Inhibitory Effects of Mulberry (*Morus alba*) Ethanolic Extract on *Streptococcus mutans* Biofilm Formation

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Abstract

Objectives: To evaluate the susceptibility of *S. mutans* (ATCC 25175) and three *S. mutans* clinical isolates from Thai children to three concentration of mulberry extract and to analyze the inhibition effect on biofilm of each strain using confocal scanning electron microscopy (CLSM).

Materials and Methods: Obtained *S. mutans* clinical isolates from children (aged 3.5-10-years) who came to the Pediatric Dental Clinic, Faculty of Dentistry, Chulalongkorn University, Bangkok. Three concentration of mulberry extract were tested (125, 250, 500 mg/ml). The Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) were carried out. Incubated all *S. mutans* strains for 5 and 10 hours to form biofilm suitable for the susceptibility tests. The biofilm inhibition effect of mulberry extract also further evaluated using CLSM. Data was expressed as mean ± standard deviation (SD). The Kruskal-Wallis Test was used to compare the experimental and control groups (significance at 95%).

Results: The MIC of mulberry extract was not able to visualize due to the darkness of the solution and precipitations. The MBC were in the range of 125-500 mg/ml. All strains tested were sensitive to all mulberry extract concentrations (> 99.88%) when compared to the controls. All strains pre form biofilm at 5 and 10 hours were inhibited after 5 and 10 minutes exposure to the mulberry extract at the concentration of 500 mg/ml which showed the percentage of inhibition at > 99.96-100% and > 99.93-100% compared to the control, respectively. All concentrations of the mulberry extract inhibited biofilm formation of all strains visualized by CLSM. The effects were dose-dependent. The 500 mg/ml concentration exhibited a significant inhibitory effects on *S. mutans* (ATCC 25175) ($p = 0.013$), *S. mutans* clinical isolate N006, N029 and N113 ($p = 0.016$) when compared to the control group.

Conclusions: Mulberry extract at the 500 mg/ml concentration showed excellent antibacterial activity

Keywords: Natural essential oil, Dental caries, *Streptococcus mutans,* Mulberry, Biofilm

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Introduction

Dental caries still remains the most prevalent disease in the world. It can initiate pain, distress, and diminish the quality of life (1). It is a multifactorial disease and no simple causation pathway (2). This disease has a complex cause involving genetic, microbial, environmental, and social and behavioral factors (3). Dental caries is anticipate by the disbiose caused in biofilm due to frequent sugar intake (2). It is a diet-driven, biofilm-mediated illness that is directly influenced by an individual's intake of fermentable carbohydrates and has unique microbial compositions linked to its onset, progression, and arrest (4). The acids released into the aqueous part of the biofilm provoke the enamel/dentine demineralization (5,6).

The oral cavity contains dental plaque, also known as biofilm, which is made up of about 800 different types of bacteria coexisting (2). More than 800 aggregated microorganisms that adhere to a surface and are encased in an extracellular matrix make up a complex microbial community known as a biofilm (3). Studies focusing on chosen acidogenic and aciduric caries involved species, which are important caries pathogens, may be determining for caries risk assessment and prevention. Evidence suggests that *Streptococcus mutans,* a bacterium frequently isolated from dental plaque, is one of the microorganisms that cause dental caries (6–9). *S. mutans* is able to colonize the oral cavity and survive in an acidic environment and specific interaction with other microorganisms colonizing the ecosystem (10). Following the cleavage of sucrose molecules by the enzyme glucosyltransferases (Gtfs), the glucose component is polymerized into adherent glucans (6). According to earlier research on Thai children, children with Early Childhood Caries (ECC) have greater levels of *S. mutans* in their plaque (7-9). Reducing the adherence of *S. mutans* to tooth surface or inhibit *S. mutans* biofilm formation might be an important method to prevent dental caries.

Alternative medicine using natural products proven to have anti-cariogenic properties is of great interest in caries research (10-12). Previous studies have been reported for its effectiveness against bacteria implanted within the biofilm (10-12). Mulberry is a deciduous tree of the genus Morus in the family Moraceae. It was originated in China and has been growing throughout Southwest Asia, Europe and South America (13). The antimicrobial activity of Morus species is mainly associated with the stem bark of the mulberry plant (14,15). The antibacterial and antifungal properties of a number of chemicals that have been isolated from different portions of the Morus plant and their crude extracts have been investigated (13). According to the investigation, the majority of the bioactive substances found in Morus plants, including terpenoids and flavonoids, have strong antibacterial properties (16–19). One of the most widely used remedies in traditional Chinese medicine is mulberry leaves *(Morus alba).* It is used to treat obesity and diabetes mellitus. Mulberries include bioactive substances called polyphenols, alkaloids, flavonoids, and polysaccharides (20). Studies have examined mulberry leaves in a variety of forms, including ethanol extract, powder, and aqueous extract, all of which indicate beneficial effects on enhancing physiological conditions (20–22). Human volunteers with diabetes and those in good health were used to examine the effects of mulberry leaf on blood glucose response. The results showed that mulberry leaf consumption considerably decreased blood glucose levels (20).

It has been demonstrated that mulberry components enhance nerve protection and cognitive function. Mulberry leaf extract and mulberry milk have been found in numerous clinical investigations to enhance cognitive function in middle-aged and older persons (20).

