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Short communication

Six new dihydrobenzofuran lignans from the branches and leaves of *Illicium wardii* and their cytotoxic activities

Feng-mei Ye[a], Yang-guo Xie[a], Jie Ren[a], Ji Ye[b], Yi-gong Guo[a], Shi-Kai Yan[a], Hui-Zi Jin[a,*], Wei-Dong Zhang[a,b,**]

[a] School of Pharmacy, Shanghai Jiao Tong University, Shanghai 200240, PR China
[b] Department of Phytochemistry, Second Military Medical University, Shanghai 200433, PR China

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ABSTRACT

Six new dihydrobenzofuran lignans, named illiciumlignans A–F (compounds **1–6**), along with 15 known compounds (**7–21**) were isolated from the branches and leaves of *Illicium wardii*. The structures of **1–6** were determined using a combination of 1D and 2D NMR, HR-ESI--MS, and CD spectroscopic data. Illiciumlignan D (**4**) is the first reported dihydrobenzofuran lignan arabinofuranoside that is derivatized with the arabinofuranose moiety on C-9′. Compounds **1–21** were evaluated for cytotoxic activity against four human cancer cell lines. Compounds **8**, **12** and **20** exhibited significant activity against human cancer cell lines (A549, SKOV3, HepG2 and HCT116), with IC_{50} values ranging from 2.7 to 14.9 μM.

1. Introduction

Illiciaceae is a single-genus family made up of the genus *Illicium*. Globally, there are about fifty species of *Illicium* known with the majority being found in the southeastern portions of Asia and America. In China, there are about twenty-eight species and two variants, and these are distributed in the southwestern part of the country. Among these species, twenty-one are found exclusively in China, and some of them are used to treat rheumatism, traumatic injury, and stomach cold vomiting in Traditional Chinese Medicine (Ye et al., 2015). *Illicium wardii* A.C. Smith, which grows as evergreen trees or shrubs, was found as bushes in uncultivated land near brooks at elevations of up to 1800–2700 m above sea level (Gao et al., 2015). Our group has studied other plants of the *Illicium* genus, but the phytochemical investigation of *I. wardii* has seldom been reported, except for the isolation of fourteen compounds from its fruits in 2007 and 2014 (Min et al., 2007; Gao et al., 2014). In this work, phytochemical investigation of the branches and leaves of *I. wardii* led to the isolation and identification of six new dihydrobenzofuran lignans (**1–6**) and 15 (**7–21**) previously known lignins. Their structures were elucidated by different spectroscopic methods including NMR, CD, IR as well as HRESIMS analysis. In addition, these twenty-one compounds were tested for cytotoxicity against four tumor cell lines using the MTT assay.

2. Results and discussion

The EtOAc-soluble portion of the 95% EtOH extract of the branches and leaves of *I. wardii* was fractioned by silica gel, MCI, Sephadex LH-20, and preparative HPLC to afford illicium lignans A–F (**1–6**), as well as acernikol (**7**) (Toshio et al., 2003), isodunnianol (**8**) (Konno et al., 1991), dihydrodehydrodiconiferylalcohol-9-*O*-β-D-xylopyranoside (**9**) (Konno et al., 1993), (7′*S*, 8′*R*)-dihydrodehydrodiconiferyl alcohol (**10**) (Seidel et al., 2000), hinokinin (**11**) (Wenkert et al., 1976), honokiol (**12**) (Sun et al., 2005), (7*S*, 8*R*)-dihydrodehydrodiconiferyl alcohol 4′-*O*-β-D-glucopyranoside (**13**) (Matsuda et al., 1996), (7′*R*, 8′*R*)-dihydrodehydrodiconiferyl alcohol-9′-*O*-β-D-glucopyranoside (**14**) (Lee et al., 2014), (7*S*, 8*R*)-dihydrodehydrodiconiferyl alcohol 9-*O*-β-D-(3″-*O*-acetyl)xylopyranoside (**15**) (Liu et al., 2011), (−)-massoniresinol (**16**) and (Shen and Theander, 1985), (7′*S*, 8′*S*)-dihydrodehydrodiconiferyl alcohol 9-*O*-β-D-glucopyranoside (**17**) (Liu et al., 2011), (+)-5′-methoxyisolariciresinol-9′-*O*-α-L-rhamnopyranoside (**18**) (Fuchino et al., 1995), 1-(4-hydroxy-3-methoxyphenol-2-[4-(3-rhamnopyrnoxy-propyl)-2-methoxyphenoxyl]-1,3- propanediol(erythro)) (**19**)

* Corresponding author.
** Corresponding author at: School of Pharmacy, Shanghai Jiao Tong University, Shanghai 200240, PR China.
E-mail addresses: kimhz@sjtu.edu.cn (H.-Z. Jin), wdzhangy@hotmail.com (W.-D. Zhang).

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(Liang et al., 2013), dunnianol (**20**) (Konno et al., 1993), and (7′*S*, 8′*S*)-dihydrodehydrodiconiferyl alcohol (**21**) (Liu et al., 2014). The known compounds were identified by comparison of their NMR, MS and CD spectroscopic data with the literature reports.

