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Lupane-type triterpenoids from the steamed leaves of *Acanthopanax koreanum* and their inhibitory effects on the LPS-stimulated pro-inflammatory cytokine production in bone marrow-derived dendritic cells

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ABSTRACT

Two new compounds, (20*R*)-3α-hydroxy-29-dimethoxylupan-23,28-dioic acid (**1**) and 3α-hydroxylup-20(29)-ene-23,28-dioic acid 28-*O*-[β-D-glucopyranosyl-(1→6)-β-D-glucopyranosyl] ester (**2**), and eight known lupane-type triterpenoids (**3**–**10**), were isolated from steamed leaves of *Acanthopanax koreanum*. Chemical structures were determined using a combination of spectroscopic analyses and chemical reactivity. Compounds **1**–**10** were evaluated for their inhibitory activities on lipopolysaccharide (LPS)-stimulated interleukin (IL)-12 production in bone marrow-derived dendritic cells (BMDCs). Compound **1** exhibited inhibitory activity with IC_{50} value of 26.5 μM on IL-12 production, compared with IC_{50} value of 29.6 μM for the positive control. Compound **1** also showed significant suppression of LPS-stimulated IL-6 and tumor necrosis factor-alpha (TNF-α) production.

Acanthopanax koreanum Nakai (Araliaceae) is an indigenous plant prevalently distributed throughout South Korea.[1] The roots and stem bark of *A. koreanum* have been traditionally administered as a tonic and sedative and have been used as a treatment for rheumatism, hepatitis, and diabetes.[2] The leaves of this plant contain diterpenoids, lignans, triterpenoids, polyacetylenes, phenylpropanoids, and flavonoids.[3] Recent pharmacological investigations of *A. koreanum* report anti-inflammatory,[4] immunostimulatory,[5] antioxidant,[6] and protein tyrosine phosphatase (PTP)-1B inhibitory activities.[7]

Innate immunity is triggered when pathogen-associated molecular patterns (PAMPs) that are shared among groups of microbial pathogens are recognized by pattern recognition receptors (PRR), such as the Toll-like receptors (TLRs), of host cells.[8] The engagement of TLR by PAMPs triggers the activation of several signal cascades, including the nuclear factor kappa B (NF-κB) and mitogen-activated protein (MAP) kinase pathways, leading to the production of pro-inflammatory cytokines. Interleukin (IL)-12 p40 is a subunit component of both IL-12 and IL-23. IL-12 is involved in type 1 helper T cell (Th1)-mediated inflammation as part of the normal immune defense, as well as inflammatory diseases, such as rheumatoid arthritis, asthma, psoriasis, and Crohn's disease.[9–11] IL-23 plays an immunological role in regulating T cells that produce other pro-inflammatory cytokines. IL-12 p40 exists in either a monomeric or homodimeric (IL-12 p80) form, each of which possesses its own bio-activity, independent of IL-12 and IL-23, including chemoattraction of macrophages and stimulated dendritic cells.[12]

The current study evaluated the potential of *A. koreanum* as a functional food with medicinal properties and, for the first time, reports anti-inflammatory effects of steamed *A. koreanum* leaves on LPS-induced pro-inflammatory cytokine production by bone marrow-derived dendritic cells (BMDCs).

A. koreanum leaves were collected in Susin Ogapi, Cheonan City, Korea, in August 2006 and taxonomically identified by one of the authors (Professor Young Ho Kim). A voucher specimen (CNU 060810) was deposited at the Herbarium of the College of Pharmacy, Chungnam National University, Korea. Oriental herbal medicines are often processed by steaming at 98–100 °C to preserve and/or improve efficacy.[13] To induce the chemical changes required to enhance the biological activities of this plant, the leaves of *A. koreanum* (4.0 kg) were processed at 120 °C for 4 h at 0.15 MPa pressure, without mixing with water, to give the steamed sample.

