

# Flavonoids such as Luteolin, Fisetin and Apigenin Are Inhibitors of Interleukin-4 and Interleukin-13 Production by Activated Human Basophils

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## Key Words

Allergy · Asthma · Basophils · Flavonoids · Interleukin-4 · Th2

## Abstract

**Background:** We have previously shown that fisetin, a flavonol, inhibits IL-4 and IL-13 synthesis by allergen- or anti-IgE-antibody-stimulated basophils. This time, we investigated the inhibition of IL-4 and IL-13 production by basophils by other flavonoids and attempted to determine the fundamental structure of flavonoids related to inhibition. We additionally investigated whether flavonoids suppress leukotriene C4 synthesis by basophils and IL-4 synthesis by T cells in response to anti-CD3 antibody. **Methods:** Highly purified peripheral basophils were stimulated for 12 h with anti-IgE antibody alone or anti-IgE antibody plus IL-3 in the presence of various concentrations of 18 different kinds of flavones and flavonols. IL-4 and IL-13 concentrations in the supernatants were then measured. Leukotriene C4 synthesis was also measured after basophils were stimulated for 1 h in the presence of flavonoids. Regarding the inhibitory activity of flavonoids on IL-4 synthesis by T cells, peripheral

blood mononuclear cells were cultured with flavonoids in anti-CD3-antibody-bound plates for 2 days. **Results:** Luteolin, fisetin and apigenin were found to be the strongest inhibitors of both IL-4 and IL-13 production by basophils but did not affect leukotriene C4 synthesis. At higher concentrations, these flavonoids suppressed IL-4 production by T cells. Based on a hierarchy of inhibitory activity, the basic structure for IL-4 inhibition by basophils was determined. **Conclusions:** Due to the inhibitory activity of flavonoids on IL-4 and IL-13 synthesis, it can be expected that the intake of flavonoids, depending on the quantity and quality, may ameliorate allergic symptoms or prevent the onset of allergic diseases.

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## Introduction

Th2 cytokines such as IL-4 and IL-13 play an important role in the pathogenesis of allergic diseases [1]. We have recently demonstrated that fisetin, a flavonol, inhibits the synthesis of these cytokines by activated basophils [2]. This time, we investigated whether 18 kinds of flavones and flavonols had an inhibitory effect on IL-4 and

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1018–2438/04/1342–0135\$21.00/0

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IL-13 synthesis or leukotriene C4 production, which is believed to be a major mediator in allergic inflammation. We also studied whether flavonoids might inhibit IL-4 production by T cells.

## Methods

### *Culture Medium*

RPMI 1640 (Nacalai Tesque, Kyoto, Japan) supplemented with 10% FCS (Dainippon Pharmaceutical, Osaka, Japan), *L*-glutamine (2 mM), 2-mercaptoethanol (0.05 mM), penicillin (100 U/ml) and streptomycin (100 µg/ml) was used as the culture medium.

### *Chemicals*

Flavonoids were purchased from Extrasynthese (Genay, France) and dissolved in methanol at a concentration of 10 mM. Anti-human IgE antibody (Ab) was obtained from DAKO (Glostrup, Denmark). Anti-human CD3 Ab was purchased from Pharmingen (San Jose, Calif., USA). IL-3 was purchased from Genzyme-Techne (Boston, Mass., USA).

### *Purification of Basophils*

Peripheral blood buffy coats from healthy transfusion donors were anticoagulated with 10 mM EDTA and mixed with the same amount of phosphate-buffered saline. This was then layered onto Ficoll-Paque PLUS (Amersham Pharmacia Biotech, Amersham, UK) and centrifuged at 400 *g* for 20 min. This was followed by negative selection using a MACS Basophil Isolation Kit (Miltenyi Biotech, Bergisch-Gladbach, Germany) as described before [2, 3]. The number of total cells and their viability were determined by staining with trypan blue solution and counted with a hemocytometer. The purity of the basophils in this fraction was assessed by examining 1,000 cells on cytopins treated with May-Grünwald stain. The basophil purity was 80–93% in four different experiments, and the contaminating cells consisted mainly of small lymphocytes and few monocytes. For convenience, these basophil-enriched fractions are henceforth simply referred to as basophils.

