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ent-Kaurenoic acids from *Mikania hirsutissima* (Compositae)

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Abstract

Four *ent*-kaurenoic acid derivatives, 2β,16α,17-trihydroxy-*ent*-kauran-19-oic acid (**1**), 3β,16α,17-trihydroxy-*ent*-kauran-19-oic acid (**2**), 11α,15β-dihydroxy-7-*O*-β-D-glucopyranosyl-*ent*-kaur-16-en-19-oic acid (**3**) and 1α,15β-dihydroxy-7-*O*-β-D-glucopyranosyl-*ent*-kaur-16-en-19-oic acid (**4**), were isolated together with five known compounds, 1,5-dicaffeoyl-quinic acid (**5**), 2-*O*-glucosyloxy-4-methoxy-cinnamic acid (**6**), phenethyl alcohol glucoside (**7**), phenethyl-1-*O*-β-D-apiofuranosyl (1→2) β-D-glucopyranoside (sayaendoside) (**8**) and 3,6-dihydroxy-β-ion-9-ol (**9**) from the 50% aqueous acetone extract of the aerial parts of *Mikania hirsutissima* DC. (Compositae). Compounds **1**–**9** were tested for their proliferative activity toward peripheral blood mononuclear cells (hPBMC); compounds **1** and **2** showed significant activity (43.8% and 36.7%, at 100 μM, respectively) on the lymphocyte.

Keywords: *Mikania hirsutissima*; Compositae; Diterpene; *ent*-kaurene; Proliferative activity

1. Introduction

Mikania hirsutissima DC. (Compositae) has been used as a folk medicine for treatment of rheumatism, diarrhea, and gout in Brazil (Hashimoto, 1996). The plant is distributed in the southwest region of South America (Oliveira, 1972). We have investigated the biological activities of this plant and found that the aqueous ethanolic extract exhibited proliferative activity toward human peripheral blood mononuclear cells (hPBMC). In this paper, we describe the isolation and structure elucidation of four new diterpenoids (**1**–**4**), together with five known compounds (**5**–**9**), from the aerial parts of *M. hirsutissima* (Fig. 1).

2. Results and discussion

Compound **1** was obtained as colorless crystals, mp 277–280 °C, $[\alpha]_D^{20}$ −40.8° ($c = 0.13$, MeOH). It was established to have a molecular formula of $C_{20}H_{32}O_5$ by HR-negative ion FAB-MS $[M-H]^-$ m/z: 351.2163 (calcd for $C_{20}H_{31}O_5$: 351.2171). The ^1H and ^{13}C NMR spectra including DEPT indicated the presence of two tertiary methyls, nine methylenes, four methines, four quaternary carbons and a carboxyl group (Tables 1 and 2). The ^1H–^1H COSY and HMQC of **1** suggested the presence of an oxymethylene [δ_H 3.40 and 3.50 (ABq, $J = 11.0$ Hz, δ_C 65.3)] group and the partial structures [A], [B] and [C] (Fig. 2). The connectivities of these partial structures and the functional groups were investigated by analysis of HMBC of **1**. As shown in Fig. 2, long range correlations were observed between the following proton and carbon signals: H-18 (CH_3 and C-3, C-4, C-5, C-19 (COOH); H-20 (CH_3 and C-1, C-5, C-9, C-10; H-15α and C-7, C-8, C-9, C-16; H-14α and C-15, C-16; H-17 and C-15, C-16, C-13. Thus, structure **1** was confirmed as shown in Fig. 2.

The relative stereochemistry of **1** was assigned by analysis of the NOESY spectrum. The H-2 signal (δ 3.83) showed a correlation with the methyl proton signal at δ 0.85 (H-20) which in turn exhibited cross-peaks with proton signals due to H-6α (δ 1.72), H-12 α (δ 1.32) and H-14 α (δ 1.69). The NOESY experiment observations were further confirmed by measurement of the NOE difference spectrum. The stereochemistry at C-16 was determined to be in an α-orientation as regards the hydroxyl group by comparison of the chemical shifts values for C-16 and C-17 with those reported in the

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literature (Harinantenaina et al., 2002). For chemical shift values of C-16 hydroxylated *ent*-kauranes, Harinantenaina et al. reported that the signals of the C-16 with a β-hydroxyl group and C-17 appeared at δ 79.7 and 70.4 while those of C-16 with an α-hydroxyl group appeared at δ 81.6 and 66.4, respectively. The chemical shift values for C-16 and C-17 of **1** were δ 81.7 and 66.5, respectively, suggesting the α-orientation of the hydroxyl group at C-16. Thus, the relative stereostructure of **1** was confirmed as shown in Fig. 3.

