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DITERPENE GLYCOSIDES DERIVED FROM *ASTER SPATHULIFOLIUS* HAVE IMMUNOMODULATORY EFFECTS

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Abstract – Senescence-associated secretory phenotype (SASP) factors, involved in inflammation and tumorigenesis, can be inhibited by CD39, which degrades extracellular ATP. In this study, we analysed the chemical composition of the extracts of *Aster spathulifolius*, a coastal medicinal plant belonging to the Asteraceae family, and thereafter, isolated and determined the structures of the active components. We also treated THP-1 cells with the *A. spathulifolius* extracts and observed CD39 expression. Nine secondary metabolites, including a new diterpene glycoside **1**, were isolated. Compounds **1** and **3**–**7** substantially promoted CD39 expression in THP-1 cells, and compound **3**, the abundant component, showed the strongest effect in this regard, possibly degrading ATP and inhibiting the SASP.

INTRODUCTION

Secreted extracellular ATP is an alarm-signalling molecule that functions when cells receive external physical or chemical stimuli.[1] The secreted extracellular ATP stimulates surrounding senescent cells and, thus, induces the senescence-associated secretory phenotype (SASP), characterized by high expression levels of various secretory factors, of which inflammatory cytokines and chemokines are the primary

components. Substances that induce the SASP are called SASP factors, some of which promote inflammation and tumorigenesis, leading to various diseases, such as chronic inflammation and cancer. The membrane protein CD39, which is abundantly expressed in the plasma membranes of human Langerhans cells, degrades extracellular ATP and inhibits the SASP to suppress inflammatory responses and modulate immune response functions.[2] Thus, its deletion induces inflammation in skin cells.[3–6] Therefore, the SASP can be suppressed by degrading extracellular ATP so as to reduce its amount and prevent its accumulation.

Recently, research on the regulation of CD39 expression using natural products has been actively conducted.[7] To identify natural organic compounds that exhibit immunomodulatory effects and promote CD39 expression, more than 200 medicinal plants were screened, and only the ethanol (EtOH) extract of the coastal plant *Aster spathulifolius* was found to be active in this regard. *A. spathulifolius* is a coastal plant of the Asteraceae family with thick and hairy leaves and stems that grow wild on the coast of southern Kyushu in Japan. *A. spathulifolius* has long been used in Korea for medicinal purposes owing to its antiviral, antiallergic, and antiobesity properties.[8,9] This plant also produces species-specific secondary metabolites, such as sesquiterpenes, diterpenes, or aromatic compounds.[10–12] Especially, many diterpene glycosides have been reported.[10,11] In Japan, an intensive study on the chemical composition of *A. spathulifolius* extracts was conducted in the 1980s.[10] However, the identification of natural organic compounds that exhibit immunomodulatory effects from this plant has not yet been reported. Therefore, in this study, we aimed to analyse the chemical composition of *A. spathulifolius* extracts, isolate active substances, determine their structures, and clarify their effects on CD39 expression.

RESULTS AND DISCUSSION

Screening of medicinal plants for CD39 expression-enhancing activity

Of the more than 200 medicinal plant extracts analysed, only the extract of *A. spathulifolius* enhanced CD39 expression level (Data is not shown). Notably, it showed a 1.93-fold increase in CD39 expression level relative to the negative control (Table 1). Thus, we concluded that *A. spathulifolius* contains chemical components that promote CD39 expression and may inhibit skin cell ageing. The extracts of all the other plants showed CD39/GAPDH values of approximately 1.00 and did not promote CD39 expression. Further, *Artemisia princeps*, *Aster hybridus*, *Carthamus tinctorius*, *Helianthus tuberosus*, and *Matricaria chamomilla*, which also belong to the Asteraceae family, did not show CD39 expression-promoting activity. This finding may be because the CD39 expression-enhancing activity of *A. spathulifolius* is due to secondary metabolites produced in a species-specific manner.

