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Original article

Effectiveness of kuromoji (*Lindera umbellata* Thunb.) extract in the prevention of influenza infection after vaccination: A randomized, double-blind, placebo-controlled, parallel-group study.

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Abstract

***Objectives*:** Although vaccination is an effective method to prevent influenza infection, current influenza vaccines have inadequate efficacy in general populations. For these reasons a supplement that prevents influenza infection is keenly anticipated. In basic research, it is demonstrated that kuromoji (*Lindera umbellata* Thunb.) extract has an effectiveness preventing influenza infection. The aim of this study was to assess whether kuromoji extract has a protective effect on influenza infection in clinical settings.

***Methods*:** In a randomized, double-blind, placebo-controlled, parallel-group study, 135 adult volunteers, all healthy nursing staff, received flu shots and participated in this study. Sixty-seven participants received test candy (containing 67 mg/day of kuromoji extract), and another 68 participants received placebo candy.

***Results*:** The test candy significantly reduced influenza infection after 12 weeks compared with the placebo candy.

***Conclusions*:** This is the first clinical study to assess the effectiveness of kuromoji extract in the prevention of influenza infection after vaccination. Our results suggest that kuromoji extract is a safe supplement for the prevention of influenza infection.

***KEY WORDS*:** kuromoji (*Lindera umbellata*), influenza, vaccination

Introduction

According to the announcement of the Ministry of Health, Labour and Welfare, seasonal influenza is raging in Japan and about 1.5 million people are infected with it every year, so influenza vaccination is recommended [1]. However, the influenza virus mutates a part of its amino-acid sequence of the surface antigen little by little, and as a result, occasionally it appears with high pathogenicity or with drug resistance. Therefore, it cannot always be said that the vaccine is an absolute preventive measure [2-4], on the contrary, it may cause pandemics [5-8]. Once influenza develops, an early symptomatic treatment is the mainstay treatment. However, appropriate prevention measures are important because it disturbs daily life.

Since 2014, the author et al. have joined with the "Strategic Innovation Promotion Program (SIP)" and have proceeded with research on new anti-glycation functional

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food together with Yomeishu Seizo Company, Limited, one of our cooperative companies (founded in 1602). As a result of the screening of more than 500 kinds of vegetables, fruits and herbs, Kuromoji (*Lindera umbellata*). a Japan-specific shrub was selected as the main object of our research [9, 10].

The branch of Kuromoji of Lauraceae (*Lindera umbellata* Thunb) is an herbal medicine called "*usho*" and its effects on the symptoms of the digestive system such as "sunstroke, colicky pain, abdominal pain, abdominal bloating and undigested food retention" are described in the Japanese and Chinese Dictionary of Medicines [11]. As an aromatic plant containing plenty of volatile components such as linalool and geraniol [12], it has been used as a toothpick since ancient times and its essential oil has been used for aromatherapy, too. Regarding non-volatile components, lots of various polyphenols including proanthocyanidin compounds (including the polymers of flavann-3-ol such as procyanidin B1, procyanidin B2 and cinnamtannin D1) and flavonols (including hyperin and quercitrin) are present [13, 14], and their medicinal benefits such as antioxidative action, antiglycation action and anti-ulcer action have been reported [9, 10, 15]. Furthermore, recently, the inhibition of the proliferation of influenza virus from Kuromoji hot water extract by plaque assey has been reported [16, 17], and its preventive effect on influenza can be expected.

Therefore, in this research, for the purpose of discussing the preventive effect of Kuromoji extract on influenza, a randomized double-blind placebo-controlled parallel-group comparison test was conducted for the purpose of discussing the preventive effect of Kuromoji extract on influenza.

Subjects and Method

Subjects

The subjects were 135 male and female nursing staff at Ehime University Hospital who received flu shots [18]. The vaccine used was made with four strains of A-type Singapore/GP1908/2015 (IVR-180) (H1N1) pdm09, A-type Hong Kong/4801/2014 (X-263) (H3N2), B-type Phuket/3073/2013 (Yamagata lineage) and B-type Texas/2/2013 (Victria lineage) selected based on the 2016/17 season Infectious Agents Surveillance Report [19, 20].

Test Design and Intake Method

In this research, a randomized double-blind placebo-controlled parallel-group comparison test was conducted. The Kuromoji extract was made as follows: The trunk and branches of Kuromoji (*Lindera umbellat*a Thunb.) were minced, added to water of ten times its volume, and thermally extracted at 95°C for 60 minutes. The extract was centrifugal-filtrated by vacuum concentrator, sterilized by a continuous sterilization device for liquids and used as a dried powder. The test product drops were made with sugar, syrup, fragrance and Kuromoji extract at a volume of 67 mg per one drop. Placebo drops were made with the same ingredients without Kuromoji extract and blended with caramel and colored the same color as the test product drop (***Table 1***).

