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Characterization of Diterpenoid Glucosides in Roasted Puer Coffee Beans

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Supporting Information

ABSTRACT: Five new diterpenoid glucosides, named mascaroside I (**1**), mascaroside II (**2**), paniculoside VI (**3**), cofaryloside I (**4**), and villanovane I (**5**), along with seven known *ent*-kaurane diterpenoid glucosides (**6**−**12**) were isolated from acetone extracts of the roasted coffee beans of *Coffea arabica* var. *yunnanensis*. Their structures were established by extensive spectroscopic analysis including 1D and 2D NMR (HSQC, HMBC, COSY, and ROESY) and by comparison with published data. Cytotoxicities evaluation of the isolates showed that they were inactive against HL-60, SMMC-7721, A-549, MCF-7, and SW480 cells.

KEYWORDS: *Coffea arabica var. yunnanensis, roasted coffee beans, diterpenoids, structural elucidation, cytotoxicities*

INTRODUCTION

Coffea is an evergreen arbor in the Rubiaceae family, which has a worldwide distribution. Yunnan province, People's Republic of China, is a well-known cultivation base of *Coffea arabica* in the world.[1] Coffee bean has been used for hundreds of years as a functional and refreshing drink. Moreover, modern pharmacological research has shown that long-term coffee intake has consistently been associated with a substantially lower incidence of some chronic diseases, such as diabetes,[2−4] Parkinson's disease,[5] and hepatopathy.[6,7] Phytochemical investigation expressed that diterpenoids are one of the major active compositions in coffee. Until now, more than 80 *ent*-kaurane diterpenoids have been isolated from green coffee, most of which were connected with sugars or the fatty chain[8] and possessed a broad spectrum of biological properties, such as anti-inflammatory,[9] antioxidant,[10] and enhanced glutathione *S*-transferase activities.[11] Besides, *ent*-kaurane diterpenoids are of great interest because of their cytotoxic potency, as indicated by some studies that maoecrystal P,[12] amethystoidin A,[13] and cisplatin showed significant cytotoxic effects against T24 cells and K569 cells with IC_{50} values of 0.051, 0.69, and 1.11 μM, respectively.

Previous investigation demonstrated that roasting has a significant effect on coffee chemical constituents.[14] Therefore, diterpenoids in roasted coffee bean could be more complex, and the occurrence of diterpenoids in roasted coffee has not been investigated so far.

Thus, the aim of the current research was to isolate and elucidate the diterpenoids in the roasted coffee bean of *C. arabica* var. *yunnanensis* from Puer and evaluate their possible cytotoxicities.

MATERIALS AND METHODS

Chemicals. Silica gel (200−300 mesh, Qingdao Marine Chemical, Inc., Qingdao, China), Lichroprep RP C-18 (40−63 μm, Merck, Darmstadt, Germany), MCI gel (75−150 μm, Mitsubishi Chemical Co., Tokyo, Japan), and Sephadex LH-20 (20−150 μm, Pharmacia) were used for column chromatography. Methanol, trichloromethane, ethyl acetate, acetone, petroleum ether, *n*-butanol (Tianjing Chemical Reagents Co., Tianjing, China), and water (Wahaha Co., Zhejiang, China) were used for eluent. Trifluoroacetic acid, hexamethyldisilazane, trimethylchlorosilane, and D-glucose were purchased from Beijing Innochem Science and Technology Co. (Beijing, China).

General Experimental Procedures. Optical rotations were obtained with a Jasco P-1020 polarimeter (Jasco Co., Tokyo, Japan). 1D and 2D NMR spectra were measured on Bruker AM-400, DRX-500, and DRX-600 spectrometers (Bruker, Zurich, Switzerland) with TMS as internal standard. ESIMS and HREIMS data were recorded on an API QSTAR time-of-flight spectrometer (PE Sciex, Redwood City, CA, USA), and infrared spectra were recorded on a Bruker Tensor-27 instrument by using KBr pellets. Preparative HPLC was performed on an Agilent HP1100 series instrument (Agilent Technologies, Waldbronn, Germany) equipped with an L-2130 pump and a UV L-2400 detector in an Agilent ZORBAX SB-C-18 column (9.4 mm × 250 mm, flow rate at 3.0 mL/min; wavelength detection at 210 nm). GC analysis was carried out on an Agilent 7820A GC system using a HP-5 (30 m × 0.32 mm × 0.25 μm) column: detection, FID; carrier gas, N_2; injection temperature, 250 °C; detection temperature, 250 °C; column temperature, 180 °C. TLC was performed on precoated TLC plates (200−250 μm thickness, F254 Si gel 60, Qingdao Marine Chemical, Inc., Qingdao, China) with compounds visualized by spraying the dried plates with 10% aqueous H_2SO_4 followed by heating until dryness.