In this study, *A. spathulifolius* leaf and flower extracts were separately used to treat THP-1 cells. The results indicated that both extracts enhanced CD39 expression similarly, and the observed effect was concentration-dependent (Figure 1). This indicated that *A. spathulifolius* exhibits the same CD39 expression-promoting activity regardless of the extraction site. Therefore, we isolated and characterized bioactive secondary metabolites to identify the active components.

Table 1. Results of the screening of Asteraceae extracts

Plant material	**CD39/GAPDH[a)]**
A. spathulifolius	1.93
A. millefolium	1.12
C. cyanus	1.07
A. princeps	1.06
C. tinctorius	1.05
M. chamomilla	1.01
A. hybridus	0.97
H. tuberosus	0.88

[a)] Values shown are those corresponding to when the CD39 expression level in the negative control THP-1 cells, to which no extract solution was added, was 1.00.

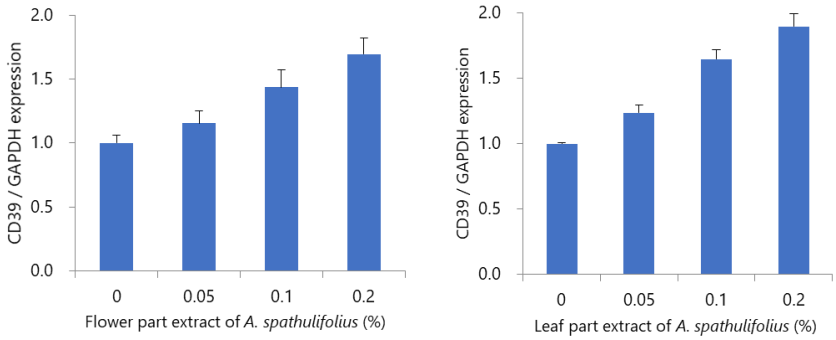


Figure 1. CD39 expression-promoting activity of *A. spathulifolius* flower and leaf extracts

Structure elucidation and identification

A. spathulifolius flowers were extracted using 90% (w/w) hydrous EtOH. Thereafter, the extract was concentrated and partitioned between ethyl acetate (EtOAc) and distilled water (H_2O), purified, and subjected to chromatography. Thus, nine secondary metabolites were obtained (Figure 2), and among them, (13*R*)-labd-14(15)-ene-8,13-diol 13-*O*-α-L-(4′-*O*-acetyl)-6′-deoxyidopyranoside (**1**), was identified as a new diterpene glycoside.

Compound **1** was obtained as a yellow oil $[\alpha]_D^{27}$ of -48.5 (*c* 0.1, $CHCl_3$). Its IR spectrum exhibited absorption bands corresponding to the hydroxy (3444 cm^{-1}) and acetoxy (1743 and 1240 cm^{-1}) groups. Its

molecular formula was $C_{28}H_{48}O_7$, determined via LC-APCI Q-TOF MS, which showed a positive ion at *m/z* 461.3269 $[M\text{-}2(H_2O)+H]^+$ (calculated for $C_{28}H_{45}O_5$, 461.3262). The ^1H and ^{13}C NMR spectra revealed 28 carbon signals (Table 2), corresponding to seven methyl (including one acetoxy group), eight methylene, eight methine groups, and five quaternary carbon atoms. Further, one ester group (δ_C 169.6), one acetal group (δ_C 95.7), two olefinic carbons (δ_C 142.3 and 115.1), and six oxygenated carbons (δ_C 81.1, 74.6, 73.0, 69.2, 68.7, and 62.1) were detected. Furthermore, two pairs of double bonds accounted for two of the five unsaturation degrees deduced from the molecular formula, indicating a tricyclic system in compound **1**. The ^1H NMR spectrum revealed a monosubstituted double bond (δ_H 5.89, 5.20, and 5.18), six singlet methyl groups (δ_H 2.11, 1.33, 1.14, 0.85, 0.77, and 0.77), one doublet methyl group (δ_H 1.14), and five oxymethine groups (δ_H 4.97, 4.87, 4.49, 3.82, and 3.52). Detailed analysis of the ^1H-^1H COSY spectrum of compound **1** allowed the construction of five spin systems: C1–C3, C5–7, C9–C12, C14–C15, and C1′–C6′ (Figure 3). HMBC between H_3-20/C-1, C-5, C-9, and C-10 revealed that C1–C3 was linked to the C5–C7 and C9–C12 units via the sp^3 quaternary carbon at C-10. Similarly, HMBC between H_3-18 and H_3-19/C-3, C-4, and C-5 indicated that the C1–C3 unit was linked to the C5–C7 unit via the sp^3 quaternary carbon at C-4 with geminal dimethyl groups H_3-18 and H_3-19. HMBC between H_3-17/C-7, C-8, and C-9 showed that the C5–C-7 unit was linked to the C9–C12 unit via the sp^3 quaternary carbon at C-8. Moreover, HMBC between H_3-16/C-12, C-13, and C-14 indicated that the C9–C12 unit was linked to the C14–C15 unit via the sp^3 quaternary carbon C-13. HMBC between H-1′/C-13 and C-5′ indicated that an ether bond linked C-1′ and C-13, and C-1′ and C-5′, forming a six-membered ring. Additionally, HMBC between H-4′ and C-7′ indicated that the acetoxy group was linked to C-4′.

