# Anti-Allergic Action of *O*-methylated EGCG in Green Tea Cultivar Benifuuki

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## ABSTRACT

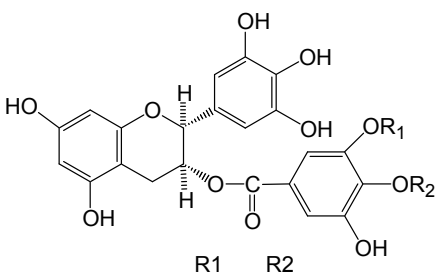
The anti-allergic effect of epigallocatechin-3-*O*-(3-*O*-methyl) gallate (EGCG3"Me) and epigallocatechin-3-*O*-(4-*O*-methyl) gallate (EGCG4"Me) isolated from Japanese or Taiwanese tea (*Camellia sinensis* L.) leaves. These catechins strongly inhibited mast cell activation and histamine release after FcepsilonRI cross-linking through the suppression of tyrosine phosphorylation (Lyn) of cellular protein kinase, and the suppression of myosin light chain phosphorylation and high-affinity IgE receptor expression via the binding to 67kDa laminin receptor. A double blind clinical study on subjects with Japanese cedar pollinosis was carried out. At the eleventh weeks after starting to intake, the most severe cedar pollen scattering period, symptoms i.e. blowing nose, itch of eyes were significantly relieved in Benifuuki group compared with placebo group. Over one consecutive month intake of Benifuuki green containing *O*-methylated catechin tea was useful for reduction of some symptoms derived from Japanese cedar pollinosis, and did not affect any normal immune response in the subjects with Japanese cedar-pollinosis. From the investigation that the effects of cultivars, tea seasons of crops and manufacturing methods, green or semi-fermented teas, made from fully-matured Benifuuki in second crop season, should be consumed. It is possible to develop functional articles such as beverage or food with this Benifuuki green tea.

Key words: *O*-methylated EGCG, 'Benifuuki' green tea (*Camellia sinensis* L.), anti-allergic action

## INTRODUCTION

Tea (*Camellia sinensis* L.) is consumed all over the world, and in large quantities in Japan and China, where it has been used for medicinal purposes for thousands of years. Tea has been found to exhibit various bioregulatory activities, such as being anti-carcinogenetic, anti-metastatic, anti-oxidative, anti-hypertensive anti-hypercholesterolemic, anti-dental caries, anti-bacterial, and to contribute to intestinal flora amelioration activity. Catechins, a group of polyphenolic compounds, have been shown to be largely responsible for these activities.

Allergy has been defined as a disease of excessive immune activity, and in Japan the morbidity of allergy is estimated to be about 30%. Many Japanese feel misgivings about the use of anti-allergic medicine as a result of side effects and mounting medical expenses, so there is a demand for the development of physiological-functional foods for allergy prevention. Anti-allergic effect is one of these functional properties in which catechins apparently play a significant role. In previous studies, we have reported that *O*-methylated forms of (-)-epigallocatechin-3-*O*-gallate (EGCG), i.e., epigallocatechin-3-*O*-(3-*O*-methyl) gallate (EGCG3"Me) and epigallocatechin-3-*O*-(4-*O*-methyl) gallate (EGCG4"Me) (Figure 1), which were contained in some cultivars of tea such as 'Benifuuki', had anti-allergic action in *in vivo* type I and type IV allergies[(1,2)]. In this report, we present evidence that EGCG3"Me has an anti-allergic activity and inhibit several protein kinases, and 'Benifuuki' green tea relieves the allergic symptoms with seasonal/perennial allergic rhinitis.




