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ANTIVIRAL ACTIVITY OF LIGNANS AND THEIR GLYCOSIDES FROM *JUSTICIA PROCUMBENS*

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Key Word Index—*Justicia procumbens*; Acanthaceae; lignan; lignan glycoside; diphyllin; justicidin; antiviral activity.

Abstract—Ten antiviral lignans, seven known (justicidins A, B, C and D, diphyllin, diphyllin apioside and diphyllin apioside-5-acetate) and three new compounds, justicidinosides A (justicidin C 6′-*O*-glucoside), B (justicidin A 6′-*O*-glucoside) and C (justicidin B 6′-*O*-glucoside), were isolated from a methanolic extract of the aerial parts of *Justicia procumbens* var. *leucantha*. Justicidins A and B, diphyllin, diphyllin apioside and diphyllin apioside-5-acetate showed strong antiviral activity (the MIC were less than 0.25 $\mu g\,ml^{-1}$, respectively) against vesicular stomatitis virus and low cytotoxicity (the MTC were larger than 31 $\mu g\,ml^{-1}$, respectively) against cultured rabbit lung cells (RL-33).

INTRODUCTION

In our screening work to find the non-nucleic acid antiviral compounds from about 500 plant materials [1, 2], we found that a methanolic extract of the aerial part of *Justicia procumbens* var. *leucantha* showed significant inhibitory activity against vesicular stomatitis virus (VSV). Whole plants of *J. procumbens* have been used as a folk medicine in China for laryngeal disease and cancer. Several naphthalide lignans have been reported as constituents of this plant [3–6]. Refinement of the antiviral fractions led to the isolation of five lignans and five lignan glycosides. Seven of these were identified as justicidins A (**1**) [4, 6], B (**2**) [4, 5], C (**3**) [4–6] and D (**4**) [4–6], diphyllin (**5**) [4, 6], diphyllin apioside (**6**) [7] and diphyllin apioside-5-acetate (**7**) [7] by spectral analysis, whereas the three new compounds were named justicidinosides A (**8**), B (**9**) and C (**10**), respectively. We report here the isolation, structural elucidation, antiviral activity against VSV and cytotoxicity against cultured rabbit lung cells (RL-33) of these lignans and their glycosides.

RESULTS AND DISCUSSION

The methanolic extract of the aerial part of *J. procumbens* L. suppressed the multiplication of VSV in the screening test. The extract was partitioned with ethyl acetate and H_2O. The ethyl acetate was evaporated and the residue was partitioned between hexane and methanol. The antiviral activity was observed in both the hexane and methanol phases, whereas the aqueous phase showed low activity. The antiviral activity of the compounds to VSV was measured by our newly developed screening method. This method enabled us to detect inhibitory effects on the replication of virus, together with virus inactivation in 96-well microtitre plates simultaneously for many samples. Furthermore, the compounds which photodynamically inactivate the virus, e.g. porphyrins [8], could be excluded by incubating the microplates under dark conditions. The active phases were subjected to silica gel column chromatography. The active fractions were purified by repeated HPLC and crystallization to yield compounds **1**–**10**.

Compounds **1**–**7** were identified as justicidins A [4, 6], B [4, 6], C [4–6] and D [4–6], diphyllin [4, 6], diphyllin apioside [7] and diphyllin apioside-5-acetate [7], respectively, by comparison of their NMR spectral data (^1H and ^{13}C) with published data and nOe difference spectroscopy.

The HRFAB mass spectrum $[M+H]^+$ *m/z* 573.1609 of **8** showed the molecular formula $C_{28}H_{28}O_{13}$. In the NMR spectra (^1H and ^{13}C) of **8**, distinctive signals due to aryl naphthalide type lignans like **1** or **3** (three methoxyls, singlet aromatic protons, a methylene at the furanone ring and a methylene of 1,3-benzodioxole) were observed, together with five aliphatic methines and a methylene due to a sugar moiety; the AB type

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signals due to the aromatic hydrogens of the 1,3-benzodioxole ring were not observed. These suggest that **8** has the carbon skeleton of the justicidins with a sugar moiety. The molecular formula and the characteristic NMR signals showed that **8** is a monoglycoside of an aryl naphthalide lignan. The coupling constants of the characteristic signals of sugar moiety and the chemical shifts of the ^{13}C NMR spectrum (Table 1) revealed that the sugar is β-glucopyranose. The assignment of the NMR signals of **8** was performed with 2D NMR experiments (^1H–^1H COSY, ^{13}C–^1H COSY, NOESY, COLOC and HMBC) (Table 1). Sugar substitution on the 1,3-benzodioxole ring (C-6″) was deduced from the nOe between the anomeric hydrogen on the β-glucopyranose (1″-H) and the aromatic hydrogen of the 1,3-benzodioxole ring (5′-H) and the ^{13}C–^1H long-range correlation between the anomeric hydrogen on the β-glucopyranose (1″-H) and C-6′. The methylene hydrogens of the furanone ring showed a nOe with 2″-H and a ^{13}C–^1H long-range correlation with C-1, suggesting that **8** is a 12-carbonyl type lactone like **3**. This led to the identification of justicidinoside A as **8**.

