

# SUPPLEMENTARY MATERIAL

## Phenolic Profiling and Bioactive Potential of *Iris bucharica*

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## Correlation Between Phenolic Composition and Antioxidant Activity

The Pearson correlation coefficient (r) between the 'Compounds Quantity' and 'Radical Scavenging Activity' of *Iris bucharica* leaves and bulbs altogether is approximately **0.314**, with a p-value of **0.043**. This indicates a **weak positive correlation** that is **statistically significant** (since  $p < 0.05$ ). The values for leaves only or bulbs only are indicated in Table S1. This means that as the quantity increases, the scavenging activity tends to increase slightly, but the relationship is not very strong (r is closer to 0 than to 1).

**Table S1. Statistically significant positive relationship between the quantity of the compound and its radical scavenging activity in *I. bucharica* leaves and bulbs (Pearson correlation coefficient).**

| COMBINING ALL KNOWN COMPOUNDS from both LEAVES AND BULBS |          |                             |                  | ONLY LEAVES        |             |                             |                       | ONLY BULBS         |             |                             |                  |
|--|----------|-----------------------------|------------------|--------------------|-------------|-----------------------------|-----------------------|--------------------|-------------|-----------------------------|------------------|
| Compound   | Quantity | Radical Scavenging Activity |                  | Compound           | Quantity    | Radical Scavenging Activity |                       | Compound           | Quantity    | Radical Scavenging Activity |                  |
| Isoorientin  | 9.45     | 3.57                        |                  | Isoorientin        | 9.45        | 3.57                        |                       | Isoorientin        | 0           | 0                           |                  |
| Isoorientin  | 10.11    | 3.84                        |                  | Isoorientin        | 10.11       | 3.84                        |                       | Isoorientin        | 0           | 0                           |                  |
| Isoorientin  | 10.75    | 4.11                        | Pearson 0.314394 | Isoorientin        | 10.75       | 4.11                        | Pearson 0.148292      | Isoorientin        | 0           | 0                           | Pearson 0.453354 |
| p-Coumaric acid  | 0.97     | 4.13                        | p = 0.043        | p-Coumaric acid    | 0.97        | 4.13                        | p = 0.521             | p-Coumaric acid    | 0           | 0                           | p = 0.039        |
| p-Coumaric acid  | 1.04     | 4.44                        |                  | p-Coumaric acid    | 1.04        | 4.44                        |                       | p-Coumaric acid    | 0           | 0                           |                  |
| p-Coumaric acid  | 1.11     | 4.75                        |                  | p-Coumaric acid    | 1.11        | 4.75                        |                       | p-Coumaric acid    | 0           | 0                           |                  |
| Ferulic acid   | 2.87     | 23.6                        |                  | Ferulic acid       | 2.87        | 23.6                        |                       | Ferulic acid       | 1.24        | 5.25                        |                  |
| Ferulic acid   | 3.07     | 25.4                        |                  | Ferulic acid       | 3.07        | 25.4                        |                       | Ferulic acid       | 1.33        | 5.65                        |                  |
| Ferulic acid   | 3.28     | 27.2                        |                  | Ferulic acid       | 3.28        | 27.2                        |                       | Ferulic acid       | 1.42        | 6.05                        |                  |
| Guajaverin   | 4.88     | 194.2                       |                  | Guajaverin         | 4.88        | 194.2                       |                       | Guajaverin         | 0           | 0                           |                  |
| Guajaverin   | 5.24     | 208.8                       |                  | Guajaverin         | 5.24        | 208.8                       |                       | Guajaverin         | 0           | 0                           |                  |
| Guajaverin   | 5.6      | 223.5                       |                  | Guajaverin         | 5.6         | 223.5                       |                       | Guajaverin         | 0           | 0                           |                  |
| Apigenin   | 0.047    | 60.1                        |                  | Apigenin           | 0.047       | 60.1                        |                       | Apigenin           | 0           | 0                           |                  |
| Apigenin   | 0.05     | 64.6                        |                  | Apigenin           | 0.05        | 64.6                        |                       | Apigenin           | 0           | 0                           |                  |
| Apigenin   | 0.053    | 69.1                        |                  | Apigenin           | 0.053       | 69.1                        |                       | Apigenin           | 0           | 0                           |                  |
| Iristectorigenin B                                       | 0.2      | 10.6                        |                  | Iristectorigenin B | 0.2         | 10.6                        |                       | Iristectorigenin B | 1.71        | 0                           |                  |
| Iristectorigenin B                                       | 0.21     | 11.4                        |                  | Iristectorigenin B | 0.21        | 11.4                        |                       | Iristectorigenin B | 1.83        | 0                           |                  |
| Iristectorigenin B                                       | 0.22     | 12.1                        |                  | Iristectorigenin B | 0.22        | 12.1                        |                       | Iristectorigenin B | 1.95        | 0                           |                  |
