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A-Type Proanthocyanidins from Lychee Seeds and Their Antioxidant and Antiviral Activities

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Two new A-type trimeric proanthocyanidins with two doubly bonded interflavanoid linkages, litchitannin A1 [epicatechin-(2β→*O*→7,4β→6)-epicatechin-(2β→*O*→7,4β→8)-catechin] (**1**) and litchitannin A2 [epicatechin-(2β→*O*→7,4β→6)-epicatechin-(2β→*O*→7,4β→6)-epicatechin] (**2**), were isolated from lychee (*Litchi chinensis* Sonn. cv. Heiye) seeds together with aesculitannin A (**3**), epicatechin-(2β→O→7,4β→8)-epiafzelechin-(4α→8)-epicatechin (**4**), proanthocyanidin A1 (**5**), proanthocyanidin A2 (**6**), proanthocyanidin A6 (**7**), epicatechin-(7,8-*bc*)-4β-(4-hydroxyphenyl)-dihydro-2(3*H*)-pyranone (**8**), and epicatechin (**9**). Their structures were elucidated on the basis of spectroscopic and chemical evidence. It is the first time that compounds **1**−**4**, **7**, and **8** have been reported in this species. Compounds **1**−**9** showed more potent antioxidant activity than L-ascorbic acid with ferric reducing antioxidant power (FRAP) values of 3.71−24.18 mmol/g and IC_{50} values of 5.25−20.07 μM toward DPPH radicals. Moreover, litchitannin A2 (**2**) was found to exhibit *in vitro* antiviral activity against coxsackie virus B3 (CVB3) and compounds **3** and **6** displayed antiherpes simplex virus 1 (HSV-1) activity.

KEYWORDS: *Litchi chinensis*; lychee seed; proanthocyanidin; antioxidant activity; antiviral activity

INTRODUCTION

Lychee (*Litchi chinensis* Sonn., Sapindaceae) has been widely cultivated as an economic crop in subtropical area for fruits. Delicious taste and attractive color of lychee fruits make them very popular in international markets. The annual output of lychee fruits in China was over 1,300 million kilograms since 2005, accounting for 60% of the whole production worldwide (*1*). Currently, lychee arils are consumed as fresh or processed fruits, whereas its byproduct pericarps and seeds are mainly discarded as a waste. The previous studies on chemical constituents and biological activities of lychee fruits are mainly focused on its pericarps, because they have been found to be a rich source of a multitude of potential antioxidants (*1*−*4*). Recent work suggested that lychee seeds could also be potentially used as a readily accessible source of natural antioxidants, which are promising as functional food ingredients or natural preservatives (*5*). Previous chemical studies on lychee seeds reported the isolation and determination of the following phenolic compounds: protocatechuic aldehyde, protocatechuic acid, (−)-epicatechin, proanthocyanidins A1 and A2, rutin, and pinocembrin 7-*O*-neohesperidoside (*6*, *7*). However, these phenolics might not be the only contributors for the high antioxidant activity of lychee seeds since our thin layer chromatography (TLC) examination of the ethanol extract revealed that they contained other phenolic constituents besides the aforementioned ones. To clarify the structures and bioactivities of these uncharacterized constituents, further chemical investigation was required. Herein we report the isolation and structure elucidation of seven A-type proanthocyanidins and two related compounds, six of which were previously uncharacterized from this species, as well as their antioxidant activity in FRAP and DPPH radical scavenging assays and *in vitro* antiviral activity against CVB-3 and HSV-1.

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MATERIALS AND METHODS

General Methods. Optical rotations were obtained on a Perkin-Elmer 343 polarimeter (Perkin-Elmer Inc., Waltham, MA). UV spectra were measured in MeOH on a Perkin-Elmer Lambda 650 UV/vis spectrophotometer. ^{1}H NMR (400 MHz) and ^{13}C NMR (100 MHz) spectra were recorded on a Bruker DRX-400 instrument (Bruker BioSpin Gmbh, Rheinstetten, Germany) in CD_3OD with the solvent residual peaks (δ_H 3.31 and δ_C 49.00 ppm) as reference. High resolution electrospray ionization mass spectra (HRESIMS) were measured on a Bruker Bio TOF IIIQ mass spectrometer (Bruker Daltonics Inc., Billerica, MA). ESIMS spectra were taken on a MDS SCIEX API 2000 LC/MS/MS apparatus (Applied Biosystems Inc., Forster, CA). Column chromatography was performed over silica gel 60 (100−200 mesh, Qingdao Haiyang Chemical Co., Ltd., Qingdao, China) and Develosil ODS (S-75 μm, Nomura Chemical Co., Ltd., Seto, Japan), respectively. High performance liquid chromatography (HPLC) was performed on a Shimadzu LC-6AD liquid chromatograph (Shimadzu Corp., Kyoto, Japan) equipped with a Shimadzu RID-10A refractive index detector, and YMC-Pack ODS-A columns (YMC Co., Ltd., Kyoto, Japan; S-5 μm, 12 nm; 250 mm × 4.6 mm and 250 mm × 20 mm i.d.) were used for analytical and preparative purposes, respectively. TLC was performed on precoated silica gel $HSGF_{254}$ plates (Yantai Jiangyou Silica

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Gel Development Co., Ltd., Yantai, China) and reversed phase (RP)-18 F_{254S} plates (Merck Japan Ltd., Tokyo, Japan), and spot detection was done under fluorescent light (λ = 254 nm) and then spraying 10% H_2SO_4 in EtOH, followed by heating. Phloroglucinol, 2,4,6-tripyridyl-*s*-triazine (TPTZ), 1,1-diphenyl-2-picrylhydrazyl (DPPH), and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma Chemical Co. (St. Louis, MO). L-Ascorbic acid, Dulbecco's modified Eagle's medium (DMEM), virazole, and acyclovir were purchased from Shanghai Boao Biotech Co., Ltd. (Shanghai, China), HyClone Laboratories Inc. (South Logan, UT), Guangzhou Shiqiao Pharmaceutical Co., Ltd. (Guangzhou, China), and Hubei Keyi Pharmaceutical Co., Ltd. (Wuhan, China), respectively.