Illiciumlignan A (**1**) was obtained as a yellowish oil with positive optical rotation ([α]20*D* +3.0°). The molecular formula was found to be ($C_{30}H_{34}O_9$), which was determined by high resolution ESI–MS analysis (*m*/*z* 561.2109 [M+Na]$^+$, 561.2095 calcd.). The ^{1}H NMR spectrum displayed signals at δ_H 6.93 (brs), 6.81 (d, *J* = 8.0, 1.7 Hz), and 6.76 (d, *J* = 8.1 Hz) suggestive of an ABX spin system and four singlets (4H) at δ_H 6.93, 6.92, 6.73 × 2 corresponding to two tetrasubstituted aromatic rings with four benzylic methines. The remaining ^{1}H NMR signals showed two &9472;O—CH—CH—CH_2—O-spin systems, three methoxy groups, and an *n*-propyl group. The ^{13}C and DEPT-NMR spectra revealed the presence of three methoxy groups, five methylenes, eleven methines, and eleven quaternary carbons (Table 1). By comparing the IR, UV and NMR spectroscopic data for **1** with the literature (Gu et al., 2008), **1** was found to be almost identical to vitrifol A, except for the configuration at C-7′, C-8′, C-7″ and C-8″. In the NOESY spectrum, the correlations between H-7′ and H-8′ as well as H-7″ and H-8″ indicated erythro arrangements for phenyl and hydroxymethylene groups at C-7′ and C-8′ and the two hydroxyl groups at C-7″ and C-8″. Additionally, the absolute configurations at C-8′, 8″ and C-7′, 7″ were elucidated as 8′ (*R*),8″(*R*) and 7′ (*R*),7″(*R*) on the basis of CD data (a positive Cotton effect at 293 nm) (Zhang et al., 2014; Xiong et al., 2011; Huang et al., 2013). On the basis of this evidence, the absolute configuration of **1** was determined and the compound was named illiciumlignan A.

Illiciumlignan B (**2**) was obtained as yellowish solid with a negative optical rotation ([α]20*D* −11.2°). **2** possessed the molecular formula ($C_{32}H_{38}O_{11}$), as revealed by high resolution ESI–MS analysis (*m*/*z* 621.2313 [M+Na]$^+$, 621.2306 calcd.). Analysis of the NMR spectra revealed that **2** was almost identical to **1**, except for the presence of an ethanoyl moiety and lack of a methoxy group. The ethanoyl moiety placement at C-9 was based upon the observation of downfield shifts assigned to H_2-9 (δ_H 4.06, t, *J* = 6.6 Hz; 3.57, t, *J* = 6.5 Hz) and C-9 (δ_C 63.4, 62.3) while comparing the ^{1}H NMR and ^{13}C NMR spectra of **2** with those of **1** (Fig. 2). The absolute configurations at C-8′, 8″ and C-7′, 7″ were identical to **1** based on the CD spectrum (positive Cotton effects at 293 and 241 nm). On the basis of the above evidence, the absolute configuration of **2** was determined and the compound was named illiciumlignan B.

Illiciumlignan C (**3**) was obtained as yellowish solid with a negative optical rotation ([α]20*D* −3.33°). The molecular formula was ($C_{25}H_{32}O_{10}$), which was ascertained via high resolution ESI–MS analysis (*m*/*z* 537.1985 [M+COOH]$^+$, 537.1978, calcd.). The ^{1}H NMR spectrum displayed signals at δ_H 6.98 (1H, brs), 6.84 (1H, d, *J* = 8.2 Hz), 6.76 (1H, d, *J* = 8.2 Hz) corresponding to an ABX spin system and two singlets (2H) at δ_H 6.77 (1H, brs, H-6) and 6.72 (1H, brs, H-2), suggestive of a tetra substituted aromatic ring with two benzylic methines. The remaining ^{1}H NMR signals showed one &9472;O—CH—CH—CH_2—O-spin system, two methoxy groups, and *n*-propyl and xylose moieties. The ^{13}C and DEPT-NMR spectrum revealed the presence of two methoxy groups, four methylenes, seven methines, seven quaternary carbons, and a xylose moiety (Table 3). Acidic hydrolysis of **3** in HCl-methanol (2 M) yielded (7′*S*, 8′*S*)-dihydrodehydrodiconiferyl alcohol and a xylose, identified as β-D-xylopyranose by GC comparison with an authentic sample and both showed the same ^{1}H NMR coupling constant [$J_{(1'',2'')}$ = 7.5 Hz] (Li et al., 1984; Jiang et al., 2004; Wang et al., 2014). The cross-peak between H-1″ and C-9′ in the HMBC spectrum indicated that the xylopyranose group is linked to C-9′.

Table 1
^{1}H-, ^{13}C- and 2D-NMR data for compound **1** recorded at 500 (^{1}H) and 125 (^{13}C, 2D) MHz in CD_3OD.

No.	1					
	δ_c	DEPT	δ_H (J in Hz)	HMBC	COSY	NOESY
1	135.6					
2	112.7	CH	6.73 brs	C-7,6,3,4		
3	144.0					
4	146.4					
5	128.4					
6	116.5	CH	6.73 brs	C-7,2,3,4		
7	31.5	CH_2	2.62 t (7.7)	C-1,2,6,8,9	H-8	
8	34.4	CH_2	1.82 m	C-1,7,9	H-7,9	H-2,6,7,9
9	60.8	CH_2	3.56 t (6.5)	C-7,8	H-8	
1′	135.4					
2′	110.4	CH	6.91 brs	C-6′,4′,3′		
3′	143.8					
4′	147.8					
5′	128.9					
6′	114.8	CH	6.93 brs ovl	C-7′,2′		
7′	87.8	CH	5.52 d (6.2)	C-9′,8′,6′,2′,1′,5,4	H-8′	H-8′,9′
8′	54.0	CH	3.50 m ovl	C-9′,7′,6′,1′,6,5,4	H-7′,9′	H-7′,9′
9′	63.6	CH_2	3.86,3.80 m	C-8′,7′,5		
1”	132.9					
2”	109.2	CH	6.93 brs	C-7”,6”,4”,3”,1”		
3”	147.8					
4”	146.0					
5”	114.4	CH	6.76 d (8.2)	C-6”,4”,3”		
6”	118.3	CH	6.81 dd (1.7,8.2)	C-7”,2”		
7”	87.9	CH	5.52 d (6.2)	C-9”,8”,6”,2”,1”,5′,4′	H-8”	H-6”,8”,9”
8”	53.8	CH	3.50 m ovl	C-9”,7”,6”,1”,6′,5′,4′	H-7”,9”	H-6′,7”,9”
9”	63.4	CH_2	3.76 m			
3-OMe	55.0	OMe	3.83 s	C-3		
3′-OMe	55.4	OMe	3.85 s	C-3′		
3”-OMe	55.3	OMe	3.80 s	C-3”		

Ovl, overlap.

