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ORIGINAL PAPER

Chemical constituents with anti-allergic activity from the root of Edulis Superba, a horticultural cultivar of *Paeonia lactiflora*

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Abstract The methanolic extract and its subfractions from the dried root of Edulis Superba, a horticultural cultivar of *Paeonia lactiflora* Pallas, showed potent anti-allergic effect as inhibition of immunoglobulin E (IgE)-mediated degranulation in rat basophil leukemia (RBL)-2H3 cells. Bioassay-guided fractionation led to the isolation of 26 compounds, including a new norneolignan glycoside, paeonibenzofuran (**1**), together with 25 known ones (**2**–**26**). The chemical structure of the new compound was elucidated on the basis of spectroscopic and chemical evidences. Among the isolated compounds, mudanpioside E (**5**) with paeoniflorin-type skeleton and quercetin (**16**) showed potent inhibitory activity against a degranulation marker, β-hexosaminidase release with IC_{50} values of 40.34 and 25.05 μM, respectively. A primary structure–activity relationship of these components was discussed.

Keywords *Paeonia lactiflora* · Horticultural cultivar · Bioactive constituents · Anti-allergic activity

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Introduction

Peony root, called "Shakuyaku" in Japanese, is prescribed as the root of *Paeonia lactiflora* Pallas with no less than 2.0 % of paeoniflorin in the Japanese Pharmacopoeia [1]. As an analgesic, antispasmodic, astringent, etc., peony root has been widely used in approximately one-third of generally used Kampo formulas in Japan [2], such as Kakkonto, Tokishakuyakusan, and Keishibukuryogan. Peony root in the Japanese market is mainly imported from China and partly produced domestically; for instance, in 2012, 1,407,825 kg of peony root was imported and 81,336 kg was domestically produced [3]. In the course of our study to search for new resources of peony root and further to promote domestic production, several horticultural cultivars of *P. lactiflora* were selected as promising candidates which have potential as a medicinal resource on the basis of genetic and chemical analyses [4, 5].

Recently, the anti-allergic activity of peony root has attracted much attention [6–8]. Type I allergic reactions are triggered by the binding of antigen to specific IgE antibody which has been produced after initial stimulation with the antigen, and then this signal sensitizes mast cells and basophils via high-affinity IgE receptor (FcεRI) to release allergic mediators, such as β-hexosaminidase, histamine, and cytokines [9, 10]. β-Hexosaminidase is considered as a degranulation marker and the inhibition of β-hexosaminidase release in rat basophil leukemia (RBL)-2H3

cells has been widely employed as an in vitro model of IgE-mediated degranulation in mast cells for screening candidates with anti-allergic drugs from natural resources [6–8, 11, 12]. We have previously reported the anti-allergic activity of the characteristic monoterpenoids from red peony root derived from wild *P. lactiflora* growing in Inner Mongolia, China [13]. In a pilot experiment, both water and methanol extracts of the root of Edulis Superba, a horticultural cultivar of *P. lactiflora*, showed relatively strong anti-allergic activity among 17 cultivars of *P. lactiflora*. Therefore, subsequent bioassay-guided fractionation of the methanol extract was conducted, which led to the isolation of 26 compounds, including one new norneolignan glycoside, and the anti-allergic activities of the isolates were further investigated.

