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Antiallergic Properties of Biflavonoids Isolated from the Flowers of *Mesua ferrea* Linn.

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Abstract: The methanolic extract from the flowers of *Mesua ferrea* Linn. (Calophyllaceae) showed significant hyaluronidase inhibitory activity. Following a bioassay-guided separation of the extract, two biflavonoids, viz., mesuaferrone-A (**1**) and mesuaferrone-B (**2**), were isolated, along with ten flavonoids (**3**–**12**), two xanthones (**13** and **14**), three triterpenes (**15**–**17**), a phenylpropanoid (**18**), and five aromatics (**19**–**24**). Among the isolates, **1** and **2** (IC_{50} = 51.1 μM and 54.7 μM, respectively) exhibited hyaluronidase inhibitory activity equivalent to that of the commercially available antiallergic agents disodium cromoglycate (64.8 μM) and ketotifen fumarate (76.5 μM). These biflavonoids (**1** and **2**) are 8-8″ linked dimers that are composed of naringenin (**1a**) or apigenin (**3**), with their corresponding monomers lacking inhibitory activity (IC_{50} > 300 μM). In addition, **1** and **2** (IC_{50} = 49.4 μM and 49.2 μM, respectively) inhibited the release of β-hexosaminidase, which is a marker of antigen-IgE-mediated degranulation, in rat basophilic leukemia (RBL-2H3) cells. These inhibitory activities were more potent than those of the antiallergic agents tranilast and ketotifen fumarate (IC_{50} = 282 μM and 158 μM, respectively), as well as one of the corresponding monomers (**1a**; IC_{50} > 100 μM). Nonetheless, these effects were weaker than those of the other monomer (**3**; IC_{50} = 6.1 μM).

Keywords: *Mesua ferrea*; mesuaferrone; biflavonoid; hyaluronidase inhibitor; degranulation inhibitor; Calophyllaceae

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1. Introduction

Mesua ferrea Linn. (Ceylon ironwood in English and locally known as "bunnak" in Thai), of the Calophyllaceae family, is a tropical tree that is widely distributed across Southeast Asia, Thailand, India, and Sri Lanka [1–5]. In traditional Indian medicine, different aerial parts of the plant and their extracts are used to manage a wide range of bodily disorders, such as the use of essential oils to treat skin diseases and rheumatism. In addition, powders from the flowers and fruit of the plant, when mixed with butter, are applied locally for the management of piles, while the seeds are used for treating pain and inflammatory conditions such as arthritis [2]. Previous studies of the chemical constituents from the rhizomes of this plant have led to the isolation and characterization of numerous compounds, including biflavonoids [4–6], xanthones [2,4,5], coumarins [4,5,7], flavanone glycosides [8], cyclohexanedione derivatives [9], triterpenes [10], and essential oil [11]. Furthermore, the biological effects of the extract and constituents have been reported, such as the antioxidant [4], antibacterial [5], anti-inflammatory [5], and antitumor [12] properties. During our characterization of the bioactive constituents of plants in Thailand [13–26],

a methanolic extract of the *M. ferrea* flower was found to inhibit hyaluronidase activity. Following the use of a bioassay-guided separation technique, two 8-8″ linked biflavonoids, mesuaferrone-A (**1**) and mesuaferrone-B (**2**), were isolated, along with ten flavonoids (**3**–**12**), two xanthones (**13** and **14**), three triterpenes (**15**–**17**), a phenylpropanoid (**18**), and five aromatics (**19**–**24**). Herein, we report on the isolation, structure elucidation, and antiallergic activities, such as the hyaluronidase and degranulation inhibitory properties, of the isolates.

2. Materials and Methods

2.1. General

The following instruments were used to obtain spectroscopic data: specific rotation, JASCO P-2200 polarimeter (JASCO Corporation, Tokyo, Japan, l = 5 cm); UV spectra, Shimadzu UV-1600 spectrometer; IR spectra, IRAffinity-1 spectrophotometer (Shimadzu Co., Kyoto, Japan); ^{1}H NMR spectra, JNM-ECA800 (800 MHz), JNM-LA500 (500 MHz), JNM-ECS400 (400 MHz), and JNM-AL400 (400 MHz) spectrometers; ^{13}C NMR spectra, JNM-ECA800 (200 MHz), JNM-LA500 (125 MHz), JNM-ECA400 (100 MHz), and JNM-AL400 (100 MHz) spectrometers (JEOL Ltd., Tokyo, Japan) by using tetramethylsilane as the internal standard; ESIMS and HRESIMS, Exactive Plus mass spectrometer (Thermo Fisher Scientific Inc., Waltham, MA, USA).

Instruments and tools for analytical determinations included the following: HPLC detectors, the Shimadzu RID-6A refractive index (RI) and SPD-10A UV-VIS detectors, and a Shodex OR-2 optical rotation detector; HPLC columns, Cosmosil 5C_{18}-MS-II (Nacalai Tesque, Inc., Kyoto, Japan), Cosmosil ΠInap (Nacalai Tesque, Inc., Kyoto, Japan), and Wakopak Navi C30-5 (FUJIFILM Wako Pure Chemical Co., Osaka, Japan). Columns with 4.6 mm i.d. × 250 mm and 20 mm i.d. × 250 mm were used for analytical and preparative purposes, respectively.

The following experimental chromatographic materials were used for column chromatography (CC): highly porous synthetic resin, Diaion HP-20 (Mitsubishi Chemical Co., Tokyo, Japan); normal-phase silica gel CC, silica gel 60 N (Kanto Chemical Co., Ltd., Tokyo, Japan; 63–210 mesh, spherical, neutral); reversed-phase ODS CC, Chromatorex ODS DM1020T (Fuji Silysia Chemical, Ltd., Aichi, Japan; 100–200 mesh); TLC, precoated TLC plates with silica gel 60F_{254} (Merck, Darmstadt, Germany, 0.25 mm) (normal-phase) and silica gel RP-18 WF_{254S} (Merck, 0.25 mm) (reversed-phase); reversed-phase HPTLC, precoated TLC plates with silica gel RP-18 WF_{254S} (Merck, 0.25 mm). Detection was performed by spraying with 1% Ce$(SO_4)_2$–10% aqueous H_2SO_4, followed by heating.