| | R1 | R2 |
|---|---|---|
| EGCG | H | H |
| EGCG3"Me | $CH_3$ | H |
| EGCG4"Me | H | $CH_3$ |

**Figure 1**. Chemical structure of *O*-methylated EGCGs and EGCG.

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## MATERIALS AND METHODS

I. *Cells, Stimulation, Histamine Analysis, Immunoblot, Immune Complex Kinase Assay*

Bone marrow cells from the femurs of NC/Nga mice were cultured in 4ng/mL of murine recombinant IL-3-containing RPMI1640 medium supplemented with 10% heat-inactivated fetal bovine serum, glutamine and 2-mercaptoethanol in humidified 5%$CO_2$ at 37˚C. More than 95% pure mast cells were obtained as bone marrow derived mast cells (BMMC) after four weeks of culture. BMMC were passively sensitized with anti- DNP mouse monoclonal IgE antibody at 37˚C overnight. After washing in $Ca^{2+}$-free Tyrode buffer, the cells were resuspended in Tyrode buffer, incubated for 20 min with samples at 37˚C, and then stimulated by DNP-BSA with $CaCl_2$ at 37˚C. Eighteen kinds of cytokines secreted into the Tyrode solution during 2 – 4 h stimulation were measured by Bio-plex protein suspension array system. To measure the histamine, the equivalent volume of 0.1 N HCl was added to the supernatant (after DNP-BSA stimulation for 10 min), and the released histamine was measured by on-column HPLC[3]. Cell pellets (after stimulation for 3 min) were lysed in ice-cold 1% Nonidet P-40-containing lysis buffer (20mM Tris-HCl, pH8.0, 0.15M NaCl, 1mM EDTA, 1mM sodium orthovanadate, 1mM PMSF, 10 μg/mL aprotinin, 10 μg/mL leupeptin , 25μM p-nitrophenyl p'-guanidinobenzoate, 1 μM pepstatin and 0.1%sodium azide, immediately after stimulation and centrifuged. For immunoprecipitation, lysates were incubated on ice with an appropriate Ab for 1.5 h, and immune complexes were recovered by brief centrifugation following 30min incubation with Pansorbin for rabbit polyclonal Abs or anti-mouse Ig-conjugated agarose for mouse mAbs. Immune complexes were washed in lysis buffer four times. Cleared cell lysates or immunoprecipitates were separated by SDS-PAGE and blotted electrophoretically to polyvinylidene difluoride (PVDF) membranes. Membranes were blocked and incubated with a primary Ab and then with a horseradish peroxidase-conjugated secondary Ab. Visualization of immunoreactive proteins was performed with enhanced chemiluminescence reagents. Immunoprecipitates were washed four times in lysis buffer and once with kinase buffer without ATP. Washed immune complexes were incubated in kinase buffer with or without exogenous substrate in the presence of [$\gamma$-$^{32}$P] ATP. Reaction products were analyzed by SDS-PAGE followed by electroblotting onto PVDF membranes and autoradiography. Phosphorylated protein bands were quantified by densitometry.

II. *Human Clinical Trial on Seasonal Allergy Rhinitis*

(1) Twenty seven Subjects who feeling stuffy nose, itch of eyes, throat pain or having sneeze strokes at the time of cedar pollen scattering, and Japanese cedar pollen specific IgE value was positive without taking treatment with a medical institution were recruited to participants in this study. The researcher who did not participate in the final examination directly divided participants to test groups, based on each cedar pollen specific IgE value of blood. Study participants were informed of all procedure and requirements for the study. All procedures were in strict compliance with study protocol, which was approved by an Institutional Review Board of National Institute of Vegetable and Tea Science on Human Research. Written informed consent was obtained from all participants. All the subjects visited hospital every 4weeks for consultation, and the blood and urine samples were taken each times to examine some hematological examination, general biochemical examination, histamine content, and IgE score. During the test period, all the subjects were required to write 'allergy daily' which came to mention about frequency of sneeze and blowing nose, stuffy nose, itch of eyes, the quantity of tears, throat pain, difficulty of common life, and the use situation of the medicine on every day in accordance with the method that Japanese Society of Allergology allergic rhinitis Committee proposed. All subjects evaluated each symptom by five phase of evaluation from 0(no symptom) to 4 (very terrible and appears the symptom all day). The diary was collected at the end of examination and we calculated nose Symptom Score and Medication Score (pattern of taking medicine), and Symptom Medication Score which added up these both score, according to practical guideline for the management of allergic rhinitis of Japan Allergy Foundation.

(2) An open-label, single-dose, randomized, parallel-group study was performed on 38 subjects with Japanese cedar pollinosis. The subjects were randomly assigned to long-term (1.5 months before pollen exposure-Apr. 8th) or short-term (after cedar pollen dispersal-Apr. 8th) drinking of a 'Benifuuki' tea drink containing 34 mg *O*-methylated EGCG per day. Each subject recorded their dairy symptom scores in a daily. The primary efficacy variable was the mean weekly nasal symptom medication score during the study period.