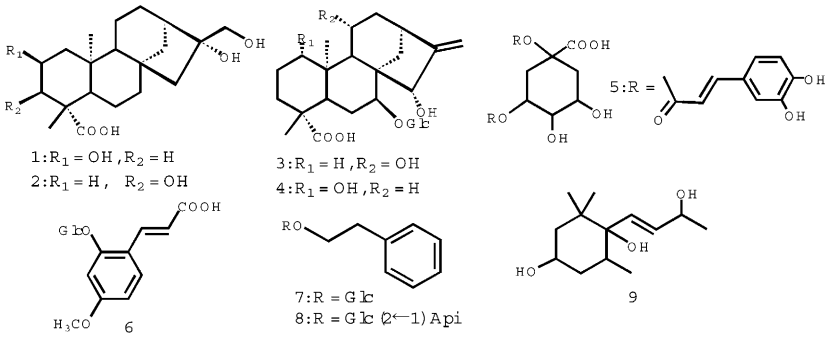

Fig. 1. Isolated compounds from *M. hirsutissima*.

Table 1
^{1}H NMR spectral data for compound **1**–**4** (400 MHz, δ)

H		**1** (DMSO-d_6)	**2** (DMSO-d_6)	**3** (Pyridine-d_5)	**4** (Pyridine-d_5)
1	α	1.99 (*dd*, 3.7, 11.5)	1.41 (*m*)	2.08 (*m*)	
	β	0.54 (*t*, 11.5)	1.15 (*dt*, 3.7, 13.2)	3.58 (*dd*, 4.4, 13.7)	3.73 (*brd*, 13.1)
2	α	3.83 (*m*)	2.00 (*dt*, 2.7, 13.2)	2.39 (*dt*, 3.7, 13.7)	2.60 (*m*)
	β		1.38 (*m*)	1.44 (*m*)	1.79 (*m*)
3	α	2.20 (*dd*, 3.4, 11.7)	3.77 (*brs*)	1.13 (*dt*,4.3, 13.1)	1.22 (*dt*, 3.6, 13.2)
	β	0.79 (*t*, 11.7)		2.48 (*m*)	2.46 (*brd*, 13.2)
4					
5		1.23 (*m*)	1.41 (*m*)	2.58 (*d*, 13.1)	2.38 (*brd*, 11.7)
6	α	1.72 (*m*)	1.69 (*dt*, 2.4, 13.2)	2.55 (*dd*, 9.7, 13.1)	2.56 (*t*, 13.9)
	β	1.65(*m*)	1.54 (*m*)	2.93 (*dd*, 4.0, 13.1)	2.89 (*brd*, 13.9)
7a	α	1.50 (*m*)	1.47 (*m*)	4.40 (*brs*)	4.28 (*brd*, 2.7)
	β	1.34 (*m*)	1.32 (*dt*, 3.7, 13.2)		
8					
9		0.92 (*d*, 8.3)	0.93 (*d*, 4.1)	2.50 (*m*)	2.24 (*brd*, 4.6)
10					
11	α	1.48 (*m*)	1.46 (*m*)	4.50 (*m*)	3.64 (*dd*, 4.6, 15.1)
	β	1.54 (*m*)	1.50 (*m*)		1.78 (*m*)
12	α	1.32 (*m*)	1.38 (*m*)	2.22 (*dt*, 1.8, 12.2)	1.51 (*m*)
	β	1.52 (*m*)	1.56 (*m*)	1.40 (*m*)	1.87 (*m*)
13		1.87 (*brs*)	1.87 (*d*, 2.7)	2.87 (*brs*)	2.81 (*brs*)
14	α	1.69 (*m*)	1.72 (*d*, 10.7)	2.09 (*brd*, 12.2)	1.94 (*brd*, 1.2)
	β	1.53 (*m*)	1.50 (*m*)	1.72 (*m*)	1.76 (*m*)
15	α	1.25 (*d*, 13.9)	1.24 (*d*, 14.1)	6.39 (*brs*, OH)	6.39 (*brs*, OH)
	β	1.39 (*d*, 13.9)	1.39 (*m*)	4.68 (*brs*)	4.68 (*brs*)
16					
17	α	3.40 (*d*, 11.0)	3.39 (*d*, 11.0)	5.19 (*brs*)	5.16 (*s*)
	β	3.50 (*d*, 11.0)	3.49 (*d*, 11.0)	5.41 (*brs*)	5.45 (*s*)
18		1.12 (*s*)	1.09 (*s*)	1.79 (*s*)	1.74 (*s*)
19					
20		0.85 (*s*)	0.85 (*s*)	1.73 (*s*)	1.61 (*s*)
Glc					
1				4.94 (*d*, 7.9)	4.93 (*d*, 7.8)
2				4.10 (*t*, 9.5)	4.07 (*dd*, 7.8, 9.2)
3				4.22 (*t*, 8.9)	4.19 (*dd*, 8.9, 9.2)
4				4.39 (*m*)	4.34 (*t*, 9.3)
5				3.87 (*ddd*, 2.4, 4.5, 9.2)	3.87 (*ddd*, 2.7, 3.2, 9.3)
6				4.42 (*dd*, 2.4, 11.3)	4.38 (*dd*, 2.7, 11.5)
				4.49 (*dd*, 4.5, 11.3)	4.44 (*dd*, 3.2, 11.5)