The steamed sample was then air-dried and extracted three times with methanol (MeOH) under reflux for 15 h, yielding 825 g of a dark, solid extract. The extract was suspended in water and partitioned with *n*-hexane and ethyl acetate (EtOAc) to give an *n*-hexane-soluble fraction (181 g), an ethyl acetate fraction (104 g), and a water-soluble fraction (540 g). The ethyl acetate extract

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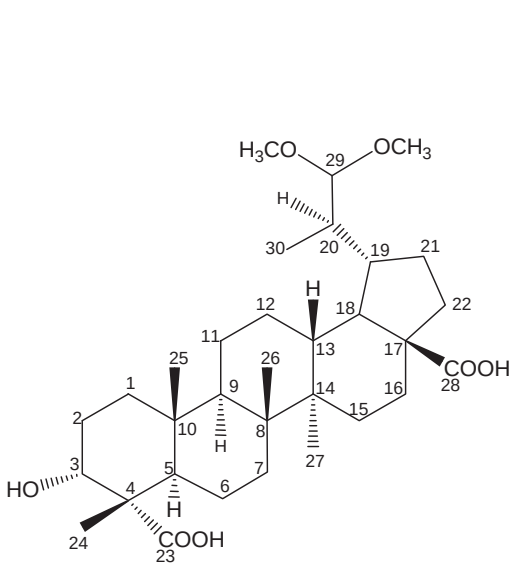

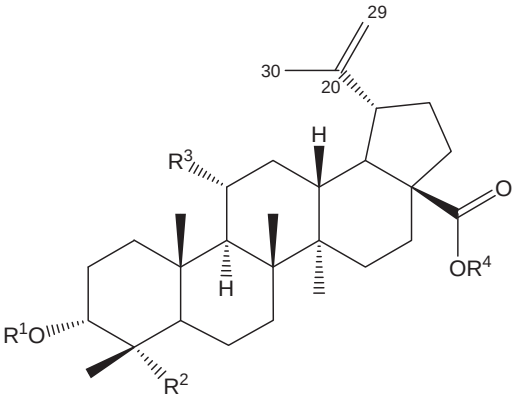

	R¹	R²	R³	R⁴
2	H	COOH	H	glc⁶-glc
3	H	COOH	H	H
4	H	COOH	H	glc⁶-glc⁴-rha
5	H	CHO	OH	H
6	H	CHO	OH	glc⁶-glc⁴-rha
7	H	CH_2OH	OH	H
8	H	CH_3	OH	H
9	glc	CH_3	OH	H
10	H	CH_3	OH	glc⁶-glc⁴-rha

Figure 1. Structures of the lupane-type triterpenoids 1–**10** (glc: β-D-glucopyranosyl and rha: α-L-rhamnopyranosyl).

(104 g) was first separated on a silica gel column and eluted with a mixture of *n*-hexane/EtOAc/MeOH (gradient from 3:1:0, 1:1:0, 0:3:1, 0:2:1, 0:1:1 v/v). Subsequent separations of certain fractions were performed on YMC RP-18 and silica gel columns[14] to ultimately yield two new compounds, (20*R*)-3α-hydroxy-29-dimethoxylupan-23,28-dioic acid (**1**) and 3α-hydroxylup-20(29)-ene-23,28-dioic acid 28-*O*-[β-D-glucopyranosyl-(1→6)-β-D-glucopyranosyl] ester (**2**), and eight known triterpenoids, 3α-hydroxy-lup-20(29)-ene-23,28-dioic acid (**3**),[15] acankoreoside A (**4**),[15] 3α,11α-dihydroxy-23-oxo-lup-20(29)-en-28-oic acid (**5**),[16] acankoreoside D (**6**),[16] 3α,11α,23-trihydroxy-lup-20(29)-en-28-oic acid (**7**),[15] impressic acid (**8**),[17] 3-*O*-β-D-glucopyranosyl 3α,11α-dihydroxylup-20(29)-en-28-oic acid (**9**),[16] and acantrifoside A (**10**)[17] (Fig. 1).

