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Review article

Antiglycative effect and Antiviral effect of Kuromoji (*Lindera umbellata* Thunb.) extract

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Abstract

Kuromoji (*Lindera umbellata* Thunb.) is a deciduous shrub of the camphoraceae family endemic to Japan. It has long been used as an ingredient for tea and essential oil, and its dried trunk branches have been used for medicinal drinks using an ingredient known as "usho," a crude drug with stomachic actions. This paper describes the antiglycative and antiviral activities of a non-volatile extract of kuromoji extract (KE), which we have recently revealed. In absorption and metabolism studies, KE was found to have inhibitory activity against the formation of advanced glycation endproducts (AGEs). AGEs accumulate between collagen fibers in the bone matrix, thus reducing bone strength, and studies using bone glycation samples suggest that KE may ameliorate the reduction in bone strength caused by glycation. Furthermore, an 8-week oral administration of KE to streptozotocin-induced diabetic rats showed that it suppressed renal inflammation in diabetic nephropathy. The antiviral action of KE was confirmed by plaque assay to act against influenza viruses even after infection and to inhibit viral growth. The TCID50 assay showed that KE has antiviral activity against adenovirus and enterovirus, which are viruses that cause colds, as well as new strains of influenza virus and their drug-resistant strains, confirming that the antiviral activity of KE has a wide spectrum of action. A randomized, double-blind, placebo-controlled, parallel-group comparative study on the effects of taking KE-containing candies showed a reduction in the incidence of influenza and in the symptomatic period of cold symptoms. KE is expected to develop as a functional food material unique to Japan in the future because of its various functionalities as described above due to its antiglycative and antiviral effects.

KEY WORDS: Kuromoji (*Lindera umbellata* Thunb.), antiglycative effect, advanced glycation endproducts (AGEs), antiviral effect, influenza virus

Introduction

Kuromoji (*Lindera umbellata* Thunb.) is a deciduous shrub of the camphoraceae family endemic to Japan, and grows widely from southern Hokkaido to Kyushu. Kuromoji has a refreshing aroma and has been used since ancient times as a material for high-quality toothpicks to be attached to Japanese traditional sweets, as well as for various other purposes, such as boiling the branches and drinking the liquid as tea. Kuromoji are steam-distilled to extract essential oil that is rich in linalool and geraniol. Kuromoji essential oil is reported to have relaxant and antibacterial effects, and is used in aromatherapy as a domestically produced essential oil [1,2].

The dried trunk and branches of Kuromoji are known as "usho," a herbal medicine, and have been used as an ingredient in folk medicines such as stomachic medicines and medicinal liquors [3,4]. Non-volatile extracts of Kuromoji branches (hereinafter referred to as KE) are rich in polyphenols such as proanthocyanidins, *i.e.*, (+)-catechin, (-)-epicatechin, procyanidin B1, procyanidin B2, and flavan-3-ol and their multimeric forms [5], and flavonoids, *i.e.*, kaempferol, quercetin, hyperin, isoquercitrin [6]. KE has been reported to possess various functional properties, including antioxidant [7], anti-ulcer [8], and immune function improvement effects [9]. In this report, we describe the antiglycative and antiviral activities of KE, which we have recently revealed.

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Kuromoji extract (KE)

KE is a dried brown powder of non-volatile extract of Kuromoji and is used as a food ingredient. KE is produced by using a 10:1 ratio of water to crushed Kuromoji stem and branches. The mixture is heated at 95°C for 60 minutes, centrifugally filtering the extract, concentrating it in a decompressor, sterilizing it by liquid continuous sterilization, followed by drying it.

Antiglycative action of KE

(1) Inhibition of advanced glycation endproduct (AGE) accumulation

The following screening test revealed that KE is a particularly useful antiglycative material among various food material extracts [10]. First, in order to screen the food materials with antiglycative effects, hot water extracts of 536 materials were examined using the AGE-forming reaction model between human serum albumin (HSA) and glucose (Glu). Next, considering the absorption of the top 93 materials in the intestinal tract, the AGE formation inhibitory activity of the culture medium permeated through the intestinal epithelial absorption model using human colon adenocarcinoma-derived cells (Caco-2 cells) was measured in the same way [10]. As a final step, considering the absorption and metabolism of the top 34 extracts, the AGE formation inhibitory activity was examined using the serum specimen 3 hours after oral administration of each test extract to rats [10].