Comparing the ^{13}C NMR spectra of the sugar moieties of compounds **1** and **4**–**7** showed that the conformation of the sugar moiety of compound **1** was likely the same as those of compounds **4**, **5**, and **7**, but different from that of compound **6**.[10,11] Therefore, chemical conversions were performed to test this hypothesis. Thus, we observed that compound **1** had a structure in which the hydroxy group at the 4′ position of compound **7** was replaced by an acetoxy group. Additionally, the specific optical rotation values of compounds **1** and **7** were similar. Their charts were matched by comparing the NMR spectra and the specific optical rotation values of synthesized **10**. Thus, compounds **1** and **7** were found to have the same absolute configuration (Scheme 1).

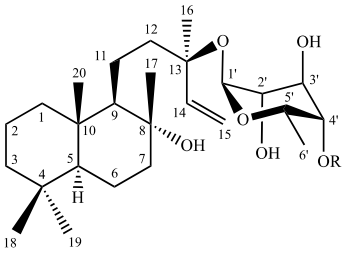
The eight compounds with known structures (Figure 2) were identified as isomanool (**2**),[13–15] sclareol (**3**),[16,17] (13*R*)-labda-7,14-diene 13-*O*-α-L-(4′-*O*-acetyl)-6′-deoxyidopyranoside (**4**),[10,11] (13*R*)-labda-7,14-diene 13-*O*-α-L-6′-deoxyidopyranoside (**5**),[10,11] (13*R*)-labda-7,14-diene 13-*O*-β-D-fucopyranoside (**6**),[11] (13*R*)-labd-14(15)-ene-8,13-diol 13-*O*-α-L-6′-deoxyidopyranoside (**7**),[10] germacrone (**8**),[18–21] and

nevadensin (**9**).[22] Compounds **2** and **3** were labdane-type diterpenes. Reportedly, compound **2**, which is primarily isolated from Asteraceae plants and liverwort[13–15] exerts a high growth inhibitory activity on malignant cells, mainly prostate cancer cells.[11] Further, compound **3**, present in several plants, among which *Salvia sclarea* is well-known,[16,17] exhibits cytotoxicity by inhibiting DNA synthesis and inducing apoptosis in human leukocytes and colon cancer cells.[23,24] Compounds **4**–**7** were identified as diterpene glycosides, while compounds **2** and **3** were identified as aglycones. Furthermore, compounds **4**–**6** are cytotoxic to human tumor cells.[10,11] A similar compound, labda-7,14-dien-(13*R*)-ol-α-L-rhamnopyranoside, was isolated from *A. oharai*.[25] Therefore, *Aster* plants specifically produce diterpene glycosides. Compound **8** is one of the major bioactive components of *Curcumae rhizoma*, exhibiting antitumor, anti-inflammatory, and antiviral activities. It is also present in *A. spathulifolius* and reduces hyperlipidemia and improves lipid metabolism in high-fat diet-induced obese mice.[26] Compound **9** was identified as a flavonoid. Many flavonoids have been isolated from herbs, such as basil, and they inhibit the activity of estragole, a carcinogen.[22] In addition, its selective inhibition of human carboxylesterase has been observed. Thus, it could be a therapeutic agent for obesity and type 2 diabetes.[27]

