Effects of Luteolin and Other Flavonoids on IgE-Mediated Allergic Reactions

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Abstract: The anti-allergic action of luteolin was investigated in the rodent experimental allergic models. In the present study, the effects of luteolin were compared to those of baicalein, quercetin, and prednisolone. Luteolin as well as baicalein, quercetin, and prednisolone inhibited the IgE antibody-mediated biphasic cutaneous reaction (immediate phase reaction and late phase reaction) in mice. However, these compounds did not affect the histamine-, serotonin-, and platelet activating factor-induced cutaneous reactions in rats. In an *in vitro* study, luteolin, baicalein, and quercetin inhibited IgE-mediated histamine release from bone marrow-derived cultured murine mast cells (BMMC) and rat peritoneal mast cells. These compounds also inhibited IgE-mediated TNF-α and IL-6 production from BMMC. From these results, luteolin inhibited the IgE-mediated biphasic cutaneous reaction mainly by the inhibition of histamine and cytokine release from mast cells, but not through mediator antagonistic effects.

Key words: Flavonoids, luteolin, biphasic cutaneous reaction, mast cells, anti-allergic activity.

Introduction

The flavonoids have long been recognized to possess anti-allergic, anti-inflammatory, anti-viral, anti-proliferative, and anti-carcinogenic activities as well as to affect some aspects of mammalian metabolism (1), (2). As for the anti-allergic action of flavonoids, most investigations were focused on the effect on acute, immediate allergic reactions *in vitro* (1–4). Recently, the biphasic allergic reactions have become of interest because of the similarity to the clinical manifestations of chronic allergic diseases. After challenge with the relevant antigen, sensitized animals and atopic individuals exhibit immediate responses, such as the appearance of wheals and flares on the skin and bronchoconstriction of the airways, and late phase responses, such as edema and erythema usually persist over a 6 to 24 h period at the challenged site of the skin and airways (5), (6). Human non-atopic subjects passively sensitized with IgE-containing sera exhibit immediate and late phase skin reactions to subsequent antigen challenge (7). We have established similar reactions in mice. The biphasic allergic cutaneous reaction was caused by painting of the antigen on the ear of mice which are passively sensitized with monoclonal anti-dinitrophenol (DNP) IgE antibody (8). The biphasic cutaneous reaction appeared with peaks at 1 h (IPR; immediate phase reaction) or 24 h (LPR; late phase reaction) after epicutaneous challenge with the antigen. IPR is mainly due to small molecule chemical mediators, such as histamine and serotonin, released from resident mast cells. LPR involves both mast cell-dependent and mast cell-independent mechanisms which are suppressed by glucocorticoids. Our previous studies also indicate that cytokines including TNF-α and IL-1β play an important role in the onset of LPR (9), (16).

In the present study, we focused on the effect of luteolin on the IgE antibody-mediated biphasic cutaneous reaction in mice. This compound had been found in *Elodea*, *Secale*, *Perilla*, and *Origanum* species (10). The reason why we have chosen luteolin is mainly due to the small amount of information available about an anti-allergic action of luteolin. Until now, it was reported that luteolin inhibited compound 48/80- and ionophore A23187-induced histamine release from rat peritoneal mast cells and tumor promoter-induced histamine release from human basophils (11), (12). As far as cytokine production from mast cells or basophils is concerned, the effect of luteolin was not at all investigated.

Materials and Methods

Agents

For the *in vivo* study, luteolin (Sigma, St. Louis, MO) biacelein (Extrasynthese, Genay, France) and quercetin (Extrasynthese) were suspended in 1% polyethylene glycol (PEG200; WAKO Pure Chemical Industries, Ltd., Osaka, Japan). The chemical structures of these compounds are shown in Table **1**, and the purities of these compounds were more than 99% (written in data sheets of each company). For the *in vitro* study, these agents were dissolved in DMSO as 100 mmol/L dilutions and stored. Histamine (dihydrochloride, Nakarai, Japan) and serotonin (creatinine sulfate, Sigma) were dissolved in physiological saline. Platelet activating factor (PAF, Sigma) was dissolved in chloroform, and then served for experiments after being diluted appropriately with physiological saline.