### *Measurement of IL-4 and IL-13 Synthesis by Basophils*

Purified basophils ( $0.4\text{--}1 \times 10^6$  cells/ml) were incubated without (equivalent amount of diluted methanol) or with flavonoid at an indicated dose for 15 min and then stimulated with anti-IgE Ab (1 µg/ml) alone or plus IL-3 (20 ng/ml) for 12 h. The concentration of anti-IgE Ab has previously been determined to induce maximal synthesis of IL-4. The supernatant was harvested and the level of IL-4 and IL-13 in the culture supernatant was measured by means of ELISA (an ultrasensitive human IL-4 or IL-13 ELISA kit, Biosource International, Camarillo, Calif., USA). The measurable range of IL-4 or IL-13 was 65–25,000 fg/ml or 12–2,500 pg/ml, respectively.

### *Measurement of Leukotriene C4 Synthesis by Basophils*

Purified basophils ( $0.4\text{--}1 \times 10^6$  cells/ml) were incubated without (equivalent amount of diluted methanol) or with flavonoid at an indicated dose for 15 min and then stimulated with anti-IgE Ab (1 µg/ml) or anti-IgE Ab plus IL-3 for 1 h. The concentration of leukotriene C4 was then measured by means of ELISA (Leukotriene C4 EIA Kit, Cayman Chemical, Ann Arbor, Mich., USA). The measurable range of leukotriene C4 was 10–1,000 pg/ml.

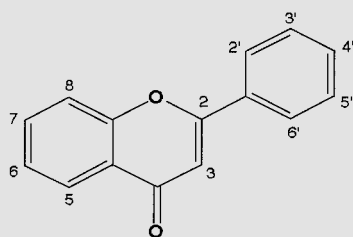
### *Measurement of IL-4 Synthesis by Peripheral Blood Mononuclear Cells*

Peripheral blood mononuclear cells from healthy volunteers were separated by Ficoll-Hypaque density centrifugation. Flat-bottom 96-well microtiter Immulon 2 plates (Dynatech Laboratories, Alexandria, Va., USA) were coated by incubation with anti-CD3 Ab (Pharmingen, 1 µg/ml in 50 µl borate-buffered saline, pH 8.5) overnight at 4°C. Wells were washed three times with HBSS. Peripheral mononuclear cells ( $10^6$  cells/ml) were then added to the wells in the absence or presence of the indicated doses of flavonoids and cultured for 48 h. The IL-4 concentration was measured using an ultrasensitive human IL-4 ELISA kit.

## Results

### *Hierarchy of Flavonoids in the Inhibitory Activity of IL-4 and IL-13 Production by Basophils*

It has previously been demonstrated that fisetin suppressed IL-4 synthesis by activated basophils and that its  $IC_{50}$  was 5.1 µM [2]. This time, we investigated whether other flavonoids might possess such inhibitory activity. The structure of 18 selected flavonoids including flavones and flavonoids is shown in figure 1. Highly purified basophils were incubated with or without various concentrations of flavonoids for 15 min and then stimulated with either anti-IgE Ab alone or anti-IgE Ab plus IL-3 for 12 h. We performed at least three independent experiments to examine the effect of each flavonoid, and the results of three flavonoids (fisetin, luteolin and apigenin) are shown in figure 2. Depending on the donors, the stimulation of basophils with anti-IgE Ab caused various amounts of IL-4 production, and in the supernatant of some donors the IL-4 concentration was hardly detectable. However, when basophils were incubated with both anti-IgE Ab and IL-3, IL-4 synthesis was consistently increased in the culture to a detectable level, as demonstrated previously [4]. Under these conditions, luteolin and apigenin showed a marked inhibition of IL-4 production and their  $IC_{50}$  was around 2–6 µM irrespective of the stimuli (anti-IgE Ab alone or anti-IgE Ab with IL-3; fig. 1). Scutellarein, 3-hydroxyflavone, kaempferol, quercetin, eriodictyol, fusticin and 7-hydroxyflavone inhibited IL-4 synthesis weakly but significantly ( $IC_{50} = 14\text{--}26.5$  µM). The other flavonoids, however, showed less activity. A comparison of the structure of these various flavonoids with their inhibitory activity determined the fundamental structure for the inhibition (fig. 1, lower panel). For maximal inhibition, hydroxylation in position 7 and 4' as well as the presence of OH in either position 3 or 5 are required. Similarly, luteolin, apigenin and fisetin suppressed IL-13 production by basophils (fig. 2) in a dose-dependent fashion.



