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NOTE

Structure–activity relationships of flavanones, flavanone glycosides, and flavones in anti-degranulation activity in rat basophilic leukemia RBL-2H3 cells

Toshiro Noshita[1] · Kaori Miura[2] · Kaoru Ikeda[2] · Hidekazu Ouchi[3] · Takuya Matsumoto[4] · Akihiro Tai[1]

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Abstract
The incidence of type I allergies, which are associated with mast cell degranulation and local inflammation, is increasing, and new treatments are needed. To date, structure–activity relationships of flavonoids in their degranulation-inhibiting activity have not been systematically characterized. In the current study, the degranulation-inhibiting activity of a series of flavonoids was evaluated. The following three observations were made: (1) the activity disappears when a sugar moiety is introduced into the A ring of the flavanone; (2) the activity depends on the number of hydroxyl groups on the B ring; (3) the activity is markedly enhanced when a double bond is introduced into the C ring. The information obtained in the current study may guide the development of a therapy for type I allergies.

Keywords Flavonoids · Anti-degranulation · Type I allergy · Structure–activity relationship · Rat basophilic leukemia RBL-2H3 cell

Introduction

Flavonoids are one of the most widespread and important plant pigments, with over 9000 naturally occurring flavonoids from various plants characterized to date [1]. New flavonoids, including crude drugs, are continuously isolated from various sources. Flavonoids have been shown to be beneficial for human health [2] and to possess a wide range of biological and pharmacological activities in vitro, such as antimicrobial [3], antioxidative [4], anti-inflammatory [5], antihypertensive [6], anti-metastatic [7], anti-hypercholesterolemic [8], hypolipidemic [9], AMP-activated protein kinase (AMPK)-inhibiting [10], and antitumor [11] activities.

Japan is currently facing the societal problem of an increasing number of cases with type I allergies. According to one report, in 2006, ca. 30% of Japanese individuals in the Tokyo metropolitan area had cedar pollinosis, one of the typical allergic diseases [12]. Antigen-induced mast cell degranulation leads to local inflammation in type I allergies; therefore, natural compounds that can attenuate this degranulation have received particular attention as possible therapeutics. Recently, we showed that methyl or ethyl etherification at the 7-OH of hesperetin, a typical flavanone of *Citrus* fruits, slightly enhanced the inhibition of antigen-induced degranulation of rat basophilic leukemia cells (RBL-2H3) [13]. The degranulation-inhibiting activity is an important index of the anti-allergy action of a drug. However, no structure–activity relationship studies on the anti-degranulation effects of flavonoids have been published, with the exception of one study on the degranulation-inhibiting activities of flavanone glycosides and their aglycon derivatives [14].

✉ Toshiro Noshita
noshita@pu-hiroshima.ac.jp

✉ Akihiro Tai
atai@pu-hiroshima.ac.jp

1 Department of Life Sciences, Faculty of Life and Environmental Sciences, Prefectural University of Hiroshima, 5562 Nanatsuka, Shobara, Hiroshima 727-0023, Japan

2 Program in Biological System Sciences, Graduate School of Comprehensive Scientific Research, Prefectural University of Hiroshima, 5562 Nanatsuka, Shobara, Hiroshima 727-0023, Japan

3 Department of Pharmacy, Faculty of Pharmacy, Kindai University, 3-4-1 Kowakae, Higashiosaka 577-8502, Japan

4 Department of Environmental Sciences, Faculty of Life and Environmental Sciences, Prefectural University of Hiroshima, 5562 Nanatsuka, Shobara, Hiroshima 727-0023, Japan

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In the current study, a series of flavonoids were tested as inhibitors of degranulation in the RBL-2H3 cell line (a mast cell model). Combined with the results of our earlier study, their structure–activity correlations have now been clarified.