The values in parentheses represent the coupling constants in Hz. The δ values are in ppm downfield from TMS.

Table 2
^{13}C NMR spectral data for compound **1**–**4** (100 MHz, δ)

C	**1** (DMSO-d_6)		**2** (DMSO-d_6)		**3** (pyridine-d_5)	**4** (pyridine-d_5)
1	49.7	(50.9)	33.7	(35.1)	43.3	82.3
2	62.2	(63.8)	26.0	(27.8)	20.2	31.3
3	46.9	(48.7)	68.8	(70.7)	38.6	36.9
4	43.9	(42.8)	46.8	(48.5)	44.3	43.8
5	55.4	(56.7)	47.7	(49.2)	47.8	45.7
6	21.7	(22.8)	21.5	(22.9)	27.3	27.5
7	41.8	(45.0)	42.0	(43.1)	85.5	86.0
8	43.9	(45.3)	44.0	(45.2)	54.0	52.8
9	55.2	(56.3)	55.3	(56.4)	53.6	49.3
10	40.3	(41.4)	40.1	(40.3)	41.5	41.5
11	18.2	(19.1)	18.1	(19.2)	69.2	20.7
12	25.7	(26.8)	25.8	(27.1)	43.1	33.9
13	44.5	(45.9)	44.5	(46.1)	42.5	43.2
14	36.8	(37.9)	36.8	(38.0)	35.6	36.2
15	52.7	(53.9)	52.8	(54.0)	79.9	80.5
16	80.4	(81.7)	80.5	(81.7)	159.8	160.2
17	65.3	(66.5)	65.3	(66.5)	107.6	107.7
18	28.5	(29.4)	24.2	(25.6)	29.7	29.1
19	178.5	(180.4)	178.8	(180.5)	181.3	180.8
20	16.4	(17.3)	15.1	(16.2)	17.3	12.9
Glc						
1′					106.8	106.8
2′					75.7	75.8
3′					78.8	78.8
4′					71.0	71.3
5′					78.4	78.3
6′					62.1	62.3

The δ values are in ppm downfield from TMS. The numbers in parentheses are δ values determined in pyridine-d_5.

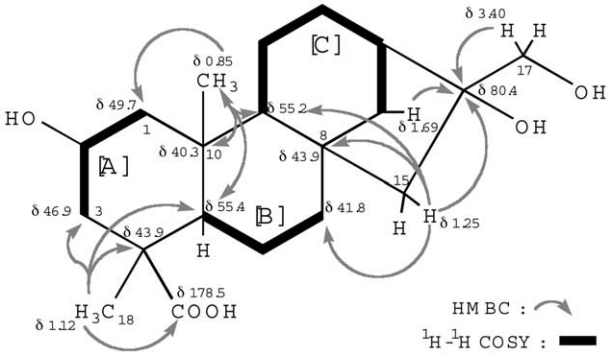

Fig. 2. Key HMBC correlations of Compound **1**.

