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Bioactive monoterpene glycosides conjugated with gallic acid from the leaves of *Eucalyptus globulus*

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Abstract

Two monoterpene glycosides, conjugated with gallic acid [globulusin A (**1**) and B (**2**)], together with four known compounds, cypellocarpin A (**3**), eucaglobulin (**4**), cuniloside (**5**) and (1*S*, 2*S*, 4*R*)-*trans*-2-hydroxy-1,8-cineole β-D-glucopyranoside (**6**), were isolated from hot-water extracts of the leaves of *Eucalyptus globulus*. The structures of compounds **1** and **2** were determined by 1D, 2D NMR and MS spectroscopic analyses. The absolute stereochemistry of **1** was determined by correlating the spectroscopic data with those of synthetic compound **6** with a known configuration. Globulusin A (**1**) and B (**2**), cypellocarpin A (**3**) and eucaglobulin (**4**), scavenged DPPH free radicals and globulusin A (**1**) showed a higher antioxidant activity than the other tested compounds, with an IC_{50} of 3.8 μM. Globulusin A (**1**) and eucaglobulin (**4**) concentration-dependently suppressed inflammatory cytokine production, tumor-necrosis factor-α and interleukin-1β in cultured human myeloma THP-1 cells co-stimulated with phorbol myristate acetate. These compounds also inhibited melanogenesis in cultured murine melanoma B16F1 cells, without any significant cytotoxicity. These results suggested that globulusin A (**1**) and eucaglobulin (**4**), which were isolated as antioxidants from *E. globulus*, also had anti-inflammatory as well as anti-melanogenesis activity.

Keywords: *Eucalyptus globulus*; Myrtaceae; Monoterpene; Glucosides; Gallic acid

1. Introduction

The genus *Eucalyptus* (Myrtaceae) is native to Australia, and this plant is mainly cultivated for paper, pharmaceutical and cosmetics industries. Several species of *Eucalyptus* are also used in traditional medicine as antiseptics, and against infections of the upper respiratory tract, such as the common cold, influenza and sinus congestion (Silva et al., 2003; Williams et al., 1998). Essential oil from this species has a therapeutic application in treatment of pulmonary infections by inhalation (Low et al., 1974), and the monoterpene extracted from *Eucalyptus citriodora*, *Eucalyptus globulus* and *Eucalyptus teretcorni* exhibit antibacterial activity (Ramezani et al., 2002). Besides antibacterial activity, the essential oil from *Eucalyptus* shows analgesic and anti-inflammatory effects (Silva et al., 2003). Phytochemical analysis has established that the genus *Eucalyptus* contains monoterpenes (Foudil-Cherif et al., 2000), cyanogenic glycosides (Gleadow and Woodrow, 2000) and the triterpene cladocalol (Benyahia et al., 2005). Since no previous studies have been reported on the biological activity related to the medicinal use of *Eucalyptus*, we chose one of major *Eucalyptus* species, *E. globulus*, and report here the isolation and structure elucidation of two new compounds (**1** and **2**), and the effects of these compounds on inflammatory cytokine production, 1,1-diphenyl-2-pycrylhydrazyl (DPPH) free radical formation and melanogenesis.

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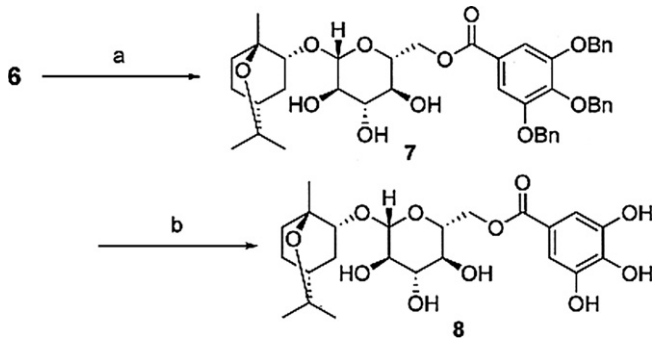
2. Results and discussion

The lyophilized powder of a hot-water extract of the leaves of *E. globulus* was absorbed on Diaion HP-20 resin, and fractions were successively eluted with aqueous MeOH. A fraction eluted with MeOH, which exhibited strong radical scavenging activities using the DPPH assay, was subjected to bioassay-guided fractionation with repeated column chromatography on silica gel and ODS, and further purified by HPLC equipped with an ODS column, to yield six compounds.

