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Three New Triterpenes from the Seeds of *Combretum quadrangulare* and Their Hepatoprotective Activity

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Three new triterpenes of the lupane type, 2α,6β-dihydroxybetulinic acid (**1**) and 6β-hydroxyhovenic acid (**2**), and an oleanane type, 6β-hydroxyarjunic acid (**3**), together with several known compounds, have been isolated from the MeOH extract of the seeds of *Combretum quadrangulare*. The structures of these compounds were elucidated on the basis of spectroscopic analysis, and their hepatoprotective activities were tested for D-GalN/TNF-α-induced cell death in primary cultured mouse hepatocytes.

Combretum quadrangulare (Combretaceae) is a tree indigenous to eastern Asia that is commonly known as "Tram bau" in Vietnam. The seeds, leaves, and stem bark of the plant have been used in Vietnamese folk medicine as an antipyretic, antidysenteric, antihepatitis, and anthelmintic agent.[1] We previously reported that the MeOH extract of the seeds of *C. quadrangulare* exhibits potent hepatoprotective activity against D-galactosamine (D-GalN)/tumor necrosis factor alpha (TNF-α)-induced cell death in primary cultured mouse hepatocytes.[2] The MeOH extract was fractionated into water- and MeOH-soluble fractions, and both of these fractions showed significant hepatoprotective activity. From the water-soluble fraction, 38 compounds including 11 new triterpene glucosides, namely, quadranosides I−XI,[2,3] and 1-*O*-galloyl-6-*O*-(4-hydroxy-3,5-dimethoxy)benzoyl-β-D-glucose,[4] were isolated, and their hepatoprotective activities reported.[5] Recently, we examined the constituents of the MeOH-soluble fraction of MeOH extract and isolated three new triterpenes together with 17 known compounds. This paper reports the structure elucidation of the three new triterpenes and the hepatoprotective activity of the constituents isolated.

By repeated column chromatography over silica gel and preparative TLC, the MeOH-soluble fraction afforded three new triterpenes (**1**−**3**) along with 17 known compounds, namely, quadranoside II (**4**),[2] arjunic acid (**5**),[6] arjunolic acid (**6**),[7] arjunglucosides I (**7**)[8] and II (**8**),[9] 19α-hydroxyasiatic acid (**9**),[10] nigaichigoside F1 (**10**),[10] quadranoside IV (**11**),[2] 2α,3β,23-trihydroxyurs-12,18-dien-28-oic acid (**12**),[11] a mixture of glucosyl 2α,3β,23-trihydroxyurs-12,18-dien-28-oate (**13**) and glucosyl 2α,3β,23-trihydroxyurs-12,19(20)-dien-28-oate (**14**),[11] β-sitosterol, β-sitosterol glucoside, (+)-catechin,[12] (+)-gallocatechin,[13] (−)-epicatehin,[13] and (−)-epigallocatechin.[14] Their structures (Chart 1) were elucidated on the basis of spectral data and comparison with literature data.

Compound **1** was obtained as a colorless amorphous solid. The pseudomolecular ion peak at *m*/*z* 487.3411 $(M-H)^-$ in its HRFABMS suggested its molecular formula to be $C_{30}H_{48}O_5$. The IR spectrum indicated the presence of hydroxyl (3450 cm^{-1}), carbonyl (1710 cm^{-1}), and olefinic (1640 cm^{-1}) groups. The ^1H NMR spectrum of **1** displayed signals corresponding to six tertiary methyls (δ_H 1.08, 1.45, 1.59, 1.68, 1.75, 1.81), two exo-olefinic protons (δ_H 4.79, 4.96), and three oxymethine protons (δ_H 3.42, 4.27, 4.81), while the ^{13}C NMR spectrum showed 30 carbon signals including six methyls, nine methylenes, eight (three of which are oxygenated) methines, and seven (including one carbonyl) quaternary carbons (Table 1). On the basis of the analysis of the $^1H-^1H$ COSY and HMQC spectra, **1** was suggested to be a lupane-type triterpene bearing three hydroxyls and a carboxyl group (C-28).[8] Two oxymethine protons at δ_H 3.42 and 4.27 showed correlations in the $^1H-^1H$ COSY spectrum, suggesting their vicinal arrangement. Furthermore, both of the protons had long-range correlations, in the FG-pulsed HMBC spectrum, with a quaternary carbon at δ_C 40.6 assigned to C-4. This indicated that the position of the two hydroxyl groups should be at C-2 and C-3. Similarly, the position of the third hydroxyl group was determined to be at C-6 from the $^1H-^1H$ COSY and the HMBC spectra (Figure 1a in Supporting Information). Accordingly, the planar structure of **1** was determined as 2,6-dihydroxybetulinic acid, which was further supported by the mass fragmentation pattern (Figure 2 in Supporting Information). The stereochemistry of **1** was determined by analysis of its coupling constants and ROESY data. The coupling constant (9.2 Hz) between H-2 and H-3 indicated the hydroxyl groups should have 2α,3β-orientation, which was further supported by the ROESY correlations of H-2 with H-1β and H_3-25 and of H-3 with H-1α (Figure 1b in Supporting Information). The broad singlet nature of H-6 suggested the hydroxyl group at C-6 should be β-oriented, which was further supported by the intense cross-peak between H-6 and H_3-23 in the ROESY spectrum. Thus, the structure of **1** was determined to be 2α,3β,6β-trihydroxylup-20(29)-en-28-oic acid, i.e., 2α,6β-dihydroxybetulinic acid.