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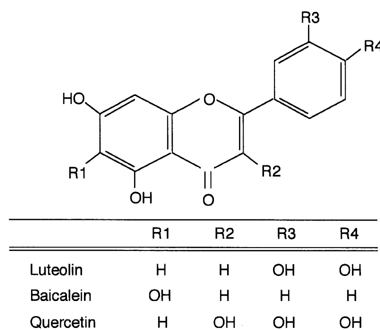
Table 1 Chemical structures of luteolin, baicalein, and quercetin.



	R1	R2	R3	R4
Luteolin	H	H	OH	OH
Baicalein	OH	H	H	H
Quercetin	H	OH	OH	OH

Antigens and antibodies

2,4-Dinitrofluorobenzene (DNFB) was purchased from Nakalai Tesque (Kyoto, Japan) and dissolved in acetone and olive oil (3:1). DNP-coupled bovine serum albumin (DNP-BSA) was prepared according to the method described by Eisen et al. (13) and was used as an antigen. The DNP-BSA preparation contained 25 or 37.5 DNP groups per BSA molecule. DNP_{25}-BSA was used for histamine release from rat peritoneal mast cells, and $DNP_{37.5}$-BSA was used for the *in vivo* study and cytokine release from bone marrow-derived mast cells (BMMC). Rat monoclonal anti-DNP IgE and mouse monoclonal anti-DNP IgE were prepared as reported previously (6), (14).

Animals

Female BALB/c mice (Japan SLC, Inc., Hamamatsu, Japan) and male Wistar rats (Japan SLC, Inc.) were housed in plastic cages in an air-conditioned room at 24 °C, fed a standard laboratory diet and given water ad libitum. All experiments were carried out following guidelines for the care and use of experimental animals formulated by the Japanese Association for Laboratory Animal Science in 1987.

Biphasic cutaneous reaction in mice

Mice were passively sensitized by an intravenous injection with 1 mL of mouse monoclonal anti-DNP IgE. Then, 24 h after the injection, the cutaneous reaction was caused by painting with 25 µL of 0.15% DNFB acetone-olive oil solution to both sides of the ears. The reaction was assessed by measuring the ear thickness using an engineer's micrometer (Upright Dial Gauge, Ozaki, Tokyo, Japan).

Mediator-induced cutaneous reactions in rats

Mediator-induced cutaneous reactions were carried out in rats as previously described (15). Briefly, 0.1 mL each of physiological saline as a control for cutaneous reaction, 3×10^{-5} g/mL histamine, 3×10^{-7} g/mL serotonin and 10^{-6} g/mL PAF were injected subcutaneously into the spots, respectively. Next, 1 mL of physiological saline containing 5 mg of Evans blue was immediately injected into the tail vein. Flavonoids were injected intraperitoneally 1 h before challenge, and prednisolone was injected intraperitoneally 2 h before challenge. Rats were sacrificed by bleeding 30 min after the induction of the reactions, and the skin with each reaction spot was cut off, from which the extravasated dye was extracted and assayed.

Histamine release from rat peritoneal mast cells

Peritoneal cells were sensitized by an intraperitoneal injection of 2 mL of 1/200 dilution of rat monoclonal anti-DNP IgE. Next day, peritoneal cells were recovered by washing the cavity with Tyrode's solution (137 mM NaCl, 2.7 mM KCl, 0.41 mM NaH_2PO_4, 1.6 mM $CaCl_2$, 1 mM $MgCl_2$, 0.1% glucose, 10 mM HEPES, 0.05% gelatin, pH 7.4) containing 5 units/mL heparin. Collected cells were washed twice and purified by density gradient centrifugation (using 60% Percoll; Pharmacia LKB, Uppsala Sweden). Only those preparations found to contain a minimum of 97% mast cells were used for our studies. Viability of cells assessed by staining with trypan blue was >97%. Tyrode's solution was used throughout the experiments. Cells were suspended at 1×10^5 cells/mL in Tyrode's solution, and were treated with several drugs for 10 min, and then were challenged with DNP-BSA_{25} at 10 µg/mL for 30 min. The concentration of histamine released into the supernatant was quantified by a method of post-column derivatization (16).