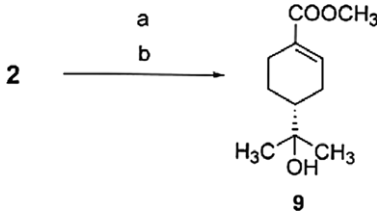
Compound **1** was obtained as a colorless solid, and its molecular formula $C_{23}H_{32}O_{11}$ was established from HRFABMS, which gave a pseudomolecular ion peak at *m/z* 483.1867 $[M-H]^-$. The presence of a galloyl group was suggested by a two-proton singlet at δ 7.04 in the ^{1}H NMR spectrum and five characteristic carbon signals (δ 110.2, 121.5, 140.4, 146.6, 168.3) in the ^{13}C NMR spectrum. The resonances between δ 3.16 and δ 4.48 were assigned to the protons of the sugar moiety. The coupling constant of the anomeric proton (δ 4.24, *d*, *J* 7.8 Hz) in the ^{1}H NMR spectrum indicated a β-configuration, and from the other coupling constants of the sugar moiety, it was clear that the sugar unit was a β-glucopyranose. In addition, the ^{1}H NMR spectrum also displayed signals for a 2-hydroxy-1,8-cineole moiety; three methyl singlets at δ 0.94, 1.04 and 1.16; three methylenes at δ 1.31, 1.42, 1.51, 1.87 (2H) and 2.21; and two methines at δ 1.42 and 3.44 (Someya et al., 2001). The presence of this moiety was supported by analysis of ^{13}C NMR and DEPT spectroscopic data. The HMBC spectrum of **1** showed the anomeric proton signal H-1′ coupled to C-2 (δ 81.4) of the 2-hydroxy-1,8-cineole moiety, and H-6′ (δ 4.42, 4.48) of the β-glucose moiety coupled to the C-1″ (δ 121.5) of the galloyl moiety. The absolute stereochemistry of **1** was elucidated by synthesis from compound **6**. In brief, compound **6** which resembled a monoterpene moiety of **1** was esterified with 3,4,5-tris(benzyloxy)benzoic acid in the presence of DCC and DMAP in dry pyridine, and then the benzyl protecting groups were removed by hydrogenolysis to obtain compound **8** (Tillekeratne et al., 2002) (see Scheme 1). All the proton signals for **8** were found to be identical to those for **1** isolated from *E. globulus*. Furthermore, the optical rotation was also identical between **8** $[\alpha]_D$ + 12.5 (*c* 0.1, MeOH) and **1** $[\alpha]_D$ + 11.3 (*c* 0.16, MeOH). Accordingly, the absolute stereochemistry of **1** was determined as 1*S*, 2*S* and 4*R*. Thus, compound **1** was determined to be (1*S*, 2*S*, 4*R*)-*trans*-2-hydroxy-1,8-cineole 2-*O*-β-D-(6′-galloyl) glucopyranoside. Based on the above data, compound **1** was assigned to globulusin A.

Compound **2** was obtained as a colorless solid, and its molecular formula $C_{23}H_{29}O_{12}$ was established from HRFABMS, which gave a pseudomolecular ion peak at *m/z* 497.1652 $[M-H]^-$. The ^{1}H NMR and ^{13}C NMR spectroscopic data of **2** were similar to those of the galloyl and β-glucopyranose moieties of **1**, which indicated that **2** also had a similarly substituted β-(6′-galloyl)glucopyranose moiety. The ^{13}C NMR and DEPT spectra showed the presence of two methyls, three methylenes, two methine groups and three quaternary carbons, including one carboxyl carbon, one olefinic carbon and one carbon bearing a hydroxyl group in the monoterpene moiety. ^{1}H–^{1}H COSY correlations were observed between H-2/H-3, H-3/H-4, H-4/H-5 and H-5/H-6. Furthermore, the HMBC spectrum of **2** showed H-2 (δ 7.06) was coupled to C-1 (δ 130.9) and C-7 (δ 168.8), and H-9 (δ 1.12) was coupled to C-4 (δ 45.6) and C-8 (δ 72.9). Thus, these 2D NMR methods established that **2** had an oleuropeic acid moiety. The HMBC spectrum of **2** showed the anomeric proton signal H-1′ (δ 4.63) coupled to C-7 of the oleuropeic acid moiety, and H-6′ (δ 4.24, 4.54) of a β-glucose moiety coupled to C-1″ (δ 175.0) of the galloyl moiety. The absolute stereochemistry of **2** was elucidated by methanolysis of **2** with 8.0% HCl in MeOH, which yielded the oleuropeic acid methyl ester (**9**) $[\alpha]_D$ + 81.0 (*c* 0.032, $CHCl_3$) (see Scheme 2). The physicochemical data of **9** were in agreement with those reported for (+)-oleuropeic acid methyl ester (Ito et al., 2000). Based on the above data, compound **2** was assigned to globulusin B.