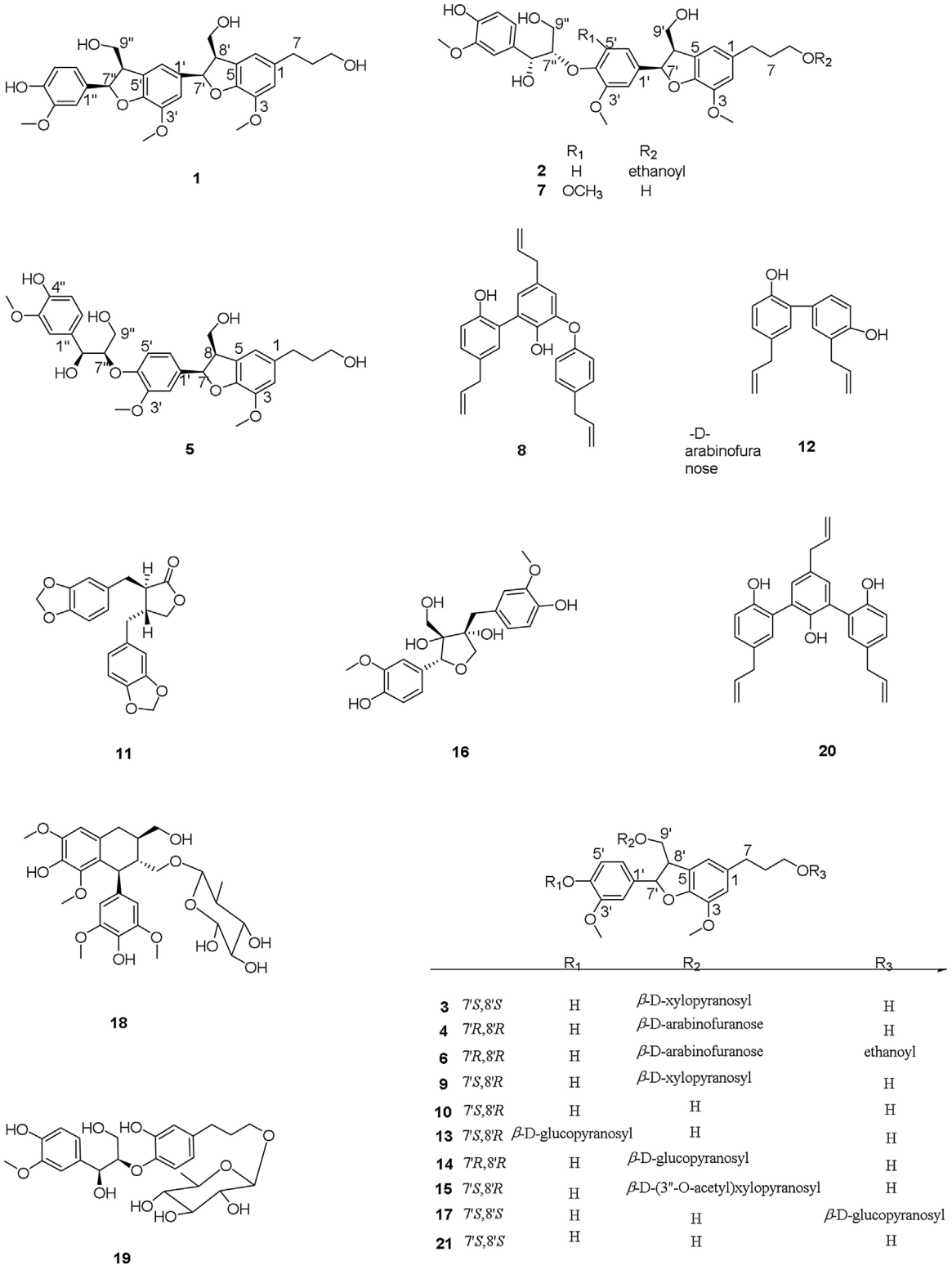

Fig. 1. Structures of compounds **1**–**21**.

By comparing IR, UV and NMR spectroscopic data for **3** with literature (Konno et al., 1993), **3** was nearly identical to 2,3-dihydro-7-methoxy-2-(4′-hydroxy-3′-methoxyphenyl)-3a-*O*-β-D-xylopyranosyloxymethyl-5- benzofuranprop-anol, except for the stereochemistry at C-7′ and C-8′. Moreover, the CD spectrum of **3** showed a negative Cotton effect at 293 nm, which indicated that the absolute configuration of the 8′-position of **3** was in the *S* orientation (Zhang et al., 2014; Xiong et al., 2011; Huang et al., 2013). In the NOESY spectrum, the correlations between H-7′ and H-8′ indicated erythro arrangements for phenyl and hydroxymethylene groups at C-7′ and C-8′, respectively. Hence, the absolute configuration at the 7′-position of **3** was in the