Mulberry extracts have demonstrated antiviral activity against several viruses, including influenza virus, SARS-CoV-2, herpes simplex virus type 1 (HSV-1), murine norovirus, and feline cupripoxvirus (13,21,22).

The objectives of this study were to evaluate the susceptibility of *S. mutans* and three clinical isolations from Thai children to different concentration of the Mulberry extract (125, 250, 500 mg/ml) and to analyze the inhibition effect on biofilm of each strain using fluorescent dyes and confocal scanning electron microscopy (CLSM).

Materials and Methods

Essential Oil Preparation

Purchased materials and reagents was described in the previous study (23). C3G in mulberry fruit extract powder was measured using an HPLC-extracted sample, as previously mentioned (23). A 0.20-µm nylon filter was used to filter the stock solution of C3G W, which was made with 0.1% phosphoric acid in water and acetonitrile (50:50, v/v) at 30°C in an ultrasonic bath to achieve a concentration of 100 µg/mL. Main components in the mulberry fruit extract (MFE) in this study are polysaccharides, flavonoids, and phenolic acids. Anthocyanin is the major constituent (23).

Subject Selection and *S. mutans* Clinical Isolation

The Ethical Human Research Committee of Chulalongkorn University's Faculty of Dentistry in Bangkok, Thailand, gave its clearance to this study (approval number: 42/2010) (24). Consent forms were signed by all kid parents and legal guardians. Every participant was free to leave the research at any moment. *S. mutans* clinical isolations were obtained from child subjects. Detail method was described in the previous study (24). Biochemical tests verified that every isolate was *S. mutans.* The standard strain of *S. mutans* (ATCC 25175) was cultivated on BHI agar or broth and incubated for 48 hours at 37°C. For 18 hours, it was supplemented with 5% CO2.

Bacteria and Culture Conditions

S. mutans (ATCC 25175) aliquot stocks should be grown at -20°C on BHI. 48 hours of incubation at 37°C with 5% CO_2 added. Three to five colonies were chosen to suspend in Todd-Hewitt broth (Difco, USA) and then incubated for a full day. Three aliquot stocks of *S. mutans* clinical isolates (N006, N029, and N113) were chosen at random and cultivated at -20°C.

Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

The broth macrodilution method was used to determine the MICs of mulberry extract. Stock solutions of oils were initially dissolved in tween 80 and 95% ethanol then a series of twofold dilutions of the mulberry extract was prepared in Brain Heart infusion broth (BHI). MIC and MBC of all strains were performed. Three to five colonies of each strain was picked and suspended in

BHI broth for 24 hours. Bacterial suspensions that had recently been cultivated in BHI were adjusted to around 10^5 CFU/mL. 100 μL of seeded broth and 100 μL of mulberry extract were added to each of the eight wells for the MIC determination. 100 μL of the seeded broth for eight wells was placed in the first test well. To increase the volume to 200 μL, 100 μL of the mulberry extract solution was added to the first well, and then 100 μL was moved to the next well. To get a two-fold serial dilution, repeat the procedure. Sterilized 96-well polysterene plate in the UV sterilization for 1 hour. Inoculated *S. mutans* into each well then vortexed for 5 mimutes and incubated for 24 hours at 37°C, supplemented with 5% CO_2. The first assay well, with no microorganism growth which contained the lowest dilution of the mulberry extract representing the MIC. Determined MBC by removed samples from the wells that showed no turbidity and dropped onto BHI plates. Incubated at 37°C for 24 hours, the minimum concentration without any visible growth was reported as the MBC.

Biofilm Susceptibility Test

To examine the effects of various concentrations of the mulberry extract on 5 and 10-hour-old biofilm and to determine whether different strains had different susceptibilities to the bactericidal activity of each concentration of the mulberry extract, a biofilm susceptibility test was performed. Todd Hewitt broth mixed with 10% (w/v) sucrose was used to dilute all strains. The starting cell density was 10^8 CFU/mL. Transferred 2 mL of diluted cells into a new tube and incubated it (please delete) for 5 and 10 hours. Following incubation, planktonic cells were eliminated by three rounds of sterile water washing. Then, 2 mL of mulberry extract were added into the same tube. After one minute exposed to the mulberry extract this was washed out. Added two milliliters of regular saline solution and ten 2x2 mm sterile beads, and vortexed for a minute. 20 mL of the fluid was pipetted and then put over BHI agar. Plate counting was used to determine the CFU counts. Every experiment was carried out in triplicate for every concentration of mulberry extract. Positive control was chlorhexidine gluconate (0.12% CHX mouthwash). Negative control was no adding antibacterial solution. The colony was calculated as a percentage compared to the control. The effective bactericidal effect was significant, at more than 99.9% compared to the control.

