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# Structural Requirements of Flavonoids for Inhibition of Antigen-Induced Degranulation, TNF-α and IL-4 Production from RBL-2H3 Cells

Hisashi Mastuda, Toshio Morikawa, Kazuho Ueda, Hiromi Managi and Masayuki Yoshikawa*

*Kyoto Pharmaceutical University, Misasagi, Yamashina-ku, Kyoto 607-8412, Japan*

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**Abstract**—To clarify the structure–activity relationships of flavonoids for antiallergic activity, the inhibitory effects of various flavonoids on the release of β-hexosaminidase, as a marker of degranulation of RBL-2H3 cells, were examined. Among them, luteolin ($IC_{50}=3.0\,\mu M$), diosmetin (2.1 μM), and fisetin (3.0 μM) were found to show potent inhibitory activity, and the results suggested the following structural requirements of flavonoids: (1) the 2-3 double bond of flavones and flavonols is essential for the activity; (2) the 3- or 7-glycoside moiety reduced the activity; (3) as the hydroxyl groups at the 3′-, 4′-, 5-, 6-, and 7-positions increased in number, the inhibitory activities become stronger; (4) the flavonols with a pyrogallol type moiety (the 3′,4′,5′-trihydroxyl groups) at the B ring exhibited less activity than those with a phenol type moiety (the 4′-hydroxyl group) or catechol type moiety (the 3′,4′-dihydroxyl groups) at the B ring; (5) the activities of flavones were stronger than those of flavonols; and (6) methylation of flavonols at the 3-position reduced the activity. However, (7) several flavones and flavonols with the 4′- and/or 7-methoxyl groups did not obey rules (3), (4), and (5). In addition, several flavonoids, that is apigenin, luteolin, diosmetin, fisetin, and quercetin, inhibited the antigen-IgE-mediated TNF-α and IL-4 production from RBL-2H3 cells, both of which participate in the late phase of type I allergic reactions.


## Introduction

Histamine, which is released from mast cells stimulated by an antigen or a degranulation inducer, is usually determined as a degranulation marker in experiments on immediate allergic reactions in vitro. β-Hexosaminidase is also stored in secretory granules of mast cells, and is released concomitantly with histamine when mast cells are immunologically activated,[1] and this enzyme activity in the medium is used as a marker of mast cell degranulation.[2] Previously, we reported the isolation and structural elucidation of various antiallergic constituents from natural medicines, such as the leaves of *Hydrangea macrophylla* var. *thunbergii*, the bark of *Myrica rubra*, the fruit of *Alpinia oxyphylla*, and the rhizome of *Hedychium coronarium*, and so on. In these cases, phenolic constituents such as isocoumarin, diarylheptanoids, and flavonoids were isolated as active components.[3] Flavonoids are widely distributed in plant kingdum, and have been recognized to have various biological activities including antiallergic actions, and various in vitro and in vivo studies of the antiallergic effects of flavonoids have been reported. However, their stucture-activity relationships were not discussed satisfactorily because of insufficient numbers of examples in vitro and/or technical limitations in vivo. To clarify the structure–activity relationships of flavonoids for antiallergic activity, we examined the effects of flavonoids on the release of β-hexosaminidase induced by dinitrophenylated bovine serum albumin (DNP-BSA) from a rat basophilic leukemia cell line (RBL-2H3) sensitized with anti-DNP IgE. In addition, the inhibitory effects of several flavonoids such as apigenin (**10**), luteolin (**13**), diosmetin (**15**), fisetin (**22**), quercetin (**23**), and myricetin (**35**) on antigen-induced production of TNF-α and IL-4, which participate in the late phase of type I allergic reaction,[4,5] from RBL-2H3 cells were examined.