Histamine release and cytokine production from bone marrow-derived mast cells (BMMC)

BMMC were obtained by culturing (in CO_2 incubator; 37 °C, 5% CO_2) bone marrow cells. Briefly, bone marrow cells were suspended at a density of 1×10^6 cells/mL in RPMI-1640 supplemented with 10% fetal calf serum (FCS; Filtron Pty Ltd., Brooklyn, Australia), 50 µM 2-mercaptoethanol, 2 mM L-glutamine, 0.1 mM non-essential amino acids, 100 units/mL penicillin, 100 µg/mL streptomycin, and 10 ng/mL recombinant murine IL-3 (Pepro Tech EC Ltd., London, England). Cells were harvested weekly and resuspended in the fresh medium. After 4 weeks, more than 99% of non-adherent cells were stained positively with alcian blue. BMMC were sensitized with a 1/10 dilution of mouse monoclonal anti-DNP IgE for 24 h, then the cells were washed and resuspended at the concentration of 2×10^6 cells/mL in Tyrode's solution. Aliquots of the cell suspension were treated with flavonoids for 10 min and then challenged with $DNP_{37.5}$-BSA for 20 min. Released histamine was quantified by the same method as for rat peritoneal mast cells. For cytokine production analysis, BMMC were suspended in the culture medium at the concentration of 5×10^5 cells/mL, aliquots of the cell suspension were challenged with $DNP_{37.5}$-BSA for 6 h. The concentrations of IL-6 and TNF-α were detected by commercial ELISA kits (ENDOGEN, Woburn, MA).

Statistical analysis

Statistical significance between control group vs. prednisolone-treated group was evaluated by means of Student's t test. Dunnett two-tailed test was employed for the evaluation between control group and flavonoid-treated groups.

Results

After challenge with DNFB on the sensitized mice ear, an almost 10% increase of ear thickness is observed at around 1 h (IPR) and 24 h (LPR). The effects of luteolin, baicalein, and quercetin on this biphasic cutaneous reaction were examined. As shown in Figure **1**, all three flavonoids inhibited IPR dose-dependently. Luteolin and baicalein inhibited IPR significantly from 0.1 mg/kg, whereas quercetin inhibited IPR significantly

at 10 mg/kg. In the case of LPR, baicalein and quercetin showed dose-dependent inhibition, while luteolin inhibited LPR in a U-shaped manner. In 1 and 10 mg/kg of administered groups, each inhibition was significant (Fig. **2**). The inhibition of IPR and LPR by quercetin was a little weaker than that of luteolin and baicalein. Prednisolone, a positive control, inhibited IPR and LPR significantly at a dose of 3 mg/kg.

The inhibition of IPR may be explained as both the inhibition of mediator release from mast cells and the antagonistic effect of the released mediators or vasoconstrictors. To clarify which is the major effect of flavonoids, we examined the effects of flavonoids on both histamine release from mast cells and mediator-induced cutaneous reactions. At first, we examined the effect of luteolin and other flavonoids on IgE-mediated histamine release from BMMC. DNP-BSA (30 ng/mL)-induced histamine release was 46.5 ± 7.2% (% of total histamine content, n = 3). When BMMC were treated with flavonoids from 1 to 100 μM, histamine release was inhibited in a dose-dependent fashion (Fig. **3A**). 10 μM of luteolin and quercetin inhibited histamine release by more than 50%, while the same concentration of baicalein could not affect it. Because we planned to investigate the mediator antagonistic effect in rats, we examined the effect of flavonoids on histamine release also in rat peritoneal mast cells. In the case of rat peritoneal mast cells, almost the same result was obtained as compared with the BMMC experiment (Fig. **3B**).