Results and discussion

Methanol extract from the root of Edulis Superba showed potent anti-allergic activity as inhibitory effects against 2,4-dinitrophenylated bovine serum albumin (DNP-BSA) stimulated β-hexosaminidase release in IgE-sensitized RBL-2H3 cells (IC_{50}: 0.72 ± 0.02 mg/ml). After partition with EtOAc and *n*-BuOH, respectively from methanol extract, both EtOAc-soluble and *n*-BuOH-soluble subfractions exhibited potent inhibitory effects against β-hexosaminidase release (IC_{50}: 0.32 ± 0.01 and 0.58 ± 0.01 mg/ml, respectively). Therefore, the EtOAc-soluble and *n*-BuOH-soluble fractions were further subjected to normal- and reversed-phase column chromatography (CC), as well as preparative high-performance liquid chromatography (HPLC) to afford a new norneolignan glycoside, paeonibenzofuran (**1**), and 25 known compounds (Fig. 1). The spectral data of compounds **2**–**26** were consistent with those reported in previous literatures; therefore, they were identified as paeoniflorin (**2**) [14], salicylpaeoniflorin (**3**) [14], oxypaeoniflorin (**4**) [14], mudanpioside E (**5**) [15], albiflorin (**6**) [14], 1,2,3,4,6-penta-*O*-galloyl-β-D-glucose (**7**) [15], (*2S*)-(−)-naringenin-5-*O*-β-D-glucopyranoside (**8**) [16], (*2R*)-(−)-naringenin-5-*O*-β-D-glucopyranoside (**9**) [16], (*2R*)-(−)-naringenin-7-*O*-β-D-glucopyranoside (**10**) [17], (+)-catechin (**11**) [17], (+)-catechin-7-*O*-gallate (**12**) [18], (+)-catechin-3-*O*-β-D-glucopyranoside (**13**) [17], (−)-epicatechin-3-*O*-gallate (**14**) [18], taxifolin-3-*O*-β-D-glucopyranoside (**15**) [19], quercetin (**16**) [20], quercetin-3-*O*-β-D-glucopyranoside (**17**) [20], neohancoside C (**18**) [21], paeonol (**19**) [22], paeonolide (**20**) [22], benzoic acid (**21**) [23], poacynose (**22**) [24], gallic acid (**23**) [22], methyl gallate (**24**) [22], 3,4-dihydro-4-hydroxy-naphthalene-2-carboxylic acid (**25**) [25], and phenyl-*O*-β-xylopyranosyl (1 → 6)-*O*-glucopyranoside (**26**) [26]. The structure of compound **1** was determined as follows.

Compound **1** was isolated as white amorphous powder. Quasimolecular ions at *m/z* 475.1632 $[M - H]^-$ and 521.1684 $[M + HCOO]^-$ in the HR-ESI-MS spectrum suggested a molecular formula of $C_{24}H_{28}O_{10}$. The IR spectrum showed absorption bands at 3397 cm^{-1} (hydroxyl group) and 1600, 1515, and 1485 cm^{-1} (aromatic ring). The ^{1}H-NMR spectrum of **1** (Table 1) presented signals attributable to a vinylic proton at δ_H 6.78 (1H, d, J = 1.2 Hz, H-3); a methoxyl signal at δ_H 3.86 (3H, s); a C3 fragment of $CH_2CH_2CH_2OH$ [δ_H 2.63 (2H, t, J = 8.0 Hz, H-8); δ_H 1.78 (2H, m, H-9); and δ_H 3.51 (2H, t, J = 6.0 Hz, H-10)]; and two sets of aromatic signals ascribable to a trisubstituted benzene ring [δ_H 6.77 (1H, d, J = 8.0 Hz, H-5′); δ_H 7.27 (1H, dd, J = 1.6, 8.0 Hz, H-6′), and δ_H 7.34 (1H, d, J = 1.6 Hz, H-2′)] and a tetrasubstituted benzene ring [δ_H 6.83 (1H, br s, H-6) and δ_H 6.94 (1H, d, J = 1.6 Hz, H-4)]; together with a series of proton signals assignable to a glucopyranosyl moiety. In addition, the acid hydrolysis of **1** yielded β-D-glucopyranose. The ^{13}C-NMR spectrum displayed 24 carbon signals, which included characteristic signals of two vinylic carbons (δ_C 100.7, C-3; δ_C 158.1, C-2), a methoxyl signal (δ_C 56.6), a C3 fragment of CH_2CH_2-CH_2OH (δ_C 33.2, C-8; δ_C 35.8, C-9; δ_C 62.5, C-10), 12 aromatic carbons (δ_C 109.6–149.3), and a set of glucopyranosyl carbons (δ_C 62.2–102.6). The NMR data mentioned above closely resembled those of 5-(3″-hydroxypropyl)-7-methoxy-2-(3′,4′-dimethoxyphenyl) benzofuran [27–30], except for the absence of signals of two methoxyl groups, showing instead a set of additional signals for a glucopyranosyl moiety. The long-range correlations between H-1″ (δ_H 5.16) and C-7 (δ_C 143.3) in the HMBC spectrum (Fig. 2) confirmed that glucosylation was at C-7 of the benzofuran skeleton. In addition, the correlation from methoxyl protons (δ_H 3.86) to C-3′ (δ_C 148.8) in the HMBC spectrum indicated that the methoxyl moiety was connected to C-3′, which was also confirmed by the NOE enhancement signal of H-2′ resulting from irradiation at methoxyl protons. The correlations from H-9 to C-5, as well as from H-8 to C-4 and C-6 in the HMBC spectrum, were clearly observed, which indicated that the C-3 unit was connected to C-5. The correlations between H-8 and H-9, H-9 and H-10, as well as H-5′ and H-6′ were clearly observed in the ^{1}H-^{1}H COSY spectrum (Fig. 2). Consequently, compound **1** was identified as 2-(4′-hydroxyl-3′-methoxyphenyl)-5-(3-hydroxypropyl)-benzofuran-7-*O*-β-D-glucopyranoside and named as paeonibenzofuran.