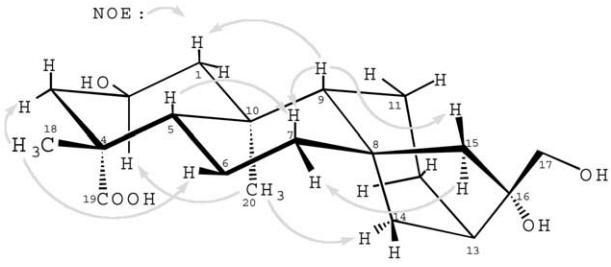

Fig. 3. Difference NOEs of Compound **1**.

Compound **2** was obtained as colorless crystals, mp 246–249 °C, $[\alpha]_D^{20}$ −55.1° (c = 0.4, MeOH). It was established to have a molecular formula of $C_{20}H_{32}O_5$ by HR-negative ion FAB-MS $[M-H]^-$ m/z: 351.2170 (calcd for $C_{20}H_{31}O_5$: 351.2171), which was the same as that of **1**. The ^{1}H and ^{13}C NMR spectra of compound **2** were very similar to those of 3α,16α,17-trihydroxy-*ent*-kauran-19-oic acid (Harinantenaina et al., 2002). The small coupling constant of H-3 (δ 3.77, *brs*) indicated that the conformation of H-3 must have an α-equatorial orientation. From these spectral data, compound **2** was determined to be 3β,16α,17-trihydroxy-*ent*-kauran-19-oic acid.

Compound **3** was obtained as a brownish amorphous powder, $[\alpha]_D^{20}$ −15.5 °C (c = 0.9, MeOH). It was established to have a molecular formula of $C_{26}H_{40}O_{10}$ by HR-negative ion FAB-MS $[M-H]^-$ m/z: 511.2546 (calcd for $C_{26}H_{39},O_{10}$ 511.2543). The ^{1}H and ^{13}C NMR spectra suggested that **3** is a kaurenoic acid glycoside [δ_H 4.94 (1H, *d*, J = 7.9 Hz); anomeric proton] having two secondary hydroxyl groups [δ_C 69.3, δ_H 4.50 (1H, m), and δ_C 79.9, δ_H 4.68 (1H, s)]. The allocation of two hydroxyl groups and the glucoside linkage were deduced by the observation of long range correlations between following proton and carbon signals in the HMBC spectrum: H-17 and C-15, C-13; H-15 and C-9, C-14, C-13, C-17; H-11 and C-8, C-9, C-10, C-12, C-13; H-7 and C-5, C-6, C-8, C-9, C-1′ [δ 106.8 (anomeric carbon)]. These spectral data indicated that the sugar moiety and two hydroxyl groups should be placed at C-7, C-11 and C-15, respectively, as shown in Fig. 4. Moreover, on acid hydrolysis, **3** afforded D-glucose which was identified by both TLC and gas chromatographic (GC) analysis of its trimethylsilyl thiazolidine derivative (Hara et al., 1987). Finally, the relative stereochemistry of **3** was determined as shown in Fig. 5

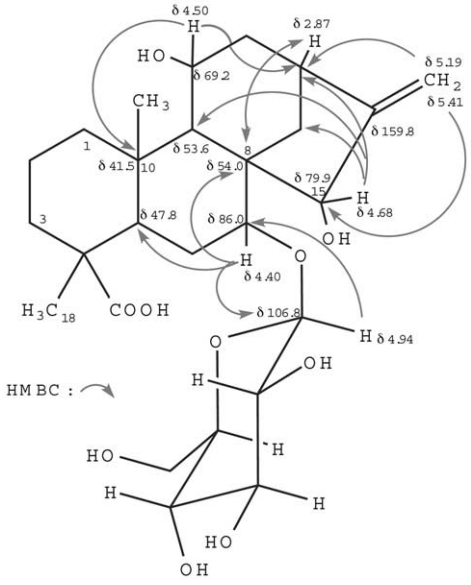

Fig. 4. Key HMBC correlations of Compound **3**.