| Irigenin   | 0.86     | 21.1                        |                  | Irigenin           | 0.86        | 21.1                        |                       | Irigenin           | 0.63        | 0                           |                  |
| Irigenin   | 0.92     | 22.7                        |                  | Irigenin           | 0.92        | 22.7                        |                       | Irigenin           | 0.68        | 0                           |                  |
| Irigenin   | 0.98     | 24.3                        |                  | Irigenin           | 0.98        | 24.3                        |                       | Irigenin           | 0.73        | 0                           |                  |
| Isoorientin  | 0        | 0                           |                  |                    |             |                             |                       |                    |             |                             |                  |
| Isoorientin  | 0        | 0                           |                  | Total TEAC         |             | 672.316 µmol/g              |                       | Total TEAC         |             | 11.8995 µmol/g              |                  |
| Isoorientin  | 0        | 0                           |                  | Total phenolics    | 4.87 ± 0.09 |                             |                       |                    | 5.74 ± 0.10 |                             |                  |
| p-Coumaric acid  | 0        | 0                           |                  |                    |             |                             |                       |                    |             |                             |                  |
| p-Coumaric acid  | 0        | 0                           |                  |                    |             |                             |                       |                    |             |                             |                  |
| p-Coumaric acid  | 0        | 0                           |                  |                    |             |                             |                       |                    |             |                             |                  |
| Ferulic acid   | 1.24     | 5.25                        |                  |                    |             |                             |                       |                    |             |                             |                  |
| Ferulic acid   | 1.33     | 5.65                        |                  | Dataset            | Pearson r   | p-value                     | Significance p < 0.05 |                    |             |                             |                  |
| Ferulic acid   | 1.42     | 6.05                        |                  | Combined           | 0.314       | 0.043                       | Significant           |                    |             |                             |                  |
| Guajaverin   | 0        | 0                           |                  | Leaves Only        | 0.148       | 0.521                       | Not Significant       |                    |             |                             |                  |
| Guajaverin   | 0        | 0                           |                  | Bulbs Only         | 0.453       | 0.039                       | Significant           |                    |             |                             |                  |
| Guajaverin   | 0        | 0                           |                  |                    |             |                             |                       |                    |             |                             |                  |
| Apigenin   | 0        | 0                           |                  |                    |             |                             |                       |                    |             |                             |                  |
| Apigenin   | 0        | 0                           |                  |                    |             |                             |                       |                    |             |                             |                  |
| Apigenin   | 0        | 0                           |                  |                    |             |                             |                       |                    |             |                             |                  |
| Iristectorigenin B                                       | 1.71     | 0                           |                  |                    |             |                             |                       |                    |             |                             |                  |
| Iristectorigenin B                                       | 1.83     | 0                           |                  |                    |             |                             |                       |                    |             |                             |                  |
| Iristectorigenin B                                       | 1.95     | 0                           |                  |                    |             |                             |                       |                    |             |                             |                  |
| Irigenin   | 0.63     | 0                           |                  |                    |             |                             |                       |                    |             |                             |                  |
| Irigenin   | 0.68     | 0                           |                  |                    |             |                             |                       |                    |             |                             |                  |
| Irigenin   | 0.73     | 0                           |                  |                    |             |                             |                       |                    |             |                             |                  |

## Statistical comparison of the physicochemical parameters and compounds content between *I. bucharica* leaves and bulbs

Physicochemical parameters showed statistically higher levels of total flavonoid content and total hydroxycinnamic acid content in the leaves, while the bulbs showed statistically higher amounts of total phenolic compounds content isoflavonoids, loss on drying, and total ash.