Materials and methods

Chemicals

Eriodictyol (**1**), eriodictyol-7-*O*-glucoside (**4**), eriocitrin (**5**), and narirutin (**9**) were purchased from Extrasynthese (Lyon, France). Hesperetin (**2**), hesperidin (**6**), and luteolin (**11**) were purchased from Cayman Chemical Co. (Ann Arbor, MI, USA), Nacalai Tesque Inc. (Kyoto, Japan), and LKT laboratories Inc. (St. Paul, MN, USA), respectively. Naringenin (**3**), neohesperidin (**7**), naringin (**8**), pinocembrin (**10**), diosmetin (**12**), and quercetin (**15**) were obtained from Sigma-Aldrich Co. (St. Louis, MO, USA). Apigenin (**13**) and chrysin (**14**) were purchased from Wako Pure Chemical Industries (Osaka, Japan). 7-*O*-Methylchrysin (**16**) and 7-*O*-methyldiosmetin (**17**) were prepared according to modified methods of Lim et al. [15] and Yang et al. [16], respectively, and their physical data were comparable. The ^{1}H and ^{13}C NMR spectra of compounds **16** and **17** were recorded using an ECA-500 spectrometer (JEOL, Tokyo, Japan) with tetramethylsilane as the internal standard. The IR spectra were obtained using a Nicolet iS10 FT-IR spectrometer (Thermo Fisher Scientific, Waltham, MA, USA) with a diamond horizontal attenuated reflectance (ATR) accessory and co-addition of 16 interferograms. Calibration models were generated using OMNIC 9.2.98 software (Thermo Fisher Scientific). Mass spectra were recorded using an API-2000 mass spectrometer (AB Sciex LLC, Framingham, MA, USA).

p-Nitrophenyl-2-acetamido-2-deoxy-β-D-glucopyranoside and the penicillin–streptomycin solution were obtained from Nacalai Tesque Inc. The monoclonal anti-dinitrophenyl (DNP) antibody, cromolyn sodium salt (DSCG), and DNP-labeled human serum albumin were purchased from Sigma-Aldrich Co. Dulbecco's modified Eagle's medium was purchased from Corning (Corning, NY, USA). Fetal bovine serum was acquired from HyClone (Logan, UT, USA).

7-*O*-Methylchrysin (16)

A mixture of chrysin (127 mg, 0.5 mmol), K_2CO_3 (83 mg, 0.6 mmol), and dimethyl sulfate (76 mg, 0.6 mmol) in 5 mL of acetone was refluxed for 1 h. Then, the reaction mixture was poured into water and extracted with CH_2Cl_2. The extract was washed with water and brine, dried over anhydrous $CaCl_2$, and concentrated under pressure. Purification of the residue by preparative thin-layer chromatography (benzene/ethyl acetate 12:1) yielded **16** (85 mg, 0.32 mmol, 63%). 7-*O*-Methylchrysin (**16**). Pale yellow crystal. M.p. 163°C (lit. [17] 164–166°C). ^{1}H NMR ($CDCl_3$, 500 MHz) δ: 3.87 (3H, s, O-CH_3), 6.36 (1H, d, J = 2.3 Hz, 6-H), 6.49 (1H, d, J = 2.3 Hz, 8-H), 6.65 (1H, s, 3-H), 7.49–7.56 (3H, m, Ar–H), 7.86–7.89 (2H, m, Ar–H). ^{13}C NMR ($CDCl_3$, 125 MHz) δ: 55.6, 92.6, 98.1, 105.6, 105.7, 126.2, 129.0, 131.2, 131.8, 157.7, 162.1, 163.9, 165.5, 182.4. IR (diamond ATR) cm^{-1}: 1663, 1606, 1586, 1493, 1350, 1201, 1158, 1039, 848, 805, 768. ESIMS *m/z*: 269.0 $[M + H]^+$.

7-*O*-Methyldiosmetin (17)

A mixture of luteolin (143 mg, 0.5 mmol), K_2CO_3 (280 mg, 2.0 mmol), and dimethyl sulfate (150 mg, 1.2 mmol) in 10 mL of acetone was refluxed for 1 h. Then, the reaction mixture was poured into water and extracted with CH_2Cl_2. The extract was washed with water and brine, dried over anhydrous $CaCl_2$, and concentrated under pressure. Purification of the residue by column chromatography on silica gel and elution with $CHCl_3$/MeOH (30:1) yielded **17** (110 mg, 0.37 mmol, 73%). 7-*O*-Methyldiosmetin (**17**). Pale yellow powder. M.p. 234–235 °C, decomp. (lit. [18] 230–232 °C, decomp.). ^{1}H NMR ($CDCl_3$:DMSO-d_6 = 2:1, 60 °C, 500 MHz) δ: 3.89 (3H, s, O-CH_3), 3.93 (3H, s, O-CH_3), 6.31 (1H, d, J = 2.1 Hz, 6-H), 6.53 (1H, d, J = 2.1 Hz, 8-H), 6.54 (1H, s, 3-H), 6.99 (1H, d, J = 8.4 Hz, 5′-H), 7.41 (1H, d, J = 2.1 Hz, 2′-H), 7.43 (1H, dd, J = 8.4, 2.1 Hz, 6′-H), 8.81 (1H, br-s, Ar-OH), 12.77 (1H, s, Ar-OH). ^{13}C NMR ($CDCl_3$/DMSO-d_6 = 2:1, 60 °C, 125 MHz) δ: 54.6, 54.8, 91.3, 96.9, 103.0, 104.2, 110.8, 112.2, 117.5, 122.6, 146.0, 150.1, 156.5, 160.8, 163.1, 164.3, 180.9. IR (diamond ATR) cm^{-1}: 3350, 1655, 1603, 1505, 1438, 1325, 1187, 1141, 1039, 813, 770. ESIMS *m/z*: 315.1 $[M + H]^+$.