Compound **9** gave a molecular formula $C_{28}H_{28}O_{13}$ (HRFAB mass spectrum $[M+H]^+$ m/z 573.1609), the same as **8**, and the NMR spectra (^1H and ^{13}C) were quite similar. The signals of a sugar moiety similar to β-glucopyranose, four aromatic protons and three methoxyl groups as in **8** were observed, together with different signals due to the furanone ring. These suggest that **9** has the same carbon skeleton and the same substituents as **8**, but the position of the carbonyl group on the furanone ring is different. The 2D NMR spectra (^1H–^1H COSY, ^{13}C–^1H COSY, NOESY, COLOC and HMBC) supported this. The nOe between 12-H and 4-OCH_3 confirmed that **9** has a 11-carbonyl type lactone. The assignment of the NMR signals are shown in Table 2, with 2D NMR correlations.

The ^1H and ^{13}C NMR spectra of **10** were very similar to those of **9** except for the lack of signals (^1H and ^{13}C) due to a methoxyl group and the appearance of a singlet ^1H due to an aromatic hydrogen. These suggest that one of the methoxyl groups on an aromatic ring of **9** was substituted by a proton on **10**. In the 2D NMR spectra of **10**, the correlations between 4-H and 5-H/12-H (NOESY), between 4-H and C-2/C-9 (COLOC), between 5-H and C-4 (COLOC) and between C-4 and 12-H (HMBC) confirmed the structure **10** for justicidinoside C (Table 3). The HRFAB mass

Table 1. ^{13}C and ^1H NMR, ^{13}C–^1H long range (COLOC and HMBC) and nOe (NOESY) data for justicidinoside A (**8**), δ (ppm) in CD_3OD [coupling constant (Hz) in parentheses]

C	^{13}C NMR	^1H NMR	^{13}C–^1H long range	NOESY
1	124.66 (*s*)		11-H, 8-H,* 2′-H*	
2	141.85 (*s*)		11-H	
3	110.73 (*s*)		11-H	
4	156.61 (*s*)		4-OCH_3, 5-H*	
5	103.43 (*s*)	7.70 (*s*)		4-OCH_3, 6-OCH_3
6	151.32 (*s*)		6-OCH_3, 5-H,* 8-H*	
7	153.86 (*s*)		7-OCH_3, 5-H,* 8-H*	
8	105.96 (*d*)	6.97 (*s*)		7-OCH_3, 2′-H
9	124.75 (*s*)		8-H	
10	135.46 (*s*)		5-H	
11	70.71 (*t*)	5.07 (*d*, 14.7)		2′-H
		5.41 (*d*, 14.7)		
12	171.80 (*s*)		11-H	
4-OCH_3	63.80 (*q*)	4.33 (3H, *s*)		5-H
6-OCH_3	56.42 (*q*)	4.01 (3H, *s*)		5-H
7-OCH_3	56.35 (*q*)	3.82 (3H, *s*)		8-H
1′	118.59 (*s*)		5′-H*	
2′	111.08 (*d*)	6.72 (*s*)		8-H, 11-H
3′	144.48 (*s*)		2′-H, 5′-H, 7′-H	
4′	150.11 (*s*)		2′-H, 5′-H, 7′-H	
5′	100.35 (*d*)	7.05 (*s*)		1″-H
6′	151.38 (*s*)		2′-H, 5′-H, 1″-H*	
7′	103.12 (*t*)	6.02 (*s*)		
		6.06 (*s*)		
1″	102.93 (*d*)	4.81 (*d*, 7.8)		5′-H
2″	74.56 (*d*)	2.99 (*dd*, 7.8, 9.3)		
3″	78.32 (*d*)	3.32–3.34 (*m*)		
4″	71.30 (*d*)	3.22 (*t*, 9.3)		
5″	78.07 (*d*)	3.32–3.34 (*m*)		
6″	62.58 (*t*)	3.60 (*dd*, 5.4, 11.7)		
		3.77 (*dd*, 2.4, 11.7)		

*Correlations were observed by HMBC.

