

Efficacy of *Moringa oleifera* Leaf Extracts against Cariogenic Biofilm

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ABSTRACT: *Moringa oleifera* leaves are beneficial for human health. Dental caries is closely related with cariogenic biofilm, which is an oral biofilm containing a high proportion of *Streptococcus mutans*. The purpose of this study was to investigate the antimicrobial effects of the *M. oleifera* leaf extracts on *S. mutans* and formation of cariogenic biofilm. Extract from *M. oleifera* leaves was derived using distilled water (DW) and ethyl alcohol (EtOH). *S. mutans* susceptibility assays were performed for each extract. Cariogenic biofilm was formed with or without DW and EtOH extract, and cariogenic biofilm was treated with both extracts. The biofilm was observed by confocal laser microscopy, and the bacteria in the biofilm were counted. Both extracts showed antimicrobial activity against *S. mutans* and inhibited formation of cariogenic biofilm. The EtOH extracts exhibited anti-biofilm activity. *M. oleifera* leaves may be potential candidates to prevent dental caries.

Keywords: *Moringa oleifera*, leaf extract, oral biofilm, antimicrobial activity

INTRODUCTION

Moringa oleifera, known as the drumstick tree or the horseradish tree, leaves and seeds are used as food supplement because they have distinctive tastes and flavors (Bhattacharya et al., 2018; Anwar et al., 2007). The leaves and the seeds can be stored for many months without any major loss of functional nutrients (Arabshahi-D et al., 2007). *M. oleifera* leaves contain β -carotene, vitamin E, and protein, and so can be used as an antioxidant, and its seeds contain bioactive related molecules, such as flavonoid, isothiocyanates, glucosinolate, and thocarbamate (Guevara et al., 1999; Paikra et al., 2017; Amaglo et al., 2010). Various studies have reported that the leaf constituents show anti-inflammatory, antibacterial, and anti-cancer efficacy (Siddhuraju and Becker, 2003; Sreelatha et al., 2011; Moyo et al., 2011). Furthermore, traditional medicines derived from plant extracts, such as moringa leaf and seed extracts, may be effective treatments, but may also be cheaper and less toxic than commercial drugs (Awaad et al., 2011). Therefore, the leaf of *M. oleifera* has potential to be used as a medicine as well as in food.

Dental caries is closely related with cariogenic biofilm, an oral biofilm containing a high proportion of *Streptococcus mutans* (Lee and Kim, 2014). Generally, oral biofilm is structurally and functionally organized and contains a

balance of normal flora and pathogenic bacteria like *S. mutans* (Marsh, 1994). The homeostasis of the bacteria proportion can break down by environmental changes, such as uptake sucrose and a decrease in pH, and the number of cariogenic bacteria may increase (Marsh, 1994; Marsh, 1991). This may cause cariogenic biofilm to be formed by the cariogenic bacteria *S. mutans*, *Streptococcus sobrinus*, and *Lactobacillus acidophilus*. Among these bacteria, *S. mutans* is particularly associated with dental caries because of its rapid metabolism and strong acid tolerance (Nakano et al., 2005). *S. mutans* also synthesizes glucan from sucrose by glucosyltransferases (Lee et al., 2012). Glucan contributes to biofilm formation, is linked to biofilm proteins, and protects bacteria in the biofilm from antimicrobial agents (Kreth et al., 2008).

The present study investigated the antimicrobial and the antibiofilm effects of *M. oleifera* leaf extracts on *S. mutans* and cariogenic biofilm.