The 26 isolated compounds were further examined for their anti-allergic activity as inhibitory effects against DNP-BSA stimulated β-hexosaminidase release in IgE-sensitized RBL-2H3 cells, and their IC_{50} values are shown in Table 2. Cell viability under stimulation was evaluated by the Cell Counting Kit (CCK)-8 assay, and all of the test compounds at several dose levels from 10.0 up to

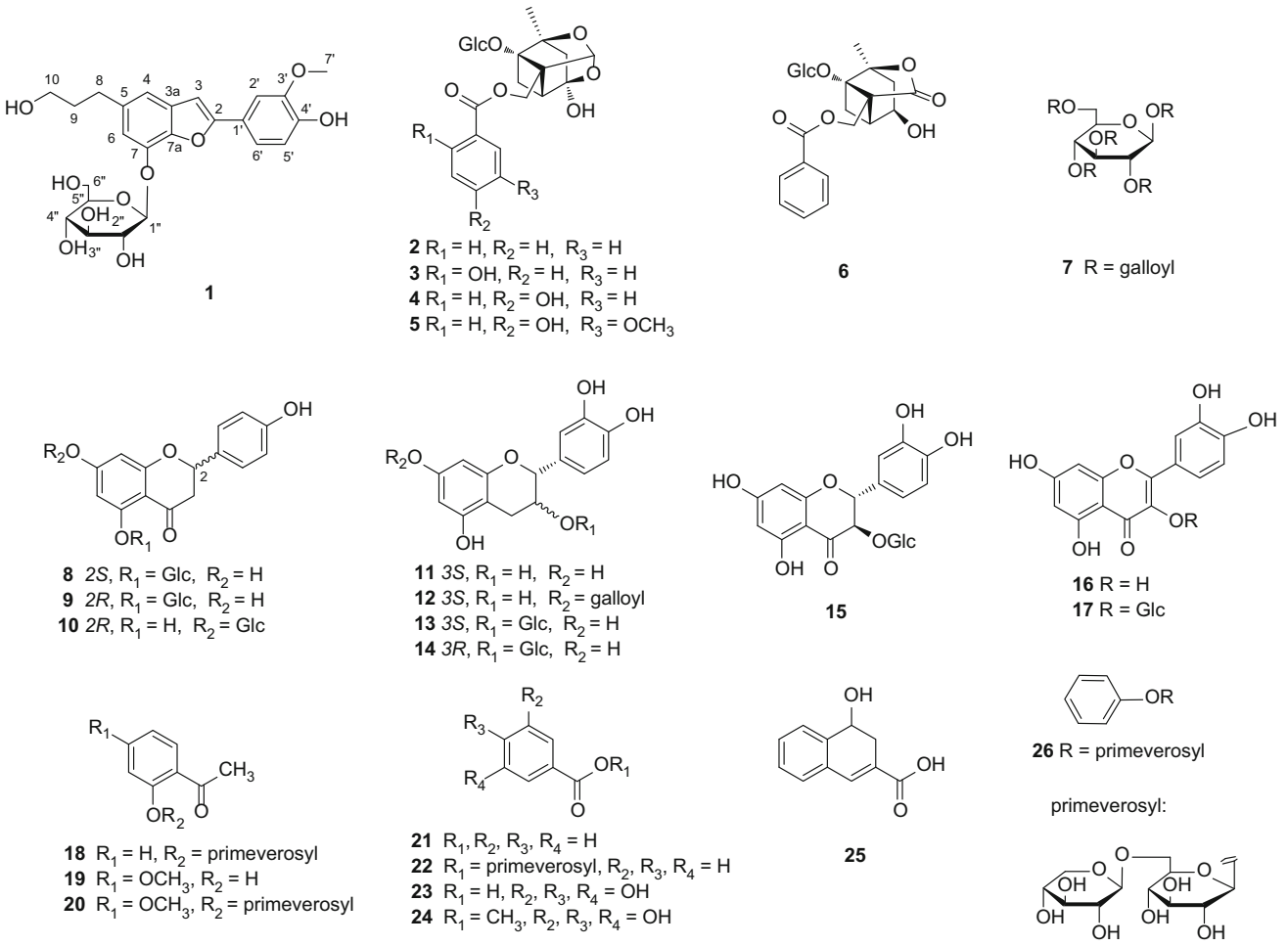

Fig. 1 Structures of the chemical constituents isolated from the root of Edulis Superba, a horticultural cultivar of *Paeonia lactiflora*

Table 1 ^{13}C-NMR (100 MHz) and ^{1}H-NMR (400 MHz) spectroscopic data of compound **1** measured in CD_3OD (δ in ppm)

1 (paeonibenzofuran)					
Position	δC	δH (*J* in Hz)	Position	δC	δH (*J* in Hz)
1			**1′**	123.8	
2	158.1		**2′**	109.6	7.34, d (1.6)
3	100.7	6.78, d (1.2)	**3′**	148.8	
3a	133.1		**4′**	149.3	
4	115.0	6.94, d (1.6)	**5′**	116.6	6.77, d (8.0)
5	139.1		**6′**	119.5	7.27, dd (1.6, 8.0)
6	113.0	6.83, br s	**1″**	102.6	5.16, d (6.8)
7	143.3		**2″**	75.0	3.50, dd (7.6, 8.8)
