Hemiterpene Glucosides with Anti-Platelet Aggregation Activities from *Ilex pubescens*

Zhi-Hong Jiang, Jing-Rong Wang, Min Li, Zhong-Qiu Liu, Ka-Yee Chau, Chi Zhao, and Liang Liu*

School of Chinese Medicine, Hong Kong Baptist University, Kowloon Tong, Hong Kong, People's Republic of China

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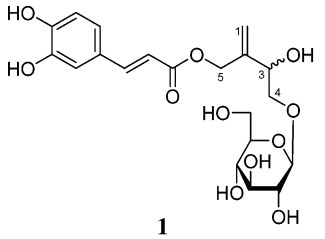
Two new hemiterpene glucosides named pubescenosides A and B were isolated from the root of *Ilex pubescens*. Their structures were elucidated on the basis of spectroscopic and chemical evidence as 2-(*trans*-caffeoyloxy)methyl-3-hydroxy-1-butene-4-*O*-*β*-D-glucopyranoside and 2-hydroxymethyl-3-caffeoyloxy-1-butene-4-*O*-*β*-D-glucopyranoside, respectively. Pharmacological investigation on pubescenosides A and B indicated that both possess potent anti-platelet aggregation activities.

"Mao-Dong-Qing", the dried root of *Ilex pubescens* Hook. et Arn. (Ilexaceae), is a Chinese herbal medicine commonly used in southern China for the treatment of cardiovascular diseases and hypercholestaemia. Previous chemical investigations have indicated the presence of triterpene saponins[1−6] and simple phenolics, i.e., 3,4-dihydroxyacetophenone, hydroquinone, scopoletin, esculetin, homovanillic acid, vomifoliol, and glaberide, in the roots and leaves of this plant.[7] Pharmacological investigation demonstrated that extracts of "Mao-Dong-Qing" not only enlarge blood vessels but also improve minicirculation, lower blood pressure, inhibit platelet aggregation, prevent thrombosis, reduce cardiac ischemia, decrease the excitation of the cardiac conduction system, and enhance anoxia resistance.[8] In regard to the inhibitory effect of "Mao-Dong-Qing" on anti-platelet activation and aggregation, it was reported that ilexonin A, a semisynthetic succinate of a pentacyclic triterpene that is the aglycone of the saponins in "Mao-Dong-Qing", could significantly inhibit platelet aggregation induced by either ADP (adenosine diphosphate) or AA (arachidonic acid), both in vivo and in vitro, and block 5-HT (5-hydroxytryptamine, serotonin) release by the platelets.[9] Aiming at identifying the active components of this Chinese medicinal herb, we focused on the isolation and purification of polar constituents with water-solubility from the *n*-butanol layer of its methanol extracts. By employing a series of column chromatographies, two pure phenolic glucosides named as pubescenosides A (**1**) and B (**2**) were isolated and purified from the *n*-butanol fraction. Their chemical structures were elucidated by means of chemical and spectroscopic methods as hemiterpene glucopyranosides acylated by caffeic acid. We further carried out the pharmacological investigation of pubescenosides A and B on anti-platelet aggregation activities. The results indicated that their effects on anti-platelet activation and aggregation are much stronger than those of aspirin and salvianolic acid B. This paper describes the isolation, structure elucidation, and biological evaluation of the two hemiterpene glucopyranosides **1** and **2**.



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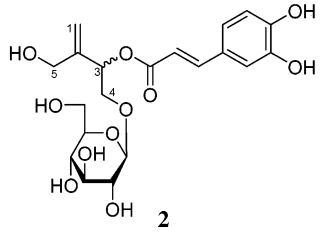

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Results and Discussion

"Mao-Dong-Qing", the root of *Ilex pubescens* Hook et Arn., purchased from Pharmacy of Chinese Medicine Clinics of Hong Kong Baptist University, was extracted with MeOH (×3) at room temperature. The extract was then subjected to liquid−liquid partition to yield four fractions, i.e., Et_2O layer, EtOAc layer, *n*-BuOH layer, and H_2O layer. Preliminary tests on these fractions indicated that some phenolic compounds positive to $FeCl_3$/EtOH reagent in thin-layer chromatography exist in the *n*-BuOH layer. Thus, the subsequent experimental procedures were concentrated on the isolation of compounds within the *n*-BuOH layer in order to obtain these polar compounds for pharmacological screening. The *n*-BuOH layer of the MeOH extract was chromatographed over a combination of MCI-gel CHP 20P, TSK Toyopearl HW-40, and ODS to yield two new compounds, pubescenosides A (**1**) and B (**2**).

