Anti-allergic Flavones from *Arthraxon hispidus*

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Bioactivity-guided fractionation for an EtOAc-soluble fraction of methanolic extract of *Arthraxon hispidus*, using primary cell assay with bone marrow-derived mast cells (BMMC), led to an isolation of six new flavones and nine known compounds. The structures of the new compounds were established by one dimensional (1D)- and 2D-NMR spectroscopic data, as luteolin 8-*C*-*β*-kerriopyranoside (1), luteolin 8-acetic acid methyl ester (2), 7-methyl-luteolin 8-*C*-*β*-(6-deoxyxylo-3-uloside) (3), apigenin 8-*C*-*α*-fucopyranoside (4), apigenin 8-*C*-*β*-fucopyranoside (5) and luteolin 8-*C*-*β*-fucopyranoside (6). All the isolates were evaluated for inhibitory activities on interleukin-6 release in the primary cultures using BMMC. Of the tested compounds, compounds 2, 3 and 10 were found to inhibit interleukin-6 release. Furthermore, compound 2 displayed inhibitory activity against prostaglandin D_2, leukotriene C_4, and *β*-hexosaminidase releases.

Key words *Arthraxon hispidus*; flavonoid; bone-marrow derived mast cell; interleukin-6; leukotriene C_4; prostaglandin D_2

The mast cells are known to mediate allergies and inflammation through the release of various mediators, such as cytokines, histamine, leukotrienes and prostaglandin D_2, involved in various diseases, including asthma, atopic dermatitis, chronic rhinitis, multiple sclerosis, and rheumatoid arthritis.[1)] Therefore, modulation of the mast cells is recognized as one of the targets of these diseases.

Arthraxon hispidus (Thunb.) Makino (Poaceae), an annual grass, grows in China, Japan, Korea, and Russia. This plant has been used for the treatment of asthma and inflammatory diseases in traditional medicine.[2)] The grass, called 'Kobunagusa' in Japanese, has been used as a source of natural dyestuff for coloring 'Kihachijo,' which is a traditional yellow silk cloth produced on Hachijo Island, Japan.[3,4)] Thus far, a few chemical investigations on *A. hispidus* have been reported on the isolation and identification of arthraxin, aconitic acid, luteolin and luteolin-7-glucoside,[4,5)] and no pharmacological evaluation on this plant has been conducted.

As part of our ongoing search for bioactive agents of plant origin,[6,7)] an EtOAc-soluble fraction of the methanolic extract of *A. hispidus* was chosen for a phytochemical and biological study due to interleukin-6 (IL-6) production inhibitory activity in an initial screening using bone marrow-derived mast cells (BMMC) stimulated by phorbol 12-myristate 13-acetate (PMA) plus A23187. Herein, we describe the isolation and structures elucidation of six new flavonoids and nine known compounds, and their inhibitory activity on IL-6 production in primary cell assay using BMMC. Further evaluation for leukotriene C_4, prostaglandin D_2, and *β*-hexosaminidase releases was carried out.

Results and Discussion

Repeated column chromatography for the ethyl acetate-soluble fraction, using Sephadex LH-20, silica gel, HPLC and high speed countercurrent chromatography, yielded seven new flavones and nine known compounds. The known compounds were identified by comparison of their spectroscopic data (^{1}H, ^{13}C, and high resolution-electrospray ionization (HR-ESI)-MS) with literature values as luteolin (**7**),[8)] luteolin 7-*O*-glucoside (**8**),[9)] apigenin 8-*C*-glucoside (**9**),[8)] tricin (**10**),[10)] tricin-4′-*O*-*erythro*-(*β*-guaiacylglyceryl) ether (**11**),[11)] 4-*O*-caffeoylquinic acid (**12**),[12)] 5-*O*-caffeoylquinic acid (**13**),[13)] 4-hydroxycinnamic acid (**14**), and ergosterol endoperoxide (**15**).[14)]

Compound **1** was obtained as a yellow powder and exhibited a pesudomolecular ion peak at *m/z* 413.0879 $[M-H]^-$(Calcd for $C_{21}H_{17}O_9$, 413.0873) in the HR-ESI-MS, corresponding to the molecular formula of $C_{21}H_{18}O_9$. ^{1}H- and ^{13}C-NMR spectroscopic data of compound **1** (Table 1) suggested a presence of luteolin skeleton in the structure. In addition, a kerriose unit was observed in the range of δ_H 1.40 to 5.22 in the ^{1}H-NMR spectrum and further comparison of the ^{13}C chemical shifts [δ_C 19.2 (C-6″), 45.2 (C-2″), 70.8 (C-1″), 78.6 (C-4″, 5″), 206.4 (C-3″)] of **1** with the published values,[15)] and the heteronuclear multiple bond correlation (HMBC) correlations of δ 5.22/78.6, 45.2 (H-1″/C-5″, C-3″, C-2″), δ 3.41/70.8, 206.4 (H-2eq″/C-1″, C-3″), δ 2.39/206.4 (H-2ax″/C-3″), δ 4.02/206.4, 78.6, 19.2 (H-4″/C-3″, C-5″, C-6″), δ 1.40/78.6, 78.6 (H-6″/C-5″, C-4″) supported the presence of kerriose. In addition, the cross-peaks of δ 5.22 to 155.4, 160.8, 104.5 (H-1″/C-9, C-7, C-8), and δ 3.41/104.5 (H-2eq″/C-8) in the HMBC confirmed that the sugar moiety was connected to C-8 through a *C*-linkage. Based on the coupling constant of H-1″ (J=12.0 Hz), the configuration was assigned as *β*-form. Therefore, compound **1** was established as luteolin 8-*C*-*β*-kerriopyranoside.