Compound **2** was also isolated as a colorless amorphous solid, and its molecular formula was determined to be $C_{30}H_{48}O_6$ by HRFABMS. The IR spectrum indicated the presence of hydroxyl (3400 cm^{-1}), carbonyl (1725 cm^{-1}), and olefinic (1635 cm^{-1}) groups. The ^1H and ^{13}C NMR spectra of **2** were similar to those of **1**, except for the presence of one more oxymethylene group (δ_H 4.06, 4.41; δ_C 66.2) instead of the methyl group in **1** (δ_H 1.45; δ_C 28.8) (Table 1). The position of the new oxymethylene group was determined to be at C-4α (i.e., C-23), on the basis of the long-range correlations between the oxymethylene protons and C-3, C-4, C-5, and C-24 in the FG-pulsed HMBC spectrum and the ROESY correlations between the oxymethylene protons (H_2-23) and H-6. Thus, the structure of

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Chart 1

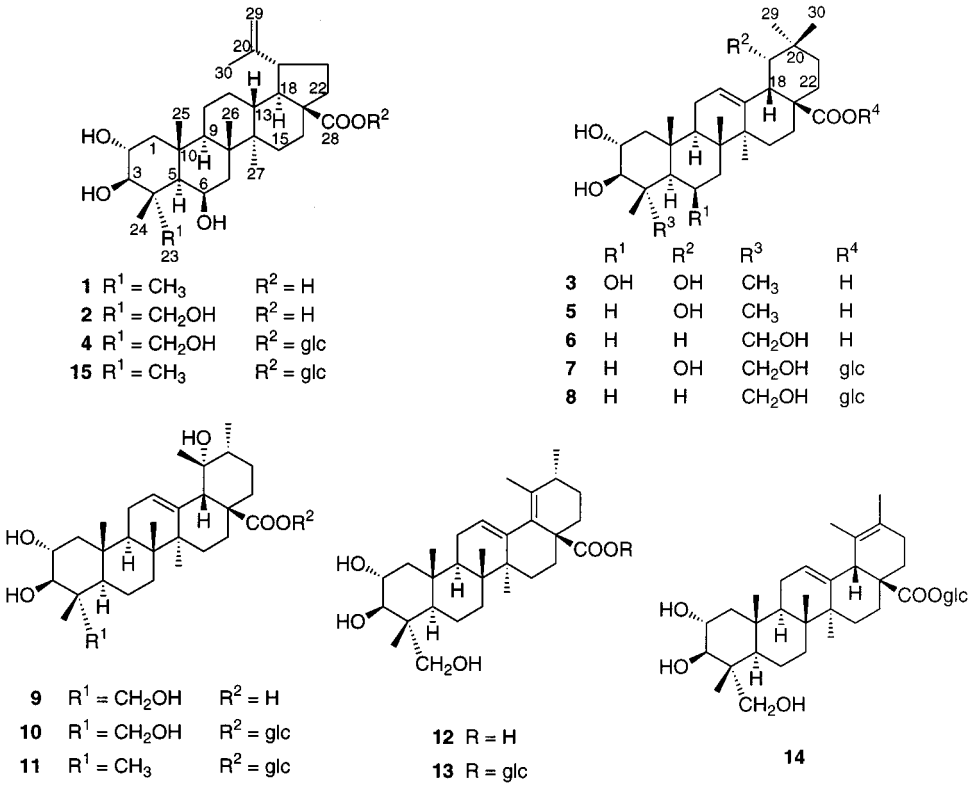

Table 1. ^{1}H and ^{13}C NMR Data of Compounds **1**−**3** in C_5D_5N[a]