MATERIALS AND METHODS

Extraction from leaf of *M. oleifera*

Ethyl alcohol (EtOH) and distilled water (DW) were used to prepare leaf extracts. Fifty grams of the powdered leaves were mixed with 250 mL of DW or EtOH, and the

Received 25 April 2019; Accepted 5 July 2019; Published online 30 September 2019

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mixture was incubated for 6 h with constant agitation. The extracts were filtrated with Whatman filter paper (Toyo Roshi Co., Tokyo, Japan). EtOH extract and DW extract were cooled and frozen at -80°C , respectively. The extracts were dried using a freeze drying machine (IIShinBioBase, Gyeonggi, Korea) and the extracts were weighed. EtOH and DW extracts were dissolved in dimethyl sulfoxide and DW at a concentration of $100\ \mu\text{g}/\text{mL}$, respectively. The extracts were filtrated with polyvinylidene fluoride (PVDF) filter (Merck Millipore Co., Darmstadt, Germany) with a pore size of $0.22\ \mu\text{m}$ and stored in 4°C until further use.

Bacterial strain and biofilm formation

S. mutans KCTC 3065 (ATCC 25175) was purchased from Korean Collection for Type Cultures (Jeongeup, Korea) and cultured in brain heart infusion (BHI) broth (BD Biosciences, San Jose, CA, USA). For cariogenic biofilm formation, unstimulated saliva was collected from 10 healthy donors, and the pooled saliva was mixed with an equal volume of BHI broth containing 2% sucrose. The mixture was centrifuged at $2,000\ \text{g}$ for 10 min to remove debris. *S. mutans* (1:400 ratio) were added into the saliva-bacteria suspension. Pooled saliva ($200\ \mu\text{L}$ and $400\ \mu\text{L}$) was dispensed onto 8-well glass plates (BD Biosciences) and 12-well polystyrene plates (SPL Lifescience, Gyeonggi, Korea), respectively, and the plates were dried at 37°C to coat the saliva. This procedure was repeated 10 times. The suspension was dispensed into a saliva-coated glass plate to observe the biofilm and a saliva-coated polystyrene 12-well plate to count bacteria in the biofilm after adding the extracts. The plates were incubated at 37°C for 3 days to form cariogenic biofilm.

Investigation of antimicrobial activity

The *S. mutans* susceptibility assay was performed according to the guidelines of the Clinical Laboratory Standard Institute (Wayne, PA, USA). The EtOH and DW extracts were diluted with phosphate buffered saline (PBS, pH 7.2) at a concentration of $50\ \mu\text{g}/\text{mL}$. BHI broth ($180\ \mu\text{L}$) was dispensed into a 96-well plate (SPL Lifescience), and the extracts were added into the 12th row well containing BHI broth. Two-fold serial dilution was performed using a multi-channel micropipette. The number of cultured *S. mutans* was counted with a bacterial counting chamber (Paul Marienfeld GmbH & Co. KG, Lauda-Königshofen, Germany) and adjusted to a concentration of 1×10^7 cells/mL with fresh BHI broth. The prepared bacterial suspension ($20\ \mu\text{L}$) was inoculated into the wells. The plate was incubated at 37°C in aerobic conditions for 24 h. The growth of *S. mutans* was calculated by measuring optical density at a wavelength of $660\ \text{nm}$ using a spectrophotometer (BioTek Instruments Inc., Winooski, VT, USA).

Investigation of cariogenic biofilm

Cariogenic biofilm was formed with or without the DW and the EtOH extracts at non-lethal concentrations for *S. mutans* ($3.2\ \mu\text{g}/\text{mL}$) to investigate inhibitory effects on biofilm formation. In a separate experiment, after formation of the cariogenic biofilm, the biofilm was treated with the extracts ($25\ \mu\text{g}/\text{mL}$) and 70% EtOH (positive control) for 1 h and washed three times with PBS. BHI broth (1 mL) was then added. The biofilm on polystyrene plates was disrupted with a scraper (Corning Co., Corning, NY, USA), transferred into a 1.5 mL tube, and was homogenized using a vortex. The suspension was serially diluted 10-fold from 10^7 to 10^6 cells with fresh BHI broth, and the diluted bacterial suspensions were spread on mitis salivarius-bacitracin agar plates (BD Biosciences) and BHI agar to count *S. mutans* and total bacteria, respectively. The agar plates were incubated at 37°C in aerobic conditions for 36 h, and the colonies were counted. To observe cariogenic biofilm, the biofilm on the glass plate was stained using a bacterial live/dead staining kit (Invitrogen, Eugene, OR, USA) and observed using a LSM 700 confocal laser scanning microscope (CLSM) (Carl Zeiss Meditec AG, Oberkochen, Germany). The biofilm was analyzed by two-laser CLSM using Z-stack scans from 0 to $30\ \mu\text{m}$, and the 3D image of the biofilm was visualized by the ZEN program (Carl Zeiss Meditec AG). Green and red colors indicated live and dead bacteria in the biofilm, respectively.

