Anti-inflammatory Flavonoid C-Glycosides from Piper aduncum Leaves

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Anti-inflammatory Flavonoid C-Glycosides from *Piper aduncum* Leaves

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Key words
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- Piperaceae
- Flavonoid C-glycoside
- IL-12 p40
- IL-6
- TNF-α
- LPS

Abstract

Four new compounds, acacetin 8-C-[β-D-apiofuranosyl-(1→2)-β-D-glucopyranoside] (**1**), 7-methoxyacacetin 8-C-[β-D-apiofuranosyl-(1→3)-β-D-glucopyranoside] (**2**), 7-methoxyacacetin 8-C-[β-D-glucopyranosyl-(1→2)-β-D-glucopyranoside] (**3**), and 4‴-O-acetylacacetin 8-C-[α-L-rhamnopyranosyl-(1→2)-β-D-glucopyranoside] (**4**), along with ten known compounds (**5**–**14**), were isolated from *Piper aduncum* leaves. The effects of these compounds on lipopolysaccharide-induced expression of the proinflammatory cytokines IL-12 p40, IL-6, and TNF-α in bone marrow-derived dendritic cells were evaluated. Compounds **2**, **3**, **6**, **8**, **9**, and **11**–**13** inhibited the production of both IL-12 p40 and IL-6, with IC_{50} values ranging from 0.35 ± 0.01 to 1.40 ± 0.04 µM and 1.22 ± 0.02 to 3.79 ± 0.10 µM, respectively. Compounds **5** and **10** only showed strong inhibition effects on the production of IL-12 p40, with IC_{50} values of 2.76 ± 0.08 and 0.39 ± 0.05 µM, respectively. However, all compounds showed weak activity or no activity on TNF-α production at the tested concentrations.

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Introduction

The usual outcome of acute inflammation is successful resolution and repair of tissue damage. However, it has also been recognized as the central cause of many prevalent diseases, including arthritis, periodontal disease, cardiovascular diseases, cancer, and Alzheimer's disease [1]. Cytokines are primarily involved in host responses to infection, immune responses, inflammation, and trauma. Several cytokines, called proinflammatory cytokines, tend to increase disease symptoms and produce fever, inflammation, tissue destruction, shock, and even death when they are administered to humans. Therefore, compounds that could block or inhibit the production of proinflammatory cytokines might be beneficial anti-inflammatory agents [2].

The genus *Piper* (Piperaceae) is well known for producing a large number of physiologically active compounds and is widely used in folklore medicine in the West Indies and Latin America. The Piperaceae family, in which the genus *Piper* belongs, comprises approximately 2000 species distributed in the tropical regions of the world [3,4]. *Piper aduncum* L. is widely used in folk medicine to treat stomachaches (Jamaica) and as an anti-inflammatory, diarrhea (Peru), and antisteptic to heal wounds (Brazil and Papua New Guinea) [3–5]. Previous phytochemical studies of *P. aduncum* reported the isolation of dihydrochalcones, chalcones, flavanones, chromene, phenylpropanoids, and benzoic acid derivatives [6–8]. Several pharmacological effects of *P. aduncum* extracts have been demonstrated, including antileishmanial, antibacterial, cytotoxic, and antifungal activities [6,9]. Furthermore, some of the isolated compounds have been shown to be active against promastigote and intracellular amastigotes, causing damaging effects on DNA, and antileishmanial, antimicrobial, molluscicidal, antitumor, and antifungal activities [8,10,11]. However, anti-inflammatory studies on *P. aduncum* are limited. To date, only an essential oil (dillapiole) of this plant was investigated for anti-inflammatory activity in the carrageenan-induced rat paw edema model [12].

As a part of our ongoing investigations on the chemical constituents of medicinal plants with

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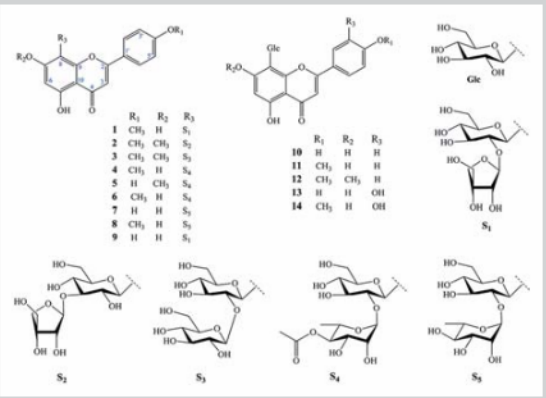

Fig. 1 Chemical structures of compounds **1**–**14** isolated from *P. aduncum* leaves. (Color figure available online only.)

anti-inflammatory activities, we found that the methanolic extract of *P. aduncum* showed significant anti-inflammatory effects *in vitro* with an inhibition value of 65.9% (20.0 µg/mL). In the present study, we report the isolation and structural elucidation of four new flavonoids (**1**–**4**) and ten known compounds (**5**–**14**) from *P. aduncum* leaves (**Fig. 1**). Also, the anti-inflammatory effects of these compounds on lipopolysaccharide (LPS)-induced expression of the proinflammatory cytokines IL-12 p40, IL-6, and TNF-α in bone marrow-derived dendritic cells (BMDCs) were evaluated.