	1		**2**		**3**	
position	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H
1	50.1	1.32 m; 2.39 dd (12.2, 4.2)	50.4	1.41 m; 2.43 dd (12.5, 4.4)	49.8	1.38 m; 2.32 m
2	69.0	4.27 td (10.5, 4.2)	69.3	4.41 m	68.8	4.28 ddd (11.0, 9.3, 4.4)
3	84.2	3.42 d (9.2)	78.4	4.24 d (9.5)	84.2	3.41 d (9.3)
4	40.6		38.5		40.8	
5	56.6	1.12 m	49.2	1.71 m	56.9	1.18 m
6	67.8	4.81 br s	67.8	5.07 br s	67.9	4.86 br s
7	42.6	1.76 m; 1.90 br d (12.0)	42.4	1.92 m	41.8	1.90 m
8	43.1		43.1		39.6	
9	51.8	1.46 m	51.9	1.42 m	49.1	2.15 m
10	38.7		40.7		38.6	
11	21.5	1.65 m	21.6	1.65 m	24.3	2.30 m
12	26.3	1.23 m; 1.99 m	26.3	1.23 m; 1.99 m	123.3	5.65 br s
13	37.8	2.88 td (11.3, 3.4)	37.8	2.89 td (11.5, 4.2)	144.3	
14	40.7		44.5		42.7	
15	30.4	1.54 m; 1.99 m	30.4	1.51 m; 1.99 m	29.1	1.30 m; 2.28 m
16	32.9	1.32 m; 2.62 br d (12.7)	32.8	1.28 m; 2.58 br d (12.7)	28.4	2.14 m; 2.84 td (13.4, 3.4)
17	56.6		56.6		46.1	
18	49.9	1.80 m	49.9	1.67 m	44.9	3.67 br s
19	47.8	3.56 td (11.7, 5.2)	47.8	3.60 td (10.9, 4.6)	81.3	3.65 br s
20	151.3		151.3		35.8	
21	31.2	1.54 m; 2.25 m	31.2	1.53 m; 2.24 m	29.3	2.01 m; 1.22 m
22	37.6	1.58 m; 2.25 m	37.5	1.58 m; 2.24 m	33.7	2.02 m; 2.18 m
23	28.8	1.45 s	66.2	4.06 d (10.3); 4.41 d (10.3)	29.1	1.46 s
24	19.1	1.75 s	19.8	1.71 s	19.2	1.78 s
25	19.3	1.59 s	15.8	1.67 s	18.4	1.75 s
26	17.1	1.68 s	17.2	1.79 s	24.9	1.67 s
27	15.2	1.08 s	15.2	1.00 s	18.3	1.66 s
28	178.8		178.8		180.8	
29	110.0	4.79 br s; 4.96 br s	110.0	4.78 br s; 4.94 br s	28.9	1.20 s
30	19.5	1.81 s	19.5	1.73 s	24.9	1.13 s

[a] *J* values (in Hz) in parentheses.

compound **2** was determined to be 2α,3β,6β,23-tetrahydroxylup-20(29)-en-28-oic acid, i.e., 6β-hydroxyhovenic acid.

Compound **3** was obtained as a colorless amorphous solid. The pseudomolecular ion peak at *m*/*z* 503.3393 $(M-H)^-$ in the HRFABMS of **3** indicated its molecular formula to be $C_{30}H_{48}O_6$. Absorption bands at 3400, 1720, and 1645 cm^{-1} in the IR spectrum of **3** suggested the presence of hydroxyl, carbonyl, and olefinic groups, respectively. The ^{1}H NMR spectrum of **3** displayed signals corresponding to seven tertiary methyls (δ_H 1.13, 1.20, 1.46, 1.66, 1.67, 1.75, 1.78), an olefin (δ_H 5.65, br s), and four oxygenated methines (δ_H 3.41, 3.65, 4.28, 4.86) (Table 1). The ^{13}C NMR