The known compounds cypellocarpin A (**3**), eucaglobulin (**4**), cuniloside (**5**) and (1*S*, 2*S*, 4*R*)-*trans*-2-hydroxy-1,8-cineole β-D-glucopyranoside (**6**) were also isolated from these fractions. They were identified by comparing their spectroscopic data with those reported in the literature (Ito et al., 2000; Hou et al., 2000; Manns and Hartmann, 1994; Someya et al., 2001). To the best of our knowledge,



Scheme 1. Synthesis of **1**. Reagents and conditions: (a) 3,4,5-tris(benzyloxy)benzoic acid, DCC, DMAP, dry pyridine, rt, 24 h (18%); (b) hydrogen, palladium/carbon, ethanol, rt, 10 h (96%).



Scheme 2. Methanolysis of **2**. Reagents and conditions: (a) 8.0% HCl/methanol, 60 °C, 12 h; (b) TMS-diazomethane, methanol, rt, 1 h.

this is the first report of compound **5** being isolated from *E. globulus*.

It is interesting that plants of the genus *Eucalyptus* have been shown to produce a number of unique structure types, gallic acid–monoterpene (Ito et al., 2000), phloroglucinol–sesquiterpene and monoterpene (Osawa and Yasuda, 1996) coupled compounds (see Fig. 1).

After establishing their structures, we first examined the suppressive effects of the isolated compounds on DPPH free radical formation. As indicated in Table 2, compounds **1**–**4** scavenged DPPH free radical in a concentration-dependent manner, and their IC_{50} was 3.8–122 μM. Of the compounds tested in this experiment, the inhibitory activity of **1** and **4** was stronger than that of the positive control ascorbic acid. No activity was observed when the DPPH solution was mixed with **5** or **6** (Table 2).

E. globulus has traditionally been used to treat inflammatory diseases (Silva et al., 2003). Therefore, the effects of isolated compounds on inflammatory cytokine production in cultured THP-1 cells were examined. It is well known that fully activated monocytes produce a large amount of inflammatory cytokines such as interleukin (IL)-1β, IL-6, IL-12 and tumor necrosis factor (TNF)-α, and these cytokines directly or indirectly cause tissue inflammation (Bornstein et al., 2004). Cytokine production of both IL-1β and TNF-α in THP-1 cells was dramatically enhanced after 24 h stimulation with non-specific monocyte activator phorbol myristate acetate (PMA) (Fig. 2). The hot-water extract of *E. globulus* reduced IL-1β and TNF-α production by 54% and 61%, respectively, at a concentration 20 μM, without any significant cytotoxicity (data not shown). The major active compounds **1** and **4** suppressed the production of these cytokines in a concentration-dependent manner (Fig. 2), whereas they showed cytotoxic activity against THP-1 cells at higher concentrations (cell viability, 71.4% at 40 μM, $P < 0.05$). Anti-inflammatory agent dexamethasone, which was used as a

Table 1
^{13}C and ^{1}H NMR spectroscopic data for **1** and **2**[a]

