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Inhibition of antigen-induced degranulation by aryl compounds isolated from the bark of *Betula platyphylla* in RBL-2H3 cells

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ABSTRACT

The methanolic extract of the bark of *Betula platyphylla* was found to suppress antigen mediated degranulation of RBL-2H3 cells. Four arylbutanoids (**1**–**4**) and eight diarylhepatonoids (**5**–**12**) were isolated from the methanolic extract using bioassay-guided fractionation. Among them, compounds **4** and **12** were isolated and assigned for the first time. Compounds **1**, **2**, **3**, **5**, **10**, and **12** showed remarkable inhibitory activity against the degranulation of RBL-2H3 by antigen stimulation in a dose dependent manner at the concentrations ranging from 10 μM to 100 μM.

Mast cells play a crucial role in the development of allergic diseases and inflammatory processes such as asthma, mastocytosis, inflammatory arthritis and autoimmune disease.[1] Various types of antigens initiate production of antigen-specific IgE antibodies that bind to high affinity IgE receptor (FcεRI) on the surface of mast cells.[2] As a result, mast cells immediately released chemical mediators such as histamine, arachidonic acid metabolites, proteases, serotonin, and heparin from the intracellular granules by exocytosis.[2] β-Hexosaminidase is also stored in the secretory granules of mast cells and released when activated mast cells degranulate. Thus many investigators have concentrated on finding effective therapeutics for allergic inflammation, using well established mast cell-dependent experimental model system.[3] The continuous rat cell line, RBL-2H3, cloned by the limited dilution technique from leukemia cells isolated from rats after treatment with the chemical carcinogen, β-chlorethylamine, have been extensively used for studying IgE–FcεRI interactions.[4] Degranulation of RBL-2H3 by antigen can be monitored following the release of granule-associated β-hexaminidase.[3]

Betula platyphylla var. *japonica* (Betulaceae), birch tree, is widely distributed in Korea, Japan, China, Sahalin, and Siberia.[5] The two main products harvested from birch without timber-cutting are sap and bark. The collected sap can be drunk as a tonic. In addition, syrups, drinks and food additives have been already commercialized from the birch sap. The bark of *B. platyphylla* has been used in folk medicine for the treatment of various inflammatory diseases including, arthritis, nephritis, dermatitis, and bronchitis.[5] Regarding the pharmacological studies of this plant, anticancer, anti-arthritis, and hepatoprotective activities have been reported.[5–7] Moreover, Kim et al. reported that the water extract of birch bark inhibits the development of atopic dermatitis like skin lesions in Nc/Nga mice.[8] Several terpenoids including betulin and phenolics such as diarylheptanoids and arylbutanoids have been reported to be isolated from the outer bark and inner bark of *B. platyphylla*, respectively.[7] In the course of searching mast cell degranulation inhibitory natural products, the methanolic extract of the bark of *B. platyphylla* significantly inhibited the release of β-hexosaminidase from antigen-stimulated RBL-2H3 cells. Accordingly, the phytochemical research to reveal the active constituents of the extract has been followed. Herein, we describe the isolation, structure elucidation and anti-degranulation activity of the isolated compounds of *B. platyphylla* using anti-DNP IgE-stimulated RBL-2H3 cells as an in vitro screening system.

The bark of *B. platyphylla* was provided by SK E&C (Korea). The samples were collected in the afforested land of SK E&C, which has more than 450,000 of *B. platyphylla* in 167.6 ha of afforestation area in Chungju. The dried plant material (5.7 kg) was ground and extracted with 80% methanol (30 L, 3 h × 4) by ultrasonication at room temperature. The methanolic extract was concentrated in vacuo to give a crude extract (948.4 g). The methanolic extract was then suspended in H_2O and partitioned successively with $CHCl_3$

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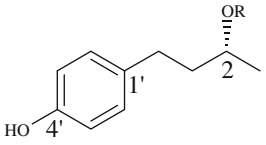
Table 1
Inhibition effect of the fractions of *B. platyphylla* against β-hexosaminidase release in RBL-2H3 cells