|                  | IL-4 inhibition |    |    |    |       |    |    |    |    |  | basophils<br>IC <sub>50</sub> (μM) | T cells<br>IC <sub>50</sub> (μM) |
|------------------|-----------------|----|----|----|-------|----|----|----|----|--|------------------------------------|----------------------------------|
|                  | 3               | 5  | 6  | 7  | 8     | 2' | 3' | 4' | 5' |  |                                    |                                  |
| Luteolin         | H               | OH | H  | OH | H     | H  | OH | OH | H  |  | 2.4                                | 10.8                             |
| Fisetin          | OH              | H  | H  | OH | H     | H  | OH | OH | H  |  | 5.1                                | 19.1                             |
| Apigenin         | H               | OH | H  | OH | H     | H  | H  | OH | H  |  | 5.7                                | 10.2                             |
| Scutellarein     | H               | OH | OH | OH | H     | H  | H  | OH | H  |  | 14.0                               | 13.5                             |
| 3-Hydroxyflavone | OH              | H  | H  | H  | H     | H  | H  | H  | H  |  | 15.0                               | 24.8                             |
| Kaempferol       | OH              | OH | H  | OH | H     | H  | H  | OH | H  |  | 15.7                               | > 30                             |
| Quercetin        | OH              | OH | H  | OH | H     | H  | H  | OH | H  |  | 18.8                               | > 30                             |
| Eriodictyol(2-3) | H               | OH | H  | OH | H     | H  | H  | OH | OH |  | 20.8                               | > 30                             |
| Fustin(2-3)      | OH              | H  | H  | OH | H     | H  | H  | OH | H  |  | 23.0                               | > 30                             |
| 7-Hydroxyflavone | H               | H  | H  | OH | H     | H  | H  | H  | H  |  | 26.5                               | > 30                             |
| Myricetin        | OH              | OH | H  | OH | H     | H  | OH | OH | OH |  | > 30                               | > 30                             |
| Galangin         | OH              | OH | H  | OH | H     | H  | H  | H  | H  |  | > 30                               | n.d.                             |
| Morin(dihydrate) | OH              | OH | H  | OH | H     | OH | H  | OH | H  |  | > 30                               | n.d.                             |
| Astragalin       | O-Glc           | OH | H  | OH | H     | H  | H  | OH | H  |  | > 30                               | n.d.                             |
| Rutin            | O-Rutinoside    | OH | H  | OH | H     | H  | OH | OH | H  |  | > 30                               | n.d.                             |
| Gossypin         | OH              | OH | H  | H  | O-Glc | H  | OH | OH | H  |  | > 30                               | n.d.                             |
| Isoquercitrin    | O-Glc           | OH | H  | OH | H     | H  | OH | OH | H  |  | > 30                               | n.d.                             |
| Myricitrin       | O-Rhamnoside    | OH | H  | OH | H     | H  | OH | OH | OH |  | > 30                               | n.d.                             |