Table 2. ^{1}H and ^{13}C NMR data (400 MHz and 100 MHz, $CDCl_3$ for **1** (δ in ppm, J in Hz))

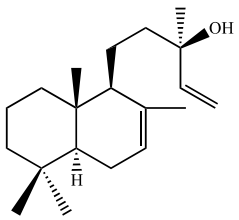
Position	δ_H (mult., J in Hz)	δ_C
1	0.92 (1H, m)	39.8
	1.57 (1H, m)	
2	1.25 (1H, m)	19.2
	1.45 (1H, m)	
3	1.14 (1H, m)	42.0
	1.70 (1H, m)	
4		33.3
5	0.90 (1H, m)	56.2
6	1.24 (1H, m)	20.5
	1.63 (1H, m)	
7	1.37 (1H, m)	44.2
	1.83 (1H, m)	
8		74.6
9	1.04 (1H, t, J=3.7)	62.1
10		39.2
11	1.24 (1H, m)	18.5
	1.56 (1H, m)	
12	1.63 (1H, m)	43.5
	1.80 (1H, m)	
13		81.1
14	5.89 (1H, dd, J=17.4, 10.5)	142.3
15	5.18 (1H, dd, J=17.4, 1.8)	115.1

	5.20 (1H, dd, *J*=10.5, 1.8)	
16	1.33 (3H, s)	22.4
17	1.14 (3H, s)	24.4
18	0.85 (3H, s)	33.5
19	0.77 (3H, s)	21.5
20	0.77 (3H, s)	15.6
1′	4.97 (1H, br d, *J*=2.3)	95.7
2′	3.52 (1H, br s)	69.2
3′	3.82 (1H, t, *J*=3.2)	68.7
4′	4.87 (1H, t, *J*=3.2)	73.0
5′	4.49 (1H, dq, *J*=6.9, 2.3)	62.1
6′	1.14 (3H, d, *J*=6.9)	15.8
7′		169.6
8′	2.11 (3H, s)	20.9



1：R = Ac

7：R = H



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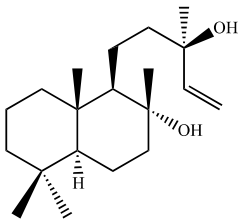

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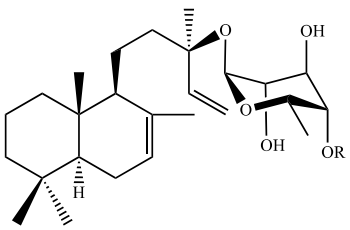
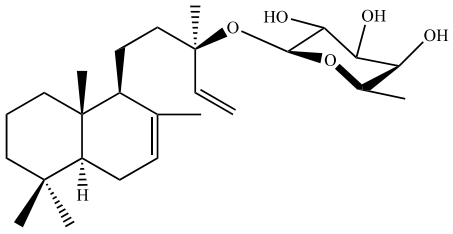


4：R = Ac

5：R = H



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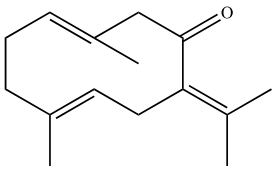

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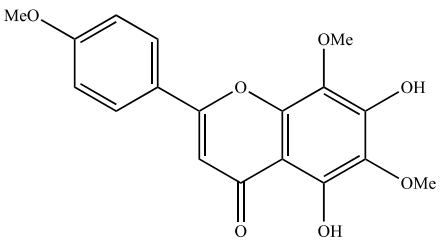

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Figure 2. Structures of compounds **1**–**9**