*Corresponding author. Tel.: +81-75-595-4633; fax: +81-75-595-4768; e-mail: shoyaku@mb.kyoto-phu.ac.jp

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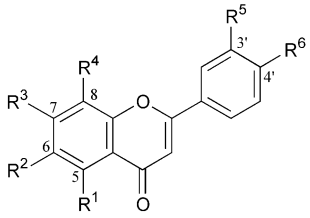
## Results and Discussion

### Structural requirements of flavonoids for inhibition of degranulation of RBL-2H3 cells

To clarify the structure–activity relationships of flavonoids for antiallergic activity, we examined the inhibitory effects of more than 50 flavonoids on the release of β-hexosaminidase from RBL-2H3 cells, which is a common screening test for the immediate phase of type I antiallergic reactions.[2] As shown in Tables 1–3, luteolin (**13**, $IC_{50} = 3.0\,\mu M$), diosmetin (**15**, $2.1\,\mu M$), fisetin (**22**, $3.0\,\mu M$), and quercetin (**23**, $4.6\,\mu M$) showed potent

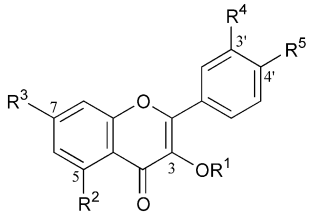
**Table 1.** Inhibitory effects of flavones on the release of β-hexosaminidase from RBL-2H3 cells




| | $R^1$ | $R^2$ | $R^3$ | $R^4$ | $R^5$ | $R^6$ | $IC_{50}$ (μM) | Enzyme inhibition (%)[a] |
|---|---|---|---|---|---|---|---|---|
| Flavone (**1**) | H | H | H | H | H | H | 44 | 2.1 |
| 7-Hydroxyflavone (**2**) | H | H | OH | H | H | H | 33 | −0.7 |
| Chrysin (**3**) | OH | H | OH | H | H | H | 20 | −3.7 |
| Tectochrysin (**4**) | OH | H | $OCH_3$ | H | H | H | 49 | 6.5 |
| 4′,7-Dihydroxyflavone (**5**) | H | H | OH | H | H | OH | 22 | 9.6 |
| 3′,4′-Dihydroxyflavone (**6**) | H | H | H | H | OH | OH | 30 | 5.0 |
| Baicalein (**7**) | OH | OH | OH | H | H | H | 16 | −4.6 |
| Baicalin (**8**) | OH | OH | O-GlcA | H | H | H | > 100 (2.0) | — |
| Wogonin (**9**) | OH | H | OH | $OCH_3$ | H | H | 29 | 10 |
| Apigenin (**10**) | OH | H | OH | H | H | OH | 6.1 | −1.7 |
| **11** | OH | H | $OCH_3$ | H | H | $OCH_3$ | > 100 (41) | — |
| 3′,4′,7-Trihydroxyflavone (**12**) | H | H | OH | H | OH | OH | 13 | 7.9 |
| Luteolin (**13**) | OH | H | OH | H | OH | OH | 3.0 | 21 |
| Luteolin 7-*O*-Glc (**14**) | OH | H | O-Glc | H | OH | OH | > 100 (19) | — |
| Diosmetin (**15**) | OH | H | OH | H | OH | $OCH_3$ | 2.1 | 4.4 |
| Pilloin (**16**) | OH | H | $OCH_3$ | H | OH | $OCH_3$ | 28 | 9.6 |
| **17** | OH | H | $OCH_3$ | H | $OCH_3$ | $OCH_3$ | 19 | 8.9 |
| **18** | $OCH_3$ | H | $OCH_3$ | H | $OCH_3$ | $OCH_3$ | 68 | 11 |

Glc, β-D-glucopyranosyl; GlcA, β-D-glucopyranosiduronic acid. Values in parentheses represent the inhibition (%) at 100 μM.
[a]Values indicate enzyme inhibition (%) against β-hexosaminidase at 100 μM.