2.2. Plant Material

The flowers of *M. ferrea* (loss on drying: 4.35% at 105 °C for 6 h) were collected from the Nakhon Si Thammarat Province of Thailand in September 2006. The plant material was identified by one of the authors (Y.P.). A voucher specimen (2006.09. Raj-07) of the plant is on file in our laboratory.

2.3. Extraction and Isolation

Dried flowers (986.2 g) of *M. ferrea* were extracted three times following reflux with methanol for 3 h. Solvent evaporation from the combined extracts under reduced pressure yielded an aqueous acetone extract (156.8 g, 15.9%). An aliquot (127.0 g) was partitioned in an EtOAc–H_2O (1:1, *v*/*v*) mixture to furnish an EtOAc-soluble fraction (75.68 g, 9.47%) and an aqueous phase. The aqueous phase was subjected to Diaion HP-20 CC (2.0 kg, H_2O→MeOH) to yield H_2O-eluted (40.89 g, 5.12%) and MeOH-eluted (10.43 g, 1.31%) fractions. An aliquot (60.20 g) of the EtOAc-soluble fraction was subjected to normal-phase silica gel CC [3.00 kg, *n*-hexane–EtOAc (20:1→5:1→1:1→1:2, *v*/*v*)→EtOAc→MeOH] to yield seven fractions [Fr. E1 (0.49 g), Fr. E2 (2.02 g), Fr. E3 (19.70 g), Fr. E4 (11.10 g), Fr. E5 (4.67 g), Fr. E6 (1.17 g), and Fr. E7 (16.10 g)]. Fraction E2 (2.02 g) was subjected to reversed-phase silica gel CC [70.0 g, MeOH–H_2O (80:20→95:5, *v*/*v*)→MeOH] to yield ten fractions [Fr. E2-1 (77.0 mg), Fr. E2-2 (163.7 mg), Fr. E2-3 (62.4 mg), Fr. E2-4 (87.1 mg), Fr. E2-5

