

Article

Lignan Glucosides from the Stem Barks of *Illicium difengpi*

Zheng-Hong Pan ^{1,*}, De-Sheng Ning ^{1,†}, Si-Si Huang ¹, Ling Cheng ^{1,2}, Meng-Wen Xia ^{1,2}, Li-Yan Peng ³ and Dian-Peng Li ^{1,*}

¹ Guangxi Key Laboratory of Functional Phytochemicals Research and Utilization, Guangxi Institute of Botany, Chinese Academy of Sciences, Guilin 541006, China; ndshgxb@sina.com (D.-S.N.); ssh520123@163.com (S.-S.H.); 15507837860@163.com (L.C.); xmw1036978486@126.com (M.-W.X.)

² Faculty of Chemistry and Chemical Engineering, Yunnan Normal University, Kunming 650500, China

³ State Key Laboratory of Phytochemistry and Plant Resources in West China, Kunming Institute of Botany, Chinese Academy of Sciences, Kunming 650204, China; pengliyan@mail.kib.ac.cn

* Correspondence: pan7260@126.com (Z.-H.P.); ldp@gxib.cn (D.-P.L.); Tel.: +86-773-355-0194 (Z.-H.P. & D.-P.L.); Fax: +86-773-355-0067 (Z.-H.P.)

† These authors contributed equally to this work.

Academic Editor: Derek J. McPhee

Received: 6 April 2016; Accepted: 4 May 2016; Published: 10 May 2016

Abstract: In this study, four new lignan glucosides, named difengpiosides A–D (1–4), were isolated from the stem barks of *Illicium difengpi*, together with seven known compounds 5–11. Their structures were identified on the basis of spectroscopic analyses (1D and 2D NMR, HRESIMS, CD) and a comparison with literature data. All the compounds were evaluated for their inhibitory effects on lipopolysaccharide (LPS)-induced nitric oxide (NO) production in RAW264.7 cells.

Keywords: *Illicium difengpi*; stem barks; lignan glucosides; difengpiosides A–D; NO inhibition

1. Introduction

Illicium difengpi K. I. B et K. I. M. (Illiciaceae), native to China, is a toxic shrub that grows in the mountainous areas of Guangxi Province. Its stem bark is listed in the Chinese Pharmacopeia as a traditional Chinese medicine to treat rheumatic arthritis [1]. Previous phytochemical studies on this plant mainly focused on the low and middle polarity components and reported the isolation of over 40 compounds, including phenylpropanoids, lignans, triterpene acids, sesquiterpenes and others [2–7]. However, there are few investigations on the polar substances of *I. difengpi*. In order to provide comprehensive chemistry support for pharmacological studies and quality control of *I. difengpi*, the present study describes the isolation and structure elucidation of four new lignan glycosides together with seven known compounds from the *n*-BuOH-soluble fraction of an EtOH extract of the stem barks of this plant, as well as their inhibitory activities against nitric oxide production in lipopolysaccharide-induced RAW264.7 cells.

2. Results and Discussion

The EtOH extract of the stem barks of *I. difengpi* were suspended in water and successively partitioned with petroleum ether, EtOAc, and *n*-BuOH. The *n*-BuOH-soluble extract was subjected to multiple chromatographic fractionations using silica gel, Sephadex LH-20, and ODS, yielding compounds 1–11 (Figure 1).

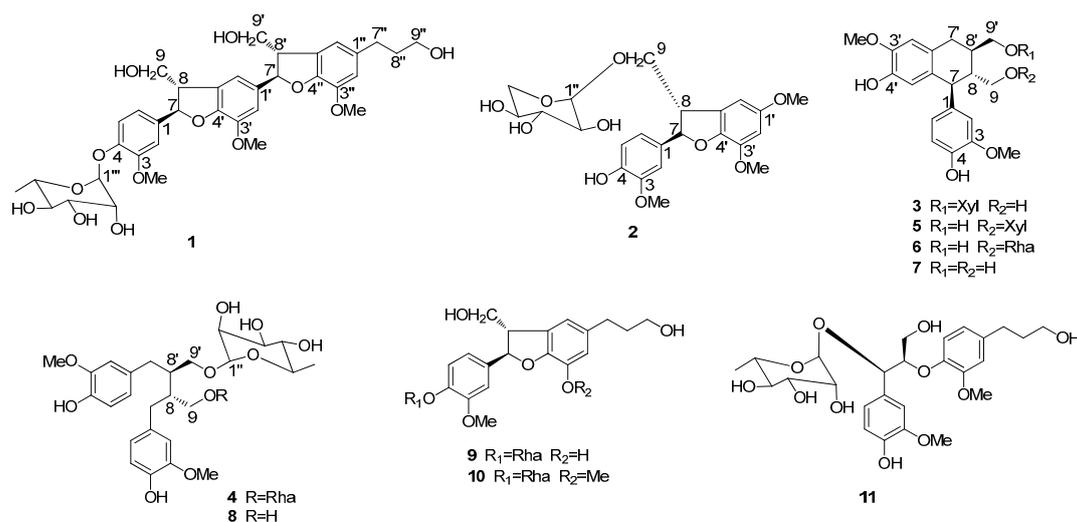


Figure 1. Chemical structures of 1–11.