Table 2. ^{13}C and ^{1}H NMR and ^{13}C–^{1}H long range (COLOC and HMBC) and nOe (NOESY) data for justicidinoside A (**9**), δ (ppm) in CD_3OD [coupling constant (Hz) in parentheses]

C	^{13}C NMR	^{1}H NMR	^{13}C–^{1}H long range	NOESY
1	131.13 (*s*)		8-H, 2′-H	
2	121.70 (*s*)		12-H	
3	125.42 (*s*)		12-H	
4	149.10 (*s*)		5-H, 12-H, 4-OCH_3	
5	101.76 (*d*)	7.54 (*s*)		4-OCH_3, 6-OCH_3
6	152.77 (*s*)		5-H, 8-H, 6-OCH_3	
7	151.51 (*s*)		5-H, 8-H, 7-OCH_3	
8	106.97 (*d*)	7.00 (*s*)		7-OCH_3, 2′-H
9	126.99 (*s*)		8-H	
10	131.56 (*s*)		5-H	
11	172.58 (*s*)		12-H	
12	68.57 (*t*)	5.63 (2H, *s*)		4-OCH_3
4-OCH_3	59.90 (*q*)	4.16 (3H, *s*)		5-H, 12-H
6-OCH_3	56.29 (*q*)	3.97 (3H, *s*)		5-H
7-OCH_3	56.12 (*q*)	3.76 (3H, *s*)		8-H
1′	119.03 (*s*)		5-H	
2′	111.13 (*d*)	6.54 (*s*)		8-H
3′	144.26 (*s*)		2′-H, 5′-H, 7′-H	
4′	149.66 (*s*)		2′-H, 5′-H, 7′-H	
5′	101.07 (*d*)	7.12 (*s*)		1″-H
6′	151.90 (*s*)		2′-H, 5′-H, 1″-H*	
7′	102.92 (*t*)	6.02 (*d*, 1.0)		
		6.05 *d*, 1.0)		
1″	103.63 (*d*)	4.71 (*d*, 7.9)		5′-H
2″	74.72 (*d*)	2.92 (*dd*, 7.9, 8.9)		
3″	77.96 (*d*)	3.28–3.35 (*m*)		
4″	71.31 (*d*)	3.20 (*t*, 9.2)		
5″	77.96 (*d*)	3.28–3.35 (*m*)		
6″	62.49 (*t*)	3.62 (*dd*, 5.6, 11.9)		
		3.82 (*dd*, 2.3, 11.9)		

*Correlation was observed by HMBC.

spectrum established the molecular formula $C_{27}H_{26}O_{12}$ ($[M+H]^+$ *m/z* 543.1503), supporting the proposed structure **10**.

The minimum inhibitory concentrations (MIC) against VSV and minimum cytotoxic concentrations (MTC) against RL-33 cells [9, 10] of the aryl naphthalide type lignans are given in Table 4. Compounds **1**, **2**, **5**, **6** and **7** showed strong antiviral activity, (the MIC were less than 0.25 $\mu g\,ml^{-1}$, respectively) and low cytotoxicity (the MTC were bigger than 31 $\mu g\,ml^{-1}$, respectively) against cultured rabbit lung cells (RL-33), whereas 6′-glucosides (**8**–**10**) and **3** and **4** showed lower antiviral activity. The antiviral activities of **2**, **5**, **6** and **7** against Sindbis virus and MCMV (Murine cytomegalovirus) which were similar inhibition to our results with VSV, have been reported [11]. The major constituent, **1**, showed similar or superior activity to these compounds against VSV. In the cytotoxicity of **1**–**5** against 9-KB (human nasopharyngeal carcinoma), the lactonized position influenced the activity [6]. On the antiviral activity against VSV, the 11-carbonyl-type compounds **1**, **2**, **5**, **6** and **7** were more effective than 12-carbonyl-type compounds **3** and **4**. The weak antiviral activity of 6′-glucosides **9** and **10** could be due to the steric bulk of the sugar moiety.

EXPERIMENTAL

Mps: uncorr.; ^{1}H and ^{13}C NMR: 500 or 270 and 125 or 68 MHz, respectively, CD_3OD, TMS as int. standard; FAB-MS and HRFAB-MS: glycerol as matrix; CC; silica gel 60 (Merck, 70–230 mesh) with $CHCl_3$ and MeOH; HPLC: ODS column (LiChrospher RP-18, Inertsil PREP-ODS, eluted with MeOH–H_2O), silica gel column (LiChroprep Si 60, eluted with $CHCl_3$–MeOH) and gel permeation column (Asahipak GS310, eluted with MeOH or $CHCl_3$) with UV detector.