The results showed that the antiglycative activity of KE was the 21st highest of 536 species in the first selection, the 6th highest of 93 species in the second selection, and the 4th highest of 34 species in the final selection. The activity in rat serum was 75% of that of aminoguanidine, a positive control. These results indicate that the high AGE formation inhibitory effect of KE is demonstrated even after absorption and metabolism *in vivo*.

Furthermore, KE has strong activity against HSA as well as collagen and elastin distributed in skin, bone and cartilage, and concurrently inhibits the formation of AGEs, *i.e.*, fluorescent AGEs, N^{ε}-(carboxymethyl) lysine (CML), and intermediate aldehydes, *i.e.*, 3 deoxyglucosone, glyoxal, methylglyoxal [7]. KE has also been shown to enhance the activity of oxidized protein hydrolase (OPH), an enzyme that degrades oxidized and glycated proteins, and to cleave the cross-linked structure of AGEs, thereby promoting their degradation [7].

The antiglycative effect of KE may inhibit the formation of AGEs that occur in multiple pathways, and may further inhibit the AGE accumulation in the tissue by degrading the AGEs that are once formed.

(2) Effects on bone glycation

It is known that the risk of bone fracture is significantly higher in diabetic patients than in non-diabetic patients [11]. The risk is thought to be independent of bone mineral density and is caused by deterioration of bone quality due to glycation [12, 13].

To reproduce the degradation of bone quality caused by this glycation, bone glycated samples were prepared, followed by evaluation for the bone strength by a three-point bending test [14]. The bone samples were prepared by immersing chicken radius bones from which the bone marrow had been removed in phosphate buffer solution with Glu and heating at 60°C for 20 days. For the 3-point bending test, a weight load was applied perpendicularly to the bone stem of the bone sample until fracture using a universal testing machine (Instron, Kawasaki, Kanagawa, Japan), and the bone displacement and maximum load at the time of fracture were measured. As a result, the load-displacement curves of the three-point bending test for both the glycated and control samples showed that the load increased proportionally to the displacement until rupture (***Fig. 1***). The displacement to rupture was significantly reduced by about 30% in the glycated bone compared to the control (***Fig. 2-a***). In contrast, the addition of KE to the buffer solution during glycation of the bone samples tended to suppress the decrease in displacement at break, although the difference was not significant ($p = 0.078$). No difference in maximum load at break was observed between the glycated bone and control (***Fig. 2-b***).

The bone matrix is known to be rich in collagen type 1 which provide suppleness and strength to bone through the formation of physiological cross-links between collagen fibers. When non-physiological, pathological cross-links are formed in these collagen fibers due to glycation, the bone loses suppleness and bone strength decreases due to deterioration of the bone matrix [13]. In a same manner, the glycated bone samples lost bone suppleness and were susceptible to fracture due to a greater load applied at a given displacement. Whereas, the addition of KE may have ameliorated the loss of bone suppleness caused by glycation.

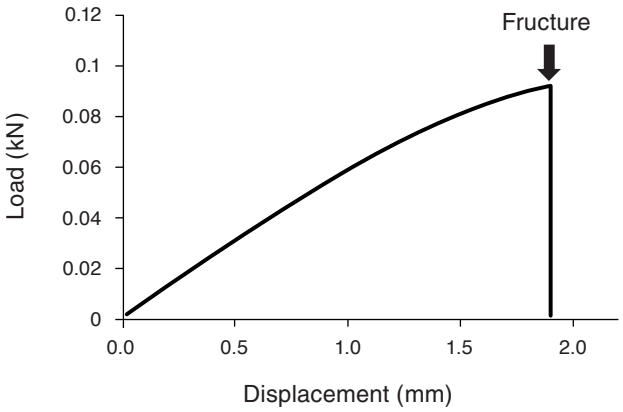

Fig. 1. **Example load-displacement plot from three-point bending test to assess bone strength (Control sample).**

(3) Action on diabetic nephropathy

Accumulation of AGEs *in vivo* is a known factor in the development of diabetic nephropathy [15]. We investigated the effect of KE on the renal inflammatory response observed in a diabetic rat model [16].