Evaluation of degranulation-inhibiting activity

The inhibitory activity of the compounds against the release of β-hexosaminidase from RBL-2H3 cells was evaluated using a modified method of Watanabe et al. [19]. RBL-2H3 cells were purchased from the JCRB Cell Bank (Osaka, Japan). Dulbecco's modified Eagle's medium containing 10% heat-inactivated fetal bovine serum, 100 U mL^{-1} penicillin, and 100 μg mL^{-1} streptomycin was used as growth medium. The cells were cultured in 96-well plates (5.0×10^4 cells/well) for 24 h at 37 °C under a humidified 5% CO_2 atmosphere. Then, they were incubated in growth medium supplemented with 50 ng mL^{-1} mouse monoclonal anti-DNP IgE for 2 h. The cells were washed with a modified Tyrode's (MT) buffer before the test compounds or wortmannin (2.5 μM) was added. The test compounds and wortmannin were dissolved in DMSO and diluted in the MT

buffer at a final DMSO concentration of 0.25%. After 20 min of incubation, DNP-labeled human serum albumin (50 ng mL^{-1} final concentration) was added to the cells, and the culture was continued for 1 h. The supernatant was then collected, and the cells were lysed with MT buffer containing 0.1% Triton X-100. β-Hexosaminidase activity in the supernatants and cell lysates was determined by the method of Demo et al. [20]. Briefly, the supernatant or cell lysate (20 µL) was mixed with 3.3 mM *p*-nitrophenyl-2-acetamide-2-deoxy-β-D-glucopyranoside (40 µL) in 100 mM citrate buffer (pH 4.5), and the mixture was incubated in a 96-well plate at 37 °C for 90 min. The reaction was terminated by the addition of 2 M glycine–NaOH buffer (pH 10.4, 40 µL), and the absorbance at 405 nm was measured using a microplate reader.

Analysis of degranulation-inhibition assay data

The results are expressed as the mean and SD from three independent cultures. Multiple data comparisons were performed by analysis of variance followed by Dunnett's test; p values below 5% were regarded statistically significant. A single asterisk (*) indicates $p < 0.05$; two asterisks (**) indicate $p < 0.01$. The differences between two samples were analyzed using an unpaired Student's t test ($^{\#}p < 0.05$, $^{\#\#}p < 0.01$).

Results and discussion

Flavonoids have the general structure of a C6–C3–C6 skeleton, consisting of two benzene rings (A and B) and a heterocyclic C ring. In the current study, the anti-degranulation effects of 17 flavonoids were evaluated using the β-hexosaminidase release assay [19]; the tested flavonoids, including flavanones, flavanone glycosides, and flavones, are shown in Fig. 1. These flavonoids were chosen so as to investigate the effects of the sugar on the A ring; the substituent on the B ring; and the double bond in the C ring.

We first investigated the effect of the presence of a sugar moiety in the flavonoid A ring on the anti-degranulation activity. As shown in Fig. 2, eriodictyol (**1**) and hesperetin (**2**) both exhibited degranulation-inhibiting activities, although these were weak. Naringenin (**3**) tended to show degranulation-inhibitory activity. In contrast, glycosides of eriodictyol (**1**) [eriodictyol-7-*O*-β-D-glucopyranoside (**4**) and eriocitrin (**5**), Fig. 2a], hesperetin (**2**) [hesperidin (**6**) and neohesperidin (**7**), Fig. 2b], and naringenin (**3**) [narirutin (**9**) and naringin (**8**), Fig. 2c] did not exhibit any observable degranulation-inhibiting activity. On the basis of these observations, it was apparent that the degranulation-inhibiting activity is lost when a sugar moiety (regardless of the type and number of sugars) is connected to the flavonoid A ring.