The structure of compound **1** was determined by spectroscopic analyses using a combination of ^{1}H and ^{13}C NMR, COSY, HMQC, HMBC, and MS spectral data, and comparison with literature data.[18] Compound **1** was obtained as a white amorphous powder. HRESIMS revealed a molecular formula of $C_{32}H_{53}O_7$, based on the experimental [M+H]+ peak at *m/z* 549.3809 (calcd 549.3713). The

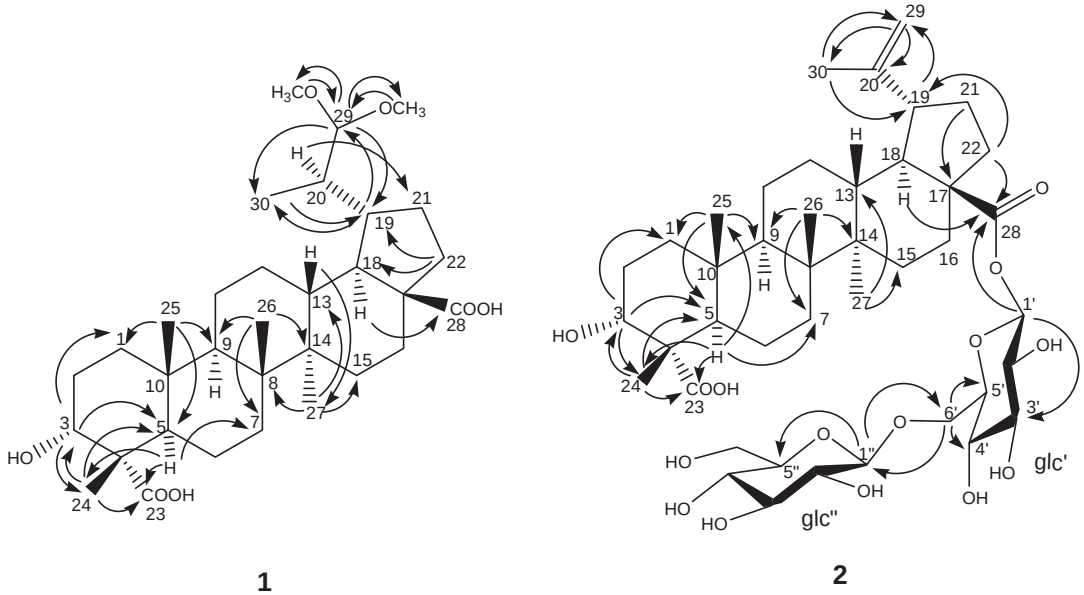

Figure 2. Selected HMBC (H→C) correlations of compounds **1** and **2**.