## RESULTS AND DISCUSSION

I. *Anti-allergic Action of O-methylated Catechins*

When EGCG3"Me and EGCG, and their C-2 epimers, GCG3"Me and GCG were compared, the anti-allergic effect of ECG3"Me was strongest, and the activity was GCG3"Me > EGCG3"Me > GCG > EGCG, in that order[4]. EGCG3"Me strongly inhibited mast cell activation through the prevention of tyrosine phosphorylation (Lyn, Syk, and Btk) of cellular protein[5], myosin light chain phosphorylation[6], and the expression of FcεRI[7]. So, it is suggested that mast cell degranulation (histamine/leukotriene release and interleukin secretion after FcεRI cross-linking) was inhibited by these preventive effects (Figure 2). The Japanese tea cultivar 'Benifuuki' was rich in EGCG3"Me. 'Benifuuki' green tea or the simultaneous addition of 'Benifuuki' green tea and ginger extract strongly inhibited inflammatory cytokines production such as TNF-$\alpha$ and MIP-1$\alpha$ after antigen sti-

mulation of BMMC. From these results, 'Benifuuki' green tea or the combination of 'Benifuuki' and ginger are expected suppressed delay type allergy by inhibition of inflammatory cytokines production[8].

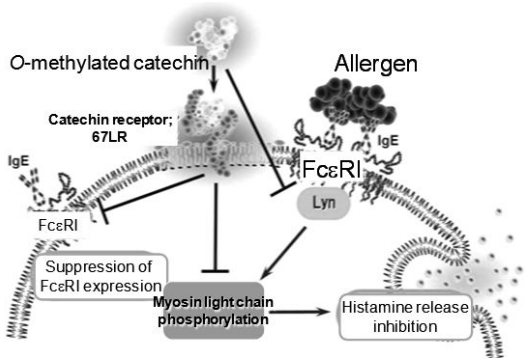


**Figure 2.** Anti-allergic model of mast cell activation by EGCG3"Me.

EGCG3"Me disappeared in black tea due to polyphenol oxidase, and the content was high in fully-matured tea leaves late in the first crop season or under the third leaf during the second crop season[9,10]. The amount of EGCG3"Me contained in the stem was low. A higher concentration of strictinin was found in younger new shoots in the first crop, and the content markedly decreased during the second crop season. Thus, to benefit from the anti-allergic benefits of EGCG3"Me, green tea should be made from fully-matured 'Benifuuki' leaves under the fourth shoot late in the first crop or under the third shoot in the second crop, refined by removing stems.

## II. *Clinical Studies of 'Benifuuki' Green Tea*

In the eleventh week after starting the study, in the most severe cedar pollen-scattering period, symptoms, i.e., nose blowing and itching the eyes, were significantly relieved in the 'Benifuuki' green tea containing EGCG3"Me intake group compared with the placebo group ('Yabukita' green tea did not contain EGCG3"Me and was used as a placebo),and the nose blowing, eye itching, and nose symptom score, and, in the eleventh and thirteenth weeks, stuffy nose, throat pain, and the nose symptom medication score, were significantly relieved in the 'Benifuuki' plus ginger extract group compared with the placebo group. These results suggested that, over one consecutive month, drinking 'Benifuuki' green tea was useful to reduce some of the symptoms of Japanese cedar pollinosis (Figure 3), and did not affect any normal immune response in subjects with seasonal rhinitis, and the ginger extract enhanced the effect of 'Benifuuki' green tea[8].

Furthermore, we evaluated the efficacy and safety of 'Benifuuki' green tea in patients with mild perennial allergic rhinitis. Seventy-five patients with mild perennial allergic rhinitis meeting the predetermined criteria for subjects were assigned to either the 'Benifuuki' green tea or 'Yabukita' green tea beverage group. The subjects took 700 mL of tea beverage (34 mg of EGCG3"Me contained in 700 mL) and recorded their nasal and ocular symptoms every day for 12 weeks, as well as visited hospital every 6 weeks for consultation and blood collection. As a result, the scores for nasal and ocular symptoms in the 'Benifuuki' group were lower than those of the 'Yabukita' group, with a significant difference in the 7th-12th weeks for nasal scores and 4th - 12th weeks for ocular scores[11]. No adverse effect was observed in physiological, hematological, and biochemical parameters, with normal immune responses of peripheral blood leukocytes, and no subjective symptom throughout the experiment. An additional study involving 9 healthy subjects without any allergic symptoms was also conducted. The subjects were given 700 mL of 'Benifuuki' green tea daily for 12 weeks, and no adverse effect was noted throughout the study. These results suggest that 'Benifuuki' green tea beverage containing *O*-methylated EGCG is useful for the treatment of mild perennial allergic rhinitis.

