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MEDICINAL FOODSTUFFS. XXVI.[1] INHIBITORS OF ALDOSE REDUCTASE AND NEW TRITERPENE AND ITS OLIGOGLYCOSIDE, CENTELLASAPOGENOL A AND CENTELLASAPONIN A, FROM *CENTELLA ASIATICA* (GOTU KOLA)

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Abstract — The methanolic extract from the aerial parts of *Centella asiatica* L. cultivated in Vietnam was found to inhibit rat lens aldose reductase. From the methanolic extract, a new olean-13-ene-type triterpene, centellasapogenol A, and its oligoglycoside, centellasaponin A, were isolated together with several known saponins and flavonoids. Their structures were elucidated on the basis of chemical and physicochemical evidence. In addition, a flavonol component, petuletin, was found to show potent inhibitory activity on aldose reductase.

The Umbelliferae plant *Centella* (*C.*) *asiatica* L. URBAN (common name, Gotu Kola) has been widely cultivated as a vegetable or spice in China, Southeast Asia, India, Sri Lanka, Africa, and Oceanic countries. The whole plants or aerial parts of *C. asiatica* has been also used to prompt bladder activity and for the treatment of physical and mental exhaustion, diabetes, eye disease, diarrhea, stomach aches, and icterus in Chinese, Indian Ayurvedic, and Indonesian traditional medicines. Recently, the aerial parts of this plant are consumed as a health food in U.S.A. and Southeast Asia countries. As the chemical constituents of *C. asiatica*, several olean-12-ene- and urs-12-ene-type triterpenes and their glycosides have been reported.[2] However, the pharmacological active constituents of this natural medicine are left uncharacterized. In the course of our characterization studies on the bioactive constituents in medicinal foodstuffs,[1,3] we found that the methanolic extract from the dried aerial parts of *C. asiatica* showed the inhibitory activity on rat lens aldose reductase, which is the key enzyme that catalyzes the reduction of glucose to sorbitol in the polyol pathway. This paper deals with the isolation and structure elucidation of a new olean-13-ene-type triterpene, centellasapogenol A (**1**), and its oligoglycoside, centellasaponin A (**2**), from the methanolic extract. Furthermore, kaempferol 3-*O*-β-D-glucuronide (**9**) and petuletin (**10**) were also isolated as aldose reductase inhibitors by a bioassay-guided separation.

The aerial parts of *C. asiatica* cultivated in Vietnam were extracted with methanol under reflux. Since the methanolic extract was found to exhibit inhibitory activity against rat lens aldose reductase, the methanolic extract was partitioned in an ethyl acetate (AcOEt)/water mixture to give an AcOEt-soluble fraction and a H_2O-soluble fraction. As is apparent from Table 1, the AcOEt-soluble fraction showed more potent inhibitory activity (IC_{50} 0.25 μg/mL) than the H_2O-soluble fraction. The AcOEt-soluble fraction was subjected to silica gel and ODS column chromatography and/or finally HPLC to furnish five constituents, centellasapogenol A (**1**, 0.007% from the natural medicine), madecassic acid (**6**,[2b] 0.034%), kaempferol 3-*O*-β-D-glucuronide (**9**,[4] 0.0017%), petuletin (**10**,[5] 0.0006%), and 2,5-dihydroxybenzoic acid (**11**,[6] 0.0004%). From the H_2O-soluble fraction, centellasaponin A (**2**, 0.0092%), madecassoside (**3**,[7] = asiaticoside A,[2g] 0.65%), asiaticoside (**4**,[2f,8] 0.078%) scheffoleoside A (**5**,[8] 0.014%), **6** (0.12%, total isolation yield was 0.15%), and asiatic acid (**7**,[2a,8] 0.0087%) were isolated using the above-mentioned chromatography.