A person who was not involved in this test, randomly allocated the subjects into two groups, test drop group and placebo drop group. The allocation table was sealed and kept until unblinding. The subjects took three drops a day, a drop after every meal from December 15, 2017 to March 15, 2018. They were given a questionnaire and it was investigated whether the subjects caught influenza, what type of influenza, period absent from work caused by influenza, existence or non-existence of common cold symptoms, duration of common cold symptoms, whether they had a fever, and throat and nasal symptoms caused by the common cold. Diagnoses of influenza infection were made based on a positive reaction by an influenza rapid diagnostic kit in medical institutions after they received a medical examination.

Safety Confirmation

The safety of the components of Kuromoji extract involved in the test product was confirmed by the following tests:

Table 1. Composition of the test products per one drop (3.8 g).

Item	Unit	Test drop	Placebo drop
Energy	kcal	14.9	15.0
Protein	g	0	0
Lipid	g	0	0
Carbohydrate	g	3.7	3.7
Water	g	0	0
Sodium	mg	0.1	0
Kuromoji extract	mg	67.0	0

Ames Test [21, 22]

The ability to induce gene mutation of Kuromoji extract was discussed in the presence and non-presence of metabolic activation of the pre-incubation method, using three strains (*Salmonella typhimurium* TA100 and TA1532, and *Eschrichia coli* WP2uvrA) detecting base substitution gene mutation and two strains (*Salmonella typhimurium* TA98 and TA1537) detecting frameshift gene mutation. As a result, regardless of the presence or absence of metabolic activation, no gene mutation inducing action was recognized in any strain.

Chromosomal Aberration Test and Micronucleus Test [23]

In order to evaluate the presence or absence of clastogenesis of Kuromoji extract in mammalian cultured cells, a chromosomal aberration test was conducted using cultured cells of the Chinese hamster (CHL/IU) in the presence of a drug-metabolizing activating enzyme system (S9 mix), (hereafter referred as to "metabolic activation method") by a 6-hour treatment or in the absence (hereafter referred as "direct method") by 6- and 24-hour treatments. The dosages were decided as follows: 400-800 μg/mL for a 6-hour treatment in the direct method, 50-350 μg/mL for a 24-hour treatment in the direct method and 200-1,200 μg/mL in the metabolic activation method.

As a result of observation of chromosome samples, no structural abnormality was observed among all tested methods. On the other hand, a significantly increased appearance rate of polyploidy abnormality was observed in all methods compared with the negative control group, and at the same time, dosage dependence was also confirmed, therefore, this test was judged to have a positive result.

In the above chromosome aberration test, the polyploidy abnormality was judged as slightly positive, so that, in compliance with the "Voluntary inspection flowchart concerning the safety of raw materials of foods in tablet and capsule forms," [24] a micronucleus test was conducted using rats as *in vivo* genotoxicity test.

Crl:CD(SD) rats (6 males per group) of 8 weeks old were orally dosed with Kuromoji extract of 750, 1,500 and 3,000 mg/kg/day, one time a day, with a time interval of 24 hours, two times in total. There was no case of death in any dosing amount during the dosing period, and no serious toxic symptom was observed. Micronucleus inducibility was negative and no inhibitory action on the growth of bone marrow cells was observed.

Toxicity Test on Rats by Repeated Oral Dosing for 13 Weeks [25, 26]

In compliance with a guideline regarding toxicity testing in cases of repeated dosing [27], a toxicity test by repeated dosing was conducted. Six Crl:CD (SD) male rats and six female rats of the first group were dosed with 120, 600 and 3,000 mg/kg/day of Kuromoji extract for 13 weeks continuously. As the results of the investigation into the toxicity of repeated dosing, there was no change suggestive of toxicity of Kuromoji extract in any of the general conditions, bodily weight, food consumption, water consumption, eye examination, blood tests, blood biochemical test, urinalysis, autopsy, organ weight or histopathological examination. From the above, the non-toxic amount of Kuromoji extract was judged as 3,000 mg/kg/day for both male rats and female rats.

Humans' Eating Experience

The branches of Kuromoji have been traditionally used for tea and recently Kuromoji tea is sold on the market in various places: however, its safety has not been reported.

Statistical Analysis

The average age and average period of cold symptoms are expressed as mean ± standard deviation and the Mann-Whitney U test was used for the comparison between groups. The χ^2 test was used for the comparisons between groups regarding gender, whether infected with influenza, common cold, having a fever, and throat or nasal symptoms caused by the common cold. SPSS 16.0 J for Windows was used for the data analysis. The significance level is 5% or less in either test.

