Methanol extract of Lonicera caerulea var. emphyllocalyx fruit has anti-motility and anti-biofilm activity against enteropathogenic Escherichia coli

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Summary

Foodborne diseases have become a worldwide problem that threatens public health and welfare. Enteropathogenic Escherichia coli (EPEC) is one of major pathogens of moderate to severe diarrhea. The increased prevalence of EPEC strains that produce extended spectrum β-lactamase (ESBL) has deepened the problem. The fruit of Lonicera caerulea var. emphyllocalyx (LCE) has been used as a traditional food preservative and medicine in northern temperate zones such as Hokkaido Island, Japan. In this study, we investigated the antibacterial effect of LCE fruit extract (LCEE) against EPEC. The antibacterial activities of LCEE were examined by bacterial growth, time-kill curve, soft-agar motility, electron microscopy, and 96 well-microplate biofilm assays. We also investigated the bacterial mRNA expression of biofilm-associated genes (fliC, csgA, and fimA) by quantitative real-time PCR assays. LCEE was found to suppress the growth, time-kill curve, and spread of EPEC. It also reduced the biofilm formation in a dose-dependent manner. Morphological analysis using transmission and scanning electron microscopy revealed that LCEE diminished the function of flagella resulting in reduced motility and biofilm formation. The mRNA expression of all three biofilm associated genes was downregulated under LCEE treatment. Extracts of the fruit of LCE inhibit the motility and biofilm formation of EPEC as a result of the inhibition of flagella development and function. We propose LCEE as a therapeutic candidate for the effective therapy of EPEC-associated infectious diseases.

Keywords: Lonicera caerulea var. emphyllocalyx, EPEC, biofilm, flagellar, electron microscopy

1. Introduction

WHO defines foodborne diseases as the infection or poisoning of human bodies, usually due to pathogens that enter the body through ingestion (1). These diseases have become a worldwide problem that threatens public health and well-being, and are therefore attracting an increasing level of attention. Along with these health issues, they also seriously impact the economic interests of society. Children are especially vulnerable to foodborne diseases; according to a WHO report, approximately 1.5 billion children aged less than 5 years contract diarrhea globally each year, resulting in more than 2.5 million deaths (1). Of these, 70% are caused by the biological contamination of foods. The morbidity of foodborne diseases in the under-5 years is higher than that of the other age groups (1). Escherichia coli represents a particular problem as a common contaminant of food, even in developed countries (2).

Enteropathogenic E. coli (EPEC) is a leading cause of moderate to severe diarrhea in young children, especially in developing countries (3). EPEC has only a human reservoir of infection (4). The increased prevalence of EPEC strains that produce extended...
spectrum β-lactamase has led to a growing interest in the development of non-antibiotic treatment options (5,6).

*Lonicera caerulea* var. *emphyllocalyx* (LCE) belongs to the honeysuckle family, and its fruits are known as edible berries. LCE grows in northern temperate zones such as Hokkaido (northern Japan), northern China, Korea, and Russia. It is commercially produced in Japan and Russia, although the fruits have been used as a traditional food preservative and medicinal agent (7). Fresh fruit juice is often used as a general strengthening agent, and therefore has potential in the treatment of stomach diseases and tonsillitis due to its antiseptic properties (8). However, the mode of action and efficacy of the juice remains unclear. In recent years, numerous studies have investigated the therapeutic effects of berries in the prevention of a range of diseases (7). In addition, herbal medicine products have invoked an increasing interest. Berries contain several important health-supporting phytochemicals that are an important part of the human diet (9). They also contain carbohydrates, lipids, proteins, organic acids, ascorbic acid, vitamin B, magnesium, phosphorus, calcium and potassium, as well as other minor compounds (10). Moreover, berries have antitumorogenic, antimicrobial, anti-inflammatory and antimutagenic properties (11). Although the antibacterial effect of LCE fruit toward *E. coli* has been demonstrated, the mode of action of LCE has yet to be elucidated completely (12). We therefore hypothesized that LCE exhibits antibacterial activity toward *E. coli*. The aim of this study was to evaluate a methanol extract of LCE fruits (LCEE) as a novel candidate for anti-EPEC therapy.