The molecular formula of compound **2** was deduced as $C_{18}H_{14}O_8$ by the observed pseudomolecular ion peak at *m/z* 357.0621 $[M-H]^-$ (Calcd for $C_{18}H_{13}O_8$, 357.0610) in the HR-ESI-MS. ^{1}H- and ^{13}C-NMR spectroscopic data of compound **2** (Table 1) had a close resemblance with those of compound **1**, except for the presence of acetic acid methyl ester resonated

The authors declare no conflict of interest.

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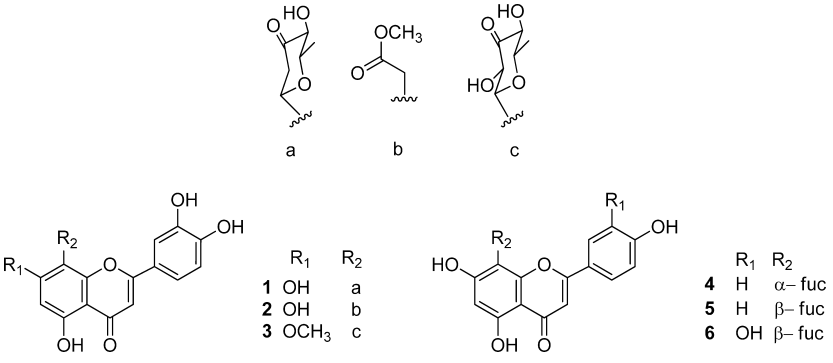
Table 1. ^{1}H- and ^{13}C-NMR Spectroscopic Data of Compounds **1**–**3** (400 MHz, DMSO-d_6)

No.	**1**		**2**		**3**	
	δ_H J (Hz)	δ_C	δ_H J (Hz)	δ_C	δ_H J (Hz)	δ_C
2		164.0, C		163.7, C		164.4, C
3	6.72 s	102.7, CH	6.67 s	102.7, CH	6.77 s	102.9, CH
4		181.8, C		181.9, C		182.1, C
5		160.8, C		160.0, C		161.8, C
6	6.28 s	98.5, CH	6.28 s	98.2, CH	6.55 s	95.1, CH
7		160.8, C		162.0, C		163.2, C
8		104.5, C		100.7, C		104.4, C
9		155.4, C		154.9, C		155.0, C
10		103.9, C		103.4, C		104.4, C
1′		121.9, C		121.6, C		121.9, C
2′	7.51 m	113.9, C	7.36 m	113.4, CH	7.50 m	114.0, CH
3′		145.9, C		145.8, C		146.0, CH
4′		149.8, C		149.8, C		149.9, CH
5′	6.91 d (8.0)	115.8, CH	6.88 d (8.0)	116.0, CH	6.92 d (8.0)	115.8, CH
6′	7.51 m	119.1, CH	7.36 m	118.9, CH	7.50 m	119.2, CH
1″	5.22 dd (12.0, 2.0)	70.8, CH		171.3, C	4.89 d (10.0)	75.1, CH
2″	3.41 t (12.0)	45.2, CH_2	3.78 s	27.9, CH_2	4.78 dd (10.0, 1.0)	74.0, CH
	2.39 dd (12.0, 2.0)					
3″		206.4, C				206.5, C
4″	4.02 d (10.0)	78.6, CH			4.08 dd (10.0, 1.0)	78.2, CH
5″	3.58 dq (10.0, 6.0)	78.6, CH			3.50 dq (10.0, 6.0)	78.7, CH
6″	1.40 d (6.0)	19.2, CH_3			1.40 d (6.0)	19.3, CH_3
OCH_3			3.62 s	51.7, CH_3	3.87 s	56.7, CH_3



at δ_H 3.78 (2H, s, H-2″), and 3.62 (3H, s, OCH_3), as well as δ_C 171.3 (COO), 27.9 (C-2″), and 51.7 (OCH_3), instead of kerriopyranoside.[16] The location of acetic acid methyl ester was assigned on C-8 by the observed HMBC correlations of δ_H 3.78 (H-4″) to δ_C 154.9 (C-9), 162.0 (C-7), and 100.7 (C-8) *via* a *C*-linkage. From the above result, compound **2** was determined to be luteolin 8-acetic acid methyl ester.

Compound **3** was obtained as a yellow powder and exhibited a pesudomolecular ion peak at *m/z* 443.0979 $[M-H]^-$ (Calcd for $C_{22}H_{19}O_{10}$, 443.0978), corresponding to the molecular formula of $C_{22}H_{20}O_{10}$ in the HR-ESI-MS. ^{1}H-NMR spectra of compound **3** (Table 1) at room temperature also showed a duplication of signals due to rotational isomers with a ratio of *ca.* 1 : 0.28, based on the integrals of H-6″. Structure elucidation of compound **3** was accomplished by the interpretation of NMR signals from the major rotamer.