Table 1. Inhibitory Effects of MeOH extract, AcOEt-, and H_2O-soluble Fractions from *C. asiatica* on Rat Lens Aldose Reductase

	IC_{50} (μg/mL)
MeOH extract	0.80
AcOEt-soluble fraction	0.25
H_2O-soluble fraction	6.7

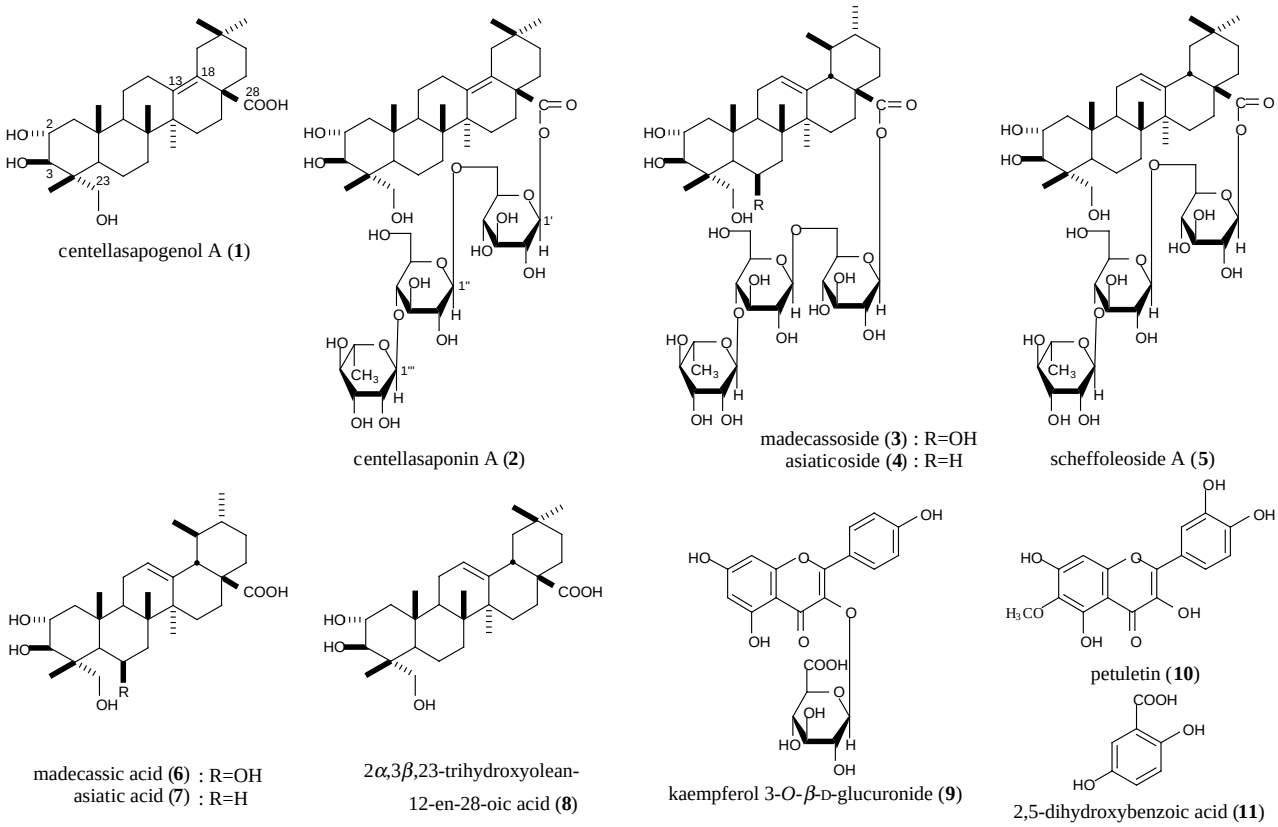
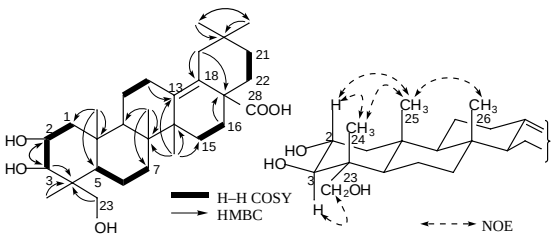


Chart 1

Structures of Centellasapogenol A (1) and Centellasaponin A (2)

