Antiviral Flavonoids from the Seeds of *Aesculus chinensis*

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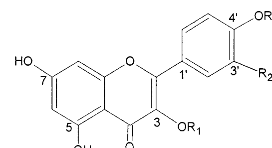
A bioassay-guided fractionation of an ethanol extract of the seeds of *Aesculus chinensis* led to the isolation of two new flavanoids (**1** and **2**), along with eight known ones (**3**−**10**). The structures of the new compounds were elucidated by spectroscopic methods including 2D NMR. All compounds were tested for antiviral activity against respiratory syncytial virus (RSV), parainfluenza virus type 3 (PIV 3), and influenza virus type A (Flu A). Compounds **1**, **2**, and **6** showed significant antiviral activities against RSV with IC_{50} values of 4.5, 6.7, and 4.1 μg/mL and selective index (SI) values of 15.8, 32, and 63.8, respectively. Compound **8** demonstrated significant antiviral activity against Flu A with an IC_{50} of 24.5 μg/mL and a SI of 16.0, respectively.

Aesculus chinensis Bge. (Hippocastanaceae) is a medicinal plant widely distributed in midwestern China. For traditional Chinese medicine, its dried ripe seeds (Suoluozi) have been used as a carminative, stomachic, and analgesic for the treatment of distension and pain in the chest and abdomen.[1] Extracts from the seeds of the horse chestnut tree (*A. hippocastanum* L.), which are composed largely of saponins and flavonoids, have been widely used to treat chronic venous insufficiency (CVI).[2,3] Although main active constituents are considered to be saponins, known as escin, some authors believe that flavonoids also contribute to the observed activities of the seed extracts from aesculus plants.[4,5]

Viruses are the leading cause of respiratory infections in children and adults and are a major cause of morbidity and mortality worldwide.[6] The screening of plants for viral growth inhibitors in vitro and the use of the ethnopharmacological approach enhance the probability of identifying new bioactive compounds. Our focus has been on inhibition of respiratory viral infections.[7−13]

In our screening program for potential antiviral agents, two new (**1**, **2**) and eight known flavonoids (**3**−**10**) were obtained from a 95% EtOH extract of the seeds of *A. chinensis*. This paper describes the structure elucidation of flavonoids **1** and **2** and reports the antiviral activities of flavonoids **1**−**10** isolated from this plant against RSV, PIV 3, and Flu A tested in vitro.



	R_1	R_2	R_3
1	β-xylopyranosyl (1→2)-α-rhamnopyranosyl (1→6)-β-glucopyranosyl	β-glucopyranosyloxy	H
2	β-xylopyranosyl (1→2)-β-glucopyranosyl	β-glucopyranosyloxy	CH_3
3	β-Xylopyranosyl (1→2)-β-glucopyranosyl	β-glucopyranosyloxy	H
4	β-xylopyranosyl (1→2)-β-glucopyranosyl	OH	H
5	β-glucopyranosyl	OH	H
6	β-xylopyranosyl (1→2)-β-glucopyranosyl	H	H
7	β-xylopyranosyl (1→2)-β-glucopyranosyl (1→6)-β-glucopyranosyl	H	H
8	β-glucopyranosyl	H	H
9	β-glucopyranosyl (1→4)-α-rhamnopyranosyl	H	H
10	β-galacopyranosyl	H	H

Results and Discussion

The 95% EtOH extracts of Suoluozi were separated as described in the Experimental Section to yield 10 compounds, **1**−**10**. The eight known compounds were identified by direct comparison with authentic specimens and published data.[14,15]

Compound **1** was isolated as a yellow powder and gave positive Molish and Mg/HCl reactions, suggesting that **1** was a flavonoid type compound. Its IR spectrum showed absorption bands due to hydroxyl, aromatic, and carbonyl functions.