Analysis of the dry water extracts revealed that *Iris* leaves (Fig. 5 and Tables 3-5) exhibit significantly stronger antioxidant activity compared to the bulbs (see statistical significance, with the letter “a” indicating a higher content). This superior activity is likely attributed to the leaves' higher phenolic content, a class of compounds well-documented for their antioxidant properties.

**Table S2. Statistical comparison of the physicochemical parameters and the quantity of compounds between *I. bucharica* leaves and bulbs, including raw data (t-test, n=3).**

|         | Trial | Phenolic Compounds | Hydroxycinnamic acids | Flavonoids | Isoflavonoids | Loss on drying | The total ash | The ash content insoluble in hydrochloric acid |
|---------|-------|--------------------|-----------------------|------------|---------------|----------------|---------------|--|
| Leaves  | 1     | 4.87               | 1.26                  | 1.4        | 2.07          | 5.19           | 3.25          | 2.15   |
| Leaves  | 2     | 5.17               | 1.4                   | 1.35       | 2.25          | 5.4            | 3.18          | 2.06   |
| Leaves  | 3     | 5.23               | b                     | 1.3        | a             | 2.3            | b             | 2.1  |
| Bulbs   | 1     | 5.74               | 0.75                  | 0.57       | 3.34          | 6.13           | 4.05          | 2.23   |
| Bulbs   | 2     | 6.1                | 0.5                   | 0.5        | 3.5           | 6.05           | 3.58          | 2.1  |
| Bulbs   | 3     | 6.15               | a                     | 0.7        | b             | 4.05           | a             | 2.12   |
| P-value |       | 0.006              | 0.0015                | 0.0002     | 0.0033        | 0.0005         | 0.0156        | 0.3867 (ns)                                    |

T test, parametric test, 95% confidence interval

| Treatment | Trial | Neochlorogenic acid | Catechin | Chlorogenic acid | Vanillic acid | Mangiferin | Epicatechin | Isoorientin | p-Coumaric acid | Ferulic acid | Vitexin | Hyperoside | Guajaverin | Tectoridin |
|-----------|-------|---------------------|----------|------------------|---------------|------------|-------------|-------------|-----------------|--------------|---------|------------|------------|------------|
| Leaves    | 1     | 0.0093              | 0.0283   | 0.142            | 0.561         | 0.040      | 0.047       | 3.050       | 0.550           | 1.100        | 0.019   | 1.000      | 3.050      | 0.000      |
| Leaves    | 2     | 0.0100              | 0.0307   | 0.151            | 0.523         | 0.038      | 0.050       | 3.250       | 0.580           | 1.150        | 0.020   | 1.070      | 3.260      | 0.000      |
| Leaves    | 3     | 0.0100              | 0.0307   | 0.151            | 0.592         | 0.042      | 0.053       | 3.440       | 0.610           | 1.220        | 0.021   | 1.140      | 3.450      | 0.000      |
| Bulbs     | 1     | 0.0000              | 0.0000   | 0.028            | 0.000         | 0.019      | 0.000       | 0.000       | 0.000           | 0.752        | 0.000   | 0.000      | 0.000      | 1.500      |
| Bulbs     | 2     | 0.0000              | b        | 0.000            | b             | 0.000      | b           | 0.000       | b               | 0.801        | b       | 0.000      | b          | 1.605      |
| Bulbs     | 3     | 0.0000              | 0.0000   | 0.031            | 0.000         | 0.021      | 0.000       | 0.000       | 0.000           | 0.845        | 0.000   | 0.000      | 0.000      | 1.695      |
| P-value   |       | <0.0001             | <0.0001  | <0.0001          | <0.0001       | 0.0001     | <0.0001     | <0.0001     | <0.0001         | 0.0012       | <0.0001 | <0.0001    | <0.0001    | <0.0001    |