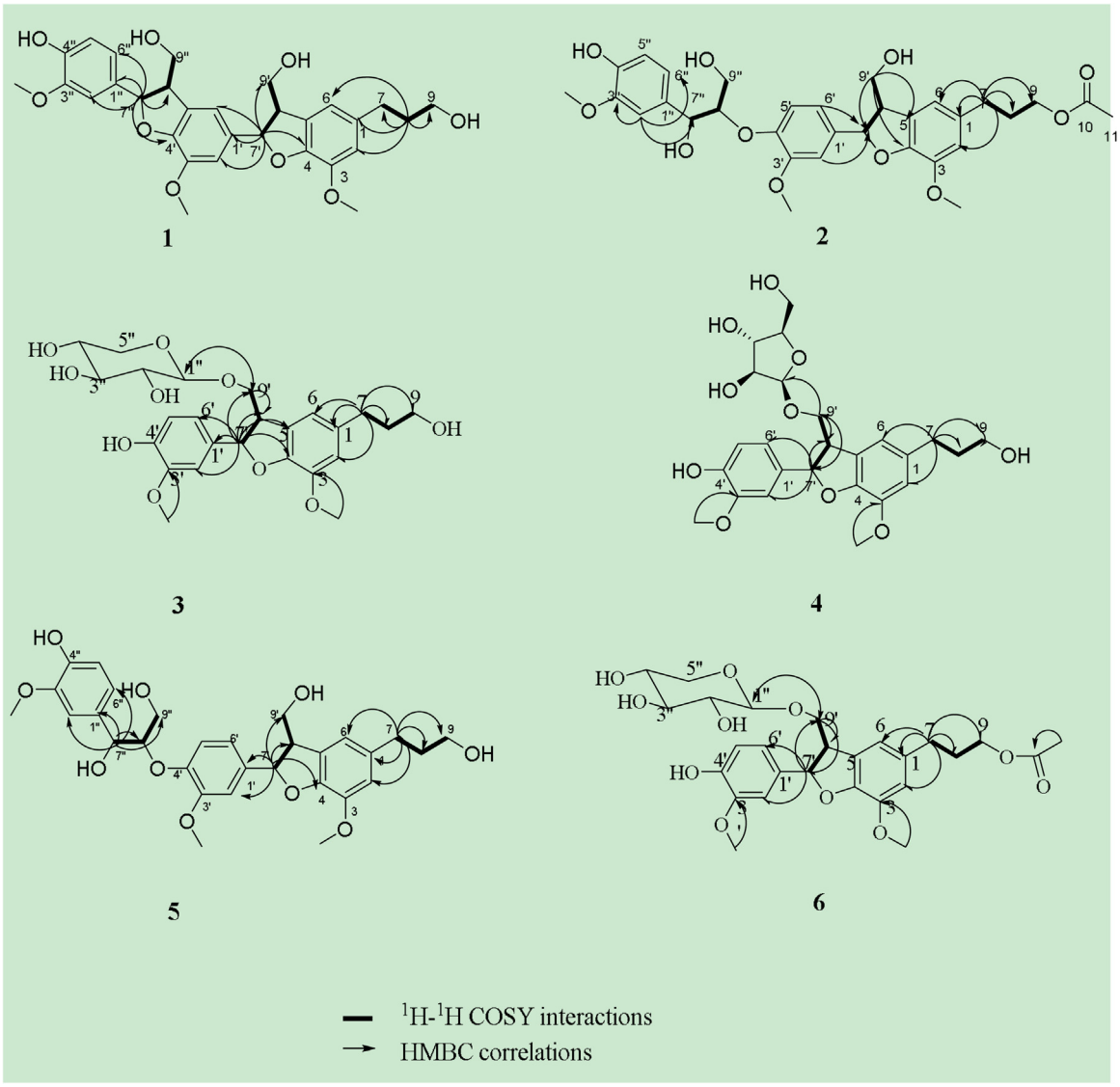

Fig. 2. Key ^{1}H–^{1}H COSY and HMBC correlations for **1**–**6**.

S orientation. On the basis of this evidence, the absolute stereochemistry of **3** was elucidated and the compound was named illiciumlignan C.

Illiciumlignan D (**4**) was obtained as yellowish solid with a negative optical rotation ([α]20*D* −26.4°). The molecular formula was found to be ($C_{25}H_{32}O_{10}$), which was determined by high resolution ESI–MS analysis(*m*/*z* 515.1888 ([M+Na]$^+$, 515.1888). Analysis of the ^{1}H and ^{13}C NMR spectra indicated the presence of the same skeleton as **3**. The major difference in the NMR spectra were β-D-arabinofuranosyl at C-9′ and the absolute configuration at C-7′ and C-8′. Acidic hydrolysis of **4** in HCl-methanol (2 M) yielded (7′*R*, 8′*R*)-dihydrodehydrodiconiferyl alcohol and arabinofuranose, which was identified as β-D-arabinofuranose by GC comparison with an authentic sample and both showed the same ^{1}H NMR coupling costant ($J_{(1'',2'')}$ = 1.4 Hz) (Kaeothip et al., 2013). The cross-peak between H-1″ and C-9′ observed in the HMBC spectrum indicated that the arabinofuranose moiety was linked to C-9′. Moreover, the CD spectrum of **4** showed a positive Cotton effect at 290 nm, which indicated that the absolute configuration of the 8′-position was in the *R* orientation (Zhang et al., 2014; Xiong et al., 2011; Huang et al., 2013). In the NOESY spectrum, the correlations between H-7′ and H-8′, indicated erythro arragements for phenyl and hydroxymethylene groups at C-7′ and C-8′, respectively (Fig. 2). Hence, the absolute configuration at the 7′-position of **4** was in the *R* orientation. Thus, the absolute configuration of **4** was elucidated and the compound was named illiciumlignan D, which is the first reported dihydrobenzofuran lignin containing an arabinofuranoside (positioned at C-9′).