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Table 1. ^{1}H NMR Data of Compounds 1−5[a]

position	1[b]	2[b]	3[c]	4[c]	5[c]
1	2.42 (d, 11.6)	2.45 (m, 9.6)	1.20 (s)	1.22 (d, 9.6)	1.31 (d, 11.4)
	2.75 (d, 11.2)	2.67 (m, 15.2)	1.88 (d, 11.4)	1.61 (s)	1.38 (d, 10.2)
2			1.49 (m, 10.8)	1.31 (d, 15.6)	1.47 (m, 9.6)
			2.16 (m, 12.0)	1.75 (m, 14.4)	1.78 (m, 10.8)
3			0.96 (m, 9.6)	0.87 (m, 8.4)	0.98 (m, 13.2)
			2.37 (br d, 13.2)	1.34 (s)	2.36 (br d, 10.8)
5	2.86 (m, 7.6)	2.87 (m, 12.0)		1.48 (m, 13.2)	2.27 (m, 12.0)
6	1.62 (s)	1.60 (m, 11.2)	5.16 (s)	1.48 (m, 12.0)	2.36 (d, 11.4)
	2.04 (m, 12.0)	2.00 (s)		1.60 (m, 11.4)	3.04 (td, 18.0)
7	2.15 (s)	1.59 (m, 9.2)	1.65 (m, 17.4)	1.08 (d, 12.6)	5.50 (d, 6.6)
	1.56 (m, 7.6)	1.78 (m, 13.2)	2.16 (s)	1.60 (m, 9.6)	
9	1.62 (br s)	2.00 (br s)	1.73 (m, 19.2)		
11	3.89 (m, 10.8)	3.60 (m, 12)	1.29 (s)	0.93 (s)	1.59 (s)
			1.52 (m, 12.0)	1.66 (d, 13.8)	1.69 (m, 14.4)
12	1.77 (m, 12.4)	1.94 (m, 11.6)	1.98 (d, 10.2)	0.87 (m, 6.0)	2.28 (s)
	2.16 (br d, 8.8)	2.12 (d, 8.8)	2.71 (m, 12.0)	1.41 (d, 12.6)	
13	2.15 (m, 7.2)	2.60 (m, 12.0)	2.54 (m, 6.0)	1.63 (br s)	
14	1.80 (m, 11.2)	1.37 (m, 16.4)	1.49 (d, 9.6)	1.41 (s)	2.17 (d, 12.0)
	2.16 (m, 12.0)	2.12 (d, 7.2)	2.37 (m, 17.4)	1.95(d, 11.2)	2.94 (d, 8.4)
15	1.40 (d, 12.4)	1.59 (m, 9.6)	2.21 (m, 11.4)	0.87 (m, 6.0)	1.35 (m, 14.4)
	2.14 (m, 8.0)	1.78 (m, 11.2)	1.98 (d, 9.6)	2.06 (d, 12.0)	1.60 (m, 12.0)
16					1.60 (m, 12.0)
					1.87 (m, 13.8)
17	3.57 (m, 7.2)	3.63 (m, 8.4)	3.60 (m, 12.0)	3.39 (td, 6.6)	3.89 (d, 12.6)
	4.77 (d, 7.6)	4.09 (d, 7.2)	3.99 (d, 6.0)	3.49 (s)	3.99 (d, 9.6)
18	6.61 (s)	6.56 (s)	1.22 (s)	1.01 (s)	1.23 (s)
19	7.80 (s)	7.75 (s)			
20	0.85 (s)	1.05 (s)	1.21(s)	0.59 (s)	1.08 (s)
1′	4.28 (m, 7.2)	4.28 (m, 7.2)	6.44 (d, 6.6)	5.79 (d, 6.6)	6.40 (d, 12.0)
2′	3.24 (m, 8.0)	3.24 (m, 8.0)	5.97 (td, 17.6)	3.55 (td, 12.6)	4.20 (td, 11.4)
3′	3.28 (m, 8.4)	3.28 (m, 8.0)	4.47 (m, 11.4)	3.36 (m, 11.4)	4.04 (m, 10.2)
4′	3.26 (m, 8.0)	3.26 (m, 8.0)	4.49 (d, 12.6)	3.44 (s)	4.39 (m, 9.6)
5′	3.37 (m, 8.0)	3.37 (m, 8.4)	4.14 (m, 6.0)	3.59 (s)	4,29 (td, 6.6)
6′	3.65 (m, 15.6)	3.65 (m, 15.6)	4.42 (m, 13.2)	3.65 (d, 6.0)	4.42 (d, 12.0)
	3.87 (br d, 12.0)	3.87 (br d, 12.0)	4.49 (m, 12.0)	3.76 (br d, 11.2)	4.47 (br d, 12.0)
2″			5.67 (d, 18.0)		
3″			8.05 (d, 18.0)	7.81 (d, 6.6)	
4″				6.71 (d, 12.0)	
5″			7.61 (s)		
6″			7.18 (s)	6.71 (d, 12.0)	
7″				7.81 (d, 6.6)	
8″			7.18 (s)		
9″			7.61 (s)		

[a] δ in parts per million, J in hertz. NMR solvent was MeOD.([b]Obtained at 400 MHz. [c]Obtained at 600 MHz.