Results and Discussion

A MeOH extract of the leaves of *P. aduncum* was suspended in H_2O and successively partitioned with CH_2Cl_2 and EtOAc. The EtOAc fraction was separated by various chromatographic experiments to afford 14 C-glycosyl flavones. Ten known compounds including isospinosin (**5**) [13], 2-O-β-D-glucosyl-8-C-β-D-glucosyl-4′-O-methylapigenin (**6**) [14], apigenin-8-C-neohesperidoside (**7**) [15], 2″-O-α-rhamnosyl-4′-O-methyl vitexin (**8**) [16], ficuflavoside (8-C-(2″-O-β-D-apiofuranosyl)-β-D-glucopyranosyl apigenin) (**9**) [17], apigenin 8-C-β-glucopyranoside (**10**) [18], cytisoside (**11**) [19], isoembigenin (**12**) [20], orientin (**13**) [21], and 4′-O-methylorientin (**14**) [22] were identified by NMR analysis as well as comparison with previous reports (**Fig. 1**). The C-glycosyl flavones were isolated for the first time from the leaves of this plant, indicating that these compounds could be considered chemotaxonomic markers for the identification of *P. aduncum*.

Compound **1** was obtained as a yellow amorphous powder. The HR-ESI-MS of **1** was consistent with the molecular formula of $C_{27}H_{30}O_{14}$. The ^{13}C NMR and DEPT spectra (**Table 1**) revealed 27 carbon signals, including 15 signals that were assigned to a flavone aglycon, as well as two anomeric and nine other oxygenated carbon signals, of which three methylenes were ascribable to C-4‴ of an apiofuranosyl unit, and C-6″ of a glucopyranosyl unit, respectively. Assignment of the protons and carbons in the aglycone moiety was done based on 2D spectra. All of the sugar proton resonances were assigned by a COSY experiment, and the corresponding ^{13}C resonances were then identified by the HMQC. The COSY spectra of compound **1** indicated two individual correlation systems of sugar units [H-1″ (δ_H 4.99)/H-2″ (δ_H 4.24)/H-3″ (δ_H 3.65)/H-4″ (δ_H 3.70)/H-5″ (δ_H 3.46)/H-6″ (δ_H 3.84 and 4.01)] and [H-1‴ (δ_H 5.16)/H-2‴ (δ_H 3.78)]. The connectivity of these fragments was determined through HMBC. The downfield shift was observed for C-2″ (δ_C 77.4), as well as HMBC correlations of H-1‴ (δ_H 5.16) with C-2″ (δ_C 77.4), and H-2″ (δ_H 4.24) with C-1‴ (δ_C 111.1), C-1″ (δ_C 73.7), and C-8 (δ_C 105.4), demonstrating the (1 → 2) linkage between the apiosyl and glucosyl units in **1**. Furthermore, acid hydrolysis of **1**, followed by TLC, GC analysis, and a comparison with authentic D-apiose, confirmed the presence of a D-apiose moiety in that compound (see Materials and Methods). A C-8 substituted acacetin structure was suggested by the ^1H (δ_H 6.25, s, H-6) and ^{13}C NMR (δ_C 105.4 for C-8 and 99.3 for C-6) signals characteristic of C-8-glycosylated flavones [23]. In addition, the HMBC correlations of H-1″ (δ_H 4.99) with C-7 (δ_C 164.1), C-8 (δ_C105.4), and C-9 (δ_C 158.4) confirmed that the disaccharide chain was bonded by a C-glycosidic linkage at the 8-position. Accordingly, compound **1** was identified as acacetin 8-C-[β-D-apiofuranosyl-(1 → 2)-β-D-glucopyranoside].