Statistical analysis

The data were analyzed non-parametrically by using the Kruskal-Wallis test after checking the data distribution using the Kolmogorov-Smirnov test. Results with a *P*-value of less than 0.05 were considered significant different. Post-hoc analyses to compare differences between individual groups were performed using the Mann-Whitney test. IBM SPSS Statistics ver. 23 (IBM, Armonk, NY, USA) was used for statistical analysis.

RESULTS AND DISCUSSION

M. oleifera leaves are beneficial for human health (Anwar et al., 2007). Therefore, the leaf powder is directly used as a food supplement. Various studies have reported that moringa leaves have anti-inflammatory, antibacterial, and anti-cancer efficacy. However, the effects of moringa leaves on oral diseases are not well known. Thus, the present study examined the preventive effects of the moringa leaf on induction of biofilm caries after forming cariogenic biofilm with salivary bacteria and *S. mutans*.

S. mutans is a Gram-positive and facultatively anaerobic bacteria, and has virulence factors such as acidogenicity and aciduricity (acid tolerance) (Lee et al., 2012).

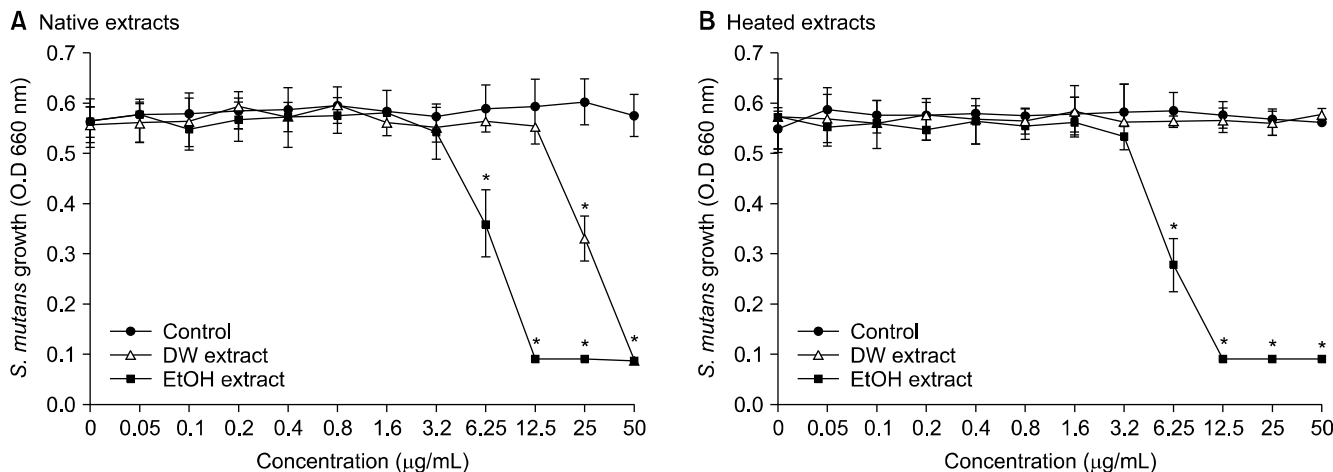


Fig. 1. The antimicrobial activity of extract from *Moringa oleifera* leaves. *Streptococcus mutans* was cultured with or without distilled water (DW) and ethanol (EtOH) extracts, and the growth of *S. mutans* was measured using spectrophotometer at 660 nm wavelength. Asterisks (*) represent statistically significant differences compared with the control group ($P < 0.05$). DW, distilled water; EtOH, ethanol.