Plant Material. The green coffee beans of *C. arabica* var. *yunnanensis* were harvested in June 2012 by Tao Zhang and then roasted at 180 °C for 20 min to yield roasted coffee beans. The plant material was identified by Ming-hua Qiu, Kunming Institute of Botany, Chinese Academy of Sciences.

Extraction and Isolation. The powder of roasted coffee beans (200−300 mesh, 23 kg) was extracted with 80% acetone (3 × 50 L) three times (two days each times). The combined acetone extracts were evaporated under reduced pressure; then the 1.8 kg residue was suspended in H_2O and extracted with petroleum ether (PE), ethyl acetate (EtOAc), and *n*-butanol, respectively. The EtOAc layer (500 g) was subjected to silica gel column chromatography (25 × 180 cm) and eluted in a step gradient manner with PE/acetone (20:1, 10:1, 5:1, 1:1, v/v) to yield four fractions: fraction 1 (22 g), fraction 2 (67 g), fraction 3 (310 g), and fraction 4 (60 g). Fraction 4 was decolorized on an MCI gel column (5.0 × 60 cm) eluting with the step gradient of MeOH/H_2O (30−80%, v/v). The 50% MeOH/H_2O fraction (8.2 g) was chromatographed over a second silica gel column (5.0 × 100 cm, eluted with the step gradient manner of $CHCl_3$/MeOH, 15:1 to 3:1, v/v) to yield five fractions (fractions 4-1−4-5). Then fraction 4-1 (600 mg) was chromatographed on a silica gel column (2.0 × 80 cm, eluted with $CHCl_3$/MeOH, 10:1, v/v) to obtain **8** (15 mg), **9** (5.1 mg), and **10** (20 mg). Fraction 4-2 (800 mg) was subjected to chromatography over RP C-18 (3.0 × 50 cm, eluted with MeOH/H_2O, 30−70%, v/v) to give five minor fractions (fractions 4-2-1−4-2-5). Fraction 4-2-2 (50 mg) was separated by preparative thin layer chromatography (P-TLC, eluted with $CHCl_3$/MeOH, 8:1, v/v) to gain **1** (5.7 mg), **2** (20 mg), and **5** (3.0 mg). Using the same methods as for fraction 4-2-2 on P-TLC with $CHCl_3$/MeOH (8:1, v/v), compounds **3** (3.6 mg) and **6** (15 mg) were obtained from fraction 4-2-3 (80 mg). Fraction 4-3 (32 mg) was separated by reverse-phase HPLC (MeOH/H_2O: 30−50%, 25 min, flow rate = 3.0 mL/min, UV 210 nm) to get **4** (2.5 mg, 10.3 min). Fraction 4-5 (600 mg) was chromatographed on a silica gel column (2.0 × 60 cm) with $CHCl_3$/MeOH (10:1, v/v) to afford three

Table 2. ^{13}C NMR and DEPT Data of Compounds 1−5[a]