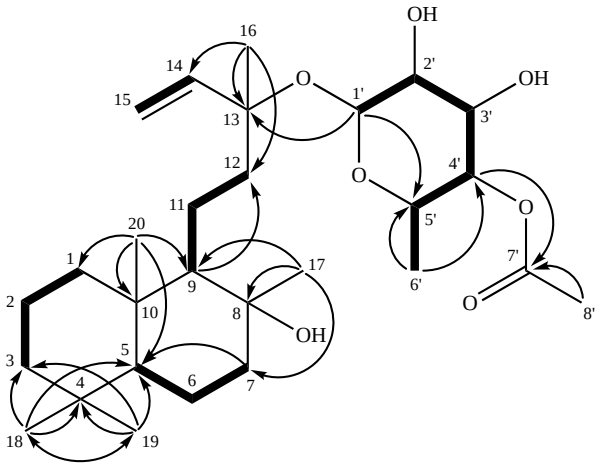
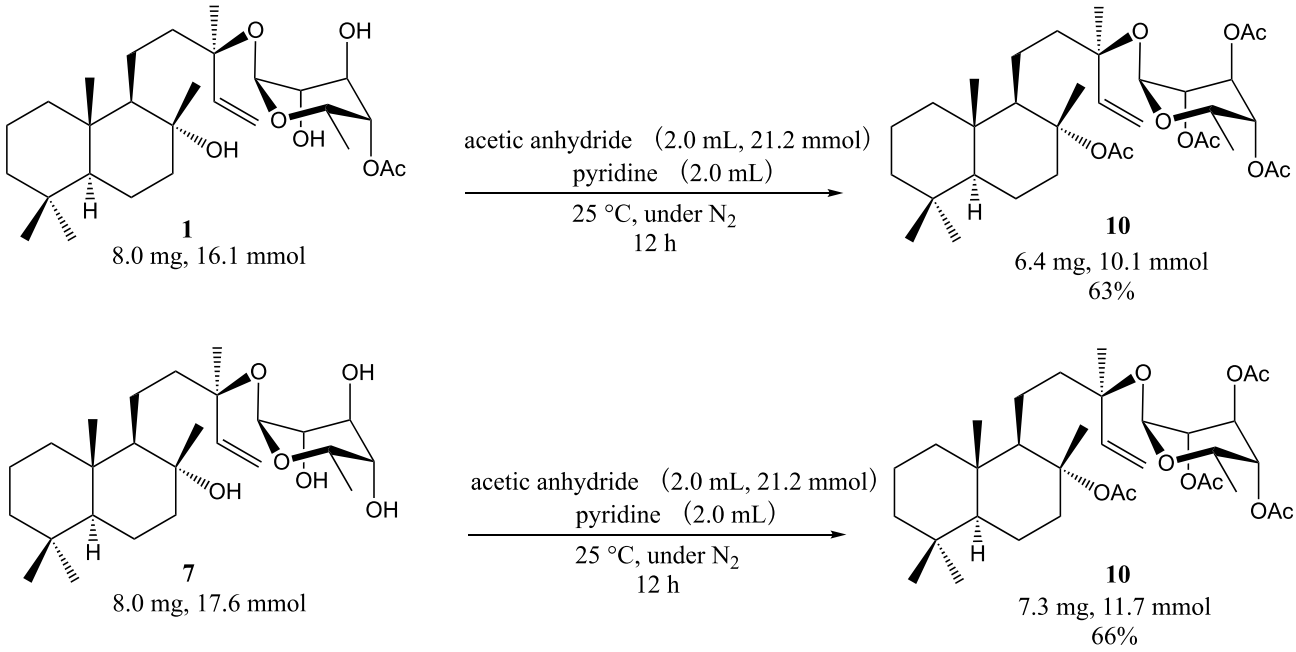


Figure 3. ^{1}H-^{1}H COSY (bold lines) and key HMBC (arrows) correlations of compound **1**



Scheme 1. Acetylation process of compounds **1** and **7** to yield **10**, respectively

CD39 expression-promoting activity

CD39 expression-promoting activity was observed in compounds **1** and **3**–**7** (Figure 4). Compound **3** was the most abundant in the *A. spathulifolius* extract and exhibited substantial CD39 expression-promoting activity at all concentrations. Therefore, we concluded that compound **3** was responsible for the CD39 expression-promoting activity of the *A. spathulifolius* extract. To verify this hypothesis, we performed a similar experiment using commercially available sclareol preparations. Our results indicated that the sclareol preparation also showed strong CD39 expression-promoting activity (Figure 5).