The ^{1}H- and ^{13}C-NMR data of compound **3** were closely similar to those of compound **1**, except for the presence of different sugar moiety and an additional methoxy group. The ^{13}C-NMR data of the sugar moiety included a carbonyl group at δ_C 206.5, four oxygenated carbons at δ_C 74.0, 75.1, 78.2, and 78.7, and a methyl group at δ_C 19.3, assignable to a 6-deoxy-ribo-3-ulose.[17] This inference was further corroborated by the careful inspection of 2D-NMR (correlated spectroscopy (COSY), heteronuclear multiple quantum coherence (HMQC), and HMBC). An anomeric proton at δ_H 4.89 (1H, d, J=10.0 Hz, H-1″) was found to be positioned in the β-form based on the coupling constant. This sugar moiety was connected to C-8 through *C*-linkage, which was supported by the HMBC correlations of δ_H 4.89 to δ_C 155.0, 163.2, 104.4 (H-1″/C-9, C-7, C-8). Thus, the structure of compound **3** was determined to be luteolin-7-methyl ether-8-*C*-β-6-deoxy-xylohexopyranos-3-uloside.

Compound **4** was obtained as a yellow powder and exhibited a pesudomolecular ion peak at *m/z* 415.1083 $[M-H]^-$ (Calcd for $C_{21}H_{19}O_9$, 415.1029), corresponding to the molecular

Table 2. ^{1}H- and ^{13}C-NMR Spectroscopic Data of Compounds **4**–**6** (400 MHz, DMSO-d_6)

No.	**4**		**5**		**6**	
	δ_H J (Hz)	δ_C	δ_H J (Hz)	δ_C	δ_H J (Hz)	δ_C
2		163.6, C		164.3, C		164.2, C
3	6.77 s	102.5, CH	6.82 s	101.9, CH	6.65 s	101.9, CH
4		182.1, C		182.3, C		182.1, C
5		160.3, C		160.4, C		160.4, C
6	6.26 s	98.4, CH	6.28 s	98.2, CH	6.26 s	98.1, CH
7		163.2, C		162.6, C		162.6, C
8		104.8, C		104.7, C		104.6, C
9		156.0, C		156.1, C		156.1, C
10		103.9, C		104.1, C		104.1, C
1′		121.6, C		121.1, C		121.2, C
2′	7.97 d (8.0)	128.5, CH	8.32 d (8.0)	129.8, CH	7.55 s	113.7, CH
3′	6.92 d (8.0)	115.8, CH	6.90 d (8.0)	116.0, CH		145.4, C
4′		161.2, C		161.2, C		149.8, C
5′	6.92 d (8.0)	115.8, CH	6.90 d (8.0)	116.0, CH	6.89 d (8.0)	116.2, CH
6′	7.97 d (8.0)	128.5, CH	8.32 d (8.0)	129.8, CH	8.03 d (8.0)	121.3, CH
1″	5.16 d (6.6)	68.7, CH	4.66 d (9.8)	73.9, CH	4.65 d (9.6)	73.9, CH
2″	4.04 br d (8.0)	68.5, CH	4.18 t (9.4)	67.9, CH	4.17 t (9.3)	67.9, CH
3″	3.26 d (8.0)	71.7, CH	3.44 m	75.7, CH	3.43 m	75.6, CH
4″	3.95 m	71.5, CH	3.68 m	71.8, CH	3.69 m	72.0, CH
5″	3.67 m	73.4, CH	3.68 m	75.1, CH	3.69 m	75.0, CH
6″	1.19 d (6.0)	18.3, CH_3	1.21 d (6.0)	17.2, CH_3	1.19 d (6.3)	17.1, CH_3