Table 1
NMR data for compounds **1** and **2** in $C5D_5N$

Position	1			2		
	δ_C[a]	δ_H (J in Hz)[b]	HMBC (H→C)	δ_C[a]	δ_H (J in Hz)[b]	HMBC (H→C)
1	32.8	1.50 m, 1.88 m[c]	5, 9	33.1	1.66 m[c], 1.84 m[c]	10
2	26.0	1.87 m[c], 2.60 m[c]		26.2	1.85 m[c], 2.67 m[c]	25
3	72.8	4.30 m	1, 4, 5, 24	71.5	4.29 m	1, 24, 5
4	51.8	—		51.9	—	
5	44.7	2.59 m[c]	1, 4, 6, 7, 9, 10, 23, 24	45.3	2.63 m[c]	4, 6, 7, 9, 10, 23, 24, 25
6	21.6	1.91 m[c]	3, 4, 8	21.7	1.98 m[c]	
7	32.8	2.64 m[c]	10	34.6	2.60 m[c]	5, 8, 9, 10, 21, 26
8	41.6	—		41.8	—	
9	50.7	1.71 m	8, 10, 11, 25, 26	51.0	1.66 m	1, 11, 26
10	38.2	—		37.4	—	
11	21.1	1.89 m[c]	8, 10, 25, 26	20.9	1.28 m, 1.49 m[c]	
12	27. 7	1.80 m[c]	9, 14	26.0	1.85 m[c]	
13	38.6	2.74 m[c]	27	38.4	2.54 m[c]	27
14	43.0	—		42.9	—	
15	30.2	1.24 m	13, 17, 27	30.1	1.27 m	13, 17
16	34.7	1.35 m		32.2	1.47 m	4, 5, 7, 8, 15, 17
17	56.7	—		57.0	—	
18	49.1	2.00 m	17, 19, 20, 28	49.8	1.66 m	13, 14, 17, 19, 20, 28
19	43.7	2.76 m[c]	18, 29, 30	47.4	3.40 m	30
20	37.6	2.22 m[c]	19, 21, 29, 30	150.9	—	
21	24.6	1.87 m[c]	17, 18	30.8	2.01 m[c], 1.56 m[c]	17
22	37.2	2.20 m[c]	17, 18, 19, 21, 28	36.9	2.21 m[c], 1.47 m[c]	18, 19, 21, 28
23	178.9	—		181.3	—	
24	17.8	1.47 s	3, 4, 5, 23	18.1	1.45 s	3, 4, 5, 23
25	16.6	1.11 s	9, 10	16.8	0.94 s	1, 5, 9, 10, 27
26	16. 6	1.13 s	7, 14	16.7	1.20 s	7, 9, 14
27	14.5	0.96 s	C7, C9, C15	14.8	0.88 s	13, 15
28	179.4	—		175.0	—	
29	107.8	4.43 d (5.5)	19, 30, OCH_3	110.0	4.84 br s, 4.70 br s	19, 30
30	16.4	0.95 d (2.8)	19, 20	19.4	1.70 s	20, 29
OCH_3	53.3	3.39 s	29			
OCH_3	54.4	3.40 s	29			
glc-1′				95.2	6.36 d (8.0)	3′, 28
glc-2′				72.9	4.10 t (8.2)	
glc-3′				78.7	4.20 m[c]	
glc-4′				71.0	4.29 m[c]	2′
glc-5′				78.3	4.12 m[c]	1′, 3′
glc-6′				69.5	4.48 dd (1.4, 11.7)	
					4.32 dd (4.8, 11.7)	4′, 5′, 1″
glc-1″				105.4	5.02 d (8.0)	6′, 5″
glc-2″				74.0	4.00 t (8.5)	5″
glc-3″				75.1	4.07 m[c]	1″, 5″
glc-4″				78.4	4.24 m[c]	2″
glc-5″				78.0	4.17 m[c]	2″, 3″, 4″
glc-6″				62.7	4.07 dd (2.1, 11.7)	
					4.20 dd (5.8, 11.7)	4″, 5″

[a] Recorded at 100 MHz.
[b] Recorded at 400 MHz.
[c] Overlapped signals. All assignments were confirmed by DEPT-135, HMQC and HMBC experiments.

