Original Article

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Methanol extract of *Lonicera caerulea* var. *emphyllocalyx* fruit has antibacterial and anti-biofilm activity against *Streptococcus pyogenes in vitro*

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Summary ***Streptococcus pyogenes*** **causes several infectious diseases such as tonsillitis, cellulitis, and streptococcal toxic shock syndrome. As antibiotics are used for the general treatment of *S. pyogenes* infection, cases of treatment failure due to drug-resistant bacteria have increased. *Lonicera caerulea* var. *emphyllocalyx* (LCE) has been used as a folk medicine in northern Japan (Hokkaido). In this study, we investigated the antibacterial effect of methanol extracts of the fruit, stem, and leaf of LCE (LCEEs) against *S. pyogenes* using disk diffusion assay. As LCEE (fruit) had the strongest antibacterial activity among the three LCEEs, we focused on functional analysis of antibacterial effects of LCEE (fruit). LCEE (fruit) suppressed the growth of *S. pyogenes* in a dose-dependent manner. Morphological analysis by transmission electron microscopy demonstrated that LCEE (fruit) damaged the shape of *S. pyogenes*. Microplate and confocal laser microscopy analysis showed that biofilm formation was also suppressed by LCEE (fruit) in a dose-dependent manner. To further evaluate the surface structure of these biofilms, we performed hydrophobic analysis, which demonstrated that LCEE (fruit) reduced the hydrophobicity of the bacterial surface structure. Our data demonstrated that LCEE (fruit) had anti-bacterial and anti-biofilm effects on *S. pyogenes in vitro*, suggesting that the direct anti-bacterial effects of the LCEE (fruit) may be useful for treatment of local *S. pyogenes* infection.**

Keywords: *Lonicera caerulea* var. *emphyllocalyx*, fruit, *Streptococcus pyogenes*, anti-bacterial effect, anti-biofilm effect

1. Introduction

Streptococcus pyogenes is a common gram-positive virulent bacterium (*1*). Because *S. pyogenes* contains various virulent factors such as streptolysin O, streptolysin S, NADase, and SpeB protease, it causes various types of infectious diseases such as pharyngitis, tonsillitis, nephritis, cellulitis, and necrotizing fasciitis (*1*). Because the antibiotic resistance rate of *S. pyogenes* has been gradually increasing worldwide (*2,3*), identification of novel anti-*S. pyogenes* therapies is of great importance.

Lonicera caerulea var. *emphyllocalyx* (LCE) belongs to the honeysuckle family (Caprifoliaceae) and the honeysuckle genus, which is best-known for having edible berries (*4*). LCE is distributed in northern temperate zones such as the northern part of Japan (Hokkaido) and the northern part of the Eurasian continent (*4*). LCE is cultivated commercially in Japan, Canada, and Russia (*4*).

LCE berries contain several beneficial phytochemicals, including carbohydrates, lipids, proteins, sugar, organic acids, vitamins, and mineral such as iron, magnesium, phosphorus, calcium, and potassium (*5*). They also

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contain phenolic acid, anthocyanins such as cyanidin 3-*O*-glucoside, flavonoids, and caffeic acid (*5*). LCE berries have anti-tumorigenic, antimicrobial, anti-inflammatory, and anti-mutagenic properties (*6*).

In Japanese traditional Kampo medicine and traditional Chinese medicine, the flower bud, stem, and leaf of *Lonicera japonica* are formulated into prescriptions for treatment of febrile common cold (*7*), joint pain (*8*), and severe viral diseases (*9,10*). In their countries of origin, honeysuckle plants have also been used as folk medicines, and fresh honeysuckle fruit juice has been used as a general strengthening drink and as a treatment for tonsillitis (*11*).

Owing to the increasing interest in the use of plants belonging to the honeysuckle family as herbal products, various studies have investigated the therapeutic effects of honeysuckle berries for prevention of a range of diseases (*12-14*). However, the mechanisms of action of honeysuckle berries has not been characterized. In this study, we hypothesized that LCE, a plant belonging to the *Lonicera* genus, may exert anti-microbial activity. Furthermore, we evaluated whether LCE is a novel candidate agent for anti-*S. pyogenes* therapy.