2. Materials and Methods

2.1. Preparation of LCEE

LCE fruits were cultivated in marshy grassland of Atsuma-Town, Hokkaido, Northern Japan in 2017. Voucher specimens were preserved in the Department of Pharmacognosy, Graduate School of Pharmaceutical Sciences, Nagoya City University. The LCEE was used in our previous study which included a fingerprint pattern and content of marker compounds (13). LCEEs were dissolved in water to a concentration of 200 mg/mL, and stored at -20°C.

2.2. Preparation of bacteria

EPEC strain E2348/69, which was kindly provided by Dr. James Kaper, was used in this study. A fresh colony was inoculated onto LB agar (Sigma-Aldrich, St. Louis, MO, USA) and cultured for 16 h at 37°C. The bacteria were harvested, centrifuged and resuspended in sterile phosphate-buffered saline (0.15 M, pH 7.2, PBS). Bacterial density was determined by measuring absorbance at 600 nm (A600). The bacterial suspension was then diluted to 10⁶ cells/mL in PBS using a standard growth curve to relate absorbance to bacterial concentration.

2.3. Growth of EPEC

Prior to broth culture analysis, the bacteria were pre-incubated in LB agar for 24 h. To assess growth-inhibitory activity, the bacteria (10⁶ cells/mL) were incubated in 5 mL polypropylene tubes (As-One, Osaka, Japan) containing 2 mL LB medium with the LCEE (0.5, 1, and 2 mg/mL). After 24 h, bacterial growth was determined by CFU counts. Aliquot of bacterial culture were plated onto LB agar for 24 h and CFU counts were evaluated. We also performed ³H-thymidine uptake assay as measurement of bacterial growth. After 24 h of pre-incubation, ³H-thymidine (2.0 Ci/mmol; PerkinElmer, MA, US) was added to LB medium in the polypropylene tubes. After culturing for 24 h, the cells were adsorbed onto 0.45 μm membrane filters (Advantech Japan, Tokyo, Japan), washed with distilled water, and then dried. The filters were transferred to vials containing liquid scintillator cocktail, and the radioactivity was measured with a liquid scintillation counter (LSC-6100, Hitachi Aloka Medical, Tokyo, Japan). All procedures were performed in triplicate.

2.4. Time-kill analysis

A time-kill analysis was performed as described previously (14). LB medium containing LCEE (0.5, 1, 2 mg/mL) or ampicillin sodium (ABPC) (50 μg/mL) (Fujifilm-Wako Pure Chemical, Osaka, Japan), was inoculated with bacterial suspension at a final concentration of 1 × 10⁶ CFU/mL in triplicate. Aliquots of undiluted and 10-fold serially diluted samples were then plated onto LB agar at both 0 and 60 min after inoculation and the plates were incubated at 37°C for 24 h. The resulting colonies (number of CFUs) were then counted.

2.5. Motility analysis of EPEC

Motility was evaluated by inoculating stationary-phase *E. coli* (10⁶ cells) onto the center of a 24-well polystyrene motility plate. LB containing 0.2% (w/v) agar and LCEE (0.5, 1, or 2 mg/mL) was then added. Plates were incubated at 37°C for 24 h, after which the diameters of the colonies were measured (an indication of bacterial migration). Motility assays were performed at least three times on separate days (15).