Compound **1** was obtained as an amorphous powder. The molecular formula was determined as $C_{36}H_{44}O_{13}$ on the basis of a HRESIMS peak at m/z 707.2623 $[M + Na]^+$ (calcd. for 707.2674), indicating 15 degrees of unsaturation. The IR spectrum showed the presence of hydroxyl (3406 cm^{-1}) and aromatic ring (1595 and 1501 cm^{-1}) groups. The $^1\text{H-NMR}$ spectrum (Table 1) displayed signals at δ_{H} 7.07 (1H, d, $J = 8.5$ Hz), 7.01 (1H, d, $J = 2.0$ Hz) and 6.89 (1H, dd, $J = 8.5, 2.0$ Hz) for an AMX aromatic spin system, and four broad singlets at δ_{H} 6.92 (2H) and 6.72 (2H) for two tetrasubstituted aromatic rings, as well as three aromatic methoxy groups at δ_{H} 3.85 (6H, s) and 3.80 (3H, s). The remaining signals indicated the presence of a sugar moiety, an *n*-propanol and two $-\text{O}-\text{CH}-\text{CH}-\text{CH}_2\text{O}-$ spin systems, which were also confirmed by the $^1\text{H}-^1\text{H}$ COSY spectrum (Figure 2). The $^{13}\text{C-NMR}$ spectrum (Table 1) showed the presence of six characteristic rhamnopyranoside signals (δ_{C} 101.5, 73.9, 72.3, 72.1, 70.9, and 18.0) and another 30 carbons, including eighteen aromatic carbon signals of three benzene rings, two oxygenated methine carbons at δ_{C} 89.2 and 88.8, three methyleneoxy carbons at δ_{C} 65.1, 64.9 and 62.3, three methoxy carbons at δ_{C} 56.9, 56.8 and 56.5, two methine carbons at δ_{C} 55.5 and 55.5, two methylene carbons at 35.8 and 32.9.

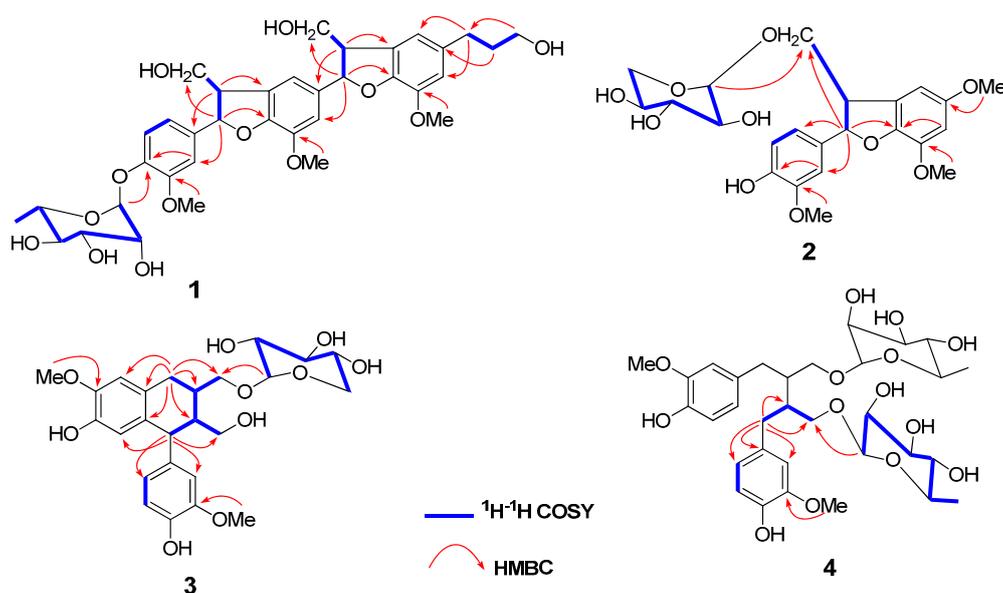


Figure 2. Key $^1\text{H}-^1\text{H}$ COSY and HMBC correlations of compounds 1–4.

Table 1. NMR data of compound **1** (in CD₃OD, *J* in Hz).

