Antiviral Flavans from the Leaves of *Pithecellobium clypearia*

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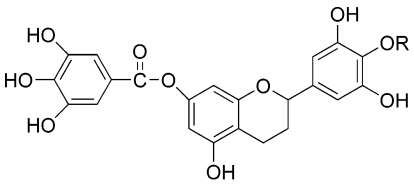
Two new antiviral flavan derivatives were isolated from a methanol extract of leaves of *Pithecellobium clypearia* as guided by antiviral assays. The structures were characterized, by spectroscopic analyses, as 7-*O*-galloyltricetifavan (**1**) and 7,4′-di-*O*-galloyltricetifavan (**2**). Cytopathic effect (CPE) reduction assay showed that both compounds **1** and **2** possess antiviral activity against respiratory syncytial virus (RSV), with 50% inhibition concentration (IC_{50}) values of 5 and 10 μg/mL, respectively; influenza A (H1N1) virus, with IC_{50} values of 15.7 and 30 μg/mL; Coxsackie B3 (Cox B3) virus, with IC_{50} values of 12.5 and 25 μg/mL, respectively; and Herpes simplex virus type 1 (HSV-1) with IC_{50} values of 30 and 20 μg/mL, respectively. Cytotoxicity evaluation using the MTT assay showed that both compounds **1** and **2** were also moderately toxic to several cultured cell lines.

Pithecellobium clypearia (Jack.) Benth (Fabaceae) is used as an herbal medicine in the treatment of respiratory tract diseases in southern China. One pharmaceutical product manufactured from the aqueous extract of the leaves and twigs of *P. clypearia* has been sold in China's market for many years. This Chinese patent medicine, which is recorded in the Pharmacopeia of China,[1] is used for the treatment of upper respiratory tract infections, pharyngitis, laryngitis, acute tonsillitis, acute gastroenteritis, and bacterial dysentery. However, little information is available on the potential biological activities and phytochemical components of *P. clypearia*. Gallic acid is the only reported component previously isolated from the plant.[2,3]

Our previous screening had shown that the aqueous extract of *P. clypearia* exhibited in vitro antiviral activity against respiratory syncytial virus (RSV) and Herpes simplex virus type 1 (HSV-1) with IC_{50} values of 31.3 and 62.5 μg/mL, respectively, and that the antiviral activity of *P. clypearia* was possibly due to its phenolic components.[4] These results led us to investigate the antiviral components of *P. clypearia*, and we have thus obtained two new active flavans (**1** and **2**). This present paper describes the bioactivity-guided fractionation, structure elucidation, and antiviral evaluation of compounds **1** and **2**.



7-O-galloyltricetiflavan (**1**): R=H
7,4'-O-di-galloyltricetiflavan (**2**): R=$COOC_6H_5$

The methanol extract of the leaves of *P. clypearia* was subjected to repeated chromatography on polyamide resin, silica gel 60, and sephadex LH-20 columns to yield compounds **1** and **2**. Isolation was guided by an in vitro antiviral assay.

Compound **1** was isolated as a light brown amorphous powder and showed a dark blue spot on a TLC plate sprayed with ferric chloride reagent, suggesting that **1** was a phenolic compound. Positive-ion FABMS of **1** yielded a quasimolecular ion peak at *m/z* $[M + H]^+$ 443. High-resolution FABMS analysis revealed the molecular formula of **1** to be $C_{22}H_{18}O_{10}$. Its IR spectrum indicated absorption peaks due to hydroxyl, carbonyl, and aromatic groups.