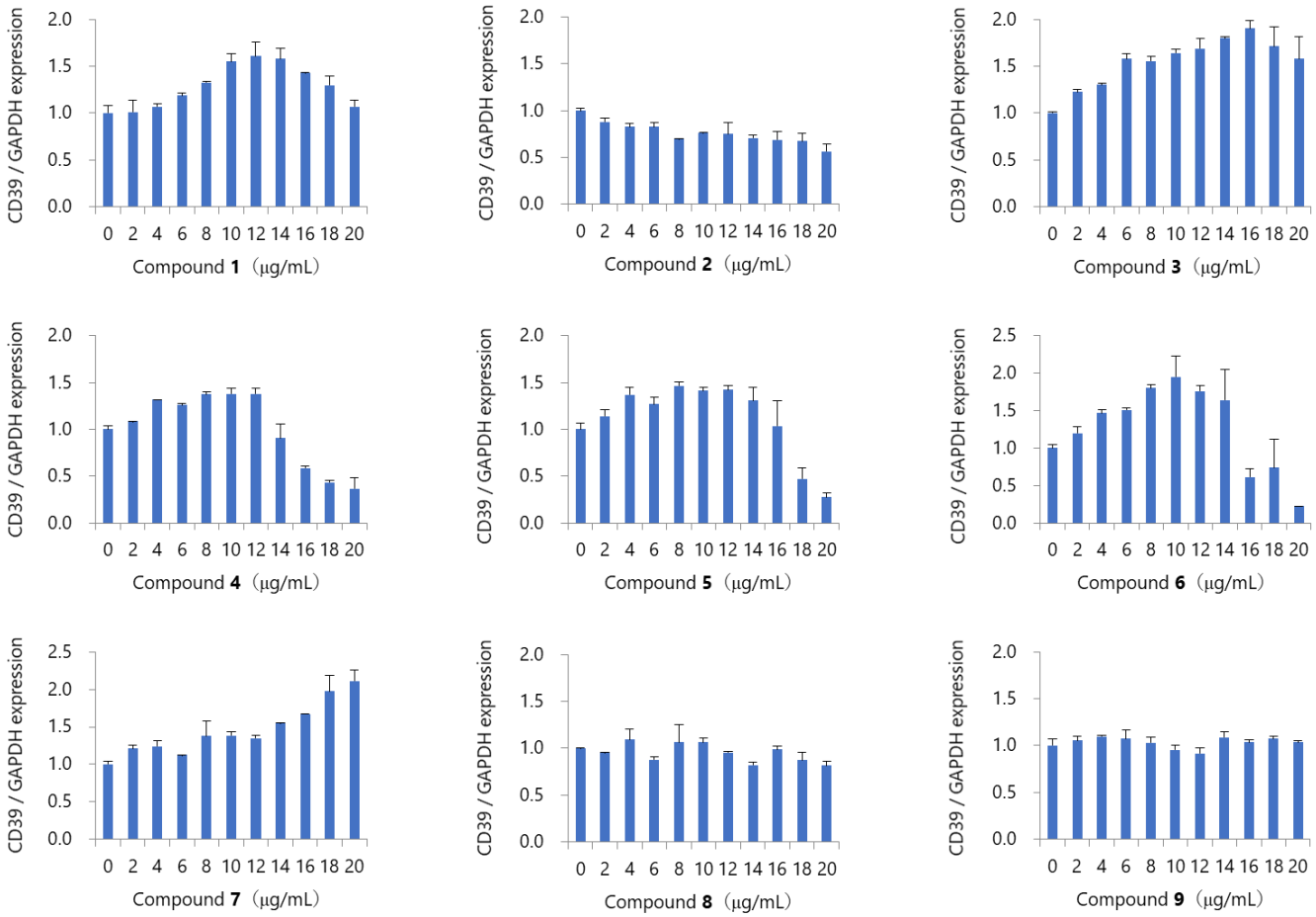


Figure 4. CD39 expression-promoting bioassay for compounds **1**–**9**

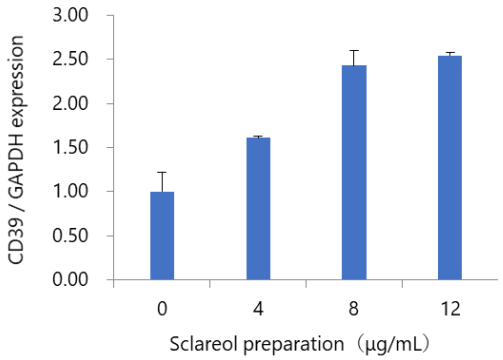


Figure 5. CD39 expression-promoting bioassay for sclareol preparation

In this study, we observed that *A. spathulifolius* extracts promote the expression of CD39, which exerts an inhibitory effect on inflammatory response in skin Langerhans cells. Therefore, we analysed the chemical components of the *A. spathulifolius* extracts and isolated nine compounds, including a new diterpene glycoside (compound **1)**. CD39 expression-promoting bioassay for these nine compounds showed remarkable activities for compounds **1**, **3**, and **7**. Particularly, compound **3** showed the strongest CD39

expression-promoting effect. Therefore, extracts of the edible plant, *A. spathulifolius* have the potential for use as a therapeutic agent. On the other hand, compound **2**, an aglycon of compounds **4**–**6**, which showed moderate biological activity, showed no biological activity. This may be due to the fact that compound **2** had the lowest polarity of these compounds.

EXPERIMENTAL

Plant Material, Extraction, and Isolation

The flowers and leaves of *A. spathulifolius*, grown by a farmer in Saga Prefecture from May to November 2021, were separately immersed and extracted in 2 L of 90% (w/w) hydrous EtOH solution. Thereafter, the crude extract was suspended in H_2O (400 mL) and partitioned four times using EtOAc (400 mL × 4). After removing the organic solvent, an EtOAc fraction (1.0 g) was obtained. Next, these flower and leaf EtOAc fractions were subjected to ^{1}H NMR profiling, and their ^{1}H NMR spectra were compared and analysed. NMR spectra were recorded on JEOL ECA 400 FT-NMR (400 MHz) spectrometers