^1H and ^{13}C NMR spectral data in Table 1 indicate that **1** was a lupane-type triterpene. The ^1H NMR spectrum of **1** contained five methyl signals at δ_H 0.95 (3H, d, J = 2.8 Hz, H-30), 0.96, 1.11, 1.13, and 1.47 (3H each, all s, H-27, 25, 26, 24), two methoxyl signals at δ_H 3.39 and 3.40 (3H each, s), an oxygenated methine proton at δ_H 4.30 (1H, m, H-3), and a dioxygenated methine proton as doublet at δ_H 4.43 (1H, d, J = 5.5 Hz, H-29). A comparison of the ^1H and ^{13}C NMR spectroscopic data for **1** with those of **3**[15] revealed strong similarities, except for signals at δ_C 37.6 and 107.8, which were assigned to C-20 and -29 based on DEPT-135, HMQC, and HMBC data. The absolute configuration at C-3 (δ_C = 72.8) was determined to be α by comparing with the chemical shifts of 3α-hydroxylup-20(29)-en-23,28-dioic acid (C-3 at δ 72.7)[15] and 3β-hydroxylup-20(29)-en-23,28-dioic acid (C-3 at δ 84.4).[19] In the NOESY spectrum, cross-peaks between H-24, H-25, and H-3 indicated that the C-24 methyl group was axial and that the carboxy group was at C-23. As shown in Figure 2, key HMBC correlations were observed between the following protons and carbon atoms: H-3 and C-1, -5, -24; H-5 and C-7, -23, -24; H-18 and C-28; H-19 and C-29, -30; H-20 and C-21; H-24 and C-3, -5, -23; H-25 and C-1, -5, 9; H-26 and C-7, -9, -14; H-27 and C-8, -13, -14, -15; H-29 and C-19, -30, –OCH_3; H-30 and C-19; and H–OCH_3 and C-29. The absolute configuration at C-20 of **1** was determined to be *R* by comparing the chemical shift of the methyl group at δ_C 16.4 (C-30) with those of closely related compounds. In the case of a lupane-type compound bearing a hydroxymethyl, aldehyde, or carboxy group at C-20, the chemical shift of methyl (C-30) is in the range of 13.0–18.0 ppm (*R* configuration) and 7.0–10.5 ppm (*S* configuration).[20,21] Compound **1** was thus identified as (20*R*)-3α-hydroxy-29-dimethoxylupan-23,28-dioic acid, and this isolate is an aglycon of acankoreoside L reported previously.[22] The possibility of chemical changes during steaming process would be considered as an artifact formed from acankoreoside L. However, so far the study on this compound has not been reported.

Compound **2**[18] was obtained as an amorphous powder with the molecular formula $C_{42}H_{67}O_{15}$, based on HRESIMS (experimental

m/z $[M+H]^+$ 811.4444, calcd 811.4402). Acid hydrolysis liberated D-glucose, which was identified by GC.[23] Based on the ^1H and ^{13}C NMR data in Table 1, compound **2** was identified as a lupane-type triterpene glucoside, including two β-D-glucopyranosyl units. The large coupling constant (J = 8.0 Hz) of the anomeric proton at δ_H 6.36 and 5.02 in the ^1H NMR spectrum of **2** indicated the β configuration. Additionally, the ^1H NMR spectrum of **2** showed signals corresponding to an aglycone component: δ_H 0.88, 0.94, 1.20, 1.45, 1.70 (3H each, all s, H-27, 25, 26, 24, 30), 4.29 (1H, m, H-3), 4.84 and 4.70 (1H each, both br s, H-29). A comparison of the ^1H and ^{13}C NMR spectroscopic data for **2** with those of acankoreoside A (**4**)[15] revealed the lack of an α-L-rhamnopyranosyl moiety in **4**. The absolute configuration at C-3 (δ_C = 71.5) was determined to be the α orientation on the basis of the above manner. In the NOESY spectrum, cross-peaks between H-24, H-25, and H-3 indicated that the C-24 methyl group was axial, so that the carboxy group at C-4 was α-orientated. The structure was further confirmed by assignment of peaks in ^1H–^1H COSY, HMQC, and HMBC spectra. As shown in Figure 2, key HMBC correlations were observed between the following protons and carbon atoms: H-1″ and C-6′; H-6′ and C-1″; H-1′ and C-28; H-18, -22 and C-28; H-3 and C-1, -24; H-5 and C-23,- 24, -25; H-19 and C-29; H-24 and C-3, -5, -23; H-25 and C-1, -5, -9; H-27 and C-7, -9, -14; H-29 and C-20, -30; H-30 and C-19, -29. Thus, compound **2** was identified as 3α-hydroxylup-20(29)-ene-23,28-dioic acid 28-*O*-[β-D-glucopyranosyl-(1→6)-β-D-glucopyranosyl] ester.