(46.0 mg), Fr. E2-6 (91.9 mg), Fr. E2-7 (192.0 mg), Fr. E2-8 [=lupeol (**15**, 430.1 mg, 0.068%)], Fr. E2-9 (133.7 mg), and Fr. E2-10 (430.8 mg)]. Fraction E2-7 (436.8 mg) was analyzed using HPLC [detection: RI, MeOH-1% aqueous AcOH (95:5, *v*/*v*)] to yield **15** (7.1 mg, 0.0011%). Fraction E3 (19.7 g) was subjected to reversed-phase silica gel CC [600.0 g, MeOH–H_2O (80:20→90:10, *v*/*v*)→MeOH] to yield eight fractions [Fr. E3-1 (78.0 mg), Fr. E3-2 (181.3 mg), Fr. E3-3 (127.2 mg), Fr. E3-4 (583.1 mg), Fr. E3-5 (193.1 mg), Fr. E3-6 (12.6 g), Fr. E3-7 (348.5 mg), and Fr. E3-8 (1.78 mg)]. Fraction E3-2 (181.3 mg) was analyzed using HPLC [detection: RI, MeOH-1% aqueous AcOH (60:40, *v*/*v*)] to yield 1,7-dihydroxyxanthone (**13**, 12.1 mg, 0.019%). Fraction E3-7 (348.5 mg) was purified by HPLC [detection: RI, MeOH-1% aqueous AcOH (95:5, *v*/*v*)] to yield betulinaldehyde (**16**, 15.5 mg, 0.0024%). Fraction E4 (11.05 g) was subjected to reversed-phase silica gel CC [330.0 g, MeOH–H_2O (80:20→90:10, *v*/*v*)→MeOH] to yield 11 fractions [Fr. E4-1 (58.1 mg), Fr. E4-2 (473.6 mg), Fr. E4-3 (189.4 mg), Fr. E4-4 (799.1 mg), Fr. E4-5 (2301.3 mg), Fr. E4-6 (72.5 mg), Fr. E4-7 (920.0 mg), E4-8 (723.5 mg), E4-9 (254.6 mg), E4-10 (1050.0 mg), and Fr. E4-11 (734.2 mg)]. Fraction E4-1 (58.1 mg) was analyzed using HPLC [detection: UV (254 nm), MeOH-1% aqueous AcOH (20:80, *v*/*v*)] to yield *p*-hydroxybenzoic acid (**19**, 20.1 mg, 0.00080%), protocatechuic acid (**20**, 18.2 mg, 0.0018%), protocatechuic aldehyde (**22**, 5.6 mg, 0.0019%), and vanillic acid (**21**, 7.8 mg, 0.00074%). Fraction E4-2 (473.6 mg) was analyzed using HPLC [detection: UV (254 nm), MeOH-1% aqueous AcOH (50:50, *v*/*v*)] to yield quercetin (**10**, 27.2 mg, 0.00428%) and *trans*-cinnamic acid (**18**, 8.1 mg, 0.00128%). Fraction E4-3 (189.4 mg) was analyzed by HPLC [detection: UV (254 nm), MeOH-1% aqueous AcOH (50:50, *v*/*v*)] to yield 1,3,7-trihydroxyxanthone (**14**, 3.2 mg, 0.0020%). Fraction E4-4 (624.1 mg) was analyzed using HPLC [detection: UV (254 nm), MeOH-1% aqueous AcOH (60:40, *v*/*v*)] to yield apigenin (**3**, 8.6 mg, 0.0020%). Fraction E4-10 (300.0 mg) was analyzed using HPLC [detection: UV (254 nm), MeOH-1% aqueous AcOH (90:10, *v*/*v*)] to yield ursolic acid (**17**, 20.1 mg, 0.0048%). Fraction E5 (4.67 g) was subjected to reversed-phase silica gel CC [150 g, MeOH–H_2O (40:60→60:40→70:30→80:20, *v*/*v*)→MeOH] to yield eight fractions [Fr. E5-1 (480.0 mg), Fr. E5-2 (250.0 mg), Fr. E5-3 (60.0 mg), Fr. E5-4 (110.0 mg), Fr. E5-5 (870.0 mg), Fr. E5-6 (420.0 mg), Fr. E5-7 (0.51 mg), and Fr. E5-8 (1.18 g)]. Fraction E5-1 (230.0 mg) was analyzed using HPLC [detection: UV (254 nm), MeOH-1% aqueous AcOH (5:95, *v*/*v*)] to yield 20 (71.0 mg, 0.0113%) and gallic acid (23, 37.6 mg, 0.0149%). Fraction E5-5 (300.0 mg) was characterized using HPLC [detection: UV (254 nm), MeOH-1% aqueous AcOH (60:40, *v*/*v*)] to yield mesuaferrone-A (**1**, 85.3 mg, 0.0387%), mesuaferrone-B (**2**, 99.8 mg, 0.0452%), and luteolin (**4**, 7.0 mg, 0.0032%). Fraction E5-6 (420 mg) was analyzed using HPLC [detection: UV (254 nm), CH_3CN-1% aqueous AcOH (50:50 *v*/*v*)] to yield **2** (13.9 mg, 0.0022%). Fraction E7 (16.1 g) was subjected to reversed-phase silica gel CC [60.0 g, MeOH–H_2O (40:60→60:40→70:30→80:20, *v*/*v*)→MeOH] to yield ten fractions [Fr. E7-1 (0.097 g), Fr. E7-2 (1.22 g), Fr. E7-3 (0.48 g), Fr. E7-4 (0.74 g), Fr. E7-5 (1.04 g), Fr. E7-6 (3.55 g), Fr. E7-7 (2.33 g), E7-8 (2.05 g), E7-9 (1.70 g), and Fr. E7-10 (1.50 g)]. Fraction E7-4 (300.0 mg) was analyzed using HPLC [detection: UV (254 nm), MeOH-1% aqueous AcOH (30:70, *v*/*v*)] to yield orientin (**6**, 13.9 mg, 0.0032%). Fraction E7-5 (500.0 mg) was characterized using HPLC [detection: UV (254 nm), MeOH-1% aqueous AcOH (30:70, *v*/*v*)] to yield vitexin (**5**, 14.6 mg, 0.0126%). Fraction E7-6 (500.0 mg) was analyzed using HPLC [detection: UV (254 nm), MeOH-1% aqueous AcOH (50:50, *v*/*v*)] to yield **5** (12.7 mg, 0.0140%), saponaretin (**7**, 89.5 mg, 0.100%), and quercetin-3-*O*-α-L-rhamnopyranoside (**12**, 135.4 mg, 0.151%). Fraction E7-7 (500.0 mg) was analyzed using HPLC [detection: UV (254 nm), MeOH-1% aqueous AcOH (40:60, *v*/*v*)] to yield **12** (17.0 mg, 0.011%) and kaempferol-3-*O*-α-L-rhamnopyranoside (**11**, 48.5 mg, 0.033%). An aliquot (8.20 g) of the MeOH-eluted fraction was subjected to reversed-phase silica gel CC [500 g, MeOH–H_2O (35:65→50:50→70:30→90:10, *v*/*v*)→EtOAc→MeOH] to yield seven fractions [Fr. M1 (0.80 g), Fr. M2 (0.78 g), Fr. M3 (1.13 g), Fr. M4 (1.72 g), Fr. M5 (1.46 g), Fr. M6 (0.39 g), and Fr. M7 (1.48 g)]. Fraction M4 (500.0 mg) was subjected to HPLC [detection: UV (254 nm), MeOH-1% aqueous AcOH (50:50, *v*/*v*)] to yield five fractions {Fr. M4-1 (13.4 mg), Fr. M4-2 (3.4 mg), Fr. M4-3 (31.2 mg), Fr. E4-4 [=5 (51.7 mg, 0.028%)], and Fr. E4-5

[=7 (11.1 g, 0.0061%)]}. Fraction M4-3-1 (31.2 mg) was analyzed using HPLC [detection: UV (254 nm), MeOH-1% aqueous AcOH (35:65, *v*/*v*)] to yield homoorientin (**8**, 3.7 mg, 0.0020%). Fraction M5 (500.0 mg) was analyzed using HPLC [detection: UV (254 nm), MeOH-1% aqueous AcOH (50:50, *v*/*v*)] to yield **5** (21.5 mg, 0.040%), **6** (10.7 mg, 0.020%), **7** (31.1 mg, 0.0582%), and apigenin-7-*O*-rutinoside (**9**, 23.7 mg, 0.044%) (Figure 1).