Fractions	Inhibition[a] (%)	
	10 μM	100 μM
Control	0.0 ± 3.9	
Quercetin[b]	32.9 ± 5.3***	
Total extract	30.8 ± 1.0***	74.3 ± 2.7***
$CHCl_3$ fraction	26.8 ± 1.2**	44.6 ± 1.3***
n-Butanol fraction	38.3 ± 4.7***	79.8 ± 1.7***
Aqueous fraction	42.5 ± 1.1***	85.5 ± 0.9***

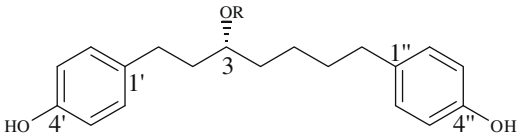
[a] Results are expressed as the mean ± standard deviation of three independent experiments, each performed using triplicate wells. Results differ significantly from control, *p <0.05, **p <0.01, and ***p <0.001, respectively.
[b] Quercetin was used as a positive control (10 μM).

and *n*-butanol. Among these fractions, the *n*-butanol fraction, which showed the most significant inhibitory effect on the release of β-hexosaminidase (Table 1), were used for the isolation of active compounds to yield four arylbutanoids (**1**–**4**) and eight diarylheptanoids (**5**–**12**) (Fig. 1). The *n*-butanol fraction (577.5 g) was eluted from Diaion HP-20 resin using water/MeOH gradient as a mobile phase to afford six fractions (B1–B6). B4 (90.7 g) was subjected to silica gel column chromatography (CC) and eluted by $CHCl_3$/MeOH/water gradient to yield 15 fractions (B4-1–B4-15).

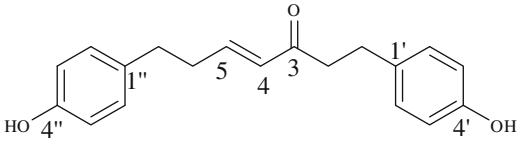
Compounds **1** (129.3 mg) and **10** (57.3 mg) were sequentially obtained from B4-4 by HPLC (Phenomenex Gemini C_{18}, 150 × 10 mm, 40% ACN in water, 1 mL/min). B4-7 was subjected to MPLC (RediSep C_{18}, 114 × 23 mm, water/MeOH gradient, 25 mL/min) to yield 13 fractions (B4-7-1–B4-7-13). Compounds **2** (50.5 mg), **6** (115.0 mg) and **11** (3.2 g) were respectively isolated from B4-7-3, B4-7-12, and B4-7-8 by recrystallization. B4-11 was divided into eight fractions (B4-11-1–B4-11-8) by MPLC (RediSep C_{18}, 114 × 23 mm, water/MeOH gradient, 25 mL/min). Compounds **3** (35.7 mg) and **7** (3.1 g) were isolated from B4-11-2 by HPLC (Phenomenex Gemini C_{18}, 150 × 10 mm, 33% ACN in water, 1 mL/min) and B4-11-7 by



1 R = H
2 R = *β*-D-glucopyranose
3 R = *β*-D-apiofuranosyl-(1→6)-*β*-D-glucopyranose
4 R =1-*O*-*β*-D-glucopyranosyl-3-hydroxy-3-methylglutaric acid



5 R = H
6 R = *β*-D-glucopyranose
7 R = *β*-D-apiofuranosyl-(1→6)-*β*-D-glucopyranose
8 R = β-D-apiofuranosyl-(1→2)-[β-D-apiofuranosyl-(1→6)]-β-D-glucopysanose



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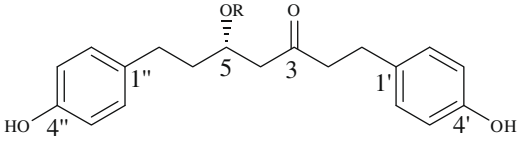

10 R = H
11 R = *β*-D-glucopyranose
12 R = β-D-apiofuranosyl-(1→2)-[β-D-apiofuranosyl-(1→6)]-β-D-glucopysanose

Figure 1. Structures of the isolated compounds (**1**–**12**).

recrystallization, respectively. B4-13 was fractionated by Sephadex LH-20 CC (MeOH) (B4-13-1–B4-13-7) and MPLC (RediSep C_{18}, 114 × 23 mm, water/MeOH gradient, 25 mL/min) (B4-13-2-1–B4-13-2-20). Compounds **4** (31.8 mg) and **8** (795.0 mg) were obtained by recrystallization of B4-13-2-5 and B4-13-2-18, respectively. From B4-13-2-14, compound **12** (4.7 mg) was isolated by HPLC (Phenomenex Gemini C_{18}, 150 × 10 mm, 24% ACN in water, 1 mL/min). Purification of B4-5 by HPLC (Phenomenex Gemini C_{18}, 150 × 10 mm, 40% ACN in water, 1 mL/min) yielded compound **5** (13.7 mg). Compound **9** (18.1 mg) was obtained from B4-6 by MPLC (RediSep C_{18}, 114 × 23 mm, water/MeOH gradient, 25 mL/min).