Saliva-Coated 24-Well Plate for Biofilm Inhibition Assay and Fluorescence Staining using Confocal Laser Scanning Microscope (CLSM)

Three volunteers (dental students who are a part of the research team) who abstained from tooth brushing for at least 6 hours prior to saliva collection. Chewing paraffin enhanced the production of saliva. The saliva samples from the 3 volunteers were pooled, centrifuged and then diluted to 1:10 with phosphate buffered saline (PBS) and filtered through a 0.22-micron membrane (filter paper, Millipore, St Louis, MO). Since *S. mutans* clings to the glycoprotein in saliva, the stimulated pooled saliva was utilized to cover the wells of a 24-well polystyrene plate (Corning Inc., Corning, NY) in order to start the biofilm formation process. Each well containing 500 μL of diluted saliva was incubated at 37°C for 24

hours and then the saliva was rinsed off. For Fluorescence Staining using Confocal Laser Scanning Microscope (CLSM) following the method as described in the previous study (10).

Statistical analysis

The mean ± standard deviation (SD) was used to display the data. Determined live (the green emission at 530 nm) and dead cells (the red emission at 630 nm) by measuring and calculated ratio. Each well was measured in triplicate. Calculated mean and SD. from the experimental and control groups were compared using the Kruskal-Wallis Test (also known as the Bonferroni and Dunn Test) (significance at 95%).

Results

Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

The MIC of mulberry extract was not able to visualize due to the darkness of the mulberry extract solution and precipitations. However, the MBC of all strain tested were in the range of 125-500 mg/ml (Table 1). The MBC of three clinical isolations were higher than the standard strain. Although the MIC was not able to identify, the MBC indicating a bactericidal action of the mulberry extract.

Table 1. Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal concentration (MBC) of mulberry extract to different strains of *Streptococcus mutans.*

***S. mutans* Strains**	**Mulberry extract concentration (mg/ml)**	
	MIC	**MBC**
S. mutans (ATCC 25175)	N/A	125
S. mutans (N006)	N/A	500
S. mutans (N029)	N/A	500
S. mutans (N113)	N/A	500

N/A=non applicable.

Biofilm Susceptibility Test

All strains of *S. mutans* tested in this study were sensitive to all concentrations (125, 250 and 500 mg/ml) of the mulberry extract (>99.88%) when compared to the controls. Highest concentration of the mulberry extract was used to demonstrate antibacterial effects on 5-hour and 10-hour-old biofilm. For 5-hour-old preformed biofilm, all strains were inhibited after 5 and 10 minutes exposure to the mulberry extract solution at the concentration of 500 mg/ml (>99.96-100%) compared to the control (Table 2, 3). Similarly, for 10-hour-old preformed biofilm, all strains were inhibited after 5 and 10 minutes exposure to the mulberry extract at the concentration of 500 mg/ml (>99.93-100%) compared to the control (Table 4, 5).

Table 2. Inhibition of 5-hour-old preformed biofilm of all *S. mutans* strains after 5 minutes exposure to the Mulberry extract (concentration of 500 mg/ml).

***S. mutans* strains**	**Control Biofilm (colony forming unit (CFU)**	**5 minute exposed to mulberry extract biofilm (CFU)**	**Reduction (%)**
S. mutans (ATCC 25175)	3.45×10^6	2.75×10^2	99.99%
S. mutans (N006)	9.0×10^4	ND	100%
S. mutan (N029)	3.0×10^5	ND	100%
S. mutans (N113)	1.65×10^5	2.0×10^2	99.88%

ND= not detect

Table 3. Inhibition of 5-hour-old preformed biofilm of all *S. mutans* strains after 10 minutes exposure to the Mulberry extract (concentration of 500 mg/ml).

***S. mutans* strains**	**control biofilm (colony forming unit (CFU)**	**10 minute exposure to mulberry extract biofilm (CFU)**	**Reduction (%)**
S. mutans (ATCC 25175)	5.3×10^4	ND	100%
S. mutans (N006)	1.5×10^4	ND	100%
S. mutan (N029)	2.0×10^4	ND	100%
S. mutans (N113)	2.5×10^4	ND	100%

ND= not detect

Table 4. Inhibition of 10-hour-old preformed biofilm of all *S. mutans* strains after 5 minutes exposure to the Mulberry extract (concentration of 500mg/ml).

***S. mutans* strains**	**control biofilm (colony forming unit (CFU)**	**5 minutes exposure to mulberry extract biofilm (CFU)**	**Reduction (%)**
S. mutans (ATCC 25175)	1.60×10^6	6.65×10^2	99.96%
S. mutans (N006)	7.75×10^5	75	99.99%
S. mutan (N029)	1.35×10^7	4.38×10^3	99.97%
S. mutans (N113)	6.75×10^4	225	99.97%

Table 5. Inhibition of 10-hour-old preformed biofilm of all *S. mutans* strains after 10 minutes exposure to the Mulberry extract (concentration of 500 mg/ml).

***S. mutans* strains**	**control biofilm (colony forming unit (CFU)**	**10 minute exposure to mulberry extract biofilm (CFU)**	**Reduction (%)**
S. mutans (ATCC 25175)	3.85×10^5	2.6×10^2	99.93%
S. mutans (N006)	3.50×10^5	ND	100%
S. mutans (N029)	9.75×10^6	3.25×10^2	99.99%
S. mutans (N113)	2.83×10^4	ND	100%

ND= not detect

Fluorescence Staining and CLSM

For the inhibition of *S. mutans* (ATCC 25175) at cells concentration of 10^8 CFU/ml, the mulberry extract at the concentration of 500 mg/ml showed the highest inhibition rate which gave the lowest percentage of live cells (31.65 ± 2.03%), followed by the concentration of 250 mg/ml (63.91 ± 7.05%), and 125 mg/ml (74.16 ± 0.85%), respectively (Fig 1). The 500 mg/ml showed significantly (p = 0.013) different inhibition rates compared to control.