To evaluate these compounds for anti-inflammatory activity, the isolates (**1**–**10**) were tested for the inhibitory effects on LPS-stimulated IL-12 p40 production in BMDCs, as described previously.[24] As a positive control, SB203580, an inhibitor of p38 MAP kinase, showed an inhibitory effect of 84.3% at a concentration of 50 μM. Among the ten compounds evaluated, compound **1** exhibited the most potent inhibitory activity, with 94.2% inhibition at 50 μM. At the same concentration, compounds **5** and **8** exhibited moderate effects with inhibition values of 57.6% and 62.6%, respectively, and the remaining compounds yielded negligible effects with inhibition values less than 50% (vehicle, 0.1% DMSO; Fig. 3A). Compound **1** also showed significant inhibition of IL-6 and TNF-α production in LPS-stimulated DC, with the inhibition values of 63.6% and 64.7%, respectively (Fig. 3B and C). Compound **8** also inhibited of TNF-α production by 67.9% in LPS-stimulated DC (Fig. 3C). To confirm the anti-inflammatory activity of the tested compounds, cell viability was simultaneously determined using a colorimetric MTT assay (Sigma, MO, USA). All of the isolates (**1**–**10**) exhibited little or no effect at the concentration tested (data not shown).

IL-12 is an inducible, heterodimeric disulfide-linked cytokine, composed of p35 and p40 subunits.[25,26] Expression of the p35 subunit is ubiquitous and the biological activity of IL-12 is regulated primarily through the induction of p40,[26] which is, in turn, regulated at the level of transcription. It should be noted that because IL-12 is a key cytokine in Th1-mediated autoimmune responses, down-regulation of IL-12 production by the isolates from steamed leaves of *A. koreanum* may ameliorate the autoimmune diseases.[27,28] Conversely, the IL-12 cytokine is a heterodimeric p70 molecule in which both subunits are co-expressed in the same cell to generate the bioactive form.[21] The regulation of IL-12 p40 production has been investigated at the transcriptional level and multiple regulatory elements have been identified, including NF-κB, Ets-2, and members of the interferon (IFN) regulatory factor (IRF)[26] and CCAAT enhancer binding protein (C/EBP) families.[28] Further studies are required to examine the anti-inflammatory action of the active lupane-type triterpene compounds (**1** and **8**). Additionally, LPS induces endotoxic (septic) shock through the overproduction of cytokines, such as IL-6 and TNF-α, by macrophages and dendritic cells. Thus, compound **1** may ameliorate both the symptom of and mortality associated with endotoxic shock.