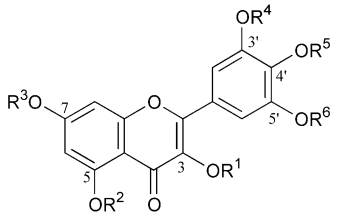
**Table 2.** Inhibitory effects of flavonols on the release of β-hexosaminidase from RBL-2H3 cells—1




| | $R^1$ | $R^2$ | $R^3$ | $R^4$ | $R^5$ | $IC_{50}$ (μM) | Enzyme inhibition (%)[a] |
|---|---|---|---|---|---|---|---|
| 3-Hydroxyflavone (**19**) | H | H | H | H | H | 69 | 2.5 |
| Izalpinin (**20**) | H | OH | $OCH_3$ | H | OH | > 100 (30) | — |
| Kaempferol (**21**) | H | OH | OH | H | OH | 14 | 4.6 |
| Fisetin (**22**) | H | H | OH | OH | OH | 3.0 | 21 |
| Quercetin (**23**) | H | OH | OH | OH | OH | 4.6 | 14 |
| Quercetin 3-*O*-Glc (**24**) | Glc | OH | OH | OH | OH | 54 | 5.0 |
| Quercetin 3-*O*-Gal (**25**) | Gal | OH | OH | OH | OH | > 100 (27) | — |
| Rhamnetin (**26**) | H | OH | $OCH_3$ | OH | OH | 21 | 0 |
| Tamarixetin (**27**) | H | OH | OH | OH | $OCH_3$ | 57 | 4.8 |
| Omburine (**28**) | H | OH | $OCH_3$ | OH | $OCH_3$ | 5.5 | 6.0 |
| **29** | $CH_3$ | OH | $OCH_3$ | OH | OH | 64 | 21 |
| Ayanin (**30**) | $CH_3$ | OH | $OCH_3$ | OH | $OCH_3$ | 18 | 2.6 |
| **31** | H | OH | $OCH_3$ | $OCH_3$ | $OCH_3$ | 6.0 | −1.7 |
| **32** | $CH_3$ | OH | $OCH_3$ | $OCH_3$ | $OCH_3$ | 25 | −0.6 |
| **33** | $CH_3$ | $OCH_3$ | $OCH_3$ | $OCH_3$ | $OCH_3$ | > 100 (36) | — |
| Rutin (**34**) | -Glc$^6$—$^1$Rha | OH | OH | OH | OH | > 100 (30) | — |

Glc, β-D-glucopyranosyl; Gal, β-D-galactopyranosyl; Rha, α-L-rhamnopyranosyl. Values in parentheses represent the inhibition (%) at 100 μM.
[a]Values indicate enzyme inhibition (%) against β-hexosaminidase at 100 μM.

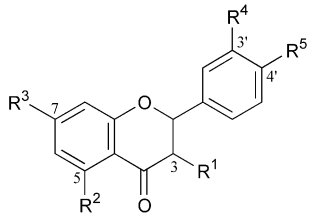
**Table 3.** Inhibitory effects of flavonols on the release of β-hexosaminidase from RBL-2H3 cells—2



| | $R^1$ | $R^2$ | $R^3$ | $R^4$ | $R^5$ | $R^6$ | $IC_{50}$ (μM) | Enzyme inhibition (%)[a] |
|---|---|---|---|---|---|---|---|---|
| Myricetin (**35**) | H | H | H | H | H | H | 23 | −4.8 |
| Mearnsetin (**36**) | H | H | H | H | $CH_3$ | H | 27 | 0.4 |
| **37** | H | H | $CH_3$ | H | H | H | 19 | 21 |
| **38** | $CH_3$ | H | $CH_3$ | H | H | H | 27 | 15 |
| **39** | H | H | $CH_3$ | H | $CH_3$ | H | 7.9 | 63 |
| **40** | H | H | $CH_3$ | $CH_3$ | $CH_3$ | H | 13 | 2.4 |
| **41** | $CH_3$ | $CH_3$ | $CH_3$ | $CH_3$ | $CH_3$ | $CH_3$ | 58 | 2.0 |
| Myricitrin (**42**) | Rha | H | H | H | H | H | >100 (14) | — |
| **43** | Rha | H | $CH_3$ | H | $CH_3$ | H | >100 (−6.2) | — |

Rha, α-L-rhamnopyranosyl. Values in parentheses represent the inhibition (%) at 100 μM.
[a]Values indicate enzyme inhibition (%) against β-hexosaminidase at 100 μM.

