic	Lav.04: T-Tropic	HIV-1 _{LAV}	HIV-1 ₁₆₁₇₋₁	HIV-1 _{N119}
2	15	15	14	21	641	185	495
AZT	77	95	85	79	72	3508	–
Nevirapine	–	–	–	–	532	156	>100000 ^a

^a The datum is taken from the literatures (Fujihashi et al., 1995; Richman et al., 1991).

polarimeter. IR spectra were recorded on a Jasco FT/IR-410 spectrometer, equipped with a Specac Silver Gate ATR system by applying a film on a Germanium plate (Jasco, Maryland). 1D and 2D NMR spectra were recorded on a Bruker DRX-500 MHz or a Bruker DPX-400 MHz or a Bruker DPX-360 MHz spectrometer (Germany). Chemical shifts (δ) were expressed in ppm with reference to the solvent signals (methanol- d_4 CD $_3$ OD: ^1H : 3.30 ppm, ^{13}C : 49.00 ppm), and coupling constants (J) are reported in Hz. All NMR experiments were obtained by using standard pulse sequences supplied by the vendor. Column chromatography was carried out on silica gel (230–400 mesh, Natland International Corporation, North Carolina). Reversed-phase flash chromatography was accomplished with RP-18 silica gel (40–63 μm , EM Science, New Jersey), and reversed-phase preparative HPLC was carried out on a Waters 600E Delivery System pump, equipped with a Waters 996 photodiode detector (Massachusetts), and a Watrex GROM-Saphir 110 C18 column (120 \AA , 12 μm , 300 \times 40 mm 2 , Switzerland) or a Phenomenex LUNA-C-18 column (120 \AA , 12 μm , 250 \times 50 mm 2 , California). Thin-layer chromatography was performed on EMD glass-backed plates coated with 0.25 mm layers of Silica gel 60 F $_{254}$ (Germany). HRTOFMS spectra were recorded on a Micromass QTOF-2 (Massachusetts) or an Agilent 6540 Q-TOF (California) or an Agilent 6460 Triple Quadrupole (California) or a Bruker Q-TOF mass spectrometer (Germany).

4.2. Plant materials

The initial collection of the root and stem sample of *Justicia gendarussa* Burm. f. (Acanthaceae) was made in 2006 in Cave of Prehistoric Man close to hotspot 33 of Cuc Phuong National Park, with geographic coordinate readings of 20° 21' 633" N 105° 38' 257" E. A larger amount of the plant sample for the current isolation work, consisting of the barks and stems (4.0 kg), was subsequently recollected from the plants located in the same area. The recollection of root and stem sample of *J. gendarussa* was made on July 15, 2008 in Cave of Prehistoric Man close to hotspot 33 of Cuc Phuong National Park, with geographic coordinate readings of 20° 17' 250" N 105° 40' 235" E. Voucher specimens (*M.V. Xinh* #742 for the initial collection and *N.M. Cuong* #2162 for the recollection) were collected and are in deposit at the Herbarium of Cuc Phuong National Park.

4.3. Extraction and isolation

The dried, milled plant material (4.0 kg) was extracted with methanol to afford 155 g of extract. A small portion of the methanol extract (5.5 g) was rapidly fractionated to 22 fractions (Fa1–Fa22) in one hour over a silica gel (10 g) column eluting with gradient CHCl $_3$, CHCl $_3$ /Me $_2$ CO, and CHCl $_3$ /MeOH solutions. Anti-HIV evaluation of the fractions determined that the fractions Fa14–Fa18 inhibited HIV replication by more than 95% at a concentration of 0.1 $\mu\text{g}/\text{mL}$ without apparent toxicity at 20 $\mu\text{g}/\text{mL}$. Scale-up fractionation of SVA5614 was then carried out in an attempt to isolate other anti-HIV active compounds in this plant. The methanol extract (140 g) was absorbed onto 214 g silica gel, and chromatographed over a silica gel column (1545 g). The column was developed by gradient elution with CHCl $_3$, CHCl $_3$ /Me $_2$ CO, and CHCl $_3$ /Me $_2$ CO/MeOH solutions to afford 40 fractions. Fraction F26 showed very similar TLC and HPLC profiles to those of Fa16. A portion (120 mg) of F26 was thus subjected preparative HPLC separation on a Phenomenex LUNA-C-18 column (solvent system: MeOH/H $_2$ O 65:35) to yield 8 fractions (F41–F48). F45 (9.7 mg) and F48 (12.9 mg) were identified as justiprocumins A (**1**) and B (**2**), respectively.