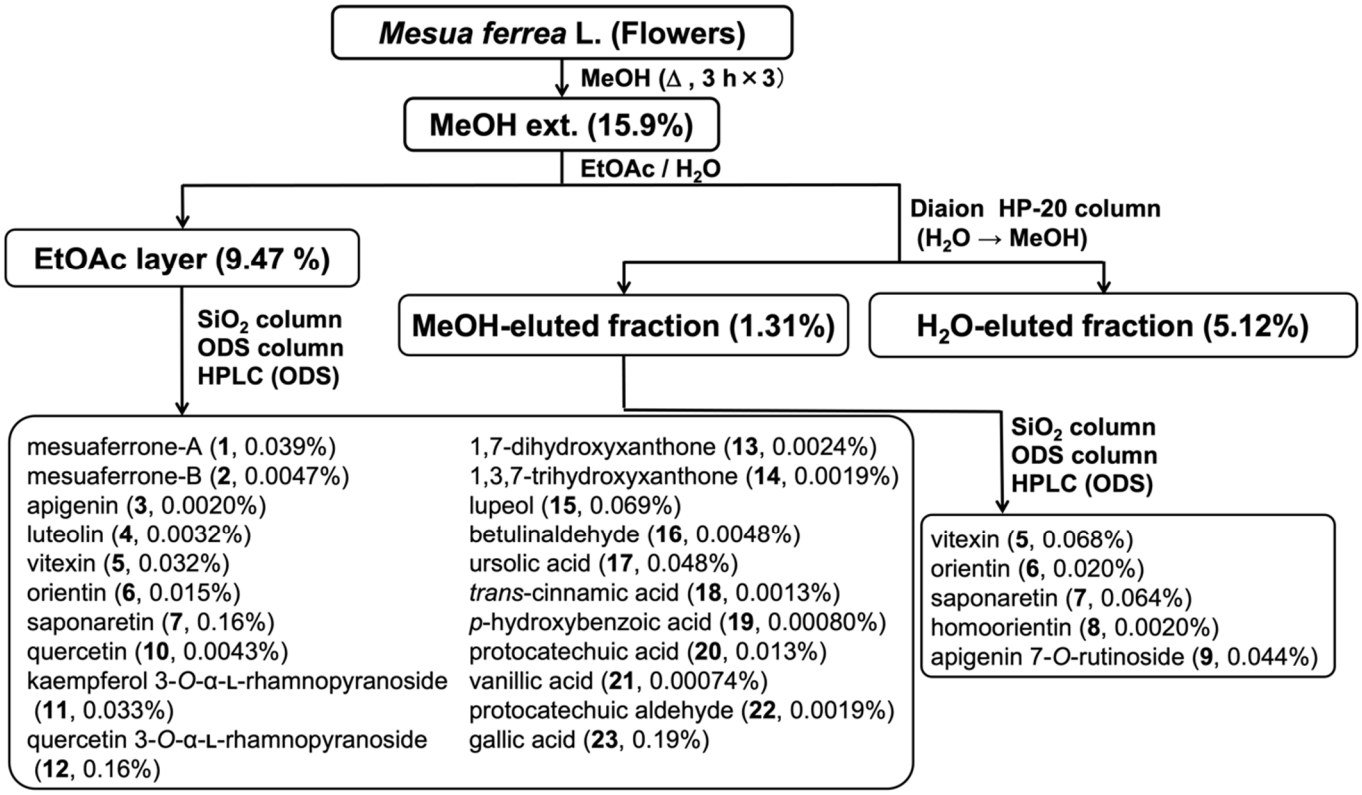

Figure 1. Isolation protocol of the chemical constituents (**1**–**23**) from the flowers of *M. ferrea*.

2.4. Hyaluronidase Inhibitory Activity

Hyaluronidase inhibitory activity was determined in accordance with a previously reported method [27], with slight modifications. Briefly, the assay was performed in 96-well microplates. Preincubation of 10 μL hyaluronidase enzyme (Type IV-S from bovine testes; 340 NF unit/mL, Sigma-Aldrich Co. LLC, St. Louis, MO, USA) or a blank buffer (0.1 M acetate buffer, pH 3.5) with 20 μL of sample or control was performed at 37 °C for 20 min. Calcium dichloride (20 μL, final concentration: 2.0 mM) was added to the buffer, and the mixture was incubated at 37 °C for 40 min. Next, 50 μL of hyaluronic acid potassium salt (final concentration: 0.6 mg/mL, Sigma-Aldrich Co. LLC, St. Louis, MO, USA) was added, and the mixture was incubated at 37 °C for 40 min. The reaction was stopped by the addition of 0.4 M NaOH (10 μL) and 0.08 M borate solution (pH 9.1, 10 μL), and was immediately heated using boiling water for 3 min. The reaction solution (20 μL) was transferred to another 96-well microplate. *p*-Dimethylaminobenzaldehyde (80 μL, final concentration: 8.0 mg/mL, Wako Pure Chemical Industries Ltd., Osaka, Japan) acetate solution was added to the reaction mixture and incubated at 37 °C for 20 min. The optical density (OD) of the reaction mixture was measured using a microplate reader (SH-9000, CORONA ELECTRIC Co., Ltd., Ibaraki, Japan) at a wavelength of 585 nm (reference 670 nm). The final concentration of dimethyl sulfoxide (DMSO) in the test solution was 1.0%, and no influence of DMSO on the inhibitory activity was detected. All experiments were performed in quadruplicate, and IC_{50} values were determined graphically. Disodium cromoglycate (DSCG), ketotifen fumarate, and tranilast, which are clinically prescribed

antiallergic medicines, were used as the reference compounds. Equation (1) below was used to calculate the percentage inhibition.

$$\text{Inhibition (\%)} = [(\text{OD (C)} - \text{OD (N)}) - (\text{OD (T)} - \text{OD (B)})]/(\text{OD (C)} - \text{OD (N)}) \times 100 \quad (1)$$

Control (C): enzyme (+), test sample (−); Test (T): enzyme (+), test sample (+); Blank (B): Missouri, enzyme (−), test sample (+); Normal (N): enzyme (−), test sample (−); OD, optical density.