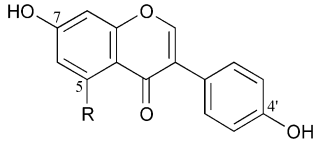
**Table 4.** Inhibitory effects of flavanones and dihydroflavonol on the release of β-hexosaminidase from RBL-2H3 cells



| | $R^1$ | $R^2$ | $R^3$ | $R^4$ | $R^5$ | $IC_{50}$ (μM) | Enzyme inhibition (%)[a] |
|---|---|---|---|---|---|---|---|
| Flavanone (**44**) | H | H | H | H | H | >100 (25) | — |
| Liquiritigenin (**45**) | H | OH | OH | H | OH | >100 (7.3) | — |
| Eriodictyol (**46**) | H | OH | OH | OH | OH | >100 (38) | — |
| Fustin (**47**) | OH | H | OH | OH | OH | >100 (8.0) | — |

Values in parentheses represent the inhibition (%) at 100 μM.
[a]Values indicate enzyme inhibition (%) against β-hexosaminidase at 100 μM.

**Table 5.** Inhibitory effects of isoflavones on the release of β-hexosaminidase from RBL-2H3 cells



| | R | $IC_{50}$ (μM) | Enzyme inhibition (%)[a] |
|---|---|---|---|
| Daidzein (**48**) | H | >100 (1.3) | — |
| Genistein (**49**) | OH | >100 (39) | — |

Values in parentheses represent the inhibition (%) at 100 μM.
[a]Values indicate enzyme inhibition (%) against β-hexosaminidase at 100 μM.

inhibitory activity (<5 μM). On the other hand, baicalin (**8**), **11**, luteolin 7-*O*-β-D-glucopyranoside (**14**), izalpinin (**20**), quercetin 3-*O*-β-D-galactopyranoside (**25**), **33**, rutin (**34**), myricitrin (**42**), and **43** lacked the activity (>100 μM). Thus, flavones and flavonols with the 3- and/or 7-glycoside moieties did not show such activity, with the exception of quercetin 3-*O*-β-D-glucopyranoside (**24**, 54 μM). In addition, the activities of the methylated derivatives were reduced, except for three examples [**15**, **39** (7.9 μM), and **40** (13 μM)].

As the hydroxyl groups of flavones at the 3′-, 4′-, 5-, 6-, and 7-positions increased in number, the inhibitory activity became stronger [flavone (**1**, 44 μM) <7-hydroxyflavone (**2**, 33 μM) <3′,4′-dihydroxyflavone (**6**, 30 μM), 4′,7-dihydroxyflavone (**5**, 22 μM), chrysin (**3**, 20 μM), <baicalein (**7**, 16 μM), 3′,4′,7-trihydroxyflavone (**12**, 13 μM), apigenin (**10**, 6.1 μM) <**13**].

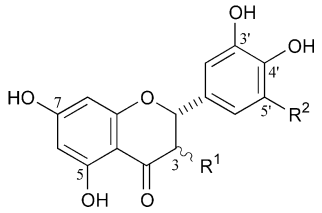
Comparison of flavones (Table 1) with flavonols (Table 2) revealed that the activities of flavones were stronger than those of flavonols [flavone (**1**, 44 μM) >3-hydroxyflavone (**19**, 69 μM), **10**>kaempferol (**21**, 14 μM), **13**>**23**, and **15**>tamarixetin (**27**, 5.5 μM)], but flavonols with the 4′,7-methoxyl groups showed stronger activities than the corresponding flavones [pilloin (**16**, 28 μM)< ombuine (**28**, 5.5 μM), **17** (19 μM)<**31** (6.0 μM)].

In most examples, as the numbers of hydroxyl groups of flavonols increased, the activity became stronger similarly to the observations in flavones. However, flavonols with the pyrogallol type moiety (the 3′,4′,5′-trihydroxyl groups) exhibited less activity than those with the catechol type moiety (the 3′,4′-dihydroxyl groups) or phenol type moiety (the 4′-hydroxyl group) [**23**>**21**>myricetin (**35**, 23 μM), **28**>**39**, **31**>**40**], although exceptions were found [rhamnetin (**26**, 21 μM)=**37** (19 μM), **29** (64 μM)<**38** (27 μM)]. Methylation of flavonols at the 3-position reduced the activity [**31** (6.1 μM)>**32** (25 μM), **37**>**38**].