Diabetic model rats were prepared by administering a single intraperitoneal dose of streptozotocin (STZ) to male Sprague Dawley (SD) rats (STZ rats). Oral administration of chromophyll extract to STZ rats was 100 mg/kg/day for the low-dose group (KE-LD group) and 300 mg/kg/day for the high-dose group (KE-HD group), and the administration period was 8 weeks. Oral administration of KE to STZ rats

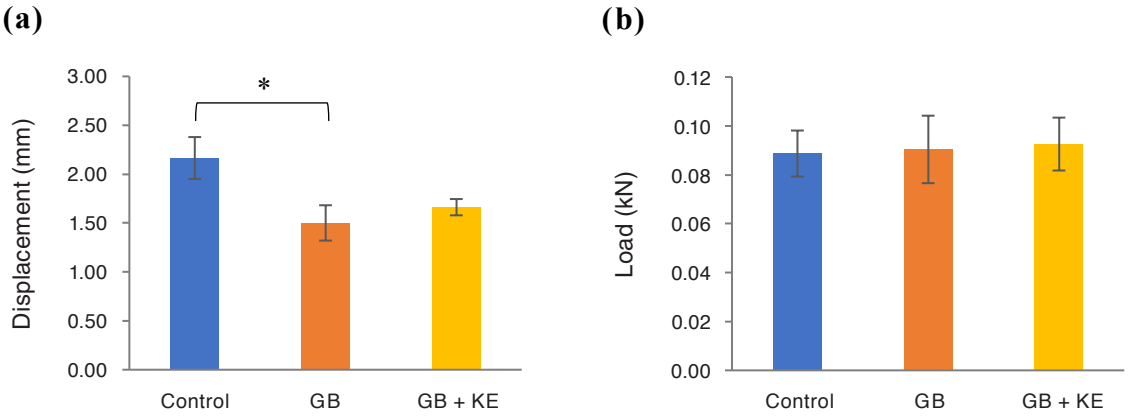

Fig. 2. **Bone strength parameters by Three-Point Bending test.**
(a) Displacement at fracture, **(b)** Load at fracture. Load was applied vertically to the shaft of bone sample at 5 mm/min. Results are expressed as mean ± SD, n = 6. * $p < 0.05$ vs Control, Statistical analysis by Mann-Whitney U test. SD, standard deviation; GB, glycated bone; KE, kuromoji extract.

was 100 mg/kg/day for the low-dose group and 300 mg/kg/day for the high-dose group, and the administration period was 8 weeks. The following three groups were designed to serve as comparison controls: normal group (SD rats were administered distilled water), Vehicle group (STZ rats were administered distilled water), and AG group (STZ rats were administered 100 mg/kg/day of aminoguanidine hydrochloride).

For each group, anti-CML autoantibody levels[17] in serum, which may reflect the formation and accumulation of AGEs *in vivo*, were measured as glycation indices. TNF-α and IL-6 levels in renal tissue supernatants were also measured as renal inflammatory indices.

As a result, the amount of serum anti-CML autoantibody in the Vehicle group was significantly higher than that in the normal group. And that in the KE-LD group, KE-HD group, and the AG group were significantly lower than the Vehicle group (***Fig. 3-a***). The tissue TNF-α amount in the Vehicle group was significantly higher than that in the control group. And that in the KE-LD group and the AG group were significantly lower than the Vehicle group (***Fig. 3-b***). IL-6 levels in the Vehicle group were not different in the normal group, while those in the KE-LD, KE-HD, and AG groups were significantly lower than the Vehicle group (***Fig. 3-c***).