The ^1H NMR and ^{13}C NMR signals of **1** were assigned according to their chemical shifts and coupling constants, as well as 2D-NMR spectra, especially HMBC analysis. In the ^1H NMR and ^{13}C NMR spectra of **1**, signals at δ_H 1.94 (1H, m), 2.14 (1H, m), 2.70 (2H, m), and 4.79 (1H, dd, 2.4, 9.8) and signals at δ_C 20.4 (t), 30.4 (t), and 79.1 (d) indicated the presence of two methylene groups and one oxygenated methine group. In the ^{13}C NMR spectrum, the presence of 11 aromatic quaternary carbon signals at δ_C 157.3 (s), 151.5 (s), 157.8 (s), 108.6 (s), 133.7 (s), 146.7 (2C, s), 134.0 (s), 120.8 (s), 146.9 (2C, s), 140.4 (s), and 167.1 (s) suggested that the structure of **1** contained three aromatic groups and one ester group. In the ^1H NMR spectrum, four aromatic proton signals at δ_H 6.15 (1H, d, 2.4), 6.18 (1H, d, 2.4), 6.40 (2H, s), and 7.15 (2H, s) indicated that there was a pair of *meta*-coupled protons. Comparison of the ^1H NMR and ^{13}C NMR spectra of **1** with those of catechin-7-galloyl ester,[5] catechin-5-galloyl ester,[6] and catechin-4′-galloyl ester[7] showed that the signals of H-3 and C-3 of **1** shifted upfield significantly. In addition, the H-3 signal of **1** corresponded to two protons. Clearly compound **1** was a galloyl-substituted flavan, similar to tricetiflavan.[8] To determine the location of the ester groups, the ^{13}C NMR spectra of equal molar samples of **1** were measured with CD_3OH and CD_3OD as solvents, respectively. Theoretically, a protonated hydroxyl being exchanged for a deuterated hydroxyl will cause a β-isotope shift of the ^{13}C NMR signal of the carbon to which the hydroxy group is attached.[9] The $\Delta\delta_C$ data compared between solvents CD_3OH and CD_3OD showed a small β-isotope shift at the esterified C-7 ($\Delta\delta$ = 0.01 ppm), whereas the hydroxylated C-5, C-3′, C-4′, C-5′, C-galloyl 3, C-galloyl 4, and C-galloyl 5 gave obvious β-isotope shifts ($\Delta\delta$ = 0.11, 0.14, 0.20, 0.14, 0.14, 0.24, 0.14 ppm, respectively). Therefore, the galloyl ester group was placed at the C-7 OH of the flavan. Consequently, compound **1** was deduced to be 7-*O*-galloyltricetiflavan, or tricetiflavan-7-*O*-gallate.

Compound **2** also was isolated as a light brown amorphous powder and showed a dark blue spot on TLC after spraying with ferric chloride reagent. Its IR spectrum was also similar to that of **1**, with hydroxyl, carbonyl, and aromatic groups indicated. Positive-ion FABMS of **2** yielded quasimolecular ion peaks at *m/z* $[M + H]^+$ 595, and high-resolution FABMS analysis revealed the

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Table 1. Antiviral Effects of Compounds **1** and **2** Isolated from *P. clypearia*

		antiviral effects			
		1	**2**	ribavirin	acyclovir
RSV (Hep-2 cells)	CC_{50} (μg/mL)	95	62.5	62.5	nd[a]
	MNCC (μg/mL)	50−25	25−12.5	nd	nd
	IC_{50} (μg/mL)	5	10	3	nd
	SI	19	6.3	20.8	nd
HSV-1 (Vero cells)	CC_{50} (μg/mL)	120	100	nd	>500
	MNCC (μg/mL)	62.5−31.3	62.5−31.3	nd	nd
	IC_{50} (μg/mL)	30	20	nd	0.25
	SI	4	5	nd	>2000
Flu A (MDCK cells)	CC_{50} (μg/mL)	110	60	nd	nd
	MNCC (μg/mL)	62.5	31.3	nd	nd
	IC_{50} (μg/mL)	15.7	30	nd	15.7
	SI	7.0	2	nd	nd
Cox B3 (Hep-2 cells)	CC_{50} (μg/mL)	95	62.5	nd	nd
	MNCC (μg/mL)	50−25	25−12.5	nd	nd
	IC_{50} (μg/mL)	12.5	25	nd	25
	SI	7.6	2.5	nd	nd

[a] nd = no detection.

molecular formula of **2** to be $C_{29}H_{22}O_{14}$. The MS analyses suggested the presence of an extra galloyl group in **2** as compared to **1**.