Plant material. Justicia procumbens L. var. *leucantha* HONDA was collected in the campus of the Tokyo University of Agriculture and Technology in October and November, 1994.

Isolation of the active compounds. The MeOH extract from the aerial part of *J. procumbens* (3.6 kg) was evapd and the residue was partitioned between EtOAc and H_2O. The EtOAc phase was evapd and repartitioned between hexane and MeOH. The antiviral activity to VSV was observed in the hexane and MeOH

Table 3. ^{13}C and ^{1}H NMR, ^{13}C–^{1}H long range (COLOC and HMBC) and nOe (NOESY) data for justicidinoside A (**10**), δ (ppm) in CD_3OD [coupling constant (Hz) in parentheses]

C	^{13}C NMR	^{1}H NMR	^{13}C–^{1}H long range	NOESY
1	136.86 (*s*)		8-H, 2′-H	
2	120.68 (*s*)		4-H, 12-H	
3	141.23 (*s*)		12-H	
4	119.73 (*d*)	7.79 (*s*)	5-H, 12-H*	5-H, 12-H
5	107.42 (*d*)	7.30 (*s*)		4-H, 6-OCH_3
6	153.10 (*s*)		5-H, 8-H, 6-OCH_3	
7	151.31 (*s*)		5-H, 8-H, 7-OCH_3	
8	106.67 (*d*)	7.04 (*s*)		7-OCH_3, 2′-H
9	129.91 (*s*)		4-H	
10	134.86 (*s*)			
11	173.04 (*s*)		12-H	
12	69.90 (*t*)	5.39 (2H, *s*)		4-H
	—			
6-OCH_3	56.38 (*q*)	3.96 (3H, *s*)		5-H
7-OCH_3	56.14 (*q*)	3.76 (3H, *s*)		8-H
1′	118.89 (*s*)		5′-H	
2′	110.88 (*d*)	6.58 (*s*)		8-H
3′	144.27 (*s*)		2′-H, 5′-H, 7′-H	
4′	149.83 (*s*)		2′-H, 5′-H, 7′-H	
5′	100.94 (*d*)	7.14 (*s*)		1″-H
6′	151.67 (*s*)		2′-H, 5′-H, 1″-H*	
7′	102.97 (*t*)	6.02 (*d*, 1.0)		
		6.05 *d*, 1.0)		
1″	103.47 (*d*)	4.76 (*d*, 7.9)		5′-H
2″	74.73 (*d*)	2.91 (*dd*, 7.9, 8.9)		
3″	77.97 (*d*)	3.28–3.38 (*m*)		
4″	71.32 (*d*)	3.20 (*t*, 9.2)		
5″	77.97 (*d*)	3.28–3.38 (*m*)		
6″	62.54 (*t*)	3.62 (*dd*, 5.9, 11.9)		
		3.83 (*dd*, 2.0, 11.9)		

*Correlations were observed by HMBC.

phases. The active phase was subjected to CC on silica gel (eluted with $CHCl_3$ and MeOH, increasing polarity). All frs were monitored by the anti-VSV assay mentioned later. Further purification of the active frs with HPLC yielded five lignans (**1–5**) and give lignan glycosides (**6–10**). The yields of **1–7** were 109.1, 78.8, 71.3, 44.6, 21.6, 23.8 and 5.8 mg, respectively.

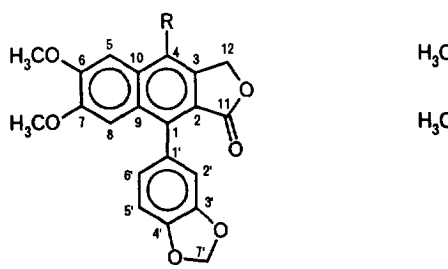
Justicidinoside A (**8**). Powder (14.9 mg), mp 160° (dec.). $[\alpha]_D^{25}$ +2.4° ($c = 0.5$, MeOH). HRFAB-MS *m/z* calc. for $C_{28}H_{29}O_{13}$ $[M+H]^+$: 573.1608, found: 573.1609. UV λ_{max}^{MeOH} nm (log ε): 257 (4.66), 304 (4.12), 356 (3.70). IR(KBr) cm^{-1}: 3405, 2735, 1743, 1507, 1488, 1260, 1212, 1064, 1035, 1002.