Pubescenoside A (**1**) was isolated as a pale yellow powder and showed positive reaction to $FeCl_3$/EtOH reagent in thin-layer chromatography. The positive ESI-Q-TOF mass spectrum of **1** gave an $[M + Na]^+$ ion at *m/z* 465.1377, indicating its molecular formula is $C_{20}H_{26}O_{11}$. Its ^1H NMR and ^{13}C NMR data (Table 1) suggested the presence of a cafffeoyl[10−12] and a *β*-glucosyl moiety [anomeric proton at δ_H 4.33 (J = 7.8 Hz) and anomeric carbon at δ_C 104.5].[13] The remaining five nonaromatic carbon signals in the ^{13}C NMR spectrum implied the presence of a hemiterpene moiety. By detailed analysis of the ^1H NMR data, the signals attributed to an exomethylene proton at δ 5.25 (1H, brs) and δ 5.34 (1H, brs) and 1,2-glycol unit as evidenced by a $^1H-^1H$ coupling between the oxygenated methine signal at δ 4.42 (1H, dd, J = 6.8, 3.6 Hz, H-3) and an oxygenated methylene signal at δ 3.91 (1H, dd, J = 10.8, 6.8 Hz, H-4) were observed. The HMBC spectra showed correlation between the exomethylene proton signal and the carbon [δ 72.3 (C-3)] bearing the secondary hydroxyl group of the 1,2-glycol unit. The $^1H-^{13}C$ long-range correlations between the exomethylene proton and the oxygenated carbon at δ 65.4 (C-5) were also confirmed in the HMBC spectrum. These correlations led to the elucidation

* To whom correspondence should be addressed. Tel: (852) 34112456. Fax: (852) 34112461. E-mail: liuliang@hkbu.edu.hk.

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Table 1. ^{1}H (500 MHz) and ^{13}C (125 MHz) NMR Data (δ) of Compounds **1** and **2** (in CD_3OD)[a]

	1		**2**	
position	C	H	C	H
1	114.6 t	5.25 br s 5.34 br s	113.3 t	5.25 d (1.0) 5.23 d (1.0)
2	145.4 s		147.1 s	
3	72.3 d	4.42 dd (6.8, 3.6)	74.6 d	5.58 dd (7.6, 3.6)
4	74.0 t	3.91 dd (10.8, 6.8) 3.76 dd (10.8, 3.6)	71.9 t	4.12 dd (11.2, 7.6) 3.85 dd (11.2, 3.6)
5	65.4 t	4.79 d (13.5) 4.73 d (13.5)	63.9 t	4.18 d (13.5) 4.14 d (13.5)
caf-1	127.4 s		127.7 s	
caf-2	115.1 d	7.04 d (2.0)	115.2 d	7.04 d (2.0)
caf-3	147.0 s		146.8 s	
caf-4	150.1 s		149.7 s	
caf-5	116.6 d	6.76 d (8.0)	117.1 d	6.77 d (8.0)
caf-6	123.1 d	6.94 dd (8.0,2.0)	123.0 d	6.95 dd (8.0, 2.0)
caf-7	147.3 d	7.56 d (15.6)	147.3 d	7.57 d (16.0)
caf-8	114.8 d	6.27 d (15.6)	115.1 d	6.28(d (16.0)
caf-9	168.8 s		168.5 s	
Glc-1	104.3 d	4.31 d (7.8)	104.5 d	4.33 d (7.8)
Glc-2	75.0 d	3.22 dd (7.8, 9.0)	75.0 d	3.18 dd (7.8, 9.0)
Glc-3	77.9 d	3.36 m	78.1 d	3.33 m
Glc-4	71.5 d	3.30 m	71.5 d	3.30 m
Glc-5	78.0 d	3.29 m	78.1 d	3.28 m
Glc-6	62.7 t	3.65 m 3.85 dd (12.0, 1.6)	62.7 d	3.65 dd (12.0, 2.4) 3.82 m

[a] Assignments were established on the basis of ^{1}H−^{1}H COSY, HSQC, and HMBC spectroscopic data.