Therefore, *S. mutans* is considered to be a cariogenic bacteria. *M. oleifera* leaf extracts were prepared with EtOH and DW, and the antimicrobial activity of the extracts against *S. mutans* was investigated. When *S. mutans* were cultivated in BHI broth in the presence or the absence of EtOH and DW extracts at the various concentrations, the EtOH extract showed stronger antimicrobial activity against *S. mutans* than the DW extract. EtOH and DW extracts significantly reduced the growth of *S. mutans* at and above concentrations of 6.25 µg/mL and 25 µg/mL, respectively ($P < 0.05$) (Fig. 1A). Also, heated EtOH extracts significantly inhibited growth of *S. mutans* at the same concentration of the native EtOH extract ($P < 0.05$). However, the heated DW extract did not impact the growth of *S. mutans* (Fig. 1B). Both the DW and EtOH extracts exhibited antimicrobial activity against *S. mutans*. However, when comparing antimicrobial activity between heated and unheated extracts, no difference was observed for the EtOH extract. Whereas, the heated DW extract did not show antimicrobial activity; therefore, a heat-unstable component of the DW extract may show antimicrobial effects.

Oral biofilm is structurally and functionally organized by the bacteria balance (Marsh, 1994). However, when the proportion of *S. mutans* is increased due to environmental changes, oral biofilm becomes a cariogenic biofilm (Marsh, 1994; Lee et al., 2012). Cariogenic biofilm is more resistant against antibiotic agents due to an abundance of glucan (Socransky and Haffajee, 2002), which acts as a physical barrier. Also, an exopolymer matrix which includes glucan, has a strong negative charge and inhibits the diffusion of antibiotic agents with positive charge into biofilm (Socransky and Haffajee, 2002). For these reasons, resistance between planktonic bacteria and biofilm bacteria against antimicrobial molecules is different. Therefore, we investigated the efficacy of our extracts

against cariogenic biofilm. In the first experiment we investigated the inhibitory effect of the extracts on the formation of cariogenic biofilm by treatment at non-lethal concentration (3.2 µg/mL) during biofilm formation. The DW and the EtOH extracts significantly inhibited cariogenic biofilm formation (Fig. 2A~C, 3A, and 3B). Furthermore, both extracts showed slight bactericidal activity (red color in the biofilm) (Fig. 2B and 2C). In the second experiment, we explored the effects of the extracts at concentrations of 25 µg/mL on removal of the cariogenic biofilm after biofilm formation. The DW extract did not impact the cariogenic biofilm, whereas the EtOH extract showed anti-biofilm activity, including biofilm removal and bactericidal activity (Fig. 2 and 4). Considering the function of glucan, these results suggest the DW extracts may contain charged and heat-unstable components, and the EtOH extracts may contain heat-stable and uncharged or negatively charged components.

M. oleifera leaves contain largely consist of protein, followed by fatty acids and phenolic molecules (Teixeira et al., 2014). Specifically, fatty acids and phenolic molecules show strong antimicrobial activity. Fatty acids containing short and long carbon chains exhibit antimicrobial activities against Gram-negative and Gram-positive bacteria, respectively (Kabara, 1980; Knapp and Melly, 1986). Furthermore, fatty acids with unsaturated carbon chain show stronger antibacterial activity than those with saturated carbon chains (Nieman, 1954). The EtOH extracts contain phenol compounds (Ndhlala et al., 2014), which show both anti-fungal and antimicrobial activities (Marrufo et al., 2013). On the basis of these studies and the results in the present study, EtOH extracts have stronger anti-biofilm activity against cariogenic biofilm than DW extracts.