7a	143.8		**3″**	78.1	3.59, dd (8.8, 9.2)
8	33.2	2.63, t (8.0)	**4″**	71.4	3.35, m
9	35.8	1.78, m	**5″**	78.3	3.33, m
10	62.5	3.51, t (6.0)	**6″**	62.2	3.62, dd (5.2, 12.0)
					3.76, dd (1.2, 12.0)
			OCH_3	56.6	3.86, s

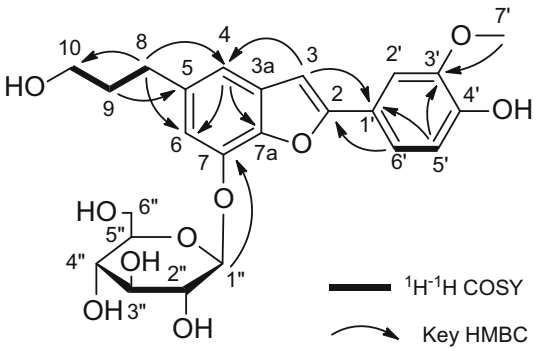

Fig. 2 Important 2D-NMR correlations of compound **1**

Table 2 Inhibitory effects of the 26 isolated components against DNP-BSA stimulated β-hexosaminidase release in IgE-sensitized RBL-2H3 cells

Compound no.	IC_{50} (μM)	Compound no.	IC_{50} (μM)
1	62.50 ± 1.8[a]	**17**	42.55 ± 2.0
2	76.02 ± 1.4	**18**	65.44 ± 2.5
3	42.22 ± 2.9	**20**	48.84 ± 1.4
4	87.11 ± 1.7	**21**	88.92 ± 2.7
5	40.34 ± 3.2	**22**	68.71 ± 3.2
7	62.00 ± 1.4	**23**	78.11 ± 3.1
8	91.40 ± 1.5	**24**	47.72 ± 1.0
9	68.36 ± 1.3	**25**	78.51 ± 2.3
10	60.70 ± 1.1	**6, 11–15, 19, 26**	NA[b]
16	25.05 ± 4.0	**Baicalein**[c]	37.78 ± 1.0

[a] The results are expressed as the mean ± standard deviation (SD); IC_{50} values were calculated by probit regression analysis of the SPSS software package (version 15.0)