Position	Globulusin A (**1**)		Globulusin B (**2**)	
	δ_C	δ_H	δ_C	δ_H
1	72.4		130.9	
2	81.4	3.44 (1H, *m*)	142.0	7.06 (1H, *m*)
3	34.7	1.51 (1H, *m*)	28.7	2.03 (1H, *m*)
		2.21 (1H, *m*)		2.36 (1H, *m*)
4	35.2	1.42 (1H, *m*)	45.6	1.56 (1H, *m*)
5	22.9	1.42 (1H, *m*)	24.6	1.21 (1H, *m*)
		1.87 (1H, *m*)		2.03 (1H, *m*)
6	26.7	1.31 (1H, *m*)	26.3	2.15 (1H, *m*)
		1.87 (1H, *m*)		2.51 (1H, *m*)
7	24.5	1.04 (3H, *s*)	168.8	
8	73.8		72.9	
9	28.8	1.16 (3H, *s*)	27.0	1.19 (3H, *s*)
10	29.0	0.94 (3H, *s*)	27.0	1.19 (3H, *s*)
1′	106.4	4.24 (1H, *d*, 7.8)	107.5	4.63 (1H, *d*, 7.8)
2′	75.4	3.16 (1H, *t*, 8.3)	74.9	3.50 (1H, *t*, 9.3)
3′	75.3	3.28 (1H, *m*)	77.5	3.45 (1H, *t*, 9.3)
4′	78.1	3.26 (1H, *m*)	71.6	3.38 (1H, *t*, 9.3)
5′	75.3	3.48 (1H, *m*)	76.4	3.62 (1H, *m*)
6′	64.9	4.42 (1H, *dd*, 2.4, 11.7)	64.4	4.24 (1H, *m*)
		4.48 (1H, *dd*, 7.8, 11.7)		4.54 (1H, *m*)
1″	121.5		125.4	
2″	110.2	7.04 (1H, *s*)	110.1	7.03 (1H, *s*)
3″	146.6		150.7	
4″	140.0		136.6	
5″	146.6		150.7	
6″	110.2	7.04 (1H, *s*)	110.1	7.03 (1H, *s*)
7″	168.3		175.0	

[a] Values in parentheses indicate coupling constants in Hz.

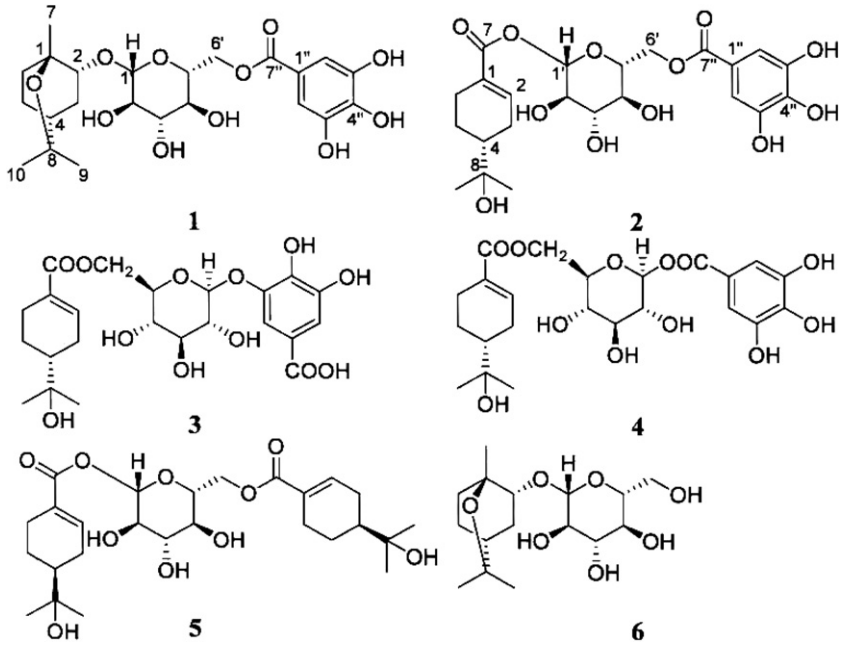


Fig. 1. Chemical structures of isolated compounds from hot-water extracts of *E. globulus*.

Table 2
DPPH radical scavenging activity of compounds isolated from hot-water extract of *E. globulus*

Compounds	IC_{50} (μM)[a]
1	4.8
2	122
3	21
4	3.8
5	na[b]
6	na
Ascorbic acid	12

[a] IC_{50} values were determined by regression analysis and expressed as the mean of three replicate measurements.
[b] na, no activity.

positive control, markedly reduced the production of both cytokines at a concentration of 2.0 μM.

Compound **1**, for which sufficient material was available, was also investigated for its inhibitory activity on melanogenesis in cultured murine melanoma B16F1 cells. Fig. 3 shows that B16F1 cells constitutively synthesized and released melanin within 48 h. Compound **1** suppressed both melanin secretion into the culture medium (Fig. 3a) and intracellular melanin synthesis (Fig. 3b), in a concentration-dependent manner. This compound did not exhibit any significant cytotoxicity to melanoma cells, even at a higher concentration of 40 μM (data not shown). Furthermore, the antioxidant ascorbic acid (100 μM) inhibited the secretion of melanin into the medium, whereas it did not affect intracellular synthesis of melanin (Fig. 3). Kojic acid, which is known to have anti-melanogenic activity (Maeda and Fukuda, 1991), inhibited melanogenesis, and the activity was similar to that of compound **1** (Fig. 3). To further investigate the detailed mechanism of the inhibition of melanogenesis by compound **1**, we tested whether the compound affected tyrosinase activity. It is well established that tyrosinase is one of the key enzymes in melanogenesis (Ito, 2003). No activity was observed when compound **1** was directly added to tyrosinase with its substrate, suggesting