2.6. Electron microscopic analysis

Bacterial morphology was investigated using a
transmission electron microscope (TEM) and scanning electron microscope (SEM). Bacteria (10⁶ cells) pre-treated with LCEE (0.5, 1, or 2 mg/mL) were cultured in LB broth for 24 h. Then, approximately one drop of the culture was applied to a Formvar/carbon-coated 300-mesh copper grid (Nissin EM, Tokyo, Japan). Excess solution was removed and 2% phosphotungstic acid (PTA) (Fujifilm-Wako Pure Chemical) was added for negative staining. The samples were then observed under a TEM (JEM1011J; JEOL, Tokyo, Japan) and digital images were taken with a MegaView Slow-scan camera (JEOL). The square of bacterial shape was measured using NIH Image J, and areas were measured at five arbitrary points in bacterial cells treated with LCEE. Preparation of samples for SEM analysis was performed at first. Briefly, bacterial cells (10⁶ cells) pre-treated with LCEE (0.5, 1, or 2 mg/mL) were cultured in LB broth for 24 h. The samples were then immediately fixed in 0.1 M phosphate buffer (pH 7.4) containing 2.5% glutaraldehyde (Nissin EM, Tokyo, Japan) for 24 h at 4°C. The samples were rinsed twice with 0.1 M phosphate buffer, further fixed using 2% osmium tetroxide (Nissin EM) for 2 h at room temperature, and finally rinsed with distilled water. Next, the specimens were dehydrated using serial concentrations of ethyl alcohol (30, 50, 70, 90, 95 and 100%) for 30 min each, followed by immersion in absolute alcohol for a further 30 min. The specimens were dried using a critical point dryer CPD300 (Leica, Wetzlar, Germany). Carbon conductive paint was used for mounting for the specimens which were coated with osmium (NL-OPC-AJ, Filgen, Nagoya, Japan). Finally, several areas of each sample were systematically scanned using a SEM (S-4800; Hitachi High-Technologies, Co, Tokyo, Japan).

2.7. Biofilm analysis

Overnight cultures of E. coli were inoculated into each of a 96-well polystyrene plate (Thermo Fisher Scientific, MA, US) to achieve a bacterial concentration of 10⁶ cells. The wells contained LB broth with or without LCEE (0.5, 1, or 2 mg/mL). Incubation was allowed at 37°C for 48 h. After removal of the media, the plates were washed three times with PBS, and adherent bacteria stained with 0.2% crystal violet at room temperature for 10 min, before being gently washed a further three times with PBS. The biofilms were quantitated by measuring absorbance at 570 nm (A570). Wells incubated without bacteria were used as controls. The absorbance values from the control wells were subtracted from the test values.

2.8. Total RNA extraction, cDNA synthesis, and quantitative real-time PCR (qPCR) analysis

The bacteria (10⁶ cells/mL) were incubated in LB medium with the LCEE (2 mg/mL) for 8 h. Bacterial cells were washed three times with PBS and RNA samples were prepared with RNeasy Protect Bacteria Mini Kit (Qiagen, Hilden, Germany) according to the enclosed protocol. RNA samples were quantitated with the NanoDrop ND-1000 (Thermo Fisher Scientific, Inc., Waltham, MA). cDNA was synthesized with ReverTra Ace qPCR RT Master Mix (Toyobo Co., Ltd., Osaka, Japan) according instruction. qPCR for the quantification of cDNA was executed with the Thunderbird qPCR Mix (Toyobo) and Applied Biosystems 7900HT Fast Real Time PCR System (Thermo Fisher Scientific) according to the procedure recommended by the manufacturer. The primers designed for qPCR are described elsewhere (16). The following PCR conditions were used: 50°C for 2 min, 95°C for 10 min; 40 cycles of 95°C for 30 s and 60°C for 1 min; followed by 95°C for 15 s, 55°C for 15 s, and 95°C for 15 s. The glyceraldehyde-3-phosphate dehydrogenase (gapA) gene was used as an internal control for normalization. The fold changes in the flagellin (fljC), major curli subunit (csgA), and major type 1 subunit fimbrin (fimA) expression levels were calculated by the comparative cycle threshold (CT) method.

2.9. Statistical analysis

Experimental data was expressed as mean ± standard deviation (S.D.). Statistical analysis was conducted using Student's t-test between two groups and Tukey's multiple comparison tests for the differences among multiple groups. A probability value less than 0.05 was considered to represent a statistical significance.

3. Results

3.1. Growth of EPEC

Initially, we examined the effect of LCEE on the growth of E. coli. Figure 1 showed the absorbance readings of 24 h culture solutions treated with different concentrations of LCEE. The data clearly showed that the decrease in absorbance is proportional to an increase in LCEE concentration. The effect of LCEE on the proliferative ability of EPEC was examined by a thymidine uptake test. Figure 2 showed the tritium value of thymidine concentration for a 24 h culture containing LCEE and thymidine. The tritium value clearly decreases in parallel with an increase in LCEE concentration. These results also confirmed that the growth of E. coli was suppressed in the presence of LCEE in a dose-dependent manner.