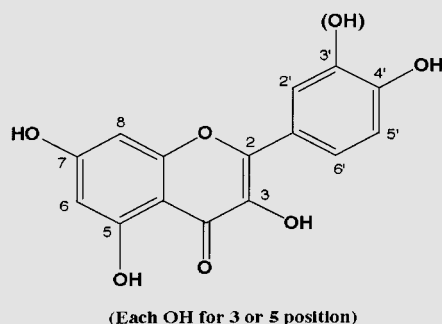


**Fig. 1.** Structure of 18 flavonoids and their inhibitory activity on IL-4 synthesis by human peripheral stimulated basophils and T cells. The lower figure shows the basic structure of flavonoids for inhibitory activity on IL-4 expression in basophils.

### Luteolin, Fisetin and Apigenin Fail to Inhibit Leukotriene C4 Synthesis by Basophils

The important role of basophils in allergic inflammation depends on their production of chemical mediators such as histamine, leukotrienes and cytokines [5–7]. Previously, several reports have demonstrated that flavonoids (including fisetin, luteolin and apigenin) inhibited histamine release by human basophils or rat mast cells at 5–30 μM [8–11]. Then we checked whether these flavonoids might inhibit another important mediator, leukotriene C4 synthesis. However, as shown in figure 2, these

three flavonoids did not suppress leukotriene C4 synthesis even at a high dose (30 μM).

### Inhibitory Activity of Flavonoids on IL-4 Synthesis by Anti-CD3-Ab-Stimulated Mononuclear Cells

Finally, we checked whether the inhibitory activity of flavonoids on IL-4 synthesis might be specific for basophils. Since T cells are IL-4-producing cells [1], peripheral blood mononuclear cells were cultured in plate-bound anti-CD3 Ab with or without various concentrations of flavonoids for 2 days, and IL-4 concentration was measured. The inhibitory activity from four independent experiments was shown as the IC<sub>50</sub> value in figure 1. Luteolin, fisetin, apigenin, scutellarein and 3-hydroxyflavone inhibited IL-4 production by stimulated T cells, but the required flavonoid dose for IL-4 suppression in T cells was higher than that in basophils (IC<sub>50</sub> = 10.8–24.8 μM).

## Discussion

On the basis of our previous study, which has shown that fisetin possesses an inhibitory activity on IL-4 and IL-13 synthesis by normal activated basophils [2], this time we screened whether 18 kinds of selected flavonoids inhibited IL-4 production by basophils. Among these flavonoids, luteolin, apigenin and fisetin are the strongest inhibitors of IL-4 and IL-13 synthesis. Scutellarein, 3-hydroxyflavone, kaempferol, quercetin, eriodictyol, fustin and 7-hydroxyflavone also inhibited IL-4 production but more weakly than luteolin, apigenin and fisetin. The comparison of the flavonoid structure with the inhibitory activity determined the fundamental structure shown in figure 1. For maximal inhibition, hydroxylation in positions 7 and 4' is essential and, additionally, the presence of OH in either position 3 or 5 is required. The major functions of basophils (and mast cells) in the development of allergic inflammation are reported to reside mainly in their capability to produce chemical mediators and cytokines [5–7, 12]. Fewtrell and Gomperts [8] first demonstrated the effect of flavone inhibition on histamine secretion by rat mast cells. Subsequently, flavonoids, including fisetin, apigenin, luteolin and quercetin, were reported to suppress histamine release by human basophils [9–11]. Therefore, three major flavonoids possess an inhibitory effect on Th2-type cytokines and histamine release but do not have an inhibitory activity on leukotriene C4 synthesis. Previously, it has been demonstrated that some flavonoids suppress cysteinyl leukotriene synthesis through an inhibition of phospholipase A<sub>2</sub> and 5-lipoxygenase [13,