T test, parametric test, 95% confidence interval

| Trial   | Mangiferin | Isoorientin | p-Coumaric acid | Ferulic acid | Guajaverin | Tectoridin | Cosmosin | Germanaism B | Kikkalidone | Genistin | Apigenin | Iristectorigenin B | Nigrin  | Irisgenin | Biochanin A | Irisolidone |
|---------|------------|-------------|-----------------|--------------|------------|------------|----------|--------------|-------------|----------|----------|--------------------|---------|-----------|-------------|-------------|
| Leaves  | 1          | 0.86        | 9.45            | 0.97         | 2.87       | 0.15       | 1.55     | 0.43         | 0.28        | 0        | 0.047    | 0.2                | 0       | 0.86      | 1.08        | 1.27        |
| Leaves  | 2          | 0.92        | a               | 10.11        | a          | 1.04       | a        | 3.07         | a           | 0.16     | a        | 0.3                | n       | 0         | 0.92        | a           |
| Leaves  | 3          | 0.98        | a               | 10.75        | a          | 1.11       | a        | 3.28         | a           | 0.17     | a        | 0.32               | s       | 0         | 0.98        | a           |
| Bulbs   | 1          | 0.33        | b               | 0            | b          | 1.24       | b        | 1.49         | b           | 0.21     | b        | 0.32               | s       | 0.49      | 0.36        | 0.25        |
| Bulbs   | 2          | 0.35        | b               | 0            | b          | 1.33       | b        | 1.6          | a           | 0        | 0.22     | b                  | 0.34    | n         | 0.52        | a           |
| Bulbs   | 3          | 0.37        | b               | 0            | b          | 1.42       | b        | 1.71         | 0           | 0.23     | 0.36     | s                  | 0.55    | 0         | 0.73        | b           |
| P-value |            | 0.0001      | <0.0001         | <0.0001      | 0.0002     | <0.0001    | <0.0001  | 0.0002       | 0.0705      | <0.0001  | <0.0001  | <0.0001            | <0.0001 | <0.0001   | 0.006       | <0.0001     |

T test, parametric test, 95% confidence interval

| Trial   | Not identified 1 | Not identified 2 | Isoorientin | p-Coumaric acid | Not identified 3 | Ferulic acid | Not identified 4 | Guajaverin | Not identified 5 | Not identified 6 | Apigenin | Not identified 7 | Iristectorigenin B | Irisgenin | Total content |
|---------|------------------|------------------|-------------|-----------------|------------------|--------------|------------------|------------|------------------|------------------|----------|------------------|--------------------|-----------|---------------|
| Leaves  | 1                | 20.7             | 0           | 3.51            | 4.15             | 18.4         | 23.6             | 165.1      | 194.2            | 0                | 0        | 60.1             | 105.5              | 21.1      | 325.4         |
| Leaves  | 2                | 22.2             | 0           | 3.84            | 4.44             | 19.8         | 25.4             | 177.5      | 208.8            | 0                | 0        | 64.6             | 111.7              | 22.7      | 372.38        |
| Leaves  | 3                | 23.7             | a           | 0               | b                | 4.11         | a                | 21.2       | a                | 27.2             | a        | 189.9            | a                  | 223.5     | a             |
| Bulbs   | 1                | 0                | 1.87        | 0               | 0                | 0            | 5.25             | 0          | 0                | 2                | 1.95     | 0                | 0                  | 0         | 11.07         |
| Bulbs   | 2                | 0                | 2.01        | 0               | 0                | 0            | 5.65             | 0          | 0                | 2.3              | 2.1      | 0                | 0                  | 0         | 12.06         |
| Bulbs   | 3                | 0                | b           | 2.15            | a                | 0            | b                | 6.05       | b                | 0                | b        | 2.15             | a                  | 2.25      | a             |
| P-value |                  | <0.0001          | <0.0001     | <0.0001         | <0.0001          | <0.0001      | 0.0001           | <0.0001    | <0.0001          | <0.0001          | <0.0001  | <0.0001          | <0.0001            | <0.0001   | <0.0001       |

T test, parametric test, 95% confidence interval

Note. Means followed by different letters within the same row are significantly different ( $p < 0.05$ ); 'ns' indicates no significant difference. The letter “a” indicating a higher content

## Materials and Methods

Details of procedures from Section 2.6 and 2.7 of the main text are described below, including HPLC methodology and bioactivity assays.