On the other hand, flavanones (**44–46**), dihydroflavonol (**47**), isoflavones (**48**, **49**), and flavan-3-ols (**50–52**) did not show such activity (>100 μM), as shown in Tables 4–6.

The effects of baicalein (**7**) and various related flavonoids on histamine release from RBL-2H3 cells were reported previously by Kawasaki et al.; they concluded that the 4′- and 5-hydroxyl groups are important for the activity and the 3- and 7-hydroxyl groups reduced the activity.[6] Cheong et al. also reported the possible relationships of structure to the inhibitory activities of flavonoids based on the results obtained with more than 20 compounds using a similar assay method by the determination of β-hexosaminidase: (1) flavones and flavonols were active, but flavanones, isoflavones, and catechins were not; (2) the 3-, 3′-, 5-, and 5′-hydroxyl groups did not alter the activity markedly; (3) the 4′-, 6-, and 7-hydroxyl groups enhanced the activity; (4) the 7-glycoside moiety decreased the activity markedly; and (5) the 2′-hydroxyl group was not better for the activity.[7]

**Table 6.** Inhibitory effects of flavan-3-ols on the release of β-hexosaminidase from RBL-2H3 cells




| | $R^1$ | $R^2$ | $IC_{50}$ (μM) | Enzyme inhibition (%)[a] |
|---|---|---|---|---|
| (+)-Catechin (**50**) | β-OH | H | >100 (2.1) | — |
| (−)-Epicatechin (**51**) | α-OH | H | >100 (−3.9) | — |
| (−)-Epigallocatechin (**52**) | α-OH | OH | >100 (0.2) | — |

Values in parentheses represent the inhibition (%) at 100 μM.
[a]Values indicate enzyme inhibition (%) against β-hexosaminidase at 100 μM.

The results of the present study supported those reported previously and indicated some additional and revised structural requirements of flavonoids for the activity: (1) the 2–3 double bond of flavones and flavonols is essential for the activity; (2) the 3- or 7-glycoside moiety reduced the activity; (3) as the hydroxyl groups at the 3′-, 4′-, 5-, 6-, and 7-positions increased in number, the inhibitory activities become stronger; (4) the flavonols with the pyrogallol type moiety (the 3′,4′,5′-trihydroxyl groups) at the B ring exhibited less activity than those with the phenol type moiety (the 4′-hydroxyl group) or catechol type moiety (the 3′,4′-dihydroxyl groups) at the B ring; (5) the activities of flavones were stronger than those of flavonols; and (6) methylation of flavonols at the 3-position reduced the activity. However, (7) several flavones and flavonols with the 4′- and/or 7-methoxyl groups did not obey rules (3), (4), and (5).

Moreover, the effects of test compounds on β-hexosaminidase activity were examined to clarify whether their effects were due to inhibition of enzyme activity or of degranulation. With the exception of **39** (enzyme inhibition, 15% at 10 μM, 39% at 30 μM, 63% at 100 μM), these active constituents did not affect the enzyme activity of β-hexosaminidase.

In conclusion, luteolin (**13**, 3.0 μM), diosmetin (**15**, 2.1 μM), and fisetin (**22**, 3.0 μM) showed potent inhibitory activity. In addition, some additional structural requirements of flavonoids for the activity were clarified.