spectrum of **3**, on the other hand, showed 30 carbon signals including seven primary, seven secondary, eight tertiary, and eight quaternary carbons (Table 1), including the typical signals (δ_C 123.3 and 144.3) for the double bond at C-12(13) of an oleanane-type triterpene.[8] The carbon signals corresponding to rings A and B, including the three oxymethine groups (δ_C 67.9, 68.8, 84.2), were identical with those of **1**, suggesting the presence of three hydroxyl groups at C-2, C-3, and C-6 (i.e., with **3** having the same rings A and B as **1**). This was also supported by the fragment ion at *m*/*z* 264 via retro-Diels−Alder reaction in the mass spectrum. In addition, the ^{1}H and ^{13}C NMR spectra of **3** indicated the presence of one more oxymethine group (δ_H 3.65, 1H, br s; δ_C 81.3). The oxymethine proton had HMBC correlations with C-17, suggesting the position to be at C-19 (Figure 3a in Supporting Information). Accordingly, the planar structure of compound **3** was determined as 2,3,6,19-tetrahydroxyolean-12-en-28-oic acid. The stereochemistry of **3** was determined from the ROESY spectrum (Figure 3b in Supporting Information). The spectrum showed correlations of H-2 with the methyl protons H_3-24 and H_3-25 and of H-3 with H-5, indicating the orientation of the hydroxyl groups to be at 2α,3*β*. The broad singlet nature of H-6 and H-19 and the ROESY correlations between H-5 and H-6, between H-6 and H_3-27, and between H-19 and H_3-30 indicated the configuration of 6-OH and 19-OH to be *β* and α, respectively. Thus, the structure of compound **3** was determined to be 2α,3*β*,6*β*,19α-tetrahydroxyolean-12-en-28-oic acid, i.e., 6*β*-hydroxyarjunic acid.

Among the isolated compounds, 13 (**4**, **7**−**11**, **13**, **14**, *β*-sitosterol glucoside, catechin, gallocatechin, epicatechin, epigallocatechin) were previously isolated from the water-soluble fraction of the MeOH extract of *C. quadrangulare* seeds.[2,3] Their hepatoprotective activities in D-GalN/TNF-α-induced cell death in primary cultured mouse hepatocytes were previously reported, and catechin derivatives seemed to play a role in the hepatoprotective effect.[5] Using the same experimental model, the newly isolated compounds (**1**−**3**, **5**, **6**, **12**, *β*-sitosterol) were tested for their hepatoprotective activities (Table 2). Among these compounds, **1**, **2**, **6**, and **12** exhibit strong hepatoprotective activities, with IC_{50} values of 60.9, 51.2, 58.4, and 51.0 *μ*M, respectively. In the same experimental model, silibinin and glycyrrizhin, clinically used drugs, had IC_{50} values of 29.9 and >200 *μ*M, respectively.[15] The active compounds are lupane-, oleanane-, and ursane-type triterpenes and catechin derivatives. The hepatoprotective activities of the lupane-type aglycones **1** and **2** and their glucosyl esters, quadranosides I (**15**) and II (**4**),[5] were found to be almost identical. In addition, an ursane-type aglycone (**12**) and its glucoside, quadranoside V,[5] also showed similar hepatoprotective activity. On the other hand, ursane-type triterpenes (**9**−**11**), with only one double bond at C-12, showed either weak or no hepatoprotective activity.[4] Thus, the aglycone part would seem to be important for hepatoprotective activity.

In conclusion, some lupane-type triterpenes have been shown to possess potent protective activity against D-GalN/TNF-α-induced cell death in primary cultured mouse hepatocytes, and the conjugated double bond within ursane-type triterpene glucosides seems to enhance the hepatoprotective activity. On the basis of these studies, the hepatoprotective activity of the MeOH-soluble fraction of the MeOH extract of seeds of *C. quadrangulare* is due to these triterpene and catechin derivatives.

Table 2. Hepatoprotective Activity of Compounds Isolated from the MeOH-Soluble Fraction of MeOH Extract of *C. quadrangulare* against D-GalN/TNF-α-Induced Cell Death in Primary Cultured Mouse Hepatocytes[a]