No.	δ_C	δ_H	No.	δ_C	δ_H
1	138.6		1''	137.1	
2	111.4	7.01 d (2.0)	2''	112.0	6.72 s
3	152.2		3''	145.3	
4	146.7		4''	147.5	
5	119.7	7.07 d (8.5)	5''	129.9	
6	119.2	6.89 dd (8.5, 2.0)	6''	117.9	6.72 s
7	88.8	5.58 d (6.0)	7''	32.9	2.61 t (7.5)
8	55.5	3.49 m	8''	35.8	1.80 m
9	65.1	3.83 m	9''	62.3	3.55 t (6.5)
1'	137.0		1'''	101.5	5.33 d (1.5)
2'	112.0	6.92 s	2'''	72.1	4.04 dd (3.5, 2.0)
3'	145.6		3'''	72.3	3.86 m
4'	149.3		4'''	73.9	3.43 t (9.5)
5'	130.1		5'''	70.9	3.78 m
6'	115.8	6.92 s	6'''	18.0	1.20 d (6.0)
7'	89.2	5.52 d (6.5)	3-MeO	56.5	3.80 s
8'	55.5	3.49 m	3'-MeO	56.8	3.85 s
9'	64.9	3.75 m	3''-MeO	56.9	3.85 s

These spectral features implied that the aglycone of **1** should be a dimer of dihydrobenzofuran neolignan and was the same as vitrifol A [8]. The rhamnose unit was located at C-4 as elucidated by the HMBC correlation between δ_H 5.33 (H-1''') and δ_C 146.7 (C-4) (Figure 2). The α -orientation of the sugar was determined by the small coupling constant ($J = 1.5$ Hz) of the anomeric proton H-1''' and its L-configuration was established by HPLC analysis after acid hydrolysis. By comparison with reported data [9], the relative configuration of H-7/H-8 and H-7'/H-8' was determined as *threo* according to their coupling constants ($J_{7,8} = 6.0$ Hz and $J_{7',8'} = 6.5$ Hz). The absolute configuration of **1** was established as 7*R*,8*S* and 7'''*R*,8'''*S* on the basis of the negative Cotton effects at 237 and 290 nm in the circular dichroism (CD) spectrum as shown in Figure S8, Supplementary Materials [10]. From the above analysis, compound **1** was determined to be vitrifol A 4- α -L-rhamnopyranoside, and named as difengpioside A. To the best of our knowledge, this is the first report of a dihydrobenzofuran sesquiliglan glucoside from the family Illiciaceae.

Compound **2** was isolated as an amorphous powder. The HRESIMS spectrum showed a peak at m/z 487.1572 [$M + Na$]⁺ (calcd. for 487.1575), corresponding to the molecular formula C₂₃H₂₈O₁₀. The IR spectrum displayed the presence of hydroxyl (3412 cm⁻¹) and aromatic ring (1595 and 1501 cm⁻¹) groups. The ¹H-NMR spectrum (Table 2) displayed AMX aromatic spin-system signals at δ_H 6.76 (1H, d, $J = 8.0$ Hz), 6.83 (1H, dd, $J = 8.0, 1.5$ Hz) and 6.97 (1H, d, $J = 1.5$ Hz), two aromatic H-atom signals at δ_H 7.02 (1H, s) and 6.56 (1H, s), three aromatic methoxy groups at δ_H 3.83 (3H, s), 3.82 (3H, s) and 3.79 (3H, s), and an anomeric proton of xylose at δ_H 4.31 (1H, d, $J = 7.5$ Hz) which indicated a β -configuration for the xylosyl moiety. The ¹³C-NMR spectrum (Table 2) showed the presence of 23 carbons including twelve aromatic carbon signals of two benzene rings, an oxygenated methine carbon at δ_C 89.0, a methyleneoxy carbon at δ_C 72.8, three methoxy carbons at δ_C 57.8, 56.6 and 56.4, and a methine carbon at δ_C 52.7. Additionally, five carbon signals (δ_C 105.0, 77.9, 74.9, 71.2, and 66.9) were ascribed to a xylosyl moiety. The NMR data were quite identical to those of the known compound 2,3-dihydro-7-methoxy-2-(4'-hydroxy-3'-methoxyphenyl)-3a-O- β -D-xylopyranosyloxymethyl-5-benzofuranpropanol [5]. The only difference lies in that the propanol moiety at C-1' of the known compound was replaced by a methoxyl group in **2**, as confirmed by the HBMC correlation from δ_H 3.82 (OCH₃) to δ_C 151.6 (C-1') (Figure 2) and the molecular formula of **2**. The xylose unit was located at C-9 as elucidated by the HMBC correlation between δ_H 4.31 (H-1'') and δ_C 72.8 (C-9) and its D-configuration was detected by direct comparison with an authentic sample on HPLC after acid hydrolysis. The absolute configurations of C-7 and

C-8 of **2** were determined as *7R,8S*, on the basis of their coupling constant ($J_{7,8} = 6.5$ Hz), indicating H-7 and H-8 to be *threo*, and the negative Cotton effects at 243 and 287 nm in the CD spectrum [10]. Compound **2** was thus identified as (2*R*,3*S*)-2,3-dihydro-5,7-dimethoxy-2-(4'-hydroxy-3'-methoxyphenyl)-3a-*O*- β -D-xylopyranosyloxymethylbenzofuran and named difengpioside B.