Positive-ion FABMS of **1** yielded quasimolecular ion peaks at *m*/*z* 927 $[M + Na]^+$ and 905 $[M + H]^+$. High-resolution MS analysis of the quasimolecular ion peaks revealed the molecular formula to be $C_{38}H_{48}O_{25}$. Complete acid hydrolysis of **1** afforded quercetin, which was identified by TLC and comparison of its NMR and IR data with those reported in the literature,[16] and glucose, xylose, and rhamnose were identified by co-TLC comparison with authentic samples. Four sugar residues were clearly indicated by signals at δ 98.1, 102.0, 104.3, and 100.2 in the ^{13}C NMR spectrum and signals at δ 5.61 (1H, d, $J = 7.0$ Hz), 4.86 (1H, d, $J = 7.5$ Hz), 4.58 (1H, d, $J = 7.5$ Hz), and 4.36 (1H, s) in the ^{1}H NMR spectrum. The above data together with the results in the 2D NMR indicated that the saccharide moiety was composed of two β-D-glucose, one β-D-xylose, and one α-L-rhamnose residue. As observed in the HMBC

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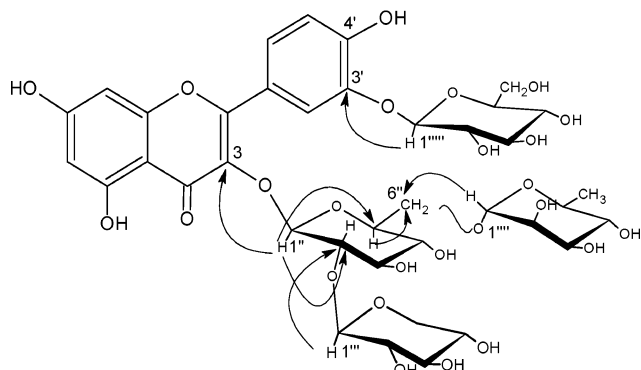

Figure 1. Key HMBC correlations of compound **1**.

spectrum (Figure 1), the long-range correlations between H-1″ (δ 5.61) of the glucose and C-3 (δ 133.0) of the aglycon, H-1‴ (δ 4.58) of the xylose and C-2″ (δ 81.5) of the glucose, H-1⁗ (δ 4.36) of the rhamnose and C-6″ (δ 65.6) of glucose, and H-1′′′′′ (δ 4.86) of another glucose and C-3′ (δ 145.1) of the aglycon established the sesquences and linkage sites of a trisaccharide chain at C-3 as [*β*-D-xylopyranosyl(1→2)]-[α-L-rhamnopyranosyl(1→6)]-*β*-D-glucopyranose. Thus, the structure of **1** was established as quercetin-3-*O*-[*β*-D-xylopyranosyl(1→2)]-[α-L-rhamnopyranosyl(1→6)]-*β*-D-glucopyranoside-3′-*O*-*β*-D-glucopyranoside, a new flavonoid named aescuflavoside.

Compound **2** gave results similar to compound **1** (data not shown) upon treatment with UV shift reagents. Its IR and NMR spectral data were also similar to those of **1**, which suggested a quercetin type aglycon with a similar substitution. Complete acid hydrolysis of **2** afforded glucose, xylose, and an aglycon that was different from quercetin. The positive-ion FABMS data 773 $[M + H]^+$, 641$[M - Xyl + H]^+$, 479$[M - Xyl - Glc + H]^+$, and a fragment at 317 $[M - Xyl - Glc - Glc + H]^+$ confirmed the above conclusion and suggested the presence of a methoxyl group connected to the aglycon. HRMS analysis revealed the molecular formula of **2** to be $C_{33}H_{40}O_{21}$. The ^{1}H and ^{13}C NMR spectra of **2** confirmed the presence of a methoxyl and the absence of a rhamnose unit compared with **1**. The positions of the methoxyl group and the glycosidic linkage were determined by HMBC correlations between the following pairs: methoxyl proton δ 3.80 and C-4′ (δ 147.3), glucosyl anomeric proton H-1″ (δ 5.74) and C-3 (δ 132.7), xylosyl anomeric proton H-1‴ (δ 4.58) and C-2″ (δ 81.5) of glucosyl, and another glucosyl anomeric proton H-1⁗ (δ 4.77) and C-3′ (δ 145.2) of the aglycon. The configuration of the anomeric carbons was defined as *β* from their proton coupling constants of 7.5 Hz (H-1″), 6.0 Hz (H-1″), and 7.5 Hz (H-1″), respectively. Therefore, the structure of **2** was established as 4′-methoxylquercetin-3-*O*-*β*-D-xylopyranosyl (1→2)-*β*-D-glucopyranoside-3′-*O*-*β*-D-glucopyranoside, a new flavonoid named aescuflavoside A.