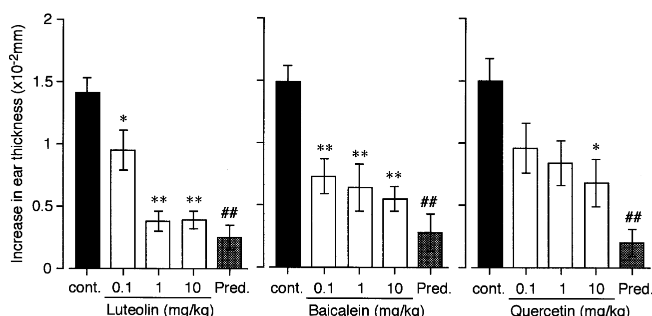


Fig. 1 Effect of flavonoids and prednisolone on IgE-mediated cutaneous reaction (IPR) in BALB/c mice. Mice received an intravenous injection of 1 mL anti-DNP IgE antibody preparation 24 h before skin testing with 0.15% DNFB. Flavonoids and prednisolone (3 mg/kg) were given intraperitoneally 1 h and 2 h prior to the antigen challenge, respectively. Ear thickness of each group was measured at 1 h after the challenge. Each value represents the mean ± SEM of 6 mice. * $p < 0.05$, ** $p < 0.01$ (Dunnett two-tailed vs. control) ## $p < 0.01$ (Student's t vs. control).

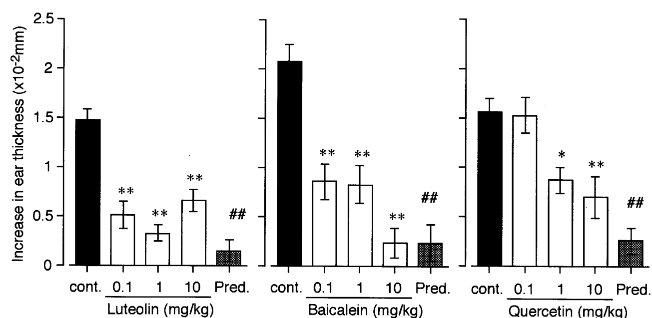


Fig. 2 Effect of flavonoids and prednisolone on IgE-mediated cutaneous reaction (LPR) in BALB/c mice. Mice received an intravenous injection of 1 mL anti-DNP IgE antibody preparation 24 h before skin testing with 0.15% DNFB. Flavonoids and prednisolone (3 mg/kg) were given intraperitoneally 1 h and 2 h prior to the antigen challenge, respectively. Ear thickness of each group was measured at 1 h after the challenge. Each value represents the mean ± SEM of 6 mice. * $p < 0.05$, ** $p < 0.01$ (Dunnett two-tailed vs. control) ## $p < 0.01$ (Student's t vs. control).

Next, we examined the effect of flavonoids on mediator-induced cutaneous reactions in rats. Histamine, serotonin, and PAF were used as mediators which might be released from resident mast cells after DNFB challenge. All three flavonoids did not affect histamine-, serotonin- and PAF-induced cutaneous reactions (Fig. **4**). Prednisolone, which exerts its effect by inhibiting plasma exudation, inhibited the mediator-induced cutaneous reaction at a dose of 3 mg/kg.

The effects of luteolin and other flavonoids on IgE-mediated IL-6 and TNF-α release from BMMC were examined. DNP-BSA (30 mg/mL)-stimulated cells release IL-6 and TNF-α (3.73 ± 0.25 ng/10^6 cells and 319 ± 44 pg/10^6 cells, respectively). Flavonoids inhibited both IL-6 and TNF-α release from BMMC in a dose-dependent manner (Fig. **5**).