Illiciumlignan E (**5**) was obtained as yellowish solid with a negative optical rotation ([α]20*D* −0.55°). The molecular formula was found to be ($C_{30}H_{36}O_{10}$), which was based on high resolution ESI–MS analysis (*m*/*z* 579.2214 [M+Na]$^+$, 579.2201 calcd.). Analysis of the NMR spectra revealed that **5** was almost identical to **7**, except for the absence of a methoxy signal. In the NOESY spectrum, the correlations observed between H-7′ and H-8′ as well as H-7″ and H-8″ indicated erythro arrangements for both the phenyl and hydroxymethylene groups at C-7′ and C-8′, and the two hydroxyl groups at C-7″ and C-8″ (Fig. 2). Moreover, the CD spectrum of **5** showed a positive Cotton effect at 290 nm and a negative Cotton effect at 240 nm, which indicated the absolute configurations of the 8′, 8″ and 7′, 7″ positions of **5** were 8′ (*R*),8″(*R*) and 7′ (*R*),7″(*S*), respectively (Zhang et al., 2014; Xiong et al., 2011; Huang et al.,

2013). Therefore, the absolute configuration of **6** was elucidated and the compound was named illiciumlignan E.

Illiciumlignan F (**6**) was obtained as yellowish crystal with a negative optical rotation ([α]20*D* −2.0°) and the molecular formula ($C_{27}H_{34}O_{11}$), which was deduced from high resolution ESI–MS analysis (*m*/*z* 579.2101 [M+COOH]$^+$, 579.2083 calcd.). Analysis of the NMR spectra revealed that **6** was almost identical with **4**, except for small differences in the chemical shifts which were attributed to the ethanoyl moiety. The ethanoyl moiety was positioned at C-9 based upon the observation of downfield shifts in the signals assigned to H_2-9 (δ_H 4.05, t, *J* = 6.6 Hz; 3.56, t, *J* = 6.4 Hz) and C-9 (δ_C 63.6, 60.8) while comparing the ^{1}H NMR and ^{13}C NMR spectra of **6** with those of **3**. In the NOESY spectrum, the correlations observed between H-7′ and H-8′ indicated erythro arrangements for phenyl and hydroxymethylene groups at C-7′ and C-8′ (Fig. 2). Moreover, the CD spectrum of **6** showed a positive Cotton effect at 293 nm, which indicated the absolute configurations of the 8′ and 7′-position of **6** were 8′ (*R*) and 7′ (*R*), respectively. Therefore, **6** was elucidated as shown and the compound was named illiciumlignan F.

Compounds **1**–**21** were tested for their cytotoxicity against human lung cancer (A549), colon cancer (HCT116), ovarian neoplasm (SKOV3), and hepatocellular carcinoma (HepG2) cell lines using the MTT assay using Doxorubicin as a positive control. The results of this survey are as follows: Doxorubicin had IC_{50} values of 0.72, 0.16, 3.09, and 0.10 μM against the A549, HCT116, SKOV3, and HepG2 cell lines, respectively. **8**, **12**, and **20** showed moderate activity against the A549 cell line with IC_{50} values of 14.88, 26.04, and 28.22 μM, respectively. **8** and **20** had better cytotoxicity against the HCT116 cell line with respective IC_{50} values of 2.78 and 2.70 μM than **10** and **12** with respective IC_{50} values of 80.47 and 20.48 μM. Compounds **4**, **5**, **8**, **10**, **18**, and **20** showed IC_{50} values ranging from 6.08 μM to 13.31 μM against the SKOV3 cell line. **8**, **12**, and **20** showed moderate activity against the HepG2 cell line with IC_{50} values of 5.65, 14.61, and 11.31 μM, respectively. None of the remaining compounds showed significant cytotoxicity against the above cell lines (IC_{50} > 100 μM) (Figs. 1 and 2, Table 4).

This study describes the isolation of six new dihydrobenzofuran lignans from the branches and leaves of *I. wardii*. The structures of these compounds were determined by extensive IR, UV, CD and NMR spectroscopic analysis. To the best of our knowledge, illiciumlignan D (**4**) is the first reported dihydrobenzofuran lignan arabinofuranoside in which the arabinofuranose moiety was derivatized on C-9′. Additionally, the cytotoxic properties of the 21 isolated compounds were assessed. Among them, compound **8** is the most promising with the most potent cytotoxicity against SKOV3 and HepG2 cell lines, while **20** is the most promising against HCT116.

Table 2
^{1}H and ^{13}C— NMR data for compounds **2** and **5**, recorded at 500 (^{1}H) and 125 (^{13}C) MHz in CD_3OD.

No.	2		5	
	δ_c	δ_H (J in Hz)	δ_c	δ_H (J in Hz)
1	134.8		135.6	
2	112.6	6.71 brs ovl	112.7	6.71 brs
3	143.9		143.8	
4	147.2		146.1	
5	128.4		128.4	
6	116.5	6.71 brs ovl	116.6	6.70 brs
7	31.5	2.63 t (7.5)	31.5	2.62 t (7.5)
8	30.3	1.92 m	34.4	1.80 m
9	63.4	4.06 t (6.6)	60.9	3.56 t (6.6)
1′	132.6		132.6	
2′	109.6	6.93 d (1.9)	109.9	6.95 brs
3′	150.5		147.3	
4′	145.5		147.5	
5′	114.2	6.90 d (8.4)	114.3	6.72 d (8.2)
6′	117.9	6.82 m ovl	118.1	6.82 m ovl
7′	87.2	5.50 d (5.5)	87.2	5.50 d (6.1)
8′	54.1	3.43 m	54.0	4.37 m
9′	63.6	3.75 m	63.6	3.81,3.75 m
1”	136.1		136.1	
2”	110.4	6.99 d (1.9)	110.4	7.00 brs
3”	147.5		150.5	
4”	146.2		145.6	
5”	117.4	6.82 m ovl	117.5	6.89 d (8.4)
6”	119.6	6.71 m ovl	119.7	6.83 m ovl
7”	72.7	4.80 d (5.9)	72.7	4.83 d (5.3)
8”	84.8	4.36 m	84.8	4.37 m
9”	60.8	3.82 m	60.9	3.78, 3.85 m
3-OMe	55.3	3.85 s	55.4	3.80 s
3′-OMe	54.9	3.74 s	55	3.74 s
5′-OMe				
3”-OMe	55.0	3.77 s	55.2	3.70 s
Me	19.4	2.02 s		
C=O	171.7			

Ovl, overlap.