Flavonoids **3**−**10** were also isolated from the seeds of *A. chinensis*. These flavonoids were all tested for their antiviral activity against RSV, PIV 3, and Flu A (H1N1) (Table 1). Compounds **2** and **6** showed significant antiviral activity against RSV with IC_{50} values of 6.7 and 4.1 *μ*g/mL and SI values of 32 and 63.8, respectively, comparable to that of ribavirin, an approved drug for the treatment of RSV infections in humans. Compound **1** also showed significant antiviral activity against RSV with an IC_{50} value of 4.5 *μ*g/mL and SI value of 15.8, respectively. Compound **2** also demonstrated moderate antiviral activities against PIV 3 with an IC_{50} value of 26.9 *μ*g/mL and SI value of 8.0, respectively. Compound **8** revealed significant antiviral activity against Flu A (H1N1) with an IC_{50} value of 24.5 *μ*g/mL and SI value of 16.0, respectively.

Experimental Section

General Experimental Procedures. Melting points were determined using a Fisher Scientific instrument and were uncorrected. Optical rotations were measured with a JASCO DIP-370 digital polarimeter. UV spectra were recorded using a Shimadzu UV-2100PC spectrophotometer. IR absorption spectra were obtained with a Shimadzu FTIR-8100 spectrometer as a film on KBr disks. NMR spectra were obtained with a Varian INOVA-500 spectrometer operating at 500 MHz for ^{1}H and 125 MHz for ^{13}C, respectively. Chemical shifts are reported in parts per million on the δ scale with TMS as the internal standard. FABMS and HRFABMS were recorded on a JEOL JMS-SX 102A mass spectrometer. Column chromatographies were performed with silica gel (Qingdao Haiyang Chemical Group Co. Ltd., Qingdao, People's Republic of China), Lichroprep RP-18 (40−63 *μ*m, Merck, Darmstadt, Germany), Resin D101 (Tianjin Agricultural Chemical Co. Ltd., Tianjin, People's Republic of China), and Sephadex LH-20 (Pharmacia Fine Chemical Co. Ltd.). For preparative HPLC [pump, Beckman 126P; detector (UV 270 nm), 125P; Beckman Separation Products] separation, an ODS column [Phenomenex LUNA 10u, C_{18} (250 × 21.2 mm i.d.)] was used. TLC was performed on Si gel 60 F_{254} (EM Science) using $CHCl_3$−MeOH (4:1, solvent A), toluene−EtOAc−MeOH (5:2:1, solvent B), and reversed-phase KC_{18} F Si gel 60 (Whatman) using MeOH−H_2O (80:20, solvent C).

Plant Material. The seeds of *A. chinensis* Bge. were collected at Luoyang, Henan Province, People's Republic of China, in September 1998. The voucher specimens (AC-1) are deposited at the Herbal Museum of National Institute for the Control of Pharmaceutical and Biological Products, State Food and Drug Administration (Beijing, People's Republic of China).

Extraction and Isolation. The powdered seeds of *A. chinenesis* Bge. (10 kg) were extracted three times with 95% EtOH under reflux for 2 h. After removal of the solvent in vacuo, the extract (1.6 kg) was further partitioned between H_2O and EtOAc to give H_2O-soluble and EtOAc-soluble fractions. The EtOAc-soluble fraction (164 g) was chromatographed over a silica gel column (4 kg) eluting with $CHCl_3$−MeOH (20:1, 15:1, 10:1, 5:1, each 5000 mL) to give four fractions (A, B, C, and D). Fraction C (16 g) was rechromatographied over a silica gel column (420 g) eluting with $CHCl_3$−MeOH (15:1, 10:1, 7:1, each 1000 mL) to give fractions C1 (1.9 g), C2 (4.8 g), and C3 (3.6 g). Further fractionation of C1 by column chromatography over Sephadex LH-20 afforded compounds **5** (42 mg), **8** (36 mg), and **10** (49 mg). Fraction C2 was separated by HPLC with a MeOH−1% HOAc gradient system (3:7−8:2) to yield compounds **6** (43 mg, 0.0043%), **3** (36 mg, 0.0036%), **4** (23 mg, 0.0023%), **7** (26 mg, 0.0026%), and **9** (22 mg, 0.0022%). Refractionation of fraction C3 using a Sephadex LH-20 column with MeOH−H_2O (1:1) yield two fractions, which were further purified repeatedly by HPLC with a MeOH−1% HOAc gradient system (2:8−7:3) to yield compounds **1** (48 mg, 0.0048%) and **2** (52 mg, 0.037%).