Discussion

In the present investigation, we have demonstrated the inhibitory action of luteolin on the IgE-mediated biphasic cutaneous reaction as well as those of baicalein and quercetin. We found that these flavonoids are one of the most potent inhibitors of this model among our previously investigated agents including anti-allergic agents, immunosuppressors, and some Chinese herbal medicines (17–19). Previously, we have reported that LPR is mainly induced by TNF-α and IL-1β re-

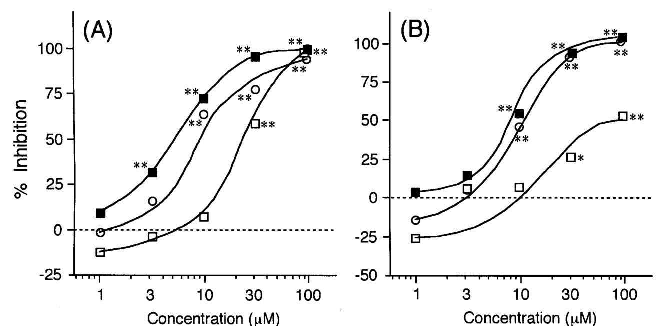


Fig. 3 Effect of flavonoids on IgE-mediated histamine release from bone marrow-derived mast cells (**A**) and rat peritoneal mast cells (**B**). **A**; 4-weeks-old bone marrow-derived mast cells (BMMC) were sensitized with mouse monoclonal anti-DNP IgE. 24 h later, BMMC were collected and adjusted to 2 × 10^6 cells/mL in Tyrode's solution. Cells were incubated with $DNP_{37.5}$-BSA (30 ng/mL) for 20 min. Luteolin (■), baicalein (□), and quercetin (○) were added 10 min before challenge. $DNP-BSA_{37.5}$-stimulated cells release histamine at a level of 46.5 ± 7.2% (% of total histamine content, n = 3). **B**; Wistar rats (body weight >200 g) were sensitized with rat monoclonal anti-DNP IgE. 48 h later, peritoneal mast cells were collected and adjusted to 1 × 10^5 cells/mL. Cells were challenged with DNP_{25}-BSA (10 μg/mL) for 20 min. Luteolin (■), baicalein (□), and quercetin (○) were added 10 min before challenge. DNP_{25}-BSA-induced histamine release was 5.8 ± 1.3% (% of total histamine content). Each value represents the mean of 3 or 4 separate experiments. * $p < 0.05$, ** $p < 0.01$ (Dunnett two-tailed vs. control).

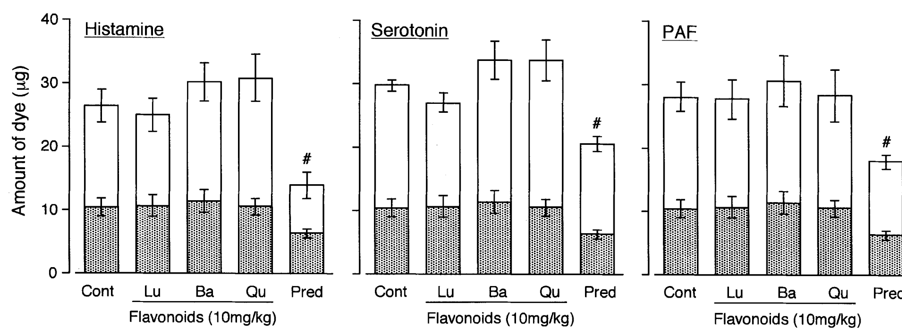


Fig. 4 Effect of flavonoids and prednisolone on mediator-induced cutaneous reactions in rats. Just before the Evans blue injection, saline, 3 × 10^{-5} g/mL histamine, 3 × 10^{-7} g/mL serotonin, and 10^{-6} g/mL PAF were injected subcutaneously into the spots, respectively. After 30 min, the amount of extravasated dye was calculated. Flavonoids and prednisolone (3 mg/kg) were given intraperitoneally 1 h and 2 h prior to the antigen challenge, respectively. Each value represents the mean ± SEM of 6 rats. Dotted columns represent the result of saline injected group as a control. * $p < 0.05$, ** $p < 0.01$ (Dunnett two-tailed vs. control) ## $p < 0.01$ (Student's t vs. control).