4.4. Justiprocumin A (**1**)

White powder; $[\alpha]_D^{20}$ -26.2° (c 0.55, MeOH); UV (MeOH) λ_{max} [AU

(absorbance units)] 201.6 (0.77), 225.2 (0.41), 261.5 (1.01), 294.0 (0.19), 314.9 (0.21), 356.1 (0.07) nm; IR (film) ν_{max} 3421 (*br*), 2924, 1745, 1622, 1507, 1480, 1456, 1435, 1391, 1341, 1262, 1229, 1168, 1066, 1034, 930, 865, 770 cm $^{-1}$; for ^1H NMR and ^{13}C NMR data, see Table 1; positive HRESIMS m/z 731.2217 [M+H] $^+$ (calcd for C $_{35}$ H $_{38}$ O $_{17}$, 731.2187).

4.5. Justiprocumin B (**2**)

White powder; $[\alpha]_D^{20}$ -32.0° (c 0.74, MeOH); UV (MeOH) λ_{max} [AU (absorbance units)] 201.6 (1.16), 226.5 (0.63), 261.5 (1.41), 293.6 (0.29), 314.9 (0.31), 356.0 (0.14) nm; IR (film) ν_{max} 3422 (*br*), 2924, 1748, 1651, 1621, 1558, 1541, 1507, 1476, 1456, 1435, 1450, 1262, 1227, 1168, 1068, 1035, 931, 863, 770 cm $^{-1}$; for ^1H NMR and ^{13}C NMR data, see Table 1; positive HRESIMS m/z 731.2216 [M+H] $^+$ (calcd for C $_{35}$ H $_{38}$ O $_{17}$, 731.2187).

4.6. "One-Stone-Two-Birds" anti-HIV evaluation assay

This protocol allows us to identify potential inhibitors for HIV replication (post-entry steps). In this system, the HIV vector pNL4-3.Luc.R.E (Connor et al., 1995; He et al., 1995), obtained through the AIDS Research and Reference Reagent Program (Division of AIDS, NIAID, NIH), 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 an SI, T-tropic virus (Michael et al., 1998) 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 (reverse transcription, integration and HIV gene expression). 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. The fractions or compounds were evaluated as follows. The stock HIV/HA virions (approximately 2×10^6 relative light units, or RLUs, on the target cells) were mixed with the individual extract first, and the mixture was incubated with the target cells in 24 well plates for 24 h (human lung cell line A549 was used since it is susceptible to HA-mediated viral entry). The final concentration of the extract was 20 $\mu\text{g}/\text{mL}$. Forty-eight (48) hours post-infection, the target cells were lysed and the luciferase activity was determined.

4.7. Anti-HIV assay against broad spectrum of HIV strains including resistant strains

Four HIV1 clinical strains, BAL and SF162 (M-tropic), BAL (T-tropic), and 89.6 (a dual tropic strain), as well as HIV-1 $_{\text{LAV}}$, NRTI (nucleoside reverse transcriptase inhibitor)-resistant isolate (HIV-1 $_{1617-1}$) and NNRTI (non-nucleoside reverse transcriptase inhibitor)-resistant isolate (HIV-1 $_{\text{N119}}$) were used in the current study. Here a standardized human peripheral blood mononuclear cell culture (PBMC) assay was used to determine the compound susceptibility of these HIV-1 strains. AZT, an anti-HIV drug in clinical use, was used as a positive control. Briefly, human PBMCs were collected from a donor and stimulated for seven days. The preparations (compound or fraction) were then added to the cultured cells at a wide range of concentrations, and the different HIV-1 strains were used to challenge the cultured cells using 96-well plates. After seven-days of incubation, the supernatants were collected and the HIV p24 levels of the infected cells were determined using a p24 antigen ELISA. The IC $_{50}$ s were calculated by

comparing p24 antigen values for the compounds (fraction)-containing wells with those for no drug control wells. In these experiments, AZT was used as a positive control.