Aescuflavoside (1): yellow powder; $[\alpha]_D^{22}$ −107.8° (*c* 1.25, MeOH); UV (MeOH) λ_{max} (log ε) 220 (3.26), 268 (3.12), 352 (2.08) nm; IR (KBr) ν_{max} 3413, 1655, 1608, 1504, 1358, 1072 cm^{-1}; ^{1}H NMR (DMSO-d_6, 500 MHz) δ 8.02 (1H, dd, *J* = 9.0, 1.5 Hz, H-6′), 7.78 (1H, d, *J* = 1.5 Hz, H-2′), 6.91 (1H, d, *J* = 9.0 Hz, H-5′), 6.50 (1H, s, H-8), 6.19 (1H, s, H-6), 5.74 (1H, d, *J* = 7.5 Hz, H-1″), 4.83 (1H, d, *J* = 7.5 Hz, H-1′′′′′), 4.58 (1H, d, *J* = 7.0 Hz, H-1‴), 4.36 (1H, d, *J* = 3.2 Hz, H-1⁗), 4.03 (1H, m, H-2⁗), 3.95 (1H, m, H-5‴a), 3.92 (1H, m, H-2″), 3.88 (1H, m, H-6′′′′′a), 3.85 (1H, m, H-3″), 3.82 (1H, m, H-6″a), 3.74 (1H, m, H-3⁗), 3.73(1H, m, H-2′′′′′), 3.66 (1H, m, H-3′′′′′), 3.64 (1H, m, H-6′′′′′b), 3.63 (1H, m, H-6″b), 3.59 (1H, m, H-4‴), 3.58 (1H, m, H-4′′′′′), 3.54 (1H, m, H-4″), 3.45 (1H, m, H-5″), 3.40 (1H, m, H-2‴), 3.39 (1H, m, H-5′′′′′), 3.37 (1H, m, H-4⁗), 3.35 (1H, m, H-3‴), 3.30 (1H, m, H-5⁗), 3.27 (1H, m, H-5‴b), 0.89

Table 1. Antiviral Activities of Compounds **1**−**10** against RSV and PIV3 on HEp2 Cells and Flu A (H1N1) on MDCK Cells (tests were performed two times and the average is included)

	RSV			PIV3			Flu A (H1N1)		
compounds	IC_{50} (μg/mL)[a]	CC_{50} (μg/mL)[b]	SI[c]	IC_{50} (μg/mL)[a]	CC_{50} (μg/mL)[b]	SI[c]	IC_{50} (μg/mL)[a]	CC_{50} (μg/mL)[b]	SI[c]
1	4.5	71.3	15.8	35.6	71.3	2.0	>100.0	107.5	<1.1
2	6.7	215.0	32.0	26.9	215.0	8.0	>500.0	322.5	<0.6
3	26.9	215.0	8.0	53.8	215.0	4.0	>500.0	>322.5	−
4	12.3	131.7	10.7	32.9	131.7	4.0	>200.0	197.5	<1.0
5	5.9	23.4	4.0	23.4	23.4	1.0	>500.0	>400.0	−
6	4.1	261.7	63.8	65.4	261.7	4.0	>100.0	46.9	<0.5
7	13.4	53.4	4.0	26.7	53.4	2.0	>100.0	106.9	<1.1
8	26.9	143.3	5.3	35.8	143.3	4.0	24.5	392.5	16.0
9	125.0	250.0	2.0	46.9	250.0	5.3	>500.0	375.0	<0.8
10	71.7	143.3	2.0	53.8	143.3	2.7	>300.0	215.0	<0.7
Ribavirin[d]	2.6	62.5	24.0	2.6	62.5	24.0	62.5	>125.0	>2.0

[a] IC_{50} is the concentration of the sample required to inhibit virus-induced CPE by 50%. [b] CC_{50} is the concentration of the 50% cytotoxic effect. [c] SI = CC_{50}/IC_{50}. [d] Positive control drug.