2.5. Inhibitory Effects on the Release of β-Hexosaminidase from RBL-2H3 Cells

Inhibitory effects on the release of β-hexosaminidase in RBL-2H3 cells [Cell No. JCRB0023, obtained from the Health Science Research Resources Bank (Osaka, Japan)] were evaluated by using a previously reported method [28,29]. Briefly, RBL-2H3 cells in 24-well plates (2×10^5 cells/well in minimal essential medium (MEM) containing 10% fetal bovine serum (FBS), penicillin (100 units/mL), and streptomycin (100 μg/mL)) were sensitized with anti-dinitrophenyl immunoglobulin E (anti-DNP IgE, 0.45 μg/mL). The cells were washed with Siraganian buffer (119 mM NaCl, 5 mM KCl, 0.4 mM $MgCl_2$, 25 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (PIPES), and 40 mM NaOH, pH 7.2) supplemented with 5.6 mM glucose, 1 mM $CaCl_2$, and 0.1% bovine serum albumin (BSA) (incubation buffer), and then incubated in 160 μL of the incubation buffer for 10 min at 37 °C. Next, 20 μL of the test sample solution was added to each well and was incubated for 10 min, followed by an addition of 20 μL of antigen (DNP-BSA, final concentration: 10 μg/mL) and incubation at 37 °C for 10 min to stimulate cell degranulation. Subsequently, the reaction was stopped by cooling in an ice bath for 10 min. The supernatant (50 μL) was then transferred into a 96-well plate and incubated with 50 μL of substrate (1 mM *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide) in 0.1 M citrate buffer (pH 4.5) at 37 °C for 1 h. The reaction was stopped by adding 200 μL of stop solution (0.1 M Na_2CO_3/$NaHCO_3$, pH 10.0). The absorbance was measured by using a microplate reader set at a wavelength of 405 nm. The test sample was dissolved in DMSO, and the solution added to incubation buffer (final DMSO concentration: 0.1%). The inhibition (%) of the release of β-hexosaminidase by the test samples was calculated using Equation (2), and IC_{50} values were determined graphically:

$$\text{Inhibition (\%)} = [1 - (\text{T} - \text{B} - \text{N})/(\text{C} - \text{N})] \times 100 \quad (2)$$

Control (C): DNP-BSA (+), test sample (−); Test (T): DNP-BSA (+), test sample (+); Blank (B): DNP-BSA (−), test sample (+); Normal (N): DNP-BSA (−), test sample (−).

To ascertain whether the antiallergic effects of the samples were due to the inhibition of β-hexosaminidase release, and not due to a false positive from the inhibition of β-hexosaminidase activity, we performed the following test. The cell suspension (5×10^7 cells) was placed in 6 mL of phosphate-buffered saline and sonicated. The solution was then centrifuged, and the supernatant was diluted with the incubation buffer and adjusted to equal the enzyme activity of the above-tested degranulation. The enzyme solution (45 μL) and test sample solution (5 μL) were transferred into a 96-well microplate, and the enzyme activity was examined as described above (Equation (2)). Under these conditions, the total β-hexosaminidase activity of the cell suspension after sonication was calculated from the cells in the control groups. Tranilast and ketotifen fumarate, which are clinically prescribed antiallergic medicines, were used as the reference compounds.

2.6. Statistics

All data are expressed as means ± standard error of the mean. One-way analysis of variance, followed by Dunnett's test, were used for statistical analysis. Probability (p) values < 0.05 were considered statistically significant.

3. Results and Discussion

3.1. Inhibitory Effects of the M. ferrea Flower Methanolic Extract and Its Fractions on Hyaluronidase

Hyaluronidases are enzymes that break down hyaluronic acid, which is a mucopolysaccharide that is related to inflammation, through the release of histamine from mast cells. Hyaluronidase inhibitors are effective therapies for the suppression of allergies and inflammation [30–32]. It is known that DSCG, which is a commercially available antiallergic agent, exhibits a strong inhibitory effect against hyaluronidase [31]. Therefore, a close relationship has been defined between allergic reactions and hyaluronidase inhibitory activity [31,32].

The dried flowers of *M. ferrea* were used to obtain a methanol extract (15.9% from the dried material). The methanol was partitioned by using EtOAc–H_2O (1:1, *v*/*v*) to yield an EtOAc-soluble fraction (9.47%) and an aqueous phase. The aqueous phase was subjected to Diaion HP-20 CC ($H_2O \rightarrow$MeOH) to yield MeOH- and H_2O-eluted fractions (5.12% and 1.31%, respectively). As shown in Table 1, the methanolic extract was found to have hyaluronidase inhibitory activity [inhibition (%): 52.1 ± 4.6 at 1000 μg/mL]. Following the use of a bioassay-guided separation procedure, the EtOAc-soluble and the MeOH-eluted fractions were found to be the active fractions (IC_{50} = 430 and 360 μg/mL, respectively), while the H_2O-eluted fraction showed no notable activity.

Table 1. Hyaluronidase inhibitory activity of the MeOH extract and its fractions obtained from flowers of *M. ferrea*.

	Inhibition (%)				
	0 μg/mL	**125 μg/mL**	**250 μg/mL**	**500 μg/mL**	**1000 μg/mL**
MeOH extract	0.0 ± 8.1	5.1 ± 6.8	10.7 ± 5.6	24.2 ± 6.4	52.1 ± 4.5 [b]
EtOAc-soluble fraction	0.0 ± 3.3	19.6 ± 7.7 [a]	27.2 ± 5.1 [b]	52.8 ± 3.8 [b]	72.0 ± 3.7 [b]
MeOH-eluted fraction	0.0 ± 8.4	16.6 ± 4.8	44.9 ± 5.1 [b]	61.9 ± 4.7 [b]	79.6 ± 1.4 [b]
H_2O-eluted fraction	0.0 ± 10.0	−5.7 ± 8.4	11.7 ± 7.7	12.1 ± 7.0	6.9 ± 7.4

Each value represents the mean ± S.E.M. (N = 4). Significantly different from the control, [a] $p < 0.05$, [b] $p < 0.01$.