2. Materials and Methods

2.1. *Preparation of samples*

LCE was harvested in a field located in Atsuma-Town, Hokkaido, northern part of Japan. Voucher specimens were kept at the Department of Pharmacognosy, Graduate School of Pharmacognosy, Nagoya City University. Six hundred thirty-three grams of the fruit (fresh), 5.6 g of the leaf (dried), and 20.9 g of the stem (dried) were soaked in 2 L, 500 mL, and 500 mL of MeOH (Fujifilm Wako Pure Chemical, Osaka, Japan), respectively, and allowed to incubate for 72 h at room temperature. Following paper filtration, equal amounts of MeOH were added to the residues, and the mixtures were allowed to incubate for 72 h. After filtration, each filtrate was combined, evaporated under reduced pressure, and then lyophilized. The weights of the LCE extracts (LCEEs) after lyophilization were 79.8 g (fruit), 1.33 g (leaf), and 0.686 g (stem). The extraction efficiencies were 12.6% (fruit), 23.8% (leaf), and 3.28% (stem). LCEEs of fruit, leaf, and stem were dissolved and suspended at a concentration of 200 mg/mL in water, 40% DMSO(Fujifilm Wako Pure Chemical), and 20% DMSO, respectively, and stored at –20°C.

Chromatographic signatures of LCEE (fruit) were created as follows. Ten milligrams of LCEE (fruit) was suspended in 1 mL of MeOH and centrifuged at $1.5 \times 10^3 \times g$ for 5 min. Twenty-five microliters of the supernatant was injected onto an HPLC with the following operating conditions: system: Shimadzu LC-$10A_{VP}$; column: TSK-GEL ODS-80_{TS} (4.6 × 250 mm, Tosoh, Tokyo); mobile phase: 0.05 M AcOH-$AcONH_4$ buffer (pH 3.6) (Fujifilm Wako Pure Chemical)/CH_3CN (Fujifilm Wako Pure Chemical) 90:10 (0 min) – 45:55 (40 min), linear gradient; flow rate: 1.0 mL/min; column temperature: 40°C; and detection wavelength: 245-600 nm using a photodiode array detector (Figure 1). Cyanidin 3-*O*-glucoside was purchased from Tokiwa Phytochemical (Sakura, Japan). LCEE (fruit) (5 µg) and cyanidin 3-*O*-glucoside (28.8, 57.5, and 115 ng) were injected onto the HPLC system described above, and absorbance was monitored at 520 nm. The retention time of cyanidin 3-*O*-glucoside was 9.0 min. The range of cyanidin 3-*O*-glucoside was calibrated by analyzing peak response using the least-squares method (r^2 = 0.997), and the concentration of cyanidin 3-*O*-glucoside in LCEE (fruit) was calculated as 1.12% (w/w).

2.2. *Bacterial strains*

S. pyogenes 1529 was isolated from a patient with

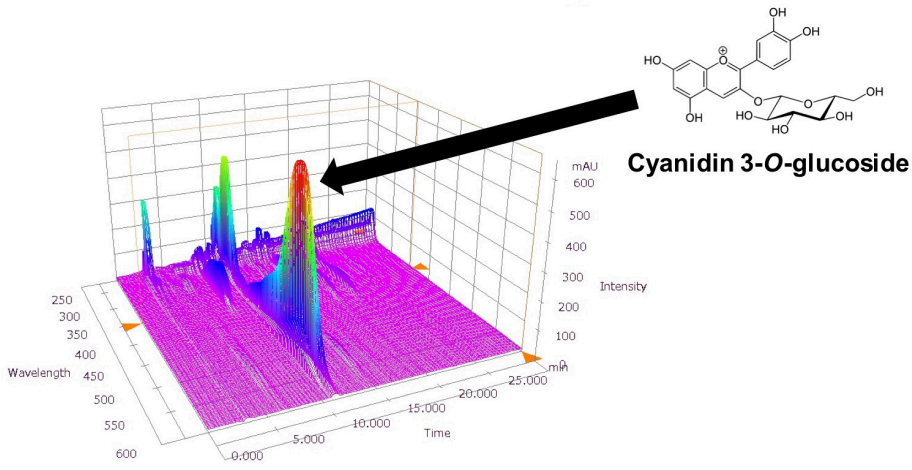

Figure 1. 3D-HPLC profile of LCEE (fruit). Fingerprint analysis of LCEE (fruit) was conducted as described in the Materials and Methods section. Cyanidin 3-*O*-glucoside was identified by comparison with the retention time and UV spectrum of its standard.