Compound **2** was obtained as a yellow amorphous powder. The HR-ESI-MS was consistent with the molecular formula of $C_{28}H_{32}O_{14}$. The ^1H and ^{13}C NMR spectra (**Table 1**) of **2** were similar to those of **1**. The differences between the spectra of compounds **1** and **2** were with regard to the presence of two methoxy groups and the linkage between two sugar units, glucose and apiose. The downfield shift of C-3″ (δ_C 84.8) and the upfield shift of C-2″ (δ_C 70.9) of the glucopyranosyl unit, in comparison with those observed in **1**, were indicative of a glycosidation at C-3″. In addition, the interglycosidic linkage of the apiofuranosyl moiety in **2** was established unambiguously by the HMBC spectrum to be at the C-3″ position of the glucopyranosyl unit based on the cross-peak between H-1‴ (δ_H 5.20) with C-3″ (δ_C 84.8) and H-3″ (δ_H 3.45) with C-1‴ (δ_C 109.8). The position of the disaccharide chain was determined as C-8 based on the HMBC correlations of the anomeric proton H-1″ (δ_H 4.76) of glucosyl with C-7 (δ_C 164.0), C-8 (δ_C 105.2), and C-9 (δ_C 155.8) of an aglycon. Two methoxy groups were located at C-7 (δ_C 164.0) and C-4′ (δ_C 163.0), as indicated by the HMBC correlations of a methoxy proton at δ_C 3.89 with C-7 (δ_C 164.0), and at δ_H 3.87 with C-4′ (δ_C 163.0), respectively. In addition, acid hydrolysis of **2**, followed by TLC, GC analysis, and a comparison with authentic D-apiose, confirmed the presence of a D-apiose moiety in **2** (see Materials and Methods). Therefore, the structure of **2** was identified as 7-methoxyacacetin 8-C-[β-D-apiofuranosyl-(1 → 3)-β-D-glucopyranoside].

Compound **3** was obtained as a yellow powder. The HR-ESI-MS spectra of **3** indicated the molecular formula of $C_{29}H_{34}O_{15}$. The ^1H and ^{13}C NMR spectra of **3** showed the similar aglycon of **2** as 7-methoxyacacetin (**Table 1**). The presence of a glucopyranosyl unit, instead of a terminal apiofuranosyl unit, was the main difference in the structure of **3**. Two individual correlations for the spin systems of H-1″/H-2″/H-3″/H-4″/H-5″/H-6″ and H-1‴/H-2‴/H-3‴/H-4‴/H-5‴/H-6‴ were observed in the COSY spectra of **3**, which supports the backbone proton assignments of the two sugar units. HMBC correlations of the anomeric proton H-1‴ (δ_H 3.87)/C-2″ (δ_C 81.3) and proton H-2″ (δ_H 4.05)/C-1‴ (δ_C 105.3), as well as the downfield shift of C-2″ (δ_C 81.3), suggested the structure of a β-D-glucopyranosyl (1 → 2) β-D-glucopyranosyl disaccharide. The position of the sugar moiety in **3** through a C-C linkage was identified as C-8 based on the HMBC correlations of the anomeric proton H-1″ (δ_H 4.83) with C-8 (δ_C 105.3), C-7 (δ_C 164.0), and C-9 (δ_C 155.9). Acid hydrolysis experiments con-

Table 1 ^{1}H and ^{13}C NMR spectroscopic data for compounds **1–3**.

Pos.	1		2		3	
	δ_H[a, c] (mult., *J* in Hz)	δ_C[a, d]	δ_H[b, c] (mult., *J* in Hz)	δ_C[b, d]	δ_H[b, c] (mult., *J* in Hz)	δ_C[b, d]
2	–	166.1	–	164.5	–	164.3
3	6.55 (s)	104.0	6.92 (s)	103.8	6.88 (s)	103.9
4	–	184.0	–	182.9	–	182.8
5	–	162.5	–	162.0	–	162.0
6	6.25 (s)	99.3	6.55 (s)	95.6	6.50 (s)	95.7
7	–	164.1	–	164.0	–	164.0
8	–	105.4	–	105.2	–	105.3
9	–	158.4	–	155.8	–	155.9
10	–	105.7	–	105.6	–	104.8
1′	–	124.7	–	123.7	–	123.8
2′	8.01 (d, 8.4)	129.8	8.16 (d, 9.0)	129.5	8.15 (d, 8.4)	129.5
3′	7.01 (d, 9.0)	115.5	7.09 (d, 9.0)	115.0	7.09 (d, 9.0)	115.0
4′	–	164.3	–	163.0	–	162.9
5′	7.01 (d, 9.0)	115.5	7.09 (d, 9.0)	115.0	7.09 (d, 9.0)	115.0
6′	8.01 (d, 8.4)	129.8	8.16 (d, 9.0)	129.5	8.15 (d, 8.4)	129.5
7-OCH_3	–	–	3.89 (s)	57.1	3.84 (s)	57.1
4′-OCH_3	3.85 (s)	56.0	3.87 (s)	56.1	3.83 (s)	56.1
1″	4.99 (d, 10.2)	73.7	4.76 (d, 10.2)	73.8	4.83 (d, 9.6)	71.9
2″	4.24 (t, 9.0)	77.4	3.99*	70.9	4.05 (t, 9.0)	81.3
3″	3.65 (t, 9.0)	80.9	3.45 (t, 8.4)	84.8	3.48*	78.8
4″	3.70 (t, 9.0)	72.2	3.49 (m)	69.3	3.45*	70.6
5″	3.46 (m)	82.8	3.29 (m)	82.3	3.23 (m)	82.4
6″	3.84*	63.0	3.59*	61.4	3.54 (m)	61.4
	4.01 (dd, 1.2, 12.0)		3.77*		3.73 (m)	
1‴	5.16 (br s)	111.1	5.20 (d, 1.8)	109.8	3.87*	105.3
2‴	3.78 (br s)	77.8	3.71*	76.4	2.71 (m)	75.0
3‴	–	80.6	–	79.5	2.40 (m)	76.6
4‴	2.55 (d, 9.6)	74.7	3.55*	74.0	2.87*	69.8
	3.15 (d, 9.6)		4.01*			
5‴	3.28 (d, 12.0)	65.9	3.36 (m)	64.0	2.87*	76.7
	3.40 (d, 12.0)					
6‴	–	–	–	–	3.06 (m)	60.7
					3.12 (m)	