Acknowledgements

This project was supported by the Research Grants Council of the Hong Kong Special Administrative Region, China (Project No. HKBU 262912), the Health and Medical Research Fund (12132161) of the Food and Health Bureau, Hong Kong SAR, Faculty Research Grants, Hong Kong Baptist University (FRG2/11-12/134 and FRG2/14-15/047), NIH Grants 3U01TW001015-10S1 and 2U01TW001015-11A1, administered by the Fogarty International Center as part of an International Cooperative Biodiversity Groups (ICBG) program, through funds from NIH, NSF, and Foreign Agricultural Service of the USDA, and Mr. Kwok Yat Wai and Madam Kwok Chung Bo Fun Graduate School Development Fund.

Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.phytochem.2017.01.005>.

References

- Barré-Sinoussi, F., Chermann, J.C., Rey, F., Nugeyre, M.T., Chamaret, S., Gruest, J., Dauguet, C., Axler-Blin, C., Vézinet-Brun, F., Rouzioux, C., Rozenbaum, L., Montagnier, W., 1983. Isolation of a T-lymphotropic retrovirus from a patient at risk for acquired immune deficiency syndrome (AIDS). *Science* 220, 868–871. CDC (Centers for Disease Control and Prevention, USA), 1981. Pneumocystis pneumonia-los angeles. *Morb. Mortal. Wkly. Rep. MMWR* 30, 250.
- Chang, C.W., Lin, M.T., Lee, S.S., Liu, K.C., Hsu, F.L., Lin, J.Y., 1995. Differential inhibition of reverse transcriptase and cellular DNA polymerase- α activities by lignans isolated from Chinese herbs, *Phyllanthus myrtifolius* Moon, and tannins from *Lonicera japonica* Thunb and *Castanopsis hystrix*. *Antivir. Res.* 27, 367–374.
- Charlton, J.L., Oleschuk, C.J., Chee, G.L., 1996. Hindered rotation in aryl-naphthalene lignans. *J. Org. Chem.* 61, 3452–3457.
- ClinicalTrials.gov, 2016a. <http://clinicaltrials.gov/ct2/results?term=calanolide> (Accessed 8 September 2016).
- ClinicalTrials.gov, 2016b. <http://clinicaltrials.gov/ct2/show/NCT00511368?term=PA457&rank=1> (Accessed 8 September 2016).
- Connor, R.L., Chen, B.K., Choe, S., Landau, N.R., 1995. Vpr is required for efficient replication of human immunodeficiency virus type-1 in mononuclear phagocytes. *Virology* 206m, 935–944.
- Decosterd, L.A., Parsons, I.C., Gustafson, K.R., Cardellina II, J.H., McMahon, J.B., Cragg, G.M., Murata, Y., Pannell, L.K., Steiner, J.R., Clardy, J., Boyd, M.R., 1993. HIV inhibitory natural products. 11. Structure, absolute stereochemistry, and synthesis of conocurvone, a potent, novel HIV-inhibitory naphthoquinone trimer from a *Conospermum* sp. *J. Am. Chem. Soc.* 115, 6673–6679.
- Eckard, P., Abel, U., Rasser, H.F., Simon, W., Sontag, B., Hansske, F.G., 2006. Natural products-based, chemically and functionally diverse libraries. In: Boldi, A.M. (Ed.), *Combinatorial Synthesis of Natural Product-based Libraries*. CRC Press, Taylor & Francis Group, Boca Raton (Chapter 5).
- Evans, F.J., Kinghorn, A.D., 1974. Ingenol from *Euphorbia desmondii*. *Phytochemistry* 13, 1011.
- Fujihashi, T., Hara, H., Sakata, T., Mori, K., Higuchi, H., Tanaka, A., Kaji, H., Kaji, A., 1995. Anti-human immunodeficiency virus (HIV) activities of halogenated gomsin J derivatives, new nonnucleoside inhibitors of HIV type 1 reverse transcriptase. *Antimicrob. Agents Chemoth.* 39, 2000–2007.
- Fujiwara, M., Ijichi, K., Tokuhisa, K., Katsuura, K., Shigeta, S., Konno, K., Wang, G.Y., Uemura, D., Yokota, T., Baba, M., 1996a. Mechanism of selective inhibition of human immunodeficiency virus by ingenol triacetate. *Antimicrob. Agents Chemoth.* 40, 271–273.