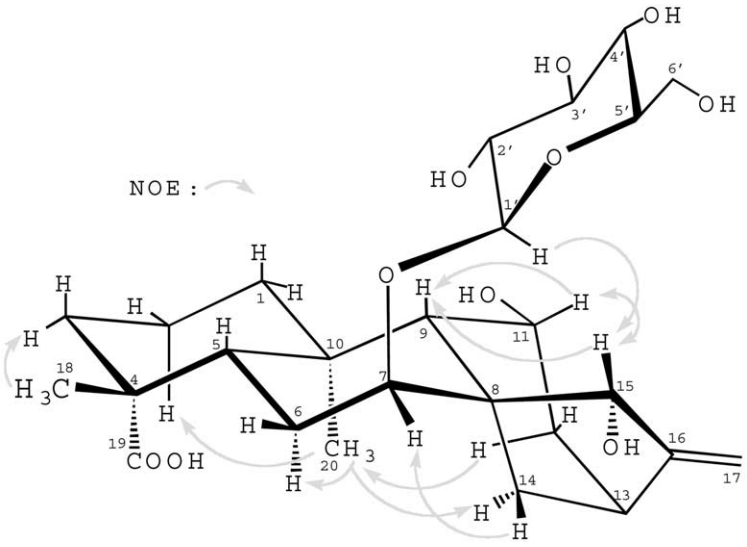

Fig. 5. Key NOE correlations of Compound **3**.

by analysis of the NOESY spectrum. Therefore, compound **3** was determined as 11α, 15α-dihydroxy-7-*O*-β-D-glucopyranosyl-*ent*-kaur-16-en-19-oic acid.

Compound **4** was obtained as brown amorphous powder, $[\alpha]_D^{24}$ −7.2 °C ($c = 0.9$, DMSO). It was established to have a molecular formula of $C_{26}H_{40}O_{10}$ by HR-negative ion FAB-MS $[M-H]^-$ m/z: 511.2548 (calcd for $C_{26}H_{39}O_{10}$: 511.2543). The ^1H NMR spectrum of compound **4** were very similar to those of compound **3**, so it was assumed that compound **4** should have the same skeleton as that of **3**. The HMBC spectrum of **4** displayed almost the same long-range correlations with those of **3** except for the appearance of the correlations between δ 1.61 (H-20) and an oxymethine carbon signal (δ 82.3, C-1). Thus, the hydroxyl group should be placed at C-1. The coupling constant of H-1β ($J = 13.1$ Hz) and NOESY spectrum indicated that the hydroxyl group at C-1 must be in the α-equatorial orientation. Thus, compound **4** was deduced as 1α,15α dihydroxy-7-*O*-β-D-glucopyranosyl-*ent*-kaur-16-en-19-oic acid.

In addition to the new compounds, five known compounds, 1,5-di-*O*-caffeoylquinic acid (**5**) (Merfort, 1992), *trans*-2-β-D-glucopyranosyloxy-4-methoxycinnamic acid (**6**) (Kanamori et al., 1993), phenethyl β-D-glucopyranoside (**7**) (Kitajima et al., 1998), phenethyl 1-*O*-β-D-apiofuranosyl (1→2) β-D-glucopyranoside (**8**) (sayaendoside) (Murakami et al., 2001), and 3,6-dihydroxy-5,6-dihydro-β-ionol (**9**) (Perez et al., 1996) were isolated and their structures were identified by comparison of their spectral data with those reported in the literature.

Table 3
Proliferative activity of **1**–**9** for hPBMC at a concentration of 100 μM

Compd	Proliferation (%)	Compd	Proliferation (%)
1	43.8	**7**	6.7
2	36.7	**8**	−16.5
3	7.1	**9**	−18.3
4	−2.0		
5	6.0	rhIL-2 (1 ng/ml)	32.8
6	2.4	Concanavalin A (1 μg/ml)	32.1

The lymphoproliferative response is one of the most widely used assay for determining immune activity (Sondel et al., 1975). All of the isolated compounds (**1**–**9**) were subjected to in vitro assay for hPBMC proliferation. The activities of compounds are summarized in Table 3. Compound **1** and **2** showed significant proliferative activity on hPBMC at 100 μM, but the other compounds did not show the effects.

3. Experimental

3.1. General

^1H and ^{13}C NMR spectra were measured on either JOEL GSX-400 (^1H, 400 MHz; ^{13}C, 100 MHz, JOEL Co., Tokyo, Japan) or Varian Mercury 300BB (^1H, 300 MHz; ^{13}C, 75 MHz, Varian Co., CA, USA) spectrometers. Chemical shift values are given in ppm using TMS as an internal standard. MS spectra were obtained using a Hitachi RMU-6M instrument, whereas optical rotations were measured on a JASCO DIP-360 polarimeter. Melting points utilized a Yanagimoto melting point apparatus and are uncorrected.