The ^{1}H NMR spectrum of **2** was similar to that of **1** except for the additional signal at δ_H 7.22 and the chemical shifts of the B-ring signals, suggesting that the extra galloyl group was located at the C-4′ OH. In the ^{13}C NMR spectrum of **2**, seven additional signals including five quaternary carbons [δ_C 120.7, 146.7 (2C), 141.3, 166.8] and two tertiary carbons [δ_C 110.9 (2C)] were found as compared with the spectrum of **1**. Consequently, compound **2** was deduced to be 7,4′-di-*O*-galloyltricetiflavan, or tricetiflavan-7,4′-di-*O*-gallate. The configuration of C-2 of compounds **1** and **2** was defined as β from their optical rotation values. Gallic acid was isolated in the present study, and its structure was identified by direct comparison with an authentic sample and published NMR data.[10]

Cytopathic effect (CPE) reduction assay showed that compounds **1** and **2** possessed a spectrum of antiviral activities against respiratory syncytial virus (RSV), influenza A (Flu A) virus, Coxsackie B3 (Cox B3) virus, and Herpes simplex virus type one (HSV-1) (Table 1). Cytotoxicity evaluation using the MTT assay showed that both compounds **1** and **2** were moderately toxic to HEp-2 cells, Vero cells, and MDCK cells, which probably resulted in their lower antiviral selective indices (SI) (Table 1). Generally, antiviral drug design may be targeted at either viral proteins or cellular proteins. The first approach is likely to yield more specific, less toxic compounds, with a narrow spectrum of antiviral activity and a higher likelihood of virus drug-resistance development, whereas the second approach might afford antiviral compounds with a broad spectrum of antiviral activity and less chance of resistance development, but higher likelihood of toxicity.[11] Our experimental results suggest that compounds **1** and **2** might exert their antiviral activities through targeting cellular proteins.

Catechin derivatives are very common in nature, and flavan-3-ol derivatives have been isolated from *Pithecellobium lobatum* previously.[7] However, the galloyl-substituted tricetiflavan, which is a catechin-like compound without C-3 OH substitution, has not been reported in the literature. As compounds **1** and **2** exhibited significant antiviral activity against several important respiratory infectious viruses, our study has thus provided partial scientific support for the traditional uses of *P. clypearia* in the treatment of various respiratory tract infections.

Experimental Section

General Experimental Procedures. NMR spectra including ^{1}H NMR, ^{13}C NMR, DEPT, HMQC, and HMBC spectra were measured on a Bruker DRX 400 spectrometer operating at 400 MHz for ^{1}H MNR and 100 MHz for ^{13}C NMR. Chemical shifts are reported in δ (ppm) with TMS as internal standard. FABMS was recorded in a nitrobenzyl alcohol matrix on a VG ZAB-HS instrument. High-resolution FABMS (HRFABMS) was recorded in a glycerol matrix on a ThermoFinnigan MAT 95 XL. IR spectra were obtained in potassium bromide (KBr) matrix on a Nicolet Magna 750 FT-IR spectrophotometer. Optical rotations were measured on a Perkin-Elmer 341 polarimeter. The isolation processes were conducted on the following chromatographic columns: polyamide resin (100 mesh, Zhejiang, China), silica gel 60 (0.063−0.200 mm, Merck, Germany), and Sephadex LH-20 (25−100 μm, Fluka, Switzerland). Thin-layer chromatography (TLC) was carried out on silica gel 60 F_{254} plates (0.2 mm thickness, 10 × 20 cm, Merck, Germany) using $CHCl_3$/MeOH/formic acid (8:2:0.5) as the developing reagent. Spots on TLC plates were detected by spraying with $FeCl_3$−C_2H_5OH reagent for phenolic compounds.