- Fujiwara, M., Ijichi, K., Tokuhisa, K., Katsuura, K., Wang, G.Y.S., Uemura, D., Shigeta, S., Konno, K., Yokota, T., Baba, M., 1996b. Ingenol derivatives are highly potent and selective inhibitors of HIV replication in vitro. *Antivir. Chem. Chemother.* 7, 230–236.
- Guan, Y.F., Wang, D.Y., Tan, G.T., Hung, N.V., Cuong, N.M., Pezzuto, J.M., Fong, H.H.S., Soejarto, D.D., Zhang, H.J., 2016. *Litsea* species as potential antiviral plant sources. *Am. J. Chin. Med.* 44, 275–290.
- He, J., Choe, S., Walker, R., Di Marzio, P., Morgan, D.O., Landau, N.R., 1995. Human immunodeficiency virus type 1 viral protein R (Vpr) arrests cells in the G2 phase of the cell cycle by inhibiting p34cdc2 activity. *J. Virol.* 69, 6705–6711.
- Hoang, V.D., Tan, G.T., Zhang, H.J., Tamez, P.A., Hung, N.V., Cuong, N.M., Soejarto, D.D., Fong, H.H.S., Pezzuto, J.M., 2002. Natural anti-HIV agents. Part I. (+)-Demethoxyepiexelsin and verticillatol from *Litsea verticillata*. *Phytochemistry* 59, 325–329.
- Japour, A.J., Mayers, D.L., Johnson, V.A., Kuritzkes, D.R., Beckett, L.A., Arduino, J.M., Lane, J., Black, R.J., Reichelderfer, P.S., D'Aquila, R.T., 1993. Standardized peripheral blood mononuclear cell culture assay for determination of drug susceptibilities of clinical human immunodeficiency virus type 1 isolates. The RV-43 Study Group, the AIDS Clinical Trials Group Virology Committee Resistance Working Group. *Antimicrob. Agents Chemoth.* 37, 1095–1101.
- Jiangsu College of New Medicine, 1986. *Dictionary of Chinese Traditional Medicine*. Shanghai Science and Technology Press, Shanghai. Item 2471.
- Kashman, Y., Gustafson, K.R., Fuller, R.W., Cardellina, J.H., McMahon, J.B., Currens, M.J., Buckheit Jr., R.W., Hughes, S.H., Cragg, G.M., Boyd, M.R., 1992. The calanolides, a novel HIV-inhibitory class of coumarin derivatives from the tropical rainforest tree, *Calophyllum lanigerum*. *J. Med. Chem.* 35, 2735–2743.
- Kavitha, K., Sridevisangeetha, K.S., Sujatha, K., Umamaheswari, 2014. Phytochemical and pharmacological profile of *Justicia gendarussa* Burm f. – review. *J. Pharm. Res.* 8, 990–997.
- Lee, J.C., Lee, C.H., Su, C.L., Huang, C.W., Liu, H.S., Lin, C.N., Won, S., 2005. Justicidin A decreases the level of cytosolic Ku70 leading to apoptosis in human colorectal cancer cells. *J. Carcinog.* 26, 1716–1730.
- Li, W.F., Zhang, J.J., Song, S., Ku, C.F., Zou, J., Li, J.X., Rong, L.J., Pan, L.T., Zhang, H.J., 2015. Henrin A: a new anti-HIV *ent*-kaurane diterpene from *Pteris henryi*. *Int. J. Mol. Sci.* 16, 27978–27987.
- Liu, G.R., Wu, J., Si, J.Y., Wang, J.M., Yang, M.H., 2008. Complete assignments of ^1H and ^{13}C NMR data for three new aryl-naphthalene lignan from *Justicia procumbens*. *Magn. Res. Chem.* 46, 283–286.
- Michael, N.L., Nelson, J.A., KewalRamani, V.N., Chang, G., O'Brien, S.J., Mascola, J.R., Volsky, B., Loulder, M., White 2nd, G.C., Littman, D.R., Swanstrom, R., O'Brien, T.R., 1998. Exclusive and persistent use of the entry coreceptor CXCR4 by human immunodeficiency virus type 1 from a subject homozygous for CCR5 delta32. *J. Vir.* 72, 6040–6047.
- Pan, W.H., Liu, K.L., Guan, Y.F., Tan, G.T., Hung, N.V., Cuong, N.M., Doejarto, D.D., Pezzuto, J.M., Fong, H.H.S., Zhang, H.J., 2014. Bioactive compounds from *Vitex leptobotrys*. *J. Nat. Prod.* 77, 663–667.
- Paval, J., Kaitheri, S.K., Potu, B.K., Govindan, S., Kumar, R.S., Narayanan, N., Moorkoth, S., 2009. Anti-arthritis potential of the plant *Justicia gendarussa* Burm F. *Clinics* 64, 357–360.