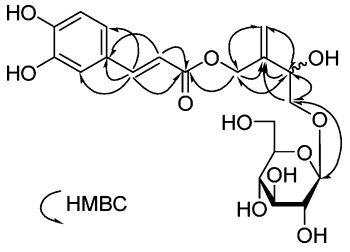

Figure 1. Selected HMBC (H to C) correlations for compound **1**.

of a 2-hydroxymethyl-3,4-dihydroxy-1-butene structure as the aglycone of **1**. The location of the caffeoyl group was determined to be at the C-5 position of the aglycone since correlations between the carbonyl carbon (δ 168.8) of the caffeoyl group and the H-5 proton at δ 4.79 and 4.73 were observed in the HMBC spectra (Figure 1). The anomeric proton of the β-glucosyl group at δ_H 4.31 (J = 7.8 Hz) showed obvious correlation with C-4 at δ_C 74.0, indicating the glucosylation position to be at C-4 of the aglycone. The acid hydrolysis of **1** afforded caffeic acid and D-glucose ($[\alpha]_D^{22} = +43.7°$), which were confirmed by HPLC comparison with authentic samples, and aglycone **1a**, 2-hydroxymethyl-3,4-dihydroxy-1-butene ($[\alpha]_D^{21} = +21.2°$), which was identified as an enantiomer of the hemiterpene isolated from *Artabostrys hexapetalus*[14] and the aglycone of the glycoside isolated from the Japanese fern *Hymenophyllum barbatum*.[15] On the basis of the above results, the structure of pubescenoside A was established as 2-(*trans*-caffeoyloxy)-methyl-3-hydroxy-1-butene-4-*O*-β-D-glucopyranoside.

Pubescenoside B (**2**) was also isolated as a pale yellow powder showing positive reaction with $FeCl_3$ in TLC. Its ESI-Q-TOF mass spectrum showed a quasimolecular ion peak at *m/z* 465.1339 (corresponding to $C_{20}H_{26}O_{11}Na$), demonstrating that it is an isomer of **1**. Careful examination of the ^{1}H NMR and ^{13}C NMR spectra of **2** suggested the existence of a caffeoyl group, a glucose moiety, and a hemiterpene moiety. These results indicated that **2** is also a hemiterpene glucoside acylated by caffeic acid. By comparing its ^{1}H NMR data with those of **1**, a large downfield shift of H-3 ($\Delta\delta_H$ = +1.16 ppm) was observed,

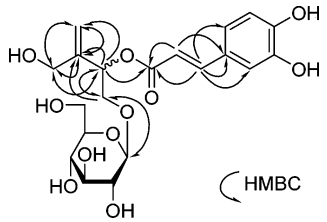

Figure 2. Selected HMBC (H to C) correlations for compound **2**.

Table 2. Activities of Pubescenosides A (**1**) and B (**2**), Salvianolic Acid B, and Aspirin on Anti-Platelet Aggregation

sample	concentration (μM)	n	platelet aggregation rate (%) ($\bar{X} \pm$ SD)
control	10	10	54.400 ± 8.592
pubescenoside A	10	10	36.550 ± 7.697[a,c,d]
pubescenoside B	10	10	27.300 ± 10.263[a,b]
aspirin	10	10	44.700 ± 4.668[a]
salvianolic acid B	10	10	39.650 ± 8.577[a]

[a] vs control, $P < 0.05$. [b] vs aspirin, $P < 0.05$. [c] vs aspirin, $P < 0.01$. [d] vs salvianolic acid B, $P<0.01$.

suggesting that the caffeoyl group is located at C-3. The HMBC experiments provided further evidence for this conclusion because a correlation between the caffeoyl carbonyl carbon (δ 168.5) and H-3 proton at δ 5.58 (dd, J = 7.6, 3.6 Hz) was observed (Figure 2). Furthermore, the anomeric proton of the β-glucosyl group at δ_H 4.33 (J = 7.8 Hz) showed strong correlation with C-4 at δ_C 71.9 in the HMBC spectrum, which determined the glucose linkage at C-4. Acid hydrolysis of **2** under similar conditions for **1** afforded caffeic acid, D-glucose ($[\alpha]_D^{22} = +33.1°$), and aglycone **1a**, which was confirmed by ^{1}H NMR data. On the above spectroscopic and chemical evidence, compound **2** was concluded to be 2-hydroxymethyl-3-caffeoyloxy-1-butene-4-*O*-β-D-glucopyranoside.

Both pubescenosides A and B are hemiterpene glucosides, whose existence is very rare in nature. No more than 40 such hemiterpenes have been isolated from plants so far. Among several types of hemiterpenes, 2-hydroxymethyl-3,4-dihydroxy-1-butene, the aglycone of pubescenosides A (**1**) and B (**2**), is especially unusual. Only three hemiterpenes possessing this structure were reported; one was isolated from an Annonaceae plant,[14] and the other two were obtained from Japanese ferns.[15] Pubescenosides A and B are the first hemiterpene glycosides with 2-hydroxymethyl-3,4-dihydroxy-1-butene as the aglycone, which is further acylated by caffeic acid.