Centellasapogenol A (**1**) with negative optical rotation ($[\alpha]_D^{28}$ –26.6°) was isolated as colorless fine crystals of mp 247—250 °C from $CHCl_3$–MeOH. In the positive and negative-ion fast atom bombardment (FAB)-MS of **1**, quasimolecular ion peaks were observed at *m/z* 511 $(M+Na)^+$ and *m/z* 487 $(M-H)^-$, respectively. High-resolution MS analysis of a quasimolecular ion peak $(M+Na)^+$ in the positive-ion FAB-MS revealed the molecular formula of **1** to be $C_{30}H_{48}O_5$. The IR spectrum of **1** showed absorption bands at 3431, 1698, and 1655 cm^{-1} ascribable to hydroxyl, carbonyl, and olefin functions. The proton and carbon signals in the ^{1}H-NMR and ^{13}C-NMR spectra of **1** were similar to those of 2α,3β,23-trihydroxyolean-12-en-28-oic acid (**8**),[8] except for the signals due to the olefin function. The ^{1}H-NMR (pyridine-d_5) and ^{13}C-NMR (Table 2) spectra of **1** showed signals assignable to six methyls [δ 0.84, 0.96, 1.06, 1.08, 1.17, 1.18 (all s, 29, 30, 24, 25, 27, and 26-H_3)], a methylene [δ 3.72, 4.20 (ABq, *J*=10.4 Hz, 23-H_2)], and two methines [δ 4.18 (d, *J*=11.0 Hz, 3-H), 4.26 (ddd, *J*=6.7, 11.0, 11.9 Hz, 2-H)] bearing a hydroxyl group together with ten methylenes (1, 6, 7, 11, 12, 15, 16, 19, 21, and 22-H_2), two methines (5, 9-H), and nine quaternary carbons (4, 8, 10, 13, 14, 17, 18, 20, and 28-C). The olean-13-ene structure of **1** was constructed on the basis of ^{1}H—^{1}H correlation spectroscopy (H—H COSY) and heteronuclear multiple bond correlation (HMBC) experiments (Figure 1). Thus, the H—H COSY experiment on **1** indicated the presence of five partial structures shown by thick lines (C-1—C-3, C-5—C-7, C-11—C-12, C-15—C-16, and C-21—C-22). In the HMBC experiment, long-range correlations were observed between the protons and carbons shown in Figure 1, so that the position of the double bond in **1** were determined to be the 13 and 18-carbons. Furthermore, the stereostructure of **1** was characterized on the basis of the nuclear Overhauser effect spectroscopy (NOESY) experiment, in which the NOE correlations were observed between the following proton pairs of **1** (25-H_3 and 2-H, 24-H_3, 26-H_3; 3-H and 23-H_2). The above-mentioned evidence led us to elucidate the structure of centellasapogenol A (**1**) to be 2α,3β,23-trihydroxyolean-13(18)-en-28-oic acid.



1 **1**

Figure 1. H–H COSY, HMBC, and NOESY Correlations of Centellasapogenol A (**1**)

Centellasaponin A (**2**) with negative optical rotation ($[\alpha]_D^{24}$ –33.4°) was also isolated as colorless fine crystals of mp 200—201 °C from $CHCl_3$–MeOH. The molecular formula $C_{48}H_{78}O_{19}$ of **2** was also determined from the positive- and negative-ion FAB-MS [*m/z* 981 $(M+Na)^+$, *m/z* 957 $(M–H)^–$] and by high-resolution MS measurement. Furthermore, fragment ion peaks at *m/z* 811 $(M–C_6H_{11}O_4)^–$ and *m/z* 487 $(M–C_{18}H_{31}O_{14})^–$, which were presumed to be derived by cleavage of the glycoside linkages at the 1‴ and 1'-protons, were observed in the negative-ion FAB-MS. The IR spectrum of **2** showed absorption bands at 3436, 1721, 1655, and 1070 cm^{-1} ascribable to hydroxyl, ester carbonyl, olefin, and ether functions. Alkaline hydrolysis of **2** with 5% aqueous sodium hydroxide provided centellasapogenol A (**1**). On acid hydrolysis with 5% aqueous sulfuric acid (H_2SO_4)–1,4-dioxane (1 : 1, v/v), **2** liberated D-glucose and L-rhamnose, which were identified by GLC analysis of their trimethylsilyl thiazolidine derivatives.[9] The ^{1}H-NMR (pyridine-d_5) and ^{13}C-NMR (Table 2) spectra of **2** showed signals assignable to six methyls [δ 0.77, 0.94, 1.01, 1.06, 1.09, 1.17 (all s, 29, 30, 25, 24, 27, and 26-H_3)], a methylene [δ 3.73, 4.17 (each m, 23-H_2)], and two methines [δ 4.18 (m, 3-H), 4.25 (m, 2-H)] bearing a hydroxyl group, two β-D-glucopyranosyl moieties [δ 4.98 (d, *J*=7.9 Hz, 1"-H), 6.24 (d, *J*=7.9 Hz, 1'-H)], and a α-L-rhamnopyranosyl moiety [δ 1.68 (d, *J*=6.1 Hz, 6‴-H), 5.79 (d, *J*=0.9 Hz, 1‴-H)]. The signals due to aglycon moiety of **2** were found to be similar to those of centellasapogenol A (**1**), whereas the signals of the trisaccharide part were superimposable on those of scheffoleoside A (**5**). The oligosaccharide structure bonding to the 28-position of the centellasapogenol A (**1**) moiety was characterized by HMBC experiments. Thus, long-range correlations were observed between the 1'-proton of the glucopyranosyl moiety and the 28-carbon of the centellasapogenol A (**1**) moiety, between the 1"-proton of the glucopyranosyl moiety and the 6'-carbon of the glucopyranosyl moiety, and between the 1‴-proton of the rhamnopyranosyl moiety and the 4"-carbon of the glucopyranosyl moiety. On the basis of this evidence, the structure of centellasaponin A (**2**) was determined to be centellasapogenol A 28-*O*-α-L-rhamnopyranosyl(1→4)-β-D-glucopyranosyl(1→6)-β-D-glucopyranoside.