The structures of the known compounds (**1**–**3**, **5**–**11**) were identified as (-)-rhododendrol (**1**), (−)-rhododendrin (**2**), apiosylrhododendrin (**3**), (-)-centrolobol (**5**), aceroside VII (**6**), aceroside VIII (**7**), (3*R*)-1,7-bis-(4-hydroxyphenyl)-3-heptanol-3-*O*-[2,6-bis-*O*-(β-D-apiofuranosyl)-β-D-glucopyranoside (**8**), 1,7-bis-(4-hydroxy phenyl)-5-hepten-3-one (**9**), platyphyllone (**10**), and platyphylloside (**11**) from spectral data comparison with those reported in the literature.[9–13]

Compound **4** was isolated as brownish syrup with negative optical rotation $[\alpha]_D^{20}$ −18.1 (*c* 2.0, MeOH). Its ESIMS exhibited a molecular ion peak at *m*/*z* 471 $[M-H]^-$, corresponding $C_{22}H_{32}O_{11}$, which was confirmed by HRFABMS at *m*/*z* 495.1823 $[M+Na]^+$ (calcd 495.1842). Signals due to a 1,4-disubstituted aromatic ring, two methylenes, one oxymethylene and one methyl group were observed in ^1H and ^{13}C NMR spectra (Table 2). Moreover, one anomeric signal was also provided by these spectral data. These signals were similar to those of rhododendron (**2**), a glycosidic arylbutanoid.[10] In the HMBC spectrum, methyl protons [δ_H 1.28 (3H, s, H-6″)] showed a cross peak with a quaternary oxymethylene carbon [δ_C 71.7 (C-3″)] linked with two methylene groups. The HMBC correlations between the protons of these two methylenes [δ_H 2.55 (2H, s, H-2″), 2.33 (1H, d, *J* = 15.3 Hz, H-4″a), 2.49 (1H, d, *J* = 15.3 Hz, H-4″b)], and two carbonyl carbons [δ_C 173.6 (C-1″), 180.9 (C-5″)] revealed the presence of 3-hydroxy-3-methylglutaric acid moiety.[14] The glucose unit was determined to be attached to 3-hydroxy-3-methylglutaric acid moiety by the cross peak in the HMBC spectrum between H-6 of glucose at δ_H 4.19 and C-1″. The HMBC cross peak between anomeric proton of glucose unit and C-2 of arylbutanoid moiety at δ_C 75.8 assigned the glycosidic linkage. From these spectroscopic data above, compound **4** was characterized as 1-[(2*R*)-4-(4-hydroxyphenyl)-2-butanol-2-*O*-β-D-glucopyranosyl]-3-hydroxyl-3-methylglutaric acid.

Compound **12** was isolated as yellowish syrup with negative optical rotation $[\alpha]_D^{20}$ −55.0 (*c* 1.0, MeOH). Its ESIMS exhibited a molecular ion peak at *m*/*z* 739 $[M-H]^-$, corresponding $C_{35}H_{48}O_{17}$, which was confirmed by HRFABMS at *m*/*z* 763.2781 $[M+Na]^+$ (calcd 763.2789). ^1H NMR and ^{13}C NMR spectra provided signals of two 1,4-disubstituted aromatic rings, five methylenes, one oxymethylene and one carbonyl group, which were similar to

Table 2
^1H and ^{13}C NMR data of compounds **4** and **12** (in CD_3OD)