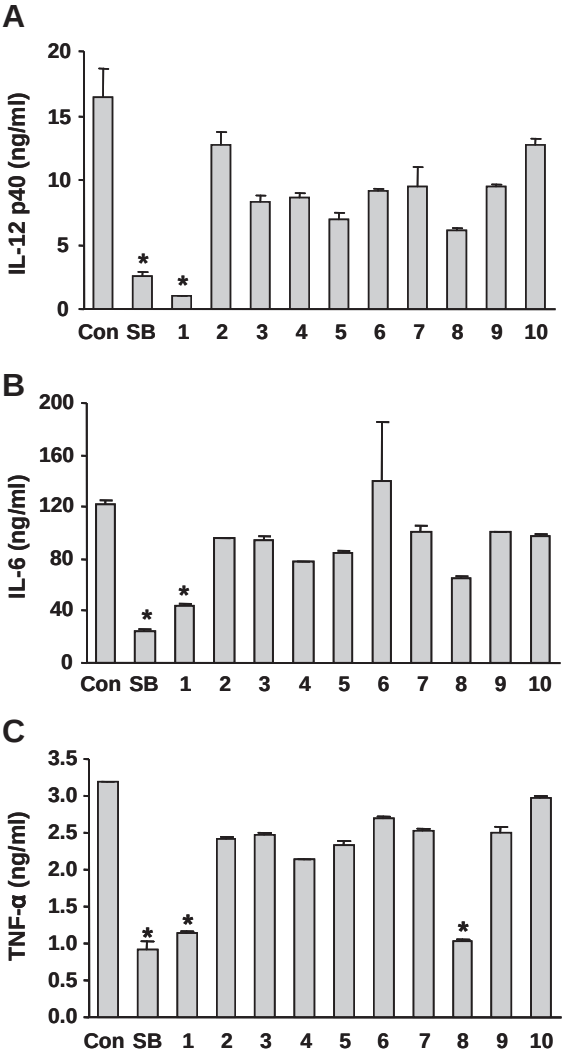

Figure 3. Effect of compounds (**1**–**10**) isolated from the steamed leaves of *A. koreanum* on IL-12 p40 (A), IL-6 (B), and TNF-α (C) production by LPS-stimulated BMDCs. DCs were treated with the compounds (50 μM) for 1 h before stimulation with LPS (10 ng/mL). Supernatants were harvested 18 h after stimulation. Concentrations of murine IL-12 p40, IL-6, and TNF-α in the culture supernatants were determined by ELISA. The data were presented as mean ± standard deviation (SD) of three independent experiments in triplicate. * P <0.05 versus DMSO-treated control (Con). SB, SB203580.

There have been some reports on describing the anti-inflammatory effects of terpenoids from *A. koreanum*. Triterpenoids, such as impressic acid (**8**), from the root of *A. koreanum* were found to inhibit NFAT transcription factor activity.[29] Also, acanthoic acid, a pimarane-type diterpene, exhibited potent inhibition of IL-8 secretion from TNF-α-stimulated human colon adenocarcinoma cells.[30] Acanthoic acid has also been shown to exert beneficial effects in experimental studies involving colitis.[31] Recent reports describe the anti-inflammatory activity of terpenoids from *A. koreanum*, which is mediated by various pro-inflammatory cytokines. These findings are consistent with the current study, in which, compound **1** was shown to inhibit the production of pro-inflammatory cytokines, IL-12, IL-6, and TNF-α in LPS-stimulated DCs. Thus, the present study suggests a potent possibility of contribution on anti-inflammatory properties by compound **1** isolated from the steamed leaves of *A. koreanum*.