[b] IC_{50} values are more than 100 μM

[c] Positive control [12, 34]

100.0 μM showed no observable cytotoxicity. Among the 26 isolated compounds, 18 compounds exhibited anti-allergic activity, with IC_{50} values ranging from 25.05 to 91.40 μM. As for the monoterpenoids, which are considered as the main and characteristic constituents of peony root, compounds **2**–**5** with paeoniflorin-type skeleton showed moderate activities, but albiflorin (**6**) with the lactone ring in the pinane aglycone exhibited no activity. Except for **5**, compounds **2**–**4** and **6** have also been isolated from red peony root in our previous paper [13]. Of the four paeoniflorin derivatives, compound **5** with 3-methoxy-4-hydroxybenzoyl moiety at C-8 showed the highest activity (IC_{50}: 40.34 μM), and compound **3** with *O*-hydroxybenzoyl moiety at C-8 exhibited higher activity than the two compounds with benzoyl (**2**) or *p*-hydroxybenzoyl moieties (**4**) at C-8. Quercetin (**16**) exhibited the most potent activity (IC_{50}: 25.05 μM), which was stronger than that of baicalein, the positive control (IC_{50}: 37.78 μM). A similar tendency has been previously reported that quercetin showed stronger activity against IgE-mediated allergic mediator release from human cultured mast cells than baicalein [31]. In addition, the other flavonol (**17**) also exhibited high inhibitory activity, followed by three flavanones (**8**–**10**), whereas four flavan-3-ols (**11**–**14**) and a flavanonol (**15**) showed no activity. These results suggested that the vinylic structure at C2-C3 of flavonols was important for the activity (**16**, **17** vs. **15**), and glycosylation of hydroxyl group at C-3 in flavonols reduced the inhibitory activity (**16** vs. **17**). Flavanones having the C-2 (*R*) configuration was more active than that of the C-2 (*S*) configuration (**9**, **10** vs. **8**). Among the three paeonol derivatives (**18**–**20**), compounds **18** and **20** with primeverosyl moiety at C-4 of aglycone exhibited inhibitory activity, but paeonol (**19**) had no activity.

To date, several studies have reported that paeoniflorin (**2**), paeonol (**19**), and 1,2,3,4,6-penta-*O*-galloyl-β-D-glucose (**7**) showed high potency of anti-allergic activity both in vitro and in vivo [6, 7]. In the present study, several other compounds belonging to monoterpenoids and flavonol, such as mudanpioside E (**5**), salicylpaeoniflorin (**3**), quercetin (**16**), etc., are also documented to make a contribution to the anti-allergic activity of peony root as inhibitory effect against IgE-mediated degranulation in RBL-2H3 cells.

Experimental

General experimental procedures

NMR spectra were recorded on a JEOL ECX-400 spectrometer (^{1}H, 400 MH_Z; ^{13}C, 100M H_Z) with TMS as an internal standard. HR-ESI-MS spectra were measured by an Accela HPLC system equipped with an Orbitrap-EX mass spectrometer (Thermo Fisher Scientific Inc., USA). IR spectra were measured by using a JASCO FT/IR-460 Plus spectrophotometer (JASCO Internal Co., Ltd., Japan). Optical rotations were detected by using a JASCO P2100 digital polarimeter (JASCO Internal Co., Ltd., Japan). CC was performed with normal-phase (Wakogel® C-200 and 300HG, Wako Pure Chemical Industries, Ltd., Japan), reverse-phase [ODS-A (12 nm, s-150 μm) and ODS-AQ-HG (12 nm, s-50 μm), YMC Co., Ltd., Japan], and Sephadex LH-20 (GE Healthcare Life Sciences, Sweden). Preparative HPLC was performed on a Waters HPLC system (Waters Co., Ltd., USA), equipped with a Delta 600 pump and 2489 UV detector by using a YMC-Pack ODS-A column (5 μm, 250 × 20 mm i.d.). TLC was carried out to monitor fractions on precoated silica gel ($60F_{254}$) or RP-18 (F_{254}) plates (0.50 mm thickness, Merck, Germany). Spots were visualized under UV (254 and 360 nm) and/or

monitored by spraying with the solution of 10 % sulfuric acid in ethanol (v/v). L-Cysteine methyl ester hydrochloride, phenyl isothiocyanate, L-(−)-glucose (purity ≥ 98 %), D-(+)-glucose (purity ≥ 98 %), and pyridine were purchased from Wako Pure Chemical Industries, Ltd.