Plant Material. Fresh fruits of *Litchi chinensis* (cv. Heiye) at commercial maturity were collected from an orchard in Luogang District, Guangzhou, China, in June of 2007. A voucher specimen was deposited at the Herbarium of South China Botanical Garden, Chinese Academy of Sciences (Guangzhou, China). The seeds were manually separated and ground to pieces with a Santronic multi food processor.

Extraction and Isolation. Fresh lychee seed pieces (6,150 g) were extracted with 95% EtOH (10 L × 3) at room temperature (25−32 °C) for 4 days each time. Concentration of the extraction solution under vacuum gave a reddish solid (934.86 g), most of which (906.34 g) was dissolved in 800 mL of water, and then sequentially fractionated with petroleum ether (800 mL × 5) and ethyl acetate (EtOAc, 800 mL × 5) to yield a petroleum ether-soluble fraction (fraction P, 38.80 g) and an EtOAc-soluble fraction (fraction E, 91.65 g) after condensation to dryness *in vacuo*. Fraction P (38.80 g) was subjected to silica gel (822 g) column (72 mm i.d. × 490 mm) chromatography eluted with an increasing polarity of $CHCl_3$/MeOH (100:0, 95:5, 9:1, 85:15, 8:2, and 7:3, vol/vol, 9.0, 11.0, 12.0, 17.5, 10.0, and 7.5 L, respectively) to give fractions P1−P11 after pooling according to their TLC profiles. Fractions P6−P7 (1.89 g), obtained from the elution of $CHCl_3$/MeOH of 9:1, were passed through a Develosil ODS (3.8 g) precolumn, and the 80% MeOH/H_2O eluate (0.42 g) was separated over a Develosil ODS (9.1 g) column (12 mm i.d. × 165 mm) eluted with a decreasing polarity of MeOH/H_2O (1:9, 2:8, 3:7, 4:6, 5:5, 6:4, 7:3, and 8:2, vol/vol, 80 mL each) to give fractions P6-1−P6-16. Fraction P6-9 (43.3 mg) was purified with HPLC using 50% MeOH/H_2O as mobile phase at the flow rate of 5 mL/min to afford **8** (t_R 30.4 min, 6.8 mg, 0.00011%). Fractions P6-3−6-6 (164.2 mg) were combined and purified with HPLC using 30% MeOH/H_2O as mobile phase at the flow rate of 5 mL/min to give **9** (t_R 35.4 min, 58.5 mg, 0.00098%). Fraction P9 (4.76 g), obtained from the elution of $CHCl_3$/MeOH (85:15), was passed through a Develosil ODS (9.6 g) precolumn, and the 80% MeOH/H_2O eluate (4.20 g) was separated over a Develosil ODS (60.2 g) column (18 mm i.d. × 480 mm) eluted with a decreasing polarity of MeOH/H_2O (1:9, 2:8, 3:7, 4:6, 5:5, 6:4, 3:7, and 8:2, vol/vol, 500 mL each) to provide fractions P9-1−P9-35. Fractions P9-8−P9-13 (628 mg) were purified with HPLC using 30% MeOH/H_2O as mobile phase at the flow rate of 5 mL/min to yield **5** (t_R 34.4 min, 70.3 mg, 0.0012%) and **6** (t_R 57.8 min, 134.6 mg, 0.0023%). Fraction E (80.28 g) was subjected to silica gel (1,600 g) column (92 mm i.d. × 590 mm) chromatography using a gradient of $CHCl_3$/MeOH (9:1, 85:15, 8:2, 7:3, and 6:4, vol/vol, 11.9, 49.7, 14.7, 11.9, and 6.3 L, respectively) as eluents, and 700 mL was collected as a fraction. Fractions 58−96 (5.69 g), obtained from the elution of $CHCl_3$/MeOH of 85:15 and 80:20, were combined and separated over a Develosil ODS (110 g) column (30 mm i.d. × 320 mm) using MeOH/H_2O (1:9, 2:8, 3:7, 4:6, 5:5, and 6:4, vol/vol, 900 mL each) as eluents, and 60 mL was collected as a subfraction. Subfractions 39−45 (265.4 mg) were purified with HPLC using CH_3CN/H_2O/AcOH (25:75:0.1, vol/vol/vol) as mobile phase at the flow rate of 5 mL/min to afford **1** (t_R 31.4 min, 38.5 mg, 0.00074%), **2** (t_R 43.1 min, 9.2 mg, 0.00018%), and **7** (t_R 28.3 min, 10.0 mg, 0.00019%). Subfraction 17 (201.1 mg) was purified with HPLC using CH_3CN/H_2O/AcOH (16:84:0.1) as mobile phase at the flow rate of 5 mL/min to yield **3** (t_R 47.9 min, 13.8 mg, 0.00026%) and **4** (t_R 43.1 min, 15.0 mg, 0.00029%).