compound	conc (*μ*M)	cell viability (% of normal)	inhibition (% of control)	IC_{50} (*μ*M)
normal		100.0 ± 8.3		
control		20.9 ± 1.2		
silibinin	100	47.9 ± 8.2[b]	129.0	29.9
	50	35.1 ± 3.5[b]	68.2	
	25	30.2 ± 3.5[b]	44.9	
1	100	47.6 ± 6.1[b]	127.5	60.9
	50	28.4 ± 1.2[b]	35.8	
	25	22.2 ± 1.4	5.9	
2	100	36.0 ± 2.1[b]	71.7	51.2
	50	30.6 ± 1.1[b]	46.2	
	25	28.6 ± 1.8[b]	36.4	
3	100	26.1 ± 2.0[b]	24.4	
	50	24.7 ± 1.6[b]	18.0	
	25	24.2 ± 0.1[b]	15.4	
5	100	27.8 ± 2.3[b]	32.9	
	50	26.6 ± 1.9[b]	27.0	
	25	26.5 ± 1.0[b]	26.7	
6	100	43.6 ± 2.1[b]	108.4	58.4
	50	28.0 ± 1.9[b]	33.8	
	25	24.9 ± 3.3[b]	19.1	
12	100	51.5 ± 1.6[b]	149.9	51.0
	50	30.8 ± 3.1[b]	47.4	
	25	29.4 ± 1.5[b]	40.6	
β-sitosterol	100	27.3 ± 3.3[b]	32.5	
	50	26.4 ± 1.0[b]	28.1	
	25	24.3 ± 0.6[b]	17.7	

[a] $p < 0.05$, $n = 4$ for sample and $n = 8$ for normal and control. [b] $p < 0.01$.

Experimental Section

General Experimental Procedures. Optical rotations were determined in MeOH on a JASCO DIP 140 digital polarimeter at 25 °C. IR spectra were recorded in KBr disks on a Shimadzu IR-408 spectrophotometer. NMR spectra were recorded in C_5D_5N containing TMS as an internal standard on a JEOL JNM-GX400 spectrometer. Mass spectra were obtained on a JEOL JMS-SX102A spectrometer using glycerol as a matrix. Column chromatography was performed with normal-phase (Fuji Silysia BW-820 MH, Aichi, Japan) and reversed-phase silica gel (Cosmosil 75C_{18}-OPN, Nacalai Tesque Inc., Kyoto, Japan). Analytical and preparative TLC were carried out on precoated Merk Kieselgel 60F_{254} (0.25 or 0.50 mm thickness) and RP-18F_{254} plates.

Plant Material. Seeds of *Combretum quadrangulare* Kurz were collected in Ho Chi Minh City, Vietnam, in January 1998. A voucher sample (TMPW 19000) is preserved in the Museum for Materia Medica, Institute of Natural Medicine, Toyama Medical and Pharmaceutical University, Toyama, Japan.

Extraction and Isolation. The dried seeds (2.25 kg) of *C. quadrangulare* were extracted with MeOH (7 L, 3 h × 2) at 80 °C, followed by removal of the solvent under reduced pressure, to yield a dried MeOH extract (748 g). The MeOH extract (700 g) was dissolved in H_2O to give a water-soluble fraction (507 g) and a MeOH-soluble residue (180 g). The MeOH-soluble residue (180 g) was subjected to silica gel column chromatography with a $CHCl_3$−MeOH gradient system to afford five fractions (fraction 1, $CHCl_3$ eluate, 8.9 g; fraction 2, 10% $CHCl_3$−MeOH eluate, 1.0 g; fraction 3, 25% $CHCl_3$−MeOH eluate, 4.4 g; fraction 4, 50% $CHCl_3$−MeOH eluate, 18.1 g; fraction 5, MeOH eluate, 100 g).

Fraction 2 (1.0 g) was chromatographed on silica gel with a H_2O−MeOH gradient system to give five subfractions. Preparative TLC with 15% MeOH−$CHCl_3$ of subfraction 3 yielded *β*-sitosterol (7.0 mg), and that of subfraction 4 gave *β*-sitosterol glucoside (15.0 mg) and 19α-hydroxyasiatic acid (**9**, 11.0 mg).

Fraction 3 (4.0 g) was applied on a silica gel column with a $CHCl_3$−MeOH gradient system, and six subfractions were collected. Preparative TLC ($CHCl_3$−MeOH−MeCN, 10:3:1) of

combined subfractions 3−5 yielded 19α-hydroxyasiatic acid (**9**, 4.5 mg) and β-sitosterol (4.0 mg), β-sitosterol glucoside (10.0 mg), and arjunic acid (**5**, 5.5 mg).

Fraction 4 (15 g) was also chromatographed on a silica gel column with $CHCl_3$−EtOAc−MeOH (3:3:1) to afford eight subfractions. Preparative TLC ($CHCl_3$−EtOAc−MeOH, 3:3:2) of subfractions 3, 5, and 6 yielded the following compounds: subfraction 3, 2α,6β-hydroxybetulinic acid (**1**, 5.5 mg) and arjunic acid (**5**, 7.0 mg); subfraction 5, 6β-hydroxyhovenic acid (**2**, 4.0 mg) and arjunolic acid (**6**, 6.5 mg); subfraction 6, 6β-hydroxyarjunic acid (**3**, 7.4 mg) and 2α,3β,23-trihydroxyursa-12,19-dien-28-oic acid (**12**, 8.5 mg).