Ethical Consideration

This research was conducted in Ehime University Hospital. The doctor in charge of this examination consolidated all operations relating to this experiment such as explanation and instruction for the study participants as well as obtaining letters of consent, conducting medical interviews, confirmation and judgment of adverse events, creation of case report forms and the management of the inspection implementation system. Before the implementation of examinations, the doctor in charge of the examination gave the subjects a briefing paper and fully explained the contents and intent of this examination and employed only the subjects from whom he could obtain letters of consent based on their free will. It was decided to deal with adverse events when needed. This research was conducted in compliance with the guidelines of the Declaration of Helslinki (reversed in WMA General Assembly in Fortaleza, 2013) and the ethical guideline on research on medical systems targeting people (announcements by the Ministry of Education, Science, Sports and culture and the Ministry of Health, Labour and Welfare). The Clinical Research Ethics Review Committee of Ehime University Hospital investigated this research and approved it (Approval No. : 1711022). This research was conducted after advance registration on the University Hospital Medical Information Network clinical trial registration system (UMIN-CT) (I<OM000030339).

Result

Subjects

As the result of randomly allocating 135 subjects, the number of subjects of the test drop intake group was 67 (three males) and that of the placebo intake group was 68 (six males); however, one subject dropped out from this group for personal reasons. During the test period, no adverse event caused by the test food was recognized.

Evaluation of the Effectiveness

The results of the questionnaires of the 67 subjects of the test drop intake group and the 67 subjects of the placebo intake group are shown in ***table 2***. No difference in age or

Table 2. Influenza prevalence and "Common cold" symptoms.

Item	Test group	Placebo group	p values
Number	67	67	
Male	3 (4.5)	6 (9.0)	0.49
Age (year)	37.9 ± 11.9	37.4 ± 10.0	0.99
Cases of influenza infection	2 (3.0)	9 (13.4)	**0.028**
Type A	0	6 (9.0)	**0.028**
Type B	2 (3.0)	3 (4.5)	1.00
"Common cold" symptoms (more than 1 episode)	17 (25.4)	16 (23.9)	0.84
Fever (>37°C)	8 (12.0)	4 (6.0)	0.23
Throat	13 (19.4)	12 (17.9)	0.82
Nose	9 (13.4)	11 (16.4)	0.63
Duration (day)	8.4 ± 4.4	11.0 ± 7.5	0.48

Results are expressed as mean ± standard deviation. Parenthesis indicates percentage values. Statistical analysis by Mann-Whitney U test or chi-square test.

sex was recognized between the two groups. The number of subjects who were infected with influenza of the test drop intake group was two (3.0%), and that of the placebo intake group was nine (13.4%); no significant difference was recognized between the two groups ($p = 0.028$). The influenza type which both of the subjects of the test drop group were infected with was type-B. Six out of the nine influenza infected subjects of the placebo intake group were Type A, and the remaining three were Type B. There was no subject who was infected with influenza more than once and the absence period of subjects of the test drop intake group was four-five days and that of the placebo intake group was two-six days; no difference was observed.

Cold symptoms not caused by influenza were also examined, but no significant differences were observed in the number of subjects who had fever, throat symptoms and nasal symptoms more than once in the periods of influenza symptoms and the presence or absence of fever, throat symptoms and nasal symptoms.

Discussion

According to the data of the Ministry of Health, Labour and Welfare, if a vaccine was not given, the incident rate of influenza was 30% and if a vaccine was given, the incidence rate was 12%. The vaccine efficacy rate that was used is reported to be 60%[1]. In this research, the influenza incident rate of the placebo intake group was 13%, so there was no difference compared with general data. On the other hand, that of the test drop intake group was 3%. This finding suggests that Kuromoji extract drops have anti-influenza activity as additional efficacy after vaccination.

As a result of the analysis based on the type of influenza virus, the type of virus which infected six out of nine influenza patients in the placebo intake group was type A, and that of the other three who were infected was Type B. Meanwhile the influenza virus type that infected two patients in the test drop intake group was Type B; therefore, the anti-influenza activity of Kuromoji extract drop against A type was remarkable. No significant differences in common cold symptoms were recognized between the two groups. The result of investigation of the rapid tests of influenza by a sentinel medical institution of the Ehime Prefecture Infectious Diseases Information Center[28] shows that, as a whole, the peak of the incidence of influenza was in the third week of January 2018 and after that, it gradually decreased. The proportion of A type influenza patients was higher from the beginning of December 2017 until the second week of January, and the proportion of B type influenza patients was higher since the third week of January, when influenza is at its peak. As for the total from December to March, in spite of the fact that the proportion of B type influenza patients was approximately 1.5 times higher, our research showed that the number of B type influenza patients of the placebo intake group was lower. As one of the reasons, because all subjects received the influenza vaccine, it can be considered that the difference in effects of the protective vaccination against A type and B type viruses reflects the result.