Plant material

The root of a cultivar of *P. lactiflora*, named Edulis Superba, was harvested from Toyama Prefectural Medicinal Plants Center, Toyama, Japan in November 2013. It was identified as a RPR type of *P. lactiflora* by genetic analysis of the nrDNA-ITS sequence [2, 4, 32]. The voucher (S78N) was deposited in the Museum of Materia Medica, Institute of Natural Medicine, University of Toyama, Japan.

Extraction and isolation

The dried root (1.2 kg) was cut into small pieces and then extracted repeatedly with methanol (3 × 2.0 L) ultrasonically for 2 h at room temperature. The MeOH extract (330.0 g) was obtained after removing the solvent under vacuum, and was then suspended in water and partitioned with EtOAc and *n*-BuOH, respectively. The anti-allergic activity assay showed that the EtOAc and *n*-BuOH-soluble fractions had potent activities against β-hexosaminidase release in IgE-sensitized RBL-2H3 cells, so the two elutions were further isolated for tracking the active constituents. EtOAc-soluble fraction (100.0 g) was subjected to normal-phase silica gel CC using $CHCl_3$–MeOH gradient elution from 100:1 to 2:1 (v/v) to obtain eight fractions (EF-1 to EF-8). EF-1 was separated by preparative HPLC eluted with isocratic acetonitrile (CH_3CN)–H_2O (30:70, v/v) to give **19** (12.0 mg). Compounds **21** (100.0 mg) from EF-5 and **7** (10.0 mg) from EF-6 were obtained by using preparative HPLC eluted with isocratic CH_3CN–H_2O (30:70, v/v). EF-7 was subjected to YMC-ODS-A CC and eluted with MeOH–H_2O (1:9 to 1:0, v/v) to give eight fractions (EF-7-1 to EF-7-8). EF-7-6 was further separated by YMC-ODS-AQ CC eluted with MeOH–H_2O (2:8 to 1:0, v/v) to give five fractions (EF-7-6-1 to EF-7-6-5). Compounds **10** (4.0 mg) from EF-7-6-4 and **11** (10.0 mg) from EF-7-6-2 were obtained by using preparative HPLC eluted with CH_3CN–H_2O (15:85, v/v). EF-7-7 was subjected to YMC-ODS-Rp18 CC eluted with MeOH–H_2O (2:8 to 1:1, v/v) to obtain seven fractions (EF-7-7-1 to EF-7-7-7). EF-7-7-1 was purified by using preparative HPLC eluted with CH_3CN–H_2O (10:90, v/v) to give **23** (11.0 mg) and **24** (8.0 mg). Compounds **2** (100.0 mg) and **6** (50.0 mg) were purified from EF-7-7-2 and EF-7-7-3 by using preparative HPLC eluted with CH_3CN–H_2O (15:85, v/v). Compounds **16** (5.0 mg) and **17** (4.0 mg) were purified from EF-7-7-4 by using the preparative HPLC eluted with CH_3CN–H_2O (12:88, v/v). EF-7-7-5 was subjected to Sephadex LH-20 CC eluted with MeOH–H_2O (6:4, v/v) to give 13 fractions (EF-7-7-5-1 to EF-7-7-5-13). Compounds **1** (5.0 mg) and **3** (3.0 mg) were obtained from EF-7-7-5-4 by using preparative HPLC eluted with CH_3CN-H_2O (25:75, v/v). Compounds **8** (8.0 mg) and **9** (8.0 mg) were obtained from EF-7-7-5-8 and EF-7-7-5-9 by using preparative HPLC eluted with CH_3CN-H_2O (20:80, v/v), respectively. In addition, the *n*-BuOH-soluble fractions (50.0 g) were subjected to Daion HP-21 CC eluted with a gradient of H_2O and MeOH as 0, 30, 60, and 100 % MeOH (v/v), respectively. The 30 % MeOH-soluble layer was subjected to YMC-ODS-A CC eluted with MeOH–H_2O (5:95 to 90:10, v/v) to obtain eight fractions (30MF-1 to 30MF-8). 30MF-4 was further subjected to YMC-ODS-RP18 CC eluted with MeOH–H_2O (1:9 to 6:4, v/v) to give six fractions (30MF-4-1 to 30MF-4-6). 30MF-4-2 was separated by using Sephadex LH-20 CC eluted with MeOH–H_2O (6:4, v/v) to give seven fractions (30MF-4-2-1 to 30MF-4-2-7). Compounds **12** (3.0 mg) and **14** (3.0 mg) were purified from 30MF-4-2-5, and **13** (20.0 mg) and **15** (4.0 mg) from 30MF-4-2-4 by using preparative HPLC eluted with CH_3CN–H_2O (12:88, v/v). 30MF-4-3 was further eluted with MeOH–H_2O (1:9 to 6:4, v/v) by using YMC-ODS-AQ CC to give four fractions (30MF-4-3-1 to 30MF-4-3-4). Compounds **25** (3.0 mg) and **26** (8.0 mg) were purified from 30MF-4-3-1 by using preparative HPLC eluted with CH_3CN–H_2O (12:88, v/v). Compounds **18** (5.0 mg) and **4** (380.0 mg) were obtained from 30MF-4-3-2 and 30MF-4-3-3, respectively, by using preparative HPLC eluted with CH_3CN–H_2O (13:87, v/v). 30MF-4-3-4 was subjected to preparative HPLC eluted with CH_3CN–H_2O (13:87, v/v) to achieve **5** (10.0 mg). Compound **20** (17.0 mg) was purified from 30MF-4-4 and 30MF-4-5 by using preparative HPLC eluted with CH_3CN–H_2O (13:87, v/v). Furthermore, 30MF-5 was further subjected to YMC-ODS-AQ CC eluted with MeOH–H_2O (1:9 to 1:0, v/v) to give nine fractions (30MF-5-1 to 30MF-5-9). 30MF-5-8 was further separated by using YMC-ODS-AQ CC eluted with MeOH–H_2O (2:8 to 1:1, v/v) to give six fractions (30MF-5-8-1 to 30MF-5-8-6). 30MF-5-8-3 was purified by using preparative HPLC eluted with CH_3CN–H_2O (13:87, v/v) to afford **20** (35.0 mg). 30MF-5-8-6 was subjected to YMC-ODS-RP18 CC eluted with MeOH–H_2O (15:85, v/v) to obtain six fractions (30MF-5-8-6-1 to 30MF-5-8-6-6). Compounds **22** (3.5 mg) and **11** (14.0 mg) were obtained from 30MF-5-8-6-3 and 30MF-5-8-6-6 by using preparative HPLC eluted with CH_3CN-0.1 % acetic acid in H_2O (12:88, v/v), respectively.