The mulberry extract at 500 mg/ml had the highest inhibition rate for *S. mutans* clinical isolate N006, resulting in the lowest percentage of live cells (37.69 ± 2.12%). This was followed by concentrations of 250 mg/ml (46.67 ± 0.47%) and 125 mg/ml (60.46 ± 1.35%), respectively (Fig 2). Comparing all concentrations to the controls, the inhibition rates varied considerably (p = 0.016).

The mulberry extract at 500 mg/ml demonstrated the highest inhibition rate for *S. mutans* clinical isolate N029, resulting in the lowest percentage of live cells (28.22 ± 8.37%). This was followed by concentrations of 250 mg/ml (48.67 ± 2.60%) and 125 mg/ml (56.75 ± 2.36%), respectively (Fig 3). Comparing all concentrations to the controls, the inhibition rates varied considerably (p = 0.016).

The mulberry extract at 500 mg/ml demonstrated the highest inhibition rate for *S. mutans* clinical isolate N113, resulting in the lowest percentage of live cells (43.57 ± 1.4%) This was followed by concentrations of 250 mg/ml (51.25 ± 1.9%) and 125 mg/ml (62.07 ± 3.19%), respectively (Fig 4). Comparing all concentrations to the controls, the inhibition rates varied considerably (p = 0.016).

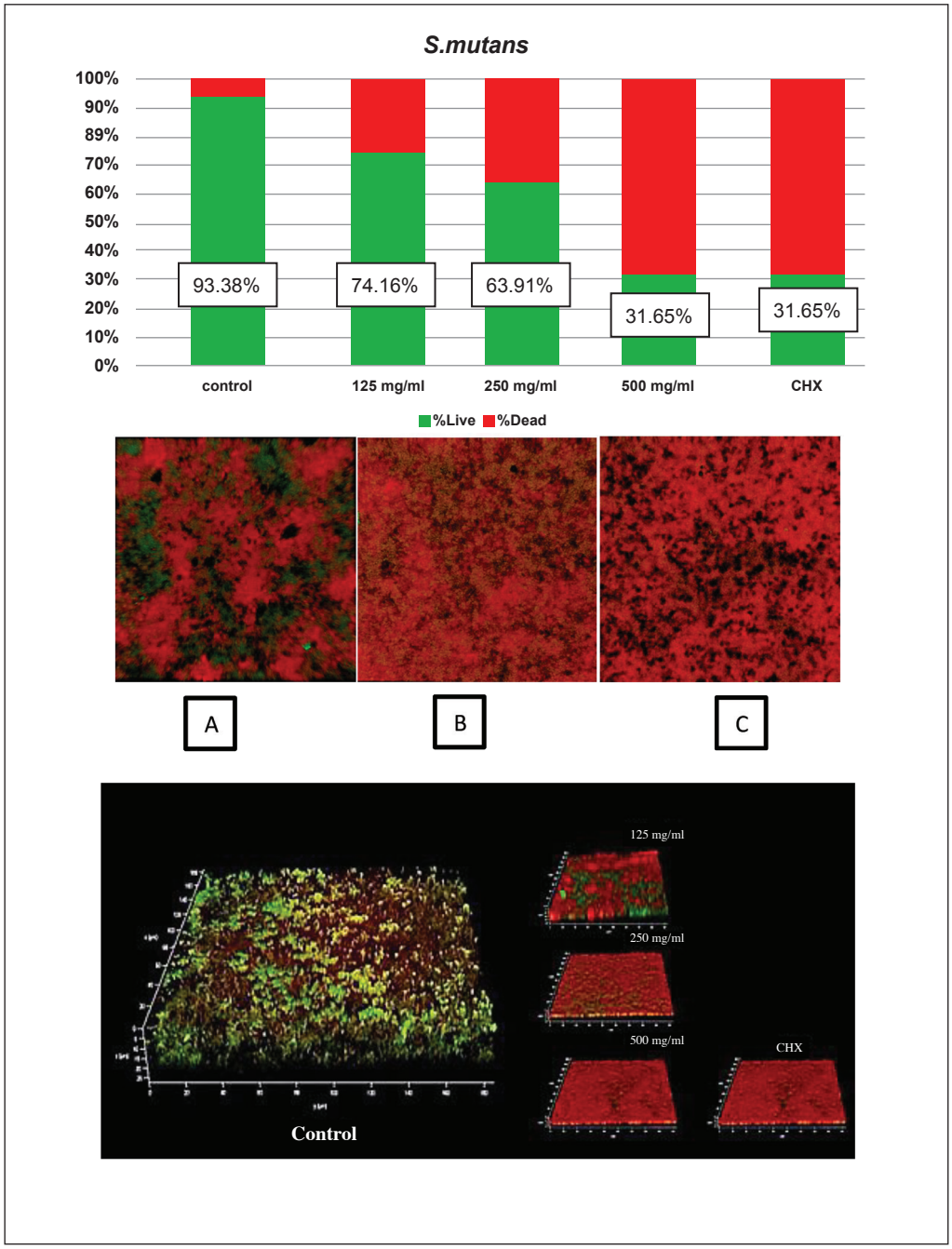

Fig 1. 24-hours *S. mutans* (ATCC 25175) biofilm formation. Live cells were stained and exhibited green fluorescence whereas dead cells exhibited red fluorescence under CLSM. Two and three dimensional pictures showed the penetration and inhibition effect of mulberry extract in all concentrations. The graph represents the percent reduction in variable cells in the *S. mutans* biofilm using different mulberry extract concentration.
(A) 125 mg/ml, (B) 250 mg/ml, (C) 500 mg/ml.