An increase in inflammatory cytokines due to infiltration of inflammatory cells such as macrophages has been reported in renal tissues of diabetic nephropathy[18]. It has been suggested that CML-modified proteins induce an inflammatory response mediated by Receptor for AGEs (RAGE) present in macrophages or other inflammatory cells[19]. KE may have reduced the accumulation of AGEs through its antiglycative properties, suppressed the activation of inflammatory cells via the AGEs/RAGE signaling pathway, and reduced the development of inflammatory reactions in the kidney.

Antiviral action of KE

(1) Antiviral action

The viral growth inhibitory activity of KE was examined using a plaque assay, and it was reported that KE inhibited plaque formation in an infection test system using influenza A virus (A/Puerto Rico/8/34) and Madin-Darby canine kidney (MDCK) cells (***Fig. 4***)[20].

The antiviral activity of KE added after viral infection was measured by the TCID50 (50% tissue culture infectious dose) method using crystal violet staining in infection test systems using three types of influenza A virus strains, A/Puerto Rico/8/34, A/Nagasaki/HA-4/2009 (a porcine-derived strain of the 2009 epidemic)[21] and A/Nagasaki/HA-58/2009 (oseltamivir-resistant strain)[21], and MDCK cells. The results showed that KE exhibited dose-dependent antiviral activity against all strains[22]. In the infection test systems using human adenovirus (Type V) and Hep2 cells, and human enterovirus (Type 71) and Vero cells, the antiviral activity when KE was added simultaneously with viral infection was measured by the TCID50 method using crystal violet staining and showed dose-dependent antiviral activity for both viruses. These results indicate that KE can inhibit the growth of influenza viruses even after infection and has a broad spectrum of activity against viruses, including mutant viruses.

(2) Antiviral effects of KE-containing candy

A randomized, double-blind, placebo-controlled, parallel-group study was conducted to investigate the effects of the consumption of 1.8% KE-containing candy on influenza incidence and cold symptoms[24,25].

Subjects were male and female nurses working at Ehime University Hospital (Matsuyama, Ehime Japan). Subjects were given 3 pieces of KE-containing candy (KE group) or candy without KE (placebo group) per day for 3 months from December 2017 to March 2018. The presence or absence of influenza, type of influenza, onset of cold symptoms (fever, throat symptoms, nasal symptoms), and duration of onset of cold symptoms were evaluated using a questionnaire.

The results showed that the number of influenza cases was significantly lower in the KE group (2 out of 67) than in the placebo group (9 out of 67, ***Table 1***)[24]. The duration of onset of cold symptoms (fever, throat symptoms, or nasal symptoms) was significantly reduced in the KE group compared to the placebo group. When the data was collected