[a] CD_3OD; [b] DMSO-d_6; [c] 600 MHz; [d] 150 MHz. *Overlapped signals. Assignments were done by DEPT, HMQC, HMBC, and COSY

firmed the presence of D-glucose in **3**. This conclusion was further supported by TLC, GC analysis, and comparisons with an authentic D-glucose sample. Thus, compound **3** was identified as 7-methoxyacacetin 8-C-[*β*-D-glucopyranosyl-(1→2)-*β*-D-glucopyranoside].

Compound **4** was obtained as yellow powder. The HR-ESI-MS spectra of **4** indicated the molecular formula of $C_{30}H_{34}O_{15}$. The ^{1}H NMR spectra of **4** showed signals of six aromatic protons, including two singlet signals, four aromatic protons, one methoxy group, two methyl groups, and two anomeric protons, together with carbinol protons, suggesting the presence of two sugar units. The ^{13}C NMR spectra displayed 30 carbon resonance signals, including 15 carbons belonging to an aglycon moiety, 12 carbons of sugar units, 1 methoxy carbon, and 2 carbons of an acetyl group.

The ^{1}H and ^{13}C NMR data showed doubling of the signals due to restricted rotation (**Table 2**). The rotameric signals show almost the same ^{1}H NMR signal intensity of 3 : 2. Hence, the rotameric partners of **4** could also be observed in the ^{13}C NMR spectrum. Because of the similar ^{1}H and ^{13}C resonance signals between **4** and **1**, the aglycon of **4** was identified as acacetin. The presence of glucose and rhamnose units was observed in the ^{1}H, ^{13}C, and COSY spectra of **4**. Furthermore, acid hydrolysis of **4**, followed by TLC, GC analysis, and a comparison with authentic L-rhamnose, confirmed the presence of an L-rhamnose moiety in **4** (see Materials and Methods). The HMBC correlations of the anomeric proton of rhamnose H-1‴ (δ_H 5.42/5.30) and C-2″ (δ_C 76.2/76.1) of glucose, proton H-2″ (δ_H 4.20) of glucose and C-1‴ (δ_C 101.3) of rhamnose, and C-8 (δ_C 105.9/105.7) of the A ring suggested the structure of an α-L-rhamnopyranosyl (1→2) *β*-D-glucopyranosyl disaccharide. The position of an acetyl group at C-4‴ was confirmed by the cross-peak observed between H-4‴ (δ_H 4.53/4.61) and C-1⁗ (δ_C 172.5), as well as the downfield shift of C-4‴ (δ_C 75.2). The C-8 substituted structure was determined by the HMBC correlation of H-1″ (δ_H 5.05) of the glucopyranosyl unit with C-8 (105.9/105.7), C-7 (δ_C 164.4/164.3), and C-9 (δ_C 157.7/156.2) of the aglycon. Therefore, compound **4** was assigned as 4‴-O-acetylacacetin 8-C-[α-L-rhamnopyranosyl-(1→2)-*β*-D-glucopyranoside].

The isolated compounds were assessed for anti-inflammatory activity on LPS-induced expression of the proinflammatory cytokines IL-12 p40, IL-6, and TNF-α by BMDCs (see Materials and Methods). Many natural products have been shown to exhibit anti-inflammatory activities by inhibiting the production of proinflammatory cytokines, including IL-12 p40, IL-6, and TNF-α, which play a crucial role in host defenses and inflammation [24]. Thus, agents that block the excessive production of these cytokines might be candidates for use in the treatment of inflam-

Table 2 ^{1}H and ^{13}C NMR spectroscopic data for compound **4** in CD_3OD.