- Ren, Y.L., Lantvit, D.D., Deng, Y., Kanaqasabai, R., Gallucci, J.C., Ninh, T.N., Chai, H.B., Soejarto, D.D., Fuchs, J.R., Yalowich, J.C., Yu, J., Swanson, S.M., Kinghorn, A.D., 2014. Potent cytotoxic aryl-naphthalene lignan lactones from *Phyllanthus poilanei*. *J. Nat. Prod.* 77, 1494–1504.
- Richman, D., Shih, C.K., Lowy, I., Rose, J., Prodanovich, S.G., Griffin, J., 1991. Human immunodeficiency virus type 1 mutants resistant to nonnucleoside inhibitors of reverse transcriptase arise in tissue culture. *Proc. Natl. Acad. Sci. U. S. A.* 88, 11241–11245.
- Rumschlag-Booms, E., Zhang, H.J., Soejarto, D.D., Fong, H.H.S., Rong, L.J., 2011. Development of an antiviral screening protocol: one-Stone-Two-Birds. *J. Antivir. Antiretrovir.* 3, 8–10.
- Soejarto, D.D., Zhang, H.J., Fong, H.H.S., Tan, G.T., Ma, C.M., Gyllenhaal, C., Riley, M.C., Kadushin, M.R., Franzblau, S.G., Bich, T.Q., Cuong, N.M., Hiep, N.T., Loc, P.K., Xuan, L.T., Hai, N.V., Hung, N.V., Chien, T.Q., Vu, B.M., Ly, H.M., Southavong, B., Sydara, K., Bouamanivong, S., Pezzuto, J.M., Rose, W., Dietzman, G., Miller, B., Thuy, T.V., 2006. "Studies on biodiversity of Vietnam and Laos" 1998-2005: examining the impact. *J. Nat. Prod.* 69, 473–481.
- Song, X., He, J., Xu, H., Hu, X.P., Wu, X.L., Wu, H.Q., Wu, X.L., Wu, H.Q., Liu, L.Z., Liao, C.H., Zeng, Y., Li, Y., Hao, Y., Xu, C.S., Fan, L., Zhang, J., Zhang, H.J., He, Z.D., 2016. The antiviral effects of acetoside and the underlying IFN- γ -inducing action. *Food Funct.* 7, 3017–3030.
- Taylor, L., 2005. *The Healing Power of Rainforest Herbs*. Square One Publishers, New York.
- Tsang, C.K., Qi, H.Y., Liu, L.F., Zheng, X.F., 2007. Targeting mammalian target of rapamycin (mTOR) for health and diseases. *Drug Dis. Today* 12, 112–124.
- Tuchinda, P., Kornsakulkarn, J., Pohmakotr, M., Kongsaree, P., Prabpai, S., Yoosook, C., Kasisit, C., Sophasan, S., Reutrakul, V., 2008. Dichapetalin-type triterpenoids and lignans from the aerial parts of *Phyllanthus acutissima*. *J. Nat. Prod.* 71, 655–663.
- UNAIDS, 2016a. *Fact Sheet 2015*. <http://www.unaids.org/en/resources/campaigns/HowAIDSchangedeverything/factsheet> (Accessed 8 September 16).
- UNAIDS, 2016b. *Global Aids Update 2016*. http://www.unaids.org/sites/default/files/media_asset/global-AIDS-update-2016_en.pdf (Accessed 8 September 2016).
- WHO (World Health Organization), 2016a. *Global Health Observatory (GHO) Data: HIV/AIDS*. <http://www.who.int/gho/hiv/en/> (Accessed 8 September 2016a).
- WHO (World Health Organization), 2016b. *Global Health Observatory (GHO) Data: the Top 10 Causes of Death*. <http://www.who.int/mediacentre/factsheets/fs310/en/> (accessed 8 September 2016).
- Yang, M.H., Wu, J., Fan, C., Zhou, Y., 2006. Complete assignments of ^1H and ^{13}C NMR data for seven aryl-naphthalene lignans from *Justicia procumbens*. *Magn. Res. Chem.* 44, 727–730.
- Zhang, H.J., Tan, G.T., Hoang, V.D., Hung, N.V., Cuong, N.M., Soejarto, D.D., Pezzuto, J.M., Fong, H.H.S., 2001. Natural anti-HIV agents Part II. Litseaverticillol A, a prototypic litseane sesquiterpene from *Litsea verticillata*. *Tetrahedron Lett.* 42, 8587–8591.
- Zhang, H.J., Tan, G.T., Hoang, V.D., Hung, N.V., Cuong, N.M., Soejarto, D.D., Pezzuto, J.M., Fong, H.H.S., 2003a. Natural anti-HIV agents. Part III. Litseaverticillols A-H, novel sesquiterpenes from *Litsea verticillata*. *Tetrahedron* 59, 141–148.
- Zhang, H.J., Tan, G.T., Santarsiero, B.D., Mesecar, A.D., Hung, N.V., Cuong, N.M.,