position	1[b]	2[b]	3[c]	4[c]	5[c]
1	54.2 (t)	54.2 (t)	40.3 (t)	33.4 (t)	33.2 (t)
2	187.9 (s)	186.5 (s)	19.7 (t)	20.1 (t)	20.4 (t)
3	147.9 (s)	147.0 (s)	37.7 (t)	39.3 (t)	39.4 (t)
4	145.0 (s)	143.9 (s)	44.4 (s)	45.5 (s)	45.0 (s)
5	45.8 (d)	46.3 (d)	156.8 (s)	50.8 (d)	47.1 (d)
6	23.4 (t)	22.7 (t)	113.5 (d)	23.2 (t)	25.2 (t)
7	36.5 (t)	36.8 (t)	29.8 (t)	38.0 (t)	119.1 (d)
8	45.3 (s)	45.1 (s)	42.2 (s)	50.9 (s)	139.8 (s)
9	62.2 (d)	56.4 (d)	46.0 (d)	78.8 (s)	42.4 (s)
10	43.8 (s)	43.9 (s)	38.1 (s)	49.1 (s)	38.2 (s)
11	66.2 (d)	77.5 (d)	29.1 (t)	30.0 (t)	23.9 (t)
12	37.5 (t)	40.4 (t)	18.2 (t)	28.2 (t)	33.3 (d)
13	46.5 (d)	43.3 (d)	43.8 (d)	45.1 (d)	75.1 (s)
14	41.2 (t)	43.8 (t)	42.9 (t)	39.5 (t)	32.6 (t)
15	52.0 (t)	53.8 (t)	54.9 (t)	48.1 (t)	43.6 (t)
16	82.4 (s)	89.6 (s)	83.7 (s)	83.1 (s)	23.7 (t)
17	75.5 (t)	73.5 (t)	67.9 (t)	66.8 (t)	69.1 (t)
18	111.5 (d)	111.3 (d)	27.1 (q)	29.3 (q)	29.8 (q)
19	150.5 (d)	150.0 (d)	175.5 (s)	178.5 (s)	177.6 (s)
20	15.7 (q)	16.9 (q)	23.0 (q)	18.6 (q)	17.1 (q)
1′	104.9 (s)	104.2 (s)	92.3 (s)	93.3 (s)	96.3 (s)
2′	75.3 (d)	74.6 (d)	72.9 (d)	74.0 (d)	74.6 (d)
3′	77.8 (d)	78.4 (d)	78.8 (d)	79.1 (d)	80.0 (d)
4′	71.8 (d)	71.2 (d)	70.0 (d)	71.2 (d)	71.4 (d)
5′	77.8 (d)	77.4 (d)	75.5 (d)	77.2 (d)	79.6 (d)
6′	62.8 (t)	62.4 (t)	60.9 (t)	62.4 (t)	62.6 (t)
1″			165.8 (s)	166.9 (s)	
2″			114.1 (d)	121.9 (s)	
3″			145.1 (d)	133.7 (d)	
4″			125.0 (s)	116.4 (d)	
5″			129.8 (d)	164.3 (s)	
6″			115.9 (d)	116.4 (d)	
7″			160.7 (s)	133.7 (d)	
8″			115.9 (d)		
9″			129.8 (d)		

[a] δ in parts per million, and J in hertz. NMR solvent was MeOD. [b] Obtained at 100 MHz. [c] Obtained at 150 MHz.

minor fractions (fractions 4-5-1−4-5-3). Fraction 4-5-2 (190 mg) was purified by use of Sephadex LH-20 (1.0 × 200 cm, eluted with MeOH, 100%, 1 L) and divided into two fractions (fractions 4-5-2-1 and 4-5-2-2), and then fraction 4-5-2-2 (160 mg) was isolated by P-TLC ($CHCl_3$/MeOH, 7:1, v/v) to gain **7** (7.1 mg), **11** (12 mg), and **12** (14 mg).

Acid Hydrolysis of Compounds 1−5.[15] Compounds **1**−**5** (each 1 mg) were dissolved in 25 mL of MeOH and refluxed with 1 N HCl (1 mL) for 3 h at 80 °C. The reaction mixture was then evaporated to dryness and diluted with H_2O (10 mL). After being extracted with EtOAc (3 × 10 mL), the aqueous layer was concentrated and compared with reference D-glucose by TLC ($CHCl_3$/MeOH, 2:1, v/v). Then 900 μL of hexamethyldisilazane/trimethylchlorosilane (2:1, v/v) was added in the aqueous layer, and the mixture was stirred at 60 °C for 30 min. The supernatant was subjected to GC analysis. Derivatives of D-glucose were detected from **1**−**5**, which were identified by comparison of their retention times with that of an authentic sample of D-glucose (9.53 min).

*Mascaroside I (**1**):* white amorphous powder; $[\alpha]^{22}_D$ −85.1 (*c* 0.16, MeOH); UV (MeOH) λ_{max} (log ε) 276 (3.86) nm; IR (KBr) ν_{max} 3418, 3132, 2926, 1660, 1471, 1436, 1411, 1387, 1365, 1331, 1314, 1237, 1208, 1165, 1131, 1076, 1047, 1031 cm^{-1}; ESIMS *m*/*z* 531 [M + Na]$^+$; HREIMS *m*/*z* 508.2308 [M]$^+$ (calcd for $C_{26}H_{36}O_{10}$, 508.2315). ^1H and ^{13}C NMR data are shown in Tables 1 and 2, respectively.

*Mascaroside II (**2**):* white amorphous powder; $[\alpha]^{25}_D$ −89.5 (*c* 0.16, MeOH); UV (MeOH) λ_{max} (log ε) 276 (3.93) nm; IR (KBr) ν_{max} 3850, 3429, 2923, 2853, 1661, 1555, 1435, 1076 cm^{-1}; ESIMS *m*/*z* 513 [M + Na]$^+$; HREIMS *m*/*z* 490.2199 [M]$^+$ (calcd for $C_{26}H_{34}O_9$, 490.2203). ^1H and ^{13}C NMR spectral data are shown in Tables 1 and 2, respectively.