### 2.6 Chromatographic Determination

#### 2.6.2 HPLC-DAD Conditions

##### 2.6.2.1 Apparatus

Separation of compounds was achieved using a Shimadzu Nexera X2 LC-30AD HPLC system (Shimadzu, Japan). The system comprises an online degasser, a quaternary pump, SIL-30AC autosampler (Shimadzu, Japan), CTO-20AC thermostat (Shimadzu, Japan), a column temperature controller, and SPD-M20A diode array detector (DAD). Other instruments used in the investigation were Ultrasonic Cleaner Set (Wise Clean WUC-A06H, Witeg Labortechnik GmbH, Germany), Libra UniBloc AUW120D (Shimadzu Analytical Scale, Japan); pH-meter – Knick Electronic Battery-operated pH Meter 911 PH (Portamess, Germany), and class A analytical vials that meet requirements of the in accordance with the requirements of the State Pharmacopoeia of Ukraine, Article 2.0, “Liquid chromatography” (2.2.29).

##### 2.6.2.2 HPLC-DAD gradient and detection parameters

Qualitative and quantitative analysis of phenolic compounds in *I. bucharica* raw materials was carried out on a Nexera X2 LC-30AD HPLC system (Shimadzu, Kyoto, Japan) liquid chromatograph equipped with a SPD-M20A diode array detector (DAD) and an ACE C18 (250 × 4.6 mm × 5 µm) column (Zorbax Eclipse Plus, Agilent, Santa Clara, CA, USA). The mobile phase consisted of two solutions: 0.1% acetic acid in water (mobile phase A) and acetonitrile P (mobile phase B) Chromatography was carried out according to the linear gradient program: 0–8 min, 5–15% B; 8–30 min, 15–20% B; 30–48 min, 20–40% B; 48–58 min, 40–50% B; 58–65 min, 50%; 65–66 min, 50–95% B. The flow rate was 1 mL/min, and the injection volume was 10 µL. The column temperature was 25 °C. Detection of compounds occurred depending on the nature of the spectrum (from 245 nm to 350 nm). The identification of all constituents was performed using HPLC analysis, which involved comparing the retention times (Rt), UV spectra, and mass spectra of the peaks in the samples with those of authentic reference compounds. The detailed chromatographic analysis conditions and the validation characteristics of the method have been previously published (1,2)

##### 2.6.2.3 Quantitative Analysis Validation Procedures

Following the United States Pharmacopeia (USP) recommendations, various analytical method validation parameters are considered, including the limit of quantification (LOQ), the limit of detection (LOD), linearity, accuracy, and repeatability (2). The responses' linearity range of the standards was obtained using ten concentration levels with two injections for each level. The seven analytes were dissolved in methanol and the stock

solutions were prepared. The stock solutions were diluted to a series of appropriate concentrations to construct the calibration curves. All calibration curves were recorded using the solutions of the reference compounds with an injection volume of 2.2  $\mu$ L. The working solution with the lowest concentration was diluted with methanol to various concentrations. These solutions were then used to determine the limits of detection (LOD) and limits of quantification (LOQ) at signal-to-noise ratios (S/N) of 3 and 10 for each compound. The repeatability was evaluated by analyzing six replicates of each preparation using HPLC (repeatability on the real sample). The main peak areas of two repeated chromatograms were used to calculate the relative standard deviation (RSD).