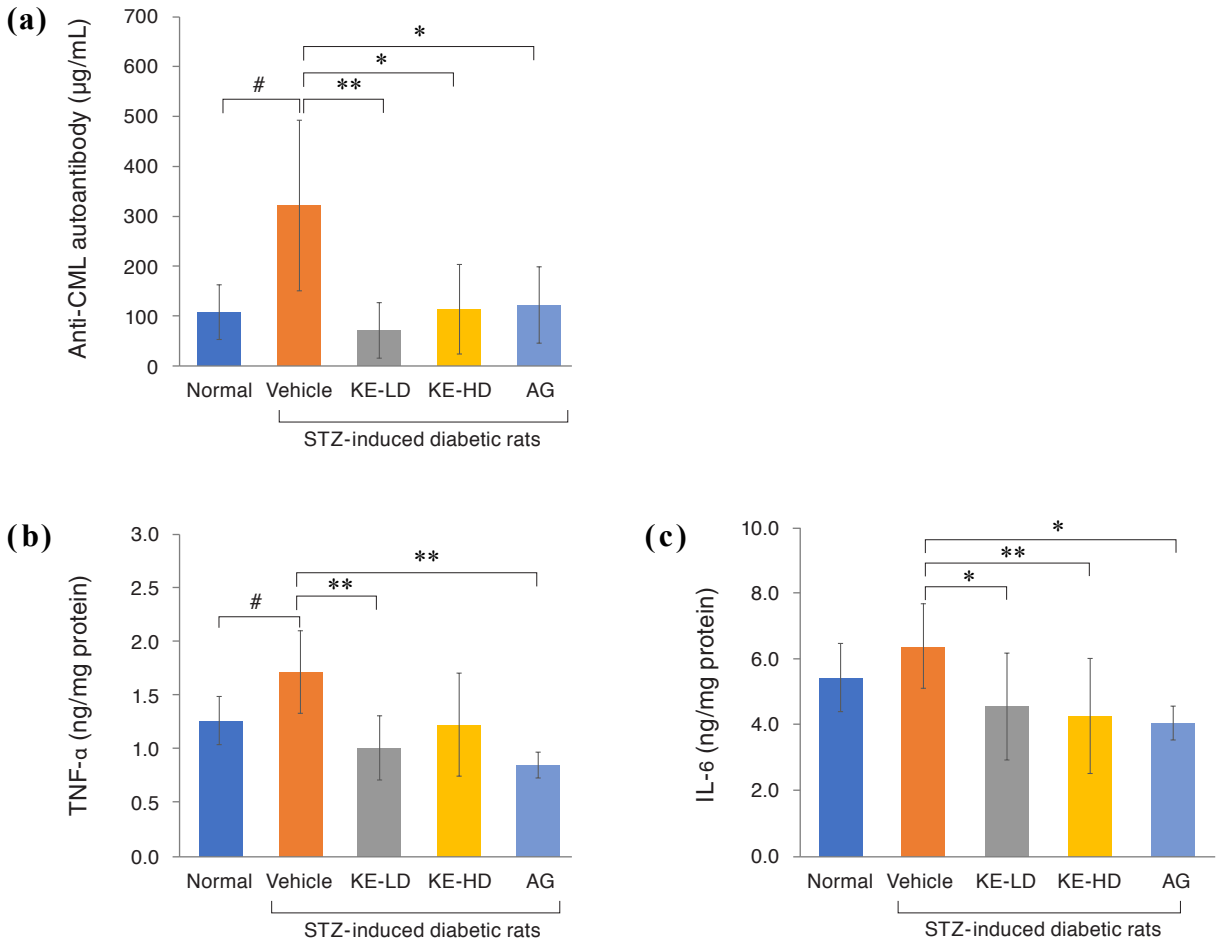

Fig. 3. **The values of blood anti-CML autoantibody and the values of kidney cytokine after treatment in the STZ rats.**
(a) Anti-CML autoantibody in serum, **(b)** TNF-α in renal tissue supernatant, **(c)** IL-6 in renal tissue supernatant. Normal, No STZ-treat and vehicle treated group; Vehicle, STZ and vehicle treated group; KE-LD, STZ and 100 mg/kg/day KE treated group; KE-HD, STZ and 300 mg/kg/day KE treated group; AG, STZ and 100 mg/kg/day AG treated group. Results are expressed as mean ± SD, n = 8, # $p < 0.05$ vs. Normal, Statistical analysis by Wilcoxon rank sum test; * $p < 0.05$, ** $p < 0.01$ vs. Vehicle, Statistical analysis by Bonferroni multiple comparison test. STZ, streptozotocin (i.p.); KE, kuromoji extract (p.o.); AG, aminoguanidine (p.o.); CML, N^{ε}-(carboxymethyl) lysine; SD, standard deviation; i.p., intraperitoneal administration; p.o., oral administration.