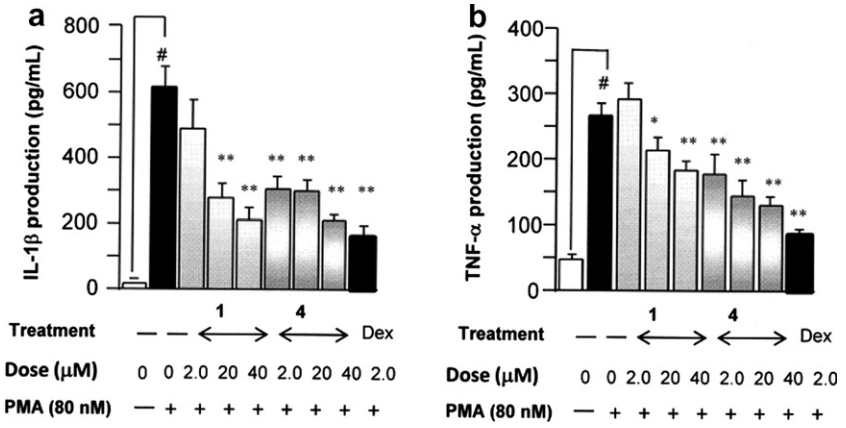


Fig. 2. Effects of globulusin A (**1**) and eucaglobulin (**4**) on inflammatory cytokine production in cultured THP-1 cells. Cells were directly treated with **1**, **4** or dexamethasone co-stimulated with 80 nM PMA. The culture supernatants were collected at 24 h after PMA stimulation and cytokine levels such as IL-1β (panel a) and TNF-α (panel b) were measured using ELISA. All data are expressed as means±SD of quadruplicate cultures. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{\#}P < 0.01$ compared with controls or normal culture.

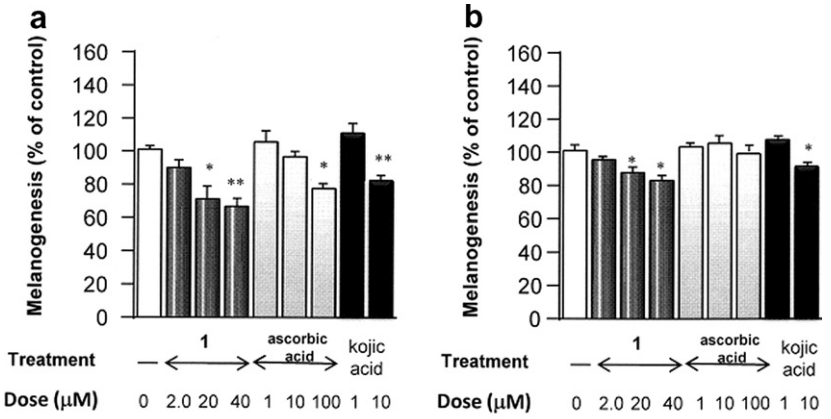


Fig. 3. Inhibitory effects of globulusin A (**1**) and antioxidant agent ascorbic acid on melanogenesis in cultured murine B16F1 melanoma cells. Cells were treated with **1,** ascorbic acid or kojic acid for 48 h. After incubation, melanin secretion (panel a) and synthesis (panel b) in cultured B16F1 were measured. All data are expressed as means ± SD of quadruplicate cultures. $^{*}P < 0.05$ and $^{**}P < 0.01$ compared with control cultures.

that the compound indirectly inhibited intracellular melanin synthesis (data not shown). Further study is required for elucidating the detailed mechanism of inhibition of melanogenesis by compound **1**.

2.1. Concluding remarks

In summary, this study demonstrated that the new monoterpene conjugates globulusin A (**1**) and eucaglobulin (**4**), which were isolated as antioxidants from *E. globulus*, had anti-inflammatory as well as anti-melanogenic activities.