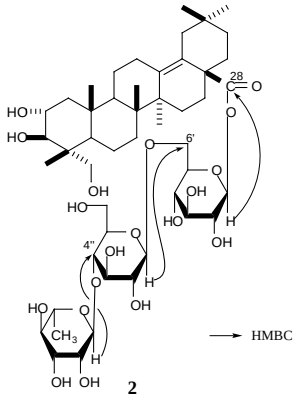


Figure 2. HMBC Correlations of Centellasaponin A (**2**)

Table 2. ^{13}C-NMR Data for Centellasapogenol A (**1**) and Centellasaponin A (**2**) (125 MHz, pryidine-d_5)

	1	**2**		**2**
C-1	48.2	48.1	Glc-1'	95.9
C-2	69.2	69.2	2'	74.0
C-3	78.5	78.8	3'	78.8
C-4	43.6	43.6	4'	71.5
C-5	48.3	48.2	5'	78.2
C-6	18.6	18.6	6'	69.6
C-7	35.1	35.0	Glc-1"	104.9
C-8	42.0	42.0	2"	75.3
C-9	51.3	51.1	3"	76.7
C-10	38.8	38.8	4"	78.8
C-11	22.3	22.2	5"	77.2
C-12	25.6	25.5	6"	61.5
C-13	137.8	138.8	Rha-1‴	102.8
C-14	44.9	44.8	2‴	72.6
C-15	27.9	27.7	3‴	72.8
C-16	33.7	33.4	4‴	74.0
C-17	49.2	49.0	5‴	70.4
C-18	128.0	128.2	6‴	18.5
C-19	41.6	41.4		
C-20	33.0	32.9		
C-21	37.5	37.1		
C-22	36.5	36.1		
C-23	66.8	66.6		
C-24	14.2	14.2		
C-25	18.5	18.5		
C-26	18.2	18.1		
C-27	21.3	21.2		
C-28	179.6	175.9		
C-29	32.4	32.3		
C-30	24.5	24.5		

Glc: β-D-glucopyranosyl
Rha: α-L-rhamnopyranosyl

Inhibitory Effects of Constituents from *C. asiatica* on Rat Lens Aldose Reductase

Aldose reductase as a key enzyme in the polyol pathway is reported to catalyze the reduction of glucose to sorbitol. Sorbitol does not readily diffuse across cell membranes, and the intercellular accumulation of sorbitol has been implicated in the chronic complications of diabetes such as cataract. Previously, we reported that several natural medicines, such as the leaves

of *Myrcia multiflora* (Myrtaceae)[10] and the flowers of *Chrysanthemum indicum* (Compositae),[11] exhibited potent inhibitory activity against rat lens aldose reductase, and several flavonoids have been isolated from their natural medicines as active compounds. Since *C. asiatica* has been used for the treatment of diabetes and eye disease in Southeast Asia countries, we examined the inhibitory activity of the extract and fractions from *C. asiatica* (*vide ante*) and also the isolated components from the active fraction (AcOEt-soluble fraction) against rat lens aldose reductase. As shown in Table 3, two flavonols, kaempferol 3-*O*-β-D-glucuronide (**9**) and petuletin (**10**) showed inhibitory activity on rat lens aldose reductase, but their activities were weaker than that of a commercial synthetic aldose reductase inhibitor, epalrestat.[12] These results indicate that flavonols are active principles of this natural medicine.