Epicatechin-(2*β*→*O*→7,4*β*→6)-epicatechin-(2*β*→*O*→7,4*β*→8)-catechin (1): off-white amorphous powder; $[\alpha]_D^{20}$ +34.0° (*c* 0.10, DMSO); UV (MeOH) λ_{max} (log ε) 284 (4.10); ^{1}H NMR data, see **Table 1**; ^{13}C NMR data, see **Table 2**; ESIMS (+) *m*/*z* 863 $[M + H]^+$, (−) *m*/*z* 861 $[M - H]^-$, 897 $[M + Cl]^-$; HRESIMS (−) *m*/*z* 861.1634 $[M - H]^-$ (calculated for $C_{45}H_{33}O_{18}^-$, 861.1667).

Table 1. ^{1}H NMR data for Compounds **1**−**4** in CD_3OD

H	1	2	3	4
2			4.97 (br s)	
3	4.07 (d, 3.4)	3.93 (d, 3.0)	4.09 (br s)	3.28 (d, 3.5)
4	4.25 (d, 3.4)	4.67 (d, 3.0)	4.50 (d, 2.7)	4.15 (d, 3.5)
6	6.06 (br s)	6.05 (d, 2.3)	6.09 (br s)	6.01 (d, 2.2)
8	6.04 (br s)	6.03 (d, 2.3)	6.01 (br s)	5.95 (d, 2.2)
12	7.14 (d, 2.0)	7.06 (d, 2.0)	7.19 (br s)	7.03 (d, 1.8)
15	6.82 (d, 8.3)	6.76 (d, 8.3)	6.78 (d, 8.1)	6.82 (d, 8.3)
16	7.02 (dd, 2.0, 8.3)	6.95 (dd, 2.0, 8.3)	6.99 (br d, 8.1)	6.85 (dd, 1.8, 8.3)
2′				5.77 (br s)
3′	4.16 (d, 3.5)	4.14 (d, 3.1)	4.21 (br s)	4.13 (br s)
4′	4.32 (d, 3.5)	4.34 (d, 3.1)	4.61 (br s)	4.57 (br s)
6′			5.92 (s)	5.80 (s)
8′	6.23 (s)	6.13 (s)		
12′	7.14 (d, 2.0)	7.46 (d, 2.0)	7.18 (br s)	7.69 (d, 8.6)
13′				6.85 (d, 8.6)
15′	6.82 (d, 8.3)	6.91 (d, 8.3)	6.81 (d, 8.1)	6.85 (d, 8.6)
16′	7.02 (dd, 2.0, 8.3)	7.50 (dd, 2.0, 8.3)	7.05 (br d, 8.1)	7.69 (d, 8.6)
2″	4.79 (d, 8.2)	4.86 (br s)	4.97 (br s)	4.39 (br s)
3″	4.09 (m)	4.17 (m)	4.25 (m)	3.86 (m)
4″	2.54 (dd, 9.1, 16.2)	2.81 (dd, 2.0, 16.9)	2.65 (m)	2.84 (2H, m)
	3.00 (dd, 5.3, 16.2)	2.99 (dd, 4.2, 16.9)	2.89 (dd, 4.1, 17.6)	
6″	6.06 (s)		6.01 (s)	6.10 (s)
8″		6.12 (s)		
12″	7.09 (d, 2.0)	6.94 (d, 1.8)	6.93 (br s)	6.83 (br s)
15″	6.89 (d, 8.3)	6.74 (d, 8.5)	6.81 (d, 8.1)	6.73 (d, 8.3)
16″	7.03 (dd, 2.0, 8.3)	6.76 (dd, 1.8, 8.5)	6.79 (br d, 8.1)	6.71 (dd, 1.6, 8.3)

Epicatechin-(2*β*→*O*→7,4*β*→6)-epicatechin-(2*β*→*O*→7,4*β*→6)-epicatechin (2): off-white amorphous powder; $[\alpha]_D^{20}$ +16.0° (*c* 0.05, DMSO); UV (MeOH) λ_{max} (log ε) 287 (4.03); ^{1}H NMR data, see **Table 1**; ^{13}C NMR data, see **Table 2**; ESIMS (+) *m*/*z* 863 $[M + H]^+$, (−) *m*/*z* 861 $[M - H]^-$, 897 $[M + Cl]^-$; HRESIMS (−) *m*/*z* 861.1651 $[M - H]^-$ (calculated for $C_{45}H_{33}O_{18}^-$, 861.1667).

Aesculitannin A (3): off-white amorphous powder; $[\alpha]_D^{20}$ +49.8° (*c* 0.17, DMSO); ^{1}H NMR data, see **Table 1**; ^{13}C NMR data, see **Table 2**; ESIMS (+) *m*/*z* 887 $[M + Na]^+$, (−) *m*/*z* 863 $[M - H]^-$.

Epicatechin-(2*β*→*O*→7,4*β*→8)-epiafzelechin-(4α→8)-epicatechin (4): off-white amorphous powder; $[\alpha]_D^{20}$ +66.5° (*c* 0.13, DMSO); ^{1}H NMR data, see **Table 1**; ^{13}C NMR data, see **Table 2**; ESIMS (+) *m*/*z* 871 $[M + Na]^+$, (−) *m*/*z* 847 $[M - H]^-$.