3.2. Time-kill analysis

We also examined the time-kill effects of LCEE on EPEC. A significant bacterial reduction was observed after 60 min of treatment with LCEE in dose-dependent
manner. However, this antibacterial effect of LCEE could not reduce the number of bacteria to less than one-tenth, such as ABPC, even if the concentration of LCEE was increased (Figure 3).

3.3. Motility analysis of EPEC

We then examined the effect of LCEE on motility, a fundamental factor that enhances the pathogenicity of bacteria. The spread of E. coli after 24 h of incubation was found to be inhibited when grown in media that contained LCEE (Figure 4A). A decrease in the diameter of the spread zone was found to parallel an increase in LCEE concentration to a significant degree (Figure 4B).

3.4. Bacterial morphology analysis of EPEC

To elucidate the mechanism of motility inhibition, we examined the effect of LCEE on E. coli morphology using electron microscopy. Negative staining revealed that the number of flagella decreased with an increase in LCEE concentration; no flagella were observed at a LCEE concentration of 1 mg/mL (Figure 5A). In addition, the morphology of E. coli also increased at a LCEE concentration of 1 mg/mL and over. The cell area of E. coli also increased significantly under LCEE treatment (Figure 5B). This observation was also corroborated by SEM, which confirmed that an LCEE concentration of 1 mg/mL led to an increase in morphology. Similarly, SEM analysis revealed...
an increase in the cell area of *E. coli* due to LCEE treatment (Figures 5C and 5D).

### 3.5. Biofilm analysis of EPEC

As flagellum was one of important factors of biofilm, the effect of LCEE on biofilm formation as another bacterial virulence was also examined. Crystal violet staining assay showed that biofilm formation decreased in parallel with an increase in LCEE levels (Figure 6A), with a corresponding reduction in the absorbance of the biofilm staining solution (Figure 6B).

### 3.6. qPCR analysis

Finally, we performed qPCR assay whether the flagella associated genes of EPEC were affected by LCEE. Our results demonstrated that all of the target genes (*fliC, csgA*, and *fimA* genes) were downregulated in expression when bacteria were treated with LCEE significantly. Decreased these genes expression with less than 2-fold lower was observed in the presence of LCEE (*p < 0.01*) (Figure 7).

### 4. Discussion

This study was designed to investigate the effect of fruit of the LCE on EPEC. Our result revealed that LCEE prepared from the fruits showed significant anti-motility and anti-biofilm actions even if the antibacterial effect of LCEE was limited compared to antibiotics. Furthermore, in morphological analysis, LCEE treated bacteria were swelling and lack of flagella, these morphological changes may contribute...
to the inhibition of bacterial motility. The LCEE fruit will be very useful agent that can be expected for future medical applications against EPEC infectious disease.

Several researchers have described the anti-biofilm effect of extracts from *Lonicera* species. The freeze-dried fruit of *Lonicera caerulea* and its phenolic fraction reduced the biofilm formation and adhesion to the artificial surface of *Candida parapsilosis*, *Staphylococcus epidermidis*, *Enterococcus faecalis*, *Streptococcus mutans* and *E. coli* (12). The fruit of *Lonicera caerulea* also had anti-bacterial and anti-biofilm effects on *Streptococcus pyogenes in vitro* (13). As extracts from *Lonicera alpigena* exhibited the biofilm inhibition, limited bacteriostatic activity was also evident (17). However, this study did not reveal the mechanism of anti-biofilm action by molecular biological analysis and morphological evaluation (12). Our analysis results focus on these points.

A biofilm is a highly structured immobile complex entrenched with bacterial communities in an extracellular matrix of exopolysaccharide, along with proteins and DNA. It is also used to describe matrix-enclosed bacterial population's adherent to each other and to surface (18). Bacterial strains that adhere onto medical devices or damaged tissue can cause persistent infections through biofilm formation. Antibiotics may not be effective when the bacteria form a biofilm even if showing a high level of activity against the planktonic bacterial cells. The formation of a bacterial biofilm often inhibits the activity of an antimicrobial agent (19). Thus, novel drugs which have the ability to target these bacteria are urgently desired.