### ***List of Identified Compounds' Mass Data***

Neochlorogenic acid:  $C_{16}H_{18}O_9$ , 354.31 g/mol

Catechin:  $C_{15}H_{14}O_6$ , 290.27 g/mol

Chlorogenic acid:  $C_{16}H_{18}O_9$ , 354.31 g/mol

Vanillic acid:  $C_8H_8O_4$ , 168.15 g/mol

Mangiferin:  $C_{19}H_{18}O_{11}$ , 422.35 g/mol

Epicatechin:  $C_{15}H_{14}O_6$ , 290.27 g/mol

Isoorientin:  $C_{21}H_{20}O_{11}$ , 448.38 g/mol

p-Coumaric acid:  $C_9H_8O_3$ , 164.16 g/mol

Ferulic acid:  $C_{10}H_{10}O_4$ , 194.18 g/mol

Vitexin:  $C_{21}H_{20}O_{10}$ , 432.38 g/mol

Hyperoside:  $C_{21}H_{20}O_{12}$ , 464.38 g/mol

Guaijaverin:  $C_{20}H_{18}O_{11}$ , 434.36 g/mol

Tectoridin:  $C_{22}H_{22}O_{11}$ , 462.41 g/mol

Nicotiflorin:  $C_{27}H_{30}O_{15}$ , 594.52 g/mol

Cosmosiin:  $C_{21}H_{20}O_{10}$ , 432.38 g/mol

Afzelin:  $C_{21}H_{20}O_{10}$ , 432.39 g/mol

Germanaism B:  $C_{23}H_{22}O_{11}$ , 474.42 g/mol

Kakkalidone:  $C_{23}H_{24}O_{11}$ , 476.43 g/mol

Genistin:  $C_{21}H_{20}O_{10}$ , 432.38 g/mol

Apigenin:  $C_{15}H_{10}O_5$ , 270.24 g/mol

Iristectorigenin B:  $C_{17}H_{14}O_7$ , 330.29 g/mol

Nigricin:  $C_{17}H_{12}O_6$ , 312.27 g/mol

Irigenin:  $C_{18}H_{16}O_8$ , 360.30 g/mol

Biochanin A:  $C_{16}H_{12}O_5$ , 284.26 g/mol

Irisolidone:  $C_{17}H_{14}O_6$ , 314.29 g/mol

## **2.7 Pharmacological Assay in Vitro**

### **2.7.4 Anti-Allergic Activity in RBL-2H3 Cells**

#### **2.7.4.1. Chemicals and Reagents**

Dulbecco's modified Eagle's medium-high glucose powder (DMEM), [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (MTT), *p*-nitrophenyl-*N*-acetyl-D-glucosaminide (*p*-NAG), penicillin and streptomycin, dexamethasone, calcium ionophore A23187, mouse anti-DNP IgE antibody, and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Moreover, fetal bovine serum (FBS) was obtained from Hyclone (Logan, UT, USA). Dinitrophenyl-conjugated bovine serum albumin (DNP-BSA) was purchased from Merck (Kenilworth, NJ, USA). Isoorientin was obtained from ChemFaces (Hubei, China). The rat basophilic leukemia (RBL-2H3) cell line, derived from mucosal mast cells, was purchased from the American Type Culture Collection. Cells were grown in DMEM medium supplemented with 10% FBS and 100 U/mL penicillin plus 100 µg/mL streptomycin. Cells were cultured in 10 cm cell culture dishes (Cellstar) at 37 °C in a humidified chamber with 5% CO<sub>2</sub>. All other chemicals and reagents were purchased at the highest possible purity and quality.

#### **2.7.4.2. Cell Culture**

The rat basophilic leukemia (RBL-2H3) cell line, derived from mucosal mast cells, was purchased from the American Type Culture Collection. Cells were grown in DMEM medium supplemented with 10% FBS and 100 U/mL penicillin plus 100 µg/mL streptomycin. Cells were cultured in 10 cm cell culture dishes (Cellstar) at 37 °C in a humidified chamber with 5% CO<sub>2</sub>.

#### **2.7.4.3. Cell viability assay**

The methylthiazolyl tetrazolium (MTT) assay was used to measure the potential toxic effect of the samples on RBL-2H3 cells (3). The degree of cell viability of each sample was calculated as the percentage of the control value (untreated cells). All experiments were repeated three times. The maximally tolerated dose of DMSO was 0.5%. It served as a control, not affecting the growth of RBL-2H3 cells. Triton X-100 (0.5% solution) was used as the positive control, resulting in the death of all cells in the well.