Acid-Catalyzed Degradation of 3 with Phloroglucinol. A mixture of compound **3** (2.5 mg) and phloroglucinol (2.5 mg) was dissolved with 1% HCl in EtOH (1.0 mL), and the resulting solution was left at room temperature for 24 h. The reaction mixture was evaporated to dryness under vacuum, and the filtrated residue in MeOH was examined by HPLC (*8*). Proanthocyanindin A2 (**6**) was detected as a product of the reaction.

FRAP Assay. This assay was carried out following a modified protocol from Griffin and Bhagooli (*9*). Briefly, 2 mL of a 10 mM TPTZ solution in 40 mM HCl plus 2.0 mL of 20 mM $FeCl_3$ and 20 mL of 300 mM acetate buffer (pH 3.6) were prepared to give a FRAP solution. A total of 20 μL of test compounds including L-ascorbic acid as a reference compound in DMSO was allowed to react with 180 μL of the freshly prepared FRAP solution for 30 min at 37 °C in the dark condition in quadruplicate. Absorbance of the resulting colored product (ferrous TPTZ complex) was measured on a Tecan Genios microplate reader (Tecan Group Ltd., Männedorf, Switzerland) at 595 nm. One milliliter of various concentrations of $FeSO_4$ plus 1 mL of 10 mM TPTZ and 10 mL of 300 mM acetate buffer (pH 3.6) were used for a calibration curve. FRAP values of test compounds were expressed as means ± standard errors (SE) mmol of Fe(II)/g in quadruplicate.

DPPH Radical Scavenging Assay. This assay was carried out according to the procedure described by Zheng et al. (*10*). Briefly, 20 μL of various concentrations of test compounds including L-ascorbic acid as a reference compound in DMSO were added to 180 μL of 0.1 mM DPPH solution in MeOH with final concentrations of 0.8, 1.6, 3.2, 6.4, and 12.8 μg/mL in triplicate. After an incubation period of 30 min at 37 °C in the darkness, the decrease in the absorbance was measured on a Tecan

Table 2. ^{13}C NMR Data for Compounds **1**−**4** in CD_3OD

C	1	2	3	4
2	100.52	99.91	77.33	100.09
3	67.48	68.72	73.37	67.36
4	29.66	29.75	37.21	29.02
5	156.64	158.06	156.90	156.95
6	98.22	97.78	96.65	98.43
7	158.28	157.88	158.25	158.00
8	96.76	96.38	98.57	96.68
9	154.08	154.32	155.05	154.32
10	103.94	103.42	103.60	105.06
11	132.21	132.64	132.20	132.62
12	115.76	115.29	115.35	115.74
13	145.67	145.96	145.98	145.96
14	146.87	146.71	145.69	146.77
15	116.46	115.63	115.85	115.90
16	119.84	119.52	119.31	120.02
2′	100.55	100.89	100.81	78.91
3′	67.39	67.60	67.71	72.69
4′	29.62	30.03	30.31	38.39
5′	150.75	149.15	149.63	155.89
6′	109.66	109.29	104.64	96.23
7′	156.25	155.66	157.80	158.42
8′	97.43	97.65	109.43	106.56
9′	152.64	154.32	154.71	151.26
10′	106.06	105.92	103.80	106.85
11′	132.02	132.36	131.14	131.32
12′	115.76	115.75	116.21	130.97
13′	145.67	145.55	145.98	116.12
14′	146.87	146.88	146.60	151.97
15′	115.76	115.70	116.21	116.12
16′	119.86	120.70	120.26	130.97
2″	83.92	79.98	81.91	80.46
3″	68.42	66.99	66.95	67.68
4″	28.86	29.32	29.55	29.97
5″	156.20	155.03	156.90	156.16
6″	96.85	109.08	96.65	96.68
7″	152.58	152.17	151.07	155.68
8″	106.32	96.25	107.90	109.00
9″	150.73	152.13	150.99	155.68
10″	103.06	103.22	103.60	100.20
11″	131.05	132.00	131.14	133.34
12″	115.76	115.21	116.21	115.90
13″	145.70	145.65	146.17	145.64
14″	146.83	145.84	146.60	145.51
15″	115.70	115.88	115.85	116.12
16″	120.85	119.35	120.81	119.55

Genios microplate reader at 515 nm. The control contained DMSO instead of sample solution, and the blank contained MeOH in place of DPPH solution. The inhibition of DPPH radicals by test compounds was calculated according to the following formula: DPPH scavenging activity (%) = [1 − (absorbance of sample − absorbance of blank)/absorbance of control] × 100. The IC_{50} (the concentration required to scavenge 50% radicals) values of test compounds toward DPPH free radical were calculated using the software of SPSS 16.0 and expressed as means ± standard errors (SE) in triplicate.

Virus and Cell Cultures. Coxsackie virus B3 (CVB3) Nancy strain and herpes simplex virus 1 (HSV-1), which were generously provided by Wuhan Institute of Virology, Chinese Academy of Sciences, Wuhan, China, were propagated in HeLa cell monolayers and stored at −80 °C until use. Viral titers were determined by tissue culture infection dose ($TCID_{50}$) assays, and the concentration of the CVB3 titer used for the infections was 100 $TCID_{50}$. HeLa cells were obtained from the American Type Culture Collection (ATCC) and routinely grown in complete medium (DMEM supplemented with 10% heat-inactivated newborn calf serum, 0.1% L-glutamine, 100 U/mL penicillin, and 0.1 mg/mL streptomycin) at 37 °C in an atmosphere of 5% CO_2. The test medium used for cytotoxic assay as well as for antiviral assay contained 2% of the appropriate serum.