Then, the effect of the structure of the B ring on the degranulation-inhibiting activity was evaluated. Eriodictyol (**1**), which has two hydroxyl groups in the B ring,

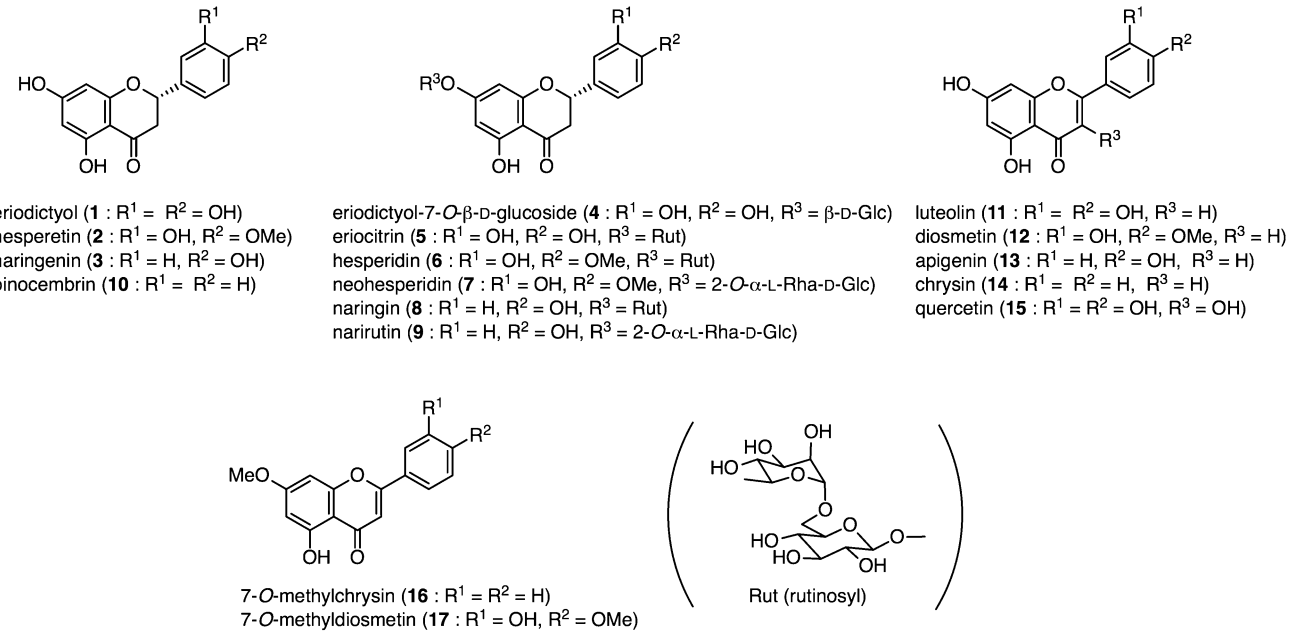

Fig. 1 Structures of flavonoids used in the current study

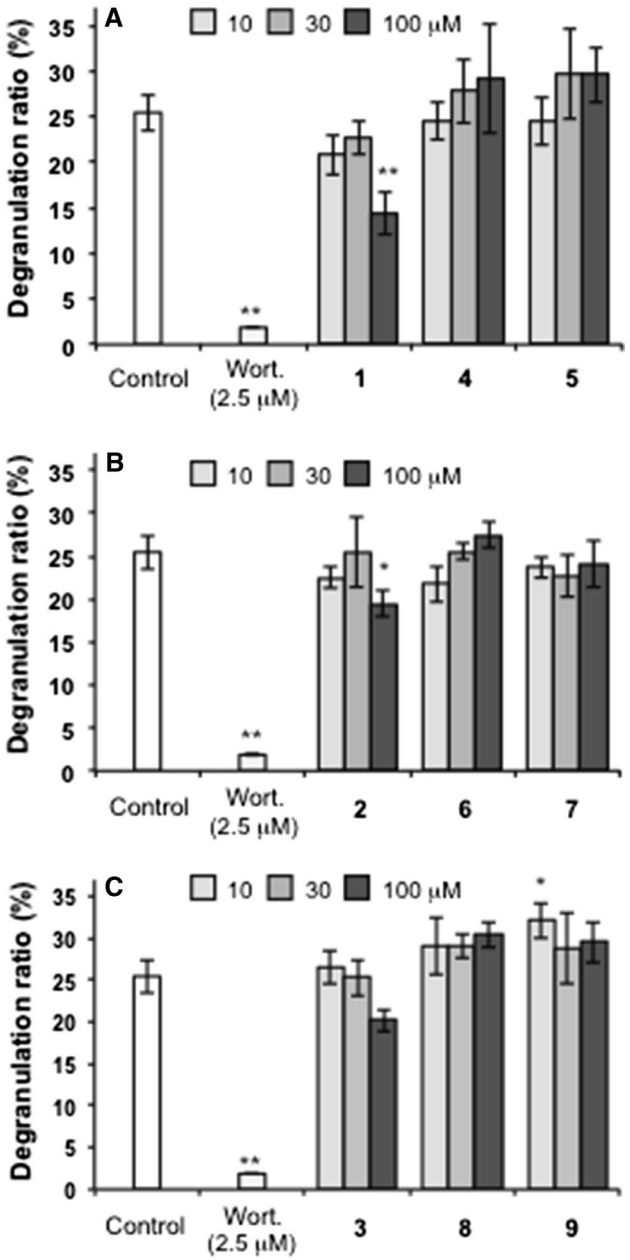

Fig. 2 Inhibitory effects of flavanones and their glycosides on antigen-induced degranulation of RBL-2H3 cells. **a** Eriodictyol (**1**) and its glycosides **4** and **5**; **b** hesperetin (**2**) and its glycosides **6** and **7**; **c** naringenin (**3**) and its glycosides **8** and **9**. Wort wortmannin. All data represent the mean ± SD of three independent cultures. *$p < 0.05$, **$p < 0.01$ (Dunnett's test), compared to the control

clearly exhibited the strongest activity, as shown in Fig. 3. Moreover, hesperetin (**2**) and naringenin (**3**), which have one hydroxyl group, exerted weak activity; no activity was observed for pinocembrin (**10**), which does not have a hydroxyl group on the B ring. These observations indicated that the degranulation-inhibiting activity of flavanones depends on the number of hydroxyl groups on the B ring.