M. oleifera leaves may be able to be used for prevention and treatment of various disease states, in addition to as a nutritional supplement. In the present study, we showed

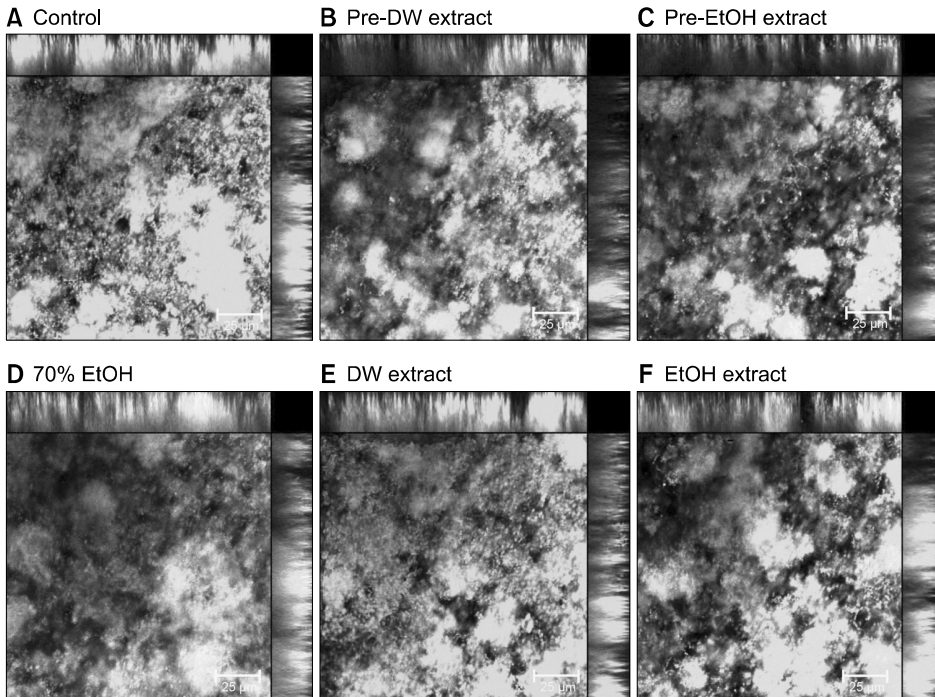


Fig. 2. Effect of extract on cariogenic biofilm. In the first experiment, cariogenic biofilm was formed with or without the *Moringa oleifera* leaf extracts at non-lethal concentration (A~C). In the second experiment, cariogenic biofilm was treated with extracts and 70% ethanol (D~F). The biofilm was observed by confocal laser scanning microscope after treatment with the live/dead staining kit. Green and red colors indicate live and dead bacteria, respectively. DW, distilled water; EtOH, ethanol.