The pharmacological effects of pubescenosides A (**1**) and B (**2**) on anti-platelet aggregation were assayed with a series of repeatable experiments of high shear stress-induced platelet activation and aggregation tests. The results (Table 2) indicated that anti-platelet aggregation activities of pubecenosides A and B are stronger than that of salvianolic acid B, an active ingredient from the Chinese herb Radix Salviae miltiorrhizae (Danshen) and aspirin, a well-known Western drug that has anti-platelet aggregation activity.

Experimental Section

General Experimental Procedures. Optical rotations were measured with a Jasco P-1010 polarmeter. ^{1}H and ^{13}C NMR data were recorded on a Bruker AV-500 (500 MHz for ^{1}H and 125 MHz for ^{13}C) and Varian Mercury-Plus 300 MHz NMR spectrometer. Coupling constants are given in Hz, and chemical shifts are represented in δ (ppm). UV spectra were obtained on a Jasco V-530 UV/vis spectrophotometer. IR spectra were run on a PerkinElmer FT/IR-GX spectrometer. HR-MS was preformed on a QSTAR Pulsar *i* TOF mass

spectrometer. HPLC was carried out on an Agilent Series 1100 HPLC equipped with an Agilent G1315A DAD detector and Alltech evaporative light scattering detector. Column chromatography was performed with MCI-gel CHP 20P (75−150 μm, Mitsubish Chemical Corporation, Japan), Chromatorex ODS (100−200 mesh, Fuji Silysia Chemical Ltd., Japan), and Toyopearl HW-40F (Tosoh Corporation, Japan). TLC was carried out on precoated Kieselgel 60 F_{254} plates (0.2 mm thick, Merck KGaA), and the spots were detected by ultraviolet (UV) illumination and by spraying with 2% ethanolic $FeCl_3$ and 10% sulfuric acid reagent.

Plant Material. "Mao-Dong-Qing" was purchased from Pharmacy of Chinese Medicine Clinics of Hong Kong Baptist University and was identified by Dr. Zhongzhen Zhao, School of Chinese Medicine, Hong Kong Baptist University. A voucher specimen was deposited in the Centre of Chinese Materia Medica Speciemen, Hong Kong Baptist University.

Extraction and Isolation. The dried plant materials (1.2 kg) were mechanically powdered and extracted with 9.6 L of MeOH (×3) at room temperature. The combined extract (68 g) was suspended in H_2O, then subjected to liquid−liquid partition by adding Et_2O (0.5 L × 3), EtOAc (0.5 L × 3), and *n*-BuOH (0.5 L × 4) successively, yielding four fractions, i.e., Et_2O layer (2.9 g), EtOAc layer (15.1 g), *n*-BuOH layer (30.7 g), and H_2O layer (20.0 g).

The *n*-BuOH layer was chromatographed over MCI-gel CHP 20P eluted with gradient MeOH in H_2O to afford six fractions. All of these fractions were monitored and detected by silica gel thin-layer chromatography [$CHCl_3$−MeOH−H_2O (8:2:0.2; 7:3:0.5)]. The fractions eluted by 50% and 60% MeOH which were positive to $FeCl_3$ reagent were further chromatographed on Chromatorex ODS (20−60% MeOH) and Toyopearl HW-40F (30−60% MeOH) to yield compounds **1** (162 mg) and **2** (256 mg).

Pubescenoside A (1): pale yellow amorphous powder. $[\alpha]_D^{21}$ −17.4° (*c* 0.1, MeOH); HRESI-Q-TOF (positive ion mode) *m/z* 465.1339 $[M + Na]^+$ (calcd for $C_{20}H_{26}O_{11}Na$ 465.1365); UV (MeOH) λ_{max} (log ϵ) 329.5 (5.04) nm; IR (KBr) ν_{max} 3400, 2935, 1694, 1607, 1521, 1448, 1370, 1283, 1163, 1114, 1075, 855, 814 cm^{-1}; ^1H and ^{13}C NMR data, Table 1.