*Paniculoside VI (**3**):* white amorphous powder; $[\alpha]^{22}_D$ −6.0 (*c* 0.27, MeOH); UV (MeOH) λ_{max} (log ε) 315 (4.03) nm; IR (KBr) ν_{max} 3431, 2924, 2853, 2070, 1722, 1630, 1605, 1514, 1461, 1378, 1262, 1167, 1070, 1034 cm^{-1}; ESIMS *m*/*z* 641 [M - H]$^+$; HREIMS *m*/*z* 642.3052 [M]$^+$ (calcd for $C_{35}H_{46}O_{11}$, 642.3040). ^1H and ^{13}C NMR spectral data are shown in Tables 1 and 2, respectively.

*Cofaryloside I (**4**):* white amorphous powder; $[\alpha]^{27}_D$ −35.1 (*c* 0.06, MeOH); UV (MeOH) λ_{max} (log ε) 259 (3.98) nm; IR (KBr) ν_{max} 3771, 3424, 2928, 2872, 2857, 1720, 1610, 1513, 1447, 1383, 1313, 1268, 1235, 1166, 1097, 1070, 1036 cm^{-1}; ESIMS *m*/*z* 657 [M + Na]$^+$; HREIMS *m*/*z* 634.3006 [M]$^+$ (calcd for $C_{33}H_{46}O_{12}$, 634.2989). ^1H and ^{13}C NMR spectral data are shown in Tables 1 and 2, respectively.

*Villanovane I (**5**):* white amorphous powder; $[\alpha]^{22}_D$ +29.3 (*c* 0.11, MeOH); UV (MeOH) λ_{max} (log ε) 318 (3.12) nm; IR (KBr) ν_{max} 3425, 2926, 2872, 1730, 1630, 1514, 1451, 1382, 1267, 1215, 1071 cm^{-1}; ESIMS *m*/*z* 519 [M + Na]$^+$; HREIMS *m*/*z* 496.2666 M$^+$ (calcd for $C_{26}H_{40}O_9$, 496.2672). ^1H and ^{13}C NMR spectral data are shown in Tables 1 and 2, respectively.

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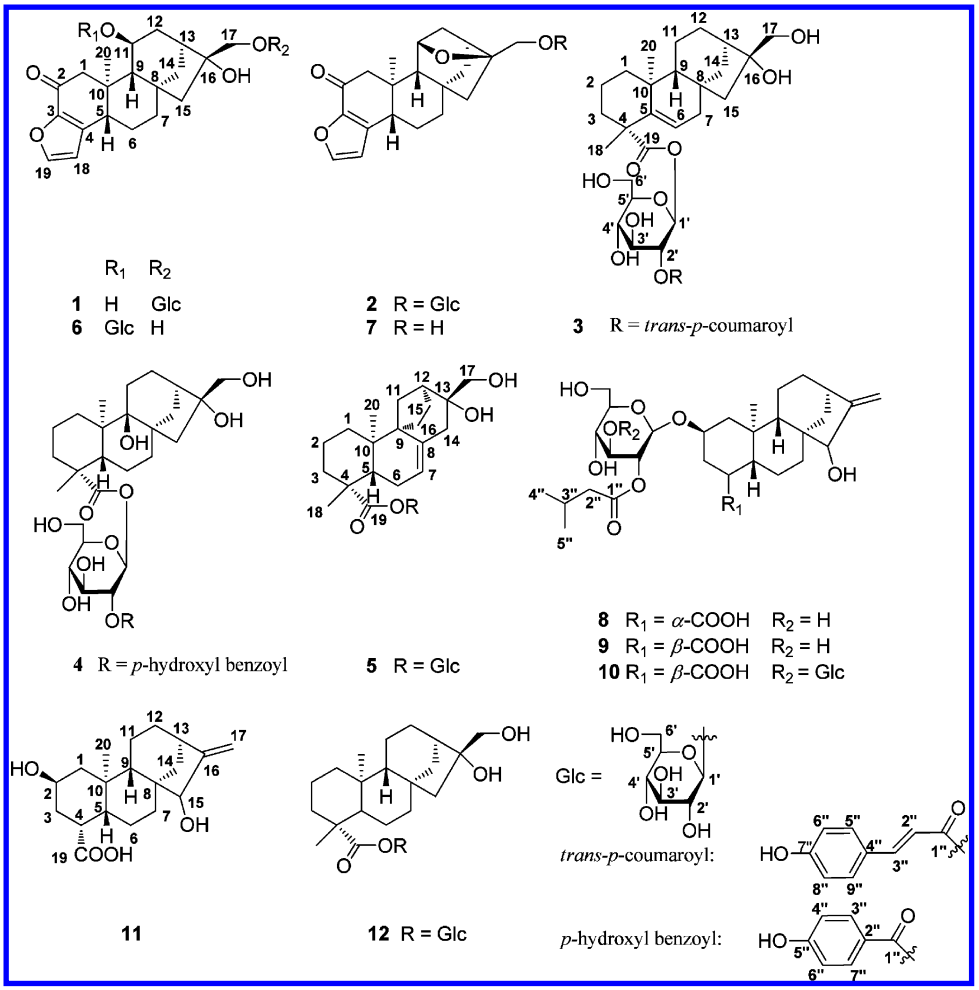

Figure 1. Structures of diterpenoid glucosides isolated from roasted coffee beans.