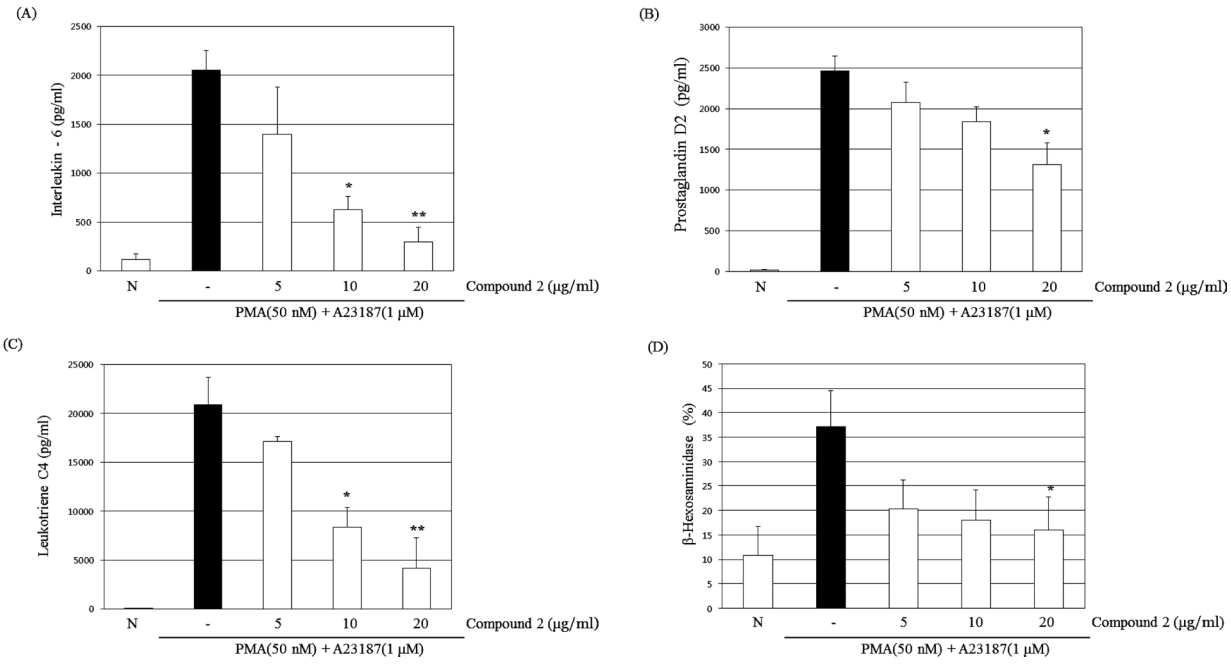


Fig. 1. Effect of Compound **2** on the Release of IL-6 (A), PGD_2 (B), LTC_4 (C), and β-Hexosaminidase (D) in PMA Plus A23187-Stimulated BMMCs

Statistical significance: *$p<0.05$ and **$p<0.005$, as compared to the PMA plus A23187 treated group. Values shown are the mean±S.E. of duplicate determinations from three separate experiments.

formula of $C_{21}H_{20}O_9$ in the HR-ESI-MS. The ^{1}H-NMR spectroscopic data (Table 2) of compound **4** provided the presence of apigenin and a sugar moiety. The ^{1}H- and ^{13}C-NMR data corresponding to a sugar moiety in compound **4** were able to assign a fucopyranoside with α configuration (H-1″ at δ_H 5.16, J=6.6 Hz), evidenced by sequential ^{1}H–^{1}H correlations in the COSY and HMBC correlations as described in Fig. 1.[18] Furthermore, HBMC correlations of δ_H 5.16 (H-1″) to δ_C 156.0 (C-9), 163.2 (C-7), and 104.8 (C-8) enabled to link the sugar moiety to C-8 through a *C*-linkage. Thus, the structure of compound **4** was determined to be an apigenin 8-*C*-α-fucopyranoside.

Compound **5** exhibited a pesudomolecular ion at the peak at m/z 415.1089 $[M-H]^-$ (Calcd for $C_{21}H_{19}O_9$, 415.1029), corresponding to the molecular formula of $C_{21}H_{20}O_9$ in the HR-ESI-MS. ^{1}H-NMR spectra of compound **5** (Table 2) at room temperature also showed a duplication of signals due to rotational isomers with a ratio of *ca.* 1 : 0.3, based on the integrals of

H-2″. Structure elucidation of compound **5** was accomplished by the interpretation of NMR signals from the major rotamer. ^{1}H- and ^{13}C-NMR data of compound **5** were similarly close to those of compound **4**, except for fucopyranoside with β configuration by the observed coupling constant (J=9.8 Hz) of H-1″ at δ_H 4.66.[18,19] The long range correlations from δ_H 4.66 (H-1″) to δ_C 156.1 (C-9), 162.6 (C-7), and 104.7 (C-8) in the HMBC confirmed that the sugar moiety was connected to C-8 through a *C*-linkage. From the above result, compound **5** was determined to be apigenin 8-*C*-β-fucopyranoside.

Compound **6** was obtained as a yellow powder and exhibited a pesudomolecular ion peak at *m/z* 431.1034 $[M-H]^-$ (Calcd for $C_{21}H_{19}O_{10}$, 431.0978) in the HR-ESI-MS, corresponding to the molecular formula of $C_{21}H_{20}O_{10}$. The ^{1}H-NMR spectra of compound **6** at room temperature displayed a duplication of signals due to the presence of rotational isomers with a ratio of *ca.* 1 : 0.7, based on the integrals of H-2″. Structure elucidation of compound **6** was accomplished by the interpretation of NMR signals from the major rotamer (Table 2).[20,21] When the NMR measurement of **6** was conducted at 60°C, its NMR signals appeared as a single set of signals, which was observed in certain flavone *C*-glycosides due to the reduction of slow correlation time as the temperature increases.[22] The ^{1}H-NMR spectrum (Table 2) showed five aromatic protons attributable to H-3, H-6, H-2′ H-5′ and H-6′ of luteolin skeleton at δ_H 6.65 (s), 6.26 (s), 7.55 (m), 6.89 (d, J=8.0 Hz), and 8.03 (m), respectively. The remaining proton signals were assigned to a fucopyranoside by aid of ^{13}C-NMR signals [δ_C 73.9 (C-1″), 67.9 (C-2″), 75.6 (C-3″), 72.0 (C-4″), 75.0 (C-5″), and 17.1 (C-6″)] contingent to these proton signals in the HMQC spectroscopy.[18] The coupling constant (J=9.6 Hz) of δ_H 4.65 indicated this sugar possessed the β-configuration.[18] Furthermore, the location of the sugar was confirmed at C-8 through a *C*-linkage by the observed HMBC correlations of δ_H 4.65 (H-1″) to δ_C 156.1 (C-9), 162.6 (C-7), and 104.6 (C-8) (Fig. 1). Taken together with all data, compound **6** was determined to be luteolin 8-*C*-β-fucopyranoside.