Table 2. NMR data of compounds **2–4** (in CD₃OD, *J* in Hz).

No.	2		3		4	
	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H
1	134.7		138.6		133.4	
2	110.6	6.97 d (1.5)	113.9	6.67 d (2.0)	113.3	6.52 s
3	149.0		148.9		148.9	
4	147.4		145.9		145.6	
5	116.1	6.76 d (8.0)	116.0	6.72 d (8.0)	115.9	6.65 d (8.5)
6	119.7	6.83 dd (8.0, 1.5)	123.2	6.59 dd (8.0, 2.0)	122.7	6.50 d (8.5)
7	89.0	5.44 d (6.5)	47.8	3.84 br d (11.5)	36.2	2.62 d (7.5)
8	52.7	3.57 dd (13.5, 6.5)	48.1	1.75 m	41.6	2.05 m
9	72.8	3.99 dd (9.5, 7.5) 3.81 overlap	61.6	3.35 dd (11.5, 3.0) 3.68 dd (11.5, 3.0)	69.4	3.34 dd (9.5, 5.5) 3.80 dd (9.5, 6.5)
1'	151.6		128.9			
2'	96.1	6.56 s	112.4	6.63 s		
3'	144.9		147.2			
4'	155.6		145.2			
5'	119.0		117.4	6.17 s		
6'	111.6	7.02 s	134.1			
7'			33.7	2.81 br d (7.5)		
8'			37.4	2.13 m		
				3.63 dd (10.0, 6.0)		
9'			73.9	3.90 dd (10.0, 6.0)		
1''	105.0	4.31 d (7.5)	105.3	4.22 d (7.5)	102.3	4.63 d (1.5)
2''	74.9	4.04 dd (8.5, 7.5)	74.9	3.19 m	72.4	3.81 dd (3.5, 1.5)
3''	77.9	3.86 dd (9.0, 4.0)	77.8	3.30 m	72.6	3.67 dd (9.5, 3.5)
4''	71.2	3.50 m	71.2	3.48 m	73.9	3.36 t (9.5)
5''	66.9	3.87 dd (11.0, 5.0) 3.20 d (11.0)	66.9	3.20 m 3.85 br d (11.5)	70.1	3.63 dd (9.5, 6.0)
6''					18.0	1.25 d (6.0)
3-MeO	56.4	3.83 s	56.3	3.77 s	56.3	3.73 s
1'-MeO	56.6	3.82 s				
3'-MeO	57.8	3.79 s	56.4	3.79 s	56.3	3.73 s

Compound **3** was obtained as an amorphous powder with the molecular formula C₂₅H₃₂O₁₀, as evidenced by HRESIMS (m/z 515.1920 [M + Na]⁺, calcd. 515.1888). The IR spectrum revealed the presence of hydroxyl (3431 cm⁻¹) and aromatic ring (1631 cm⁻¹) groups. Five typical carbon signals (δ_C 105.3, 77.8, 74.9, 71.2, and 66.9) was assigned to a xylose moiety and its β -orientation was determined by the large coupling constant ($J = 7.5$ Hz) of the anomeric proton at δ_H 4.22. The ¹³C-NMR (Table 2) and HSQC spectra revealed that the remaining signals of **3** contains twelve aromatic carbons of three benzene rings, three methylene carbons including two oxygenated ones at δ_C 73.9 and 61.6, two methoxy carbons at δ_C 56.4 and 56.3, three methine carbons at δ_C 48.1, 47.8 and 37.4. The above data were very similar to those of (+)-isolariciresinol 9-*O*- β -D-xylopyranoside (**5**) [11], except that the difference in the chemical shifts of C-9 and C-9'. This indicated the xylosyl moiety was positioned at C-9', which was further supported by HMBC correlation observed between H-1'' (δ_H 4.22) and C-9' (δ_C 73.9). The configuration of the xylosyl unit was established as D by HPLC analysis after acid hydrolysis. The absolute configuration of the chiral centers was established to be the same as that of (+)-isolariciresinol from the results of CD spectral analysis [12]. Therefore, compound **3** was elucidated as (+)-isolariciresinol-9'-*O*- β -D-xylopyranoside, with the trivial name difengpioside C. It is worth noting that although the literature had been reported a compound with the same name as **3** [13],

analysis of NMR spectra revealed that the compound actually was identical with (+)-isolariciresinol 9-O- β -D-xylopyranoside (**5**).