Justicidinoside B (**9**). Powder (21.6 mg), mp 164–165° (dec.). $[\alpha]_D^{25}$ −5.9° ($c = 0.5$, MeOH). HRFAB-MS *m/z* calc. for $C_{28}H_{29}O_{13}$ $[M+H]^+$: 573.1608, found: 573.1609. UV λ_{max}^{MeOH} nm (log ε): 234 (4.49), 262 (4.71), 304 (4.14), 352 (3.73). IR(KBr) cm^{-1}: 3408, 2938, 1751, 1508, 1490, 1260, 1214, 1165, 1072, 1038, 1007.

Justicidinoside C (**10**). Powder (22.0 mg), mp 166° (dec.). $[\alpha]_D^{25}$ −15.2° ($c = 0.5$, MeOH). HRFAB-MS *m/z* calc. for $C_{27}H_{26}O_{12}$ $[M+H]^+$: 543.1503, found: 543.1503. UV λ_{max}^{MeOH} nm (log ε): 258 (4.68), 300 (4.04), 347 (3.64). IR(KBr) cm^{-1}: 3402, 2930, 1749, 1507, 1480, 1260, 1219, 1160, 1072, 1038, 1010.

Virus and cell cultures. The Indiana strain of VSV was propagated in human embryonic lung fibroblast (HEL) cells and stored at −70° until use. RL-33 cells were grown in Eagle's minimum essential medium (MEM) supplemented with 0.11% $NaHCO_3$, 8% bovine serum and antibiotics (penicillin 100 U ml^{-1} and streptomycin 100 $\mu g\ ml^{-1}$).

Titration and screening tests for antiviral activity. The antiviral activity tests have been performed in 96-well microtitre plates (Falcon). Test samples were dissolved in DMSO to prepare appropriate concns (usually 10–20 mg ml^{-1}). Starting materials for the assay were diluted with MEM containing 0.11% $NaHCO_3$ at 1/10 respectively just before testing. Serial 2-fold dilutions were made in 96-well microplates by a 50-μl system in each dilution step using an 8-channel pipette (Titertek). 50 μl of VSV (300 $TCID_{50}$/50 μl) was challenged to all wells except cell control. The plates were mixed well using a plate shaker, incubated for 1 hr at 36° under dark condition with a black cover, 100 μl of RL-33 cell suspension (5×10^5 cell ml^{-1}) in MEM containing 0.11% $NaHCO_3$, and 5% foetal bovine serum was added to all wells. The plates were incubated for 24 hr in a 36° CO_2 incubator. The end



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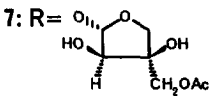
5: R=OH



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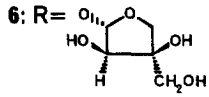

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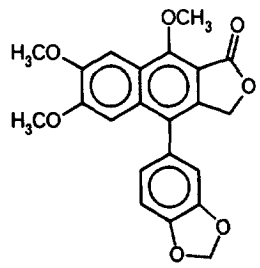

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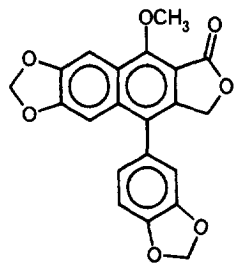

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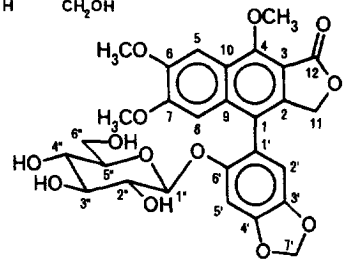

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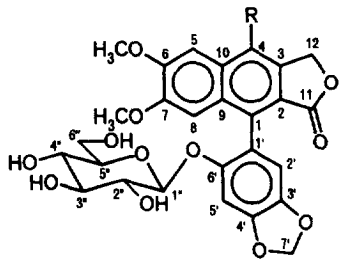

9: R=OCH_3
10: R=H

Table 4. Antiviral activity against VSV (MIC) and cytotoxicity (MTC) against RL-33 cells (μg/ml)

Compound	MIC	MTC
Justicidin A (**1**)	0.13	63.0
Justicidin B (**2**)	≧0.06	31.0
Justicidin C (**3**)	16.0	63.0
Justicidin D (**4**)	16.0	63.0
Diphyllin (**5**)	0.25	63.0
Diphyllin apioside (**6**)	0.25	63.0
Diphyllin apioside-acetate (**7**)	0.13	63.0
Justicidinoside A (**8**)	16.0	125.0
Justicidinoside B (**9**)	125.0	250.0
Justicidinoside C (**10**)	125.0	125.0

point of antiviral activity was taken at highest sample dilutions (lowest concns), which did not show any cytopathic effect by microscopic observation.

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