using $CDCl_3$ (Merck, Darmstadt, Germany) as solvent and tetramethylsilane as the internal standard. Both fractions exhibited similar spectra; therefore, the fractionation of the EtOAc fraction of the flower extract was performed, yielding a large amount of the fraction. This was followed by chromatography on a silica gel column and elution with *n*-hexane/EtOAc with increasing polarity (*n*-hexane/EtOAc: 9:1, 8:2, 7:3, 5:5, 100% EtOAc, and 100% methanol (MeOH)) to yield six fractions (1–6). Fraction 2 was subjected to iterative preparative TLC with toluene to yield compounds **2** (12.3 mg) and **8** (21.4 mg). Fraction 4 was subjected to iterative preparative TLC with *n*-hexane/EtOAc (2:1) to yield compounds **3** (94.3 mg), **4** (17.9 mg), and **5** (23.3 mg). Similarly, fraction 5 was subjected to iterative preparative TLC using *n*-hexane/EtOAc (3:2) to yield compounds **6** (30.3 mg) and **9** (6.1 mg) and to reversed-phase preparative TLC using MeOH/H_2O (3:2) to yield **1** (4.4 mg). Fraction 6 was also subjected to iterative preparative TLC using $CHCl_3$/MeOH (9:1) to yield compound **7** (23.9 mg). Preparative TLC was performed using silica gel glass plates (Merck, Kieselgel 60 F_{254}), reversed-phase preparative TLC was performed using silica gel glass plates (Merck, Kieselgel 60 RP-18 F_{254}s), and column chromatography (CC) was performed using silica gel (Merck, Kieselgel 60, 70–230 mesh). The optical rotations of the compounds were measured using a Jasco P-1010 digital polarimeter (Jasco, Tokyo, Japan). The isolated compounds were subjected to IR spectroscopy using a Jasco FT/IR-6100 spectrophotometer. High-resolution mass spectra were acquired using an LC-quadrupole time of flight MS (Bruker Daltonics "Q-Tof Impact II" + Shimadzu LC system).