3. Experimental

3.1. General

Optical rotations were determined with a Horiba SEPA-3000 high-sensitivity polarimeter. UV spectra were measured on a Shimadzu UV-1600 UV–visible spectrometer. IR spectra were recorded on a Shimadzu IR-460 IR spectrophotometer, whereas NMR spectra were obtained using a JEOL GSX-500 spectrometer in CD_3OD and $CDCl_3$. Chemical shifts were referenced to the residual solvent peaks CD_3OD (δ_H 3.30 and δ_C 49.8) and $CDCl_3$ (δ_H 7.24 and δ_C 77.0). Mass spectra were measured on a JEOL SX-102 mass spectrometer. Reversed-phase HPLC was performed on RP-23 (5 μm, Waters). Diaion HP-20 (Mitsubishi), silica gel (63–210 μm, Kantou) and ODS (63–212 μm, Wako) were used for open column chromatography. TLC was performed on silica gel 60 F_{254} (Merck) and RP-18 F_{254S} (Merck).

3.2. Plant material

Dried leaves of *E. globulus* used in this study were kindly provided and taxonomically identified by Ichimaru Pharcos, and deposited in a database at the Graduate School of Natural Science and Technology, Kanazawa University, Japan under registration number S-2006-07.

3.3. Extraction and isolation

The dried leaves (1.0 kg) of *E. globulus* were extracted with hot-water (3 l) at 90 °C for 30 min. After filtration and removal of the solvent by freeze-drying, a residual powder (135.8 g) was obtained, which was passed through a Diaion HP-20 column with a step-wise gradient of aqueous MeOH (0, 20 and 100%). A fraction eluted with 100% MeOH was subjected to silica gel CC with gradient mixtures of EtOAc and MeOH, to give five fractions. A biological fraction was further separated by ODS CC with a step-wise gradient of aqueous MeOH (30, 50 and 100%), and each fraction was purified by ODS HPLC with 45% MeOH in H_2O and 25% CH_3CN in H_2O to give globulusin A (**1**) (4.3 mg), globulusin B (**2**) (5.6 mg), cypellocarpin A (**3**) (15.3 mg), eucaglobulin (**4**) (13.0 mg), cuniloside (**5**) (115 mg), and (1*S*, 2*S*, 4*R*)-*trans*-2-hydroxy-1,8-cineole β-D-glucopyranoside (**6**) (457 mg), respectively.

3.4. Globulusin A (*1*)

Colorless solid; $[\alpha]_D + 11.3$ (MeOH, *c*0.16); UV (MeOH) λ_{max} ($\log\varepsilon$) 277 (3.42), 219 (3.74) nm; IR ν_{max} (KBr) 3344, 2945, 2833, 1556, 1448, 1419, 1114, 1028, 669, 652 cm^{-1}; for ^1H NMR spectroscopic data (500 MHz, CD_3OD) and ^{13}C NMR spectral data (125 MHz, CD_3OD), see Table 1; HRFABMS *m/z* 483.1867 $[M-H]^-$ (calculated for $C_{23}H_{31}O_{11}$, 483.1867).

3.5. Synthesis of 7

To an ice-cold solution of **6** (33 mg, 0.1 mmol), 3,4,5-tris(benzyloxy)benzoic acid (152 mg, 0.3 mmol) and DMAP (12 mg, 0.1 mmol) in dry pyridine (2.5 ml) was added DCC (62 mg, 0.3 mmol), and the mixture was stirred at room temperature for 24 h. MeOH (1.0 ml) was added dropwise to the reaction mixture, stirred for 15 min, and then diluted with EtOAc (5.0 ml). Dicyclohexylurea formed was removed by filtration off and washed thoroughly with EtOAc (10 ml). The filtrate was concentrated, and the residue was purified by silica gel flash CC (eluting with $CHCl_3$/MeOH 14:1) to yield **7** (8.2 mg) as a colorless oil. $[\alpha]_D - 9.9$ ($CHCl_3$, *c*0.6); ^1H NMR (500 MHz, $CDCl_3$) δ 1.02 (3H, *s*), 1.09 (3H, *s*), 1.18 (3H, *s*), 1.34 (1H, *m*), 1.48 (1H, *m*), 1.60 (1H, *m*), 1.68 (1H, *m*), 1.79–1.91 (2H, *m*), 2.32 (1H, *m*), 3.38 (1H, *t*, *J* 8.3 Hz), 3.40 (1H, *t*, *J* 9.3 Hz), 3.52–3.58 (3H, *m*), 4.33 (1H, *d*, *J* 7.3 Hz), 4.49 (1H, *dd*, *J* 2.4, 11.7 Hz), 4.70 (1H, *dd*, *J* 5.4, 11.7 Hz), 5.10 (6H, br *s*), 7.32–7.40 (15H, *m*); ^{13}C NMR (125 MHz, $CDCl_3$) δ 21.9, 24.5, 25.7, 28.5, 28.8, 33.5, 33.9, 64.0, 70.4, 71.3, 71.6, 73.4, 74.0, 74.0, 75.2, 76.1, 80.6, 104.5, 109.4, 124.7, 127.6, 128.0, 128.1, 128.2, 128.5, 128.6, 136.6, 137.3, 142.8, 152.5, 166.6; HREIMS *m/z* 754.3363 $[M]^+$ (calculated for $C_{44}H_{50}O_{11}$, 754.3354).