## Effects of several flavonoids on antigen-induced TNF-α and IL-4 production from RBL-2H3 cells

Recently, the biphasic allergic reaction mediated by antigen-IgE antibody has been reported. After challenge with an antigen, sensitized animals and atopic individuals exhibit immediate responses, such as the appearance of wheals and fares on the skin and bronchoconstriction of the airways, and late-phase responses such as edema and erythema usually persist over a 6–24 h period at the site of challenge in the skin and airways.[4,8] The immediate responses are mainly due to small molecule chemical mediators (e.g., histamine, serotonin) from mast cells. Mast cells also produce cytokines including TNF-α, IL-4, and IL-5, and these cytokines play an important role in the late phase reactions.[4,5] However, there have been only a few reports about the inhibitory effects of flavonoids such as luteolin (**13**) and quercetin (**23**) on TNF-α release from mast cells.[4]

In the present study, the effects of several flavones and flavonols, which exhibited strong inhibitory effects against the release of β-hexosaminidase, on concentrations of TNF-α and IL-4 in the medium 4 h after challenge in RBL-2H3 cells were examined. In agreement with previous reports,[4] luteolin (**13**) and quercetin (**23**) inhibited release of TNF-α with $IC_{50}$ values of 5.8 and 5.6 μM, respectively. Apigenin (**10**, $IC_{50}$ = 5.3 μM), diosmetin (**15**, 5.3 μM), and fisetin (**22**, 4.2 μM) also showed a significant inhibitory effect (Table 7). These compounds (**10**, **13**, **15**, **22**, **23**) inhibited release of IL-4 with $IC_{50}$ values of 3.6, 3.7, 3.0, 3.8, and 7.5 μM, respectively. However, myricetin (**35**), which exhibited

**Table 7.** Effects of flavonoids (**10**, **13**, **22**, **23**, **35**) on the release of TNF-α and IL-4 from RBL-2H3 cells

| | Conc. of test sample (μm) | | | | | | $IC_{50}$ (μM) |
|---|---|---|---|---|---|---|---|
| | 0 | 1 | 3 | 10 | 30 | 100 | |
| | | | | TNF-α (inhibition %) | | | |
| Apigenin (**10**) | 0.0±5.6 | −1.9±6.1 | 26.8±4.3** | 96.3±7.6** | 113.9±5.4** | — | 5.3 |
| Luteolin (**13**) | 0.0±3.1 | — | 25.0±3.0** | 89.0±1.3** | 101.3±1.2** | — | 5.8 |
| Diosmetin (**15**) | 0.0±2.9 | 5.1±1.8 | 29.5±4.6** | 91.0±1.2** | 99.0±1.2** | — | 5.3 |
| Fisetin (**22**) | 0.0±1.8 | 2.0±6.1 | 34.4±1.9** | 80.1±0.9** | 95.5±1.3** | — | 4.2 |
| Quercetin (**23**) | 0.0±4.2 | 11.1±6.3 | 30.9±3.8** | 67.2±1.4** | 86.8±0.7** | — | 5.6 |
| Myricetin (**35**) | 0.0±11.9 | — | — | 5.1±2.7 | 8.6±3.4 | 21.0±5.2 | >100 |
| | | | | IL-4 (inhibition %) | | | |
| Apigenin (**10**) | 0.0±2.5 | — | 45.3±0.6** | 75.7±1.8** | 96.9±0.1** | — | 3.6 |
| Luteolin (**13**) | 0.0±2.1 | 36.5±0.5** | 41.6±1.3** | 89.4±1.0** | 99.2±0.2** | — | 3.7 |
| Diosmetin (**15**) | 0.0±2.3 | 32.3±1.9** | 47.9±2.0** | 88.3±1.0** | 98.8±0.1** | — | 3.0 |
| Fisetin (**22**) | 0.0±2.6 | 33.3±1.2** | 37.4±2.6** | 75.9±2.7** | 99.1±0.1** | — | 3.8 |
| Quercetin (**23**) | 0.0±3.7 | 29.3±2.4** | 35.6±1.8** | 54.3±1.8** | 88.7±0.4** | — | 7.5 |
| Myricetin (**35**) | 0.0±2.5 | — | 24.8±0.5** | 32.9±0.8** | 40.5±1.0** | 53.5±3.0** | 74 |

Each value represents the mean±SEM ($N$ = 3 or 4). Significantly different from control, $^{**}p < 0.01$.

weak activity against the release of β-hexosaminidase, showed a weak inhibitory effect against release of IL-4, but not against TNF-α. These findings suggested that these active flavonoids (**10**, **13**, **15**, **22**, **23**) are effective against the late-phase reactions.