streptococcal toxic shock in Japan (*15*). A fresh colony was inoculated overnight on Trypticase soy agar with 5% sheep blood (Nippon Becton Dickinson Co., Ltd., Tokyo, Japan) and cultured for 16 h at 37°C in an atmosphere of 5% CO_2. The bacteria were harvested, centrifuged, and resuspended in sterile phosphate-buffered saline (0.15 M, pH 7.2, PBS) (Fujifilm Wako Pure Chemical). Bacterial density was determined by measuring the absorbance at 600 nm (A_{600}). The bacterial suspension was then diluted with PBS to 1.0×10^9 colony forming units (CFU)/mL using a standard growth curve to determine bacterial concentration from A_{600} values.

2.3. *Disk diffusion assay*

Disk diffusion assay was performed with some modifications (*3*). Sterile paper disks (GE healthcare Japan, Tokyo, Japan) were impregnated with LCEEs (7.5 mg/disk) and dried at room temperature. Kirby-Bauer Disk 'EIKEN' containing penicillin G (10 U = 6 μg/disk) (Eiken Kagaku, Tokyo, Japan) was used as a positive control. *S. pyogenes* colonies cultured overnight on Trypticase soy agar with 5% sheep blood were collected into Todd Hewitt broth (Difco Laboratories Inc., Detroit, MI, USA). 1.0×10^9 CFU/mL of *S. pyogenes* was inoculated on another Trypticase soy agar plate with 5% sheep blood. The paper disks were placed on the plates and were incubated at 37°C in a 5% CO_2 atmosphere for 24 h. Inhibitory zone diameters were measured.

2.4. *Evaluation of growth in vitro*

S. pyogenes (1×10^6 CFU) was cultured in Todd Hewitt broth with 0.3% yeast extract (Difco) (THY broth) and LCEE (fruit) at 37°C in an atmosphere of 5% CO_2 for 24 h. Turbidity was estimated by measuring the absorbance at 600 nm.

2.5. *Biofilm assay*

Biofilm assay was performed with some modifications (*16*). Overnight cultures of *S. pyogenes* (1×10^6 CFU) were seeded onto 96-well polystyrene plates (Thermo Fisher Scientific, MA, USA), which were then incubated in THY broth with or without LCEE (fruit) at 37°C for 48 h. After removal of the media, the plates were washed three times with PBS, and adherent bacteria were stained with 0.2% safranin red (Fujifilm Wako Pure Chemical) at room temperature for 10 min, after which, they were gently washed three times with PBS. Each biofilm was quantitated by measuring the absorbance at 490 nm (A_{490}). Wells incubated without bacteria were used as blanks. The absorbance of blank wells was subtracted from the test values. For confocal microscopic observations, *S. pyogenes* was grown on glass coverslips in 24-well polystyrene plates (Thermo Fisher Scientific) at 37°C for 48 h. After removing the media, the wells were washed three times with PBS and stained with fluorescein isothiocyanate isomer (FITC) (Fujifilm Wako Pure Chemical). Images were collected using an LSM 510 confocal laser microscope (Carl Zeiss, Oberkochen, Germany). Three-dimensional images were created from Z-stack images using Imaris software (Carl Zeiss).

2.6. *Bacterial morphological investigation*

Bacterial morphological analysis using transmission electron microscopy (TEM) JEM1011J (JEOL, Tokyo, Japan) was performed with slight modifications (*17*). *S. pyogenes* strains treated with LCEE were cultured in THY broth for 24 h. For negative staining, approximately one drop of the bacterial culture was applied onto a 300-mesh carbon formvar copper grid (Nisshin EM, Tokyo, Japan). Excess solution was removed and negative staining was performed using 2% phosphotungstic acid (PTA) (Fujifilm Wako Pure Chemical). The samples were visualized using transmission electron microscopy. Digital images were collected using a Mega View Slow-scan camera (JEOL).