3.6. Synthesis of *8*

Palladium hydroxide on carbon (6.0 mg) was added to a solution of **7** (6.0 mg) in EtOH (1.0 ml) at room temperature. The reaction mixture was stirred for 12 h at room temperature under hydrogen. After filtration of the mixture and evaporation of the solvent, the residue was purified by silica gel flash CC (eluting with $CHCl_3$/MeOH 4:1) and ODS HPLC (eluting with 25% CH_3CN) to yield **8** (3.4 mg) as a colorless solid. $[\alpha]_D + 12.5$ (MeOH, *c*0.1); ^1H NMR (500 MHz, CD_3OD) δ 0.99 (3H, *s*), 1.09 (3H, s), 1.20 (3H, *s*), 1.35 (1H, *m*), 1.46 (2H, *m*), 1.55 (1H, *m*), 1.93 (2H, *m*), 2.25 (1H, *m*), 3.20 (1H, *t*, *J* 8.3 Hz), 3.31 (2H, *m*), 3.48 (1H, *m*), 3.57 (1H, *m*), 4.28 (1H, *d*, *J* 7.8 Hz), 4.46 (1H, *d*, *J* 11.7 Hz), 4.52 (1H, *dd*, *J* 7.8, 11.7 Hz), 7.08 (2H, *s*); HRFABMS *m/z* 483.1868 $[M-H]^-$ (calculated for $C_{23}H_{31}O_{11}$, 483.1867).

3.7. Globulusin B (2)

Colorless solid; $[\alpha]_D$ 20.0 (MeOH, *c*0.04); UV (MeOH) λ_{max} (log ε) 290 (2.88), 209 (3.89) nm; IR ν_{max} (KBr) 3167, 2918, 2833, 1448, 1421, 1115, 1013, 650 cm^{-1}; for ^1H NMR spectroscopic data (500 MHz, CD_3OD) and ^{13}C NMR spectral data (125 MHz, CD_3OD), see Table 1; HRFABMS *m/z* 497.1659 $[M-H]^-$ (calculated for $C_{23}H_{29}O_{12}$, 497.1652).

3.8. Methanolysis of 2

A solution of **2** (3.0 mg) in 8.0% HCl/MeOH (3.0 ml) was left standing at 60 °C for 12 h. After addition of three drops of 1 N NaOH, the concentrated solution was treated with TMS/diazomethane/Et_2O (1.8 ml) in MeOH (1.0 ml) at room temperature for 1 h and evaporated. Following dilution with $CHCl_3$ and water, the layers were separated. After evaporation of the solvent layer, the residue was purified by silica gel preparative TLC ($CHCl_3$/MeOH 14:1) to yield **9** (0.8 mg) as a colorless oil. $[\alpha]_D$ + 81.0 ($CHCl_3$, *c*0.032); ^1H NMR (500 MHz, $CDCl_3$) δ 1.22 (3H, *s*), 1.23 (3H, *s*), 1.25 (1H, *m*), 1.50 (1H, *m*), 2.02 (2H, *m*), 2.17 (1H, *m*), 2.33 (1H, *m*), 2.50 (1H, *m*), 3.71 (3H, *s*), 6.95 (1H, *m*). NMR spectroscopic data were in agreement with those reported for (+)-oleuropeic acid methyl ester (Ito et al., 2000).

3.9. 1,1-Diphenyl-2-pycrylhydrazyl (DPPH) free radical scavenging assay

The DPPH assay was performed by a method previously reported (Marsden, 1958; Zhang et al., 2007). In brief, 100 μl test samples at different concentrations in EtOH and 100 μl DPPH (Sigma) in EtOH (200 μl) were added to 96-well microtiter plates. The plate was shaken for 1 min by a plate shaker, and incubated for 30 min at room temperature in the dark. After incubation, the absorbance was recorded at 517 nm. The tested samples at different concentrations without DPPH solution were used as controls to eliminate the influence of the sample color. Ascorbic acid was used as positive control, and DPPH solution in EtOH served as a negative control. All tests were independently performed in triplicate and the free radical scavenging activity of tested samples was compared in terms of IC_{50} value.