Cytotoxicity Assays. The cytotoxicities of compounds **1**−**5** were evaluated against HL-60, SMMC-7721, A-549, MCF-7, and SW480 cells by using the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] method.[16] All cells were cultured in RPMI-1640 or DMEM medium (Hyclone, Logan, UT, USA), supplemented with 10% fetal bovine serum (Hyclone) at 37 °C in a humidified atmosphere with 5% CO_2. After seeding of 1×10^5 cells/mL in a 96-well cell culture plate for 12 h before test compound addition, 100 μL of sample or standard agent was placed in each well and incubated at 37 °C for 3 days. After incubation, MTT (100 μg) was added to each well, and the incubation was continued for 4 h at 37 °C. The cells were lysed with 100 μL of 20% SDS−50% DMF after removal of 100 μL of medium. The optical density of the lysate was measured at 595 nm in a 96-well microtiter plate reader (Bio-Rad 680). The IC_{50} value of each compound was calculated by using Reed and Muench's method.[17]

RESULTS AND DISCUSSION

Structure Elucidation. The acetone extract of roasted coffee beans was extracted with ether (PE), ethyl acetate (EtOAc), and *n*-butanol. The EtOAc layer was fractionated by silica gel column chromatography in a step gradient of PE/acetone to yield four fractions, and then fraction 4 (PE/acetone, 1:1, v/v) was further purified successively on silica gel, Sephadex LH-20, and RP C-18 columns, respectively. As a result, the diterpenoid glucosides **1**−**12** were obtained in pure forms (Figure 1), of which **1**−**5** are reported for the first time in this study. Their structures were established by interpretation and full assignments of 1D and 2D NMR spectroscopic data and comparison with literature data.

Compound **1** was isolated as a white amorphous powder. The molecular formula $C_{26}H_{36}O_{10}$ was deduced from the molecular ion peak at m/z $[M]^+$ 508.2308 (calcd for $C_{26}H_{36}O_{10}$, 508.2315) in HREIMS. The IR spectrum indicated that **1** possessed hydroxyl (3418 cm^{-1}) and α,β-unsaturated ketone (1660 cm^{-1}) groups. The ^1H NMR spectrum (Table 1) of **1** displayed characteristic signals for one methyl (δ_H 0.85, s, H-20), two oxymethylenes [δ_H 3.57 (m, J = 7.2 Hz, H-17a), 4.77 (d, J = 7.6 Hz, H-17b); δ_H 3.65 (m, J = 15.6 Hz, H-6′a), 3.87 (d, J = 12.0 Hz, H-6′b)], and five oxygenated methines. The ^{13}C NMR spectrum (Table 2) showed 26 carbon resonances, attributed to a monosaccharide and an aglycone moiety. The monosaccharide was identified as glucose on the basis of comparison of chemical shifts to those found in the literature.[18] Its relative configuration was confirmed as β on the basis of the coupling constant (δ_H 4.28, m, J = 7.2 Hz, H-1′) in the ^1H NMR spectrum. The configuration of glucose was identified as D-form by acid hydrolysis. The other 20 carbon signals of the aglycone moiety were classified by ^{13}C NMR as 1 methyl, 7 methylenes (1 oxygenated), 6 methines (including 1 oxygenated, 2 olefinic), and 6 quaternary carbons (including 1 carbonyl, 2 olefinic, and 1 oxygenated). These data indicated that **1** resembled mascaroside,[19] except that a methylene (δ_C 52.0, C-15) in **1** replaced the oxymethine in mascaroside at C-15, which was further confirmed by the heteronuclear multiple-

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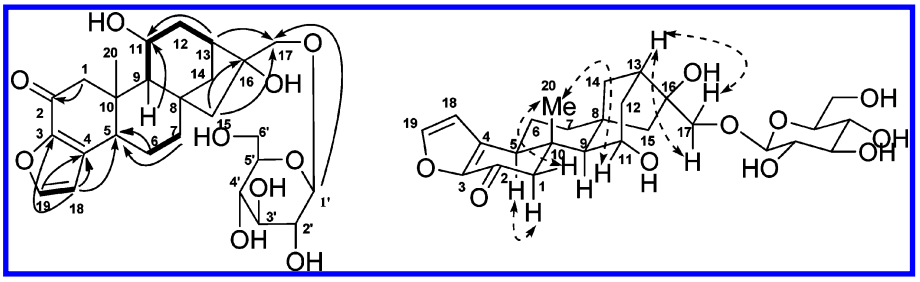

Figure 2. Key HMBC (H→C), ^{1}H–^{1}H COSY (bold), and ROESY (H←- -→H) correlations of **1**.

bond correlations (HMBC) from H_2-15 to C-9, C-13, and C-17 (Figure 2). Meanwhile, the HMBC correlations from H_2-17 to C-13, C-16, and C-1′ suggested the glucose group was coupled to C-17. The relative configuration of **1** was determined by the rotating frame Overhauser effect spectroscopy (ROESY) spectrum. On biogenetic grounds, compound **1** was confirmed as an *ent*-kauranoid with the configuration of H-20 being α-orientated. The key ROESY correlations of H-11/H-20 and H-13/H_2-17 (Figure 2) were assigned H-11 and OH-16 as α-orientated. Thus, the structure of **1** was assigned as mascaroside I.