Position	Compound **4**		Position	Compound **12**	
	δ_H	δ_C		δ_H	δ_C
Aglycone			*Aglycone*		
1	1.17 (3H, d, 6.2)	20.9	1	2.73 (2H, br s)	30.7
2	3.83 (1H, m)	75.8	2	2.73 (2H, br s)	47.0
3	1.67 (1H, m)	41.2	3		212.4
	1.81 (1H, m)				
4	2.57 (2H, m)	32.4	4	2.57 (1H, m)	50.7
1′		135.3	5	4.16 (1H, q, 6.3)	76.0
2′	7.01 (1H, d, 8.4)	131.2	6	1.74 (1H, m)	39.7
3′	6.67 (1H, d, 8.4)	116.8	7	2.57 (2H, m)	32.4
4′		157.0	1′, 1″		134.0, 135.3
5′	6.67 (1H, d, 8.4)	116.8	2′, 2″	6.99 (1H, d, 8.4)	131.2, 131.3
				7.00 (1H, d, 8.3)	
6′	7.01 (1H, d, 8.4)	131.2	3′, 3″	6.67 (2H, d, 8.3)	116.9, 117.0
Glucose			4′, 4″		157.0, 157.4
1	4.32 (1H, d, 7.8)	103.0	5′, 5″	6.67 (2H, d, 8.3)	116.9, 117.0
2	3.20 (1H, m)	75.9	6′, 6″	6.99 (1H, d, 8.4)	131.2, 131.3
				7.00 (1H, d, 8.3)	
3	3.36 (1H, m)	78.6	*Glucose*		
4	3.36 (1H, m)	72.4	1	4.32 (1H, d, 7.7)	102.6
5	3.44 (1H, m)	75.9	2	3.31 (1H, m)	80.1
6	4.19 (1H, dd, 11.9, 5.56)				
	4.45 (1H, dd, 11.9, 1.8)	65.5	3	3.42 (1H, m)	79.2
3-Hydroxy-3-methyl glutaric acid			4	3.31 (1H, m)	72.4
1″		173.6	5	3.31 (1H, m)	77.4
2″	2.55 (2H, s)	48.0	6	3.63 (1H, m)	69.2
				3.97 (1H, d, 11.0)	
3″		71.7	*Apiose* (1 → 6)		
4″	2.33 (1H, d, 15.3)	48.7	1	5.00 (1H, d, 2.1)	111.7
	2.49 (1H, d, 15.3)				
5″		180.9	2	3.92 (1H, m)	78.8
6″	1.28 (3H, s)	28.7	3		81.4
			4	3.71 (1H, d, 9.6)	75.9
				3.92 (1H, m)	
			5	3.55 (2H, d, 4.1)	66.6
			Apiose (1 → 2)		
			1	5.32 (1H, d, 1.6)	111.6
			2	3.92 (1H, m)	78.8
			3		81.4
			4	3.72 (1H, d, 9.6)	75.9
				4.02 (1H, d, 9.5)	
			5	3.58 (2H, br s)	66.8

those of platyphyllone (**10**), a diarylheptanoid (Table 2).[13] The rest of signals [δ_H 4.32 (1H, d, J = 7.7 Hz), 5.00 (1H, d, J = 2.1 Hz), 5.32 (1H, d, J = 1.6 Hz)] implied the existence of one pyranose and two furanoses. From their chemical shifts and coupling constants, these sugars were characterized as one β-D-glucopyranose and two β-D-apiofuranose.[12] The glucose unit was determined to be attached to diarylheptanoid moiety by the cross peak in the HMBC spectrum between the anomeric proton at δ_H 4.32 of glucose with C-5 at δ_C 76.0. The HMBC correlation between the two anomeric signals of apioses and two carbon signals of glucose [δ_C 69.2 (C-6), 80.1 (C-2)] revealed that the two apioses formed a (1 → 2) and a (1 → 6) glycosidic linkages with the glucose unit, respectively.[12] Therefore, compound **12** was identified as (5*S*)-5-hydroxy-1,7-bis-(4-hydroxyphenyl)-3-heptanone-5-*O*-[2,6-bis-*O*-(β-D-apiofuranosyl)-β-D-glucopyranoside].