The purity of each compound was determined by an HPLC-DAD system, and all of them had purities higher than 93.4 % based on the detection data at four wavelengths (210, 230, 254, and 280 nm).

Paeonibenzofuran (1)

White amorphous powder; $[\alpha]_D^{20}$ −44.77° ($c = 0.11$, MeOH); IR (KBr): ν_{max} 3397, 1600, 1515, 1485, 1286, 1065, 864 cm^{-1}; ^1H and ^{13}C-NMR data are shown in Table 1; HR-ESI-MS: *m/z* 475.1628 $[M–H]^-$, 521.1677 $[M + HCOO]^-$ (calcd. for $C_{24}H_{28}O_{10}$, 476.4731).

Acid hydrolysis and HPLC analysis of compound 1

Acid hydrolysis of compound **1** was carried out using a procedure similar to a previous report [33]. Briefly, compound **1** (1.0 mg) was hydrolyzed by heating in 1.0 M HCl (0.2 ml) at 90 °C in a stoppered vial for 2 h and neutralized with Amberlite IRA-400. After drying in vacuo, the residue was dissolved in pyridine (0.1 ml) containing L-cysteine methyl ester hydrochloride (0.5 mg) and heated at 60 °C for 1 h, and then a 0.1-ml solution of phenyl isothiocyanate (0.5 mg) was added to the mixture and heated at 60 °C for 1 h. The reaction solution was directly analyzed by an HPLC-PDA system to determine the D/L configuration of sugar moieties by comparing their retention time (t_R) with these authentic D- and L-glucose, D- and L-xylose derivatives (t_R: D-glucose, 21.05 min; L-glucose, 22.02 min; D-xylose, 25.10 min; and L-xylose, 27.05 min).