2.7. *Analysis of cell surface hydrophobicity*

Cell surface hydrophobicity was evaluated using the hexadecane method with a minor modification (*16*). *S. pyogenes* treated with LCEE (fruit) was grown to the exponential phase and suspended in PBS, and A_{600} was adjusted to 1.0. After addition of 200 μL of n-hexadecane (Fujifilm Wako Pure Chemical) to 2 mL of bacterial suspensions in glass tubes, the A_{600} value of the lower aqueous phase was measured. Then, the tubes were vigorously vortexed for 2 min, followed by 10 min of incubation at room temperature to allow for phase separation, and the A_{600} value of the lower aqueous phase was measured. Hydrophobicity was calculated using the following equation: percent hydrophobicity = $[1 - (A_{600}$ after vortex/A_{600} before vortex$)] \times 100$.

2.8. *Statistical analysis*

Statistical analysis was performed using Student's *t*-test for two groups, and Bonferroni-Dunnett's multiple comparison *t*-test to evaluate differences among multiple groups. *P*-values less than 0.05 were considered statistically significant.

3. Results

3.1. *Evaluation of growth inhibitory effect of LCEE (fruit) against S. pyogenes in vitro*

We evaluated the antibacterial activities of the three LCEEs (fruit, leaf, and stem) by Kirby-Bauer disk diffusion assay (Figure 2). Treatment with each

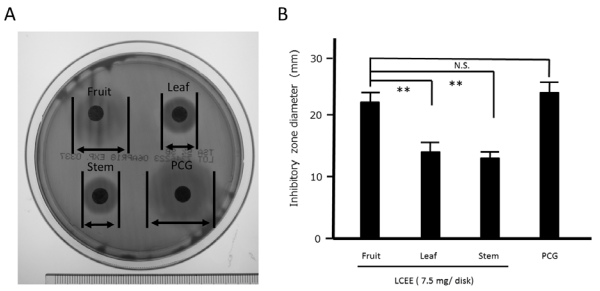

Figure 2. Antibacterial activities of the extracts of fruit, leaf, and stem of LCE (LCEEs). (A) Paper disks, 12 cm in diameter, containing 7.5 mg/disk of LCEEs (fruit, leaf, and stem) or 6 μg/disk of penicillin G (PCG) were placed on *S. pyogenes* cultured on trypticase soy agar with 5% sheep blood, and incubated for 24 h. Arrows show the inhibitory zones resulting from LCEEs or PCG. **(B)** The sizes of the inhibitory zones were measured. Data were expressed as mean ± S.D. (n = 6). N.S. not significant, *p < 0.05 and **p < 0.01 by Bonferroni-Dunnett's multiple t-test.

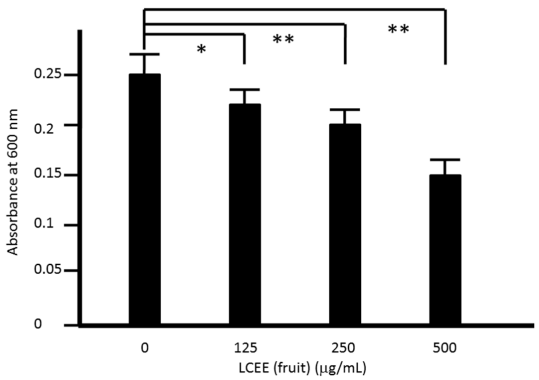

Figure 3. Bacterial growth inhibitory activity of LCEE (fruit). Numerical values represent extract concentrations. LCEE (fruit) was added in Todd Hewitt broth, and growth of *S. pyogenes* was measured for 24 h at 600 nm. Data were expressed as mean ± S.D. (n = 6). *p < 0.05 and ** < 0.01 by Bonferroni-Dunnett's multiple t-test.

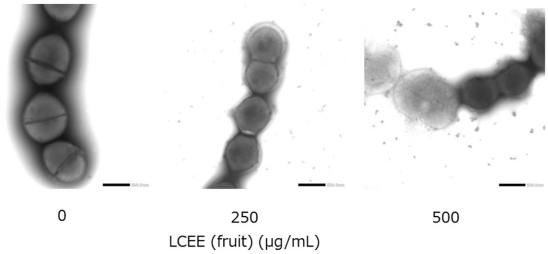

Figure 4. Morphological change of *S. pyogenes* treated with LCEE (fruit). Representative electron microscopy image of *S. pyogenes* cultured for 24 h with or without LCEE using negative staining.