Compound **4**, isolated as an amorphous powder, had the molecular formula C₃₂H₄₆O₁₄ deduced from HRESIMS m/z 677.2778 [M + Na]⁺ (calcd. for 677.2780). The IR spectrum revealed the presence of hydroxyl (3426 cm⁻¹) and aromatic ring (1631 cm⁻¹) groups. The ¹H-NMR spectrum (Table 2) showed signals of 1,3,4-trisubstituted aromatic ring at δ_H 6.65 (1H, d, J = 8.5 Hz), 6.52 (1H, s) and 6.50 (1H, d, J = 8.5 Hz), a methoxy group at δ_H 3.73 (3H, s), and an anomeric proton of rhamnose at δ_H 4.63 (1H, d, J = 1.5 Hz) which indicated an α -configuration for the sugar moiety. Sixteen carbon signals, including six aromatic carbons, two methylene carbons (including one oxygenated), one methine carbon, one methoxy carbon and six rhamnopyranosyl signals, were supported by the ¹³C-NMR (Table 2) and HSQC spectra. The above spectral data combined with the molecular formula indicated **4** possessed a highly symmetrical skeleton. Acid hydrolysis of **4** liberated the L-rhamnose moiety, which was determined by HPLC analysis. The HMBC correlation between δ_H 4.63 (H-1'') and δ_C 69.4 (C-9) confirmed that L-rhamnose was linked to C-9. The NMR data of **4** were very similar to those of secoisolariciresinol diglucoside [14], except for the sugar moieties, suggesting that **4** was a diphenylbutane-type lignan dirhamnoside. The symmetrical structure feature of **4** indicated its configuration should be either 8S, 8'S or 8R, 8'R. Since two negative Cotton effects at 228 and 280 nm were observed in the CD spectrum [15], the absolute configuration of **4** was determined to be 8R, 8'R. Thus, compound **4** was elucidated as (-)-secoisolariciresinol 9,9'-di-O- α -L-rhamnopyranoside, which was named as difengpioside D.

The known compounds were identified as (+)-isolariciresinol 9-O- β -D-xylopyranoside (**5**) [11], avicularin (**6**) [16], (+)-isolariciresinol (**7**) [12], (-)-secoisolariciresinol-O- α -L-rhamnopyranoside (**8**) [17], 7S,8R-*threo*-3',9,9'-trihydroxy-3-methoxy-4',7-epoxyneolignan-4-O- α -L-rhamnopyranoside (**9**) [5], icariside E4 (**10**) [5], *threo*-4,9,9'-trihydroxy-3,3'-dimethoxy-8-O-4'-neolignan 7-O- α -rhamno-pyranoside (**11**) [18], respectively, by comparison of the spectroscopic data with those reported in the literature.

Inhibitors of NO release are considered as potential anti-inflammatory agents [19]. Since the stem barks of *Illicium difengpi* have been applied for the treatment of rheumatic arthritis in China, the isolated compounds were evaluated for their effects on the inhibition of NO production in LPS-activated RAW264.7 cells. As shown in Table 3, the dihydrobenzofuran-type (**1**, **2**, **9**, and **10**) and aryltetralin-type (**3**, **5**, **6**, and **7**) lignan glycosides exhibited weak inhibitory effect against NO with inhibition ratios in the range of 3.29% to 10.53% at a concentration of 25 μ M, while the dibenzylbutane-type lignan glycosides **4** and **8** and neolignan glucoside **11** showed no inhibitory effect at the same concentration.

Table 3. Inhibitory effects of compounds 1–11 on NO production.

Compound	Conc. (μ M)	Inhibitory Rate (%)
1	25	7.24
2	25	5.69
3	25	4.61
4	25	-0.65
5	25	10.53
6	25	3.95
7	25	3.29
8	25	1.32
9	25	3.30
10	25	4.05
11	25	0.66
MG132 ^a	0.125	91.19

^a Positive control, carbobenzoxy-leu-leu-leucinal.

3. Materials and Methods

3.1. General Information

Melting points were obtained on an X-4 micro melting point apparatus (Shanghai Jingke Scientific Instrument Co., Ltd, Shanghai, China). Optical rotations were measured with a P-1020 polarimeter (JASCO, Tokyo, Japan). UV spectra were obtained on a UV-2401A spectrophotometer (Shimadzu, Kyoto, Japan). CD spectra were recorded on a J-810 CD spectrometer (JASCO, Tokyo, Japan). IR spectra were measured in a FTS-135 spectrometer (Bio-Rad, Richmond, CA, USA) with KBr pellets. HRESIMS were recorded on a LCMS-IT-TOF spectrometer (Shimadzu, Kyoto, Japan). The NMR spectra were recorded on a DRX-500 spectrometer (Bruker Co., Ettlingen, Germany) with TMS as internal standard, and chemical shifts (δ) were expressed in ppm with reference to the solvent signals. Silica gel (200–300 mesh; Qingdao Marine Chemical Inc., Qingdao, China), D101 macroporous resin (Nankai University, Tianjin, China), ODS (40–63 μm ; Merck, Darmstadt, Germany), and Sephadex LH-20 (Amersham Pharmacia Biotech, Uppsala, Sweden) were used for column chromatography. Semipreparative HPLC was performed on an Agilent 1200 apparatus equipped with a UV detector and a Zorbax SB-C-18 (9.4 mm \times 25 cm, Agilent Technologies, Santa Clara, CA, USA) column. Analytical HPLC was performed on a Shimadzu HPLC system equipped with a refractive index detector and a CARBOsep COREGEL-87C Ca^+ (7.8 \times 300 mm, 9 μm , Transgenomic Inc., Omaha, NE, USA) column. Fractions were monitored by TLC and spots were visualized by heating silica gel plates sprayed with 10% H_2SO_4 in EtOH. Solvents were distilled before use.