3.10. Inhibitory effects on inflammatory cytokine production in cultured human myeloma THP-1 cells

Cells suspended in RPMI-1640 medium (containing 5% fetal bovine serum) were seeded at 2.0×10^5 cells/ml into 96-well tissue culture plates, and then incubated for 24 h at 37 °C in a humidified CO_2 incubator. Fresh medium was then replaced, and cells were further incubated for 24 h with test sample or positive control dexamethasone (dissolved in DMSO, final concentration 0.1% (v/v)) co-stimulated with 80 nM PMA (dissolved in EtOH, final concentration 0.1% (v/v)). To measure cytokine production in cultured THP-1 cells, culture supernatants were collected at 24 h and stored at −70 °C until use. The level of IL-1β and TNF-α in supernatants was measured by enzyme-linked immunosorbent assay (ELISA) using commercial kits for these cytokines (Cytoscreen; Biosource) according to the manufacturer's instructions.

3.11. Inhibitory effects on melanogenesis in cultured murine melanoma B16F1 cells

The content of melanin in cultured B16F1 melanoma cells was measured according to a previously described method (Hill et al., 1989; Matsuda et al., 2004). Briefly, cultured B16 mouse melanoma cells were trypsinized (0.25% trypsin and 0.1% EDTA at 37 °C for 5–10 min). Cells (2.5×10^4 cells/well in 980 μl DMEM) were inoculated with a pipette into 24-well plates and incubated for 24 h at 37 °C in a CO_2 incubator. After 24 h incubation, 20 μl of each sample solution was added to each well in triplicate, and the 24-well plate was incubated was incubated for 2 days at 37 °C in a CO_2 incubator. Test samples and theophylline were dissolved in DMSO/phosphate-buffered saline (PBS) (1:1, v/v), and then diluted with DMEM to an appropriate concentration. The final concentration of DMSO was 0.03%. In the control group, DMSO/ PBS (1:1, v/v) solution, diluted with Dulbecco's Modified Eagle's Medium (DMEM) to 0.03% of the final DMSO concentration was used instead of the sample solution. After incubation, the culture medium was removed by a pipette, and assayed for extracellular melanin, as described below. The remaining melanoma cells were trypsinized (0.25% trypsin and 0.1% EDTA at 37 °C for 5–10 min) and washed with 100 μl PBS. The cells were digested by the addition of 400 μl 1 N NaOH, and left standing for 16 h at room temperature. The optical density at 475 nm of the resulting solution was measured, and the amount of intracellular melanin was calculated. The culture medium was centrifuged (600*g*, 10 min at 4 °C) to give a supernatant. One milliliter of a mixture of 0.4 M 2-[4-(2-Hydroxyethyl)-1-piperazinyl]ethanesulfonic acid (HEPES) buffer (pH 6.8) and EtOH (9:1, v/v) was added to 1 ml supernatant. The optical density at 475 nm of the resulting solution was measured. For the second experiment, tyrosinase activity was measured according to the method of Mason and Peterson (1965). Tyrosinase (Sigma) obtained from mushroom was used (50,000 U/mg). A sample of 0.5 ml enzyme solution (135 U/ml) was incubated with 0.5 ml globulusin A at 25 °C for 10 min, and 0.03% L-Dopa (0.5 ml) was then added. After 15 min, tyrosinase activity was determined from the absorbance at 475 nm.

3.12. Cytotoxic activity

In order to measure the cytotoxic activity, cell suspensions (180 μl) were seeded in 96-well plates at 1.0×10^5 cells per well with tested compounds (20 μl) added from DMSO stock solutions. After 3 days in culture, adherent (B16F1) or non-adherent cells (THP-1) were incubated with MTT

(10 μl, 4 h) and subsequently solubilized in 10% SDS/DMF solution (100 μl, 10 h). The absorbance was measured at 570 nm using a microplate reader.

3.13. Statistical analysis

The mean cytokine production and ratio (percentage of control) of melanogenesis were considered as a single datum point for analysis of results from at least three or four independent experiments. All data are expressed as the mean ± SD. Statistical significance was determined by Dunnett's multiple test after one-way analysis of variance (ANOVA) with comparison to a control group, and the differences were consider significant at $P < 0.05$.

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