Plant Material. Leaves of *P. clypearia* were collected in Guangzhou, People's Republic of China, and authenticated by Mr. Zheng-Qiu Mai, a senior pharmacist of Guangzhou Branch, Chinese Medicinal Material Company, Guangzhou, China. A voucher specimen was deposited in Laboratory 208 of the Institute of Chinese Medicine, the Chinese University of Hong Kong, with accession number 2082002013.

Chemicals. Gallic acid was obtained from the National Institute for the Control of Pharmaceutical and Biological Products, State Food and Drug Administration, Beijing, People's Republic of China. Analytical reagent grade solvents, including $CHCl_3$, formic acid, MeOH, EtOH, and dimethylsulfoxide (DMSO) was purchased from Labscan Asia Co. (Bangkok, Thailand). Ribavirin, purchased from Sigma Chemical Co., was used as the positive control in antiviral assays against RSV, Flu A, and Cox B3. Acyclovir, purchased from the Welcome Foundation Ltd., was used as the positive control in the antiviral assay against HSV-1. MTT (3-[4,5-dimethyl-thiazol-2-yl]-2,5-diphenyl tetrazolium bromide) was purchased from Sigma Chemical Co.

Extraction and Isolation. Dried and cut leaves of *P. clypearia* (70 g) were soaked in MeOH (1 L) at room temperature for 5 days. The MeOH extract was evaporated at 40 °C under reduced pressure, and 17.7 g of crude extract was obtained. Part of the crude extract (3.5 g) was subjected to a polyamide (90 g) column, equilibrated with H_2O overnight. The column was successively eluted by 20% EtOH, 40% EtOH, 60% EtOH, 80% EtOH, and MeOH to afford five fractions: A (199.3 mg), B (516.1 mg), C (790.5 mg), D (577.6 mg), E (104.4 mg). Anti-RSV assays showed that fractions C and D were active fractions, and these fractions were further separated using a silica gel column. Fraction C (700 mg) was chromatographed on a silica gel (30 g) column eluted with a solvent system of $CHCl_3$/MeOH/formic acid. Fractions (5 mL each) were collected and monitored by silica gel TLC. Identical fractions were combined and dried under vacuum to afford 18 mg of gallic acid. The identical fractions from 85 to 100 mL were combined, dried at 40 °C under vacuum, and further purified using a Sephadex LH-20 column eluted with $CHCl_3$/MeOH (3:7) to afford 78 mg of compound **1**. Fraction D (300 mg) was also subjected to a silica gel (10 g) column eluted with a solvent system of $CHCl_3$/MeOH/formic acid (9:1:0.05). Fractions from 150 to 200 mL were combined, dried

at 40 °C under vacuum, and further purified using a Sephadex LH-20 column eluted with $CHCl_3$/MeOH (3:7) to afford 39.7 mg of compound **2**.