Flagella as a bacterial virulence factor have three mechanisms of involvement in biofilm formation. Firstly, flagella are required for physical attachment to abiotic surfaces, thus promoting the initiation of biofilm formation. Secondly, motility may enable a bacterium to reach the surface or developing biofilm. Thirdly, motility is thought to allow the bacteria to move within a developing biofilm, thereby enhancing growth and spread of the biofilm (20). Moreover, the process of chemotaxis allows bacteria to swim toward nutrients that are present on a surface. Motility promotes initial cell-to-surface contact and may also contribute to the spread of a growing biofilm along an abiotic surface (18). Flagellated bacteria can adapt their locomotion machinery to achieve a specialized form of flagellum-driven motility when grown on solid surfaces. This motility is characterized by a multicellular movement of bacteria that migrate over solid substrates in groups of tightly bound cells (14). This kind of movement is dependent on the ability of surface-adhering bacteria to undergo a differentiation process characterized by the production of more flagellated than planktonic cells. Thus, this behavioral response to the surface provides flagellated bacteria with the ability to act as a multicellular community that can rapidly colonize nutrient-rich solid substrates (15). Most of the motility-negative mutants tested
were found to be severely defective in their ability to form a biofilm. This mechanism is mainly related to the gene cluster including *flIC*. Thus, we tried to check the mRNA expression of biofilm-associated genes under LCEE treatment. Our study showed that genes involved in adhesion (*csgA* and *fimA*), and motility (*flIC*) were expressed significantly lower in EPEC in the presence of LCEE. Expression of *csgA* in bacterial cells that adhered to cell cultures was similar to that observed for bacteria on abiotic surfaces. This structure might play an important role in increasing bacterial attachment. Other reports have shown that curli fimbriae are also required for biofilm formation and bacterial adhesion of planktonic cells during the process of *E. coli* biofilm formation (21,22). Furthermore, this curli fimbriae associated gene, the *fimA* gene, was possible involved in initial attachment. Previous studies have also demonstrated that *fimA* induction in planktonic cells under LCEE treatment. The decrease in those gene expression may induce the disappearance of flagella and the like, thereby reducing bacterial motility and biofilm formation. Our results highlight the crucial role of motility in biofilm formation; the non-motile strains were unable to form biofilms. LCEE-treated EPEC cells that lack flagella have a reduced ability to form a biofilm.

Several previous reports have also described the antibacterial effect of extracts of *Lonicera* species on *E. coli*. The monoterpenoids isolated from the dried flower buds of *L. japonica* exhibited antibacterial activities against *E. coli* (25). Xiong demonstrated that these compounds were identified as 3, 5-bis-O-caffeoyl quinic acid, 4, 5-bis-O-cafeoylquinic acid, luteoloside, 3-O-cafeoylquinic acid and secoxyloganin (26). Yang demonstrated an enhanced antimicrobial activity of silver nanoparticles- *L. japonica* combo against *E. coli* (27). Myricetin and quercitrin, derived from cranberries, can significantly decrease uropathogenic *E. coli* biofilm formation, while dihydroferulic acid glucuronide, procyanidin A dimer, quercetin glucoside, myricetin, and prodelphinidin B are known to decrease surface hydrophobicity (28). Although the growth or the production of bacterial effector proteins of the type III secretion system of EPEC was unaffected by proanthocyanidins, inhibition of EPEC pedestal formation was observed (29). Previous study demonstrated the antibacterial effect of cranberry against uropathogenic *E. coli*. It showed that the expression of the *flIC* gene in this bacterial species is inhibited by proanthocyanidins. It explained that bacterial motilities were hindered in the presence of proanthocyanidins (30). LCEE also suppresses the motility and biofilm formation of *E. coli*, however, a full mechanism of activity toward the bacteria cannot be confirmed at present. Proanthocyanidins from LCEE may be as deeply involved in this mechanism of action as cranberry. Further clarification of the mechanism of action of these compounds would facilitate their development as novel antimicrobial agents.

In conclusion, the extracts of the fruit of LCE inhibit the motility and biofilm formation of EPEC as a result of the inhibition of flagella development and function. We propose LCEE as a therapeutic candidate for the effective therapy of EPEC-associated infectious diseases.

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**References**


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