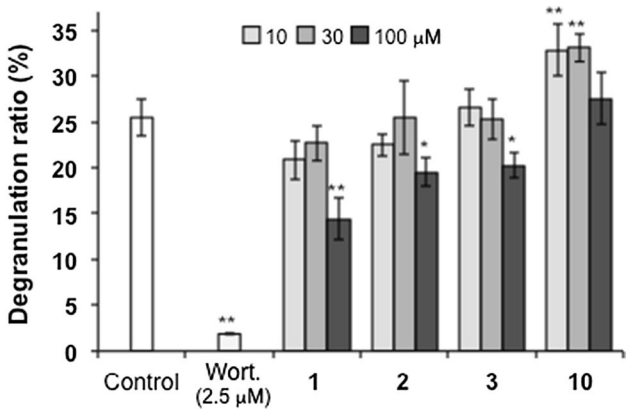

Fig. 3 Inhibitory effects of flavanones with a hydroxyl substitution on the B ring on antigen-induced degranulation of RBL-2H3 cells. Wort wortmannin. All data represent the mean ± SD of three independent cultures. *$p < 0.05$, **$p < 0.01$ (Dunnett's test), compared to the control

Next, the effect of the structure of the C ring on the degranulation-inhibiting activity was investigated. As shown in Fig. 4, the degranulation-inhibiting activity markedly increased when a double bond was present between positions C-2 and C-3 of the C ring. Luteolin (**11**), which has a double bond, exhibited very strong degranulation-inhibiting activity at a concentration of 10 μM, whereas eriodictyol (**1**), which does not possess the double bond, showed little activity. Similar differences in activity were observed for other flavanones and their corresponding flavones [**2** vs. **12**, **3** vs. apigenin (**13**), and **10** vs. chrysin (**14**)]. On the other hand, the introduction of a hydroxyl group at the C-3 position of the C ring did not affect the activity as there was no apparent difference in activity between luteolin (**11**) and