The HREIMS data for compound **2** indicated a molecular formula of $C_{26}H_{34}O_9$. Analysis of the 1D NMR data of **2** and those of **1** (Tables 1 and 2) revealed that they were comparable. However, the carbon signals of C-11 (δ_C 77.5 for **2**, 66.2 for **1**) and C-16 (δ_C 89.6 for **2**, 82.4 for **1**) were significantly downfield shifted in **2**, and **2** being 18 mass units less than **1** suggested the presence of the ether between C-11 and C-16 in **2**. The HMBC correlations of H-11 with C-9, C-13, and C-16, of H-13 with C-16, and of H_2-15 with C-16 provided important evidence for the above inference. The glucose group was positioned at C-17 on the basis of the HMBC correlation from H_2-17 to C-13, C-16, and C-1′. Furthermore, major correlations of H-20/H-11, H-13/H_2-17 were observed in the ROESY spectrum of **2**, establishing H-11 and H_2-17 as being α-oriented. Therefore, the structure of compound **2** was identified as mascaroside II.

Compound **3**, a white amorphous powder, displayed a $[M]^+$ ion at *m*/*z* 642.3052 (calcd for $C_{35}H_{46}O_{11}$, 642.3040) in HREIMS, consistent with the molecular formula of $C_{35}H_{46}O_{11}$, indicating 13 degrees of unsaturation. IR absorption bands at 3431 and 1630 cm^{-1} were attributed to hydroxyl and double-bond groups. A *p*-coumaroyl group was confirmed by comparison of the MS and 1D NMR data of **3** with those of laevissioside A;[20] its double bond was suggested as *trans* due to the coupling constant (J = 18.0 Hz). The ^{13}C NMR spectrum (Table 2) showed the remaining 26 characteristic carbon signals for diterpenoid and β-glucose. Meanwhile, 20 carbon signals of the diterpenoid were attributed to 2 singlet methyls, 9 methylenes (1 oxygenated), 3 methines (1 olefinic), and 6 quaternary carbons (1 carboxyl, 1 oxygenated, 1 olefinic). These data revealed that **3** was similar to paniculoside IV,[21] except for the presence of two olefinic carbons (δ_C 156.8, C-5, and δ_C 113.5, C-6) in **3**. In the HMBC spectrum, the protons of C-7, C-20, and C-3 all correlated with the sp^2 quaternary carbon (δ_C 156.8, C-5), and the proton of sp^2 methine showed correlations with C-4 and C-10. Therefore, the position of the double bond was between C-5 and C-6. The β-glucose group was confirmed at C-19 by HMBC correlations of H-1′ to C-19. The HMBC correlation from H- 2′ to C-1″ (Figure 3) established that the *trans*-*p*-coumaroyl group was at C-2′. The

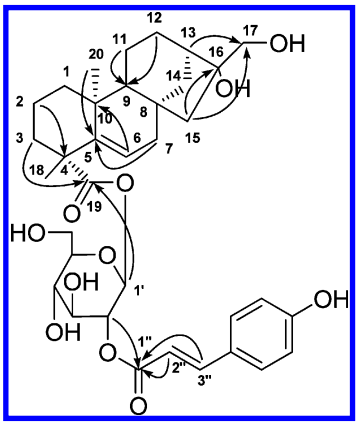

Figure 3. Key HMBC (H→C) correlations of **3**.

ROESY correlation of H-20/H-1′ assigned the relative configuration of CH_3-18 as β. Finally, the structure of **3** was confirmed as paniculoside VI.