Table 3. Inhibitory Activity of Constituents from *C. asiatica* on Rat Lens Aldose Reductase

Compounds	IC_{50} (μM)
Centellasapogenol A (**1**)	300
Kaempferol 3-*O*-β-D-glucuronide (**9**)	5.1
Petuletin (**10**)	0.68
2,5-Dihydroxybenzoic acid (**11**)	120
Epalrestat	0.072

EXPERIMENTAL

The following instruments were used to obtain physical data : melting points, Yanagimoto micro hot-stage apparatus (uncorrected); specific rotations, Horiba SEPA-300 digital polarimeter (*l*=5 cm); IR spectra, Shimadzu FTIR-8100 spectrophotometer; ^{1}H-NMR spectra, JNM-LA500 (500 MHz) spectrometer; ^{13}C-NMR spectra, JNM-LA500 (125 MHz) spectrometer with tetramethylsilane as an internal standard; MS and high-resolution MS, JEOL JMS-SX 102A mass spectrometer.

The following experimental conditions were used for chromatography : ordinary-phase column chromatography; Silica gel BW-200 (Fuji Silysia Chemical, Ltd., 150—350 mesh), reversed-phase column chromatography; Chromatorex ODS DM1020T (Fuji Silysia Chemical, Ltd., 100—200 mesh): TLC, pre-coated TLC plates with Silica gel 60F_{254} (Merck, 0.25 mm) (normal-phase) and Silica gel RP-18 F_{254S} (Merck, 0.25 mm) (reversed-phase); HPTLC, pre-coated TLC plates with Silica gel RP-18 WF_{254S} (Merck, 0.25 mm) (reversed-phase). Detection was done by spraying with 1% $Ce(SO_4)_2$-10% aqueous H_2SO_4, followed by heating.

Extraction and Isolation of Centellasapogenol A (1) and Centellasaponin A (2)

The dried aerial parts of *C. asiatica* (3.0 kg, cultivated in Vietnam) were finely cut and extracted with methanol under reflux for 3 h (3 times). Evaporation of the solvent under reduced pressure gave the MeOH extract (545 g). The MeOH extract (300 g) was partitioned in an AcOEt—H_2O (1 : 1) mixture. Removal of the solvent under reduced pressure from the AcOEt- and H_2O-soluble fractions yielded 92 g and 208 g of residues, respectively.

The AcOEt-soluble fraction (92 g) was subjected to ordinary-phase silica gel column chromatography [2.7 kg, *n*-hexane–AcOEt (30 : 1 → 10 : 1 → 1 : 1) → AcOEt → $CHCl_3$–MeOH–H_2O (10 : 3 : 1, lower layer → 6 : 4 : 1) → MeOH] to afford eight fractions [fr. 1 (1.9 g), fr. 2 (6.2 g), fr. 3 (1.8 g),fr. 4 (28.0 g), fr. 5 (1.3 g), fr. 6 (2.5 g), fr. 7 (40.0 g), fr. 8 (10.3 g)]. Fraction 8 (10.3 g) was further subjected to ordinary-phase silica gel column chromatography [500 g, $CHCl_3$–MeOH–H_2O (30 : 3 : 1, lower layer → 10 : 3 : 1, lower layer → 6 : 4 : 1) → MeOH] furnished ten fractions (fr. 8–1—8–10). Fraction 8–5 (1.5 g) was purified by reversed-phase silica gel column chromatography [60 g, MeOH–H_2O (70 : 30 → 90 : 10) → MeOH] to give centellasapogenol A (**1**, 12 mg, 0.007%) and madecassic acid (**6**, 546 mg, 0.034%). Fraction 8–9 (1.3 g) was subjected to reversed-phase silica gel column chromatography [40 g, MeOH–H_2O (50 : 50 → 70 : 30 → 90 : 10) → MeOH] and finally HPLC [YMC-Pack ODS-A, CH_3CN–1% aqueous H_2O (25 : 75 v/v)] to give kaempferol 3-*O*-β-D-glucuronide (**9**, 28 mg, 0.0017%), petuletin (**10**, 10 mg, 0.0006%), and 2,5-dihydroxybenzoic acid (**11**, 7 mg, 0.0004%).