LCEE on trypticase soy agar with 5% sheep blood inhibited bacterial growth by forming inhibitory zones (Figure 2A). Each LCEE showed significant antibacterial activity against the *S. pyogenes* 1529 strain. In particular, LCEE (fruit) showed the strongest antibacterial activity among the three LCEEs (p < 0.01), as evidenced by the largest inhibitory zone observed for this extract (Figure 2B). There were no significant differences in inhibitory zone sizes between 7.5 mg/disk of LCEE (fruit) and 6 μg/disk of penicillin G. Thus, we focused on analysis of LCEE (fruit) in further investigations because LCEE (fruit) had the greatest antibacterial effect among the three LCEEs. Using the liquid dilution method to evaluate antibacterial activity, bacterial growth inhibition by LCEE was measured after 24 h. As a result, we confirmed that LCEE (fruit) had inhibited *S. pyogenes* growth after 24 h in a concentration-dependent manner (Figure 3).

3.2. *Bacterial morphological investigation*

To confirm whether bacterial growth inhibition by LCEE was due to growth arrest or morphological disruption, we performed morphological analysis of *S. pyogenes* treated with LCEE (fruit). Negative staining analysis revealed that the shape of the bacterial cells became irregular in response to LCEE treatment. Local collapse of bacterial cell walls occurred in response to 250 μg/mL LCEE, and cell membrane irregularity and thinning occurred in response to 500 μg/mL LCEE. Finally, the cells collapsed and the bacterial contents were released into the extracellular environment. This morphological change occurred in a concentration-dependent manner (Figure 4).

3.3. *Biofilm analysis by microplate assay and confocal laser scanning microscopy*

Bacteria form biofilms to protect themselves from external stimuli (*16*). To evaluate whether LCEE (fruit) could inhibit biofilm formation, *S. pyogenes* was grown in THY broth with LCEE (fruit), and the ability to form biofilm on polystyrene plates was assessed using safranin red staining. Bacterial biofilms not exposed to LCEE were stained deep red. In contrast, formation of biofilms was suppressed by LCEE treatment, resulting in thinning of the red staining. Thus, a significant inhibitory effect of LCEE on *S. pyogenes* biofilm formation was observed after 48 h (p < 0.01) (Figure 5A). Next, we evaluated absorbance of safranin red to quantify bacterial biofilms. LCEE (fruit) at concentrations above 125 μg/mL significantly inhibited *S. pyogenes* biofilm formation (p < 0.01) (Figure 5B). Thus, we confirmed that LCEE (fruit) inhibited biofilm formation in a concentration-dependent manner. To confirm the results obtained using safranin red staining, *S. pyogenes* biofilms treated/untreated with LCEE (fruit) for 48 h were stained with FITC dye and visualized using confocal laser scanning microscopy (Figure 5C). The obtained Z-stack images were converted into three-dimensional images. The biofilms formed by the control showed a multi-layered surface-adhered cluster reflecting a mature biofilm. In contrast, the LCEE (fruit)-treated strains showed a lack of biofilm extracellular matrix. Overall, the findings from safranin red staining and microscopic three-dimensional