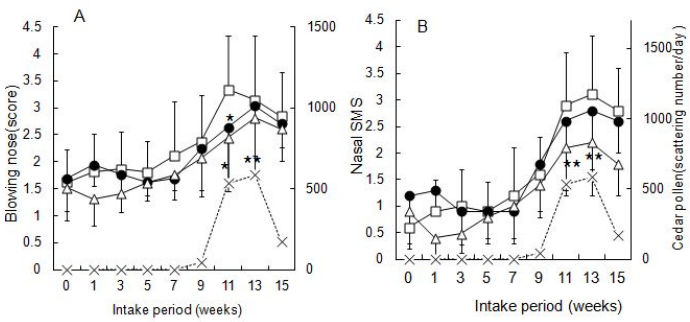


**Figure 3.** The effects of 'Benifuuki' green tea plus ginger extract on the symptom score of seasonal allergic rhinitis. All subjects drank 1.5 g of each tea powder: 'Benifuuki' green tea (●), 'Benifuuki' green tea containing 30 mg of ginger extract (△), and 'Yabukita' green tea (□), with water twice a day for 13 weeks. Each point represents the average of nine subjects every two weeks, and the cross-vertical bars represent SD of the mean. ■: Cedar pollen scattering number. (A) Blowing nose (0 (0 times)-4 (more than 21 times)), (B) nose symptom medication score.
*·** Significantly different from the placebo ('Yabukita') group (*$p < 0.05$, ** $p < 0.01$).

The nasal symptom score (nose blowing) in the long-term intake group was significantly lower than that of the short-term intake group at the peak of pollen dispersal (Figure 4). The symptom scores for throat pain, nose-blowing, tears, and hindrance to activities of daily living were significantly better in the long-term group than the short-term group. In particular, the differences in the symptom scores for throat pain and nose-blowing between the 2 groups were marked. We conclude that drinking 'Benifuuki' tea for 1.5 months prior to the cedar pollen season is effective in reducing symptom scores for Japanese cedar pollinosis[12].

After drinking 'Benifuuki' green tea containing 43.5 mg of EGCG and 8.5 mg of EGCG3"Me, the AUC (area under the drug concentration time curve; min·μg/mL) of EGCG was 6.72 ± 2.87 and that of EGCG3"Me was 8.48

± 2.54 in healthy human volunteers. Though the dose of EGCG was 5.1-times that of EGCG3"Me, the AUC of EGCG3"Me was higher than that of EGCG[8]. It was suggested that EGCG3"Me was absorbed easier than EGCG that the metabolic clearance rate of EGCG3"Me was slower than that of EGCG, and that these actions were related to the strong *in vivo* allergic effects.

Moreover, when 27 atopic dermatitis patients (moderate severity) applied 'Benifuuki' tea extract-containing cream to the affected parts for 8 weeks, the symptoms significantly improved, and the consumption of corticosteroid significantly decreased compared with that of the base cream[13].

## CONCLUSIONS

In Japan, based on these results, a bottled drink and snack containing 'Benifuuki' green tea are now sold by food manufacturers. It is possible to develop useful functional (anti-allergic) articles such as beverage or food with this 'Benifuuki' green tea which is rich in EGCG3"Me.

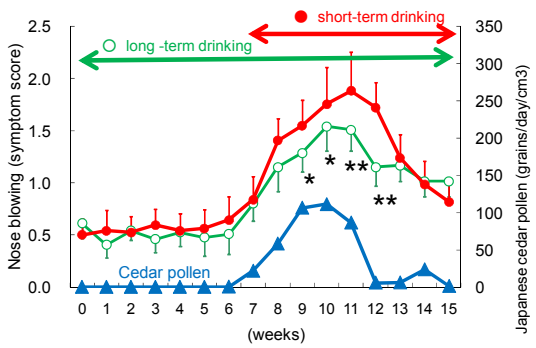


**Figure 4**. Mean nasal symptom score of the changes in 1-week symptoms of subjects given 'Benifuuki' green tea in the long-term and short-term intake groups.

A comparison between the long-term intake group and the short-term intake group was performed using the Mann-Whitney U test. ** $p < 0.01$, * $p < 0.05$ vs. another group.

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