## Experimental

### Preparations of flavonoids

All test flavonoids were prepared with some chemical modifications, as described previously.[9]

### Inhibitory effects on the release of β-hexosaminidase from RBL-2H3 cells

Inhibitory effects of test samples on the release of β-hexosaminidase from RBL-2H3 cells were evaluated by the method reported previously.[3d,3e] Briefly, RBL-2H3 cells dispensed into 24-well plates at a concentration of $2\times10^5$ cells/well using Eagle's Minimum Essential Medium (MEM) containing 10% fetal calf serum, penicillin (100 units/mL), streptomycin (100 μg/mL), and anti-DNP IgE (0.45 μg/mL), and incubated overnight at 37 °C in 5% $CO_2$ for sensitization of the cells. Then, cells were washed twice with 500 μL of 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 of siraganian buffer (supplemented with 5.6 mM glucose, 1 mM $CaCl_2$, and 0.1% BSA) for an additional 10 min at 37 °C. Then, aliquots of 20 μL of test sample solution were added to each well and incubated for 10 min, followed by addition of 20 μL of antigen (DNP-BSA, final concentration was 10 μg/mL) at 37 °C for 10 min to stimulate the cells to evoke allergic reactions (degranulation). The reaction was stopped by cooling in an ice bath for 10 min. The supernatant (50 μL) was transferred into a 96-well microplate 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 with a microplate reader at 405 nm. The test sample was dissolved in dimethylsulfoxide (DMSO), and the solution was added to siraganian buffer (final DMSO concentration was 0.1%).

The inhibition % of the release of β-hexosaminidase by the test samples was calculated by the following equation, and $IC_{50}$ values were determined graphically:

$$\text{inhibition (\%)} = \left(1 - \frac{T - B - N}{C - N}\right) \times 100$$

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

Under these conditions, it was calculated that 40–50% of β-hexosaminidase was released from the cells in the control groups by determination of the total β-hexosaminidase activity after sonication of the cell suspension.

### β-Hexosaminidase inhibitory activity

The cell suspension ($5\times10^7$ cells) in 6 mL of PBS was sonicated. The solution was then centrifuged and the supernatant was diluted with siraganian buffer and adjusted to equal the enzyme activity of the degranulation test described above. The enzyme solution (45 μL) and test sample solution (5 μL) were transferred into a 96-well microplate and incubated with 50 μL of the substrate solution at 37 °C for 1 h. The reaction was stopped by adding 200 μL of the stop solution. The absorbance was measured using a microplate reader at 405 nm.

### Inhibitory effect on antigen-induced TNF-α and IL-4 production from RBL-2H3 cells

RBL-2H3 cells ($2\times10^5$ cells/well) were sensitized with anti-DNP IgE as described above. The cells were washed twice with 500 μL of MEM containing 10% fetal calf serum, penicillin (100 units/mL), and streptomycin (100 μg/mL), and exchanged with 320 μL of the fresh medium. Then, 40 μL of test sample solution and 40 μL of antigen (DNP-BSA, final concentration was 10 μg/mL) were added to each well and incubated at 37 °C for 4 h. The supernatant (50 μL) was transferred into a 96-well ELISA plate and TNF-α and IL-4 concentrations were determined using commercial kits (TNF-α, rat, ELISA system, code 2734; IL-4, rat, ELISA system, code 2737, Amersham Pharmacia Biotech Co., Ltd.). The test samples were dissolved in DMSO, and the solution was added to MEM (final DMSO concentration was 0.1%).

To estimate the production of TNF-α or IL-4 from cells, the same procedure was followed, but without addition of antigen and IgE (Normal). Thus, the inhibition % of the production of TNF-α or IL-4 by the test sample was calculated by the following equation, and $IC_{50}$ values were determined graphically:

$$\text{inhibition (\%)} = \left(1 - \frac{T - N}{C - N}\right) \times 100$$

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

### Statistics

Values are expressed as means ± SEM. One-way analysis of variance followed by Dunnett's test was used for statistical analysis.

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