Pos.	Aglycon		No.	Sugar moiety	
	δ_H[a] (mult., *J* in Hz)	δ_C[b]		δ_H[a] (mult., *J* in Hz)	δ_C[b]
2	–	166.0	1″	5.05 (d,	73.7
		165.3		9.6)	75.1
3	6.60 (s)	104.0	2″	4.20 (dd,	76.2
				7.8, 9.6)	76.1
4	–	184.0	3″	3.67 (m)	81.7
					81.4
5	–	162.9	4″	3.68*	72.3
		162.7			72.0
6	6.28 (s)	99.8	5″	3.49 (m)	83.0
	6.23 (s)	101.1			82.9
7	–	164.4	6″	4.01*	63.0
		164.3		3.84*	62.5
8	–	105.9	1‴	5.42 (br s)	101.3
		105.7		5.30 (br s)	
9	–	157.7	2‴	3.57 (t, 9.6)	71.7
		156.2		3.67*	71.5
10	–	105.5	3‴	3.48 (dd, 3.6, 9.6)	69.9
				2.97 (dd, 3.6, 9.6)	
1′	–	124.4	4‴	4.53 (t, 9.6)	75.2
				4.61 (t, 9.6)	
2′	8.03 (d, 9.0)	129.9	5‴	2.31 (m)	67.2
	7.85 (d, 8.4)	129.3		2.05 (m)	66.7
3′	7.03 (d, 9.0)	115.6	6‴	0.67 (d, 6.0)	17.9
	6.98 (d, 8.4)	115.5		0.50 (d, 6.0)	17.8
4′	–	164.2	1⁗	–	172.5
		164.1			
5′	7.03 (d, 9.0)	115.6	2⁗	2.02 (s)	20.9
	6.98 (d, 8.4)	115.5		1.91 (s)	20.9
6′	8.03 (d, 9.0)	129.9			
	7.85 (d, 8.4)	129.3			
4′-OCH_3	3.86 (s)	56.0			
	3.84 (s)				

[a] 600 MHz; [b] 150 MHz. *Overlapped signals. Assignments were done by DEPT, HMQC, HMBC, and COSY

matory diseases. BMDCs were pretreated with the isolated compounds for 18 h, and then viability was measured using the MTT assay. At a concentration of 5.0 µM, compound **4** showed toxicity. Other compounds did not exhibit significant cytotoxic effects (data not shown). Thus, the effects of compounds **1**–**3** and **5**–**14** on LPS-stimulated production of proinflammatory cytokines, including IL-12 p40, IL-6, and TNF-α, in BMDCs were evaluated at concentrations of 0.2, 0.5, 1.0, 2.0, and 5.0 µM. SB203580, a bicyclic imidazole compound and an inhibitor of p38 mitogen-activated protein (MAP) kinase [25], was used as a positive control, with IC_{50} values of 5.00 ± 0.16, 3.50 ± 0.12, and 7.20 ± 0.13 µM for IL-12 p40, IL-6, and TNF-α, respectively. Compounds **2, 3, 5, 6**, and **8**–**13** potently inhibited IL-12 p40 production in LPS-stimulated BMDCs with IC_{50} values ranging from 0.35 ± 0.01 to 2.76 ± 0.08 µM (**Table 3** and **Fig. 2A**). Among them, compound **9** showed the greatest inhibitory activity, up to 83.74% at a concentration of 5.0 µM and 42.66% after dilution to 0.2 µM. Other compounds were weak or inactive (**Fig. 2A**).

To investigate the effects of the tested compounds on other proinflammatory cytokines, their effects on IL-6 production were measured. Compounds **2, 3, 6, 8, 9**, and **11**–**13** showed potent inhibition of IL-6 production, with IC_{50} values ranging from 1.22 ± 0.02 to 3.79 ± 0.10 µM (**Table 3**). Compound **3** showed the greatest inhibition of IL-6 production, with 61.63% at a concentration of 5.0 µM and 37.47% when diluted to 0.2 µM (**Fig. 2B**). Compounds **5** and **10** showed moderate activity, with inhibition percentages ranging from 4.11% to 39.11% at the tested concentrations. Other compounds were inactive. In addition, the isolated compounds were also evaluated for TNF-α production. However, all of them showed weak activity or were inactive at the tested concentrations from 0.2 to 5.0 µM (**Fig. 2C**). As shown in **Table 3**, compounds **2, 3, 6, 8, 9**, and **11**–**13** inhibited the production of both IL-12 p40 and IL-6. Compounds **5** and **10** showed potent inhibitory effects only on the production of IL-12 p40. Compounds **1, 7**, and **14** were inactive in terms of production of both IL-12 p40 and IL-6.

Flavonoids, one of the most widespread groups of natural products, possess a wide variety of pharmacological and biochemical properties, including antioxidant, antimicrobial, antiallergenic, and anti-inflammatory effects. Among them, flavonoid C-glycosides such as vitexin, isoorientin, orientin, and isovitexin were shown to have significant anti-inflammatory activity [26, 27]. Therefore, our results were in agreement with those from previous studies. This study has provided evidence of the anti-inflammatory properties of flavonoid C-glycosides that could be promising in the development of anti-inflammatory agents.