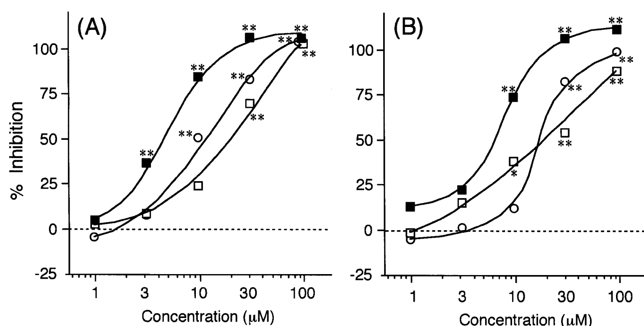


Fig. 5 Effect of flavonoids on IgE-mediated TNF-α (**A**) and IL-6 (**B**) production from bone marrow-derived mast cells (BMMC). 4 weeks old BMMC were sensitized with mouse monoclonal anti-DNP IgE. 24 h later, BMMC were collected and adjusted to 5 × 10^5 cells/mL in culture medium. Cells were incubated with $DNP_{37.5}$-BSA (30 mg/mL) for 6 h. Luteolin (■), baicalein (□), and quercetin (○) were added 10 min before challenge. $DNP_{37.5}$-BSA-stimulated cells release TNF-α and IL-6 at 319 ± 44 pg/10^6 cells and 3.73 ± 0.25 ng/10^6 cells, respectively. Each value represents the mean ± SEM of 3 experiments. * $p < 0.05$, ** $p < 0.01$ (Dunnett two-tailed vs. control).

leased from mast cells or FcεRI bearing cells (6), (9). We also demonstrated that the expression of TNF-α and IL-1β mRNA in the ear after DNFB challenge occurred, but these cytokine mRNA-expressing cells have not yet been clarified. On the other hand, the expression of IL-6 mRNA was detected in mast cells selectively (unpublished data). To analyze the mechanisms of LPR inhibition, we observed the inhibitory effects of flavonoids on IL-6 and TNF-α release from BMMC. The inhibition of IL-6 release may demonstrate that the activation of mast cells is suppressed. Furthermore, the inhibition of TNF-α release from mast cells was considered to be a major factor for the inhibition of LPR. From these results, mast cells are the target of luteolin and the other two flavonoids in LPR inhibition. Moreover, some reports show that a kind of flavonoids inhibited TNF-α and IL-1β-induced adhesion molecule expression in endothelial cells, which represent inflammatory cells migrating to inflammatory loci (20), (21). Therefore, we could assume that such an effect of flavonoids might contribute, in a part, to the LPR inhibition.

IPR is induced by chemical mediators, such as histamine released from resident mast cells (19). As for IPR inhibition, flavonoids may contribute not only to the inhibition of mediator release from mast cells but also to the antagonistic effect of the released mediators or vasoconstrictors. Thus, the effect of luteolin and the other two flavonoids was studied on histamine release from BMMC/rat peritoneal mast cells and the mediator-induced cutaneous reactions in rats. Biphasic cutaneous reaction and BMMC experiments were carried out in mice, but the mediator-induced cutaneous reaction was examined in rats because of its sensitivity to the lack of mediators. Luteolin, baicalein, and quercetin inhibited histamine release, but not mediator-induced cutaneous reactions. These results indicate that flavonoids directly act on mast cells, and that the mast cell is the target of flavonoids in IPR inhibition.

As already described, flavonoids are known to have multipotential activities (1), (2). Luteolin, a compound we focused on, inhibited both IPR and LPR, at the same magnitude as baicalein. However, the effect of quercetin was slightly weaker when compared with those of luteolin and baicalein. On the other hand, luteolin and quercetin inhibited mediator release from BMMC/rat peritoneal mast cells more strongly than baicalein. Analysis of structure-activity data revealed a model of the minimal essential features required for PKC inhibition by flavonoids (22), (23), luteolin and quercetin complied with these essential features but baicalein did not. These reports may explain why luteolin and quercetin have stronger anti-allergic activities than baicalein *in vitro*. We do not know why the efficacies of flavonoids *in vivo* and *in vitro* are not in parallel, but the absorption or/and metabolism pathways of the flavonoids may be related. Nevertheless, luteolin showed powerful inhibitory effects in both *in vivo* and *in vitro* systems, and the degree of LPR inhibition was almost the same as that of prednisolone. This study shows that luteolin is a promising agent for the prevention of allergic inflammatory diseases.