Cytotoxicity Assay. Cytotoxicity was determined using the MTT method as previously described by Mosmann (*11*). Briefly, HeLa cells were seeded in 96-well plates and grown to monolayers. After removal of the growth medium (DMEM maintenance with 10% newborn calf serum), the test compound solutions were added (100 μL each well) at serial 2-fold dilutions in the test medium. The plates were incubated at 37 °C for 48 h, and 20 μL of MTT (0.5 mg/mL, in phosphate-buffered saline) was added to each well and allowed to react for 4 h. After removal of the supernatant, 100 μL of DMSO was added to each well. Plates were incubated at room temperature for 30 min, and the optical density (OD) was measured at the dual wavelengths of 570 and 630 nm on an Emax precision microplate reader (Bio-Rad Co., Hercules, CA). The 50% cytotoxic concentration (CC_{50}) was defined as the concentration required to reduce the viability of untreated cell cultures by 50%. The mean dose−response curve of at least three tests was used to calculate the CC_{50}.

Anti-CVB3 Activity Assay. Anti-CVB3 activity was evaluated by the cytopathic effect (CPE) inhibitory assay previously described (*12*). HeLa cells were grown in 96-well plates and allowed to form monolayers. Then, 50 μL of 100 $TCID_{50}$ viral suspensions and an equal volume of serial 2-fold dilutions of test compounds at doses below CC_{50} were added to each well. Noninfected and infected cells without test compounds served as cell and virus controls, respectively. Virazole was used as a reference compound. When virus control showed a maximum CPE, the antiviral effect was determined using the MTT assay as described above, and the IC_{50} values (the concentration required to reduce 50% of CPE) were calculated. The therapeutic index (TI) was determined from the CC_{50}/IC_{50} ratio.

Anti-HSV-1 Activity Assay. Anti-HSV-1 activity was evaluated with the plaque reduction assay as previously described (*13*). Vero cells were seeded into 24-well culture plates. After 24 h incubation, cells were infected with 30 plaque forming units (PFU) HSV-1 in the presence of different concentrations of test compounds diluted with cell sustainable medium. Dilution medium without test compounds was used as the control. Acyclovir was used as a reference compound. Each well was overlaid with medium containing 1% of methylcellulose, and the plate was incubated for 72 h. Then, the cell monolayer was fixed and stained with formalin and crystal violet, respectively. Viral plaques were counted under a binocular microscope. The concentration required to reduce plaque formation by 100% relative to the control was estimated from graphic plots and defined as 100% inhibitory concentration.

RESULTS AND DISCUSSION

The EtOH extract of lychee seeds was initially partitioned between water and petroleum ether and EtOAc sequentially. The resulting fractions were separated over silica gel column chromatography, Develosil ODS column chromatography and HPLC preparation and yielded seven A-type proanthocyanidins including four trimers (**1**−**4**) and three dimers (**5**−**7**) in addition to two related compounds (**8** and **9**). Their chemical structures were determined on the basis of spectroscopic analysis and comparison of their data to the reported values in literatures. Compounds **1** and **2** are new trimeric proanthocyanindins possessing two doubly bonded interflavanoid linkages.

Compound **1** was obtained as an off-white amorphous powder with a molecular formula of $C_{45}H_{34}O_{18}$, which was determined from its ^{13}C NMR and ESIMS spectra as well as the HRESIMS peak of $[M - H]^-$ at m/z 861.1634. The ^1H NMR spectrum (**Table 1**) displayed two AB coupling systems at δ 4.07 and 4.25 (both d, J = 3.4 Hz, H-3 and H-4) and δ 4.16 and 4.32 (both d, J = 3.5 Hz, H-3′ and H-4′) due to the heterocyclic rings of A-type proanthocyanidins, two *meta*-coupling broad singlets at δ 6.06 and 6.04 (H-6 and H-8), two aromatic singlets at δ 6.23 and 6.06 (H-8′ and H-6″), and three ABX coupling systems at δ 6.82 (2H, d, J = 8.3 Hz, H-15 and H-15′), 6.89 (1H, d, J = 8.3 Hz, H-15″), 7.02 (2H, dd, J = 2.0, 8.3 Hz, H-16 and H-16′), 7.03 (1H, dd, J = 2.0, 8.3 Hz, H-16″), 7.09 (1H, d, J = 2.0 Hz, H-12″) and 7.14 (2H, d, J = 2.0 Hz, H-12 and H-12′), indicating that **1** was an A-type trimeric proanthocyanidin composed of three flavan-3-ol units. In the ^{13}C NMR spectrum (**Table 2**), two characteristic ketal