Materials and Methods

General experimental procedures

Optical rotation was recorded on a JASCO DIP-370 automatic digital polarimeter. The NMR spectra were measured using a JEOL ECA 600 spectrometer with TMS as the internal standard. The ESI mass spectra were performed on an AGILENT 1100 LC-MSD trap spectrometer. The HR-ESI-MS were obtained from an Agilent 6530 Accurate-Mass Q-TOF LC/MS system. GC used an instrument (SHIMADZU GC-14B). Silica gel (70–230, 230–400 mesh, Merck) and YMC RP-18 resins (75 µm, Fuji Silysia Chemical Ltd.) were used as absorbents in the CC. TLC plates (silica gel 60 F_{254} and RP-18 F_{254}, 0.25 µm, Merck) were purchased from Merck KGaA. Spots were detected under UV radiation (254 and 365 nm) and by spraying the plates with 10% H_2SO_4, followed by heating with a heat gun. Other chemical reagents and standard compounds were purchased from Sigma-Aldrich.

Plant material

The leaves of *P. aduncum* were collected from Coblong-Bandung, West Java, Indonesia in September 2014 and taxonomically identified by the staff at the Herbarium Laboratory, Department of Biology, School of Life Sciences and Technology, Bandung Institute of Technology, Bandung, West Java, Indonesia. A voucher specimen (BIT-1481) was deposited at the Herbarium of the Department of Biology, School of Life Sciences and Technology, Bandung Institute of Technology.

Table 3 Anti-inflammatory effects of selected compounds on LPS-stimulated BMDCs.

Compounds	IC_{50} values (μM)	
	IL-12 p40	IL-6
2	0.87 ± 0.06	1.54 ± 0.09
3	0.56 ± 0.01	1.22 ± 0.02
5	2.76 ± 0.08	> 5.0
6	0.41 ± 0.02	3.17 ± 0.23
8	1.40 ± 0.04	2.95 ± 0.17
9	0.35 ± 0.01	3.79 ± 0.10
10	0.39 ± 0.02	> 5.0
11	1.26 ± 0.02	2.07 ± 0.21
12	0.56 ± 0.03	2.27 ± 0.05
13	0.39 ± 0.05	3.71 ± 0.04
SB203580 [a]	5.00 ± 0.16	3.50 ± 0.12

[a] SB203580 was used as a positive control

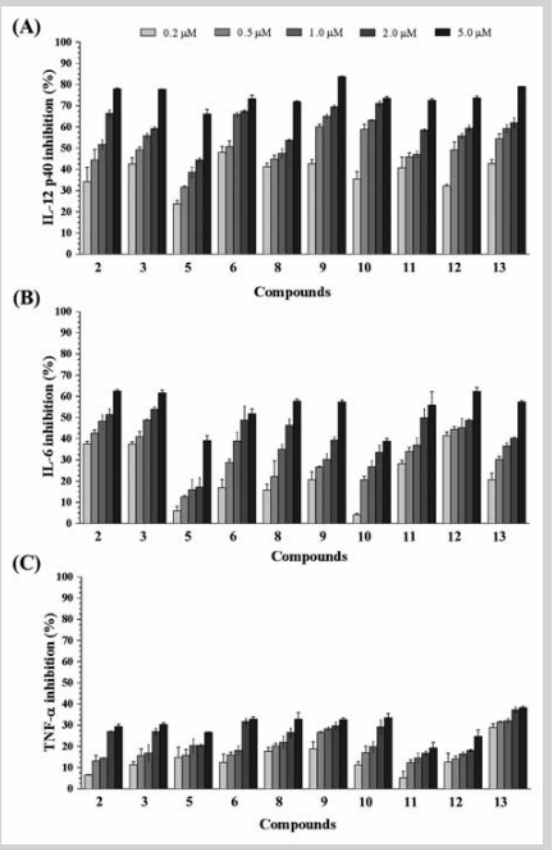

Fig. 2 **A** Effect of compounds **2, 3, 5, 6**, and **8–13** on IL-12 p40 production by LPS-stimulated BMDCs. **B** Effect of compounds **2, 3, 5, 6**, and **8–13** on IL-6 production by LPS-stimulated BMDCs. **C** Effect of compounds **2, 3, 5, 6**, and **8–13** on TNF-α production by LPS-stimulated BMDCs. The data are presented as inhibition rate (%). Data represent the mean ± SD of at least three independent experiments performed in triplicate. Statistical significance is indicated as determined by one-way ANOVA followed by Dunnett's multiple comparison test, $p < 0.05$, using GraphPad Prism 6.

Extraction and isolation

The dried leaves of *P. aduncum* (3.2 kg) were extracted with 70% ethanol (10 L × 3 times) under reflux condition. Evaporation of the solvent under reduced pressure gave the crude extract (379 g), which was suspended in H_2O and successively separated with CH_2Cl_2 and EtOAc to yield CH_2Cl_2 (93 g) and EtOAc extracts (27 g).