All the isolates were evaluated for their inhibitory activities on IL-6 release in PMA+A23187 induced-BMMC and the results were described in the S Table 1 of supporting materials. Of the tested compounds, compounds **2** (0.001%, yield from EtOAc extract), **3** (0.016%), and **10** (0.0006%) exhibited significantly inhibitory activities with 88, 77, and 74%, respectively, at a concentration of 20 μg/mL. Even though compounds **7** (0.0062%) and **8** (0.038%) also inhibited IL-6 release, cytotoxic effects of these compounds on BMMC were observed. Furthermore, the most potent compound **2** was tested for its inhibitory activities on the production of leukotriene C_4 and prostaglandin D_4, and β-hexosaminidase in the range 5–20 μg/mL (Fig. 1). This compound was found to suppress leukotriene C_4 and prostaglandin D_4 release *via* suppression of 5-lipoxygenase and cyclooxygenase-2 expression, respectively (Fig. 2). In addition, it was assess whether or not compound **2** modulated mast cell degranulation stimulated by A23187 which was known to increase the intracellular calcium level and induce degranulation.[24] As shown in Fig. 1, inhibition of β-hexosaminidase release, a marker of histamine release, by compound **2** was observed. Thus, flavones including **2**, **3** and **10** present in this plant may partially be responsible for anti-allergic activity of the extract of *A. hispidus*.

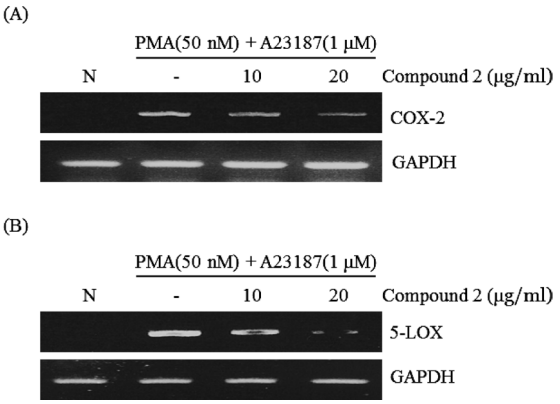


Fig. 2. Effect of Compound **2** on the Expression of COX-2 (A) and 5-LOX (B) in PMA Plus A23187-Stimulated BMMCs

Experimental

General Procedures Melting points were measured on Yanagimoto micro hot-stage melting point apparatus and were uncorrected. Optical rotations were measured with a JASCO DIP-370 digital polarimeter. UV spectra were recorded on a UV-2450 spectrometer (Shimadzu). NMR spectra were recorded on a Varian UNITY 400 spectrometer, with the chemical shifts being represented as parts per million (ppm, δ). Column chromatography was carried out on silica-gel (Silica-P Hash Silica Gel, 0.040–0.063 mm, Silicycle), RP (Cosmosil 75C18-Prep) and Sephadex LH-20 (Pharmacia) columns. Thin layer chromatography (TLC) was performed on pre-coated Silica-gel 60 F_{254} (25 TLC Plates 20×20 cm, Merck) and RP-18 F_{254s} plates (25 TLC Plates 20×20 cm, Merck). HPLC was carried out with a model Finnigan ChromQuest (Thermo). This system was equipped with Finnigan Surveyor LC Pump Plus (Thermo), Finnigan Surveyor Autosampler Plus (Thermo), and Finnigan Surveyor PDA Plus Detector (Thermo). Synergi polar-RP 80A (4 μm, 150×21.2 mm, Phenomenex, U.S.A.) and Pursuit C18 (5 μm, 150×21.2 mm, Varian, U.S.A.) were used for HPLC columns. Ultra performance liquid chromatography (UPLC) was carried out with a model Waters ACQUITY UPLC® System with PDA Detector. Column: ACQUITY UPLC®BEH C18 1.7 μm 2.1×10 mm. Prep HPLC: Preparative HPLC was carried out with a model Dynamic Mixer 811B (Gilson), 305 Pump (Gilson), 306 Pump (Gilson), and UV/VIS-155 Detector (Gilson). High-Speed Counter Current Chromatography (HSCCC): Semi-preparative HSCCC was carried out with a model TBE-1000A (Shanghai, Tauto Biotech, China). This system was equipped with TBP5002 pump (Shanghai, Tauto Biotech, China), Varian 385-LC ELS Detector (Varian), and Autochro-Win software (version 2.0, Younglin-Tech, Korea). HR-ESI-MS were measured on a Waters Q-Tof Premier spectrometer, with MassLyne V4.1 software. Column: ACQUITY UPLC®BEH C18 1.7 μm 2.1×10 mm.