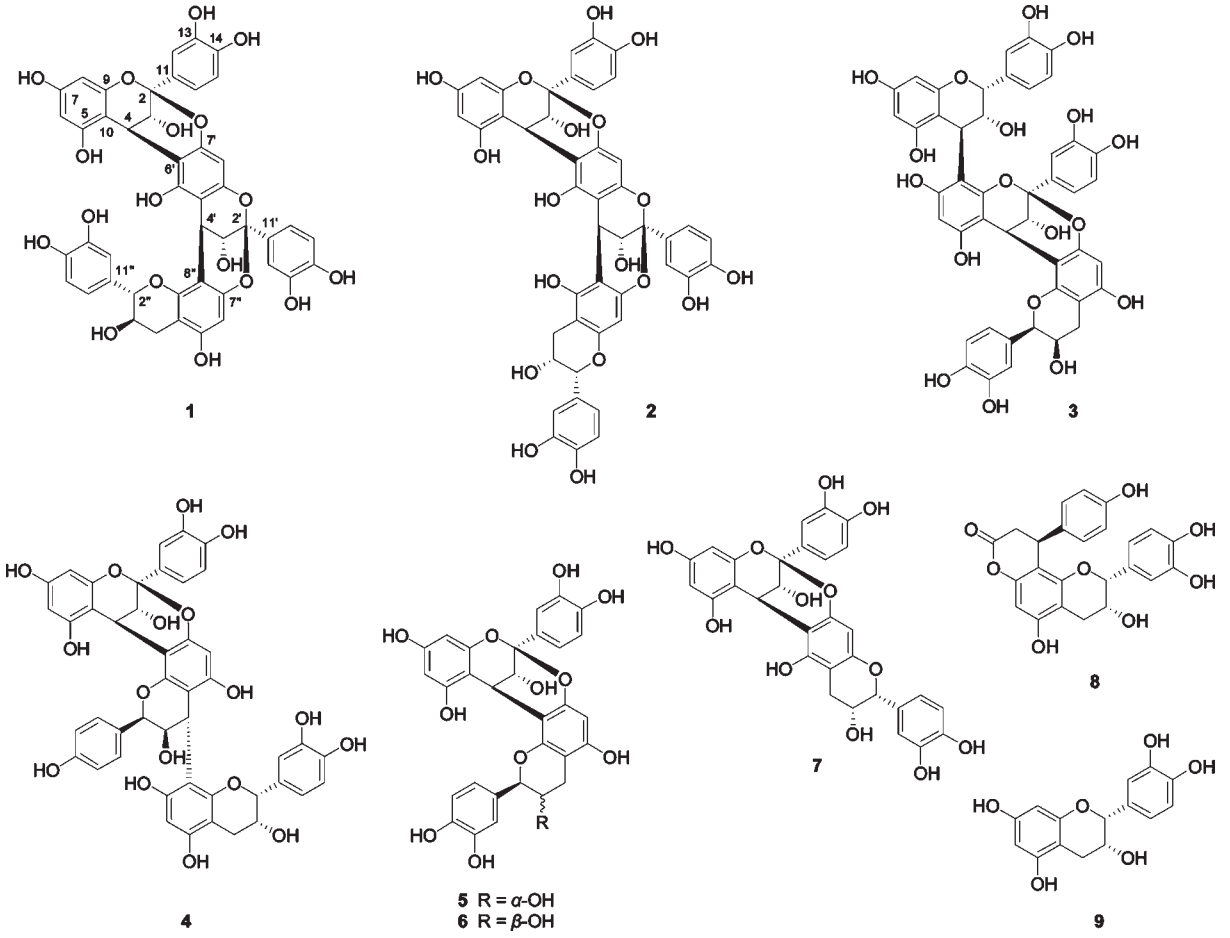

Figure 1. Structures of **1**−**9** from *L. chinensis* seeds.

carbons at δ 100.52 and 100.55 assignable to C-2 and C-2′ indicated the presence of two doubly linked bonds (*14*). Seven upfield signals from δ 28.86 to 83.92 were attributable to other alphatic carbons 2, 3, and 4 in each unit. The carbon signals at δ 83.92 (C-2″), 68.48 (C-3″), and 28.86 (C-4″) in combination with their respective proton signals at δ 4.79 (1H, d, J = 8.2 Hz, H-2″), 4.09 (m, H-3″), 2.54 (dd, J = 9.1, 16.2 Hz, H-4″a), and 3.00 (dd, J = 5.3, 16.2 Hz, H-4″b), which were very close to those of the lower unit in proanthocyanidin A-1 (**5**), suggested that the terminal unit in **1** was catechin (*15*). Moreover, the chemical shifts signaled at δ 67.48 and 67.39 assignable to C-3 and C-3′ indicated that the upper and middle units in the molecule were epicatechins (*1*, *14*). The most downfield carbon signals from δ 145.67 to 158.28 were due to the phenolic carbons 5, 7, 9, 13, and 14 in three units. The resonances from δ 96.76 to 109.66 excluding δ 100.52 and 100.55 were ascribed to the phenolic carbons 6, 8, and 10 of each unit. Finally, the remaining signals from δ 115.70 to 132.21 were attributable to carbons 11, 12, 15, and 16 in each unit. The ^{1}H and ^{13}C NMR signal patterns of the lower unit and the heterocyclic ring in middle unit of **1** were quite close to those of **5**, suggesting that the middle and lower units of **1** were the same as **5**. The double linkages between the upper and middle units were deduced to be 2β→O→7 and C-4→C-6 on the basis of the carbon chemical shift for C-6′ at δ 109.66. The C-6 and C-8 chemical shifts of the extending unit of A-type proanthocyandin with [2β→O→7,4β→8] linkages (*ca.* δ 107.0 for C-8) are distinguished from those with [2β→O→7,4β→6] linkages (*ca.* δ 108.8 for C-6) (*11*). Therefore, compound **1** was determined to be epicatechin-(2β→O→7,4β→6)-epicatechin-(2β→*O*→7,4β→8)-catechin (**Figure 1**), which was a new A-type trimeric proanthocyanidin and trivially named litchitannin A1.