3.2. Plant Material

The stem barks of *Illicium difengpi* were collected from Longzhou County, Guangxi Province, China, in October 2010 and identified by Prof. H. Tang. A voucher specimen (CTM201002) was deposited at the Guangxi Key Laboratory of Functional Phytochemicals Research and Utilization, Guangxi Institute of Botany, China.

3.3. Extraction and Isolation

The air-dried stem barks of *I. difengpi* (14 kg) were extracted with 95% EtOH (20 L \times 3) at room temperature for 24 h. The dried EtOH extract (1.3 kg) was suspended in H_2O and then partitioned successively with petroleum ether (1.5 L \times 3), EtOAc (1.5 L \times 3) and *n*-BuOH (1.5 L \times 3). The *n*-butanol portion (540 g) was applied to a D101 macroporous resin column eluted with water, 10%, 30%, 60%, 90% and 100% methanol. The 30% methanol fraction (32 g) was further purified by an ODS column and a preparative HPLC (CH_3CN – H_2O , 15:85) to yield compounds **1** (9 mg) and **4** (12 mg). The 60% methanol fraction (26 g) was subjected to a Sephadex LH-20 column eluted with MeOH to give five fractions (Fr. 1–5). Fr.3 was further separated by an ODS column and preparative HPLC (CH_3CN – H_2O , 15:85 to 30:70) to obtain compounds **8** (25 mg), **10** (30 mg) and **11** (8 mg). Fr.4 was subjected to silica gel column chromatography (CHCl_3 –MeOH– H_2O , 5:1:0.1 to 3:1:0.1) and preparative HPLC (CH_3CN – H_2O , 15:85 to 30:70) to obtain compounds **2** (6 mg), **3** (10 mg), **5** (35 mg), **6** (33 mg) and **9** (40 mg). The 90% methanol fraction (12 g) was further purified by silica gel column chromatography (CHCl_3 –MeOH, 5:1 to 0:1) and preparative HPLC (CH_3CN – H_2O , 30:70) to yield compound **7** (10 mg).

3.4. Compound Characterization

Difengpioside A (**1**): Amorphous powder; $[\alpha]_{\text{D}}^{20} +25$ (c 0.2, MeOH); IR (KBr) ν_{max} : 3406, 2933, 1609, 1511, 1267, 1030 cm^{-1} ; UV (MeOH) λ_{max} (log ϵ) nm: 204 (4.47), 281 (3.55); CD (MeOH, nm) λ_{max} ($\Delta\epsilon$) 290 (–3.18), 254 (–2.55), 237 (–3.27); ^1H and ^{13}C -NMR data, see Table 1; HRESIMS m/z 707.2581 $[\text{M} + \text{Na}]^+$ (calcd for $\text{C}_{36}\text{H}_{44}\text{O}_{13}\text{Na}$, 707.2674).

Difengpioside B (**2**): white amorphous powder; $[\alpha]_{\text{D}}^{20} +21$ (c 0.2, MeOH); IR (KBr) ν_{max} : 3412, 2937, 1595, 1501, 1461, 1124, 1034 cm^{-1} ; UV (MeOH) λ_{max} (log ϵ) nm: 206 (4.87), 281 (3.58); CD (MeOH,

nm) λ_{\max} ($\Delta\epsilon$) 287 (−3.57), 254 (−3.39), 243 (−4.10), 213 (−2.90); ^1H and ^{13}C -NMR data, see Table 2; HRESIMS m/z 487.1572 $[\text{M} + \text{Na}]^+$ (calcd for $\text{C}_{23}\text{H}_{28}\text{O}_{10}\text{Na}$, 487.1575).

Difengpioside C (3): white amorphous powder; $[\alpha]_{\text{D}}^{20}$ +16 (c 0.2, MeOH); IR (KBr) ν_{\max} : 3431, 2924, 1631, 1384, 1049 cm^{-1} ; UV (MeOH) λ_{\max} ($\log\epsilon$) nm: 204 (3.45), 281 (2.59); CD (MeOH, nm) λ_{\max} ($\Delta\epsilon$) 292 (−0.21), 277 (0.16), 239 (0.20), 213 (0.56); ^1H and ^{13}C -NMR data, see Table 2; HRESIMS m/z 515.1967 $[\text{M} + \text{Na}]^+$ (calcd for $\text{C}_{25}\text{H}_{32}\text{O}_{10}\text{Na}$, 515.1888).