Fraction 5 (25.0 g) was again chromatographed on silica gel with $CHCl_3$−EtOAc−MeOH (3:3:1 and then 3:3:2), and nine subfractions were collected. Further Cosmosil 75C_{18}-OPN column chromatography and preparative TLC (MeOH−MeCN−H_2O, 1:1:1) of subfrations 3−6 yielded the following compounds: subfraction 3, quadranoside II (**4**, 5.5 mg) and nigaichigoside F1 (**10**, 8.5 mg); subfraction 4, (+)-catechin (5.0 mg), (+)-gallocatechin (3.5 mg), (−)-epicatechin (7.5 mg), and (−)-epigallocatechin (**20**, 6.0 mg); subfraction 5, quadranoside IV (**11**, 12.0 mg) and a mixture (1:1, 5.0 mg) of 2α,3β,23-trihydroxyursa-12,18-dien-28-oic acid β-D-glucopyranosyl ester (**13**) and 2α,3β,23-trihydroxyursa-12,19(20)-dien-28-oic acid β-D-glucopyranosyl ester (**14**); subfraction 6, arjunglucosides I (**7**, 8.0 mg) and II (**5**, 12.0 mg).

2α,6β-Dihydroxybetulinic acid (**1**): colorless amorphous solid; $[\alpha]_D^{25}$ +1.5° (*c* 0.400, MeOH); IR (KBr) ν_{max} 3450, 1710, 1640 cm^{-1}; ^1H and ^{13}C NMR, see Table 2; HRFABMS *m/z* 487.3411 [calcd for $C_{30}H_{47}O_5$ $(M-H)^-$, 487.3423]; EIMS (probe) 70 eV, *m/z* (rel int) 488 $[M]^+$ (31), 470 $[M - H_2O]^+$ (33), 452 $[M - 2H_2O]^+$ (24), 442 $[M - COOH_2]^+$ (47), 424 $[M - 3H_2O]^+$ (66), 248 (54), 239 (9), 221 (43), 219 (44), 203 (82), 185 (48), 175 (73), 173 (50).

6β-Hydroxyhovenic acid (**2**): colorless amorphous solid; $[\alpha]_D^{25}$ +28.0° (*c* 0.113, MeOH); IR (KBr) ν_{max} 3400, 1725, 1635 cm^{-1}; ^1H and ^{13}C NMR, see Table 2; HRFABMS *m/z* 503.3390 [calcd for $C_{30}H_{47}O_6$ $(M - H)^-$, 503.3373]; EIMS (probe) 70 eV, *m/z* (rel int) 504 $[M]^+$ (15), 486 $[M - H_2O]^+$ (14), 468 $[M - 2H_2O]^+$ (82), 458 $[M - COOH]^+$ (39), 450 $[M - 3H_2O]^+$ (10), 255 (10), 237 (11), 219 (35), 201 (100), 173 (78).

6β-Hydroxyarjunic acid (**3**): colorless amorphous solid; $[\alpha]_D^{25}$ +75.9° (*c* 0.026, MeOH); IR (KBr) ν_{max} 3400, 1720, 1645 cm^{-1}; ^1H and ^{13}C NMR, see Table 2; HRFABMS *m/z* 503.3393 [calcd for $C_{30}H_{47}O_6$ $(M - H)^-$; 503.3372]; EIMS (probe) 70 eV, *m/z* (rel int) 504 $[M]^+$ (2), 486 $[M - H_2O]^+$ (7), 468 $[M - 2H_2O]^+$ (5), 458 $[M - CO_2]^+$ (13), 440 $[M - H_2O - CO_2]^+$ (11), 264 (96), 246 (100), 231 (46), 201 (94).

Hepatoprotective Activity. The hepatoprotective activities of the extract, fractions, and isolated compounds were tested according to the previous reports.[2,5]

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Supporting Information Available: Figure 1 showing significant correlations observed in the HMBC (a) and in the ROESY (b) spectra of **1**, Figure 2 showing mass fragments corresponding to compound **1**, and Figure 3 showing significant correlations observed in the HMBC (a) and in the ROESY (b) spectra of **3**. This material is available free of charge via the Internet at http://pubs.acs.org.

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