7-*O*-Galloyltricetifavan (1): light brown amorphous powder; $[\alpha]^{20}_D$ −5.3 (*c* 0.8, MeOH); IR (KBr) ν_{max} 3,411 (br), 2,931, 1,703, 1,616, 1,540, 1,448, 1,349 (br), 1,213 (br), 1,128, 1,072, 1,032, 851, 734 cm^{-1}; ^1H NMR (CD_3OD, 400 MHz) δ 7.15 (2H, d, 2.0, H-galloyl 2, 6), 6.40 (2H, d, 2.4, H-2′, 6′), 6.18 (1H, d, 2.4, H-6), 6.15 (1H, d, 2.4, H-8), 4.79 (1H, dd, 2.4, 9.8, H-2), 2.70 (2H, m, H-4), 2.14 (1H, m, H-3b), 1.94 (1H, m, H-3a); ^{13}C NMR (CD_3OD, 100 MHz) δ 167.11 (C, C-galloyl 7), 157.77 (C, C-9), 157.29 (C, C-5), 151.47 (C, C-7), 146.91 (C, C-galloyl 3, 5), 146.65 (C, C-3′, 5′), 140.45 (C, C-galloyl 4), 134.08 (C, C-1′), 133.73 (C, C-4′), 120.80 (C, C-galloyl 1), 110.55 (CH, C-galloyl 2, 6), 108.66 (C, C-10), 106.25 (CH, C-2′, 6′), 102.44 (CH, C-8), 101.51 (CH, C-6), 79.08 (CH, C-2), 30.41 (CH_2, C-4), 20.38 (CH_2, C-3); ^{13}C NMR (CD_3OH, 100 MHz) δ 167.10 (C, C-galloyl 7), 157.76 (C, C-9), 157.40 (C, C-5), 151.46 (C, C-7), 147.05 (C, C-galloyl 3, 5), 146.79 (C, C-3′, 5′), 140.69 (C, C-galloyl 4), 134.01 (C, C-1′), 133.93 (C, C-4'), 120.71 (C, C-galloyl 1), 110.59 (CH, C-galloyl 2, 6), 108.68 (C, C-10), 106.30 (CH, C-2′, 6′), 102.41 (CH, C-8), 101.59 (CH, C-6), 79.07 (CH, C-2), 30.41 (CH_2, C-4), 20.38 (CH_2, C-3); the ^{13}C NMR signals measured in CD_3OH solvent were referred to the C-3 signal of δ_C 20.38 ppm or the C-4 signal of δ_C 30.41 ppm; FABMS *m*/*z* 443 $[M + H]^+$; HRFABMS *m*/*z* 441.0812 $[M - H]^-$ (calcd for $C_{22}H_{18}O_{10}$, 441.0827).

7,4′-Di-*O*-galloyltricetifavan (2): light brown amorphous powder; $[\alpha]^{20}_D$ −10 (MeOH; *c* 0.4); IR (KBr) ν_{max} 3,399 (br), 1707, 1612, 1533, 1447, 1350 (br), 1208 (br), 1129, 1078, 1034, 864, 759 cm^{-1}; ^1H NMR (CD_3OD, 400 MHz) δ 7.22 (2H, s, H-galloyl 2′, 6′, connected in B ring of the flavan), 7.15 (2H, s, H-galloyl 2, 6, connected in A ring of the flavan), 6.64 (2H, s, H-2′, 6′), 6.18 (2H, s, H-6, 8), 4.87 (1H, H-2, overlaps with the signal of solvent), 2.72 (2H, m, H-4), 2.21 (1H, m, H-3b), 1.96 (1H, m, H-3a); ^{13}C NMR (CD_3OD, 100 MHz) δ 167.1 (CH, C-galloyl 7), 166.8 (CH, C-galloyl 7′), 157.5 (C, C-9), 157.3 (C, C-5), 151.5 (C, C-7), 146.7 (C, C-galloyl 3′ 5′), 146.5 (C, C-galloyl 3, 5), 151.5 (C, C-3′, 5′), 141.3 (C, C-galloyl 4′), 140.6 (C, C-galloyl 4), 134.0 (C, C-1′), 128.0 (C, C-4'), 120.7 (C, C-galloyl 1, 1′), 110.9 (CH, C-galloyl 2′, 6′), 110.7 (CH, C-galloyl 2, 6), 108.6 (C, C-10), 106.2 (CH, C-2′, 6′), 102.5 (CH, C-8), 101.6 (CH, C-6), 78.6 (CH, C-2), 30.7 (CH_2, C-4), 20.4 (CH_2, C-3); FABMS *m*/*z* 595 $[M + H]^+$; HRFABMS *m*/*z* 593.0971 $[M - H]^-$ (calcd for $C_{29}H_{22}O_{14}$, 593.0937).

For antiviral and cytotoxic tests, the fractions and pure compounds were dissolved in DMSO to reach the concentration of 40 mg/mL. The DMSO solutions were then added dropwise to the maintenance medium to reach the concentrations of 1000 and 100 μg/mL for cytotoxic and antiviral tests, respectively. The maintenance medium contained an antibiotic (gentamicin) in order to avoid sterilization of the tested solutions.