Solvents for extraction and isolation (methanol, acetone, ethyl acetate, chloroform, *n*-hexane) were obtained from Duksan Chemical Co. (Korea) and Wintech (Korea), and used after redistillation. HPLC solvents were purchased from Duksan Chemical Co. (Korea) and Win-tech (Korea). UPLC solvents were purchased from Win-tech. LC-MS solvents (Acetonitrile for LC-MS, Chromasolv®) were purchased from Fluka. Solvents for NMR were purchased from CellBio (Korea).

Plant Material Whole plant of *Arthraxon hispidus*

(Thunb.) Makino was collected at Chungnam, Korea, in September 2006 and was identified by Drs. Joong-Ku Lee and Shin-Ho Kang, senior researchers at KRIBB (Korea Research Institute of Bioscience and Biotechnology). A voucher specimen (accession number 0007378) has been deposited at the Plant Extract Bank, KRIBB, Daejeon, Korea.

Extraction and Isolation The dried whole plant of *A. hispidus* (18 kg) was extracted with MeOH (30 L) three times at room temperature and concentrated under reduced pressure to obtain MeOH extract (1.5 kg). The MeOH extract was suspended in hot H_2O and partitioned with *n*-hexane, ethyl acetate, and butanol, sequentially. Each solvent fraction was evaporated to give a residue of 152.7 g, 451.9 g and 364.3 g, respectively.

The ethyl acetate fraction was selected for the follow-up isolation work due to its anti-allergic effect (64% inhibition of IL-6 production at a concentration of 20 μg/mL) in the initial screen using BMMC assay system. The EtOAc-soluble fraction was chromatographed on a silica gel column eluting with a gradient mixture of hexane–EtOAc (5 : 1, 3 : 1, 1 : 1), and then with a gradient of increasing polarity EtOAc–MeOH (10 : 1, 5 : 1, 3 : 1, 1 : 1), $CHCl_3$–MeOH (30 : 1, 10 : 1, 5 : 1, 1 : 1) and $CHCl_3$–MeOH–H_2O (75 : 25 : 1) to afford 16 fractions (AE1–AE16). AE7 was chromatographed on a silica gel column eluting with a gradient mixture of hexane–EtOAc (20 : 1, 15 : 1, 10 : 1) to give compound **15** (9 mg, 0.002% yield from EtOAc extract). AE11 was subjected to a RP-18 column chromatography eluting with MeOH–H_2O (50 : 50, 60 : 40, 80 : 20, 100 : 0) to give five sub-fractions (AE11A–AE11E). AE12 was chromatographed on a silica gel column eluting with a gradient mixture of hexane–EtOAc (5 : 1, 1 : 1) and with a gradient of increasing polarity $CHCl_3$–MeOH (50 : 1, 40 : 1, 30 : 1, 20 : 1, 5 : 1, 1 : 1, 0 : 100) to yield nine sub-fractions (AE12A to AE12I). AE12I was chromatographed on a silica gel column eluting with a gradient mixture of $CHCl_3$–MeOH (30 : 1, 20 : 1, 15 : 1, 10 : 1, MeOH) to yield seven sub-fractions (AE12I-1 to AE12I-7). AE12I-2 fraction gave five sub-fractions (AE12I-2-a–AE12I-2-e) by RP-VLC (reversed-phase vacuum liquid chromatography) eluting with a gradient mixture of MeOH–H_2O (40 : 60, 80 : 20, 100 : 0). From the fifth sub-fraction (AE12I-2-e), compound **10** (2.5 mg, 0.0006%) was obtained by preparative HPLC (Pursuit C18, MeOH–H_2O, 60 : 40, flow rate: 10 mL/min). AE12I-2-d was chromatographed on HSCCC ($CHCl_3$–isopropanol–MeOH–H_2O=4 : 0.25 : 3 : 2 v/v, upper phase for stationary phase, lower phase for mobile phase, flow rate: 2.5 mL/min, 500 rpm, forward) to yield three sub-fractions (AH12I-2-d-CIMW-A–AH12I-2-d-CIMW-C). AE12I-3 was subjected to RP-VLC using a gradient mixture of MeOH–H_2O (30 : 70, 40 : 60, 50 : 50, 60 : 40, 80 : 20, 100 : 0) to yield seven sub-fractions (AH12I-3-a–AH12I-3-g). Combined AH12I-3-d and AH12I-3-a was subjected to preparative HPLC (Synergi polar-RP 80A, MeOH–H_2O, 60 : 40, flow rate: 10 mL/min) to give compound **11** (1.5 mg, 0.0003%). AE12I-4 was subjected to RP-VLC using a gradient mixture of MeOH–H_2O (40 : 60, 50 : 50, 60 : 40, 80 : 20, 100 : 0) to yield six sub-fractions (AH12I-4-a–AH12I-4-f). AH12I-4-f was chromatographed to RP with CH_3CN–H_2O (30 : 70) to give compound **14** (53 mg, 0.012%). AE12I-5 was subjected to RP-VLC eluting with a gradient mixture of MeOH–H_2O (40 : 60, 50 : 50, 60 : 40, 80 : 20, MeOH) to yield five sub-fractions (AE12I-5-a to AE13I-5-e). The second sub-fraction (AE12I-5-b) was applied on a Sephadex LH-20, to give 18 sub-fractions. These sub-fractions were subjected to preparative HPLC (Synergi polar-RP 80A, CH_3CN–H_2O, 30 : 70, flow rate: 10 mL/min) to obtain compounds **7** (28 mg, 0.0062%), **1** (9 mg, 0.002%), **2** (4.6 mg, 0.001%), and **3** (72.8 mg, 0.016%). AE13 was subjected to RP-VLC, eluted with a gradient mixture of MeOH–H_2O (30 : 70, 40 : 60, 50 : 50, 80 : 20, 100 : 0), to yield five sub-fractions (AE13A to AE13E). AE13B-2 was subjected to Sephadex LH-20 (MeOH–H_2O=70 : 30) to give 18 sub-fractions (AE13B-2-a–AE13B-2-t). The seventh sub-fraction (AE13B-2-g) was subjected to preparative HPLC (Synergi polar-RP 80A, CH_3CN–H_2O, 30 : 70–36 : 74, flow rate: 10 mL/min) to give compound **3** (28.7 mg, 0.0064%). AE13B-2-i, AE13B-2-j, AE13B-2-k and AE13B-2-l were subjected to HSCCC (hexane–EtOAc–MeOH–H_2O=1 : 5 : 3 : 5 v/v, upper phase for stationary phase, lower phase for mobile phase, flow rate: 5 mL/min, 500 rpm, forward), to give compounds **5** (233 mg, 0.052%), **8** (16.9 mg, 0.038%), **9** (13.6 mg, 0.03%), **6** (410 mg, 0.091%), and **4** (23.9 mg, 0.0053%), respectively. AE15 was subjected to RP-VLC eluting with a gradient mixture of MeOH–H_2O (10 : 90, 20 : 80, 30 : 70, 80 : 20, 100 : 0) to yield five sub-fractions (AE15A–AE15E). Compounds **12** (2 mg, 0.0004%) and **13** (12.6 mg, 0.0028%) were isolated by preparative HPLC (Synergi polar-RP 80A, MeOH–H_2O=60 : 40, flow rate: 5 mL/min) from sub-fraction AE15B.