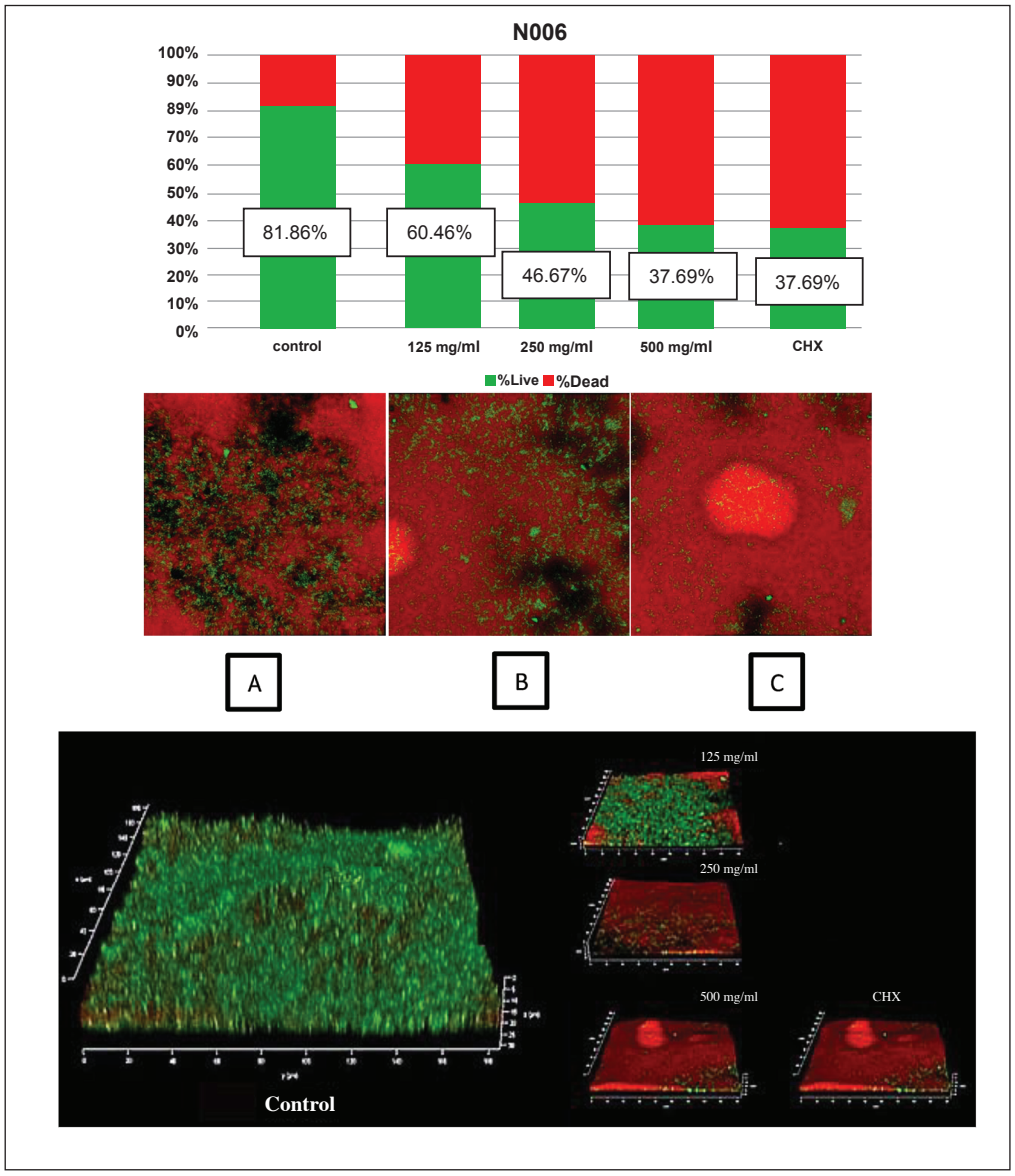

Fig 2. 24-hours *S. mutans* (N006) biofilm formation. Live cells were stained and exhibited green fluorescence whereas dead cells exhibited red fluorescence under CLSM. Two and three dimensional pictures showed the penetration and inhibition effect of mulberry extract in all concentrations. The graph represents the percent reduction in variable cells in the *S. mutans* biofilm using different mulberry extract concentration. (A) 125 mg/ml, (B) 250 mg/ml, (C) 500 mg/ml.