Synthesis of Compound 10

Acetic anhydride (2.0 mL, 21.2 mmol; FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) was added to compounds **1** (8.0 mg, 16.1 mmol) and **7** (8.0 mg, 17.6 mmol). Dehydrated pyridine (2.0 mL; FUJIFILM Wako Pure Chemical Corporation) was also added, and the mixtures were stirred at 25 °C for

12 h under a N_2 atmosphere with light shielding. After this period, the reaction was quenched by adding toluene (10 mL), and the reaction solution was poured into *n*-hexane (100 mL). Thereafter, the solvent was distilled, and the remaining residue was acetylated. The solution was then subjected to preparative TLC using *n*-hexane/EtOAc (2:1) to yield **10** (from **1**: 6.4 mg, 10.1 mmol, 63%; from **7**: 7.3 mg, 11.7 mmol, 66%).

CD39 Expression-Promoting Bioassay

Compounds **1**–**9** and the standard were dissolved in absolute EtOH to prepare 10 mg/mL sample solutions. Further, approximately 1×10^5 THP-1 cells (TIB-202; American Type Culture Collection, Manassas, VA, USA) were seeded in each well (3.34 mL/well) of a 24-well cell culture plate, and 998 µL of RPMI-1640 medium containing 10% foetal bovine serum was added. Thereafter, the cells were cultured for 24 h. Next, compounds **1**–**9** and absolute EtOH were each mixed in the required amounts and added to the wells in 2 µL portions. The concentration of each compound ranged from 0 to 20 µg/mL. The cells were then cultured for 24 h. Some of the cells were cultured with the addition of 50% (w/w) aqueous EtOH (2 µL) solution as the negative control. After culturing, total RNA was extracted from the THP-1 cells using the FastGene™ RNA Basic Kit (Nippon Genetics, Tokyo, Japan). cDNA was then synthesized from the total RNA using ReverTra Ace® qPCR RT Master Mix (Toyobo, Osaka, Japan). CD39 expression levels were then measured via qPCR using a LightCycler® Nano (Roche, Basel, Switzerland) and PrimeTime™ Gene Expression Master Mix (Integrated DNA Technologies (IDT), Coralville, IA, USA). Probe/primer sets for the following genes were used: CD39 (left primer 5′-TTGGGGCATTTTCAGCTTTTTAC-3′; right primer 5′-TCTCCTTTACTCCAGCGTAAGATG-3′; probe 5′-/56-FAM/ATCTCCTCC/ZEN/CAAGGCTGAGCACAGAACTTT/3IABkFQ/-3′) and GAPDH (predesigned by IDT, cat no: Hs.PT.39a.22214836).

(13*R*)-Labd-14(15)-ene-8,13-diol 13-*O*-α-L-(4′-*O*-acetyl)-6′-deoxyidopyranoside (1): yellow oil; $[\alpha]_D^{27}$ –48.5 (*c* 0.1, $CHCl_3$); IR (liquid film, ν_{max}, cm^{-1}): 3444, 2925, 1743, 1463, 1387, 1240, 1046, 937, and 758; ^1H and ^{13}C NMR spectral data: see Table 2; LC-APCI Q-TOF MS: *m/z* 461.3269 $[M\text{-}2(H_2O)+H]^+$ (calcd for $C_{28}H_{45}O_5$, 461.3262).

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