3.2. Chemical Constituents of the M. ferrea Flower

The EtOAc-soluble fraction was subjected to normal-phase silica gel and reversed-phase ODS column CC, and finally to HPLC to obtain the following: two 8-8″ linked biflavonoids, mesuaferrone-A (**1**, 0.039%) [33] and mesuaferrone-B (**2**, 0.0047%) [34]; eight flavonoids, apigenin (**3**, 0.0020%), luteolin (**4**, 0.0032%), vitexin (**5**, 0.032%) [35], orientin (**6**, 0.015%) [36], saponaretin (**7**, 0.16%) [37], quercetin (**10**, 0.0043%), kaempferol 3-*O*-α-L-rhamnopyranoside (**11**, 0.033%) [38], and quercetin 3-*O*-α-L-rhamnopyranoside (**12**, 0.16%) [39]; two xanthones, 1,7-dihydroxyxanthone (**13**, 0.0024%) [40] and 1,3,7-trihydroxyxanthone (**14**, 0.0019%) [41]; three triterpenes, lupeol (**15**, 0.069%) [42], betulinaldehyde (**16**, 0.0048%) [43], and ursolic acid (**17**, 0.048%) [44]; a phenylpropanoid, *trans*-cinnamic acid (**18**, 0.0013%); and five aromatics, *p*-hydroxybenzoic acid (**19**, 0.0008%), protocatechuic acid (**20**, 0.013%), vanillic acid (**21**, 0.00074%), protocatechuic aldehyde (**22**, 0.0019%) [45], and gallic acid (**23**, 0.19%). From the MeOH-eluted fraction, five flavonoids, **5** (0.068%), **6** (0.020%), **7** (0.064%), homoorientin (**8**, 0.0020%) [46], and apigenin 7-*O*-rutinoside (**9**, 0.044%) [47], were isolated (Figure 2 and Table S1). The isolates were identified by a comparison of their physical and spectral data with those of commercially available samples (**3**, **4**, **10**, **18**–**21**, and **23**), or with reported values [33–47].

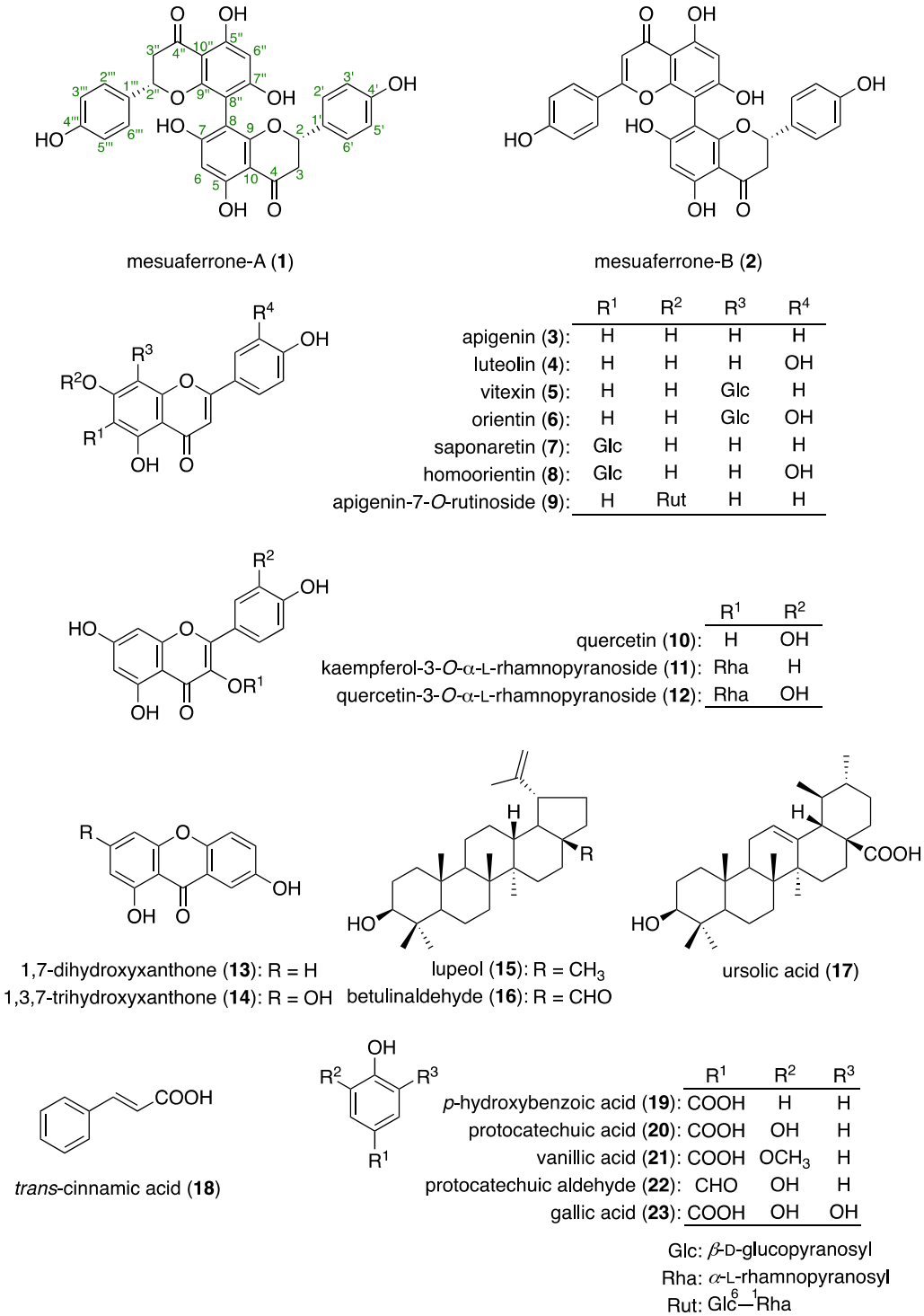

Figure 2. Isolates (**1**–**23**) from the flowers of *M. ferrea*.