Luteolin 8-*C*-*β*-Kerriopyranoside (**1**): Yellow powder; mp 157–159°C; $[\alpha]_D^{25}$ +64.4° (*c*=0.1, MeOH); UV (MeOH) λ_{max} (log ε) 296 (3.83), 303 (3.83), 349 (4.13) nm; HR-ESI-MS *m/z* 413.0879 $[M-H]^-$ (Calcd for $C_{21}H_{17}O_9$, 413.0873). For ^{1}H-(400 MHz) and ^{13}C-NMR (100 MHz) spectroscopic data of **1**, see Table 1.

Luteolin 8-Acetic Acid Methyl Ester (**2**): Yellow powder; mp 152–154°C; UV (MeOH) λ_{max} (log ε) 248 (4.01), 270 (4.03), 349 (4.11) nm; HR-ESI-MS *m/z* 357.0621 $[M-H]^-$ (Calcd for $C_{18}H_{13}O_8$, 357.0610). For ^{1}H- (400 MHz) and ^{13}C-NMR (100 MHz) spectroscopic data of **2**, see Table 1.

7-Methyl-luteolin 8-*C*-*β*-(6-Deoxy-xylo-3-uloside) (**3**): Yellow powder; mp 160–162°C; $[\alpha]_D^{25}$ +58.5° (*c*=0.1, MeOH); UV (MeOH) λ_{max} (log ε) 256 (4.21), 349 (4.27) nm; HR-ESI-MS *m/z* 443.0979 $[M-H]^-$ (Calcd for $C_{22}H_{19}O_{10}$, 443.0978). For ^{1}H- (400 MHz) and ^{13}C-NMR (100 MHz) spectroscopic data of **3**, see Table 1.

Apigenin 8-*C*-*α*-Fucopyranoside (**4**): Yellow powder; mp 168–170°C; $[\alpha]_D^{25}$ +16.3° (*c*=0.1, MeOH); UV (MeOH) λ_{max} (log ε) 270 (4.21), 328 (4.21) nm; HR-ESI-MS *m/z* 415.1083 $[M-H]^-$ (Calcd for $C_{21}H_{19}O_9$, 415.1029). For ^{1}H- (400 MHz) and ^{13}C-NMR (100 MHz) spectroscopic data of **4**, see Table 2.

Apigenin 8-*C*-*β*-Fucopyranoside (**5**): Yellow powder; mp 167–168°C; $[\alpha]_D^{25}$ +58.7° (*c*=0.1, MeOH); UV (MeOH) λ_{max} (log ε) 269 (4.11), 337 (4.18) nm; HR-ESI-MS *m/z* 415.1089 $[M-H]^-$ (Calcd for $C_{21}H_{19}O_9$, 415.1029). For ^{1}H- (400 MHz) and ^{13}C-NMR (100 MHz) spectroscopic data of **5**, see Table 2.

Luteolin 8-*C*-*β*-Fucopyranoside (**6**): Yellow powder; mp 186–188°C; $[\alpha]_D^{25}$ +73.7° (*c*=0.1, MeOH); UV (MeOH) λ_{max} (log ε) 270 (4.12), 309 (3.90), 354 (4.24) nm. HR-ESI-MS: 431.1034 $[M-H]^-$ (Calcd for $C_{21}H_{19}O_{10}$, 431.0978). For ^{1}H-(400 MHz) and ^{13}C-NMR (100 MHz) spectroscopic data of **6**, see Table 2.