Table 3
^{1}H and ^{13}C— NMR data for compounds **3**, **4** and **6**, recorded at 500 (^{1}H) and 125 (^{13}C) MHz in CD_3OD.

No.	3		4		6	
	δc	δ_H (*J* in Hz)	δc	δ_H (*J* in Hz)	δc	$\boldsymbol{\delta}_H$ (*J* in Hz)
1	133.2		135.5		133.1	
2	112.8	6.72 brs	112.7	6.71 brs	112.7	6.71 d (1.5)
3	143.8		143.8		143.8	
4	146.1		146.1		146.2	
5	128.2		128.2		128.2	
6	116.7	6.77 brs	116.6	6.72 brs	116.7	6.76 brs
7	31.4	2.62 t (7.6)	31.5	2.61 t (6.4)	31.5	2.63 t (7.3)
8	34.3	1.82 m	34.4	1.81 m	30.3	1.91 m
9	60.8	3.56 t (6.4)	60.8	3.56 t (6.5)	63.6	4.05 t (6.6)
1′	135.5		133.2		134.8	
2′	109.3	6.98 brs	109.2	6.96 d (1.7)	109.2	6.97 d (1.9)
3′	147.6		147.7		147.6	
4′	146.0		146.1		146.1	
5′	114.7	6.76 d (8.2)	114.7	6.76 d (8.2)	114.6	6.75 d (8.2)
6′	118.3	6.84 d (8.2)	118.3	6.82 dd (8.2,1.9)	118.3	6.83 dd (8.1,1.9)
7′	87.8	5.55 d (6.3)	87.9	5.49 d (6.4)	87.8	5.54 d (6.4)
8′	51.5	3.62 m	51.5	3.62 m	51.5	3.61 m
9′	70.7	4.00,3.83 m	69.0	3.90,3.71 m	70.9	4.00,3.83 m
1”	103.3	4.27 d (6.9)	107.9	4.93 d (1.4)	103.5	4.29 d (7.5)
2”	72.9	3.20 m ovl	82.1	4.01 m	73.5	3.20 m ovl
3”	77.3	3.33 m	77.4	3.85 m	76.5	3.33 m
4”	68.2	3.50 m	84.3	3.93 m	69.8	3.50 m
5”	65.6	3.87,3.20 m	61.6	3.63, 3.75 m	65.6	3.88, 3.20 m
3-OMe	55.3	3.85 s	55.1	3.83 s	55.3	3.85 s
3′-OMe	55.0	3.82 s	55.4	3.80 s	55.0	3.81 s
Me					19.4	2.03 s
C=O					171.7	

Ovl, overlap.

3. Experimental

3.1. General experimental procedures

IR spectra were obtained on Bruker FTIR Vector 22 spectrometer. UV spectra were acquired in MeOH on SHIMADZU UV-2550 spectrometer. Optical rotations were measured on Jasco P-2000 Polarimeter. Melting points were carried out on XT4A micro-melting point apparatus. ESIMS spectra were performed using an Agilent 1100 series mass and Autospec-Ultima ETOF apparatus, while high resolution ESI–MS spectra were recorded on Q-TOF micro-mass spectrometer (Waters, USA). ^{1}H and ^{13}C NMR spectra were acquired in CD_3OD on Bruker Avance-500 spectrometers (^{1}H at 500 Hz and ^{13}C at 125 MHz). TLC analysis was performed on $HSGF_{254}$ silica gel plates (10–40 μm, Yantai, China), and compounds were visualized by spraying the dried plates with 10% H_2SO_4, followed by heating, or using iodine vapor. Sephadex LH-20

(GE Healthcare Bio-Sciences AB, Sweden) was used and column chromatography (CC) was performed on silica gel (200–300 mesh, Yantai, China). GC data were recorded on an Agilent N6890 instrument. A preparative column (Shimadzu PRC-ODS EV0233, C_{18}, 5 μm, 250 × 21.2 mm, 500 μL/inj.) was used for preparative HPLC (Shimadzu LC-20A).

3.2. Plant material

The branches and leaves of *I. wardii* were collected in Nujiang County of Yunnan Province, PR China in August 2011 and were subsequently authenticated by Prof. Zhou Yuan-chuan, the director of Nujiang Nationality Medicine Plants Institution. An authentic specimen (No.201108GSBJ) was deposited at the School of Pharmacy, Shanghai Jiao Tong University.