The H_2O-soluble fraction (208 g) was subjected to reversed-phase silica gel column chromatography [3.0 kg, H_2O → MeOH → $CHCl_3$–MeOH–H_2O (6 : 4 : 1)] to afford five fractions [fr. 1 (95.1 g), fr. 2 (67.5 g), fr. 3 (8.9 g),fr. 4 (27.5 g), fr. 5 (9.0 g)].

Fraction 4 (27.5 g) was further subjected to reversed-phase silica gel column chromatography [550 g, MeOH–H_2O (50 : 50 → 60 : 40 → 70 : 30 → 80 : 20 → MeOH) to give seven fractions [fr. 4–1 (2.0 g), fr. 4–2 (14.5 g), fr. 4–3 (1.0 g),fr. 4–4 (0.5 g), fr. 4–5 (5.0 g), fr. 4–6 (1.1 g), fr. 4–7 (3.4 g)]. Fraction 4–2 (1.9 g) was purified by HPLC [YMC-Pack ODS-A, MeOH–H_2O (60 : 40 v/v)] to give centellasaponin A (**2**, 20 mg, 0.0092%), madecassoside (**3**, 1420 mg, 0.65%), asiaticoside (**4**, 170 mg, 0.078%), and scheffoleoside A (**5**, 30 mg, 0.014%). Fraction 4–3 (886 mg) was purified by HPLC [YMC-Pack ODS-A, MeOH–H_2O (65 : 35 v/v)] to give centellasaponin A (**2**, 13 mg, 0.00092%), asiaticoside (**4**, 28 mg, 0.0018%), Fraction 4–4 (0.4 g) was subjected to HPLC [YMC-Pack ODS-A, MeOH–H_2O (75 : 25 v/v)] to furnish madecassic acid (**6**, 158 mg, 0.012%). Fraction 4–5 (5.0 g) was subjected to ordinary-phase silica gel column chromatography [250 g, $CHCl_3$–MeOH–H_2O (30 : 3 : 1, lower layer → 10 : 3 : 1, lower layer) → MeOH] to yield madecassic acid (**6**, 1.8 g, 0.11%) and asiatic acid (**7**, 144 mg, 0.0087%). These known constituents were identified by comparison of their physical data with those of commercial standard sample (**11**)[6] or with reported values.[2,4–8]

Centellasapogenol A (**1**): colorless fine crystals, mp 247—250 °C (from $CHCl_3$–MeOH), $[\alpha]_D^{28}$ –26.6° (*c*=0.1, MeOH). High-resolution positive-ion FAB-MS: Calcd for $C_{30}H_{48}O_5Na$ $(M+Na)^+$: 511.3420. Found: 511.3399. IR (KBr): 3431, 2944, 1698, 1655, 1046 cm^{-1}. ^{1}H-NMR (pyridine-d_5, 500 MHz) δ: 0.84, 0.96, 1.06, 1.08, 1.17, 1.18 (3H each, all s, 29, 30, 24, 25, 27, and 26-H_3), 2.18, 2.60 (1H each, both m, 19-H_2), 3.72, 4.20 (2H, ABq, *J*=10.4 Hz, 23-H_2), 4.18 (1H, d, *J*=11.0 Hz, 3-H), 4.26 (1H, ddd, *J*=6.7, 11.0, 11.9 Hz, 2-H). ^{13}C-NMR (pyridine-d_5, 125 MHz) δc: given in Table 2. Positive-ion FAB-MS: *m/z* 511 $(M+Na)^+$. Negative-ion FAB-MS: *m/z* 487 $(M-H)^-$.