- Soejarto, D.D., Pezzuto, J.M., Fong, H.H.S., 2003b. New sesquiterpenes from *Litsea verticillata*. *J. Nat. Prod.* 66, 609–615.
- Zhang, H.J., Tan, G.T., Hoang, V.D., Hung, N.V., Cuong, N.M., Soejarto, D.D., Pezzuto, J.M., Fong, H.H.S., 2003c. Natural anti-HIV agents. Part IV. Anti-HIV constituents from *Vatica cinerea*. *J. Nat. Prod.* 66, 263–268.
- Zhang, H.J., Sydara, K., Tan, G.T., Ma, C.Y., Southavong, B., Soejarto, D.D., Pezzuto, J.M., Fong, H.H.S., 2004. Bioactive constituents from *Asparagus cochinchinensis*. *J. Nat. Prod.* 67, 194–200.
- Zhang, H.J., Hung, N.V., Cuong, N.M., Soejarto, D.D., Pezzuto, J.M., Fong, H.H.S., Tan, G.T., 2005. Sesquiterpenes and butenolides, natural anti-HIV constituents from *Litsea verticillata*. *Planta Med.* 71, 452–457.
- Zhang, H.J., Rothwangl, K., Mesecar, A.D., Sabahi, A.A., Rong, L.J., Fong, H.H.S., 2009. Lamiridosins, hepatitis C virus entry inhibitors from *Lamium album*. *J. Nat. Prod.* 72, 2158–2162.
- Zhang, Y.L., Bao, F.K., Hu, J.J., Liang, S.W., Zhang, Y., Du, G.H., Zhang, C.J., Cheng, Y.X., 2007. Antibacterial lignans and triterpenoids from *Rostellularia procumbens*. *Planta Med.* 73, 1596–1599.