#### **2.7.4.4. Degranulation $\beta$ -hexosaminidase assay induced by A23187 and antigen**

RBL-2H3 cells were dispensed into the 96-well plate at a density of  $2 \times 10^4$  cells/well (A23187-induced assay) or 48-well plate at a density of  $3 \times 10^4$  cells/well (antigen-induced assay) (4,5). Cells were incubated at 37 °C in 5% CO<sub>2</sub> for at least 5 hours to allow them to adhere entirely to the bottom of the wells. Cells were washed with PBS, and various concentrations of the samples or medium (untreated control) were added to each well (100 µL), followed by 20 hours of incubation at 37 °C in 5% CO<sub>2</sub>. Dexamethasone (10 nM) was used as

a positive control. The cells for antigen-induced assay were sensitized with anti-DNP IgE (0.1 µg/mL) for 2 h. Afterwards, the cells were washed by pre-warmed Tyrode's buffer (135 mM NaCl, 5 mM KCl, 1.8 mM CaCl<sub>2</sub>, 1.0 mM MgCl<sub>2</sub>, 5.6 mM glucose, 20 mM HEPES, and 1 mg/mL BSA at pH 7.4) and were stimulated by calcium ionophore A23187 (1 µM) or cross-linking antigen DNP-BSA (100 ng/mL) diluted in Tyrode's buffer. The cells were incubated at 37 °C in 5% CO<sub>2</sub> for 1 hour. Unstimulated cells were either lysed with a 0.5% Triton X-100 solution for the total amount of β-hexosaminidase release or left untreated for the spontaneous release of β-hexosaminidase. Stimulated but untreated cells served as the control. Then, aliquots of the supernatants (50 µL) collected from the control and experimental wells were incubated with an equal volume (50 µL) of 1 µM of *p*-NAG (*p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide) prepared in 0.1 M citrate buffer (pH 4.5) serving as the substrate for the released β-hexosaminidase. After 1 hour of incubation at 37 °C, the reaction was quenched by the addition of 100 µL of stop buffer (0.1 M Na<sub>2</sub>/NaHCO<sub>3</sub>, pH 10.0). Absorbance was measured at 405 nm on a microplate reader. The inhibition percentage of β-hexosaminidase release from RBL-2H3 cells was calculated as the percentage of the control value (untreated stimulated cells) using the following equation:

$$\text{Inhibition (\%)} = \left[ 1 - \frac{(\text{OD}_{\text{sample}} - \text{OD}_{\text{spontaneous}})}{(\text{OD}_{\text{control}} - \text{OD}_{\text{spontaneous}})} \right] \times 100$$

### 2.7.5. Anti-Inflammatory Activity in Human Neutrophils

#### 2.7.5.1. Preparation of human neutrophils

Blood was collected from healthy human donors (20-35 years old) via venipuncture using a protocol approved by the Institutional Review Board at Chang Gung Memorial Hospital (IRB number: 201902217A3). Neutrophils were isolated using a standard method as previously described (6).

#### 2.7.5.2. Measurement of superoxide generation

The inhibition of superoxide anion generation was measured by the reduction of ferricytochrome *c*, as previously described (7). Neutrophils ( $6 \times 10^5$ /mL) supplemented with 0.6 mg/mL ferricytochrome *c* and 1 mM Ca<sup>2+</sup> were equilibrated at 37 °C for 2 min and then incubated with the tested compounds or DMSO (control) for 5 min. Genistein served as a positive control. Cells were activated with formyl-methionyl-leucyl-phenylalanine (fMLF, 100 nM)/cytochalasin B (CB, 1 µg/mL) for 10 min. The absorbance was continuously monitored at 550 nm in a double-beam, six-cell-positioned spectrophotometer Hitachi U-3010 with constant stirring (Hitachi Inc., Tokyo, Japan). Calculations were based on the differences in absorbance with and without superoxide dismutase (SOD, 100 U/mL) divided by the extinction coefficient for the reduction of ferricytochrome *c* ( $\epsilon = 21.1/\text{mM}/10 \text{ mm}$ ).

#### 2.7.5.3. Measurement of elastase release

Elastase release, representing degranulation from azurophilic granules, was evaluated as described before (8). Neutrophils were equilibrated with MeO-Suc-Ala-Ala-Pro-Val-p-nitroanilide (100  $\mu$ M), an elastase substrate, at 37 °C for 2 min and then incubated with the drugs for 5 min. Genistein served as a positive control. Cells were activated by 100 nM fMLF and 0.5  $\mu$ g/mL CB, and changes in absorbance at 405 nm were continuously monitored to track elastase release. The results were expressed as the percent of the initial rate of elastase release in the fMLF/CB-activated drug-free control system.

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