The EtOAc extract (27 g) was fractionated on a silica gel CC eluting with gradient solvent systems of CH_2Cl_2-MeOH (0–100% MeOH, stepwise) to obtain six fractions (C.1 through C.6). Compound **12** (20 mg) was isolated from fraction C.3 by YMC reverse-phase (RP)-18 CC using MeOH-H_2O (1/1, v/v) as the eluent, and further purified by YMC RP-18 CC eluting with acetone-H_2O (1/2, v/v). Fraction C.4 was separated on silica gel CC eluting with CH_2Cl_2-MeOH-H_2O (4/1/0.1, v/v/v) to give six subfractions (C.4.1 through C.4.6). Compounds **2** (10 mg), **3** (70 mg), and **11** (38 mg) were separated from fraction C.4.2 by silica gel CC using CH_2Cl_2-MeOH (6/1, v/v) and further purified by Sephadex® LH-20 CC using MeOH-H_2O (1/1, v/v) as the eluent. Fraction C.4.4 was fractionated on YMC RP-18 CC eluting with MeOH-H_2O (1/2, v/v) to obtain three fractions (C.4.4.1 through C.4.4.3). Compounds **1** (12 mg) and **14** (10 mg) were obtained from fraction C.4.4.2 by Sephadex® LH-20 CC eluting with MeOH-H_2O (1/1, v/v). Fraction C.5 was isolated on YMC RP-18 CC eluting with MeOH-H_2O (1/2, v/v) to obtain four fractions (C.5.1 through C.5.4). Similarly, compound **13** (40 mg) was afforded from the fraction C.5.1 by Sephadex® LH-20 CC using MeOH-H_2O (1/1, v/v) as the eluent. Compounds **5** (8 mg), **9** (9 mg), and **10** (7 mg) were isolated from fraction C.5.2 by Sephadex® LH-20 CC eluting with MeOH-H_2O (1/1, v/v). And finally, fraction C.5.4 were separated by Sephadex® LH-20 CC using MeOH-H_2O (1/1, v/v) and further purified by silica gel CC using CH_2Cl_2-MeOH-H_2O (4/1/0.1, v/v/v) as eluents to afford compounds **4** (20 mg), **6** (10 mg), **7** (15 mg), and **8** (100 mg).

Acacetin 8-C-[β-D-apiofuranosyl-(1 → 2)-β-D-glucopyranoside] (**1**): Yellow amorphous powder; $[\alpha]_D^{25}$: −65.3° (*c* 0.15, MeOH); IR (KBr) ν_{max}: 3367, 2925, 1650, 1602, 1561, 1496, 1070, and 1000 cm^{-1}; UV (MeOH) λ_{max} (log ε) 329 (4.39), 270 (4.31) nm; HR-ESI-MS *m/z*: 579.1726 $[M + H]^+$ (Calcd. $C_{27}H_{31}O_{14}$ for 579.1714) and 601.1544 $[M + Na]^+$ (Calcd. $C_{27}H_{30}NaO_{14}$ for 601.1533); ^1H NMR (600 MHz, CD_3OD) and ^{13}C NMR (150 MHz, CD_3OD) are given in **Table 1**.

7-Methoxyacacetin 8-C-[β-D-apiofuranosyl-(1 → 3)-β-D-glucopyranoside] (**2**): Yellow amorphous powder; $[\alpha]_D^{25}$: −75.8° (*c* 0.15, MeOH); IR (KBr) ν_{max}: 3375, 2931, 1650, 1602, 1570, 1523, 1459, 1350, and 1055 cm^{-1}; UV (MeOH) λ_{max} (log ε) 331 (4.42), 272 (4.32) nm. HR-ESI-MS *m/z*: 615.1685 $[M + Na]^+$ (Calcd. $C_{28}H_{32}NaO_{14}$ for 615.1684); ^1H NMR (600 MHz, DMSO-d_6) and ^{13}C NMR (150 MHz, DMSO-d_6) are given in **Table 1**.

7-Methoxyacacetin 8-C-[β-D-glucopyranosyl-(1 → 2)-β-D-glucopyranoside] (**3**): Yellow powder; $[\alpha]_D^{25}$: −49.7° (*c* 0.1, MeOH); IR (KBr) ν_{max}: 3369, 2925, 1651, 1610, 1570, 1480, 1100, and 1040 cm^{-1}; UV (MeOH) λ_{max} (log ε) 332 (4.43), 268 (4.27) nm. HR-ESI-MS *m/z*: 623.1979 $[M + H]^+$ (Calcd. $C_{29}H_{35}O_{15}$ for 623.1970), 645.1800 $[M + Na]^+$ (Calcd. $C_{29}H_{34}NaO_{15}$ for 645.1790); ^1H NMR (600 MHz, DMSO-d_6) and ^{13}C NMR (150 MHz, DMSO-d_6) are given in **Table 1**.