3.2. Plant material

The plant material was purchased from LABORATORIO FARMAERVAS LTDA. in Sao Paulo,

Brazil and a voucher specimen (YF0030) was deposited in the herbarium of College of Pharmacy, Nihon University.

3.3. Extraction and isolation of compounds (1–9)

The aerial part of air-dried *M. hirsutissima* (2 kg) was extracted with acetone–H_2O (1:1, v/v, 2 l×3) under ultrasonication at room temperature. The aqueous acetone extract was then concentrated under reduced pressure to give an oily material (55.0 g), this being applied to a Diaion HP-20 resin (Nippon Rensui Co., Tokyo) columun which was eluted successively with H_2O (3 l) and then with stepwise gradients of H_2O–MeOH [20% MeOH (4 l), 40% MeOH (5 l), 70% MeOH (5 l)]. MeOH (5 l) and acetone (6 l). The 70% aq. MeOH fraction (6.3 g) was concentrated under reduced pressure to afford a brown material which was separated by HPLC (Shiseido CAPCELL PAK ODS–Ph, 20 i.d.×250 mm) with CH_3CN–H_2O (15:85, v/v) to give five fractions (Fr.I-1–I-5). Fraction I-3 (0.5 g) was purified by HPLC [Shiseido CAPCELL PAK ODS-Ph 10 i.d.×250 mm, CH_3CN–0.1% aq. CH_3COOH (15:85, v/v), flow rate; 2 ml/min] to give compound **1** (14.7 mg) and **2** (4.1 mg). The 40% aq. MeOH fraction (3.0 g) was concentrated under reduced pressure to give a brown material which was purified by HPLC (Shiseido CAPCELL PAK ODS–Ph 20 i.d.×250 mm) with CH_3CN–H_2O (15:85, v/v) to give four fractions Fr.II-1–II-4). Fraction II-1 (0.2 g) was purified by HPLC [Shiseido CAPCELL PAK ODS-Ph 10 i.d.×250 mm, CH_3CN–0.1% aq. CH_3COOH (20:80, v/v), flow rate; 2 ml/min, monitored at 254 nm] to give compound **6** (5.1 mg) and **5** (21 mg). Fraction II-3 (0.2 g) was purified by prep. HPLC [Shiseido CAPCELL PAK ODS-Ph 10 i.d.×250 mm,CH_3CN-0.1% aq. CH_3COOH (10:90, v/v), 2 ml/min, monitored at 254 nm] to give compound **9** (2.0 mg), **8** (4.0 mg) and **7** (6.0 mg). The 20% aq. MeOH fraction (2.2 g) was concentrated under reduced pressure to leave a brown material which was purified by HPLC (Develosil RPAQUEOUS C-30, 10 i.d.×250 mm) using MeOH–H_2O (35:65) to give compound **3** (15.0 mg) and **4** (6.2 mg).

3.4. Acid hydrolysis of 3 and 4

Each sample [0.8 mg (**3**), 0.6 mg (**4**)] was heated in 1M HCl (0.2 ml) at 100 °C for 3 h, with the reaction mixtures neutralized by individually passing each through an ion-exchange resin (Amberlite IRA 400, OH^- form) column. Each water-eluate was concentrated, dissolved in pyridine (0.1 ml), and then treated with 0.1 M L-cysteine methyl ester hydrochloride in pyridine (0.2 ml), with the mixture kept at 60 °C for 1 h. After each solvent was evaporated under reduced pressure, the residues were trimethylsilylated with hexamethyl disilazane-trimethylchlorosilane (HMDS-TMCS; A5604; TMS-HT, TOKYO KASEI CO, Tokyo, Japan) (0.1 ml) at 40 °C for 1 h. The TMS derivatives of the monosaccharide were identified as glucose by GC analysis with standard D-glucose (t_R 18.7mm).