Preparation and Activation of Bone Marrow-Derived Mast Cells (BMMC) The bone marrow cells from male Balb/cJ mice were cultured for up to 10 weeks in 50% en-

riched medium (RPMI 1640 containing 2 mM L-glutamine, 0.1 mM nonessential amino acids, antibiotics, and 10% fetal calf serum) and 50% WEHI-3 cell conditioned medium as a source of IL-3. After 3 weeks >98% of the cells were bone marrow-derived mast cells (BMMC) when checked by the previously described procedure.[23)]

Determination of Interleukin-6 Levels The cells were seeded at 1×10^6/mL per well in 24 well tissue culture plates and pretreated with indicated concentration of *A. hispidus* for 30 min before stimulation. The supernatant was decanted into a new microcentrifuge tube, and the amount of IL-6 was determined using the enzyme-linked immunosorbent assay (ELISA) kit according to the procedure described by the manufacturer (BD Bioscience, U.S.A.). All subsequent steps took place at room temperature, and all standards and samples were assayed in duplicate.

Determination of Prostaglandin D_2 (PGD_2) and Leukotriene C_4 (LTC_4) Levels The BMMCs were activated with PMA (50 nM) plus A23187 (1 μM) at 37°C for 1 h in the presence or absence of compounds dissolved in phosphate buffered saline (PBS). The supernatants were isolated for further analysis by enzyme immunoassay (EIA). PGD_2 and LTC_4 were determined using an EIA kit (Cayman Chemical, Ann Arbor, MI, U.S.A.). Data are reported as the arithmetic mean of triplicate determinations.

Assay of β-Hexosaminidase Release β-Hexosaminidase (β-HEX), a marker of mast cell degranulation, was quantitated by spectrophotometric analysis of the hydrolysis of *p*-nitrophenyl-2-acetamido-2-deoxy-β-D-glucopyranoside hydrolysis (Sigma Chemical Co.). Briefly, after harvesting the supernatant, the cells were lysed in the same volume of the medium by freezing and thawing three times. A 10 mL of the BMMC lysate or supernatant samples were mixed with 50 mL of β-HEX substrate solution (1.3 mg/mL *p*-nitrophenyl-2-acetamido-2-deoxy-β-D-glucopyranoside in 100 mM sodium citrate, pH 4.5) in each well of 96-well plates and then incubated at 37°C for 60 min. The reaction was stopped by adding 150 μL of 0.2 M glycine–NaOH (pH 10.7). The absorbance at 410 nm was measured in a micro plate reader. A percentage of β-HEX released into the supernatant was calculated by the following formula: $[S/(S-P)]\times100$, where S and P are the β-HEX contents of the supernatants and cell pellets, respectively.

RNA Extraction and Reverse Transcription-Polymerase Chain Reaction (RT-PCR) Total cellular RNA was isolated using an easy-BLUE™ RNA extraction kit according to the manufacturer's instructions. Briefly, the total RNA (2 μg) was converted to cDNA by treatment with 200 units of reverse transcriptase and 500 ng of oligo-dT primer in 50 mM Tris–HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 10 mM dithiothreitol (DTT), and 1 mM deoxyribonucleotide triphosphates (dNTPs) at 42°C for 1 h. The reaction was then stopped by incubating the solution at 70°C for 15 min, after which, 3 μL of the cDNA mixture was used for enzymatic amplification. PCR was then performed using a reaction mixture comprised of 50 mM KCl, 10 mM Tris–HCl (pH 8.3), 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 2.5 units of Taq DNA polymerase, and 0.1 μM each of primers specific for cyclooxygenase-2 (COX-2), 5-LOX and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The amplification conditions were as follows: denaturation at 94°C for 3 min for the first cycle and then 30 cycles of 94°C for 45 s, annealing of COX-2 at 55°C for 45 s annealing of 5-LOX at 55°C for 45 s annealing of IL-6 at 60°C for 45 s with a final extension at 72°C for 7 min. The PCR products were then electrophoresed on a 1.5% agarose gel and stained with ethidium bromide. The primers used were 5′-GCC CAA CAA TAC AAA TGA CCC TA-3′ (sense) and 5′-TCC TGT TGT TTC TAT TTC CTT TGT-3′(antisense) for IL-6, 5′-CTC AGT TTG TTG AGT CAT TC-3′ (sense) and 5′-CAT TGA TGG TGG CTG TTT TG-3′(antisense) for COX-2, 5′-CTA GAG CGG CAG CTC AGT TT-3′ (sense) and 5′-GTG ATC GTT TGA TGG ACG TG-3′ (antisense) for 5-LOX, and lastly, 5′-CCC ATC ACC ATC TTC CAG-3′ (sense) and 5′-ATG ACC TTG CCC ACA GCC-3′ (antisense) for GAPDH.

Statistical Analysis The data from the experiments are presented as the mean±S.E.M. The level of statistical significance was determined by the analysis of variance (ANOVA), followed by Dunnett's *t*-test for multiple comparisons. p Values of less than 0.05 were considered to be significant.

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