The molecular formula of **4**, $C_{33}H_{46}O_{12}$, was determined by HREIMS (*m*/*z* 634.3006, calcd 634.2989). The IR absorption bands at 3424, 2928, and 1610 cm^{-1} suggested the presence of hydroxyl, carbonyl, and double-bond groups. The 1D NMR spectrum shows seven carbon signals (δ_C 166.9, C-1″; δ_C 164.3, C-2″; δ_C 133.7, C-3″; δ_C 133.7, C-4″; δ_C 121.9, C-5″; δ_C 116.4, C-6″; δ_C 116.4, C-7″) and two aromatic signals at δ_H 7.81 (2H, d, J = 6.6 Hz, H-2″ and H-6″), 6.71 (2H, d, J = 12.0 Hz, H-3″ and H-5″), indicating the presence of a *p*-hydroxybenzoyl group. In addition, the ^{13}C NMR spectrum exhibited another 26 carbon signals for diterpenoid and β-glucose. Comparison of the NMR data of the diterpenoid moiety of **4** with those of cofaryloside indicated they were similar.[22] In the HMBC spectrum, correlation from H-1′ to C-19 suggested the β-glucose group was linked to C-19. HMBC correlation from H-2′ (δ_H 3.55) to C-1″ confirmed the *p*-hydroxybenzoyl group at C-2′. The similar ROESY correlations with **3** indicated the relative configuration of CH_3-18 to be β. Therefore, compound **4** was assigned as cofaryloside I.

Compound **5** was obtained as a white powder, and its molecular formula was deduced as $C_{26}H_{40}O_9$ from HREIMS (*m*/*z* 496.2259, calcd 496.2672). The ^{13}C NMR spectrum (Table 2) of **5** displayed 20 carbon signals for diterpenoid, attributed to 2 methyls, 9 methylenes (1 oxygenated), 3 methines (1 olefinic), and 6 quaternary carbons (1 olefinic, 1 oxygenated, 1 carboxyl), suggesting **5** may be analogous to **4**. However, this assignment was in disagreement with HMBC correlations between H-20 and two quaternary sp^3 carbons (δ_C 38.2, C-10 and δ_C 42.4, C-9). Furthermore, comparison of chemical shifts of **5** showed resemblance to villanovane,[23] which was confirmed by the HMBC correlations from H-7, H-11, H-12, H_2-14, H_2-15, and H_2-16 to C-9, and the ^{1}H–^{1}H

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correlation spectrometry (COSY) correlations of H-11, H-15/H-12, and H-15/H-16 (Figure 4).

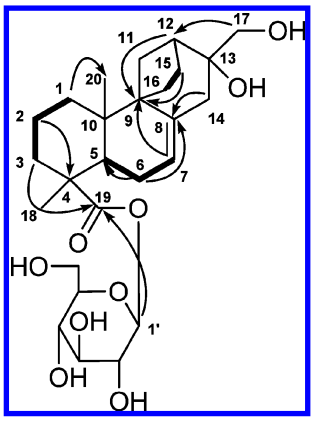

Figure 4. Key HMBC (H→C) and ^{1}H−^{1}H COSY (bold) correlations of **5**.

Comparison of 1D NMR data of **5** with those of villanovane suggested a double-bond and a glucose group were present in **5**, which were consistent with the IR absorption bands at ν_{max} 1631 and 3425 cm^{-1}. The relative configuration of glucose was confirmed as β-based on a coupling constant of 12 Hz for H-1′ (δ_H 6.40). Its position was confirmed at C-19 on the basis of HMBC correlation from H-1′ to C-19. The olefinic group involving C-7 and C-8 was supported by the HMBC correlations from H-5 and H_2-6 to C-7. Furthermore, the key correlations of H-12/H-20 and H-5/H-18 in the ROESY spectrum assigned the relative configurations of C-15, C-16, and C-18 as β. Hence, compound **5** was characterized as villanovane I.

The known compounds isolated were identified by comparison of their NMR data with literature data as mozambioside (**6**),[24] bengalensol (**7**),[25] 19-*norkaur*-16-en-18-oic acid-15-hydroxy-2-[[2-*O*-(3-methyl-1-oxobutyl)-β-D-glucopyranosyl]oxy]-(2β,4α,15α)- (**8**),[26] 19-*norkaur*-16-en-18-oic acid-15-hydroxy-2-[[2-*O*-(3-methyl-1-oxobutyl)-β-D-glucopyranosyl]oxy]-(2β,4β,15α)- (**9**),[26] 19-*norkaur*-16-en-18-oic acid-2-[[3-*O*-β-D-glucopyranosyl-2-*O*-(3-methyl-1-oxobutyl)-β-D-glucopyranosyl]oxy]-15-hydroxy-(2β,4α,15α)- (**10**),[26] 2β,16α,17-trihydroxy-*ent*-kauran-19-oic acid (**11**),[27] and paniculoside IV (**12**).[21]

The evaluation of cytotoxicities of compounds **1**−**12** showed that they were inactive against all test cells.

In conclusion, the present investigation on diterpenoid glucosides has revealed a wide structural diversity and upgrades our knowledge on the composition of roasted coffee. This is the first report about *ent*-atisans from Rubiaceae. Our work lays the chemical foundation for further bioactive research of coffee.

■ ASSOCIATED CONTENT

Supporting Information

^{1}H and ^{13}C NMR, DEPT, HSQC, HMBC, ^{1}H−^{1}H COSY, and ROESY spectra of compounds **1**−**5**. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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