Looking through the data at the incidence rate of 2017/2018 in Ehime Prefecture, the number of cases of A type influenza was larger than that of B type. Due to the fact that the number of cases of A type was larger than that of B type in the placebo intake group, it is possible that the effect of the vaccine used this time was weak against A type. Given that the A type virus is more infectious, and the effect of the vaccine is not sufficient, the significance of the additional

effects of Kuromoji extract is great.

Regarding B type influenza, the vaccine used in this study was quadrivalent vaccine responding to Phuket/3073/2013 (Yamagata lineage) and Texas/2/2013 (Victria lineage) as B type strain. In the 2010s, a mixed epidemic of Yamagata lineage B type influenza virus and Victoria lineage B type influenza virus was recognized. The mismatch between vaccine strains for trivalent influenza vaccine and epidemic strain became a problem, and as a result, in the seasons of 2015/2016, a quadrivalent vaccine was introduced in Japan [32, 33]. Even though a quadrivalent vaccine improved its effect against B type influenza more than the trivalent influenza vaccine [31], there were some reports saying that it has room for improvement [32, 33]. According to the investigation in Mie Prefecture in FY2015 (including dialysis patients), those who developed influenza after receiving the vaccine were 161 out of 1,380, and the number of patients with A type influenza was 43, and that of those with B type influenza was 10 [33]. The enforcement of measures against B type influenza infection should be continuously maintained.

Recently, anti-viral activities in food ingredients have been reported, such as inactivating activity of grape seed extract against feline calicivirus [34], which is an alternate virus of the human norovirus, inactivating activity of guava extract against influenza virus and inactivating activity of persimmon extract against herpes and vesicular stomatitis viruses [36]. The active ingredient common in these foods is polyphenol and it is considered to have a nonspecific activity against viruses. Furthermore, not only the inactivation of proanthocyanidin against a virus, but also its inhibitory activity on virus growth in cells have been reported [37, 38]. Kuromoji contains proanthocyanidins including polymers of flavann-3-ols such procyanidin B1, procyanidin B2 and cinnamtannin D1 [13]. The extract from Kuromoji and proanthocyanidin fractions not only have inactivation but also an inhibitory action on virus growth, and it is reported that these are non-specific activities [13].

Clinical research on the anti-influenza actions of food ingredients have also been reported. An examination was conducted where 297 members of the same occupational group were divided into two groups and they gargled with black tea extract for five months. The influenza-infected persons in the test group were 35.1% and those in the control group were 48.8%, so a significant inhibitory action was recognized [39]. There is a report that the effect of gargling with green tea extract is not different from that with city water [40]. However, as the result of a recent meta-analysis, there are reports that gargling with green tea extract or its ingredients reduce the risk of influenza infection by 30% more than gargling with water or no gargling [41, 42], so that suggests that food ingredient's actions on a part of the pharynx can prevent influenza infection. However, as virus infiltration into cells after virus infection in the upper airway is very rapid, you have to gargle at short intervals; otherwise it cannot become assured as preventive measures with high possibility. As Kuromoji extract drop used in this research includes proanthocyanidins, it is considered that it has growth inhibitory activity, and in this research, it remained even after influenza infection. Furthermore, due to the form of drop, it is expected that its ingredients remain in the pharynx longer than by gargling, so it is considered to be an effective measure.

Limitation of Research

As all subjects had received the influenza vaccine, the influenza preventive effect of the test product alone could not be verified. Therefore, the influenza preventive effect of test products is regarded as the effect in addition to the influenza vaccine. In this research, different efficacies of the test product on A type and B type influenza were recognized and its efficacy only on A type influenza was confirmed. The reason could not be verified in this research.

Evaluation of Safety

Regarding the safety of Kuromoji extract, the following were confirmed: there was no abnormality detected in a reverse mutation test using bacteria, there was no inducibility of chromosome structural abnormality in a chromosomal aberration test using mammalian cultured cells, there was no inducibility of chromosome abnormality and no bone marrow suppression in a micronucleus inducing action against rat's bone marrow immature red blood cells and suppression action on the growth of bone marrow cells and there was no abnormality in the toxicity test by repeated oral dosing for 13 weeks. From the fact that no adverse event was recognized from the intake of test drops including Kuromoji extract under the conditions of this research, it is considered that it is sufficiently secure.

Conclusion

From the result of this research, it was suggested that the intake of Kuromoji extract drops possibly reduces the number of influenza patients. Furthermore, it was considered that it leads to the prevention of seasonal influenza variously mutating every year by making the virus inactive non-specifically and inhibiting the growth of the virus.

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Conflict of Interest Statement

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