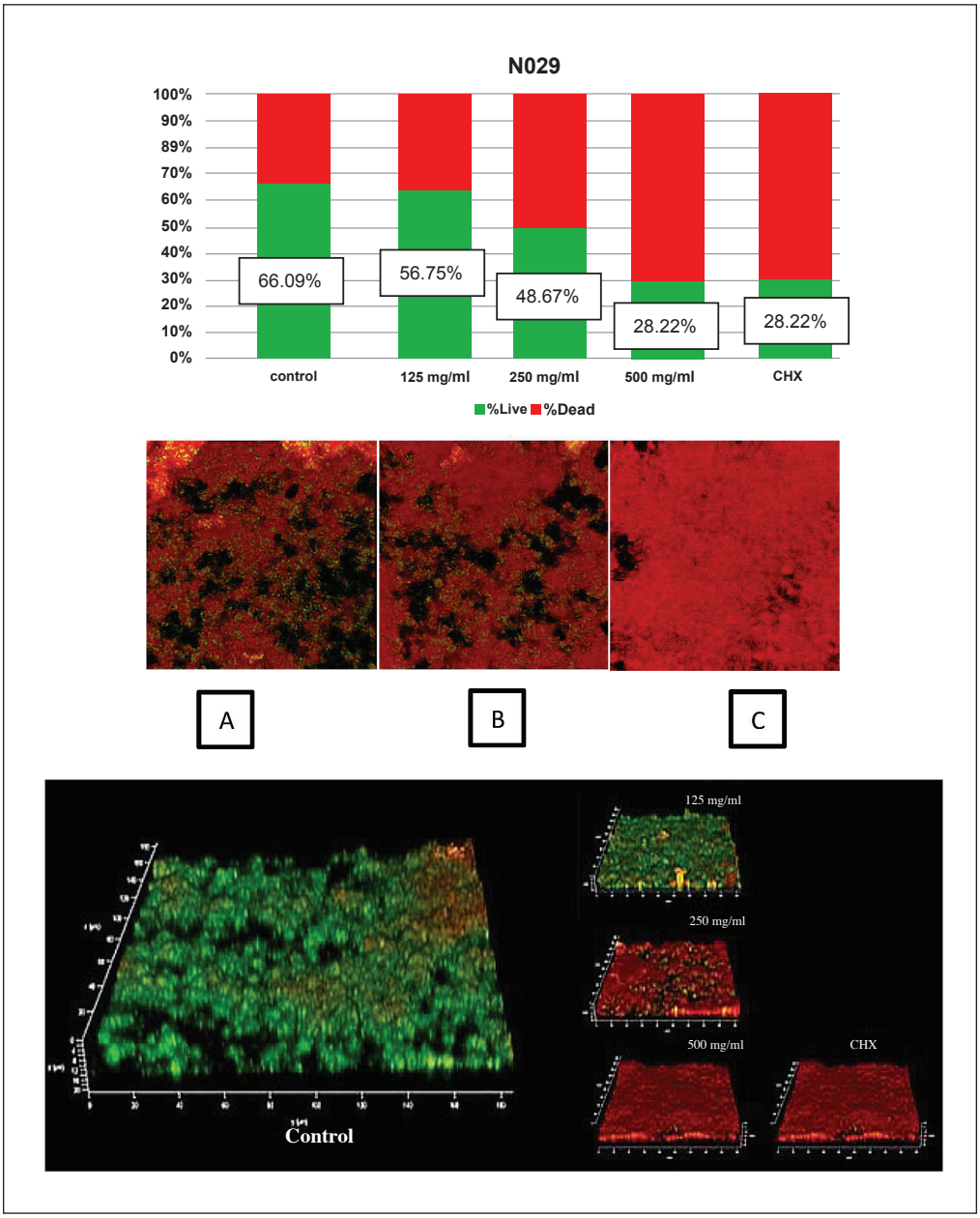

Fig 3. 24-hours *S. mutans* (N029) biofilm formation. Live cells were stained and exhibited green fluorescence whereas dead cells exhibited red fluorescence under CLSM. Two and three dimensional pictures showed the penetration and inhibition effect of mulberry extract in all concentrations. The graph represents the percent reduction in variable cells in the *S. mutans* biofilm using different mulberry extract concentration.
(A) 125 mg/ml, (B) 250 mg/ml, (C) 500 mg/ml.