3.5. 2β,6α,17-Trihydroxy-ent-kauran-19-oic acid (1)

Colorless crystals (MeOH), mp 277–280 °C, $[\alpha]_D^{20}$ −40.8 °C (*c* 0.13, MeOH); For ^1H NMR and ^{13}C NMR spectra: see Tables 1 and 2; Negative HR-FAB-MS, *m/z*: $[M-H]^-$ 351.2163 (calcd for $C_{20}H_{31}O_5$: 351.2171). Negative FAB-MS, *m/z* (rel. int.): 351 $[M-H]^-$ (37), 275 (36), 183 (100), 91 (99), 72 (81), 61 (99).

3.6. 3β,16α,17-Trihydroxy-ent-kauran-19-oic acid (2)

Colorless crystals (MeOH), mp 246–249 °C $[\alpha]_D^{20}$ −55.1 °C (*c* 0.4, MeOH); For ^1H NMR and ^{13}C NMR spectra: see Tables 1 and 2; Negative HR-FAB-MS, *m/z*: $[M-H]^-$ 351.2170 (calcd for $C_{20}H_{31}O_5$: 351.2171). Negative FAB-MS, *m/z* (rel. int.): 351 $[M-H]^-$ (16), 275 (51), 183 (100), 91 (99), 72 (86), 61 (99).

3.7. 11α,15α-Dihydroxy-7-O-β-D-glucopyranosyl-ent-kaur-16-en-19-oic acid (3)

Brownish amorphous powder. $[\alpha]_D^{20}$ −15.5 °C (*c* 0.9, MeOH); For ^1H NMR and ^{13}C NMR spectra: see Tables 1 and 2; Negative HR-FAB-MS, *m/z*: $[M-H]^-$ 511.2546 (calcd for $C_{26}H_{39}O_{10}$: 511.2543). Negative FAB-MS, *m/z* (rel. int.): 511 $[M-H]^-$ (19), 331 (10), 275 (66), 183 (100), 164 (26), 151 (42), 137 (25), 119 (19), 107 (43). 275 (66), 183 (100), 164 (26), 151 (42), 137 (25), 119 (19), 107 (43).

3.8. 1α,15α-Dihydroxy-7-O-β-D-glucopyranosyl-ent-kaur-16-en-19-oic acid (4)

Brownish amorphous powder. $[\alpha]_D^{24}$ −7.2 °C (*c* 0.9, DMSO); For ^1H NMR and ^{13}C NMR spectra: see Tables 1 and 2; Negative HR-FAB-MS, *m/z*: $[M-H]^-$ 511.2548 (calcd for $C_{26}H_{39}O_{10}$: 511.2543). Negative FAB-MS, *m/z* (rel. int.): 511 $[M-H]^-$ (57), 331 (36), 197 (34), 183 (100), 179 (90), 151 (33), 135 (24), 119 (78), 113 (39).

4. Bioassay

4.1. Preparation of human peripheral blood mononuclear cell (hPBMC)

Human peripheral blood mononuclear cell (hPBMC) obtained from healthy human volunteers were isolated from heparinized venous blood by density gradient

sedimentation using Ficoll-Hypaque (Pharmacia Biotech; Ficoll-Paque R PLUS) and cells were collected from the interface, and then washed twice and resuspended in RPMI 1640 (Sigma) supplemented with 10% FBS (ICN, Ohio, USA), 2 mM L-glutamine, 100 U/ml penicillin and 100 mg/ml streptomycin (Mediatech cellgroR, Japan). Cells obtained by this procedure were >99% viable as determined by trypan blue dye exclusion.

4.2. Proliferation for hPBMC

Cells were seeded in 96-well plates (IWAKI, Tokyo, Japan) at final density of $0.8–1.2\times10^6$ cells/ml and incubated for 72 h in a continuous flow of 95% air and 5% CO_2 at 37 °C, in the presence or absence of plant extracts and isolated compounds. Every sample was assayed in quadruplet, and each experiment was performed with mononuclear cells from two to five donors. At the end of culturing, a 10 μl aliquot of Alamer Blue reagent (BIOSORCE INTERNATIONAL) was added to the cultures in the 96-well plates. After 4 h incubation, the optical density (OD) was determined at 570 nm with a reference wavelength of 595 nm on a microplate reader (Model 3550MICROPLATE READER; BIO-RAD Tokyo). Recombinant human IL-2 and concanavalin A were used as positive controls, and showed proliferative activity by 32.8% (1 ng/ml) and 32.1% (1 μg/ml), respectively, under the above conditions.

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