In conclusion, we demonstrated that the naturally occurring flavonoids, luteolin and the other two flavonoids, inhibited both IPR and LPR of the mouse biphasic cutaneous reaction. These compounds are likely to have multiple actions at different points of the stimulus-secretion pathway amongst which are steps for mediator release from mast cells. We conclude that these results are due to the suppression of mast cell mediator release such as histamine and TNF-α by multipotential flavonoids.

References

[1] Middleton EJ, Kandaswami C. in: The Flavonoids, Harborne JB, ed.,: pp. 619 – 652, Chapman & Hall, London 1993
[2] Middleton EJ, Kandaswami C. Biochem. Pharmacol. 1992; 43: 1167 – 1179
[3] Foreman JC, J. Allergy Clin. Immunol. 1984; 73: 769 – 774
[4] Middleton EJ, Drzewiecki G. Biochem. Pharmacol. 1984; 33: 3333 – 3338
[5] Charlesworth EN. J. Allergy Clin. Immunol. 1994; 94: 1240 – 1250
[6] Takeda H, Kogame A, Tanaka H, Nagai H. Prostaglandins. 1997; 54: 805 – 820
[7] Dvoracek JE, Yunginger JW, Kern EB, Hyatt RE, Gleich GJ. J. Allergy Clin. Immunol. 1984; 73: 363 – 368
[8] Nagai H, Sakurai T, Inagaki N, Mori H. Biol. Pharm. Bull. 1995; 18: 239 – 245
[9] Sakurai T, Inagaki N, Nagai H. Life Sci. 1994; 54: PL291 – 295
[10] Sanchez de Rojas VR, Somoza B, Ortega T, Villar AM. Planta Med. 1996; 62: 272 – 274
[11] Middleton EJ, Fujiki H, Savliwala M, Drzewiecki G. Biochem. Pharmacol. 1987; 36: 2048 – 2052
[12] Bronner C, Landry Y. Agents Actions. 1985; 16: 157 – 151
[13] Eisen HN, Belman S, Carsten ME. J. Am. Chem. Soc. 1953; 75: 4583 – 4585
[14] Inagaki N, Kawasaki H, Ueno M, Nagai H, Koda A. Life Sci. 1994; 54: 1403 – 1409
[15] Koda A, Miura T, Inagaki N, Sakamoto O, Arimura A, Nagai H, Mori H. Int. Arch. Allergy Appl. Immunol. 1990; 92: 209 – 216
[16] Yamatodani A, Fukuda H, Wada H, Iwaeda T. J. Chromatogr. 1985; 344: 115 – 123
[17] Watanabe C, Hase K, Oku T, Koizumi F, Kadota S, Nagai H, Namba T, Saiki I. Planta Med. 1998; 64: 12 – 17
[18] Inagaki N, Sakurai T, Abe T, Musoh K, Kawasaki H, Tsunematsu M, Nagai H. Life Sci. 1998; 63: 145 – 150
[19] Inagaki N, Tsunematsu M, Sakurai T, Matsuo A, Nagai H. Gen. Pharmacol. 1997; 28: 93 – 97
[20] Kimura Y, Matsushita N, Okuda H. J. Ethnopharmacol. 1997; 57: 63 – 67
[21] Gerritsen ME, Carley WW, Ranges GE, Shen CP, Phan SA, Ligon GF, Perry CA. Am. J. Pathol. 1995; 147: 278 – 292
[22] Agullo G, Gamet PL, Manenti S, Viala C, Remesy C, Chap H, Payrastre B. Biochem. Pharmacol. 1997; 53: 1649 – 1657
[23] Ferriola PC, Cody V, Middleton EJ. Biochem. Pharmacol. 1989; 38: 1617 – 1624

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