Compound **2** was obtained as an off-white amorphous powder with the same molecular formula as **1**, which meant a trimeric proanthocyanidin composed of three flavan-3-ol units. The ^{13}C NMR spectrum (**Table 2**) showed two ketal carbon signals at δ 99.91 (C-2) and 100.89 (C-2′), indicating two doubly bonded interflavanoid linkages in the molecule (*14*). The AB systems at δ 3.93 and 4.67 (both d, J = 3.0 Hz, H-3 and H-4) and δ 4.14 and 4.34 (both d, J = 3.1 Hz, H-3′ and H-4′) and a broad singlet at δ 4.86 (H-2″) in the ^{1}H NMR spectrum (**Table 1**) in combination with the carbon signals at δ 68.72 (C-3), 67.60 (C-3′), 79.98 (C-2″), and 66.99 (C-3″) of the heterocyclic rings in each unit suggested that **2** consisted of only epicatechin units (*1*). The two doubly linked bonds between the upper and middle units and between the middle and lower units were confirmed to be both 2β→O→7 and 4β→6 on the basis of the carbon signals at δ 109.29 (C-6′) and 109.08 (C-6″) and comparison of its ^{1}H and ^{13}C NMR data with those of epicatechin-(2β→O→7,4β→6)-epicatechin-(2β→*O*→7,4β→8)-epicatechin (*14*). Consequently, compound **2** was characterized as epicatechin-(2β→*O*→7,4β→6)-epicatechin-(2β→*O*→7,4β→6)-epicatechin (**Figure 1**), which was also a new A-type trimeric proanthocyanidin and trivially named litchitannin A2.

As for the trimeric proanthocyanidins possessing two doubly bonded interflavanoid linkages, except for compounds **1** and **2** from lychee seeds in the present paper, epicatechin-(2β→*O*→7,4β→6)-epicatechin-(2β→*O*→7,4β→8)-epicatechin from the barks of *Parameria laevigata* (*14*), a mixture of pavetannin B7 [epicatechin-(2β→*O*→7,4β→8)-*ent*-epicatechin-(2α→*O*→7,4α→8)-*ent*-catechin]

and pavetannin B8 [epicatechin-(2β→*O*→7,4β→8)-epicatechin-(2β→*O*→7,4β→8)-*ent*-catechin] from the stem barks of *Pavetta owariensis* (*15*), aesculitannin C [epicatechin-(2β→*O*→7,4β→8)-epicatechin-(2α→*O*→7,4α→8)-epicatechin] from the stems of *Ecdysanthera utilis* (*16*) and the seed shells of *Aesculus hippocastanum* (*17*), and aesculitannin D [epicatechin-(2β→*O*→7,4β→8)-epicatechin-(2α→*O*→7,4α→8)-*ent*-catechin from the seed shells of *A. hippocastanum* (*17*) have so far been reported.

Compound **3** exhibited an ion peak at *m*/*z* 863 $[M - H]^-$ in the ESIMS spectrum, which was assumed to be a trimeric proanthocyanidin. Acid-catalyzed degradation of **3** with phloroglucinol yielded proanthocyanidin A2 (**6**), indicating that **3** contained the same moiety as **6**. The carbon signals at δ 77.33 (C-2), 73.37 (C-3), 37.21 (C-4), and 103.60 (C-10) were similar to those of proanthocyanidin B2 [epicatechin-(4β→8)-epicatechin] (*18*), suggesting that an epicatechin was the extender unit attached to **6** via a 4β→8 interflavanoid bond (*8*, *19*). Thus, compound **3** was determined as aesculitannin A [epicatechin-(4β→8)-epicatechin-(2β→*O*→7,4β→8)-epicatechin] (**Figure 1**), which was previously isolated from cranberry fruits (*8*) and the seed shells of *Aesculus hippocastanum* (*17*), but no complete NMR data was given in the literature.

Compound **4** showed an ion peak at *m*/*z* 847 $[M - H]^-$ in the ESIMS spectrum, 16 mass units less than that of **3**, suggesting that **4** was a trimeric proanthocyanidin with an afzelechin or epiafzelechin unit, which was evident from its ^{1}H NMR signals (**Table 1**) at δ 7.69 (2H, *J* = 8.6 Hz, H-12′ and H-16′) and 6.85 (2H, *J* = 8.6 Hz, H-13′ and H-15′) and ^{13}C NMR signals at δ 130.97 (2C, C-12′ and C-16′) and 116.12 (2C, C-13′ and C-15′) (**Table 2**). In addition, two broad proton singlets at δ 4.13 and 4.57 (H-2′ and H-3′) and carbon chemical shifts at δ 78.91 and 72.69 (C-2′ and C-3′) indicated that it was an epiafzelechin rather than afzelechin unit (*20*, *21*). Other constitution units and their interflavonoid linkages in the molecule were readily deduced from its proton and carbon data. Therefore, compound **4** was elucidated to be epicatechin-(2β→*O*→7,4β→8)-epiafzelechin-(4α→8)-epicatechin (**Figure 1**), a constituent previously described in *Dicranopteris pedata*, but only with carbon chemical shifts in the heterocyclic rings reported (*20*).