*3.3. Inhibitory Effects of M. ferrea Flower Isolates (***1**–**23***) on Hyaluronidase*

In previous studies of compounds from natural medicines that possess hyaluronidase inhibitory activity, it was reported that a phenylethanoid glycoside that was isolated from the flowers of *Mimusops elengi* L. (Sapotaceae) [20], aporphine- and benzylisoquinoline-type alkaloids from the flowers of *Nelumbo nucifera* Gaertn. (Nelumbonaceae) [27], and iridoids from the rhizomes of *Picrorhiza kurroa* Royle ex Benth. (Plantaginaceae) [48] possess this property. To add on to these findings, the hyaluronidase inhibitory activity of the isolates from the flowers of *M. ferrea* were examined. Among the isolates, the two 8-8″

linked biflavonoids mesuaferrone-A (**1**, IC_{50} = 51.1 μM) and B (**2**, IC_{50} = 54.7 μM) exhibited hyaluronidase inhibitory activity that was equivalent to that of the commercial antiallergic agents DSCG (64.8 μM) and ketotifen fumarate (76.5 μM), which are therapeutically effective owing to their inhibitory activity on degranulation [49] (Table 2). These biflavonoids (**1** and **2**) are 8-8″ linked dimers that are composed of naringenin (**1a**) or apigenin (**3**). However, their corresponding monomers did not show similarly potent inhibitory activity (IC_{50} > 300 μM).

Table 2. Inhibitory effects of the isolates (**1**–**23**) from the flowers of *M. ferrea* on hyaluronidase.

	Inhibition (%)					**IC50**
	0 μM	12.5 μM	25 μM	50 μM	100 μM	(μM)
mesuaferrone-A (**1**)	0.0 ± 8.1	10.2 ± 14.6	26.5 ± 7.3	48.8 ± 6.8 [b]	71.1 ± 1.4 [b]	51.1
mesuaferrone-B (**2**)	0.0 ± 4.3	7.3 ± 1.9	23.6 ± 2.5 [b]	46.6 ± 0.9 [b]	54.5 ± 1.4 [b]	54.7
	Inhibition (%)					**IC50**
	0 μM	32.5 μM	75 μM	150 μM	300 μM	(μM)
naringenin (**1a**)	0.0 ± 8.8	−0.4 ± 5.3	−8.8 ± 2.0	0.4 ± 2.2	20.5 ± 1.7	—
apigenin (**3**)	0.0 ± 6.2	15.8 ± 7.7	27.5 ± 8.1	32.9 ± 8.2	38.3 ± 6.4 [b]	—
luteolin (**4**)	0.0 ± 4.6	5.7 ± 2.8	8.9 ± 3.8	10.5 ± 3.5	12.3 ± 3.4	—
vitexin (**5**)	0.0 ± 8.3	0.4 ± 8.2	−7.0 ± 7.9	−11.7 ± 3.8	−10.9 ± 5.2	—
orientin (**6**)	0.0 ± 9.3	4.9 ± 5.9	5.9 ± 3.2	9.0 ± 2.9	13.2 ± 2.6	—
saponaretin (**7**)	0.0 ± 3.5	1.1 ± 2.0	4.8 ± 3.1	4.6 ± 3.0	2.2 ± 2.4	—
homoorientin (**8**)	0.0 ± 7.9	−7.1 ± 8.5	−2.9 ± 7.6	−5.0 ± 4.0	1.7 ± 5.9	—
apigenin 7-O-Rut (**9**)	0.0 ± 7.6	4.4 ± 4.5	3.8 ± 2.1	2.5 ± 3.1	1.3 ± 2.4	—
quercetin (**10**)	0.0 ± 3.7	−1.0 ± 1.4	−4.3 ± 5.2	−4.3 ± 3.8	0.7 ± 4.5	—
kaempferol 3-O-Rha (**11**)	0.0 ± 1.2	0.5 ± 2.5	−4.7 ± 3.6	−1.2 ± 3.7	6.3 ± 3.1	—
quercetin 3-O-Rha (**12**)	0.0 ± 4.3	1.4 ± 4.2	4.0 ± 2.3	5.9 ± 3.3	13.2 ± 2.6	—
1,7-dihydroxyxthantone (**13**)	0.0 ± 3.0	3.3 ± 3.0	0.4 ± 1.4	−1.8 ± 3.6	5.4 ± 3.9	—
1,3,7-trihydroxyxthantone (**14**)	0.0 ± 7.7	7.8 ± 2.7	4.7 ± 1.8	11.1 ± 2.7	18.4 ± 1.9	—
lupeol (**15**)	0.0 ± 1.1	10.3 ± 3.2	9.4 ± 2.8	0.8 ± 2.2	−0.4 ± 6.2	—
betulinaldehyde (**16**)	0.0 ± 3.6	−3.9 ± 2.1	0.2 ± 1.9	1.0 ± 1.9	5.3 ± 5.6	—
ursolic acid (**17**)	0.0 ± 1.3	0.7 ± 1.5	0.5 ± 0.7	2.4 ± 1.5	−5.7 ± 2.8	—
trans-cinnamic acid (**18**)	0.0 ± 1.4	2.4 ± 0.9	−1.7 ± 4.7	−4.0 ± 5.1	−1.2 ± 5.0	—
p-hydroxybenzoic acid (**19**)	0.0 ± 5.0	−2.8 ± 2.2	−0.7 ± 1.4	2.6 ± 2.7	−0.2 ± 4.3	—
protocatechuic acid (**20**)	0.0 ± 2.5	1.0 ± 2.5	−2.1 ± 1.5	4.0 ± 2.3	5.6 ± 4.2	—
vanillic acid (**21**)	0.0 ± 3.8	4.0 ± 1.4	2.3 ± 3.1	−0.7 ± 3.3	5.7 ± 3.2	—
protocatechuic aldehyde (**22**)	0.0 ± 1.0	0.7 ± 1.8	4.1 ± 1.1	4.7 ± 3.0	1.5 ± 0.8	—
gallic acid (**23**)	0.0 ± 1.4	−0.6 ± 1.6	−0.3 ± 3.1	3.6 ± 3.5	4.4 ± 4.2	—
disodium cromoglycate [48]	0.0 ± 2.0	4.0 ± 2.4	14.4 ± 0.4 [a]	39.0 ± 4.9 [b]	69.1 ± 2.2 [b]	64.8
ketotifen fumarate [48]	0.0 ± 6.1	11.9 ± 1.9	26.7 ± 4.9	36.4 ± 2.9 [b]	54.6 ± 2.5 [b]	76.5

Each value represents the mean ± S.E.M. (N = 4). Significantly different from the control, [a] $p < 0.05$, [b] $p < 0.01$.