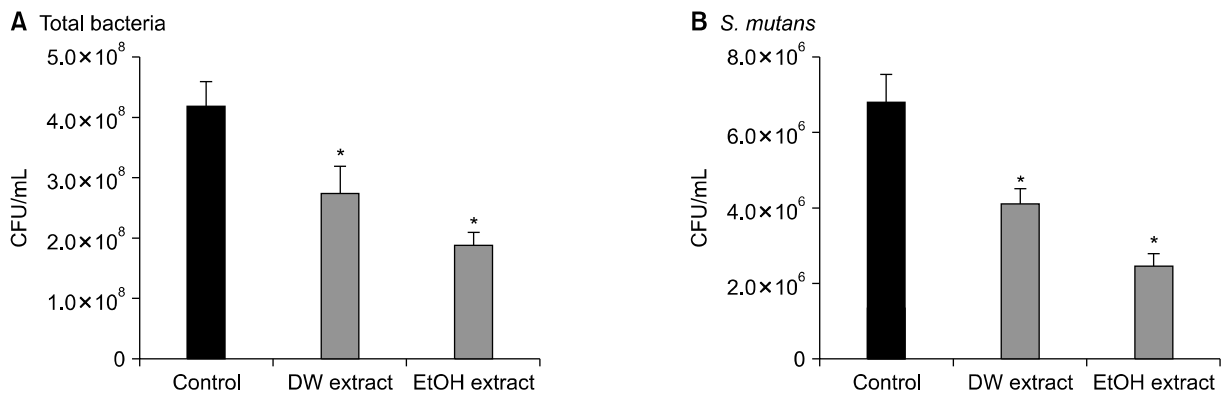


Fig. 3. Inhibition of cariogenic biofilm formation by *Moringa oleifera* leaf extracts. Cariogenic biofilm was formed with or without the at a non-lethal concentration, and was disrupted with a scraper. The bacteria in the biofilm were resuspended in brain heart infusion (BHI) broth and spread on mitis salivarius-bacitracin and BHI agar plates to count *Streptococcus mutans* and total bacteria, respectively. Asterisks (*) represent statistically significant differences compared with the control group ($P < 0.05$). CFU, colony-forming unit; DW, distilled water; EtOH, ethanol.

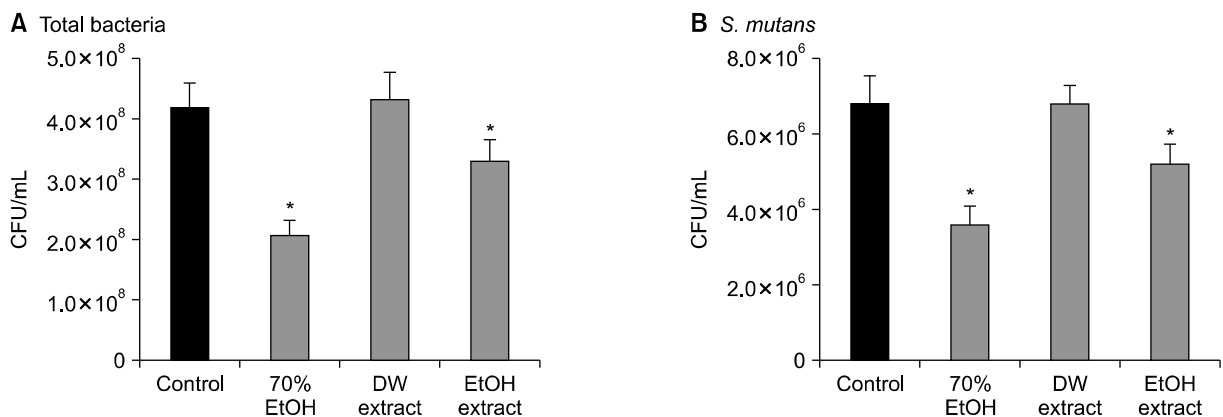


Fig. 4. Anti-biofilm effects of *Moringa oleifera* leaf extracts on cariogenic biofilm. Cariogenic biofilm was formed and treated with extracts for 10 min, and the biofilm was disrupted with a scraper. The bacteria in the biofilm were resuspended with brain heart infusion (BHI) broth and spread on mitis salivarius-bacitracin and BHI agar plate to count *Streptococcus mutans* and total bacteria, respectively. Asterisks (*) represent statistically significant differences compared with the control group ($P < 0.05$). CFU, colony-forming unit; DW, distilled water; EtOH, ethanol.

that the leaf extracts have antimicrobial activities against cariogenic bacteria and biofilm. *M. oleifera* EtOH extracts may be more effective against caries-related bacteria and biofilm compared with the DW extracts. This study was the first to examine the antimicrobial activity of *M. oleifera* leaves against oral biofilm. These results suggest that *M. oleifera* leaves may potential candidates to prevent dental caries.

ACKNOWLEDGEMENTS

This work was supported by the 2019 Research Fund of Ulsan College.

AUTHOR DISCLOSURE STATEMENT

The author declares no conflict of interest.

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