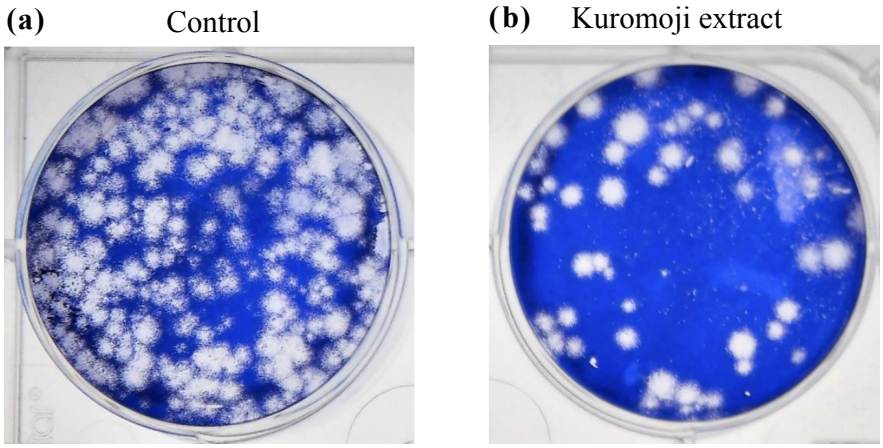

Fig. 4. **Inhibition on plaque formation by influenza virus of Kuromoji extract (KE).**
KE was added to monolayers of MDCK cells 1 hour after inoculation with influenza virus (A/Puerto Rico/8/34), and the cells were incubated for 72 hours and stained with crystal violet.

Table 1. Number of Influenza cases.

	Placebo candy group (n = 67)	Kuromoji candy group (n = 67)
Influenza prevalence (n (%))	9 (13.4)	2 (3.0)*
Type A (n (%))	6 (9.0)	0 (0)*
Type B (n (%))	3 (4.5)	2 (3.0)

* $p < 0.05$ vs Placebo group, Statistical analysis by chi-square test.

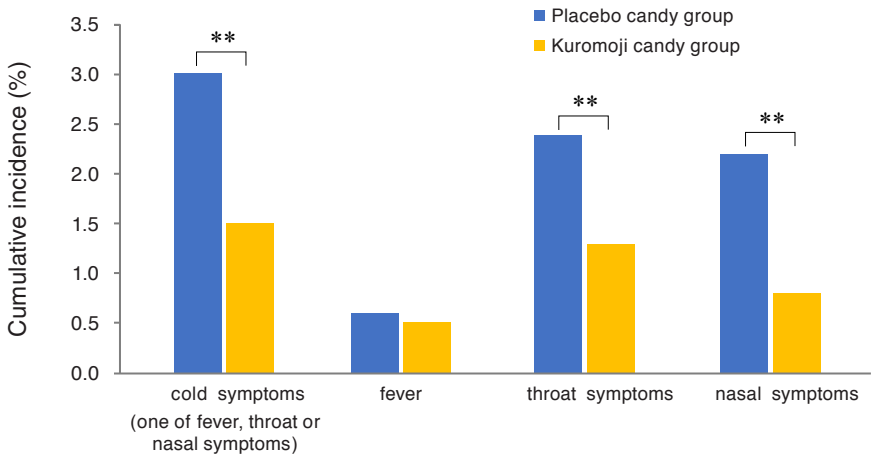

Fig. 5. **The cumulative incidence of common cold symptoms (one of fever, throat or nasal symptoms) and each symptom during 75 days.**

** $p < 0.001$ vs Placebo group, Statistical analysis by log-rank test.

over a period of 75 days, excluding the period after March, when pollen dispersal in the test area increased sharply, the decrease in the duration of onset on cold symptoms was more pronounced than for the 90-day data, and the duration of onset of throat and nasal symptoms were reduced significantly (***Fig. 5***)[25].

The above results indicate that taking three KE-candy per day can be expected to assist prevention of influenza infection and shorten the symptomatic period.

Mechanism of antiglycative and antiviral effects of KE

In a living body, proteins react with reducing sugars in a non-enzymatic manner and undergo various glycation intermediates such as aldehydes, leading to the formation of AGEs. AGE-modified proteins lead to protein browning, hardening due to cross-link formation, and inflammation induced by RAGE. These are known as exacerbating factors of aging, including age-related diseases such as diabetic complications, aging of skin appearance[26] and bone fragility[27]. To suppress AGE accumulation, it is important to inhibit AGE formation in the body and to promote their degradation. KE is characterized by its AGE inhibitory activity in tests considering absorption and metabolism, and its ability to inhibit AGE formation through multiple pathways by suppressing the formation of multiple intermediate aldehydes.