All the fractions and isolated compounds (**1**–**12**), together with quercetin (10 μM) as a positive control, were examined for their inhibitory effect on the release of β-hexosaminidase from RBL-2H3 cells. RBL-2H3 cells were obtained from Korean Cell Line Bank (Seoul, Korea) and grown in DEME supplemented with 10% fetal bovine serum, 100 IU/mL penicillin and 100 μg/mL streptomycin at 37 °C in a humidified incubator with a 5% CO_2/95% air atmosphere. RBL-2H3 cells in 48-well plates [1.5×10^5 cells/well] were sensitized with anti-DNP IgE (final concentration, 90 ng/mL). The cells were washed with Siraganian buffer for two times and then incubated in 160 μL of incubation buffer for 30 min at 37 °C. After that, 20 μL of test sample solution was added to each well and incubated for 20 min, followed by an addition of 20 μL of DNP–BSA (final concentration, 200 ng/mL) at 37 °C for 20 min. The reaction was stopped by cooling in an ice bath for 10 min. The collected media was centrifuged at 3000 rpm for 3 min. The supernatant (50 μL) was transferred into 96-well plate and incubated with 50 μL of *p*-NAG at 37 °C for 1 h. The reaction was stopped by 200 μL of carbonate buffer. The absorbance was measured with a microplate reader at 405 nm. The test sample was dissolved in DMSO (final concentration in cultures <0.1%). The inhibition (%) was calculated as [1 − (absorbance of DNP–BSA + test sample treated − absorbance of blank − absorbance of non-DNP–BSA treated)/(absorbance of DNP–BSA treated − absorbance of non-DNP-BSA treated)] × 100. The inhibitory effect of the fractions was tested at the concentrations of 10 and 100 μg/mL, respectively. The inhibitory activity was expressed as mean ± standard deviation. The evaluation of statistical significance was determined by an 'one-way ANOVA' test using computerized statistical package. The data were considered to be statistically significant if the probability had a value of 0.05 or less.

All the isolated compounds statistically inhibited the release of β-hexosaminidase induced by antigen stimulation in RBL-2H3 cells (Table 3). These results are in accordance with the previous reports describing the anti-degranulation activity of diarylheptanoids.[15,16] Among them, compounds **1**, **2**, **3**, **5**, **10**, and **12** showed remarkable inhibitory effect on β-hexosaminidase activity at the concentration of 100 μM (p <0.001). The inhibitory activity of the three active arylbutanoids, compounds **1**, **2**, and **3**, was more potent than that of diarylheptanoids (**10**, **12**) except compound **5**. Compounds **1**, **2** and **5** were more effective than quercetin, the positive control, at the same concentration, 10 μM. Among the diarylhaptanoids, the presence of carbonyl group at C3 and *O*-glycoside at C5, and elimination of hydroxyl group at C5 could not contribute to the inhibition of anti-DNP IgE-mediated degranulation of RBL-2H3.

Table 3
Inhibition effect of the isolated compounds of *B. platyphylla* against β-hexosaminidase release in RBL-2H3 cells

Compounds	Inhibition[a] (%)	
	10 μM	100 μM
Control	0.0 ± 3.9	
Quercetin[b]	32.9 ± 5.3***	
1	29.7 ± 3.1**	36.5 ± 5.5***
2	25.0 ± 5.4**	42.3 ± 1.6***
3	25.4 ± 5.6**	26.7 ± 2.4***
4	26.6 ± 4.0**	20.0 ± 7.9*
5	19.22 ± 6.5*	31.5 ± 2.0***
6	16.2 ± 3.5**	23.2 ± 2.9**
7	11.7 ± 4.2*	22.4 ± 3.1**
8	22.1 ± 4.2*	23.2 ± 1.1**
9	14.4 ± 5.5*	18.8 ± 8.8*
10	23.7 ± 0.2**	24.9 ± 2.6***
11	16.7 ± 4.8*	20.5 ± 1.8**
12	11.8 ± 0.6*	23.4 ± 1.3***

[a] Results are expressed as the mean ± standard deviation of three independent experiments, each performed using triplicate wells. Results differ significantly from control, *p <0.05, **p <0.01, and ***p <0.001, respectively.
[b] Quercetin was used as a positive control (10 μM).

Taken together, the arylbutanoids and diarylheptanoids isolated from the bark of *B. platyphylla* exerted an inhibitory effect on antigen-stimulated degranulation. These results suggested the bark of *B. platyphylla* as an additional natural resource for the treatment of IgE–FcεRI interaction related allergic disorders.

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Supplementary data

Supplementary data (^{1}H, ^{13}C NMR spectra and HMBC spectrum of compounds **4** and **12**) associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2010.03.053.

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