3.4. Inhibitory Effects of **1** *and* **2** *on the Release of β-Hexosaminidase in RBL-2H3 Cells*

Basophils and mast cells play important roles in both the immediate- and late-phase reactions of type I allergies. The aggregation of high-affinity Fcε receptor I (FcεRI) by antigens results in tyrosine phosphorylation, Ca^{2+} release from intracellular Ca^{2+} stores, and Ca^{2+} influx via release-activated Ca^{2+} channels. The levels of intracellular free Ca^{2+} ($[Ca^{2+}]_i$) play an essential role in the degranulation process [49–52]. Histamine, which is a chemical mediator that is released from mast cells and basophils when they are stimulated by an immunoglobulin E (IgE)-antigen complex or a degranulation inducer, is usually considered to be a degranulation marker of immediate allergic reactions in in vitro experiments. β-Hexosaminidase is also stored in the secretory granules of cells and is released concomitantly with histamine when the cells are immunologically activated. Therefore, the enzymatic activity of β-hexosaminidase can be used as a marker for the degranulation of the cells [53]. Previously, our studies on compounds from natural medicines

that possess degranulation inhibitory activity have shown that phenylpropanoids [54], neolignans [49], flavonoids [52,55], stilbenoids [28,56,57], diarylheptanoids [29,58,59], terpenoids [55,60–63], and alkaloids [64,65] have this property. In addition, it has been found that there is a close relationship between allergic reactions and hyaluronidase inhibitory activity [31,32]. Therefore, the degranulation inhibitory activities of mesuaferrone-A (**1**) and mesuaferrone-B (**2**), which have hyaluronidase activity, were examined. Our findings show that **1** (IC_{50} = 49.4 μM) and **2** (C_{50} = 49.2 μM) inhibited the release of β-hexosaminidase, which is a marker of antigen-IgE-mediated degranulation in RBL-2H3 cells (Table 3). These inhibitory activities were more potent than those of tranilast (282 μM), ketotifen fumarate (158 μM), and one of the corresponding monomers (**1a**; >100 μM), but they were weaker than that of the other monomer (**3**; 6.1 μM). Next, to confirm that these observations were definitely the result of inhibiting the release of β-hexosaminidase, and not a false positive from inhibiting the enzymatic activity of β-hexosaminidase, the effects of the abovementioned active degranulation inhibitors on the enzyme activity were examined. The results show that none of the investigated molecules significantly inhibited β-hexosaminidase activity at a concentration of 100 μM (data not shown).

Table 3. Inhibitory effects of mesuaferrone-A (**1**) and B (**2**) on the release of β-hexosaminidase in RBL-2H3 cells.

	Inhibition (%)					**IC_{50}**
	0 μM	**3 μM**	**10 μM**	**30 μM**	**100 μM**	**(μM)**
mesuaferrone-A (**1**)	0.0 ± 7.1	24.9 ± 6.6 [a]	24.8 ± 1.6 [a]	37.8 ± 5.6 [b]	86.0 ± 7.8 [b]	49.4
mesuaferrone-B (**2**)	0.0 ± 9.5	13.8 ± 5.3	4.2 ± 4.4	5.6 ± 6.2	113.0 ± 10.1 [b]	49.2
	Inhibition (%)					**IC_{50}**
	0 μM	**30 μM**	**100 μM**	**300 μM**	**1000 μM**	**(μM)**
tranilast [49]	0.0 ± 1.7	8.2 ± 1.8	22.4 ± 2.5 [a]	56.9 ± 3.4 [b]	75.0 ± 0.6 [b]	282
ketotifen fumarate [49]	0.0 ± 1.8	7.7 ± 1.5	27.6 ± 2.2 [a]	80.7 ± 1.8 [b]	100.7 ± 1.1 [b]	158

Each value represents the mean ± S.E.M. (N = 4). Significantly different from the control, [a] $p < 0.05$, [b] $p < 0.01$.

4. Conclusions

In conclusion, we found that the methanolic extract of the flowers of M. ferrea inhibits the enzymatic activity of hyaluronidase. Through a bioassay-guided separation of the extract, two biflavonoids, mesuaferrone-A (**1**) and mesuaferrone-B (**2**), were isolated, along with ten flavonoids (**3**–**12**), two xanthones (**13** and **14**), three triterpenes (**15**–**17**), a phenylpropanoid (**18**), and five aromatics (**19**–**24**). Among the isolates, the biflavonoids mesuaferrone-A (**1**, IC_{50} = 51.1 μM) and B (**2**, IC_{50} = 54.7 μM) were identified as the active constituents. Their inhibitory activities were equivalent to those of the antiallergic medicines DSCG (64.8 μM) and ketotifen fumarate (76.5 μM). As for the corresponding monomer flavonoids, naringenin (**1a**) or apigenin (**3**), they did not show similar inhibitory activity (IC_{50} > 300 μM). In addition, **1** (IC_{50} = 49.4 μM) and **2** (IC_{50} = 49.2 μM) were found to possess degranulation inhibitory activities. These inhibitory activities were more potent than those of the antiallergic medicines tranilast (IC_{50} = 282 μM) and ketotifen fumarate (158 μM), and one of the corresponding monomers (**1a**; >100 μM), but they were weaker than that of the other monomer (**3**; 6.1 μM). These results suggest that the presence of a biflavonoid skeleton may be important for antiallergic properties through both the hyaluronidase and degranulation inhibitory pathways. Further studies on the mechanisms of action of these constituents, as well as the associated structural requirements, are in progress.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/xxx/s1. Figures S1–S10: NMR spectra of mesuaferrone-A (**1**) and mesuaferrone-B (**2**); and Table S1: List of the isolates (**1**–**23**) from the flowers of *M. ferrea*.

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