Centellasaponin A (**2**): colorless fine crystals, mp 200—201 °C (from $CHCl_3$–MeOH), $[\alpha]_D^{24}$ –33.4° (*c*=0.3, pyridine). High-resolution positive-ion FAB-MS: Calcd for $C_{48}H_{78}O_{19}Na$ $(M+Na)^+$: 981.5035. Found: 981.5048. IR (KBr): 3436, 2937, 1721, 1655, 1070 cm^{-1}. ^{1}H-NMR (pyridine-d_5, 500 MHz) δ: 0.77, 0.94, 1.01, 1.06, 1.09, 1.17 (3H each, all s, 29, 30, 25, 24, 27, and 26-H_3), 1.68 (3H, d, *J*=6.1 Hz, 6'''-H), 1.84, 2.74 (1H each, both m, 12-H_2), 2.17, 2.52 (1H each, both m, 19-H_2), 3.73, 4.17 (1H each, both m, 23-H_2), 4.18 (1H, m, 3-H), 4.25 (1H, m, 2-H), 4.98 (1H, d, *J*=7.9 Hz, 1''-H), 5.79 (1H, d, *J*=0.9 Hz, 1'''-H), 6.24 (1H, d, *J*=7.9 Hz, 1'-H). ^{13}C-NMR (pyridine-d_5, 125 MHz) δc: given in Table 2. Positive-ion FAB-MS: *m/z* 981 $(M+Na)^+$. Negative-ion FAB-MS: *m/z* 957 $(M-H)^-$, 811 $(M-C_6H_{11}O_4)^-$, 487 $(M-C_{18}H_{31}O_{14})^-$.

Alkaline Hydrolysis of Centellasaponin A (2)

A solution of cantellasaponin A (**2**, 7 mg) in 5% aqueous sodium hydroxide (0.5 mL) was heated under reflux for 1 h. After cooling, the reaction mixture was neutralized with Dowex HCR-W2 (H^+ form) and the resin was filtered. After removal of the solvent under reduced pressure, the residue was subjected to ordinary-phase silica gel column chromatography [0.5 g, $CHCl_3$–MeOH–H_2O (10 : 3 : 1, lower layer)] to furnish centellasapogenol A (**1**, 3 mg, 80%), which was identified by comparison of its physical and spectral data ($[\alpha]_D$, IR, ^{1}H-NMR, ^{13}C-NMR) with an authentic sample.

Acid Hydrolysis of Centellasaponin A (2)

A solution of centellasaponin A (**2**, 5 mg) in 5% aq. H_2SO_4–1,4-dioxane (1 : 1, v/v, 1 mL) was heated under reflux for 1 h. After cooling, the reaction mixture was neutralized with Amberlite IRA-400 (OH^- form) and the resin was filtered. After removal of the solvent under reduced pressure, the residue was passed through a Sep-Pak C18 cartridge using H_2O and MeOH. The H_2O eluate was concentrated under reduced pressure and the residue was treated with L-cysteine methyl ester hydrochloride (0.01 mL) in pyridine (0.02 mL) at 60 °C for 1 h. The solution was then treated with *N,O*-bis(trimethylsilyl)trifluoroacetamide (0.01 mL) at 60 °C for 1 h. The supernatant was subjected to GLC to identify the derivatives of D-glucose (i) and L-rhamnose (ii). GLC conditions: column, Supelco^{TM}-1, 0.25 mm (i.d.) x 30 m; injection temperature, 230 °C; detector temperature 230 °C; column temperature, 230 °C; He flow rate 15 mL/min; t_R, i: 24.4 min; ii: 15.4 min.

Bioassay

Aldose reductase activity was assayed by the method described in a previous paper.[10,11] The supernatant fluid of rat lens homogenate was used as the crude enzyme. The incubation mixture contained 135 mM Na, K-phosphate buffer (pH 7.0), 100 mM Li_2SO_4, 0.03 mM NADPH, 1 mM DL-glyceraldehyde as a substrate, and 100 μL of enzyme fraction, with or without 25 μL of sample solution, in a total volume of 0.5 mL. The reaction was initiated by the addition of NADPH at 30 °C. After 30 min, the reaction was stopped by the addition of 150 μL 0.5 M HCl. Then, 0.5 mL 6 M NaOH containing 10 mM imidazole was added, and the solution was heated at 60 °C for 10 min to convert NADP to a fluorescent product. Fluorescence was measured using a spectrofluorometer (Type 650-10, Hitachi, Japan) at an excitation wavelength of 360 nm and an emission wavelength of 460 nm.

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