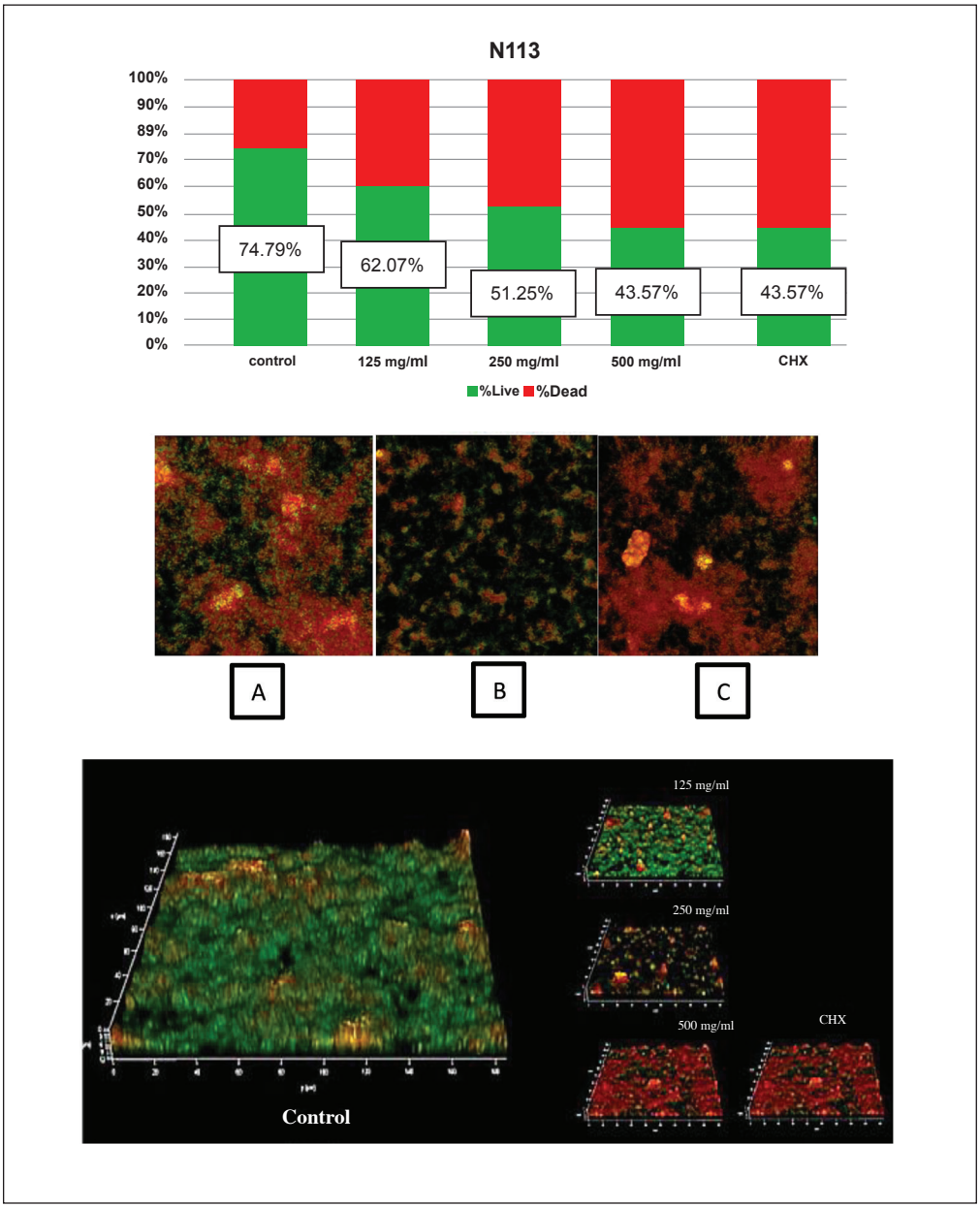

Fig 4. 24-hours *S. mutans* (N113) biofilm formation. Live cells were stained and exhibited green fluorescence whereas dead cells exhibited red fluorescence under CLSM. Two and three dimensional pictures showed the penetration and inhibition effect of mulberry extract in all concentrations. The graph represents the percent reduction in variable cells in the *S. mutans* biofilm using different mulberry extract concentration. (A) 125 mg/ml, (B) 250 mg/ml, (C) 500 mg/ml.

Discussion

To test the efficacy of antibacterial of plant-derived oil extraction on single bacterial specie establish an important pre-requisite before testing its effects on preparations containing multiple bacterial species. It has been well demonstrated that *S. mutans* is the major etiological bacteria in dental caries (6). Biofilm formed by single specie is advantageous to study the mechanisms of action of antibacterial therapeutic agents. The efficacy of ethanolic extract from mulberry against *S. mutans* allowing for the possibility of decrease costs to prevent dental caries in Thailand. Brushing is usually followed by 30 seconds of application of a unique antimicrobial mouthwash as part of a plaque removal routine. Chlorhexidine gluconate (CHX) has been ranked as a gold standard among antibacterial agents effective in reducing oral biofilm including the effects on *S. mutans,* have been elucidated largely by in vitro culture methods (25,26). However, few studies investigated the effect of 0.12% CHX mouthwash if use twice daily for 7 days, on the abundance of bacteria colonizing the tongue in healthy individuals (26). The results showed that CHX reduced species richness and variety while increasing the abundance of Gram-negative bacteria, especially those in the phylum Bacteroidetes (Capnocytophaga). They also found that CHX lowered the bacteria especially *Prevotellaceae,* while increased *Proteobacteria* (including *Neiseriaceae*) and *Streptococcus* after using CHX (26).