3.3. Extraction and isolation

The dried branches and leaves of *I. wardii* (17.4 kg) were pulverized and extracted with 95% EtOH under reflux for 3×2 h. The combined EtOH extracts were concentrated in vacuo to yield the crude material (1.6 kg), which was successfully portioned with petroleum ether (5 L × 4), methylene chloride (5 L × 4), and ethyl acetate (5 L × 4). The CH_2Cl_2 fraction (100.0 g) was subjected to CC on silica gel (1200 g, 100–200 mesh, 100 mm × 60 cm) with a CH_2Cl_2/MeOH (100/1–2/1, v/v) gradient eluent to obtain fourteen subfractions (Fr.1–Fr.14). Compound **8** (10.0 mg) was obtained from Fr.1 (10.0 g) after CC on silica gel (140 g, 200–300 mesh, 60 mm × 60 cm, CH_2Cl_2/MeOH 20/1) and recrystallized in methanol. Fr.4 (6.0 g) was purified over Sephadex LH-20 (5.0 × 150 cm, MeOH, 1 mL/min) followed by preparative HPLC (C_{18}, 5 μm, 250 × 21.2 mm, 500 μL/inj, flow rate 8.0 mL/min) to yield **7** (25.0 mg, MeOH/H_2O 45/55), **1** (16.0 mg, MeOH/H_2O 45/55), **9** (20.0 mg, MeOH/H_2O 45/55), **10** (50.0 mg, MeOH/H_2O 45/55), **11** (12.0 mg, MeOH/H_2O 75/25), and **12** (12.0 mg, MeOH/H_2O 85/15). Fr.5 (4.0 g) was purified using MCI CC (3.0 × 60 cm, MeOH/H_2O 90/10) followed by preparative HPLC to yield **13** (30.0 mg, MeOH/H_2O 40/60), **14** (15.0 mg, MeOH/H_2O 40/60), **15** (45.0 mg, MeOH/H_2O 70/30) and **20** (20.0 mg, MeOH/H_2O 85/15). The EtOAc fraction (100 g) was subjected to CC on silica gel (1200 g, 100–200 mesh, 100 mm × 60 cm) with a CH_2Cl_2/MeOH (100/1–2/1, v/v) gradient eluent to obtain ten subfractions (Fr.1– Fr.10). Fr.6 (5.0 g), Fr.7 (3.0 g) and Fr.8 (4.0 g) were purified over MCI CC (3.0 × 60 cm, MeOH/H_2O 90/10) followed by Sephadex LH-20 (5.0 × 150 cm, MeOH, 1 mL/min). Then, the Fr.6 was subjected to preparative HPLC (C_{18}, 5 μm, 250 × 21.2 mm, 500 μL/inj, flow rate 8.0 mL/min, MeOH—H_2O) to yield **2** (7.0 mg, MeOH/H_2O 60/40), **4** (20.0 mg, MeOH/H_2O 40/60), **5** (30.0 mg, MeOH/H_2O 40/60), and **6** (6.0 mg, MeOH/H_2O 60/40). Compounds **3** (6.0 mg, MeOH/H_2O 35/65), **16** (20.0 mg, MeOH/H_2O 40/60), and **17** (8.0 mg, MeOH/H_2O 50/50) were obtained from Fr.7 by preparative HPLC. Fr.8 was subjected to preparative HPLC (C_{18}, 5 μm, 250 × 21.2 mm, 500 μL/inj, flow rate 8.0 mL/min) to yield **18** (4.0 mg, MeOH/H_2O 30/70), **19** (8.0 mg, MeOH/H_2O 35/65) and **21** (6.0 mg, MeOH/H_2O 45/55).

Table 4
IC_{50} values of compounds **1**–**21** against four human tumor cell lines.

Compounds	IC_{50} (μM)			
	A549	HCT116	SKOV3	HepG2
1	>100.00	>100.00	>100.00	>100.00
2	>100.00	>100.00	>100.00	>100.00
3	>100.00	>100.00	>100.00	>100.00
4	>100.00	>100.00	8.67	>100.00
5	>100.00	>100.00	10.66	>100.00
6	>100.00	>100.00	>100.00	>100.00
7	>100.00	>100.00	>100.00	>100.00
8	14.88	2.78	6.08	5.65
10	>100.00	80.47	9.36	>100.00
12	26.04	20.48	36.25	14.61
18	>100.00	>100.00	11.47	>100.00
20	28.22	2.70	13.31	11.31
DOX	0.72	0.16	3.09	0.10

Doxorubicin (DOX) was used as a positive control.

3.4. Acid hydrolysis of compounds *3*, *4*, *6*

Each compound (5.0 mg) was refluxed in 2 M HCl/MeOH (v/v 1/1, 2 mL) at 60 °C for 3 h, and the solution was evaporated under N_2. The residue was dissolved in pyridine (100 μL), 1 M L-cysteine methyl ester hydrochloride (100 μL) was added, and the mixture was heated to 60 °C for 2 h. After the reaction mixture was dried in vacuo, the trimethylsilylation reagent HMDS-TMCS (hexamethyldisilazane/Me_3SiCl/pyridine 2/1/10) was added, and heating at 60 °C was continued for another 2 h. Finally, the mixture was partitioned between hexane and H_2O (2 mL each), and the hexane extract was analyzed by gas chromatography (GC) under the following conditions: column temperature, 255 °C; injection temperature, 255 °C; flow rate 1.0 mL/min; N_2 carrier gas. In the acid hydrolysate of **4**, identification of D-arabinofuranose and D-xylopyranose was carried out by comparison of the retention time with those of authentic samples prepared in a similar way. t_R: 6.8 min (D-arabinofuranose), 9.7 min (D-xylopyranose) (Li et al., 1984; Wang et al., 2014).

3.5. Structural elucidation of new products

Illiciumlignan A (**1**): Yellowish oil, $C_{30}H_{34}O_9$, ESI–MS *m/z*: 561 $[M+Na]^+$, HR-ESI–MS: *m/z* 561.2109 ($[M+Na]^+$, calcd. 561.2095); UV (MeOH) λ_{max} (log ε):217 (2.05), 281 (0.54) nm, [α]20*D* +3.0 (*c* 0.1, MeOH); CD [MeOH *c* 0.005, Δε (nm)], −1.85 (201), +1.19 (209), +2.18 (220), +5.19 (245), +1.52 (292); IR (KBr) υ_{max} 3434, 2924, 1619, 1499, 1463, 1383, 1275, 1211, 1141, 1032, 813 cm^{-1}; ^1H NMR (CD_3OD, 500 MHz) and ^{13}C NMR data (CD_3OD, 125 MHz) (Table 1).