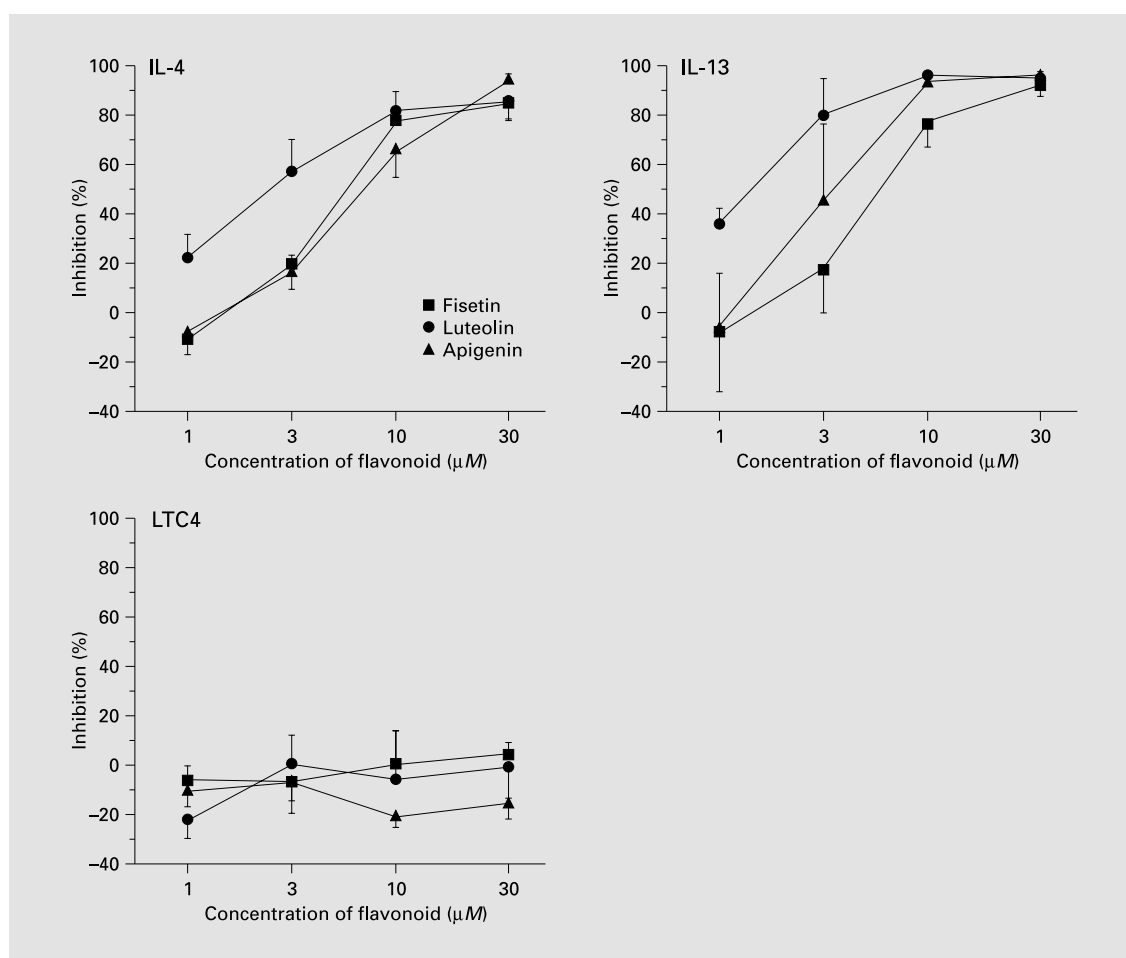


**Fig. 2.** Fisetin, luteolin and apigenin inhibit IL-4 and IL-13 synthesis by basophils in a dose-dependent fashion, but fail to inhibit leukotriene C4 synthesis. The data are shown as the mean inhibition from four different donors.