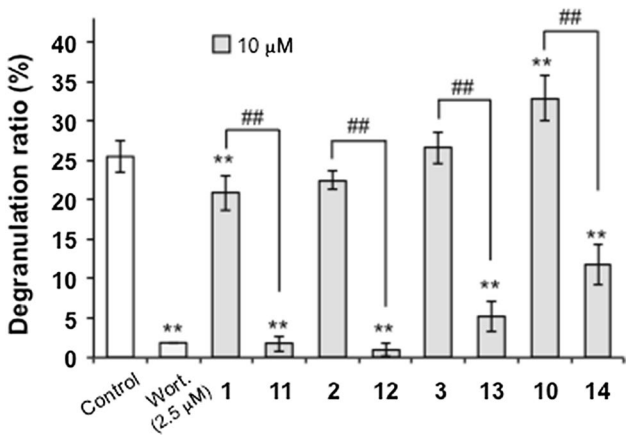

Fig. 4 Inhibitory effects of flavanones and flavones on antigen-induced degranulation of RBL-2H3 cells. Wort wortmannin. All data represent the mean ± SD of three independent cultures. **$p < 0.01$ (Dunnett's test), compared to the control. $^{\#\#}p < 0.01$ (*t* test), flavanones vs. flavones

quercetin (**15**) (data not shown). For the flavones, the activity depended on the number of hydroxyl groups on the B ring, which was also observed for the flavanones (activity: **11** = **12** > **13** > **14**). On the basis of these results, when the structure of the B ring was the same, the flavone showed a considerably stronger degranulation-inhibiting activity than the flavanone (Fig. 4). In other words, the double bond of the C ring of the flavonoid is important for the degranulation-inhibiting activity. In addition, the activity depends on the number of hydroxyl groups of the B ring in both the flavanone and the flavone (Figs. 3, 4), and these two effects [the effect of the double bond of the C ring and the effect of the hydroxyl group(s) in the B ring] are additive.

We recently reported that the methylation or ethylation of the 7-*O*-hydroxyl group clearly increases the degranulation-inhibiting activity of hesperetin [13]; thus, two 7-*O*-methylated derivatives, 7-*O*-methylchrysin (**16**) and 7-*O*-methyldiosmetin (**17**), were synthesized, and the effects of the methylation on their activity were examined. Comparison of the degranulation-inhibiting activities of **14** and 7-*O*-methylchrysin (**16**) confirmed that methylation of the C-7 hydroxyl group potentiated the activity (Fig. 5). In contrast, 7-*O*-methyldiosmetin (**17**) showed a weaker activity than diosmetin (**12**), as shown in Fig. 5. In other words, the effect of 7-*O*-methylation of the A ring of flavones was different from that of the introduction of a sugar moiety on the A ring of flavanones. The degranulation-inhibiting activities were lost following glycosidation of the A ring, as shown in Fig. 2, but in the case of 7-*O*-methylation, the effect was not as clear-cut (Fig. 5). Thus, an investigation of additional compounds is necessary to reach a conclusion about the effect of 7-*O*-methylation of the A ring of flavonoids on the degranulation-inhibiting activity.

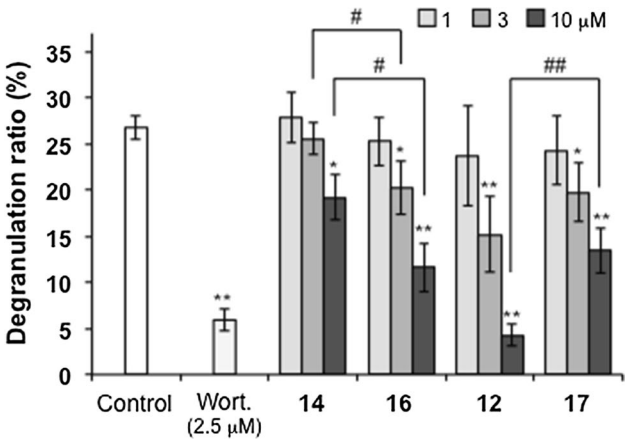

Fig. 5 Inhibitory effects of 7-*O*-methylated flavones on antigen-induced degranulation of RBL-2H3 cells. Wort wortmannin. All data represent the mean ± SD of three independent cultures. $^{*}p < 0.05$, $^{**}p < 0.01$ (Dunnett's test) compared to the control. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ (*t* test)

In conclusion, the inhibiting activity of 17 flavonoids against antigen-induced degranulation of rat basophilic leukemia cells (RBL-2H3) was examined, and several structural requirements for the activity were clarified. The experiments revealed that the inhibitory activity was lost when a sugar unit was coupled to the A ring of the flavanone and that the activity depended on the number of hydroxyl groups on the B ring. The inhibiting activities of flavones were remarkably more pronounced than those of flavanones, indicating the importance of the double bond in the C ring. The spatial configuration of flavones and flavanones is considerably different, resulting in differences in activity. The findings of this study might be helpful in designing lead compounds for the development of therapeutics for type I allergies.

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