Illiciumlignan B (**2**): Yellowish solid, $C_{32}H_{38}O_{11}$, m.p. 105.4–106.2 °C, ESI–MS: *m/z* 621 $[M+Na]^+$, HR-ESI–MS: *m/z* 621.2313 ($[M+Na]^+$, calcd. 621.2306); UV (MeOH) λ_{max} (log ε): 214 (1.95), 230 (1.51), 282 (0.55) nm, [α]20 D −11.2 (*c* 0.13, MeOH); CD [MeOH *c* 0.005, Δε (nm)]: +3.76 (202), +2.55 (212), +1.34 (240), 0.63 (292); IR (KBr) υ_{max} 3432, 2932, 1733, 1607, 1514, 1456, 1429, 1367, 1266, 1213, 1141, 1032 cm^{-1}; ^1H NMR (CD_3OD, 500 MHz) and ^{13}C NMR data (CD_3OD, 125 MHz) (Table 2).

Illiciumlignan C (**3**): Yellowish solid, $C_{25}H_{32}O_{10}$, m.p. 95.7–96.6 °C, ESI–MS *m/z*: 515 $[M+Na]^+$, HR-ESI–MS: *m/z* 537.1985 ($[M+COOH]^+$, calcd. 537.1978); UV (MeOH) λ_{max} (log ε): 213 (1.74), 230 (1.72), 284 (0.43) nm, [α]20*D* −3.33 (*c* 0.21, MeOH); CD [MeOH *c* 0.005, Δε (nm)]: +5.52 (212), −4.55 (242), −2.55 (292); IR (KBr) υ_{max} 3415, 2924, 1607,1517, 1499, 1452, 1430, 1384, 1215, 1211, 1040 cm^{-1}; ^1H NMR (CD_3OD, 500 MHz) and ^{13}C NMR data (CD_3OD, 125 MHz) (Table 3).

Illiciumlignan D (**4**): Yellowish solid, $C_{25}H_{32}O_{10}$, m.p. 84.4–85.3 °C, ESI–MS *m/z*: 515 $[M+Na]^+$, HR-ESI–MS: *m/z* 515.1888 ($[M+Na]^+$, calcd. 515.1888); UV (MeOH) λ_{max} (log ε): 214 (1.98), 232 (1.58), 283 (0.61) nm; [α]20*D* −26.4 (*c* 0.22, MeOH); CD [MeOH *c* 0.005, Δε (nm)]: −3.19 (202), −1.24 (223), +4.41 (240), +2.03 (290); IR (KBr) υ_{max} 3419, 2936, 1607, 1518, 1498, 1455, 1275, 1211, 1141, 1034 cm^{-1}; ^1H NMR(CD_3OD, 500 MHz) and ^{13}C NMR data (CD_3OD, 125 MHz) (Table 3).

Illiciumlignan E (**5**): Yellowish solid, $C_{30}H_{36}O_{10}$, m.p. 90.2–91.0 °C, ESI–MS: *m/z* 579 $[M+Na]^+$, HR-ESI–MS: *m/z* 579.2214 ($[M+Na]^+$, calcd. 579.2201); UV (MeOH) λ_{max} (log ε): 214 (2.01), 230

(1.72), 283 (0.62) nm; [α]20*D* −0.55 (*c* 0.91, MeOH); CD [MeOH *c* 0.005, Δε (nm)]: +0.83 (223), −1.30 (240), +1.44 (290); IR (KBr) υ_{max} 3417, 2934, 1605, 1513, 1463, 1428, 1269, 1210, 1139, 1029 cm^{-1}; ^1H NMR (CD_3OD, 500 MHz) and ^{13}C NMR data (CD_3OD, 125 MHz) (Table 2).

Illiciumlignan F (**6**): Yellowish crystal, $C_{27}H_{34}O_{11}$, m.p. 99.3–100.2 °C, ESI–MS *m*/*z*: 579 $[M+Na]^+$, HR-ESI–MS: *m*/*z* 579.2101 ($[M+Na]^+$, calcd. 579.2083); UV (MeOH) λ_{max} (log ε): 208 (0.69), 236 (0.21), 283 (0.08) nm; [α]20*D* −2.0 (*c* 0.1, MeOH); CD [MeOH *c* 0.005, Δε (nm)]: −3.62 (202), +1.95 (216), −1.11 (241), +0.39 (294); IR (KBr) υ_{max} 3420, 2923, 2851, 1733, 1608, 1518, 1498, 1457, 1434, 1368, 1269, 1211, 1142, 1038 cm^{-1}; ^1H NMR (CD_3OD, 500 MHz) and ^{13}C NMR data (CD_3OD, 125 MHz) (Table 3).

3.6. Cytotoxicity assay

Compounds **1**–**21** were tested for their cytotoxicity against human lung cancer (A549), colon cancer (HCT116), ovarian neoplasm (SKOV3), and hepatocellular carcinoma (HepG2) cell lines using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-*2H*-terazolium bromide) colorimetric assay (Alley et al., 1988; Deng et al., 2014). The cells were cultured in RPMI-1640 medium for 4 h and 100 μL of cell suspension ($4–5\times10^4$ cells/ml) were put into each well of a 96-well microplate and incubated at 37 °C, 5% CO_2 for 24 h 10 μL of sample was added into each well and incubated at the above conditions for 72 h, and then 20 μL MTT was added for 4 h. The final concentrations of the compounds were 100, 10, 1, 0.1, 0.01, and 0.001 μM. After removing the medium, DMSO (100 μL/well) was added to the microplate and the plate was shaken for 10 min. The absorbance was measured on a Varioskan Flash (Thermo Scientific) at a wavelength of 570 nm. The test substances were dissolved in DMSO and Doxorubicin was used as a positive control (Table 4).

Author contributions

Feng-mei Ye and Yang-Guo Xie contributed equally to this work and should be considered co-first authors.

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Appendix A. Supplementary data

Supplementary data associated with this article, including the HRESIMS, 1D and 2D NMR data for compounds **2**–**7**, can be found, in the online version, at http://dx.doi.org/10.1016/j.phytol.2016.07.010.

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