Difengpioside D (4): white amorphous powder; $[\alpha]_{\text{D}}^{20}$ −32 (c 0.2, MeOH); IR (KBr) ν_{\max} : 3426, 2932, 1631, 1141 cm^{-1} ; UV (MeOH) λ_{\max} ($\log\epsilon$) nm: 204 (4.68), 281 (3.64); CD (MeOH, nm) λ_{\max} ($\Delta\epsilon$) 280 (−0.89), 228 (−1.98), 211 (−5.35); ^1H - and ^{13}C -NMR data, see Table 2; HRESIMS m/z 677.2778 $[\text{M} + \text{Na}]^+$ (calcd for $\text{C}_{32}\text{H}_{46}\text{O}_{14}\text{Na}$, 677.2780).

3.5. Determination of the Absolute Configuration of the Sugars in Compounds 1–4

Each compound (1.5 mg) was dissolved in 0.5 M HCl (0.2 mL) and heated at 80 °C for 2 h. The mixture was desalinated by passing through columns of anion and cation exchange resin and then filtered. The filtrate was directly analyzed by a Shimadzu HPLC system equipped with a refractive index detector and a CARBOsep COREGEL-87C Ca^+ column (7.8 × 300 mm, 9 μm , Transgenomic Inc.) at 85 °C with elution of HPLC grade water for 18 min at a flow rate of 0.5 mL/min. The injection volume was 20 μL . The standards L-rhamnose and D-xylose were treated by the same water and chromatographic conditions. The sugars from each compound were identified by comparison of their retention times with those for authentic standards (t_{R} : 13.67 min for L-rhamnose, 13.13 min for D-xylose).

3.6. NO Production Inhibition Assay

Assays for NO production were carried out as previously described [20]. Briefly, RAW 264.7 macrophages were harvested and seeded in 96-well plates (3×10^4 cells/well) for measurement of NO production. The plates were pretreated with various samples for 30 min and then incubated with 1 $\mu\text{g}/\text{mL}$ LPS for 24 h. The inhibitory effects of the isolated compounds on NO production were evaluated by using the Griess reagent.

4. Conclusions

Eleven compounds, including four new lignan glucosides 1–4, named difengpiosides A–D, were isolated from the *n*-BuOH-soluble fraction of an EtOH extract of the stem barks of *I. difengpi*. All compounds showed weak or no inhibitory activities against NO production at the concentration of 25 μM , indicating that the polar constituents of this plant showed no inflammatory activity *in vitro*.

Supplementary Materials: Supplementary materials can be accessed at: <http://www.mdpi.com/1420-3049/21/5/607/s1>. HRESIMS, 1D- and 2D-NMR, IR, and CD spectra of four new compounds 1–4.

Acknowledgments: This work was supported by the Guangxi Scientific Research and Technology Development Program (14125008-2-23), the Science Research Foundation of Guangxi Institute of Botany (13015), the Foundation of Guangxi Key Laboratory of Functional Phytochemicals Research and Utilization (ZRJJ2014-2), and the Bagui Scholar Program of Guangxi.

Author Contributions: Zheng-Hong Pan and Dian-Peng Li organized and designed the experiments; Zheng-Hong Pan wrote the manuscript; De-Sheng Ning, Si-Si Huang, Ling Cheng, and Meng-Wen Xia performed the experiments; De-Sheng Ning conducted NMR measurement and analyzed the data; Li-Yan Peng helped to evaluate the bioactivities.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Editorial Committee of Chinese Pharmacopoeia. *Chinese Pharmacopoeia*, 2015 ed.; China Medical Science Press: Beijing, China, 2015; p. 123.