Measurement of β-hexosaminidase release in IgE-sensitized RBL-2H3 cells

The inhibitory effects of the test samples against DNP-BSA stimulated β-hexosaminidase release in IgE-sensitized RBL-2H3 cells (Cell No. RCB2782, RIKEN Cell Bank, Tsukuba, Japan) were evaluated using a previously reported method [34]. Briefly, RBL-2H3 cells were dispended into 96-well culture plates at a concentration of 4×10^5 cells/well using Eagle's Minimum Essential Medium (MEM, Gibco, Thermo Fisher Scientific Inc., USA) supplemented with 10 % fetal bovine serum (FBS, 12003C, Sigma-Aldrich Co. LLC., USA), penicillin (100 U/ml, Sigma-Aldrich), and streptomycin (100 μg/ml, Sigma-Aldrich), and cultured overnight at 37 °C in 5 % CO_2. After washing with phosphate-buffered saline (PBS, Gibco), the cells were sensitized with 0.5 μg/ml of anti-DNP IgE (D8406, Sigma-Aldrich) for 24 h, and then washed twice with 200 μl Siraganian buffer [119 mM NaCl, 5 mM KCl, 0.4 mM $MgCl_2$, 25 mM piperazine-*N*,*N*'-*bis* (2-ethanesulfonic acid) (PIPES), 40 mM NaOH; pH 7.2] and incubated in 160 μl Siraganian (+) buffer [added 5.6 mM glucose, 1 mM $CaCl_2$, and 0.1 % bovine serum albumin (BSA, A7030, Sigma-Aldrich) in Siraganian buffer; pH 7.2] for 10 min at 37 °C. Subsequently, 50 μl of the test sample solution was added to each well and incubated for 30 min, followed by 50 μl DNP-BSA (1.0 μg/ml, A23018, Thermo Fisher Scientific Inc.) as an antigen for 1 h to activate cells and to evoke allergic reactions (degranulation). The reaction was stopped by cooling in an ice bath for 10 min, and 100 μl of the supernatant were transferred to a 96-well plate and incubated with 50 μl of substrate (3.3 mM *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide) for 1 h at 37 °C. The reaction was stopped by adding 100 μl of alkaline buffer (0.1 M $NaHCO_3$ and 0.1 M Na_2CO_3; pH 10) and cooled in an ice bath for 10 min. The absorbance at 405 nm was measured using a microplate reader (Infinite® F200, Switzerland). The test samples were dissolved in dimethylsulfoxide (DMSO) and Siraganian (+) buffer (the final DMSO concentration was less than 0.1 %).

The inhibitory activity against β-hexosaminidase release of the test samples was calculated using the following equation:

$$\text{Inhibition } (\%) = [1 - (\text{At} - \text{Ab} - \text{An})/(\text{Ac} - \text{An})] \times 100$$

where:

At Test, DNP-BSA (+), sample (+)
Ab Blank, DNP-BSA (−), sample (+)
An Normal, DNP-BSA (−), sample (−)
Ac Control, DNP-BSA (+), sample (−)

The results were expressed as the mean ± standard deviation (SD) for three independent experiments. One-way analysis of variance (ANOVA) followed by post hoc Dunnett's test or the Chi-square test were used for statistical analysis, and IC_{50} values were calculated by using SPSS software (version 15.0, IBM, USA).

Measurement of cell viability

The cytotoxicity of extract, fractions, and isolated compounds on RBL-2H3 cells was evaluated with CCK-8 (Dojindo Laboratories Co., Ltd., Japan), according to the manufacturer's instructions. Briefly, anti-DNP IgE-sensitized cells were treated with various sample solutions and stimulated by DNP-BSA as described above. After washing with PBS, 100 μl 10 % FBS-MEM containing 10 μl WST-8 was added to each well and incubated for 3 h. Then, the absorbance at 450 nm was measured by using a microplate reader. The percentage of cell viability was calculated according to the following formula: Cell viability (%) = (Mean absorbance of test samples/Mean absorbance of vehicle control) × 100.

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