Acid Hydrolysis of Pubescenoside A.[16] A solution of **1** (10 mg) in 2 N HCl (4 mL) was heated at 90 °C for 3 h. The reaction mixture was neutralized with 5% NaOH and then extracted with EtOAc. Chromatography and co-chromatography of the EtOAc extract with caffeic acid by silica gel TLC [$CHCl_3$−EtOAc−toluene−HCOOH−MeOH (15:20:10:10:1), R_f = 0.45] and HPLC [Alltech Altima C18 (4.6 × 250 mm); 0−30% (40 min) CH_3CN in CH_3CN−H_2O−HCOOH (10:90:0.4); flow rate 1.0 mL/min; detection wavelength UV 280 nm, t_R = 15.60 min] confirmed the existence of caffeic acid. The water layer was evaporated to dryness and chromatographed on silica gel using $CHCl_3$−MeOH−H_2O (9:1:0.1, 8:2:0.2, 7:3:0.5) as the mobile phase to give compound **1a** (1 mg) and D-(+)-glucose, which was identified by HPLC [Alltech Prevail Amino Column (2.1 × 150 mm), solvent system CH_3CN−H_2O (90:10 v/v) at flow rate of 0.3 mL/min, detection: ELSD detector (tube temperature 95 °C, N_2 gas at flow rate of 1.0 mL/min), t_R = 11.83 min (t_R of standard sugars mannose, glucose, and galactose are 10.49, 11.78, and 12.48 min, respectively] and co-chromatography with authentic D-(+)-glucose. The D-configuration of the glucose was confirmed by its $[\alpha]_D^{22}$ +43.7° [(*c* 0.04, H_2O), determined after being dissolved in H_2O for 24 h; the concentration of glucose was determined by HPLC].

2-Hydroxymethyl-3, 4-dihydroxy-1-butene (**1a**): colorless oil; $[\alpha]_D^{21}$ +21.2°(*c* 0.04, MeOH); ^1H NMR (300 MHz, C_5D_5N) δ: 5.63 (2H, s, H-5), 4.91 (1H, dd, J = 5.2, 7.2 Hz, H-2), 4.71 (2H, s, H-4), 4.24 (1H, dd, J = 10.8, 5.2 Hz, H-1), 4.16 (dd, J = 10.8, 7.2 Hz, H-1).

Pubescenoside B (2): pale yellow amorphous powder; $[\alpha]_D^{21}$ −28.7° (*c* 0.1, MeOH); HRESI-Q-TOF (positive ion mode) *m/z* 465.1377 $(M + Na)^+$ (calcd for $C_{20}H_{26}O_{11}Na$ 465.1365); UV (MeOH) λ_{max} (logϵ) 328.5 (4.85) nm; IR (KBr) ν_{max} 3400, 2936, 1694, 1601, 1517, 1449, 1334, 1281, 1163, 1118, 1075, 855, 816 cm^{-1}; ^1H and ^{13}C NMR data, Table 1.

Acid Hydrolysis of Pubescenoside B. Compound **2** (15 mg) was hydrolyzed in a manner similar to that described for **1**, yielding caffeic acid, D-(+)-glucose {$[\alpha]_D^{21}$ +33.1° (*c* 0.1, H_2O), in H_2O for 24 h}, and **1a**, which was identified by comparing its ^1H NMR data with literature data.[13]

Preparation of Platelet-Rich Plasma (PRP). SD rats (350−450 g) were used as blood donors to collect anticoagulated blood. Collected blood samples were immediately transferred into a plastic tube with anticoagulant (1/10 volume of 3.8% trisodium citrate, pH 7.4). The platelet-rich plasma (PRP) was then prepared by centrifuging the blood at 300 rpm for 15 min. The platelet concentration was adjusted to a level of $5 \times 10^5/\mu$L by addition of homologous platelet-poor plasma (PPP) obtained after further centrifugation of blood at 1500 rpm for 15 min. For getting sufficient amount of PRP, at least two rats were sacrificed each time to obtain a pooled PRP sample.

Shear-Induced Platelet Aggregation (SIPA). Adjusted PRP was divided into different groups according to the experimental protocol and mixed with certain doses of pubescenosides A (**1**), B (**2**), aspirin, and salvianolic acid B. HAAke Rheometer RS 600 (Thermo Haake Corp., Ltd., Germany) with sensor C60/0.5° was employed as shear generator. The rheometer has the benefit of controlling shear stress and with an accuracy of 1 μm in the automatic adjustment of the gap between cone and plate. Shear program for PRP: preheating sample at 37 °C, increasing stress level from 0 to 15 Pa in the duration of 30 s, and then maintaining the stress level at 15 Pa for 360 s. After shear test, PRP was transferred to a platelet aggregometer (Chrono-Log aggregometer, Model 560 CA, Chrono-Log Corp., Led.), and SIPA was determined by turbidity. Since platelet-poor plasma (PPP) and the pre-shear PRP were used as the turbidity scales of 100% and 0% aggregation, accordingly, the aggregative degree of a post-shear PRP could be measured.

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Supporting Information Available: ^1H NMR, ^{13}C NMR, ^1H−^1H COSY, HSQC, HMBC, and HR-MS spectra of compounds **1** and **2**. This material is available free of charge via the Internet at http://pubs.acs.org.

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