14]. Quercetin, as well as quercetagenin, kaempferol-3-*O*-galactoside and scutellarein were reported to be inhibitors of phospholipase A<sub>2</sub> [15, 16], whereas cirsiolol is shown to inhibit 5-lipoxygenase [17]. However, in human basophils, luteolin, fisetin and apigenin did not inhibit anti-IgE-Ab-induced leukotriene C4 synthesis.

The precise mechanisms through which they inhibit cytokine synthesis and histamine release in response to cross-linking of high affinity IgE receptor (FcεRI) remain to be elucidated. Previously, we demonstrated that fisetin inhibited nuclear localization of nuclear factor of activated T cells by a A23187-stimulated basophilic cell line, KU812 [2], but studies determining whether these low-molecular-weight compounds might affect other signaling cascades initiated by high-affinity IgE receptors are now

in progress. It should be pointed out here that cyclosporin A or FK506, a calcineurin inhibitor, has been shown to suppress cytokine production and histamine release [18–21], but their effect on leukotriene C4 production is controversial [22]. Although T cells appear to be more resistant to these flavonoids in the synthesis of IL-4 than basophils, T cells were generally shown to be more sensitive to cyclosporin A than basophils. Thus, it is possible that in addition to inhibition of nuclear factor of activated T-cell activation, other mechanisms may contribute to flavonoid inhibition of IL-4 synthesis by basophils.

Flavonoids are naturally and ubiquitously included in plant foods and drinks and are therefore common components of our diet [23]. Whether the worldwide increase in the prevalence of allergic diseases is directly associated

with diet is unknown, but a recently reported population-based case-control study found that consumption of apples or red wine was negatively associated with asthma prevalence or severity, respectively, possibly due to the protective effect of flavonoids [24]. In addition, a cohort study in Finland demonstrated a lower asthma incidence for higher total flavonoid intakes (relative risk = 0.65,  $p = 0.04$ ), mainly in the form of apples and oranges [25]. Thus these epidemiological studies raise the possibility that an intake of substantial amounts of flavonoids may ameliorate allergic symptoms as complementary and alternative medicine and prevent the onset of asthma. In this regard, we have also reported that in an atopic dermatitis mouse model, NC/Nga, administration of astragalin, a glycosylated kaempferol, showed both preventive and ameliorative effects on dermatitis [26, 27]. Based on the anti-allergic activity of flavonoids, an appropriate intake of such flavonoids as luteolin, fisetin and apigenin is strongly recommended.

In the past, the average intake of five selected flavonols and flavones, e.g. quercetin, kaempferol, myricetin, apigenin and luteolin, was 23 mg/day, of which quercetin contributed 16 mg/day in The Netherlands [28]. In 17 international subjects, intakes of these five flavonols and flavones were variable from 5.1 to 77 mg/day [29]. Querce-

tin is found in abundance in onion, kale, broccoli, apples and oranges, while kale, endive and other leafy vegetables have been shown to contain substantial amounts of kaempferol [30]. It should also be noted that celery contains exceptionally large quantities of apigenin (17–750 mg/kg) and luteolin (22–200 mg/kg) [31], both of which strongly inhibit Th2 cytokine expression by basophils. Luteolin is also included in bird chili (1,035 mg/kg), onion leaves (391 mg/kg), belimbi fruit (202 mg/kg) and belimbi leaves (464 mg/kg) in Malaysian edible plants [32]. Apigenin is detected in substantial amounts (272–579 mg/kg) in bell pepper, belimbi fruit and guava [32]. Recently, it has been reported that fisetin is found in strawberries (160 mg/kg) and apples (27 mg/kg) [33].

So far, diet therapy for allergic diseases has not been established except for avoidance of food allergens in cases where patients were sensitized to foods. *Lactobacillus GG* is promising since it was shown to be effective in the prevention of early atopic eczema in children at high risk, when it was administered prenatally to mothers and postnatally to their infants [34]. Similarly it is expected that, depending on the quantity as well as the quality, the intake of flavonoids may alleviate allergic symptoms and even prevent allergic diseases [35].

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