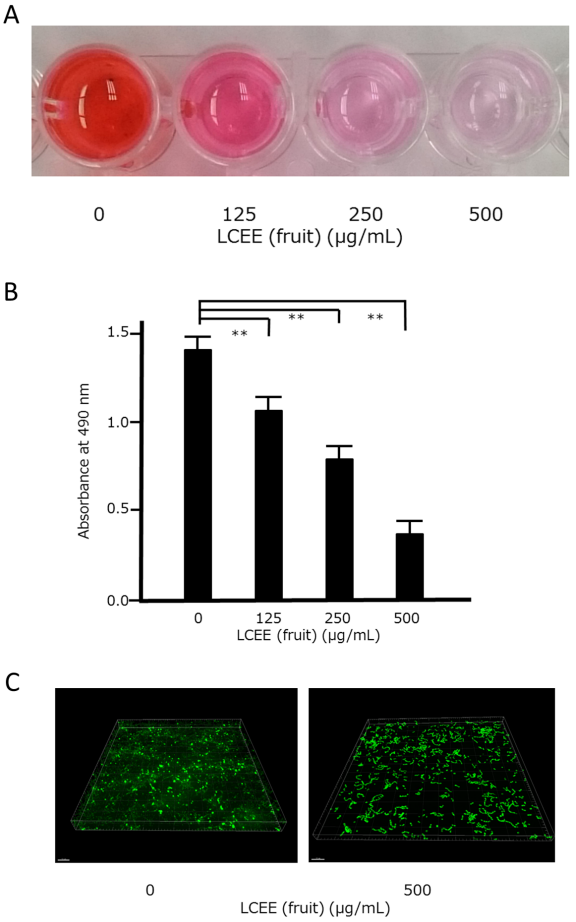

Figure 5. LCEE (fruit) inhibited *S. pyogenes* biofilm formation. (A) Image of a microplate. **(B)** *S. pyogenes* was cultured for 48 h with or without LCEE (fruit), and anti-biofilm activity of LCEE was quantified by safranin red adsorption at 490 nm. Data shown represent the mean ± S.D. ($n = 6$). $^{**}p < 0.01$ by Bonferroni-Dunnett's multiple *t*-test. **(C)** 3D analysis of biofilm formation of *S. pyogenes* treated with LCEE (fruit). Bacteria were cultured with LCEE (fruit) under static conditions at 37°C for 48 h in THY. Biofilms were stained with FITC. Three-dimensional images were reconstructed from confocal optical sections using Imaris software.

observations were consistent in showing that LCEE (fruit) inhibited biofilm formation.

3.4. *Bacterial cell surface hydrophobicity*

Based on the observation that LCEE altered bacterial morphology, we hypothesized that LCEE influenced bacterial surface hydrophobicity. As bacterial cell surface hydrophobicity affects the formation of bacterial biofilms (*16*), we evaluated the hydrophobicity of the strains using the n-hexadecane method. Surface hydrophobicity was markedly reduced in *S. pyogenes* treated with LCEE compared to that of the untreated group ($p < 0.01$) (Figure 6).

4. Discussion

In this study, we demonstrated that LCEE (fruit)

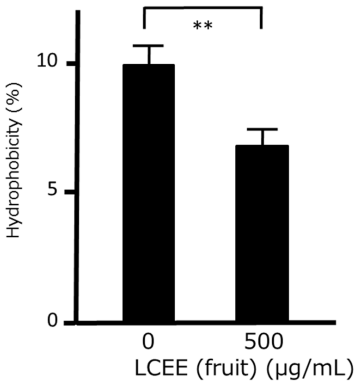

Figure 6. Hydrophobicity of *S. pyogenes* treated with LCEE (fruit). Cell surface hydrophobicity of *S. pyogenes* treated/untreated with LCEE (fruit) in the exponential phase (A_{600} of 1.0) was determined. The value of the control strain was set at 100%. Data shown represent the mean ± S.D. ($n = 6$). $^{**}p < 0.01$ by Student's *t*-test.

showed antibacterial and anti-biofilm activities. LCEE (fruit) inhibited bacterial growth and biofilm formation, and caused bacterial collapse in concentration-dependent manners. Adhesion to host tissues and biofilm formation are important steps in bacterial infections (*16*). A previous study showed that LCEE (fruit), which is rich in polyphenols, suppressed biofilm formation of *Staphylococcus epidermidis*, *Enterococcus faecalis*, and *Streptococcus mutans* (*18*). This study did not include *S. pyogenes* (*18*). Anthocyanins and proanthocyanidins have antibacterial properties and can inhibit adhesion of bacteria to mucosal membranes (*19*). Although organic acids such as succinic, malic, lactic, tartaric, citric, and acetic acid exerted antibiotic activity against streptococci, synthetic mixtures of organic acids tested at the concentrations found in wine had greater antibacterial activity than the beverages, indicating that in wine, mixed organic acids are inhibited by other components (*20*). Since wine is made directly from fruit, the antimicrobial effects of the organic acids in LCEE (fruit) may also be inhibited by other components. Although we did not evaluate the effects of specific vitamins or minerals on streptococci, several reports have indicated that anthocyanin and phenolic acid are involved in the antibacterial effect of LCEE (*4-6,18*). Although we used cyanidin-3-glucoside as a representative component of LCEE when we performed 3D-HPLC analysis (*18*), we did not evaluate the effects of cyanidin-3-glucoside on streptococci. Future studies should focus on the effects of individual components of LCEE on streptococci. The hydrophobic properties of bacterial cell surfaces play important roles in host cell-bacterium interactions (*21*). A previous study showed that plant extracts altered bacterial hydrophobicity by suppressing the formation of biofilms (*22*). Similarly, LCEE (fruit) decreased the cell surface hydrophobicity of *S. pyogenes* to approximately 2/3 of the hydrophobicity of untreated cells. Thus, LCEE (fruit) may exhibit anti-

biofilm activity. Moreover, reduced hydrophobicity may increase exposure of bacteria to antibiotics due to elimination of biofilms.