4‴-O-Acetylacacetin 8-C-[α-L-rhamnopyranosyl-(1 → 2)-β-D-glucopyranoside] (**4**): Yellow powder; $[\alpha]_D^{25}$: −56.0° (*c* 0.15, MeOH); IR (KBr) ν_{max}: 3414, 2936, 1704, 1650, 1570, 1490, 1446, 1350, and 1045 cm^{-1}; UV (MeOH) λ_{max} (log ε) 334 (4.44), 271 (4.28)

nm. HR-ESI-MS *m/z*: 635.1975 $[M + H]^+$ (Calcd. $C_{30}H_{35}O_{15}$ for 635.1970) and 657.1794 $[M + Na]^+$ (Calcd. $C_{30}H_{34}NaO_{15}$ for 657.1790); ^1H NMR (600 MHz, CD_3OD) and ^{13}C NMR (150 MHz, CD_3OD) are given in **Table 2**.

Acid hydrolysis and sugar identification

Each compound (2.0 mg) was dissolved in 1.0 N HCl (dioxane/H_2O, 1 : 1, v/v, 1.0 mL) and then heated to 80 °C in a water bath for 3.5 h. The acidic solution was neutralized with silver carbonate and the solvent thoroughly driven out under N_2 gas overnight. After extraction with ethyl acetate, the aqueous layer was first TLC analyzed [individual and co-analysis with standard sample: glucose (R_f 0.29), rhamnose (R_f 0.57), and apiose (R_f 0.59); $CHCl_3$/MeOH/H_2O, 3 : 2 : 0.3] and then concentrated to dryness using N_2 gas. The residue was dissolved in 0.1 mL of dry pyridine, and then L-cysteine methyl ester hydrochloride in pyridine (0.06 M, 0.1 mL) was added to the solution. The reaction mixture was heated at 60 °C for 2 h, and 0.1 mL of trimethylsilylimidazole solution was added, followed by heating at 60 °C for 1.5 h. The dried product was partitioned with *n*-hexane and H_2O (0.1 mL, each), and the organic layer was analyzed by GC; Column: SPB-1 (0.25 mm × 30 m); detector FID, column temp 210 °C, injector temperature 270 °C, detector temperature 300 °C, carrier gas He. The absolute configuration of the monosaccharide was confirmed to be D-glucose, L-rhamnose, and D-apiose by comparison of the retention time of the monosaccharide derivative (t_R14.11, 4.50, and 4.67 min, respectively) with that of authentic sugar derivative samples prepared in the same manner (the retention times of standards D-glucose, L-rhamnose, and D-apiose were 14.12, 4.50, and 4.68 min, respectively.

Cell culture and measurement of cytokine production

BMDCs were grown from wild-type C57BL/6 mice (Orient Bio Inc.) as previously described [28]. All animal procedures were approved and performed according to the guidelines of the Institutional Animal Care and Use Committee of Jeju National University (#2010–0028). Briefly, the mouse tibia and femur were obtained by flushing with Dulbecco's modified Eagle's medium to yield bone marrow cells. The cells were cultured in RPMI 1640 medium containing 10% heat-inactivated FBS (Gibco), 50.0 µM β-mercaptoethanol, and 2 mM glutamine supplemented with 3% J558 L hybridoma cell culture supernatant containing granulocyte-macrophage colony stimulating factor. The culture medium was replaced with fresh medium every second day. On day 6 of the culture, non-adherent cells and loosely adherent DC aggregates were harvested, washed, and resuspended in RPMI 1640 supplemented with 5% FBS.

The BMDCs were incubated in 48-well plates in 0.5 mL containing 1×10^5 cells per well, and then treated with the isolated compounds at different concentrations for 1 h before stimulation with 10.0 ng/mL LPS from Salmonella Minnesota (Alexis). Supernatants were harvested 18 h after stimulation. Concentrations of compounds IL-12 p40, IL-6, and TNF-α in the culture supernatant were determined by ELISA (BD PharMingen) according to the manufacturer's instructions. SB203580 was the product of Calbiochem.

The inhibitory activity *(I)* was expressed as the inhibition rate (%), which was calculated from the following formula:

$$I = \frac{Cdcv - Cdcc}{Cdcv} \times 100$$

Where: *Cdcv*: Cytokine level (ng/mL) in vehicle-treated DC; *Cdcc*: Cytokine level (ng/mL) in compound-treated DC.

Statistical analysis

All data represent the mean ± SD of at least three independent experiments performed in triplicate. Statistical significance is indicated as determined by one-way ANOVA followed by Dunnett's multiple comparison test, $p < 0.05$, using the GraphPad Prism 6.01 program (GraphPad Software Inc.).

Supporting information

Spectral data of compounds **1**–**4** are available as Supporting Information.

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Conflict of Interest

The authors declare no conflict of interest.

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PAGE 1

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PAGE 3

PAGE 4

PAGE 5

PAGE 6

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