(3H, d, J = 5.7 Hz, CH_3); ^{13}C NMR (DMSO-d_6, 125 MHz) δ 177.2 (C-4), 164.2 (C-7), 161.0 (C-5), 156.3 (C-9), 155.2 (C-2), 149.7 (C-4′), 145.1 (C-3′), 133.0 (C-3), 125.5 (C-6′), 121.1 (C-1′), 116.4 (C-2′), 115.6 (C-5′), 104.3 (C-1′′′), 103.7 (C-10), 102.3 (C-1′′′′′), 100.2 (C-1′′′′), 98.6 (C-6), 98.0 (C-1′′), 93.8 (C-8), 81.5 (C-2′′), 76.9 (C-3′′′′′), 76.7 (C-5′′′′′), 76.0 (C-5′′ and C-3′′′), 75.8 (C-3′′), 73.8 (C-2′′′), 73.7 (C-2′′′′′), 73.3 (C-4′′′′), 71.8 (C-3′′′′), 70.3 (C-2′′′′), 69.6 (C-4′′′, C-5′′′′ and C-4′′′′′), 68.1 (C-4′′), 65.9 (C-5′′′), 65.6 (C-6′′), 60.6 (C-6′′′′′), 17.6 (C-6′′′′); FABMS m/z 927 $[M + Na]^+$, 905 $[M + H]^+$, 773 $[M - Xyl + H]^+$, 627 $[M - Xyl - Rha + H]^+$, 465 $[M - Xyl - Rha - Glc + H]^+$, 302 $[M - Xyl - Rha - Glc - Glc]^+$; HRFABMS m/z 927.2389 $[M + Na]^+$ (calcd for $C_{38}H_{48}O_{25}Na$, 927.2386).

Aescuflavoside A (2): yellow powder; $[\alpha]_D^{22}$ −102.1° (c 0.25, MeOH); UV (MeOH) λ_{max} (log ε) 217 (3.28), 269 (3.13), 358 (2.04) nm; IR (KBr) ν_{max} 3494, 1653, 1607, 1490, 1348, 1074 cm^{-1}; ^1H NMR (DMSO-d_6, 500 MHz) δ 7.95 (1H, dd, J = 8.0, 2.0 Hz, H-6′), 7.78 (1H, d, J = 2.0 Hz, H-2′), 6.89 (1H, d, J = 8.0 Hz, H-5′), 6.46 (1H, d, J = 2.0 Hz, H-8), 6.17 (1H, d, J = 2.0 Hz, H-6), 5.61 (1H, d, J = 7.0 Hz, H-1′′), 4.58 (1H, d, J = 7.5 Hz, H-1′′′), 4.36 (1H, br. s, H-1′′′′), 3.93 (2H, m, H-2′′ and H-5′′′a), 3.88 (1H, m, H-6′′′′a), 3.76 (1H, m, H-6′′a), 3.73 (1H, m, H-2′′′′), 3.71 (1H, m, H-3′′), 3.69 (1H, m, H-6′′b), 3.66 (1H, m, H-3′′′′), 3.64 (1H, m, H-6′′′′b), 3.58 (1H, m, H-4′′′′), 3.56 (1H, m, H-4′′), 3.49 (1H, m, H-4′′′), 3.44 (1H, m, H-5′′), 3.43 (1H, m, H-2′′′), 3.39 (1H, m, H-5′′′′), 3.38 (1H, m, H-3′′′), 3.25 (1H, m, H-5′′′b); ^{13}C NMR (DMSO-d_6, 125 MHz) δ 177.1 (C-4), 165.1 (C-7), 161.0 (C-5), 156.1 (C-9), 154.7 (C-2), 147.3 (C-4′), 145.2 (C-3′), 132.7 (C-3), 125.8 (C-6′), 122.9 (C-1′), 116.3 (C-2′), 115.0 (C-5′), 104.4 (C-1′′′), 103.3 (C-10), 102.3 (C-1′′′′), 97.8 (C-6), 98.3 (C-1′′), 93.9 (C-8), 81.5 (C-2′′), 77.1 (C-3′′), 76.5 (C-5′′′′), 76.1 (C-3′′′′), 75.8 (C-5′′), 74.5 (C-3′′′), 73.8 (C-2′′′′), 73.3 (C-2′′′), 73.3 (C-4′′′′), 69.9 (C-4′′′), 69.5 (C-4′′′′), 69.4 (C-4′′), 65.7 (C-5′′′), 63.4 (C-6′′′′), 60.6 (C-6′′); FABMS m/z 773 $[M + H]^+$, 641 $[M - Xyl + H]^+$, 479 $[M - Xyl - Glc + H]^+$, 317 $[M - Xyl - Glc - Glc + H]^+$; HRFABMS m/z 795.1965 $[M + Na]^+$ (calcd for $C_{33}H_{40}O_{21}Na$, 795.1963).