Acknowledgments

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14. *Isolation*: The ethyl acetate extract (104 g) was separated on a silica gel column and eluted with a mixture of *n*-hexane/EtOAc/MeOH (gradient from 3:1:0, 1:1:0, 0:3:1, 0:2:1, 0:1:1 v/v), yielding 18 fractions, F1–F18. Fraction F5 (4.5 g) was further separated on an YMC RP-18 column using a MeOH/H_2O (4:1 v/v) as an eluent to yield five fractions, F5A–F5E. Further purification of the fractions F5B (146.2 mg) and F5C (280.5 mg) on silica gel columns with CH_2Cl_2/MeOH (9:1, v/v) as an eluent resulted in the isolation of compounds **5** (23.1 mg) and **8** (14.7 mg). Fraction F7 (5.2 g) was further separated on an YMC RP-18 column with MeOH/H_2O (4:1 v/v) to give eight fractions F7A–F7H. Fraction F7B (130.7 mg), F7D (40.1 mg), and F7F (86.4 mg) were purified on silica gel columns and eluted with *n*-hexane/EtOAc (4:1, v/v) to give **1** (20.0 mg), **3** (16.9 mg), and **7** (26.0 mg), respectively. Fraction F9 (6.0 g) was further separated on an YMC RP-18 column and eluted with a mixture of MeOH and H_2O (7:3 v/v), followed by purification using a silica gel column eluted with chloroform/MeOH/H_2O (7.5:2.5:0.2 v/v) to yield **2** (38 mg) and **9** (74.6 mg). The F15 fraction (6.0 g) was separated on an YMC RP-18 column using MeOH/H_2O (3:2 v/v) to yield seven fractions, F15A–F15G. F15B (531 mg), F15D (622 mg), and F15F (540 mg) were purified on silica gel column eluted with chloroform/MeOH/H_2O (7.5:2.5:0.2 v/v) to afford compounds **4** (100.3 mg), **6** (118.4 mg), and **10** (184.8 mg), respectively.
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18. (20*R*)-3α-Hydroxy-29-dimethoxylupan-23,28-dioic acid (**1**): white amorphous powder; $[\alpha]_D^{20}$ −8.2 (*c* 0.2, MeOH); ^{1}H NMR (C_5D_5N, 400 MHz) and ^{13}C NMR (C_5D_5N, 100 MHz), see Table 1; HRESIMS *m*/*z* 549.3809 $[M+H]^+$ (calcd for $C_{32}H_{53}O_7$, 549.3713). 3α-Hydroxylup-20(29)-ene-23,28-dioic acid 28-*O*-[β-D-glucopyranosyl-(1→6)-β-D-glucopyranosyl] ester (**2**): white amorphous powder; $[\alpha]_D^{20}$ −32.1 (*c* 0.1, MeOH); ^{1}H NMR (C_5D_5N, 400 MHz) and ^{13}C NMR (C_5D_5N, 100 MHz), see Table 1; HRESIMS *m*/*z* 811.4444 $[M+H]^+$ (calcd for $C_{42}H_{67}O_{15}$, 811.4402).
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23. *Acid hydrolysis and sugar determination of compound* **2**: Compound **2** (3.0 mg), dissolved in 1.0 M HCl (3.0 mL), was heated under reflux for 4 h. The reaction mixture was then concentrated to dryness under reduced pressure. The residue was extracted with EtOAc and H_2O (5 mL each, 3 times). Next, the sugar residue, obtained by concentration of the water layer, was dissolved in dry pyridine (0.1 mL) and L-cysteine methyl ester hydrochloride in pyridine (0.06 M, 0.1 mL) was added to the solution. After heating the reaction mixture at 60 °C for 2 h, 0.1 mL of trimethylsilylimidazole was added. Heating at 60 °C was continued for another 2 h, and the reaction mixture was evaporated to give a dried product, which was then partitioned between hexane and H_2O. The hexane layer was analyzed by GC (Shimadzu-2010, Tokyo, Japan) using a DB-05 capillary column (0.5 mm ID × 30 m length; column temperature: 210 °C; detector temperature: 300 °C; injector temperature: 270 °C; He gas flow rate: 30 mL/min). The peak of the hydrolysate was detected at 14.12 min for D-glucose. The retention times of the authentic samples (Sigma-Aldrich), after being treated in a similar manner, were 14.12 min (D-glucose), and 14.25 min (L-glucose).
24. *Cell cultures and measurements of cytokine production*: BMDCs were grown from wild-type C57BL/6 mice (Taconic Farm, NY, USA). Briefly, bone marrow from the tibia and femur was obtained by flushing with DMEM and bone marrow cells were cultured in RPMI 1640 medium containing 10% heat-inactivated fetal bovine serum (FBS; Gibco, NY, USA), 50 μM of 2-ME, and 2 mM of glutamine, supplemented with 3% J558L hybridoma cell culture supernatant containing granulocyte-macrophage colony-stimulating factor (GM-CSF). The culture medium containing GM-CSF was replaced every other day. At day 6 of culture, non-adherent cells and loosely adherent DC aggregates were harvested, washed, and resuspended in RPMI 1640, supplemented with 5% FBS. DCs were incubated in 48-well plates at a density of 1×10^5 cells/0.5 mL and then treated with the isolated compounds at the concentration of 50 μM for 1 h before stimulation with 10 ng/mL of LPS from *Salmonella minnesota* (Alexis, NY, USA). Supernatants were harvested 16 h after stimulation. Concentrations of murine IL-12 p40, IL-6, and TNF-α in the culture supernatants were determined by ELISA (BD PharMingen, CA, USA) according to the manufacturer's protocol. All experiments were performed at least three times. Data are presented as the mean and the standard deviation (SD) of three independent experiments. Statistical significance was determined with Student's *t* test. *P* values of <0.05 were deemed to indicate statistical.
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