Several investigations have demonstrated the antibacterial effects of fruits on *S. pyogenes in vitro*. A previous study showed that crude extracts of *C. fistula* fruit exhibited moderate to strong antibacterial activity against *S. pyogenes* (*23*). Another study showed that a novel anti-bacterial peptide, brucin, which is specific to S. pyogenes was produced from the dried fruit protein of *Brucea javanica* (*24*). A mixture of active peptides was prepared from the fruit protein *in vitro* by pepsin hydrolysis, and its inhibitory activity toward gram-positive bacteria was higher than that of penicillin G and chloramphenicol (*24*). Other studies showed that 80% ethanol extracts of *Moringa oleifera*, *Limnophila aromatica*, *Terminalia chebula*, and *Phyllanthus emblica* had anti-bacterial activities against human virulent bacteria, including *S. pyogenes* (*25*). Our study was the first to show that that LCEE (fruit) may be effective against *S. pyogenes*.

Several reports have demonstrated the antibacterial effects of polyphenol compounds contained in LCEE (fruit) (*26*). DNA repair mutant gram-negative bacteria were significantly affected by phenolic compounds (*26*). This strain lacks a DNA repair mechanism, and was therefore expected to be more sensitive than the wild-type strain to damage caused by mutagenic agents (*26*). The chemical group of anthocyanidins, and the individual compounds quercetin and chlorogenic acid, suppressed the growth of the DNA repair mutant, but did not suppress growth of the wild-type (*26*). These results suggested that the anti-bacterial activities of these compounds occurred through interactions with DNA (*26*). Furthermore, antimicrobial action may also occur through genotoxicity. Small phenolic compounds such as carvacrol and thymol exhibited bacterial inhibitory effects by disrupting outer membranes (*27*). The flavonoid myricetin clearly inhibited growth of the *Lactococcus* and *Enterococcus* species (*28*). Based on these results, the degree of hydroxylation might affect the antimicrobial activity of pure phenolic compounds (*29*).

The antimicrobial activities of naturally-occurring phenols from fermented fruit juices such as wine have been widely studied (*30*), but little is known about the anti-microbial capacity of phenols present in berries. Cranberry and blueberry extracts rich in anthocyanins inhibited gram-negative bacteria (*26*). The antibacterial properties of cranberry juice have been extensively demonstrated (*31*) and these effects may be associated with inhibition of *E. coli* adherence to mucosal surfaces (*32*). However, berry extracts mainly inhibited the growth of gram-negative bacteria but had no effects on gram-positive bacteria (*25*). The anti-bacterial effects of LCEE (fruit) against S. pyogenes, a gram-positive bacterium, may distinguish these extracts from other berries. Furthermore, the bacterial morphological changes induced by LCEE (fruit), as evaluated by electron microscopy, were similar to those observed with cranberries (*33*).

Phenolic compounds affected the growth of different bacterial species by different mechanisms, which are not well-understood. Anti-adhesion may also be a mechanism of antimicrobial activity of berry compounds. Future studies should be performed to improve the understanding of these antimicrobial mechanisms.

5. Conclusions

In summary, LCEE (fruit) exerted anti-bacterial and anti-biofilm effects on *S. pyogenes in vitro*. Based on these results, the direct antibacterial effects of LCEE (fruit) may be useful for topical therapies, such as gargling. We propose that LCEE (fruit) is a novel therapeutic candidate for treatment of diseases resulting from *S. pyogenes* infections.

Conflicts of Interest

This study was partly supported by JSPS KAKENHI Grant Number JP 16K09251, and academic donations from both the Research Foundation for Oriental Medicine, and Atsuma town. There has been no other financial support for this work that could have influenced its outcome.

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