Acid Hydrolysis of Flavonoids 1 and 2. A solution of flavonoid (10 mg each) in MeOH (1 mL) was treated with 20% aqueous H_2SO_4 (1 mL), and the mixture was heated under reflux for 4 h. It was then neutralized with saturated $NaHCO_3$ and extracted three times with EtOAc. The H_2O layer was then condensed and subjected to paper chromatography (n-BuOH−H_2O−HOAc, 4:2:1) together with authentic samples. The hydrolysates were coincident with authentic sugars and aglycons.

Materials, Viruses, and Cells. Dulbecco's modified Eagle's medium (DMEM) and phosphate-buffered saline (PBS) were purchased from Sigma Co. Trypsin-EDTA (×10) and trypsin (1:250) were from Gibco Co. Fetal bovine serum (FBS) was from Biofluids Inc. and ribavirin from Sigma Chemical Co.

RSV strain Long, PIV 3, MDCK cells, and HEp 2 cells were obtained from American Type Culture Collection. Flu A (H_1N_1) strain was obtained from Guangzhou Province, People's Republic of China.

Cytotoxicity Assay. Cytotoxicity was measured by the cytopathic effect assay (CPE).[7−9] Cell toxicity was monitored by determining the effect of **1**−**10** on cell morphology and cell viability. Serial 2-fold dilutions of **1**−**10** were added to confluent cell monolayers, and the cells were cultivated at 37 °C for 2−5 days. The morphology of the cells was inspected daily and observed for microscopically detectable alterations, including the loss of monolayer, rounding, shrinking of the cells, granulation, and vacuolization in the cytoplasm. The cytopathic effect was scored (scores: 0 = 0% CPE, 1 = 0−25% CPE, 2 = 25−50% CPE, 3 = 50−75% CPE, 4 = 75−100% CPE). The 50% toxic concentration (CC_{50}), the concentration required to cause visible changes in 50% of intact cells, was estimated from graphic plots. The maximal noncytotoxic concentration (MNCC) was determined as the maximal concentration of the natural products that did not exert a toxic effect detected by microscopic monitoring.

Cytopathic Effect Reduction Assay. The antiviral activity of **1**−**10** against viruses was measured by the CPE inhibition assay.[7−9] Twofold serial dilutions of **1**−**10** were seeded into cell monolayers cultivated in 96-well culture plates, using the MNCC as the higher concentration. An infection control was made in the absence of test compounds. An equal volume of virus suspension (100 $TCID_{50}$/mL) was added to the cell monolayers. The plates were incubated at 37 °C in a humidified CO_2 atmosphere (5% CO_2) for 2−5 days. After that, CPE was observed. The virus-induced CPE was scored as described above in the cytotoxicity assay. The reduction of virus multiplication was calculated as percent of virus control (%virus control = $CPE_{exp}/CPE_{virus\ control} \times 100$). The concentration reducing CPE by 50% with respect to virus control was estimated from graphic plots and was defined as 50% inhibited concentration (IC_{50}) expressed in μg/mL. The selective index (SI) was calculated from the ratio CC_{50}/IC_{50}.

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