The other known compounds were identified as proanthocyanidin A1 (**5**) (*22*), proanthocyanidin A2 (**6**) (*14*, *22*), proanthocyanidin A6 (**7**) (*14*), epicatechin-(7,8-*bc*)-4β-(4-hydroxyphenyl)-dihydro-2(3*H*)-pyranone (**8**) (*23*), and epicatechin (**9**) (*24*) (**Figure 1**) by comparison of their spectroscopic data to those reported in the literature. Among the known ones, compounds **3**, **4**, **7**, and **8** were isolated from this species for the first time.

Antioxidant Activity. The antioxidant activity of compounds **1**−**9** isolated from lychee seed was determined using two different methods, i.e., the FRAP assay and DPPH radical scavenging assay. As shown in **Table 3**, the FRAP values of nine compounds ranged from 3.71 to 24.18 mmol/g and their IC_{50} values toward DPPH radicals ranged from 5.25 to 20.07 μM, which were more potent than the reference compound, L-ascorbic acid, of which FRAP value and IC_{50} value toward DPPH radicals were 2.67 mmol/g and 45.36 μM, respectively. It was reported that IC_{50} values of epicatechin and L-ascorbic acid toward DPPH radicals were 2.2 and 17 μM (*25*), which were consistent with our test results. The antioxidant activity of the newly characterized compounds (**1**−**4**, **7**, and **8**) were comparable to those of three known ones, i.e., proanthocyanidins A1 (**5**) and A2 (**6**) and epicatechin (**9**). To the best of our knowledge, literature concerning the antioxidant activity of these individual A-type proanthocyanidins is very scarce (*1*, *26*).

Antiviral Activity. Compounds **1**−**7** and **9** were tested for *in vitro* antiviral activity against CVB3 and HSV-1 using the cytopathic effect (CPE) inhibitory assay and the plaque reduction assay, respectively. As shown in **Table 4**, litchitannin A2 (**2**) was found to display *in vitro* anti-CVB3 activity with IC_{50} and TI (CC_{50}/IC_{50}) values of 35.2 μg/mL and 3.2, which was comparable to virazole, of which IC_{50} and TI values were 69.2 μg/mL and 6.2. Meanwhile, aesculitannin A (**3**) and proanthocyanidin A2 (**6**) exhibited weak anti-HSV-1 activity with IC_{50} values of 27.1 and 18.9 μg/mL and TI values of 2.0 and 3.0, respectively, which were much weaker than acyclovir, of which IC_{50} and TI values were 1.3 μg/mL and 506.9. Whereas the other tested compounds did not show *in vitro* antiviral activity against CVB3 and HSV-1.

In conclusion, five A-type proanthocyanidins including two new ones, litchitannin A1 (**1**) and litchitannin A2 (**2**), aesculitannin A (**3**), epicatechin-(2β→O→7,4β→8)-epiafzelechin-(4α→8)-epicatechin (**4**), and proanthocyanidin A6 (**7**), and one phenylpropanoid-epicatechin, epicatechin-(7,8-*bc*)-4β-(4-hydroxyphenyl)-dihydro-2(3*H*)-pyranone (**8**), were obtained in purity

Table 3. Antioxidant Activity of Compounds **1**−**9** Using FRAP and DPPH Assays

compound	FRAP (mmol/g)	DPPH (IC_{50}, μM)
1	4.97 ± 0.11	5.25 ± 0.23
2	24.18 ± 0.92	12.61 ± 1.96
3	5.29 ± 0.03	5.57 ± 0.14
4	4.23 ± 0.06	9.65 ± 0.10
5	13.96 ± 0.97	9.53 ± 0.56
6	6.84 ± 0.22	7.17 ± 0.17
7	5.19 ± 0.25	8.66 ± 0.49
8	3.71 ± 0.17	20.07 ± 0.17
9	5.86 ± 0.14	9.55 ± 0.24
L-ascorbic acid	2.67 ± 0.10	45.36 ± 0.98

Table 4. Anti-CVB3 and Anti-HSV-1 Activities of Compounds **1**−**7** and **9**[a]

	anti-CVB3 activity			anti-HSV-1 activity		
compound	TC_{50} (μg/mL)	IC_{50} (μg/mL)	TI (CC_{50}/IC_{50})	TC_{50} (μg/mL)	IC_{50} (μg/mL)	TI (CC_{50}/IC_{50})
1	62.5	>200		44.3	>200	
2	111.3	35.2	3.2	>200	>200	
3	117.8	>200		55.3	27.1	2.0
4	187.8	>200		90.0	>200	
5	>200	>200		40.8	>200	
6	110.0	>200		57.0	18.9	3.0
7	91.8	>200		70.2	>200	
9	121.0	>200		>200	>200	
virazole	426.0	69.2	6.2			
acyclovir				659	1.3	506.9

[a] All the data represent mean values for three independent experiments.

from lychee seeds for the first time, of which antioxidant activity were comparable to previously characterized proanthocyanidins A1 (**5**) and A2 (**6**) and epicatechin (**9**), suggesting that these newly characterized proanthocyanidins could also be contributors to the antioxidant activity of lychee seeds. In addition, litchitannin A2 (**2**) was found to exhibit *in vitro* anti-CVB3 activity, whereas compounds **3** and **6** were active against HSV-1.

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