Antiviral Evaluation. Viruses and Cells. Respiratory syncytial virus (RSV, Long strain) and influenza A (H1N1) virus (A/NWS/33 strain) were purchased from the American Type Culture Collection (ATCC). Herpes simplex virus type 1 (HSV-1, 15577 strain) was kindly provided by Prof. Spencer H. S. Lee of the Department of Microbiology and Immunology, Dalhousie University, Halifax, Canada. Coxsackie B3 (Cox B3) virus was kindly provided by Prof. Jiuxiang Li of Guangzhou (Jinan) Biomedicine Research & Development Center, Jinan University, Guangzhou, People's Republic of China. All virus stocks were stored at −70 °C until use.

Vero cells (African green monkey kidney cell line), HEp-2 cells (human larynx epidermoid carcinoma cell line), and MDCK cells (Madin-Darby canine kidney cell line) were used for culturing HSV-1, RSV, Cox B3, and Flu A, respectively. All cells were bought from ATCC and were grown in Eagle's minimum essential medium (MEM) (Gibco) supplemented with 10% fetal bovine serum (FBS) (Gibco), 25 μg/mL gentamicin (Sigma), and 200 mM L-glutamine (Sigma) (growth medium). HSV-1-infected cells (Vero cells), RSV-infected cells (HEp-2 cells), and Cox B3-infected cells (HEp-2 cells) were maintained in MEM with 1% FBS, 25 μg/mL gentamicin, and 200 mM L-glutamine (maintenance medium). Flu A-infected cells (MDCK cells) were maintained in maintenance medium supplemented with 1% of TPCK-treated typsin (Sigma). All the cells were cultured at 37 °C in a humidified atmosphere supplied with 5% CO_2.

Cytotoxicity Assay. The MTT (3-[4,5-dimethyl-thiazol-2-yl]-2,5-diphenyl tetrazolium bromide) reduction assay[12] was adopted to test the cytotoxicity of fractions and isolated compounds. In the assay, different concentrations of samples (0.1 mL) were applied to the wells of a 96-well plate containing a confluent cell monolayer in triplicate, while the dilution medium without the sample was used as the control. After 3 days of incubation, 12 μL of the MTT solution [5 mg/mL in phosphate-buffered saline (PBS)] was added to each well. The trays were further incubated for 3 h to allow MTT formazan formation. After removing the medium, 100 μL of DMSO was added to dissolve the formazan crystals. After 15 min, the contents in the wells were homogenized on a microplate shaker. The optical densities (OD) were then read on a microplate spectrophotometer at double wavelengths of 540 and 690 nm. The median cytotoxic concentration (CC_{50}) was calculated as the concentration of sample that decreased the number of viable cells to 50% of the cell control through the OD values of viable cells in comparison with nonviable cells.

Cytopathic Effect (CPE) Reduction Assay. The CPE reduction assay was adopted to monitor the isolation and fractionation processes of the plant extracts and test the antiviral activity of isolated compounds.[13,14] In brief, to confluent cell monolayers in a 96-well plate were added simultaneously 0.1 mL of 100 $TCID_{50}$ virus suspensions and 0.1 mL of serial 2-fold dilutions of sample. As the virus control and cell control, virus suspension and maintenance medium without sample were added, respectively. The plates were incubated at 37 °C in a humidified CO_2 atmosphere for 4−6 days. The virus-induced CPE was scored in comparison with the virus control and cell control (scores: 0 = 0% CPE, 1 = 0−25% CPE, 2 = 25−50% CPE, 3 = 50−75% CPE, 4 = 75−100% CPE). The concentration that reduced 50% of CPE with respect to virus control was estimated from the plots of the data and was defined as 50% inhibitory concentration (IC_{50}). The selective index (SI), which is an important parameter to evaluate the antiviral activity, was calculated from the ratio CC_{50}/IC_{50}.[15]

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