Plant-derived essential oil is ideal for use in oral health care products such as toothpastes, mouthwashes, spray and gels, because of their non-toxic and antiseptic properties (11-13). The arylbenzofurans isolated from Morus species, including Moracin C and Moracin M, showed promising antibacterial activity against methicillin-resistant *Staphylococcus aureus, Klebsiella pneumoniae, and Pseudomonas aeruginosa* (17). Previous studies have shown that mulberry fruits may have anti-inflammatory, hypoglycemic, immunomodulatory, antioxidant, and analgesic properties (14-16,18). One of the studies testing Mulberry leaves which was extracted by 85% methanol using ultrasonic extraction method and using disk diffusion and agar plate dilution methods, results showed *S. mutans* (KCTC 3065) growth inhibition rate was over 87.54% after 6 hours compared with control (19). There were several Mulberry extract studies in Thailand. Our previous pilot study found that the minimum bactericidal concentration (MBC) of ethanolic extract from mulberry fruit *(Morus nigra)* against *S. mutans* and *S. sobrinus* was 125 mg/ml. Another study by Suriyaprom and colleagues using well diffusion method showed that the ethanolic extract from mulberry fruit at the concentration of 500 mg/ml had antibacterial properties against Gram-positive bacteria; *Staphylococcus.* aureus and Gram-negative bacteria; *Escherichia coli, Salmonella typhi, Shigella dysenteriae* and *Vibrio cholerae,* with diameters of the inhibition zones as high as 23.50, 15.50, 16.50, 25.67 and 16 mm, respectively when compared to the positive control which was gentamicin 1 mg/ml. In this study, our results were in line with previous studies aforementioned (27).

In addition, this study evaluated the sensitivity of *S. mutans* (ATCC 25175) and three *S. mutans* clinical isolations from Thai children to the mulberry extract at the concentration of 500 mg/ml showing bactericidal effects on 5 hours and 10 hours pre-formed biofilm. This finding supports the idea that biofilms form in three

distinct stages, with accumulation occurring between 0 and 5 hours, active accumulation at 4–20 hour, and a slower accumulation with plateau-phase (28). The 5 hours biofilm was in the first stage, whereas the 10 hours biofilm was in the second stage. The 10 hours biofilm was more complex and resistant to the antibacterial treatment because of a rapid increase in adherence. An excellent activity of the mulberry extract against different strains of *S. mutans* was demonstrated in this study even in 10 hours pre-formed biofilm, the antibacterial effect was obvious.

The mechanism of the inhibitory effect of the mulberry extract is not fully comprehend. However, the active ingredients that exert antibacterial effects might be flavonoids which can be found in mulberry leaves, fruits and roots (18). It has the ability to destroy bacterial cell walls (18). Another active ingredient is tannin which can be found in the mulberry leaves and fruits which can cause leakage of bacterial cell walls (29,30). In addition, phenolic acid which can be found in mulberry leaves, fruits and roots can denature bacterial proteins (31). Other active ingredient such as deoxynojirimycin (DNJ) (a potent glucosidase inhibitor) which can be found in mulberry leaves, fruits and roots can inhibit glucan formation (32-34). Another active ingredient is Kuwanon G which can be found in mulberry roots. It has properties to inhibit the growth of *S. mutans* by destroying cell walls and cytoplasm concentrations (34,35). In this study, 10 hour *S. mutans* pre formed biofilm was inhibited implying that the mulberry extract showed inhibition effect at the active accumulation and plateau phases which involve cascade active synthesis of glucan (33,36). Thus, mulberry extract might either inhibit glucan synthesis or reduce cells glucan binding.

Confocal laser scanning microscopy (CLSM) has been used to evaluate bacterial viability on biofilm (37,38). When apply fluorescence dyes on the biofilm, it can differentiate live and dead bacteria, allowing bacteria to be distinguished according to cytoplasmic membrane permeability (37). In addition, CLSM is able to penetrate in a horizontal plane (X-Y axes) and evaluate the depth of biofilm from the outer part to the inner part and can capture a series of image-scans showing changes in the viability of the bacterial cells over time, allowing for the real-time visualization of death of the microorganisms possible (37,38).

In this study, biofilm was stained with fluorescence dye. Live cells and dead cells demonstrated green (SYTO9) and red colours (propidium iodide), respectively. Our results showed that when evaluating 3 dimension images using CLSM, the mulberry extract at the concentration of 500 and 250 mg/ml was able to penetrate the biofilm to the deepest layer and in all directions of biofilm structure of *S. mutans* (ATCC 25175), clinical isolate N006 and N029. This result is consistent with a prior study that demonstrated the efficacy of a range of dental treatments, such as toothpaste dentifrice slurries and mouthwashes containing essential oils. According to their CLSM results, a mouthwash containing a blend of essential oils killed bacteria in biofilm within the first 30 seconds and penetrated biofilm in all directions (39). In this study, we demonstrated an excellent activity of mulberry extract against different strains *S. mutans* at various concentrations.

Interestingly, each strain showed different pattern of mature biofilm but the antibacterial and penetrating activities of mulberry extract were able to inhibit biofilm in all strains.

This study's limitation is the *in vitro* results from one species of biofilm. The actual oral environment with multi-species dental plaque with structural complexity and dynamic conditions formed in the mouth cavity may not be represented by this pre-established bacterial biofilm. We recommend to further test the different responses of dental plaque to this mulberry extract in the toxicity aspect in the future before starting the clinical trial study. However, the results from this study could be applied to clinical use. Development of the mulberry extract oral spray may lead to the alternative prevention method for dental caries.

In conclusion, the mulberry extract at the various concentrations effectively inhibited biofilm formation of *S. mutans* standard strain and three clinical isolates *in vitro*. These mulberry extract demonstrated satisfying results and need further clinical studies to verify its efficacy against dental caries.

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