2. Huang, P.; Xi, Z.M.; Zheng, X.Z.; Lai, M.X.; Zhong, X.Q. Studies on the chemical constituents of the Traditional Chinese Medicine “DIFENGPI”. *Acta Pharm. Sin.* **1996**, *31*, 278–281.
3. Huang, P.; Xi, Z.M.; Zheng, X.Z.; Lai, M.X.; Zhong, X.Q. Triterpene acids from the barks of *Illicium difengpi*. *Acta Pharm. Sin.* **1997**, *32*, 704–707.
4. Kouno, I.; Yanagida, Y.; Shimono, S.; Shintomi, M.; Yang, C.S. Phenylpropanoids from the barks of *Illicium difengpi*. *Chem. Pharm. Bull.* **1992**, *40*, 2461–2464. [[CrossRef](#)]
5. Kouno, I.; Yanagida, Y.; Shimono, S.; Shintomi, M.; Ito, Y.; Yang, C.S. Neolignans and a phenylpropanoid glucoside from *Illicium difengpi*. *Phytochemistry* **1993**, *32*, 1573–1577. [[CrossRef](#)]
6. Fang, L.; Du, D.; Ding, G.Z.; Si, Y.K.; Yu, S.S.; Liu, Y.; Wang, W.J.; Ma, S.G.; Xu, S.; Qu, J.; et al. Neolignans and glycosides from the stem barks of *Illicium difengpi*. *J. Nat. Prod.* **2010**, *73*, 818–824. [[CrossRef](#)] [[PubMed](#)]
7. Fang, L.; Wang, X.J.; Ma, S.G.; Yu, S.S. A new sesquiterpene lactone and a new aromatic glycoside from *Illicium difengpi*. *Acta Pharm. Sin. B* **2011**, *1*, 178–183. [[CrossRef](#)]
8. Gu, Q.; Zhang, X.M.; Zhou, J.; Qiu, S.X.; Chen, J.J. One new dihydrobenzofuran lignan from *Vitex trifolia*. *J. Asian Nat. Prod. Res.* **2008**, *10*, 499–502. [[CrossRef](#)] [[PubMed](#)]
9. Li, S.; Iliefski, T.; Lundquist, K.; Wallis, A.F.A. Reassignment of relative stereochemistry at C-7 and C-8 in arylcoumaran neolignans. *Phytochemistry* **1997**, *46*, 929–934. [[CrossRef](#)]
10. Kim, T.H.; Ito, H.; Hayashi, K.; Hasegawa, T.; Machiguchi, T.; Yoshida, T. Aromatic constituents from the heartwood of *Santalum album* L. *Chem. Pharm. Bull.* **2005**, *53*, 641–644. [[CrossRef](#)] [[PubMed](#)]
11. Zhang, J.J.; Ma, Y.P.; Yuan, J.Z.; Sun, Q.S. Isolation and identification of lignans from pine needles of *Pinus koraiensis* Sieb. et Zucc. *J. Shenyang Pharm. Univ.* **2010**, *27*, 797–802.
12. Baderschneider, M.; Winterhalter, P. Isolation and characterization of novel benzoates, cinnamates, flavonoids, and lignans from Riesling wine and screening for antioxidant activity. *J. Agric. Food Chem.* **2001**, *49*, 2788–2798. [[CrossRef](#)] [[PubMed](#)]
13. Bu, P.B.; Li, Y.R.; Jiang, M.; Wang, X.L.; Wang, F.; Lin, S.; Zhu, C.G.; Shi, J.G. Lignans from *Machilus robusta*. *Chin. J. Chin. Mater. Med.* **2013**, *38*, 1740–1746.
14. Qiu, S.X.; Lu, Z.Z.; Luyengi, L.; Lee, S.K.; Pezzuto, J.M.; Farnsworth, N.R.; Thompson, L.U.; Fong, H.H.S. Isolation and characterization of Flaxseed (*Linum usitatissimum*) constituents. *Pharm. Biol.* **1999**, *37*, 1–7. [[CrossRef](#)]
15. Xie, L.H.; Akao, T.; Hamasaki, K.; Deyama, T.; Hattori, M. Biotransformation of pinoresinol diglucoside to mammalian lignans by human intestinal microflora, and isolation of enterococcus faecalis strain PDG-1 responsible for the transformation of (1)-pinoresinol to (1)-lariciresinol. *Chem. Pharm. Bull.* **2003**, *51*, 508–515. [[CrossRef](#)] [[PubMed](#)]
16. Liu, J.F.; Zhang, X.M.; Shi, Y.; Jiang, Z.Y.; Ma, Y.B.; Chen, J.J. Chemical constituents from rhizomes of *Illicium henryi*. *Chin. J. Chin. Mater. Med.* **2010**, *35*, 2281–2284.
17. Chen, X.C.; Jia, Z.J. Two new glycosides from *Rubus amabilis*. *Chinese Chem. Lett.* **2000**, *11*, 897–900.
18. Liu, J.F.; Jiang, Z.Y.; Geng, C.A.; Zhang, Q.; Shi, Y.; Ma, Y.B.; Zhang, X.M.; Chen, J.J. Two new lignans and anti-HBV constituents from *Illicium henryi*. *Chem. Biodivers.* **2011**, *8*, 692–698. [[CrossRef](#)] [[PubMed](#)]
19. Kerwin, J.F., Jr.; Lancaster, J.R., Jr.; Feldman, P.L. Nitric oxide: A new paradigm for second messengers. *J. Med. Chem.* **1995**, *38*, 4343–4362. [[CrossRef](#)] [[PubMed](#)]
20. Zhang, L.; Dong, L.; Huang, J.; Liu, M.S.; Li, G.Y.; Zhang, C.; Zhang, K.; Wang, J.H. 3, 4-seco-Labdane diterpenoids from the leaves of *Callicarpa nudiflora* and their inhibitory effects on nitric oxide production. *Fitoterapia* **2013**, *89*, 218–223. [[CrossRef](#)] [[PubMed](#)]

Sample Availability: Samples of the compounds 1–11 are not available from the authors.



© 2016 by the authors; licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC-BY) license (<http://creativecommons.org/licenses/by/4.0/>).