KE may also inhibit renal inflammation in diabetic nephropathy and bone quality deterioration caused by glycation. As for the antiglycative activity components, (+)-Catechin, procyanidin B1, and procyanidin B2 in KE have been reported to contribute significantly to the activity[28]. These low-molecular-weight flavan-3-ols have strong antiglycative activity, and their activity was maintained even after permeabilization of Caco2 cells, suggesting that they are some of the major active components in KE *in vivo*.

Exploratory studies have been conducted on the antiglycative effects of food ingredients in a wide range of food groups, including teas[29], herbs[30], spices[31], vegetables[32], and fruits[33]. Among the many antiglycative ingredients that have shown efficacy in clinical trials are mangosteen peel extract for improving skin water content and vascular stiffness[34] and a mixed herbal extract of dokudami, Roman chamomile, hawthorn, and grape leaves (AG Herb Mix) for improving skin elasticity[35]. In particular, mangosteen pericarp extract has been put to practical use as a food with functional claims that retains skin moisture through its antiglycative mechanism. KE is expected to be applied to functional foods

as a material that shows effects on renal inflammation and bone quality due to its antiglycative action.

Influenza causes systemic symptoms, *i.e.*, high fever, headache, arthralgia, fatigue, and upper respiratory tract symptoms, *i.e.*, cough, rhinorrhea, and can lead to fatal pneumonia and encephalopathy when severe in infants and the elderly[36]. The primary target sites of influenza virus infection in the early stages are the epithelial cells of the upper and lower airways, including the pharyngeal mucosa[37]. Influenza is being mitigated by vaccination, however the emergence of new strains due to mutation reduces its effectiveness. Cold syndrome (so-called "common cold") has upper respiratory tract symptoms, *i.e.*, sneezing, rhinorrhea, nasal obstruction as its main symptoms, and symptoms usually peak on the second or third day of onset, with symptoms becoming mild within one week to 10 days[38]. KE showed antiviral activity against influenza virus as well as against adenovirus and enterovirus, which are viruses that cause colds. It was also effective against the new strain of influenza virus that caused a pandemic in 2009 and its drug-resistant strain. KE is characterized by its antiviral activity against several types of viruses as described above, therefore it is desirable to identify the active ingredients in order to elucidate the mechanism of this activity.

The proanthocyanidin fraction of KE has been reported to have influenza virus growth inhibitory activity[22], and further studies are expected to identify more antiviral active molecules. As an example of a clinical trial demonstrating the inhibitory effect of a food ingredient on influenza infection, a five-month gargle test using black tea extract showed that the influenza incidence rate was 35% in the test group versus 48% in the control group, indicating a significant inhibitory effect[39]. Black tea extract has antiviral activity, and it is thought that influenza infection was suppressed by the direct action of its antiviral component on the target site of infection, the pharynx, through gargling.

Consumption of KE-containing candies had been shown to suppress the incidence of influenza and shortened the symptomatic period of cold symptoms. Influenza virus infection is established promptly after contact with target cells, but KE has growth inhibitory activity against influenza viruses, therefore it may have been effective even after influenza infection. Ingestion of the KE-candies was considered to be a more effective method of exerting antiviral effects because it prolonged the residence time of the active ingredients in the pharynx when ingested, compared to gargling.

Conclusion

Kuromoji trees grow throughout Japan and have long been used for crafts, tea, essential oil in everyday life. However, they are often treated as miscellaneous trees not as forest resources, and it is difficult to say that the resources are being effectively utilized. On the other hand, the various functional properties of kuromoji have been elucidated in recent years, and its utilization is expected to further expand in the future, possibly leading to the revitalization of the forest industry. The future development of kuromoji as a functional food material is expected.

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Declaration of conflict of interest

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